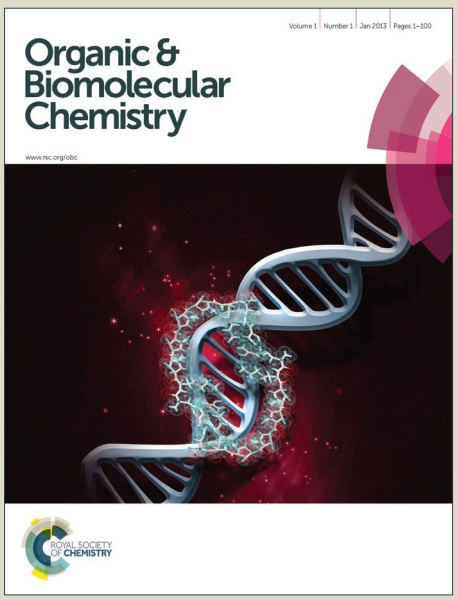
Organic & Biomolecular Chemistry

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Organic and Biomolecular Chemistry

COMMUNICATION

Synthesis and biophysical properties of (L)-aTNA based G-Quadruplexes

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Vipin Kumar and Kurt V. Gothelf

Novel G-quadruplex structures are constructed by acyclic (L)-threninol nucleic acid and their synthesis and biophysical properties are described. Pyrene excimer fluorescence and circular dichroism (CD) data revealed that four strands of aTNA are oriented in antiparallel direction.

Acyclic oligonucleotides[1] are playing a very important role for the development of antisense techniques[2] and biosensors[3]. The interest in investigating acyclic oligonucleotides has increased due to their easy chemical synthesis, stability against nucleases[2, 3a] and high chemical stability at low pH[4]. Naturally occurring deoxyribonucleic acid (DNA) and ribose nucleic acid (RNA) have been shown to form various unusual structures like the A-motif, i-motif and G-quadruplex[5]. These structures have been utilized for the construction of nano devices and sensors. Guanine-rich oligonucleotides can self-assemble in G-tetrads, where four guanine nucleotides interact by Hoogsteen hydrogen bonds, which results in the G-quadruplex[6] (Fig. 1). Monovalent cations are required for further stabilization of G-Quadruplex structures. Of the acyclic oligonucleotides peptide nucleic acid (PNA) has also been explored for the construction of G-quadruplex structures[7]. PNA does not contain phosphodiester bonds and therefore shows poor aqueous solubility. Only butyl nucleic acid (BuNA) which contains phosphodiester linkages has been reported as an acyclic scaffold for the A-motif[4]. Due to the flexible backbone, BuNA was unable to hybridize with DNA or RNA[1c].

Recently, we showed that poly pyrimidine (L)-aTNA is able to make highly stable triple helix with single stranded poly purine DNA or RNA (Fig. 1).[1e] Here we extend our investigation in order to construct the (L)-aTNA based G-quadruplex structures. In this investigation we have found that aTNA forms stable G-quadruplexes in both intermolecular and intramolecular forms. Furthermore, G-rich aTNA could be very good candidate in the future for the charge transport, since it has been shown that G-quadruplexes are conducting[8].

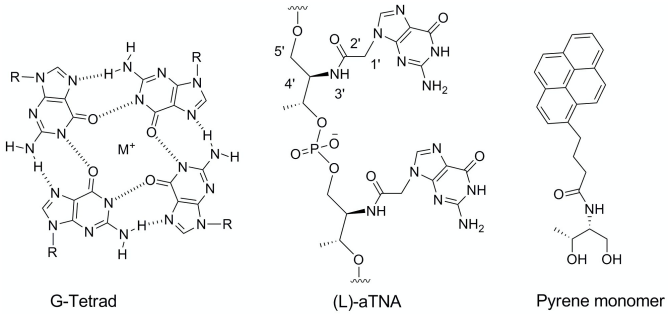


Figure 1: Structure of the G-tetrad, acyclic (L)-threoninol nucleic acid (L-aTNA) and pyrene on aTNA scaffold.

Synthesis of the guanine (L)-aTNA phosphoramidite was carried out in four steps with good yields (ESI, S1, S2). DNA and aTNA oligonucleotides were synthesized and analysed by liquid chromatography–mass spectrometry (LC-MS) (ESI, S3, S4).

Table 1: Non-Fluorescent aTNA and DNA oligonucleotide sequences[a]

Code	Sequences	Type
ON-1	5′-ggggggggggT-3′	(L)-aTNA
ON-2	5′-gggggggT-3′	(L)-aTNA
ON-3	5′-gggtttgggtttgggtttgggT-3′	(L)-aTNA
ON-4	5′-GGGTTTGGGTTTGGGTTTGGGT-3′	DNA
ON-5	5′-ggttggtgtggttgg-4′	(L)-aTNA
ON-6	5′-gggggggCCCCCCCT-3′	(L)-aTNA-DNA
ON-7	5′-CCCCCCCCCCC-3′	DNA
ON-8	5′-ccccccccccT-3′	(L)-aTNA
ON-9	5′-CCCAAACCCAAACCCAAACCCA-3′	DNA

[a]Small letters indicate (L)-aTNA nucleotides and capital letters indicate DNA nucleotides.

First, the existence of (L)-aTNA based G-quadruplex (Q-aTNA) was verified by melting curve monitoring at 295 nm, where the inverse sigmoidal thermal denaturation is characteristic for G-quadruplex structures.[6a] Figure 2 shows the UV-melting profile of ON-1 (Table 1) in 10 mM phosphate buffer containing 100 mM potassium chloride at pH 7.4, which exhibited a melting temperature at 70 °C.

Danish National Research Foundation Center for DNA Nanotechnology, iNANO and Department of Chemistry, Aarhus University, 8000 Aarhus C, Denmark, E-mail: kvg@chem.au.dk, Fax: 86196199.

† Footnotes relating to the title and/or authors should appear here. Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

A decrease in absorbance was observed by increasing the temperature. In addition, the melting temperature of ON-2 was determined to be 47 °C (Fig. 2). These data indicate that sequences containing a stretch of aTNA guanine nucleotides are able to form G-tetrad. The presence of salts is also an important parameter in order to obtain G-quadruplex structure and in particular potassium ions are known to stabilize quadruplexes[6d]. Therefore, the stability of the structures was also investigated in Milli-Q water (no added salt). ON-2 showed increase in the absorbance at wavelength 295 nm by increasing the temperature, which reveals that ON-2 exists as random coil in the absence of a buffer and potassium ions (data not shown). However, ON-1 showed a distinct melting curve in water and the melting temperature was determined to be 33 °C (ESI, Figure S5.1). Next, we investigated the folding of the ON-3 sequence composed of four stretches of "ggg", forming the G-quartet core with loops of "ttt" nucleotides. The sequence showed a thermal stability at T_m = 51 °C (Fig. 2). Interestingly, this presumable intramolecular folding of ON-3 was stable in Milli-Q water (T_m = 37 °C) (ESI, Figure S5.2) (Table 2). All the thermal denaturation curves were observed to be reversible at 1 °C/ min heating and cooling rate with low hysteresis of 3 °C lower annealing temperature. These studies indicate that a stretch of guanine nucleotides of aTNA has a property of self-assembling in quadruplex structure. In addition an oligo strand a containing chimera of aTNA and DNA (ON-6) was investigated but no melting transition was observed at wavelength 295 nm while stable duplex was observed at 260 nm (ESI, Figure S5.3).

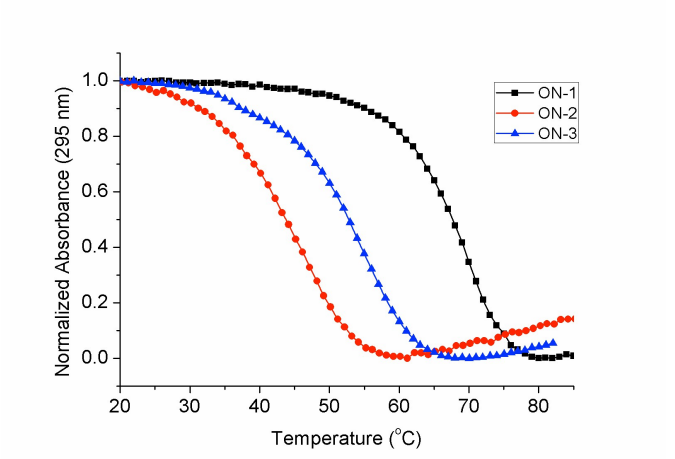


Figure 2: Thermal denaturation profiles at wavelength 295 nm of 4 µM oligos ON-1, ON-2 or ON-3 was used in 10 mM potassium phosphate buffer containing 100 mM potassium chloride at pH 7.4.

Table 2: Thermal stabilities of Q-aTNA measured at 295 nm.

Code	Sequences	T_m (°C)[a]	T_m (°C)[b]
ON-1	5'-gggggggggggT-3'	70	33
ON-2	5'-ggggggT-3'	47	Nd
ON-3	5'-gggtttgggtttgggtttgggT-3'	51	37

[a]Condition: 10 mM phosphate buffer, 100 mM KCl at pH 7.4, [b]Condition: Milli-Q water (no added salt). Nd = No melting was observed.

The secondary structures of G-rich oligonucleotides have been investigated by circular dichroism (CD) under various conditions.[9] In phosphate buffer, ON-1 and ON-2 showed positive Cotton effects at 255 nm and 210 nm with small shoulder peak at 289 nm and negative Cotton effects at 273 nm and 236 nm. Interestingly, ON-3 containing the four "ggg" domains with loops of "ttt" nucleotides displayed the same CD pattern which can be interpreted as the intramolecular Q-aTNA. CD was also recorded in Milli-Q water for ON-1, ON-2 and ON-3. ON-2 was observed as a random coil, whereas ON-1 showed weak CD profile, which further confirms the UV-melting data (ESI, Figure S5.4). However, a weak structure was observed for ON-2 in 10 mM tris-HCl buffer at pH 7.4, where melting temperature was to be determined as 29 °C (ESI, Figure S5.5)[6d].

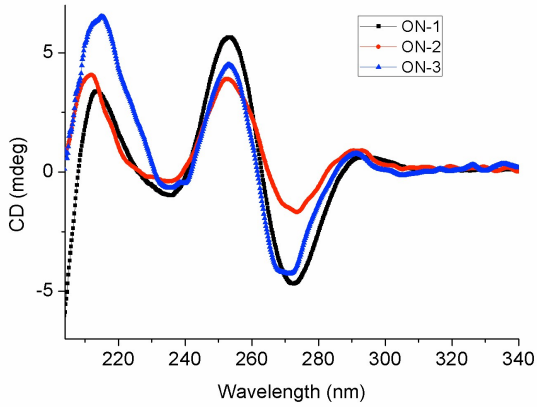


Figure 3: CD profiles of G-Quadruplex (L)-aTNA at 22 °C. Each oligonucleotide with 2 µM concentration in 10 mM phosphate buffer at pH 7.4 containing 100 mM potassium chloride.

In Milli-Q water, ON-3 exhibited similar CD pattern with weak negative cotton effect at 272 nm, which demonstrates that its folding was less dependent on salt as compared to ON-1 and ON-2 (ESI, Figure S5.6). In addition, ON-3 in unbuffered water containing 100 mM potassium chloride exhibited similar melting temperature (51 °C) (ESI, Figure S5.7). This clearly data indicate that folding of ON-3 is independent of counter ions (buffer). On the contrary DNA based intramolecular G-quadruplex sequence (ON-4) exhibited denatured structure when CD was recorded in Milli-Q water as compared to buffer (ESI, Figure S5.8). Moreover, increased absorbance was observed when UV-melting was performed at 295 nm in unbuffered water. The DNA analogs $d(G)_7$ or $d(G)_{11}$ were difficult to synthesize, therefore we were unable to compare with aTNA. Furthermore, we also tested a short sequence (ON-5) of aTNA, which was expected to fold in a G-quadruplex, where two tetrads stacking allow structure formation. In 10 mM phosphate buffer with 100 mM potassium chloride, ON-5 did not display secondary structure as confirmed by CD (ESI, Figure S5.9). This study revealed that three tetrads of guanine nucleotides may be the minimum requirement for the existence of intramolecular aTNA G-quadruplex structure. It should be noted here that PNA has not been reported to form intramolecular G-quadruplex.

Formation of more different G-quadruplex structures of ON-1 and ON-3 cannot be rule out. We therefore investigated ON-1 and ON-3 by non-denatured electro mobility gel shift assay (EMSA), which showed only a single band for both sequences. The mobility of the

band for ON-1 is around 25 base pair (bp) relative to the ladder in lane 3, which corresponds to an intermolecular Q-quadruplex formed out by 4 strands of the 12 nucleotides long ON-1 (Fig. 4, Lane 2 and 4).

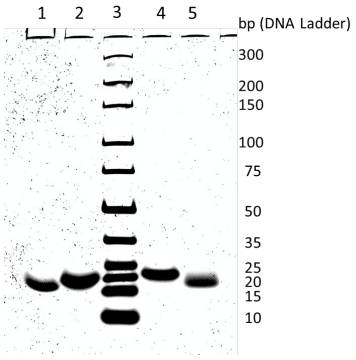


Figure 4: Electro mobility gel shift assay (EMSA). Samples were incubated overnight in Milli-Q water(Lane 1, 2) and 10 mM phosphate buffer, 100 mM potassium chloride at pH 7.4 (Lane 4, 5) with 140 pmol for ON-1 (Lane 2, 4) and 70 pmol for ON-3 (Lane 1, 5). Non-denatured 7 % polyacrylamide gel was run in TBE buffer containing 50 mM potassium chloride at 110 V for 3 hours at low temperature (≈ 15 °C). The gel was stained by SYBR Gold.

The higher mobility band of ON-3 (22 nucleotides) suggests that ON-3 folds in an intramolecularly G-quadruplex (Fig. 4, Lane 1 and 5). In addition, when gel was run at low voltage and low temperature for long time, a low mobility band was observed for ON-3 (ESI, Figure S5.10a, Lane 5). This low mobility complex might be due to weak intermolecular interactions. In addition, we also compared the G-quadruplex of DNA (ON-4) with aTNA (ON-3) by EMSA, which showed similar band mobility (ESI, Figure S5.10b, Lane 3 and 4), therefore confirm the intramolecular folding, since ON-4 is known to fold in intramolecular G-quadruplex[6e]. Furthermore, the DNA strand (ON-4) gave rise to a second band with high mobility, which may be the denatured structure. Furthermore, intramolecular structure formation was also confirmed by concentration dependent thermal denaturation studies of ON-3, which showed that the melting temperature was concentration independent (ESI, Figure S5.7). It is important to note that both intramolecular and intermolecular Q-aTNA showed similar melting temperature in the presence of sodium or potassium ions (data not shown). In contrast it has been shown that DNA based G-quadruplex are highly dependent on potassium than sodium ions[6e]. Next, we investigated the interaction of the Q-aTNA with complementary strands in 10 mM phosphate buffer containing 100 mM potassium chloride at pH 7. ON-1, ON-2 and ON-3 were mixed with their complementary DNA strands, where melting temperatures at wavelength 260 nm was to be determined as 80 °C, 61 °C and 71 °C respectively (ESI, Figure S5.11, 12, 13). In addition for these duplexes no sigmoidal melting curve was observed at wavelength at 295 nm, which indicated the heteroduplex (aTNA/DNA) formation and absence of Q-aTNA (ESI, Figure S5.14). Furthermore, CD-melting data showed highly stable homoduplexes (aTNA/aTNA) of ON-1/ON-8 (T_m = 92 °C) and ON-2/c_7dT (T_m = 70 °C) (ESI, Figure S5.15). These studies revealed that Q-aTNA structures can be directed to form the duplex by the addition of complementary DNA or aTNA strands. CD spectra were also recorded for homoduplex and heteroduplex structures. Oligonucleotides ON-1/ON-7 and ON-6 showed positive Cotton effects around 267 nm and negative Cotton effect at 225 nm. The ON-1/ON-8 duplex displayed positive CD band at 267 nm and 204 nm with negative bands at 285 nm and 255 nm. Heteroduplex of ON-3/ON-9 exhibited Cotton effects at 262 nm and 203 nm with negative Cotton effects at 238 nm (Fig. 5).

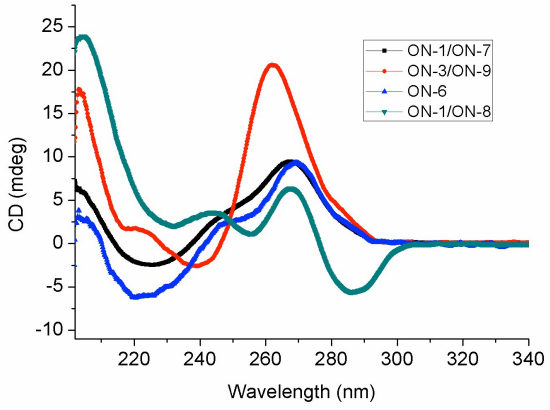


Figure 5: CD profiles of homoduplex aTNA/aTNA, heteroduplex of DNA/aTNA and self-complementary at 22 °C. Each oligonucleotide with 1 µM concentration in 10 mM potassium phosphate buffer at pH 7.4 containing 100 mM potassium chloride.

The formation of intermolecular G-quadruplexes with DNA strands generally assemble with the strands in a parallel orientation. In the literature it has been shown that pyrene excimer fluorescence can be used for the investigation of strand orientation in DNA G-quadruplexes.[10] Therefore, we investigated ON-10 and ON-13 sequences (Table 3), where pyrene moieties were installed at the 5'-end and at both the ends respectively. First, we investigated ON-10 which contains pyrene at the 5'-end. The sample (ON-10) in phosphate buffer was excited at 340 nm and monitored from 350 nm to 670 nm. However, no excimer emission at around 494 nm was displayed, while monomer fluorescence at wavelength 378 nm was observed (Fig. 6).

Table 3: Fluorescent (L)-aTNA and DNA oligonucleotides[a]

Code	Sequences	Type
ON-10	5'-Pyggggggggggt-3'	(L)-aTNA
ON-11	5'Py-ccccccccccT-3'	(L)-aTNA
ON-12	5'-PyCCCCCCCCCCC-3'	DNA
ON-13	5'-PyggggggggggPyT-3'	(L)-aTNA

[a]Small letters indicate (L)-aTNA nucleotides whereas capital letters indicate DNA nucleotides. Py = Pyrene nucleotide

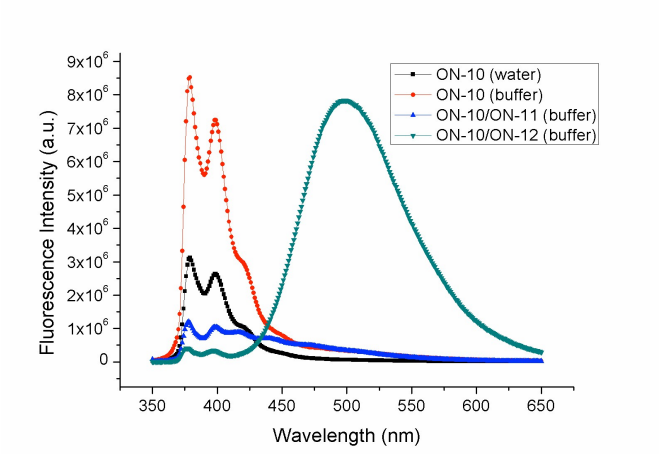


Figure 6: Fluorescence emission spectra of ON-10 (0.5 µM) and with their DNA or aTNA complementary strands (0.5 µM). Samples were incubated overnight at 22 °C in water (Milli-Q) or 10 mM potassium phosphate buffer containing 100 mM potassium chloride at pH 7.4). Duplex of ON-10/ON-12 were measured at 0.25 µM concentration.

Moreover, the fluorescence intensity of ON-10 was decreased in Milli-Q water (no added salt) due to a less stable Q-aTNA structure, which can allow thymine nucleotide to interact with pyrene[11]. The fluorescence intensity was further decreased for the antiparallel duplex formation of ON-10 with ON-11. This fluorescence quenching[11] can be expected due the presence of thymine nucleotide at the 3'-ends of ON-10 and ON-11. When ON-10 was mixed with ON-12, a strong excimer emission at 494 nm was observed which corresponds to the parallel heteroduplex[1f] (Fig. 6). The absence of excimer emission from ON-10 is ascribed to the formation of an intermolecular G-quadruplex in which the four strands have an antiparallel orientation. This places two pairs of pyrenes in diagonal positions at each end of the G-quadruplex. Since a short three-carbon linker on (L)-aTNA scaffold is used for linking pyrene (Fig. 1) on the 5'-position of ON-10, the pairs of pyrene are not capable of establishing the close contact required or excimer formation.

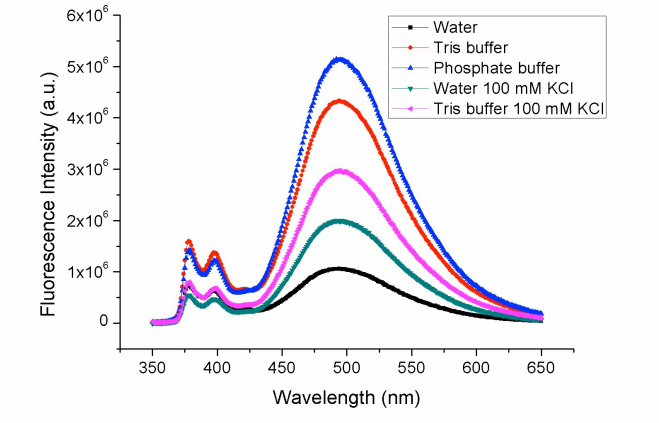


Figure 7: Bis-pyrene excimer fluorescence spectra of ON-13 (0.5 µM concentration) under different conditions. Water (Milli-Q), 10 mM Tris-HCl buffer at pH 7.4, 10 mM potassium phosphate buffer containing 100 mM potassium chloride at pH 7.4, Milli-Q water containing 100 mM potassium chloride and 10 mM Tris-HCl buffer containing 100 mM potassium chloride. Samples were incubated overnight at room temperature and excited at wavelength 340 nm.

In order to verify the formation of the quadruplex by excimer formation, we investigated the fluorescence properties of ON-13 (Table 3), which contains pyrene moieties at the 5' and 3'-ends. The ON-13 sequence gives rise to a strong excimer fluorescence band around 494 nm, which demonstrates that Q-aTNA is composed of antiparallel strands arrangement (Fig. 7). In this case four pyrenes are located at each end of the G-quadruplex and they can pairwise interact with the nearest neighbour to form an excimer. Moreover, less intense excimer fluorescence was observed in 10 mM tris buffer at pH 7.4 without potassium salt (Fig. 7). When ON-13 was examined in Milli-Q water (no added salt), a weak excimer effect was observed. Furthermore, addition of potassium chloride salt in water and tris buffer improved the excimer fluorescence effect (Fig. 7) where the ratio of excimer to monomer fluorescence intensity (I_{ex}/I_m) was equivalent in phosphate buffer. This data suggest that four strands of ON-13 are oriented in antiparallel direction, which favours excimer formation.

In the previous studies G-Quadruplex DNA has been used as a sensor for the potassium ions.[10] We therefore, investigated the effect of potassium chloride on ON-13. The ON-13 was dissolved in Milli-Q water and different amount of potassium chloride was added (ESI, Figure S5.16). The ratio I_{ex}/I_m increased with increasing concentration potassium chloride, which revealed the sensitivity of ON-13 from 0 mM to 5 mM concentration of potassium chloride (ESI, Figure S5.17). Unlike DNA, RNA the aTNA structures turned out to form G-quadruplexes in the absence of potassium and other salts, which is remarkable.

In conclusion, we have synthesized (L)-aTNA based G-Quadruplexes. UV-melting, CD, EMSA and fluorescence studies showed that G-rich aTNA can fold as intermolecular and intramolecular G-quadruplex structures depending on the sequence. Thermal denaturation studies demonstrated that intramolecular Q-aTNA is more stable than intermolecular Q-aTNA. Importantly, aTNA (ON-3) folds into the intramolecular G-quadruplex in Milli-Q water, while DNA (ON-4) was observed unfolded under this condition. Furthermore, these acyclic backbones have reported to be very stable against nucleases.[2,3a] These studies may provide a new platform, where (L)-aTNA can find its applications for the development of nanodevices or nanostructures.

The authors acknowledge financial support from the Danish National Research Foundation (DNRF 81).

Notes and references

1 (a) L. Zhang, A. Peritz and E. Meggers, *J. Am. Chem. Soc.*, 2005, **127**, 4174-4175; (b) H. Asanuma, T. Toda, K. Murayama, X. Liang and H. Kashida, *J. Am. Chem. Soc.*, 2010, **132**, 14702-14703; (c) V. Kumar, K. R. Gore, P. I. Pradeepkumar and V. Kesavan, *Org. & Biomol. Chem.*, 2013, **11**, 5853-5865; (d) P. Li, J. Sun, M. Su, X. Yang and X. Tang, *Org. & Biomol. Chem.*, 2014, **12**, 2263-2272; (e) V. Kumar, V. Kesavan and K. V. Gothelf, *Org. & Biomol. Chem.*, 2015, **13**, 2366-2374; (f) K. Murayama, H. Kashida and H. Asanuma, *Chem. Commun.*, 2015, **51**, 6500-6503.
2 (a) Y. Kamiya, J. Takai, H. Ito, K. Murayama, H. Kashida and H. Asanuma, *ChemBioChem*, 2014, **15**, 2549-2555; (b) A. Alagia,

M. Terrazas and R. Eritja, *Molecules*, 2014, **19**, 17872; (d) A. Alagia, M. Terrazas and R. Eritja, Molecules, 2015, **20**, 7602.
3 (a) H. Asanuma, M. Akahane, R. Niwa, H. Kashida and Y. Kamiya, *Angew. Chem. Int. Ed*., 2015, **54**, 4315-4319; (b) K. Murayama, Y. Kamiya, H. Kashida and H. Asanuma, *ChemBioChem*, 2015, 16, 1298-1301.
4 V. Kumar and V. Kesavan, *RSC Adv*., 2013, **3**, 19330-19340.
5 (a) P. Alberti, A. Bourdoncle, B. Sacca, L. Lacroix, J.-L. Mergny, *Org. Biomol. Chem*., 2006, **4**, 3383-3391; (b) J. Choi, T. Majima, *Chem. Soc. Rev*., 2011, **40**, 5893-5909; (c) Y. Krishnan, F. C. Simmel, *Angew. Chem. Int. Ed*., 2011, **50**, 3124-3156; (d) Wang, F.; Liu, X.; Willner, I. *Angew. Chem. Int. Ed.* 2015, 54, 1098.
6 (a) J.-L. Mergny, A.-T. Phan and L. Lacroix, *FEBS Letters*, 1998, **435**, 74-78S; (b) Burge, G. N. Parkinson, P. Hazel, A. K. Todd and S. Neidle, Nucleic Acids Res., 2006, **34**, 5402-5415; (c) J. L. Huppert, *Chem. Soc. Rev*., 2008, **37**, 1375-1384; (d) B. G. Kim, Y. L. Shek and T. V. Chalikian, *Biophys. Chem*., 2013, **184**, 95-100; (e) A. Guédin, J. Gros, P. Alberti and J.-L. Mergny, *Nucleic Acids Res*., 2010, **38**, 7858-7868.
7 (a) B. Datta, C. Schmitt and B. A. Armitage, *J. Am. Chem. Soc*., 2003, **125**, 4111-4118; (b) Y. Krishnan-Ghosh, E. Stephens and S. Balasubramanian, *J. Am. Chem. Soc.*, 2004, **126**, 5944-5945; (c) B. Datta, M. E. Bier, S. Roy and B. A. Armitage, *J. Am. Chem. Soc*., 2005, **127**, 4199-4207; (d) A. Paul, P. Sengupta, Y. Krishnan and S. Ladame, *Chem. Eur. J*., 2008, **14**, 8682-8689; (e) S. Lusvarghi, C. T. Murphy, S. Roy, F. A. Tanious, I. Sacui, W. D. Wilson, D. H. Ly and B. A. Armitage, *J. Am. Chem. Soc*., 2009, **131**, 18415-18424; (f) N. K. Sharma and K. N. Ganesh, *Org. & Biomol. Chem*., 2011, **9**, 725-729.
8 (a) S.-P. Liu, S. H. Weisbrod, Z. Tang, A. Marx, E. Scheer and A. Erbe, *Angew. Chem. Int. Ed.*, 2010, **49**, 3313-3316; (b) G. I. Livshits, A. Stern, D. Rotem, N. Borovok, G. Eidelshtein, A. Migliore, E. Penzo, S. J. Wind, R. Di Felice, S. S. Skourtis, J. C. Cuevas, L. Gurevich, A. B. Kotlyar and D. Porath, *Nat. Nanotechnol.*, 2014, **9**, 1040-1046; (c) L. Xiang, J. L. Palma, C. Bruot, V. Mujica, M. A. Ratner and N. Tao, *Nat. Chem.*, 2015, **7**, 221-226.
9 S. Paramasivan, I. Rujan and P. H. Bolton, *Methods*, 2007, **43**, 324-331.
10 (a) D. Lewis, *Bioconjugate Chem.*, 2007, **18**, 1213-1217; (b) S. Nagatoishi, T. Nojima, B. Juskowiak and S. Takenaka, *Angew. Chem. Int. Ed*., 2005, 44, 5067-5070.
11 (a) Y. Saito, Y. Miyauchi, A. Okamoto and I. Saito, *Chem. Commun.*, 2004, 1704-1705; (b) J. N. Wilson, Y. Cho, S. Tan, A. Cuppoletti and E. T. Kool, *ChemBioChem*, 2008, **9**, 279-285.