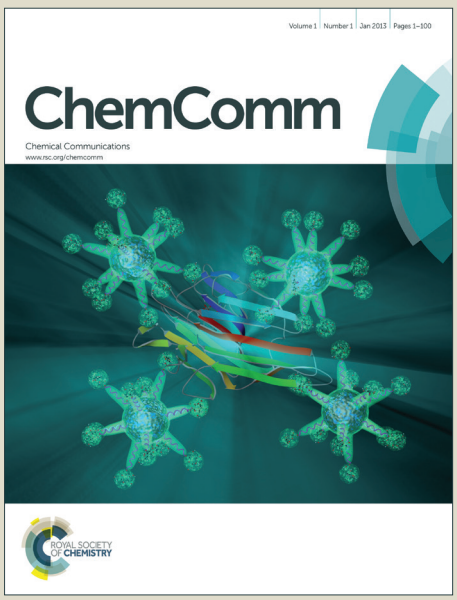
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

RSCPublishing

COMMUNICATION

Single Thiazole Orange Forms Exciplex in DNA i-motif

Cite this: DOI: 10.1039/xoxxoooooox

Baochang Xu, Xiangyang Wu, Edwin K. L. Yeow and Fangwei Shao*

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

Fluorescent exciplex of thiazole orange (TO) is formed in a single-dye conjugated DNA i-motif. The exciplex fluorescence exhibits large Stoke shift, high quantum yield, robust response to pH oscillation and little structural disturbance to DNA quadruplex, which can be used to monitor the folding of high-order DNA structure.

Nucleic acid conjugated fluorescent excimers/exciplexes exhibit unique absorption and emission spectra often with unusual large Stokes shifts and hence have great potentials in the application of biosensing and bioimaging.[1] Excimer/exciplex based probes allow the free and target-bound probes to be analyzed under different fluorescent channels as a result of the emission wavelength shifts and/or color changes between fluorochrome monomer and excimer/exciplex. Thus, the detection sensitivity could be significantly improved due to the little background interference between the distinct statuses of biomolecules.[2] Notably, the majority of excimer/exciplex systems on nucleic acids are assembled by incorporating multiple chromophores to DNA scaffolds.[3] Fluorochromes with fused aromatic rings, such as pyrenes and perylenes, are commonly used to form excimers/exciplexes in DNA conjugates for sensing of nucleic acids, proteins and small molecules.[4] However the excitation wavelength falls in high energy UV regions and limits the applications of these systems in cellular bioimaging. Whereas, upon DNA hybridization, interstranded thiazole orange (TO) dimer can exhibit an excimer fluorescence with excitation in visible region and is more suitable for biological relevant devices and protocols.[5] Though as a quencher-free system, these excimer/exciplex systems can be constructed by as few as only one type of fluorochrome, the synthetic difficulties and high preparation cost of incorporating multiple dye molecules to DNA strands cannot be ignored. More importantly, the folding pathways and structures of nucleic acids could be significantly disturbed in the multiple-dye excimer/ exciplex systems, which may deprive the excimers/ exciplexes the abilities to probe the high order structures of DNA and nucleic acid-protein interactions. Exciplex systems with single dye molecule, whereas, would be highly desirable due to the minimum disturbance on the intrinsic properties of nucleic acids, while the merits of the exciplex emission are remained. Exciplex emission between pyrene and guanine in DNA scaffolds has been observed, though the small bathochromicity and UV incident wavelength of the exciplex emission are not ideal.[6]

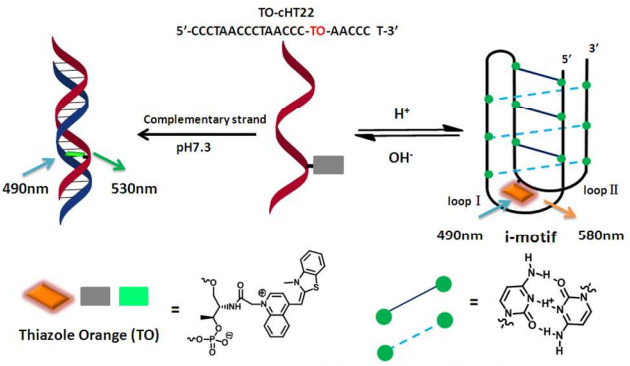


Figure 1. TO-cHT22 forms TO exciplex in i-motif and TO monomer in duplex, respectively. **TO-cHT22** is shown as a red ribbon (*middle*) along with the sequence. Orange diamond and gray or green rectangles represent tethered TO (structure at *right top*) in exciplex, quenched and monomer fluorescent states, respectively. In i-motif, cytosines are shown as green balls. Structure of hemi-protonated cytosine-cytosine base pairs are shown at *right bottom*.

DNA quadruplexes folding into defined three dimensional structures with noncanonical base spatial orientations could be an ideal scaffold to form excimer/exciplex via dye-dye or dye-DNA π-stacking.[6b,7] I-motif is a DNA quadruplex structure formed by cytosine-rich sequences under acidic pH.[8] In i-motif, DNA tetraplex is stabilized by hemiprotonated cytosine-cytosine (CH^+-C) pairs and three lateral loops (Figure 1). The fact that i-motif folding sequences are widely present in oncogenic regions and human telomeric DNA suggests that the formation of i-motif may be associated with the regulation of oncogene expression at the transcription level.[9] Besides, i-motif structures have also been applied as building blocks in the assembly of biosensors, nanoclusters, hydrogels and nanodevices, because of their rapid and reversible response to the variation of pH.[10] The unique spatial orientations of dual loop region in i-motif supply a defined scaffold to form fluorescent exciplex between dye molecule and nucleobases. Herein, we report an example of fluorescent exciplex formed by

single thiazole orange (TO) covalently conjugating to DNA i-motif. Upon exciting with visible light, the single TO molecule emits orange-color exciplex fluorescence in i-motif structure and green emission as a TO monomer in duplex DNA.

A single TO molecule is incorporated to telomeric sequence **TO-cHT22** (shown in Figure 1) via solid phase DNA synthesis[11] to substitute a thymine (T_{16}) and is purified by HPLC (as shown in Figure S1 and sequence in Table S1). UV spectrum of **TO-cHT22** shows characteristic absorption of both DNA at 260 nm and TO at 513 nm, respectively (Figure S2). D-threoninol is used to covalently tether TO to DNA backbone through phosphodiester bond. The acyclic linkage here bestows good flexibility on TO, which allows optimal stacking between the dye molecule and nucleic bases, and diminishes the disturbance of base surrogate to the folding structure of i-motif.[12]

Under acidic pH, the CD spectrum of **TO-cHT22** features a positive peak at 286 nm and a negative peak around 255 nm (Figure S3). The fact that the CD spectrum of **TO-cHT22** at pH 5.0 superimposes on that of the wild type telomeric C-rich sequence, **cHT22**, indicates that negligible disturbance to the formation and pH sensitivity of i-motif is induced by incorporating one TO molecule to the dual-lateral loop region in the tetraplex i-motif. At neutral pH, **TO-cHT22** exhibits characteristic CD spectra of a random coil or B-form helix in the absence or presence of the complementary G-rich strands, **HT22**, respectively (Figure S3).

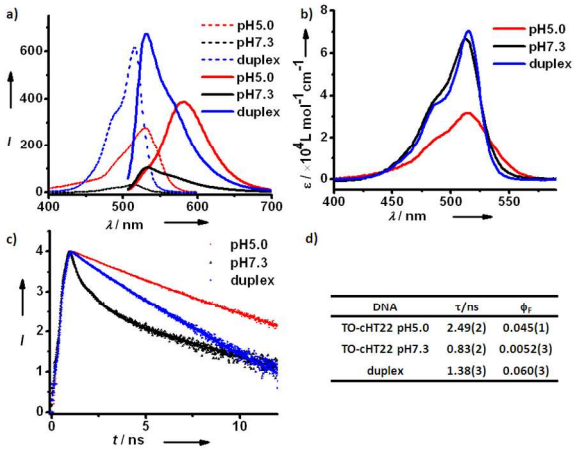


Figure 2. Optical spectra and parameters of **TO-cHT22**. a). Fluorescence emission (solid) and excitation (dot) spectra; b). UV-vis absorption spectra; c). Time-dependent fluorescence decay spectra; d). Lifetimes (τ) and quantum yields (ϕ_F) of **TO-cHT22** in i-motif structure at pH 5.0 (red), random coil (blue) and duplex (black, **TO-cHT22** + **HT22**) at pH 7.3.

Upon folding into i-motif at pH 5.0, the fluorescence emission of **TO-cHT22** exhibits a band around 580 nm and a Stokes shift of 90 nm is achieved (Figure 2a). The intense fluorescent signal at 580 nm obtains a quantum yield of 0.045 and is comparable to that of TO in duplex (0.060), which the emission band is peaked at 530 nm. Once the solution is neutralized to pH 7.3, **TO-cHT22** emission is significantly quenched to a quantum yield as low as 0.0052 and exhibits the same band at 530 nm as TO in the duplex DNA (Figure 2d). The fluorescent peak of **TO-cHT22** in i-motif is unambiguously distinguished from the typical green emission of TO monomer with Stokes shift of 50 nm. The similar bathochromic phenomenon is also observed in the fluorescent excitation spectra (Figure 2a). The maximum of excitation spectrum of **TO-cHT22** at pH 5.0 exhibits a red shift of approximately 15 nm comparing to that of **TO-cHT22** in duplex and in random coil at pH7.3 (at 515 nm). Both emission and excitation spectra of **TO-cHT22** indicate that TO forms a new excitation state in i-motif structure, which can be tentatively assigned to an exciplex state, since TO excimer formed by two diagonal TO in duplex DNA exhibits an identical emission band at 580 nm.[5]

The UV absorption spectra of **TO-cHT22** have shown similar features to the corresponding excitation spectra. **TO-cHT22** exhibits an absorption band with maximum at approximately 513 nm in random coil and duplex form (Figure 2b). After forming i-motif structure at pH 5.0, the absorption intensity around 513 nm decreases to half of that under neutral pH. The hypochromicity in TO absorbance indicates a strong hydrophobic stacking between thiazole orange and the vicinal nucleobases in i-motif. The attenuation in UV absorption of fluorochromes has also been observed in several DNA-conjugated excimer/exciplex systems[6b,7,13] and is ascribed to the π-electron stacking between dye molecules or between dye and nucleobases. Furthermore, the absorption band of **TO-cHT22** motif extends to longer wavelength up to 575 nm. The concurrent enhancement at yellow-green visible region (525-575 nm) in both absorption and excitation spectra demonstrates once more that a new exciplex state of the cyanine dye exists in i-motif structure, presumably due to the unique binding/stacking of TO molecule with neighboring nucleobases, which are absent from duplex or random coil DNA with less rigid local structures.

The time-resolved fluorescence decay is performed to further verify the formation of exciplex (Figure 2c). The fluorescence lifetime (τ) of TO in i-motif structure is observed as 2.49 ns, and is significantly prolonged comparing to that of TO in random coil state (τ = 0.83 ns) and in duplex (τ = 1.38 ns), which are similar to the reported values of TO monomer (Figure 2d).[5, 14] τ of TO exciplex formed in i-motif is slightly smaller than that of the dual-TO excimer in duplex DNA.[5] This could be due to the fact that the exciplex in current system is formed by the π-stacking between single fluorochrome and neighboring nucleobases, instead of stacking between fluorochrome homo- or heterodimers. The electronic stacking between TO and nucleobases may not be as optimal as that between dual TO molecules when they intercalate into base pairs[5] and hence slightly smaller τ of TO exciplex is observed in current system. Based on above observations, a TO exciplex is unambiguously formed when **TO-cHT22** folds into i-motif structure. To our knowledge, exciplex formed in single TO conjugated DNA structures has not been reported yet to date.

In order to better elucidate the origin of exciplex formation in i-motif structure, we used thymine to replace the four adenines (A5, 6, 17, 18) in dual loop region of i-motif one at each time and prepared four **TO-cHT22** analogues (**5T**, **6T**, **17T** and **18T**, sequences in Table S1). TO exciplex is likely formed by the π-electron coupling or stacking between the dye molecule and nucleobases in close vicinity. According to the NMR structure of i-motif **cHT22** (PDB: 1ELN),[15] the four adenines (A5, 6, 17, 18) in the dual loop region have the accessibility to stack and electronic couple with TO molecule. Thymine is chosen as the substituent for adenine, because thymine as a pyrimidine, can minimize the electron stacking and coupling that TO receives from adenine, due to the smaller aromatic area and lower efficiency in energy/electron transfer than adenine,[16] while the original folding of **TO-cHT22** has minimum disturbance. If the adenine under questioning is indispensable to the exciplex formation, the exciplex fluorescence of the corresponding thymine substituted sequence will be significantly compromised, if not eliminated completely. Under acidic pH 5.0 and neutral pH 7.3, CD spectra of the four thymine substituted **TO-DNA** (nT, n = 5, 6, 17, 18) feature similar to those of **TO-cHT22** in i-motif structure and random coil, respectively (Figure S4), which indicates that negligible

perturbation to the formation of i-motif is induced by replacing one adenine with thymine at the dual-lateral loop region.

UV absorption spectra of nT strands have shown similar features as those of **TO-cHT22** (Figure S5). A hypochromicity at TO absorption band is observed when the sequences fold into i-motif from random coil state. Noteworthy, a red shift featured in exciplex formation is also observed in the UV absorption spectrum of **17T** at pH 5.0, though the phenomenon is less pronounced than that of **TO-cHT22**.

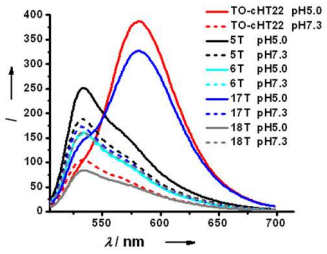


Figure 3. Fluorescence emission spectra of T-substituted **TO-cHT22** analogues in i-motif state at pH 5.0 (solid line) and in random coil state at pH7.3 (dash).

The fluorescent emission spectra of thymine substituted **TO-cHT22** reveal the roles of adenines (A5, 6, 17, 18) in the formation of TO exciplex. As shown in Figure 3, **17T**, the sequence which the adenine adjacent to TO molecule is replaced by a thymine, maintains the exciplex emission, though the intensity is attenuated slightly. The fluorescent emission at 580 nm, hypochromicity and bathochromicity in UV absorption spectrum of **17T** demonstrate that the adenine (A17), though adjacent to TO, is likely not the nucleobase to form exciplex with the dye molecule directly, but rather contributes merely as an ancillary component to assist the dye and other vicinal nucleobases to enter a well-defined spatial arrangements for exciplex formation. Surprisingly, substituting either one of other three adenines (A5, A6 and A18) in the dual loop region induces a complete elimination of exciplex emission, which implies these three adenines play essential roles in either lock TO conformation or π-stacking with TO to form exciplex, if not both.

The monomer emission spectra allow us to investigate the structural and electronic effects of A5, A6 and A18 to the exciplex formation. Upon folding into i-motif at pH 5.0, **5T**, instead of exciplex, exhibits monomer fluorescence of TO, and shows 30% of intensity increase comparing to that of its random coil state at pH 7.3. Monomer emission is restored in the replacement of A5, indicating TO molecule can retain good stacking with nucleobases in dual loop region, likely in a fashion similar to TO in duplex DNA, and achieve monomer emission. Hence, A5 may contribute mainly to the π-electron stacking/coupling with TO to form exciplex state, since in the absence of A5, only exciplex breaks down, but not the torsional rigidity of the methine center in TO molecule induced by the base stacking. Whereas, the T-substitutions of A6 and A18 eliminate the exciplex fluorescence entirely. More interestingly, even no apparent enhancement of the monomer fluorescence is observed upon the formation of i-motif. The significant attenuation of exciplex and monomer fluorescence in **6T** and **18T** indicates that A6 and A18 could be essential to establish the optimum structural arrangement between TO and the adenines in dual loops, and hence contribute to stabilizing the exciplex conformation. Although the current data cannot rule out the possibility that the interactions between terminal base pair, CH^+-C, and TO molecule may contribute to the TO exciplex formation, the facts that adenines have significantly higher aromatic stacking ability than pyrimidines (A > T, C)[16], and that the fluorescence of **TO-cHT22** is significantly altered by replacing the loop adenines, imply that A5, A6 and A18 in the dual loop region play essential roles in either structural and/or π-stacking interactions to form exciplex with TO molecule within the i-motif scaffold.

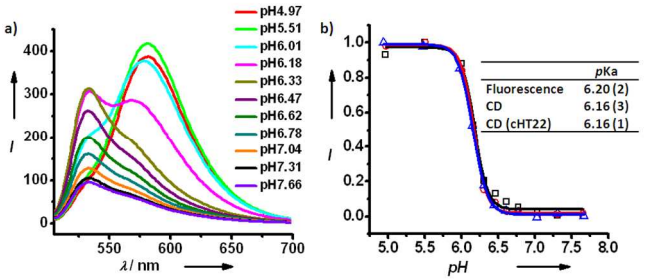


Figure 4. pH-dependent fluorescent spectra (a) of TO exciplex in **TO-cHT22** yield the dissociation constant (*p*Ka) (b) of i-motif. Fluorescence intensity at 625nm (□), CD intensity at 286nm of **TO-cHT22** (○) and **cHT22** (Δ) under different pHs are fitted to sigmoidal curves to obtain *p*Ka (*inset table*).

The exciplex fluorescence of **TO-cHT22** can be used to monitor the formation and dissociation of i-motif. Upon titration from acidic to basic pH, exciplex emission around 580 nm dissolves and monomer emission around 530 nm emerges. The CD spectra in Figure S6 indicate that, like control DNA, **cHT22**, **TO-cHT22** undergoes a collapse of i-motif along with the corresponding pH neutralization. Fluorescent signal at 625 nm of TO exciplex features a sigmoidal transition according to the variation of pH, which is similar to the transition of the characteristic CD signals of i-motif at 286 nm (Figure 4). Dissociation constants (*p*Ka) of i-motif **TO-cHT22** are derived from the exciplex fluorescence as 6.20 and from CD signals as 6.16, respectively. Similar *p*Ka value is obtained from the unmodified i-motif **cHT22** via CD spectroscopy. The variations of *p*Ka values either between the modified and unmodified i-motif or between the two experimental methods are all within the uncertainty of the data. Hence, the fluorescence intensity of exciplex can loyally reflect the unfolding of i-motif as well as the conventional CD signals. **TO-cHT22** with only single TO modification has the same sensitivity on pH dependence as the unmodified sequence, indicating that the single base surrogate introduces negligible disturbance to the folding and unfolding of i-motif. The unique exciplex affords **TO-cHT22** a capability to monitor the folding and unfolding pathway of human telomeric i-motif via an orange emission, which is well separated from the monomer fluorescence, commonly used to track the hybridization of duplex DNA.

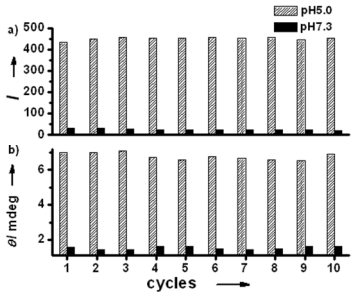


Figure 5. Cycles of exciplex formation and dissociation upon pH titration between 5.0 and 7.3 observed by fluorescent emission and CD spectra.

The robustness of the exciplex system is demonstrated by monitoring both the exciplex fluorescence and CD intensity of i-motif structure upon cycling pH between 5.0 and 7.3. In Figure 5, the fluorescence intensity at 580 nm peaks at pH 5.0 and minimizes at pH 7.3, which is in good concurrence with the association and dissociation of i-motif indicated by the cyclical oscillations of CD intensity at 286 nm. Over 10 cycles, no

attenuation on the intensity of exciplex emission is observed and full quenching of monomer emission is achieved at pH 7.3 to maintain a low background noise level. Meanwhile, the CD intensity of i-motif at 286 nm also keeps the amplitude between i-motif and random coil state after 10 cycles. The synchronization of exciplex emission and i-motif formation confirms the structural dependence of exciplex formation and shows the potential of monitoring i-motif folding pathways by exciplex emission. Evidently, the accumulating salt concentration from neutralization exhibits little interference to the efficiency of exciplex system, which endows the system a great potential for fluorescent nucleic acid probes and pH-driven nanodevices.

Using DNA i-motif as a scaffold, a fluorescent exciplex is constructed between nucleobases and single TO surrogate. The i-motif dependent formation of exciplex is robust and exhibits large Stokes shift of nearly 90 nm and decent quantum yield near 600 nm. Thymine substituted TO-DNA strands shows that four adenines in the dual loop region contribute to the exciplex formation by either defining the spatial conformation and/or to π-electron stacking with the dye molecule. This result supplies a new insight to fabricate exciplex system with single dye surrogate and nucleobases in high-order structures of nucleic acids. The mono-modification on DNA strands shows little disturbance to the structure of nucleic acids and hence bestows the exciplex system promising potentials to investigate the folding pathway and kinetics of DNA quadruplexes, and to design fluorogenic probes for the study of protein-nucleic acids interactions.

The authors would like to thank Professor H. Asanuma from Nagoya University, Japan for the advices on the syntheses of TO modified DNA sequence. We also thank the financial support of Nanyang Assistant Professor Fellowship (M4080531) and Singapore MOE AcRF Tier 2 grant (M4020163).

Notes and references

[a] *Division of Chemistry and Biological Chemistry, Nanyang Technological University, 21 Nanyang Link, Singapore 637371. Fax: (65)6791-1961; Tel: (65) 6592-2511; E-mail: fwshao@ntu.edu.sg.*

† Electronic Supplementary Information (ESI) available: Experimental procedures and characterization for the preparation of TO modified DNA sequences and spectroscopic measurements, UV-vis and CD spectra. See DOI: 10.1039/b000000x/

1. Y. N. Teo and E. T. Kool, *Chem. Rev.*, 2012, **112**, 4221-4245.
2. (*a*) A. A. Marti, S. Jockusch, N. Stevens, J. Y. Ju and N. J. Turro, *Acc. Chem. Res.*, 2007, **40**, 402-409; (*b*) C. M. Wang, C. C. Wu, Y. Chen, Y. L. Song, W. H. Tan and C. J. Yang, *Curr. Org. Chem.*, 2011, **15**, 465-476.
3. (*a*) F. D. Lewis, Y. F. Zhang and R. L. Letsinger, *J. Am. Chem. Soc.*, 1997, **119**, 5451-5452; (*b*) A. Mahara, R. Iwase, T. Sakamoto, K. Yamana, T. Yamaoka and A. Murakami, *Angew. Chem., Int. Ed.*, 2002, **41**, 3648-3650; (*c*) K. Fujimoto, H. Shimizu and M. Inouye, *J. Org. Chem.*, 2004, **69**, 3271-3275; (*d*) C. J. Yang, S. Jockusch, M. Vicens, N. J. Turro and W. H. Tan, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 17278-17283; (*e*) R. Haner, S. M. Biner, S. M. Langenegger, T. Meng and V. L. Malinovskii, *Angew. Chem., Int. Ed.*, 2010, **49**, 1227-1230; (*f*) H. Kashida, N. Kondo, K. Sekiguchi and H. Asanuma, *Chem. Commun.*, 2011, **47**, 6404-6406.
4. (a) A. Okamoto, T. Ichiba and I. Saito, *J. Am. Chem. Soc.*, 2004, **126**, 8364-8365; (*b*) K. Yamana, T. Iwai, Y. Ohtani, S. Sato, M. Nakamura and H. Nakano, *Bioconjugate Chem.*, 2002, **13**, 1266-1273;
5. (a) S. Berndl and H. A. Wagenknecht, *Angew. Chem., Int. Ed.*, 2009, **48**, 2418-2421. (b) F. Hovelmann, I. Gaspar, A. Ephrussi and O. Seitz *J. Am. Chem. Soc.*, 2013, **135**, 19025-32. (c) S. Kummer, A. Knoll, E. Socher, L. Bethge, A. Herrmann and O. Seitz, *Angew. Chem.* 2011, **123**,1972-1975; *Angew. Chem. Int. Ed.* 2011, **50**,1931-1934.
6. (*a*) T. Kawai, M. Ikegami and T. Arai, *Chem. Commun.*, 2004, 824-825; (*b*) Y. J. Seo, I. J. Lee, J. W. Yi and B. H. Kim, *Chem. Commun.*, 2007, 2817-2819.
7. (*a*) I. J. Lee and B. H. Kim, *Chem. Commun.*, 2012, **48**, 2074-2076; (*b*) J. W. Park, Y. J. Seo and B. H. Kim, *Chem. Commun.*, 2014, **50**, 52-54; (*c*) S. Nagatoishi, T. Nojima, B. Juskowiak and S. Takenaka, *Angew. Chem., Int. Ed.*, 2005, **44**, 5067-5070.
8. M. Gueron and J. L. Leroy, *Curr. Opin. Struct. Biol.*, 2000, **10**, 326-331.
9. (*a*) T. A. Brooks, S. Kendrick and L. Hurley, *FEBS J.*, 2010, **277**, 3459-3469; (*b*) J. A. Brazier, A. Shah and G. D. Brown, *Chem. Commun.*, 2012, **48**, 10739-10741.
10. (*a*) H. Liu and D. S. Liu, *Chem. Commun.*, 2009, 2625-2636; (*b*) C. Teller and I. Willner, *Curr. Opin. Biotechnol.*, 2010, **21**, 376-391; (*c*) J. Choi and T. Majima, *Chem. Soc. Rev.*, 2011, **40**, 5893-5909.
11. (*a*) H. Asanuma, X. Liang, H. Nishioka, D. Matsunaga, M. Liu and M. Komiyama, *Nat. Protoc.*, 2007, **2**, 203-212; (*b*) Y. Hara, T. Fujii, H. Kashida, K. Sekiguchi, X. G. Liang, K. Niwa, T. Takase, Y. Yoshida and H. Asanuma, *Angew. Chem., Int. Ed.*, 2010, **49**, 5502-5506.
12. H. Kashida, X. G. Liang and H. Asanuma, *Curr. Org. Chem.*, 2009, **13**, 1065-1084.
13. (*a*) I. J. Lee, J. W. Yi and B. H. Kim, *Chem. Commun.*, 2009, 5383-5385; (*b*) P. P. Neelakandan, Z. Z. Pan, M. Hariharan, Y. Zheng, H. Weissman, B. Rybtchinski and F. D. Lewis, *J. Am. Chem. Soc.*, 2010, **132**, 15808-15813.
14. T. L. Netzel, K. Nafisi, M. Zhao, J. R. Lenhard and I. Johnson, *J. Phys. Chem.*, 1995, **99**, 17936-17947.
15. A. T. Phan, M. Gueron and J. L. Leroy, *J. Mol. Biol.*, 2000, **299**, 123-144.
16. K. M. Guckian, B. A. Schweitzer, R. X. F. Ren, C. J. Sheils, D. C. Tahmassebi and E. T. Kool, *J. Am. Chem. Soc.*, 2000, **122**, 2213-2222.

