Bioorganic & Medicinal Chemistry Letters 25 (2015) 333–336

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Polymerase-mediated high-density incorporation of amphiphilic functionalities into DNA: Enhancement of nuclease resistance and stability in human serum

Hiroto Fujita [a], Kohsuke Nakajima [a], Yuuya Kasahara [a,b], Hiroaki Ozaki [a], Masayasu Kuwahara [a,*]

[a] Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin-cho, Kiryu, Gunma 376-8515, Japan
[b] National Institute of Biomedical Innovation (NIBIO), 7-6-8 Asagi, Saito, Ibaraki, Osaka 567-0085, Japan

ARTICLE INFO

Article history:
Received 1 July 2014
Revised 11 November 2014
Accepted 13 November 2014
Available online 20 November 2014

Keywords:
Amphiphilic functionality
Biostability
DNA polymerase
High-density incorporation
Modified nucleoside triphosphate

ABSTRACT

Modified oligodeoxyribonucleotides (mdODNs) bearing multiple copies of an amphiphilic functional group were enzymatically synthesized by simultaneous incorporation of base-modified 5′-triphosphate analogs of 2′-deoxyguanosine (dG^{am}TP), 2′-deoxyuridine (dU^{am}TP), 2′-deoxyadenosine (dA^{am}TP), and 2′-deoxycytosine (dC^{am}TP). The amphiphilic functionality, that is, (*E*)-38,53-dioxo-2,5,8,11,14,17,20,23,26,29,32,35-dodecaoxa-39,52-diazapentapentacont-54-en-55-yl group, consists of the water soluble dodeca(ethylene glycol) chain and the hydrophobic dodecyl chain. An enzymatically synthesized ODN, composed of a 20-mer 5′-terminal segment containing 2′-*O*,4′-C-methylene-bridged/linked bicyclic ribonucleotide (B/L nucleotide) and a 12-mer 3′-terminal segment containing the nucleobase-modified analogs, exhibits very high resistance against phosphodiesterase I and is stable in human serum for a longer period when compared with ODN, where the 12-mer 3′-terminal segment contains unmodified nucleotides.

© 2014 Elsevier Ltd. All rights reserved.

Interest in functionalizing DNA with aliphatic groups has recently grown owing to its potential in a wide-ranging field of applications, which include drug delivery,[1] cell adhesion,[2] bioimaging,[3] development of membrane-bound receptors,[4] liposome tagging,[5] and for the generation of functional thin films.[6] Such mdODNs are generally prepared using solid-phase phosphoramidite-based ODN synthesis. There are no reports on the enzymatic synthesis of mdODNs containing long aliphatic chains.[7] A factor hampering the enzymatic syntheses of such mdODNs is the lack of water-solubility of the modified monomer triphosphates and polymerized mdODN products that contain long aliphatic chains. Hence we first synthesized 7-substituted 7-deazapurine nucleoside triphosphates dG^{am}TP and dA^{am}TP, and 5-substituted pyrimidine nucleoside triphosphates dU^{am}TP and dC^{am}TP (Fig. 1). Although the structure of linker arm in base-modified functionalities can greatly affect their incorporation efficiency,[8] the (*E*)-3-oxoprop-1-en-1-yl linker was adopted based on the results of our previous studies.[9]

Furthermore, we evaluated if these analogs were treated as substrates in the polymerization reaction catalyzed by *KOD Dash* DNA polymerase (also called *KOD XL* DNA polymerase),[10] which is recognized for its ability to synthesize mdODNs. As shown in Figures S1–S5, inclusion of a single modified nucleotide (G^{am}, U^{am}, A^{am}, or C^{am}) in the reaction mixture for primer extension (PEX) readily affords an mdODN that migrates much slower than the corresponding ODN containing all natural nucleotides in gel electrophoresis. Multiple/successive incorporations of multiple modified nucleotides are known to be inefficient when compared with incorporation of a single modified nucleotide. To evaluate if all four modified nucleotides can be incorporated in a single experiment, PEX experiments were conducted using the four modified triphosphates (dN^{am}TPs) instead of the four natural ones (dNTPs). As shown in Figure 2, replacement of two, three, or four natural dNTPs with the corresponding dN^{am}TPs in PEX experiments provided full-length extended products (lanes 5–7) in a manner similar to that produced on the replacement of dGTP alone with dG^{am}TP (lane 4). Similar to the gel behavior of single nucleotide-incorporated ODNs, band-shifts were also observed for ODNs containing multiple modified nucleotides; the product ODNs migrated slower as the number of modified nucleotides replaced in PEX reaction increased. Thus, as observed by the migration of ODNs in lane 7, multiple copies of the amphiphilic functionalities were successfully incorporated by PEX to extend a primer by 12 modified nucleotides.

* Corresponding author. Tel.: +81 277 30 1222; fax: +81 277 30 1222.
E-mail address: mkuwa@gunma-u.ac.jp (M. Kuwahara).

http://dx.doi.org/10.1016/j.bmcl.2014.11.037
0960-894X/© 2014 Elsevier Ltd. All rights reserved.

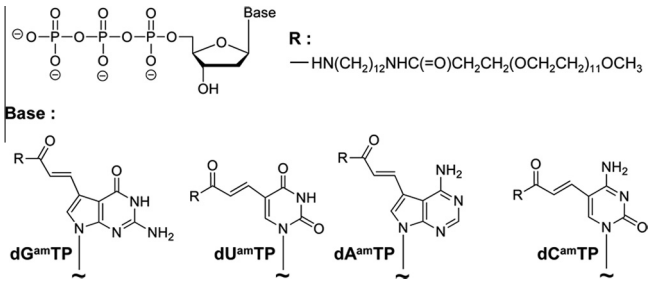


Figure 1. Chemical structures of the modified monomer triphosphates.

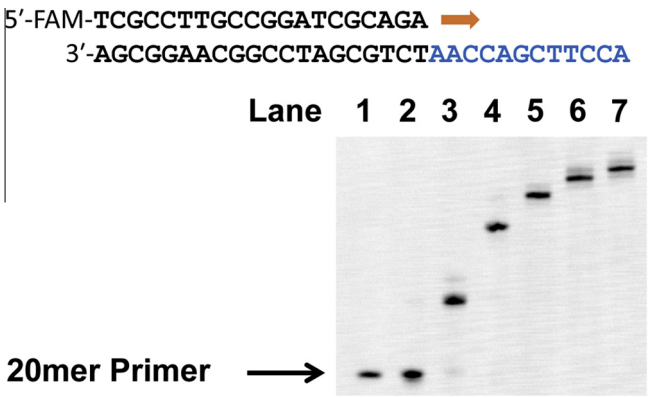


Figure 2. Gel analysis of the products of PEX experiments performed with the four modified monomer triphosphates. Lane 1: primer only; lane 2: reaction mixture without any monomer triphosphates; lane 3: reaction containing all four natural dNTPs; lane 4: reaction containing dG^{am}TP, TTP, dATP, and dCTP; lane 5: reaction containing dG^{am}TP, dU^{am}TP, dATP, and dCTP; lane 6: reaction containing dG^{am}TP, dU^{am}TP, dA^{am}TP, and dCTP; and lane 7: reaction containing four modified dN^{am}TPs.

Polymerase chain reaction (PCR) can exponentially amplify a double-stranded DNA (dsDNA) template. In addition, PCR can readily produce long dsDNA [more than several hundred base pairs (bp)]; it is a task that remains a challenge for chemical syntheses. However, in contrast to PEX, PCR-generated strands must act as templates during the course of amplification. Therefore, PCR can afford low yields of mdODN, even when the modified triphosphates are good substrates in PEX. Therefore, we evaluated the ability of dN^{am}TPs to participate in PCR using the plasmid template pUC18 with appropriate forward and reverse primers. When PCR is conducted with dNTPs, these primers provide an ODN of 500-bp length. An attempt with simultaneous inclusion of all four dN^{am}TPs during PCR did not yield the desired product. Addition of natural dNTPs into the reaction mixture can sometimes yield the PCR product. Reaction mixtures containing substrates at the dN^{am}TPs:dNTPs molar ratio of 5:95, 10:90, 15:85, and 20:80 provided PCR products, whereas a mixture with the dN^{am}TPs:dNTPs molar ratio

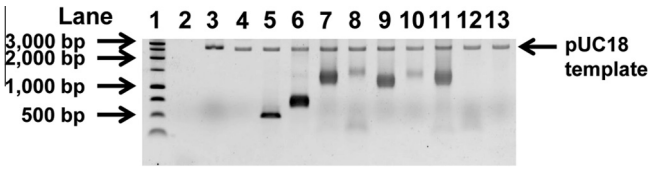


Figure 3. Gel analysis of the products of PCR experiments performed with the four modified monomer triphosphates. Lane 1: 1-kb DNA ladder marker; lane 2: primer only; lane 3: template only; lane 4: reaction in the absence of monomer triphosphates; and lane 5: reaction containing the four natural dNTPs. PCR performed in the presence of both natural and modified substrates, with the dN^{am}TPs:dNTPs molar ratio of lane 6: 5:95; lane 7: 10:90; lane 8: 15:85; lane 9: 15:85 in 5% acetonitrile; lane 10: 20:80; lane 11: 20:80 in 5% acetonitrile; lane 12: 25:75; and lane 13: 25:75 in 5% acetonitrile.

of 25:75 did not (Figs. 3 and S6). Slower migrating bands of ODNs containing modified nucleotides (N^{am}) are observed (lanes 6–11), however, mobilities of ODN products formed when the reaction mixture containing 10% or more of dN^{am}TP were comparable (lanes 7, 8, and 10). These results indicate that the ratio of N^{am} incorporation is saturated when the dN^{am}TP content is approximately 10%; further increase in the content of dN^{am}TPs does not increase the ratio of incorporation. Inclusion of acetonitrile (5%, v/v) in the reaction mixture leads to an apparent improvement in the product yield; however, it is likely to diminish the amount of N^{am}s incorporated because the product bands migrate at a rate that is faster compared with that observed for products isolated from acetonitrile-free reaction mixtures (lanes 9 and 11). On using the agarose gel electrophoresis-purified PCR-amplified mdODN as a template in a new PCR reaction in the presence of four dNTPs, natural dsODN of 500-bp length was reproduced (Fig. S7). Thus, a natural dsODN that was further amplified using a PCR-amplified mdODN as a template could preserve a sequence identical to that in the original template (used in the initial PCR), which was identified by sequencing (Figs. S8 and S9).

As shown in Figure 4, mdODN shows high thermostability (T_m = 72 °C), even in sodium phosphate buffer with low salt concentration (ca. 17 mM Na^+). This value is comparable with that of the corresponding natural dsODN (T_m = 76 °C), indicating that mdODN forms a stable duplex in aqueous buffer at body temperature (37 °C). Thus, these results indicate that multiple amphiphilic functionalities can readily be incorporated into long dsDNA by including dN^{am}TPs in PCR. Furthermore, the functionalities present in mdODN can be easily 'taken off and added on' while retaining sequence information and double helical conformation through PCR-mediated replications.

We also investigated the effects of a single insertion of the amphiphilic functionality on short DNA duplex thermostability (Fig. S10). This showed that the amphiphilic functionality reduced duplex thermostabilities in sodium phosphate buffers with both low (approximately 17 mM Na^+) and normal (approximately 116 mM Na^+) salt concentrations, but did not significantly affect duplex formation.

We then investigated the effects of modification on biostability, which is a critical feature of modified nucleic acids when considered for biological and medical applications. Chemical and structural modifications introduced in the phosphate or sugar moiety, such as phosphorothioates and B/L nucleotides, significantly enhance biostability of ODN.[11] Modifications in nucleobase, which are located away from the phosphodiester bonds, are not likely to impart resistance against nucleases. However, flexible bulky

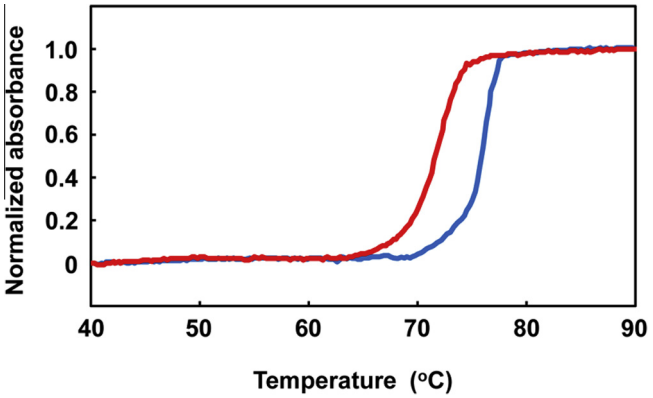


Figure 4. UV melting curves of 500-bp natural ODN (blue) and 500-bp modified ODN (red) in 10 mM phosphate buffer (pH 7.0) containing ca. 17 mM Na^+, monitored at 260 nm. The modified ODN was synthesized by PCR using a mixture of modified and natural dNTPs (dN^{am}TPs:dNTPs molar ratio of 10:90).

hydrophobic chain-containing functionalities on the several nucleobases can form a large hydrophobic domain around ODN strands, which can prevent the nucleases from accessing the phosphodiester backbone. To verify this hypothesis, we enzymatically synthesized two B/L nucleotide-containing ODNs. While the 5′-terminal initial sequence of both ODNs are same (i.e., 5′-FAM-TC*G* C*C*T TG*C* CG*G* AT*C* G*C*A GA), the 3′-terminal of one has a 12-mer nucleotide tail containing nucleobase-modified nucleotides (ODN^{bmd}: 5′-FAM-TC*G* C*C*T TG*C* CG*G* AT*C* G*C*A GAU^{am} U^{am}G^{am}G^{am} U^{am}C^{am}G^{am} A^{am}A^{am}G^{am} G^{am}U^{am}-3′) while the other has a 12-mer nucleotide containing natural nucleotides (ODN^{ntl}: 5′-FAM-TC*G* C*C*T TG*C* CG*G* AT*C* G*C*A GAT TGG TCG AAG GT-3′). Here FAM represents 6-carboxyfluorescein, and *A*, *G*, *C*, and *T* represent B/L nucleotides bearing adenine, guanine, 5-methylcytosine, and thymine base, respectively. As shown in Figure 5, ODN^{ntl} is rapidly degraded by phosphodiesterase I (*Crotalus adamanteus* venom; SVPD) and no intact full-length ODN^{ntl} is observed after 30 min. Owing to the resistance of B/L nucleotides to nucleases, a 15-mer undegraded fragment remained as the major product after 120 min of incubation. Under identical reaction conditions, >95% of ODN^{bmd} remained intact after 120 min. A faint band representing a 19-mer fragment was observed (Fig. 5B); this fragment was obtained on enzymatic hydrolysis of the 3′,5′-phosphodiester linkage between the two successive natural nucleotides, G-19 and A-20. The band intensity of the 19-mer fragment gradually increased as the reaction progressed; however, no bands representing fragments that were 20–31 nucleotides long were observed on the gel. These results indicate that the 19-mer fragment is generated by endonuclease activity, not by the 3′ to 5′exonuclease activity that is prominently associated with SVPD. Thus, excellent resistance to a high concentration of the nuclease SVPD (10 μU/μL) was demonstrated by a 12-mer ODN containing 12 successive N^{am}s at 37 °C.

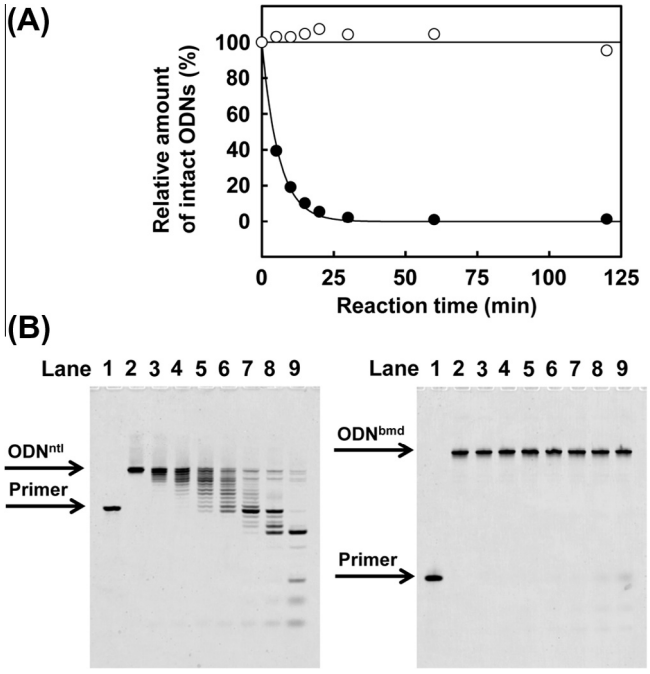


Figure 5. (A) Time-dependent degradation of ODNs catalyzed by phosphodiesterase I; ODN^{ntl} (closed circles) and ODN^{bmd} (open circles). The reaction time (in min) is shown on the *x*-axis and the relative amount of intact ODNs (%) is shown on the *y*-axis. Initial quantities of the substrate were assumed to be at 100% in each reaction mixture. (B) Degradation of ODN^{ntl} (left) and ODN^{bmd} (right) on incubation with phosphodiesterase I analyzed by gel electrophoresis. Primer only (lane 1). The reaction mixtures at 0, 5, 10, 15, 20, 30, 60, and 120 min were loaded in wells corresponding to lanes 2, 3, 4, 5, 6, 7, 8, and 9, respectively.

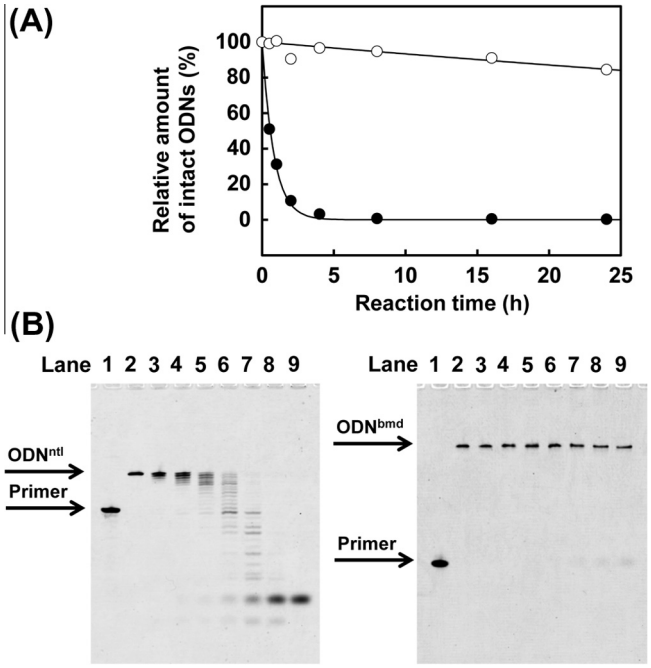


Figure 6. (A) Time-dependent degradation of ODNs in 80% v/v human serum; ODN^{ntl} (closed circles) and ODN^{bmd} (open circles). The reaction time (in hour) is shown on the *x*-axis and the relative amount of intact ODNs (%) is shown on the *y*-axis. Initial quantities of the ODNs were assumed to be at 100% in each reaction mixture. (B) Degradation of ODN^{ntl} (left) and ODN^{bmd} (right) in 80% v/v human serum analyzed by gel electrophoresis. Primer only (lane 1). The reaction mixtures at 0, 0.5, 1, 2, 4, 8, 16, and 24 h were loaded in wells corresponding to lanes 2, 3, 4, 5, 6, 7, 8, and 9, respectively.

Stability of ODN^{bmd} in 80% human serum (v/v) was assessed by comparing the stability of ODN^{ntl} under similar conditions. As shown in Figure 6A, the half-life of the intact ODN^{bmd} is estimated to be 100 h, while that of ODN^{ntl} is 0.57 h. The 19-mer fragment resulting from ODN^{bmd} is faintly observed in the reactions incubated for 8, 16, and 24 h (lanes 7–9 in the picture on the right); other fragments (20–31-mers) were not observed on the gel (Fig. 6B). In addition, the 19-mer fragment is the major product of ODN^{ntl} degradation after 4 h of incubation (lane 6 in the picture on the left); however, this fragment is gradually and completely degraded over the duration of the assay (lanes 7–9 in the picture on the left). Thus, ODN^{bmd} is found to be more stable than ODN^{ntl} toward degradation by both exo- and endonucleases present in human serum at 37 °C, which is a result that is consistent with those of the analysis using SVPD.

In conclusion, by tethering the dodeca(ethylene glycol) group to the amino terminus of the modified nucleobase, multiple hydrophobic aliphatic chains (C_{12}) are densely incorporated in a section of the 3′-terminus of a short ODN by PEX. Extension of the primer by incorporating 12 modified nucleotides markedly elevates nuclease resistance and biostability, which is demonstrated in the assays with SVPD and human serum. Furthermore, PCR successfully amplified a long dsODN (500 bp) when the content of the modified triphosohates (dN^{am}TPs) in natural triphosphates (dNTPs) was approximately 10%. The modified ODN PCR product formed a stable duplex; however, the inclusion of multiple modified bases decreased the duplex thermostability in a neutral buffer with low salt concentration, presumably because perturbation of the hydration network surrounding the duplex by the incorporated hydrophobic aliphatic chains. These results indicate that the folding/unfolding of DNA or formation/deformation of nanostructures can be controlled by external stimuli or changes in milieu, such

as light irradiation, redox, pH, and ligand concentration. We are currently developing amphiphilic triphosphate analogs containing various trigger functionalities in the base moiety. Furthermore, selection of modified DNA aptamers containing such analogs is being attempted.

Acknowledgments

This study was partly supported by a Grant for Adaptable and Seamless Technology Transfer Program through Target-driven R & D, No. AS2525029M, from Japan Science and Technology Agency (JST), and by a Grant-in-Aid for Scientific Research (C), No. 25350962, from the Japan Society for the Promotion of Science (JSPS).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.11.037.

References and notes

1. (a) Shi, J.; Xiao, Z.; Votruba, A. R.; Vilos, C.; Farokhzad, O. C. *Angew. Chem., Int. Ed.* **2011**, *50*, 7027; (b) Alemdaroglu, F. E.; Alemdaroglu, N. C.; Langguth, P.; Herrmann, A. *Adv. Mater.* **2008**, *20*, 899.
2. (a) Selden, N. S.; Todhunter, M. E.; Jee, N. Y.; Liu, J. S.; Broaders, K. E.; Gartner, Z. J. *J. Am. Chem. Soc.* **2012**, *134*, 765; (b) Takemoto, N.; Teramura, Y.; Iwata, H. *Bioconjugate Chem.* **2011**, *22*, 673.
3. (a) Loew, M.; Springer, R.; Scolari, S.; Altenbrunn, F.; Seitz, O.; Liebscher, J.; Huster, D.; Herrmann, A.; Arbuzova, A. *J. Am. Chem. Soc.* **2010**, *132*, 16066; (b) Seferos, D. S.; Giljohann, D. A.; Hill, H. D.; Prigodich, A. E.; Mirkin, C. A. *J. Am. Chem. Soc.* **2007**, *129*, 15477.
4. Teramura, Y.; Minh, L. N.; Kawamoto, T.; Iwata, H. *Bioconjugate Chem.* **2010**, *21*, 792.
5. (a) Dhule, S. S.; Penfornis, P.; He, J.; Harris, M. R.; Terry, T.; John, V.; Pochampally, R. *Mol. Pharm.* **2014**, *11*, 417; (b) Chan, Y. H.; van Lengerich, B.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 979.
6. Keller, R.; Kwak, M.; de Vries, J. W.; Sawaryn, C.; Wang, J.; Anaya, M.; Mullen, K.; Butt, H. J.; Herrmann, A.; Berger, R. *Colloids Surf. B Biointerfaces* **2013**, *111C*, 439.
7. (a) Stewart, G.; Hira, T.; Higgins, A.; Smith, C. P.; McLaughlin, J. T. *Am. J. Physiol. Cell Physiol.* **2006**, *290*, C785; (b) Yoshina-Ishii, C.; Miller, G. P.; Kraft, M. L.; Kool, E. T.; Boxer, S. G. *J. Am. Chem. Soc.* **2005**, *127*, 1356; (c) Briscoe, C. P.; Tadayyon, M.; Andrews, J. L.; Benson, W. G.; Chambers, J. K.; Eilert, M. M.; Ellis, C.; Elshourbagy, N. A.; Goetz, A. S.; Minnick, D. T.; Murdock, P. R.; Sauls, H. R., Jr.; Shabon, U.; Spinage, L. D.; Strum, J. C.; Szekeres, P. G.; Tan, K. B.; Way, J. M.; Ignar, D. M.; Wilson, S.; Muir, A. I. *J. Biol. Chem.* **2003**, *278*, 11303.
8. (a) Kuwahara, M.; Nagashima, J.; Hasegawa, M.; Tamura, T.; Kitagata, R.; Hanawa, K.; Hososhima, S.; Kasamatsu, T.; Ozaki, H.; Sawai, H. *Nucleic Acids Res.* **2006**, *34*, 5383; (b) Jager, S.; Rasched, G.; Kornreich-Leshem, H.; Engeser, M.; Thum, O.; Famulok, M. *J. Am. Chem. Soc.* **2005**, *127*, 15071; (c) Tasara, T.; Angerer, B.; Damond, M.; Winter, H.; Dorhofer, S.; Hubscher, U.; Amacker, M. *Nucleic Acids Res.* **2003**, *31*, 2636; (d) Thum, O.; Jager, S.; Famulok, M. *Angew. Chem., Int. Ed.* **2001**, *40*, 3990; (d) Lee, S. E.; Sidorov, A.; Gourlain, T.; Mignet, N.; Thorpe, S. J.; Brazier, J. A.; Dickman, M. J.; Hornby, D. P.; Grasby, J. A.; Williams, D. M. *Nucleic Acids Res.* **2001**, *29*, 1565.
9. (a) Imaizumi, Y.; Kasahara, Y.; Fujita, H.; Kitadume, S.; Ozaki, H.; Endoh, T.; Kuwahara, M.; Sugimoto, N. *J. Am. Chem. Soc.* **2013**, *135*, 9412; (b) Kasahara, Y.; Irisawa, Y.; Fujita, H.; Yahara, A.; Ozaki, H.; Obika, S.; Kuwahara, M. *Anal. Chem.* **2013**, *85*, 4961; (c) Kajiyama, T.; Kuwahara, M.; Goto, M.; Kambara, H. *Anal. Biochem.* **2011**, *416*, 8.
10. (a) Vanikova, Z.; Hocek, M. *Angew. Chem., Int. Ed.* **2014**, *53*, 6734; (b) Kasahara, Y.; Irisawa, Y.; Ozaki, H.; Obika, S.; Kuwahara, M. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 1288; (c) Goubet, A.; Chardon, A.; Kumar, P.; Sharma, P. K.; Veedu, R. N. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 761; (d) Kuwahara, M.; Takano, Y.; Kasahara, Y.; Nara, H.; Ozaki, H.; Sawai, H.; Sugiyama, A.; Obika, S. *Molecules* **2010**, *15*, 8229; (e) Vaught, J. D.; Bock, C.; Carter, J.; Fitzwater, T.; Otis, M.; Schneider, D.; Rolando, J.; Waugh, S.; Wilcox, S. K.; Eaton, B. E. *J. Am. Chem. Soc.* **2010**, *132*, 4141; (f) Veedu, R. N.; Vester, B.; Wengel, J. *Org. Biomol. Chem.* **2009**, *7*, 1404; (g) Loakes, D.; Gallego, J.; Pinheiro, V. B.; Kool, E. T.; Holliger, P. *J. Am. Chem. Soc.* **2009**, *131*, 14827; (h) Kuwahara, M.; Obika, S.; Nagashima, J.; Ohta, Y.; Suto, Y.; Ozaki, H.; Sawai, H.; Imanishi, T. *Nucleic Acids Res.* **2008**, *36*, 4257; (i) Ohmichi, T.; Kuwahara, M.; Sasaki, N.; Hasegawa, M.; Nishikata, T.; Sawai, H.; Sugimoto, N. *Angew. Chem., Int. Ed.* **2005**, *44*, 6682; (j) Sawai, H.; Ozaki, A. N.; Satoh, F.; Ohbayashi, T.; Masud, M. M.; Ozaki, H. *Chem. Commun.* **2001**, 2604.
11. (a) Nielsen, P. E. *Artif. DNA PNA XNA* **2012**, *3*, 1; (b) Selvam, C.; Thomas, S.; Abbott, J.; Kennedy, S. D.; Rozners, E. *Angew. Chem., Int. Ed.* **2011**, *50*, 2068; (c) Campbell, M. A.; Wengel, J. *Chem. Soc. Rev.* **2011**, *40*, 5680; (d) Rahman, S. M.; Seki, S.; Obika, S.; Yoshikawa, H.; Miyashita, K.; Imanishi, T. *J. Am. Chem. Soc.* **2008**, *130*, 4886; (e) Rahman, S. M.; Seki, S.; Obika, S.; Haitani, S.; Miyashita, K.; Imanishi, T. *Angew. Chem., Int. Ed.* **2007**, *46*, 4306.