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■ Oligonucleotides

2-Allyl- and Propargylamino-dATPs for Site-Specific Enzymatic Introduction of a Single Modification in the Minor Groove of DNA

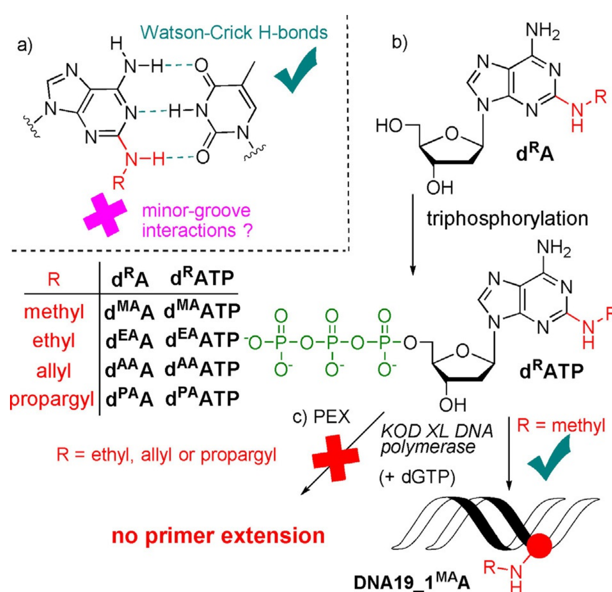
Ján Matyašovský,[a, b] Radek Pohl,[a] and Michal Hocek*[a, b]

Abstract: A series of 2-alkylamino-2′-deoxyadenosine triphosphates (dATP) was prepared and found to be substrates for the Therminator DNA polymerase, which incorporated only one modified nucleotide into the primer. Using a template encoding for two consecutive adenines, conditions were found for incorporation of either one or two modified nucleotides. In all cases, addition of a mixture of natural dNTPs led to primer extension resulting in site-specific single modification of DNA in the minor groove. The allylamino-substituted DNA was used for the thiol-ene addition, whereas the propargylamino-DNA for the CuAAC click reaction was used to label the DNA with a fluorescent dye in the minor groove. The approach was used to construct FRET probes for detection of oligonucleotides.

Functionalized oligonucleotides (ONs) or DNA are frequently used in chemical biology, diagnostics, or material science.[1] Modifications at the nucleobase are typically attached to position 5 of pyrimidines or to position 7 of 7-deazapurines to point out into the major groove of DNA. The corresponding "major groove" substituted 2′-deoxyribonucleoside triphosphates (dNTPs) are good substrates for DNA polymerases in enzymatic synthesis of the modified DNA.[2–4] Polymerase construction of ONs or DNA containing a single modified base at a specific single position is more difficult but even that has been achieved[5] through a single-nucleotide incorporation (if the modified nucleobase is followed by a different base) followed by primer extension (PEX) or through a PEX using a one-nucleotide longer template, magnetoseparation, reannealing with long template and another PEX (in case the modified base is followed by the same non-modified base).

Enzymatic synthesis of DNA modified in the minor groove is yet underdeveloped and has mostly been reported with some sugar-modified derivatives.[6] We have recently found[7] that some dATP derivatives bearing small substituents (Me, vinyl, ethynyl) at position 2 are still substrates for some DNA polymerases suitable for enzymatic synthesis of minor-groove modified ONs or DNA and the vinyl or ethynyl groups can be used for postsynthetic labeling through thiol-ene or CuAAC reactions.[7] Shortly thereafter, Gowda et al.[8] reported that N^2-alkylguanine dNTPs were not substrates for most DNA polymerases except for pol κ and they used this enzyme for their incorporation to DNA. The minor groove sites of the nucleobases are crucial for Watson–Crick base-pairing as well as for interactions with polymerase important for extension of the chain.[9] We envisaged that some 2-alkylamino-adenine derivatives might still form the H-bonds with T and partially inhibit the extension through disruption of the minor-groove interactions (Scheme 1 a) and, therefore, be candidates for single-nucleotide extension.

We prepared a small series of four 2-alkylamino-dATP derivatives (**d^{R}ATPs**) by triphosphorylation[10] of the corresponding 2′-deoxy-ribonucleosides (**d^{R}As**, Scheme 1 b; for details of their synthesis, see the Supporting Information). They were then



Scheme 1. (a) Watson–Crick base pairing of 2-alkylaminoadenine with T; (b) Synthesis of **d^{R}ATPs** and attempted PEX incorporation to DNA.

[a] J. Matyašovský, Dr. R. Pohl, Prof. Dr. M. Hocek
Institute of Organic Chemistry and Biochemistry
Czech Academy of Sciences
Flemingovo nam. 2, 16610 Prague 6 (Czech Republic)
E-mail: hocek@uochb.cas.cz
Homepage: http://www.uochb.cas.cz/hocekgroup

[b] J. Matyašovský, Prof. Dr. M. Hocek
Department of Organic Chemistry, Faculty of Science
Charles University in Prague, Hlavova 8, 12843 Prague 2 (Czech Republic)

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tested as substrates for DNA polymerases in PEX experiments in presence of KOD XL or Vent(*exo*-) polymerase. In all cases, only the 2-methylamino derivative **d^{MA}ATP** worked as substrate, whereas all the bulkier ones did not give any primer extension (Scheme 1 c, Figure 1 a). Then, we tested the **d^{R}ATP**s in single-nucleotide extension of a 15-mer primer using Therminator DNA polymerase and a 16-mer template. To our delight, all of them were substrates for this enzyme and gave the extended primer (Figure 1 b).

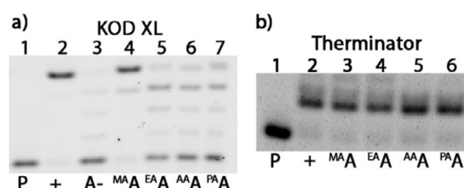
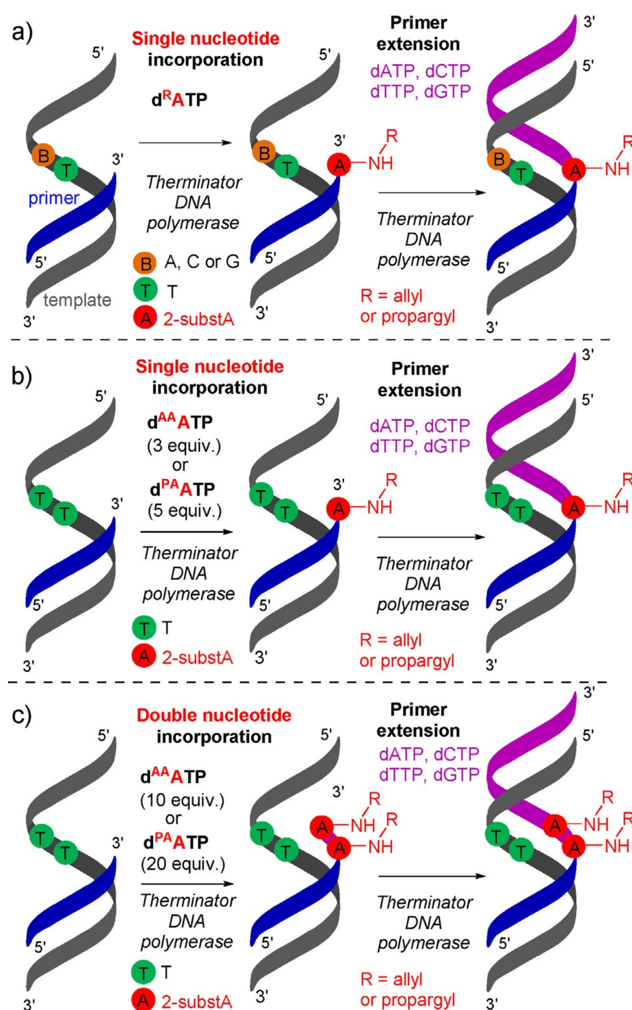


Figure 1. Denaturing PAGE of PEX experiments using Prim15-FAM and all modified **d^{R}ATP**s (a) in the presence of *KOD XL* DNA polymerase and Temp19 and (b) in the presence of Therminator DNA polymerase and Temp16. Lanes 1, P: primer; lanes 2, +: products of PEX with natural dNTPs; lane (a) 3, A−: product of PEX with dGTP only; lanes (a) 4–7 and (b) 3–6, ^{R}A: products of PEX with (a) dGTP and functionalized **d^{R}ATP** and (b) with **d^{R}ATP** only.

Therefore, we then tested the single-nucleotide extension (SNE) using longer 31-mer templates with clickable allylamino and propargylamino dATP derivatives **d^{AA}ATP** and **d^{PA}ATP** (Scheme 2). For completion of the SNE we used 50–100 equiv of the **d^{R}ATP**s on analytical scale or 3–20 equiv on a semi-preparative scale (concentrated solutions); see Tables S2 and S3 in the Supporting Information. In all cases, we observed clean products of SNE without significant formation of longer ONs (Figure 2 a,b). Interestingly, if the template contained two consecutive thymines encoding for extension of primer with two adenines, we were able to incorporate just one (Scheme 2 b, Figure 2 c) or two consecutive **d^{R}A** nucleotides (Scheme 2 c, Figure 2 d) depending on the excess of the **d^{R}ATP**. In all cases, the addition of a large excess of a mixture of all four natural dNTPs led to PEX giving full length DNA containing one or two **d^{R}A** nucleotides incorporated in the $n+1$ (and $n+2$) position(s) on the primer (Scheme 2, Figure 2 a–d, lanes 9 and 10).[11] Similar to our previous paper,[7] we performed post-synthetic modification of the allylamino-linked DNA through thiol-ene reaction with coumarine-methylthiol (CM-SH) and of the propargylamino-linked DNA through CuAAC reaction with Cy5-azide (Scheme 3). Structures and identity of all modified ONs were confirmed by MALDI analysis of samples prepared by SNE and PEX (and post-synthetic transformation) using biotinylated template followed by magnetoseparation[12] on streptavidin magnetic beads (Table S4 in the Supporting Information). We also studied the influence of the minor-groove modifications on the stability of the DNA duplex. The melting temperatures were in most cases similar (±0.6 °C) to natural DNA, indicating that the additional H-bond compensates for the steric effect of the modification (Table S5 in the Supporting Information).



Scheme 2. Single (a,b) or double (c) nucleotide extension followed by PEX.

After the SNE, because of some remaining **d^{R}ATP**s in the reaction mixture, it was important to rule out that they become partially incorporated into the other part of the ON during the PEX in the presence of excess of natural dNTPs. Therefore, we ran PEX reactions using 31-mer template in the presence of all four natural dNTPs and in the absence or in the presence of 5–20 equiv of **d^{AA}ATP** or **d^{PA}ATP**. The PEX products were then treated with CM-SH or Cy5-N_3 in the same way as above and emission spectra of the purified products were recorded. Figure S11 in the Supporting Information shows that the fluorescence of the PEX products formed in the presence of **d^{R}ATP**s was negligible (the same level as fluorescence of natural DNA treated with the same dyes) confirming that there was no significant incorporation of **d^{R}A** nucleotides in the PEX in competition with dATP.

To show the application potential of this novel approach for site-specific minor-groove modification of ONs, we tested it on construction of FRET probes using a combination of Cy3 and Cy5.[13,14] First, we prepared two complementary 31-mer ONs, one bearing a Cy3 and one Cy5, through SNE with **d^{PA}ATP**, followed by PEX, magnetoseparation, and CuAAC reaction with the corresponding Cy3-N_3 or Cy5-N_3. By annealing them and ir-

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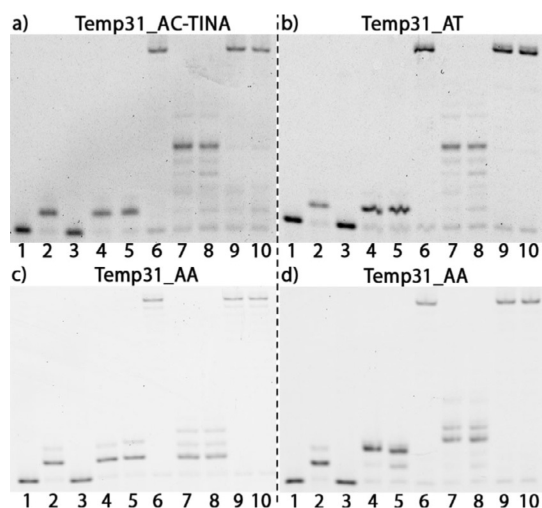
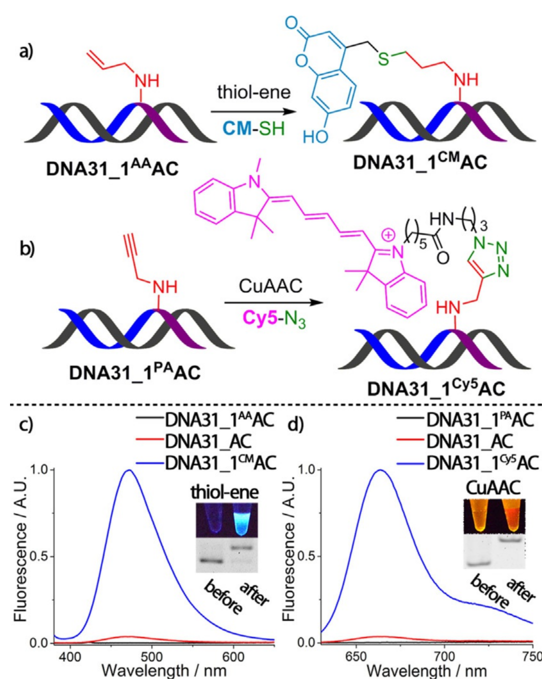


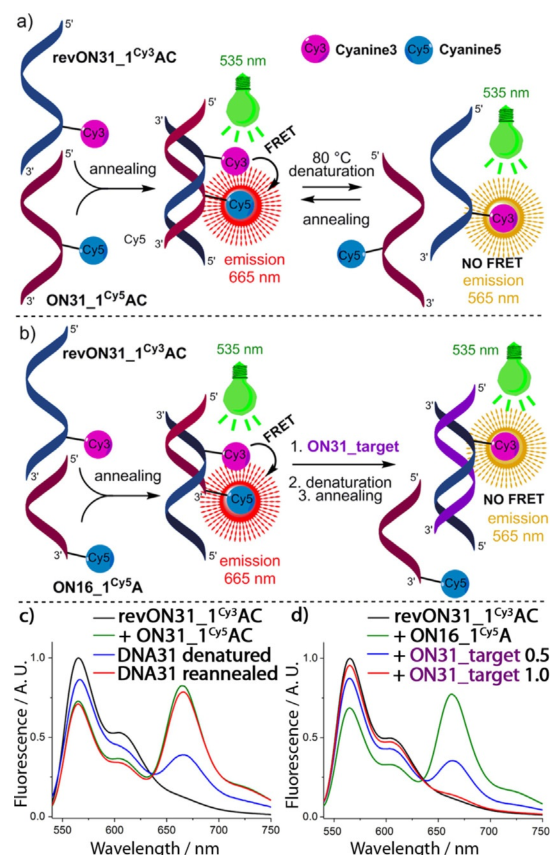
Figure 2. Denaturing PAGE of enzymatic synthesis of ONs bearing (a–c) a single modification, or (d) two subsequent modifications in the minor groove using Therminator DNA polymerase, Prim15-FAM and (a) Temp31_AC-TINA, (b) Temp31_AT, and (c,d) Temp31_AA. Lanes 1: primer; lanes 2: 16-mer ON standard; lanes 3: negative control for SNE (no dNTPs present); lanes 4,5: SNE with $\mathbf{d^{AA}ATP}$ or $\mathbf{d^{PA}ATP}$; lanes 6: positive control–product of PEX with natural dNTPs; lanes 7,8: PEX (in the presence of dGTP, dCTP, and dTTP; absence of dATP) after SNE with either $\mathbf{d^{AA}ATP}$ or $\mathbf{d^{PA}ATP}$; lanes 9,10: products of PEX (with all natural dNTPs present) after SNE with either $\mathbf{d^{AA}ATP}$ or $\mathbf{d^{PA}ATP}$.



Scheme 3. Post-synthetic modification of allylamino- (a) or propargylamino-linked (b) DNA with fluorescent thiol or azide and emission spectra of the products of the thiol-ene reaction with CM-SH (c) or CuAAC reaction with Cy5-N_3 (d).

radiation with 535 nm light, we observed FRET and emission at 665 nm. When the DNA was heated at 80 °C, partial denaturation occurred and the FRET was ceased resulting in emission at 565 nm only. By reannealing at ambient temperature, the FRET was restored (Scheme 4a,c). Then, we tested construction of a FRET probe for detection of an ON. By the same approach as above, we prepared a Cy3-linked 31-mer ON and a complementary Cy5-linked 16-mer ON. Their annealing formed the FRET probe, which displayed emission at 665 nm when irradiated at 535 nm. By addition of a 31-mer ON fully complementary to the Cy3-linked 31-mer probe, heating and cooling, we observed ceasing of the FRET and emission at 565 nm (Scheme 4 b,d).

In conclusion, we found that 2-alkylamino-dATP derivatives are poor substrates for DNA polymerases in PEX but one (or two) modified nucleotide(s) can be efficiently incorporated by the Therminator DNA polymerase in SNE. The SNE using either $\mathbf{d^{AA}ATP}$ or $\mathbf{d^{PA}ATP}$ followed by PEX with natural dNTPs can be used for construction of DNA bearing single modification at a specific single position in the minor groove. To the best of our knowledge, this is the first enzymatic method for site-specific single modification of the minor groove of DNA. Allylamino- or propargylamino-linked DNAs can be efficiently labeled through thiol-ene or CuAAC reactions with thiols or azides. When this method is applied using biotinylated template and magnetoseparation, single-stranded ONs bearing one modification can be prepared. Applicability of the approach has been demon-



Scheme 4. (a) FRET during the hybridization and denaturation of Cy3- and Cy5-modified complementary ONs; (b) construction of FRET probe for detection of an ON based on annealing of a Cy3-linked 31-mer ON with a Cy5-linked complementary 16-mer ON (showing FRET and emission at 665 nm) and its re-hybridization with target ON (exerting no FRET and emission at 565 nm); (c,d) the corresponding emission spectra.

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strated by construction of FRET probes for detection of ONs, but one can envisage many other applications, including so far unknown minor-groove cross-linking of DNA with proteins[15] or minor-groove caging of DNA for regulation of interactions with proteins.[16] Studies along these lines are under way.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

Keywords: DNA · fluorescent probes · nucleotides · oligonucleotides · polymerases

[1] *Modified Nucleic Acids, Nucleic Acids and Molecular Biology Series*, Vol. 31 (Eds.: K. Nakatani, Y. Tor), Springer, **2016**, pp. 1 – 276.
[2] Reviews: a) A. Hottin, A. Marx, *Acc. Chem. Res.* **2016**, *49*, 418 – 427; b) M. Hollenstein, *Molecules* **2012**, *17*, 13569 – 13591; c) M. Hocek, *J. Org. Chem.* **2014**, *79*, 9914 – 9921; d) M. Kuwahara, N. Sugimoto, *Molecules* **2010**, *15*, 5423 – 5444.
[3] a) S. Obeid, A. Baccaro, W. Welte, K. Diederichs, A. Marx, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 21327 – 21331; b) K. Bergen, A. L. Steck, S. Strütt, A. Baccaro, W. Welte, K. Diederichs, A. Marx, *J. Am. Chem. Soc.* **2012**, *134*, 11840 – 11843; c) P. Kielkowski, J. Fanfrlík, M. Hocek, *Angew. Chem. Int. Ed.* **2014**, *53*, 7552 – 7555; *Angew. Chem.* **2014**, *126*, 7682 – 7685; d) H. Cahová, A. Panattoni, P. Kielkowski, J. Fanfrlík, M. Hocek, *ACS Chem. Biol.* **2016**, *11*, 3165 – 3171; e) A. Hottin, K. Betz, K. Diederichs, A. Marx, *Chem. Eur. J.* **2017**, *23*, 2109 – 2118.
[4] Recent examples: a) M. Hollenstein, *Chem. Eur. J.* **2012**, *18*, 13320 – 13330; b) X. Ren, A. H. El-Sagheer, T. Brown, *Analyst* **2015**, *140*, 2671 – 2678; c) D. Dziuba, P. Jurkiewicz, M. Cebecauer, M. Hof, M. Hocek, *Angew. Chem. Int. Ed.* **2016**, *55*, 174 – 178; *Angew. Chem.* **2016**, *128*, 182 – 186; d) D. Dziuba, P. Pospíšil, J. Matyašovský, J. Brynda, D. Nachtigallová, L. Rulíšek, R. Pohl, M. Hof, M. Hocek, *Chem. Sci.* **2016**, *7*, 5775 – 5785; e) M. Welter, D. Verga, A. Marx, *Angew. Chem. Int. Ed.* **2016**, *55*, 10131 – 10135; *Angew. Chem.* **2016**, *128*, 10286 – 10290; f) M. Merkel, S. Arndt, D. Ploschik, G. B. Cserép, U. Wenge, P. Kele, H.-A. Wagenknecht, *J. Org. Chem.* **2016**, *81*, 7527 – 7538; g) H. Hoshino, Y. Kasahara, H. Fujita, M. Kuwahara, K. Morihiro, S.-I. Tsunoda, S. Obika, *Bioorg. Med. Chem. Lett.* **2016**, *26*, 530 – 533; h) M. Ortiz, A. M. Debela, M. Svobodova, S. Thorimbert, D. Lesage, R. B. Cole, B. Hasenknopf, C. K. O'Sullivan, *Chem. Eur. J.* **2017**, *23*, 10597 – 10603; i) M. Yamabe, K. Kaihatsu, Y. Ebara, *Bioconjugate Chem.* **2018**, *29*, 1490 – 1494.
[5] P. Ménová, H. Cahová, M. Plucnara, L. Havran, M. Fojta, M. Hocek, *Chem. Commun.* **2013**, *49*, 4652 – 4654.
[6] a) U. Wenge, T. Ehrenschwender, H. A. Wagenknecht, *Bioconjugate Chem.* **2013**, *24*, 301 – 304; b) L. H. Lauridsen, J. A. Rothnagel, R. N. Veedu, *ChemBioChem* **2012**, *13*, 19 – 25; c) M. Kuwahara, S. Obika, J. Nagashima, Y. Ohta, Y. Suto, H. Ozaki, H. Sawai, T. Imanishi, *Nucleic Acids Res.* **2008**, *36*, 4257 – 4265; d) T. Chen, N. Hongdilokkul, Z. Liu, R. Adhikary, S. S. Tsuen, F. E. Romesberg, *Nat. Chem.* **2016**, *8*, 556 – 562; e) A. Marx, M. P. MacWilliams, T. A. Bickle, U. Schwitter, B. Giese, *J. Am. Chem. Soc.* **1997**, *119*, 1131 – 1132.
[7] J. Matyašovský, P. Perlíková, V. Malnuit, R. Pohl, M. Hocek, *Angew. Chem. Int. Ed.* **2016**, *55*, 15856 – 15859; *Angew. Chem.* **2016**, *128*, 16088 – 16091.
[8] A. S. P. Gowda, M. Lee, T. E. Spratt, *Angew. Chem. Int. Ed.* **2017**, *56*, 2628 – 2631; *Angew. Chem.* **2017**, *129*, 2672 – 2675.
[9] S. Matsuda, A. M. Leconte, F. E. Romesberg, *J. Am. Chem. Soc.* **2007**, *129*, 5551 – 5557.
[10] T. Kovács, L. Ötvös, *Tetrahedron Lett.* **1988**, *29*, 4525 – 4528.
[11] In some cases, the PEX was conducted using a 31-nucleotide template modified with TINA at 3′-end to prevent non-templated incorporation: P. Güixens-Gallardo, M. Hocek, P. Perlíková, *Bioorg. Med. Chem. Lett.* **2016**, *26*, 288 – 291.
[12] P. Brázdilová, M. Vrábel, R. Pohl, H. Pivonková, L. Havran, M. Hocek, M. Fojta, *Chem. Eur. J.* **2007**, *13*, 9527 – 9533.
[13] Examples of DNA FRET probes using combination of Cy3 and Cy5: a) T. Ha, I. Rasnik, W. Cheng, H. P. Babcock, G. H. Gauss, T. M. Lohman, S. Chu, *Nature* **2002**, *419*, 638 – 641; b) C. R. Sabanayagam, J. S. Eid, A. Meller, *J. Chem. Phys.* **2005**, *123*, 224708.
[14] Other examples of nucleic acids FRET probes and related applications: a) Y. Xie, A. V. Dix, Y. Tor, *J. Am. Chem. Soc.* **2009**, *131*, 17605 – 17614; b) C. Holzhauser, H. Wagenknecht, *ChemBioChem* **2012**, *13*, 1136 – 1138; c) C. Holzhauser, M. M. Rubner, H. Wagenknecht, *Photochem. Photobiol. Sci.* **2013**, *12*, 722 – 724; d) H. Walter, J. Bauer, J. Steinmeyer, A. Kuzuya, C. M. Niemeyer, H. Wagenknecht, *Nano Lett.* **2017**, *17*, 2467 – 2472.
[15] Examples of DNA probes for cross-linking with proteins in major groove: a) M. Winnacker, S. Breeger, R. Strasser, T. Carell, *ChemBioChem* **2009**, *10*, 109 – 118; b) S. Wickramaratne, S. Mukherjee, P. W. Villalta, O. D. Schärer, N. Y. Tretyakova, *Bioconjugate Chem.* **2013**, *24*, 1496 – 1506; c) J. Dadová, P. Orság, R. Pohl, M. Brázdová, M. Fojta, M. Hocek, *Angew. Chem. Int. Ed.* **2013**, *52*, 10515 – 10518; *Angew. Chem.* **2013**, *125*, 10709 – 10712; d) P. Pande, S. Ji, S. Mukherjee, O. D. Schärer, N. Y. Tretyakova, A. K. Basu, *Chem. Res. Toxicol.* **2017**, *30*, 669 – 677.
[16] Examples of major-groove DNA caging and regulation of protein binding: a) P. Kielkowski, H. Macíčková-Cahová, R. Pohl, M. Hocek, *Angew. Chem. Int. Ed.* **2011**, *50*, 8727 – 8730; *Angew. Chem.* **2011**, *123*, 8886 – 8889; b) Z. Vaníková, M. Hocek, *Angew. Chem. Int. Ed.* **2014**, *53*, 6734 – 6737; *Angew. Chem.* **2014**, *126*, 6852 – 6855; c) M. Slavíčková, M. Janoušková, A. Šimonová, H. Cahová, M. Kambová, H. Šanderová, L. Krásný, M. Hocek, *Chem. Eur. J.* **2018**, *24*, 8311 – 8314.

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