"Clickable" LNA/DNA probes for fluorescence sensing of nucleic acids and autoimmune antibodies†

Anna S. Jørgensen, Pankaj Gupta, Jesper Wengel and I. Kira Astakhova*

Herein we describe fluorescent oligonucleotides prepared by click chemistry between novel alkyne-modified locked nucleic acid (LNA) strands and a series of fluorescent azides for homogeneous (all-in-solution) detection of nucleic acids and autoimmune antibodies.

Several autoimmune disorders are characterized by production of antibodies against single- and double-stranded DNA. If not diagnosed and treated early, the autoimmune conditions can lead to serious health deterioration and even mortality.† The sequence-specific autoimmune antibodies (autoantibodies) against single-stranded DNA have been thoroughly studied.‡ In turn, non-sequence-specific autoantibodies against double-stranded DNA, a hallmark of autoimmune conditions such as antiphospholipid syndrome and systemic lupus erythematosus (SLE), have not been studied in detail.¶

Generally, monitoring interactions of nucleic acids by fluorescence is a convenient method in modern bioanalysis and can be performed under native conditions without additional equipment or procedures. Currently, fluorescent oligonucleotides containing bright cyanine and xanthene dyes are often applied in bioanalysis of nucleic acids§ and proteins, including antibodies.¶ Furthermore, affinity-enhancing locked nucleic acids containing 2’-amino-LNA monomers with fluorescent polyaromatic hydrocarbons (PAHs) attached at the 2’-amino group provide high target binding affinity and selectivity, remarkable fluorescence quantum yields and brightness values.¶ Another appealing aspect of LNA/DNA probes is their structural aspects of the interactions clarified by the developed fluorescence assay.

Previously, satisfactory biosensing properties of fluorescent probes labelled at the 2’-position of uridine by CuAAC reactions were demonstrated.¶ In the present study we designed alkyne-LNA monomer M1 which combines the unique bicyclic structure of 2’-amino-LNA§ with a terminal alkyne group, allowing post-synthetic attachment of different tags by click chemistry.

Monomer M1 was incorporated into oligonucleotides using the phosphoramidite building block 3 which was prepared starting from the corresponding 2’-amino-5’-O-dimethoxytrityl protected LNA nucleoside 1 in two steps with 52% overall yield. Subsequent automated DNA synthesis furnished modified 21-mer oligonucleotides similar to those previously used in studies of 2’-alkyne-uridine (Scheme 1 and Table 1).8 After purification and characterization by ion-exchange (IE) HPLC and MALDI-MS (ESI†), ON1–ON4 were subjected to CuAAC reactions with fluorescent azides of three important classes: xanthene 5–8 (Scheme 1, Table S1, ESI†), cyanines Cy3 and Cy5 (6 and 7, respectively), and PAH perylene 8 (Table S1, ESI†).
Table 1 Sequences of oligodeoxyribonucleotides (ONs) and thermal denaturation temperatures of the duplexes prepared in this study

| ON  | Sequence, 5'→3' | Target |
|-----|----------------|--------|
| DNA | TGCACCTATGCTCTGATCAT | DNA    |
| ON1 | TGCACCTATGCTCTGATCAT | DNA    |
| ON2 | TGCACCTATGCTCTGATCAT | DNA    |
| ON3 | TGCACCTATGCTCTGATCAT | DNA    |
| ON4 | TGCACCTATGCTCTGATCAT | DNA    |
| ON5 | TGCACCTATGCTCTGATCAT | DNA    |
| ON6 | TGCACCTATGCTCTGATCAT | DNA    |
| ON7 | TGCACCTATGCTCTGATCAT | DNA    |
| ON8 | TGCACCTATGCTCTGATCAT | DNA    |
| ON9 | TGCACCTATGCTCTGATCAT | DNA    |
| ON10 | TGCACCTATGCTCTGATCAT | DNA    |
| ON11 | TGCACCTATGCTCTGATCAT | DNA    |
| ON12 | TGCACCTATGCTCTGATCAT | DNA    |
| ON13 | TGCACCTATGCTCTGATCAT | DNA    |
| ON14 | TGCACCTATGCTCTGATCAT | DNA    |
| ON15 | TGCACCTATGCTCTGATCAT | DNA    |
| ON16 | TGCACCTATGCTCTGATCAT | DNA    |
| ON17 | TGCACCTATGCTCTGATCAT | DNA    |
| ON18 | TGCACCTATGCTCTGATCAT | DNA    |
| ON19 | TGCACCTATGCTCTGATCAT | DNA    |
| ON20 | TGCACCTATGCTCTGATCAT | DNA    |

\[ T_m/\Delta T_m (^\circ C) \]

- DNA
- RNA

\[ \Delta T_m (^\circ C) \] (ESI).

Conditions of the click chemistry were adjusted for each azide, taking into account the high hydrophobicity of the cyanine and perylene dyes, giving 62–81% yields of the products in ≥95% purity as determined by HPLC (Table S2, ESI†).

Binding affinity of ON5–ON20 to complementary and mismatched DNA/RNA targets was evaluated in a medium salt phosphate buffer ([Na⁺] = 110 mM, pH 7.0) by thermal denaturation \( (T_m) \) measurements monitoring absorbance at 260 nm and at the characteristic fluorophores' wavelength (Tables S3–S5, ESI†). First, the resulting \( T_m \) values were similar at both wavelengths suggesting high sensitivity of the dyes to hybridization. Second, affinity enhancing LNA monomers resulted in high \( T_m \) values of the duplexes with complementary DNA/RNA suggesting that the fluorophores are tolerated within the double strands as described earlier for other fluorophores.8,9 Further, especially high target binding was displayed by the probes having M2 \( \Delta T_m \) (up to +13 °C for three incorporations), most likely provided by additional interactions involving the attached perylene groups. Third, thermal denaturation values for the selected probes ON7 and ON19 were decreased by 8–18 °C in the presence of a single mismatch in target DNA/RNA, confirming high binding selectivity for the examined probes (Table S4, ESI†) based on their attractive fluorescence properties as potential biosensors described below).

Notably, hybridization resulted in hypochromic shift of absorbance maxima by the monomer M2 \( \Delta A_{\max}^{abs} \) (7–9 nm), confirming placement of the xanthene within the minor groove of the duplexes.8 In turn, M2–M5 showed minor changes in absorbance peaks upon binding DNA/RNA \( \Delta A_{\max} \) (1–3 nm), although in the case of the cyanine monomers M2–M3 the ratio between two visible absorbance bands (RIII) increased upon hybridization. According to the literature, this indicates reduced dye interactions upon hybridization and their aggregation within single-stranded probes (Fig. S5, ESI†, e.g. RI/II 1.0 and 2.3 for ON16 and ON16:RNA, respectively).

Fig. 1 Representative fluorescence intensities of the probes (ss) and duplexes compared to 5'-labelled LNA/DNA references. Spectra were obtained in a medium salt buffer at 19 °C using 1.0 μM of fluorophores. Reference A: 5'-FAM-d(TGC ACT)sis; reference B: 5'-ROX-d(TGC ACT)sis. The probes were selected based on their attractive fluorescence properties as potential biosensors described below).

Fig. 2 Fluorescence detection of monoclonal autoantibodies (ESI†).
and brightness values, which are important properties for their application in various bioanalytical assays. Finally, fluorescence intensities of duplexes formed by ON7 and ON19 decreased 1.9–9.9 times in the presence of single-base mismatches in target DNA/RNA (Table S4, ESI†). Decreased emission in the presence of a mismatch is most likely caused by high sensitivity of the fluorescence of the internally incorporated monomers M₂ and M₃ to the local microenvironment within the biomolecules.⁸

An important advantage of synthetic oligonucleotides within molecular diagnostics of proteins (so-called aptasensing approach) and immunimoaming techniques is their high specificity in binding a target.¹⁰ To assess the potential of the novel probes in diagnostics of clinically important proteins, fluorescence homogenous detection of human autoantibodies against double-stranded DNA was performed. Single-stranded ON7–ON8 and their duplexes with complementary DNA/RNA were incubated with commercially available human monoclonal autoantibodies dsDNA-mAb32 and dsDNA-mAb33, which were recently studied by surface plasmon resonance (SPR).¹⁰ The subtypes of the monoclonal antibodies were IgG1 (dsDNA-mAb33) and IgG3 (dsDNA-mAb32), and both antibodies have been used as serological parameters in diagnostics of SLE.¹¹

Incubation was then performed in a medium salt phosphate buffer at 37 °C for 1 h followed by analysis after 2 h at 19 °C (ESI†). In order to evaluate the probes' specificity bovine serum albumin (BSA) and non-specific isotype antibodies IgG1 and IgG3 were used as references.¹² Unlike single-stranded ON7 and other examined complexes, ON7:DNA showed 5.7-fold increase of fluorescence at 530 nm when binding dsDNA-mAb33, and 4.2-fold greater fluorescence than in the presence of dsDNA-mAb32, BSA or IgG controls (Fig. 2). Previously SPR studies indicated weaker binding for dsDNA-mAb33 compared to dsDNA-mAb32 by a 24 bp DNA duplex \((k_{\text{obs}} \sim 6.5 \times 10^{-4} \text{ s}^{-1} \text{ and } 0.5 \times 10^{-3} \text{ s}^{-1}, \text{respectively})\).¹⁰ Thus, the binding pattern of ON7:DNA implies that chemical modification might change binding properties of nucleic acids to target proteins. In contrast to ON7:RNA and triply modified ON8:DNA/RNA, little to no fluorescence signal of interaction with BSA or non-specific isotype IgGs was observed for ON7:DNA, confirming high binding selectivity for the latter complex (Table S7, ESI†). According to our molecular models, triple incorporation of the xanthene dyes \(\text{M}^3\) results in high surface hydrophobicity of the duplexes, which may account for their non-specific binding to BSA [ESI†; Fig. S7a]. In turn, effective recognition of dsDNA-mAb33 is provided by steric and chemical complementarity of the unmodified internal region of ON7:DNA and the variable region of the autoantibody's heavy chain, accompanied by effective hydrogen bonding (Fig. S7b, ESI†) compared to c and d.

We speculate that similarly to hybridization described above, target binding results in positioning of the xanthenes in a less polar environment compared to the initial nucleic acid complex resulting in an increased fluorescence.

Finally, the limit of target detection (LOD) for ON7:DNA was determined to be below 4.6 μg mL⁻¹ of dsDNA-mAb33 (Fig. S8, ESI†). This is comparable with currently applied enzyme-linked immunosorbent assay (ELISA), immunofluorescence tests (LOD approx. 1–2 μg mL⁻¹),¹³ and other fluorescent aptasensors.¹³ Notably, being compared to voltage current and electrochemical methods, homogeneous detection is robust, rapid and does not affect interacting surfaces of the biomolecules which can be detected without the need for additional steps and reagents.¹³,¹⁴

In conclusion, the click approach presented here efficiently yields probes with various dyes attached internally at the 2'-amino-position of 2'-amino-LNA monomers. This approach provides a reliable foundation for simple and efficient preparation of a library of fluorescent probes, screening and identification of several bright oligonucleotides with high target binding affinity and specificity. As demonstrated by our initial studies, potential applications of these probes include a wide range of fluorescence assays including, but not limited to, live-cell nucleic acid imaging, aptasensing and nucleic acid diagnostics. Moreover, the 2'-alkynyl group in the LNA/DNA strands is available for the attachment of other tags such as carbohydrates, lipids, co-factors and cell-penetrating peptides. In this context we believe that “clickable” LNADNA probes offer appealing opportunities for developing efficient tools for biosensing, pharmacological or nano-production purposes.

The authors would like to acknowledge financial support from The Sapere Aude programme of The Danish Council for Independent Research, THE VILLUM FOUNDATION and The European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/ERC Grant agreement No. 268776.

Notes and references

1 B. Giannakopoulos, F. Passam, Y. Ioannou and S. A. Krilis, Blood, 2009, 113, 985.
2 P. C. Ackroyd, J. Cleary and G. D. Glick, Biochemistry, 2001, 40, 2911.
3 M. D. Blower, E. Feric, K. Weis and R. Heald, J. Cell Biol., 2007, 179, 1365.
4 S. C. B. Gopinath, K. Awazu and M. Fujinami, Sensors, 2012, 12, 2136.
5 I. V. Astakhova, D. Lindegaard, A. D. Malakhov, V. A. Korkush and J. Wengel, Chem. Commun., 2010, 46, 8362; M. E. Östergaard and P. J. Hrdlicka, Chem. Soc. Rev., 2011, 40, 5771.
6 K. K. Karlsen and J. Wengel, Nucleic Acid Ther., 2012, 22, 366.
7 V. C. Spiteri and J. E. Moses, Angew. Chem., Int. Ed., 2010, 49, 31.
8 L. K. Astakhova and J. Wengel, Chem.–Eur. J., 2013, 19, 1112; M. M. Rubner, C. Holzhauser, P. R. Bohlandner and H.-A. Wagenknecht, Chem.–Eur. J., 2012, 18, 1299; S. P. Sau and P. J. Hrdlicka, J. Org. Chem., 2012, 77, 5; A. H. El-Sagheer and T. Brown, Acc. Chem. Res., 2012, 45, 1258.
9 F. Würtz, T. E. Kaiser and C. R. Saha-Möller, Angew. Chem., Int. Ed., 2011, 50, 3376.
10 A. Buhl, S. Page, N. H. H. Heegaard, P. von Landenburg and P. B. Luppa, Biosens. Bioelectron., 2009, 25, 198.
11 T. H. Winkler, S. Jahn and J. R. Kalden, Clin. Exp. Immunol., 1991, 85, 379; dsDNA-mAb32 and dsDNA-mAb33 correspond to clones 32.B9 and 33.H11, respectively.
12 Y. Zhang and X. Sun, Chem. Commun., 2011, 47, 3927.
13 C.-H. Leung, D.-S.-H. Chan, H.-Z. He, Z. Cheng, H. Yang and D.-L. Ma, Nucleic Acids Res., 2012, 40, 941.
14 L. Wang, Q. Zheng, Q. Zhang, H. Xu, J. Tong, C. Zhu and Y. Wan, Oncol. Lett., 2012, 4, 935; S. Xie, Y. Chai, R. Yuan, L. Bai, Y. Yuan and Y. Wang, Anal. Chim. Acta, 2012, 735, 46.