Synthetic LNA/DNA Nano-scaffolds for Highly Efficient Diagnostics of Nucleic Acids and Autoimmune Antibodies

Prof. Dr. I. Kira Astakhova

Nucleic Acid Center, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, DK-5230 Odense M, Denmark, ias@sdu.dk

ABSTRACT

Herein novel fluorescent oligonucleotides for homogeneous (all-in-solution) detection of nucleic acids and autoimmune antibodies (autoantibodies) are described. The probes are prepared by highly efficient copper-catalyzed click chemistry between novel alkyne-modified locked nucleic acid (LNA) strands and a series of fluorescent azides. The multiply labeled fluorescent LNA/DNA probes prepared herein generally display high binding affinity to complementary DNA/RNA, high quantum yields and, hence, high fluorescence brightness values. With the novel fluorescent probes, specific sensing of the monoclonal human autoantibody is achieved. It makes the novel "clickable" LNA/DNA complexes a very promising tool in molecular diagnostics of both nucleic acids and autoantibodies against DNA. The latter are produced under several autoimmune conditions including antiphospholipide syndrome and systemic lupus erythematosus.

Keywords: nanotechnology, biology, diagnostics, medicine

INTRODUCTION

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 [1]. 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 antiphospholipide syndrome and systemic lupus erythematosus, have not been studied in detail [2]. Diagnostics of these conditions is still based on clinical manifestations, since currently applied heterogeneous natural biomarkers often provide false results, poor sensitivity and low to no reproducibility of laboratory tests [1,2]. Such luck of knowledge and efficient assays targeting clinically important biomolecules inspired us to prepare synthetic nano-scaffolds specifically targeting nucleic acids and autoimmune antibodies.

Monitoring interactions of biomolecules by optical methods such as fluorescence is a convenient method in modern bioanalysis and can be performed under native conditions without additional equipment or procedures. Homogeneous (all-in-solution) biosensing requires use of fluorescent dyes that are photostable and chemically stable and simultaneously provide high sensitivity of detection and long wavelength emission compared to cells autofluorescence at approx. 380 nm. Currently, fluorescent oligonucleotide probes conjugated with bright cyanine and xanthene dyes are often applied in detection of nucleic acids and proteins, including antibodies [3,4]. Moreover, the diagnostic probes have to bind target biomolecules with high affinity and specificity. These properties have been demonstrated by affinity-enhancing locked nucleic acids containing 2′-amino-LNA monomers with fluorescent polyaromatic hydrocarbons (PAHs) attached at the 2′-amino group (Chart 1) [5]. Therefore, LNAs are very promising for diagnostics and research on diverse nucleic acid targets. Another potent aspect of LNA/DNA mixmer probes is their very promising properties as aptamers in binding various protein targets [6].

Generally, synthetic fluorescent 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
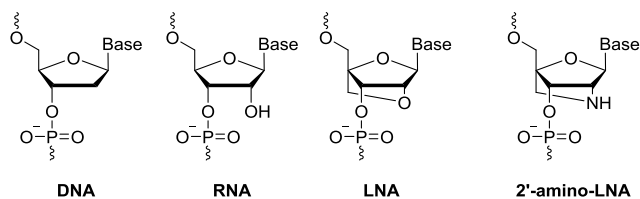
Chart 1. Chemical structures of DNA, RNA, LNA and 2'-amino-LNA nucleotide units.

group provide high target binding affinity and selectivity, remarkable fluorescence quantum yields and brightness values [7]. Another appealing aspect of LNA/DNA probes is their high potential as aptamers in selective binding of diverse proteins [8]. Herein we describe novel fluorescent oligonucleotides for homogeneous (all-in-solution) detection of nucleic acids and autoimmune antibodies. The synthetic scaffolds are prepared by efficient click chemistry between novel alkyne-modified locked nucleic acid (LNA) strands and a series of fluorescent azides (Scheme 1) [8].

RESULTS AND DISCUSSION

First, we designed alkyne-LNA monomer M^1 which combines the bicyclic skeleton of 2′-amino-LNA with a terminal alkyne group, allowing post-synthetic attachment of different groups using e.g. click chemistry (Scheme 1). Monomer M^1 was incorporated into oligonucleotides using the phosphoramidite reagent 3 which was prepared starting from the 2′-amino-5′-O-dimethoxytrityl protected LNA nucleoside 1 in two steps with 52% overall yield [8]. Subsequent automated DNA synthesis furnished modified 21-mer oligonucleotides which were subjected to CuAAC reactions with fluorescent azides of three important classes: xanthene 5-R110 (5), cyanines Cy3 and Cy5 (6 and 7, respectively), and PAH perylene 8. The multiply labeled fluorescent LNA/DNA molecules prepared herein generally displayed high binding affinity to complementary DNA/RNA, high quantum yields and, hence, high fluorescence brightness values (Φ_F 0.54−1.00 and fluorescence brightness (FB) values up to 80) [8].

Upon binding complementary targets by the probes ON7 and ON8, up to 7.8-fold light-up of fluorescence was observed at λ^{fl}_{max} ~ 530 nm, accompanied by Φ_F 0.22−0.45 and FB values up to 77 of the corresponding duplexes (for the sequences, see legend to Figure 1). Being compared to previously reported fluorescent probes, ON7 and ON8 display superiority in binding affinity, fluorescence quantum
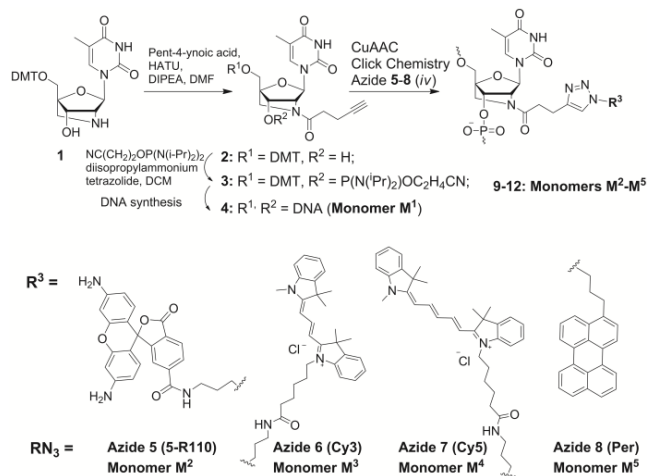
Scheme 1. Synthesis of alkyne-LNA scaffold M^1 and incorporation of monomers M^1–M^5 into synthetic oligonucleotides.

yield and brightness values, which is an important advantage for their application in various bioanalytical assays [9-12]. In turn, perylene modified probes show extraordinary high photostability and binding affinity to complementary DNA/RNA, which makes them attractive for appealing *in vitro* and *in vivo* imaging techniques [13-17].

Next, we assessed the potential of the novel double-stranded LNA/DNA constructs in diagnostics of clinically important protein targets by fluorescence homogenous detection of human autoantibodies against double-stranded DNA. 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), and have been used as serological parameter in the in vitro diagnosis of SLE [18,19]. In order to evaluate the probes' specificity BSA protein and isotype human antibodies were used as controls. Unlike single-stranded LNA/DNA and other examined complexes, a particular double-stranded LNA/DNA scaffold with double incorporation of xanthene dyes showed a 5.7-fold increase of fluorescence at 530 nm when binding monoclonal antibody dsDNA-mAb33, and 4.2-fold greater fluorescence than in the presence of dsDNA-mAb32, bovine serum albumin (BSA) or non-specific isotype IgG controls (Figure 1). Low to no fluorescence signal of interaction with the controls confirmed high specificity for the prepared nucleic acid complex compared to e.g. single-stranded ON7, ON7:RNA and triply modified ON8:DNA/RNA. According to molecular models, effective recognition of dsDNA-mAb33 is provided by steric and chemical complementarity of the
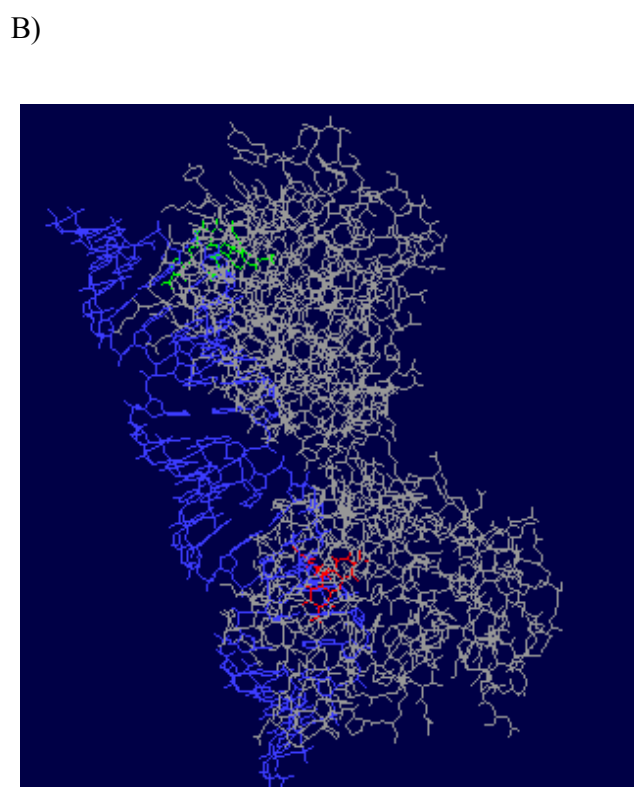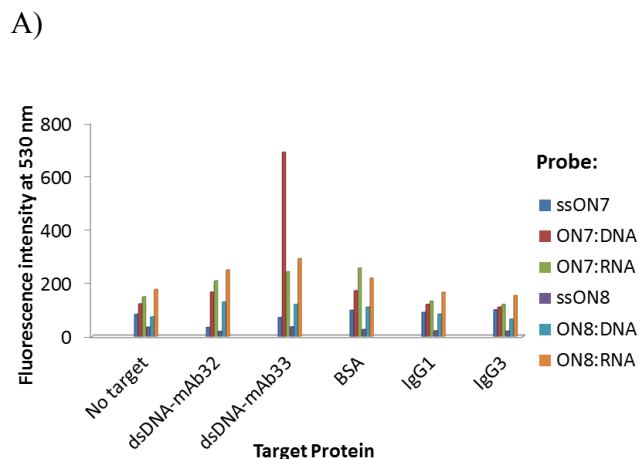
A)

B)

Figure 1. (A) Fluorescence detection of monoclonal autoantibodies (dsDNA-mAbs) compared to control proteins; **ON7**: 5'-d(TGCAC**M²**CTATGTCTGTA**M²**CAT); **ON8**: 5'-d(TGCAC**M²**CTATG**M²**CTGTA**M²**CAT); **DNA**: 5'-d(ATGATACAGACATAGAGTGCA); **RNA**: 5'-d(AUGAUACAGACAUAGAGUGCA). (B) Representations of binding modes between fluorescent nucleic acid complex **ON7**:DNA binding variable region of dsDNA-mAb33 (indicated in blue and white, respectively; fluorophores are shown in red and green), resulting in light-up of fluorescence.

unmodified internal region of the LNA/DNA scaffold and the variable region of the autoantibody's heavy chain, accompanied by effective hydrogen bonding (Figure 1B). We speculate that similarly to hybridization with nucleic acid targets, 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 [8].

Finally, limit of target detection (LOD) for the LNA/DNA scaffold was determined to be below 4.6 µg/mL of dsDNA-mAb33 [8]. This is comparable with a currently applied enzyme-linked immunosorbent assay (ELISA), immunofluorescence tests (LOD approx. 1−2 µg/mL), and other fluorescent aptasensors [1,20]. 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 [20-22].

CONCLUSIONS AND PERSPECTIVE

As demonstrated herein, our approach provides a reliable foundation for simple and efficient preparation of bright fluorescent oligonucleotides with high target binding affinity and specificity. Potential applications of the "clickable" LNA/DNA nano-scaffolds include a wide range of fluorescence assays including, but not limited to, live-cell nucleic acid imaging, aptasensing and nucleic acid diagnostics [8].

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