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Synthetic Oligonucleotide Probes for Detection of Autoimmune Antibodies

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Abstract

In this review, applications of synthetic oligonucleotide probes in diagnostics and studies of autoimmune antibodies against double-stranded DNA (anti-dsDNA) are described. As outlined herein, synthetic oligonucleotides and monoclonal antibodies provide appealing opportunities to develop standard simple assays for detection of anti-dsDNAs. Recent examples of anti-dsDNA detection using synthetic nucleic acid antigens include those applying surface plasmon resonance (SPR), antibody microarrays and homogeneous, or all-in-solution, detection with fluorescently-labelled probes. Since sequences of the applied nucleic acid antigens and monoclonal antibodies are known, the major benefit of these assays is the ability for the first time to clearly define the structural factors that govern the formation and stability of anti-dsDNA complexes. This is an essential first step toward understanding immune-complex mediated tissue injury in autoimmune conditions such as systemic lupus erythematosus (SLE) and arthritis, which involve production of pathogenic anti-dsDNAs. Moreover, synthetic nucleic acids and monoclonal antibodies have been demonstrated as key tools for the development of novel nucleic acid sensors for serotyping, along with studies of specificity and avidity of the antibody-dsDNA binding process. Among other antigens, fluorescent oligonucleotides prepared by click chemistry between novel alkyne-modified locked nucleic acid (LNA) strands and a series of fluorescent azides are proved to be very promising tools for efficient homogeneous detection of anti-dsDNAs.

Keywords: Autoantibody; Double-stranded DNA; Systemic lupus erythematosus; Oligonucleotide; Fluorescence; Diagnostics

Introduction

Autoimmunity occurs when antibodies attack one's own cells, which is a necessary natural mechanism when it comes to eliminating damaged or cancerous cells. However, if the control factors such as those encoded by DNA of the components of the immune system are incorrect, the immune system attacks healthy cells [1]. This condition occurs in certain autoimmune disorders including antiphospholipid syndrome, systemic lupus erythematosus (SLE), arthritis and diabetes. Notably, multiple autoimmune disorders are characterized by production of autoantibodies against single- and double-stranded DNA. If not diagnosed and treated early, these autoimmune conditions can lead to serious health deterioration and even mortality [1]. The sequence-specific autoimmune antibodies (autoantibodies) against single-stranded DNA (anti-ssDNAs) have been thoroughly studied [2]. In turn, non-sequence-specific anti-dsDNAs, a hallmark of SLE, have not been studied in detail [1].

In current diagnostics of anti-dsDNAs, natural heterogeneous DNA molecules are applied [3,4]. This results in poor reproducibility of assays and significant disagreement in results of different research groups and diagnostic laboratories. Natural molecules that are heterogeneous in sequence contain impurities and rapidly degrade under storage conditions, resulting in the aforementioned difficulties in their diagnostic applications. Finally, an additional visualization reagent, such as anti-antibody conjugate containing a fluorescent dye or enzyme substrate, has to be applied for detection of the target. These conjugates are expensive and they also rapidly degrade under storage conditions.

Recent developments of synthetic biology allow preparation of synthetic oligonucleotides containing various modifications for detection of nucleic acids and proteins [5-9]. An important advantage of synthetic oligonucleotides within molecular diagnostics of proteins (so-called aptasensing approach) and immunoimaging techniques are their high specificity in binding a target [10]. Furthermore, synthetic oligonucleotides can be stored at low temperature for years without changing the structure and immunogenity[5-9]. Finally, clear information on the sequence of oligonucleotide allows standardization of the assay in which it is applied.

The ability to directly sense autoantibodies by one-step interaction with a sensor is a very attractive diagnostic approach (Figure 1) [10,11]. Synthetic nucleic acids containing ultra-sensitive fluorophores, such as polyaromatic hydrocarbons (PAHs), are promising candidates for this purpose, since optical properties of PAHs are dramatically affected by polarity of the microenvironment and presence of quenchers [12-16].

In this review, current diagnostics of anti-dsDNAs using synthetic oligonucleotides is described. In doing this, structural and clinical aspects of the interaction between the synthetic nucleic acid antigens and autoantibodies are reviewed. Finally, application of fluorescently labelled oligonucleotide analogues in recently introduced direct sensing approaches is a special focus of this review.

Diagnostics of Autoantibodies against DNA Using Natural Nucleic Acids

The most frequently associated nucleic acid autoantigens include nucleosomes, histones, and double stranded DNA [1,2,17]. Like all antibody tests, DNA antibody assays are conducted either by

immunofluorescence or by the simpler enzyme-linked immunosorbent assay (ELISA) test. Though the former is a more reliable test for autoimmune antibodies, the ELISA test is more preferred in clinics due to its low cost [18]. In the ELISA test nucleic acid antigen is immobilized on solid support (i.e. a plastic plate), and subjected to interaction with human serum. Thereafter, a secondary antibody against anti-dsDNA is applied. This antibody contains a substrate for an enzyme which creates color change upon interaction allowing detection of the primary antibody by colorimetry[19]. In the current ELISA and immune-fluorescent tests, natural heterogeneous DNA molecules are applied as antigens [20]. Recently, it was shown that anti-dsDNA antibodies do show preference for binding certain native double stranded DNA (dsDNA) fragments [20]. Clearly, sequence specificity of anti-dsDNAs can be validated only by using DNA antigens with known sequence. Moreover, anti-DNA avidity varies for different patients: it is noteworthy that patients with only low avidity anti-DNA in their circulation develop a more mild form of SLE, whereas the avidity of autoantibodies increases during the course of their diseases. These factors make ELISA in its standard form unsuitable as a diagnostic tool for anti-dsDNAs[21]. In turn, rapidly developing fluorescent techniques opens up new possibilities for diagnostics and studies of autoimmune antibodies of various avidity by the direct sensing approach (Figure 1) [5-11].

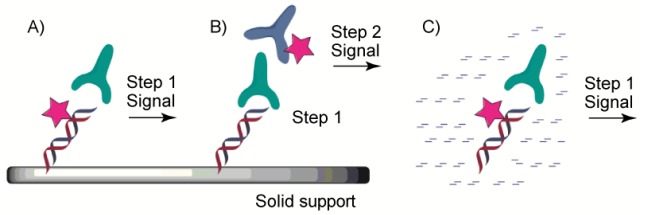

Figure 1: Representation of diagnostic assays for detection of autoimmune antibodies: direct **(A)** and indirect **(B)** solid-phase methods, and direct homogeneous assay **(C)** Sensor molecules are indicated as a star.

Synthetic Oligonucleotides in Indirect Sensing of Autoantibodies

Defining the structural and biochemical factors that govern the formation and stability of anti-DNA complexes is an essential first step toward understanding immune-complex mediated tissue injury in SLE and arthritis. Although there are numerous examples of the application of native dsDNA molecules as non-ELISA sensors of autoimmune antibodies [22], using synthetic stable analogues with a known sequence could bring several benefits in this field. Furthermore, testing of a new antigen using monoclonal autoantibodies is a key step for development of novel nucleic acid sensors, along with studies of specificity and avidity of the antibody-DNA binding process [23]. Having studied interactions of anti-ssDNA antibodies and diverse synthetic oligonucleotides, Glick and co-workers suggested a concept of induced conformational fit in antibody-DNA complexes [24-29]. Moreover, anti-ssDNAs often show a preference for either thymine or guanine. However, no sequence-specificity has been clearly demonstrated for anti-dsDNA when interacting with autoantibodies from either monoclonal or polyclonal sources [29]. The authors propose that low affinity of the synthetic DNA homopolymers that have been used as ligands in these studies might be an obstacle for targeting anti-dsDNAs. Furthermore, cross-reactivity with other non-related antibodies resulted in problems for validation of results using model nucleic acids [29].

Immobilization of DNA on solid support leads to functional diagnostic materials which can be used for detection and studies of autoimmune antibody-DNA complexes in various assays [30-35]. Thus, using a series of monoclonal anti-dsDNA antibodies and synthetic DNA strands [23], Buhl and co-workers developed an optical biosensor as a possible new standard for laboratory tests [36]. In this study recently, a recently introduced surface plasmon resonance (SPR) biosensor chip containing synthetic DNA molecules was applied for binding studies between dsDNA and anti-dsDNA autoantibodies. In doing this, two human and one murine monoclonal anti-dsDNA antibodies were characterized by measuring the kinetic on- and off-rates by using the biosensor and calculating avidity as the ratio of these. Obtained dissociation constants were verified by an independent method and inhibition experiments in order to determine the reactivity to DNA of various length and composition. While all mAbs exhibited comparable avidities, which could be confirmed by gel shift experiments, one of them proved to have slower association and dissociation kinetics. This was the only mAb providing positive results in the Farr radioimmunoassay (Farr RIA). Conducting inhibition experiments with ss- and ds-oligonucleotides 10, 24 and 42 bp in length, the mAbs acted substantially different. This study demonstrated how putative standards for the anti-dsDNA determination can be characterized using SPR biosensor technology. These results suggest that the kinetic rate constants seem to be decisive in explaining the behavior of mAbs. The authors underline that different reactivity to various DNA species should be taken into account with respect to varying DNA sources in commonly used laboratory assays [36].

The use of antibody-based miniaturized devices for microbiological applications is still a rather poorly investigated in the era of more developed molecular amplification techniques. One appealing approach for serotyping is antibody microarray, in which antibodies are immobilized on a solid-surface and then subjected to human serum to be analyzed [37]. This type of assays allows rapid validation of antibody spectrum in serum, determination of avidity and cross-reactivity. Recently, a novel antibody microarray for *Streptococcus pneumoniae* serotyping was developed, by printing pneumococcal serotype-specific antibodies on multi-well slides [38]. The microarray showed high specificity when tested against reference and clinical *S. pneumoniae* isolates and therefore can be applicable as a faster, cost-effective and accurate serotyping technique for pneumococcal and other epidemiological studies, including autoimmune conditions [38].

Finally, diverse nano-particles have been successfully applied in the development of new serotyping strategies based on synthetic unmodified dsDNA antigens [39-41]. For example, Baker and co-workers investigated the time-dependent strand displacement activity of several targets with dsDNA probes of varying affinity [41]. In this work the relative affinity of various probes was altered through choices in hybridization length (11–15 bases) and the selective inclusion of center mismatches in the duplexes. The dsDNA molecules were immobilized on microspheres, whereas the soluble, 15 base-long complementary sequence was presented either alone as a short target strand or as a recognition region embedded within a longer target strand. Remarkably, strand displacement activity of the longer targets was slower compared to the shorter targets, but still successful. Additionally, the longer targets exhibited modest differences in the

observed displacement rates, depending on the location of recognition segment within the long target. Similar assays can be applied for studies of the antibody-dsDNA binding kinetics and also to determine essential probe sequence design parameters for efficient target binding [41].

Fluorescent Oligonucleotides in Direct Sensing of Autoantibodies in Solution

Generally, monitoring interactions of biomolecules 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 [42,43]. Furthermore, locked nucleic acids containing 2′-amino-modification (2'-amino-LNA) labelled with various fluorescent dyes at the 2′-amino group provide high target binding affinity and selectivity, remarkable fluorescence quantum yields and brightness values (Figure 2) [10]. Another appealing aspect of LNA/DNA probes is their high potential as aptamers in selective binding of diverse proteins [10,44]. Being incorporated into synthetic oligonucleotides, LNAs induce high thermal stabilities of dsDNA complexes and also improve binding selectivity of nucleic acid and protein targets by the LNA/DNA chimera [45-47]. Notably, alkyne-LNA monomer **1** combines the unique bicyclic structure of 2′-amino-LNA with a terminal alkyne group, allowing postsynthetic attachment of different tags by convenient click chemistry [48,49]. In our recent research, the LNA/DNA monomer **1** was incorporated into 21-nt long synthetic oligonucleotides which were later labelled with commercially available xanthene, cyanine and PAH azides**2–5** (Figure 2 and Table 1) [49]. The click chemistry protocol is very simple and provides high yields of the products (62–81%) without need in additional purification steps, i. e. ≥95% purity as determined by ion-exchange IE HPLC.

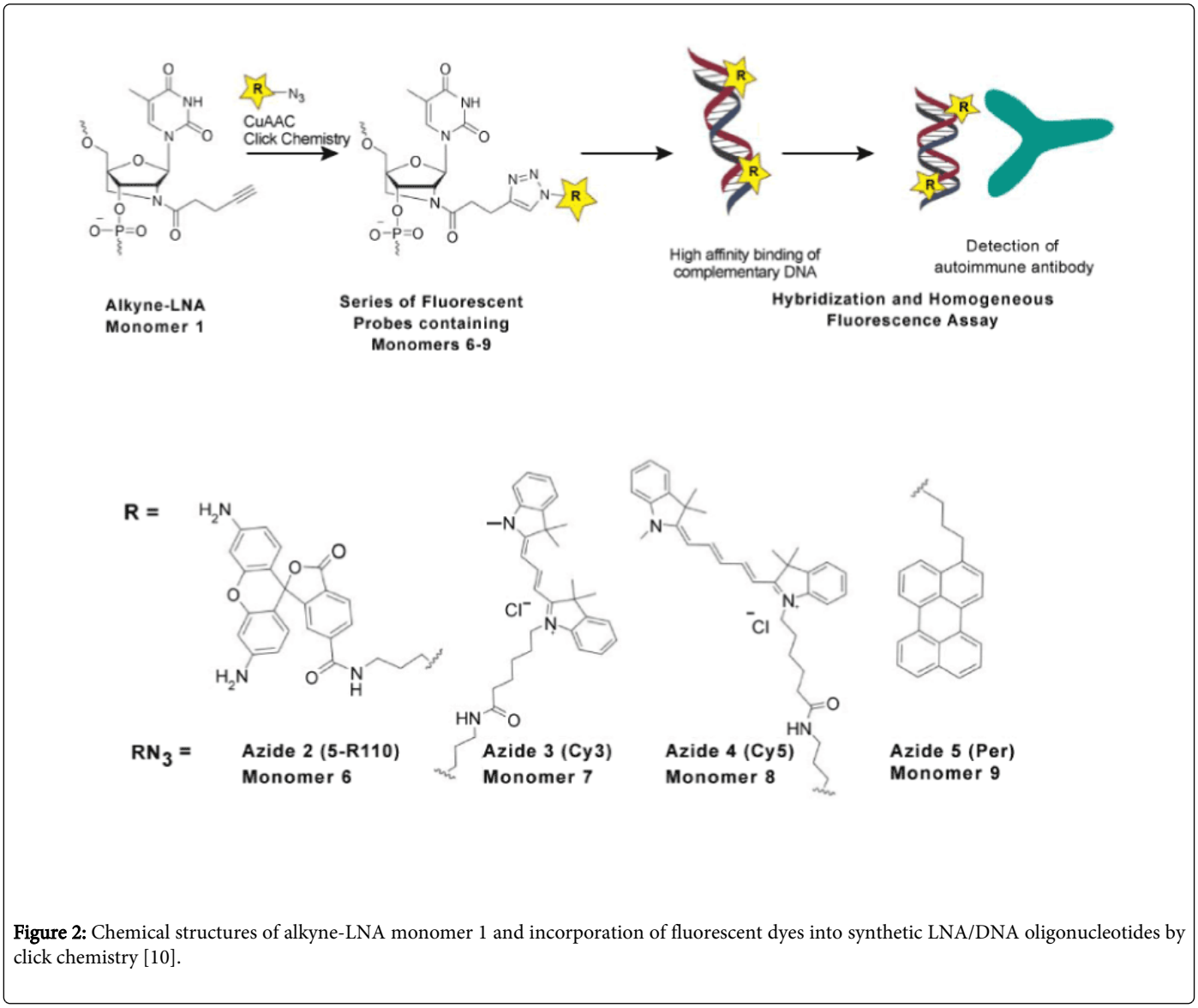

Figure 2: Chemical structures of alkyne-LNA monomer 1 and incorporation of fluorescent dyes into synthetic LNA/DNA oligonucleotides by click chemistry [10].

Probe#	Sequence, 5′→3′	T_m/ ΔT_m, °CTarget	
		DNA	RNA
DNA^{ref}	d(TGCACTCTATGTCTGTATCAT)	59.0	60.5
P1	d(TGCAC**(1)**CTATGTCTGTA**(1)**CAT)	63.0/+4.0	68.0/+7.5
P2	d(TGCAC**(1)**CTATG**(1)**CTGTA**(1)**CAT)	65.0/+6.0	71.0/+10.5
P3	d(TGCAC**(6)**CTATGTCTGTA**(6)**CAT)	58.0/-1.0	64.0/+3.5
P4	d(TGCAC**(6)**CTATG**(6)**CTGTA**(6)**CAT)	58.0/-1.0	66.0/+5.5
P5	d(TGCAC**(7)**CTATGTCTGTA**(7)**CAT)	63.0/+4.0	65.0/+4.5
P6	d(TGCAC**(7)**CTATG**(7)**CTGTA**(7)**CAT)	65.0/+6.0	66.5/+6.0
P7	d(TGCAC**(9)**CTATGTCTGTA**(9)**CAT)	67.0/+8.0	67.0/+6.5
P8	d(TGCAC**(9)**CTATG**(9)**CTGTA**(9)**CAT)	72.0/+13.0	70.0/+9.5

Table 1: Representative sequences of LNA/DNA probes and thermal denaturation temperatures of the duplexes applied in homogeneous sensing of anti-dsDNAs[10].

[a]Thermal denaturation temperatures T_m(°C)/change in $T_m$relative to corresponding unmodified duplex (ds**DNA**^{ref}or **DNA**^{ref}:RNA), ΔT_m(°C). $T_m$values measured as the maximum of the first derivatives of the melting curves (A_{260}vs temperature). For chemical structures of modified monomers see Figure 2. Complementary DNA: 5'-d(ATG ATA CAG ACA TAG AGT GCA); complementary RNA: 5'-r(AUG AUA CAG ACA UAG AGU GCA).

Binding affinity of fluorescent LNA/DNA probes to complementary DNA/RNA targets was evaluated in a medium salt phosphate buffer (110 mM [Na^+], pH 7.0) and showed increased thermal denaturation (T_m) values for the modified duplexes compared to the reference (Table 1). This is an important result for application of the formed duplexes in diagnostics with shorter incubation steps at elevated temperatures and in various buffer systems [50,51]. Next, to assess the potential of the novel probes in diagnostics of clinically important autoantibodies, fluorescence homogeneous detection of human anti-dsDNAs was performed (Figure 3) [10]. The duplexes for this assay were chosen based on documented brightness of fluorescence and sensitivity of the attached dyes to hybridization. Single-stranded probes **P3–P4** 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 SPR [23,36]. The subtypes of the monoclonal antibodies were IgG1 (dsDNA-mAb33) and IgG3 (dsDNA-mAb32), and both antibodies have been used as a serological parameter in diagnostics of SLE [51]. Unlike single-stranded **P3** and other examined complexes, **P3:**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 (Figure 3). SPR studies showed a weaker binding for dsDNA-mAb33 compared to dsDNA-mAb32 by a 24 bp DNA duplex ($(k_d)^{obs}$~ $6.5\times10^{-3}s^{-1}$and $0.5\times10^{-3}s^{-1}$, respectively) [36]. Thus, avidity of **P3:**DNA implies that chemical modification might change the binding preferences of nucleic acids to target proteins. On the contrary to P3:RNA and triply modified **P4:**DNA/RNA, little to no fluorescence signal of interaction with BSA or non-specific isotypeIgGs was observed for **P3:**DNA (Figure 3). This confirms high binding selectivity for the selected LNA/DNA complex. According to molecular models, recognition of dsDNA-mAb33 is provided by steric and chemical complementarity of the unmodified internal segment of **P3:**DNA and the variable region of autoantibody's heavy chain, accompanied by effective hydrogen bonding [10]. Furthermore, we speculate that target binding results in positioning of the xanthene dyes in a less polar environment compared to the initial nucleic acid complex resulting in an increased fluorescence [10,52].

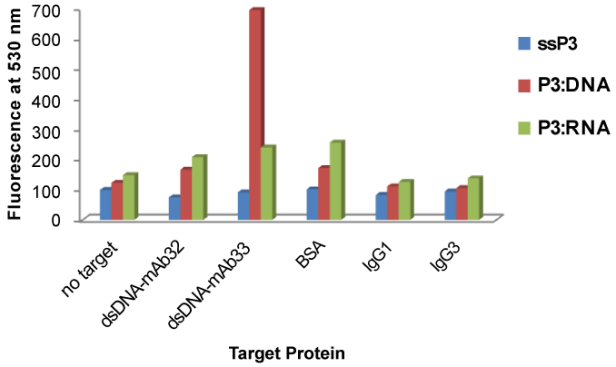

Figure 3: Fluorescence homogeneous detection of monoclonal autoantibodies (dsDNA-mAb32 and dsDNA-mAb33) compared to control proteins (BSA, IgG1 and IgG3) in a medium salt buffer (110 mM[Na^+] at 19°C using 500 nM probes [10]. Sequences of the sensor molecules are listed in Table 1. Incubation was performed by adding 0.5×10^5IU of the target autoantibody to a solution of corresponding nucleic acid complex. As a reference, the BSA, IgG1 or IgG3 stock solution was used in similar incubation reactions with the probes. Incubation was performed at 37°C for 3 h. Upon cooling to ambient temperature over 2-4 h, the resulting solutions were analysed by fluorescence spectroscopy using excitation wavelength 500 nm and monitoring fluorescence at 530 nm.

As a last aspect, limit of target detection (LOD) for **P3:**DNA was determined to be below 4.6 μg/mL of dsDNA-mAb33. This is comparable with currently applied ELISA, immunofluorescence tests (LOD approx. 1-2 μg/mL), and other fluorescent aptasensors[53]. 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 [54,55].

Conclusion and Perspective

Synthetic oligonucleotides have much to offer to diagnostics of autoimmune antibodies, making the current challenging detection highly reproducible and reliable. Recent examples of such biosensors include synthetic unmodified dsDNA probes in SPR and microarray assays, and synthetic oligonucleotides prepared by the click approach between alkyne-derivatives of locked nucleic acids (LNA) and various dyes. As confirmed by these and other reports, synthetic oligonucleotides provide a reliable foundation for robust and efficient detection of anti-dsDNAs with the advantages of stability, high target binding affinity and specificity.

Rapid development of synthetic biology and imaging techniques allows rational design and preparation of advanced nucleic acid sensors addressing diverse research and clinical tasks on autoimmune diseases. Advanced computational chemistry integrated with existing empirical data plays an important role for this field, since molecular simulations, supported by recent studies on antibody-DNA binding process, provide structural considerations for the new synthetic sensors with high target selectivity, brightness and binding affinity [56]. Another vital aspect for development of new biosensors is a close interdisciplinary collaboration between synthetic chemists and clinical community [57]. Finally, and as it is outlined in this review, when these collaborations are established, rationally designed synthetic nucleic acids offer great opportunities for diagnostics and medicine of the future.

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