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Oligonucleotide-based systems: DNA, microRNAs, DNA/RNA aptamers

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There are an increasing number of applications that have been developed for oligonucleotide-based biosensing systems in genetics and biomedicine. Oligonucleotide-based biosensors are those where the probe to capture the analyte is a strand of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) or a synthetic analogue of naturally occurring nucleic acids. This review will shed light on various types of nucleic acids such as DNA and RNA (particularly microRNAs), their role and their application in biosensing. It will also cover DNA/RNA aptamers, which can be used as bioreceptors for a wide range of targets such as proteins, small molecules, bacteria and even cells. It will also highlight how the invention of synthetic oligonucleotides such as peptide nucleic acid (PNA) or locked nucleic acid (LNA) has pushed the limits of molecular biology and biosensor development to new perspectives. These technologies are very promising albeit still in need of development in order to bridge the gap between the laboratory-based status and the reality of biomedical applications.

Introduction

Oligonucleotides are unmodified or chemically modified polymers (DNA or RNA) that are relatively small (12–25-mer) and introduce an expanded range of applications in molecular genetics research and forensics. The suffix 'mer' is often used (derived from the Greek for 'part') to denote the length of an oligonucleotide. In the natural world, oligonucleotides exist mainly as small non-coding RNAs (e.g. microRNAs (miRNAs)). Such oligonucleotides are commonly synthesized using solid-phase chemistry. Chemical modifications of the sugar–phosphate backbone or the bases are often used to increase the stability and half-life of oligonucleotides. In general, oligonucleotides work by hybridizing to their complementary sequences. They are used in many different ways including as 'primers' in polymerase chain reaction (PCR), as 'probes' in microarray analysis or *in situ* hybridization, and in biosensing applications. The explosion of knowledge regarding gene expression and gene regulation mechanisms has led to many new opportunities to develop oligonucleotide-based technologies. In order to appreciate these opportunities, this review begins with a brief overview of the increasing complexity of gene expression.

The complexities of gene expression: a brief summary

The last few decades have witnessed an explosion of knowledge in the field of molecular biology. It is increasingly evident that evolution has generated an astonishingly complex set of interconnected processes through which gene expression can be regulated. The purpose of this section is to provide a general overview of gene expression and to highlight the importance of RNA-centred processes in gene regulation.

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In the early days of molecular biology, it was thought that transcriptional control was the main mechanism through which gene expression is controlled. However, it is abundantly clear that epigenetics (including chemical modifications of DNA and of histones, affecting how DNA is packaged) and post-transcriptional control (pre-mRNA processing, mRNA localization, translation and stability) are equally important. It is also apparent that all of these processes are highly interconnected. What follows is a brief overview of the complexities of gene expression. We draw particular attention to two topics that have attracted a lot of interest recently: alternative splicing and non-coding RNAs.

Chromatin, transcription and post-transcriptional regulation

The regulation of transcription is a fundamental aspect of gene regulation. After all, to be expressed, genes need to be transcribed, producing either a messenger RNA (mRNA) or a non-coding RNA. Promoters and distal enhancer sequences facilitate the recruitment of RNA polymerases and the process of transcription, in which DNA is unwound and one strand copied into a complementary RNA transcript, begins at discrete start sites in the genome. *In vivo* DNA does not exist in a 'naked' state; instead, it is packaged by proteins into structures called nucleosomes by proteins called histones. DNA that is packaged by proteins such as histones (or by protamines in sperm cells) is called chromatin. In order for transcription to occur, the chromatin structure often needs to be loosened in a process called chromatin remodelling. This remodelling occurs following the covalent modification of histones by enzymes including histone acetyltransferases, deacetylases, protein kinases and methyltransferases. DNA can also be modified directly at CpG (a cytosine followed by a guanine nucleotide) positions by the methylation of cytosine to 5-methylcytosine. Methylated DNA tends to be associated with genes that are less transcriptionally active. Chromatin remodelling and DNA modifications, both in response to physiological, developmental and environmental cues, do not alter the actual base sequence of DNA [1]. However they do alter gene expression in a reversible manner in a process called epigenetics ('epi' is from the Greek for 'outside of').

The products of transcription, in other words RNA molecules, are called transcripts; in general, transcripts need to be processed. The precursor of mRNA, pre-mRNA, needs to be modified at the 5′ end. This consists of a series of covalent modifications to the first base (the so-called trimethyl m^7G cap). The 5′ cap facilitates nuclear export, mRNA translation and mRNA stability. Pre-mRNA is also cleaved at the 3′ end (defining the end of the transcript) and polyadenylated. Further complexity arises from the process of pre-mRNA splicing, discovered in the late 1970s. In pre-mRNA splicing, sequences known as introns are precisely removed (spliced) and exons joined together to form the mature mRNA. Collectively, capping, splicing, cleavage and polyadenylation are known as pre-mRNA processing. They mostly occur co-transcriptionally, i.e. while the RNA polymerase is still working its way along the gene. The C-terminus of RNA polymerase II is able to recruit pre-mRNA processing factors through its heavily phosphorylated C-terminal domain (CTD). The process, called alternative splicing, in which exons are joined together in different ways, results in several transcripts with potentially different coding potential.

There are several other regulatory processes that occur. RNA editing involves the direct chemical modification of RNA bases (e.g. adenine to inosine) potentially altering coding potential. mRNA export is a regulated process; incorrectly processed mRNAs are retained in the nucleus. In the cytoplasm, mRNA translation, mRNA localization and mRNA stability are all highly regulated. Localization allows mRNAs to be delivered to and translated in discrete parts of the cytoplasm where the protein is needed, such as an axon or lamellipodia. Translation consists of three steps, initiation, elongation and termination, each of which is highly regulated. The extent to which an mRNA is translated affects how much protein is made. Further complexity arises from the presence of occasional alternative translation start codons. mRNAs have a half-life; some are more stable than others and an mRNA's half-life has a bearing on how much protein is made. Degradation of mRNA is also highly regulated in response to developmental and physiological cues.

It is quite clear that transcription control is only one of many layers of regulation. Gene expression is a highly complex process. Many proteins that are involved in gene regulation are multifunctional, facilitating connections between different steps of gene expression. In the biosensing field there are opportunities to exploit this complexity. We can illustrate this point by turning our attention to two specific examples: alternative splicing and non-coding RNAs.

Alternative splicing of pre-mRNA: one gene, multiple transcripts

When pre-mRNA splicing was first observed in eukaryotic viruses, the discoverers had no idea that over 94% of human genes would turn out to be alternatively spliced [3,4]. Alternative splicing is the process whereby exons are not always joined together in the same way. The main types of alternative splicing are intron retention (sometimes introns

are not spliced out), cassette exons (exons can be skipped entirely) and alternative 5′ and 3′ splice sites (in which the boundaries of the exons can change). Through alternative splicing, genes can express dozens of splice isoforms. Genes can even produce thousands of transcripts; a famous example is the fruitfly *Dscam* gene [5]. The effect of alternative splicing is to augment the size of the proteome very significantly. Splice isoforms often have antagonistic functions and their expression is regulated by proteins called splice factors. In the biosensing field the existence of multiple, often biologically distinct, splice isoforms provides the opportunity to develop ways to detect and measure levels of specific splice isoforms.

The latter point can be illustrated by the example of the *HER2* gene. *HER2* is a member of the human epidermal growth factor receptor family. It is overexpressed in a subset of breast cancers and is considered one of the most notable breast cancer biomarkers. The HER2 protein is targeted by the drug Herceptin, a monoclonal antibody that binds to HER2 and prevents it from receiving growth signals. However, multiple HER2 splice isoforms have been reported. Retention of intron 8 introduces a premature stop codon that results in a truncated inhibitory HER2 isoform called herstatin. The isoform HER2Δ16 is produced by skipping exon 16, which is 48 bases long and contributes 16 amino acids in frame to the extracellular domain. HER2Δ16 forms a constitutively active dimer that is implicated in Herceptin resistance [6]. The problem with Herceptin is that it does not discriminate between HER2 splice isoforms. Thus if a patient's tumour is already prone to skipping exon 16, resistance to Herceptin could arise relatively quickly. Current diagnostic kits in clinical practice only detect overall *HER2* expression, but they do not measure how it is alternatively spliced. The same issue applies to several existing cancer therapies: they do not discriminate splice isoforms.

There is undoubtedly a need to develop tools that permit the 'biosensing' of splice isoforms of key biomarkers such as HER2. There are also significant opportunities to use antisense oligonucleotides in order to manipulate alternative splicing *in vivo*. For example, splice-switching oligonucleotides (SSOs) can be designed to block specific 5′ and 3′ splice sites facilitating the skipping of disease-associated exons. Antisense oligonucleotides can be used to correct splicing defects associated with diseases such as spinal muscular atrophy or Duchenne muscular dystrophy. The following example illustrates how oligonucleotides can be potentially used as anticancer agents. Signal transducer and activator of transcription 3 (STAT3) is involved in the activation of several oncogenic pathways. The splice variant STAT3β arises from an alternative 3′ splice site in exon 23; it encodes a truncated pro-apoptotic isoform (in contrast with the oncogenic STAT3α isoform). A phosphorodiamidate morpholino antisense oligonucleotide was targeted to a 'splice enhancer' sequence that resulted in increased STAT3β expression causing tumour regression in a mouse xenograft model [7]. Several hurdles need to be overcome including the problems associated with specificity and the need for efficient delivery to target tissues. However, it is reasonable to predict that antisense oligonucleotides will become mainstream pharmacological agents.

The increasing importance of non-coding RNAs

One of the most unexpected recent developments in molecular biology is the finding that the genome is rather promiscuous in its transcription, producing a staggering number of transcripts that do not encode proteins [8–10]. These are known as non-coding RNAs. Non-coding RNAs include ribosomal RNA (rRNA) and transfer RNAs (tRNA) required for translation. They also include small non-coding RNAs involved in the processing of other RNAs (for example, small nuclear RNAs (snRNAs) involved in splicing and small nucleolar RNAs (snoRNAs) in pre-rRNA processing). These have been known for a long time; however, more recently, several other types of non-coding RNA have been discovered. They are subdivided into short and long non-coding RNAs. The (somewhat arbitrary) definition of a long non-coding RNA is that it is more than 200 nucleotides in length.

Long non-coding RNAs (also known as megaRNAs or lncRNAs) are best known for their involvement in epigenetic regulation [11]. Long non-coding RNAs are particularly well suited to epigenetic regulation because they can bind to complementary target sequences in the genome. After binding their genome targets, they help recruit proteins that modify chromatin. A well-known long non-coding RNA called *XIST* (X-inactive specific transcript) provides the molecular basis of X-chromosome inactivation in female mammals [12].

Short non-coding RNAs include miRNAs are, when fully processed, quite small (21 nucleotides). They work by binding to target mRNAs, generally (but not exclusively) to 3′UTRs (the untranslated sequence that follows the stop codon in mRNAs). miRNAs are involved in plant and animal physiology and development. They work by repressing translation or even causing the targeted degradation of mRNAs (Figure 1). The net effect is that miRNAs significantly reduce the amount of protein produced by an mRNA; in other words, they repress gene expression post-transcriptionally. Each miRNA is thought to regulate multiple genes in complex regulatory networks. The use of deep sequencing techniques has recently shown that there are even more miRNAs in the human genome than originally

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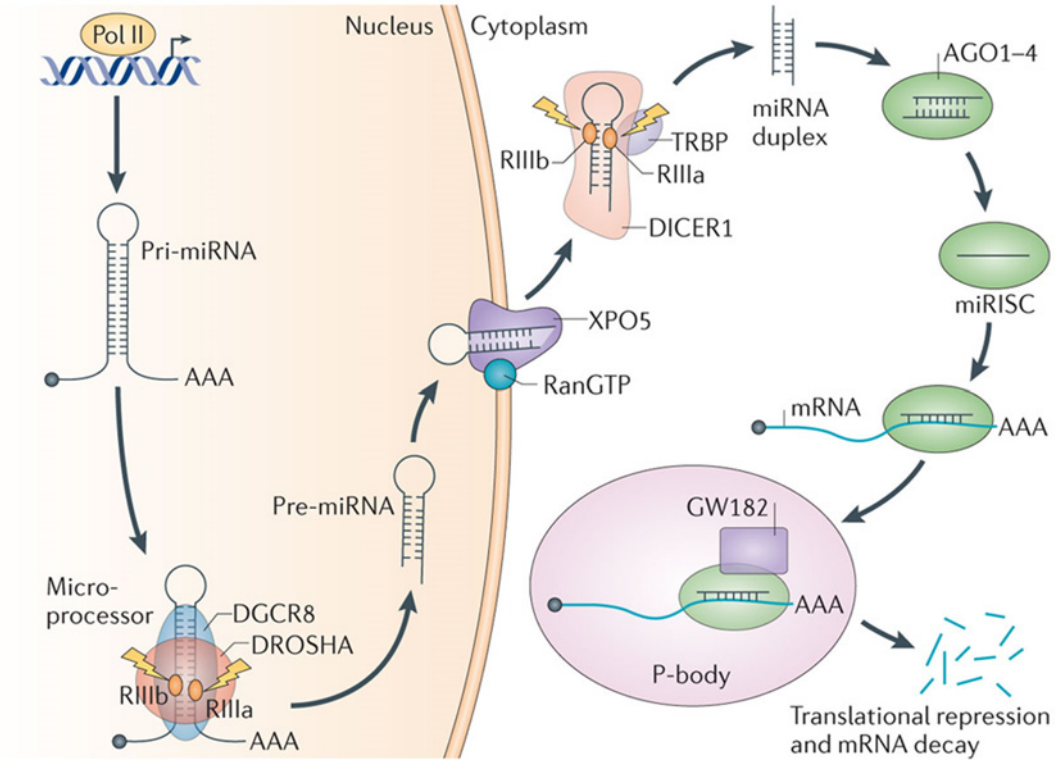

Figure 1. Biogenesis and function of miRNAs

miRNAs are transcribed by RNA polymerase II into primary transcripts called pri-miRNAs which fold into a stem–loop structure. They are trimmed in the nucleus by the Microprocessor complex into pre-mRNAs. The latter are then exported with the aid of exportin 5. In the cytoplasm the enzyme Dicer further trims the pre-miRNAs into a mature miRNA duplex. The duplex is then complexed by Argonaute and other proteins forming mature microribonucleoprotein (miRNP) complexes (also referred to as miRNA-induced silencing complex (miRISC)) in which only the guide strand of the miRNA is retained. The guide strand is able to bind to mRNA targets in the cytoplasm. miRNAs are also found in processing bodies (P-bodies), cytoplasmic granules involved in mRNA turnover. (Reproduced from [2] by permission from Macmillan Publishers Ltd, copyright 2015.)

thought [13]. Aberrant miRNA expression is associated with several pathological states. miRNAs can be detected in blood and urine [14] providing the framework for the development of new biosensing technology based on antisense oligonucleotides that are complementary miRNAs associated with disease.

As we have seen in this section, gene expression is an increasingly complex matter. There are several opportunities to exploit the advantages of oligonucleotides. They can be used to modify and monitor gene expression both in basic research and in gene therapy. Oligonucleotides have been used to repress mRNA translation binding mRNAs directly or to block miRNAs, or even to modify pre-mRNA splicing in the nucleus. We now turn our attention to DNA aptamers, discussing how they can be modified and used in a variety of biosensing applications.

DNA aptamers

An emerging class of protein-binding oligonucleotides are aptamers. These are single-stranded DNA or RNA sequences that are deliberately designed to bind to a particular molecule (including proteins) with high specificity and affinity. They are considered as alternatives to antibodies, where they can bind to their targets by undergoing conformational changes [15]. An aptamer for a specific target is derived through selective rounds of binding followed by amplification using the technique known as SELEX (systematic evolution of ligands by exponential enrichment). SELEX has been used to determine which DNA or RNA species are bound by proteins of interest. One of the most

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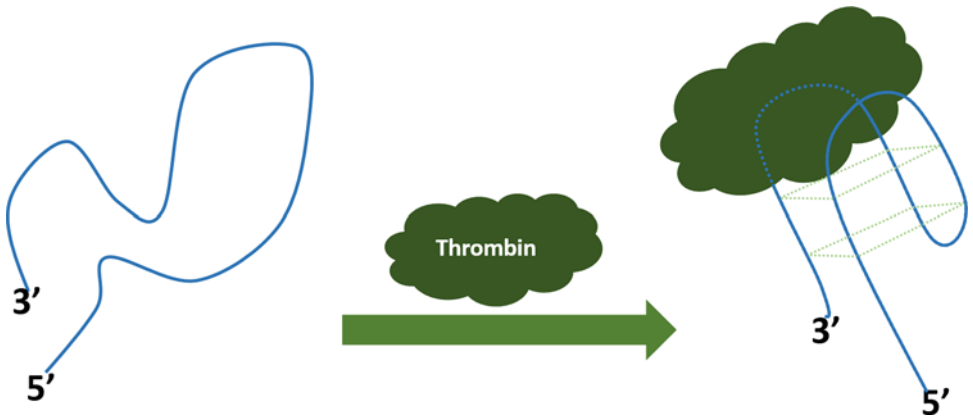

Figure 2. Change in conformation from a single-stranded DNA aptamer to a quadruplex structure upon specific binding with thrombin

widely studied DNA aptamers was raised against the protein thrombin. On recognizing thrombin, it forms a signature quadruplex structure by capturing the protein. The thrombin protein is 'trapped' within the structure and therein it stabilizes (Figure 2).

Aptamers have many advantages over antibodies making them very important molecular tools for both diagnostics and therapeutics. There has been an intense interest in the in-depth understanding of ligand binding and conformational properties. This has led to a range of bioassay methods that rely on aptamers as bioreceptors. Aptamers are currently widely used in drug-delivery applications along with a new emerging application as bioreceptors in bioassays and biosensors (termed 'aptasensors'). Aptamers can be exploited in different methodologies such as electrochemical, optical or mass sensitive [16,17]. Although aptamers have many advantages over antibodies, they still face some challenges relating to nuclease degradation or reduced binding efficiency because of DNA/RNA-binding proteins in the blood.

Artificial oligonucleotide analogues

In recent years, there is hardly a field in biology in which the potential of using synthetic oligonucleotides has not been explored. The reason for such a turnover is mainly due to the emergence of different molecular cloning techniques along with the simultaneous development of varied methods for efficient oligonucleotide synthesis. The primary motivations behind these developments for biochemists have been not only the huge biological potential but also immense demand for synthetic oligonucleotides. Synthetic oligonucleotides or nucleic acid analogues are compounds which are structurally similar to naturally occurring RNA or DNA. Some of the artificial nucleic acids include peptide nucleic acid (PNA), locked nucleic acid (LNA), glycol nucleic acid (GNA) and therose nucleic acid (TNA), which differ from naturally occurring RNA or DNA in the backbone structure of the molecule. Consequently, the availability of these synthetic oligonucleotides has led to a revolution in solving molecular biology problems along with promising applications in biosensing. The next section will give an overview on how synthetic oligonucleotides, such as PNA and LNA, can be transferred from in-solution application to on-surface biosensing applications.

Peptide nucleic acids

PNA was first invented by Nielsen et al. in 1991 [18]. PNA is a DNA analogue where the sugar–phosphate backbone of DNA is replaced by a backbone comprising of repeated units of *N*-(2-aminoethyl)glycine units via an amide linkage (Figure 3A). Such a modification changes the negative charges of the DNA sugar–phosphate backbone to a neutral charge of the peptide-like backbone. In a PNA, the four naturally occurring nucleobases, namely adenine, cytosine, guanine and thymine, are connected to the central amine of the peptide backbone via a methylene bridge and a carbonyl group. Therefore, PNA sequences are depicted like any peptide with an N-terminus at the left end position and a C-terminus on the right end position.

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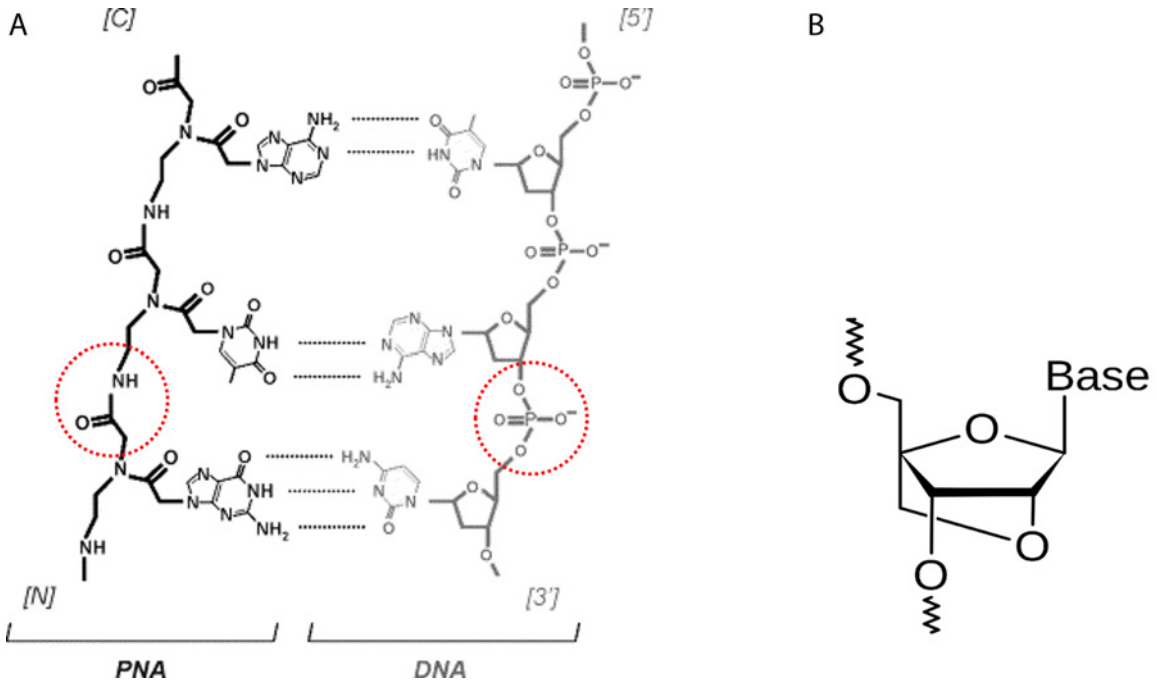

Figure 3. Chemical structure of PNA and LNA

(**A**) Chemical model of a PNA molecule (sequence N-GTA-C) hybridized in antiparallel orientation with its complementary DNA (sequence 5′-TAC-3′). The dotted line indicates the hydrogen bonding between complementary nucleobases. (**B**) Chemical structure of an LNA monomer. (Adapted from [26] with permission from Springer Science and Business Media.)

Since PNA has a neutral charge and proper interbase spacing, PNA can bind to its complementary DNA sequence with higher affinity and specificity following the rules of Watson–Crick base pairing [19]. This is because of the reduced electrostatic repulsion between PNA and DNA compared with DNA and DNA. It also results in reduced melting temperatures of the PNA–DNA duplex leading to higher thermal stability. Moreover, it has also been demonstrated that the stability of a PNA–DNA duplex is essentially independent of the ionic strength of the buffer in which hybridization is performed [20].

Because of its unique physico-chemical and biochemical properties, PNA as a bioreceptor opens up many applications (biological and diagnostic) which would not be achievable with naturally occurring oligonucleotides. Many reports in the literature show how PNA has been exploited to detect miRNAs/DNA in biological samples. A range of electrochemical techniques have been successfully applied for PNA-based biosensing [21–22]. For example, Keighley et al. [22] demonstrate how the neutral charge of PNA can be exploited with electrochemical impedance spectroscopy: whereas DNA probes on an electrode offer high resistance to negatively charged redox markers in solution [23], neutral PNA probes do not; upon binding with the complementary strand, a massive increase in resistance was observed because of the addition of negative charges and increased binding efficiency compared with DNA–DNA.

Locked nucleic acids

LNAs are another class of synthetic nucleotides which is often referred to as inaccessible RNA. LNA was first synthesized by both Obika et al. [24] and Koshkin et al. [25] in 1997. It consists of a modified RNA nucleotide, where the ribose moiety is modified with an extra bridge that connects the 2′ oxygen and 4′ carbon (Figure 3B). Such a linkage via a methylene bridge restricts (locks) the ribofuranose into the 3′-endo conformation, which is responsible for LNA–DNA or LNA–RNA heteroduplexes [26]. Interestingly, LNA nucleotides can be easily mixed with DNA or RNA molecules in the oligonucleotide sequence, which greatly improves the thermal stability of LNA–DNA or LNA–miRNA duplexes. LNA, like DNA, forms specific base pairing with complementary DNA/RNA sequences following Watson–Crick rules. By introducing LNA molecules, the melting temperature can be increased by 2.0–6.0 °C per LNA monomer for an LNA–DNA duplex and 3.0–9.6 °C for an LNA–RNA duplex [27]. It has been reported that

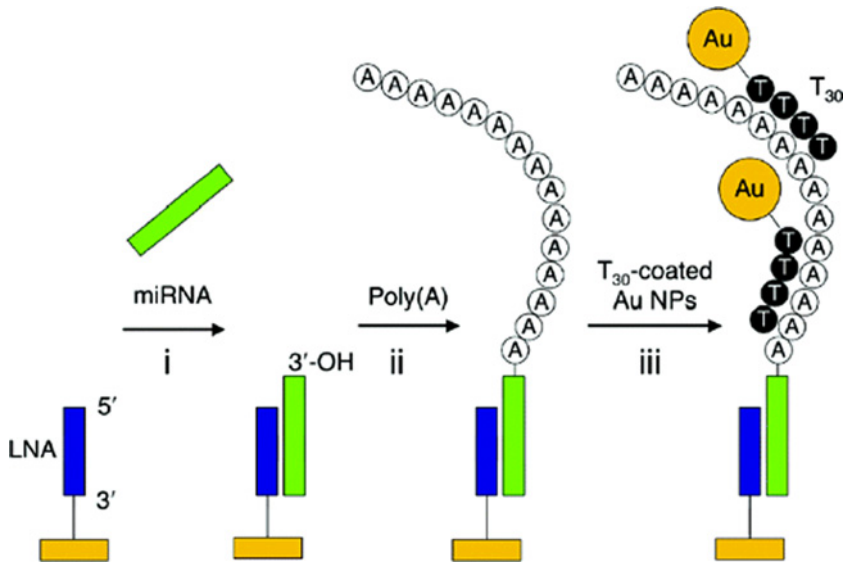

Figure 4. Schematic diagram showing the detection of miRNAs with nanoparticle amplified SPR detection

(**i**) specific hybridization of miRNA on to a complementary LNA array; (**ii**) addition of poly(A) tails to the surface-bound miRNAs using poly(A) polymerase enzyme; and (**iii**) hybridization of T_{30}-coated gold nanoparticles (Au NPs) to the poly(A) tails. (Reproduced with permission from [29], copyright 2006 American Chemical Society.)

LNA forms the strongest duplexes with RNAs which has opened new doors for miRNA technology along with DNA detection [28] and biosensors.

LNA has been used as a probe for microarray technology for the detection of multiple miRNAs via a novel approach, achieving limits of detection in the attomolar range [29]. Figure 4 depicts this novel approach where the enzyme reaction is combined with nanoparticle amplification using surface plasmon resonance (SPR) as a detection technique. Briefly, a poly(A) polymerase was used to extend the miRNAs bound to LNA on the surface with a poly(A) tail. Later, gold nanoparticles modified with poly(T) tails were hybridized with the poly(A) tail which could then be detected using SPR.

Although PNA and LNA have been shown to have many advantages over naturally occurring DNA or RNA, they still suffer from limitations that include the constraints with length of the sequence and composition of bases that can affect the stability of PNA or LNA. This is one of the main reasons PNA- or LNA-based microarrays are currently not used as high-throughput biosensors. Nonetheless, biosensors based on artificial analogues are still in the early stage of development and exciting new developments are expected as these become more mature.

Conclusion

The use of natural and synthetic nucleotides is still developing and paves the way towards advanced biosensor development. It can be seen from the literature how researchers from different fields are coming together to realize high-throughput oligonucleotide-based biosensors for use with complex matrix samples, e.g. clinical or environmental. The ease of manipulation of oligonucleotides, controlled surface chemistry approaches and 'straightforward' charge distribution, makes them optimal bioreceptors for biosensing applications.

Developments in biochemistry and molecular biology have led to a deeper understanding of the role of oligonucleotides and showed that the functions they play are far greater than originally expected. This leads to new worlds of biosensing applications, where oligonucleotide-based biosensing approaches can have an unparalleled impact on clinical diagnosis, prognosis and monitoring, as well as environmental and food control monitoring.

The increasing demand for enhanced efficiency and to overcome some of the drawbacks of using naturally occurring oligonucleotides has enabled biochemists to come up with synthetic analogues such as PNA and LNA, which have further increased the prospects of novel biosensing approaches.

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Summary

- DNA-based biosensors can be used for a wide range of applications, from genetic identification to pathogen detection and disease diagnosis in biomedical, environmental and forensic applications.
- The negative charge of DNA or RNA strands makes them ideal biorecognition and/or target elements in a range of electrochemical biosensing approaches.
- MicroRNAs can provide detailed 'fingerprints' of diseases and medical conditions.
- DNA/RNA aptamers are single-stranded oligonucleotides that can bind to a range of biomolecules with very high affinity through conformational changes.
- Aptamers can replace antibodies in biosensor development, providing a new way to control bioreceptor immobilization, density and orientation on to surfaces, and hence the viability of sensitive sensors.
- Peptide nucleic acids and locked nucleic acids are examples of novel synthetic DNA/RNA analogues that can provide higher sensitivity and selectivity in biosensors.

Abbreviations

HER2, human epidermal growth factor receptor 2; LNA, locked nucleic acid; PNA, peptide nucleic acid; SELEX, systematic evolution of ligands by exponential enrichment; SPR, surface plasmon resonance; STAT3, signal transducer and activator of transcription 3.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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