

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

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Abstract

There is 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 DNA, RNA or a synthetic analogue to naturally occurring nucleic acids. This chapter will draw light upon various types of nucleic acids such as DNA, RNA (particularly microRNAs), their role and their application in biosensing. Also, it will cover DNA/RNA aptamers, which can be used as bioreceptors to a wide range of targets such as proteins, small molecules, bacteria and even cells. It will also highlight how the invention of synthetic oligonucleotides like PNA or 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 lab-based status and the reality of biomedical applications.

Keywords: Oligonucleotides, microRNAs, aptamers, peptide nucleic acids, locked nucleic acids, biosensing

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Introduction

Oligonucleotides are unmodified or chemically modified polymers (DNA or RNA) that are relatively small (12-25-mer) introducing an expanded range of applications in molecular genetics research and in 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 noncoding RNAs (e.g. microRNAs). 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 the 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* hybridisation, and in biosensing applications. The explosion of knowledge about gene expression and gene regulation mechanisms has led to many new opportunities to develop oligonucleotide-based technologies. In order to appreciate these opportunities, this chapter begins with a brief overview of the increasing complexity of gene expression.

The **complexities** of gene expression: a brief summary

The last decades have witnessed an explosion of knowledge in 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.**

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 posttranscriptional control (pre-mRNA processing, mRNA localisation, 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 attention particularly to two topics that have attracted a lot of interest recently: alternative splicing and noncoding 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 noncoding 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 acetyl transferases, deacetylases, protein kinases and methyltransferases. DNA can also be modified directly at CpG positions by 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 messenger RNA, 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. Messenger RNAs 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 noncoding 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), 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 fruit fly *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 in the biosensing field 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**. *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 *HER2* Δ 16 is produced by skipping exon 16. Exon 16 is 48 bases long and contributes 16 amino-acids in frame to the extracellular domain. *HER2* Δ 16 **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 Duchennes Muscular Dystrophy. The following example illustrates how oligonucleotides can be potentially used as anticancer agents. *STAT3* is involved in the activation of several

oncogenic pathways. The splice variant STAT3 β arises from an alternative 3' splice site in exon 23; it encodes a truncated pro-apoptotic isoform (in contrast to the oncogenic STAT3 α isoform). A phosphorodiamidate morpholino antisense oligonucleotide was targeted to a 'splice enhancer' sequence that resulted in increased STAT3 β 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 in the future antisense oligonucleotides become mainstream pharmacological agents.

The increasing importance of noncoding 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 noncoding RNAs. Noncoding RNAs include ribosomal RNA (rRNA) and transfer RNAs (tRNA) required for translation. They also include small noncoding RNAs involved in the processing of other RNAs (for example, snRNAs involved in splicing and snoRNAs in pre-rRNA processing). These have been known for a long time; however more recently, several other types of noncoding RNA have been discovered. They are subdivided into short and long noncoding RNAs. The (somewhat arbitrary) definition of a long noncoding RNA is that it is more than 200 nucleotides in length.

Long noncoding RNAs (also known as megaRNAs or lncRNAs) are best known for their involvement in epigenetic regulation [11]. Long noncoding 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 lncRNA called *XIST* (X-inactive specific transcript) provides the molecular basis of X chromosome inactivation in female mammals [12].

Short noncoding RNAs include the microRNAs. MicroRNAs (often abbreviated to miRNA) 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). MicroRNAs 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 microRNAs 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 thought [13]. Aberrant miRNA expression is associated with several pathological states. MicroRNAs 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 microRNAs associated with disease.

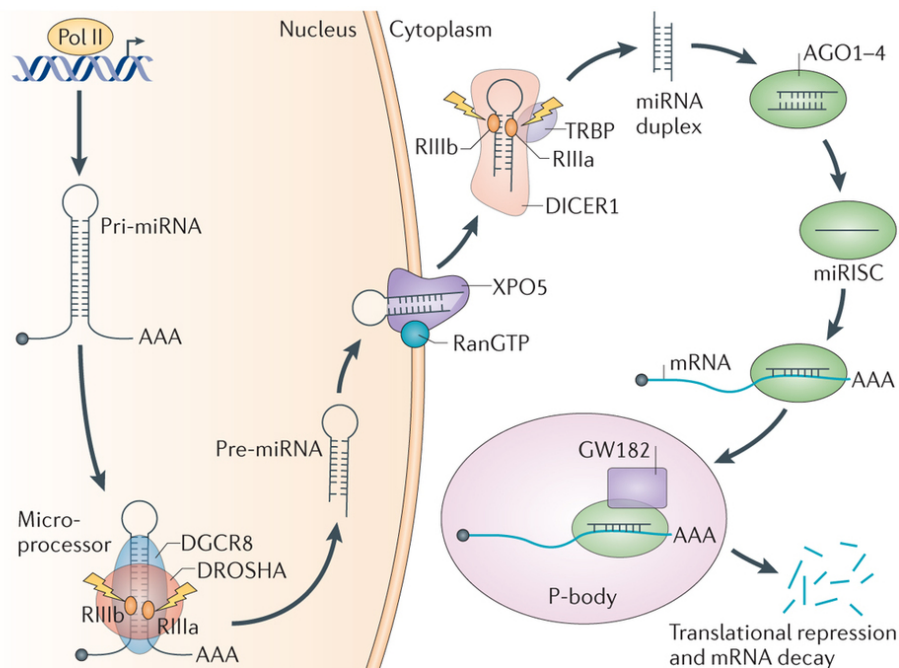


Figure 1. Biogenesis and function of microRNAs. MicroRNAs are transcribed by RNA polymerase II into primary transcripts called pri-miRNAs which fold into a stemloop structure. They are trimmed in the nucleus by a microprocessor complex into pre-mRNAs. The latter are then exported with the aid of exportin 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 miRNP complexes (also referred to as 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 P-bodies, cytoplasmic granules involved in mRNA turnover. (Reproduced from [2] by permission from Macmillan Publishers Ltd, copyright 2015).

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, and 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 in 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 widely studied DNA aptamers was raised against the protein thrombin. On recognising thrombin, it forms a signature quadruplex structure by capturing the protein. The thrombin protein is “trapped” within the structure and therein it stabilises (figure 2).

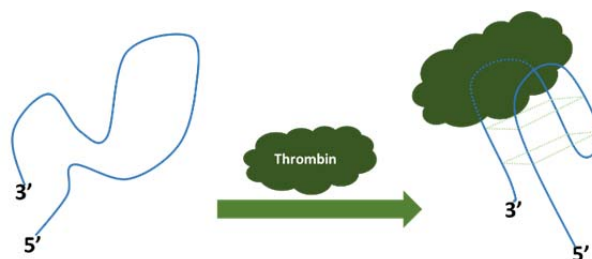


Figure 2 - Change in conformation from single stranded DNA aptamer to a quadruplex structure upon specific binding with thrombin

Aptamers have many advantages over antibodies making them very important molecular tools for both diagnostics and therapeutics. There has been an intense interest in understanding the in-depth 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 application along with a new emerging application as bioreceptors in bioassays and in biosensors (termed “aptasensors”). Aptamers can be exploited in different methodologies such as electrochemical, optical, mass sensitive, etc [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 simultaneous development of varied methods for efficient oligonucleotide synthesis. The primary motivations behind these developments for the 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 acids (PNA), locked nucleic acids (LNA), glycol nucleic acids (GNA) and theros nucleic acids (TNA), which differ from naturally occurring RNA or DNA in the backbone structure of the molecule. Consequently, availability of these synthetic oligonucleotides has led to a revolution in solving molecular biology problems along with promising applications in biosensing. This section will give an overview on how such synthetic oligonucleotides such as PNA and LNA can be transferred from in-solution application to on-surface biosensing applications.

Peptide Nucleic Acids

Peptide Nucleic Acid (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 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 N-terminus at the left end position and C-terminus on the right end position.

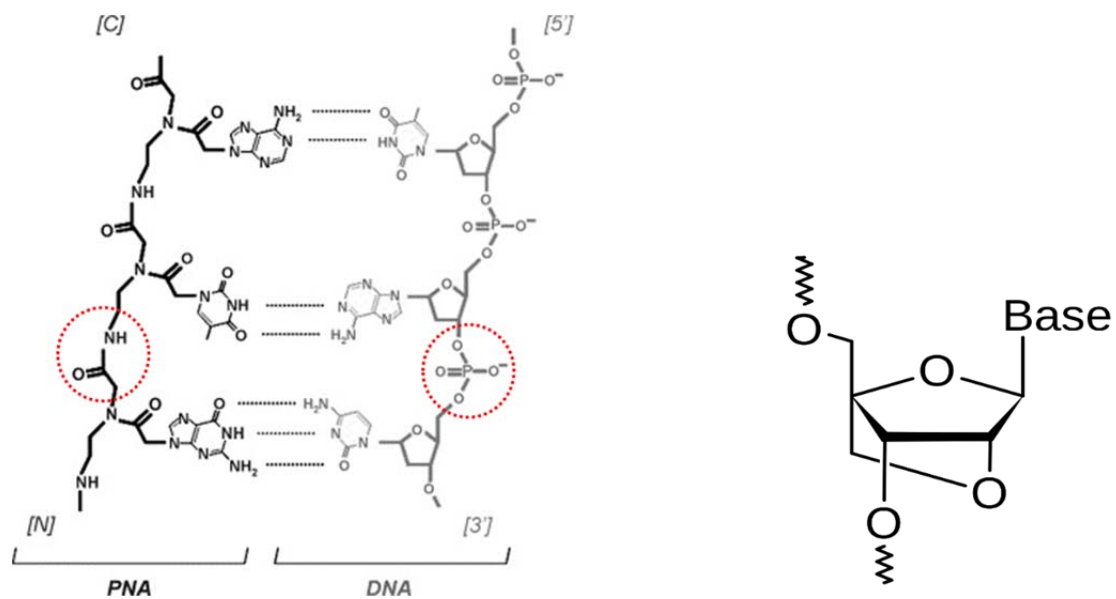


Figure 3. A) Chemical model of a PNA molecule (sequence N-GTA-C) hybridized in antiparallel orientation with its complementary DNA (sequence 5'-TAC-3'). The dotted line indicates the hydrogen bonding between complementary nucleobases. B) Chemical structure of an LNA monomer. (Adapted from [26] with permission 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 reduced electrostatic repulsion between PNA/DNA as compared to DNA/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 hybridisation is performed [20].

Because of its unique physicochemical and biochemical properties, PNA as a bioreceptor opens up many applications (biological and diagnostic) which would not be achievable with naturally occurring oligonucleotides. Many literature reports 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] demonstrated how the neutral charge of PNA can be exploited with electrochemical impedance spectroscopy: while DNA probes on an electrode offer high resistance to negatively charged redox markers in solution [23], neutral PNA probes don't.; upon binding with the complementary strand, a massive increase in resistance was observed because of addition of negative charges and increased binding efficiency as compared to DNA/DNA.

Locked Nucleic Acids

Locked nucleic acids (LNA) are another class of synthetic nucleotides which is often referred to as inaccessible RNA. LNA was first synthesized by both Imanishi *et al.* and Wengel *et al.* in 1997 [24,25]. 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, form specific base pairing with complementary DNA/RNA sequences following Watson-Crick rules. By introduction of LNA molecules, the melting temperature can be increased by 2.0 to 6.0 °C per LNA monomer for LNA/DNA duplex and 3.0–9.6 °C for LNA/RNA duplex [27]. It has been reported that 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 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.

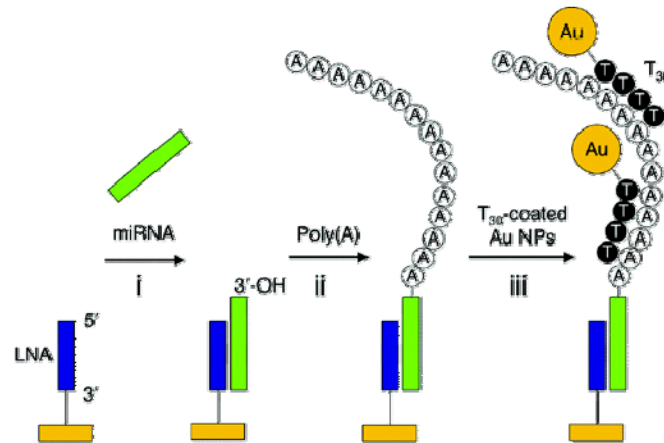


Figure 4. Schematic showing the detection of microRNAs with nanoparticle amplified SPR detection: (i) specific hybridization of miRNA onto a complementary LNA array; (ii) addition of poly(A) tails to the surface-bound miRNAs using poly(A) polymerase enzyme; and (iii) hybridization of T₃₀-coated gold nanoparticles (Au NPs) to the poly(A) tails. (Reproduced with permission from [29], copyright 2006 American Chemical Society).

Although PNA and LNA have 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 why PNA or LNA based microarrays are currently not used as high-throughput biosensors. Nonetheless, biosensors based on artificial analogues are still in their 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 paving the way towards advanced biosensor development. It can be seen from literature how researchers from different fields are coming together to realise high-throughput oligonucleotide-based biosensors for use with complex-matrix samples such as 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 in 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.

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 stands 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 on biosensor development, providing a new way to control bioreceptor immobilization, density and orientation onto 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.

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Michael Lodomery read his first degree at the University of Melbourne where he majored in Genetics. He then did a PhD at the University of St Andrews, studying the protein composition of stored maternal mRNAs in *Xenopus laevis* oocytes. He stayed in St Andrews for his first postdoctoral job, funded by the Wellcome Trust, to work on the RNA helicase Xp54. His second postdoctoral appointment was at the MRC Human Genetics Unit, initially funded by Kidney Research UK, working on the posttranscriptional roles of the WT1 transcription factor. He was then appointed Senior Lecturer at the University of the West of England in Bristol, and he is now an Associate Professor in Molecular Genetics.