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REVIEW

# Applications of peptide nucleic acids (PNAs) and locked nucleic acids (LNAs) in biosensor development

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**Abstract** Nucleic acid biosensors have a growing number of applications in genetics and biomedicine. This contribution is a critical review of the current state of the art concerning the use of nucleic acid analogues, in particular peptide nucleic acids (PNA) and locked nucleic acids (LNA), for the development of high-performance affinity biosensors. Both PNA and LNA have outstanding affinity for natural nucleic acids, and the destabilizing effect of base mismatches in PNA- or LNA-containing heterodimers is much higher than in double-stranded DNA or RNA. Therefore, PNA- and LNA-based biosensors have unprecedented sensitivity and specificity, with special applicability in DNA genotyping. Herein, the most relevant PNA- and LNA-based biosensors are presented, and their advantages and their current limitations are discussed. Some of the reviewed technology, while promising, still needs to bridge the gap between experimental status and the harder reality of biotechnological or biomedical applications.

**Keywords** Nucleic acid analogue · DNA · Hybridization · SNP · Microarray · Self-assembled monolayer

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## Introduction

Among the different definitions of biosensors that have been elaborated in recent decades, an updated version of that selected by the International Union of Pure and Applied Chemistry (IUPAC) in 1999 [1] is: "a biosensor is a compact analytical device incorporating a biological or biologically derived sensing element, either integrated within or intimately associated with a physicochemical transducer" [2]. The two main families of biosensors currently used are based on bio-affinity and bio-catalytic processes involving different types of bioreceptor or "probe" molecule (for example proteins, natural and artificial nucleic acids, or carbohydrates), combinations of these, and macromolecular assemblies and even whole cells or fragments of tissues [3]. Nucleic acid based biosensors are those in which the probe molecule is DNA, RNA, or a synthetic polymer analogous to natural nucleic acids [4]. Most of the current nucleic acid based biosensors exploit their base pair hybridization properties, although some use aptamers as the biosensing element [5]. Aptamers are single-stranded nucleic acids with a specific three-dimensional structure that are able to specifically recognize their targets by means of molecular interactions analogous to those operating in antibody–antigen pairs [6, 7].

Nucleic acid immobilization on the biosensor surface is an important initial step that affects the overall performance of the sensor. In general, nucleic acids are immobilized onto solid surfaces in such a way that a signal is obtained only if they react with their specific target molecules. Hence, experimental conditions must be adjusted for every application, and a large choice of immobilization methods can be used. These include covalent binding (immobilization onto the surface via one end of the nucleic acid molecule, e.g., an epoxy-modified surface that binds to a 5′-amino-modified DNA oligonucleotide), non-covalent binding (e.g., affinity

binding based on the strong avidin–biotin system), and chemisorption (e.g., formation of self-assembled monolayers—SAMs, adsorption of thiolated oligonucleotides on gold surfaces, etc.) [4]. Regarding transduction systems, current nucleic acid biosensors benefit from the sensitivity and specificity offered by optimized electrochemical, electrical, optical, mechanical, acoustic, or thermal methods [8, 9].

Nucleic acid biosensors are used in different fields of genomics including genotyping and gene-expression studies [5, 10]. Some of their current applications take advantage of the development of different families of nucleic acid analogues, which overcome specific limitations of natural nucleic acids for biosensing. In particular, the use of peptide nucleic acid (PNA) and locked nucleic acid (LNA) probes enables high biosensor sensitivity and specificity to be achieved, although the specific features of these polymeric molecules also introduce some limitations in their use. The unique physicochemical nature of the peptidomimetic, neutral PNA backbone have promoted the use of PNA oligomers as capture probes in electrochemical, optoelectronic, and microarray-based biosensors, and in other types of sensor. In turn, LNA-based biosensors benefit from the restricted conformation of LNA monomers and the possibility of designing chimeric molecules which contain both LNA nucleotides and DNA or RNA nucleotides. In the sections below, the main physicochemical features of the nucleic acid analogues and their usefulness in biosensing are discussed, the most relevant achievements in PNA and LNA-based biosensors are critically reviewed, and a comparative analysis of the relative biosensing potential of PNA and LNA is provided. Finally, current challenges and future trends in the field are emphasised.

## Nucleic acid analogues and their applications in biosensing

Several families of nucleic acid analogues have been synthesized in recent decades by incorporation of artificial nucleobases into their natural backbones (reviewed elsewhere [11, 12]) and by replacing their ribose phosphate backbone either by combinations of other sugars and linkage isomers or by short linear motifs of glycerol or glycine derivatives. Investigation of nucleic acid analogues with alternative polymeric backbones was initiated in the early 1980s. The objective was to synthesize polymeric molecules containing nucleobases whose spacing and conformation enabled the formation of heteroduplexes with DNA or RNA by specific base pairing. One of the first molecules developed was a glycerol-derived nucleic acid (GNA), the backbone of which is composed of phosphodiester-linked acyclic glycerol units (Fig. 1a) [13]. Despite basic and technological interest in this acyclic three-carbon sugar-containing analogue [14], its usefulness for biosensing remains to be proved.

Alternative backbones for nucleic acid analogues have been obtained by using sugar motifs other than deoxyribose or ribose. Among these, pyranosyl-RNA (p-RNA) is an artificial analogue that contains six-membered, β-D-ribopyranosyl instead of ribofuranosyl units (Fig. 1b) [15]. p-RNA is capable of forming duplexes with natural RNA in antiparallel orientation [16, 17], although biosensors based on p-RNA have not yet been developed. In turn, threose nucleic acid (TNA) is an analogue based on α-L-threofuranosyl units joined by 3′→2′ phosphodiester linkages (Fig. 1c) [18]. Because threose is one of the two four-carbon monosaccharides, TNA is the simplest of all potential sugar-containing nucleic acids. TNA hybridizes efficiently with DNA and RNA in a sequence-specific manner, and, therefore, could be a good candidate for the development of biosensors in the near future.

Other nucleic acid analogues have been synthesized that contain conformational restricted sugar motifs; these include the bicyclo-DNA and tricyclo-DNA families [19, 20]. The most biotechnologically relevant representative of this family is LNA, a polymer of 2′-*O*,4′-*C*-methylene-linked β-D-ribonucleotide monomers (Fig. 1d) [21, 22]. The linkage of the 2′-*O* and the 4′-*C* atoms via a methylene bridge restricts or "locks" the ribofuranose into the 3′-*endo* conformation, which is responsible for the A conformation of the LNA/DNA and LNA/RNA heteroduplexes [23]. LNA has been reported to form the strongest duplexes with complementary RNA so far described, and it also has very high affinity for DNA [22, 24, 25]. This has encouraged the development of LNA-based biosensors, as will be discussed in a specific section of this review.

Polymeric backbones for nucleic acid mimics have also been produced by replacement of phosphate by pyrophosphate, polyphosphate, or alkylphosphate, and by sulfones or other sulfur-containing linkers [26–28]. A different approach was followed in the synthesis of PNA, an analogue whose backbone lacks both the sugar-based and the phosphate-related groups typical of natural nucleic acids and most of their artificial mimics. PNA is the result of polymerization of *N*-(2-aminoethyl)glycine units, each nucleobase being connected to the peptidomimetic structure by a methylencarbonyl linkage (Fig. 1e) [29]. Thus, PNA combines nucleic acid features with others typical of peptides and proteins. It has unique physicochemical properties, being an achiral, uncharged polymer [30, 31] capable of strongly and specifically binding to complementary targets (DNA, RNA, or PNA) according to the Watson–Crick rules for base-pairing [32]. The outstanding usefulness of PNA oligomers as probe molecules for biosensor development is described in the next section. Recently, a novel PNA-related molecule termed "thioester PNA" (tPNA) has been developed, which combines side-chain protein functionality with the capacity of base-pairing with natural nucleic acids [33].

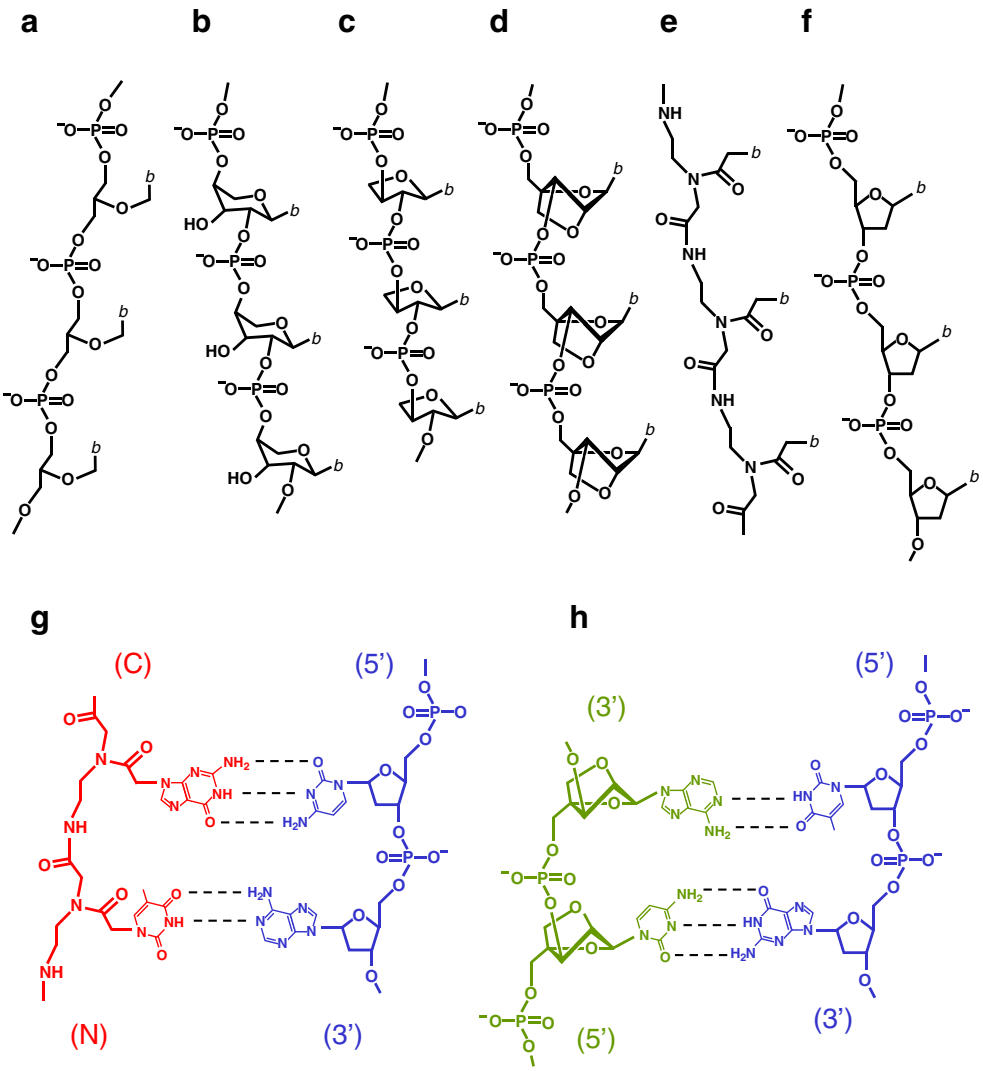


**Fig. 1** Schematic representation of the molecular backbones of five relevant nucleic acid analogues (the letter *b* denotes the position of the nucleobase): glycerol-derived nucleic acid, GNA (**a**); pyranosyl-RNA, p-RNA (**b**); threose nucleic acid, TNA (**c**); locked nucleic acid, LNA (**d**); peptide nucleic acid, PNA (**e**). The structure of ssDNA has been included for comparison (**f**). Schematic chemical model of PNA (*red*) and LNA (*green*) hybridized with DNA (*blue*) in antiparallel orientation (**g** and **h**, respectively), with the hydrogen bonding between complementary nucleobases depicted by *dotted lines*

In summary, the most useful nucleic acid analogues for biosensing are PNA and LNA. Therefore, this review will focus on the physicochemical features of these two artificial polymers and will emphasize the most relevant applications (and current limitations) of PNA and LNA-based biosensors.

## PNA-based biosensors

PNA has high affinity for its complementary DNA or RNA molecules, mainly because of the lack of electrostatic repulsion between the uncharged PNA backbone and that of the natural nucleic acid. Since PNA was designed by Nielsen et al. in 1991 [29], it has been evident that for most of the sequences investigated any single-stranded (ss) PNA oligomer had greater affinity for its complementary DNA molecule than the equivalent ssDNA strain for the same target. At moderate salt concentrations, the thermal stabilities increase in the order: DNA/DNA <PNA/DNA <PNA/RNA <PNA/PNA [34]. In all cases, the hybridization in the antiparallel orientation (the amino terminus of the PNA facing the 3′ end of the DNA or RNA; Fig. 1g) is more stable, although sequence-specific binding in the parallel orientation is also possible [30, 32]. The thermodynamics of hybridization of PNA/DNA heteroduplexes have been investigated in solution by use of absorption hypochromicity melting curves and isothermal titration calorimetry. For perfectly sequence-matched duplexes of different lengths (6–20 bp) and sequences, the average free energy of binding ($\Delta G$) per base pair was determined to be $-6.5\pm0.3$ kJ mol$^{-1}$ [34] (Table 1).

X-ray crystallography, nuclear magnetic resonance, fluorescence energy transfer, and other, complementary techniques have shown that the typical structures of PNA/DNA heteroduplexes are extended double helices whose features are intermediate between those of the A and B forms of dsDNA. Thus, the PNA/DNA duplex has a helix diameter of 2.3 nm and a helical rise of 4.2 nm with 13 bp per turn [31, 34, 35]. The structures of the PNA/RNA heteroduplex in solution [36] and the crystal structure of the PNA/PNA

**Table 1** Main thermodynamic properties relevant to PNA and LNA hybridization with natural nucleic acids (6 to 20-mers, at micromolar levels) in solution. Comparable DNA data (for hybridization in 1 mol $L^{-1}$ $Na^{+}$) are included

| | PNA | LNA | DNA |
|---|---|---|---|
| $\Delta G$ (average) per base pair in duplexes with DNA (kJ $mol^{-1}$) | −6.5 (at 25 °C) [34] | −4.4 (at 37 °C) [126]<br>−7.2 (at 5 °C) [127] | −6.2 (at 37 °C) [126]<br>−5.9 (at 25 °C) [129] |
| $\Delta T_m$ (range) per monomer in duplexes with DNA (°C) | 4.6–4.9 (antiparallel)<br>3.7–3.8 (parallel) [30] | −0.3–7.3 [126] | 2.0–4.8 [23, 30, 130, 131] |
| $\Delta T_m$ (range) per monomer in duplexes with RNA (°C) | 4.8 (antiparallel)<br>3.4 (parallel) [30] | 3.0–9.6 [22] | 2.0–4.0 [23, 130, 131] |
| Decrease in $T_m$ (range) per base mismatch in duplexes with DNA (°C) | 8–20 [30]<br>15–20 [34] | 1–8 [22, 128] | 1–9 [132] |

homoduplex [37] have also been resolved. Also, because of the high affinity of PNA for DNA, the so called "triplex forming" homopyrimidine ssPNA oligomers are capable of hybridizing to double-stranded (ds) DNA molecules by a mechanism known as "strand invasion" [38]. The interaction of PNA with DNA and RNA is highly specific, and for virtually all base-pair mismatches the decrease in thermal stability is greater for the PNA/DNA or PNA/RNA heteroduplexes than for the corresponding homoduplexes [30]. Particularly relevant for biosensing applications in the field of DNA genotyping, the melting temperature (Tm) of 9 to 12-mer PNA/DNA duplexes with a single base mismatch drops in the range of 15–20 °C relative to that of the perfectly complementary sequence [34].

PNA has outstanding chemical and thermal stability [29, 30] and is insensitive to enzymatic biodegradation by nucleases or peptidases [39]. Additionally, the uncharged nature of its peptidomimetic backbone makes PNA/DNA hybridization highly insensitive to changes in pH or ionic strength [29, 40]. In turn, the interaction of PNA with surfaces has been investigated from both basic and technological perspectives. Thiol-modified PNA oligomers have unprecedented capability for self-assembly on gold surfaces, adopting a standing-up conformation [41]. SAMs of PNA on surfaces tend to interact specifically with complementary nucleic acid molecules [42, 43], and are, therefore, useful for biosensing applications [44]. This behaviour has also been observed with unmodified PNA oligomers on functionalized silicon surfaces [45].

All these features make PNA an optimum probe molecule for development of different kinds of affinity biosensor. The rest of this section comprises a critical, non-comprehensive review of the main electrochemical, optoelectronic, and microarray-based biosensors developed so far, followed by some relevant examples of other types of biosensor.

## Electrochemical

Electrochemical biosensors have several advantages over those using alternative transduction systems, because they are easy to miniaturize, simple, rapid, and inexpensive [8, 9]. Consequently, and because of the physicochemical features of PNA, there has been substantial interest in developing PNA-based electrochemical sensors for different biochemical and biotechnological applications [46]. The advantages of using PNA as recognition elements in electrochemical biosensors were first reported by Wang et al. in 1996 [47]. In their approach, a 15-mer PNA probe was adsorbed on to a carbon-paste electrode transducer, and the formation of the PNA/DNA hybrid was detected by its exposure to a solution of a redox indicator. The hybridization response was almost independent of the ionic strength and hybridization temperature although a fair detection limit of $5\times10^{-9}$ mol $L^{-1}$ was achieved. Following this achievement, the same group was able to detect a specific mutation in the p53 gene, thus showing the potential of PNA-based biosensors for mutation screening and single-nucleotide polymorphism (SNP) mapping. In their work, when the biosensor (containing a 17-mer PNA oligo as the probe element) was hybridized to a single-base mismatch DNA oligomer used as the mutant target, the hybridization signal was only 3% that of the perfect matching hybridization. In turn, the unspecific hybridization signal was a 91% using an equivalent DNA-coated electrode. However, those responses were achieved for very high concentrations of the target (a minimum of $6\times10^{-7}$ mol $L^{-1}$) [48]. A $6\times10^{-10}$ mol $L^{-1}$ detection limit has recently been reported by Raaof et al. for detection of p53 gene mutations [49] using methylene blue as an electrochemical indicator. Although effective discrimination against a SNP-containing DNA target was achieved, the authors did not test their biosensor with PCR amplicons or real samples. Hejazi et al. [50] developed an electrochemical DNA biosensor which relies on self-assembly on to the electrode surface of a 14-mer PNA probe containing a specific sequence of the hepatitis C virus (HCV) genome. The calculated detection limit was $5.7\times10^{-11}$ mol $L^{-1}$, although the linear range was $1–50\times10^{-9}$ mol $L^{-1}$ and neither PCR nor real samples were used. This group also reported [51] use of the same PNA probe for

direct detection of the complementary sequence present in dsDNA oligos via triplex formation, achieving a detection limit of $1.8\times10^{-12}$ mol $L^{-1}$ under the same conditions. Furthermore, they have recently reported the detection of SNPs in different PCR samples with high sensitivity (detection limit $4.8\times10^{-12}$ mol $L^{-1}$), although the hybridization time was too long—up to 15 h was needed [52].

Luo et al. [53] described the multiplex detection of sequence-specific DNA without requiring probe immobilization but, instead, using a PNA-labelled probe with an electroactive indicator and a negatively charged indium tin oxide (ITO) electrode. When the DNA target was hybridized with the PNA probe, the electrostatic repulsion between the negative backbone of the DNA in the PNA/DNA duplex and the negative surface of the electrode prevented the electroactive indicator from approaching the electrode, thus resulting in a substantially suppressed electrochemical signal. The authors reported that SNP detection was achieved within minutes at 37 °C, and that the sensor can operate in multiplex format by using different PNA probes labelled with distinguishable electroactive indicators. In our view, such a requirement will probably be a challenging step for developing a highly multiplexed biosensor, and improvement of the sensitivity is also essential. The same group reported further data about this methodology after monitoring PNA/DNA hybrid dissociation in real-time at different temperatures [54]. Hüsken et al. [55] reported a new design consisting of two electrochemically distinguishable ferrocenyl (Fc)–PNA conjugates that were simultaneously immobilized on to a gold electrode. Upon DNA hybridization, each one selectively induced specific changes in the electrochemical response. Nevertheless, the biosensor was tested with DNA oligonucleotides at extremely high concentrations ($5\times10^{-5}$ mol $L^{-1}$) and reaction for 4 h was required, evidence of a lack of sensitivity that must be addressed for real applicability of this technique.

Inspired by the fact that PNA oligomers cannot function as primers for DNA polymerases, Kerman et al. [56] used a PNA probe to block a PCR amplification process involved in an electrochemical biosensor. The specificity of PNA-mediated PCR clamping is such that two alleles of the alcohol dehydrogenase gene that differ by one SNP only could be discriminated. When a mutation exists in the queried gene, the "PCR clamping PNA probe" does not bind to that region and the PCR takes place, resulting in amplification of the dsDNA. Then, a "capture PNA probe" attached to the surface of the glassy carbon electrode (GCE) binds to its complementary sequence on the amplicon, and the subsequent accumulation of $[Co(NH_3)_6]^{3+}$ on the sensor surface (by electrostatic binding to the PNA/DNA duplex) results in a higher current signal. In contrast, in the presence of the *wild type* gene, the PCR clamping PNA probe binds strongly to its fully complementary DNA strand and effectively blocks the PCR amplification, which results in less accumulation of $[Co(NH_3)_6]^{3+}$ on the sensor surface and a lower current signal. Despite the good performance of this biosensor, further improvement of its detection level are required before it is chosen in preference to other previously reported electrochemical systems.

Many other reports on PNA-based electrochemical biosensors have been published in the last decade. Some of these describe new detection schemes or novel strategies for probe immobilization, and multiplexing formats or sensitivity improvements even working with real samples. Nonetheless, a large fraction of these articles deal with proof of concept devices, far removed from real-life or commercial applications for which flexibility, easy handling, and high sensitivity are always required. We will, therefore, focus on the few articles that, in our view, have real potential applicability in their current format. Among these, Fang et al. [57] described an electrocatalytic reporter system with PNA probes immobilized on an electrode consisting of three-dimensional gold nanowires. The biosensor was used to detect a newly identified cancer biomarker at concentrations of $10^{-13}$ mol $L^{-1}$ RNA, even in the presence of a large excess of non-complementary sequences. In addition, the sensor detected $10^{-8}$ g mRNA isolated from cell lines and $10^{-7}$ g total RNA from patient tissue samples. This PNA–nanowire system was one of the first electrochemical sensors that detected specific mRNAs in unamplified clinical samples.

Other PNA-based biosensors have been reported for the detection of micro-RNAs (miRNA), a large and growing class of 18 to 24-nt-long, non-coding RNA molecules which are highly important in the regulation of gene expression and, thus, constitute new targets in drug discovery. These biosensors overcome the limitations associated with conventional, DNA-based detection systems for miRNA, most of which relied on prior fluorescent labelling of the target sample. In a relevant example, Wu et al. [58] recently reported a highly sensitive and label-free method for direct detection of miRNA by means of PNA-functionalized silicon nanowires (SiNWs). The sensor is capable of detecting target miRNA at concentrations of $10^{-15}$ mol $L^{-1}$, and it discriminates fully matched PNA/miRNA duplexes from base-mismatched duplexes. More importantly, the SiNW biosensor detects a specific miRNA in a heterogeneous sample containing the total RNA extracted from HeLa cells. This method therefore has potential diagnostic applications in early detection of miRNA as a cancer biomarker. Recently, Gao et al. [59] investigated the detection of miRNA by use of PNA-based electrochemical biosensors, without the need for PCR amplification or ligation steps. The authors reached a limit of detection of $10^{-14}$ mol $L^{-1}$ and a linear current–concentration relationship up to $10^{-11}$ mol $L^{-1}$ [60]. Nevertheless, although the detection scheme is simple and

the background signal is low, more than 90 min is needed to perform the hybridization assay.

Combined use of PNA-based biosensors and specific aptamers was reported by Le Floch et al. [61], who developed a strategy for label-free detection of a protein using a specific ssDNA aptamer. In their approach, the human α-thrombin aptamer X1 was added in excess to a solution in which such a protein was present at an unknown concentration, and then the S1 DNase was added to specifically hydrolyse the unprotected, free aptamer. Finally, the bound aptamers were released from the protein by heating the solution, and were electrochemically detected by use of a gold electrode grafted with PNA probes complementary to the aptamer sequence. With this strategy, human α-thrombin concentrations could be measured, although with an unsatisfactory detection limit of $7.5\times10^{-8}$ mol $L^{-1}$.

In a different application, Kong et al. [62] reported ultrasensitive electrical detection using PNA probes immobilized on the gaps of a pair of finger microelectrodes. This biosensor was hybridized with target DNA and, subsequently, pectin molecules were introduced into the DNA strand of the PNA/DNA duplex by use of zirconium phosphate and zirconium carbonate chemistry; the pectin molecules were then oxidized by periodate in acetate buffer. The oxidized, attached pectin molecules act as a catalyst to accelerate the reduction of ammoniacal silver ion to form silver nanoparticles, which then span the gap of the interdigited microelectrode. The conductance of the metallic nanoparticles directly correlated with the amount of the hybridized DNA, and $3\times10^{-15}$ mol $L^{-1}$ sensitivity was achieved under optimum conditions. These authors also reported a second version of their sub-microgapped system, in which haematin rather than pectin was inserted in the DNA strand by use of the same chemistry [63]; they achieved sensitivity of $1\times10^{-15}$ mol $L^{-1}$. Although both systems are very sensitive, enable mutation screening, and have multiplexing potential, the assay format used involves too many steps, and as a result is complicated and time-consuming. Additionally, detection of DNA in real samples (PCR and/or real samples) is also missed.

Another biosensor based on gapped electrodes, developed by Fang et al. [64], enabled detection of DNA oligonucleotides with sensitivity of $5\times10^{-14}$ mol $L^{-1}$. In this approach (Fig. 2), capped gold nanoparticles (NP) interacted with $Zr^{4+}$ ions and formed an aggregate which was, in turn, used as a conductive tag for electrical detection of DNA. PNA immobilized in the gap as the capture probe provided the discriminating location of the conductive tag formed from two comb-shaped electrodes separated by silicon dioxide as insulating material. Upon hybridization with target DNA, its negative backbone reached the gap and interacted with the $Zr^{4+}$ linker of the aggregate of the NPs, thus modifying the conductance between the two comb-shaped electrodes. The signal correlated directly with the amount of hybridized DNA and, therefore, with the concentration of target DNA in the sample. The authors suggested this approach could be generalized for detection of other DNA molecules by using appropriate and complementary PNA sequences in a multiplexed scheme, although detection of DNA in real samples remains to be investigated.

## Optoelectronic

As an alternative to conventional methods, piezoelectric biosensors, for example the quartz-crystal microbalance (QCM) seem to be suitable for monitoring hybridization of nucleic acids in solution [65]. With this objective, different groups have demonstrated that PNA probes immobilized on a QCM transducer enable screening of functionally relevant single mutations of the p53 gene [48, 66]. In another recent application, Yao et al. [67] constructed a PNA-based, QCM biosensor for label-free and real-time monitoring of the hybridization of hepatitis B virus (HBV) genomic DNA without previous PCR amplification. The detection limit (three times the noise signal) was $8.6\times10^{-12}$ g $L^{-1}$ and, working with clinical samples, the specificity was found to be extremely high (94.44%) compared with the reference method of real time PCR.

The higher mismatch discrimination efficiency of PNA probes compared with their equivalent DNA sequences, already documented by Nielsen's group when PNA was synthesized [29, 30], was confirmed by Lao et al. taking together QCM and surface plasmon resonance (SPR) measurements [68]. SPR is a label-free, optical detection method that measures the change in refractive index after hybridization of a target to the probe immobilized on a gold surface:

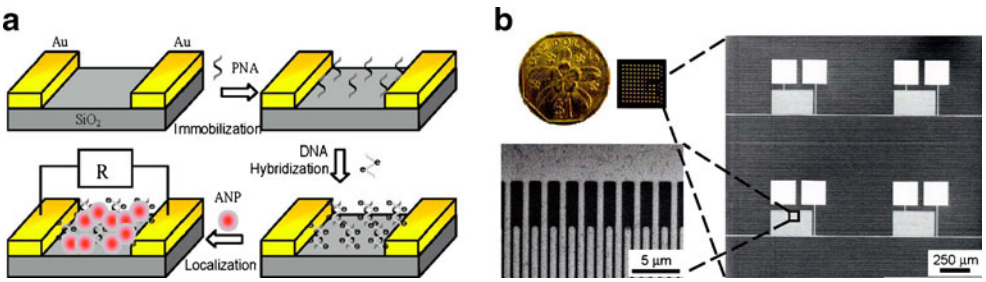


**Fig. 2** Schematic drawing of the biosensing mechanism proposed by Fang et al. [64] (**a**) and the structure of the biosensor (**b**). Figure reproduced with permission from Analytical Chemistry

on hybridization the refractive index shifts, causing a change in the surface plasmon wave. SPR can be used to monitor biological interactions in real time, a distinct advantage over other detection systems [69, 70].

One of the first SPR applications using PNA probes was reported by Sawata et al. [71]. They showed the hybridization of PCR products in a sample volume of 30 μL and with a detection limit of $7.5\times10^{-9}$ mol $L^{-1}$ over a range of 4–16 × $10^{-8}$ mol $L^{-1}$. Although the sensitivity is not as impressive as in other reported methods, the experiments were carried out with PCR amplicons and the analysis only took 10 min, enabling fast and accurate detection of the DNA encoding the verotoxin 2 of *E. coli*. Kinetic data have been reported for some SPR-based applications, and DNA and PNA probes have been compared from an analytical perspective. For instance, Prabhakar et al. [72] quantified the values of the association and dissociation rate constants ($K_a$ and $K_d$) for the DNA complementary sequence for PNA/Au ($8.5\times10^{4}$ $m^{-1}s^{-1}$ and $3.6\times10^{-3}$ $s^{-1}$, respectively) and DNA/Au ($2.5\times10^{4}$ $m^{-1}s^{-1}$ and $1.1\times10^{-3}$ $s^{-1}$) bioelectrode, thus demonstrating that the results were threefold better when PNA probes were used. Furthermore, no binding with the single-base mismatched DNA target was observed for the PNA–Au bioelectrode. Other groups further improved SPR measurements by introducing chemical modifications to PNA probes [73, 74] and achieved better stability and reusability of the sensors. Likewise, dsDNA has been detected by use of a duplex invasion method [75], and localized SPR has been used by Endo et al. [76] to detect $6.7\times10^{-13}$ mol $L^{-1}$ ssDNA with base mismatch specificity. Additional reports have described the detection of *E. coli* ribosomal RNA [77], and the development of a signal-amplification strategy that uses DNA-templated polyaniline deposition [78].

SPR imaging (SPRI) is emerging as a versatile method for detecting interactions of biomolecules in a microarray format. With that purpose, D'Agata et al. [79] reported the use of PNA probes for NP-enhanced SPRI detection of DNA sequences, achieving a detection limit of $10^{-15}$ mol $L^{-1}$ and SNP specificity. Another strategy based on NPs and optical detection systems was reported by Pita et al. [80]. They attached PNA probes to gold-covered magnetic NPs, and their hybridization with specific ssDNA oligomers was measured using rhodamine 6G as fluorescent marker. The optimum single base-mismatch specificity was achieved, although the sensitivity should be improved and the usefulness of these mobile biosensors with real samples remains to be investigated.

## Microarrays

Microarrays, also called "biochips", are analytical devices based on the covalent immobilization of thousands of probe molecules (nucleic acids, proteins, and others) on a solid substrate (chemically modified glass, silicon, gold, etc.). The probe molecules are arranged in miniaturized bidimensional arrays of dots, typically 10 to 150 μm in diameter. The sample to be analysed is fluorescently labelled and hybridized to the microarray, and the specific target–probe interactions are detected by means of a high-resolution scanner. Microarrays provided the possibility of performing high-throughput analysis, dramatically increasing the speed and performance of experimental work in genomics and proteomics [81, 82]. Nucleic acid microarray technology was initiated in the 1990s [83] and enables the production of biochips by two alternative strategies: in-situ synthesis of short oligonucleotide probes using photolithographic technology or mechanical deposition of pre-synthesized probe molecules on to the solid support [84]. Despite its broad applicability in biology, classical microarray technology has some technical limitations, mainly imposed by the need for fluorescent labelling of the sample to be analysed. This has triggered the development of alternative, non-optical microarray-based detection techniques that avoid fluorescent labelling of the target DNA. Some of these rely on the use of nucleic acid analogues as probe molecules.

Soon after DNA microarrays were available, the improved stability of PNA and its unique hybridization features encouraged the development of PNA-based microarrays. The peptidomimetic nature of the PNA backbone also enables label-free monitoring of DNA hybridization, by use of analytical techniques that detect either physicochemical signatures of the phosphate and/or sugars present in DNA and RNA or the net increase in negative charge that occurs upon hybridization. This was soon evaluated for PNA microarray-based detection of unlabelled DNA molecules [85, 86], thus circumventing one of the aforementioned limitations of DNA microarrays.

PNA microarrays can be produced either by spotting prefabricated PNA oligomers onto solid supports or by parallel in-situ synthesis of high-density PNA library arrays on porous support media [87–89]. Brand et al. [90] used a combination of the two approaches in the production of PNA microarrays capable of detecting single-base mismatches in either fluorescently labelled or unlabelled DNA oligonucleotide target molecules. The best results were obtained by label-free detection methods, as we will discuss in the next section. Several applications of PNA microarrays have been reported in the last decade, some of which will be discussed here. Song et al. [91] compared the results of their previously released PNA-based array (PANArray HPV) [92] with those obtained by means of a commercial DNA microarray-based kit for detection and genotyping of human papillomavirus (HPV). Analysis of 741 prospectively collected clinical samples showed that the PANArray HPV test resulted in greater HPV-positivity than did the DNA chip test, although the difference was not statistically significant. However, it was confirmed by DNA sequencing that the

frequency of false-positive or false-negative results was much lower for the PANArray HPV test.

A novel application of PNA microarrays was reported by Jang et al. [93] who detected HBV mutations related to antiviral resistance in 68 clinical DNA samples. PNA probes were designed to pick up mutations associated with resistance to the antiviral drugs lamivudine, adefovir, and entecavir. The PNA array was sensitive enough to hybridize to amounts of fluorescently labelled, viral DNA as low as 100 copies $mL^{-1}$. Interestingly, minority mutants present at 5% of the virus population where detected if the total HBV DNA concentration was greater than $10^4$ copies $mL^{-1}$. With regard to its specificity for identifying the correct viral mutants, results from use of the PNA array were highly concordant (98.3%) with those from direct sequencing of the mutant HBV genomic DNA.

Calabretta et al. [94] explored PNA patterning by microcontact printing (μCP) and demonstrated that the resulting PNA microarrays can be used to distinguish among fully matched, singly base-mismatched, and non-complementary DNA strands. Moreover, the ability of PNA to self-assemble on surfaces has been exploited to immobilize libraries of peptides or small molecules in microarray format [95], thus expanding the biosensing potential of PNA-based biochips. Despite these achievements with PNA-based arrays, it must be noted that, as we will discuss in the last section of this review, several problems are delaying their use as alternatives to high-performance DNA microarrays.

## Other PNA-based biosensors

Additional PNA-based biosensing methods have been developed in the last decade; some are proof of concept studies whereas others have promising applicability in biotechnology and/or biomedicine. Mass spectrometry (MS), specifically matrix-assisted laser desorption/ionization time-of-flight MS (MALDI–TOF MS) is an accurate and sensitive method for molecular weight and sequence determination for different kinds of polymeric biomolecules, with relevant applications in genomics [96]. The strong peptidomimetic backbone of PNA molecules make PNA probes resistant to fragmentation during the MALDI process, resulting in high-quality performance in MALDI-TOF experiments designed to characterize DNA targets. Ross et al. [97] discriminated among human genomic single mutants by use of a procedure involving PNA hybridization to PCR-amplified DNA, followed by MALDI-TOF analysis. Brandt et al. [90] synthesised PNA probes on filter-bottom microtitre plates and attached them without any further purification step to microarray surfaces by use of different chemistry. Direct detection of the hybridization of unlabelled DNA was achieved by TOF secondary-ion MS. The authors reported that, using thiol-modified PNAs on maleimide surfaces, unprecedented sensitivities in the $10^{-18}$ mol $L^{-1}$ range could be obtained, with enough specificity for detection of SNPs. Nevertheless, these detection limits were achieved by use of DNA oligonucleotides as target molecules, and the hybridization of PCR samples or natural DNA has not been assayed. Other PNA-based microarrays with MS readout have been reported, for example those used for the evaluation of DNA methylation markers in tumour tissue [98], gene diagnostics [99], or protein profiling [100]. Although this technique is very sensitive, it is not cost-effective, the apparatus is not portable, and highly trained and experienced personnel are required to analyse the results. Therefore, it is not a useful option for point-of-care utilization.

The outstanding capacity of thiol-modified PNA oligomers for self-assembly on gold surfaces (Fig. 3a) [41] encouraged the use of surface science characterization techniques [101] to assess the usefulness of such ordered layers as biosensors of DNA hybridization. In particular, Briones et al. [41, 42] used X-ray photoemission spectroscopy (XPS) to chemically characterize the PNA SAM before and after the hybridization of target DNA molecules. High-resolution XPS enabled qualitative and quantitative analysis of the N1s and P2p core level peaks on the biosensor surface, the intensity of which increased and appeared, respectively, on DNA hybridization (Fig. 3b). The optimum concentration of the PNA probes for formation of bioactive monolayers with optimum coverage was in the range 0.1 to $1\times10^{-6}$ mol $L^{-1}$. The specificity of this biosensor enabled base-mismatch detection in oligonucleotide DNA targets corresponding to viral genes, among them that encoding the reverse transcriptase of human immunodeficiency virus type 1 (HIV-1). These results showed the usefulness of PNA-based biosensors and surface characterization techniques for detection and SNP mapping of label-free nucleic acid targets of biomedical relevance [44]. Although technologically relevant, these reports can be regarded as proof of concept, because the maximum sensitivity of the method has not yet been reported, and PCR or natural DNA molecules have not been used as targets. They also require ultra-high vacuum technology, which is only available in specialized laboratories.

Technological limitations related to the need for XPS analysis encouraged the use of an optical technique such as infrared (IR) spectroscopy, in particular reflection absorption IR spectroscopy (RAIRS), for studying the hybridization of DNA targets to SAMs of PNA probes adsorbed on metal surfaces [102]. With this objective, Mateo-Martí et al. [43] confirmed for PNA layers that coverage and molecular orientation are optimum at $1\times10^{-6}$ mol $L^{-1}$. The neighbouring PNA molecules are stabilized by intermolecular interactions via non-complementary base-pairing, because the layer tends to interact specifically with complementary DNA molecules in solution. This knowledge was used by the same authors [103] to develop a PNA-based biosensor of DNA oligonucleotides. By means of RAIRS, several distinct vibrational

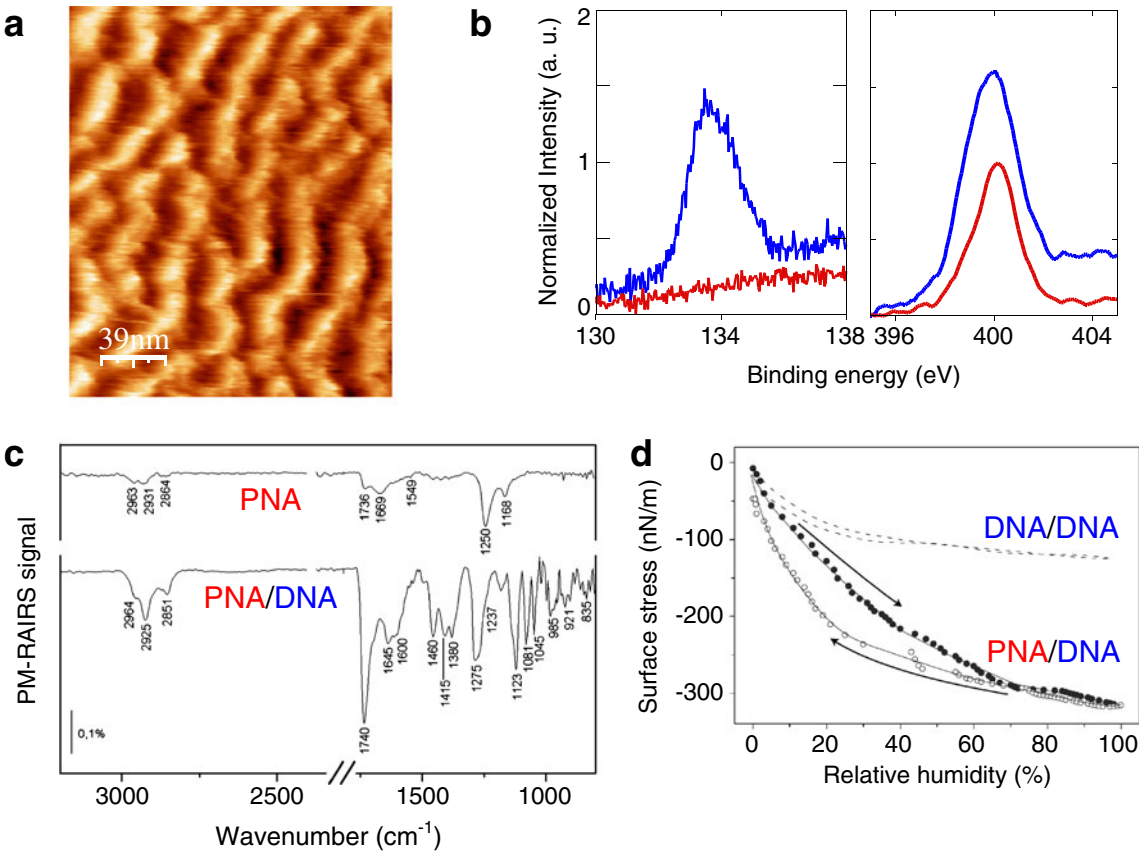


**Fig. 3** Biosensors based on self-assembled monolayers of PNA on gold surfaces (adapted from Refs. [41, 103, 104]). (**a**) AFM image recorded in air of the SAM of PNA formed upon immobilization of the oligomer at 1 μmol $L^{-1}$ concentration; (**b**) XPS spectra, normalized to the Au4f peak, of the P2p and N1s core level peaks of the PNA monolayer before (*red curve*) and after (*blue curve*) hybridization to the fully complementary DNA target molecule; (**c**) PM-RAIRS spectra of the SAM of PNA before (*upper curve*) and after (*lower curve*) hybridization with complementary DNA; (**d**). Surface stress variation during a hydration/dehydration cycle for a gold-coated silicon cantilever sensitized with DNA (*upper curve*) or PNA (*lower curve*) upon hybridization of complementary DNA

features corresponding to the chemical groups present in the deoxyribose and phosphate groups of the target were detected upon specific DNA hybridization (Fig. 3c).

A novel biosensor based on PNA SAMs on gold surfaces was described by Mertens et al. [104]. They developed a nanomechanical sensor relying on the adsorption of water molecules in sub-nanometre channels present within the layers of either ssPNA or ssDNA probe molecules adsorbed on gold-covered, silicon microcantilevers. They found that the surface stress changed dramatically when the layer interacted with complementary DNA molecules. Although the hydration-dependent, repulsive steric forces were qualitatively similar in the PNA/DNA and DNA/DNA-hybridized microcantilevers, the response was threefold higher in the PNA-based system than in the DNA-based system (Fig. 3d). The sensitivity of this nanomechanical biosensor was in the $10^{-15}$ mol $L^{-1}$ range and it was used for SNP mapping at room temperature, with the ability to detect minority target DNA molecules at the 0.1% level in the sample investigated. Nevertheless, DNA oligonucleotides rather than PCR amplicons or natural samples have been used as target molecules. Also, the potential of the technique for multiplex detection of different DNA sequences remains unknown.

## LNA-based biosensors

Locked nucleic acids (LNA) were synthesized by the Imanishi and Wengel groups in 1997 and 1998, respectively [105, 106]. Since then, LNA have attracted much attention and helped to improve the sensitivity and specificity of FISH-related methods, real-time PCR, microarrays, and other molecular biology techniques based on oligonucleotides. As previously discussed, the restricted 3′-*endo* conformation of the ribose ring drastically reduces the conformational flexibility of LNA. Nevertheless, this artificial nucleic acid is fully able to form specific base pairs with DNA and RNA according to Watson–Crick rules (Fig. 1h) [21, 22]. LNA has high affinity for complementary sequences present in natural nucleic acids. The $T_m$ increase for LNA–DNA hybridization in solution ranges from 2.0 to 6.0 °C per LNA monomer

[107], and this rises to 3.0–9.6 °C for LNA/RNA duplexes (Table 1). It has been shown that both the highest $T_m$ increase per LNA nucleotide and the best mismatch discrimination are achieved for short LNA oligomers, typically shorter than 10 bases [25]. Additionally, LNA phosphoramidites and their oligomers are commercially available, and LNA nucleotides can be mixed with those of the natural nucleic acids for polymerizing combined, heterogeneous probe molecules. This makes LNA a very flexible tool in biotechnology, nucleic acid diagnostics, and nucleic acid-based therapeutics.

LNAs have many other excellent properties for biosensor development, for example low toxicity, resistance to nuclease digestion, enhanced triplex formation when hybridized to dsDNA, and synthesis by standard chemical methods (reviewed elsewhere [23, 108, 109]). In particular, surface-immobilized LNA oligonucleotides could constitute optimum probes for nucleic acid characterization in microarrays, because current procedures for microarray production need only minimal adjustment when LNA probes are used. In a relevant example reported by Fang et al. [110], LNA microarrays were used to detect multiple miRNAs by means of a novel approach that combines the surface reaction of poly(A) polymerase (which creates poly(A) tracks on miRNAs specifically hybridized to surface bound LNAs), the further adsorption on the poly(A) tails of DNA-modified NPs, and the final detection of the hybridization points by nanoparticle-amplified SPRI. Although this multi-step assay is rather complex and laborious, it has an outstanding limit of detection of approximately $10^{-18}$ mol $L^{-1}$. Nevertheless, Diercks et al. [111] reported controversial results in which LNAs did not improve DNA properties in microarray-based biosensors and, indeed, resulted in worse specificity, sensitivity, and stability.

An early example of the usefulness of LNA oligonucleotides in biosensing is the screening for the factor V Leiden mutation by Orum et al. [112]. In these experiments, 8-mer LNA capture probes (complementary to either the *wild type* or the mutated sequence) were covalently attached to individual wells of a microtitre plate. Subsequently, hybridization of PCR amplicons was colorimetrically tested with an ELISA-like assay. Because of its reproducibly the method detected both factor V homozygotes and heterozygotes with excellent sensitivity and specificity and, moreover, the results were in 100% concordance with those from the PCR-RFLP reference method. This was the first demonstration that LNAs can effectively and reproducibly capture PCR amplicons in a simple solid-phase hybridization assay. Soon after, Simeonov et al. [113] used short LNAs for efficient SNP scoring by means of fluorescence polarization (FP) detection. LNA probes were fluorescently labelled and their hybridization to target DNAs was followed by measuring the FP of the dyes. The formation of perfectly complementary LNA/DNA duplexes gave rise to significant FP increases, whereas the presence of single mismatches resulted in very small or no changes of FP. This was a significant achievement in detection of SNPs, although its multiplexing could be complicated because different dyes must be used for every SNP screened.

LNA has been used to overcome some of the traditional limitations of molecular beacons (MB). Wang et al. [114] engineered MB with a LNA backbone to generate novel probes with higher thermostability, enhanced mutant selectivity, nuclease resistance, and reduction of false positive signals, even in complex biological environments. They obtained improved results compared with the original MB, although the observed kinetics were too slow and some improvements in LNA design (length and G/C content) should be addressed. Martinez et al. [115] studied the performance of such LNA-based MB after their immobilization on to a glass surface. They achieved a signal-to-background ratio of 25, with detection limits reaching $10^{-9}$ mol $L^{-1}$. The authors recognized that the kinetics of LNA-based MB were much slower than expected. Consequently, they recommended use of LNA nucleotides exclusively in the unpaired hybridization region of the MB, and not in the stem region, which should be easily opened on target hybridization. In that sense, Han et al. [116] designed a DNA hairpin containing a 19-mer loop and a six base-pair stem. They placed a triplet of LNA nucleotides close to the centre of the loop, surrounding the potential single-base mismatch site. Hybridization of the immobilized, LNA-bearing MB to its specific target DNA led to clear variations of the film thickness, a property that could be directly measured by use of atomic-force microscopy (AFM) and nanolithography. The measured thickness increase was three times larger (4.5 nm vs 1.5 nm) when a fully complementary target instead of a single-base mismatched target was hybridized. This technique requires substantial adjustment because of thermal drifting and, consequently, although excellent discrimination results are obtained, it still needs further technical improvements to enable SNP screening in a miniaturized array format.

Electrochemical biosensors have also benefited from the LNA potential for specific target recognition. Chen et al. [117] used a 18-mer LNA-modified capture probe for hybridization with the BCR/ABL fusion gene to detect chronic myelogenous leukaemia. Differential pulse voltammetry was used to monitor the hybridization reaction on the capture probe electrode; response was a linear function of complementary ssDNA concentration in the range $10^{-11}$ to $10^{-12}$ mol $L^{-1}$, and the detection limit was $9.4\times10^{-13}$ mol $L^{-1}$. Later, these authors used a thio-modified hairpin LNA as the capture probe immobilized on a nanogold (NG)/poly-eriochrome black T film-modified GCE [118], although they did not improve their previous results. They also attempted detection of promyelocytic leukaemia/retinoic acid receptor

alpha by use of sandwich detection scheme [119]. Their approach involved a pair of LNA probes: a capture probe immobilized on to the electrode surface and a biotinylated reporter probe as an affinity tag for streptavidin–horseradish peroxidase (HRP) (Fig. 4). A detection limit of $7.4\times10^{-14}$ mol $L^{-1}$ and a dynamic range of $10^{-11}$ to $10^{-12}$ mol $L^{-1}$ were achieved, a slight improvement of their previous results at the expense of a more complicated detection scheme. Recently, this group [120] dually labelled the LNA hairpin probe with biotin (for streptavidin-based immobilization) and a carboxyfluorescein (FAM) molecule (as an affinity tag for HRP). The immobilized hairpin probe suffers a significant conformational change upon target hybridization, separating FAM from the electrode and making it accessible to the anti-FAM-HRP antibody. This biosensor enabled specific SNP detection and could be used to detect $8.3\times10^{-14}$ mol $L^{-1}$ target DNA in real samples, thus constituting a good example of the usefulness of LNA-based probes. Nevertheless, complicated detection schemes could be challenging when trying to apply them in commercial biosensors.

In a different approach, Berti et al. [121] combined the remarkable properties of carbon nanotubes with the high stability of LNA probes. The resulting biosensor was applied to the detection of a PCR amplicon belonging to a region of the CB2 cannabinoid receptor gene. A linear response was obtained over a wide concentration range ($0–100\times10^{-9}$ mol $L^{-1}$), and a detection limit of $4\times10^{-10}$ mol $L^{-1}$ was achieved, far from the $\sim10^{-15}$ mol $L^{-1}$ value obtained in the most sensitive assay reported by Lin's group [120]. A comparative study of the properties of PNA and LNA as capture probes for development of an electrochemical hybridization assay has been carried out by Mascini's group [122]. With this objective, streptavidin-coated paramagnetic micro-beads were used as solid phase to immobilize biotinylated DNA, PNA, and LNA capture probes complementary to DNA and RNA target oligonucleotides. Detection limits for the DNA target were 1.52, 1.18, and $0.91\times10^{-10}$ mol $L^{-1}$ (DNA, PNA, and LNA probes, respectively). For the RNA target, they were even smaller: 5.1, 6.0, and $7.8\times10^{-11}$ mol $L^{-1}$, respectively. Thus, similar sensitivity (and reproducibility) were found for the three probe molecules. However, this experiment did not check the performance of the biosensor for SNP detection, an application likely to reveal the advantages of PNA and LNA probes over DNA probes in such a biosensor.

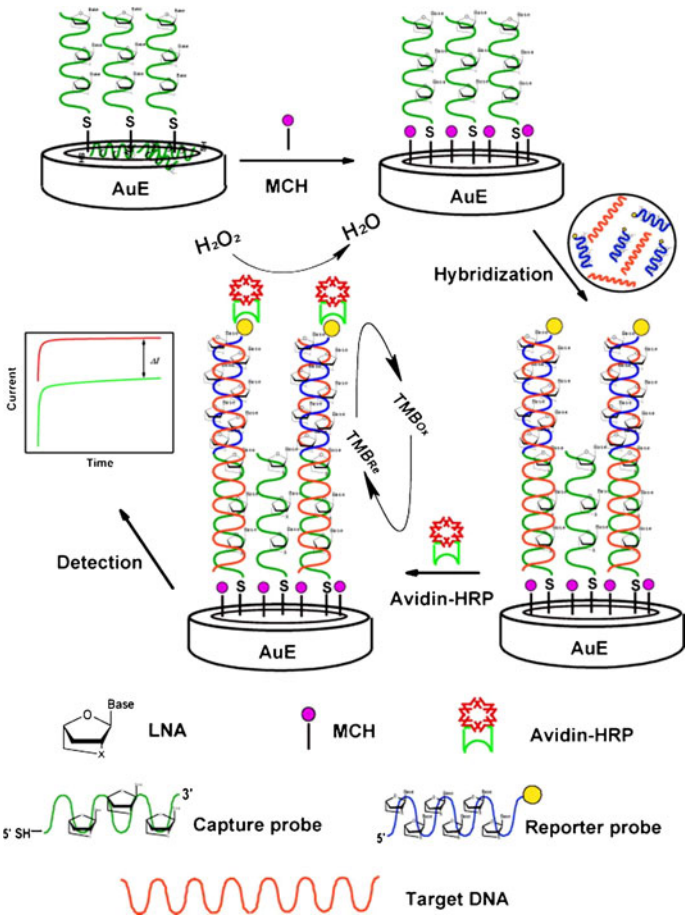


**Fig. 4** Schematic diagram of fabrication of the sandwich-mode electrochemical LNA biosensor reported by Wang et al. [119]. Figure reproduced with permission from Elsevier

LNA nucleotides have also been introduced in DNA or RNA aptamers developed for biosensing applications, because "locked" nucleotides not only increase the thermal stability of the aptamer but also improve its in vivo resistance to nuclease digestion. Darfeuille et al. [123] studied the effect of incorporation of LNA nucleotides into the RNA aptamer specific to the HIV-1 TAR RNA element. Although most of their efforts resulted in non-functional chimeric nucleic acids, they succeeded in developing an aptamer in which RNA and LNA nucleotides were interspersed and whose affinity for TAR was similar to that of the parent RNA aptamer. This example emphasized that incorporation of LNA into aptamers can increase their stability and/or nuclease resistance without necessarily reducing their affinity for the target molecule. Indeed, when LNA nucleotides are introduced into the aptamer, both affinity decreases (e.g., in the α-thrombin aptamer [124]) and increases (e.g., in the α-avidin aptamer [125]) have been reported. These contradictory results make evident the current need for systematic studies with the purpose of determining the (either universal or case-dependent) factors affecting the affinity of an aptamer for its target molecule when LNA nucleotides are introduced into the sequence. This would certainly help to improve the performance of LNA-containing aptamer biosensors.

## Comparison of the biosensing potential of PNA and LNA

Among the nucleic acid analogues developed so far, PNA and LNA have relevant advantages (and some limitations) compared with DNA for designing probe molecules useful in biosensing applications. The main properties of PNA and LNA regarding their hybridization with natural nucleic acids are summarized in Table 1. The values listed in the table have been obtained in solution (data taken from Refs. [22, 30, 34, 126–128]) and, being strictly thermodynamic, they reflect the strength of nucleobase pairing together with the effect of the molecular backbones along the hybridizing nucleic acid strains. Therefore, the values are assumed to be independent of attachment of one end of the nucleic acid analogue to any biosensor surface. This notwithstanding, it is clear that the overall behaviour of nucleic acid hybridization in bulk solution is different from that in the proximity of a surface, because of kinetic factors dependent, among other issues, on the overall accessibility of the immobilized PNA or LNA probe to the DNA or RNA target molecule present in the surrounding solution. Although probe–target accessibility is affected by the particular geometry of each biosensor, it is generally recommended (and this is the strategy used in most biosensors) to add a link or spacer molecule to the immobilized PNA or LNA probe to physically separate the hybridization sequence from the biosensing surface, thus avoiding or limiting steric hindrance during the process.

The common advantages of PNA and LNA over DNA for their use as probe molecules in biosensors include:

1. the greater thermodynamic stability of PNA and LNA-containing heteroduplexes (namely, PNA/DNA, PNA/RNA, LNA/DNA, and LNA/RNA) compared with the corresponding homoduplexes formed by natural nucleic acids (DNA/DNA, DNA/RNA or RNA/RNA) enables the use of PNA or LNA probes that are shorter than the equivalent DNA or RNA probes;
2. the higher destabilizing effect of base mismatches in PNA or LNA-containing heterodimers improves discrimination in genotyping; and
3. the high chemical stability of PNA (and, to a lesser extent, LNA) probes and their resistance to enzymatic degradation enables the use of PNA or LNA-based biosensors with a broad range of biological samples.

Also, both analogues are commercially available (with some sequence limitations discussed in the next section) and can be used in any molecular biology or analytical chemistry laboratory.

Nevertheless, there are also important differences between PNA and LNA which affect the limit of detection and dynamic range obtained when they are used in biosensors (Table 2). Whereas LNA has a negatively charged phosphate–sugar backbone, the uncharged nature of the PNA backbone enables hybridization with DNA or RNA molecules under low or no salt conditions, thus hindering the formation of potentially interfering secondary structures in the targets. The neutral backbone of the PNA monomers makes this analogue an optimum probe molecule for electrochemical biosensing, as documented by the growing number of reported applications. Also, because of the different electrical nature of their polymeric backbones, LNA do not have the remarkable strand-invasion properties of PNA and its hybridization to dsDNA targets is less efficient.

Another critical difference is that PNAs are assembled using standard peptide synthesis procedures and, consequently, it is much easier to append peptide motifs on to PNA molecules than on to LNA oligomers. In turn, LNAs are polymerized by use of conventional phosphoramidite chemistry, and individual LNA oligomers are commercially available and can be combined with DNA, RNA, and 2′-*O*-Me-RNA monomers, thus furnishing chimeric molecules with different applications. In particular, this has led to the possibility of in vitro selection of DNA or RNA aptamers that contain (or, alternatively, are further modified with) LNA oligomers, thus constituting very useful probes for developing affinity biosensors.

## Current challenges and future trends

A common disadvantage of PNA and LNA- based biosensors arises from the several sequence limitations in the synthesis of these two nucleic acid analogues. In particular, the design of PNA oligomers is constrained by four requirements:

1. PNA length must comprise between 6 and 18 monomers;
2. to impair PNA aggregation, sequences with a purine content higher than 60% must be avoided;
3. for the same reason, the maximum sequence of purines is four in a row (for consecutive Gs, this value is reduced to three); and
4. because of the strength of PNA–PNA interactions, self complementary sequences (inverse repeats, palindromes, or hairpins) must be avoided if they involve six or more consecutive monomers (four or more consecutive Gs or Cs).

In turn, the following design guidelines should be followed for LNA:

1. sequences of more than four LNA nucleotides must be avoided, except when very short (9 or 10 nt) DNA or RNA oligonucleotides are designed;
2. sequences of three or more Gs or Cs must be avoided;
3. GC content must be kept between 30 and 60%; and
4. to avoid LNA–LNA interactions, LNA sequences with potential self-complementarity or cross-hybridization must be discarded.

These limitations in the sequences of the PNA or LNA oligomers that can be synthesized and used as capture probes in biosensors can obviously impair the detection of some mutations in genes of interest. Therefore, some of the multiplexing applications claimed by several authors seem unrealistic, because some oligomers required for them could not be synthesized.

This is one of the main reasons why, until now, PNA or LNA-based microarrays are not high-throughput biosensors with widespread applicability in biotechnology or in the clinical setting, despite their high sensitivity for target DNA oligonucleotides and their specificity for SNP genotyping of clinical samples. Also, although the in-situ synthesis of PNA oligomers on surfaces has been successfully achieved, this technology is much less developed than the synthesis of DNA (or LNA) oligonucleotides using standard phosphoramidite chemistry and photolithographic technology. Moreover, the current price of any oligomer (especially true for PNA) is much higher than that of a DNA oligomer with the same sequence; this can be a serious obstacle if hundreds or thousands of them are required for microarray construction. For these practical reasons, current DNA-based biochips (produced by several biotechnological companies, and optimized for different applications) are currently more useful than those based on any of their synthetic analogues. Nevertheless, research and industry initiatives are in progress with the intention of capturing, in the near future, a portion of the array market currently served by DNA arrays.

Other current challenges faced by PNA and LNA-based biosensors arise because they are still at an early stage of development compared with DNA-based biosensors. Therefore, as we have critically reviewed in the previous sections, a large fraction of the published results have been obtained using proof-of-concept devices (some involving very complicated and time-consuming assays) far from commercial applications useful in biotechnology or biomedicine. In particular, not all the developed biosensors are sufficiently specific to detect point mutations in target DNA molecules present in complex mixtures, a feature currently required for efficient SNP mapping. Regarding sensitivity, the best reported PNA or LNA-based electrochemical biosensors (and at least one example of a PNA-based nanomechanical biosensor) provide detection limits of approximately $10^{-15}$ mol $L^{-1}$, and some microarrays (either PNA-based in combination with MS detection, or LNA-based combined with nanoparticle-amplified SPRI) enable detection of target DNA at $10^{-18}$ mol $L^{-1}$ (Table 2). Although these values are truly remarkable they have, in general, been obtained under laboratory conditions using short DNA oligomers as target molecules, and little or no information is yet available on biosensor sensitivity with real samples (for example PCR amplicons and/or complex clinical DNA extracts).

Therefore, future work in this field should include systematic study of the performance of the (either already reported or novel) biosensors with natural samples. Also, the reproducibility of the results (not always reported in the reviewed literature) and the reusability of the sensor (already demonstrated for PNA-based SPR applications) remains to be proved for a large fraction of the cases. Moreover, on the basis of the current successful examples discussed above, it would be desirable to develop improved biosensors based on the immobilization of specific DNA or RNA aptamers containing some LNA monomers at selected positions, thus combining the advantages of highly specific affinity recognition (that should be preserved or even increased upon the incorporation of LNA nucleotides) and resistance to chemical or biological degradation.

## Conclusions

Different families of PNA and LNA-based biosensors have been developed in the last two decades, and have applicability in a growing number of research fields. It is now evident that, as summarized in the previous sections, although PNA and LNA share some similarities, there are also important

**Table 2** Summary of the most outstanding applications of PNA and LNA-based biosensors

| Nucleic acid analogue | Detection method | Target | Limit of detection | Dynamic range | Comments | Ref. |
|---|---|---|---|---|---|---|
| PNA | Electrochemical (redox indicator) | dsDNA oligomers (HCV SNP) | $1.8\times10^{-12}$ mol $L^{-1}$ | $1\times10^{-11}$–$1\times10^{-8}$ mol $L^{-1}$ | Calculated (non-experimental) detection limit | [51] |
| | | PCR amplicon | $4.8\times10^{-12}$ mol $L^{-1}$ | $1\times10^{-11}$–$1\times10^{-9}$ mol $L^{-1}$ | SNP discrimination | [52] |
| | Electrochemical (nanowire) | DNA (oligomer) and cellular RNA | $10^{-13}$ mol $L^{-1}$ (DNA oligo) $10^{-7}$ g (total RNA) | $1\times10^{-12}$–$1\times10^{-10}$ mol $L^{-1}$ | Real samples for tumour biomarker detection | [57] |
| | | miRNA from HeLa cells | $10^{-15}$ mol $L^{-1}$ | $10^{-15}$–$10^{-9}$ mol $L^{-1}$ | SNP discrimination/label-free | [58] |
| | Electrical (nanowire) | DNA oligomer | $5\times10^{-14}$ mol $L^{-1}$ | $10^{-14}$–$10^{-11}$ mol $L^{-1}$ | Nanoparticle amplified. Large sample volume (5 mL) | [64] |
| | Optoelectronic (QCM) | HBV genomic DNA (no PCR) | $8.6\times10^{-12}$ g $L^{-1}$ | $10^{-12}$–$10^{-6}$ g $L^{-1}$ | Detection limit as three times the noise signal. SNP detection | [67] |
| | Optoelectronic (SPR) | DNA (PCR) encoding the verotoxin 2 of *E. coli* | $7.5\times10^{-9}$ mol $L^{-1}$ | $4$–$16\times10^{-8}$ mol $L^{-1}$ | SNP discrimination/label-free | [71] |
| | Optoelectronic (localized SPR) | ssDNA (21-mer oligo and PCR-derived, TNF-α gene) | $6.7\times10^{-13}$ mol $L^{-1}$ | $10^{-15}$–$10^{-6}$ mol $L^{-1}$ | SNP discrimination in real sample/ label-free | [76] |
| | Optoelectronic (SPRI) | DNA oligomer | $10^{-15}$ mol $L^{-1}$ | $1$–$500\times10^{-15}$ mol $L^{-1}$ | SNP discrimination | [79] |
| | Microarray (fluorescent) | HBV genomic DNA from real samples | $10^{2}$ copies $mL^{-1}$ | $10^{1}$–$10^{8}$ copies $mL^{-1}$ | Detection of 5% minority mutants in $10^{4}$ copies $mL^{-1}$ | [93] |
| | Microarray (MALDI–TOF) | DNA oligomer | $10^{-18}$ mol $L^{-1}$ | N.D. | SNP discrimination/label-free | [90] |
| | Nanomechanical | DNA oligomer | $10^{-15}$ mol $L^{-1}$ | $10^{-15}$–$10^{-9}$ mol $L^{-1}$ | SNP discrimination/label-free | [104] |
| LNA | Optoelectronic (SPRI)/ microarray | miRNA | $10^{-18}$ mol $L^{-1}$ ($5\times10^{-21}$ moles) | $1\times10^{-12}$–$2\times10^{-9}$ mol $L^{-1}$ | Nanoparticle amplified | [110] |
| | Electrochemical (redox indicator) | DNA oligomers/PCR product (BCR/ABL gene) | $9.4\times10^{-13}$ mol $L^{-1}$ | $1$–$11\times10^{-12}$ mol $L^{-1}$ | SNP discrimination with real samples | [117] |
| | Electrochemical | PCR real sample | $8.3\times10^{-14}$ mol $L^{-1}$ | $10^{-7}$–$10^{-1}$ | SNP discrimination, real sample in serum | [120] |
| DNA/PNA/LNA | Electrochemical | DNA oligomer<br>RNA oligomer | $1.52/1.18/0.91\times10^{-10}$ mol $L^{-1}$<br>$5.1/6.0/7.8\times10^{-11}$ mol $L^{-1}$ | $0$–$2\times10^{-9}$ mol $L^{-1}$ | Sensitivity comparison | [122] |

N.D., not determined

differences between them that suggest the use of one or the other depending on the desired application. Also, both analogues have specific disadvantages compared with use of DNA for biosensor development, in particular for the construction of high-throughput microarrays. Also, the sensitivity and specificity of most PNA and LNA-based biosensors remain to be assessed with natural samples.

It is expected that further studies comparing the performance of PNA and LNA probes in distinct biosensors will provide additional information on their practical usefulness and limitations. Optimistically, some of the current or future PNA and/or LNA-based technology will lead to the development of novel, simple, and inexpensive biosensors, with high sensitivity, specificity, and reproducibility. These would complement the DNA-based sensors currently available, and provide a growing range of analytical tools with applicability in the different disciplines of biotechnology and medicine.

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