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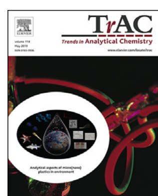
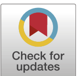


Recent advances on application of peptide nucleic acids as a bioreceptor in biosensors development



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ABSTRACT

The analogs of DNA are unique biomedical tools that are broadly utilized to develop different types of biosensors. Peptide nucleic acids (PNA) are an individual and notable class of nucleic acid analogs due to their unique, novel physicochemical and biochemical characteristics, stability and resistance to nuclease and protease enzymes, significant interactions with complementary strands and remarkable hybridization attributes. Therefore, they are employed in the preparation and fabrication of various types of functional biosensors. In other words, immobilization of PNA as an appropriate diagnostic probe on the surface of electrochemical and optical converters lead to the fabrication of PNA-based biosensors which could be applied for sensitive and selective determination of diverse analytes. In this article, we are purposed to demonstrate an overview of physicochemical and biochemical features, modifications of peptide nucleic acid backbone and its usages as a bio-receptor in the PNA-based biosensors in recognition of viruses, RNA, bacteria, DNA, SNP (single-nucleotide polymorphism) and etc.

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1. Introduction PNA

Nucleic acids (DNA and RNA) are the biomolecules providing the basis of effective structures for function of all known cell complexes and transfer information through replication, transcription and translation processes. Therefore, they are used in different fields, including nanotechnology, research, diagnosis and treatment [1–3]. The most well-known and useful nucleic acid analogs are LNA (locked nucleic acid, negative charged, an RNA analog), PNA (peptide nucleic acid, uncharged), and PTO (phosphorothioate oligonucleotide, negative charged). Peptide nucleic acid (PNA) is a DNA analog that for the first time, in the 1990s, introduced by the efforts of the organic chemist Ole Buchardt's and biochemist Peter Nielsen's groups. According to the flow linear dichroism examination, the PBG (α-helical poly-γ-benzylglutamate) with aromatic chromophores could lead to the creation of stacked complexes. It was suggested that α-helical poly-γ-benzylglutamate with alternative nucleobases and acridine moieties could selectively connect to the DNA sequences through merging Hoogsteen base pair structure and in the major groove intercalate with the helical backbone. The *ad interim* name was given to the suggested peptide nucleobase combination which was PNA or peptide nucleic acid. These days, replacement of natural nucleic acid's sugar-phosphate backbone with a synthetic peptide which is commonly structured from units of N-(2-amino-ethyl)-glycine, results in formation of uncharged and achiral mimic named PNA (peptide nucleic acid) [4–6]. The peptide nucleic acid's backbone is composed of repeated N-(2-aminoethyl) glycine units. Pyrimidines and purines are bound to this backbone via the methylene carbonyl. PNAs are achiral and non-ionic molecules with no sensitivity towards hydrolytic enzymatic cleavage. Unlike analogs of DNA or DNA itself, there are no phosphate groups or moieties of pentose sugar in PNAs. In spite of all these changes, the PNA has the ability to bind to the specific

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sequence of RNA and DNA through obeying hydrogen binding rule (Watson-Crick) (Fig. 1) [7,8]. Since the backbone of PNA has no charged phosphate group, therefore, there is no electrostatic repulsion which leads to augmentation of stronger connection between PNA and DNA strands in comparison to DNA/DNA duplex. The thermal stability of DNA/PNA duplex is higher than the DNA/DNA duplex with same length and also less influenced by the great ionic strength of the medium. Moreover, the stability of DNA/PNA hybrid is very sensitive to the existence of a single mismatched base pair. The two strands of DNA/PNA hybrid could be formed in both parallel and anti-parallel way, which is more stable in anti-parallel form with regard to the achiral backbone of peptide nucleic acid (PNA) [9].

Since enzymes are substrate specific, it is not simple to detect backbone of PNA through using proteases or nucleases, therefore, this leads to resistance of them towards enzymatic degradation and stability of them towards a broad range of pH [10]. Contrary to DNA with a regular phosphodiester backbone, the backbone of PNA is composed of 2-aminoethylglycine in which moieties of methylene carbonyl serve as a linker of nucleotide bases to the backbone. This characteristic of achiral peptide nucleic acid makes its synthesis, simple without the need for any stereoselectivity. Peptide nucleic acid oligomers can be synthesized by utilization of monomers of PNA and (methylbenzhydryl) amine polystyrene resin for the solid support through standard solid-phase procedures [11]. The aim of this review is to focus on PNA physicochemical characteristics and its diverse applications. We discuss about applications of PNA in designing various types of biosensors.

2. Physicochemical properties of PNA

Due to the significant advantages of nucleic oligomers like resistance against nucleases and proteases, great affinity for interaction, thermal stability, powerful strand invasion capability and strong binding at high concentration of salt, they are also employed in biological fields. DNA/PNA and RNA/PNA duplexes have great thermal stability in comparison to DNA/RNA and DNA/DNA duplexes, which is due to the absence of charge repulsion between RNA or DNA strand and neutral strands of PNA [12,13]. At low ionic strength peptide nucleic acid can attach to object sequence in temperatures where hybridization of DNA is vigorously inhibited. Therefore, the appropriate ionic strength could be beneficial for design of protocols where competing RNA or DNA is exist in the specimen or where probed nucleic acid involves secondary structure in greater levels [14]. The neutral molecules of PNA have less solubility in water than DNA. The solubility of these molecules is also dependent on the ratio of pyrimidine/purine and lengths of the oligomer. Negative charges in the DNA/PNA chimeras may also result in increment of water solubility, also some modifications, including incorporation of lysine (positively charged) and increase the solubility of water [12]. A summary of the structural and physicochemical characteristics of this analog is demonstrated in Table 1.

Fig. 1. A schema of PNA and DNA structure. The N-(2-aminoethyl)-glycine backbone of PNA (black) is hybridized in antiparallel direction by deoxyribose-phosphate backbone of DNA (gray). Point lines indicate the hydrogen linkage between the bases [8].

3. Modifications of nucleobases and PNA backbone

For synthesis of PNA molecule, the nucleobases which are not natural could also be used in order to obtain more stable duplex/triplex and create new identifiable motifs with potential for diagnosis and research applications. N4-benzoylcytosine [15] and Hypoxanthine [16] can be mentioned as modified nucleobases. Thiouracil as well 2,6-diaminopurine as a base pair has application in identifying DNA/PNA which lastly caused to double duplex invasion phenomenon. In addition, the modified nucleobase tricyclic G-clamp led to increment of stability of duplex through base stacking and base pairing while the presence of positive-charged side chains led to increment of solubility [17]. Moreover, other modified nucleobases including; 2-amino pyridine [9], thio-pseudo isocytosine [18] and mono-m- (guanidinoethoxy) phenyl] pyrrolocytosine [19] have the ability in determining RNA editing, targeting the region of RNA duplex, and increased selectivity for RNA, respectively.

Besides modification of nucleobases, the usual PNA monomer's backbone has been exposed to a diversity of rational alterations with an understandable approach of relations of structure. For applications such as molecular biology, diagnostics and medicine, PNA have some limitations like low membrane permeability, low solubility and ambiguity in orientation of DNA binding [20]. Chemical alterations in PNA were performed by adding different subgroups, configurationally restricted cyclic backbones or changed nucleobases. For instance, the replacement of the amide group in structures leads to decrease of binding tendency, which confirms the importance of restricted flexibility of the peptide nucleic acid backbone. A cyclic group like cyclohexyl, which was incorporated to boost the rigidity, led to a reduction in affinity towards binding [20], while adding compounds such as prolyl [21], (2S, 5R)-aminoethyl pipecolyl [22] and cyclopentyl [23] derivatives increased the affinity towards binding. Additionally, charged peptide nucleic acids have been advanced to increase the cellular delivery and solubility by introduction of groups such as guanidium [24] and phosphate [25] in the backbone. Table 2 demonstrates examples of nucleobase and PNA backbone variations and their effects on PNA characteristics.

3.1. Applications of PNA

Peptide nucleic acid biomolecules serve a powerful role in molecular genetics, identification and act as a diagnostic tool for genetic analysis in the advancement of factors of gene therapy. *In vitro* studies demonstrated that PNAs could cause inhibition of both gene transcription and translation, which confirm their application as

Table 1
Structural and physical and chemical properties of DNA and analog PNA.

Deoxyribonucleic acid (DNA)	Peptide nucleic acid (PNA)
Sugar-phosphate backbone	Peptide backbone (monomer: N-(2-aminoethyl) glycine)
Negative charge	Non-charge
Stability of DNA/DNA duplex at high salt concentration	More stability of PNA/DNA duplex at high salt concentrations
Sensitivity to protease and nuclease enzymes	Resistance to protease and nuclease enzymes
Sensitivity to pH changes	Stability in the wide range of pH

antisense and antigene agents. Nucleic acids which are termed antisense oligonucleotides attach to the complementary sequence mRNA in the target gene and lead to a reduction or prevention of the related protein production [34]. Conjugation of antisense peptide nucleic acid to the carrier peptide $(RXR)_4$ could be employed as a therapeutic platform for targeting variety of *P. aeruginosa* genes [35]. Conjugation of antisense peptide nucleic acids (APNAs) to the $(KFF)_3K$ peptide caused to targeting the growth of significant gene gyrA in Acinetobacter baumannii and displayed vigorous effects of inhibition with lowest bactericidal and inhibitory concentration of 10 and 5 μM, respectively [36]. For investigating the clinical usages in proliferative fibrotic disorders, it was discovered that as an antigene reagent, PNA caused to decline of gene's mRNA levels, thereby decreasing the production of type I collagen by fibroblast cells [37]. Additionally, antigene PNAs (agPNAs) could also be utilized for the object of silence expression with siRNAs or mRNA targeted peptide nucleic acids. AgPNAs lead to blockage of gene expression and exploration of chromosomal DNA [38]. For several hybridization applications of analogous, peptide nucleic acids can play roles as synthetic or natural DNA probes with more specificity and powerful binding. During the past years, the application of PNA as a probe draw a lot of interest for *in situ* hybridization tests. Due to growth *in situ* specificity and neutral backbone of PNA probes, needing for low concentration and speed of hybridization, they are very efficient in the fluorescent *in situ* hybridization application (FISH). The formed photosensitive structures based on PNA, as a result of PNA-DNA hybridization, have valuable potential in biotechnology, research, nanotechnology, medicine and diagnostics [39,40]. Polymer molecules of PNA could also utilize for the development of a variety of biosensors which will describe in below section.

4. PNA-based biosensors

Biosensors as an analytical tools are composed of three parts: A) biological, biochemical or chemical recognition element (bioreceptor) which has the capability to interact directly or indirectly with target analyte, B) a signal transducer which cause to conversion of biological responses into a measurable electrical signal; and C) an amplifier leading to amplification of signal [41,42]. Biosensors result in integration of attractive sensitivity and specificity of biology with transducer for conveying simple, easy-to-sue format of complex bioanalytical measurements. They have a variety of diverse applications in various fields, including environmental monitoring, drug control, food safety, biodefense and protection [43–45]. With regard to the type of transducer, the biosensors are categorized into five classes: i) Calorimetric, ii) Thermometric, iii) Mass-based, iv) Optical; and v) Electrochemical. Bioreceptors such as antibodies, enzymes, antigens, tissues, nucleic acids and whole cells have responsibility to attach target to the sensor and determining it through utilizing a biochemical mechanism [46,47].

Application of nucleic acids as a bioreceptor lead to increment of biochips potential and DNA-based biosensors. Nucleic acid bioreceptors are employed for identification of pathogens with regard to complementary base pairs. Because of simplicity, low-cost and being rapid, DNA-based biosensors are broadly applied for pathogen detection. Nucleic acid-based biosensors have drawn lots of attention because of their usefulness in various fields. Biosensors based on DNA commonly employed for specific and special nucleic acid sequence detection that are of considerable significance, especially in clinical diagnosis [48]. The use of peptide nucleic acids (PNA) as a bioreceptor has resulted in DNA biosensors progress. Furthermore, PNA as a probe has benefits like detecting single base mismatch, good hybridization characteristics and enhanced enzymatic and chemical stability associated with DNA [49]. The importance of PNA oligomers application as a probe in the development of biosensors described in the below section.

The modified peptide nucleic acid structures enhance the modulation and binding of PNA probes attributes which cause to facilitation of modern biosensing platforms generation [50]. Great linking of peptide nucleic acid oligomers has led to advancement of novel PNA applications, notably as a diagnostic probe. A significant step in designing PNA-based biosensors is the nucleic acid immobilization on solid surface, which leads to production of signals. For this purpose, various methods of immobilization, including chemical absorption, non-covalent binding and covalent binding can be utilized [51]. Moreover, sever absorption of soluble peptide nucleic acid oligomers on a variety of substrate materials permits non-covalent immobilization of peptide nucleic acids on different biosensor surfaces. Peptide nucleic acids boost the biosensors selectivity and specificity thanks to their unique physicochemical characteristics, so making use of these oligomers as a probe is beneficial for the development of optical, microarray, electrochemical and other biosensors.

4.1. PNA-based electrochemical biosensor

Electrochemical biosensors are broadly employed for detection of a variety kinds of analytes due to their profits like fastness, miniaturization, simplicity, low-cost and great sensitivity [52]. Formation of the compound at the biosensor surface from binding of bioreceptor and biomolecule results in detectable changes, converted into quantitative potentiometric, impedametric or amperometric signal [53]. The electrochemical biosensors are multifunctional instruments with electrochemical transducers such as voltammetric, impedimetric, amperometric or conductometric [53]. PNA-based electrochemical biosensors have been improved due to physicochemical features of peptide nucleic acid that are used in many fields [54]. In recent years, PNA has been utilized as a vital platform for detecting nucleic acid quickly and sensitively. Table 3 summarizes different electrochemical biosensors based on PNA.

4.1.1. PNA-based electrochemical biosensor for detection of DNA

As a bio-receptor, usage of PNA in biosensors was firstly developed by Wang et al., in 1996. In this research, by absorption of 15-mer peptide nucleic acid probe onto a carbon paste electrode and utilization of $Co(phen)_3^{3+}$, redox indicator, a DNA/PNA hybrid was formed. The response of hybridization is not dependent to ionic intensity and temperature and the obtained LOD (low detection

Table 2
Nucleobase and PNA backbone modifications and their effects on properties.

Structure	Modified nucleobase	Effects	Ref.
	6-Thioguanine	Reduced 8.5°C in Tm because of PNA-DNA heteroduplex, has less impact on the parallel complex stability	[26]
	Thiouracil	Attack on dsDNA (antigene application)	[27]
	Thiazole	The formation of a PNA probe capable of detecting hybridization through fluorescence increase	[28]
	2-Amino purine	Can form a hydrogen bond in the reverse Watson-Crick model with U and T, and also its intrinsic fluorescence can be used in kinetic studies of the hybridization processes with complementary nucleic acid	[29]
	Pseudoiso-cytosine	Mimics the C+ identification motif for triplex structure	[27]
	2,6-Diamino purine	Increasing selectivity and affinity for thymine	[27]
Structure	**Modified PNA backbone**	**Effects**	**Ref.**
	α-Amino methylene PNA	Increase cellular absorption	[27,28]
	γ-Amino methylene PNA	Enhanced binding PNA to DNA	[27,28]
	Pyrrolidine PNA	The formation of highly stable complexes with RNA and DNA	[21]
	Amino-ethylprolyl PNA	It has significant biophysical characteristic for the stabilization of the triplex	[21]
	Amino-prolyl PNA	Consolidation of PNA-DNA hybrid	[21]
	Diethylene glycol PNA	Improve hybridization by increasing water solubility	[29]

(continued on next page)

Table 2 (*continued*)

Structure	Modified nucleobase	Effects	Ref.
	β-methyl PNA (S and R forms)	PNA usually with on S-form chiral parts was suitable for forming a right-handed hybrid duplex with DNA	[30]
	α-lysine PNA	Stabilize the duplex of DNA-PNA	[24]
	γ-Amino propylene PNA	More duplex thermal stability of RNA-PNA duplex (Tm). More duplex DNA-PNA stability than duplex RNA-PNA (ΔTm).	[31]
	α-Guanyl-ated PNA	Significant cellular absorption by maintaining the identification of the Watson Creek	[32]
	Thiol modified PNA	No significant effect on the RNA-PNA duplex	[33]

Table 3
Reported PNA-based electrochemical biosensors.

Method	Target	LOD	Linear range	Ref.
Electrochemical	dsDNA oligomers (HCV SNP)	1.8×10^{-12} mol L^{-1}	1×10^{-11}–1×10^{-8} mol L^{-1}	[70]
Electrochemical	PCR amplicon	4.8×10^{-12} mol L^{-1}	1×10^{-11}–1×10^{-9} mol L^{-1}	[71]
Electrochemical	DNA (oligomer) and cellular RNA	10^{-13} mol L^{-1} (DNA oligo), 10^{-7} g (total RNA)	1×10^{-12}–1×10^{-10} mol L^{-1}	[72]
Impedimetric and square-wave voltammetry	miR-145	0.37 fM	1 fM–100 nM	[73]
Impedimetric	miR let-7a, let-7b, let-7c	0.50 fM	–	[74]
Impedimetric	single-strand DNA sequences	–	–	[75]
Electrochemical	BRAF and KRAS DNA mutations	1 fg μL^{-1}	–	[76]
Electrochemical	BRAF and KRAS DNA mutations	1 fg μL^{-1}	–	[77]
Electrochemical	pHCV3a	5.7×10^{-11} M	–	[78]
Electrochemical	M. Tuberculosis	8.948×10^{-13} M	$1x10^{-11}$–$1x10^{-7}$ M	[79]
Electrochemical	DNA hybridization	5.6×10^{-10} M	5.0×10^{-9}–2.5×10^{-7}M (R = 0.9940)	[80]
Electrochemical	Hg^{2+}	4.5 nmol L^{-1}	5–500 nmol L^{-1}	[81]
Electrochemical	M. Tuberculosis	0.1 fM (PNA/Fe_3O_4-GOPS/ITO bioelectrode), 0.1 pM (PNA-GOPS/ITO bioelectrode)	–	[82]
Voltammetric	DNA hybridization	2 pM	–	[83]
Voltammetric	short sequence of p53	6.82×10^{-10} M	–	[84]

NH2-GO/QDs: functionalized graphene oxide/CdS quantum dots.
pHCV3a: hepatiti C virus genotype 3a.
GOPS: 3-glycidoxypropyltrimethoxysilane.
ITO: indium-tin-oxide.
Fe_3O_4: iron-oxide.

limit) was 5×10^{-9} mol L^{-1} [55]. Hashimoto and co-workers immobilized a peptide nucleic acid probe on a 20-channel Au electrode for c-Ki-*ras* (cancer gene) determination. After the reaction of PNA with target DNA, it was incubated in a DNA connector solution (Hoechst 33258). This solution was attached to DNA-PNA hybrid minor groove and the anodic current evaluated using LSV (linear sweep voltammetry). Anodic current expanded by increment of *ras* gene's PCR product concentration from 10^{11}-10^{15} copy/mL (Fig. 2) [56].

Goda and colleagues designed a method for determination of DNA hybridization through utilizing a PNA probe. In order to evaluate the sensitivity enhancement through immobilized PNA

probes on the surface of electrode for potentiometric detection of DNA, a comparative examination was done about DNA and PNA probe hybridization on a 10-channel Au electrodes microarray. Alterations in density of charge were converted to potentiometric signals through an impedance electrometer utilization. The charge read-out permits multi-parallel, reagent-less and label-free determination of targets without any optical cooperation. The probe length variations between 15 and 22 mer impressively affected on DNA and PNA sensors sensitivity [58]. Cai et al., employed a FET (field-effect transistor) biosensor for label-free DNA/PNA hybridization determination through utilization of a transducer based on RGO (reduced graphene oxide). The designed biosensor had similarity to analogous DNA-modified biosensors with higher specificity and sensitivity, because of the high affinity of PNA towards complementary DNA. The obtained linear range was between 1.0×10^{-14} mol L^{-1}–1.0×10^{-9} mol L^{-1}. This technique had the capability for detecting one-base mismatch with great specificity. Moreover, it has been indicated that the developed biosensor could be utilized for several times [59].

Gaiji et al., developed a biosensor based on FND (ferrocene moiteties) for detection of DNA-PNA hybridization. 6-mercapto-1-hexanol (MCH) and PNA immobilized on the gold electrode surface. Hybridization of ssDNA was investigated by the addition of FND, which had the capability of binding to the duplex of DNA-PNA, and the electrochemical signal of FND was utilized for monitoring of DNA. Developed biosensor indicated a good linear range vary from 1 fM-100 nM and limit of detection was 11.68 fM [60]. Reisberg and co-workers also designed a PNA-based electrochemical biosensor for DNA hybridization determination. Immobilization of PNA occurred through covalent bond on the carbon electrode surface modified by electroactive polymer quinone. Details of the chemical structure of this polymer shown in Fig. 3. Quinone peak current enhanced because of hybridization of probe with target DNA. The detection limit was approximately 10 nM [61].

4.1.2. PNA-based electrochemical biosensor for detection of SNP

The determination of SNPs (single-nucleotide polymorphisms) employing peptide nucleic acid probes have the benefits of the important physicochemical and structural differences between the complete hybrids and single-nucleotide polymorphisms in PNA-DNA and DNA-DNA duplexes [62]. Hamidi-Asl et al., designed an electrochemical biosensor to detect single nucleotide polymorphism in the p53 gene through utilization of ssPNA as a recognition component. DPV (Differential pulse voltammetry) was applied for evaluating the interaction between oligonucleotides and redox index, MB (methylene blue). The detection of target molecule stemmed from hybridization of dsDNA with ss-PNA through hydrogen-bonded Watson Crick and non-Watson Crick. In the designed biosensor, the detection limit (LOD) was approximately 4.15×10^{-12} mol L^{-1} with 1.0×10^{-11} to 5.0×10^{-5} mol L^{-1} linear range. Also selectivity of biosensor was recognized via application of non-complementary double strand (ds) oligonucleotides and the results indicated that the biosensor has the capability to recognize single base mismatch directly on ds-oligonucleotide [63]. Arami and co-workers immobilized the 13-mer ssPNA probe on the surface of the gold electrode in order to design a PNA based electrochemical biosensor for SNP detection in the UGT1A9 gene promoter. The rate of hybridization was estimated based on difference between DPV signal of MB stored on probe-Au electrode and probe-sequence DcUG275 (target)-Au electrode. The PNA modified Au electrode in optimal conditions had the capability to identify point mutations and hybridization. The obtained LOD of biosensor was 22 nm [64]. Luo et and colleagues developed an electrochemical strategy for multiple SNP and specific DNA sequence detection via employing negative charge indium tin oxide electrode (ITO) and the neutral charge PNA labeled with Fc and MB (electroactive indicator), respectively. It is remarkable to mention that there was no need to immobilize a probe in this technique. The electrostatic repulsion between the duplex of DNA-PNA negative backbone was resulted from hybridization and the ITO electrode negative surface was prevented the electroactive indicator from electrode and led to suppression of the electrochemical signal (Fig. 4). Due to the immobilization-free characteristic of this strategy, there was no interference in SNPs and DNAs simultaneous detection and the SNP identified at 37°C within minutes [65].

By taking into consideration that the peptide nucleic acid molecule cannot serve as a primer for DNA polymerase because of its unique structure and formation into consideration, Kerman et al., utilized this for determination of sensitive hybridization in an electrochemical biosensor. PNA was utilized for blocking the amplifying action of PCR involved in the biosensor. The mutation in the gene was prevented PCR clamping peptide nucleic acid probe from binding, thus PCR was carried out and double strand-DNA amplified. DPV was used to evaluate the electrostatic interaction between neutral peptide nucleic acid molecules and negative DNA with redox-active metal cation $[Co(NH_3)_6]^{+3}$ (cobalt (III) hexamine). Attachment of PNA probe onto the surface of GCE (glassy carbon electrode) resulted in $[Co(NH_3)_6]^{+3}$ accumulation at the surface of sensor and the current signal boosted. In contrast, the coupling action of PNA probe with complementary DNA strand in the existence of wild gene was led to blockage of PCR amplification process and finally the reduction of $[Co(NH_3)_6]^{3+}$ accumulation on the surface of sensor and the current signal reduction [66].

4.1.3. PNA-based electrochemical biosensor for detection of virus

Other applications of PNA biosensors are in the field of virus detection. Jampasa and co-workers developed an acpc-PNA (anthraquinone-labeled pyrrolidinyl peptide nucleic acid)-based electrochemical biosensor for HPV (human papilloma virus)

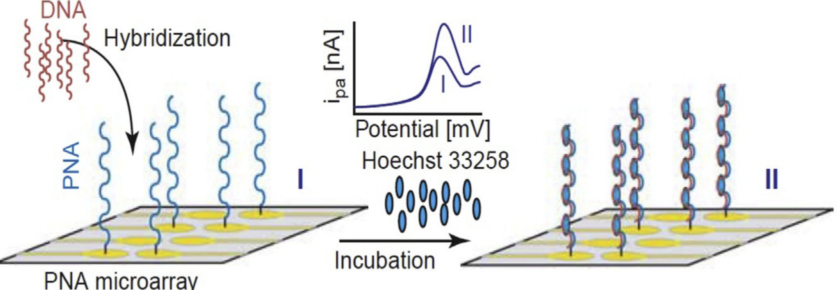

Fig. 2. Electrochemical determination. PNAs are immobilized on the Au electrodes of chip surface. After hybridization of PNAs with DNAs, the Hoechst 33258 links to the DNA-PNA hybrids. The i_{pa} (anodic peak current) is measured after attachment of Hoechst 33258 to the DNA-PNA hybrid by LSV (linear sweep voltammogram) [57].

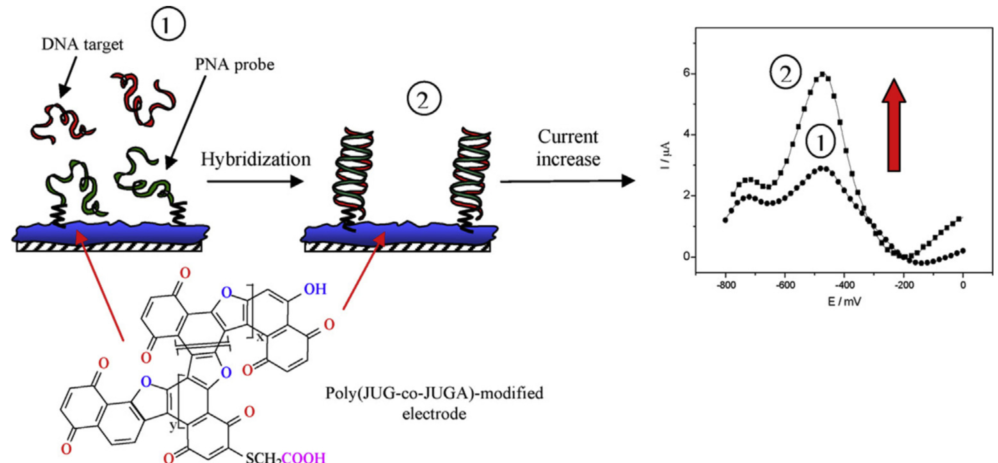

Fig. 3. A schema illustrating electrochemical PNA-based biosensor for determination of DNA hybridization, including three steps: 1) polymer building 2) hybridization reaction 3) transduction Process [61].

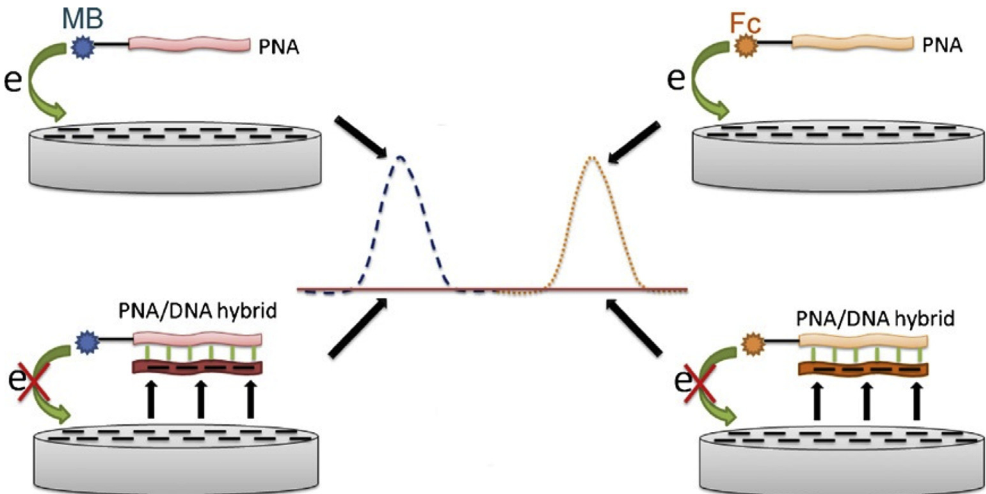

Fig. 4. A schema illustrating detection of DNA through immobilization-free multiplex electrochemical biosensor. Fc and MB labeled PNA probes are freely distributed on the ITO electrodes and converted to the electrochemical signal. The electrostatic repulsion between the negative DNA-PNA duplex backbone created by hybridization with target DNAs and ITO electrodes delayed the approach of the electroactive marker to the electrode and caused a significant reduction in the electrochemical signal [65].

DNA 16 type detection. The 14-mer acpc-peptide nucleic acid probe from N-terminal was attached to the redox active label-AQ (anthraquinone) through an amide bond and as a cross-linker C-terminal of the reside-lysine using glutaraldehyde was immobilized on a chitosan modified screen-printed carbon electrode. These tests were carried out through applying SWV (square wave voltammetry). The quantitation limit and LOD were 14 and 4 nM, respectively with 0.02–12.0 mM linear range. Results showed that the designed biosensor with great sensitivity and specificity needs a low amount of specimen. Preparing electrode with ease and the ability of detecting DNA from PCR samples was another plus point of this device [67]. Another paper-based electrochemical biosensor developed by Teengam and co-workers through the immobilization of the acpc-PNA probe (AQ-PNA) on a G-PANI (graphene-polyaniline modified) electrode for HPV type 16 detection. In order to modify the working electrode, the G-PANI composite solution was applied as an ink for inkjet printer. The immobilization of AQ-PNA probes on the surface of electrode performed through electrostatic attraction and evaluated by EIS (electrochemical impedance spectroscopy). The AQ-labeled electrochemical signal using SWV before and after hybridization was applied for detection of DNA target with the same sequence DNA type 16 HPV through a paper-based biosensor. Results indicated that the hybridization was led to a reduction in current because of the electron transfer inhibition from the AQ label to the surface of electrode by a duplex PNA-DNA. In optimal conditions, limit of detection was 2.3 nM and the obtained linear range was 10–200 nM [68].

Regarding the role of HCV (hepatitis C virus) in the prevalence of chronic liver disease, the detection of this virus is very significant. Ahour and colleagues developed an electrochemical biosensor using a PNA probe for detection of ds-Pl (double strand plasmid) directly and without any requirement for target plasmid denaturation. The modification of Au electrode with 6-mercapto-1-hexanol was followed by monolayer self-assembly of cysteine conjugated with a 20-mer peptide nucleic acid probe that complements the HCV core/E1 region and by attaching to the double strand plasmid was led to the creation of PNA/double strand plasmid structure. The

results showed the linear range of 10–300 pg/ml and LOD of 9.5 pg/ml [69].

4.2. PNA-based optical biosensor

Optical biosensors which are often proposed as an alternative to the electrochemical methods mentioned above. Some qualities like being real-time, label-free and direct determination of several biological and chemical materials gives them definite advantage over conventional analytical techniques. Optical biosensors have different types such as adsorption, luminescence, fluorescence, optical fibers and Surface Plasmon Resonance (SPR) [47]. Several studies have been performed about the application of PNA-based optical biosensors over the past decades. Table 4 summarized the PNA-based optical biosensors used for detection of various analytes.

4.2.1. PNA-based optical biosensor for detection of miRNA

Nucleic acids are utilized as a target for development of biosensors as an important tool for disease diagnosis. miRNA (micro-RNA), non-coding and short sequences, regulates the function of mRNAs molecules which are efficient in cellular activity and act as biomarkers for early detection of cancer in plasma, serum and whole blood. Different variety of peptide nucleic acid-based optical biosensors have been applied for micro-RNA detection [85]. Yildiz and co-workers designed a paper-based biosensor for naked eye determination of miRNA related to lung cancer (mir21) through immobilization of peptide nucleic acid on PVDF (poly (3-alkoxy-4-methylthiophene)) paper impregnated with luminescence reporter, PT (poly (vinylidene fluoride)). The basis for recognition of miRNA was the generation of mi-RNA/PT duplex structures and PT/hybrid PNA-mi-RNA triplex via creating two various optical signals was led to facilitation of diagnosis through naked eyes. The developed platform was capable of sensitive, fast and selective detection of miRNA at the clinical level without requiring complex and expensive tools and equipment [86]. A new probe designed through utilization of f-CNNS (functionalization of carbon nitride nanosheet) with Cy5-PNA (Cy5-labeled peptide nucleic acid) and folate for detecting intracellular and cancer cell micro-RNAs by Liao and colleagues. Quenching the Cy5 fluorescence was done through interaction of CNNS and Cy5-labeled peptide nucleic acid and existence of folate were led to inducement of probe specificity towards folate acceptor highly-expressed cells. The identification of peptide nucleic acid to complementary miRNA was led to the release of hybridization product from surface of CNNS, which was resulted in recovery of fluorescence and introduction of special strategy for miRNA sensing [87].

In order to detect multiple miRNAs (miR-96, miR-125b and miR-21) in live cancer cells Wu et al., designed a biosensor through combination of NMOF (nanoporous metal-organic frameworks) with PNA-labeled fluorophores such as cyanine 5 (Cy5)-PNA96, 6-carboxy-X-rhodamine (ROX)-PNA125b, and carboxyfluorescein (FAM)-PNA21. Hybridization PNA with target miRNA was caused by its separation from NMOF and increment of NMOF fluorescence features. The results exhibited that the optimum fluorescence quenching of 50 pmol of Cy5-PNA96, ROX-PNA125b, and FAM-PNA21 could be attained within 10 min by presenting 8 μL, 16 μL and 10 μL of 20 μg/ml nanoporous metal-organic frameworks, respectively. The developed biosensor had the capability to quantitatively and specifically detect multiple miRNAs in live cancer cells and permits *in situ* controlling of spatiotemporal alterations in expression of miRNAs [88].

4.2.2. PNA-based optical biosensor for detection of bacteria

Peptide nucleic acid-based biosensors have an application in the identification of pathogens with excessive stability and enhance the affinity towards target sequences. Recently, many studies have been done in the realm of PNA-based sensors application for bacteria detection. Joung and co-workers designed SPR (surface plasmon resonance) biosensor by employing PNA probe for greatly sensitive detection of *E. coli* 16S rRNA. Immobilization of non-specific and specific sequences of peptide nucleic acid probes against 16s rRNA of *E. coli* and *Staphylococcus aureus* occurred through neutravidin-biotin interaction on the SPR sensor chip surface. The cationic AuNPs (gold nanoparticle) were applied for amplification of signal using 16s rRNA/PNA probe ion interactions. The obtained results demonstrated the detection of *S. aureus* without rRNA purification and LOD for *E. coli* rRNA was 58.2 ± 1.37 pg mL^{-1}. The whole analysis procedure showed in Fig. 5 [89].

Chaumpluk and colleagues also designed simple and fast technique for the fluorescence determination of *E. coli* in fresh mango slices by hybridization of aeg-PNA (N-(2-aminoethyl) glycine peptide nucleic acids) with amplified DNA product on the malB target

Table 4
PNA-based optical biosensors.

Method	Target	Limit of detection	Linear range	Ref.
Fluorescence	miRNA	1.0×10^{-12} mol L^{-1}	1.0×10^{-14} mol L^{-1} to 1.0×10^{-9} mol L^{-1}	[95]
SPR	16s rRNA	58.2 ± 1.37 pg mL^{-1}	–	[89]
Fluorescence	*E. coli* DNA	10^2 copies/50 g	–	[90]
SPR	genomic DNA of MT	PNA/Au E (1.0 ng ml^{-1}), DNA/Au E (3.0 ng ml^{-1})	–	[91]
SPR	DNA of MT	0.26 pM	–	[92]
Localized SPR	E542K, E545K, methylation in PIK3CA gene	50 fM	–	[96]
SPR	DNA (PCR) encoding the verotoxin 2 of *E. coli*	7.5×10^{-9} mol L^{-1}	$4–16 \times 10^{-8}$ mol L^{-1}	[97]
Localized SPR (LSPR)	ssDNA (21-mer oligo and PCR-derived, TNF-α gene)	6.7×10^{-13} mol L^{-1}	10^{-15}–10^{-6} mol L^{-1}	[98]
SPR	DNA oligomer	10^{-15} mol L^{-1}	$1–500 \times 10^{-15}$ mol L^{-1}	[99]
SPR	DNA hybridization	about 0.2 μM	–	[100]
LSPR	DNA hybridization	1 fM	100 pM–100 nM	[101]
SPR	point mutation in W1282X	–	–	[8]
microstructuredoptical fiber (MOF)	DNA	–	–	[102]
MOF	Genomic DNA	–	–	[103]
MOF	DNA	–	–	[104]
MOF	DNA	–	–	[105]

gene through the LAMP (loop-mediated isothermal amplification) strategy. N-(2-aminoethyl) glycine peptide nucleic acids, aeg-PNAs, were derived from analogous oligonucleotide with series of N-aminoethylglycine monomers in the backbone. After hybridization with aeg-PNA, measurement of DNA signal was done by utilizing UV light source fluorescence visualization. This method had limit of detection at 100 copies of *E. coli* DNA per 50 g of the sample [90]. In another research, immobilization of cys (cysteine) modified NH2-ended peptide nucleic acid probe and 5-thiol-ended labeled DNA probe specific to *Mycobacterium tuberculosis* on the gold surface employed for determination of one-base mismatch, non-complementary and complementary target sequence in DNA of *Mycobacterium tuberculosis* via SPR. The Au/PNA and Au/DNA electrodes were recognized through contact angle, AFM (atomic force microscopy), EIS and CV (cyclic voltammetric) methods, respectively. Hybridization results demonstrated that there was not any connection between Au/PNA and Au/DNA electrodes and non-complementary target. The Au/PNA electrode acts more effectively than Au/DNA electrode for the one-base mismatch sequence detection. The detection limit of Au/PNA electrode (1.0 ng ml^{-1}) was better than Au/DNA electrode (3.0 ng ml^{-1}). The kd (dissociation rate constant) and ka (association) of complementary sequence of the Au/PNA electrode calculated to be 3.6×10^{-3} s^{-1} and 8.5×10^{4} m^{-1} s^{-1}, respectively [91]. Silvestri et al., tried to detect the MT (*Mycobacterium tuberculosis*) DNA via a PNA probe and utilizing azimuthally controlled grating-coupled SPR method. The peptide nucleic acid-based sensor layer was optimized by monitoring the composition of sensor surface with anti-deposition PEO (poly ethylene oxide) layer and a PNA probe. The sensor response was firstly evaluated in the existence of non-complementary and complementary sequences and then in the existence of amplified PCR specimens. The fluorescence-based microarray strategy was utilized as a control. The 8.9 pM LOD obtained for the complementary MT sequence by the fluorescence method which was 30 times more than that obtained by the SPR (0.26 pM). The achieved LOQ from the SPR was 2.56 pM, while it was 31.9 pM for the fluorescence method which indicated low detection limit of the SPR sensor [92].

4.2.3. PNA-based optical biosensor for detection of DNA hybridization and point mutation

By applying SPR technique, RNA or DNA hybridization could be detected with attached PNA probes to the flat surface. The interface between specimen (fluid) and chip is illuminated by utilizing polarized light, the hybridization of PNA and DNA causes alteration of index of array refraction. Changing the refractive index is associated to the alterations of mass at the surface of chip. This method can control the hybridization process real time during a test [57]. Cheperaporn Ananthanawat and co-workers fixed an acpc-PNA probe through streptavidin-biotin interaction on the surface of the SPR chips for detecting DNA hybridization. In order to evaluate the mismatch detection specificity in target DNA, the effect of target DNA concentration and ionic strength on hybridization of probes, binding orientation to target were formed and compared. Results exhibited that acpc-PNA had great sensitivity toward mismatches, less dependency upon ionic intensity and selectable antiparallel attachment in comparison to other probes. Regarding to these distinct features, acpc-PNA can be served as a probe in diagnostic and clinical sensors [93]. Yasunobu Sato et al., utilized 15-mer PNA and DNA probes immobilized on the surface of SPR in order to recognize G-G mismatch in the codon 2 of the K-ras gene for early cancer detection. The 15-mer peptide nucleic acid probe had great capability to identify mismatch in 11-mer target DNA at 40 and 25°C. But in the time of applying DNA as a probe, only at 40°C detection was possible, which confirms the melting temperature differences between the probe and the target that plays as a key factor in point mutation detection. These results exhibited that temperature, length of analyte and surrounded environment of ligand were significant in the single-base mismatch detection [94].

4.3. PNA-based microarray biosensor (biochips)

Biochips are analytical instruments based on probe molecules immobilization via covalent binding onto a solid material such as silicon, gold, modified glass and so on. The probe molecules are immobilized in two-dimensional miniaturized arrays of dots, usually with 10–150 μm diameter. By labeling target with fluorescence material and after hybridizing in microarray, the special probe-target interaction is detected via utilizing a high resolution scanner. Microarrays lead to an increment of efficiency and speed of proteomic and genomic investigations and offer high-performance analysis [106]. For the first time the nucleic acid microarray method was used in 1990 [107]. Biochips are produced by two alternative techniques including photolithographic method for *in situ* short oligonucleotide probes synthesis or pre-synthesized probe's mechanical deposition on a solid supporter [108]. Despite pervasive applications in biology, some limitations of classic microarray methods are needed for labeling target specimens with fluorescent materials. This leads to the advancement of alternative non-optical diagnostic techniques based on microarray that eliminate the

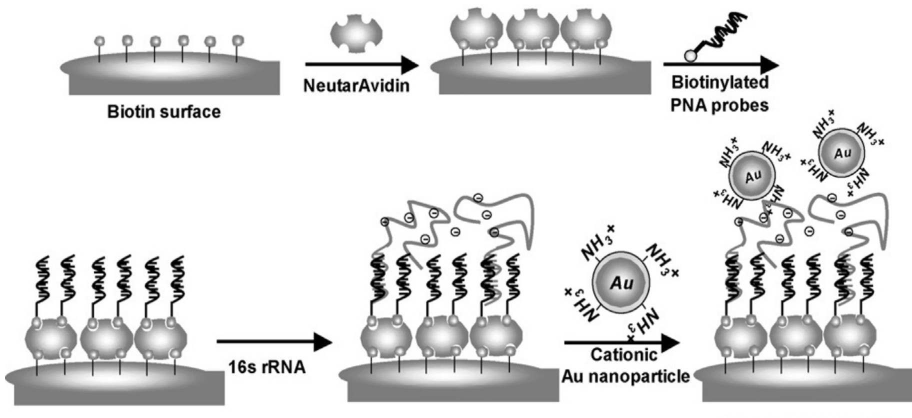

Fig. 5. An illustration of PNA probe/16s rRNA hybridization on a gold chip and amplification of signal through ion interactions with cationic AuNPs [89].

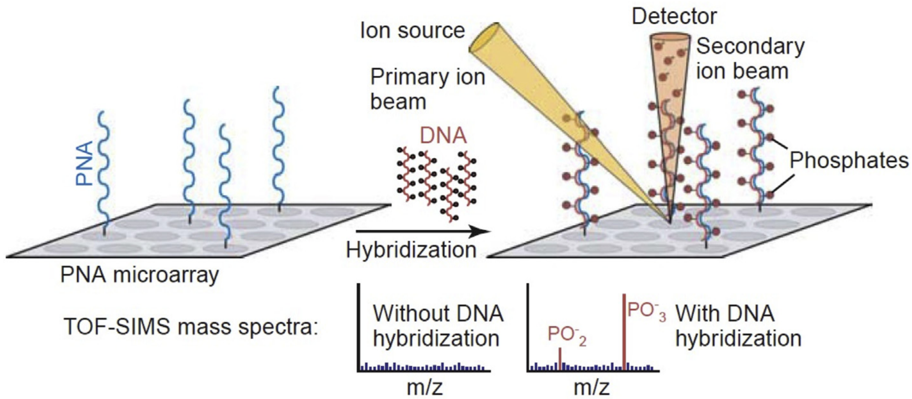

Fig. 6. TOF-SIMS examination of PNA microarrays. Phosphate ions are released as a result of bang on the initial ion beam to the surface. Therefore, PO_2^- and PO_3^- separated from the DNA backbone are visible after hybridization [57].

requirement of labeled-target molecules. The development of some of these are based on the application of analogs nucleic acid as a probe. In the view of more stability and novel hybridization attributes of peptide nucleic acid molecules, PNA-based microarrays were developed. Also, label-free DNA hybridization controlling via employing analytical strategies which has physicochemical impacts on phosphate or sugar in DNA and RNA or increment of negative charge during hybridization is possible with regard to the features of peptide nucleic acid backbone. Therefore, PNA-based microarray methodology eliminates one of the DNA microarray limitations and applied for detecting unlabeled DNA [109]. Brand et al., fabricated PNA microarrays for detection of unlabeled and labeled target with fluorescent through parallel synthesis of probe and full-length molecules selective coupling. Synthesis of PNAs was done via an automated procedure in filter-bottom microtitre plates. After separating the produced molecules from solid surface, they were connected to the surface of microarray through their terminal amino group. Therefore, the full-length PNA molecules were connected whereas generating incomplete molecules during synthesis were not coupled [110]. Better specificity and selectivity was achieved by utilizing thiol-modified PNA on maleimide surfaces. By employing MALDI-TOF mass spectrometry, the binding selectivity was analyzed through isolation of attached PNAs to the microarray surface. Unlabeled molecular hybridization was evaluated by phosphate (PO_2^- and PO_3^-) detection, which was an integral part of the DNA. Hence, it could be recognized the unlabeled DNA by combining PNA microarrays and TOF-SIMS (time-of-flight-secondary ion mass spectrometry) (Fig. 6) [57].

Jang and co-workers designed an array for determination of point mutations related to resistant antiviral agents (adefovir, lamivudine and entecavir) in the HBV (hepatitis B virus) in 68 clinical DNA specimens by using the PNA probe. The PNA array had the ability of 100 copies/ml detection. It is remarkable that when the virus whole DNA concentration was higher than 10^4 copies/ml, the PNA array could be recognized mutations in more than 5% of virus populations. Comparison of the results of the PNA array analysis with direct sequencing of hepatitis B virus genome mutation results demonstrated that PNA array accurately identified viral mutations and had 98.3% higher coordination with direct sequencing in the detection of antiviral resistant mutations [111]. Calabretta et al., immobilized PNAs on to surfaces through utilizing reactive μCP (microcontact printing). Firstly, surfaces were modified with aldehyde groups for reacting with amino-terminal groups of peptide nucleic acids. While, pattering PNAs were labeled with fluorescein by reactive μCP through employing oxygenoxidized polydimethylsiloxane stamps, construction and characterization of homogeneous arrays was done by optical methods. The surface of PNA patterns was hybridized with mismatched-dye labeled and complementary oligonucleotides for evaluating their capability to detect DNA sequences. Consequently, this method was utilized for making chips through spotting a peptide nucleic acid microarray onto a flat poly(dimethylsiloxane) (PDMS) stamp. The chips employed for detection of SNP (single nucleotide polymorphism) on the oligonucleotides [112].

5. Conclusion

The growth of biosensors has extreme selectivity and sensitivity. It has cardinal significance in application of drug discovery, genomics and clinical diagnostics. DNA-based biosensors have many applications in detection of different variety of analytes such as metal ions, proteins, simple organic composites and special nucleic acid sequences. However, in sensors which DNA is utilized as a bioreactor hold crucial disadvantages, including shortage of being resistant to restrictive enzymes and restricted capability to recognize single-base mismatches. Therefore, in order to eradicate these disadvantages, it is possible to utilize nucleic acid analogs such as peptide nucleic acid as a bioreactor in biosensors. PNA is a new type of compound which is employed for different applications in biology. It specifically interacts with RNA and DNA and their biological and chemical stability make them hopeful and promising in a broad range of applications. The primary advantage of peptide nucleic acid is a combination of various attributes which are greatly alike to those of natural DNA with more properties that are quite different. Improvement of peptide nucleic acid technology is acquiring momentum and many applications profit from the unique characteristics of the PNA. Peptide nucleic acid has found more exciting novel and very strong detection techniques, like 'biosensors' are being applied. According to the biological and chemical results of hybrid formation, peptide nucleic acid has a unique status in comparison to other derivatives of nucleic acids and could make an effect, especially in the fields of assays based on probe, antisense and antigene therapy. There will be very anticipation in the near future. with the cooperation of chemists and biologists, peptide nucleic acid can employ in completely its enormous practical potential. In this review, we summarized combination of PNA with biosensing platforms for sensitive detection of miRNA, viruses, DNA, bacteria and etc. Our main aim was to demonstrate the application of PNA probes in biosensing which could lead to a selective and specific determination of target analytes. Moreover, hybridization of PNA and ssDNA is firmly dependent on the presence of a single base mismatch between the

target sequence and probe. Consequently, PNA-based sensors are extremely accurate than biosensors which are based on DNA. These attributes open new windows for improvement and application of peptide nucleic acid-based biosensors.

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