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Application strategies of peptide nucleic acids toward electrochemical nucleic acid sensors

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Abstract

Peptide nucleic acids (PNAs) have attracted tremendous interest in the fabrication of highly sensitive electrochemical nucleic acid biosensor due to their higher stability and increased sensitivity than common DNA probes. The neutral pseudopeptide backbone of PNAs not only makes the PNA/DNA duplexes more stable but also provides many opportunities to construct ultrasensitive nucleic acid sensors. This review presents the details of various constructed protocols of PNA-based electrochemical nucleic acid sensor. The crucial factors, origin, and development of PNA, immobilization methods of PNA probes and signal generation mechanisms, are discussed. This review aims to provide a reference for ultrasensitive PNA electrochemical biosensor preparation.

Key words: signal amplification, PNA, electrochemical biosensor, labels, nucleic acid detection

1. Introduction

Recently, the recognition of disease status and the identification of microbial species at the biomolecular level have attracted wide attention from researchers, which is of great importance to improve the efficacy of pharmaceutical treatment and reduce the mortality[1-3]. Nucleic acid is one of the important biomolecules due to its high specificity and great influence on health.

The electrochemical detection of nucleic acid is a popular method because of its advantages of fast detection speed, simple operation, and no need for costly instruments[2, 4-6]. Electrochemical biosensors are highly integrated analysis devices that integrate specific identification and conversion components to convert biological signals into electronic signals[7, 8]. The strong recognition ability of

specific identifiers is an important guarantee for the excellent performance of electrochemical biosensors.

The common specific identifier of the gene sensor is DNA sequence, called DNA probe, which can specifically recognize the nucleic acid target by Watson–Crick base pairing. The advantages of DNA probe are good selectivity, reliable synthetic technology, and low cost. However, DNA probe and target DNA are negatively charged, and electrostatic repulsion reduces the hybridization efficiency of the probe and the stability of the hybrid[9]. Moreover, DNA probe is susceptible to be degraded by enzyme and lose recognition ability. To address these problems, researchers try to synthesize nucleic acid analogues.

In these analogues, the ribose phosphate backbone of nucleic acid is replaced by other sugars or polypeptide, such as glycerol nucleic acid[10], pyranosyl-RNA[11], bicyclo-DNA[12, 13], 2′-O,4′-C-methylene-linked β -D-ribonucleotide polymer (locked nucleic acid)[11, 14], and N-(2-aminoethyl)glycine polymer (peptide nucleic acid [PNA])[15, 16]. In addition, nucleobases are incorporated into the pseudo nucleic acid backbone, which confers synthetic analogues to form heteroduplexes with DNA or RNA by base pairing. Among these analogues, PNA is a widely used, promising specific probe of nucleic acid due to its inherent structure and characteristics.

At the end of the 20th century, the PNA probe was introduced into the hybridization assays[17]. PNA is a nucleic acid analogue with a neutral N-(2-aminoethyl) glycine-based pseudopeptide backbone, in which the monomers are linked via peptide bonds[15, 16]. It can form a more stable heteronucleic acid double chains with DNA/RNA through Watson–Crick base pairs than that of DNA/DNA, DNA/RNA, and RNA/RNA duplex[18, 19]. For single nucleotide polymorphism detection, the melting temperature change of PNA/DNA heteroduplexes is more substantial than that of DNA/DNA duplexes (about 15 °C versus 4 °C), thus demonstrating a better selectivity of PNA-based probe[20, 21].

The outstanding performance of PNA in terms of chemical stability, specificity, and modifiability makes it have great potential in disease diagnosis and treatment[22]. In the last decades, numerous PNA-based sensors were reported, and the potential of PNA in detection gradually emerged. Recently, Moccia et al. reported the emerging technologies of PNA-based biosensors, involving electrochemical PNA biosensors, optical PNA biosensors, and crystal microbalance PNA biosensors[23]. The typical examples of the above types of sensors were introduced from the aspects

of PNA fixed method, signal generation mechanism, and detection performance. However, no special discussion focused on the methods of probe immobilization and amplification strategies of electroanalytical signal.

For the construction of PNA-based electrochemical nucleic acid sensors, first, the structure of PNA and its physicochemical properties in solution, such as solubility in water[24] and rigidity of backbone[25], have to be learned. High solubility in analysis medium is the premise that PNAs can hybridize with the target nucleic acid, and the moderate rigidity of PNA structure makes the PNA–target hybrid more stable. Second, the immobilization of PNA probe is a crucial starting step of sensor preparation, which affects overall detection performance. How to immobilize PNAs on the sensor surface without damaging the performance of probe detection is very important. Third, the generation mechanism of detection signal is the core of electrochemical sensing detection system. Scientific, cleverly designed detection schemes is an effective way to achieve ultrasensitive detection.

The utilization of PNA characteristics to construct electrochemical biosensor is a promising way to improve the detection limit and sensitivity of the electrochemical nucleic acid sensor. However, no review of signal amplification strategies has been reported so far for PNA-based electrochemical biosensor. Here, an overview of recent advances in PNA-based electrochemical nucleic acid sensors is reported. The origin and development of PNA, immobilization methods of PNA probes, and signal generation mechanisms are summarized.

2. Peptide nucleic acids

Since PNA was synthesized by Nielsen and Egholm[24], it has attracted great interest from researchers. PNA is an uncharged polymer composed of pseudopeptide backbone (N-(2-aminoethyl) glycyl units) and natural nucleobases, and these two components are connected through methylenecarbonyl linkers[26]. A typical chemical structure of PNA with a non-charged N-(2-aminoethyl) glycine unit was shown in Figure 1a. PNA is not a natural nucleic acid. Therefore, PNAs are not recognized and degraded by nucleases and proteases[27, 28], which is conducive to keeping the recognition ability well in detection and retaining signal stability in post processing, such as washing and drying[29].

The superior performance of PNAs in affinity and selectivity to complementary DNA/RNA

sequence makes it possible for ultrasensitive detection using biosensors. However, unmodified PNA has several disadvantages, which represent serious obstacles to its applications, such as poor solubility in water[30], low cellular uptake[31], and more pronounced local aggregation on the sensor surface[32].

Several efforts have been made to solve these problems. Researchers reported that the water solubility of PNAs could be considerably improved by the modification of $-NH_2$[33-35] and $-OH$[36]. In the research of Zanardi et al., four lysine residues (lys4) were introduced into homothymine PNA decamer to improve PNA solubility. The analysis of spectrophotometrical results at 260 nm indicated that PNA-lys4 is nine times more soluble than unmodified PNA[37]. –OH could be introduced by the incorporation of L-serine, in which the –OH could form hydrogen bonds with water molecules and subsequently enhance the solubility of PNA[36]. Sahu et al. successfully introduced a small, hydrophilic (R)-diethylene glycol to the γ-backbone to improve water solubility[38]. This PNA could be synthesized from a relatively cheap Boc-L-serine. Zhou et al. showed that the cellular uptake rate could be increased by the introduction of guanidinium group into the PNA backbone[39]. The improvement of PNAs involved in these studies is mainly based on the introduction of side chain functional groups or related derivatives. It has the advantages of high feasibility and remarkable improvement in chemical properties. The performance of PNAs has been improved by introducing side chain groups, but several challenges need to be addressed for selective binding of parallel or antiparallel targets and further improvement of binding affinity[40].

To improve the affinity of PNAs with DNA or RNA further, researchers designed and synthesized chiral PNA monomers[41]. Chiral PNA sequence could be synthesized and adjusted by different chiral monomers to improve the selectivity and affinity of the PNA probe. The sequences composed of chiral monomers are relatively rigid, which can avoid the entropy loss of chemical bond rotation in the PNA backbone[42]. The decrease of entropy loss not only enhances hybrid stability but also improves hybridization dynamics.

The investigation of PNA backbone demonstrated three common sites for the incorporation of functional groups, namely α, β, and γ. The installation of a chiral center at γ position induces helical organization in PNA oligomer, enhancing the thermodynamic stability of PNA[43, 44]. Whether PNA is preorganized into a right-handed helix or a left-handed helix is closely related to the monomer configuration. The PNA synthesized from L-amino acids adopts a right helix structure,

and that made from D-amino acids adopts a left helix structure. Right-handed helical γ PNA presents high affinity and selectivity[38]. For the modifications of chiral PNA[45], an open chain chiral PNA monomer was obtained by functionalized substitution one or more of the positions (α, β, γ), which was shown in areas with a light yellow background of Figure 1b. Methylene[46, 47], ethylidene[48, 49] or butylene[50] bridges the two sites (α and β′, γ and β′, γ and α′, β and β′, α and γ, β and γ carbon) to obtain chiral PNA monomer with a ring (five-membered or six-membered ring), which was shown in the areas with light green background of Figure 1b. More details about chiral PNAs can be found in these literature[51-54].

Figure 1

3. Immobilization of PNA probe

Immobilization of PNA probes is the first and important step for applications of electrochemical biosensor. In electrochemical detection, the probes are immobilized on the sensor surface before detection, and then nucleic acid hybridization occurs at the solid–liquid interface. Different from reaction in solution, the surface-based sensor shows good performance in high sensitivity and reduced interference[7, 55]. In several studies, the "arm" molecule, a nonspecific nucleic acid sequence or C_6 chain, was attached to the fixed end of the probe to reduce the adverse effect of the sensing surface on probe hybridization performance. However, the hybridization between the immobilized probe and the target still suffers from sterical interference between probes[56].

The research suggested that the high surface density of probe leads to the deviation of hybridization kinetics from the theoretical value[32, 57]. An excessively high probe density would reduce the accessibility of the analyte toward the probe surface. This behavior is due to the steric hindrance of probes and the electrostatic repulsion generated from the formed hybrid[58]. Low-density probes can weaken the adverse effects of adjacent probes on hybridization, but the price paid is the reduction of target binding sites[59]. The control of probe density is an important, challenging work for the fabrication of electrochemical biosensor[60, 61].

The conformation of attachment probes and nonspecific adsorption affect the hybridization efficiency as well. Figure 2 shows two possible attachment states, standing up and lying down, when

the probes are immobilized on the sensor surface[62-64]. The research of Hook et al. indicated that multiple contact sites between "lying down" probes and substrate would reduce subsequent hybridization efficiency[65]. Therefore, a suitable immobilization method is also crucial for improving hybridization efficiency, which plays a fundamental influence on stable, sensitive detection.

In this section, the immobilization of PNA-based electrochemical biosensor is summarized in two aspects, immobilization methods and substrate properties. The crucial issues, conformation of attachment probe, and control of probe density are discussed in immobilization methods. The construction procedures, mechanism, and detection limits of typical construction scheme are described in detail.

Figure 2

3.1 Immobilization methods

Several methods can be used for probe immobilization including covalent binding[66], noncovalent binding, chemisorption, and electrostatic adsorption. Covalent binding is usually carried out by modifying a linker group or a nature group ($-NH_3$ and $-COOH$) on an end of PNAs, and the group reacts with the corresponding group on the substrate, such as $-COOH$, $-NH_3$, and $-CHO$. Avidin–biotin binding is a typical example of noncovalent binding, which shows good stability and specificity. The operation of physical adsorption is simple, but the number of attached probes and probe density cannot be controlled accurately, such as self-assembled monolayer on gold (Au) surface[67]. The electrostatic adsorption of probe immobilization was directly used in DNA sensor[68]. For the application of this method in PNA immobilization, the PNA must be premodified with charged groups due to its neutral properties. Figure 3a shows that the N-terminus of PNA was modified with a negatively charged amino acid, and then the PNA was attached to positively charged substrate through electrostatic attraction[69]. Several examples of PNA probe immobilization methods are shown in Table 1.

Acylation is a common reaction for the immobilization of PNA on glass substrates, such as $-COOH$ and $-NH_2$[70, 71], $-NH_2$, and $-CHO$[72]. Therefore, chemically functionalized surface[73] and silanization on SiO_2[74] are common operations in the pretreatment of SiO_2 substrate. Macanovic and coworkers proposed that PNAs could covalently link to the Si/SiO_2 substrate by prefunctionalizing

the substrate with 3-glycidoxypropyltrimethoxysilane (GPTS)[75, 76]. In the presence of hydrolyzable methoxy group in GPTS, silane can be covalently bonded to the oxide surface through siloxane bonds. Another probe immobilization method on SiO_2 substrate is based on the formation of secondary amine between an epoxysilane monolayer and the 3'amino linkage of probe[74], as shown in Figure 3b. The preparation is easy and without premodification of PNA probes because the epoxide functional group could bind to the PNAs[77]. Amidation reaction was also used in glassy carbon electrode[78] and screen printed carbon electrode[79], and the details are shown in Table 1.

For gold[80] or gold-coating[81] electrode surface, a typical immobilization method is to modify the PNA probes with thiolate (−SH), an then the probe is immobilized through the Au-S bond[82]. Au-S contacts were confirmed by Inkpen et al. as physical adsorption using numerous control experiments, conductance noise analysis, and transport calculations based on density functional theory[67]. The schematic Au-S is shown in Figure 3c(Ⅰ)[83]. As reported, free thiol and disulfide can react with gold surface spontaneously and rapidly[84], but thiol-bound oligonucleotides would dissociate from the surface in the presence of competing thiol reagents or high temperature (> 60 °C)[85]. To improve the stability of coupling compounds, dithiane-bridged conjugates (shown Figure 3c(Ⅱ))[86] and the connection with three sulfur groups (shown in Figure 3c(Ⅲ))[85] were proposed to immobilize oligonucleotides. In the research of Zanardi et al., the Au-N interaction was reported as a feasible approach to attach PNA on gold substrate[37]. These amines were introduced in PNA synthesis, without additional modification during immobilization. Much work has been done to clarify the mechanism of Au-N interaction, but no clear mechanism has been found[87, 88]. Zanardi et al. found that the adsorbed amine does not modify the surface of gold by the spectrum test[37]. Au-N interaction is probably physical adsorption.

Another research of self-assembled monolayers of thiol-derivatized PNAs on gold surface indicated that the concentration of PNA probe solution remarkably affects its orientation on the surface, and the orientation cannot be adjusted with adsorption time[89]. The reorientation of the molecule from lying down to standing up geometry can be accomplished by increasing the concentration from 0.01 μM to 10 μM[89]. A similar observation was reported in the work of Briones et al.[90] In probe immobilization, low-concentration probes contribute a strong interaction between side chains and surface, while the main interaction for high-concentration probes is adjacent chains

rather than the interaction between side chains and surface. Therefore, attention must be paid to the concentration of self-assembly solution in probe immobilization.

However, the research of Höök et al. suggested that PNA/DNA probes immobilized directly on bare gold surface by Au-S bond are less efficient than those immobilized with streptavidin–biotin coupling[65].

The interaction between biotin and streptavidin is an important method to immobilize PNA probes. Biotin is a small molecule that has a high binding affinity with avidin or streptavidin, which could be used to modify oligonucleotides without damaging hybridization ability[91, 92]. As reported by Kerman et al.[15], 5'-end of PNA was labeled with biotin, and superparamagnetic iron oxide beads were coated with streptavidin. The PNAs were successfully immobilized on the beads through the stable linkage between biotin and streptavidin, which is a noncovalent interaction and dissociation constant (Kd) is 10^{-14}–10^{-15}mol/L. Every streptavidin has four biotin binding sites, and the shape of binding sites are complementary to one another within two kinds of molecules[32], as shown in Figure 3d. The use of biotinylated PNA as a capture probe seems to reduce structural interference and improve hybridization efficiency with the target[93].

Figure 3

For the regulation of probe density, researchers proposed that the surface density of probe could be controlled by changing the deposition time of the probe[94] or adjusting the concentrations of the probe buffer[95]. However, accurately controlling probe density is impossible, and characterizing the prepared probe density in these methods is difficult. Movilli et al. developed a strategy to adjust the probe density on the biosensor surface through the modified poly(L-lysine) (PLL) with maleimide[96]. First, a predeposited layer of PLL-ethylene glycol-maleimide was fabricated. Then, thiol-functionalized PNA probes were coupled to the deposited layer by the interaction between maleimide and thiol. In this case, the surface density of probes could be easily adjusted with the changing amount of maleimide groups in the preceding synthetic step of PLL. The scheme is shown in Figure 4a[96].The relationship between probe density and PLL grafting density was evaluated by the function relation between Mal and complementary DNA using quartz crystal microbalance. The absolute probe density values on the biosensor surface were determined by cyclic voltammetry (CV) experiments using methylene blue-functionalized DNA.

Another problem that should be noted is that the neutral charge of PNA brings more difficulties

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in achieving a homogeneously diluted layer than negatively charged DNA. The absence of electrostatic repulsion may result in local crowding of PNA probes[97-99]. In the above method, the average density of the probe could be easily adjusted, but it cannot control the local density effectively. The local area with high probe density tends to nonspecific adsorption of probes through base pair interaction, which will not effectively hybridize targets and further decrease detection sensitivity[100]. To address this issue, Yao et al. hybridized PNA probes with short complementary DNA sequence to introduce the charge into the probe prior to probe immobilization[32]. Then, the short DNA sequence was removed after the prehybrid was evenly immobilized on the surface. Simon and coworkers took a similar strategy to improve hybridization efficiency[101], and the scheme is shown in Figure 4b. An additional advantage of prehybrid immobilization is the minimization of nonspecific adsorption. The possibility of nonspecific adsorption between the substrate and bases was eliminated because the bases of double-stranded DNA were occupied by the hydrogen bond between complementary chains[32, 101].

Figure 4

Table 1. Examples of PNA probe immobilization methods

Immobilization method	Type of substrate	Key points of immobilization strategy	Ref.
Covalent bonding	Glassy carbon electrode	Amidation reaction; The free terminal amine group of PNA reacts with the NHS-activated ester on poly(5-hydroxy-1, 4-naphthoquinone-co-5-hydroxy-3-thioaceticacid-1,4-naphtho-quinone)-electrode.	Reisberg et al.[78]
	Single-walled carbon nanotube	Amidation reaction between -COOH and $-NH_2$. $-COOH$ or NH_2 was modified on one end of PNA, and another group was modified on the single-walled carbon nanotube.	Jung et al.[70, 71]
	Glass/ SiO_2	The formation of secondary amine; Amine linker on the end of PNA reacts with	Lamture et al.[74]

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		epoxide monolayer coated on SiO_2.	
	Glass/ SiO_2	Reaction between amino group and aldehyde group; Modify the silicon surface with aldehyde organic monolayer. Then PNA with N-terminus reacts with aldehyde group, and subsequently PNA was immobilized.	Rogero et al.[72]
	Gold	Reaction between thiol-reactive maleimide and thiol group; Modify the electrode with poly(L-lysine) polymers containing thiol-reactive maleimide, and then the thiol-modified peptide nucleic acid reacts with maleimide.	Jacopo et al.[96]
	Screen printed carbon electrode	Amidation reaction between -COOH and $–NH_2$; Firstly, Graphene oxide(GO) was functionalized with $–NH_2$. Secondly, NH_2-GO and quantum dots were deposited onto screen printed carbon electrode. Lastly, PNA with –COOH reacts with the $–NH_2$ on electrode.	Mohd Hazani Mat Zaid et al.[79]
	Graphene-based electrode	Pyrenebutanoic acid succinimidyl ester (PASE) was used as a cross linker to immobilize PNA on the graphene surface. PASE was attached to graphene surface by π-π stacking interaction.	Zheng et al.[66]
Non-covalent bonding	Gold	Streptavidin-biotin interaction; PNA with a biotin moiety at the 5'-end or the N-terminus assembled on the streptavidin coated gold electrode.	Yao et al.[92]

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	Iron oxide beads	Streptavidin-biotin interaction; PNA with biotin was attached to strepavidin coated beads.	Kerman et al.[15]
Physical adsorption	Gold or gold-coating electrode	Au-S bond; The free terminal -SH of PNA attached to gold electrode surface.	Liu et al[82]
	Gold	Au-N interaction; The amine radical in PNA interacts with the gold surface.	Zanardi et al.[37]
Electrostatic attraction	Paper-based electrode/Graphene-polyaniline modification layer	Modify the screen printing electrode with graphene-polyaniline, which is positively charged. Then the PNA with a negatively charged amino acid at the N-terminus was attracted and immobilized on the electrode.	Teengam et al.[69]

3.2 Substrate properties

As mentioned above, the probes were required to be preimmobilized on the electrode surface for surface-based sensor. Therefore, the geometric structure, biocompatibility, and electrochemical properties of the substrate have an important effect on the detection results. The utilization of materials with a great surface area and a high conductivity is a promising strategy to improve detection sensitivity[102]. Examples of improved detection performance were summarized by adjusting the substrate structure and materials. Several of them have not been reported by PNA probes but can be applied to PNA detection system in the future.

Commonly used working electrodes are gold electrode[103], carbon paste electrodes (CPE)[104], and glassy carbon electrodes[105]. The surface of these electrodes is a smooth plane. The research focus of these electrodes is to enhance stability and conductivity. Hamidi-Asl et al. prepared a CPE modified with Au and platinum (Pt) nanoparticles (Au-Pt-CPE), which was used to immobilize PNA probe related to the p53 gene[106]. The resistance of Au-Pt-CPE was considerably decreased from 810.78 Ω (bare CPE) to 71.58 Ω. In this section, the influence of the micro nano structure of the substrate surface on the detection performance of the probes is mainly reviewed. More planar

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electrodes are not discussed.

Many researchers place utmost effort into the development of substrate with nanostructure, in which the small curvature radius of the immobilization surface contributes to accessibility during hybridization[107, 108]. In the comparative test of smooth surface and nanostructure surfaces, the CV signal of gold-nanostructured surface is about 1.35 times higher than that of smooth surface for 2 nM of target solution[109]. In the research of Wang and coworkers, an electrode with gold nanostructure was easily fabricated by the deposition method[110]. The detection limit of the obtained nanostructure biosensor was 1 pM using $[Ru(NH_3)_6]^{3+}$ as a redox indicator, which is about three orders of magnitude higher than that of planar gold electrode.

Deposition of nanoparticles on the substrate is a common method to fabricate composite electrode substrate. Nanoparticles possess several attractive physical and chemical properties that make them the best candidates for the preparation of micro nano structures to improve the detection ability of biosensor[111]. The introduction of nano materials not only improves the material properties of the electrode but also provides a better surface morphology for probe immobilization and target hybridization[112, 113].

Bao and coworkers successfully fabricated a gold nanoparticle/polypyrrole-reduced graphene oxide nanocomposite as a substrate for probe immobilization[114]. The CV measurements indicated that the peak current of electrode with nanocomposite is about twofold higher than that of bare electrode.

Other researchers also showed that the deposition of bimetallic nanoparticles is an effective way to improve the performance of the sensor, such as Au/Pt nanoparticles[68] and Ag/Pt nanoparticles[106]. Hamidi-Asl et al. investigated CPE performance before and after modification with mixing of 15% Ag and Pt nanoparticles[106]. The study demonstrated that the current of 15% Pt-CPE is about two folds higher than the current of bare CPE, the current of 15% Ag-CPE is about 10-fold higher than the current of bare CPE, and the current of 15% Ag/Pt-CPE is 14-fold higher than the current of bare CPE. The results show that the conductivity of nanocomposite modified electrode is remarkably higher than that of single metal modified electrode and bare electrode. Reducing the surface electron transfer resistance is the main driving force to improve the performance of the sensor.

Several researchers used paramagnetic microbeads as a substitute solid support in probe

immobilization. An evident advantage of magnetic beads as solid support is that hybridization detection on the surface of beads can substantially inhibit nonspecific adsorption errors[115, 116]. Serena et al. successfully immobilized biotinylated DNA, LNA, or PNA capture probes on streptavidin-coated paramagnetic micro beads, and the constructed biosensor could accurately recognize the target sequence via complementary base pairing. Based on beads, a label-free detection of DNA test was carried by using chronopotentio-metric adsorptive stripping analysis[115]. The detection limit was 160 pM. Additionally, the inherent magnetism of the beads contributed to separation and collection of the PNA probes during incubation and washing step.

In addition to nanoparticles, other nanomaterials, such as nanorods and nanotubes, are used in electrochemical biosensors.

Guan et al. developed a glucose biosensor based on the multiwall carbon nanotube modifier and glucose oxidase[117]. Since the reduction of electrons transfer resistance between the modified electrode and the mediator, the peak separation of cyclic voltammogram decreased from 750 mV to 45 mV. It clearly demonstrated that the biosensor with nanostructured electrode performs better in sensitivity and linear response range than the conventional planar electrode biosensor. The improved electron transfer efficiency with nanostructure was further evaluated by Chen and coworkers[118]. Nanomaterials exhibit superior property of electron transfer on electrochemical transducer surfaces, which is favorable for signal amplification[119]. Moreover, in the presence of microstructure, the required analyte diffusion distances are reduced in the presence of micro and nano scale structures, resulting in fast response times[109, 120].

Moreover, good electrical conductivity and surface characteristics of the nanostructure allow increasing the signal/noise ratio of the biosensor[121]. A large specific surface area of the nanostructure offers larger suitable areas for probe immobilization with respect to a smooth surface[109]. In several cases, nanostructured metal surfaces were developed to increase the number of immobilized probes in the biosensor[120].

4. Mechanisms of signal generation

The efficient conversion of hybridization event into electrical signals is the core of sensor design.

According to the mechanisms of signal generation, it is mainly divided into direct signals (oxidation of guanine or adenine in sequence) and indirect signals (various labels, such as $Ru(NH_3)_6$ and Meldola's Blue). Representative examples are presented in Table 2.

4.1 Direct signal detection

PNA can directly invade the double chain and form a stable triple chain structure[122]. Based on this feature, Ahmadi and Ahour constructed a PNA-based electrochemical biosensor for direct recognition of ds-DNA[123]. After interaction between PNA probes and ds-DNA, the probes detached from the electrode, resulting in a decrease of guanine oxidation. The detection limit was 1.3 pM. On the contrary, Hamidi-Asl et al. successfully utilized the enhancement of guanine oxidation current after duplex formation between the probe and target to analyze the hybridization event on sensor surface[68]. The detection limit of their sensor was 53.10 $pg\mu L^{-1}$.

Detection systems based on direct signal have the advantages of simple operation procedure of electrode preparation, low cost, and without requirement of the label preparation and additional chemical modification. However, in most cases, the detection sensitivity of direct detection is lower than that of the system involved labels. In this section, several construction schemes of PNA probes that are different from DNA probes are mainly reviewed, focusing on indirect signal detection. The details are as follows.

4.2 Indirect signal detection

For nucleic acid sensor, the application of labels is an effective method to enhance detection signal. In general, labels have a unique detectability or an interaction with other components to produce a strong detection signal. In the past decades, numerous types of labels were developed in biosensor, such as enzyme, inorganic catalytic label, electroactive indicator, metal nanoparticles, and electroactive polymer[124, 125].

For further improvement of sensor, all behaviors should be considered, such as introduced methods of labels, stability of labels, signal amplification ability, and the influence of labels on hybridization performance. In this part, the labels used in PNA-based electrochemical sensor and

several DNA-labels that might be utilized for PNA-based electrochemical sensor in the future are reviewed.

4.2.1 Direct detection of redox labels

Designing PNA sensors by using the characteristic of neutral charge backbone of PNAs (different from DNA probe) is an important idea. In detection, the PNA probe modified sensor surface gradually changes from a no-charged state into negative charge state with the hybridization between the PNAs and the target. Therefore, several positively charged electroactive indicators can be selectively introduced to the sensor surface after hybridization, such as $[Co(phen)_3]^{3+}$[126] and $Ru(NH_3)_6$[127, 128].

Wang et al. immobilized a single-stranded probe on the surface of GCE-biosensor to capture targets. With hybridization proceeding, the negative charge density on the sensor surface increased gradually, resulting in an increased concentration of attached $[Co(phen)_3]^{3+}$. The local concentration of $[Co(phen)_3]^{3+}$ is positively correlated with the increased concentrations of anionic phosphates on the electrode surface. As a result, the signal of p53 target is easily obtained by chronopotentiogram measurement[126]. Several researchers constructed an electrochemical catalytic system to amplify the detection signal further. As the application of $Ru(NH_3)_6^{3+}$and $Fe(CN)_6^{3-}$, the Ru^{3+} electron acceptor is reduced on the electrode surface and then reoxidized by excess Fe^{3+}, making it a catalytic electro chemical process[127, 128].

In addition, several indicators were not introduced through electrostatic interaction but also play a similar role in detection. Meldola‘s Blue (MB), 7-dimethyl-amino-1,2-benzophenoxazinium salt is a popular indicator, which could be introduced by intercalation into the minor groove of long dsDNA polymers by π-π stacking interaction[114]. Arami et al. prepared a biosensor for UGT1A9 gene based on 13-mer PNA probes and MB indicator[129]. The target was analyzed by differential pulse voltammetry of MB. The detection limit was 22 nM.

The signal generated in this type of sensor is the direct redox signal of labels. The amounts of attached labels depend on the concentration of the target. Therefore, detection signal is usually not strong enough when target concentration is extremely low.

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4.2.2 Detection based on inorganic composite labels

In addition to the feature of uncharged of PNAs, constructing a detection system using the lack of phosphate groups in the PNA backbone, such as phosphate-Zr^{4+}-carboxylate chemistry, is also an effective approach.

Zhang et al. constructed a detection system with the combination of PNA capture probes and signal amplified transduction material Ti_3C_2MXenes[130]. After the hybridization on the network electrode, the Ti_3C_2 MXenes were introduced to the gaps by strong interactions between phosphate groups of the target and zirconium-cross-linked Ti_3C_2MXenes, thus enhancing the conductance between the electrodes. The detection limit was 20 CFU mL^{-1}, and the required time was 2 h. A similar deposition of silver was reported by Park and coworkers; the gap was connected by silver deposition, thus improving conductivity[131].

In recent years, the excellent physical properties of metal nanoparticles, such as scattering coefficients, large extinction, catalytic activity, and surface electronics, have aroused many researchers' interests[132]. In several cases, the binding of oligonucleotides functionalized with gold nanoparticles could introduce more remarkable conductivity changes into target-probe binding events[133].

Silver nanoparticles (AgNPs) have been widely used in electrochemical biosensor due to their unique electrochemical and photophysical properties, which could substantially improve the performance of biosensor. DNA-templated silver nanoparticles (AgNPs), as a new method to apply AgNPs in biosensor, emerged in the detection of pathogenic bacteria[134] and biological macromolecules[135]. Liu et al. wrapped polymer chains with metallic AgNPs via silver mirror reaction; as a result, target DNA fragments were labeled by numerous electroactive AgNPs[82]. This dual-signal amplification system, thermally initiated SI-RAFT polymerization, and DNA-templated AgNPs contributed a 2–1800-fold reduction in the detection limit compared with other electrochemical DNA assays.

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4.2.3 Detection based on electroactive polymer labels

Electroactive polymer is an attractive label in electrochemical sensor; the related sensor is highly selective, and holds high anti-interference capability[136]. Electroactive polymer in practical applications can be divided into two categories, in-situ growth of polymer and direct introduction of the presynthesized polymer.

For in-situ grown polymers, one case is that the hybrid provides the attachment sites of the initiator but does not participate in the reaction itself.

Qian and He constructed a signal amplification system based on the introduction of polylysine (PLL) macroinitiator[58]. The application of PLL endows more than one initiator on a single DNA identification event, and it presents a 60-fold enhancement in DNA detection limit over the single-initiator-labeled target DNA detection system. The details are shown in Figure 5a, where part of PLL's side chains are modified by initiators, and the residual amino groups drive the PLL to PNA/DNA hybrid via electrostatic attraction between negatively charged PNA/DNA duplex and positively charged PLL.

Fan and coworkers designed a target-guided formation of conducting polymer nanowires in nanogaps[137]. After the target was captured by the PNA probe, the electrostatic interaction between cationic aniline molecules and anionic phosphate groups in miRNA was designed to deposit polyaniline nanowires on the PNA–target hybrid. Polyaniline nanowires formed an electrically conducting nanowire network in the gaps and remarkably enhanced conductance, hence directly analyzing the target miRNA in the sample solution. Compared with control miRNA, the conductance of the biosensor with the deposition of polyaniline nanowire improved by about seven fold in the presence of 2.0 pM of complementary targets. The detection limit was 5.0 fM. The fabrication is shown in Figure 5b.

In the research of Hu and coworkers, the target DNA was captured on the electrode surface by the preimmobilized PNA probe, which contributed to the formation of high density of PNA/DNA heteroduplexes on the electrode surface. After probe immobilization, atom transfer radical polymerization (ATRP) initiators were attached to the sensor surface via the phosphate-Zr^{4+}-carboxylate chemistry[136]. When a negative potential was applied to the working electrode, the activators can be reduced to initiate the polymerization for the in situ growth of electroactive

polymers. The growth of polymers introduced numerous electroactive probes, which directly contributed a high detection signal. The detection limit was 0.47 fM.

A similar method was reported by Zhao and coworkers; they fabricated an electrochemical biosensor via a dual-signal amplification strategy of polysaccharide and electrochemically mediated atom transfer radical polymerization (eATRP)[138]. The constructed sensor can detect as low as 9.04 aM tDNA, and the sensor could be applied to detect double-stranded DNA directly. The detection limit was 0.12 fM.

ATRP is a new active polymerization[139, 140], which is widely used in surface modification as a result of its accurately controlled molecular weight[141]. Zheng and coworkers developed a dual atom transfer radical polymerization to detect ultralow concentration of DNA[142]. After the reaction of ATRP-1, the peak current of ferrocene increased 10-fold, and the subsequent oxidation current of ferrocene increased about three times with the reaction of eATRP-2. The detection limit of this signal amplification strategy was 25 aM, which is considerably lower than other reports summarized in Zheng et al.[142]. The experimental procedures are as follows: A thiol group modified PNA probe was self-assembled on the electrode to work as capture probes, and then the target was captured to form heteroduplexes and subsequently remove the unbound targets. The initiator of α-bromoisobutyric acid followed to trigger the first ATRP, which was attached to PNA/DNA heteroduplexes via coordination of Zr^{+}. The absence of phosphate groups of PNA ensured that the attachment of Zr^{+} only occurred on the target DNA. The product of ATRP1 was one of the initiators of the second ATRP. The monomers of the second ATRP were modified with ferrocene. With the reaction of ATRP, many electroactive probes were attached to the electrode surface.

Another case of in-situ grown polymers is that nucleic acid itself participates in the polymerization reaction. As reported by Gao et al.[143], they first labeled the detection probe with horseradish peroxidase (HRP), and then the HRP was introduced to the sensor surface forming a sandwich structure via the hybridization between detection probe and PNA/DNA heteroduplex, in which HRP served as catalyst and initiator. The generated sensor surface was immersed in the blend of aniline and H_2O_2. Subsequently, the polyaniline was selectively coupled on the template of hybridized nucleic acid molecules. The obtained electroactive complex of PANi and nucleic acid allowed the ultrasensitive quantification analysis of nucleic acids. The detection limit was 1 fM. The scheme is shown in Figure 5c.

In most cases, the introduction of polymers requires catalysts, such as enzymes or heavy metal catalysts. However, the high cost of enzyme[144, 145] and potential pollution of heavy metal catalyst[145, 146] greatly limit its application. One of the reversible addition fragmentation chain transfer (RAFT) approaches, a versatile methodology for complex polymeric architecture synthesis[147], exactly solves the above problems in electrochemical signal amplification. As reported by Liu et al., a thermally initiated reversible addition fragmentation chain transfer (SI-RAFT) was successfully applied in electrochemical detection, which was a more environmentally friendly, simple operation than other polymerization synthesis methods[82]. Apart from SI-RAFT, they coupled DNA-templated metal deposition[148] into the biosensor, in which the detection limit was 5.6 aM. Electrochemical polymerization is also a method of in-situ synthesis of polymers with less interference.

Figure 5

Table 2 Examples of sensors with different signal generation mechanisms

Signal type		Target	LOD	Ref.
Direct signal	Guanine oxidation signal	pBKC84	1.3 pM	Ahmadi et al.[123]
	Guanine oxidation signal	p53 gene	53.10 pgμL−1	Hamidi-Asl et al.[68]
Indirect signal	$Ru(NH_3)_6$	23S rRNA	∽5 aM	Gasparac et al.[127]
	Meldola's Blue	UGT1A9 gene	22 nM	Arami et al. [129]
	Ti_3C_2MXenes	16S rDNA	20 CFU mL^{-1}	Zhang et al.[130]
	Conducting polymer nanowires	Synthetic 22-mer DNA samples	5 fM	Fan et al.[149]
	Atom transfer radical polymerization of ferrocenylmethyl methacrylate	CYFRA 21–1 DNA	9.04 aM	Zhao et al. [138]

5. Others

Increasing target concentration is an effective method to solve this problem. Various amplification methods, such as polymerase chain reaction (PCR), rolling-circle amplification, and loop-mediated

isothermal amplification, are the main means to increase target concentration. The main research content of target amplification is target DNA, which is inconsistent with the focus of this review. It will not be discussed in the text. The details could be found in the supplementary file.

Compared with the DNA probe, a unique feature of PNA sequence is that it cannot work as a primer extended by DNA polymerase[150]. Nielsen et al. indicated that the binding of PNA to PCR primer site can effectively block the formation of a PCR product[151], and it was called "PCR clamping"[152, 153]. Taking advantage of this feature of PNA, the target sequence can be selectively amplified or suppressed, hence observing a different signal between target and nontarget sequence[154, 155]. Several researchers developed a detection method of point mutations in Ki-ras with the utilization of wild-type-specific PNA as competitors to mutation-specific primers[156, 157]. The analysis time of this method was reduced from 2 days to 1 hour with respect to the time of restriction fragment length polymorphism analysis. The research of Sotlar et al. showed that the addition of 0.75 μM PNA oligomer to suppress 100 ng of wild-type DNA amplification resulted in the detection limit of c-kit mutations reaching 1:2000[158].

Conclusion

The utilization of PNA-based biosensor as a powerful tool enables the feasibility of nucleic acid diagnosis in genetics, pathology, criminology, and food safety. Learning the preparation scheme of PNA electrochemical nucleic acid sensor is of great importance to widen the application fields of PNAs. This review discusses PNA-based electrochemical nucleic acid sensors in detail from three perspectives: development of PNAs, immobilization methods of PNA probes, and signal generation mechanisms. For representative research, the preparation procedures, mechanisms advantages, and detection limits are presented. The review offers a simple, accurate strategy for the construction of PNA electrochemical nucleic acid sensor.

Author contributions

Qingteng Lai：conceptualization, resources, investigation, formal analysis, methodology, writing

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– original draft, writing – review and editing.

Zhengchun Liu：funding acquisition, supervision, writing – review and editing.

Wei Chen：investigation, formal analysis.

Yanke Zhang：formal analysis, methodology.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Supplementary Material

Details of target amplification method.

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Application strategies of peptide nucleic acids toward electrochemical nucleic acid sensors

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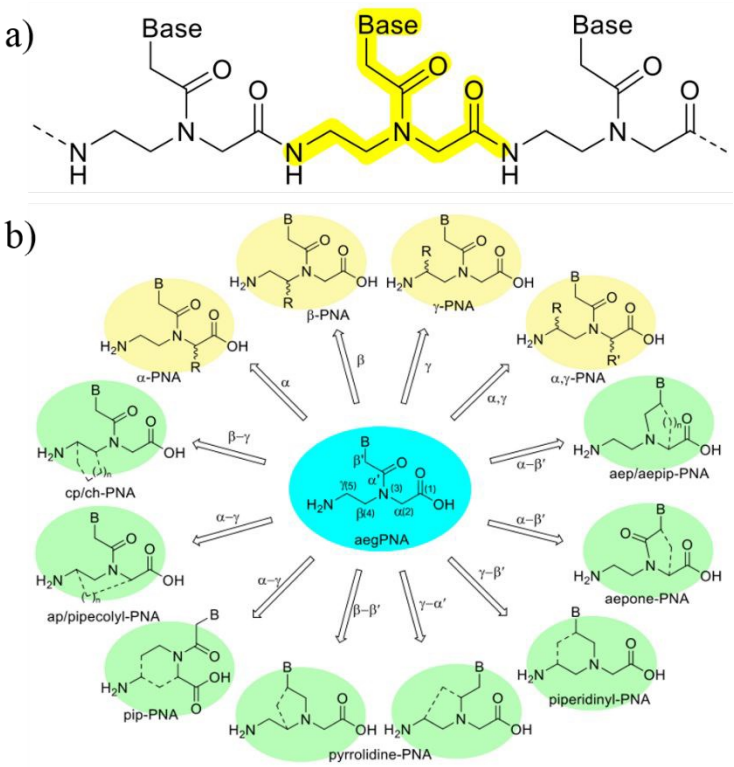

Fig.1 Chemical structure of PNA monomer. (a) N-(2-aminoethyl) glycine unit of PNA backbone, (b) Modifications of chiral PNA monomer.

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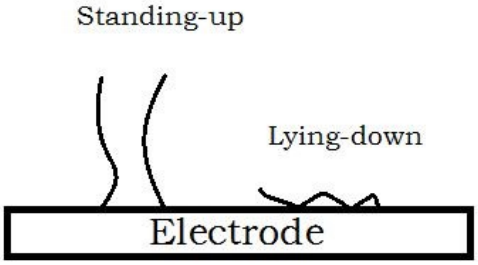

Fig.2 The conformation of immobilized probe on sensor surface

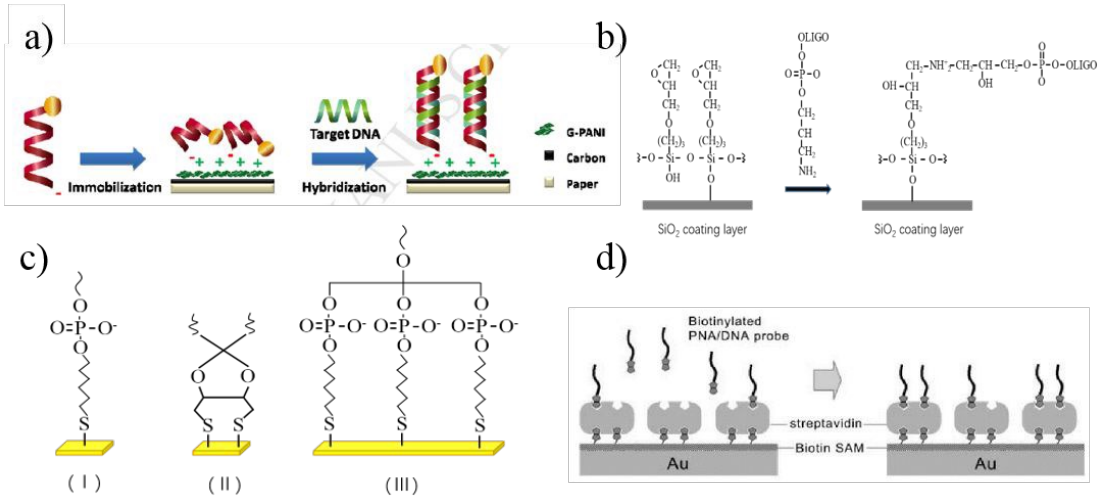

Fig.3 Immobilization methods of PNA probe. (a) Immobilization of PNA probe on paper-based sensor with electrostatic attraction[69]; (b) Attachment occurs by secondary amine formation between an epoxysilane monolayer and the 3'amino linkage[74]. (c)Conjugating oligonucleotides to gold nanoparticles by Au-S bond[83]; (d)The immobilization of probe through the interaction of streptavidin and biotin[32]

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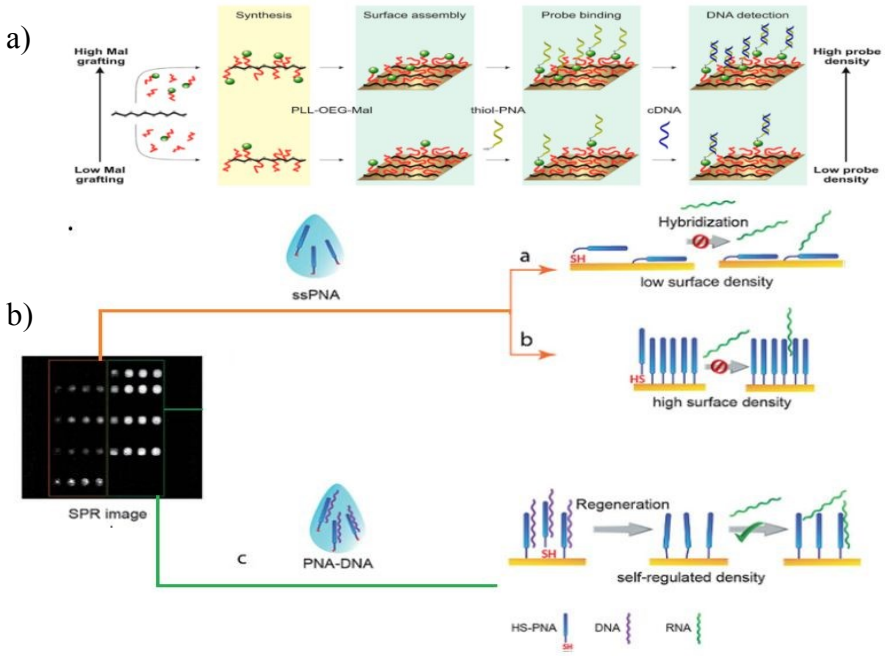

Fig.4 (a)Scheme showing the control of the probe density with the synthesis of PLL-OEG-Mal polymers[96]; (b)Immobilization of ssPNA and PNA prehybridized with complementary DNA as well as their expected effects in terms of subsequent DNA hybridization. The SPR image shows side-by-side microspots made using the two strategies (as indicated) with the intensity of the spots being indicative of the amount of RNA bound[101].

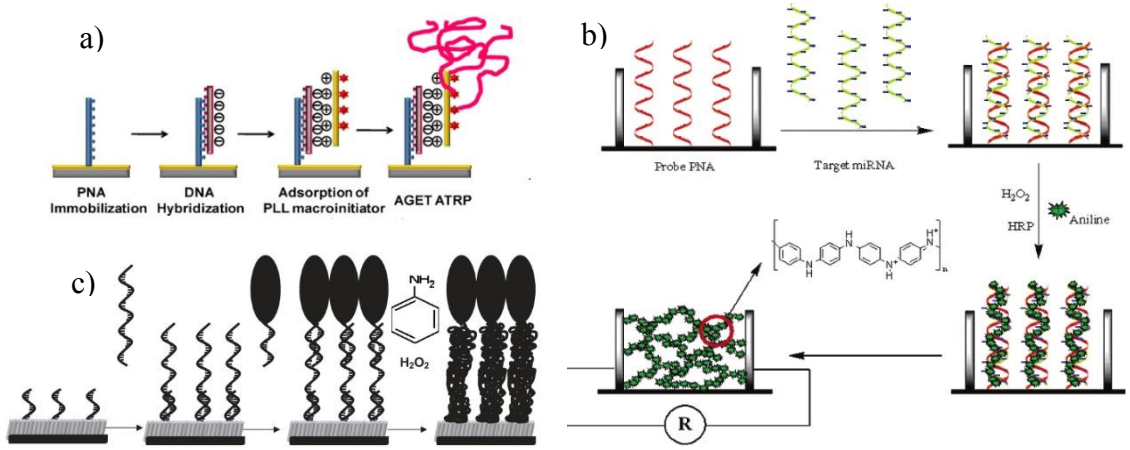

Fig.5 DNA detection using PLL macroinitiators for signal amplification[58]; (b) Schematic representation of the nucleic acid biosensor based on enzyme-catalyzed template-guided deposition of conducting polymers[137]; (c) Selective coupling of polyaniline to nucleic acid hybrid[143].