Received: 24 March 2021 | Revised: 22 June 2021 | Accepted: 22 June 2021

DOI: 10.1002/bip.23464

REVIEW

Biopolymers WILEY

Application of peptide nucleic acid in electrochemical nucleic acid biosensors

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Funding information
National Natural Science Foundation of China, Grant/Award Numbers: 21890740, 9195401, 21890742, 21974068

Abstract

The early diagnosis of major diseases, such as malignant tumors, has always been an important field of research. Through screening, early detection of such diseases, and timely and effective treatment can significantly improve the survival rate of patients and reduce medical costs. Therefore, the development of a simple detection method with high sensitivity and strong specificity, and that is low cost is of great significance for the diagnosis and prognosis of the disease. Electrochemical DNA biosensing analysis is a technology based on Watson Crick base complementary pairing, which uses the capture probe of a known sequence to specifically recognize the target DNA and detect its concentration. Because of its advantages of low cost, simple operation, portability, and easy miniaturization, it has been widely researched and has become a cutting-edge topic in the field of biochemical analysis and precision medicine. However, the existing methods for electrochemical DNA biosensing analysis have some shortcomings, such as poor stability and specificity of capture probes, insufficient detection sensitivity, and long detection cycles. In this review, we focus on improving the sensitivity and practicability of electrochemical DNA biosensing analysis methods and summarize a series of research work carried out by using electrically neutral peptide nucleic acid as an immobilized capture probe.

KEYWORDS
ATRP, biosensor, nucleic acid, peptide nucleic acid, RAFT

1 | INTRODUCTION

With the increasing demand for DNA detection in various fields such as drug detection and early disease diagnosis, various biological analysis methods have developed rapidly.[1–4] However, traditional gel electrophoresis is tedious and time consuming. In this case, DNA biosensors based on base pair emerge.[5,6] DNA biosensor is a kind of sensor device that can convert the presence of target DNA into detectable electrical,[7] optical,[8–11] acoustic,[12] and other signals.[13,14] As shown in Figure 1, DNA biosensor detects the hybridization reaction of nucleic acid, and its structure includes a target DNA recognition element and a signal transducer.[15,16] Among them, DNA molecular recognition element is usually composed of a probe fixed on the transducer and some other auxiliary materials, which can specifically recognize and hybridize target sequences. The signal detected by the recognition element is further converted into the response that can be recognized and measured by the signal element, then according to the change of the signal value before and after hybridization, the target DNA can be accurately quantified.[17,18]

Among them, electrochemical DNA biosensor is favored by scientists due to its unique characteristics of high sensitivity, high efficiency, and high precision. Although many electrochemical platforms have been constructed to detect specific gene sequences, from clinical perspective, there are still some defects, such as poor stability and specificity of capture probes, insufficient detection sensitivity, complex preparation processes, or long detection cycle. To improve the practicability of electrochemical DNA biosensor, a series of works have been reported.

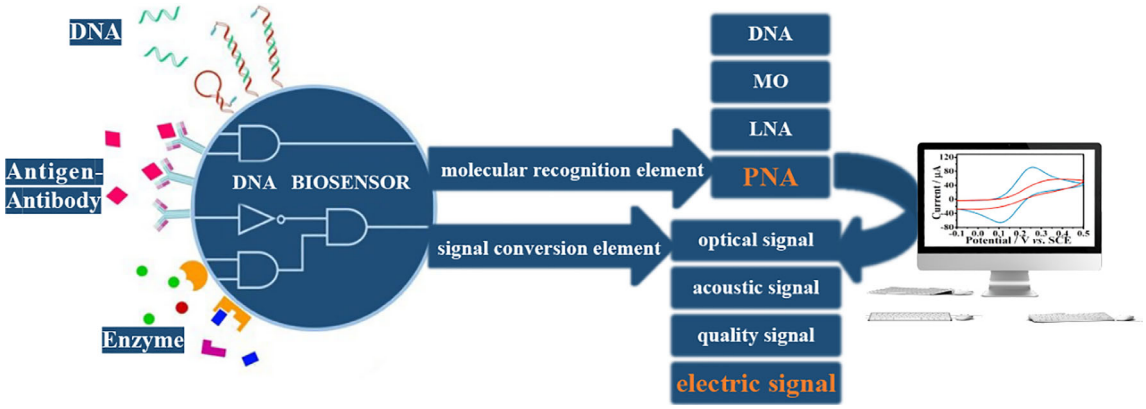

FIGURE 1 Basic structure of electrochemical DNA biosensor: contains molecular recognition components and signal conversion components. This article mainly uses peptide nucleic acid (PNA) as molecular recognition components and electrochemical workstations as signal conversion components

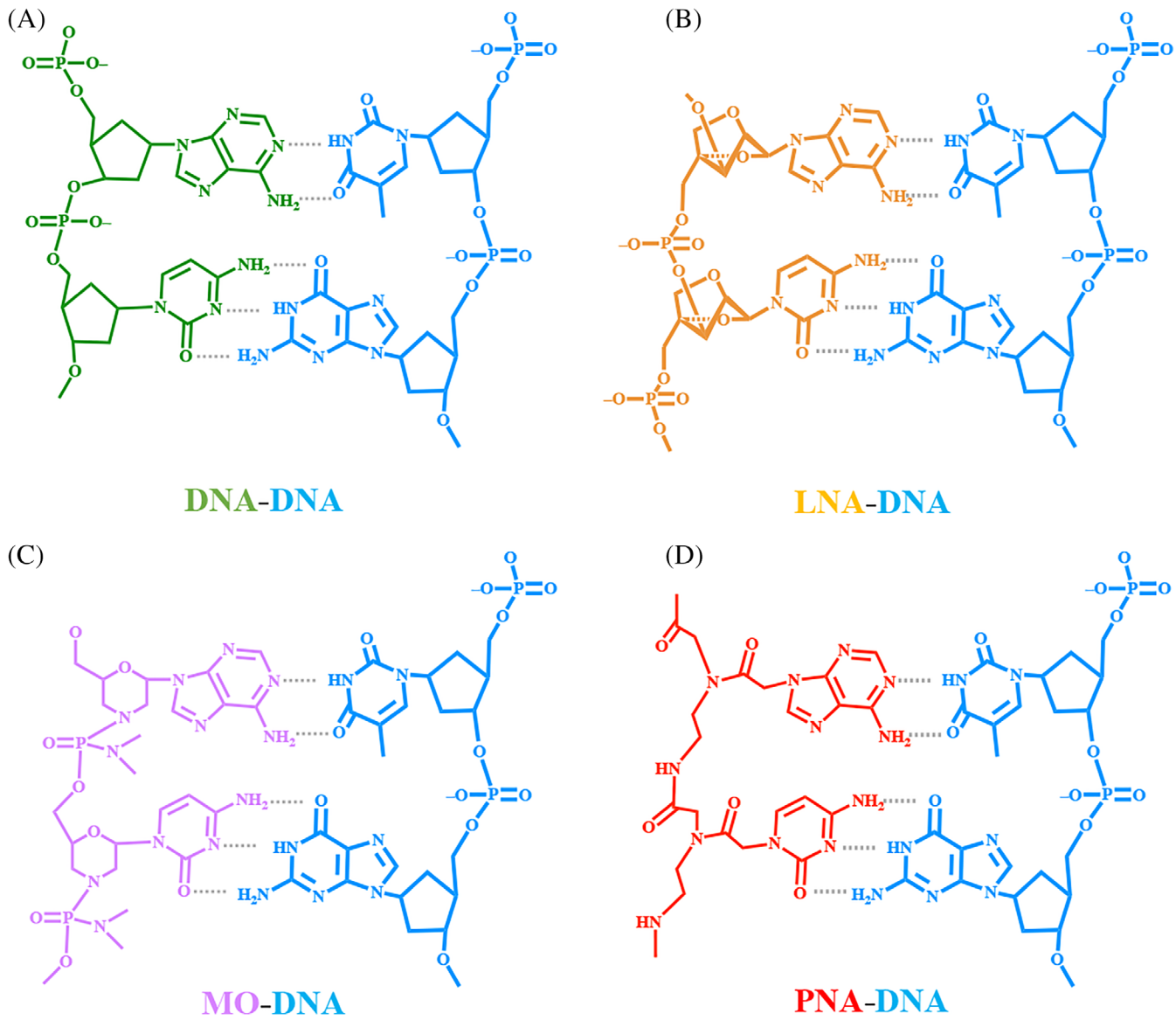

FIGURE 2 (A) The structure of DNA/DNA double helix; (B) the structure of locked nucleic acid (LNA)/DNA heteroduplex; (C) the structure of morpholine oligonucleotide (MO)/DNA heteroduplex; and (D) the structure of peptide nucleic acid (PNA)/DNA heteroduplex

The detection of specific DNA fragments of diseases, especially the detection of single nucleotide polymorphisms (SNPs), is of great value for the early diagnosis and genotyping of diseases.[19] In order to make more selective analysis and realize SNPs analysis for electrochemical DNA biosensor, recently, researchers have carried out a lot of research work based on a series of new capture probes.[20] In addition to DNA probes, common hybridization probes include morpholine oligonucleotide (MO), locked nucleic acid (LNA), and peptide nucleic acid (PNA) as shown in Figure 2. These hybridization probes are all DNA analogs. MO is a kind of non-ionic artificial nucleic acid composed of morpholine ring, phosphoryl diamine, and nucleic acid bases, designed and synthesized by Summerton.[21] MO has good water solubility, resistance to enzymatic degradation, high hybridization specificity, and cell activity, but it will break in strong acid solutions.[22] LNA is an RNA derivative, that is, the 2′O and 4′C of β-D-ribofuranose in the RNA structure form an oxymethylene "bridge" through shrinkage.[23–25] The synthesis method of LNA is similar to that of DNA, the backbone of LNA is negatively charged, and its solubility is similar to that of DNA.[23,26–28] Therefore, it is not conducive to be distinguished by positively charged substances in the process of DNA analysis.

To solve the above problems, our team and collaborators proposed using PNA as a capture probe and carried out a series of research work.[29–34] PNA, a class of DNA analogs that replace the sugar phosphate backbone with a polypeptide backbone,[35] is a new nucleic acid sequence-specific reagent that Danish organic chemist Ole Buchardt and biochemist Peter Nielsen began to study with great concentration in the 1980s.[36] Different from the hybridization between DNA/DNA or DNA/RNA, PNA has the following advantages:

1. PNA molecules are electrically neutral, and there is no electrostatic repulsion when hybridizing with electronegative complementary DNA. Moreover, it is easy to form a more stable double-stranded complex with higher hybridization efficiency. What is more, the formed double helix has higher thermal stability.[35,37,38]
2. PNA has stronger specificity when hybridizing with DNA, and can accurately distinguish single-base mismatches, and is suitable for SNPs analysis.[35]
3. The amide bond in the PNA backbone is different from the peptide bond in protein or the phosphodiester bond in DNA, and its biological properties are quite stable, so it is not easy to be hydrolyzed by proteases or nucleases, and have strong anti-enzymatic ability.[23,39]
4. The combination of PNA and DNA has nothing to do with the ionic strength of the buffer, and it can hybridize with the corresponding DNA in a low ionic strength environment.[37,38] In the absence of Mg^{2+}, PNA can also specifically hybridize with complementary DNA.[40]

Therefore, PNA as a capture probe has obvious advantages in genetic diagnosis, point mutation, gene sequence, and structure analysis of diseases.

In recent years, PNA has been widely applied to the construction of DNA sensors, and its unique properties make it an excellent capture probe.[41–44] As the molecular tools and probes of biosensors, PNA has made great contributions to molecular diagnosis and detection. Joung *et al*.[45] combined the PNA probe with the surface plasmon resonance sensor (SPR), and used the PNA-SPR method to determine the 16s rRNA of *Escherichia coli*. After hybridization, the hybridization signal was amplified by Au cations and detected. The sensitivity of the experimental results was 58.2 ± 1.37 pg/mL, which overcomes the problem of low sensitivity of DNA probe to directly detect 16s rRNA. Kaisti *et al*.[46] used PNA to hybridize DNA with stronger specificity and developed a new type of field-effect transistor (FET) sensor based on standard electrical components for DNA detection. The sensor can accurately distinguish single-base mismatches, has low cost, and can perform real-time detection of labeled PNA-DNA hybridization. In addition, when the salt ion concentration is about 10 mM, PNA probes can directly hybridize with double-stranded DNA sequences, while DNA probes must be denatured to single-stranded before hybridization, the study shows.[47] At the same time, the PNA chain with high-purine can combine with two high-pyrimidine DNA chains to form a triple helix structure. Based on this, Xu *et al*.[48] reported a label-free colorimetric sensor platform that uses DNA-PNA-DNA as the triple helix molecular switch to detect microRNA in 2020, with a detection limit of 0.18 nM. The development of new biosensors is to provide new tools for clinical specimen analysis of disease biomarkers, and the disease biomarkers concentration in body fluids ranges from nanomolar (nM) to attomolar (aM).[49] Therefore, in the absence of a more highly sensitive detector, we need to develop better detection methods to deal with this problem.

Based on this, with the many advantages of PNA capture probes as the research core, we and our collaborators have developed two types of electrochemical DNA biosensors.[30,32,50–52] As shown in Figure 3, one is to directly couple the electroactive probes to the captured target DNA for rapid and highly selective electrochemical biosensor analysis; the other is that when PNA and DNA are hybridized, numerous electroactive probes are polymerized on the electrode surface with the help of various signal amplification effects, and the target DNA is subjected to highly sensitive and selective electrochemical biosensing analysis. In this review, we focus on improving the sensitivity and practicability of electrochemical DNA biosensing analysis methods and summarize a series of research works carried out by using electrically neutral PNA as an immobilized capture probe.

2 | WITHOUT SIGNAL AMPLIFICATION SENSOR

In electrochemical DNA biosensing, after the target DNA is hybridized with the immobilized capture probe, the target DNA can be quantitatively analyzed by detecting the signal intensity or change value of the quantitatively labeled electroactive probe. For example, ferrocene (Fc), methylene blue (MB), and so on[53,54] have been widely used as

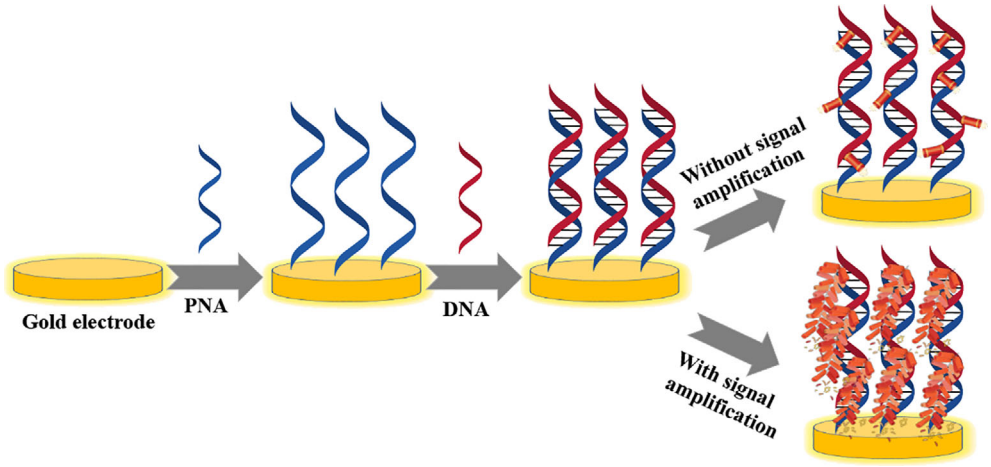

FIGURE 3 Schematic diagram of two types of biosensors triggered by peptide nucleic acid (PNA) as a capture probe in DNA detection

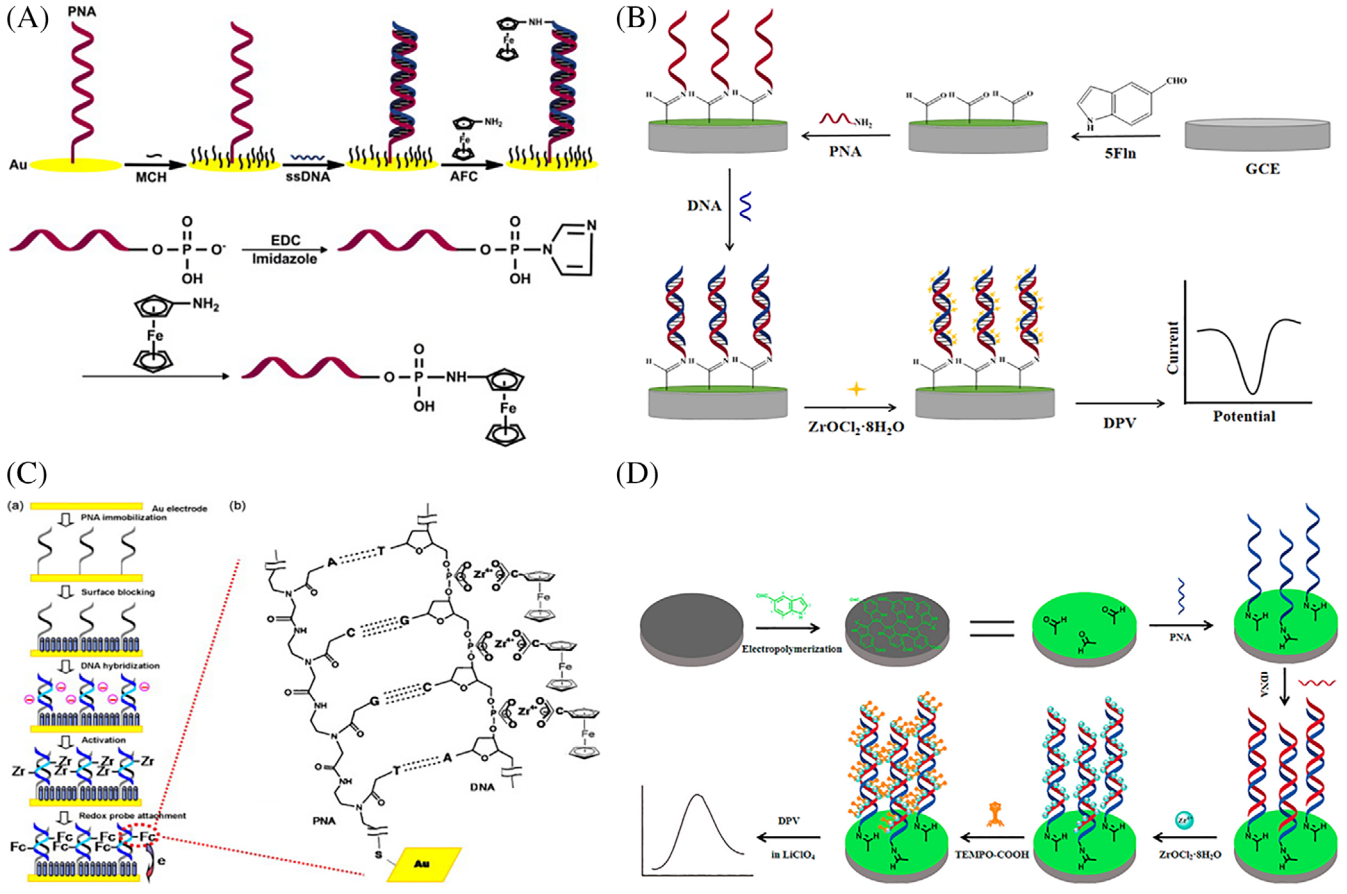

FIGURE 4 The mechanism diagram of a DNA biosensor without signal amplification strategy using peptide nucleic acid (PNA) as the capture probe. (A) The schematic diagram of biosensor construction by directly modifying amino ferrocene to the end of the captured target DNA through a one-step coupling reaction mediated by carbodiimide chemistry. (B) The schematic diagram of electrochemical DNA detection using Zr^{4+} as an electroactive probe. (C) The principle diagram of DNA detection using carboxy ferrocene as the signal molecule. (D) The principle diagram of direct detection of DNA with tempo as electrochemical marker

electroactive probes for electrochemical biosensing analysis of DNA due to their excellent redox properties. In order to introduce Fc and other electroactive probes into the electrochemical sensor analysis platform, the main method is to use hairpin DNA,[55,56] single-stranded DNA[57] modified with electroactive probes such as Fc, or directly label Fc and other electroactive probes to the target DNA by

synthesis. However, some disadvantages do exist for these methods, such as complex synthesis, separation and purification process, and expensive prices.[58] For this reason, a series of methods were invented in our lab to directly modify the electroactive probe to the captured target DNA.[29,33,52] Electron transfer is achieved through redox media, and hybridization detection is performed by means of these electrochemically active indicators that selectively bind to DNA.

We use PNA and Fc as capture probes and electroactive probes, respectively, to detect target DNA through a one-step coupling reaction mediated by carbodiimide chemistry (CDI).[52] A fairly simple electrochemical method was established for rapid and highly selective biosensor analysis of target DNA. As shown in Figure 4A, the PNA probe is fixed on the surface of the gold electrode by self-assembly, and after hybridization with the target DNA, the amino ferrocene (AFc) is directly modified to the 5′ end of the target DNA through one-step coupling reaction under the action of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and imidazole. Finally, the quantitatively labeled Fc was detected by differential pulse voltammetry (DPV). Since the target DNA and Fc are coupled in a certain stoichiometric ratio, the quantitative detection of target DNA can be realized by detecting Fc. Under the optimal conditions, this method can be used for rapid sensor analysis of target DNA concentration in the range of 0.1-100 nM, with a detection limit of 93 pM. It can detect the target DNA effectively, but takes nearly 2 hours for DNA and AFc coupling. Detection time is a key factor that limits the speed and convenience of electrochemical biosensors. Only when the desired results are obtained in a short time can the purpose of rapid screening be achieved. This method is not suitable for real-time detection, thus new rapid and sensitive methods need to be developed.

In the sensor development, a faster and more convenient method was developed to shorten the detection time. Some electroactive active cations were introduced to directly bind to the negatively charged DNA phosphate backbone. We found that zirconium compounds are rich in content, non-toxic, cheap and stable, and it has good redox properties.[59] Therefore, zirconium ions (Zr^{4+}) as redox probes were adopted (Figure 4B).[29] At the same time, because PNA has stronger specificity for hybridizing, it can accurately distinguish single-base mismatches. Therefore, PNA was used as a capture probe to electrochemically detect DNA, which has achieved good results. Moreover, in order to evaluate the applicability of this DNA biosensor in the analysis of complex biological samples, experiments were carried out with 5% and 10% normal human serum (NHS) as the biological matrix, respectively. The results show that this strategy has good analytical performance in complex serum samples and has clinical analysis potential. However, the background signal limits the sensitivity of electrochemical DNA sensors, especially when the concentration of the target DNA is very low, it is very difficult to distinguish the electrical signal generated by DNA hybridization from the background signal of non-hybridization. And if the background signal is too large, it is difficult to obtain higher sensitivity. Although this method can be carried out quickly, the background signal is too high, which is not conducive to the highly sensitive detection of DNA.

While retaining the advantage that Zr^{4+} can specifically bind to the DNA phosphate backbone without binding to PNA, it is necessary to eliminate the background signal. Fang[60] and Wang[33] proposed DNA biosensors using carboxyferrocene and stable free radical TEMPO as electrochemical markers, respectively. As shown in Figure 4C, Fang *et al.*[60] fixed the PNA probe on the surface of the gold electrode via self-assembly, and after hybridizing with the target DNA, the carboxylferrocene was connected to the PNA/DNA heteroduplex formed via the hybridization by means of the coordinated bonding of Zr^{4+}. Finally, through the detection of Fc, the quantitative detection of target DNA can be achieved. Similarly, Wang *et al.*[33] used the stable free radical of carboxyl oxynitride (TEMPO-COOH) as a new electrochemical marker for the first time, prepared an electrochemical biosensor by immobilizing the capture probe PNA and using the coordination of Zr^{4+} with phosphate and carboxyl groups. Next, DPV was used to measure the electrochemical signal of TEMPO related to the target DNA concentration. In addition, the detection principle, optimization of key factors, and performance analysis of the sensor were discussed. Under the best conditions, the electrochemical DNA biosensor has a good linear relationship in the range of 10 pM to 100 nM, and the detection limit is 2.57 pM. In addition, due to the non-charged characteristics of PNA, Ali *et al.*[61] designed a simple nanofluid sensor for DNA detection. The sensor modified PNA on the surface of conical nanochannel via carbodiimide coupling chemistry. With the help of PNA and DNA hybridization, it has stronger specificity and can accurately distinguish single-base mismatches for specific detection of target DNA. The sensor utilizes the advantage of PNA being electrically neutral, which makes the current signal of PNA modified nanochannel significantly reduced, which is beneficial to distinguish target DNA during detection.

Due to the many advantages of PNA, DNA biosensors using PNA as capture probes have been widely used in electrochemistry, and we have also found many interesting applications of PNA in other fields. As shown in Figure 5A, Xing *et al.*[62] provided a fast, simple, and label-free colorimetric method for detecting target DNA. This method uses PNA to accurately distinguish the characteristics of single-base mismatches and applies it to the analysis of SNPs. Because PNA can effectively identify mismatched DNA, when a mismatch occurs when PNA binds to DNA, a single exonuclease starts to cut the DNA into small fragments. While when PNA and DNA are completely complementary, DNA fragments can be completely preserved under the protection of PNA. Finally, based on the aggregation-inducing effect of PNA on gold nanoparticles (AuNP), an effective colorimetric analysis of the target DNA was carried out. The detection limit of this method in cell lysate reaches 2.3 nM, which proves the application prospect of this method in clinical detection. Subsequently, Xu *et al.*[63] reported a new type of light scattering (LS) biosensor using a similar principle (Figure 5B). However, they used the aggregation effect of PNA on single-wall nanotube (SWNT) to effectively detect the target DNA via LS. Compared with the aggregation effect of AuNP, SWNT is more convenient and effective in DNA analysis due to its water solubility and low price. The double-stranded DNA was detected by using the advantage of advantages that there is no electrostatic rejection when

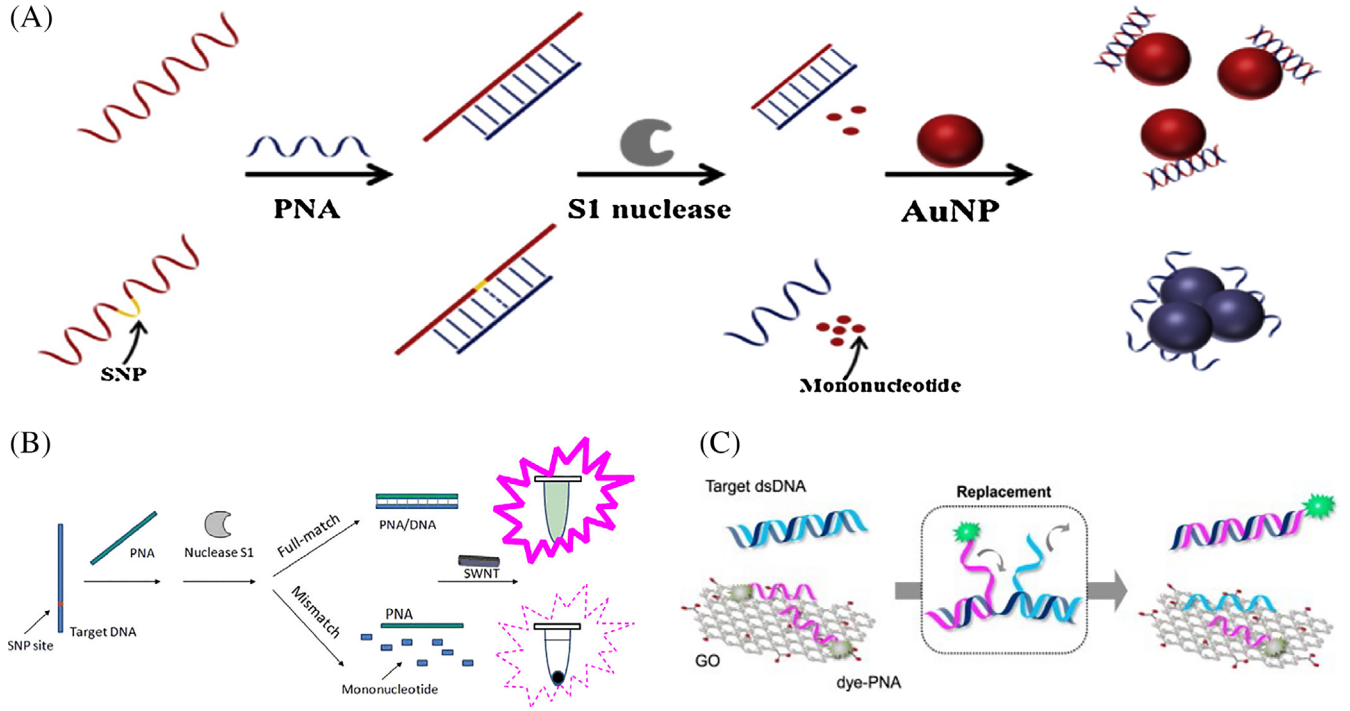

FIGURE 5 The mechanism diagram of a DNA biosensor without signal amplification strategy using peptide nucleic acid (PNA) as the capture probe in another field. (A) The schematic diagram of gold nanoparticles (AuNP)-mediated colorimetry for target DNA detection. (B) The schematic diagram of single-wall nanotube (SWNT)-mediated light scattering biosensor for selective detection of DNA. (C) The principle diagram of the graphene oxide biosensor based on the PNA/DNA hybridization reaction for DNA detection

the PNA is hybridized with the complementary DNA with electronegativity, which is easy to form a more stable double chain complex. As shown in Figure 5C, Lee *et al.*[64] designed a DNA biosensor with low background signal and high sensitivity through the selective quenching response of graphene oxide to fluorescent dye modification. This method has been used to detect a variety of targets by changing PNA sequence and fluorescent dye, and has achieved good results, which has an important application prospect in the future personalized medical detection.

The electrochemical DNA biosensor without signal amplification is favored by scientific researchers due to its advantages of simple and fast preparation. However, most of its sensitivity cannot meet the needs of clinical research, so it is necessary to develop DNA biosensors that can meet the needs of clinical testing.

3 | WITH SIGNAL AMPLIFICATION SENSOR

To meet the requirements for the detection of trace and ultra-trace biomolecules in analytical methods, it is of great significance to develop electrochemical biosensors with good selectivity, high sensitivity, and rapid response. In the previous discussion, we have known that compared with other capture probes, PNA has stronger specificity when hybridizing with DNA, which can accurately distinguish single-base mismatches, and is suitable for SNPs analysis.[52,60] Therefore, in the follow-up research, we continue to use PNA as the capture probe. In addition, we can also see that the biosensor in the previous study has a very short production process, which greatly shortens the detection time compared with the existing hospital detection.[29,33,52,60] Then, to improve detection sensitivity, signal amplification is the most common strategy in biosensors. For example, Fu *et al.*[65] introduced the catalytic hairpin assembly amplification strategy into the PNA-mediated DNA biosensor for highly sensitive detection of the target. In addition, in order to avoid false positives phenomenon, they effectively used Fc and MB as dual signals to signal the target. Compared with the detection, the detection limit reached 2.49 fM. Moreover, Fu *et al.* also detected the tumor cell lysates and achieved satisfactory results.

In order to achieve a lower detection limit for the detection of target DNA, ultra-sensitive detection of trace targets can be effectively performed. In recent years, with the rapid development of polymer material technology and the rise of various biotechnologies, a variety of signal amplification strategies have emerged.[66,67] Hereafter we briefly introduce the application of various polymer material signal amplification methods in DNA biosensors.

3.1 | ATRP-mediated DNA biosensor

Polymers are generally formed of thousands of small molecular monomers via polymerization or polycondensation, and usually contain

more active functional groups or active units. Therefore, a great number of electroactive probes can be introduced on the surface of the electrode via modifying the polymer to perform high-sensitivity electrochemical biosensing analysis of DNA. Based on this principle, a variety of signal amplification strategies based on polymeric materials have been reported. In 1995, Matyjaszewski reported atom transfer radical polymerization (ATRP) in which transition metal complexes were used as catalysts and organic halides were used as initiators to initiate free radical polymerization of vinyl monomers.[68,69] This kind of polymerization can not only obtain polymers with narrow molecular-weight distribution, controllable molecular weight, and clear structure, but also have many types of polymerizable monomers, and the reaction conditions are mild and easy to control. Therefore, this signal amplification strategy based on ATRP will have a broad application prospect in the highly sensitive detection of biomolecules.

Based on this consideration, in 2017, we proposed using PNA as a capture probe and ATRP-mediated polymers as a simple and efficient signal amplification strategy for highly sensitive and highly selective electrochemical biosensor analysis of target DNA.[70] As shown in Figure 6A, mercapto group-modified PNA was used as a capture probe to capture target DNA; α-bromophenylacetic acid (BPAA) and ferrocenylmethyl methacrylate (FMMA) were used as initiators and monomers of ATRP, respectively, to polymerize a large number of electroactive probes on the electrode surface by means of surface-initiated electrochemically mediated ATRP (SI-eATRP). Under the optimal conditions, this method can be used for sensing analysis of target DNA concentration in the range of 0.1 fM to 0.1 nM, and the detection limit is 0.072 fM. To further improve the sensitivity of DNA biosensors, in 2019, Sun *et al.*[30] proposed a more sensitive DNA detection method, which uses SI-eATRP signal amplification and DNA as a template of metallic silver deposition. As shown in Figure 6B, this method uses PNA as the capture probe. In the presence of the target DNA, Zr^{4+} can bind to the phosphate on the DNA. Then Zr^{4+} and BPAA were combined to initiate the ATRP reaction, and a large amount of glycosyloxyethyl methacrylates (GEMAs) was polymerized on the electrode surface. Then the polymerized sugar

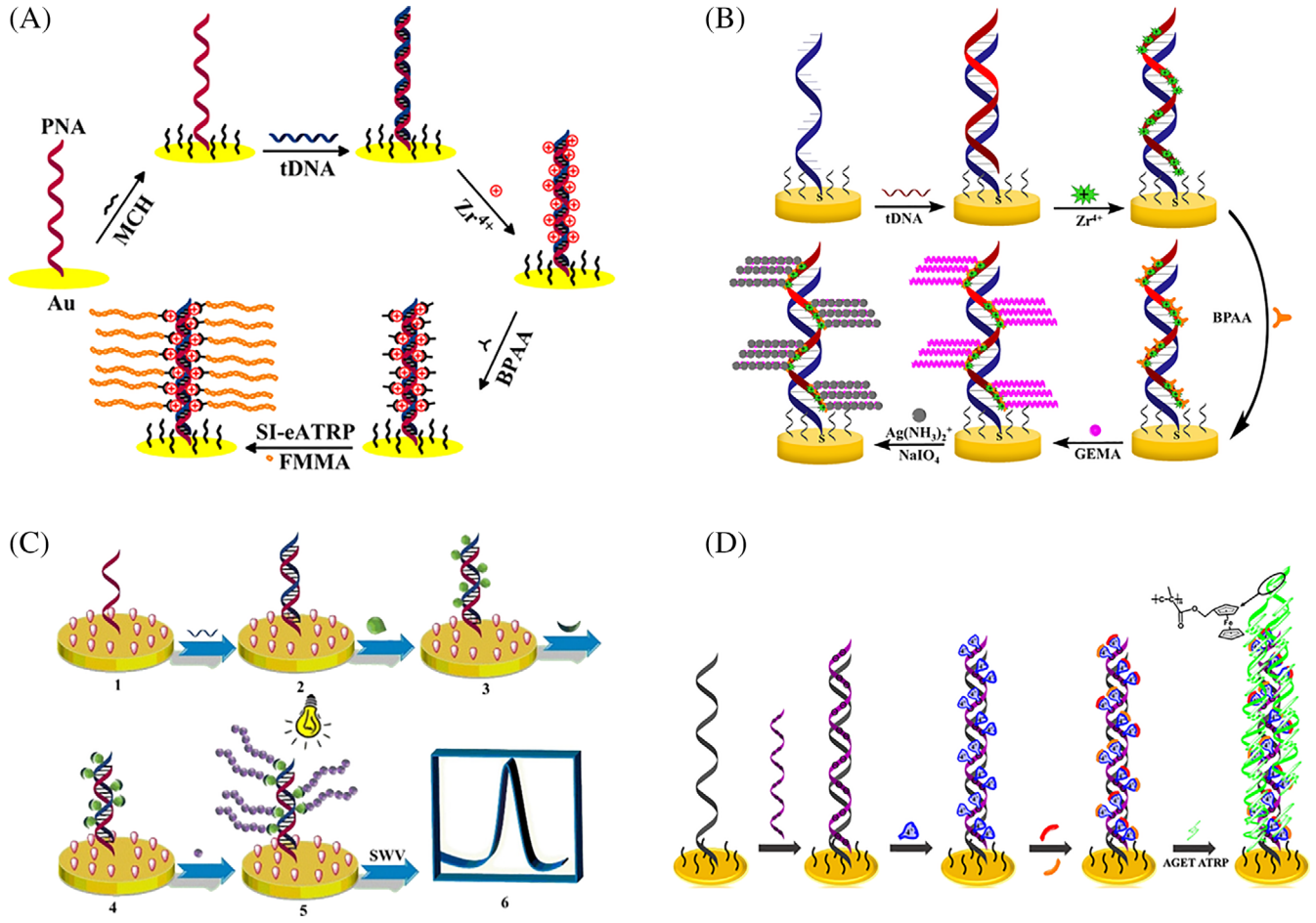

FIGURE 6 The mechanism diagram of DNA biosensor using peptide nucleic acid (PNA) as the capture probe and atom transfer radical polymerization (ATRP) as the amplification strategy. (A) The principle diagram of the biosensor structure that uses surface-initiated electrochemically mediated ATRP (SI-eATRP) to gather ferrocenylmethyl methacrylate (FMMA) on the electrode surface to detect DNA. (B) The schematic diagram of the dual signal amplification strategy of combining SI-eATRP and DNA as a template for metal silver deposition for DNA detection. (C) Schematic diagram of SI-photo-ATRP is used as the signal amplification strategy to realize DNA detection. (D) The schematic diagram of a DNA biosensor that MnTBAP as mimics enzyme-catalyzed ATRP to achieve signal amplification strategy

was reduced to polymerized aldehyde by $NaIO_4$, and silver mirror reaction was used to deposit silver on the electrode surface. The amount of silver nanoparticles (AgNPs) proportional to the target DNA concentration was quantified by DPV. In this work, it has been demonstrated that the self-assembled PNA electrochemical biosensor can be applied to the detection of highly sensitive DNA with a detection limit as low as 4.725 aM.

However, SI-eATRP requires an electrochemical workstation, so it cannot initiate polymerization in large quantities, which is limited to a certain extent in the application of biosensors. In addition, this reaction requires metals such as copper as a catalyst. It is easy to remain in the polymer during the polymerization process, which is difficult to purify; and the metal catalyst is not environmentally friendly enough. Based on this, Liu[34] and Sun[31] successively proposed photocatalytic ATRP (photo-ATRP) and enzyme-catalyzed ATRP for highly sensitive detection of DNA. Liu reported that a biosensor using PNA as a capture probe and metal-free photo-ATRP as an amplification strategy was used for the detection of DNA sequences related to lung cancer.[34] As shown in Figure 6C, BPAA and Eosin Y (EY) were used as initiators and catalysts for ATRP, and a large amount of FMMA was polymerized on the target DNA backbone by surface-initiated photo-ATRP (SI-photo-ATRP) under the irradiation of 470 nm light-emitting diode (LED) array on the surface of the gold electrode. Under the optimal conditions, this strategy can detect target DNA concentrations ranging from 0.1 pM to 10 nM, with the detection limit of 1.4 fM. The measured signal value of the sensor can reach 89.7% when placed in an environment of 4 °C for 2 weeks, showing good stability. To further get rid of additional equipment such as electrochemical workstations and LED-light that participate in the preparation of DNA biosensors, in 2020, Sun *et al*. proposed a green, environmentally friendly, and ultra-sensitive DNA biosensor that can be prepared in high throughput at room temperature without additional equipment.[31] As shown in Figure 6D, the biosensor is based on mimic enzyme-mediated ATRP as a signal amplification strategy, and PNA is used as a capture probe for quantitative detection of DNA sequences related to tumor diseases. In this strategy, when the target DNA is captured by PNA, BPAA, and Mn(III) meso-Tetra (4-carboxyphenyl) porphine chloride (MnTBAP) act as the initiator and catalyst of ATRP, respectively, and bind to the PNA/DNA heteroduplex simultaneously through Zr^{4+}. Finally, a large amount of FMMA was polymerized on the electrode surface through enzyme-catalyzed ATRP to complete the preparation of the biosensor. Under the optimal conditions, this strategy can detect target DNA concentrations ranging from 100 aM to 100 pM, with the detection limit of 11.79 aM. In addition, the DNA biosensor also obtained satisfactory results in diluted healthy NHS.

ATRP, as a polymer polymerization technology, is often used to prepare polymers with narrow molecular weight distribution, end functional groups, and hyperbranched polymers. With its many advantages in polymer polymerization, ATRP, as a signal amplification method, can effectively improve the detection sensitivity and realize the early diagnosis of diseases, which is of great significance. However, there are still some problems in the application of ATRP in biosensors, such as a long time and many steps in biosensor preparation, which reduces the efficiency of clinical use; in addition, most ATRP reactions require metal salts as catalysts, which is not good for environmental protection. Therefore, a more environmentally friendly and convenient high-sensitivity biosensor should be developed for clinical detection of target DNA.

3.2 | RAFT-mediated DNA biosensor

Although signal amplification is certainly an effective means to improve DNA biosensors, the methods have limitations. For example, the use of enzymes will lead to high costs,[71] PCR requires amplification instruments,[72,73] and ATRP needs metal salts as catalysts, which is not environmentally friendly.[74] Based on the above problems, we proposed a series of electrochemical DNA detection methods combined with reversible addition-fragmentation chain transfer (RAFT), which can overcome these shortcomings while retaining the advantages of high efficiency and high precision.

In 1998, Moad and colleagues reported a new controllable free radical polymerization reaction, that is, RAFT living radical polymerization reaction using dithioester derivatives.[75] Once the RAFT polymerization method was reported, it immediately attracted widespread attention. The strategy of amplifying the detection signal via the RAFT polymerization method is quite effective. RAFT, as an emerging polymerization method, has been applied in many fields, which can be used to prepare coatings for automobiles and other vehicles; The block, star, and graft polymers prepared via RAFT polymerization can be used as amphiphilic polymers, dispersants, and electronic devices. Based on the many advantages of RAFT, the strategy of amplifying the detection signal through the RAFT polymerization method has also received widespread attention. A large number of detection probes can be directly introduced or numerous active sites are available for subsequent modification of detection probes via labeling polymer materials.[76] Therefore, this signal amplification strategy based on RAFT will have a very broad application prospect in the highly sensitive detection of biomolecules.

In 2018, Hu started the study of RAFT application in biosensors in our laboratory by using PNA as a capture probe during his PhD study, and then corresponding satisfactory results were obtained and published.[77,78] As shown in Figure 7A, in order to further improve the sensitivity of the biosensor, we then report a new type of sensor. It modifies PNA to the surface of magnetic beads as a capture probe and uses RAFT signal amplification strategy for DNA detection. The sensor can realize simple, fast, low-cost, highly sensitive, and highly selective detection of DNA, with a detection limit of 0.02 fM. Among them, CPAD and fluorescein-*o*-acrylate (FA) are used as CTAs and monomers of RAFT.[79] Next, in 2019, Sun *et al*.[32] combined electrochemical-mediated RAFT with silver nano-plating technology to achieve a dual-signal amplification strategy for effective detection of target DNA. This method breaks the detection limit of a DNA biosensor based only on RAFT amplification strategy and can regulate the molecular weight range of RAFT products by controlling the magnitude of the externally applied voltage or current as shown in

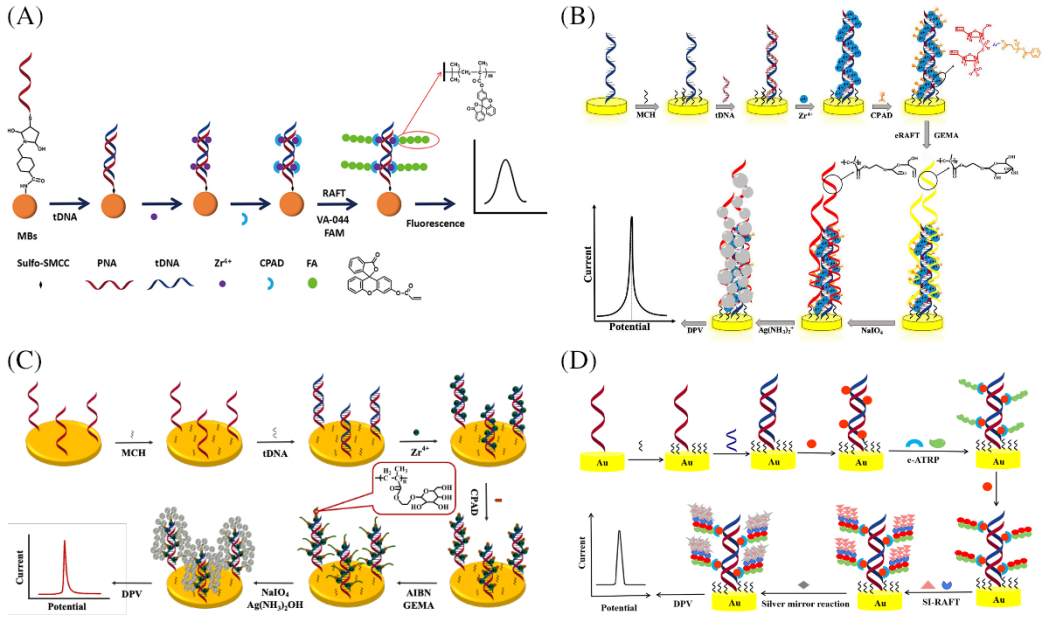

FIGURE 7 The mechanism diagram of DNA biosensor using peptide nucleic acid (PNA) as the capture probe and reversible addition-fragmentation chain transfer (RAFT) as the amplification strategy. (A) The principle diagram of a biosensor for DNA detection that uses RAFT to aggregate fluorescein-*o*-acrylate (FA) onto a PNA/DNA heteroduplex. (B) Schematic illustration of the electrochemical DNA detection, the dual-signal amplification strategy process of combining electrochemically mediated surface-initiated reversible addition-fragmentation chain transfer (SI-eRAFT), and metal silver deposition with DNA as a template. (C) Schematic diagram of DNA electrochemical biosensing analysis based on thermally induced SI-RAFT and nano-silver coating. (D) The schematic diagram of the biosensor structure that combines surface-initiated electrochemically mediated ATRP (SI-eATRP), thermally induced RAFT, and silver nano-plating technology to achieve a three-time signal amplification strategy to detect DNA

Figure 7B. First, the thiol-modified PNA capture probe is bound to the surface of the gold electrode through the Au-S bond. Then, many phosphate groups were captured on the surface of gold electrode by hybridization of target DNA with PNA. Therefore, CPAD can be captured by the phosphoric acid-Zr^{4+}-carboxylic acid structure and a large amount of GEMAs can be captured during the *in situ* electrochemically mediated RAFT (eRAFT) process. Afterwards, the hydroxyl groups of the GEMA monomer are oxidized to aldehyde groups through the reaction with sodium periodate ($NaIO_4$). Finally, the metallic AgNPs are deposited on the polymer chains to form a film through the silver mirror reaction. In this way, the target DNA fragments are labeled with numerous Ag nanoparticles with good electrochemical signals, which significantly improve the sensitivity of this DNA analysis method. The detection limit of this method when detecting target DNA is as low as 5.4 aM. In order to study the storage, Sun *et al*. stored the sensor in a closed and dark environment at 4 °C. After 2 weeks of storage, no significant changes were detected in the electrochemical signal of the sensor. Therefore, this analytical method has satisfactory storage stability.

In subsequent attempts, we found that thermally induced RAFT has many advantages over eRAFT, which is easy to operate. It only needs to be heated to decompose the initiator to generate free radicals, which can trigger RAFT. Moreover, it does not need constant voltage applied by the electrochemical workstation, which is more convenient for batch experiment operation and greatly saves time. In addition, because the electrolytic cell needs to be continuously applied with nitrogen (N_2) to maintain an oxygen-free environment to avoid the deactivation of free radicals, while the thermal reaction only needs to replace the air in the closed environment with N_2 before the reaction starts. Compared with the open environment of the electrolytic cell, thermal initiation is more convenient to react in a sealed environment. Based on these advantages, Liu *et al*.[50] proposed an electrochemical biosensor for DNA analysis using PNA as a capture probe, thermally triggered RAFT, and nano-silver coating as a dual signal amplification strategy. As shown in Figure 7C, the PNA-modified electrode acts as a specific probe, which can accurately identify and capture target DNA. The CPAD was immobilized on the target DNA through the coordination of carboxylate and phosphate with Zr^{4+} ions to act as a chain transfer agent for RAFT. Subsequently, AIBN was used as a thermal initiator to bring, a large amount of GEMAs to the electrode surface by heating. Then, the hydroxyl groups on the GEMA were oxidized to aldehyde groups via $NaIO_4$. Finally, through the silver mirror reaction, AgNPs wrapped the polymer chains to form a silver nano-coating film. So, the target DNA fragment was labeled with considerable AgNPs with good electrochemical signal. This results in significant amplification of the electrochemical signal. Therefore, compared with other electrochemical DNA detection methods, this method can improve the detection sensitivity of electrochemical signals to a higher level, and can detect about 34 DNA molecules in a 10-μL serum sample. In addition, Liu *et al*. also explored the selectivity of the biosensor, and obtained the detection signals of single-base mismatched DNA, three-base mismatched DNA, and control DNA via

DPV. The results showed that the signal of single-base mismatched DNA and three-base mismatched DNA was only 24% and 12.4% of the target DNA, which showed that the DNA biosensor had high specificity and selectivity.

To further improve the sensitivity of detection and achieve the purpose of single-molecule detection. We proposed the combination of electrochemically mediated ATRP, thermally triggered RAFT, and silver nano-coating technology to realize a three-time signal amplification strategy.[51] As shown in Figure 7D, PNA is used as a capture probe. In the presence of target DNA, lots of AgNPs are deposited on the electrode surface through ATRP, RAFT, and silver nano-coating technology. Through the electrochemical determination of AgNPs, ultra-sensitive detection of CYFRA21-1 DNA can be achieved, with a detection limit as low as 0.487 aM, which means that about three DNA molecules are detected in a 10-μL serum sample. Through our combination of various signal amplification strategies, the detection of target DNA has reached an ultra-sensitive level.

The powerful ability of RAFT to control the molecular weight of the polymer is of great significance for biomolecule detection and biomarker material synthesis, which can provide breakthrough development for biosensors and nucleic acid detection. In the preparation process of the biosensor, the generation rate of free radicals plays a vital role in the RAFT reaction. It not only affects the polymerization time, but also determines the molecular weight distribution range of the polymer chain and the polymerization yield. Therefore, in future work, if we can conduct more detailed studies on the relationship between the initiation conditions of various initiators (such as the temperature of thermal initiator and the decomposition voltage of electrical initiator) and the rate of free radical generation and the concentration of other polymer components, it will greatly improve the application efficiency of the RAFT method in DNA biosensors. In addition, it can also improve research efficiency, save costs, and reduce pollution during sensor preparation. However, compared with the sensor without signal amplification, RAFT-mediated biosensor has some disadvantages, such as relatively complicated experimental operation, long detection time. Therefore, our future work will focus on how to make the RAFT operating system more convenient and shorten the response time. In short, RAFT is a good signal amplification strategy in DNA biosensors.

4 | CONCLUSIONS AND PERSPECTIVE

In view of the many shortcomings of the existing electrochemical DNA biosensing analysis methods, for example, poor stability and specificity of capture probes, insufficient detection sensitivity, complex preparation processes, and long detection cycles, a series of research work focusing on improving the practicality of electrochemical DNA biosensing analysis methods, using the neutral nucleic PNA as an immobilized capture probe has been carried out. In this review, we have discussed two types of PNA-mediated biosensors. On one hand, with the help of the coordination chemistry of $Zr^{4+}$and CDIs chemistry, the electroactive probe is directly coupled to the target DNA captured by the PNA probe, and a variety of simple and convenient electrochemical methods has been established to realize the rapid and highly selective detection of DNA biosensor. On the other hand, with the aid of ATRP and RAFT-mediated *in situ* signal amplification strategies, a large number of electroactive substances have been introduced into biosensors, realizing the ultra-sensitive and highly selective analysis of target DNA. The powerful control ability of ATRP and RAFT on the molecular weight of products is of great significance to the detection of biomolecules and the synthesis of biomarkers, and will provide breakthrough developments for biosensors, nucleic acid detection, drug delivery, and the synthesis of various materials. In addition, combining materials with excellent labeling properties will bring creative development to the fields of nucleic acid detection, early diagnosis, and treatment of diseases. These methods have greatly expanded the application potential of electrochemical DNA biosensing analysis in the field of early diagnosis of diseases. However, these established methods are currently only suitable for the sensing analysis of a single target DNA sample and do not meet the needs of direct detection of actual samples (such as clinical serum samples) and on-site "real-time detection." Therefore, further improvements to the practicability of sensors, and development of a more sensitive and practical DNA biosensor are required. PNA shows excellent potential to meet the needs of on-site "real-time detection." We believe future research work based on PNA-mediated electrochemical DNA biosensing analysis will mainly focus on fast and convenient, miniaturization, low cost, ultra-sensitivity, high specificity, high stability, high throughput, and real-time online detection.

ACKNOWLEDGMENT

This research was financially supported by the National Natural Science Foundation of China (Grant Nos. 21974068, 21890742, 9195401, and 21890740).

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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REFERENCES

[1] W. Vercoutere, M. Akeson, *Curr. Opin. Chem. Biol.* **2002**, *6*, 816.
[2] H. H. Thorp, *Trends Biotechnol.* **1998**, *16*, 117.
[3] S. J. Salipante, D. R. Hoogestraat, D. J. SenGupta, D. Murphey, K. Panayides, E. Hamilton, I. Castañeda-Sánchez, J. Kennedy, P. W. Monsaas, L. Mendoza, K. Stephens, J. J. Dunn, B. T. Cookson, *J. Clin. Microbiol.* **2012**, *50*, 1480.
[4] P. F. Thomsen, J. Kielgast, L. L. Iversen, C. Wiuf, M. Rasmussen, M. T. P. Gilbert, L. Orlando, E. Willerslev, *Mol. Ecol.* **2012**, *21*, 2565.
[5] G. Chiti, G. Marrazza, M. Mascini, *Anal. Chim. Acta* **2001**, *427*, 155.
[6] K. Kerman, M. Kobayashi, E. Tamiya, *Meas. Sci. Technol.* **2003**, *15*, R1.

[7] H. Sun, Y. Qiu, Q. Liu, Q. Wang, Y. Huang, D. Wen, X. Zhang, Q. Liu, G. Liu, J. Kong, *Biosens. Bioelectron.* **2019**, *131*, 193.
[8] H. J. Parab, C. Jung, J.-H. Lee, H. G. Park, *Biosens. Bioelectron.* **2010**, *26*, 667.
[9] D. Santano, A. Urrutia, C. R. Zamarreño, I. D. Villar, *IEEE Sens. J.* **2020**, *21*, 1.
[10] J. Peng, Q. Huang, W. Zhuge, Y. Liu, C. Zhang, W. Yang, G. Xiang, *Biosens. Bioelectron.* **2018**, *106*, 212.
[11] J. Peng, Q. Huang, Y. Liu, P. Liu, C. Zhang, *Sens. Actuators B* **2019**, *294*, 157.
[12] H.-L. Cai, Y. Yang, Y.-H. Zhang, C.-J. Zhou, C.-R. Guo, J. Liu, T.-L. Ren, *Mod. Phys. Lett. B* **2014**, *28*, 1450056.
[13] H. Li, S. Xiao, D. Yao, M. H.-W. Lam, H. Liang, *Chem. Commun.* **2015**, *51*, 4670.
[14] J. Y. Peng, C. T. Hou, X. X. Liu, H. B. Li, X. Y. Hu, *Talanta* **2011**, *86*, 227.
[15] D. R. Thévenot, K. Toth, R. A. Durst, G. S. Wilson, *Biosens. Bioelectron.* **2001**, *16*, 121.
[16] Ü. Anik, Y. Tepeli, M. Sayhi, J. Nsiri, M. F. Diouani, *Analyst* **2018**, *143*, 150.
[17] S. Kumar, N. Verma, A. K. Singh, *Sens. Actuators B* **2017**, *240*, 248.
[18] A. Sassolas, B. D. Leca-Bouvier, L. J. Blum, *Chem. Rev.* **2008**, *108*, 109.
[19] G. Liu, R. Lao, L. Xu, Q. Xu, L. Li, M. Zhang, S. Song, C. Fan, *Biosens. Bioelectron.* **2013**, *42*, 516.
[20] F. Le Floch, H.-A. Ho, P. Harding-Lepage, M. Bédard, R. Neagu-Plesu, M. Leclerc, *Adv. Mater.* **2005**, *17*, 1251.
[21] J. Summerton, D. Weller, *Antisense Nucleic Acid Drug Dev.* **1997**, *7*, 187.
[22] R. M. Hudziak, E. Barofsky, D. F. Barofsky, D. L. Weller, S.-B. Huang, D. D. Weller, *Antisense Nucleic Acid Drug Dev.* **1996**, *6*, 267.
[23] C. Briones, M. Moreno, *Anal. Bioanal. Chem.* **2012**, *402*, 3071.
[24] A. A. Koshkin, S. K. Singh, P. Nielsen, V. K. Rajwanshi, R. Kumar, M. Meldgaard, C. E. Olsen, J. Wengel, *Tetrahedron* **1998**, *54*, 3607.
[25] B. Vester, J. Wengel, *Biochemistry* **2004**, *43*, 13233.
[26] D. A. Braasch, D. R. Corey, *Chem. Biol.* **2001**, *8*, 1.
[27] P. M. McTigue, R. J. Peterson, J. D. Kahn, *Biochemistry* **2004**, *43*, 5388.
[28] R. Owczarzy, Y. You, C. L. Groth, A. V. Tataurov, *Biochemistry* **2011**, *50*, 9352.
[29] Y. Han, F. An, J. Liu, J. Kong, X. Zhang, *New J. Chem.* **2020**, *44*, 20770.
[30] H. Sun, J. Kong, Q. Wang, Q. Liu, X. Zhang, *ACS Appl. Mater. Interfaces* **2019**, *11*, 27568.
[31] H. Sun, Y. Qiu, Y. Lu, J. Kong, X. Zhang, *Chem. Commun.* **2020**, *56*, 6636.
[32] H. Sun, W. Xu, B. Liu, Q. Liu, Q. Wang, L. Li, J. Kong, X. Zhang, *Anal. Chem.* **2019**, *91*, 9198.
[33] C. Wang, J. Liu, J. Kong, X. Zhang, *Anal. Chim. Acta* **2020**, *1136*, 19.
[34] Q. Liu, L. Jian, R. Liu, H. Yang, J. Kong, X. Zhang, *Chem. Eur. J.* **2020**, *26*, 1633.
[35] M. Egholm, O. Buchardt, L. Christensen, C. Behrens, S. M. Freier, D. A. Driver, R. H. Berg, S. K. Kim, B. Norden, P. E. Nielsen, *Nature* **1993**, *365*, 566.
[36] P. Nielsen, M. Egholm, R. Berg, O. Buchardt, *Science* **1991**, *254*, 1497.
[37] F. P. Schwarz, S. Robinson, J. M. Butler, *Nucleic Acids Res.* **1999**, *27*, 4792.
[38] J. Liu, L. Tiefenauer, S. Tian, P. E. Nielsen, W. Knoll, *Anal. Chem.* **2006**, *78*, 470.
[39] J. Chun, T. Hla, K. R. Lynch, S. Spiegel, W. H. Moolenaar, *Pharmacol. Rev.* **2010**, *62*, 579.
[40] K. I. Izvolsky, V. V. Demidov, P. E. Nielsen, M. D. Frank-Kamenetskii, *Biochemistry* **2000**, *39*, 10908.
[41] B. Piro, V. Noël, S. Reisberg, in *RNA and DNA Diagnostics* (Eds: V. A. Erdmann, S. Jurga, J. Barciszewski), Springer International, Cham **2015**, p. 47.
[42] S. Cauteruccio, E. Licandro, M. Panigati, G. D'Alfonso, S. Maiorana, *Coord. Chem. Rev.* **2019**, *386*, 119.
[43] P. Anstaett, G. Gasser, *Chimia (Aarau)* **2014**, *68*, 264.
[44] H. Sun, L. Qian, J. Kong, X. Zhang, *Sens. Actuators B* **2021**, *337*, 129791.
[45] H.-A. Joung, N.-R. Lee, S. K. Lee, J. Ahn, Y. B. Shin, H.-S. Choi, C.-S. Lee, S. Kim, M.-G. Kim, *Anal. Chim. Acta* **2008**, *630*, 168.
[46] M. Kaisti, A. Kerko, E. Aarikka, P. Saviranta, Z. Boeva, T. Soukka, A. Lehmusvuori, *Sci. Rep.* **2017**, *7*, 15734.
[47] A. Geiger, A. Lester, J. Kleiber, H. Ørum, *Nucleosides Nucleotides* **1998**, *17*, 1717.
[48] M. Xu, P. Fu, S. Xing, Y. Zhao, C. Zhao, *ChemBioChem* **2020**, *21*, 2667.
[49] S. O. Kelley, *ACS Sensors* **2017**, *2*, 193.
[50] B. Liu, H. Sun, L. Li, J. Zhang, J. Kong, X. Zhang, *Microchim. Acta* **2019**, *187*, 35.
[51] J. Li, L. Zhao, D. Wen, X. Li, H. Yang, D. Wang, J. Kong, *Anal. Bioanal. Chem.* **2020**, *412*, 4155.
[52] Q. Hu, X. Deng, X. Yu, J. Kong, X. Zhang, *Biosens. Bioelectron.* **2015**, *65*, 71.
[53] F. Liu, Q. Xu, W. Huang, Z. Zhang, G. Xiang, C. Zhang, C. Liang, H. Lian, J. Peng, *Electrochim. Acta* **2019**, *295*, 615.
[54] J. Peng, C. Hou, X. Hu, *Sens. Actuators B* **2012**, *169*, 81.
[55] X. Miao, X. Guo, Z. Xiao, L. Ling, *Biosens. Bioelectron.* **2014**, *59*, 54.
[56] Y. Qian, C. Wang, F. Gao, *Talanta* **2014**, *130*, 33.
[57] W. Wei, Q. Ni, Y. Pu, L. Yin, S. Liu, *J. Electroanal. Chem.* **2014**, *714–715*, 25.
[58] C. Xu, H. Cai, P. He, Y. Fang, *Analyst* **2001**, *126*, 62.
[59] J. Liu, J. Song, T. Qi, C. Zhang, J. Qu, *Adv. Powder Technol.* **2016**, *27*, 1.
[60] C. Fang, H. Ji, W. Y. Karen, S. R. M. Rafei, *Biosens. Bioelectron.* **2011**, *26*, 2670.
[61] M. Ali, R. Neumann, W. Ensinger, *ACS Nano* **2010**, *4*, 7267.
[62] S. Xing, X. Xu, P. Fu, M. Xu, T. Gao, X. Zhang, C. Zhao, *Colloids Surf. B* **2019**, *181*, 333.
[63] W. Xu, S. Xing, X. Xu, M. Xu, P. Fu, T. Gao, C. Zhao, *ACS Omega* **2018**, *3*, 17835.
[64] J. Lee, I.-S. Park, E. Jung, Y. Lee, D.-H. Min, *Biosens. Bioelectron.* **2014**, *62*, 140.
[65] P. Fu, S. Xing, M. Xu, Y. Zhao, C. Zhao, *Sens. Actuators B* **2020**, *305*, 127545.
[66] P. Harding Lepage, R. Peytavi, M. G. Bergeron, M. Leclerc, *Anal. Chem.* **2011**, *83*, 8086.
[67] Y. Wu, S. Liu, L. He, *Anal. Chem.* **2009**, *81*, 7015.
[68] J.-S. Wang, K. Matyjaszewski, *J. Am. Chem. Soc.* **1995**, *117*, 5614.
[69] J.-S. Wang, K. Matyjaszewski, *Macromolecules* **1995**, *28*, 7901.
[70] Q. Hu, Q. Wang, G. Sun, J. Kong, X. Zhang, *Anal. Chem.* **2017**, *89*, 9253.
[71] H. Tan, S. Yang, G. Shen, R. Yu, Z. Wu, *Angew. Chem. Int. Ed.* **2010**, *49*, 8608.
[72] F. Patolsky, Y. Weizmann, I. Willner, *J. Am. Chem. Soc.* **2002**, *124*, 770.
[73] G. Wu, N. Yang, T. Zhang, Z. Wang, X. Lu, J. Kang, *Sens. Actuators B* **2011**, *160*, 598.
[74] A. Anastasaki, V. Nikolaou, F. Brandford-Adams, G. Nurumbetov, Q. Zhang, G. J. Clarkson, D. J. Fox, P. Wilson, K. Kempe, D. M. Haddleton, *Chem. Commun.* **2015**, *51*, 5626.
[75] J. Chiefari, Y. K. Chong, F. Ercole, J. Krstina, J. Jeffery, T. P. T. Le, R. T. A. Mayadunne, G. F. Meijs, C. L. Moad, G. Moad, E. Rizzardo, S. H. Thang, *Macromolecules* **1998**, *31*, 5559.
[76] G. Moad, E. Rizzardo, S. H. Thang, *Aust. J. Chem.* **2009**, *62*, 1402.

[77] Q. Hu, D. Han, S. Gan, Y. Bao, L. Niu, *Anal. Chem.* **2018**, *90*, 12207.
[78] Q. Hu, J. Kong, D. Han, L. Niu, X. Zhang, *ACS Sensors* **2019**, *4*, 235.
[79] W. Liu, L. Ma, Z. Guo, T. Liu, Y. Liu, D. Wang, J. Kong, *Anal. Sci.* **2020**, *36*, 681.

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How to cite this article: H. Sun, J. Kong, X. Zhang, *Biopolymers* **2021**, e23464. https://doi.org/10.1002/bip.23464