Analytica Chimica Acta 1136 (2020) 19–24

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Nitronyl nitroxide monoradical TEMPO as new electrochemical label for ultrasensitive detection of nucleic acids

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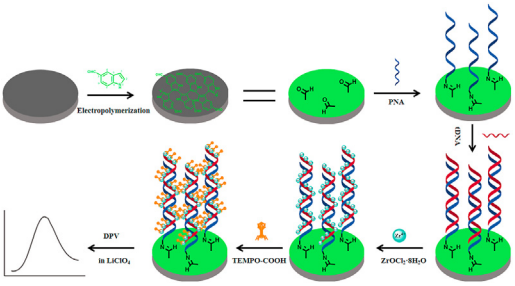
HIGHLIGHTS

- A novel electrochemical biosensor is developed for nucleic acids detection.
- The biosensor is based on nitronyl nitroxide TEMPO as an electrochemical label.
- Stable free radical TEMPO as electrochemical detection signal is proposed firstly.
- The biosensor shows high sensitivity, specificity and simple manufacturability.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 14 July 2020
Received in revised form
17 August 2020
Accepted 19 August 2020
Available online 28 August 2020

Keywords:
Electrochemical biosensor
Nitronyl nitroxide
Stable free radical
New electrochemical label
Nucleic acids detection

ABSTRACT

In this work, a novel electrochemical biosensor based on nitronyl nitroxide monoradical 2,2,6,6-tetramethylpiperidine 1-Oxyl (TEMPO) as new electrochemical label for facile nucleic acids detection is developed. This fast and convenient functional microelectrode was designed by fixing the capture probe peptide nucleic acid (PNA) and using the coordination interaction of Zr^{4+} with both phosphate groups and carboxyl groups. Differential pulse voltammetry (DPV) was used to study the oxidation current of TEMPO which was combined with the electrode surface and labeled. TEMPO electrochemical signal related to target deoxyribonucleic acid (tDNA) concentration was finally detected when tDNA was added on the surface of glassy carbon electrode (GCE). The detection principle, optimization of key factors and performance analysis of the biosensor are also discussed. A great linear relation is acquired within the scope of 10 pM−100 nM under optimal conditions and the detection limit of this experiment is calculated as low as 2.57 pM ($R^2 = 0.996$). In addition, complex serum samples were used to explore the practical application of this experiment. The results show the developed electrochemical DNA biosensor has wide application prospects in nucleic acids detection and clinical analysis.

1. Introduction

With the development of scientific technology, the recognition of disease status at the biomolecular level has attracted wide attention from researchers [1]. Among biomolecules, nucleic acids are the most frequently investigated. Small changes in nucleic acids sequences can lead to a major change in biological medicine and

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https://doi.org/10.1016/j.aca.2020.08.035
0003-2670/

biology. Biomarkers of different diseases in the diagnosis and treatment of cancer screening have important effects, especially in the detection of early stage cancer patients' bodies with low levels of nucleic acids. Convenient, rapid and sensitive nucleic acids detection plays an important role in disease diagnosis. However, traditional methods have disadvantages of high cost, complex experiment process and low accuracy [2]. Electrochemical detection is widely used in detecting DNA [3], RNA [4], proteins [5], and toxins [6] because of its advantages of fast detection speed, simple method and no need for complicated and costly instruments. In addition, the practicability of electrochemical technology and electrical analysis equipment in the study of DNA damage [7] and the interaction between DNA and various genotoxic drugs and pollutants have been fully confirmed. These trends make electrochemical DNA biosensor closer to practical biological applications [8]. By monitoring electroactive probes labeling, there are usually two events in the quantitative analysis of DNA biosensor: biometric and biotransformation [9,10]. The former provides selectivity because of specific DNA hybridization. The latter converts biological signals into electrochemical signals. Therefore, various DNA biosensors based on biometric and biotransformation strategies [11,12] emerge as the times require.

A vast variety of stable nitroxyl free radicals have been synthesized since their first appearance in literature approximately one hundred years ago [13]. Stable radicals with unique paramagnetism and redox properties have attracted much attention [14]. TEMPO and a series of its derivatives belong to a class of substances, which refer to as nitroxyl or nitroxide radicals. The great stability of TEMPO is principally caused by three reasons: (i) out of proportion with the corresponding nitro and hydroxylamine due to deficiency of α hydrogen atoms; (ii) delocalized motion of uncoupled electrons on a nitrogen-oxygen bond; (iii) steric inhibition of methyl groups [15,16]. The experiments of catalytic conversion and electrosynthesis with the participation of TEMPO have the advantage of low pollution [17–20]. TEMPO was also used as spin labeling in electron spin resonance (ESR) [21,22] and was applied to the chemical industry because of its high efficiency and low environmental pollution [23–26]. The electrochemical properties of TEMPO and its use in electrocatalytic reactions have given rise to synthetically useful processes for small-molecule and polymer syntheses [27]. It is the first time to report TEMPO as a new electrochemical label for electrochemical nucleic acids detection.

Herein, an electrochemical DNA biosensor based on stable free radical TEMPO as new electrochemical label is developed. In this experiment, only five simple steps were needed to label TEMPO on the surface of GCE as an electrochemical signal. DPV scanning in the electrochemical workstation was used to scan the modified and labeled electrode surface to obtain the oxidation current of TEMPO. TEMPO is oxidized to $TEMPO^+$ in electrolytes when a current from negative to positive is applied. The principle of oxidation of TEMPO into $TEMPO^+$ is shown in Schematic diagram 1. It can be seen from Fig. S2, the potential of oxidation peak current of TEMPO is about 0.58 V. The target nucleic acid was detected by observing the peak position and peak value of the oxidation current of TEMPO. The label TEMPO has high electrochemical activity, which shows good performances in electrochemical nucleic acids detection. TEMPO is chosen as a new electrochemical label for the following reasons: (i) it shows clear electrochemical signal in aqueous solution; (ii) it is soluble and has advantages of high stability, low pollution and high utilization rate [28,29]. TEMPO is hydrophilic when compared with common redox materials such as ferrocene and MB, and the experimental steps are more convenient than those of metal nanoparticles [30–32].

2. Experimental sections

2.1. Reagents and apparatus

Relevant information can be found in Supplementary Materials.

2.2. Pretreatment and surface functionalization of GCE

For the accuracy of the experiment, GCE was polished on the micro cloth pad with alumina slurry (1, 0.3 and 0.05 μm). After polishing for 5 min, GCE was cleaned with ultrapure water and ethanol continuously, and dried with N_2. 0.05 M indole-5-carboxaldehyde (5FIn) and 0.1 M tetrabutylammonium tetrafluoroborate (TBATFB) were dissolved in acetonitrile (ACN) and GCE was immersed in this electrolyte [33]. Thus poly (5FIn) (P5FIn) films containing aldehyde groups were formed on the surface of GCE by electropolymerization. The amount of P5FIn films containing aldehyde groups was regulated by monitoring the amount of charge in electrochemical experiments. In order to remove extra adsorbed electrolyte solution, monomers and oligomers, the prepared GCE was rinsed with ultrapure water.

2.3. Fabrication of the proposed electrochemical DNA biosensor

First of all, 0.5 μM PNA was dropped onto modified GCE of aldehyde groups. GCE was incubated for 2 h in an incubator at 37 °C subsequently. After that, GCE was washed with ultrapure water. Then, 10 μL tDNA dissolving in 0.1 M phosphate buffer saline (PBS, pH = 7.0) was dripped onto GCE surface at 37 °C. After hybridization of PNA and tDNA for 2 h, GCE was rinsed thoroughly with 0.1 M PBS to ensure that there was no residual nonspecific adsorbed tDNA. Then, GCE was immersed in 5.0 mM $ZrOCl_2$ solution (20% ethanol) for 30 min, and zirconium was bound to the phosphate of tDNA through the chemical interaction between zirconium and phosphate [2]. Then GCE was rinsed with 20% ethanol and ultrapure water. Subsequently, GCE was immersed in 1.0 mM 4-Carboxy-TEMPO (TEMPO-COOH) solution (20% ethanol) at 37 °C for 60 min to load stable free radical label TEMPO. Finally, the electrode modified with TEMPO-COOH/Zr^{4+}/tDNA/PNA/P5FIn was obtained and was thoroughly cleaned with ethanol and ultrapure water. The electrode was dried with N_2 before electrochemical measurements.

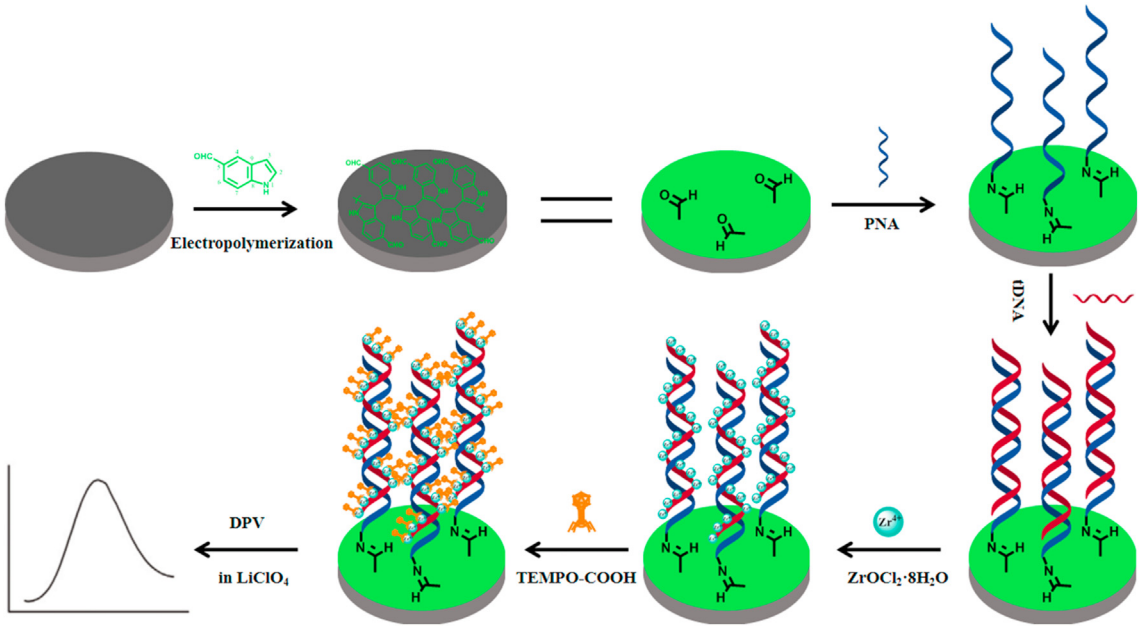
2.4. Electrochemical measurements

0.5 M $LiClO_4$ was used as electrolyte solution to soak the assembled electrochemical DNA biosensor for detecting nucleic acids. The electrochemical signal of TEMPO-COOH was measured by DPV and the potential scanning range was 0–1 V. The experimental parameters used in the experiment are: 50 mV of the pulse amplitude, 0.2 s of the pulse width, 4 mV of the potential increment, 0.5 s of the pulse period and 60 s quiet time.

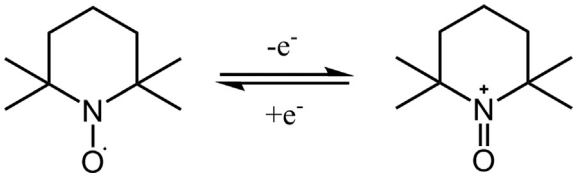
3. Results and discussion

3.1. Principle of the strategy

The principle of this strategy for detecting nucleic acids is illustrated by Scheme 1. Firstly, the surface of GCE was modified with aldehyde groups by electropolymerization and then PNA with amino groups was fixed on the GCE containing aldehyde groups by aldimine condensation [33]. PNA in blank control group had no available connection position to connect with Zr^{4+} because there were no phosphate groups on the PNA skeleton, so the subsequent loading of TEMPO was avoided. Secondly, GCE-PNA-tDNA was

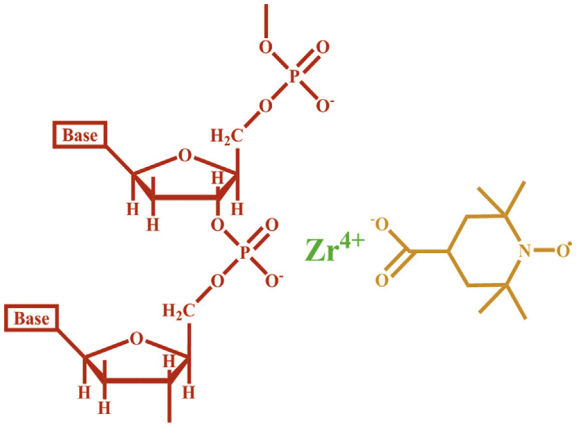


Scheme 1. Schematic representation of the proposed strategy for nucleic acids detection.



Schematic diagram 1. Schematic diagram of oxidation of TEMPO into $TEMPO^+$.

formed and phosphate groups were supplied by tDNA to be introduced into the assembly when PNA hybridized with tDNA. Thirdly, the connecting ion of Zr^{4+} had a strong chemical coordination interaction with both phosphate groups and carboxyl groups which were provided by tDNA and TEMPO-COOH, respectively. As shown in Schematic diagram 2, a molecular structure assembled by DNA-Zr-TEMPO is formed. Thus, an assembly of GCE-PNA-DNA-Zr-TEMPO was formed and numerous stable free radical labels TEMPO can be used to detect nucleic acids simply, quickly and sensitively. ch3.



Schematic diagram 2. Schematic diagram of a molecular structure assembled by DNA-Zr-TEMPO.

3.2. Feasibility of the strategy

DPV was utilized to scan different modified surfaces of GCE to verify the feasibility of the assay based on nitronyl nitroxide monoradical electrochemical label TEMPO. The experimental results are shown in Fig. 1A, without adding P5FIn films (a), PNA (b), tDNA (c), Zr^{4+} (d) or TEMPO-COOH (e), no electrochemical signal can be detected. However, when all steps are modified on the GCE surface (f), an obvious oxidation peak appears at the potential around 0.58 V, which is interpreted as the electrochemical oxidation of TEMPO into $TEMPO^+$ (more details can be found in Supplementary Materials). The above results illustrate that the strategy is feasible for nucleic acids detection and each step is essential. More importantly, only after the hybridization is completed and the label TEMPO is successfully labeled on the hybrid chain, the oxidation current will appear, which strongly guarantees the reliability of the response signal.

3.3. Electrochemical characterizations

The electrochemical characterizations of the modified GCE were characterized by CV at scanning rates of 10, 25, 50, 75, 100, 150 and 200 mV s^{-1}. The illustration in Fig. 1B shows that the peak current of anode (red line) increases linearly and that of cathode (black line) decreases linearly with the increase of square root of scanning rate. This indicates that stable free radical label TEMPO had been deposited on tDNA through chemical bonding rather than physical adsorption and the redox process of TEMPO was not diffusion dependent. Therefore, this strategy for nucleic acids detection was reliable.

Electrochemical impedance spectroscopy (EIS) is usually utilized to study electrochemical behaviors of modified electrodes and has high sensitiveness to the micro difference of the electrode surface. The diameter of the semicircle in the impedance high frequency area is numerically equivalent to the charge transfer resistance (R_{ct}). The Impedance diagrams were obtained in 0.1 M KNO_3 containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ and the experimental

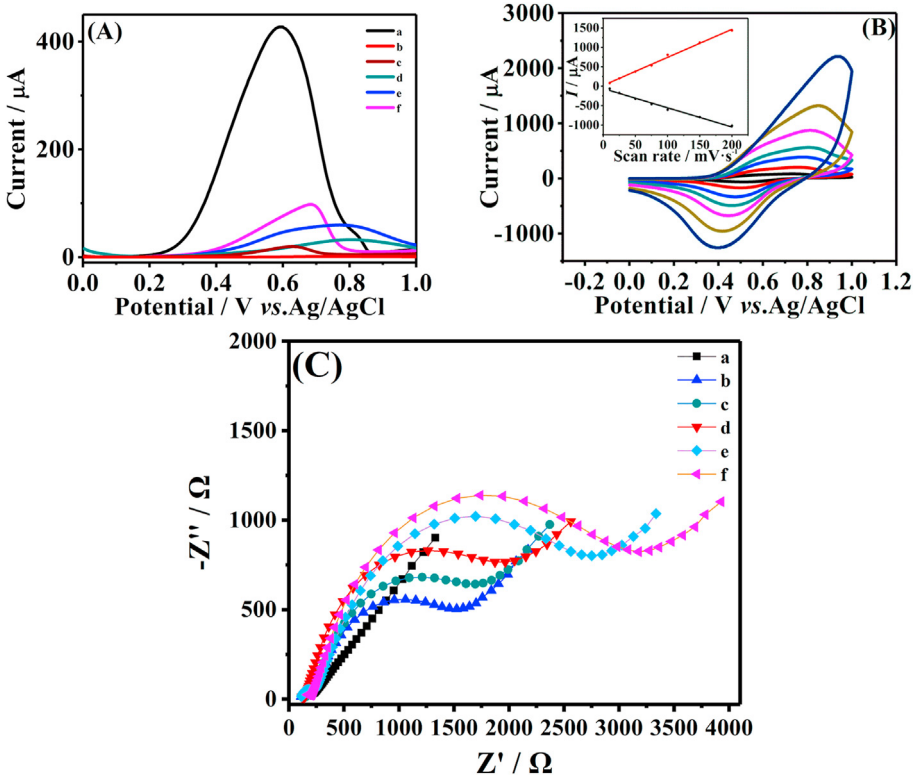


Fig. 1. (A) Figures of DPV of GCE with different modified surfaces in 0.5 M $LiClO_4$. (a) completely assembled electrodes, (b) without P5FIn films, (c) without PNA, (d) without tDNA, (e) without Zr^{4+}, (f) without TEMPO-COOH. (B) Figures of cyclic voltammetry (CV) of modified GCE with different scanning speeds in 0.5 M $LiClO_4$. The red and black dots in the inset represent anode peak current and cathode peak current at different scanning rates, respectively. (C) Impedance diagrams of GCE with different modified surfaces: (a) bare GCE, (b) GCE/P5FIn films, (c) GCE/P5FIn/PNA, (d) GCE/P5FIn/PNA/tDNA, (e) GCE/P5FIn/PNA/tDNA/Zr^{4+}, (f) GCE/P5FIn/PNA/tDNA/Zr^{4+}/TEMPO-COOH.

parameters were: open circuit potential: 0.18 V; quiet time: 60 s; frequency range: 100 kHz to 0.1 Hz; voltage amplitude: 5 mV. The bare GCE exhibited the smallest semicircle because of the rapid electron-transfer process (Fig. 1C, a). The R_{ct} of GCE/P5FIn films was markedly increased (Fig. 1C, b) because the electropolymerization of P5FIn films caused an increase of steric hindrance. When the immobilization of PNA was finished, the R_{ct} increased (Fig. 1C, c) due to the obstruction of PNA on the interface electron transfer process. After hybridization, the R_{ct} increased (Fig. 1C and d) due to the phosphate of tDNA being negatively charged, which blocked $[Fe(CN)_6]^{3-/4-}$ from reaching the surface of GCE. When Zr^{4+} was successfully added, the R_{ct} increased (Fig. 1C, e) because of the electrostatic interaction between Zr^{4+} with positive charges and $[Fe(CN)_6]^{3-/4-}$ with negative charges. Finally, the spatial effect of covalently labeled TEMPO-COOH led to a further increase of R_{ct} (Fig. 1C, f). Thus, Fig. 1C reveals the variations of R_{ct} of gradually modified GCE surfaces, which shows that electrons transfer between the GCE surface and the electrolyte is gradually blocked with the gradual modification of experimental materials, indicating that every step of the biosensor modification process has been completed successfully and this strategy is effective.

3.4. Optimizations of experimental conditions

Two experimental parameters were optimized: (a) TEMPO-COOH concentration; (b) TEMPO-COOH time. Experimental details are included in Supplementary Materials. The optimal experimental results can be obtained by selecting the following experimental conditions: (a) TEMPO-COOH concentration: 1.0 mM; (b) TEMPO-COOH time: 60 min.

3.5. Detection limit

As Fig. 2A shows, with the increasing of tDNA concentration, the peak current of corresponding potential also increases. Therefore, our experiment can exclude false positive results. It can be seen from Fig. 2B that electrochemical signals of TEMPO-COOH increase linearly with the increases of the logarithm of tDNA in the concentration scope of $10–10^5$ pM. The linear regression equation is: I (μA) = 103.37 + 69.48 lg [C_{tDNA}/pM] (R^2 = 0.996), and the detection limit of this experiment is calculated as low as 2.57 pM (tDNA concentration of signal peak height when the standard deviation of baseline noise is 3 times). The detection result of our electrochemical biosensor was compared with those of other references, which confirmed our electrochemical biosensor had the advantages of fast, convenient and high sensitivity (Table S1).

3.6. Selectivity, repeatability and storage stability

The selectivity of the experiment was verified by using tDNA, three-base-mismatched ssDNA (TBM) and control ssDNA (cDNA). Fig. 3A shows the electrochemical current of detecting TBM is 20.4% of detecting tDNA. The electrochemical current of detecting cDNA is very low and approaches the background noise signal. This difference is caused by the hybridization efficiency between different kinds of ssDNA and PNA capture probe. Therefore, due to the specificity of PNA, the electrochemical DNA biosensor has high selectivity, and has a great application prospect in nucleic acids detection. Here, the repeatability and storage stability of this experiment were also confirmed. The experiment was repeated five times with three electrodes for five consecutive days, and the concentration of tDNA was 100 nM with the same other conditions.

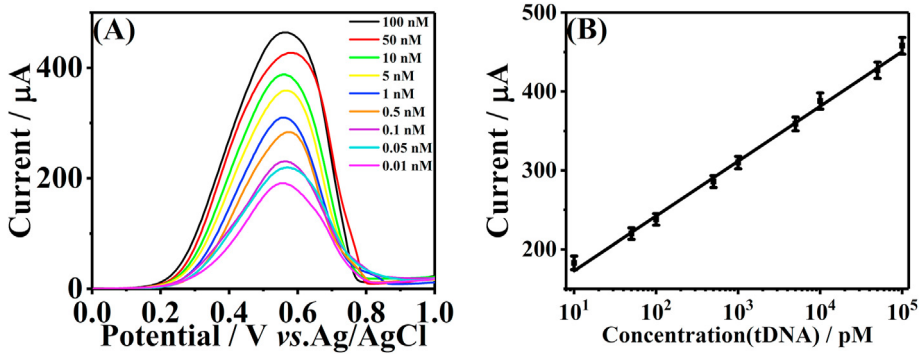


Fig. 2. (A) The DP voltammograms of the electrodes modified with different concentrations of tDNA. (B) The linear relationship between the peak current of TEMPO-COOH and the logarithm of different concentrations of tDNA. The points in Fig. 2B represent the peak current of TEMPO-COOH. Error bars represent the standard deviations of six independent measurements.

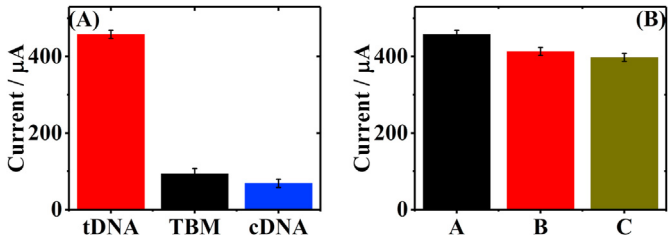


Fig. 3. (A) Histogram of DPV of different kinds of ssDNA, the concentrations of them are all 100 nM. (B) The histogram of the current amount detected by DPV of 100 nM tDNA in (A) 0.1 M PBS, (B) 1% serum sample, (C) 5% serum sample. The histogram values in Fig. 3 represent the peak current of TEMPO-COOH under different conditions. Error bars represent the standard deviations of six independent measurements.

The coefficient of variation of this electrochemical biosensor was 4.6%, which indicated this method was repeatable. After the identical modification method of two groups of electrodes, one group was immediately detected by DPV scanning and the other group was kept in a closed surrounding at 4 °C. Two weeks later, the retention rate of electrochemical signals was observed to be as high as 94.7% which concluded the modified electrode was judged to have satisfying storage stability.

3.7. Analysis of complex biological samples

The analytical performance of this method on real samples was investigated by using 1% and 5% normal human serum (NHS) as serum samples, respectively. The serum sample solutions were formed by doping 100 nM tDNA into 1% and 5% NHS, while the control sample solution was made of tDNA with the same concentration in 0.1 M PBS. There are many interfering substances in NHS. The DPV current analysis in Fig. 3B illustrates that the current intensity detected in 1% NHS and 5% NHS are 90.2% and 86.8% of that in 0.1 M PBS, respectively. The results confirm that tDNA in biological samples can also be detected, indicating that the biosensor has good anti-interference ability and can exclude the influence of interfering substances in NHS. In order to evaluate the reliability and applicability of this method in real serum samples, we prepared different concentrations of tDNA (50 nM, 5 nM, and 0.5 nM) samples in 5% NHS for labeling recovery experiments. As shown in Table S2, the recoveries ranged from 98.4% to 96.8% and the relative standard deviation (RSD) was 3.7%–4.8%. The results indicated that the recovery rate of the samples was excellent and the biosensor had good reliability and applicability. Hence, this method can be used for nucleic acids detection in normal human serum biological samples and has broad clinical application prospects.

4. Conclusions

In summary, a novel electrochemical biosensor based on nitronyl nitroxide monoradical TEMPO as new electrochemical label in detecting nucleic acids rapidly and sensitively is constructed. Stable free radical TEMPO as an electrochemical detection signal is proposed firstly. It is proved that TEMPO has good electrochemical redox characteristics. Under optimal experimental conditions, the biosensor can detect the minimum 2.57 pM tDNA. The electrochemical biosensor is successfully applied to the quantitative analysis and detection of special sequences DNA in serum samples, which provides good practicability for nucleic acids detection. This method has many advantages of saving time, labor and cost, good repeatability and stability, and also has a broad application prospect in nucleic acids detection.

CRediT authorship contribution statement

Chen Wang: Methodology, Formal analysis, Investigation, Visualization, Writing - original draft. **Jingliang Liu:** Investigation, Visualization, Writing - review & editing. **Jinming Kong:** Conceptualization, Project administration. **Xueji Zhang:** Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 21974068, 21890740).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.08.035.

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