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Electrochemical detection of sequence-specific DNA based on formation of G-quadruplex-hemin through continuous hybridization chain reaction

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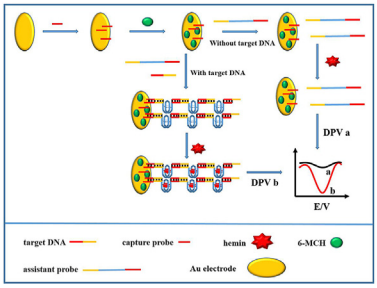
HIGHLIGHTS

- A DNA sensor based on hybridization chain reaction and G-quadruplex-hemin complex.
- High sensitive detection of HIV DNA was achieved with the detection limit down to fM level.
- The DNA sensor can discriminate mismatched sequences of HIV DNA sequence.
- The DNA sensor was applied in detection of the target DNA in serum.

GRAPHICAL ABSTRACT



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ABSTRACT

A high-sensitive detection of sequence-specific DNA was established based on the formation of G-quadruplex-hemin complex through continuous hybridization chain reaction (HCR). Taking HIV DNA sequence as an example, a capture probe complementary to part of HIV DNA was firstly self-assembled onto the surface of Au electrode. Then a specially designed assistant probe with both terminals complementary to the target DNA and a G-quadruplex-forming sequence in the center was introduced into the detection solution. In the presence of both the target DNA and the assistant probe, the target DNA can be captured on the electrode surface and then a continuous HCR can be conducted due to the mutual recognition of the target DNA and the assistant probe, leading to the formation of a large number of G-quadruplex on the electrode surface. With the help of hemin, a pronounced electrochemical signal can be observed in differential pulse voltammetry (DPV), due to the formation of G-quadruplex-hemin complex. The peak current is linearly related with the logarithm of the concentration of the target DNA in the range from 10 fM to 10 pM. The electrochemical sensor has high selectivity to clearly discriminate single-base mismatched and three-base mismatched sequences from the original HIV DNA sequence. Moreover, the established DNA sensor was challenged by detection of HIV DNA in human serum samples, which showed the low detection limit of 6.3 fM. Thus it has great application prospect in the field of clinical diagnosis and environmental monitoring.

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1. Introduction

With the development of medical science, DNA detection plays an important role in pathogen analysis [1], genetic disorder diagnosis [2] and forensic tests [3]. Sensitive and low-cost detection of sequence-specific DNA can facilitate early diagnosis, reduce the health risk of diseases or pollution, with affordable cost to the people in low income countries and regions [3]. Up to now, different techniques have been employed for DNA detection, such as fluorescence [4], electrochemistry [5], electrochemiluminescence [6], chemiluminescence [7], quartz-crystal microbalance [8], and surface plasmon resonance technique [9]. Among these techniques, electrochemical DNA sensors possess several outstanding characteristics, such as high-sensitive, high-selective, simple and easy to miniaturization, and therefore have been widely used in different scenarios [9–11].

To achieve sufficient sensitivity during electrochemical detection, an appropriate signal amplification strategy appears to be the most significant [12,13]. The commonly used signal amplification includes nanomaterials-based techniques and DNA amplification-based techniques. The former techniques generally utilize various nanomaterials as carriers of signal labels. The high-load signal labels owing to the high specific area of nanomaterials offers efficient amplification of detectable signals [14–16]. However, the stability of nanomaterials sometimes is not satisfactory and the steps for the preparation of nanomaterials can be tedious. The latter techniques including polymerase chain reaction [17], hybridization chain reaction [18,19], strand displacement amplification [20,21], and rolling circle amplification [22,23], amplify the output signals based on DNA hybridization and amplification reaction either under the catalysis of enzymes or in enzyme-free mode. Polymerase chain reaction requires specific enzyme to realize the rapid synthesis and amplification of the target DNA. Strand displacement amplification requires DNA polymerase and forms new DNA long chain under the action of primers, which can replace the target DNA. Rolling circle amplification utilizes circular DNA as template and stretches out many complement repetitive DNA sequence under the catalysis of DNA polymerase. Both these amplification techniques involve DNA polymerase. The certain reaction conditions of enzymes can limit the application of such techniques. Whereas hybridization chain reaction (HCR) can be conducted without the involvement of enzymes [24]. Using the target DNA as the evoked agent, hybridization chain reaction can promote signal amplification and improve detection sensitivity through proper design [25]. Different readout signals can be produced after hybridization chain reaction depending on the labels used [26,27], among which the electrochemical detection attracted wide interest owing to its outstanding characteristics as described above.

G-quadruplex is a guanine-rich DNA sequence that can fold into spatial structure in the presence of K^+ and the structure can strongly combine with hemin to form G-quadruplex-hemin complex [28]. Owing to its unique features, such as relatively easy to label, low cost, more stable against hydrolysis and heat treatment, low nonspecific adsorption, and typical electrochemical property, G-quadruplex-hemin complex that consists of hemin intercalated in a G-quadruplex structure becomes a frequently used signal label in biosensors [29–32].

Herein, we fabricated a simple yet ultrasensitive electrochemical DNA biosensor. Taking HIV DNA sequence as an example, the sensor coupled the hybridization chain reaction between the target sequence and the assistant probe with the formation of signal-generating G-quadruplex-hemin complex. The rationally designed probes triggered the signal amplification procedure. Thus high sensitivity as well as high specificity of the sensor were achieved.

2. Experimental

2.1. Materials and reagents

6-mercaptohexanol (6-MCH) and hemin were purchased from Sigma-Aldrich. Human serum was purchased from Beijing Solarbio Science & Technology Co., Ltd. 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid sodium salt (HEPES) was obtained from Sangon Biotech Co., Ltd. Other chemicals were of analytical grade. All solutions were prepared with ultrapure water (18.2 MΩ cm) obtained from a Millipore water purification system.

All oligonucleotides were synthesized and HPLC-purified by Sangon Biotech Co., Ltd. They were diluted to 100 μM with 10 mM PBS buffer (pH7.4) containing 1 M NaCl and 1 mM $MgCl_2$. The sequences of the oligonucleotides were as below.

Capture probe: 5′-HS-$(CH_2)_6$-TT*TAGTACTGGTG* -3′

Assistant probe: 5′-AAATTGCTGCCTTTGGGTAGGGCGGGTTGGG C*TTAGTAC TGGTG*-3′

HIV DNA: 5′-GGCAGCAATTT*CACCAGTACTA*-3′

Single-base mismatched HIV DNA: 5′-GGCAGCAATTT**G**ACCAG-TACTA-3′

Three-base mismatched HIV DNA: 5′-GGCAGCAATT**AGT**CCAG-TACTA-3′

The italic letters and the underlined letters are the complementary sequences, and the bold letters are the mismatched bases.

2.2. Preparation and modification of Au electrode

Au electrode (3 mm in diameter) was polished carefully with alumina slurry (0.3 and 0.05 μm) and then sonicated in ultrapure water and ethanol for 3 min, respectively. Then the polished electrode was electrochemically activated by cyclic scanning in the potential range from −0.2 to +1.5 V in 0.5 M H_2SO_4. Thereafter the electrode was dried at nitrogen atmosphere.

The pretreated Au electrode was immersed in 100 μL of 0.3 μM capture probe for 6 h, allowing the capture probe to be attached to the surface of Au electrode via Au-S bond. Then the capture probe-modified electrode was washed with 10 mM PBS buffer, dried with nitrogen, and then immersed in 200 μL of 2 mM 6-MCH for 4 h to block the nonspecific adsorption sites on electrode surface. Finally, the electrode was thoroughly washed with 10 mM PBS buffer and dried with nitrogen.

2.3. Fabrication of the electrochemical DNA biosensor

Samples with different concentration of HIV DNA were mixed with 0.8 μM assistant probe in 100 μL hybridization solution, which were heated to 95 °C for 5 min, and cooled down to room temperature. The capture probe-modified Au electrode was then immersed into the hybridization solution and incubated for 2 h to allow the complete hybridization among the complementary sequences of the capture probe, the target DNA and the assistant probe. After hybridization, the electrode was immersed into 200 μL G-quadruplex-forming solution (10 mM HEPES containing 50 mM KCl, pH 8.0) for 30 min to allow the formation of the spatial structure of G-quadruplex. After that, 2 μL of 20 mM hemin (dissolved in DMSO) was added into the G-quadruplex-forming solution and incubation for additional 1 h to form G-quadruplex-hemin complex.

The electrochemical measurements were conducted on a CHI 660e electrochemical workstation. Differential pulse voltammetry (DPV) was conducted in 20 mM HEPES buffer containing 20 mM KCl (pH 8.0) in the potential range from −0.6 to −0.15 V.

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3. Results and discussion

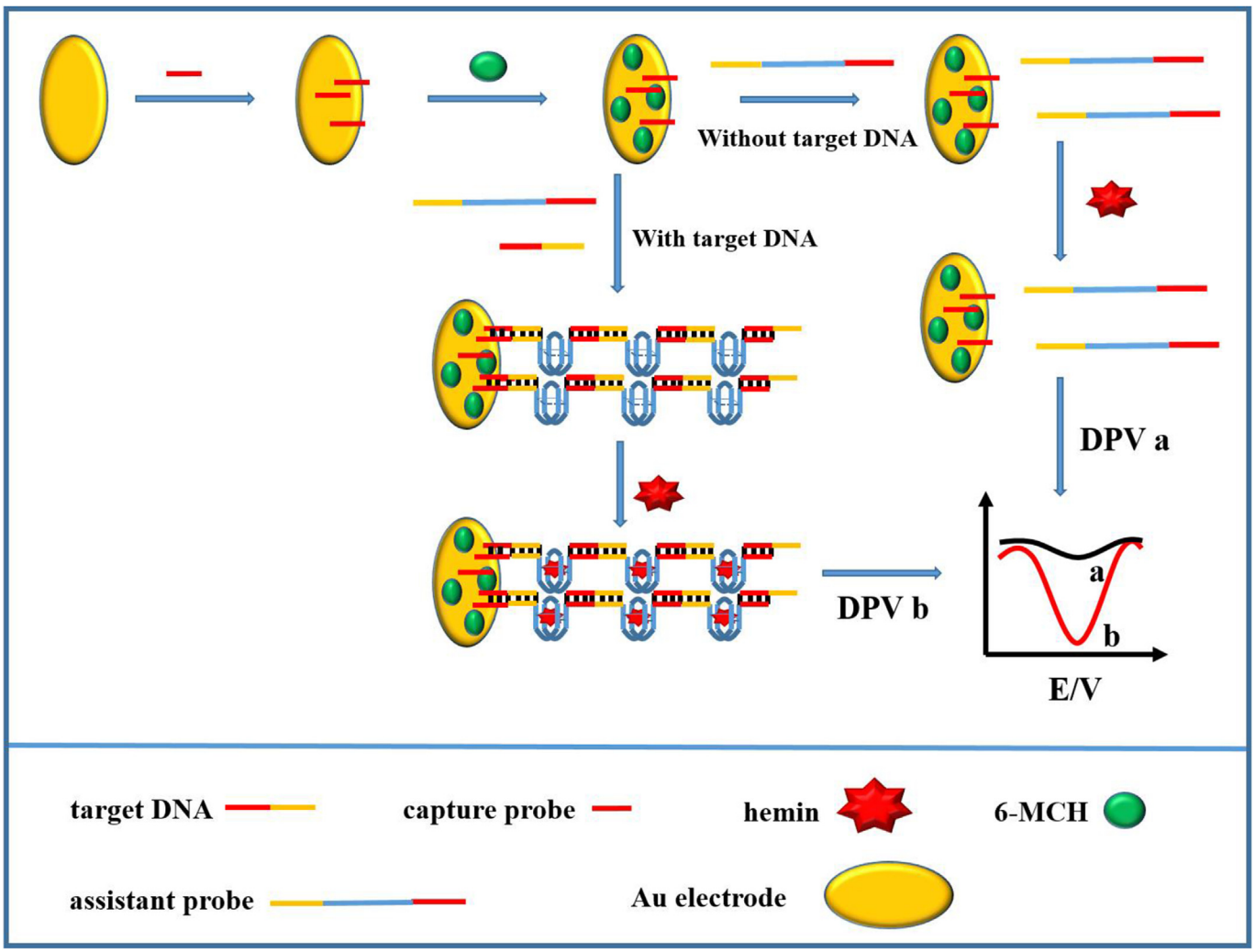
3.1. The principle of the electrochemical DNA biosensor

The principle of the designed electrochemical DNA sensor is depicted in Scheme 1. A capture probe with a thiol group at 5′-end and complementary to part of HIV DNA was designed and self-assembled onto the surface of Au electrode via Au-S bond. Then a special assistant probe with both terminals complementary to the target DNA and a G-quadruplex-forming sequence in the center was also designed. When the target DNA and the assistant probe were introduced into the hybridization system and incubated with the capture probe-modified Au electrode, the target DNA can be captured onto the electrode surface and further conduct a continuous hybridization chain reaction due to the mutual recognition between the target DNA and the assistant probe. Thus a large number of G-quadruplex can be introduced onto the electrode surface, which can produce pronounced electrochemical signal by using differential pulse voltammetry (DPV) in the presence of hemin, owing to the formation of G-quadruplex-hemin complex. The value of peak current is related with the amount of G-quadruplex-hemin complex formed on the electrode, and therefore the concentration of the target DNA.

To verify the feasibility of the principle, the modification of the electrode and the electrochemical signal in different electrode configuration were characterized.

Electrochemical impedance spectroscopy (EIS) can effectively characterize the electron transfer rate between the electrode and solution species, and therefore the modification procedure of the electrode. As shown in Fig. 1A, compared with bare Au electrode (curve a), the electron transfer resistance (R_{et}) of the capture probe-modified electrode is apparently increased (curve b), due to the electrostatic repulsion of the negatively charged capture probe against $Fe(CN)_6^{4-/3-}$. When the electrode is further blocked with 6-MCH, R_{et} is significantly increased (curve c). After the hybridization with the target DNA, R_{et} is further slightly increased (curve d). These results confirm the successful modification of the electrode, which was ready for detection of the target DNA.

The principle of the proposed DNA sensor is based on the formation of numerous G-quadruplex-hemin complexes through a continuous hybridization chain reaction (HCR) between the target DNA and the assistant probe. The electroactive G-quadruplex-hemin complex is then used as output signal tag. In order to test if the continuous HCR occurred and the middle part of the assistant probe folded into G-quadruplex and further combined with hemin, a set of experiments were carried out by employing different combination of the components (0.5 μM target DNA, 0.5 μM assistant probe and 0.5 μM hemin), and thereafter DPV was conducted and the



Scheme 1. Principle of the electrochemical detection of HIV DNA based on G-quadruplex-hemin complex.

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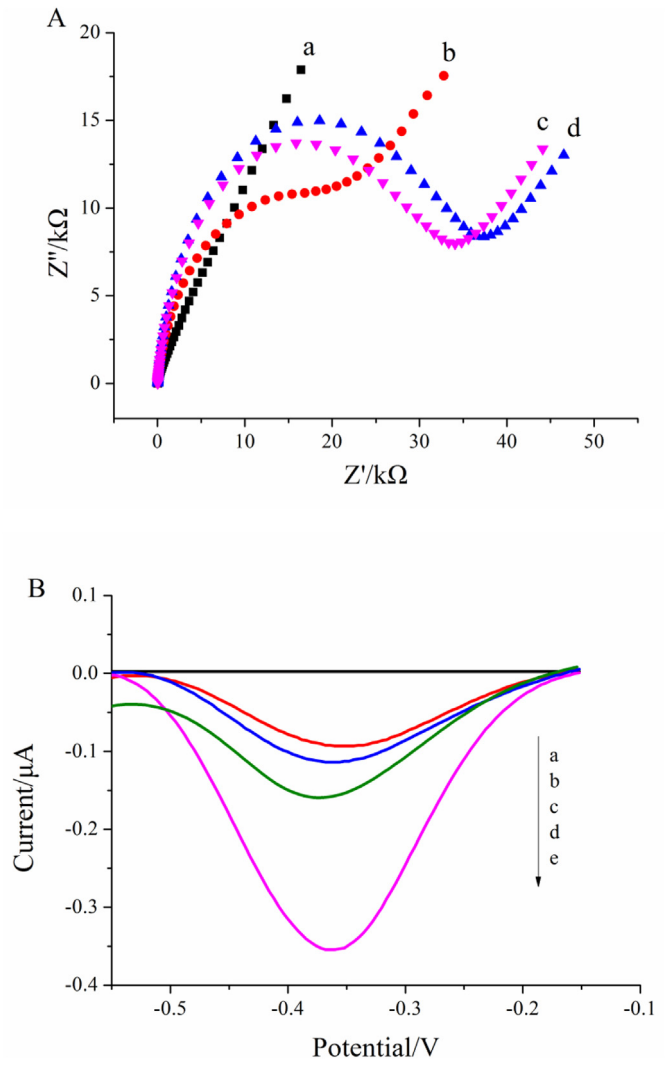

Fig. 1. (A) Characterization of the modification of the electrode by EIS: bare Au electrode (a); capture probe-modified Au electrode (b); 6-MCH-blocked capture probe-modified Au electrode (c); after hybridization with the target DNA (d). (B) DPV curves obtained in the presence of assistant probe and target DNA (a); target DNA and hemin (b); assistant probe and hemin (c); one layer of target DNA, assistant probe and hemin by non-HCR approach (d); assistant probe, target DNA and hemin by HCR approach (e).

electrochemical signals were recorded, as shown in Fig. 1B. In the coexistence of the target DNA, the assistant probe and hemin, a pronounced peak can be observed in DPV curve (curve e), which can be attributed to the formation of G-quadruplex-hemin complex. Therefore, the assistant probe was successfully immobilized onto the electrode surface through the mutual recognition between the target DNA and the assistant probe. In control groups, the absence of either the target DNA (curve c) or the assistant probe (curve b) leads to intensive decrease of peak current in DPV curves. The observable background peak is attributed to the electrochemical response of hemin on the electrode, whose peak current is much lower than those from G-quadruplex-hemin complex. Meanwhile, the DPV curve in the absence of hemin was also recorded (curve a). Although G-quadruplex structure can be formed on the electrode surface in the presence of both the target DNA and the assistant probe, no observable peak can be identified in DPV, indicating G-quadruplex itself cannot produce electrochemical signal without the help of hemin. To verify the signal amplification of HCR, a non-HCR control experiment was carried out through incubation of the capture probe-modified electrode in turn with the target DNA and the assistant probe separately. Thus only one layer of signal-generating stand was introduced on the electrode. The peak current in DPV (curve d) is apparently higher than those of in the absence of the target, the assistant probe or hemin, yet much lower than that of HCR was conducted (curve e). The amplification factor of HCR was estimated to be 8.48, through the comparison of the peak current in curve d with that of curve e in Fig. 1B. Moreover, the relationship between the increment in the electrochemical response and the accumulation of the layers of G-quadruplex-hemin complex on the electrode was further investigated. The scheme of layer-by-layer modification of the electrode with G-quadruplex-hemin complex was described in the Supplementary material and shown in Fig. S1. And the relationship between the increment of the current and the increase of the layers was shown in Fig. S2. Therefore, HCR due to the mutual recognition of the target DNA and the assistant probe can greatly improve the sensitivity of the detection. To sum up, the principle of the DNA sensor is feasible.

3.2. Optimization of the detection conditions

In order to obtain satisfactory performance of the electrochemical DNA biosensor, several key factors, including the concentration of the capture probe, the incubation time of the capture probe and the concentration of the assistant probe were optimized.

The coverage of the capture probe attached to the surface of Au electrode is related with the concentration of the capture probe used to modify the electrode and the incubation time of modification, and has significant influence on the capturing of the target DNA and the assistant probe, and therefore the performance and reproducibility of the detection. The effect of the concentration of the capture probe was firstly investigated. Different concentrations of the capture probe ranging from 0.05 μM to 0.5 μM were used to modify bare Au electrodes respectively, and then the capture probe-modified electrodes were incubated with 0.5 μM target DNA and 0.5 μM assistant probe, followed by incubation with 200 μM hemin. Finally DPV was performed and the peak current in DPV curves was plotted to the concentration of the capture probe used to modify the electrode. As shown in Fig. 2A, the peak current increases with the increased concentration of the capture probe. However, the peak current no longer increases when the concentration reaches 0.3 μM. Therefore, 0.3 μM capture probe was chosen to modify Au electrode.

The incubation time of the capture probe was then investigated. Different incubation time ranging from 2 h to 6 h was used to modify Au electrode at the fixed concentration of the capture probe. Then the capture probe-modified electrodes were incubated with 0.5 μM target DNA and 0.5 μM assistant probe, followed by incubation with 200 μM hemin. As shown in Fig. 2B, the peak current in recorded DPV curves increases with the extended incubation time. As the incubation time reaches 6 h, the increase of peak current levels off, indicating the saturation of the modification. Therefore, the incubation time for modification of the capture probe was fixed at 6 h.

The assistant probe can not only hybridize with the target DNA, but also fold into G-quadruplex structure on the electrode surface, which is associated with the current response. The effect of the concentration of the assistant probe was then investigated by addition of the assistant probe ranging from 0.2 μM to 1.0 μM to the hybridization solution. Then DPV was performed and the peak current in DPV curves was plotted to the concentration of the assistant probe. As shown in Fig. 2C, the peak current continuously increases when the concentration of the assistant probe is lower than 0.8 μM, and then levels off. Thus 0.8 μM is the optimal

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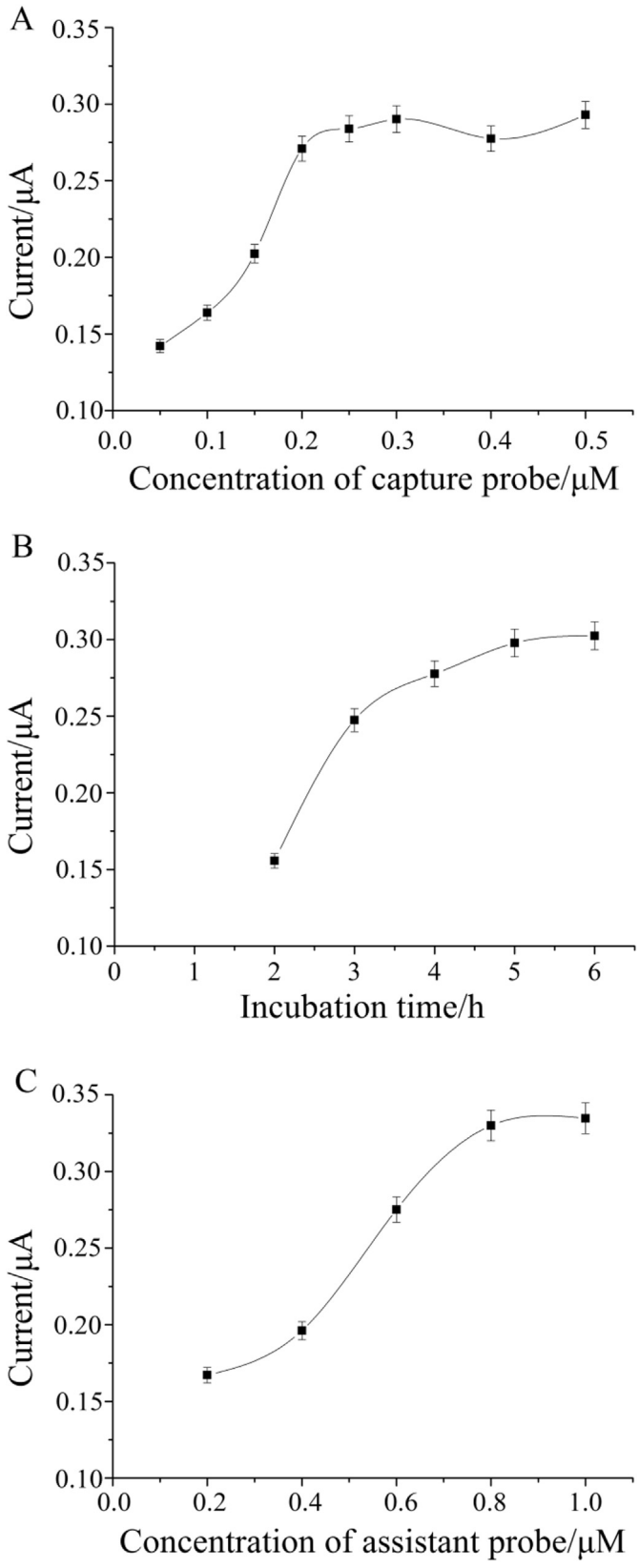

Fig. 2. The influence of (A) concentration of capture probe, (B) incubation time of capture probe, and (C) concentration of assistant probe on the peak current in DPV.

concentration of the assistant probe.

3.3. The performance of the electrochemical DNA biosensor

Under the optimal conditions, the sensitivity and dynamic response range of the electrochemical DNA biosensor were investigated. A series of different concentrations of HIV DNA were mixed and annealed with 0.8 μM assistant probe in hybridization solution. Then the capture probe-modified electrode was immersed into the hybridization solution and transferred into G-quadruplex-forming solution thereafter. After the introduction of hemin, the DPV curves of the electrodes were recorded. As shown in Fig. 3A, the peak current in DPV increases with the increased concentration of HIV DNA in the range from 10 fM to 1 nM. The relationship between the peak current in DPV and the concentration of HIV DNA was studied and shown in Fig. 3B. A linear relationship between the peak current and the logarithm of the concentration of HIV DNA can be derived in the range from 10 fM to 10 pM (Fig. 3B, inset). The linear regression equation is $y = 0.0216 \log C + 0.1900$ ($R^2 = 0.9968$), where C represents the concentration of the target DNA (pM), and y represents the peak current (μA). The detection limit of the

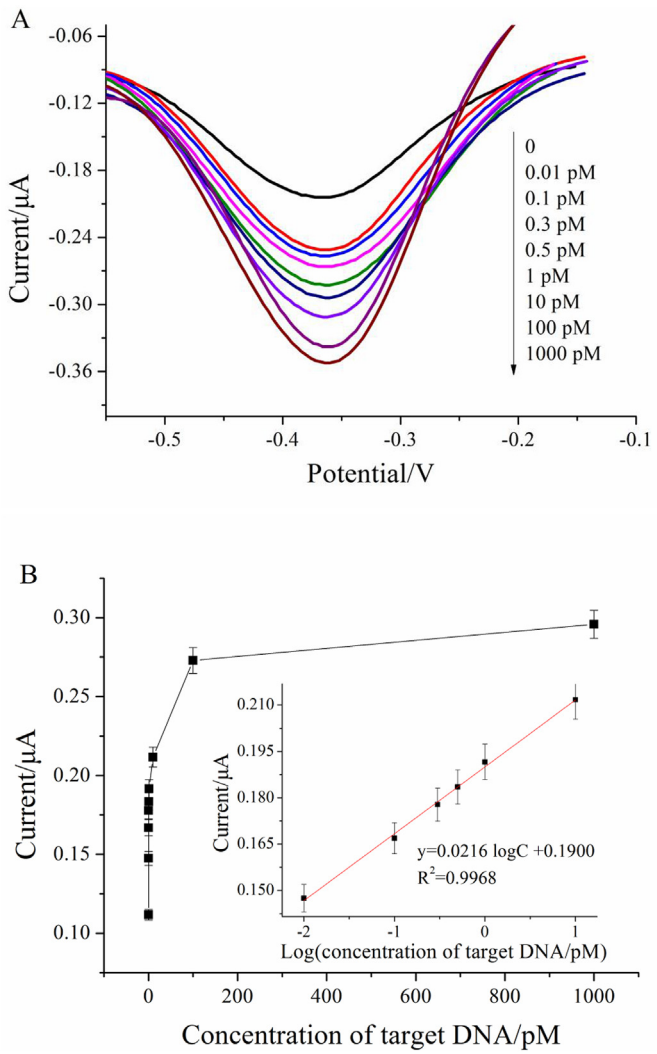

Fig. 3. (A) DPV curves obtained in the presence of different concentration of target DNA; (B) The relationship between the peak current in DPV and the concentration of target DNA; Inset shows the linear relationship between the peak current and the logarithm of the concentration of target DNA.

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Table 1
Comparison of the proposed method with other reported methods for DNA/RNA detection.

Methods	Targets	Detection range	Detection limit	References
Hairpin probes-based HCR fluorescent assay	miRNAs miR-21 and let-7a	0–200 pM	0.18 pM	[33]
Hairpin probes-based HCR MB-assisted DPV	miRNA let-7a	1–800 pM	1 pM	[34]
Hairpin probes-based HCR and RuHex-assisted DPV	BRCA-1	1 aM–10pM	1 aM	[35]
Hairpin probes-based HCR and G-quadruplex/hemin	miRNA-21	100 fM~10 nM	36.2 fM	[36]
HCR and AuNPs-based electrogenerated chemiluminescence	HIV-1	0.02–1.0 pM	5 fM	[37]
Impedimetric assay based on graphene-Nafion composite film	HIV-1	100 fM~1 pM	23 fM	[38]
Exonuclease III-assisted and G-quadruplex/hemin-based assay	HIV DNA	0.01–10 pM	4.83 fM	[39]
DNA tetrahedron and star trigon nanostructures-based DPV assay	HIV DNA	0.01–10 pM	1 fM	[40]
Closed bipolar electrochemistry integrated with electrogenerated chemiluminescence detection	HIV DNA	0.1–300 nM	30 pM	[41]
HCR and G-quadruplex/hemin-based assay	HIV DNA	10 fM~10pM	3.8/6.3 fM	This work

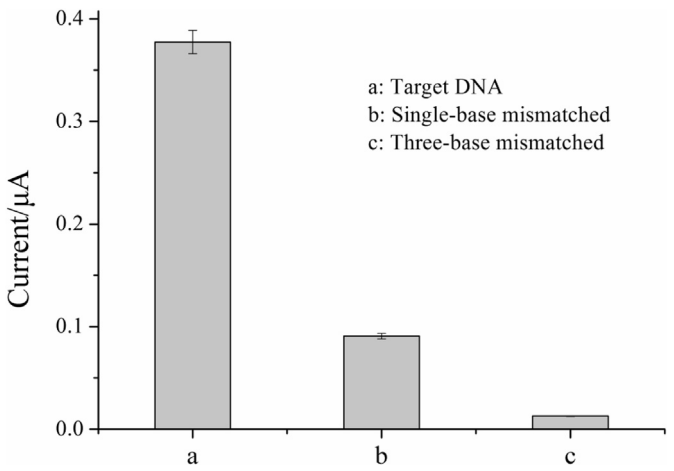

Fig. 4. The histogram of the current response in the presence of target DNA and mismatched sequences.

electrochemical sensor for HIV DNA is estimated to be 3.8 fM (3 × standard deviation of the blank signal/sensitivity). The ultrahigh sensitivity of the proposed sensor can be attributed to the numerous G-quadruplex-hemin complexes which are introduced to the electrode surface based on the mutual recognition of the target DNA and the assistant probe and the continuous HCR. Compared with those reported detection methods for sequence-specific DNA or RNA, including HIV DNA (Table 1), although the detection limit of this work is not the lowest, only one assistant probe is required for the formation of signal structure, which reduces the complexity of the detection system and shortens the operation procedure. Nevertheless, the detection limit at fM level is satisfactory for practice purpose.

The specificity of the DNA sensor reflects the ability to discriminate the target DNA from other sequences with slight differences. Thus the specificity of the constructed DNA biosensor was checked by comparing the current response of the target DNA to those of mismatched sequences, such as single-base and three-base mismatched sequences. As shown in Fig. 4, the peak current of the target sequence is much higher than those of the mismatched sequences. Only 23.6% of the peak current remains for the single-base mismatched sequence, whereas the peak current of the three-base mismatched sequence is negligible. Therefore, the constructed electrochemical DNA biosensor has excellent specificity.

3.4. Detection of the target DNA in serum samples

Serum testing is frequently used to obtain preliminary clinical diagnosis for diseases based on specific biomarkers. In order to evaluate the practicability of the constructed electrochemical DNA biosensor, it was challenged by human serum samples. Different concentration of HIV DNA was added in healthy human serum. Then the serum samples were centrifuged at 10 000 rpm for 3 min, followed by dilution with 10 mM PBS buffer for 5 folds. The determination was carried out under the same conditions as described above.

The sensitivity and dynamic response range of the electrochemical DNA biosensor for serum samples were also investigated.

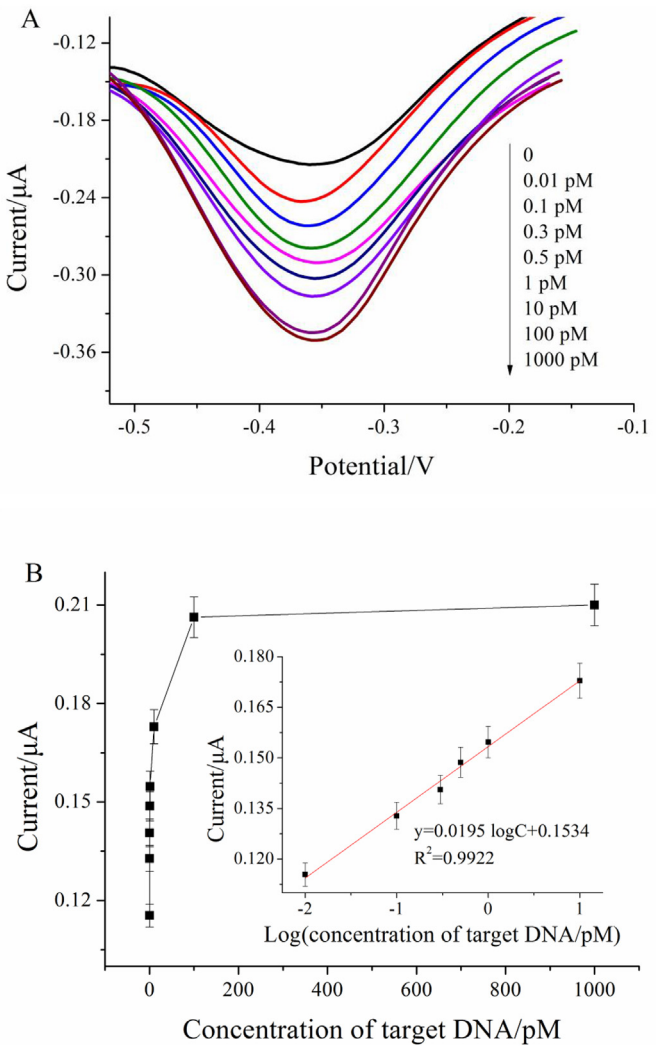

Fig. 5. (A) DPV curves obtained in the presence of different concentration of target DNA in serum samples; (B) The relationship between the peak current in DPV and the concentration of target DNA in serum samples; Inset shows the linear relationship between the peak current and the logarithm of the concentration of target DNA.

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As shown in Fig. 5A, the peak current in DPV increases with the increased concentration of HIV DNA in serum. The relationship between the peak current and the concentration of HIV DNA in serum is shown in Fig. 5B. A linear relationship between the peak current and the logarithm of the concentration of HIV DNA can be derived in the range from 10 fM to 10 pM (Fig. 5B, inset), with the detection limit of 6.3 fM (3 × standard deviation of the blank signal/sensitivity). The linear regression equation is $y = 0.0195 \log C + 0.1534$ ($R^2 = 0.9922$), where y and C have the same meanings as above. The similar calibration curve and detection limit of HIV DNA in serum samples to those of standard DNA samples indicate that serum hardly disturbs the detection of the target DNA.

4. Conclusion

In summary, an ultrasensitive electrochemical DNA biosensor was fabricated to detect sequence-specific DNA. Taking HIV DNA as an example, with rationally designed capture probe and assistant probe, numerous G-quadruplex was introduced to the electrode surface through continuous hybridization chain reaction. Then the electrochemical signal of G-quadruplex-hemin complex was utilized to quantify the concentration of the target DNA. With the amplification effect of HCR, the fabricated DNA biosensor exhibited ultrahigh sensitivity. Meanwhile, the discrimination of single-base and three-base mismatched sequences verified the specificity of the sensor. Moreover, the constructed DNA sensor was further used to detect HIV DNA in serum samples and therefore it may have great application prospect in the fields of early diagnosis of gene-related diseases and environmental monitoring.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.02.076.

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