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A dual signal amplification strategy based on scanning electrochemical microscopy for DNA biosensing using a PEDOT-modified ultramicroelectrode and long self-assembled DNA concatemers

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ABSTRACT

A PEDOT-modified Pt microelectrode was used in SECM for DNA biosensing and combined with long self-assembled DNA concatemers to realize dual signal amplification. The approach curve could also be plotted for tip location using this modified microelectrode with no impact on the catalytic effect so that both signal amplification and tip locating were achieved in one single tip. The detection limit of this method reached 0.076 fM, providing an ultra-sensitive DNA biosensing platform. Furthermore, this strategy could be applied in scanning a DNA chip, showing great potential in high-throughput bioanalysis with high sensitivity.

1. Introduction

The mutation of human genes has been proven to be related to predisposition to different types of diseases. Thus, the detection of specific DNA has become a popular research direction, and many kinds of analytical methods have been applied in this important field of study [1–4]. Over the past three decades, many DNA biosensors have been designed and established based on scanning electrochemical microscopy (SECM) since the invention of this technique by Bard and his coworkers [5–8]. It is known that in traditional electrochemical methods, the element for signal amplification is usually modified on the electrode surface, which might hinder electron transfer between the electrode and electrolytes in solution, thus weakening the effect of signal amplification. However, SECM allows the separation of the signal carrier (i.e., the substrate electrode) and the signal receiver (i.e., the ultramicroelectrode, known as the tip). Therefore, the element for signal amplification on the substrate would not influence electron transfer. SECM has been widely exploited for biosensing, but most of the studies focused on creating amplifying platforms on the surface of the substrate electrode because it is difficult to plot approach curves for the tip location with a modified tip. Therefore, some groups have fabricated parallel dual microelectrodes for signal recording and tip approach [9–11]. However, the manufacturing process and operation are both complicated. Hence, a multifunctional single tip with both signal amplification and tip approaching abilities is needed.

Conducting polymers have been highlighted because of their unique surface structures for probe immobilization and their excellent electrochemical properties for detection. Poly(3,4-ethylenedioxythiophene) (PEDOT), polymerized from 3,4-ethylenedioxythiophene (EDOT), has received much attention for its fascinating conductivity and favorable stability [12–14]. Moreover, this polymer has exhibited a unique catalytic effect towards some redox molecules, such as hydroquinone/benzoquinone redox couples [15]. Besides, PEDOT has been frequently used for the detection of biomolecules, such as ascorbic acid and dopamine, [16–18] and also in SECM-based sensing researches [19–22]. However, there have been no reports on PEDOT-modified tips in SECM for dually catalytic biosensing.

Herein, an ultrasensitive method based on SECM with dual signal amplification for DNA biosensing has been developed using a PEDOT-modified tip and long self-assembled DNA concatemers. Hydroquinone was first oxidized on the substrate, catalysed by horseradish peroxidase (HRP) labelled on DNA concatemers, and its oxidative product, benzoquinone, was then reduced back to hydroquinone by the biased modified tip. This reduction reaction on the tip was catalysed by the modified layer, PEDOT, so that the tip current was amplified twice due to these two catalytic process stated above (Scheme 1). The PEODT-modified tip could also be used to generate approach curves in a redox couple solutions, such as ferrocenyl methanol, without damage. Based on the dual signal amplification, a linear correlation between the signal current and the concentrations of the target DNA was established in the range of 1 fM to 100 fM, and the detection limit was 0.076 fM, as estimated by the 3σ rule. This strategy holds great potential for the

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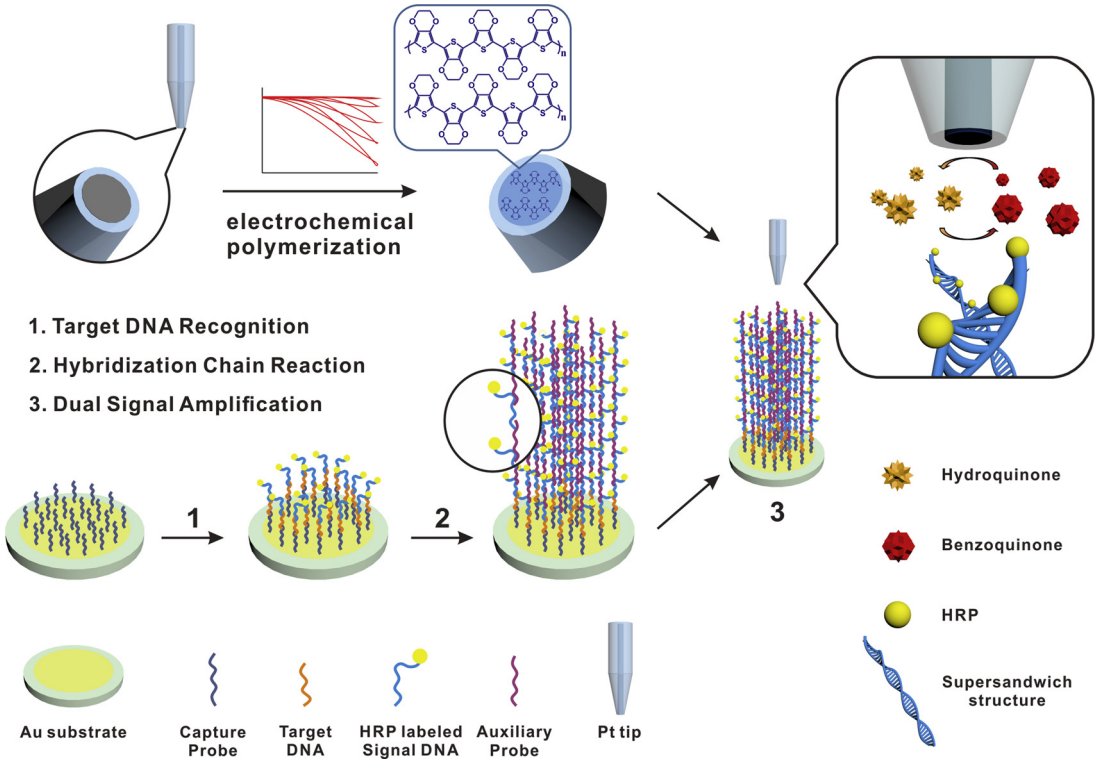

Scheme 1. Schematic illustration of SECM detection with dual signal amplification.

detection of other biomolecules and high-throughput scanning of biomolecular arrays.

2. Material and methods

2.1. Reagents and materials

The HPLC-purified oligonucleotides used in this work were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and are listed in Table S1. Bovine serum albumin (BSA) was also supplied by Sangon Biotech Co., Ltd. (Shanghai, China). 3,4-Ethylenedioxythiophene (EDOT), benzoquinone (BQ), hydroquinone (H_2Q), acetonitrile, 98% sulfuric acid (H_2SO_4) and 30% hydrogen peroxide (H_2O_2) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). (3-Aminopropyl)trimethoxysilane (APTM) was supplied by Sigma-Aldrich (Shanghai, China). Ferrocene methanol (FcMeOH) was obtained from Tokyo Chemical Industry (TCI), and streptavidin-labelled horseradish peroxidase (HRP) was provided by Beyotime Institute of Biotechnology (Shanghai, China). Phosphate-buffered saline (PBS, 0.1 M, pH 7.4) was prepared using Na_2HPO_4, NaH_2PO_4 and KCl. Water-based solutions were prepared with ultrapure water from a Millipore Milli-Q water purification system.

2.2. Electrochemical polymerization process and characterization of the PEDOT-modified ultramicroelectrode

All electrochemical experiments were performed with a CHI 660C electrochemical workstation (CH Instruments Co., Shanghai, China). Before electrochemical polymerization, a Pt ultramicroelectrode was first activated by 20 cycles of cyclic voltammetry scanning from −0.25 V to 1.20 V vs. Ag/AgCl (saturated KCl) in 0.5 M H_2SO_4 at 50 mV/s. Then, another 20 cycles between -1.5 V and 0.2 V vs Ag/AgCl were performed on the ultramicroelectrode in an acetonitrile solution to decrease the amount of water molecules adsorbed on the Pt surface, which could inhibit the formation of PEDOT films by reacting with the cation radicals, $EDOT^+$, produced during electropolymerization with the application of a high positive potential [23,24]. Afterwards, the electropolymerization proceeded in acetonitrile containing EDOT and $NaClO_4$ with a typical three-electrode configuration: a 10 μm Pt ultramicroelectrode as the working electrode, a Ag/AgCl (saturated KCl) electrode as the reference electrode, and a Pt wire as the counter electrode. This electropolymerization process was realized with cyclic voltammetry scanning in the range of 0.8–1.4 V because the oxidation potential of EDOT is 1.2 V. To achieve the best catalytic effect of PEDOT towards benzoquinone, the concentration of EDOT, the number of cycles, the sweep rate in cyclic voltammetry for electropolymerization, and the existence of the initiator $NaClO_4$ were optimized by investigating the catalytic effect in 0.1 M PBS (pH = 7.4) containing 1 mM benzoquinone (BQ) via cyclic voltammetry in the range of -0.2 V to 0.3 V. Scanning electron microscopy (SEM) was used for the characterization of electropolymerization on a Pt ultramicroelectrode. This modified tip could be stored at room temperature within a few days.

2.3. Self-assembly of long DNA concatemers on the SECM substrate

According to the previous work of our group, [25] the capture probe (CP) was first immobilized on the gold substrate by immersing the activated gold substrate into PBS (pH = 7.4) containing 1 μM CP for 12 h. After rinsing with PBS to remove the unbound CP, the modified substrate was dipped into PBS (pH = 7.4) containing the target DNA (TD) at different concentrations for 1 h, followed by hybridization with 1 μM signal DNA (SD) for 1 h. Then, the long DNA concatemers were self-assembled by the immersion of modified substrate in PBS containing 2 μM SD and 2 μM auxiliary probe (AP) for 5 h with the subsequent attachment of HRP through specific binding of streptavidin-biotin. All procedures were performed at room temperature.

2.4. SECM measurement using the PEDOT-modified ultramicroelectrode

SECM measurements were performed using a CHI 920C scanning electrochemical microscope (CH Instruments, Austin, TX, USA). Z-approach curves were obtained in 0.1 M PBS (pH = 7.4) containing 1 mM

H_2Q and 1 mM H_2O_2 on the long DNA concatemer self-assembled substrate with a three-electrode configuration: the unmodified or modified ultramicroelectrode as the working electrode, a Ag/AgCl electrode as the reference electrode and a Pt wire as the counter electrode. To determine the distance between the tip and substrate, approach curves were recorded using the unmodified and modified ultramicroelectrodes in a 1 mM FcMeOH water-based solution containing 0.1 M KCl before the SECM measurement. All detections were performed at room temperature.

2.5. SECM imaging of DNA biochips

A simple DNA biochip was fabricated according to the reported steps [26]. In brief, a slide treated with piranha solution was modified by an aldehyde group from APTMS, followed by the self-assembly of long DNA concatemers. The tip potential was held at -0.15 V with a 10 μm distance between the tip and the substrate, as determined from the approach curves for SECM imaging. All procedures were performed at room temperature.

3. Results and discussion

3.1. Characterization of polymerization process and modified tip

The polymerization process of PEDOT from EDOT is illustrated in Scheme S1. The polymerization of 3.4-ethylenedioxythiophene (EDOT) was achieved on a 10 μm Pt ultramicroelectrode by cyclic voltammetry (Fig. 1A). The monomer, EDOT, was oxidized above 1.2 V and then polymerized to form PEDOT (Scheme S1) in the presence of the initiator $NaClO_4$, which also served as the supporting electrolyte. The anodic current increased from cycle to cycle due to the growth of the conductive PEDOT film, which confirmed the formation of the polymer and its conductivity. In addition, the active surface of the tip increased as the polymerization process proceeded, resulting in an increased current. On the other hand, the oxidation potential became more negative, mainly because the generated oligomers could be oxidized more easily than the monomer [27].

The PEDOT-modified tip was then used to record cyclic voltammograms of 1 mM benzoquinone (BQ) in 0.1 M PBS (pH = 7.4) (Fig. 1B, red curve). Compared to the response obtained by the unmodified Pt tip (Fig. 1B, black curve), an obvious shift to a more positive potential (nearly 150 mV) occurred, and the reductive diffusion current was enhanced, demonstrating the catalytic effect of PEDOT towards BQ. This effect may be caused by a π-π interaction and the formation of a donor/acceptor complex between BQ and PEDOT [15]. The former could promote the electron transfer reaction, and the latter could increase the rate of the reaction, in which the oxidized PEDOT behaved as a Lewis acid, and the electron pair was provided by the oxygen atom in BQ.

3.2. Optimization of tip-modified condition and the stability of the modified tip

To achieve the best performance of the PEDOT-modified tip, the polymerization conditions were optimized. EDOT (1.5 μM) in acetonitrile and 3 cycles of cyclic voltammetry scanning were determined to be the optimized conditions because a higher concentration of EDOT and more cycles could generate more PEDOT, which would wrap the cusp of the tip, including the glass sealed area, and lead to a non-disc-like tip surface and further violation of diffusion dynamics. The SEM images (Fig. S2A and B) has shown that the PEDOT deposit protruded a little (4–5 μm) above the tip (Fig. S2D), but it did not wrap its glass-sealed area under the optimized conditions. Also, the red "S-shape" curve in Fig. 1B (similar curves were also exhibited in Figs. 2B and 3 B, corresponding to FcMeOH) verified that the diffusion dynamics of BQ's reduction was not influenced. Furthermore, a contrast experiment with and without sodium perchlorate ($NaClO_4$), the initiator and supporting electrolyte, was performed, and the results (Fig. S1A, C and D) showed that the response of the modified tip without $NaClO_4$ (blue curve) had nearly no difference compared with that of the unmodified tip (black curve). However, an excellent catalytic effect emerged with the participation of $NaClO_4$ (red curve) and demonstrated the necessity of $NaClO_4$ during the electropolymerization process. The sweep rates during the electropolymerization process were also considered. As shown in Fig. S1B, the catalytic effect of the modified tip using a sweep rate of 10 mV/s is superior to that using a sweep rate of 50 mV/s, mainly because slower sweeping in CV polymerization with the same number of cycles could produce a more porous polymerized layer on the Pt surface, which was displayed in the SEM image of Fig. S2, thus improving the catalytic effect. In summary, the optimized polymerization was achieved in 1.5 μM EDOT in acetonitrile containing 0.1 M $NaClO_4$ with 3 cycles of cyclic voltammetry at a sweep rate of 10 mV/s.

The stability of the modified tip was evaluated by cyclic voltammetry scanning for 100 cycles in 0.1 M PBS (pH = 7.4) containing 1 mM BQ (Fig. 2A). Obviously, the modified tip presents almost no decrease in the reductive diffusion current after 100 cycles, indicating excellent stability, probably because PEDOT is very stable in aqueous electrolytes [28] and cannot fall off the Pt surface after a long electrochemical process. However, after only 10 cycles of cyclic voltammetry scanning, the diffusion current of the unmodified tip decreased (Fig. 2B), mainly due to the decreased active area of the easily absorbed Pt surface by the absorption of species in solution, such as dissolved oxygen.

3.3. Feedback mode achieved by the modified tip

The modified tip was further exploited in the feedback mode of SECM over an insulating spot on the good substrate. Fig. 3A exhibits two approach curves in 0.1 M KCl containing 1 mM FcMeOH with the modified tip (red curve) and unmodified tip (blue curve), in which the distance between tip and substrate was calculated using the theoretical

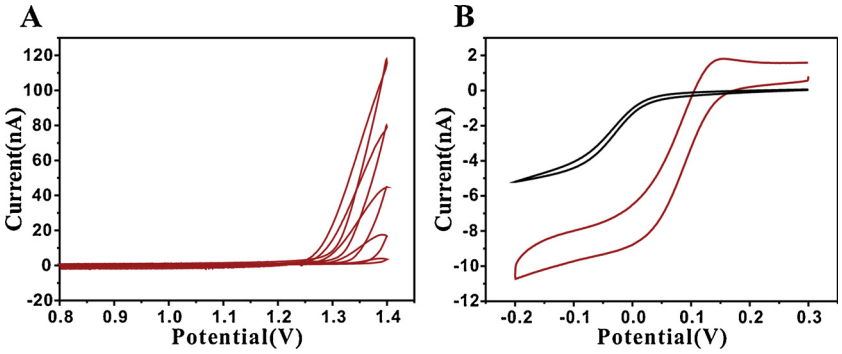

Fig. 1. A. Cyclic voltammograms of polymerization at a 10 μm Pt ultramicroelectrode in an acetonitrile solution containing 1.5 mM EDOT and 0.1 M $NaClO_4$. **B.** Cyclic voltammograms of a Pt ultramicroelectrode before (black) and after (red) electropolymerization in response to 1 mM benzoquinone in 0.1 M PBS (pH = 7.4) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

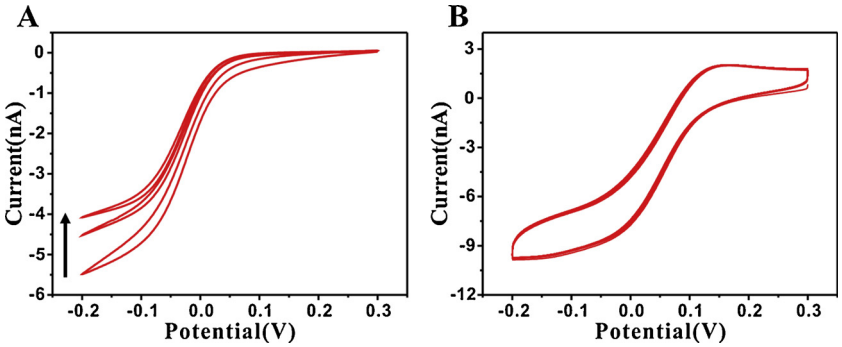

Fig. 2. A. Ten cycles of cyclic voltammetry scanning obtained with a Pt ultramicroelectrode in 0.1 M PBS (pH = 7.4) containing 1 mM benzoquinone. From top to bottom: first cycle, fourth cycle and tenth cycle. **B.** One hundred cycles of cyclic voltammetry scanning obtained with PEDOT-modified Pt ultramicroelectrode in 0.1 M PBS (pH = 7.4) containing 1 mM benzoquinone.

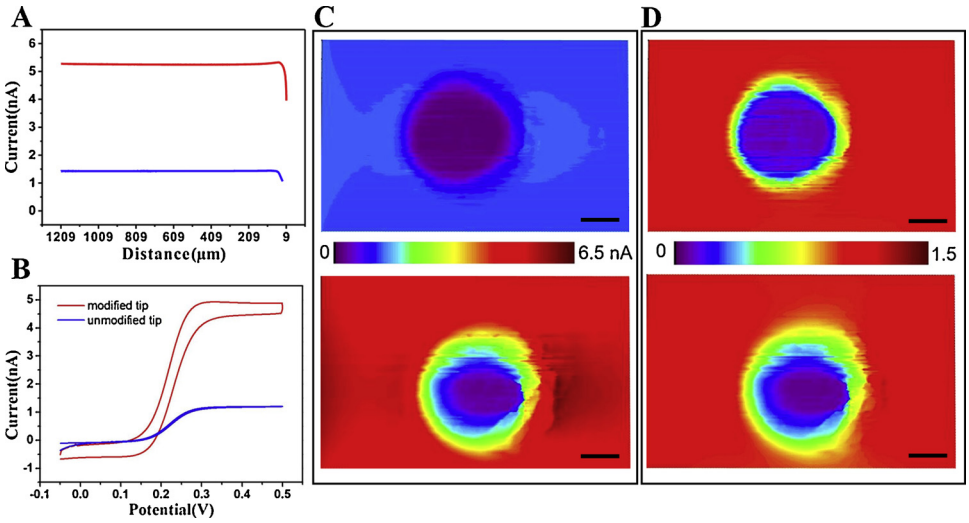

Fig. 3. A. Approach curves in 0.1 M KCl containing 1 mM FcMeOH achieved by 10 μm Pt ultramicroelectrodes with (red) and without (blue) PEDOT modification. **B.** Cyclic voltammetry of a Pt ultramicroelectrode with (red) and without (blue) PEDOT modification. **C.** SECM images of an insulting spot on gold substrate, presenting the absolute values of current. **D.** SECM images of an insulting spot on gold substrate, presenting the normalized current. The scale bar: 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

formula stated by Bard and his co-workers [29]. Obviously, a negative feedback curve was shown using the unmodified tip due to the insulating properties of the spot. While, a tiny positive feedback was presented when the modified tip was used. This is because the approach curves were recorded at the insulting part near the conductive part of the gold substrate and a little FcMeOH reduced by the bipolar gold substrate might be oxidized again on the protruding conductive part of the modified tip, since the effect of hindering the diffusion of electrolyte (FcMeOH) to the tip by the sealed glass was weakened by the protruding conductive deposit (Fig. S2D). Besides, the current of the modified tip has a much higher intensity than that of the unmodified tip because the protruding layer increases the effective radius of the tip. The cyclic voltammetric curves of 1 mM FcMeOH obtained by these two tips (Fig. 3B) also exhibit the difference of current intensity.

The SECM imaging towards an insulting spot on gold substrate was further performed. Fig. 3C (origin SECM data is showed in Fig. S3) exhibits the positive and negative feedback images using the Pt tip (upper) and the PEDOT-modified tip (below). It could be observed that the conductive and insulting parts were distinguished clearly in both two graphs, demonstrating the feasibility to apply this modified tip for imaging in feedback mode. However, the current obtained by the modified tip is definitely larger than the unmodified one. After the normalization to the diffusion current, these two graphs exhibit almost the same signal intensity (Fig. 3D, 3D graph in Fig. S4), indicating that the PEDOT layer had no catalytic effect on FcMeOH, which was also verified by Fig. 3B, since no positive or negative shift of half-wave potential occurred according to the comparison between those two cyclic voltammetric curves. Consequently, this modified tip could be used in feedback mode of SECM and the porous PEDOT layer could enhance the current intensity but had no catalytic effect on FcMeOH. Moreover, the application of this modified tip in feedback mode could benefit both signal acquisition and distance verification, avoiding the complex fabrication steps of a dual-electrode system but realizing signal amplification.

3.4. Dual-catalytic signal amplification

Another signal amplification element in this strategy is the long DNA concatemers self-assembled on the substrate in SECM, and these concatemers linked numerous HRPs to catalyse the oxidation of H_2Q to BQ [25]. The generated BQ would be reduced by the tip, displaying an obvious substrate generating-tip collecting mode approach curve (Fig. 4A). The tip was held at -0.15 V, which was a positive shift from -0.4 V in our previous work [25,30] due to the catalytic effect of PEDOT towards the reduction of BQ, and positioned at 500 μm above the fabricated substrate, followed by the approach of the tip to the substrate. In the presence of 1 μM target DNA, the long concatemers on the substrate result in a nearly 4-fold enhancement of the current detected by the unmodified tip (blue curve, single signal amplification) compared with that detected by the tip modified with a traditional sandwich structure formed by the self-assembly of a capture probe (CP), target DNA (TD) and HRP-labelled signal DNA (SD) (black curve, no signal amplification). This enhancement is due to the increased amount of HRP associated with the long DNA-concatemer structure, which improves the catalytic effect. With further signal amplification by the PEDOT-modified tip (red curve, dual signal amplification), the current is 15 times larger than that for the non-amplified signal and 3.5 times larger than that with single signal amplification. In the absence of TD (Fig. 4B), the long DNA concatemers could not be immobilized on the substrate, so there was no HRP to catalyse the oxidation of H_2Q; thus, no signal current was detected. Therefore, the combination of the modified tip with long DNA concatemers could amplify the signal

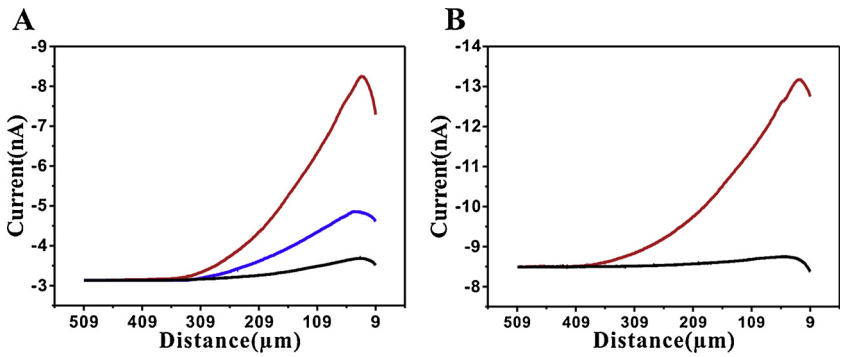

Fig. 4. A. Approach curves obtained by the unmodified tip on a traditional sandwich-modified substrate **(black** curve), the long DNA concatemer-modified substrate (blue curve) and the PEDOT-modified tip on the long DNA concatemer-modified substrate (red curve) in 0.1 M PBS (pH = 7.4) containing 1 mM H_2Q and 1 mM H_2O_2. **B.** Approach curves of the DNA biosensing platform based on dual signal amplification with (red) and without **(black)** 1 μM target DNA in 0.1 M PBS (pH = 7.4) containing 1 mM H_2Q and 1 mM H_2O_2 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

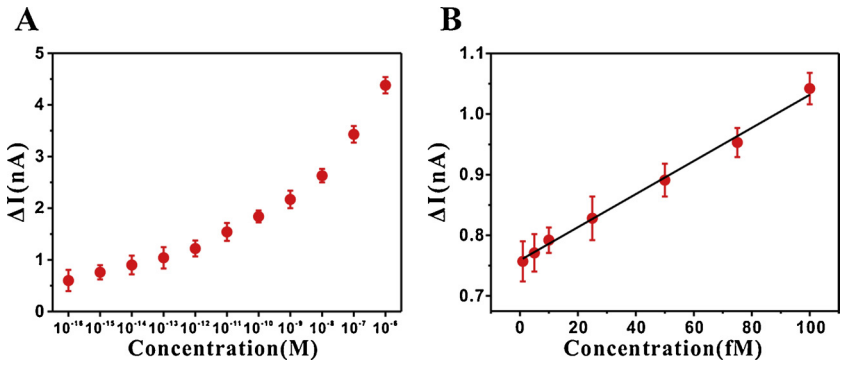

Fig. 5. A. Signal current (Δ*I*) values varying with the concentrations of target DNA from 0.1 fM to 1 μM. **B.** Linear correlation between the signal current and target DNA concentrations from 1 fM to 100 fM. Error bars represent the standard deviation of three experiments.

Table 1
Comparison of detection limit between this work and others.

Methods of amplification	Detection limit	Reference
Hairpin probe and enzymatic amplification using SECM	17 pM	[31]
Structure-aid DNA walker with enzymatic recycling	1 fM	[32]
Nanocatalyze using DNA-conjugated Au nanoparticles	1 fM	[33]
Exonuclease III-based and gold nanoparticle-assisted	33 pM	[34]
PEDOT-based catalyze and long self-assembled DNA concatemers using SECM	0.076fM	This work

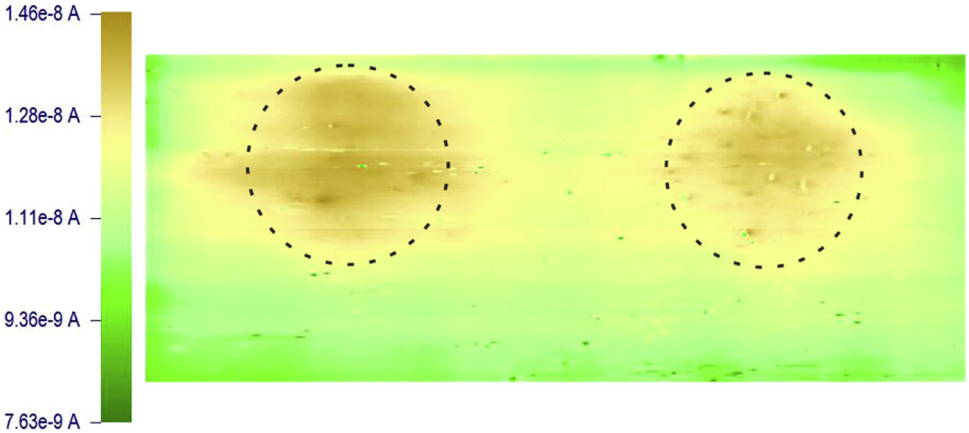

Fig. 6. Imaging of the DNA biochip in the presence of 1 nM target DNA using the PEDOT-modified tip. The area of the fabricated DNA spot is marked by the black dotted circle.

effectively, enhancing the sensitivity of determination. It should be stated that other mediators, such as ferrocene derivatives or 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), can also act as electron shuttle for HRP, but the PEDOT-modified tip has no catalytic effect on their redox reactions, resulting in the loss of the meaning to modify the tip. Therefore, benzoquinone, which could be catalytically reduced by PEDOT-modified tip, was used as the mediator in this work

3.5. Quantitative measurement of target DNA

The analytical performance of this DNA biosensing platform was investigated by the approach curves obtained with target DNA at

different concentrations in 0.1 M PBS (pH = 7.4) containing 1 mM H_2Q and 1 mM H_2O_2. As shown in Fig. S3, the positive feedback current increases with increasing TD concentration, which is associated with the amount of HRP linked to the long DNA concatemers. To more accurately demonstrate the relationship between the current and the concentration of TD, the signal current (ΔI) was defined as the difference between the peak values of the positive feedback current and the steady current. It is clear that the increased concentration of TD enhances the signal value in the range from 0.1 fM to 1 μM (Fig. 5A, 3D graph was shown in Fig. S5). Furthermore, a linear correlation could be established between the signal current values and the concentrations of TD ranging from 1 fM to 100 fM (Fig. 5B). The deduced regression equation is $\Delta I = 0.0027c_{TD} + 0.7587$ ($R = 0.9969$), where c_{TD} is the concentration of TD. The detection limit is 0.076 fM, as estimated by the 3σ rule, where σ is the standard deviation of the signal in the blank solution, displaying a more excellent sensitivity than most existing DNA biosensing platforms as shown in Table 1 [31–34].

3.6. SECM imaging of DNA biochips

The PEDOT-modified tip was further exploited for the simultaneous scanning of multiple spots on a biochip. For demonstration, a simple DNA biochip with two spots was fabricated as reported previously [26]. After positioning the modified tip at 10 μm above the biochip, obvious positive feedback was obtained for both spots (Fig. 6), indicating that this modified tip has great potential in high-throughput bioanalysis.

4. Conclusions

In conclusion, a dual signal amplification strategy based on SECM has been developed for DNA detection using a PEDOT-modified Pt ultramicroelectrode and long self-assembled DNA concatemers. Under the optimal conditions of polymerization, a PEDOT film was modified on the Pt tip, and a good catalytic effect towards the reduction of benzoquinone was realized. The detection limit of this method could reach 0.076 fM, providing better sensitivity than that achieved with most DNA biosensing strategies. Additionally, approach curves for verifying the tip position could be generated with this modified tip. Furthermore, this method could be applied in scanning a DNA chip, showing great potential for use in high-throughput bioanalysis with high sensitivity.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2019.03.128.

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