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An ultrasensitive scanning electrochemical microscopy (SECM)-based DNA biosensing platform amplified with the long self-assembled DNA concatemers

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ABSTRACT

The long DNA concatemers have been well developed to fabricate various biosensing platforms for the signal amplification. Herein, this signal amplification strategy was firstly used for an ultrasensitive scanning electrochemical microscopy (SECM)-based DNA biosensing platform. This platform was constructed through the hybridization of target DNA (TD) with thiol-tethered DNA capture probes (CP), immobilized on the gold substrate surface, and biotinylated DNA signal probes (SP), which formed then the long DNA concatemers through the continuous self-assembly with alternating DNA auxiliary probes (AP). The streptavidin-horseradish peroxidase (HRP) was linked to the long DNA concatemers through biotin-streptavidin interaction. In the HRP-catalyzed reaction, hydroquinone (H_2Q) was oxidized to benzoquinone (BQ) with H_2O_2 at the modified substrate surface where sequence-specific hybridization had occurred, and the BQ generated could be monitored by a SECM tip. This platform exhibited a low detection limit of 0.18 aM estimated by the 3σ rule. Combined with DNA microarrays, four kinds of TDs (100 fM) as the models were detected simultaneously by using this proposed strategy, which also demonstrated sufficient selectivity to distinguish specific DNA sequences and good reproducibility. This method opens a promising direction to improve the SECM sensitivity for high-throughput DNA detection.

1. Introduction

Detection toward specific DNA sequences is greatly important in many research fields, such as virus detection, drug development, clinical treatment and early diagnosis of genetic diseases [1–4]. Most recently, the interest to the development of high-throughput DNA biosensing platforms based on DNA microarrays for a large number of genes to be studied simultaneously has dramatically increased [5–12]. Most of these methods rely on fluorescence, raman scattering spectroscopy and chemiluminescence. Compared to these optical assays, electrochemical detection methods are particularly attractive with the remarkable advantages, such as high sensitivity, rapid response time, low cost and the possibility of miniaturization. However, it is difficult for conventional electrochemical DNA biosensing platforms to achieve the high-throughput DNA analysis, because the working electrode is not only used as DNA probes supporter, but also as electrochemical signal reporter.

Scanning electrochemical microscopy (SECM) is expected to overcome this difficulty, as the signal species are monitored by the Pt microelectrode rather than the modified substrate, realizing the separation of supporter and inceptor. In other words, the conductivity of the SECM substrate becomes non-essential, so that the insulating substrates, such as glass slides, can be also employed as supporters for the DNA probes immobilization [13–17]. Recently, the SECM imaging of DNA microarrays for high-throughput DNA detection has been demonstrated by various researches [18–23]. However, the sensitivity of present detection methods is still limited. Till now, different signal amplification strategies have been developed to improve the sensitivity of SECM-based DNA detection. Wain and Zhou obtained an enhanced tip current by the regeneration of ferrocyanide, used as the SECM mediator, to image hybridized DNA, combined with methylene blue as a redox-active intercalator [24]. Palchetti et al. used alkaline phosphatase to biocatalyze precipitation of insoluble and insulating products, changing the conductivity of the substrate surface where specific DNA sequences hybridization had occurred, which could be sensitively detected by the SECM tip [25]. Yu and co-workers reported a novel scheme using SECM for DNA imaging based on streptavidin-horseradish peroxidase (HRP) as the signal

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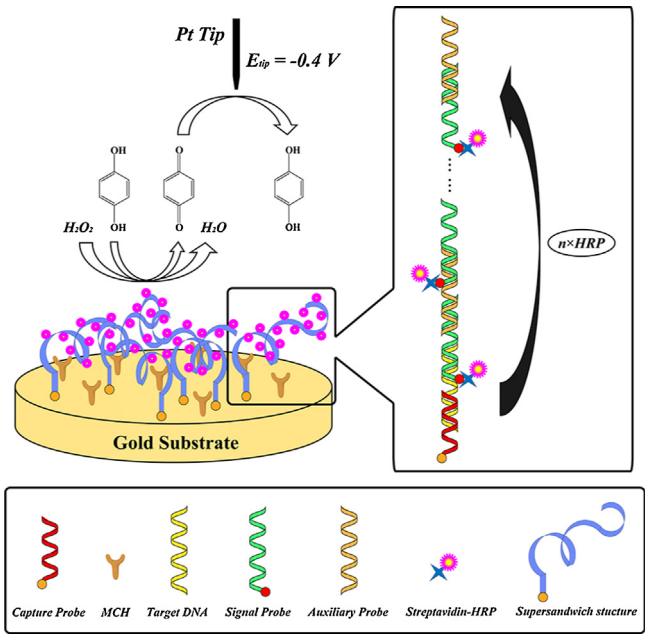
amplification element [26]. Our group achieved the qualitative and quantitative DNA detection by a "sandwich" DNA biosensing platform amplified with HRP-wrapped SiO_2 nanoparticles [27].

In recent years, there have been ever-growing interests in the development of ultrasensitive electrochemical DNA biosensing platforms amplified with the supersandwich DNA structure, which is based on the long self-assembled DNA concatemers with a large number of electrochemically active molecules [28–32]. The amplified signal by the long DNA concatemers realizes a significantly higher gain than traditional sandwich assay. In addition, long DNA comcatamers-based signal amplification does not need the multiple steps required by most amplification assays, such as those based on nanoparticles, quantum dots, supramolecular polymerization. However, these conventional electrochemical DNA biosensing platforms are all structured on the electrode surface. As a result, the efficiency of electron transfer between working electrode and electrochemically active molecules modified on the electrode surface would be greatly diminished caused by the hindrance of the long DNA concatemers, limiting the total signal gain and thus the detection sensitivity. For the SECM-based DNA biosensing platform, there is no need to consider the efficiency of electron transfer mentioned above, since the employment of microelectrode achieved the separation of supporter and inceptor.

Herein, we describe a novel SECM-based DNA biosensing platform amplified with the long self-assembled DNA concatemers in the substrate generation and tip collection (SG/TC) mode of SECM, as shown in Scheme 1. The long DNA concatemers were formed through the continuous self-assembly between alternating biotinylated DNA signal probes (SP) and DNA auxiliary probes (AP). Thus, plenty of biotin molecules would be along with each DNA concatemer. In the presence of target DNA (TD), one terminus of TD could hybridize with the thiol-tethered DNA capture probes (CP) immobilized on the gold substrate surface, and the other terminus could hybridize with SP. When SP and AP were added, the long DNA concatemers could be automatically developed. The streptavidin-HRP could be linked to the long DNA concatemers through streptavidin-biotin interaction. As a result, the TD could trigger the linkage of the long DNA concatemers with a great deal of HRP to the gold substrate to produce a significant amplified electrochemical signal. In the SECM detection, z-approach curves were employed for TD detection. Hydroquinone (H_2Q) was oxidized to benzoquinone (BQ) by the catalysis of HRP in the presence of H_2O_2 at the modified substrate surface where sequence-specific hybridization had occurred, and the generated BQ corresponding to the amount of TD was monitored by a Pt tip. The resulting DNA biosensing platform exhibited a low detection limit of 0.18 aM estimated by the 3σ rule. This method holds great potential for improving the SECM sensitivity of high-throughput DNA detection.



Scheme 1. Schematic of the fabrication and SECM detection of the proposed DNA biosensing platform.

2. Experimental

2.1. Reagents and materials

The HPLC-purified oligonucleotides used in the present study were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and used as received. The sequences of oligonucleotides were listed in the Supplementary data (Table S1). Tris(hydroxymethyl) aminomethane (Tris), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 6-mercapto-1-hexanol (MCH) and bovine serum albumin (BSA) were also supplied by Sangon Biotech Co., Ltd. (Shanghai, China). Ferrocenemethanol (FMA), hydroquinone (H_2Q) and Tween-20 were purchased from Sigma–Aldrich (Shanghai, China). Streptavidin-HRP was obtained from Beyotime Institute of Biotechnology (Shanghai, China). The DNA microarrays were supported by Shanghai Biotechnology Co., Ltd. (Shanghai, China), which was detailed in the Supplementary data, and used as received. Other chemicals employed were all of analytical grade and provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All of the solutions were prepared with ulrapure water from a Millipore Milli-Q water purification system with the resistivity of 18.2 MΩ cm.

2.2. Immobilization of CP on the gold substrate surface

The gold substrate (2 mm diameter) was polished with alumina powder (0.05 μm) to obtain a mirror surface followed by ultrasonic cleaning with water, ethanol and water for 5 min respectively. The substrate surface was then electrochemically cleaned in 0.5 M H_2SO_4 solution within a potential window between −0.2 and +1.5 V at a scan rate of 100 mV s^{-1} to remove any remaining impurities. The immobilization of CP on the treated substrate surface was performed in I-buffer (10 mM Tris-HCl, 500 mM NaCl and 10 μM TCEP, pH 7.4) containing 1 μM CP for 10 h at room temperature. After rinsed with washing buffer (10 mM Tris-HCl, 500 mM NaCl, 1 mM $MgCl_2$ and 1 vol% Tween-20, pH 7.4) to remove the unbound CP, the modified substrate surface was passivated with MCH solution (10 mM Tris–HCl, 500 mM NaCl, 1 mM $MgCl_2$ and 2 mM MCH, pH 7.4) for 1 h at room temperature to remove the nonspecific DNA adsorption and optimize the orientation of the CP to make later hybridization easier. Finally, the resulting substrate was rinsed and ready for the next step.

2.3. Fabrication of the proposed DNA biosensing platform

The CP-modified substrate surface was dipped into H-buffer (10 mM Tris–HCl, 500 mM NaCl and 1 mM $MgCl_2$, pH 7.4) containing TD at different concentrations for 1 h at room temperature. After rinsed, the target-recognized substrate was submerged into H-buffer containing 1 μM SP for 1 h at room temperature. Afterward, the substrate was immersed in H-buffer containing 2 μM SP and 2 μM AP for 5 h at room temperature. When the self-assembly of the long DNA concatemers finished, the assembled substrate was again thoroughly rinsed to remove any nonspecifically adsorbed sequences and blocked with BSA solution

(10 mM Tris–HCl, 500 mM NaCl, 1 mM $MgCl_2$ and 1 wt% BSA, pH 7.4) for 1 h at room temperature. To attach the HRP, the above substrate was incubated in streptavidin-HRP solution (1:100 dilution from the stock solution using H-buffer) for 0.5 h at room temperature. Prior to use, the fabricated substrate was rinsed.

2.4. SECM measurement

SECM measurement was performed with a CHI 920C scanning electrochemical microscope (CH Instruments, Austin, TX, USA). The SECM set-up consists of a piezo positioner, a controller which can move the tip in three dimensions, and a bipotentiostat. Z-approach curves and SECM images were obtained in 0.1 M PBS (pH 7.4) containing 1 mM H_2Q, 1 mM H_2O_2 and 0.1% Triton X-100 with a typical three-electrode configuration: the amperometric SECM tip – a Pt microelectrode (25 μm in diameter) as working electrode, a Ag/AgCl (3 M KCl) electrode as reference electrode, and a Pt wire as counter electrode. As previously reported by our group [18], the addition of 0.1% Triton X-100 was used to avoid the passivation of the tip during detection, which could keep the tip clean by preventing the adsorption of generated redox species. The modified gold surface prepared above and fabricated DNA microarray were used as the substrates in z-approach curves and SECM imaging respectively. All the measurement was performed at room temperature.

3. Results and discussion

3.1. Electrochemical characterization of the fabricated DNA biosensing platform by z-approach curves

On the DNA biosensing platform, HRP catalyzed the oxidation of H_2Q to BQ with H_2O_2 at the modified substrate surface and z-approach curves were obtained through detecting the reduction current of BQ generated in HRP-catalyzed reaction by Pt tip held at −0.4 V. The tip current would increase with the tip approaching to the modified substrate surface, since the concentration of generated BQ was the function of tip-substrate distance (d) due to its diffusion from the modified surface to the Pt tip. The tip current arrived at the highest value (i_H) when the tip was in very close proximity of the modified substrate surface. The difference between i_H and the steady-state current was called signal current (i_T). Prior to each z-approach curve, the tip was precisely positioned at the same place, 1500 μm away from the substrate surface, which was achieved using the positive feedback mode as follows: When the tip current was up 25% from the initial current, indicating that the tip was ~0.25 tip radius away from the substrate surface, the tip was stopped, and then retreated 1497 μm. The positive feedback mode was performed in 1 mM FMA solution, with holding the tip potential at +0.45 V for electrochemical oxidation of FMA.

The feasibility of the fabricated DNA biosensing platform was firstly examined and shown in Fig. 1. The CP was attached to the gold substrate by gold-thiol interaction at the beginning of fabricating this DNA biosensing platform and no signal current could be found (Fig. 1A, curve a). In the absence of TD, the long self-assembled DNA concatemers by the added SP and AP in the solution could not link to the modified surface. Consequently, a very small signal current could be observed due to the nonspecific adsorption of SP (Fig. 1A, curve b). While, in the presence of TD, the long DNA concatemers containing numerous biotin-tethered SP molecules were firmly immobilized on the modified surface via TD. Then, a large amount of HRP bound to the long DNA concatemers yielded a magnificently amplified signal current (Fig. 1A, curve d), whereas no signal current was observed when no HRP was linked to the long DNA concatemers (Fig. 1B, curve a). Under the same

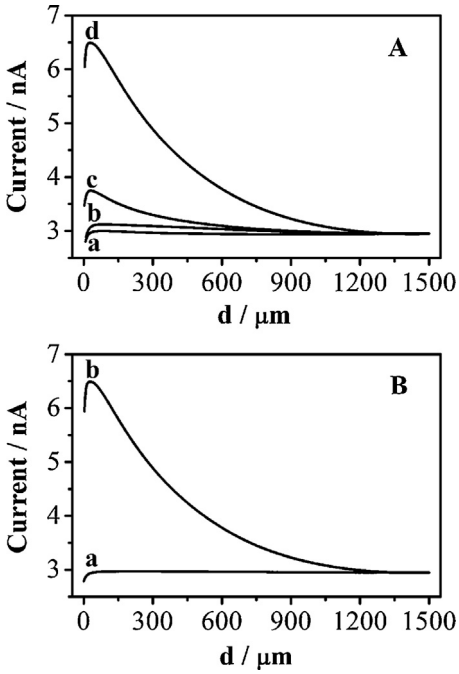


Fig. 1. Z-approach curves of the fabricated DNA biosensing platform. (A) The substrate surface was modified with HRP and different oligonucleotides: (a) CP, (b) CP, SP and AP, (c) CP, TD and SP, (d) CP, TD, SP and AP. (B) CP, TD and long DNA concatemers modified substrate surface was linked with (curve b) and without (curve a) HRP. The concentrations of TD involved in (A) and (B) were 1 nM. E_{tip} = −0.4 V. Electrolyte: 0.1 M PBS (pH 7.4) containing 1 mM H_2Q, 1 mM H_2O_2 and 0.1% Triton X-100. Approach rate: 25 μm s^{-1}.

experimental conditions, the traditional sandwich assay produced a much smaller signal current (Fig. 1A, curve c), because 1:1 ratio of SP to TD limited the amount of HRP linked to the substrate, which contrasted sharply with the supersandwich assay.

3.2. Effects of the long DNA concatemers on the signal amplification

The signal amplification of the proposed DNA biosensing platform was based on the HRP linked to the long DNA concatemers. Accordingly, the longer the self-assembled DNA concatemers was developed, the better the signal amplification effect would be produced. The length of DNA concatemers, we found, was closely related to the self-assembly time and the concentrations of SP and AP. Fig. 2A shows the effect of the self-assembly time of SP (2 μM) and AP (2 μM) from 0 to 8 h on signal current for 1 nM TD. Obviously, the signal current increased rapidly with the self-assembly time in the range of 0–5 h. It suggested that DNA concatemers would be longer with the increased self-assembly time of SP and AP, which was in agreement with the gel-electrophoresis results of the long DNA concatemers (Fig. S1a-d in the Supplementary data). However, the signal current did not further increase as the self-assembly time extended to 8 h. It was due to the slow growth of DNA concatemers, confirmed by the gel-electrophoresis results of the long DNA concatemers (Fig. S1d and e in the Supplementary data). Thus, 5 h was the optimum self-assembly time of SP and AP. The effect of SP and AP concentrations on signal current was also examined, as shown in Fig. 2B. The signal current increased significantly as the concentrations of SP and AP up to 2 μM. However, the signal current almost kept constant when the concentrations of SP and AP higher than 2 μM. Therefore, the optimized concentrations of SP and AP were 2 μM. Under the selected experimental conditions, the formed long self-assembled DNA concatemers produced a ladder of different lengths with the maximum in the range 750 to 1000 base pairs (Fig. S1d in the

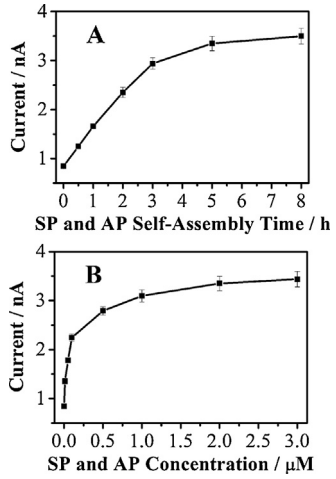


Fig. 2. (A) Effect of the self-assembly time for detection of 1 nM TD. Both SP and AP were of the same concentration (2 μM). The measurements were performed after the self-assembly time of 0, 0.5, 1, 2, 3, 5 and 8 h for long DNA concatemers. (B) Effect of the concentrations of SP and AP for detection of 1 nM TD. The self-assembly time for the long DNA concatemers was 5 h. Both SP and AP were of the same concentration. Different concentrations of SP and AP were used (0, 0.01, 0.05, 0.1, 0.5, 1, 2 and 3 μM). The illustrated error bars represent the standard deviation of three repetitive measurements.

Supplementary data). According to the number of base pairs in one unit, the HRP linked to single self-assembled DNA concatemer was calculated as 23 to 30 at most.

The signal amplification effect of the long DNA concatemers on the fabricated SECM-based DNA biosensing platform was compared with that on conventional electrochemical method, based on the ratio i_T/i_0 (where "i_0" stands for the signal current when the self-assembly time of SP and AP was 0 h). The DPV (differential pulse voltammetry) responses from the conventional electrochemical method was obtained with a potential window of 0.3–0.6 V in 0.1 M PBS (pH 7.4) containing 1 mM H_2Q, 1 mM H_2O_2 and 0.1% Triton X-100. The fabrication process of modified electrode was the same as that of the substrate fabricated for the proposed SECM-based DNA biosensing platform. From Fig. 3, it could be found that the value of i_T/i_0 obtained from the SECM-based DNA biosensing platform increased as the self-assembly time changed from 0 to 5 h, and then did not increase obviously as the self-assembly time extended to 8 h (red curve). While, using conventional electrochemical method, the value of i_T/i_0 increased with smaller rate until the self-assembly time was up to 2 h and then tended to be constant (blue curve). It could be probably explained that the two roles of the working electrode, both as DNA probes supporter and electrochemical signal reporter, would influence each other, so that the long DNA concatemers on the working electrode, lengthening with the increased self-assembly time, reduced the electron transfer efficiency between electrochemically active molecules and working electrode. Thus, the signal amplification effect of the long DNA concatemers on the conventional electrochemical method was limited, even a little decrease occurred after the length of DNA concatemers increasing to a certain extent. While, using SECM, this problem could be avoided.

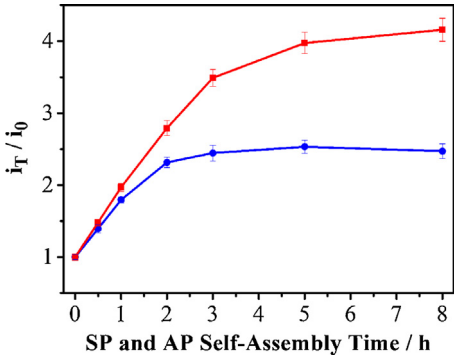


Fig. 3. The values of i_T/i_0, acquired by the z-approach curves and DPV responses were obtained from the SECM-based DNA biosensing platform (red curve) and the conventional electrochemical detection (blue curve) with different self-assembly time of SP and AP. Both of them were performed in 0.1 M PBS (pH 7.4) containing 1 mM H_2Q, 1 mM H_2O_2 and 0.1% Triton X-100. Error bars represent the standard deviation of three experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Analytical performance of the fabricated SECM-based DNA biosensing platform

The sensitivity of the fabricated DNA biosensing platform was investigated by z-approach curves with the SG/TC mode in 0.1 M PBS (pH 7.4) containing 1 mM H_2Q, 1 mM H_2O_2 and 0.1% Triton X-100. The difference between i_H and the steady current was called signal current (i_T), which was related to the TD concentration. As illustrated in Fig. 4A, it is clear that the signal current increased with the addition of TD at different concentrations from 1 aM to 1 nM, associated with the quantity of HRP bound to the long DNA concatemers. Fig. 4B shows that the signal current exhibited a linear correlation to the logarithm of the TD concentration ranging from 1 aM to 1 nM. Regression equation was $I = 0.3229 \lg c_{TD} + 6.3393$ ($R = 0.9955$), where I is the signal current (nA), and c_{TD} is the concentration of target DNA in M. The limit of detection and the limit of quantification for the proposed scheme were 0.18 aM estimated by the 3σ rule and 0.58 aM estimated by the 10σ rule, respectively (where σ is the standard deviation of a blank solution, n = 11). The detection limit was much lower than those of the existing SECM-based DNA biosensing platforms. Furthermore, the fabricated DNA biosensing platform was superior

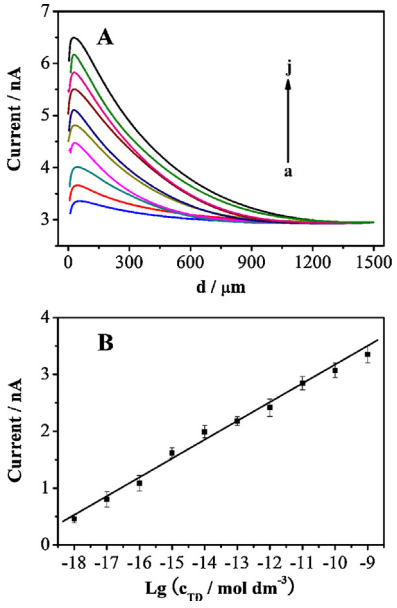


Fig. 4. (A) Z-approach curves on the fabricated DNA biosensing platform toward the detection of TD at different concentrations: (a) 1 aM, (b) 10 aM, (c) 100 aM, (d) 1 fM, (e) 10 fM, (f) 100 fM, (g) 1 pM, (h) 10 pM, (i) 100 pM and (j) 1 nM. $E_{tip} = -0.4$ V. Electrolyte: 0.1 M PBS (pH 7.4) containing 1 mM H_2Q, 1 mM H_2O_2 and 0.1% Triton X-100. Approach rate: 25 μm s^{-1}. (B) The linear plots of signal current vs the logarithm of TD concentration. Error bars represent the standard deviation of three experiments.

on the linear range of detection (Table S2 in the supplementary data).

To test the reproducibility of the method, three substrate electrodes were prepared with the same procedures, and the estimated RSD is 4.4% for the determination of 1 nM TD under the same conditions. The stability of the fabricated DNA biosensing platform was also checked. Three independent experiments demonstrated that this platform could retain about 93.6% of its initial response toward 1 nM TD after its storage in the refrigerator at 4 °C for 7 days, showing a relatively robust stability. We further challenged the detection toward TD spiked in relatively complex biological matrix (1:5 dilution of human serum). Comparable responses were obtained for the detection of TD in both buffer and real samples (Fig. S2 in the Supplementary data), indicating the potential for real analytical application.

3.4. SECM imaging of the DNA microarray

The DNA microarray could allowed a large number of genes to be analyzed simultaneously. In this study, SECM imaging was x,y-scanning in 0.1 M PBS (pH 7.4) containing 1 mM H_2Q, 1 mM H_2O_2 and 0.1% Triton X-100, with the Pt tip biased at −0.4 V. The generated BQ by the oxidation of H_2Q in the HRP-catalyzed reaction with H_2O_2 at each spot on DNA microarray could be detected by the amperometric reduction at the movable Pt tip. Therefore, the Pt tip could produce concentration maps above the fabricated DNA microarray. Before the SECM imaging, a horizontal substrate surface was required to make sure that the tip could scan at a constant height and a stable background would be obtained, which was detailed in the Supplementary data.

As illustrated in Fig. 5A, a DNA microarray was structured by spotting four kinds of DNA capture probes (CP1-4) on the glass substrate in one row. When the DNA microarray hybridized with four different TDs (TD1-4, 100 fM) and the HRP-linked long DNA concatemers, remarkable signal current distinguished from the background in the central part of the DNA spots was obtained. A horizontal band around the spots was created owing to the BQ diffusion influenced by the tip movement along the x-axis from left to right. Fig. 5B shows that when the DNA microarray was only hybridized with TD1 and TD3 (100 fM), the signal current was found only at Spot 1 and 3, indicating that the HRP catalytic reaction did not occur at the spots without target DNA. It provides a good selectivity for the high-throughput detection of specific DNA sequences.

The sequence-specificity of the proposed SECM-based DNA biosensing platform was also explored by structuring a DNA microarray with capture probe (CP') and the mismatched sequences from CP' by one base (1MC), three bases (3MC) and

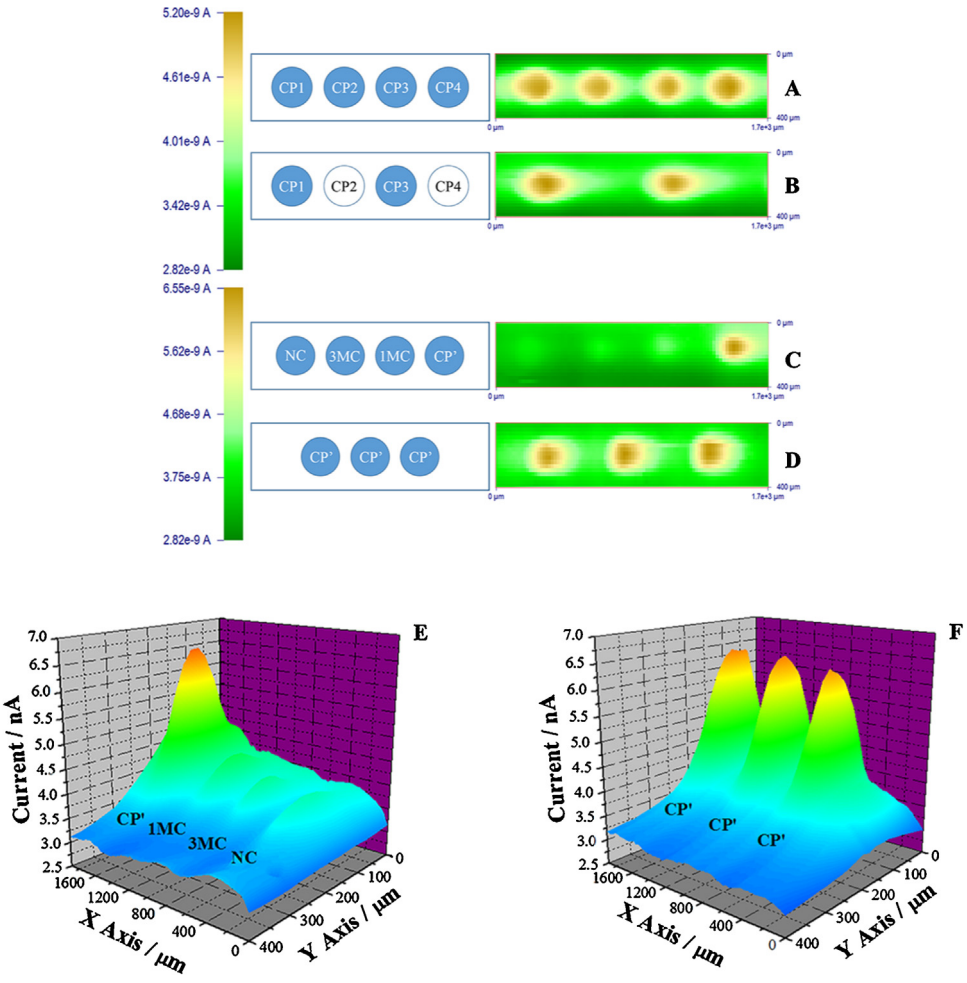


Fig. 5. SECM imaging of DNA microarray with four different DNA spots (CP1-4) hybridized with: (A) TD1-TD4, (B) TD1 and TD3. The concentrations of the TD involved in (A) and (B) were all 100 fM. (C) SECM imaging of DNA microarray with CP', 1MC, 3MC and NC as capture probes on four spots respectively. (D) SECM imaging of DNA microarray with CP' immobilized on three spots. (E) and (F) represent the 3-D plots of the same with (C) and (D), respectively. The concentrations of the TD involved in (C) and (D) were 1 nM. E_{tip} = −0.4 V. Electrolyte: 0.1 M PBS (pH 7.4) containing 1 mM H_2Q, 1 mM H_2O_2 and 0.1% Triton X-100. Scan rate: 100 μm s^{-1}. Distance between the tip and the substrate surface: 20 μm.

all bases CP' (NC), which were immobilized on four spots respectively in one row. When the DNA microarray hybridized with TD (1 nM), forming the HRP-linked long DNA concatemers, it could be seen from Fig. 5C that the signal current obtained on Spot CP' was approximately five times that on Spot 1MC, even much higher than that on Spot 3MC and Spot NC. Thus, it has enough selectivity to discriminate the DNA sequences containing the base mismatches in different numbers. The reproducibility of the proposed SECM-based biosensing platform was then estimated through a DNA microarray with CP' immobilized on three spots in one row (Fig. 5D). It is clear that the signal current of these three spots were very reproducible.

4. Conclusions

An ultrasensitive detection toward target DNA was achieved by the proposed SECM-based DNA biosensing platform with the long self-assembled DNA concatemers as the carrier for signal amplification. Under the optimized self-assembly time (5 h) and the concentrations of SP (2 μM) and AP (2 μM), this newly proposed DNA biosensing platform exhibited a low detection limit of 0.18 aM estimated by the 3σ rule, superior to most of existing SECM-based DNA biosensing platforms. Compared with conventional electrochemical method, the SECM-based DNA biosensing platform obtained much more amplified signal by the long DNA concatemers. Moreover, using this proposed strategy, four kinds of TDs (100 fM) as the models were detected simultaneously through SECM imaging of the fabricated DNA microarrays with sufficient selectivity to distinguish specific DNA sequences and good reproducibility. Therefore, this detection strategy has a great potential for the SECM sensitivity improvement of high-throughput DNA detection.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.electacta.2015.12.102.

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