Biosensors and Bioelectronics 25 (2010) 1953–1957

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Scanning electrochemical microscopy assay of DNA based on hairpin probe and enzymatic amplification biosensor

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ARTICLE INFO

Article history:
Received 18 November 2009
Received in revised form
27 December 2009
Accepted 11 January 2010
Available online 18 January 2010

Keywords:
Hairpin probe
Enzyme catalytic reaction
Ultramicroelectrode
Scanning electrochemical microscopy
DNA imaging

ABSTRACT

A novel scheme for scanning electrochemical microscopy (SECM) assay of DNA based on hairpin probe and enzymatic amplification biosensor was described. In this method, streptavidin–horseradish peroxidase (HRP) was captured by double-stranded DNA (ds-DNA) modified gold substrate via biotin–streptavidin interaction after hybridization of target DNA to the immobilized hairpin probe functioned with a biotin at its 3′ end. In the presence of H_2O_2, hydroquinone (H_2Q) was oxidized to benzoquinone (BQ) at the modified substrate surface through the HRP catalytic reaction, and the generated BQ corresponding to the amount of target DNA was reduced in solution by a SECM tip. The resulting reduction current allowed concentration detection of target DNA and SECM imaging of hybridization between the target DNA and the immobilized hairpin probe. The detection limit of this method was as low as 17 pM for complementary target DNA and it had good selectivity to discriminate between the complementary sequence and one containing base mismatches.

1. Introduction

For DNA detection, electrochemical methods have many advantages such as rapid detection time, simplicity of operation and the possibility of miniaturization. These methods are generally considered cost-effective alternatives to conventional optical assays (Aoki et al., 2000; Bakker, 2004; Boon et al., 2000; Del Giallo et al., 2005; Xiao et al., 2006). In recent years, considerable efforts have been made to bring down the detection limit and improve the selectivity of electrochemical DNA biosensors to enable the genoassays be suitable for various applications in clinical diagnosis, forensic analysis and environmental control (De-Los-Santos-Álvarez et al., 2004; Drummond et al., 2003). In this respect, electrochemical DNA biosensors based on enzymatic amplification and binding-induced unfolding of hairpin probe appear to be a promising means. The employment of an enzyme for signal amplification helps to improve the sensitivity, and the hairpin probe is shown to be extraordinary stable and high selective in the detection of target DNA (Fan et al., 2003; Immoos et al., 2004a,b; Lucarelli et al., 2005, 2006; Munge et al., 2005; Patolsky et al., 2002, 2003; Wang et al., 2002a). However, the conventional three electrodes electrochemical methods have a common problem that the signaling species produced in proportion to the amount of target DNA are detected on the non-reproducible sensor substrate surface resulted from hybridization of the immobilized capture probe to the different concentration target DNAs. This common problem causes uncertainty in precise concentration determination and restrains achieving lower detection limits.

Scanning electrochemical microscopy (SECM) is a scanning probe technique based on Faradaic current changes when an ultramicroelectrode is moved across the surface of a sample (Bard et al., 1991). It has been shown to be a powerful technique for studying immobilized biomolecules and biological processes (Amemiya et al., 2006; Bard et al., 1991; Gyurcsányi et al., 2004; Turcu et al., 2005; Zhang et al., 2006). The imaging of immobilized DNA arrays using SECM has been demonstrated by various authors (Fortin et al., 2006; Gyurcsányi et al., 2004; Komatsu et al., 2006; Liu et al., 2005; Palchetti et al., 2007; Turcu et al., 2004a,b; Wain and Zhou, 2008; Wang and Zhou, 2002; Wang et al., 2002b; Yamashita et al., 2001), and it has been considered to be capable of affording the opportunity for detecting base pair mismatches in a rapid manner (Wain and Zhou, 2008). Moreover, compared with the conventional electrochemical methods, SECM possesses an apparent advantage that the use of ultramicroelectrode under steady-state conditions overcomes many typical problems, including the effects of the resistive potential drop in solution (iR drop), charging current (Lu et al., 2007), so that the problematic background currents due to double-layer charging and contributions from possible absorbates can be eliminated, which brings about lower detection limit and higher sensitivity in electrochemical assay. Therefore, it is interesting to explore whether the ultramicroelectrode of SECM could be used to replace the modified electrode surface for quantifying signaling

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doi:10.1016/j.bios.2010.01.013

species to circumvent the aforementioned troublesome problem in the conventional electrochemical DNA biosensors.

In this paper, a Pt ultramicroelectrode of SECM applied as a reproducible and sensitive probe for detecting the signaling species was added into the conventional three electrodes electrochemical DNA biosensing system, and a novel scheme for sensitive concentration detection and imagining of DNA using SECM was developed.

2. Experimental

2.1. Chemicals and materials

All oligonucleotides in this work were obtained from Takara Biotechnology Co. Ltd. (DaLian, China), and were used as received. The sequences for both probe and targets are shown in Table 1. The hairpin probe was modified with a hexanethiol group at its 5′ end and a biotin at the 3′ end. The 31-base oligonucleotide sequence was the same as that in the work of Miranda-Castro et al. (2007). Target oligonucleotides included the complementary 22-base sequence and sequence with two base mismatches. Tween-20 and bovine serum albumin (BSA) were supplied by Dingguo Biologic Products (Beijing). Streptavidin labeled horseradish peroxidase (HRP) and 6-mercaptohexanol (MCH) were products of Sigma. H_2Q, BQ, H_2O_2 (30%) and other reagents were all of analytical grade. The buffer solution of 0.01 M sodium phosphate buffer saline (PBS) of pH 7.4 was used as incubating and washing buffer. All solutions were prepared with 18.2 MΩ cm ultrapure water.

2.2. Protocol of the SECM assay

The protocol was as follows: hairpin probe modified with a hexanethiol group at its 5′ end and a biotin at its 3′ end was firstly immobilized on gold electrode through mixing self-assembly with mercaptohexanol (MCH). The hybridization of target DNA to the immobilized capture probe caused conformational change of the hairpin probe, triggering the attached biotin group away from the electrode surface. Further specific binding between the conformation-triggered biotin and streptavidin–horseradish peroxidase (HRP) resulted in the immobilization of HRP. In the presence of hydrogen peroxide (H_2O_2), the immobilized HRP converted enzyme substrate hydroquinone (H_2Q) to benzoquinone (BQ), and then the enzymatic product BQ corresponding to the amount of target DNA was monitored by a Pt tip instead of the modified gold electrode with a substrate generation and tip collection (SG/TC) mode of SECM (Scheme 1).

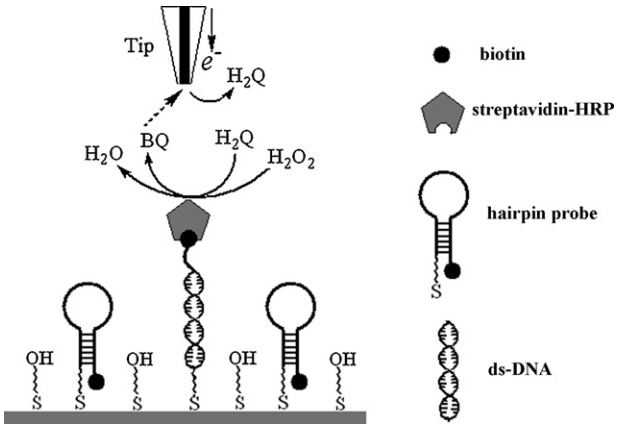
2.3. Preparation of modified electrodes

The gold disk electrodes (of 2 mm diameter) were polished sequentially with 1.0, 0.3 and 0.05 μm alumina slurries, and then thoroughly ultrasonicated with ethanol and ultrapure water. Prior to hairpin probe immobilization, electrodes were electrochemically cleaned in 1 M H_2SO_4 by cyclic voltammetry between 0 and 1.6 V *vs*. Ag/AgCl, until a steady-state redox wave of polycrystalline gold was obtained.

The self-assembly process was performed according to the optimal procedure of our previous investigation (Mao et al., 2008) with minor modification. Firstly, 20 μL mixture solution of 1 μM hairpin probe and 5 μM MCH was dropped on the cleaned gold electrode in a humidified chamber. Twelve hours later, the substrate was thoroughly rinsed with hot PBS solution (50 °C) to remove any nonbonded DNA. For the purpose of measuring approach curves and detecting concentration of target DNA, hybridization to the immobilized hairpin probe was carried out by dropping 20 μL of variable concentration target DNA (either the complement or one containing two base mismatches) on the hairpin probe modified substrate in a humidified chamber at 37 °C for 2 h. After incubation, the surfaces were rinsed with PBS and submerged in a PBS solution of 1% BSA for 1 h to block the active sites. After that, 20 μL of 1:500 diluted streptavidin–HRP containing 1% BSA was dropped on the electrode surface and incubated at 4 °C for 35 min. Then the electrodes were rinsed with PBS solution of 2% Tween-20 and soaked in PBS at 4 °C prior to use. For SECM imaging experiments, the localized hybridization scheme was applied as follows: after the gold electrode being fully covered by the mixture self-assembly monolayers, a micropipettor was employed to load 100 nL solution of target DNA on the center of the gold electrode surface and incubated at 37 °C for 2 h in a humidified chamber. In this way, a ds-DNA spot of about 1000 μm diameter resulted from the localized hybridization was prepared successfully. The following fabrication procedures were the same as that mentioned above.

Table 1
Hairpin probe and target DNA sequences.

Name	Sequence
Hairpin probe	5′-SH$(CH_2)_6$-CGGCCAGTATTATCTGAC-CGTCCCATGGCCG-$(CH_2)_6$-biotin-3′
Complement	3′-GGTCATAATAGACTGGCAGGGT-5′
Two base mismatch	3′-GGTCATAATAGACTGGACGGGT-5′



Scheme 1. Schematic representation of the SECM detection methodology.

2.4. SECM experiments

Electrochemical and SECM experiments were performed with a CHI 900B scanning electrochemical microscope (CH Instruments, Austin, TX, USA). Polycrystalline, 2-mm-diameter gold disk electrode was used for hairpin probe immobilization and as the substrate in SECM measurements. A Pt ultramicroelectrode of 10 μm diameter severed as the SECM tip. The auxiliary and reference electrodes were a Pt wire and an Ag/AgCl (3 M KCl) electrode, respectively. Prior to each measurement, the Pt tip was precisely positioned at the same position over the substrate surface using the negative feedback mode, which was carried out by monitoring the anodic current at 0.8 V for electrochemical oxidation of 1 mM H_2Q in pH 7.4 PBS solution and fitting the normalized experimental approach curve to the theoretical curve for negative feedback behavior (*vide infra*). After positing the Pt tip at the predetermined distance from the substrate surface, H_2O_2 was then added into the above solution with a final concentration of 1 mM and allowed to react with H_2Q for 6 min via the HRP catalytic reaction before measurement. The Pt tip was biased at −0.3 V to reduce the enzymatic product BQ in current–time curve recording and SECM imaging.

3. Results and discussion

3.1. Electrochemical behavior of H_2Q and BQ

In the proposed SECM scheme, the enzyme substrate H_2Q was oxidized via the HRP catalytic reaction at the modified gold electrode, and the generated BQ was collected by the Pt tip with a reduction potential. Before carrying out SECM experiments, the electrochemical behavior of H_2Q and BQ at the Pt tip was investigated. Fig. 1 shows the cyclic voltammetry of 1 mM H_2Q and 1 mM BQ in PBS buffer with a 10 μm Pt tip. It indicated that H_2Q began to be oxidized to BQ at the potential of 0.4 V and the steady-state oxidization current occurred at the potentials more positive than 0.6 V. BQ began to be reduced to H_2Q at the potential of 0 V and the steady-state reduction current was observed at the potentials more negative than −0.2 V. Therefore, the Pt tip was hold at +0.8 V to oxidate H_2Q in approach curve measurements and at −0.3 V for detecting BQ in SECM imaging and concentration detecting, respectively.

3.2. Position of Pt tip over the modified substrate electrode

When the SG/TC mode was used for imaging and detecting concentration of DNA, the SECM tip should be placed at the same position over the substrate electrode to obtain reliable results, and this was achieved by measuring approach curves over the modified substrate electrode. The substrate electrode was immersed face up in a 1 mM H_2Q solution of pH 7.4 PBS in the absence of H_2O_2, and a 10 μm Pt tip was positioned close to the surface. The tip was held at a potential of 0.8 V, which was sufficient to lead to the diffusion controlled oxidization of H_2Q to BQ according to the voltammogram in Fig. 1. The limiting current ($i_{T,\infty}$) was measured as a function of distance (d) from the unbiased modified substrate surface (Fig. 2(a)). It was clear that the tip current decreased at all tip–substrate distances, which exhibited a negative feedback. Such behavior was characteristic of an insulating substrate surface, indicating that diffusion of H_2Q from the solution bulk to the SECM tip was blocked upon approach and BQ generated on the tip could not be reduced back to H_2Q at the unbiased modified substrate surface. As shown in Fig. 2(a), the negative feedback curve fitted well to the theoretically calculated curve based on negative feedback for pure insulator (Cornut and Lefrou, 2007). The excellent agreement between experimental and theoretical approach curves allowed positioning the SECM tip over the modified substrate electrode at a predetermined distance.

For DNA imaging and concentration detecting, H_2O_2 was then added in the above solution to induce the HRP catalytic reaction, and H_2Q could be oxidized to generate BQ at the modified substrate electrode surface. As BQ would diffuse through the tip–substrate gap before it was detected by the tip, the concentration profiles of BQ in the test solution should be taken into account. In this regard, the tip current was the function of the tip–substrate distance. Meanwhile, diffusion of reactants (H_2Q and H_2O_2) and generated BQ from the outside solution to the tip–substrate gap would be restricted when the gap distance was short. This means that the distance between the tip and the modified substrate electrode was very critical for the proposed measurement. Therefore, the approach of Pt tip to the modified gold electrode surface was performed after H_2Q reacting with H_2O_2 for about 6 min via the HRP catalytic reaction. The Pt tip was held at a potential of −0.3 V, which was sufficient to bring about the diffusion controlled reduction of BQ to H_2Q. The resulting curve is presented in Fig. 2(b).

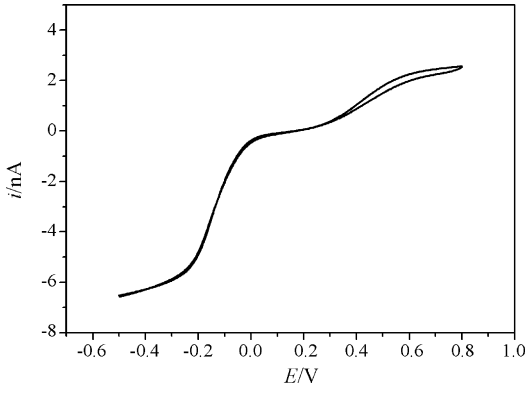


Fig. 1. Cyclic voltammetry of the Pt tip in pH 7.4 PBS solution containing 1 mM H_2Q and 1 mM BQ.

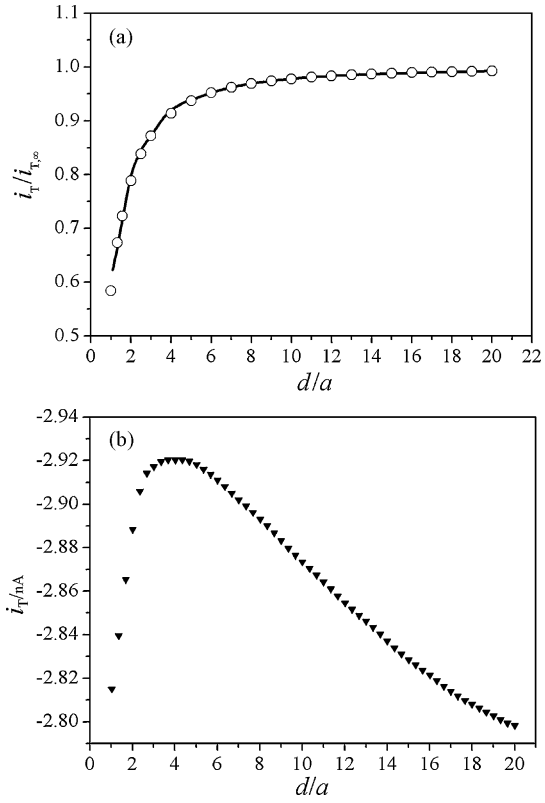


Fig. 2. (a) SECM approach curves obtained at the unbiased ds-DNA modified gold substrate in pH 7.4 PBS solution containing 1 mM H_2Q. Circles show the theoretical curve for negative feedback over an insulator. i_T and d are normalized with respect to the bulk solution limiting current, $i_{T,\infty}$, and the tip radius, a, respectively. Approach rate: 2 μm s^{-1}. (b) Dependence of the Pt tip currents on the tip–substrate distance in pH 7.4 PBS containing 1 mM H_2Q and 1 mM H_2O_2. Approach rate: 2 μm s^{-1}.

It was clear that the tip current dropped with the increase of the normalized tip–substrate distance (d/a) within 5 (25 μm) to 20 (100 μm). When the tip–substrate distance was shorter than 3a (15 μm), a drastic tip current decrease appeared, which should be mainly resulted from the inhibition of diffusion of enzymatic products BQ from outside solution to Pt tip caused by the blocking of negative feedback modified substrate electrode. Additionally, at the short tip–substrate distance, the diffusion of enzyme substrates of H_2Q and H_2O_2 to the tip–substrate gap was also blocked. In the range of 3a (15 μm) to 5a (25 μm), the tip current was relatively steady and less sensitive to the change of tip–substrate distance. This indicated that detection of BQ generated by the HRP catalytic reaction in this relatively large range could give reproducible signal even the tip–substrate distance changed slightly.

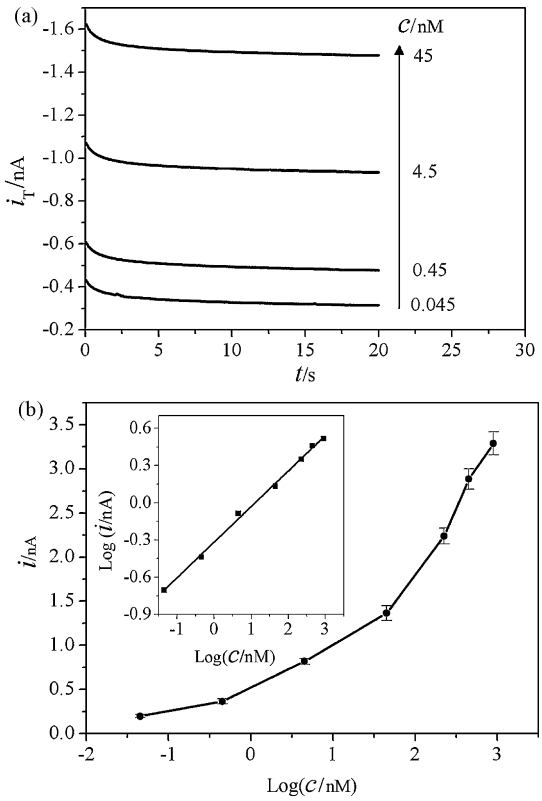


Fig. 3. (a) Current–time responses of the Pt tip for complementary target DNA of different concentrations. (b) Dependence of the Pt tip currents taken at the polarization time of 10 s on concentration of the complementary target DNA.

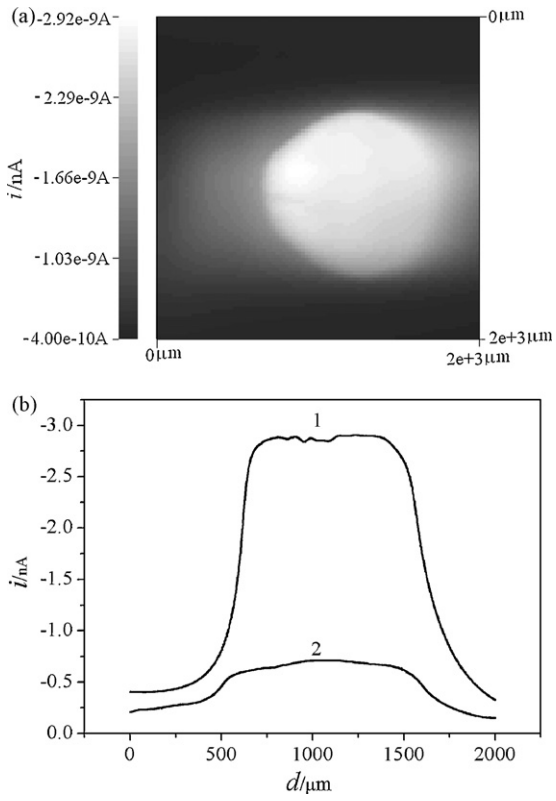


Fig. 4. (a) SECM image of a ds-DNA spot fabricated by 450 nM complementary target DNA. Scan rate: 50 μm s^{-1}. (b) SECM scan curves across the ds-DNA spots fabricated by 450 nM complementary (1) and two base mismatches (2) targets. Scan rate: 30 μm s^{-1}.

3.3. Detecting concentration of complementary target DNA

At an optimized tip–substrate distance of 15 μm, the dependence of the tip reduction current of BQ generated by the HRP catalytic reaction on the concentration of complementary target DNA was investigated in detail. Fig. 3(a) shows the amperometric response profiles for different concentrations of complementary target DNA. As a reduction potential of −0.3 V was applied to the Pt tip, the tip current dropped significantly in the first 2 s as a consequence of the quick decrease of charging current and the depletion of the previously accumulated BQ in the tip-distance gap. After that the tip current reached a relative steady-state value when the diffusion of BQ from the solution outside of the tip-distance gap was equal to the consumption of BQ on the tip. Therefore, the steady-state tip current values taken at the polarization time of 10 s were adopted as the corresponding reduction current responses for different concentrations of complementary target DNA in the quantification determination.

Fig. 3(b) shows the relative tip current increases for complementary target DNA of different concentrations. The response signal increased with the increasing of target concentration in the range of 0.045 nM to 900 nM. As shown in the inset figure, the logarithm of tip current increase was in proportion to the logarithm of target concentration with a coefficient of 0.997. The background tip current, obtained without adding complementary target DNA was -0.13 ± 0.05 nA. Then the limit of detection for the proposed scheme was 0.017 nM as estimated by the 3σ rule (MacDougall and Crummett, 1980). Compared with the conventional electrochemical methods using hairpin probes for detecting target DNA (Mao et al., 2008; Miranda-Castro et al., 2007), the lower detection limit was achieved with the proposed SECM scheme. This improved analytical performance should mainly originate from the SG/TC mode of SECM, as the use of an ultramicroelectrode instead of the modified substrate electrode to detect the enzymatic products under steady-state conditions could eliminate problematic background current, increase the signal to noise ratio by allowing any change in the faradic current attributed to the enzyme catalytic reaction to be determined (Roberts et al., 2007). To test the reproducibility of the method, four substrate electrodes were prepared with the same procedure, and the estimated RSD is 3.6% for determination of 45 nM complementary target under the same conditions.

3.4. Imaging of local DNA hybridization using collection mode of SECM

In imaging experiments, the Pt tip was biased at −0.3 V and scanned at a constant height of 15 μm above the modified substrate. The SECM imaging of a ds-DNA spot fabricated with 450 nM complementary target ss-DNA was shown in Fig. 4(a). The appearance of the ring-shaped spot with high reduction current was attributed by the HRP catalytic reaction, which indicated the presence of ds-DNA resulted from the localized hybridization of the complementary target to the immobilized hairpin probe.

As mentioned in the literature (Wain and Zhou, 2008), SECM imaging could provide the chance of discriminating between immobilized ss-DNA and ds-DNA, and detecting base pair mismatches in ds-DNA. Then, we compared SECM scan curve measurements for two spots of one fabricated with 450 nM complementary target and another with 450 nM target containing two mismatch bases. The corresponding SECM scan curves across the spots are shown in Fig. 4(b). For the spot fabricated with 450 nM complementary targets, the platform peak reduction current was about −2.90 nA, which was approximately four times larger than that for the spot fabricated using target containing two mismatch bases. Such obvious difference should result from its different

hybridization efficiencies of MB probe to complementary target DNA and two base mismatches target DNA. In addition, the baseline current of the SECM scan cure for the spot fabricated with 450 nM complementary target was a little higher than that of two base mismatches target, which should be a result from the diffusion of BQ toward the bulk solution.

4. Conclusions

In this work, the SG/TC mode of SECM was introduced to the conventional electrochemical DNA biosensor combining with hairpin probe and enzymatic amplification for sensitive concentration determination and imaging of DNA. Through applying a proper potential to a SECM microelectrode tip positioned at an optimal distance over the modified substrate, the signaling products in proportion to the target DNA could be probed sensitively, and ds-DNA spot imaging could be accomplished successfully by scanning the microelectrode tip across the modified surfaces. Compared with conventional electrochemical DNA biosensors, the developed SECM scheme provided lower detection limit, allowed discrimination between ss- and ds-DNA and showed significant promise in the sensing of base mismatches in ds-DNA. Additionally, as the signal species are detected by the SECM microelectrode tip rather than the modified substrate, the conductivity of the modified substrate becomes non-essential, many other low cost substrates such as glass slide, silicon wafer and nylon membrane can be employed as supports for the DNA probes immobilization, which will enlarge the scope of electrochemical DNA biosensors.

Acknowledgement

This work was supported by “973” National Key Basic Research Program of China (2007CB310500).

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