Feature Article

# DNA Sensor for Recognition of Native Yeast DNA Sequence with Methylene Blue as an Electrochemical Hybridization Indicator

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**Abstract**

An electrochemical DNA sensor based on the recognition of single-stranded yeast DNA (ssDNA) immobilized on gold electrode to its complementary ssDNA (cDNA) is presented for hybridization detection. The yeast single-stranded DNA was covalently bound on a self-assembled 3-mercaptopropionic acid monolayer by using water soluble *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) as a linker. The self-assembled monolayer prevented the nonspecific adsorption of long DNA chain on the electrode surface. The covalently immobilized ssDNA could selectively hybridize with cDNA in solution to form double-stranded DNA (dsDNA) on the surface. The increase of peak currents of methylene blue (MB), an electroactive indicator, upon the hybridization of immobilized ssDNA with cDNA in the solution was observed, which was used to monitor the recognition of yeast DNA sequence. The interaction of immobilized ssDNA and dsDNA with MB also resulted in a transition of electrode process from diffusion-controlled to surface-controlled process. The adsorption constants of methylene blue on ssDNA and dsDNA modified gold electrode surface were found to be $(3.3 \pm 0.3) \times 10^3\ \mathrm{M}^{-1}$ and $(6.6 \pm 0.4) \times 10^3\ \mathrm{M}^{-1}$, respectively, indicating a higher affinity of dsDNA to MB.

**Keywords:** Sensors, DNA sensors, Chemically modified electrodes, Methylene blue, Self-assembled monolayers, Hybridization, Yeast DNA.

## 1. Introduction

The detection technique of DNA sequence has been rapidly developing [1 – 4] for its theoretical and practical significance in many fields [5 – 7]. The method to detect DNA sequence was first based on a radioisotope technique [3]. With the establishment of many new non-radioactive procedures [8 – 10], especially the appearance of various DNA biosensors [11 – 13], the traditional radioactive methods have been gradually replaced due to the radioactive injury and the requirement of fast sequence detection. The electrochemical DNA hybridization sensor is an important area of DNA biosensors. It introduces DNA hybridization to the electrode surface to detect the hybridization procedure with the aid of an electroactive indicator. The electrochemical signal depends on both the surface hybridization and the interaction of immobilized DNA with the indicator. The immobilization of ssDNA on the electrode surface is a key process for the preparation of electrochemical DNA biosensors.

Several methods have been used to immobilize the DNA probe on the solid surface [14, 15]. The direct adsorption of ssDNA on electrode surface could obtain a stable layer to both acid and stock of long time [16]. Another was the application of Avidin-Biotin System for the immobilization of biotinylated ssDNA molecular beacon on a solid surface [17]. A method based on the incorporation of $C_{16}$ alkyl chain terminated DNA into the lipid membrane on electrode surface was also presented to detect DNA hybridization [18]. Recently, the covalent bonding of DNA to the electrode surface has been developed as a usually used method for the preparation of DNA sensors [19 – 21]. This technique usually uses a short ssODN (15 – 20 nucleotides long) as a DNA probe which is immobilized on electrode surface to create a DNA recognition element. For example, a 20-mer DNA probe including a mercaptohexyl group at the 5′-phosphate-end was synthesized to form the self-assembled monolayer of DNA on gold surface through the gold-sulfur bond [19]. Millan et al. covalently immobilized oligo- and polydeoxynucleotides on the surfaces of oxidized glassy carbon electrodes (-COOH formation) using water-soluble carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) coupling reagents [22]. In this article, with the help of EDC and NHS, the native yeast single-stranded DNA (about $1.35 \times 10^7$ base pairs) is immobilized at a gold electrode by the covalent bonding of deoxyguanosine (dG) residues of DNA with carboxyl groups in a self-assembled monolayer of 3-mercaptopropionic acid [22]. Comparatively, the immobilization of single-stranded yeast DNA is more convenient and avoids the synthesis and sulfhydryl modification of oligonucleotides, which is tedious, time-consuming and costly. On the other hand, the presence of a self-assembled monolayer can also prevent the nonspecific adsorption of long DNA chain on the electrode surface during the immobilization and hybridization procedures.

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In general, there are two kinds of DNA hybridization electroactive indicators: metal coordination complexes and intercalating organic compounds. Many transition metal complexes, notably Co, Fe, Ru and Os complexes [22–27], with aromatic ligands can intercalate into the double helical DNA. The electroactive dyes such as acridine orange [28], Hoechst 33258 [19] and benzyl viologen [29] have also been used as indicators of surface DNA hybridization. The phenothiazinium dye methylene blue (MB) showed very good voltammetric behavior [30–32]. At the same time it was optically proved to be a good intercalator and could interact with calf thymus DNA in an intercalation mode [33–36]. Barton et al. [37] used a 15-base pair oligonucleotides modified electrode to study the intercalation of MB to dsDNA on the electrode surface. Ozsoz et al. [38] observed a difference between the voltammetric signals of MB at single- and double-stranded calf thymus DNA modified carbon paste electrodes and thought that MB was a promising novel hybridization indicator of DNA electrochemical biosensors. They then used MB as hybridization indicator to detect short DNA sequences related to the hepatitis B virus at a 29-mer synthetic oligonucleotide adsorbed carbon paste electrode [39]. Sadik and co-workers [40] reported an electrochemical DNA biosensor prepared on a gold electrode through specific adsorption of 17-mer DNA probe and observed the increase in peak current of MB upon the adsorption of a probe and the hybridization with cDNA. Here, we use MB as an electrochemical indicator to monitor the recognition of immobilized yeast DNA to yeast DNA sequence. As control, this biosensor does not show response to calf thymus ssDNA, which has the fully mismatch sequence to yeast DNA.

## 2. Experimental

### 2.1. Chemicals

Tris-(hydroxymethyl)aminomethane (Tris, Sigma), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, Merck), *N*-hydroxysuccinimide (NHS, Aldrich) and 3-mercaptopropionic acid (Aldrich) were used without further purification. Methylene blue (MB) (Shanghai Chemical Reagent Factory), yeast DNA ($1.35 \times 10^7$ base pairs, Shanghai Changyang Pharmaceutical Factory) and calf thymus DNA (Sigma) were used as received. DNA solution (20 μg/mL, ca. $6.11 \times 10^{-5}$ M in nucleotide phosphate determined by UV spectrometry) including 50 mM NaCl and 5 mM Tris at pH 7.1 gave a $A_{260}/A_{280}$ (absorbance at 260 nm and 280 nm, respectively) ratio of 1.9. Thus the DNA was sufficiently pure [41]. The single-stranded DNA (ssDNA) was obtained by heating native double-stranded DNA (dsDNA) in a 100 °C water bath for 10 min and then rapidly cooling in an ice-water bath. Other chemicals were of analytical reagent grade. Double-distilled water was used in all experiments.

### 2.2. Apparatus

Electrochemical experiments were carried out with a BAS-100B electrochemical analyzer (BAS Inc, USA) and a standard three-electrode system containing the modified or unmodified gold disk (diameter ca. 0.5 mm) as working, the Ag /AgCl (3M NaCl) as reference and a platinum wire as counter electrode. Unless otherwise indicated, all experimental solutions were deaerated with highly purified nitrogen for 10 min and kept in nitrogen atmosphere during the experimental processes. The cyclic voltammetric experiments were performed at 100 mV/s and room temperature.

### 2.3. Construction of DNA Sensors

Gold electrodes were prepared by sealing polycrystalline gold wires (> 99.99%) in soft glass tubes. The gold electrodes were abraded with successively finer grades of SiC paper and polished to a mirror-like suface with 0.3 and 0.05 μm alumina slurry on microcloth pads (Buehler). The used gold disk electrodes were first heated in a boiling mixed solution of $H_2O_2$ (30%) and concentrated $H_2SO_4$ (3:7) for 5 min to eliminate the former adsorption layer. After further dipping in $HNO_3$ (1:1) for 30 min, the electrode was rinsed with water and then cleaned ultrasonically. The 3-mercaptopropionic acid monolayer was first prepared by soaking a freshly treated electrode in 1% 3-mercaptopropionic acid acetone solution for 24 h and then thoroughly rinsing the electrode with acetone and water to remove the physically adsorbed 3-mercaptopropionic acid. This monolayer modified gold electrode is denoted as SAM/Au, as shown in Figure 1.

After being immersed in a pH 7.0, 20 mM phosphate buffer (PB) solution containing 5 mM EDC and 8 mM NHS for 15 min, the SAM/Au electrode was contacted with ssDNA solution (pH 7.0) for 12 h at 25 °C. Under this condition the covalent bonding occurred between carboxyl

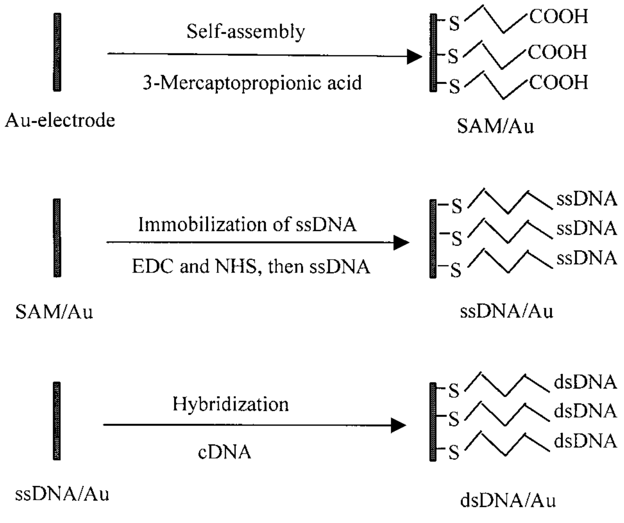

Fig. 1. Scheme for the preparation procedure of DNA sensor.

groups and deoxyguanosine (dG) residues of DNA and the hybrid reaction could be neglected due the low hybridization rate. Thus, a ssDNA-modified electrode (ssDNA/Au) was obtained after being washed thoroughly with water.

The ssDNA/Au was further hybridized with 100 μg/mL cDNA (the same heat-denatured yeast DNA) in 0.3 M NaCl + 30 mM sodium citrate solution (2 × SSC buffer, pH 7.0) with stirring for 1 h at 70 °C and then cooling gradually to 25 °C [42]. Thus the dsDNA modified electrode (dsDNA/Au) was obtained. The dsDNA/Au was rinsed with water and stored at 4 °C in the solution of pH 8.0 10 mM Tris including 1.0 mM EDTA. The procedures of ssDNA immobilization on the gold electrode and hybridization with cDNA are shown in Figure 1.

The control experiments of ssDNA immobilization were performed with the above-mentioned process without the activation step of EDC/NHS. The ssDNA/Au was hybridized with two kinds of DNA: the complementary ssDNA (cDNA) and the heat-denatured calf thymus ssDNA as control.

## 3. Results and Discussion

### 3.1. Interaction Between MB and Single- or Double-Stranded Yeast DNA

The cyclic voltammogram of MB at pH 7.2 on the bare gold electrode (Fig. 2A) shows a pair of symmetrical redox peaks with the anodic peak potential of ( − 198 ± 2) mV and the peak-to-peak separation of 35 mV at 100 mV $s^{-1}$. The electrode reaction follows a process of two electrons and one proton at pH 7.2 [32].

After 200 μg/mL ssDNA or dsDNA was added in pH 7.2 PB solution containing 75 μM MB, the anodic peak current ($i_{pa}$) of MB decreased by 26.5% and 34.5%, respectively. The presence of both ssDNA and dsDNA did not change the peak potentials of MB redox process. The experimental results are shown in Table 1. The decrease in the peak current resulted from the decrease of free MB concentration due to the formation of MB-ssDNA or MB-dsDNA. The decrease of peak currents upon addition of dsDNA was more than that of ssDNA, indicating a higher affinity of dsDNA to MB, which could be used to distinguish the dsDNA and ssDNA. So MB is an appropriate indicator for the electrochemical detection of DNA sequence.

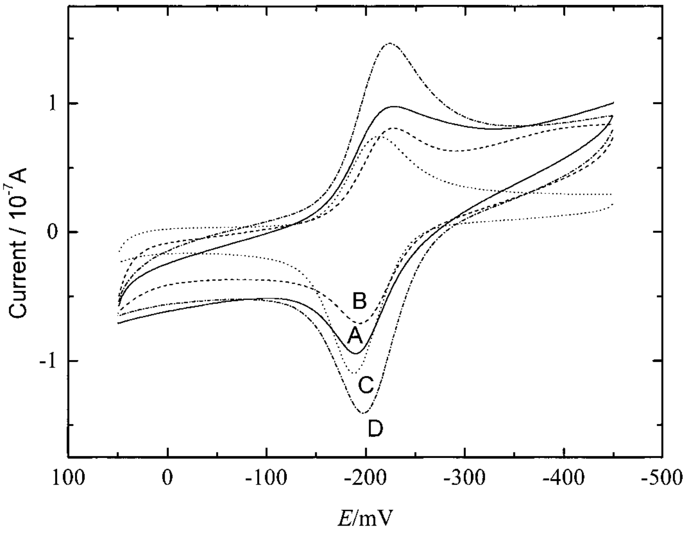

Fig. 2. Cyclic voltammograms of 50 μM MB in pH 7.2 50 mM Tris + 20 mM NaCl solution at the bare gold (A, solid), SAM/Au (B, dash), ssDNA/Au (C, dot) and dsDNA/Au (D dash dot) electrodes at 100 mV/s.

Table 1. Peak current and potential of 75 μM MB in the absence and presence of ssDNA or dsDNA.

| | $i_{pa}$ *(nA)* | *Decrease of* $i_{pa}$ | $E_{pa}$ *(mV)* |
|---|---|---|---|
| MB | 90.9 ± 0.1 | 34.5 ± 0.3% | − (198 ± 2) |
| MB + dsDNA | 59.5 ± 0.2 | | − (198 ± 2) |
| MB | 89.9 ± 0.1 | 26.5 ± 0.3% | − (198 ± 2) |
| MB + ssDNA | 66.1 ± 0.2 | | − (198 ± 2) |

### 3.2. Response of MB at ssDNA Immobilized Gold Electrode

Figure 2A and B display the decrease of both the anodic and cathodic peak currents of MB after the self-assembled monolayer of 3-mercaptopropionic acid is formed on the gold electrode surface. Both peak potentials and peak-to-peak separation almost keep at constant values. Thus, the electrode reaction mechanism does not change. The decrease of peak currents is due to the presence of 3-mercaptopropionic acid self-assembled monolayer which hinders the diffusion of MB molecules to electrode surface in the electrode process.

With the help of EDC and NHS, ssDNA was immobilized on SAM/Au through deoxyguanosine (dG) residues of DNA to form ssDNA-SAM surface (ss-DNA/Au). The oxidation peak current of MB at ssDNA/Au increased significantly (Fig. 2B and C). The redox peak currents of MB at SAM/Au electrode were almost identical, but those of ssDNA/Au electrode were different, indicating the different actions of ssDNA/Au to the oxidation and reduction states of MB. As a neutral alkali, the reduction state of MB could form the hydrogen bond with the immobilized ssDNA more easily than its oxidation state with a positive charge, so more reduction-state MB molecules could approach to electrode surface or be complexed by immobilized ssDNA, producing greater oxidation peak current. It was noted that the redox peak potentials of MB moved in a positive direction and the peak-to-peak separation decreased from 31 to 23 mV after ssDNA were immobilized on SAM/Au. Thus, the MB electrochemistry transferred from diffusion-controlled electrode process to a process involving a surface reactant.

The control experiments for ssDNA/Au preparation without EDC/NHS activation showed that the oxidation peak current of MB did not increase and the peak potentials of MB did not change. Although DNA could be adsorbed on the gold surface [16], the nonspecific adsorption of ssDNA at SAM/Au surface was negligible due to the presence of 3-mercaptopropionic acid SAM which resulted in a repellency between negative-charge monolayer and ssDNA at pH 7.0. The repellency prevented nonspecific adsorption of ssDNA as observed at a two-component film containing a thiol-derived ssDNA probe and a diluent thiol [43].

### 3.3. Hybridization of Immobilized ssDNA

Figure 2D shows the cyclic voltammogram of MB on dsDNA/Au. In comparison with that on ssDNA/Au (Fig. 2C), the peak currents of MB further increase after hybridization of the immobilized ssDNA with 100 μg/mL cDNA in 2 × SSC buffer. The cathodic peak current of MB at dsDNA/Au increases by about 80% when comparing with that at ssDNA/Au. The enhancement of peak current indicates that more MB molecules are preconcentrated on the dsDNA/Au surface, or more MB molecules are bound by immobilized dsDNA.

As control, the DNA sensors were used to detect calf thymus ssDNA (with the different sequence from yeast DNA). After the same treatment process as hybridization of cDNA, the CV of MB at the treated electrode gave redox peak currents and potentials similar to those at ssDNA/Au in spite of the change in the charge current due to the heating treatment. Thus, neither contribution of nonspecific adsorption of calf thymus ssDNA nor hybridization of ssDNA/Au with calf thymus ssDNA was observed. In other words, the contribution of nonspecific binding of calf thymus ssDNA during the treatment process could be ignored.

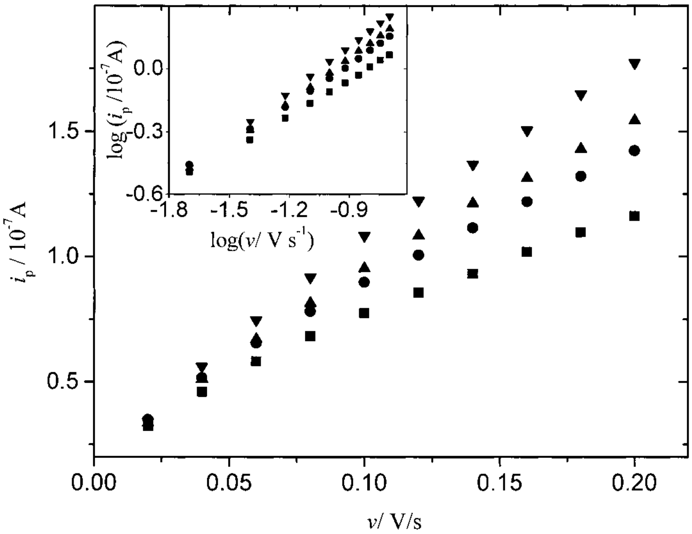

Fig. 3. Plots of peak currents vs. scan rate at bare gold (■), SAM/Au (●), ssDNA/Au (▲) and dsDNA/Au (▼) electrodes. Inset: plots of log$i$ vs. log$v$. The slope of their regression equations is 0.56, 0.61, 0.67 and 0.72, respectively. Other conditions are the same as Fig. 2.

These results provide a method to recognize the surface hybridization of ssDNA with cDNA, producing a kind of efficient DNA biosensor for the sequence detection of cDNA. Here the concentration of 100 μg/mL cDNA with $1.35 \times 10^7$ base pairs corresponds $3.06 \times 10^{-4}$ M in nucleotide phosphate or $1.36 \times 10^{10}$ cDNA/mL. Thus the sensitivity of the hybridization sensors is rather high.

### 3.4. Effect of Scan Rate on Peak Current of MB

The relations between the anodic peak currents of MB at bare gold, SAM/Au, ssDNA/Au and dsDNA/Au electrodes and the scan rates in the range of 20 to 200 mV/s are shown in Figure 3. No linear relation is observed at all these electrodes. The plots of log $i_p$ vs. log $v$ at these electrodes show the expected linear relations with different slopes between 0.5 and 1.0, which indicate ideal diffusion-controlled process and surface-controlled process, respectively. The slope increases gradually with the processing of modification. The plot of $i_p$ vs. $v$ at a bare electrode is similar to the square root relation of scan rate, and the slope of log $i_p$ vs. log $v$ is 0.56. Considering the peak-to-peak separation of $(31 \pm 3)$ mV at the scan rates less than 100 mV $s^{-1}$ and the electron transfer number of 2, the electrode reaction at bare gold electrode is a diffusion-controlled process. Similarly, the electrode process of MB at SAM/Au is also a diffusion-controlled process.

The slope of log $i_p$ vs. log $v$ at ssDNA/Au and dsDNA/Au electrodes is 0.61 and 0.72, respectively. Thus, the electrode reaction is a combination of diffusion-controlled and surface-controlled processes. The surface-controlled process is caused by the electrode reaction of MB bounding to the dsDNA. The slope of 0.72 at the dsDNA/Au electrode is closer to the slope of the ideal surface-controlled process. Thus the contribution of the surface-controlled process at the dsDNA/Au electrode is larger than at the ssDNA/Au electrode and more MB molecules are bound to the dsDNA/Au electrode surface. The combination of diffusion-controlled and surface-controlled processes primarily comes from a mixture of free and bound MB on the electrode surface [23]. With the further procedure of modification and hybridization, more MB molecules are bound on the surface, resulting in that the peak-to-peak separation of the cyclic voltammogram becomes less than 29 mV (valve for a diffusion-controlled electrode reaction).

### 3.5. Dependence of Peak Currents on MB Concentration

The cyclic voltammograms of MB at different concentrations on dsDNA/Au electrode are shown in Figure 4. With increasing MB concentration, the peak current increases and then trends to saturation value, as expected shape of Langmuir adsorption. Thus the adsorption thermodynamics follows the equation: $c/i_p = 1/(Ki_{p,\max}) + c/i_{p,\max}$, where $i_p$, $i_{p,\max}$, $c$ and $K$ is the peak current, maximum peak current, MB concentration and the adsorption constant of MB at

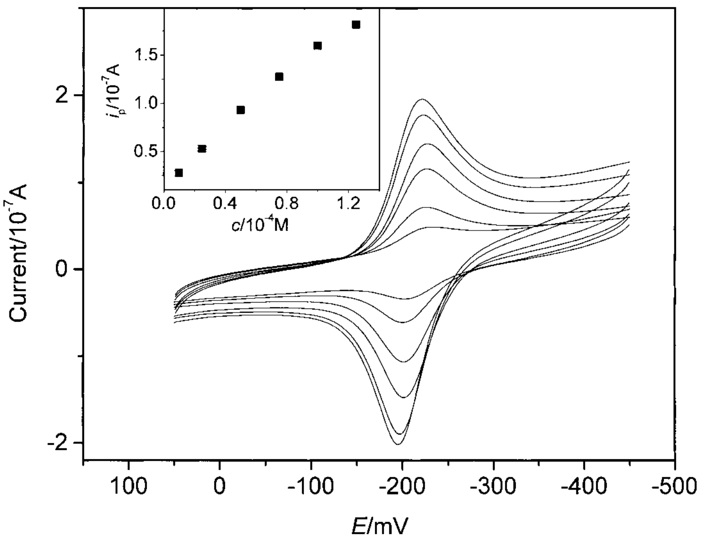

Fig. 4. Cyclic voltammograms of 10, 25, 50, 75, 100 and 125 μM MB (from internal to external) at dsDNA/Au electrode at 100 mV/s. Supporting electrolyte is the same as Figure 2.

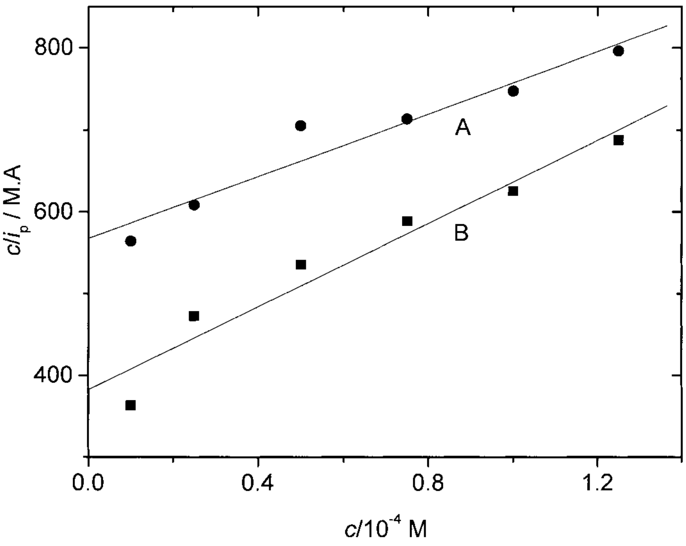

Fig. 5. Plot of $c_{MB}/i_p$ vs. $c_{MB}$ at ssDNA/Au (A) and dsDNA/Au (B) electrodes with pH 7.2 50 mM Tris + 20 mM NaCl as supporting electrolyte.

ssDNA/Au or dsDNA/Au surface. From the slope and intercept of $c/i_p$ vs. $c$ the maximum peak current, $i_{p,max}$, and the adsorption constant, $K$, can be obtained.

The plots of $c/i_p$ vs. $c$ at two electrodes are shown in Figure 5. From the linear regression equation, the adsorption constants of MB at ssDNA/Au and dsDNA/Au electrode are calculated to be $(3.3 \pm 0.3) \times 10^3$ $M^{-1}$ and $(6.6 \pm 0.4) \times 10^3$ $M^{-1}$, respectively. These values are less than the association constant of $6 \times 10^4$ $M^{-1}$ for the complex of MB with double-stranded calf thymus DNA in solution [33] and $3.8 \times 10^6$ $M^{-1}$ for the complex of MB with dsDNA mentioned in the literature [37]. The similar difference of association constants in homogeneous solution and interfacial system has also been noted for the complex of Co(phen)$_3^{3+}$ with dsDNA [22, 44]. A value of the association constant was reported to be $1.74 \times 10^3$ $M^{-1}$ for the complex of Co(phen)$_3^{3+}$ with immobilized dspoly(dG)poly(dc) [22], while $1.6 \times 10^4$ $M^{-1}$ for the complex of Co(phen)$_3^{3+}$ with double-stranded calf thymus DNA in homogeneous solution was given [44]. Thus, the binding constant between DNA and indicator depends on both the variety of DNA and the experimental conditions such as ionic strength, concentration of indicator or DNA, pH value of solution and the microenvironment, even for a simple model intercalator, MB [36]. In this experiment, the low binding constant may be due to the defect of ssDNA monolayer, its partial hybridization with complementary DNA strands and the covering of active binding sites resulted from the agglomeration or partial distortion of long chain DNA molecules. These factors result in the decrease of binding sites for MB.

The different adsorption constants of MB at ssDNA/Au and dsDNA/Au indicate that the complex of MB with dsDNA is more stable than that with ssDNA, which is most likely due to higher affinity of MB for the duplex of hybridized dsDNA.

## 4. Conclusions

The native DNA probe has been employed to prepare the electrochemical DNA sensor by the surface derivation of SAM, which avoids the synthesis and sulfhydryl modification of oligonucleotides for preparation of DNA probe. This sensor provides a simple method to recognize yeast cDNA sequence and can be used to detect the hybridization of cDNA with a rather high sensitivity. The detection procedure has been accomplished by the significant increase in peak current of an electroactive intercalator, MB, upon the hybridization of cDNA. Furthermore, the presence of SAM prevents the nonspecific adsorption of long DNA chain on the electrode surface during the immobilization and hybridization procedures, thus improving the reliability for detection of the specific DNA sequence. This would be valuable in the design and development of DNA sensors.

## 5. Acknowledgements

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