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Original Paper

Electrochemical Biosensor for Polycyclic Organic Compounds Screening Based on Methylene Blue Incorporated DNA Polyion Complex Modified Electrode

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Abstract

A reagent-less electrochemical DNA biosensor for rapid non-electroactive polycyclic organic compounds (POCs) screening and detection was proposed. In this method, methylene blue (MB) was incorporated into DNA/chitosan polyion complex membrane and then modified onto a glassy carbon electrode (GCE).The electrochemical analysis for the prepared DNA-MB/chitosan/GCE showed that the modified electrode exhibited high electrochemical activity and stability. The addition of tetracycline hydrochloride (TC), a model analyte of non-electroactive POCs, resulted in an obvious peak current decrease in DNA-MB/chitosan/GCE, and this electrochemical response was affected by the DNA type and MB/DNA ratio in the modified electrodes. Ultraviolet–visible (UV-vis) absorption spectroscopy was utilized to furthermore investigate the interaction between TC and DNA-MB/chitosan/GCE. As a result, a competitive interaction and displacement effect between TC and the intercalated MB was proposed. In our condition, the prepared DNA-MB/chitosan/GCE showed high sensitivity to POCs and had almost no response to common interferences. Besides, the good stability and reproducibility of the prepared electrode made it suitable for practical use.

Keywords Polycyclic organic compounds, electrochemical DNA biosensor, toxicity evaluation, environmental monitoring

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Introduction

Polycyclic organic compounds (POCs), including polycyclic aromatic hydrocarbons, substituted aromatic hydrocarbons and heterocyclic aromatic compounds, are ubiquitous in the natural world. Many of these compounds are produced during the combustion of carbonaceous materials and exhibit pronounced carcinogenic, mutagenic, and teratogenic effects in biological systems.[1-3] On the other hand, some of POCs are utilized as drugs for different application areas, for example, tetracycline antibiotics are heavily used as veterinary therapeutics and growth promoters for animals.[4] Exposures to low-level of such antibiotics in the environment have raised significant concerns of the toxic effect as well as the transfer and spread of antibiotic resistant genes among microorganisms.[5-7] As the dynamic monitoring of POCs concentration is of acute important, developing a simple and rapid method for POCs detection is essential and valued. Conventional techniques for POCs detection including high performance liquid chromatography,[8] gas chromatography–mass spectrometry,[9] surface-enhanced Raman scattering spectroscopy,[10] ultraviolet–visible spectroscopy,[11] solid-phase microextraction,[12] provide high sensitivity and low interference, however, are time consuming and more importantly, require complex and expensive equipments operated by professional technicians.

POCs are toxic because most of them interact with deoxyribonucleic acid (DNA). Actually, binding interaction with DNA is the first step in the DNA-damaging action and genotoxic processes of many POCs.[13] Therefore, DNA-based analytical methods should be an ideal alternative for POCs screening and detection with high selectivity due to the excellent molecular recognition property of DNA. Among these methods, electrochemical DNA

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biosensors are continually attracted attentions as they are cost-effective, fast and portable detection, which make in situ and real time monitoring possible, without extensive and time consuming sample preparation.[14-17] Electrochemical DNA-biosensors were proposed for detecting several analytes, such as intercalating compounds, toxin, hydrazine compounds, metals and an antibody. A common method for POCs screening was based on electrochemical DNA biosensor by direct monitoring the electrochemical signal of DNA, particularly the guanine bases, which can be oxidized on a suitable solid electrode.[18-20] However, the electrochemical oxidation of guanosine frequently requires high electrode potentials (*ca.* 1.06 V vs. SCE at pH 7),[21] the situation leads to co-oxidation of other electroactive interferences. Besides, since the limited amount of guanine base in the DNA modified electrodes, the response signals of these types of electrochemical DNA biosensor were relatively poor.

On the other hand, the electrochemical signal of POCs interact with DNA can be amplified through some mediators with excellent electroactivity. Guo and his coworkers proposed an electrochemical replacement method to investigate the interaction between DNA and POCs, using a DNA film deposited indium tin oxide electrode and Ru(2,2′-bipyridine)$_2$-(dipyrido[3,2-a:2′,3′-c]phenazine)$(BF_4)_2$, a redox-active DNA intercalator, as the electrochemical indicator.[22, 23] Similar method also has been reported by Wu et al, who developed an electrochemical DNA biosensor for chlorinated benzenes pollutants, based on DNA modified Au electrode and redox-active methylene blue, which was used to amplify the interaction signal, while the mechanism was unclear.[24] In these methods, the target analytes must be mixed with electrochemical indicator in solution and then reacted with the DNA film, which was pre-modified onto a suitable electrode surface, thus, the

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electrode preparation protocol and measurement process was relatively complicated.

Recently, our group reported a DNA-Cu(II) complex exhibited electrocatalytic activity to H_2O_2 [25] and finally developed an amperometric assay for drug-DNA interactions based on an inhibitory effect of antimararial drug (quinacrine).[26] The DNA-Cu(II) complex was stably immobilized onto glassy carbon (GC) electrodes by a polycations materials of poly(allylamine). In this work, the technique was further developed to construct a DNA-methylene blue (MB)/chitosan polyion complex membrane for rapid detection of POCs. Here, MB was utilized as an electrochemical active mediator, which intercalative binds to DNA and forms a biorecognized complex. The DNA-MB complex then was immobilized onto GC electrodes with the aid of chitosan, which is an attractive biocompatible, biodegradable, and nontoxic natural biopolymer.[27] Electrostatic interactions between DNA and chitosan were proposed to fabricate a stable polyion complex film.[28-31] The finally fabricated POCs biosensor showed high sensitivity, selectivity and high storage stability, which was suitable for rapid POCs screening and detection.

Experimental

Reagents and materials

Three kinds of DNA samples, calf thymus double-stranded DNA (cDNA), herring sperm double-stranded DNA (hDNA) and salmon taste double-stranded DNA (sDNA) were obtained from Sigma (USA). Poly-L-lys and methylene blue (MB) was obtained from Amresco (USA) and chitosan (Mw. ~ 200,000) was purchased from Sinopharm Chemical Reagent Co (China). Tetracycline hydrochloride (TC), ethidium bromide (EB), and quinacrine dihydrochloride

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(QC) are purchased from Sigma (USA). All other chemicals used in this study were of analytical grade unless otherwise specified and all solutions were prepared using distilled water.

Preparation of DNA based polyion complex membrane modified GC electrode

A GC electrode was polished before each experiment with alumina powder (Al_2O_3, 1.0 and 0.05 μm in turn) and chamois leather to achieve fine mirror finish. Then the GC electrode was thoroughly washed with distilled water, and allowed to dry at room temperature. 20 μL of 0.1 M phosphate buffer (pH 7.0) containing DNA-MB mixture, which was prepared by mixing 500 μL of 1mg mL^{-1} DNA and 500 μL of 0.1 mM MB for about 30 min, and 10 μL of aqueous chitosan solution were dropped onto the electrode surface .Then electrode was dried at room temperature for 20 h. Before electrochemical measurements, the resulting electrode (DNA-MB/chitosan/GCE) was thoroughly rinsed with distilled water and immersed into a 0.1 M PB (pH 7.0) for about 5 h to remove physically adsorbed species. For control experiments, the electrodes were prepared in a similar manner using corresponding solutions.

Electrochemical measurement

All electrochemical experiments were carried out with a three-electrode system comprising a GC electrode (3 mm diameter, CHI, USA) as a working electrode, an Ag/AgCl (sat. KCl, CHI, USA) reference electrode and a platinum wire auxiliary electrode (1 mm diameter, CHI, USA). All potentials are referred to the reference electrode in this paper. A 0.1 M PB (pH 7) was used as an electrolyte solution. Cyclic voltammetry (CV) and

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differential pulse voltammetry (DPV) were performed with an electrochemical analyzer (CHI 823, Shanghai Chenhua Instrument, Inc., China). All measurements were carried out at room temperature. Prior to the measurements, the electrolyte solutions were thoroughly deoxygenated by bubbling N_2 into the electrolyte for at least 30 min.

For ultraviolet–visible (UV-vis) absorption spectroscopy measurement, the prepared samples solutions were detected by a UV/VIR/Nir spectrophotometer (LaTBda 900, PerkinElmer, USA) at room temperature.

Detection of polycyclic organic compounds with DNA–MB/chitosan/GC biosensor

Taking TC as an example, the detection was performed by adding different concentrations of TC standard solution into 0.1 M phosphate buffer (pH 7.0) under room temperature. Subsequently, electrochemical measurements were performed on the DNA–MB/chitosan/GC biosensor. For a better comparison of the tested compound, the decrease percentage of peak current (D_{pc}) is very useful to evaluate these analytes.[24,31] The D_{pc} was calculated as follows: $D_{pc} = 100\% \times (i_0 - i_c)/ i_0$, where i_0 and i_c were the peak currents of DNA–MB/chitosan/GC biosensor before and after treatment with the analyte, respectively.

Results and Discussions

Electrochemical characteristics of cDNA-MB/chitosan/GC electrode

The electrochemical properties of various modified electrodes were investigated by CVs in N_2-saturated phosphate buffer (0.1 M, pH 7.0) under room temperature (Fig. 1A). A pair of well-characterized redox peaks were observed at cDNA–MB/chitosan/GCE (vs. Ag/AgCl)

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with peak to peak separation (ΔE) of 65 mV. The cathodic and anodic peak currents densities are approximately equal, which indicated a revisable reaction occurred at present modified electrode. However, such redox pair cannot be observed at a cDNA/chitosan/GCE or a bare GC electrode, thus it could be concluded that the redox waves must be ascribed only to the MB. No obvious peak current density decrease was observed at cDNA-MB/chitosan/GCE after multi-scans (at least 50 cycles) implied high stability of the prepared polyion complex membrane (Figure S1 A, Supporting Information). The formal potential (E^0) of the cDNA–MB/chitosan/GCE was calculated as *ca.* −206 mV, which was −40 mV more negative than that of free MB at a bare GCE (data not shown). It has been reported that the reduction potential of intercalated MB in cDNA is cathodically shifted from the value in aqueous solution.[32] Thus, it is reasonable to assume that the intercalative interaction between the MB and the cDNA existed in the prepared cDNA-MB/chitosan membrane.

The influence of scan rates on the peak current density of cDNA-MB/chitosan/GCE was investigated as shown in Fig. S1 B. Both anodic peak current (I_a) and cathodic peak current (I_c) increased linearly with increasing scan rates from 5 to 500 mV s^{-1}(Fig. 1 B). It means that the electrochemical behavior of cDNA-MB/chitosan/GCE has the typical property of a surface-confined electrode process, as expected that MB was tightly intercalated into the cDNA. This result was different to that as reported in our previous work, in which a diffusion controlled process was observed at cDNA-MB/polyamine complex membrane modified Au electrode.[33] It's reported that MB combine to DNA with two main model: loosely bound with A-T pairs, probably in the major groove, and intercalated with G-C pairs.[34] In the present work, the loosely bound MB, which usually exhibited a diffusion controlled property, might

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be almost removed and only the one which intercalated with G-C pairs remained and shown surface controlled property. The electron transfer rate constant was calculated as 2.06 s^{-1}, which is smaller but comparable to that of free MB, indicated high electron transfer rate of MB with electrode although intercalated into DNA helix and immobilized with chitosan on the electrode surface. The surface coverage of the electrochemically active MB (Γ) was estimated to be $1.2 \pm 0.1 \times 10^{-11}$ mol cm^{-2} at DNA–MB/chitosan/GCE based on the equation of $Q = nFA\Gamma$, where, Q is the quantity of electricity, n is the charge of the redox reaction (in this case, $n = 2$), F is the Faraday constant and A is the area of the electrode surface (in this case, $A = 0.07$ cm^2).

Electrochemical responding of cDNA-MB/chitosan/GCE to TC

TC is a broad spectrum antibiotics and known to interactively bind to DNA.[35] However, in the present potential range (from –0.4 to 0.1 V vs. Ag|AgCl), neither pristine TC nor DNA-TC complex gave any detectable electrochemical signal at a bare GC electrode (data not shown). Interestingly, in the case of cDNA-MB/chitosan/GCE, obvious peak current density reduction (both anodic and cathodic) was observed in 10 seconds after the addition of TC (Figure S2) (Supporting Information). The decreased current density depended on the concentration of TC added. Control experiments without DNA shown no statistical significance current-change after the addition of TC means the absence of interaction between TC and MB (data not shown). These results indicated the interactions between TC and DNA, and the signals were well amplified by those intercalated MB which act as electrochemical active indicators.

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DPV is a more sensitive electrochemical method than CV. The current responses of cDNA-MB/chitosan/GCE to TC, therefore, were further monitored by DPV (Figure 2 A). As expected, after the addition of TC, a much more obvious peak current reduction on the prepared cDNA-MB/chitosan/GC electrode was observed. With the TC concentration (c_{TC}) from 0 to 0.5 mM, the peak current density (at −0.2 V vs. Ag/AgCl) decreased from 5.5 ± 0.2 to 4.5 ± 0.2 μA cm^{-2} (Figure. 2 A). Finally, the added 2.50 mM TC caused a decrease percentage of peak current density (D_{pc},%) of more than 50.

To confirm the essential of DNA in the modified electrode, hDNA, sDNA and poly-L-lys were also utilized respectively to fabricated different polyion complex membrane modified electrodes. Figure S2 (Supporting Information) compares the electrochemical responds of these modified electrodes to 2.5 mM TC. Almost no peak current density change was observed at poly-L-lys-MB/chitosan/GC electrode indicated the absence of interaction between TC and the modified electrode. On the other hand, at all DNA-based modified electrodes, the addition of drug induced obvious peak current density reduction, among which, however most significant at cDNA-based modified electrode was observed. DNA from different sources must have different nucleotide sequence,[36] which might affect the bind of MB as well as the drug (here, refer to the TC). We just simply reported the result in the present paper. However, studies on the investigation of details about sequence specify of DNA to MB and TC bound are still on the way, and synthesis of a specific poly-nucleotide sequence with high response to TC is expected in the future.

The prepared cDNA-MB/chitosan/GCE therefore is suitable for rapid TC detection. Considering the fact that the composition of modified electrode may affect the biosensing

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performance, the ratio of MB to cDNA was examined. cDNA monomer unit was calculated to be 330 as average molecular weight of nucleotide one base unit. The mole ratios of MB monomer unit to cDNA monomer unit were set as 0.03, 0.06, 0.24, 0.48 and 1.20. Figure S3 (Supporting Information) shows the effect of the MB/DNA ratio on the initial peak current densities and the current response to 2.5 mM TC. Both initial peak current densities and the current response increased with the increase of MB/DNA ratio and reached plateau at the ratio more than 0.48. Figure 2B shows the dependency of D_{pc} to 2.5 mM TC on the MB : cDNA ratio at modified electrode. As a result, the maximum D_{pc},% value of 71 ± 1 was obtained at the MB : cDNA ratio of 0.48.

Attempting to understand the interaction between cDNA-MB and TC

The reduction of peak current density, therefore was proposed to be caused by the addition of TC, which interact with cDNA by following possible ways: 1) broken the DNA helix thus the intercalated MB was released to electrolyte; 2) bind to cDNA and block the electron transfer between MB and electrode;[26] 3) competitively bind to cDNA so that MB was replaced and released to electrolyte.[22] Unfortunately, no more details could be obtained from the current electrochemical experiments, therefore, UV-vis spectroscopy was carried out to deeply investigate the interaction between TC and cDNA-MB complex.

Figure 3 shows the absorption spectrum of free MB (curve a), cDNA-MB complex in the absence (curve b) and presence of TC (curve c and d) in the phosphate buffer (0.1 M, pH 7.0), respectively. In the case of free MB (0.01mM), an undisturbed absorption peak was observed at 664 nm, where both cDNA and TC do not absorb appreciably (Figure S4) (Supporting

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Information). Therefore, this peak was selected to study the interaction between cDNA-MB and TC. As shown in Figure 3, as 0.01mg mL^{-1} cDNA was added into above MB solution, the absorbance at 664 nm sharply decreased from 0.93 to 0.35 (curve b). According to the literature, such obvious hypochromic effects should be ascribed to be the intercalation of MB into cDNA.[34] However, the absorbance at 664 nm back to 0.63 after the addition of 0.25 mM TC into the above cDNA-MB solution, and as the concentration of TC reached to 2.5 mM, the absorbance at 664 nm recover to the initial level of only free MB exist. Neither obvious peak-shift nor new absorption peak was observed implied no byproduct generation, therefore, such hyperchromic effect might be owing to the increase of free MB in the solution.

Taken together, extra addition TC could induce the release of MB from DNA-MB complex then cause the peak current reduction of cDNA-MB/chitosan/GCE and the absorbance increase at 664 nm in solution. As mentioned above, MB release from DNA-MB complex could be two possible ways: 1) TC induce the DNA helix broken thus MB was free; and 2) intercalated MB was replaced by TC. According to the literature, TC also intercalative binds to DNA and form a TC-DNA binary complex.[35] Therefore, we proposed here that (a part of) TC might bind to DNA at the same site of the indicator-MB and compete with MB for the DNA binding site which induces the release of MB. The similar replacement effect of DNA-binding compounds on the electrochemical response of electrochemical indicator has been reported.[22, 23]

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cDNA-MB/chitosan/GCE for rapid POCs screening

The proposed cDNA-MB/chitosan/GCE electrodes therefore were utilized for rapid

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detection of POCs. The characteristics of the biosensor were investigated by DPVs under the optimum conditions obtained above. Figure 4 shows the dependence of the peak current density reduction (at −0.2 V) on the concentration of different POCs. For all three POCs examined, the addition of drugs induced obvious peak current decrease although the sensitives are different. As a result, in the present conditions, D_{pc},% of 99, 95 and 76 were obtained for 1 mM of EB, QC and TC, respectively. The limited detection concentration of the three kind of POCs was 5 μM (S/N > 3), which is comparable to that of the previous reports.[10, 26] On the other hand, almost no signal could be measured for several non-POCs indicated that the proposed DNA biosensor of high selectivity and anti-interference (Figure 5).

The long-term storage stability of the prepared biosensor was investigated by stored at 4 °C for two weeks. The current response to 1 mM TC still remained above 90%. The relative standard deviation (LSD,%) of three independent prepared biosensors response to 1 mM TC was 5.1%. The results verify the acceptable stability and reproducibility of the DNA-based polyion complex membrane biosensor.

Conclusion

In summary, a simple and sensitive electrochemical method was proposed for rapid detection of non-electroactive poly-cyclic organic compounds (POCs). In this method, a polyion complex membrane modified electrode was firstly constructed based on the electrostatic interaction between DNA and chitosan. Here, DNA acts as bio-recognized reagent for selective combine to POCs. MB was employed as electrochemical active indicator to amplify

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the interaction between DNA and POCs. Competitive interaction and displacement effects between POCs and MB bind to DNA was proposed and investigated by UV-vis spectroscopy. The finally optimized DNA-MB/chitosan/GC electrode exhibited high stability, high selectivity to POCs and anti-interferences to common non-POCs.

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Supporting Information

Additional electrochemical and spectroscopic data are provided in the supporting information. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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Figure Captions

Figure 1 A) CVs of bare GCE (a), DNA/chitosan/GCE (b), and DNA-MB/chitosan/GCE (c) with υ= 50 mV s^{-1}. B) The plot of peak current density (I) against scan rate (υ) for DNA-MB/chitosan/GCE. All measurements were carried out in N_2-saturated phosphate buffer (0.1 mM, pH 7) and under room temperature.

Fig. 2 A) DPVs of DNA-MB/chitosan/GCE in pH 7 phosphate buffer (0.1 mM) containing different TC concentration (from a to g are 0, 0.25, 0.50, 1.00, 1.50, 2.00, 2.50 mM, respectively). B) Dependence of $D_{pc,}$ to 2.5 mM TC on the MB/DNA ratio. All measurements were carried out in N_2-saturated phosphate buffer (0.1 mM, pH 7) and under room temperature.

Fig. 3 UV-vis characterization of different solution. Curves: (a) free MB ; (b) DNA+MB; (c) DNA+MB added TC with concentration of 0.25 mM and (d) 2.50 mM,. The concentrations of DNA and MB in all solutions are 0.01 mg mL^{-1} and 0.01 mM.

Fig. 4 Dependence of D_{pc} on the concentration of TC, EB and QC.

Fig. 5 Response comparison of DNA-MB/chitosan/GCE for screening 6 kinds of representative analytes. Six kinds of compounds, from 1 to 6, were TC, glucose, uric acid, ascorbic acid, NaCl and ethanol, respectively. The concentration of TC was 1 mM and others were 2.5 mM.

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Figure 1

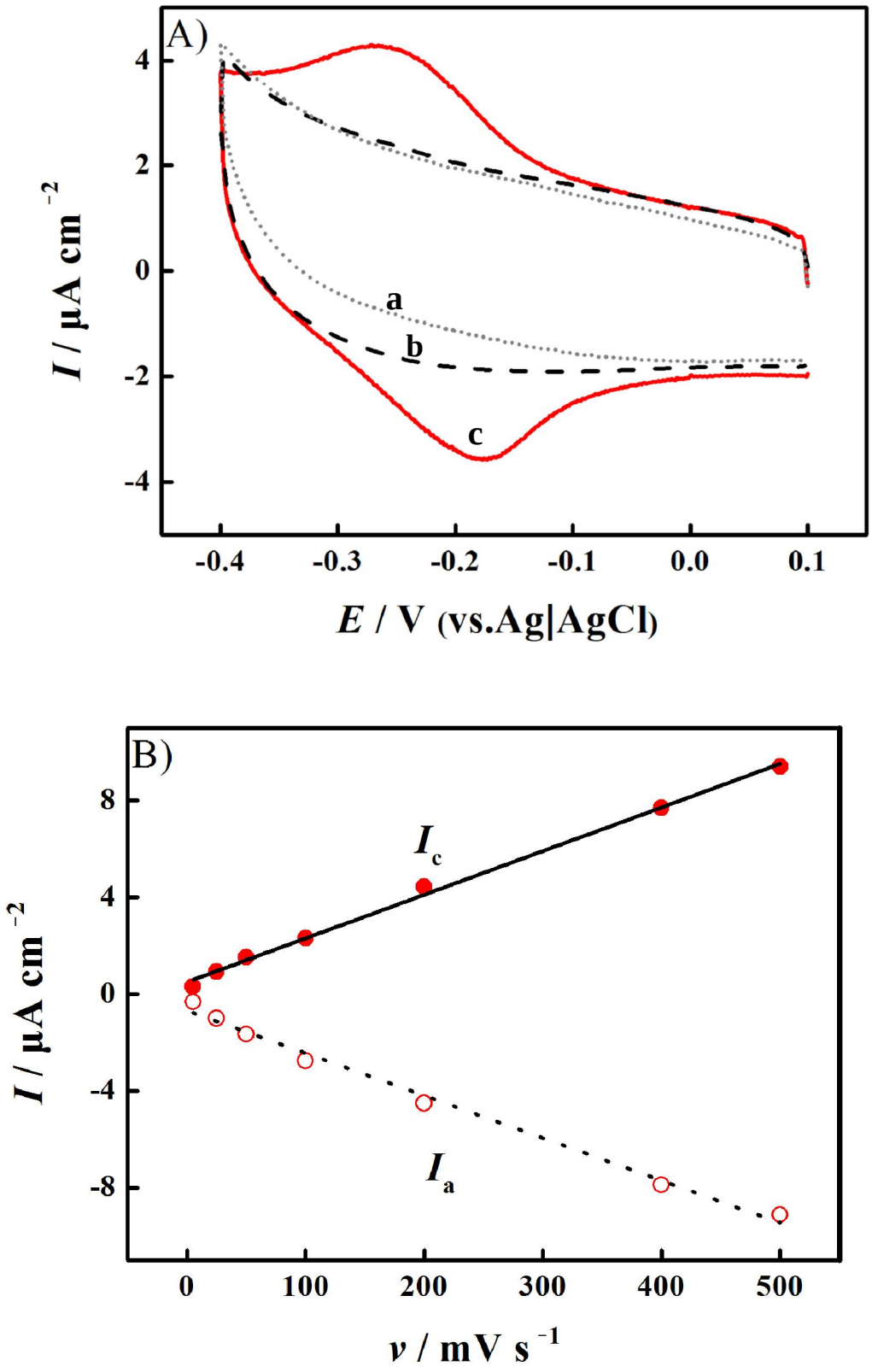

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Figure 2

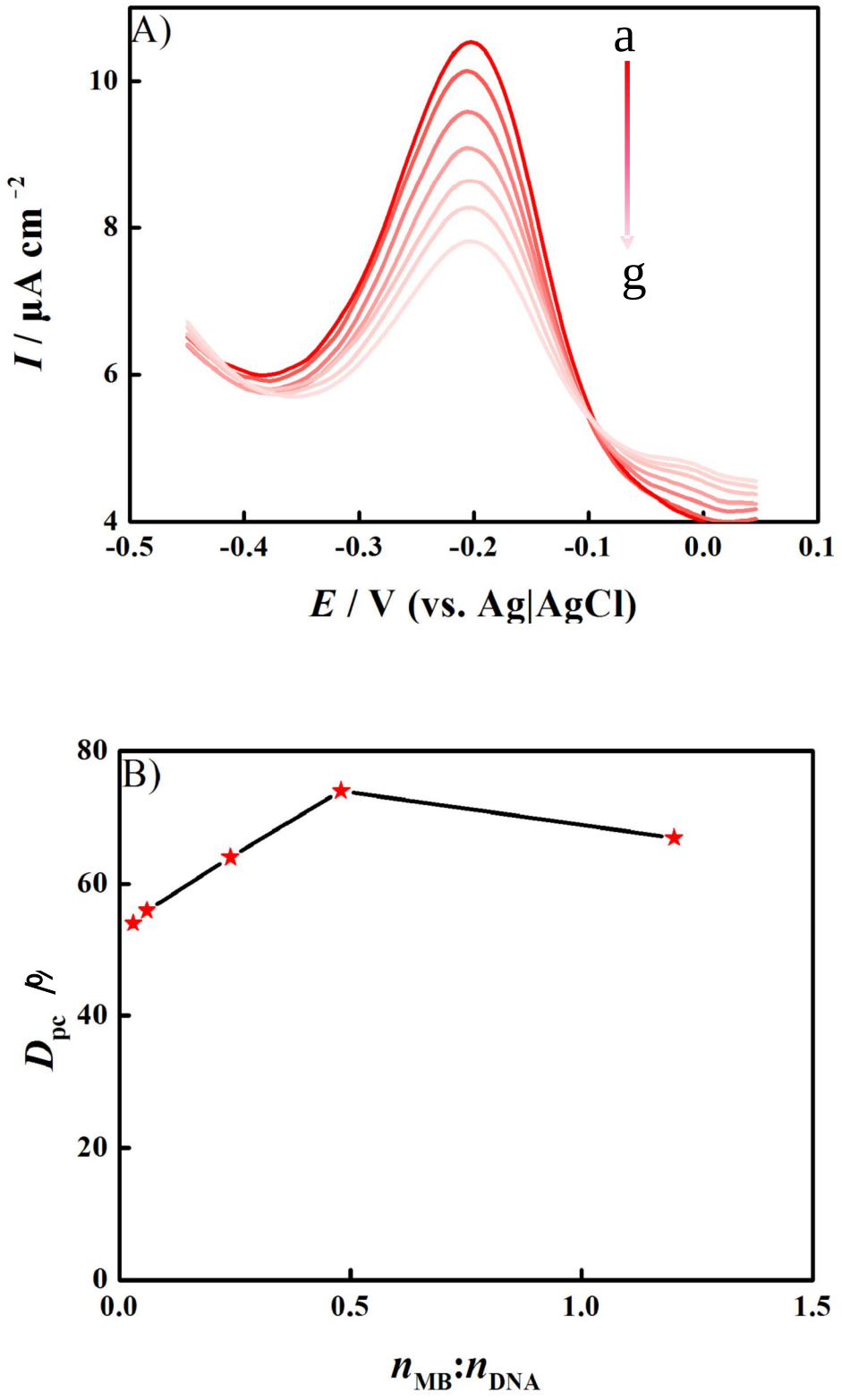

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Figure 3

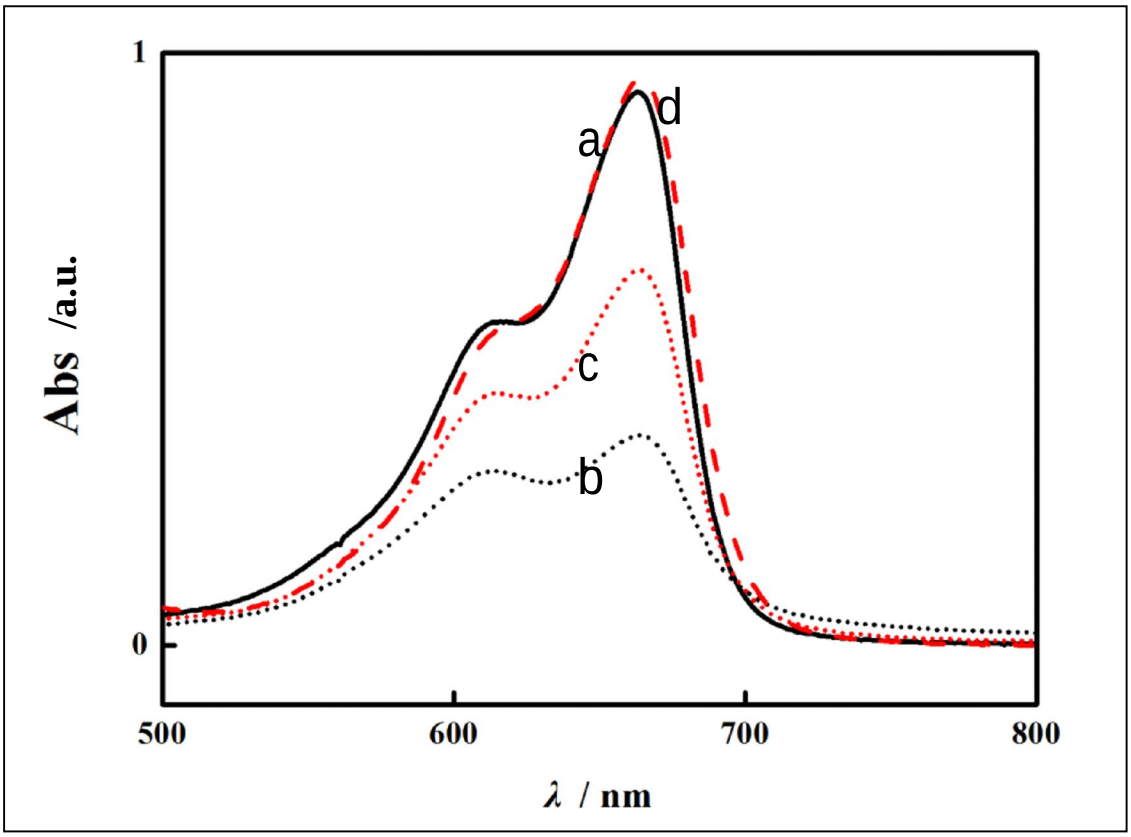

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Figure 4

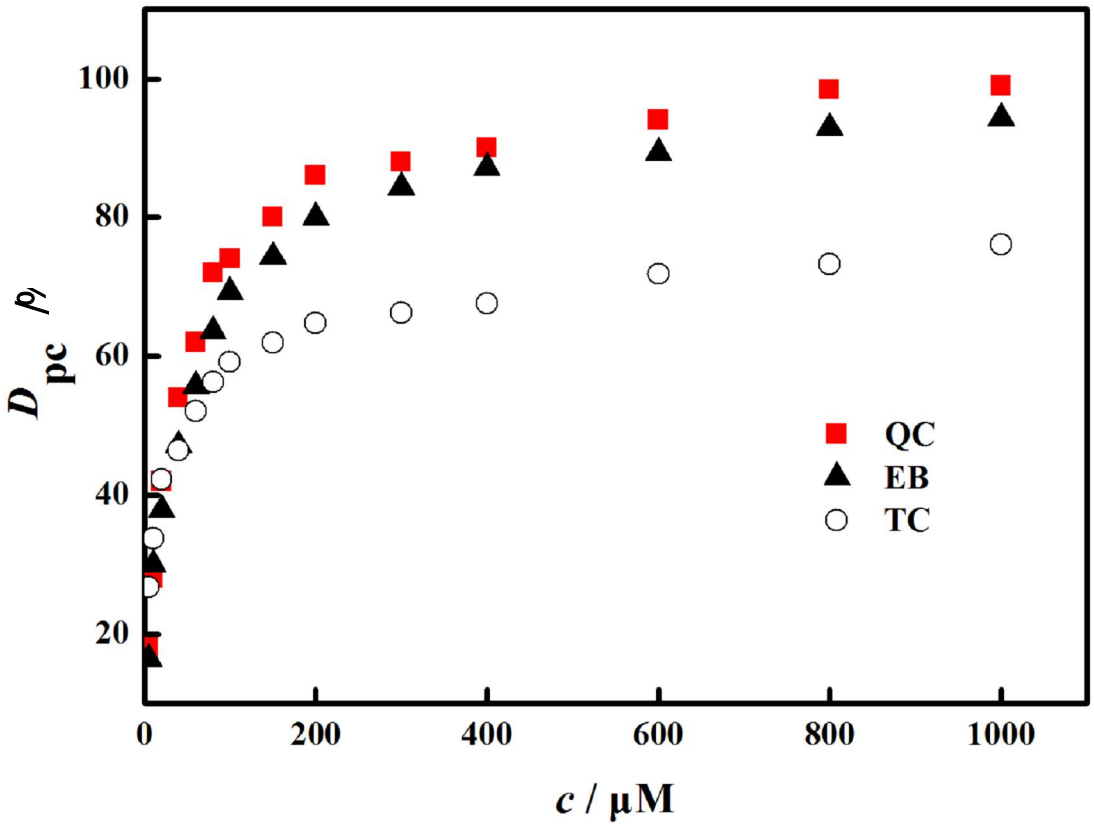

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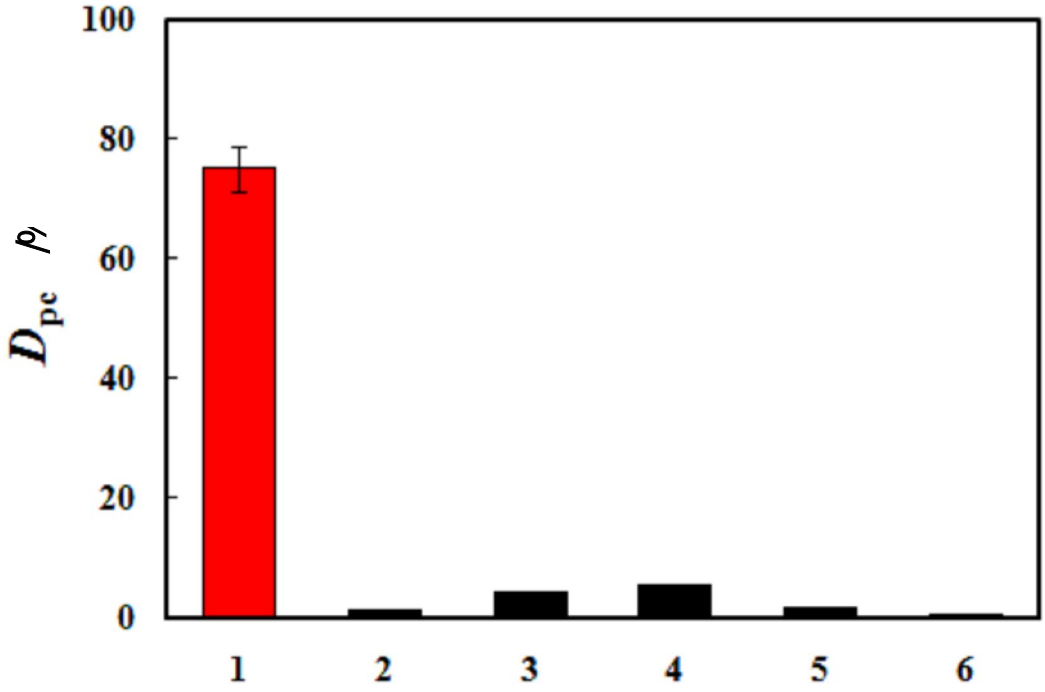
Figure 5



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