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Novel amperometric assay for drug–DNA interaction based on an inhibitory effect on an electrocatalytic activity of DNA–Cu(II) complex

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ABSTRACT

A novel strategy of amperometric assay for drug–dsDNA interactions was developed based on an inhibitory effect of antimararial drug (quinacrine) on an electrocatalytic activity of DNA–Cu(II) complex. In this method, a DNA–Cu(II) complex immobilized DNA/polyallylamine(PAA) polyion complex membrane was used as a sensing element. The electrocatalytic activity of a DNA–Cu(II) complex for hydrogen peroxide reduction was reversibly inhibited by electron blocking effect of quinacrine–dsDNA interaction and this inhibitory effect was amplified by the hydrogen peroxide reduction. This phenomenon was utilized for development of a novel amperometric biosensor for DNA-binding drug. From the amperometric current–time curves, the response time of the sensor to 20 μM quinacrine was obtained about 20 s, and the detection limit of the quinacrine was found to be 10 μM estimated to a signal-to-noise ratio of 3.0. Based on the change of steady-state catalytic current, the kinetic analysis of drug–dsDNA interaction can be done in a similar manner of enzyme inhibition, and the binding constant of the quinacrine with DNA can be calculated. This measurement method would be useful for screening of wide variety of DNA-binding drugs and highly toxic pollutants.

1. Introduction

Interaction between DNA and other molecules is an important subject in life science, for example, transcription and transformation of DNA in vivo, mutation of genes, and function of some DNA-targeting drugs. In order to evaluate these interactions, a number of methods have been used so far, i.e., footprinting, affinity cleavage, UV/visible spectroscopy, Raman spectroscopy, NMR, X-ray crystallography, calorimetric method, quartz crystal microbalance (QCM), surface plasmon resonance (SPR) and fluorescence, etc. (Dervan, 2001; Graves and Velea, 2000; Tse and Boger, 2004; Boger et al., 2001). On the other hand, electrochemical detection offers great advantages, such as rapid, simple, and low-cost. The electrochemical DNA biosensors using DNA-probe modified electrodes are new research area in analytical chemistry (Palecek and Fojta, 2001; Wang, 2000; Drummond et al., 2003). A number of single-stranded DNA-probe-modified electrodes have been proposed for detecting sequence-selective hybridization and point mutation of gene (Wang, 1999; Gooding, 2002; Ihara et al., 1997; Kelley et al., 1999). Based on the molecular recognition property of double stranded DNA, several kinds of electrochemical DNA-biosensors were proposed for detecting some DNA-binding compounds, such as intercalating compounds, a toxin, hydrazine compounds, metals and an antibody (Erdem and Ozsoz, 2002; Yang et al., 1998; Pang and Abruna, 1998; Wang et al., 1996a, 1996b; Maeda et al., 1992, 1994; Nakano et al., 1997; K'Owino et al., 2003).

Differing from the enzyme sensors, DNA itself generally has no catalytic activity (except for deoxyribozyme, ribozyme). It is important to know how the DNA–analyte interaction is translated into output sensor signals. Especially, in the case that the target species are not redox active, the signal transduction strategy of DNA–analyte interaction is important for design and construction of the dsDNA-based electrochemical biosensors. Wang et al. reported the novel DNA biosensor for quantitative detection of highly toxic hydrazines and aromatic amines, based on monitoring changes in the potentiometric stripping peaks associated with the oxidation of the guanine residue (Wang et al., 1996a,b). They used dsDNA-coated carbon paste electrode, and this method required no label or indicator. Compared to potentiometric stripping analysis, cyclic voltammetry is more popular electrochemical technique. Maeda et al. firstly proposed a strategy of voltammetric dsDNA-biosensor for DNA-binding drug and metal ions, based on "ion channel effect" (Maeda et al., 1992, 1994; Nakano et al., 1997). They synthesized thiol-modified dsDNA, and dsDNA-modified electrode was prepared based on a sulfur-gold linkage. In this sensor, the

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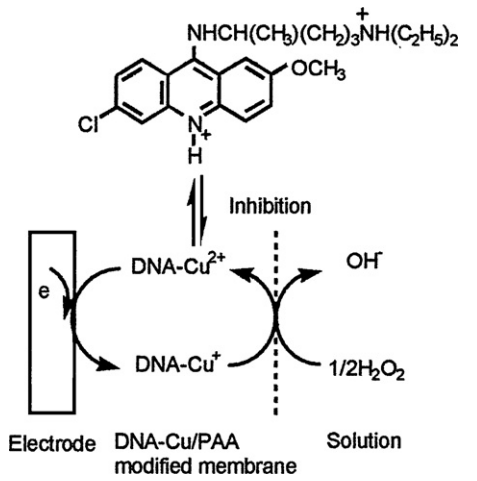

Scheme 1. Electron transfer scheme of DNA–Cu(II)/PAA/GC electrode catalyzed hydrogen peroxide reduction in the presence of DNA-binding drug (quinacrine).

binding of positively charged analytes leads to increasing in a permeability of negatively charged reporter molecules [$Fe(CN)_6^{4-/3-}$] through a DNA-layer. K'Owino et al. (2003) reported another strategy of votammmetric dsDNA biosensor for cisplatin, which was based on "site blocking effect" on an electron transfer from DNA-bound Ag^{+1} to the electrode surface. They modified biotin-tagged DNA onto the avidine-adsorbed Ag-deposited Au electrode. More recently, Wang et al. (2008) developed a "electrochemical displacement method" for the investigation of polycyclic organic DNA binders. In this method, a DNA film was deposited on an indium tin oxide electrode surface, and an electroactive DNA intercalator $Ru(bpy)_2$-(dppz)$(BF_4)_2$(bpy) 2,2′-bipyridine, dppz) dipyrido [3,2-a:2′,3′-c] phenazine) used as a indicator competes with an polycyclic organic compound in the same binding site of DNA, resulting in a reduction of its electrochemical signal. However, these methods require pre-modification of DNA (SH and avidine) and/or activation of the electrode surface, thus the preparation protocol is relatively complicated.

It is known that enzyme is frequently inhibited selectively by low concentrations of certain chemical substances: for example, acetylcholine esterase is inhibited by some kinds of pesticides (Liu and Lin, 2006); tyrosinase is inhibited by organophosphate pesticides (Tanimoto de Albuquerque and Ferreira, 2007); peroxidase is inhibited by cyanide (Shan et al., 2004); and laccase is inhibited by azide (Trudeau et al., 1997). Based on these inhibitory effects, various enzymatic sensors have been commonly used for inhibitor determinations (Amine et al., 2006). In previous work, we found that DNA intercalator can inhibit the thiol oxidation catalyzed by the DNA–Cu(II) complex, and an oxygen electrode-based DNA-binding drug sensor was developed (Hasebe et al., 1997). However, the response time of oxygen electrode was relatively slow and the drift of current baseline affects the sensitivity. In addition, the oxygen concentration in the sample seriously influences upper limit of linearity. If the electrochemical signal can be obtained directly from the DNA–Cu(II) complex, the development of a novel electrocatalytic DNA-based biosensor for DNA intercalator would be expected.

In this paper, we report a novel amperometric assay of drug–dsDNA interactions based on the catalytic activity of a DNA–Cu(II) complex-catalyzed charge transport (shown in Scheme 1). DNA–Cu(II) complex was immobilized on a glassy carbon electrode surface by the polycations materials of poly(allylamine). This method does not require any modification of DNA and an electrode surface. Thus the preparation of the sensor is much easy as compared with other voltammetric DNA biosensors. As reported previously, this DNA–Cu(II)/PAA membrane-modified GC electrode (DNA–Cu(II)/PAA/GC electrode) exhibited an excellent electrocatalytic activity for the reduction of hydrogen peroxide (H_2O_2), based on $Cu^{2+/1+}$-based catalytic mechanism (Hasebe and Gu, 2005; Gu and Hasebe, 2006). We found that the steady-state current response of the DNA–Cu(II)/PAA/GC electrode for H_2O_2 reduction was reversibly diminished by quinacrine, which is a typical DNA-binding antimalarial drug. This behavior allowed not only quantitative detection of quinacrine but also kinetic analysis of quinacrine–dsDNA interaction based on a steady-state current, in a similar manner of interference-based enzyme electrode for enzymatic inhibitors.

2. Experimental

2.1. Reagents and materials

The reagents and chemicals used in this investigation are as follow: double-stranded DNA (from Salmon testes, average molecular weight, ca. 200–3000 bp: evaluated by agarose-gel electrophoresis) was obtained from Worthington Biochemical Co. $CuCl_2{\cdot}2H_2O$, and 30% hydrogen peroxide were purchased from Wako. Quinacrine was purchased from Sigma. Poly(allylamine) hydrochloride (PAA; average M_w ~10,000) was purchased from Nittobo Co. Poly-L-glutamic acid sodium salt (M_w 17,000) was purchased from Sigma-Aldrich Co. All the other chemicals were of analytical grade and used without further purification. Double-distilled water was used in preparing buffer and standard solutions. A solution of H_2O_2 was prepared daily.

2.2. Preparation of DNA–Cu(II)/PAA/GC electrode

The DNA–Cu(II)/PAA/GC electrode was prepared in similar manner previously reported by us (Hasebe and Gu, 2005). 1 mg/ml dsDNA and 3 mM $CuCl_2$ were mixed for 1 h. Then 40 μl of DNA and $CuCl_2$ mixture and 20 μl of 1 mg/ml PAA were placed on the electrode surface. The electrode was kept for one day under a 500 ml beaker at room temperature (~20 °C). Before used the electrode was soaked in 5 mM phosphate buffer (pH 7.0) for 1 h. The electrode was stored in the refrigerator at 4 °C when not in used.

2.3. Electrochemical measurements

Cyclic voltammetric (CV) measurements were carried out with a CHI 612A workstation (CHI Instruments, Inc.) using a three electrodes system. The working electrode was a resulting DNA–Cu(II)/PAA/GC electrode. A platinum wire electrode (1 mm diameter) and an Ag/AgCl (sat. KCl) electrode was used as auxiliary and reference electrodes, respectively. The constant-potential amperomeric measurements were carried out by using potentiostat (LC4C/BAS) equipped with X-t recorder with stirring the 10 ml of electrolyte solution gently. All electrochemical experiments were carried out in 10 ml of 5 mM phosphate buffer (pH 7.0) and the solutions were thoroughly deoxygenated by bubbling nitrogen though the solution for at least 10 min.

2.4. Quartz crystal microbalance measurements

Quartz crystal microbalance (QCM) measurement was performed with a quartz chemical analyzer (QCA917, SEIKO EG&G) at 9 MHz using A-T cut quartz (diameter, 5 mm) and an Au electrode (area, 0.2 cm^2). The DNA–Cu(II)/PAA, DNA/PAA and poly-glutamate/PAA polyion complex membranes for QCM measurements were prepared on the Au electrode surface in similar manner in of preparation of the DNA–Cu(II)/PAA/GC electrode.

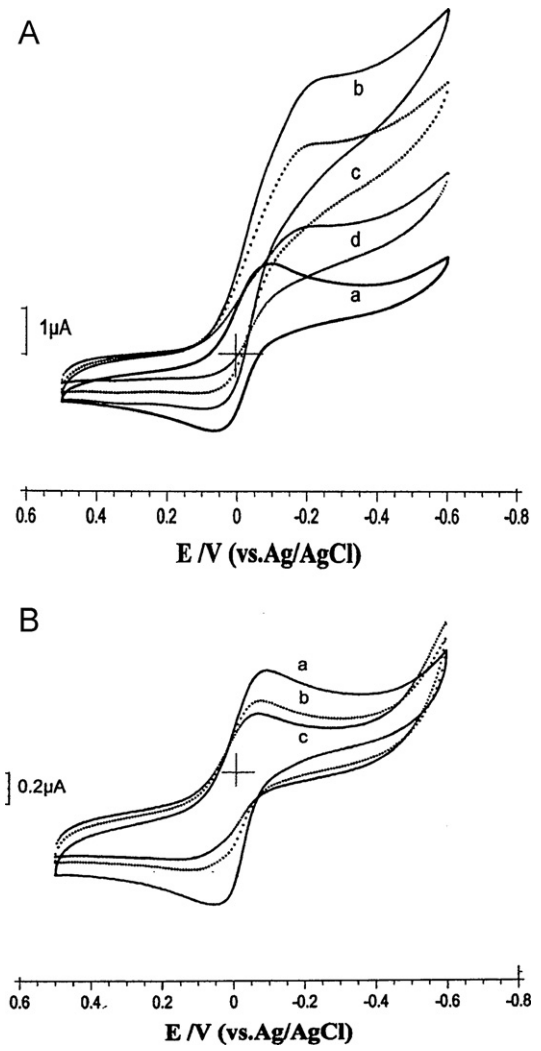

Fig. 1. Cyclic voltammograms of the DNA–Cu(II)/PAA/GC electrode in deoxygenated 5 mM phosphate buffer solutions (pH 7) before (B) and after (A) the addition of 100 μM H_2O_2 (b–d). (A) Quinacrine concentration was 0 (a and b), 200 μM (c) and 500 μM (d). (B) Quinacrine concentration was 0 (a), 200 μM (b) and 500 μM (c). Scan rate was 50 mV/s.

2.5. Circular dichroism spectra

Circular dichroism (CD) spectra of the DNA–Cu(II)/PAA film in the presence of quinacrine was measured by using a JASCO J-725 spectropolarimeter ($\Delta\lambda$ resolution, 1 nm at 25 °C; JASCO., Tokyo). The optical density of the samples varied between $0.4 \leq OD \leq 0.7$ at λ_{max} 204–310 nm. The DNA–Cu(II)/PAA film for the CD measurement was prepared on a quartz plate surface in similar manner of preparation of the DNA–Cu modified membrane. The film was soaked with quinacrine solution for one hour before the CD measurements.

3. Results and discussion

3.1. Electrochemical behaviors of DNA–Cu(II)/PAA/GCE binding with quinacrine

It is known that copper ions bind strongly to dsDNA and form stable DNA–Cu(II) complex (Bregadze, 1996; Gao et al., 1993; Geierstanger et al., 1991). Sigel et al. have already reported that the DNA–Cu(II) complex has "peroxidase-like" catalytic activity (Sigel et al., 1967). Fig. 1A shows the cyclic voltammograms of the DNA–Cu(II)/PAA/GC electrode in deoxygenated 5 mM phosphate buffer solutions (pH 7.0). A well-defined and reversible redox couple observed at 0 V vs. Ag/AgCl (line *a*) was attributed to Cu(II)/Cu(I) redox couple (Gu and Hasebe, 2006). When 0.1 mM H_2O_2 was introduced into the solution, the CV response changed; significant increase of cathodic current and completely disappearance of cathodic current (line *d*). This was attributed to electrocatalytic reduction of H_2O_2 as reported previously (Gu and Hasebe, 2006). This catalytic current diminished in the presence of quinacrine (line *c* and *d*), suggesting that the quinacrine prohibited the electrocatalytic activity of the DNA–Cu(II)/PAA/GC electrode. Fig. 1B shows the cyclic voltammograms of the DNA–Cu(II)/PAA/GC electrode without H_2O_2. The peak currents of Cu(II)/Cu(I) redox couple without H_2O_2 also decreased in the presence of quinacrine, but the current change was relatively small and un-definitive as compared with that of the catalytic current, which indicate that the blocking effect of quinacrine on the charge transfer of copper ion was amplified via the catalytic cycle associated with the H_2O_2 reduction. The use of "blocking effect" of DNA-intercalators upon the "electrocatalytic activity" of DNA-bound metal ion has not been reported until now in our knowledge.

3.2. QCM study on quinarine intercalation with DNA–Cu(II)/PAA membrane

Quinacrine is a well-known antimalarial drug that strongly intercalates into the DNA base stack (Nastasi et al., 1976; Wilson and Lopp, 1979). There have been several reports on the intercalation of various dye molecules to self-standing dsDNA–cation complex films, including the cast film of the DNA-lipid (dialkylamphiphile) complex (Okahata et al., 1993) and layer-by-layer dsDNA/PAA film (Lang and Liu, 1999; Jiang and Liu, 2004). We confirmed the binding of quinacrine to DNA–Cu(II)/PAA films by quartz crystal microbalance. After resonance frequency (RF) had reached to a steady-state value in aqueous solution in gentle stirring, concentrated quinacrine stock solutions were added to the solution in a stepwise manner. Fig. 2A shows the relationship between the frequency change and quinacrine concentration obtained by measuring the DNA/PAA, DNA–Cu(II)/PAA and poly-glutamate/PAA membrane. For the DNA–Cu(II)/PAA and the DNA/PAA membrane, the RF decreased by each addition of quinacrine (0.2 mM). The saturated behavior was observed at quinacrine concentration more than 1 mM. Narrow linear range of the DNA–Cu(II)/PAA membrane may be attributed to the limited binding site of quinarine as compared with the DNA/PAA membrane. In contrast, control experiments using a poly-glutamate/PAA membrane exhibited no apparent response. From these results, it indicated that quiacrine binds with DNA in the DNA film, and it is reasonable to assume that the inhibitory response of the DNA–Cu(II)/PAA-GC electrode as shown in Scheme 1 is originated from the binding interaction of quniacrine to DNA.

3.3. Amperometric response of quinacrine

Compared with the cyclic voltammerty, constant-potential amperometry is more attractive for an evaluation of kinetics of continuous inhibition properties. Fig. 3A shows typical current–time profile of the DNA–Cu(II)/PAA/GC electrode at a fixed applied potential of −0.2 V (vs. Ag/AgCl). After the current baseline had been established with buffer solution, 100 μM of H_2O_2 was added into the measuring cell. Then, aliquot of stock solution of quinacrine was added stepwise to electrolyte buffer. The addition of quinacrine (20 μM) induced a decrease in the current, which reached to another steady-state current after 20 s. A saturated behavior was observed at quinacrine concentration

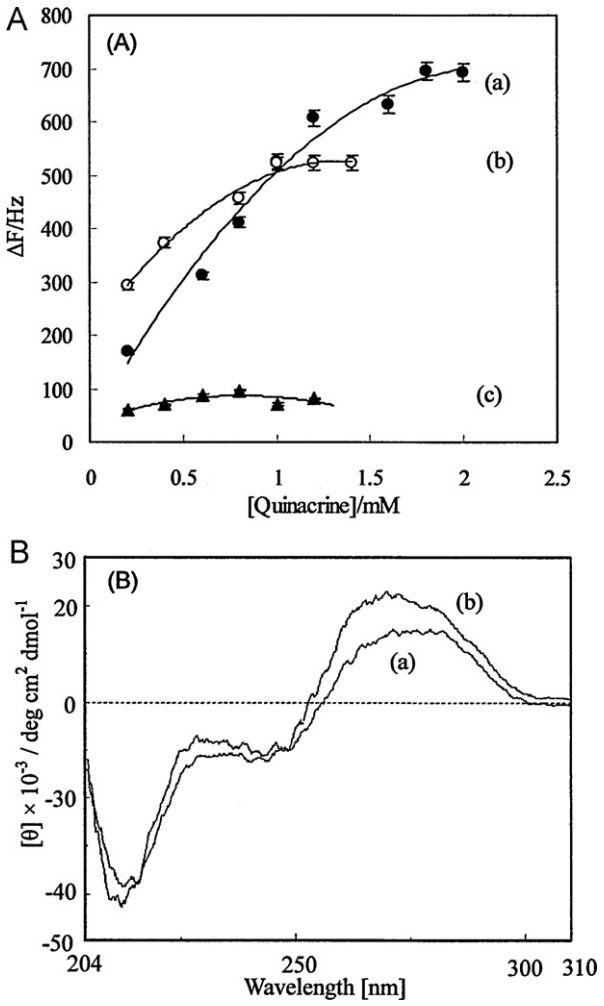

Fig. 2. (A) The relationship between the frequency change and quinacrine concentration obtained by measuring the DNA/PAA (a), DNA–Cu(II)/PAA (b) and poly-L-glutamic acid-PAA (c) membrane. (B) CD spectra of the DNA–Cu(II)/PAA membrane in the absence (a) and the presence (b) of 20 μM quinarine in wet state.

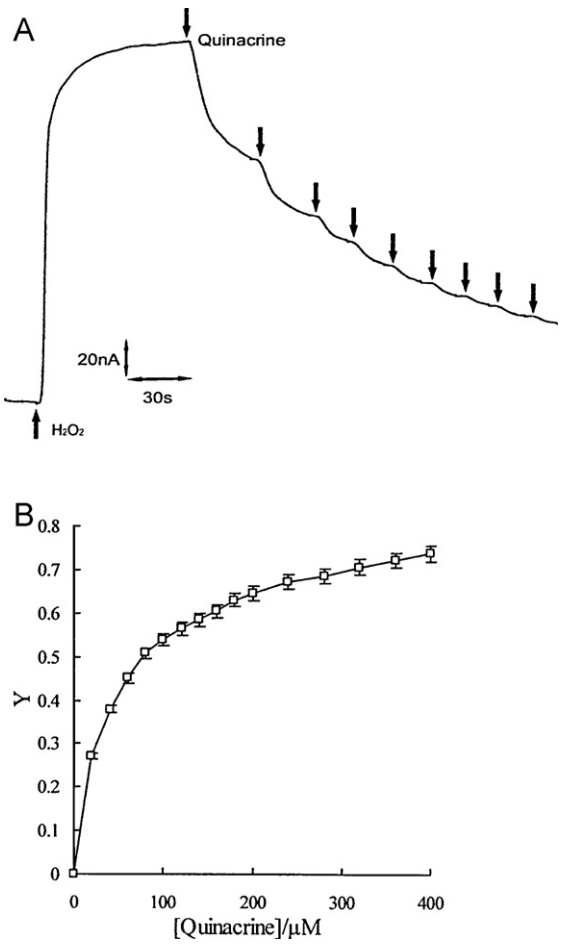

Fig. 3. (A) Steady-state current response of the DNA–Cu(II)/PAA/GC electrode upon the successive addition of 20 μM quinacrine in the presence of 100 μM H_2O_2. (B) Calibration graph for detecting quinarine, plotting the *Y* value against the quinacrine concentrations. The *Y* value (% inhibition) is a ratio of the current decrease to the original current (no inhibition) under the steady-state.

more than 300 μM. The electrode was then transferred to fresh buffer, and the response for same concentration of H_2O_2 was measured. It was found that about 88% of initial catalytic current was recovered, which is similar to the result that without quinacrine (Gu and Hasebe, 2006). This result indicates that the inhibition of quinacrine was reversible. Fig. 3B plots the ratio of the current decrease to the original current (no inhibition) (*Y*) against the quinacrine concentration. A 50% inhibition was observed at quinacrine concentration ca. 80 μM. The detection limit of the quinacrine was found to be 10 μM estimated to a signal-to-noise ratio of 3.0 and the relative standard deviation (RSD) for three replicate determinations of 20 μM quinacrine was 1.89%.

Similar experiments were done in the presence of different concentration of H_2O_2 (50, 100, 200 and 500 μM). The inhibition current of quinacrine is depended on the substrate concentration, while inhibition degree (*Y*) is not influence by substrate concentration (Fig. 4A). The inhibitory behaviors seemed essentially similar in all cases, in spite of the difference in H_2O_2 concentrations, and 50% inhibitions were obtained at quinacrine concentrations from 50 μM to 80 μM. Fig. 4B shows the quinacrine inhibition degree in presence of 0.1 mM H_2O_2 by using DNA–Cu(II)/PAA membrane with different concentration of copper ion. Both the substrate (H_2O_2) and inhibition degree increases upon increasing the copper ion concentration in the film, indicating that the inhibition effect of quinacrine on the H_2O_2 reduction reaction was proportional to the catalytic activity of the DNA–Cu complex.

3.4. Kinetic study

To obtain further information about the inhibition mechanism of quinacrine on the electrocatalytic activity of DNA–Cu(II) complex, enzyme-kinetics was applied to this system, although the reaction mechanism of this DNA–Cu(II)/PAA/GC electrode is not necessarily the same as conventional enzyme-electrodes. The amperometric responses of the DNA–Cu(II)/PAA/GC electrode were measured as a function of H_2O_2 concentration for several concentrations of quinacrine. Fig. 5A shows the electrochemical Lineweaver–Burk plots (double-reciprocal plots, $1/I$ vs. $1/[H_2O_2]$) corresponding to 0, 10, 40, 100 μM of quinacrine. *I* is the steady-state current. The slope of the fitting straight line increased with increasing in the quinacrine concentration, and each line seemed to cross at *X* axis. These findings suggest that quinacrine inhibits the electrocatalytic current response according to a non-competitive-type inhibition pattern, which the inhibition does not affect substrate binding, but the inhibitor binding hampers catalysis (Tipton, 1996). This assumption is not in conflict with the result that 50% inhibition scarcely depended on the concentration of H_2O_2.

Fig. 5B illustrates the electrochemical Dixon plot (relationship between the quinacrine concentration and the reciprocal of

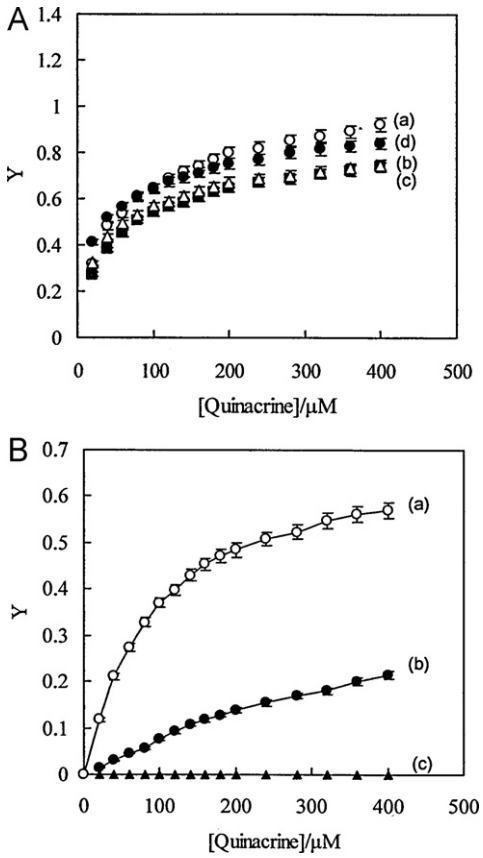

Fig. 4. (A) Relationship between the *Y* value and quinacrine concentration in the presence of 0.05 mM (a), 0.1 mM (b), 0.2 mM (c) and 0.5 mM (d) H_2O_2 on successive additions of 20 μM quinarine. (B) Relationship between the *Y* value and quinarine concentration in the presence of 0.1 mM H_2O_2 for DNA–Cu(II)/PAA/GC electrode prepared by using $CuCl_2$ concentration of 0.1 mM (a), 1 mM (b)and 3 mM (c). Other experimental conditions are same as Fig. 3.

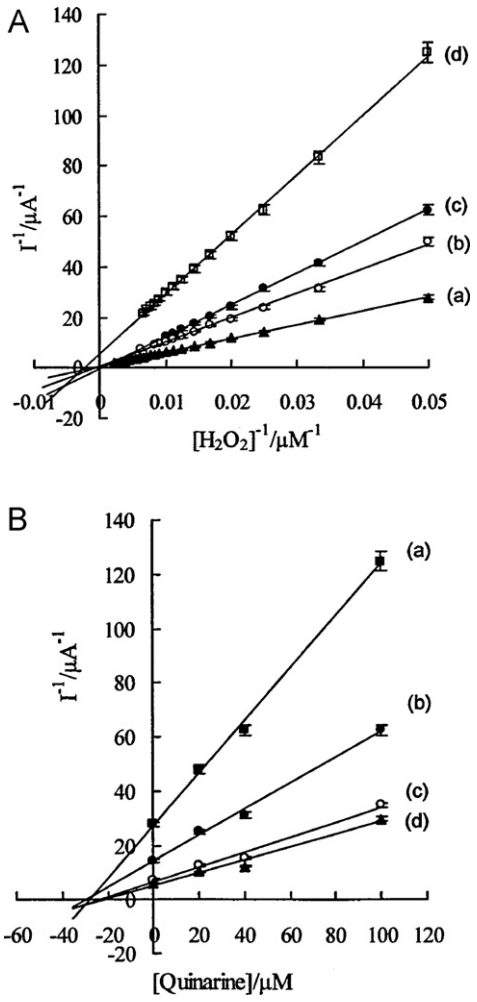

Fig. 5. (A) Electrochemical Lineweaver–Burk plots for the DNA–Cu(II)/PAA-GC electrode with H_2O_2 as a substrate, without quinarine (inhibitor) (a), with 10 μM (b), 40 μM (c), and 100 μM (d) quinacrine. (B) Electrochemical Dixon plots for the DNA–Cu(II)/PAA/GC electrode. The concentrations of H_2O_2 are 20 μM (a), 40 μM (b), 80 μM (c) and 150 μM (d). Other experimental conditions are same as Fig. 3.

the catalytic current for H_2O_2). From the intersection of approximation straight lines on *X* axis, an apparent inhibition constant (K_i) of the quinacrine to the DNA–Cu(II)/PAA film was calculated to be 2.56×10^{-5} M (this value is average of four *X* sections). From this value, apparent binding constant of quinacrine to the DNA–Cu(II)/PAA membrane was estimated to be 3.9×10^4 M^{-1}. This value is lower than the reported values for DNA solution (1.5×10^6 M^{-1}) (Gao et al., 1993). Such a small binding constant of quinacrine to the DNA–Cu(II)/PAA film may be attributed to the polyion-complex formation with PAA.

It is known that copper ion [both Cu(II) and Cu(I)] binds strongly to DNA, primarily through the N7 of guanine (Gao et al., 1993; Geierstanger et al., 1991). It has been reported that quinacrine is bound closer (intercalated) to A-T base pairs than to G-C base pairs in the DNA (Nastasi et al., 1976; Wilson and Lopp, 1979). If the quinacrine intercalates to the DNA in the DNA–Cu(II)/PAA membrane, it is reasonable to assume that the binding site of quinacrine (inhibitor) and the hydrogen peroxide (substrate) is different. A preliminary study on circular dichroism (CD) measurement of the DNA–Cu(II)/PAA film was carried out. Fig. 2B shows the CD spectrum of DNA–Cu(II)/PAA film in wet condition. In the absence of quinacrine, there are one positive band at about 274 nm and negative bands at about 240 nm, which are essentially the same as that of B-form structure of native DNA in aqueous solution (Mahadevan and Palaniandavar, 1998). In the presence of quinacrine, the intensity of positive band increased, which suggests that quinarine intercalated into the base pairs of DNA in the DNA–Cu(II)/PAA film (Lang and Liu, 1999).

4. Conclusion

In summary, we developed a novel methodology of amperometric assay of drug–DNA interaction, by using the DNA–Cu(II)/PAA membrane-modified electrode. This method is based on an inhibitory effect of quinacrine toward the electrocatalytic behavior of copper ion incorporated in the DNA/PAA membrane. This measurement concept is similar to the interference-based enzyme electrode for detecting enzymatic inhibitors. Preliminary experiments indicated that the DNA-binding molecules other than quinacrine (e.g., crystal violet, tetracycline and ethidium bromide) similarly diminished the steady-state current response of this electrode. Thus, this method would be useful for screening of wide variety of DNA-binding drugs and highly toxic pollutants. Further, this method does not require any modification of DNA and an electrode surface. Thus the preparation of the sensor is much easy as compared with other voltammetric DNA biosensors.

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