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Electrochemical determination of interaction parameters for DNA and mitoxantrone in an irreversible redox process

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Abstract

Mitoxantrone (MXT), an anti-tumor antibiotic, shows irreversible electrochemical behavior at a waxed graphite electrode in a 0.05 M Tris–HCl buffer (pH 7.4) solution. The interaction between MXT and calf thymus DNA (ctDNA) in solution has been studied using cyclic voltammetry. An electrochemical equation suitable for examining the binding of irreversibly electroactive molecules to DNA is established. Determination of diffusion coefficients of both free and binding MXT (D_f, D_b), the binding constant (K) and binding site size (s base pairs per molecule, bp) of MXT with DNA was performed on the basis of the equation. A nonlinear fit analysis of the experimental data yielded: $D_f = 3.76\times10^{-5}$ cm^2 s^{-1}, $D_b = 2.73\times10^{-7}$ cm^2 s^{-1}, $K = 8.7\times10^{9}$ cm^3 mol^{-1}, $s = 2.8$ bp. The results demonstrate that MXT binds tightly to ctDNA and covers three base pairs. The anthraquinone of MXT, which is a planar heterocyclic ring, intercalates between the DNA's base pairs. The two aminoethylamino side-chains of the drug fit to the major groove reinforce the combination of MXT and DNA. The results show that MXT is a DNA intercalator with a high binding constant compared to those of other anthraquinones.
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Keywords: DNA; Mitoxantrone; Irreversible electrochemical process; Voltammetry; Intercalation

1. Introduction

Currently, most tests for the interaction of DNA with other molecules are based on fluorescence signals [1–5]. Although the technique is extremely sensitive and quantitative, it requires excitation lasers and reading by spectroscopic instruments. A direct electric reading to study the interaction of DNA and other compounds appears much more elegant and sensitive because the electrical signal can be monitored without regard to the clarity or turbidity of a sample. Also it doesn't require expensive equipment to detect electric signal.

A number of research groups are closing in electrical detection of these interactions [6–16]. Some papers were devoted to voltammetric studies of the interaction of metal chelates with DNA [6–8,14]. These investigations indicated that rather straightforward electrochemical method could be employed to characterize the intercalative interaction between DNA and metal complexes or other

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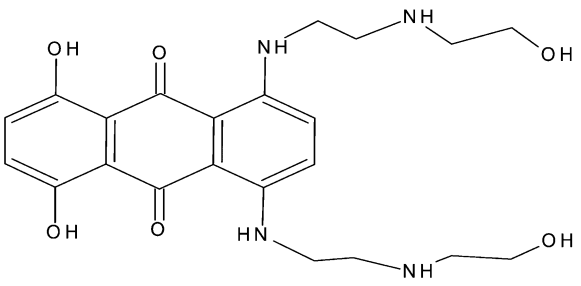

Fig. 1. The structure of MXT.

electroactive species to yield estimation of the binding constant and the size of binding site. The interaction of daunomycin, an anti-tumor drug, with double-stranded calf thymus DNA (ctDNA) was studied in solution using voltammetry [12,13]. These studies provide a useful aid to investigate binding model of small molecules with DNA. Bard et al. have studied the interaction of DNA with redox-active compounds using an electrochemical method and established a redox current equation for DNA complex, which is suitable for a reversible process [6–8]. Electrochemical research about the interaction of DNA and electroactive molecules in irreversible processes has not been reported. However, many DNA-targeting drugs (such as some new chemotherapeutic agents) show irreversibility in electrochemical reaction. For irreversible processes, the redox current is different, because the electron transfer coefficient (β) of these compounds plays an important role in electrochemical reaction. This paper presents an electrochemical equation of redox current suitable for irreversible process in the solution containing DNA and targeting molecules. The main parameters of interaction could be determined simultaneously by simple voltammetric experiments on the basis of the equation.

Mitoxantrone (MXT) is an aminoanthraquinone-containing compound used as an anti-tumor antibiotic for leukemia and breast cancer treatment, due to its combination with DNA [17,18]. The structure of MXT is given in Fig. 1. Different methods, such as electrical linear dichroism [19], in vitro transcription assay [20], surface-enhanced Raman scattering analysis [21], molecule dynamics simulation [22] and surface electrochemical technique [23] have been used to examine the interaction. However, the results are different. The mechanism of anti-tumor activity is still not completely understood [24]. In this experiment, MXT selected as a targeting molecule was examined for its ability to bind to DNA by electrochemical method. The irreversible electrochemical behavior of the drug and its binding to ctDNA was studied in cyclic voltammetry. The diffusion coefficients for both of free and binding MXT (D_f, D_b), the binding constant (K) and the binding site size (s, bp) of MXT with DNA were determined, and the mechanism of the interaction was explored.

2. Experimental

Electrochemical experiments were carried out on a BAS-100B/W electrochemical analyzer (Bioanalytical Systems Inc.) with a 10-ml cell. A waxed graphite electrode with geometric area of 0.283 cm^2 was used as the working electrode. An Ag/AgCl electrode was used as the reference electrode, and a platinum wire as the counter electrode.

MXT was obtained from Sigma Chemical Company and the stock solution was prepared by directly dissolving it in a 0.05 M Tris–HCl buffer (pH 7.4) solution, stored at 4 °C under dark condition and used up in 1 week. ctDNA purchased from Sigma Chemical Company and the stock solution was prepared in triply distilled water. Solutions of DNA ($\approx 10^{-5}$ M in nucleotide phosphate, NP) gave ratios of optical density at 260 and 280 nm, OD_{260}/OD_{280}, of $\approx 1.8–1.9$, indicating that the DNA was sufficiently free of protein. Stock solutions (the concentrations determined by the absorbance at 260 nm in nucleotide phosphate) were stored at 4 °C and used up in 3 days. Other chemicals were of analytical reagent grade. Triply distilled water was used for all solutions.

All voltammetric experiments were performed in a 0.05 M Tris–HCl buffer solution (pH 7.4). The working electrode was polished using alumina powder and thoroughly washed with purified water between measurements. Unless otherwise indicated, cyclic voltammetry was performed with a scan rate of 100 mV s^{-1}.

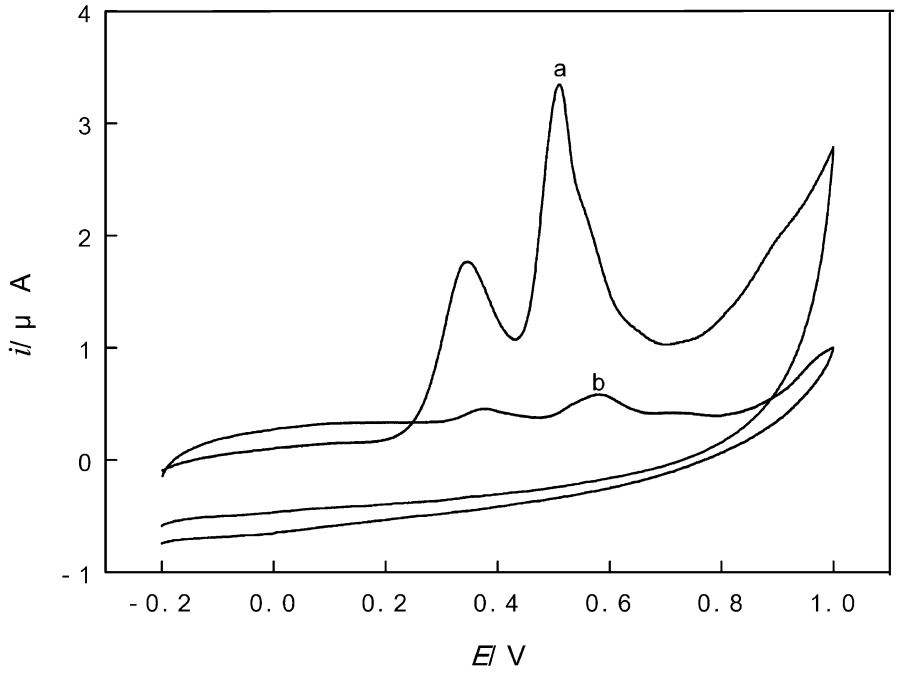

Fig. 2. Cyclic voltammograms of MXT in 0.05 M Tris–HCl (pH 7.4) solution. *Scan rate:* 100 mV s^{-1}. (a) 4.0×10^{-6} M MXT; (b) 4.0×10^{-6} M MXT + 2.0×10^{-4} M ctDNA.

All experiments were carried out at ambient temperature of the laboratory (23–27 °C). All data, unless specified otherwise, were the average of three to five repetitive measurements.

The ORIGIN software version 5.0 was used for linear regression analysis of i_{pa} vs. $\nu^{1/2}$ and E_{pa} vs. ln ν plots, extrapolating E_{pa} vs. ν plot to $\nu=0$ for determining the formal potential E^0, and non-linear regression analysis of experimental data based on the deduced electrochemical equation.

3. Results and discussion

3.1. Irreversible electrochemical process of MXT

The cyclic voltammogram of MXT in 0.05 M Tris–HCl buffer (pH 7.4) shows that the oxidation at a waxed graphite electrode is an irreversible process (Fig. 2a). The first oxidation peak at 0.35 V corresponds to the oxidation of the hydroxyl substituents at positions 5 and 8 and the second peak at 0.51 V corresponds to the oxidation of the aminoalkyl substituents after a tautomeric structural rearrangements. Each of the steps involves two-electron transfer [17,25]. Unless otherwise indicated, the second oxidation peak was chosen in all subsequent experiments, as it was more sensitive than the first one. The peak current of MXT could be described by the classical equation for irreversible process [26].

3.2. Interaction of MXT and DNA

In the presence of DNA (the ratio of total concentration of NP to total concentration of MXT, $R=50$), both anodic peaks of MXT sharply decrease in peak current and slightly shift towards the positive direction in peak potential (Fig. 2b). The peak current hardly decreases and the peak potential does not shift when increasing the MXT concentration ($R>50$), suggesting that there are almost no free MXT in the solution when $R\geqslant50$. In order to demonstrate that the decrease in current is due to the slow diffusion rate of MXT–DNA

Table 1
Voltammetric behavior of MXT and MXT–DNA

ν (V s^{-1})	i_{pa} (μA)	i_{pa}[a] (μA)	E_{pa} (V)	E_{pa}[a] (V)
0.01	0.757	0.0617	0.479	0.554
0.02	1.09	0.0816	0.487	0.562
0.05	1.61	0.124	0.500	0.575
0.1	2.17	0.182	0.509	0.584
0.2	3.00	0.250	0.518	0.593
0.5	4.93	0.408	–	–

Supporting electrolyte: 0.05 M Tris–HCl (pH 7.4) solution, $c_{MXT}=4.0\times10^{-6}$ M.
[a] DNA addition, $R=50$.

complex, not due to the increased viscosity of the solution or the blockage of the electrode surface by DNA adsorption, a special CV experiment was designed in a $K_4Fe(CN)_6$ solution with the absence and presence of DNA. In these solutions, the ions of $Fe(CN)_6^{4-}$ did not interact with DNA, because of coulombic repulsion between their negative charges. In the absence of DNA, a normal voltammetric peak of $Fe(CN)_6^{4-}$ was observed ($E_{pa}=410$ mV and $i_{pa}=6.1\times10^{-7}$ A). Upon an addition of DNA ($R=40$), only the peak current (i_{pa}) decreased a little (5.4×10^{-7} A). It showed that the addition of DNA only slightly affected the current and there was no shift of peak potential. Thus, there was no obvious effect on the diffusion from the changed viscosity of solution and the DNA adsorption. A great decrease in current and a shift in potential in above CV experiments could be attributed to the slow diffusion rate of MXT–DNA complex with large molecular weight.

The UV absorption spectra of MXT and its complex with DNA are also investigated. Both of two maximum absorption peaks of the free MXT shifted from 610 to 624 nm and 659 to 682 nm (red shift) respectively, when the DNA is added enough, indicating the interaction between MTX and dsDNA.

Both peak potentials (E_{pa}) and peak currents (i_{pa}) of MXT and MXT–DNA complex at the waxed graphite electrode, were examined as a function of scan rate (ν). A summary of voltammetric data was given in Table 1. There is a linear relationship between i_{pa} and $\nu^{1/2}$ if the electroactive species does diffuse to the electrode (26),

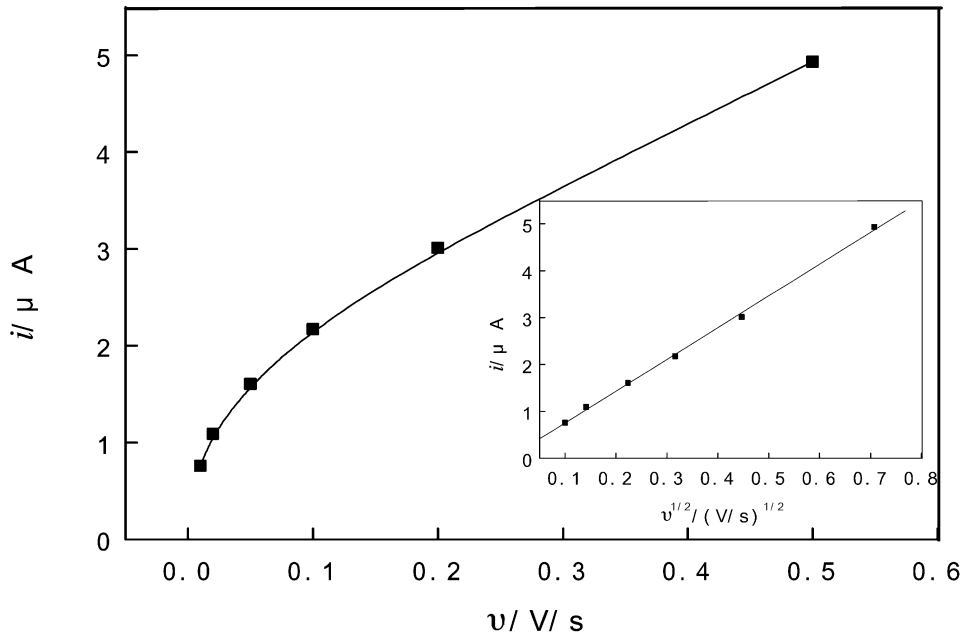

Fig. 3. Relationship between i_{pa} and ν or $\nu^{1/2}$ for 4.0×10^{-6} M MXT in 0.05 M Tris–HCl (pH 7.4) solution.

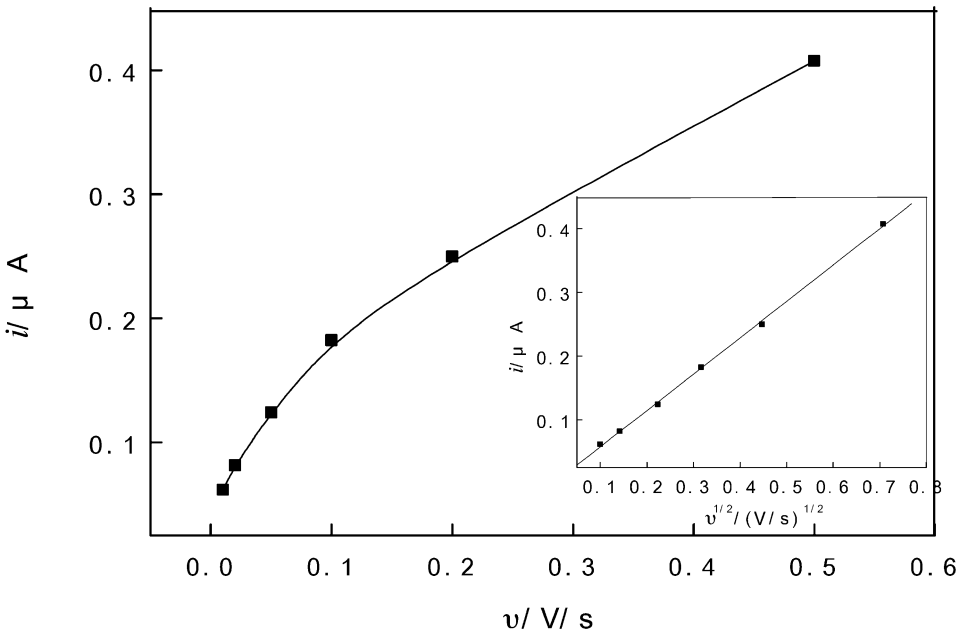

Fig. 4. Relationship between i_{pa} and ν or $\nu^{1/2}$ for 4.0×10^{-6} M MXT + 2.0×10^{-4} M ctDNA in 0.05 M Tris–HCl (pH 7.4) solution.

whereas the species adsorbed on the electrode surface shows a linear plot of i_{pa} vs. ν. Plots of i_{pa} vs. ν and i_{pa} vs. $\nu^{1/2}$ for MXT and MXT–DNA complex are shown in Figs. 3 and 4, respectively. Both plots of i_{pa} vs. $\nu^{1/2}$ for MXT and MXT-DNA complex are linear in the figures, demonstrating that the main mass transport of MXT and the complex to the electrode surface is from the diffusion.

3.3. Measurement of electron transfer coefficient

The relationship between E_{pa} and ln ν, over the range of 0.01–1.00 V, was discussed as following. A classical equation of peak potential for an irreversible electrode process is used here [27]:

$$E_{pa} = E^0 + \mathrm{RT}/(\beta nF)\{0.780 + 0.5 \ln [\beta nD\mathrm{F}\nu/(\mathrm{RT})] - \ln k_s\} \quad (1)$$

where k_s is the standard rate constant of surface reaction and β is the electron transfer coefficient. According to Eq. (1), the curve of E_{pa} vs. ln ν should be linear (Figs. 5 and 6). βn can be obtained from the slope of the curve and k_s can be calculated from the intercept, if the values of E^0 and D are known. The value of E^0 in Eq. (1) can be obtained from the intercept of E_{pa} vs. ν plot on the ordinate by extrapolating the line to $\nu = 0$. The diffusion coefficient (D) can obtain from nonlinear analysis of binding data later. In the absence of DNA, the slope of $E_{pa} \sim \ln \nu$ plot was 0.0132, and the β_f was calculated: $(\beta n)_f = 1.02$, i.e., $\beta_f = 0.51$ ($n = 2$; [17,25]). In the presence of DNA, the slope of $E_{pa} \sim \ln \nu$ plot was 0.0130, and $(\beta n)_b = 0.98$, i.e., $\beta_b = 0.49$ ($n = 2$; [17,25]).

3.4. Current equation of targeting molecule–DNA interaction suitable for irreversible redox processes

If an electroactive molecule nonspecifically reacts with a DNA duplex at a binding site, which is composed of s bases or base pairs, the reaction can be expressed as follows:

$$\mathrm{EM} + \mathrm{S} = \mathrm{EM} - \mathrm{S} \quad (2)$$

here EM, S and EM − S symbolize the electroac-

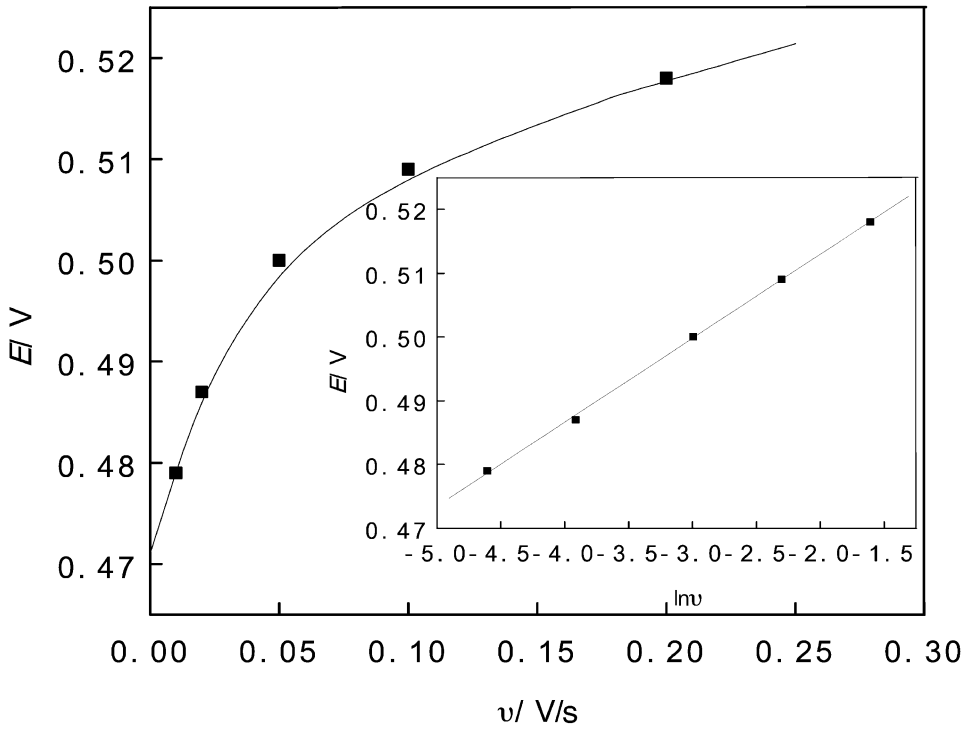

Fig. 5. Relationship between E_{pa} and v or ln v for 4.0×10^{-6} M MXT in 0.05 M Tris–HCl (pH 7.4) solution.

tive compound, binding site, and electroactive compound–DNA complex, respectively. The total concentration of the electroactive molecule, C_t, is therefore obtained:

$$C_t = C_b + C_f \quad (3)$$

here C_b and C_f, represent the equilibrium concentrations of EM in EM−S, free EM, respectively. The binding constant, K, is described by the following form:

$$K = C_b/(C_f C_s) \quad (4)$$

where C_s represents the equilibrium concentration of free S. The average number of binding sites (x) along a DNA duplex molecule with an average total number of base pairs L can be described by the following form:

$$x = L/s \quad (5)$$

Here, s is the binding site size (base pairs, bp) of the electroactive molecule interacting with DNA. It means that the number of DNA base pairs is occupied (or covered) by a binding molecule. Thus, the total concentration of binding sites (xC_{DNA}) can be expressed as follows:

$$xC_{DNA} = C_b + C_s \quad (6)$$

where $C_{DNA} = C_{NP}/(2L)$. Here, C_{NP} represents the concentration of nucleotide phosphate, which is determined by the UV absorption at 260 nm. The ratio of the nucleotide phosphate concentration and the total concentration of electroactive molecule can be defined as R:

$$R = C_{NP}/C_t \quad (7)$$

CV experiments were carried out, in which the value of R was varied. Two limit regions were found in the experiment. With large values of R, the current was primarily attributed to EM–DNA

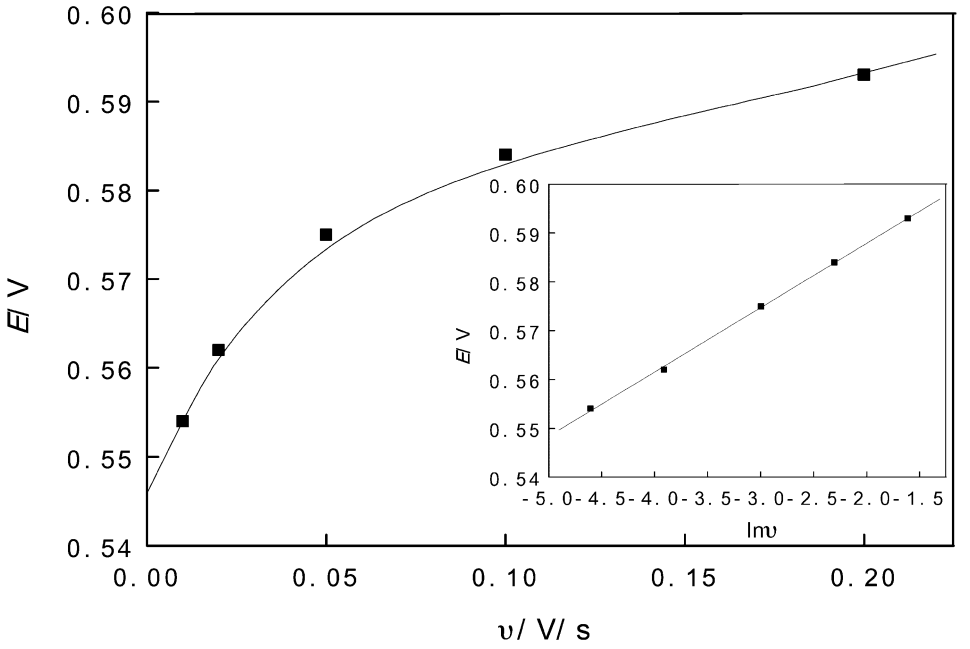

Fig. 6. Relationship between E_{pa} and ν or ln ν for 4.0×10^{-6} M MXT + 2.0×10^{-4} M ctDNA in 0.05 M Tris–HCl (pH 7.4) solution.

Table 2
Effects of added MXT to DNA solution on peak currents

No.	NP (μM)	MXT (μM)	R (C_{NP}/C_t)	i_{pa} (μA)
1	9.09	0.500	18.18	0.0478
2	9.09	0.645	14.12	0.0832
3	9.09	1.00	9.09	0.217
4	9.09	2.00	4.55	0.474
5	9.09	4.00	2.27	0.990
6	9.09	5.20	1.75	1.68
7	9.09	8.00	1.14	2.23

complex; and with $R=0$, the total contribution to i_{pa} was from free EM. For an irreversible reaction in CV at 25 °C, the total anodic current (i_{pa}) under the fixed potential with any R can be calculated:

$$i_{pa}=B[(\beta n)_f^{1/2}D_f^{1/2}C_f+(\beta n)_b^{1/2}D_b^{1/2}C_b] \quad (8)$$

here $B=2.99\times10^5 nA\nu^{1/2}$ [26]. Making appropriate substitutions and eliminating C_t in the equations of Eqs. (3)–(8), an equation of i_{pa} was obtained:

$$i_{pa}=B\{(\beta n)_f^{1/2}D_f^{1/2}C_{NP}/R+[(\beta n)_b^{1/2}D_b^{1/2}-(\beta n)_f^{1/2}D_f^{1/2}][b-(b^2-2K^2C_{NP}^2/R/s)^{1/2}]/(2K)\} \quad (9)$$

where $b=1+KC_{NP}/R+KC_{NP}/(2s)$.

Eq. (9) is valid for the assumption of noncooperative, non-specific binding to DNA with the existence of one type of discreet binding site. Because i_{pa}, C_{NP} and R are experimentally measurable quantities, the diffusion coefficients of EM and EM–DNA (D_f, D_b), the binding constant (K) and binding site size (s, bp) of EM–DNA can be obtained by nonlinear regression analysis of the experimental data (i_{pa} and R) according to the equation.

3.5. Determination of interaction parameters of MXT–DNA complex

A series of CV experiments in an aqueous medium with the same amount of DNA and different amount of MXT, were carried out in order to study the interaction of MXT with ctDNA using Eq. (9). The total current of the oxidation

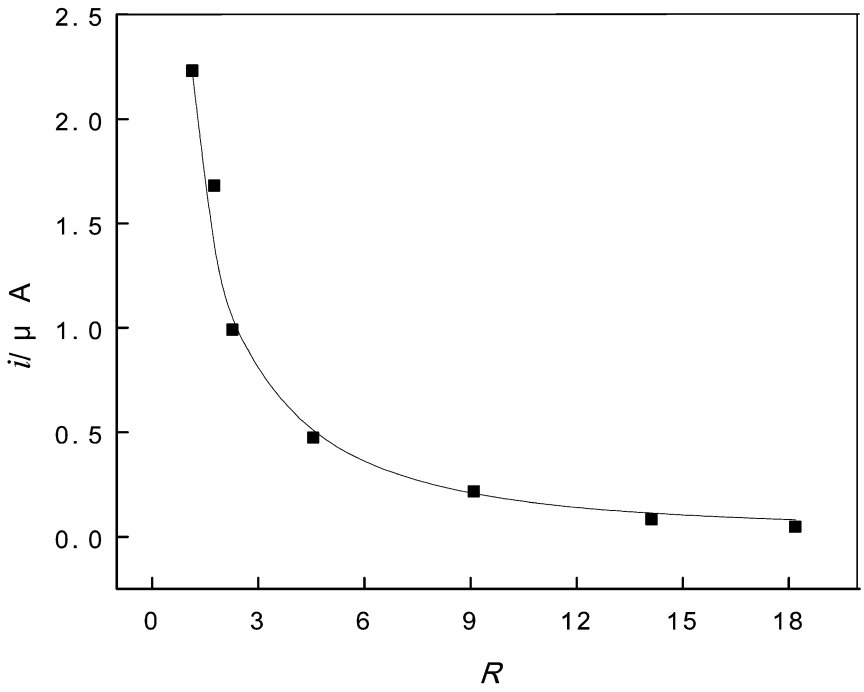

Fig. 7. Binding curve of MXT with DNA in 0.05 M Tris–HCl (pH 7.4) solution. *Scan rate:* 100 mV s^{-1}.

of MXT was detected when each of binding equilibriums were reached. The experimental data were shown in Table 2. A nonlinear fit analysis of the data to Eq. (9) yielded the binding curve shown in Fig. 7 and the following results were obtained: $D_f = 3.76\times10^{-5}$ $cm^2\,s^{-1}$, $D_b = 2.73\times10^{-7}$ $cm^2\,s^{-1}$, $K = 8.7\times10^9$ $cm^3\,mol^{-1}$, $s = 2.8$.

3.6. Determination of k_s of MXT and MXT–DNA complex

According to Eq. (1), the curve of E_{pa} vs. ln ν should be linear and the standard rate constant of surface reaction k_s can be calculated from the intercept, if the values of E^0 and D are known. In the absence of DNA, $(E^0)_f = 0.472$ V, the intercept was 0.539 V (Fig. 5), $D_f = 3.76\times10^{-5}$ $cm^2\,s^{-1}$, and the $(k_s)_f$ of 0.0061 cm s^{-1} was calculated. In the presence of DNA, $(E^0)_b = 0.546$ V, the intercept was 0.614 V (Fig. 6), $D_b = 2.73\times10^{-7}$ $cm^2\,s^{-1}$, and the $(k_s)_b$ of 0.0016 cm s^{-1} was calculated. The results suggest that the apparent diffusion coefficient and the standard rate constant of MXT decreased after an addition of an excess of DNA.

3.7. Interaction of MXT and ctDNA

The binding constant of MXT and ctDNA ($k = 8.7\times10^9$ $cm^3\,mol^{-1}$), which is larger than that of other anthraquinones [28], and the binding site size ($s = 2.8$ bp) demonstrate that MXT binds tightly to three base pairs of ctDNA.

According to the simulation of ACD/CHEMSKETCH software (ACD/Labs Products), while the anthraquinone of MXT, a planar heterocyclic ring, intercalates the ctDNA, the two aminoethylamino side-chains of MXT shape the helix (0.8 nm in diameter in average). In general, major grooves of DNA double helix are 1.2 nm in diameter; minor grooves are 0.6 nm in diameter. For energetically favorable orientation the anthraquinone of MXT is perpendicular to the direction of inter-base hydrogen bonds, i.e., the anthraquinone intercalates between the base pairs of DNA,

and two helically shaped side-chains of MXT fit to the major groove of the duplex DNA and reinforce the combination with DNA. This may explain why the binding constant of MXT is higher than those of other anthraquinones [28]. The binding site size (2.8 bp), which is just suitable for the length of one MXT molecule, indicates that MXT binds to DNA and covers three base pairs.

4. Conclusion

This paper presents an electrochemical equation suitable for irreversible redox processes to examine the interaction of DNA and targeting molecules in solution for the first time. MXT, showing irreversible electrochemical process in cyclic voltammetry, was selected as the targeting molecule. The D_f and D_b for free and binding MXT, K and s of MXT with DNA were determined simultaneously. It shows that the electrochemical research for this kind of interaction is sensitive, fast and economical. The equation offers great promise for further study of interaction of DNA and more electroactive drugs.

Acknowledgments

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