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Electrochemical studies on the binding of a carcinogenic anthraquinone dye, *Purpurin* (C.I. 58 205) with DNA

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ABSTRACT

The interaction of the anthraquininoid dye, *Purpurin* (C.I. 58 205) with fish-sperm DNA was electrochemically investigated in 0.02 mol L^{-1} pH 7.0 Britton–Robinson (B–R) buffer. After interaction with DNA, the irreversible oxidation peak of the dye decreased and underwent a positive shift of potential, suggesting intercalation between the dye and DNA, this being consistent with fluorescence quenching results. The binding constant (K) and binding site size (s) of the two species were 9.6×10^6 L mol^{-1} and 1.7 respectively, as determined using voltammetric titration, indicating that the dye was strongly bound to the DNA.

1. Introduction

Anthraquinones enjoy widespread applications such as in food coloring, textile dyeing, paints and medical treatments [1,2]. However, as little information on their potentially hazardous effects on humans has been published [3] it was required to determine the correlation between anthraquinones and carcinogenesis. For this, *Purpurin* (PP, C.I. 58 205), a naturally occurring anthraquinone colorant found in species of madder root (*Rubia tinctorum*), was selected for investigation. Marczylo et al. [4] found that the presence of Purpurin was responsible for a marked inhibition of mutagenicity induced by food-derived heterocyclic amines. In contrast, it has been reported that the dye has mutagenic activity [5] and can cause urinary bladder tumors (papilloma and carcinoma) in rats [6]. However, the detailed mechanism of its carcinogenesis is unclear. As DNA is often the target for many tumorigenic and mutagenic molecules [7,8], clarification on the interactions between this typical anthraquininoid dyes with DNA might help understand the toxicity of this type of compound.

Compared with many established technologies for investigating the interaction of DNA and small molecules, such as UV–Vis, FTIR, NMR, ESR and Raman, electrochemistry offers the advantages of high efficiency, convenience, simplicity and low cost [9–11]. Additionally, in view of the similarity between electrochemical reactions that occur at the electrode/solution interface and real reactions that take place in the living cell *in vivo*, knowledge of the electrochemical mechanism of the interaction of a biomacromolecule with its binder could provide useful pharmacological and toxicity information [12,13].

This paper concerns an electrochemical study of the mechanism of the interactions that occur between PP and DNA at a molecular level so as to obtain information about the toxicity of PP. The results show that PP can intercalate into the base pairs of DNA and form a new complex, thereby demonstrating that the approach adopted may offer potential for the interpretation of the toxicity of PP and its derivatives.

2. Experimental

2.1. General

Voltammetry was performed using a CHI 832 electrochemical analysis system (CHI Instrument, China) equipped with a three-electrode system that comprised a bare glassy carbon electrode (GCE, $\Phi = 3$ mm) as working electrode, a saturated calomel electrode (SCE) as reference electrode and a platinum wire as auxiliary electrode. Fluorescence was measured using an Hitachi F-4500 fluorospectrophotometer (Japan).

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Native fish-sperm DNA from Beijing Baitai Biochemistry Technology Company (China) was used as received. Stock solutions of DNA was prepared by dissolving an appropriate amount of DNA in doubly distilled water and were stored at 4 °C. The ratio of the absorbance at 260 and 280 nm (A_{260}/A_{280}) was found to be ~1.89, indicating that the DNA was sufficiently free from protein. Denatured single-stranded DNA was produced by thermal denaturation [14] namely, 0.5 g L^{-1} native DNA solution was heated in a water bath at 100 °C for ~8 min and then was rapidly cooled in an ice bath. The molar concentration of native DNA in nucleotide phosphate and also of single-stranded DNA in the base were determined spectrophotometrically at 260 nm using molar extinction coefficient of 6600 L mol^{-1} cm^{-1} for native DNA and 8250 L mol^{-1} cm^{-1} for denatured DNA, respectively [15]. Unless specified otherwise, the term of DNA refers to native DNA. The dye (PP) was purchased from Shanghai Chemical Reagent Company of Chinese Medical Group (China). A stock solution of PP was prepared by dissolving the dye in 100 mL of a 1:1 mixture of distilled water:ethanol. All other reagents purchased commercially were of analytical grade; double distilled water was used throughout.

2.2. Fluorescent and electrochemical determination

The fluorescence titration of PP with DNA in 0.02 mol L^{-1} pH 7.0 B–R was carried out by monitoring the emission intensity at 580 nm. The excitation wavelength was fixed at 520 nm and the emission range adjusted before measurements. Electrochemical studies of the interaction of PP with DNA were carried out using the following procedures: Appropriate amounts of DNA, PP and B–R buffer were successively added to a colorimetric tube and then transferred to a 10 mL electrochemical cell after reaction for 30 min at ambient temperature. Before measurement, the working electrode of GCE was polished to a mirror-like surface using a 0.3 mm and 0.05 mm α-alumina slurry and then ultrasonicated for 2 min in doubly distilled water. Cyclic voltammetry (CV) was carried out in the range 0.5 to −0.2 V and differential pulse voltammetry (DPV) was undertaken from −0.12 to 0.3 V with an increment potential of 4 mV, amplitude of 50 mV, pulse width of 50 ms and a pulse period of 200 ms.

3. Results and discussion

3.1. Cyclic voltammetry

Fig. 1 shows the typical CVs of PP interaction with DNA in 0.02 mol L^{-1} pH 7.0 B–R buffer solution. As showed in Fig. 1, PP had a pair of asymmetric redox peaks at +0.087 V and +0.175 V, respectively on GCE. The ratio of the oxidation peak current (I_{pa}) to the reduction peak current (I_{pc}) was determined to be about 40, suggesting that the electrochemical process of PP was irreversible. As the oxidation peak was more sensitive than the reduction peak, the former one was chosen as the analysis signal for further studies. Acidity experiments showed that the pH values of B–R buffer had significant effects on the electrochemical response of PP. Over the range from pH 3.0–7.0, the oxidation peak currents of PP increased gradually with the increase of pH values, and then diminished when pH values were upon 8.0 (data not shown). Additionally, with the decrease of the acidity, the oxidation peak potentials (E_{pa}) shifted positively, suggesting that protons were involved in the electrochemical reaction [16].

When 3.3×10^{-5} mol L^{-1} DNA was added into PP solution and then voltammetrically detected under the same conditions, the oxidation peak current decreased obviously accompanied by a positive shift of the peak potential (Fig. 1b), which suggested that PP had been bound to DNA via an intercalative mode [17], and this result could be well testified by the following fluorescent experiments.

It was clearly observed that PP had an emission peak centered at 580 nm when exited at 520 nm (Fig. 2a). When increasing concentrations of DNA were present, the fluorescence intensities of PP were found to be substantially quenched (curves b–f in Fig. 2). It has been reported that when a chromphore was intercalated into the adjacent base pairs, the photoelectrons would transfer from the bases of DNA to the excited state of the intercalator, and resulted in the quenching of the fluorescent intensity of the chromphore [18,19]. So the intercalative binding mode of PP with DNA could also be obtained through this spectroscopic method.

Additionally, for an irreversible oxidation process, the number of electron transferred (n) could be obtained by Eq. (1) [16]:

$$|E_{pa} - E_{pa/2}| = 1.857RT/\alpha nF \quad (1)$$

Where $E_{pa/2}$ was the half peak potential, α the electron transfer coefficient (generally, $0.3 < \alpha < 0.7$), F Faraday constant (96 487 Coulombs mol^{-1}), R universal gas constant (8.314 J K^{-1} mol^{-1}), T Kelvin temperature (K). In the present study, a value of 45 mV for $|E_{pa} - E_{pa/2}|$ obtained from Fig. 1a yielded the value of n of 2.1 (≈ 2) when α was assumed to be 0.5 for a totally irreversible process according to Eq. (1) [16].

Also, according to the value of $|E_p - E_{pa/2}|$ in Fig. 1b and Eq. (1), the number of electron transfer was determined to be about 2, suggesting that PP–DNA complex also underwent a two electron transfer process.

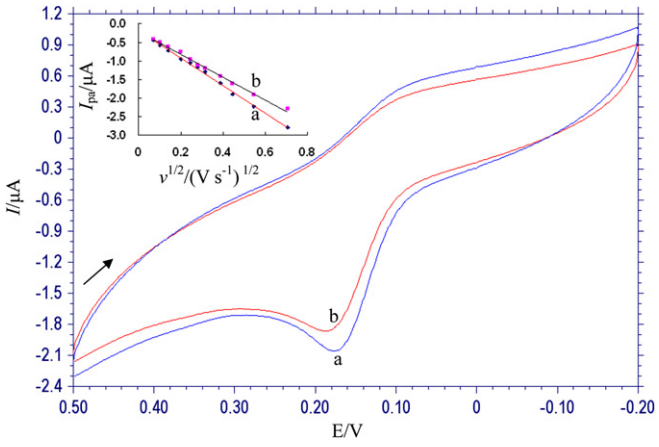

Fig. 1. Cyclic voltammograms of 1.0×10^{-4} mol L^{-1} PP without (a) and with (b) interaction with 3.3×10^{-5} mol L^{-1} DNA in 0.02 mol L^{-1} pH 7.0 B–R buffer solution. Inset: Plots of I_{pa} versus $v^{1/2}$ for PP (a) and PP–DNA complex (b).

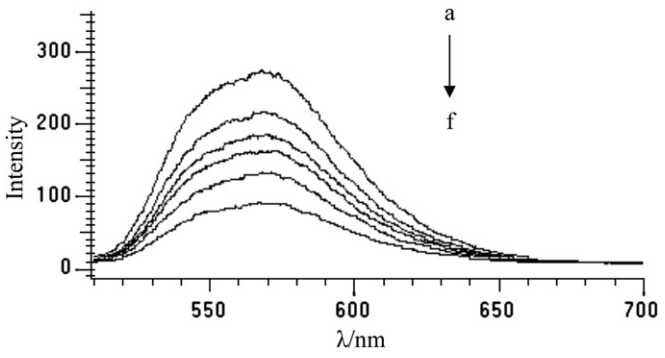

Fig. 2. Fluorescent spectra of 1.0×10^{-4} mol L^{-1} PP in the presence of 0 (a), 6.6×10^{-5} (b), 1.2×10^{-4} (c), 1.9×10^{-4} (d), 2.6×10^{-4} (e), 3.2×10^{-4} (f) mol L^{-1} DNA in 0.02 mol L^{-1} pH 7.0 B–R.

Some experimental parameters such as the absolute interaction time, ionic strength (μ) and scan rate (v) were also exploited to investigate the binding of PP and DNA. In pH 7.0 B–R buffer solution, DNA was added into PP solution and then voltammetrically detected at regular intervals, it was obtained that the oxidation peak of PP decreased gradually with the increase of the reaction time and become a stable state at 30 min, indicating that the absolute interaction between PP and DNA has been achieved. The effects of the ionic strength controlled by adding KCl on the interaction of PP and DNA were also investigated. It was found that in the range from 0 to 30 mmol L^{-1}, the oxidation peak potentials of PP shifted positively with the increase of ionic strength (Fig. 3), suggesting that the electrostatic interaction between PP and DNA was weakened by the added KCl [17]. This could be explained by the ionic shielding effect of the added K^+ on the negatively charged DNA [17]. When μ was upon 30 mmol L^{-1}, the peak potentials became constant values (Fig. 3), indicating that PP could no longer interact with DNA electrostatically at high ionic strength. So the dash line in Fig. 3 could divide the binding mode of PP and DNA into two parts: the mixture of electrostatic and intercalative modes ($\mu < 30$ mmol L^{-1}) and the only intercalative mode ($\mu \geq 30$ mmol L^{-1}). Additionally, on the time scale of the experiment, the peak potentials were more positive than the free PP, also demonstrating that the main binding mode of PP to DNA was controlled by intercalation at any ionic strength [17].

The effects of scan rate (v) on the electrochemical signals such as the peak currents (I_{pa}) and peak potentials (E_{pa}) were further studied to assess the interaction of PP with DNA.

It was well found that, whether DNA was present or not, the CV curves of PP varied regularly with the increase of scan rate, and for both PP and PP–DNA systems, the plots of oxidation peak currents had good linear relationships with the square root of scan rate ($v^{1/2}$) (inset of Fig. 1), suggesting that the electrochemical processes of PP and PP–DNA complex were both controlled by diffusion [16]. The regression equations were $I_{pa}/\mu A = -3.73v^{1/2}/(V\ s^{-1})^{1/2} - 0.174$ ($r = 0.998$) and $I_{pa}/\mu A = -3.05v^{1/2}/(V\ s^{-1})^{1/2} - 0.211$ ($r = 0.997$), respectively for free PP and PP–DNA complex. Obviously, the slop of PP–DNA system was much smaller than that of free PP, indicating that DNA-bound form of PP diffused more slowly than free PP. Thus, the reason that caused the decrease of currents of PP upon DNA addition could be assigned to the diffusion of an equilibrium mixture of free and DNA-bound PP to the electrode surface [17].

The change of electron-transfer rate constant (K_s) of electroactive molecule after interaction with DNA had often been used to probe the binding nature of small molecules with DNA [20,21]. In this paper, the K_s of DNA-bound PP was also determined and compared with that of free PP. Fig. 4 shows the relationships of the oxidation peak potentials (E_{pa}) of free (curve a) and DNA-bound PP (curve b) with scan rate (v). It could be well observed that the peak potentials shifted positively with the increase of scan rate for both of the systems. The formal potentials ($E^{0'}_{pa}$) of free and DNA-bound PP were then obtained to be +0.128 V and +0.140 V, respectively, via prolonging the E_{pa}–v curves to E_{pa} axis. Additionally, it was observed that E_{pa} had good linear relationships with the logarithm of scan rate (ln v) for the two systems (Fig. 5), which was in accordance with the following Laviron's Eq. (2) for an irreversible oxidation process [22]:

$$E_{pa} = E^{0'}_{pa} + (RT/(1-\alpha)nF)\ln((1-\alpha)nF/RTK_s) + (RT/(1-\alpha)nF)\ln v \quad (2)$$

Thus, from the intercepts of E_{pa}–ln v and the values of $E^{0'}_{pa}$, the values of K_s were calculated to be 0.15 s^{-1} and 0.14 s^{-1}, respectively for PP and PP–DNA complex. This result showed that, after interaction with DNA, the constants of electron-transfer rate (K_s) of PP–DNA complex was close to free PP, *i.e.*, PP–DNA complex kept the electroactivity of free PP in homogeneous solution.

3.2. Differential pulse voltammetry (DPV)

DPV was further applied to investigate the interaction mechanism and binding parameters of the PP with DNA. As showed in Fig. 6, PP had a sensitive DPV oxidation peak in the scan range (curve a), and this peak was attenuated gradually with the increase of DNA concentrations, testifying the interaction of PP and DNA. When DNA was upon 1.6×10^{-4} mol L^{-1}, the peak current of PP became steady and minimum value (curve b), indicating that PP had been fully bound by DNA. The attenuated values of the PP peak currents (ΔI_{pa}) had good linearity with the concentration of DNA in range from 0.66 to 9.9 μM with a linear regression equation of $\Delta I_{pa}/\mu A = 1.426C_{DNA}/(10^{-4}\ M) + 0.102$, $\gamma = 0.988$ (data not shown), providing a possibility for quantitative determination of DNA via using PP an electroactive probe.

Moreover, after interaction with excess native DNA, the peak potential of PP showed a limiting positive shift of +8 mV (curve b). According to the previous report [17], if both the oxidized and

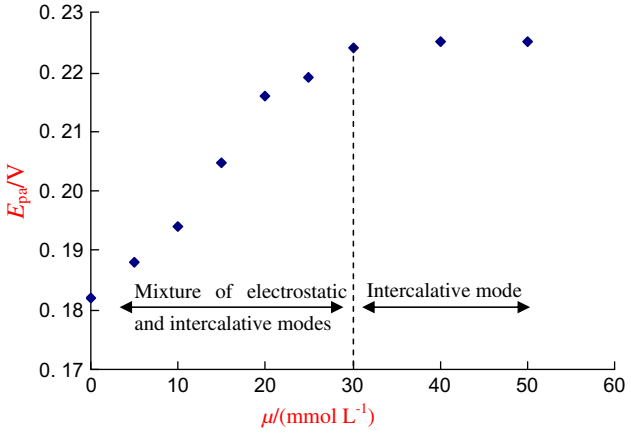

Fig. 3. Effects of ionic strength (μ) on the oxidation peak potentials (E_{pa}) of PP–DNA system. The conditions were the same with Fig. 1.

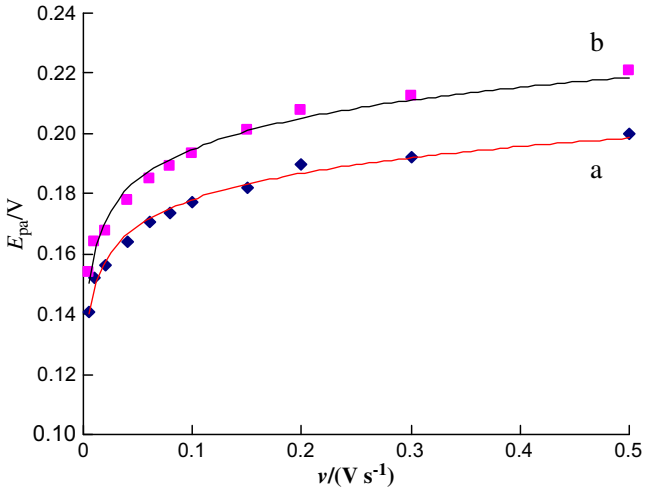

Fig. 4. Relationships between the oxidation peak potentials (E_{pa}) and the scan rate (v) for PP (a) and PP–DNA complex (b). The other conditions were the same with Fig. 1.

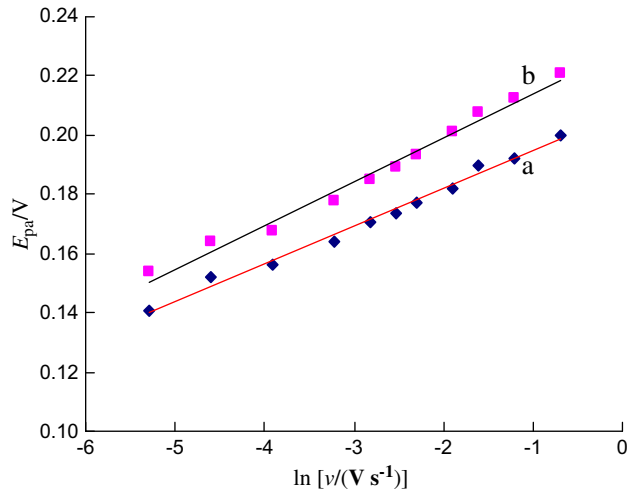

Fig. 5. Relationships between the oxidation peak potentials (E_{pa}) and the logarithm of scan rate (ln v). The other conditions were the same as in Fig. 1.

reduced forms associated with DNA, the corresponding equilibrium constants for each oxidation state binding to DNA could be calculated via the following Eq. (3):

$$\Delta E^0 = E_b^{0'} - E_f^{0'} = (RT/nF)\ln(K_R/K_O) \quad (3)$$

Where $E_b^{0'}$ and $E_f^{0'}$, the formal potentials for DNA-bound and free forms of PP, respectively, were determined by the formula of $E^{0'} = E_{pa} + \Delta E_p/2$, ($E_{pa}$, the DPV peak potential; ΔE_p, the pulse amplitude); K_O and K_R the binding constants of oxidized and reduced forms to DNA, respectively.

Thus, for the limiting potential shift of +8 mV after interaction with excess DNA, the ratio of the binding constants (K_R/K_O) could be calculated to be 1.8, indicating that the reduced form of PP (PP_{Red}) bound to DNA as strongly as 2 times of the oxidation form (PP_{ox}). This result was consistent with the characteristic of an intercalation mode [17].

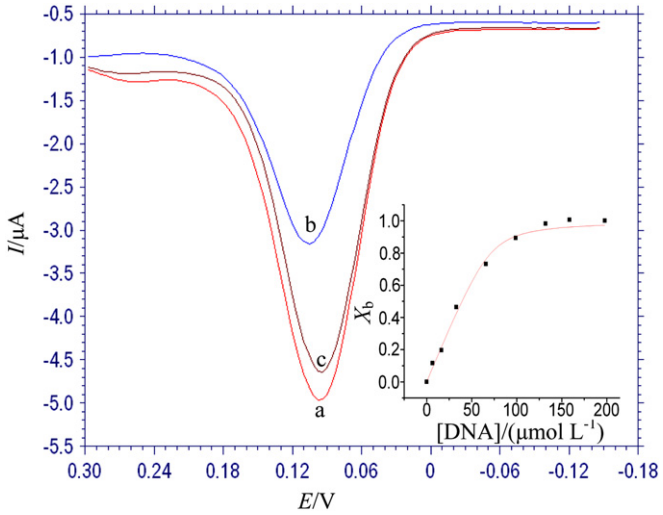

Fig. 6. Differential pulse voltammograms of 2.0×10^{-5} mol L^{-1} PP in the absence (a) and presence of 1.6×10^{-4} mol L^{-1} native DNA (b) and 1.6×10^{-4} mol L^{-1} denatured DNA (c) in 0.02 mol L^{-1} pH 7.0 B–R buffer. Inset: Plots of the bound mole fraction of 2.0×10^{-5} mol L^{-1} PP upon titration with DNA in 0.02 mol L^{-1} pH 7.0 B–R, and the associated non-linear least squares fits to Eqs. (3)–(5).

As a control experiment, the interaction of PP with denatured single-stranded DNA was also investigated. It was found that, absolutely different with the case of native double-stranded DNA as showed in Fig. 6b, the obtained signal showed minor change compared with free PP (curve c), indicating that the interaction between the dye with denatured DNA was very weak. This could be ascribed to the change of the DNA structure after denaturation. For the denatured DNA, the hydrogen bonding between two associated strands was destroyed and the two strands were separated into two "random-coil" states, resulting in the extinction of intercalation sites for the external molecules. The larger decrease of the electrochemical signals after interaction with native DNA also further proved the existence of intercalative mode between PP and native double-stranded DNA.

Additionally, the binding site size (s) and binding constant (K) of PP with DNA were calculated via voltammetric titration method. Assuming that free small molecules and DNA-binding sites were in equilibrium with the bound species, then an expression for the bound fraction as a function of the association constant, K, and the binding site size, s, could be derived from the following equilibrium constant expressions [23].

$$X_b = \left\{b - \left(b^2 - 2K^2C_t[\mathrm{DNA}]/s\right)^{1/2}\right\}/2KC_t \quad (4)$$

$$b = 1 + KC_t + K[\mathrm{DNA}]/2s \quad (5)$$

Where X_b is the bound mole fraction, C_t the total concentration of small molecule, and [DNA] was the DNA concentration. Eq. (4) was valid for noncooperative, nonspecific binding. Values for the binding constant and the binding site size were determined by non-linear regression analysis of bound fraction versus nucleotide phosphate profiles. The bound fraction of redox molecule was determined from the oxidation current during the titration of PP with DNA. The redox current diminished gradually upon the addition of DNA because the effective diffusion coefficient of the redox molecule was lowered by binding to DNA as discussed in CV section. The bound mole fraction, assuming rapid exchange between free and bound states on the voltammetric time scale, was given by a ratio of squared current differences:

$$X_b = (I^2 - I_o^2)/(I_{sat}^2 - I_o^2) \quad (6)$$

Where I_o is the redox current in the absence of DNA and I_{sat} was the current at complete saturation.

Thus, X_b values corresponding to the different concentrations of DNA were showed as the dots in inset of Fig. 6. A non-linear fitting analysis was applied to yield binding curves of PP to DNA according to Eq. (4)–(6). The binding parameters of binding constant (K) and binding site size (s) were then obtained to be 9.6×10^6 L mol^{-1} and 1.7, respectively, which indicated that one PP molecule covered about 2 nucleotides with a strong affinity.

4. Conclusions

As an important member of the anthraquinone dye, PP has been reported to display mutagenic action, which suggested that information relating to both the nature and strength of the interaction between DNA and the dye might be helpful in understanding the toxicity mechanism of PP. This electrochemical study shows that PP binds to DNA mainly via typical intercalation; the corresponding binding constant (K) and binding site size (s), as determined by voltammetric titration and non-linear fitting analysis suggest that one PP molecule interacts with 2 base pairs of DNA with a binding strength of 9.6×10^6 L mol^{-1}.

Acknowledgments

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