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ORIGINAL PAPER

Electrochemical studies of calcium dobesilate and interaction with DNA

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Abstract The electrochemical behavior of calcium dobesilate (CD) and its interaction with DNA were explored using voltammetry and UV spectroscopy. The results show that CD could interact with DNA molecules by intercalation, forming a non-electroactive complex. CD has excellent electrochemical activity on a gold nanoparticle-modified glassy carbon electrode with a couple of redox peaks. In the presence of DNA, the peak current of CD decreases and the peak potential is shifted positively, but no new peak appears. The binding of CD with DNA, analyzed in terms of the cooperative Hill model, yields an association constant of $2.63\times10^{3}\,L\cdot mol^{-1}$ and a Hill coefficient of $m\approx2$. These results may serve as a reference for in vivo investigation of the interaction of CD with DNA base pairs in living cells.

Keywords Calcium dobesilate · Gold nanoparticles · Modified electrode · DNA interaction

Introduction

The interaction between drugs and DNA is an important fundamental issue in life process, and it is crucial for gene therapy due to correlation with the mechanisms of drug and gene delivery systems. Intercalation, groove binding, and electrostatic interaction are the three major binding modes of small molecules to DNA [1]. Numerous analytical techniques, including UV spectrophotometry, circular dichroism spectroscopy, fluorescence spectroscopy, nuclear magnetic resonance, luminescence, and so on [2–5], have been used to study these interactions.

Recently, electrochemical methods have been increasingly used to investigate the interaction of DNA with other molecules, which is emerging as an actively developing field [6–8] thanks to the simple operation, high sensitivity, and low cost. Bard and co-workers pioneered the electrochemical study of the interaction of metal complex with DNA [9, 10]. The interactions of drugs such as ofloxacin [11], aloe-emodin [12], morin [13], gallic acid [14], berberine [15], and rutin [16] with DNA have been studied in connection with their electrochemical behavior. And extensive efforts have been devoted to studying the interaction of drugs with DNA at electrodes, hoping to provide basic information for optimizing the biological utilization and pharmaceutical science. Moreover, in vivo electrochemical technique has also been used to investigate the interaction between drugs and DNA by simulating the real actions occurring in living cells [17].

Calcium dobesilate (CD), i.e., calcium 2,5-dihydroxybenzenesulfonate, is a vascular protecting agent commonly used in the treatment of diabetic retinopathy and chronic venous insufficiency. Synthesized CD has therapeutic effect in microcirculatory disorders by reducing capillary permeability, inhibiting platelet aggregation and thrombus formation, lowering blood hyperviscosity, and increasing red cell flexibility [18, 19]. The electrochemical behavior of CD has been reported recently [20–22]. However, no report is currently available about the study of the interaction of CD with DNA using electrochemical methods.

Gold nanoparticles (AuNPs) are becoming increasingly attractive for use in biosensors, because they not only have nanoscale dimensions comparable to that of biomacromo-

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lecules but also have size-dependent optical and electronic properties [23]. Besides, AuNPs modified electrodes have many advantages such as favorable electronic properties, ease of biomolecule attachment, and electrocatalytic effects [24–26]. Therefore, glassy carbon electrodes modified by AuNPs were used to study the electrochemical behavior of CD in the present work. The voltammetric properties of CD at AuNPs modified glassy carbon (AuNPs/GC) electrode were examined. The interaction of CD with DNA was investigated using cyclic voltammogram (CV), differential pulse voltammetry (DPV) and spectroscopic technique. The binding stoichiometry and association equilibrium constant of DNA with CD were detected. And the binding mechanism for CD and DNA was preliminarily discussed.

Experimental

Reagents and chemicals

Herring sperm DNA was purchased from Sigma (http://www.sigmaaldrich.com). Stock solutions of DNA were prepared with phosphate buffer solution. It was stored at 4°C and used for not more than 5 days. Calcium dobesilate was purchased from Juye Lingfeng Chemical Materials Company Ltd (Shandong, China). Hydrogen tetrachloroaurate ($HAuCl_4 \cdot 3H_2O$) was purchased from Alfa Aesar (http://www.alfa.com). All other reagents are of analytical reagent grade and used as received. All measurements were the average of at least three repeat measurements performed at ambient temperature.

Apparatus

Electrochemical experiments were carried out using a computer controlled model CHI-660 electrochemical workstation (CH Instrument Inc., USA) with a conventional three-electrode system. A bare or modified GC electrode (diameter 3 mm) was used as the working electrode, a platinum wire as the counter electrode, and a Ag/AgCl (3.3 M KCl) electrode as the reference electrode. All potentials were reported with respect to the reference. The absorption spectra were obtained using a Hitachi-4100 spectrophotometer (Tokyo, Japan).

Procedure

Single-stranded DNA (ssDNA) was produced by heating double stranded DNA (dsDNA) solution in water bath at 100°C for 30 min, then promptly cooling it in ice bath. In the following investigation, all DNA were dsDNA except otherwise statement. Pretreatment of GC electrode was carried out according to normal method. The pretreated clean electrodes were immersed into a solution of $HAuCl_4$ (0.2 mg mL^{-1}). Electrochemical deposition was conducted at −200 mV in single potential time base mode, where $AuCl_4^-$ was reduced to AuNPs as described previously [27], forming the AuNPs/GC electrode after removing adsorbent by washing with water. The electrode was refreshed as mentioned above after each measurement.

In 0.1 M phosphate buffer solution (pH 6.0), an appropriate amount of CD solution and DNA were added in sequence and mixed homogeneously. The voltammograms were scanned in a potential range of −0.6~0.8 V (vs. Ag/AgCl).

Results and discussion

Electrochemical behavior of CD at the AuNPs/GC electrode

The electrochemical behavior of CD in 0.1 M phosphate buffer solution (pH 6.0) was investigated. Figure 1 shows the CV of CD on a bare GC electrode (Fig. 1b) and AuNPs/GC electrode (Fig. 1c). When the bare electrode was used, a pair of very small redox peak was recorded. The redox wave of CD at a bare GC electrode is very broad and has a ΔE_p value of 0.39 V. Here ΔE_p is defined as the difference between the anodic peak potential E_{pa} (0.29 V) and the cathodic peak potential E_{pc} (−0.10 V), i.e., $\Delta E_p = E_{pa} - E_{pc}$. Contrary to the above, no redox peak appeared on CVs in the blank buffer solution as measured with the AuNPs/GC electrode (see Fig. 1a) and a pair of well defined reversible peak appeared at the AuNPs/GC electrode (Fig. 1c), indicating that AuNPs is non-electroactive in the selected potential range. The negatively shifted E_{pa} (0.06 V) and positively shifted E_{pc} (0.00 V) result in a much smaller ΔE_p value (0.06 V). Moreover, the responses of the peak

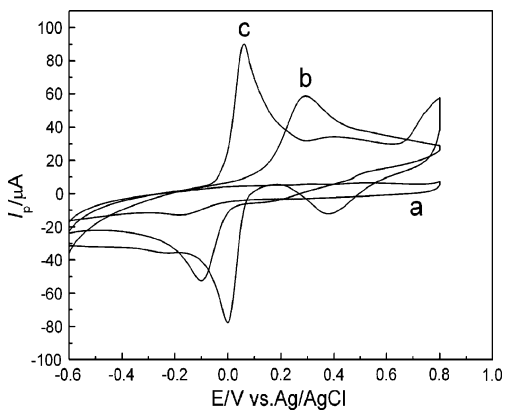

Fig. 1 CVs of 1 mM CD buffer solution alone (**a**) and with bare GC electrode (**b**) and AuNPs/GC electrode (**c**) in 0.1 M phosphate buffer solution (pH 6.0). Scan rate: 0.1 $V \cdot s^{-1}$

currents obviously increased, since AuNPs are similar to a conducting wire or an electron conducting tunnel, making it easier for electron transfer to take place. In other words, AuNPs might have electrocatalysis to the oxidation of CD, and CD containing negative charges on surface could be preferentially adsorbed on positively charged AuNPs/GC electrode in phosphate buffer solution.

The effect of pH on the redox peak currents and peak potentials of CD was also studied, and it was found that the potentials of both redox peaks shifted in the negative direction with increasing pH from 2.0 to 6.0. Investigating the variation of the potential E_p (taken as the average of E_{pc} and E_{pa}, unit mV) with pH can provide valuable information on the electrode reaction process. Based on our experimental data, a linear regression equation E_p=−50.7 pH+365 (correlation coefficient R=−0.998) was obtained, which indicates that the uptake of electron was accompanied by equal number of protons. In order to simulate physiological environment, the 0.1 M phosphate buffer solution (pH 6.0) was directly chosen as a supporting electrolyte. Since the phosphate buffer solution is prone to oxidation and turns red at pH=7.0, its pH value was kept as 6.0 during the experiments.

The effect of scan rate on the peak current of CD was also examined. As shown in Fig. 2, with the increase of the scan rate, the peak current (I_{pa}, I_{pc}) increased. The anodic peak potentials shifted gradually towards positive direction and the cathodic peak potentials shifted towards negative direction. The peak current was proportional to the root of scan rate over a range of 0.05 to 0.5 V·s^{-1}. And the regression equations can be expressed as: I_{pa} (mA)=0.0125+0.3105$\upsilon^{1/2}$ (V·s^{-1}), R=0.998; I_{pc} (mA)=0.0920–0.5090$\upsilon^{1/2}$ (V·s^{-1}), R=−0.996. The above results indicate that the electrode reactions of CD at the AuNPs/GC electrode are diffusion-controlled processes. The relative standard deviation (RSD) of 1 mM CD (n=6) the value of anodic peak current on the AuNPs/GC electrode was 2.3%, which showed excellent stability and reproducibility.

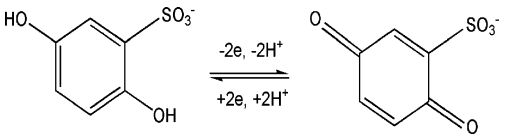

Fig. 3 The proposed mechanism of electrode process of CD

From the CV curve of CD at the AuNPs/GC electrode, an E_{pa} value of 0.060 V and an $E_{pa/2}$ value of 0.019 V were obtained. For a reversible electrochemical system, assuming electronic transfer coefficient α=0.5, according to αn=0.048/(E_{pa}−$E_{pa/2}$) [28], the electron transfer number (n) was calculated to be approximately 2 in the present study. This is similar to that previously reported by Wang et al. [22]. Therefore, the proposed mechanism for CD can be described as in Fig. 3.

Electrochemical behavior of CD in the presence of DNA

Cyclic voltammetry and differential pulse voltammetry were employed for investigating the interaction between CD and DNA, where the well-defined electrochemistry of CD could provide a way to probe the CD-DNA interaction. Figure 4 shows the CVs of CD in the absence and presence of DNA. It can be seen that a couple of reversible redox peaks appeared for CD (Fig. 4a). As for the voltammogram of CD interacting with DNA (Fig. 4b), the duration over which CD is in contact with DNA has a great influence on both the stability of the peak and value of peak current. Thus the optimal rest time was chosen as 5 min, and the accumulation time was chosen as only 2 s so as to eliminate the influence of the competitive adsorption between CD and DNA at the electrode surface. In fact, under the conditions of a lower species concentration and larger electrode area, and shorter accumulation

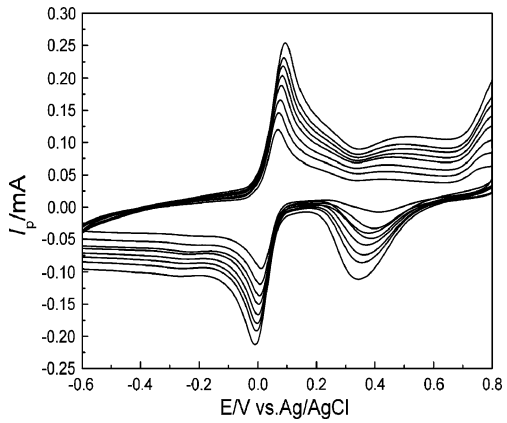

Fig. 2 Influence of scan rate on the peak potential and peak current of 1.2 mM CD. Scan rate from inner to outer: 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, and 0.50 V·s^{-1}

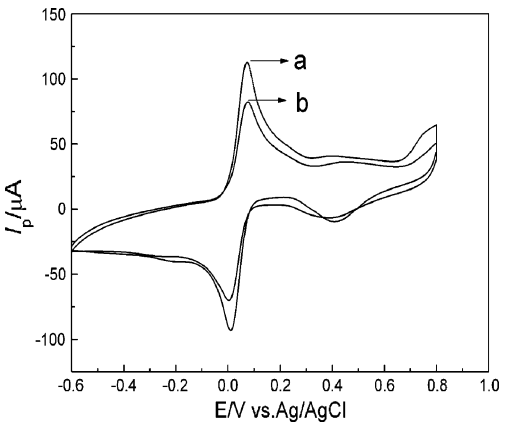

Fig. 4 CVs of 1.2 mM CD in the absence (**a**) and presence (**b**) of 16 μg·mL^{-1} DNA. Accumulation time: 2 s; Scan rate: 0.1 V·s^{-1}

time as well, the so-called competitive adsorption hardly takes place. As shown in Fig. 4b, both the cathodic and anodic peak currents were decreased, indicating that an electrochemically non-active complex could have been formed. Because CD may combine with DNA via either intercalation or outside binding, it is difficult for the complexed CD to contact with the surface of AuNPs/GC electrode and not to be reduced thereon, thus electrochemically non-active complex is generated. However, the formation of complex results in decrease of the equilibrium concentration of CD in solution, leading to a decrease of the peak current. Since DPV technique can be used to investigate the interaction between CD and DNA at a higher sensitivity and better peak resolution, the phenomena mentioned above were further studied by DPV. Figure 5 shows the DPV curves of 7×10^{-5}M CD in the absence (Fig. 5a) and presence (Fig. 5b, c, d and e) of DNA. The peak current in the presence of DNA is obviously weaker than that in the absence of DNA. And interestingly, the decrease of peak current of 7×10^{-5}M CD is proportional to the DNA concentration in a range of 0.8~3.2 μg·mL^{-1}, while the regression equation can be expressed as ΔI_p (μA)=3.13+6.79C (μg·mL^{-1}, R=0.997). This, hopefully, could be used to determine the concentration of DNA.

At the same time, the addition of DNA makes the peak potential shift towards positive direction, confirming the dominance of intercalative interaction between CD and DNA [9]. In addition, the plot of I_p versus $\nu^{1/2}$ is linear with respect to the addition of DNA, indicating that the electron transfer process involves diffusion of substances with DNA.

Chronocoulometry was used to determine the diffusion coefficient for free CD or CD-DNA complex. The chronocoulometric response of diffusion reactant can be described as below [29]:

$$Q = 2nFAc(Dt)^{1/2}/\pi^{1/2} + Q_{dl} + Q_{ads} \quad (1)$$

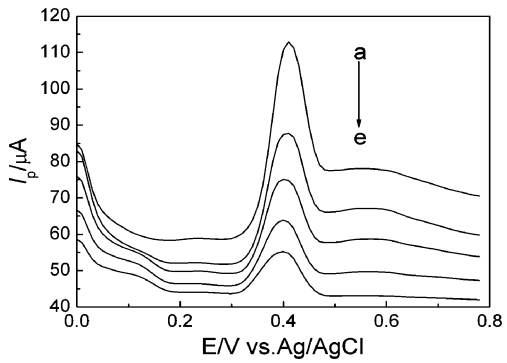

Fig. 5 DPV curves of CD with different concentrations of DNA in solution. Curve a: 7.0×10^{-5}M CD; b: a+0.8 μg·mL^{-1} DNA; c: a+1.6 μg·mL^{-1} DNA; d: a+2.4 μg·mL^{-1} DNA; and e: a +3.2 μg·mL^{-1} DNA

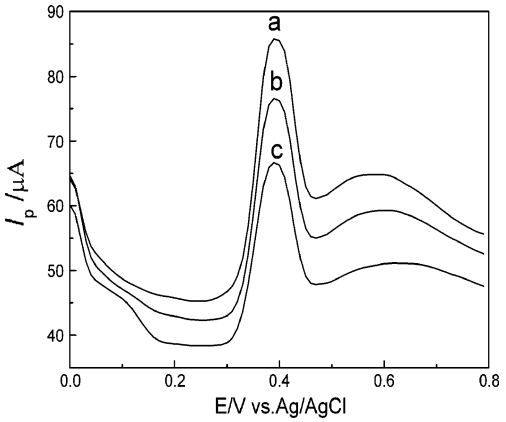

Fig. 6 DPV curves of CD with dsDNA and ssDNA in solution. a: 5.0×10^{-5}M CD; b: a+0.2 μg·mL^{-1} dsDNA; and c: a+0.2 μg·mL^{-1} ssDNA

where n is the number of electrons transferred in reaction, F is the Faraday constant (96,487 C·mol^{-1}), A is the surface area of the working electrode, c is the concentration of reactant, D is the diffusion coefficient of reactant in buffer, t is the pulse width, Q_{dl} is the double-layer charge (integration of charging current), and Q_{ads} is the faradaic component due to the adsorbed species. Based on experimental data, the linear regression equations related to CD and CD-DNA complex can be expressed as Q (μC)= −15.82+88.02 $t^{1/2}$ (S$^{1/2}$), R^2=0.996; Q (μC)=−12.40+75.01 $t^{1/2}$ (S$^{1/2}$), R^2=0.992, respectively. According to Eq. (1), the diffusion coefficient D can be determined from the slope of Q *vs.* $t^{1/2}$ plot if we know n, c and A. The slope of Q *vs.* $t^{1/2}$ plot decreased when DNA was added, indicating a decrease in diffusion rate. Therefore, the diffusion coefficient of 2.24×10^{-5}cm^2·s^{-1} was obtained for free CD and of 1.63×10^{-5}cm^2·s^{-1} for the CD-DNA complex.

Comparison of the interaction of CD with dsDNA and ssDNA

In order to further demonstrate the interaction of CD with DNA, 0.2 μg·mL^{-1} dsDNA (Fig. 6b) and 0.2 μg·mL^{-1} ssDNA (Fig. 6c) were added into 5.0×10^{-5}M CD solution (Fig. 6a), respectively. Interestingly, CD showed discrimination between dsDNA and ssDNA. Namely, although the peak current of CD decreased in both cases, it decreased more sharply with ssDNA, indicating that CD interacted with ssDNA more strongly than with dsDNA.

UV spectroscopic study on the interaction between CD and DNA

In order to validate the mechanism described above, UV-vis absorption spectra of CD in the presence of an increasing

amount of DNA were investigated. In the UV-visible absorption spectra, signatures of intercalative binding, where the planar aromatic ring system inserts itself between the base pairs of DNA, are hypochromism and red shift [30]. As shown in Fig. 7, the addition of DNA to CD solution led to significant differences in the UV-vis absorption spectra as compared with that of CD alone. Namely, the absorption peaks of CD-DNA show a hypochromicity and a 1 nm of red shift with respect to the absorption bands of CD at 221 and 300 nm. Therefore, it can be concluded that binding occurred between DNA and CD via preferential intercalation, which provides further evidences to the mechanism proposed earlier for the electrochemical interaction between CD and DNA.

Determination of the stoichiometric coefficient and association constant between CD and DNA

According to the method of Li [31], it is assumed that CD and DNA only produce a single complex DNA·CD_m, with the reaction schemes as follows:

$$\mathrm{DNA} + \mathrm{mCD} = \mathrm{DNA} \cdot \mathrm{CD}_m \tag{2}$$

In terms of the overall Hill cooperativity model, the fraction of CD bound to DNA is given by:

$$f = [\mathrm{DNA} \cdot \mathrm{CD}_m]/[\mathrm{DNA} \cdot \mathrm{CD_m}]_{\max} = [\mathrm{CD}]^{\mathrm{m}}/\left([\mathrm{CD}]^{\mathrm{m}} + K_{\mathrm{d}}^{\mathrm{m}}\right) \tag{3}$$

where $[\mathrm{DNA}\cdot\mathrm{CD}_m]_{\max}$ represents the maximum concentration of complexed DNA, and [CD] is the concentration of free CD. Then the following formula can be deduced:

$$K_{\mathrm{d}}^{\mathrm{m}} = [\mathrm{CD}]^{\mathrm{m}}(1-f)/f \tag{4}$$

where K_{d} is the equilibrium dissociation constant and m the Hill coefficient. Note that $K_{\mathrm{d}}=[\mathrm{CD}]_{0.5}$ at f=0.5, i.e., at a half occupation. The binding constant K_{a} is given by the reciprocity: $K_{\mathrm{a}}=K_{\mathrm{d}}^{-1}$.

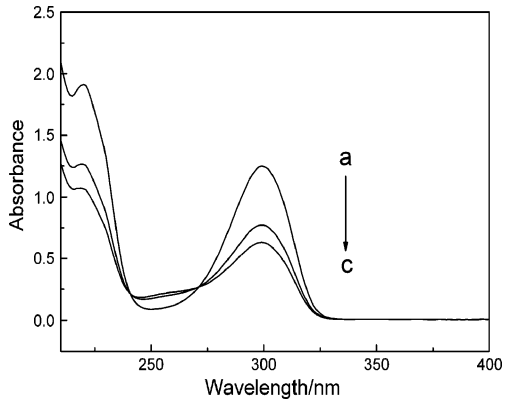

Fig. 7 UV-vis absorption spectra of 1×10^{-4}M CD as functions of increasing concentration of DNA at a: 0, b: 20, and c: 30 μg·mL^{-1}

Mass conservation dictates that the concentration of free DNA is available from the DNA after binding:

$$[\mathrm{DNA}] = [\mathrm{DNA} \cdot \mathrm{CD_m}]_{\max} - [\mathrm{DNA} \cdot \mathrm{CD}_m] \tag{5}$$

For the same reason, we have:

$$[\mathrm{CD}] = [\mathrm{CD}]_0 - m[\mathrm{DNA} \cdot \mathrm{CD}_m] \tag{6}$$

where $[\mathrm{CD}]_0$ is the total concentration of CD. Under the given experimental conditions, the current I is given as:

$$I = k \cdot [\mathrm{CD}] \tag{7}$$

$$\Delta I = I_{(\mathrm{CD})0} - I_{(\mathrm{CD})} \tag{8}$$

Insertion of Eqs. (6) and (7) into Eq. (8) yields:

$$\Delta I = k \cdot \left([\mathrm{CD}]_0 - [\mathrm{CD}]\right) = k \cdot m \cdot [\mathrm{DNA} \cdot \mathrm{CD}_m] \tag{9}$$

$$\Delta I_{\max} = k \cdot m \cdot [\mathrm{DNA} \cdot \mathrm{CD_m}]_{\max} \tag{10}$$

From Eq. (4), we obtain the following equation:

$$\log[f/(1-f)] = m\ \log\ K_{\mathrm{a}} + m\ \log[\mathrm{CD}] \tag{11}$$

Insert Eqs. (9) and (10) into Eq. (11) to yield:

$$\log[\Delta/(\Delta I_{\max} - \Delta I)] = m\ \log\ K_{\mathrm{a}} + m\ \log[\mathrm{CD}] \tag{12}$$

The plot of log $[\Delta I/(\Delta I_{\max}-\Delta I)]$ should be linear with log [CD]. Keeping the DNA concentration and pH constant and changing the concentration of CD from 4.0×10^{-4} to 1.2×10^{-3}M, the linear regression equation log $[\Delta I/(\Delta I_{\max}-\Delta I)]$=1.98log K_{a}+6.77, R^2=0.980. The slope and the intercept were1.98 and 6.77, respectively, which means $m\approx2$ and $K_{\mathrm{a}}=2.63\times10^3$L/mol, implying that only one compound is formed. Thus in terms of stoichiometry, the cooperative binding of CD with DNA is dominated by the cooperation of one CD with at least 2 base pair unit.

Conclusions

The electrochemical behavior of drug CD and its interaction with DNA were studied by electrochemical and spectroscopic methods. Results indicate that the interaction of CD with DNA is dominated by cooperative intercalation, and it can be quantified in terms of the Hill model of cooperative interactions. The redox current of CD was remarkably enhanced on a AuNPs/GC electrode, and the binding interaction of CD with DNA produced a new complex in buffer solution, resulting in decrease of the redox peak current of CD. The present research could serve as a reference for the in vivo investigation of CD with DNA base pairs in living cells.

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