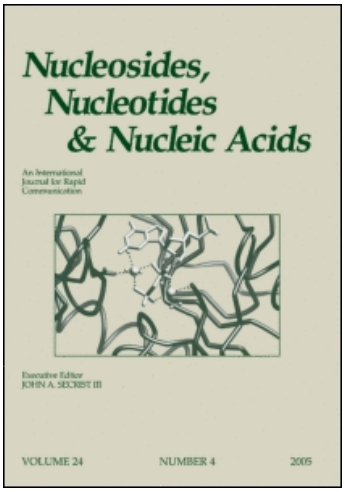

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Study on the Interaction between the Inclusion Complex of Hematoxylin with β-Cyclodextrin and DNA

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STUDY ON THE INTERACTION BETWEEN THE INCLUSION COMPLEX OF HEMATOXYLIN WITH β-CYCLODEXTRIN AND DNA

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□ *Ultraviolet-visible (UV-vis) spectra, fluorescence spectra, electrochemistry, and the thermodynamic method were used to discuss the interaction mode between the inclusion complex of hematoxylin with β-cyclodextrin and herring sperm DNA. On the condition of physiological pH, the result showed that hematoxylin and β-cyclodextrin formed an inclusion complex with binding ratio* $n_{hematoxylin}$:$n_{\beta\text{-}cyclodextrin}$ = 1:1. *The interaction mode between β-cyclodextrin-hematoxylin and DNA was a mixed binding, which contained intercalation and electrostatic mode. The binding ratio between β-cyclodextrin-hematoxylin and DNA was* $n_{\beta\text{-}cyclodextrin\text{-}hematoxylin}$:$n_{DNA}$ = 2:1, *binding constant was* $K^{\ominus}_{298.15K} = 5.29 \times 10^4\ L{\cdot}mol^{-1}$, *and entropy worked as driven force in this action.*

Keywords Spectrometry; β-cyclodextrin; hematoxylin; inclusion complex; herring sperm DNA

INTRODUCTION

Current studies on supramolecular interactions of organic dyes with biological molecules are significant to understand the functions of biomacromolecules.[1] Generally, the interactions of organic dyes with DNA involve three binding modes: namely, intercalative binding that dyes intercalate into the base pairs of nucleic acids,[2] groove binding in which the dyes bound on nucleic acids are located in the major or minor groove,[3] and electrostatic interaction.

Cyclodextrins (CDs) are macrocyclic glucose oligomers capable of binding a variety of guest molecules inside their hydrophobic cavities altering the

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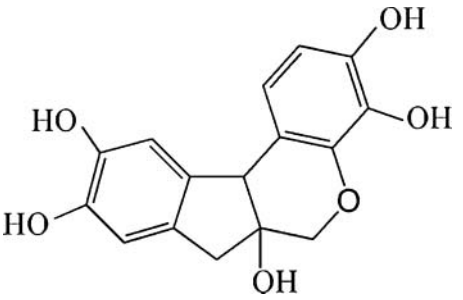

FIGURE 1 Molecular structure of hematoxylin.

immediate environment of the reacting species.[4,5] Because of the unique properties, CDs have been widely used as novel media for photophysical and photochemical studies and building blocks for supramolecular structures and functional units as well as in various fields of industries.[6] As is well known, the hydrophobic effect can impel the hydrophobic groups of the guest inside the cavity of CD and the hydrophilic groups outside. If several charged groups have been linked onto the edge of CD, the characteristics of parent CDs will be changed. If the hydrophilic groups are just those which interact with or intercalate into DNA, the inclusion action of β-CD must affect the interaction in some degree. Both DNA and CD are common in having hydrophilic coat/hydrophobic core structure. Thus, an aromatic ring stacking between nucleobase pairs or incorporated in CD cavity is the main driving force for the binding of an intercalator into double stranded DNA and a guest molecule to CD, respectively.[7] These prompted us to investigate the complexing properties of CD and DNA as hosts for a guest. Hematoxylin (HE) is a biological dye that can form hydrogen bonds with base pairs. (Its structure is shown in Figure 1.) In this study, CDs were used to change the microenvironment of HE molecule that interacts with DNA. Through comparing the difference, it is concluded that HE interacts with DNA by "intercalative binding" while β-CD-HE with DNA by "intercalative and electrostatic binging."

EXPERIMENTS AND METHODS

Materials

Herring sperm DNA was purchased from Sigma Biological Co. (USA) and used as received. Purity of DNA was checked by monitoring the ratio of absorbance at 260–280 nm. The ratio was 1.89, indicating the DNA was free from protein. The DNA was dissolved in doubly distilled deionized water with 50 mM NaCl and dialyzed for 48 hours against a Tris-HCl buffer solution at 277 K. The concentration of DNA stock solution was determined according to the absorbance at 260 nm by using the extinction coefficients of 6600 $(\text{mol}\cdot\text{cm})^{-1}$.

All of the samples were dissolved in Tris-HCl buffer (pH7.40, examined by acidometer). Tris was purchased from Tianjin Kemi'ou Chemical Reagents Center, China. Neutral red (NR), hematoxylin, and β-cyclodextrin were purchased from Sichuan Chengdu China Kelong Chemical Plant (A.R.), China. Other reagents were at least analytical grade, and were used without further purification.

Instruments

The absorption spectra were recorded on an UV-3150 spectrophotometer (Shimadzu, Japan). The fluorescence spectra were recorded on a PE LS55 spectrofluorophotometer (Perkin Elmer, USA). The cyclic voltammetry were performed on the PARSTAT 2273 Electrochemical system (Princeton Applied Research, USA), with a three-electrode system: the working electrode—a glassy carbon, the reference electrode—calomel, and the counter electrode—a platinum wire. The pH was recorded on a pHS-2C acidometer (China). All of the spectroscopic work was carried out at pH 7.40 remained by a Tris–HCl buffer.

Procedures

Samples for absorption and fluorescence were prepared by mixing known amounts of stock solutions of hematoxylin (HE), DNA and β-cyclodextrin (β-CD) in Tris-HCl buffer (pH 7.40) and diluted to the required concentrations. The absorption and fluorescence titrations were performed by keeping the concentration of HE constant while varying the concentration of β-CD, or keeping the concentration of β-CD-HE inclusion complex constant while varying the concentration of DNA. All the absorption measurements were made against the blank solution. In fluorescence mode, both excitation and emission bandwidths were set at 5 nm, $\lambda_{ex} = 411$ nm. 1.0-cm path-length quartz cuvettes were used for absorption and fluorescence measurements.

Electrochemical experiments were carried out with a conventional three-electrode system. The glassy carbon (GC) electrode was used as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl electrode as the reference electrode. The GC electrode surface was polished first with Al_2O_3 polishing powder, and then it was cleaned ultrasonically for 5 minutes in doubly distilled water. In Tris-HCl buffer solution (pH 7.4), an appropriate amount of β-CD-HE solution and DNA were added in sequence and mixed homogeneously. The voltammograms were scanned in a potential range of $-0.3.0 \sim 0.3$ V (vs. Ag/AgCl), with a scan rate of 0.05 V/S.

RESULTS AND DISCUSSION

Formation of β-CD-HE Inclusion Complex

Cramer[8] was the first to discover that that cyclodextrin could affect the ultraviolet-visible (UV-vis) spectra of organic molecules, and proposed that the spectral changes were because of the high electron density in cyclodextrin cavity induced the electrons of guest molecules to move. As shown in Figure 2A, with increasing concentration of β-CD, hematoxylin absorbance intensities showed a regular increase, indicating the formation of an inclusion complex between β-CD and HE. In order to determine the stoichiometry of the formation of β-CD-HE complex, the mole ratio experiment[9] was done at the peak 562 nm. The absorption spectra of HE upon increasing the concentration of β-CD were record at 562 nm, and then the graph was plotted by mole ratio method. The mole ratio plots of HE with β-CD were shown in Figure 2B. The binding ratio of the complex was: $n_{\beta\text{-CD}}$:$n_{\text{HE}} = 1{:}1$. The inclusion constant (K) is an important parameter to represent the inclusion capacity, which can be determined by the double-reciprocal method using the following equation[10,11]:

$$1/\triangle A = 1/(A_0 - A) = [1/(a \cdot K_{\text{f}})] \cdot (1/c_{\beta-CD}) + (1/a) \cdot c_{\text{HE}}, \qquad (1)$$

where A_0 and A are the absorbencies of HE in the absence and in the presence of β-CD, respectively. α is the constant. K_{f} is the binding constant between β-CD and HE, $c_{\beta\text{-CD}}$ is the concentration of β-CD. The binding constants were calculated from the ratio of the intercept on the vertical (Figure 3): $K_{\text{f}} = 294.12\ \text{L}\cdot\text{mol}^{-1}$.

The Interaction of β-CD-HE with DNA

Absorption Spectra Studies

UV-vis absorption spectra of β-CD-HE inclusion complex at various concentrations of DNA were obtained (Figure 4). Generally, red shift and hypochromic effect are observed in the absorption spectra of small molecules if they intercalate with DNA, and red shift and hypochromic effect are unremarkable when groove binding or electrostatic interaction takes place. With the addition of DNA, it can be seen that the absorption peaks of the inclusion complex exhibited regular decrease, and a new absorption peak is observed at 660 nm. The isochromatic point at 570 nm confirms that a new complex of DNA-β-CD-HE is formed.[12] The intercalative binding of β-CD-HE to a DNA helix has been characterized by large changes in the absorbance and an appreciable shift in wavelength due to the interaction of a DNA π stack and HE π system.

In order to determine the stoichiometry of the formation of DNA-β-CD-HE inclusion complex, the mole ratio method experiment was done at the

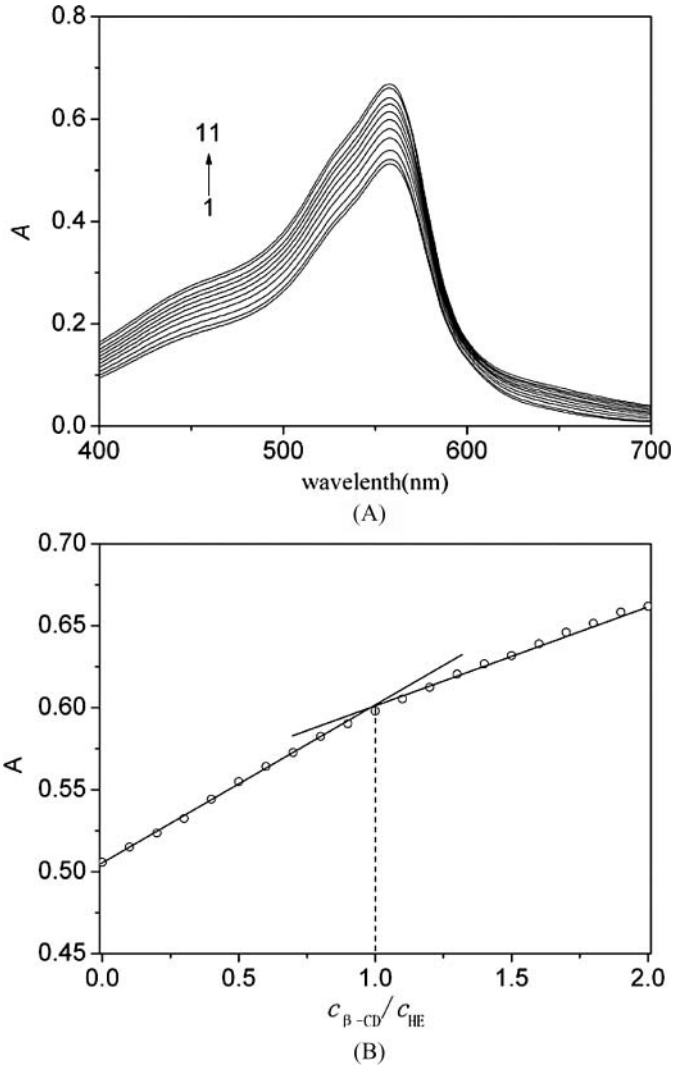

FIGURE 2 A) UV-vis absorption spectra of HE in different concentrations of β-CD (pH7.40). From curve 1–11, $c_{HE} = 1.00 \times 10^{-4}$ mol·L^{-1}, $c_{\beta\text{-CD}} = 0.00, 0.06, 0.12, 0.18, 0.24, 0.30, 0.36, 0.42, 0.48, 0.54, 0.60 \times 10^{-3}$ mol·L^{-1}, respectively. B) Mole ratio plots of HE and β-CD (pH 7.40; $\lambda = 562$ nm); $c_{HE} = 1.00 \times 10^{-4}$ mol·L^{-1}.

peak 660 nm. The Mole ratio plots of DNA with β-CD-HE were shown in Figure 5. The binding ratio of the complex was: $n_{\beta\text{-CD-HE}}$:$n_{DNA} = 2$:1. While it was reported[13] that the binding ratio between HE and DNA was n_{HE}: $n_{DNA} = 3$:1, it showed that the hydrophobic environment of β-CD changed the interaction between HE and DNA, therefore the binding sites decreased. According to Lambert-Beer law:

$$A = \varepsilon bc, \tag{2}$$

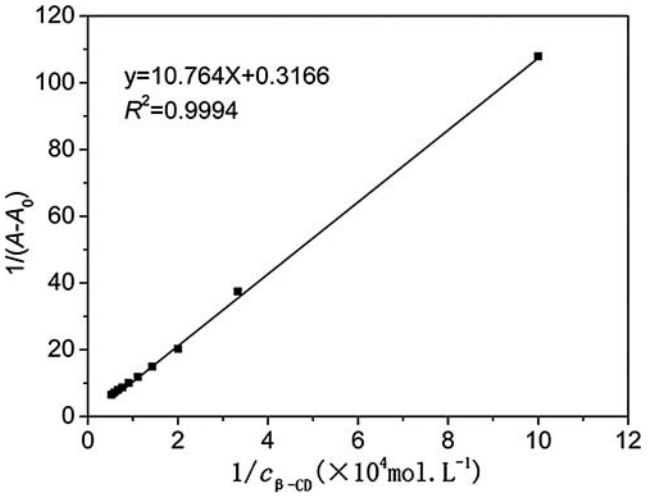

FIGURE 3 Double reciprocal curves for HE to β-CD in a tris-HCl buffer (pH 7.40); $c_{HE} = 1.00 \times 10^{-4}$ mol·L^{-1}.

where A is the absorbance of the DNA-β-CD-HE; ε is the molar absorptivity of DNA-β-CD-HE; c is the concentration of DNA-β-CD-HE. The apparent mol absorption coefficient of DNA -β-CD-HE was counted: $\varepsilon = 5.00 \times 10^5$ L·mol^{-1}·cm^{-1}.

The absorption relationship between the complex and DNA was expressed by double reciprocal equation:

$$1/(A_0 - A) = 1/A_0 + 1/(K \times A_0 \times c_{\text{DNA}}), \tag{3}$$

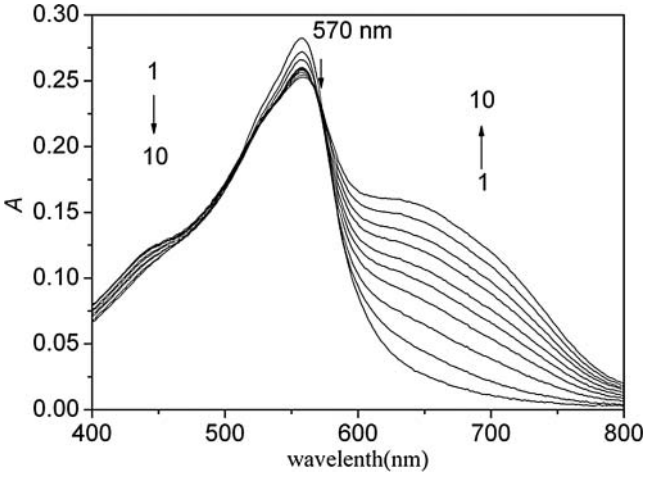

FIGURE 4 UV-vis absorption spectra of β-CD-HE in different concentrations of DNA (pH 7.40). From curve 1–10, $c_{\beta\text{-CD-HE}} = 5.00 \times 10^{-6}$ mol·L^{-1}; $c_{\text{DNA}} = 0.00, 0.06, 0.12, 0.18, 0.24, 0.30, 0.36, 0.42, 0.48, 0.54 \times 10^{-4}$ mol·L^{-1}, respectively.

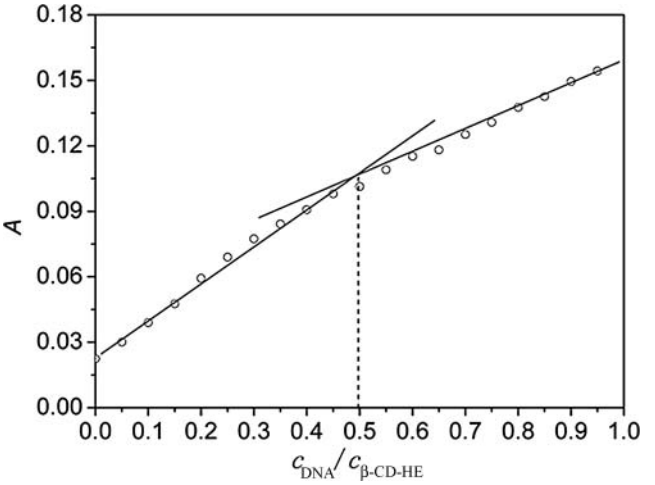

FIGURE 5 Mole ratio plots of β-CD-HE and DNA in a tris-HCl buffer (pH 7.40; $\lambda = 660$ nm); $c_{\beta\text{-CD-HE}} = 5.00 \times 10^{-6}$ mol·L^{-1}.

where A_0 and A are the absorbance of β-CD-HE in the absence and in the presence of DNA, respectively. K is the binding constant between β-CD-HE and DNA, c_{DNA} is the concentration of DNA.

The double reciprocal plots of $1/(A_0-A)$ versus $1/c_{DNA}$ were linear (at 298.15 K and 310.15 K, respectively.), and the binding constants were calculated from the ratio of the intercept on the vertical (Figure 6): $K^{\ominus}_{298.15K} =$

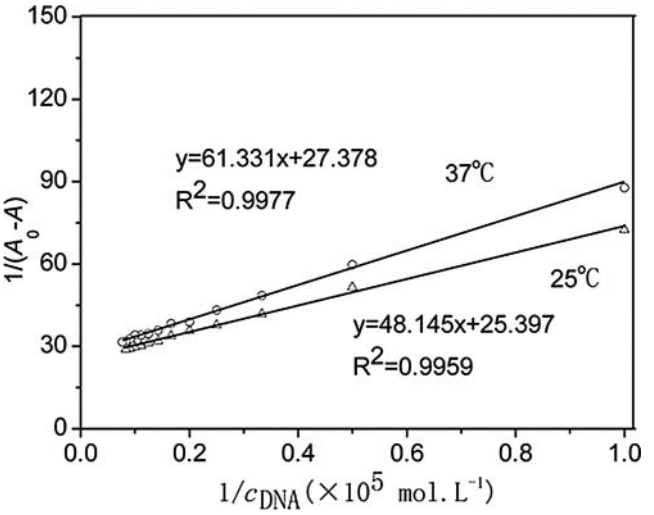

FIGURE 6 Double reciprocal plots for DNA-β-CD-HE in a tris-HCl buffer (pH 7.40) at 25 and 37°C, respectively; $c_{\beta\text{-CD-HE}} = 5.00 \times 10^{-6}$ mol·L^{-1}.

5.29×10^4 L·mol^{-1}, $K^{\ominus}_{310.15K} = 4.46 \times 10^4$ L·mol^{-1}. According to Thermodynamic equation of $K^{\ominus}$, $\triangle_r H^{\ominus}_m$, $\triangle_r G^{\ominus}_m$, $\triangle_r S_m{}^{\ominus}$ and T:

$$\ln K^{\ominus}_2/K^{\ominus}_1 = -\triangle_r H^{\ominus}_m(1/T_2 - 1/T_1)/R \quad (4)$$

$$\triangle_r G^{\ominus}_m = -RT\ln K^{\ominus} \quad (5)$$

$$\triangle_r G_m, T^{\ominus} = \triangle_r H^{\ominus}_m - T\triangle_r S^{\ominus}_m, \quad (6)$$

where $K^{\ominus}_1$ refers to standard binding constant of β-CD-HE and DNA at 298.15 K, $K^{\ominus}_2$ refers to standard binding constant of β-CD-HE and DNA at 310.15 K. T_1 is 298.15 K, T_2 is 310.15 K, $\triangle_r H^{\ominus}_m$ is standard molar reaction enthalpy. $\triangle_r G^{\ominus}_m$ refers to the standard molar reaction Gibbs free energy. $\triangle_r S^{\ominus}_m$ refers to the standard molar reaction entropy. Then $\triangle_r H^{\ominus}_m = 2.50 \times 10^5$ J·mol^{-1} is deduced. The positive result shows that it is an endothermic reaction that temperature enhancement redounded to reaction processes. The $\triangle_r G^{\ominus}_{m298.15K} = -2.70 \times 10^4$ J·mol^{-1}. The negative result shows spontaneous interaction tide between β-CD-HE and DNA. The $\triangle_r S^{\ominus}_m$ is 98.91 J·mol^{-1}·K^{-1}. The results suggest that the process of interaction of β-CD-HE and herring-sperm DNA is driven by entropy.[14,15]

Fluorescence Measurements

Fluorescence titration was a useful method in the studies of binding properties of small molecules to DNA.[16,17] The fluorescence measurements were carried out with neutral red (NR) as probe. Neutral Red (NR) is a planar phenazine dye. In recent years, the interaction of the fluorescent NR dye with DNA has been demonstrated by spectrophotometric method. Compared with a common fluorimetric probe, ethidium bromide (EB), the NR dye offers lower toxicity, higher stability and convenience of use. In addition, its solution remains stable for up to 2 years.[18,19] In this work, NR was selected as the probe, because of its conjugated planar, it can insert between two adjacent base pairs in a DNA helix to get the fluorescence intensity remarkably increased. If β-CD-HE has the same binding mode with DNA as well as NR, there is a competition mode between NR and β-CD-HE with DNA. So the fluorescence spectra will be changed.[20,21] The influence on emission spectra of NR to DNA-β-CD-HE was shown in Figure 7. It can be seen that the new fluorescence peak at 620 nm increases, while the peak at 520 nm decreases upon the addition of NR. The new isosbestic point at 550 nm confirms the interaction between NR and DNA. Figure 8 showed the fluorescence of DNA-NR in different concentrations of β-CD-HE.

It can be seen that the fluorescence peak at 620 nm is gradually quenched by the adding of β-CD-HE while the peak at 520 nm increases, at the same time the isosbestic point also appeares at 585 nm. The fluorescence spectra of Figures 7 and 8 show that the reaction competition between NR and β-CD-HE with DNA are remarkable. According to the intercalation binding

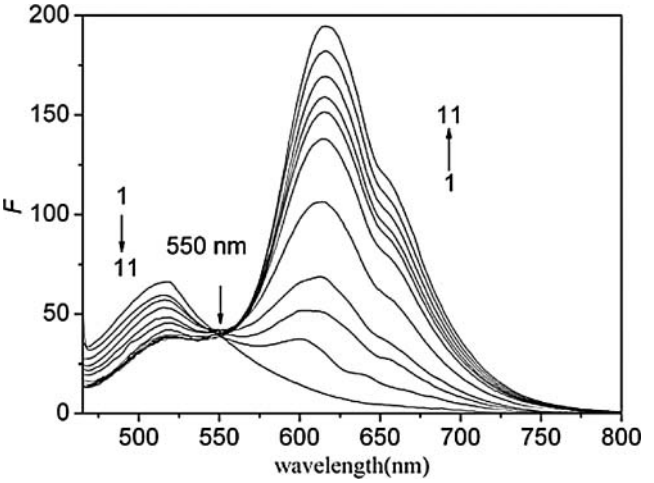

FIGURE 7 Emission spectra of DNA-β-CD-HE mixture in different concentrations of NR (pH 7.40; λ_{ex} = 411 nm). From curve 1–11, $c_{\text{DNA-}\beta\text{-CD-HE}} = 2.50 \times 10^{-6}$ mol·L^{-1}; $c_{\text{NR}} = 0.00$, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10 $\times 10^{-4}$ mol·L^{-1}, respectively.

mode between NR and DNA, intercalation binding mode between β-CD-HE and DNA is basically confirmed.[22]

The binding mode between small molecules and DNA can be determined using the Scatchard's procedure.[23] Scatchard equation expresses the binding of NR-DNA in the presence of β-CD-HE:

$$r/c = K\,(n - r), \tag{7}$$

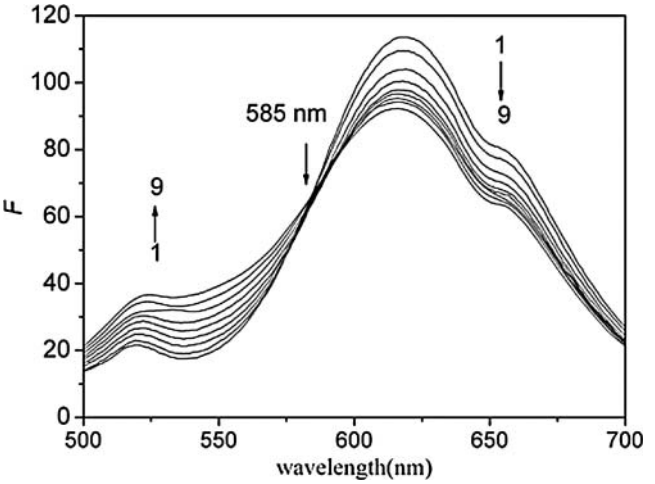

FIGURE 8 Emission spectra of DNA–NR mixture in different concentrations of β-CD-HE (pH 7.40; λ_{ex} = 411 nm). From curves 1–9, $c_{\text{DNA–NR}} = 1.00 \times 10^{-4}$ mol·L^{-1}; $c_{\beta\text{-CD-HE}} = 0.00$, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40 $\times 10^{-6}$ mol·L^{-1}, respectively.

where r is in moles of NR bound per mole of DNA, c is the molar concentration of free NR, n is binding site multiplicity per class of binding sites and K is the association binding constant of NR with DNA. Generally, it is regarded as an intercalation binding mode if the values of n are same in the presence and absence of the inclusion complex, and it is regarded as a dis-intercalation binding mode if the values of K are same. And it is regarded as mix binding mode including dis-intercalation and intercalation binding if both the values of n and K are changed.[24]

In order to investigate the effect of electrostatic force on the interaction between β-CD-HE and DNA, two groups of buffers in presence and absence of NaCl as a contrast were constructed (Figures 9A and 9B). From the Scatachard plot, we can get the value of K and n. The results were shown in Table 1.

As shown in Table 1, it can be seen that both values of n and K change with the different concentrations of β-CD-HE. The variation of the parameter n and K suggest a mix interaction herein, which contained intercalation and nonintercalation. It also observed that both n and K change again in presence of NaCl. It may because that the existence of Na^+ interfered with electrostatic interaction by combining with the negative polyphosphate skeleton of DNA, and tightened the DNA-chain, so that the complex was hard to intercalate in. These results prove that β-CD-HE interacte with DNA by some static electronic effects.

Cyclic Voltammetry

Cyclic voltammetry (CV) has proved to be a very sensitive analytical technique to detemine changes in redox behavior of biological dye in the presence of biologically important molecules.[25,26] The redox behavior of biological dye is very sensitive to the coordination surrounding of biological dye, therefore, the interaction of β-CD-HE with DNA can be detected using this technique.[27]

Normally, the peak potential shifts positively, indicating the mode between small molecules and DNA are hydrophobic interaction, when the

TABLE 1 Data of Scatchard equation of the interaction between β-CD-HE and DNA

Curve	$c_{\beta\text{-CD-HE}}/c_{\text{DNA}}$	NaCl %	Scatchard	$K/(\text{L}\cdot\text{mol}^{-1})$	n
a.	0.00	0.50	$0.18 \times 10^4 - 1.17 \times 10^5$x	1.17×10^5	0.015
		0	$2.53 \times 10^4 - 4.22 \times 10^5$x	4.22×10^5	0.059
b.	0.40	0.50	$1.08 \times 10^4 - 3.02 \times 10^5$x	3.02×10^5	0.036
		0	$4.59 \times 10^4 - 6.63 \times 10^5$x	6.63×10^5	0.069
c.	0.80	0.50	$3.49 \times 10^4 - 6.90 \times 10^5$x	6.90×10^5	0.051
		0	$7.37 \times 10^4 - 10.61 \times 10^5$x	10.61×10^5	0.069
d.	1.20	0.50	$2.87 \times 10^4 - 2.61 \times 10^5$x	2.61×10^5	0.019
		0	$9.20 \times 10^4 - 10.57 \times 10^5$x	10.57×10^5	0.087

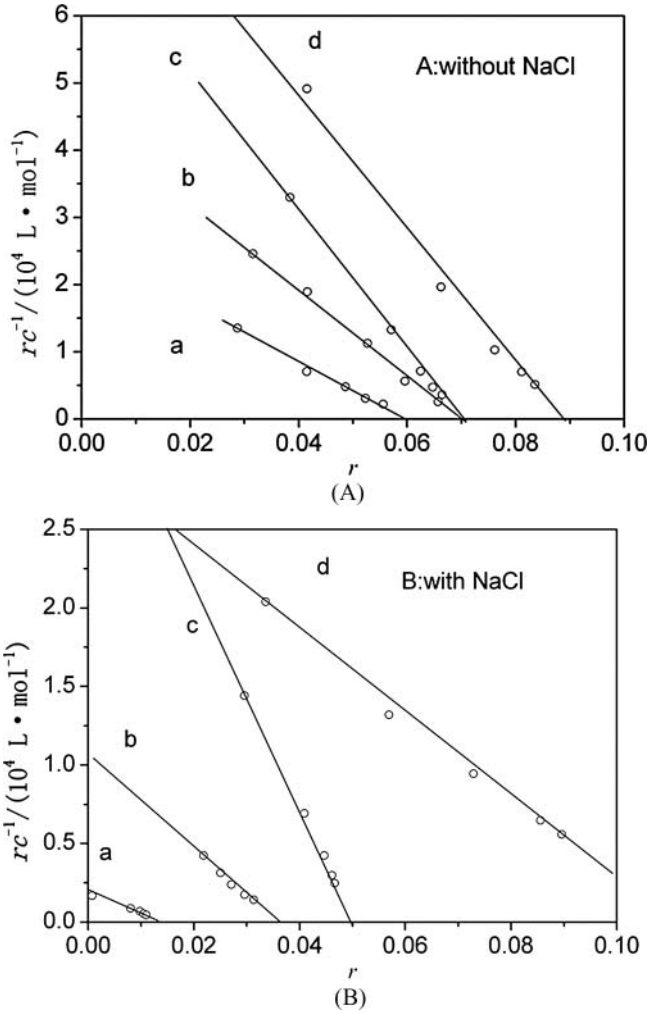

FIGURE 9 A) Scatchard plots of DNA-β-CD-HE in different concentrations of NR (without NaCl; pH 7.40). $c_{DNA} = 1.00 \times 10^{-5}$ mol·L^{-1}; $c_{NR} = 3.00 \times 10^{-4}$mol·L^{-1}; Rt = $c_{\beta\text{-CD-HE}}/c_{DNA}$; RT = a:0.00, b:0.40, c:0.80, d:1.20. B) Scatchard plots of DNA-β-CD-HE in different concentrations of NR (with NaCl; pH 7.40). $c_{DNA} = 1.00 \times 10^{-5}$ mol·L^{-1}; $c_{NR} = 3.00 \times 10^{-4}$mol·L^{-1}; Rt = $c_{\beta\text{-CD-HE}}/c_{DNA}$; RT = a:0.00, b:0.40, c:0.80, d:1.20.

peak potential shifts negatively, it occurs electrostatic interaction. Figure 10 exhibits the cyclic voltammogram of DNA-β-CD-HE in tris-HCl buffer solution (pH 7.4). The electrochemical properties of β-CD-HE in the absence and presence of DNA were studied within −0.30V to 0.30 V, sweep rate at 0.05 V·S^{-1}. Under the selected conditions, a well-defined oxidation peak was observed at 0.175 V. With the addition of DNA, peak current revealed a positive shift to 0.188 V. These results suggested the existence of intercalation between β-CD-HE and DNA.

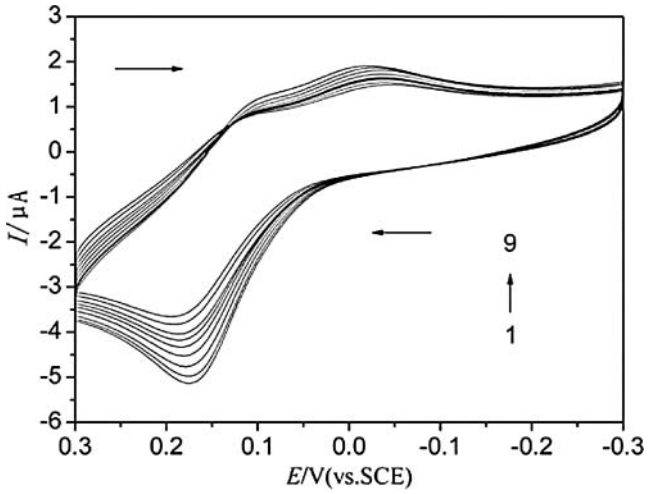

FIGURE 10 Cyclic voltammograms of β-CD-HE in the absence and presence of DNA; From curve 1–9, $c_{\beta\text{-CD-HE}} = 5.00 \times 10^{-5}$ mol·L^{-1}; $c_{\text{DNA}} = 0.000, 0.075, 0.150, 0.225, 0.300, 0.375, 0.450, 0.525, 0.600 \times 10^{-4}$ mol·L^{-1}, respectively.

CONCLUSIONS

By means of ultraviolet and thermodynamic methods, the results indicated that β-CD-HE inclusion complex can bind to DNA, red shift was observed in the absorption spectra. These results further supported by fluorescence spectra and electrochemistry where the binding modes of β-CD-HE to DNA were electrostatic mode and intercalation binding. The molecular structure of hematoxylin containing binary phenol ring can insert to DNA molecule. These results strongly supported the idea that hematoxylin could be useful for design of new and more efficient drugs targeted to DNA.

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