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Direct probing of the folding/unfolding event of bovine hemoglobin at montmorillonite clay modified electrode by adsorptive-transfer voltammetry

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ABSTRACT

An efficient way was proposed for probing the folding/unfolding event of bovine hemoglobin (Hb) through adsorptive-transfer voltammetry. Hb molecules in native and pre-unfolded in different urea conditions for 23 h were adsorbed onto the montmorillonite clay modified glassy carbon electrode (Hb/clay/GCE and uHb/clay/GCE, respectively). Cyclic voltammograms of Hb/clay/GCE and uHb/clay/GCE showed that the unfolding of Hb caused great change in the direct electron transfer between the heme irons within Hb and electrode surface, which was facilitated on clay film. From the amount of the electroactive Hb (W_{Hbe}) and the adsorbed Hb (Γ) on clay per unit mass, the minimal electroactive portion (MEP) of the adsorbed Hb was calculated to assess the unfolding state of Hb. With the increase of urea concentration, MEP showed a sigmoid curve. Thermodynamic parameters related to the unfolding event of Hb were also obtained based on the linear free energy model (LEM), including the free energy of folding in water ($\Delta G_U{}^{water}$), the slope of the Santoro–Bolen equation (m), and the urea concentration required in for achieving half of the total change (S_m) in the unfolding curves. This work gave the first try for investigating protein unfolding at nano-materials modified electrode using adsorptive-transfer voltammetry, which improved the sensitivity of analysis and avoided the disadvantages involved in the existing electrochemical methods for protein unfolding. The proposed method will benefit the electrochemical studies of protein.

1. Introduction

The investigation of protein folding/unfolding has drawn increasing interest because of its great importance in providing knowledge of the relationship between the structure and the function of protein, and in better understanding the reasons of some diseases, such as Alzheimer's disease, Parkinson's disease, systemic amyloidoses, and many other abnormalities [1,2]. Although several techniques have been developed and utilized to probe protein conformation and folding/unfolding state, including fluorescence, circular dichroism (CD), UV–vis absorption, IR/Raman and mass spectroscopy [3–6], electrochemical methods play a considerable role due to their high sensitivity, rapid analysis, low instrument cost [1,7–9], and the capability of achieving kinetic and thermodynamic information on protein unfolding [10–12].

Currently, electrochemical investigations on the folding/unfolding of proteins mainly focus on metalloproteins, such as cytochrome *c* (cyt. *c*), myoglobin and hemoglobin [1,7,9,13–19]. The heme irons in these proteins are used as endogenous electroactive probes [1,18,19]. Proteins are commonly pre-unfolded by a certain denaturant of high concentration. The conformational transition information of protein is therefore directly monitored at a bare or modified electrode [1,18] in the denaturing solution through the change of the redox behavior of heme irons. However, several potential disadvantages are involved in these approaches. For instance, the addition of concentrated denaturant increases the viscosity of protein solution, which restricts the current response of heme irons. The influence from the trace of oxygen in protein solution is hardly removed because lots of foam are formed as deaerating the protein solution [9]. The direct electrochemical response of heme irons in many proteins is hardly achieved since heme irons are usually deeply buried into the hydrophobic core of polypeptides within protein molecules. Therefore, low sensitivity always limits these methods.

It is well known that nano-scale or biocompatible materials can greatly promote the redox reaction of heme irons [20–24]. Besides, our previous work indicated that adsorptive-transfer voltammetry, which is to adsorb the analyte at an electrode, and transfer the electrode (with the adsorbed layer) in a medium solution to perform voltammetric analysis, can easily avoid the influence of high viscosity and solve the difficulty of deaerating protein solution [25]. Therefore, by integration of nano-materials modified electrode with adsorptive-transfer process, the sensitivity of the

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electrochemical analysis of protein folding/unfolding can be confessedly improved without involving those problems mentioned above. However, two issues are mostly concerned in the use of this approach: the possible changes in the structures of protein during the adsorption onto nano-scale materials, and the amount of the adsorbed protein under different denaturing conditions.

In this work, the folding/unfolding of bovine hemoglobin (Hb) was directly investigated at montmorillonite clay modify glassy carbon electrode (clay/GCE) by adsorptive-transfer voltammetry. As shown in Scheme 1, native or urea unfolded Hb (uHb) was adsorbed onto clay/GCE (Hb/clay/GCE and uHb/clay/GCE, respectively). The possible structural change of proteins after their adsorption was addressed by UV–vis, and the variation of the adsorption amount of Hb under different denaturing conditions was calibrated by the minimal electroactive portion (MEP) of adsorbed uHb, in which both the amount of the electroactive Hb (W_{Hbe}) and the total adsorbed Hb (Γ) on clay per unit mass were considered. The conformational transition of Hb induced by urea was revealed, and the thermodynamic parameters related to the unfolding event of Hb were obtained and compared with those from spectral techniques specifically.

2. Experimental

2.1. Chemicals and Reagents

Bovine Hb (lyophilized) was purchased from Sigma and used without further purification. Urea was obtained from Fluka (puriss. p.a., ACS ≥ 99.5%). All other chemicals were of analytical grade.

Phosphate buffer solution (PBS, 0.1 mol L^{-1}, pH 7.0) was prepared from the stock solutions of Na_2HPO_4 and NaH_2PO_4 (0.1 mol L^{-1}), and adjusted to the pH value. Urea solutions with different concentrations (0–9 mol L^{-1}) were prepared by dissolving desirable amount of urea in PBS (0.1 mol L^{-1}, pH 7.0). Montmorillonite clay suspension (1 mg mL^{-1}) was prepared by dispersing clay (1 mg) in doubly distilled water (1 mL) with ultrasonication and resonicated before use. All aqueous solutions were prepared with doubly distilled water.

2.2. Electrochemical measurements

Glassy carbon electrode (GCE, 3 mm in diameter) was polished successively with 1.0, 0.3, and 0.05 μm alumina powders, and sonicated in 1:1 nitric acid, ethanol, and doubly distilled water, respectively. Montmorillonite clay suspension (5 μL) was then carefully dropped onto the surface of GCE and dried at room temperature (clay/GCE). Before electrochemical experiments, Hb solutions (4 mg mL^{-1}) containing different concentrations of urea (0–9 mol L^{-1}) were prepared. The Hb solutions were incubated at 4 °C for 23 h. Then the clay/GCE was immersed into the Hb solutions for 1 h to adsorb Hb on clay/GCE (uHb/clay/GCE). After extensively rinsed with doubly distilled water, the resulting electrodes were ready to be measured.

Cyclic voltammetry (CV) was performed with a CHI 660C electrochemical workstation (Shanghai, China). Electrochemical impedance spectroscopy (EIS) was performed with a μ-Autolab type III electrochemical workstation (EcoChemie, Netherlands). A conventional three-electrode system was used with an Ag/AgCl (3 mol L^{-1} KCl) electrode as the reference electrode, a platinum wire as the counter electrode, and a modified GCE as the working electrode. All CV measurements were performed in deaerated PBS (0.1 mol L^{-1}, pH 7.0) at room temperature. A nitrogen environment was kept over solutions in the cell for protecting the solution from oxygen. EIS of the modified GCE was obtained in 0.1 mol L^{-1} KCl containing 1 mmol L^{-1} $K_3Fe(CN)_6$. The polarized voltage was +0.2 V. The frequency range was from 0.1 to 10,000 Hz with amplitude of 10 mV.

2.3. Spectral measurements

UV–vis absorption spectra were recorded with a Shimadzu UV-3150 UV-vis-NIR spectrophotometer. Hb and Hb/clay thin films were prepared by casting 600 μL Hb solution (4 mg mL^{-1}) and Hb/clay solution (4 mg mL^{-1} Hb in 1 mg mL^{-1} clay suspension) onto quartz slide, separately. The thin film UV–vis spectra of Hb and Hb/clay were obtained in the wavelength of 360–460 nm at the scan rate of 200 nm min^{-1}. Fluorescence measurements were carried out with a Shimadzu RF-5301PC spectrofluorimeter using 280 nm as the excitation wavelength, and the emission spectra were in the range of 300–400 nm.

For all spectral measurements (except the thin film UV–vis), Hb was incubated in urea (0–9 mol L^{-1}) for 24 h at 4 °C to reach equilibrium. In order to obtain better spectra, all Hb solutions were diluted before measurements, and the time between the measurements and dilution was controlled within seconds. The concentrations of Hb for UV–vis and fluorescence measurements were 50 and 200 mg L^{-1}, respectively. All spectral measurements were carried out at room temperature.

2.4. The measurements of the amount of the Hb adsorbed on clay and the electroactive Hb on the clay/GCE at each urea concentration

The amount of Hb adsorbed on clay at each urea concentration was estimated as following: firstly, Hb solutions (50 mg L^{-1} Hb in 4 mL urea solution with different concentrations from 0 to 9 mol L^{-1}) were prepared and incubated for 24 h at 4 °C. The maximum absorbance of these Hb solutions at 405 nm was measured by UV–vis as A_o. Secondly, Hb solutions (50 mg L^{-1} Hb in 4 mL urea with different concentrations from 0 to 9 mol L^{-1}) were prepared and incubated for 23 h at 4 °C. These Hb solutions were then added with clay (10 mg) and gently shaking for 1 h. The suspensions were centrifuged at 3000 rpm for 30 min and the maximum absorbance of the supernatant at 405 nm was measured by UV–vis as A_x. The amount of the Hb adsorbed on clay per unit mass (Γ) was estimated according to Eq. (1) [26]:

$$\Gamma = \frac{C_o(1 - (A_x/A_o))V}{m_{clay}} \quad (1)$$

where C_o represents the concentration of Hb in the absence of clay. A_x and A_o represent the absorbance of Hb at 405 nm of UV–vis spectra in the presence and absence of clay. V is the volume of Hb solution. m_{clay} stands for the amounts of clay used to adsorb Hb.

The amount of electroactive Hb adsorbed on clay per unit mass (W_{Hbe}) was estimated according to Eq. (2):

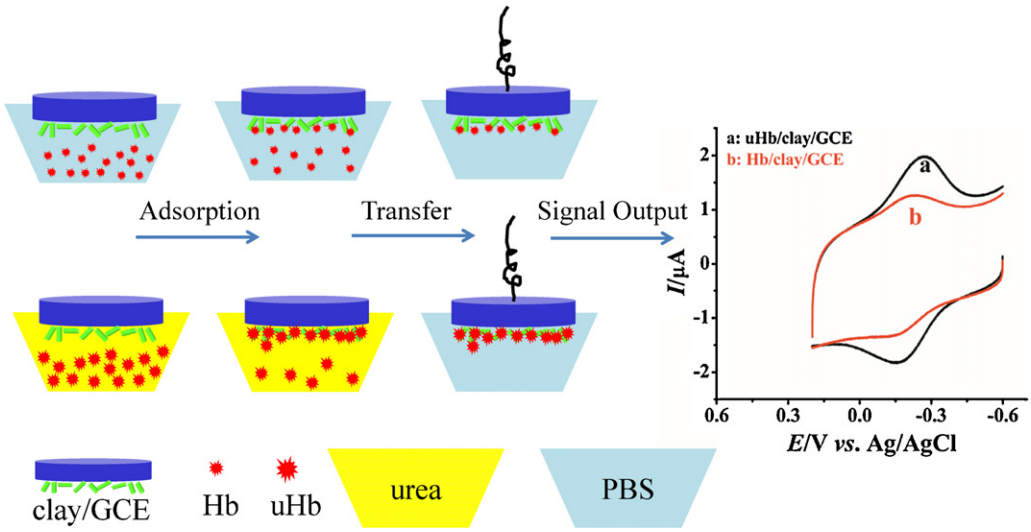
$$W_{Hb_e} = \frac{(Q/nF) \times 64,500}{m_{clay}} \quad (2)$$

where Q is the integrated area of cathodic peaks within CVs of Hb in the presence of urea at the scan rate of 0.1 V s^{-1}. n is the number of electron transfer that participated in the redox reaction. F is the Faraday constant. The constant of 64,500 is the molecular weight of Hb. m_{clay} stands for the amounts of clay immobilized on the surface of GCE.

3. Results and discussion

3.1. Thin-film UV–vis spectra of Hb and Hb/clay

It is known that UV–vis spectra in the Söret band can provide the conformational information of the heme group region in proteins



Scheme 1. The scheme of the electrochemical investigation of Hb unfolding at clay/GCE by adsorptive-transfer voltammetry.

[27,28]. Therefore, in order to evaluate the possible conformational change of Hb after adsorbed onto clay, Hb and Hb/clay in neutral phosphate buffer solution (PBS) were casted on quartz glass slides for measurements. As shown in Fig. 1, both Hb and clay/Hb films gave the same maximum absorbance of Söret bands at 410 nm (solid line and dashed line), indicating that the adsorption of Hb onto clay did not cause obvious conformational change around the heme group region of Hb. The result is in agreement with the previous reports. Montmorillonite clay possesses a composite surface and abundant negative charges, which allow Hb adsorbing on clay through hydrophilic and hydrophobic interactions [26]. The clay-related films can stabilize the active sites of the heme groups in Hb and greatly promote their direct electron transfer [29–32].

3.2. Electrochemical behaviors of Hb/clay/GCE

Fig. 2 showed the electrochemical impedance spectra (EIS) and cyclic voltammograms (CV) of different electrodes. Small interfacial electron transfer resistance (R_{ct}) of 474 Ω was found at bare GCE (Fig. 2A, curve a). After the GCE was modified by clay, no obvious semi-circle in the high frequency region of EIS was found (Fig. 2A, curve b). The decrease of the R_{ct} indicated that clay facilitated the electron transfer. Meanwhile, no obvious peak current was found in deaerated PBS (0.1 mol L^{-1}, pH 7.0) before and after clay/GCE was incubated in 9 mol L^{-1} urea (Fig. 2B, curves a and b). The observations were in consistence with the previous report [33]. After the Hb in neutral PBS was adsorbed onto the clay/GCE (Hb/clay/GCE), the R_{ct} increased to 817 Ω (Fig. 2A, curve c), and a pair of weak redox peaks with the formal potential ($E^{o'}$) of −0.181 V and the peak-to-peak separation (ΔE_p) of 66 mV were observed (Fig. 2B, curve c). The redox couples at −0.181 V were attributed to the redox reaction of heme Fe^{III}/Fe^{II} in Hb [29]. Hb in neutral condition is in its native structure. The heme group in each subunit of Hb is buried in the hydrophobic pocket with a fifth coordinative bond to the residue of polypeptide (His-F8) [34,35]. Therefore, the long distance between heme groups and electrode, and the large steric hindrance of native Hb resulted in the poor electrochemical response and the increase of R_{ct}. After Hb was incubated in 9 mol L^{-1} urea for 23 h and adsorbed onto the clay/GCE, the R_{ct} was further increased to 1714 Ω (Fig. 2A, curve d). Moreover, the peak current (I_p) increased greatly with a negative shift of $E^{o'}$ to −0.211 V and an enlarged ΔE_p to 108 mV (Fig. 2B, curve d). The incubation of Hb in urea caused changes of the native secondary and tertiary structure surrounding the heme group, and enhancement of the water solubility of hydrophobic side chains. The change of the coordinating environment of heme irons resulted in the change of $E^{o'}$ [36]. Although the direct electron transfer between the heme groups of Hb and clay-related film can be facilitated, the ΔE_p was larger than that of Hb/clay/GCE, indicating the increase of the interfacial resistance, which was in agreement with the results from EIS (Fig. 2A, curve d). Moreover, the I_p of uHb/clay/GCE was larger than that of Hb/clay/GCE, suggesting that the amount of uHb adsorbed on clay/GCE might be more than that of the native Hb adsorbed on clay/GCE (which was clarified in the following experiments).

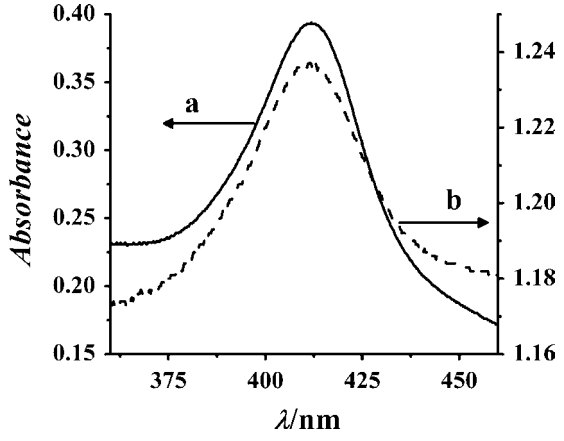


Fig. 1. The thin-film UV–vis spectra of Hb (solid line) and clay/Hb (dashed line) on quartz glass slides.

The dependence of the cathodic peak current (I_{pc}) of uHb/clay/GCE on the scan rate was shown in Fig. 2C. The I_{pc} increased linearly with the increase of scan rate from 0.1 to 0.7 V s^{-1}, indicating that the redox reaction on the uHb/clay/GCE was a surface-controlled process. Fig. 2D shows the direct electrochemical response of uHb/clay/GCE during forty cycles of successive scans. The I_{pc} shows no much variation, indicating that the adsorption of Hb onto the clay/GCE was quite stable.

3.3. Electrochemical investigation of Hb unfolding

The I_{pc} of the uHb on clay/GCE was influenced by two factors: the total amount of Hb (Γ) and the electroactive amounts of Hb (W_{Hbe}) that adsorbed on clay/GCE. Therefore, in order to eliminate

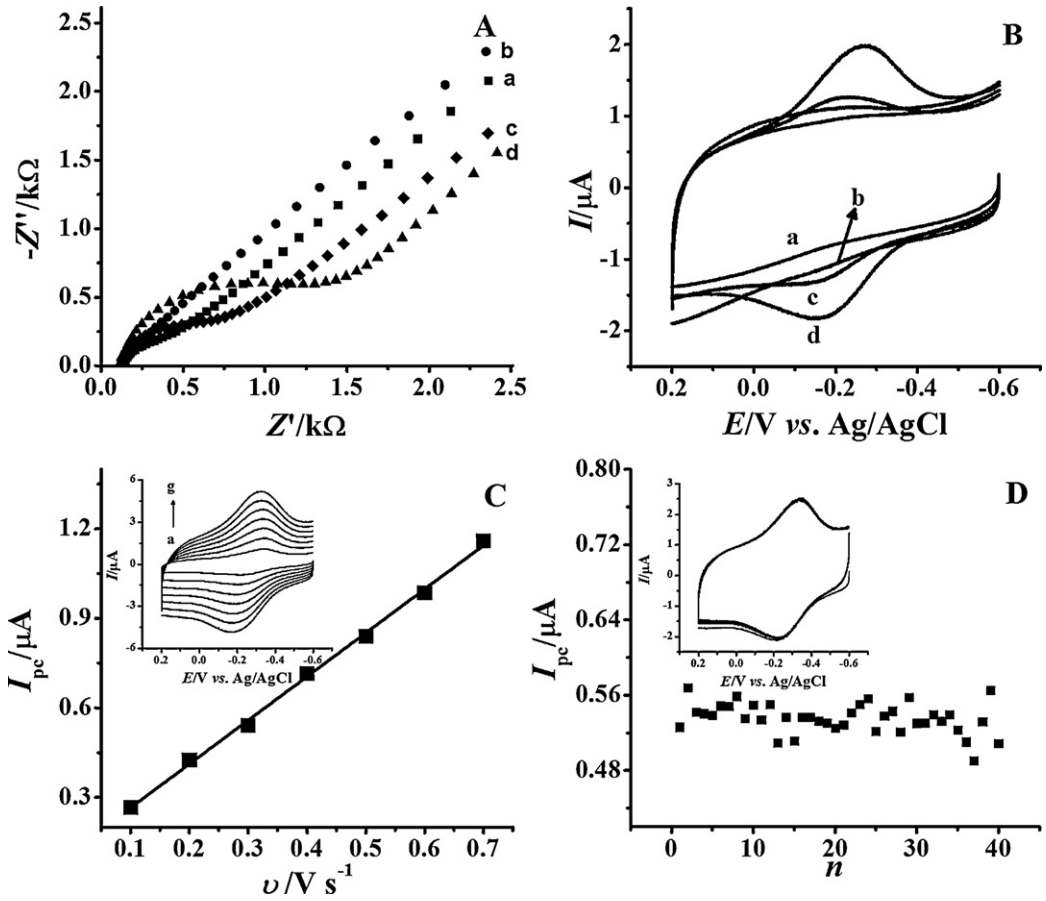


Fig. 2. (A) EIS characterization of bare GCE (a), clay/GCE (b), Hb/clay/GCE (c), and uHb/clay/GCE (d) in 1 mmol L^{-1} $K_3Fe(CN)_6$ containing 0.1 mol L^{-1} KCl. The polarized voltage was +0.2 V. The frequency range was from 0.1 to 10,000 Hz with amplitude of 10 mV. (B) CVs of clay/GCE (a), clay/GCE (b) after treated with 9 mol L^{-1} urea, Hb/clay/GCE (c), and uHb/clay/GCE in pH 7.0 PBS (d) at scan rate of 0.3 V s^{-1}. (C) The dependent plots of I_{pc} on the scan rate, inset: the CVs of the uHb/clay/GCE at different scan rate of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 V s^{-1}, respectively (from a to g). (D) The dependence of the I_{pc} of uHb/clay/GCE on the scanning cycles (n), inset: the CVs of the uHb/clay/GCE for forty cycles of successive scans at the scan rate of 0.3 V s^{-1}. The uHb/clay/GCE was prepared by adsorption of the Hb that pre-unfolded in 9 mol L^{-1} urea for 23 h.

the possible influence from the variation of the amount of adsorbed Hb on clay/GCE, and precisely quantitate the effects of urea concentration on the unfolding of Hb, it was necessary to specify the MEP of adsorbed Hb according to Eq. (3):

$$\text{MEP} = \frac{W_{\text{Hb}_e}}{\Gamma} \tag{3}$$

The W_{Hbe} values were obtained from the CVs of uHb/clay/GCE (Fig. 3A), in which Hb was unfolded by urea of different concentration. By the integration the cathodic peak of each CV, the W_{Hbe} enhanced with the increase of urea concentration up to 6 mol L^{-1}. After the urea concentration was higher than 6 mol L^{-1}, the W_{Hbe} showed slight decrease (Fig. 3B).

The Γ at each urea concentration was investigated from the UV–vis spectra of Hb in urea solution in the absence and presence of known amount of clay. As shown in Fig. 4A, in the absence of clay, the native Hb showed a maximum absorbance at 405 nm (curve a). The intensity of the absorption spectra kept constant up to 2 mol L^{-1} urea (curves a–c). Further increasing the urea concentration from 2 to 6 mol L^{-1} caused progressive decrease in the absorbance (curves

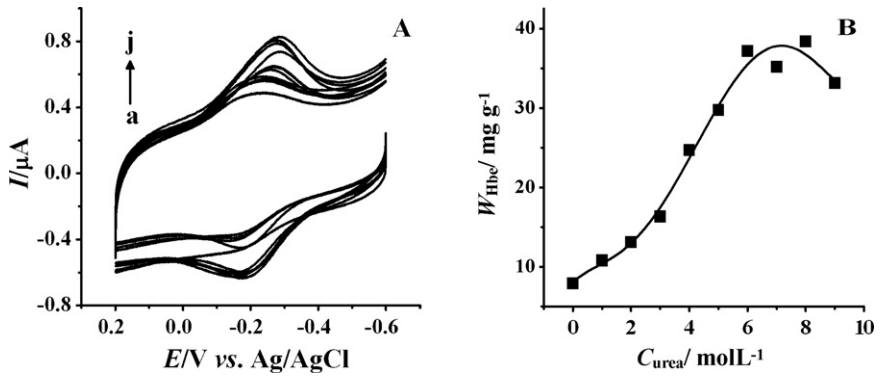


Fig. 3. (A) The CVs of uHb/clay/GCE, in which Hb was unfolded by 0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 mol L^{-1} urea, respectively (from a to j). The scan rate was 0.1 V s^{-1}. (B) The dependence of W_{Hbe} on the concentration of urea.

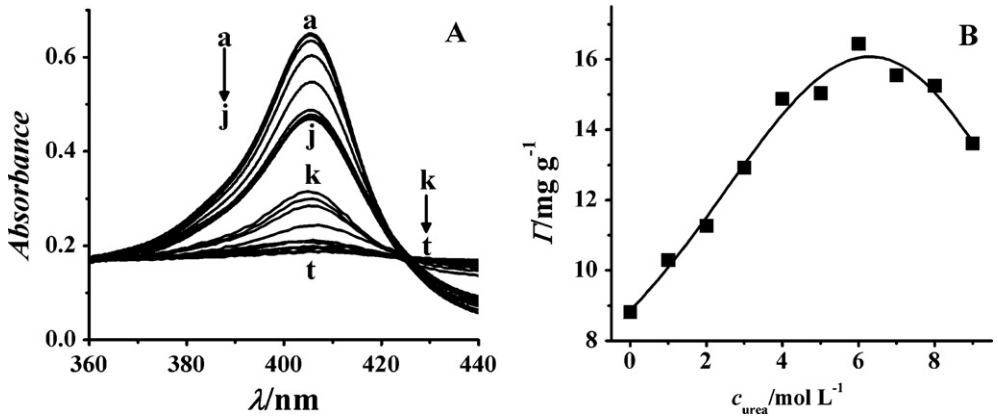


Fig. 4. (A) From a to j, UV–vis spectra of Hb incubated in the presence of 0–9 mol L^{-1} urea in the absence of clay. From k to t, UV–vis spectra of Hb incubated in the presence of 0–9 mol L^{-1} urea containing 10 mg clay. The concentration of Hb is 50 mg L^{-1}. (B) The relationship between Γ on clay and the concentration of urea.

c–g). Between 6 and 9 mol L^{-1} urea, the absorption spectra did not show obvious change (curves g–j). Moreover, the maximum absorption wavelength kept constant under various urea concentrations, indicating the heme remained attached to globin at the native site when Hb was unfolded by urea. In contrast, in the presence of clay, the corresponding UV–vis absorption of Hb decreased significantly with the increase of urea concentration (Fig. 4A, curves k–t). The dependence of Γ on the concentration of urea was shown in Fig. 4B. With the increase of urea concentration, the Γ increased concomitantly and reached the maximum when the concentration of urea was 6 mol L^{-1}. Then the Γ decreased slightly as the concentrations of urea increased.

Therefore, both the W_{Hbe} and the Γ of Hb onto clay/GCE changed under different urea concentration. The MEP indicated the changes of the electroactive Hb relative to adsorbed Hb on clay/GCE, which calibrated the inflexion of adsorbing amount of Hb and reflected the structural changes of Hb induced by denaturant. Fig. 5 showed the dependence of the MEP of the adsorbed Hb at clay/GCE on the urea concentration. When the concentration of urea was lower than 3 mol L^{-1}, the MEP showed minor changes, indicating that the tetrahedral structure of Hb had not obvious changes under these conditions. However, the MEP increased markedly as the concentration of urea increased from 3 to 6 mol L^{-1}. The aromatic amino acid residues of Hb became more and more exposed with the enhancement of the solubilization of Hb induced by urea. The amount of α-helix in Hb was reduced (Supplementary data, Fig. S1). The tetrahedral structure of Hb was destroyed gradually, which caused the decrease of the hindrance of polypeptide chain. Therefore, the amount of heme groups facing to the clay/GCE surface was increased. When the concentration of urea was higher than 6 mol L^{-1} (6–9 mol L^{-1}), the MEP reached a plateau and no obvious changes were observed, indicating the Hb achieved a stable unfolded state. A transition midpoint (S_m), which is the concentration of urea required to achieve half of the total change of the unfolding curve, was obtained to be 4.22 mol L^{-1}.

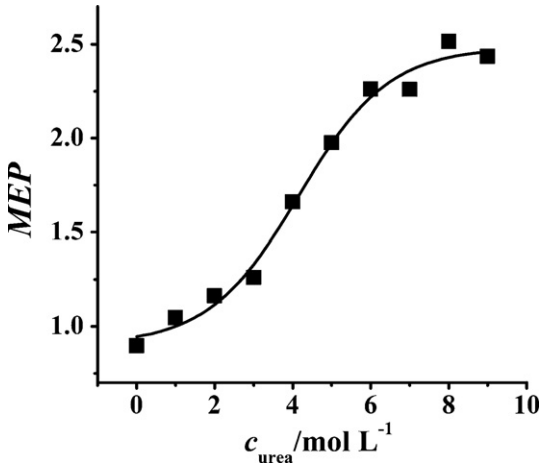


Fig. 5. The dependence of the MEP of the adsorbed Hb on the concentration of urea.

3.4. Comparison of the unfolding events of Hb investigated by electrochemistry and fluorescence

As shown in Fig. 6A, the tryptophan (Trp) residues in native Hb produced a maximum emission peak at 339 nm (curve a). When the urea concentration increased from 0 to 9 mol L^{-1}, the fluorescence intensity of Trp increased gradually, and the maximum emission wavelength shifted slightly to 336 nm (curves a–j), indicating that the presence of urea caused changes of hydrophobicity in the vicinity of Trp residues. In native Hb, the αTrp14, βTrp15, and βTrp37 are located in the vicinity of heme, their fluorescence is strongly quenched by Förster resonance energy transfer [5,37–39]. With the increase of urea concentration, the conformational change of Hb led to the increase of the distance between Trp and heme, accordingly the energy transfer efficiency was reduced, and the fluorescence intensity increased gradually [5,40].

The unfolding curves of Hb from electrochemistry and fluorescence showed similar shape (Fig. 6B). In order to further evaluate the results from different techniques, a linear free energy model (LEM) was used [41,42]:

$$f_U\ (\%) = \frac{y - y_N}{y_U - y_N} \times 100\% \quad (4)$$

$$K_{eq} = \frac{f_U}{f_N} = \frac{f_U}{1 - f_U} \quad (5)$$

$$\Delta G_U = -RT \ \ln \ K_{eq} \quad (6)$$

$$\Delta G_U = \Delta G_U^{water} - m[D] \quad (7)$$

where y represents the spectral intensity/MEP value in a particular unfolding condition, y_N and y_U are the spectral intensity/MEP value in the native and unfolded state of Hb. The unfolded percentage of the native Hb (f_N) is 0% while that of the fully unfolded Hb (f_U) is 100%. K_{eq} is the equilibrium constant between the folded and unfolded state of Hb. ΔG_U is the change in the free energy of the folding Hb in a certain unfolding condition. $\Delta G_U{}^{water}$ is the change in the free energy of the folding Hb in water. m, which can index the change in solvent exposure during the transition and therefore the compactness of Hb, is obtained from the slope of the Santoro-Bolen equation [43–45]. [D] is the concentration of urea. Accordingly, sim-

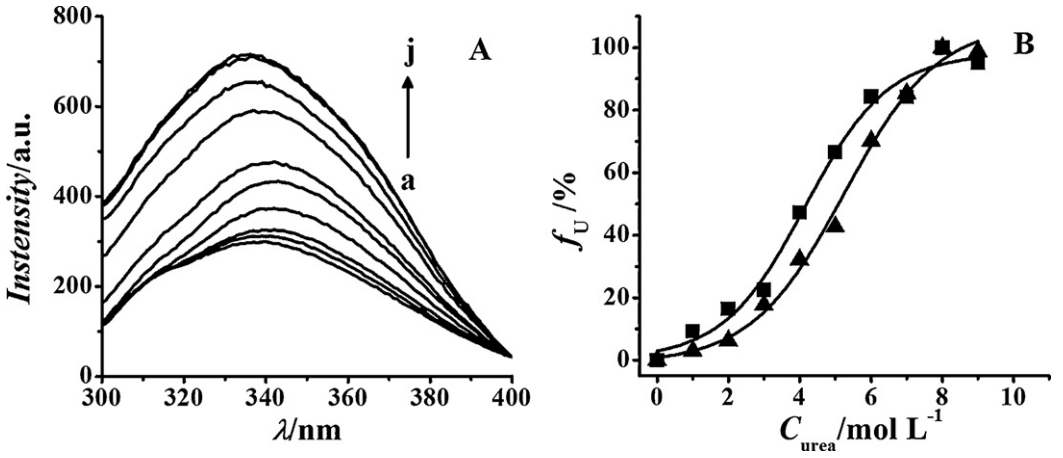


Fig. 6. (A) The intrinsic fluorescence emission spectra of Trp within Hb unfolded by 0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 mol L^{-1} urea, respectively (a to j). The concentration of Hb was 200 mg L^{-1}. The excitation wavelength was 280 nm. (B) The unfolding curves of Hb obtained from electrochemistry (■) and intrinsic Trp fluorescence at 336 nm (▲) according to LEM model.

Table 1
The comparisons of S_m, m, and ΔG_U^{water} obtained from electrochemical and fluorescence.

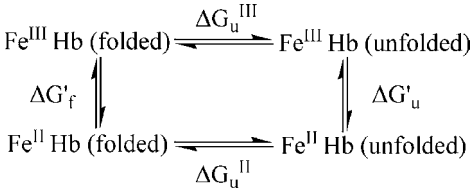
Characterization method	S_m (mol L^{-1})	m (kJ mol^{-2})	ΔG_U^{water} (kJ mol^{-1})
Electrochemistry	4.22	1.977	8.114
Trp Fluorescence	5.03	2.125	10.68

ilar thermodynamic parameters of S_m, m and ΔG_U^{water} of Hb were obtained from electrochemistry and fluorescence (Table 1).

The small discrepancies of S_m, m, and ΔG_U^{water} from electrochemistry and fluorescence might be caused by the change of the interfacial interaction between uHb and clay that tend to destabilize the conformation of Hb and make the surface of Hb less buried. Consequently, the Hb was less stable in water. The conclusions were further investigated by Langmuir and Freunlich models (Supplementary data, Fig. S2).

3.5. Establishment of thermodynamic cycle

As reported previously [10,46,47], the unfolding of Hb induced by urea is reversible. Therefore, a thermodynamic cycle was established as following:



By combining the electrochemical data with the free energy changes in Hb unfolding process, the different stability of reduced and oxidized Hb induced by urea can be assessed from the cycle. According to $\Delta G_f' - \Delta G_u' = -nF\ (\Delta E^{\circ\prime}) = -nF\ (E_f^{\circ\prime} - E_u^{\circ\prime})$, the difference of the free energies between unfolding the oxidized and the reduced Hb ($\Delta\Delta G_u = \Delta G_u^{III} - \Delta G_u^{II}$) was estimated to be -3 kJ mol^{-1}. Consequently, when treated with urea at neutral pH, the reduced Fe^{II} Hb was more stable than the oxidized Fe^{III} Hb, which may be caused by the different coordination environments of Fe^{II} and Fe^{III} in the native Hb.

4. Conclusions

An efficient electrochemical approach was first proposed for investigation of Hb unfolding by integration of nano-material modified GCE and adsorptive-transfer voltammetry. By monitoring the minimal electroactive portion of adsorbed uHb onto montmorillonite clay modify GCE, the effects of urea concentration on the conformation of Hb were precisely valuated. Moreover, the sensitivity of analyzing the unfolding process of Hb was improved with overcoming the disadvantages in the existing electrochemical methods. The proposed method will confessedly advance the electrochemical studies of protein.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.12.038.

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