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Studies on the interaction of protein with acid chrome blue K by electrochemical method and its analytical application

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Abstract

An electrochemical investigation on the interaction of acid chrome blue K (ACBK) with protein on the mercury electrode with different electrochemical methods such as cyclic voltammetry and linear sweep voltammetry was reported in this paper. In pH 3.0 Britton–Robinson (B–R) buffer solution, ACBK has an irrevisible voltammetric reductive peak at −0.23 V (vs. SCE). The addition of human serum albumin (HSA) into the ACBK solution resulted in the decrease of reductive peak currents without the change of the peak potential and no new peaks appeared on the cyclic voltammogram. In the absence and presence of HSA, the electrochemical parameters such as the formal potential E^0, the electrode reaction standard rate constant k_s and the charge transfer coefficient α of the interaction system were calculated and the results showed that there were no significant changes between each other. Thus, the interaction of ACBK with protein forms an electro-inactive supramolecular bio-complex, which induces the decrease of the free concentration of ACBK in the reaction solution, and the decrease of the reductive peak current of ACBK. The binding constant and the binding ratio are calculated as 1.29×10^8 and 1:2, respectively, and the interaction mechanism is discussed. Based on the binding reaction, this new electrochemical method is further applied to the determination of HSA with the linear range from 3.0–20.0 mg/L and the linear regression equation as $\Delta Ip''(nA)=10.08+19.90$ C (mg/L). This method was further applied to determinate the content of protein in the healthy human serum samples with the results in good agreement with the traditional Coomassie brilliant blue G-250 spectrophotometric method.

Keywords: Acid chrome blue K; Serum albumin; Voltammetry; Interaction; Protein assay

1. Introduction

The interactions of small molecules with biomolecules such as proteins and nucleic acid have aroused great interest among chemists and biologists. The study of supramolecular interaction between them is useful for understanding the structures and functions of biomolecules. The quantitative determination of proteins is very important in clinical tests and biological techniques because it is often used as a reference for the measurement of other components in biological systems. The traditionally used methods include the Coomassie brilliant blue G-250 [1], Lowry method [2], and bromophenol blue method [3]. In recent years, many dye-binding methods have been widely proposed for protein determination by spectrophotometry [4,5], fluorometry [6,7], light scattering technique [8,9] and electrochemical method [10,11].

Compared with a spectroscopic method, electrochemical assay is simple, reliable and practical with low detection limit and wide dynamic range. Because electrochemical reaction occurs on the electrode/liquid surface, it is especially suitable for small amounts of sample. Electrochemical methods are useful techniques for the study of the interaction of small molecules with biomolecules, and have been widely used to investigate the binding reaction of DNA

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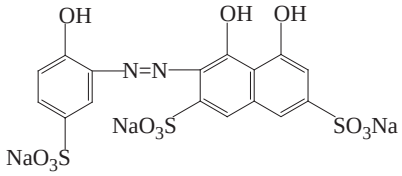

Fig. 1. The molecular structure of ACBK.

with drugs and metal complexes [12–14]. Feng et al. reported the electrochemical responses of prophyrin with DNA and further applied to the determination of DNA [15]. Sun et al. has applied beryllon III as an electrochemical probe for the detection of different proteins such as human serum albumin (HSA) and bovine serum albumin (BSA) by linear sweep voltammetry with satisfactorily results [16]. Luo et al. studied the polarographic behavior of Co(II)–BSA or Co(II)–HSA complex in the presence of a guanidine modifier and applied to the protein determination [17]. However, compared with other analytical technique, the related reports are few to our knowledge. Since electrochemical methods are useful in research of the electrochemical transfer process of biological procedure with high sensitivity and wide dynamic range, it is a necessary to investigate the binding reaction with protein by this method.

Acid chrome blue K (ACBK) has been used as the spectrophotometric reagent for protein assay [18]. In this paper, electrochemical studies on the interaction of ACBK with human serum albumin (HSA) at the mercury electrode are reported. ACBK is an azo dye (molecular structure shown as in Fig. 1) and can take place redox reaction at the mercury working electrode. The sulfonic and hydroxyl groups in ACBK molecular structure, which are dissociated at pH 3.0 buffer solution, are in negatively charged and easily bound to the positively charged groups such as protonated amino group in the HSA (isoelectric point, p*I* 4.7). In our experiments, the electrochemical studies of the interaction of ACBK with protein at the mercury electrode are reported according to the changes of electrochemical responses of the reaction solution. The electrochemical parameters of ACBK reaction system were calculated and the conditions of the binding reaction and the electrochemical determination were optimized. Under the optimal conditions, the present method was further applied to the determination of the content of human serum albumin in human serum samples with satisfactory results.

2. Experimental

2.1. Apparatus

All the cyclic voltammetric experiments were carried out using a DS model 2004 electrochemical analyzer (Shandong Dongsheng Electronic Instrument, China) with a DS-991 static mercury drop electrode (Shandong Dongsheng Electronic Instrument, China) as working electrode, a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode. The second order derivative linear sweep voltammetric determination was obtained on a model JP-303 polarographic analyzer (Chengdu Apparatus Factory, China) with a traditional three-electrode system composed of a dropping mercury electrode (DME) as working electrode, a saturated calomel electrode (SCE) as reference electrode and a platinum wire electrode as auxiliary electrode. UV–Visible absorption spectra were recorded by a Cary model 50 probe spectrophotometer (Varian, Australia). The values of buffer pH were measured with a pHS-25 acidimeter (Shanghai Leici Instrument Factory, China). All the experiments were carried out at 25 °C±2 °C.

2.2. Reagents

Human serum albumin (HSA, 99%, Shanghai Biomedical Products Research Institute) was used as received without further purification. The 1.0 g/L stock solution of different proteins was prepared by directly dissolving it in doubly distilled water from all-quartz still and stored at 4 °C. The working solutions were obtained by diluting the stock solution with water. 1.0×10^{-3} mol/L acid chrome blue K solution (ACBK, Tianjin No.1 Chemical Reagent Factory) was used as stock solution and diluted to the working concentration when used. 0.2 mol/L Britton–Robinson (B–R) buffer solution was used to control the pH of the tested solutions. The Coomassie brilliant blue G-250 (CBB G-250, Shanghai Chemical Reagent Company) solution for spectrophotometric determination of protein was prepared according to the common procedure. All other reagents used were of analytical reagents grade and doubly distilled water was used throughout.

2.3. Procedure

Into a 10-ml volumetric flask were added 2.5 ml of 1.0×10^{-4} mol/L ACBK, 1.5 ml of 0.2 mol/L B–R (pH 3.0) buffer solution and an appropriate amount of standard HSA solution or human serum sample solution. The mixture was diluted to 10 ml with doubly distilled water and mixed homogeneously. After reaction at 25 °C for 10 min, the voltammetric peak current (Ip) was recorded in the potential range from 0 mV to −800 mV (vs. SCE). Under the same conditions, the mixture without the addition of HSA was used as the blank solution and the voltammetric peak current (Ip_0) was obtained. The differences of peak current ($\Delta Ip = Ip_0 - Ip$) were used to show the changes of electrochemical responses of the reaction system.

For sample determination, healthy human sera, which were kindly provided by the Hospital of Qingdao University of Science and Technology, were diluted 1000-fold with water. From comparison, the CBB G-250 spectrophotomet-

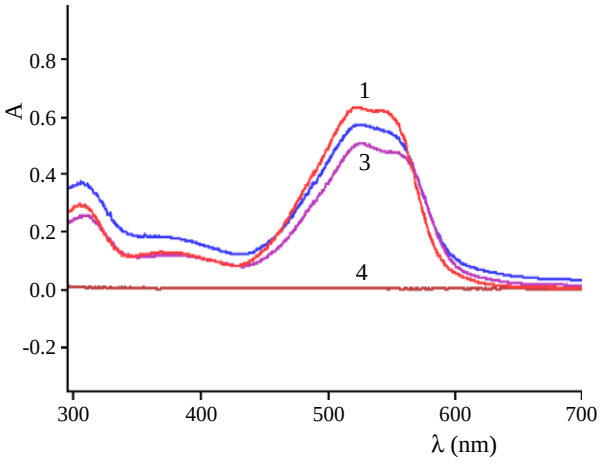

Fig. 2. UV–Vis absorption spectra of ACBK–HSA reaction system. (1) pH 3.0 B–R+2.5×10^{-5} mol/L ACBK; (2) 1+40.0 mg/L HSA; (3) 1+100.0 mg/L HSA; (4) pH 3.0 B–R+100.0 mg/L HSA.

ric method was used to determine the protein concentration in the same samples.

3. Results and discussion

3.1. UV–Vis absorption spectra

Fig. 2 shows the UV–Vis absorption spectra of ACBK in the absence and presence of HSA. In pH 3.0 B–R buffer solution and in the scanning range of 300–700 nm, ACBK has a maximum absorption peak at 526 nm (curve 1) and HSA has no absorption (curve 4). When HSA was mixed with ACBK, the absorbance of ACBK at 526 nm decreased without the movement of maximum absorption wavenumber (curve 2, 3). The more the protein added, the greater the absorbance decreased, which indicated that a binding reaction between ACBK and HSA had taken place and a new complex was formed on this experimental condition.

3.2. Cyclic voltammograms of ACBK and its mixture with HSA

ACBK is an electrochemical active dye with azo group in its molecular structure (shown in Fig. 1), so it can be easily reduced on the Hg electrode. It has been widely used as a chelating reagent in polarographic adsorption wave for the determination of metals. The cyclic voltammogram of ACBK was recorded under the selected conditions and the result was shown in Fig. 3. ACBK had a reductive peak at −0.23 V (vs. SCE) and did not have any oxidative peak in the potential range of −0.8–0 V, which indicated that the electrochemical behavior of ACBK on Hg electrode was irreversible at pH 3.0 B–R buffer solution. The multi-sweep cyclic voltammograms (shown in Fig. 3) indicated that with the increase of the scanning cycle, the reductive peak currents decreased greatly, showing the strong adsorption of ACBK on the Hg electrode. The relation of the reductive peak potential against pH of buffer solution was investigated. With the increase of buffer pH, the peak potential moved negatively and the linear relation was observed in the pH range of 1.5–6.0,which indicated that there was hydrogen ion participating in the electrode reaction.

Fig. 4 is the cyclic voltammograms of ACBK in the absence and presence of HSA. When HSA was added into ACBK solution, the reductive peak current of ACBK decreased greatly without the shift of peak potential and no new reductive peaks appeared in the same scan potential range. The results also showed that there were interactions between HSA and ACBK.

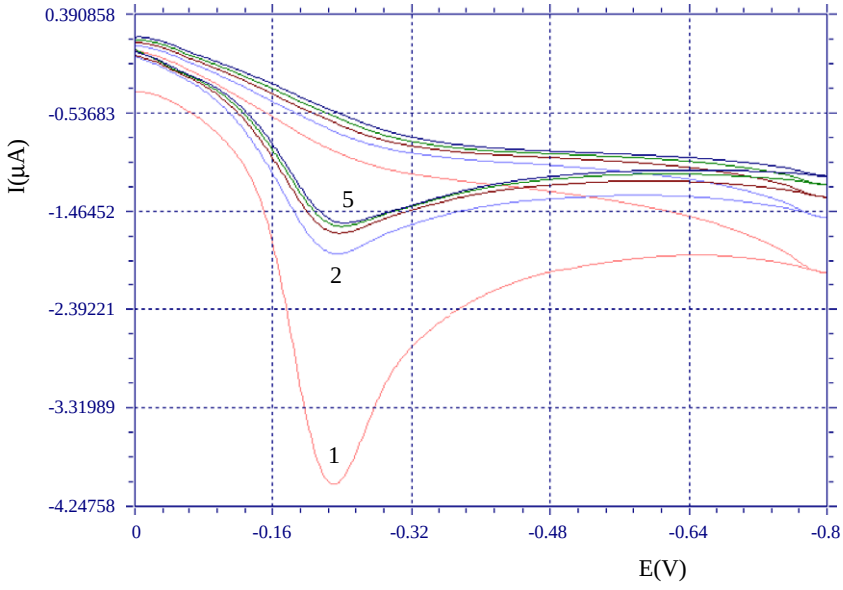

Fig. 3. The multi-sweep cyclic voltammograms of ACBK. 1.5 mL pH 3.0 B–R buffer+2.5 × 10^{-5} mol/L ACBK; scan rate: 100.0 mV/s.

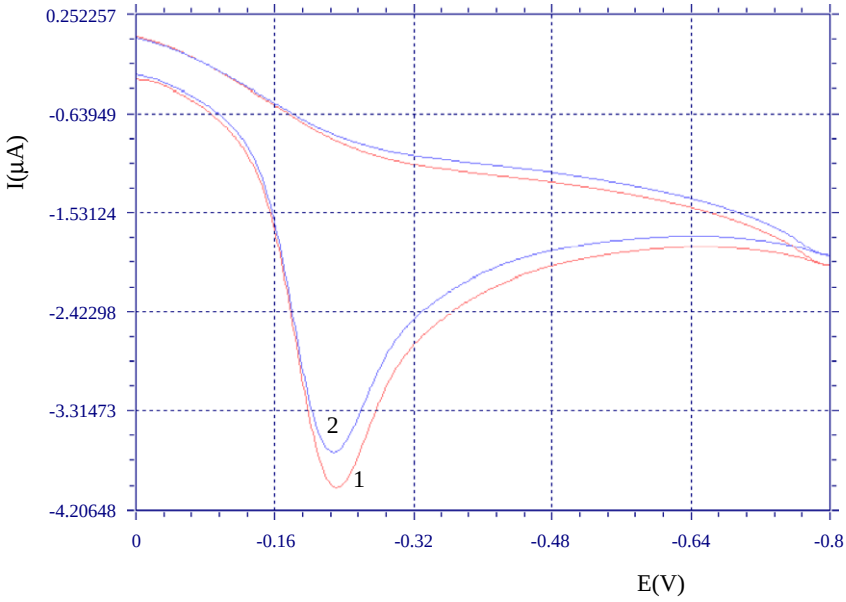

Fig. 4. Cyclic voltammograms of ACBK–HSA binding reaction solution. (1) 2.5×10^{-5} mol/L ACBK in pH 3.0 B–R buffer; (2) 1+3.0 mg/L HSA, scan rate: 100.0 mV/s.

3.3. Comparison of electrochemical parameters of ACBK in the absence and presence of HSA

In order to investigate the reaction mechanisms of ACBK–HSA reaction solution, the variation of electrochemical parameters of ACBK in the absence and presence of HSA was calculated and compared. Because of the strong adsorption behavior and the irreversible electrode process of the reductive reaction of ACBK on the mercury electrode, the following Laviron's equation [19] may be used to calculate the electrochemical parameters of the reduction reaction of ACBK on mercury electrode,

$$\mathrm{Ep} = E^0 + \mathrm{RT}/(\alpha nF)[\ln[(\mathrm{RT}k_s)/(\alpha nF)] - \ln v] \quad (1)$$

where α is the electron transfer coefficient, k_s is the standard rate constant of the surface reaction, v is the scan rate and E^0 is the formal potential.

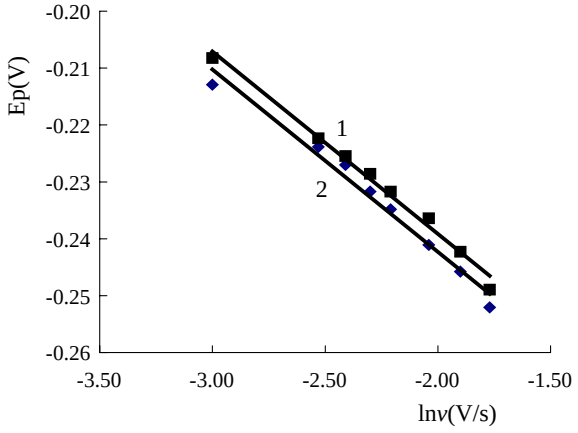

Fig. 5. Semilogarithmic dependence of the peak potential Ep on the potential scan rate (ln$v$). (1) pH 3.0 B–R buffer+2.0×10^{-5} mol/L ACBK; (2) 1+3.0 mg/L HSA.

According to Eq. (1), if the E^0 is known, Ep is linearly related to ln$v$ and the αn value can be calculated from its slope and k_s from the intercept. The E^0 value can be deduced from the intercept of Ep vs. v plot on the ordinate by extrapolating the line to $v=0$. According to this method, the electrochemical parameters were calculated for ACBK–HSA reaction system.

The plots of Ep of ACBK solution vs. ln$v$ are shown in Fig. 5 (curve l), which is a well-defined straight line. From the slope, the αn value of ACBK can be determined, and from the intercepts, the k_s value can be calculated, if the value of E^0 is known. The values E^0 of ACBK can be determined from curve 1 of Fig. 6. The intercepts of the Ep vs. v plots of ACBK and in Fig. 6 on the ordinate by extrapolating the line to $v=0$ represent the E^0 values of ACBK.

Because the electrochemical behavior of ACBK–HSA reaction solution is also an irreversible electrode processes, so the Laviron's equation was also used to calculate the electrochemical parameters of the ACBK–HSA reaction

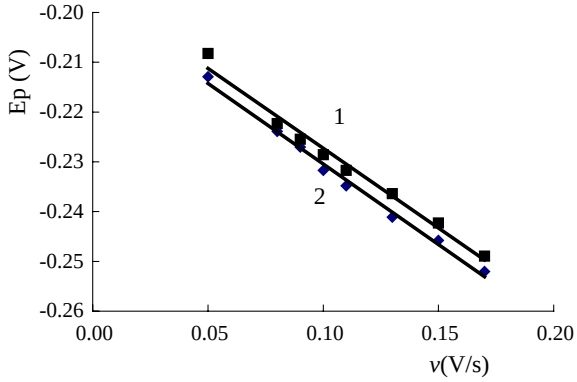

Fig. 6. Dependence of the peak potential Ep on the potential scan rate (v). (1) pH 3.0 B–R buffer+2.0×10^{-5} mol/L ACBK; (2) 1+3.0 mg/L HSA.

Table 1
The electrochemical parameters of ACBK in the absence and presence of HSA

Parameters	ACBK	ACBK–HSA
E^0 (V)	− 0.198	− 0.195
αn	0.799	0.802
ks (s^{-1})	1.064	1.074

solution. With the same method, the results were also got from Fig. 5 (curve 2) and Fig. 6 (curve 2). All the results of these two reaction solutions were compared and listed in Table 1. Obviously, the values of αn and k_s of ACBK in the absence and presence of HSA have not significant changes.

As for the reason of the decrease of the reductive peak current without the change of the peak potential after the reaction of ACBK with HSA, there maybe three probable different explanations: (1) the competitive adsorption between the ACBK and HSA on the mercury electrode; (2) the formation of electrochemical active supramolecular complex and the changes of electrochemical parameters, such as diffusional coefficient decrease responsible for the decrease of peak current; (3) the formation of electro-inactive complex and no changes of electrochemical processes. Li et al. have studied the interaction of many electro-active small molecules such as 9,10-anthraquinone, tetraphenylporphyrin tetrasulfonate (TPPS) with different proteins such as hemoglobin, albumin and antibody [20–22]. The results showed that in such lower concentration of protein and shorter accumulated time, the coverage of electrode surface only accounts for about 10% or less of the total electrode area, so the competitive absorption between small molecule and protein can hardly exist. Since the electrochemical parameters of this reaction system have not changed significantly, so the ACBK interacting with HSA formed an electro-inactive supramolecular complex, which could not be reduced on the Hg electrode surface. In the presence of HSA, the equilibrium concentration of free ACBK in solution decreased, which resulted in the decrease of the peak current.

3.4. Measurement of stoichiometry of HSA–mACBK supramolecular complex

With references to the method proposed by Li and Min [23], the combining number and the equilibrium constant between ACBK and HSA complex can be calculated based on the changes of reductive peak current. It was assumed that ACBK and HSA produced the single complex of HSA–mACBK.

$$\mathrm{HSA} + \mathrm{mACBK} \leftrightarrow \mathrm{HSA} - \mathrm{mACBK}$$

The equilibrium constant is expressed as follows:

$$\beta_s = \frac{[\mathrm{HSA} - \mathrm{mACBK}]}{[\mathrm{HSA}][\mathrm{ACBK}]^m} \quad (2)$$

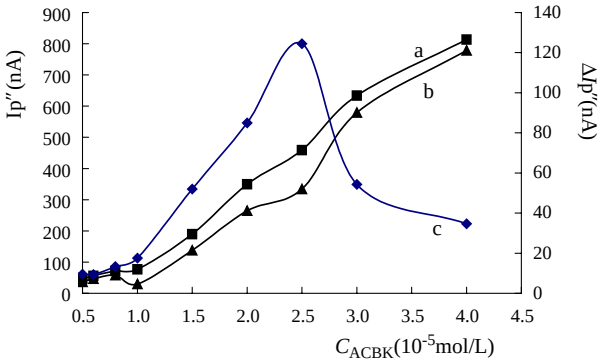

Fig. 7. Relationship between Ip″ and C_{ACBK}(a, b), ΔIp″ and C_{ACBK}(c). (a) C_{HSA}=0; (b) C_{HSA}=10.0 mg/L; (c) ΔIp″=Ipa″–Ipb″.

And the following equation can be deduced:

$$\Delta I_{max} = kC_{HSA} \quad (3)$$

$$\Delta I = k[\mathrm{HSA} - \mathrm{mACBK}] \quad (4)$$

$$[\mathrm{HSA}] + [\mathrm{HSA} - \mathrm{mACBK}] = C_{HSA} \quad (5)$$

Therefore:

$$\Delta I_{max} - \Delta I = k(C_{HSA} - [\mathrm{HSA} - \mathrm{mACBK}]) = k[\mathrm{HSA}] \quad (6)$$

Introducing Eqs. (2), (4) and (6) gives:

$$1/\Delta I = 1/\Delta I_{max} + (1/\beta \Delta I_{max})(1/[\mathrm{ACBK}]^m) \quad (7)$$

or

$$lg[\Delta I/(\Delta I_{max} - \Delta I)] = lg\beta_s + mlg[\mathrm{ACBK}] \quad (8)$$

Where ΔI is the peak current difference between the presence and absence of HSA and ΔI_{max} corresponds to the obtained value when the concentration of ACBK is extremely higher than that of HSA. C_{HSA}, [HSA], [HSA–mACBK] are corresponding to the total, free and bound concentration of protein in the solution, respectively.

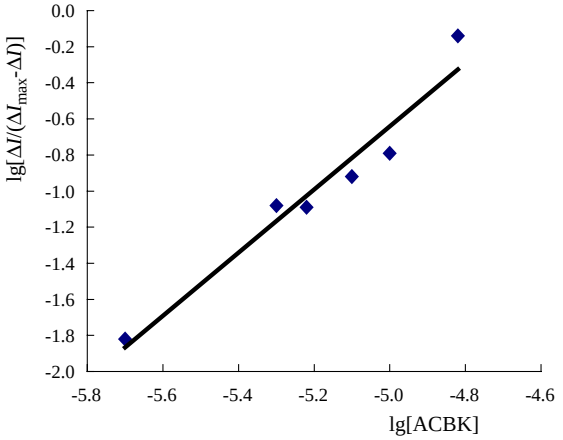

Fig. 8. Relationship between lg[ΔI/(ΔI_{max}−ΔI)] and lg[ACBK].

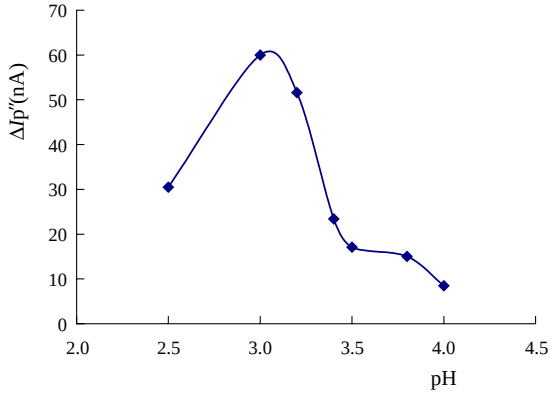

Fig. 9. The influence of pH on the peak current 2.0×10^{-5} mol/L ACBK+10.0 mg/L HSA in different pH B–R buffer solution.

For minimizing the reading error, the second order derivative linear sweep polarographic peaks were used. In Fig. 7, curve a is the relationship of Ip″ with the concentration of ACBK, curve b represents the change of peak current after the addition of 10.0 mg/L HSA on varying the concentration of ACBK, and curve c is the differences between curve a and curve b, which represents the relationship between ΔIp″ (Ipa″–Ipb″) and the concentration of ACBK. From Eq. (8), the relation of lg[ΔI/($\Delta I_{max}-\Delta I$)] with lg[ACBK] was calculated and plotted in Fig. 8. From the intercept and slope $m=2$ and $\beta_s=1.29\times10^8$ were deduced, which indicated that a stable 1:2 complex of HSA–2ACBK was formed in the desired concentration range of ACBK with respect to the concentration of HSA.

3.5. Analytical application

3.5.1. Optimal of reaction conditions

The pH of buffer solution greatly influences the binding reaction and the optimal reaction pH was selected in the pH range of 2.5–4.0, the results were showed in Fig. 9 and at pH 3.0, the difference of peak current reached its maximum, so pH 3.0 buffer solution was used throughout in this experiment. Different buffers such as B–R, HOAc–NaOAc, NH_3-NH_4Cl were tested and in B–R buffer solution, the response was the maximum. So the pH 3.0 B–R buffer solution is recommended in this paper.

Table 3
The results for the determination of HSA in human serum samples

Sample	This method (g/L)	RSD (%, $n=3$)	Recovery (%)	CBB G-250 method (g/L)	RSD (%, $n=3$)
1	52.0	1.90	99.5	44.7	1.77
2	30.2	5.40	100.8	32.7	3.42
3	39.8	4.02	108.5	36.6	2.32

After ACBK and HSA were mixed, the difference of peak currents reached the maximum after reaction for about 5 min and remained unchanged for at least 2 h. Therefore, the system gives enough time for routine measurements. The effect of the reaction temperature on the interaction was tested at 15 °C, 25 °C, 30 °C and 37 °C, respectively. The results showed that there were no obvious differences among them. So the temperature had little influence on the binding reaction and room temperature (25 °C±2 °C) was used throughout. Different adding orders of ACBK, HSA and B–R buffer were tested and the results showed that the best addition sequences were ACBK, B–R buffer and protein. This result indicated that the electronic coupling made ACBK bind to HSA.

The scanning rate and the standing time of the mercury drop for the assay were studied. The peak current increased with the increase of potential scanning rate within 350–950 mV/s and the mercury drop standing time from 4 to 16 s. When the dropping mercury time was more than 16 s, the mercury drop would fall down. But after 13 s, the shape of the peak was not good, so the scanning rate and standing time are selected as 850 mV/s and 11 s, respectively.

3.5.2. Interference of coexisting substances

The effect of coexisting substances such as metal ions, amino acids, carbohydrates on the determination method was tested by premixing HSA with the interference substances. Table 2 shows that the commonly observed metal ions in blood sample such as Ca^{2+}, Mg^{2+}, Cu^{2+} etc. can be allowed with higher concentrations.

3.5.3. Working curves of HSA

Under the selected optimal conditions, the decrease of the peak current was linear with the addition of HSA and a good linearity was got in the concentration range of 3.0–20.0 mg/L with the linear regression equation as ΔIp″(nA)=10.08+

Table 2
Effect of coexisting substances on the determination of 10.0 mg/L HSA

Coexisting substance	Concentration (mol/L)	Relative error (%)	Coexisting substance	Concentration (μg/mL)	Relative error (%)
$MgSO_4$	2.0×10^{-4}	3.19	L-Lysine	5.0	3.50
$Pb(NO_3)_2$	2.0×10^{-4}	4.10	L-Valine	5.0	2.51
$Fe_2(SO_4)_3$	2.0×10^{-4}	4.51	L-Glutamine	5.0	−0.65
$CuSO_4$	2.0×10^{-4}	2.21	L-Arginine	5.0	3.62
$MnSO_4$	2.0×10^{-4}	2.31	L-Leucine	5.0	−3.06
$CaCl_2$	2.0×10^{-4}	2.67	L-Serine	5.0	2.32
KCl	5.0×10^{-4}	2.69	L-Cysteine	5.0	6.09
$CoCl_2$	2.0×10^{-4}	1.25	L-Glutamic acid	5.0	2.12
$ZnCl_2$	2.0×10^{-4}	3.26	Glucose	50.0	−2.75

19.90 C (mg/L), ($n=7$, $\gamma=0.991$). The detection limit (3σ) was 1.93 mg/L. So the sensitivity of ACBK method for HSA determination is enough for routine detection.

3.5.4. Sample determination

The proposed method was further applied to determine the albumin content in healthy human serum sample and the results are listed in Table 3. By comparing the results with generally used Coomassie brilliant blue G-250 (CBB G-250) spectrophotometric method, it is clear that this new electrochemical detection method is reliable, practical and reproducible.

4. Conclusion

Generally speaking, there are two kinds of reaction modes between small molecules with protein, one is the electrostatic binding and the other is hydrophobic interaction. In pH 3.0 acidic solution the lysine, arginine and other amino acid residues in the HSA (isoelectric point p$I=4.7$) molecular chains are positively charged, while the ACBK species are negatively charged. So it is possible that ACBK interacts with HSA by electrostatic attraction and other weak response such as ionic, van der Waals and hydrogen bonding etc. to form an electro-inactive supramolecular complex.

By using electrochemical method and based on the experimental results, we suppose that HSA interacts with ACBK to form an electrochemical inactive 1:2 complex, which results in the decrease of peak current of ACBK. The decrease of peak current can be further applied to determine the concentration of HSA with good selectivity and low detection limit.

Acknowledgements

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