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Electrochemical determination of proteins using arsenazo I as voltammetric probe

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A new electrochemical method is proposed for the determination of trace amounts of proteins using arsenazo I as a voltammetric probe. At *p*H 1.5 Britton-Robinson buffer solution, arsenazo I has a sensitive second order derivative linear sweep voltammetric reductive peak at -0.23 V (vs. SCE). The addition of human serum albumin into the dye solution decreases the reductive peak current without change of peak potential, indicating that HSA can interact with arsenazo I to form a supramolecular complex. The decrease of peak current is proportional to the concentration of HSA, which can be further used for the HSA determination. The optimal conditions of the binding reaction and the electrochemical detection have been investigated. The binding constant (β_s) and the binding number (m) of human serum albumin with arsenazo I have been calculated as $\beta_s = 3.9\times10^{17}$ and m= 4. No changes are observed in the electrochemical parameters of the reaction system indicating that an electro-inactive complex is formed in the mixed solutions.

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Several analytical method based on spectrophotometry have been reported for protein assay, e.g., Lowry, Bradford, Coomassie brilliant blue G 250, Bromophenol blue, etc. However, these methods suffer from the disadvantages of low sensitivity, poor selectivity or a complicated procedure. Other analytical procedures have also been reported, including fluorometry[1], light scattering technique[2], chemiluminescence[3] and electroanalysis[4-6]. Most of these are based on the binding of dyes or metal complex with proteins.

Compared to the above methods, electroanalytical methods are simple, reliable and practical with low detection limit and wide dynamic range. Since the electrode reactions take place at the electrode/liquid surface, it is suitable for small amounts of samples. Guo *et al.*[7] have investigated the catalytical hydrogen wave of protein in the presence of different kinds of oxidants such as KIO_3, H_2O_2, $KClO_3$, etc. The binding interaction of small molecules with proteins is also of great interests for both therapeutic and chemical reasons. Zhang *et al.*[8] studied the interaction of porphyrin with different kinds of proteins such as albumin and hemoglobin. Sun and Jiao[9] have reported some organic probes such as alizarin red S, beryllon III for the electrochemical detection of HSA. Cerda *et al.*[10] proposed the electrochemical studies on complex of Re (V) with protein.

Arsenazo I (AAI) is a commonly used chromogenic dye and has been used for the determination of some rare earth metals and proteins with spectrophotometry[11] and light scattering technique[12]. In the present study AAI has been used as an electrochemical probe for the determination of proteins. Based on its interaction with HSA which cause the decrease of peak current of AAI at -0.23 V (vs. SCE), a sensitive voltammetric method for protein determination is proposed and has been successfully applied for the determination of HSA in synthetic samples. The stoichiometry of HSA-AAI has been calculated and the interaction mechanism has been discussed.

Experimental

Human serum albumin (HSA, 99%, Shanghai Biomedical Products Research Institute), bovine serum albumin (BSA, 99%, Sigma), bovine hemoglobin (BHb, 99%, Tianjin Chuanye Biochemical Products Company) were used without further dilution. A 1.0 g/L stock solution of protein was prepared by dissolving it in water and stored at 4°C. Stock solution of arsenazo I (1.0×10^{-3} mol/L Chemical Reagent of Huadong Normal University) was prepared by dissolving 0.0592 g AAI in water and diluting up to the mark in a 100 mL calibrated flask. Britton-Robinson (B-R) buffer (0.2 mol/L) was used to adjust the *p*H of the test solution. The stock and working solutions were stored at 4°C. All other reagents were of analytical reagent grade and doubly distilled water was used throughout.

The second order derivative linear sweep voltammetric measurements were made on a JP-303 polarographic analyzer (Chengdu Apparatus Factory, China) with a traditional three-electrode system consisting of a drop mercury working electrode (DME), a platinum wire counter electrode and a

saturated calomel reference electrode (SCE). A Cary 50 probe UV-vis spectrophotometer (Varian, Australia) was used to record the UV-vis absorption spectra. A *p*HS-25 acidimeter (Shanghai Leici Instrument Factory, China) was used for measuring the *p*H of the solutions. All the experiments were carried out at 25±2°C unless stated otherwise.

Procedure

In a 10 mL calibrated tube, 2.5 mL of 0.2 mol/L B-R buffer, 0.5 mL of 1.0×10^{-3} mol/L AAI solution and an appropriate amount of HSA solution were taken. The mixture was diluted up to the mask with doubly distilled water and shaken. The blank solution was also prepared in the same manner without addition of HSA. The linear sweep voltammograms were scanned in the potential range from 0 to -0.8 V and the reductive peak current of AAI at -0.23 V (vs. SCE) was measured. The decrease of peak current was calculated as $\Delta I_p'' = I_{p0}'' - I_p''$, where I_{p0}'' and I_p'' are the peak current of the solution before and after the addition of HSA, respectively.

Results and discussion

The absorption spectra of AAI in the absence and presence of HSA were studied. At *p*H 1.5 B-R buffer solution and wavelength range 300 - 760 nm, HSA does not have any absorbance while AAI has maximum absorption at 515 nm. On mixing AAI with HSA, the absorbance decrease slowly with no change in λ_{max}. With increase in the HSA added, the greater was the decrease in absorbance, indicating the interaction of AAI with HSA. However, the change in the absorbance value with HSA concentration is too small to explore a relative sensitive analytical procedure.

Figure 1 shows typical second order derivative linear sweep voltammograms of interaction of AAI with HSA. In the selected potential range, B-R buffer does not have any electrochemical response (curve 1). AAI has a sensitive voltammetric response at -0.23 V (vs. SCE), which is attributed to the electrode reduction of azo group on the mercury electrode (curve 2). After the addition of HSA in the AAI solution, the peak current decreased greatly without any shift of peak potentials and the appearances of new peaks (curve 3). This indicates that an interaction has taken place in the mixture. This decrease of the reductive peak current can be further used for establishing a sensitive analytical method for estimation of protein.

The effect of acidity on the peak current of the interaction was studied in the *p*H range 1.3 - 3.0. Peak current reached a maximum at *p*H 1.5, hence *p*H 1.5 was selected for the assay. In the acidic buffer solution, HSA is negatively charged (p*I*=4.8) while AAI in positively charged, which is suitable for the electrostatic binding reaction. In a final 10 mL solution, volume of 0.2 mol/L B-R buffer solution required was selected as 2.5 mL.

The effect of the concentration of AAI in the range of 1.0×10^{-5} - 1.0×10^{-4} mol/L on the peak current was studied with 20.0 mg/L HSA. Results show that when the concentration of AAI was 5.0×10^{-5} mol/L, the difference of the peak current reached its maximum; hence 5.0×10^{-5} mol/L AAI was used throughout.

Different adding sequence of HSA, AAI and B-R buffer were tested under the optimal conditions. The results show that the best addition sequences was B-R buffer, AAI and HSA. This also indicates that the electronic coupling made AAI bind to HSA. The effect of incubation time on the peak current was tested. It was found that the binding reaction reached equilibrium in about 20 minutes when it attained a constant value. The reaction temperature had little influence on the difference of peak current in the range of 10 - 40°C. Therefore, 25°C was used throughout. The response remained stable for about 2 hours, indicating that this system was stable for routine applications.

The scan rate and the dropping mercury standing time of the instrument for the assay were studied. The

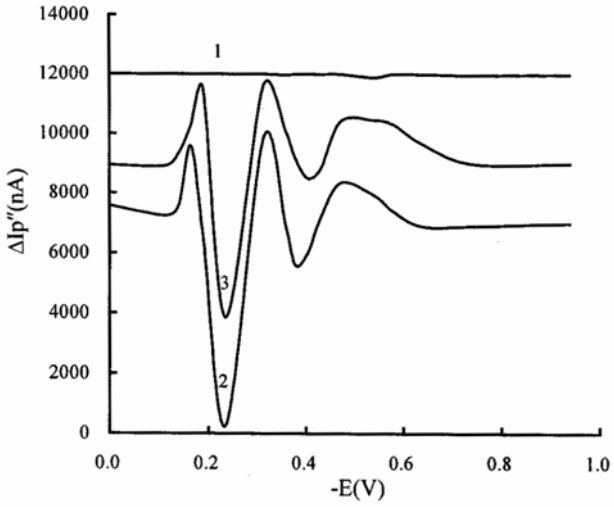

Fig. 1— Second order derivative linear sweep voltammograms of AAI-HSA interaction system. [curve 1, *p*H 1.5 B-R buffer; curve 2, as for 1 + 5.0×10^{-5} mol/L AAI; 3, as for 2 + 20.0 mg/L HSA].

peak current increased with the increase of potential scan rate in the range 200 - 1000 mV/s and the mercury drop standing time from 5 - 21 s. When the dropping mercury time was more than 21 s, the mercury drop would fall down naturally. Hence, the scan rate and the stand time were selected as 1000 mV/s and 20 s, respectively.

The effect of ionic strength on the AAI-HSA system was examined by the addition of different amounts of NaCl. Results show that the addition of high concentrations of NaCl had significant influence on the interaction. The peak current decreased with the increase of ionic strength, indicating that the increase of salt concentration hinders the binding reaction of AAI with HSA. The results prove that electrostatic attraction is responsible for the binding reaction.

Under the optimal conditions, the effect of coexisting metal ions and amino acids on the determination of 50.0 mg/L HSA were investigated (Table 1). Most of these do not interfere with the determination of HSA. Therefore, the method can be directly applied to the sample determination.

Under the optimal conditions, the proposed voltammetric method was applied to the determination of proteins such as HSA, BSA and BHb. The analytical parameters for different kinds of proteins are shown in Table 2. Since different proteins have different isoelectric points, their responses were also different. At the same time, the weight, size and shape of the biomolecules are also different, hence the signals for the different proteins are distinct. The limit of detection is calculated using following formula: $C_L=kS_{b1}/S$, where C_L is the limit of detection, k is a constant related to the confidence level, (as per IUPAC, k=3, 99% confidence), S_{b1} is the standard deviation of ten blank solution measurements (no protein added) and S is the slope of the calibration graph.

Synthetic samples of HSA, containing metal ions and amino acids were analyzed by the proposed method. Results show that HSA in synthetic samples can be determined with satisfactory results, with the recovery was in the range of 96.4 - 102.3% (Table 3).

The cyclic voltammetric experiments of AAI and its mixture with HSA were carried out. Results show that AAI has an irreversible electrode process on the mercury electrode with characteristic strong adsorption. The results are similar to those of other azo substances such as Orange G and Beryllon III. The following Laviron's equation[13] can thus be used to evaluate the kinetic constants of AAI in the absence and presence of HSA,

$$E_p = E^0 + RT/(\alpha nF)[\ln [(RTk_s)/(\alpha nF)] - \ln v]$$

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

Table 1—Effect of foreign substances on the determination of HSA [HSA= 50.0 mg/L]

Coexisting comp.	Conc. (mg/L)	Relative error (%)	Coexisting ion	Conc. (μmol/L)	Relative error (%)
L-Glutamine	3.0	0.10	Mn^{2+}	5.0	2.19
L-Valine	3.0	-3.14	Cu^{2+}	5.0	-2.30
L-Leucine	3.0	-1.37	Ca^{2+}	5.0	1.09
L-Cystine	3.0	0.95	Sn^{2+}	5.0	-2.12
L-Arginine	3.0	-2.67	Zn^{2+}	5.0	2.40
Glucose	3.0	0.85	Mg^{2+}	5.0	2.79
Citric acid	3.0	1.31	Co^{2+}	5.0	-0.59

Table 2—Analytical parameters for different proteins

Protein	Standard regression equation	Linear range (mg/L)	Detection limit (mg/L)	Regression coeff. (γ)
HSA	$\Delta I_p''$(nA)=95.76C(mg/L) − 417.24	6.0 - 60.0	2.80	0.994
BSA	$\Delta I_p''$(nA)=121.71C(mg/L) − 898.18	6.0 - 60.0	1.79	0.992
BHb	$\Delta I_p''$(nA)=109.36C(mg/L) − 590.94	6.0 - 70.0	1.97	0.996

Table 3—Analysis of HSA in synthetic samples (n= 5)

Sample	Foreign coexisting comp.[a]	HSA (mg/L)		Recovery (%)	RSD (%)
		Added	Found		
1	Co^{2+}, Zn^{2+}, citric acid, *L*-Glutamine	40.0	40.9	102.3	2.25
2	Mn^{2+}, Mg^{2+}, *L*-Leucine, *L*-Valine	20.0	20.4	101.8	1.61
3	Co^{2+}, Zn^{2+}, citric acid, *L*-Glutamine	20.0	19.3	96.4	4.37

[a] *L*-Glutamine, *L*-Leucine, *L*-Valine , citric acid 0.1 mg/L; Co^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+} 0.1 μmol/L

Table 4—Electrochemical parameters of AAI in the absence and presence of HSA

Parameters	AAI	AAI - HSA
E^0 (V)	-0.150	-0.153
αn	0.513	0.505
ks (s^{-1})	3.772	3.745

According to above equation, if the E^0 is known, αn and k_s can be got from the slope and the intercept of the linear plot of E_p versus ln ν. The E^0 value can be deduced from the intercept of plot of E_p versus ν by extrapolating the line to ν=0. Based on this, the electrochemical parameters of AAI and AAI-HSA solutions have been calculated respectively, and listed in Table 4.

The values of αn and k_s of AAI with or without HSA do not have distinct changes. It may be thus concluded that the reaction of AAI with HSA gives an electro-inactive complex, which can not be reduced on the mercury electrode, resulting in a decrease in the free concentration of AAI in the mixed solution, and also a decrease in the peak current.

The stoichiometry of the HSA-AAI complex was calculated by voltammetric data. It was assumed that AAI interacting with HSA only formed a single complex HSA-mAAI[14, 15]. The binding number (m) and the equilibrium constant (β_s) of the binding reaction can be deduced as follows:

$$\text{HSA} + \text{m AAI} \leftrightarrow \text{HSA-m AAI} \quad \ldots (1)$$

The equilibrium constant was deduced as follows:

$$\beta_S = \frac{[\text{HSA-mAAI}]}{[\text{HSA}][\text{AAI}]^m} \quad \ldots (2)$$

$$\text{Since } \Delta I_{max} = kC_{HAS} \quad \ldots (3)$$

$$\Delta I = k[\text{HSA-m AAI}] \quad \ldots (4)$$

$$[\text{HSA}] + [\text{HSA-m AAI}] = C_{HSA} \quad \ldots (5)$$

Therefore,

$$\Delta I_{max} - \Delta I = k(C_{HSA} - [\text{HSA-m AAI}]) = k[\text{HSA}] \quad \ldots (6)$$

Equations (2), (4) and (6) give

$$lg[\Delta I/(\Delta I_{max} - \Delta I)] = lg\beta_s + m\,lg[\text{AAI}] \quad \ldots (7)$$

where ΔI is the difference between the peak current of sample and blanks, ΔI_{max} corresponds the maximum value of difference of peak current, C_{HSA}, [HSA-mAAI] and [HSA] correspond to the total, bound and free concentration of HSA in the solution, respectively.

From Equation (7), the linear plot of $lg[\Delta I/(\Delta I_{max} - \Delta I)]$ versus lg[AAI] gave a linear regression equation as $lg[\Delta I/(\Delta I_{max} - \Delta I)] = 3.9\ lg[\text{AAI}] + 17.58$. From the intercept and the slope m=4 and $\beta_s = 3.83 \times 10^{17}$ were deduced, which indicates that a stable 1:4 complex of HSA-4AAI was formed under the above experimental conditions.

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