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Application of Arsenazo III for the Polarographic Detection of Proteins

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In this paper, a diazo dye of arsenazo III (AAIII) was selected as a new electrochemical probe for the determination of proteins. In Britton-Robinson (B-R) buffer solution of pH 2.4, AAIII had a sensitive second order derivative linear sweep voltammetric reductive peak at -0.39 V (*vs*. SCE). After the addition of human serum albumin (HSA) into AAIII solution, an interaction was taken place in the mixed solution and a biosupramolecular complex was formed, which resulted in the decreased reductive peak currents of AAIII. Based on the observed decrease in peak current, a sensitive electrochemical method was proposed for the determination of different proteins such as HSA, bovine serum albumin (BSA) and bovine hemoglobin (BHb). The optimal conditions for the interaction and the interfering effects of coexisting substances on the detection were investigated. The proposed method was successfully applied to the determination of HSA in synthetic samples with the recoveries in the range of 99.13 -100.50%. The stoichiometry of HSA-AAIII biocomplex was calculated by voltammetric data with a binding number of 2 and a binding constant of 7.53×10^9.

Keywords: Arsenazo III, Human serum albumin, Linear sweep voltammetry, Interaction, Protein assay

INTRODUCTION

Protein is a very important biomolecule and the determination of microamount of protein is essential in the field of bioanalytical chemistry and clinical analysis [1]. Many analytical methods have been proposed for protein determination [2-5] including spectrophotometry, fluorometry, light scattering technique, electroanalytical method, *etc*. Generally speaking, the spectrophotometric methods are often used because of their simplicity and easy performance. Most of these are based on the dye-protein interaction [6,7]. Recently, several new spectrophotometric probes including trypan blue [8], fast green FCF [9], polychrome blue B [10], arsenazo-DBS [11] have been proposed for the detection of protein. Electroanalysis is an important method for the investigation of the interaction of small molecules with biomacromolecules, which can provide the electron transfer information about bioprocesses. Different types of biomolecules such as DNA, proteins and glycosaminoglycans (GAGs) have been studied by electrochemical methods. Pang *et al*. have studied the interaction of DNA and redox-active complexes such as $[Co(phen)_3]^{3+/2+}$, $[Co(bpy)_3]^{3+/2+}$ [12]. Tamiya *et al*. established an electrochemical DNA quantification method based on aggregation of DNA induced by Hoechst 33258 [13]. Ni *et al*. studied the interaction of DNA and riboflavin, which resulted in the decrease of peak current and positive shift of peak potentials [14]. Mehmet used a local anaesthetics procanine as an electrochemical probe for the determination of DNA [15]. Our group has also proposed some electrochemical methods for DNA detection by using

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toluidine blue [16], methyl violet [17] and a new Co(II) complex, $Co(bbt)_2Cl_2$ [18]. The electrochemistry of protein can also provide basic information about the structure and function of protein. Hu *et al*. have investigated the direct electrochemistry of protein on different kinds of modified electrodes [19]. Sun *et al*. have also applied some electroactive organic substances such as beryllon III [20], acidic chrome blue K [21] and amaranth [22] for the detection of proteins.

In this paper, arsenazo III as a diazo dye (Fig. 1) was used as an electrochemical probe for the study of the protein-dye interaction. Arsenazo III is an electroactive substance which has also been used as a spectrophotometric probe for the determination of protein [23]. In Britton-Robinson (B-R) buffer solution of pH 2.4, the electrochemical reductive peak of AAIII appeared at -0.39 V (*vs*. SCE), which greatly decreased by the addition of proteins such as human serum albumin (HSA), bovine serum albumin (BSA) and bovine hemoglobin (BHb). The decrease of peak current was linear with the concentration of different kinds of proteins, and was successfully applied to the protein sample determinations. The stoichiometry of HSA-AAIII complex was also evaluated by the proposed electrochemical method.

EXPERIMENTAL

Apparatus and Reagents

All the electrochemical experiments were carried out on a JP 303 polarographic analyzer (Chengdu Apparatus Factory, China) with the 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 as counter electrode. Absorption spectra were recorded on a Cary 50 Probe UV-Vis. spectrophotometer (Varian, Australia). All pH values were controlled by a pHS-25C acidimeter (Shanghai Leici Instrument Factory, China).

Human serum albumin (HSA, Shanghai Bioproduct Company), bovine serum albumin (BSA, Beijing Shuangxuan Biochemical Products Factory), bovine hemoglobin (BHb, Tianjin Chuanye Biochemical Products Company) were used as received without any further purification. A 1.0 g l^{-1} stocking solution was prepared by directly dissolving the protein in water and kept at 4 ^{o}C. The working solution was

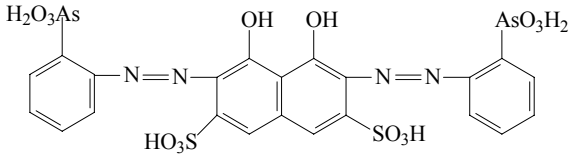

Fig. 1. The molecular structure of arsenazo III.

diluted to the certain concentration just before use. A 1.0 × 10^{-3} M arsenazo III (AAIII, Chemical Factory of East China Normal University) solution were prepared by dissolving 0.0776 g AAIII in 100 ml water. A series of 0.2 M Britton-Robinson (B-R) buffer solution was used to control the acidity of buffer solution. All the chemicals used were of analytical grades and doubly distilled water was used throughout.

Procedure

In a 10 ml colorimetric tube, the solutions were added in the following sequence, 1.5 ml of 1.0 × 10^{-4} M AAIII, 1.5 ml of 0.2 M B-R buffer (pH 2.4) and an appropriate amount of HSA solution. The mixture was diluted to 10 ml with doubly distilled water and shaken thoroughly. The blank solution was also prepared by the same procedure without the addition of HSA. After reaction at 25 ^{o}C for 10 min, the second order derivative linear sweep polarographic detection were carried out within the potential range from 0 to -0.70 V. The polarographic reductive peak potential was appeared at -0.39 V (*vs*. SCE) and the peak current (Ip) of the reaction solution was recorded. Under the same conditions, the peak current of blank solution (Ip_0) was also recorded and the difference in the reductive peak current ($\Delta Ip = Ip_0 - Ip$) was used for quantitative analysis.

RESULTS AND DISCUSSION

Second Order Derivative Linear Sweep Polarography

In this paper the second order derivative linear sweep polarography is used throughout in all the experiments, which is more sensitive and selective than the traditional linear sweep polarography and a very sharp peak shape polarographic curve can be achieved. The experimental results of the relationship between the d^2i/dE^2 with E can also be

automatically recorded by the JP 303 polarographic analyzer. Thus, the peak current value measured is demonstrated as Ip″, which means the d^2i/dE^2 value. Figure 2 shows typical second order derivative linear sweep voltammograms of AAIII-HSA system. Curve 1 is the voltammogram of B-R buffer solution without the electrochemical response. Curve 2 is the linear sweep voltammogram of AAIII in B-R buffer solution of pH 2.4, which has a sensitive reductive peak at -0.39 V (*vs*. SCE). This reduction peak was due to the electrochemical reduction of azo group in AAIII molecule on the mercury electrode. Curve 3 is the voltammogram of AAIII-HSA system. As seen, addition of HSA into AAIII solution, the reductive peak current decreased considerably, while the peak potential did not change. The observed changes of electrochemical response indicates that AAIII has interacted with protein in the selected buffer solution, which resulted in a decrease in free concentration of AAIII in solution and, consequently, a decrease in peak current. At the selected conditions, the decrease of peak current was proportional to the HSA concentration, which was used for establishing a sensitive analytical method for proteins.

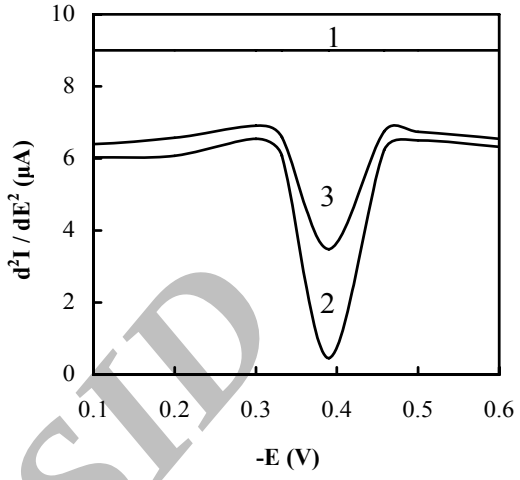

Fig. 2. Second order derivative linear sweep voltammograms For AAIII-HSA reaction system: (1) B-R buffer solution of pH 2.4, (2) $1 + 1.5 \times 10^{-5}$ M AAIII, (3) 2 + 10.0 mg l^{-1} HSA.

Optimization of General Procedures

Effect of acidity and buffer medium. The pH of buffer solution greatly influenced the binding reaction. Figure 3 shows the influence of pH on the ΔIp″ of the AAIII-HSA reaction system. The pH of buffer solution was studied in the range from 1.5-4.5 and the results show that the highest difference in peak current is obtained at pH 2.4; so this pH was selected for further experiment. In a final 10 ml solution, 1.5 ml of B-R buffer solution of pH 2.4 was found to be suitable. Because the isoelectric point of HSA (p*I*) is at 4.7, in the selected acidic pH buffer solution, the lysine, arginine and other amino acid residues in the HSA molecules are positively charged. While the $-SO_3H$, $-AsO_3H$ and -OH groups on AAIII molecules are dissociated and the free AAIII molecule is negatively charged, which make it easy for the two reactants to bind together by electrostatic attraction; thus, the pH of solution is expected to largely influence the binding reaction. Different types of buffers such as B-R, HOAc-NaOAc and phosphate buffer were tested and the results indicated that the

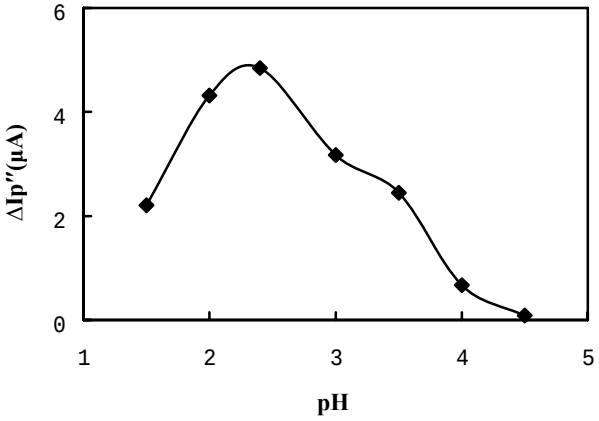

Fig. 3. The influence of pH on the binding interaction. Conditions: 1.5×10^{-5} MAAIII + 20.0 mg l^{-1} HSA in B-R buffer solution of varying pH.

best shape and current for the resulting voltammograms is obtained in the B-R buffer medium.

Effect of AAIII **concentration.** The concentration of AAIII influenced the sensitivity and the electrochemical response. As shown in Fig. 4, the peak current in the absence (1) and presence (2) of 10.0 mg l^{-1} of HSA increased with

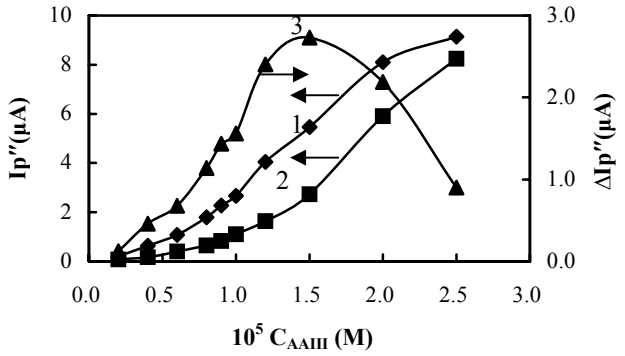

Fig. 4. Relationship between Ip″ and ΔIp″ and AAIII concentration in B-R buffer solution of pH 2.4: (1) C_{HSA} = 0, (2) C_{HSA} = 10.0 mg l^{-1}, (3) ΔIp″ = Ip_1″ - Ip_2″.

increasing AAIII concentration, the observed current being lower in the presence of the protein, as expected. Meanwhile, the difference in peak current (ΔIp″) increased with the increase in AAIII concentration and reached a maximum value when the AAIII concentration was 1.5 × 10^{-5} M (curve 3). However, by further increase in the concentration of AAIII, the value of ΔIp″ is decreased, indicating that the HSA molecules have completely interacted with AAIII.

Effect of adding sequence of reagents. The effect of change in adding sequence of reagents on the peak current was studied. The results indicated that maximum response is obtained after protein was added into a mixture of AAIII and B-R buffer, which also emphasized the fact that the binding of AAIII to HSA is occurred electrostatically.

Effect of reaction time and temperature. After mixing AAIII with protein at pH 2.4, the change in peak current reached its maximum within 10 min and remained constant for about 2 h, indicating that the reaction system possesses ample time for routine applications.

Four temperature levels (20, 25, 37 and 40 °C) were tested to investigate the influences of temperature on the reaction system. The results showed that no distinct difference was appeared after 10 min reaction, which indicated that the binding reaction had reached its equilibrium at the selected conditions; thus, 25 °C was used as reaction temperature.

Instrumental Conditions

The effects of instrumental conditions such as scan rate and mercury drop standing time were optimized. The peak current increased with increasing scan rate from 200 to 1000 mV s^{-1} and the standing time from 3 to17 s. Therefore, the scan rate and standing time were selected as 1000 mV s^{-1} and 17 s, respectively, so that the value of the peak current was maximum.

Effect of Ionic Strength

The effect of ionic strength on the formation of AAIII-HSA complex was studied by addition of different concentrations of NaCl, from 0.02 to 0.5 M to the reaction solution. The results indicated that the difference in peak current of the complex firstly remained constant in the range of 0.02-0.08 M, and decreased slowly with further increase in ionic strength. It is thus obvious that the ionic strength of medium has a large effect on the electrostatic attraction of AAIII and HSA, which shielded the binding of AAIII with HSA at high concentration. For further experiments, the ionic strength of buffer solution was fixed at 0.03 M.

Effect of Coexisting Substances

The influences of various metal ions, amino acids, glucose and urea were tested with the general procedure by premixing HSA with interfering substances. As shown in Table 1, the coexisting substances at low concentrations seldom affected the binding reaction, and the relative error was found to be smaller than 5%.

Calibration Graphs and Sensitivity

The calibration graphs for determination of HSA, BSA and BHb were constructed under the optimal conditions. All the analytical parameters were listed in Table 2. Compared with some other reported spectrophotometric and electrochemical methods, the proposed method showed comparable sensitivity for protein determination [6,7,20-22]. The detection limits were evaluated at a S_0/S of 3, where 3 is the factor at a 99% confidential level, S_0 is the standard deviation of blank measurements without HSA (n = 9) and S is the slope of calibration curves.

Synthetic Sample Determination

The HSA content of three synthetic samples containing HSA and different coexisting substances were measured by the

Table 1. Effect of Coexisting Substances on the Determination of 10.0 mg l^{-1} HSA

Coexisting substances	Concentration (mg l^{-1})	Relative error ΔIp″ (%)	Coexisting substances	Concentration (μM)	Relative error ΔIp″ (%)
L-Serine	0.5	1.83	Cu^{2+}	0.5	3.36
L-Tyrosine	0.5	-1.40	Mn^{2+}	0.5	1.72
L-Leucine	0.5	1.19	Ca^{2+}	0.5	-0.52
L-Arginine	0.5	4.60	Sn^{2+}	0.5	-4.93
L-Valine	0.5	4.95	Zn^{2+}	0.5	-1.57
Glycine	0.5	1.12	Mg^{2+}	0.5	1.97
6-Amino caproic	0.5	-4.39	Co^{2+}	0.5	1.50
Urea	0.5	1.30	Glucose	0.5 (mg l^{-1})	-3.86

Table 2. Analytical Parameters of Calibrations Curves for the Determination of Different Proteins

Protein	Linear range (mg l^{-1})	Standard regression equation	Regression coefficient (γ)	Detection limits (mg l^{-1})
HSA	3.0~15.0	ΔIp″ (nA) = 212.53C (mg l^{-1}) + 1026.50	0.993	0.53
BSA	2.0~20.0	ΔIp″ (nA) = 185.40C (mg l^{-1}) + 928.79	0.992	0.39
BHb	0.8~12.0	ΔIp″ (nA) = 263.69C (mg l^{-1}) + 744.31	0.997	0.42

Table 3. Determination Results of HSA in Synthetic Samples (n = 5)

Sample	Coexisting substances	HSA added (mg l^{-1})	HSA found (mg l^{-1})	RSD (%)	Recovery (%)
1	L-Serine, Glycine, Mn^{2+}, Mg^{2+}	5.00	4.97	2.61	99.4
2	L-Tyrosine, Glucose, Zn^{2+}, Co^{2+}	10.00	10.05	3.73	100.5
3	L-Leucine, Urea, Ca^{2+}, Cu^{2+}	15.00	14.87	1.85	99.1

*Concentration of the coexisting substances: L-Serine, L-Tyrosine, L-Leucine, Glycine, Glucose, Urea: 0.5 mg l^{-1}; Ca^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+}: 0.5 μM.

proposed method and the results are summarized in Table 3. It can be seen that the proposed method is reliable, sensitive, reproducible and practical.

Stoichiometry of HSA-AAIII Supramolecular Complex

According to the method proposed by Li [24], the binding number m and the equilibrium constant β_s of HSA-AAIII complex can be calculated by the change in reductive peak current. It was assumed that AAIII interacted with HSA to form a single complex of $HSA(AAIII)_m$.

$$HSA + mAAIII = HSA(AAIII)_m$$

The equilibrium constant is evaluated from equation:

$$\beta_s = [HSA(AAIII)_m]/[HSA][AAIII]^m \quad (1)$$

The following equation can be deduced step by step:

$$\Delta I_{max} = k\ C_{HSA} \quad (2)$$

$$\Delta I = k\ [HSA(AAIII)_m] \quad (3)$$

$$[HSA] + [HSA(AAIII)_m] = C_{HSA} \quad (4)$$

Therefore:

$$\Delta I_{max} - \Delta I = k\ (C_{HSA} - [HSA(AAIII)_m]) = k\ [HSA] \quad (5)$$

Introducing equations (1), (3) and (5) gives:

$$\log[\Delta I/(\Delta I_{max} - \Delta I)] = m\log[AAIII] + \log\beta_s \quad (6)$$

where, ΔI is the difference in peak current before and after addition of HSA, ΔI_{max} corresponded to the obtained value when the concentration of AAIII was extremely higher than that of HSA. C_{HSA}, $[HSA(AAIII)_m]$ and [HSA] are the total, bound and free concentration of HSA in solution, respectively. From the equation (6), the plot of $\log[\Delta I/(\Delta I_{max} - \Delta I)]$ *vs.* log[AAIII] resulted a linear graph with a regression equation of $\log[\Delta I/(\Delta I_{max} - \Delta I)] = 1.96\ \log[AAIII] + 9.88$ ($n = 7$, $\gamma = 0.995$). The values of m and β_s, evaluated from the intercept and slope of the linear plot, respectively, were found to be $m = 2.0$ and $\beta_s = 7.53 \times 10^9$, which indicated that a stable 1:2 complex of $HSA(AAIII)_m$ was formed under the selected conditions.

UV-Vis Absorption Spectra

The interaction of AAIII with HSA was also checked by UV-Vis spectrophotometric method. Figure 5 shows the UV-Vis absorption spectra of AAIII before and after addition of two different concentrations of HSA in a B-R buffer solution of pH 2.4, at a spectral region of 300-700 nm. As seen, AAIII has a maximum absorption peak at 541 nm (curve 1). After addition of 50.0 mg l^{-1} and 100.0 mg l^{-1} HSA into AAIII solution, the absorbance value of AAIII decreased gradually with a red shift in maximum absorption wavelength of 550 nm and 552 nm (curve 2, 3), respectively. The more HSA added, the greater the absorbance decreased. This phenomenon could also prove that HSA has interacted with AAIII to form a biocomplex in the selected conditions [25].

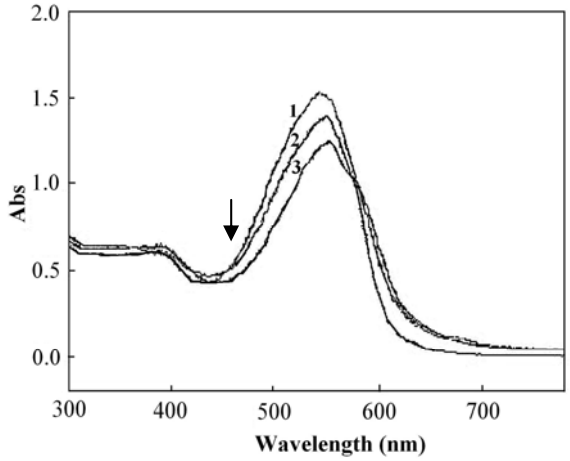

Fig. 5. UV-Vis absorption spectra of AAIII and HSA: (1) pH 2.4 B-R buffer + 8.0×10^{-5} M AAIII, (2) 1 + 50.0 mg l^{-1}, (3) 100.0 mg l^{-1} HSA.

CONCLUSIONS

In this paper the interaction of arsenazo III with different proteins was studied by linear sweep polarography. In a B-R buffer solution of pH 2.4, the $-SO_3H$, $-AsO_3H$ groups in AAIII molecular structure are negatively charged and protein is in positively charged, so that they can interact electroestatically to form a supramolecular complex, which greatly decreased the linear sweep polarographic reductive peak current of AAIII at -0.39 V. Based on the observed decrease in peak current, a new electrochemical method for low level determination of proteins was established. This assay was convenient, sensitive, simple to use and suitable for practical applications.

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