Technical Note

POLAROGRAPHIC DETERMINATION OF CHONDROITIN SULFATE BASED ON ITS INTERACTION WITH CRYSTAL VIOLET

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***Abstract*—Electrochemical determination of chondroitin sulfate (CS) was established with crystal violet. Under the optimal conditions, the decrease of the current was proportional to CS concentration in the range from 1.5 to 20.0 mg/L. The method was successfully applied to determine the CS content in synthetic samples.**

***Keywords*—chondroitin sulfate, crystal violet, linear sweep polarography, interaction**

I. INTRODUCTION

Chondroitin sulfate (CS) is one important member of glycosaminoglycans (GAGs) family with alternating *D*-glucuronic acid and *N*-acetylated *D*-galactosamine residues (Maruyama *et al.*, 1998). In recent years, CS has attracted much attention as it shows many important biological and pharmacological activities (Mourano *et al.*, 1996). For example, CS had been used as an effective medicine for the treatment of rheumatism, arthritis, nephritis, lumbago, gastric ulcer and AIDS (Zhu *et al.*, 2000). So it is important to establish a sensitive method for CS determination. At present, some methods had been proposed to detect CS such as spectrophotometry (Chen *et al.*, 2006), HPLC (Kaoshiishi *et al.*, 1998), ion chromatography (Plaas *et al*., 1996) and electrophoretic methods (Payan *et al.*, 1998). But there are seldom reports about the electrochemical methods for CS detection.

Electrochemical methods have been successfully utilized to the determination of different kinds of biomolecules such as DNA, proteins, enzymes and heparin with the advantages such as higher sensitivity, wider linear range, faster response and cheaper instruments. For example, Hu *et al.* (2006) investigated the electrochemical behavior of malachite green with dsDNA and detected the content of DNA. Kobayashi *et al.* (2004) reported the interaction of Hoechst 33258 with DNA in solution by linear sweep voltammetry and further used for electrochemical DNA quantification. Peng *et al.* (2007) studied the interaction of toluidine blue with heparin by cyclic voltammetry. Sun *et al.* (2006, 2007) proposed electrochemical probes for the determination of biomolecules such as DNA (Sun *et al.*, 2006) and protein (Sun *et al.*, 2007). Based on the changes of electrochemical response, the proposed method was sensitive, reliable and successfully applied to the samples determination.

In this work, crystal violet (CV) was selected as the electrochemical probe to investigate the binding reaction with CS and further used to the detection of CS. CV is a cationic dye and had been used as color reagent in the determination of DNA (Huang *et al.*, 2001) and heparin (Xu *et al.*, 2002). Under the selected conditions, CS was in negatively charged and could easily interact with the cationic dye of CV. The interaction was monitored by linear sweep polarography and the electrochemical response of CV was decreased with the increase of CS concentration. The proposed method was further used for CS determination in synthetic samples.

II. METHODS

A. Apparatus and reagents

The second order derivative linear sweep polarographly was performed on a JP-303 polarographic analyzer (Chengdu Apparatus Factory, China) with a traditional three-electrode system composed of a dropping mercury working electrode (DME), a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode. A Cary 50 probe spectrophotometer (Varian Company, Australia) was used to record the UV-Vis absorption spectra. All the experiments were carried out at 25±1ºC.

Chondroitin sulfate (CS, 99%, Shandong Linyitianli Biochemical Company, China) was used as received. A 1.0 mg/mL CS stock solution was prepared by directly dissolving 0.1000 g of CS into water, then diluted in a 100 mL calibration flask and stored at 4 ºC. The working solutions were obtained by diluting the stock solution with water. A 1.0×10^{-3} mol/L of crystal violet (CV, Shanghai Chemical Reagent Company, China) solution was prepared by dissolving 0.04080 g CV in water and diluted to 100 mL. 0.2 mol/L Britton-Robinson (B-R) buffer solution was used to control the acidity of reaction solution. All of other reagents used were of analytical reagent grade and doubly distilled water was used throughout.

B. Procedures

Into a dry 10 mL calibrated tube 1.5 mL of pH 2.5 B-R buffer solution, 0.2 mL of 1.0×10^{-4} mol/L CV and different amounts of CS were added in sequences. The mixtures were diluted to the mark with water, mixed

homogeneously and allowed to react at 25 ºC for 15 min. Then the solution was transferred to a 10 mL electrochemical cell and the second order derivative linear sweep polarographic curve was recorded in the potential range from -0.4 to -1.0 V (vs. SCE). The reductive peak current (ip″) of CV at -0.70 V (vs. SCE) was measured. Under the same condition, the peak current (ip_0″) of CV solution without the addition of CS was also recorded and the difference of peak current (Δip″= ip_0″- ip″) was used to determine the concentration of CS.

C. Results and Discussion

UV-Vis absorption spectra

Figure 1 showed the UV-Vis absorption spectra of CV in the absence and presence of different concentrations of CS in pH 2.5 B-R buffer. In the wavelength range from 300 to 800 nm no absorption peak appeared for CS solution (curve 1) and CV had a maximum absorption peak at 590 nm (curve 2). After the addition of CS into CV solution, the absorbance at 590 nm decreased without new absorption peak appeared (curves 3 and 4). The more the CS added into the solution, the greater the absorbance at 590 nm decreased, which indicated the interaction of CV and CS in the acidic solution.

Second order derivative linear sweep polarogram

The second order derivative linear sweep polarograms of B-R, CV and their mixture with CS were shown in Fig. 2. Curve 1 was the polarogram of B-R buffer and no polarographic peak appeared, which indicated that no electroactive substances existed. Curve 2 was the polarogram of the CV solution, a well-defined polarographic reductive peak appeared at -0.70 V (vs. SCE), which was due to the reduction of CV molecules on the mercury electrode. Curve 3, 4 and 5 were the polarograms of the mixture of different concentration of CS with CV. Owing to the interaction of CS with CV, the concentration of free CV in the mixture solution decreased, so the reductive peak current decreased correspondingly. The decrease of peak current was proportional to the concentration of CS, which could be further used for the CS determination.

The electrochemical behaviors of CV had been investigated on different working electrodes including glassy carbon electrode (Perekotii *et al.*, 2002) and dropping mercury electrode (DME) (Li and Jiang, 2005). CV exhibited an irreversible adsorptive reductive wave with two protons and two electrons transferred on the DME. Based on the sensitive response of CV on the DME, it was selected as the electrochemical indicator for CS determination in this paper.

Optimal of the reaction conditions

The influences of the reaction conditions such as the acidity of buffer solution, the CV concentration, reaction time, the instrumental conditions and the ion strength etc. were carefully investigated.

The effect of pH on the difference of peak current (Δip″) was investigated by keeping CS and CV concentration constant and changing the pH in the range from 1.0 to 6.0. The result showed that the value of Δip″

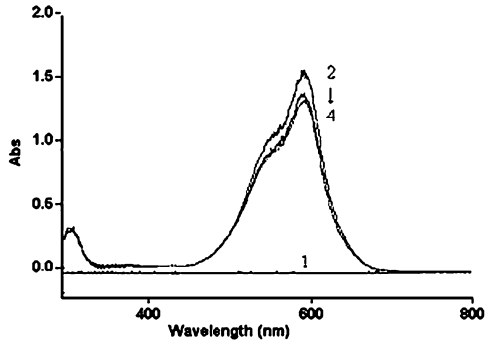

Figure 1. UV-Vis absorption spectra of CV-CS system.
Condition: 1.pH 2.5 B-R buffer + 20.0 mg/L CS; 2. pH 2.5 B-R buffer + 2.0×10^{-5} mol/L CV; 3. 2 + 5.0 mg/L CS; 4. 2 + 10.0 mg/L CS

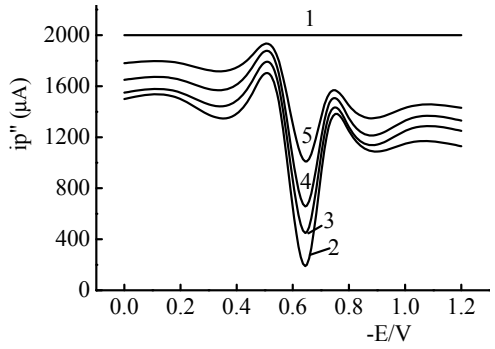

Figure 2. Second order derivative linear sweep polarogramof CV-CS reaction system.
Condition: 1. pH 2.5 B-R; $2.1+2.0\times10^{-5}$ mol/L CV; 3. 2+ 6.0 mg/L CS; 4.2+10.0 mg/L CS; 5.2+20.0 mg/L CS.

reached its maximum at pH 2.5, so pH 2.5 was selected for this assay. In the acidic buffer solution, the positively charged CV can easily interact with negatively charged CS to form a supramolecular biocomplex. The volume of B-R buffer added in the final solution was investigated in the range of 1.0~5.0 mL and 1.5 mL was suitable for the following procedures.

The effect of CV concentration on the value of Δip″ was studied with 20.0 mg/L CS. The results showed that the value of Δip″ reached the maximum at the CV concentration of 2.0×10^{-5} mol/L and then remained constant, which indicated the binding reaction reached its equilibrium. So 2.0×10^{-5} mol/L CV was recommended.

After mixing CV with CS, the value of Δip″ reached maximum within 15 min and remained constant for about 2 hours. So the system gave enough time for routine determination.

The effect of the reaction temperature on the interaction was tested in the range of 10~40ºC. There were no obviously differences of Δip″ in the selected temperature range. So the reaction temperature had little influences on the interaction and 25ºC was used throughout.

The effect of instrumental conditions, such as the scan rate and the dropping mercury standing time (the lifetime of the mercury drop), was also tested. The results indicated that the value of Δip″ reached the maximum when the scan rate was at 700 mV/s. The differ-

ence of peak current increased with the increase of the dropping mercury standing time from 4 to 15 s, but the mercury drop would fall down naturally when the dropping mercury standing time exceeded 15 s. So the scan rate and the mercury drop standing time were selected as 700 mV/s and 14 s, respectively.

The effect of NaCl concentration was examined to show the influence of ionic strength. The presence of NaCl had significant influences on the interaction and the value of Δip″ decreased with the increase of salt concentration in the range of 0.01~0.2 mol/L. The result proved that the interaction of CV with CS was mainly caused by electrostatic attraction. The electrostatic shielding effect of the charges on binding reaction with the increase of Na^+ concentration was unbeneficial to the formation of CS-CV complex.

Influences of coexisting substances

The influences of commonly coexisting substances on the determination of 20.0 mg/mL CS were also tested with the results listed in Table 1. It can be seen that different substances such as metal ions, amino acids and glucose had little effects on the determination. So this method could be directly applied to the biological sample determination.

Working curve

Under the optimal conditions, a calibration curve was obtained in the CS concentration range of 1.5~20.0 mg/L with the linear regression equation as Δip″(nA)=258.54 C (mg/L)-58.79 (n=9, γ=0.991). The relative standard deviation (RSD) for eleven parallel determinations of 10.0 mg/L CS was 2.21 % and the detection limit was calculated as 0.30 mg/L (3σ).

Sample determinations

Three synthetic samples were analyzed by the proposed method with the results listed in Table 2. It can be seen that this new method was practical and reliable to determine CS in synthetic samples with the recovery in the range of 91.1 %~94.0 %.

Stoichiometry of CS-CV complex

The composition of the supramolecular complex and the equilibrium constant was determined based on a reported method (Li and Min, 1989). It was presumed that interaction of CV with CS only formed a single complex of CS-mCV. The binding number (m) and the equilibrium constant (βs) of the binding reaction could be calculated from the following equations:

$$CS+mCV \rightarrow CS\text{-}mCV \quad (1)$$

The equilibrium constant was deduced as follows:

$$\beta_s = \frac{[CS\text{-}mCV]}{[CS][CV]^m} \quad (2)$$

Since

$$\Delta i_{max}=kC_{CS}, \quad (3)$$

$$\Delta i=k[CS\text{-}mCV], \quad (4)$$

$$[CS]+[CS\text{-}mCV]=C_{CS}. \quad (5)$$

Therefore:

$$\Delta i_{max}-\Delta i=k(C_{CS}-[CS\text{-}mCV])=[CS]. \quad (6)$$

Introducing equations (2), (4) and (6) gave:

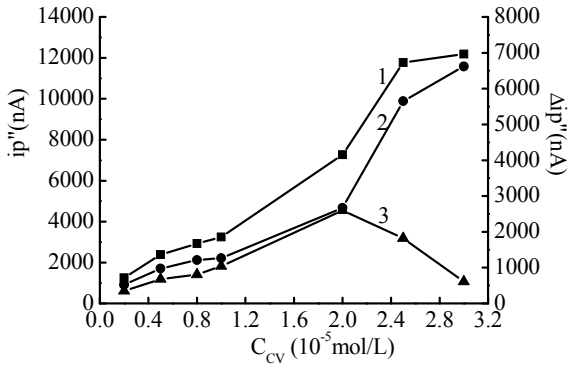

Figure 3. Relationship between ip″ and C_{CV} (1, 2), Δip″ and C_{CV} (3).
Condition: 1. C_{CS}=0; 2. C_{CS}=10.0 mg/L; 3. $\Delta ip''=ip_1''-ip_2''$

$$\log[\Delta i/(\Delta i_{max}-\Delta i)]=\log\beta s+m\log[CV]. \quad (7)$$

Where Δi is the difference of peak current in the presence and absence of CS and Δi_{max} corresponds to the obtained value when the concentration of CV is extremely higher than that of CS. C_{CS}, [CS], [CS-mCV] are corresponding to the total, free and bound concentration of CS in the solution, respectively.

Figure 3 showed the relationship between ip″, Δip″ with the concentration of CV. By using the Eq. (7) the relationship of $\log[\Delta i/(\Delta i_{max}-\Delta i)]$ with log[CV] was calculated. From the intercept and the slope the values of m≈1 and $\beta s=2.75\times10^4$ were deduced, which indicated that a 1:1 complex of CS-CV was formed in the selected conditions.

III. CONCLUSIONS

In this paper an electroanalytical method for the determination of CS was described by using CV as an electrochemical probe. The addition of CS into CV solution caused the decrease of reductive peak current, which was due to the formation of a new supramolecular complex by electrostatic interaction of negatively charged CS with positively charged CV in the solution. The binding parameters of CS with CV were calculated by the electrochemical data and the established method could be further applied to determine microamount of CS with satisfactory results.

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Table 1 Effect of foreign substances on the determination of 20.0 mg/L CS.

Coexisting substances	Concentration (mmol/L)	Relative error (%)	Coexisting Substances	Concentration (mg/L)	Relative error (%)
Cu^{2+}	1.0×10^{-6}	-4.20	L-Tyrosine	2.0	-2.88
Ca^{2+}	1.0×10^{-6}	-3.52	L-Arginine	2.0	-4.41
Mn^{2+}	1.0×10^{-6}	-4.12	L-Glutamine	2.0	-4.96
Zn^{2+}	1.0×10^{-6}	-1.96	L-Leucine	2.0	-4.92
Fe^{3+}	1.0×10^{-6}	-5.77	L- Cysteine	2.0	-3.22
Mg^{2+}	1.0×10^{-6}	-1.27	L- Cystine	2.0	-5.35
Glucose	2.0 mg/L	0.58	Glycin	2.0	-5.11
SDS	2.0 mg/L	-3.72	Glutamine	2.0	2.99

SDS: sodium dodecyl sulfate.

Table 2 Determination results of CS in synthetic samples (n=5).

Samples	Coexisting substances	Added (mg/L)	Found (mg/L)	RSD (%)	Recovery (%)
1	L-Tyrosine, L-Cysteine, Zn^{2+}, Mg^{2+}	20.00	18.98	4.70	94.9
2	L-Cysteine, Glucose, Zn^{2+}, Ca^{2+}	20.00	18.42	2.94	91.1
3	L-Tyrosine, Glucose, Mg^{2+}, Ca^{2+}	20.00	18.32	2.58	91.6

Coexisting substances: L-Tyrosine, L-Cysteine, Glucose: 2.0 mg/L; Mg^{2+}, Zn^{2+}, Ca^{2+}:3.0×10^{-6} mol/L

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