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Voltammetric Investigation on Interaction of Hyaluronic Acid with Crystal Violet and its Analytical Application

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ABSTRACT: In this paper, the interaction of hyaluronic acid (HA) with crystal violet (CV) was investigated carefully by linear sweep voltammetry on the dropping mercury working electrode (DME). In pH 5.0 Britton-Robinson (B-R) buffer solution, CV has a sensitive, well-defined second order derivative linear sweep voltammetric reductive wave at –0.85 V (*vs*. SCE). After adding a certain amount of HA into CV solution, the reductive peak current decreased without any shift of reductive peak potential. Based on the difference in the reductive peak current, a new voltammetric method for the detection of HA was established. The reaction conditions and the electrochemical determination were studied and optimized. Under the optimized conditions, the decrease of peak current showed a good linear relationship with the HA concentration in the range from 10.0 to 40.0 mg/L. The linear regression equation was got as $\Delta ip''(nA)= 84.07\ C–527.86$ (mg/L) (n=8, γ=0.997) and the detection limit was calculated as 2.65 mg/L (3σ). This new established method was further used to HA determination in the synthetic samples with satisfactory results and good recovery. The stoichiometry of CV-HA complex was calculated and the binding mechanism was also discussed by the electrochemical data.

Keywords: Hyaluronic acid; Crystal violet; Voltammetry; Binding reaction

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1. INTRODUCTION

As a member of naturally occurring glycosaminoglycan, hyaluronic acid (HA) is consisted of alternating disaccharide units of β-(1-3)-N-acetyl-D-glucoscmine and β-(1-4)-D-glucuronic acid [1]. Owing to the advantages of biodegradability, good biocompatibility, water retention, non-toxic, anti-inflammatory etc. which has been used for various medical applications such as arthritis treatment, ocular surgery, heart surgery, tissue augmentation, and so on [2]. The efficacy of HA mostly relies on its linear negatively charged sugar chain, and it also affected by its molecular weight [3]. Furthermore, the level of HA in serum is a sensitive marker of liver cell function and can be used for the clinical diagnosis [4]. Therefore, to establish a reliable, accurate quantitative determination method of HA is very important in the field of bio-analytical chemistry. At present, the analytical methods for the determination of HA main contains immunochemistry [5] enzyme-linked immunosorbent assay [6], electrophoresis [7] spectrophotometry [8], high-performance liquid chromatography [9], resonance rayleigh scattering technique [10, 11] etc. Owing to the advantages of lower detection limit and wider linear range, electrochemical methods have been successfully used for the determination of different kinds of biomolecules such as DNA [12-14], protein [15, 16], heparin [17, 18] and chondroitin sulfate [19,]. Also a small amount of sample is needed because the electrochemical reaction is often taken place on the interface of the electrode and the solution.

Crystal violet (CV) is a triphenylmethane dye and the molecular structure of CV was shown in Fig. 1 (B), which had been used as electrochemical probe [20] and modified material [21] in electro-analytical methods. But to our knowledge no reports had been proposed to determine HA by CV by the electrochemical method. In this work, CV was selected as an electrochemical probe to study the binding reaction with HA and further used to determine HA by linear sweep voltammetry (LSV). The experimental results indicated that CV could interact with HA by the electrostatic force to form a supramolecular complex, which resulted in the decrease of the voltammetric response of CV. The decrease of the peak current is directly proportional to the HA concentration, thus a new sensitive analytical method was established for HA determination.

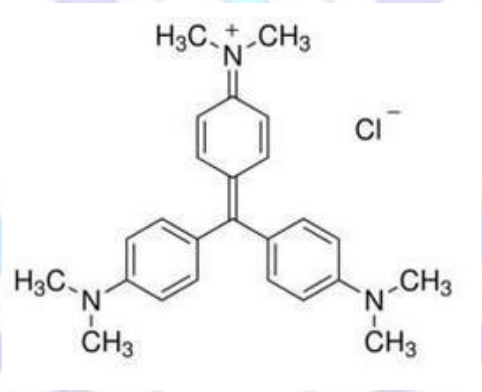

Fig. 1 The molecular structure of CV

2. EXPERIMENTAL

2.1 Chemicals and Apparatus

Hyaluronic acid (HA, purity, 99%, average molar mass of 1.5×10^6, Shandong Huayuan Biological Engineering Company, China) was used as received without further purification. The 1.0 g/L stock solution of HA was prepared by dissolving 0.1000 g of HA in water, diluted to 100 mL and stored at 4°C. The working solutions were obtained by diluting the stock solution with water. A 1.0×10^{-3} mol/L crystal violet (CV, Shanghai Chemical Reagent Plant, China) solution was prepared by dissolving 0.0408 g CV in water and diluted to 100 mL. 0.2 mol/L Britton-Robinson (B-R) buffer with different pH value used as the supporting electrolyte prepared by mixing 12.35 g

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H_3BO_3, 13.55 mL 85 % H_3PO_4, and 11.80 mL HOAc, diluted to1000 mL and adjusted by 0.2 mol/L NaOH. All of the other reagents used were of analytical reagents grade without further purification and doubly distilled water was used throughout.

Linear sweep voltammetric experiments were actualized by using a model JP-303 polarographic analyzer (Chengdu Apparatus Factory, China). The conventional three-electrode system of a dropping mercury working electrode (DME) which served as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the auxiliary electrode. UV-Vis absorption spectra were recorded by a UV-2550 probe spectrophotometer (Shimadzu, Japan). In this study, all the pH values were measured by a PHS-3C meter (Hangzhou Orion Instrument Co. Ltd., China). The experiments were all performed at 25±1 ºC.

2.2 Procedure

Into a dry 10.0 mL colorimetric tube solutions were added in the following orders: 3.0 mL of pH 5.0 B-R buffer, 2.0 mL of 1.0×10^{-4} mol/L CV and an appropriate amount of HA solution. The mixtures were diluted to the mark with water, mixed homogeneously and allowed to react for 20 min at 25 ºC. The second order derivative linear sweep voltammetric curves of the mixture solution were recorded in the potential range of 0 ~ -1000 mV and the reductive peak currents at -0.85 V (*vs*. SCE) were measured. The values of the peak current of CV solution and the CV-HA reaction solution were noted as ip0'' and ip'', respectively. The difference of peak current ($\Delta ip''=ip_0''-ip''$) was used to determine the concentration of HA.

3. RESULTS AND DISCUSSION

3.1 UV-Vis absorption spectra

The UV-Vis absorption spectra of CV and the interaction solution with different amount of HA in pH 5.0 B-R buffer solution were showed in Fig. 2. It can be seen that CV had a maximum absorption peak at 526 nm (curve 1). After the addition of 50.0 mg/L of HA into CV solution, the absorption peak value decreased without any wavelength shift (curve 2). The decrease of the absorption spectra indicated that an interaction had taken place in the mixture solution.

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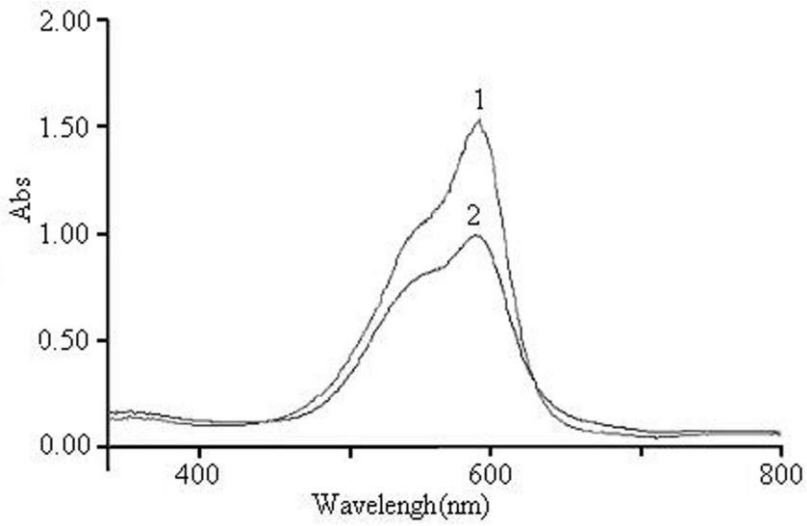

Fig. 2 The UV-Vis absorption spectra of CV-HA reaction system

Curve: 1.pH 5.0 B-R buffer +2.0×10^{-5} mol/L CV; 2.2+50.0 mg/L HA

3.2 Linear sweep voltammogram

In order to improve the sensitivity, a second order derivative linear sweep voltammetry was used throughout the experiments and the typical voltammograms of HA-CV interaction system were showed in Fig. 3. Curve 1 was the voltammogram of B-R buffer solution, which showed no electrochemical response in the scanning range. Curve 2 was that of CV in pH 5.0 B-R buffer solution, a well-defined voltammetric reductive peak at -0.85 V (vs. SCE) appeared, which was due to the electrochemical reduction of CV on the mercury electrode. Curves 3 and 4 were that of the interaction solution of CV with different amounts of HA. When HA was added into CV solution, the reductive peak current decreased without the shift of the peak potential, which demonstated that CV interacted with HA and a non-electrochemical active supramolecular biocomplex was formed in the solution. So the free concentration of CV in the solution was decreased and consequently provoking a decrease in the peak current. The difference of peak current was related to the HA concentration, which could be further used for the sensitive determination of HA.

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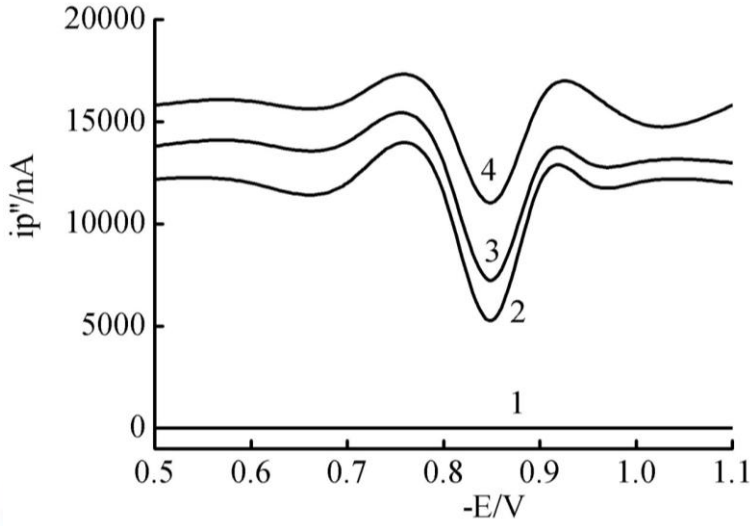

Fig. 3 Second order derivative linear sweep voltammograms of CV-HA reaction system.

Curve: 1. pH 4.5 B-R buffer; 2.1+2.0×10^{-5} mol/L CV, 3.2+20.0 mg/L HA, 4.2+50.0 mg/L HA

3.3 Optimal of the reaction conditions

The interferences of experimental conditions on the reaction such as buffer acidity, buffer volume, the CV concentration, the adding order and reaction time etc. were studied carefully by changing only one factor and keeping the others constant.

The effect of buffer acidity on the difference of peak current (Δip″) was investigated by changing the buffer pH in the range from 2.0 to 6.0. The result indicated that the value of Δip″ reached its maximum at pH 5.0, so pH 5.0 was selected in this experiment. When the volume of the B-R buffer was 3.0 mL in a final 10 mL volume of reaction solution, the value of Δip″ reached a maximum, and 3.0 mL was selected for the following procedures.

The influence of CV concentration on the on the value of Δip″ was studied in the range from 1.0×10^{-6} mol/L to 5.0×10^{-5} mol/L and the results indicated that the value of Δip″ reached a maximum when the concentration of CV was at 2.0×10^{-5} mol/L, so this value was employed in the assay.

The effect of adding order of B-R buffer, CV and HA was tested and B-R buffer, CV and HA was selected as the optimal adding order, which indicted that the electrostatic attraction existed in the mixture solution.

The influence of reaction temperature on the interaction was also tested in the range from 10 to 40 °C and the results showed that there were no obviously differences on Δip″ in the selected temperature range; so all experiments were carried out at 25 °C. The value of Δip″ reached a maximum about 30 min and remained stable for more than 2 hours. Therefore the system can be used for routine experiments with ample time.

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The instrumental conditions such as the scan rate and the dropping mercury standing time were also investigated within 100~1000 mV/s and 1s to 13 s, respectively. The value of Δip″ reached its maximum when the potential scan rate was at 900 mV/s and the dropping mercury standing time was at 11s. So the scan rate and the standing time were selected as 1000 mV/s and 11 s, respectively.

3.4 Working curve

As seen in Fig. 4, under the optimal conditions, a linear relationship between the decrease of the reductive peak current and the HA concentration was obtained in the range from 10.0 mg/L to 40.0 mg/L. The linear regression equation was got as Δip″(nA)=84.07 C-527.86 (mg/L) (n=8，γ=0.997) and the detection limit was calculated as 2.65 mg/L (3σ). The relative standard deviation (RSD) for 10 parallel determinations of 25.0 mg/L HA was 0.78 %.

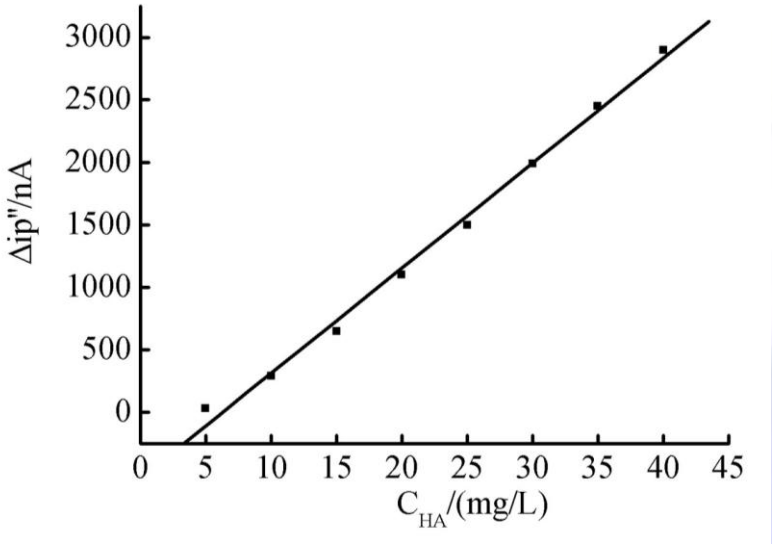

Fig.4 Relationship between Δip" and C_{HA}

Curve: pH 5.0 B-R buffer 3.0 mL +2.0×10^{-5} mol/L CV+ different amount of HA

3.5 Influence of foreign substances

The influences of various coexisting substances such as metal ions, amino acids *etc.* on the determination of HA was carried out and the results were listed in Table 1. It can be seen that most of them had little effects on the detection of HA except β-CD and DNA, which may be due to the dissociation of them in the solution and hold positively charged, then competed the interaction of HA with CV.

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Table 1 Effect of foreign substances on the determination of 100.0 mg/L HA (n=3)

Substance	Concentraion mol/L	Relative error %	Substance	Concentration mg/L	Relative error %
Co^{2+}	1.0×10^{-5}	-4.83	L- Leucine	10.0	-2.40
Ni^{2+}	1.0×10^{-5}	-3.62	L-Arginine	10.0	2.19
Cu^{2+}	1.0×10^{-5}	-2.82	L-Serine	10.0	0.47
Mg^{2+}	1.0×10^{-5}	2.82	L-Tyrosine	10.0	2.10
Pb^{2+}	1.0×10^{-5}	1.61	Citric acid	10.0	-0.60
Hg^{2+}	1.0×10^{-5}	4.03	L-Valine	10.0	1.98
Ba^{2+}	1.0×10^{-5}	1.61	DNA	10.0	43.78
Fe^{3+}	1.0×10^{-5}	2.56	β-CD	10.0	-48.59

* DNA (deoxyribonucleic acid), β-CD (β-cyclodextrin)

3.6 Analysis of synthetic samples

In order to test the proposed method[22], three synthetic samples was prepared by adding different amounts of amino acids, metal ions into 25.0 mg/L HA solution, which were further determined according to the general procedure with the results given in Table 2. It can be seen that this new method was practical and reliable to determine HA in synthetic samples with high accuracy and precision.

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

Sample	Coexisting substance	Added mg/L	Found mg/L	RSD %	Recovery %
1	L-Tyrosine, L-Arginine , Pb^{2+}, Mg^{2+}	25.00	25.86	2.18	103.44
2	L-Tyrosine, L-Serine , Mg^{2+}, Co^{2+}	25.00	26.04	1.22	104.16
3	L-Arginine, Citric acid , Ba^{2+}, Mg^{2+}	25.00	25.56	2.31	102.24
4	L-Serine, L-Valine , Ba^{2+}, Co^{2+}	25.00	25.81	1.52	103.24

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3.7 Stoichiometry of HA–CV supramolecular complex

According to the method [22], the binding number (*m*) and the equilibrium constant (β_S) of the supramolecular complex were measured. It was presumed that CV interacting with HA repeated unit only formed a single complex of HA-mCV. The value of *m* and β_S between HA unit and CV could be calculated from the following equations:

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

The equilibrium constant (β_S) is deduced as follows:

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

The following equations can be deduced step by step:

$$\Delta i_{max} = k\, C_{HA} \quad (3)$$

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

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

Therefore:

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

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

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

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

The relationship between ip", Δip" (ip_0"-ip") with the CV concentration was shown in Fig. 4. The relationship of $\log[\Delta i/(\Delta i_{max}-\Delta i)]$ versus log[CV] was calculated according to equation (7) and a linear regression equation was obtained as $\log[\Delta i/(\Delta i_{max}-\Delta i)]=2.85\log[CV]+14.35$ (*n*=5, *γ*=0.987). From the intercept and the slope, the value of $m \approx 3.0$ and $\beta_S = 2.24\times10^{14}$ were deduced, which indicated that a stable complex of HA-3CV was formed in the selected conditions.

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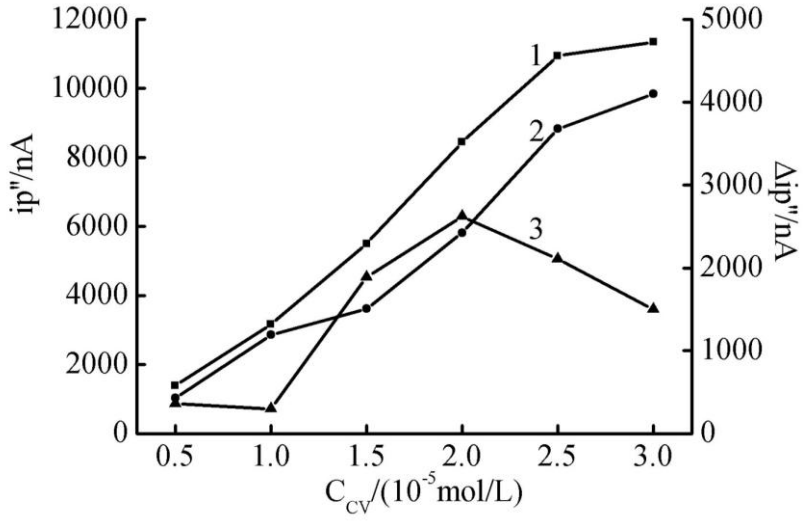

Fig. 4 Relationship between ip" and C_{CV} (curve 1,2), Δip" and C_{CV} (curve 3)

Curve: 1.C_{HA}=0; 2.C_{HA}=25.0 mg/L; 3. $\Delta ip" = ip_0" - ip"$

4. CONCLUSIONS

In this paper, the behavior of CV and the interaction of CV with HA were investigated by linear sweep voltammetric method. The results showed that CV had a sensitive linear sweep voltammetric peak at -0.85 V, and the decrease of reductive peak current was directly proportional to the HA concentration, which indicated a new supramolecular complex was formed. The interaction mechanism was also discussed and the proposed method was successfully applied to HA synthetic samples detection with satisfactory results.

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