Bull. Chem. Soc. Ethiop. **2008**, 22(2), 165-172. ISSN 1011-3924
Printed in Ethiopia

VOLTAMMETRIC DETERMINATION OF HEPARIN BASED ON ITS INTERACTION WITH MALACHITE GREEN

Xueliang Niu[1], Weili Zhang[2], Na Zhao[1] and Wei Sun[1*]

[1]College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, China
[2]Department of Basic Medicine, Shangdong Wanjie Medical College, Zibo 255213, China

(Received June 10, 2007; revised September 22, 2007)

ABSTRACT. In this paper malachite green (MG) was used as a bioprobe to determine heparin concentration by linear sweep voltammetry on the dropping mercury working electrode (DME). In Britton-Robinson (B-R) buffer solution of pH 1.5, MG had a well-defined second order derivative linear sweep voltammetric reductive peak at –0.618 V (vs. SCE). After the addition of heparin into the MG solution, the reductive peak current decreased apparently without the movement of peak potential. Based on the difference of the peak current, a new voltammetric method for the determination of heparin was established. The conditions for the binding reaction and the electrochemical detection were optimized. Under the selected experimental conditions the difference of peak current was directly proportional to the concentration of heparin in the range from 0.3 to 10.0 mg/L with the linear regression equation as $\Delta ip''$ (nA) = 360.19 C (mg/L) + 178.88 (n = 15, γ = 0.998) and the detection limit as 0.28 mg/L (3σ). The effects of coexisting substances such as metal ions, amino acids on the determination of heparin were investigated and the results showed that this method had good selectivity. This method was further applied to determine the heparin content in heparin sodium injection samples with satisfactory results and good recovery. The stoichiometry of the biocomplex was calculated by the electrochemical method and the binding mechanism was further discussed.

KEY WORDS: Heparin, Malachite green, Voltammetry, Binding reaction

INTRODUCTION

Heparin is a kind of commonly used glycosaminoglycans (GAGs), which contains glucosamine-*N*-sulfate and uronic acid with lots of sulfate, carboxyl and acetyl residues. Heparin is often found in blood vessels, liver capsule, lung, skin, intestine and the peritoneal wall and the average molecular mass of heparin is 12000 Da. In clinical therapy heparin is often used as anticoagulant and it also has other functions such as antithrombotic, antilipemic, antiatherosclerosis, antiphlogistic and antiallergic activities [1]. Therefore, it is very important to establish a sensitive method for the determination of the heparin concentration in pharmaceutical analysis and biochemistry.

At present, many analytical methods have been proposed for the detection of heparin, including UV-Vis spectrophotometry [2-5], light scattering technique [6], HPLC [7], electrophoretic method [8], flow injection analysis [9], surface plasmon resonance sensor analysis [10], piezoelectric quartz crystal sensor [11] and electroanalytical method [12]. For example, Jiao *et al.* investigated the interaction between some cationic dyes such as azure A, methylene blue and azure B with heparin by absorption spectrophotometry [13]. Sun *et al.* applied brilliant cresyl blue and neutral red for spectrophotometric determination of heparin [14, 15]. Liu *et al.* established a resonance rayleigh scattering technique to the heparin determination by using some basic diphenylnaphthylmethane dyes such as Victoria blue 4R, Victoria blue B [16-18]. Meyeroff *et al.* had reported the potentiometric sensors for heparin by some specific formulated polymer membrane modified electrodes [19-22]. Sun *et al.* used voltammetric method for heparin determination using some dyes such as methyl violet, netural red**,** light green and crystal violet [23-26]. Compared with other analytical methods electrochemical method is

*Corresponding author. E-mail: sunwei@qust.edu.cn

useful with the advantages of higher sensitivity, wider liner range, faster response and cheaper instruments. Since the electrochemical reaction occurs at the electrode surface, the measurement need small amounts of samples and this method has received more attentions in bioanalytical chemistry recently.

In this paper, a triphenylmethane cationic dye of malachite green (MG) was selected to investigate the interaction with heparin by linear sweep voltammetry. MG is commonly used as an effect additive for the treatment of external fungal and protozoan infections of fish. But MG has the potential of carcinogenicity and terotogenicity. So it is important to investigate the interaction of MG with biomacromolecules. MG had been used as the electrochemical probe for the determination of DNA [27]. But no reports had been proposed by electrochemical method with MG for the study of heparin to our knowledge. The molecular structure of MG is shown in Figure 1. The experimental results showed that it could strongly interact with heparin by electrostatic force and an electroinactive supramolecular complex was formed, which resulted in the decrease of the voltammetric response of MG. The optimal conditions were selected and based on the decrease of peak currents of MG, a determination method for heparin by linear sweep voltammetry was further developed.

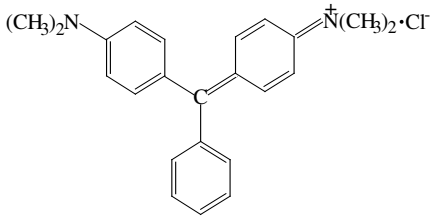

Figure 1. The molecular structural formula of malachite green (MG).

EXPERIMENTAL

Apparatus

All the linear sweep voltammetric experiments were carried out on a model JP-303 polarographic analyzer (Chengdu Apparatus Factory, China) with the traditional three-electrode system consisting 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-Vis absorption spectra were recorded by a Cary 50 probe spectrophotometer (Varian, Australia). All the values of pH were measured by a pHS-25 acidimeter (Shanghai Leici Instrument Factory, China). The experiments were carried out at 25 ± 1 ºC.

Reagents

Malachite green (MG, Shanghai Xinzhong Chemical Plant, China) and heparin sodium (140 IU/mg, Shanghai Chemical Reagent Company, China) were used as received without further purification. A 1.0 mg/mL stock solution of heparin was prepared in water and stored at 4 ºC. The working solutions were obtained by diluting the stock solution with water. The heparin sodium injection samples were purchased from Tianjing Biochemical Pharmaceutical Factory of China (20031003) with the specified amount of heparin as 12500 IU/2 mL. A 0.2 M Britton-Robinson (B-R) buffer solution was used to control the pH of the tested solutions. All the reagents used were of analytical reagent grade and doubly distilled water was used throughout.

Procedure

Into a dry 10.0 mL colorimetric tube, solutions were added in the following order: 1.0 mL of pH 1.5 B-R buffer, 4.0 mL of 1.0 x 10^{-4} M MG and an appropriate amount of heparin solution. The mixtures were diluted to the mark with water and mixed homogeneously. After reacted at 25 ºC for 30 min, the linear sweep voltammetric curves were recorded in the potential range of 0 to -800 mV. The peak currents at -0.618 V (vs. SCE) were measured with ip_0'' for the reagent blank and ip″ for the heparin-MG reaction solution. The difference of peak current ($\Delta ip'' = ip_0'' - ip''$) was used to detect the concentration of heparin.

RESULTS AND DISCUSSION

UV-Vis absorption spectra

The UV-Vis asorption spectra of MG and its mixture with heparin were recorded in the range of 300-700 nm. As shown in Figure 2, MG had three absorption peaks with wavelengths at 318 nm, 428 nm and 615 nm, respectively, in pH 1.5 B-R buffer solution (curve 1). After mixing heparin with MG, the absorbance at 615 nm decreased (curve 2 and 3) and no new absorption peaks appeared in the wavelength range. The more the heparin added, the greater the absorbance decreased, which indicated that MG had interacted with heparin in the mixed solution.

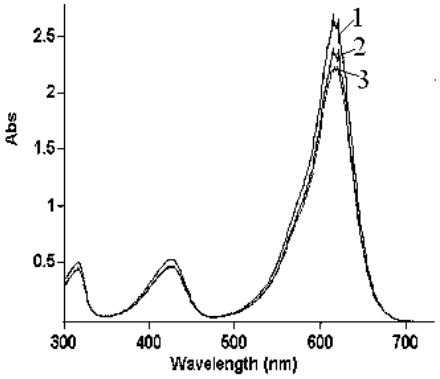

Figure 2. UV-Vis absorption spectra of MG interaction with heparin. 1. pH 1.5 B-R + 4.0 x 10^{-5} M MG; 2. 1 + 20.0 mg/L heparin; 3. 1 + 50.0 mg/L heparin.

Second order derivative linear sweep voltammogram

Figure 3 showed the typical second order derivative linear sweep voltammetric curves of the heparin-MG reaction system. Curve 1 was the voltammogram of B-R buffer solution without any voltammetric peaks. Curve 2 was the voltammogram of MG solution, it had a well-defined voltammetric reductive peak at -0.618 V (vs. SCE), which was due to the reduction of MG on the mercury electrode. Curve 3-5 were the voltammograms of the mixture of heparin with MG. Owing to the interaction of heparin with MG, the concentration of free MG in solution was decreased, so the reductive peak current decreased correspondingly. The more the heparin added, the higher the peak current decreased. The difference of peak current was proportional to the concentration of heparin, which could be further used for the detection of heparin.

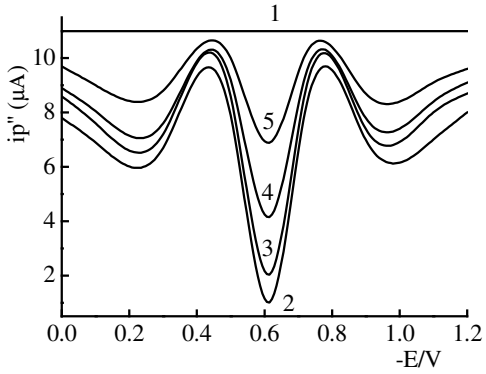

Figure 3. Second order derivative linear sweep voltammetric curves of MG-heparin interaction system. Reaction condition: 1. pH 1.5 B-R; 2. 1 + 4.0 x 10^{-5} M MG; 3. 2 + 2.0 mg/L heparin; 4. 2 + 5.0 mg/L heparin; 5. 2 + 10.0 mg/L heparin.

The relationships of reductive peak current with the scan rate were examined in the absence and presence of heparin and the plots were showed in Figure 4. It can be seen that regardless of whether heparin was present or not, the reductive peak current obtained from the MG and MG-heparin reaction solution showed linear dependence on the square root of the scan rate, which indicated that, the electrode process was all controlled by diffusion mass transport of the electroactive species to the mercury electrode in the absence and presence of heparin. The slope of the linear relation of ip″ *vs.* $v^{1/2}$ without heparin, 6.70 μA/$(V/s)^{1/2}$, was more than that with heparin, 3.67 μA/$(V/s)^{1/2}$, indicating that the diffusion coefficient of the free MG was larger than that of MG-heparin complex.

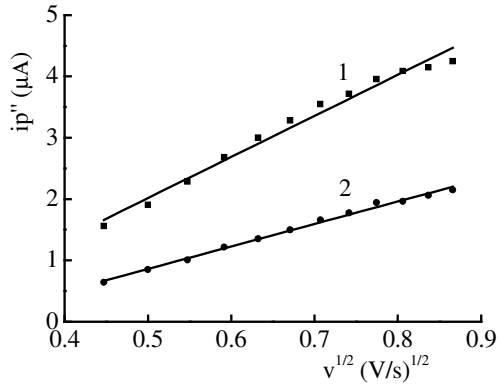

Figure 4. The dependence of the peak current on the square root of the scan rate. Condition: 1. pH 1.5 B-R + 4.0 x 10^{-5} M MG; 2. 1 + 10.0 mg/L heparin.

Optimal of general procedure

The acidity of buffer solution greatly influences the binding reaction of MG with heparin. The dependence of Δip″ on the pH of the B-R buffer solution was obtained in the pH range from 1.5 to 6.0. The results indicated that the value of Δip″ decreased as the pH increased, so pH 1.5 was

chosen for the assay. Heparin has linear and negatively charged sugar chains in its molecular structure, which consists of three *O*-sulfate groups, two *N*-sulfate groups and two carboxyl groups per tetrasaccharide unit. Owing to the presence of sulfate and carboxyl groups, it has a high anionic charge density. In the selected pH 1.5 B-R buffer solution, the *O*-sulfate and *N*-sulfate groups were completely dissociated and the whole heparin molecule was in negatively charged, while cationic dyes of MG species were in positively charged, therefore they were bound together by electrostatic forces to form a supermolecular complex, which resulted in the decrease of the free concentration of MG in solution and the decrease of the peak current of the heparin-MG reaction solution. And in a final 10 mL volume, 1.0 mL of the B-R buffer was suitable for interaction.

The effect of MG concentration on the difference of peak current was studied with 10.0 mg/L heparin. The results indicated that when the MG concentration was at 4.0×10^{-5} M, the difference of peak current reached the maximum. So a final MG concentration of 4.0×10^{-5} M was recommended in this paper.

After mixing heparin with MG, the binding reaction occurred rapidly. The difference of peak currents reached the maximum within 30 min and remained unchanged for at least 2 hours. Therefore, this system gave enough time for routine measurement.

The effect of reaction temperature on the difference of peak current was tested at 15, 25, 30, 35, and 40 ºC, respectively. The results showed that there were no obvious differences among them and 25 ºC was used throughout.

The scanning rate and the mercury drop standing time of the instrument for the assay were studied. The peak current reached its maximum at 650 mV/s, so 650 mV/s was selected as the scan rate for detection. The dropping time mercury standing time for the assay was also optimized and selected at 10 s.

Calibration curve and detection limit

Under the optimal conditions, a calibration curve for heparin was obtained between the difference of the peak current and heparin concentration in the range of 0.3 - 10.0 mg/L with the linear regression equation as $\Delta ip''(nA) = 360.19\ C + 178.88$ (mg/L) ($n = 15$ $\gamma = 0.998$). The detection limit for heparin was calculated as 0.28 mg/L with the equation of $LOD = 3S_0/S$, where 3 is the factor at the 99 % confidence level, S_0 is the standard deviation of the black measurement, and S is the slope of the calibration curve.

The comparison between the results of this paper and some reported papers for the determination of heparin was shown in Table 1. Although the sensitivity of this method was not higher than that of resonance Rayleigh scattering technique, it could be seen that this method had a preferable detection limit and linear range. Therefore this method was valuable for routine measurements.

Table 1. Comparison of linear range and limit of detection of this method with other reported methods.

Method	Linear range (mg/L)	LOD (mg/L)	Reference
Surface plasmon resonance sensor analysis	1.26-12.6	1.26	[10]
Spectrophotometry	0.6-6.0	0.173	[14]
Resonance Rayleigh scattering technique	0-0.4	0.00335	[16]
Linear sweep voltammetry	0.2-4.0	0.072	[23]
	2.0-10.0	1.34	[24]
	0.8-20.0	0.28	[25]
	0.1-8.0	0.50	[26]
This method	0.3-10.0	0.28	This paper

Effect of coexisting substances

The effect of foreign substances such as metal ions, amino acids, glucose, etc. on the determination of 10.0 mg/L heparin was tested. As shown in Table 2, most of the cations and amino acids had little influences on the determination of heparin. But some ionic surfactants such as cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), *β*-cyclodextrin (*β*-CD) and bovine hemoglobin (BHb) showed great influences on the interaction, which may be caused by the absorption of them on the surface of mercury electrode.

Table 2. Influence of coexisting substances on the determination of 10.0 mg/L heparin.

Coexisting substance	Concentration (mg/L)	Relative error Δip″ (%)	Coexisting substance	Concentration (mg/L)	Relative error Δip″ (%)
Glycine	10.0	0.48	Cu^{2+}	0.64	-2.96
L-Serine	10.0	2.75	Ba^{2+}	1.37	2.04
L-Arginine	10.0	-0.31	Ni^{2+}	0.59	4.06
L-Leucine	10.0	3.07	Mg^{2+}	0.24	-2.50
L-Valine	10.0	0.59	Hg^{2+}	2.00	1.68
L-Glutamine	10.0	0.59	Co^{2+}	0.59	2.86
Citric acid	10.0	-1.56	Pb^{2+}	2.07	-1.61
Glucose	10.0	2.37	CTAB	3.64	-67.01
DNA	10.0	-0.23	*β*-CD	1.14	-181.83
SDS	1.0 %	-7.88	BHb	64.50	-89.00

Sample determination and recovery test

The procedure for sample determination was as follows: a 1.00 mL portion of heparin sodium injection solution was transferred into a 1000 mL calibrated flask and was diluted to the mark with water. A 0.5 mL amount of the diluted solution was used in the general procedure. The results of determination and recovery test were listed in Table 3 and Table 4. It can be seen that this linear sweep voltammetric method was practical and reliable for the sample determination.

Table 3. Results of the determination of heparin in heparin sodium injection.

Sample No.	Single determination (IU/mL)					Average (IU/mL)	RSD (%)	Specified (IU/mL)
	1	2	3	4	5			
20031003	6318	6424	6682	6389	6602	6483	2.37	6250

Table 4. Recovery test of the determination of heparin in samples.

Sample No.	Original (mg/L)	Added (mg/L)	Single determination (mg/L)					Averaged (mg/L)	Recovery (%)
			1	2	3	4	5		
20031003	2.026	1.000	3.174	2.982	2.949	2.896	3.102	3.021	99.45

Determination of the stoichiometry of heparin-MG complex

To determine the composition of the supermolecular complex and the equilibrium constant, the following method was used [28], which was assumed that MG and heparin only formed a single complex heparin-mMG. The binding number (m) and the equilibrium constant (β_s) of the binding reaction could be deduced from the following equations:

$$\text{Heparin} + m\text{MG} \leftrightarrow \text{Heparin-}m\text{MG} \quad (1)$$

The equilibrium constant could be obtained:

$$\beta_s = \frac{[\text{Heparin-}m\text{MG}]}{[\text{Heparin}][\text{MG}]^m} \quad (2)$$

Because of:

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

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

$$[\text{Heparin}] + [\text{Heparin - mMG}] = C_{Heparin} \quad (5)$$

Therefore:

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

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

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

Where Δi was the difference of peak current in the presence and absence of heparin, Δi_{max} corresponded to the maximum of difference of peak current. $C_{Heparin}$, [Heparin], [Heparin-mMG] were corresponding to the total, free and bound concentration of heparin in the solution, respectively.

Figure 5 showed the relationship between ip″ and the concentration of MG as well as $\Delta ip''(ip_1'' - ip_2'')$ and the concentration of MG. Curve 1 was the relationship of ip″ with the concentration of MG., it can be seen that the peak current increased with the increase of the MG concentration. Curve 2 represented the change of ip″ after the addition of 10.0 mg/L heparin on varying the concentration of MG. The peak current was smaller than that of MG, which was due to the interaction of MG with heparin. Curve 3 showed the differences between $\Delta ip''(ip_1'' - ip_2'')$ and the concentration of MG. From the equation (7) the relation of $\log [\Delta i/(\Delta i_{max} - \Delta i)]$ with $\log [\text{MG}]$ was calculated with the regression equation as $\log [\Delta i/(\Delta i_{max} - \Delta i)] = 1.87 \log [\text{MG}] + 9.07$. From the intercept and the slope $m \approx 2$ and $\beta_s = 1.18 \times 10^9$ were deduced, which indicated that a stable 1:2 complex of heparin-2MG was formed under the selected conditions.

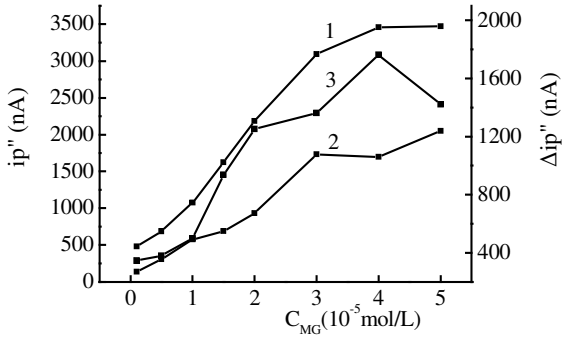

Figure 5. Relationship between ip″ and C_{MG} (1, 2), $\Delta ip''$ and C_{MG} (3). Condition: 1. $C_{Heparin} = 0$; 2. $C_{Heparin}$=10.0 mg/L; 3. $\Delta ip'' = ip_1'' - ip_2''$.

CONCLUSIONS

A linear sweep voltammetric method for heparin determination was established by using MG as bioprobe. Under the selected conditions the addition of heparin into MG solution can result in the decrease of reductive peak current of MG and further used for heparin detection. The proposed method was successfully applied to heparin sodium injection sample detection with satisfactory results.

AKNOWLEDGEMENTS

Grants from the Doctoral Foundation of QUST (0022125) and the Experimental Project of Shandong Provincal Education Department supported this project.

REFERENCES

1. He, Q.; Jiao, Q.C. *Biomedicine Assay*, Chemical Industrial Press, China: Beijing, **2003**, p 258.
2. Liu, S.P.; Xu, H.; Luo, H.Q. *Chin. J. Anal. Chem.* **2002**, 30, 712.
3. Jiao, Q.C.; Liu, Q.; Sun, C.; He, H. *Talanta* **1999**, 48, 1095.
4. Band, P.; Lukton, A. *Anal. Biochem.* **1982**, 120, 19.
5. Jiao, Q.C.; Liu, Q. *Spectrochim. Acta A* **1999**, 55, 1667.
6. Liu, S.P.; Luo, H.Q.; Li, N.B.; Liu, Z.F.; Zheng, W.X. *Anal. Chem.* **2001**, 73, 3907.
7. Mao, P.; Huang, X.L.; Li, C.Z. *Chin. J. Med. Lab. Sci.* **1995**, 18, 358.
8. Takagaki, K.; Munakata, H.; Nakamura, T.; Endo, M. *Bunseki Kagaku* **1995**, 44, 789.
9. Nemcova, I.; Rychlovsky, P.; Havelcova, M.; Brabcova M. *Anal. Chim. Acta* **1999**, 401, 223.
10. Gaus, K.; Hall, E.A.H.; *Biosens. Bioelectron* **1998**, 13, 1307.
11. Cheng, T.J.; Lin, T.M.; Wu, T.H.; Chang, H.C.; *Anal. Chim. Acta* **2001**, 432, 101.
12. Bakker, B. Fu, E., Yun, J. H., Yang, V. C., Meyerhoff, M. E. *Anal. Chem.* **1994**, 66, 2250.
13. Jiao, Q.C.; Liu, Q. *Anal. Lett.* **1998**, 31, 1311.
14. Sun, W.; Jiao, K.; Niu, X.L.; Lu, L.D. *Spectroscopy Spectral. Anal.* **2005**, 25, 1322.
15. Sun, W.; Ding, Y.Q.; Rao, J.; Jiao, K. *Spectroscopy Spectral. Anal.* **2006**, 26, 1322.
16. Liu, S.P.; Luo, H.Q.; Li, N.B.; Liu, Z.F.; Zheng, W.X. *Anal. Chem.* **2001**, 73, 3907.
17. Liu, S.P.; Luo, H.Q.; Li, N.B.; Liu, Z.F. *Chin. J. Chem.* **2003**, 21, 423.
18. Luo, H.Q.; Liu, S.P.; Li, N.B.; Liu, Z.F. *Anal. Chim. Acta* **2002**, 468, 275.
19. Ma, S.C.; Yang, V.C.; Fu, B.; Meyerhoff, M.E. *Anal. Chem.* **1993**, 65, 2078.
20. Yang, V.C.; Ma, S.C.; Liu, D.; Brown, R.B.; Meyerhoff, M.E. *ASAIO J.* **1993**, 39, M195.
21. Yun, J.H.; Ma, S.C.; Fu, B.; Yang, V.C.; Meyerhoff, M. E. *Electroanalysis* **1993**, 5, 719.
22. Yun, J.H.; Han, I.S.; Chang, L.C.; Ramamurthy, N.; Meyerhoff, M.E.; Yang, V. C. *Pharm. Sci. Technol. Today* **1999**, 2, 102.
23. Sun, W.; Jiao, K.; Han, J.Y.; Lu, L.D. *Anal. Lett.* **2005**, 38, 1137.
24. Sun, W.; Jiao, K.; Ding, Y.Q. *Acta Chim. Sin.* **2006**, 64, 397.
25. Sun, W.; Ding, Y.Q.; Wang, Q. X.; Jiao, K. *Electroanalysis* **2006**, 18, 1114.
26. Sun, W.; Ding, Y.Q.; Jiao, K. *J. Anal. Chem.* **2006**, 61, 359.
27. Hu, X.; Jiao, K.; Sun, W.; You, J.Y. *Electroanalysis* **2006**, 18, 613.
28. Wang, X.L.; Sun, W.; Jiao, K.; Zhang, K.T. *Chem. J. Chin. Univ.* **2004**, 25, 1448.