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Determination of Bovine Serum Albumin and Metronidazole by Electrochemical and Fluorescent Methods

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Poly(L-tyrosine)-graphene modified glassy carbon electrode (PLT-GN/GCE) was prepared by cyclic voltammetric method. Electrochemical method, fluorescence and UV-visible spectroscopy were applied to study the interaction of metronidazole and bovine serum albumin. Fluorescence spectrum of bovine serum albumin in the presence of metronidazole clearly shows that metronidazole acts as a quencher and it is a static quenching process. From the experimental data of electrochemical and fluorescent experiment, the binding constants were deduced to be 5.481×10^4 and 5.369×10^4 L mol^{-1}, respectively and the numbers of binding site were 1.422 and 1.468 at 25 °C, respectively. The calibration plots for bovine serum albumin with electrochemical method and for metronidazole with fluorescent method were in the range of 5×10^{-7} to 1×10^{-4} and 2×10^{-7} to 7×10^{-5} mol L^{-1}, respectively and the detection limits for bovine serum albumin and metronidazole were 1×10^{-7} and 5×10^{-8} mol L^{-1}, respectively. The method had been successfully applied to the determination of metronidazole and bovine serum albumin in samples with satisfactory results.

Keywords: Metronidazole, Bovine serum albumin, Graphene, L-Tyrosine, Determination.

INTRODUCTION

Graphene (GN) is the thinnest and the strongest material ever measured. As good charge carriers, graphene exhibits an astonishing electronic quality because of its special honeycombed flat network structure caused by sp^2-hybridized carbon atoms. Graphene also possesses a variety of exceptionally high crystal and electronic quality[1]. Some biological and chemical sensors combining graphene with other materials such as enzyme[2], amino acid[3,4] and nanoparticles[5,6], perform excellent properties to detect biomolecules and chemicals[7,8]. Tyrosine and multi-walled carbon nanotubes composite film modified electrode had been fabricated and used as electrochemical sensor for simultaneous determination of ascorbic acid, dopamine and uric acid[9]. Similarly, in present work, by virtue of electrochemical polymerization, poly(L-tyrosine)-graphene modified glassy carbon electrode (PLT-GN/GCE) was fabricated.

Proteins are essential biomacromolecules in cells and blood plasma, they are also fundamental substances to transfer various biological functions[10]. As a representative carrier protein, serum albumin is key to store and transport endogenous metabolites and exogenous drug molecules. Serum albumin has multiple lipophilic binding sites, which make itself to be a target molecule[11-13]. It is crucial to figure out the action mechanism and characters of serum albumin and drugs. As a kind of antiprotozoal and antibacterial drug, metronidazole (MET) is usually adopted in the preventive and therapeutic process of protozoal diseases such as trichomoniasis and giardiasis, in addition, it is used as veterinary antibiotics. More importantly, metronidazole lead to mutagenic effects on bacteria and does harm to nervous system[14]. Bovine serum albumin (BSA) is a typical serum albumin and is often applied to investigate drug action mechanism. If we understand the characterization of the interaction of bovine serum albumin and metronidazole and develop a stable, sensitive and selective sensor for bovine serum albumin and metronidazole, there will be considerable importance in clinical analysis and instruction of reagent dosage. Various methods for the determination of bovine serum albumin and metronidazole have been reported, such as electrochemistry[15-17], UV-visible spectrophotometry[18], high performance liquid chromatography[19], resonance light-scattering technique[20] *etc*. However, most of these methods have problems such as expensive instruments and complex operations. In present paper, the characterization of the interactions of bovine serum albumin and metronidazole were investigated by means of electrochemical and spectroscopic methods. Additionally, a novel analytical method was successfully applied to the determination of metronidazole and bovine serum albumin in samples with satisfactory results.

EXPERIMENTAL

FP-6500 fluorescence spectrofluorometer (Jasco, Japan); UV-3600 UV-VIS-NIR spectrophotometer (Shimadzu, Japan); BAS100B/W electrochemical workstation (BAS, USA). A conventional three-electrode system was used for all electrochemical experiments, which consisted of an Ag/AgCl as reference electrode, a platinum wire as auxiliary electrode, a bare GCE (3 mm in radius) or a modified GCE as working electrode. All pH measurements were performed using a PHS-3C digital pH meter (Shanghai Leici Device Works, Shanghai, China). A KQ-250B ultrasonic washer (Kunshan Ultrasonic Instrument Works, Kunshan, China) was used to wash the electrode.

L-Tyrosine, bovine serum albumin and metronidazole were purchased from Aladdin-Reagent Company (Shanghai, China). Graphene dispersion (2 mg mL^{-1}; dispersing agent: water; radius: 0.5-2 μm; thickness: 0.8-1.2 nm; single layer ratio: 80 %; purity: 98 %) was obtained from XFNANO Materials Technology Company (Nanjing, China). All other chemicals used were of analytical grade. Double distilled water was used throughout the experiments. The pH of the PBS solutions was adjusted with 0.1 M H_3PO_4 and 0.1 M NaOH. All experiments were carried out at room temperature (25 °C).

For fluorescent analysis, 2 mL PBS buffered solution, an appropriate amount of metronidazole and bovine serum albumin standard solution were added into a 10 mL volumetric flask, then diluted with double distilled water to 10 mL. The fluorescence spectrophotometer was set up with excitation wavelength at 282 nm and emission wavelength at 342 nm. The width of excitation and emission slit were both 10 nm. The solution was allowed to wait for 5 min for full interaction and then was scanned from 283 nm to 500 nm. For electrochemical analysis, the solution was poured into an electrolytic cell, then cyclic voltammetry (CV) or square wave voltammetry (SWV) were employed to study the electrochemical behavior. In addition, square wave voltammetry was run under electrochemical paraments with high potential of -0.05 V, low potential of -1.1 V, step E of 5 mV, amplitude of 100 mV, frequency of 5Hz and samples period of 16. All experiments above were conducted at 298 K.

RESULTS AND DISCUSSION

Preparation of PLT-GN/GCE: Prior to its modification, the bare glassy carbon electrode was polished with 0.05 μm α-alumina powder and rinsed with 1:1 HNO_3 solution, ethanol and doubly distilled water for 5 min successively. To prepare the solution for PLT-GN film, in a 10 mL volumetric flask, 2.5 mL graphene dispersion, 5.0 mL L-tyrosine saturated solution, 1 mL PBS (pH 5.0) were added to form an aqueous solution, diluted with double distilled water to 10 mL, followed by ultrasonication for 10 min, then poured into a electrolytic cell. Finally, the polymeric film was electropolymerized by cyclic sweeping from 2.3 to -1.0 V at a scan rate of 0.08 V s^{-1} for 8 cycles. After polymerization, the modified electrode was rinsed with doubly distilled water and air-dried. The PLT-GN/GCE was successfully prepared. For a comparison, poly(L-tyrosine) modified electrode (PLT/GCE) without graphene was fabricated under the same conditions.

Electrochemical behavior of metronidazole and MET-BSA at different electrodes: Fig. 1 shows the square wave voltammetry responses to metronidazole (a) and MET-BSA (b) in PBS (pH 8.0) at the bare GCE (a), PLT/GCE (b) and PLT-GN/GCE (c). Metronidazole and MET-BSA show broad cathodic peaks and slight peak currents at bare GCE. However, the bad characteristic response got improved after PLT or PLT-GN were modified on GCE, especially PLT-GN/GCE. And PLT-GN/GCE gave the cathodic peaks of metronidazole and MET-BSA at -0.570 and -0.575 V, respectively.

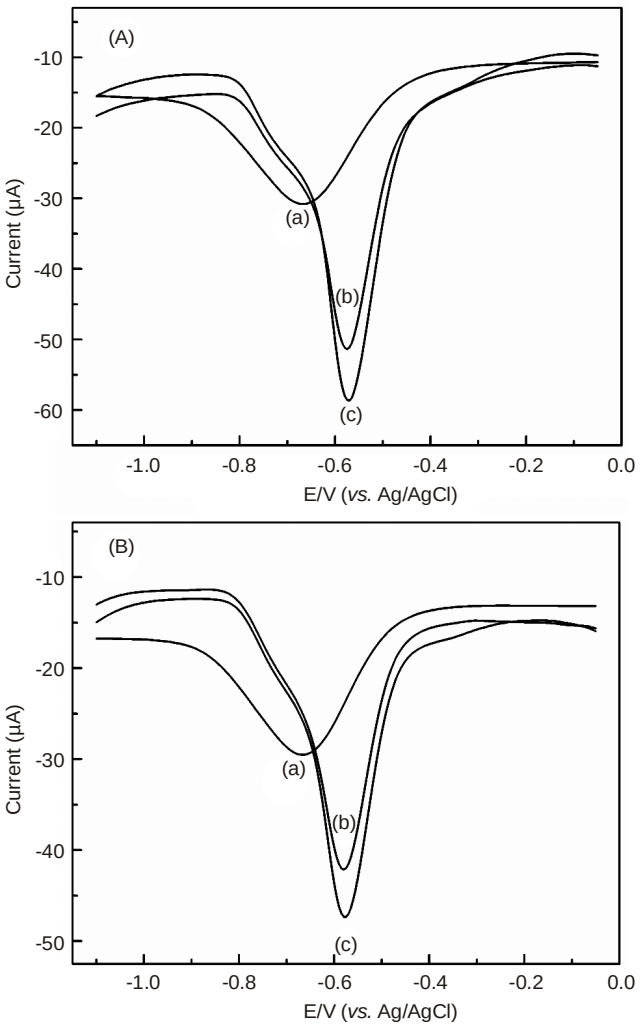

Fig. 1. Square wave voltammetrys of MET (A) and MET-BSA (B) at bare GCE (a), PLT/GCE (b) and PLT-GN/GCE (c) $C_{MET} = 5 \times 10^{-5}$ mol L^{-1}; $C_{BSA} = 2 \times 10^{-5}$ mol L^{-1}

Interactions of bovine serum albumin and metronidazole: Fig. 2 describes the square wave voltammetry responses of metronidazole (A) and MET-BSA (B) on PLT-GN/GCE in PBS (pH 8.0). The peak current of metronidazole obviously declined after bovine serum albumin being added, suggesting some interactions existed between bovine serum albumin and metronidazole.

Protein usually launches strong endogenous fluorescence because of their tryptophan and tyrosine residues. Fig. 3 depicts the fluorescence spectra of the interactions of bovine serum albumin and metronidazole. Under the optimal acidity condition of pH 6.5, bovine serum albumin produced maximum

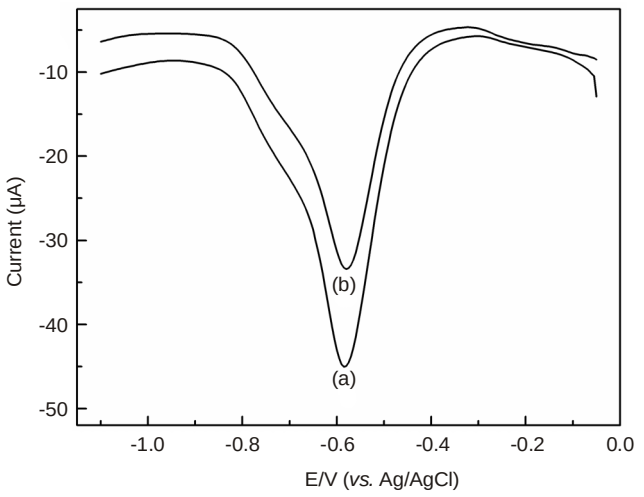

Fig. 2. SWVs of MET (a) and MET-BSA (b) at PLT-GN/GCE $C_{MET} = 5 \times 10^{-5}$ mol L^{-1}; $C_{BSA} = 2 \times 10^{-5}$ mol L^{-1}

fluorescence emission peak at 342 nm. Keeping the concentration of bovine serum albumin changeless and with the concentration of metronidazole increasing, the endogenous fluorescence intensity of bovine serum albumin gradually declined. Furthermore, a slight red-shift of fluorescence peak occured, it revealed that bovine serum albumin and metronidazole interacted with each other.

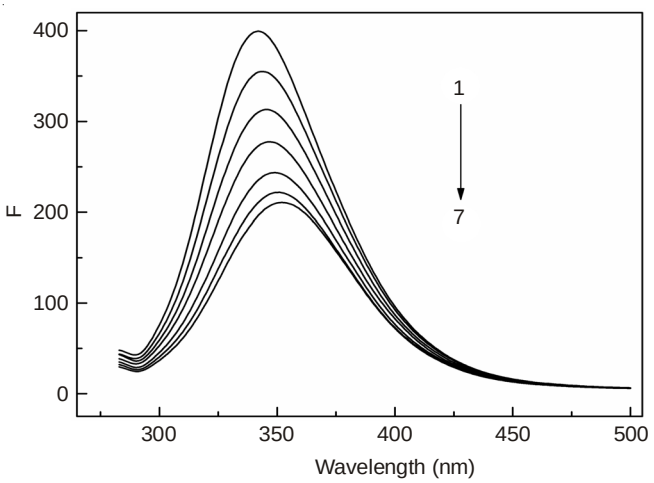

Fig. 3. Fluorescence quenching of bovine serum albumin in the presence of metronidazole $C_{BSA} = 2 \times 10^{-6}$ mol L^{-1}; C_{MET}(1-7) = 0, 1×10^{-5}, 2×10^{-5}, 3×10^{-5}, 4×10^{-5}, 5×10^{-5} and 6.50×10^{-5} mol L^{-1}

UV-visible absorption measurement is a simple but effective method in detecting complex formation. Fig. 4 is UV-visible absorption spectra of metronidazole (a), bovine serum albumin (b) and their mixed solution (c). In the individual measurement of ultraviolet light, metronidazole has a strong absorption response at 318 nm and bovine serum albumin at 278 nm in PBS (pH 6.5). In the mixture, however, two absorbance peaks appeared at 318 and 283 nm. Compared with the individual absorbance value of metronidazole and bovine serum albumin, the values of the two absorption peak in mixed solution were not additivity. This finding also elucidated that metronidazole and bovine serum albumin proceeded with some interactions[21]. It presumably due to the binding of metronidazole and bovine serum albumin and a new complex formation

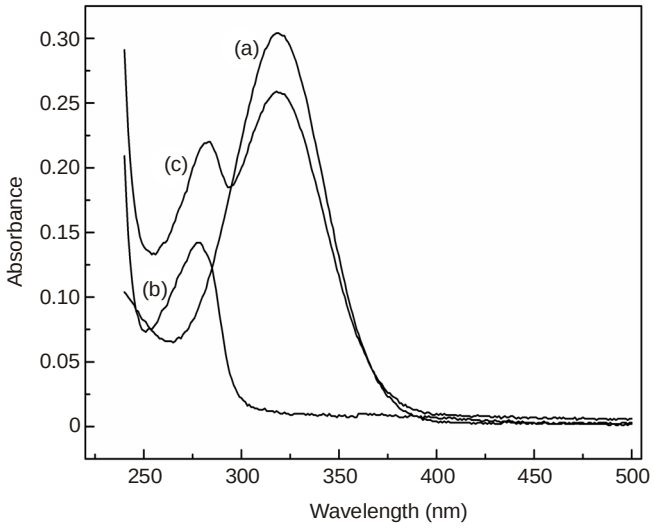

Fig. 4. UV-visible absorption spectra of metronidazole (a), bovine serum albumin (b) and their mixed solution(c) $C_{MET} = 2.50 \times 10^{-5}$ mol L^{-1}; $C_{BSA} = 2 \times 10^{-6}$ mol L^{-1}

being synthesized. As such, the absorbance of metronidazole declined, that of bovine serum albumin increased.

Fluorescence quenching mechanism of bovine serum albumin and metronidazole: The Stern-Volmer equation: $F_o/F = 1 + K_q\tau_o C_{(Q)} = 1 + K_{sv}C_{(Q)}$, was utilized to explore the quenching mechanism qualitatively, herein, F_o and F are fluorescence intensity, with and without quenching reagent, respectively, τ_o is the average fluorescence lifetime (about 10^{-8} s) of biological macromolecules, K_q is the quenching rate constant, K_{sv} is the Stern-Volmer quenching constant and $C_{(Q)}$ is the concentration of quencher[22].

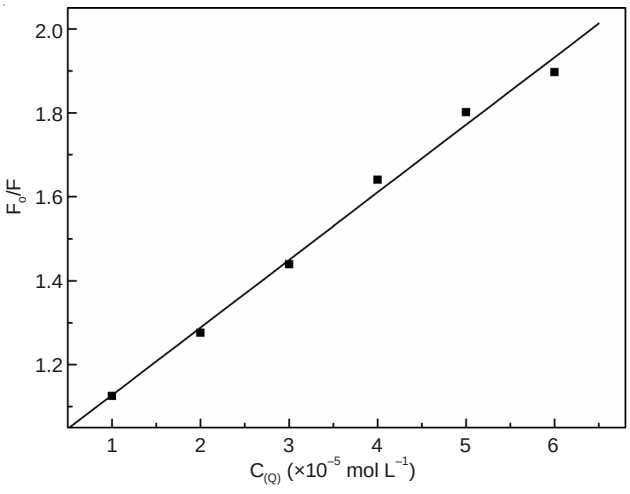

Fig. 5. Stern-Volmer plot of bovine serum albumin with metronidazole $C_{BSA} = 2 \times 10^{-6}$ mol L^{-1}; $C_{MET} = 1 \times 10^{-5}$, 2×10^{-5}, 3×10^{-5}, 4×10^{-5}, 5×10^{-5} and 6.50×10^{-5} mol L^{-1}; pH 6.5

From Fig. 5, an equation was obtained, $F_0/F = 0.9664 + 1.610 \times 10^4\ C_{(Q)}$, r = 0.9964, where K_{sv} was 1.610×10^4 L mol^{-1} and K_q was calculated as 3.61×10^{12} L mol^{-1} s^{-1} that was much higher than 2×10^{10} L mol^{-1} s^{-1} (the maximum scatter collision quenching constant of diverse kinds of quenchers for biopolymers fluorescence), it further confirmed that the effect observed is static quenching[22], indicating the existence of static quenching.

The fluorescence intensity follows the relationships which can be described in the equations shown as log [(F_0-F)/F] = log K + n log $C_{(Q)}$, where K is the binding constant of metronidazole and bovine serum albumin and n is the number of binding sites[23]. Fig. 6 exhibits a plot of log [(F_0-F)/F] = 4.746 + 1.128 log $C_{(Q)}$; r = 0.9981, then K was calculated to be 5.369×10^4 L mol^{-1} and n was calculated as 1.468.

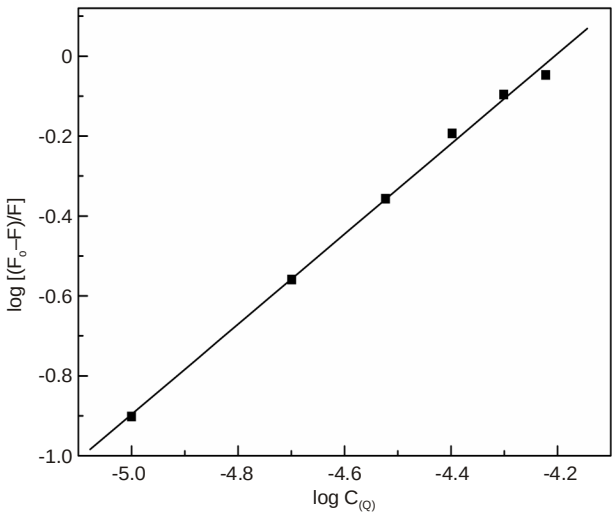

Fig. 6. Plot of log [(F_0-F)/F] and log $C_{(Q)}$ $C_{BSA} = 2 \times 10^{-6}$ mol L^{-1}; $C_{MET} = 1 \times 10^{-5}$, 2×10^{-5}, 3×10^{-5}, 4×10^{-5}, 5×10^{-5} and 6.50×10^{-5} mol L^{-1}; pH 6.5

Electrochemical mechanism of bovine serum albumin and metronidazole: In most of the cases, the electrolyte pH is an important parameter to the electrochemical reaction. Fig. 7 demonstrates the square wave voltammetry curves of metronidazole (A) and MET-BSA (B) from pH 4.5 to 9, the cathodic peak potentials shift in negative direction with an increase of pH, showing that protons have taken part in electrode reactions. The cathodic peak currents of metronidazole and MET-BSA both increase up to pH 6.5 and then decrease, however, the current difference between metronidazole and MET-BSA in same pH reaches maximum at pH 8.0. Table-1 gives their relationship between peak potential and pH.

To investigate the effect of scan rates on the response of metronidazole (A) and MET-BSA (B) in PBS (pH 6.5), respectively, cyclic voltammetry (CV) were performed at various potential scan rates. As presented in Fig. 8, the cathodic peak potential is shifted to negative region with an increasing scan

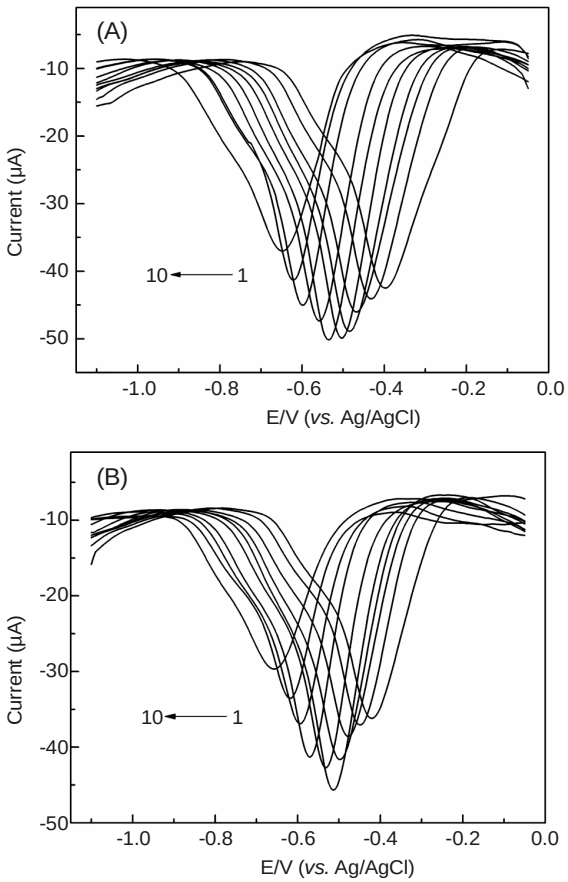

Fig. 7. Square wave voltammetrys of metronidazole (A), MET-BSA (B) with different pH from 1 to 10:pH = 4.5, 5, 5.5, 6.5, 6.5, 7.0, 7.5, 8, 8.5, 9 $C_{MET} = 5 \times 10^{-5}$ mol L^{-1}; $C_{BSA} = 2 \times 10^{-5}$ mol L^{-1}

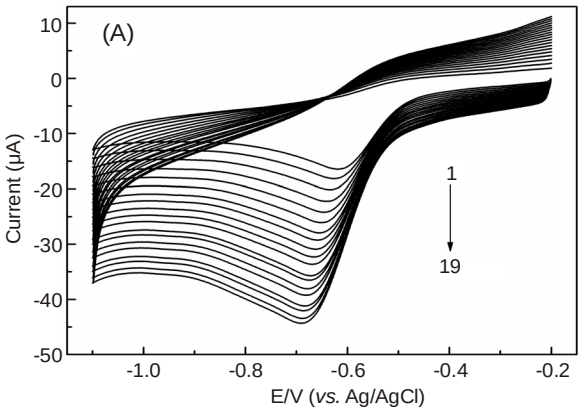

TABLE-1
RELATIONSHIP BETWEEN PEAK POTENTIAL AND pH OF METRONIDAZOLE AND MET-BSA

Analyte	pH range	Linear regression equation (E:V)	Correlation coefficient
Metronidazole	4.5-9.0	E = -0.1253 – 0.06018 pH	0.9923
MET-BSA		E = -0.1966 – 0.04976 pH	0.9908

TABLE-2
RELATIONSHIP BETWEEN SCAN RATE AND PEAK CURRENT, PEAK POTENTIAL

Analyte	Relationship between scan rate and peak current			Relationship between scan rate and peak potential		
	Scan rate range (V s^{-1})	Linear regression equation (I: μA; v: V s^{-1})	Correlation coefficient	Scan rate range (V s^{-1})	Linear regression equation (E: V; v: V s^{-1})	Correlation coefficient
Metronidazole	0.04-0.40	log I = 1.711 + 0.5142 log v	0.9942	0.04-0.30	E = -0.7087 – 0.03019 ln v	0.9961
MET-BSA		log I = 1.731 + 0.6892 log v	0.9967		E = -0.7313 – 0.03228 ln v	0.9927

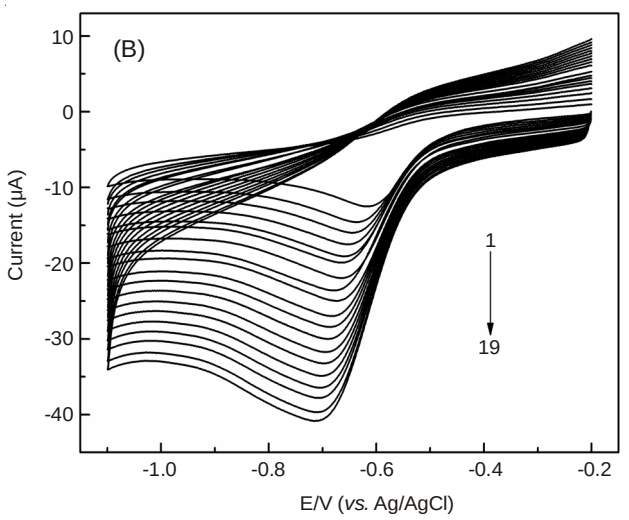

Fig. 8. Cyclic voltammetrys for metronidazole (A), MET-BSA (B) with different scan rate from 1 to 19: 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, 0.20, 0.22, 0.24, 0.26, 0.28, 0.30, 0.32, 0.34, 0.36, 0.38, 0.40 V s^{-1} $C_{MET} = 5 \times 10^{-5}$ mol L^{-1}; $C_{BSA} = 2 \times 10^{-5}$ mol L^{-1}

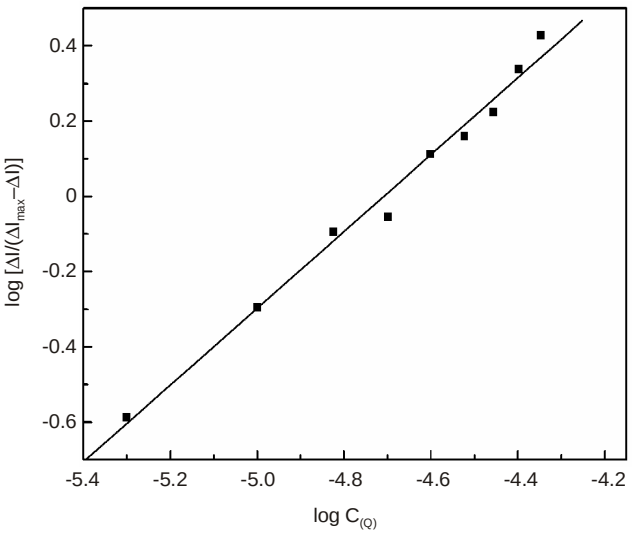

Fig. 9. Plot of log $[\Delta I/(\Delta I_{max}-\Delta I)]$ and log $C_{(Q)}$ $C_{BSA} = 2 \times 10^{-5}$ mol L^{-1}; $C_{MET} = 5 \times 10^{-6}$, 1×10^{-5}, 1.50×10^{-5}, 2×10^{-5}, 2.50×10^{-5}, 3×10^{-5}, 3.50×10^{-5}, 4×10^{-5} and 4.50×10^{-5} mol L^{-1}; pH 6.5

rate. Furthermore, the cathodic peak current is elevated in the meantime. Table-2 lists the relationships between scan rate and peak current, peak potential.

In the linear relationship with the regression equations of the logarithm of the peak current *versus* the logarithm of scan rate, the slope value of metronidazole approximates 0.5, whereas metronidazole-BSA is between 0.5 and 1, which clearly illustrates that the reduction of metronidazole was diffusion controlled process and metronidazole-BSA was simultaneously controlled by diffusion and adsorption. According to literature[24], the real reaction electron number of metronidazole and metronidazole-BSA were estimated to be 1.702 and 1.562, respectively. Compared with the value of metronidazole without bovine serum albumin, the real reaction electron number of metronidazole-BSA decreased and it could be ascribed to the combination of metronidazole and bovine serum albumin.

Supposing that metronidazole and bovine serum albumin generated a new simple compound, denoted as BSA-m.MET, the binding constant β of metronidazole and bovine serum albumin, the number of binding sites m could be deduced by right of formula $\log [\Delta I/(\Delta I_{max}-\Delta I)] = \log \beta + m \log C_{(Q)}$, wherein, ΔI is the difference between peak currents with and without quenching reagent, ΔI_{max} is the maximum among ΔI, $C_{(Q)}$ is the concentration of quencher[25]. Fig. 9 gives a plot of $\log [\Delta I/(\Delta I_{max}-\Delta I)] = 4.762 + 1.012 \log C_{(Q)}$, r = 0.9929, then β was calculated to be 5.481×10^4 L mol^{-1} and m was calculated as 1.412. The outcomes matched those in fluorometric analysis.

Linear regression equation of determination of metronidazole and bovine serum albumin: Metronidazole was measured by fluorescence spectroscopy with a fixed concentration of bovine serum albumin for 2×10^{-6} mol L^{-1} and bovine serum albumin was determined using square wave voltammetry with a quantified concentration of metronidazole for 5×10^{-5} mol L^{-1}. Table-3 shows the results based on experiments.

Reproducibility and stability: A mixed solution containing 5×10^{-5} mol L^{-1} metronidazole and 2×10^{-5} mol L^{-1} bovine serum albumin was measured by square wave voltammetry and another mixture solution consisted of 2×10^{-5} mol L^{-1} metronidazole and 2×10^{-6} mol L^{-1} bovine serum albumin was fluorescence spectroscopy detected. After twenty parallel experiments, the relative standard deviations (RSD) in square wave voltammetry and fluorescent method were 3.2 % and 2.7 %, respectively, which suggested remarkable reproducibility. The modified electrode was stored in humidity environment at room temperature for 15 days, it could retain 93 % of its original response and the similar shape of the original curves, demonstrating an acceptable storage stability of the PLT-GN/GCE.

Interference: The tolerance limit was taken as the maximum concentration of the foreign substances that caused an approximately ± 5 % relative error in the determination. A mixed solution consisted of 5×10^{-5} mol L^{-1} metronidazole and 2×10^{-5} mol L^{-1} bovine serum albumin was square wave voltammetry measured, starch, L-histidine, L-leucine, L-arginine, L-glutamic acid, L-serine, L-aspartate, Al^{3+}, Na^+, K^+, Mg^{2+}, Ca^{2+} (≥ 1 mg, not to ceiling); Cr^{6+} (0.3 mg); Fe^{2+} (0.4 mg); Ag^+, Cu^{2+} (0.5 mg) did not interfere with the square wave voltammetry responses. Another mixed solution containing 2×10^{-5} mol L^{-1} metronidazole and 2×10^{-6} mol L^{-1} bovine serum albumin was fluorescence spectroscopy determined,

TABLE-3
LINEAR REGRESSION EQUATION OF DETERMINATION OF METRONIDAZOLE AND BOVINE SERUM ALBUMIN

Analyte	pH	Linear range (mol L^{-1})	Linear regression equation (I: μA; C: mol L^{-1})	Correlation coefficient	Detection limit (mol L^{-1})
Metronidazole	6.5	2.00×10^{-7}-7.00×10^{-5}	$\Delta F = 1.587 + 3.062 \times 10^6$ C	0.9921	5.00×10^{-8}
Bovine serum albumin	8.0	5.00×10^{-7}-1.00×10^{-5}	$\Delta I = 1.650 + 1.051 \times 10^6$ C	0.9924	1.00×10^{-7}
		1.00×10^{-5}-1.00×10^{-4}	$\Delta I = 11.04 + 1.456 \times 10^5$ C	0.9908	

TABLE-4
ANALYTICAL RESULTS OF TABLET AND ARTIFICIAL SAMPLE (n = 6)

Sample	Analyte	Recommended (mol L^{-1})	Measured value (mol L^{-1})	RSD (%)	Added (mol L^{-1})	Recovery (%)
Tablet	Metronidazole	2.50×10^{-5}	2.39×10^{-5}	4.31	2.50×10^{-5}	102
Artificial sample	Bovine serum albumin	5.00×10^{-6}	5.08×10^{-6}	3.71	5.00×10^{-6}	105

starch, L-histidine, L-leucine, L-arginine, L-glutamic acid, L-serine, L-aspartate, Na^+, K^+, Mg^{2+}, Ca^{2+}(≥ 1 mg, not to ceiling); Cr^{6+} (2 μg); Cu^{2+}, Ag^+ (3 μg); Fe^{2+} (20 μg); Al^{3+} (30 μg) had no influence on fluorescence intensity.

Sample analysis: Table-4 lists the determination results of metronidazole in tablet and bovine serum albumin in artificial sample with the method above.

Conclusions

In present work, electrochemistry, fluorescence spectroscopy and UV spectra were applied to investigate the interplay of bovine serum albumin and metronidazole, confirming a binding effect exists between bovine serum albumin and metronidazole. On the basis of their interaction, a novel analytical method was established and successfully applied to the determination of metronidazole and bovine serum albumin. In addition, PLT-GN/GCE could be a promising candidate applicable for a wide range of electrochemical sensor and biosensor applications.

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