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Studies on electrochemical oxidation of azithromycin and its interaction with bovine serum albumin

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Abstract

A novel nanoparticle film modified electrode has been constructed using a glassy carbon electrode (GCE) coated with a carbon nanotube-dihexadecylphosphate (DHP) film. This modified electrode exhibits an enhanced effectiveness for the oxidation of azithromycin. A method is also described for the evaluation of azithromycin–bovine serum albumin (BSA) interaction. The electrochemical behavior of azithromycin as well as its interaction with BSA at this nanoparticle film electrode has been investigated by cyclic voltammetry, linear sweep voltammetry, differential pulse voltammetry and chronocoulometry. The binding number and association constant between azithromycin and bovine serum albumin have been obtained.
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Keywords: Bovine serum albumin; Azithromycin; Multiwall carbon nanotube; Electrochemistry; Modified electrode

1. Introduction

Azithromycin is a 15-cyclic lactone antibiotic (Scheme 1) and semisynthetic erythromycin derivative. It exhibits some advantages over erythromycin including better oral bioavailability, higher tissue concentrations, and fewer side effects. Azithromycin is active against some Gram-positive and Gram-negative microorganisms and plays its role by binding to the 50*S* subunit of the bacterial ribosome. This action influences the microbial protein synthesis by preventing transpeptidation and translocation processes. Thus, azithromycin has been used to treat respiratory infections, skin and soft-tissue infections and some sexually transmitted diseases [1].

Azithromycin has been analyzed using a microbiological method [2] and a high performance liquid chromatography procedure [3,4]. Only two electrochemical methods have been described in the literature. One is based on the reduction of azithromycin in a strongly basic media at a mercury electrode [5], however, a strong basic media is not suitable to study the interaction between drug and protein. The other is a voltammetric assay of a pharmaceutical dose of azithromycin at a glassy carbon electrode (GCE) [6] but in this case, the interaction of azithromycin and BSA could not be observed clearly because of its low sensitivity on the bare GCE. Thus, electrochemical methods for the highly sensitive detection of azithromycin and the characterization of azithromycin and bovine serum albumin (BSA) interaction is seldom found in literature.

In this paper, the preparation method for a multi-wall carbon nanotube (MWNTs) modified electrode is described. The use of a MWNTs-modified glassy carbon electrode greatly enhances the electrochemical response of azithromycin and the electrode exhibited the advantages of high reproducibility and reusability. Based on these advantages, the interaction of azithromycin with BSA was investigated. Electrochemical parameters of azithromycin were reported and its oxidative mechanism was also proposed. Based on the decrease of peak current of azithromycin when BSA was mixed, the binding number and association constant between BSA and azithromycin were determined by electrochemical method.

2. Experimental

2.1. Reagents

Azithromycin dihydrate was purchased from National Institute for the Control of Pharmaceutical and Biological

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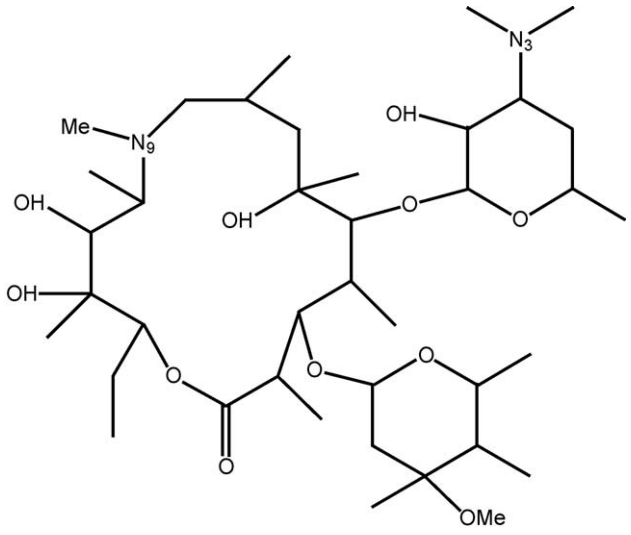


Scheme 1. Structural formula of azithromycin.

Products (Beijing, China). Its stock solution (7.6×10^{-3} M) was prepared with methanol and stored in a refrigerator at 4 °C. Bovine serum albumin, purchased from Shanghai Boao Biochemistry Science and Technology, was prepared by directly dissolving in 0.06 M phosphate buffer (pH 7.0) and stored in a refrigerator at 4 °C. The phosphate solution containing 0.06 M Na_2HPO_4 and 0.06 M NaH_2PO_4 was used as buffer. The value of pH was adjusted by adding appropriate NaOH and HCl solution. Dihexadecylphosphate (DHP) was purchased from Fluka. All other chemicals were analytical grade and all the solutions were prepared from doubly distilled water.

The multi-wall carbon nanotubes (obtained from the Institute of nanometer materials, Central China Normal University, China) were synthesized by a catalytic pyrolysis method and purified with concentrated HNO_3 [7].

2.2. Apparatus

All electrochemical measurements were performed with a computer controlled Model CHI830A electrochemical analyzer (Chenghua Instrument, Shanghai, China). A three-electrode cell was employed with a platinum wire as counter electrode, a saturated calomel electrode (SCE) as reference electrode and a multiwall carbon nanotube modified glassy electrode as working electrode. All potentials were quoted with respect to SCE. Tu-1901 UV–VIS Spectrophotometer (Beijing Purkinje General Instrument, China) was used for spectrophotometric investigation.

2.3. Preparation of the MWNTs-modified electrode

A glassy carbon electrode of 3 mm diameter was used. It was polished with 0.3 μm and 0.05 μm alumna slurry in sequence, then sonicated in ethanol and doubly distilled water, respectively. Multi-wall carbon nanotube (MWNTs, 5 mg) and dihexadecylphosphate (DHP, 5 mg) were added into 5 ml doubly distilled water. A well-dispersed of MWNTs-DHP solution was obtained by ultrasonication. The GCE was coated by dropping 5 μl dispersion of MWNTs and dried under an infrared lamp. The freshly prepared MWNTs-modified electrodes were activated in 0.06 M phosphate buffer (pH 7.0) by cyclic scans from −0.2 to 1.2 V successively and continued until the shape of the cyclic curve no longer changed. After each measurement, the electrode was refreshed by potential scans mentioned above.

2.4. Procedure

The supporting electrolyte for electrochemical measurements performed in this study was 0.06 M phosphate buffer (pH 7.0) and the scanning potential values were always from −0.2 to 1.2 V. In the experiments to determine binding number and binding constant between azithromycin and BSA, a two-step procedure was developed to avoid the competitive adsorption between azithromycin and BSA at the electrode surface. First, the electrode was immersed in a solution containing azithromycin for 2 min. The electrode was withdrawn from this bath and washed to minimize the amount of adhering solution by phosphate buffer (pH 7.0). Then the electrode was dipped into the cell containing phosphate buffer and the oxidation peak current of azithromycin was obtained in the absence of BSA.

To obtain the peak current of azithromycin in the presence of BSA, the azithromycin is initially adsorbed onto the electrode surface for 2 min. After that the electrode is placed into a stirred BSA solution (pH 7.0) for 2 min to assure complete electrode/solution interaction. The electrode was withdrawn from this bath and washed thoroughly by phosphate buffer (pH 7.0). Finally, the electrode was dipped into a fresh phosphate buffer and the oxidation peak current of azithromycin was recorded.

3. Results and discussion

3.1. Electrochemical behavior of azithromycin

Fig. 1 shows the cyclic voltammograms of azithromycin on a MWNTs-modified GCE (a, b) and a bare GCE (c) with a peak potential of +0.84 V. During the experiments both the modified GCE and the bare GCE were activated in the same scan potential range. To prove the enhancement effect of MWNTs for the oxidation of azithromycin, a comparison experiment was also performed at the activated DHP-modified GCE and no increase in the peak current is observed. Higher peak current (Fig. 1a) and larger oxidation charge amount Q (Fig. 2) were achieved with longer accumulation time t_{acc}, which revealed that azithromycin may be preconcentrated onto the modified electrode surface. The enhancement effect of MWNTs may contribute to the strong adsorption of azithromycin onto the MWNTs-modified

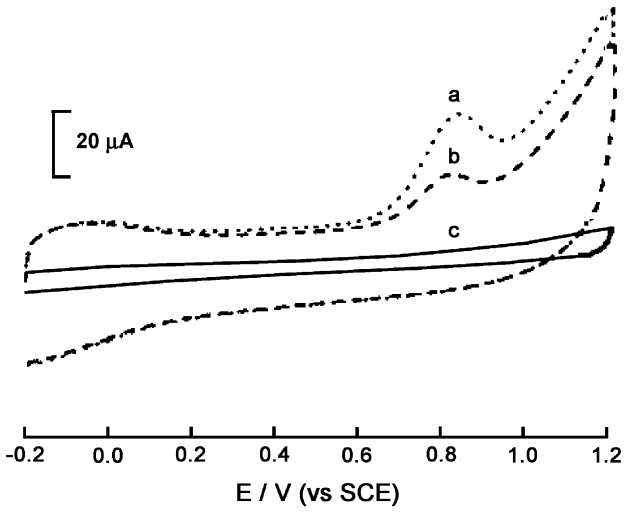


Fig. 1. Cyclic voltammograms of 1.53×10^{-5} M azithromycin at bare GCE with $t_{acc} = 90$ s (c); MWNTs-modified GCE with $t_{acc} = 30$ s (b) and $t_{acc} = 90$ s (a) in 0.06 M phosphate buffer (pH 7.0). The potential scan rate is 100 mV s^{-1}.

electrode surface. The reproducibility and reusability of MWNT-modified GCE for azithromycin were investigated. The relative standard deviation obtained from 10 replicate measurements of the regeneration, rinsing, preconcentration and determination procedures was 1.32%.

The effect of potential scan rate on the peak current of azithromycin was evaluated by linear sweep voltammetry. The result showed that the peak current was proportional to the square root of the scan rate over the 10 to 200 mV s^{-1} range, which indicated that electrode process is diffusion controlled. As the scan rate increased from 200 to 500 mV s^{-1}, the peak current was proportional to the scan rate, suggesting that an adsorption-controlled process is involved.

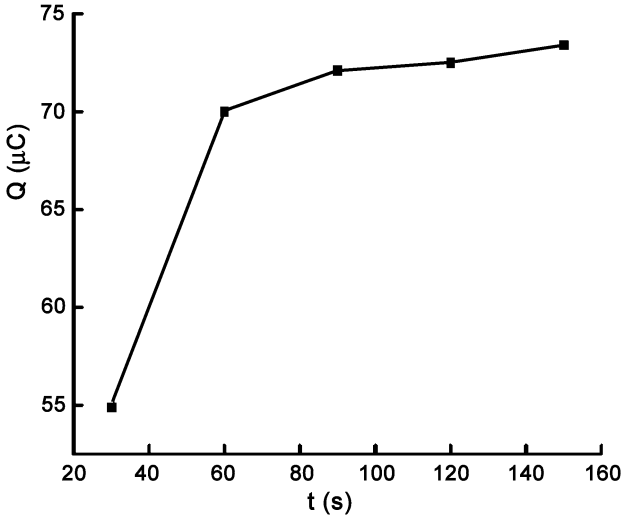


Fig. 2. Dependence of Q on the accumulation time, t_{acc}, in 0.06 M phosphate buffer (pH 7.0). The potential scan rate is 100 mV s^{-1}.

The electrooxidation of azithromycin was also studied over pH range of 4.5–11 in phosphate buffer by differential pulse voltammetry. The peak definition was best when using 50 mV pulse amplitude, 30 ms pulse width and 20 mV s^{-1} scan rate. The experimental results showed that the wave was well developed at pH>5. The peak current reaches the maximum at pH 7.0 (inset in Fig. 3), and the pH value is similar to physiological pH, thus, this pH value was adopted in the following experiment. At pH>9, the E_p is pH-independent (Fig. 3). The linear regions of the E_p vs. pH plot intersect at about 8.5, corresponding to the pK_a values of the dimethylamino group on the sugar moiety of azithromycin. The anodic peak potential of azithromycin shifted linearly towards negative values when the pH is increased by 0.121 V/pH. Based on the Eq. (1), the number of hydrogen ions taking part in the electrode reaction was estimated as 1.

In aqueous solution, for the reaction

$$\mathrm{Ox} + ne + q\mathrm{H}^{+} = \mathrm{Red}$$

The $E_{1/2}$ value of the reaction is estimated by the following equations [8] and [9]:

$$E_{1/2} = E^{0'} + 2.303(qRT/\alpha_a nF)\log H^{+} \quad (1)$$

where $E_{1/2}$ is the half wave potential, $E^{0'}$ is the formal potential, α_a is the transfer coefficient, q and n are the number of the protons and electrons involved in the reaction, respectively. The value of $n\alpha_a$ was determined as 0.48 (see below). Other terms have their usual meanings.

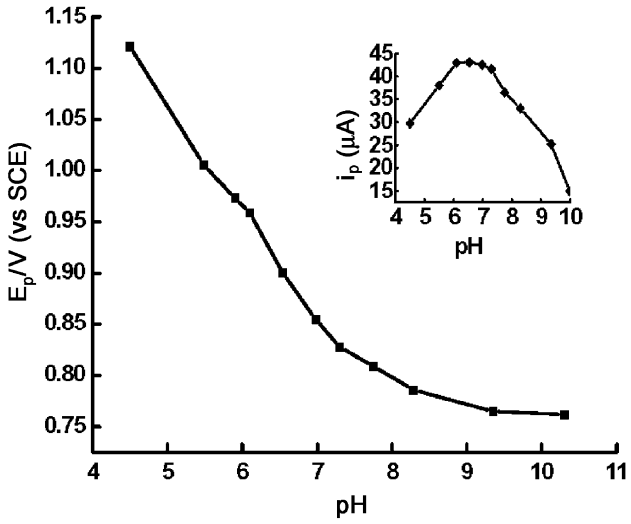


Fig. 3. Effect of pH on peak potential for 1.53×10^{-5} M azithromycin in phosphate buffer by means of differential pulse voltammetry at MWNTs-modified electrode. Inset: variation of peak current, i_p (μA), with different pH.

3.2. Adsorption of azithromycin measured by single-potential step chronocoulometry

The chronocoulometric method was applied to determine the diffusion coefficient D and Q_{ads} of azithromycin on the MWNTs-modified electrode, according to the formula given by Anson [10]

$$Q = 2nFAC(Dt)^{1/2}/\pi^{1/2} + Q_{dl} + Q_{ads} \quad (2)$$

Q_{dl} is double-layer charge and Q_{ads} is the faradaic charge due to the oxidation of adsorbed azithromycin. The plot of Q vs. $t^{1/2}$ should be linear. The values of D and Q_{ads} can be obtained from the slope and intercept. In our experiment the double layer charge Q_{dl} in 0.06 M phosphate (pH 7.0) supporting electrolyte was 11.53 μC (Fig. 4a). When 3.82×10^{-5} M azithromycin was added to the solution, chronocoulometry was carried out with different accumulation time. The experiment results showed that the intercept and slope of the plot Q vs. $t^{1/2}$ remained the same with the accumulation time when the t_{acc} was more than 1.5 min. This is consistent with the results in Fig. 2 and indicated that azithromycin was saturate on the electrode surface when the t_{acc} was 1.5 min. The values of the slope and Q_{ads} in Fig. 4c are 3.3×10^{-4} and 7.3×10^{-5} C, respectively. The surface concentration corresponding to a monolayer at the electrode surface, Γ^s, can be obtained by the Eq. (3).

$$\Gamma^s = Q_{ads}/nFA \quad (3)$$

The active area of the electrode surface, A, was determined in 5 mM $K_4[Fe(CN)_6]$ solution as 7.2×10^{-2} cm^2. Thus, the diffusion coefficient D and Γ^s were calculated to be 1.25×10^{-6} cm^2 s^{-1} and 1.08×10^{-8} mol cm^{-2}, respectively.

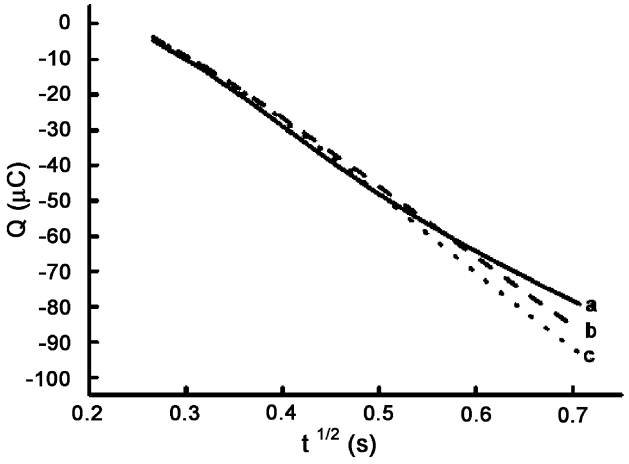


Fig. 4. The plot of $Q \sim t^{1/2}$ for a single-potential step chronocoulometric experiment in phosphate buffer (pH 7.0) in absence of azithromycin (a) and in the presence of 3.82×10^{-5} M azithromycin with $t_{acc} = 30$ s (b) and $t_{acc} = 120$ s (c): initial, −0.2 V; potential stepped to 1.2 V.

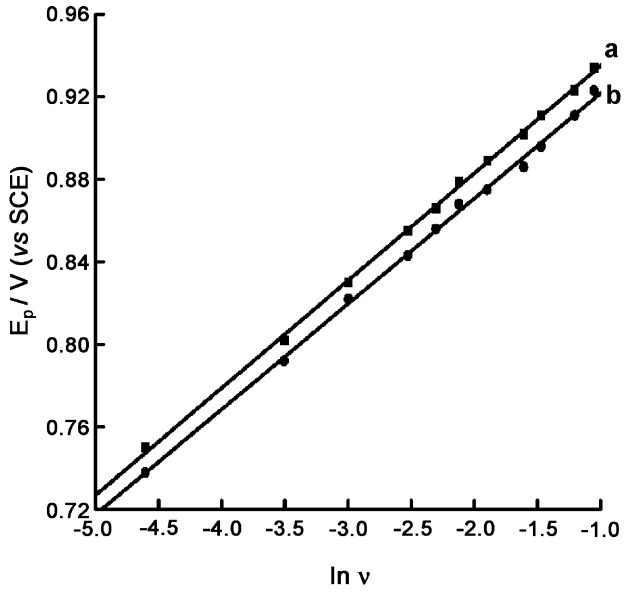


Fig. 5. Dependence of peak potential E_p on the potential scan rate ln$v$: 3.82×10^{-5} M azithromycin (a); a + 2×10^{-6} M BSA (b) in pH 7.0 phosphate buffer.

3.3. Kinetic nature for azithromycin

Figs. 1 and 2 demonstrate the electrode reaction of azithromycin is an irreversible surface reaction. For the irreversible surface electrochemical reaction, the relationship between the peak potential E_p and the scan rate v in the linear sweep voltammogram is expressed in the Eq. (4) by Laviron [11]:

$$E_p = E^{0'} + (RT/\alpha_a nF)\ln(RTk_s/\alpha_a nF) - (RT/\alpha_a nF)\ln v \quad (4)$$

where α_a is the transfer coefficient, k_s, the standard rate constant of the surface reaction, n is the number of electrons involved in the reaction and $E^{0'}$ is the formal potential.

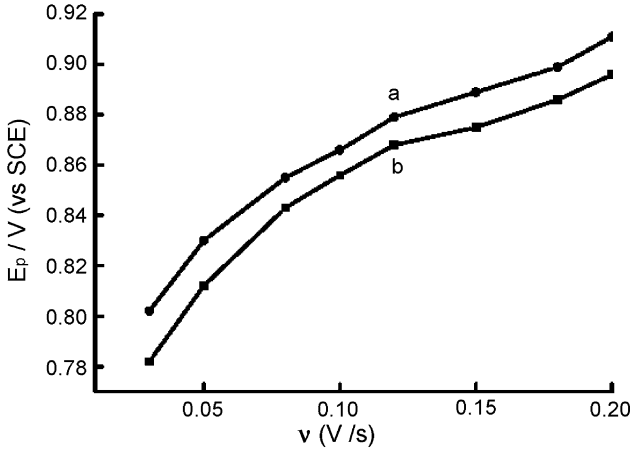


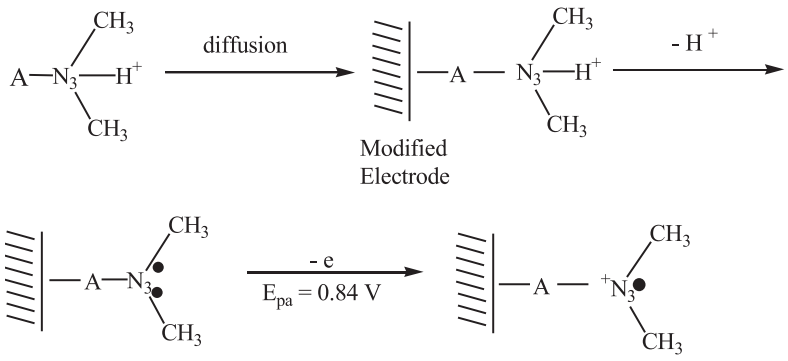
Fig. 6. Dependence of peak potential E_p on the potential scan rate v: for (a) 3.82×10^{-5} M azithromycin pH 7.0 phosphate buffer; (b) a + 2×10^{-6} M BSA.

According to Eq. (4), the plot of E_p vs. ln$v$ is linear with a slope that allows $n\alpha_a$ to be determined, and an intercept from which k_s can be calculated if the value of $E^{0'}$ is known. The value of $E^{0'}$ in Eq. (4) can be obtained from the intercept of the E_p vs. v curve by extrapolation to the vertical axis at $v = 0$.

Linear sweep voltammogram data was obtained from a 0.06 M Na_2HPO_4–NaH_2PO_4 buffer (pH 7.0) containing 3.82×10^{-5} M azithromycin by varying the scan rate from 30 to 500 mV s^{-1} and reported as plots of E_p vs. ln$v$ (Fig. 5) and E_p vs. v (Fig. 6). From slope (0.05278), intercept (0.953 V) and $E^{0'}$ (0.797) values, the value of $n\alpha_a = 0.48$ and $k_s = 345$ s^{-1} were calculated. Since α_a is assumed to be 0.5 in a totally irreversible electrode process, these results demonstrate that one electron is involved in the oxidation of azithromycin.

3.4. The electrode reaction mechanism of azithromycin

Based on the above discussions, the electrode reaction mechanism of azithromycin can be developed. Even though azithromycin hydrophobicity depends on the masking of its ionizable functions by the methyl-groups, at pH 7.0 azithromycin is protonated at both N_9 and N_3 and is a very hydrophobic molecule [12]. Since carbon nanotubes are also hydrophobic, azithromycin is strongly adsorbed on the surface of the electrode and oxidation half reactions are facilitated. Among azithromycin's various functional groups, the amine group is most easily oxidized. Dialkylamines oxidize to form a radical cation by the loss of one electron [13]. Since N_9 is situated in the macrocyclic lactone ring, its lone pair electron(s) is not easily lost. The structurally analogous drug, erythromycin, which has no nitrogen atom in the macrocyclic lactone ring, exhibits similar voltammetric behavior [14]. Thus, the observed oxidation peak is attributed to one of the electrons in N_3's lone pair set. At pH < pK_a, N_3 is initially deprotonated with subsequent lose of one electron to form a radical cation. Schematically, it can be expressed as:



3.5. Studies of the interaction between azithromycin and BSA

Serum album is the principle protein component in plasma and is remarkable for its power to bind a great variety of molecules, including bilirubin, fatty acids, metal ions, tryptophan, and numerous drugs. Investigation of the mechanism of drug binding to serum albumin is indispensable for understanding the transport function of albumin. Several methods have been used to study drug and protein interaction. Fluorometry technique involves the detection of the change in the fluorescence intensity of protein or a fluorescent probe when drug bounds to protein [15]. The disadvantage of the technique is that the protein must be fluorescent or the drug and the probe must share the same binding sites on the protein. Some indirect methods such as equilibrium dialysis, ultrafiltration, and gel filtration have also been used [16]. The main drawback of these techniques is the disturbance of the drug–protein equilibrium caused by the separation of the free drug from the complexes. Potentiometry, based on direct measurements of the electrode potential caused by free ionic drug, has also been used to study ionic drugs binding to proteins [17]. The main limitations of the method are its applicability only to ionic drugs and the interferences of some similar ions. In the present work, the binding of azithromycin with BSA was demonstrated by spectrometry and some binding parameters were measured by voltammetry.

3.5.1. UV–VIS absorption spectra

Fig. 7 shows the UV–VIS absorption spectra of azithromycin and its mixture with BSA in phosphate buffer (pH 7.0), which is obtained by keeping the azithromycin concentration constant and changing the BSA concentration. In the wavelength range from 190 to 300 nm, azithromycin has a maximum absorption at 210 nm (curve a). With the addition of BSA, the absorbance of azithromycin at 210 nm disappears and a new maximum absorption peak is formed at 230 nm (curve b). With the increasing addition of BSA, the maximum absorption peak is red shifted and the absorption increases (curve c). The changes of absorption

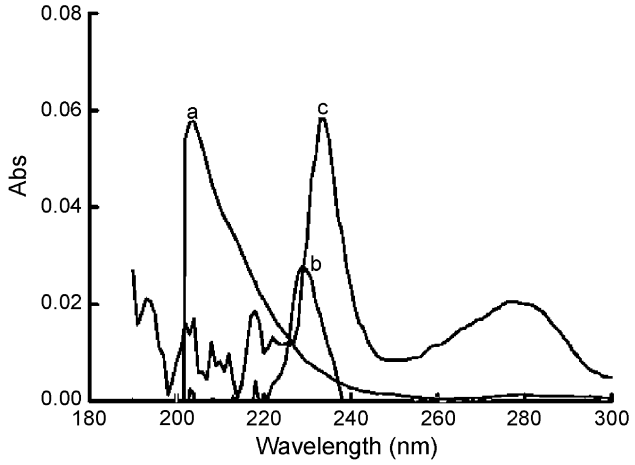


Fig. 7. Absorption spectra of azithromycin bound to BSA at pH 7.0. Azithromycin (a); Azithromycin–BSA complex: $C_{AZI}=3.82\times10^{-4}$ M, $C_{BSA}=5\times10^{-6}$ M (b); Azithromycin–BSA complex: $C_{AZI}=3.82\times10^{-4}$ M, $C_{BSA}=1\times10^{-5}$ M (c).

spectra indicate that there are binding interactions between azithromycin and BSA.

3.5.2. Electrochemical behavior of azithromycin in the presence of BSA

Fig. 8 displays cyclic voltammograms of 3.82×10^{-5} M azithromycin in the absence (curve a) and presence of 2×10^{-6} M BSA (curve b) and 4×10^{-6} M BSA (curve c). It can be seen that the oxidation current of azithromycin decreases obviously in the presence of BSA, but the oxidation potential does not change and no new peaks are found in the same potential range. The α_a of azithromycin

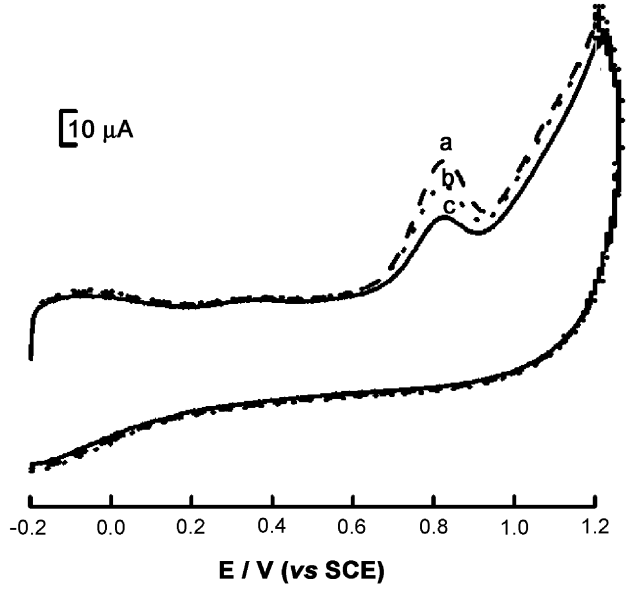


Fig. 8. Cyclic voltammograms for: 3.82×10^{-5} M azithromycin (a); 3.82×10^{-5} M azithromycin + 2×10^{-6} M BSA (b); 3.82×10^{-5} M azithromycin + 4×10^{-6} M BSA (c). The potential scan rate is 100 mV s^{-1}, $t_{acc}=2$ min.

mixed with BSA is 0.49 and the k_s is calculated to be 371 s^{-1} from Figs. 5 and 6 (slope 0.05229, intercept 0.942 V, $E^{0\prime}$ 0.782 V). Comparing with those of azithromycin (0.48, 345 s^{-1}), these parameters have no obvious change. It can be concluded that it is still azithromycin but not BSA–*m*Azithromycin complex taking the electrode reaction.

It is difficult for azithromycin in the complex to make contact with the electrode surface and subsequently be reduced at that surface. Furthermore, the free concentration of azithromycin on the electrode surface is decreased, thus the oxidation current of azithromycin is reduced. The reproducibility of the curves in Fig. 8 was also investigated. Keeping the concentration of BSA and azithromycin constant, the RSD of the value of peak current was obtained as 2.03% for 10 replicate measurements.

3.5.3. Discussion of the interaction mechanism of azithromycin and BSA

The results indicated that there were interactions between BSA and azithromycin and the complex BSA–*m*Azithromycin was formed. In the pH 7.0, which is higher than the isoelectric point of BSA (pI = 4.8), BSA is negatively charged, while azithromycin is protonated and positively charged. Thus, azithromycin and BSA would bind together by electrostatic attractive strength. Furthermore, the weak interactions such as ionic, van der Waals, hydrogen bonding and hydrophobic would also be helpful to form the complex. It is known that azithromycin is hydrophobic and insoluble in serum. Its binding to BSA facilitates its solubility in blood and its transportation by blood circulation.

3.5.4. Determination of the binding constant and binding number between azithromycin and BSA

According to the method of Li [18], it is assumed that BSA and azithromycin only produce a single complex BSA–*m*Azithromycin. The binding number, *m*, and the

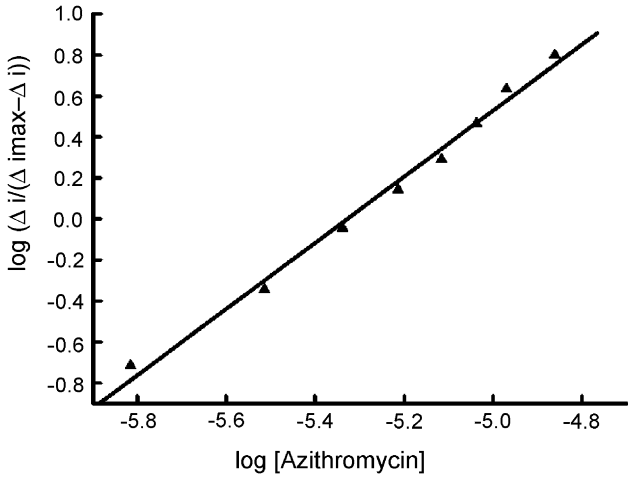


Fig. 9. Dependence of log ($\Delta i/(\Delta i_{max}-\Delta i)$) on the log [Azithromycin].

binding constant, β_s, between BSA and azithromycin could be determined as:

$$\mathrm{BSA} + m\mathrm{Azithromycin} \leftrightarrow \mathrm{BSA} - m\mathrm{Azithromycin}$$

The equilibrium constant β_s can be obtained:

$$\beta_s = [\mathrm{BSA} - m\mathrm{Azithromycin}]/([\mathrm{BSA}][\mathrm{Azithromycin}]^m) \quad (5)$$

The following equation can be deduced:

$$\Delta i_{\max} = kC_{\mathrm{BSA}} \quad (6)$$

$$\Delta i = k[\mathrm{BSA} - m\mathrm{Azithromycin}] \quad (7)$$

$$[\mathrm{BSA}] + [\mathrm{BSA} - m\mathrm{Azithromycin}] = C_{\mathrm{BSA}} \quad (8)$$

$$\Delta i_{\max} - \Delta I = k(C_{\mathrm{BSA}} - [\mathrm{BSA} - m\mathrm{Azithromycin}]) = k[\mathrm{BSA}] \quad (9)$$

Based on the Eqs. (5), (7) and (9), the following equation can be obtained:

$$\log[\Delta_i/(\Delta_{max} - \Delta_i)] = \log\beta_s + m\log[\mathrm{Azithromycin}] \quad (10)$$

where Δi is the peak current change of the same concentration of azithromycin in the absence and presence of BSA, $\Delta i_{\max}$ is the maximum peak current change. C_{BSA}, [BSA−*m*Azithromycin], [BSA] are referring to the total, drug-bound and free concentration of protein in solution, respectively.

The plot of log $(\Delta i/(\Delta i_{\max} - \Delta i))$ should be linear with log [Azithromycin]. Keeping the BSA concentration at 2.0×10^{-6} M and pH constant and changing the concentration of azithromycin from 1.53×10^{-6} to 1.38×10^{-5} M, the plot in Fig. 9 was obtained with the mean value of the peak current change by seven replicate measurements. The slope and the intercept were 1.7 and 8.64, respectively, which means $m \approx 2$ and $\log \beta_s = 8.64$. Thus, azithromycin binding to BSA forms a 2:1 complex of BSA−2Azithromycin.

4. Conclusion

In this paper, the electrochemical behavior of azithromycin at MWNTs-modified electrode was investigated and electrochemical parameters were reported. Based on the experimental results, the electrode reaction mechanism of azithromycin was proposed. Most important of all, the interaction of azithromycin with bovine serum albumin was studied and binding number and binding constant were obtained. It is helpful for us to understand the pharmacokinectics of azithromycin.

Acknowledgements

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