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Insertion Study of Adriamycin and Quelamycin into Bilayer Lipid Membrane

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Adriamycin is an antitumor agent and its amphipathic characteristic makes interaction at the level of the cellular surface possible. In this paper, we studied the insertion behaviour of adriamycin and its metallic derivative, triferric adriamycin *i.e.*, quelamycin, into lipid membrane with vesicles and supported bilayer lipid membrane as model membranes formed by neutral lipids. Observed from UV-VIS absorption spectroscopy, quelamycin could interact with vesicles in adriamycin-iron complex form. In the fluorescence measurements, adriamycin and triferric adriamycin could insert into the bilayer membrane of vesicles. The concentration effect of adriamycin on the supported bilayer lipid membrane and comparison of the penetration ability between adriamycin and quelamycin were investigated through electrochemical methods with $Fe(CN)_6^{4-/3-}$ as marker ion.

Key Words: Adriamycin, Quelamycin, Insertion, Bilayer lipid membrane, Electrochemical methods.

INTRODUCTION

Adriamycin (ADM, generic name, doxorubicin), a cytotoxic anthracycline antibiotic isolated from cultures of *Streptomyces peucetius* var. *caesius*, is a chemotherapeutic agent that is used specifically in the treatment of many types of cancer. It interferes with the multiplication of cancer cells and slows or stops their growth and spread in the body. Adriamycin with an anthracycline structure consists of an aglycon (a dihydroxyanthraquinone nucleus, namely adriamycinone) and links with a glycosidic bond to an amino sugar (daunosamine)[1]. The molecular structure of adriamycin is presented in Fig. 1. Adriamycin has several possible metal ion binding sites including the sugar amino groups, the quinone-hydroquinone functionalities and the side-chain carbonyls[2]. Quelamycin (QUM) is a metallic derivative of adriamycin made by chelation of adriamycin with three ferric ions at neutral pH. The complexation sites are most likely two ferric ions chelated by four oxygens of the adriamycinone ring system and the third ion coordinating to the amino sugar. This derivative maintains considerable antitumor activity with less general, hematologic and cardiac toxicity than adriamycin[3].

The adriamycin is an amphiphilic antitumor agent with the anthracycline ring lipophilic and the saturated end of the ring system containing abundant hydroxyl groups adjacent to the amino sugar, producing a hydrophilic center. The amphipathic characteristic of drugs makes interaction at the

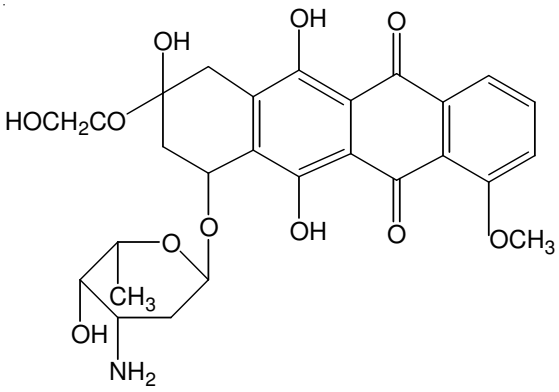


Fig. 1. Molecular structure of adriamycin

level of the cellular surface possible[4,5]. Because the surface membrane of the cell is the first barrier encountered in the treatment of cancer, anthracyclines interacting with model and natural membrane systems play an important role in the understanding of the bioactivity properties of these drugs[6]. The interaction process involves modulation of membrane fluidity, transport of small molecules and ions across membranes, phospholipid organization in the membrane and so on, which are believed to have some impacts either on its antitumoral effect or on its stability inside lipid vesicles.

Because biomembranes are too complex, there has been extensive interest in employing simple models to *mimic* biological cell membranes, including vesicles as curved bilayer,

monolayer, cast film, bilayer lipid membrane (BLM), supported bilayer lipid membrane (s-BLM), *etc*. The s-BLM has been applied more and more considerable in the field of membrane research, for having many advantages of ease and reproducibility of preparation, long-term mechanical stability, availability to electrochemical analysis and surface detection[7]. In this paper, we investigated the insertion effect of adriamycin and quelamycin with vesicles and s-BLM as *mimetic* model membranes by spectroscopic and electrochemical methods, respectively.

EXPERIMENTAL

Dipalmitoylphosphatidylcholine (DPPC) was purchased from sigma (USA) and used without further purification. Adriamycin hydrochloride was a generous gift from Zhejiang Hisun Pharmaceutical Co. Ltd. (Zhejiang, China). Quelamycin, chelating adriamycin with three ferric ions at neutral pH, was prepared by following procedure mentioned in the reference[3]. Stock solutions were prepared in 10 mM phosphate buffer solution (PBS) at pH = 7.0 and stored in a refrigerator in the dark. Analytical-grade potassium ferricyanide and ferrocyanide was obtained from Beijing Chemical Reagent Factory (Beijing, China). Other chemicals were of the highest quality as possible as obtained. Water purified by Milli-Q water system was used throughout the experiments.

Preparation of dipalmitoylphosphatidylcholine vesicles: Vesicles of DPPC were obtained by the sonication method. Briefly, DPPC was dissolved in chloroform and dried under nitrogen stream. Subsequently, the dried lipid film was hydrated in PBS. The hydrated film was sonicated for several hours until a clear suspension was obtained. Throughout the process, temperature was held above the phase transition temperature (T_m) of DPPC. After centrifugation, the supernatant was used and annealed above the T_m for about 1 h then cooled to ambient temperature prior to use.

Spectra measurements: A Cary 500 Scan UV-VIS-NIR spectrophotometer (Varian, USA) was employed for absorption spectra measurements. The sample solutions contained 21.6 μM drugs in the presence of DPPC vesicles. The reference solution was comprised of the same amount of DPPC vesicles. The absorption spectrum of the sample solution was measured against the reference solution using 1 cm light-pathlength cuvette.

Fluorescence spectra were carried out on a LS55 luminescence spectrometer (Perkin Elmer, USA). The excitation wavelength was set at 470 nm and the emission spectrum was monitored from 500-700 nm. Fluorescence grade quartz cuvette (1 cm light-pathlength) was used with the fluorescence measurements.

Method for s-BLM formation: Method for the formation of s-BLM on glassy carbon electrode (GCE) was described in the references of Wu *et al.*[8], Huang *et al.*[9] and Ye *et al.*[10]. DPPC was dissolved in chloroform to give a final concentration of 2.5 mg/mL, which was called BLM-forming solution. First, the electrode was polished with 1.0, 0.3 and 0.05 μm alumina slurry, respectively and then washed in an ultrasonic bath with pure water to remove any adhesive particles. Subsequently, in order to polarize the electrode, it was immersed in the 0.1 M KCl solution and the potential was held at 1500 mV for 3 min. After polarization, the electrode was dried under purified nitrogen stream. Then a drop of lipid solution was added to the surface of GCE by a microsyringe and the electrode was immediately transferred into the 0.1 M KCl solution, in which the bilayer lipid membrane was formed spontaneously.

Electrochemical measurements: All cyclic voltammetry (CV) experiments were performed on CHI900 electrochemistry station (CHI, USA). Electrochemical impedance spectroscopy (EIS) was carried out with an Autolab PGSTAT30 (Eco Chemie B.V. Utrecht, Netherlands) in the frequency range from 0.1-10 KHz with signal amplitude of 10 mV. A standard three-electrode system was used for the experiments with a platinum wire as counter electrode, an Ag/AgCl (KCl-saturated) electrode as reference electrode with respect to which all potentials were reported and a glassy carbon electrode as working electrode.

RESULTS AND DISCUSSION

UV-VIS absorption spectra: The UV-VIS absorption spectra of adriamycin (a) and quelamycin (b) with the effect of DPPC vesicles were recorded in Fig. 2A. As can be seen in curve b, the spectrum of quelamycin in the presence of vesicles exhibited a lowered absorption intensity in the 475-500 nm range and a shoulder band at *ca.* 605 nm compared to curve a. The appearance of the shoulder band is characteristic of adriamycin-Fe^{3+} complex[11]. The above results showed that qualemycin was stable to hold in the adriamycin-Fe^{3+} complex form during the interaction with the lipid bilayer in aqueous dispersions.

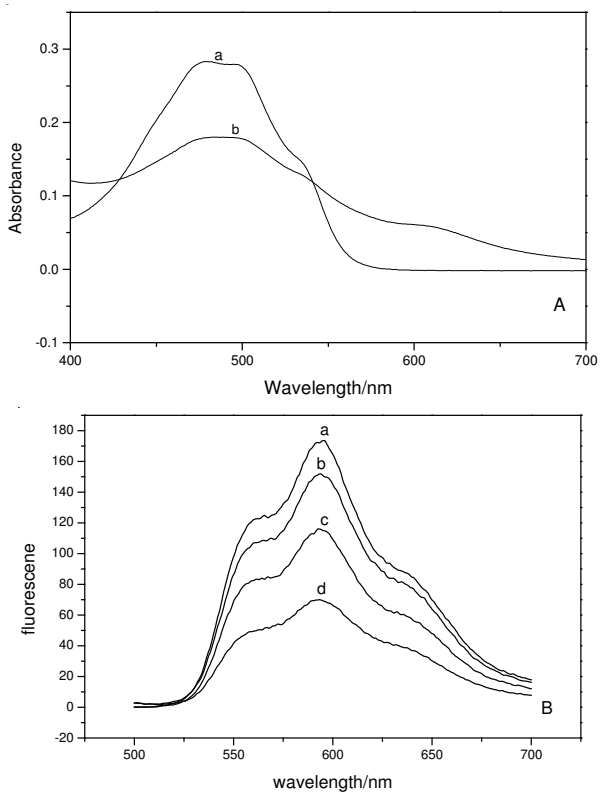


Fig. 2. UV-VIS absorption spectra (A): a. ADM; b. QUM in the presence of vesicles and fluorescence spectra (B): a. ADM; b. a + DPPC vesicles; c. QUM; d. c + DPPC vesicles

Fluorescence spectra: Adriamycin molecule possesses a fluorescent hydroxy-substituted anthraquinone chromophore and the fluorescence spectrum of adriamycin is well-known[12]. In this section, we exploited the intrinsic fluorescence properties of the anthracycline drugs to study the various properties of free and vesicles bilayer membrane-bound drugs. From the fluorescence spectra presented in Fig. 2B, binding of the two drugs to the sonicated lipid vesicles (curves b and d) resulted in a slight shift of the emission spectra to shorter wavelengths compared to free drugs (curves a and c), indicative of the drugs relocation to a less polar environment. A change in the fluorescence intensity also accompanied the binding, which showed a more deep bury of the drugs. The results suggested that adriamycin and quelamycin could insert into the curved bilayer membrane of DPPC vesicles.

Characterization of s-BLM: We judged the formation of bilayer lipid membrane supported on the surface of the GCE by means of cyclic voltammetry and electrical impedance spectroscopy recording the change in the bare electrode with modified electrode.

Fig. 3A shows the cyclic voltammetric responses taken at the bare GCE (curve a) and the coated GCE with DPPC membrane (curve b). Comparing curve b with curve a, we could find that a pair of well-defined reversible waves of $Fe(CN)_6^{4-/3-}$ ion couple was obtained at bare GCE; however, after the electrode was modified, the reversible waves disappeared. The BLM coated on the electrode almost completely suppressed the electron transfer between the solution and the GCE, which was consistent with previous results and implied the successful formation of the s-BLM on the surface of the GCE[8,10,13].

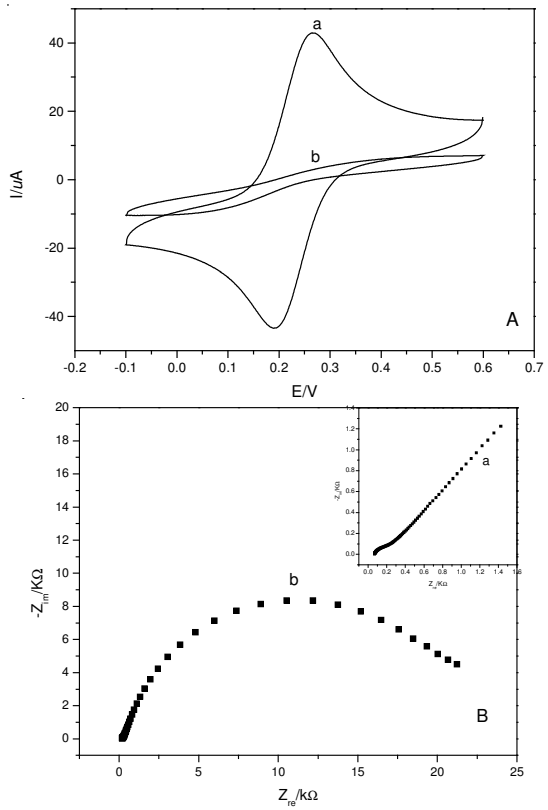


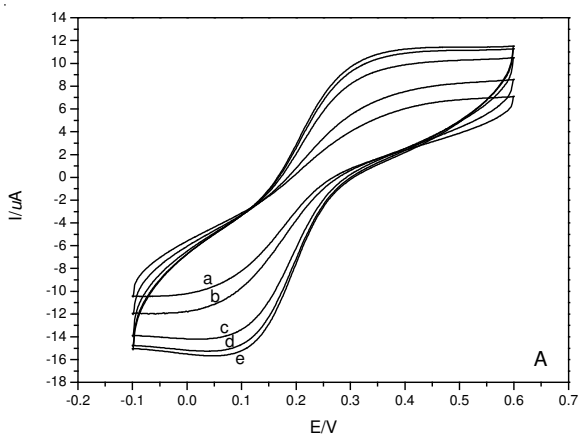
Fig. 3. Cyclic voltammograms (A) and impedance spectroscopy plots (B) of (a) bare GCE; (b) GCE modified with lipid membrane in 1 mM $Fe(CN)_6^{4-/3-}$ solution with PBS as supporting electrolyte. Scan rate: 50 mV s^{-1}

Fig. 3B illustrates the results of impedance spectroscopy measurements on the bare electrode (plot a) and the modified electrode (plot b) in 1 mM $Fe(CN)_6^{4-/3-}$ solution, which are measured at the formal potential of the system. It is expressed as Nyquist plots, in which the complex impedance can be presented as the sum of the real, Z_{re} and imaginary, Z_{im} components that originate mainly from the resistance and capacitance of the cell, respectively. It could be seen from plot a that the bare GCE exhibited an almost straight line that was characteristic of a diffusional limiting step of the electrochemical process[14]. With respect to the coated electrode, significant difference in impedance spectroscopy was observed from plot b, which showed a part of single semicircle meaning a kinetic limiting step of charge transfer process at a smooth electrode surface[15]. It could be seen that the presence of membrane caused the charge transfer resistance to increase and the double-layer capacitance to decrease compared with that of the bare GCE, further proving the formation of a bilayer lipid membrane.

The result observed from impedance spectroscopy was consistent with that obtained from the cyclic voltammetry measurements. It could be concluded that the bilayer lipid membrane was constructed on the surface of the GCE successfully.

Insertion of ADM into s-BLM: Electrochemical methods can provide an initial assessment of membrane permeability and serves as a sensitive probe of structural changes in membrane[16,17]. Thus, we studied the interaction between ADM and s-BLM through CV and EIS.

The cyclic voltammograms taken at the coated GCE by DPPC BLM in 1 mM $Fe(CN)_6^{4-/3-}$ solution containing different concentrations of ADM is presented in Fig. 4A. After the interaction between adriamycin and lipid membrane reaching equilibrium, the CV plots were recorded. Observed from curve a to e, the redox peak current increased obviously with the increasing concentrations of adriamycin and reached finally a plateau. The results implied that adriamycin could induce the local lipid disorder with the insertion into the bilayer of s-BLM and the information about membrane change was quantitatively transduced into a electrochemical signal which corresponded to the amount of $Fe(CN)_6^{4-/3-}$ allowed to access the electrode. However, there were limited lipid molecules coated on the surface of the GCE, thus the site of adriamycin interacting with lipid membrane was also limited. When the sites were all occupied, the redox peak current reached a plateau.



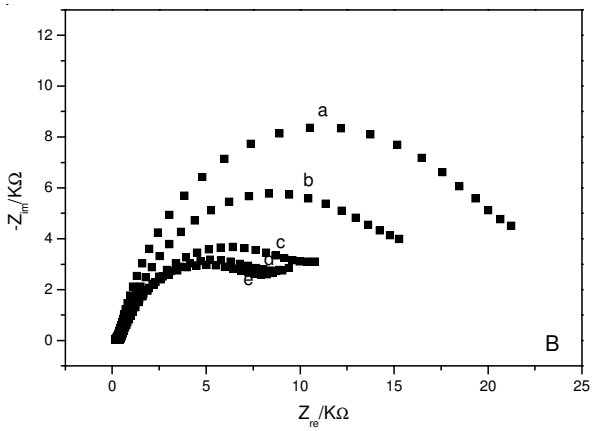


Fig. 4. Cyclic voltammetric response (A) and impedance spectroscopy plots (B) on the GCE modified with BLM after interacting with different concentrations of ADM: (a) 0, (b) 1.7, (c) 4.3, (d) 8.6, (e) 12.9 μM. Scan rate: 50 mV s^{-1}

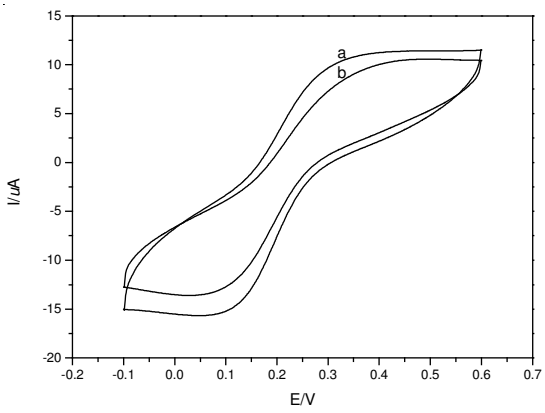


Fig. 5. Cyclic voltammetric response of 1 mM $Fe(CN)_6^{4-/3-}$ on the GCE modified with BLM containing 12.9 μM: (a) ADM; (b) QUM. Scan rate: 50 mV s^{-1}

The EIS was based on the charge-transfer kinetics of the $Fe(CN)_6^{4-/3-}$ redox couple and could be used to monitor the alteration of the interfacial interaction between drug and lipid membrane[18]. Compared to the s-BLM modified electrode surface, the drug interacting with lipid membrane on the electrode surface altered the capacitance and interfacial electron resistance. Thus, we evaluated the electron transfer kinetics and the membrane compactness taking EIS as a complementary technique to CV.

Fig. 4B shows the EIS measurements in solution with different concentrations of adriamycin. Observed from the Nyquist plots, the semicircles dominated by the interfacial mass transfer of the redox probe were sinking gradually coupled with the smaller diameters with increasing concentration of adriamycin. This indicated that the electrode kinetics became quicker as the s-BLM interacting with drug. Under the effect of adriamycin, a considerable decrease in the charge-transfer resistance featured a change from a dielectric surface to a surface with defects permeating through lipid membrane to the GCE electrode[9,14]. Obviously, we could obtain the result that the increased bilayer permeability on the s-BLM after it interacted with adriamycin was consistent with the result obtained from the cyclic voltammetry measurements.

Comparison of the interaction of adriamycin and quelamycin with s-BLM: From foregoing spectroscopy experimental results, both ADM and QUM had the ability to insert into the curved bilayer lipid membrane as the model of vesicles. But we didn't deduce the magnitude of action ability of the two drugs from the change of the fluorescence intensity, for the lack of regularity in response of ADM fluorescence[12]. Thus, we studied the cyclic voltammetric behaviour of ADM and ADM-iron complex at the same concertration of 12.9 μM interacting with the s-BLM on the GCE electrode presented in Fig. 5. Observed from these peak currents, under the same condition, ADM possessing a higher peak current had the stronger interaction ability with the s-BLM. This result could be involved in the low toxicity of quelamycin.

The perturbation of phospholipid bilayer membrane induced by ADM depends on the composition of lipid. The affinity of adriamycin for lipid membrane which bears no net charge is moderate. In contrast, adriamycin interacts strongly with posititively or negatively charged lipid membrane[19]. In the binding of anthracyclines with membranes, both electrostatic and hydrophobic forces appear to be necessary for the stabilization of the drug in the lipid bilayer. The affinity for neutral lipid membrane appears to be dominated by hydrophobic interaction between the aglycon portion of the drug molecule and the acyl chains of the membrane[20].

Adriamycin in the hydrochloride form contains a charged tertiary amine and can establish electrostatic interaction, but due to its structure it can also behave as a hydrophobic substance and insert into the hydrophobic core of the lipid layer. The increasing insertion of drug into the lipid bilayer can induce local disorder of regular packed membrane, which allowed the electron transfer between the solution and the GCE and led to increase the bilayer membrane permeability. Because the interaction sites between the drug and lipid molecules were limited, finally, the permeability change reached equilibrium. Under the circumstance of interaction dominated by hydrophobic force, the more hydrophibic drugs were apparently buried deeper in the membrane. Structural change in the aglycon portion of the anthracycline ring chelated with ferric ion, which increased the polarity of drug, would result in a reduced membrane penetration and binding ability.

Conclusion

In the present paper, we studied the insertion behaviour of adriamycin and its metallic derivative, triferric adriamycin, into bilayer lipid membrane with vesicles and s-BLM as model membranes. Observed from UV-VIS absorption spectroscopy, quelamycin interacted with vesicles in adriamycin-iron complex form. In the fluorescence measurements, adriamycin and triferric adriamycin could insert into the bilayer membrane of vesicles. The concentration effect of adriamycin on s-BLM and comparison of the penetration ability between adriamycin and quelamycin were investigated through cyclic voltammetry and impedance spectroscopy measurements with $Fe(CN)_6^{4-/3-}$ as marker ion.

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