

Effect of Amphotericin B antibiotic on the properties of model lipid membrane

S Kiryakova, M Dencheva-Zarkova, J Genova

Institute of Solid State Physics, Bulgarian Academy of Sciences, 72 Tzarigradsko
Chaussee, Blvd., 1784 Sofia, Bulgaria

Corresponding author e-mail: sophia_kiryakova@abv.bg

Abstract. Model membranes formed from natural and synthetic lipids are an interesting object for scientific investigations due to their similarity to biological cell membrane and their simple structure with controlled composition and properties. Amphotericin B is an important polyene antifungal antibiotic, used for treatment of systemic fungal infections. It is known from the literature that the studied antibiotic has a substantial effect on the transmembrane ionic channel structures. When applied to the lipid membranes it has the tendency to create pores and in this way to affect the structure and the properties of the membrane lipid bilayer.

In this work the thermally induced shape fluctuations of giant quasi-spherical liposomes have been used to study the influence of polyene antibiotic amphotericin B on the elastic properties of model lipid membranes. It has been shown experimentally that the presence of 3 mol % of AmB in the lipid membrane reduces the bending elasticity of the lipid membrane for both studied cases: pure SOPC membrane and mixed SOPC-Cholesterol membrane.

Interaction of the amphotericin B with bilayer lipid membranes containing channels have been studied in this work. Model membranes were self-assembled using the patch-clamp and tip-dip patch clamp technique. We have found that amphotericin B is an ionophore and reduces the resistance of the lipid bilayer.

1. Introduction

Infections as a consequence of weakened immunity defences, such as from AIDS or from many cancer therapies, have renewed the scientific studies on polyene antibiotic amphotericin B (AmB). The structure of amphotericin B is given in figure 1. This antibiotic is the most common antifungal agent used to treat deep-seated mycotic infections [1].

In biological systems, AmB acts by forming ion channels that induce ion leakage across lipid membranes [2]. The formation of these channels is highly dependent on the presence of cholesterol (figure 1B) in the membrane [3]. It has been suggested that the channels are composed of AmB – cholesterol complexes made of superimposed antibiotic and cholesterol molecules. The polar groups on these molecules face toward the interior of the channel where they interact with water molecules, while the hydrophobic groups interact with the aliphatic chains of membrane phospholipids [4].

However, it has been later indicated that the mechanism of action of AmB is similar for both mammalian and fungal cells. The ion leakage that leads to the cell's death has the same effect on both



types of cells because of their similar structure. Despite its toxicity this antibiotic is still irreplaceable in many micotic therapies because of its wide spectrum of application.

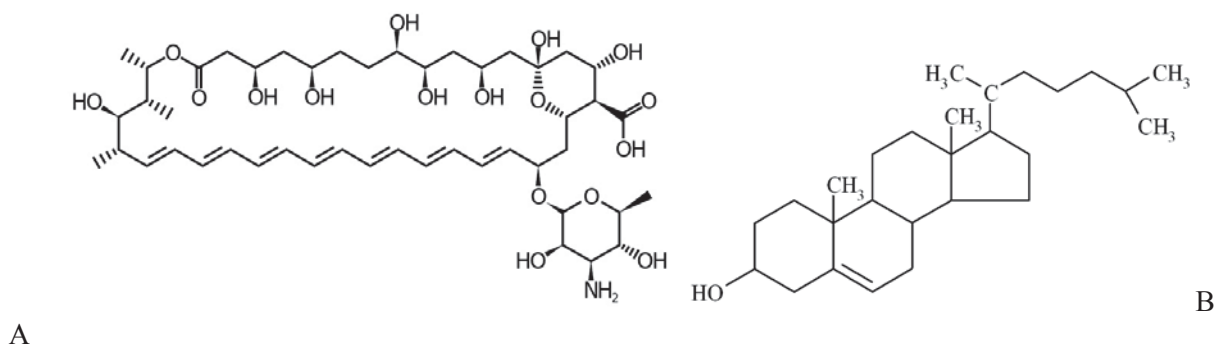


Figure 1. Structure of (A)- antibiotic Amphotericin B and (B)- cholesterol.

The membranes of living cells are very complex structures composed of lipids, proteins, sugars and cholesterol. The main role of the membranes in the cell functioning is to separate the interior of the cell from the outside environment. The protoplasm inside the cells consists of many organelles (most of them membrane structures) responsible for the cell's life and functioning. They make the living cell even a more complex subject of research.

Artificially created lipid vesicles mimic the cells and their membranes. The bilayer membrane of lipid vesicles presents a simple model for investigation of membrane properties and can be formed from various lipids with controlled composition in controlled environment. With better understanding of the mechanism of AmB action on model lipid membranes we will have a clearer image on how this antibiotic interacts with living cells and their membranes.

The aim of the present study is to investigate the effect amphotericin B (AmB) and cholesterol on the properties of lipid membranes formed from natural and synthetic lipids.

2. Materials and methods

L- α -lecithin from soybean (choline content 20%), amphotericin B, HEPES, KCl (p.a.) and cholesterol (5-Cholesten-3 β -ol) were obtained from Sigma. SOPC (1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (C18:0/C18:1)) was purchased from Avanti Polar Lipids Inc., USA).

Methanol (p.a.), n-propanol (p.a.) and n-hexane (p.a.) were purchased from Valerus Co. All the chemicals were used without any further purification.

The vesicles for thermal shape fluctuation analysis were prepared via modified electroformation method [5]. The lipid was dissolved in chloroform 1 mg/ml. The cholesterol was dissolved in methanol 2 mg/ml. AmB was dissolved in n-propanol:distilled water (4:6, v/v). The final lipid-cholesterol solution for electroformation was prepared by mixing SOPC and cholesterol to obtain 40 mol % concentration of cholesterol in the lipid membrane. The lipid (lipid/cholesterol) solution was laid on the glasses of the electroformation cell and put under vacuum. After the entire evaporation of the solvent AmB solution was laid above the lipid layer and again put under vacuum for solvent evaporation. The electroformation cell, used for all experimental procedures contained two glasses, coated with transparent conductor, indium tin oxide (ITO; thickness of 100 ± 20 nm, resistivity of $100 \Omega/\square$) acting as electrodes and a PDMS (polydimethylsiloxane) spacer.

After the entire evaporation of the solvent the experimental cell was assembled and filled with double distilled water. A low frequency (10 Hz) sinusoidal alternative voltage was applied (1.5 V PP) to the

conductive glasses overnight. This procedure led to the formation of vesicles, appropriate for our experiment. We have chosen giant (diameter of the order of 20–40 μm) fluctuating vesicles without any visible defects.

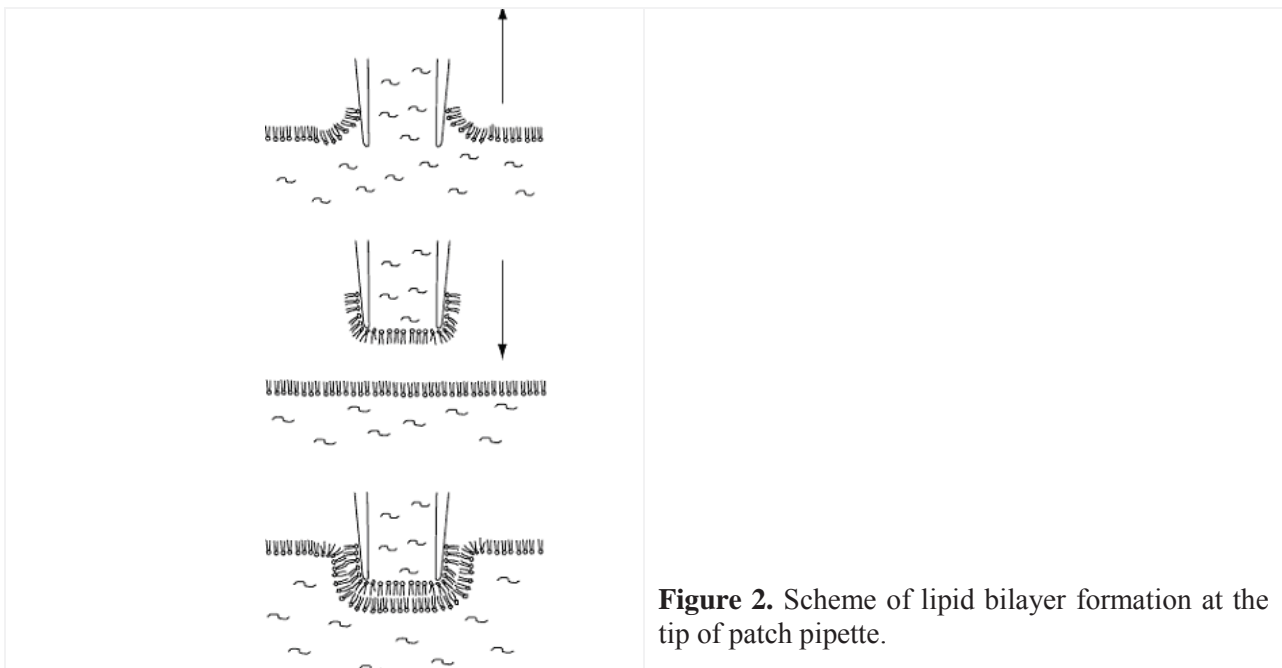
Thermally induced shape fluctuation method [6, 7] was used to investigate the influence of AmB and combination of AmB and cholesterol on the bending elasticity of model lipid membranes.

The samples of the fluctuating giant vesicles were observed under phase contrast microscope (Axiovert 100, Zeiss, Germany, oil immersion objective Ph3 100x magnification). The experimental equipment was improved using stroboscopic illumination, based on xenon flash lamp L6604 Hamamatsu, Japan) with damping vibration system, short light pulses (less than 3–4 μs long full width at half maximum) and high input energy (2 J) [8].

The obtained digital data was further recorded on the hard disk drive of the PC. Every second an image was acquired and recorded till the total number of images reaches a preliminary given value (about 400).

Further details on the contour determination, mean squared amplitudes calculation and fitting procedure to determine the bending elastic modulus, k_c , and the dimensionless membrane tension $\bar{\sigma}$, can be found in the articles of Genova et al. [9, 10].

For the patch-clamp experiments lipid bilayers were self-assembled at the tips of patch pipettes, using the tip-dip patch clamp technique [11], from monolayers formed by spreading L- α -lecithin from soybean in n-hexane (10 mg/ml) onto the surface of electrolyte solutions contained in Petri dishes (figure 2).



Patch pipettes (tip diameter 1–2 μm) and Petri dishes (10 cm^2 area) were filled with aqueous solutions of KCl (0.1 M) buffered with HEPES (0.01 M) at pH 7. Only bilayers with seal resistances $> 1\text{G}\Omega$ were used. AmB dissolved in n-propanol:distilled water (4:6, v/v), was added to the Petri dish after bilayer formation to a final concentration of 4×10^{-8} g/ml.

Solutions of cholesterol (2 mg/ml) were added to the Petri dish to final concentrations in the micromolar range.

Single AmB channel currents were monitored using a patch clamp amplifier Model 2400 (A-M Systems, Inc.) and patch clamp software. The currents were stored on a PC hard disc with 1 ms time resolution. All measurements were performed at room temperature ($\sim 22^\circ\text{C}$).

3. Results and discussion

3.1. Thermal shape fluctuations

The analysis of thermally induced shape fluctuations of giant vesicles is used to study the influence of amphotericin B and cholesterol on the bending elasticity of lipid membrane. The analysis of thermally induced shape fluctuations of giant liposomes is a classical method for studying the elastic properties of lipid membranes [6, 7]. It is based on the fact that under the Brownian motions of water molecules, bombarding the lipid membrane in water environment the lipid vesicle constantly changes its shape. Such fluctuations of the biological or model membranes are a part of the phenomena, describing the deviation of some physical properties from their equilibrium state. Strong qualification criteria for every recorded image in the image sequence and for the quality of the vesicle as a whole are imposed [10]. The white noise contribution to the fluctuation amplitudes is calculated [10] and taken into account.

The obtained experimental data for the bending elasticity modulus (k_c) for pure SOPC membrane, SOPC membrane, containing 3 mol % AmB, SOPC:Cholesterol=6:4 membrane, SOPC:Cholesterol=6:4 membrane containing 3 mol % AmB are presented in table 1.

Table 1. Bending elasticity modulus of SOPC membrane, containing AmB, cholesterol, and combination of AmB and cholesterol. The first column gives information on the studied system, the second column shows the mean weighted value of the bending elasticity modulus and the third column shows the number of vesicles over which the mean values are calculated.

System	Weighted mean value of the bending elasticity modulus $\bar{k}_c \times 10^{-19} (J)$	Number of vesicles
SOPC	1.88 \pm 0.17	6
SOPC+3mol% AmB	1.56 \pm 0.05	10
SOPC/Cholesterol=6:4	3.63 \pm 0.28	9
SOPC/Cholesterol=6:4 +3mol% AmB	3.03 \pm 0.19	12

The values of the bending elasticity modulus of each studied system are calculated as a weighted average value of about 6-12 vesicles. The value of the bending elasticity modulus of pure SOPC membrane in double distilled water, measured by the same method is given for comparison [10]. The measured bending elastic modulus for 40 mol% cholesterol in SOPC membrane is in good agreement with the values reported in the literature showing the tendency of cholesterol to increase the bending elastic modulus i.e. to stiffen the membrane [12-15]. All the experiments are performed in double distilled

water environment. As it can be seen from the obtained results, the bending elasticity modulus decreases in the presence of AmB in comparison with that of pure lipid membrane for both SOPC membrane and SOPC:Cholesterol=6:4 membrane. This result is in good agreement with the theoretical predictions [16] that show that for all the admixtures, influencing the elastic modulus of lipid membranes at low concentrations in the membranes reduction of the bending elastic modulus should be observed.

3.2. Patch clamp technique

Lecithin bilayers were used to study the effect of cholesterol on AmB channels formed by several molecules self-assembled into a “bundle” structure which traverses the lipid bilayer (figure 3). Incorporation of AmB molecules into the hydrophobic core of the membrane results in the formation of molecular aggregates, which probably take the form of hydrophilic pores composed of six to nine molecules.

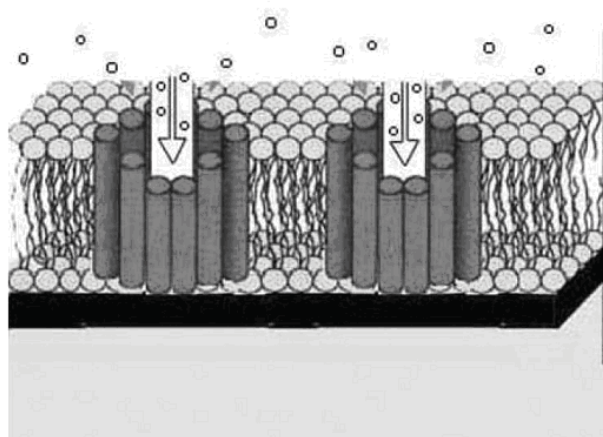


Figure 3. AmB ion channel in phosphatidylcholine bilayer model membrane.

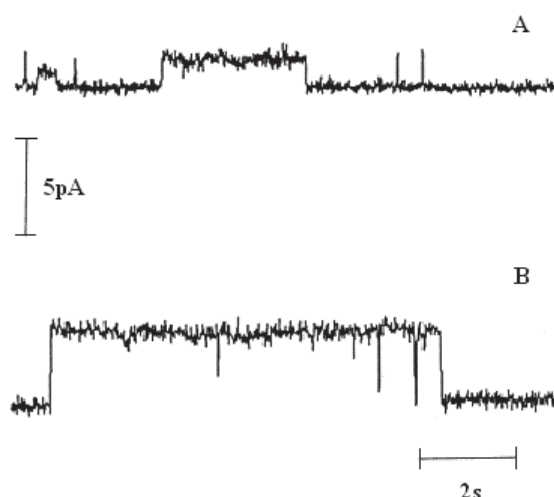


Figure 4. Records of AmB channel currents in the absence (A) and presence (B) of cholesterol in lecithin membranes (bath 100 mM KCl; pH 7.0; ± 150 mV holding potential). The concentration of cholesterol is 40 molar % (B).

Figure 4 shows an example of AmB channels recorded in the absence or presence of cholesterol in lecithin membranes. In the same lipid membrane, single-channel currents appeared at ~ 60 min after addition of amphotericin B.

In the absence of cholesterol (figure 4A), AmB channel openings were alternated with relatively long closings. We observed that amphotericin B is an ionophore and reduces the electrical resistance of the lipid membrane. AmB and cholesterol form ion channels with millisecond dwell times (figure 4B). The volt-ampere (I–V) characteristics were linear over a range of ± 150 mV. We have shown that AmB - cholesterol complexes can produce highly conductive ion channels in phospholipid membranes [17]. Ion channels with high conductivity have great potential for application in fabrication of new types of biosensors on solid supports.

3. Conclusions

Thermally induced shape fluctuations were used to study the influence of Amphotericin B on the elastic properties of pure lipid and cholesterol-containing membranes. It was shown that in both studied systems (pure SOPC and SOPC - Cholesterol membrane) the addition of 3 mol % Amphotericin B reduces significantly the bending elasticity modulus of the membrane.

Patch-clamp technique measurements showed that in the absence of cholesterol AmB channel openings were alternated with relatively long closings. Amphotericin B reduces the electrical resistance of the lipid membrane. AmB and cholesterol interact to form ion channels in the lipid membrane with millisecond dwell times. The obtained volt-ampere characteristics were linear over a range of ± 150 mV.

Acknowledgements

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