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Spectroscopic studies of molecular organization of antibiotic amphotericin B in monolayers and dipalmitoylphosphatidylcholine lipid multibilayers

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ABSTRACT

Amphotericin B (AmB) is considered the gold-standard in the treatment of serious systemic mycoses despite its numerous adverse effects. Both the mechanism of antifungal action and the toxicity of this drug are dependent on its molecular organization. The effect of AmB on the organization of lipid membranes formed with dipalmitoylphosphatidylcholine (DPPC) was studied with application of the Langmuir–Blodgett technique and ATR-FTIR spectroscopy. The aim of this research was to analyze the physical interactions leading to the formation of aggregated forms of AmB molecules in one-component monolayers and lipid multibilayers. Analysis of FTIR spectra of two-component multibilayers suggests the possibility the mutual reorientation of the amino-sugar moiety (mycosamine) and macrolide ring. This effect may be significant in the explanation of the aggregation processes of AmB in biological systems.

1. Introduction

Amphotericin B (AmB) is the metabolite *Streptomyces nodosus* and is one of the oldest polyene antibiotic that has been clinically used in the treatment of serious systemic fungal infections for more than 50 years [1]. Its structure was established in 1970 [2]. Numerous scientific works concerning its pharmacological properties and healing effects, as well as its toxicity, have been published over the course of recent years testifying to the importance of this drug in modern medicine [2–7]. The application of AmB in the treatment of severe mycosis of internal organs occurring in the aftermath of AIDS [8] and internal organ transplants is now experiencing its renaissance [2]. AmB induces many side effects such as nephrotoxic activity in the human organism. Additionally, AmB displays hepatotoxic activity, and in higher doses neurotoxic and hemolytic activities [9]. As it has been concluded from many studies, the selectivity of AmB molecules with respect to lipid membranes of fungi is mainly connected with the presence of a particular types of sterols which occur in the cellular membranes of both fungi (ergosterol) and mammals (cholesterol). The most popular concept regarding the effect of AmB on the biomembranes is directly associated with the formation of transmembrane pores which are able to cause the leakage of ions (K^+, Na^+, H^+, Cl^-) and small organic molecules from inside the cell and, in consequence, lead to the cell's death [10–14].

The membrane ionic channels formed by the AmB and sterol molecules are regarded as a crucial element of the antifungal mode of action of this drug. The stoichiometry, topology, and structure of the channel model were proposed by DeKruiff and Demel [10]. The interaction of AmB molecules with the lipid monolayers on the surface is connected with the fact that molecules of this drug form monolayers at the air/water interface. The same behavior of AmB molecules is expected at the water/membrane interface (membrane surfaces) [15]. It is considered that an AmB single molecule (monomeric forms) is not able to pass through a membrane. However, the associated species of AmB (dimers and N-aggregates) can interact with a membrane surface and then penetrate it. To pass through a membrane requires structural rearrangement of AmB molecules (horizontal and vertical position in respect to the membrane surface) and exposition of hydrophobic polyene fragment of molecule into the membrane environment [15].

The application of atomic force microscopy (AFM) studies allowed for the illustration of porous structures formed from AmB in a monolayer containing 90 mol% antibiotic and 10 mol% DPPC. The size of the pore was estimated at ~17 Å while the internal diameter came out to ~6 Å [16]. These results were confirmed by the spectroscopic data using the exciton splitting theory [17]. The low-angle technique of X-ray diffraction was also employed in the aim of examining the orientation of the AmB molecules in the lipid membrane [18]. Both the vertical and horizontal orientation of the molecules relative to the lipid membrane was asserted on the basis of analysis of the electron density profile. Such an orientation was also confirmed by ATR-FTIR linear dichroism spectroscopic studies of two-compound monolayers consisting of lipids and sterols [19].

The aspect of the amphiphilic molecular structure and the presence of carboxyl and amino groups (which are charged at neutral pH) are important for the physicochemical properties of AmB, and it

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has been significant in determining the way of thinking about the drug's mode of action. Both the pharmacological activity and the toxic side effects are strongly dependent on the molecular organization of AmB. A rigid polyene subunit limited in internal rotation, with atoms of hybridization sp^2 containing seven conjugated double bonds can be distinguished in the macrolactone ring of AmB molecule (see Fig. 1). Mycosamine in the pyranose form is glycosidically linked to the hydroxyl group at C-19 of the macrolactone ring of AmB [20]. The mycosamine moiety may change its position with respect to the macrolide ring [21]. The change of spatial arrangement is a result of the protonization of the carboxyl group in an acidic environment (pH<5) [22,23]. The orientation of mycosamine relative to the macrolactone ring plays a crucial role in the interaction of AmB with sterols, ergosterol in particular [21], in the facilitation of channel formation [12] as well as in the selective toxicity of the compound [24]. Although conformation analysis of individual AmB molecules indicates that the relative freedom of the mycosamine moiety around C19-O42 and O42-C43 (the torsion angles Φ and Ψ respectively, see Fig. 1) is restricted owing to the steric hindrance [25]. It is possible to distinguish two *open* and *closed* conformations defined by spatial orientation of angles Φ i Ψ. In the open conformation (Φ ~ −150°, Ψ~ −180°) groups —COO^- i —NH_3^+ are spatially isolated and exhibit a tendency to form intermolecular hydrogen bonds while the conformer is stabilized by electrostatic interaction with the solvent in the compound with the strongest hydration of separated ionized groups. However, in the closed conformation (Φ ~ −60°, Ψ~ −180°) it is stabilized mainly by the electrostatic contribution to the intramolecular enthalpy, arising from the short distance between the oppositely charged groups present in the AmB's polar head (—COO^- and —NH_3^+) [12,24,26,27]. The possibilities of interaction between other molecules are limited and they can only create intramolecular hydrogen bonds with the carboxyl group and water molecules [26,28,29]. Molecular dynamic methods indicate that the open geometry becomes the dominant formation in the membrane environment [24]. These effects may be of significant importance for the biological activity of the compound [30]. The understanding of spectroscopic effects related to AmB aggregation may help to understand structural changes, including mycosamine rotation, which appear during supramolecular complex formation not only in model systems, but also in biological systems as well.

2. Materials and methods

Dipalmitoylphosphatidylcholine (DPPC) and crystalline amphotericin B (AmB) were purchased from Sigma Chem. Co. (USA). AmB was dissolved in 40% 2-propanol and then centrifuged for 15 min at 15,000*g* in order to remove micro crystals of the drug still remaining in the sample. AmB was further purified by means of HPLC on YMC C-30 coated phase reversed column (length 250 mm, internal diameter 4.6 mm) with 40% 2-propanol in H_2O as a mobile phase. The final concentration of AmB was calculated from the absorption spectra on the basis of the molar extinction coefficient 1.3×10^5 M^{-1} cm^{-1} in the 0–0 absorption maximum at 408 nm. Chemicals were stored under argon in a deepfreeze.

The measurements of the monomolecular layers were carried out in a laminar hood purged with N_2 (relative humidity of 80%). Monomolecular layers of AmB were formed at the air–water interface. As a first step, AmB solution was spread from a solution to many places at the subphase (mQ water). Monomolecular layers were formed in a Teflon trough (282 mm×75 mm) and were compressed along the long side at a speed of 15 mm/min. Surface pressure was monitored by a computer-controlled tensiometer, model KSV, Helsinki, Finland. Monomolecular layers were deposited onto a solid support by means of the Langmuir–Blodgett technique (L–B films), with a speed of lift of 5 mm/min at a constant, computer-controlled surface pressure. In order to remove water residuals, thin L–B films were placed in a vacuum for 1 h. Monolayer compression and deposition was carried out at 24±1 °C.

Oriented multibilayers consisting of 50 DPPC bilayers were prepared according to the procedure described previously [31].

Electronic absorption spectra were recorded with a Cary 300 Bio UV-Vis spectrophotometer from Varian (Australia). Infrared absorption measurements (ATR-FTIR) were recorded with a Fourier-transform infrared spectrometer, model Bio-Rad FTS 185, equipped with a MCT detector and KBr beamsplitter. Prior to measurements the instrument was purged with CO_2-free dry air for 40 min. The attenuated total reflection (ATR) configuration was used with a 10-reflection Ge crystal (45° cut). Typically, 200 interferograms were collected, Fourier transformed and averaged. Absorption spectra in the region between 4000 to 600 cm^{-1}, at a resolution of one data point per 2 cm^{-1}, were obtained using a clear crystal as the background. The ATR crystals were first purged with organic solvents and then for 30 min with a "Harric" Plasma Cleaner. Spectral analysis was performed with Grams/AI software from ThermoGalactic Industries (USA).

3. Results and discussion

3.1. Aggregation effect of amphotericin B in monomolecular layers: electronic absorption spectroscopic studies

Van der Waals interactions of the AmB chromophores induce hypsochromic and bathochromic shifts in the electronic absorption spectrum which are connected with the aggregation processes of antibiotic molecules (e.g. creation of dimers) [16,32]. On the basis of Langmuir monolayers formed at the air-water interface, it is affirmed that at surface pressure close to 10 mN/m, AmB molecules have horizontal orientation, while at about 25 mN/m a vertical orientation with respect to the water surface (π-A isotherm, inset on Fig. 2) [16]. The Langmuir–Blodgett technique (L–B) was applied for the better explanation of the aggregation process between single AmB molecules in one-component monolayers. The absorption maximum centered at 401 nm (see Fig. 2) is related to the horizontally orientated forms of AmB. Upon higher surface pressure values (25 mN/m) it is considered

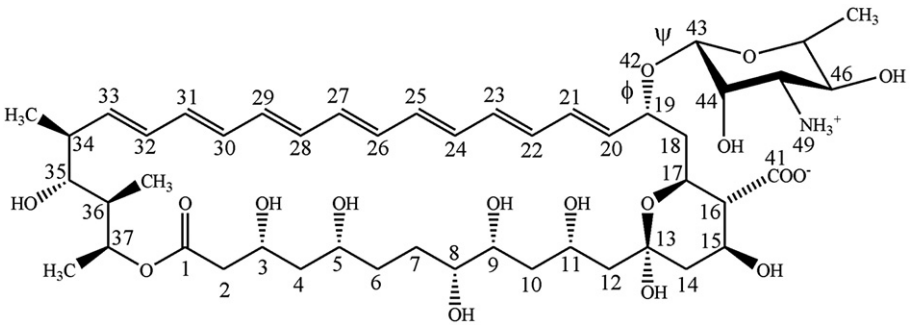

Fig. 1. Chemical structures of Amphotericin B.

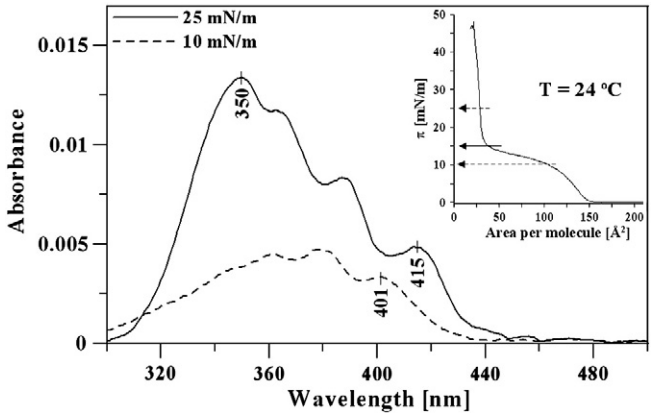

Fig. 2. Electronic absorption spectra of AmB monolayers formed from the sample dissolved in solution of 2-propanol/water (4/6, v:v) at the air–water interface. The monolayers were deposited at two sides of the quartz plate by means of the L–B technique. Surface pressure of monolayers was stabilized at 10 mN/m (dash line) and 25 mN/m (solid line). The inset presents the π-A isotherm of AmB monolayer. The dotted arrows indicate the surface pressures of L–B depositions. The solid arrow is connoted with reorientation of AmB molecules on the subphase, see Fig. 6. Temperature, 24 °C.

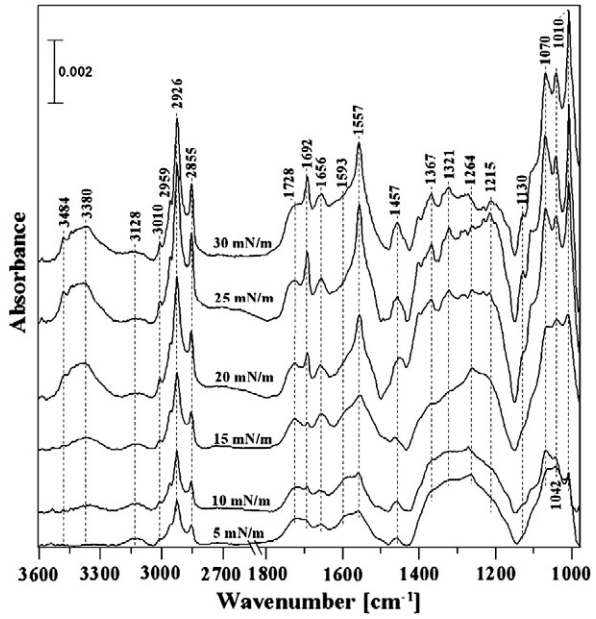

Fig. 3. ATR-FTIR absorption spectra of AmB monolayers formed from the sample dissolved in solution of 2-propanol/water (4/6, v:v) at the air-water interface. The monolayers were deposited at two sides of a Ge crystal by means of the L–B technique. Surface pressure of monolayers was stabilized at the surface pressure within 5–30 mN/m range. Temperature 24 °C.

that the spectrum with the band centered at 350 nm is related to vertically orientated forms with respect to the experimental data [16,33]. This hypsochromic spectral shift effect is connected with the aggregation processes of AmB molecules at the interface [34]. The exciton splitting theory can be applied to the analysis of the formation of molecular oligomeric forms of AmB and enable the calculation of the distances between adjacent chromophores [17,32,35,36]. The hypsochromic shift, in accordance with the aforementioned theory, is connected with the forming of H-type aggregated molecular structures (*card pack*, the transition dipole moments of molecules are orientated parallel and the out of the plane of aggregate) [17,30,36]. The spectral shift value of the 0-0 vibronic transition for the monomeric form at 401 nm (24938 cm^{-1}) to 350 nm (28571 cm^{-1}) is $\Delta\nu = 3633\ cm^{-1}$. It is possible to calculate the distances between the centers of interacting chromophores leading to the observed spectral effect on the basis of the exciton splitting theory [17]. Supposing for dimers ($N=2$) the distance between the centers of chromophores was calculated which came out to 4.4 Å. Whereas for N-aggregate structures (pores consisting of 8 molecules) it was approximately 5.6 Å [16,37]. The molecular structure of the AmB pores was confirmed by atomic force microscopy (AFM) [16]. The results obtained from AFM indicated the co-existence of two kinds of molecular distribution of AmB in the monolayer: homogenous spacing and the formation of cylindrical structures with a diameter of approximately 17 Å [16,37]. The aggregated AmB form is also represented in Fig. 2 by a bathochromically shifted band centered at 415 nm (so-called J-aggregates) [36]. It is presumed that AmB molecule interactions, for example in dimers, are most likely for those for which electronic transition from the ground level to the bottom of the exitonic band is forbidden [35,38]. Upon such an assumption it is crucial to stress that the dipole moments of the neighboring AmB molecules are not orientated parallel and that the value of the spectral shift is related to the distances between chromophores of molecules in the idealized model above [17,36]. As pointed out above, higher surface pressures (above 15 mN/m) in monolayer induce AmB aggregation [16,34]. FTIR spectroscopy was applied in order to more deeply understand the mechanisms of molecular aggregation of AmB, which was discussed above.

3.2. Langmiur–Blodgett films of AmB: ATR-FTIR studies

Fig. 3 presents ATR-FTIR spectra of AmB monolayers deposited onto the surface of a Ge crystal using the Langmuir–Blodgett technique upon surface pressures of 5, 10, 15, 20, 25 and 30 mN/m. The electronic absorption spectra presented above in Fig. 2 exhibits hypsochromic shift that is related to the molecular aggregation effect. Analysis of the FTIR spectra provides a possibility to more deeply understand AmB aggregation processes in monolayers [34]. AmB molecules are orientated horizontally at the interface at the range of surface pressures from 0 to 15 mN/m and vertically at surface pressure over 15 mN/m (the π-A isotherm, Fig. 2) [16,19]. The manner of intermolecular interaction of the AmB molecules is manifested by the spectral changes connected with the increasing of the surface pressure [34]. The spectral region between 1500 and 1800 cm^{-1} represents the stretching vibrations of the C O, —COO^-, —NH_3^+ and C C groups. As presented in Table 1, spectral changes in the region from 1500 to 1800 cm^{-1} were observed at increasing surface pressure from 5 to 30 mN/m. The absorbance of the bands centered at 1692 cm^{-1} and 1557 cm^{-1} increase during the rise of surface pressure. Dependence of intensity of the ratio of these absorption bands (1692 cm^{-1} and 1557 cm^{-1}) on surface pressure is presented in Fig. 4. As can be observed, the dependence indicates a minimum value upon pressure of 15 mN/m, in which the AmB molecules change their orientation from horizontal to vertical (see Fig. 2) [34]. The reorientation of AmB molecules to vertical position on the surface indicates the possibility of creation molecular pores. As can be seen in Fig. 3, the increase of the band in the range 3300–3500 cm^{-1} is characteristic for hydrogen-bonded AmB molecules (—OH···HO—, in polyalcohol chain of molecules). The band centered at 1728 cm^{-1} presented in Fig. 3 is attributed to the stretching vibration of the C O group in the ester band (atom C1, see structure of molecule in Fig. 1). The inductive influence of oxygen in the ester fragment of the molecule causes the increase of frequency of the C O group, resulting in changes of electron density and bond lengths. In CCl_4, the frequency of vibrations of the C O group is registered at 1745 cm^{-1} (Fig. 5, panel A). It must be added that the carboxyl group is not ionized in CCl_4, and the 1745 cm^{-1} line has been assigned to the carboxyl group coupled with the ester groups. Analysis of AmB in KBr disc [39] indicates the presence of —COO^- vibrations in the spectrum, which confirm asymmetrical and symmetrical stretching vibrations at 1692 cm^{-1} and 1401 cm^{-1}, respectively (Fig. 5, panel B) [40]. For

Table 1
The position of FTIR vibrations of AmB in KBr, CCl_4 and monolayers.

Wavenumber [cm^{-1}]			Assignment*
KBr	CCl_4	Monolayer	
851	852		δ (—CH), δ_s(—COO^-)
887	890		γ(—CH) + δ(—CH_3)
1010		1010	δ(C—C—H) for chromophore, γ(—CH) in *trans*-polyene
1040		1042	γ(N—H_2), ν_s(C—O—C) pyranose ring
1070	1085	1070	ν_{as}(C—O)
1131		1130	
1187	1167		ν_{as}(C—O—C) for β-glycosidic linkage, (C—O—C=O)
1203		1215	ν(C—O—C) for ester + δ(OH)
		1264	δ(CH_2)
1322		1321	
1338		1325	δ (CH_3) + δ(OH),
	1377	1367	
1401			ν_s (—COO^-), δ(—CH)
1449	1462	1457	δ_{as}(CH_2, CH_3)
1558		1557	δ_s(—NH_3^+) + ν_{as} COO^-
		1593	ν(C=C)
1635	1604		δ_{as}(—NH_3^+)
1692	1652	1692	ν_{as} (—COO^-)
1716	1745	1728	ν_{as}(C=O) for ester, ν(C=O) for saturated carboxylic group
	2800 - 3000		ν_{s+as}(CH_2, CH_3) + ν(CH) in polyene
3377		3380	ν(—OH), ν(N—H)

*ν—stretching mode , δ—bending in plane, γ—bending out of plane, s—symmetric vibrations, as—asymmetric vibrations.

the purpose of facilitating further discussion, AmB vibration frequencies in CCl_4, KBr and in monolayers formed from the pure compound are presented in Table 1. It can be seen that the wide band centered at 1728 cm^{-1} does not shift towards lower frequencies with the rise of surface pressure (see Fig. 3). It is observed, however, that a rather sharp and intensive band centered at 1692 cm^{-1} does appear, the origin of which has to be related to the aggregation of AmB molecules in the monolayer. As one of the possible explanations may assume, the saturated polyalcohol chain of AmB is immersed in the aqueous medium and forms hydrogen bonds with it upon low surface pressures. As

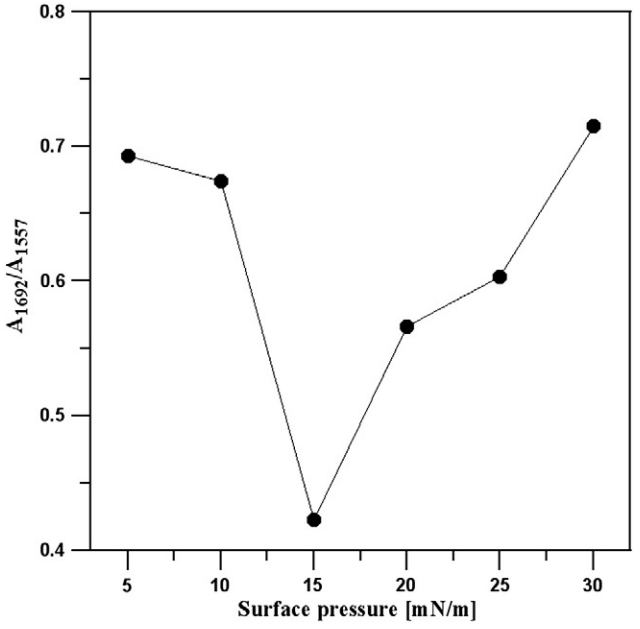

Fig. 4. Surface pressure dependence of the ratio of absorbance centered at 1692 cm^{-1} and 1557 cm^{-1} (see Fig. 3).

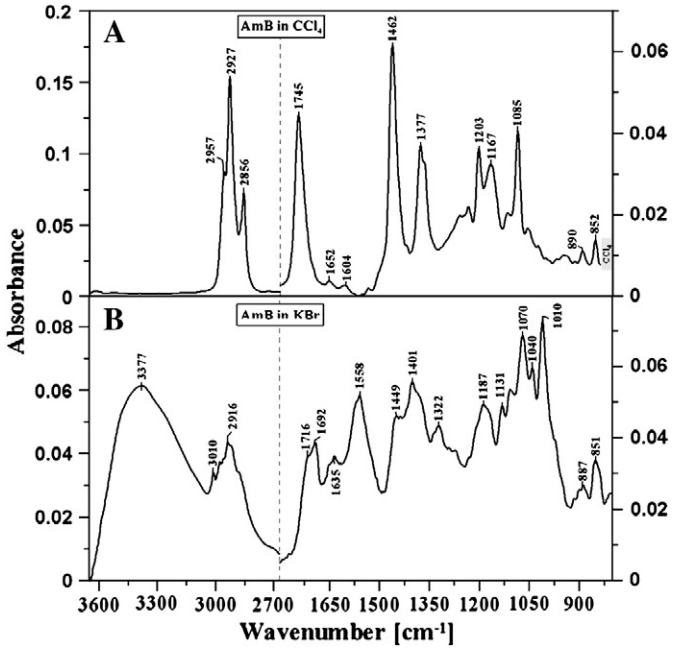

Fig. 5. ATR-FTIR absorption spectra of AmB dissolved in CCl_4 (Panel A) and KBr disc (Panel B). Measurements of AmB in CCl_4 were carried out on the solvents using a trough HATR Ge crystal plate for liquids.

presented in the hypothetical model (see Fig. 6), the increase of surface pressure causes the orientation of AmB molecules to switch from horizontal to vertical. Upon higher surface pressures, the vertical orientation in the monolayer leads to the creation of dimers as well as N-aggregate forms of AmB [16]. In respect to the origin of discussed bands cantered at 1692 cm^{-1} and 1557 cm^{-1} was carried out the simulation via Hyperchem 7.5 software, the distance between the —COO^- and —NH_3^+ groups with horizontal molecular organization came out to ~3 Å. The increase of surface pressure together with the switch to vertical orientation may result in mycosamine rotation, the distance between the groups increases to ~5 Å [21]. A change in distance of this magnitude can make a significant contribution to the electrostatic interaction between the groups, which should also affect spectral change in the ascribed range for the —COO^- and —NH_3^+ (at 1692 cm^{-1} and 1557 cm^{-1}) groups. The molecular reorientation from the horizontal to the vertical position is strongly endothermic with a high energy requirement necessary for breaking the hydrogen bonds between the hydroxyl groups from polyalcohol chain of AmB and water molecules [41]. In this case the vertical positioning and the solvation effects of the —COO^- and —NH_3^+ groups will comprise the energetic gain. Furthermore, it is necessary to add that the rise in surface pressure leads to a change in enthalpy in the solvation processes (resulting from the molecular reorientation) and structural arrangement (entropy decreases). Therefore, it seems that AmB molecules should be organized at the interface horizontally to the surface of lipid membranes in biological systems and that each switch of orientation to vertical requires additional energy [19]. The wide band in the 1500–1600 cm^{-1} range has been assigned to the C C stretching vibration coupled with the in plane —NH_3^+ bending absorption. The sharp band with a maximum centered at 1557 cm^{-1} arising together with the increase in surface pressure should be attributed to the vibrations of the —NH_3^+ group. It should be also added that the symmetrical deformational vibrations of —NH_3^+ in the amino acids in the zwitterion form appear in the range between 1550 and 1485 cm^{-1}. The increase in distances between net electrical charges of the —COO^- and —NH_3^+ groups (see Fig. 6) results in greater freedom of their vibrations. The effect of the rise of absorbance at 1692 cm^{-1} assigned to the vibrations of the —COO^- group related to the increase in surface pressure can be

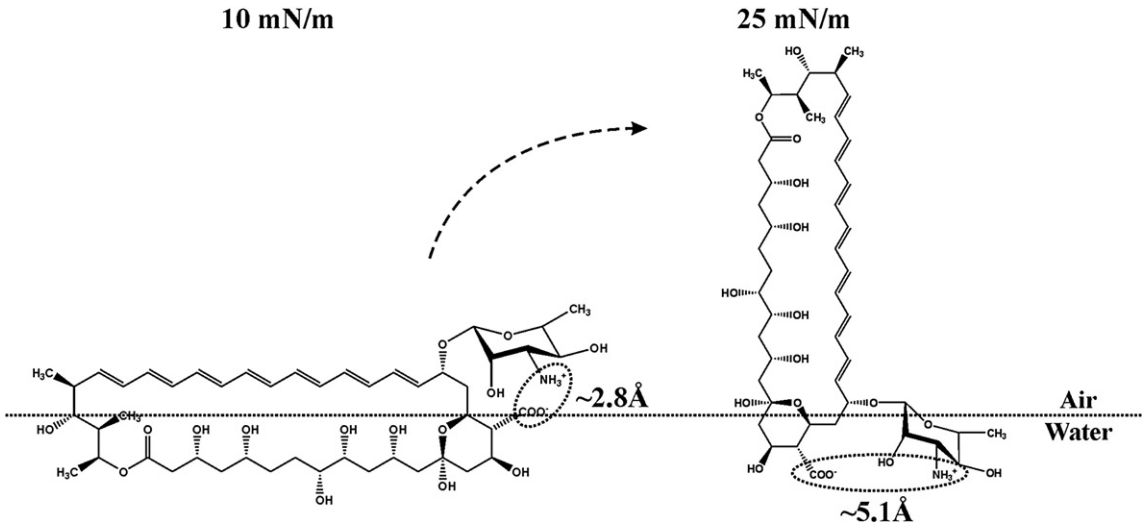

Fig. 6. Schematic hypothetical arrangement of amphotericin B molecule at the air-water interface indicated at low and high surface pressure. The distances between $—COO^-$ and $—NH_3^+$ groups for the polar head of AmB are indicated.

explained by changes of dipole moment transition in this group. It has a direct effect on the enhancement of the oscillator's strength, which is related to the increase of absorbance. Differences in distance between the ionized groups also cause the aminosugar to acquire greater freedom of rotation around the β-glycosidic linkage, see Fig. 1, (C19-O42-C43). This is indicated by the changes related to the increase in absorption in the 1100–950 cm^{-1} frequency range, characteristic for vibrations of C—O—C bonds. Studies of monolayers were carried out with the use of water (mQ) at pH = 5 (near the isoelectric point for AmB) while AmB was dissolved in a mixture of 2-propanol/water (4/6, v/v). AmB appears as a zwitterion in these conditions [23]. On the basis of research with the Raman spectroscopic study, the origin of the band with a maximum centered at 1010 cm^{-1} is related to the skeletal vibrations of the C—C—H (bending deformational in plane) connected with changes (bending distortion) in the C C—C of chromophoric chain [42,43]. Considering the AmB aggregation process in the monolayers, it must be emphasized that the change of surface pressures leads to the emerging of the molecular fragments with ester bonds from water. Further compression to higher surface pressure causes the molecules of the compound to come into close contact, which may facilitate the intermolecular interaction via hydrogen bonds formation (the wide band centered at 3380 cm^{-1}). The band that appears at 1656 cm^{-1} is most likely related to the aforementioned formation process of hydrogen bonds between water molecules and the carboxyl group of AmB. It should be added that in this region asymmetrical deformational vibrations of the $—NH_3^+$ group are also observed, but this band is usually very weak. The spectral shift from 1692 cm^{-1} to 1656 cm^{-1} is close to 36 cm^{-1} and corresponds to the typical values for hydrogen bonds. The bands in the 1330–1040 cm^{-1} range are assigned to the stretching and deformational vibrations of C—O and C—H out of plane (*trans*-polyene) bonds, which further indicates their participation in the aggregation process. At low surface pressures values, AmB molecules are orientated horizontally in the monolayer and can form dimeric structures through electrostatic interactions of the $—COO^-$ and $—NH_3^+$ groups and van der Waals interaction between chromophores (which is indicated by the electronic absorption spectra, Fig. 2). When the monolayer is compressed to higher value of surface pressures, molecular reorientation causes increased distance between these groups. The molecular interactions at surface pressure close to 25 mN/m are related to the creation of highly aggregated forms [34]. Such a molecular rearrangement connected with progressive changes of molecules orientation from a horizontal to a vertical position on the water surface corresponds to the creation of molecular pores [10,17]. The arising pore is built of a few molecules (e.g. 8) such that the hydroxyl —OH groups are pointing to the center and the hydrophobic heptaene chain to the outside of the pore [30]. The band of C C stretching vibrations appears along with the increase of absorbance in the region of 1600–1500 cm^{-1} (wide band, also assigned to vibrations of the $—NH_3^+$ group). The increase of absorbance in the frequency range of 1100–950 cm^{-1} upon surface pressure of 20 mN/m (the vertical orientation of AmB molecules) is also related to C—O—C vibrations in both the β-glycosydic linkage and the pyranose ring [44–47]. The band related to symmetrical deformation vibrations of the methylene substituent with a maximum at 1457 cm^{-1} is characteristic for scissoring vibrations. Asymmetrical and symmetrical C—H (alkyl) stretching vibrations appear as intense bands with maxima within the 2800–3000 cm^{-1} range. The effect of the increase of intensity of the band centered at 2957 cm^{-1} is related to asymmetrical stretching CH_3 vibrations in the AmB spectra in CCl_4 (see Fig. 5). The band centered at 1377 cm^{-1} is assigned to symmetrical vibration of $\delta(CH_3)$ coupled with δ(OH) in the non intermolecular hydrogen bonded polyalcohol chain, in CCl_4, this is quite likely. The band is not initially very intense during the monolayers compression but in the condensed state of the monolayer (high surface pressure) it appears at 1367 cm^{-1}. In the AmB condensed monolayer by compressing, the frequency of symmetrical deformation vibrations of the methyl group is related to the aggregation effects of the AmB molecules. With these spectral effects, one cannot exclude the participation of the methyl groups of the AmB molecule in the association process, particularly in hydrophobic interactions [48]. The formation of molecular pores is related to the influence of the hydroxyl AmB groups, whose bands appear in the region of deformational vibrations of the C—O bond. Stretching vibrations of C—O coupled with deformational vibrations of the —OH group decrease intensity in CCl_4. They are shifted towards lower frequencies in pure AmB monolayers (1215 cm^{-1}), while centered at 1187 cm^{-1} in KBr. This shift is related to the involvement of the —OH group in the creation of hydrogen bonds between AmB molecules in molecular pores [10].

3.3. DPPC multibilayers containing AmB: studies by means of ATR-FTIR spectroscopy

ATR-FTIR multilayer spectra formed from 50 bilayers, prepared with DPPC lipid and AmB (5 mole% AmB in DPPC) as well as multilayers formed from pure DPPC are presented in Fig. 7A. A differential spectrum (subtracted spectrum: 5 mole% AmB in DPPC minus pure DPPC) is presented in panel B (Fig. 7). For the purpose of considering what is presented above, a 5 mole% concentration was selected in the case of the distinctly visible aggregation effects of AmB in lipid membranes [17,35]. It is therefore possible to analysis of the

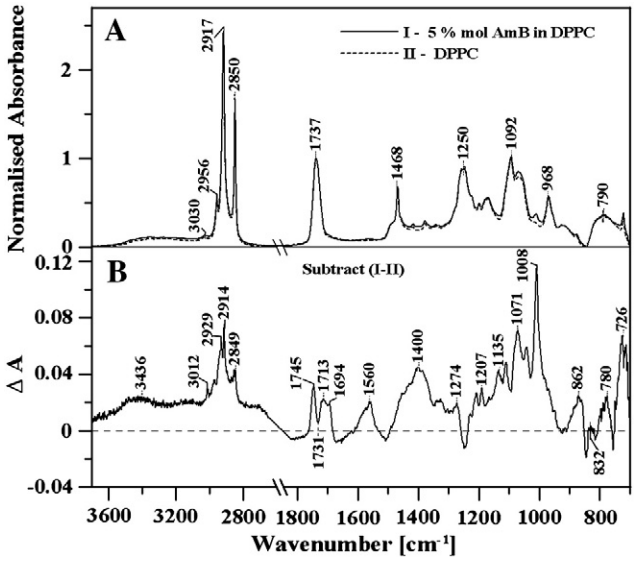

Fig. 7. ATR-FTIR absorption spectra recorded from the 50 bilayers formed from 5 mol% of AmB in DPPC (solid line) and pure DPPC bilayers (dashed line) formed at the same condition (Panel A), deposited at the Ge crystal. Note that the spectra were normalized in the maximum of the band at 1737 cm^{-1}. Panel B presents differential spectrum obtained by the subtraction of two-component multibilayers composed of AmB and DPPC from pure DPPC sample. Temperature, 24 °C.

aggregation of AmB molecules in the lipid multibilayers formed from DPPC [35,42]. Electronic absorption spectroscopic studies indicate the aggregation processes of AmB in lipid multibilayers. FTIR spectroscopy was carried out in order to better understand the mechanisms of these processes [31,35,49–51].

As presented in Fig. 7, changes in the differential FTIR spectrum are visible in the entire spectral region. Noteworthy is the wide band with its center-wavelength position at 1560 cm^{-1}, which as mentioned above is related to the C C stretching vibrations and coupled with—NH_3^+ deformational vibrations. The band centered at 1400 cm^{-1} is assigned to symmetrical stretching vibrations of the C—O in the —COO^- group. It should be added that this frequency is also considered for CH_3 deformational vibrations which does not occur in the spectrum of pure DPPC. This insight is unusually helpful in the analysis of the molecular organization of AmB in this lipid [19]. This result unambiguously indicates the participation of the carboxylic group in the process of AmB molecular aggregation in the lipid membrane. We assume the electrostatic interactions of the —COO^- and —NH_3^+ groups from AmB as well as —N^+ $(CH_3)_3$ and —PO_2^- from DPPC to be the likely mechanism [16,35], which have already been proposed in the theoretical calculation proposed by Bagiński et al [52]. The sharp intensive band centered at 1008 cm^{-1} is assigned to the C—C—H skeletal vibrations in AmB chromophore which can be related to the aggregation of AmB molecules [42]. The band in the spectral region of 1600–1800 cm^{-1} is assigned to the stretching vibrations of the C O in the lipid's ester group of DPPC. The band position centered at 1694 cm^{-1} (see also Fig. 3) is assigned to the asymmetrical stretching vibrations of the —COO^- group. These results confirm the participation of the —COO^- and —NH_3^+ groups and the C C conjugated bonds from chromophore (by van der Waals interactions) in the aggregation process of AmB molecules in the lipid membranes [19]. The wide band with its center-wavelength position at 3436 cm^{-1} is related to the —OH and —NH_3^+ vibrations also indicate the aggregation of AmB molecules in the multibilayer. A strong wide band centered at 1250 cm^{-1} (upper panel in Fig. 7) is attributed to the asymmetrical stretching vibrations of the —PO_2^- group in DPPC [53]. Symmetrical vibrations of —PO_2^- group appear in the spectral region of 1095–1085 cm^{-1}. The incorporation of AmB molecules into lipid multibilayers does not lead to significant changes in the region of its vibrations, neither in monolayers nor lipid multibilayers [19]. This effect is consistent with similar results obtained for multibilayers formed from AmB and EYPC where the influence of compound concentration on these band intensities was reported [18]. These properties may explain the effect of choline —$N^+(CH_3)_3$ charge on the dissociation of the phosphoric acid residue (lack of electrostatic interaction, P-OH group is not ionized). These changes may decrease the possibility of interactions between lipids and AmB molecules. It must be added that the —PO_2^- group is an analogue (isostructure) of the —COO^- group, which should undoubtedly facilitate the interaction of AmB with lipids. The changes in the differential spectrum in the range of asymmetrical stretching vibrations of the —$N^+(CH_3)_3$ group (centered at 3012 cm^{-1}) and symmetrical mode from the region of ~1400 cm^{-1} are related to AmB interaction with that group. The band centered at 1071 cm^{-1} is assigned to the C—O—C symmetrical stretching vibrations (see Fig. 1, C19, O42, O43) linking mycosamine moiety to the macrolactone ring of AmB. These results allow us to conclude that AmB molecules mainly effect the hydrophilic (choline) part of lipid head group [19,54]. The existence of an elution band centered at 1694 cm^{-1} in the differential spectrum, whose origin is attributed to the asymmetric vibrations of the carboxylic group, indicates its involvement in the aggregation process.

4. Conclusion

On the basis of the findings presented in this study, it is important to note that AmB molecules form aggregated structures at the air/water interface. The first stage is a dimerization process at low surface pressures, when molecules are orientated horizontally on the surface. In the next stage there is association of AmB dimers in the monolayer that are vertically orientated on the water surface, which is forced by the rise of the surface pressure. The aggregation process of AmB in the monolayer is closely related to the interaction of functional groups of the AmB molecule such as: —COO^-, —NH_3^+, —OH and C C bonds. Interaction of AmB zwitterions, as results from earlier considerations, has a significant effect on the aggregation process of the molecules. Elements of resemblance of the fragment of FTIR spectra of AmB in the compressed monolayer and lipid multibilayer allow us to conclude an analogous aggregation mechanism of the antibiotic in both systems. The involvement of the —COO^- and —NH_3^+ groups in the aggregation process affects AmB molecules in both the monolayer and the lipid membrane. The spectral effects attributed to mycosamine moiety position with regard to macrolide ring related to the AmB aggregation process allow us to assume that aminosugar can freely rotate with respect to the macrolide ring of AmB. This property is important in the molecular interaction process of AmB with lipids and is crucial for the illumination of the mechanisms of interaction of the antibiotic's molecules with cellular membranes.

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