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Molecular organization of antifungal antibiotic amphotericin B in lipid monolayers studied by means of Fluorescence Lifetime Imaging Microscopy

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ABSTRACT

Amphotericin B (AmB) is a life-saving polyene antibiotic used to treat deep-seated mycotic infections. Both the mode of therapeutic action as well as toxic side effects are directly dependent on molecular organization of the drug. Binding of AmB to lipid monolayers formed with dipalmitoylphosphatidylcholine, pure and containing 40 mol% cholesterol or ergosterol, the sterols of human and fungi respectively, has been examined by means of Fluorescence Lifetime Imaging Microscopy. AmB emits fluorescence with the characteristic lifetimes dependent on actual molecular organization: $\tau_{M2} \leq 10$ ps and $\tau_{M1} = 0.35$ ns in the monomeric state, the emission from the S_2 and the S_1 states respectively and $\tau_D = 14$ ns and $\tau_A = 3.5$ ns in the form of a dimer and associated dimers respectively. Analysis of the Langmuir–Blodgett films reveals that AmB binds to the lipid membranes and to the cholesterol-containing lipid membranes preferentially in the form of associated dimers. The same form of AmB appears in the membranes containing ergosterol but additionally the monomers and dimers of the drug. can be observed, which can severely affect molecular organization of the lipid membrane. The results are discussed in terms of selectivity of AmB towards the ergosterol-containing biomembranes of fungi.

1. Introduction

Amphotericin B (AmB, Fig. 1) is a life-saving polyene antibiotic used to treat deep-seated mycotic infections [1,2]. Despite very strong side effects and toxicity to patients AmB is constantly being applied owing to its pharmaceutical effectiveness [3]. Severe mycotic infections accompanying AIDS are frequently treated with AmB formulations. Both the pharmacologic as well as toxic side effects of AmB are associated with specific molecular organization of the drug. Efforts of multiple research laboratories in the world are directed toward elaboration of a formulation of the drug characterized by minimized toxic side effects but still preserving the antibiotic activity toward fungi. In this respect, understanding of molecular mechanisms of biological action of AmB seems to be a primary target [1,2].

According to a popular conviction AmB present in biomembranes associates into molecular aggregates in the form of transmembrane pores that affect physiological ion transport [4–8]. The concept that such structures are formed more efficiently in the presence of ergosterol, the sterol present in the membranes of fungi, than in the presence of cholesterol is a key paradigm of selectivity of AmB. It has been also proposed that selectivity toward cells of fungi is based upon a difference of the radii of porous structures of AmB binding ergosterol and cholesterol [6,9]. On the other hand, very recent reports show that alternatively both the biological action of the drug as well as toxic side effects may be directly related to the effect of AmB on physical properties of the membranes [10–13]. The ^{1}H NMR technique studies demonstrated that the polar headgroup region of the membranes, rather than the hydrophobic core, is a predominant site of binding of AmB from the water phase [12]. Analysis of the effect of AmB on structural and dynamic properties of lipid membranes carried out with application of SANS (small angle neutron scattering), ^{1}H NMR and FTIR techniques showed that AmB molecules, even bound to the lipid polar groups, influence motional freedom of acyl lipid chains [11–13]. This effect is particularly pronounced in the case of the lipid membranes containing ergosterol but less in the membranes containing cholesterol. Linear dichroism studies carried out with UV–Vis and FTIR absorption spectroscopy techniques revealed also that the membrane-bound AmB molecules are distributed between two pools: one oriented parallel and the other one perpendicular with respect

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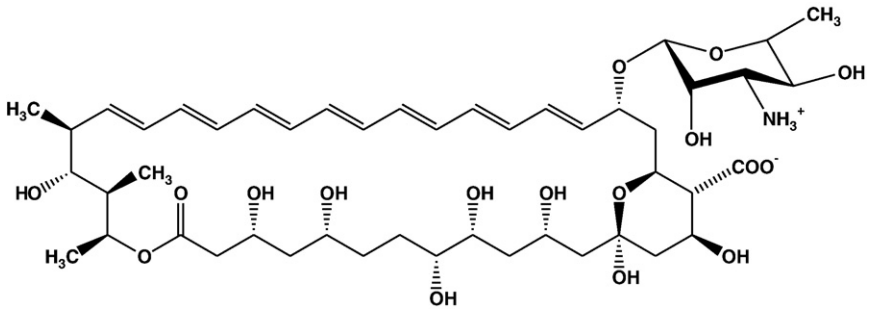

Fig. 1. Chemical structure of amphotericin B.

to the plane of the membrane [13–15]. It appeared that a laterally-oriented pool was particularly large in the membranes containing ergosterol and that such orientation of the drug had strongest effect on the membrane organization [13]. According to our earlier studies, AmB is able to form hydrophilic pores which can disturb transmembrane ion transport [16], indeed, but the results of the most recent studies, outlined above, seem to suggest that molecular mode of action of AmB with respect to biomembranes is primarily based on interference with the structural and dynamic properties of the lipid bilayers. This hypothesis requires further verification. In particular the finding that the effect of AmB on membranes is totally dependent on molecular organization of the drug (monomers and dimers display the opposite effect as compared to the larger aggregates) awaits interpretation and explanation at the molecular level. AmB at low concentrations in the lipid phase restricts the transmembrane proton transfer but acts in the opposite direction at higher concentrations (molar fractions above 3 mol%) [17].

According to our earlier report [15], confirmed recently by Stoodley et al. [18], AmB displays measurable fluoresce which can be analyzed, providing that fluorescing contaminations are separated [19]. Despite relatively low quantum yield fluorescence can be applied to distinguish different organization forms of molecules of the drug in different environments (even in not transparent natural samples). In particular, the presence of AmB dimers can be detected by means of fluorescence spectroscopy, owing to distinguished spectral signature. Detection of AmB dimers by means of other spectroscopic techniques is extremely difficult, due to spectral overlap with large aggregated structures. It appeared only possible, so far, in a very simple and well defined model system such as a monomolecular film [20]. Interestingly, very recent reports show that, most probably, more complex molecular structures of the drug are formed out of dimers [21].

In the present work we apply the fluorescence lifetime measurements and FLIM (Fluorescence Lifetime Imaging Microscopy) in order to understand more deeply molecular mechanisms responsible for the effect of AmB on biomembranes as well as toxic side effects and selectivity toward fungi.

2. Materials and methods

2.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC), cholesterol, ergosterol 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 15000 ×g in order to remove micro crystals of the drug that may still remain 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 $1.3\times10^5\ M^{-1}\ cm^{-1}$ in the 0–0 absorption maximum at 408 nm.

2.2. Monomolecular layers

AmB was dissolved in water alkalized to pH 12 with KOH and then centrifuged for 15 min at 15000 ×g in order to remove microcrystals of the drug, still remained in the sample. A stock solution of AmB was adjusted to 1 mg/ml. Lipids (DPPC, cholesterol and ergosterol) were dissolved in chloroform. Monomolecular layers of pure DPPC and two-component monolayers composed of 60 mol% DPPC and 40 mol% of sterols were formed at the air–water interface. Monolayers were formed at the air–water interface and the water subphase was buffered with 10 mM Hepes, pH 7.2. Monomolecular layers were formed in computer-controlled Minitrough2 system from KSV Helsinki (Finland) composed of a Wilhelmy type tensiometer and of a Teflon trough (364 mm × 75 mm). Monolayers were compressed along the long side with a speed of 15 mm/min. After compression of the lipid monolayers to the surface pressure of 22 mN/m the solution of monomeric AmB in water alkalized to pH 12 was injected into the water subphase, beneath the monolayer. Final concentration of AmB in the subphase was 2.2 μM. Monomolecular layers were deposited to non-fluorescent quartz glass slides (10 mm × 20 mm) by means of the Langmuir–Blodgett technique (L–B films), with a speed of the lift 5 mm/min at a constant, computer-controlled surface pressure which

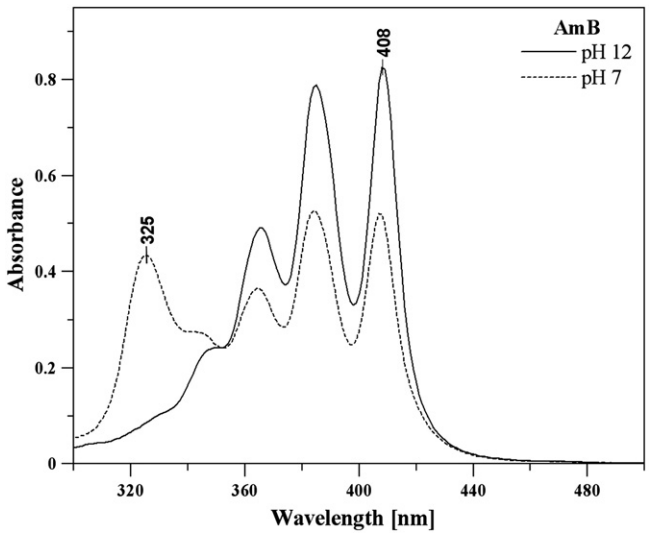

Fig. 2. Electronic absorption spectra of amphotericin B in water medium alkalized with KOH to pH 12 and 7, indicated. Sample concentration 6.2 μM.

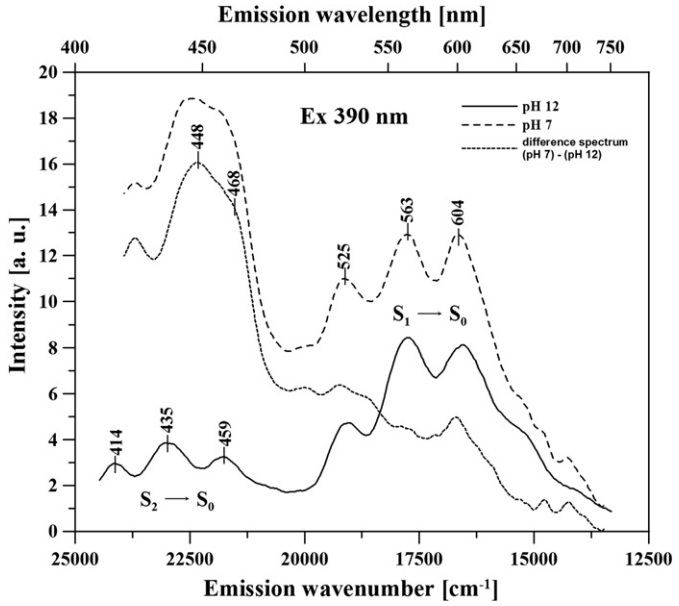

Fig. 3. Fluorescence emission spectra of amphotericin B dissolved in water medium at pH 12, pH 7 and the difference spectrum, indicated. Excitation was at 390 nm. Two emission bands visible in monomeric AmB (at pH 12) represent fluorescence emission from the S_1 and S_2 energy levels, as indicated. The two bands of the fluorescence emitted from the AmB sample at pH 7 correspond to the de-excitation of molecular dimers (in the spectral region 430–480 nm) and larger aggregated structures (emission above 500 nm). The AmB aggregate-related bands are represented by the difference spectrum. Sample concentration 3.7 μM.

was the surface pressure at which deposition was initiated. Monolayer compression and deposition was carried out at 25 ± 1 °C.

2.3. Spectroscopic measurements

Electronic absorption spectra were recorded with a Cary 300 Bio UV–Vis spectrophotometer from Varian (Australia). Fluorescence spectra were recorded with Eclipse spectrofluorometer from Varian (Australia). Fluorescence decay kinetics were measured with Fluo Time 200 time correlated single photon counting system from PicoQuant Inc. (Berlin, Germany) equipped with pulsed laser diodes and LEDs excitation sources, and ultra fast MCP detector. In the case of temperature-dependent fluorescence lifetime measurements the K2 Multifrequency Cross-Correlation Phase and Modulation fluorometer (ISS, USA) was applied. Fluorescence Lifetime Imaging Microscope (FLIM) images were collected with the MT 200 system from PicoQuant Inc. (Berlin, Germany) coupled with Olympus IX71 objective and Perkin Elmer APD (SPCM-AQR-14) detection system. The details of fluorescence lifetime and FLIM measurements were in more detailed form described previously [22]. In order to avoid possible parasite fluorescence signal originating from a glass support or lipid contaminations all the FLIM recordings were performed with the laser excitation at 405 nm. Such excitation wavelength assures also that traces of short polyenes, that could be possibly present in the AmB preparation, despite the HPLC purification [23], cannot interfere with AmB fluorescence experiments. On the other hand, owing to the fact that the H-type molecular assemblies of AmB are not characterized by rigorously parallel orientation of the dipole transitions of neighboring molecules, the electronic transitions from the ground energy level of AmB to the lowest excitonic energy levels of the dimeric and aggregated structures are slightly allowed [20,21]. This provides possibility to excite all the molecular organization forms of AmB at wavelengths longer than 390 nm.

3. Results and discussion

Fig. 2 presents the electronic absorption spectrum of AmB dissolved in water medium alkalized to pH 12, in order to assure monomeric organization of the drug [21]. The main absorption band, with the 0–0 vibrational maximum at 405 nm, corresponds to the electronic transition from the ground energy level (1^1A_g) to the so-called S_2 energy level (1^1B_u) [15,21]. The direct electronic transition from the S_0 to the S_1 energy level (2^1A_g) is optically forbidden due to the symmetry reasons. Nevertheless, emission from both the energy levels, S_1 and S_2, can be observed after excitation of the S_2 state of AmB: the direct emission and also the emission from the S_1 state after the non-radiative $S_2 \rightarrow S_1$ relaxation (see Fig. 3). All those processes are displayed in the energy level diagram presented in Fig. 4.

Fluorescence lifetime measurements of monomeric AmB excited at 405 nm show the single decay component characterized by $\tau_{M2} \leq$ 10 ps (the time resolution limit of the apparatus is below 10 ps [22]),

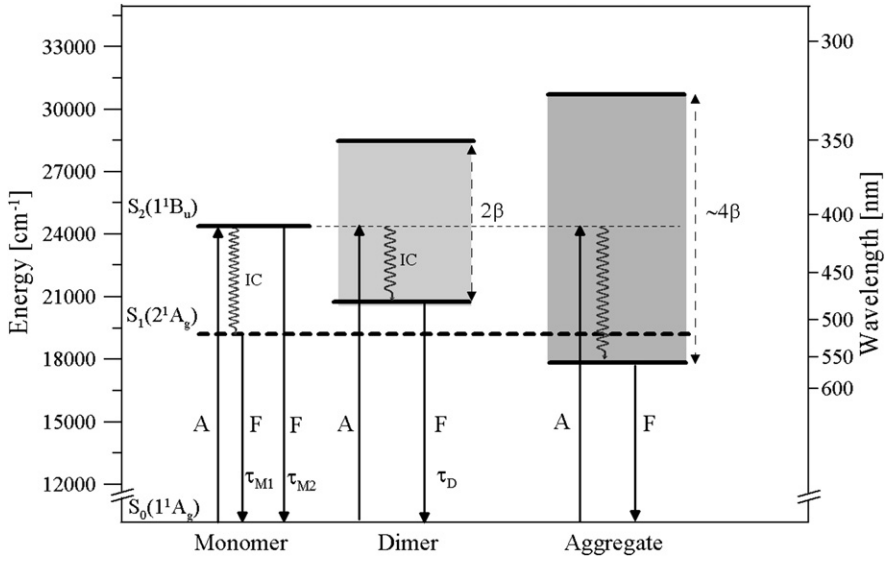

Fig. 4. Energy level diagram of amphotericin B in monomeric, dimeric and aggregated state. Position of the energy levels based on the spectroscopic data discussed in the text. A denotes light absorption, F fluorescence, IC internal non-radiative conversion. Shaded areas represent vibronic energy levels.

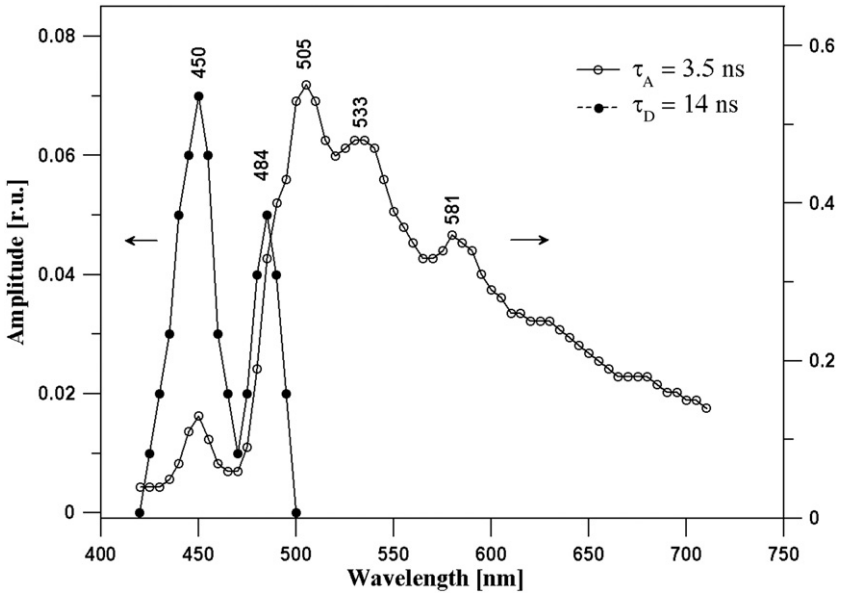

Fig. 5. Emission wavelength dependencies of the relative amplitude of the fluorescence lifetime components as indicated. Excitation wavelength 405 nm.

while detected at 430 nm, and the single decay component characterized by $\tau_{M1} = 0.35$ ns, while detected at 705 nm. Since those detection wavelengths correspond directly to light emission from the S_2 state (430 nm) and the S_1 state (705 nm) the fluorescence lifetime parameters determined can be unequivocally assigned as representing the radiative de-excitation of those energy states. The characteristic fluorescence lifetime parameters determined here correspond well to the fluorescence lifetimes reported for the S_1 and S_2 emission from the polyenes formed by 7 conjugated double bonds [24–27].

AmB dissolved in water medium remains in a partially aggregated state in a broad pH range below 9.0 [21]. Owing to this fact, the electronic absorption spectrum of AmB recorded in water medium (pH 7.0) represents more complex structure than the spectrum of the drug in the monomeric state (Fig. 2). In particular, the short wavelength spectral components represent the high energy excitonic states characteristic of the dimeric and also more complex organization forms of AmB [21,28] (see Fig. 4). Excitation of AmB in such a sample at 340 nm results in fluorescence characterized by different lifetime components $\tau_D = 14$ ns (92%) and $\tau_A = 3.5$ ns (8%) while detected at 430 nm and very similar lifetimes and relative fractions while detected at 490 nm. Owing to the fact that the excitation wavelength corresponds to the maximum absorption of AmB the dimeric structure and the fluorescence emission detection was at the energies much higher than it might be expected for an emission from large aggregates of AmB (fluorescence emission expected from the "bottom" of the excitonic band, see Fig. 4) both the fluorescence lifetime components can be assigned to the de-excitation of the dimeric structures. Excitation of AmB in the sample at pH 7.0, containing both the monomeric and oligomeric spectral forms (see Fig. 2), at 405 nm gives rise to fluorescence characterized by the same lifetime components as monomeric AmB but additional components $\tau_D =$ 14 ns and $\tau_A = 3.5$ ns have been found, the same as in the case of excitation at 340 nm, in relative amplitudes dependent on actual emission wavelength. Fig. 5 presents the spectral distribution of relative fractions of both the components assigned to fluorescence of AmB dimers. The fact that the shorter component (3.5 ns) is also detected in the longer wavelength spectral region suggests that this particular component may represent a molecular fraction of the AmB dimers quenched, for example as a result of association of dimers into more complex molecular structures (pairs of dimers, etc.). The amplitudes of the major fluorescence lifetime components assigned to monomers ($\tau_{M1} = 0.35$ ns) and dimers ($\tau_D = 14$ ns) in solution of AmB in 40% 2-propanol at different temperatures were analyzed in terms of the Van't Hoff equation [15] (Fig. 6). As can be seen, the dimer association energy determined in the present work on the basis of the fluorescence lifetime measurements, 5.9 kJ/mol, is very close to the one reported previously [15].

The results of research on incorporation of AmB into lipid membranes (briefly outlined in the Introduction section) let draw relatively complex picture, with fraction of molecules oriented in the plane of the membrane and perpendicular to it and with molecules penetrating the hydrophobic core of the membrane and anchored in the polar headgroup region. A basic and important information which is still missing is molecular organization of AmB spontaneously bound to the lipid membrane from the water phase. In the present work we applied the Fluorescence Lifetime Imaging Microscopy (FLIM) to address this problem. Monomolecular layers composed of DPPC

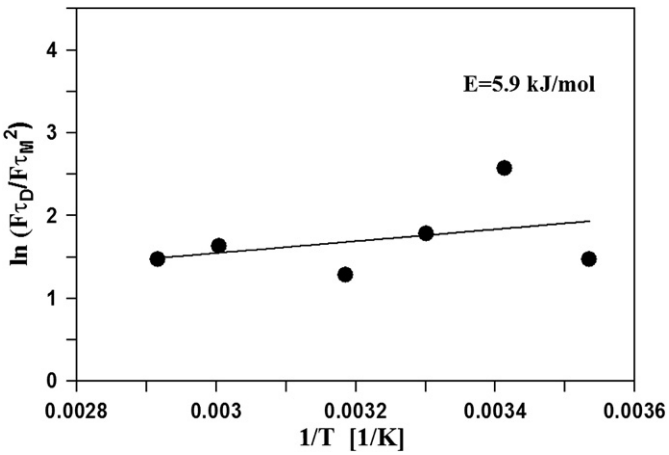

Fig. 6. Van't Hoff plot of the ratio of amplitudes of the fluorescence decay components assigned to the AmB dimers ($\tau_D = 14$ ns) and monomers ($\tau_M = 0.35$ ns). The sample was in 40% 2-propanol. The intensity average lifetime amplitudes were calculated according to the equation $F = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i}$.

and containing cholesterol or ergosterol, the sterol present in the organisms of fungi, have been subjected to interaction with AmB dissolved in the water subphase and then deposited to glass support by means of the Langmuir–Blodgett technique. Such a protocol yields efficient binding of AmB to the lipid membranes as described in our previous work [13]. Fig. 7 presents the electronic absorption spectra of such films. Despite very low optical density of the samples it can be concluded that organization of AmB in the membranes is heterogeneous, what is manifested by relative complexity of the spectra (compare with Fig. 2). The highest absorbance level recorded in the samples containing ergosterol, reflects that binding of AmB to the membranes from the water phase is most effective in such a system. Despite that, even in the case of the ergosterol-containing membranes the signal to noise ratio in the spectra recorded, is not high enough to allow precise analysis of molecular organization of AmB. Fig. 8 presents the FLIM images based on the fluorescence lifetimes of AmB bound to the lipid monolayers formed with pure DPPC and containing 40 mol% of cholesterol or ergosterol. As can be seen, organization of AmB in all the systems studied is highly heterogeneous. In pure DPPC (Fig. 8A), fluorescence lifetime analysis shows that entire AmB associated with the membrane remains in the form of molecular assemblies: no lifetime components shorter than 1 ns, diagnostic of monomers, and longer than 13 ns, diagnostic of separated dimers, have been detected. Very similar molecular organization of AmB has been observed in the DPPC membrane containing cholesterol (Fig. 8B). Interestingly, in the case of the membranes containing ergosterol (Fig. 8C), the distribution of AmB is much more homogeneous than in the case of the other systems discussed above. Moreover, the short lifetime components (below 1 ns) indicate presence of AmB in the monomeric form and the long lifetime components (above 13 ns) indicate presence of the AmB dimers, in addition to the most abundant more complex molecular structures. The presence of such long lifetime components may be alternatively interpreted in terms of shortening of the conjugation length, e.g. by sample degradation or formation of intrinsic π-type hydrogen bond with mycosamine which can gain relative rotational freedom [21]. On the other hand, the fact that the excitation wavelength was at 405 nm, definitively beyond the absorption of polyenes shorter than heptaene, contradicts such an interpretation. The fluorescence lifetime analysis shows that AmB binds to the membranes containing ergosterol as a monomer. The presence of AmB in the monomeric form in the ergosterol-containing membranes can be also deduced directly from the absorption spectra (Fig. 7) and is manifested by very clear vibronic structure characteristic of monomers (Fig. 2).

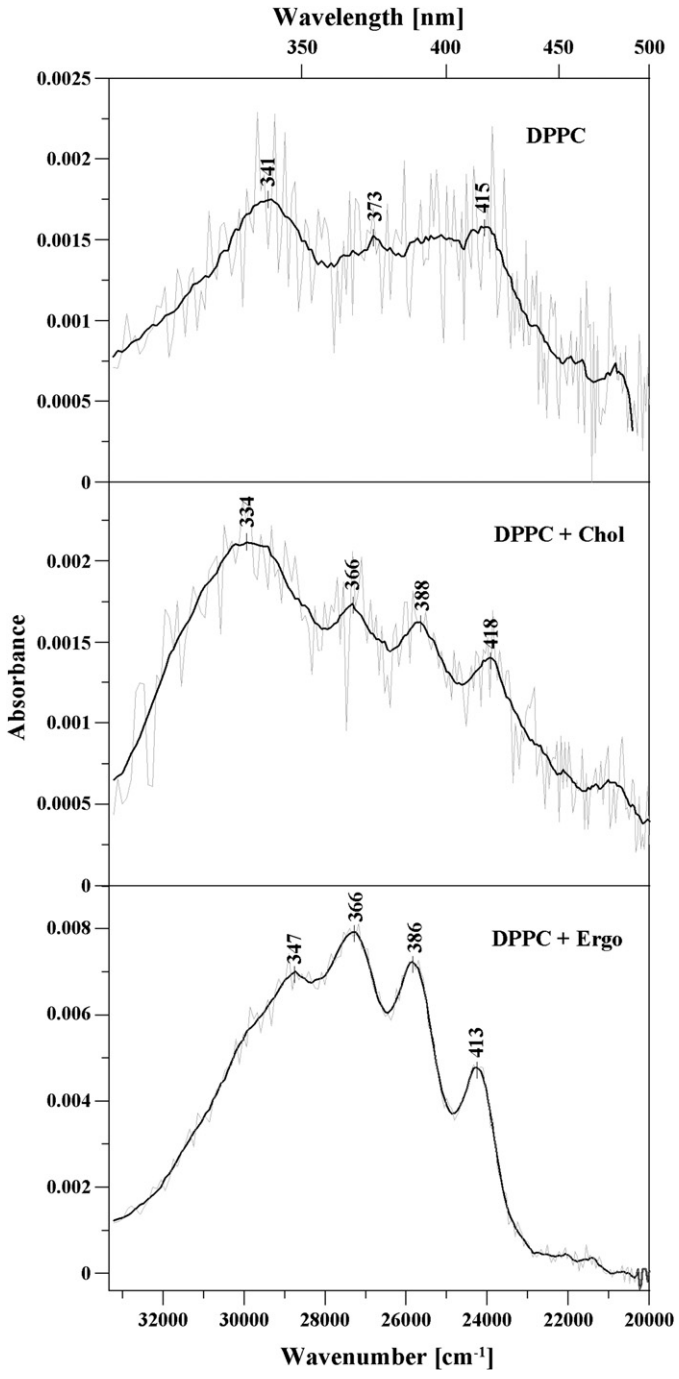

Fig. 7. Electronic absorption spectra of two monomolecular layers, each deposited at the opposite side of the quartz glass sidle by means of the Langmuir–Blodgett technique from the monomolecular layers. Monolayers were formed with pure DPPC. DPPC + 40 mol% cholesterol and DPPC + 40 mol% ergosterol, indicated. 40 min before the film deposition the solution of monomeric AmB, prepared in water alkalized to pH 12 with KOH, was injected beneath the lipid layer (into the water subphase). In order to increase the signal the sample was tilted by 45° (the angle between the axis normal to the plane of the sample and the direction of the measuring light beam). Original spectra are drawn with gray lines and smoothed spectra are drawn with black lines.

FTIR measurements show that majority of the AmB molecules which bind to the membranes are localized in the polar headgroup region [13]. The results of the linear dichroism–FTIR measurements are consistent with the microscopic picture according to which the molecules of the membrane-bound AmB are distributed among two orientational fractions: one horizontal and one vertical with respect to the plane of the membrane [13]. The presence of ergosterol has a pronounced effect in the increase in population of the fraction of horizontally bound AmB (85% vs. 59% in the pure membrane) [13]. The more preferred horizontal orientation of AmB molecules in the ergosterol-containing membranes coincides with the observation made in the present study that the drug appears also in the monomeric form in such a system. It is therefore possible that molecules of monomeric AmB are anchored in the polar headgroup zone and are able to adopt exclusively the lateral orientation with respect to the plane of the membrane.

The results of the experiments reported in this work seem to be particularly interesting in terms of understanding differences in molecular activity of AmB towards lipid membranes containing cholesterol and the membranes of fungi containing ergosterol. AmB monomers and dimers are believed to bear most powerful potential in severely disturbing physiological biomembranes activity: monomers, owing to the amphiphilic character of the molecule (see Fig. 1) that cannot fit into the lipid membrane without disturbing its organization and dimers owing to the potential of formation of hydrophilic membrane channels [20]. In our opinion, such a finding sheds light on the molecular mechanisms of selectivity of AmB towards the

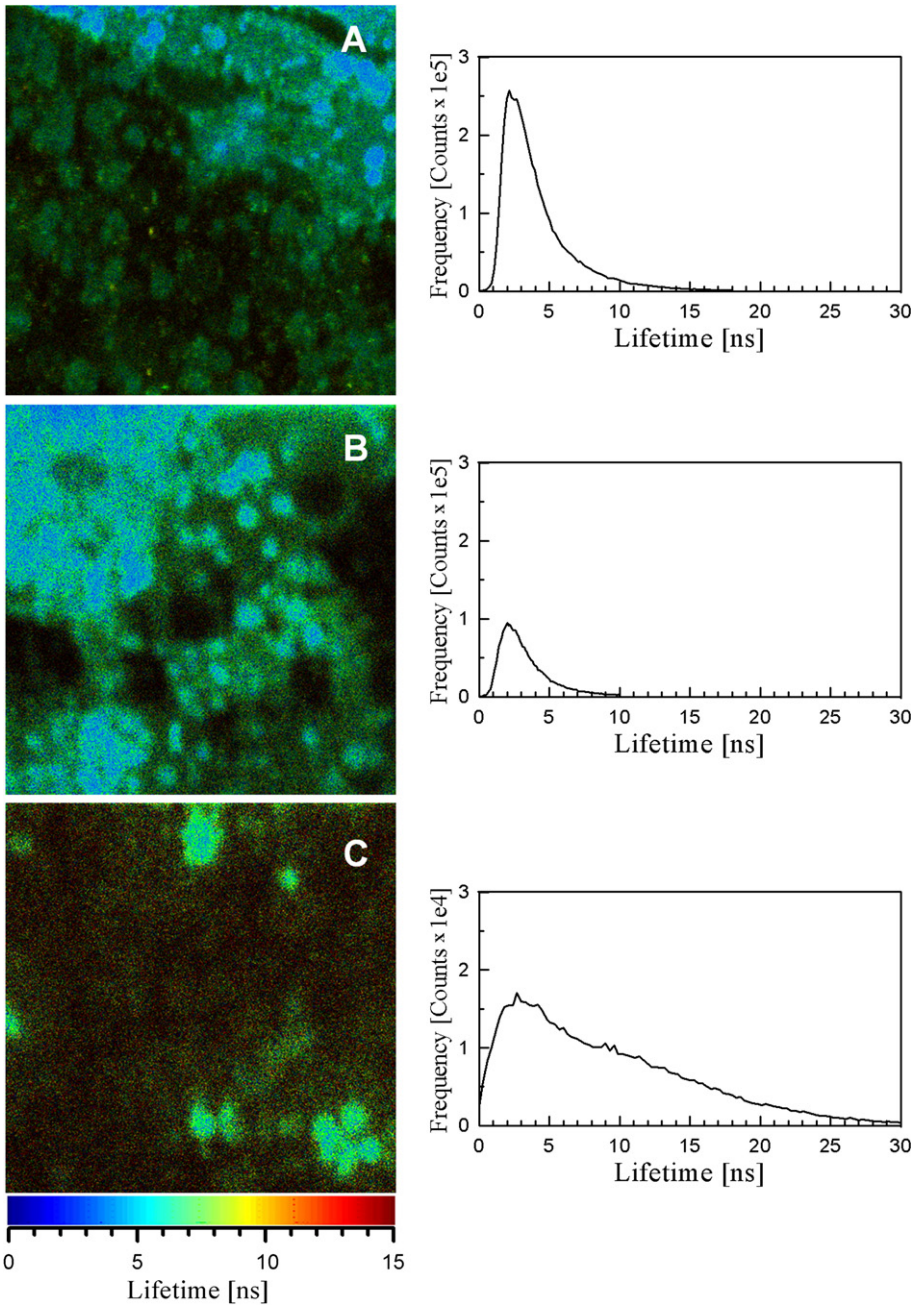

Fig. 8. FLIM images (20×20 μm) of monomolecular layers formed at the argon–water interface and deposited to glass support by Langmuir–Blodgett technique. Monolayers were formed with pure DPPC (A), DPPC + 40 mol% cholesterol (B) and DPPC + 40 mol% ergosterol (C). 40 min before the film deposition the solution of monomeric AmB, prepared in water alkalized to pH 12 with KOH, was injected beneath the lipid layer (into the water subphase). Fluorescence was excited at 405 nm and recorded with application of the 475 nm long-pass filter. Control experiments prove that the fluorescence recorded originates from AmB. False colors represent lifetimes, as indicated. The histograms shown in the right panels present fluorescence lifetime distributions corresponding to whole 20×20 μm field-of-view. Note, that only in the case of the ergosterol-containing membrane light-emitting AmB species display the short lifetimes characteristic of monomers and the long lifetimes characteristic of dimers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

biomembranes of fungi and opens new perspectives in preparation of formulations with enhanced therapeutic effect.

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