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Interactions of Amphotericin B derivative of low toxicity with biological membrane components—the Langmuir monolayer approach

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Abstract

Amphotericin B (AmB)–a polyene macrolide antibiotic–exhibits strong antifungal activity, however, is known to be very toxic to mammalian cells. In order to decrease AmB toxicity, a number of its derivatives have been synthesized. Basing on in vitro and in vivo research, it was evidenced that one of AmB derivatives, namely *N*-methyl-*N*-D-fructopyranosylamphotericin B methyl ester (in short MF-AME) retained most of the antifungal activity of the parent antibiotic, however, exhibited dramatically lower animal toxicity. Therefore, MF-AME seems to be a very promising modification product of AmB. However, further development of this derivative as potential new antifungal drug requires the elucidation of its molecular mechanism of reduced toxicity, which was the aim of the present investigations. Our studies were based on examining the binding energies by determining the strength of interaction between MF-AME and membrane sterols (ergosterol-fungi sterol, and cholesterol-mammalian sterol) and DPPC (model membrane phospholipid) using the Langmuir monolayer technique, which serves as a model of cellular membrane. Our results revealed that at low concentration the affinity of MF-AME to ergosterol is considerably stronger as compared to cholesterol, which correlates with the improved selective toxicity of this drug. It is of importance that the presence of phospholipids is essential since–due to very strong interactions between MF-AME and DPPC–the antibiotic used in higher concentration is "immobilized" by DPPC molecules, which reduces the concentration of free antibiotic, thus enabling it to selectively interact with both sterols.

Keywords: Langmuir monolayers; Air–water interface; Mixed monolayers; Interactions; Amphotericin B derivatives

1. Introduction

The technique of monomolecular layers formed at the aqueous solution–air interface, termed Langmuir (or insoluble, spread, floating) monolayers/films can be applied to build up model biological membranes for various applications [1]. The use of the Langmuir method allows for a precise and continuous control of such parameters as molecular packing, physical state, lateral pressure, composition and quality of the surface. In particular, mixed monolayers composed of constituents of biological membranes, such as phospholipids, sterols and glycolipids, provide a highly informative approach for studying intermolecular interactions between membrane components, usually phospholipids, and biomolecules, for example, various drugs [2–5], hormones [6,7], enzymes [8–11], proteins [12–21], etc. Such investigations may be of much help in elucidating a mode of action of many physiological active compounds [22–29]. An excellent review article by Maget-Dana [30] provides a brief description of the monolayer technique and summarizes its biomedical applications. However, one has to be aware of different possible errors in applying the Langmuir monolayer method, which has been described and analyzed in details in Ref. [31].

To study the interactions with the Langmuir technique, two different methods can be applied.

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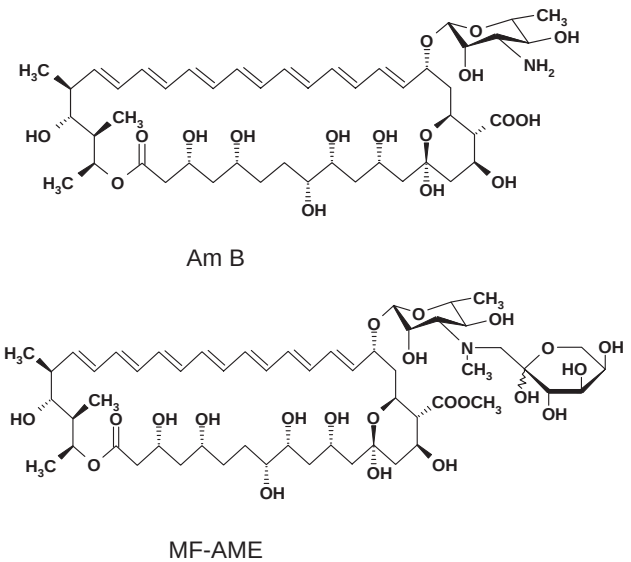
The first one, appropriate for biomolecules that are water soluble, is based on spreading of a monolayer from phospholipids/sterols and its subsequent compression on the top of aqueous subphase containing the dissolved biomolecule (see, for example, [2,3]). Alternatively, a solution with the biomolecule is injected into bulk water after the monolayer has been spread and compressed to a particular surface pressure value (see, for example, [32]). The biomolecules diffuse towards the interface and incorporate into the monolayer. The change in surface pressure, due to the penetration of biomolecules into the floating film, is monitored and can be taken as a measure of interactions between the molecules.

Another approach, useful for water insoluble biomolecules, is based on co-spreading of phospholipid/sterol molecules and the biomolecules on the water surface as a mixed Langmuir monolayer [5,24–29]. Upon varying the proportion of both types of molecules in the monolayer and analyzing the stability and miscibility of the investigated mixed system with the simple functions or thermodynamic parameters, one may infer on the nature and strength of interaction between film molecules [33].

Recently, the Langmuir monolayer technique has successfully been applied to study the mechanism of selective toxicity of antifungal antibiotic, amphotericin B (AmB). This important clinical drug induces differential membrane permeability changes in ergosterol- and cholesterol-containing fungal and mammalian cells, respectively (reviewed in [34,35]). The Langmuir monolayer studies were of help in understanding the mechanism of a higher affinity of AmB towards ergosterol as compared to cholesterol (which has been confirmed both in mixed monolayers [24–26] and in penetration experiments [22,32], and to get insight into the reduced toxicity of liposomal formulations of AmB [5,36].



Scheme 1. The chemical structure of AmB and MF-AME.

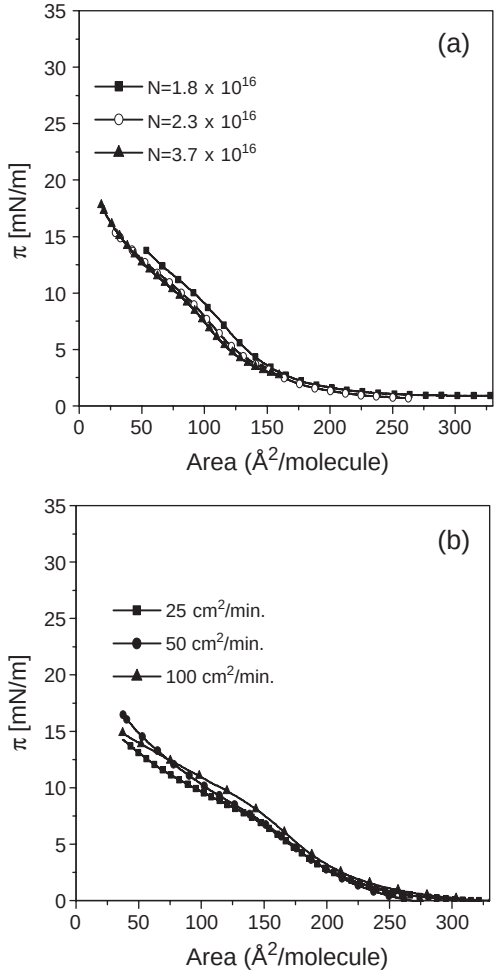


Fig. 1. (a–b) The influence of the number of deposited molecules (a) and compression speed (b) on the surface pressure (π)–area (A) isotherms of MF-AME spread on water at 20 °C.

The undesirable toxicity of AmB to host mammalian cells [34] has stimulated the efforts to overcome this problem. Although liposomal formulations of AmB were found to be much less toxic than conventional adminis-

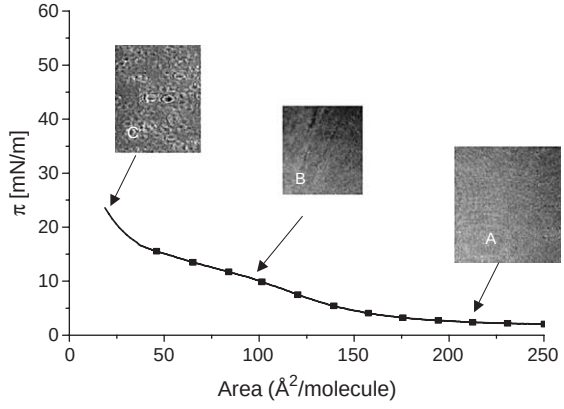


Fig. 2. Surface pressure (π)–area (A) isotherm of MF-AME monolayer spread on water subphase together with BAM images taken at different stages of film compression. Inset: compression modulus (C_s^{-1}) vs. surface pressure (π) dependence.

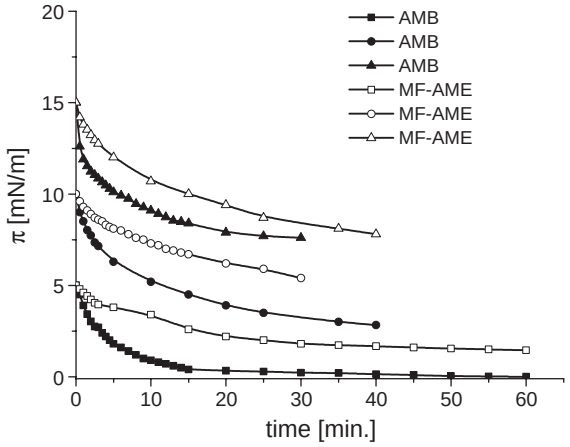


Fig. 3. The stability of MF-AME and AmB monolayers at different surface pressures.

tration forms (colloidal suspension with sodium deoxycholate: *Fungizone*) [37], the cost of medical treatment with liposomal AmB is very high and significantly limits its general clinical use.

Recently, AmB derivatives of reduced toxicity have been synthesized [38–42]. The chemical alteration was based on modifying the carboxyl and/or amino group of the native AmB. The highest selectivity was exhibited by so-called *derivatives of second generation*, characterised by the presence of bulky substituent inducing the steric hindrance effect. Among them, the most promising was *N*-methyl-*N*-D-fructopyranosylamphotericin B methyl ester (abbreviated as MF-AME) [42], the chemical structure of which is shown in Scheme 1. Basing on research carried out in vitro and in vivo, it was observed that MF-AME retained most of the antifungal activity of the parent antibiotic, however, exhibited dramatically lower animal toxicity.

One of the most widely accepted hypothesis on selective toxicity of AmB holds that the toxicity of AmB is due to the close similarity of the membrane-located target of this drug in fungi (ergosterol) and in mammalian organisms (cholesterol) [34,35,43]. According to this model, AmB forms complexes with membrane sterols, which associate into transmembrane channels through which uncontrolled migration of ions and small molecules occurs, causing cell death. Recent molecular dynamic studies provide new information regarding the structure of AmB/sterols channels [44]. Comparative analysis of the simulation data revealed that the kind of a sterol affects the properties of AmB membrane channels. The main difference between AmB/cholesterol and AmB/ergosterol pores lies in a larger diameter of channels formed in fungi cells causing, as a result, more efficient transmission of ions. Moreover, ergosterol-containing channels are more stable due to more favourable van der Waals interactions between AmB and ergosterol as compared to AmB/cholesterol. Despite of a slightly higher AmB affinity to fungal ergosterol vs. mammalian cholesterol, AmB induces comparable effects in both organisms, and this explains its toxicity in host organisms. Following this hypothesis, one may expect that the reduced toxicity of MF-AME should be due to its increased differential affinity towards cholesterol and ergosterol. Since nobody so far has examined the differences in MF-AME/sterol binding energies, herein we have performed experiments that enable us to verify the above assumption. For this purpose, we have investigated mixed Langmuir monolayers formed by MF-

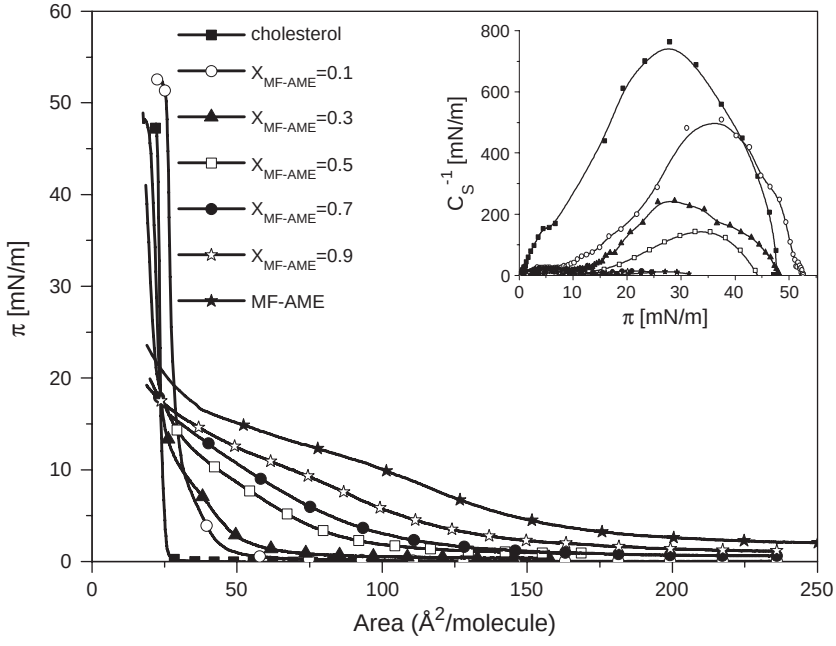


Fig. 4. Surface pressure–area (π–A) isotherms of MF-AME, cholesterol and their mixtures spread on water at 20 °C. Inset: The respective compression modulus (C_s^{-1}) vs. surface pressure (π) dependencies.

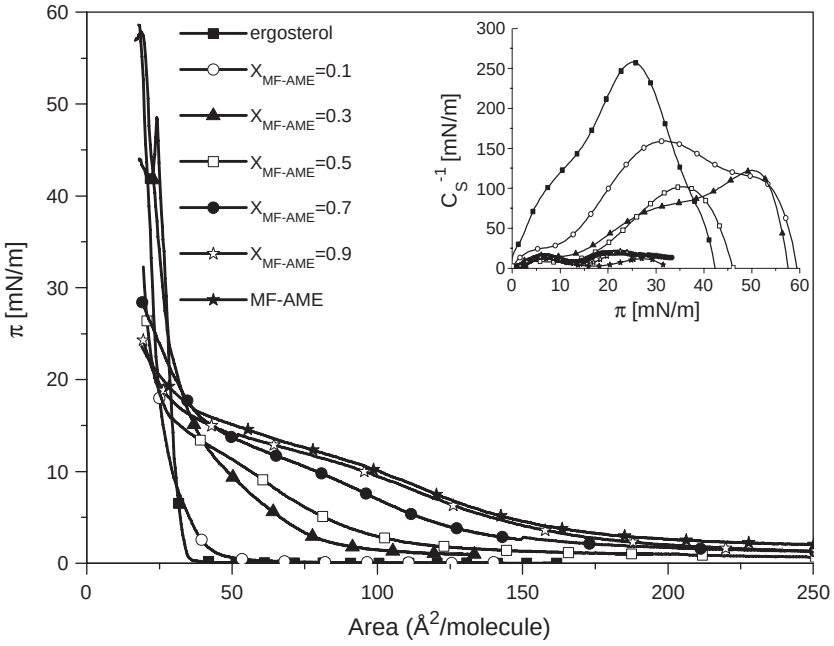


Fig. 5. Surface pressure–area (π–A) isotherms of MF-AME, ergosterol and their mixtures spread on water at 20 °C. Inset: The respective compression modulus (C_s^{-1}) vs. surface pressure (π) dependencies.

AME and cholesterol or ergosterol, basing on surface pressure measurements and analyzing isothermal compressibility (C_s), the mean area per molecule (A_{12}), excess free energy of mixing (ΔG^{Exc}) and total free energy of mixing (ΔG^{M}). To get insight into the role of phospholipids in the MF-AME–sterol interactions, the mixed monolayers of the antibiotic and DPPC have also been performed and analyzed.

2. Experimental

N-Methyl-*N*-D-fructopyranosylamphotericin B methyl ester (MF-AME) was synthesized in the Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology (Poland) [42]. The yellow-colored compound as a free base is water insoluble, however, forms water soluble salts with acids. Cholesterol (+99%) and ergosterol (+97%) were purchased from Aldrich and Fluka, respectively. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (synthetic, 99%) was purchased from Sigma. Stock solutions of MF-AME and sterols prior to experiments were prepared in *N*,*N*-dimethylformamide (p.a., POCh, Poland) in the concentration of ca. 0.2 mg/mL, and kept in a fridge (at 4 °C). DPPC was dissolved in chloroform:ethanol 4:1 v/v mixture spreading solutions were deposited onto the water subphase with the Hamilton microsyringe, precise to 2.0 μL. After spreading, the monolayers were left to equilibrate for ca. 5 min before the compression was initiated with the barrier spread of 50 cm^2/min, unless otherwise specified. π–A isotherms were recorded with a NIMA (UK) Langmuir trough (total area = 590 cm^2) placed on an anti-vibration table. Surface pressure was measured with the accuracy of ±0.1 mN/m using a Wilhelmy plate made of filter paper (ashless Whatman Chr1) connected to an electrobalance. The subphase temperature (20 °C) was controlled thermostatically to within 0.1 °C by a circulating water system. In order to investigate the influence of the subphase pH, the monolayers were spread on Theorell–Stenhagen buffer subphase [45] (ionic strength <0.1), which was prepared with double distilled water. For a direct visualization of the monolayer structure, Brewster Angle Microscope (mini-BAM, NFT, Germany) was applied.

3. Results and discussion

3.1. Monolayers formed by pure MF-AME

In the first step of our investigations, the influence of the number of deposited molecules and the compression speed on

Table 1
Compression modulus (C_s^{-1} max.) values for the investigated mixed monolayers of MF-AME and sterols

X_{MF-AME}	MF-AME/cholesterol	MF-AME/ergosterol
	C_s^{-1} max. [mN/m]	C_s^{-1} max. [mN/m]
Sterol	771.0	251.1
0.1	609.0	158.4
0.3	241.0	123.4
0.5	145.0	102.0
0.7	12.4/14.9	15.0/20.0
0.9	13.0	14.0/21.8
MF-AME	12.0/12.3	12.0/12.3

the isotherms of MF-AME have been examined. Fig. 1a–b present the π–A isotherms of MF-AME monolayer obtained by spreading different number of molecules (1.8×10^{16}, 2.3×10^{16} and 3.7×10^{16}) applying the same compression speed, namely 50 cm^2/min (Fig. 1a), or using different barrier speed while keeping the same (1.8×10^{16}) number of deposited molecules (Fig. 1b). These experiments have been carried out on the aqueous subphase (pH $\approx$ 6) at 20 °C. It is evident that the surface concentration of MF-AME within the investigated range has practically no influence on the shape of π–A isotherms. However, with the increase of surface density of MF-AME molecules, a non-zero initial surface pressure is observed. Regarding the compression speed effect, only an increased velocity (of 100 cm^2/min) causes some kind of the isotherm's shape deformation.

The isotherm of MF-AME (Fig. 2) starts to rise at about 250 $Å^2$/molecule. Upon compression, surface pressure increases monotonically until $\pi \approx 10$ mN/m. Afterwards, the isotherm changes the slope and a diffused pseudo-plateau is observed, which spans over the area of 100–50

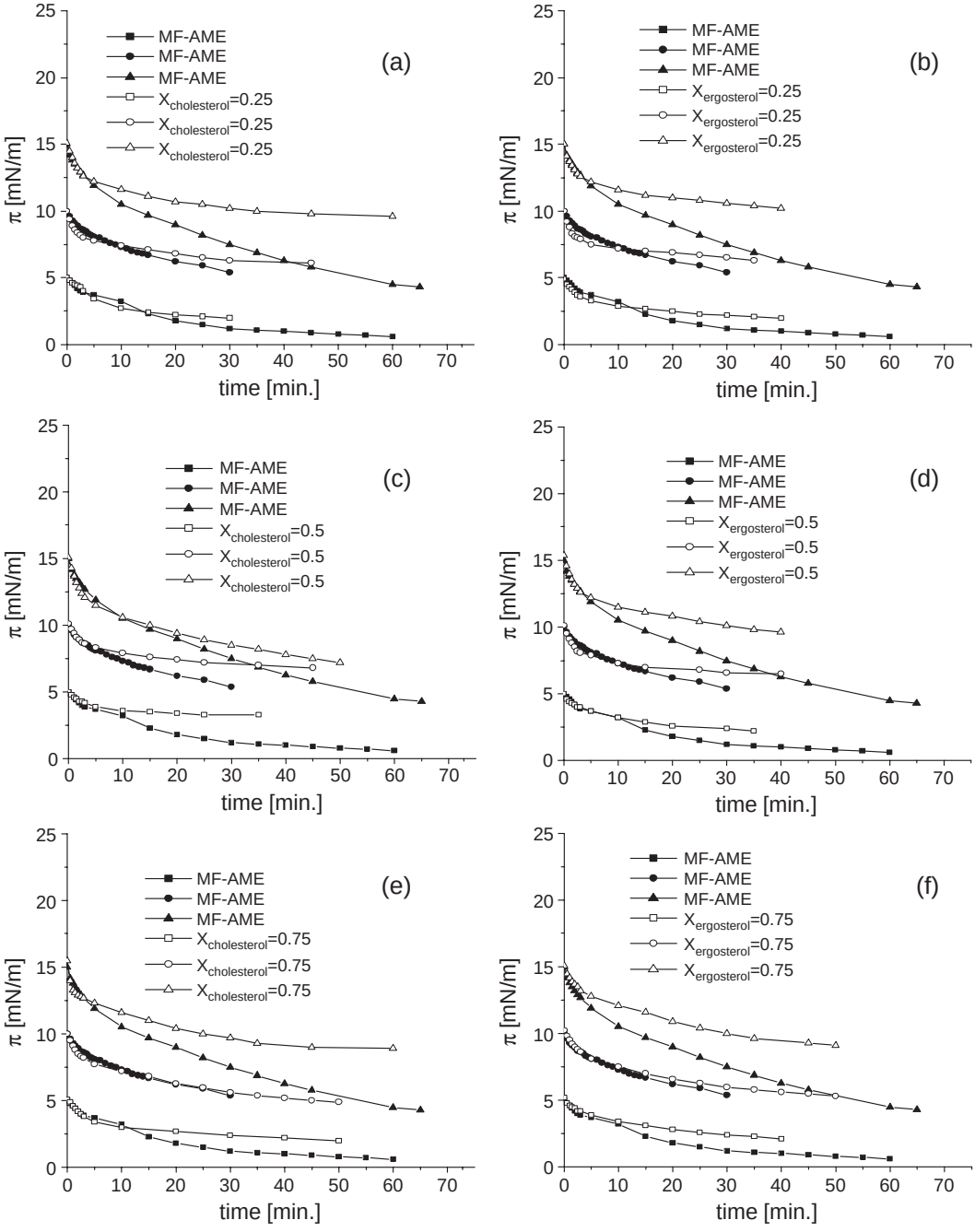


Fig. 6. (a–f) The stability of MF-AME/sterols mixed monolayers at different surface pressures.

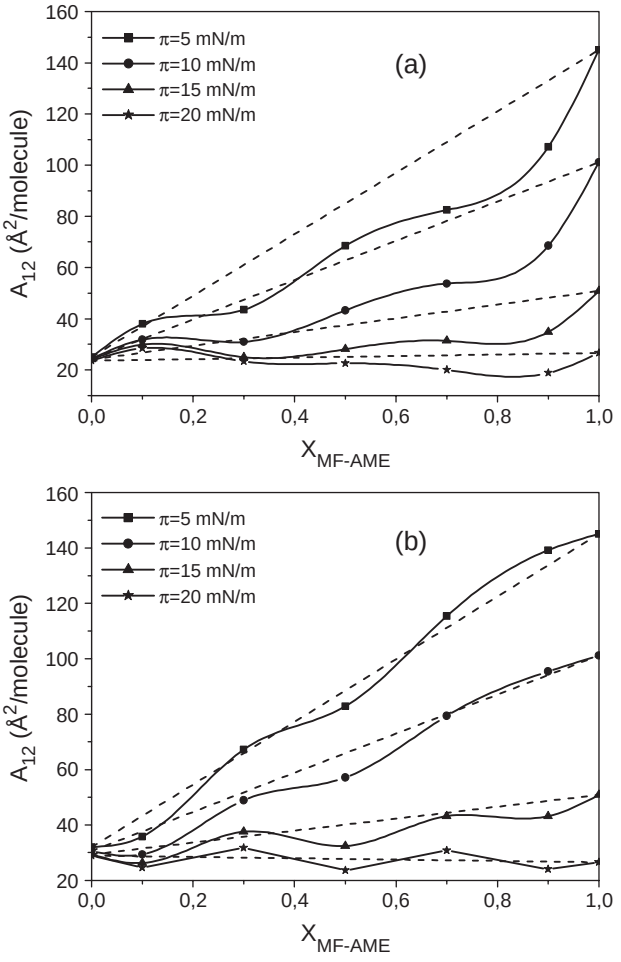


Fig. 7. (a–b) Mean molecular area (A_{12}) vs. composition plots for mixtures of MF-AME and cholesterol (a) and ergosterol (b) at different constant surface pressures.

Å^2/molecule. At large molecular areas, the monolayer is completely homogeneous, without any detectable domains (photo A); however, at the plateau region, some stripe-shaped structures start to appear (B). Upon further compression, surface pressure increases again, and no film collapse is observed even at such small area/molecule as few Angstroms. In the post-plateau region, a number of round-shaped, bright domains are visible (C). The shape of MF-AME isotherm is typical for a fluid-like monolayer.

On the other hand, the characteristic feature of the pressure/area (π/A) isotherm of the parent, unsubstituted AmB is a transition plateau that separates two regions of liquid-expanded (LE) and liquid-condensed (LC) state [46]. It has been supposed [22,47] that in the liquid-expanded region, molecules are lying flat on the surface (with the limiting area of ca. 180 Å^2/molecule as calculated from molecular models), linked to the surface water by hydrogen bonds, whereas in the liquid-condensed region, the molecules are oriented vertically (with the area of 55 Å^2/molecule), and most of the hydrogen bonds are broken. The phase transition was interpreted as being due to the changes in molecules orientation from horizontal to vertical position [22,47]. We have also performed semi-empirical calculations of cross-sectional areas for MF-AME using a HyperChem computer program [48]. For horizontally oriented molecule, the cross-sectional area was calculated to be 242 Å^2, while vertically oriented MF-AME occupies 79 Å^2. Therefore, it is possible to adopt similar interpretation as for AmB, i.e. at large molecular areas, in the pre-plateau region, MF-AME molecules have enough room to lie flat on the surface, however, upon reaching the pseudo-plateau, molecules start to change their orientation from horizontal to an upright position. Beyond the transition, the mean molecular area is too small for a monomolecular layer to exist. However, no film collapse into 3D structures is observed on the π–A isotherm. This implies that in the post-plateau region, MF-AME undergoes dissolution into bulk water phase.

Both AmB and MF-AME at large areas per molecule, in the pre-plateau region, adopt horizontal orientation on the water surface. Such a hydrogen-bonded monolayer should be stable, in contrary to the film compressed to the pressure above the transition, where most of the hydrogen bonds

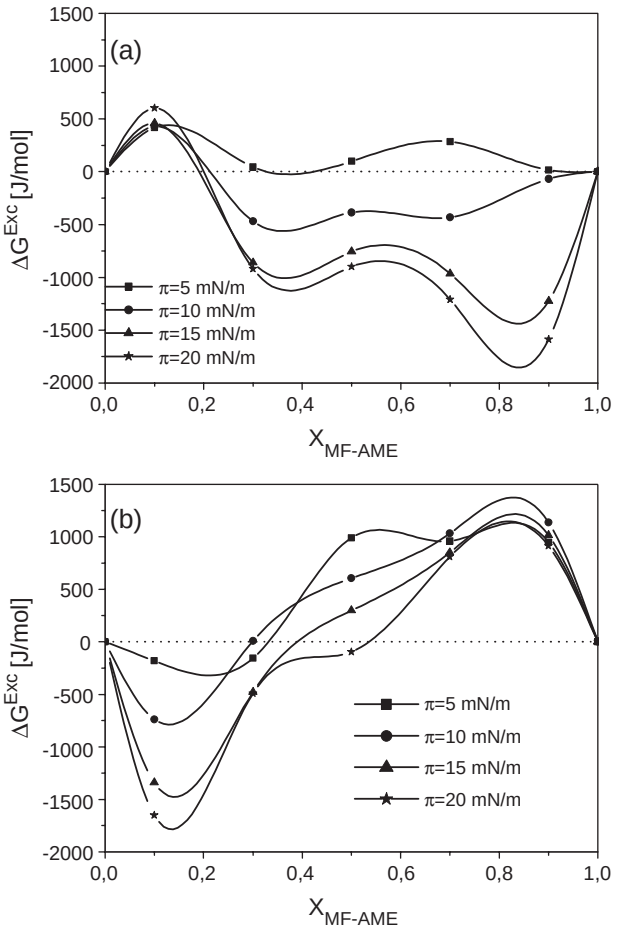


Fig. 8. (a–b) Excess free energy of mixing (ΔG^{Exc}) vs. composition plots for mixtures of MF-AME and cholesterol (a) and ergosterol (b) at different constant surface pressures.

between vertically oriented molecules and water are broken. To verify this hypothesis, we have performed stability experiments. Namely, the monolayer was compressed to a particular value of surface pressure, corresponding to the pre-plateau, plateau and post-plateau region (5, 10 and 15 mN/m, respectively). Then, the barrier was stopped and changes of the surface pressure with time were recorded. The obtained results are presented in Fig. 3. For the purpose of comparison, the results for AmB (under the same experimental conditions) have also been included in the same plot. It is evident that MF-AME is more stable as compared to amphotericin B. For example, in the pre-plateau region (at 5 mN/m) after 5 min of observation, a 64.2% and a 23.8% decrease of the initial surface pressure value for AMB and MF-AME, respectively, was observed. At both 5 and 10 mN/m, surface pressure stabilizes after ca. 20 min; while in the post-plateau region, surface pressure decreases gradually with time, which confirms the dissolution of molecules into the bulk water phase.

3.2. Mixed monolayers of MF-AME and sterols

Mixed MF-AME/sterol monolayers have been formed after spreading an appropriate amount of respective mixed solution onto the water surface so that the number of molecules deposited on the surface was always kept constant. Figs. 4 and 5 present the pressure/area isotherms for the pure components as well as mixed films. The isotherms for mixtures lie in-between those for pure components and their shape changes from this for sterol to that characteristic for MF-AME upon increasing the amount of the antibiotic in the mixture.

The influence of the presence of sterols on the state of MF-AME monolayers can be analyzed on the basis of compression modulus (elasticity), C_s^{-1} (defined as $C_s^{-1}=-A(d\pi/dA)$ [49]) vs. surface pressure plots (Figs. 4 and 5, inset). Generally, the addition of a sterol (which forms a solid-type monolayer) to MF-AME monolayer causes the increase of mixed monolayer's elasticity (C_s^{-1}) (see Table 1). Simultaneously, upon the addition of a sterol to MF-AME monolayer, the film stability increases. As evidenced in Fig. 6a–b, even a small content of a sterol in the mixed monolayer ($X_{sterol}=0.25$) improves the stability.

Because the mixed films were found to be stable, basing on the experimental π–A isotherms, we were able to analyze the interactions between film molecules. In general, the interactions between components of the mixed monolayer can be studied from the point of view of their miscibility and stability.

One of the methods to examine the miscibility of film components in the monolayer is to calculate the mean area per molecule in the mixed film (A_{12}) (defined as: $A_{12}=A_1X_1+A_2X_2$, where A_i is the mean molecular area of pure component i (1 or 2) at the relevant surface pressure and X_i is the mole fraction of component i in the mixed film). It is known [33] that a linear dependence of a simple functions, such as $A_{12}=f(X_i)$, indicates either the ideal mixing of non-interacting molecules or their complete immiscibility. Any deviations from the straight line prove the miscibility between film components and their non-ideal behavior. Quantitatively these deviations can be described with the excess area values (A^{exc}), which are defined as: $A^{exc}=A_{12}-(A_1X_1+A_2X_2)$.

Fig. 7a and b show the $A_{12}=f(X_i)$ dependencies for the investigated mixtures at different values of surface pressure, namely: $\pi=5$, 10, 15, 20 mN/m. The observed deviations from linearity prove that the investigated compounds are miscible and interact in mixed monolayers. For MF-AME/cholesterol system (Fig. 7a), at low antibiotic content, a positive deviations from ideal behavior appear. This suggests that for such a composition, the interactions between MF-AME and cholesterol in a mixed monolayer are weaker as compared to the interactions between molecules in the respective pure films. For mixed films richer in MF-AME, negative deviations from the linearity, suggesting attractive interactions between molecules in the mixed film, occur. The system of MF-AME/ergosterol

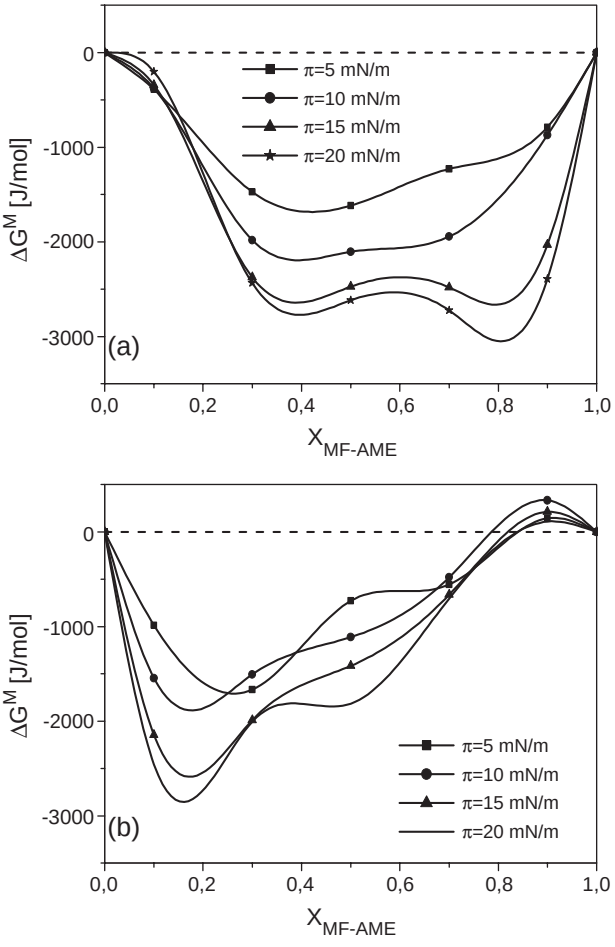


Fig. 9. (a–b) Total free energy of mixing (ΔG^M) vs. composition plots for mixtures of MF-AME and cholesterol (a) and ergosterol (b) at different constant surface pressures.

behaves differently (Fig. 7b), i.e. for low MF-AME content ($X_{\text{MF-AME}} \leq 0.3$) the negative deviations can be observed. Further increase of the antibiotic amount in the mixed monolayer causes the interactions to become less attractive.

The thermodynamic stability of a mixed monolayer and a quantitative analysis of intermolecular interactions can be done with the excess free energy of mixing (ΔG^{Exc}) values [33], defined as:

$$\Delta G^{\text{Exc}} = N \int_0^{\pi} (A_{12} - X_1 A_1 - X_2 A_2) \mathrm{d}\pi \quad (1)$$

Fig. 8a–b present $\Delta G^{\text{Exc}} = f(X_{\text{MF-AME}})$ dependencies for the investigated mixtures. The positive values of ΔG^{Exc}, which can be observed for low content of MF-AME in the mixtures with cholesterol and for high mole ratios of the antibiotic in the other system (MF-AME/ergosterol), suggest lower films stability in these regions. On the other hand, negative values of ΔG^{Exc} reflect high thermodynamic stability of mixed monolayers and prove attractive interactions between film components.

Comparing the plots of the ΔG^{Exc} for MF-AME mixtures with sterols, it is evident that the strongest interactions between molecules occur in the low antibiotic proportion ($X_{\text{MF-AME}} \leq 0.3$) for its mixtures with ergosterol. Interestingly, in the same mole fraction region, mixtures with cholesterol exhibit the weakest attractive interactions. In the light of this, it is possible to conclude that MF-AME, in small amounts, shows significantly higher affinity towards ergosterol (fungal sterol) as compared to cholesterol (mammalian sterol).

For a better examination of the monolayers stability, the total free energy of mixing (ΔG^{M}) values, defined by the following equation (Eq. (2)) [33]:

$$\Delta G^{\text{M}} = \Delta G^{\text{Exc}} + \Delta G^{\text{id}} \quad (2)$$

where

$$\Delta G^{\text{id}} = RT(X_1 \ln X_1 + X_2 \ln X_2) \quad (3)$$

have been calculated. The results of calculation of ΔG^{M} vs. mole fraction of MF-AME ($X_{\text{MF-AME}}$) at different values of surface pressure are shown in Fig. 9a–b. Analyzing Figs. 8a and 9a, it can be noticed that although ΔG^{Exc} is positive in low MF-AME content in the mixtures with cholesterol, ΔG^{M} is negative within the whole range of $X_{\text{MF-AME}}$. Regarding the mixtures with ergosterol, ΔG^{M} is also negative, except for slightly positive values at high MF-AME proportions. These results prove that the 2D mixed state (MF-AME/sterol) is thermodynamically more stable and more favourable than the corresponding unmixed state. The most negative values of ΔG^{M} for both mixed systems are of the same order (ca. −3000 J/mol), however, the corresponding mixed films compositions are quite different.

3.3. Mixed monolayers of MF-AME and DPPC

Since in biological membranes, sterols are always associated with phospholipids, and moreover, as it has already been mentioned above, one of the methods to reduce AmB toxicity is its incorporation in liposomes, we have also

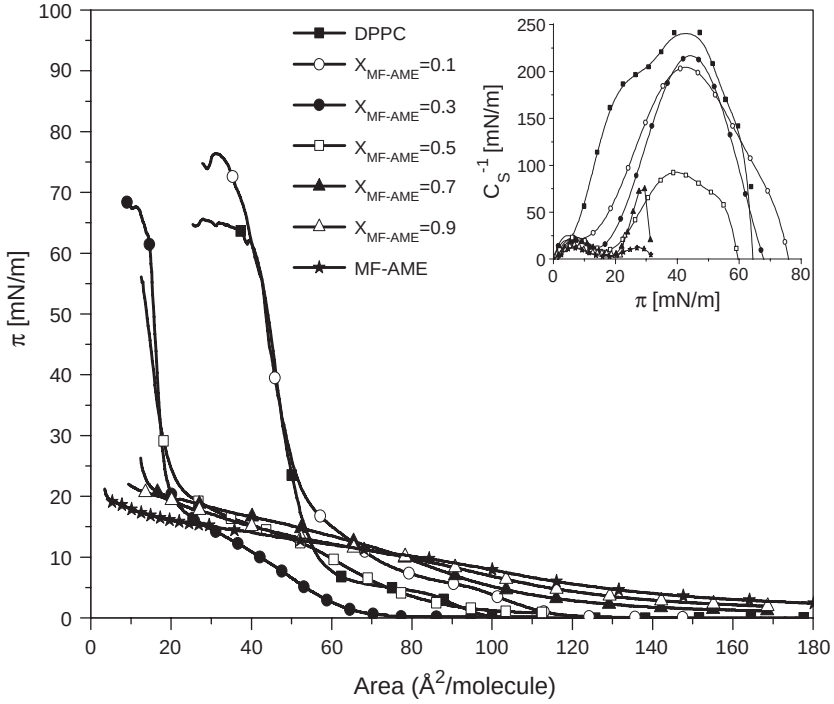


Fig. 10. Surface pressure–area (π–A) isotherms of MF-AME, DPPC their mixtures spread on water at 20 °C. Inset: The respective compression modulus (C_s^{-1}) vs. surface pressure (π) dependencies.

undertook studies on the phospholipid/MF-AME interactions. For our investigation, DPPC has been chosen, which serves as a model membrane phospholipid [50]. Fig. 10 presents π/A isotherms for mixtures of DPPC and MF-AME. As it can be seen, the isotherms for $X_{\text{MF-AME}}=0.3$ and 0.5 are shifted to smaller mean molecular areas as compared to both pure components. The isotherms for all the remaining mixtures lie in-between those for pure components. The corresponding compression modulus vs. surface pressure dependencies are shown in the inset of Fig. 10. It is clear that upon compression DPPC, monolayer changes its state from a liquid expanded to a liquid condensed. The increase of MF-AME content in the investigated mixed monolayers causes the decrease of C_s^{-1}. Similarly to mixed MF-AME/sterol monolayers, we have also analyzed the stability of mixtures of MF-AME with DPPC (Fig. 11). It is evident that upon incorporation of a phospholipid into MF-AME monolayer, its stability increases. The miscibility and interactions between both components have been examined with the same procedure as described above. As can be noticed (Fig. 12a), negative deviations from the straight lines on the $A_{12}=f(X_{1,2})$ plots are observed within the whole composition range except for mixtures of low MF-AME proportions (below 0.2) at $\pi=5$ and 10 mN/m. The $A_{12}=f(X_{1,2})$ dependencies prove that the investigated compounds mix and interact in monolayers in the whole range of mole fractions. The course of $\Delta G^{\text{Exc}}=f(X_{\text{MF-AME}})$ (Fig. 12b) indicates the existence of strong attractive interactions between DPPC and the antibiotic in mixed films for $X_{\text{MF-AME}}\geq 0.2$. The strongest negative values of ΔG^{Exc} occur at $X_{\text{MF-AME}}=0.33$ and indicate the film composition of the highest stability. High stabilities of DPPC/MF-AME mixtures are additionally proved with ΔG^{M} vs. $X_{\text{MF-AME}}$ dependencies, which are negative within the whole composition range (Fig. 12c).

4. Conclusions

Studies which have been carried out on biological materials [42,51–53] indicate that MF-AME, which is a sterically hindered second generation amphotericin B derivative with substituted both amino and carboxyl groups, is practically non-toxic as compared to native AmB. The mechanism leading to its reduced toxicity is therefore of great interest. These investigations suggest that

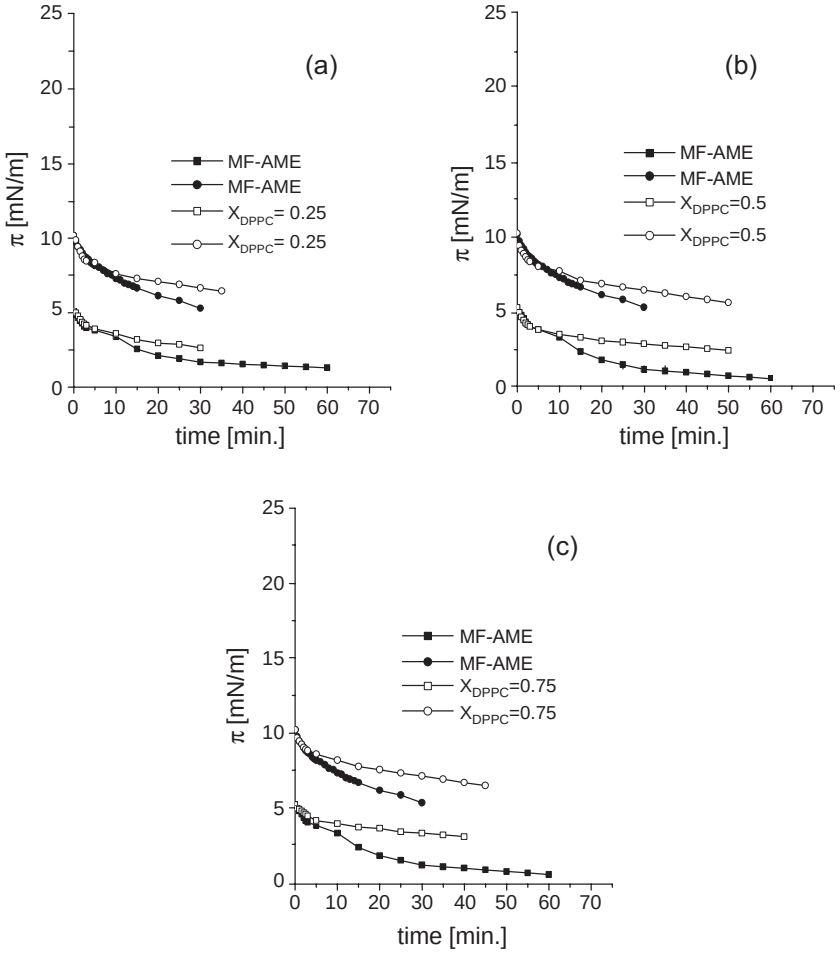


Fig. 11. The stability of MF-AME/DPPC mixed monolayers at different surface pressures.

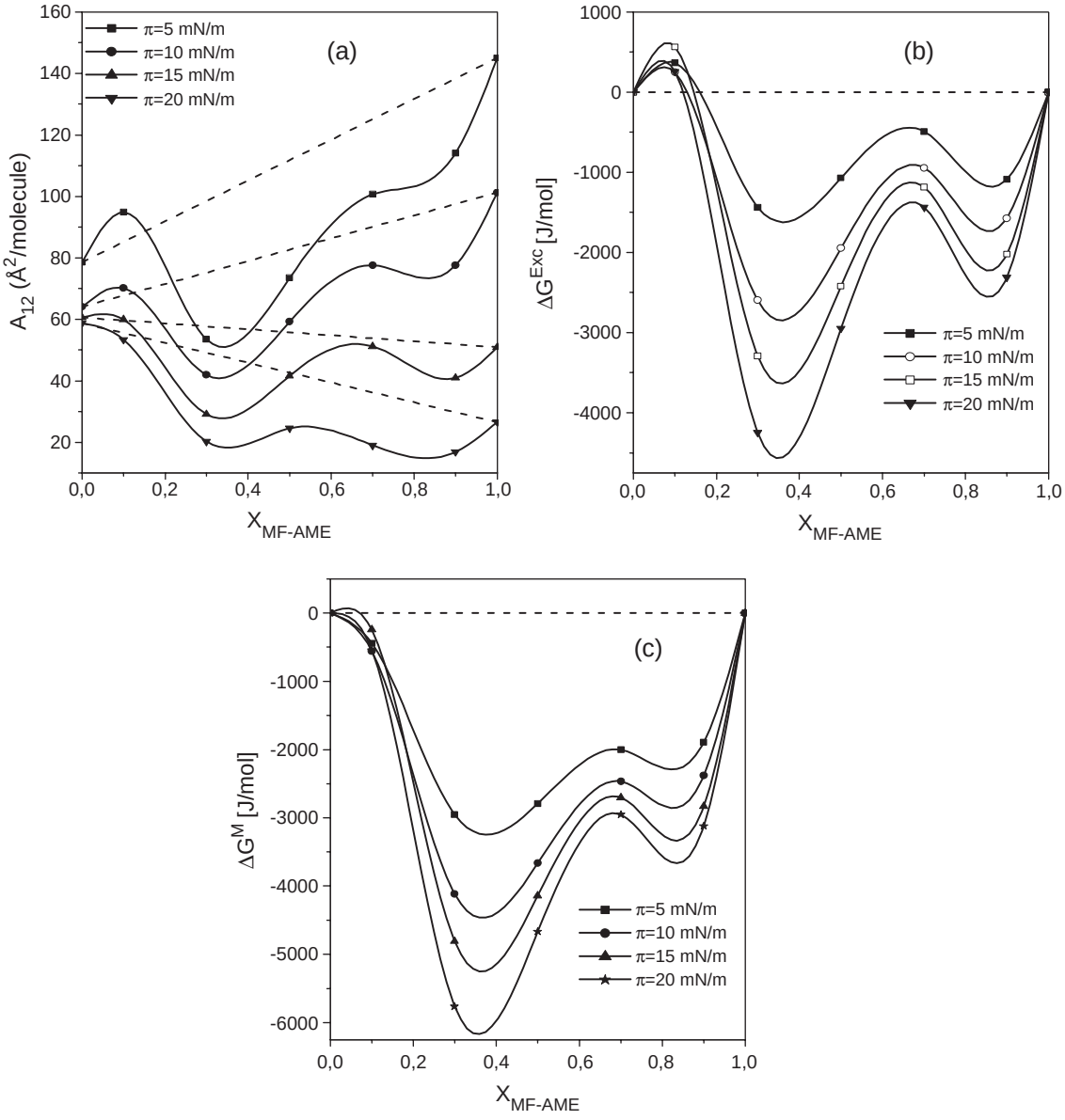


Fig. 12. (a–c) Mean molecular area (A_{12}) (a), excess free energy of mixing ΔG^{Exc} (b), and total free energy of mixing ΔG^{M} (c) vs. composition plots for mixtures of MF-AME and DPPC.

MF-AME as well as other AmB derivatives of low toxicity is less effective than AmB in pores formation in mammalian cell membrane. The monolayers studies presented herein allow us to explain the mechanism of low toxicity of MF-AME.

Firstly, considering the interactions between MF-AME and sterols, it is clear that for low mole fractions of the antibiotic, its affinity to ergosterol is considerably stronger vs. cholesterol, which might explain improved selective toxicity of MF-AME towards fungi and its non-toxicity to host cells. Such a different behavior of the investigated antibiotic towards both sterols is probably due to differences in the chemical structure of cholesterol and ergosterol. Namely, ergosterol possesses two additional double bonds (one in the side chain and the other in the sterol ring) as well as an additional methyl group in the side chain as compared to cholesterol. Especially, the presence of an unsaturated bond in the hydrocarbon chain makes the molecule more rigid and hinders its conformational changes [54]. As a result, the side hydrocarbon tail, which is stiffer and more elongated than that of cholesterol, protrudes out of the cyclic part of the molecule and therefore the cross-sectional area is larger for ergosterol (Å46 ^{2}, as calculated with the Hyper-Chem computer modelling programme [48]) than for cholesterol (Å38 ^{2}). In consequence, the monolayer of ergosterol is less packed as compared to cholesterol (which is confirmed by lower compression modulus values). Therefore looser monolayer from ergosterol enables more favourable molecular packing of the bulky MF-AME molecule, contrary to rigid and closely packed film from

cholesterol. This has a strong impact on the short-range (van der Waals) interactions between the antibiotic and sterol molecules, reflected in stronger interactions between MF-AME and ergosterol. This effect is especially pronounced at low antibiotic content in the mixed monolayer.

Comparing our results with AmB/sterol mixtures in monolayers [24–26], one may conclude that: (i) since the strength of interactions between AmB and cholesterol is comparable to that between AmB and ergosterol, AmB exerts toxic effects in both fungi and mammalian cells, and (ii) since the strongest interactions with ergosterol are observed for $X_{AmB}=0.5–0.7$ (vs. $X_{MF\text{-}AME}=0.2$), it may be expected that significantly higher amount of AmB is required for its fungicidal effect as compared to MF-AME. Studies on mixed monolayers of DPPC/MF-AME prove that in this region of low MF-AME content, the interactions between DPPC and MF-AME are not important, in contrary to the remaining mixtures. Namely, for $X_{MF\text{-}AME}$ above 0.2, the interactions with DPPC become significant, and their strength exceeds those between the antibiotic and sterols (in the case of which the minimum value of ΔG^{Exc} does not exceed −2000 J/mol). The strongest interactions ($\Delta G^{Exc}=-4600$ J/mol) occur at $X_{MF\text{-}AME}=0.33$, which suggest a stable complex formation between MF-AME and DPPC of the 1:2 stoichiometry. It is worthy mentioning that for mixed monolayers of AmB and DPPC (experiments have been performed under the same experimental conditions as in the present work), the strongest interactions were found to occur at 1:1 mixed film composition, however, their strength is nearly seven times lower (unpublished results). Therefore it seems that in the presence of phospholipids, at higher MF-AME concentration, the antibiotic is "immobilized" by its interactions with DPPC, which reduces the concentration of free MF-AME that is capable of interacting with membrane sterols. The small amount of free MF-AME may still be sufficient for fungicidal effect, while in the host cells this concentration of the antibiotic is too low to induce toxic effect.

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