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Interaction between nystatin and natural membrane lipids in Langmuir monolayers—The role of a phospholipid in the mechanism of polyenes mode of action

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Abstract

Nystatin (NYS), a polyene antifungal antibiotic, has been investigated in Langmuir monolayers alone and in mixtures with mammalian and fungi membrane sterols (cholesterol and ergosterol, respectively) as well as with a model phospholipid (DPPC). The interactions between film molecules have been examined both in a qualitative and quantitative way with the excess area per molecule (A^{Exc}), excess free energy of mixing (ΔG^{Exc}) and the interaction parameter (α). The obtained results have been compared with those previously reported for another polyene antimycotic: amphotericin B (AmB) mixed with lipids. Higher affinity of NYS has been observed for ergosterol vs. cholesterol, however, the strongest attractions were found for its mixtures with DPPC. The obtained results have been verified with biological studies reported previously for both antibiotics (NYS and AmB). A thorough analysis of the Langmuir experiment results performed for both polyenes enabled us to conclude that the presence of DPPC can be considered as a key factor affecting their antifungal activity as well as their toxicity towards host cells.

Keywords: Nystatin (NYS); Polyene mechanism of action; Langmuir monolayers; Interaction parameter

1. Introduction

Polyene antibiotics nystatin (NYS) and amphotericin B (AmB) have been incessantly used in the treatment of topical (NYS) and systemic (AmB) fungal infections for more than 50 years now. The advantage of administering these compounds, which are more efficient and not replaceable with other agents belonging to different families of antifungal compounds, e.g. azoles, is their wide spectrum of activity towards pathogenic fungi and yeasts. However, their application is accompanied by serious side effects, resulting from compositional similarity between host and fungi cells [1]. This issue will be discussed later on.

Nystatin was discovered in 1944 by Elisabeth Hazen and isolated from *Streptomyces nodosus* by Rachel Brown. Their research was published in 1950, and the isolated agent was refereed as AN No. 48240. Later it was called *fungicidin*, while its commercial formulation — *mycostatin*. Finally, it was named *nystatin* from the New York State (Department of Health), where both researchers worked [2]. The chemical structure of nystatin is presented in Scheme 1. This molecule possesses an amphipathic structure. The system of four conjugated double bonds forms a hydrophobic (apolar) part, while on the other side of the ring, the hydroxyl, carboxyl and keto groups form hydrophilic (polar) part of nystatin molecule. Moreover, a mycosamine moiety is linked to the macrolacton ring [2,3]. The number of conjugated double bonds differentiates tetraene nystatin from heptaene amphotericin B.

As soon as the therapeutic properties of nystatin isolated from *S. nodosus* were found, the explanation of its mechanism of action attracted a lot of attention. First experiments, which cast some light on the mode of antifungal action of polyenes, were performed at the end of the 1950s. It has been proved that these antibiotics interact specifically with lipids present in the cellular membrane of a sensitive organism. Since bacteria, which are unaffected by polyenes, does not possess sterols in their cells, while polyene-sensitive fungi contain sterols in their

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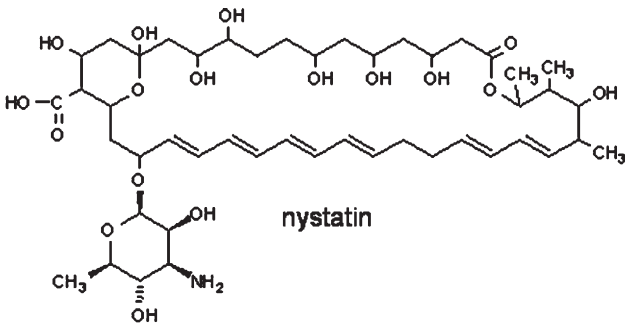

Scheme 1. The chemical structure of nystatin.

membrane, it has been proposed that the action of these antibiotics is limited to organisms, which possess sterols in their cell membrane composition [3–7]. The mechanism of action of polyene antibiotics was further explained more precisely by DeKruiff and Demel [8], who proposed that polyenes form specific complexes with membrane sterols, and associate into transmembrane channels, or pores that permit a loss of ions and small molecules from the cell, causing its damage and finally death. This mode of action is considered to be most probable and is now widely accepted. However, it is worth mentioning that there are still some controversies, especially regarding the role of other membrane components in polyene antifungal activity. First of them appeared in 1963, when Goldfine and Ellis [9] proved that polyene-insensitive bacteria, which do not have sterols in their membranes, are unable to synthetize lecithins. On the contrary, polyene-sensitive organisms have in their membrane composition not only sterols, but also lecithins, and therefore the latter should also be taken into consideration when looking for the polyene mode of action. However, further experiments have indicated that the antibiotic action is more efficient in the presence of sterols [10–13]. On the other hand, it has been reported that polyenes are able to form pores in membranes devoid of sterols [14,15], however, the formed channels are not responsible for their antifungal activity [10,11]. Thus, although the role of sterols in polyene mechanism of action is known to be important, the question regarding the influence of phospholipids on polyene activity is still unsolved.

The most serious problem directly involved in the mechanism of action of these antifungal agents is their toxicity. The similarity between fungal and mammalian membranes, namely the presence of sterols in both types of cells (cholesterol in mammalian and ergosterol in fungi), causes the polyenes to form pores that affects the permeability of both pathogenic as well as host cells. However, ergosterol-containing membranes are more sensitive to nystatin than those possessing cholesterol [2,6,16], and therefore nystatin can be therapeutically applied in the treatment of fungal infections. However, due to low absorption of nystatin from the gut and a high toxicity when administrated intraperitoneally and intravenously, its application is limited rather to oral and topical therapy [3,17–19]. Nystatin spectrum of antifungal activity is broader as compared to amphotericin B, and therefore the intravenous administration of the former antibiotic could be more effective. Also, it has been found that nystatin can be incorporated into liposomes (Nyotran), and in this form it does not loose its therapeutic properties, while its toxicity is profoundly reduced. The liposomal formulation of nystatin is as effective as liposomal amphotericin B, and even more active than amphotericin B deoxycholate or amphotericin B lipid complex. In addition, what is of great importance, nystatin in this form acts also on amphotericin B-resistant infections [20–26].

Since the mechanism of action of nystatin (and other polyenes) has not been thoroughly elucidated so far, there are still experiments being performed on the influence of polyenes on natural membrane properties and pores formation [18,27–29]. Recently, interesting results regarding the interaction between liposomes, imitating fungi and mammalian membranes, as well as the interaction between antibiotic and phospholipids vesicles have been presented [30,31]. The authors propose [31] that the antifungal mechanism of nystatin is comprised of two steps, depending on the antibiotic concentration, and that the interaction between nystatin and ergosterol-containing liposomes is different from those containing cholesterol.

In this paper, the results of investigations on the interaction between nystatin and mammalian and fungi sterols (cholesterol and ergosterol, respectively) as well as a model phospholipid (DPPC) have been presented, using the Langmuir monolayer technique [32]. This experimental technique has been previously used for investigation of the mechanism of action of other polyene antibiotic namely amphotericin B [33–41], however, to the best of our knowledge, such experiments have not been yet performed for nystatin. Previously [42] we examined the film-forming properties of pure nystatin, especially its stability at the air/water interface. Improved stability of mixed monolayers in comparison to pure nystatin proves that this antibiotic can be investigated in 2D mixtures with sterols and phospholipids at physiological conditions. We do believe that the investigations presented herein together with results of our previous studies regarding amphotericin B derivatives will give a better insight into the understanding of the effect of polyenes on natural membrane components.

2. Experimental

Nystatin dihydrate was purchased from Fluka, 99% in the form of a yellow powder, while cholesterol (+99%) and ergosterol (+97%) were purchased from Aldrich and Fluka, respectively. L-α Phosphatidylcholine dipalmitoyl (DPPC) (synthetic, 99%) was purchased from Sigma. Stock solutions of all the investigated compounds were prepared in *N*,*N*-dimethylformamide (p.a., POCh, Poland) prior to experiments in the concentration of ca. 0.2 mg/ml, and were stored without the access of light in a desiccator placed in a fridge (at 4 °C). 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 20 cm^2/min. π–*A* isotherms were recorded with a NIMA (U.K.) Langmuir trough (total area = 300 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. All the surface pressure/area isotherms reported here are the averages of at least three experiments. The subphase temperature (20 °C) was controlled thermostatically to within 0.1 °C by a circulating water system.

3. Results

A thorough characteristic of Langmuir monolayers formed by the antifungal antibiotic – nystatin – has been presented in our former contribution [42]. It has been found that this antibiotic at the air/water interface built monolayers of a liquid expanded (LE) character.

The interactions in mixed Langmuir monolayers can be studied from the point of view of miscibility of monolayer components, based on the analysis of π–A isotherms [43]. Thus, in the first step of our studies, the isotherms for one-component (nystatin and respective lipids) as well as mixed antibiotic/lipids monolayers have been recorded (Fig. 1).

As regards mixed films containing sterols, the isotherms for mixed monolayers lie in-between those for pure nystatin and respective sterols. However, π–A curves for mixtures of antibiotic/ergosterol, especially of lower antibiotic content, are more shifted towards pure ergosterol isotherm as compared to nystatin/cholesterol mixtures. Additionally, the isotherms for nystatin/cholesterol mixed system possess, even at low nystatin mole fraction, characteristic plateau region, which changes position with the antibiotic content in a mixed monolayer. This phenomenon also differentiates this system from that containing fungi sterol, in which plateau appears only for monolayers containing nystatin in excess ($X_{NYS} \geq 0.5$). This indicates a stronger effect of nystatin on fungi sterol monolayers. The isotherms recorded for nystatin/DPPC mixed monolayers are shown in Fig. 3c. As seen, only π–A curves for film of nystatin mole fraction $X_{NYS} \leq 0.3$ lie in-between the isotherms for pure phospholipid and the antibiotic, while those for $X_{NYS} \geq 0.5$ are strongly shifted towards smaller areas, which suggests the existence of strong intermolecular interaction in this composition region.

The influence of lipids on the physical state of nystatin monolayer can be verified based on the analysis of compression modulus values for mixed films. The compression modulus is defined as follows (Eq. (1)) [44]

$$C_s^{-1} = -A(d\pi/dA) \quad (1)$$

and was obtained by numerical calculation of the first derivative from the isotherm data points. The C_s^{-1} vs. π plots have been presented in Fig. 2. The addition of a sterol or a phospholipid (which form more condensed monolayers than NYS) into the antibiotic film causes an increase of C_s^{-1} values, causing the mixed film to be more rigid and closely packed. Generally, sterols condense expanded nystatin monolayers, however, this effect seems to be stronger for ergosterol-containing films (see Fig. 2a and b). These results, together with the observation regarding the isotherm course and

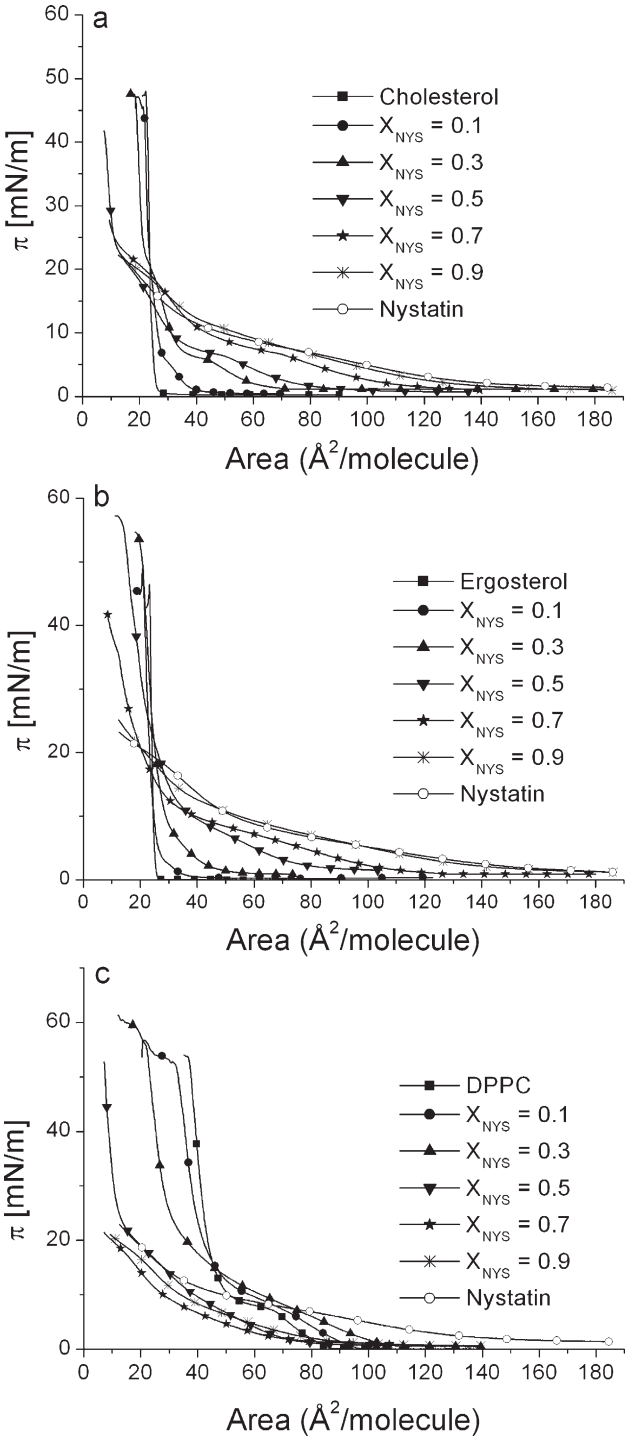

Fig. 1. Surface pressure–area (π–A) isotherms of mixed monolayers: nystatin/cholesterol (a), nystatin/ergosterol (b), nystatin/DPPC (c) spread on water at 20 °C.

characteristic and the fact that stronger contraction of the mean molecular area is observed for isotherms recorded for ergosterol-containing mixtures, indicate stronger interactions between the antibiotic and fungi sterol.

A thorough qualitative and quantitative analysis of the interaction between molecules in mixed monolayers have been done based on calculations of the excess area A^{Exc} and the

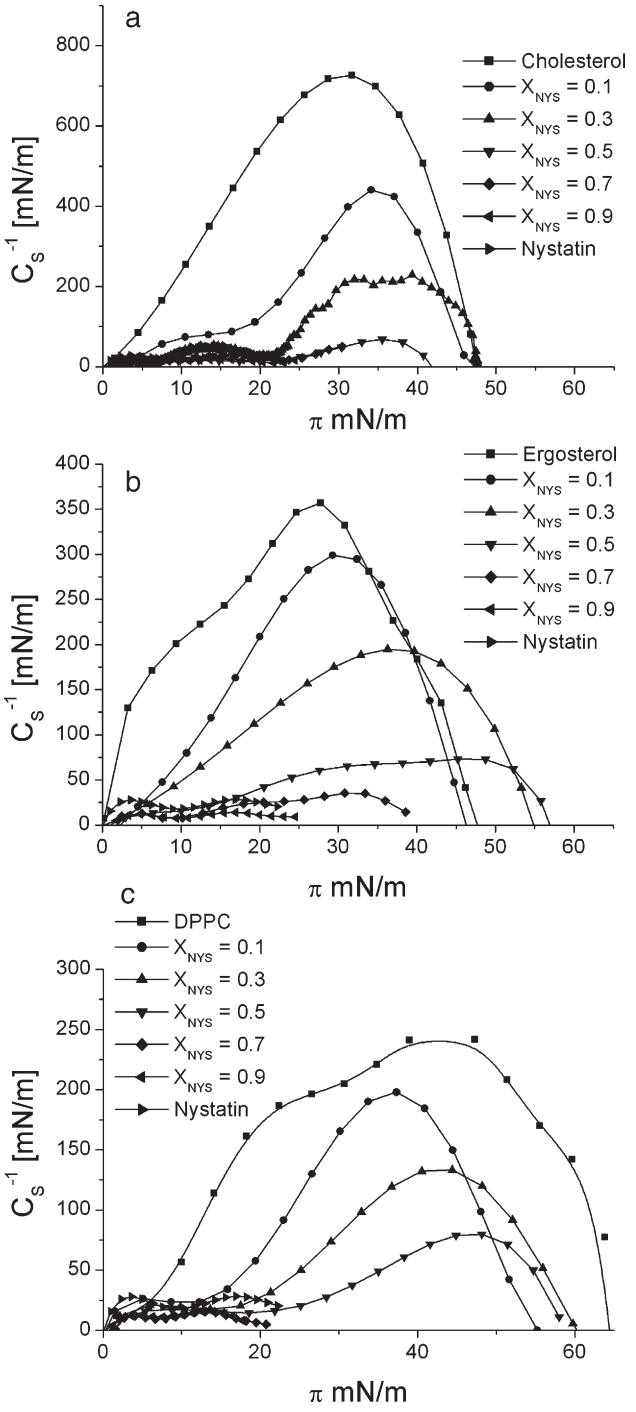

Fig. 2. The compression modulus (C_s^{-1}) vs. surface pressure (π) dependencies for mixed monolayers: nystatin/cholesterol (a), nystatin/ergosterol (b), nystatin/DPPC (c).

excess Gibbs energy of mixing ΔG^{Exc}, respectively. The excess area is expressed by the formula (Eq. (2)) [45]

$$A^{\mathrm{Exc}} = A_{12} - (A_1X_1 + A_2X_2) \tag{2}$$

where A_{12} is the mean area per molecule in mixed monolayer at constant surface pressure, A_1, A_2 are the molecular area of single component at the same surface pressure and X_1, X_2 are the mole fractions of components 1 and 2 in mixed film.

The values of ΔG^{Exc} can be evaluated directly from π–A isotherms using following equation (Eq. (3)) [45]

$$\Delta G^{\mathrm{Exc}} = N\int_0^{\pi}(A_{12} - X_1A_1 - X_2A_2)\mathrm{d}\pi \tag{3}$$

Analyzing A^{Exc} and ΔG^{Exc} values as a function of the film composition it is possible to draw conclusions regarding the miscibility between monolayer components as well as the kind and strength of interaction between them [32,45].

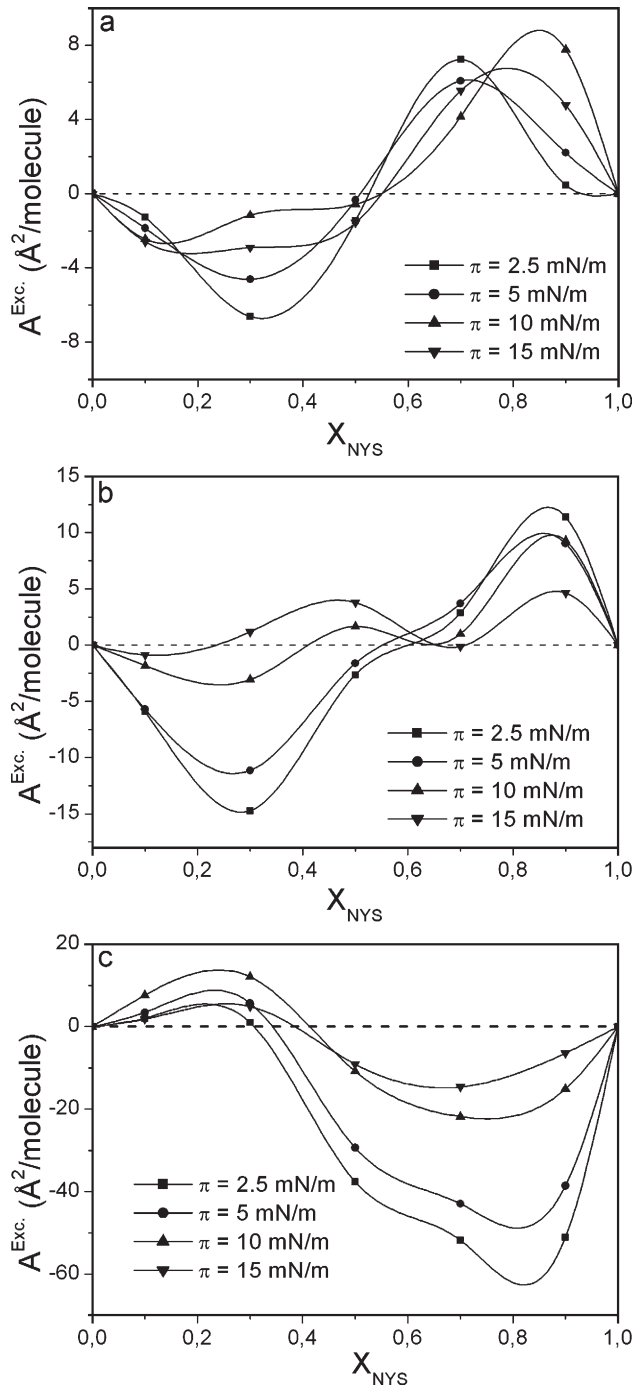

Fig. 3. Excess area (A^{Exc}) vs. composition plots for mixtures of nystatin/cholesterol (a), nystatin/ergosterol (b), nystatin/DPPC at different constant surface pressures.

In Fig. 3, A^{Exc} vs. nystatin mole fraction plots for mixtures containing antibiotic and cholesterol (a), ergosterol (b) and DPPC (c) at different values of surface pressure, namely: π=2.5, 5,10 and 15 mN/m, are presented. As it can be seen, there is no linearity between the area available for a molecule and the mole fraction of nystatin. Therefore it is evident that the investigated compounds are miscible and interact in mixed monolayer in whole range of antibiotic mole fraction and surface pressures. However, the kind of interactions depends on the monolayer composition. At low content of nystatin in sterol-containing monolayers ($X_{NYS} \leq 0.5$) (Fig. 3a, b), a negative deviation from ideality, suggesting attractive interaction between molecules, are observed. Further increase of nystatin content in mixed monolayer results in positive deviations from linearity. This proves that the molecular interactions in this region are less attractive as compared to ideally mixed monolayers. Also in the case of mixed monolayers of nystatin with phospholipid, the kind of interactions between molecules in mixed films depends on monolayer composition; however, some oppose effects with relation to nystatin/sterols mixtures can be observed (Fig. 3c). It means that the negative deviations from linearity appear for monolayers of $X_{NYS} > 0.4$, while at lower antibiotic content, positive deviations and less attractive forces between molecules exist.

For a quantitative analysis of the interaction between nystatin and lipids, the excess Gibbs energy of mixing ΔG^{Exc} for mixtures of different composition at constant surface pressures (π=2.5, 5, 10 and 15 mN/m) were obtained and the interaction parameter α was calculated (Eq. (4)) [45]:

$$\alpha = \frac{\Delta G^{Exc}}{RT(X_1 X_2^2 + X_2 X_1^2)} \quad (4)$$

where R is the gas constant and T is the absolute temperature. The positive sign of α (or ΔG^{Exc}) indicates that the mixed monolayer is more expanded and the molecular interactions are less attractive as compared to ideally mixed monolayers, while negative values suggest attractive interaction between monolayer components and its higher thermodynamic stability. The ΔG^{Exc} values obtained for investigated mixed systems are compiled in Table 1, while α was presented as a function of monolayer composition in Fig. 4a–c. Analyzing these parameters obtained for all the investigated mixed systems as a function of mixed monolayer composition, two characteristic regions of interaction between nystatin and respective lipids can be noticed, namely the first one, at low antibiotic content, in which both ΔG^{Exc} as well as α parameter are negative for mixtures with sterol and positive for mixed monolayers containing phospholipid, and the other one, at higher antibiotic mole fraction, where the excess Gibbs energy of mixing values and interaction parameter are positive for mixtures with sterol and negative for mixtures with DPPC. As regards mixed monolayers of nystatin and sterols, the composition range, in which attractive interaction between monolayers components appears, is nearly the same for mammalian and fungi sterol and ranges between 0.1 and 0.6 of nystatin mole fraction. However, the values of ΔG^{Exc} as well as α are nearly two times more negative for mixed monolayers containing ergosterol vs. cholesterol. Interestingly, at higher X_{NYS}, the attractions between the antibiotic and both sterols decrease, while a strong affinity towards phospholipid can be observed. It is worth pointing out significantly higher absolute values of ΔG^{Exc} for mixtures with DPPC as compared to nystatin/sterol systems in this composition regions, where the attraction between film molecules are observed ($X_{NYS} > 0.4$ for mixtures with DPPC and X_{NYS}=0.1–0.6 for sterol-containing systems).

In order to obtain information of mixed monolayers stability, the values of total free energy of mixing ΔG^{M} (Eq. (5)) have been calculated:

$$\Delta G^{M} = \Delta G^{Exc} + \Delta G^{id} \quad (5)$$

where

$$\Delta G^{id} = RT(X_1 \ln X_1 + X_2 \ln X_2) \quad (6)$$

The ΔG^{M} vs. nystatin mole fraction dependencies are shown in Fig. 5a–c.

Analyzing Fig. 5a it can be noticed that the ΔG^{M} values are negative for all the investigated mixed monolayers within the whole range of nystatin content; independently of surface pressure. This proves that the 2D mixed state is thermodynamically more stable and thus, more favorable than the corresponding unmixed state. Moreover, the mixed monolayers containing sterols are of the same stability in wide and nearly the same composition range namely $X_{NYS} \approx 0.3 \div 0.65$. As

Table 1
Excess Gibbs energy of mixing ΔG^{Exc} values for mixed monolayers of nystatin with cholesterol, ergosterol and DPPC at different surface pressures

X_{NYS}	ΔG^{Exc} [J/mol]											
	Cholesterol				Ergosterol				DPPC			
	π [mN/m]				π [mN/m]				π [mN/m]			
	2.5	5	10	15	2.5	5	10	15	2.5	5	10	15
0.1	−82	−88	−68	−131	50.1	−56	−173	−365	153	203	363	495
0.3	−21	−0.5	−62	−179	−85	−300	−540	−491	63	116	344	612
0.5	262	185	−25	−223	−176	−224	−239	−224	−313	−806	−1424	−1700
0.7	5	111	283	356	−61	−12	59	−2	−382	−1080	−2074	−2588
0.9	427	461	611	745	320	461	702	868	−50	−650	−1495	−1787

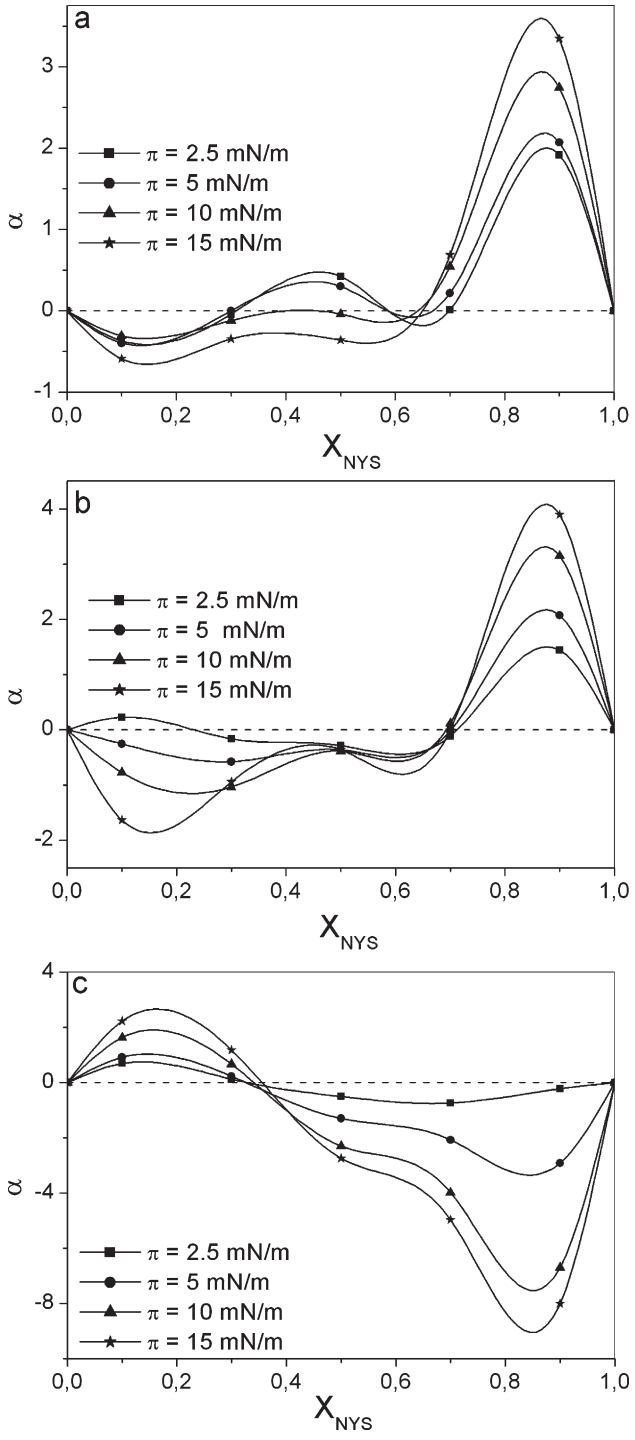

Fig. 4. α parameter vs. composition plots for mixtures of nystatin/cholesterol (a), nystatin/ergosterol (b), nystatin/DPPC at different constant surface pressures.

regards monolayers of antibiotic and DPPC, the highest stability is found when the antibiotic content is about 0.7.

4. Discussion

The results of investigations presented herein provide both quantitative and qualitative analysis of the interaction between polyene antibiotic – nystatin – and natural (mammalian and fungi) membrane components in mixed Langmuir monolayers and allow us to draw some general conclusions, which can be of help in understanding the role of phospholipids in polyene mechanism of action.

It has been found that although the interaction between nystatin and both sterols are attractive in nearly the same monolayer composition range, they are stronger in monolayers containing ergosterol vs. cholesterol-containing films. These stronger interactions between components in mixed monolayers containing fungi sterol can be attributed to structural differences

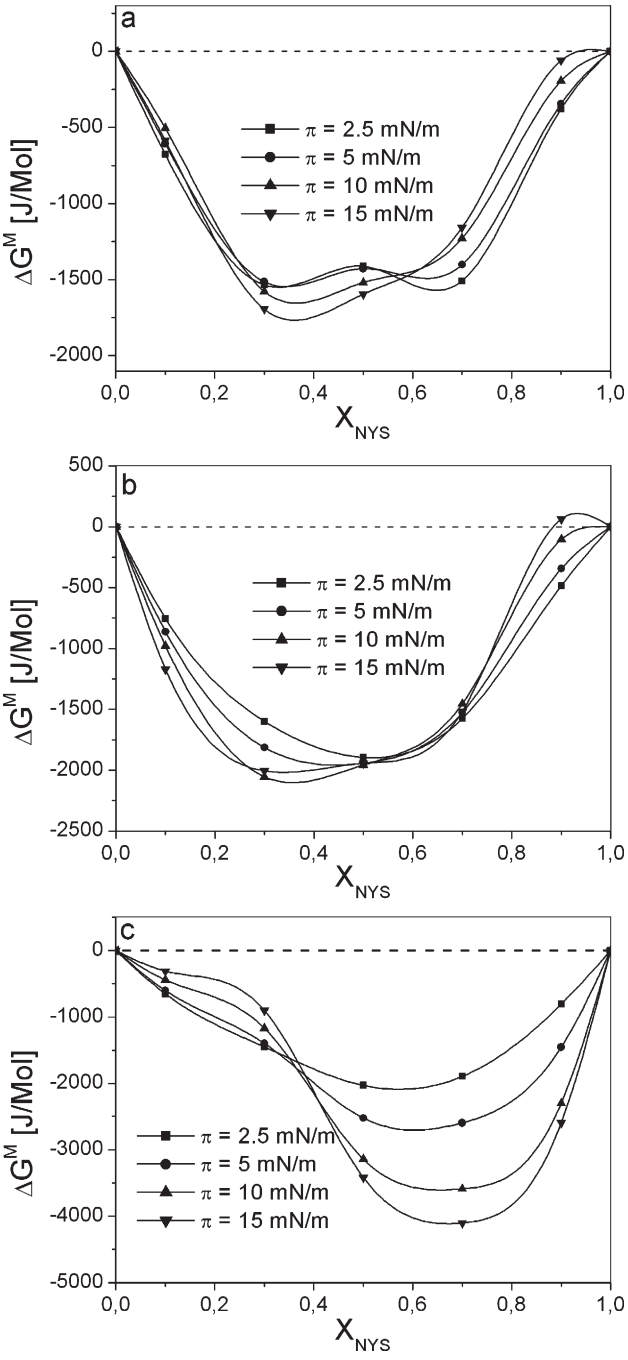

Fig. 5. Total free energy of mixing (ΔG^M) vs. composition plots for mixtures of nystatin/cholesterol (a), nystatin/ergosterol (b), nystatin/DPPC at different constant surface pressures.

between mammalian and fungi sterol. Ergosterol, in comparison with cholesterol, possesses additional double bonds in the side chain and the other one in the sterol ring as well as an additional methyl group in the side chain. The presence of an unsaturated bond in the hydrocarbon chain makes the molecule more rigid and hinders its conformational changes and the side hydrocarbon tail, which is stiffer and more elongated than that of cholesterol, protrudes out of the cyclic part of the molecule. As has been precisely described previously [46] the monolayer from ergosterol is looser as compared to cholesterol, which gives possibility of more favorable, as compared to rigid and closely packed cholesterol film, molecular packing of large nystatin molecule.

Interestingly, the affinity of the other polyene: amphotericin B (AmB) to both sterols is comparable in a broad range of mixed film compositions [47]; however, as compared to nystatin, the interactions between AmB and fungi sterol are weaker. On the other hand, the biological studies indicate that amphotericin B is more active towards fungi than the investigated here nystatin, as lower concentration of amphotericin B is required to cause the same effect on different pathogenic fungi as compared to nystatin [20,25,48]. Although at first sight these reports are at variance with our results; however, the situation changes if the interactions between nystatin and both kinds of lipids (sterols and DPPC) are analyzed together and compared to the results for AmB/lipid systems. As it has been found [47], the interaction between amphotericin B and DPPC are stronger than with sterols, however, in comparison with those presented herein for nystatin/DPPC monolayers it is evident that the affinity of the latter antibiotic to the investigated phospholipid is about 3 times higher than AmB. In our opinion, these interactions are crucial for understanding the action of polyenes in a membrane. We suggest that the antibiotic/phospholipid interaction reduce the concentration of free antibiotic, which is capable of interacting with membrane sterols, decreasing in this way its therapeutic action.

Based on our monolayer experiments, considering NYS/cholesterol interactions and comparing them with those for AmB, it is possible to discuss the toxicity problem of both polyenes. It has been found that the free energy of mixing values, in the same range of monolayer composition, for nystatin/cholesterol mixtures are only insignificantly lower than those for AmB/cholesterol monolayers (minimum of $\Delta G^{Exc} \approx -200$ and -300 J/mol for nystatin and amphotericin B, respectively). This suggests rather comparable effect of both antibiotics on mammalian cells. However, biological studies [16] indicate that the antibiotic concentration required for 50% hemolysis of erythrocytes of mice blood are significantly different (6 and 80 μg/ml for amphotericin B and nystatin, respectively). Our suggestion regarding the role of phospholipids in polyene action in natural membrane allows explaining also lower toxicity of nystatin in comparison with amphotericin B. Namely, the phospholipid/cholesterol interactions prevent this sterol from forming complexes with the antibiotic. Since the interactions between DPPC and nystatin are significantly stronger as compared to AmB, thus, as proven by the above-mentioned biological studies, nystatin is less toxic towards mammalian cell.

It is worth discussing here one more effect, which is of importance for a better understanding of polyene affinity towards sterols. It is well known that phospholipids are always associated with sterols in biological membranes, and therefore when analyzing the sterol/antibiotic interactions, the interactions between DPPC – the investigated here model phospholipid – and sterols need also to be taken into account. The investigations carried out by other authors prove the existence of stronger interactions and significantly higher stability of mixed DPPC and cholesterol monolayers as compared to DPPC/ergosterol system [35]. Therefore, it may be supposed that strong interaction between DPPC and mammalian sterol prevent interaction of antibiotic/cholesterol and "naturally" increases selectivity of antibiotics, however, without altering its antifungal properties. It should be stressed that the influence of the above-mentioned DPPC/cholesterol interactions is the same for both antibiotics.

Our conclusion regarding the role of phospholipids in polyene mechanism of action is in agreement with suggestions of other authors. Namely, it has been found that phosphatides compete with sterols in cell membranes and decrease the polyene activity by reduction of the effective antibiotic concentration due to a complex formation [49–51].

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