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The negligible effects of the antifungal natamycin on cholesterol-dipalmitoyl phosphatidylcholine monolayers may explain its low oral and topical toxicity for mammals

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ABSTRACT

Natamycin is an effective, broad spectrum antifungal with no reported resistance, in contrast to most antimicrobials. It also exhibits reduced (oral and topical) toxicity to humans, which is probably associated with the lack of effects on mammalian cell membranes. In this paper we employ Langmuir monolayers to mimic a cell membrane, whose properties are interrogated with various techniques. We found that natamycin has negligible effects on Langmuir monolayers of dipalmitoyl phosphatidylcholine (DPPC), but it strongly affects cholesterol monolayers. Natamycin causes the surface pressure isotherm of a cholesterol monolayer to expand even at high surface pressures since it penetrates into the hydrophobic chains. It also reduces the compressibility modulus, probably because natamycin disturbs the organization of the cholesterol molecules, as inferred with polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS). In mixed cholesterol/DPPC monolayers, strong effects from natamycin were only observed when the cholesterol concentration was 50 mol% or higher, well above its concentration in a mammalian cell membrane. For a sterol concentration that mimics a real cell membrane in mammals, i.e. with 25 mol% of cholesterol, the effects were negligible, which may explain why natamycin has low toxicity when ingested and/or employed to treat superficial fungal infections.

1. Introduction

Diseases caused by opportunistic fungal infections have become a major health problem owing to the increasing number of individuals whose immune system has been compromised by HIV infection or by administration of immunosuppressive drugs during cancer treatment and organ transplants [1,2]. This has motivated the development of new antifungal drugs, including polyene antibiotics [3], that are natural drugs typically produced by *Streptomyces* spp. [4]. Examples of these polyenes are amphotericin B, nystatin and natamycin, which are effective antifungals with no reported resistance since their discovery 50 years ago, in contrast to most antimicrobials. The mechanism of action appears to be connected with their high affinity for sterols [5], but controversies exist about the precise molecular-level mechanism [6], especially with regard to interaction with cell membranes. The activity of amphotericin B and nystatin was normally attributed to malfunction and/or disruption (leakage) of the lipid bilayer promoted by their binding to the biomembrane sterols [7], inducing pores or channels and disturbing the selective permeability of the membrane [1,2,8–11]. Recent findings have cast doubt on this hypothesis since a modified amphotericin exhibited antifungal activity without being able to form pores or channels [6]. As for natamycin, this formation of pores or channels is not applicable [6,12], and therefore other mechanisms must prevail [11,13,14].

In this study we try to explain the low toxicity of natamycin to mammalian cells, in spite of its affinity to cholesterol, by investigating the interaction with cell membrane models. We use Langmuir monolayers to mimic a cell membrane for two main reasons, namely the ability to probe molecular-level interactions with vibrational spectroscopy and the possibility of controlling the model membrane composition. Understanding the interaction with membranes is crucial because natamycin is a macrolide polyene antifungal with broad spectrum activity, which is only possible if the action involves the membrane. Natamycin was first isolated

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in 1955 from a soil sample in Natal province, South Africa, produced by the bacteria strain named *Streptomyces natalensis*. It is poorly soluble in water, almost insoluble in nonpolar solvents. Natamycin powder is stable in the dark, with no loss of activity, but it is degraded in aqueous suspensions if exposed to UV-light, oxidants and heavy metals [15,16]. When applied to food surfaces, natamycin does not affect the food quality (color, texture and flavor), being safe for consumption because its oral absorption is negligible [16–18]. Due to its low toxicity, natamycin is approved as a natural food additive, can be used topically for treating mycoses [19,20], and is the only topical antifungal approved by the US Food and Drug Administration (FDA) for topical ophthalmic use [20–22].

Macrolide polyene antibiotics have been successfully studied with the Langmuir monolayer technique [10,23–30]. Here we used binary mixtures of phosphatidylcholine (PC) and cholesterol (Chol), which are the most abundant phospholipid and sterol in mammalian membranes [28,31–33], as model membrane, in addition to neat monolayers of dipalmitoyl phosphatidylcholine (DPPC) and cholesterol for comparison.

2. Materials and methods

2.1. Adsorption kinetics and surface pressure isotherms

Polyene antifungal natamycin was supplied by Fluka, cholesterol was purchased from Sigma-Aldrich and synthetic phospholipid dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids. Fig. 1 displays the chemical structure of the three compounds. DPPC was chosen for this study because phosphatidylcholine (PC) is the most abundant component in membranes of mammals, being an important constituent of the alveolar fluid [34]. All reagents were used without further purification. Spreading stock solutions (0.5 mg/mL) for the lipids were made daily by dissolving each compound separately in chloroform, with mixtures being produced in the desired proportions. Aqueous solution/suspension of natamycin is quite stable [15], which is why aqueous formulations are used in therapeutic applications [20–22] and as food additives [16–18]. In ultrapure water (pH = 5.5–6) natamycin molecules are slightly positively charged, since their isoelectric point is at pH = 6.5 [15,35]. Although natamycin solubility in water has been estimated to be 0.03–1.0 g/L [15,35], our preliminary investigations led to irreproducible results (data not shown) when 0.04 g/L was used for the stock solution. This could be associated with aggregation, since a broadening was observed in the UV–vis. bands for natamycin at 0.04 g/L in Fig. S1 in the Supporting information. Therefore, all experiments were performed with 0.02 g/L (black line) for the natamycin stock solution. The subphase employed was made with ultrapure water (pH = 5.5–6) at 21 ± 0.5 °C.

The kinetics of adsorption for natamycin at the air/water interface, which could contain a lipid monolayer, was investigated using a cylindrical Teflon container with a diameter of 4 cm and a volume of 10 mL adapted on a KSV mini trough. Surface pressure was measured with the Wilhelmy method [10]. Aliquots from lipid stock solutions were spread on the water surface, with a volume chosen to reach the desired final pressure. Natamycin was either injected into the subphase or dissolved in the subphase from the start. Surface pressure isotherms for the lipid monolayers were carried out in a mini KSV Langmuir Teflon trough with 250 mL volume. The number of natamycin molecules dissolved in the subphase (0.15 μmol) was about 3 times the number of lipid molecules spread (0.054 μmol) at the air–water interface. This total amount of natamycin (0.4 μg/mL) in the subphase is well below the safe limit established by USA FDA (20 ppm) [35] and it is also about ten times below the minimum inhibitory concentration (MIC) [19–21,47,48]. Since significant oxidation of cholesterol molecules at the interface may occur after 40 min [32,36,37], the isotherm experiments were performed within 40 min of monolayer spreading. A waiting time of 20 min elapsed for chloroform evaporation and monolayer stabilization. The scan rate for the symmetric barrier compression was 30 cm^2/min. At least three isotherms were acquired for each composition to ensure reproducibility, and the curves shown are representative.

2.2. Analysis of isotherms

The mechanical properties of the monolayers were analyzed by calculating the compression modulus C_s^{-1} from the first derivative (1) [10,23–28,31,38,39]

$$C_s^{-1} = -A\left(\frac{\partial \pi}{\partial A}\right)_T \quad (1)$$

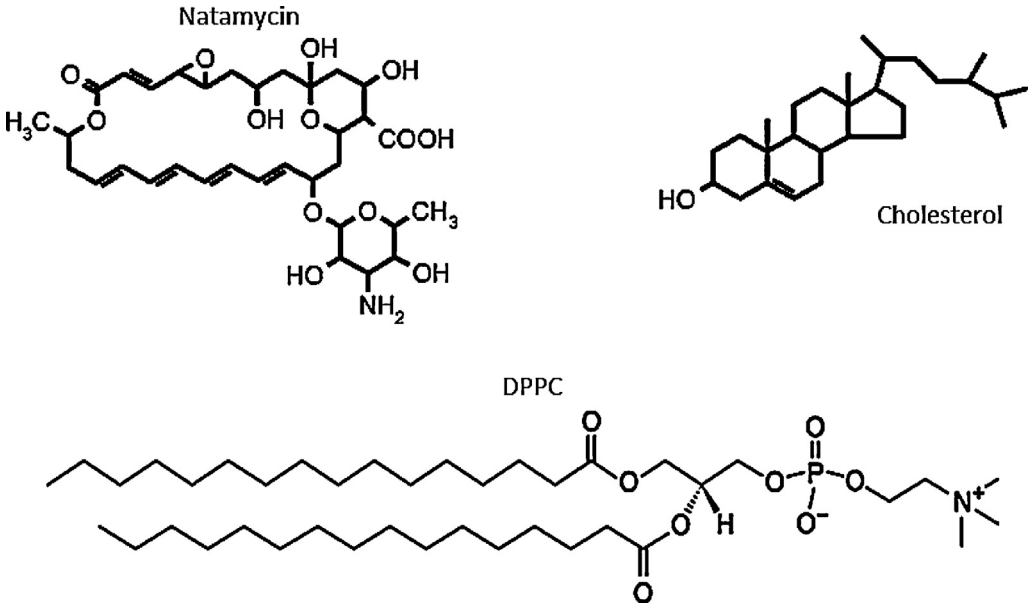

Fig. 1. Chemical structures of the antifungal natamycin, the sterol cholesterol and the phospholipid dipalmitoylphosphatidylcholine (DPPC).

where A is the area per molecule at pressure π. According to Davis and Rideal [40] the monolayer phases can be identified by the compression modulus values: liquid-expanded phase for C_s^{-1} = 12.5–50 mN/m, liquid phase for C_s^{-1} = 50–100 mN/m, liquid-condensed for C_s^{-1} = 100–250 mN/m and condensed (solid) for C_s^{-1} > 250 mN/m. The higher C_s^{-1} the less elastic the monolayer is. The collapse pressure is defined at C_s^{-1} = 0.

The thermodynamic properties (stability and miscibility) of the mixed monolayers were studied by calculating the excess free energy of mixing (ΔG_{exc}) at a given surface pressure (π) defined in the following equation [10,23,26–28,31,38,41,42]

$$\Delta G_{exc} = N \int_0^{\pi} (A_{12} - A_1X_1 + A_2X_2)\, d\pi \quad (2)$$

where N is the Avogrado's number; A_{12} is the measured area per molecule for a mixed monolayer; A_1 and A_2 are the molecular areas for each pure component, with X_1 and X_2 being their respective mole fractions. Non-zero values for ΔG_{exc} indicate molecular-level interactions, which may be attractive or repulsive depending on whether ΔG_{exc} is negative or positive. Hence, the positive sign of the ΔG_{exc} value indicates segregation of the components, while the most negative ΔG_{exc} characterizes the most thermodynamically stable monolayer, of which the great miscibility of components is secured [10,26,28,42].

2.3. Brewster angle microscopy (BAM)

The monolayer morphology was monitored using a Brewster angle microscope (BAM), model BAM 2 Plus (Nano Film Technology, Germany). The microscope (light source and camera) was coupled to a NIMA Langmuir trough, and the compression speed used during the imaging procedure was the same as for recording the surface pressure isotherms. In this experimental set-up [23,24,31,43,44], the *p*-polarized light beam incident at the Brewster angle (about 53°) on a clean water surface is not reflected. On the other hand, differences in the refraction index owing to monolayer formation leads to reflected light, thus forming an Image [23,24,31,43,44]. In the gray scale, the darkest regions represent the clean water surface (no light reflection), while the brightest correspond to monolayer domains [23,24,31,43,44].

2.4. PM-IRRAS analysis

The spectra for polarization-modulated infrared reflection-absorption spectroscopy (PM-IRRAS) were obtained using a KSV PMI 550 instrument (KSV, Finland) coupled to a mini KSV Langmuir trough. The polarized infrared beam was positioned to reach the monolayer at an incident angle of 80°, relative to the normal water surface. The total acquisition time for each spectrum was 5 min and the compression speed was the same in the surface pressure isotherms. This experimental set-up is optimized to study Langmuir films at the air–water interface [38,39,44–46], since the signal-to-noise ratio is magnified and the interference from water vapor and carbon dioxide is minimized with the modulation of the polarization. Under the conditions used, positive bands in the spectra indicate transition moments preferentially on the surface plane, whereas negative bands indicate preferential orientation perpendicular to the surface.

3. Results and discussion

3.1. Effect of natamycin on DPPC and cholesterol monolayers

Our adsorption kinetics results (Fig. S2A–C) are attached in the Supporting Information. Natamycin has no surface activity for the concentrations used in this work, which is inferred from the lack of changes in surface tension (Fig. S2A) for aqueous solutions. This is consistent with the results obtained by Demel et al. [30] who showed that, compared with other polyenes (amphotericin B, nystatin and filipin), only natamycin spread at the air–water interface exhibited a poor quality isotherm, with little increase in surface pressure (~5 mN/m), even when compression was carried out until reaching a very low area per molecule (~8 Å^2) [29]. Natamycin has apparently no affinity toward DPPC, as no adsorption was observed according to the surface pressure measurements in Fig. S2B, in the Supporting information. In contrast, the surface pressure of a cholesterol monolayer changed with time, owing to adsorption of natamycin, as indicated in Fig. S2C especially for already-prepared subphases containing natamycin.

The lack of affinity toward DPPC and the strong effect on cholesterol, inferred from the adsorption kinetics results above, are confirmed with the surface pressure isotherms in Fig. 2. While natamycin had no effect on a DPPC monolayer, a large shift occurred for the cholesterol monolayer, and this is indicated quantitatively in the extrapolated areas in Table 1. The lack of interaction with DPPC is consistent with several pieces of evidence in the literature, for natamycin in aqueous solutions did not affect phosphocholine (PC) spherules [47] and DPPC unilamellar vesicles [11,12].

As for the effect on cholesterol monolayers, in addition to expanding the surface pressure isotherm, natamycin causes the compressibility modulus to decrease considerably, as shown in the inset of Fig. 2. Therefore, natamycin penetrates into the cholesterol

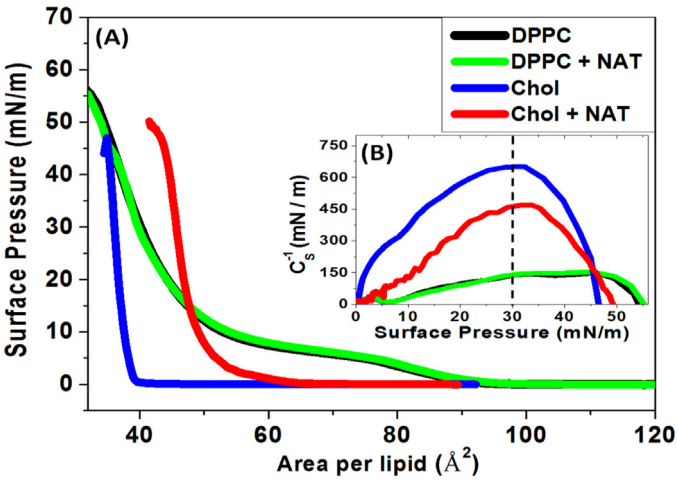

Fig. 2. Surface pressure (π) vs. mean molecular area compression isotherms for DPPC and cholesterol spread at air/water interface. The subphase used was ultrapure water, pH 5.8, T = 21 °C, with or without addition of natamycin. The inset shows the in-plane elasticity (C_s^{-1}) values versus surface pressure for the monolayers, with C_s^{-1} being calculated from the isotherms using Eq. (1).

Table 1
Values for area per molecule, collapse pressure and in-plane elasticity (C_s^{-1}) for DPPC, cholesterol monolayers and their binary mixture with and without natamycin in the subphase.

Monolayer	Area per molecule (±2 Å^2)	C_s^{-1} (π = 30 mN/m)
DPPC	52	138
+Natamycin	53	140
X_{CHOL} = 0.25	46	172
+Natamycin	47	163
X_{CHOL} = 0.5	43	358
+Natamycin	47	192
X_{CHOL} = 0.75	41	474
+Natamycin	44	469
Cholesterol	38	650
+Natamycin	49	463

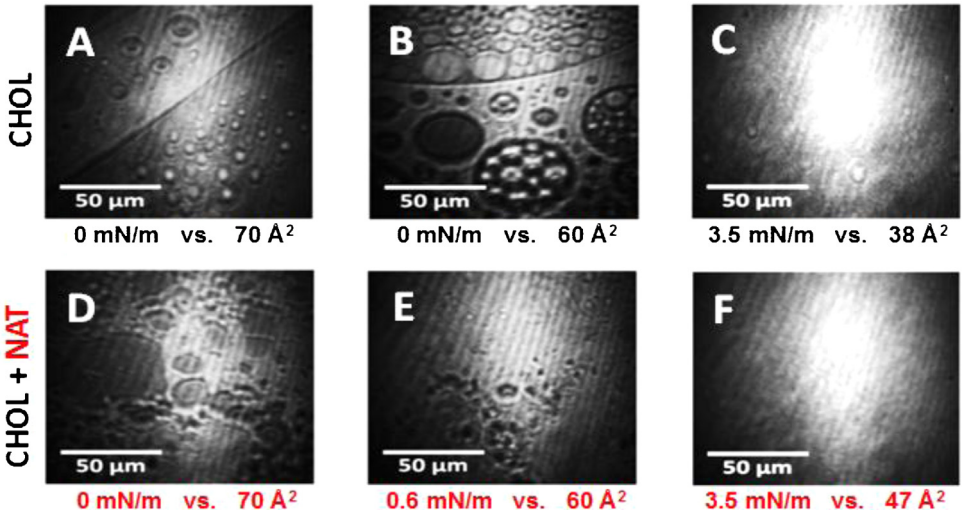

Fig. 3. Brewster angle microscopy (BAM) images for cholesterol monolayers in the absence or in the presence of natamycin at various surface pressures (mN/m) and areas per lipid (Å²).

monolayer and turns it less rigid. In subsidiary experiments we observed that natamycin expands the monolayer of the negatively charged dipalmitoyl phosphatidylglycerol (DPPG), but to a much lesser extent than it does for cholesterol (Fig. S3 in the Supporting information).

Fig. S4 in the Supporting information shows two sets of images (A–C) and (D–F) corresponding, respectively, to BAM images for DPPC monolayer spread at the surface of a clean water subphase and of a subphase containing natamycin. The latter did not alter the morphology of a DPPC monolayer made up of characteristic domains owing to coexistence of the liquid-expanded (dark regions) and liquid-condensed (bright regions) phases [32,34] (Fig. S4A, B, D and E), then evolving to a continuous, bright film when the liquid-condensed phase dominates (Fig. S4C and F). The domains in cholesterol monolayers are not as well defined as in DPPC, but one may nevertheless note that the overall morphology is significantly affected by natamycin. For an area per lipid of 70 Å² in Fig. 3A, the surface pressure is zero for a cholesterol monolayer and, as expected, the morphology is characteristic of the gas phase containing circular domains, while the addition of natamycin in Fig. 3D causes a predominance of clusters of irregularly-shaped domains. The morphology of cholesterol monolayers at fixed areas per molecule are also affected by the expansion effect caused by natamycin. For instance, at 60 Å² the cholesterol monolayer containing natamycin appeared almost fully continuous (Fig. 3E), while pure cholesterol films are still segregated (Fig. 3B).

In contrast, according to Fig. 4, natamycin promoted changes in the PM-IRRAS spectra of a cholesterol monolayer; though the shifts were almost within the resolution of the instrument, clear trends were observed. At 5 mN/m in Fig. 4A, there is an increase for both asymmetric (2937 cm^{-1} shifted to 2944 cm^{-1}) and symmetric stretch of CH_2 groups (2851 cm^{-1} shifted to 2856 cm^{-1}), and for the symmetric stretch of CH_3 (2886 cm^{-1} shifted to 2890 cm^{-1}); at 10 mN/m in Fig. 4B, the vibration frequencies increased for both symmetric stretch of CH_2 (2851 cm^{-1} shifted to 2853 cm^{-1}) and CH_3 (2886 cm^{-1} shifted to 2890 cm^{-1}), while the relative intensity between the asymmetric stretch bands of CH_2 (2934 cm^{-1}) and CH_3(2953 cm^{-1}) decreased. Because intensity variations are correlated to changes in molecular orientation [38,48], one may infer that natamycin disturbs the organization of cholesterol molecules, probably by increasing the distance between them. Surprisingly, at 30 mN/m (Fig. 4C), which corresponds to the pressure in a real membrane [49,50], no significant change is noted for any of the

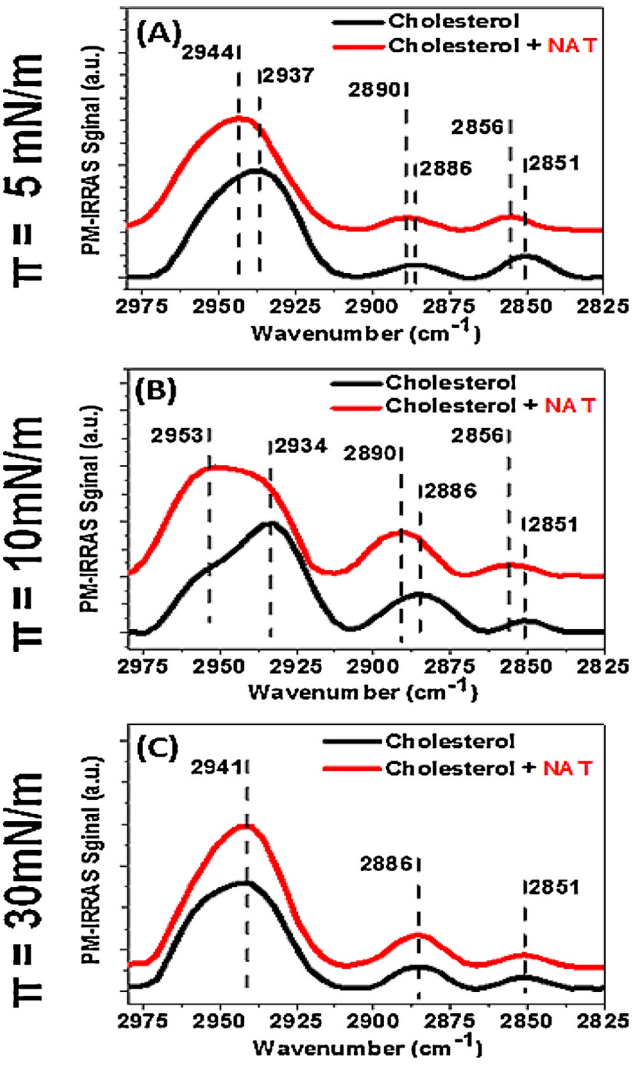

Fig. 4. PM-IRRAS spectra obtained for cholesterol monolayer at (A) 5 mN/m, (B) 10 mN/m and (C) 30 mN/m.

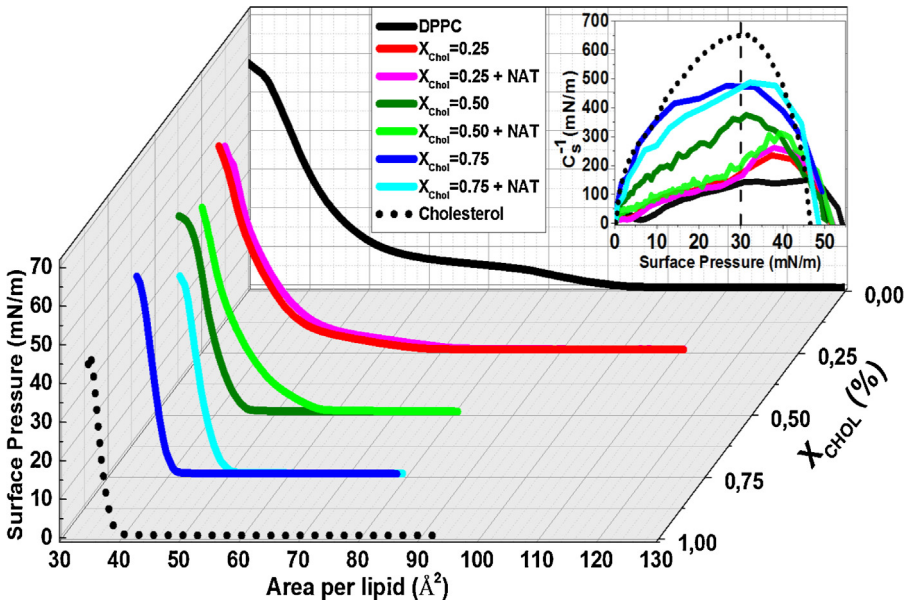

Fig. 5. Surface pressure (π) vs. mean molecular area (A) isotherms for DPPC and cholesterol and their mixtures (cholesterol mol fraction, X_{Chol}) spread at the air/water interface. The subphase used was ultrapure water, pH 5.8, $T = 21\,^{\circ}C$, with or without the addition of natamycin. The inset shows the in-plane elasticity (C_s^{-1}) versus surface pressure obtained from the data in Fig. 6 using Eq. (1).

bands. This lack of change may perhaps be related to the large rigidity (i.e. compression modulus) for cholesterol in a condensed state, which makes it difficult to observe dynamic changes in the vibrational spectra.

3.2. Effect of natamycin on DPPC–cholesterol mixed monolayers

Cholesterol is known to induce condensation in DPPC monolayers [31,32,38], which was indeed observed in the surface pressure (π)–area per molecule (A) isotherms in Fig. 5. The incorporation of natamycin in the subphase caused reduction in the condensing effect, i.e. a shift to larger areas per lipid, especially at medium-high concentrations of cholesterol.

This behavior is quantified in Table 1 with the extrapolated areas to zero pressure, in addition to the in-plane elasticity (C_s^{-1}) taken from the inset in Fig. 5. As expected, the effect from natamycin on a monolayer that mimics the sterol composition of a mammalian membrane [33] ($X_{Chol} = 0.25$) is very small and the pressure dependence for C_s^{-1} is practically the same as for the monolayer without natamycin. One could expect the largest effects to take place for $X_{Chol} = 0.75$, which does not occur probably because the amount of cholesterol that may penetrate into a DPPC monolayer is limited. For PC bilayers, for instance, above 37.5 mol% domains of cholesterol plaques are formed [51]. The changes induced by natamycin on the DPPC monolayer with $X_{Chol} = 0.5$ were significant, especially in the in-plane elasticity. These results are consistent with atomic force microscopy (AFM) data taken with mixed DPPC/cholesterol Langmuir–Blodgett (LB) films [52]. The films containing 10–30 mol% of cholesterol were smooth, densely packed, while for 50 mol% of cholesterol the film was rough owing to the decreased solubility of cholesterol in the DPPC monolayer.

From a thermodynamic viewpoint, another way to assess the condensing effect from cholesterol is to calculate the excess free energy of mixing (ΔG_{exc}), which is negative as shown in Fig. 6A for various cholesterol concentrations. These negative ΔG_{exc} values gradually increase with the surface pressure and indicate stronger van der Waals interactions (higher packing and miscibility) [10,26] between DPPC and cholesterol molecules. Moreover, the minimum

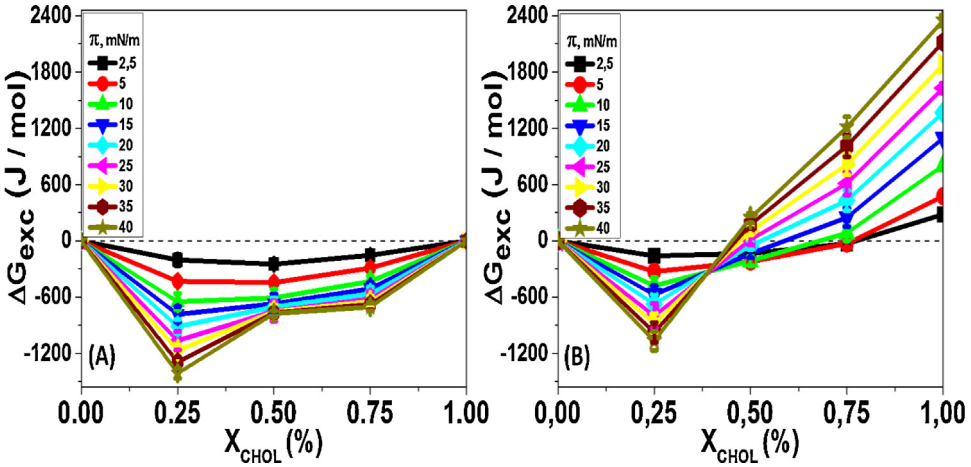

Fig. 6. Excess free energy of mixing (ΔG_{exc}) versus film composition (cholesterol mol fraction, X_{Chol}) at different surface pressures for DPPC/cholesterol mixtures spread on the surface of (A) ultrapure water and (B) aqueous solution of natamycin. Error bars represent the standard deviation for measurements taken in triplicate ($n = 3$).

ΔG_{exc} occurs at $X_{Chol} = 0.25$ and, therefore corresponds to the mixture of the highest thermodynamic stability [28], the cholesterol content of which is below the 37.5 mol% limit for solubility already mentioned [51]. The effect from natamycin is shown in Fig. 6B, where the condensing effect is diminished (ΔG_{exc} decreased) for all mixtures, but is reverted (positive ΔG_{exc} values) only at higher cholesterol contents ($X_{Chol} = 0.50$, 0.75 and 1.0). For these high concentrations, the expansion caused by natamycin overtakes the condensing effect from cholesterol on DPPC.

4. Conclusions

Natamycin does not form a Gibbs monolayer, adopting a preferential location in the bulk as suggested by Weissmann and Sessa [47]. Natamycin does not affect DPPC monolayers but causes large expansion in cholesterol monolayers. For DPPC-cholesterol mixtures, natamycin only has considerable effects when the cholesterol concentration is large (50 mol% or higher). For the mixture mimicking a real cell membrane in mammals, i.e. with ca. 25 mol% of cholesterol [33], its great thermodynamic stability (higher packing) seems to protect it from natamycin effects. This explains why natamycin administration (oral and topical) is safe for mammals [16,22,35]. Additionally, the interaction of natamycin with DPPG was found to be weaker than with cholesterol. One may conclude, therefore, that only membranes rich in sterols, cholesterol in our case, are amenable to significant action from natamycin.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2014.06.058.

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