Molecular organization of antibiotic amphotericin B in dipalmitoylphosphatidylcholine monolayers induced by K⁺ and Na⁺ ions: The Langmuir technique study

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The effect of potassium (K⁺) and sodium (Na⁺) ions on the self-association of antibiotic amphotericin B (AmB) in the lipid membrane was reported. Mixed Langmuir monolayers of AmB and dipalmitoylphosphatidylcholine (DPPC) were investigated by recording surface pressure–area isotherms spread on aqueous buffers containing physiological concentration of K⁺ and Na⁺ ions. The analyses of the π–A isotherms and compressional modulus curves indicate the interactions in the AmB–DPPC system. The strength of the AmB–DPPC interactions and the stability of the mixed monolayers were examined on the basis of the excess free energy of mixing values. The obtained results proved a high affinity of AmB towards lipids induced by the presence of K⁺ than Na⁺ ions. The most stable monolayers in the presence of K⁺ and Na⁺ ions were formed by AmB and DPPC with the 1:1 and 2:1 stoichiometry. The understanding of the AmB aggregation processes at the molecular level should contribute to elucidate the mechanisms of action and toxicity of this widely used drug. The presented results are potentially valuable in respect to develop more efficient and less toxic AmB formulations.

1. Introduction

The amphiphilic polyene antibiotic amphotericin B (AmB) is currently the drug of choice in the treatment of severe fungal infections despite its undesirable side effects. The toxicity of the drug has been related to its low solubility due to the formulation of self-associated species called molecular aggregates. According to the general conviction, the biological action of the drug is based on the formation of membrane pores that considerably affect physiological ion transport, especially K⁺ ions [1–3]. On the basis of commonly accepted hypothesis — the disturbance of membrane barrier function is made by creating transmembrane pores or channels [4,5]. The molecules of AmB adopt a quasi parallel orientation with their polar sides (the polyhydroxyl groups) facing the interior of such formed pores and the hydrophobic parts interacting with the lipids of the cellular membrane [6,7]. Further this model was completed in many experimental studies [8,9] both in theoretical studies on chemical structure and molecular properties of these channels [10,11]. It was postulated that the possibilities of chemotherapeutical activity of AmB are the result of interaction with biological membrane and it is based on higher affinity of the antibiotic towards ergosterol-containing fungal cells compared to cholesterol-containing mammalian cells [12–16]. The application of atomic force microscopy (AFM) studies allowed illustrating the porous structures formed from AmB in a monolayer containing 90 mol% of antibiotic and 10 mol% of DPPC. The size of the pore was estimated at −17 Å while the internal diameter came out to −6 Å [17].

Although there are numerous studies of interactions between AmB and model phospholipid membranes, the complete interpretation of these mechanisms is still unknown. AmB might be entering into bilayer membrane system which is dependent on its concentration and self-association but it is also possible in sterol-free membranes [18,19]. However, sterols seem to be necessary to stabilize the channels [20,21]. AmB may strongly interact with phospholipid molecules to form a stoichiometric complex 1:1. It has been postulated that there are interactions between the conjugated chain of antibiotic and the methylene groups of lipid acyl chains, while the sugar moiety interacts with the phosphate head group by the formation of a hydrogen bond [19,21]. The channel activity of the antibiotic is caused not only by the presence of sterols but it is also connected with the interaction of other membranes components. The possibility of the interactions of AmB with the surface of phospholipid bilayer is dependent on the properties of lipid polar heads, the length and the degree of the saturation of PC acyl chains, as well [22,23].

Analyzing of the pressure–area isotherm of the mixed monolayers, Minones et al. [23] reported the existence of stronger interactions
between AmB and the saturated phospholipid as compared to unsaturated one. The nature of these interactions revealed the influence of apolar parts of the phospholipid chains on the stoichiometry of AmB–phospholipid complexes [23,24]. The greatest interactions in the AmB–DPPC system were found to occur for a 2:1 mixture and a stable complex was composed of two horizontally oriented AmB molecules and one DPPC molecule in a vertical position [24]. The occurrence of stronger interaction between AmB and phospholipids than between sterols and membranes plays a key role in the biological activity of the compound. It leads to diminish the effective concentration of the antibiotics molecules in membranes which might interact with membrane sterols [22,25].

Overall, our previous works presented that the K⁺ and Na⁺ ions can affect the molecular organization of AmB in substantially different ways. The obtained results with the application of different spectroscopic techniques, such as electronic absorption spectroscopy, Raman and FTIR, indicated the influence of K⁺ ions on the aggregate levels of AmB molecules [26,27]. It was also proposed that AmB may have a direct influence on the ATP–proton pump, in the case of the cells of fungi, or it may inhibit the ATP (Na⁺ – K⁺) activity in the animal cells [28]. On the other hand, it was observed that the enrichment of fungal cell cultures with K⁺ cations exhibits protective properties against the toxic activity of AmB [29]. The latest reports indicated that high sodium intake (≈4 mEq/kg) per day might be associated with lower nephrotoxicity in extremely premature infants treated with AmB [30].

In this work we present results regarding the significant interaction in mixed monolayers between AmB and DPPC formed at the air–water solution interface in the presence of the K⁺ and Na⁺ ions.

2. Experimental section

2.1. Materials and methods

Amphotericin B in crystalline form and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma-Aldrich (Poland). The AmB was dissolved in deionized (mQ) water, alkalized to pH 12 with KOH or NaOH and then centrifuged for 15 min at 15,000 × g in order to remove micro-crystals of the drug still remaining in the sample. The phospholipid has been dissolved in chloroform/methanol 9:1 v/v mixture. AmB was further purified by means of HPLC on YMC C-30 (Europe GmbH, Germany) coated phase reversed column (length 250 mm, internal diameter 4.6 mm) with 40% 2-propanol in H₂O as a mobile phase. The final concentration of AmB was calculated from the absorption spectra on the basis of the molar extinction coefficient (0–0 absorption maximum at 408 nm) ε₄₀₈ = 1.08 × 10²³ M⁻¹ cm⁻¹ and ε₄₀₈ = 1.6 × 10²³ M⁻¹ cm⁻¹ in the cases of the sample dissolved in alkalized water with KOH and NaOH, respectively. Hepes sodium and potassium salt were purchased from Sigma Chem. Co. (analytical grade). A buffer solution (subphase) of potassium and sodium Hepes (10 mM) was adjusted to pH=7.4 with 1 M HCl. The subphase temperature (24±1 °C) was controlled by a Poly Science thermociculator through.

2.2. Data analysis

To better characterize the influence of lipid on the physical state of antibiotic monolayer the compression modulus Cs⁻¹ for the mixed films has been investigated, defined according to (Eq. (1)) [31,32]

\[
Cs^{-1} = -\frac{d\pi}{d\Gamma}
\]  

(1)

where A is the area per molecule at the indicated surface pressure \( \pi \). The compression modulus is obtained by numerical calculation of the first derivative from the π–A isotherm datapoints and plotted as a function of surface pressure [33]. A characteristic minimum on the \( \pi^{-1} = f(\pi) \) graph is used to identify the phase transition. The value of surface pressure at \( \pi^{-1} = 0 \) indicates the occurrence of collapse π_{coll}. The interactions between compounds in the mixed monolayers and their miscibility have been also analyzed for their mutual miscibility in accordance with the additivity rule [34].

Based on the mean molecular surface \( A_{12} \) as a function of \( (X_{12}) \) it was possible to determine whether two components are immiscible or ideally miscible, because deviations from the ideal mixture show the nonlinearity dependence of \( A_{12} = f(X_{12}) \) [22,34–36]. For the ideal mixing, the mean area per molecule, \( A_{12} \) is defined according to (Eq. (2)) [35]:

\[
A_{12} = A_1X_1 + A_2X_2
\]  

(2)

where \( X_1, X_2 \) are the molar fractions of components 1 and 2 in the mixed monolayer, and \( A_1, A_2 \) are the molecular areas of single components at the same surface pressure.

The excess of free energy of mixing \( \Delta G_{EXC} \) is used as an indication of intermolecular interactions and the stability of the mixed films. The value of \( \Delta G_{EXC} \) has been calculated as the compression work difference between ideal and real monolayer mixtures directly from the π–A isotherms using Eq. (3) as follows:

\[
\Delta G_{EXC} = N \int_0^\infty \left( A_{12} - X_1A_1 - X_2A_2 \right) d\pi
\]  

(3)

where: \( N \) is Avogadro’s number [34,35,37]. The negative sign of \( \Delta G_{EXC} \) is considered as a criterion of monolayer’s stability while a positive value may suggest the phase separation in the monolayer [35].

3. Results and discussion

Due to amphiphilic properties of AmB, it creates monomolecular layers at the air–water interface. Using the Langmuir monomolecular technique makes it possible to carry out detailed analysis of molecular organizations of AmB both in a monolayer model system and aqueous solution. Fig. 1 presents the surface pressure–area (π–A) isotherms for pure AmB monolayer spread on the 10 mM buffer subphase (pH 7.4, potassium and sodium Hepes according to the type of experiments, respectively). The shape of the AmB compression isotherm is very close to the isotherms previously reported [17,38–43]. It exhibits a typical plateau region in the range of molecular areas from 100 Å² to 40 Å², which is attributed to the reorientation of the AmB molecules from a horizontal to a vertical position [38,39,43,44]. The extrapolation of the linear parts of the isotherms in the regions corresponding to the expanded and condensed states of AmB monolayers allows to know the values of the limiting area occupied by the molecules in its horizontal (A_h) and vertical (A_v) orientation with relation to the surface of the water. All these parameters together with the surface pressures both at the beginning and at the end of the transition region are shown in Table 1. The surface pressures of the plateaus in Fig. 1 are
in the range of 10–12 mN/m. The molecular reorientation from the horizontal to vertical position in case of AmB is endothermic process which requires a high energy [39]. In the horizontal orientation AmB molecules are anchored to the interface by the hydroxyl groups. During compression the hydrogen bonds are significantly reduced and the molecules are oriented vertical (anchored by the polar heads, the inset of Fig. 1B). The results obtained by Brewster angle microscopy show that during monolayer compression their domain-like structure is transformed into a homogeneous image as a result of the reorientation of the molecules [24]. Minones et al. [42] have observed a 3-fold increase in the relative film thickness during compression the monolayer from expanded to a condensed state and the ratio of the cross-sectional area of AmB in this two orientations might be also the aggregation process. When the monolayer is compressed the molecules of AmB are anchoring to the interface by the polar head and such setting gives the possibility of chromophores interaction and creating the H-aggregated forms (card pack) [45].

limiting molecular areas obtained from fitting the isotherms in extremely packed monolayers are as follows: $27.0 \pm 1.2 \text{Å}^2/$molecule and $31.2 \pm 0.6 \text{Å}^2/$molecule for AmB in the presence of Na+ and K+ ions, respectively (see Table 1). The values are comparable to previously published data although the slight differences are the result of using different solvents as well as the rate of compression [39,40]. The most remarkable differences were observed in the horizontal position. In the presence of K+ ions the limiting molecular area ($157 \pm 4 \text{Å}^2/$molecule) is bigger than in the Na+ ions’ environment ($148 \pm 2 \text{Å}^2/$molecule). Taking into account these values it can be concluded that the AmB molecules create the aggregated forms on the surface whose size is bigger in the presence of K+ ions. The orientation of AmB in the monolayer is connected with the interaction of the –COO− and –NH3+ groups as well as –OH groups in the polyhydroxyl part of molecule [43]. The ionized –COO− group is subjected to greater exposure to the aqueous phase than –NH3+ due to stronger its hydration, which orients the macrolide ring closer to the polar environment. With the increase in surface pressure it is also possible to change the distance between these groups both in the horizontal orientation and the vertical one [43]. Due to the changes in the interactions between the K+ and Na+ ions and the –COO− group, the molecules of AmB might interact differently with the surface. This can result in different areas occupied by single molecules of AmB in the presence of these ions. In the presence of K+ or Na+ ions, the –COO− group may generate specific ionic interactions as a result of weak ion pairs that was presented in our previous works [27,46]. The results of the experimental research relative to the molecular dynamics indicated that the strength of ion pairing with the –COO− group decreases in the sequence Na+ > K+ [47,48]. On the other hand, we suggested a higher preference of K+ over Na+ ions to an anionic site the –COO− group on the assumption that the binding force comes mainly from electrostatic interaction and it is related to the difference in the size of these two cations. In such a case, the ion of smallest hydration radius (which corresponds to the greatest radius of the non-hydrated ion) is able to attract the negative attachment site so that the binding of K+ will be stronger [46].

![Fig. 1. The surface pressure–area isotherms of AmB spread on sodium buffer subphase (solid line) and potassium buffer subphase (dashed line). The linear fits to the linear portions of the isotherms of compression extrapolated to zero surface pressure point to the specific molecular areas in a horizontal position ($A_h$) and in a vertical position ($A_v$). The inset presents a model of reorientation of AmB molecules on the subphase. Temperature 24 °C.](image)

<table>
<thead>
<tr>
<th>AmB + K+</th>
<th>$A_h$ [Å²/molecule]</th>
<th>$\pi_c$ [mN/m]</th>
<th>$A_v$ [Å²/molecule]</th>
<th>$\pi_c$ [mN/m]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmB + Na+</td>
<td>148.0 ± 2</td>
<td>10.3 ± 0.2</td>
<td>27.0 ± 1.2</td>
<td>11.2 ± 0.1</td>
</tr>
<tr>
<td>AmB</td>
<td>148.0</td>
<td>10</td>
<td>35.0</td>
<td>12</td>
</tr>
</tbody>
</table>

- Amphotericin B before deposition onto buffer subphase (10 mM potassium Hepes, pH 7.4) was dissolved at pH 12.6 with 1 M KOH.
- Amphotericin B before deposition onto buffer subphase (10 mM sodium Hepes, pH 7.4) was dissolved at pH 12.6 with 1 M NaOH.
- Reported date, AmB before deposition onto water subphase was dissolved in 2-propanol/water (v:v, 4:6) [17,46].

The average SD ± of six experiments.
**Fig. 2** shows the π–A isotherms for the pure phospholipid, pure AmB and the mixtures of AmB–DPPC which contain AmB in the molar concentration from 10% to 90% (these values are equal with molar fraction X$_{AmB}$ = 0.1–0.9). These monolayers were spread on potassium subphase (10 mM Hepes buffer solution with pH 7.4, Fig. 2A and B) and sodium subphase (10 mM Hepes buffer solution with pH 7.4, Fig. 2C and D). The formed monolayer of pure DPPC exhibits at the surface pressure of 6 mN/m, a characteristic LE–LC phase transition evidenced as a plateau [38,39,44]. When the AmB is incorporated into the lipid monolayer, the phase transition is gradually shifted in the direction of the higher surface pressure (ca. 11 mN/m). When the amount of AmB is higher (X$_{AmB}$ > 0.5), the biggest changes in the plateau shape concern the monolayer compressed in the presence of Na$^+$ ions. In the K$^+$ ions’ environment they occur in similar range to pure compound.

For a more detailed analysis of the physical state of the monolayer and to know the influence of AmB on the condensation of model lipid membranes, the values of compression modulus C$_{3r}^{-1}$ as a function of surface pressure have been calculated (Eq. (1), see Fig. 3). According to Davis et al. [31] these values were typical of monolayers in liquid state. Two minima are observed in C$_{3r}^{-1}$ curves. The first minimum (M$_0$) shown in Fig. 3A and C for pure DPPC (curves 1) refers to the above mentioned transition from liquid expanded phase to liquid condensed phase. During the incorporation of AmB into lipid monolayer this minimum gradually disappears and a new one appears (M$_1$) at higher surface pressures close to 11 mN/m. A fairly distinguished discontinuity at 16–20 mN/m is also observed for the mixtures of X$_{AmB}$ = 0.5–0.7 both in the presence of K$^+$ and Na$^+$ ions. However, this minimum in the Na$^+$ ions’ environment is reduced to a small inflection. It might be due to the reorientation of AmB molecules at high surface pressures or its partial dissolution into the aqueous subphase [24]. For mixtures of X$_{AmB}$ = 0.9 as well as the single-component monolayers of pure AmB, three regions can be noticed (Fig. 3B, curves 2 and 3). The first region corresponds to the monolayers in the liquid expanded phase, the second, is connected with the liquid condensed phase characterized by high value of C$_{3r}^{-1}$. The third region is represented by a wide plateau and is connected with the LE–LC transition at the range of 10–11 mN/m [17,24]. For the mixed monolayers spread on buffer containing Na$^+$ ions, the regions mentioned above were not distinguished. The monolayer of pure AmB exhibits also a small inflection in the range of 44–50 mN/m. The presence of this point at such high surface pressure may be connected with the disorganization of the molecular structures in a form of pores [49]. AFM microscopy revealed the formation of cylindrical pore-like structures in the topography of the two-component monolayers composed of 90 mol% AmB and 10 mol% DPPC [17]. The structures formed during compression become disordered upon further compression of the monolayer. It was suggested by Gagoś et al. [49] that the symmetrically ordered structures of AmB are not stable at the surface pressures above 20 mN/m and other membrane cell components (e.g. sterols) could play a role in the stabilization of molecular pores. Finally, it may be stated that the incorporation of AmB into the DPPC monolayers causes film expansion and the disappearance of the phase transition for pure phospholipid [24]. It is probably
associated with an ordering effect of DPPC with respect to AmB [45,50,51].

In Table 2, the surface pressures of phase transitions and the values of the compression modules $C_{\pi}^{-1}$ obtained for the $\pi$–$A$ isotherms, shown in Fig. 2, were compiled. The increasing amounts of AmB in the mixed monolayers cause the decrease in the value of $C_{\pi}^{-1}$ compared to pure DPPC that means a lower rigidity of obtained monolayers. This decrease is especially noticed for the mixed films at the range of $X_{\text{AmB}} = 0.1$–$0.7$. Interestingly, that high molar fractions of AmB (90 mol%) decrease the value of $C_{\pi}^{-1}$ compared to pure antibiotic. The mentioned effect was distinctly noticed in the presence of the K$^+$ ions.

However, the mixed monolayers show the phase transition at higher surface pressure than the phase transition of pure DPPC (~6 mN/m) and they have got similar values like pure AmB (~11 mN/m). This behavior indicates the existence of molecular interactions between the components and their miscibility at the air–water interface. It is also ascribed to the molecular reorientation of antibiotic in the mixed films but this process may be limited by the presence of phospholipid. The molecules of AmB interact with the polar headgroups of DPPC and they may be organized in this region [16,43]. In this case, applying the Crisp phase rule [37] $F = C - P + 1$ (where $F$ is the number of degree of freedom of the system, $C$ is the number of components and $P$ describes the number of phases involved) to the transition region is helpful to estimate the number of surface phases in equilibrium and the miscibility or immiscibility of mixture components. According to this, there are two different behaviors depending on the composition and surface pressure of the mixed monolayers which are independent on the presence of K$^+$ and Na$^+$ ions, see Fig. 4. In the range of composition $X_{\text{AmB}} = 0$–$0.5$ and at surface pressures $<10$ mN/m the components are miscible. Since the mixed monolayers consisting of two film-forming component, $C = 2$, and the fact that the surface pressure corresponding to the LE-LC phase transition varies with the composition of the mixed system, $F = 1$ (see Fig. 4 and Table 2). Therefore, there are two surface phases in equilibrium. The phase $P_1$ (below the surface pressure corresponding the transition region) composed of AmB–DPPC complexes with 1:1 stoichiometry, where both components are horizontally oriented on the subphase, and by molecules of DPPC in excess, with their hydrocarbon chains tilted towards the water surface. The phase $P_2$

---

Table 2

<table>
<thead>
<tr>
<th>AmB (X_{AmB})</th>
<th>M_0</th>
<th>M_1</th>
<th>$C_{\pi}^{-1}_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ K$^+$</td>
<td>+ Na$^+$</td>
<td>+ K$^+$</td>
</tr>
<tr>
<td>0 (DPPC)</td>
<td>6.4</td>
<td>6.4</td>
<td>145.0</td>
</tr>
<tr>
<td>0.1</td>
<td>9.3</td>
<td>9.5</td>
<td>11.5</td>
</tr>
<tr>
<td>0.3</td>
<td>7.3</td>
<td>8.3</td>
<td>11.3</td>
</tr>
<tr>
<td>0.5</td>
<td>10.4</td>
<td>10.8</td>
<td>76.0</td>
</tr>
<tr>
<td>0.7</td>
<td>10.5</td>
<td>11.0</td>
<td>59.5</td>
</tr>
<tr>
<td>0.9</td>
<td>10.4</td>
<td>10.6</td>
<td>36.6</td>
</tr>
<tr>
<td>1 (AmB)</td>
<td>10.2</td>
<td>10.2</td>
<td>44.4</td>
</tr>
</tbody>
</table>
observed only the minimum $M_1$, where $C_s$ transition pressures as a function of the composition of AmB (A), 23 mN/m (B), 30 mN/m (C), and 45 mN/m (D). The mixed monolayers of AmB and DPPC at different surface pressures: 12 mN/m (E). Plot of phase transition pressures as a function of the composition of AmB–DPPC mixed monolayers. $M_0$ corresponds to the LE–LC phase transition for pure DPPC and $M_1$ denotes the phase transition of mixed monolayers.

The results of the calculations based on Eq. (3), in the form of $\Delta G^{\text{exc}} = f(X_{\text{AmB}})$ dependencies for two-component monolayers were presented in Fig. 6. As can be seen, at lower surface pressures (5 and 10 mN/m), $\Delta G^{\text{exc}}$ is positive in the whole range of mixture compositions in the presence of K$^+$ ions in subphase (Fig. 5A). A different tendency can be observed at higher surface pressures (20 mN/m).

(above the surface pressure corresponding to that transition), formed by the 1:1 “complex” and by DPPC molecules with their hydrocarbon chains more or less vertically oriented on the water as a result of the horizontal–vertical orientation change of molecules along the LE–LC phase transition. In the range of composition $X_{\text{AmB}} = 0.5$–1 was observed only the minimum $M_1$, where $C_s^{-1}$ and the surface pressure values remain constant with the composition of the mixtures (see line $M_1$, Fig. 4), which is characteristic of mixed systems with immiscible components. The application of the Crisp phase rule to the phase transition of the AmB ($M_1$) proves the existence of three phases in equilibrium in this situation. Indeed, $C = 2$ and $F = 0$ because the surface pressure corresponding to this minimum does not vary with the film-forming composition. So, in this case $P = 3$. The first one, $P_1$, was described above (at the surface pressure corresponding to the AmB phase transition). There are also two other separate phases $P_2$ and $P_3$ which are formed by the segregated components of the complex, that is, by phospholipid molecules, vertically oriented, and by AmB, also vertically oriented.

The plots of the mean molecular area ($A_{12}$) as a function of the molar fractions $X_{\text{AmB}}$ of AmB (Eq. (2)) are presented in Fig. 5. At surface pressures 5 and 10 mN/m, the deviations from the ideality are positive for mixtures of $X_{\text{AmB}} = 0.1$ and 0.7 in the presence of K$^+$ ions (Fig. 5A). However, in the presence of Na$^+$ (Fig. 5B) the positive deviations from the ideal behavior were observed when $X_{\text{AmB}} = 0.3$. In contrast, at high surface pressures (20 mN/m), the obtained results of mean molecular area almost coincide with the theoretical value calculated on the basis of the additivity rule (shown as dashed lines). This fact shows that the components of the mixed films are only partially miscible or immiscible. However, these values are uncertain due to the increased likelihood of instability of the AmB monolayers [24,44,52]. On the other hand, as can be seen from Fig. 5A, in the presence of K$^+$ ions slight negative deviations from the additivity rule were observed in the range of $X_{\text{AmB}} = 0.1$–0.7 at higher surface pressures (20 mN/m).

The values of $\Delta G^{\text{exc}}$ were negative in the whole range of $X_{\text{AmB}}$, showing a minimum value when $X_{\text{AmB}} = 0.5$ at ca. $-0.545$ kJ/mol (Fig. 6A). The presence of this minimum proves that the most stable mixed monolayers of AmB–DPPC were formed at 1:1 stoichiometry and suggests strong interactions between molecules. Similar results regarding the interactions between AmB and phospholipid were also found by Balakrishnan et al. [19,25]. The compound might be involved in the complex formation with DPPC via the intermolecular hydrogen bonds with head groups of lipid and it is localized mostly in this polar region [16]. The strength of the interaction between the molecules of AmB and DPPC in the presence of K$^+$ ions is further confirmed by the fact that in similar studies conducted by Hac-Wydro et al. [25] but for the mixed monolayer spread only on water subphase, the value of $\Delta G^{\text{exc}}$ was higher and reached ca. $-0.7$ kJ/mol.

Analyzing the mixed monolayers spread on buffer subphase containing Na$^+$ ions, the different tendency of $\Delta G^{\text{exc}}$ vs. $X_{\text{AmB}}$ dependencies was found (see Fig. 6B). In the monolayers of medium AmB content ($X_{\text{AmB}} = 0.3$–0.5), the excess free energy of mixing was positive in the whole range of surface pressures. Stronger interactions associated with the appearance of minima in the analyzed dependencies related to low content of AmB ($X_{\text{AmB}} = 0.1$) and at above 50 mol.% of antibiotic in the mixture. The most negative value of $\Delta G^{\text{exc}}$ for a wide minimum is achieved at $X_{\text{AmB}} = 0.7$ ($\Delta G^{\text{exc}}$ ca. $-0.309$ kJ/mol), this suggests that stronger attractions and the stability of AmB–DPPC complex can be approximated to a 2:1

![Fig. 4](image-url)  The values of compression modulus $C_s^{-1}$ vs. the molar fraction of AmB ($X_{\text{AmB}}$) for mixed monolayers of AmB and DPPC at different surface pressures: 12 mN/m (A), 23 mN/m (B), 30 mN/m (C), and 45 mN/m (D).

![Fig. 5](image-url)  Mean molecular area ($A_{12}$) as a function of the molar fraction of AmB ($X_{\text{AmB}}$) for the mixed monolayers of AmB and DPPC. The mixed films spread on potassium buffer subphase (A) and sodium buffer subphase (B).
and Na\(^+\) (Fig. 6B) reveals higher stability for the monolayers spread on K\(^+\)-containing subphase. The differences in the interactions of AmB with phospholipid in the mixed monolayers spread on different kinds of subphase under the same experimental conditions could be also connected with the influence of aforementioned ions on the formation of aggregated structures. Our previous findings which carried out the application of spectroscopic methods have indicated that monovalent ions can affect the molecular organization of AmB in substantially different ways, and that the K\(^+\) ions exhibited stronger ionic binding affinity to the –COO\(^–\) group of AmB relative to Na\(^+\) [26,27]. A considerably stronger effect of K\(^+\) ions on the aggregation level of AmB is mainly related to the difference in the size of these two cations [26]. Although the monolayer experiments for two-component system composed of AmB and DPPC have been already reported [24,25], in none of these studies AmB was dissolved at pH 12 with KOH or NaOH. In the context of this, it may be proposed that the spreading solvent as well as different subphase affecting the limiting molecular areas determines the surface occupied by the single molecule of AmB and the stoichiometry of the antibiotic–phospholipid complexes. Differences in the strength of interactions with DPPC exist in the whole range of AmB concentrations and they are much stronger in the presence of K\(^+\) ions.

4. Conclusions

The differences in the interactions between AmB and DPPC in the presence of K\(^+\) and Na\(^+\) ions may result from the existence of different influences of these ions on the molecular organization of drug. These effects can be associated with differences in the sizes of these ions. The strength of these interactions is significantly weaker for the mixed monolayers spread on Na\(^+\)-containing subphase. The presence of K\(^+\) ions can be an important element in facilitating the interaction of molecules with the model lipid membranes and enhance the efficiency of transmembrane transport of these ions without interaction with sterols. In light of this discussion, it seems likely that K\(^+\) ions bind more efficiently with AmB than Na\(^+\) ions.

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