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Studies of the interactions of ursane-type bioactive terpenes with the model of *Escherichia coli* inner membrane—Langmuir monolayer approach

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ABSTRACT

Pentacyclic triterpenes (PT), ursolic acid (Urs), and α-amyrin (AMalf) are natural products exhibiting broad spectrum of antibacterial activity. These compounds are membrane-active and can disorder bacterial membranes when incorporated; however, the exact mechanism of their membrane activity is unknown. In our studies, we applied Langmuir monolayer technique supported by Brewster angle microscopy to model the interactions of the selected PT with the lipid matrix of *E. coli* inner membrane. As the model membrane, we applied mixtures (75/25 mole/.mole %) of the representative *Escherichia coli* phosphatidylethanolamine (POPE), with the cardiolipin (ECCL) or phosphatidylglycerol (ECPG) extracted from the *E. coli* inner membrane. On the basis of the recorded isotherms, we performed thermodynamic analysis and calculated free energy of mixing ΔG^{exc}. It turned out that the phospholipids forming the inner membrane of *E. coli* are ideally miscible, whereas in binary systems composed of PT and POPE, negative deviations from ideality indicating attractive interactions between the investigated PT and POPE molecules were observed. On the other hand, in ternary systems composed of PT, POPE and one of the *E. coli* anionic phospholipids large positive changes in ΔG^{exc} were observed. Thus, both PT exhibit disorganizing effect on the model *E. coli* membrane. It was also proved that at low terpene proportion, AMalf can be more active than Urs. However, at higher proportion Urs incorporation can lead to the disintegration of cardiolipin-rich domains present in bacterial membrane.

1. Introduction

Multi-drug resistance (MDR) of pathogenic bacteria is considered an increasing hazard for public health [1]. MDR bacteria are the main cause of nosocomial infections and severe complications in the therapies of contagious diseases [2]. One of the reasons of MDR development is the overuse of antibiotics in different fields of medicine and industry [3]. Thus, the application of non-antibiotic drugs exhibiting antibacterial activity can effectively combat the MDR phenomenon [4,5]. Plant secondary metabolites such as alkaloids, saponins, glycosides, and terpenes exhibit such activity and are investigated as potential drugs [6]. Among these compounds, pentacyclic triterpenes (PT) turned out to be the active components of different medicinal plant extracts applied in traditional medicine in the therapy of bacterial infections [7]. The natural PT can be divided on three main groups: lupanes, ursanes, and oleananes [8] (structures in Supplementary Materials). The derivatives of the ground pentacyclic triterpene alkanes differ in their antibacterial potential. Oleananes and ursanes were proved to exhibit moderate or high antibacterial activity in numerous studies performed on different bacterial strains *in vitro* [9–13]. In contrast, it was proved that antimicrobial activity of lupanes is limited [2]. The exact mechanism of PT bioactivity has not been entirely elucidated and different hypotheses function in scientific literature. In some studies, PT were stated to interact with the bacterial genetic material leading to serious alterations of gene expression [14,15]. Other studies indicated the effect of PT on the enzymes active in the synthesis of peptydoglicam [16] or proved the PT-inhibition of other enzymes important in the metabolism of the bacterial cell [17]. However, it is also highly probable that PT interacts directly with some target molecules in the membrane and disturb its structure and function.

Among Gram-negative bacteria *Escherichia coli* is treated as the model organism, and the structure of its membranes is in detail described in scientific literature. The lipid matrix of *E. coli* inner membrane is composed in 75% of phosphatidylethanolamines (PE), 20% of phosphatidylglycerols (PG), and 5% of cardiolipins [18]. The anionic phospholipids PG and CL are not distributed uniformly in the membrane, and CL-rich domains were observed in different regions of the cell [19,20]. For the purpose of *E. coli* membrane modeling, the Langmuir monolayer method was often applied as multiple experimental factors can be strictly controlled during the studies [21–23]. The

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Langmuir technique was successfully applied in studies assessing the influence of different drugs on *E. coli* model membranes [22–26]. Different lipids were considered as *E. coli* model membrane: some authors applied the total extract of *E. coli* polar lipids [21,22,26], others investigated one-component phospholipid monolayers as an extreme simplification of the lipid matrix [23]. There were also attempts to re-create the composition of the membrane applying synthetic phospholipids [22,27].

In our studies, we applied the Langmuir monolayer technique for the studies of the interactions of two representatives of ursane PT: α-amyrin (AMalf) and ursolic acid (Urs) with the model inner membrane of *E. coli*. We proposed two different binary systems as the model membrane: the first containing 75% of PE and 25% of PG (EcoliPG) and a second mimicking the CL-rich domains containing 75% of PE and 25% of CL (EcoliCL). Multiple studies proved that the fatty acids containing cyclopropane ring play an important role in the structure of bacterial lipids [28–30]. Therefore, we decided to apply CL and PG extracted from *E. coli* membrane, as both of them contain such a fatty acid rest in their hydrophobic moieties. As PE we applied 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), as it was proved that the ratio of saturated to unsaturated phospholipids in *E. coli* PE is approximately 1:1 and that palmitic and oleoic acid dominate over other fatty acid rests [31]. We decided to investigate ternary Langmuir monolayers composed of two *E. coli* phospholipids and one of the terpenes. In the studies, we changed the terpene/phospholipids proportion in the whole range of mole ratios, but in all experiment, the mutual POPE/anionic phospholipids ratio of 75%/25% was preserved. For all the monolayers, surface pressure (π)–mean molecular area (A) isotherms were recorded, and the monolayers were visualized upon their compression by Brewster angle microscopy (BAM). On the basis of the π-A isotherms recorded for the ternary systems, the thermodynamic analysis of the excess free energy of mixing was performed. In our studies, we tried to elucidate the following problems: What is the influence of the incorporation of PT on the structure of a model bacterial membrane? Do PT mix with the phospholipids or separate forming a different phase? Do PT discriminate over PG and CL? Are there important differences in the interactions of AMalf and Urs with the artificial membranes? Which of the terpenes is more active as antibacterial membrane-specific drug?

2. Experimental

2.1. Materials

Phospholipids: POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) (CAS 26662-94-2) synthetic sample, ECCL (CAS 796964-05-01), and ECPG (CAS 796963-92-3). Cardiolipin and phosphatidylglycerol isolated from *E. coli* membrane were purchased from Avanti Polar Lipids. α-Amyrin (99%) (CAS 638-95-9) and ursolic acid (99%) (77-52-1) were supplied by Sigma Aldrich. The structures of the investigated compounds are depicted in Scheme 1.

Solvents: Chloroform (HPLC grade 99%, stabilized by ethanol 1%) and methanol (HPLC grade 99%) were purchased from Sigma-Aldrich.

Water: Ultrapure water of the resistivity ≥18.2 MΩ · cm was produced in our laboratory with the application of Millipore Synergy system. Water pH was controlled before every experiment, and it ranged from 6.5 to 7.

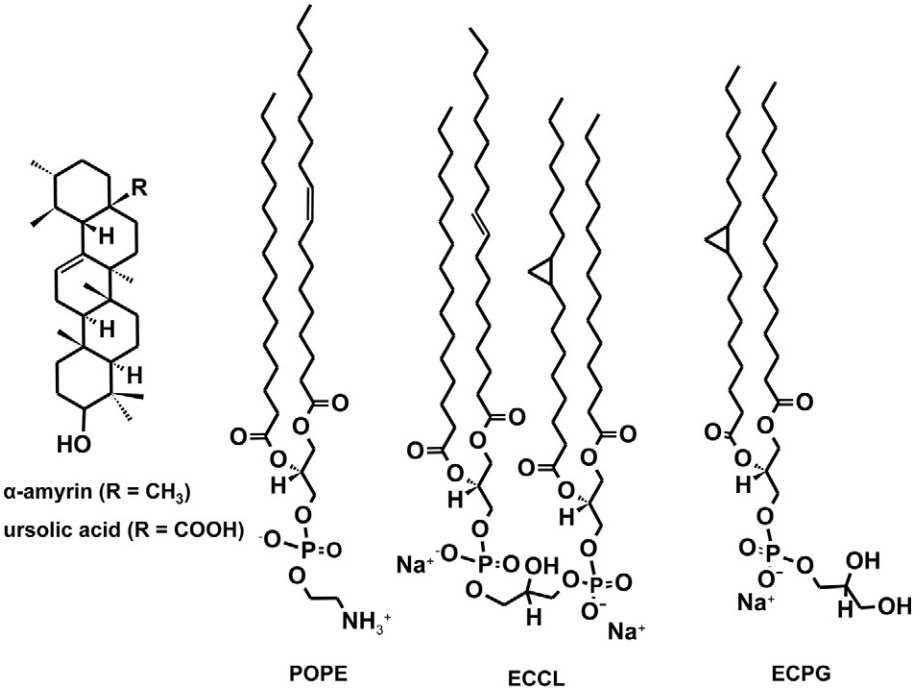
2.2. Solutions

All the solutions of the applied investigated phospholipids and terpenes were prepared in chloroform/methanol 9/1 v/v mixture. The one-component stock solutions were kept refrigerated and were mixed in required proportions to obtain the binary and ternary solutions just before the experiments. The concentration of the solutions was ca. 0.2–0.25 mg/ml, which recalculated to mol/dm^3 gives ca. $4.4 \cdot 10^{-4}$ M for the terpenes, ca. $2.7 \cdot 10^{-4}$ M for the double-chained phospholipids, and ca. $1.4 \cdot 10^{-4}$ M for cardiolipin. The more in-detail description of the solution preparation and of the applied surface proportions (mole ratios) of the monolayer components are included in the Supplementary Materials.

2.3. Methods

2.3.1. Langmuir technique

In our experiments, we applied KSV-NIMA (Helsinki, Finland) double-barrier Langmuir trough of the nominal area of 273 cm^2. BAM experiments were performed on a larger instrument of the area 840 cm^2



Scheme 1. Structural formulas of the investigated compounds.

designated by KSV for microscopic experiments. The troughs are prepared from one block of Teflon and equipped with two Delrin barriers enabling symmetrical compression of the monolayer. The troughs were carefully cleaned and rinsed before each experiment, which guaranteed high reproducibility of the π-A isotherms (±1 Å^2). The required volumes of the chloroform/methanol solutions of the investigated compounds were deposited at the air/water interface by the application of Hamilton microsyringe. Ten minutes were left for the solvent evaporation after which the monolayers were compressed with the constant compression speed of 1 cm·min^{-1} per barrier. This value of compression speed was acquired after a series of experiments in which the influence of compression speed on the investigated monolayers was checked. The measurement of the π-A isotherm was repeated for a monolayer of a given surfactant proportions at least three times and the isotherms presented in Supplementary Materials are averages of at least 3 separate curves. Surface pressure was monitored with 1 s log with the application of the Wilhelmy plate method. The plate was made of ashless Whatman filtration paper. The accuracy of the measurements was ±0.1 mN/m. All the experiments were performed at constant temperature of 20 °C, which was controlled to ±0.1 °C by the Julabo circulating bath. For each binary or ternary system, 5 mole proportions of the components were investigated. Cardiolipin has the molecular cross-section approximately two times greater than the double-chained phospholipids; thus, it was reasonable to have fixed surface proportions of the components and not the mole ratios. The investigated surface proportions of terpenes to phospholipids molecules were 1/4, 1/2, 1/1, 2/1, and 4/1. The recalculation of the surface proportions to mole ratios of the components is explained in detail in the Supplementary materials.

2.3.2. Brewster angle microscopy

Brewster angle microscopy experiments were performed with ultraBAM instrument (Accurion GmbH, Goettingen, Germany) equipped with a 50 mW laser emitting p-polarized light at a wavelength of 658 nm, a 10× magnification objective, polarizer, analyzer, and a CCD camera. The spatial resolution of the BAM was 2 μm. The instrument was coupled with the KSV 2000 Langmuir trough and installed on an antivibration table.

3. Results and discussion

3.1. One-component monolayers and the binary POPE/ECCL and POPE/ECPG systems

The paper concerns ternary model systems; however, their discussion would be difficult without the presentation of the isotherms registered for one-component monolayers and for the binary lipid–lipid monolayers mimicking the *E. coli* membrane. The isotherms together with corresponding C_S^{-1}–π curves are presented in Fig. 1.

POPE, the main phospholipid of *E. coli* inner membrane [18], is an example of mixed acyl (saturated-unsaturated) lipid, for which a phase transition from the liquid expanded to liquid condensed phase is observed. The phase transition at 20 °C is located at a quite high surface pressure of ca. 35 mN/m. The monolayers of *E. coli* anionic phospholipids ECCL and ECPG have an expanded character which is reflected in the course of the π-A isotherms and C_S^{-1} values, which hardly achieve 100 mN/m. ECCL can be approximately treated as a dimer of ECPG, so it is obvious that the lift-off area (A_0) observed for ECCL (190 Å^2/molecule) is approximately two times greater than for ECPG (103 Å^2/molecule). The isotherms of the monolayers mimicking *E. coli* inner membrane EcoliCL and EcoliPG are similar with the isotherm of POPE, which constitute 75% of the mixtures. There is also a phase transition visible in the course of these isotherms. The isotherm of EcoliPG virtually overlap with POPE; the courses of the curves are different only at the highest surface pressures. The monolayer of AMalf achieves a solid state just at the beginning of surface pressure rise, which finds its manifestation in very high values of C_S^{-1} exceeding 400 mN/m. The course of the isotherm recorded for ursolic acid together with rather low values of compression modulus indicates the expanded state of the monolayer. However, such a conclusion is wrong as it is visible in BAM photos. The monolayer is composed of large condensed domains surrounded by more expanded regions. As it was previously postulated by us and other authors, the presence of two different phases is strictly connected with the bolaamphiphilic character of the pentacyclic triterpene acids [32,33]. All the monolayers were visualized upon their compression and the representative BAM images are gathered in Fig. 2.

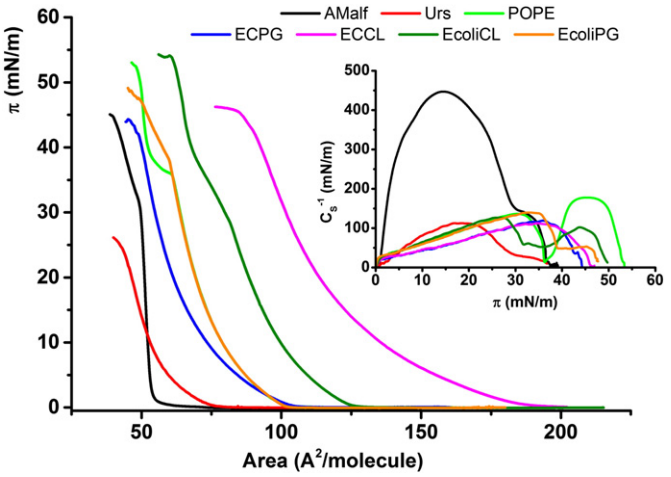


Fig. 1. π-A isotherms and C_S^{-1}-π (inset) curves for the investigated compounds.

As it was mentioned above, the monolayers of ECCL and ECPG had expanded character. In BAM images, they were homogenous from the beginning of their compression till collapse. Similarly, but of different reasons, the monolayers of AMalf were homogeneous. The films were solid within the whole range of surface pressures; no cracks were observed upon the compression in BAM photos. In contrast, in the films of Urs large solid domains were visible just at the beginning of compression (Fig. 2a). The number of the domains grew upon the compression, but till the collapse the monolayer was not homogeneous. The monolayers of POPE were homogeneous until the transition surface pressure of 34 mN/m at which condensed domains appeared. With further surface pressure rise the domains grew up and at 40 mN/m they merged forming uniform film. Regarding the systems imitating *E. coli* inner membrane, they were homogenous till the LE-LC transition visible in the course of the isotherms. Small condensed domains appeared at higher surface pressures (ca. 40 mN/m) than in the case of POPE.

3.2. Binary systems of POPE/ECCL and POPE/ECPG

The lipid matrix of *E. coli* inner membrane contains 75% of PE (represented statistically by POPE) and 25% of anionic lipids. At the beginning of the study, it was necessary to investigate the mutual interactions of the zwitterionic/anionic lipids in the pairs POPE/ECCL and POPE/ECPG. The isotherms and compression moduli for both systems are gathered in supporting materials (Fig. S1). Here we focus our attention on the dependences derived from the π-A isotherms: maximal C_S^{-1}–X(POPE) plots (Fig. 3) and excess free energy of mixing (ΔG^{exc})–component mole ratio plots (Fig. 4).

As it is shown in Fig. 4 for the systems containing bacterial phospholipids, in POPE/ECCL and POPE/ECPG, the values of ΔG^{exc} are close to 0. In the system, POPE/ECCL only at the highest X(POPE) slight positive deviation is observed, whereas in the system POPE/ECPG some oscillations of ΔG^{exc} can be noticed. However, the oscillations are not significant, as the values of ΔG^{exc} change between −150 and 200 J/mol. ΔG^{exc} values close to 0 can indicate either ideal miscibility or ideal immiscibility in a given system [34,35]. Here we opt for the first option due to the following reasons: In *E. coli* membranes, ECCL and ECPG are immersed in the

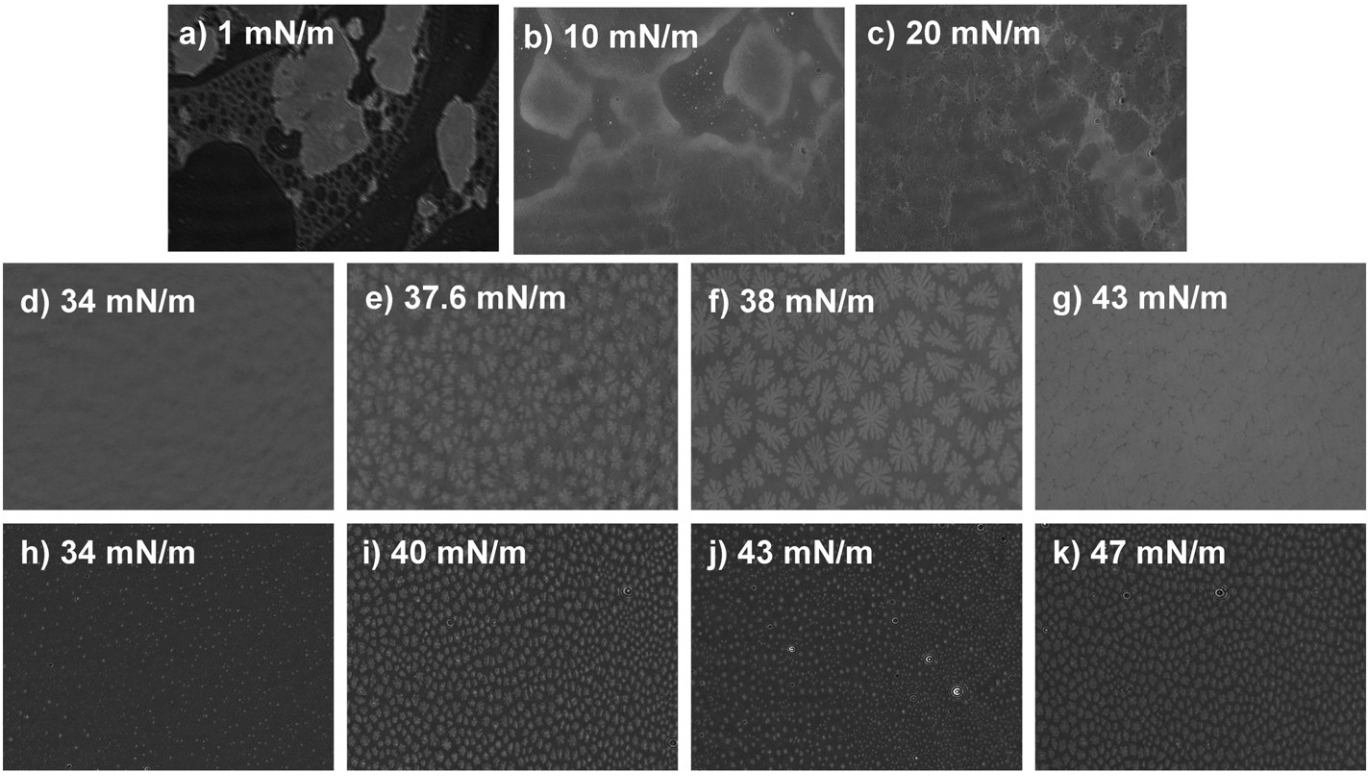


Fig. 2. Representative BAM images for the monolayers of (a–c) Urs, (d–g) POPE, (h, i) EcoliCL, (j, k) EcoliPG.

matrix of POPE, which dominates among the membrane lipids. CL can form separate domains, but it is rather caused by its interactions with specific proteins and not simple immiscibility with POPE. Moreover, immiscibility in binary Langmuir monolayers is observed mainly in the case when at least one of the components can form LC domains. For two lipids being at 20 °C in an LE state, the probability of phase separation is low. Moreover, we do not observe any symptoms of phase separation for these systems in BAM images, even at high surface pressures. Mixing of POPE with ECCL or ECPG does not affect monolayer condensation, as the maximal C_S^{-1} values observed at different component proportions were practically identical. This observation also proves the mutual miscibility of the lipids in the binary systems. All the above data can be concluded by the statement that POPE and the anionic *E. coli* phospholipids are ideally miscible regardless the film composition.

In our studies, POPE was for the first time mixed with a bacterial PG possessing the cyclopropane ring in its hydrophobic moiety. However, different PE/PG mixed films are known from the scientific literature. In particular, the dipalmitoyl-PE (DPPE) and dipalmitoyl-PG (DPPG) mixtures were applied as a simplified model of bacterial membrane [36,37].

3.3. Binary mixtures of terpenes with POPE

The second step of the studies of binary mixtures was the investigation of the interactions of AMalf and Urs with POPE. The resultant π-A

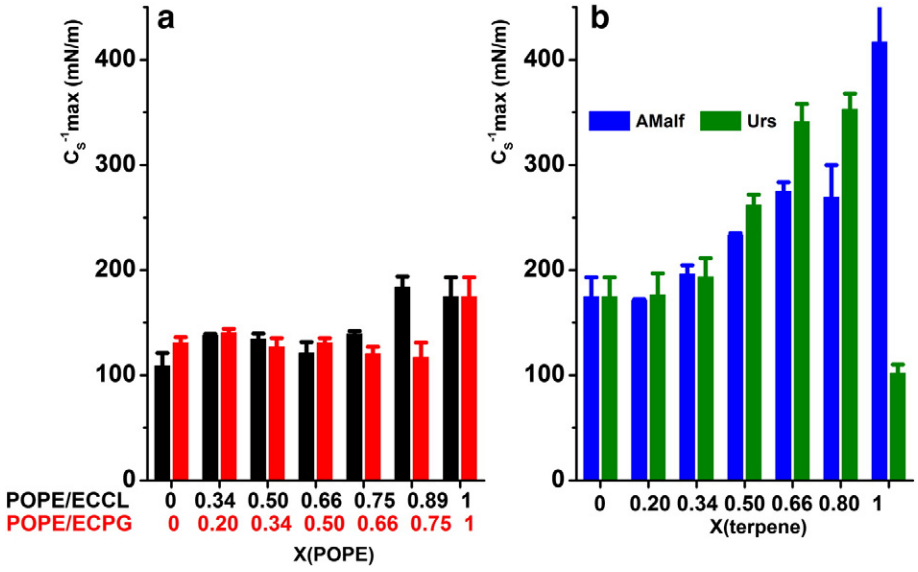


Fig. 3. C_S^{-1} max–X(POPE) (a) and C_S^{-1} max–X(terpene) (b) for the investigated binary systems.

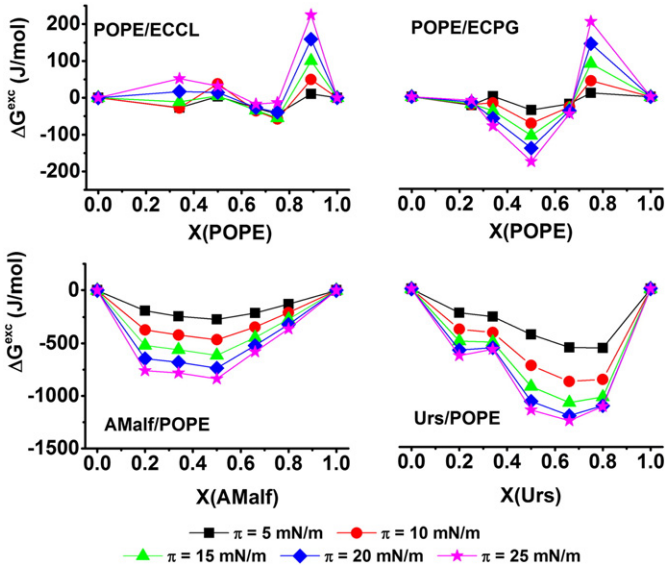


Fig. 4. ΔG^{exc}–X(POPE) (up) and ΔG^{exc}–X(terpene) (down) plots for the investigated binary systems.

isotherms and C_S^{-1}- π curves are gathered in Supplementary materials (Fig. S2). In Fig. 3b, the C_S^{-1} max–X(terpene) plots are shown, whereas the ΔG^{exc} –X(terpene) dependences are gathered in Fig. 4(c,d). In both systems with POPE, ΔG^{exc} has negative sign for all component proportions and the absolute values are high. Thus, the interactions in the pairs AMalf/POPE and Urs/POPE are more energetically favorable than between the same molecules in one-component monolayers. The condensation of the monolayers in the system AMalf/POPE grows gradually with X(AMalf), whereas in the system Urs/POPE, it is comparable for lower X(Urs). For X(Urs) = 0.66 and 0.8, a sudden jump in the condensation (Fig. 3b) is observed. The C_S^{-1} achieves values higher than 300 mN/m, which proves that the state of the monolayers can be classified as solid. All the monolayers in the binary systems containing POPE were visualized by BAM; however, regardless the composition and mutual proportion of the components, the films were homogeneous from the beginning of compression till the collapse. All the results described here corroborate the mutual miscibility of the investigated terpenes and POPE in binary monolayers within the whole range of surface proportions.

POPE had not previously been mixed with PT in Langmuir monolayers; however, the results for the binary mixtures containing animal or plant sterols can be found in scientific literature [38,39]. Sterols and PT are both products of squalene cyclization, which are structurally similar [40] membrane active compounds, so it could be expected that the results obtained in our studies and these from scientific literature are qualitatively similar. The feature which is common in these binary mixtures is the significant condensation of the monolayers observed at high sterol (terpene) proportions. However, the comparison of here investigated systems and these taken from the literature is not so straightforward. The cited authors investigated the pairs DPPE/cholesterol [39] or DPPE/sitostanol [38] in which both components form solid monolayers. In our case, POPE forms liquid expanded films, so we observe here a strong ordering effect exerted by PT on the monolayer of a mixed acyl phospholipid.

The attractive interactions find also their manifestation in the negative ΔG^{exc} values observed in the systems AMalf/POPE and Urs/POPE. It seems that effective van der Waals interactions between the hydrophobic moieties of the PT and the fatty acid chains of POPE are responsible for the observed phenomena. It should also be underlined that the ΔG^{exc} absolute values are higher in the system Urs/POPE than in the system AMalf/POPE, so it can be assumed that Urs has higher affinity to POPE than AMalf. POPE is the main phospholipid of *E. coli* membrane, so if this conclusion is true, Urs could be more effectively accumulated in the membranes than AMalf.

3.4. Ternary systems

After the thorough analysis of the binary systems, we moved to the monolayers of mixed lipids mimicking *E. coli* membrane. Similar with the above-described experiments, we prepared ternary monolayers containing the mixture of two phospholipids (POPE + ECCL or POPE + ECPG) and one of the investigated terpenes. π-A isotherms and C_S^{-1}–π curves are presented in supporting materials (Fig. S3 and Fig. S4, respectively), whereas here we present the data derived from the isotherms: C_S^{-1} max–X(terpene) curves are shown in Fig. 5 and the ΔG^{exc}–X(terpene) dependences are gathered in Fig. 6.

Regarding the C_S^{-1} max–X(terpene) plots, the condensation of the ternary monolayers remains mutually comparable for most of surface proportions and the maximal values of C_S^{-1} range from 150 to 200 mN/m. Only at the highest X(terpene) proportion of 0.8 much higher values of C_S^{-1} max can be noticed. It was interesting to compare the fluidity of the monolayers in the ternary and binary systems. Thus, we calculated the differences in the maximal C_S^{-1} at a given X(terpene) between the ternary and the binary systems (Fig. 7).

For the majority of surface proportions, the appearance of ECCL and ECPG in the monolayer leads to the lowering of monolayer condensation. It is especially noticeable in the systems containing Urs and ECCL at the terpene/phospholipids 1:1 proportion and in the systems containing Urs and ECPG at the 2/1 proportion. Lowering of film condensation is the result of the decrease of lateral order within the film. This effect can have both entropic and energetic character. Regarding the energetic effects, it was proved in our previous studies that the interactions between AMalf and Urs with *E. coli* anionic phospholipids is energetically unfavorable [41]. The terpene molecules can escape from the monolayer at some surface pressure to avoid the contact with ECCL and ECPG. This leads to the formation of small 3D terpene-rich domains. The presence of such domains within the monolayer would severely decrease its condensation. The size of the multilayer nuclei can be below the BAM microscope resolution; however in some cases we observed such nuclei (see Fig. S5 in Supplementary materials).

Regarding the ΔG^{exc}–X(terpene) dependences in three of the ternary systems positive values of ΔG^{exc} are observed, only in the system Urs/EcoliPG ΔG^{exc} acquires negative sign for all the terpene proportions. Positive values of ΔG^{exc} prove that the interactions of AMalf and Urs with the lipid mixture mimicking *E. coli* inner membrane are not energetically beneficial. Only for the system Urs/EcoliPG, it can be inferred from the ΔG^{exc}–X(Urs) curve that the intermolecular interactions are energetically favorable. Similar with the C_S^{-1} max values, it was also interesting to compare the ΔG^{exc} values in the binary and ternary systems. Fig. 8 illustrates such comparison at two selected π values of 10 and 20 mN/m:

All the above bar plots at both selected surface pressures indicate that the shift from binary to ternary systems, that is, the appearance of *E. coli* anionic phospholipids leads to profound increase of ΔG^{exc} values. For the terpene/phospholipids 1/1, 2/1 and 4/1 at π = 20 mN/m the increase of ΔG^{exc} is ca. 750 mN/m (with the exception of the systems containing Urs and ECCL where the differences were much higher). It should be underlined that at the proportions 2/1 and 4/1 the participation of *E. coli* lipids in the ternary systems is limited to 8% and 6%, respectively, so even such small content of ECCL and ECPG leads to significant destabilization of the terpene/POPE system. Interpreting the above effect, we should take under consideration the possible synergisms in the ternary systems. It can be supposed that there is a kind of competition between the PT and the *E. coli* anionic phospholipids as the close contact with POPE molecules is energetically favorable for both of them. The presence of PT in the inner *E. coli* membrane can directly and indirectly be connected with the antibacterial activity of PT. The attractive interactions between PT and POPE can lead to the disintegration

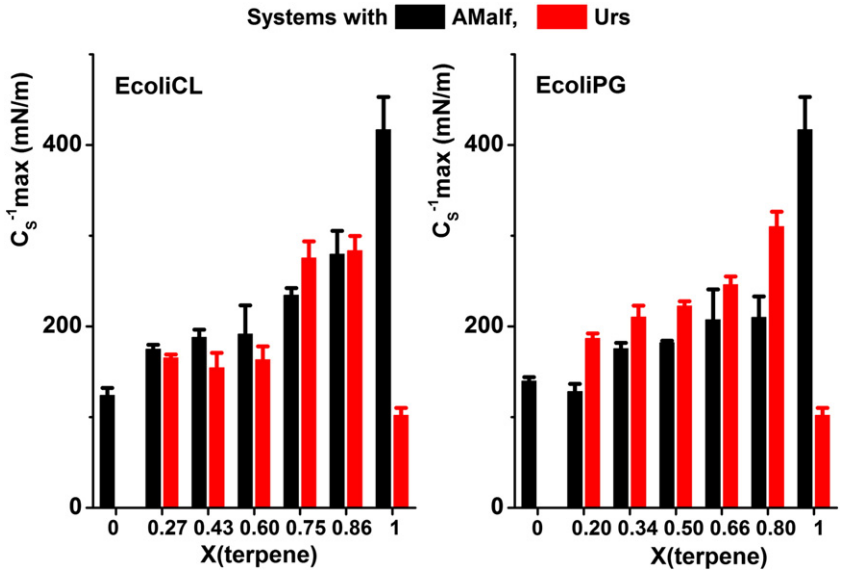


Fig. 5. C_S^{-1} max–X(terpene) plots for the investigated ternary systems.

of CL-rich domains and by this affect the energy generation in the inner membrane as it was proved that such domains are crucial for vital processes of the bacterial cell [42,43]. On the other hand, it was proved that the mutual PE/PG 4/1 proportion and the formation of PE/PG domains are crucial for the development of bacterial resistance to different drugs [37]. Thus, the interference of PT with such structures can indirectly lead to the increase of membrane permeability and facilitate the transport of different drugs into the cell. On the basis of Figs. 6 and 8, it is also possible to speculate which of the terpenes has a more destructive effect on the membrane; thus, it can be a more effective antibacterial drug. It can be seen that at the lowest terpene proportion the highest positive changes in ΔG^{exc} are observed in the system AMalf/EcoliPG. ECPG is the main anionic phospholipid of *E. coli* membrane constituting 20% of all the membrane lipids, which is rather uniformly distributed in the POPE matrix [18]; thus, it seems that the incorporation of even small amounts of AMalf into the inner membrane can severely affect its structure. The results of the experiments performed on model systems indicate that AMalf is more effective in this action than its oxidized derivative Urs. The PT adsorbed in bacterial membrane has not to be necessarily uniformly distributed, and it is possible that in some membrane regions the local concentrations of PT are higher. So if accumulation of PT in cardiolipin-rich domains is possible, Urs would be more destructive than AMalf as it can be seen in Fig. 8. At greater X(Urs), the values of ΔG^{exc} are ca. 1500 J/mol higher in the ternary system Urs/EcoliCL than in the binary system Urs/POPE.

It was also checked if the ternary monolayers have different texture than the previously observed for one and two-component films. Thus, all the monolayers within the ternary systems were visualized by BAM upon their compression. All the monolayers in the systems with Urs were homogenous till their collapse and no textures could be observed. The situation was similar regarding the systems with AMalf. However, at certain surface pressure value, usually higher than 20 mN/m, suddenly a large number of bright points appeared in the photos proving the separation of a 3D phase (Fig. S5). As the monolayers of the investigated phospholipids have expanded characters, it can be assumed that the small 3D nuclei are composed solely of AMalf or that

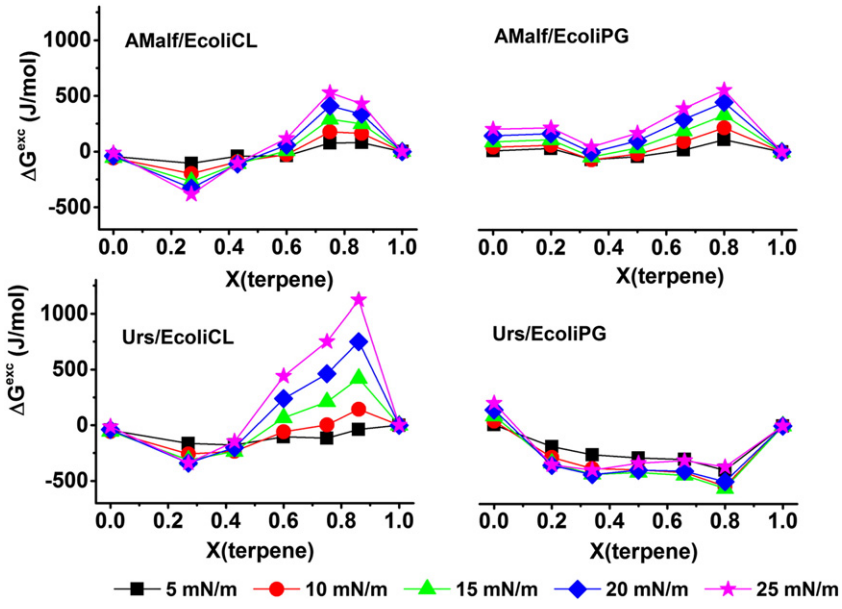


Fig. 6. ΔG^{exc}–X(terpene) dependences for the ternary systems.

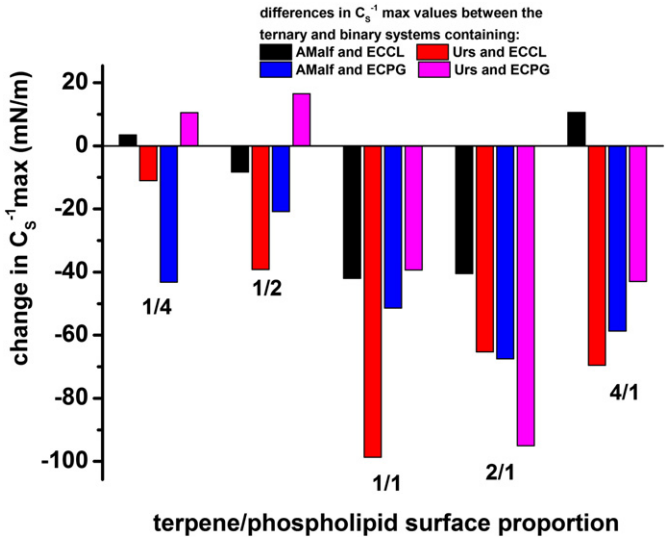


Fig. 7. Differences in the C_S^{-1} max values between the ternary systems and the binary systems containing POPE and one of the terpenes.

it is an AMalf-rich phase. There is practically no manifestation of the phase separation in the π-A isotherms and C_S^{-1}–π curves, which means that the monolayers are metastable at high surface pressures and that the growth of the 3D nuclei is slow as compared with the compression rate. The separation of small 3D nuclei was not observed for the lowest AMalf proportion (1/4 terpene/lipid). Regarding the separation of 3D phase the systems AMalf/EcoliCL and AMalf/EcoliPG behaved very similarly. Similar phenomena were also observed in model systems containing membrane phospholipids and cholesterol or plant stanol [38]. The excretion of AMalf-rich domains can also be important in the discussion of the membrane activity of PT. The POPE/ECCL and POPE/ECPG interactions are energetically favorable, so the elimination of the terpene from the monolayer at a certain surface pressure can also be beneficial. The AMalf crystallites present in the membrane environment can be also of importance as far as the antibacterial activity of PT is concerned.

4. Conclusions

Our studies focused on the investigation of the interactions of selected bioactive pentacyclic triterpenes with the phospholipids of inner *E. coli* membrane. In the first step, the binary films of the main phospholipids of *E. coli* membrane: POPE, ECCL, and ECPG were investigated. It turned out that POPE is ideally miscible both with ECCL and ECPG at any mutual proportions of the components. In contrast to previous studies performed on PE and PG of saturated acyl chains [36], our results do not indicate that in the systems POPE/ECCL and POPE/ECPG some proportions of the lipids are thermodynamically preferred or that a complex of fixed stoichiometry is formed between them. In the second step of our studies, the binary systems composed of the main *E. coli* membrane phospholipid POPE and the investigated PT: AMalf or Urs were investigated. It was proved that the applied terpenes are miscible with POPE in Langmuir monolayers for all the investigated proportions. ΔG^{exc} in both systems acquired negative sign and the absolute values were large. Both PT behaved in the monolayers of POPE similar with cholesterol, exerting condensing effect on the films of this mixed acyl PE. After the thorough characterization of the binary systems, we followed to the studies of the interactions of the selected PT with *E. coli* membrane-mimicking lipid mixtures. It turned out that both AMalf and Urs can exert destructive effect on the model membranes. In three ternary systems, ΔG^{exc} vales were positive for all compositions, only in the system Urs/EcoliPG the sign of ΔG^{exc} was negative. It was more reasonable to discuss the changes in ΔG^{exc} between the ternary and binary systems that explicitly ΔG^{exc} in ternary systems. Such approach indicated that the changes of ΔG^{exc} in all the investigated ternary systems are significant and have positive sign. This effect was explained taking into consideration the beneficial interactions of bacterial PE and PG in the inner membrane. PT when incorporated into the membrane can interfere with the hydrogen bond formation between the PE and PG headgroups as well as disorganize the packing of the phospholipids hydrophobic chains. It is also possible that PT not only disturb the PE-PG

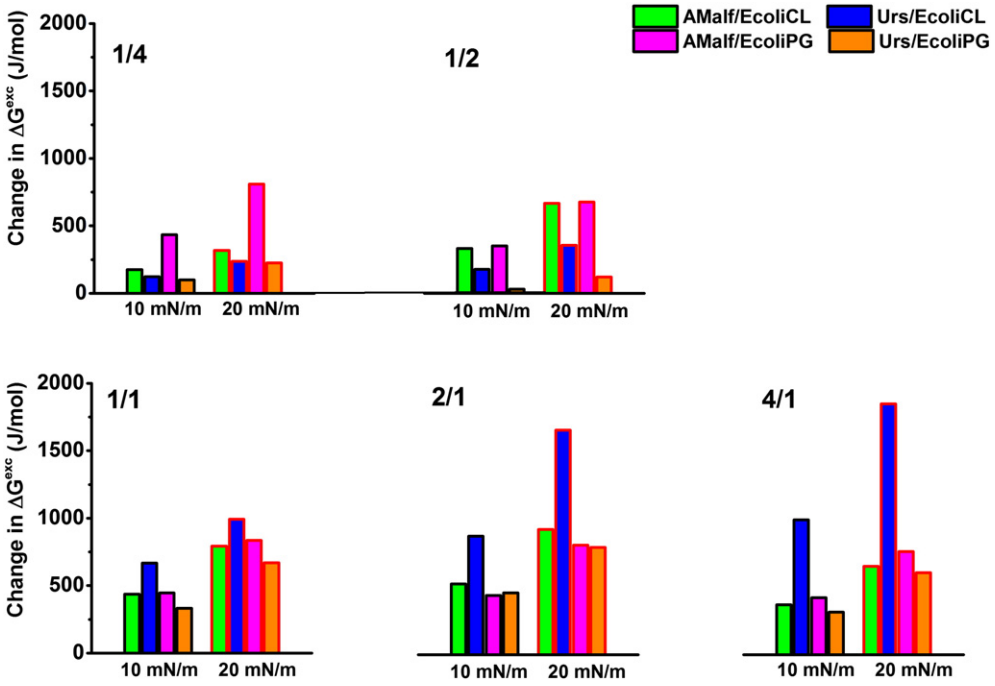


Fig. 8. Differences in ΔG^{exc} values between the ternary and binary systems containing POPE and terpenes. The 1/4 to 4/1 ratios in the panels indicate the terpene/mixed phospholipid surface proportion.

packing but also alter locally the PE/PG mutual proportion making the bacteria more susceptible to antibiotics. It seems that at low proportion, AMalf is more destructive to the bacterial membrane organization than Urs. If the terpenes can be accumulated in the cardiolipin-rich domains, then our experiments performed on model systems indicate that Urs can be more effective than AMalf. On the other hand, the BAM experiments proved that at higher proportion AMalf can be expulsed from the *E. coli* model membranes, both enriched in ECPG and ECCL, and form extrusions on the membrane surface. Such 3D domains can also adversely affect the organization of bacterial membrane. Thus, it seems that even at the membrane level the mechanism of PT action is multifactor. On the basis of the presented studies, it can be speculated that AMalf is more destructive to *E. coli* inner membranes than Urs; however, further studies are needed to explore the questions on more elaborated model systems.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.10.024.

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