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Regular Article

The influence of steroidal and triterpenoid saponins on monolayer models of the outer leaflets of human erythrocytes, *E. coli* and *S. cerevisiae* cell membranes

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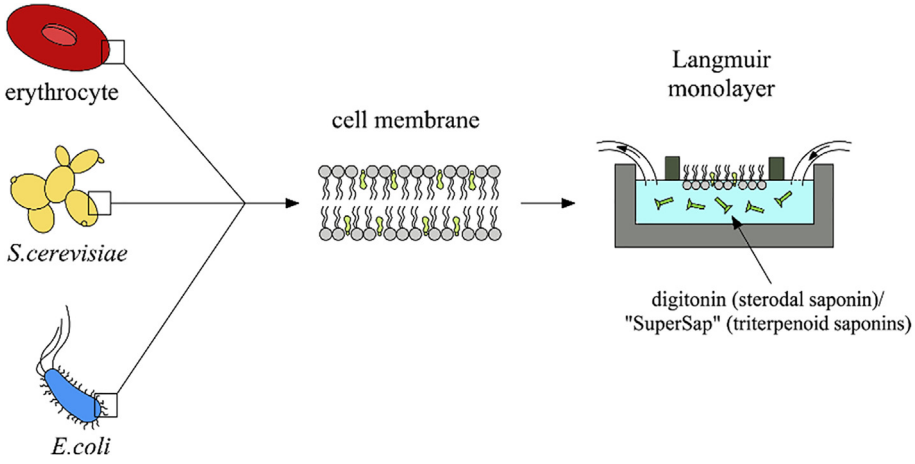
GRAPHICAL ABSTRACT



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ABSTRACT

The present paper discusses the use of monolayers of lipid mixtures mimicking the composition of biological membranes of bacteria, erythrocyte and yeast in the context of the anti-bacterial, hemolytic and anti-fungal activity of saponins. Saponins are plant-produced glycosidic biosurfactants with either steroidal or triterpenoidal aglycone. In the present study we used digitonin as a representative steroidal saponin (extracted from *Digitalis purpurea*) and a mixture of triterpenoid saponins from *Quillaja saponaria* Molina. The effect of saponins was studied first on monolayers consisting of single lipids characteristic for the given type of biological membrane, and then - on model mixed lipid monolayers. Finally, the monolayers were formed from total lipid extracts of natural cell membranes (*E. coli* and *S. cerevisiae*) to verify the results obtained in the simplified models. The effect of saponins on monolayers was studied by a combination of surface pressure relaxation, infrared reflection – absorption spectroscopy (IRRAS) and fluorescence microscopy. In line with expectations, sterols (cholesterol and ergosterol) play a major role in the

Abbreviations: CL, cardiolipin; DPPC, 1,2-dipalmitoyl-*sn-glycero*-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn-glycero*-3-phosphoethanolamine; DPPS, 1,2-dipalmitoyl-*sn-glycero*-3-phosphoserine; DPPG, 1,2-dipalmitoyl-*sn-glycero*-3-phosphoglycerol; DPPI, 1,2-dipalmitoyl-*sn-glycero*-3-phospho-(1′-myo-inositol) (ammonium salt); Erg, ergosterol; Chol, cholesterol; SM, sphingomyelin; TopFluor PC, 1-palmitoyl-2-(dipyrromethenebordifluoride) undecanoyl-*sn-glycero*-3-phosphocholine; LC, liquid-condensed phase; LE, liquid-expanded phase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; GPC, glycerophosphatidylcholine; QBS, *quillaja* bark saponin; IRRAS, infrared reflection-absorption spectroscopy; FM, fluorescence microscopy.

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Langmuir monolayer
IRRAS

saponin-lipid interactions in monolayers, which may explain especially the hemolytic and antifungal properties of saponins. In contrast, bacterial membranes are devoid of sterols, although the presence of similar compounds may be responsible for their affinity to saponins. Nevertheless, the effect of saponins on bacterial models is less pronounced than for the erythrocyte or fungal ones.

1. Introduction

Although the influence of saponins on biological membranes was described for the first time already at the beginning of the 20th century, their detailed mechanism of action is still not known to this day [1]. Saponins as natural surfactants (so-called biosurfactants) are very interesting object of research due to the wide spectrum of biological properties [2]. They include anti-inflammatory, anti-fungal, anti-bacterial, immunomodulatory, hypolipidemic, antiviral, or anti-cancer activities, to name just a few [3–8]. Despite numerous potential pharmacological applications of saponins, there is still lack of detailed information confirming their efficacy and mechanism of action. In addition, some saponins display hemolytic activity, precluding their use as pharmaceuticals at this stage. The hypothesis most often quoted in the literature concerns destruction of the cell membrane by engaging sterols constituting the membrane (e.g. cholesterol in erythrocytes or ergosterol in fungi) into adducts with saponins [9–14]. Saponins were shown to play a significant role in limiting growth of Gram positive bacteria, such as *Mycobacterium tuberculosis* [15], *Bacillus subtilis, Staphylococcus aureus* [16], as well as Gram negative ones: *Ruminobacter amylophilus* and *Prevotella bryantii* [17]. Nonetheless, in the case of *Escherichia coli* [18] and *Selenomonas ruminantium* [17] an opposite effect (i.e. enhancement of bacterial growth upon addition of saponins) was observed. In spite of numerous literature reports describing bacteriostatic or anti-bacterial action of saponins, their molecular details have not been fully clarified so far. Although the fluidity of bacterial cell membranes is not regulated by sterols (like in the case of erythrocytes or fungi), their role is assumed by some structurally similar compounds – hopanoids [19]. It is thus possible that these compounds may play the key role in bacterial activity of saponins. It should also be emphasized that saponins exhibit a great diversity of chemical structures of their aglycone part, the amount and length of the sugar chains (glycone part), as well as the position of their attachment to the aglycone part. All these parameters affect both physicochemical and biological properties of individual saponins.

To date, most studies on the potential pharmacological activity of saponins were carried out using whole-cell biological methods. Nevertheless, these studies cannot provide a clear molecular information about the mechanism of action of saponins on the membrane. Instead, only an overall cell response can be analyzed, e.g. an ultimate halting of its life cycle as a result of many interrelated stimuli. An alternative to experiments using living cells or tissues is the use of model lipid analogues of biological membranes in the form of Langmuir monolayers or bilayers (liposomes, supported lipid bilayers or black lipid membranes). One of the major advantages of these model lipid system is that they can be easily characterized with a number of surface-characterization techniques (surface pressure and potential, surface rheology, Brewster and fluorescence microscopies, neutron and X-ray scattering, ellipsometry, or reflectance (UV, IR) spectroscopies). In addition, both the composition and molecular density of Langmuir monolayers can be easily controlled. The model lipid systems, thanks to their simplicity, can provide useful hints about the interactions of real biological membranes with active substances of potential drugs, like saponins.

With this in mind, we have decided to study the influence of two different saponin biosurfactants (steroidal and triterpenoidal) on model lipid Langmuir monolayers simulating different cell membranes (of erythrocytes, bacteria and yeasts). In order to elucidate the effect of both saponins on model monolayers, surface pressure relaxation measurements were conducted. To gain additional information about the changes of packing and orientation of lipid molecules in the monolayers after contact with saponins, IRRAS (Infrared Reflection Absorption Spectroscopy) measurements were then carried out. Finally, the morphological changes in the mixed monolayers exposed to the saponins were imaged using fluorescence microscopy.

2. Experimental procedures

2.1. Materials

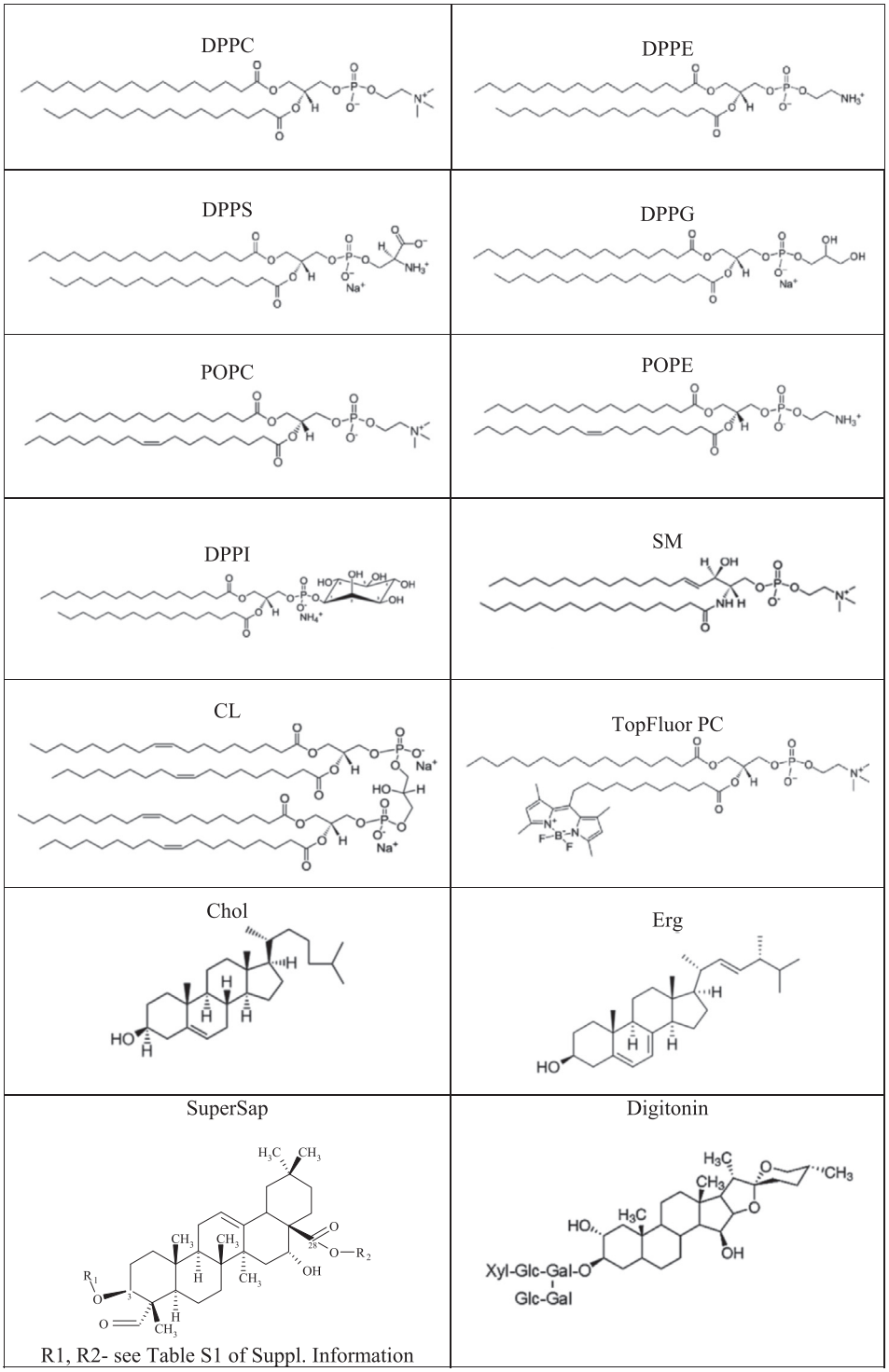
Digitonin (Table 1, M_w = 1229 g/mol, cat. No. 4005) was purchased from Carl Roth and *Quillaja* bark saponin mixtures (see Table 1 for a general structure and Table S1 in Supporting Materials for the structure of selected identified saponins, M_w = 1650 g/mol, which will be referred in this paper as "SuperSap") was obtained from Desert King Inc. The composition of "SuperSap" products was already discussed in [20] and the chemical structures of the identified saponins are presented in Table S1 in the Supplementary Materials. Digitonin and "SuperSap" were used without further purification, and both were dissolved in phosphate buffer (pH 7). In the case of digitonin, boiling was necessary to enable dissolution of the saponin.

Lipids from the following commercial sources were used throughout the study (see Table 1 for the abbreviations):

- CL, Avanti Polar Lipids, cat. no. 710335P; purity > 99%;
- DPPC, Sigma Aldrich, cat. no. P0763; purity ≥ 99%;
- DPPE, Avanti Polar Lipids, cat. no. 850705P; purity > 99%;
- DPPS, Avanti Polar Lipids, cat. no. 840037P; purity > 99%;
- DPPG, Avanti Polar Lipids, cat. no. 840455X, purity > 99%;
- DPPI, Avanti Polar Lipids, cat. no. 850141P, purity > 99%;
- Ergosterol, Sigma Aldrich, nr kar. E6510, purity ≥ 75%;
- Cholesterol, Sigma Aldrich, cat. no. 26732, purity ≥ 99%;
- SM, Avanti Polar Lipids, cat. no. 860061P, purity > 99%;
- TopFluor PC, Avanti Polar Lipids, cat. no. 810281C, purity > 99%;
- E. coli Extract Total, Avanti Polar Lipids, cat. no. 100500P, no information about purity;
- Yeast Extract Total, Avanti Polar Lipids, cat. no. 190000, no information about purity.

Spreading solutions of DPPC, CL, SM, cholesterol, ergosterol were prepared in chloroform. TopFluor PC, POPC, POPE and DPPI were dissolved in chloroform/methanol 9:1 (v/v), and DPPE, DPPG and DPPS were prepared in chloroform/ methanol/ water 65:35:8 (v/v/v). Solvents used in the experiments (chloroform

Table 1
Chemical structures of the lipids and biosurfactants used in the experiments.



(purity ≥ 99.8%) and methanol (purity ≥ 99.9%)) were purchased from Sigma-Aldrich and used without further purification. Milli-Q water (Millipore) with a resistivity of 18.2 MΩ cm was used for all experiments. The subphase was a phosphate buffer in which the concentration of dihydrate of sodium hydrogen orthophosphate was 0.89 g/l and that of dihydrate of sodium dihydrogen orthophosphate – 0.78 g/l (pH 7, ionic strength, I = 10 mM). Both salts were purchased from Sigma-Aldrich.

The measurements were carried out with monolayers consisting of single lipids and of their mixtures, mimicking the composition of outer leaflets of the natural cell membranes – erythrocytes, bacteria and yeast (see Table 2 for compositions of all 2-, 3- and 4-component lipid mixtures). This allowed us to assess whether digitonin or "SuperSap" has any specific affinity for the above-mentioned lipids. To verify the results obtained with the artificially reconstructed lipid mixtures, the monolayers were also prepared

Table 2
Compositions of 2-, 3- and 4-component lipid mixtures used for the IRRAS measurements [%].

lipid	DPPC	DPPS	DPPE	DPPG	POPC	POPE	DPPI	SM	CL	Chol	Erg
lipid mixture											
9 chol:10POPC	0	0	0	0	53	0	0	0	0	47	0
9 chol:10POPE	0	0	0	0	0	53	0	0	0	47	0
9 chol:10SM	0	0	0	0	0	0	0	53	0	47	0
9chol: 10(POPE:SM)	0	0	0	0	0	11	0	42	0	47	0
9chol: 10(POPC:SM)	0	0	0	0	23	0	0	30	0	47	0
9chol: 10(POPC:SM:POPE)	0	0	0	0	21	7	0	25	0	47	0
Erg:DPPE:DPPC:DPPS:DPPI:SM	23	6	22	0	0	0	15	18	0	0	16
DPPE:DPPG:CL	0	0	75	20	0	0	0	0	5	0	0

from commercially available extracts of natural cell membranes – bacteria (*E. coli*) and yeast (*S. cerevisiae*), respectively. The composition of the extracts is shown in Fig. 1. Both extracts were purchased from Avanti Polar Lipids and dissolved in chloroform as recommended by the producer.

2.2. Measurements and instruments

The surface pressure over time (Π-t) dependence was measured using a home-built Langmuir trough dedicated to the exchange of the subphase, with an active area of 194 cm^2. A lipid(s) solution dissolved in an appropriate solvent was deposited onto the phosphate buffer with a Hamilton microsyringe and left for evaporation for 15 min before the compression (with a rate of 10 mm/min) started. The monolayers were pre-compressed to the initial surface pressure (Π_0 = 30 mN/m), and then the subphase (phosphate buffer), was exchanged for the corresponding biosurfactant solution by means of a peristaltic pump (Gilson Minipuls 3) with a flow rate of 2 mL/min. The surface pressure was measured to an accuracy of ± 0.1 mN/m using a filter paper plate (Whatman Chr1) connected to the Langmuir balance. Each measurement was repeated at least twice.

To collect the structural information about the bare lipid monolayers and monolayers in the presence of the biosurfactants in the subphase, infrared reflection – absorption spectroscopy (IRRAS) was used. IRRAS measurements were made using a Bruker IFS66 spectrometer equipped with a mercury-cadmium-tellurium detector (MCT). All spectra were recorded at a resolution of 8 cm^{-1}. The IR beam used in the experiments was s- and p- polarized and the angle of incidence was 40°. The measurements were carried out in a home-built Langmuir trough equipped with two separate compartments, where one was filled with buffer, constituting the subphase, while the other contained a subphase to which a lipid solution dissolved in a volatile organic solvent was dropped. The Langmuir trough was shuttled between the two separated compartments, so that the IR beam illuminated at first the reference (subphase, spectrum R_0) and thereafter the sample (subphase with the monolayer, spectrum R). As a result, the obtained RA (reflection-absorption) is plotted as -log (R/ R_0), and thus it is possible to eliminate the strong absorption bands coming from water. The instrument setup is placed in a sealed container (an external air/water reflection unit XA-511, Bruker, Germany) to guarantee a constant vapor atmosphere. The details about IRRAS can be found in literature [21–24]. In experiments with lipid monolayers on a saponin-free subphase (monolayer characterization), the IRRAS spectra were obtained at constant surface pressure (30 mN/m), controlled by moving the barriers in a feedback mode. In contrast, in the experiments with the lipid monolayers compressed "chemically" by addition of the respective saponins to the subphase, the constant area mode was employed. The uncertainties provided in the figures and tables were estimated from the uncertainties of the IRRA spectra fitting. Selected measurements were repeated up to 4 times.

Fluorescence microscopy (FM) was used to visualize phase transitions in the monolayers. An OLYMPUS BX51WI epifluorescence microscope with a U-MWB2 filter was employed for this purpose. The microphotographs were taken with a CCD camera and analyzed with the cellSens software. In order to highlight morphological changes in the monolayers, a small amount (0.5 mol%) of a fluorescent phospholipid (TopFluor PC) was added. The FM measurements were performed in duplicate.

3. Results

3.1. The influence of saponins on lipid monolayers mimicking the outer layer of the cell membranes of erythrocytes

The effect of "SuperSap" (a highly purified extract from *Quillaja saponaria* Molina tree containing different triterpenoid saponins)

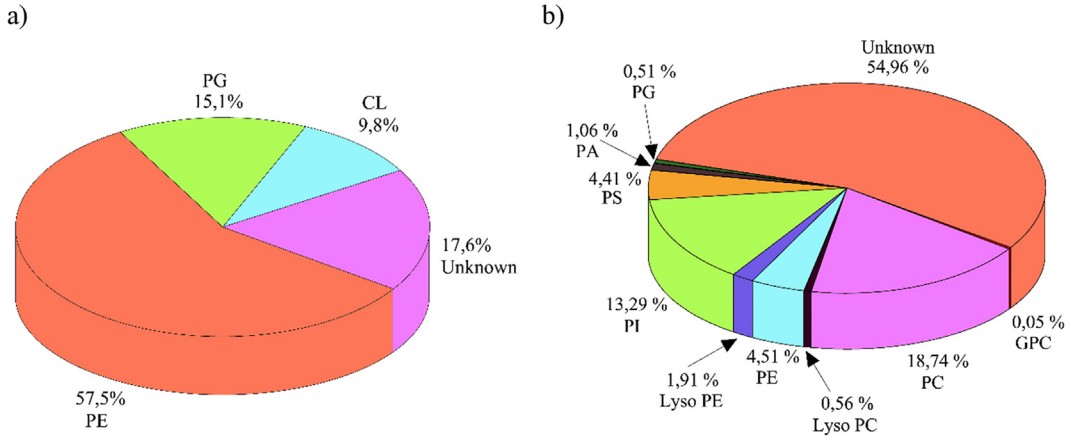


Fig. 1. *E. coli* (a) and *S. cerevisiae* (b) phospholipid profiles of the total lipid extracts used in the experiments. Charts constructed using the composition provided by the producer (Avanti Polar Lipids).

and digitonin (steroidal saponin) on the Langmuir monolayers mimicking the outer layer of red blood cell (erythrocyte) membranes was studied with help of Langmuir monolayers. They were prepared by mixing the appropriate amounts of cholesterol, palmitoyl-oleoyl phosphatidylcholine (POPC), sphingomyelin (SM) and palmitoyl-oleoyl phosphoethanolamine (POPE). The initial studies were carried out with the monolayers consisting of single lipids. In the next stage, the binary, ternary, quaternary mixtures of these lipids were used, where the molar ratio of the sum of phospholipids and sphingolipids to cholesterol was always maintained at 10:9 (mol/mol), corresponding to the high cholesterol content in erythrocytes [13].

In an attempt to establish the structural information about each bare monolayer and the same monolayer exposed to the action of digitonin or "SuperSap", infrared reflection–absorption spectroscopy (IRRAS) was used. In the experiments where pure lipids or their mixtures were characterized, the Langmuir films were symmetrically compressed by two barriers to the desired surface pressure (i.e. 2, 5, 10, 15, 20, 25, 30 and 35 mN/m) and then the IR spectrum was recorded. For studying the effect of saponins, the spectra were recorded first for the monolayer on the phosphate buffer initially compressed to Π = 30 mN/m, and then after exchanging the subphase with the digitonin or "SuperSap" solution for 6000 s. The IRRA spectra were also recorded for the Gibbs layers of digitonin and "SuperSap" at the same final concentration of the saponins in the subphase ($3 \cdot 10^{-6}$ M). In line with our previous measurements, the initial surface pressure of 30 mN/m was chosen because the packing of lipid molecules in monolayers at surface pressures between 30 and 35 mN/m is generally believed to resemble that in real biological bilayers [25].

Fig. 2 presents the wavenumbers of the asymmetric $-CH_2$ stretching vibration (ν_{as} CH_2) for all lipids and their mixtures versus surface pressure. The ν_{as} CH_2 values are sensitive to the ordering of the lipid acyl chains and are often taken as a direct measure of the monolayer phase state. The acyl chains with ν_{as} CH_2 > 2924-cm^{-1} are considered as being in a liquid-expanded (LE) state, where the *gauche* conformation dominates, while for ν_{as} CH_2 < 2920 cm^{-1}, the *trans* conformers dominate in the acyl chains (liquid-condensed, LC) [26]. In the case of unsaturated lipids (POPE and POPC), ν_{as} CH_2 > 2924 cm^{-1} shows that the acyl chains are in the all-*gauche* conformation in the whole range of the employed surface pressures. This does not change significantly even in presence of high content of cholesterol (47 mol%), known for its

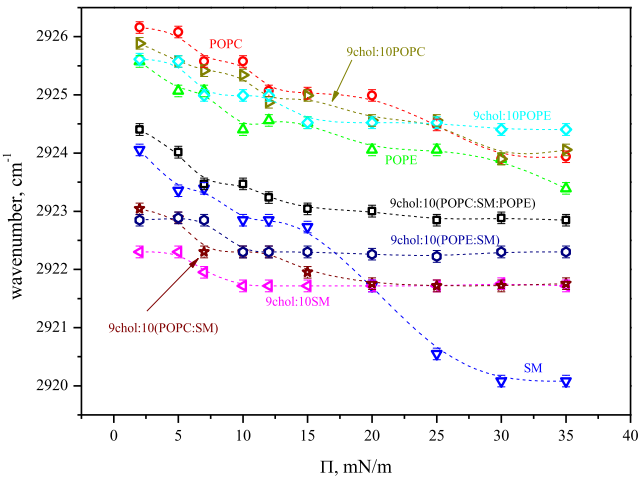


Fig. 2. Position of the asymmetric –CH_2 – stretching vibration of the lipid monolayers mimicking the outer leaflet of erythrocyte's bilayer on phosphate buffer at 21 °C as a function of the surface pressure applied by barriers (mechanical compression).

"condensing effect" on disordered lipids. The latter can only be observed in mixtures with sphingomyelin (SM) which exhibits LE-LC phase transition around 20 mN/m [27]. For a binary system SM:cholesterol [9chol:10SM] at low surface pressure, Π < 20 mN/m (corresponding to the LE phase of SM) the presence of cholesterol shifts the ν_{as} CH_2 wavenumbers towards lower values, ~ 2922 cm^{-1}. This indicates a reduction in the number of *gauche* conformers in proportion to *trans* conformers in the hydrocarbon chains of SM in presence of cholesterol. Further compression of this monolayer ([9chol:10SM]) above Π = 20 mN/m (corresponding to the LC phase of SM) reverses the trend: the wavenumbers of ν_{as} CH_2 in pure SM monolayers are lower than in mixtures with cholesterol. Hence, in highly compressed mixed monolayers, cholesterol molecules hinder ordering of sphingomyelin. Consequently, the LE-LC phase transition disappears.

The condensing effect of cholesterol is also observed for both ternary mixtures involving cholesterol, sphingomyelin and a phospholipid – [9chol:10(POPE:SM)] and [9chol:10(POPC:SM)]. In the case of a quaternary mixture (containing cholesterol, SM and both unsaturated phospholipids: POPC and POPE - [9chol:10(POPC:SM:POPE)]), the wavenumbers are in the range typical for the liquid-ordered phases, yet slightly higher compared with the binary system [9chol:10SM]. As expected, the addition of the unsaturated lipids leads to a higher degree of disorder in the quaternary mixture.

Knowing the ordering of each lipid monolayer at the given surface pressure achieved by mechanical compression (Fig. 2), we first analyzed the effect of flow on representative single-component lipid monolayers in order to facilitate the subsequent analysis of the effect of saponins introduced in a flow-through mode. Two lipids with unsaturated chains (POPE, POPC), SM and one saturated lipid (DPPC) were chosen for this purpose. All monolayers were compressed to Π_0 = 30 mN/m and the changes of surface pressure and wavenumbers for ν_{as} CH_2 were monitored during the buffer recirculation. For the monolayers of POPE, POPC and SM (Figs. S4, S5, S6, S7 in the Supplementary Materials) surface pressure reached the final values of 33.2 mN/m, 29.1 mN/m, and 16.9 mN/m, respectively. At the same time, the wavenumbers of the stretching vibrations of the asymmetric methylene groups (ν_{as} CH_2) of POPC and POPE increased with respect to the values for Π_0 = 30 mN/m (up to ca. 2926 cm^{-1} and ca. 2925 cm^{-1}, respectively). This would suggest an increase of the disorder in the acyl chains of POPE and POPC without a significant reduction of surface pressure. The opposite was observed for SM, where despite a huge reduction of surface pressure, the acyl chain ordering did not change significantly (ν_{as} CH_2 = 2920 cm^{-1}). On the other hand, it should be noted that the IR band intensities of ν_{as} CH_2, ν C=O and ν_{as} PO_2- were not reduced (Figs. S4, S5, S6 in the Supplementary Materials), suggesting that the adsorbed lipid amounts do not change significantly after the buffer recirculation. Thus, the accompanying relatively large reduction of surface pressure for SM is probably a consequence of a steepness of the surface pressure isotherm in this region. All this taken together brings us to the conclusion that the shear induced by the flow of the subphase might alter organization of the unsaturated lipids, facilitating their relaxation. On the other hand, an example of DPPC shows that this does not have to be the case for saturated phospholipids with well described LE and LC phases (Fig. S8 in the Supplementary Materials). To illustrate the resistance of a DPPC monolayer to the subphase flow, the former was first compressed to Π_0 = 30 mN/m and the IRRA spectrum was recorded at constant surface pressure, providing the value ν_{as} CH_2 = 2920 cm^{-1}. Immediately afterwards the subphase (water) recirculation was started with fixed barriers positions. The spectrum recorded at the end (after 2 h) showed only a slight reduction of the ν_{as} CH_2 wavenumber (down to 2919.7 cm^{-1}) accompanied by a tiny drop of surface pressure

(Π = 28 mN/m). Re-compression of the monolayer back to Π = 30 mN/m immediately restored the ν_{as} CH_2 wavenumber (2920 cm^{-1}). The whole recirculation cycle was repeated again, producing the same tiny reduction of ν_{as} CH_2 wavenumber (without any noticeable reduction of the band intensities) and even less surface pressure drop (by 0.3 mN/m). Hence the DPPC monolayer proved to be resistant to the subphase flow. The tiny surface pressure drop was caused probably by the remainders of the LE phase that were easily removed by re-compression. The comparison of the POPE, POPC, SM and DPPC behavior shows that the susceptibility of the phospholipid monolayers to the flow of the subphase depends mostly on the saturation of the acyl chains of the lipid. The most probable physical causes of the observed increased disordering and reduction of the surface pressure without significant reduction of the adsorbed amount are relaxation of the monolayers, solubilization of the lipids in the subphase and their chemical degradation. When the rate of initial compression is higher than the rate of the respective phase transition, certain amounts of the less ordered molecules might be trapped during compression and could relax slowly at later stages. The subphase flow probably increases the rate of this relaxation. Given the fact that the slope of the SM compression isotherm is high around Π = 30 mN/m [28], even tiny changes of the adsorbed amount could result in large drops of surface pressure.

In the next step, the effect of saponins introduced in a flow-through mode was analyzed for each single-, two-, three- and a four-component monolayer (pre-compressed to Π_0 = 30 mN/m). Due to the presence of unsaturated phospholipids (POPE and POPC) and 47% content of cholesterol, the mixed lipid monolayers were very fragile and could be easily collapsed. For this reason the concentration of the biosurfactants introduced into the subphase were reduced with respect to our previous experiments [29]). In all experiments with erythrocyte model monolayers the final saponin concentration was fixed at $3 \cdot 10^{-6}$ M, which is well below the cmc (between $2{,}5 \cdot 10^{-4}$ M and $5 \cdot 10^{-4}$ M for digitonin [30] and $4 \cdot 10^{-4}$ M for "SuperSap" [31]). At this concentration, the Gibbs layers (free adsorption to the bare water/air interface) of the two saponins reached final surface pressures (Fig S2 in the Supplementary Materials) of Π = 3.2 mN/m (digitonin) and Π = 16.8 mN/m ("SuperSap"). Table 3 shows the values of surface pressure after the relaxation process, as well as the values of ν_{as} CH_2 before and after the addition of saponins to the subphase.

Both digitonin and "SuperSap" affect the single-component monolayers in a similar way. For the pure phospholipid monolayers, both saponins only slightly affected the surface pressure (slight reduction for POPC and slight increase for POPE), while for SM a clear reduction of Π was observed for "SuperSap" and even more for digitonin. For all three lipids the extent of changes was, however, comparable to that observed for the reference experiments with the flow of saponin-free subphase (only buffer, see above). Similarly, the wavenumbers of ν_{as} CH_2 were close to the values similar to those observed during the buffer recirculation (shifted to higher values for POPE and POPC and practically unaffected for the SM monolayer). The observed changes were thus probably not related to any interaction with the subphase components but only to the subphase flow.

When comparing the effect of the two biosurfactants on the cholesterol-containing monolayers, it is worth noting that digitonin caused a far more pronounced increase in surface pressure compared to "SuperSap" (Table 3). This might seem surprising given the fact that the equilibrium surface pressure of the analogous Gibbs layers (spontaneous adsorption in the absence of lipids) of digitonin is only 3.2 mN/m, much less than for "SuperSap" (16.8 mN/m). Apparently, the surface activity of digitonin is lower than that of "SuperSap" but its high affinity to cholesterol attracts it strongly to the cholesterol containing monolayers. This holds true also for the binary, ternary, quaternary monolayers, where cholesterol constitutes 47% of the lipids (Table 2). For example, surface pressure of the ternary mixture [9chol:10(POPC: SM: POPE)] in contact with digitonin rises up to 52.3 mN/m, whereas for the same system with "SuperSap" - only to 35.8 mN/m. The presence of digitonin reduces the wavenumber of ν_{as} CH_2 indicating the increased ordering of the monolayer. By contrast, in the case of "SuperSap" a slight increase in the wavenumber of ν_{as} CH_2 was observed, indicating increased disorder of monolayers. Overall, as long as the monolayer contains cholesterol, the effect of the saponins is rather insensitive to the nature of the remaining lipids. Consequently, also for the four-component monolayer, which is supposed to mimic most closely the outer layer of the natural membrane of the erythrocytes, the presence of digitonin in the subphase increases surface pressure and ordering of the lipids, while "SuperSap" rather disorders the lipids.

In view of the dominant role of cholesterol, the monolayers devoid of any other lipids were probed for their response to both saponins. Unfortunately, even the minute amount of digitonin employed in the study ($3 \cdot 10^{-6}$ M) collapsed the bare cholesterol monolayer within the first minutes after its introduction into the subphase. The monolayer did, however, survive the contact with "SuperSap" at the same concentration, probably because of the lower affinity of the triterpenoid saponins to cholesterol. In this case surface pressure was raised up to 45.7 mN/m, i.e. ca. 10 mN/m higher than for the ternary and quaternary lipid mixtures with 47% of cholesterol.

The effect of saponins on the monolayers containing the lipids representative of erythrocytes was in parallel probed using fluorescence microscopy (FM). However, for almost all monolayers

Table 3
Surface pressure for monolayers imitating the outer layer of erythrocytes initially pre-compressed to Π_0 = 30 mN/m and exposed to the action of biosurfactants (C = $3 \cdot 10^{-6}$ M) in the subphase and the wavenumbers for the – CH_2 asymmetric stretching vibration of the alkyl chains of the lipids in monolayers before and after the addition of saponins to the subphase.

Composition and ν_{as} CH_2 (at 30 mN/m) of monolayers	Π after adding "SuperSap", mN/m	ν_{as} CH_2 after adding "SuperSap", cm^{-1}	Π after adding Digitonin, mN/m	ν_{as} CH_2 after adding Digitonin, cm^{-1}
POPC ν_{as} CH_2 = 2923.9 cm^{-1}	25.6	2926.7	27.8	2926.6
POPE ν_{as} CH_2 = 2923.7 cm^{-1}	31.0	2925.1	32.6	2925.8
SM ν_{as} CH_2 = 2920.2 cm^{-1}	21.5	2920.3	15.6	2920.2
Cholesterol	45.7	–	Collapse	–
9 chol:10POPC ν_{as} CH_2 = 2924.5 cm^{-1}	38.6	2926.9	43.5	2924.5
9 chol:10POPE ν_{as} CH_2 = 2924.6 cm^{-1}	39.6	2926.4	46.4	2923.9
9chol:10SM ν_{as} CH_2 = 2921.7 cm^{-1}	34.8	2922.3	49.9	2921.0
9chol:10(POPE:SM) ν_{as} CH_2 = 2922.3 cm^{-1}	35.1	2922.5	46.3	2922.1
9chol:10(POPC:SM) ν_{as} CH_2 = 2921.7 cm^{-1}	32.5	2922.3	40.4	2921.0
9chol:10(POPC:SM:POPE) ν_{as} CH_2 = 2922.8 cm^{-1}	35.8	2924.0	52.3	2921.5

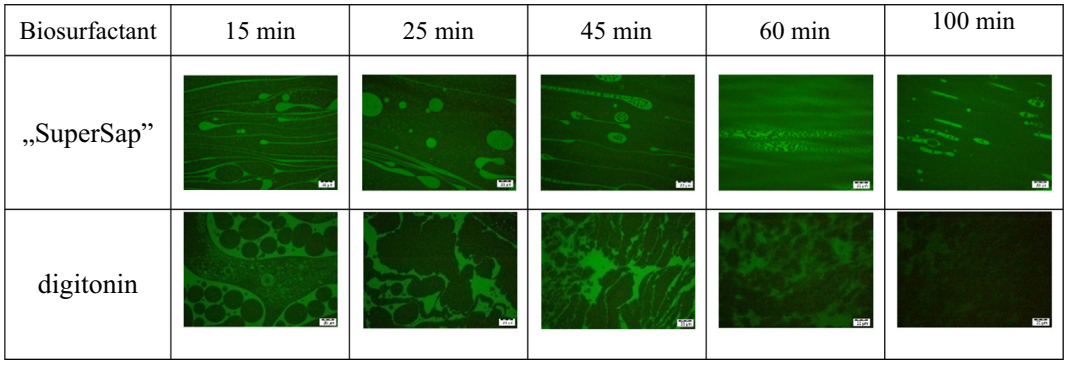


Fig. 3. Fluorescence microphotographs of Langmuir monolayers of [9chol:10(POPC:SM)] during the subphase exchange for “SuperSap” and digitonin solutions.

detection of any changes was hindered by either the lack of phase transitions or the good miscibility between the components even in the presence of saponins. Only for one ternary lipid mixture [9chol:10(POPC:SM)] was it possible to observe changes during the subphase exchange for the appropriate biosurfactant solution (Fig. 3). The fluorophore is attached to one of the acyl chains of the probe lipid (TopFluor PC), rendering it sensitive to the packing of acyl chains of the neighboring lipids. The fluorescence probe is preferentially partitioned in the less ordered LE phase, hence the brighter appearance of the latter in the corresponding FM images. Oppositely, the darker regions correspond to the more ordered LC phase, from which the probe is excluded. The effect of digitonin is clearly than for “SuperSap”. During circulation of the former, movements of the whole LC phase lobes (dark regions) could be observed. The system appeared generally more viscous and approximately 30 min after the beginning of the subphase exchange with digitonin, the image ceased to move along the flow. This observation is in line with our previous observations by Grazing Incidence X-ray Diffraction (GIXD) suggesting formation of a crystalline phase of digitonin-cholesterol adducts [29].

3.2. The influence of saponins on lipid monolayers mimicking the outer layers of cell membranes of bacteria and yeast

In the second part, we describe the effect of the two saponins on model monolayers prepared from the lipids characteristic for bacteria and yeast, in the context of the anti-bacterial and anti-fungal activity of saponins. The experiments were performed using the same experimental techniques as described in the previous section, on two types of monolayers. First, the lipid monolayers were prepared from individual lipids and their mixtures (which will be referred as *E. coli reconstructed* or *Yeast reconstructed*), based on the literature data concerning the lipid compositions of the outer layers of bacterial and yeast cell membranes. In the second part, the commercially available total lipid extracts from *E. coli* bacteria (which will be referred as “*E. coli extract Avanti*”) and *S. cerevisiae* yeast (“*Yeast extract Avanti*”) were used. On the basis of preliminary experiments (results not shown), the saponin concentration was increased with respect to the part concerning erythrocytes to c = 10^{-4} M, the same as used in our previous study [29]). The increase was necessary because of a much lower cholesterol content and the consequent lower surface pressure response to saponins. The Gibbs layers of digitonin and “SuperSap” at c = 10^{-4} M display equilibrium surface pressures of Π = 23.7 mN/m and 28.6 mN/m, respectively (Fig S3 in the Supplementary Materials).

3.2.1. Model yeast membranes

On the basis of the literature data about the external layer of the yeast cells membrane composition [32–35] we proposed a model consisting of a mixture of ergosterol, DPPE, DPPC, DPPS, DPPI and SM, with a molar ratio of 16:22:23:6:15:18. In the first part, the surface pressure dependence of the ordering of individual lipids and their mixtures in monolayers was assessed using IRRAS by analyzing the changes of ν_{as} CH_2 in acyl chains of the lipids. The IRRA spectra were recorded up to Π = 35 mN/m for sphingomyelin (SM) and all phospholipids used for the preparation of the reconstructed outer layer of yeast cell membrane (DPPC, DPPI, DPPS, DPPE), for their mixture (containing additionally ergosterol), as well as for the *Yeast extract Avanti*. The surface pressure dependencies of the wavenumber for the ν_{as} CH_2 are presented in Fig. 4.

For DPPC at low surface pressures (<5 mN/m), high values of the ν_{as} CH_2 wavenumbers (above 2924 cm^{-1}) indicate the occurrence of mainly *gauche* conformers, typical for the LE phase. At surface pressure above 15 mN/m, ordering of the palmitoyl chains in the DPPC monolayer suggests domination of the LC phase (wavenumbers below 2920 cm^{-1}). In the intermediate region (5–15 mN/m), the values of wavenumbers between 2924 and 2920 cm^{-1} indicate the co-existence of both phases (LE-LC) [36]. Analogously, for DPPI at surface pressures below 15 mN/m, ν_{as} CH_2 exceeding 2924 cm^{-1} suggest the prevalence the LE phase. However, in comparison to DPPC, the ordering of the monolayer increases much less steeply at surface pressures above Π = 12 mN/m, indicating coexistence of the LE and LC phases in a wider range of surface pressures. For sphingomyelin, the values between 2924 and 2920 cm^{-1} testify the co-existence of LE-LC phase in the whole range of the employed surface pressures. In contrast, for both DPPS and DPPE

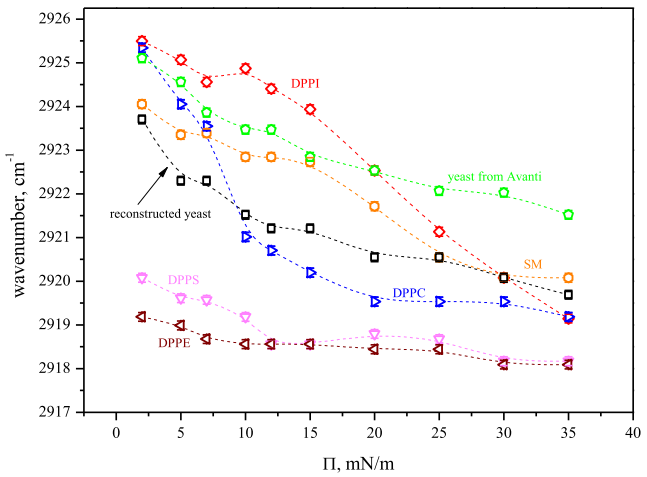


Fig. 4. Position of the asymmetric –CH_2 – stretching vibration of the lipid monolayers on phosphate buffer at 21 °C as a function of the surface pressure applied by barriers (mechanical compression).

the low values of ν_{as} CH_2 wavenumbers (<2020 cm^{-1}) at all employed surface pressures point to the all-*trans* conformation of the palmitoyl chains (LC phase).

The reconstructed mixed lipid model of the outer leaflet of the yeast cell membrane, consisting of ergosterol, DPPE, DPPC, DPPS, DPPI and SM and the total lipid extract were characterized analogously. The analysis of IRRAS data from Fig. 4 shows that the reconstructed mixed monolayer may exist in both the LE (at very low surface pressures) and the LC state, with a rather wide range of LE-LC coexistence. Interestingly, the shape of the ν_{as} CH_2 vs. Π is similar for the *Yeast extract Avanti*, but the whole curve is shifted to higher wavenumbers. This suggests that the acyl chains in the total lipid extract are more disordered than in our reconstructed model. According to the information provided by the producer, the commercially available total lipid extract from *S. cerevisiae* contains PG, PS, PI, PE, PC and PA lipids (see Fig. 1 for the quantitative composition), which account for only 45.04% of the total lipids. The remaining content is not specified, but from the literature it is clear that it should include sterols, of which the major one should be ergosterol. The latter plays a role analogous to cholesterol in mammalian cells, stabilizing and providing flexibility to yeast cell membranes and its content in the yeast membrane was reported as 13% [37,38]. The observed difference between the two mixed lipid monolayers might thus be related to the difference in ergosterol content, but also to the presence of other sterols in the unspecified lipid fraction of the extract. Another possible explanation might be a higher extent of unsaturation of the lipids in the latter – according to the producer more than half of the fatty acid chains in the extract are unsaturated.

For all single lipid monolayers exposed to "SuperSap" or digitonin, surface pressure either remained unchanged or increased with respect to the initial situation (Π_0 = 30 mN/m, see Table 4). In general, "SuperSap" raises the surface pressure to higher values than digitonin, with the exception of DPPI, where the increase of surface pressure is comparably high. This may indicate an attractive interaction of both saponins with the inositol headgroup. The rather weak response of the pre-compressed DPPE and especially DPPS monolayers is somehow surprising in view of our previous study on the effect of digitonin on uncompressed phospholipid monolayers [39]. Even though the ordering of the palmitoyl chains of DPPE and DPPS is very similar at Π = 0 mN/m (previous study) and at Π = 30 mN/m (this study), the uncompressed monolayers were undergoing a peculiar phase transition from LC to LE with increasing surface pressure (due to the presence of digitonin). The same monolayers, when compressed mechanically to Π = 30 mN/m are no longer capable of interacting with saponins. Even though the ordering of the dipalmitoyl chains is comparable at high and low surface pressures (as suggested by the weak slope of the curves in Fig. 4), certainly the van der Waals attraction between the acyl chains is stronger under the presently employed conditions. This makes the penetration by saponins much more difficult than for the uncompressed monolayers studied earlier in Ref. [39]. The increase of surface pressure induced by the presence of saponins is accompanied by a minor reduction of ν_{as} CH_2 wavenumbers, suggesting a slight increase of the lipid chains ordering, in line with the data in Fig. 4. Not surprisingly, the highest reduction can be noticed for DPPI which displays the steepest ν_{as} CH_2 vs. Π slope. The weak effect of both saponins on SM monolayers has already been discussed in the previous chapter. In all cases the unchanged intensity of $-CH_2$ peaks combined with the lack of changes in ν C=O and ν_{as} PO_2- moieties confirms that the lipids are not removed by the biosurfactant solutions, despite their relatively high concentration (C = 10^{-4} M).

When comparing the effect of the biosurfactants on two monolayers imitating the outer layer of yeast cells – the *Yeast extract Avanti* and *Yeast reconstructed*, it can be noticed that the ordering of the acyl chains in both cases slightly increases, analogously to the single lipids. Interestingly, both "SuperSap" and digitonin affect more strongly the surface pressure of the reconstructed monolayer than that of the total lipid extract one. In view of the results from the previous chapter and the literature on interactions between saponins and sterols [11,40,41], the discrepancy between the two monolayers could probably be explained by differences in the amount of ergosterol (13% in the reconstructed mixture vs. unknown amount in the total extract).

In order to verify whether ergosterol could indeed interact with the saponins in a similar way as cholesterol, a mixed DPPC:ergosterol monolayer was prepared. The DPPC:sterol molar ratio was set to 10:9 (note that the experiments with higher cholesterol content are not feasible because of the surface pressure instabilities and monolayer collapse [29]). The monolayer was pre-compressed to the initial surface pressure of Π_0 = 32.5 mN/m and the subphase was exchanged for digitonin to reach the final concentration of 10^{-4} M. The increase of surface pressure observed for the DPPC:ergosterol mixture (Π = 51.4 mN/m) was very similar to that observed previously for the DPPC:cholesterol under the same conditions (Π = 55 mN/m [29]). This confirms that the lower amount of ergosterol (or other sterols) in the *Yeast extract Avanti* could be responsible for its lower response to the saponins, as compared to the reconstructed monolayer.

Further characterization of the influence of biosurfactants on the morphology of the monolayers imitating the yeast cell membrane (*Yeast reconstructed* and *Yeast extract Avanti*) was carried out using fluorescence microscopy. The images were taken during the subphase exchange process for 100 min (Table S2 in the Supplementary Materials). The microscopic patterns of both monolayers are not significantly affected by the presence of biosurfactants. Only for the reconstructed lipid mixture penetrated by digitonin some bright spots could be observed. Their appearance was similar to the crystallites observed previously for cholesterol-rich mixed monolayers [29].

Table 4
Surface pressure for monolayers imitating the outer layer of yeast cell membrane initially pre-compressed to Π_0 = 30 mN/m and exposed to the action of biosurfactants (C = 10^{-4} M) in the subphase and the wavenumbers for the $-CH_2$ asymmetric stretching vibration of the alkyl chains of the lipids in monolayers before and after the addition of saponins to the subphase.

Composition and ν_{as} CH_2 (at 30 mN/m) of monolayers	Π after adding "SuperSap", mN/m	vas CH_2 after adding "SuperSap", cm^{-1}	Π after adding Digitonin, mN/m	ν_{as} CH_2 after adding Digitonin, cm^{-1}
DPPS ν_{as} CH_2 = 2918.5 cm^{-1}	33.9	2917.9	30.0	2918.1
DPPI ν_{as} CH_2 = 2920.6 cm^{-1}	41.1	2918.6	41.3	2919.1
DPPE ν_{as} CH_2 = 2918.3 cm^{-1}	40.4	2917.9	30.7	2917.9
SM ν_{as} CH_2 = 2920.3 cm^{-1}	36.3	2919.5	33.7	2919.5
DPPC ν_{as} CH_2 = 2919.7 cm^{-1}	36.8	2919.5	33.5	2919.7
Yeast extract Avanti ν_{as} CH_2 = 2922.2 cm^{-1}	33.7	2920.1	36.6	2921.7
Yeast reconstructed (Erg:DPPE:DPPC:DPPS:DPPI:SM) ν_{as} CH_2 = 2920.0 cm^{-1}	39.0	2918.6	40.0	2918.7

3.2.2. Model bacterial membranes

The single lipids, their mixture forming a reconstructed outer leaflet of the bacterial membrane and the *E. coli extract Avanti* were characterized first using IRRAS, as described in the previous chapters. The results collected in Fig. 5 show a variety of phase behavior for different constituents and mixtures. Cardiolipin (CL) belonging to diphosphatidylglycerols, a lipid characteristic to mitochondrial and bacterial membranes is a bulky molecule with four acyl chains. The derivative used in the present study features one unsaturated bond in each acyl chain, which introduces high disorder (ν_{as} CH_2 > 2925 cm^{-1}). On the other extreme, DPPE is capable of packing its two saturated palmitoyl chains effectively even at low surface pressures, as discussed above for the yeast membrane (ν_{as} CH_2 < 2919.5 cm^{-1}). For DPPG, the wavenumbers for ν_{as} CH_2 span a range between CL (at low surface pressures) and DPPE (for high surface pressures), indicating the presence of LE and LC, as well as their coexistence within the applied surface pressure range. It is worth noting that in our previous study, the DPPG monolayers on pure water showed high ordering in the whole range of surface pressures (ν_{as} CH_2 ≈ 2919 cm^{-1} - only LC phase) [39]. This difference stems most likely from the fact that DPPG is a negatively charged lipid and might be partially negatively charged at pH 7, employed in the present study. The electrostatic repulsion hinders effective packing of the molecules, hence the presence of LE and LE-LC phases at lower surface pressures. The repulsion is sufficiently weak that it can be overcome by mechanical compression, and for Π > 25 mN/m the LC phase can be reached even on the phosphate buffer.

The reconstructed outer layer bacterial cell used in the present study consisted of two saturated (DPPE and DPPG) and one unsaturated (CL) phospholipids at the molar ratio of 15:4:1 (DPPE:DPPG:CL) [19,42,43]. Since the saturated lipids comprise 95% of the molecules in the monolayer, it is not surprising that their phase behavior dominates that of the mixture. Consequently, the reconstructed bacterial membrane model in the whole range of applied surface pressures is in the LC phase (ν_{as} CH_2 < 2919 cm^{-1}). The opposite behavior can be observed for the monolayer prepared from *E. coli extract Avanti* (ν_{as} CH_2 > 2924 cm^{-1}). According to the producer, *E. coli* extract is composed of three identified phospholipids: PE, PG, CL, and an "unknown" fraction, accounting for 17.6% of the phospholipids (see Fig. 1 for a detailed composition). The producer does not, however, provide any information on the degree of unsaturation in the fatty acid chains of individual phospholipids, or other lipids present in the extract. From the values of ν_{as} CH_2 exceeding 2924 cm^{-1}, it can be presumed that the phospholipid chains in the extract are mostly unsaturated.

The monolayers of single phospholipids exposed to the biosurfactants do not show pronounced changes of surface pressure. For DPPE and DPPG both "SuperSap" and digitonin induce a slight decrease of ν_{as} CH_2, pointing to an increased ordering due to penetration of saponins (Table 5). The opposite effect is observed for cardiolipin (CL), suggesting further disordering. The effect on both the *E. coli reconstructed* and *E. coli extract Avanti* lipid monolayers is even less pronounced, and it can be safely stated that the saponins employed in this study at concentration of 10^{-4} M do not affect these model monolayers, except for one combination: digitonin and *E. coli extract Avanti*. The lack of significant changes in the fluorescence microscopic images (Table S3 in the Supplementary Materials) further supports the hypothesis of generally weak effect of saponins on these models.

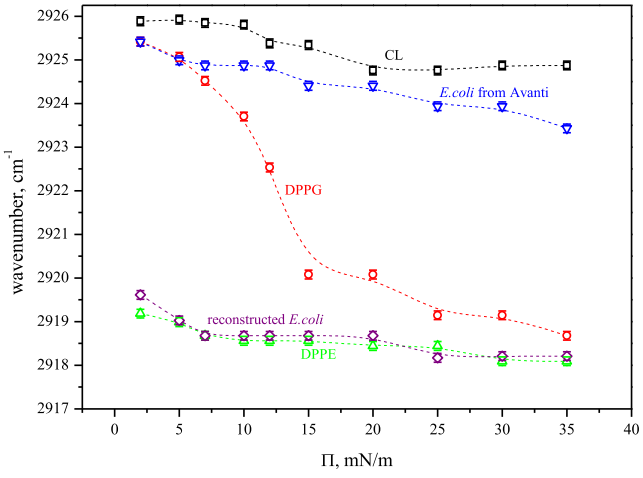


Fig. 5. Position of the asymmetric –CH_2 – stretching vibration of monolayers on phosphate buffer at 21 °C as a function of the surface pressure applied by mechanical compression.

4. Discussion

In general, the sterol-containing monolayers proved to be more susceptible to the action of saponins as compared to those without cholesterol or ergosterol. In the case of the erythrocyte membrane models, containing as much as 47% of cholesterol, the saponin concentration had to be reduced down to $3 \cdot 10^{-6}$ M in order to prevent the monolayer collapse during the subphase exchange. The present results confirm that the hemolytic activity of saponins is most likely related to a saponin-cholesterol interaction. The higher increase of surface pressure for the [9chol:10(POPC:SM:POPE)] monolayer exposed to digitonin than to "SuperSap" is in line with a higher hemolytic activity of the steroidal saponin [44–47]. Ergosterol present in the yeast cell membranes plays a role similar to cholesterol in the mammalian cells and displays comparable affinity to saponins. The response of the reconstructed outer layer of yeast membrane to digitonin and "SuperSap" was, however, smaller than that for the erythrocyte membrane model, even though the saponin concentration was increased over 30 times (to 10^{-4} M). The main reason for that is a much smaller sterol content in the yeast model (13% mol in the reconstructed model, and probably even less in the *Yeast extract Avanti*).

Table 5
Surface pressure for monolayers imitating the outer layer of *E. coli* initially pre-compressed to Π_0 = 30 mN/m and exposed to the action of biosurfactants (C = 10^{-4} M) in the subphase and the wavenumbers for the –CH2 asymmetric stretching vibration of the alkyl chains of the lipids in monolayers before and after the addition of saponins to the subphase.

Composition and ν_{as} CH_2 (at 30 mN/m) of monolayers	Π after adding "SuperSap", mN/m	ν_{as} CH_2 after adding "SuperSap", cm^{-1}	Π after adding Digitonin, mN/m	ν_{as} CH_2 after adding Digitonin, cm^{-1}
DPPG ν_{as} CH_2 = 2919.5 cm^{-1}	33.7	2918.1	31.6	2918.7
CL ν_{as} CH_2 = 2925.0 cm^{-1}	33.2	2927.1	33.2	2927.2
DPPE ν_{as} CH_2 = 2918.3 cm^{-1}	40.4	2917.9	30.7	2917.9
E. coli extract Avanti ν_{as} CH_2 = 2924.1 cm^{-1}	33.6	2924.9	37.5	2925.1
E. coli reconstructed DPPE:DPPG:CL ν_{as} CH_2 = 2918.2 cm^{-1}	33.5	2917.9	29.3	2918.2

On the other hand, bacterial membranes are completely devoid of sterols, and so was our reconstructed bacterial cell outer membrane model. Consequently, practically no effect of the saponins on the reconstructed bacterial membrane model was observed, in contrast to the total lipid extract of *E. coli* cell membrane, where a relatively high increase in surface pressure was noticed (especially for digitonin). It is thus likely that some unspecified lipid components, e.g. hopanoids, present in the extract are responsible for the high affinity of the model to steroidal saponins.

The present results show that a steroidal saponin (digitonin) differs significantly from the mixture of triterpenoid saponins present in "SuperSap" in its effect on lipid monolayers. It should be noted that the aglycon structure is not the only difference between digitonin and "SuperSap" - the former being a monodesmoside (all sugars attached to the same carbon atom of the aglycon), while the latter - a mixture of mostly bidesmosidic saponins (two sugar chains attached on opposite sides of the aglycon). Digitonin increases surface pressure of its solutions to a less extent than "SuperSap" does, but the trend usually reverses in the presence of lipids on the surface.

Saponins are well known to form complexes with cholesterol, and the strength of this interaction is generally higher for steroidal saponins. In the present system, a huge increase in surface pressure was indeed observed for digitonin, significantly higher than for "SuperSap". In our previous paper, we have shown that upon penetrating the compressed mixed DPPC-cholesterol monolayers, digitonin can substitute the phospholipid, forming a highly ordered digitonin-cholesterol adduct. Consequently, DPPC can be released from cholesterol and its ordering increases (note that cholesterol orders liquid-like monolayers but disorders condensed phospholipid monolayers) [29]. A similar effect is probably responsible for the behavior observed for the present monolayers, as suggested by the shift of the asymmetrical CH_2 peaks to lower wavenumbers. On the other hand, the bidesmosidic "SuperSap" may probably have more problems to accommodate in the crowded mixed lipid, which combined with its lower affinity to cholesterol, might explain its lower effect on surface pressure and lipid ordering.

5. Conclusions

In the present study, the effect of two different saponin biosurfactants on Langmuir monolayers simulating the outer layers of the bacteria, erythrocyte and yeast was examined with help of surface pressure relaxation, fluorescence microscopy and Infrared Reflection Absorption Spectroscopy (IRRAS) measurements. The conclusions of the present study can be summarized as follows:

1) Both digitonin (steroidal saponin) and "SuperSap" (a mixture of triterpenoidal saponins from *Quillaja saponaria* Molina) affect the single phospholipid monolayers in a comparable way at low concentrations.
2) Digitonin is capable of significantly increasing surface pressure of monolayers containing cholesterol even at concentrations at which it is weakly surface active on its own. The effect of "SuperSap" is less pronounced, despite its higher surface activity.
3) The high sterol content in an erythrocyte cell membrane (47%) is probably responsible for their high affinity to saponins. The presence of phospholipids does not seem to affect these interactions, although their ordering is altered to different extents.
4) The monodesmosidic steroidal digitonin can easily bind to cholesterol, helping to release phospholipids from their adducts with the sterol. This increases surface pressure and ordering of the phospholipid acyl chains. In contrast, bidesmosidic triterpenoidal "SuperSap" with lower affinity to cholesterol and more bulky sugar chains shows much less increase of surface pressure and a rather weak effect on the acyl chain ordering.
5) Analogously to mammalian cells, the affinity of saponins to the yeast model monolayers seems to be dictated by the presence of sterols, e.g. ergosterol. Due to the lower sterol content as compared to e.g. erythrocytes, the respective yeast models (*Yeast reconstructed* and *Yeast extract Avanti*) monolayers are less affected.
6) Bacterial membranes do not possess any sterols, hence negligible response of our reconstructed bacterial model to both saponins has been observed. However, the total lipid extract from *E.coli* shows some sensitivity to saponins, probably related to unspecified sterol-like components, for example hopanoids.
7) At high surface pressures the saturated phospholipids forming well-ordered monolayers (LC) in the whole surface pressure range (DPPE, DPPS) do not interact strongly with the saponins, probably because of the hindered access to the monolayer components. This is in contrast to our previous observations with the uncompressed monolayers of the same phospholipids, where digitonin could easily bind and affect the ordering of the acyl chains (in some cases even inducing an unprecedented LC-LE phase transition with increasing surface pressure) [39].
8) The POPE and POPC phospholipids, as well as sphingomyelin (SM) may undergo slow relaxation that could be affected even by a flow of the subphase during the subphase exchange.

The picture provided by the IRRAS measurements described above complements our previous conclusions from the Langmuir monolayer relaxation, Grazing Incidence Angle X-ray Diffraction (GIXD) and Neutron Reflectivity (NR) studies [29]. Especially the latter technique may provide molecular details of interfacial layers on a molecular scale and has been proven recently very useful in solving similar problems [48]. Due to a high extent of hydration of saponin glycone parts, obtaining a more detailed picture is, however, difficult without isotopic substitution [49–51]. In the future we plan to investigate, using NR, the interaction of lipids in a monolayer with partially deuterated biosynthetic saponins from another saponin-rich plant - *Saponaria officinalis*.

CRediT authorship contribution statement

M. Orczyk: Investigation, Data curation, Conceptualization, Methodology, Writing - original draft. **K. Wojciechowski:** Conceptualization, Writing - review & editing. **G. Brezesinski:** Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2019.12.014.

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