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Physicochemical and electrical properties of DPPC bilayer membranes in the presence of oleanolic or asiatic acid

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This study aimed to investigate the effect of selected compounds from the group of triterpene sapogenins on model phosphatidylcholine membranes. Two types of biological membrane model systems were used in the work, i.e., liposomes (microelectrophoresis method) and spherical bilayers (interfacial tension method). Each model was modified with the tested sapogenin compounds, and the change in their physicochemical and electrical parameters was analyzed. Parameters characterizing the equilibrium in the membrane of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)-oleanolic acid (OA) and DPPC-asiatic acid (AA) were determined). Based on the Young-Laplace equation, the interfacial tensions of spherical lipid bilayers were measured. The formation of 1:1 complexes was assumed in the DPPC-OA and DPPC-AA membrane systems, and the parameters characterizing the interactions in the formed complexes were calculated. Microelectrophoresis was used to study the surface charge density of lipid membranes. These values were obtained from electrophoretic mobility data using Smoluchowsky's equation. The influence of pH on the electrolyte solution and the composition of the membranes was investigated. The results indicate that modifying DPPC membranes with selected triterpene sapogenins, both OA and AA, causes changes in the surface charge density and shifts of the isoelectric point. Data presented in this work, obtained through mathematical derivation and confirmed experimentally, are of great importance for interpreting phenomena occurring in lipid membranes. A quantitative description of equilibria between phosphatidylcholine and sapogenins lets us understand the processes on the membrane surface. The equilibria are particularly significant from the standpoint of cell functioning. Phosphatidylcholine-sapogenin interactions modulate a range of physicochemical properties of membranes, and they are important in the course of the multiple processes involving membranes in the living cell (e.g., transport mechanism).

Keywords Interfacial tension, Microelectrophoresis, Lipid bilayer, Liposomes, Triterpene sapogenins, Oleanolic acid, Asiatic acid, DPPC, Complex formation equilibria

A cell membrane, the plasma membrane, surrounds each living cell. This biological membrane mainly consists of a double layer of amphiphilic phospholipids, which is why it is called a phospholipid bilayer[1]. In addition to one of the membrane's most essential functions, isolating the cell from the outside world, the transport of substances in and out of the cell is also fundamental. The membrane enables the delivery of substances, e.g., drugs, to the cell, and by creating surrogate models of the membrane, detailed control of the parameters responsible for proper transport is possible. The membrane is semi-permeable and selectively permeable because, to a limited extent, it can freely pass a substance (molecule or ion) or prevent it from entering the cell. The transfer of substances through the membrane takes place thanks to the porosomes, which it contains, and thanks to the various protein molecules, channels, and pumps that transport substances to and from the cell. Cell surface membranes also contain sugars and receptor proteins that enable cells to detect external signaling molecules such as hormones[2,3]. Their study is complicated because natural plasma membranes contain a complex, heterogeneous protein-lipid structure. To study this highly complex membrane environment, modeling of native membranes has been developed over the years. The most common systems include lipid monolayers, vesicles, and supported lipid

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bilayers (SLBs)[4]. As mentioned earlier, membranes are mainly composed of phospholipids, the representative of which is phosphatidylcholine, commonly found in all animal cell membranes[5]. It is a lipid with a neutral charge, into which other compounds can be easily incorporated and stable connections can be created. Due to these features, phosphatidylcholine is one of the most frequently chosen materials for making model cell membranes.

The aim of this study was to investigate the effect of selected compounds from the triterpene sapogenins group on bilayer phosphatidylcholine membrane models. Saponins, as well as their aglycone parts, have a number of biological and physicochemical properties. A very important feature of this group of compounds is its medicinal properties, especially anticancer properties. There are many reports in the literature on the effect of saponins on cells, e.g., cancer cells, but less attention is paid to sapogenins themselves. Our research therefore focuses on understanding the effect of these compounds on physicochemical parameters describing model bilayers.

Saponins are a diverse group of secondary metabolites that occur mainly in plants. However, their presence has also been detected in marine echinoderms, sponges, soft corals, and small fish[6,7]. One of the groups of these compounds is the triterpene saponins. They are usually divided into two classes, pentacyclic and tetracyclic, each including many aglycone types. Triterpene saponins have an aglycone composed of 4–5 connected carbon rings. The most common, however, are those with a pentacyclic system with 30 carbon atoms, i.e., aglycones of the type of oleanolic acid ((β-amarin), arsenic acid ((α-amarin), and lupine, as well as compounds with a four-ring structure, e.g., dammaran. Conjugated glycosyl groups include glucose, rhamnose, galactose, arabinose, xylose, and ribose. Sugar chains can be linear or branched and consist of a maximum of eleven monosaccharide units. Although in the case of triterpene monodesmosides, the most common binding site is the ether bond at C-3 (less often at C-23 or the ester bond at C-28), in bidesmosides, the sugar chain attachment sites are usually C-3 + C-28 or C-3 + C-30. Most of these saponins are bidesmosides. Monodesmoside saponins are less common[8–10]. Saponins are very popular due to their extraordinary biological and pharmacological properties. The bioactivity of saponin mixtures or individual saponins in vitro and in vivo includes cytotoxic, immunomodulatory, hepatoprotective, anti-osteoporotic, antiviral, antifungal, and anthelmintic effects. Clinical studies suggest that saponins lower blood lipids, reduce cancer risk, and lower blood glucose levels. A diet rich in saponins can inhibit dental caries and platelet aggregation, treat hypercalciuria in humans, and act as an antidote to acute lead poisoning[11,12].

The literature also indicates the potential anticancer effect of saponins through their cytotoxic, cytostatic, pro-apoptotic, and anti-invasive effects. At high concentrations (above 100 μM), saponins exert cytotoxic and hemolytic effects through the permeability of cell membranes. In the presence of low concentrations of saponins, inhibition of cancer cell proliferation, induction of apoptosis, and reduced cell invasiveness are observed. Saponins may also influence the expression of genes associated with malignancy. These changes are directly related to the invasive phenotype of cancer cells. There are also reports that some saponins also have anti-inflammatory and cytoprotective effects on healthy cells. It is worth noting that triterpene saponins show selectivity of action on cancer and normal cells of the body and are also highly effective in inhibiting carcinogenesis[13–15].

Sapogenins are divided into triterpene and steroidal, as in the case of saponins, and it is the aglycone that determines whether a saponin belongs to a given group of compounds. In this study, two compounds from the group of triterpene sapogenins were used for research: oleanolic acid (Fig. 1a) and Asian acid (Fig. 1b).

Oleanolic acid (OA, oleic acid, (3β)-hydroxy-olean-12-en-28-oic acid) is an organic compound, a pentacyclic triterpene of the β-Amarin type. It consists of five six-carbon aromatic rings (Fig. 1a). In its structure, this compound has seven methyl groups, two of which are attached at the C4 and C20 positions[16]. OA is found in large amounts in plants from the *Oleaceae* family, such as *Olea europaea*. Olive leaves are particularly rich in OA, constituting about 3.5% of the dry weight of this raw material. This acid can also be obtained from garden thyme, cloves, apple trees, loquats, grapes, or elderberries. Its presence has also been demonstrated in waxes found on the epidermis of various leaves and fruits[16–18]. This compound interferes with several stages of development of various types of cancer. It has been indicated as an effective ingredient in pharmaceutical preparations for treating non-lymphocytic leukemia or skin cancer treatment. OA also has cytotoxic effects in many cancer cell lines, such as oral, esophageal, liver, colorectal, ovarian, breast, and lung cancers[18,19].

Asiatic acid (2α, 23-dihydroxy ursolic acid, AA) is an organic chemical compound classified as pentacyclic triterpenes. However, unlike OA, AA has an α-amarin-type skeleton, an ursolic acid derivative. Structurally, AA has three hydroxyl groups at the C-2, C-3, and C-23 positions, an olefinic group at the C-12 position, and a carboxylic acid group at the C-28 position (Fig. 1b). Like other compounds of this type, AA is poorly soluble in water, but has good solubility in physiological saline solution and organic solvents. This sapogenin is the aglycone

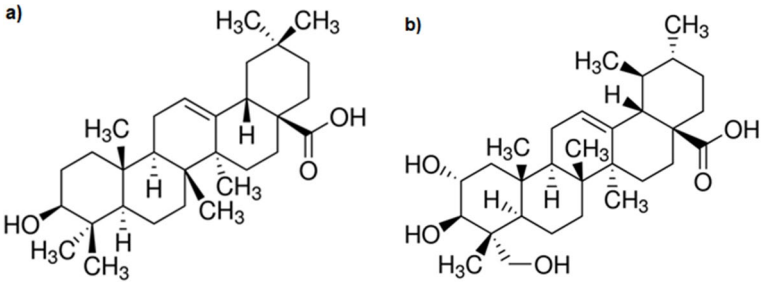

Fig. 1. Structures of oleanolic acid (**a**) and asiatic acid (**b**). Olives and olive trees are the main sources of OA. However, AA is found in high content in *Centella asiatica*, which is called *Gotu Kola*.

form of asiaticoside and is readily formed by hydrolysis of the sugar moiety of the asiaticoside structure under acidic conditions[20,21]. AA has been found in over fifty plant species, and its content is highest in leaves, flowers, and above-ground parts, i.e., bark or stem, as well as in roots and rhizomes. This substance is the main bioactive component of a perennial herb called *Centella asiatica*, or *Gotu Kola*. Centella asiatica is a frost-resistant perennial plant from the *Apiaceae* family of flowering plants, originating from Southeast Asian countries. In traditional Asian medicine, it has been used for thousands of years to treat a variety of diseases, as evidenced by reports of its presence in the list of "Miraculous elixirs of life" medicines and the "Sushruta Samhita," historical medicine texts from traditional Chinese and Indian medicine, respectively, dating back over 2,000 years ago. It is an herb used in traditional medicine from Sri Lanka, Java, and other Indonesian islands. This plant has been recommended for treating various skin diseases, such as leprosy, lupus, varicose ulcers, eczema, and . AA has several biological and pharmacological activities. It has been found to increase collagen synthesis, which is important in wound healing. Moreover, this compound has particular anticancer, anti-inflammatory, and antidiabetic properties, has antioxidant, hepatoprotective, and neuroprotective properties, and prevents hepatitis C virus[20–22].

The research included in this work continues previous research on the interaction of model membranes composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine with sapogenins. This work aims to analyze the impact of these sapogenins on the interfacial tension and the surface charge density of the model cell membranes (spherical bilayer lipid membranes (a) and liposomes (b), Fig. 2). We analyze the influence of the composition of DPPC-OA and DPPC-AA systems on the interfacial tension in the possible concentration range. We also analyze the impact of the pH of the electrolyte solution and the membrane composition on the surface charge of the model membrane to better describe the interactions in DPPC membranes after modification with sapogenins.

Since physicochemical and electrical parameters affect the interactions between membranes and biologically active compounds, the data presented below can help to understand the mechanisms of membrane binding and transport. Of particular importance for understanding membrane surface phenomena is their quantitative assessment, which is necessary for a complete interpretation. We achieve this by applying simple mathematical relationships for theoretical models related to the quantitative description of phenomena between membrane components and between them and the environment. This allows us to verify the experimental and theoretical data through theoretical descriptions of complex formation. We then use this data to quantitatively determine parameters describing equilibria, i.e., interface tension, surface charge density, stability constants, and complex formation energies. Notably, there are no precise data on stability constants for complex formation in the literature, so our group is the first to determine and present these parameters using various research methodologies.

Because physicochemical and electrical parameters influence the interactions between membranes and biologically active compounds, the data presented below may help understand the mechanisms of membrane binding and transport.

Results and discussion

Interfacial tension experiment and theoretical considerations

We have considered the case when the components of the lipid bilayer of a two-component membrane, e.g., DPPC (component 1) and triterpene sapogenin (component 2), do not form chemical compounds, where the interactions between the membrane components can then be described[23,24]:

$$\gamma_1 m_1 A_1 + \gamma_2 m_2 A_2 = \gamma \tag{1}$$

$$\frac{m_1}{m_1 + m_2} = x_1 \tag{2}$$

$$x_1 + x_2 = 1 \tag{3}$$

where A_1^{-1} and A_2^{-1} (mol·m^{-2}) are the surface concentrations of bilayer components 1 and 2, m_1 and m_2 (mol·m^{-2}) are the quantities of bilayer components 1 and 2 per unit area of the membrane, γ_1 and γ_2 (N·m^{-1}) are the interfacial tensions of membranes formed from pure components 1 and 2, γ (N·m^{-1}) is the measured interfacial tension of the bilayer membrane, and x_1 and x_2 are the solution mole fractions of bilayer components 1 and 2.

A linear equation is obtained by elimination of parameters m_1 and m_2:

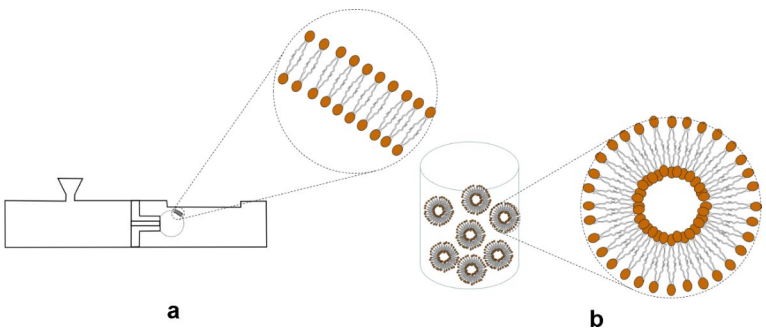

Fig. 2. Spherical lipid membrane (**a**) and liposome (**b**).

$$(\gamma - \gamma_1)\, x_1 = \frac{A_2}{A_1}\,(\gamma_2 - \gamma)\, x_2 \tag{4}$$

To the fact that the first stability constant in complexes, as the essential one, is usually the biggest and should be taken into consideration[25], existence of 1:1 complexes in phospholipid-sapogenin system was assumed. This assumption was verified experimentally using interfacial tension method (the interface tension values reported below refer to the one side of bilayer membrane surface area unit).

Then, the set of Eqs. (1–3) are modified because the interfacial tension is the sum of the contributions of all compounds and the equilibrium between the individual components that make up the complex can be written as follows[26]:

$$\underset{\text{(Component 1)}}{\text{A}} + \underset{\text{(Component 2)}}{\text{B}} \Leftrightarrow \underset{\text{(Complex)}}{\text{A}}$$

$$\gamma_1 m_1 A_1 + \gamma_2 m_2 A_2 + \gamma_3 m_3 A_3 = \gamma \tag{5}$$

$$K = \frac{m_3}{m_1 m_2} \tag{6}$$

$$\frac{m_1 + m_3}{m_1 + m_2 + 2m_3} = x_1 \tag{7}$$

$$x_1 + x_2 = 1 \tag{8}$$

where A_1^{-1}, A_2^{-1}, A_3^{-1} (mol·m^{-2}) are the surface concentrations of bilayer components 1, 2 and complex, m_1, m_2, m_3 (mol·m^{-2}) are the quantities of bilayer components 1, 2 and complex per unit area of the membrane, γ_1, γ_2, γ_3 (N·m^{-1}) are the interfacial tensions of membranes formed from pure components 1 and 2 and complex, γ (N·m^{-1}) is the measured interfacial tension of the bilayer membrane, K_1 is stability constant of the complex, and x_1, x_2 are the solution mole fractions of bilayer components 1 and 2.

Elimination of m_1, m_2, m_3 yields to basic equation describing the interaction between components 1 and 2 (formed the 1:1 complex) can be written as follows:

$$\begin{aligned}&[(\gamma - \gamma_1)\, B_2 x_1 + (\gamma - \gamma_2)\, B_1 x_2]\,[(\gamma_3 - \gamma_1)\, B_2 x_1 + (\gamma_3 - \gamma_2)\, B_1 x_2 + (\gamma_1 - \gamma_2)\,(x_1 - x_2)]\\ &= K A_3^{-1} B_1 B_2\,[(\gamma - \gamma_1)\,(x_2 - x_1) + (\gamma_3 - \gamma)\, B_1 x_2]\,[(\gamma - \gamma_2)\,(x_1 - x_2) + (\gamma_3 - \gamma)\, B_2 x_1]\end{aligned} \tag{9}$$

where $B_1 = \frac{A_3}{A_1}$ and $B_2 = \frac{A_3}{A_2}$.

Equation (9) is the equation of a second degree with respect to γ, to the complex composition as well as with respect to the constants: γ_1, γ_2, γ_3, B_1, B_2. Opening of the parentheses results in a great complexity of the equation, and is troublesome when directly applied to the determination of constants. The constants mentioned above can be determined in individual cases using simplified forms of this equation.

Equation (9) can be simplified, assuming that the stability constant of the resulting complex has a high value. The use of this simplification results in linear behavior for small ($x_2 < x_1$) and large ($x_2 > x_1$) x_2 values.

$$(\gamma_1 - \gamma)\,\frac{x_1 - x_2}{x_2} = -B_1\gamma_3 + B_1 \tag{10}$$

$$(\gamma_2 - \gamma)\,\frac{x_2 - x_1}{x_1} = -B_2\gamma_3 + B_2 \tag{11}$$

Equation (9) can be simplified in some other way. In the case where $x_1 = x_2$, it assumes the form (12) can be determined the stability constant for the complex:

$$\begin{aligned}K\left(A_1^{-1}\right)^2\left(A_2^{-1}\right)^2\left(A_3^{-1}\right)^{-1}(\gamma - \gamma_3)^2 = &\left[\gamma_2 A_1^{-1} + \gamma_1 A_2^{-1} - \gamma\left(A_1^{-1} + A_2^{-1}\right)\right]\left(\gamma_2 A_1^{-1} + \gamma_1 A_2^{-1}\right)\\ &- \left[\gamma_2 A_1^{-1} + \gamma_1 A_2^{-1} - \gamma\left(A_1^{-1} + A_2^{-1}\right)\right]\left(A_1^{-1} + A_2^{-1}\right)\gamma_3\end{aligned} \tag{12}$$

The parameters describing the complex determined from Eqs. (9) and (12) could be applied to the calculation of theoretical points using Eqs. (13) presented below (the agreement between theoretical and experimental values means that the system is well described by the equations above):

$$\begin{aligned}&K A_1^{-1} A_2^{-1}\,(a_1 + a_2)\,(a_3 - a_1)\,\gamma^2 + \left[K A_1^{-1} A_2^{-1}\,(\gamma_1 a_1 - \gamma_3 a_3)\,(a_1 + a_2)\right.\\ &\left. - K A_1^{-1} A_2^{-1}\,(\gamma_2 a_1 + \gamma_3 a_2)\,(a_3 - a_1) + a_4 A_3^{-1}\,(a_3 + a_2)\right]\gamma\\ &+ K A_1^{-1} A_2^{-1} a_3 \gamma_3\,(\gamma_3 a_2 + \gamma_1 a_2) - K A_1^{-1} A_2^{-1} a_1 \gamma_1\,(a_1\gamma_2 + a_2\gamma_3) - a_4 A_3^{-1}\,(\gamma_2 a_3 + \gamma_1 a_2) = 0\end{aligned} \tag{13}$$

where:

$$a_1 = A_3^{-1}\,(x_2 - x_1)$$

$$a_2 = A_2^{-1} x_1$$

$$a_3 = A_1^{-1} x_2$$

$$a_4 = \left[A_3^{-1}(\gamma_1 - \gamma_2)(x_2 - x_1) + (\gamma_1 - \gamma_3)x_1 A_2^{-1} + (\gamma_2 - \gamma_3)x_2 A_1^{-1}\right]$$

In the case of the two-component systems, it was assumed that forming a 1:1 complex explains the deviation from the additivity rule. Theoretical curves were obtained using the parameters characterizing complexes, such as the stability constants, molecular areas, and interfacial tension values. The accuracy of the presented models was verified by comparison with experimental data.

The complex formation energy of the DPPC-OA and DPPC-AA systems can be determined from Eq. (14), knowing the stability constant of the obtained complexes and the physicochemical constants (the gas constant and the measurement temperature). Knowledge of this thermodynamic function provides information concerning the nature and type of bonding in the tested systems and groups taking part in the complex-forming reactions.

$$-\log K = \frac{\Delta G^0}{2.3RT} \tag{14}$$

where K ($m^2 \cdot mol^{-1}$) is the stability constant of the DPPC-OA or DPPC-AA complex, ΔG^0($J \cdot mol^{-1}$) is the DPPC-OA or DPPC-AA complex formation energy, R ($J \cdot mol^{-1} \cdot K^{-1}$) is the gas constant, and T (K) is the temperature in Kelvin.

Figure 3 shows graphs for two phosphatidylcholine-sapogenin systems: the DPPC-OA (Fig. 3a) and DPPC-AA (Fig. 3b). Without interactions between the membrane components, the functions described by Eq. (4) should give straight lines. The graphs show a lack of straightness, suggesting that complex structures are formed in the studied systems.

Since the thesis about no complex formation in the tested systems was incorrect, the formation of complexes in bilayers was assumed. According to literature data, complexes in a 1:1 ratio create the most durable structures with maximum durability[25]. Previous studies of monolayer and bilayer lipid models with sapogenins showed the formation of complexes with a stoichiometric ratio of 1:1[27–29]. These complexes were characterized by high

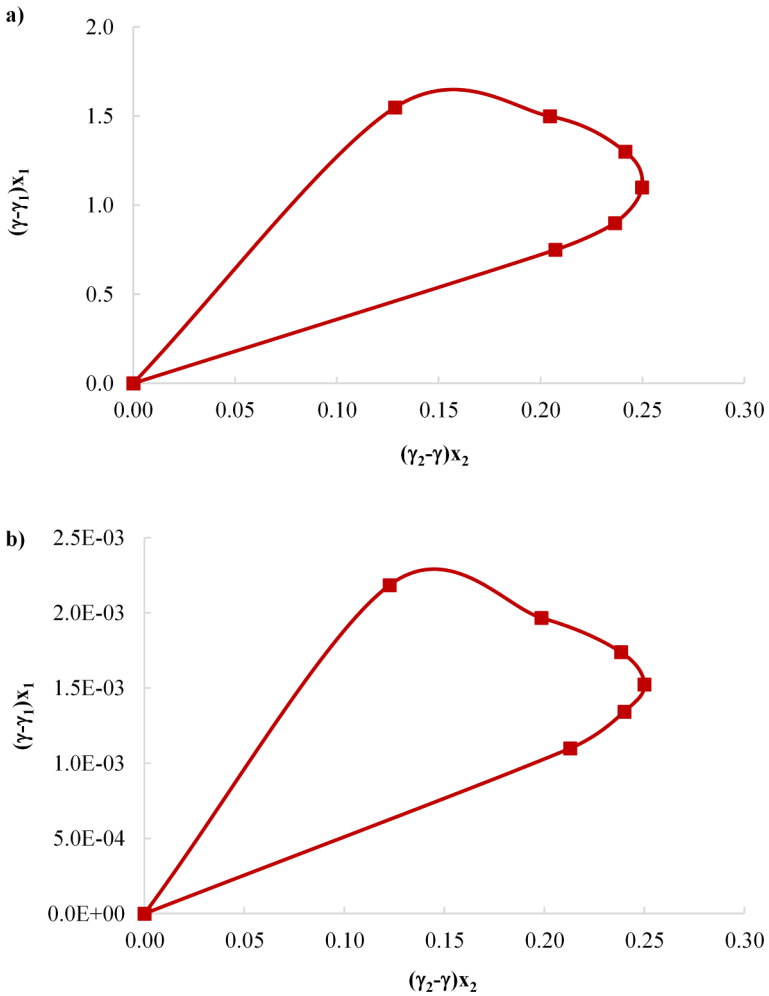

Fig. 3. Plot of Eq. (4) for DPPC-OA (**a**) and DPPC-AA (**b**), where x_1 and x_2 are the mole fractions of components 1 and 2 (DPPC and OA or AA, respectively), showing interfacial tension measurements.

stability constants of 10^5–10^6, indicating a stable connection of these compounds. Based on previous studies, complexes can also be formed in models of lipid bilayers modified with other sapogenins.

The dependence of bilayer interfacial tension vs. membrane composition for the DPPC-OA and DPPC-AA membrane (Fig. 4) was analyzed in the possible concentration range.

The interfacial tension of the DPPC-OA and DPPC-AA membrane was determined vs. the composition with 60% ($x_2 \sim 0.7$) of the sapogenine content because only with such contents of component 2 (OA/AA) with DPPC was a bilayer created. The determination of interfacial tension for bilayers composed of pure sapogenins was unsuccessful. The pure DPPC interfacial tension value (component 1), γ1, was determined directly and presented[26], equal to $1.62 \pm 0.12 \times 10^{-3}$ N·m^{-1}. Since OA and AA (component 2) do not create a bilayer, no accurate interfacial tension data exists in the literature. However, to describe the course of the experimental plot, the γ_2 value for the pure component is necessary. Therefore, the hypothetical interfacial tension data for the sapogenin bilayers were calculated by adjusting the experimental curve with the polynomial of the other mark, extrapolating the $x_2 = 1$ value (Fig. 4). These values were – 0.13 mN m^{-1} and 0.11 mN m^{-1}, respectively. The values of interfacial tensions for both compounds were very low or negative, proving that bilayers of the mentioned sapogenins are not formed under such conditions.

Phosphatidylcholine-sapogenins complex

As previously shown, interactions occur between DPPC and the tested sapogenins, indicating complex formation (Fig. 3). In previous publications, we demonstrated the formation of a 1:1 complex in DPPC-steroid sapogenin in single-layer[27–29] and bilayer[30] systems with high stability constant values. Due to this, we assumed that this type of complex may also be formed by components of lipid bilayers containing the tested triterpene sapogenins.

The graphs of Eqs. (10) and (11) are shown in Fig. 5a. The values B_1 (3.026) and B_2 (1.393) were obtained from the slopeslines' of the lines. The intersections of the straight lines with the ordinate give $-B_1\gamma_3$ and $-B_2\gamma_3$, which can be used to determine γ_3, the interfacial tension of the DPPC-OA complex. The average value obtained in this way was 1.14×10^{-3} N·m^{-1}. Determination of the interfacial tension as a function of composition enabled the calculation of surface concentrations for bilayers formed from DPPC and OA/AA. At least one of these

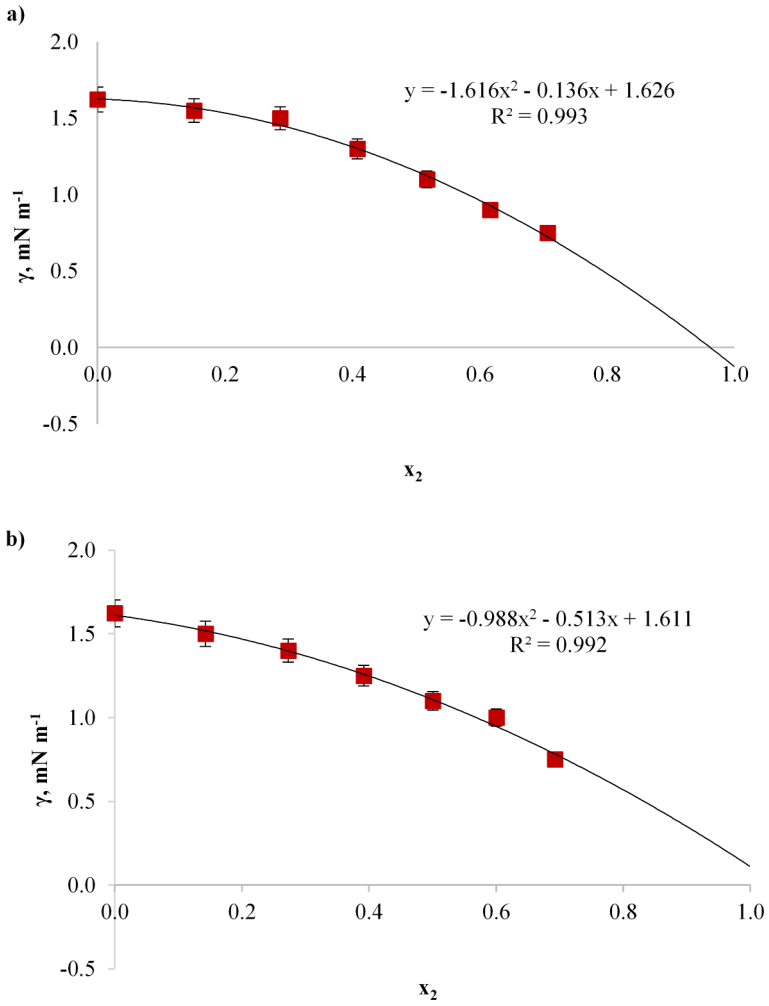

Fig. 4. DPPC-OA (**a**), and DPPC-AA (**b**) interfacial tension values vs. mole fraction x_2 (component 2); the theoretical values are marked by curves and the experimental values by points.

calculations is necessary to determine the value of A_3^{-1}. The surface areas occupied by DPPC, OA, and AA are 46 ± 0.5, 54 ± 0.5, and 56 ± 0.6 Å^2·molecule^{-1}[28], respectively. Based on this, we can determine that the area occupied by one DPPC-OA complex, A_3^{-1}, was approximately 107 Å^2·molecule^{-1} (1.55×10^{-6} mol·m^{-2}). The value is larger than the sum of the areas occupied by individual DPPC and OA molecules.

In the case of the DPPC-AA system (Fig. 5b), the obtained values for Eqs. (10) and (11) were 3.052 and 1.359, respectively, but the obtained value for interfacial tension for the mentioned system was 1.34×10^{-3} N·m^{-1}. The area occupied by a single DPPC-AA molecule complex was approximately 110 Å^2·molecule^{-1} (1.83×10^{-6} mol·m^{-2}), and the value of the area is lower than the sum of the areas occupied by single DPPC and AA molecules (112 Å^2·molecule^{-1}).

Based on the fact that the obtained value is relatively high, it can be concluded that a 1:1 complex was formed in the DPPC-sapogenin mixed bilayers. The K values obtained for DPPC-AA and DPPC-OA monolayers (1.03×10^6 m^2 mol^{-1} and 5.97×10^5 m^2 mol^{-1}, respectively) are comparable to the K values for bilayers[29].

The stability constant and formation energy of complexes

The stability constants of the formed DPPC–SAP complexes were calculated according to formula (12)[26], where it was assumed that a complex is formed in the systems with a stoichiometry of 1:1, i.e., the share of DPPC and SAP in the bilayer is equal ($x_1 = x_2 = 0.5$). The Gibbs free energy of complexes was calculated from Eq. (14). For the mixture of phosphatidylcholine with oleanolic acid (DPPC–OA), the surface concentration of the complex was $1.55 \cdot 10^{-6}$ mol m^{-2} (surface area 107 Å^2 molecule^{-1}), the stability constant of the resulting complex $K_{PC\text{-}OA} = 9.40 \cdot 10^5$ m^2 mol^{-1}. In the case of the phosphatidylcholine – asiatic acid system (DPPC–OA), the surface concentration of the complex was $1.50 \cdot 10^{-6}$ mol m^{-2} (surface area 110 Å^2 molecule^{-1}), the stability constant of the resulting $K_{DPPC\text{-}AA}$ complex = 7.42 10^5 m^2 mol^{-1}.

The energy values of complex formation for the DPPC-OA and DPPC-AA complexes were equal to −34.04 ± 1.05 and −33.45 ± 0.91 kJ·mol^{-1}, respectively. Table 1 presents some physicochemical values for DPPC-OA and DPPC-AA complexes.

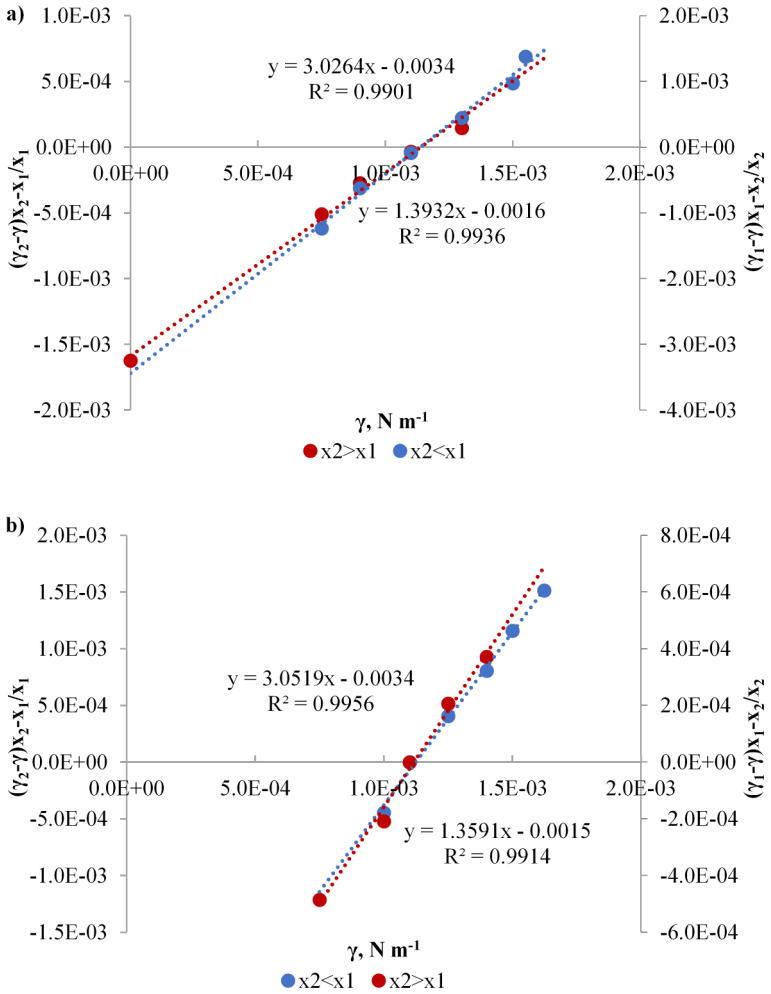

Fig. 5. Graph showing Eqs. (10) and (11) for determining B_1, B_2, and γ: parameters: (**a**) the DPPC-OA system and (**b**) the DPPC-AA system.

Examined system	Surface area occupied by one molecule of complex [Å^2 molecule^{-1}]	Stability constant of studied complex [m^2 mol^{-1}]	Complex formation energy (Gibbs free energy) [kJ mol^{-1}]
DPPC–OA	107.0 ± 1.1	9.40×10^5	-34.04 ± 1.05
DPPC–AA	110.0 ± 1.1	7.42×10^5	- 33.45 ± 0.91

Table 1. Some physicochemical values for the obtained complexes.

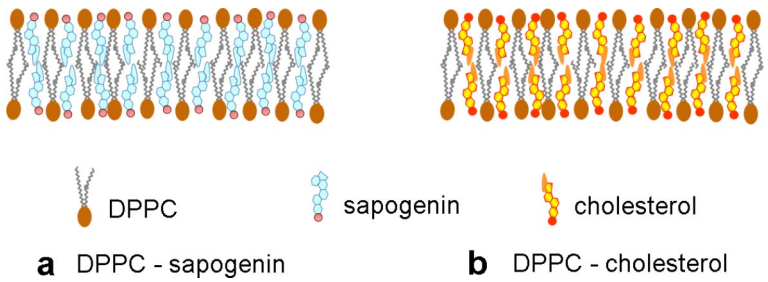

Fig. 6. Schematic models of the lipid bilayer structure of DPPC-sapogenin (**a**) and DPPC-cholesterol (**b**) complexes.

During our investigations, we assumed the formation of a DPPC–sapogenin complex in the monolayer[29] and bilayer (this paper). These complexes arise by producing a connection between the $-\overset{(+)}{N}(CH_3)_3$ group from the molecule of the phosphatidylcholine and $-COO^{(-)}$ groups of sapogenin. The dissociation constants of the $-\overset{(+)}{N}(CH_3)_3$ group from the PC and $-COO^{(-)}$ groups of the sapogenin are equal to $10^{-5.7}$[31] and about 10^{-5}[32], respectively.

The experimentally obtained values for the area occupied by the DPPC–sapogenin complex for the mixed monolayer are 88.8 ± 0.9 Å^2 molecule^{-1} for DPPC-OA and 77.9 ± 0.8 Å^2 molecule^{-1} for DPPC-AA, and the value obtained for the area occupied by the DPPC-OA and DPPC-AA, complex in the bilayer are 107 ± 1.1 and 110 ± 1.1 Å^2 molecule^{-1}, respectively. This difference is likely connected to the arrangement of DPPC molecules in this complex and is connected to the structural construction of such complexes in the bilayers. In[33], we suggested an arrangement of DPPC molecules in the bilayer membrane at pH > 5. In these media, one particle from the DPPC molecules in the bilayer (orientated in this way) has two straightened chains; however, the next molecule of DPPC has one straightened and another chain fastened to the membrane surface. An association of ions occurs under such conditions with OH- from the electrolyte solution. These ions were previously characterized in[33]. These ions are strongly solvated and produce a separation of phosphatidylcholine particles in the bilayer, which increases the surface occupied by a single DPPC molecule.

Schematic model of the spherical lipid bilayer structure of DPPC-sapogenin and DPPC-cholesterol are presented in Fig. 6. The presence of both sapogenin and cholesterol in the bilayer does not modify the orientation of the lipid heads because the molecules are parallel with the DPPC chains and their hydroxyl groups are oriented at the level of the lipid's carbonyl groups (Fig. 6). The stability constant values obtained for DPPC-sapogenin 1:1 complex have a similar order of magnitude (~ 10^5–10^6) compared to DPPC-cholesterol 1:1 complex[26,34].

Microelectrophoretic experiments

The effect of the triterpene sapogenins on the surface charge density and zeta potential of DPPC membranes was studied. Both one- and two-component liposomes were formed. The surface charge densities and zeta potential values of DPPC-OA and DPPC-AA systems were drawn vs. pH (ranging from 2 to 11) and the molar composition of the components in the membranes (Figs. 7a and b, respectively).

Figures 7a and b show the effect of liposome composition and pH value on the surface charge density and zeta potential values of the DPPC-OA and DPPC-AA bilayers, respectively. Additionally, the figures show data obtained for pure DPPC membranes. Detailed data are summarized in Tables 2 and 3. In the DPPC-OA system, with the increase in the OA content in the DPPC membrane, the isoelectric point shifts towards lower pH values (from pH = 3.70 for the DPPC membrane to pH = 3.03 for the DPPC–OA 1:3). Moreover, the presence of oleanolic acid in the lipid membrane results in a significant decrease in the surface density of the positive charge of the membrane at low pH values and an increase in the surface density of negative charge at high pH values.

In the case of the DPPC-AA system, it can be seen that as the AA content in the DPPC membrane increases, the isoelectric point shifts towards lower pH values (from pH = 3.70 for the DPPC membrane to pH = 2.50 for the DPPC-AA membrane 1:3). Moreover, similarly to the case of DPPC–OA, the presence of asiatic acid in the membrane also results in a significant decrease in the surface density of the positive charge of the membrane in the low pH range and an increase in the surface density of the negative charge at high pH values.

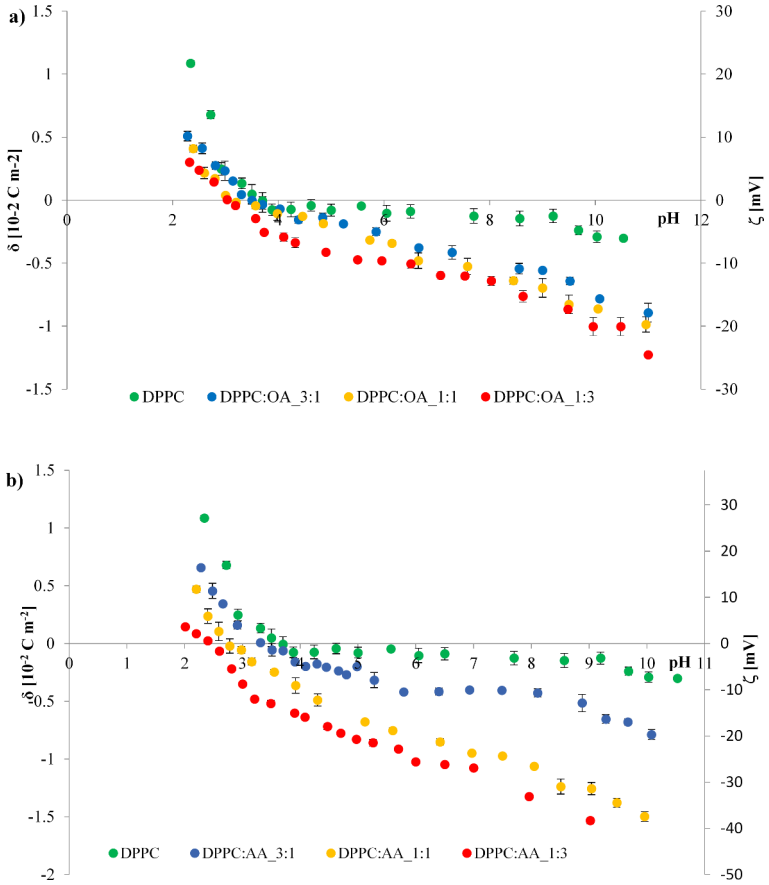

Fig. 7. Dependence of the DPPC:OA (**a**) and DPPC:AA (**b**) membranes surface charge densities and zeta potential vs. the pH of the electrolyte solution.

Examined system	Isoelectric point	Surface charge density δ [10^{-2} C m^{-2}]		Zeta potential ζ [mV]	
		pH values ~ 2	pH values ~ 10	pH values ~ 2	pH values ~ 10
DPPC	3.70	1.09 ± 0.03	-0.30 ± 0.02	22.0 ± 0.6	-6.0 ± 0.4
DPPC – OA (3:1)	3.57	0.51 ± 0.04	-0.89 ± 0.07	10.3 ± 0.5	-17.8 ± 1.4
DPPC – OA (1:1)	3.19	0.41 ± 0.03	-0.98 ± 0.06	8.3 ± 0.6	-19.6 ± 1.2
DPPC – OA (1:3)	3.03	0.30 ± 0.01	-1.23 ± 0.02	6.1 ± 0.2	-24.6 ± 0.4

Table 2. Membrane surface charge densities, zeta potential and isoelectric points values for the DPPC-OA system.

Examined system	Isoelectric point	Surface charge density δ [10^{-2} C m^{-2}]		Zeta potential ζ [mV]	
		pH values ~ 2	pH values ~ 10	pH values ~ 2	pH values ~ 10
DPPC	3.70	1.09 ± 0.03	-0.30 ± 0.02	22.0 ± 0.6	-6.0 ± 0.4
DPPC – AA (3:1)	3.31	0.66 ± 0.02	-0.79 ± 0.04	13.3 ± 0.4	-15.8 ± 0.8
DPPC – AA (1:1)	2.78	0.47 ± 0.03	-1.50 ± 0.04	9.5 ± 0.6	-30.0 ± 1.0

Table 3. Membrane surface charge densities, potential zeta and isoelectric points values for the DPPC-AA system.

The hydrodynamic nanoparticle size measurements were taken in triplicate, and determined values are reported along with the polydispersity index (PDI) in Table 4. All samples showed mean particle diameter sizes ranging from 250.20 ± 65.02 nm to 342.76 ± 85.02, with PDI ranging from 0.070 to 0.137. It was observed that, on the one hand, the hydrodynamic particle size was dependent on the liposome composition and had the highest value in the 1:1 ratio (DPPC and OA or AA). Furthermore, PDI showed a monodisperse, homogeneous particle distribution in all measured samples.

Using the microelectrophoresis method, the influence of selected triterpene sapogenins on the surface charge density of the phosphatidylcholine membrane was analyzed. Electrophoretic mobility measurements, based on of which the surface charge density was determined, were carried out as a function of the pH of the electrolyte solution.

After conducting the experiments, it was noticed that the presence of each of the tested sapogenins (oleanolic acid and asiatic acid) in the phosphatidylcholine membrane causes changes in the values of the analyzed electrical parameter in the entire pH range tested.

In the case of liposomal membranes containing triterpene sapogenins, a change in the position of the isoelectric point on the curve can be observed (Figs. 7a and b). With the increase in the content of both acids in the liposomal DPPC membrane, the isoelectric point was shifted towards lower pH values for both tested systems. The presence of individual acids in phosphatidylcholine membranes causes significant changes in the surface charge density of the DPPC membrane in the tested pH range.

Materials and methods

Materials

Membrane forming materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (semisynthetic, ≥ 99%), oleanolic acid (OA), and asiatic acid (AA), in purity ≥ 97%, were provided by Sigma-Aldrich (St. Louis, MO, USA) and used without extra purifications. The molecular weights of DPPC, OA, and AA were approximately determined to be 734.04, 488.70, and 456.70 g mol^{-1}, respectively.

Electrolyte solutions

The electrolyte solutions for interfacial tension measurements (0.1 M potassium chloride) and microelectrophoretic measurements (0.155 M sodium chloride) were prepared using ultrapure water (purified by Hydrolab HLP 5UV) at 20 °C.

Spherical bilayers preparation

The solutions spherical bilayers forming were prepared by dissolving the compounds in chloroform (anhydrous, ≥ 99%, Sigma-Aldrich, St. Louis, MO, USA) in the appropriate weight ratios, and then the solvent was evaporated under inert gas. Bilayer solutions (20 mg · cm^{-3}) were prepared by dissolving the test components (DPPC, OA, AA) in n-decane and butanol (anhydrous, ≥ 99%) in a ratio of 20:1. During the bilayer formation, the organic solvents were removed, and the membrane composition remained in the same proportions as in the stock solution. Besides measurements, the solutions were stored at 4 °C.

Liposomes preparation

Liposome-forming solutions (10 mg · cm^{-3}) were prepared by dissolving tested substances (DPPC, OA, AA) in chloroform (anhydrous, ≥ 99%). The formed solutions were mixed in various molar ratios (3: 1; 1: 1; 1: 3). The chloroform evaporated under an argon stream to give a dry residue. The obtained residue was combined with the electrolyte solution (0.155 M NaCl), thus obtaining solutions ready for the sonication process. Liposomes were prepared by direct sonication of a suspension (ultrasound generator UD 20, Techpan, Poland). This operation was repeated five times for 90 s. In this process, thermal energy is released; hence, the sonication reactions are carried out in an ice bath.

Interfacial tension measurements

The interfacial tension measurements were made using a technique based on Young's and Laplace's equation (Eq. 15)[35]. Using the following equation, the lipid bilayer's interfacial tension, γ, can be determined from

Examined system	Hydrodymanic size [nm]	PDI
DPPC	250.20 ± 65.02	0.070
DPPC – OA (3:1)	290.09 ± 75.42	0.115
DPPC – OA (1:1)	330.12 ± 85.82	0.137
DPPC – OA (1:3)	282.76 ± 73.52	0.123
DPPC – AA (3:1)	297.12 ± 77.22	0.118
DPPC – AA (1:1)	342.76 ± 85.02	0.129
DPPC – AA (1:3)	296.18 ± 77.08	0.121

Table 4. Liposomes hydrodynamic size and polydispersity values (PDI) for the DPPC, DPPC-AA, and DPPC-OA systems at pH = 7.4.

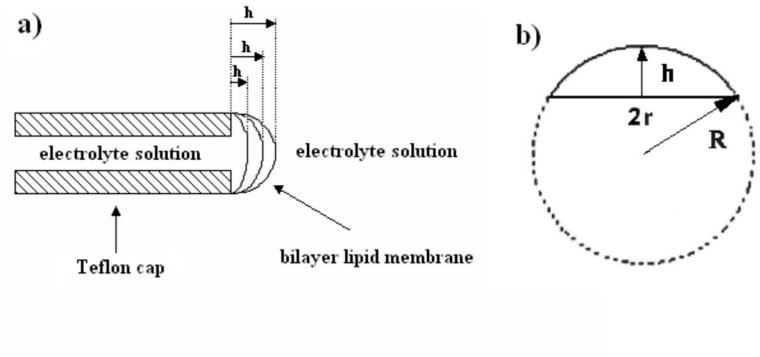

Fig. 8. The scheme presented the determination of the radius of curvature of the lipid bilayer during interfacial tension measurement, where R- radius of curvature; r – radius of the Teflon cap; h – convexity of the lipid membrane. The radius of curvature was calculated from the equation: $R = r^2 + h^2/2\,h$.

measurements of the radius of curvature, *R*, of the convex surface formed by the pressure difference *Δp* applied on both sides.

$$2\gamma = R\Delta p \tag{15}$$

The detailed structure and principle of operation of the measuring equipment have been described previously[23,26,36]. It consisted of a measuring vessel, a VEB manometer for applying and measuring the pressure difference on both sides of the membrane, and a computer program for determining the radius of curvature of spherical bilayers.

The main element of the apparatus was a glass measuring vessel divided into two chambers by a partition. The element in which spherical bilayers were produced by the Mueller-Rudin[26] method was a Teflon cap with a diameter of 1.5 mm embedded in the partition. Both chambers were filled with an electrolyte solution. A micropipette was used to introduce the membrane-forming solution to the flat wall of the Teflon element. To better visualize the forming spherical lipid bilayers, a CCD camera, and backlight were built in, which allowed for a more precise measurement of the radius of curvature of the lipid bilayer membrane.

The convexity of the bilayer was measured with an accuracy of 0.05 mm using the optical part of the measuring apparatus consisting of a CCD camera and a monitor. The growth stages of the bilayers were monitored using a microscope in reflected light with a high-brightness yellow LED source. The microscope and the LED were mounted on supports, which allowed the illuminator, measuring vessel and microscope to be placed on the optical axis. Then, the radii of curvature of the formed bilayers were determined using the obtained values of the convexity of the lipid bowls and the diameter of the Teflon element on which the membranes were formed, corresponding to the diameter of the lipid bowl. The radius of curvature (R) was obtained using this value and the diameter of the Teflon cap (r), corresponding to the diameter of the lipid bowl and the convexity of the bilayer, as shown in diagrams a) and b) presented in Fig. 8. The excess pressure (Δp) causing the convexity of the bilayer was determined using a manometer. Then, the interfacial tension was calculated according to the Young–Laplace equation (Eq. 15).

The interfacial tension of single-component and two-component bilayers was measured 12–15 times. Approximately ten readings of the lipid cap diameter were taken for each bilayer. These measurements were taken over the entire range of vesicle sizes, from very small lipid cap diameter values to almost equal to the radius of the Teflon cap. Arithmetic means, and standard deviations were calculated from all instrument readings. Measurements with the preparation of the electrolyte solution were performed 2–3 times to check the repeatability of these determinations. All presented experiments were performed at 20 ∘C.

Microelectrophoretic measurements

The electrophoretic mobility and zeta potential values of liposomes were obtained using the laser Doppler Micro-electrophoresis (LDE) technique with a high-performance Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) analyzer. All measurements were performed vs. pH using a WTW InoLab pH 720 laboratory meter (WTW, *Weinheim,* Germany). Liposomes suspended in the electrolyte solution (0.155 mol·dm^{-3} NaCl) were titrated to the given pH (range 2–10, every ± 0.3 units) with hydrochloric acid or sodium hydroxide. Six measurements were performed (each consisting of 100–200 runs, duration of 5 s) for each pH value for each sample. The experiments were performed three times. The membrane surface charge densities were calculated with Eq. 16 from the electrophoretic mobility data[37]:

$$\delta = \frac{\eta \cdot u}{d} \tag{16}$$

where *u* is the electrophoretic mobility, η is the viscosity of the solution, and *d* is the thickness of the diffuse layer.

Conclusions

The results presented in this work concern the physicochemical and electrical properties of models of phospholipid membranes modified with selected triterpene sapogenin. They indicating special interactions between the components of these membranes and between the components and the solution surrounding them.

The surface charge density of liposomes made of DPPC depends mainly on modifying their structure, in this case, on the tested triterpene sapogenins and the pH value of the electrolyte solution. A shift in isoelectric points was also observed. The excellent agreement between theoretical and experimental data confirmed the assumption of forming a 1:1 complex in the lipid bilayer during the interfacial tension measurements. The lack of discrepancy between theoretical and experimental points indicates that our theoretical model can sufficiently describe the interactions in DPPC-sapogenin bilayers. Moreover, the mathematically calculated and experimentally confirmed data collected here are important for understanding various phenomena in which lipid bilayers participate.

Literature reports extensively describe sapogenins' biological and pharmacological properties, including anticancer properties. Because the sapogenins selected for research in this study significantly influence the change in the electrical and physicochemical parameters of the tested model phosphatidylcholine membranes, it can be concluded that they may also significantly influence the physicochemical parameters of the membranes of diseased cells. The obtained research results confirm the formation of stable DPPC-SAP complexes and a significant change in the surface charge density of liposomes, allowing for far-reaching conclusions to be drawn about the possibility of using selected sapogenins as potential components of therapeutic agents. Because sapogenins significantly influence the balance of phosphatidylcholine in the tested model membranes and, at the same time, exhibit numerous pharmaceutical properties, i.e., antibacterial, anti-inflammatory, antifungal, and anticancer, it is reasonable to assume that they may also have a positive effect on the natural membranes of diseased cells. The research results also allow us to conclude about the possibility of using these sapogenins as potential components of medicinal products.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

Author Contributions: Conceptualization, A.D.P.; methodology, A.D.P, K.K.; formal analysis, K.K.; writing—original draft preparation, K.K, M.G., W.U., and A.D.P.; writing—review and editing, K.K, M.G., W.U., and A.D.P.; visualization, W.U., M.G; supervision, A.D.P. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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