Task XVIII

DETERMINATION OF π-A ISOTHERMS FOR LANGMUIR MONOLAYERS AT THE LIQUID-GAS INTERFACE

I. Aim of the experiment

The purpose of this task is to determine the dependence of surface pressure as a function of surface area per molecule at the constant temperature (π-A isotherm) for the Langmuir monolayers formed by the selected membrane lipids at the water-air interface.

II. Introduction

1. Membrane lipids - structure and classification.
2. Methods of obtaining model biological membranes.
3. Langmuir monolayers.
4. Determination of surface tension and pressure using the Wilhelmy plate method.
5. Determination of π-A isotherms.
6. Determination of the physical state of model biological membranes.

Bibliography:

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2. A. Chyla, *Warstwy Langmuira-Blodgett i ich zastosowanie w elektronice molekularnej*, Oficyna Wydawnicza Politechniki Wrocławskiej, Wrocław, 2004.
3. M. Bryszewska, W. Leyko, *Biofizyka dla biologów*, PWN Warszawa, 1997.
4. E. T. Dutkiewicz, *Fizykochemia powierzchni*, WNT Warszawa, 1998.
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III. Theory

III. 1. Biological membranes - Introduction

Biological membranes are structures that build the cells of all organisms: prokaryotes, eukaryotes, animals and plants. Despite the great diversity of organisms and structures in which they occur, their functions and structure are universal. Membranes limit cell compartments and cellular organelles, which allows for the control of their composition, regulate the transport of specific molecules between the intra- and extracellular environments as well as the cytoplasm and cellular compartments, responsible for selective permeability, participate in the spread of information through conformational changes of individual components, create optimal conditions for the action of ion pumps, receptors and selected enzymes. Most biochemical processes take place with the membranes participation.

In 1972, in order to illustrate the structure of a biological membrane, Singer and Nicolson proposed the fluid mosaic model (Fig. 1), which assumes that the membrane is a liquid mosaic of lipids in the form of a bimolecular layer in which integral proteins are embedded and peripheral (surface).

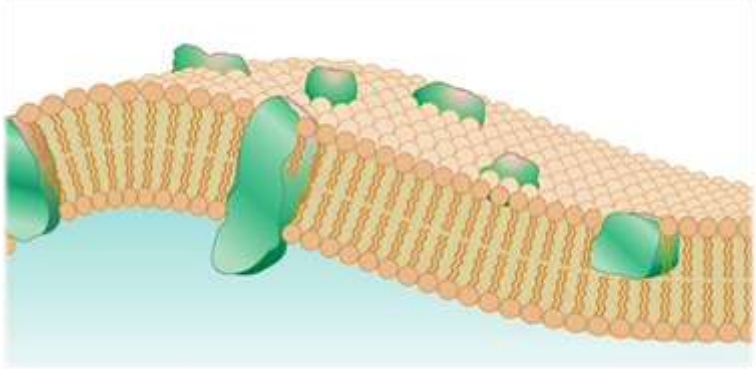
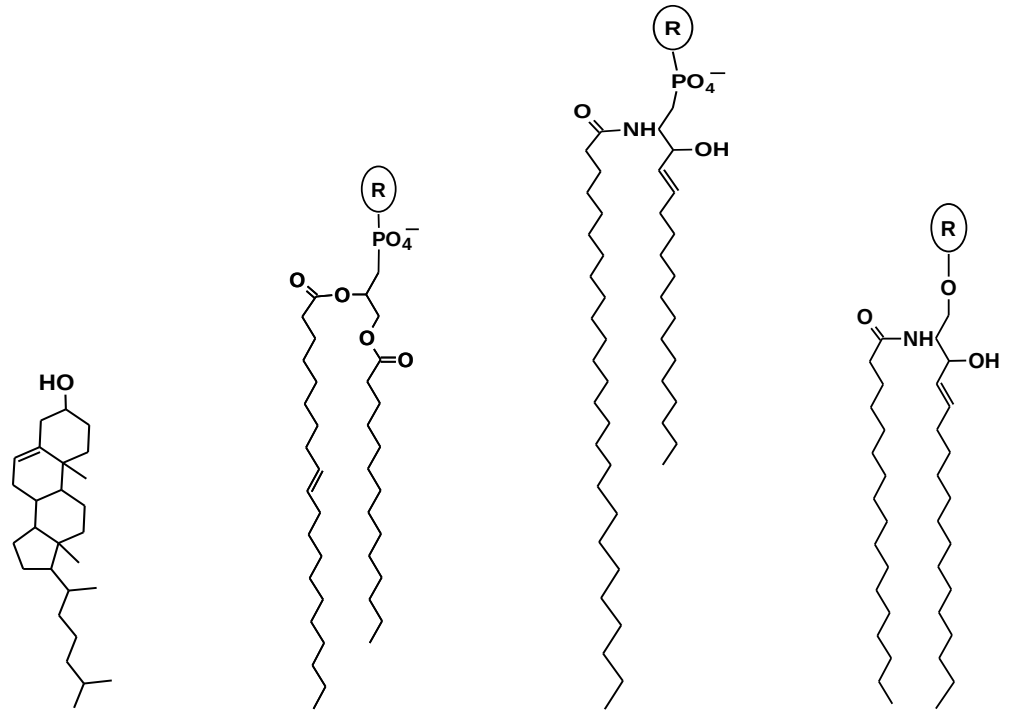


Fig. 1. Model of the fluid protein-lipid mosaic according to Singer and Nicolson.

A very important feature of the cell membrane is its fluidity (characterized by the inverse of viscosity), which reflects the mobility of lipids and the ordering of the acyl chains of fatty acids. It depends on the phase of membrane lipids, lipid composition and interactions between components. The fluidity of the membrane determines susceptibility to deformation, osmotic and mechanical stability, oxygen diffusion and the action of membrane receptors as well as determines the activity of membrane enzymes.

The main building blocks of membranes are lipids and proteins. The core of biological membranes is a lipid bilayer composed mainly of phospholipids (such as phosphatidylcholine, commonly known as lecithin, phosphatidylserine, phosphatidylethanolamine), glycolipids and sterols (Fig. 2).



cholesterol phospholipid sphingolipid glycolipid

Fig. 2. Lipid structures: cholesterol, phospholipid (R – nitrogen base: choline, serine or ethanolamine), sphingolipid (R – choline in sphingomyelin), glycolipid (R – saccharide residue: glucose, galactose).

Phospholipids, building cell membranes, play a significant role in the intercellular transport, in controlling the spread of information and in energy conversion processes. The proportion of individual lipids depends on the type of membrane. In addition, both monolayers belonging to the same membrane contain different types of lipids, the so-called asymmetry of the lipid composition of the membrane, which determines its specific functioning.

Membrane lipids (phospho- and glycolipids) are characterized by a clearly developed hydrophobic part, which consists of two hydrocarbon chains and a hydrophilic part (polar head). The remaining lipids do not show the ability to form stable liquid crystal bilayers in the aqueous environment and occur as admixtures of membrane lipids. A bilayer formed from a mixture of lipids has the ability of lateral phase separation and creation of domains (rafts) understood as areas of molecular order in which lipids of only one type predominate.

III. 2. Physicochemical properties of lipids

Lipids are biochemical compounds that are hardly soluble or insoluble in water, which results from the presence of a large number of $>CH_2$ groups in their molecules. However, they also have polar or ionic groups (amphiphilic molecules). Owing to their amphiphilicity, lipids, especially phospholipids, are surface-active compounds that, when adsorbed on the surface of water, reduce its surface tension. Due to the interactions with water, non-polar and polar lipids are distinguished. Non-polar lipids do not dissolve in water even to a minimal degree, and when applied to its surface, they form lenses - they do not dissolve, e.g. waxes. There are three classes of polar lipids:

– insoluble and non-swelling lipids in water, which form monomolecular layers on the surface of water, e.g. fats, long-chain fatty acids, alcohols, amines, cholesterol;

– insoluble lipids but swellable in water (incorporating certain amounts of water into liquid crystal structures), they spread on the surface of water forming monomolecular layers and in bulk solutions they form liquid crystals with lamellar or hexagonal structures, e.g. lecithins (phosphatidylcholines), the main components of membranes;

– lipids soluble in water, at low concentrations they form monomer solutions and after exceeding a certain concentration – micellar solutions, e.g. soaps, lysolecithin, detergents.

In the aqueous solutions, lipids show the ability to form a variety of aggregation structures (so-called lipid polymorphism) depending on the type of molecules, concentration, degree of hydration as well as temperature, pH and ionic strength of the external environment (Fig. 3). Following the concept of molecular shape, according to which lipid molecules can be assigned different shapes based on the ratio of the cross-sectional area of the polar head to the cross-section of hydrophobic hydrocarbon chains, phosphatidylcholine (PC) assumes the shape of a cylinder, and its preferred aggregation form is a planar bilayer.

On the other hand, lysophospholipids (LPC) with the inverted cone structure organize into micelles and phosphatidylethanolamine (PE) with the cone-shaped molecules forms reverse micelles (Fig. 3). Temperature has a great influence on the physicochemical properties of lipid bilayers. Most lipids have the ability to exist in the gel or liquid crystal phase (Fig. 3). Biological membranes are believed to exist in the liquid crystalline phase at physiological temperatures for a given type of cells.

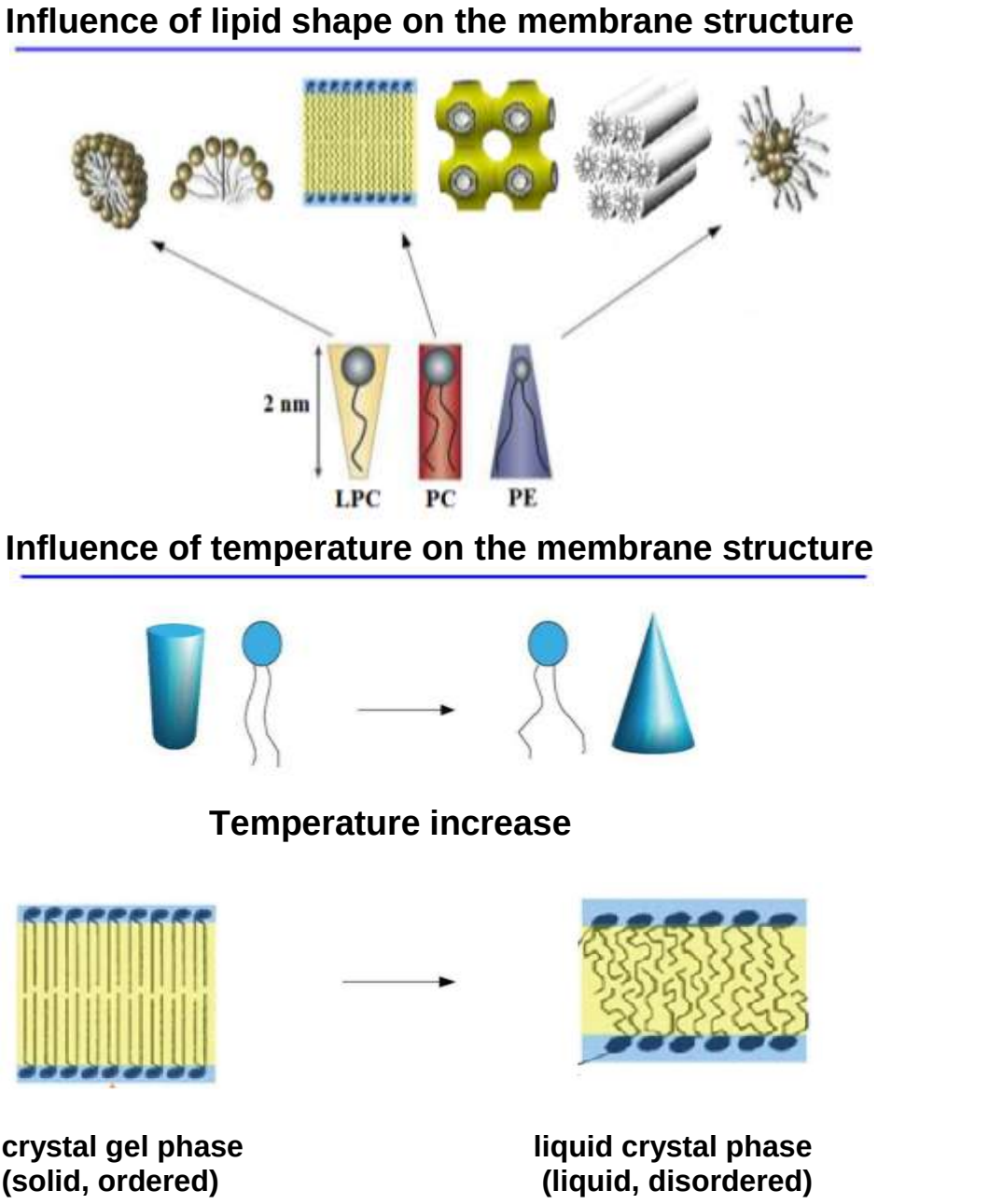


Fig. 3. Influence of the shape of lipid molecules and temperature on the structure of the membrane.

Moreover, in some lipids (phosphatidylcholines), as a result of the melting of acyl chains, one phase transition (folded phase) occurs in the case of dipalmitoylphosphatidylcholine (DPPC) at a temperature of approx. 41^{o}C. Figure 4 summarizes the gel-liquid crystal transition temperatures for selected phosphatidylcholines along with the structural formulae of the compounds.

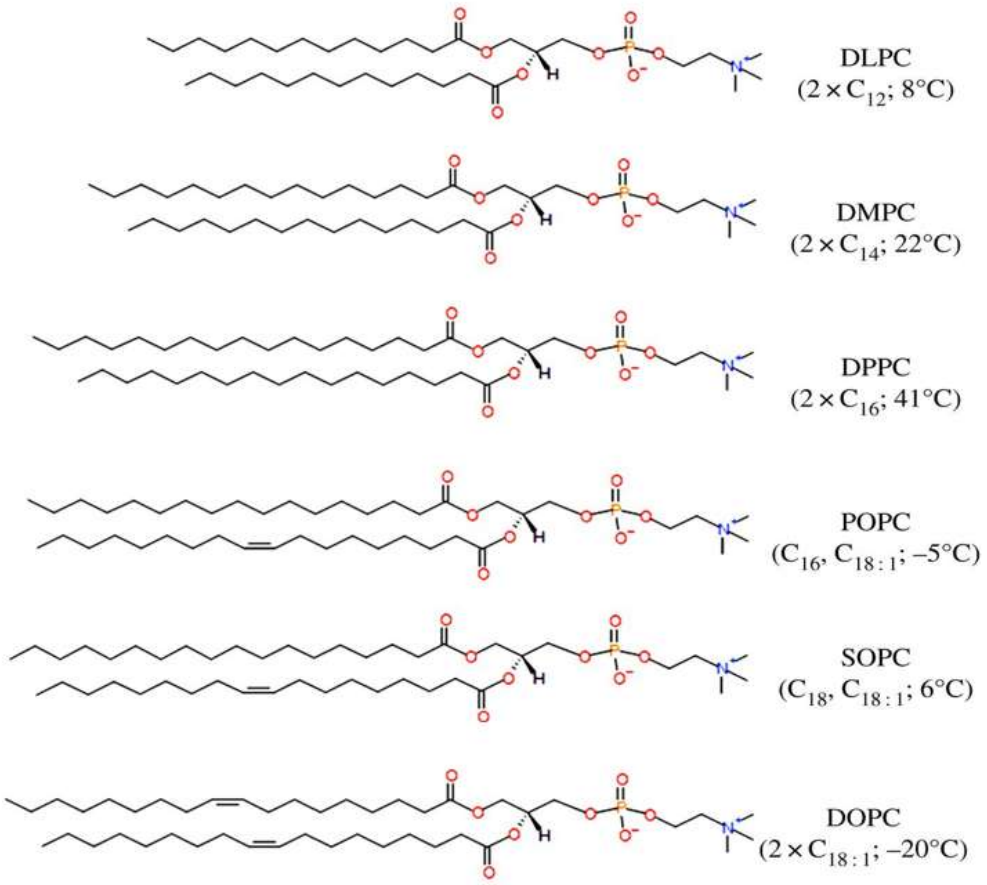


Fig. 4. Structural formulae and melting points of the gel-liquid crystal for phosphatidylcholines (PC) differing in the length of hydrocarbon chains and the type of bonds, where: O – the oleic acid chain, L – the lauric acid chain, M – the myristic acid chain, P – the palmitic acid chain, S – the stearic acid chain.

III. 3. Model systems of biological membranes

Of all the lipids, phospholipids are characterized by the best amphiphilic properties. Being a universal element of biological membranes, they also become the most frequently used lipids in the study of model membranes that mimic biological membranes.

III. 3.1. Monomolecular surface layers

The amphiphilicity of lipids, which are the main components of biological membranes, makes it possible to study their properties at interfaces. The surface Langmuir layers formed at the water-air interface are a particularly frequently used system. The example of the use of monomolecular surface layers are the studies on the mechanism of action of antibiotics, e.g. filipin, nystatin, amphotericin B, which show significant toxicity to fungal cells and red blood cells, but do not act on bacterial cells; research on the effects of anti-cancer drugs; research to elucidate the role of pulmonary surfactant in the alveoli; development of the mecha-

nism of enzymatic hydrolysis in lipid monolayers. The method of single-molecular membranes formed from ordered and oriented molecules can be helpful in comparing the results of the studies carried out on a given substance in the bulk solution, particularly in terms of the influence of the molecules orientation towards their activity or optical properties.

III. 3.2. Formation of Langmuir monolayers

The Langmuir monolayer is a two-dimensional film with a thickness of one molecule, formed on the surface of a liquid, usually water, called a subphase or subpase by insoluble or slightly soluble compounds in it. The thermodynamic state and quality of the Langmuir monolayer are determined by the compression isotherm, presenting the relationship between the surface pressure π and the surface area of the subpase per single molecule in the monolayer (A). Isotherms are determined using the Langmuir or Langmuir-Blodgett trough, the diagram of which is shown in Figure 5.

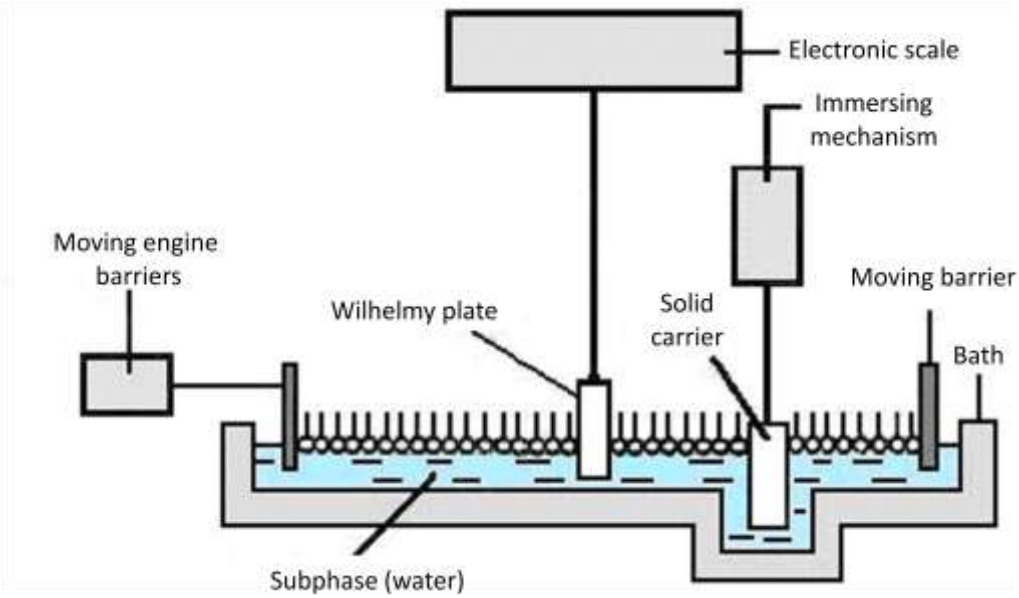


Fig. 5. Diagram of the Langmuir-Blodgett trough containing the Wilhelmy plate for measuring surface pressure using a balance and a surface tension probe.

In the classic Langmuir monolayer experiment, a known amount of an amphiphilic substance dissolved in a water-immiscible volatile organic solvent is applied by means of a microsyringe to the surface of the subphase filling the Teflon, thermostatic trough. After evaporation of the solvent, the resulting layer is compressed owing to the movement of the barriers towards the trough center. The barriers are controlled by a motor and move at a controlled speed. At the same time, a computer connected to the trough registers a compression isotherm showing the dependence of surface pressure as a function of surface area per one molecule in the monolayer.

The measurement of surface pressure is most often performed using the Wilhelmy method, in which a well-wettable plate made of platinum, mica, quartz or filter paper is placed perpendicularly to the surface of the subpase. The plate is hung on a hook connected by a wire to the device that measures a force equivalent to the surface tension. The repeatability of isotherms depends strictly on the selection of appropriate experimental conditions, e.g. temperature, pH of the subpase, compression rate, and reagent purity.

III. 3.3. $\pi - A$ isotherms

Monomolecular layers on the water surface can be in different phases, in a sense analogous to the states of aggregation of three-dimensional phases: gaseous (G), liquid (L) and solid (S). There were also discovered three additional states: the liquid-expanded (LE), the intermediate state (I) between the expanded liquid and the liquid phases characterized by smaller compressibility than the liquid, and the superfluid phase (LS) intermediate between the state of the liquid-condensed (LC) and the solid (S) (Fig. 6).

Each phase of the monolayer is strictly defined by the dependence of the surface pressure on the mean molecular area and the specific arrangement of the molecules. With very large specific areas (A>4nm^2/molecule), the monolayer behaves like a two-dimensional gas, the molecules of the compound forming it are located at considerable distances from each other, practically not interacting with each other and the hydrocarbon chains are usually parallel oriented towards the water surface (Fig. 6a). The isotherm is horizontal with the changes in the surface pressure smaller than 0.5 mN/m. Reducing the surface area per molecule as a result of the monolayer compression leads to tighter packing of molecules on the surface of the subphase and transition of the layer to the two-dimensional liquid-expanded state (LE). Then the intermolecular distances decrease and the hydrophobic hydrocarbon chains start to "stand up" from the water surface (Fig. 6 b). However, the molecules retain a great deal of freedom of movement. The surface pressure increases and there is a sharp change in the slope of the isotherm, indicating a phase transition from the gaseous state into the liquid-expanded (LE).

In this phase both the conformational transformations of molecules and the formation of domain structures occur. Further compression of the monolayer by the trough barriers causes another phase transition from the liquid-expanded phase (LE) to the liquid-condensed phase (LC), where all molecules are equally oriented and densely packed (Fig. 6 d). The LC phase is characterized by small compressibility because the surface area per molecule is slightly dependent on the surface pressure. The LE-LC region is that of two phase's coexistence (Fig. 6c). For very high surface pressures, the hydrocarbon chains are tightly packed and the layer is in the solid phase (S) considered as a two-dimensional crystal (Fig. 6 e). Further compression of the monolayer leads to its collapse due to the mechanical instability and the formation of multilayer structures (Fig. 6 f), which is manifested by a rapid decrease in the surface pressure. The pressure at which the monolayer collapses is strictly dependent on the temperature, pH of the subphase, and compression rate.

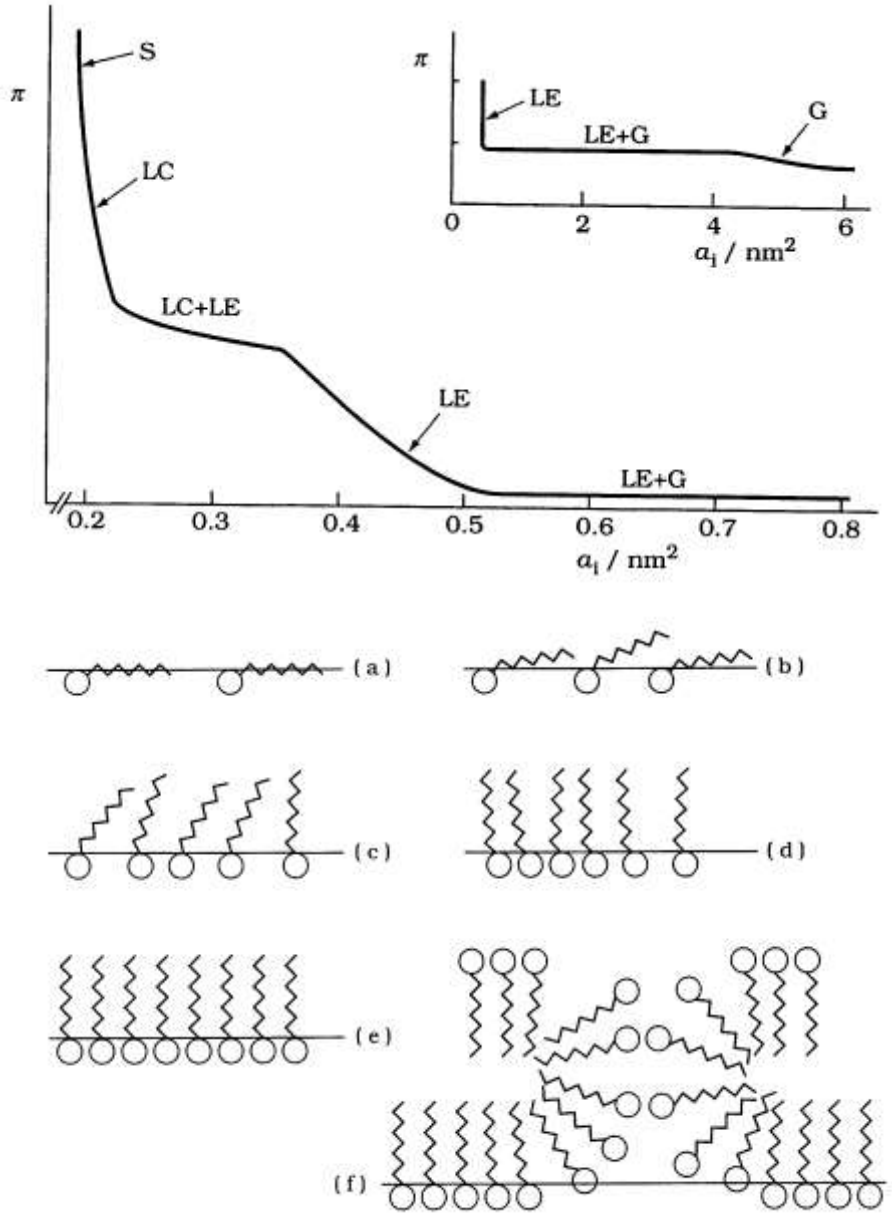


Fig. 6. Generalized $\pi - A$ sotherm of a lipid monolayer showing two-dimensional phases that can occur during the monolayer compression: gas phase (G), liquid-expanded (LE), liquid-condensed (LC) and solid (S) along with the model orientation of molecules in the individual phases: (a)G, (b)LE, (c)LE-LC, (d)LC, (e)S, (f) collapse of the monolayer.

The $\pi - A$ isotherm provides information on the stability of the monolayer on the sub-phase surface, reorientation of molecules, phase transitions and conformational changes. The presence of unsaturated bonds in the molecule affects the shape of the isotherm. As a result of blocking the rotation process in molecules, monolayers are more disordered and fluid.

Based on the course of the $\pi - A$ isotherms, it is possible to determine the surface area A_0, which corresponds to the area per molecule at which an increase in the surface pressure can already be detected, i.e. approx. 0.5 mN/m, and corresponds to the phase transition from the gas phase to the liquid-expanded phase. The most packed state of a given monolayer is characterized by the limit area A_{lim}, which is determined by extrapolation of the linear part of the isotherm to the zero value of the surface pressure. The collapse pressure, π_c of the monolayer is determined by projecting the intersection of the lines extending the isotherm below and above its inflection on the Y axis. The graphical method of determining the above parameters is shown in Figure 7.

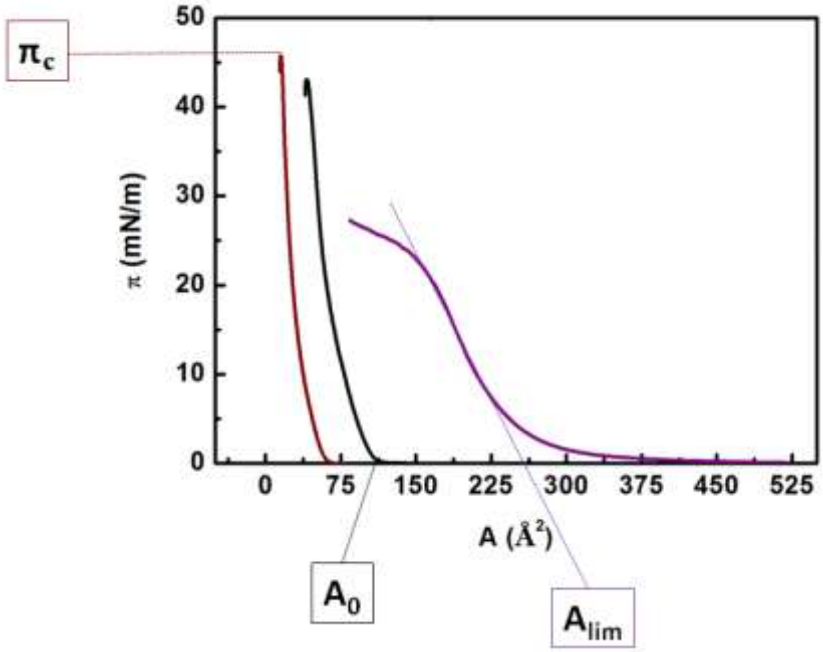


Fig. 7. Graphical presentation of the method of determining the values of the A_0 and A_{lim} and the monolayer collapse pressure, π_c.

III. 3.4. Compressibility of monolayers

The additional parameter used in the characteristics of monolayers is compressibility which can be calculated directly from the course of the $\pi - A$ isotherm on the basis of the following relationship:

$$C_s = -\frac{1}{A}\left(\frac{dA}{d\pi}\right)_T \tag{1}$$

However, the reciprocal of compressibility, i.e. **compression modulus** or elasticity, is most often used and expressed as:

$$C_s^{-1} = -A\left(\frac{d\pi}{dA}\right)_T \tag{2}$$

According to the Davies and Rideal criterion, depending on the value of the compression modulus, monolayers can be assigned a specific physical state (Table I). Generally, the higher the compression modulus, the more condensed and ordered the monolayer.

Table I. Physical state of monolayers according to the Davies and Rideal criterion.

C_S^{-1} [mN/m]	Physical state of monolayer
<12.5	Gas (G)
12.5-50.0	Liquid-expanded (LE)
50.0-100.0	LE/LC intermediate phase
100.0-250.0	Liquid-condensed (LC)
>250.0	Solid (S)

The dependencies $C_S^{-1} = f(\pi)$ make it possible to track changes in the packing and ordering of molecules during the film compression and to determine the value of the LE-LC phase transition pressure in the course of the $\pi - A$ isotherm, which appears as a characteristic minimum on the modulus-pressure diagram. For better illustration of this relationship, both functions have been summarized in a common graph (Fig. 8).

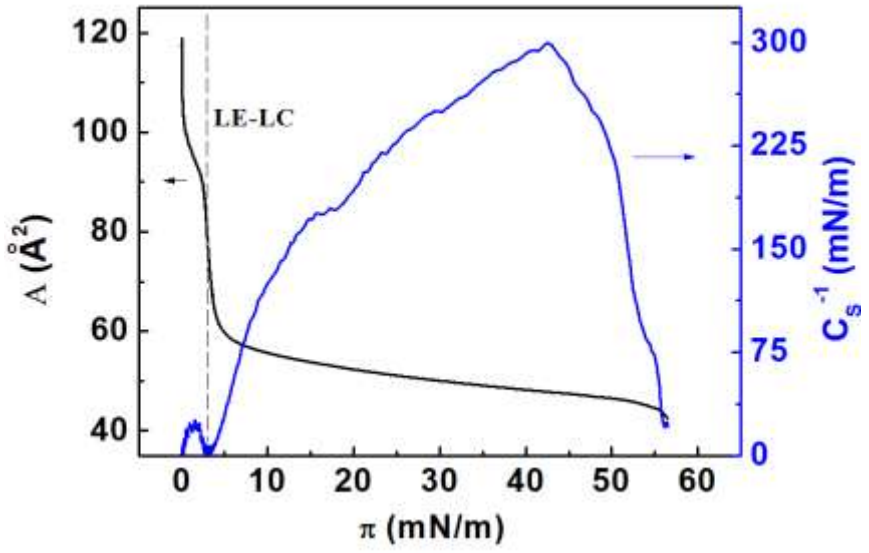


Fig. 8. Dependency statement $A - \pi$ and $C_S^{-1} - \pi$.

IV. Experimental

A. Apparatus and materials

1. Apparatus:

- Langmuir-Blodgett KSV 2000 Standard trough with the area of 780 cm^2, equipped with two barriers and the platinum Wilhelmy plate (perimeter 39.24 mm) for the surface pressure measurement, coupled with a computer,
- antivibration table,
- Lauda thermostat,
- Air Liquide water pump,
- Bunsen gas burner, Labrant,
- dark glass bottles with caps with the capacity of 5 cm^3 – 3 pcs.,
- beakers with the capacity of 50 cm^3 – 3 pcs.,
- automatic pipette with the 1 cm^3 tip – 1 piece,
- Hamilton microsyringe with the capacity of 100 µL – 1 item,
- tweezers – 1 pc.,
- spoon-spatula – 3 pcs.

2. Reagents:

- 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), ≥ 99%,
- 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), ≥ 99%,
- cholesterol (Chol), ≥ 99%,
- acetone, pure p.a.,
- methanol, pure p.a.,
- chloroform, pure p.a.,
- deionized and demineralized water from the Milli-Q system with the resistance of 18.2 MΩcm and pH 5.6.

3. Accessories:

- dust-free wipes,
- powder-free nitrile gloves,
- filter paper,
- parafilm.

B. Task scheme

1. Preparation of lipid solutions in chloroform.
2. Preparation of the Langmuir trough for measurements.
3. Creation of the Langmuir monolayers of the tested lipids.
4. Determination of $\pi - A$ isotherms during the symmetric compression process.

C. Apparatus and software service

The Langmuir-Blodgett trough (Fig. 9) is a device coupled with a computer, controlled by the KSV NIMA LB software.

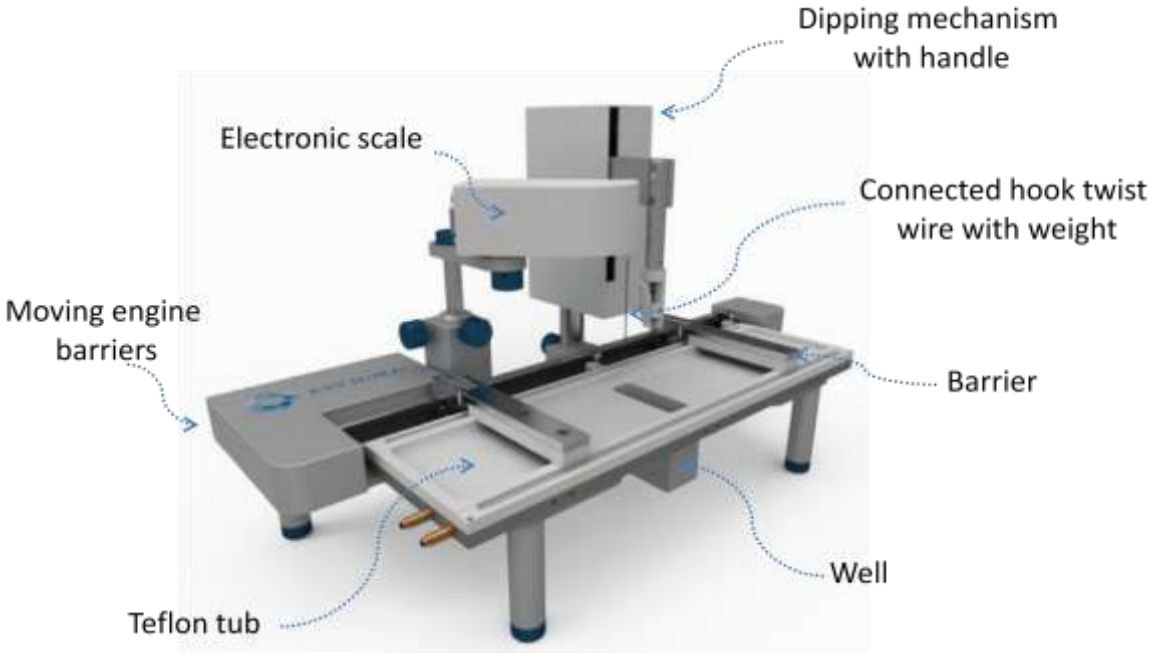


Fig. 9. Langmuir-Blodgett KSV NIMA Standard trough.

After starting the computer, turn on the power supply and click the  *Device Server* icon. A window with the main menu of the KSV NIMA LB software appears (Fig. 10):

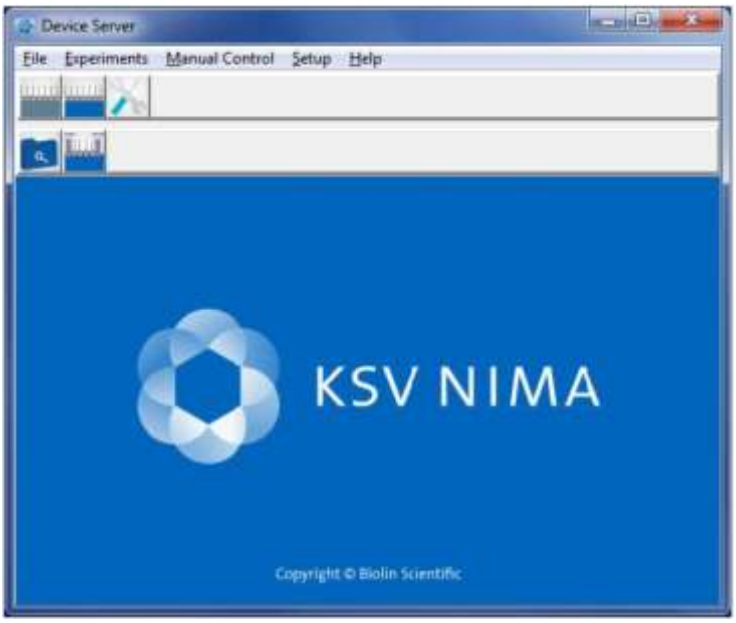


Fig. 10. *Device Server* window with the main menu of the KSV NIMA LB software.

There are five icons for the quick access to various software options. Select Manual Control to control balance and barriers. There is also a preview of the surface pressure, the position of the barriers and the speed of their movement (Fig. 11).

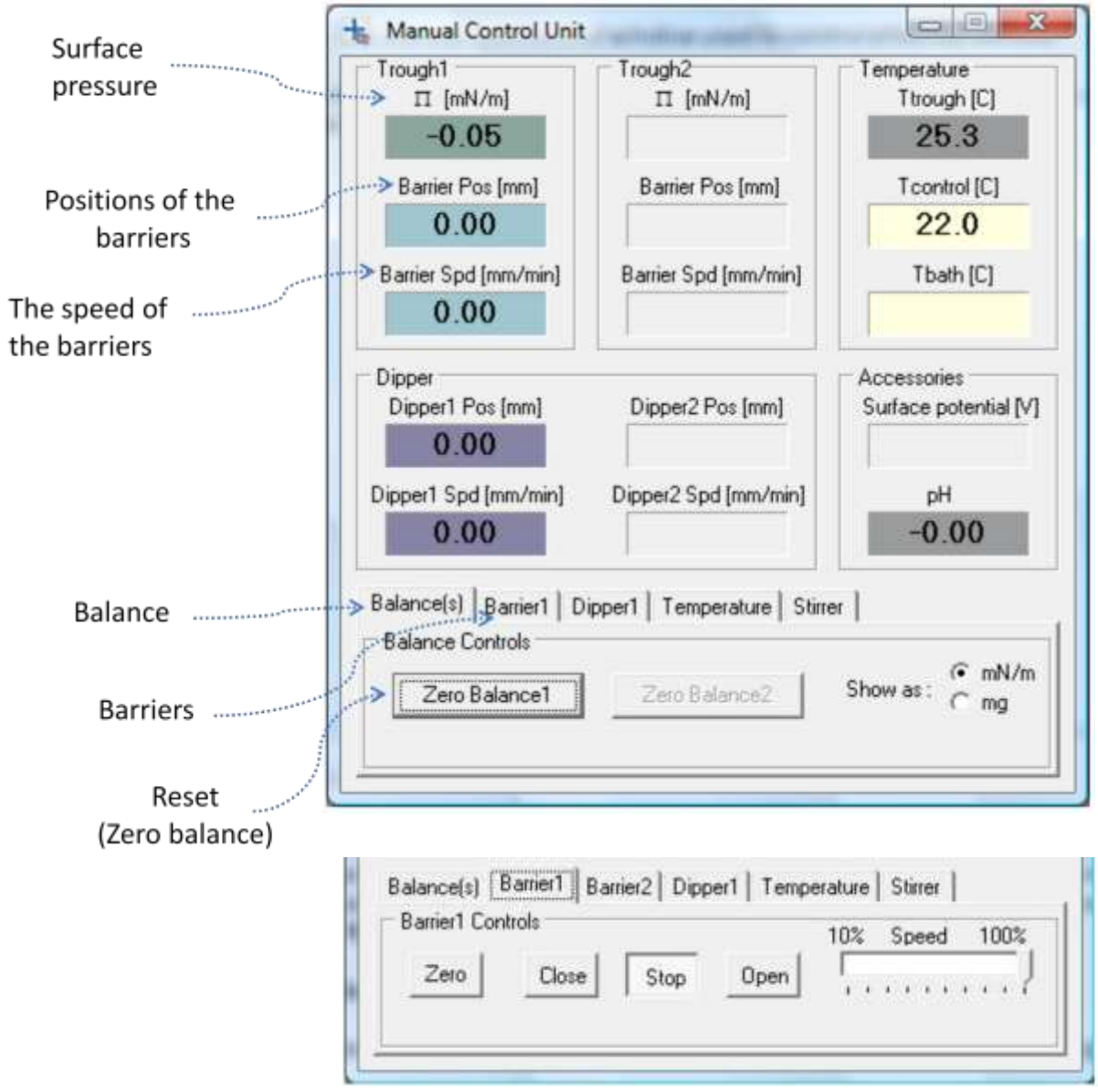


Fig. 11. *Manual Control* unit window used to control devices linked to the computer.

Start a new experiment by clicking on the icon  *LB Control Software* (Fig. 12).

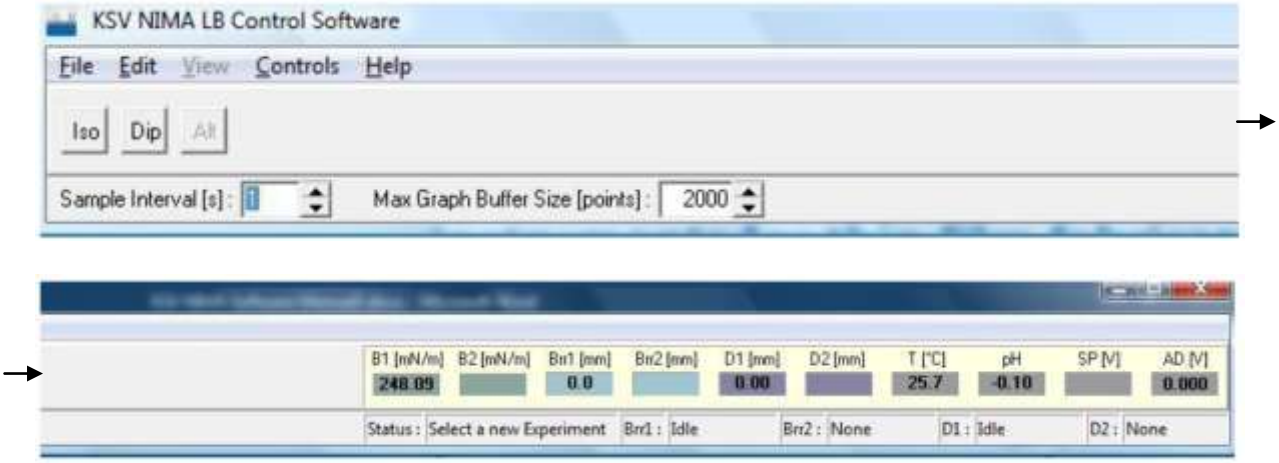


Fig. 12. Window *KSV NIMA LB Control* Software.

In the window that appears, select the *Iso* option. The *Experimental Setup* window will be displayed, which should be filled in with basic information about the experiment (Fig. 13).

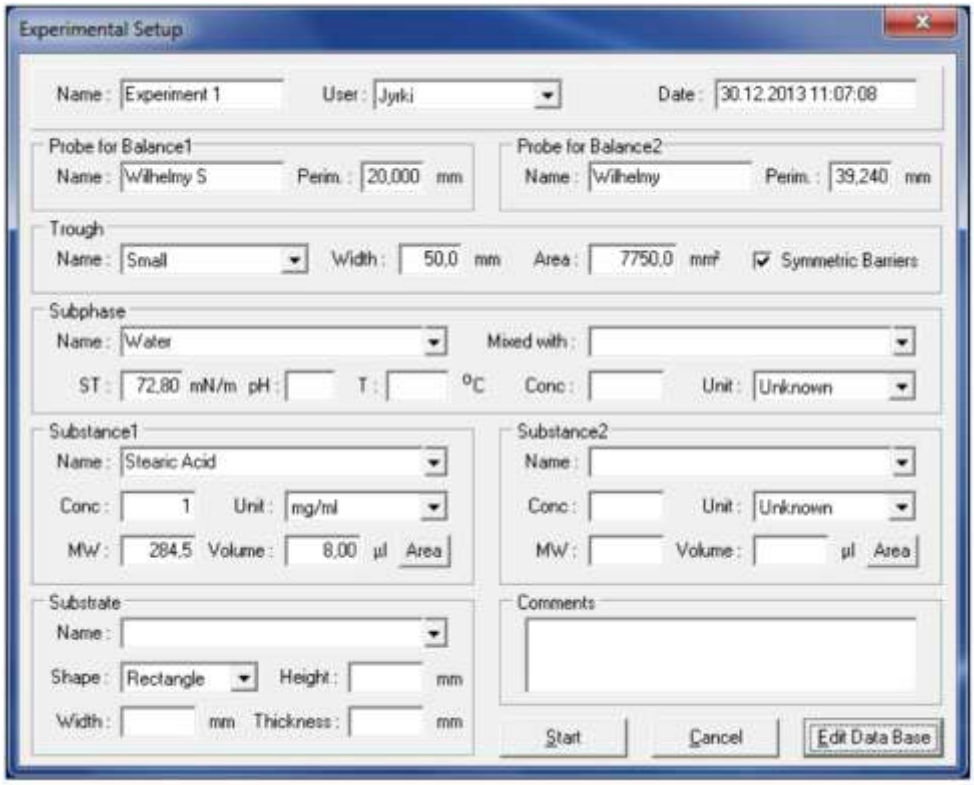


Fig. 13. Window *Experimental Setup* contains basic information about the experiment.

Pressing the *Start* button will automatically generate the *Trough Controls* window, which allows to set detailed measurement parameters, including the compression method, recording options, compression parameters (target surface pressure and barrier speed), (Fig. 14).

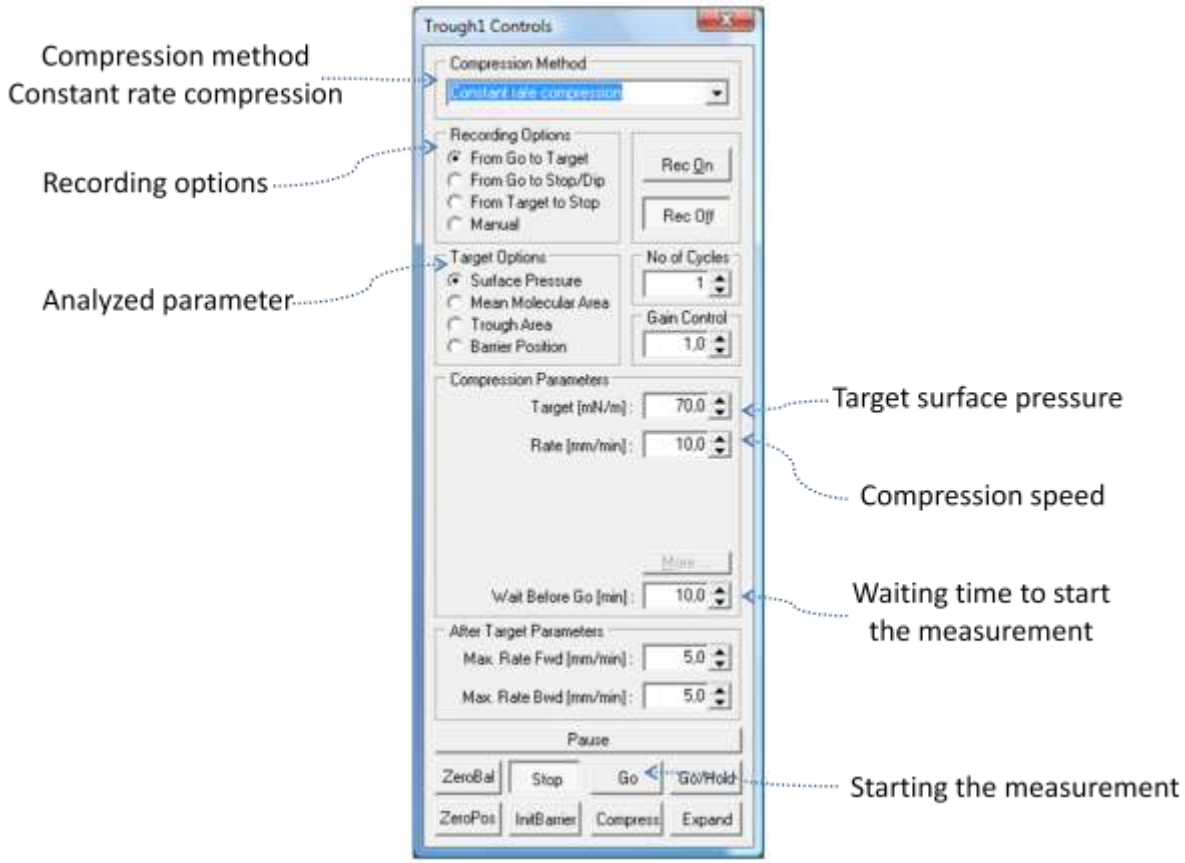


Fig. 14. Window *Trough Controls* allows determining the exact measurement parameters.

During the experiment, the formation of an isotherm can be observed on the monitor screen $\pi - A$ (Fig. 15). To complete the measurement, click *Stop* and save the data.

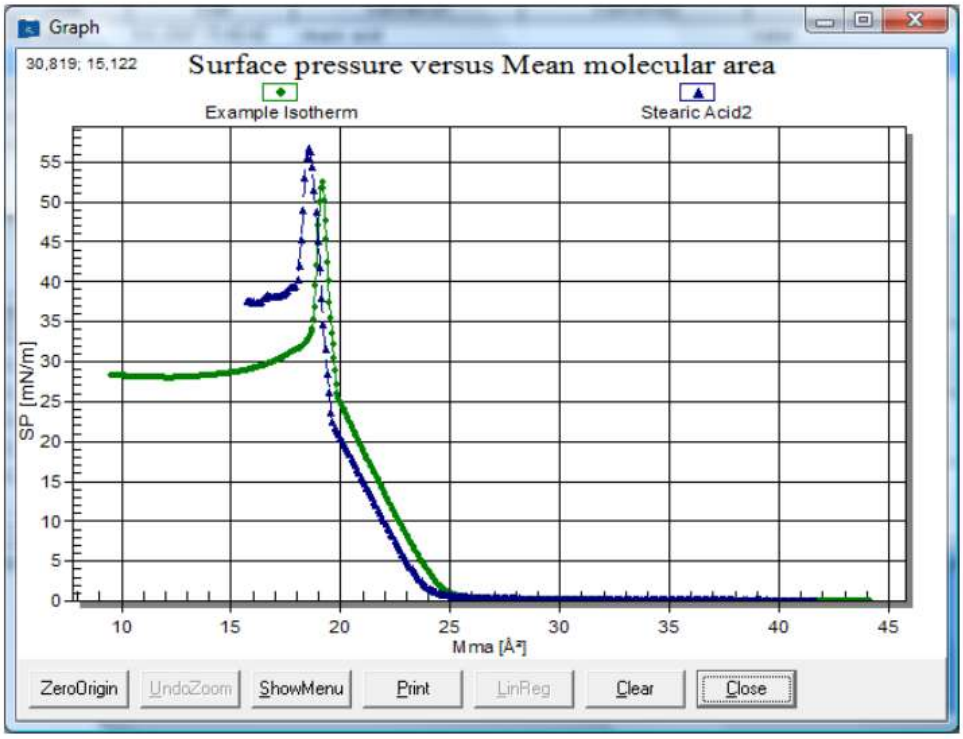


Fig. 15. Exemplary $\pi - A$ isotherms.

Saved results can be found in the folder  *Browse LB measurement* (Fig. 16). Highlight the measurement, select *Copy Data*, and paste the data into Notepad or an Origin/Excel file.

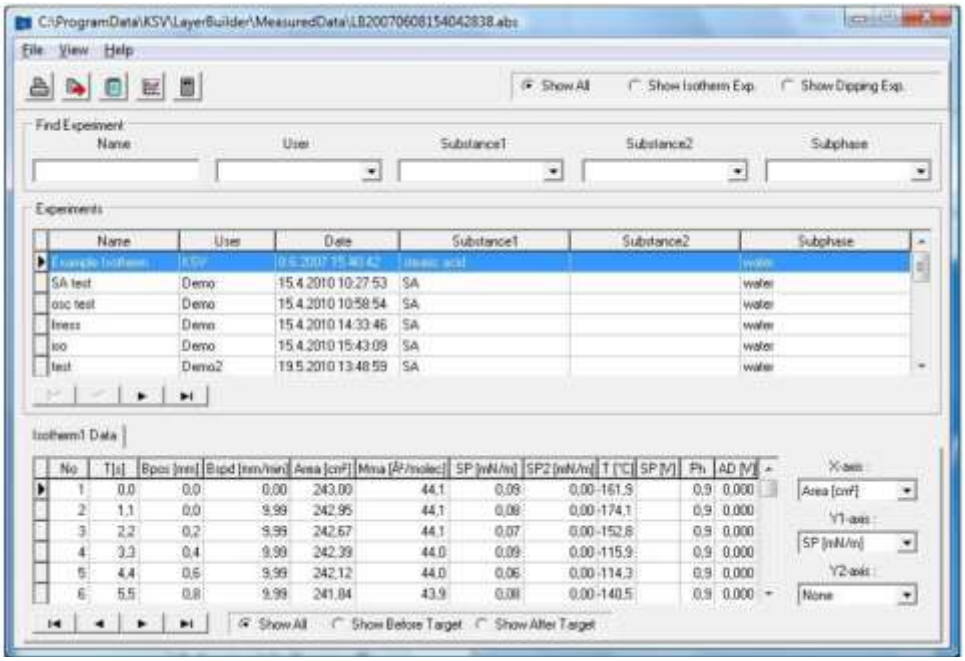


Fig. 16. List of the obtained data.

D. Program of activity

Preparation of solutions. Make solutions of lipids in chloroform. For this purpose, weigh about 1 mg of a given lipid using an analytical balance (Sartorius) into 5 cm^3 bottle of dark glass, and then dissolve the sample in the amount of chloroform to obtain a solution of the 1 mg/cm^3 concentration. Prepare all lipid solutions in the same way. Close the bottles with solutions tightly with caps and protect them against evaporation of the solvent by wrapping them with parafilm, and then store them under cover in chloroform atmosphere.

Preparation of the trough for measurements. Activate the thermostat connected to the Langmuir trough, setting the temperature to 20°C. Before the measurements, clean the Teflon trough and the barriers made of polyacetal with dust-free wipes soaked in acetone, and after evaporation of the solvent (approx. 10 minutes) with wipes soaked in methanol, using powder-free nitrile gloves and tweezers. After 10 minutes, rinse the trough and barriers several times with ultrapure water, which should be removed from the trough using a water pump equipped with a plastic, replaceable tip. Then pour water so that a convex meniscus is formed and clean the surface of the subphase by sucking out any impurities while the barriers move towards the trough center. The water level after cleaning should be equal to the edges of the trough.

Checking the cleanliness of the surface. In order to check the purity of the subphase, a platinum Wilhelmy plate with the dimensions of 10x19.62 mm^2 should be rinsed in methanol and water, fired three times in the burner flame and hung on the scale hook in such a way that 1/3 of the plate is immersed in the subphase. Then, reset the scale and the bars in the Open position, start compression. The surface pressure is measured by weighing a platinum plate immersed in the subpase. If changes in the surface tension during the movement of the barriers towards the center of the trough do not exceed 0.3 mN/m, the surface of the subpase is considered clean and after opening the barriers, the main measurement starts. Otherwise, cleaning must be repeated.

Main measurement. On the cleaned surface of the subpase, after resetting (zero balance) the surface pressure and the position of the barriers, drop the appropriate volume of the lipid solution using the Hamilton microsyringe with a Teflon plunger tip. The dosed volume should be selected in the KSV NIMA LB program based on the molar mass of the compound and the concentration of the solution. Typically, the volume is in the range of 50 to 100 μL. After applying the chloroform solution to the surface of the water, wait 10 minutes for the solvent to evaporate, then start compression at the rate of 10 mm/min. At the same time, the process is recorded in the form of the surface pressure dependence on the subphase surface per single molecule in the monolayer ($\pi - A$ isotherms), visible on the screen of a computer connected to the trough. After completing the measurement, remove the contents of the trough and clean it according to the above described procedure. Repeat for other lipids.

Pour the organic solutions remaining after the measurements into a bottle marked "**Chloroform BEAKERS**". Rinse the bottles with screw caps three times with fresh portions of chloroform and allow drying.

E. Results and discussion

The obtained experimental data should be transferred to Origin or Excel and processed according to the listed points.

1. Draw the $\pi - A$ isotherms by plotting the surface area per molecule, A, in Å^2 on the abscissa, and the corresponding surface pressure, π on the ordinate. Include the isotherms of the monolayers of the tested lipids on one summary chart (as in Fig. 7).
2. Using the curves, determine the area A_0, A_{lim} and the collapse pressure, π_c of the monolayers graphically (Fig. 7) and summarize them in Table II.

   Table II. Physicochemical parameters of membranes determined on the basis of isotherms $\pi - A$.

Type of monolayer	A_0 [Å^2]	A_{lim} [Å^2]	π_c [mN/m]	$C_S^{-1}(\pi)$ [mN/m]	Physical state
DPPC DOPC Chol					

3. Using the measurement data of the recorded $\pi - A$ isotherms for the individual monolayers, calculate the compressibility modulus, C_S^{-1} in mN/m, according to **Equation 2**, where: π – the surface pressure expressed in mN/m, A – surface area per molecule in Å^2, and T – constant measurement temperature.
4. Make the graphs $C_S^{-1} - \pi$, (mN/m), placing the surface pressure on the abscissa axis, and on the ordinate axis corresponding compression modulus values, C_S^{-1} (mN/m), as shown in Figure 8. Plot the curves on the common graph.
5. Determine the maximum value of the compression modulus and the corresponding surface pressure. On this basis, determine the physical state of the monolayers according to the Davies and Rideal criterion. Put the results in Table II.
6. Determine in which case(s) the LE-LC phase transition occurs.
7. Compare the parameter values determined from the $\pi - A$ isotherms for three model membranes (Table II) and interpret the relationships in connection with the compounds structure.