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Biomedical applications of the Langmuir monolayer technique♣

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The modeling of natural membranes is a consequence of their complex structure. The paper describes different approaches used to model biomembranes, and emphasizes the advantages of applying the Langmuir monolayer technique in this aspect.

1. INTRODUCTION

The technique of monomolecular layers formed at the aqueous solution-air interface, termed Langmuir (or insoluble, spread, floating) monolayers (films) is based on spreading an aliquot of an amphiphile of interest in organic, volatile and water-immiscible solvent (such as chloroform) on water surface. After solvent evaporation, the free surface is entirely covered by a monomolecular layer of an amphiphile, which can be compressed to the desired surface pressure/mean molecular area by sliding barriers, using the Langmuir trough [1]. The use of the Langmuir method allows for a continuous control of both quality of the surface and such parameters as molecular packing, physical state, lateral pressure and composition. A thorough understanding of monolayer behavior at the free water surface is essential for exploiting so-called Langmuir-Blodgett (LB) technique [2], which basically involves the formation of a monolayer film on water with its subsequent transfer (either by vertical or horizontal dipping) onto a solid substrate, as a viable route for making highly ordered, defectless ultrathin films with controllable molecular orientation, thickness and architecture. These outstanding opportunities of the LB method have led to an

♣ Paper dedicated to Professor Emil Chibowski on the occasion of his 65th birthday

international effort to exploit such films in optical devices, highly specific chemical sensors or molecular electronics.

It is quite understandable that the application of Langmuir films, because of the fact that they are formed on water, is much more limited as compared to LB films. However, there is a field, in which they are very important, i.e. biomedical sciences, like biology, pharmacy and medicine. Lipids monolayers form an excellent model of one leaflet of a cellular membrane and therefore the Langmuir technique is successfully applied to studying the properties of biomembranes, various processes occurring on membrane level or the interactions between membrane components. Such a technique is also useful tool for investigating of the mechanism of action of amphiphilic drugs active on membrane level or the effect of other biomolecules on biomembrane. This paper is aimed at providing examples for successful applications of the Langmuir technique in this area.

2. MODELING OF A CELLULAR MEMBRANE

The natural membrane is composed of different class of lipids (mainly phospholipids, sphingolipids and sterols) and proteins organized into the structure described generally by so-called fluid-mosaic model proposed by Singer and Nicolson in 1972 [3,4]. The framework of membrane builds a lipid bilayer, which is a “fluid” part of this structure. The “mosaic”, on the other hand, is made by proteins embedded into the lipids’ framework. Proteins either penetrate the bilayer (integral proteins) or are localized on the surface of leaflets. The latter may be located loosely on the surface layer (peripheral proteins) or bound covalently to membrane lipids (lipid-anchored proteins). The fluid-mosaic model represents general structure of a biomembrane; however, its organization is still being investigated. For example, the research performed in last decades proved the existence of microdomains enriched in cholesterol, called “lipid rafts” [5]. This finding significantly changed previously accepted view on homogeneous distribution of lipids in natural membranes. The cellular membrane is characterized by a highly dynamic structure, in which both phospholipids and proteins are mobile and able to interact. Membrane is also asymmetric in structure – this means that the composition of the two membrane layers is different. Generally, in the outer layer phosphatidylcholines and sphingomyelins are mainly present, while the inner layer contains phosphatidylethanolamines and phosphatidylserines [4]. Obviously, the composition of a membrane (lipids type and the lipid-to-protein proportion) depends on the species of organism, kind of an organ and tissue, type of a cell or, within the cell, on the type of organelle.

The cellular membrane is not only a physical barrier separating the inside of the cell from the outside, but allows cells to selectively interact with their environment [4,6]. Apart from its importance in vast array of cellular processes (such as ions and metabolites transport, communication and regulation processes), it makes a site of a number of drugs acting at the membrane level of a living cell. Antimicrobial peptides, such as alamethicin [7] or gramicidin [8], polyene macrolide antibiotics (for example amphotericin B or nystatin [9]) or alkyl-lysophospholipids (a new generation anticancer drugs, like miltefosine [10] or edelfosine [11]) are good examples of molecules, the physiological activity of which occurs at a lipid membrane interface. For these particular kinds of drugs, studies of their influence on the cell membranes are of utmost importance. For this purpose, several approaches are possible. The drug-membrane interactions can be investigated with living cells or natural membranes isolated from cells. However, the foregoing methods are characterized by complexity of both the experimental procedures and the results obtained, and moreover, provide only global information on the membrane. Therefore they are unsuitable for studying specific aspects of a given phenomena occurring at membrane level (e.g. lipid – protein or drug – lipid interactions). For these kinds of investigation the best choice is to use one of membrane models. They are briefly discussed below.

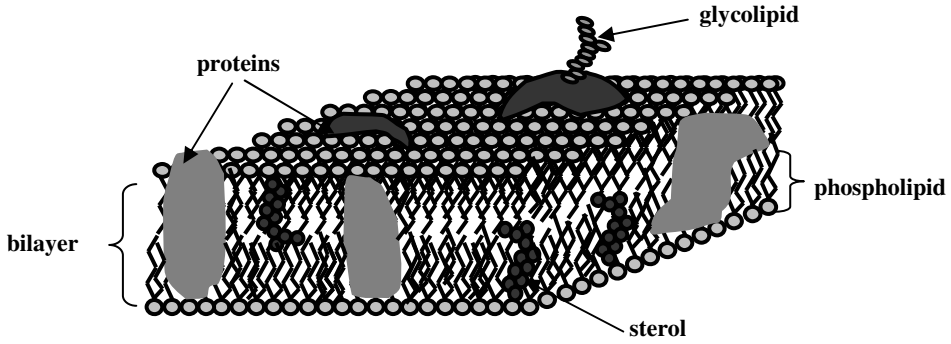

Fig. 1. Schematic representation of a biomembrane structure, according to the fluid-mosaic model.

The most widely used membrane models are lipid vesicles (liposomes), which consist of an aqueous space closed in a lipid bilayer(s) (Figure 2). Depending on the number of layers surrounding the aqueous core the liposomes are classified as multillamelar vesicles and unilamellar vesicles [12,13].

These structures were discovered in the sixties of the preceding century upon microscopic observation of phospholipids dispersion in water [12,13]. Originally

prepared liposomes were formed from natural phospholipids. Later, other amphiphatic compounds were found to be useful for making liposomes and artificial vesicles were prepared from synthetic amphiphiles. Currently, depending either on the type of an amphiphile used or on the application of vesicles, the liposomes are specifically named (niosomes, nanoparticles, nanospheres) [12,13]. To distinguish the artificial liposomes formed by surfactants from those prepared from natural phospholipids, the former are called "*vesicles*", while the name "*liposomes*" is reserved for the latter systems [14].

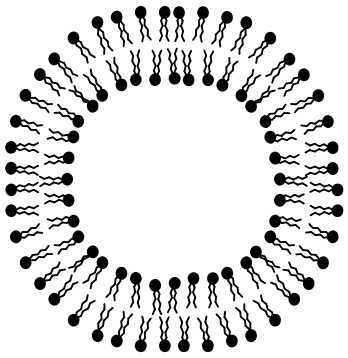

Fig. 2. Scheme of a lipid vesicle.

Liposomes formed from phospholipids can successfully mimic the dynamic, fluid and semi-permeable natural bilayer[15]. Therefore, vesicles – in general- are widely used to study the properties of various type of membranes, cellular processes (e.g. endo- and exocytosis, cell lysis, transport phenomena) or protein-membrane lipid interactions [16,17], effect of biomolecules on phospholipid bilayer [18-20] or the interaction of drugs with membrane components [21-23]. Although initially liposomes were used mainly to model the natural membranes, with time their application has significantly broadened and now they are also applied in medicine and pharmacy (drug delivery, preparation of less toxic drug formulations [24,25]), in medical diagnostics, gene therapy, cosmetics, food-industry, or in the environmental protection [26-28].

Although liposomes are easy to prepare and allow various spectroscopic measurements, they suffer from several limitations. Firstly, the range over which the lipid concentration can be varied without changing the surface curvature and physical state is limited. It is not possible to regulate lipid lateral packing density and lipid composition independently. Moreover, the physical state of compositionally identical vesicles depends on the method of preparation. It is also difficult to prepare a homogeneous (in size and layer number) vesicle

suspension and to avoid a spontaneous fusion. Another severe disadvantage is a small curvature radius that imposes strong constraints at the polar head level.
Monomolecular (Langmuir) films formed at the free surface of aqueous solutions overcome all the above limitations as it has already been mentioned in the *Introduction*. In addition, with the Langmuir technique contrary to liposomes, it is possible to mimic similar conditions as in cellular membrane. It was found that the pressure in biological membranes corresponds to the surface pressure of 30-35 mN/m in the Langmuir experiment [29].

The use of monolayers as a membrane model system will be discussed separately in the following paragraph. Before this, however, it is worthy mentioning another membrane model, namely *black lipids membranes* (BLM), so-called *planar lipid bilayers* (phospholipid molecules suspended over aperture between two solutions phases; (Figure 3)), the formation of which were originally reported at the same time as the lipid vesicles were discovered [30].

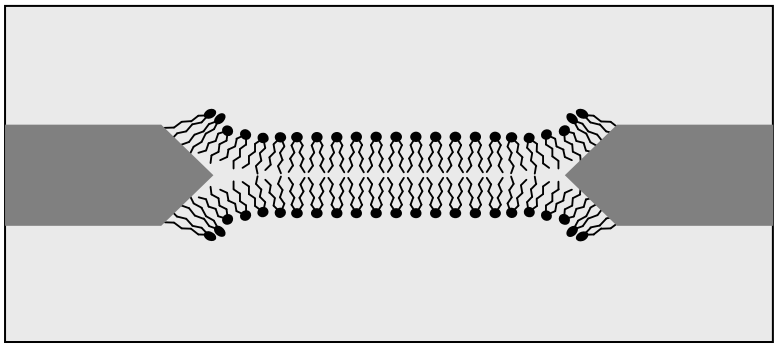

Fig. 3. Scheme of a black lipid membrane (BLM).

Although this kind of model membrane is certainly less popular as compared to liposomes, due to its rather low stability and drastically limited number of methods suitable for their analysis, it found application in studying the formation of pores in phospholipid bilayer by selected biomolecules [31,32]. In order to broaden the spectrum of experimental methods for BLM analysis, the phospholipids bilayers have been deposited onto solid supports to form a group of membrane models called *surface-confined membrane systems* [33], which include the following: *solid-supported lipid membranes, hybrid bilayers* or *polymer-cushioned lipid bilayers* (Figure 4) [33-35].

A phospholipid bilayer supported by solid substrates (*solid-supported lipid membranes*) (Figure 4 A) can be obtained by spontaneous spreading of vesicles onto solid surfaces or by transfer of the lipid monolayer formed at the air-water interface to a solid support (Langmuir-Blodgett technique). The combination of these two techniques is also frequently applied [30].

The *solid-supported lipid bilayers* are more stable as compared to *black lipids membranes*. The problem is the limited number of solid surfaces suitable for supporting lipids and the existence of unfavourable interactions between membrane components and solid surfaces. Similar disadvantages concern also the *hybrid bilayers*, which are layers of phospholipids deposited on the top of self-assembled (SAM) monolayers formed on metal surfaces. As regards SAM monolayers, most frequently applied are thiols chemisorbed on metal surfaces (like gold); other alternatives are SAM silanes chemisorbed on glass supports. To avoid the influence from a supporting substrate and minimize the interactions with the substrate, the polymer film (e.g. cellulose, chitosan, polyelectrolites) can be introduced in-between [35]. However, these systems, named *polymer-cushioned lipid bilayers*, are known of defective structure and low stability. Detailed description of the properties of the surface - confined membrane systems is presented in an excellent review by E. Castellana and P. Cremer [30]. The foregoing model systems have been applied to studying the formation of lipid rafts in membranes [36,37], charge transport properties of proteins [38], or the influence of protein on lipid bilayers [39].

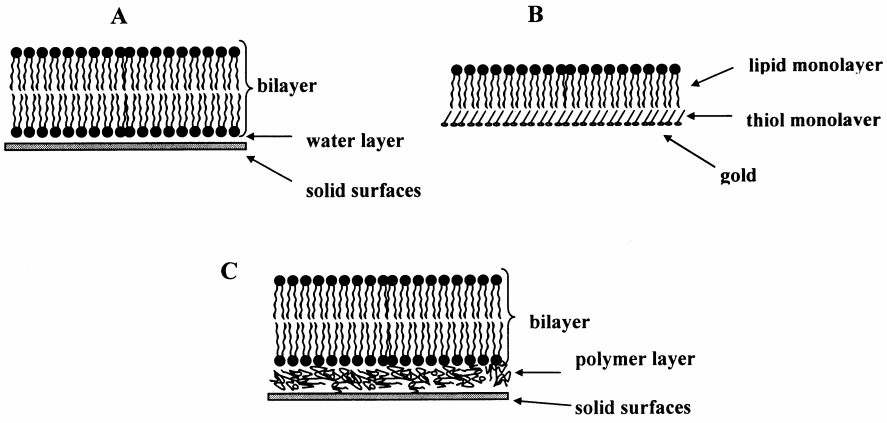

Fig. 4. The surface-confined membrane systems: A) solid-supported lipid membranes; B) hybrid membranes; C) polymer-cushioned lipid bilayers

3. LANGMUIR MONOLAYERS AS MODEL OF MEMBRANES

The Langmuir monolayer technique is of special importance as regards modelling of natural membranes. To prove, a bilayer structure of the cellular membrane was just evidenced with the Langmuir method [4, 40].

One – or multicomponent Langmuir monolayers formed at the air/water interface by membrane lipids serve as a simple model for studying the properties of individual membrane leaflets keeping their asymmetry and individuality (Figure 5) [41].

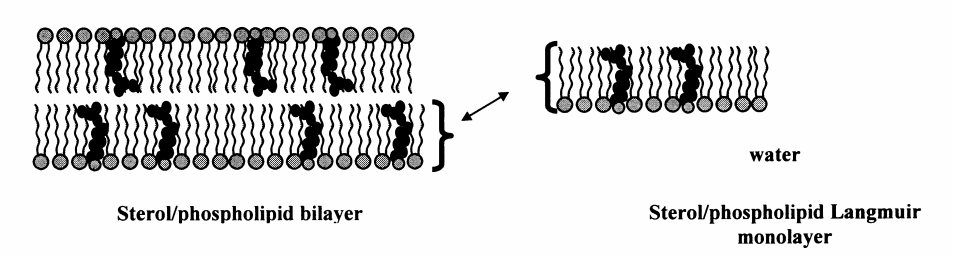

Fig. 5. A Langmuir monolayer as a model of membrane leaflet.

By mixing the components in a certain proportion it is possible to study various types of membranes. Additionally, by mixing lipids and film-forming amphiphatic drugs or other molecules acting on membrane level in a Langmuir monolayer, one may investigate the effects of these bioactive components on membrane organization. Depending on the kind of a biomolecule, two different methods can be applied.

For water soluble biomolecules the experiment is based on the compression of a monolayer mimicking the natural membrane (composed of membrane components, usually lipids, in a certain proportion) formed on the subphase containing the dissolved biomolecule (see for example [42]. It is also frequent that a solution containing the investigated biomolecule is injected into bulk water, underneath the previously formed monolayer, compressed to a particular surface pressure value. The dissolved biomolecule incorporate into the monolayer (see for example [43]). The penetration of biomolecules into the floating film causes the changes of the surface pressure values, which is monitored and can be taken as a measure of interactions between the molecules.

When water-insoluble biomolecules are investigated, the approach is based on forming mixed Langmuir monolayer by co-spreading of membrane components and biomolecules onto water surface [44,45]. Changing the proportion of monolayer components and analyzing the stability and miscibility of the investigated mixed system with the simple functions or thermodynamic parameters, the nature and strength of interaction between film molecules can be estimated.

In general, the basis of the analysis of the interactions between monolayer components is the surface pressure (π) – area (A) isotherms recorded upon film compression. The analysis of the shape, course and position of the π/A curves provides information on the monolayer state, area per film component or phase transitions. The isotherms are also a starting point for calculation of the compression modulus, area per molecule and excess area, excess thermodynamic functions of mixing (free energy of mixing, total free energy of mixing, excess entropy and enthalpy). Basing on the foregoing parameters it is possible to conclude on the monolayer organisation, its state, phase separation, film stability, and on the interactions between film components. Different approaches used to characterize molecular interactions in monolayers are reviewed in Ref. [46], while the paper by Gong *et al.* [47] can serve as a representative paper, providing very useful experimental examples of the analysis of intermolecular interactions.

Although the Langmuir monolayer technique allows mimicking the membrane leaflets only independently, there is a strong correlation between monolayers and bilayers prepared from cellular membrane components. It was found [48] that the lipid monolayers and bilayers reveal similar properties (pressure, area per lipid molecule, phase transition, elastic compressibility) at surface pressures of 30–35 mN/m. Therefore, the results of Langmuir monolayer experiments can be successfully linked to a bilayer system and provide essential information not only on the behaviour of individual membrane components and membrane properties, but also allow explaining the influence of biomolecules (e.g. drugs, hormones) on model membranes by verifying the affinity of these compounds to the respective membrane components and calculating their interactions. Some examples of the application of the Langmuir technique to modelling of natural membranes are presented below.

4. THE STUDY OF THE DRUGS MODE OF ACTION

A good example of amphiphatic drugs, extensively investigated with the Langmuir monolayers technique are antifungal polyene antibiotics, represented by amphotericin B (AmB). The mechanism of AmB activity has not been clearly elucidated yet, however, the most widely accepted view holds that this polyene antibiotic acts at the membrane level, forming membrane channels (pores) by interacting with fungi membrane sterol (ergosterol), [49], which provokes the lysis and finally death of fungi cells. Although AmB was found to be toxic also to host cells, is still in clinical use since there are no alternative drugs of such a broad spectrum of antifungal activity. Therefore, a lot of effort was put to decrease the toxicity of polyenes. Over the years a number of AmB's derivatives have been synthesized [50-54] which were proved to be of decreased toxicity,

together with AmB's liposomal formulations. However, the following problems concerning AmB remained unsolved: 1) its exact mechanism of action, 2) the reason of its toxicity, 3) the mechanism of decreased toxicity found for AmB' s liposomal formulations and some of AmB derivatives. The Langmuir monolayer technique helped in understanding of all the above questions. Namely, studies of the Langmuir monolayers of AmB mixed with fungi sterol (ergosterol) and mammalian sterol (cholesterol) proved that AmB interactions with both sterols are of nearly similar order [55]. The observed low selectivity of AmB toward both sterols explains AmB toxicity also to host cells, containing cholesterol. Studies of the mixed systems of phospholipids and both sterols proved stronger interactions of DPPC-cholesterol *vs* DPPC-ergosterol [43]. This implies that in the presence of AmB, the antibiotic molecules can bound easier to ergosterol than to cholesterol, which explains slightly stronger affinity of AmB to ergosterol as compared to cholesterol, and in consequence, its increased toxicity towards fungi cells (containing ergosterol) in comparison to mammalian cells (containing cholesterol).

To explain the mechanism of lower toxicity of liposomal formulation of AmB and reduced toxicity of some of AmB derivatives, the study of the interactions between series of polyenes and phospholipids [55-63] were found to be of much help. It came out that the antibiotic/phospholipid interactions affect the activity and toxicity of the investigated drugs. Basing on Langmuir monolayers experiments, the role of phospholipids in polyenes activity on both fungi and human membranes was explained [55,59-63] and the mechanism of reduced toxicity of polyenes liposomal formulation was suggested [57].

Similar experiments as performed for antifungal drugs are being carried out for many other physiological compounds of amphiphatic structure, possessing anti-inflammatory, analgesic or anticancer effect [42,64,65]. As far as antitumor drugs are concerned, recently the scientists focused their attention on the group of alkyl-lysophospholipids (ALP) represented by miltefosine (hexadecylphosphocholine), edelfosine (1-O-octadecyl-2-O-methyl-rac-glycero-3-,phosphocholine) as well as by perifosine (octadecylpipiridine) and ilmofosine (1-hexadecylthio-2-methoxymethyl-rac-glycero-3-phosphocholine) [66]. These compounds induce cell apoptosis, however, they differ from other chemotherapeutics because they do not affect DNA, but target a cellular membrane [67]. Although the mechanism of action of alkylphospholipids has not been elucidated so far, a number of hypotheses concerning activity of these compounds (e.g. the inhibition of phosphatidylcholines and sphingomyelin synthesis [68], accumulation in plasma membrane [69], inhibition of cellular enzymes [70] or incorporation into lipid rafts of tumor cells [68]) prove that they act on membrane level [67]. The cellular membrane plays a fundamental role in ALPs'

mode of action due to the fact that these compounds exhibit a phospholipids-like structure and can build-in natural membrane.

Current research concentrates on clarification of a significance of respective membrane components in the mechanism of action of these drugs. Recently, series of experiments have been done for monolayers containing miltefosine or edelfosine mixed with major membrane lipids: cholesterol, phosphocholines and phosphatidylethanolamines [71-76]. The obtained results show that membrane phospholipids (DPPC, OPPE) at physiological conditions (pH = 6) interact weakly with both miltefosine and edelfosine [73,74] which implies that they are of low importance as regards their mechanism of action. On the other hand, ALPs interact strongly with membrane sterol (cholesterol) [71,72,75] and form surface complexes [71,75] These results allowed understanding *in vitro* experiments on cell cultures, which showed that the presence of cholesterol in excess lowers the uptake of ALPs.

5. THE INFLUENCE OF BIOMOLECULES ON MEMBRANES

Studies on the mechanism of action of drugs and their interactions with membrane components are not the only examples of biomedical application of the Langmuir monolayer technique. Apart from drugs, there is a large group of membrane active compounds, the effect of which on the membrane can be verified with the Langmuir films method.

Carotenoids, comprising nearly 600 fat-soluble pigments, are examples of intensively studied biomolecules. Unfortunately carotenoids can be synthesized only by plants and microorganism and therefore the presence of these compounds in human organism is dependent on the diet. Within this group, the investigations are focused mainly on β-carotene, lycopene, luteine and zeaxanthin. Carotenoids possess antioxidant properties, are precursors of vitamin A and its derivatives and have beneficial influence on human organism. They prevent coronary vascular diseases, cancers, positively affect the immune system and prevent eye diseases. The classification of carotenoids and their influence on human organism were described in review article by Krinsky and Johnson [77]. Carotenoids play also an important role on biomembranes and this aspect was discussed by Gruszecki and Strzałka in their review article [78]. In membranes, carotenoids ensure protection against oxidation of unsaturated chains of membrane lipids and in this way prevent cell damage. However, the incorporation of carotenoids, depending on the structure of the molecule, may change membrane properties e.g. permeability, fluidity or molecular packing. The Langmuir monolayer experiments from carotenoids involved mainly studies on the interactions between these compounds and membrane phosholipids [79,80]. These investigations provided both the characteristics of the properties

of carotenoids monolayers and thermodynamic description of the interactions between carotenoids and membrane lipids, analysed in relation to the structure of the investigated carotenoids. Basing on these results, the localization and orientation of the respective carotenoids in membrane were discussed and differences in physiological activity of these compounds were explained [80]. Similar experiments were performed for another well known antioxidant, namely vitamin E (tocopherol) [81,82].

Both carotenoids and vitamin E are plant-derived compounds favourably influencing the human body. Another group of compounds derived from plant and possessing positive effect on human health are unsaturated fatty acids. These compounds generally prevent heart diseases and possess anticancer and antimicrobial properties. Polyunsaturated fatty acids: linoleic and α-linolenic (omega-6 and omega-3 group, respectively) are known as the "essential" fatty acids because they cannot be synthesized by human body and are derived only from the diet. The fatty acids are also necessary to synthesis of the lipids forming cellular membranes. With the Langmuir monolayer technique the interactions between cholesterol and various saturated and unsaturated fatty acids were studied in the context of anticholesterolemic properties of these compounds [83]. In addition, the influence of fatty acids on model cholesterol/phospholipids membranes was investigated [84]. It is known that fatty acids are building blocks of phospholipids molecules, however, are also present in membranes in non-esterified form ("free" fatty acids). The experiments performed in ternary mixtures allow explaining the reasons of such a low proportion of non-esterified fatty acids in membranes.

The capability of many biomolecules to form monomolecular layers at the air/water interface opens the way to study their behaviour on the membrane level. As indicated in this article, the Langmuir monolayer technique is a potent method for mimicking of cellular membranes.

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