STRUCTURE AND PHYSICAL PROPERTIES OF BIOMEMBRANES AND MODEL MEMBRANES

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Biomembranes belong to the most important structures of the cell and the cell organels. They play not only structural role of the barrier separating the external and internal part of the membrane but contain also various functional molecules, like receptors, ionic channels, carriers and enzymes. The cell membrane also preserves non-equillibrium state in a cell which is crucial for maintaining its excitability and other signaling functions. The growing interest to the biomembranes is also due to their unique physical properties. From physical point of view the biomembranes, that are composed of lipid bilayer into which are incorporated integral proteins and on their surface are anchored peripheral proteins and polysaccharides, represent liquid scrystal of smectic type. The biomembranes are characterized by anisotropy of structural and physical properties. The complex structure of biomembranes makes the study of their physical properties rather difficult. Therefore several model systems that mimic the structure of biomembranes were developed. Among them the lipid monolayers at an air-water interphase, bilayer lipid membranes (BLM), supported bilayer lipid membranes (sBLM) and liposomes are most known. This work is focused on the introduction into the "physical word" of the biomembranes and their models. After introduction to the membrane structure and the history of its establishment, the physical properties of the biomembranes and their models areare stepwise presented. The most focus is on the properties of lipid monolayers, BLM, sBLM and liposomes that were most detailed studied. This contribution has tutorial character that may be usefull for undergraduate and graduate students in the area of biophysics, biochemistry, molecular biology and bioengineering, however it contains also original work of the author and his co-worker and PhD students, that may be usefull also for specialists working in the field of biomembranes and model membranes.

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1 Introduction

Biological membrane is one of the most important cell structure. It represents an envelop of the cell with unique barrier function, that provide directional transport of species into the cell and waste and toxic compounds out of the cell. In addition, the low permeability of the membrane for charged particles, e.g. ions, allowing to maintain non equilibrium ion distribution between extra cellular and cytoplasmic side of the cell, which is crucial for cell function. Destruction of the membrane resulted in establishment of equilibrium and cell apoptosis. The membrane with supported protein net - glycocalix is responsible for the cell shape and owing to the viscoelasticity also for reversible changes of this shape during cell function. Biomembranes provide, however not only structural and barrier functions. They contain integral and peripheral proteins, that are responsible for communication of the cell with surrounding environment, i.e. they have receptor

function and are responsible also for transfer of the signals into the cell by means of sophisticated signaling pathways. In a membrane also several catalytical processes are concentrated, for example the energy transduction connected with synthesis of energetically reach molecule adenosine thriphosphate (ATP). From physical point of view the biomembrane represent anisotropic and inhomogeneous structure with properties typical to liquid crystals of smectic type. Due to rather complicated structure, anisotropy and inhomogeneity the study of the physical and electrochemical properties of the biomembranes is difficult. Therefore several models of biomembranes have been developed, that including micelles, monolayers, lipid bilayers, liposomes and also solid supported lipid films. These structures allowing to varry in large scale the lipid composition and allowing to incorporate integral or peripheral proteins. Thus, the model membranes can be constructed in a way that mimics the structure and properties of biomembranes. During last decades the unique properties of lipid membranes allowed to fabricate biocompatible and biofunctional interfaces on a solid surfaces. These supported lipid films allowing to immobilize various functional macromolecules, like enzymes, antibodies, receptors and nucleic acids without lost of their native conformation, selectivity, sensitivity and catalytic activity. These systems allowing to perform stress-free analyzing of interaction with the membrane of various pharmacological drugs and thus to understand the effect of these compounds on the cell behavior. The lipid films are self assembling structures. This unique property can be utilized in fabrication of smart biosensors with excellent sensitivity and selectivity.

This work is devoted to introduce into the peculiarities of these exiting structures and to demonstrate unique physical properties of biomembranes and their models. The reader is step wisely introduced into to the peculiarity of the membrane structure and how this structure has been historically established. Novel knowledge obtained in recent studies is presented. The chemical composition of biomembranes is then showed. Model structures, allowing to study physical properties of the membranes, such are monolayers, bilayers, liposomes and supported membranes are described together with typical methods using for their study. Important phenomena and properties, such are electrical and mechanical stability, electroporation, membrane thermodynamics and mechanics, protein-lipid interactions, membrane potentials, electroporation, ionic transport, cell receptors and signaling are considered. Examples of the application of supported lipid membranes in fundamental studies, nanotechnology and in construction of biosensors are presented as well.

2 Membrane structure and composition

2.1 Membrane structure

Biological membranes are one of the most important structural and functional components of the cell. They fulfill a number of important functions [1-4]:

- 1. structural they surround the cell cytoplasm and give a certain form to the cell and its organelles.
- 2. barrier they secure the passing into and out of the cell only of necessary ions, low molecular compounds, proteins etc.



Fig. 1. The model of the structure of biomembrane.

- 3. contact they perform the contacts of cells between each other by means of specific structures.
- 4. receptors they are susceptible to different signals from surrounding environment by means of special protein structures incorporated into the membrane. These signals could be light, mechanical deformations, specific substances etc.
- 5. transport they provide the active and passive transmembrane membrane transport of ions and transport as well as transport of electrons in mitochondria and chloroplasts.

The structure of biomembranes has several common features (Fig. 1). Their basis is a lipid bilayer composed of lipid molecules into which are incorporated peripheral and integral proteins. They are supported or covered by structural proteins, such are e.g. spectrin net in erythrocytes [2], or bacterial S-proteins [5]. The outer part of the bacterial and plant membranes are also covered by polysaccharides [2]. The lipid matrix provides the integrity of the membrane, electrical isolation, and the possibility of self assembly of the corresponding protein structure in the membrane. The proteins determine the fulfillment of the specific functions by the membrane. In particular the integral proteins, which penetrate through the membrane are for example ionic channels or the proteins that provide active ionic transport – ATPases, etc. In normal, physiological conditions the lipid bilayer is in liquid-crystalline state. From physical point of view the biomembrane represent the liquid crystal of a smectic type [6]. The thickness of biomembranes varied between 5-10 nm and is considerable less then the dimension of the cells (typically several μ m). The variations in biomembrane thickness is mostly due to the integral, peripheral and structural proteins as well as due to presence of lipopolysaccharides and glycopolysaccharides [2].

Due to small thickness of the membranes the discovery of the membrane and study of its structure and properties was not easy. The progress in understanding of the peculiarities of the membrane structure and properties was directly connected with the progress in physics. The appearance of light microscope and especially considerable progress in fabrication of optical



Fig. 2. Brief schematic historical overview of the models of biomembranes. (a) – lipid bilayer according to Gortel and Grendel [8], (b) – Model by Danielli and Dawson [10], (c) - unitary model of biomebrane by Robetson [13], (d) - micellar model proposed by Lucy [14].

lenses in 17 century allowed to perform first observation of the cell structure by Hooke in 1662. However the further progress was rather slow and only in 1831 Brown showed existence of nucleus in a cell [7]. Then the cell theory, one of the fundamental concept of biology, has been formulated by botanist Schleiden (1838) and zoologist Schwann (1839) [2]. However, at this time even hypothesis on the existence of the membrane did not exist. The existence of the membrane surrounding the cell has been proposed only in 1855 by Negeli, who observed that undamaged cells can change the volume upon the changes of osmotic pressure of surrounding environment. These experiments have been continued by Overton [1], who showed that non polar molecules penetrate easier through the cell membrane then polar molecules. Results of these experiments allowed him to raise the hypothesis that the membrane structure has a lipid nature. Further development of the concept of membrane structure has been achieved thanks to work by Gorter and Grendel [8] at the first third of XX. century. This time was characterized by excellent achievement in the study of monomolecular layers at an air-water interface performed mostly due to the work by Langmuir and co-workers [9,10]. Gorter and Grendel [8] used this approach. They extracted lipids from erythrocytes and showed that the area of the monomolecular layer formed by lipids on an air-water interface is twice of the area of erythrocyte cell. This resulted in the concept that the biomembrane is composed of the two monomolecular lipid layers (Fig. 2a).

Despite certain errors in their study, that consisted in underestimation of concentration of lipids (they used acetone for extraction of lipids, which however does not allow to extract all lipids) and area of erythrocytes (they determined the area from dry cells) as well as that they

Piomambrana	% of dry weight				
Biomemorane	proteins	lipids	hydrocarbons		
Myelin	18	79	3		
Human erythrocytes	49	43	8		
Outer segment of retina rood	51	49	-		
Mitochondria of the rat liver	-	-	-		
Inner membranes	76	24	2 <		
Outer membranes	52	48	-		
Salmonella typhimurium	-	-	-		
Inner membranes	65	35	-		
Outer membranes	44	20	37		

rub. 1. The composition of biomemorales [1].	Tab. 1. The c	composition	of biomembranes	[1].
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did not consider the presence of proteins, the bilayer concept of biomembrane structure was accepted by wide scientific community. It is now clear that if they will perform the experiment correctly the interpretation will be different. It is known now that the membrane of erythrocyte is composed only by 43 % of lipids, while the rest, 49 % are the proteins and 8 % the hydrocarbons (see Table 1).

The assumption that also the proteins are connected with the membranes has been raised 10 years later by Danielli and co-workers [11] due to necessity to explain substantially lower surface tension of biomembranes in comparison with pure lipid monolayers at air-water interface. For example the surface tension of the membrane of the cell of sea urchin was approx. 0.2 mN m^{-1} , while that for the monolayers of fatty acids at air-water interface was between 10–15 mN m⁻¹ [12]. Further studies also showed that addition of proteins into the water subphase resulted in decrease of surface tension of lipid monolayers. It has been therefore proposed that the globular proteins are connected with both surfaces of the lipid bilayer (Fig. 2b).

Direct approval of the existence of biomembrane has been possible only after discovery of electron microscopy and its application in biology in the 50th of the XX. century. The first micrographs performed by electron microscopy showed that the cell is surrounded by thin membrane of a thickness approx. 6-10 nm. This membrane was composed of three layers. Two high electron density layers of a thickness approx. 2 nm were separated by low electron density layer of a thickness 3.5 nm [13]. Similar structure has been observed also in most of intracellular organelles [14]. It has been proposed that the high electron density layers correspond to the region of polar head groups of phospholipids layer covered by proteins, while low electron density layer corresponds to the hydrophobic part of the lipid bilayer (see [7] for electron micrograph of cell structures). On the base of these results Robertson [15] proposed the elementary model of the asymmetric cell membrane. According to this model the lipid bilayer is covered by the layer of the proteins in a β -conformation that are adjacent to the polar part of the membrane due to electrostatic interactions. The asymmetry is due to the fact that outer monolayer is covered by glycoproteins (Fig. 2c). Further Lucy (see Ref. [12]) proposed the membrane model composed of the micelles covered by proteins (Fig. 2d). However, this model can not explain rather small

conductivity of bilayer lipid membranes (BLM) determined by Mueller et al. [16]. This and further studies showed that the specific conductance of BLM is in the range 10^{-6} - 10^{-10} Ω^{-1} cm⁻² [17,18].

High resolution electron microscopy as well as improved methods of preparation ultra thin samples allowed to obtain the micrographs of cell membranes with additional structural details. It has been shown the existence of channels in a membrane as well as mosaic structure of the cell surface. The analysis of these results allowed to propose so called fluid-mosaic model of biomembrane [19,20]. According to this model (Fig. 1) the membrane is composed of the lipid bilayer with incorporated proteins. The model allowed to explain particularly the dependence of the activity of membrane proteins on the physical state of membrane as well as existence of the membrane viscosity. Further protein-crystallic model proposed by Vanderkooi and Green (see Refs. [11,21]) differs from fluid-mosaic model only by postulating the existence in a membrane of a rigid protein structure. Currently various modification of fluid-mosaic models are used for description of biomembrane structure. However, the studies of physical properties of biomembranes and lipid bilayers revealed that the mobility of some membrane proteins is strongly restricted. Also the concept of continuous lipid bilayer is certain simplification. The experimental and theoretical studies performed recently have shown that the lateral structure of lipid bilayer is dynamics and heterogeneous and is characterized by lipid-domain formation with different mobility of lipids and proteins [1,2].

Specific interactions between membrane components lead to selective orientation and segregation of the lipids and proteins in the plane of the membrane. There are the lipid clusters composed of up to several hundreds of molecules. The existence of long-range superstructure was observed on model membrane systems by several methods, e.g. by scanning tunneling microscopy [22]. Aggregates of proteins also occure in biomembranes. A typical example of this phenomenon is the aggregation of integral protein - bacteriorhodopsin in membranes [23]. The two-dimensional matrix of a biomembrane probably consists of patches of phospholipid molecules in different degrees of conformational disorder. Under certain conditions, the bilayer organization can be interrupted by nonbilayer phases [24] as well as by bilayer phases of different composition, and by regions of mismatch between coexisting phases. Such features within the organization of a membrane can have different life-times, and may be formed as a response to environmental and metabolic perturbations [4]. Several types of molecular motions of lipids and proteins are experienced by the components within the membrane: rotation of molecules along their axes perpendicular to the plane of the membrane occurs every 0.1–100 nsec for lipids and 0.01-100 msec for proteins; segmental motion of acyl chains (0.01-1 nsec) gives rise to an increased disorder toward the center of the membrane; translational motion of molecules in the plane of the membrane occurs with a lateral diffusion coefficient of 10^{-10} to 10^{-8} cm² sec⁻¹. The dynamical properties of the membrane components differ more than what would be expected only on the basis of their size.

The biomembranes are composed of lipids, proteins and hydrocarbons. The proteins and lipids represent the main part of the biomembrane. The content of hydrocarbons usually does not surpass 10 %. Hydrocarbons are mostly covalently bounded to the lipids (glycolipids) or to the proteins (glycoproteins). Both glycolipids and glycoproteins play important role in the cell recognition. The hydrocarbons are localized at outer part of all biomembranes and thus together with the different chemical composition of lipids at both membrane monolayers contribute to the membrane asymmetry. Membrane is asymmetrical also in respect of proteins. The content of

proteins in the membrane varies from almost 20 % for membranes of myelin to almost 80 % for inner membranes of mitochondria (Table 1).

2.2 Membrane lipids

Lipid bilayer is a self assembled structure formed from lipids in an aqueous environment. This is the result of the hydrophobic effect, whereby the non polar acyl chains of lipids and the non polar amino acid residues in proteins tend to be squeezed away from the aqueous phase. There exist more then 100 various phospholipids that differ by their polar head groups and the composition of hydrophobic chains. Lipids can be divided on three main classes: phospholipids, glycolipids and sterols.

Phospholipids are most frequently occurred lipids of cell membranes. They are divided on two main classes: glycerophospholipids and sphingophospholipids (derivatives of ceramide and sphingomyelin). The glycerophospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylglycerol (PG)) have similar structure, which consist of polar head group and two hydrophobic chains of fatty acids that are connected to the glycerol backbone (Fig. 3). The main representatives of phospholipids are shown in Fig. 4. The structures of glycerophospholipids and sphingophospholipids differ considerably in the interfacial and hydrophobic part (compare structure of PC and SM on Fig. 4). The most common base in mammalian SM is sphingosine (1,3-dihydroxy-2-amino-4octadecene), with a trans-double bond between C4 and C5 atoms. Phospholipids play dominantly structural role in the membrane. They participate on formation of the barrier for passive translocation of ions and other species through the membrane and provide special environment for function of membrane proteins. However, certain lipids have also functional role. Typical examples are phosphatidylinositol and sphingomyelin. Phosphatidylinositol is localized in cytoplasmic side of the membrane and is important for cell signalling. Sphingomyelin (SM) is important component of eukaryotic cell. SM has the cylindrical shape like PC, that help to minimize free energy in formation of lipid bilayers. However, in addition to the structural role, it participates also in cell signaling (see part 10.2). Products of SM metabolism, like ceramide sphingosine, sphingosine-1-phosphate and diacylglycerol, are important cellular effectors and give SM a role in cellular functions like apoptosis, ageing and development [25]. SM forms more stable complexes with cholesterol in comparison with other phospholipids. Results obtained during the last decade show a substantial lateral organization of both lipids and proteins in biomembranes. Sphingolipids, including SM, together with cholesterol, have been shown as important factors in formation of lateral domains or "rafts" formation in biological membranes. These domains have been suggested to take part in cellular processes, such as signal transduction, membrane tracking and protein sorting. The formation of lateral "rafts" in biological membranes is supposed to be driven by lipid-lipid interactions, which are largely dependent on the structure and biophysical properties of the lipid components [25,26].

Glycolipids are localized exclusively at extracellular side of plasmatic membrane. The sugar residues of glycolipids are therefore exposed to the external part of the cell and create the protective film, which surrounding most of the living cells. Glycolipids are represented by cerebrosides, sulphatides and gangliosides. As an example, monogalactosyldiglyceride (MG) and galactosylceramide (GC) are shown on Fig. 4.

Sterols of the membranes are constructed on the base of sterol backbone. Among sterols the



Fig. 3. The molecule of glycerophospholipid - phosphatidylcholine. (a) – schematic representation, (b) – chemical structure. The glycerophospholipid is composed of five parts: hydrophilic choline head group is connected through the phosphate residue to the glycerol backbone. The glycerol is connected with two hydrocarbon chain of fatty acids. This part create the hydrophobic part of the phospholipid. At the place of double bond the hydrophobic chain is tilted in respect to the direction normal to the membrane (Reproduced by permission from Ref. [2]).

cholesterol (CH) is typical only for living cells, but bacterial and plant cells do not contain this sterol. The plants contains stigmasterol (ST), and microorganisms – ergosterol (ES) (Fig. 5).

Lipid composition of various living cells is presented in Table 2. We can see that the basic lipids are phosphatidylcholine and phosphatidylethanolamine. Glycolipids are occurred in larger extent in a membrane of myeline.

There exist high variety of fatty acids in phospholipids. However, mostly two or three types of fatty acids are dominant in cell membranes. In higher plants there are mostly palmitic, oleyl and linoleyl acids. The stearoyl acid practically does not occurred there. The cell of living organisms contains in addition to palmitic and oleyl acids also the fatty acids with larger number of carbons – 20 and more. As a rule they are composed of even number of carbon atoms. The unsat-

Glycerophospholipids



Fig. 4. Structural formula of main lipids presented in biomembranes. PC - phosphatidylcholine, PE - phosphatidylethanolamine, PS - phosphatidylserine, PI - phosphatidylinositol, PG - phosphatidylglycerol, SM - sphingomyelin, MG - monogalactosyldiglyceride, GC - galactosylceramide, DPG - diphosphatidylglycerol (cardiolipin).



Fig. 5. Structural formula of main sterols presented in biomembranes. CH – cholesterol, ST – stigmasterol, ES - ergosterol.

Lipids	Plasma	Nucleus	Mitochon-	Myeline	Erythro-	E. Coli
	membrane		dria		cytes	
Phosphatidylcholine		44.0	37.5	10	19.0	0
Sphingomyelin		3.0	0	8.5	17.5	0
Phosphatidylethanolamine		16.5	28.5	20.0	18.0	65.0
Phosphatidylserine	7.0	3.5	0	8.5	8.5	0
Phosphatidylinositol	3.0	6.0	2.5	1.0	1.0	0
Lisophosphatidylcholine	2.5	1.0	0	-	-	_
Phosphatidylglycerol	18.5	-	_	-	-	18.0
Diphosphatidylglycerol	12.0	1.0	14.0	0	0	12.0
Other phospholipids	11.5	-	_	-	-	_
Cholesterol	19.5	10.0	_	26.0	25.0	0
Cholesterol esters	2.5	1.0	2.5	-	-	_
Fatty acids	6.0	9.0	_	-	-	-
Glycolipids	-	-	-	26.0	10.0	0
Other lipids	15.0	5.5	15.0	0.5	1.5	_

Tab. 2. Lipid composition of cells (% of the total mass of all lipids).

urated fatty acids contain double bonds almost exclusively in a cis-conformation. An example of fatty acid composition in an erythrocyte membrane of man is presented on Table 3. The phospho-

Fatty acid	PC	PE	PS	SM
C 16:0	34	29	14	28
C 18:0	13	9	36	7
C 18:1	22	22	15	6
C 18:2	18	6	7	2
C 20:4	6	18	21	8
C 24:0	_	I	I	20
C 24:1	_	-	_	14

Tab. 3. Fatty acid composition of the phospholipids of erythrocyte. PC – phosphatidylcholines, PE – phosphatidylethanolamines, PS- phosphatidylserines, SM - sphingomyelines.

lipids composed of unsaturated fatty acids are typical for biomembranes. Appearance of only one double bond in one chain of fatty acid considerably decreases the phase transition temperature from gel to liquid-crystalline state of phospholipid. For example dipalmitoylphosphatidylcholine (DPPC) composed of two saturated fatty acids - palmitic acids (16 carbons) has main phase transition temperature at approx. 41 °C. However, palmitoyloleylphosphatidylcholine (POPC) which differ from DPPC only by one double bond in one fatty acids the biomembrane at physiological temperatures are in a liquid-crystalline state.

In addition to above mentioned lipids there exist also another lipids that occurred less frequently in a membranes. Among these lipids we can mention plasmalogens. In the molecule of plasmalogen instead of acyl group at the first carbon atom of glycerol there is aldehyde group. Another phospholipid – cardiolipin (diphosphatidylglycerol) (Fig. 4) is important component of the membranes of mitochondria. It has been found that in cyanobacteria the nitrogen is replaced by sulphate and thus creating sulphophospholipids. These organisms are able to produce sulphocholine from cysteine and metionine, which protects cyanobacteria, so they can not be utilized by other organisms in sea. The membranes of the thermophilic and methane producing bacteria contain diphytanoyl glycerolethers. The fatty acid chains of these lipids are covalently connected in a middle side of the membrane. This resulted in highest stability of the lipid bilayer that protect the membrane from disruption at higher temperatures as well as against dissolution effect of methanol.

Distribution of the lipids in a membrane is highly asymmetric. The glycolipids are exclusively located at outer monolayer of the membrane. The assymmetry in a distribution of phospholipids in the membrane of erythrocyte is shown on Fig. 6. In the outer monolayer are localized the majority of two choline containing phospholipids, sphingomyelin and phosphatidylcholine. The two amino – phospholipids are predominantly (phosphatidylethanolamine) or even exclusively (phosphatidylserine) localized in the cytoplasmic half of the bilayer [27]. The uncatalyzed exchange of lipid molecules between monolayers is very slow and probably does not exist for proteins. Transbilayer movement is energetically unfavorable because it requires the insertion of the polar groups into the nonpolar region and the exposure of the apolar groups to the polar region. Slow uncatalyzed transbilayer movement (the so called flip-flop) of some phospholipid



Fig. 6. Transbilayer distribution of phospholipids in the human erythrocyte membrane. TPL - total phospholipid, SM - sphingomyelin, PC - phosphatidylcholine, PE - phosphatidyletanolamine and PS - phosphatidylserine. (Reproduced by permission from Ref. [27]).

has half-time value of 3 to 27 h (phosphatidylcholine), whereas certain phospholipids (phosphatidylethanolamine) are subject to an ATP-dependent "flippase" – catalyzed inward movement with a half-time of approximately 30 min. Probably much faster is the transbilayer movement of cholesterol, revealing half-time value in order of seconds.

All membrane components are recycled many times during the life of the cell. The life time of the phospholipids depends on the intensity of function of the membrane. For example the life time of phosphatidylcholine in a myeline membrane is 2 months, while in membrane of mitochondria, where extensive oxidative processes take place it is only 2 weeks.

2.3 Membrane proteins

Membrane proteins play important functional role in a cell. They form ionic channels, transporters, receptors and enzymes. Certain proteins play also structural roles. An example is spectrin net located in cytoplasmic side of the membrane (Fig. 7) [2]. The membrane proteins are divided into three main groups – peripheral proteins, integral proteins (see Fig. 1) and structural proteins. The enzymes are most widespread proteins in a membranes. They can be both integral (ATPases) and peripheral (acetylcholinesterase, phosphatases). The receptors as well as immunoproteins could also be peripheral or integral. The receptor proteins are usually connected with additional proteins in cytoplasmic side of the membrane for transfer the signal inside the cell. Among these proteins the G-proteins play important role in cell signaling. Cell signaling and the role of G-proteins will be described in more detail below (see part 10.2).

Peripheral proteins, e.g. cytochrom c, are localized at the membrane surface and are connected with a membrane either by means of electrostatic interactions or they contain short hy-



Fig. 7. Membrane proteins and their functions: A - ionic channel, B - Diffusion transporter; C - Receptor, D - Enzyme (transforms substrate S to the product P).

drobhobic chain, that allows to anchor peripheral protein to the membrane. Peripheral proteins can be isolated by means of changes the pH or ionic strength.

Integral proteins, e.g. glycophorin, Na,K ATPase, bacteriorhodopsin etc. are translocated across the lipid bilayer. In addition to hydrophilic part, which contacted with water environment, they are characterized also by hydrophobic part, that contacted with hydrophobic interior of the membrane. Integral proteins have various degree of complexity and can pass through the membrane only once (glycophorin), or several time (bacteriorhodopsin - this protein 7 times cross the membrane [2]). Integral proteins are more tightly connected with the membrane then peripheral proteins. The membrane architecture as well as functioning of membrane proteins is determined by protein-lipid interactions. This question will be considered below (part 6.3). The isolation of integral proteins is more difficult in comparison with peripheral proteins. For this purpose it is necessary to use organic solvents or detergents. As we already mentioned, organic solvents, like the mixture of chloroform/methanol, allows to isolate integral proteins, however, after the isolation the proteins may lost the activity. They can be use, however, for structural studies, but usually are not suitable for functional studies. Most common is isolation of integral proteins by detergents. Among detergents, the ionic detergent, sodium dodecylsulphate, sodium cholate or non ionic detergent, like Triton X-100 are most common. Application of sodium cholate and Triton X-100 is, however most soft for preserving the function of proteins. The role of the detergents consists in disturbing the lipid bilayer and in formation of detergent-protein complexes, that are soluble in water. Also the lipid molecules forms complexes with detergents. The molecules of detergent are of conical shape. Therefore in a water they form micelles. The process of solubilization of integral proteins is showed on Fig. 8. The disadvantage of application of detergents, however consist in the fact that detergents remain adjacent to the proteins, therefore additional methods of purification should be used to receive pure protein fraction. In recent time the synthetic methods are most preferable in synthesis of short peptides for model studies and biotechnologies for production of some proteins of high purity.



Fig. 8. Scheme of the isolation of integral proteins by detergent.



Fig. 9. The schematic representation of cytoskeleton of erythrocyte membrane.

Structural proteins form the membrane cytoskeleton. An example is the spectrin localized at cytoplasmic side of erythrocyte membrane. The structural proteins do not belong exactly to the membrane proteins, but are connected to the membrane through integral proteins. For example the band III protein of erythrocytes is connected with small protein ankerine. The spectrin threads are connected to the ankerin (Fig. 9). The spectrin net together with microtubules and microfilaments protect the cell against changes of the shape or changes of the cell volume. The main protein of cytoskeleton is tubuline. Tubuline is able to form aggregates and forms tube-like structures. The integrity of these structures is possible only at very low concentration of Ca^{2+} ions (usually 10^{-7} M). Increase of calcium concentration in cytoplasmic side of the cell can destroy the cytoskeleton. Therefore Ca^{2+} pumps continuously provide very low level of calcium in a cell by either removing it into cytoplasmic reticulum or outside the cell. Cytoskeleton considerable stabilizes the integrity of the cell membrane. Important factor of this stabilization are also intercellular contacts that are created by collagen. All living cell except erythrocytes and lymphocytes have the cell envelope – the glycokalix.



Fig. 10. Incorporation of synthesized proteins into the membrane. A – signal peptide, B - signal peptide connected with receptor, C -receptor facilitate the incorporation of the peptide into the membrane, D – after incorporation of the peptide, the signal part is removed and polysaccharides are attached, E – ribosome is separated from the membrane. (According to Ref. [28]).

The life time of membrane proteins is from 2 to 5 days. Therefore there exist mechanism that provides transport of newly synthesized membrane proteins to the membrane. The synthesis of the protein is started at the ribosomes inside of the endoplasmatic reticulum (Fig. 10). The growth of the polypeptide chain starts from N-terminal. First the special sequence of the chain is synthesized that is recognized by membrane receptor. As soon as the polypeptide chain is sufficiently long, it separates from the receptor, but preserve the connection with ribosome. After the synthesis the protein is separated from ribosome [28].

3 Models of the membrane structure

The study of physical properties of the biomembranes is rather difficult due to small size of the cell (typically several μ m), small thickness (5-10 nm), considerable inhomogeneity and anisotropy. In addition it is difficult to study separately the properties of the lipid bilayers and influence of proteins to the bilayer. Therefore biophysical studies of the membrane properties have been performed on various models of membrane structure, such are lipid monolayers, multilayers, bilayer lipid membranes (BLM), multi- or unilamellar vesicles and supported bilayer lipid membranes (sBLM).

Historically the first models of membrane structure were the lipid monolayers, that played considerable role in establishing the bilayer nature of biomembranes at the first third of the XX century. The stable bilayer lipid membranes have been reported in 1962 by Mueller and co-workers [29]. Finally in 1965 Bangham with co-workers [30] discovered the liposomes, that becomes most popular and most widely used model system for study the physical properties



Fig. 11. a) Expanded and condensed monolayer on a water surface. b) Scheme of deposition of the monolayers onto a hydrophobic surface (e.g. mica). The immersed surface of the surface becomes hydrophilic after deposition of the first layer and becomes hydrophobic after deposition of the 2 nd layer.

of biomembranes. Lipid membranes on a solid support has been reported by McConnel et al. in 1988 [31]. Below we present the methods of formation and basic physical and structural properties of these systems.

3.1 Lipid monolayers

Lipid monolayers are formed spontaneously at an air-water interface. This is due to the amphiphilic nature of the lipids. When lipids are dissolved in a non-aqueous volatile solvent and introduced onto a polar liquid surface, the solvent will evaporate leaving the lipid molecules oriented at the liquid-gas interface. The polar head groups pulling the molecule into the bulk of the water and the hydrophobic chains are oriented into the air. Sweeping a barrier over the water surface causes the molecules to come closer together and eventually to form compressed and ordered monolayer – much like forcing together balls on a billiard or pool table (Fig. 11a).

The formation of thin oil films on an air-water interface has been firstly reported in XVIII. century by Benjamin Franklin. During his visit London in 1773 he observed that one teaspoon of oil sputtered on a water had calming influence over half of an acre (2000 m²) of water. Taking into account the volume of oil 5 ml this would mean that a film thickness 0.25 μ m (about 100 layers) was covering this surface. Franklin reported his finding to the Royal Society of London in 1774. The investigation of the properties of oil films on an air-water interface has been started by Agnes Pockels with very simple trough in her kitchen. She reported her results in letter to Lord Rayleight and published in Nature in 1891. She as a first performed experiments with monolayers using the barrier.

Considerable progress in physical studies of monolayers has been achieved thanks to the work by Irving Langmuir. Langmuir studied the relationship between the pressure and area on an aqueous surface. Further Katherine Blodgett, who worked with Langmuir, developed the

technique of transferring the films onto solid substrates (Fig. 11b). A brief history of Langmuir-Blodgett films has been published by Gaines [32].

Below we consider basic principle of monolayer thermodynamics and the properties of lipid monolayers at an air-water interface. We also include basic principles of dipole potential and Maxwell displacement currents of the monolayers. The basic physical value that characterizes the lipid monolayer is surface tension γ .

3.1.1 Surface tension

The changes of internal energy at the solid-liquid interface is characterized by equation [33]

$$dU = TdS + \sum \mu_i dn_i - PdV + \gamma dA,\tag{1}$$

where U is the internal energy of the system, S is the entropy, μ_i and n_i are the chemical potential and the mole number of component *i*, respectively, A is total interfacial area and γ is the surface tension of the interface.

Since the free Gibbs energy G = U - TS + pV, it follows that at constant pressure, p, and using the surface excess quantities

$$dG^{ex} = -S^{ex}dT + \sum \mu_i dn_i^{ex} + \gamma dA \tag{2}$$

and

$$\gamma = \left| \frac{dG^{ex}}{dA} \right|_{T,p,n_i}.$$
(3)

In the case of a pure liquid in an equilibrium with its saturated vapor, the surface tension is also equal to the surface excess of the Helmholtz free energy (F = G - pV) per unit area

$$\gamma_0 = F_0^{ex} / A. \tag{4}$$

To illustrate the physical meaning of the surface tension, let us consider the lipid film at the air water interface of the Langmuir-Blodgett trough (Fig. 12a). The lipid molecules in the insoluble monolayer can move only parallel of the water surface. The molecules hit against movable barrier creating the surface pressure π . Then the work done in extending the movable barrier a distance dx is: $\pi l dx$. On the other side, the change of surface energy at the exchange of lipid monolayer on purely water surface is $(\gamma_0 - \gamma) l dx$, where γ_0 and γ are surface tension of the water and monolayer, respectively. Thus,

$$\pi = \text{Force}/l = \gamma_0 - \gamma. \tag{5}$$

The surface tension is then a force per length unit expressed as Nm⁻¹. As an example, the surface tension of pure water, $\gamma_0 = 72.75 \text{ mN m}^{-1}$ [34]. A fluid interface, such as air/water interface, has the advantage of being a plane interface. The changes in interfacial free energy of this interface can be estimated by measurement of surface pressure. The most common method of measurement the surface pressure is the Wilhelmy method [35]. According to this method a thin plate, usually made of glass, mica, platinum or filter paper, is partially immersed in the liquid phase and is connected to an electromicrobalance. The forces acting on the plate are its weight



Fig. 12. a) Langmuir-Blodgett trough with lipid monolayer; b) schematic representation of the principle of the measurement the surface pressure. For description see the text.

 F_p and surface tension effects downward, and Archimedes buoyancy F_a upward (Fig. 12b). The net downward force is

$$F = F_p + 2\gamma(w+t)\cos\Theta - F_a,\tag{6}$$

where w and t ($t \ll w$) are the width and the thickness of the plane, respectively, and Θ is the contact angle of the liquid with solid plate. If the plate is completely wetted, the contact angle $\Theta = 0$ and $\cos \Theta = 1$, so that

$$F = F_p + 2\gamma w - F_a. \tag{7}$$

When the composition of the interface varies, F_p and F_a (provided the plate is maintained in a fixed position) stay constant, then $\Delta F = 2w(\gamma_{solution} - \gamma_{water}) = -2w\pi$ and

$$\pi = -\Delta F/2w. \tag{8}$$



Fig. 13. Scheme of the Maxwell displacement currents (MDC) experimental set-up in the a) gas state and b) solid state of the lipid monolayer. The monolayer is formed between the upper electrode, suspended approx 1.5 mm above the subphase surface (1) and electrode immersed in the subphase (2). Molecules are forced to change of their orientation, which is accompanied by the flow of the current. Currents induced during compression are detected by sensitive electrometer (3). Change of the orientation of the molecules results in the change of the vertical component of dipole moment (M_1 and M_2 correspond to dipole moments at the gas and solid states, respectively).

3.1.2 Dipole potential

The dipole potential ΔV of the monolayer is difference between the potential of a monolayer and that of clean subphase. The measurement of dipole potential allows to analyze changes in the orientation of the molecular dipoles in the monolayer during compression. Presence of the monolayer between electrodes causes changes of the potential based on the Helmholtz equation

$$\Delta V = \mu_n / (A \varepsilon_r \varepsilon_o), \tag{9}$$

where ε_r and ε_o are the relative dielectric constant of the air ($\varepsilon_r \approx 1$) and the permittivity of vacuum, respectively, μ_n is the normal component of the dipole moment of the molecule and A is the molecular area. The dipole potential was measured by means of vibrating plate method [36] using high sensitive electrostatic voltmeter 320C and electrode 3250 (Kelvin probe) (TREK Inc., USA), that allowed measuring dipole potential with an accuracy of 1 mV. The dipole potential of the phospholipid monolayers is in the order of several hundred mD [36].

3.1.3 Maxwell diplacement current

The Maxwell displacement current (MDC) represents the sum of the contributions coming from changes of the orientations of the molecules (dM_z) , changes of the number of the molecules under the upper electrode (dN) (Fig. 13) and changes of the surface potential of the pure subphase $(d\phi)$

$$\frac{dQ}{dt} = I = \frac{N}{d} \frac{dM_Z}{dt} + \frac{M_Z}{d} \frac{dN}{dt} + \frac{\varepsilon_0 S}{d} \frac{d\phi}{dt},\tag{10}$$

where d is the distance between electrode 1 and water surface, S is the area of electrode 1 and ε_0 is the dielectric constant of vacuum [37]. Because the surface potential of the pure subphase

is constant during the experiment, only first two components participate on the MDC. Apparatus for measurement MDC is based on two parallel electrodes. One is immersed in the water and grounded and the second is placed in the air approx. 1.5 mm above the subphase and electrically shielded. The area of the top electrode is usually approx. 20 cm². Current can be measured by sensitive electrometer e.g. Keithley 617 (Keithley Instruments, USA) (see e.g. [38]). The MDC is usually recorded simultaneously with surface pressure – area isotherms.

The MDC technique is sensitive only to dynamic charge processes, which in described experimental setup are caused by lateral compression of the lipid monolayer. Therefore any timeindependent charge (mainly structured water layer and additional substances in subphase) distributed near/at the interface has no effect on the MDC. In comparison with conventional electrical measurements of surface potential (by the Kelvin probe method) MDC technique is of considerable advantage for time-depended studies of reorientation of the molecules in the monolayer. Dipole moment projection analysis by MDC method is very sensitive for evaluation of molecular orientation (so-called 'order parameter') as well as electric state and/or conformation changes of the molecule. This method can be rather informative for study the phospholipid/protein phase transitions and influence of monolayer composition on the intermolecular interactions. In contrast with surface pressure – area isotherm analysis, MDC measurement is extremely sensitive also in the low surface pressure area, where other methods such are excess area, Gibbs free energy, elastic modulus are much less informative.

3.1.4 Properties of lipid monolayers

The phospholipids form stable monolayers at an air-water interface. The forces between the polar head groups are of electrostatic nature and are proportional to $1/r^2$ (r is the intermolecular separation). The forces between hydrocarbon chains are due to van der Waal's interactions and are proportional to $1/r^6$ (attractive forces) and $1/r^{12}$ (repulsive forces). Thus, the interactions in the subphase are of longer range than those in superphase. When the lipids are spread at sufficiently large surface and no external pressure is applied to the monolayer, the molecules behave as a two dimensional gas (Region G on Fig. 14), which can be described by equation

 $\pi A = kT,\tag{11}$

where π is the surface pressure, A the molecular area, k is the Boltzman constant and T is the thermodynamic temperature. Further compression resulted in the film ordering that behave like two dimensional liquid. This liquid expanded state (L-E) is shown on Fig. 14. With continued compression the L-E state turns into liquid condensing state (L-C). Further compression resulted in solid state of the film (S). This solid state is characterized by a steep and usually linear relationship between surface pressure and molecular area. The collapse pressure, π_C , is reached at further compression at which the film irretrievably loses its monomolecular form. The forces exerted upon it become too strong for confinement in two-dimensions and molecules are ejected out of the monolayer plane into either the subphase (more hydrophilic molecules) or the superphase (more hydrophobic molecules). However, collapse is not uniform across the monolayer but is usually initiated near the leading edge of the barrier or at discontinuities in the trough-such are corners or the Wilhelmy plate. Usually a collapsed film will consist of large areas of uncollapsed monolayer contained the islands of collapsed regions. The value of the collapse pressure varies depending on the phopsholipid structure and temperature. For simple saturated fatty acid the



Fig. 14. (a) Compression isotherm of the monolayer: G – gass state; L-E liquid expanded state; L-C liquid condensed state; S solid state; C – collapse; π_C collapse pressure; π_t transition pressure (at the beginning of the L-E-LC transition); At mean area at π_t , A_0 limiting area; A_S , area in the solid state. The arrow indicates the direction of the compression. (b) Schematic representation of the monolayer structures at different state.

collapse pressure can be in excess of 50 mN m⁻¹ which is equivalent to about 200 atmospheres if extrapolated to three dimensions.

The $\pi - A$ isotherms for real monolayers can be well described by two-dimensional analog of van der Waal's equation

$$\left(\pi + \frac{a}{A^2}\right)(A - b) = kT,\tag{12}$$

where a is the van der Waal's constant that characterizes the intermolecular interaction, b is the effective area of molecule cross-section ($b \approx A_0$).

Ouantitative information can be obtained on the molecular dimensions and shape of the molecules under study. When the monolayer is in two-dimensional solid (S) or liquid condensed state (L-C) the molecules are relatively well oriented and closely packed and the zero pressure molecular area can be obtaining by extrapolating the slope of solid (A_S) or liquid-condensed (A_0) phase to zero pressure – the point at which these lines crosses the x-axis. This point correspond to the hypothetical area occupied by one molecule in either solid or liquid-condensed state (Fig. 14). The shapes of the $\pi - A$ isotherms of lipid monolayers depends on temperature. This is shown on Fig. 15 where the general character of $\pi - A$ isotherms for monolayers formed from phosphatidylcholine composed of saturated fatty acid is presented. The isotherm labeled T7 represent monolayers at liquid crystalline state. The curve labeled T6 represents a lipid at the transition temperature. The transition of the lipid occurs very close to the collapse pressure. The other curves represent monolayers at lower temperatures. They exhibit monolayer phase transition (region tending toward horizontal) from fluid to rigid films at pressures that are lower, the lower the temperature. T1 represents an isotherm that is almost entirely of a solid film [39]. The phase transition in a monolayer could take place also in a constant pressure. For example in a narrow temperature interval (~ 2 °C) in a monolayer composed of myristine acid the phase



Fig. 15. Relationship between surface pressure and the area per molecule for lipid monolayers at an airwater interface (According to [39] reproduced by permission).

transition from condensed to a liquid expanded state takes place. This transition is accompanied by increase of the area per molecule since 0.21 nm^2 to 0.4 nm^2 [18]. The calculation of thermodynamic parameters of the monolayers at phase transitions is discussed in Ref. [40].

The $\pi - A$ isotherms can be effectively used for estimation the area per molecule of phospholipid. This area depends on both the structure and charge of head group as well as on length and degree of saturation of the hydrocarbon chains. As an example, the Fig. 16 represents the $\pi - A$ isotherms of lipid monolayers composed of different phosphatidylcholines: dioleoyl phosphatidylcholine (DOPC) has both chains unsaturated, soy bean phosphatidylcholine (SBPC) has polyunsaturated fatty acids chains, egg phosphatidylcholine (eggPC) has 50 % saturated and 50 % unsaturated chains and dipalmitoylphosphatidylcholine (DPPC) has both chains saturated. It is evident that, at any fixed surface pressure the area per molecule is in following order: DOPC > SBPC > eggPC > DPPC. Figure 17 schematically illustrates the area per molecular distance was calculated to be 10.6 Å, 10.0 Å, 9.7 Å and 8.1 Å (1 Å = 0.1 nm) at a surface pressure of 20 mN m⁻¹ (1 mN m⁻¹ = 1 dyn.cm⁻¹) [41]. Thus, a change in the saturation of the fatty acid resulted in changes of intermolecular distance in the monolayer.



Fig. 16. Surface pressure-area isotherms of \Box - DOPC, \bullet - SBPC, \triangle - eggPC and \circ - DPPC (According to [41] reproduced by permission).



Fig. 17. Schematic representation of the area per molecule and intermolecular distance on DOPC, SBPC, eggPC and DPPC based on the data plotted in Fig. 16. (According to [41] reproduced by permission).

If the monolayer is composed of the mixture of different phospholipids, then depending on the structure of phospholipids the monolayer could be less or more densely packed. Obviously, in a constant surface pressure and in the case of ideal miscibility or in the case of lack of miscibility the plot of the average area per molecule as a function of concentration of the one of the component should be straight line

$$A_{12} = xA_1 + (1 - x)A_2, (13)$$

where x is the mole fraction of the component A_1 and A_1 and A_2 are extrapolated to the "zeropressure" areas for corresponding phospholipids. Any deviation from linearity indicates changes in the miscibility of the monolayer components and can indicate of formation supermolecular assemblies or domains. These domains can be observable by fluorescence microscopy [42]. The lipid monolayers can be effectively use also for study the mechanisms of protein-lipid interactions [35], the mechanism of functioning of phospholipases [43]. Wide application of monolayers are connected with nanotechnologies. The Langmuir-Blodgett method of deposition of lipid monolayers on a solid support allowing to prepare biosensors composed of thin films as well as to use another powerful techniques for study the physical and structural properties of thin films, such are Fourier transform infrared spectroscopy (FTIR), atomic force microscopy (AFM), scanning tunneling microscopy (STM) and other methods (see Ref. [44] for application of lipid monolayers).

The exact correspondence of the properties of lipid monolayers to the properties of biomembranes is, however, still under discussion [6] and is particularly connected with selection of the surface pressure of monolayers that most adequately corresponds to that of bilayers as well as with the questions concerning the mechanisms of interaction between two monolayers that create the bilayers. Marsh suggested that π should be in range 30–35 mN m⁻¹ [45]. However, the main transition temperature, T_M , for DPPC then occurs about 5 °C to low compared to bilayers. Other authors suggest $\pi = 50 \text{ mN m}^{-1}$ [46]. This give correct T_M , but the area per molecule at T = 50 °C for DPPC monolayers is less then that of bilayers [6]. The prediction of bilayer properties using monolayers could be most correct in the case when no specific interaction exists between two monolayers. There is, however evidence, that such interaction should exists [6]. Despite of this, it is obvious a great advantage of application of monolayers for study the surface properties of lipid membranes. The monolayers allows to measure much easier the area per phospholipids then bilayers. It is also easy to measure dipole potential of the monolayers and to study the adsorption processes at the water-monolayer interface [47]. This advantage together with other new applications in nanotechnologies show that lipid monolayers represent very attractive object for physics as well as for bioelectrochemistry. The study of the thermodynamic properties of lipid monolayers can be performed by precise Langmuir-Blodget through (e.g. NIMA Technology Ltd. produces of through of different size including tensiometers and deepers for deposition films on a solid support [48]).

Monolayer technique allowing to study not only pure lipid monolayers but also their mixtures with proteins and short peptides (see Ref. [35] for review). They are also usefull for study the peculiarities of DNA hybridization at the surfaces [49] as well as for study the properties of the lipid monolayers with incorporated artificial receptors, e.g. calixarenes [50]. Other applications, including the study of the mechanisms of enzymatic reactions at interfaces can be found in Ref. [51].

3.1.5 Properties of peptide monolayers

It is interesting that also proteins or short peptides can form stable monolayers at an air-water interface [35]. As an example we will present results obtained recently in our laboratory that describe the thermodynamic and electric properties of monolayers formed by gramicidin A [52].

Gramicidin A (gA) is bacterial peptide composed of 15 amino acid residues secreted from Bacilus brevis. Due to its relatively simple structure it is one of the best studied short peptide and it is often used as a model of integral membrane protein [53,54]. The sequence of gA was for the first time reported by Sarges and Witkop [55] and is as follows: formyl-L-Val¹-D-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp¹⁵-ethanolamine. This structure has specific combination of altering L- and D- hydrophobic residues that is responsible for helical conformation of gA. It is known that gA easily incorporates into the bilayer lipid membrane (BLM). Extensive studies of gA in BLM revealed that in lipid environment this peptide maintains single-stranded $\beta^{6,3}$ conformation [56]. In a lipid bilayer two gA monomers are oriented with their N-terminals (formyl end) head-to-head, resulting in the formation of the ion channel that spanning the membrane. According to NMR studies the length of gA in DMPC bilayers is 2.5 nm [57]. Cross sectional area of gA in these bilayers was estimated as 2.5 nm² [58]. Inner diameter of such a channel is ~ 0.4 nm [59]. The channel is permeable for monovalent, but not for divalent cations [60]. However, in solution and depending on surrounding solvent as well as in a crystalline form, several different interwined helical dimers were reported. These double helical dimers were found also in vesicles of polyunsaturated lipids as well as in planar bilayer lipid membranes at certain conditions (see Ref. [54] for review). Among various secondary structures the double-strand intervined $\beta^{5,6}$ helix is of special interest. It has similar length (2.6–3.0 nm) like gA channel in $\beta^{6,3}$ conformation, and thus represent structural alternative to dimer model of the gA channel in a membrane.

Due to amphiphilic properties, gA forms stable monolayers at an air-water interface. Detailed analysis of PM-IRRAS spectra at several fixed area per molecules in combination with X-ray reflectivity results showed, that at relatively large molecular areas gA is in disordered secondary structure, however at smaller molecular areas it adopts $\beta^{5,6}$ helical conformation with 30° orientation with respect to the direction perpendicular to a monolayer [61]. Application of PM-IRRAS methods to gA in a lipid monolayers revealed, that gA could be in $\beta^{6,3}$ conformation [62]. However, final conformation of gA depends on gA/phospholipid ratio. The $\beta^{6,3}$ conformation is preferred at low gramicidin content in a lipid bilayer [53].

The properties of mixed gA – phospholipid monolayers are rather complex. It has been found that gA aggregates in the dipalmitoyl phosphatidylcholine (DPPC) monolayers in a gel state at very low concentrations (8×10^{-4} mol%). The aggregation was accompanied by formation of flat subunits with "doughnut" shape (up to ~ 150 nm in diameter). Thermodynamic studies of such a system revealed maximum of miscibility of both DPPC and gA at ~ 28 mol% of gA [63]. In this work it was confirmed, that smallest aggregation unit of gA is hexamer, surrounded by 16 lipid molecules.

Important peculiarity of gA is connected with existence of dipole moment caused by presence of tryptophan residues and their indole dipoles. These dipoles interact with dipoles of polar groups of phospholipids and water molecules at a bilayer-water interface [64,65]. Reorientation of the gA and lipid molecules occurred during compression of monolayers resulted in changes of dipole potential, which can be monitored by means of Kelvin probe [66-68]. In Section 3.1.3.



Fig. 18. a) The plot of surface pressure b) dipole potential (curve 1) and dipole moment (curve 2) as a function of mean molecular area for monolayers of pure gA. $T = 18 \text{ }^{\circ}\text{C}$ [52].

we shortly introduced the contactless method for study the physical properties of monolayers – the technique based on measurement Maxwell's displacement currents (MDCs). MDCs are generated during compression of the monolayer. This method is suitable to study reorientation of the molecules in the lipid monolayers. Although MDC technique is relatively widespread, only recently it has been applied for study the reorientation of peptides in lipid monolayers [52].

It is expected that formation of secondary structure of gA in a monolayer should be accompanied by changes of dipole moments of molecules and thus should be monitored by MDC technique.

3.1.5.1 Pressure-area isotherms of gA monolayers

As we mentioned above, gA forms stable monolayers at the water subphase. It is seen on Fig. 18a, where the plot of pressure vs. area per molecule is presented for gA monolayer at T = 18 °C. Pressure-area isotherm has typical shape and is in agreement with previously published results (see e.g. Ref. [61]). It is seen that gaseous phase (G) at relatively low pressure at which the area per molecule is $> 7 \text{ nm}^2$ is transferred to a liquid expanded phase (L-E). Further compression of the monolayer is accompanied by characteristic shoulder with clearly visible plateau at surface pressure $\pi \sim 14$ mN m⁻¹. Detailed analysis of the data obtained by PM-IRRAS and X-ray reflectivity studies [61] allowed to connect physical properties of the gA monolayers with changes of secondary structure of gA molecules. The first increase of surface pressure between 0 to 10 mN m⁻¹ was attributed to formation of interwined β -helix from disordered secondary structure. At the plateau region between $10-20 \text{ mN m}^{-1}$ only density of gA monolayer increases, but molecules are oriented parallel to the monolayer. Sharp increase of surface pressure above 20 mN m⁻¹ was attributed to tilting of the β -helix. The collapse of the gA monolayer took place at approx. 46 mN m⁻¹, which suggest very good monolayer stability. Extrapolation of the part of the isotherm corresponding to the solid state (at surface pressure above 30 mN m⁻¹) to the zero surface pressure allows to estimate molecular area per gA molecule, which is approx. 2 nm²·molecule⁻¹. This value is in good agreement with the results obtained by the other authors [63]. The pressure-area isotherms were recorded also at higher temperature 28 °C and had similar shape and properties like that for 18 °C (results are not shown). It is interesting that similar shape of the area-pressure isotherm was observed also for other short α -helical peptide L₂₄ [69]. It is likely, that the transition from disordered to ordered secondary structure for peptides of comparable length took place at similar surface pressures.

3.1.5.2 Dipole potential of gA monolayers

The plot of dipole potential vs. area per molecule (curve 1) is presented on Fig. 18b. It is seen that dipole potential monotonously increases with decreasing the area per molecule until $A \sim 1.7 \text{ nm}^2$, when sharper increase of the dipole potential starts. The start of this increase correspond to transition of monolayer into a well ordered film. In this region gA molecules are very close to each other and due to the existence of dipoles of tryptophan at ethanolamine end, the gA molecules interact more strongly. Therefore the final value of the dipole potential reflects not only the changes of the orientation, but also interactions between gA molecules. Using the dipole potential it is possible to estimate dipole moment, μ_n , of gA (see Eq. (9)). In assumption that the relative dielectric constant of the medium (air) is 1, the Eq. (9) can be transformed according to Gaines [70] into the form

$$\Delta V = 12\pi\mu_n/A,\tag{14}$$

where ΔV is dipole potential in mV, A is the area per molecule in Å²/molecule and μ_n is the dipole moment in milliDebye (mD) units. The plot of dipole moment of gA as a function of mean molecular area is shown on Fig. 18b (curve 2). The dipole moment at the area per molecule of 2.5 nm², when gA has already well formed secondary structure is around 0.5 D. This value is similar to dipole moment of phospholipids [36]. Small dipole moments at the beginning of compression are due to not established secondary structure of gA as well as due to random orientation of gA molecules. The region of small growth of dipole moment corresponds to the shoulder at $\pi - A$ isotherm, when gA has already established secondary structure. More detailed information on the kinetics of changes of dipole moment during compression can be obtained by means of MDC method.

3.1.5.3 MDC of gA monolayers

The plots of MDC as a function of area per molecule for pure gA monolayer during three compression-expansion cycles are presented on Fig. 19. Only curves corresponding to compression are showed for simplicity. The monolayer was compressed only up to surface pressure 35 mN m^{-1} at which the molecules are tightly packed, but were still in monolayer form. One can see from Fig. 19 the sharp increase of the current at beginning of the compression that can be connected with the condensation of the gA, formation of ordered secondary structure as well as with certain reorientation of gA molecules during compression. The increase of the number of the molecules under the electrode could also contribute to this effect. Please note that in contrast with the MDC, there is no sharp increase of dipole potential at the beginning of compression (Fig. 18b). This is connected with the fact that MDC is more sensitive to reorientation of dipole



Fig. 19. Maxwell displacement current versus mean molecular area for pure gA monolayer for three subsequent compressions up to 30 mN/m. The number of compression is showed at corresponding curve. $T = 18 \degree C$ [52].

moments of molecules in comparison with dipole potential measurements. Further increase of the current starting from 6 nm^2 ·molecule⁻¹ can be connected with increase of the number of molecules under the electrode. The reason of the "hairy" shape of the first compression curve is probably due to not well-ordered monolayer. Subsequent expansion, followed by compressions, resulted better ordering of gA monolayers. Both curves corresponding to the second and the third compressions have higher and more narrow peak at the region $\sim 7.2 \text{ nm}^2 \cdot \text{molecule}^{-1}$. This is probably due to better-oriented molecules, and hence better distribution of gA, under the electrode, comparing to the first compression. On the other side, increase of the current at the lower molecular areas is not so dramatic in comparison with first compression, but slowly increases up to $\sim 4 \text{ nm}^2$ ·molecule⁻¹, when the current reaches steady state value of approx. $20 \times 10^{-15} \text{ A}$ (i.e. 20 fA). The MDC is rather low. However, the background current for a pure water subphase without monolayer is around 1–3 fA, i.e. more then one order of magnitude lower in comparison with those for monolayer. Detailed analysis performed earlier [71] showed, that generation of the MDC is not connected with such phenomena as motion of intrinsic ions or ionization of the monolayer and is attributed to reorientation of dipole moments of the molecules from which the monolayer is composed.

More detailed insight into the current – area relation can be seen in Fig. 20 where both surface pressure and current are plotted as a function of area for the third compression – expansion cycle. The stability of the monolayer and the process of the reversibility are proved by almost the same shape of the isotherms corresponding to both compression and expansion. The same holds for the behavior of the MDC, while the shape of the current – area curve at the expansion is the mirror picture of the curve at the compression.

The current starts to grow immediately from the beginning of the compression, although the



Fig. 20. Surface pressure (dashed line) and Maxwell displacement current (full line) versus mean molecular area of pure gA monolayers for compression and expansion cycles. The direction of compression and expansion is showed by arrows. $T = 18 \,^{\circ}$ C [52].

surface pressure is still zero. As it was already mentioned above, current – area plot can be divided on two parts – first one at the higher areas comes from the formation of ordered secondary structure and possible changes of orientation of the gA molecules as well as due to increased number of the molecules under the electrode. Second one at the lower areas is due to change of the number of the molecules under the electrode. It is likely, that biphasic changes of the current at the beginning of the compression could be connected with formation of secondary structure of gA, namely interwined $\beta^{5,6}$ helix. The current starts to be constant at ~ 4 nm²/molecule, at the area that is characterized by the appearing the plateau on the isotherm. We should, however note, that exact type of secondary structure can be determined by spectroscopic, but not by MDC method. According to Ref. [61], at this region the secondary structure of gA is already established and the molecules are uniformly ordered in parallel to a monolayer. The sharp increase of the current after 2 nm²/molecule can be connected with changes of orientation of gA. This is in good agreement with the results obtained by PM-IRRAS and X-ray reflectivity studies [61].

3.1.5.4 Dipole moment of gA

The obtained values of MDC allowing to estimate dipole moment of gA molecules. The vertical component of the molecular electric dipole moment is proportional to the changes in the induced charge on the electrode 1 (Fig. 13) and can be expressed as follows

$$M_z = \frac{G}{N} \int_0^t I(t)dt, \tag{15}$$

where G is geometric factor (in ideal case it is equal to distance from electrode 1 to the subphase [71]) and N is the number of the molecules under the electrode 1 (see Fig. 13). Generally,



Fig. 21. Dipole moment versus mean molecular area for gA monolayer for three subsequent compressions calculated from MDC presented on Fig. 19. The number of compression is showed at corresponding curve. $T = 18 \text{ }^{\circ}\text{C}$ [52].

geometric factor has complicated form and depends on the shape of the electrode, distribution of the charges, shielding etc. Therefore, determination of the dipole moment requires exact knowledge on the geometric factor. In order to avoid problems with geometric factor we expressed the values of dipole moments in arbitrary units (a.u.).

Figure 21 shows the plot of dipole moment vs. area for gA monolayer calculated using the data presented on Fig. 19. We can see that the curve 1 corresponding to first compression is rather different from that corresponding to second and third compressions. The shape of the curve 1 reflects complicated process of the formation of the monolayer. After the preparation of the monolayer the molecules are randomly distributed and the transitions from one structural state to another do not occur at one moment, but in small steps. These steps are well resolved at areas 5 and 4.7 nm^2 ·molecule⁻¹. The dipole moment reaches maximum at the area of 3 nm^2 ·molecule⁻¹. The shape of the curves of the subsequent compressions is different from that corresponding to the first compression. It can be expected that after first compression – expansion cycle certain part of the molecules is not oriented parallel to the surface but molecules are tilted under some angle from the position normal to the surface. It is also possible, that molecules form small aggregates that are stable after compression. On the other side, because at finishing of the first compression (not exceeding the collapse pressure) well ordered monolayer is formed, consecutive expansion spreading the molecules more uniformly, i.e. they are in similar distance from each other. Therefore, further compressions are characterized by more continuous increase of the number of molecules under the electrode, as well as more uniform uprising of the molecules. Better stability of the monolayer can be seen from the shape of the curves corresponding to the second and the third compression. The reorientation of the molecules is more fluent.

From comparison of the plot of both the surface pressure and dipole moment versus mean molecular area of gA monolayer (Figs. 20 and 21) it can be see, that the dipole moment increases with decreasing of mean molecular area until $\sim 7 \text{ nm}^2/\text{molecule}$ when the surface pressure starts to increase. The dipole moment projection remains constant between 5,2-7 nm²/molecule when the monolayer is in the liquid expanded (L - E) state. In line with above discussion it is likely that ordered secondary structure of gA is formed at the mean molecular area of 7 $\text{nm}^2 \cdot \text{molecule}^{-1}$. At the region 5.2–7 nm²·molecule⁻¹ the interwined $\beta^{5,6}$ helix is already established and compression of the monolaver resulted only in more tightly packed molecules, but does not change of gA secondary structure. Phase transition from L - E to liquid- condensed state (L - C) state caused further increase of the molecule packing, however increase of dipole moment in this region revealed only changes in gA orientation. The dipole moment reaches maximum at the same area 3.6 nm²·molecule⁻¹, where the inflexed point of the plateau is situated. Next phase transition form L - C to solid (S) phase caused dramatic decrease of the dipole moment. We assume that this decrease is not caused by the collapse of the monolayer (monolayer was compressed up to 30 mN m⁻¹, i.e. far below from the critical pressure ~ 45 mN m⁻¹), but by another reorientation of the molecule.

3.2 Bilayer lipid membranes (BLM)

3.2.1 Formation and electrical properties of BLM

The stable bilayer lipid membranes (BLM) have been reported in 1962 by Mueller and coworkers [29]. Due to amphiphilic nature of phospholipids, they spontaneously form the lipid bilayers in a water phase. In experiments by Mueller et al. the BLM has been formed from crude fraction of phospholipids in a circular holes of a relatively small diameter (0.8–2.5 mm) in a Teflon cup immersed in larger glass compartment filed by electrolyte (Fig. 22).

Small amount of the phospholipids dissolved in hydrocarbon solvent (e.g. n-heptane or n-decane) can be placed to the hole using simple brush or Pasteur pipette. Immersion of the drop of phospholipid to a hole resulted formation of relatively thick lipid film with a thickness > 100 nm. The behavior of this thick film is determined by differences in hydrostatic pressures in its peripheral part (Plateau-Gibbs border) and in a central part that is relatively flat. According to Laplace law the differences in the hydrodynamic pressure between inner and outer phases is determined by equation

$$\Delta p = \gamma \left(\frac{1}{R_1} + \frac{1}{R_2}\right),\tag{16}$$

where R_1 and R_2 are inner and outer radius of the surface curvature and γ is surface tension. In a central part of the membrane the radius of curvature is close to the infinity, i.e. $R_1 = R_2 \rightarrow \infty$. Therefore the pressure differences is close to the zero: $\Delta p = 0$. However, the pressure at the central part of Plateau – Gibbs border is lower then that in a water phase, i.e. $\Delta p < 0$. Therefore the solvent will move from thin-flat part of the BLM to the Plateau-Gibbs border. This will cause further thinning of the membrane (Fig. 22). This process can be observed also visually in reflected light. Thick films are colored, like oil films on a water surface. As soon as the films become thinner the black spots start to appear. From observation of thin films formation follows that the black spots forms non uniformly and not symmetrically. As soon as the films



Fig. 22. a) The scheme of measurement current across BLM using current to voltage converter and the scheme of formation of BLM. b) electrical scheme of the system BLM-electrolyte-electrodes, R_m – membrane resistance, C_m – membrane capacitance, R_e – resistance of electrodes and electrolyte.

became thinner also the van der Waal's forces start to play considerable role in film formation. The resulted BLM is characterized by bilayer part surrounded by Plateau-Gibbs border (Fig. 22). The formation of BLM can be easily detected by measurement electrical properties of the film – conductance and capacitance.

BLM conductivity can be easily measured by current to voltage converter (Fig. 22). The dc voltage (of a relatively small amplitude $V_0=50-100 \text{ mV}$) is applied to the BLM through e.g. Ag/AgCl electrodes (usually immersed into a salt agar bridges). One electrode is connected to the high resistance input (usually > $10^{12}\Omega$) of the operational amplifier (e.g. Analog Devices AD 548J). The current *i* flowing through feed back resistor R_i is transformed into the output voltage V according to the equation: $i = VR_i^{-1}$. Thus, measuring the output voltage by e.g. milivoltmeter, chart recorder or connecting the output of the amplifier into the A/D converter and then to a computer, it is possible to determine the current *i* and its changes with time. Knowing the amplitude of applied voltage V_0 , it is then possible to determine membrane specific conductance $g = i/V_0A = V/V_0R_iA$ (A is the area of BLM that can be determined by microscope). (The principles of operation the amplifiers with negative feed back are described in [72]). The experiments on the study of membrane conductivity could be performed by simple apparatus based on operational amplifier with rather low cost. The care should be on proper shielding of the measuring chamber due to rather small current that are flowing through BLM (typically of the order of pA). It is, however possible to use commercial instrument, e.g. Keithley 6512 (USA) that can be connected on line with PC through KPC-488.2AT Hi Speed IEEE-Interface board. BLM is char-

acterized by rather low specific conductivity which is in the range $10^{-6}-10^{-10} \Omega^{-1} \text{cm}^{-2}$, but the typical value is about $10^{-8} \Omega^1 \text{cm}^{-2}$ [18]. This phenomenon is connected with low dielectric permittivity of the inner, hydrophobic part of the membrane. The relative dielectric permittivity of this part is similar to that characteristic fro n-alkanes: 2.1. The mechanisms of conductivity of BLM will be discussed below (see part 10).

BLM electrical capacitance is important value that characterizes the dilectric and geometric properties of the membrane. Usually it is specific capacitance that is used for characterization of the BLM

$$C_S = \frac{C}{A} = \frac{\varepsilon \,\varepsilon_0}{d},\tag{17}$$

where A is the area of bilayer part of BLM, d is the thickness of hydrophobic part of BLM, $\varepsilon \approx 2.1$ is relative dielectric permittivity and $\varepsilon_0 = 8.85 \times 10^{-12}$ F m⁻¹ is permittivity of free space (vacuum). The simple electrical model of the membrane can be represented by capacitor connected in parallel with resistance. The consideration of the resistance of electrolyte and measured electrodes can be taking into account as a resistance connected in series (Fig. 22b). The membrane capacitance can be measured by an alternating current bridge [73]. However, the most precise measurement of conductance and capacitance can be performed by measurement of the complex impedance of the BLM [73,74]. The theory of the impedance is described in detail in many textbooks, monographs or reviews, see e.g. [73,74]). Here we restrict ourselves by consideration of basic phenomena.

Impedance measurements are made by applying an alternating (a.c.) current of known circular frequency ω and a small amplitude i_0 to a system and measuring the amplitude V_0 and the phase difference φ of the electrical potential. The impedance is usually represented by absolute value of the impedance and the phase

$$|\tilde{Z}| = \frac{V_0}{i_0}$$
 and $\angle \tilde{Z} = \varphi.$ (18)

In cartesian coordinates, impedance becomes a complex number

$$\tilde{Z} = R + jX, \quad \text{where} \quad j = \sqrt{-1}.$$
 (19)

The real and imaginary part of \tilde{Z} describe the resistance (R) and reactance (X) respectively and can be represented by appropriate electrical circuit elements. In the case of unmodified BLM, i.e. when the membrane resistance is considerably higher then the absolute value of reactance, the equivalent electrical circuit can be simplified and reduced by resistance in series with capacitance (Fig. 23c). For this circuit the complex impedance

$$\tilde{Z} = R - \frac{j}{\omega C}.$$
(20)

The absolute value of impedance is $\tilde{Z} = \sqrt{R^2 + (\omega C)^{-2}}$ and the phase $\varphi = \arctan(\omega RC)^{-1}$. The value of \tilde{Z} and φ are usually presented as a function of frequency, i.e. so-called Bode plot. In this plot \tilde{Z} should decrease with increasing the frequency and approaching R at high frequencies (Fig. 23a). At lower frequencies the phase φ is equal 90° as for pure capacitor, but at higher frequencies it decrease dramatically approaching 0 as for pure resistance (Fig. 23b). The typical bode plot for unmodified BLM from diphytanoyl phosphatidylcholine (Avanti Polar



Fig. 23. Impedance spectra of the BLM formed from diphytanoyl phosphatidylcholine in n-decane in a 10 mmol·l⁻¹ KCl. Bode plot of the absolute value of (a) impedance, (b) phase. The points are experimental value and line represent fit according to equivalent electrical circuit (c).

Lipids, Inc, USA) (lipid was dissolved in n-decane (Fluka) in a concentration 30 mg ml⁻¹) is presented in Fig. 23a. The points are the experimental values measured by electrochemical analyzer Autolab PGSTAT 12 equipped with FRA 2 module (Eco Chemie, The Netherlands) and the line represents the fit according to the equivalent circuit (Fig. 23c). The BLM was formed in a circular hole of a diameter approx. 0.8 mm in 10 mmol 1^{-1} KCl. The typical values for R (i.e. resistance of electrolyte and electrodes) and C (i.e. the membrane capacitance) were 15 k Ω and 1.67 nF, respectively. If the BLM is modified by compounds, e.g. channel formers or carriers, that increase its conductivity, the equivalent electrical circuit is more complex [74,75]. The application of impedance spectroscopy allowing to obtain not only the basic electrical parameters of the BLM, but also allowing to analyze the mechanisms of ionic transport (see e.g. [75] and reference herein). Currently there exist several producers of sophisticated electrochemical or impedance analyzers, that can be successfully used for the study the impedance of BLM. Among others, already mentioned Autolab PGSTAT 12 with FRA 2 module is universal high quality instrument allowing not only measurement of the impedance spectra with high sensitivity in a wide range of frequencies (1 mHz to 1 MHz) but also complete electrochemical parameters of the system. Solartron (UK), BAS-Zahner (USA) and CH Instruments Inc. (USA) produce electrochemical and impedance analyzers, that can also be used for the study of BLM electrical properties.

The specific capacitance and the thickness of BLM depend on the content of organic solvent (e.g. n-heptane or n-hexane). In addition, using the solvent with larger hydrocarbon chains, e.g.
n-hexadecane or squalene [76,77], it is possible to obtain thinner membrane. This is because due to sterical restrictions the larger molecules of solvent are squeezed out of the bilayer to its border part. There were developed various methods how to make membrane thinner and to approach its properties to the bilayer of the biomembrane, which obviously does not contain the solvent (except minor content of fatty acids that appear due to action of phospholipases). In Ref. [76] these methods are described in details. In addition to approach connected with using the hydrocarbon solvent with larger length of hydrocarbon chains, it is also possible to freeze out of the solvent, i.e. n-hexadecane. This solvent is characterized by phase transition temperature from liquid to solid state below approx. 15.5 °C. Therefore if the surrounding electrolyte will be slowly cooled, it is possible to crystallize the solvent, that is moved from bilayer to the Plateau-Gibbs border of the membrane [77,78]. The method of "drying" the membrane developed by Rovin [79] is based on using two types of solvent for formation of BLM – dioxane and n-octane in different ratios. Dioxane is solvent which is dissolved both in water and in the n-alkanes. Therefore if the BLM will be formed from the mixture of lipids and a mixture of the solvents, one of which will be dioxane, the dioxane will be leaved out from the bilayer to the surrounding electrolyte and membrane specific capacitance will increase.

In 1972 Montal and Mueller [80] proposed the method of formation the BLM from monolayers. According to this method the Teflon cup divided by the wall with circular hole of a diameter approx. 0.3 mm is filed by electrolyte just below the lower hole orifice. Then the small amount of lipid dissolved in chloroform is added at water surface at both compartment of the Teflon cup. After the chloroform is evaporated (after approx. 10 min) the level of the water phase is increased by slow addition of electrolyte using the syringes. As soon as the level of the electrolyte surpass the upper orifice of the hole the BLM is formed. It is necessary to note that the membrane is not exactly without the solvent, because for membrane stability it is necessary that Plateau-Gibbs border is present. This is reached by addition of small amount of the lipids dissolved in n-hexadecane on the hole orifice.

Other method of formation the BLM and that contained proteins has been proposed by Schindler [81]. This method is certain modification of that proposed by Montal and Mueller [80] and consisting in different formation of the monolayers. For this purpose the liposomes or proteoliposomes are added to both compartment of Teflon cup. The lipid monolayers are formed due to the fact that the liposomes, when contacted with hydrophobic – air interface of a low surface pressure are broken and the lipids form monolayer at the air-water interface. Then the process of formation BLM is analogical to that by Montal and Mueller. Using the various method of formation BLM it is possible considerable change the thickness and the specific capacitance of BLM as it is revealed from Table 4. With increasing the length of the hydrocarbon solvent the thickness of the membrane decrease independently on the lipid composition or cholesterol content. The lipid composition affects only the interval of the changes of the thickness with changes the number of carbons of n-alkanes [18]. Thus, by means of variation of the hydrocarbon solvent and the kind of phospholipids it is possible to obtain BLM with desired thickness.

The electrical and other physical properties of BLM formed from natural phospholipids are similar to that of biomembranes (Table 5). Unmodified BLM are characterized by low conductivity. They do not reveal any metabolic activity and are not selective for transport of ions such are biomembranes. However, BLM can be modified by channel formers, carriers or receptors, that allowing to provide sensitivity and selectivity of ionic transport or ligand-receptor interactions, i.e. like for biomembranes. Also at presence of various modificators the conductivity of

Number of carbon	Specific capacitance,	Thickness, nm	Reference
atoms in n-alkane	$10^{-3} \mathrm{F}\mathrm{m}^{-2}$		
8	3.77	4.93	[82]
10	3.74	4.97	[82]
12	4.22	4.46	[82]
14	4.86	3.82	[82]
16	6.24	2.98	[83]
Solvent free	7.28	2.56	[82,83]

Tab. 4. The specific capacitance and the thickness of BLM formed from dioleoylphosphatidyl choline (DOPC) using the method of Mueller et al. [29] and n-alkanes of various length as well as that for solvent-free BLM prepared according to method of Montal and Mueller [80].

Tab. 5. Comparison of the properties of BLM and biomembranes.

Properties	Biomembranes	BLM	
Thickness, nm	6.0–10.0	2.5-8.0	
Surface tension, mN.m $^{-1}$	0.03-3.0	0.2-6.0	
Conductivity, Ω^{-1} cm ⁻²	$10^{-2} - 10^{-5}$	$10^{-6} - 10^{-10}$	
Specific electrical capacity, 10^{-3} F m ⁻²	5-13	2-10	
Breakdown voltage, mV	100	150-300	
Refractive index	1.6	1.56–1.66	
Permeability for water, $\mu m s^{-1}$	0.5-400	31.7	
Energy of activation for water permeability kJ mol ^{-1}	40.3	53.3	
Ionic selectivity P_{K+}/P_{Na+}	1-25	5.4–9.0	

BLM usually increases. These effects together with several similar parameters evidence that the properties of lipid bilayers are close to that of biomembranes. Therefore the study of physical properties of BLM has important significant for understanding the properties of biomembranes.

3.2.2 Stability of BLM. Electrical breakdown and electroporation

The main problem in application of BLM in electrochemical studies is their relatively low stability. Even in the case of relatively low potential difference across the BLM comparable with that occurred in biomembranes, i.e. $\Delta V = 0.1V$, and due to small thickness ($d \approx 10^{-8}$ m), rather large electrical field exists across the membrane: $E = \Delta V/h \approx 10^7$ V m⁻¹. Thus, even small decrease in the membrane thickness, e.g. due to the thermal fluctuations, resulted in increase of electrical field, that can induce the electrical breakdown of the membrane. The theory of the electrical breakdown of BLM has been developed mostly due to work by Chizmadzhev and coworkers [84]. They have found that the mean life time of the BLM in an electric field decreases with increasing the voltage difference across BLM. The membrane breakdown is connected with appearance of the conductive pores in a lipid bilayer due to membrane electrostriction, i.e. compressive force $F = \varepsilon_m \varepsilon_0 V^2/(2d^2)$, that originated due to the applied voltage, V [85]. Let us consider the changes of the energy of the membrane if the amphiphilic pore of cylindrical shape with radius r appears. The changes of the energy of the pore can be described by equation

$$E = 2\pi r \gamma_l d - \pi r^2 \gamma - \Delta C V^2 / 2, \tag{21}$$

where d is the membrane thickness, r is the radius of the pore, γ_l is the coefficient of linear tension of the pore, γ is the surface tension of the membrane, ΔC are the changes of electrical capacitance and V is the potential difference across the membrane. First term in Eq. (21) is connected with the changes of the energy of the membrane due to appearance of the surface of the cylinder between the membrane and the pore. Second term means the decrease of the surface energy due to decrease of the membrane surface, that is equal to the area of the cross section of the cylinder. The third term is connected with changes of the energy of capacitor due to the changes of dielectric permittivity of the membrane interior (the dielectric part with a dielectric permittivity $\varepsilon_m \approx 2.1$ is replaced by water with dielectric permittivity of water phase $\varepsilon_w \approx 80$). The changes of electrical capacitance can be expressed as $\Delta C = \pi r^2 C_s(\varepsilon_w/\varepsilon_m - 1)$, where C_s is the specific capacitance of the membrane, i.e. the capacitance per unit area. The Eq. (21) can be therefore transformed to

$$E = 2\pi r \gamma_l d - \pi r^2 (\gamma + C V^2 / 2), \tag{22}$$

where $C = C_s(\varepsilon_w/\varepsilon_m - 1)$. As soon as the pore radius increases its energy should change non monotonously and can be described by a curve with a maximum as it is displayed on a Fig. 24a. It is clear from the figure that the membrane defect of a small radius have tendency to diminish. However the pores with radius larger then certain critical value r_c will increase irreversibly and cause the membrane breakdown. The value of critical radius can be find by derivation of the energy dE/dr = 0 and is described by equation [86]

$$r_c = \gamma_l d / (\gamma + C V^2 / 2). \tag{23}$$

The value of r_c are of the order of the membrane thickness, i.e. several nm. After substitution of Eq. (23) into Eq. (22) we can obtain dependence of energy of the pore on the membrane potential

$$E = (\gamma_l d)^2 / (\gamma + CV^2/2).$$
(24)

Thus, application of the potential to the membrane will result decrease of the potential barrier and the probability of the membrane breakdown will increase (Fig. 24a). Above we considered simplified situation, when the breakdown of the membrane is connected with formation of amphiphilic pore. As a matter of fact, owing to thermal fluctuations of lipid molecules the hydrophobic pores are formed prior the hydrophilic one (Fig. 24c). When hydrophobic pore exceed this critical radius, a reorientation of the lipids converts the pores into the hydrophilic ones (Fig. 24c) [87]. The dependence of the energy of pore formation as a function of the radius is in this case more complicated (Fig. 24b).

The study of the BLM breakdown has great significance for understanding the processes such are membrane fusion, lysis and apoptosis of cells that may involve an opening of a lipid pore to join the volumes initially separated membranes. Experimental studies revealed two types of



Fig. 24. The dependence of the energy of the pore in a membrane on its radius in the case of (a) hydrophilic pore, (b) hydrophobic pore is formed first and then transformed into the hydrophilic one at certain critical radius. (c) the schematic representation of the transformation of hydrophobic pore into hydrophilic one (Adopted from [93] by permission).

BLM behavior under the electrical stress: reversible and irreversible electrical breakdown. Irreversible breakdown was observed for membranes of any lipid composition. It is accompanied by rapid increase of membrane conductance and resulted in mechanical breakdown of BLM [88]. However, for BLM of a specific lipid composition, e.g. oxidized cholesterol, or at the phase transition of phospholipids [89] a reversible pores were observed when the membrane was exposed to a short pulse of high electric field. In this case even after five to six order of increase the conductance, it then drops to the initial level upon voltage decrease [87,90]. It has been assumed, that in the case of irreversible breakdown few pores are formed before the first of them reaches a critical radius and starts irreversible expansion leading to membrane rupture. In the case of reversible breakdown a large population of pores accumulates under high voltage before the BLM rupture. Recently it has been observed that application of the voltage (150–500 mV) to a BLM resulted in fast transition between different conductance levels reflecting opening and closing of metastable pores [91]. The mean life time of the pore was 3 ms at V = 250 mV, however the pores with longer life time, up to 1 s were observed as well. Based on the conductance value and its dependence of the ion size, the radius of the average pore of a 0.5 nS conductance was estimated as ~ 1 nm. This pore might involve only ~ 100 lipid molecules, which corresponds to less then 10^{-8} % of all lipids in the BLMs of 1 mm² area. The metastable lipidic pores identified in this study most probably correspond to the small metastable lipid pores whose existence was assumed earlier to explain the accumulation of very large number of pores during reversible electroporation of BLM modified by uranyl ions [87]. The physical structure of non conducting pre-pores remains unknown. Opening and expansion of conductive hydrophilic pores, i.e. pores with the edges formed by polar head groups of phospholipids, is assumed to be preceded by formation of very small and short-time hydrophobic pores with the edge formed by hydrocarbon chains of the lipids. Evolution of a hydrophobic pores into a hydrophilic pore involves reorientation of the polar heads of the lipids from the surface of bilayer to the edge of the pore (Fig. 24). Melikov et al [91] assumed, that the pre-pores correspond to small clusters of lipids with their polar heads trapped inside the hydrophobic interior of the membrane upon closing of the hydrophilic or partially hydrophilic pore. Interaction between the lipid polar heads in the same cluster can increase the lifetime of the cluster and thus, stabilize the pre-pore state. Alternatively the pre-pore state can correspond to a cluster of water molecules trapped inside a hydrophobic interior. Such clusters of lipid polar head or water molecules will then transform back into a small hydrophilic pore.

Changes of conductance of the membrane following application of external voltage, i.e. electropermeability, has great practical significance. By means of application of external field it is possible to incorporate DNA or drugs into the cells. This so-called electroporation has been known for several decades and has been reviewed in several papers (see e.g. [92,93]). This process has been studied in most detail using BLM [93]. It has been shown that the charged ions or small molecules can be driven through BLM by electromigration. The mechanism of electroporation of larger molecules, like proteins is however not clear yet. The peculiarities of the electropermeability of tissues, e.g. human skin under application of electrical field revealed similar behavior like that of BLM. However, the theory of electroporation of these complex structures remains still incomplete (see e.g. [94]). For deep look into the problem of electroporation on a molecular level we recommend to reader the extensive review by Weaver and Chizmadzhev [93].

The electroporation is of great practical significance for example in gene therapy. Gene therapy is based on incorporation of DNA into the cell for the purpose of inducing synthesis of required protein or in order to stop expression of not desired gene. The later case is known as an antisense therapy and is based on incorporation of short DNA sequences complementary to the part of not desired gene or to mRNA (see [95] and reference herein). Because nucleic acids are negatively charged, there are difficulties of their transfer through plasmatic membrane. Therefore specific vectors are necessary to facilitate this translocation. So far mostly two types of vectors were used. Virus vectors have been found as most effective. However, due to problems with their possible infectivity or even cancerogeneity [96], their practical application is limited. Liposomes, especially composed of cationic lipids or cationic polymers belong to the second type of the vectors [95,97]. They allowing to encapsulate desired gene or to bind it at the liposome or polymer surface and then transport into the cell cytoplasm fusion or cell endocytosis. DNA molecules in these complexes are protected against degradation by endonucleases. Recently a new type of the vector has been studied in detail by various biophysical methods. This vector is based on chemically modified cationic derivative of antibiotic amphotericin B (AmB): 3-dimethylaminopropyl amide (AMA). Similarly like AmB, AMA consists of a rigid elongated skeleton containing a polyene part which is hydrophobic and responsible for incorporation into the hydrophobic part of the membrane. This part favorably interacts with sterols (cholesterol or ergosterol) and thus AMA complexes create pores in a lipid bilayer with similar properties like AmB [98]. Polar part of AMA contains two positive charges, which facilitate the formation of complexes between AMA with oligonucleotides (ODN). Both AmB and AMA form ionic channels in bilayer lipid membranes (BLM). In contrast with AMA, AmB is neutral molecule.

However, earlier it has been shown that presence of AmB enhances the transfer of DNA into the aucariotic cells of animals. However, such a transfer has not been confirmed for human cells and it has not been prove the formation of complexes between AmB and DNA [99,100].

In contrast, AMA interacts with plazmid DNA as well as with oligonucleotides by means of electrostatic interactions. The studies of interaction of AMA with plazmid DNA were performed with of 4356 kb plazmid DNA that contained green fluorescence protein (GFP) gene. By means of fluorescence microscopy it has been shown, that AMA allowing expression of this gene in NIH/3T3 cells. For this transfer the charge ratio ODN/AMA was 0.33. I.e. the complex was positively charged [101]. Biological effectivity of AMA as a vector for anti-complementary oligonucleotides, that should block the translation of mRNA was approved using 20-mer ODN. This ODN was complementary to the sequence of mRNA, that coded human MDR1 (multidrug resistance phenotype). The ODN was marked by fluorescence probe and its increased concentration was showed in cells. It has been also observed considerable decrease of the expression of the protein Pgp, which is coded by MDR1 gene in G815-MDR-3T3 cells. The optimal charge ratio of the ODN/AMA complexes was in this case 2, which means that the complex was negatively charged. Thus, even negatively charged complex could be translocated into the cell. This is advantageous, because lower AMA concentration can be used. Detailed study of the formation of the ODN/AMA complexes has been performed by optical spectroscopy and electron microscopy methods using 27-mer single stranded oligonucleotide. It has been shown, that at low AMA concentration ($< 1\mu$ M) the condensation of these molecules on the ODN took place. At higher concentration (> 100μ M) and for ODN/AMA negative charge ratio > 1 the circular dichroism (CD) spectra revealed on reorganization of free self-associated AMA complexes into smaller one decorating the ODN molecule. Electron microscopy study evidences that the size of ODN/AMA complexes is rather large with dimension up to 650 nm and different shape (round shaped or rodlike structure) depending on the charge ratio ODN/AMA [102]. AMA as well as ODN/AMA complexes interact with lipid monolayers and with unilamellar vesicles [103]. The adsorption of the complexes to the lipid monolayer or bilayer decreased with increasing the negative charge of the complexes.

It is, however not clear yet, whether the ODN/AMA complexes could translocate through lipid bilayer and how this translocation depends on the charge ratio of ODN/AMA. If such a translocation exists, it is not clear what are the mechanisms, considering large size of ODN/AMA complexes. We therefore used BLMs and studied their conductance at presence of AMA or ODN/AMA complexes in a different charge ratios [104]. The conductivity gives certain information about translocation of the complexes. As ODN we used 20 mer single stranded DNA of following sequence: 5' - TGT ATA GCT AAA GTA GGC GC – 3' obtained from Generi Biotech (Czech Republic).

The BLMs were formed according to the method of Mueller et al. [16] on a circular hole (diameter 0.8 mm) in a wall of Teflon cell, that divided the cell into two identical compartments with a volume of approx. 1.5 ml each. The cell was filed with buffer composed of 100 mM NaCl + 10 mM Tris-HCl + 1 mM EDTA (pH 7.6). The BLM were formed from a lipid solution composed of 1,2-diphytanoyl-sn-glycero-3-phosphatidylcholine (DPhPC) (Avanti Polar Lipids Inc, USA) and cholesterol (4:1 w/w) dissolved in n-decane (concentration 20 mg ml⁻¹). The conductivity was measured using computer controlled electrometer Keithley 6512 (USA). The dc voltage of the amplitude of 50 mV was applied from dc source to a membrane through Ag/AgCl electrode placed in a salt agar bridge.



Fig. 25. Relative changes of BLM conductance $\Delta g/g_0$ as a function of concentration of AMA or ODN/AMA complexes at different charge ratio σ (see inset) ($\Delta g = g - g_0$, where g_0 is the BLM conductance prior and g after addition of AMA or ODN/AMA complexes) and following addition of Mg²⁺ ions into the both membrane sides in a final concentration of 5 mM.

The relative changes of the BLM conductance as a function of the concentration of AMA and/or ODN/AMA complexes in various charge ratios are shown on Fig. 25. We can see that practically no changes of conductance take place with increased concentration of pure AMA. However at the charge ratio $\sigma = 0.1$ for ODN/AMA complex, i.e. the complex was positively charged, a considerable increase of conductivity took place. For less positively charged complex of ODN/AMA ($\sigma = 0.44$) the changes of conductance were less expressed. However, even for negatively charged complex ($\sigma = 4$) an increase of the BLM conductivity takes place. Additional increase of conductivity was observed following addition of Mg²⁺ ions in a final concentration of 5 mM to the both side of BLM. It is seen from Fig. 25, that in this case the increase of conductivity was slightly higher for negatively charged complex of ODN/AMA. The obtained results may evidence on translocation of ODN/AMA complexes across BLM. However, one can not be excluded, that these complexes induce only perturbance of BLM structure and thus increase of permeability of BLM for ions.

3.3 Supported bilayer lipid membranes

As we already mentioned in a section devoted to the lipid monolayers, using Langmuir-Blodget technique, it is possible to obtain *supported lipid membranes*. The simple method of formation of lipid film on a metal support has been proposed by Tien and Salamon [105]. The silver wire of a diameter approx. 0.3 mm coated by Teflon was immersed into the lipid solution in n-decane. Then the tip of the wire was cutted by sharp knife and immediately immersed into the electrolyte, where the formation of thin film occurred spontaneously. The disadvantage of this method consist in the fact that the film formed on rather rough metal surface is inhomogeneous and is composed of monolayers, bilayers or even multilayers [106]. Application of dc voltage during film formation resulted improvement of the film characteristics and the membrane became more homogeneous [107]. However, most homogeneous films can be obtained using smooth



Fig. 26. Schematic representation of sBLM and tBLM: (a) sBLM formed by chemisorption of alkylthiols on a gold. tBLM are characterized by hydrophilic spacer between gold and lipid bilayer, that can be formed by specially synthesized lipids with hydrophilic spacer (b), or by lipopetide layer (c) and/or by polymer layer (d).

gold surface with chemisorbed alkanethiols. According to this method, the surface of the gold is accurately cleaned and then immersed in a solution of alkanethiol, e.g. hexadecane thiol. Thiols provide almost covalent binding of the alkanethiol to a gold and the hydrophobic chains of hexadecane thiols will create densely packed hydrophobic surface (Fig. 26a). The tilting of the chains away from the surface normal occurs because the spacing of the three-fold hollow sites on the Au (111) surface, into which the –SH head groups fit, is slightly larger than the optimal van der Waals distance between adjacent hydrocarbon chains: by tilting at an angle of 20–25 degrees, the chains adjust their spacing to optimize the van der Waals interaction [108]. In open circuit the process of formation alkane thiol film lasts about 12 hours. However, application of dc voltage with amplitude 0.6 mV (positive terminal on a gold) resulted fast (few minutes) formation of homogeneous alkanethiol monolayer [109]. Second monolayer can be formed e.g. by Langmuir-Blodget technique, immersion of the gold electrode into the lipid solution or by liposome fusion

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[110]. The supported lipid membranes (sBLM) are considerably more stable then BLM [111-113]. While breakdown voltage of BLM is less then 300 mV, that for sBLM can reach more then 1 V [110]. The disadvantage of sBLMs consist in the fact that the alkanethiol monolayer is closely adjacent to a gold. Therefore it is impossible to incorporate e.g large integral proteins into these membranes and also impossible to use these membranes for study the mechanism of ionic transport due to lack of water phase between the bilayers and gold. This drawback has been solved by development of so called tethered membranes (tBLM). tBLM are similar to sBLM, however, instead of alkanethiols, specially synthesized molecules with hydrophilic spacer are used for the formation of the monolayer tethered to a gold support (Fig. 26b). The hydrophilic space between gold and BLM can be provided also by lipopetide layer (Fig. 26c) and/or by polymer layer (Fig. 26d). [113,114]. The sBLM and tBLM, due to their high stability and due to the unique properties that mimics the real biomembrane can be used in nanotechnologies, especially in development of sensitive biosensors (see part 11).

3.4 Liposomes

Liposomes (vesicles) are widely used models of lipid bilayer of biomembranes. The formation of liposomes has been firstly reported by Bangham [30]. They are formed from water dispersions of the lipids by various methods. Depending on the method of formation one can obtain multilamellar or unilamellar liposomes of a different size. Application of liposomes as a model of biomembranes has been discussed in large number of review and monographs, see e.g. [115]. The methods of liposome preparation are considered in Ref. [116]. Multilamellar liposomes are the simplest structures in respect of the preparation. Typically the desired amount of the lipid is dissolved in chloroform or in a mixture chloroform-methanol. This mixture is allowed to evaporate under the stream of nitrogen in a glass volume of a spherical shape. For this purpose usually rotary evaporator is used in order to make thin film of the lipids on a large surface of the glass. After the thin film is formed, it is then hydrated by addition of desired volume of the water or buffer. The dispersion of lipid in a water is then vigorously vortexing during several minutes at temperature higher than the temperature of phase transition of phospholipids. The concentration of lipids varied from method to method, but typically it is few mg.ml⁻¹. For example precise DSC calorimetry requires concentration of lipids about 0.5 mg ml $^{-1}$, but densitometry requires larger concentration – around 5 mg ml $^{-1}$. The multilamellar liposomes are rather large and their diameter is several μ m. The advantage of these liposomes is that they consist of large number of bilayers and are characterized by high degree of cooperativity in comparison with unilamellar liposomes. This cooperativity is expressed for example by more narrow interval of phase transition temperature. Disadvantage of multilamellar liposomes consists in their size inhomogeneity as well as in relatively fast saturation. They are therefore not suitable in experimental set up, that does not allow stirring of the solution.

Unilamellar liposomes can be prepared by various methods. The simplest one consists in sonication of the multilamellar liposomes by ultrasound [117] in an ultrasonic bath. This method resulted in formation of relatively small liposomes of a diameter approx. 20 nm. The disadvantage of this method is not uniform diameter of liposomes as well as possible content of certain amount of multilamellar liposomes. In addition, application of ultrasound could result in damage of the lipids. As a result also the free fatty acids could appear in a solution. Other methods are based on fast injection of ethanol solution of lipids into the buffer [118] or by dialysis of the

water dispersions of micelles composed of the lipids and detergents [119]. Rather useful method has been proposed by McDonald et al [120] that consist in formation of vesicles by extrusion of multilamellar vesicles through polycarbonate films. Depending on the size of the pores in a film the liposomes can be prepared of desired diameter, usually from 50 nm to several μ m. Commercial kits for preparation unilamellar liposomes are currently available, e.g. Avestin Inc. (Canada). The liposomes prepared by extrusion methods are rather homogeneous in the size. Liposomes can be modified by various compounds, e.g. peripheral or integral proteins. In the case of integral proteins the liposomes are prepared from a water dispersion of lipids that contain desired concentration of proteins (see e.g. [121,122]).

4 Ordering, conformation and molecular dynamics of lipid bilayers

The lipid bilayers represent liquid crystals of a smectic type. They are characterized by relative fast lateral diffusion of lipids and about 600 times slower diffusion of proteins (the typical diffusion coefficients for lipids and proteins are: $D_{LIP} = 6 \times 10^{-12} \text{ m}^2 \text{s}^{-1}$, $D_{PROT} = 10^{-14} \text{ m}^2 \text{s}^{-1}$, respectively). In direction perpendicular to the membrane plane, the lipid bilayer is characterized by certain ordering that depends on conformation of hydrocarbon chains of phospholipids. This conformation depends on temperature and lipid bilayer undergous through phase transitions.

4.1 Structural parameters of lipid bilayers measured by X-ray diffraction

The basic structural parameters of the membranes can be determined by *X-ray or neutron diffraction methods*. X-rays are electromagnetic radiation with typical photon energies in the range 100 eV – 100 keV. For diffraction applications, only short wavelength X-rays (hard X-rays) in the range of a few angstroms to 0.1 Å (intensities vary between 1 keV to 120 keV) are used. Because the wavelength of X-rays is comparable to the size of atoms and molecules, they are ideally suited for probing the structural arrangements of atoms and molecules in wide range of materials. The energetic X-rays can penetrate deep into the materials and provide information about the bulk structure [123]. X-rays are produced generally by either X-ray tubes or synchrotron radiation. In recent years synchrotron facilities have become widely used as preferred sources for X-ray diffraction measurements. Synchrotron sources are thousands to million times more intense than laboratory X-ray tubes.

X-rays primarily interact with electrons and atoms. Diffracted waves from different atoms can interfere with each other and the resultant intensity distribution is strongly modulated by this interaction. If the atoms are arranged in a periodic fashion, as in crystals, the diffracted waves will consist of sharp interference maxima (peaks) with the same symmetry as in the distribution of atoms. Measuring the diffraction pattern therefore allows one to deduce the distribution of atoms in a material. The peaks in a X-ray diffraction pattern are directly related to the atomic distances. Let us consider an incident X-ray beam interacting with the atoms arranged in a periodic manner as shown in Fig. 27. The atoms are located periodically in parallel planes. For a given set of lattice plane with and inter-plane distance of d, the condition for a diffraction (peak) to occur can be simply written as

 $2d\sin\Theta = n\lambda$

(25)



Fig. 27. Schematic representation of diffraction X-ray beams on a two plains of two dimensional atomic lattice.

which is known as the Bragg's law after W.L. Bragg, who first proposed it. In the Eq. (25), λ is the wavelength of the X-ray, Θ the scattering angle, and n is an integer representing the order of diffraction peaks. The Bragg's law is one of most important laws used for interpreting X-ray and neutron diffraction data.

In contrast with X-rays, the neutrons have one great advantage, because deuteration dramatically changes the scattering of neutrons. Specific deuteration of component parts of the lipid, such as selected methylene, therefore provides a localized contrast agent that leaves the system physically and chemically nearly equivalent [124]. The disadvantage of neutrons to X-ray diffraction is that neutron beams are much weaker and there are fewer sources of neutrons.

In analogy with classical X-ray analysis, it is assumed that determination of bilayer structure means doing crystallography. It is, however important to note, that fully hydrated bilayers, even in a gel condition are far from crystalline state. The contrast is more remarkable for fluid, L_{α} phase where the hydrocarbon chains are conformationally disordered. The differences between crystalline structures and hydrated bilayers are particularly due to high content of water, which allows for increased fluctuations. Therefore, due to fluctuations, it is impossible to determine structure of biomembranes at atomic level. However, bilayers in multilamellar vesicles (MLV), that are most often use in diffraction studies, are isotropically oriented in space and therefore give so called powder patterns. The term "powder" really means that the crystalline domains are randomly oriented in the sample. Therefore when the 2-D diffraction pattern is recorded, it shows concentric rings of scattering peaks, corresponding to the various d spacing in the crystal lattice. The MLVs are characterized of a variety of sizes. Each bilayer is influenced by its neighbors. It is assumed, that MLVs are "onion like", consisting of closed concentric spheres. Because lipid exchange between bilayers and solvent is slow, it is likely that the number of lipids in each bilayer remains constant over fairly long time [6]. The advantage of MLVs is that they do not have to be especially oriented in an X-ray beam (as a mater of fact, they can not be oriented). However, only small fraction of the lipid in a powder sample diffracts from a given beam, so intensities are weak. In addition, due to short range fluctuations (intrinsic fluctuations related to a single bilayer) and especially due to long range fluctuations (fluctuations in the relative positions of unit cell [125] (Fig. 28), the electron density profiles obtained by X-ray diffraction are broad.

The typical electron density profile obtained on MLV composed of DPPC is shown on Fig. 29



Fig. 28. Schematic representation of long range fluctuations in a MLV (According to [6] reproduced by permission).



Fig. 29. Representation of structure of DPPC in the L_{α} fluid phase. (a) Probability distribution functions for different component groups from MD simulations [126] and the downward pointing arrows show the peak locations determined by neutron diffraction with 25 % water [124]. (b) electron density profile from X-ray studies (c) The schematic representation of the head group and the hydrophobic region of the bilayers. The version on the left monolayer is a simple three compartment representation. The version on the right monolayer is a more realistic representation of the interfacial head group region. D_C is the experimentally determined Gibbs dividing surface for the hydrocarbon region. (According to [6] reproduced by permission).

together with schematic representation of bilayer regions corresponded to head groups and hydrocarbon chains. The full thickness of the layer is composed of the thickness of bilayer region D_B and the thickness of water region D_W : $D = D_B + D_W \cdot D_B$ is defined as $D_B = 2V_L/A$, where V_L is the volume of lipid and A is its area. $D_W = 2n_W V_W / A$, where V_W is volume of water molecule and n_W is the number of water molecules/lipid. The volume V_L of the lipid is divided into head groups D_H and hydrocarbon chains region D_C : $D_B = 2(D_H + D_C)$. The D_C includes the hydrocarbon chain carbons except for the carbonyl carbon, which has substantial hydrophilic character. For DPPC the hydrophobic core therefore consists of 14 methylenes and one terminal methyl on each of two chains. The determination of the D_C value is important for analysis of the mechanism of protein-lipid interactions, where the interactions between hydrophobic parts of protein and the lipids plays considerable role. The head group of phospholipid consists of remaining part of the lipid. Electron density profile provides good measure for the location of the phosphate groups. Information about z-coordinates of other groups has been obtained using neutron diffraction. Because the thermal fluctuations, the position of the atoms in the lipid molecule can be described by a broad statistical distribution functions, that can be obtained by molecular dynamics simulations) (MD) (Fig. 29) [126].

MD becomes rather attractive, because allowing to obtain much greater details than can be obtained experimentally. These details can be even guide for interpretation of experimental results. The latest structural parameters for fully hydrated lipid bilayers of most frequently used composition are showed in Tab. 6.

4.2 Interaction between bilayers

We mentioned above that long-range fluctuations of bilayers cause less precision in determination the structure of bilayers by X-ray diffraction method. These fluctuations cause interaction between bilayers. However, two bilayers close to each other fluctuate less then that at a short distance. When bilayers are on a close distance, then suppression of fluctuation take place and resulted to decrease of the entropy. This causes increase of the free energy of the system. According to Helfrich [127], the free energy of fluctuations increases with decreasing separation distance $D_{W'}$ and depends on bending elasticity modulus K_c of bilayers

$$F_{fl} = 0.42 \frac{(kT)^2}{K_c D_W^2}.$$
(26)

The mechanisms of interbilayer interactions can be studied by X-ray diffraction [6] or by surface force method [128]. X-ray diffraction studies are based on measurement of the changes in bilayer structural parameters following application of osmotic pressure. It is expected, that removal of the water from interbilayer space as a result of increased osmotic pressure should squeeze membranes together and in addition should result in decrease of the area per molecule (or increase the membrane thickness) [129]. The changes in the thickness are, however, rather small ~ 0.12 nm when osmotic pressure is increased from 0 to 5.6×10^6 N m⁻² [6]. The analysis showed, that hydration and undulation forces as well as van der Waals attractive forces contribute to the total interbilayer pressure. The hydration pressure is dominant for interbilayer distances 0.5–1.3 nm, the undulation pressure is major at larger distances and van der Waals forces at lower spacing.

Tab. 6. Structural parameters of fully hydrated lipid bilayers (according to [6]). V_L – lipid molecular volume, D – lamellar repeat spacing (see Fig. 29), A – average interfacial area/lipid, V_C – sum of volumes of chain methylenes and methyls, V_{CH3} – volume of methyl, D_C – thickness of hydrocarbon core ($D_C = V_C/A$), D_{HH} – head group peak-peak distance, D_B – Gibbs-Luzzati bilayer thickness ($D_B = 2V_L/A$), D_W – Gibbs-Luzzati water thickness ($D_W = D - D_B$), $D_{H'}$ – steric head group thickness ($D_{H'} = (D_{B'}/2) - D_C$), $D_{B'}$ – steric layer thickness ($D_{B'} = 2(D_C + D_{H'})$, $D_{W'}$ – steric water thickness ($D_{W'} = D - D_{B'}$), n_W – number of water molecules/lipid ($n_W = AD_W/2V_W$), $n_{W'}$ – number of waters between D_C and $D_{B'}/2(n_{W'} = AD_H - V_H)/V_W$).

Lipid	DPPC	DPPC	DMPC	DOPC	EPC	DLPE	DLPE
Temperature	20 °C	50 °C	30 °C	30 °C	30 °C	20 °C	35 °C
V_L (Å ³)	1144	1232	1101	1303	1261	863	907
D (Å)	63.5	67	62.7	63.1	66.3	50.6	45.8
A (Å ²)	47.9	64	59.6	72.5	69.4	41.0	51.2
V_C (Å ³ /region)	825	913	782	984	942	611	655
V_{CH3} (Å ³ /group)	25.9	28.7	28.1	28.3	-	26.0	27.3
$2D_C$ (Å)	34.4	28.5	26.2	27.1	27.1	30.0	25.8
D_{HH} (Å)	44.2	38.3	36.0	36.9	36.9	39.8	35.6
D_B (Å)	47.8	38.5	36.9	35.9	36.3	42.1	35.4
D_W (Å)	15.7	28.5	25.8	27.2	30.0	8.5	10.4
$D_{H'}$ (Å)	9.0	9.0	9.0	9.0	9.0	8.5	8.5
$D_{B'}$ (Å)	52.4	46.5	44.2	45.1	45.1	47.0	42.8
$D_{W'}$ (Å)	11.1	20.5	18.5	18.0	21.2	5.6	5.0
n_W	12.6	30.1	25.6	32.8	34.7	5.8	8.8
$n_{W'}$	3.7	8.6	7.2	11.1	10.2	2.0	4.7

Interbilayer interactions depends on the kind of phospholipids. For charged lipids in low salt concentrations one should consider also electrostatic interactions. Glycolipid bilayers are characterized by more complex interactions and include also strong adhesive forces due to saccharide head groups [129].

4.3 Dynamics and order parameter of bilayers determined by EPR and NMR spectroscopy and by optical spectroscopy methods

Electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR) and optical spectroscopy methods allowing to obtain information about lipid chain configuration and dynamics and to study the mechanisms of protein-lipid interactions. The theory of these methods is well described in literature (see e.g. [130]).

The radio spectroscopic methods (EPR, NMR) are based on interaction of electron spins (EPR) or proton spins (NMR) with magnetic field. If the spins are not paired, the spin of electrons or overall spin of charged nucleus generate a magnetic dipole along the spin axis. The magnitudes of these dipoles are of fundamental properties of electrons and nuclei and are called electron (μ_e)



Fig. 30. (a) Structural formulas and location of the spin labels $I_{1,14}$ and $I_{12,3}$ in a membrane and (b) their EPR spectra.

and nuclear (μ_N) magnetic moments, respectively. If single electron or single protons are placed in a static magnetic field H, the energy of the spins will be splitted on two energy levels. The energies of these levels are $-\frac{1}{2}g\beta H$ and $\frac{1}{2}g\beta H$, respectively. So the difference between upper and lower energy levels is $\Delta W = g\beta H$. where g is magnetogyric ratio and depends on the nature of paramagnetic particle. For electron $g \approx 2$. $\beta = 0.977 \times 10^{-23} J/T$ is Bohr's magneton. The number of electrons that will occupy the lower energy level is N_1 and that for upper level N_2 . The ratio $N_1/N_2 = e^{\Delta W/kT} = e^{g\beta B/kT} > 1$. Thus, the number of electrons at the lower energy level will be higher then in upper one. If now alternating electromagnetic field with a frequency ν will be directed perpendicular to the magnetic field H, the electrons from lower level will be moved to the upper energy level, i.e. the system will adsorb the energy. The maximum of energy absorption will take place at resonance conditions. In the case of EPR, this resonance frequency is

$$\nu_{RE} = g\beta H/h,\tag{27}$$

where h is Planck's constant. It is more convenient to keep frequency constant and change the magnetic field intensity, H. In this case the resonance energy absorption will take place at

$$H_{RE} = h\nu/g\beta. \tag{28}$$

The EPR spectra represent the dependence of the intensity of absorption on the magnetic field strength. Because the phospholipid molecules are diamagnetic, they must be labeled by spin labels. Alternatively, spin probes can be used for incorporation into the lipid membranes in order to measure EPR signal. Figure 30 shows the structural formulas of two typical spin probes with different location of nitroxyl radical. It is also shown the location of the probes in

the membrane and the spectra of the probes. The EPR spectrum of quickly rotating $I_{1,14}$ (1-doxylstearat) represents a triplet involving narrow components. Any slowing down of the label rotation or any movement anisotropy is associated with visible change in the nitroxyl radical spectrum. It is typical for spin label $I_{12,3}$ (12-doxylstearat) the nitroxyl radical of which is located closely to the polar head group region, which is more ordered than region of inner hydrophobic region of the membrane.

The EPR spectrum and its components are dependent on the anisotropy of the environment as well as on the ordering, micro viscosity, surface charge, etc. Thus, increased environmental anisotropy broadens spectral lines which become more distant from each other. Parameter A_{II} , distance between outer maxima (Fig. 30) is sometime employed as it depends on the label rotation speed and on the degree of the label orientation in respect to membrane surface. Also the relationship of amplitudes of the individual spectrum components is changed. Changes in environmental anisotropy are followed by changes in rotational diffusion of the label. Quantitatively, rotational diffusion is characterized by the rotational correlation time τ_R

$$\tau_R^{-1} \sim 2\pi (A_{zz} - A_{xx})/h,$$
(29)

where $(A_{zz} - A_{xx})$ is the maximum anisotropy of the ¹⁴N-hyperfine splitting of the nitroxyl radical [131]. The characteristic rotational times for lipids in fluid state are around 10^{-9} s. Characteristic time for lipids restricted by interactions with proteins are in region $\tau_R \sim 1-5 \times 10^{-8}$ s [132].

The ordering parameter S is an informative characteristic of EPR spectra. This parameter characterizes the degree of ordering of the bilayer. The parameter S is determined by [133]

$$S = \frac{A_{\rm II} - A_{\perp}}{2A_{xx} - (A_{xx} + A_{yy})/2} \frac{A_{xx} + A_{yy} + A_{zz}}{A_{\rm II}/2 + A_{\perp}},$$
(30)

where $A_{xx} = A_{yy} = 0.58$ mT, $A_{zz} = 3.1$ mT. The order parameter depends on the structural state and composition of the bilayer. In general the parameter S decreases toward the center of bilayer, which is consistent with increased mobility of the hydrocarbon chains toward the methyl groups (Fig. 31) [134].

Despite a number of advantages of EPR method of spin probes, the question may arise about possible disturbance of the membrane by spin probe. This problem does not exist for *NMR*. This is due to the fact that many nucleus have their own magnetic moment, which is sensitive to their surrounding, i.e. they allow to measure NMR spectra. The resonance condition for NMR is given by relation

$$H_{RN} = \nu h/g_N \mu_N,\tag{31}$$

where g_N is the nucleus magnetogyric ratio and μ_N is nuclear magneton. The magnetic moment is typical for nuclei ¹H, ¹⁵N, ¹⁹F, ³¹P etc, but not for ⁴He, ¹⁴O, ¹²C. In biological objects there are many protons, which give possibility to apply this method for investigation of ordering and dynamics of biomembranes. The spectral lines depends on the chemical structure of the atoms. This is so-called chemical shift. Unfortunately, large number of chemically different protons have similar value of chemical shift. This problem can be solved by selective deuteration of the molecules, e.g. lipids using isotopes ²H or ¹³C. If hydrophobic chains of phospholipids are predeuterated, the order parameter *S* can be determined. This parameter decreases toward center of the bilayer similarly like in the case of EPR method.



Fig. 31. The plot of the ordering parameter S on the position of hydrocarbon, n, for fully hydrated multilayers of A – eggPC and B - eggPC + cholesterol (molar ratio 2:1) (According to [134] reproduced by permission).

The study of structure and dynamics of molecules has been successfully performed using ³¹P NMR. The nuclei ³¹P are often present in various biological molecules. These spectra are sensitive to the structure of lipid bilayer. This is due to the fact that lipid phosphorus exhibits a large chemical shift anisotropy. In a large liquid crystalline systems, such are biomembrane (linear dimensions > 200 nm) the rotation of the lipid molecule along its long axis is only partially averaged. Therefore instead of narrow component the NMR spectrum is broadened and is composed of a low field shoulder and high field peak (Fig. 32). This shape of ³¹P NMR spectra is typical for large multilayer systems, e.g. MLVs. In contrast with MLVs, in small sonicated unilamellar vesicles (ULV) the fast diffusions of the lipid molecules producing line-narrowing effect. The narrow NMR spectra are typical also for micelles, cubic or rhombic lipid phases [135,136]. However, when lipids are in H_{II} hexagonal phase (Fig. 32), additional motional averaging take place due to lateral diffusion around small (2 nm diameter) aqueous channels. This effect resulted in characteristic ³¹P NMR line shape with reverse asymmetry compared to the bilayer spectra (Fig. 32) [136]. Thus the ³¹P NMR spectra can be used for study the formation of non lamellar phases in a lipid systems. The non lamellar systems – hexagonal (H_{II}) phases can appear in initially lamellar phases of lipid bilayers composed of phosphatidylethanolamines at higher temperatures. The transport of ions can also induce the H_{II} phase [137]. Non lamellar phases can appear also around proteins in a membrane [138].

The analysis of the NMR spectra revealed that at liquid crystalline state of the bilayer fast rotation, characterized by relaxation time $\tau_R \leq 10^{-7} \text{ s}^{-1}$ take place. At temperature of phase transition of phospholipids from less ordered liquid crystalline phase to more ordered gel phase at lower temperature, the micro viscosity of the membrane increases. This is reflected by increasing the time of molecule rotation $\tau_R > 10^{-5}$ s. However, the mobility of polar head groups of phospholipids are less sensitive to the phase transition in comparison with hydrocarbon chains



Fig. 32. The schematic structure of some phospholipid phases and their ³¹P NMR spectra (Reproduced by permission from Ref. [135]).

and the relaxation time of the rotation of the head groups is less affected by temperature. The sensitivity of ³¹P NMR to a structural state of the membrane makes this method sensitive tool for study the phase transitions in lipid bilayers. For example, the half width $\Delta\Theta_{1/2}$ of the sharp peak of the ³¹PNMR spectra of MLVs is influenced by dipole-dipole interactions between phospholipid head groups. This interaction changes during phase transition of bilayer. Therefore, the phase transition can be studied by measurement of the dependence of the $\Delta\Theta_{1/2}$ on the temperature [139].

Among optical spectroscopy methods the *fluorescent spectroscopy* is especially suitable for study the physical properties of lipid bilayers. The phospholipids do not reveal fluorescence, therefore they should be labeled by fluorescence labels, or fluorescence probes should be used for monitor the membrane properties, because the probes are sensitive to their surrounding. Currently there exist a large variety of fluorescent probes, that can be used for study various aspects of membrane biophysics and bioelectrochemistry. The amphiphilic probes sensitive to the membrane anisotropy (e.g. 1,6 diphenylhexatriene (DPH)) or potential sensitive styryl dyes (e.g. RH 421 or di-8-ANEPS) can serve as an example.

The application of fluorescent probes to study ordering and dynamics of the membrane is based on measurement fluorescence intensities $I_{||}$ and I_{\perp} in two directions of polarization with



Fig. 33. The temperature dependence of the fluorescence anisotropy, r, for fluorescence probe DPH in DPPC vesicles (\circ), DPPC in complex with DNA (\Box) and DPPC in complex of dextrane sulphate (\triangle). In last two cases also magnesium ions were presented in electrolyte. All compounds were in equimolar concentrations 2.5×10^{-4} mol 1^{-1} and the concentration of the fluorescence probe was 2×10^{-7} mol $^{-1}$ (Reproduced with permission from Ref. [141]).

respect to the direction of exciting beam, respectively. The fluorescence anisotropy is characterized by parameter r

$$r = \frac{I_{\rm II} - I_{\perp}}{I_{\rm I} + 2I_{\perp}}.\tag{32}$$

Thus, if the probe is in fully isotropic environment, then r = 0. The membrane, however represent anisotropic body, therefore $r \neq 0$ and their fluorescence anisotropy will depend on the membrane structural state. The anisotropy measured in a steady-state conditions can be presented as

$$r = \frac{r_0 - r_\infty}{1 + \tau/\tau_c} + r_\infty,\tag{33}$$

where r_0 and r_∞ are the initial and limiting values of time-resolved anisotropy, τ_R is correlation time and τ is life time of fluorescence probe. The parameters r_0 and r_∞ are connected with the order parameter S [140]

$$S^2 = r_\infty / r_0. \tag{34}$$

The fluorescence spectroscopy is useful for study temperature phase transitions and for the mechanisms of interaction of various species with lipid bilayer. As an example on Fig. 33 there is a plot of fluorescence anisotropy on the temperature obtained with DPH dye in vesicles composed of DPPC and in complexes of DPPC with DNA from salmon sperm and with dextran sulphate in presence of magnesium ions. For pure DPPC vesicles the parameter r sharply changes with increasing the temperature at the region of pretransition ($T \approx 35$ °C) and at main transition



Fig. 34. Fluorescence excitation spectra of RH421 in the aqueous solution of 0.2 mg/ml DPPC vesicles in the control (full curve) and in the presence of 0.5 μ M gramicidin A (dotted curve) or 10 μ M phlorentin (dashed curve) (Reproduced by permission from Ref. [144]).

temperature ($T \approx 41$ °C). For the complex DPPC+DNA +MgCl₂ only monotonous decrease of r was obtained. It is possible that interaction of DNA with the head groups of DPPC resulted partial denaturation of DNA and as a result more hydrophobic bases interact with hydrophobic part of the bilayer. Interaction of dextran sulphate (DS) with DPPC is rather strong as it is evident from Fig. 33. It is seen that DS induced second phase transition at temperature higher then main transition of phospholipids. This effect may be due to the formation of hydrogen bonds between hydroxyl groups of DS and the carbonyl groups of DPPC [141].

Potential sensitive dyes, like RH421 or di-8-ANEPS can be use as a sensitive tool for monitor changes of membrane potential. They revealed so called electrochromic effect, i.e. the electric field caused shift in their fluorescence spectra [142].

The styryl days, like RH421, are amphiphilic with partition coefficient $C_{lipid}/C_{water} > 10^5$ [143]. Due to amphiphilic nature of the dye, its polar part is located at the region of the polar head groups of phospholipids, while the chromophore and the hydrophobic tail of molecule are located at hydrophobic part of the membrane. The sensitivity of the dye to the changes of dipole potential of the membrane is illustrated on Fig. 34, where the normalized fluorescence excitation spectra of RH 421 in DPPC vesicles with and without gramicidin (5 μ mol l⁻¹) and/or phloretin (10 μ mol l⁻¹) are presented. The shift of the spectra evidences that both species changes the dipole potential of the DPPC bilayer [144]. Quantitative information of the changes of dipole

potential can be obtained by determination of the ratio of fluorescence intensities detected at two excitation wavelength on the blue and red edges of the excitation spectra [144]. For RH421 dye it is ratio: $R = I_{440}/I_{540}$, where indexes correspond to the wavelength of excitation beam. For example, Shapovalov et al. [144] showed, that gramicidin A resulted pronounced decrease of the R value with increasing concentration of this channel former. It has been supposed that gramicidin reduces the existing positive dipole potential of bilayers by inducing reorientation of dipole-carrying groups (cholines or hydrated waters).

The ion translocation could change the local electric field in the membrane [145], therefore the electrochromic effect of styryl dyes can be used for study the charge movement across membranes with incorporated ionic pumps [145].

If the membrane contained fluorescence dyes is shortly illuminated by intensive laser beam, the dyes at light exposed area lost of the fluorescence. The fluorescence of this part, however, start to increase with time due to diffusion of undamaged dyes from surrounding membrane surface, that was not illuminated by the laser. This method, that is called photo-bleaching, allowing to determine coefficients of lateral diffusion of lipid bilayers ($6 \times 10^{-12} \text{ m}^2 \text{ s}^{-1}$) or proteins ($10^{-14} \text{ m}^2 \text{ s}^{-1}$). Large variety of modern laser spectroscopic methods, such are Raman spectroscopy, time resolved nano- and picosecond spectroscopy are also of considerable interest in membrane studies [146].

5 Phase transitions in lipid bilayers

The bilayer lipid membranes can exist in different phases depending on the water content and temperature. Transition between phases can be induced by varying either the lipid concentration (lyotropic mechanism) or temperature (thermotropic mechanism). For biomembranes the particular interest are transitions involving the lamellar or bilayer lipid phase. The phase transition processes typical for biomembranes are connected with hydrocarbon chain – melting transitions and in transitions to non-lamellar phases. The chain-melting transition is based on configuration entropy of the hydrocarbon chains. The driving force for transition from lamellar to non-lamellar phase is the tendency to spontaneous curvature of the bilayer phase. This process has great significant for cell or vesicle fusion [147].

The various phases has been classified by nomenclature proposed by Luzatti [148]. Latin letter characterizes the type of long-range order: L – one dimensional lamellar, H – two dimensional hexagonal, P – two dimensional oblique, Q – three dimensional cubic, C – three dimensional crystalline. A lower-case Greek subscript characterizes the short-range conformation of the hydrocarbon chains: α – disordered (fluid), β – ordered untilted (gel), β' – ordered tilted (gel). Roman numeral subscript is used to characterize the content of structure element: I – paraffin in water (normal), II – water in paraffin (inverted).

5.1 Lyotropic and thermotropic transitions

Lyotropic transitions. In general, lyotropic transition between single phases will take place via two co-existing phases. The lyotropic mesomorphism found in lipid-water system can be schematically represented by diagram (Fig. 35) [147,149]. At very low lipid concentration, below the critical micelle concentration (cmc), the lipid is in form of monomers. At concentration



Fig. 35. Schematic lyotropic lipid-water phase diagram for phospholipids. This is high temperature section of the temperature-composition, and is intended primarily to indicate phase transitions induced by varying water content. Hatched areas indicate two-phase domains. The left-hand side of the diagram (from the L_{α} phase to higher water content) is representative of single-chain phospholipids (V/Al < 1 – here V is volume, A is area and l is the effective length of phospholipid molecule), and the right-hand side (from L_{α} phase to lower water content) is representative of two chain phospholipids (V/Al > 1). (Reproduced by permission from Ref. [147]).

higher then cmc the lipids form normal micelles (M_I) . With further increasing the lipid concentration the system is transformed first to normal hexagonal phase (H_I) (probably via intermediate cubic phase Q'_I). H_I is then transformed to the normal cubic phase (Q_I) and then to lamellar phase (L_{α}) . This sequence is typical for single chain phospholipids. For two chain phospholipids at their higher concentration the lamellar phase is transformed into an inverted cubic phase (Q_{II}) and then to and inverted hexagonal phase (H_{II}) .

Thermotropic transitions. Because in excess of water the composition is fixed a single phase can exist over range of temperatures. However, two phases can coexist only at fixed temperature. Therefore, sharp thermotropic phase transition occurs at certain temperature. In general, the sequence of thermotropic transitions of hydrated phospholipids can be presented by following scheme

$$L_{c} \xrightarrow{T_{s}} L_{\beta} \xrightarrow{T_{p}} P_{\beta'} \xrightarrow{T_{t}} L_{\alpha} \xrightarrow{T_{h}} Q_{II} \to H_{II} \xrightarrow{T_{l}} M_{II}.$$
(35)

With increasing the temperature, at T_s the subtransition from crystalline phase (L_c) to hydrated lamellar gel phase (L_β) take place. The lipid chains can be tilted or not tilted with respect to the bilayer normal. Then, at temperature T_p , the pretransition from low temperature gel phase to an intermediate ripple phase $(P_{\beta'})$ take place. Then the main transition from gel to fluid lamellar phase (L_{α}) occurs at the temperature T_t . The phase L_{α} is characterized by disordered lipid chains. With increasing the temperature the fluid phase undergo further transitions. First to inverted cubic phase (Q_{II}) and then into inverted hexagonal phase (H_{II}) . The final stage is the transition into the inverted micellar phase (M_{II}) . This phase is characterizes as an immiscible oil in excess water. Not all the phases and transitions mentioned above should appear for a single phospholipid [147].

5.2 Thermodynamics of phase transition

At the phase transition temperature the structure and properties of the lipid bilayer change sharply within narrow temperature interval – usually less then 0.1 °C. The changes of ordering of lipid bilayer are connected with changes of entropy of the system. In addition there are sharp changes of the volume of phospholipids. This is typical feature of the first order transition. The change of Gibbs energy at the transition temperature (T_t) is zero, i.e. $\Delta G = \Delta H_t - T_t \Delta S_t = 0$ and thus the changes of entropy of the system at phase transition temperature are connected with changes of the system enthalpy

$$\Delta S_t = \Delta H_t / T_t. \tag{36}$$

For a first order transition, the change in transition temperature with pressure, P, should be directly related to the change in volume, ΔV_t , at the transition, via Clausius-Clapeyron equation

$$\frac{dT_t}{dP} = N_A \Delta V / \Delta S_t, \tag{37}$$

where ΔS_t is the transition entropy and N_A is Avogadro's number. This relation is valid for phospholipid bilayers [150]. Similarly, the change in transition temperature in response to an isotropic membrane tension γ , is related to the changes in bilayer area ΔA_t at the phase transition

$$\frac{dT_t}{d\gamma} = 2N_A \Delta A / \Delta S_t,\tag{38}$$

where the factor of 2 allows for the two halves of the bilayer. This equation has been verified in Ref. [151].

Various effects, particularly connected with the composition of aqueous phase, e.g. ionic strength can induce the shift in phase transition temperature (see [147]). For charged lipids, shift in transition temperature arise from difference in surface charge density in the two phases. This shift can be estimated using electrostatic double layer theory (see below)

$$\Delta T_t^{el} = -\frac{\varepsilon}{\pi} \left(\frac{kT}{e}\right)^2 N_A \kappa \left(\sqrt{1 + \left(\frac{\sigma}{c}\right)^2} - 1\right) \frac{\Delta A_t}{\Delta S_t},\tag{39}$$

where $\kappa = \sqrt{8\pi N_A e^2 I/1000\varepsilon kT}$, is the reciprocal Debye screening length and $c = kT\varepsilon\kappa/2\pi e$. The electrostatic shift is determined mostly by the charge density, σ , the ionic strength, I, and by the change in the area/molecule, ΔA_t . The experimental determination of the phase transition temperature due to the electric effects has been performed by Träuble et al. [152].

Experimentally the phase transition can be determined by measuring the transition enthalpy by scanning calorimetry. The calorimetric properties can be presented as a specific heat capacity



Fig. 36. Calorimetric and specific volume properties as a function of temperature for multilamellar vesicles of DPPC. (a) - specific partial heat capacity (the entire heat absorption peak, reduced 100-fold, is shown at the bottom), (b) - Specific partial enthalpy, (c) - specific partial volume. The temperature dependencies of undisturbed states are extrapolated by dashed straight lines. (Reproduced by permission from Ref. [154]).

or changes the enthalpy as a function of temperature. An example of the calorimetric properties of multilamellar vesicles of DPPC is shown in Fig. 36 together with a plot of specific volume as a function of temperature. The specific volume, $V = [1 - (\rho - \rho_0)/c]/\rho_0$ of phospholipid can be determined on the base of the precise measurement of the density, ρ , using e.g. vibrating tube principle [153]. Here ρ is the density of the lipid solution, ρ_0 is the density of the buffer and c is the concentration of lipids. The low temperature peak of heat capacity at T = 35 °C is connected with pretransition, i.e. transition from ripple gel phase to lamellar gel phase. The narrow peak at $T_t = 41.2$ °C is connected with main transition from gel to fluid lamellar phase. From the changes of specific volume we can see sharp changes at main transition temperature confirming the first order of the main transition.

5.3 Trans-gauche isomerization

Microscopically the chain melting is connected with rotation around carbon bonds of hydrocarbon chains of phospholipids. The lowest energy holds for trans and highest for cis conformation of the chains. At gel state the rotation is restricted and the saturated chains are in trans confor-



Fig. 37. The conformation of hydrocarbon chain of phospholipid. A – trans configuration, B – gauche-trans-gauche conformation, C – cis-trans-gauche configuration.

mation. When temperature approaching the phase transition region the probability of rotation increases. The rotation by 120° relatively to trans conformation resulted in formation of gauche (+) or gauche (-) conformation. Energetically the gauche conformation does not differ substantially from trans conformation (2-3 kJ mol⁻¹), however these two conformations are separated by relatively high energetical barrier (12–17 kJ mol⁻¹). The appearance of gauche (+) conformation caused sterical difficulties in a bilayer. However, subsequent gauche (-) rotation resulted in diminish of sterical repulsion. As a result the gauche (+)-gauche (-) rotation the kink conformation of lipid chain appears (Fig. 37). In this case the space configuration of the chain is preserved, but the chain is shorter by 0.127 nm and the cross-sectional area increases. The phase transition in lipid bilayer from gel to liquid state is therefore accompanied by decreasing the thickness and increasing the area per molecule. The volume of phospholipid changes in lesser extend. The presence of unsaturated phospholipids caused considerable increasing the probability of trans-gauche isomerization and therefore the phase transition temperature decreases. In order to estimate the effectivity of trans-gauche isomerization let us compare the frequency of torsional oscillations of C-C bonds ($\sim 7 \times 10^{12} \text{ s}^{-1}$) with the frequency of appearance the gauche conformation at the room temperature (≈ 300 K). Considering that the energetic barrier separating the trans and gauche conformation is $\Delta E = 12 \text{ kJ mol}^{-1}$ we have: $\nu = (kT/h) \exp[-\Delta E/RT] \approx 10^{10} \text{ s}^{-1}$ [86]. Thus, the gauche conformation appears with a high frequency due to torsional oscillations. In the fluid state the kink can move along the chain due to synchronous rotation by 120° of corresponding C-C bond. The shift to the neighboring position is of the order of $\Delta L = 0.13$ nm. The shift of the king can be considered as one dimensional diffusion along the chain. This diffusion can be characterized by diffusion coefficient $D_k = 0.5\nu(\Delta L)^2 \approx 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (here we assumed that the frequency of the jump of the kink is of the order of 10^{10} s⁻¹). This value practically coincides with the diffusion of oxygen, water or small molecules of non electrolytes through lipid bilayer. Thus it can be assumed that the transport of some species through the lipid bilayer can be due to appearance of free volume in a membrane formed by kinks.

5.4 Order parameter

High mobility of hydrocarbon chains allowing to determine only average, most probable orientation of the chains. Even in a gel state there exist conformational mobility of the chains that increases toward the center of the bilayer. In order to describe the shift of the orientation of the chain from the direction normal to the bilayer surface there is usually used the order parameter S_n

$$S_n = \frac{3}{2}\overline{\cos^2}\Theta_n - \frac{1}{2},\tag{40}$$

where Θ_n is the angle between the normal of the bilayer and the normal to the plane formed by two vectors of C-H bonds of the n-segment of the hydrocarbon chain. Obviously for ideally ordered chain S = 1 and that for disordered phase S = 0. The order parameter of the bilayer is almost constant up to 8-9 methyl segment, but decreases substantially after this segment. This has been established both by NMR and EPR spectroscopy [6,132]. It is possible, that the initial segments of the hydrocarbon chain provide cohesion interaction between the chains, that is, in addition to hydrophobic interaction, necessary for preserving the bilayer integrity. It is interesting that in most of the natural phospholipids the double bonds in an unsaturated fatty acids occure after 9 carbon atom, and thus did not decrease the ordering of the densely packed starting parts of the chains.

5.5 Cooperativity of phase transition

The chain-melting transition of phospholipids with saturated hydrocarbon chains is highly cooperative with transition width that can be less than 0.1 °C. Phenomenological theory of the cooperativity of the phase transition has been formulated on the basis of coexistence of the clusters of lipid molecules. The cooperativity of the transition is evaluated as the ratio between the Van't Hoff enthalpy to the calorimetric enthalpy: $\Delta H_{VH}/\Delta H_{CAL} = 1/\sqrt{\sigma_0}$, where σ_0 is the parameter of cooperativity and the value $1/\sqrt{\sigma_0}$ is the size of cooperativity unit ($\sigma_0 = 1$ correspond to non cooperative behavior, while value $\sigma_0 < 1$ represent cooperative behavior. Lesser value of the σ_0 the higher cooperativity of the system). The Van't Hoff enthalpy can be approximately estimated from the half width of the transition $\Delta T_{1/2}$. $\Delta H_{VH} \cong 7T_t^2/\Delta T_{1/2}$ [86]. For example, the number of lipid molecules in a cooperative units for DPPC was estimated as 70 ± 10 and that for DMPC 200±40. Due to thermal fluctuations the liquid phase born in a gel phase. At the phase transition temperature both gel and liquid phase coexist. With increasing the temperature the number of molecules in a gel phase dramatically decreases. The fluctuations in cluster size take place throughout the transition [155].

5.6 Theory of phase transitions

The simplest theory of phase transition in a lipid bilayers has been raised by J. Nagle [156] and is based on the ordering-disordering transition with assumption of existence of trans-gauche

conformations in each hydrocarbon chain (see above). The temperature of phase transition calculated on the base of this theory was close to that obtained by calorimetry. In the model by Marelja [157] the energy of the chain with configuration i in a bilayer is determined as follows

$$E^i = E^i_{in} + E^i_{disp} + pA^i, (41)$$

where E_{in}^i is the energy of the chain connected with trans-gauche-transition. The second term E_{dis}^i is connected with intermolecular disperse interactions and the third term (pA^i) is connected with existence of the lateral pressure in a bilayer due to steric repulsions, electrostatic interactions and hydrophobic effect. The model of Marelja allowing to calculate the phase transition temperature and the enthalpy of the transition as well as the basic geometrical parameters of the chains in a good agreement with experiments.

The phase transitions in a bilayer can be described in general also by phenomenological theory by Landau [158]. This theory allowing to calculate free energy nearby the phase transition temperature

$$G_{\eta} = a_1 \eta + \frac{1}{2} a_2 \eta^2 - \frac{1}{3} a_3 \eta^3 + \frac{1}{4} a_4 \eta^4, \tag{42}$$

where η is ordering parameter, $a_1 = p(A_f - A_g)$, p is the lateral pressure, A_f and A_g are the area per lipid molecule in liquid and in a gel state, respectively. Coefficients a_2 , a_3 and a_4 can be found from the dependence of T_t and η on the lateral pressure (p). The parameters of the phase transition can be found from the minima of the function $G_{\eta}(T, \eta)$. In the theory of Landau the ordering parameter is determined through area per molecule in a transition temperature

$$\eta = \frac{A_f - A}{A_f - A_g},\tag{43}$$

where A is the real area per molecule in a bilayer. Using the Landau theory it has been possible to estimate the influence of cholesterol and proteins on a phase transition temperature. The results of these works are in agreement with the theory developed by Marelja.

The microscopic Pink lattice model [159] is based on description of conformational properties of acyl chain by a small number of conformational states. The conformational chain variables are coupled by hydrophobic anisotropic van der Waals interactions. The interaction between the hydrophilic moieties is modeled by a Coulomb-type force or simply by an effective intrinsic lateral pressure.

Detailed description of the phase transitions in lipid bilayers is given in book by Cevc and Marsh [3].

6 Mechanical properties of lipid bilayers

6.1 Anisotropy of mechanical properties of lipid bilayer

Viscoelastic properties have a significant role in allowing biomembranes to perform different functions. Together with cytoskeleton, viscoelasticity determines the cell shape and the transfer of mechanical deformation from mechanoreceptors to sensitive centers. Moreower during conformational changes of the proteins also the physical properties of the membrane could change.



Fig. 38. Schematic representation of the membrane deformation. Arrows indicate the application of mechanical stress. For explanation, see the text.

These changes could be described by means of macroscopic approach using the theory of elasticity of solid bodies and liquid crystals. The structure of lipid bilayer is simpler than those of biomembranes. However even the structure of BLM has clearly expressed anisotropy. This leads to strong anisotropy of membrane viscoelastic properties and requires description of bilayer properties by several elasticity modules. The difficulties of describing of membrane elasticity are still not exhausted by this phenomenon. The behavior of deformable solid bodies is described by the theory of elasticity. The principal differences of biomembranes from the classical objects of this theory are as follows. The membrane thickness is very small and is between 20-200 atomic size. Therefore the influence of microheterogeneity of each atomic layer on membrane properties can be substantial. In this case the macroscopic parameters of the membrane, which are the result of the averaging of their properties over the environment, can considerably differ from the corresponding parameters of the membrane at the level of some distinguished layers. On the other hand 20–50 atomic layers is too large a value to enable their description by equations of the theory of elasticity for each layer. Therefore there exist a number of models of biomembranes as elastic bodies, which average the properties of biomembranes by certain numbers of such layers [160-164]. However, for subsequent analysis it is necessary to introduce a macroscopic description of membrane as an elastic body and generalize it to account for its viscous properties. This analysis has been performed in monograph [76]. It has been shown that understanding of the membrane as a viscoelastic body requires analysis of membrane deformation in different directions (Fig. 38): (1) volume compressibility; (2) area compressibility; (3) unilateral extension along membrane plane; (4) transversal compression. The mechanical parameters that characterize membrane deformability listed above are volume compressibility modulus K and Young moduli of elasticity E_{II} , E_{10} and E_{\perp} , respectively. These parameters are defined as follows

$$K = -p/(\Delta V/V), \quad E_{II} = \sigma_x/(\Delta A/A) = 2\sigma_x/(\Delta C/C),$$

$$E_{10} = \sigma_x/U_{xx}, \qquad E_{\perp} = -p/U_{zz} = 2p/(\Delta C/C),$$
(44)

where σ_x is the mechanical stress along the membrane plane, p is the pressure compressing the membrane, U_{zz} and U_{xx} are relative membrane deformation in transversal direction and along the membrane plane, $\Delta V/V$, $\Delta A/A$, $\Delta C/C$ are the relative changes of the volume, area and electrical capacitance, respectively. Owing to small dimensions of the membrane, special methods were developed for measurement the elasticity modules. Below we briefly describe the basic methods of measurement the elasticity moduli of lipid bilayer and will show their typical properties. Mechanical properties of lipid bilayers have been described in detail elsewhere [76].

Transversal elasticity modulus E_{\perp} . Transversal deformation, i.e. parameter $U_{zz} = \Delta d/d$ (d is the membrane thickness) can not be measured directly due to small thickness of the membrane and extremely small changes of the thickness upon deformation. Therefore the transversal deformation is determined mostly from the measurement of the changes of the membrane electrical capacitance. In the case of isovoluminous deformation, i.e. when the volume compressibility, K, is much higher then E_{\perp} (this has been certainly showed experimentally [76]): $\Delta d/d = -\Delta C/2C$ (i.e. decrease of thickness resulted in increase of membrane capacitance). In transversal direction, the membrane can not be deformed by mechanical pressure. However, because the membrane behaves electrically as a capacitor, when voltage is applied to the BLM, it will compress the membrane with an electrostriction pressure $p = \frac{C_S V^2}{2d} (C_S \text{ is the}$ specific capacitance of the membrane $C_S = C/A$ and V is the applied voltage). Therefore $E_{\perp} = -p/(\Delta d/d) = 2p/(\Delta C/C)$. For measurement of the changes of capacitance a special method is required as well. This is connected with inhomogeneity of the membrane and with the presence of thick Plateau-Gibbs border. For example, if the dc voltage will be applied to BLM and the capacitance will be measured e.g. by capacitance meter, then the measured changes $\Delta C/C$ will be not only due to the changes of the thickness. Other factors such are rebuilding of new bilayer parts from the Plateau-Gibbs border will also contribute to the ΔC value. As a result the determined elasticity modulus will be underestimated in comparison with their real value. This particularly explains the underestimated values of transversal elasticity modulus in earlier works (see [76] for review). Therefore, special electrostriction method based on measurement of the amplitude of higher current harmonics has been developed [165]. This method as well as its application to various BLM systems has been described in detail in Ref. [76]. Briefly, if an alternating voltage of amplitude V is applied to the BLM through electrodes (e.g. Ag/AgClelectrodes), due to the nonlinear dependence of capacitance on the voltage ($C = C_0(1 + \alpha V^2)$), where C_0 is the capacitance at V = 0 and α is the electrostriction coefficient), the higher current harmonics with frequencies 2f, 3f, etc. amplitude I_2 , I_3 , etc. respectively, will be generated in addition to the basic first current harmonic (frequency f) of an amplitude I_1 . The measurements of these amplitudes allowed us to determine various parameters, particularly the absolute value of elasticity modulus

$$\left|\tilde{E}_{\perp}\right| = E_{\perp} = 3C_s U_0^2 I_1 / (4dI_3),\tag{45}$$

where C_s is the specific capacitance of the membrane. If in addition to the amplitude also the phase shift, φ , between first and third current harmonic is measured, then coefficient of dynamic viscosity, η , can be determined

$$\eta = E_{\perp} \sin \varphi / (2\pi f) \tag{46}$$

(see [76] for detailed description of the method and experimental set up). The elasticity modulus \tilde{E}_{\perp} is a complex value, which reflects the viscoelastic nature of BLM deformation. Due to viscoelasticity the elastic modulus should depend on the frequency of deformation. This has been



Fig. 39. Frequency dependence of (a) the elasticity modulus, E_{\perp} , and (b) phase shift, φ , for a BLM of eggPC in various hydrocarbon solvents: (1) n-heptane, (2) n-decane, (3) n-hexadecane. (4) – eggPC+cholesterol (1:1 w/w) in n-hexadecane.

approved by the current harmonic method [76]. The example of the plot of elasticity modulus and phase shift as a function of frequency of deformation is presented on Fig. 39 for BLM of egg phosphatidylcholine (egg PC) contained various solvent (n-heptane, n-decane o n-hexadecane) as well as for those containing cholesterol. We can see that for BLM contained n-hexadecane (curve 3) the value of E_{\perp} is much higher in comparison with those contained n-heptane or n-decane. This is connected with the amount of the solvent in lipid bilayer, which is lower for nhexadecane. We can see that presence of cholesterol caused further increase of elasticity modulus and substantially minimized the dispersion of this value at lower frequency of deformation. For comparison, the approximate values of elasticity moduli for rubber and bond are 10^4 and 10^9 Pa, respectively. Interestingly, the elasticity modulus of BLM composed of eggPC + cholesterol without solvent is close to those for bond [76].

The frequency dependence of elasticity modulus can be explained by simple rheological scheme (Fig. 40c). This scheme consists of two elastic elements with moduli E_0 and E_∞ , and one viscosity element with the coefficient viscosity η . The elasticity modulus E_0 characterizes the mechanical properties of BLM at lower frequencies, while E_∞ represents the elasticity modulus at higher frequency. At highest frequency of deformation the viscous element does not follow the changes of membrane thickness. Therefore the elasticity of BLM is determined by the value of E_∞ . As soon as the frequency decreases the elasticity element start to contribute



Fig. 40. Comparison of theoretical and experimentally obtained frequency dependence of the (a) dynamic modulus E_{\perp} and the (b) angle of losses φ , calculated for one relaxation time according to the scheme (c). Experiment – experimentally obtained dependence for BLM of eggPC in n-hexadecane (see also Fig. 39, curve 3); theoretical curves with model parameter E_0/E_{∞} : 1–0; 2–0.05; 3–0.1.

into the membrane deformation and the elasticity modulus decreases. At very low frequency the viscous frictions is rather small and the elasticity is determined mostly by the value of E_0 .

In the case $E_{\infty} \gg E_0$ the dependence of elasticity modulus E_{\perp} on frequency can be described by equation with one relaxation time τ_R

$$E_{\perp} \approx E_0 + i\omega\tau_R E_{\infty} / (1 + i\omega\tau_R), \tag{47}$$

where $\tau_R \approx \eta/E_\infty$. The experimental dependence of elasticity modulus, E_\perp , and the angle of losses, φ (Fig. 39, curves 3) was used to estimate parameters E_o, E_∞ and τ_R , using relationship (47). At $E_0 \ll E_\infty$ the shape of the curves is determined by the parameter E_0/E_∞ only. The curves are shifted along axes 2f. Fig. 40 shows that the theoretical curves (1–3) satisfactorily approximate the experimentally obtained dependence except for the high frequency part. The approximation of the angle of losses φ is less satisfactory. If elasticity characterized by the modulus of elasticity E_0 is absent, then the dependence $\varphi(2f)$ (Fig. 40b, curve 1) decreases at high frequencies, like the experimentally observed dependence. However, curve $\varphi(2f)$ lacks a maximum. If $E_0 \neq 0$, a maximum occurs within an interval of 40-320 Hz. With the increasing ratio E_0/E_∞ , the maximum becomes lower and shifts towards higher frequencies. At high frequencies, changes in the angle of losses at various ratios E_0/E_∞ are relatively small. The existence of a maximum on curve $\varphi(2f)$ is explained by the fact that BLM elasticity at low and high frequencies is determined by elastic elements (E_0 or E_∞), and thus $\varphi(2f)\approx 0$. In the



Fig. 41. Frequency dependencies of an imaginary part of an elasticity modulus $\text{Im}\tilde{E}_{\perp}$. 1 – theoretical curve calculated for one relaxation time using equation (47). 2 – $\text{Im}\tilde{E}_{\perp}$ for eggPC in n-heptane, 3 – $\text{Im}\tilde{E}_{\perp}$ for eggPC in n-heptane.

interval between low and high frequencies, it is the viscosity element 3 which also contribute to the determination of BLM viscosity. Consequently, the values of the angle φ within this interval range between 0 and 90°.

Parameters $E_{\infty} \sim 10^8$ Pa and $E_0 \sim 0.1 E_{\infty}$ can be easily obtained from Fig. 40a. Parameter τ_R can be evaluated at the frequency $(2f)_{1/2}$: at this frequency E_{\perp} decreases to one half as compared to E_{∞} . For curve 1, $\tau_R \approx 0.576/2\pi(2f)_{1/2} \approx 0.57$ ms. Hence, this simple model enables us to qualitatively explain the experimental results (Fig. 39), namely the strong initial growth of parameter E_{\perp} with the increasing frequency and a gradual retardation of this growth at higher frequencies. This model also explains the decrease of the angle of losses at low and high frequencies, and allows us to evaluate parameters E_0 , E_{∞} and τ_R for BLM composed of eggPC and n-hexadecane. The above model, however, fails to explain the gradual, although slow, growth of E_{\perp} with increasing frequency within the interval of small dispersion, and the very small decrease of φ within this interval. Moreover, a comparison of Figs. 40a and 40b shows that a better quantitative coincidence of calculations with one of the experimentally obtained curves, $\varphi(2f)$. It is entirely unclear how to express experimentally observed dependencies $E_{\perp}(2f)$ for membranes contained solvent within the above simple model with one relaxation time.

This fact is best documented by showing the theoretical curve for $\operatorname{Im} \tilde{E}_{\perp}$ together with experimental curves 2 and 3 (Fig. 41) for $\operatorname{Im} \tilde{E}_{\perp}$ (BLM of eggPC in n-decane or n-hexadecane, respectively, see Fig. 39). Curve 1 ($\operatorname{Im} \tilde{E}_{\perp}$) constructed by relationship (47) for one relaxation time has a maximum at $2\pi(2f)_{1/2}\tau_R = 1$, and is much more narrowed than the experimentally obtained relationship. The experimental relationship can be obtained by superposition of several curves of the same type as 1, shifted along the frequency axis. The standard generalization of the theory in this case, as accepted by rheology, consists in a further improvement of the model (the addition of other elastic and viscosity elements) with an introduction of a spectrum of relax-



Fig. 42. Inhomogeneous mechanical models of BLM and the nature of membrane deformation upon transversal compression. The dashed line shows the position of membrane parts following compression. (a) scheme of a three-layer BLM structure on cross-section; (b) sandwich with entirely adhering layers; (c) sandwich with free gliding of layers; (d) sandwich with microinhomogeneities and adhering layers. A – bilayer, B – microinhomogeneity with diameter r_n ; (e) and (f) schemes of planar and roughed BLM, respectively. (Reproduced by permission from Ref. [76]).

ation times [166]. Fig. 41 shows that that a spectrum of relaxation times between 1–0.01 ms is characteristic for BLMs of various composition. This fact adds a qualitative nature to a viscosity evaluation. In spite of this, in evaluating the initial directives of the frequency dependence of E_{\perp} , with $E_{\perp} \sim \omega \eta$, effective values of $\eta \sim 10^3$ Pa s can be obtained for eggPC in n-decane and 10^5 Pa s for eggPC in n-hexadecane.

For the three layer model of lipid bilayer (Fig. 42), the reological scheme of BLM (Fig. 40c) may have a simple structural interpretation. The elastic element with a large value of $E_{\perp} = E_{\infty}$ consists of two elements describing the elasticity of outer layers, and limiting elasticity of the inner layer at higher frequencies. Element with small value of elasticity, i.e. E_0 , corresponds to the inner layer. Upon compression, hydrocarbon chains get reorganized in this element, which is naturally associated with the overcoming of the potential barriers of moving chains, i.e. with viscosity forces. Viscosity forces are allowed for by element with viscosity η . Monotonous growth of E_{\perp} in parallel with frequency is due to the fact that within the low frequency interval chain rebuilding may be completed within the pressure change period. However, at higher frequencies, this rebuilding duration is longer than the pressure change period. Consequently, identical pressure values results in weaker deformation at higher frequencies, and thus is associated with larger values for moduli of elasticity.

The question arises, what are the causes underlying the considerable differences in E_{\perp} for BLMs of various thickness. In thinner membranes (e.g. BLM in n-hexadecane), hydrocarbon chains of lipid molecules touch each other in the inner membrane layer, and are sufficiently closely ordered. In thicker membranes (e.g. BLM in n-heptane or n-decane) with large amounts of solvent in the membrane, this ordering is less compact. Qualitatively, the dependence of E_{\perp} on membrane thickness may be associated with both, the closeness of chain ordering in contact area and their ability to bend and rotate. With dense chain ordering, pressure applied

to the membrane in the direction perpendicular to the surface, is able to induce only a weak compression of the bilayer. With a looser ordering the chains may change their conformation and orientation due to pressure, and a considerable decrease of bilayer thickness may result. Hence, thicker BLMs should have lower E_{\perp} than thinner membranes. Obviously, the ordering density also determines the range of relaxation times: with looser chain ordering in BLMs, processes may take a relatively short time to be completed. It is therefore, not surprising that the range of relaxation times for a BLM with n-decane is in the region of shorter times than in the case of the BLM with n-hexadecane.

The elasticity modulus E_{\perp} is extremely sensitive to the lipid composition and content of cholesterol or other sterols. For example, E_{\perp} value increases with increasing the length of hydrocarbon chains of phospholipids, that evidence on higher order of hydrophobic part of the membrane due to more extensive hydrophobic interaction between the phosholipids chains [76]. On the other hand E_{\perp} decreases with increasing of the degree of unsaturation of fatty acids, that evidence on decrease the membrane ordering [167]. This elasticity modulus changes considerably upon interaction with BLM of low molecular compounds, e.g. local anesthetic or macromolecules, e.g. integral or peripheral proteins [76]. The method of measurement E_{\perp} has been applied also to the supported lipid membranes and gave possibility to study the affinity interactions [168] or the interaction with BLM of nucleic acids and their complexes with cationic surfactants [169].

The area expansion modulus, E_{II} , can be determined by the method of micropipet pressurization of giant bilayer vesicles [151], or by determination of the changes of electrical capacitance during periodical deformation of spherical BLM [160]. The value of E_{II} can be measured only in a limited range of frequencies (5–10 Hz). The typical values of E_{II} of BLM with the hydrocarbon solvent were in the range 10^7-10^8 Pa, which is more then 10 time higher then the values of E_{\perp} for similar BLM composition at lowest frequency of deformation (20 Hz). The area expansion modulus is less sensitive to the lipid composition and does not significantly depend on the length of hydrocarbon chains of phospholipids and degree of their unsaturation [170].

The elasticity modulus E_{10} has been measured upon longitudinal distension of cylindrical BLM formed between two circles, with one of them oscillating and the other one being attached to an ergometer. Values $E_{10} \ge 10^6$ Pa have been obtained for membranes of various composition. They were independent of frequency over an interval of 30–200 Hz, i.e. they are determined by bilayer elasticity rather then viscosity [171].

Modulus of volume compressibility K has been measured by determination of sound velocity in suspension of small unilamellar liposomes. Values of $K = (1.70 \pm 0.17) \times 10^9$ Pa have been determined by this method for liposomes composed of egg phosphatidylcholine. The values of a similar order have been obtained also on large unilamellar liposomes composed of polyunsaturated fatty acids [167]. Using the measurement the elasticity modulus K, the mechanic and thermodynamic properties of liposomes of various composition, and that contained cholesterol [172] or modified by proteins [173] can be studied.

The experiments on determination various elasticity moduli revealed, that these values can be estimated only in a limited range of frequencies: $E_{II} - 5-10$ Hz, $E_{10} - 2-300$ Hz, $E_{\perp} - 20$ Hz– 15 kHz and K - 7 MHz [76]. However these values can be approximated to the frequency range 10–200 Hz [76]. It has been shown, that following inequalities hold for these elasticity modules: E_{10} , $E_{\perp} \ll E_{II} \ll K$. Thus the BLM represent anisotropic viscoelastic body. The corresponding model of BLM deformation should fulfill the above unequalities.

6.2 The model of elastic bilayer

It has been shown [76], that the mechanical properties of BLM can not be described by isotropic mechanical models proposed by Wobshall [160] or by Evans and Skalak [174]. Recently discussed brush model of the membrane mechanics composed of two isotropic layers well describe the behavior of area expansion and bending elasticity modules [170]. However the model does not provide information about distribution of chain across bilayer and thus, does not consider anisotropy of mechanical properties in transversal direction. The three layer model of deformation has been assumed for description of BLM anisotropy by Passechnik [164]. The two outer layers (thickness h_1) have a modulus of elasticity $E^{(1)}$, and the inner layer (thickness h_2) has a modulus of elasticity $E^{(2)} << E^{(1)}$, like a sandwich (Fig. 42).

Mechanical stress in membrane plane (measurement of E_{II}) deforming the layers with a large elastic modulus $(E^{(1)})$ and the stress perpendicular to the membrane plane (measurement of E_{\perp}) deforming the "soft" layer modulus $(E^{(2)})$. Therefore one can expect, that $E_{\perp} \ll E_{II}$. Deformation of the "sandwich" depends on the degree of adhering of layers. From the analysis performed in [76] follows, that the three layer model with different degree of layer adherence (i.e. sandwich with fully adhered layers, Fig. 42b, and sandwich with free gliding layers, Fig. 42c) can describe only the properties of the small parts of a BLM. To describe the deformation of all BLMs these parts must be separated by the regions into which a "superfluous" matter of bilayer, which is squeezed out with the transversal compression of the BLM, will be adsorbed. For this purpose microinhomogeneities with thickness not surpassing that of BLM were included in the model [164]. As in models a-c (Fig. 42), the model with inhomogeneities consists of an inner layer, the elasticity of which is considerably less than that of external ones. Obviously, the microinhomogeneities represent metastable formations originating at the moment of membrane formation due to the fact that the solvent is unable to leave the BLM volume quickly and must be located somewhere. The cross section size of microinhomogeneities must be comparable with membrane thickness d. In this case the microinhomogeneities do not contribute to the electrical capacitance of the BLM: $C \sim d^{-1}$, i.e. as without microinhomogeneities (see [76]). The hypothesis about the existence of microinhomogeneities allow us to explain why transversal compression of a BLM by an electrical field leads to changes in membrane capacitance. In this case mainly the inner "soft" layer is deformed. Moreover, deformation is isovoluminous [160]. And this leads to a bulging of the matter of the inner layer from the planar parts A to the microinhomogeneities B (dashed line in a Fig. 42), in that it become "invisible" (these parts do not contribute to the capacitance). For the planar bilayer (Fig. 42c) the increase of capacitance in one place is compensated by its decrease in another place. Thus, the mechanical properties of a BLM can be qualitatively described by a three-layer elastic model composed of anisotropy elements with defects. More detailed analysis of the three-layer model of BLM elasticity is given in Ref. [76].

6.3 Mechanical properties of lipid bilayers and protein-lipid interactions

Protein-lipid interactions play an essential role in the functioning of biomembranes [175]. The specificity of these interactions is, however, under discussion. Most probably the exact lipid composition is not so essential. For example changes of the fatty acid chain composition caused by diet has no injurious effect on cell function. However, dietary induced changes in lipid com-

position are limited: some features of the fatty acyl chain composition are maintained constant, like chain length in the range between, typically C16 and C20, with about half the chains being saturated and half unsaturated. This means that overall features of the lipid composition, such are length and saturation of fatty acid are likely to be important for the membrane properties [176]. The length and saturation of fatty acid are responsible for the creation of certain thickness of the membrane and its physical state, which are important factors that determining the protein lipid interactions [175,176]. In certain case also the structure of the polar part of the phospholipids play role in protein-lipid interactions. There is, however, evidence that small number of special lipids is important for function of the protein. For example for Ca^{2+} ATPase it is phosphatidylinositol 4-phosphate. The binding of this lipid resulted in increasing the activity of the calcium pump by twice [176]. Specific activity to cardiolipin has been observed for cytochrome c oxidase [132].

The functioning of membrane proteins which is accompanied by changes in their conformation, could influence the structure and physical properties of the surrounding lipid environment. The interaction of proteins with membranes is provided both by electrostatic forces (mainly peripheral proteins) and by hydrophobic interactions (integral proteins).

The structure and dynamic aspect of protein-lipid interactions can be investigated directly by various physical methods. The EPR spectra reveal a reduction in mobility of the spin-labeled lipid chains on the binding of peripheral proteins to negatively charged lipid bilayers. Integral proteins induce a more direct motional restriction of the spin-labeled lipid chains, allowing the stochiometry and specificity of the interaction, and the lipid exchange rate at the protein interface, to be determined by EPR spectra. In this way a population of very slowly exchanging cardiolipin associated with the mitochondrial ADP-ATP carrier has been identified (see [177] for review). Fluorescence spectroscopy is also effective for the study of protein-lipid interactions. In particular Rehorek et al. [178] showed that as a result of conformational changes of the integral protein, bacteriorhodopsin, the ordering of the lipid bilayer increases and a transmission of conformational energy occurs over the distance more then 4.5 nm. Mechanical properties of the membranes are also very sensitive to the conformational changes in lipid bilayers. The influence of bacteriorhodopsin on the structural state of spacious regions of planar bilayer lipid membranes (BLMs) was shown by means of measurement of elasticity modulus E_{\perp} [179]. It was shown, that the area of a lipid bilayer with an altered structure per one cluster consisting of three bacteriorhodopsin molecules surpasses 2800 nm². Moreover, as a result of the illumination of BLM modified by bacteriorhodopsin a considerable increase of E_{\perp} occurred (more then 5 times) with a further saturation on a stable level. This condition was preserved for several hours after the illumination was switched off. This shows the possibility of mechanical energy accumulation in membrane.

For the analysis of the mechanism of protein lipid interaction the thermodynamics and mechanics properties of lipid bilayers and proteoliposomes are important. Owing to the possible different geometry of the hydrophobic moiety of proteins and that of lipids, as well as to the action of electrostatic and elastic forces, regions of altered structure may arise around protein molecules [76,180]. The formation of similar regions may represent one of the reason for the occurrence of long-distance interactions in membranes. Very likely, hydrophobic interactions play the key role in the establishment of links between integral proteins and lipids. The rigid hydrophobic parts of membrane-spanning proteins cause a deformation of the hydrophobic lipid chains due to length matching. This leads to the stretching or compression of the hydrocarbon lipid chains depending on the relation of the hydrophobic part of proteins and the surrounding


Fig. 43. Schematic representation of lipid-mediated protein-protein interactions induced by hydrophobic mismatch. (a) A matched membrane. (b,c) Hydrophobic mismatch of the same sense resulted in lipid-mediated attractive forces. (d) A case of hydrophobic mismatch of opposite sense resulted in repulsive forces between proteins (Reproduced by permission from Ref. [182]).

lipids [181]. Distortion of the membrane by proteins may cause lipid-mediated attractive or repulsive forces between proteins. The possible situations are presented in Fig. 43. Due to changes of the ordering of the lipid bilayer the increase or decrease of phase transition temperature, as well as changes of membrane mechanical properties take place.

Due to considerable problems with the isolation and purification of integral proteins and with the determination of their structure, only a few proteins were analyzed so far in respect of their influence on the thermodynamic and mechanical properties of lipid bilayers. Using the mattress model of Mouritsen and Bloom [181] as well as Landau-de Gennes theory of elasticity of liquid crystals it was possible to explain satisfactorily the changes in the temperature of phase transition of proteoliposomes containing membrane bound reaction center protein (RC) and antenna protein (LHCP) [183,184]. The role of elastic forces was, however studied only in small number of works and the mechanism of its action is not clear yet. In this section we will briefly report the results of analysis of the mechanisms of protein-lipid interaction based on the knowledge of thermodynamic and mechanics properties of the lipid bilayers with incorporated bacteriorhodopsin. Detailed consideration of the theory of the mechanisms of protein-lipid interactions is given in Refs. [76,175].

As we mentioned above, the incorporation of the protein into the lipid bilayer leads to a distorted region of the membrane. This leads to changes in phase transition temperature ΔT that is function of protein concentration and according to Ref. [183] can be determined by the expression

$$\Delta T = 8\xi^2 (2r_0/\xi + 1) [2(d^f - d_p)/(d^f - d^g) - 1]x_p, \tag{48}$$

where ξ is characteristic decay length, r_0 is the radius of bacteriorhodopsin (BR) molecule, d^f and d^g are the length of hydrocarbon chains of phospholipids in a fluid or gel state, respectively,



Fig. 44. (a) Schematic cross-section of an integral protein in a phospholipid membrane. a is the half-bilayer thickness and r_0 is the radius of protein. (b) Components of membrane distortion that contribute to the free energy (Adapted from Ref. [186], reproduced by permission).

 d_p is the length of the hydrophobic moiety of BR and x_p is molar ratio of BR and phospholipid (number of BR molecules/number of phospholipid molecules). Parameter $\xi(T)$ is not measurable in the experiment. To determine its quantity as well as to determine the energy of elastic membrane deformation around protein we have used the algorithm described in Refs. [185,186] for the numerical calculation of the mechanics energy of the membrane around the ionic channel. Fig. 44 shows a schematic cross-section of integral protein in a membrane. The free energy change unit per area in the cylindrical polar coordinates is

$$F = 2\pi \int r dr [E_{\perp} u^2/a + aK_1 (u'/r + u'')^2 + \gamma (u')^2].$$
(49)

Here, E_{\perp} , K_1 and γ are the elasticity modulus of transversal compression, splay and surface tension, respectively. To determine the minimum energy conformation, we minimize the free energy with respect to the variation in u (x,y) and get the linear differential equation [186]

$$K_1(u'/r^3 - u''/r^2 + 2u'''/r + u^{IV}) - (\gamma/a)(u'/r + u'') + (E_{\perp}/a^2)u = 0.$$
(50)

The equation (50) can be solved numerically using algorithm described by Pereyra [187]. Parameter ξ can be determined from the minima of the free energy of the system with the assumption of exponential decay of perturbation

$$U(r) = u_0 \exp[-(r - r_0)/\xi].$$
(51)

In calculations the elastic parameters and the thickness of hydrophobic part typical for DMPC bilayers in gel (g) and fluid (f) state has been used: $E_{\perp}^g = 7.28 \times 10^{-9}$ dyn Å⁻², $E_{\perp}^f =$

Tab. 7. The results of calculation of the minima of deformation energy F_{min} , decay length ξ and ξ' and corresponding radial range of the distorted region $(r - r_0)$ (index e denotes for exact solution of Eq. (50) for the BR-DMPC system (see the text).

Phase state	F_{min}, \mathbf{kT}	$(r-r_0)_e, \text{\AA}$	<i>ξ</i> , Å	F_{min}, \mathbf{kT}	$(r - r_0), Å$	<i>ξ</i> ', Å
Gel	1.33	62.5	16.2	74	10.48	48.5
Fluid	1.68	55	13.5	62.5	9.6	40

 3.76×10^{-9} dyn Å⁻² (from measurements of solvent free BLM [188]), $\gamma^d = 15 \times 10^{-8}$ dyn Å⁻¹, $\gamma^f = 3 \times 10^{-8}$ dyn Å⁻¹ (measurements on liposomes [189]), $K_1 = 10^{-6}$ dyn (a typical value for smectic mesophases [190]). The parameters that characterize the BR molecule are: $d_p \approx 30$ Å and $r_0 = 17.5$ Å [191]. The thickness of hydrophobic part of lipid bilayer in a gel and liquid crystalline state were as follows: $d^g = 34.2$ Å $d^f = 22.8$ Å, respectively (see Ref. [121] for method of calculations).

The results of calculations of deformation energy, characteristic decay lengths ξ and ξ' (see below) and the range of the distortion region $r - r_0$ in the gel and fluid state of lipid bilayers of DMPC contained of BR are shown in Table 7. The dependence of u(r), that represents the profile of the distorted region of the membrane around the protein in the fluid state, is shown in Fig. 45. The exponential shape of the deformation (curve A) obtained using Eq. (51) and the value $\xi = 16.8$ Å (determined from the minima of free energy of the system) have a considerably larger range then those determined from the exact solution of Eq. (50) (curve B). Interestingly that the curve 2 is not exactly the exponential function in both gel and fluid state. Using the



Fig. 45. Geometry of the distortion region of a lipid bilayer around BR. A – u(r) calculated according to Eq. (50) from the minima $F = F(\xi)$; B – u(r) from the exact solution of Eq. (50); C – backward transformation of exact solution to the exponential function (see the text). (Reproduced by permission from Ref. [76]).



Fig. 46. Dependence of phase transition temperature T_c of proteoliposomes of DMPC containing BR on molar ratio of BR/DMPC (x_p). 1 – DSC experiment. Theoretical calculations: 2 - ξ = 16.2 Å, N = 1; 3- ξ = 16.2 Å, N = 3; 4- ξ = 10.48 Å, N = 1; 5- ξ = 10.48 Å, N = 3. (Reproduced by permission from Ref. [76]).

backward transformation of the exact solution to the exponential function we have obtained the new value of $\xi' = 9.6$ Å. The range of deformation is in this case (curve C) about half as large in comparison with exact solution (curve B). The remarkable region in the Fig. 45 is the section when all three curves crossed. One can assume, that this point determines the minimal distance from BR. From which differences between exact solution of u = u(r) and assumed exponential decay of perturbation start. The region between the BR surface to the point of differentiation can be considered as the region of the immobilized influence of protein on its lipid environment.

Parameters of distorted regions (see Table 7) allows us to calculate the changes in the phase transition temperature in dependence on the BR concentration x_p , by means of Eq. (48). In calculation we used a similar method as Peschke et al. [183], which considers the aggregation of LHCP. The numbers of BR monomers in purple membrane clusters is known from RTG analysis (N = 3) [192]. Therefore we have modified the equation (5.5) for all possible combinations of parameters and created dependencies of T_c on x_p (Fig. 46). In the case of $\xi'^g = 10.48$ Å, and assuming the aggregation of BR to trimers, we have obtained a surprisingly good agreement with the experiment. Similar results were obtained also for the gel state of the membrane.

The obtained values evidences, that at phase transition from gel to fluid state the increase of deformation energy of the system BR-DMPC takes place (see Table 7). This means that the ordering of hydrocarbon chains of lipids around protein in the fluid state increases, i.e. protein stabilizes phospholipid molecules in its environment. As a result the increase of hydrocarbon mismatch take place. The comparison of the mean thickness of hydrophobic part of the membrane $d_L = (d^g + d^f)/2d_p$ gives $d_L < d_p$. We can, thus expect the increase of phase transition temperature T_c in the BR-DMPC system and this is in agreement with the experimental results [193]. Thus BR influences its lipid environment at large distances - at least 12–20 nm in a diameter. For the geometry and the range of deformation, not only is the size of the hydrophobic mismatch important. The determination of the characteristic decay length of the perturbation that depends on elastic parameters of the membrane is important as well.

In addition to native integral proteins also their model, e.g. α -helical peptides are used. Using these model systems it has been confirmed, that the mismatch between the hydrophobic moiety of the protein and lipid bilayer resulted in changes of thermodynamic properties of the lipid bilayer [121]. Experiments with simple peptides incorporated into the lipid membranes of different hydrophobic thickness revealed, that a long peptide can incorporate into either thick or a thin bilayer, in the later case by tilting. In contrast short peptides can not incorporate into too thick membrane and instead will form aggregates [176,194,195]. The possibility of peptide tilting in thin bilayers has been demonstrated also by molecular dynamic simulation method [196,197].

While considerable attention of experimental and theoretical works was focused on the problem of the interaction of integral proteins with lipids, smaller theoretical works are known for the analysis of the lateral organization of peripheral proteins. However, there is considerable amount of papers focused on the organization of biopolymers and receptors on lipid monolayers [35,198]. This is particularly connected with development of biosensors. Experimental aspects of the interaction of peripheral proteins with membrane surface are reviewed also in paper by Kinnunen et al. [199]. This topic is rather attractive area also in connection with adsorption of DNA to the membrane surface that can be considered as an initial step for subsequent translocation of this molecule into the cell, e.g. by means of electroporation. In these systems the membrane elasticity plays important role. It has been shown that interaction of DNA and its complexes with cationic surfactants resulted in considerable changes of BLM elasticity [169]. Changes of E_{\perp} of supported BLM has been observed also during adsorption of model α -helical peptides to the membrane surface [69].

Theoretical works focused on analysis of the mechanisms of interaction of peripheral proteins with membrane surface has been reviewed in paper by Gil et al. [175]. Various approaches in this analysis include for example molecular dynamic simulations of association of peripheral proteins with fully hydrated lipid membranes. This method has been applied to study the interaction of phospholipase A_2 (PA) with membrane surface [200]. The authors obtained detailed information on PA conformation and analyzed also the enzymatic activity of this protein. Another approach has been developed by Heimburg and Marsh [201] and is connected with expression of isotherms for binding of adsorption of charged proteins (e.g. cytochrome C (cytC)) to a charged surface (e.g. dioleoyl phosphatidyglycerol bilayers). It has been found that the cross-sectional area of cytC is equivalent to 12 lipids in a fluid bilayer and that the charge of the protein in a membrane is lower in comparison with net charge of native protein in a solution. Currently one of the most effective approach is connected with application of Monte Carlo simulations [202]. This method was applied to the study of aggregation of cytC in a dimyristoyl phosphatidyglycerol bilayers and revealed also high potential in analysis of protein-induced phase separation in binary lipid mixtures where protein prefer lipids of certain configuration.

The binding of proteins to the membrane surface may result changes in surface potential of the BLM as well as changes in dynamics of reorientation of dipole moments connected with head group of phospholipids. The fundamentals of the membrane potentials and dipole relaxation will be considered below.

7 Membrane potentials

7.1 Diffusion potential

Unmodified bilayer lipid membranes represent an insulating layer with very low permeability for ions or other charged molecules. However, BLM can be modified by ionic channels or carriers that allows to transport charged species by means of diffusion either by means of a gradient of concentration or by means of a gradient of electric potential or both (see part 8). In the case of potential gradient it is so-called diffusion membrane potential, or Nernst potential, that is driving force of the ionic transport across the membrane. The Nernst potential is determined as difference between potential inside and outside the cell (or inner and outer side of the membrane)

$$\Delta \varphi = \varphi_{in} - \varphi_{out} = -\frac{RT}{z_i F} \ln \frac{C_{in}}{C_{out}},\tag{52}$$

where R is the gas constant, T is temperature, F is Faraday constant, z is valence of ion i, C_{in} and C_{out} are the concentration of ions i at inner and outer side of the membrane, respectively.

For measurement of diffusion potential on a BLM, the electrometers with high input resistance should be used. But even simple pH meter can be apply for this purposes, assuming that two reference electrodes (e.g. Ag/AgCl) should be used.

7.2 Electrostatic potentials

In addition to the diffusion potential there exists membrane potential between the polar part of the membrane and the bulk of electrolyte, so called *border or electrostatic potential*. The electrostatic potential at the membrane-solution interface is composed of two major components stemming from surface charges and dipoles, respectively (see for example [76, 203-207]). In the case of charged lipid molecules there is a *diffuse ionic double layer potential* or *surface potential* originating from fixed charge layer in combination with ions from the adjacent aqueous electrolyte solution. Its maximal value relative to the bulk solution lies just at the interface and can be described approximately by either Gouy-Chapman or Stern models (see e.g. [47,203]). In this section we will use the Gouy-Chapman potential E_{GC} . In the polar head group region there is a further potential jump, E_d , resulting from the molecular dipoles of the lipids themselves or from oriented water molecules. This is so-called *dipole potential*. Further charge and dipole contributions may come from adsorbed species. The total surface potential, Φ_m , is given by sum $\Phi_m = E_{GC} + E_d$. Experimentally, E_{GC} and E_d can be distinguished by dependence of E_{GC} on ionic strength. Fig. 47 gives a schematic representation of the electrostatic potential across a bilayer. The difference in the heights of the two corners at zero applied voltage, $\Delta \Phi_m$, is equal to the difference of the surface potentials of the two side of the bilayer

$$\Delta \Phi_m = \Delta E_{GC} + \Delta E_d. \tag{53}$$

Therefore $\Delta \Phi_m$ is measure of the asymmetry of the electrostatic potentials associated with the membrane. Surface potentials are either localized strictly within the surface region (E_d) or extend at most to a limited distance from it (E_{GC}) . At equilibrium it is not possible to make a direct measurement of these potentials with electrodes in the bulk phase except at zero ionic strength.



Fig. 47. Schematic representation of electrostatic potentials at the membrane-solution interfaces. The fixed charge surface potential (Gouy-Chapman potential E_{GC}) and surface dipole potential, E_d , are shown. Indexes 1 and 2 refer to the two sides of the membrane. The total surface potential on each side of the membrane, Φ_m is given by $E_{GC} + E_d$. The difference of the surface potential on the two sides, $\Delta \Phi_m = \Delta E_{GC} + \Delta E_d$ is called the trans-membrane potential or "internal" potential. U_a is the externally applied dc voltage. (Reproduced by permission from Ref. [76]).

7.2.1 Gouy-Chapman potential and the determination of surface charge density σ

The electrostatic potential at a charged surface in contact with electrolyte reflects both the surface charge density and the redistribution of ions in the electrolyte solution in the presence of this potential. A description of the system is based on the Boltzmann equation to describe the concentration of each ionic species as a function of the electrostatic potential, and the Poisson equation to describe the Coulomb interaction between the ions (see [203]). Using the appropriate boundary conditions to solve the integral equations leads to the Gouy-Chapman equation

$$\sigma = \sqrt{2\varepsilon\varepsilon_0 RT} \sum_i c_i(\omega) \left[\exp\left(-\frac{z_i F E_{GC}}{RT}\right) - 1 \right],\tag{54}$$

where $c_i(\omega)$ is the bulk concentration of species "i" with charge z_i . Other symbols have their usual meaning. For the general case with multivalent ions present there is no explicit solution for E_{GC} as a function of σ , but if only univalent ions are present the Gouy-Chapman potential, adapted to convenient units and at T = 25 °C, is given by

$$E_{GC}(mV) = 50.8 \ln\left[s + \sqrt{s^2 + 1}\right],$$
(55)

where $s = 1.36\sigma/\sqrt{c}$ and σ is in elementary charges per nm² and c denotes concentration of the 1:1 electrolyte in mol l⁻¹. It is evident from this equation that the surface potential resulting from a given charge density depends on the ionic strength of the solution: higher ionic strengths are said to shield the surface charge, resulting in a lower surface potential. Although it is not possible to measure the surface charge density of a membrane directly, it is possible to determine σ by measuring the change in the Gouy-Chapman potential upon a change of the ionic strength. For this purpose the membrane is usually formed at relatively low ionic strength (e.g., 10 mmol l⁻¹), and the transmembrane potential is measured. Subsequently the ionic strength on one side of the



Fig. 48. Relationship between the Gouy-Chapman fixed charge surface potential and the membrane surface charge density σ (e nm⁻², where e is the elementary charge). For a given value of the membrane charge density, the Gouy-Chapman fixed charge surface potential will be reduced when the ionic strength is increased (upper curve: 10 mmol l⁻¹, lower curve, 110 mmol l⁻¹). Concentrations refer to 1:1 salt. The dotted curve represents the difference between the curves at 10 mmol l⁻¹ and 110 mmol l⁻¹. Point A corresponds to the shielding experiment described in Ref. [207]. ($\Delta U_{Cmin} = 20.6 \text{ mV}, \sigma = 8 \times 10^{-2} \text{ e nm}^{-2}$). (Reproduced by permission from Ref. [76]).

membrane is increased, e.g. to 110 mmol l^{-1} , by addition of a small amount of concentrated electrolyte, and the transmembrane potential is again determined. The change in transmembrane potential measured in such a shielding experiment is just ΔE_{GC} , as the dipole potential E_d is insensitive to the ionic strength (see [208]). Curves showing E_{GC} vs. reciprocal surface charge density for two different ionic strengths, as well a plot of the difference between these curves, are shown in Fig. 48. Given the measured ΔE_{GC} for the given charge on ionic strength, the surface charge density can be read directly of such a plot. If the ΔE_{GC} is known with an accuracy of about 1 mV then the minimal surface charge density that can be detected in 10 mmol l^{-1} electrolyte is about 1.4×10^{-3} elementary charges nm⁻² (see Fig. 48, upper curve). The biomembranes are negatively charged due to presence of negatively charged phospholipids as well as due to the negative charge of proteins. Also many adsorbed polyelectrolytes have isoelectric points below neutral pH [206]. The surface charge density of biomembranes is usually between -0.17 e nm⁻² (frog node) [209] to -2.3 e nm⁻² (crayfish) [210] (e is elementary or electron charge: 1.602×10^{-19} C). This is different with the model membranes, where the charge density can vary in large extend and can be both positive or negative depending on lipid used: $2.5 \ge \sigma \ge -2.5 \text{ e nm}^{-2}$ [208].

A qualitative interpretation of E_d is generally given in terms of analogy with a condenser: a polarized molecule with an effective dipole moment M = qd is similar to two conducting phases separated by distance d and enclosing a charge density q. The dipole moment is expressed as Debye (D). Then the potential difference ΔE_d is given by

$$\Delta E_d = 4\pi q d/\varepsilon,\tag{56}$$

where ε is the dielectric constant. If there is an array of n dipoles per unit area,

$$\Delta E_d = 4\pi n M_\perp / \varepsilon, \tag{57}$$

where M_{\perp} is the normal component of the dipole moment to the surface.

There are several contributions to ΔE_d : (1) the change induced by reorientation of the water dipoles in the presence of the monolayer forming molecules; (2) the dipoles of the monolayer forming molecules, namely that of polar head groups of phospholipids and that of the alkyl part, which can be located in different dielectric constant media, then

$$\Delta E_d = 4\pi \sum (nM_\perp/\varepsilon). \tag{58}$$

The dipole potential of monolayers, i.e. boundary potential between the hydrocarbon center of the membrane and the bulk aqueous phase is typically several hundred of millivolts [36].

7.2.2 Surface potentials and diffusion potentials

It is worth emphasizing the difference between the transmembrane potentials and membrane potentials ("diffusion potentials") resulting from selective permeability mechanisms (see section 9.1). In contrast to surface potentials, diffusion potentials are due to a concentration difference between the aqueous phases and are thus a bulk property that can be measured directly. Surface potentials and diffusion potentials are completely independent conceptually and easily distinguished experimentally. An example of a non-zero transmembrane difference of surface potential ($\Delta \Phi_m$) for which no potential difference can be detected between the bulk phases is presented in the paper by Schoch et al. [211], where a charged membrane, made permeable to monovalent cations by nonactin, is asymmetrically shielded by calcium ions. This results in a non-zero transmembrane potential $\Delta \Phi_m$, yet no bulk potential difference, as evidenced by a current-voltage curve that passes through the origin. The opposite situation, with $\Delta \Phi_m = 0$ but a non-zero diffusion potential, would be found, for example with a membrane made of neutral lipids in presence of nonactin and a gradient of monovalent cations. With no current flowing in the external circuit a diffusion potential would establish itself across the membrane, i.e. the current-voltage curve for this system would pass through i = 0 at a voltage equal to the diffusion potential. A case which both $\Delta \Phi_m$ and diffusion potential are non-zero is, of course, also possible.

7.3 Methods of surface potential measurements

7.3.1 Measurements on monolayers or vesicles

The dipole potential can be measured by various methods, such are ionizing electrode method or vibrating plate method [36]. TREK Inc. (USA) [212] produces high sensitive electrostatic voltmeters, e.g. model 320C that in connection with electrode model 3250 can measure the surface potential with an accuracy of 1 mV. The sensitive electrode is electromechanically vibrated to produce capacitive modulation between the electrode and the test surface. If the voltage on the test surface is different than the voltage on the reference surface (probe housing), an AC signal is induced upon the electrode by virtue of this modulation in the presence of the electrostatic field. The amplitude and phase of this AC signal are related to the magnitude and polarity of the difference in potential between the test surface and the probe housing. The TREK electrostatic voltmeter can be directly connected to the electronic unit of NIMA trough [48], so fully computer controlled measurement of surface potential under compression of monolayer can be performed. For micelles and vesicles other methods for estimation of the various membrane potentials using non-electrode techniques have been developed (e.g. [145,205,213]). These methods are based on utilizing the molecular probes. The properties of the probes are sensitive to the transmembrane potential (see [206] and reference herein for more details).

Rather popular method for study the electrostatic membrane potential is based on measurement of so-called ξ -potential. In this case the mobility of the charged lipid vesicles in external electric field is determined. The charged vesicles placed into the external electric field E_{ex} begin to move due to the electrostatic force and drag part of the diffuse double layer with them. The ξ -potential is defined as the electrostatic potential at the plane of shear between the membrane associated and stationary part of the double layer. An analytical expression for ξ -potential has been found only for particles that do not interact with the solvent. When e.g. membrane vesicle of a radius r is placed in an electric field E_{ex} , it will move with constant speed v, because of the balance between electrical force and the resistance of the medium with viscosity η . The value of the ξ -potential can be then calculated according to the Smoluchovsky equation

$$\xi = \frac{4\pi\eta v}{\varepsilon E_{ex}} \tag{59}$$

(see [206] for the theory). Usually $\xi \leq \Phi_m$, however the differences between the potentials ξ and Φ_m will decrease with decreasing of potential gradient $(d\Phi_m/dx)$, i.e. this differences should decrease in diluted solutions. However, the uncertainty associated with the thickness of double layer d_{ξ} at which the potential is determined is the main drawback of the electrophoretic potential measurements. Typically, a value $d_{\xi} = 0.2$ nm is assumed. However, the value of d_{ξ} certainly depends on the interfacial membrane structure and hydration, owing to the fact that the plane of shear lies in the region where specific lipid-water effects dominate. Thus, it is probable that the actual value d_{ξ} changes with the membrane or solvent composition, temperature etc. The experimentally observed increase of the membrane hydration at the main temperature phase transition of the lipids offers the explanation for the observed increase of ξ -potential at the phase transition (see [206] for more details).

7.3.2 Measurements on planar bilayers

Direct measurement of the surface potential of planar bilayers is not possible, as mentioned above. It is however, possible to measure the difference of the surface potential, $\Delta \Phi_m$. By varying the ionic strength on each side of the membrane the fixed charge surface potentials, and thus the surface charge density, can be determined for the two sides independently (see section 7.2.1). The first determination of the surface potentials of BLMs were based on the current-voltage curves measured in the presence of an ion carrier. A method based on the dependence of membrane capacitance on the transmembrane potential (electrostriction), and therefore independent of any transport mechanisms, was introduced in the mid 1970's [211,214-216], The advantages and limitations of various approaches will now be considered in detail.

1. Current-voltage characteristics. A complete description of the I - V curves for carriermediated ion transport involves both the surface potential depicted in Fig. 47 and the Born energy of the charged species in the hydrophobic interior of the bilayer [217]. The theoretical basis for such analysis was described in 1970 [218], and many groups have published work in this field (e.g. [219-222]). All interpretation is based on a more or less appropriate model to describe the potential energy barrier to carrier transport. An exact fit of the measured I - V curves can be a laborious procedure, and the detailed shape of the curve can easily be influenced by further factors such as bilayer compressibility [223]. Nevertheless the surface potentials determined in this way have generally been found to be consistent with the prediction of Gouy-Chapman theory. While the method is experimentally relative simple, and can yield information on both E_{GC} and E_d , it harbors several disadvantages in practice. 1. A single, well-defined transport mechanism must be present. This usually means that a carrier substance must be added, which may limit the kind of experiment possible. 2. The exact results depend on the model and correction factors applied. 3. Each determination requires a measurement of the I-V characteristic and subsequent numerical analysis. This makes it impractical for the continuous automatic monitoring of surface potentials at frequent intervals [207].

2. Techniques based on electrostriction. The techniques discussed in this section are based on the compression of BLMs by a transmembrane electric field. The maximal bilayer thickness, corresponding to minimal capacitance, is found when the potential difference across the core of the bilayer is zero. (This "core" corresponds to the hydrophobic region of the BLM: given the low dielectic constant and relative thickness of this layer, it will provide the main contribution to the overall membrane capacitance). As this core or "internal" potential depends on both the intrinsic surface potentials of the bilayer itself (fixed charge and dipole potentials) and the externally applied potential, a charge in the surface potentials can be compensated by an equal but oppositely directed external field. The externally applied voltage needed to achieve minimal capacitance has been designated the "capacitance minimization potential" (U_{Cmin} , [214]) and is given directly by

$$U_{Cmin} = -(\Delta E_{GC} + \Delta E_d). \tag{60}$$

The accuracy with which U_{Cmin} can be determined depends on the amplitude of the membrane compressibility coefficient: the bigger this parameter the easier the measurement becomes.

The first report on the use of electrostriction for the determination of BLM surface potentials was given in 1976 [214] and described in detail in Refs. [211,216]. Experimentally a small ac signal (e.g. 1 kHz, amplitude 10–25 mV) was superimposed on an adjustable bias voltage. A continuous measurement of BLM capacitance was provided by a rectification of the 90⁰ component of the ac BLM current, thereby allowing operation even at relatively large background conductances. The bias voltage is varied symmetrically in small steps (e.g., $\Delta U = \pm 25$ mV) around a holding potential, U_h , which is then adjusted to give equal measured C(U) values at $U_h \pm \Delta U$. This corresponds to a discrete sampling of the C(U) curve to determine the applied voltage at which the bilayer capacitance has its minimum value. Automatic monitoring of $\Delta \Phi_m$ has been achieved with either analog feedback circuitry or a microcomputer. The noise level of U_{Cmin} was found to be 1–2 mV ptp for solvent free BLMs (apparatus time constant 3 sec, see e.g. [224]).

In 1978 Alvarez and Latorre [215] reported the measurement of BLM surface potentials of solvent free BLMs. Membrane capacitance was measured by recording the transient charging current following a small step change in applied potential. To enhance the resolution, the charging current of a matched R-C model circuit was subtracted from the BLM charging current using analog circuitry. Signal-to-noise was improved by digital signal averaging techniques. With a voltage step of 10 mV the authors were easily able to detect changes (of 0.01 %) in BLM capacitance for bilayers having a coefficient of electrostriction of 0.02 V^{-2} . As reported by the authors

the measurements may take up to 25 sec per point (512 repetitions at 20 repetitions per sec). With large voltage jump the time per point could be reduced considerably, but this may not be desirable in many situations.

The method based on determination membrane potential using current harmonics was described in 1980 [216] and is based on the generation of current harmonics when a sinusoidal voltage is applied to the membrane [76]. When membrane potential U_1 is present and ac voltage with an amplitude U_0 and frequency f is applied to the BLM, then in addition to the third current harmonics with amplitude I_3 and frequency 3f also the second current harmonics with amplitude I_2 and frequency 2f is generated. The surface potential can be then determined from equation

$$\Phi_m = -U_1 + U_0 I_2 / (4I_3). \tag{61}$$

The membrane potential can be determined either by compensation of the amplitude of second current harmonic by external voltage [216] or by measurement of amplitudes of both second and third current harmonics [76]. For measurement using second current harmonic method an ac frequency of 1 kHz has generally been used [216,225]. The optimal amplitude is determined by the BLM compressibility, but lies in the range 10–50 mV, As a rule, with a machine time constant 1 sec, the noise level correspond to about ~ 1 mV for membranes having a coefficient of electrostriction down to 10^{-3} V⁻² at frequency 1 kHz. The compressibility of most BLMs is considerably higher than this. Care must be taken when BLMs have a voltage-dependent conductivity, i.e., a non-linear current-voltage characteristics, as this can also lead to the generation of second current harmonics. In this case the use of a phase sensitive detector rather than just a tuned amplifier could increase the range of application of the technique.

8 Dielectric relaxation

The dielectric relaxation method is based on analysis of the time course of changes in the capacitance following sudden changes in the voltage applied across the bilayer [226]. Using this method, one can obtain information about, e.g., reorientation of molecular dipoles and cluster formation. A symmetric voltage-jump (-V to +V) across a bilayer can orient naturally occurring dipoles. The magnitude and time course of these effects will depend on the structure of the bilayer and bulk phase. Such effects will also be reflected by changes in membrane capacitance. The capacitance of the bilayer depends on dielectric constant ε of bilayer material, membrane area (A) and thickness of the membrane d (see section 3.2). An electric field could affect all these parameters, each must be considered separately. This has been done by Sargent [226] and Hianik and Passechnik [76]. As it has been shown by Sargent [226], no correlation was found between the normalized dielectric relaxation parameters and membrane area, showing that the effects were indeed related to the bilayer rather than to the border region.

8.1 The basic principles of the measurement of dielectric relaxation

The dielectric relaxation is determined by measuring the time course of the displacement current following a step change in potential. A detailed description of the construction and operation of the apparatus is given elsewhere [226]. Briefly a positive voltage is applied to the electrodes at time t = 0. This caused a large charging current (I_0) to flow, which decays with a time constant of $R_s C_m$, $(R_s = \text{solution+electrode resistance}, C_m = \text{membrane capacitance})$. In addition,

Tab. 8. Relaxation times of the reorientation of dipole moments of BLM prepared from soya bean phosphatidylcholine (SBPC) and sBLM prepared from SBPC on a tip of Teflon coated stainless steel wire or on a smooth gold surface covered by alkylthiol [110].

Membrane system	$ au_{1,\mu}$ s	$ au_{2,} \mu { m s}$	${ au}_{3,} \mu { m s}$	$ au_{4,} \mu { m s}$	$ au_{5,} \mu { m s}$	$ au_{6,} \mu { m s}$
BLM in n-decane	6.9±1.9	18±6				
sBLM in n-decane	5.9 ± 0.4	13.6 ± 2.0	27.6±3.6	51.7±4.2	85±9	$120{\pm}12$
(stainless steel wire)						
sBLM with alkylthiol	$40{\pm}4.0$	290±100	1903±760			
(thin gold layer)						

there may be relaxation currents caused by voltage or time-dependent changes in C_m , and a dc component through R_m . At time t = 0 a negative voltage is applied to an R-C analog circuit which models the parameters of the experimental system. In this way a current is generated that is equal in magnitude but opposite in sign so that generated by the membrane charging current and the dc component. The currents from both circuits are combined, resulting in a canceling of the charging peaks and dc currents.

Any capacitive relaxation is assumed to have an exponential time courses

$$I(t) \cong \sum_{i} I_0^i e^{-t/\tau i}.$$
(62)

The amplitudes are only meaningful when normalized in some manner: as an initial trial, the values were expressed per unit area, of which the simplest measure is the membrane capacity at zero voltage $C(0) = C'_m$. Thus it is convenient to present relaxation amplitude (I_r^i) as a fractional or per cent changes in capacitance, for which the complete expression is

$$I_{r}^{i} \equiv \Delta C_{i} / C_{m}^{\prime} = I_{0}^{i} \tau_{i} / (V_{0} C_{m}^{\prime}).$$
(63)

This is related to the "dielectric increment" through the dielectric constant. The latter is, however, not known for all the conditions met. Therefore the phenomenological "relaxation amplitude" was used for analysis [226]. The resolution of the apparatus allows the detection of ΔC_i of about 1 pF with time constant $\tau > 1 \mu s$. All relaxation phenomena reported here are considerably slower than this, so that no inaccuracy is introduced from this source.

8.2 Application of the method of dielectric relaxation to BLM and sBLM

The method of dielectric relaxation allows one to study dynamic properties of BLM and sBLM and binding the macromolecules on the membrane surface. The method of dielectric relaxation allows us to determine the characteristic time of the reorientation of dipole moments of phospholipid head groups. Due to the domain structure of lipid bilayers and different size of the clusters, we can expect different collective movement of the reoriented dipole moments following the application of symmetrical voltage jumps to the membrane.

The results of determination of relaxation times for various BLM and sBLM systems are presented in Table 8. We can see considerable differences of relaxation times between different

System	$ au_1, \mu { m s}$	$ au_2, \mu { m s}$	$ au_3, \mu { m s}$
BLM	5.3±1.0	-	-
B-BLM	5.0 ± 0.3	26.1±5.0	115±27
B-BLM+A-GOX	16.5 ± 1.0	26.5±1.0	505±16

membrane systems. While free standing BLMs are characterized by two relaxation times, the dynamics of sBLMs formed on the tip of stainless steel wire can be characterized by up to 6 relaxation times. The reason for the increase of the number of relaxation components can be due to physical and chemical adsorption of phospholipids on the metal support. This adsorption could result in the different degree of the immobilization of lipid molecules and thus can lead to the appearance of different and more relaxation times. A similar result is also seen for membranes formed on thin gold layers with alkylthiols. In this case, the number of relaxation components is lower. However, a further increase of the duration of relaxation times takes place. This provides evidence about the strong restriction of movements of dipole moments. The result is in agreement with the increased values of elasticity modulus of the latter membrane system in comparison with that of sBLMs formed on wires.

The physical origins for different relaxation times for BLMs have been analyzed by Sargent [226]. It was suggested that the fastest relaxation times (several μ s to tens of μ s) could correspond to small amplitude reorientations of individual dipoles about an axis lying in the plane of the membrane, while the times of about hundred μ s reflect a rotational reorientation of individual molecules. Slow relaxation components (several hundred μ s to ms) probably indicate the reorientation movements of domains or clusters of dipoles in the membrane plane. For comparison, an NMR study by Davis [227] gave dipole correlation times of 1-5 μ s for lecithin vesicles, which is consistent with the results obtained from conventional BLMs as used in our experiments. The relaxation times in μ s and ms range obtained by various techniques on phospholipid bilayers were recently reported by Laggner and Kriechbaum [228].

Dielectric relaxation experiments allowed us to study the binding of enzymatic complexes on the membrane surface and confirmed the strong binding of the avidin-GOX complex to the biotinylated membranes. These experiments were performed on a free-standing BLM. We checked in a stepwise manner how dipole relaxation times of phospholipid head groups changed upon the modification of lipids and membranes. The results obtained are summarized in Table 9.

In this experiment current relaxation curves were averaged and the standard deviation taken as the experimental uncertainty. Native BLMs formed from crude ox brain extract exhibited one relaxation time of $(5 \pm 1) \mu s$. Additional relaxation components $[(115 \pm 27)\mu s \text{ and } (26 \pm 1)\mu s]$ appeared in BLMs modified by biotin. Addition of the avidin-GOX complex to the electrolyte (final concentration 30 nM) on both sides of the biotinylated BLM resulted in the appearance of a slow component $(505 \pm 16)\mu s$. The appearance of this slow component presumably represents a collective motion of coupled dipole moments and reflects clustering in the membrane, induced by binding of the A-GOX complex [229].

Considerable changes of relaxation times have been observed also when short oligonuclotides modified by palmitic acid were incorporated into the BLM and sBLM as well as during hybridization with complementary oligonucleotide chain at the membrane surface [230]. Dielectric relaxation methods can be very useful also for study of binding of various macromolecules on the membrane surface, such are short peptides [231] and local anesthetics [232].

9 Transport through the membranes

The important function of biomembranes consists in regulation of the transport of ions or other molecules, e.g. nutrients into the cell or waste products and toxic substances out of the cell. Thanks to the membranes, all cells maintain concentration gradients of various metabolites across plasma membranes and membranes of cell organelles. Transport of the species across the membrane can be performed by passive diffusion or by facilitated diffusion. In later case the compounds either diffuses through a channel forming protein or are carried by a carrier proteins [233].

9.1 Passive diffusion

Passive diffusion is simplest transport process. The driving force of this process is gradient of concentration of species across the membrane or a gradient of membrane potentials or both. I.e. species are transported by passive diffusion from region of higher concentrations to the lower one or from region of higher potential to lower potential. The equilibrium condition is reached when concentrations or potentials are equal at both membrane sides.

Let us consider the transport of charged molecules, e.g. ions through the semi permeable membrane. The consideration can be performed on the base of electrochemical potential $\tilde{\mu}$. For diluted solutions (typically $C_i < 0.1 \text{ mol } 1^{-1}$)

$$\tilde{\mu} = \mu_0 + RT \ln C_i + z_i F \varphi, \tag{64}$$

where μ_0 is standard chemical potential (i.e. chemical potential of 1 mole species), C_i is the concentration of molecules or ions, φ is the potential, other parameters have their usual meanings. The flux j_i of the ions across the membrane can be determined from Teorrell equation

$$j_i = -uC_i \frac{d\tilde{\mu}}{dx},\tag{65}$$

where u is the mobility of the ions expressed in m²s⁻¹V⁻¹. Substituting Eq. (64) into Eq. (65) we obtain

$$j_i = -uRT\frac{dC_i}{dx} - uC_i z_i F\frac{d\varphi}{dx},\tag{66}$$

which is the Nernst-Planck equation. If electrically uncharged particles are transported through the membrane or if $d\varphi/dx = 0$, then

$$j_i = -uRT \frac{dC_i}{dx} = -D\nabla C_i, \tag{67}$$



Fig. 49. Schematic representation of the ion flux across the membrane.

where D = uRT is diffusion coefficient expressed in m²s⁻¹ and Eq. (67) represent the expression of first Fick law for diffusion. If concentrations of species at two membrane sides are C_1^M and C_2^M , respectively (Fig. 49)

$$j_i = -D\frac{C_2^M - C_1^M}{d},$$
(68)

where d is the membrane thickness. Because it is difficult to determine concentrations C_1^M and C_2^M , for practical purposes the following equation is used

$$j_i = -P_i(C_2 - C_1), (69)$$

where P_i is the permeability coefficient (usually expressed in cm s⁻¹). P_i is a constant that describes how easily the molecules leaves the water solvent and cross the hydrophobic barrier presented by the membranes. P_i depends on properties of the membrane and on transported species. If we consider the concentrations of species in a membrane proportional to the concentrations at membrane surface, then

$$C_1^M = kC_1$$
 and $C_2^M = kC_2$, (70)

where k is partition coefficient. Substituting Eq. (70) to Eq. (68) we obtain

$$j_i = -\frac{Dk}{d}(C_2 - C_1).$$
(71)

Thus, P_i depends on three quantities: (a) partition coefficient k, which is the ratio of the solubility of the molecule in a membrane to the solubility in water, (b) the diffusion coefficient, D, which describes the rate of diffusion of the molecule in the membrane and (c) on the membrane thickness, d. Thus, the flux of the molecules through the membrane increases with increasing the solubility of the molecules in a membrane, with increasing the diffusion coefficient and with decreasing the membrane thickness. The permeability coefficients for polar species are shown in Table 10.

9.2 Facilitated diffusion of charged species across membranes

The lipid bilayers has very low permeability for charged particles, e.g. ions (see part 3.2.1). This is due to low dielectric permittivity of the hydrophobic core of the membrane ($\varepsilon \cong 2$) which is

Mambrana	Permeability coefficient, $cm.s^{-1}$					
Weinbrane	Na ⁺	K^+	Cl ⁻	H_2O	Glucose	
Phosphatidylcholine	$< 1.2 \times 10^{-14}$		$< 5.5 \times 10^{-11}$	$<4.4\times10^{-5}$	2.5×10^{-10}	
(egg)						
Phosphatidylserine	$< 1.2 \times 10^{-13}$	$< 9 \times 10^{-13}$	$< 1.5 \times 10^{-11}$	_	4×10^{-10}	
Phosphatidylserine:	$< 5 \times 10^{-14}$	$< 5 \times 10^{-14}$	$< 3.7 \times 10^{-12}$	_	1.7×10^{-11}	
cholesterol (1:1)						
Human erythrocytes	$< 1 \times 10^{-16}$	$< 2.4 \times 10^{-10}$	$< 1.4 \times 10^{-4}$	$< 5 \times 10^{-5}$	2×10^{-5}	
Frog erythrocytes	$< 1.4 \times 10^{-7}$	$< 1.6 \times 10^{-7}$	$< 9.5 \times 10^{-8}$		—	
Dog erythrocytes	_		_	$< 5 \times 10^{-5}$	_	

Tab. 10. Permeability coefficients for polar solutes across bilayers and biomembranes. Adopted from [4,233].

not favor for incorporation of charged particles. The partition coefficient of the particles between lipid and water phase can be estimated according to following equation

$$k = \exp(-\Delta W/RT),\tag{72}$$

where ΔW is energy connected with transfer of the one mole of the charged species from the water to the membrane. According to the Born theory

$$\Delta W = \frac{(zF)^2}{2r} \left[\frac{1}{\varepsilon} - \frac{1}{\varepsilon_w} \right],\tag{73}$$

where r is the radius of charged particle, e.g. ions, z is ion valence, ε , ε_w are dielectric permittivities of hydrophobic part of the membrane and water, respectively. For practical calculations $\frac{(zF)^2}{2r} = 68.2z^2/r$ and ΔW is expressed in kJ/mol and ion radius is in nm. The changes of energy for transfer of Na⁺ (r = 0.095 nm) from water ($\varepsilon_w = 81$) to the membrane ($\varepsilon \approx 2$) is 350 kJ/mol. This means that there exists substantial energetic barrier for transfer of ion from water into the membrane. However, energy of ion in a membrane decreases due to at least 4 effects [234]: 1. membrane has finite thickness. 2. ions can form pairs. 3. The pores (channels) of high dielectric permittivity can be incorporated in a membrane. 4. Ions can be transported by carriers, that increase effective radius of the ion. These effects are schematically showed at Fig. 50. Let us consider these effects step wisely. Due to finite thickness of the membrane and due to image forces (i.e. polarization) the electrostatic energy of ion in a membrane decreases (Fig. 50a). Due to this effect the energy of ion in membrane decreases by a value $\sim r/d$, i.e. by few per cent. The height of energy barrier is, however, still of at least several hundreds of kJ.mol $^{-1}$. The formation of ionic pairs (Fig. 50b) could result in maximum two fold decrease of the energy. The pores of high dielectric permittivity could considerably decrease the energy of ion in a membrane (Fig. 50c). Finally, several compounds, e.g. valinomycin, serve as carriers of ions (Fig. 50d) [235,236]. The energy of the complex of ions of a radius r with a carrier of a



Fig. 50. Schematic representation of the ion in a membrane: (a) effect of the image forces; (b) formation of ionic pairs from cations (I^+) and anions (I^-) , (c) channel and (d) carrier in a membrane.

radius b can be calculated according to the Bohr's equation

$$\Delta W = \frac{(zF)^2}{2} \left[\frac{1}{b\varepsilon} - \frac{b-r}{rb\varepsilon_b} \right],\tag{74}$$

where ε_b is effective dielectric permittivity of inner part of the complex. If the $\varepsilon_b \gg \varepsilon$ and $b \gg r$, then $\Delta W = (zF)^2/(2b\varepsilon)$. For example, the energy of the complex of carrier + ion in a membrane with $\varepsilon \approx 2$ will be 34 kJ mol⁻¹, which is about 10 fold less then free energy of ion with a radius 0.1 nm in a membrane without carrier.

Passive and facilitated diffusion of ions by carriers are characterized by different shape of dependence of the flux on the concentration of diffused molecules. In the case of passive diffusion, the flux increases linearly with concentration of ions, while saturation of the flux take place at higher concentration of ions in the case of carrier-mediated transport. Let us consider some peculiarities of channel-forming molecules and carriers.

9.2.1 Ionic channels

Among channel-forming ionophores the gramicidin A (GRA) is most well known and has been studied in details since 1971 [237,238]. Gramicidin has been isolated from *Bacillus brevis*. GRA is a small peptide composed of 15 alternating L- and D- amino acids residues, a formyl group at N-terminus (HEAD) and an ethanolamine at the C-terminus (END). It forms ionic channels with high specificity to monovalent cations. On the basis of the analysis of the results obtained by NMR technique, it has been proposed that structure of GRA channel is dimmer composed of two β -helices monomer connected by they head-to-head, i.e. by their formyl groups [239]. Further it has been established by NMR technique, that helices are right handed [240]. The helix is unusual with 6.3 residues per turn and a central hole approximately 0.4 nm in diameter. A certain controversy still remains because there is an alternative structure that could provide a pore large enough to transport ions – the right-handed double stranded helical dimmer structure. This structure, however predominates in an organic solvent. It has been also observed that GRA form stable monolayer in an air-water interphase, in which it is in double helical form [241]



Fig. 51. (a) Schematic picture of gramicidin channel in a membrane. (b) current fluctuations in a BLM contained gramicidin channels.

(see part 3.1.5). However, in a lipid bilayers GRA channels are most probably in a dimmer form [238]. This has been confirmed also by molecular dynamics simulations, that showed that GRA dimmer is connected with 16 lipid molecules [242,243].

The conductance and kinetics of association and dissociation of gramicidin channels in a BLM has been extensively studied [237]. It is possible to observe discrete jumps of BLM conductivity $\sim 40 \text{ pS}$ in 0.1 mol l⁻¹ KCl at rather low concentrations of gramicidin ($< 10^{-12} \text{ mol } l^{-1}$). The current fluctuations can be connected with association of two monomers diffusing laterally in BLM monolayers and/or with dissociation of these dimmers (Fig. 51). The fluctuation of the current, however with a much larger time of the conductance state, has been observed also for dimmers composed of covalently connected monomers of the gramicidin A. In this case the fluctuations of conductivity are connected with the fluctuation of the thickness of the membrane. Early studies of ionic channels formed by GRA have already established that the current-voltage characteristics (IVC) of GRA modified BLM are non-linear. The non-linearity depends on electrolyte concentration and composition [237]. The quantitative method of determination of degree of non-linearity of GRA channels has been developed by Flerov et al. [244]. The method consists in the measurements of the higher current harmonics generated in membranes modified by GRA [76]. It has been found that the measurement of IVC non linearity using this method enables the investigation of the mutual interaction of ionic channels, changes of the kinetics of membrane properties during the formation of the membrane, influence of lipid environment on the channel properties [245]. This method offers a possibility to verify the theoretical models of ion transport through gramicidin channels [76].

In contrast with gramicidin, alamethicin molecules form channels of different diameter depending on the number of alamethicin molecules involved. It has been established, that alamethicin channel can have up to 7 conducting states [246]. Amphotericin is another channelforming compound. It has lower conductivity then GRA channels and is formed in presence of cholesterol that stabilizes the channel structure [247].

Recently a variety of additional natural peptides have been identified and showed channel



Fig. 52. The chemical structure of the valinomycin.

properties. Among these peptides are e.g. mellitin – a bee venom toxin peptide of 26 residues and cecropins – induced in *Hyalophora cecropia*. These peptides form α -helical aggregates in membranes, creating an ion channel in the center of the aggregate. The common feature of these peptides is their amphiphilic character, with polar residues clustered on one face of the helix and non polar residues contacted with hydrophobic part of a membrane [233].

9.2.2 Ionic carriers

The most detailed studied ionic carrier is valinomycin (VAL) isolated from *Streptomyces fulvissimus*. VAL has cyclic structure containing 12 units made from 4 different residues. Two are amino acids (L-valine and D-valine), two other residues, L-lactate and D- hydroxyisovalerate, contribute ester linkages (Fig. 52). The polar groups of the VAL structure are positioned toward the center of the ring, whereas the non polar groups are directed outward from the ring. The hydrophobic exterior of VAL interact favorably with hydrophobic core of the membrane, while the central carbonyl groups surround the K⁺ ion, shielding it from contact with non polar solvent. K⁺ - VAL complex freely diffuses across biological membranes and causes passive K⁺ transport (up to 10 000 K⁺/sec). For comparison, the GRA channel transports around 10⁷ ions per second, however it is less selective to K⁺ ions then VAL [233]. VAL selectivity binds the K⁺ and Rb⁺ cations, but has about thousand fold lower affinity to Na⁺ and Li⁺ ions. It is connected with the fact, that considerably more energy is required to desolvate smaller ions then larger ions. For example, the hydration energy for Na⁺ is -300 kJ mol⁻¹, while -230 kJ mol⁻¹ for K⁺ ions. Other mobile carrier ionophores include monensin and nonactin. The unifying feature of their structure is similar to VAL - polar inner part and hydrophobic outer part.

9.3 Mechanisms of ionic transport

There are two basic approaches for consideration the mechanisms of ionic transport across the membrane: 1. diffusion and 2. discrete mechanism.

1. Diffusion mechanism is based on application Nernst-Planck equation. This approach is based on assumption of constant field across the membrane, i.e. $d\varphi/dx = const$. This condition is fulfilled for thin membranes and for thick diffusion double electric layer. In this case the Nernst-Planck equation (67) has form of linear differential equation

$$\frac{dC}{dx} + AC = -B,\tag{75}$$

where $A = zF\varphi/(RTd)$, $B = j_i(uRT)$. The solution of Eq. (75) represents dependence of ion flux on membrane potential and the ion concentrations at both membrane sides C_1 and C_2 (see Fig. 49)

$$j_i = \frac{z_i F P \varphi}{RT} \frac{C_1 - C_2 \exp[z_i F \varphi/(RT)]}{1 - \exp[z_i F \varphi/(RT)]},\tag{76}$$

where P = uRkT/d is coefficient of permeability (see part 9.1). Thus, the Eq. (76) derived by Goldman (see Ref. [248]) allowing to estimate passive ionic transport in the case of known concentration of ions at both membrane side and for known value of permeability coefficient, P.

The Goldman's equation assumes non-linear dependence of transmembrane ion flux on membrane potential. Nonlinearity increases with increasing the ion gradient. This dependence is linear only when $C_1 = C_2$ and at very high values of membrane potential. At equilibrium condition, i.e. when $j_i = 0$, we obtain from Eq. (76) the known Nernst equation: $\varphi = (RT/(z_iF)) \ln(C_1/C_2)$. The non linearity of IVC is connected with influence of the electric field on the distribution of ions inside the membrane.

The constant field approach has been used for explanation of non linearity of IVC of GRA channels (see [76] for more details).

2. Discrete description of ionic transport. This approach takes into account the membrane inhomogeneity and ion-ion interactions in a channel. (Note that in diffusion approach membrane is considered as homogeneous and the ions do not interact inside the membrane). The analysis of the ion transport is based on Eyring theory of the kinetics of chemical reactions. Here we consider simple case of the transport of ions through ionic channel that is characterizes by three energetic barriers. Two barriers are located at the membrane border and the barrier responsible for ion selectivity is located in the central part of the membrane (Fig. 53). If the ion transfer is limited by central barrier, then unidirectional ion fluxes are determined as follows

$$\vec{j}_i = C_1 \nu A \exp[-z_i F \varphi/(2RT)]
\vec{j}_i = C_2 \nu A \exp[z_i F \varphi/(2RT)]$$
(77)

where A is constant and $\nu = \exp[-E/(RT)]$ is frequency of ion transfer across energetic barrier with a height E. Resulting current across the membrane will be

$$I_i = zF(j_i - \bar{j}_i) = z_i F\nu[C_2 \exp(z_i \psi/2) - C_1 \exp(-z_i \psi/2)],$$
(78)



Fig. 53. (a) Three barrier model of ionic channel and (b) the shape of current-voltage characteristics: ion transport is determining by A. central and B. side barriers.

where $\psi = F\varphi/(RT)$. For symmetrical ion concentrations $C_1 = C_2 = C$, the IVC will be

$$I_{i} = z_{i} F \nu C[\exp(z_{i} \psi/2) - \exp(-z_{i} \psi/2)] = z_{i} F \nu C \sinh(z_{i} \psi/2).$$
(79)

Thus, if the current is determined by the ion transfer across the central barrier, the IVC has shape of hyperbolic sinus. The conductivity will increase with increasing the membrane potentials. The shape of IVC allowing to determine which barriers are crucial for ion transport. This is illustrated on Fig. 53. We can see, that if transport is determined by central barrier, the IVC is super linear, but it is sub linear when side barriers are determining (see [86,234] for more details).

9.4 Active transport systems

Passive and facilitated diffusion transports are driven by concentration or potential gradients across the membrane. However, other transport processes in biological membranes are driven in an energetic sense. They transport species from low to high concentration regions, and thus maintain the non-equilibrium conditions that are crucial for living systems. These processes are energy consumable. The most common energy input for active transport is ATP hydrolysis, tightly coupled to the transport event. Light energy is other source for active transport, e.g. proton flux in *Halobacterium salinarum* (previously *Halobacterium halobium*) or in thylacoid or mitochondria membranes. The hydrolysis of one ATP molecule causes transport of four protons from the exoplasmic to the cytoplasmic side of thylacoid membrane. In Ca-ATPase the ATP hydrolysis is coupled with transport of two Ca²⁺ ions. In the case of Na⁺, K⁺-ATPase two ions of K⁺ are transported inside and three Na⁺ ions outside the cell following hydrolysis of one ATP molecule.

Most of these ATPases, i.e ionic pumps transfer different ions in opposite direction such as Na⁺ vs K⁺, H⁺ vs. K⁺ or Ca²⁺ vs H⁺. This process has been called Ping-Pong [249]. Each "half-cycle" consists of an ordered sequence of experimentally identified steps: ion binding, ion occlusion (together with ATPase phosphorylation and dephosphorylation), transition between both principal conformations $E_1 \rightarrow E_2$ and vice versa, ion deocclusion and release to



Fig. 54. Schematic representation of H⁺-ATPase in a thlylacoid membrane. The subunit F_1 is responsible for ATP hydrolysis, while subunit F_0 forms proton channel.

the aqueous phase on the other side of the membrane. Recent investigation revealed that a charge movement occurred mainly during the ion binding and release steps. Those reaction steps are called "electrogenic" (see [143] and reference herein for details).

The study of the mechanisms of active transport is rather complicated. It is also due to the fact, that the structure of ATPases is not known yet in sufficient details, although substantial progress has been achieved in the study of the structure and conformation of Ca²⁺-ATPase [250]. The ATPase behaves like a molecular motor. Hydrolysis of ATP resulted also in rotation of the F_1 subunit (i.e. the subunit where active side for hydrolysis of ATP are located) (Fig. 54) [251]. The activity of Na⁺, K⁺-ATPase [252] and also Ca²⁺-ATPase [176,195] can be regulated by phospholipids. The mechanisms of functioning of ATPases are studied usually by means of their reconstitution into the lipid vesicles [143], BLM [76,253] or supported lipid bilayers [254]. In the first case usually the potential sensitive fluorescence probes are used, while in later cases the electrostriction and impedance spectroscopy methods are convenient. Recently, using the ultrasonic velocimetry and densitometry method, we showed that incorporation of Na⁺, K⁺ - ATPase into the lipid vesicles of DOPC decreases their compressibility. Further decrease of compressibility has been observed after addition of ATP in a concentration range 1–5 mmol.1⁻¹ This evidences about considerable influence of ATPase on physical properties of the lipid membrane [255].

10 Membrane receptors and cell signaling

The interaction of organisms with surrounding environment is realized by receptors. For each type of the physical or chemical signal there exist special receptors localized in a plasma membrane or receptor proteins inside the cells. Light receptors provide visual orientation of the organisms, thermal receptors are sensitive to the temperature changes, mechano- and barro- receptors are responsible for sensing mechanical tension and the pressure. Mechano receptors are responsible also for tactile sensitivity that allows to distinguish shapes, textures and movements.

The principles based on mechano-electrical transduction are responsible also for registration of sound. Chemo receptors are responsible for distinguishing tastes and smells.

All receptor systems have common features. Receptors are incorporated into the cell membranes. In all cases the receptors translate the stimulus into an electrochemical potential changes, which in turn result in generation of action potential in adjacent neurons. The intensity of a stimulus is determined by the number and frequency of action potentials produced by sensory system. Special type of receptors — hormonal receptors — are responsible for transduction of the signal into the cell.

In the case of physical reception it is necessary to analyze the mechanisms of interaction of physical signal with receptor. This interaction is usually accompanied by changes of conformation of the receptor, that should affect the physical properties of the membrane, e.g. permeability and/or compressibility. Therefore, for understanding the molecular mechanisms of reception it is necessary to study physical properties of the membranes. For chemical receptors the important steps that should be analyzed consisting in diffusion of the ligand molecule to the receptor, binding of ligand with receptor and finally transduction of the signal from the binding event inside the cell through a complex signal pathways.

Let us consider first the basic features of physical reception.

10.1 Physical reception

The common feature of physical reception, e.g. visual, mechano-, barro- and thermo- is that the external signal resulted finally in opening of ionic channels in a membrane. The molecular mechanisms of visual reception was described in Refs. [233,236]. The basic transducing molecule in visual reception is rhodopsin. Using the model system – bacteriorhodopsin incorporated into BLM – it has been shown, that illumination of this light-sensitive membrane resulted in considerable increase of the elasticity modulus in direction perpendicular to the membrane plane, E_{\perp} . This effect has been connected with the influence of the conformational changes of bacteriorhodopsin on large regions of lipid bilayers. These results evidence on existence the links between functional state of the sensing macromolecule and physical state of the surrounding membrane [76].

Here we briefly describe the basic principles of mechanoreception. In 1972 V.I. Passechnik [256] proposed hypothesis, that the ionic channels with fluctuating conductivity can serve as the transducers of membrane mechanical deformation into electric response of the cell. The existence of such channels in mechanoreceptor cells was experimentally found later in 1977 [257]. The conception raised by Passechnik allows one to introduce an elementary mechanoreceptor unit — elementary mechanosensitive center (EMC) — which is composed of an ion channel and a part of a receptor membrane attached to it. The EMC function consists of the change of the mean ion channel conductivity as a result of the deformation of this part of the membrane. This concept has been proved in a detailed study of Passechnik and his co-workers, which is summarized in Ref. [76].

10.2 Principles of hormonal reception

The fact that receptors are incorporated in lipid bilayers has great significance for enhancement of diffusion of ligand to the receptor. Adam and Delbrück (see [86]) estimated the time of diffusion

of the molecule to the target of a radius "a" from the space of a radius "b". They showed, that the time $\tau^{(i)}$ is function not only the ratio b/a, but also the space dimension

$$\mathbf{r}^{(i)} = (b^2/D^{(i)})f^{(i)}(b/a),\tag{80}$$

where *i* means the uni- (1), di- (2) or three- (3) dimensional space. In a first stage the ligand moves to the receptor by means of three dimensional diffusion. As soon as it reaches the membrane, it continues to diffuse to the receptor binding site by means of two dimensional diffusion. The estimations showed that at certain conditions $10^2 < b/a < 10^4$ and $10^3 < D^{(2)}/D^{(3)} < 1$, $\tau^{(2)}/\tau^{(3)} < 1$. Thus, the existence of receptors in a membrane enhances interaction of ligand with receptor due to faster moving by means of two dimensional diffusion.

The formation of ligand (L) – receptor (R) complex (LR) is the next step in hormone reception. This step can be analyzed in analogy with substrate-enzyme interaction. The formation of ligand-receptor complex

$$L + R \leftrightarrow LR \tag{81}$$

is characterized by affinity constant $K \sim 10^8 - 10^{11} \text{ mol}^{-1}$ l, which is usually higher than that for substrate enzyme complexes. Let us consider general peculiarities of ligand receptor binding, when n ligands interacts with m binding sites. The ligand can exist in bound (**B**) or in a free (F) state. Therefore the concentration of ligand, $[L_i]$ will be

$$[L_i] = [F_i] + \sum_{j=1}^m [B_{ij}]; \qquad i = 1, \dots, n.$$
(82)

In analogy, receptor can also be in the state B_{ij} , i.e. in complex with ligand and in a free state r_{ij} . Thus, the concentration of j-binding site $[R_j]$ will be

$$[R_j] = [r_j] + \sum_{i=1}^n [B_{ij}]; \qquad j = 1, \dots, m.$$
(83)

At equilibrium $(F_i + r_j \leftrightarrow B_{ij})$ the affinity constant K_{ij} is

$$K_{ij} = \frac{[B_{ij}]}{[F_i][r_j]}; \qquad i = 1, \dots, n; \quad j = 1, \dots, m.$$
(84)

Substituting value $[B_{ij}]$ from Eq. (84) to Eqs.(82) and (83) we obtain

$$[L_i] = [F_i] + \sum_{j=1}^m K_{ij}[r_j][F_i]; \qquad i = 1, \dots, n,$$
(85)

$$[R_j] = [r_j] + \sum_{j=1}^m K_{ij}[r_j][F_i]; \qquad j = 1, \dots, m.$$
(86)

Then from Eq. (86) we have

$$[r_j] = [R_j]/(1 + \sum_{j=1}^m K_{ij}[F_i]); \qquad j = 1, \dots, m$$
(87)

and substituting value $[r_j]$ to Eq. (85) we obtain

$$[L_i] = [F_i] + (\sum_{j=1}^m K_{ij}[R_j][F_i]) / (1 + \sum_{i=1}^n K_{ij}[F_i]).$$
(88)

If we use the ratio $k_i = \sum_{j=1}^{m} [B_{ij}]/[F_i] = [L_i]/F_i] - 1$, then the Eq. (88) can be transformed as follows

$$k_i = \sum_{j=1}^{m} K_{ij}[R_j] / (1 + \sum_{i=1}^{n} K_{ij}[F_i]).$$
(89)

In simplest case, i.e. one type of ligand (i = 1) and one type of receptor (j = 1) we obtain

$$k = K[R]/(1 + K[F])$$
(90)

and taking into account that [F] = [B]/k, we obtain Scatchard equation

$$k = [B]/[F] = K[R] - K[B].$$
(91)

In coordinates [B]/[F], [R] the Eq. (91) can be expressed by straight line, that allowing to determine concentration of receptors [R] as well as affinity constant K. Usually the situation is more complex and requiring analysis of several types of binding sites and several types of ligands. For this purpose it is necessary to solve numerically the Eq. (89).

Next step in hormonal reception consists in transduction of the signal originating from the binding of ligand to its receptor into the cell. The complex of reactions accompanying signal transduction across the membrane have been described in details (see e.g. [2,233,258]). In general, the hormone interaction with receptor incorporated in plasma membrane mobilizes various second messengers: cyclic nucleotides, Ca^{2+} ions, ceramide (appeared due to phospholipase action on sphingomyelin) and other substances, that activate or inhibit enzymes inside the cell. The receptors are usually connected with other signaling structures at cytoplasmic side of the cell, e.g. G-proteins, tyrosin kinase or oligomeric ion channels, that mediated the signal transduction. As an example we consider the adenylat-cyclase-initiated cAMP signal transduction pathway with participation of G-protein. This pathway is schematically showed on Fig. 55. The binding of ligand to the receptor activates G-protein (typically it is heterotrimer composed of three subunits, α , β and γ). This activation resulted in hydrolysis of GTP to GDP at α subunit of G-protein. As a result α subunit dissociate from the G-protein complex and activates adenylat cyclase. Adenylat cyclase then converts cytoplasmic ATP into camp and cAMP then activates protein kinase, that in turn is able to modulate the gene expression.

10.3 Taste and smell reception

These types of reception are responsible for recognition of tastes and smells of various substances. It is assumed, that smell reception is based on recognition of molecular structure, i.e. the receptor responsible for specific smell has cavity that exactly corresponds to the shape of the molecule that is detected [259]. Some pheromone receptors can respond to their volatile ligands



Fig. 55. Schematic representation of the adenylate cyclase signal transduction pathway. For explanation see the text.

in a quantal way. For example the moth, *Bombyx mori*, responds to single molecules, or at most very few molecules of the pheromone bombykol, by increasing its wingheat frequency [260].

The taste reception is based on molecular recognition. The acidic taste is determined by protons, salt taste by anions, like Cl⁻. Sweet and bitter taste originating from interaction with receptor of species of different structure. The progress in investigation of the mechanisms of taste reception during last years was connected with isolation of proteins, e.g. monellin, that inducing sweet taste. Using these proteins it has been possible to demonstrate specificity of interaction of this ligands with taste receptors.

In taste and smell reception the binding of species to the receptors is also transformed into the electrical signals. However, the mechanisms of these transductions are still unknown (see [261] for review of exsisting hypothesis).

10.4 Artificial receptors

In addition to natural receptors, currently a considerable interest is focused on development of artificial receptors. It is connected with development of receptor based biosensors. Among artificial receptors the calix[n]arenes are of special interest. They represent macrocyclic aromatic molecules which originate from the synthesis of the phenols and aldehydes, whereas [n] refer to the number of the phenol aromatic cycles in the molecule. In calixarenes, phenolic subunits are bridged via methyl groups in meta position. This provides of the characteristic vase-like shape of the calixarene molecule [262]. In addition, macrocycles with subunits like resorcine or pyrrol have been successfully synthesized [263-265]. Due to the presence of the hydrophobic cavity, formed by the phenolic units, calixarenes are being used for detection of a wide range of compounds such as metal ions, biomacromolecules, etc. Moreover, easy modification of



Fig. 56. Chemical structures of calix[4]resorcinarene.

the side groups allows one to prepare tailor-made calixarenes with high affinity for the specific target molecules. Therefore, these recognizing features of calixarenes can be used as a basis for development of biosensors.

Monolayers formed by calix[n]arenes have been studied either on the water subphase [265-268] or on the subphases containing various cations [262,269]. In addition, calix[4] arenes monolayers have been also successfully employed for the detection of monomeric nucleosides in the subphase [270]. Recently, considerable attention was focused on the development of the methods of detection of the neurotransmitters adrenaline, dopamine or ephedrine, which trigger the metabolism of the lipids and sugars, followed by their utilization in the energetic processes. Uncontrolled imposition of these catecholamines acting as the doping and their difficult detection has provoked International Olympic Federation into the search for the rapid and foolproof methods, which could estimate the concentrations of the catecholamines in the urine. Synthetic calix [4] resorcinarene (Fig. 56) was extensively studied in this respect. It has been shown, that the biosensor based on the solid supported lipid membranes with incorporated calix[4]resorcinarene is highly sensitive towards catecholamines with detection limit in μ M concentration range [271]. These sensors were selective and did not reveal any significant interferences with other compounds (e.g. ascorbic acid, lactose, urea etc.) [271]. Despite these extensive studies, the mechanisms of interaction of catecholamines with calix[4]resorcinarene and the mechanisms of interaction of this artificial receptor with phospholipids is still under investigations.

In our recent work [272] we studied the properties of the monolayers formed by calix[4]resorcinarene and also the properties of mixed calix[4]resorcinarene-phospholipid monolayers formed at the water subphase or subphase containing various concentrations of dopamine. The binding of dopamine resulted in change of the shape of the surface pressure isotherms, as well as changes of the dipole potential, which indicate the charge redistribution upon the dopamine binding, as well as the reorganization of the molecular structure of the monolayers. This is seen on Fig. 57, when the plot of the surface pressure and dipole potential as a function of mean molecular area is presented for different dopamine concentration in water subphase. Increasing concentration of dopamine produces a shift of the isotherms toward higher areas.

This effect is accompanied by an increase of the mean molecular area of calix[4]resorcinarene. The values of the mean molecular areas are reported in Table 11. As it can be seen in Table 11, the mean molecular area of calix[4]resorcinarene increases at the presence of 1 mM of dopamine by approx. 7 % (the differences between the molecular area without dopamine and at certain dopamine concentration were statistically significant according to the Student's t test (p < 0.001)).



Fig. 57. The plot of surface pressure (a) and dipole potential (b) as a function of mean molecular area for monolayers of calix[4]resorcinarene at water subphase (1) and those contained dopamine in concentration: (2) - 10; (3) - 100 and $(4) - 1000 \ \mu M$, respectively. (Reproduced by permission from Ref. [272]).

Tab. 11. Mean molecular area, A and compressibility modulus, C_S^{-1} for the monolayers of calix[4]resorcinarene formed at pure water subphase and those contained various concentration of dopamine. Results represent mean±SD determined from 6 independent experiments. The differences between mean molecular areas are statistically significant according to the Student's test (P < 0.001) Adopted from Ref. [272].

Dopamine, μM	A (nm ² /molecule)	C_{S}^{-1} (mN/m)		
0	1.52 ± 0.02	$178.4\pm4,\!1$		
10	1.56 ± 0.02	$176.7\pm4,0$		
100	1.59 ± 0.01	$171.2\pm5,\!5$		
1000	1.64 ± 0.02	$165.2\pm5,\!3$		

Increase of dopamine concentration causes also a shift of the potential-area dependencies to higher dipole potentials at low pressure region (Fig. 57b). However at higher surface pressure and at presence of dopamine, the dipole potentials are higher than those without dopamine (~ 120– 130 mV). The tendency of the dipole potentials to grow since the very beginning of the compression can be related to the binding of dopamine to the monolayer. The p K_a value of dopamine in a water subphase is 8.87 [273]. Therefore at pH of water subphase used in experiments (pH \approx 6), the amino group of dopamine is positively charged and has affinity to the –OH groups of the calix[4]resorcinarene molecules exposed into the water subphase. This p K_a value could in principle shift down when dopamine bind to a monolayer. However, despite this possibility we suppose that the electrostatic interactions between dopamine and the calix[4]resorcinarene hydroxyl groups are the main reason of the changes of dipole potential. From Fig. 57b, we can also see that before the main raise of the dipole potential certain irregularity took place at ~ 2.1 nm²/molecule. The magnitude of this irregularity increases with increasing the dopamine



Fig. 58. The plot of mean molecular area as a function of dopamine concentration for monolayers composed of calix[4]resorcinarene formed on water subphase. (Reproduced by permission from Ref. [272]).

concentration. This effect may be associated with changes of the calix[4]resorcinarene structure due to dopamine binding.

Using the data presented on Fig. 57a we ploted of mean molecular area as a function of dopamine concentration (Fig. 58). It is seen from Fig. 58 that this plot represents a typical Langmuir isotherm. There is tendency to saturation at higher concentrations of dopamine and the interaction between dopamine molecules and the receptors is much stronger than those between dopamine molecules at the surface of the monolayer (see [73] for theory of specific adsorption). The dissociation constant, K_D was determined according to equation $A = A_{max}c/(K_D + c)$, where A_{max} is the maximal molecular area and c is the dopamine concentration and was found ca. 310 nM. This is much higher in comparison with e.g. the binding affinity of antibodies or DNA aptamers, which are in the range of 1–100 nM [274].

More detailed information about the physical properties of the monolayer can be achieved from the compressibility modulus, C_S^{-1} , defined as [275]

$$C_s^{-1} = -A\left(\frac{\delta\pi}{\delta A}\right). \tag{92}$$

According to Ref. [275], compressibility modulus values between 0 to 12.5 mN/m refer to the gas phase of the films, from 12.5 to 50 mN/m to the liquid – expanded (L-E) films, from 100 to 250 mN/m to the liquid – condensed (L-C) films and the values above 250 mN/m are typical for the solid films. Using Eq. (92), we determined the compressibility modulus for condensed state of the monolayers. The quantity $\left(\frac{\delta\pi}{\delta A}\right)$ is the slope of the monolayer and the area A corresponds to the mean molecular area at condensed state of the monolayer (Table 11). As it can be seen from the Table 11, all the investigated monolayers are characterized by compressibility modulus > 165 mN/m. This proves the condensed state of the monolayers. It is also seen in Table 11 that with increasing of dopamine concentration, the values of C_S^{-1} tend to decrease (although the statistical analysis using Student's t test proved the significant differences (p < 0.001) between the C_S^{-1} values only at 0 and 1000 μ M of dopamine). This suggests that changes of the conformation of the confo

mation of the calix[4]resorcinarene due to binding of dopamine caused more flexible (i.e. more compressible) monolayer.

The artificial receptors are novel structures with high perspective for practical application in biosensors. For examples monolayer formed by calix[4]resorcinarene can be deposited on a solid surface and the detection of dopamine with the calixarénu film can be determined by various method, e.g. using mass detection, amperometric or impedance spectroscopy methods.

11 Lipid film coated electrodes

Interest to the lipid film coated electrodes (known also as supported lipid membranes (sBLM)) as a tool for construction of high sensitive and selective biosensors is considerable. Lipid films protect the solid support from undesirable interefences and thus minimize the redox processes at the electrode surface. On the other hand the lipid film that mimics the properties of biomembranes represents convenient immobilization matrix, that preserve the conformational freedom of attached macromolecules, such are enzymes, antibodies or nucleic acids. The lipid film, due to its insulation properties represent high barrier for transfer of charged particles to the electrode. This disadvantage, can be, however overcome by modification of the film by electron or ion carriers. Lipid films can be prepared with high scale of lipids of various structures and thus allowing to construct biosensing system with desirable surface properties. In contrast with, so called free standig BLM, lipid coated electrodes can be stable even at air. So they can be investigated also by other then electrochemical methods, such are e.g. atomic force microscopy (AFM), surface tunneling microscopy (STM) or scanning electrochemical microscopy (SECM). Due to similar chemical composition, structural and physical properties the sBLM can serve also as a convenient model for biomembranes. The method of preparation of sBLM and so caled tethered BLM (tBLM) has been reported in part 3.2.2 (see also reviews [110,168,276]).

11.1 Modification of lipid film coated electrodes by functional macromolecules

Incorporation of functional macromolecules, e.g. enzymes, antibodies or nucleic acids into lipid layers or immobilization onto sBLMs or liposomes is a crucial stage in the preparation of biosensor. The method of immobilization should fulfill certain requirements: 1. Stability of lipid-macromolecule complex for sufficient time; 2. Optimal conformational lability of macro-molecules; 3. Access to reactive sites of enzymes, antibodies or receptors.

For integral proteins or receptors, which contain hydrophobic constituents, the incorporation into the membranes can be done either by means of mixture of the proteins with lipids in the membrane-forming solution, or by fusion of proteoliposomes with lipid layers. Mixtures of proteins with lipids in solution have been successfully used for incorporation of bacteriorhodopsin [277] and antibodies [278]. Antibodies were also immobilized to the filter supported bilayers [279]. Vesicle fusion for incorporation of proteins has also been extensively reported (e.g. bovine serum albumin [280], acetylcholinesterase [281,282], cholera toxin [282], bacteriorhodopsin [277,283], cytochrome oxidase, nicotinic acetylcholine receptor [280] and H⁺-ATPase [284].

Another immobilization method which has important practical applications has been developed by Wilchek [285]. This method consists in use of the high affinity of streptavidin and/or avidin to biotin. Thus, if a streptavidin or avidin modified macromolecule is added to a sBLM that is prepared from biotinylated phospholipids, a stable complex between protein and phospholipid is formed. The first sBLM biosensors based on immobilization of enzyme – glucose oxidase using streptavidin-biotin technology or avidin-biotin technology have been reported in Refs. [286,287]. Similar method has been used also for immobilization of antibodies to the sBLM [288].

A further novel approach of immobilization consists in using a bacterial glycoproteins so called S-layer as matrix for immobilization of protein macromolecules [289].

For immobilization of oligonucleotides to sBLM the effective approach consists in modification of short sequence of single stranded DNA by hydrophobic chain, e.g. palmitic acid [232,290] or cholesterol [291].

11.2 Bioelectrochemical and analytical applications of lipid coated electrodes

Lipid films coated electrodes are new tool for fundamental and applied research in bioelectrochemistry, biophysics and analytical chemistry. Concerning the fundamental studies the advantage of these systems consists in their high stability and availability to apply additional techniques, that can not be used for classical model of biomembranes, e.g. BLM and liposomes. There are for example thickness shear mode technique (TSM), surphase plasmon resonance (SPR), elipsometry and all types of image techniques, such are AFM, STM and SECM. Lipid films allow incorporation of integral and peripheral proteins, proteolipidic receptor complexes, that allows to mimics the structure and properties of biomembranes. As a matter of fact the biomembranes are supported by spectrin net or glycocalix, therefore selection of appropriate support, e.g. agar gel or polymers can serve as suitable model of biomembranes supported on glycocalix.

Supported lipid films revealed unique properties also for applications in analytical chemistry. They allowing to immobilize on their surface enzymes, antibodies, artificial receptors, nucleic acids, DNA/RNA aptamers and thus to use these systems as a biosensors. Variation of lipid composition allowing to select conditions that allow to avoid unspecific interactions with the sensor surface of various undesirable interferences.

The application of supported lipid films in the fundamental research has been reviewed in paper by Sackmann [113] and Knoll [254]. Application of sBLM both in fundamental and applied studies has been reviewed also in Refs. [110,168,276,292].

Unmodified supported lipid films can be rather useful for study the adsorption/desorption processes of various compounds that are dissolved in lipids, e.g. detergents or natural surfactants, like saponin [293] as well as can be used for determination of activity of phospholipases. This has been shown in paper by Mirsky et al. [294]. The approach used in this work was based on the fact that hydrolysis of the substrate, mediated by phospholipase A_2 leads to formation of water soluble products from water insoluble substrate, i.e. phospholipid monolayer. The action of phospholipase thus resulted in removing certain part of lipid monolayer from alkylthiol supported lipid film, that is monitored as increase of capacitance of the layer adjacent to the solid electrode.

Unmodified lipid coated electrodes can be successfully used also for the study of the mechanisms of interaction of nucleic acids with lipid layer and due to high stability of the supported lipid films, these systems open new routs for study of the mechanisms of electroporation of nucleic acids. As an example is the paper by Schouten et al. [295]. They prepared cationic bilayer adsorbed in self-assembled monolayer (SAM) of alkylthiols terminated by negative charged groups of carboxylic acid. Using the SPR method, they showed, that cationic lipids formed bilayers at the top of SAM of a thickness 3.2 to 3.3 nm. By means of photo bleaching method they showed that the layers were homogeneous and relatively immobile. DNA interacts with cationic lipids by physical adsorption. It has been shown in this work, that DNA forms a layer of 0.8 nm. Recently [169] the method of electrostriction has been applied to study the interaction with sBLM cationic surfactant hexadecylamine (HDA), HDA-DNA and DNA-Mg²⁺ complexes. Interaction of HDA with sBLM resulted in decrease of membrane capacitance and two-directional effect on elasticity modulus, E_{\perp} , (increase or decrease), which can be caused by different aggregation state of surfactant at the surface of sBLM. In contrast with effect of HDA, the complexes of HDA-DNA resulted, in most cases, increase of elasticity modulus and increase of membrane capacitance, which can be caused by incorporation of these complexes into the hydrophobic interior of the membrane. Certain part of these complexes can be, however, adsorbed on the sBLM surface. DNA itself does not cause substantial changes of physical properties of sBLM; however, addition of bivalent cations Mg^{2+} to the electrolyte-contained DNA caused substantial increase of elasticity modulus and surface potential. These changes were, however, much slower than that observed for HDA-DNA complexes, which can be caused by slow competitive exchange between Na⁺ and Mg²⁺ ions.

Supported lipid films are also rather perspective tool for study the mechanisms of proteinlipid interactions [69] as well as for reconstitution of ATPases and study the mechanisms of functioning these ionic pumps [284].

For specific response of lipid coated electrodes to various low and high molecular weight species the lipid film should be modified by ionic channels [296,297] and carriers [298,299], specific receptors [300,301], antibodies [302-304], of nucleic acids [230,291,305]. In this part we will show two examples of application of sBLM in bioelectrochemistry: 1-sBLM modified by carriers and ionic channels and 2-sBLM as an enzymatic electrodes. Another applications can be find in above mentioned reviews, e.g. [110,168,254,276,292].

11.2.1 Supported lipid films modified by carriers and ionic channels

In the first studies of sBLM formed according to the method developed by Tien and Salamon [105], there was attempt to check, whether sBLM could be modified by ionic carriers, e.g. valinomycin in order to use this system as an ion-selective electrode. Successful attempt in this direction was demonstrated in paper by Snejdarkova et al. [299]. They showed that modification by valinomycin really induced increase of conductivity of lipid film at the stainless-steel support. The problem, however remains with presence of metal support, that does not allowing to further diffusion of the ions. This problem has been solved in paper by Ziegler [306], who used agar supported lipid films and successfully demonstrated ionic channel characteristics similar to that observed for free standing membranes. These agar-supported lipid films has been used also for study the interaction of short peptides with lipid film [307]. The advantage of these system in addition to their high stability is that they require minimal volume of buffer – around 50–100 μ l, which is at least 10 times lower than that used in typical experiments with free standing BLM. This, of course considerably safe the amount of species used. In addition, agar supported films represents bilayers systems, which is not the case of the lipid films supported on a tip of the metal wire. As we already mentioned at the beginning of this chapter (section 3.2.3) lipid films formed on a tip of the metal wire contain structural defects and may be composed of monolayers, bilayers or even multilayers. Unfortunately, the main disadvantage of agar supported films is their lower stability in comparison with metal supported films. Therefore further attempts was focused on development of the supported lipid systems, that will contain hydrophilic spacer between metal and lipid film, which can be filed by electrolyte and thus represent more suitable model of biomembrane.

The problem has been successfully solved by Cornel et al. [308], that developed sophisticated lipid bilayer system supported on a gold layer, but contained hydrophilic spacer between gold and bilayer that allowed the buffer to be localized here. This system has demonstrated high sensitivity to dissociation of ionic channels following disruption of gramicidin dimmers by specific interaction with antigens [308] or during hybridization of nucleic acid at the lipid film surface [309].

11.2.2 Electrodes coated by lipid films with immobilized enzymes

Lipid coated electrodes represent unique tool for study the mechanisms of enzymatic reactions at the surfaces and for construction of enzymatic sensors. The enzyme can be incorporated into the lipid films by means of dissolution of enzyme molecules in lipid solution from which the film is prepared or by immobilization of the enzyme at the lipid film surface. In this respect the pioneering work by Snejdarkova et al. have great significant for further progress in this field. In these work either streptavidin [286] or avidin [310] have been used for immobilization of glucose oxidase (GOX) to the lipid film surface prepared on a tip of freshly cutted stainless-steel wire coated by insulating polymer or Teflon. Further this approach has been used also for immobilization of another enzymes, e.g. urease on a polypyrrol film [311], or bi-enzyme system contained of acetylcholinesterase and choline oxidase [312].

Let us show peculiarities of lipid-coated electrodes modified by enzymes using an example of most detailed studied system composed of GOX attached to the supported lipid film lipid using avidin –biotin technology [286,310,313,314].

In order to prepare lipid film+GOX coated solid electrode mostly the surface of freshly cutted tip of Teflon coated stainless steel [313] or platinum wires [315] were used. The formation of the film is rather simple and is based on the technique developed by Tien and Salamon [105]. The clean wire is immersed into the drop of lipid solution dissolved in n-decane-butanol mixture (8:1 volume/volume). Then the tip is cutted by sharp scalpel and immersed in a buffer (typically 0.1 mol 1^{-1} KCl + 10 mmol 1^{-1} Tris/HCl, pH 7.0). Note that pH of electrolyte has considerable influence on GOX activity, therefore it is important to work in a buffer condition with optimal pH (approx. pH 7). In a buffer the self assembled process of formation the lipid film takes place. Phospholipids isolated from crude ox brain extract (COB) are convenient for formation of these films. These lipids can be modified by D-biotin-N-hydroxy-succinimide ester [286]. In order to use avidin-biotin technology it is also important to modify enzyme by avidin or streptavidin. For this purpose the method well described in Ref. [285] can be used. This method is based on formation of avidin-GOX (A-GOX) conjugates by cross-linking with glutaraldehyde. The modification of the film surface contained biotinylated phospholipids consists in immersion of lipid coated wire into the A-GOX buffer solution. The kinetics of this process can be studied using e.g. electrostriction method. It is expected, that immobilization of A-GOX to the lipid film surface will result in strong binding of avidin to the biotin sides. This binding is noncovalent, but very



Fig. 59. (a) Time course of changes of elasticity modulus E_{\perp} (trace A) and electrical capacitance (trace B); (b) membrane surface potential $\Delta \Phi_m$ fro sBLM from COB extract modified by biotin following addition of A-GOX at a final concentration of 30 nmol 1^{-1} and 60 nmol 1^{-1} (The moment of addition of A-GOX) is indicated by arrows). (Reproduced by permission from Ref. [229]).

robust having a dissociation constant 10^{-15} mol l^{-1} . In addition there exist strong interaction between avidin molecules at the surface of the film. All these processes result in stabilization of the structure of the film. This stabilization partially causes restriction of the mobility of the lipids, that in turn influence on the mechanical properties of the lipid film. We can therefore expect substantial influence of binding process on the mechanical properties of the film. In addition GOX is negatively charged. Therefore the binding process should result in changes of surface potential of the film. These phenomena have been observed experimentally [313]. An example is showed on Fig. 59. We can see that chelation-like interaction of A-GOX with surface of biotinylated lipid film resulted in increase of elastic modulus E_{\perp} , decrease of membrane capacitance, C and increase of surface potential $\Delta \Phi_m$. The saturation of 30 nmol 1^{-1} . The restriction of the mobility of phospholipids following the adsorption of the conjugate A-GOX resulted also in increase of relaxation time of reorientation dipole moments, as has been showed above (see part 8.2 and Tab. 9).

The detection of glucose in a buffer can be performed amperometrically [316], e.g. based on anodic reoxidation of enzyme usually at potential approx +0.6 V (positive terminal on an working electrode)

$$GOX(FAD) + glucose \rightarrow GOX(FADH) + gluconolactone$$
 (93)

$$GOX(FADH_2) + O_2 \rightarrow GOX(FAD) + 2H^+ + 2e^-$$
(94)

Thus, the changes of the current in a system A-GOX electrode-reference electrode is a measure of the concentration of glucose degraded by enzymes at the surface of lipid film. As we



Fig. 60. Calibration plot at three different potential values for biosensor with a BLM formed on a Nafion layer containing Fc compared with a calibration plot for a biosensor with a BLM on a Nafion layer without Fc at +0.6 V. Measurement carried out in 0.1 mol 1^{-1} phosphate buffer of pH 7.2. The platinum wire of a diameter 0.6 mm coated by low conducting polyoxyphenylene film was used as a support for the films (Reproduced by permission from Ref. [315]).

mentioned above, the lipid film is poorly permeable by charged particles, e.g. ions or electrons. Therefore for increasing the sensitivity it is necessary to modify the lipid film by electron carrier, e.g. tetracyanoquinodimethane (TCNO) [313] or tetrathiofulvalene (TTF) [317]. The sensor response following addition of glucose is in order of 1 min. The plot of the current as a function of glucose concentration has typical shape expected for enzymatic reactions followed by Michaelis-Menten kinetics, i.e. it is curve with saturation (Fig. 60). The sensitivity of the sensor response depends on the potential applied as well as on modification of the supporting layer by mediator. The dependence of the current versus concentration of glucose can be linearized using the Lineweaver-Burk plot, i.e. plot of $(I - I_{max})/(I - I_0)$ versus glucose concentration. Here I_0 is the current in absence of glucose, I is the current at certain glucose concentration and I_{max} is the current at saturation, i.e. at high glucose concentration. The standard analysis of this plot allowing to determine Michaelis constant, K_M . For example for lipid film based enzyme electrode reported in paper [313], the $K_M = 0.66 \pm 0.18 \text{ mmol } l^{-1}$ which is close to the value obtained by Bartlett et al. [316] ($K_M = 1 \text{ mmol } l^{-1}$) for the enzyme electrode with GOX modified by ferrocene derivatives and immobilized on glassy carbon electrode, i.e. no lipid film was present. Higher value of K_M has been, however obtained when lipid film was modified by TCNQ ($K_M = 14.45 \pm 1.32 \text{ mmol } l^{-1}$) or when GOX was formed on the film composed of lecithin and polypyrrole [318] ($K_M = 13.1 \text{ mmol } l^{-1}$). The above values of K_M were, however,
lower then that for free GOX in solution (33 mmol l^{-1} , see [319]), which may be connected with slower rate of glucose oxidation at the amphiphilic surface as it is revealed from considerably lower value of enzyme turnover (around 1.1 s⁻¹ for lipid coated electrodes [313] and 10^{-3} to 10^{-2} s⁻¹ for GOX immobilized on glassy carbon electrode [316]) in comparison with that for a free GOX in a solution (approx. 340 s⁻¹ [319]). Relatively low enzyme turnover as well as the sensitivity of the sensor based on lipid could evidence about certain unfolding of the enzyme at more hydrophobic surface [320].

Recently performed analysis of the properties of GOX sensor based on lipid films prepared on various supports, including stainless steel, platinum, polypyrrol and Nafion films modified by ferrocene showed, that the best sensitivity and best resistance to various interferences (ascorbic acid, paracetamol, uric acid) has been obtained by GOX sensor formed on the base of lipid films supported on Nafion film with incorporated ferrocene (Fc). Nafion film was supported on a tip of platinum wire coated by insulating polymer [315]. The presence of the mediator – Fc – entrapped into Nafion film allowed substantially increase sensitivity of the sensor (17.7 μ A mmol 1⁻¹ cm⁻²), which is almost 1000 time more in comparison with the sensor prepared on the base of lipid film on a stainless steel support. In addition, the presence of mediator allowed to decrease the potential for oxidation H₂O₂ to +0.4 instead of +0.6 for films without mediator (Fig. 60), similarly to the systems modified by TCNQ [313]. This allowed considerably reduce action of various interferences. The sensor was rather stable. Despite the fact that at +0.4 V during the first three days of use the sensitivity decreased almost by 40 %, during subsequent 3 weeks the sensor was stable. These unique properties open possibilities also for practical application of the lipid film based sensors in a complex biological liquids.

Thus, in addition to practical applications of self assembled structures on a solid support, these systems allowing to use various powerful physical techniques for study the adsorption of enzymes, antibodies or nucleic acids to the lipid films. Supported lipid film with immobilized proteins represents also certain biomimetic structure, modeling, e.g. lipid membrane with immobilized peripheral proteins. These structures are crucial for study the problems connected with interaction between biological and non biological interfaces. Due to the fact, that a considerable part of biochemical processes in cells take place at the membranes and their surface, the biomimetic structures that allow to study these processes should have increasing importance [321-323].

12 Conclusion

In this work I have attempted to present an introduction to the physical properties of biomembranes and their models. It is obvious, that these objects that seem to be rather simply in a brief view are rather complex in a more deep analysis. The dimension of the most cells is in micrometer scale, while the thickness of biomembranes is in the scale of several nanometers. It is likely that with applications of novel technical tools for the study of "nano word" a new exiting knowledge on these structures will appear. The supported lipid membranes with incorporated receptors, enzymes, antibodies or nucleic acids can also be rather useful for development of the biosensors that mimic the peculiarities of natural membranes. The growing interest to the application of biomimetic structures in biosensing could result in apparance of devices with application in medical diagnostics, food industry as well as for environmental monitoring. Acknowledgement: The author is grateful to Agency for Promotion Research and Development (Contracts No. APVT-51-013904), Grant agency VEGA (Projects No. 1/1015/04) and to Ministry of Education of Slovak Republic (Project in framework of Slovak-Greece bilateral collaboration in Science and Technology) for financial support.

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