VILNIUS UNIVERSITY

Tadas Ragaliauskas

Immobilization of lipid membranes on the planar surfaces. Surface plasmon resonance study

Summary of doctoral dissertation
Physical sciences, biochemistry (04 P)

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VILNIAUS UNIVERSITETAS

Tadas Ragaliauskas

Lipidinių membranų imobilizavimas ant paviršiaus ir jų tyrimas paviršiaus plazmonų rezonanso metodu

Daktaro disertacija

Fiziniai mokslai, biochemija (04 P)

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CONTENTS

INTRODUCTION

Phospholipid membranes are complex and self-assembled systems, one of their roles is to protect a cell's inner content against external factors such as toxins. Thus, the cell membrane studies are significant in medicine, physiology etc. However, there is a lack of full knowledge of the mechanisms of their function. To solve this issue variety of lipid membrane models were introduced [1]. And over the last decades, there were created and developed several models such as: support bilayer lipid membranes (sBLM) [2–4], hybrid bilayer lipid membranes (hBLM) [5,6], lipid unilamellar liposomes [7]. Recently, a new promising platform – tethered bilayer membranes (tBLMs) was built up, it appeared to be a stable and suitable tool for artificial membranes studies [8–13]. tBLMs provide excellent stability as they are covalently linked membrane bilayers to a solid support: the inner leaflet of the membrane is bound to the solid support via a short spacer group. The ability of the functional incorporation of various proteins has been shown in the similar systems.

The planar surfaces, particularly, if they are electrically conducting, are advantageous due to possibility to use surface sensitive techniques, such as: atomic force microscopy [14], quartz crystal microbalance [15], neutron reflectometry [16] and electrochemical impedance spectroscopy [17]. In this doctoral thesis, the design and development of tBLMs are described and a possible application of these models for incorporation and detection of proteins (peptides) is introduced. The tBLMs were analyzed mainly by surface plasmon resonance (SPR) technique.

Pore – forming toxins (PFT), as the name suggests, disrupt the selective influx and efflux of ions across the plasma membrane by inserting a transmembrane pore. Ion channels, in general, play diverse roles in the cells and organelles transport of specific ions and macromolecules. Pneumolysin (PLY) [18] and vaginolysin (VLY) [19] – are cholesterol dependent cytolysins

produced by *Streptococcus pneumoniae* and *Gardnerella vaginalis,* respectively. The effects of PFTs directly related with the interaction between toxin and phospholipid membrane. In this work, were demonstrated the possibilities of electrochemical impedance spectroscopy (EIS) and SPR to detect toxins as well as to study the interactions of toxins with artificial tethered bilayer membranes.

The aim of this work: to characterize tethered bilayer lipid membranes (tBLMs) and adapt them for protein – lipid interactions study.

The objectives of the work:

1. To develop a new method of lipid layer formation.
2. To determinate sensitivity of SPR technique to lipids, cholesterols and thiols.
3. To determinate an area per anchor compound: WC14 and dHC18.
4. To determinate an amount of cholesterol in the lipid membranes.
5. A) To determinate a relationship between toxins activity and the amount of cholesterol in membrane.

   B) To determinate a detection capabilities of formed toxin pores in the membrane by EIS and SPR.

The novelty of the work

Lipid membrane models have been developed for many years, but their actual composition is still unclear: which part of the surface the anchor or backfiller molecules are occupied; which fraction is filled by lipids. The issue of that was the absence of a quick and convenient method to form the lipid layers. Thus, in this work a new method for lipid layers formation has been adapted from fusion of multilamellar liposomes (MLV). In this case, MLV can be prepared within 30 minutes without any expensive treatment such as extrusion or powerful ultrasonic bath. Besides, for the first time the new preparation method allowed to monitor a formation of lipid membrane by

surface plasmon resonance and to determinate the exact sensitivity of SPR technique to lipids. In the result, the precise composition of model lipid bilayer was established.

The use of tBLMs in the research of pore toxin formation often raises the question: which part of the adsorbed toxin inserts in the membrane bilayer? In this work, for the first time the dependence of protein concentration, lipid membrane composition and cholesterol on the detection vary was showed.

ACRONYMS

SAM	*self – assembled monolayer*
sBLM	*support bilayer lipid membrane*
hBLM	*hybrid bilayer lipid membrane*
tBLM	*tethered bilayer lipid membrane*
βME	*β – mercaptoethanol*
DOPC	*1,2 – dioleoyl – sn – glycero – 3 – phosphatidilcholine*
Chol	*cholesterol*
SPR	*surface plasmon resonance*
EIS	*electrochemical impedance sprectroscopy*
PLY	*pneumolysin*
VLY	*vaginolysin*
ODT	*octadecanethiol*
WC14	*20 – tetradecyloxy – 3,6,9,12,15,18,22 – heptaoxahexatricontane – 1 – thiol*
dHC18	*20 – (Z – octadec – 9 – enyloxy) – 3, 6, 9, 12, 15, 18, 22 – heptaoxatetracont – 31 – ene – 1 – dithiol*
MβCD	*methyl – β – cyclodextrin*
MβCD – Chol	*methyl – β – cyclodextrin and cholesterol complex*
$\Delta\alpha$	*SPR angle shift, m° (milidegree)*
n	*refractive index*
Δm	*mass amount, ng/cm²*
d	*layer thickness, nm or Å*
t	*time, s*
φ	*phase, °*
c	*concentration, mol/l or M*
Y	*admittance, S or S · cm²*
f_{min}	*EIS frequency at phase minimum, Hz*
n	*amount of „active“ proteins, %*
S	*SPR sensitivity to material, ng/(cm² · m°)*
CDC	*cholesterol – dependent cytolysins*
MLV	*multilamellar lipid vesicles*

MATERIALS

Mili-Q purified water	– dist. H_2O (Milli-Q plius, USA)
Sodium chloride	– NaCl (FLUKA, Switzerland);
Monosodium phosphate	– $NaH_2PO_4{\cdot}2H_2O$ (FLUKA, Switzerland)
Ethanol	– EtOH (AB „Vilniaus degtinė“, Lithuania)
β – mercaptoethanol	– βME (Sigma–Aldrich, Germany);
di β – mercaptoethanol	– dβME (FMTC, Lithuania);
chloroform	– $CHCl_3$ (Sigma–Aldrich, Germany);
methyl – β – cyclodextrin	– MβCD (Sigma-Aldrich, USA);
sulfuric acid	– H_2SO_4 (Reachem, Slovakia);
sodium hydroxide	– NaOH (Sigma–Aldrich, Germany)

Lipids:

1,2 – dioleoyl – sn – glycero – 3 – phosphatidilcholine (DOPC)
Cholesterol (Chol)

All lipids were obtained from Avanti Polar Lipid, USA.

Anchor molecules:

20 – tetradecyloxy – 3,6,9,12,15,18,22 – heptaoxahexatricontane – 1 – thiol (WC14);
Z – 20 – (Z – octadec – 9 – enyloxy) – 3,6,9,12,15,18,22 – heptaoxatetracont – 31 – ene – 1 – dithiol (dHC18);

These compounds were synthesized by dr. D. J. Vanderah at National Institute of Standards and Technology, Gaithersburg, MD, USA.

Proteins:

Pneumolysin and vaginolysin – were donated by Dr. Aurelija Žvirblienė, from the Institute of Biotechnology, Vilnius University.

METHODS

Multilamellar vesicles preparation (MLV). Vesicle suspensions were prepared from DOPC or mixed with Chol. The lipids were dissolved in chloroform to a concentration of 10 mM. Typically, an aliquot of this lipid solution (~ 10 mL) was transferred to a separate vial and chloroform evaporated under a continuous flow of nitrogen until a thin, a uniform lipid film formed (~ 20-30 min). The dried lipid film was resuspended in buffer solution (0.1 M NaCl, 0.01 M $NaH_2PO_4{\cdot}2H_2O$) to a 1 mM total lipid concentration. Using an automatic pipette (1 mL tip) slowly (1 cycle per second) aspirated and dispensed until the lipid film on the vial walls dispersed and the solution became milky (~ 50 aspiration-dispense cycles to obtain a homogenous, though, milky opaque preparation). After that preparation, these vesicle compositions can be stored at room temperature for 1 month. However, before each experiment the lipid mixture in buffer must be mixed again (usually, 5-10 aspiration-dispense cycles) to fully restore the functional properties.

Surface plasmon resonance (SPR). SPR measurements were conducted on Autolab Twingle system (Eco Chemie B.V., The Netherlands) equipped with a flow-through cell (volume – 175 μL). The unit performs SPR spectra recording at fixed wavelength of 670 nm. It automatically follows the position of an incidence angle (ranging from 62 to 78 degrees) in 0,1 milidegree (m°) resolution. Model F34 refrigerating/heating circulator (Julabo, DE) was used to stabilize temperature at 21 ± 0.1 °C. Before each experiment, the baseline in buffer solution was recorded. All measurements were carried out at stopped-flow conditions.

Electrochemical impedance spectroscopy (EIS). EIS measurements were carried out using Autolab PGSTAT302N electrochemical workstation (Metrohm, Netherlands). The EI spectra were recorded in a

potentiostatic mode with 10 mV AC perturbation voltage at 0 V vs. Ag|AgCl|NaCl (sat), with the potential +197 mV respective to the standard hydrogen electrode. Measurements were carried out in a SPR cell with two channels. Each cell had 7.8 mm^2 surface of the working electrode at the bottom. The measurements were carried out in the frequency range from 0.5 Hz to 100 kHz unless otherwise indicated. Data analysis was carried out by Zview 2.8 software package.

RESULTS AND DISCUSSION

1. A new method of lipid layer formation

Several methodologies are used to form tBLMs. One of them is a rapid solvent exchange (RSE), one of the most widely used [20–22]. RSE bilayers exhibit excellent electrical sealing properties and longevity. The formation process is fast, but the real-time monitoring of the bilayer formation is impossible. In addition, the RSE methodology allows creating lipid layers from limited phospholipid compositions because it requires tBLMs forming lipids to be soluble in the exchangeable solvent, this is not always possible.

In the literature are described several attempts to use vesicle fusion for the formation of the tBLMs. In some cases suspensions of small unilamellar vesicles have been utilized to accomplish tBLMs [23]. However, the complete and electrically insulating bilayers were formed only if densely packed, mono-component SAM anchors were used. Densely packed constructs are similar to the hybrid bilayer membranes, which exhibit limited ability to functionally reconstitute transmembrane proteins due to dense inner leaflet. Moreover, vesicles can also be fused to less dense, sparse tether systems made of one or two components – a hydrophobic lipid like anchor and a shorter hydrophilic backfiller. However, the electrical resistance of tBLMs deteriorates quickly with tether density. While the number of defects may not necessarily solely determine the measured resistance of tBLMs, decrease in the tether density most likely causes increased defectiveness. Another important drawback of vesicle fusion to sparsely-populated anchors is that hours of incubation in the vesicle solution to achieve full surface coverage and low residual conductance (high tBLM resistance) are required. From a practical standpoint, fast and reproducible formation of tBLMs is needed for a quick activation of tBLMs – based devices, for example sensors.

To date, most of the studies employed monodisperse, small unilamellar vesicle (SUV) compositions to accomplish supported bilayers by vesicle fusion. In this work it was discovered that without compromising their integrity, tBLMs or another lipid membrane models can be accomplished through vesicle fusion within less than 60 min on sparsely populated anchor SAMs, using MLV that can be prepared within minutes without any special treatment such as freeze-thaw cycles or extrusion. Such bilayers exhibit low defect densities, comparable to or exceeding those achievable by RSE on the same anchor SAMs. Importantly, bilayers formed by the MLV fusion are suitable for the reconstitution and detection of transmembrane proteins, including large multimeric, cholesterol dependent toxins, such as pneumolysin or vaginolysin and possibly any other membrane damaging protein complex.

2. Determination sensitivity of SPR to lipids and thiols

Surface plasmon resonance – a method to detect small molecules on the surface, approach based on the change of refractive index. This method is popular in the world, but it has the main disadvantage – in many laboratories, the SPR devices use different prisms and wavelength. For example, if the prism is made of BK7 (n=1.515) glass and the wavelength of incident light is 635 nm, then the angular shift due to a protein binding layer (n= 1.5) of 3 nm on a gold sensor chip is 750 m°. If keeping everything the same, except replacing the BK7 glass prism with a SF10 glass (n=1.723) prism, then the same protein binding layer leads to an angular shift of 350 m° (a weaker response). The same situation is with wavelength. Therefore, it is important to determinate sensitivity of various materials.

2.1 Thiol

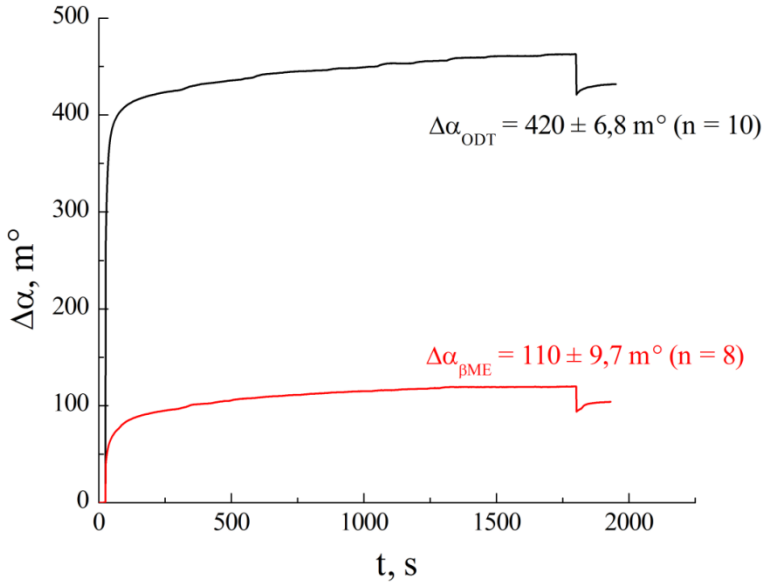

Figure 1. *SPR monitoring of self – assembled monolayers formation: black curve – ODT; red curve – βME. Thiol concentration in ethanol solution – 0.1 mM.*

To determinate sensitivity of SPR to thiols, it was first necessary to form homogeneous monolayers. Thus, firstly was formed self – assembled monolayer from the solutions of ODT and βME in ethanol (c = 0.1 mM).

Figure 1 represents the SPR sensograms of the SAMs formationsFigure 1. SPR monitoring of self – assembled monolayers formation: black curve – ODT; red curve – βME. As can be seen the changes of SPR angles in both cases differ: $\Delta\alpha_{ODT}$ = 420 m°; $\Delta\alpha_{\beta ME}$ = 110 m°. The difference between these two measurements is likely the result of inequality of their chain lengths. In order to prove this result, a three – stage calculation (TSC) algorithm is applied, which shows the transition from mass quantity to the refractive index on the surface. In the first stage, the number of thiol molecules per occupied surface in monolayer case is calculated, if an area per one ODT molecule is 22.5 Å^2 [24]. The result is obtained, that there are $4.44 \cdot 10^{14}$ molecules in 1 cm^2 or 211 ng/cm^2. In the second stage, the amount per surface area (Δm) of organic material localized on the surface can be estimated by the equation [25]:

$$\Delta m = d \frac{n_{layer} - n_{liquid}}{dn/dC} \quad (1)$$

where d is the thickness of the absorbed organic material layer, n_{layer} is the refractive index of the absorption layer, n_{liquid} is the refractive index of the liquid, and dn/dC is the incremental change in refractive index due to molecules concentration. To estimate the amount of material associated with the formation of SAM, the following parameters were used: n_{ODT} – 1.5 [26], n_{EtOH} – 1.36, dn/dC – 0.16 ml/g and d_{ODT} – 24 Å [27]. Thus, 1 eq. returns Δm value – 211 ng/cm^2. In addition, in the final stage, the numbers from 1 eq. (n_{ODT}, n_{EtOH} and d_{ODT}) were used to estimate the SPR shift response of 420 m° per ODT monolayer, using Winspall 3.02 software and the parameters are listed in Table 1. Consequently, SPR sensitivity in mass units is 1 m° per 0.502 ng/cm^2 of thiol material.

Table 1. Parameters used to model SPR shift.

Layer	Media	n	k	d (Å)
1	Prism BK7	1.518	0	-
2	Titan film	2.3829	3.0414	7
3	Gold film	0.188	3.77	510
4	SAM layer	1.5	0	24
6	Ethanol	1.36	0	-

2.2 Lipid

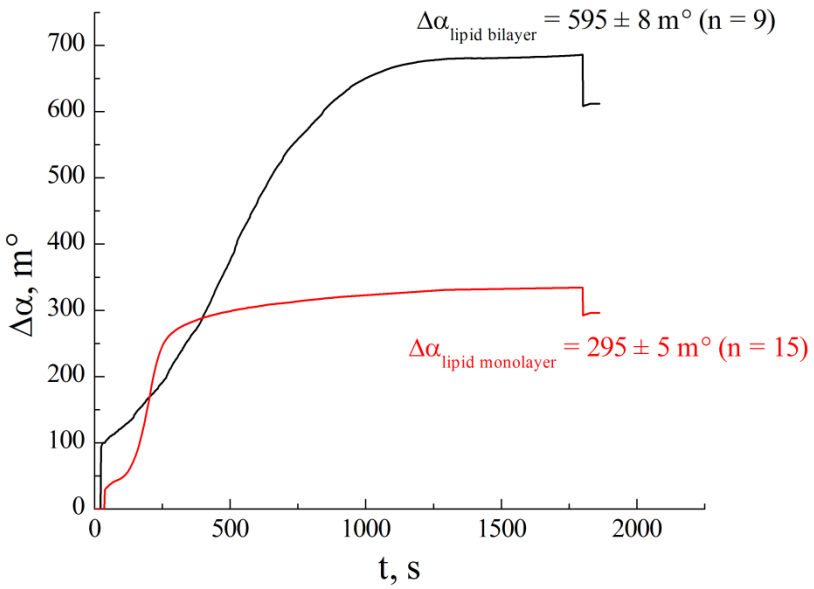

Figure 2. *SPR sensograms of lipid (DOPC) layers formation on self-assembled monolayers of: black curve – ODT; red curve – βME upon exposure to MLV liposome preparation. Lipid concentration in MLV – 1 mM.*

Figure 2 displays SPR curves of the formation of monolayer and bilayer by fusion of DOPC MLV on hydrophobic ODT and hydrophilic βME surfaces. On the hydroxyl – terminated SAMs, fusion produces a layer twice as thick as that on the hydrophobic ODT surface. The near saturation level is achieving within 400 – 500 s and around 1000 s on ODT and βME, respectively. The average SPR angle shifts after washing of the cell at t=1800 s was 295 ± 4 m° (n=15) on ODT and 595 ± 7 m° (n=6) on βME.

To quantify the amount of the lipid material transferred from MLV to self-assembled monolayer, TSC algorithm was used again. Therefore, the first stage: if A_{DOPC} = 72 Å^2 [28], then Δm_{DOPC} = 181 ng/cm^2. The second stage: if n_{DOPC} – 1.478 [29], n_{buff} – 1.335, dn/dC – 0.138 ml/g, d_{DOPC} – 1.85 nm and after that the calculation obtains the same value of Δm_{DOPC} = 181 ng/cm^2. The third stage, by using fitting data SPR angle changes in 290 m° per monolayer of DOPC. Consequently, SPR sensitivity in mass units is 1 m° per 0.62 ng/cm^2 of lipid material.

2.3 Cholesterol

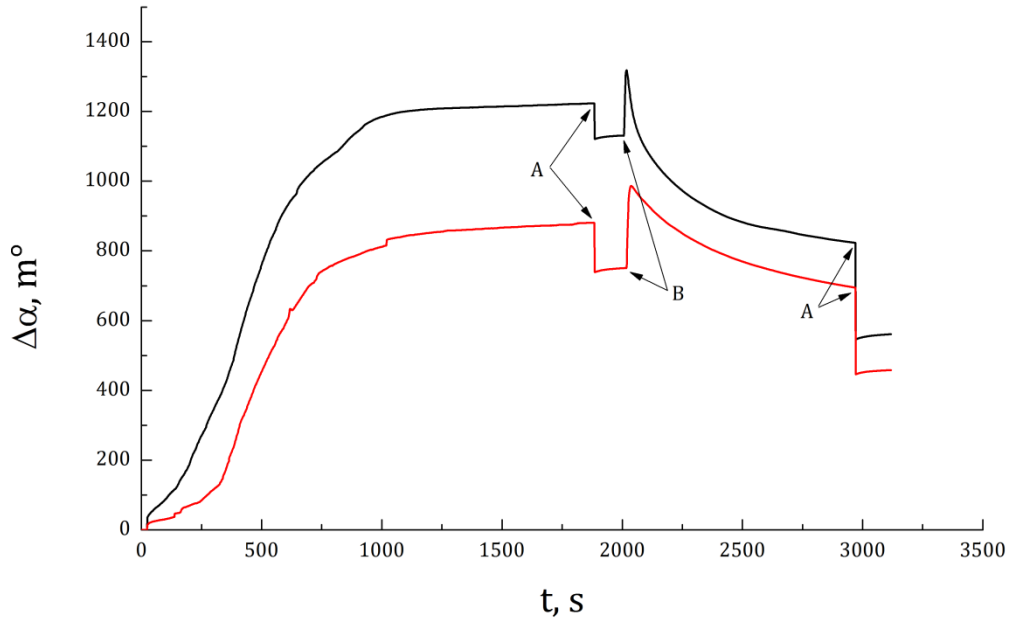

Figure 3. *SPR sensograms of lipid layers formation on βME monolayers from various MLV compositions: black curve – DOPC:Chol 60%:40%; red curve –DOPC:Chol 80%:20% preparation. A – washing, B – injection of MβCD. Total lipid concentration in MLV – 1 mM.*

Figure 3 presents SPR results of the bilayer's formation by fusion of DOPC and Chol MVL on hydrophilic βME. After first washing (Figure 3, A arrows) SPR angle changes by: $\Delta\alpha_{DOPC:Chol(20\%)}$ = 750 m° and $\Delta\alpha_{DOPC:Chol(40\%)}$ = 1100 m°. It is not clear why these differences appear. Accordingly, latterly MβCD solution added to the SPR cell (Figure 3, B arrows). This is a cyclic oligosaccharide, which can extract cholesterol from lipid membrane [30]. After cholesterol depletion SPR angle decreases by $\Delta\alpha_{DOPC:Chol(20\%)}$ = 450 m° and $\Delta\alpha_{DOPC:Chol(40\%)}$ = 560 m°, respectively (Figure 3, second washing).

It is known, that lipids can exist in the several crystalline phase, which occurs in lipid mixtures combining cholesterol with a phospholipid [31]. Taking this into account: DOPC:Chol (20%) membrane is in liquid disordered (L_D) phase, DOPC:Chol (40%) – liquid ordered (L_O) phase. The main difference between these two phases is, that in L_D phase DOPC molecule occupies 72.5 Å^2, and in L_O phase the area of DOPC depends on cholesterol content. Thus, in DOPC:Chol (20%) membrane surface coverage of DOPC

should be 76% (450 m°/590 m°), the remaining 24% corresponds to cholesterol. Which is equivalent to $1.19 \cdot 10^{14}$ Chol molecules (1 cm^2*0.24/40 $Å^2$) or S_{Chol} = 0.25 ng/(cm^2·m°). In L_O case, the area per DOPC molecule is unknown as it decreased. Due to known sensitivity of SPR, the actual amount of cholesterol can be determined. Hence, the surface filling by cholesterol in the lipid layers formed from DOPC:Chol (40%) MLV is ~44%. Currently, when the amount of DOPC molecules is known ($\Delta\alpha_{DOPC}$ = 550 m° and S_{DOPC} = 0.62 ng/(cm^2·m°)), the number of DOPC molecules can be divided by occupied surface area (56%) and the area of DOPC is determined A_{DOPC} = 43 $Å^2$. In the conclusion, the increasing of cholesterol amount in MLV affects not only cholesterol level in the lipid bilayer, but also structural parameters of the DOPC molecule itself [32].

3. Determination of the area per anchor compounds: WC14 and dHC18

The tethered bilayer lipid membranes consist of three types of molecules: a backfiller, an anchor compound and a lipid (Figure 4). The anchor compound is a lipid analog, where hydrophobic part is carbohydrate tails, hydrophylic part – ethylenoxide chain with sulfur in the end. Surface concentration of the anchor molecules is controled by the backfiller, which usually has ethylenoxide group and sulfur. Lower membrane leaflet is formed by the anchor compound and backfiller, which depending of the quantity of the anchor molecules may also be a lipid. Upper membrane leaflet consists of a lipid. So, the main objective of this part – to determinate a distribution of the anchor and backfiller molecules on the surface, and an area of per molecule of the anchor compound.

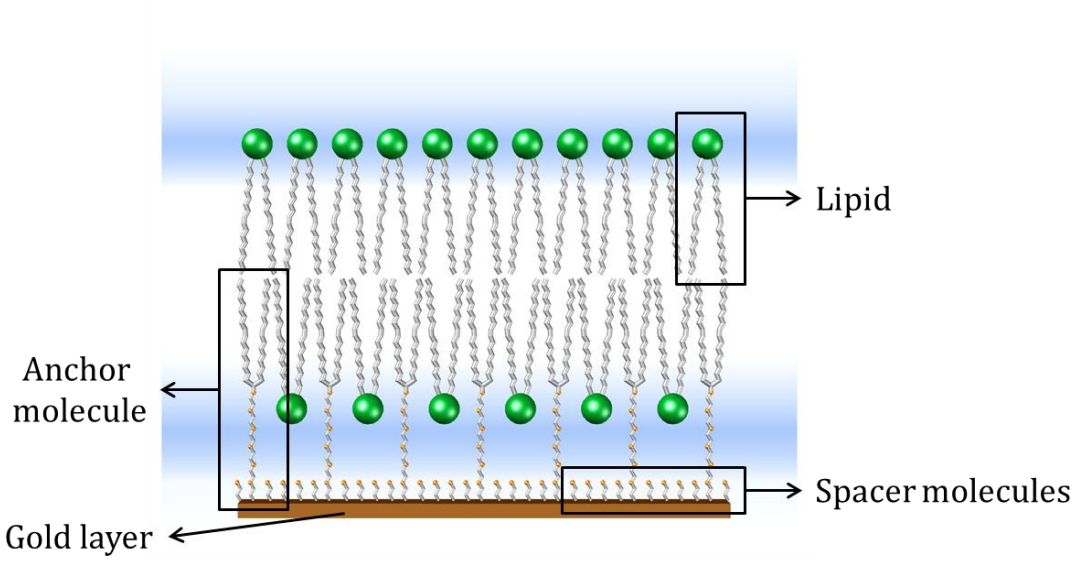

Figure 4. *tBLM structure.*

3.1 Thiol part

The SPR cell with coated Au wafers was filled with ethanolic solutions of the pure tether compound WC14 or various mole fraction mixtures of WC14 and βME (c_{total} = 0.1 mM), expressed here after as x %, where x is

the mole fraction of the tether compound multiplied by 100 %. Figure 5 presents the formation of mixed self-assembled monolayers by SPR sensograms. As can be seen, the SPR angle tends to change in the depend manner to the tether compound concentration. Although WC14 is a thiol, but in this case TSC algorithm is not applicable, as an area per tether compound and the fraction occupied by βME molecules are unknown. For that reason, firstly, the monolayer of WC14 (100%) molecules will be analyzed.

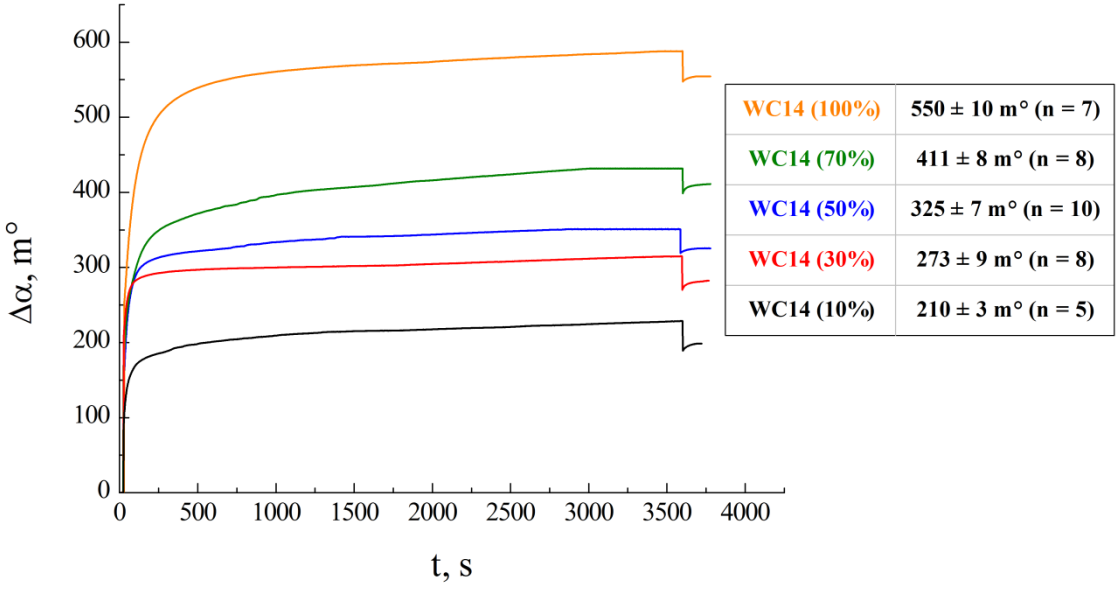

***Figure 5**. SPR sensograms of mixed self - assembled monolayers from various WC14 (x%) composition solutions in ethanol. Total thiol concentration in solution – 0.1 mM.*

SPR data can be represented as summarized data of each investigative object's layer: if object has k layers, the sum of each SPR angle shift $\Delta\alpha_k$ gives a total – $\Delta\alpha$. Hereby, WC14 monolayer can be analyzed: first, it is divided into two groups upper part – hydrophobic tails of a tether compound and lower part – hydrophilic ethylenoxide group of the tether compound. As tails are very similar to the ODT molecules and occupy the same area, it means that $2.22 \cdot 10^{14}$ WC14 molecules (because one WC14 molecule has two tails) are on 1 cm^2 surface. If partial molecular weight of this compound part is 467 g/mol, then Δm = 171 ng/cm^2. In ODT experiment the SPR sensitivity of thiol material was determined and $\Delta\alpha_{tails}$ – 340 m°. The

remaining 210 m° should belong to hydrophilic ethylenoxide group. This group consists of 6 ethylenoxide (EO) fragments and one sulfur group in the end. However, this group can be splitted into 5 EO fragments and 1 EO fragment with attached sulfur. The latter part becomes similar to βME (**βME$_{WC14}$**). It follows that there are $2.22 \cdot 10^{14}$ **βME$_{WC14}$** molecules and then $\Delta\alpha_{\beta MEWC14}$ – 55 m° (full monolayer of βME has $2.22 \cdot 10^{14}$ molecules and $\Delta\alpha_{\beta ME}$ = 110 m°). The remaining angle of SPR (155 m°) belongs to 5 ethylenoxide fragments. Finally, SPR sensitivity of anchors was determined – 0.51 ng/(cm^2·m°).

Therefore, in all SAM mixtures the full coverage of the first monolayer will consist of 1 EO fragments, which correspond to a full monolayer of βME, $\Delta\alpha_{\beta ME}$ = 110 m°. In addition, if this value subtracted from the total SPR angle in each mixed SAM, then the remaining value will belong only to tethered compound without **βME$_{WC14}$** fragment, which can be calculated in the same way as in ODT case. Equations:

$$N_{ink} = \frac{(\Delta\alpha - \Delta\alpha_{skied}) \cdot S}{M} \cdot N_A \qquad (2)$$

$$N_{skied} = N - N_{ink} \qquad (3)$$

where $\Delta\alpha$ – total SPR angle change, $\Delta\alpha_{skied.}$ – SPR angle change of a full monolayer of the backfiller, M – molecular weight of tether compound without backfiller's fragment, S – SPR sensitivity to tethered compound, N_A – Avogadro's number, N – a number of backfiller molecules in full monolayer. Thus, to determine each mixed monolayer actual composition the following parameters will be used: $\Delta\alpha_{\beta ME}$ – 110 m°; M – 687 g/mol; S – 0.51 ng/(cm^2·m°) ir N – $4.65 \cdot 10^{14}$ cm^{-2} and 2 and 3 equations (2 table).

2 table. Tether compound WC14 and backfiller βME surface concentration dependence of the volumetric concentration

C_{WC14}, %	WC14		βME	
	N_{ink}, mol. sk.·10^{14}	N_{ink}, %	N_{skied}, mol. sk. ·10^{14}	N_{skied}, %
10	0,447	9,6	4,2	90,4
30	0,729	15,7	3,92	84,3
50	0,961	20,7	3,69	79,3
70	1,346	28,9	3,3	71,1
100*	2,22	47,7	2,43	52,3

3.2 Lipid part

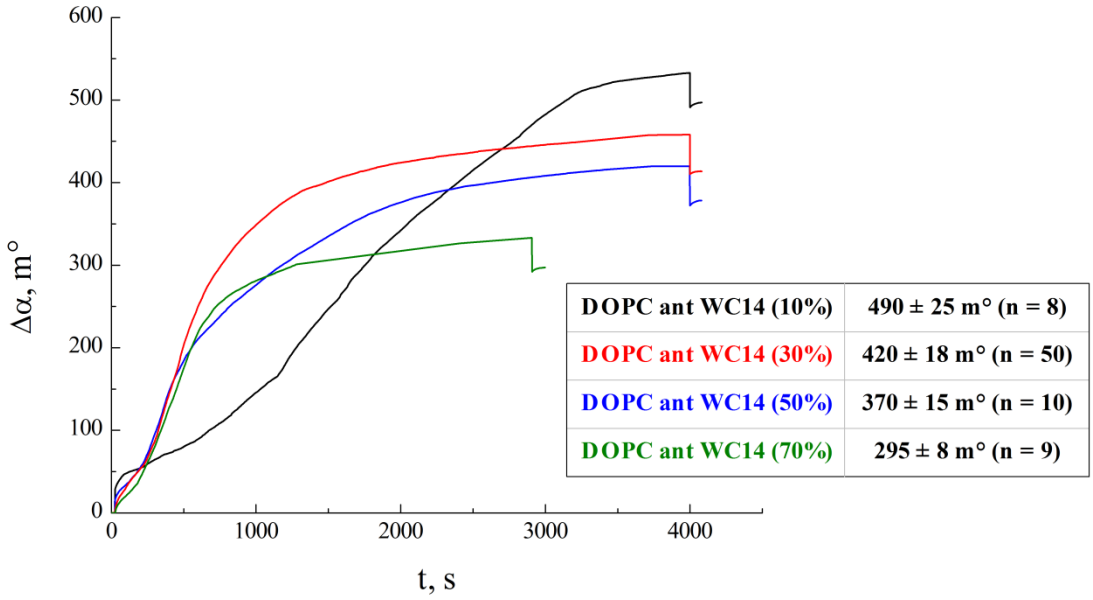

***Figure 6**. SPR sensograms of DOPC tBLMs formation on various WC14/βME SAMs. Total lipid concentration in MLV – 1 mM.*

Vesicle fusion directly observed by SPR. The addition of DOPC MLV triggered immediately the increase of SPR signal (Figure 6). The angle of the SPR signal variation was dependent on the composition of the mixed WC14/βME SAMs. The calculated coefficient for the SPR shift allows to quantitatively estimate the transferred amount of phospholipid material from liposomes to the surface. In particular, the density of the phospholipid layer proximal to a surface and the completeness of the tBLMs can be estimated. Figure 6 indicates that the SPR angle variation during vesicle fusion is inversely proportional to the density of the anchor SAM. The fraction of the transferable DOPC in the proximal leaflet of the tBLMs can estimated. Assuming that the

290 m° SPR angle shift corresponds to a formation of a distal monolayer of tBLMs, the rest of the SPR angle shift measures the amount of DOPC located in the proximal leaflet. Then the amount of the DOPC transferred to the proximal leaflet can be calculated from the difference between total SPR shift less 290 m°.

For example, WC14 (30%) has 420 m° lipids, where distal monolayer has 295 m° and rest of the SPR angle shift measures the amount of DOPC located in the proximal leaflet (125 m°). In this case, the proximal leaflet would fill 43% of the surface (125 m°/295 m° * 100% = 43%). Therefore, anchor molecules (WC14) would occupy remaining part of surface (57%). From the previous measurement is known, that this mixed SAM has $7.29 \cdot 10^{13}$ anchor molecules, while an area per tether compound is ~ 78 Å^2 (1 cm^2 * 57% / $7.29 \cdot 10^{13}$). The same procedure was accomplished with other mix SAM and data is displayed in Table 3.

Table 3. Molar fractions of anchors WC14 and lipids in tBLMs

C_{WC14}, %	A_{WC14}, Å^2	N_{WC14}, 10^{14} cm^{-2}
10%	78	0.447
30%	78	0.729
50%	78	0.961
70%	74	1.346
100%	45	2.22

The same measurements and calculations were carried out with another tether anchor compound, HC18 (Table 4).

Table 4. Molar fractions of anchors dHC18 and lipids in tBLMs

C_{HC18}, %	A_{HC18}, Å^2	N_{HC18}, $\cdot 10^{14}$ cm^{-2}
10%	103	0.348
40%	103	0.637
70%	103	0.85
100%	60	1.672

The peculiarities of tBLM structure. From experimental data it has been identified, that $A_{WC14(100\%)}$ = 45 Å^2 and $A_{WC14(30\%)}$ = 78 Å^2. Moreover, as can be seen, A_{WC14} (100% -> 30%) increases ~ 1.75 time. The same result is

obtained with dHC18 compound, where $A_{dHC18(100\%)}$ = 60 Å^2 and $A_{dHC18(40\%)}$ = 105 Å^2 and their ratio ~ 1.75. So, the question arises: why do an area of both compounds (WC14 and dHC18) increas 1.75 time in the forming process of lipid layer?

Firsts, it needs to focus attention to the fact, that the changes of the area per anchor compounds occurs, when interfering lipids are included. Thus, there is the lower membrane leaflet composed of lipids and anchor molecules. Both compounds have two parts: hydrophilic and hydrophobic. Hydrophobic part is the same – both have hydrocarbons chains. There is a different situation in the case of hydrophilic part: a lipid has phosphocholine group, the anchor compound - ethylene oxide chain. It could be, that between phosphocholine group and ethylene oxide repulsive forces are dominative, because both molecules are "not enough" hydrophilic and have different hydrophilic degrees. Thus, the distance between two anchor molecules should be sufficient (Figure 7, distance B) and wider than an area of the anchor molecule, that the incorporated lipid could find room among them.

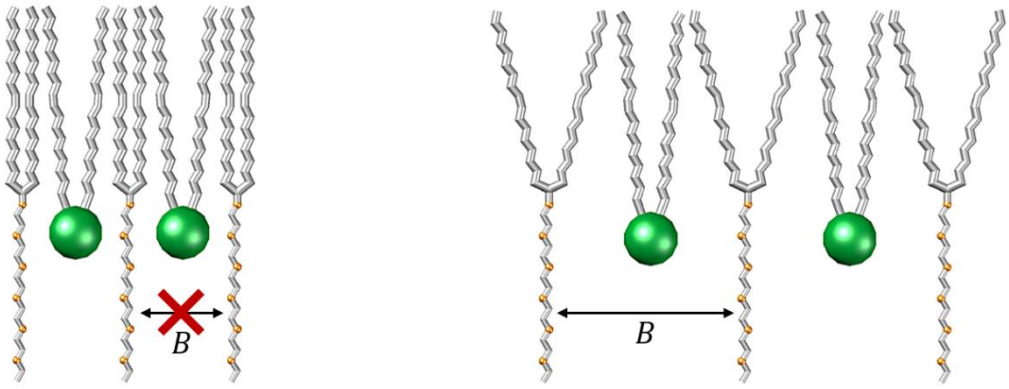

Figure 7. *The influence of lipids on the anchor molecules.*

4. Determination and control of cholesterol level in the lipid layers

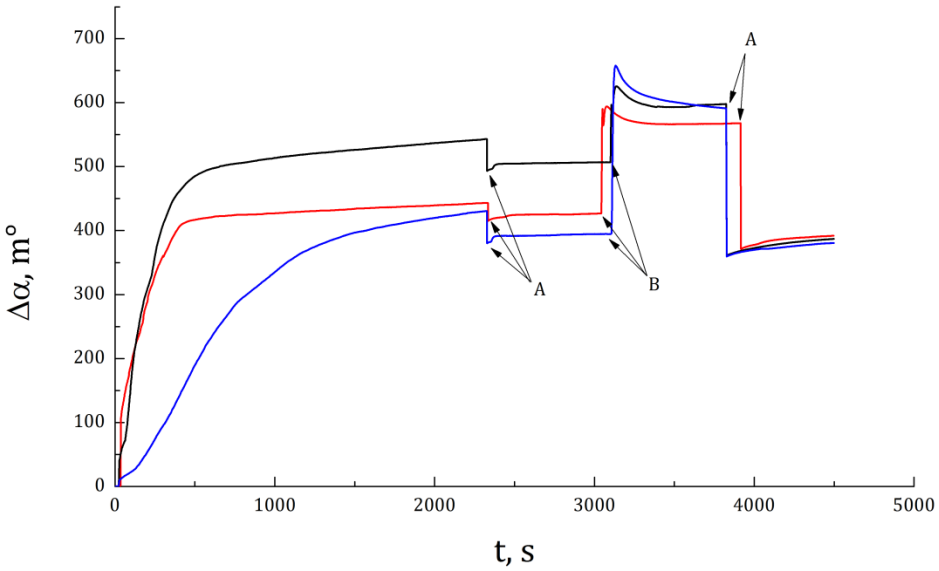

***Figure 8**. SPR sensograms of tBLM formation on dHC18 (30%) SAMs from various MLV composition: blue line – DOPC, red line – DOPC:Chol 80%:20%, black line – DOPC:Chol 60%:40%. Total lipid concentration in MLV – 1 mM. A – washing, B – MβCD injection (c = 2 mM).*

Figure 8 displays SPR sensograms of the formation of bilayer by fusion of various MLV compositions on dHC18 (30%) surfaces. It shows, that the total SPR angle change between MLV compositions ($\Delta\alpha_{DOPC:Chol\,(40\%)}$ = 520 m°, $\Delta\alpha_{DOPC:Chol\,(20\%)}$ = 440 m°) differs less than on βME surface ($\Delta\alpha_{DOPC:Chol\,(40\%)}$ = 1100 m°, $\Delta\alpha_{DOPC:Chol\,(20\%)}$ = 750 m°). And after MβCD injection SPR angle decays only $\Delta\alpha_{DOPC:Chol\,(40\%)}$ = 100 m° and $\Delta\alpha_{DOPC:Chol\,(20\%)}$ = 50 m°, respectively (Figure 8, second washing). If in the sBLM, the ratio of cholesterol and DOPC was 0.4, here the ratio decreases to 0.2. The reason of this, probably, that not all cholesterol molecules transferred from MLV into the surface due to unknown properties of the mixed SAM.

Thus, to load intentionally more cholesterol into membrane MβCD – Chol complex was used [33,34]. This complex was injected into bilayer, which was formed only from DOPC MLV. In figure 9 it shown, that loaded cholesterol amount is inversely proportional to complex concentration in the measurement cell. The hypothesis is that cholesterol and metil – β – cyclodextrin are in the equilibrium [35]:

$$2M\beta CD + Chol \overset{K}{\leftrightarrow} [M\beta CD_2 - Chol]$$

This reaction equation shows that the higher concentration of MβCD, the more complex may occur. Hence, the lower concentration of the complex (c ⩽ 1 mM) is injected (concentration of stock solution – 20 mM), the higher amount of cholesterol transferred to lipid membrane due to shifting of equilibrium to the left.

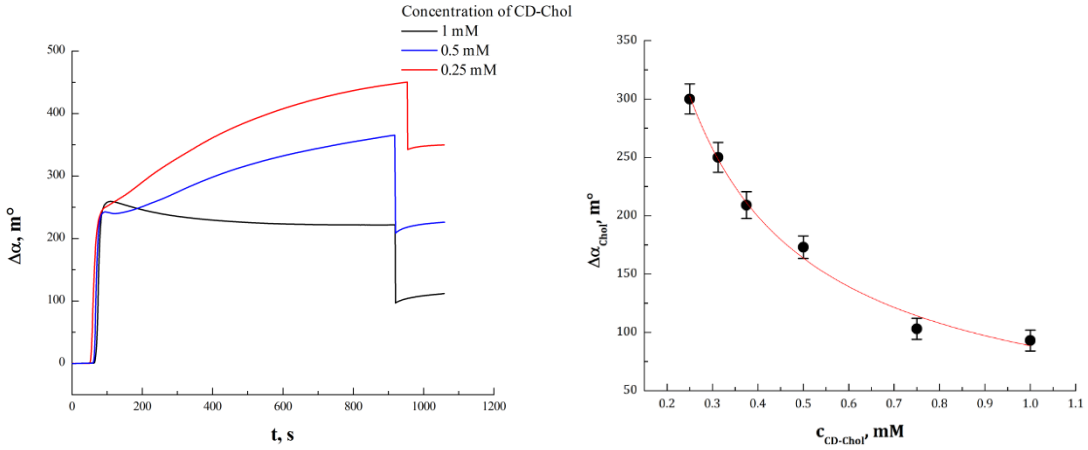

Figure 9. *Left: SPR sensograms of loading of cholesterol to tBLMs (DOPC) from MβCD – Chol complex. Right: the dependence of loading cholesterol on the concentration of MβCD – Chol complex.*

5. The investigation of pore forming toxins interaction with membranes

The cholesterol – dependent cytolysins (CDCs) constitute a large family of pore – forming toxins that functioning exclusively on a cholesterol containing membranes. The CDC cytolytic mechanism requires the presence of cholesterol in the membrane and cholesterol has been considered to be a receptor for the CDCs. In this study, two members of this family were examined, pneumolysin (PLY) and vaginolysin (VLY), in particular, their ability to reconstitute into tBLMs. Both proteins are toxins that incorporate spontaneously into cholesterol containing membranes by the formation of large β-barrel pores. Their functional reconstitution into the bilayer should lead to a drastic decrease in the membrane resistance. Therefore, it should be easily monitored by EIS and SPR techniques.

5.1 The interaction of pneumolysin with tBLM

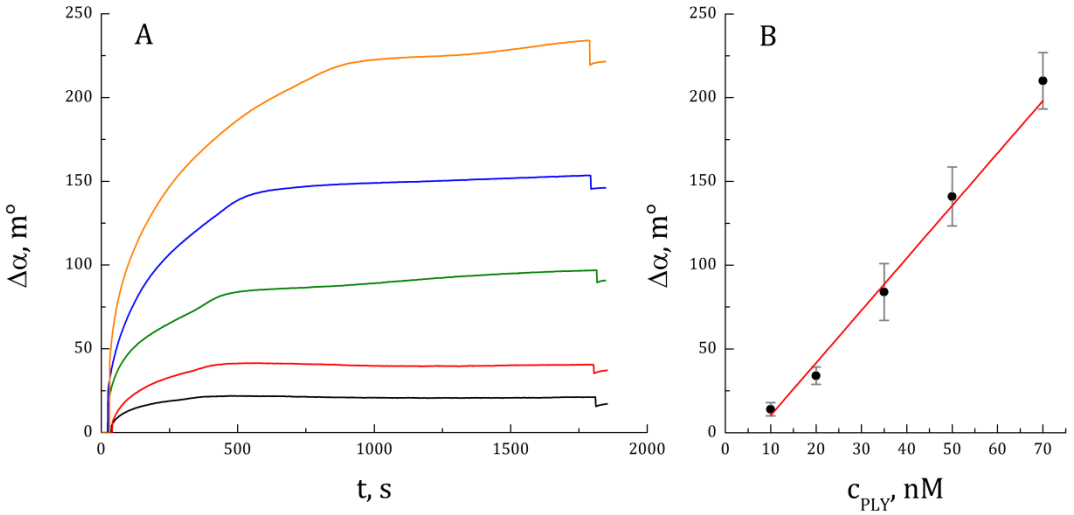

Figure 10. *SPR sensograms of PLY adsorption on tBLM, formated from DOPC:Chol 60mol%:40mol% MLV on mixed SAM (dHC18:dβME = 30% : 70%), as a function of PLY concentration in the measurement cell: black line – 10 nM, red line – 20 nM, green line – 35 nM, blue line – 50 nM and yellow line – 70 nM.*

Figure 10 presents SPR sensograms of PLY adsorption on the lipid surface. As can be seen, the amount of protein on the surface depends on the protein concentration in the cell. In addition, this dependence described by following equation:

$$\Delta\alpha_{PLY} = 3{,}7\, c_{PLY} - 21 \qquad (4)$$

However, from this equation the number of inserted and formed pores into membrane cannot be determined. Thus, another method is applicable – electrochemical impedance spectroscopy (EIS), which can show, how lipid membrane is damaged by pore forming toxin. Figure 11 shows the typical EIS spectra of PLY effect on tBLMs. Bode diagrams indicate, as expected, a decrease in the membrane admittance (Y) at low frequencies and continuous shift of the phase minimum with increase of PLY concentration.

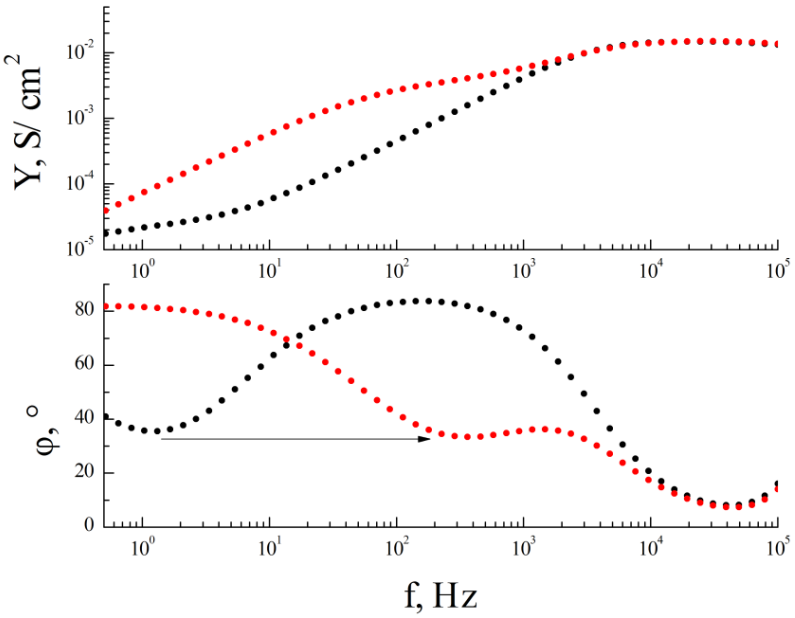

Figure 11. *Bode plot of conducting tBLM: black – initial curve, red – 30 min after PLY of 10 nM injected. tBLM from DOPC:Chol 60%:40% MLV on mixed SAM (dHC18:dβME = 30% : 70%)*

Data on Figure 11 show the dislocation of the phase minimum f_{min} (from low frequencies to high frequencies) before and after membrane damage. The movement of f_{min} can be associated with the numbers of toxin pores determined by equation [36]:

$$\lg(N_{def}) = 0{,}8644 \lg(f_{min}) - 1{,}142 \qquad (5)$$

Hence, by using this equation and EIS data the amount of "active" proteins (n_{PLY}) in the membrane calculated by:

$$n_{PLY} = \frac{m_{EIS}}{m_{SPR}} \times 100\% \qquad (6)$$

where m_{SPR} – adsorbed protein from SPR data, m_{EIS} – protein, which constituted into the pore in the membrane. Data are displayed in Figure 12 on the right axis. Thus, the detection limit of EIS method is up to 50 ng of protein (maximum changes of phase minimum is 3000 Hz and n > 80 %), while SPR detects higher amounts of material on the surface.

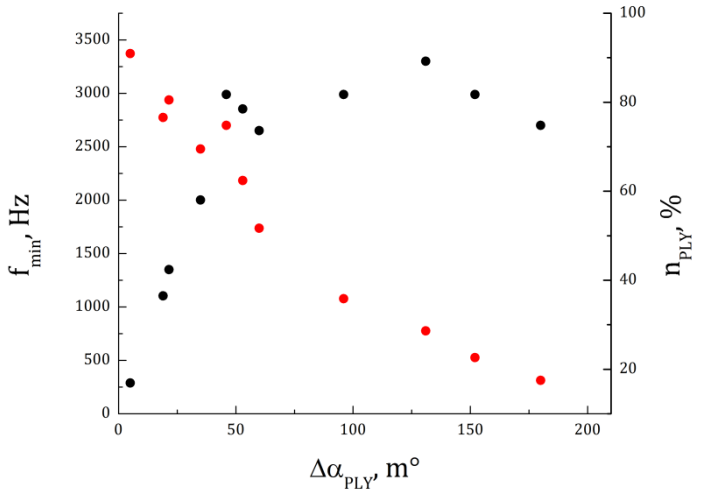

Figure 12. *f_{min} (left axis and black dots) and „active" proteins (right axis and red dots) dependence of PLY amount on the lipid surface. tBLM from DOPC:Chol 60%:40% MLV fusion on mixed SAM (dHC18:dβME = 30% : 70%)*

Another aim of this part was to investigate the relationship between protein amount and cholesterol level in the membrane. The cholesterol level was controlled by MβCD – Chol complex and PLY concentration was kept in the cell steady – 50 nM. Thus, in Figure 13 presented data show, that the amount of adsorbed protein is not proportional to cholesterol level in the membrane. Moreover, protein monomer binds to the lipid surface, then $\Delta\alpha_{Chol}$ > 80 m°. However, another important point, that further cholesterol razing almost has no effect to amount of bounded protein.

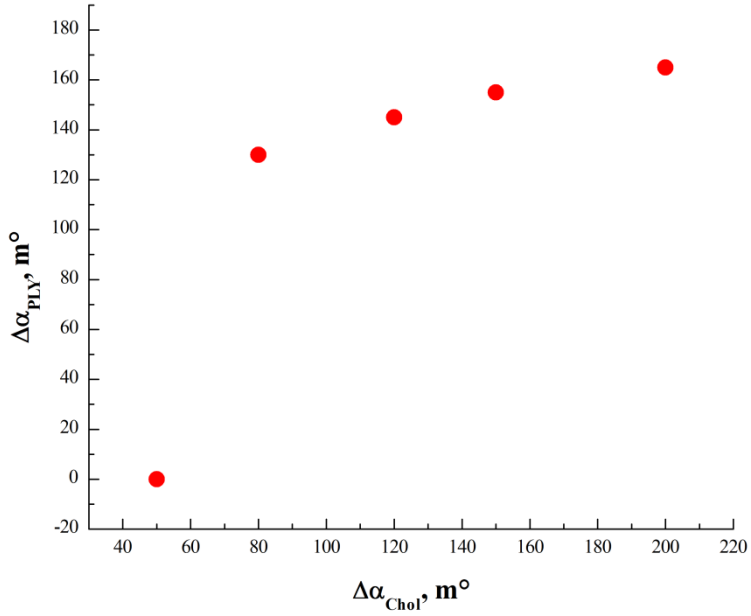

Figure 13. *The dependence of pneumolysin absorption to cholesterol amount in the lipid layer.*

5.2 The interaction of vaginolysin with tBLM

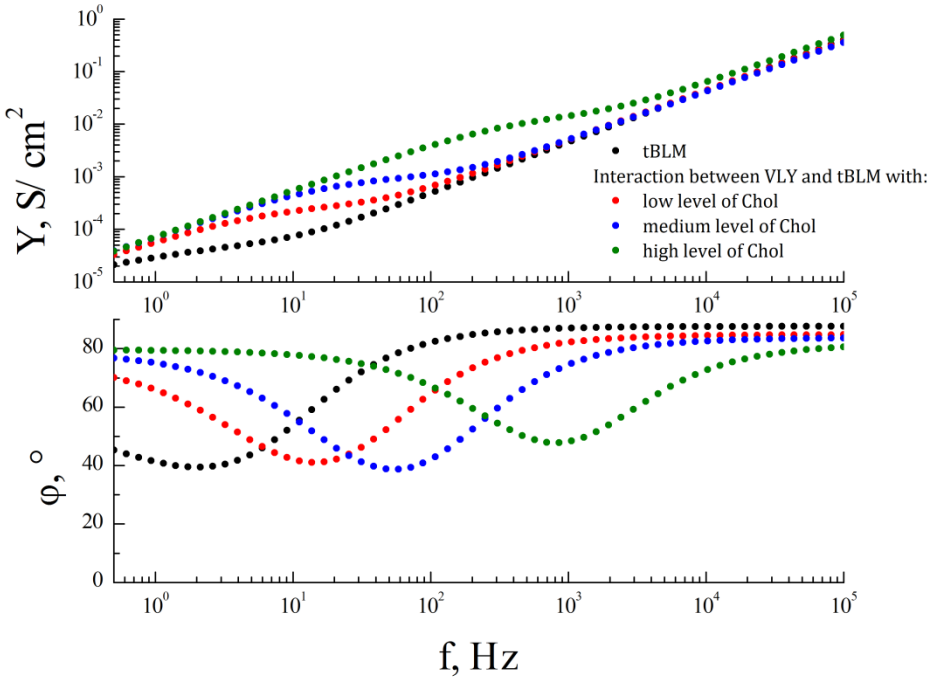

Figure 14. *The modulus and phase angle of EIS spectra of tBLM after VLY (c = 10 nM) injection. tBLM was formed by DOPC MLV and modified by MβCD – Chol complex in various cholesterol level on mixed SAM (dHC18 (40%): red dot – low level, blue dot – medium level, green dot – high level of cholesterol. The spectra were measured 30 min after the addition of VLY to final concentration.*

The interaction between lipid membrane and VLY is not visible in the DOPC - Chol 40% membrane due to low cholesterol level. Thus, the amount of cholesterol was controlled by MβCD – Chol complex. For convenience, the lipid layers with cholesterol were divided into three levels depending on the calculated amount of cholesterol in the membrane: low level ($\Delta\alpha_{Chol}$ = 130 – 180 m°), medium level ($\Delta\alpha_{Chol}$ = 190 – 250 m°) and high level ($\Delta\alpha_{Chol}$ = 270 – 320 m°). The effect of reconstitution of VLY (c = 10 nM) into the phospholipid membrane is shown in Figure 14, the toxin action is readily seen from the changes of EIS plots. In Bode plot, it shown, that the number of defects depends on the amount of cholesterol in the membrane: the higher content of cholesterol, the greater change of phase minimum in the frequency range.

Moreover, results obtained with SPR are more complicated. Figure 15 shows how the amount of protein is changing depending on cholesterol level. Still, these results do not correlate with EIS data. $\Delta\alpha_{VLY}$ value is lower in the membrane with high cholesterol level than in low or medium cholesterol level, though it should be vice versa. The explanation of this mismatched result is the hypothesis, that protein, in order to form a pore, have to displace lipids. As known, that lipids in membrane can change own area, hence, while cholesterol is loading into membrane, the occupied area of DOPC molecule is decreasing. In the case of membrane with high cholesterol level, due to dense cohesion, there is no space to insert additional material. In other words, for VLY to form pore and to get more room some lipids have to be force out.

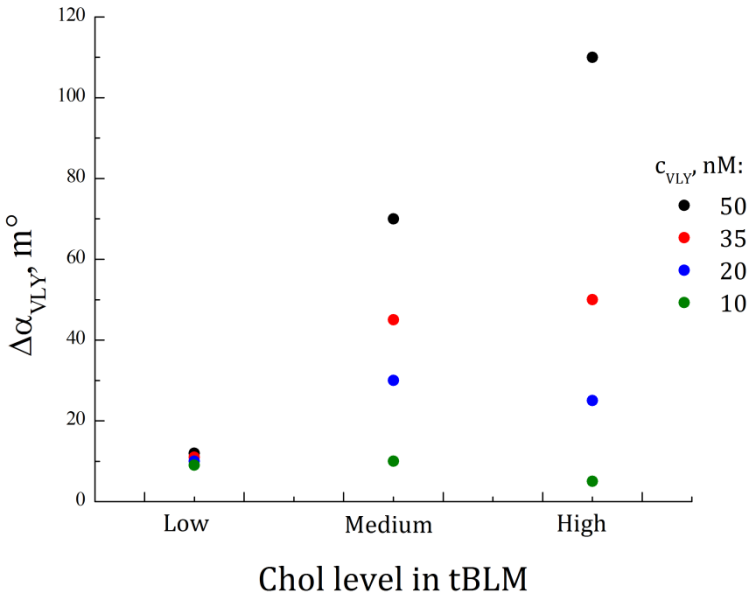

Figure 15. *The dependence of vaginolysin absorption on the level of cholesterol level in the membrane.*

CONCLUSIONS

1. The new method of lipid layer formation – by fusion of multilamellar liposomes was developed.
2. The sensitivity of surface plasmon resonance (SPR) to lipids, cholesterols and thiols was determined: S_{lipid} = 0.62 ng/(cm²·m°); S_{Chol} = 0.25 ng/(cm²·m°); S_{tether} = 0,25 ng/(cm²·m°), respectively.
3. The area of anchor molecules WC14 and dHC18 was determined, in presence of unsaturated phospholipid: A_{WC14} = 78 Å²; A_{dHC18} = 105 Å².
4. A) If the ratio of cholesterol/non – saturated phospholipid in multilamellar lipid liposomes is 0.4, then in tethered lipid bilayer is 0.2.
   B) Loaded cholesterol amount is inversely proportional to the concentration of metil – β – cyclodextrin and cholesterol complex in the measurement cell.
5. A) The detection limit of defects (from pore forming toxins) by electrochemical impedance spectroscopy method depends on concentration of the tether compound on the surface, while SPR detects higher amounts of adsorbed material on the surface. However, the limitation of SPR is analyzing of interaction of pore forming toxins with densely packed lipid membranes (with high level of cholesterol).
   B) Pneumolysin monomer binds to the lipid surface, when there is 15% cholesterol in the membrane, vaginolysin – 30% cholesterol, moreover further cholesterol razing has effect only to the bounded amount of vaginolysin.

List of publications

1. Rakovska, B., Ragaliauskas, T., Mickevicius, M., Jankunec, M., Niaura, G., Vanderah, D. J., & Valincius, G. (2015). ***Structure and Function of the Membrane Anchoring Self-Assembled Monolayers***. *Langmuir*, *31*(2), 846-857.

2. Ragaliauskas, T., Mickevicius, M., Budvytyte, R., Niaura, G., Carbonnier, B., & Valincius, G. (2014). ***Adsorption of β-amyloid oligomers on octadecanethiol monolayers.*** *Journal of colloid and interface science*, *425*, 159-167.

3. Mindaugas Mickevicius, Tadas Ragaliauskas, Bozena Rakovska, Tadas Penkauskas, David J. Vanderah, Frank Heinrich and Gintaras Valincius. (2017). ***Fast formation of low-defect-density tethered bilayers by fusion of multilamellar vesicles***. *BBA Biomembranes. Accepted manuscript*.

List of patents

1. Mindaugas Mickevicius, Tadas Ragaliauskas, David J. Vanderah and Gintaras Valincius. ***Paviršiuje imobilizuotų fosfolipidinių bisluoksnių membranų (tBLM) gavimo būdas.*** Application number: 2015 080. Application: Vilniaus universitetas. Legal status: application published.

REFERENCES

1. Mueller P, Rudin DO, Tien HT, Wescott WC. ***Reconstitution of cell membrane structure in vitro and its transformation into an excitable system***. *Nature*. 1962;194(4832):979-980. doi:10.1038/194979a0.

2. Brian AA, McConnell HM. ***Allogeneic stumulation of cytotoxic T cells by supported planar membranes***. *Proc Natl Acad Sci USA*. 1984;81(October):6159-6163.

3. Tamm LK, McConnell HM. ***Supported phospholipid bilayers***. *Biophys J*. 1985;47(1):105-113. doi:10.1016/S0006-3495(85)83882-0.

4. Lang H, Duschl C, Grätzel M, Vogel H. ***Self-assembly of thiolipid molecular layers on gold surfaces: optical and electrochemical characterization***. *Thin Solid Films*. 1992;210-211:818-821. doi:10.1016/0040-6090(92)90413-6.

5. Plant AL. ***Supported Hybrid Bilayer Membranes as Rugged Cell Membrane Mimics***. *Langmuir*. 1999;15(15):5128-5135. doi:10.1021/la981662t.

6. Plant a L. ***Self-Assembled Phospholipid Alkanethiol Biomimetic Bilayers on Gold***. *Langmuir*. 1993;9(11):2764-2767. doi:Doi 10.1021/La00035a004.

7. Bangham AD, Hill MW, Miller NGA. *Methods in Membrane Biology*. (Korn ED, ed.). Boston, MA: Springer US; 1974. doi:10.1007/978-1-4615-7422-4.

8. Raguse B, Braach-Maksvytis V, Cornell BA, et al. ***Tethered lipid bilayer membranes: formation and ionic reservoir characterization***. *Langmuir*. 1998;14(3):648-659. doi:10.1021/la9711239.

9. Lang H, Duschl C, Vogel H. ***A new class of thiolipids for the attachment of lipid bilayers on gold surfaces***. *Langmuir*. 1994;10(1):197-210. doi:10.1021/la00013a029.

10. He L, Robertson JWF, Li J, et al. ***Tethered bilayer lipid membranes based on monolayers of thiolipids mixed with a complementary dilution molecule 1 Incorporation of channel peptides***. *Langmuir*. 2005;21(25):11666-11672. doi:10.1021/la051771p.

11. Achalkumar AS, Bushby RJ, Evans SD, et al. ***Cholesterol-based anchors and tethers for phospholipid bilayers and for model biological membranes***. *Soft Matter*. 2010;6(24):6036. doi:10.1039/c0sm00030b.

12. Atanasov V, Knorr N, Duran RS, et al. ***Membrane on a Chip: A Functional Tethered Lipid Bilayer Membrane on Silicon Oxide Surfaces***. *Biophys J*. 2005;89(3):1780-1788. doi:10.1529/biophysj.105.061374.

13. Atanasov V, Atanasova PP, Vockenroth IK, Knorr N, Köper I. ***A molecular toolkit for highly insulating tethered bilayer lipid membranes on various substrates***. *Bioconjug Chem*. 2006;17(3):631-637. doi:10.1021/bc050328n.

14. Lin W-C, Blanchette CD, Ratto T V, Longo ML. ***Lipid asymmetry in***

DLPC/DSPC-supported lipid bilayers: a combined AFM and fluorescence microscopy study. *Biophys J*. 2006;90(1):228-237. doi:10.1529/biophysj.105.067066.

15. Cho N-J, Frank CW, Kasemo B, Höök F. ***Quartz crystal microbalance with dissipation monitoring of supported lipid bilayers on various substrates***. *Nat Protoc*. 2010;5(6):1096-1106. doi:10.1038/nprot.2010.65.

16. Koenig BW, Krueger S, Orts WJ, et al. ***Neutron Reflectivity and Atomic Force Microscopy Studies of a Lipid Bilayer in Water Adsorbed to the Surface of a Silicon Single Crystal***. *Langmuir*. 1996;12(5):1343-1350. doi:10.1021/la950580r.

17. Giess F, Friedrich MG, Heberle J, Naumann RL, Knoll W. ***The protein-tethered lipid bilayer: a novel mimic of the biological membrane***. *Biophys J*. 2004;87(5):3213-3220. doi:10.1529/biophysj.104.046169.

18. Boulnois GJ, Paton JC, Mitchell TJ, Andrew PW. ***Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of Streptococcus pneumoniae***. *Mol Microbiol*. 1991;5(11):2611-2616. doi:10.1111/j.1365-2958.1991.tb01969.x.

19. Gelber SE, Aguilar JL, Lewis KLT, Ratner AJ. ***Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from Gardnerella vaginalis***. *J Bacteriol*. 2008;190(11):3896-3903. doi:10.1128/JB.01965-07.

20. Cornell BA, Braach-Maksvytis VL, King LG, et al. ***A biosensor that uses ion-channel switches***. *Nature*. 1997;387(6633):580-583. doi:10.1038/42432.

21. Budvytyte R, Valincius G, Niaura G, et al. ***Structure and properties of tethered bilayer lipid membranes with unsaturated anchor molecules***. *Langmuir*. 2013;29(27):8645-8656. doi:10.1021/la401132c.

22. McGillivray DJ, Valincius G, Vanderah DJ, et al. ***Molecular-scale structural and functional characterization of sparsely tethered bilayer lipid membranes***. *Biointerphases*. 2007;2(1):21-33. doi:10.1116/1.2709308.

23. McConnell HM, Watts TH, Weis RM, Brian AA. ***Supported planar membranes in studies of cell-cell recognition in the immune system***. *BBA - Rev Biomembr*. 1986;864(1):95-106. doi:10.1016/0304-4157(86)90016-X.

24. Cubukcu E, Zhang S, Park Y-S, Bartal G, Zhang X. ***Split ring resonator sensors for infrared detection of single molecular monolayers***. *Appl Phys Lett*. 2009;95(4):43113. doi:10.1063/1.3194154.

25. De Feijter JA, Benjamins J, Veer FA. ***Ellipsometry as a tool to study the adsorption behavior of synthetic and biopolymers at the air???water interface***. *Biopolymers*. 1978;17(7):1759-1772. doi:10.1002/bip.1978.360170711.

26. Evans SD, Flynn TM, Ulman A. ***Self-Assembled Multilayer Formation on Predefined Templates***. *Langmuir*. 1995;11(10):3811-3814.

27. Milkani E, Khaing AM, Huang F, et al. ***Immobilization of acetylcholinesterase in lipid membranes deposited on self-assembled monolayers***. *Langmuir*. 2010;26(24):18884-18892. doi:10.1021/la103333c.

28. Tristram-Nagle S, Petrache HI, Nagle JF. ***Structure and interactions of fully hydrated dioleoylphosphatidylcholine bilayers***. *Biophys J*. 1998;75(2):917-925. doi:10.1016/S0006-3495(98)77580-0.

29. Erbe A, Sigel R. ***Tilt angle of lipid acyl chains in unilamellar vesicles determined by ellipsometric light scattering***. *Eur Phys J E Soft Matter*. 2007;22(4):303-309. doi:10.1140/epje/e2007-00038-5.

30. Szejtli J. ***Introduction and General Overview of Cyclodextrin Chemistry***. *Chem Rev*. 1998;98(97):1743-1753. doi:10.1021/CR970022C.

31. May S. ***Trans-monolayer coupling of fluid domains in lipid bilayers***. *Soft Matter*. 2009;5(17):3148. doi:10.1039/b901647c.

32. Alwarawrah M, Dai J, Huang J. ***A molecular view of the cholesterol condensing effect in DOPC lipid bilayers***. *J Phys Chem B*. 2010;114(22):7516-7523. doi:10.1021/jp101415g.

33. Visco I, Chiantia S, Schwille P. ***Asymmetric supported lipid bilayer formation via methyl-β-cyclodextrin mediated lipid exchange: influence of asymmetry on lipid dynamics and phase behavior***. *Langmuir*. 2014;30(25):7475-7484. doi:10.1021/la500468r.

34. Cheng H-T, Megha, London E. ***Preparation and properties of asymmetric vesicles that mimic cell membranes: effect upon lipid raft formation and transmembrane helix orientation***. *J Biol Chem*. 2009;284(10):6079-6092. doi:10.1074/jbc.M806077200.

35. Higuchi T., Connors KA. ***Phase-solubility techniques***. 1965;4(0):212-217.

36. Valincius G, Mickevicius M. ***Chapter Two – Tethered Phospholipid Bilayer Membranes: An Interpretation of the Electrochemical Impedance Response***. In: *Advances in Planar Lipid Bilayers and Liposomes*. Vol 21. ; 2015:27-61. doi:10.1016/bs.adplan.2015.01.003.

Lipidinių membranų imobilizavimas ant paviršiaus ir jų tyrimas paviršiaus plazmonų rezonanso metodu

Reziumė

Šioje disertacijoje pagrindinis dėmesys yra skiriamas ant kieto paviršiaus imobilizuotoms dirbtinėms fosfolipidinėms membranoms. Viena pagrindinių šių membranų pritaikymo sričių – sąveikos su įvairiais baltymais tyrimai, t.y. imobilizuotos fosfolipidinės membranos turėtų tarnauti kaip biojutikliai. Tačiau šių biojutiklių pritaikymo galimybės vis dar nėra pilnai žinomos, kur tenka susidurti su keliais iššūkiais: 1) šio objekto struktūros nustatymui šiuo metu taikomas brangus ir ne kiekvienoje laboratorijoje randamas neutronų reflektometrijos metodas; 2) pakankamai sudėtingas šių lipidinių struktūrų paruošimas. Taigi, šiame darbe buvo pritaikytas paprastesnis tyrimo metodas – paviršiaus plazmonų rezonansas (SPR), bei pademonstruota, kaip, taikant SPR metodą, galima nuodugniai ištirti šių biojutiklių sudėtį bei struktūrą. Taip pat pritaikytas paprastesnis dirbtinių membranų paruošimo metodas – daugiasluoksnės liposomos, kurių paruošimas nereikalauja daug laiko ir ypatingos technikos. Šiame darbe nemažas dėmesys skirtas ir vieno svarbiausio ląstelės membranose sutinkamo lipido – cholesterolio kiekio reguliavimui šiuose biojutikliuose, nes cholesterolis taip pat yra atsakingas ir už kai kurių baltymų sąveiką su membranomis. Tai ypatingai susiję su poras formuojančiais toksinais, kurie geba sudaryti įvairias pažaidas membranoje, taip sutrikdant ląstelės gyvavimo ciklą, kartais užsibaigiančią mirtimi. Ir kaip tik atskleidžiama, kaip cholesterolio kiekis įtakoja toksinų aktyvumą membranose.

Darbo išvados:

1) Sukurtas lipidinių dvisluoksnių prijungimo prie paviršiaus metodas, besiremiantis daugiasluoksnių liposomų suliejimu.

2) Nustatytas paviršiaus plazmonų rezonanso metodo jautrumas inkariniam junginiui, lipidui ir cholesteroliui, kurie atitinkamai lygūs: $S_{ink.\ j.} = 0{,}51$ ng/(cm2·m°); $S_{lipidų} = 0{,}62$ ng/(cm2·m°); $S_{chol} = 0{,}25$ ng/(cm2·m°)

3) Nustatytas inkarinių junginių užimamas plotas vidiniame prikabintos membranos sluoksnyje, kai tarp jų yra įsiterpusios nesotaus fosfatidilcholino molekulės: $A_{WC14} = 78$ Å^2; $A_{dHC18} = 105$ Å^2

4) A) Nustatyta, kad cholesterolio/nesotaus fosfatidilcholino santykis prikabintos membranos modelyje siekia tik 0,2, kai daugiasluoksnėse liposomose šis santykis yra 0,4.

B) Nustatyta, kad įkraunamo cholesterolio kiekis atvirkščiai proporcingas ciklodekstrino ir cholesterolio komplekso koncentracijai tirpale.

5) A) Buvo parodyta, kad membranos pažaidos masto detektavimas elektrocheminio impedanso spektroskopijos metodu priklauso nuo inkarinio junginio paviršinio tankio, kai paviršiaus plazmonų rezonanso metodu galima

detektuoti neribotą kiekį baltymo. Paviršiaus plazmonų rezonanso metodas netinkamas tirti nuo cholesterolio priklausomų toksinų sąveiką tankiose membranose.

B) Buvo parodyta, kad pneumolizino sąveika su membrana prasideda, kai membranoje yra 15% cholesterolio, vaginolizino – 30% cholesterolio.

Curriculum Vitae

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