CHEMIJA. 2022. Vol. 33. No. 4. P. 103–110

Formation of mixed hybrid bilayer lipid membrane on transparent thin film oxide electrodes

---

Inga Gabriūnaitė,

Eimantas Zolubas,

Aušra Valiūnienė*

Department of Physical Chemistry,
Faculty of Chemistry and Geosciences,
Vilnius University, 24 Naugarduko Street,
03225 Vilnius, Lithuania

In this work, we attempted to design phospholipid bilayers tethered to semiconducting metal oxide films. The phospholipid layers assembled on compact monocomponent self-assembled monolayers (SAM) on oxide films were accomplished earlier; however, previous studies indicated a poor ability of these layers to functionally reconstitute transmembrane proteins. Commercially available fluorine doped tin oxide (FTO) sheet glass was chosen as a model solid support. FTO surface was functionalised utilising by the mixture of the long and short chain hydrophobic silanes. Specifically, octadecyltrichlorosilane (OTS) and vinyltrimethoxysilane (VTS) were used to assemble mixed silane-based SAMs on FTO. Exposure of mixed SAMs to a solution of multilamellar vesicles of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 40% cholesterol triggered the changes of electrochemical parameters commensurate with the formation of additional dielectric layers of phospholipids.

Keywords: thin films, tin oxide, silane, self-assembled monolayer, electrochemical impedance spectroscopy

---

INTRODUCTION

The phospholipid bilayer surrounds every living cell providing compartmentalisation in living organisms. It ensures the control of material flow in and out of a cell. These membranes can be of diverse compositions, which in turn facilitate their specific functions. One such compositional branch can be investigated by creating the hybrid bilayer – a two-layer molecular system, which has a very compact, nearly crystalline hydrophobic layer proximal to a surface. To study membrane proteins, it is important to establish that the membrane has water containing space on its both sides. This structure imitates native environment for membrane proteins, therefore they could be integrated into the membrane bilayer and exhibit the same properties, as it would in any living cell. The distal layers in these systems may exhibit certain fluidity, thus mimicking the phospholipid bilayer in organisms. Usage of long anchoring molecules to form tethered bilayers offers this solution.

To assemble both hybrid and tethered bilayers various solid supports are used: sputtered Au and Ti/TiO_2[1–3], polished Ti [4], Si/SiO_2 [5], SnO_2 doped with either fluorine, cadmium or indium [6–8], aluminium [9, 10]. Most of these surfaces (except for SiO_2) exhibit a good electric conductance, thus providing application of the electrochemical techniques for protein membrane interaction studies. After vesicles fusion, the phospholipid membrane exhibits a low number of defect density [11], is sensitive to low concentrations of toxins [12] and could be applied for different protein immobilisation [8].

Long chain alkylsilanes were proposed for monolayer formation on oxide surfaces [13]. The head group of such material is composed of a Si atom with usually three halogens or alkoxy groups. During the hydrolysis reaction between silane and oxide surface strong Si–O–Sn bonds are

---

[*] Corresponding author. Email: ausra.valiuniene@chf.vu.lt

formed which contribute to robust SAM formation [13]. One important advantage of silanised oxide surfaces is suitability for multi-cycle hybrid bilayer lipid membrane formation and regeneration [4, 6, 14, 15], particularly due to the strength of the Si–O–Sn bond. In contrary, for the gold/alkylthiol system, the phospholipid bilayer could not be immobilised several times because a mixed monolayer rearranges to packed islands of long alkylchain molecules up to 72 h exposure to an aqueous solution [2].

Previously, we have shown that tin oxide substrate could be successfully silanised for phospholipid bilayer formation [6, 7] using octadecyltrichlorosilane SAM with a subsequent vesicle fusion and formation of 60 mol% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)/40 mol% cholesterol bilayer. However, such solid supported membranes exhibited hybrid bilayer properties, which precluded the reconstitution of transmembrane pore-forming proteins.

In the current study, following the methodology which was employed for the assembly of tethered bilayers on thin gold film supports [3], we raised the research question if sufficient fluidity of bilayers immobilised on the metal oxide surface can be achieved by designing the mixed hybrid phospholipid bilayer membrane (mhBLM) [14]. In particular, by designing the mixed self-assembled silane monolayers consisting of short chain spacers and long alkyl chain anchor molecules we expected that such anchor layer would allow the formation of tethered bilayer providing a sufficient flexibility of the proximal to a surface phospholipid monolayer for the functional reconstitution of transmembrane proteins. As an anchor molecule we have chosen commercially available silanes: octadecyltrichlorosilane (OTS) and backfilling molecules vinyltrimethoxysilane (VTS). Commercially available fluorine-doped tin oxide (FTO) was used as semiconductor metal oxide substrate. Hybrid systems consisting of a wide gap metal oxide semiconductor and biomimetic phospholipid bilayer may be an attractive combination in designing novel hybrid photovoltaic systems. Moreover, metal/metal oxide surfaces are promising substrates for tethered bilayer membrane formation due to its biocompatibility and reusability, therefore our investigations presented in this work could be of great importance in improving studies of phospholipid membranes immobilised on solid surfaces. The objective of the current study was to determine if surface constructs of phospholipid membranes immobilised on the FTO surface can be used for the functional reconstitution of transmembrane proteins.

EXPERIMENTAL

Preparation of FTO electrodes

FTO covered glass was used as a substrate. FTO sheets (300 × 300 × 2 mm) were purchased from Sigma-Aldrich and cut into smaller pieces (1 × 3 cm). Electrodes were ultrasonically washed for 10 min in each: (i) 2% Micro 90 cleaning solution (Sigma Aldrich), (ii) deionized water and (iii) 2-propanol (S-A). Afterwards, they were dried with nitrogen gas flow.

Functionalization of FTO electrodes

FTO was functionalised with OTS (Aldrich) and VTS (Alfa Aesar) silanes at different molar ratios while keeping the total concentration of 2.5 mM. Silanisation solution was prepared in heptane heated to 60°C. The FTO slide was immersed in a silanisation solution in a vertical position and incubated at 60°C for 60 min while mixing. Finally, the FTO slide was rinsed with clean heptane and dried in a stream of nitrogen.

Phospholipid bilayer formation

mhBLM was formed by the vesicles fusion method. The procedure of vesicles solution preparation was done according to the previously published papers [7, 11]. Briefly, the vesicle solution was made by mixing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Lipids Alabaster, USA) and cholesterol (Avanti Lipids Alabaster, USA) in molar ratio 6:4 in a phosphate buffer solution (PBS) pH 4.5 (0.1 M NaCl (Reachem Slovakia p. a.), 0.01 M NaH_2PO_4 (Reachem Slovakia, p. a.)) for a total concentration of 1 mM. It was injected on SAM during electrochemical measurements.

Contact angle measurements

Measurements were carried out using a Theta Lite optical tensiometer from Biolin Scientific (Finland). Contact angles of 5 μL water droplets were measured on the FTO surface at 10 different spots and then averaged.

Electrochemical measurements

Electrochemical impedance spectroscopy (EIS) measurements were recorded using either a Zennium electrochemical workstation (Zahner GmbH, Germany, frequency range 0.1 Hz – 100 kHz) or a fast Fourier transform (FFT) electrochemical impedance spectrometer EIS-128/16 (University of Kiel, Germany) constructed by G. Popkirov (frequency range 1.5 Hz – 50 kHz) [16, 17]. Cyclic voltammetry curves were recorded with a μautolab (Type III) potentiostat/galvanostat.

All electrochemical measurements took place in 21 ± 2°C PBS at pH 7.1, using a three electrode cell, which contains a platinum wire counter electrode, an Ag/AgCl, $NaCl_{sat}$ reference electrode, and an FTO glass slide of 0.32 cm^2 geometric surface area as a working electrode. All potentials given in this article are with reference to an Ag/AgCl, $NaCl_{sat}$ electrode.

RESULTS AND DISCUSSION

To attest for the hydrophobic properties of mixed self-assembled monolayers consisting of a spacer (VTS) and an anchoring unit (OTS) on FTO, surface wetting characteristics – contact angles of 5 μL water droplet on differently modified FTO surfaces – were measured. Before the silanisation procedure, the pristine FTO surface exhibits hydrophilic properties reaching a contact angle of 44° ± 8° (Fig. 1). After the silanisation with 100% OTS, CA values increased to 124° ± 4° (Fig. 1). However, the CA values decreased down to 79° ± 8° with increasing the VTS molar ratio in the silanisation solution (Fig. 1). Therefore, CA data showed that increasing short alkylchain VTS in the silanisation solution, decreases the contact angle on the silanised FTO surface accordingly to the spacer unit molar ratio in the silanisation solution. A similar tendency in CA evolution is observed on gold electrodes, using a mixture of a spacer unit and long alkylchain thiols [7]. The silanisation of FTO surface with OTS:VTS 4:6, 6:4, 8:2 and 10:0 molar ratios causes CA values to reach higher than 100° value. This result indicates that SAM is hydrophobic enough for a successful formation of the phospholipid membrane [18].

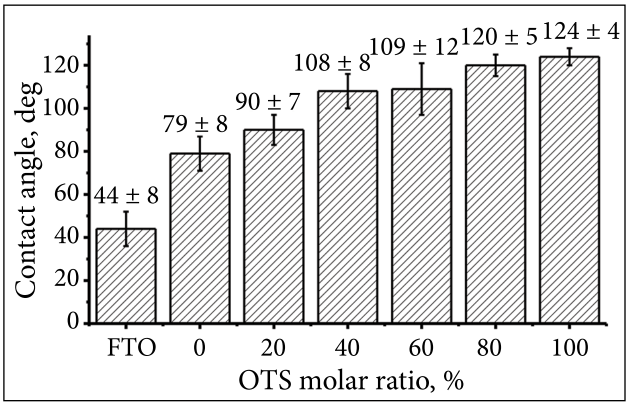

Fig. 1. Contact angle measurements of the mixed OTS:VTS SAM on the FTO electrode

Along with CA, the EIS method can be used to determine the heterogeneity of a mixed self-assembled monolayer as well as phospholipid bilayers on FTO (Fig. 2). EI spectra were presented in the Cole–Cole plot that shows capacitive features of the electrode: the radius of semicircle provides information about the double layer capacitance [19]. The semi-circular shape of the Cole–Cole plot shows that the surface of FTO exhibits a near ideal capacitive behaviour [20]. The capacitance of a bare FTO surface before the silanisation as established earlier is 8.8 ± 0.3 μF cm^{-2} [6]. It follows from the data in Fig. 2 (filled circles) that the silanisation of the FTO surface decreases the complex capacitance at all compositions of OTS:VTS, and the decrease of capacitance depends on the composition of SAM. With increasing the amount of OTS in the silanisation solution, consistently lower complex capacitance values are observed (Fig. 2, filled circles): from 6.9 ± 0.6 μF cm^{-2} (for OTS:VTS 0:10 molar ratio) to 3.4 ± 0.9 μF cm^{-2} (for OTS:VTS 10:0 molar ratio). Knowing that the obtained complex capacitance values from the Cole–Cole plot depend on the thickness of a dielectric layer, it can be concluded that by adjusting molar ratios of OTS (anchors) and VTS (backfillers) in the silanisation solution the thickness of SAM is appropriately adjusted, as well [21].

In the following experiments, the mixed hybrid bilayer lipid membrane was formed using the vesicles fusion method. The observed complex capacitance changes are dependent on anchor/backfiller molecular ratios (Fig. 2, open circles). Typical spectra for the bilayer formation signatures were observed on 0–20% backfiller SAMs (Fig. 2e, f). Upon further surface dilution of OTS by VTS (Fig. 2a–d), the complex capacitance curves exhibited marginal changes, even though

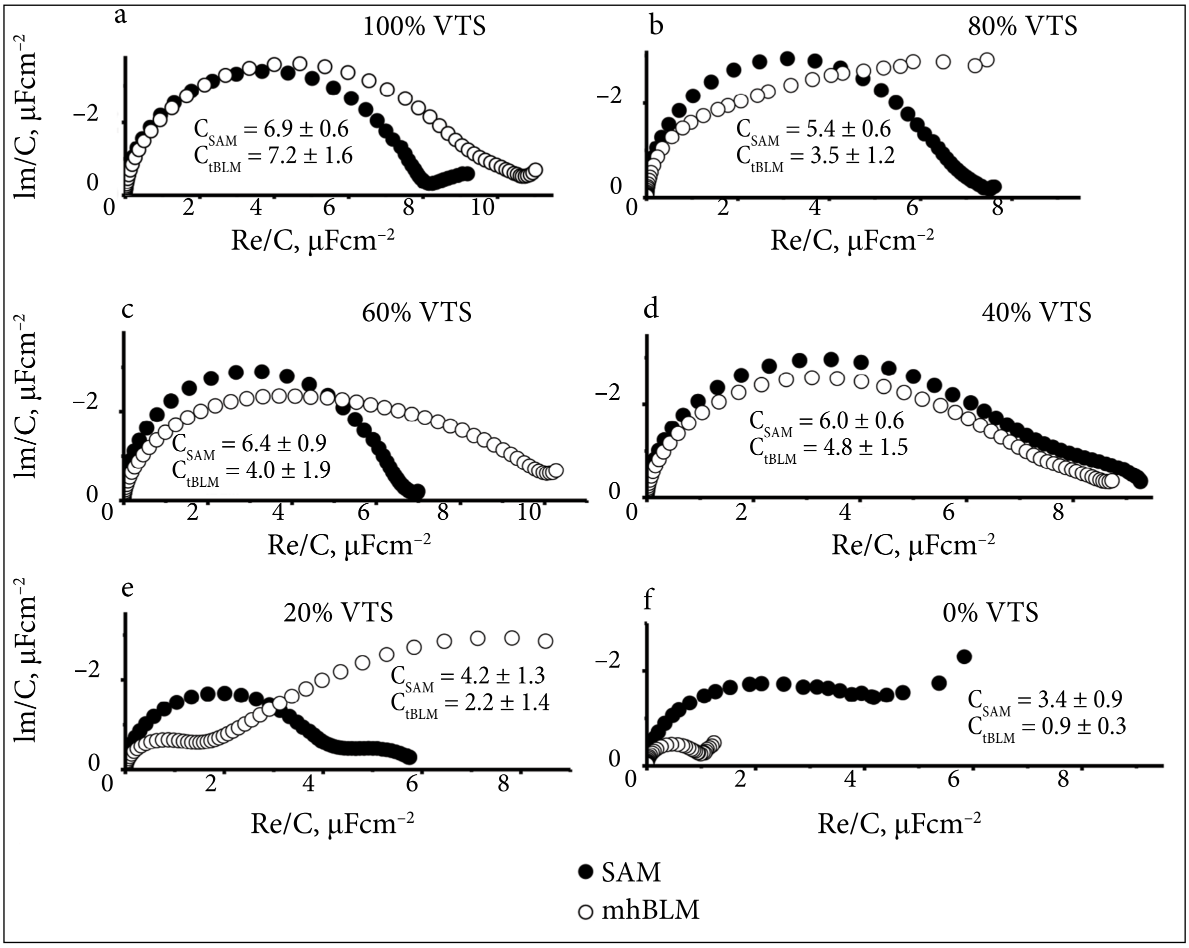

Fig. 2. EIS Cole–Cole plots of the FTO electrodes after functionalisation with the mixed OTS:VTS SAMs (filled circles) and after 1 h incubation of SAMs in DOPC:Chol(40%) vesicle solution (open circles). Molar ratios of OTS:VTS in silanisation solutions: (a) 0:10, (b) 2:8, (c) 4:6, (d) 6:4, (e) 8:2, (f) 10:0. Insets: values of complex capacitances after SAM and tBLM formation in µF cm^{-2} units. Bias potential 0 V vs the Ag/AgCl, NaCl_{sat} electrode

some of them (e.g. Fig. 2c) exhibited a shape of overlapping semi-circles indicating the existence of bilayer with a high number of defects, where the substantial part of the surface is free of phospholipids [22]. Taken together, the Cole–Cole spectra attest for the formation of phospholipid overlayers on the FTO surfaces silanised by mixed silane SAMs; however, intact bilayers can be expected only in SAMs silanised using mixtures with <20% (mol) backfillers.

Because a significant defectness can be expected, one may apply cyclic voltammetry to probe the direct electron transfer processes through the phospholipid layers on mixed silane SAMs.

Cyclic voltammograms in a 10 mM ferri/ferrocyanide redox couple solution were recorded using a FTO electrode functionalised with different molar ratio of VTS backfillers and OTS anchors. The voltammograms on mixed SAMs (Fig. 3, continuous lines) and phospholipid layers (Fig. 3, dashed lines) showed the effect of SAMs and the fused phospholipid layer on the electron transfer properties. As the fraction of OTS increases, the redox peaks, generally, tend to decrease and move away from each other until they totally disappear (Fig. 3d, e, f). Such evolution attests for the increased blockage of the direct electron transfer from the redox species to and from the electrode. The fusion of vesicles and formation of phospholipid layers atop of anchor SAMs affects voltammograms in a similar way shifting current peaks apart. After vesicle fusion, if densities of OTS are high enough (low densities of backfillers), full blockage occurs. The full blockage occurs at 0% of the VTS backfiller. The increase of electron transfer blockage after the vesicle fusion confirms the formation of phospholipid overlayers even in sparsely tethered systems.

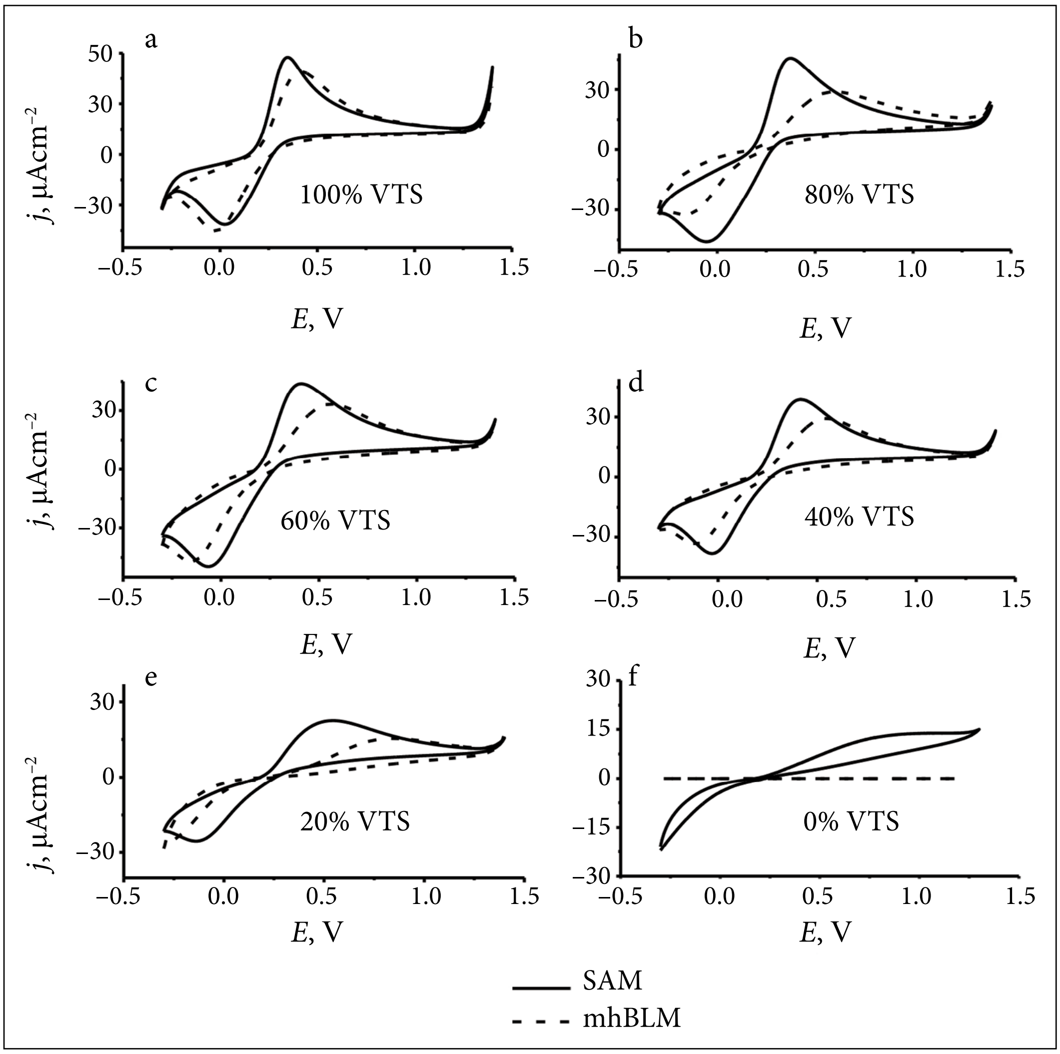

Fig. 3. Cyclic voltammograms recorded in 10 mM $K_4(Fe(CN)_6)$ and $K_3(Fe(CN)_6)$ solution (in PBS pH 7.1) using the FTO electrode after silanisation (continuous line) in the solution containing different OTS:VTS molar ratios: (a) 0:10, (b) 2:8, (c) 4:6, (d) 6:4, (e) 8:2, (f) 10:0 and after 1 h of DOPC:Chol (40%) vesicle fusion (dashed line). Potentials are with reference to the Ag/AgICl, $NaCl_{sat}$ electrode

The cyclic voltammetry experiment reveals subtle differences of the physical properties of backfillers. For example, the electron transfer constant calculated according to Klinger and Kochi [23] is $28{\cdot}10^{-6}$ cm s^{-1} for 100% VTS SAMs (Table) and it decreases when decreasing the VTS molar ratio in the silanisation solution ($1.1{\cdot}10^{-6}$ cm s^{-1} for 10% VTS). The data presented in the Table coincides with the CA data in Fig. 1 and show that the increasing amount of OTS anchor on the surface leads to a higher surface hydrophobicity and an increased electron transfer blockage. A similar trend is observed in gold/thiol mixed SAMs [18]. Vesicle fusion triggers the further blockage of electron transfer (Table). The electron transfer rate constant decreases by more than an order of a magnitude; however, the redox current peaks are still distinguishable up to a OTS:VTS molar ratio 60:40 (Fig. 3d).

Table. **Series of electron transfer constants calculated from the data in Fig. 3**

Molar ratio of backfiller/anchor, %	Electron transfer rate constant, $\cdot 10^{-6}$ cm s^{-1}	
	OTS /VTS SAMs	After vesicle fusion
0:100	28	9.4
10:90	11	0.4
20:80	7.4	0.6
40:60	9.3	1.1
80:20	1.1	n/a
100:0	n/a	n/a

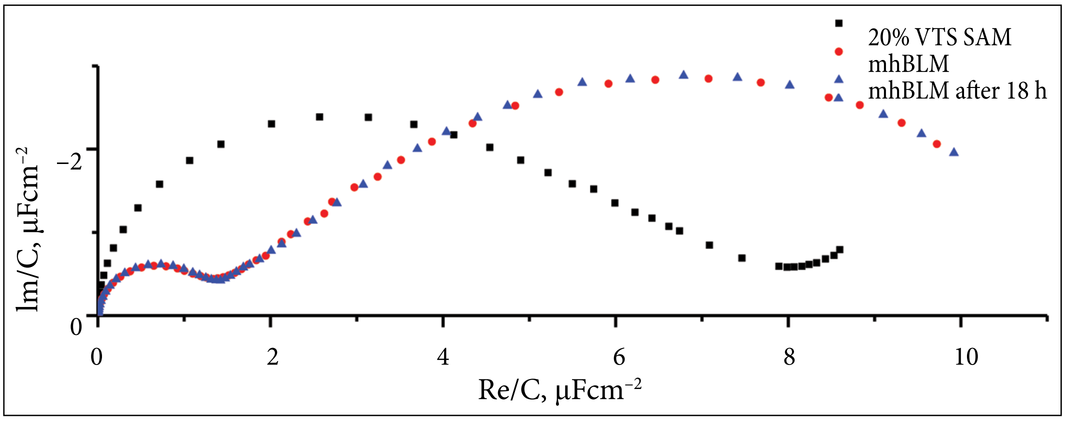

Fig. 4. EI spectra of the OTS:VTS 80:20 molar ratio SAM (black squares); immediatly after DOPC:Chol(40%) mhBLM formation (red circles) and after 18 h of DOPC:Chol (40%) mhBLM formation (blue tringles). Bias potential 0 V vs the Ag/AgCl, $NaCl_{sat}$ electrode

In Figs 2 and 3 we have proven that it is possible to form mhBLM on mixed OTS:VTS SAM, in particular, using the 8:2 molar ratio of OTS:VTS. Therefore, in the following experiment, we show the stability of formed mhBLM. When incorporating proteins into lipid membranes, it is convenient to know that the membrane is stable and it does not deteriorate over time. As seen in Fig. 4, properties of mhBLM does not change over 18 h. Previously, a similar experiment was carried out using methyltrichlorosilane (MTS) as a backfiller [14]. The authors showed (see supporting information [14]) that the membrane is stable over several hours and pore forming toxins can be incorporated into the membrane. Therefore, mhBLMs obtained in this study can be applied for the incorporation of pore forming toxins as well.

Finally, the formed mhBLM was probed with melittin (Mel) toxin to test the biocompatibility of formed membrane with proteins. Melittin is the main component of bee venom [24]. When interacting with the membrane it usually oligomerises into a tetramer and forms a channel inside the membrane [25]. As it can be seen in Fig. 5, after 1 h 30 min of 100 melittin insertion, complex capacitance slightly increases. This is explained by the melittin interaction with mhBLM: melittin formed into pores inside the membrane, increasing the number of defects. This observation confirms the biocompatibility of formed mhBLM, thus it can be successfully applied as a platform for membrane protein studies.

The experimental results of the current study confirm the possibility to design mixed monolayers

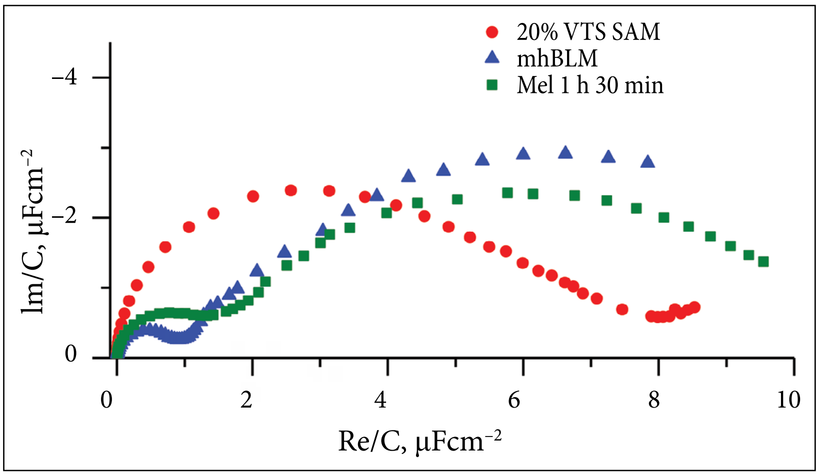

Fig. 5. EI spectra of the OTS:VTS 8:2 molar ratio SAM (red circles); DOPC:Chol(40%) mhBLM (blue triangles); after 1 h 30 min insertion of 100 nM melittin. Bias potential 0 V vs Ag/AgCl, $NaCl_{sat}$ electrode

on metal oxide surfaces, specifically, on FTO, which are capable of fusing multilamellar vesicles and formation of the phospholipid layers. The continuous variation of the molecular ratios of OTS and VTS leads to a gradual increase (decrease) of a long chain molecular anchor, in our case, the OTS amount on the surface. Such increase was attested both by the contact angle and electrochemical impedance spectroscopy data as it was the case with the gold film supported tBLMs [18] on the octadecanethiol (ODT) monolayer [18].

Even though EIS data provide direct evidence of a vesicle fusion on (interaction with) anchor SAMs, rather high values of complex capacitances (>0.9 μF cm^{-2}) point out to the absence of complete, defect-free phospholipid bilayers. A significant decrease in the complex capacitance determined for 80% OTS SAM indicated a successful formation of the bilayer; however, the blockage of the electron transfer in the ferri/ferrocyanide redox system was far from complete. In this regard, our data parallels the one obtained for octadecanethiol supported phospholipid layers on gold [26]. However, in this research developed mhBLM on the FTO surface exhibits a high stability and it can be applied for the incorporation of different proteins (enzymes, toxins, etc.).

ACKNOWLEDGEMENTS

This article is dedicated to the anniversary of Prof. Rimantas Ramanauskas.

Received 5 October 2022
Accepted 13 October 2022

References

1. G. Valincius, F. Heinrich, R. Budvytyte, et al., *Biophys. J.*, **95**, 4845 (2008).
2. B. Rakovska, T. Ragaliauskas, M. Mickevicius, et al., *Langmuir*, **31**, 846 (2015).
3. A. Valiūnienė, T. Petrulionienė, I. Balevičiūtė, L. Mikoliūnaitė, G. Valinčius, *Chem. Phys. Lipids,* **202**, 62 (2017).
4. T. Sabirovas, A. Valiūnienė, G. Valincius, *J. Electrochem. Soc.*, **165**, G109 (2018).
5. S. N. Ratnayaka, R. J. Wysocki, S. S. Saavedra, *J. Colloid Interface Sci.*, **327**, 63 (2008).
6. I. Gabriunaite, A. Valiūnienė, G. Valincius, *Electrochim. Acta*, **283**, 1351 (2018).
7. A. Valiūnienė, Ž. Margarian, I. Gabriūnaitė, et al., *J. Electrochem. Soc.*, **163**, H762 (2016).
8. K. Kumar, C. S. Tang, F. F. Rossetti, et al., *Lab Chip*, **9**, 718 (2009).
9. J. A. Jackman, S. R. Tabaei, Z. Zhao, S. Yorulmaz, N.-J. Cho, *ACS Appl. Mater. Interfaces*, **7**, 959 (2015).
10. T. Sabirovas, A. Valiūnienė, G. Valincius, *Sci. Rep.*, **11**, 1 (2021).
11. T. Ragaliauskas, M. Mickevicius, B. Rakovska, et al., *Biochim. Biophys. Acta-Biomembr.*, **1859**, 669 (2017).
12. G. Valincius, M. Mickevicius, T. Penkauskas, M. Jankunec, *Electrochim. Acta*, **222**, 904 (2016).
13. S. R. Wasserman, Y. T. Tao, G. M. Whitesides, *Langmuir*, **5**, 1074 (1989).
14. I. Gabriunaite, A. Valiūnienė, T. Sabirovas, G. Valincius, *Electroanalysis*, **33**, 1315 (2021).
15. T. Sabirovas, A. Valiūnienė, I. Gabriunaite, G. Valincius, *Biochim. Biophys. Acta-Biomembr.*, **1862** (2020).
16. G. S. Popkirov, R. N. Schindler, *Rev. Sci. Instrum.*, **63**, 5366 (1992).
17. G. S. Popkirov, R. N. Schindler, *Electrochim. Acta*, **38**, 861 (1993).
18. D. J. Mcgillivray, G. Valincius, D. J. Vanderah, et al., *Biointerphases*, **2**, 21 (2007).
19. G. Valincius, M. Mickevicius, in: *Advances in Planar Lipid Bilayers Liposomes*, Elsevier B. V. (2015).
20. M. Itagaki, S. Suzuki, I. Shitanda, K. Watanabe, *Electrochemistry*, **75**, 649 (2007).
21. A. J. Bard, L. R. Faulkner, *Electrochemical Methods: Fundamentals and Applications*, John Wiley and Sons (2008).
22. K. J. Kwak, G. Valincius, W. C. Liao, et al., *Langmuir*, **26**, 18199 (2010).
23. R. J. Klingler, J. K. Kochi, *J. Phys. Chem.*, **85**, 1731 (1981).
24. J. Chen, S. M. Guan, W. Sun, H. Fu, *Neurosci. Bull.*, **32**, 265 (2016).
25. M. T. Tosteson, D. C. Tosteson, *Biophys. J.*, **36**, 109 (1981).
26. S. A. Glazier, D. J. Vanderah, A. L. Plant, et al., *Langmuir*, **16**, 10428 (2000).

Inga Gabriūnaitė, Eimantas Zolubas, Aušra Valiūnienė

MIŠRIOS HIBRIDINĖS DVISLUOKSNĖS LIPIDINĖS MEMBRANOS SUFORMAVIMAS ANT SKAIDRAUS PLONASLUOKSNIO OKSIDO ELEKTRODO

Santrauka

Šiame darbe buvo formuojamos ir tiriamos fosfolipidinės bisluoksnės membranos (FBM) ant mišriu savitvarkiu monosluoksniu (SAM) modifikuoto puslaidininkio metalo oksidinio paviršiaus. Ankstesniuose tyrimuose FBM buvo formuojamos ant monokomponentinių SAM, tačiau jos pasižymėjo prastu suderinamumu su transmembraniniais baltymais. Todėl šiame darbe buvo naudojami mišrūs SAM siekiant suformuoti funkcionalią FBM, į kurią galėtų įsiterpti skirtingi membraniniai baltymai.

Puslaidininkis plonų plėvelių elektrodas buvo parinktas fluoru legiruotas alavo oksidas (FTO), nes jis yra komerciškai prieinamas ir pigus (palyginti su kitu plačiai naudojamu indžiu legiruotu alavo oksidu). FTO paviršius buvo funkcionalizuojamas naudojant ilgos ir trumpos grandinės hidrofobinių silanų mišinį: oktadeciltrichlorsilaną (OTS) ir viniltrimetoksisilaną (VTS). FBM buvo suformuotos vezikulių liejimo metodu. Vezikulių tirpalą sudarė 60 % 1,2-dioleoil-sn-glicero-3-fosfocholino (DOPC) ir 40 % cholesterolio, kurių suminė koncentracija siekė 1 mM.

Tyrimai atlikti naudojantis kontaktinio kampo, ciklinės voltamperometrijos (CV) ir elektrocheminio impedanso spektroskopijos metodais (EIS). Gauti rezultatai patvirtino apie hidrofobinio SAM susiformavimą, naudojant skirtingų molinių santykių OTS ir VTS tirpalus. FBM pavyko sėkmingai suformuoti ant OTS:VTS 8:2 molinio santykio SAM. Nustatyta, kad suformuota mišri hibridinė dvisluoksnė lipidų membrana yra stabili vandeninėje terpėje, todėl gali būti pritaikyta įvairių membraninių baltymų tyrimuose.