PHOSPHOLIPID LAYER STABILIZATION via Yb(III) ON ITIES AND FACILITATED K(I) TRANSPORT

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The stabilization of lecithin layer physically adsorbed on the interface between two immiscible electrolyte solutions (ITIES) using addition of Yb^{3+} to the aqueous phase was studied using cyclic voltammetry. Stability of this layer was evaluated according to the time of the layer formation; the optimal time amounted to 180 s. The stability of the layer as a function of the potential applied on the system during the layer formation was investigated. The behavior of the monolayer during the layer polarization was studied by the facilitated K^+ transport using dibenzo-18-crown-6. The stoichiometry of Yb/anion/lecithin complexes was investigated using electrospray ionization mass spectrometry (ESI-MS).

Keywords: Phospholipids; Membranes; Zwitterions; Ytterbium; Potassium; Electrochemistry; Artificial membranes; Cyclic voltammetry; Dipalmitoylphosphatidylcholine; Polarization; Electrospray ionization mass spectrometry (ESI-MS).

All living cells of plants and of animals (and of some viruses too) are surrounded by lipid bilayer membranes. These membranes are flat sheets that form a continuous barrier around cells. Similar lipid bilayers surround the sub-cellular compartments[1]. The phospholipids are the basic building elements of most natural membranes[1–3]. Lipid bilayers (LBs) (or phospholipid bilayers (PLBs)) are thin, flat membranes consisting of two layers of lipid molecules, with their hydrophobic parts, usually fatty acid tails, directed toward the center of the membrane, and with hydrophilic parts located at the inner and outer borders[4]. The LB is the barrier that keeps ions, complexes

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and a great variety of molecules at places, where they are needed, and prevents them from diffusing into areas, where they should not be. PLBs are ideally suited to this role because, even though they are only a few nanometers thick (6–10 nm), they are impermeable to most water-soluble (hydrophilic) molecules[5,6].

The basic brick stones for the formation of the real LB are the phospholipids (as diacylphosphatidylcholine or diacylphosphatidylinositol, etc.), which are composed of two fatty acid tails, phosphate group and choline, inositol, serine, etc., groups. Because the real cell membranes are very complicated, we have started to study the above mentioned processes using model membranes which should represent, in the first approximation, the real protoplast membrane[1,3,4,7,8].

For understanding of the fundamental process of ion transfer reactions at biomembranes, the ion transfer processes across a phospholipid mono-layer adsorbed at a liquid|liquid interface have been investigated[9,10]. The interface between two immiscible electrolyte solutions (ITIES) can be seen as an analogy with the interface of solid electrode|electrolyte solution[9–21].

In particular, the effect of a lipid layer on the ion transfer kinetics has been noticed[10,22]. A polarizable interface can be formed as a combination of two different and mutually immiscible electrolytes[16]. A reference interface (non-polarizable) can be formed using two electrolytes with conjoint ion[16].

The stability of the formed PL monolayer on ITIES seems to be the crucial problem, because only in time relatively stable monolayer can be utilized for more complicated experiments. For these purposes the additions of a multivalent cation have been applied. The binding of a multivalent cation to a PL membrane promotes a structural change of the membrane, which has been investigated by nuclear magnetic resonance[23,24], calorimetry[25], spectrophotometry[26], atomic force microscopy[27] and electrochemistry at lipid-supported electrodes[28,29]. However, the serious effect of the multivalent cation on the permeability across the PC monolayer adsorbed at a liquid|liquid interface has not been observed (e.g. ref.[30]).

The ion transfer across a lipid monolayer at a liquid|liquid interface has been frequently and very intensively investigated in the past years (e.g. refs[31–34]). The transfer rate is dependent on the number of ions in the compact layer which is given by the distribution function with the meaning of density function of the ion location in the surface of maximal approach. The density function depends on the ion concentration in one of the phases (e.g. aqueous phase). The rate of the ion transfer from one phase to other phase is also given by the difference of electrochemical energies of the ion in the aqueous and organic phase. The rate of ion transfer depends on the temperature as well.

As it was mentioned above, different types of phosphatidylcholine proved to be the most suitable for the formation of the PL monolayers on ITIES in such experiments[10]. Various ways of facilitating of the transporting processes of cations across the interface, e.g., utilization of ionophores (valinomycin[31,32], dibenzo-18-crown-6 (DB18C6)[10,35]), have been reported.

The effect of lanthanide ion (Ln^{III}), on the inhibition of ion transfer across a PL monolayer was investigated voltammetrically[10], and it was demonstrated that the blocking effect and the electrochemical stability of the barrier layer were controlled by pH or the kind and concentration of the multivalent cation. According to Maeda et al.[10], the blocking effect (expressed approximately on the base of the decrease of voltammetric current) is enhanced in the order $La^{3+} < Eu^{3+} < Yb^{3+}$.

The most electrochemical methods are available to study the above mentioned processes, e.g., refs[9–21]. Similarly as in other cases, cyclic voltammetry proved to be a promising technique allowing investigation of the transport of charged particles across a polarizable interface. Electrospray ionization mass spectrometry (ESI-MS) can be very suitable for revealing the structure and the ratio of formed complexes among PL, added cations (in case of this manuscript Yb) and electrolyte ions.

EXPERIMENTAL

Voltammetric Studies

The reagent grade chemicals were supplied by Fluka AG. As supporting electrolytes of the aqueous phase, 0.1 M KCl, 0.1 M KCl + 0.01 M HCl, and a mixture of H_2SO_4 and K_2SO_4 were used. Tetrapentylammonium tetraphenylborate (TPeATPB) – the supporting electrolyte of the organic phase – was prepared by direct synthesis in equimolar solid mixture of tetrapentylammonium chloride (TPeACl) and sodium tetraphenylborate (NaTPB) and by subsequent chloroform extraction and following purification processes (described in ref.[19]). 1,2-Dichlorethane (DCE) was used as the organic solvent. 0.01 M TPeACl in the aqueous solution was used as an electrolyte of the silver chloride reference electrode in the organic phase.

Dipalmitoylphosphatidylcholine (DPPC; L-α-lecithin) has been frequently used for the formation of the artificial phospholipid monolayers (e.g. refs[18,19,36,37]) as well as bilayers (e.g. refs[1–4,7,38–40]). The phospholipid monolayer on the interface of the system studied in this manuscript was prepared by addition of 40 μM DPPC [41]. The stabilization of the physically adsorbed phospholipid was enabled by ytterbium ions dissociated from 4 mM $YbCl_3$ which was added into the aqueous phase. Potassium transport was facilitated by 0.5 mM dibenzo-18-crown-6 (DB18C6). Tetraethylammonium chloride (TEACl) was used as a reference cation[42] according to which was determined the zero Galvani potential difference to 0.386 V.

The contribution of the liquid junction potential (arises owing to separation of RE1) was experimentally determined to 0.044 V (data not shown). The system with separated RE1 has the zero Galvani potential difference at 0.341 V.

The charge transport across the aqueous/organic interface[16] and its inhibition after formation of the phospholipid monolayer at the interface were studied using cyclic voltammetry in four electrode (silver chloride electrode as electrodes of organic and aqueous phases, platinum wire as auxiliary electrodes of organic and aqueous phases) arrangement (potentiostat PAR, EG&G Princeton Applied Research, USA with software 270/250, 1994 EG&G Instruments Inc., was used[43]) using cell described in refs[6,44] with a flat interface of 5.15×10^{-5} m^2 area.

ESI-MS Studies

Electrospray ionization mass spectrometry (ESI-MS) was used to determine the stoichiometry of Yb/anion/lecithin complexes. The experiments were performed with a Finnigan LCQ Advantage ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) fitted with an electrospray ionization source. Diluted solution of lecithin and $YbCl_3$ in acetonitrile/water (1:1 v/v) was introduced into the ESI source via a fused-silica capillary at a flow rate of 0.6 ml h^{-1}. Nitrogen was used as the nebulizer gas. The operating conditions were set as follows: spray voltage 5.6 kV, capillary voltage 55 V, heated capillary temperature 250 °C, sheath gas flow rate, and auxiliary gas flow rate 10–50 arbitrary units for positive mode regime and spray voltage 5.2 kV, capillary voltage –15 V, heated capillary temperature 250 °C, sheath gas flow rate, and auxiliary gas flow rate 10–50 arbitrary units for negative mode regime. Mass spectra were recorded from *m/z* 50 to 2000.

The model solutions were prepared from $YbCl_3$ salt (trace metals basis grade), lecithin, HPLC-grade acetonitrile (all Sigma Aldrich, Czech Republic), and deionized water (>18 MΩ). The stock solutions were equimolar in $YbCl_3$ and lecithin (both 2.5×10^{-6} mol l^{-1}) and diluted to the desired concentrations.

All other reagents were grade chemicals (Fluka AG) or chemicals synthesized by ourselves and diluted by deionized water (>18 MΩ). All measurements were carried out under laboratory conditions (25 °C, atm. pressure).

RESULTS AND DISCUSSION

Phospholipid Layer Formation

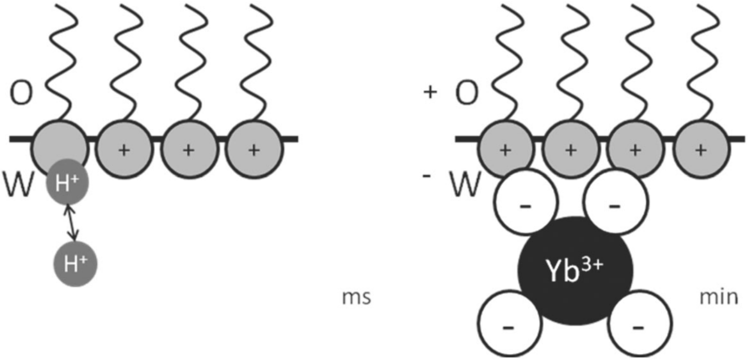
On the basis of previous results[18,19] the formation of a DPPC layer at the interface of two immiscible phases was studied. The phospholipid layer on ITIES is formed in three consequent steps (Eq. (*1*)) with different time constants (Scheme 1)

$$L^{\pm}_{(o)} \xleftrightarrow{1} L^{\pm}_{(ads)} + H^{+}_{(w)} \xleftrightarrow{2} HL^{+}_{(ads)} \xleftrightarrow{3} HL^{+}_{(o)} \qquad (1)$$

where $L^{\pm}$ stands for zwitterionic form of DPPC and HL^{+} for protonated DPPC, (o) for organic phase, (w) for water phase and (ads) for the adsorbed form.

In step 1, the physical adsorption has to be realized. This process can be described by the Fick law of diffusion, and therefore, it depends on phospholipid concentration. Using the lecithin concentration of 40 μmol l^{-1}, this process is relatively fast (of the order of ms [41]). (2) Then, in step 2, the zwitterionic lecithin is protonated by a surface reaction with H^{+} included in water phase; by this way the positively charged part of DPPC is localized into water phase. Step 2 is supported by inserted negative potential in interface, because according to the convention at negative potentials the water on the interface with organic phase carries negative charge. Step 3 describes desorption of protonated DPPC into organic phase in positive potentials area, because the positive charge of water pushes out DPPC carrying positive charge. Additionally, it is important to notice that DPPC as zwitterion is not able to desorb even at positive potential (used in our work) because it is stabilized by its negative charge on phosphate group (which is not protonated in neutral milieu). (3) Subsequently, the adsorbed phospholipid layer is stabilized from the aqueous phase side by $[Yb^{3+}(A^{n-})_x]^{(3-nx)}$, where A substitutes an anion. This process is also diffusion controlled and depends on the applied potential. However, due to the steric effects, the formation of ytterbium/lecithin complexes is more time demanding (of the order of min).

The layer, which was formed, was not a monolayer, because the charge of a monolayer is very small, and to register its desorption high polarization rates (around 1–5 V s^{-1}) are required. Low polarization rates (mostly 50 to 100 mV s^{-1}) were sufficient to study the DPPC layer in our case. According to this evidence, we supposed that various micellar structures, and multilayers were expected. The influence of time on the phospholipid membrane formation (so-called holding time) is shown in Fig. 1, where it is possible to see the increasing height of the lecithin desorption peak in time. The shape of the desorption peak is important in order to understand, which process might occur simultaneously with the desorption process. Usually, the desorption peak is very narrow and sharp[18]. Nevertheless, the anodic peak recorded in Fig. 1 is relatively broad and it shifts to more positive potentials with increasing holding time. That may be caused by additional reaction process involved, which is extended by the increasing amount of adsorbed lecithin. Suspicious reaction is the reaction with negatively charged ytterbium salts and clusters with anions (as is mentioned below). This reaction has a stabilization effect on the lecithin layer. Furthermore, it also fulfills the criterion that the increasing holding increases the amount of Yb/anion/lecithin clusters and the layer is more stable (the desorption peak is shifting to more positive potentials with increasing amount of Yb-anion/lecithin clusters).



SCHEME 1
The phospholipid layer formation and stabilization using Yb^{3+} cations in acidic pH (1.4) in area of negative potentials

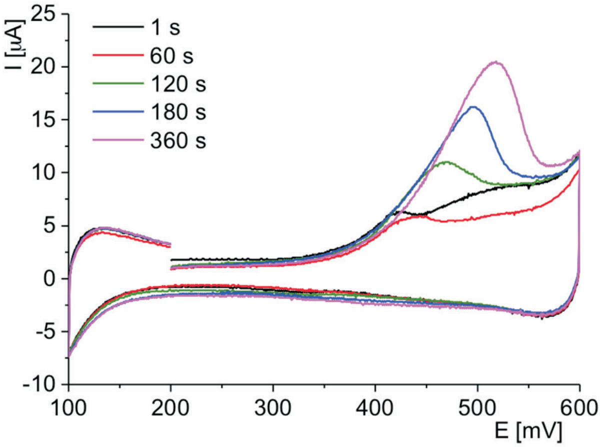


FIG. 1
Influence of holding time (1–360 s) on the compactness of lecithin layer expressed as a desorption peak of phospholipid in cyclic voltammetry; $c(H_2SO_4)$ = 21 mmol l^{-1}, pH 1.4, $c(YbCl_3)$ = 4 mmol l^{-1}, c(DB18C6) = 0.5 mmol l^{-1}, DPPC 40 μmol l^{-1}, scan rate 100 mV s^{-1}, E_{in} = 200 mV, E_{fin} = 600 mV

The formation of phospholipid layer depends on the holding potential too (Fig. 2). With the increasing holding potential, the amount of adsorbed and stabilized lecithin on ITIES decreases. This evidence may elucidate the mechanism of lecithin layer stabilization. In neutral milieu lecithin exists as a zwitterion with negatively charged part (phosphoric group) a positively charged part (amino group). In acidic pH the phosphate group is protonated and lecithin carries positive charge on its hydrophilic part. This hydrophilic part (N^+) is oriented into the aqueous phase; this orientation is stabilized/destabilized by inserted potential. The stabilization effect on lecithin layer is bigger at negative potentials of the system, because at negative potentials water carries negative charge. Likewise at positive potentials protonated lecithin would be displaced by repulsive forces.

The Transport of K^+

The phospholipid layer stability can be demonstrated on K^+ transport from the aqueous phase to the organic phase (Fig. 3). The stability of the layer is illustrated by K^+ transport hindrance. K^+ transport is facilitated (the potential range is not sufficient for spontaneous transport) using DB18C6. It is known[18] that lecithin layer is not able to protect transport of small molecules, but of big molecules only (such as DB18C6). The approach of

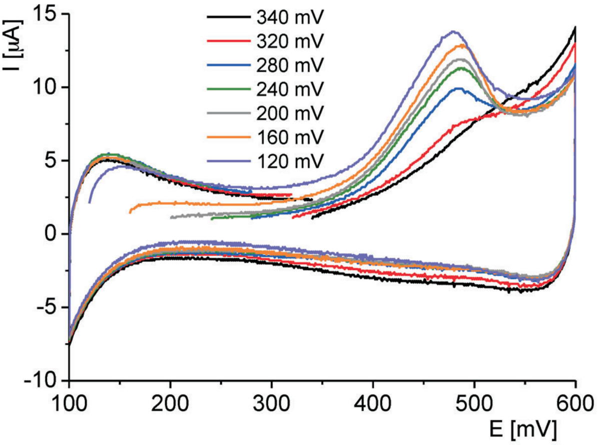


FIG. 2

Influence of holding potential on the phospholipid desorption peak at holding time 180 s in cyclic voltammetry. $c(H_2SO_4)$ = 21 mmol l^{-1}, pH 1.4, $c(YbCl_3)$ = 4 mmol l^{-1}, c(DB18C6) = 0.5 mmol l^{-1}, DPPC 40 μmol l^{-1}, scan rate 100 mV s^{-1}, E_{fin} = 600 mV. Zero Galvani potential difference of the system was at the potential of reference electrodes +341 mV

DB18C6 to the ITIES is the limiting factor of K^+ transport in this potential window.

While the compactness of the layer is sufficient (60 s is sufficient), the layer is capable to displace the K^+ transport from the aqueous to the organic phase for almost 70 mV. In the anodic part of the CV, beside the lecithin desorption peak, the peak corresponding to the K^+ transport can be registered. The transport of K^+ is hindered by the increasing compactness of the formed membrane. In a more detailed view on cyclic voltammograms, another more positively situated anodic peak can be registered.

Behind (in a more positive area) the K^+ peak of its facilitated transport via DB18C6, there is a small peak, which increases with increasing holding time. Moreover, for the holding time 60 to 180 s can be observed the reverse (60 mV shifted) peak of this process. These broad peaks may correspond to the desorption of lecithin in the form of various more complicated aggregates or clusters which also fulfills the criteria of one electron transport (Scheme 2).

The impact of applied potential during the holding time (180 s) on the compactness of the layer is demonstrated by hindrance of the facilitated transport of K^+ (Fig. 4). The compactness as well as the adsorbed amount of lecithin decrease with increasing holding potential (Fig. 2). However, the

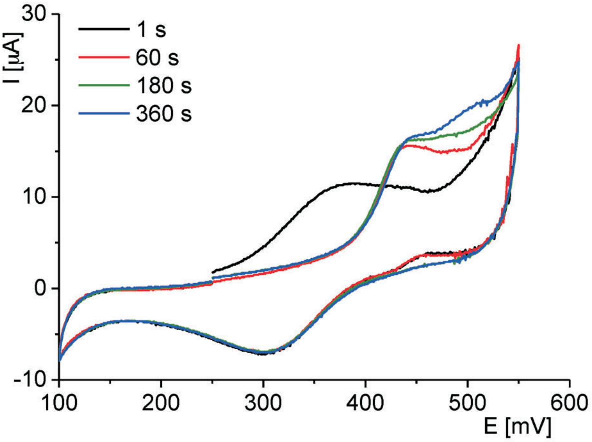
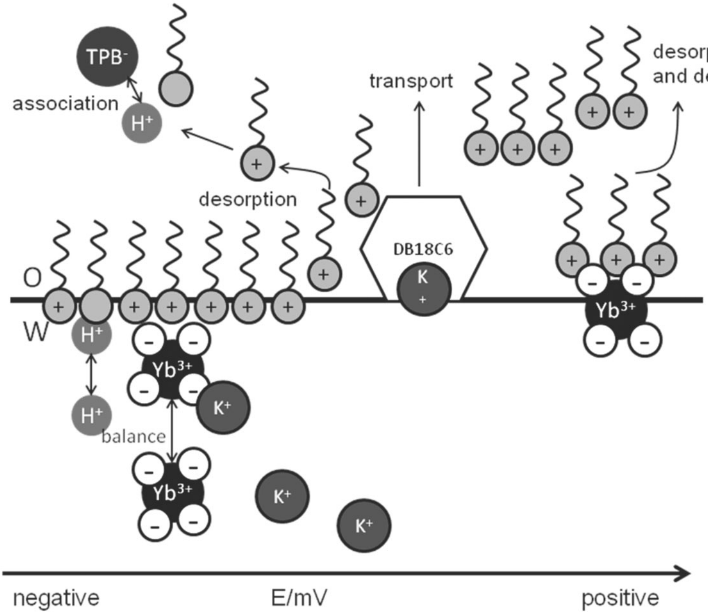


FIG. 3
Influence of holding time (1–360 s) on the compactness of lecithin layer expressed as a desorption peak of phospholipid in cyclic voltammetry with facilitated potassium transport; $c(H_2SO_4)$ = 21 mmol l^{-1}, pH 1.4, $c(YbCl_3)$ = 4 mmol l^{-1}, c(DB18C6) = 0.5 mmol l^{-1}, DPPC 40 µmol l^{-1}, $c(K_2SO_4)$ = 50 µmol l^{-1}, scan rate 50 mV s^{-1}, E_{in} = 240 mV, E_{fin} = 550 mV



SCHEME 2

The model of the phospholipid layer behavior during potential changes from more negative to more positive potentials

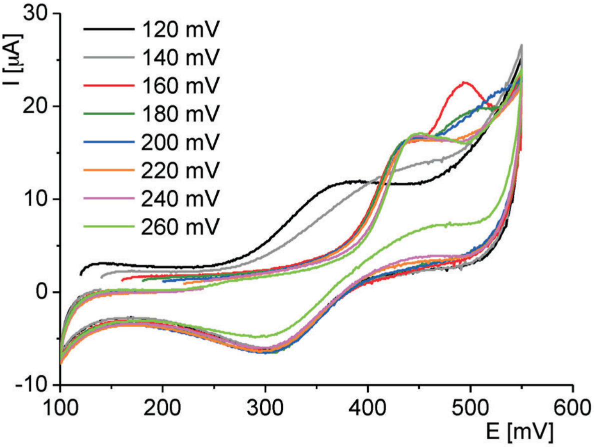


FIG. 4

Influence of holding potential on the compactness of lecithin layer. $c(H_2SO_4)$ = 21 mmol l^{-1}, pH 1.4, $c(YbCl_3)$ = 4 mmol l^{-1}, c(DB18C6) = 0.5 mmol l^{-1}, DPPC 40 µmol l^{-1}, $c(K_2SO_4)$ = 50 µmol l^{-1}, scan rate 50 mV s^{-1}, E_{fin} = 550 mV. Zero Galvani potential difference at the potential of reference electrodes +341 mV first peak, which features the retardation of K^+ transport, displays practically the same trend for all holding potentials. The second peak (explained by more complicated desorption of lecithin) depends dramatically on holding potentials, the second peak is the biggest for the holding potential 160 mV and then decreases with increasing potential (at least for holding potential of 200 mV it has a significant shape). It signifies that the second peak is related with the amount of adsorbed lecithin on the ITIES.

First of all, it is important to notice the topology of lecithin layer desorption at positive potentials. At negative potentials the position of lecithin is stabilized via negative charge of the aqueous phase of ITIES. On the other hand, at positive potentials the aqueous side of ITIES is positively charged. Consequently, at first, lecithin (as well as its clusters) is repulsively displaced from the nearest area of ITIES. Lecithin is pushed out of the interface and is forced into layers above (if we consider the lecithin barriers as a multilayer structure). This may be explained by a more complicated process of lecithin layer desorption which can be demonstrated by the shift of lecithin desorption peak to more positive potentials with increasing holding time (i.e., increasing amount of adsorbed lecithin during longer time) (Fig. 1). The clusters decay during the desorption process; however, they probably include more than one molecule of lecithin. According to this, the second peak can be also caused by desorption of lecithin conglomerates.

We also have to explain why the layer does not exist if the holding potential is below 140 mV. While the system is approaching more negative values such as 100 mV, the diffusion of $TPeA^+$ (the cation of organic phase base electrolyte) approximates the diffusion-limited current. It means that the interface is strongly affected by the $TPeA^+$ transport from organic to aqueous phase; in this way the adsorption of DPPC is disabled.

With this information at hand, we can postulate that the artificial layer is destroyed by potentially ordered decay of Yb/anion/lecithin cluster and desorption of lecithin from inner parts of the layer in the form of separate molecules as well as of various aggregates or clusters (Scheme 2).

As it was mentioned above, the stability of the phospholipid layer is given by the amount of the ytterbium/lecithin complexes. The number of the particles passing across the interface is proportional to the area of the desorption peak and can be calculated according to the equation 1 [45]

$$c_{(\mathrm{Lads\ -\ complex})} = area/FS \tag{2}$$

where *area* is the peak area of the phospholipid desorption peak (in our experiments, the integration was calculated using OriginPro 8.0, OriginLab), *S* is the cell area (5.15×10^{-5} m^2) and *F* is the Faraday constant.

Figure 5 illustrates how the holding potential affects the concentration of lecithin on ITIES. First, the protonated lecithin (positively charged) is stabilized on the ITIES by the negative charged placed on the water side of the interface. Second, the protonated lecithin is enabled to bind with anions included in the water phase and via the anions the lecithin is coupled with ytterbium cation as is summarized in Eq. (*1*).

From the linear dependence (Fig. 5) of the lecithin surface concentration (μmol cm^{-2}) on holding potential (mV), a simple equation was derived, which serves for calculation: $c = (-3.88 \pm 0.46)E_{in} + (555 \pm 61)$.

Figure 6 shows the positive and the negative mode mass spectrum of a dilute mixture of lecithin (L) and $YbCl_3$ in acetonitrile/water. There were observed: (i) adduct of lecithin with H^+ (LH^+ *m/z* 734.5 and L_2H^+ *m/z* 1468), (ii) adduct of lecithin with chloride anion (LCl^- *m/z* 768.5 and L_2Cl^- *m/z* 1502 – not shown) and (iii) clusters of ytterbium with chloride anions $[Yb(Cl)_4]^-$ *m/z* 310. All listed *m/z* values refer to the isotopes (^{1}H, ^{12}C, ^{14}N, ^{16}O, ^{31}P, ^{35}Cl and ^{170}Yb).

In acidic pH are expectable metal and bimetal clusters originating from the metal salt origin precursor[46,47].

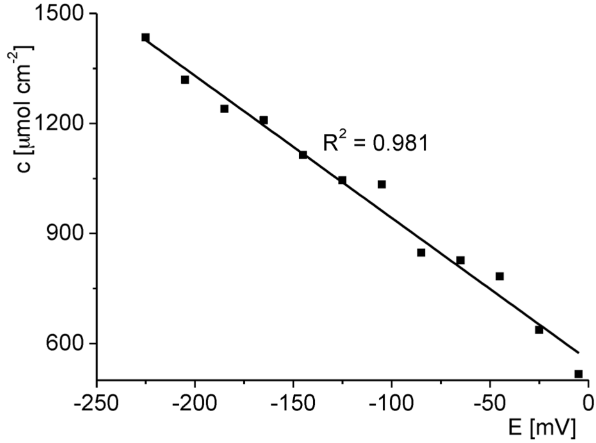


FIG. 5
Dependence of lecithin concentration at ITIES (μmol m^{-2}) on the holding potential (mV) at holding time 180 s. The holding potentials are recalculated to the potential of standard hydrogen electrode

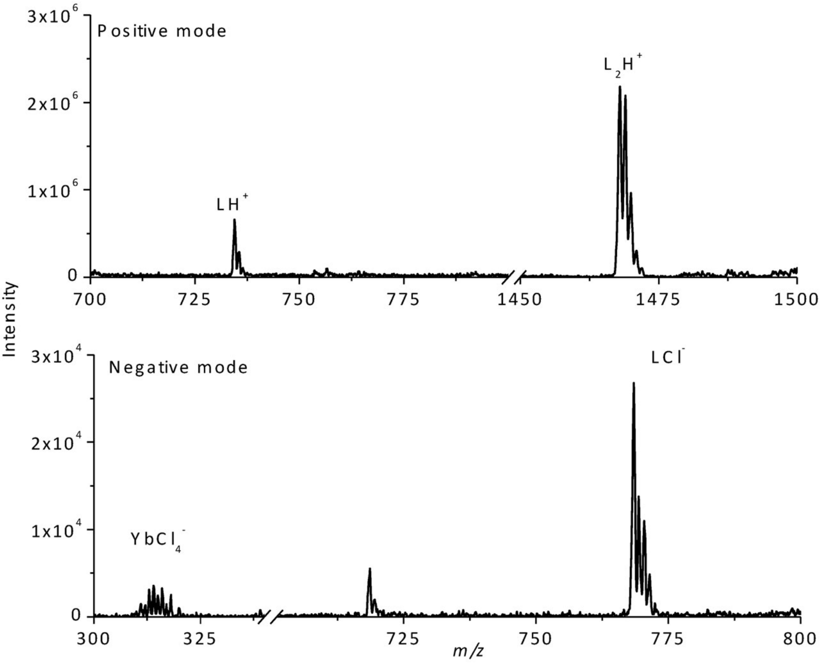


FIG. 6
Positive mode (top) and negative mode (bottom) ESI mass spectra in the range from *m/z* 700 to 1500 of an equimolar mixture of lecithin (L) and $YbCl_3$ (both 1.25×10^{-6} mol l^{-1}) acetonitrile/water (1:1 v/v)

CONCLUSIONS

The DPPC layer is formed on ITIES, while the phosphate group of lecithin is protonated from the water side of ITIES (relatively acid pH around 1.4 is required). The polar part of lecithin is immersed into the aqueous phase and the NH_3^+ group is available to the created complexes with $[Yb^{3+}(A^{n-})_x]^{(3-nx)}$.

The existence of lecithin clusters with ytterbium is allowed by the presence of the above mentioned negatively charged complexes of ytterbium with the present anions. Practically no free Yb^{3+} was observed in the mass spectra. The capability of lecithin to couple with anion was proved by the observation of LCl^- and L_2Cl^- compounds.

The creation of the DPPC is supported by (i) holding time; longer holding time yields to multilayer and micellar structures, and (ii) holding potential. More negative potentials are supportive because of a stabilizing effect of negative charge on water side of the ITIES, which stabilizes the topological orientation of a lecithin molecule.

In holding potentials (non-recalculated on the zero Galvani potential) below 140 mV the adsorption of lecithin on ITIES is disabled by the $TPeA^+$ transport from organic to aqueous phase. The holding potential 160 mV was considered the best potential for DPPC layer creation.

The ability of DPPC layer to protect big molecules approach and transport was proved with DB18C6 facilitated transport of K^+ from water phase to organic phase. The enhanced K^+ transport was about 70 mV retarded to more positive potentials compared with non-hindrance transport.

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