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# Potential controlled adsorption and lateral mobility of DOPC on polycrystalline gold – an EQCM and in situ fluorescence microscopy study

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**Abstract**

The adsorption of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) from solution of unilamellar vesicles on polarized gold surfaces is directed by the surface charge of the gold substrate. The electrochemical quartz crystal microbalance (EQCM) data indicate the existence of two different adsorption patterns. If the surface charge of the gold surface exceeds ca. 8 $\mu C\,cm^{-2}$, the determined adsorbed phospholipid mass indicates the presence of a ''bi-layer-like'' system. The existence of an adsorbed layer composed of intact vesicles remains possible, if the adsorption proceeds at potentials close to the potential of zero charge (*pzc*). The lateral mobility of the phospholipid molecules in the adsorbed layers was investigated by fluorescence recovery after photobleaching (FRAP) approach. The diffusion coefficient of the DOPC molecules in the adsorbed layers ranged between 1.0 and $2.5 \times 10^{-12}\ cm^2\ s^{-1}$, which is about 4 magnitudes smaller than in intact mica-supported DOPC bi-layers in the liquid-crystalline state. The diffusion coefficients found in the ''bi-layer-like'' system are ca. 3–5 times slower than in the layer adsorbed at *pzc*. The mobility of the phospholipid molecules decreases with increasing surface charge of the substrate.


*Keywords:* Phospholipid adsorption; EQCM; In situ fluorescence microscopy

## 1. Introduction

Structure and properties of biological membranes represent one of the vigorously studied fields due to the importance of these systems to understand the processes proceeding in living organisms. To rationalize the membranes' behavior there were formulated several simplified models enabling for quantitative characterization. Phospholipid bi-layers supported on solid surfaces represent one group of such model system. The supported phospholipid bi-layers are important not only from theoretical point of view, but they were also used in biosensors, surface modification of implantates or of catalytic interfaces [1].

The supported phospholipid bi-layers can be prepared either by the Langmuir–Blodgett method [2] or by spontaneous fusion of phospholipid vesicles [3–7]. The later approach became more popular mainly owing to experimental simplicity [5–15] and as substrate were used metals [5,6,8–10,13], oxides [5,6] or modified conductive substrates [5,11,12]. The formation of supported phospholipid bi-layers on conductive substrates is particularly interesting, since one can use an external electrical field to control some important factors affecting the adsorption process, like surface charge of the substrate. The effect of external polarization on the mode of phospholipid–metal interaction was first reported by Miller [13] and later by Nelson [14] and by Chen and Abruña for adsorption on mercury and gold amalgam [9]. This approach was later extended to single crystal gold surfaces [8,10]. In these reports the phospholipid molecules formed either adsorbed bi-layers [8,10] or mono-layers [9] which changed its orientation towards

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the metal surface depending on the applied potential. Contradictory results were reported by Keller and Kasemo who described an adsorption of intact vesicles on oxidized gold surface [5].

In this paper we present the results of systematic study of the effect of external electrical field (applied potential) on the DOPC vesicle fusion on gold and on the mobility of adsorbed DOPC molecules. The electrochemical quartz crystal microbalance (EQCM) and fluorescence recovery after photobleaching (FRAP) measurements were used to elucidate the form of adsorbed phospholipid film on gold surface at different potentials.

## 2. Experimental

### 2.1. Chemicals

The 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids; Alabaster, USA); Rhodamin Red-X 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rhodamin Red-X DHPE) (Molecular Probes; Leiden, The Netherlands); sodium perchlorate monohydrate (Fluka, p. a. quality); perchloric acid (Aldrich, 70%) and chloroform (Aldrich, HPLC grade $\geqslant$99.8%) were used as received. The water used in all experiments was of Millipore Milli Q quality.

### 2.2. Preparation of solution of unilamellar lipid vesicles

The solution of unilamellar phospholipid vesicles was prepared as described in Ref. [7]. First the mixture of Rhodamin Red-X DHPE and DOPC in chloroform in molecular ratio $1{:}10^6$ was prepared. The solvent was evaporated under flowing nitrogen. Dry mixture of phospholipids was resuspended in 0.1 M solution of sodium perchlorate to obtain lipid suspension of lipid concentration 1 mM. This suspension was sonicated for 15 min, yielding a solution of small unilamellar vesicles of the hydrodynamic radius approximately 20 nm as determined by Dynamic Light Scattering (DLS) [7].

### 2.3. Measurement of DOPC adsorption

The adsorption of DOPC was studied on a polycrystalline gold forming one of the contacts of a 10 MHz AT-cut quartz crystal (ICMFG Oklahoma, USA). The gold substrate was of keyhole shape; the gold was sputtered directly on quartz without Ti or Cr under-layers. Piezoactive area of the crystal was 0.22 $cm^2$. The gold contact of the quartz crystal was in all experiments connected as a working electrode. The experiments were performed in a one-compartment glass cell using three electrode arrangement with Pt wire auxiliary and Ag-pseudoreference electrode. The potential of the Ag-pseudoreference electrode was checked with respect to the saturated Ag/AgCl electrode and all potentials were recalculated to the Ag/AgCl scale. The potential of the gold electrode was controlled using PAR 263 A potentiostat. The frequency change of the quartz crystal was monitored using in house built EQCM circuitry designed according that of Bruckenstein [15]. Crystal impedance was measured using 8712ET RF Network Analyzer (Agilent Technologies). The surface of the gold was cleaned by cycling between $-0.2$ and 1.4 V in 0.1 M solution of perchloric acid prior to each adsorption experiment. The cycling was performed until a steady cyclic voltammogram and $\Delta f$ vs. $E$ curves identical with those presented by Bruckenstein and Shay [16] were obtained. The clean crystal was then immersed into 1 ml of 0.1 M solution of sodium perchlorate left to equilibrate for 5 min at given potential and then 450 μl of solution of unilamellar DOPC vesicles was added.

### 2.4. Determination of diffusion coefficient

The lateral mobility of the adsorbed phospholipid molecules was studied using fluorescence microscopy. Diffusion coefficient of DOPC adsorbed on gold was determined by the fluorescence recovery after photobleaching (FRAP) [17] method. The mobility of the DOPC vesicles in solution was determined by the fluorescence correlation microscopy (FCS) approach. FCS was also used to determine the radius of the bleached spot necessary to evaluate the FRAP data. For this purpose a FCS experiment on a 5 nM rhodamine 6G aqueous solution with known diffusion coefficient $D = 2.8 \times 10^{-8}\ m^2\ s^{-1}$ was performed. For the used optical setup the radius of the photobleached spot $\omega$ was determined to be $\omega = 2.375 \times 10^{-7}$ m [7].

The fluorescence microscopy measurements were performed using a Confocor 1 (Carl Zeiss GmbH, Jena; Evotec Biosystems GmbH, Hamburg) consisting of a confocal microscope Axiovert 100 containing a 40x water immersion objective, a 1.2 numerical aperture, and an He–Ne laser as the excitation source (used excitation wavelength 543 nm). The emission was filtered via a band-pass filter transmitting light between 550 and 600 nm. The fluorescence signal was detected by an avalanche photodiode (EG-SPCM 200). In the case of FCS measurements the signal was processed online with an ALV-500E digital correlator board (ALV, Langen, Germany).

The samples for measurement of diffusion in adsorbed phospholipid films were prepared by injection of unilamellar vesicle solution (see above) into electrochemical cell containing clean gold electrode polarized at given potential. The adsorbed film was formed for 90 min. The vesicles were then washed out of the cell by 0.1 M solution of sodium perchlorate. The washing of vesicles proceeded in stepwise manner to ensure the control of the electrode potential during entire procedure.

## 3. Results and discussion

### 3.1. Quartz crystal microbalance experiments

Typical frequency change vs. time dependence of the quartz crystal following the injection of DOPC vesicles into

the system is shown in Fig. 1a. The crystal frequency monotonously decreases which indicates that the crystal mass may increase due to phospholipid adsorption. The total frequency changes obtained in DOPC adsorption experiments performed at different potentials are summarized in Fig. 1b. The applied potential has an effect on the total frequency change following the DOPC vesicle injection. The observed $-\Delta f$ vs. $E$ curve shows pronounced maximum at potential of ca. 50 mV (vs. Ag/AgCl). It ought to be noted that the potential at which the maximum frequency change was observed corresponds to the potential of zero charge (*pzc*) of the bare gold in perchlorate containing solutions [18]. At potentials positive to the *pzc* the frequency change caused by phospholipid adsorption decreases until it reaches a plateau at ca. 250 mV (vs. Ag/AgCl). At potentials more negative to the *pzc* of bare gold the frequency change also decreases, however, no plateau formation is observed within studied potential range. The monotonous course of the QCM frequency signal, which is in accordance with the behavior observed previously on noble metal substrates [6], indicates that the rupture of the vesicles on the electrically charged gold substrate has (in given experimental arrangement) comparable or faster kinetics than the adsorption of intact vesicles.

The observed frequency change may be in the first approximation converted into mass change using Sauerbrey's equation:

$$\Delta f = \left(\frac{2nf_0^2}{\rho^{1/2}\mu^{1/2}}\right)\Delta m \quad (1)$$

where $n$ is the number of harmonics, $f_0$ is the fundamental frequency of the crystal and $\rho$ and $\mu$ represent the density and shear modulus of the quartz.

The mass change values obtained from the measured data using Eq. (1) range between 0.328 μg cm$^{-2}$ (at $E =$ −100 mV) and 0.459 μg cm$^{-2}$ (at $E > 250$ mV) with a maximum of 1.148 μg cm$^{-2}$ (at $E = 50$ mV).

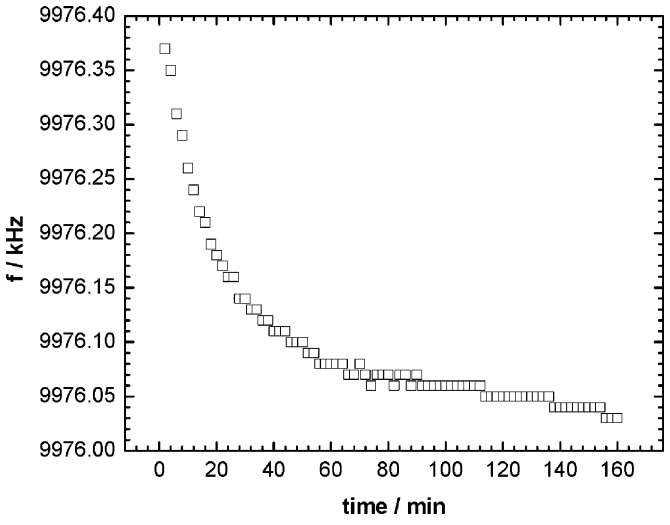


Fig. 1a. Typical frequency change vs. time curve during adsorption of DOPC molecules. The data were recorded for adsorption of DOPC molecules on gold at potential 50 mV.

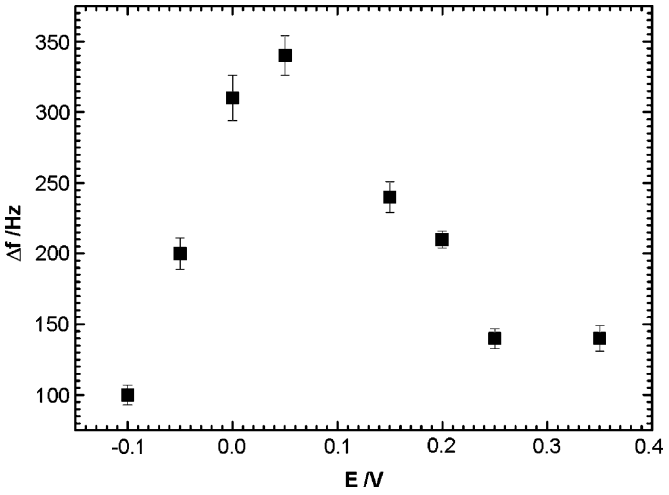


Fig. 1b. Total frequency change at different potentials. The data were extracted from EQCM experiments.

The apparent surface coverage observed for adsorption at *pzc* gives reasonable agreement with that reported by Keller and Kasemo for intact vesicle adsorption [5]. The apparent surface coverage, which we observed at potentials sufficiently departed from *pzc*, on the other hand, seems to conform to the model expecting the formation of "bi-layer-like" system [19]. The apparent surface coverage observed at negative potentials is also comparable with that reported for bi-layers formed at an oxide substrate [5]. Such a behavior indicates, that the intact DOPC vesicles may adsorb at the gold substrate if the vesicles get into contact with substrate bearing negligible electrical charge. That would restrict the vesicle adsorption only to potentials close to the *pzc*. A contact of vesicles with gold surface either positively or negatively charged results in fusion of vesicles into a "bi-layer-like" or "multi-layer-like" system. Complete fusion of the vesicles into such a system can be observed on positively polarized substrate when the surface charge density exceeds ca. 8 μC cm$^{-2}$ (onset of the plateau in Fig. 1b). In the potential region between *pzc* and the plateau onset potential the mass change data may be interpreted in terms of a coexistence of both vesicles and "bi-layer-like" system on the surface. Similar process can be expected also on negatively charged surface, the transition between vesicle and bi-layer formation is, however, less pronounced.

This model of the DOPC adsorption on gold seems to be both plausible and in accordance with previously published results. Despite that the observed EQCM data do not represent unambiguous reading of the mass of the adsorbate, since the Sauerbrey equation holds only if there is no change in the visco-elastic properties of the medium in direct contact with the oscillating crystal. Therefore the validity of the Sauerbrey equation needs to be substantiated by measuring, e.g., the impedance of the crystal during

the experiment. Approximating the oscillating crystal with an equivalent circuit depicted in Fig. 2 [20] we can say that the applicability of Sauerbrey's equation is justified only if there is no change in real part of the oscillating crystal admittance during experiment. In the case when real part of the crystal admittance varies the Sauerbrey's equation returns either an underestimate (real part of the crystal admittance decreases) or an overestimate (real part of the crystal admittance increases) of the actual mass change. A typical course of the $\Delta f_{\mathrm{fwhm}}$ (full-width at half maximum) of the real part of the oscillating crystal admittance during a vesicle adsorption experiment is shown in Fig. 3a. The dependence of the total change of the $\Delta f_{\mathrm{fwhm}}$ for sorption experiments carried out at different potentials is shown in Fig. 3b.

As follows from Fig. 3 the real part of crystal admittance increases during the exposition of gold to DOPC vesicles. This trend indicates that the visco-elastic properties of the medium in contact with the gold surface, change upon exposure to phospholipids. Since the vesicle concentration is too low to alter the bulk viscosity of the liquid phase, the viscosity change most likely results form the viscosity of the adsorbed phospholipid film. The observed $\Delta f_{\mathrm{fwhm}}$ values

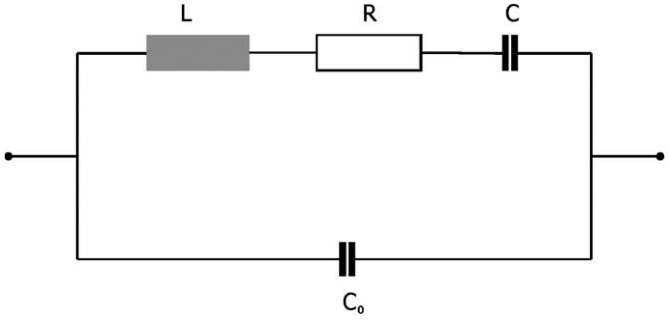


Fig. 2. Butterworth–Van–Dyke equivalent circuit of oscillating crystal.

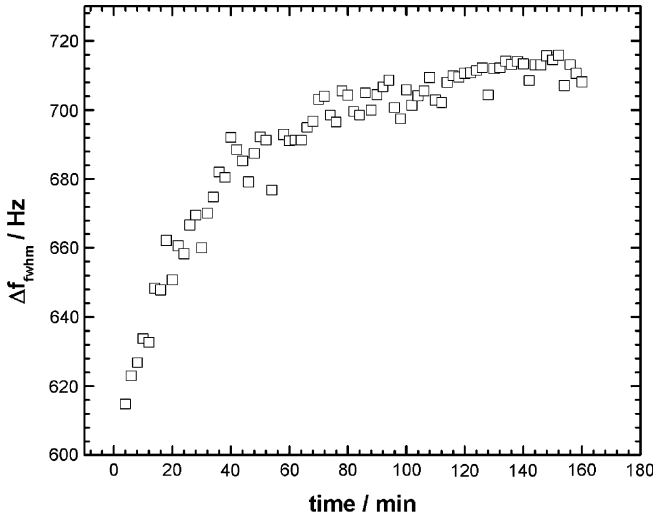


Fig. 3a. The characteristic $\Delta f_{\mathrm{fwhm}}$ vs. time curve recorded during DOPC adsorption at constant potential. Data were obtained at $E = 50$ mV.

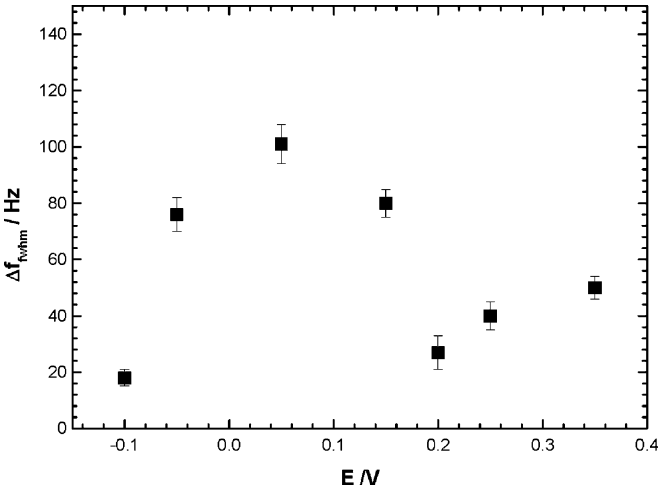


Fig. 3b. The total change of $\Delta f_{\mathrm{fwhm}}$ as a function of the applied potential.

indicate that the $R$ increases by 3–10% (the initial $\Delta f_{\mathrm{fwhm}}$ was between 600 and 800 Hz) with pronounced maximum at $E$ equal to 50 mV, i.e. at the *pzc* of bare gold. It means that the phospholipid layers adsorbed on charged gold are more rigid than those on substrate bearing no net electrical charge.

The degree by which the EQCM deviates from the actual adsorbed phospholipids mass can be obtained be comparing the experimental EQCM readings with simple predictions based on the literature data published on DOPC adsorption previously. Assuming that the adsorption intersection of DOPC molecule is primarily affected by the actual surface charge density of the support we can use in the first approximation the characteristics of a bi-layer formed on glass [21] to check the reliability of our EQCM readings measured for the adsorbed system formed at potentials sufficiently negative to *pzc* (the adsorption cross-section of the DOPC molecule is in this case 72.1 $\mathrm{Å}^2$, and the bi-layer thickness is approximately 36 Å) [21]. Anticipating the relative coverage of the substrate to be comparable with that reported by Lipkowski's group [8] (i.e. ca. 80%) we obtain theoretical surface coverage of a DOPC bi-layer 0.304 $\mu g\,cm^{-2}$. This value agrees within 8% with our value based on the EQCM data for films adsorbed on negatively charged gold. The experimental EQCM data are as expected an overestimate of actual film mass. The distortion caused by the viscosity of the DOPC lipid layer is not a dramatic one and the EQCM reading is acceptable estimate of the adsorbed lipid layer mass.

In the case of films formed at potentials sufficiently positive to *pzc* the comparison of theoretically calculated bi-layer mass and experimentally found EQCM data is less satisfactory (discrepancy ca. 50%). This discrepancy can be attributed to lower rigidity of bi-layer-like films formed on positively charged substrate (the increase of the real part of the crystal admittance is 2–3 times higher than in the previous case). Similar problems one encounters attempting

to analyze the frequency change data connected with adsorption at *pzc*. Anticipating the given vesicle radius and maximum attainable surface coverage corresponding to close cubic packing (ccp) arrangement one obtains theoretical mass of DOPC vesicles equal to 0.719 μg $cm^{-2}$. The observed difference between the theoretical and experimental surface coverage values can be attributed to the viscosity of the adsorbed layer.

### 3.2. Fluorescence measurements

Assuming the electrostatic control of the DOPC adsorption process suggested by QCM data one has to expect that the electrostatic interactions between charged substrate and adsorbed phospholipids molecules affect the properties of the adsorbed film like, e.g. lateral mobility of the molecules within the adsorbed film. That lateral mobility can be assessed by measurement of, e.g., lateral diffusion of the fluorescence dye labeled DOPC molecules.

The applicability of fluorescence based methods to study the processes proceeding in vicinity of metal surfaces is complicated by the energy transfer between excited state of the dye and surface plasmons of the metal surface (so called Foerster type quenching) which negatively affects the actual lifetime of the fluorescence label [22,23]. The theory of the Foerster type quenching predicts that no fluorescence of the label should be observable from the fluorescence molecules closer to the metal surface than 2 nm [23,24]. Certain controversy, however, exits on the thickness of the layer with suppressed fluorescence, which may according to literatures run from as little as 2 nm [23–25] to up to 50 nm [22,26]. The DOPC layer prepared according to procedure described above gave measurable fluorescence from entire gold surface. Thus, we were able to apply the method of fluorescence recovery after photobleaching for the characterization of the mobility of labeled lipid molecules within the adsorbed systems. The fluorescence intensity also varied laterally on the surface (usual fluctuation of the measured signal was within 20%) due to the inhomogeneity of the gold surface. Keeping in mind that morphology of the adsorbed DOPC film proposed from the QCM data should change from a bi-layer-like arrangement to the intact vesicles and back to a bi-layer-like arrangement as the electrode potential changes from negative to positive values one has to assume that the thickness of the adsorbed DOPC layer should vary between approximately 5 nm (bi-layer-like arrangement) and 20 nm (intact vesicles). Therefore we have to assume that the Foerster type quenching in our experimental arrangement does not cause complete quenching of the fluorescence in volume which is expected to be occupied by the adsorbed DOPC. It should be mentioned, that in contrast to intact DOPC bi-layers adsorbed on, e.g. mica [7], the determination of diffusion coefficient in DOPC film on polarized gold by confocal fluorescence spectroscopy (FCS) failed due to extensive photobleaching at laser power of the order of tens of microwatts. This finding indicates that the lateral lipid mobility in our systems is significantly slower than in intact "liquid-crystalline" bi-layers [7]. Thus, diffusion coefficients were determined by fluorescence recovery after photobleaching (FRAP) experiments. Typical curve recorded at FRAP experiment is shown in Fig. 4a. The observed data were normalized and fitted to the function:

$$F(t) = F_0 + (F_\infty - F_0) \cdot (1 - e^{-kt}), \quad (2)$$

where $F(t)$ represents the fluorescence intensity measured at time $t$, $F_0$ means fluorescence intensity measured immediately after bleaching, $F_\infty$ is the asymptotic value to which the fluorescence intensity recovers and $k$ is first-order constant of fluorescence recovery [17].

The diffusion coefficient ($D$) of the DOPC can be evaluated from the halftime of the recovery function:

$$D = 0.224 \cdot \omega^2 / t_{1/2}, \quad (3)$$

where $\omega$ is radius of the bleached spot (in our case $\omega = 2.375 \times 10^{-5}$ cm).

It needs to be stressed that the diffusion coefficients calculated using Eq. (3) represent formal values physical meaning of which may differ depending on the actual mechanism of the fluorescence recovery (see below). The effect of the applied potential on the lateral diffusion coefficient of the adsorbed DOPC molecules is shown in Fig. 4b. The observed values of the diffusion coefficient range between 1.0 and $2.5 \times 10^{-12}$ $cm^2$ $s^{-1}$. These values are by 4–5 orders of magnitude slower than those observed for DOPC vesicles in solution ($1.3 \times 10^{-7}$ $cm^2$ $s^{-1}$; according to FCS measurement) or for "liquid-crystalline-state" DOPC bi-layers on mica [7]. They may be compared to diffusion coefficient estimates for "gel-state" DPPC bi-layers at room temperature ($<1.0 \times 10^{-10}$ $cm^2$ $s^{-1}$) determined by FRAP experiments [27].

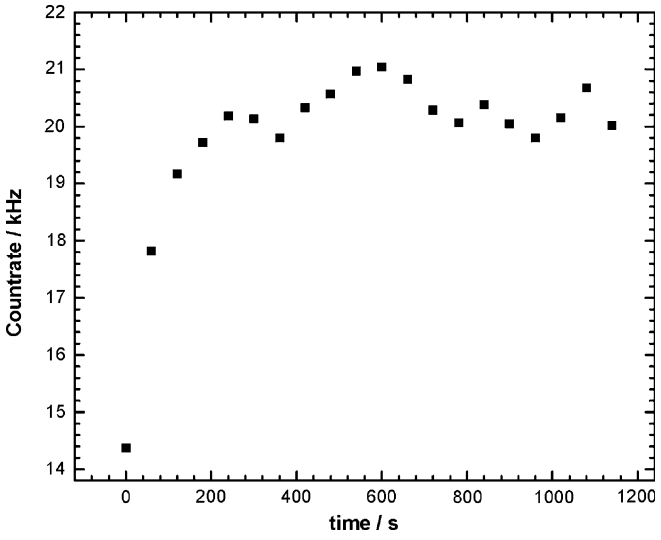


Fig. 4a. Typical fluorescence recovery curve recorded during FRAP experiments. The data were recorded on DOPC film adsorbed on gold at potential −50 mV. The potential was held constant during whole experiment.

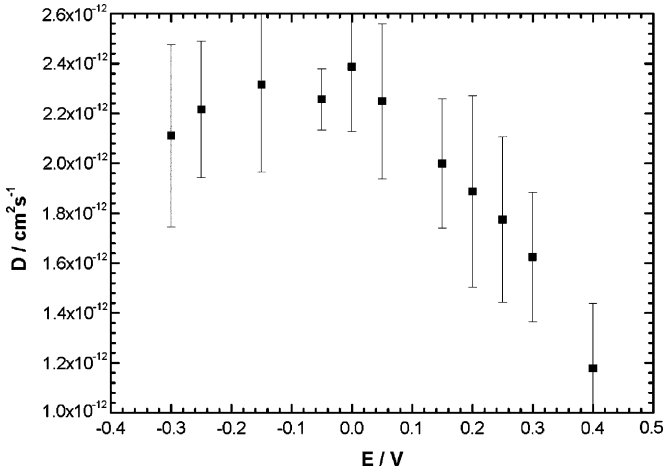


Fig. 4b. Lateral diffusion coefficients of DOPC molecules in the adsorbed film at different potentials. The data were extracted from FRAP experiments.

Low values of the measured diffusion coefficients seem to be in accordance with the interpretation of the QCM data suggesting the formation of bi-layer like arrangements by interaction of DOPC vesicles with charged gold surface. Keeping in mind that the lifetime of the fluorescence decreases with decreasing distance from the metal surface due to Foerster type quenching, one has to assume that the measured fluorescence signal is dominated by the fluorophores most departed from the metal surface. In the similar manner one has to expect that mobility of the DOPC molecules in double-to multilayer arrangements increases with increasing multilayer thickness due to weaker interactions with the surface. Assuming electrostatic nature of the interaction between the DOPC and substrate (which changes inversely with square of the distance between DOPC and surface) we have to expect that the mobility of the DOPC in a multilayer will drop dramatically when the number the of layers approaches 2. Therefore low values of diffusion coefficients may be taken as an indirect evidence of bi-layer-like arrangement formation.

As follows from Fig. 4b the FRAP based diffusion coefficients increase with decreasing charge of the substrate and show a maximum at potentials close to *pzc*. Such a comparison needs to be taken with care since we cannot assume the same mechanism of the fluorescence recovery for the bi-layer-like and vesicle based layers. The situation in the case of the adsorbed bi-layers is relatively simple. We have to assume that the measured fluorescence originates exclusively from the top layer of the arrangement (please note that the DOPC used in the study is head labeled and head also represents the active site for the interaction with the surface) and the most likely mode of lateral movement of the DOPC molecules in the adsorbed layer is a simple diffusion within each individual layer. Therefore we may expect that the measured diffusion coefficient represents mobility of the molecules in the top layer only and contains no direct information about the bottom DOPC layer.

The physical meaning of the apparent diffusion coefficient in the case of adsorbed vesicles is less evident. As follows from geometric analysis of the experiment one sees that the radius of the bleached spot (240 nm) is significantly larger than that of the individual vesicle (20 nm). In contrast to the bi-layer-like arrangements the fluorescence recovery of the adsorbed vesicles may originate either from diffusion of DOPC molecules within a vesicle (intra-vesicular transport) or from the mobility (e.g. rotation) of entire vesicles (inter-vesicular diffusion). In addition to it, one should keep in mind, that the fluorescence lifetime of the labeled DOPC molecules in the vesicle depends on the distance of the fluorophore from the metal surface. Consequently we may reasonably expect that the diffusion coefficient based on FRAP data will be dependent on the vesicle size. The measured diffusion coefficient then does not represent a true diffusion coefficient, but rather an effective proportionality constant. Therefore direct comparison of the formal diffusion data obtained for vesicle based and bi-layer-like systems should be avoided.

Despite the ambiguity of the diffusion related data one can compare the lateral mobility of DOPC in bi-layer-like systems formed at positively and negatively charged surface. As follows from the Fig. 4b the DOPC is less mobile in bi-layer-like systems formed at positively ($D \approx 1.0 \times 10^{-12}$ cm$^2$ s$^{-1}$) charged gold than in those formed on negatively ($D \approx 2.0 \times 10^{-12}$ cm$^2$ s$^{-1}$) charged gold. The observed tendency in lateral mobility reflects the difference in coulombic interactions between the substrate and adsorbed DOPC. The lower mobility of DOPC in bi-layers formed on positively charged surface ($E > pzc$) reflects probably higher localization of the negative charge on the phosphate group of the phospholipid head. It also shows that the information about rigidity of the adsorbed layers extractable from EQCM measurements reflects rather the rigidity of the DOPC molecule dangling into the solution than the strength of the interaction between DOPC and gold substrate.

## 4. Conclusions

The DOPC vesicles spontaneously fuse into a bi-layer resembling arrangement when they are in contact with positively or negatively charged gold surface. A contact of DOPC vesicle with uncharged gold surface results in adsorption of intact vesicles. The arrangements formed on negatively charged surface are more rigid than those formed on positively charged gold surface. In the case of negligible surface charge on the gold (i.e. at potentials close to *pzc*) the EQCM data do not allow unambiguous interpretation of frequency change data. The lateral mobility of DOPC molecules in adsorbed layers is 4–5 orders of magnitude lower than that in fluid DOPC bi-layers or in solution. This behavior reflects different strength of the DOPC substrate interaction due to different localization of the charge on the DOPC adsorbed on positively and negatively charged substrate.

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