Supported phospholipid bilayers with chlorophyll for optoelectronic devices

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Supported phospholipid bilayers (SPB) with chlorophyll *a* (Chla) constitute a new approach in using the lipids and Chla as promising biological materials for optoelectronic applications. The objective of the present work was to obtain supported Chla-lipid membranes by using liposomes with Chla incorporated in the bilayer and to characterize the structures by using optical methods. Chla, was used as spectral marker and molecular sensor in VIS absorption and emission studies and the kinetics of the lipid deposition was investigated by using an optical based method - Surface Plasmon Resonance (SPR). The type and dimension of lipid vesicles and the presence of Chla proved to be important factors for the kinetics of phospholipid vesicles deposition.

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1. Introduction

The liposomes, popular membrane model systems, can be prepared using natural constituents and the liposome membrane forms a bilayer structure which is almost identical to the lipid portion of the natural cell membranes. The similarity between liposomes and natural membranes can be increased by chemical modification of the liposome membrane and can be exploited in different applications, mimicking successfully the behaviour of natural membranes [1]. The liposomes with Chlorophyll a (Chla) incorporated in the lipid bilayer are excellent models for bio-membranes, specifically for photosynthetic membranes and were successfully used to study the influence of different agents on the bilayer structure at molecular level [2.3]. The strong visible absorption and fluorescence of Chla allow its use as a sensor for the interactions at molecular level in model membranes, as well as a photo-sensitizer in light conversion devices [4]. The deposition of liposomes on solid surfaces, by different techniques, conducted to a different approach: supported lipid bilayers (SLB). These models of biological membranes deposited on solid supports have become very popular, both for studying basic membrane processes and possible biotechnological applications [5, 6]. A multitude of methods, based mainly on optical and mechanical sensing principles, have been developed, e.g. atomic force microscopy (AFM) [7], Surface Plasmon Resonance (SPR) [8-11], quartz crystal microbalance [12], fluorescence [13]. The SPR investigation method is a valuable kinetic method that offers information on the processes of interaction between substrates and analytes and liposomes are suitable models for the investigation [8-11]. Due to the difficulty of the studies on liposomes deposited on gold surfaces, different approaches have been done to evaluate such surfaces, e.g. immobilization on ordered structures of the bilayered lipids [12].

The objective of our work was to obtain Supported Phospholipid Bilayers (SPB) with Chla incorporated and to characterize the structures by using optical methods: absorption and emission in VIS and SPR. Chla was used successfully as a marker for monitoring the SPB formation.

Once Chla was incorporated in the phospholipid bilayers, the direct interaction of the supported lipid artificial membranes with the SPR gold surface was studied. The Chla proved to be a good sensor for the lipid structures and a promising partner for lipids in artificial light bioconversion systems.

2. Experimental (materials and methods)

2.1 Materials and reagents

Dimyristoyl phosphatidylcholine (DMPC, Tc = 23^0C) and dipalmitoyl phosphatidylcholine (DPPC, Tc = 41.4-41.7 ^{0}C) were purchased from Sigma Aldrich (Germany) and used without further purification. The phosphate buffer solution (PBS) (NaH2PO4-K2HPO4), pH = 7.3 - 7.4 1/15M was prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity $\leq$ 0.1 μS cm-1). Chla was extracted from fresh spinach leaves by a chromatographic method using a powdered sugar column and organic solvents (petroleum ether, n-propanol and ethanol) as mobile phase, according to the procedure of Strain and Svec, (1966). The purity and concentration of Chla were monitored by using VIS absorption and emission spectra of Chla in diethyl ether and spectral criteria.

2.2 Liposomes preparation

Liposomes were prepared according to the thin-film hydration method [14] with little modifications. The lipid DMPC was dissolved in chloroform together with Chla (Chla/lipid molar ratio = 1/100). Chloroform solvent, from solutions containing lipid and Chla in specified concentration, was removed in a rotary evaporator Heildolph 94200, 60-90 rpm (Bioblock Scientific, Illkirch Cedex 67403 France), using a vacuum pump. The lipid films were hydrated, during 1 hour, in PBS pH = 7.3 – 7.4. The resulted suspensions of multilamellar vesicles were mechanically mixed with Vibrax stirrer, 200 rpm (Milian USA, OHIO 43230 USA) and then sonicated, 15 min, using a titanium probe sonicator Hielscher, UP 100H (Hielscher Ultrasonics GmbH, 14513 Teltow, Germany) to produce small unilamellar vesicles (SUVs) as stated in the literature [14]. Spectral criteria in UV-VIS range of absorption developed for Chla liposomes were used to rapid check the dimension and unilamellarity of liposomes [15]. All the operations have been performed above the critical temperature (Tc) of the phospholipids (lipids being in the liquid crystal phase, the probability to obtain liposomes without defects is higher). The suspensions were centrifuged 45 min, 20000g, with SIGMA 2-16 K centrifuge (SciQuip Ltd Merrington, Shrewsbury Shropshire SY4 3QJ UK) in order to remove the titanium traces and to obtain homogeneous vesicle populations. Only the supernatants were used in investigations. All the operations have been done in the dark, in order to prevent Chla photo-oxidation.

2.3 SPB formation

Chla-SPBs have been obtained by the vesicle spreading (adsorption) method. A drop (50-100 µl) of Chla-SUVs suspended in PBS was spread on different solid supports: silicon wafers and semitransparent ITO glass. The solid supports were washed with ethanol and acetone and dried under a stream of nitrogen gas, prior the spreading of the liposome suspension. The formation of Chla-SPBs was accomplished by keeping the supports, in the dark, at room temperature, for minimum 6 hours.

2.4 Spectral measurements

The optical absorption spectra were obtained on a double beam UV-VIS spectrophotometer Lambda 2S Perkin Elmer & PECSS software. Fluorescence experiments in steady state were done using Perkin Elmer LS 55 fluorescence spectrometer (Waltham, Massachusetts 02451, USA), fitted with a biokinetic accessory providing continuous monitoring of the temperature in the cuvette and stirring of liposome suspension. The LS 55 fluorescence spectrometer was equipped with R928 photomultiplier, red-sensitive photomultiplier fitted for collection of emission data above 630 nm. The excitation spectra are automatically corrected. Slits of 5 nm were used both for excitation and emission. Wavelength accuracy of the instrument is ± 1.0 nm and wavelength reproducibility is ± 0.5 nm. An ultra thermostat/circulator Julabo ED 5 (Julabo Labortechnik GmbH 77960 Seelbach/Germany) with accuracy of ± 0.3 K ensured the desired constant temperature in the cuvette, during all fluorescence measurements. A front face geometry was used, collecting the emission at 45^o from the solid support. Procession of all spectral data has been done using the software Origin 8.0.

2.5 The SPR measurements

The SPR measurements were done using a Autolab Springle instrument from EcoChemie. The SPR laser 670nm spot was focused on the gold surface (having a 2 mm^2 area), on which the experimental data were determined. Prior to the beginning of measurements, the cuvette was rinsed with 100 µl of distilled water. The minimum SPR signal obtained during the rinse process with distilled water was used for setting the range scale for the upcoming measurements. Successively, the automated pipette of the SPR instrument was used to insert 100 µl of Chl liposome suspension into the apparatus cuvette. The processing of the sample's SPR data was done using the EcoChemie's Kinetic Evaluation 5.0 software.

The SPR measurements were done at or above the lipid phase transition temperature (T_c) for each liposome type. In some cases the temperature influence was monitored.

The experimental measurements done with Autolab Springle equipment where processed in order to remove experimental variations. A blank measurement was done using only the buffer, afterwards using it as blank subtraction to remove the buffer signal in all the other experiments. Furthermore, the measured values were normalized in order to set a baseline to 0 milli degree (m^0) for the measured response of the SPR.

2.6 DLS measurements

The hydrodynamic diameters of the liposomes, suspended in buffer, were measured by dynamic light scattering (DLS), using a Zetasizer Nano ZS from Malvern Instruments. Light scattering measurements have been performed at 25^oC. Hydrodynamic measurements were carried out at 90^o angle, without dilution of the samples.

3. Results

Si wafers proved to be the best solid support for Chl-SLB formation. ITO proved to be also a good support, having the advantage of high transparency for VIS absorption spectra measurements.

The Chla absorption and emission spectra have been recorded in order to monitor the deposition of Chla-liposomes on solid supports. In Fig. 1 are presented the absorption spectra of Chla both in the liposome suspension and on ITO covered by Chla-SPB.

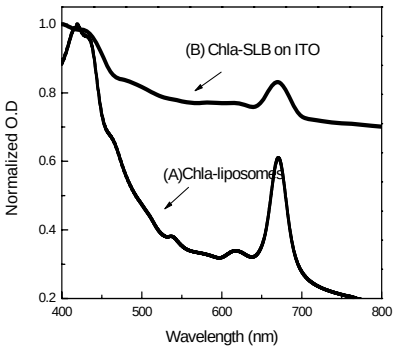


Fig. 1. VIS absorption spectra of Chla incorporated in DMPC liposomes (A) and DMPC SPB on ITO (B).

All O.D. were normalized as regarding the maximum in the respective absorption spectrum. The spectra are very similar as regarding the shape and peak positions. The difference (regarding the normalized O.D.) is a consequence of the different extent of light scattering and possible reflections. The position of the characteristic red absorption maximum of Chla is at 670 nm in both spectra, leading to the conclusion that after SPB formation, Chla is deposited on the support together with the phospholipids and much more, Chla remains in the same position in the supported lipid bilayer as in liposomes. Also the emission spectra of Chla-liposomes in suspension, as compared with the Chla-SPBs on ITO, presented in Fig. 2, supports the above conclusion.

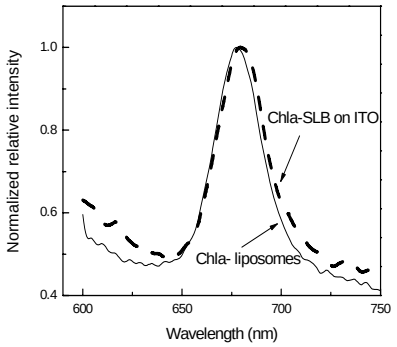


Fig. 2. Emission spectra (λexc = 430 nm) of Chla incorporated in DMPC liposomes (continuous line) and DMPC SPB on ITO (dashed line).

In order to find about the kinetics of Chla - liposomes adhesion process to solid substrates, Surface Plasmon Resonance (SPR) experiments have been done. As already mentioned, the Autolab Springle SPR instrument used in our experiments is equipped with 2 mm^2 area of gold surface. Therefore, in SPR experiments, a different type of solid support was used. In Fig. 3 (a and b) the SPR response (in mili degrees) is presented in the case of the 2 types of Chla liposomes: Chla-DMPC and Chla-DPPC liposomes, repectively.

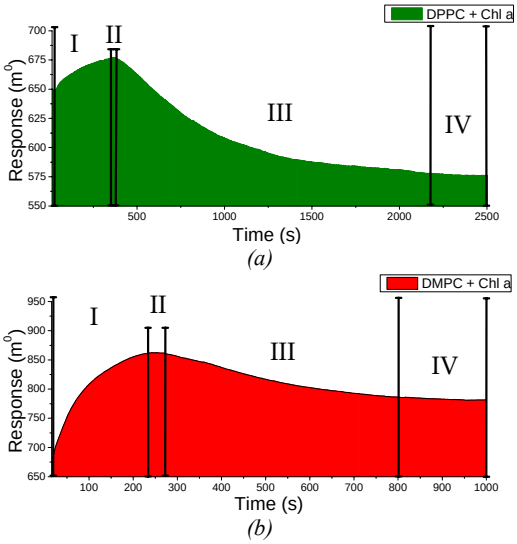


Fig. 3. SPR signal for Chla-DPPC (a) and Chla-DMPC (b) at lipid transition temperature.

As a common feature of the SPR response over a time period of 20-40 minutes, a kinetics with 4 phases for both DMPC and DPPC Chla liposomes has been observed. In the Phase I, the SPR response is presenting a rapid increase, followed by a short stationary phase (II). A slow decrease of the SPR response corresponds to the Phase III and finally, a "plateau" is reached (Phase IV). Fig. 4 presents comparatively the SPR signal in the case of the 2 types of Chla - liposomes, over the whole period of the experiments (2 hours).

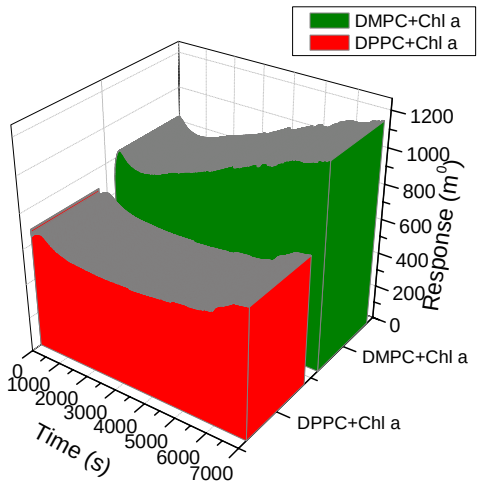


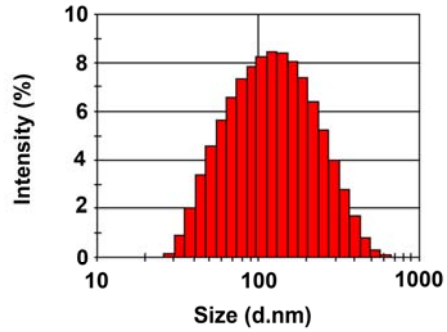
Fig. 4. SPR signal for Chla-DPPC and Chla-DMPC over a period of 2 hours.

A continuos increase of the SPR signal can be observed, suggesting a continuous deposition on the surface after the corresponding "plateau" phase for each type of liposomes.

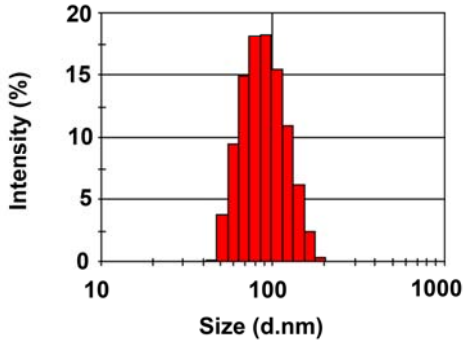
A general overview over the kinetics for the two types of Chl a liposomes, reveals faster process in the case of

Chla - DMPC liposomes than in the case of Chla - DPPC, with a steeper increase of the angular response, as seen in Fig. 3.

In order to explain the observed difference in the SPR response for the two types of liposomes, the size estimation of liposomes was done by dynamic light scattering (DLS). The size distribution for both types of liposomes (Chla-DPPC and Chla-DMPC) determined by DLS is shown in Fig. 5. Assuming a spherical shape for the liposomes, the diameter varied over a quite large range in the case of Chla-DMPC liposomes. The average diameter was 103 nm for Chla-DMPC liposomes and 86.2 nm for Chla-DPPC liposomes. Therefore, the Chla-DPPC liposomes are smaller as compared with Chla-DMPC liposomes.



(a)



(b)

Fig. 5. Size distribution of Chla-DMPC (up) and Chla-DPPC(down) liposomes in PBS solution obtained from DLS experiments.

The dependence of the SPR signal upon temperature is presented in Fig. 6, in the case of Chla – DPPC liposomes. The temperature of 25,8^{0}C is below the T_c of DPPC and 42,8^{0}C is just above T_c of the lipid (T_c of DPPC is 41.4- 41,7^{0}C).

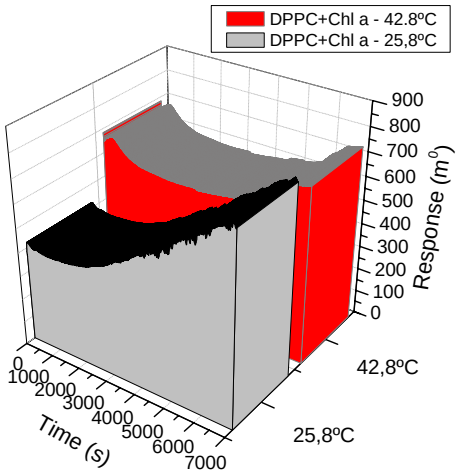


Fig. 6. SPR signal for the DPPC+Chl a liposomes with respect to the temperature.

4. Discussion

The adsorption and fusion of small unilamellar vesicles is one of the easiest and versatile means for forming solid supported phospholipid bilayer [7]. The most accepted model for SPB formation considers different events that could take place: interaction of vesicles with other vesicles and with the substrate; fusion and rupture of vesicles/ rupture of individual vesicles; transformation of surface bound vesicles in a continuous supported membrane [5]. Previous experiments [3, 16] lead to the conclusion that Chla is incorporated in the phospholipid bilayers in monomeric form, with the porphyrin ring in the vicinity of lipid polar heads, at the interface with water. The absorption red peak position and emission maximum of Chla remained unchanged during the deposition of the phospholipid bilayer on ITO, in the case of both DMPC and DPPC. This means that the process of lipid bilayer deposition/adhesion does not induce changes on the pigment positioning within bilayer. It is possible to do also the affirmation that the whole process of SPB formation (fusion/rupture/ adhesion) does not induce major defects in the phospholipid bilayer with consequences on the Chla incorporation.

In order to find the optimum liposome concentration (number of liposomes/ml or lipid molar concentration) for SPB formation, the dependence of the relative intensity of Chla emission (λexc=430 nm, λem= 678 nm) in Chla-SUVs upon SUVs concentration in the suspension was measured. The quenching of Chla fluorescence was observed when lipid molar concentration was higher than 0.3 mM. Among the possible mechanisms, responsible for Chla fluorescence quenching, the transfer of energy is more probable in case that SUVs are close enough or even are suffering a fusion/rupture process. The lipid concentration of 0.5 mM was used for SPB formation in all done experiments; at this concentration the quenching

of fluorescence was notable and therefore the probability of vesicle fusion/rupture was high.

The gold surfaces are reported as poor substrates for supporting the liposomes. In order to use investigation techniques based on gold-surface, supplementary functionalization was needed [17]. The functionalization was done by using a alkanethiol, or a hydrophobic hairy-rod polymer [18], using charged molecules [19] or by anchoring lipid bilayer on a hydrophilic surface [20, 21]. The SPR responses presented in Figures 3, 4 and 6 are pleading for the fact that the Chla - liposomes were deposited on gold-surface, forming SPB. A sort of "functionalization" was obtained by incorporation of Chla in the phospholipid bilayer. The changes in the overall structure and electrostatic charge of the liposomes due to the incorporation of chlorophyll in the phospholipids bilayer resulted in overall electrostatic change in the bilayer and its interaction with the gold substrate.

The model of the SLB formation, reviewed by E.T.Castellana et al [5], was used for the interpretation of the SPR experimental data measured when using Chla-DPPC and Chla-DMPC liposomes.

The initial phase (I), presenting an increase of the SPR signal, could be well associated to the adsorption of vesicles on the gold surface. A process of interaction and fusion can be associated to the short stationary phase, corresponding to the second phase (II). After the fusion, a release of the internal mass of the liposmes is expected, due to the bilayer rupture. As expected, a decrease of the SPR signal is observed - the third marked region (III) for both types of liposomes. A final "plateau" is reached (IV), corresponding probably to the covering of the entire area of the gold surface with the phospholipid bilayer (the formation of the continuous supported membrane).

Further studies are needed to prove that the IV stage is reached when the entire area of the gold surface is covered. Ralph et al [6] determined an approximate duration of 34 min for DOPS (dioleoylphosphatidylserine) for deposition of liposomes on mica, value that is between our experimental values of 17 min and 42 min for the Chla-DMPC and Chla -DPPC, respectively.

The difference of Phase III duration in the case of the 2 lipids (DMPC and DPPC) is well explained by the liposome size difference. The size of Chla-DPPC liposomes being smaller than that of Chla-DMPC liposomes, a longer time duration is necessary to cover the entire gold surface (~2000 sec versus ~500 sec, respectively).

Also in Fig. 4, there are notable differences between the process kinetics when using the 2 types of liposomes. An increase of the SPR signal can be observed, suggesting a continuous deposition on the surface after the corresponding "plateau" phase. The process of adhesion of other membranes to the first deposited bilayer could explain well the difference. The smaller Chla-DPPC vesicles will need more time to be added on a limited surface area, on top of the first deposited bilayer, as comparing with larger Chla-DMPC vesicles.

The SPR measurements at 25,8^{0}C (Fig. 6) show an increase of the signal with a steepness profile, but lacking the profile associated to the adhesion-fusion-rupture mechanism. This behavior is expected, as the liposome suspension is in a gel phase at this temperature, characterized by a closed pack of the hydrocarbon chains, rigid phase. The liposome with low membrane fluidity likely showed intact immobilization on a solid surface [12].

Chla-SPBs on n-dopped silicon wafers have been covered by a semitransparent gold electrode (1.5 mm^2 measurable area), thus manufacturing a photovoltaic device. Our preliminary photo-electrical measurements (the photocurrent plotted as function of the wavelength of the illumination light) are pleading for an effect of photosensitization due to Chla at Chla /Si hetero-junction in a Si/ Chla-SLB photovoltaic device.

5. Conclusions

Si wafers and ITO proved to be suitable solid support for Chl-SLB formation by a simple and versatile method. Chla was used with success as spectral marker and molecular sensor in VIS absorption and emission studies on liposomes and Chla-SPB. The kinetics of the Chla-liposomes deposition was investigated by SPR technique without the need of additional functionalization, due to the incorporation of chlorophyll. The measured SPR data confirms a mechanism of an initial adhesion on the surface and a final rupture, continued with additional deposition of the liposomes on the surface.

By manufacturing a photovoltaic device, based on Chla-SPB deposited on Si wafer, some promising preliminary results have been obtained. In conclusion, the light interaction with Chla, inserted in a lipid moiety (SPB) similar to photosynthetic membranes, could be exploited in applications, as well as Chla spectral properties could be successfully used to monitor the SPB formation.

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