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Improved accuracy and reproducibility of spontaneous liposome leakage measurements by the use of supported lipid bilayer-modified quartz cuvettes

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ABSTRACT

Recent studies have revealed avid interactions between liposomes and several solid materials, such as quartz, polystyrene (PS) and poly(methyl methacrylate) (PMMA), commonly found in cuvettes used for spectroscopic measurements. These interactions risk leading to detrimental changes in liposome structure and integrity that, if overlooked, may compromise the measurements. In case of leakage experiments based on probing the spontaneous release of encapsulated hydrophilic markers, the liposome-cuvette interactions may result in the recording of erroneously high degrees of leakage. In the present study we investigate the possibilities of preventing unwanted liposome-cuvette interactions through the use of quartz cuvettes passivated with supported lipid bilayers (SLBs). The results show that this strategy leads to higher reproducibility and significantly improved accuracy of the leakage measurements. The usefulness of the method is validated in comparative experiments focused on how changes in temperature and lipid phase state, as well as inclusion of poly(ethylene glycol)-conjugated lipids (PEG-lipids), affect the release of liposome encapsulated carboxyfluorescein (CF).

1. Introduction

The permeability of liposomes to hydrophilic probes depends on inherent physical properties of their membranes, such as the lipid packing order and prevalence of packing defects. Depending on the nature of the probe, electrostatic properties of the membrane may also have a decisive influence. Hence, the permeability is affected by numerous factors, including lipid composition and phase state, as well as the size and surface charge of the liposomes [1,2]. In line with this, changes in the chemical nature of lipids brought about by minor alterations of the carbon chains or the lipid headgroup, or by the inclusion of, e.g., PEG-lipids have been shown to affect the permeability [1,3–8]. In addition to lipid derived intrinsic properties, various extrinsic factors, such as the presence of fusogenic cations, like Ca^{2+}, or pore forming peptides can have a profound effect on the membrane's barrier properties and permeability to hydrophilic agents [9,10]. The barrier properties are as a rule also sensitive to temperature changes.[1,6,11].

Experiments designed to probe permeability and barrier properties of lipid membranes typically involve studies in which the rate of release/leakage of liposome-encapsulated hydrophilic self-quenching fluorescent probes, often calcein or carboxyfluorescein (CF), are determined [10]. The results of such spectroscopic measurements risk being compromised by side processes occurring at the cuvette-solution interface. More specifically, previous studies have confirmed that attractive interactions between the liposomes and the cuvette material can lead to strong adhesion, and, in some cases, also rupturing of the liposomes [12,13]. As a consequence of these processes, the affected liposomes prematurely release part, or all, of their encapsulated content, which, in turn, may result in the recording of an erroneously high spontaneous leakage. The extent and outcome of the liposome-cuvette interaction varies depending on the liposome phase state and the type of cuvette material used. In case of quartz cuvettes detrimental interactions can in some cases be avoided by providing the cuvette walls with a passivating layer of PEG [12]. This strategy is not efficient, however, when working with liposomes in the liquid disordered phase-state. Recent investigations reveal moreover that supplementing such liposomes with PEG-lipids is not a viable means of preventing their interaction with quartz/silica surfaces. Thus, attractive PEG-surface interactions lead to adhesion, rupture and spreading of the PEGylated liposomes on the solid material [14].

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In the present study we explored a novel cuvette pre-treatment protocol designed with the aim to mitigate the unwanted side processes at the cuvette-solution interface. To this end, we took advantage of the ease by which POPC liposomes adhere, rupture and spread on silica/quartz surfaces [12,13], and devised a method for providing the inner walls of quartz cuvettes with a passivating SLB. The performance and versatility of the SLB-passivated cuvettes was tested and compared to that of untreated quartz cuvettes in a series of leakage experiments performed under different conditions.

2. Materials and methods

2.1. Chemicals

1-palmitoyl-2-oleoyl-sn-glycero-phosphocholin (POPC), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)− 2000] (DSPE-PEG2000) were obtained as a kind gift from Lipoid GmbH (Ludwigshafen, Germany). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)− 5000] (DSPE-PEG5000) ammonium salt was purchased from Avanti polar lipids (Alabaster, AL, USA). Cholesterol (chol, ultra pure) was obtained from VWR (Leuven, Belgium). 4-(2-hydroxyethyl)− 1-piperazineethanesulfonic acid (HEPES) and 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid (TES) were purchased from Sigma Aldrich (Steinheim, Germany). Polyethylene glycol tert-octylphenyl ether (Triton™ X-100) and 5(6)-carboxyfluorescein (CF, 99 %) were purchased from Acros Organics (New Jersey, USA). Chloroform (pro analysis) and methanol (pro analysis) were purchased from Merck KGaG (Darmstadt, Germany). All aqueous solutions were prepared using deionized water (18.2 M Ω cm) from a Milli-Q plus system from Millipore (Bedford, MA, USA).

2.2. Liposome preparation

Liposome components were weighed or pipetted from stock solutions in methanol or chloroform. The lipid film was obtained by evaporation of the organic solvent with a gentle stream of nitrogen and then by removing the residual solvent in a vacuum oven (Lab instruments IL, USA) over night. The lipid film was suspended in either HEPES buffered saline (10 mM, 150 mM NaCl, pH = 7.4, HBS) or in a CF solution (100 mM CF, 10 mM TES buffer, pH = 7.4). The samples were at least 5 and up to 10 freeze-thawed depending on the complexity of lipid composition (freezing with liquid Nitrogen and thawing in a water bath). Subsequently the samples were further processed by extruding them for 31 times through a 100 nm pore size filter from Whatman plc (Kent, UK).

After the extrusion the liposomes prepared in CF solution were stored for 24 h at room temperature to reach an equilibrium state before starting the experiment.[15].

2.3. Preparation of SLB passivated cuvettes

The SLB-passivated cuvettes were prepared by adding carboxyfluorescein-free POPC liposomes (12 μM, dispersed in HBS buffered-saline) into clean quartz cuvettes (Quartz SUPRASIL®, Hellma Analytics, Mühlheim, Germany). The dispersion was constantly stirred at room temperature for at least one hour to allow the liposomes to adhere, rupture and spread on the quartz surface. The lipid concentration 12 μM was chosen to obtain full coverage of the cuvette walls within a reasonable time, while at the same time avoid scattering and inner filter effects from remaining non-ruptured liposomes. Assuming a lipid headgroup area of 63 $Å^2$ for POPC [16] and a cuvette area corresponding to 9 cm^2, it can be calculated that about 20 % of the added liposomes are consumed during the SLB coating.

2.4. Liposome leakage measurements

Liposomes were prepared in CF solution and separated from unencapsulated dye by using a gel filtration column (PD-10) from GE-Healthcare (Uppsala, Sweden) equilibrated with HBS. Thereafter the samples were diluted to 12 μM to ensure a direct proportionality between fluorescence readings and free CF concentration. The samples were transferred into untreated or SLB-passivated quartz cuvettes (Quartz SUPRASIL®, Hellma Analytics, Mühlheim, Germany). The change in fluorescence signal over time was recorded with a Fluorolog®− 3 from Horiba (Kyōto, Japan) operating in the right-angle mode. The excitation and emission wavelengths were set to 495 nm and 520 nm, respectively. All measurements were carried out at either 25 or 37 °C. The experiments were performed with a stirring rate set to 300 rpm, except for liposomes in the liquid disordered phase state, in which case a stirring rate of 900 rpm was employed. Different batches of the liposomes were measured simultaneously in bare and SLB-passivated cuvettes by use of a temperature controlled four-position turret sample changer. The degree of leakage over time ($X_{CF_{rel}}(t)$) was calculated with the following equation:

$$X_{CF_{rel}}(t) = \frac{I(t) - I_0}{I_{tot} - I_0}$$

where $I(t)$ is the time-dependent fluorescence intensity, I_0 is the intensity upon start of the experiment and I_{tot} is the maximum intensity obtained after adding 50 μL of 200 mM Triton X-100 solution to completely solubilize the liposomes. In case of liposomes in the liquid ordered phase state, a step including a brief tip sonication (MSE Soniprep 150 Ultrasonic Disintegrator) of the dispersion was added before the maximum fluorescence signal was recorded in order to ensure the complete release of CF from the liposomes.

2.5. Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) characterizations

The interactions of the different liposomes with silica and SLB-passivated silica was monitored with the help of a Quartz Crystal Microbalance with Dissipation monitoring from Q-sense (QCM-D E1, Gothenburg, Sweden). The lipid concentration was set to 1 g/L for all experiments unless stated otherwise. Silica (Q-sense, Gothenburg, Sweden) sensors were used. The frequency and dissipation signals were monitored at the fundamental sensor frequency (5 MHz), as well as the 3rd, 5th, 7th, 9th, 11th, and 13th overtones.

The sensors were cleaned before each experiment according to the procedure suggested by the provider. Briefly, the silica sensors were first cleaned in a UV/Ozone chamber from BioForce Nanosciences Inc. (Ames, IA, USA), then immersion of the sensors for 30 min into a 2 % SDS-solution followed by rinsing the sensor with Milli-Q water and drying it with nitrogen and then an additional 30 min treatment in the UV/ozone chamber.

2.6. Data analysis

At least four and up to seventeen repetitions of each experiment were performed, compressing at least three and up to eight independent preparations for each composition. All experiments were fitted to the bi-exponential model described by Agmo Hernández et al. [15]:

$$x_{CF_{rel}}(t) = 1 - A_1 e^{-k_1 t} - A_2 e^{-k_2 t}$$

Where A_1 and A_2 are pre-exponential factors that depend on the initial conditions of the experiment (among others, the age of the liposomes and the current degree of leakage at the beginning of the measurement) and fulfil the condition $A_1 + A_2 = 1$. The rate constant k_1 and k_2 are roughly defined as the short- and long-term leakage rate constants respectively. The model has been shown to describe the long-term

spontaneous leakage of liposomes more accurately than single exponential models, and is based on the assumption of two leakage mechanism occurring simultaneously (leakage through membrane defects and leakage caused by diffusion over the membrane) For details about the theoretical background of the equation, see Agmo Hernandez et al.[15].

To correct for differences in the initial conditions among repetitions of the experiments the experimental data obtained for a given composition and cuvette surface material (quartz or SLB-passivated quartz) was adjusted in the time axis to correspond to the average value of A_1 for that set of experiments. The adjusted data from the different repetitions was then weight-averaged (weights given by the quality of the fitting and defined as the inverse of the mean square error). The figures shown in the result sections show the averaged curves and the standard error of the mean.

Comparisons between datasets were performed via 2-way ANOVA (with replications) with the analysed factors being time and a given parameter of interest (either cuvette material, temperature or PEGylation). All differences between datasets discussed in the text, even if described as slight differences, were determined to be statistically significant ($p < 0.01$).

3. Results and discussion

3.1. Reducing the liposome-cuvette interactions through the use of SLBs

As mentioned in the introduction, phospholipid liposomes are susceptible to interactions with quartz/silica surfaces [17]. Depending on the lipid composition and environmental conditions, such as temperature and pH, these interactions may lead to adhesion, destabilization, and, in some cases, also rupture of the liposomes [12–14,17]. Hence, important features of the liposomes, like their ability to retain encapsulated material, can be severely affected by the interactions. In order to avoid biased results and erroneous conclusions, it is therefore important to take potential liposome-surface interactions into consideration when conducting liposome leakage experiments in quartz cuvettes. Moreover, when possible, measures should be taken to avoid or mitigate detrimental interaction between the liposomes and the cuvette material.

In search for an effective and versatile method to minimize unwanted liposome-cuvette interactions, we developed in the current study a novel cuvette pre-treatment protocol. The aim of this protocol was to provide the inner walls of quartz cuvettes with passivating supported lipid bilayers (SLBs). In order to investigate the potential and feasibility of the envisioned cuvette pre-treatment protocol, the QCM-D technique was first used to explore how the presence of a SLB affects the liposome-surface interactions. Due to the chemical similarity to quartz, silica sensors were employed for these investigations. In these experiments, POPC liposomes were first run over the sensor, and, after a rinsing step with buffer, a second injection of POPC liposomes was performed (Fig. 1).

The concomitant and typical changes in frequency and dissipation observed during the initial part of the experiment indicates that the first injection of POPC liposomes resulted in the successful formation of a SLB on the sensor surface. The rupture and spreading of POPC liposomes (Fig. 1) is indicated by the decrease and then increase in frequency stabilizing around − 26 Hz, as well as the increase and then decrease in dissipation. The application of a second batch of POPC liposomes after the successful formation of a POPC-SLB resulted in negligible liposome surface interactions. The absence of any significant changes upon injection of the second liposome dose verifies that the presence of the SLB effectively prevented any further interactions between the liposomes and the sensor surface. Subsequent experiments in which the POPC liposomes were replaced by other types of liposomes showed that SLB-treated silica sensors were similarly inert to interactions with POPC liposomes supplemented with PEG-conjugated lipids (Supplementary Material, Fig. S1). The same was observed for DPPC liposomes in the gel phase state and DSPC:chol liposomes in the liquid ordered phase state

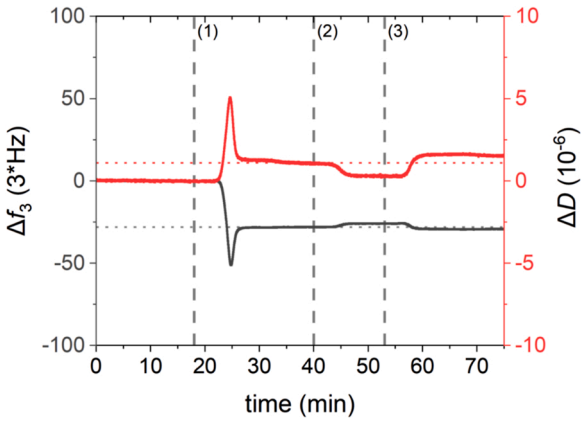

Fig. 1. QCM-D measurement of silica sensor (1) interacting with a solution containing POPC-liposomes, forming a POPC-SLB; (2) as the sensor was then rinsed with a buffer solution; (3) as a fresh batch of POPC-liposomes is inserted into the system. Changes in frequency (grey line) and dissipation (red line) were investigated.

(Fig. S2-S3), which did not interact with the POPC-SLB passivated surface.

The QCM-D experiments suggest that by pre-incubating quartz cuvettes with POPC liposomes it should be possible to coat the cuvette walls with passivating SLBs that prevent the adhesion of liposomes. Hence, we designed a protocol for such pre-treatment of quartz cuvettes (for details see Section 3.3) Of note, to ensure retained integrity of the SLB, and also for practical reasons, the POPC liposome dispersion used for the cuvette pre-treatment is not removed from the cuvette. Instead, a volume of a concentrated stock solution containing the CF-loaded liposomes of interest is added to the dispersion. Thus, when employing the SLB-coated cuvettes a population of unruptured POPC liposomes is likely to coexist with the liposomes under investigation. This population of liposomes is very small, however, and unlikely to affect the leakage experiments.

3.2. Employing SLB-passivated cuvettes for liposome leakage measurements

3.2.1. Liposomes in the liquid disordered phase state

To investigate the potential of the SLB-passivated cuvettes we carried out some comparative experiments in which the CF-leakage from pure and DSPE-PEG5000 supplemented POPC liposomes ($T_m = -2$ °C) were measured in quartz and SLB-passivated quartz cuvettes. The results from these experiments are shown in Fig. 2.

As can be appreciated in Fig. 2, the use of SLB-passivated cuvettes leads to better reproducibility and an overall lower degree of leakage for both liposome compositions, although this observation is more obvious and conclusive for the PEG-lipid-supplemented liposomes. It is to be noted, however, that the initial fast leakage observed for POPC liposomes in quartz cuvettes is absent when the cuvette is passivated by an SLB. This effect is more clearly visible through a comparison of the estimated initial rates of leakage (Supplementary Material, Fig. S5). As reported previously [13], the initial fast leakage observed in quartz cuvettes is likely to arise from liposome rupture and spreading on the quartz surface. The results in Fig. 2 suggest that this effect is reduced or eliminated if the cuvette is already passivated by an SLB.

Concerning the PEG-lipid-supplemented liposomes, we have reported in a recent publication that the inclusion of PEG-lipids leads to enhanced adhesion and rupture of liposomes to silica and quartz at the studied temperature.[14] This phenomenon can explain the higher degree of leakage observed when employing the unmodified cuvettes. By modifying the cuvettes with an SLB, the leakage is greatly reduced, and the experiments are also much more reproducible.

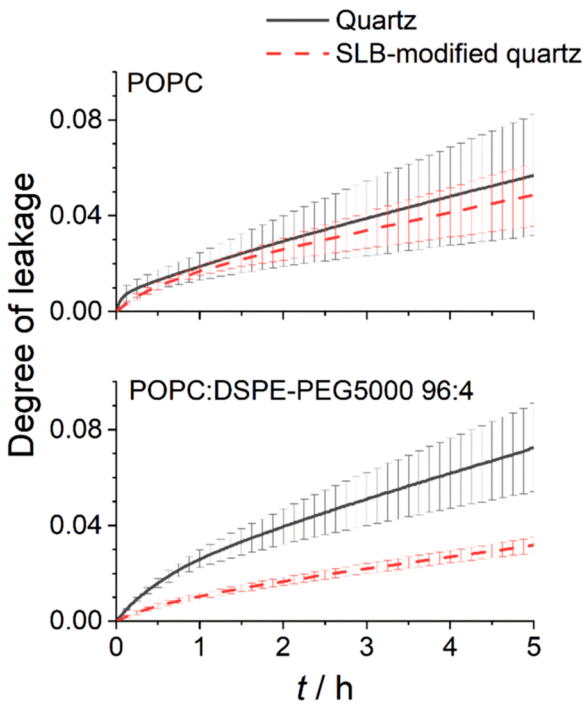

Fig. 2. Leakage assays of (top) POPC and (bottom) POPC:DSPE-PEG5000 (96:4 mol %) liposomes. The degree of leakage was measured in quartz (grey, solid line) and, SLB-passivated quartz (red, dashed line) at a temperature of 25 °C. The bars represent the standard error of the mean for at least four repetitions of each experiment.

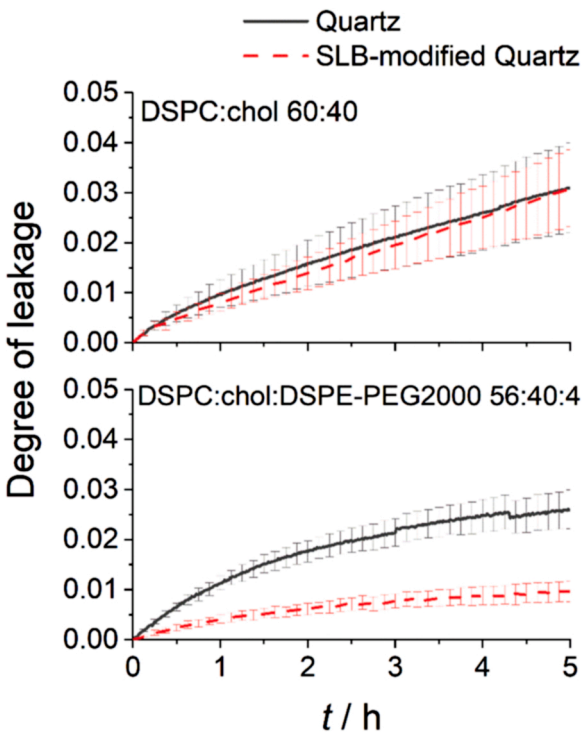

Fig. 3. Leakage assays of (top) DSPC:chol 60:40 and (bottom) DSPC:chol:DSPE-PEG2000 (56:40:4 mol %) liposomes. The degree of leakage was measured in quartz (grey, solid line) and SLB-passivated quartz (red, dashed line) at a temperature of 25 °C. The bars represent the standard error of the mean for at least nine repetitions of each experiment.

The results depicted in Fig. 2 also show that, if measured in unmodified quartz cuvettes, the PEGylated POPC liposomes would seem to leak slightly faster than their non-PEGylated counterparts. If the experiments are, on the other hand, performed using the SLB-passivated cuvettes, it is clearly appreciated that the PEG-lipid-supplemented liposomes leak much more slowly. In the former case, the results are likely affected by the adhesion and spreading processes occurring simultaneously with the spontaneous leakage, whereas in the latter case only the spontaneous leakage is being measured. These results illustrate thus the importance of avoiding liposome-cuvette interactions and suggest that modification with an SLB is a suitable way to achieve this. The slower leakage of PEGylated liposomes compared to the bare liposomes is in agreement with previous studies.[5–8,18–20].

The results of the leakage experiments shown in Fig. 2 demonstrate the importance of a proper selection of cuvette material and support the choice of SLB-passivated quartz cuvettes for experiments involving both PEGylated and non-PEGylated POPC liposomes.

3.2.2. *Liposomes in the liquid ordered phase state*

In order to confirm the applicability of the method, liposomes with different compositions were investigated. To begin with, liposomes composed of DSPC:chol with a molar ratio of 60:40 were selected. Mixing long-chain phosphatidylcholines and cholesterol at this ratio results in bilayers in the liquid-ordered phase state[21]. For comparison, PEGylated liposomes (DSPC:chol:DSPE-PEG2000) with a molar ratio of 56:40:4 were also tested. The results from the leakage measurements are summarized in Fig. 3.

As observed in Fig. 3, the passivation of the cuvette with a POPC-SLB had very little effect on the leakage profile from DSPC:chol liposomes, although a slight improvement in reproducibility and a slightly slower leakage are appreciated. On the other hand, for the PEGylated variant of the liposomes, the passivation of the cuvette resulted in a marked decrease in the degree of leakage and a considerable improvement in the reproducibility of the measurements. For this latter case, it is likely that the previously reported PEG-quartz interactions are reduced or avoided. The rupturing or spreading of liposomes on the silica surface is thereby decreased or removed. As for POPC liposomes, it is observed that the PEGylated liposomes appear to leak more slowly than their non-PEGylated counterparts if the experiments are performed on SLB-passivated cuvettes. This effect of PEGylation is not observed in bare quartz cuvettes, likely because the effect of adhesion and spreading of both PEGylated and non-PEGylated liposomes dominate the leakage measurements. This constitutes therefore a further example of the applicability of the SLB-passivated cuvettes to identify and characterize the effect of liposome modifications on their spontaneous leakage behaviour.

It is interesting that experiments with non-PEGylated DSPC:chol liposomes seem to be unaffected by the presence of the SLB. To further explore whether the SLB could have any effect on the results obtained with these liposomes, the experiments were repeated at 37 ℃ for both, PEGylated and non-PEGylated liposomes. The results are summarized in Fig. 4. As can be observed in Fig. 4, at this higher temperature there is a distinct effect of the SLB-passivation in the leakage profile of non-PEGylated DSPC:chol liposomes. Somewhat surprising, it appears that the passivation with an SLB leads to a higher degree of leakage and lower reproducibility. However, it is noted that experiments performed in bare quartz cuvettes would indicate that increasing the temperature slows down the spontaneous leakage process. This type of behaviour is clearly not compatible with the well documented inverse proportionality between temperature and lipid packing order [2]. On the other hand, the experiments performed in SLB-passivated cuvettes clearly show the expected increase in the rate of leakage upon increasing the temperature. The same observation is valid for the PEGylated liposomes, where a higher leakage rate at higher temperatures is observed clearly only when the cuvette is passivated. Once again, the results suggest that experiments performed in SLB-passivated cuvettes are more likely to reflect the actual spontaneous leakage behaviour. The question remains,

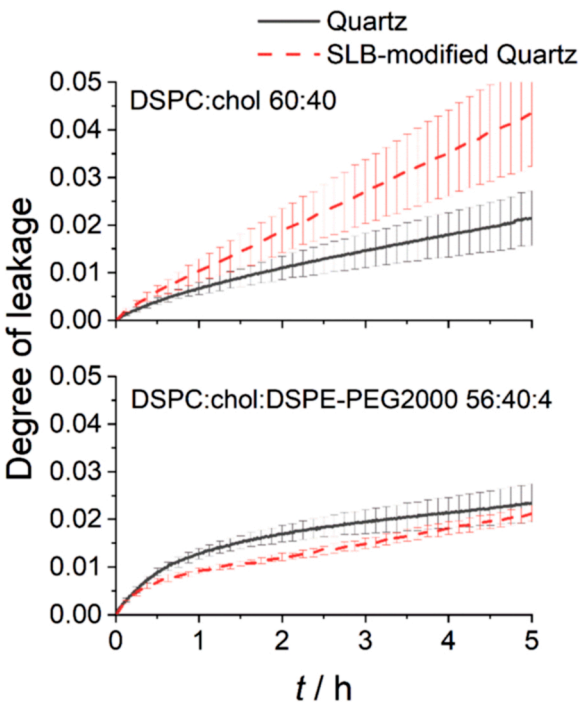

Fig. 4. Leakage assays of (top) DSPC:chol 60:40 and (bottom) DSPC:chol:DSPE-PEG2000 (56:40:4 mol %) liposomes. The degree of leakage was measured in quartz (grey, solid line), SLB-passivated quartz (red, dashed line) at a temperature of 37 °C. The bars represent the standard error of the mean for at last seven repetitions of each experiment. To facilitate comparisons, the scale of the y-axis is the same as for Fig. 3.

however, about why the leakage from non-PEGylated liposomes seems to be slowed down or unaffected by higher temperatures when the experiments are performed in bare cuvettes. Although we can only speculate about it, a possible reason could be that the adhesion of liposomes to the cuvette surface is less favourable when the temperature increases, leading to less liposomes rupturing onto the surface and reducing thus the amount of CF leaked. This, however, does not explain why the liposomes seem to leak more slowly when the experiments are performed in a bare cuvette compared to an SLB-passivated cuvette at 37 ℃. It can be speculated that some liposomes attach to the surface and remain intact. Intact liposomes attached to surfaces have been previously shown to leak more slowly than liposomes in dispersion, likely because the substrate blocks the defects on the liposome surface through which fast leakage may occur [15]. This could explain the decreased leakage observed in the Fig. 4, although, unfortunately, the experiments provide no evidence to confirm this hypothesis.

In short, the results obtained with DSPC:chol liposomes (both PEGylated and non-PEGylated) suggest that passivation of the cuvette surface with a POPC-SLB decreases unwanted interactions between the liposomes and the surface. Indeed, data collected by QCM-D showed negligible interactions between the liposomes and SLB-passivated silica sensors at both, 25 and 37 °C (Fig. S3 and S4).

3.2.3. *Liposomes in the gel phase state*

The usefulness of the SLB-passivation to study leakage behaviour of gel phase state liposomes was characterized by following the spontaneous leakage of DPPC liposomes (T_m = 41 °C) at 25 ℃ (Fig. 5). For comparison, PEGylated gel phase liposomes (DPPC:DSPE-PEG2000) were also tested. The results are summarized in Fig. 5. As observed in Fig. 5, passivation of the quartz cuvette surface results in a lower degree of leakage from DPPC liposomes, although the reproducibility of the experiment is not greatly improved. It has been previously reported that the leakage from DPPC liposomes in quartz cuvettes is several times

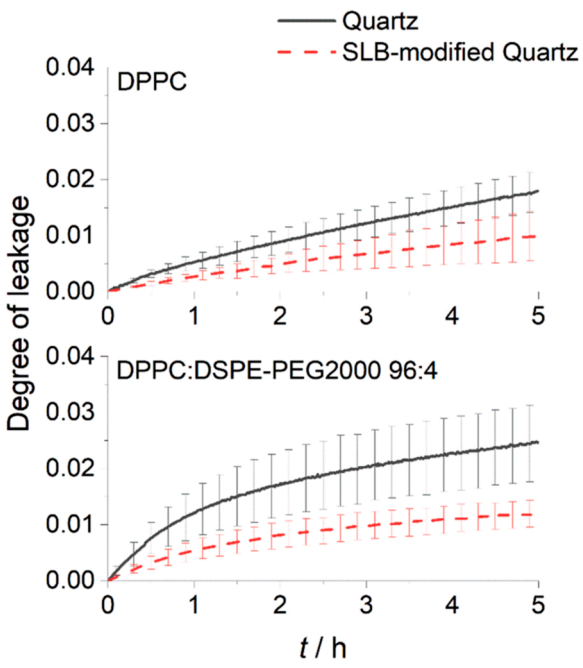

Fig. 5. Leakage assays of (top) DPPC and (bottom) DPPC:DSPE-PEG2000 (96:4 mol %) liposomes. The degree of leakage was measured in quartz (grey, solid line), SLB-passivated quartz (red, dashed line) at a temperature of 25 °C. The bars represent the standard error of the mean for four repetitions of each experiment.

faster than in, e.g., polystyrene cuvettes, likely because the liposomes adhere to the quartz surface where they are then shattered by the effect of stirring. Coating the quartz surface with a POPC-SLB seems to prevent this attachment, leading to leakage degrees similar to those observed in polystyrene or in quartz cuvettes in the absence of stirring [13]. The passivation of the cuvette with a SLB resulted in a marked decrease in the degree of leakage and a considerable improvement in the reproducibility of the measurements for the PEGylated variant of the DPPC liposomes. It is likely that the PEG-quartz interactions are reduced or avoided. The rupturing and spreading of the PEGylated liposomes on the silica surface (as described in a recent publication [14]) is thereby decreased or avoided.

Unlike the liquid-ordered and liquid-disordered phase states it is not possible to appreciate any discernible differences between PEGylated and non-PEGylated liposomes in SLB-passivated quartz cuvettes. Given the low degree of leakage observed for these gel phase liposomes longer measurement intervals would be necessary to obtain noticeable differences. It can be concluded that results obtained with gel phase liposomes (both PEGylated and non-PEGylated) suggest that passivation of the cuvette surface with a POPC-SLB decreases unwanted interactions between the liposomes and the surface.

4. Conclusions

The experiments show that artifacts in leakage measurements arising from liposome-cuvette interactions can be greatly reduced by passivating the cuvette surface with a SLB composed of POPC. This complements other approaches previously suggest in studies published by our lab, namely, the use of alternative cuvette materials (such as polystyrene) or the passivation of the cuvette by a layer of PEG. It is worth mentioning, however, that previous approaches have been found to be useful only for certain types of liposomes (see compilation in Table 1). For example, replacing quartz with polystyrene has been shown to improve the results obtained with liquid disordered and gel phase liposomes, but it fails with liposomes in the liquid ordered phase state or

Table 1
Recommended cuvette type or modification of quartz cuvette to be applied for minimizing experimental artifacts in spontaneous leakage experiments.

	Phase state of the liposomes					
	Liquid disordered		Liquid ordered		Gel	
Type of cuvette	naked	PEGylated	naked	PEGylated	naked	PEGylated
Polystyrene[a]	✓				✓	
PEG-passivated quartz[b]			✓	✓		
SLB-passivated quartz[c]	✓	✓	✓	✓	✓	✓

[a] data from Ref. [13]
[b] data from Ref. [12]
[c] data from the current study

with liposomes containing PEG-lipids [12,13]. Passivation of the cuvette surface with a layer of PEG, on the other hand, has been shown to be useful for the complementary cases (PEGylated liposomes and liposomes in the liquid ordered phase state), but not suitable for studies with liposomes in the liquid disordered or gel phase state. A clear and distinct advantage of the SLB passivation approach is the fact that, according to the presented results, it allows for direct measurements of spontaneous leakage from liposomes in all the most common phase states (liquid disordered, liquid ordered and gel), as well as from PEG-lipid-supplemented liposomes. Avoiding artifact and improving the reproducibility by using SLB passivated cuvettes allows also identifying differences between the spontaneous leakage behaviour of different samples even if the measurement times are relatively short (~5 h in comparison with the >10 h measurement times typically used in previous reports) [1,4,6,7,10,13,15], as illustrated by our results.

It should be pointed out, however, that the SLB-passivation approach is not a comprehensive solution suitable for all kinds of leakage experiments. Measurements of detergent- or peptide-induced leakage would for instance be greatly affected by the presence of the SLB and the excess remaining POPC liposomes in dispersion, and other approaches should therefore be used for this kind of experiments. When it comes to measurements of spontaneous leakage the use of SLB-passivated cuvettes seems, however, to be an effective and universal approach to avoid unwanted interactions and artifacts.

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CRediT authorship contribution statement

Philipp Grad: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Data curation, Writing – original draft, Writing – review & editing. **Víctor Agmo Hernández:** Conceptualization, Methodology, Formal analysis, Visualization, Writing – review & editing. **Katarina Edwards:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfb.2022.113022.

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