Avoiding artifacts in liposome leakage measurements via cuvette- and liposome-surface modifications

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ABSTRACT

The barrier properties of lipid membranes are often determined by investigating their solute permeability with the help of spectroscopic methods and the use of liposome-encapsulated self-quenching fluorescent dyes, for example, Carboxyfluorescein (CF). It was shown previously that liposome-surface interactions, and thus the choice of cuvette material, influence the result of such spectroscopic permeability/experiment leakage experiments. In this work, we explore different methods to minimize the artifacts observed in spontaneous leakage measurements performed with cholesterol-containing liposomes. The spontaneous leakage of CF from liposomes with different composition and surface properties is monitored in cuvettes composed of quartz, polystyrene (PS), and Poly(methyl methacrylate) (PMMA). Our results show that significantly different leakage profiles are recorded for the exact same liposome batch depending on the cuvette material used. Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D) experiments indicate that these discrepancies likely arise from side processes occurring at the solution-cuvette interface, mainly, the attaching and spreading of liposomes. Further, we show that in some cases it is possible to minimize liposome-cuvette interactions, and reduce the experimental artifacts, by supplementing the liposomes with polyethylene glycol (PEG)-grafted lipids or gangliosides, and/or by pre-adsorbing free PEG to the cuvette walls. The collected data suggest that quartz cuvettes modified by adsorption of PEG8000 are suitable for spontaneous leakage experiments with POPC:cholesterol-based liposomes, while other cuvette materials perform poorly in the same experiments.

1. Introduction

Liposomes are phospholipid vesicles commonly used for pharmaceutical applications, as well as for the modeling of biological membranes. Data and knowledge concerning inherent membrane properties, such as the permeability towards solutes, are important for these applications. The solute permeability of lipid membranes is linked to various physical parameters, such as the lipid packing order and membrane fluidity, as well as to the number and extent of packing defects (Lande et al. 1995, Agmo Hernández et al. 2011, Angelini et al. 2011, Agmo Hernández et al. 2015, Wang et al. 2019).

The membrane permeability towards solutes is often characterized by measuring the release of self-quenching hydrophilic fluorescent probes, such as carboxyfluorescein (CF) or calcine. In these experiments, the fluorophore is encapsulated at high concentrations within the liposomes, and thereby self-quenched. Upon release, it will be diluted in the bulk solution and the fluorescence increase can be monitored (Weinstein et al. 1977). The fluorescence intensity of the leaked fluorophore correlates linearly with its concentration when the latter is sufficiently low (Chen and Knutson 1988, Shimanouchi et al. 2009).

Cuvettes are inevitable elements in spectroscopic experiments and can be composed of various materials, such as quartz or different polymers. For leakage experiments, the excitation and emission wavelength of CF are in the visible range and therefore most cuvette materials are deemed suitable. A preference for quartz can be explained by its superiority with regard to the wide range of optical transmission and solvent compatibility. Furthermore, quartz cuvettes can be reused. Plastic cuvettes, made from, for example, polystyrene (PS) and poly(methyl methacrylate) (PMMA), are often limited to the visible range and to aqueous solutions but offer the advantage of being cheap. The cuvette materials also differ in their surface properties and temperature resistance (Overway 2017). The influence of the cuvette material on the actual measurements has to date received only limited attention. Predotova et al. (2011), reported on the effects
of the cuvette surface material on gas concentration measurements. Adhesion effects lead to varying results when using different cuvette materials (Predotova et al. 2011). The physical properties of cuvette surfaces have also been shown to affect the measurement of enzyme activity, as studied by Cattoir et al. (2013).

In a previous study by Eriksson and Agmo Hernández (2018), it was shown that the choice of cuvette material is an important factor to consider for liposome leakage assay experiments, since the liposomes may be prone to interact with the surface of the cuvette. Liposomes are indeed known to interact with hydrophilic surfaces, including quartz, in a manner that leads to that the liposomes either adsorb in their intact form or rupture and spread into supported bilayers (Johnson et al. 2002, Richter et al. 2006, Biswas et al. 2018). On the other hand, the interaction of liposomes with hydrophobic surfaces often results in the formation of adsorbed monolayers (Hellberg et al. 2002). On the other hand, the interaction of liposomes with hydrophobic surfaces often results in the formation of adsorbed monolayers (Hellberg et al. 2002, Agmo Hernández 2013). In spite of this, the nature of the cuvette material is seldom considered or reported in the literature.

In this study, we have followed up on the findings reported by Eriksson and Agmo Hernández (2018) and explored how modifications of the liposomes and/or the cuvettes can be used to minimize the appearance of artifacts during spontaneous leakage measurements. To this end, we studied liposomes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 40 mol% cholesterol (chol). POPC:chol liposomes were chosen partly due to their anomalous behavior in the previous study (Eriksson and Agmo Hernández 2018), but also because of their high stability and low permeability, as compared to pure POPC liposomes, render them interesting as vehicles for drug delivery. Chol-containing POPC membranes are furthermore highly relevant from a biological perspective. The surface properties of the liposomes were modified by the addition of 1,2-distearyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG(2000)) or gangliosides (porcine ganglioside extract). Cuvettes made from three different materials, that is, quartz, PS, and PMMA, were explored in the investigations. These cuvette materials were chosen due to their different surface properties with regard to surface roughness and hydrophobicity/hydrophilicity. PS is a hydrophobic material (Zheng et al. 2006) whereas PMMA has some water adsorbing capacity and thereby a more hydrophilic character (Ayme et al. 1992, N’Diaye et al. 2012). Finally, quartz and silica have a distinct hydrophilic character compared to the two polymers (Cryan et al. 2019). Complementary to the leakage measurements, the liposome–surface interactions were explored with the help of a quartz crystal microbalance with dissipation monitoring (QCM-D).

2. Materials and methods

2.1. Chemicals

The total ganglioside extract (Brain, Porcine Ammonium salt) was purchased from Avanti Polar lipids (Alabaster, US). 1-palmitoyl-2-oleoyl-sn-glycero-phosphocholin (POPC) and 1,2-

distearyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ammonium salt (DSPE-PEG2000) were obtained as a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Chol was obtained from W.R. Polylthylene glycol 8000 (PEG8000), polyethylene glycol tert-octylphenyl ether (Triton X-100), 5(6)-carboxyfluorescein (CF), 4’-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2’-(2-Hydroxy-1,1-bis(hydroxymethyl)ethylamino)ethanesulfonic acid (TES) and sodium chloride were purchased from Sigma Aldrich (Steinheim, Germany). Chlroform (pro analysis) and methanol (pro analysis) were purchased from Merck KGaG (Darmstadt, Germany). All aqueous solutions were prepared using deionized water (18.2 MΩ cm) from a Milli-Q plus system from Millipore (Bedford, USA). Deconex 11 was purchased from Borer Chemie AG (Zuchwil, Switzerland).

2.2. Liposomes preparation

The desired lipids were weighed or pipetted from stock solutions in chloroform or methanol, and subsequently dissolved in chloroform. A lipid film was obtained by evaporation of the organic solvent, first with a gentle stream of Nitrogen then removing the residual solvent in a vacuum oven from Lab instruments (IL, USA) overnight. The lipid film was suspended in a CF solution (100 mM CF, 10 mM TES buffer, pH = 7.4). The liposomes were prepared by 10 freeze-thawing cycles (freezing with liquid Nitrogen and thawing with a water bath at 60°C) followed by 31 times of extrusion through a 100 nm pore size filter from Whatman plc (Kent, UK). Finally, the samples were stored for 24 h in the dark and at room temperature to reach an equilibrium state of the liposomes before starting the experiment (Agmo Hernández et al. 2011).

2.3. Leakage measurements

Liposomes prepared in a CF solution were separated from a free dye by using a PD-10 gel filtration column from GE-Healthcare (Uppsala, Sweden) equilibrated with HEPES buffer solution (HEPES 10 mM, 150 mM NaCl, pH = 7.4) isotonic with the encapsulated CF solution. Thereafter the samples were diluted to 12 μM lipid to ensure proportionality between fluorescence readings and concentration. Subsequently, the samples were transferred into a cuvette of choice and the experiments were started. One of the following cuvettes with the dimensions 1 × 1 cm was used: Polystyrene (PS, KARTELL S.p.A., Noviglio, Italy), Poly(methyl methacrylate) (PMMA, Kartell S.p.A., Noviglio, Italy) or quartz cuvettes (Quartz SUPRASIL®, Hellma Analytics, Mühlheim, Germany). The fluorescence signal was monitored with a Fluorolog®-3 from Horiba (Kyoto, Japan) in the right-angle mode. The excitation was set to 495 nm and the emission to 520 nm. The spontaneous leakage experiments were run either 5 or 15 h with continuous stirring unless otherwise stated, and measuring the intensity every 24 s at 20°C. The degree of leakage over time \(I_{CF_{rel}}(t)\) was calculated with the following equation:

\[
\frac{I(t) - I_0}{I_{tot} - I_0}
\]
l(t) is the time-dependent fluorescence intensity, \( l_0 \) is the intensity upon start of the experiment and \( l_{\text{tot}} \) is the maximum intensity obtained by adding 50 \( \mu \)L of 200 mM Triton X-100 solution to the sample to obtain complete solubilization of the liposomes.

The effect of the magnetic stirrer was investigated by comparison of the different degrees of leakage at varying stirring speeds.

### 2.4. Fitting of leakage results

The data obtained were fitted to the bi-exponential model described by Agmo Hernández et al. (2011):

\[
x_{\text{CF,fit}}(t) = 1 - A_1 e^{-k_1 t} - A_2 e^{-k_2 t}
\]

Where \( A_1 \) and \( A_2 \) are preexponential factors that depend on the initial conditions of the experiment and fulfill the condition \( A_1 + A_2 = 1 \). In turn, \( k_1 \) and \( k_2 \) can be 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 mechanisms 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 Hernández et al. (2011).

The fitting parameters obtained for repetitions of each measurement were then averaged (weighted average based on the quality of the fitting, defined by the mean standard error of the fitting). The fitting parameters were obtained from at least 3 repetitions for each sample. The curves shown in Figures 1, 5, 8, and 9 are calculated from the averaged fitting parameters.

### 2.5. QCM-D characterizations

The spreading and adhesion of the different liposomes on various surfaces was monitored with the help of a Quartz Crystal Microbalance with Dissipation monitoring from Q-sense (QCM-D E1, Gothenburg, Sweden). Liposomes from the same batch and with the same concentration as for the fluorescence measurements were used. Silica (Q-sense, Gothenburg, Sweden) and custom PS and PMMA coated sensors were used. The polymer-coated sensors were produced by spin-coating with a VCT-100 from MTI corporation (Richmond, CA, US). Small amounts of 0.5 wt% solutions of PMMA or PS in toluene were applied to gold QCM-D sensors. The sensors were subsequently stored in the vacuum oven for 24 h.

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, Iowa), 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. The PS and PMMA coated sensors were first immersed into a 1% Deconex 11 solution at 30 °C for 30 min followed by immersion for at least 2 h into Milli-Q water with a subsequent rinsing it with 99.5% ethanol and finally drying the sensor with nitrogen.

The sensors were mounted into the QCM-D system and rinsed with HEPES-buffer until equilibration was reached. After a stable baseline was obtained the system was loaded with the liposomes with a flow rate of 150 \( \mu \)L/min for 5 min. Then the flow rate was reduced to 50 \( \mu \)L/min and the suspension was recirculated until the response stabilized. The temperature was kept at 20 °C to have similar conditions as for the fluorescence leakage measurements.

The experiments conducted at higher flow rates were realized as follows. Here the sensors were mounted into the QCM-D system and rinsing it with HEPES-buffer until equilibration was reached. After a stable baseline was obtained the system was loaded with the liposomes with a flow rate of 150 \( \mu \)L/min for 5 min. Then the flow rate was increased to 400 \( \mu \)L/min and the suspension was recirculated until the response stabilized. The temperature was kept at 20 °C to have similar conditions as for the fluorescence leakage measurements.

Experiments with PEG8000-modified surfaces were conducted by mounting the sensor into the QCM-D system and rinsing it with HEPES-buffer until equilibration was reached. After a stable baseline was obtained the system was loaded with HEPES-buffer containing 1 wt% of PEG8000 for 5 min and then recirculated for 30 min. Thereafter the system was again rinsed with HEPES buffer for 15 min and after a stable baseline was obtained the system was loaded with the liposomes with a flow rate of 150 \( \mu \)L/min for 5 min. Then the flow rate was reduced to 50 \( \mu \)L/min and the suspension was recirculated until the response stabilized. The temperature was kept at 20 °C to have similar conditions as for the fluorescence leakage measurements.

### 3. Results and discussion

#### 3.1. The effect of cuvette materials and stirring speed on the apparent rate of spontaneous leakage of CF from POPC:chol liposomes

The release of CF from POPC:chol liposomes (Figure 1) was followed at two different stirring speeds (300 and 900 rpm, respectively) using quartz, PS and PMMA cuvettes. To ensure comparability between data obtained with the different cuvettes, a 4-cuvette holder was employed and experiments involving all three cuvette materials were performed simultaneously using the same liposome batch. Figure 1 shows the obtained experimental curves (averaged from repetitions of the experiments). Table 1 shows the corresponding averaged fitting parameters using the bi-exponential model described in Materials and Methods (Section 2.4).

As can be observed, the leakage profile and values of the fitting parameters vary greatly depending on the cuvette material used. The large error margins determined for the fitting parameters (Table 1) illustrate moreover a poor reproducibility of the experimental results. Interestingly, for all three cuvette materials, the stirring rate affects the leakage profile (Figure 1) and the fitting parameters (Table 1). This effect is particularly clear in the case of PS cuvettes, where the...
degree of leakage after 15 h is twice as high at 900 rpm compared to 300 rpm. A similar positive relationship between the degree of leakage and stirring rate has previously been reported for DPPC liposomes studied in quartz cuvettes and was proposed to arise from the stirrer-induced shattering of liposomes at the cuvette surface (Eriksson and Agmo Hernández 2018). Indeed, experiments performed in PS cuvettes without any stirring resulted in a much slower long-term leakage rate (see Figure S1 in the supplementary data). The rather modest effect of the stirring rate on the leakage profiles recorded for POPC:chol liposomes in quartz and PMMA cuvettes observed in the current study may be due to experimental variations, rather than a specific effect of the stirring rate.

In any case, the large differences in behavior observed when using different cuvette materials strongly suggest that the phenomenon being monitored cannot be the same in all cases. That is, not all experiments report on the actual spontaneous leakage behavior. The poor reproducibility of the experiments suggests that none of the curves displayed in Figure 1 correctly illustrate the actual spontaneous leakage behavior.

As previously demonstrated (Eriksson and Agmo Hernández 2018), interactions between the liposomes and the surface of the used cuvettes may lead to variations in the observed leakage profiles. Hence, the interaction of the POPC:chol liposomes with solid surfaces similar to those of the cuvettes was characterized with the help of QCM-D. Sensors coated with PS, PMMA, or silica were employed in these experiments. Silica was used to mimic the chemically similar quartz surface.

In the case of silica surfaces (Figure 2(a)), liposome adhesion first causes large changes in frequency and dissipation (suggesting the adhesion of an intact lipid layer), followed by a decrease of the observed signals (suggesting rupture of the liposomes and release of the encapsulated water). The fact that the dissipation does not go back to zero suggest, however, that the rupture is only partial. The initial attachment and rupture observed in the QCM-D experiments can be coupled to the fast initial CF release observed in the leakage experiments in quartz cuvettes (see inset of Figure 1). Once the surface is coated by a supported lipid bilayer and a few intact liposomes, no more interaction between the remaining liposomes and the surface is expected, resulting thus in a much slower leakage (corresponding to actual spontaneous leakage), as observed in Figure 1.

In the case of PMMA (Figure 2(c)), a slower attachment than seen with silica (Figure 2(a)) is observed. Given that the final values of frequency and dissipation are very similar in the silica and PMMA cases, it is likely that the interaction with the latter material also results in a surface partially coated with a supported lipid bilayer and a few intact liposomes. As the adhesion occurs much more slowly, the initial accumulation of intact liposomes observed for silica is not detected with PMMA. Similar to the case when using quartz cuvettes, the initial fast leakage (inset of Figure 1) observed at 300 rpm can be explained by the release of CF upon rupture of the liposomes. At 900 rpm, the initial leakage rate is lower, probably because the increased stirring prevents the comparatively weakly interacting liposomes from attaching to the PMMA surface.

The QCM-D results obtained on PS surfaces cannot, however, directly account for the observed leakage behavior. Figure 2(b) suggests that the liposomes do not interact with the surface, and, therefore, no surface-induced leakage should be observed. It is in this context worth noting that in the case of chol-free POPC and DPPC liposomes it has previously been established that no surface-induced but only spontaneous leakage is observed in PS cuvettes (Eriksson and Agmo Hernández 2018). These findings seem to disagree with the fast and stirring-rate-dependent leakage observed from the POPC:chol liposomes investigated in the current study (Figure 1). Interestingly, studies based on the use of polystyrene nanoparticles have shown that POPC:chol liposomes indeed do attach to polystyrene surfaces. The attachment proceeds, however, very slowly (Agmo Hernández et al. 2015). A possible explanation to reconcile the results from the QCM-D and the leakage experiments is that attachment of the liposomes to the QCM-D sensors occurs very slowly and is observed mainly as a long-term drift of the baseline. The attachment of liposomes would, however, cause the fast leakage observed if the liposomes are immediately and constantly shattered by the stirring instead of (as in the case of PMMA) being washed away by the generated currents. A constant shattering of the liposomes would moreover explain the high degree of leakage observed at late time points. The very strong dependence of the leakage profile on the stirring rate supports this hypothesis. The reason
behind the opposite effect of the stirring rate in PMMA and PS cuvettes is likely linked to the strength by which the liposomes adhere to the two different materials. While the data retrieved in the current study suggest that the interaction forces are weak in the case of PMMA, previous investigations have revealed rather strong interactions between phospholipid liposomes and hydrophobic surfaces composed of PS. Thus, DPPC, POPC, as well as POPC:chol liposomes have been shown to adhere to PS nanoparticles in an irreversible manner (Agmo Hernández 2013, Agmo Hernández et al. 2015). In the case of DPPC, results from DLS and cryo-TEM investigations suggest that the attractive hydrophobic interactions between the lipid tails and the surface are strong enough that the adhered liposomes eventually rupture and spread in the form of a lipid monolayer on the particle surface (Agmo Hernández 2013).

In summary, it is likely that none of the experiments shown in Figure 1 actually measures the spontaneous leakage of CF from the liposomes. Given that chol-modified liposomes are of great importance for several applications; an approach that would allow a reliable measurement of the spontaneous leakage from such liposomes is highly desired. Since the discussed experimental artifacts seem to arise due to the liposome–cuvette interactions, strategies that minimize or counteract these interactions may allow for accurate characterization of the spontaneous leakage phenomenon. In the following section, we explore if modifications of the liposome surface can be used as a means to decrease liposome attachment/rupture on the different cuvette materials.

### 3.2. Effect of modification of the liposome surface

The incorporation of polyethylene glycol grafted lipids (PEG-lipids) in the lipid membrane is widely used to sterically stabilize liposomes. The protective PEG-layer increases liposome colloidal stability and also helps to prevent detrimental interactions between liposomes and, for example, blood proteins. Indeed, PEGylation of lipid nanocarriers is very common in order to prolong their blood circulation time (Blume and Cevc 1990, Klibanov et al. 1990, Santos et al. 2007).

Another kind of lipids that have been used to generate repulsive surface forces and prolong the circulation time of liposomes are gangliosides (Allen and Chonn 1987). Gangliosides belong to the group of glycosphingolipids, and are composed of a ceramide linked to an oligosaccharide containing at least one N-acetylneuraminic acid (sialic acid). Gangliosides are most abundant in neural cells and the lipids show extensive diversity in their carbohydrate moiety. The pK_a value of the sialic acid is around 2.6 and gangliosides are thus negatively charged at physiological pH (Kolter 2012, Yu et al. 2013). As a consequence of their polymeric and charged nature, gangliosides are potentially capable of stabilizing liposomes by both electrostatic and steric mechanisms.

Herein, we tested the effect of including 4 mol% of PEGylated-lipids or gangliosides in the POPC:chol liposomal formulations. The leakage of CF from the liposomes was measured and analyzed in the same way as for the unmodified liposomes. The results show that there is a great variation in the leakage rate and profile depending on the combination of surface modification and cuvette material used. The stirring rate also plays a role for some of the combinations. The fitting parameters for all combinations are summarised in Table S1 in the supporting material.

As can be observed, the fitting parameters vary in a very wide interval. For k_1, the interval extends from a value of $(0.87 ± 0.46) \times 10^{-4}$ s$^{-1}$ (obtained for unmodified POPC:chol liposomes in a quartz cuvette, stirring 300 rpm) up to a value of $(19.05 ± 1.33) \times 10^{-5}$ s$^{-1}$ (obtained for liposomes modified with PEG lipids and measured in a polystyrene cuvette at 900 rpm). The corresponding interval limits of k_2 are

![Figure 2. QCM-D recorded changes in frequency and dissipation upon interaction of POPC:chol liposomes with (a) silica (b) PS or (c) PMMA sensors.](image-url)
(0.10 ± 0.77) × 10^{-6} s^{-1} and (5.22 ± 1.00) × 10^{-6} s^{-1}. Both limiting values are obtained at the same conditions as the respective limiting values of \( k_1 \). Concerning \( A_1 \), the parameter varies in an interval between 0.0052 ± 0.0008 (obtained for ganglioside-modified liposomes in a quartz cuvette stirred at 300 rpm) and 0.1088 ± 0.092 (obtained for unmodified POPC:chol liposomes in a quartz cuvette, stirred at 300 rpm).

Interestingly, the obtained fitting parameters are not distributed evenly or randomly within the established intervals. On the contrary, three very clear subgroups are identified for each of the rate constants, while two are observed for the preexponential factor (see Figure S2(a–c) in the Supplementary material). This suggests that, depending on the combination of liposome modifications, cuvette materials, and stirring speeds, different processes are being monitored. Figure 3 shows a map of the obtained fitting parameters, where different groups can be identified.

As can be appreciated in Figure 3, the most important factor determining the regions is which the fitting parameters are found in the cuvette material, with the experiments performed in quartz found mainly on the bottom-left corner of the \( k_2 \) vs \( k_1 \) map (region a1), while experiments performed in PS are mostly found in the opposite corner (regions b–3 and c–3). The latter suggests that, regardless of the liposome–surface modifications, unwanted interactions between the liposomes and the PS surface occur. For experiments performed in PMMA, the rate of stirring plays an important role. At 300 rpm, most points measured in PMMA lie in region c–1 of the top diagram and c–x of the bottom diagram (group c1x). At a stirring rate of 900 rpm, all points are displaced leftwards towards region a–1, meaning that the initial leakage becomes slower upon increasing the stirring rate. This observation is in agreement with the hypothesis proposed above: liposomes adhere and spread at the surface, and this adhesion is hindered by a faster stirring rate. The modifications introduced to the liposome surface do not appear to have any effect. The fact that the \( k_2 \) values in most experiments performed in PMMA cuvettes fall into group 1, suggests that, after the formation of a supported lipid bilayer on the cuvette surface, only the long-term spontaneous leakage is measured.

In the case of quartz cuvettes, the leakage profile observed at 300 rpm for PEGylated liposomes falls into groups b–2 and b–x, while at 900 rpm the leakage profile falls into a–1 and a–x, meaning that the initial leakage becomes slower, similarly to what is observed in experiments performed in PMMA cuvettes. It is known that PEG and PEGylated lipid nanoparticles can adsorb on silica (Nawal et al. 2007, Zetterberg et al. 2016) and therefore interactions between the PEGylated nanocarriers and quartz cuvettes are likely to occur. As for PMMA cuvettes, the results suggest that increasing the stirring rate prevents the adhesion of the liposomes to the surface. The results also suggest that, when rupture and spreading occurs, the surface of the cuvette becomes passivated and only long-term spontaneous leakage is measured, as indicated by the fact that the \( k_2 \) values for all experiments performed in quartz cuvettes are found in Group 1.

Ganglioside-modified liposomes in quartz cuvettes constitute an interesting case. Here, the results from the measurements fall into groups a–1 and a–x (i.e. group a1x) regardless of the stirring rate. Furthermore, as can be appreciated in Figure S2(d,e) in the supporting information, the experiments show very good reproducibility at both stirring speeds, as indicated by the very small error margins in all fitted parameters. These observations suggest that there are no artifacts arising from liposome–surface interactions.

In order to get further insight into the proposed artifacts (or lack of them) arising from attachment and/or spreading of liposomes onto the different cuvette materials, the liposome–surface interactions were again investigated with QCM-D (Figure 4). As for the case of bare liposomes, PEGylated liposomes attached and accumulated on silica sensors, followed by an (at least partial) rupturing process. The attachment of ganglioside-modified liposomes proceeds more slowly, indicating a weak interaction. In line with our findings, Jordan et al. have reported on the ability of gangliosides to weaken the interaction between dioleoylphosphatidylcholine liposomes and silica sensors (Jordan 2019). The decreased liposome–silica interaction most likely stems from electrostatic repulsion between the sialic acid moieties in the liposomes and the negatively charged silica surface. As a consequence, the adhesion/rupturing of liposomes on the silica is hindered or, at least, slowed down. Moreover, it can be argued that the hydration repulsive force and electrostatic repulsion are enhanced by the presence of gangliosides and therefore lower adhesion energy is expected for the vesicles (Mcintosh and Simón 1986). The observations made from the QCM-D experiments agree with the lack of surface-induced artifacts in the spontaneous leakage measurements performed with ganglioside-modified liposomes. In the case of PMMA sensors, the signal recorded for PEG-modified liposomes suggests the attachment of intact liposomes (very high dissipation/frequency ratio). A similar profile is observed for ganglioside-modified liposomes. Both observations agree also with the leakage profiles recorded. In the case of PS sensors, similar profiles are observed for PEG- and ganglioside-modified liposomes. Given that the leakage profile is also very similar to that of the bare liposomes, the same conclusion can be reached: liposomes attach very slowly onto the cuvette material, where they are shattered by convection currents and/or rupture as a direct effect of the stirring.

The only experiments in which the surface–liposome interaction does not seem to affect the leakage profile are the experiments performed with ganglioside-modified liposomes on quartz cuvettes (Figure 3(a–d), black triangles). The samples can be found in the group a1x irrespective of the stirring rate. It can thus be assumed that these are the only experiments in which the actual spontaneous leakage is being monitored. For all other experiments, there are several indications of biased results: too large rate constants (either for the short-term or the long-term leakage or both), too large \( A_1 \) values (suggesting a large fraction of the liposomes releasing their contents fast) or poor reproducibility (suggesting that variables not controlled have an influence on the results). Thus, a first suggestion that can be put forward when working with POPC:chol, and related liposomes in the liquid-ordered phase state, is to modify their surface with...
gangliosides and to work with quartz cuvettes, thus avoiding surface-induced artifacts. Depending on the purpose of the investigations, this may not, however, be an ideal solution. The introduction of charged groups changes the surface properties of the liposomes and may thus affect their interaction with other molecules of interest, hence limiting their applications in induced leakage studies. Related to this problem is the issue that one cannot guarantee that the properties of ganglioside-modified membranes are the same as those of bare, unmodified liposomes. It can also be argued that the observed attachment of ganglioside-modified liposomes to silica, although probably very weak, can lead to rupture and spreading, especially at low stirring rates, thus affecting the results. In summary, although it is likely that most artifacts are avoided when using ganglioside-modified liposomes, they are not a universal solution. In the next section, we focus on the possibility of modifying the cuvette surfaces instead of the liposomes themselves, thus overcoming some of the issues raised here.

3.3. Passivation of the cuvette surfaces by PEG8000

Previous reports have shown that PEG (Agmo Hernández et al. 2015) and PEGylated lipid nanocarriers (Zetterberg et al. 2016) adsorb strongly on silica surfaces. Therefore, we hypothesized that treating quartz cuvettes with a PEG8000 solution would coat the cuvette surface with a PEG layer that could ‘passivate’ it and prevent it from interacting with the liposomes. In support of this hypothesis, we have in connection with previous studies found that the silica sensors used in QCM-D experiments involving PEGylated nanoparticles are unusable for subsequent experiments unless thoroughly cleaned. Furthermore, Bridgett et al. (Bridgett et al. 1992) have shown, that the adsorption of Pluronics, a class of tri-block copolymers consisting of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide), can significantly reduce the adhesion of bacteria onto a PS surface. For the reasons explained below, it is plausible to assume that satisfactory polymer adsorption occurs also in the absence of the highly hydrophobic middle block found in Pluronics.

Previous reports suggest that PEG can adsorb on both hydrophobic and hydrophilic surfaces (Bodratti et al. 2017). The interactions with hydrophilic surfaces are driven by hydrogen bonds and Lewis acid-base interactions (Mathur and Moudgil 1997). Hydrophobic surfaces interact with the ethylene (−CH2−CH2−)x subunits (Char et al. 1989). Experiments based on the use of pyrene-labeled PEG polymers in combination with either colloidal silica or PS particles showed differences in adsorption behavior (Char et al. 1988, 1989). The interaction with silica caused the polymer to have

![Figure 3. Distribution of the rate constants (k1 and k2, top) and pre-exponential factor (A1, bottom) fitted for experiments performed with stirring at 300 rpm (left) and 900 rpm (right). The color of the symbols represents the cuvette material: black: quartz; red: PS, and blue: PMMA. The shape of the symbols represent the composition of the liposomes: squares: non-modified; circles: PEG-modified, and triangles: Ganglioside-modified POPC:chol liposomes. Error margins have been omitted for clarity. A complete figure (including all experiments and error margins) can be found in Figure S2(d) in the Supporting Information.](image-url)
a flatter conformation compared to the looped conformation found on the hydrophobic PS surface (Char et al. 1989, Wind and Killmann 1998). The prevalence of loops and tails on hydrophobic surfaces causes an enhanced hydrodynamic layer with more material accumulated on the surface compared to the reduced layer on hydrophilic surfaces caused by the more prevalent train configuration and the flatter polymer conformation (Wind and Killmann 1998, Bodratti et al. 2017).

Based on the above discussion, the experiments described in the previous section were repeated on cuvettes that were in advance incubated with a solution of PEG8000. This relatively large molecular weight of the polymer was used in order to decrease the risk that the polymer would desorb from the surfaces during the experiments (Kling and Ploehn 1998).

Figure 5 shows the leakage profiles obtained for POPC:chol liposomes in passivated quartz, PS, and PMMA cuvettes. As can be noted, the leakage is strongly reduced as compared to the case when using bare cuvettes, and the role of the stirring speed is less noticeable. Furthermore, experiments performed using passivated PMMA and passivated quartz cuvettes correspond well with each other. The leakage measured in passivated PS cuvettes is markedly different, however, from that observed when employing the other cuvette materials. Suggesting incomplete passivation of the surface, likely because of a lower affinity of PEG for PS than for the more hydrophilic PMMA and silica. Table 2 shows the fitting parameters obtained. Comparing the data from Tables 1 and 2, it can be appreciated that the parameter that is mainly affected by the cuvette passivation is $A_1$. A possible interpretation of this observation is that the
artifacts arising from surface-liposome interactions are not completely avoided (thus the negligible effect in $k_1$ and $k_2$), but the surface available for interactions is significantly decreased (thus the large decrease in the $A_1$ values).

The combined effect of liposome and cuvette modifications was also studied. The leakage from liposomes modified with PEG-lipids or gangliosides was measured in all three kinds of passivated cuvettes at varying stirring rates. As with the bare cuvettes, the values of the fitting parameters were spread over large intervals, but with three clear groups being observed for $k_1$ and $k_2$, and two clear groups been identified for $A_1$. Figure 6 shows a map of the obtained results. Error margins have been omitted for clarity. A full representation of the data is included in Figure S2(d,e) in the Supplementary material.

Surprisingly, similarly to the bare cuvettes, the most important factor determining the region in which the experiments are found in the cuvette material, meaning that, either the passivation of the surfaces is incomplete or that the properties of the adsorbed PEG layer differ depending on the nature of the surface material. Modified PS cuvettes still dominate the top-right part of the map, indicating strong interactions of the liposomes with the surface. In modified PMMA cuvettes the role of stirring is opposite to that found in the bare cuvettes: increasing the stirring rate displaces the experimental points to the right (from region b-1 to region c-1), meaning an increase in the rate of initial leakage. It is,
however, to be noted that the error margins for these measurements are rather large (see Figure S1(d,e) in the Supplementary material), and there is no significant difference between the obtained parameters.

For the purpose of this report, however, the most interesting experiments are the ones performed in passivated quartz cuvettes. As can be observed in the figure all experiments in these cuvettes lie in regions a-1 and a-x (a1x), regardless of the stirring rate. The experiments also show very good reproducibility. It is, therefore, safe to suggest that, in these experiments, the actual spontaneous leakage of the liposomes is measured, free from artifacts arising from liposome–surface interactions.

In order to obtain further information about the liposome–surface interaction, the silica sensors were passivated with PEG8000 before the samples were applied (Figure 7). The passivation of the sensors changed the liposome–surface interactions with the sensors. Bare liposomes attached to the sensor without any rupturing being observed, meaning that the liposome–surface interaction is weaker. The reduced interaction with the PEG8000-passivated surface agrees well with the observations from the leakage experiments. Liposomes supplemented with 4 mol% of PEG-lipids also displayed reduced attachment to the passivated sensor as compared to the untreated sensor (Figure 4(a)). The weaker interaction observed for the PEGylated, as compared to the naked, POPC:chol liposomes with the sensor can be explained by the additional steric stabilization obtained through the surface grafted PEG2000 polymers. Ganglioside-supplemented liposomes showed a negligible interaction with the passivated sensor (Figure 7(c)), suggesting a further reduction of the weak interaction observed for the bare sensor (Figure 4(b)). The very weak interaction between ganglioside modified liposomes and PEG-modified silica agrees with the low leakage recorded for these liposomes in the passivated quartz cuvettes (Figure 8).

3.4. Spontaneous leakage from POPC:chol-based liposomes

Based on the results discussed above, only a handful of combinations of liposome–cuvette material can be used in order to accurately measure the spontaneous leakage of CF from the former. Assuming that spontaneous leakage is always and continuously taking place in all experiments (usually simultaneously with other processes), the measurements that monitor spontaneous leakage alone should be the ones with the lowest leakage rate. Moreover, as long as the stirring speed is high enough to keep the suspension homogeneous,
there should not be an effect of the stirring speed on the rate of spontaneous leakage. Thus, we propose that only those experiments that fall into group a1x (i.e. small values for $k_1$, $k_2$ and $A_1$ at both stirring speeds) are actually monitoring spontaneous leakage alone. We further propose that experiments falling into region 1 independently of the stirring rate (regardless of whether they fall into categories a, b, c, x or y) are suitable to monitor the long-term spontaneous leakage rate. The reason for this is that a supported lipid bilayer/monolayer is likely formed on the cuvette surface, which prevents further liposome–surface interactions.

**Table 3** summarises the groups to which each modification/cuvette experiment belongs. Figure S2(f) in the supporting information shows selected curves illustrating the differences between the different groups. The measurements that provide information about the actual spontaneous leakage are highlighted.

The averaged fitting parameters and the calculated spontaneous leakage curves are shown in Figure 8. The calculated curves correspond very well with the actual measurements performed in passivated Quartz cuvettes (see Figure S2 in the Supplementary information). It is observed that PEGylation of the liposomes has a negligible effect on the spontaneous leakage, while the modification with gangliosides results in a slightly slower long-term leakage, although the differences are not statistically significant.

### 3.5. The effect of cuvette passivation for POPC and DPPC liposomes

In order to probe the versatility of the method based on PEG8000-passivated quartz cuvettes, we conducted further experiments in which the POPC:chol liposomes were replaced by liposomes in the liquid crystalline- and gel-phase states. We choose POPC and DPPC liposomes for this purpose due to the following reasons. In a previous study (Eriksson and Agmo Hernández 2018) it was reported that whereas the use of PS cuvettes allowed for artifact-free leakage measurements, liposome–surface interactions interfered with the measurements in quartz cuvettes. More specifically, POPC liposomes were found to attach, rupture, and spread on the cuvette walls, whereas DPPC liposomes displayed stirrer-induced leakage following surface attachment. PEG8000-passivation of the cuvettes could potentially prevent the liposomes from attaching to quartz and thus mitigate the unwanted leakage.

In Figure 9(a) the leakage profiles of POPC liposomes measured in untreated and passivated quartz cuvettes are shown. Unlike POPC:chol no significant reduction in leakage was observed for POPC liposomes measured in passivated cuvettes. The reproducibility was not significantly improved and therefore PS cuvettes still remain the preferred choice for POPC liposomes. Leakage graphs without error bars can be found in the Supplementary materials Figure S3(a).

DPPC liposomes showed similar behavior and the leakage profiles did not show a significant difference between the untreated and passivated quartz cuvettes (Figure 9(b)). The measurements performed in passivated quartz cuvettes showed however an improved reproducibility. The reproducibility for these experiments is almost as good as for experiments performed with PS cuvettes. Leakage graphs without error bars can be found in the Supplementary materials Figure S3(b).

The liposome–surface interactions were monitored with QCM-D (Figure 10) and measurements with silica sensors were compared to experiments with PEG8000 passivated sensors. The POPC liposomes displayed similar interaction profiles with bare (Figure 10(a)) and passivated (Figure 10(c)) silica, in agreement with the lack of effect of PEG-coating on
the leakage measurements. Given that POPC liposomes are much softer than POPC:chol or DPPC liposomes, it is likely that these liposomes can adhere and spread on top of the PEG-layer, in agreement with the QCM-D results.

The comparative QCM-D experiments of DPPC liposomes with bare silica (Figure 10(b)) and passivated silica (Figure 10(d)) sensors showed a negligible difference in the adhesion behavior, agreeing also with the non-significant effect of cuvette passivation on the leakage profile (Figure 9(b)).

In contrast to the POPC:chol, the passivation of the quartz cuvettes with PEG8000 did not result in a significantly improved leakage behavior for POPC or DPPC liposomes. The large standard deviation leads to an overlap between the leakage profiles of samples measured in untreated and passivated cuvettes. For POPC liposomes the PS cuvettes are still showing the best performance and good reproducibility. For DPPC liposomes the passivated quartz cuvettes can be considered as a better choice instead of quartz cuvettes due to improved reproducibility.

4. Summary and conclusion

Data collected in the present study show that POPC:chol liposomes are prone to interact with three commonly used cuvette materials, that is, quartz, PMMA, and PS. As a consequence, lipid leakage assays based on the use of self-quenching fluorescent dyes like carboxyfluorescein (CF) can be affected by side processes occurring at the cuvette–solution interface. Incorporation of PEG-lipids or gangliosides in the liposome membrane is, with the possible exception of ganglioside-modified liposomes in quartz cuvettes, not a viable means to completely avoid the attractive interactions and the associated artifacts in the leakage experiments. Our results suggest, however, that the problems can be circumvented by using quartz cuvettes pre-treated with a solution of PEG8000. Noteworthy, such passivated quartz cuvettes were found to be unsuitable for leakage measurements of POPC liposomes in the liquid disordered-phase state. For these types of liposomes unbiased and reproducible results may instead be obtained with PS cuvettes, as shown previously (Eriksson and Agmo Hernández 2018).

The differences in the interaction behavior noted for liposomes in different phase-states are interesting from a fundamental point of view. Data collected in the current study does not allow putting forward a plausible general hypothesis, beyond pure speculation, and further investigations on this matter are clearly warranted. Nonetheless, it is clear that interactions at the cuvette–solution interface can have an important impact on liposome leakage studies, and that experimental artifacts can be avoided, or greatly reduced, by a conscious choice or adequate pre-treatment of the cuvette material.

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