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Effects of gangliosides and PEG-lipids on the structure, properties and interactions of lipid self-assemblies

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Abstract
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When developing or utilizing lipid-based nanocarriers detailed structural characterization of the lipid self-assemblies, as well as in depth knowledge and control of their interaction with solids materials, is necessary to understand the behaviour. Disregarding one of the parameters can lead to misinterpretation of the results due to non-uniform samples or experimental artifacts caused by undesirable interactions with solid surfaces.

Work included in this thesis show that gangliosides promote structural transitions of PEGylated liposomes to bilayer disks. The results suggest that the proposed ability of gangliosides to attenuate the anti-PEG immune response could be coupled to their ability to promote disk-formation.

The results of this thesis further emphasize the importance of processes taking place at the solution-solid interface between self-assembled lipid particles and solid surfaces. Silica surfaces were here of particular interest, and the results showed that PEGylated lipid nanocarriers, such as liposomes and lipodisks, spontaneously attach to the material. It was further shown that an elevation of the temperature can lead to irreversible structural changes, such as the formation of supported lipid bilayers. Interestingly, the investigations revealed that defect free supported lipid bilayers (SLB) can be formed from liposomes in the gel phase.

The processes at the solution-solid interface are of relevance if the solute permeability of lipid membranes are investigated with the help of liposomes in combination with spectroscopic methods. Experimental artifacts resulting from processes at the solution-cuvette interface affect the measurements and impair the reliability of the results. In order to solve this issue we explored two methods to passivated the cuvette interface, and thus prevent, or minimize, the attractive interactions between the lipid particles and the cuvette walls. In the first case a PEG-polymer and in the second a SLB was used. Both methods have their individual advantages and our findings highlight the importance of a conscious selection of the experimental procedure.

Keywords: Lipid self-assemblies, solution-solid interface, liposomes, lipodisks, lipid bilayers, liposome permeability, cuvettes

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Für Nadine

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Grad, P.**, Gedda, L., Edwards, K. (2020) Effect of gangliosides on structure and integrity of polyethylene glycol (PEG)-stabilized liposomes. *Journal of Colloid and Interface Science*, 578: 281–289

II. **Grad, P.**, Edwards, K., Agmo Hernández, V. (2021) Adhesion and structural changes of PEGylated lipid nanocarriers on silica surfaces. *Physchem*, 1(2): 133-151

III. **Grad, P.**, Agmo Hernández, V., Edwards, K. (2021) Avoiding artifacts in liposome leakage measurements via cuvette- and liposome-surface modifications. *Journal of Liposome Research*, in print

IV. **Grad, P.**, Agmo Hernández, V., Edwards, K. (2022) Improved accuracy and reproducibility of liposome leakage measurements by the use of supported lipid bilayer-modified quartz cuvettes. Manuscript

Contribution report

The author wishes to clarify his contributions to the research presented in Papers I–IV

I. I participated in planning the experiments together with LG and KE. I prepared all samples and conducted all experiments except the cryo-TEM experiments. I participated in the data analysis of all experiments. I participated in writing the first draft with KE and editing the manuscript with LG and KE.

II. I planned most of the experiments together with VAH and KE. I prepared most of the samples and conducted most of the QCM-D experiments. I participated in the data analysis together with VAH and KE. I wrote the first draft together with VAH. I edited the manuscript together with VAH and KE.

III. I planned the experiments together with VAH and KE. I prepared all samples and conducted all experiments. I analysed the data together with VAH and KE. I wrote the first draft together with VAH. I edited the manuscript together with VAH and KE.

IV. I planned the experiments together with VAH and KE. I prepared all samples and conducted all experiments. I analysed the data together with KE. I wrote the first draft together with VAH. I edited the manuscript together with VAH and KE.

Papers not included in this thesis

i. Eriksson, E. K., Edwards, K., **Grad, P.**, Gedda, L., Agmo Hernández, V. (2019) Osmoprotective effect of ubiquinone in lipid vesicles modelling the E. coli plasma membrane. *Biochimica et Biophysica Acta (BBA) – Biomembranes*, 1861(7): 1388–1396

Contents

Abbreviations

A	
ABC	Accelerated blood clearance
C	
CF	5(6)-Carboxyfluorescein
Chol	Cholesterol
CPK	Space-filling model
CPP	Critical packing parameter
Cryo-TEM	Cryo-transmission electron microscopy
D	
DLS	Dynamic light scattering
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine
DSPC	1,2-distearoyl-sn-glycero-3-phosphatidylcholine
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)]
G	
GM1	Monosialoganglioside
H	
HEPC	Hydrogenated egg phosphatidylcholine
I	
IgM	Immunoglobulin M
L	
l_d	Liquid disordered
l_o	Liquid ordered
M	
MP-SPR	Multi-parametric surface plasmon resonance
MPS	Mononuclear phagocyte system
N	
NPS	Nanoplasmonic sensing
P	
PEG-lipid	Polyethylene glycol-conjugated lipids
PEG	Polyethylene glycol
PMMA	Poly(methyl methacrylate)
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine
PS	Polystyrene

Q	
QCM-D	Quartz crystal microbalance with dissipation monitoring
R	
RES	Reticuloendothelial system
RT	Room temperature
S	
SLB	Supported lipid bilayer
s_o	Solid ordered
SPR	Surface plasmon resonance
T	
T_m	Transition temperature

1. Introduction

1.1 Lipid nanoparticles

The universe of self-assembled lipid aggregates comprises a wide range of different structures. The research presented in this thesis mainly focuses on liposomes and lipodisks, which are described in the following sections. A short introduction for a basic understanding of how and why these lipid self-assemblies are formed is provided, based on the concept of critical packing parameters. Lipids can be considered as building blocks with specific geometric limitations. They are able to form certain preferred structures and are limited to such structures.

1.1.1 Critical packing parameters

All the lipid dispersions referred to in this thesis are diluted systems with low concentrations. The formed assemblies are affected by thermodynamic and intra-aggregate forces. More concentrated systems also involve interaggregate forces. These interactions can result in the formation of ordered mesophase structures.[1]

The possible structures formed by lipids are determined by their packing properties. These properties can be described by the critical packing parameter (CPP) of the lipids and depend on various factors such as the optimal headgroup area a_0, the volume of the hydrocarbon chain v and the maximum effective length $l_{\max}$ of this chain.[1]

$$\mathrm{CPP} = \frac{v}{a_0 \cdot l_{\max}} \tag{1}$$

Interactions at the headgroup and the area of the hydrocarbon chains determine the structures the molecules can assemble into.[1] Lipids with a large hydrated headgroup, such as polyethylene glycol-conjugated lipids (PEG-lipids) or gangliosides, have a CPP with values between $\frac{1}{3}$ and $\frac{1}{2}$. Both of these lipid types are relevant for this thesis and will be discussed in later chapters. Other lipids with smaller headgroups can have truncated cone (CPP=0.5-1) or cylindrical (CPP=1) shapes.[1]

Different lipid-based structures formed by lipids relevant for this thesis are shown in *Figure 1*. Conical lipids such as PEG-lipids form micelles (*Figure*

1A), whereas liposomes are formed by cylindrical lipids (*Figure 1B*). On the other hand, lipodisks require the right combination of conical and cylindrical lipids in order to form (*Figure 1C*). The variety of different structures that can be formed is immense. Nevertheless, it should be noted that entropy favours the structure with the lowest aggregation number.[1]

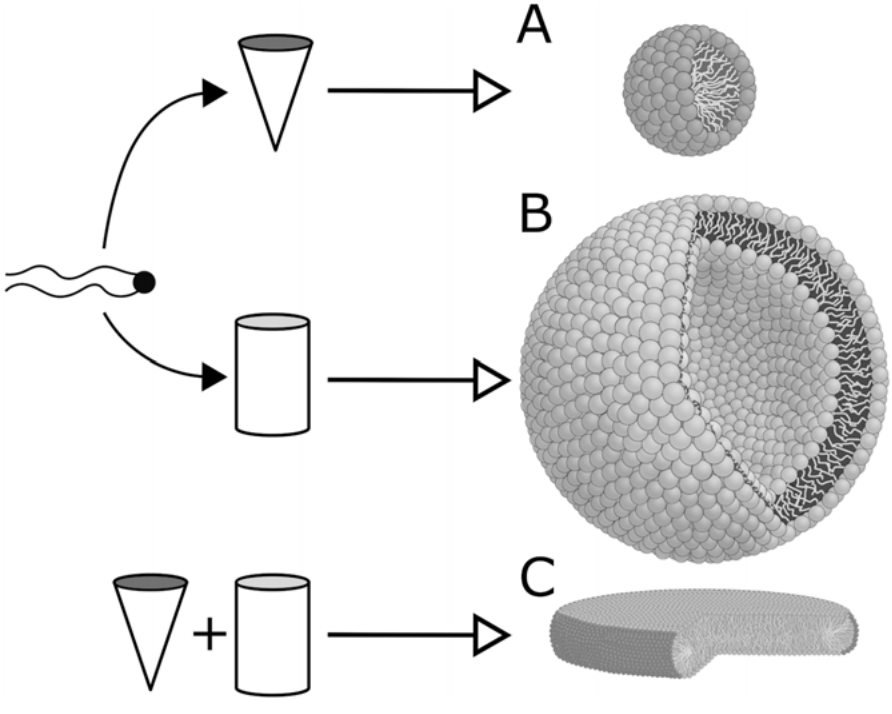


Figure 1 A schematic example of structures formed by lipids with different geometrical shapes, such as conical and cylindrical shapes.

Liposomes will be discussed first. They are lipid structures that are used in pharmaceutical applications, such as drug delivery vehicles.

1.1.2 Liposomes

Liposomes are self-enclosed lipid bilayers that are spherically shaped with an aqueous core (*Figure 2A*). Depending on the type of preparation, it is also possible to obtain multilamellar vesicles with an onion-like layering of several lipid bilayers (*Figure 2B and C*).

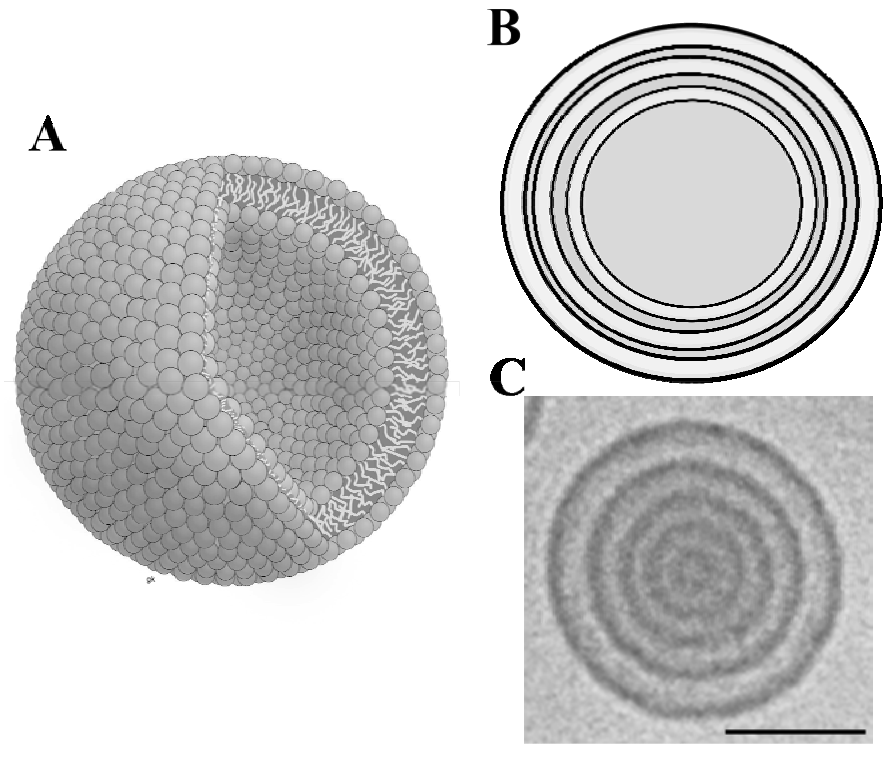


Figure 2 (A) Schematic illustration of a liposome with a lipid bilayer and an aqueous core. (B) Schematic illustration of a multilamellar liposome. (C) Cryo-transmission electron microscopy (TEM) image of a multilamellar liposome. (Size bar: 50 nm)

The versatility of these lipid particles as nanocarriers is due to their liquid-filled structure. Hydrophobic substances can be taken up by the lipid membrane, while hydrophilic substances are encapsulated in the aqueous core. Liposomes' areas of application are as versatile as the lipid nanoparticles themselves and include food, cosmetic and pharmaceutical industries.[2-4] Liposomes dedicated to drug delivery now cover a wide range of approved and potential products.[2, 5-8]

Liposomes form when a lamellar liquid crystalline phase is dispersed in excess water and larger bilayers are broken up due to an external force. The external energy can be mechanical in nature, such as extrusion through small pore filters, or acoustic, such as sonication. Several factors can influence the liposome-formation process, such as temperature and lipid composition. In particular, the lipid composition is important because it determines the bending elasticity and mechanical cohesivity of the lipid bilayer.[9] Depending on the preparation method, the radii of these self-assembled structures can range from ~20 nm to several micrometres.

The size of liposomes is not stable over time, as liposomes change their appearance. This is because liposomes are not thermodynamically stable and

therefore aggregate or fuse over time.[10] In this way, lamellar structures with decreased curvature are formed and, after some time, a precipitate may form. Liposomes' instability is related to by the bending elastic energy required to form the curved bilayer of a liposome. Curved bilayers have a higher free energy compared with a flat bilayer, and each fusion event lowers the excess bending energy. Nevertheless, fusion is entropically unfavourable.[10] Liposomes are therefore kinetically trapped as nonequilibrium structures with what may be a relatively long lifetime.[10, 11] In this context, it is worth mentioning an interdependent process – namely, colloidal stability, which will be further discussed in Section 1.2.

1.1.3 Lipodisks

Lipodisks are flat bilayer disks (*Figure 3*) that are formed through the addition of micelle-forming amphiphiles to membrane forming lipids.[12, 13]

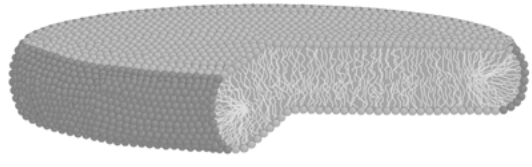


Figure 3 Schematic illustration of a lipodisk composed of bilayer-forming lipids (light green) and micelle-forming lipids (dark green).

For example, PEG-conjugated lipids are useful for preparing lipodisks due to their high spontaneous curvature and limited solubility in the flat part of a lipodisk. Exceeding the solubility limit results in the formation of either structures with high curvature, such as mixed micelles or bilayer disks, which require component segregation; however, both processes come at a cost.[14-16] On the one hand, the formation of mixed micelles has a bending energy penalty, whereas component segregation in lipodisks is unfavourable from an entropic point of view. The bending modulus and the spontaneous curvature of bilayer-forming lipids tip the scale in one way or the other.[13, 14, 17]

The first lipodisks can form above a molar ratio of 5 mol% of PEGylated lipids, and a complete transformation is usually obtained at around 20 mol%.[12, 15, 17] The comparably high molar ratios of PEG-conjugated lipids in lipodisks result in a high colloidal stability, which will be further discussed in the next section.

1.2 Polymer-stabilized lipid nanoparticles

The colloidal stability of dispersed lipid nanoparticles depends on the attractive and repulsive forces between the particles. A lack of repulsive contribution results in a nonequilibrium state in which the system tends to aggregate or fuse over time to reach a thermodynamically stable state. Lipid particles can be stabilized if they have a charged surface and the electrostatic double-layer repulsion exceeds the attractive forces. In that case, however, the particles are sensitive to higher ionic strengths. The aggregation and fusion of non-charged liposomes can be slowed down by other repulsive forces, such as hydration forces, protrusions and undulations.[18]

Additional problems arise if lipid particles are used *in vivo*. The interaction of liposomes with lipoproteins results in the disintegration of the former. [19] Furthermore, a correlation between drug release and exposure to serum proteins has been reported.[20, 21] Another issue is the rapid clearance of liposomes from circulation due to uptake into the cells of the mononuclear phagocyte system.[22] Nevertheless, there is some possibility of improving the *in vivo* stability and the colloidal stability of the system via steric stabilization, which can be provided by lipid-grafted polymers, such as polyethylene glycol (PEG).[9]

1.2.1 The application of polyethylene glycol

The addition of PEG-grafted lipids (PEG-lipids), as displayed in *Figure 4*, is a common tool to modify lipid nanoparticles.[23, 24] The presence of PEG-lipids usually prolongs the blood circulation time due to the reduction of two effects: (1) nonspecific hydrophobic interaction with reticuloendothelial system (RES) cells and (2) opsonization reactions.[25, 26] Opsonization is an immune process of the complement system, and opsonins are proteins that bind to foreign particles and then induce phagocytosis by means of phagocytes.[27] Coating a surface with PEG increases its hydrophilicity and thereby reduces nonspecific interactions with RES cells; it also sterically prevents the attachment of opsonins.[25, 26]

However, the blood survival times upon repeated administration of PEGylated liposomes can be reduced by rapid elimination, due to a phenomenon known as accelerated blood clearance (ABC).[28] The ABC phenomenon is an immune response caused by PEG that results in a rapid removal of liposomes due to the production of anti-PEG immunoglobulins M (IgM). The initial dose of PEGylated liposomes induces the production of anti-PEG IgM, which selectively bind to the surface of subsequently administered PEGylated liposomes. This process results in activation of the complement system and clearance of the liposomes.[28-30]

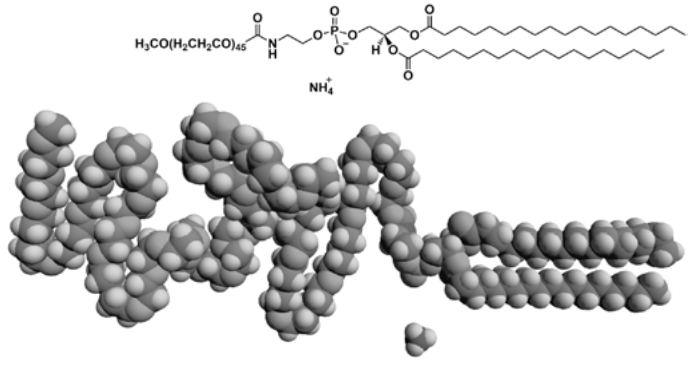


Figure 4 Chemical structures and space-filling (CPK) model of a PEGylated lipid (DSPE-PEG2000).

One proposed method to attenuate the ABC phenomenon is the addition of higher concentrations of PEG-lipids to liposomes.[31] This approach is debatable, however, due to the risk of causing the formation of non-liposomal self-assembled lipid structures as discussed in Section 1.1.2. Another proposed method to attenuate anti-PEG immunity is the addition of gangliosides to PEGylated liposomes.[32, 33]

1.2.2 The application of gangliosides

The discovery of the previously mentioned ABC phenomenon (Section 1.2.1) [28] has led to the exploration of additional lipids for the surface modification of lipid particles. Gangliosides are expressed by all tissues and are known to modulate the immune system.[34] They are glycosphingolipids composed of ceramide linked to an oligosaccharide containing a sialic acid (*Figure 5*). The sialic acid is negatively charged at most physiological pH values.[35] Similar to the case of PEG-lipids, the addition of gangliosides to liposomes promotes prolonged blood circulation times. The modified liposomes exhibit reduced uptake by the RES.[36] However, it has been shown that PEG-lipid-supplemented liposomes are superior to their ganglioside-supplemented counterparts in terms of prolonged blood circulation times, which explains the prevalence of PEGylated liposomes and the undetermined effects of gangliosides on the structure of liposomes.[25] To the best of our knowledge, our research group is the first to describe the additive effects of PEG-lipids and gangliosides.

The insertion of gangliosides into PEGylated liposomes has recently been proposed as a method to attenuate anti-PEG immunity.[33] This behaviour has been explained as being due to the capacity of gangliosides to induce immunological tolerance to PEG. This antigen tolerance is believed to be a result of gangliosides' role as Siglec (sialic-acid-binding immunoglobulin-like lectins) ligands, which are known to be regulators of the immune system.[37] Several

studies support this hypothesis and have shown that the simultaneous presence of antigens and Siglec ligands on nanoparticles induces antigen-specific tolerance.[38-40]

Like PEG-lipids, gangliosides are micelle-forming lipids. Their addition to lipid formulations may thus be hypothesized to cause structural effects comparable to those observed for PEG-lipids. However, the micelles formed by PEG-lipids and gangliosides present certain differences. Micelles formed by PEG-lipids are spherical whereas most gangliosides form micelles with an oblate shape [41-43]. The non-spherical shape is a result of the smaller area occupied by the headgroup of each molecule on the micelle's surface.[41]

The structural effect caused by the addition of gangliosides to PEGylated liposomes is investigated in Paper I.

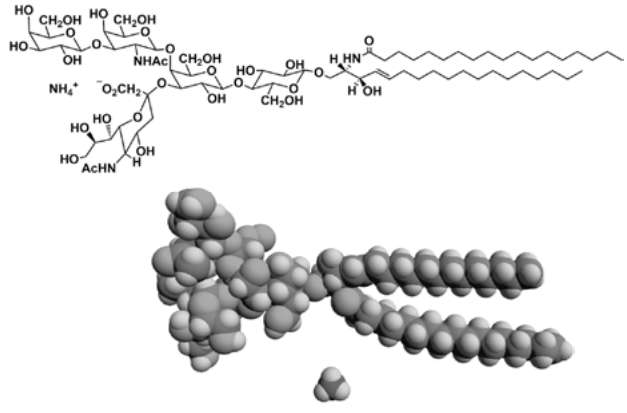


Figure 5 Chemical structure and CPK model of a ganglioside (GM1).

1.3 Properties of lipid bilayers

Colloidal stability is not the only parameter of interest if lipid particles are to be used as drug delivery vehicles. A hydrophilic drug can be stored in the aqueous compartment of liposomes. In this case, a relevant factor is the permeability of the lipid membrane towards the hydrophilic solute of interest. Permeability can be related to different lipid membrane properties such as the phase state.

1.3.1 Phase states of lipid bilayers

Water-lipid systems have a complex dependence on parameters such as temperature and water content, and are known for their lyotropic phase behaviour.[44, 45] The systems of importance to this thesis are diluted lipid-water

systems; therefore, thermotropic behaviour is a key focus. This section discusses liquid-disordered, liquid-ordered and gel phase states.

The phase state of a lipid bilayer is dictated by the molecular nature of the lipid, such as the length of the hydrocarbon chains, number of unsaturated bonds and specific properties of the lipid head group.[44] These parameters influence the main transition temperature, T_m, which is the temperature at which a phase change is induced. This phase change is a first-order transition from a solid-ordered (s_o, gel) to liquid-disordered (l_d, lamellar liquid crystalline) phase. Below the transition temperature, the fatty-acid chains are all trans and fully stretched and the lipid molecules have ordered fatty-acid chains arranged in a regular structure.[45] The bilayer is therefore fully extended, the cross-sectional area of the molecules is minimal and the intramolecular and intermolecular motions are highly restricted. Lipid bilayers in the liquid-disordered phase contain gauche rotational conformers that are coupled to an increased cross-sectional area of lipid molecules. This results in decreased bilayer thickness and increased rates of intramolecular and intermolecular motion of the lipid molecules.[46]

Transition temperature is an important parameter, and interesting morphological changes can occur when liposomes are prepared above their transition temperature and then exposed to a temperature below their T_m. This is illustrated in *Figure 6*, which shows liposomes composed of hydrogenated egg phosphatidylcholine (HEPC) that display a polygonal appearance. The polygonal structure is likely a result of the formation of flat bilayers in the gel phase, connected by highly curved patches of fluid lipids.[47]

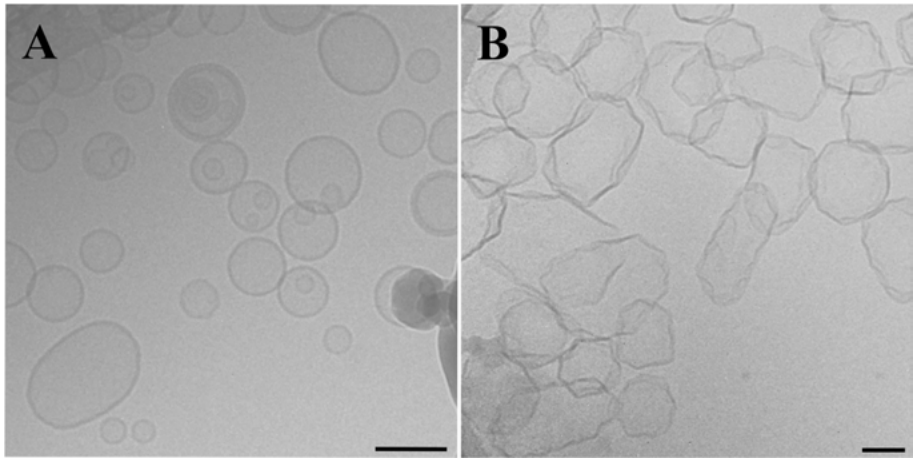


Figure 6 Cryo-TEM images of (A) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) liposomes in the liquid-disordered and (B) hydrogenated egg phosphatidylcholine (HEPC) liposomes in the gel phase. (Scale bar = 100 nm)

Cholesterol (*Figure 7*) is one of the major components of eukaryotic cells and has a drastic effect on the physical characteristics of lipid membranes composed of phosphatidylcholine (PC)-lipids.[48, 49] Concentrations of cholesterol greater than about 25%–40 mol%, depending on the lipid species, cause the lipid membrane to transform into the so-called liquid-ordered phase, l_o.[50-52] These l_o membranes have interesting properties such as a high lateral mobility coupled with a positional disorder similar to that of l_d membranes, while possessing a higher stiffness similar to that of s_o membranes.[45]

The addition of cholesterol to gel-phase bilayers breaks the lateral packing and induces disorder. An opposite ordering effect can be observed when cholesterol is added to liquid-disordered bilayers.[45, 48, 49]

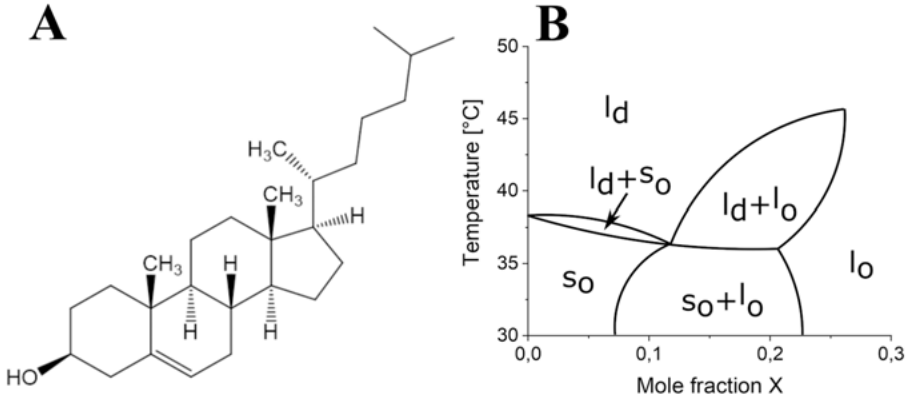


Figure 7 (A) Molecular structure of cholesterol; (B) a generic phase diagram showing the phases found in phospholipid-cholesterol mixtures. (Adapted from Refs. [49, 53].)

1.3.2 Permeability of lipid membranes

The permeability of lipid membranes towards hydrophilic solutes is an innate property that depends on the chemical nature of the lipids. Structural aspects of the hydrocarbon chains and the headgroups can influence the permeability. The lipid packing and the interaction between the headgroups are also important parameters.[54] The thermotropic behaviour of lipid membranes is a direct result of these parameters and affects the lipid membranes' permeability towards hydrophilic compounds. Liposomes in the liquid-disordered phase show higher degrees of leakage (i.e. higher permeability) than gel-phase liposomes.[55, 56] A profound decrease in the diffusion of hydrophilic compounds can be obtained by the addition of cholesterol.[54] The permeability is reduced by an increase in the mechanical rigidity, thickness and cohesiveness of lipid membranes in the liquid crystalline state.[57] Electrostatic interactions between the headgroups and cohesive forces of the fatty-acid chains can create permeation barriers. Furthermore, the thickness of the lipid membrane is an important factor, and investigations have shown that a decrease in

bilayer thickness results in an increased flux across the lipid membrane.[58, 59]

Although membrane permeability is usually investigated with fluorescent probes, other techniques are also available, such as dynamic dialysis, Franz cell diffusion and theoretical simulations, as described in Ref. [60] and the references therein. The leakage assay used for the investigations in this thesis, which is described in Section 2.2.1, is based on the use of fluorescence spectroscopy to measure the release of a liposome-encapsulated probe. When employing spectroscopic methods, it is important to note that an investigation of lipid membrane properties can be affected by interactions between the liposomes and the solid surfaces of cuvettes.[61] It is therefore important to investigate these interactions and their influence on the measured membrane permeability.

1.4 Interaction of lipid nanoparticles with different surfaces

An investigation of the interactions between solid surfaces and lipid particles can reveal processes that occur at interfaces and affinities between liposomes and different surface materials. These interactions can be studied with a plethora of techniques. In this thesis, the main technology employed was a quartz crystal microbalance with dissipation monitoring (QCM-D), which will be further discussed in Section 2.3.1.

Relevant materials to investigate regarding their interactions with lipid nanoparticles include silica, polystyrene (PS) and poly(methyl methacrylate) (PMMA). These materials are of interest not only because cuvettes for spectroscopic experiments are often composed of them but also due to their differences in hydrophilic/hydrophobic properties, with hydrophilic silica [62] at one end of the spectrum and hydrophobic polystyrene (PS) at the other.[63] In comparison PMMA is a material with intermediate properties.[64]

1.4.1 Silica surfaces and lipid nanocarriers

Interactions between silica surfaces and lipid nanoparticles have previously been investigated using various techniques, such as cryo-transmission electron microscopy (TEM), QCM-D, ellipsometry, atomic force microscopy and fluorescence assays.[65-71] Depending on the nanoparticle's composition, such interactions can lead to different possible processes at the solid surface. The adsorption and spontaneous rupture of vesicles on the surface is one possible process that results in the formation of a supported lipid bilayer (SLB).[67, 72-74] It is also possible for incomplete SLBs to be formed, with some remaining intact vesicles present at the surface. If the particles are resistant to

rupturing, then the reversible and irreversible adsorption of intact vesicles can occur, depending on the vesicles strength of interaction. Negligible adsorption can be observed if the affinity of the vesicles for the surface is low.[73, 75] The parameters responsible for this complex interaction behaviour include bilayer composition, vesicle size, membrane surface charge density, pH, type of ion and temperature. [66, 68, 73, 75-78]

The use of PEG-conjugated lipids to increase the colloidal stability of lipid nanoparticles also affects the nanoparticles' interaction with silica. The protruding polymer affects the nanoparticles' interaction with the surface and can lead to the adsorption of PEG-grafted lipid nanoparticles onto the silica surface.[79] This interaction of silica with a non-ionic polymers such as PEG is another parameter that must be investigated in order for PEGylated lipid particles to be used as drug delivery vehicles, as the particles' integrity might be affected by storage in silica-based containers. Although some studies [80-83] on this topic have been published during the last few decades, the mechanisms behind PEG-silica interactions are still not fully understood. Such interactions appear to be a result of two processes: (1) hydrogen bonding between the silanol units of the silica and the ethoxy units of the PEG; and (2) hydrophobic interactions between PEG segments and siloxane moieties.[79, 82, 84]

1.4.2 PS and PMMA surfaces and lipid nanocarriers

In comparison with the numerous studies on interactions between lipid nanoparticles and silica, less attention has been paid to interactions between lipid nanoparticles and polymers such as PS and PMMA. Some studies have investigated the interactions between lipid particles and PS surfaces using atomic force microscopy [69] or QCM-D [85]. Unlike the bilayers that form on hydrophilic materials, monolayers form on hydrophobic surfaces if the lipid particles are disrupted.[86-89] Alternatively, intact vesicles can adsorb onto the surface.[70] The interactions between PMMA and lipid nanoparticles [90] have received even less attention.

The insertion of PEG-lipids into liposomes affects the interaction between the lipid nanoparticles and hydrophobic surfaces. This observation agrees with findings on the interactions between PS and non-ionic surfactants containing ethylene oxide groups.[91] It has been shown that a fraction of the non-ionic surfactant strongly binds to the PS surface. The non-ionic surfactant is adsorbed flatly onto the PS surface, with both the aliphatic and the polar part interacting with the PS. This process is described as being reversible, with a very slow desorption.[91]

1.5 Thesis layout

The overall aim of this thesis is to investigate the effects of gangliosides and PEG-lipids on the structure, properties and interactions of lipid self-assemblies. In Paper I, the objective is to study the structural transformations of liposomes by the addition of gangliosides, PEG-lipids and a combination of both. In Paper II, the interactions between PEGylated lipid nanocarriers and silica surfaces are studied, and the difference in interactions between PEGylated and non-PEGylated liposomes with silica surfaces is presented. Furthermore, this study reveals differences between the interaction profile of liposomes and that of lipodisks and proposes an explanation. In Paper III, the interactions of liposomes with different cuvette materials are investigated in order to reduce experimental artifacts in leakage studies. The interactions are modified by supplementing the liposomes with gangliosides and PEG-lipids. However, a more promising approach is found to be modification of the cuvette surface with adsorbed PEG.

Finally, in Paper IV, we follow up on Paper III and develop a method to reduce experimental artifacts in leakage studies of liposomes in the liquid-disordered phase state. The new pre-treatment protocol provides the inner walls of quartz cuvettes with a passivating SLB to reduce unwanted side processes.

2. Experimental techniques

2.1 Cryogenic transmission electron microscopy

Cryo-TEM is a powerful method for investigating lipid particles in aqueous media. [92, 93] Vitrification of the sample by rapidly freezing it is an important step of the preparation, as it facilitates the formation of a non-crystalline, amorphous water matrix that enables visualization without additional preparatory requirements, such as drying, staining or labelling. An electron beam is then transmitted through the sample and thereby interacts with the sample. Differences in the scattering of the electrons result in the formation of a two-dimensional (2D) image, due to contrast differences between the water matrix and lipid aggregates (*Figure 8*).[93, 94]

Cryo-TEM requires vitrified films that are within a certain range of thickness. The films should be thick enough to contain the lipid aggregates, yet thin enough to minimize background electron scattering.[93] A small drop of the sample is deposited on a grid which is covered by a holey polymer film. Excess liquid is removed with a filter paper to prevent the formation of thick films. The sample is then vitrified through immersion into liquid ethane, kept at ~100 K. The procedure is performed in a humidity and temperature-controlled chamber. A protective nitrogen atmosphere is maintained during the transfer to the electron microscope. Analysis of the sample is conducted under high-vacuum conditions.[93] The resolution of cryo-TEM permits the investigation of particles as small as micelles and liposomes up to a diameter of approximately 500 nm. Larger particles are excluded due to the limited thickness of the film.

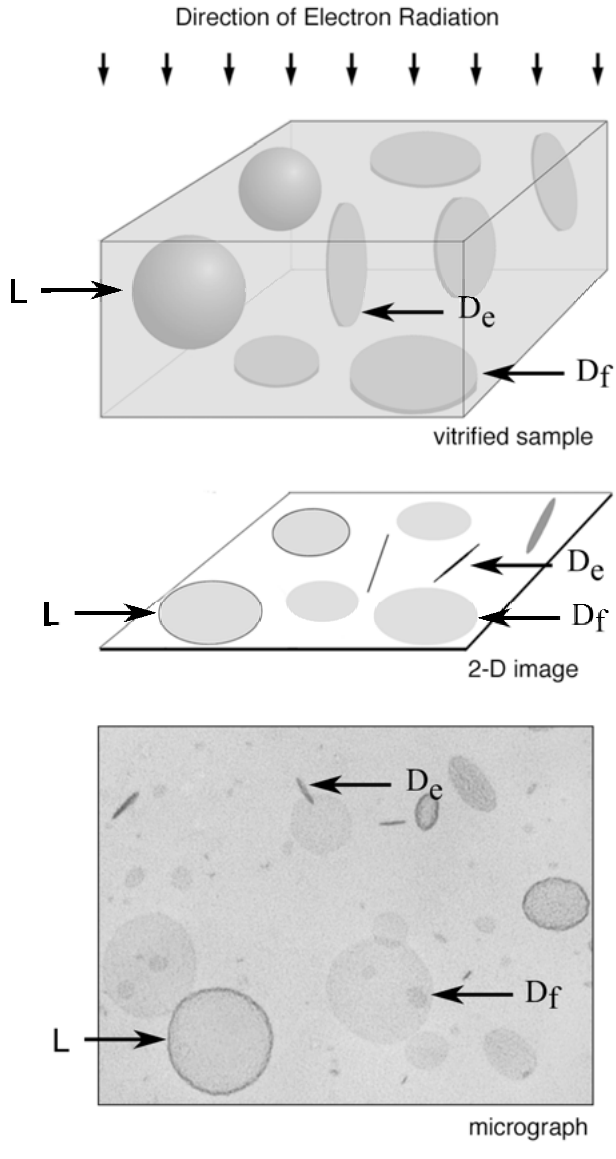


Figure 8 Interpretation of cryo-TEM images from a vitrified sample (top) to an imaginary 2D image (centre), and then to the resulting micrograph (bottom). Different self-assembled lipid structures are indicated by arrows: *L* points to a liposome, D_e to an edge-on lipodisk and D_f to a face-on lipodisk.

Interpreting the results of cryo-TEM requires a knowledge of common artifacts such as overlaying ice crystals, perturbation of the sample as a result of extended electron beam exposure and the high surface-to-volume ratios of the sample films. An understanding of the latter is particularly important, as it

causes the preparations to be sensitive to evaporation, which changes the salinity. High humidity in the preparation chamber is thus essential to prevent effects caused by increasing osmolarity, since liposomes tend to deform under osmotic stress (*Figure 9*). [93, 94] When interpreting the micrographs, it is possible to differentiate between liposomes and lipodisks, as shown in *Figure 8*.

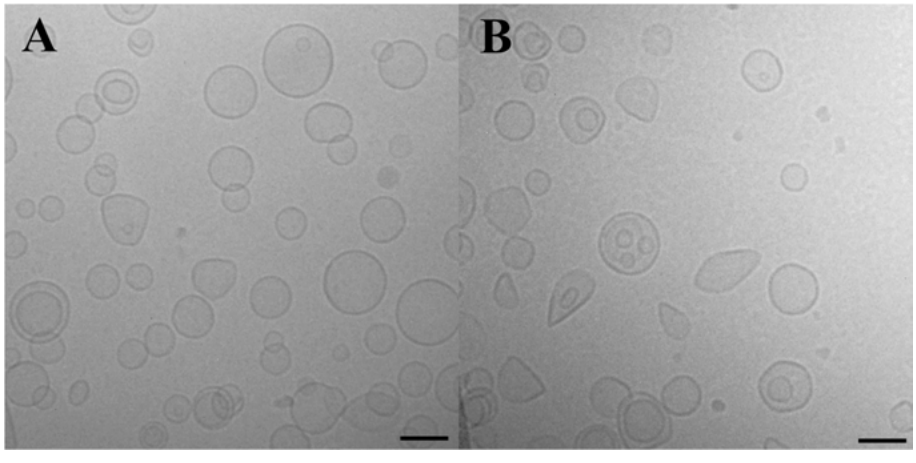


Figure 9 Cryo-TEM images of POPE:POPG:Cardiolipin liposomes with a molar composition of 75:19:6. (A) Unstressed liposomes. (B) Liposomes stressed by increased salinity. (Scale bar = 100 nm)

Cryo-TEM was used in Paper I to investigate the structural transitions of self-assembled lipid structures composed of HEPC and cholesterol (Chol) as a result of the addition of gangliosides. The effect of gangliosides in the presence of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG(2000)) in these lipid dispersions was also investigated.

2.2 Dynamic light scattering

Dynamic light scattering (DLS) is a technique used to obtain information about particle size and size distribution. The DLS instrument monitors the temporal fluctuation of the scattered light intensity I_s as a result of the random motion of dispersed particles due to Brownian motion. The intensity fluctuations are analysed with an intensity autocorrelation function that decays in the time domain and is commonly referred to as the decay time, τ. The form of this function is shown in *Figure 10A*.[95-97]

Short time intervals $t_0 + \Delta t$ are correlated due to small changes in the particles' location. For longer time intervals $t_0 + \Delta t$, the correlation to t_0 will be

lost. The rate of decorrelation is thus determined by the translational motion of the particles and described by the autocorrelation function. Smaller particles cause a faster decorrelation of the scattered intensity trace than larger particles, due to their faster dynamics. The autocorrelation function is related to the translational diffusion coefficient, D, for monodisperse and spherical particles. The hydrodynamic diameter R_h of the particle can then be obtained with the help of D, by using the Stokes-Einstein equation [95-97]:

$$R_h = \frac{k_B T}{6\pi\eta D} \tag{2}$$

where k_B is the Boltzmann constant, T is the temperature and η is the viscosity of the sample. An example of a size distribution is shown in *Figure 10B*.

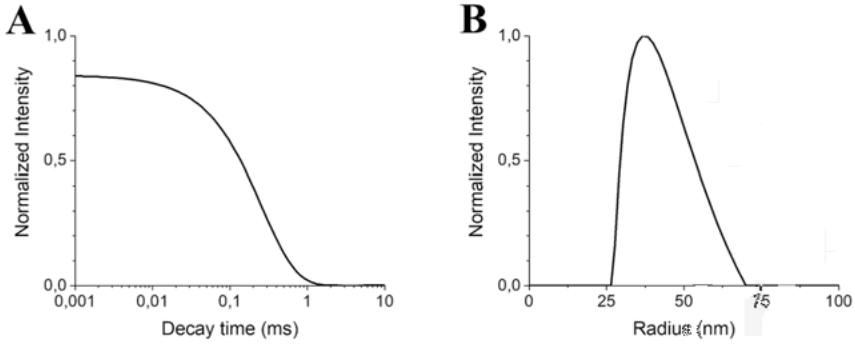


Figure 10 Experimental DLS data of a liposomal sample composed of DSPC:Cholesterol:DSPE-PEG2000 with a molar fraction of 56:40:4. The following data is acquired: (A) the intensity autocorrelation function, which can then be fitted to obtain (B) the size distribution of the sample.

This technique is powerful for monodisperse samples; on the other hand, multidisperse samples require a more complex data interpretation. In the latter case, the autocorrelation function is determined collectively by the diffusion of particles of different sizes. Here, the inherently varying scatter intensity of particles, which depends on the size, shape and number of particles, presents a problem. In particular, size is an important parameter, and the intensity of scattered light is proportional to the sixth power of the particle radius (R^6). Moreover, non-spherical particles cannot be unambiguously described using this technique. The hydrodynamic radius calculated is that of a sphere with the same translational diffusion speed as the particle.[95, 97]

Different size distributions can be a useful tool for the interpretation of polydisperse samples. DLS instruments can usually reveal the intensity, volume and number size distributions. The intensity distribution is the fundamental size distribution based on the intensity of the light scattered by the particles.

Both the volume and number size distributions are derived from the intensity distribution using Mie theory. The volume distribution is equivalent to the mass or weight distribution. The number size distribution can be of relevance if particles of different sizes are measured. The previously mentioned radius-dependent scattering intensity results in a 1,000,000 times higher scatter intensity for a particle with a diameter of 50 nm compared with a 5 nm particle. The number size distribution translates the raw data into the relative proportion of differently sized particles based on their size. Number and volume distributions should be used with care, because the calculated means and widths are less reliable than the raw data based intensity distribution.[95, 97]

Before and during an experiment, certain practical parameters must be considered. In this thesis, it is relevant to ensure the thermal equilibration of the laser. Furthermore, the DLS technique is sensitive to dust or small populations of very large particles. The sample should be thermally equilibrated to prevent gradients.[97]

DLS was used in Paper I to investigate the size distribution of liposome dispersions composed of HEPC:Chol:DSPE-PEG2000 with a molar fraction of 62:33:5, supplemented with different amounts of gangliosides. With an increasing molar fraction of gangliosides, the sample size distribution became broader, and the average hydrodynamic diameter shrank. The abovementioned issues, which are known to occur in both multi-disperse samples and non-spherical particles, require consideration. In this case, samples with different radii and different structural characteristics were present such as spherical liposomes, discoidal lipodisks and (sometimes) micelles. Investigation of the content of the samples by means of cryo-TEM analysis was therefore important.

2.3 Fluorescence assays

Liposome leakage

As mentioned earlier, the permeability of lipid membranes to hydrophilic probes can be used as an indirect measurement of physical parameters such as lipid packing order, membrane fluidity and totality of packing defects.[98-100] Therefore, the liposomal release of a self-quenching and hydrophilic fluorescent dye, such as 5(6)-carboxyfluorescein (CF), is commonly monitored. The compound is 97%–98% quenched at high concentrations, mainly due to the formation of non-fluorescent dimers.[101]

The standard procedure requires the preparation of liposomes in the self-quenching concentration range such as 100 mM CF in a 10 mM buffer solution at pH=7.4. The unencapsulated fluorescent probe is then replaced by an isotonic buffer solution through gel filtration.[102] The self-quenching property of CF at high concentrations minimizes the fluorescent signal from the dye

encapsulated in the liposomes. The leakage of the probe through the lipid membrane results in an increasing fluorescent signal of released dye diluted in the large volume of solution outside of the liposomes.[103] A schematic illustration of this process is shown in *Figure 11*.

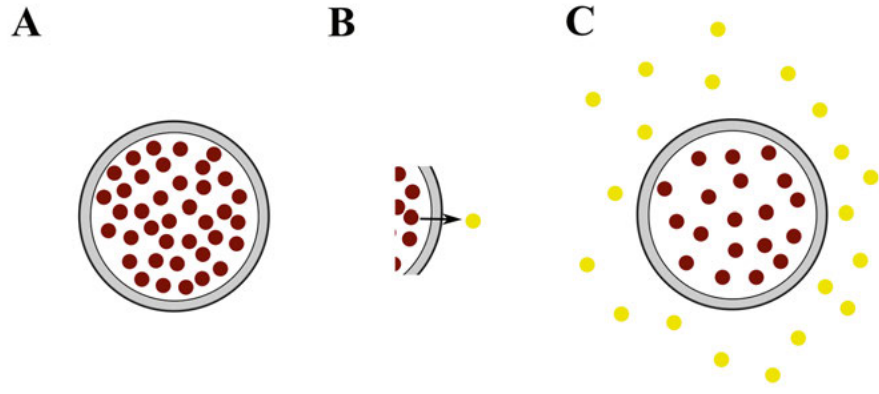


Figure 11 Schematic illustration of the leakage of a self-quenching fluorescent probe from liposomes. (A) A high concentration of the quenched dye (red dots) is encapsulated by a liposome. (B) Leakage of the dye occurs over time through the lipid bilayer, and fluorescence emerges due to the dilution and dequenching (yellow dots) of CF in the surrounding solution. (C) A decreasing concentration of CF in the liposome is coupled with an increasing amount outside of the liposome.

It is important to maintain the concentration-fluorescence relationship within the linear range. In the case of liposomes with a diameter of around 100 nm, a recommended concentration range is between 3–30 μM.[102] The maximum fluorescent signal I_{tot} can be obtained through the addition of a surfactant, such as Triton X-100. The degree of leakage can be determined using the following equation:

$$\chi_{\mathrm{CF_{rel}}} = \frac{I(t) - I_0}{I_{\mathrm{tot}} - I_0} \tag{3}$$

The time-dependent fluorescence intensity I(t) and the initial fluorescence intensity I_0 are obtained during the experiment, whereas lysis of the liposomes is required to obtain I_{tot}, the fluorescence intensity at 100% leakage.

In Papers III and IV, the spontaneous leakage of CF-filled liposomes is monitored as an indicator of liposome-surface-induced processes between the liposomes and the cuvette materials. These processes can comprise the attachment, rupturing and spreading of the liposomes, resulting in the release of encapsulated dye.

2.4 Surface-sensitive techniques

Quartz crystal microbalance with dissipation monitoring

The behaviour of liposomes at interfaces can be monitored through the use of surface-sensitive techniques such as a quartz crystal microbalance with dissipation monitoring (QCM-D). This technology detects mass changes at the sensor surface in real time at a nanoscale resolution.[104-106] For example, the theoretical sensitivity of a 5 MHz crystal is 17.7 ng/(cm^2·Hz). Moreover, changes in dissipation factor are captured, revealing information about the viscoelastic properties of the sample.[107]

The sensor is an important part of this technology and is composed of a thin quartz crystal. The crystal has deposited electrodes on each side and a coating of a desired material on the side facing the sample.[106, 107] Examples of coatings include silica, gold or polymers such as PS and PMMA. Through the application of voltage, the crystal is excited to resonance. This behaviour is described as the inverse piezoelectric effect. The resonance frequency f_0 depends on the mass per unit area.[106] Changes in the latter as a result of the adsorbed material cause changes in the resonance frequency Δf.

The viscoelasticity of an attached layer can be described by its energy-dissipating behaviour. Rigid materials have a low energy dissipation ΔD, whereas viscoelastic layers show a higher energy loss.[108, 109] The energy loss can be used to differentiate liposomes from attached lipid monolayers or bilayers, due to the differences in their viscoelastic properties. Liposomes are usually softer compared with the stiffer attached lipid layers (*Figure 12*).[105, 107]

The experimental data presented in *Figure 12* is a simplified representation of the data obtained from a QCM-D experiment. Here, the third overtones for the changes in both frequency and dissipation are displayed. The instrument collects the data from the fundamental oscillation frequency f_0 and several overtones, which can be useful for determining the mass of an immobilized film. Agmo Hernández et al. [110] proposed a useful and rapid method to quantify this immobilized mass in the case of thin viscoelastic films. The advantage of this method is that it does not require any previous knowledge of the film's density, shear modulus or viscosity. The method is based on the quantitative mass determination proposed by Voinova et al.[111] for the formation of viscoelastic films on QCM-D sensors. It has been stated that the viscoelastic properties of the formed film are affected by the overtone-dependent shifts of Δf and ΔD.[111]

The change in Δf due to the adsorption of a thin viscoelastic film in a bulk solution can be described by the following equation:

$$\Delta f = -\frac{m_\mathrm{d} n f_0}{t_\mathrm{q}\rho_\mathrm{q}} + \frac{4\pi h_\mathrm{l}\eta_\mathrm{w}\rho_\mathrm{w}\eta_\mathrm{l}(nf_0)^3}{t_\mathrm{q}\rho_\mathrm{q}\left(\mu_\mathrm{l}^2 + 4\pi^2\eta_\mathrm{l}^2(nf_0)^2\right)} \tag{4}$$

where the parameter m_d is the adsorbed mass surface density, n represents the overtone number, t_q describes the thickness and ρ_q represents the density of the quartz crystal. In addition, h_l describes the thickness of the adsorbed layer, η_w is the viscosity of the bulk solution, ρ_w is the density of the bulk solution, η_l is the viscosity of the adsorbed layer and μ_l is the elastic modulus of the adsorbed layer.[111]

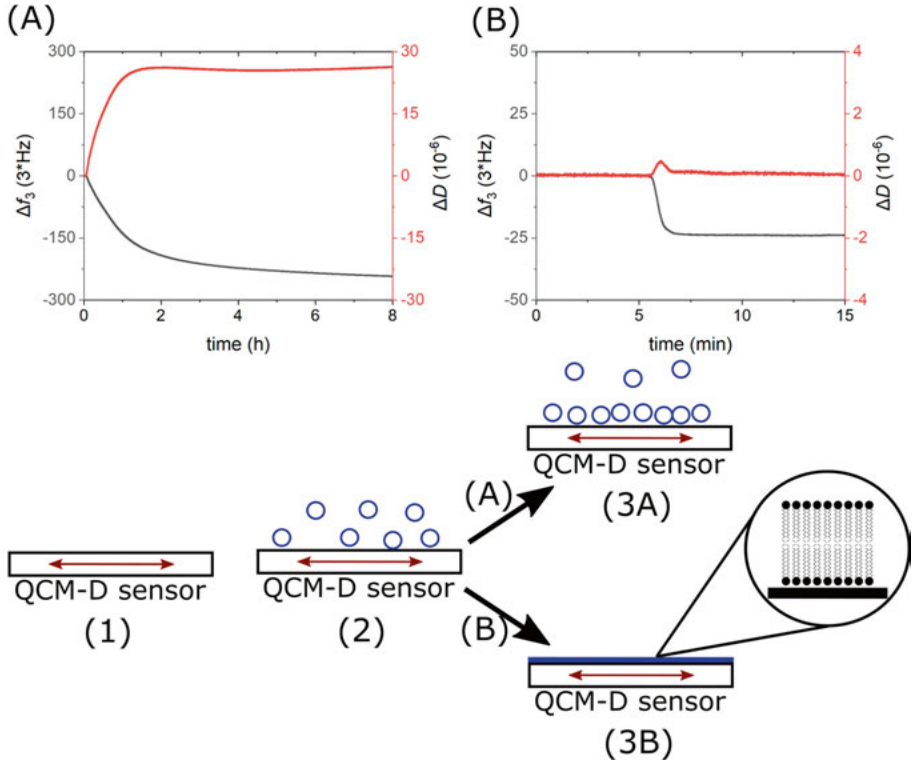


Figure 12 QCM-D graphs of (A) DPPC liposomes and (B) POPC liposomes interacting with a silica sensor at 20°C. The different liposomes exhibit different behaviour at the sensor surface. (1) Initially, the sensor resonates in the buffer solution; (2) then, the liposomes are applied. (3A) In the case of the DPPC liposomes, intact liposomes attach to the surface and stay there. (3B) In the case of the POPC liposomes, a spreading and rupturing process starts, resulting in an SLB covering the sensor.

The change in ΔD can be described by the following equation:

$$\Delta D = -\frac{4\pi h_\mathrm{l}\eta_\mathrm{w}\rho_\mathrm{w}\mu_\mathrm{l}nf_0}{t_\mathrm{q}\rho_\mathrm{q}\left(\mu_\mathrm{l}^2 + 4\pi^2\eta_\mathrm{l}^2(nf_0)^2\right)} \tag{5}$$

Agmo Hernández et al. [110] showed that the combination of equations (4) and (5) results in the following linearized equation:

$$\frac{\Delta f}{n} = -\frac{m_\mathrm{d}f_0}{t_\mathrm{q}\rho_\mathrm{q}} + \frac{\pi\eta_\mathrm{l}(f_0)^2}{\mu_\mathrm{l}} \cdot (n\Delta D) \tag{6}$$

A plot of $\Delta f n^{-1}$ against $n\Delta D$ should therefore result in a line with an intercept equal to $(m_\mathrm{d}f_0)\left(t_\mathrm{q}\rho_\mathrm{q}\right)^{-1}$. This relation is true for samples that fulfil the thin-film assumption.

QCM-D is used in Papers II and III to investigate the interactions at the liquid-solid interface between lipid aggregates and solid surfaces. In Paper II, the behaviour of PEGylated liposomes and lipodisks at the solution-silica interface is studied, where the behaviour of the liposomes is compared with that of non-PEGylated liposomes. Furthermore, temperature-induced processes of PEGylated liposomes at the solution-silica interface are studied. In Paper III, the processes at the solution-sensor interface are studied in order to investigate the behaviour of liposomes towards surfaces with different chemical compositions, such as PS, PMMA and silica.

3. Results and discussion

3.1 Paper I: Effect of gangliosides on structure and integrity of polyethylene glycol (PEG)-stabilized liposomes

In Paper I, the effect of gangliosides on the structure and integrity of PEGylated liposomes was investigated. To this end, different molar fractions of ganglioside mixtures were added to formulations containing 5 mol% of DSPE-PEG2000 and structural effects were studied using cryo-TEM and DLS. The PEGylation of lipid particles has been demonstrated to be a good method to improve the blood survival time of liposomes. More recent studies have shown, however, that the repeated administration of PEGylated lipid nanocarriers can lead to rapid elimination *in vivo*. This process, which is referred to as the ABC phenomenon, is believed to be an immune response to PEG. PEGylated liposomes initiate the production of anti-PEG immunoglobulin M (IgM).[28-30, 112] In order to mitigate the anti-PEG IgM response, several modifications have been conducted, including changes in size, structure, PEG surface density and chemical composition.[30] In particular, the latter has been shown to provide leverage, and the incorporation of gangliosides appears to attenuate anti-PEG immunity.[31-33]

Like PEG-conjugated lipids, gangliosides are micelle forming and can thereby induce structural alterations in liposomes. Although such processes are worth considering, little information is available regarding these structural transformations. Therefore, in Paper I, we investigated the alterations induced by the supplementation of liposomal preparations with PEG-lipids and/or gangliosides.

3.1.1 Structural effects induced by the addition of PEG-lipids

First, the structural effects on liposomes due to the addition of DSPE-PEG2000 were investigated. Liposomes composed of hydrogenated egg phosphatidylcholine (HEPC) and 33 mol% of cholesterol, supplemented with different molar fractions of DSPE-PEG2000 (5–40 mol%) were investigated (*Figure 13*).

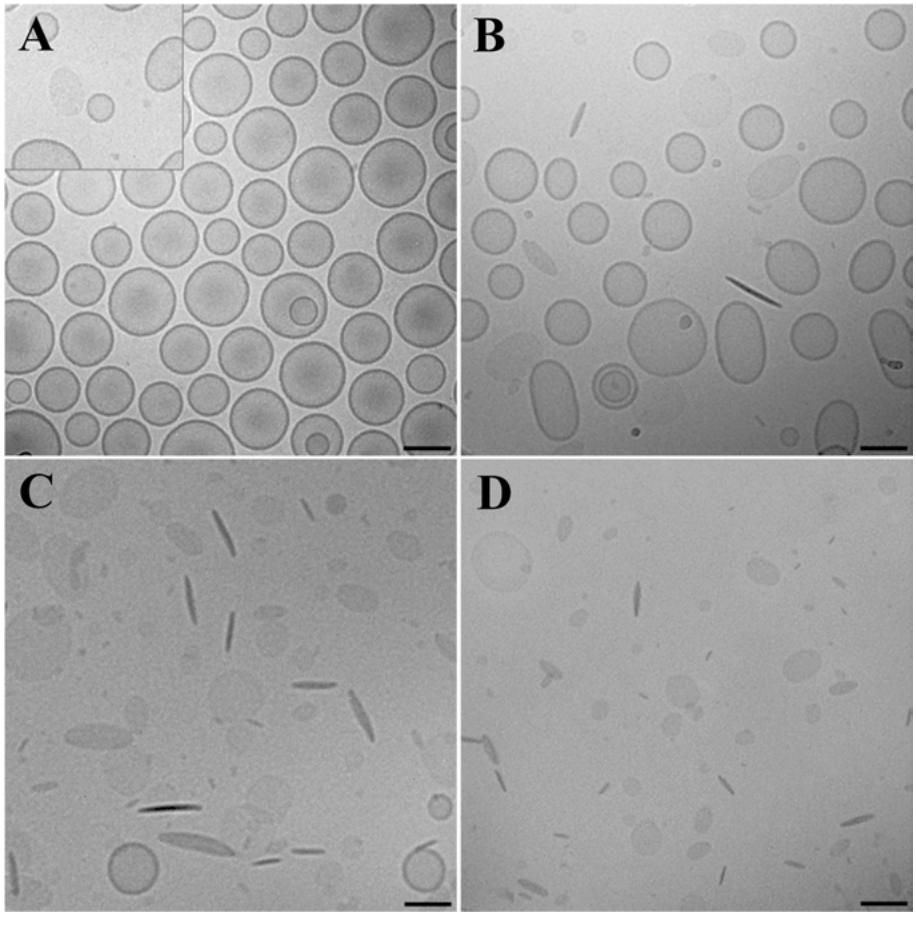


Figure 13 Cryo-TEM images of the lipid structures of samples composed of HEPC:Chol:DSPE-PEG2000 with a molar composition of 67 – *x*:33:*x*. The different micrographs correspond to different molar fractions of DSPE-PEG2000: (A) *x*=5, (B) *x*=10, (C) *x*=15 and (D) *x*=40. (Scale bar = 100 nm)

The samples containing 5 mol% of PEG-lipids were mainly composed of liposomes. However, a few lipodisks coexisted with the liposomes (*Figure 13A*). As the molar fraction of the PEG-lipids increased, a growing number of discoidal structures (i.e. lipodisks) appeared at the expense of liposomes (*Figure 13B*). Lipodisks dominated the samples containing more than 15 mol% of

PEG-lipids (*Figure 13C*). Samples containing 20 mol% of PEG-lipids were completely dominated by lipodisks with only few liposomes present. Furthermore, a correlation was found between the decreasing size of the lipodisks and the increasing molar fraction of PEG-lipids. A further increase of the PEG-lipids to 40 mol% (*Figure 13D*) resulted in the formation of mixed micelles similar in size to the micelles composed solely of PEG-lipids.

3.1.2 Structural effects caused by the addition of gangliosides

Next, the focus of the study was shifted to gangliosides and the structural effects due to their incorporation into liposomes consisting of HEPC and 33 mol% of cholesterol. To this end, liposomal formulations were supplemented with molar fractions ranging from 5 to 40 mol% of gangliosides (*Figure 14*).

An analysis of the micrographs revealed certain similarities but also differences compared with the samples containing DSPE-PEG2000. Similar to the sample supplemented with 5 mol% of PEG-lipids, the sample containing 5 mol% of gangliosides was dominated by liposomes (*Figure 14A*). However, the liposomes were larger on average in the ganglioside mixture. Increasing the amount of gangliosides to 10 mol% (*Figure 14B*) mainly resulted in larger liposomes. The sample preparation had not been modified and the liposomes were extruded through a 100 nm pore filter; thus, the larger dimensions of the liposomes appeared to be correlated with the addition of gangliosides. The liposomes' apparently larger size might be due to the fluidizing effect of gangliosides, as reported by Fricke and Dimova.[113]

The sample supplemented with 15 mol% of gangliosides (*Figure 14C*) contained a considerable fraction of lipodisks; however, a significant fraction of the sample was still composed of liposomes, and a direct comparison with the sample containing 15 mol% of DSPE-PEG2000 revealed the lower potency of gangliosides in inducing the formation of lipodisks. The gangliosides appeared to be less potent than DSPE-PEG2000 in driving lipid assemblies into structures with high average curvature. Increasing the concentration of gangliosides to 40 mol% (*Figure 14D*) resulted in a sample that mainly consisted of small lipodisks, with few liposomes.

The structural transformations that were induced by the incorporation of PEG-lipids or gangliosides raised the question of whether the simultaneous presence of both lipids would result in additive effects.

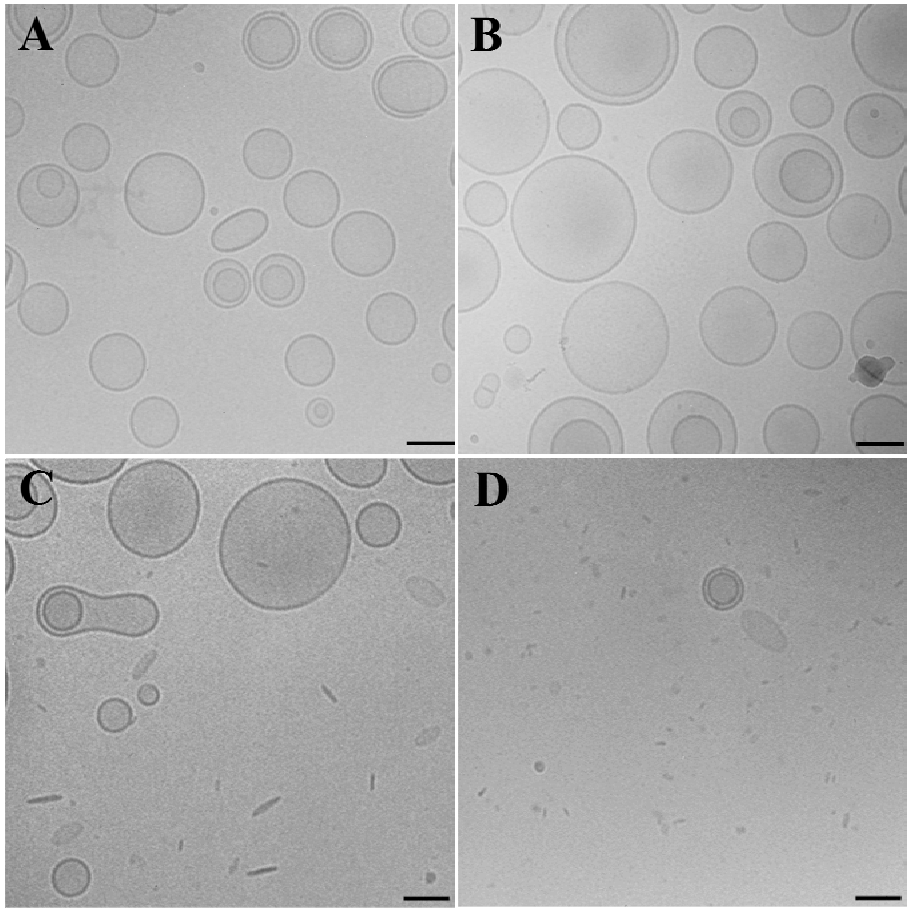


Figure 14 Cryo-TEM images of the lipid structures of samples composed of HEPC:Chol:Gangliosides with a molar composition of 67 –*y*:33:*y*. The different micrographs correspond to different molar fractions of gangliosides of (A) *y*=5, (B) *y*=10, (C) *y*=15 and (D) *y*=40. (Scale bar = 100 nm)

3.1.3 Structural effects induced by the addition of both PEG-lipids and gangliosides

To investigate the structural effects induced by the combined addition of PEG-lipids and gangliosides, samples containing both were compared with samples with increasing molar fractions of DSPE-PEG2000. A sample containing 20 mol% of this PEG-lipid was found to be mainly composed of lipodisks. This information coupled with the intermediate structures present in the sample, as discussed previously (*Figure 13*), assisted in gaining an understanding of the transformations induced by a combination of PEG-lipids and gangliosides.

In order to investigate the structural transformations, comparative DLS experiments were conducted with the different samples (*Figure 15*). It should be

noted that lipodisks are non-spherical particles and cannot be unambiguously described using DLS, as discussed in Section 2.2. Due to the presence of particles with different sizes, number-weighted distributions were used.

The experiments showed that increasing the molar fraction of PEG-lipids resulted in a decrease in the size of the observed particles (*Figure 15A*). The sample containing 20 mol% of PEG-lipids was dominated by lipodisks. In comparison, the sample composed of PEGylated liposomes supplemented with gangliosides (*Figure 15B*) showed a reduction in the particle size distribution profile as the molar fraction of gangliosides increased.

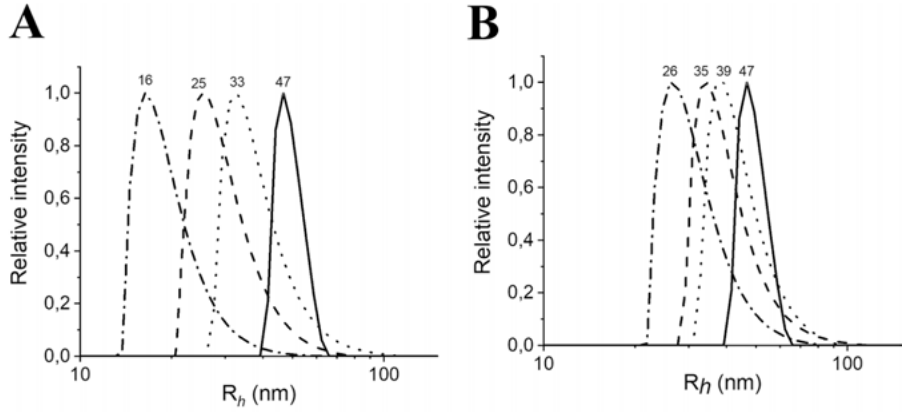


Figure 15 Number-weighted distribution of data from the DLS measurements obtained from the samples: (A) HEPC:Chol:DSPE-PEG2000 with a molar composition of 67 – *x*:33:*x*, where (——) represents *x*=5, (···) represents *x*=10, (– –) represents *x*=15 and (–·–) represents *x*=20. (B) HEPC:Chol:DSPE-PEG2000:gangliosides with a molar composition of 62 – *y*:33:5:*y*, where (——) represents *y*=0, (···) represents *y*=2.5, (– –) represents *y*=5 and (–·–) represents *y*=10. (Total lipid concentration = 1 mM)

Complementary experiments with cryo-TEM showed that the simultaneous presence of gangliosides and PEG-lipids induced the formation of lipodisks (*Figure 16*). The addition of 2.5 mol% of gangliosides to liposomes composed of HEPC, 33 mol% of cholesterol and 5 mol% of DSPE-PEG2000 induced the formation of large lipodisks (*Figure 16A*). Increasing the molar fraction of gangliosides to 5 mol% (*Figure 16B*) and 10 mol% (*Figure 16C*) resulted in an increasing number of lipodisks at the expense of liposomes. However, the diameter of both the disks and the liposomes decreased as the molar fraction of gangliosides increased. At a molar fraction of 15 mol% of gangliosides (*Figure 16D*), the sample was dominated by disks, with only a few small liposomes.

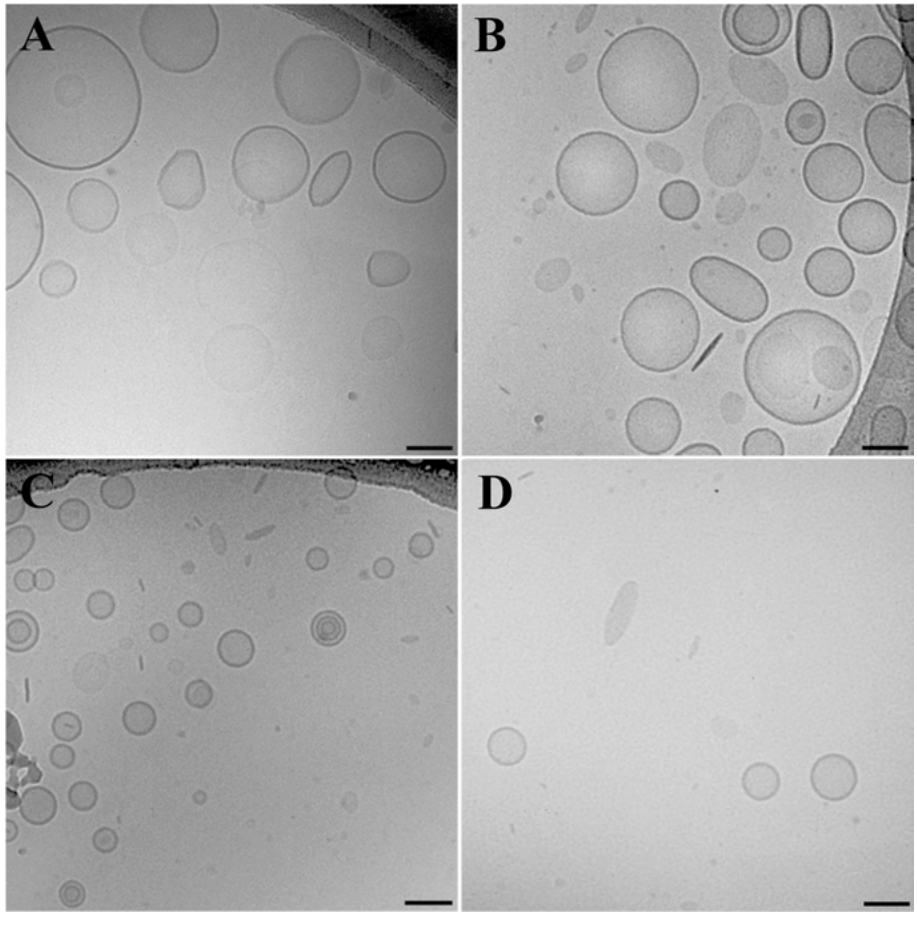


Figure 16 Cryo-TEM images of the lipid structures of samples composed of HEPC:Chol:DSPE-PEG2000 with a molar composition of 62 – *z*:33:5:*z*. The different micrographs correspond to different molar fractions of gangliosides: (A) *z*=2.5, (B) *z*=5, (C) *z*=10 and (D) *z*=15. (Scale bar = 100 nm)

In this way, it was found that gangliosides and PEG-lipids have an additive effect. It is clear that only a very small amount of gangliosides can be added to formulations containing 5 mol% of PEG-lipids without resulting in a significant amount of discoidal structures. Further investigations showed that formulations with 2.5 mol% of gangliosides and as low as 2.5 mol% of PEG-lipids already exhibited a small population of discoidal particles (*Figure 17*). These samples confirmed the strong tendency of gangliosides to induce the formation of discoidal particles in combination with PEG-lipids. Gangliosides have a lower potency than PEG-lipids in forming lipid assemblies with high average curvature. Nevertheless, the addition of gangliosides to samples containing PEG-lipids must be done carefully, due to the additive effect of these lipids.

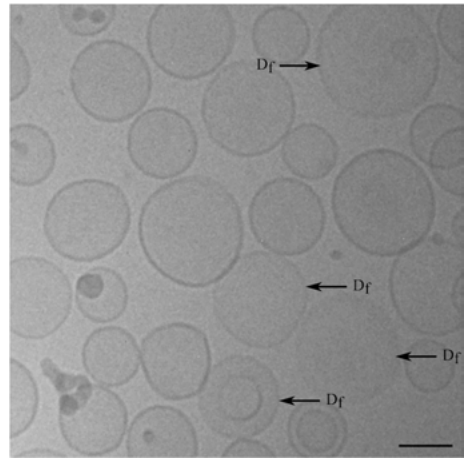


Figure 17 Cryo-TEM images of the lipid structures of a sample composed of HEPC:Chol:DSPE-PEG2000:gangliosides with a molar composition of 62:33:2.5:2.5. The lipodisks are indicated by arrows, whereas D_f points towards face-on lipodisks. (Scale bar = 100 nm)

3.1.4 Concluding remarks

The results of this study raise questions regarding the cause of the attenuation of the immunogenic effect that occurs when gangliosides are added to PEGylated liposomes. This paper showed that few – if any – gangliosides can be added to liposomal formulations with 5 mol% of PEG-lipids without inducing the formation of discoidal particles.

Moreover, there is reason to believe that the presence of discoidal particles correlates with a reduced production of anti-PEG IgM. The observation of a decreasing immune response with an increasing molar fraction of PEG-lipids from 5 to 15 mol% [31] correlates with transformations from liposome- to lipodisk-dominated samples (*Figure 13A-C*). A similar correlation was observed for samples supplemented with both gangliosides and PEG-lipids. A study [114] reported that micelles are not immunogenic and that mixed micelles induce a weaker anti-PEG IgM production compared with larger lipid particles. The complex reasons for this phenomenon are not yet known, and the mechanism is worth elucidating. A potential reason might be the significantly higher PEG surface coverage of micelles and disks compared with liposomes. Further investigations are necessary to draw more conclusions.

3.2 Paper II: Adhesion and structural changes of PEGylated lipid nanocarriers on silica surfaces

The lipid assemblies discussed in Paper I can differ in terms of properties other than their geometric shapes – for example, they can differ in their interactions with the surfaces of their containers. Such containers are usually cuvettes or vials composed of quartz, which is chemically similar to silica. The interactions of PEGylated lipid nanocarriers and solid silica surfaces have been observed in several experiments as described in a previous publication from our lab [115]. The research presented in Paper II takes these findings as a starting point, along with prior knowledge of the interactions that occur between silica and PEG [116]. Thus, in Paper II, we investigated the question of how PEGylated lipid nanocarriers interact with silica surfaces. We examined the binding of liposomes and lipodisks to silica, as well as their organization and structural transformation on the solid silica surface as a result of this binding.

3.2.1 Interaction of lipodisks with silica surfaces

Our investigations showed that lipodisks spontaneously adhere onto silica surfaces at room temperature (RT) and cannot be removed by rinsing. It can therefore be hypothesized that this attachment is irreversible. This conclusion is based on QCM-D measurements supported by multi-parametric surface plasmon resonance (MP-SPR) results. It should be noted that the two methods deliver complementary results. MP-SPR is an optical and real-time surface-sensitive technology that utilizes surface plasmon resonance.[117] This is a different physical principle than that utilized by QCM-D; the water associated with the lipids is excluded from the mass determination in MP-SPR.[118]

The measurements were performed with lipodisks containing 20 mol% of DSPE-PEG(*X*), where *X*=1000, 2000 or 5000. The lipodisks adsorbed flatly onto the silica sensor (*Figure 18*) according to the large $\Delta f \Delta D^{-1}$ ratio, indicating that a rigid film formed on the QCM-D sensor. The MP-SPR data supported this hypothesis by revealing that the film thickness was in the thin-film range, at between 6–10 nm.

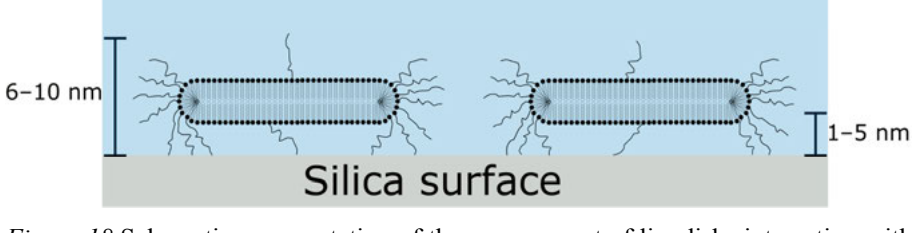


Figure 18 Schematic representation of the arrangement of lipodisks interacting with a silica surface. Measurements were performed at 21°C.

Isothermal measurements performed at different fixed temperatures returned several interesting results. First, a lower immobilized mass was observed for samples measured at 27°C compared with 21°C. Second, for temperatures above 32°C, the lipodisks first adhered to the silica and then spontaneously detached. Third, experiments performed at 37°C showed the same behaviour but with faster kinetics. For all experiments above 32°C, a second addition of fresh lipodisks did not result in the adsorption of more material onto the silica. These observations lead to the conclusion that an immobilized film spontaneously forms on the surface due to a high affinity between the silica surface and PEGylated particles. In addition, the film forms irreversibly due to the presence of PEG-lipids remaining on the surface of the silica, which "passivate" the surface (*Figure 19*). A comparison between the measurements performed at 21°C and 27°C shows that a lower recorded mass was observed at the higher temperature. This is a result of a lower water content of the adsorbed film.

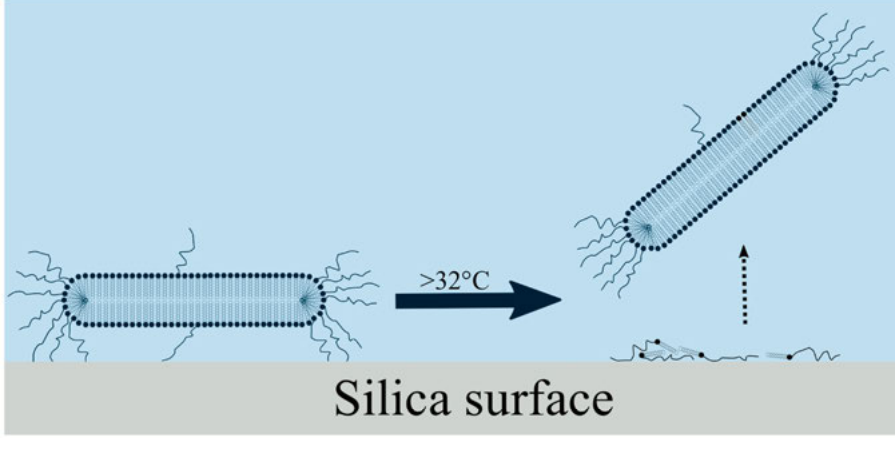


Figure 19 Schematic representation of the temperature-induced rearrangement of lipodisks interacting with a silica surface at temperatures >32°C. Lipodisks spontaneously detach from the surface leaving some adsorbed PEG-lipids behind.

This behaviour is probably caused by the strong attraction between the PEG polymers and the silica. It is plausible to assume that temperature-induced processes are involved; in fact, it has been suggested that PEG experiences conformational changes at 35°C.[119] According to previous reports, at higher temperatures, PEG transforms into an amphiphilic coil-like conformation, which has an increased affinity for a silica surface.[119, 120] This hypothesis is supported by the findings from our experiments in which lipodisks were subjected to a temperature programme (*Figure 20*). We found that temperatures greater than 32°C resulted in a drop in the immobilized mass of the material adsorbed onto the silica. A subsequent reduction in the temperature did not result in a recovery of the original signal.

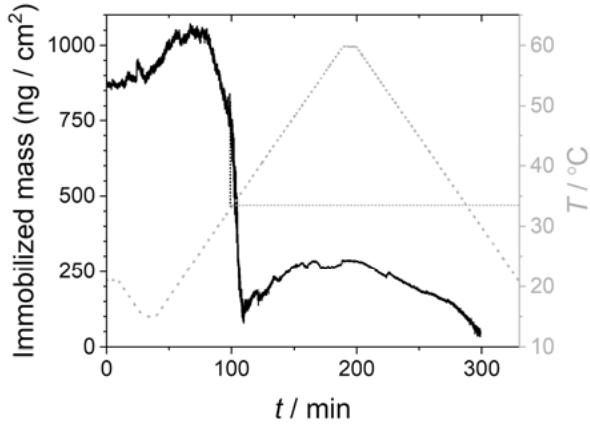


Figure 20 Temperature-induced changes of the immobilized mass of a film initially consisting of lipodisks at 21°C. The lipodisks were composed of DSPC:DSPE-PEG2000 with a molar fraction of 80:20.

3.2.2 Effect of the hydration layer on the interaction of lipodisks with silica

The release of lipodisks at temperatures above 32°C and the presence of a remaining film on the silica indicates a strong interaction between silica and the PEG polymers. This interaction is strong enough to remove the hydration layer on the silica surface and release the monovalent counterions. It is therefore important to investigate the influence of the hydration layer on silica in order to more fully understand the interaction between silica and PEG. Therefore, the monovalent counterions were replaced by multivalent calcium ions. The presence of multivalent calcium ions leads to a thicker Stern layer on silica and stronger hydration forces. This resulted in decreased interactions between the PEG and the silica. Increasing the hydration repulsive force through multivalent ions resulted in weakened interactions between the silica and the lipodisks.

3.2.3 Interaction of liposomes with silica surfaces

Our observations of the interactions between lipodisks and silica surfaces raised the question of whether similar processes occur in the interactions between liposomes and silica surfaces. First, it is important to highlight the differences between lipodisks and liposomes. Apart from the obvious differences in structure between the spherical and hollow liposomes and the discoidal lipodisks, the liposomes under study contained a lower molar fraction of PEG-conjugated lipids. The samples discussed in this section contained 4 mol% of

DSPE-PEG2000, which was significantly less than the lipodisks, which contained 20 mol%. The low molar fraction was important to prevent the liposomes from transforming into lipodisks, as discussed in Section 1.1.2 and Paper I. The following experiments were performed with liposomes in the liquid-disordered, liquid-ordered and gel phase states, respectively.

First, the results for soft liposomes in the liquid-disordered phase state will be discussed. The interactions between silica surfaces and pure or PEG-lipid-supplemented POPC liposomes were investigated. Both kinds of liposomes showed a similar behaviour of adhesion, rupturing and spreading on the solid silica surface. The PEGylated liposomes demonstrated slower kinetics, most likely as a result of steric hindrance due to the surface-grafted PEG. Subjecting the PEGylated liposomes to a temperature programme resulted in an irreversible change of the immobilized structures at temperatures greater than 32°C, similar to the observations made with lipodisks. The original immobilized mass could not be recovered after the temperature programme.

The focus was then shifted to liposomes in the gel phase – namely, DPPC and DSPC liposomes. The liposomes in the gel phase adhered to the silica surfaces and remained there intact. This behaviour, which differed from that of the liposomes in the liquid-disordered phase state, can be attributed to the higher membrane rigidity and stiffness of gel-phase liposomes. Applying a temperature programme to the liposomes resulted in the rupture and spreading of the gel-phase liposomes at the main phase transition temperature (T_m) of 41°C for DPPC and 55°C for DSPC. However, some liposomes were found to remain intact at the silica surface.

The PEG-lipid-supplemented liposomes exhibited a different behaviour; the immobilized mass decreased with increasing temperature and dropped at 35°C (*Figure 21*). After that, a relatively stable immobilized mass was observed. PEG-lipid supplementation seems to facilitate the rupturing and spreading of liposomes at temperatures below their T_m. This process raises questions regarding the interactions between the liposomes and the solid silica surface. A nano-plasmonic sensing (NPS) and QCM-D coupled instrument was used to reveal the processes occurring at the surface. The data suggested that the water molecules at the surface of the silica was displaced due to a structural rearrangement of the polymer. The rupturing and spreading of the liposomes led to the formation of defect-free bilayers in the gel phase. The reason for this behaviour might be that the process of deforming and breaking the liposome membrane is energetically more favourable than removing the PEG-lipids simultaneously from the membrane.

Finally, the PEGylated liposomes in the liquid-ordered phase displayed a slower adhesion to the solid silica surface than the normal liposomes. This behaviour is similar to the samples in the liquid-disordered and gel phase in the isothermal experiments monitored by QCM-D. Subjecting the samples to a temperature programme resulted in negligible changes being observed for bare liposomes, whereas rupturing and spreading were observed for

PEGylated liposomes when the temperature reached 33°C. The latter behaviour is most likely a result of silica-PEG interactions, because the liposomes under study in the liquid-ordered phase state do not experience a phase transition at this temperature.

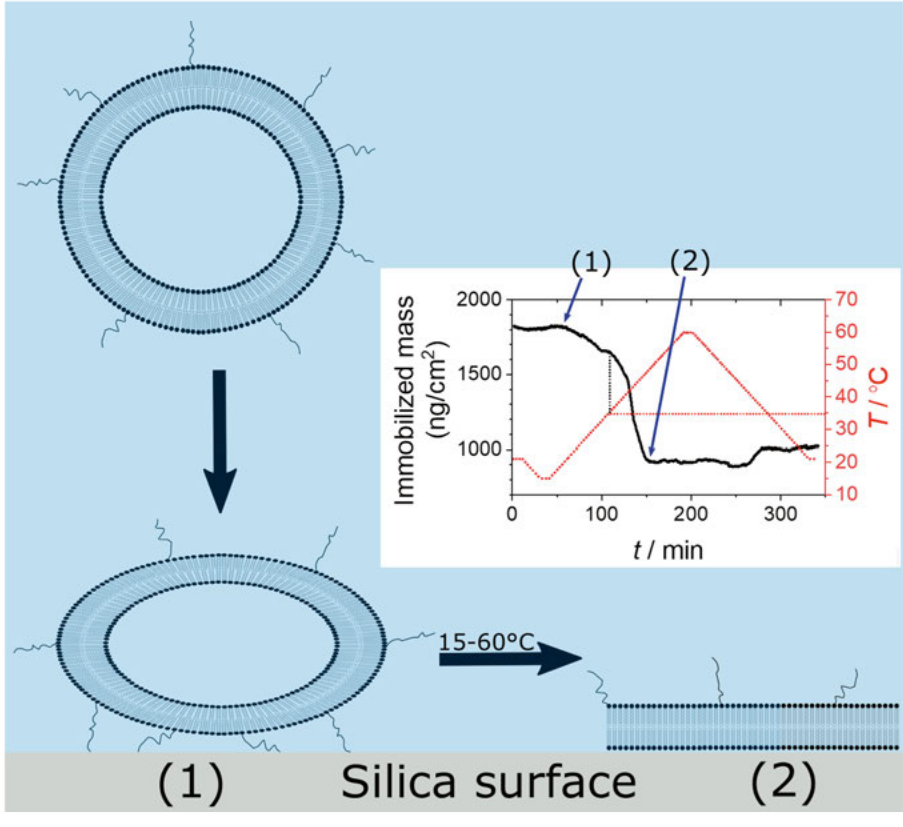


Figure 21 Schematic representation of the temperature-induced formation of a supported lipid bilayer (SLB) on the silica surface from PEG-lipid-supplemented liposomes in the gel phase. (1) Liposomes attach to the silica surface and rupture as the temperature is increased. (2) An SLB is formed.

3.2.4 Concluding remarks

The results presented in Paper II reveal the spontaneous attachment of the PEGylated liposomes and lipodisks to silica surfaces at RT. Lipodisks bind parallel to the surface and remain intact. In contrast, liposomes display ambivalent behaviour, in that immobilized layers of intact liposomes are formed by the gel and liquid-ordered phase state liposomes, while SLB are formed by liposomes in the liquid-disordered phase state. However, when the temperature increased, the behaviour of the liposomes and lipodisks changed.

At temperatures greater than 32°C, irreversible changes were observed in the immobilized PEGylated liposomes and lipodisks. This behaviour seemed to be due to a high affinity between PEGylated lipid particles and the silica surface. The PEGylated liposomes tended to form SLBs, even below their main transition temperature. This behaviour is probably due to a conformational change of the PEG from helical to coil-like, which results in a decreased affinity for water [119] coupled with an increasing affinity for silica [120]. The strong attraction between the PEG polymers on the surface of the nanocarriers and the silica surface leads to the rupture of liposomes and the removal of PEG-lipids from the lipodisks. It seems to be possible for the PEG chains to remove or replace the hydration layer on the silica surface. Increasing the temperature results in a stronger affinity between the polymer and the water-depleted silica surface. When immobilization of PEGylated lipid nanocarriers was directly attempted at 40°C, no interactions were observed likely because the hydration layer on the silica surface was not removed.

3.3 Paper III: Avoiding artifacts in liposome leakage measurements via cuvette- and liposome-surface modifications

The interactions between PEGylated lipid nanocarriers and silica surfaces that were examined in Paper II are relevant for spectroscopic leakage and permeability experiments with liposomes. In line with this, an earlier study showed that the cuvette material and rate of stirring affected the results obtained during leakage experiments;[85] in particular, liposomes composed of POPC and cholesterol (Chol) with a molar composition of 60:40 presented anomalous behaviour.[85] In Paper III we built upon these findings to investigate and discuss possibilities to minimize artifacts. Artifacts are often a result of liposome-surface interactions. In order to minimize artifacts, we explored two methods: (1) the liposome surface was modified by supplementation with DSPE-PEG2000 or gangliosides; and (2) different cuvette materials such as quartz, PS and PMMA were explored, and their surface was modified with a surface-adsorbed PEG8000 polymer.

First, the influence of different cuvette materials and stirring speed will be presented in order to demonstrate the different results that can be obtained just by changing these two parameters in a leakage experiment.

3.3.1 Influence of cuvette material and stirring speed

Initial experiments conducted with POPC:Chol liposomes with a molar composition of 60:40 in different cuvettes showed poor reproducibility (*Figure 22*). It was apparent from the results that stirring had a modest effect on the degree of leakage from liposomes measured in quartz and PMMA cuvettes; yet, a higher degree of leakage was obtained from measurements in PS cuvettes at 900 rpm as compared with 300 rpm. Additional experiments performed without stirring showed a lesser degree of leakage.

An almost identical average degree of leakage, albeit with less reproducibility between experiments, was obtained for measurements with an increased stirring rate performed in quartz cuvettes. The poor reproducibility is most likely a result of interactions between liposomes and the quartz surface. In order to investigate these interactions QCM-D experiments were performed, which can reveal the attachment and partial rupture of liposomes on the silica surface. The similar average degree of leakage of the experiments performed at 300 and 900 rpm suggested that negligible interaction occurred after the quartz surface was completely coated with an SLB. The lack of reproducibility at higher stirring rates might be the result of convection-induced disruption of liposomes.

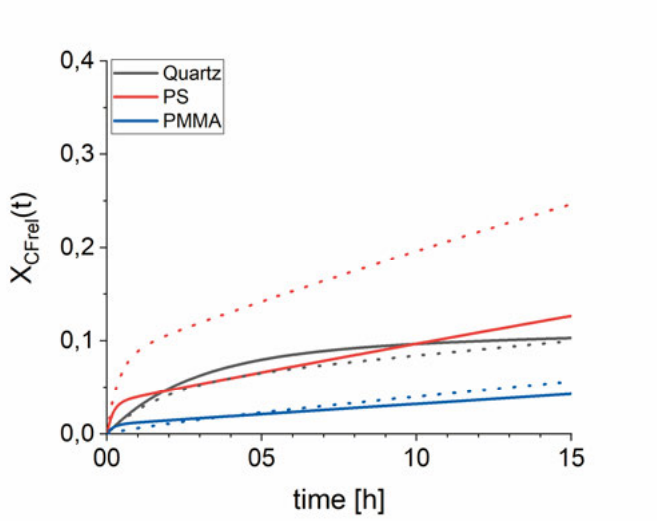


Figure 22 Degree of leakage of CF from POPC:Chol liposomes with a molar ratio of 60:40 obtained over 15 h. Measurements were performed in quartz, PS and PMMA cuvettes with stirring speeds set to 300 (solid lines) and 900 (dotted lines) rpm. Measurements were conducted at 20°C.

The samples measured in PMMA cuvettes exhibited an opposite trend: interestingly, increasing the stirring rate improved the reproducibility. These interactions were investigated with QCM-D, and the results indicated that the cuvette had a partially coated surface with a few intact liposomes. The interactions between the liposomes and PMMA appeared to be weak, and increasing the stirring speed might prevent interactions between the liposomes and the cuvette surface. The proposed interaction mechanisms of POPC:Chol liposomes based on the QCM-D and leakage measurements are summarized in *Figure 23*.

In order to reduce the interactions between liposomes and the surface of the cuvettes, various approaches were investigated. The first approach involved the incorporation of polymer-conjugated lipids such as PEG-lipids, or lipids with bulky and negatively charged headgroups, such as gangliosides, into the liposomes.

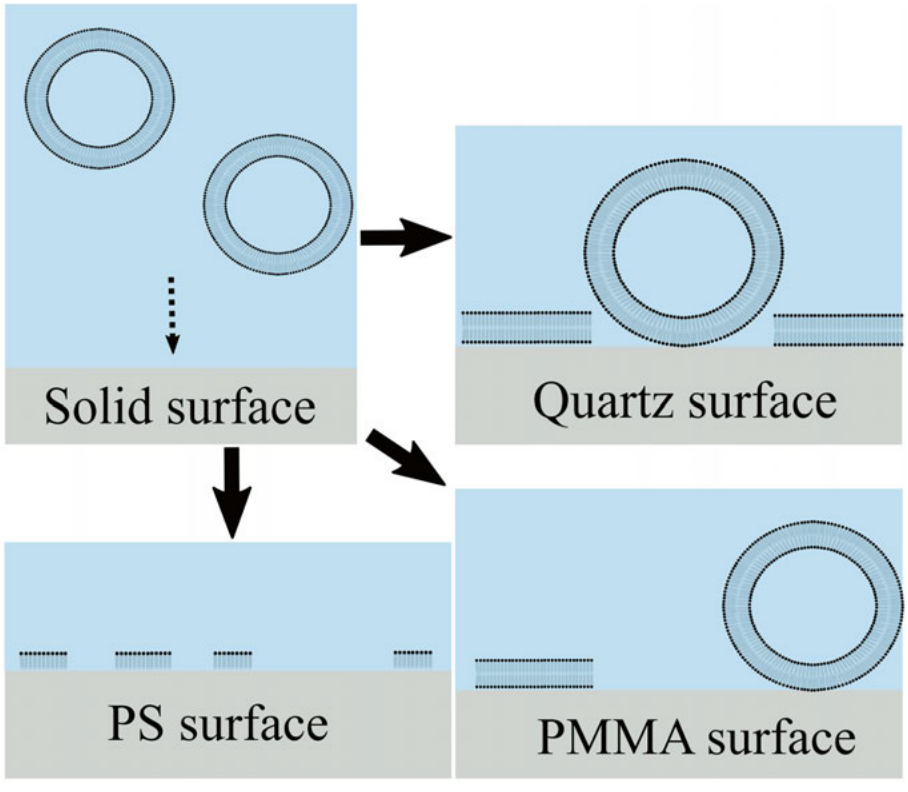


Figure 23 Schematic illustration of the proposed mechanisms at the interface of quartz, PS and PMMA cuvettes due to interactions between the solid cuvette surface and POPC:Chol liposomes with a molar ratio of 60:40. These interactions result in the following phenomena: (1) Partial rupture on the quartz, with SLBs and intact liposomes present; (2) the formation of incomplete monolayers on PS; and (3) a partially coated surface with intact liposomes and SLBs on PMMA.

3.3.2 Influence of liposome surface modifications

The addition of PEG-conjugated lipids is a common procedure to increase the colloidal stability of liposomes.[25, 121] Improved colloidal stability can also be obtained by the addition of gangliosides, which has been partially attributed to the presence of sialic acid at the surface of liposomes.[36] Both lipids change the surface properties of liposomes, making it reasonable to expect that their interactions with solid surfaces will also be affected. In this study, we investigated the interactions between liposomes supplemented with 4 mol% of DSPE-PEG2000 or gangliosides, or a mixture of both species, and the solid surfaces of quartz, PS and PMMA cuvettes.

The number of samples and the different parameters required a detailed analysis; therefore, the spontaneous leakage behaviour of the samples was fitted with a bi-exponential model described by Agmo Hernández et al.[99] This

model assumes that there is an intrinsic heterogeneity of the liposome suspensions related to the distribution, formation and consumption of hydrophobic sites on the liposome surfaces.[99] The model also describes the equilibrium composition of liposome suspensions and the time necessary to reach it:

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

where $x_{\mathrm{CF_{rel}}}(t)$ describes the degree of leakage, and A_1 and A_2 are pre-exponential factors that depend on the initial conditions of the experiment and fulfil the requirement $A_1 + A_2 = 1$. An example of initial conditions might be unequilibrated liposomes. The rate constants k_1 and k_2 respectively describe the short- and long-term leakage rates.

The data analysis revealed experimental artifacts for the liposomes measured in PS cuvettes (*Figure 24*), independent of their surface modification. Eriksson and Agmo Hernández [61] proposed a mechanism of constantly shattered liposomes on the hydrophobic surface, which agrees with our results obtained for the ganglioside- and PEG-lipid-modified liposomes. The liposomes seem to attach slowly and are shattered by convection. QCM-D measurements indicate a negligible interaction with the sensor surface for both compositions. However, the comparably high degree of leakage observed for the liposomes (*Figure 24A*) indicates that interactions do indeed take place with the surfaces of the PS cuvettes. A possible explanation for the QCM-D results is that the slow attachment is observed as a long-term drift of the baseline.

Leakage measurements in PMMA cuvettes (*Figure 24*) reveal a similar behaviour for surface-modified liposomes in comparison with non-modified liposomes. As in the aforementioned observations described in Section 3.3.1, a higher stirring rate results in lower degrees of leakage. These findings support the proposed hypothesis of hindered adhesion on PMMA due to an elevated stirring rate. The QCM-D results coupled with the leakage experiments suggest that the surface is partially passivated by the adsorption of intact liposomes; hence, only long-term spontaneous leakage is measured.

The measurements performed in quartz cuvettes (*Figure 24*) revealed an interesting behaviour. Supplementation of the liposomes with either PEG-lipids or gangliosides reduced the degree of leakage and improved the reproducibility. The QCM-D experiments revealed an interaction between the PEGylated liposomes and the silica surface, also resulting in a partial rupturing process. Similar results were observed and reported in Paper II. The ganglioside-modified liposomes exhibited a slow and weak interaction with the quartz cuvette, which might be due to electrostatic repulsion and hydration repulsive forces hindering and slowing down the interaction of the liposomes with silica. The measurements with ganglioside-supplemented liposomes thus showed no artifacts.

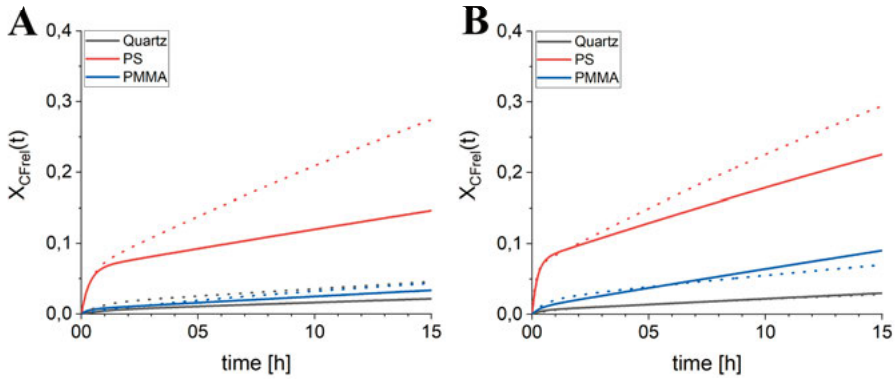


Figure 24 Degree of leakage of CF from (A) POPC:Chol:DSPE-PEG2000 and (B) POPC:Chol:Gang liposomes with a molar ratio of 56:40:4 obtained over 15 h. Measurements were performed in quartz, PS and PMMA cuvettes with stirring speeds set to 300 (solid lines) and 900 (dotted lines) rpm. Measurements were conducted at 20°C.

Unlike ganglioside-supplemented liposomes, when measured in quartz cuvettes, bare or PEG-lipid-supplemented liposomes showed either too-large rate constants for short- or long-term leakage or too-high values for the pre-exponential factor, thereby indicating a fraction of liposomes displaying a fast content-release. However, due to the inferior blood circulation times [25], ganglioside-supplemented liposomes are of less interest for pharmaceutical applications than PEG-lipid-supplemented liposomes. The proposed interaction mechanisms of PEGylated liposomes (*Figure 25 A–C*) and ganglioside-supplemented liposomes (*Figure 25 D–F*) based on QCM-D and leakage measurements are presented in *Figure 25*.

It can be concluded that, with the exception of ganglioside-supplemented liposomes in quartz cuvettes, modifying the surface of liposomes leads only partially to a reduction of the artifacts obtained during leakage assays. Another option to improve the quality and reproducibility of measurements is to reduce liposome-cuvette interactions by modifying the surface of the cuvette.

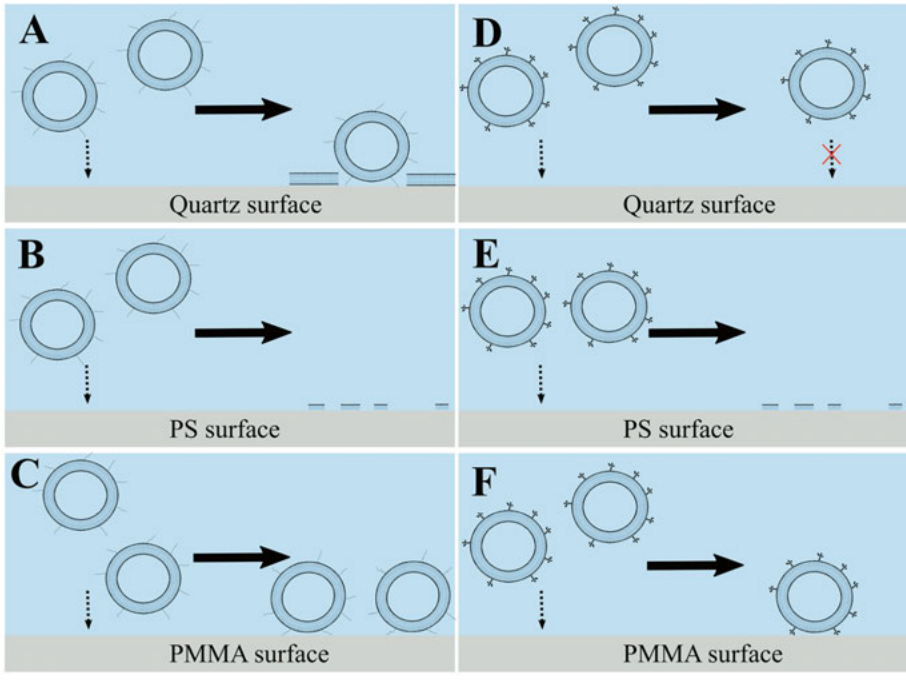


Figure 25 Schematic illustration of the proposed mechanisms at the interface of (A,D) quartz, (B,E) PS and (C,F) PMMA cuvettes due to interactions between the solid cuvette surface and (A,B,C) POPC:Chol:DSPE-PEG2000 and (D,E,F) POPC:Chol:Gang liposomes with molar ratios of 56:40:4.

3.3.3 Effect of surface-adsorbed PEG8000

The PEGylated liposomes of interest in this thesis exhibit biased leakage profiles due to spontaneous adhesion and partial rupture processes. In order to minimize surface interactions and thereby reduce experiment-related artifacts, we took advantage of a property of PEG that was observed in Paper II. In that work, the spontaneous and irreversible attachment of PEG-lipids onto silica due to high-affinity interaction was observed to result in a passivation of the surface and reduced interactions of subsequent lipid-particle applications. The PEG-lipids were replaced by a longer chain PEG polymer to reduce potential detachment of the polymers from the silica surface. The cuvettes were incubated with a solution containing PEG8000, and the polymer spontaneously attached to the silica surface. The results are discussed in the next section.

The potential surface-passivating ability of PEG8000 was also investigated for surfaces composed of PS and PMMA. PEG is mildly amphiphilic due to its molecular structure, which is comprised of hydrophobic CH_2–CH_2 and hydrophilic ether oxygen segments. Therefore, PEG can adsorb onto both hydrophilic and hydrophobic surfaces.[122] Interactions between colloidal PS particles and PEG have been reported.[80]

Initial experiments with PEG-passivated quartz, PS and PMMA cuvettes (*Figure 26*) revealed reduced degrees of leakage compared with measurements in unmodified cuvettes (*Figure 22*). Furthermore, the passivation minimized stirring-induced effects. The less pronounced effect of this passivation on PS can be explained by the lower affinity of PEG8000 for the hydrophobic surface of PS, compared with the more hydrophilic surfaces of PMMA and quartz.

A more detailed analysis of the data after fitting revealed that passivation of the cuvette surface mainly affected the pre-exponential factor A_1 due to the significantly reduced interface available for interactions after PEG8000 had been adsorbed onto the surface. However, the rate constants k_1 and k_2 are less affected; therefore, residual interactions between liposomes and the surface can be expected.

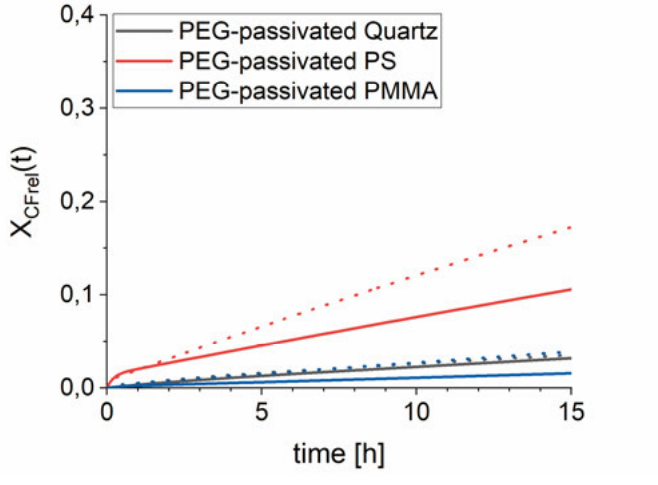


Figure 26 Degree of leakage of CF from POPC:Chol liposomes with a molar ratio of 60:40 obtained after 15 h of measurement. The measurements were performed in PEG8000-passivated quartz, PS and PMMA cuvettes with stirring speeds set to 300 (solid line) and 900 rpm (dotted line). Measurements were conducted at 20°C.

The measurements performed in PEG8000-passivated quartz cuvettes (*Figure 26*) show good reproducibility. The data from fitting further suggests that artifact-free spontaneous leakage was measured for all experiments. In order to further investigate these interactions, QCM-D measurement was set up. The silica sensor was passivated with a PEG8000-containing solution prior to the addition of liposomes, which resulted in a weaker interaction between the liposomes and the sensor, compared with the interaction between the same liposomes and a bare silica sensor. Intact liposomes interacted with the passivated silica sensor without rupturing. Similar behaviour was observed for

PEGylated liposomes. Moreover, the naked and PEG-lipid supplemented liposomes had a very similar degree of leakage. Ganglioside-supplemented liposomes displayed negligible interactions with the PEG8000-passivated silica sensor, which agrees with the slightly lower degree of leakage observed for these types of liposomes (*Figure 27*).

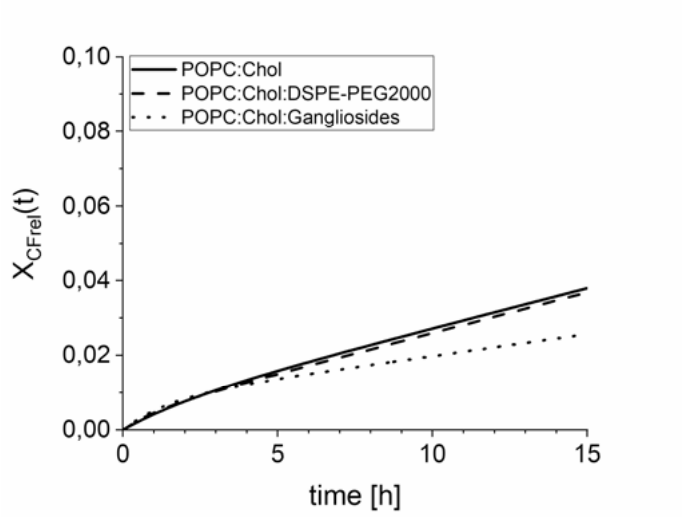


Figure 27 Degree of leakage of CF from liposomes composed of POPC:Chol (60:40 mol%; solid line), POPC:Chol:DSPE-PEG2000 (56:40:4 mol%; dashed line) and POPC:Chol:Gang (56:40:4 mol%; dotted line) obtained after 15 h of measurement. Measurements were performed in quartz cuvettes passivated with PEG8000, with stirring speeds set to 300 and 900 rpm. Measurements were conducted at 20°C.

3.3.4 Concluding remarks

The leakage experiments performed with different cuvettes (quartz, PS and PMMA) highlighted the influence of processes at the solution-cuvette interface. Modification of the liposomes' surface with DSPE-PEG2000 and gangliosides helped to minimize surface interactions. Adsorption of PEG8000 onto the surface of the cuvettes minimized the leakage-dependent artifacts even further (*Figure 28*). The experiments performed in PEG8000-passivated quartz cuvettes showed good reproducibility and comparability between liposomes supplemented with minor amounts of different lipids such as DSPE-PEG2000 and gangliosides.

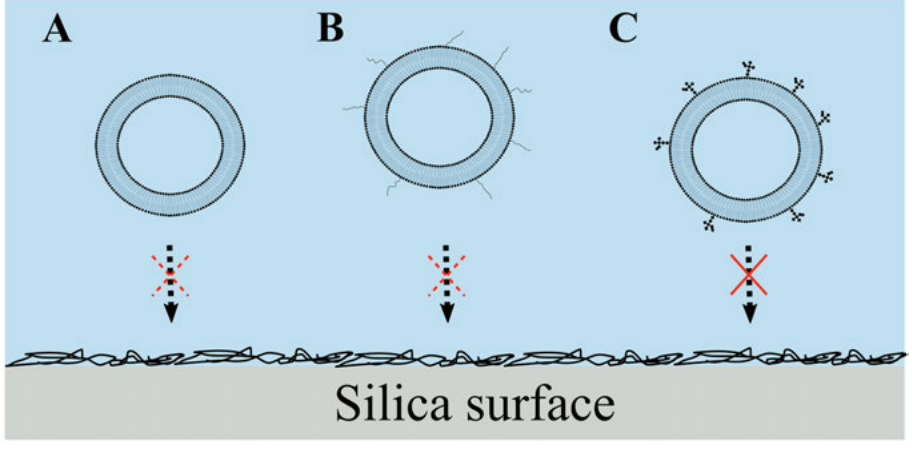


Figure 28 Schematic illustration of the proposed interaction at the interface of a PEG8000-passivated quartz cuvette with (A) POPC:Chol liposomes with a molar ratio of 60:40; (B) POPC:Chol:DSPE-PEG2000 with a molar ratio of 56:40:4; and (C) POPC:Chol:Gang liposomes with a molar ratio of 56:40:4. Dashed red crosses indicate a weak interaction and solid red crosses indicate an insignificant interaction.

3.4 Paper IV: Improved accuracy and reproducibility of liposome leakage measurements by the use of supported lipid bilayer-modified quartz cuvettes

The passivation of cuvettes with a layer of the polymer PEG, as presented in Paper III, was found to be a useful method for measurements of spontaneous leakage involving PEGylated liposomes and liposomes in the liquid-ordered phase state. However, artifact-free measurements could not be achieved with liposomes in the liquid-disordered and gel phase states. Hence, further research was conducted to obtain a more universal method that would be suitable for liposomes in these phase states as well. In Paper IV, we developed a novel cuvette pre-treatment protocol with the aim of mitigating unwanted side processes at the cuvette-solution interface. The inner walls of the quartz cuvettes were passivated with an SLB composed of POPC lipids, and the performance and versatility of the method were then tested with liposomes in different phase states.

3.4.1 Reduction of liposome-cuvette interactions with an SLB

The interactions between liposomes and solid surfaces composed of quartz or silica lead to liposome adhesion, destabilization and occasional rupture.[61, 73, 123, 124] These interactions can lead to biased results and erroneous conclusions if they are not taken into consideration when liposome leakage experiments are conducted. We envisioned that an effective and versatile method to minimize unwanted liposome-cuvette interactions could be based on passivation of the inner walls of quartz cuvettes with an SLB. The potential and feasibility of this method was first investigated by means of QCM-D (*Figure 29*). POPC liposomes were initially run over the sensor; then, after a rinsing step with buffer, a second injection of POPC liposomes was performed.

The changes in frequency and dissipation observed during the initial part of the experiment indicate that the first injection of POPC liposomes resulted in the successful formation of an SLB on the sensor surface. In brief, POPC liposomes were inserted into the QCM-D system and formed an SLB by rupturing and spreading on the silica surface. The rupturing and spreading of the POPC liposomes (*Figure 29*) is indicated by the decrease and then increase in frequency, which stabilizes at around –26 Hz, as well as by the increase and subsequent decrease in dissipation. The application of a second batch of POPC liposomes after the successful formation of a POPC-SLB results in negligible liposome surface interactions. The absence of any significant changes upon the 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 with other types of liposomes showed that the SLB-treated silica sensors were similarly inert to interactions with liposomes in the liquid-ordered and gel phase state.

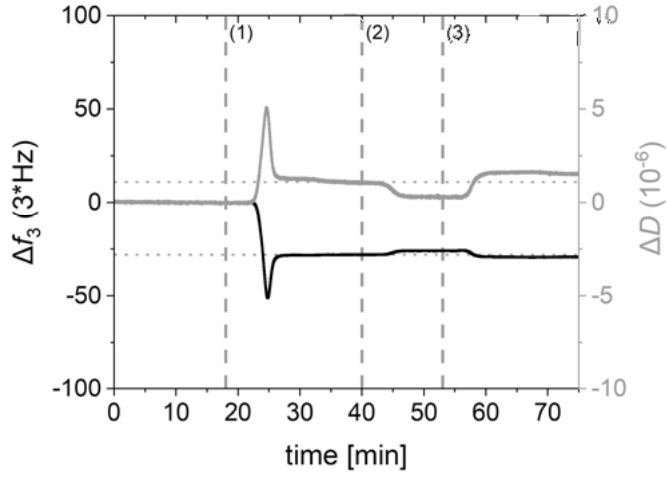


Figure 29 QCM-D measurement of a silica sensor (1) interacting with a solution containing POPC liposomes, forming a POPC-SLB; (2) as the sensor is rinsed with a buffer solution; (3) as a fresh batch of POPC liposomes is inserted into the system. Changes in frequency (black line) and dissipation (grey line) were investigated.

3.4.2 Employing SLB-passivated cuvettes for liposome leakage measurements

The potential and versatility of the SLB-passivated quartz cuvettes was first investigated with liposomes in the liquid-disordered phase state. Comparative experiments were performed with POPC and DSPE-PEG5000-supplemented POPC liposomes (*Figure 30*).

As evident in *Figure 30*, the use of SLB-passivated cuvettes resulted in improved reproducibility and a lower degree of leakage for both compositions. The initial fast leakage that was observed for POPC liposomes in quartz cuvettes, which is linked to the rupture and spreading of liposomes [61], is absent in the SLB-passivated cuvettes. The presence of the SLB seems to inhibit this initial fast leakage effect. The measurement of PEGylated liposomes in SLB-passivated cuvettes results in improved reproducibility and a reduced degree of leakage. A comparison between the POPC and PEG-lipid-supplemented liposomes revealed a lower degree of leakage for the latter but only in the SLB-passivated cuvettes. These results agree with previous observations.[125-131]

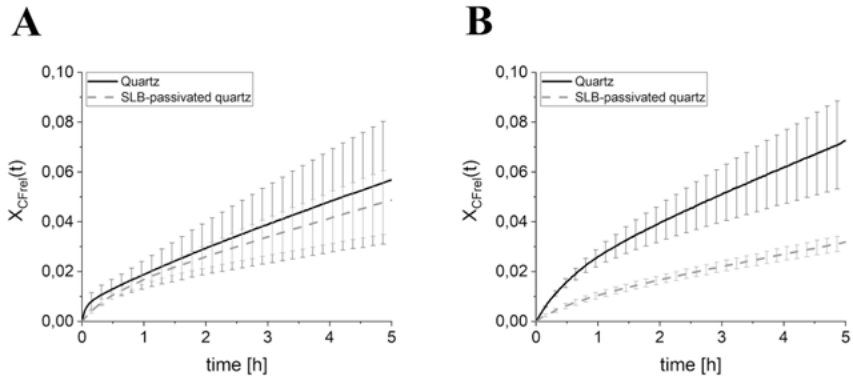


Figure 30 Leakage assays of (A) POPC and (B) POPC:DSPE-PEG5000 (96:4 mol%) liposomes. The degree of leakage was measured in quartz (black, solid line) and SLB-passivated quartz (grey, dashed line) at a temperature of 25°C. The bars represent the standard error of the mean for at last four repetitions of each experiment

Next, the method was tested with liposomes in the liquid-ordered phase state. Bare and PEG-lipid-supplemented liposomes were measured (*Figure 31*) in unmodified and SLB-passivated cuvettes. Passivation of the cuvette had only a minor effect on the leakage of DSPC:Chol liposomes, but had significant effect with PEGylated liposomes. It is likely that the previously reported interactions between silica and PEG-lipids [124] could be avoided by using SLB-passivated cuvettes. Only the measurements performed in SLB-modified cuvettes revealed a lower degree of leakage with PEG-lipid-supplemented liposomes compared with DSPC:Chol liposomes.

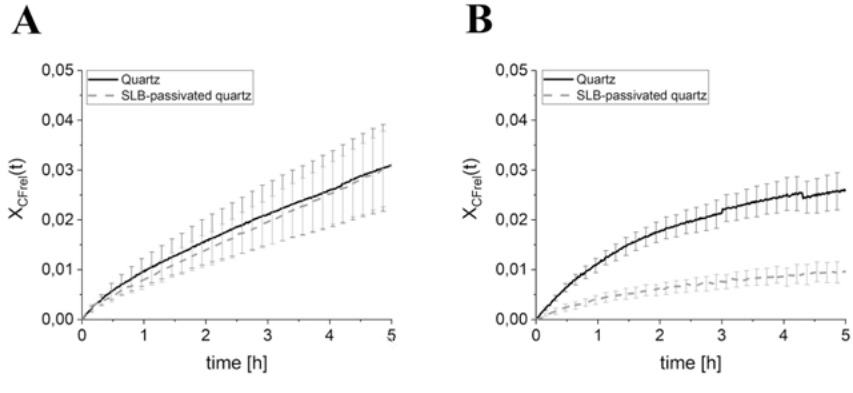


Figure 31 Leakage assays of (A) DSPC:cholesterol (60:40 mol%) and (B) DSPC:cholesterol:DSPE-PEG2000 (56:40:4 mol%) liposomes. The degree of leakage was measured in quartz (black, solid line) and SLB-passivated quartz (grey, dashed line) at a temperature of 25°C. The bars represent the standard error of the mean for at last nine repetitions of each experiment.

Further experiments to investigate the applicability of this method were then performed with liposomes in the gel phase (*Figure 32*). The passivation of quartz cuvettes with an SLB resulted in a lower degree of leakage compared with that observed with unmodified cuvettes for DPPC and PEGylated DPPC liposomes. The presence of SLB prevents or hinders the interactions between the liposomes and the cuvette surface. For DPPC liposomes a similar degree of leakage has previously been observed for measurements conducted in PS cuvettes.[61] The PEGylated liposomes displayed a similar behaviour as PEGylated liposomes in the liquid-disordered and liquid-ordered phase state and it is likely that the previously reported interactions between silica and PEG-lipids [124] could be avoided by using SLB-passivated cuvettes.

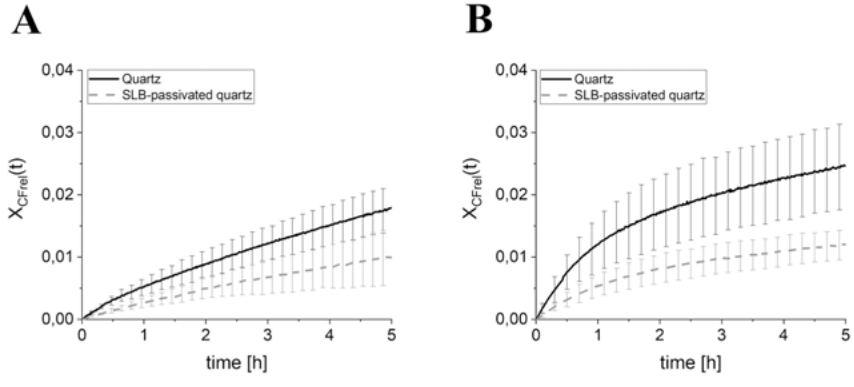


Figure 32 Leakage assays of (A) DPPC and (B) DPPC:DSPE-PEG2000 (96:4 mol%) liposomes. The degree of leakage was measured in quartz (black, solid line) and SLB-passivated quartz (grey, dashed line) at a temperature of 25°C. The bars represent the standard error of the mean for four repetitions of each experiment.

3.4.3 Concluding remarks

The results demonstrate that passivating the inner walls of cuvettes with an SLB in experiments of spontaneous leakage reduces the experimental artifacts arising from liposome-cuvette interactions. This method complements other approaches, such as the use of different cuvette materials [61] or the passivation of the cuvette surface with PEG, as presented in Paper III. An advantage of the SLB method is its applicability for measurements of spontaneous leakage of liposomes in the most common phase states, and for PEG-lipid-supplemented liposomes. It is worth to mention that previous approaches have been only useful for certain types of liposomes (*Figure 33*).

It should be noted that the proposed method is not a universal solution and does not render the previous results from Paper III unnecessary; rather, it expands the scope of application. SLB-passivated cuvettes would be a poor

choice for measurements focused on the leakage-promoting effects of membranolytic peptides or surfactants. An awareness of interactions at the solution-solid interface and careful selection of an appropriate means of reducing such interactions when necessary are therefore important when conducting spectroscopic measurements.

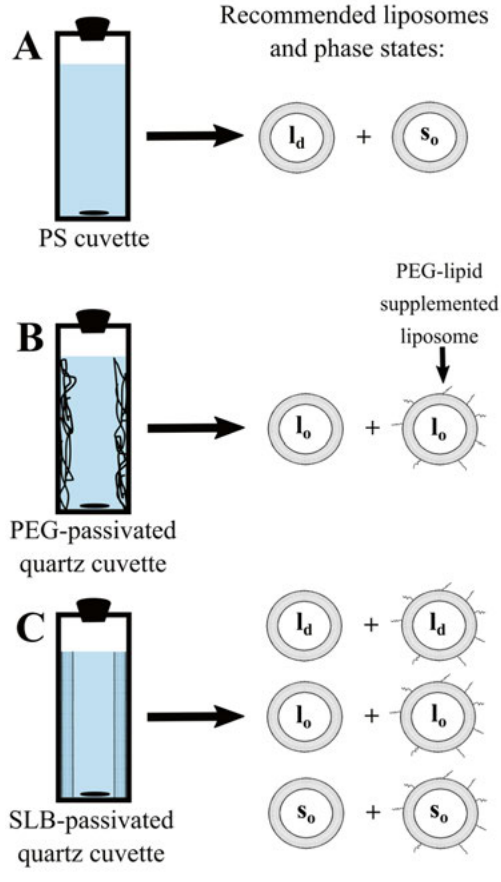


Figure 33 Recommended cuvette type or modification of the quartz cuvette for minimizing or preventing experimental artifacts in experiments of spontaneous leakage. Liposome phase states are liquid-disordered (l_d), liquid-ordered (l_d) and gel (s_o). The recommendation is based on the results presented in [61], Paper III and Paper IV.

4. Conclusions and future perspectives

The findings of this thesis illustrate the importance of obtaining detailed structural characterizations of lipid self-assemblies, and of establishing an in-depth knowledge and control of their interactions with solid materials. For example, our results suggest that the proposed ability of gangliosides to attenuate the anti-PEG immune response could be linked to gangliosides' ability to promote disk-formation rather than their capacity as a Siglec ligand.

Furthermore, the results obtained in this thesis emphasize the importance of understanding the processes that occur at the solution-solid interface between self-aggregated lipid particles and silica surfaces. This work showed that PEGylated lipid nanocarriers such as liposomes and lipodisks, spontaneously attach to silica surfaces. This interaction is strong enough to displace the hydration layer of silica, resulting in the formation of immobilized nanoparticle films. Increasing the temperature can then lead to irreversible changes due to stronger interactions between the silica and PEG. Interestingly, the findings showed that defect-free SLBs can be formed with liposomes in the gel phase. Potential applications include the sorting of nanoparticles, the enrichment of PEGylated protein and the passivation of silica surfaces. In fact, the latter found an application in Paper III.

Furthermore, this work showed that processes at the solution-solid interface between self-aggregated lipid particles and surfaces with different chemical properties (hydrophilic to hydrophobic) are relevant when investigating the solute permeability of lipid membranes by means of spectroscopic methods. More specifically, experimental artifacts can arise during spectroscopic measurements due to side processes occurring at the solution-cuvette interface. The passivation of cuvette surfaces with the polymer PEG is proposed as an effective method to minimize attractive interactions between the surfaces of a quartz cuvette and liposomes. In this way, the associated artifacts in leakage measurements can be reduced.

Nevertheless, PEG-passivated quartz cuvettes are not an all-in-one solution. The passivation of quartz cuvettes with an SLB was therefore proposed in Paper IV, as this method expands the scope of application. It should be noted that the results obtained with SLB-passivated quartz cuvettes do not render the passivation of quartz with PEG unnecessary. For example, SLB-passivated cuvettes are a poor choice for measurements focused on the leakage-promoting effect of membranolytic peptides or surfactants. Therefore,

these results emphasize the importance of consciously selecting an appropriate means of reducing interactions at the solution-solid interface.

The results presented in this thesis highlight the importance of detailed structural characterizations of lipid self-assemblies. Future experiments are necessary to investigate the combined effect of PEG-lipids and gangliosides in order to corroborate or disprove the proposed ability of gangliosides to attenuate the immunogenicity of PEGylated liposomes. Moreover, additional studies, such as immunological experiments, are necessary to investigate the hypothesis of the reduced anti-PEG immune response of PEG-stabilized lipodisks compared with PEGylated liposomes.

To summarize, the findings of this thesis indicate the importance of obtaining detailed knowledge and control of the interactions that may occur between lipid self-assemblies and solid surfaces. Although possible methods to control these interactions are proposed herein, more studies are necessary in order to fully understand these interactions and avoid interaction-related artifacts. For example, additional studies could include complementary surface-sensitive techniques, such as ellipsometry, NPS, neutron and/or X-ray reflectometry, atomic force microscopy and simulations. It would also be interesting to extend such studies to include more complex lipid self-assemblies, such as model membranes with compositions designed to mimic those of biological membranes, or with proteins included as components.

5. Popular Science Summary

Similar to Lego bricks, lipids are nature's building blocks, forming the body's cell walls, among other important biological structures. When dispersed in water, lipids usually form structures by themselves, where the type of structure formed depends on the geometries of the building blocks. The shape of the building blocks can vary, and geometries such as conical or cylindrical structures are common. Micelles, liposomes and lipodisks are examples of the self-assembled structures relevant in this thesis (*Figure 34*).

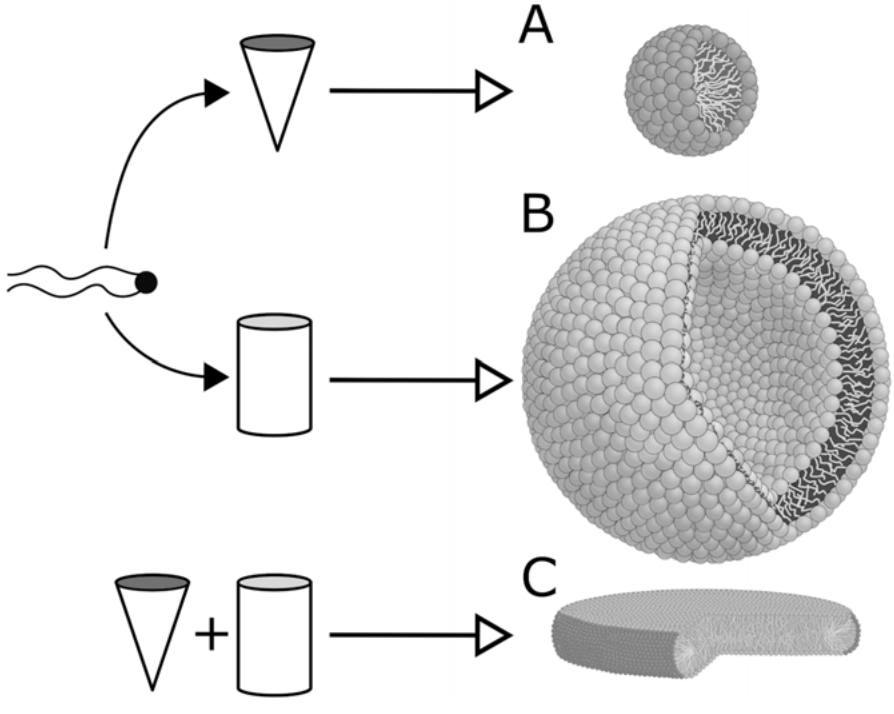


Figure 34 A schematic example of self-assembled structures, such as conical and cylindrical, formed by lipids with different critical packing parameters. (A) micelles, (B) liposomes and (C) lipodisks.

In particular, lipodisks exemplify the modularity of lipids as building blocks and the structures that can be formed due to the combination of different lipids.

Small lipodisks are composed of cylindrical and conical, polymer-coupled lipids. In this thesis, the polymer used was the water-soluble PEG; therefore, the polymer-coupled lipids in this case are abbreviated as 'PEG-lipids'. An insufficient fraction of PEG-lipids can result in the formation of liposomes whereas a greater proportion of PEG-lipids will lead to the formation of micelles. It was therefore of important to select an appropriate proportion of PEG-lipids in order to obtain a sample with ideally uniform particles that have similar properties. The careful selection of different lipids and their composition can lead to the formation of specifically shaped particles with selected properties.

The first publication associated with this thesis, Paper I, investigated the structural effects caused by the combination of different lipids, with PEG-lipids and/or gangliosides being added to the samples. Gangliosides are lipids that are composed of a sugar headgroup coupled to two hydrocarbon chains. This study showed that gangliosides can induce the formation of discoidal lipid assemblies. Gangliosides exhibit certain transformational capabilities, similar to those of the abovementioned PEG-lipids. Further investigations showed that the simultaneous presence of both gangliosides and PEG-lipids can promote important and previously undocumented structural effects. This study exemplifies the importance of properly analysing and characterizing samples.

The results of this thesis demonstrate that interactions between lipid particles and their surroundings – such as the solid surfaces of their containers – can lead to anomalous behaviour. The second publication, Paper II, analysed the interaction between lipid particles and a hydrophilic (attracted to water) surface – in this case, silica. The study showed that the polymer PEG can influence the interactions between lipid self-assemblies and the hydrophilic silica surface. Samples with added PEG-lipids attached strongly to the silica surfaces. Increasing the temperature led to irreversible changes that depended on the type of lipid particle. The interaction between silica and lipodisk containing samples, with a higher fraction of PEG-lipids, results in the loss of PEG-lipids, whereas liposomes with a lower PEG-lipid content tended to rupture. The latter process was not observed for PEG-free samples.

Differences in the interactions between lipid particles and solid surfaces can become important when analysing mixtures of particles. Such mixtures can be (sometimes unintentionally) complex, with liposomes, micelles and lipodisks present. These particles may have unequal affinities to surfaces or may be affected differently by interactions with surfaces. It is therefore advisable to use a well-characterized sample with, ideally, uniform particles. Moreover, is it important to consider relevant processes resulting from the interactions between lipid particles and solid surfaces.

Interactions between lipid self-assemblies such as liposomes, and solid surfaces can be problematic if contact between them is inevitable, which may

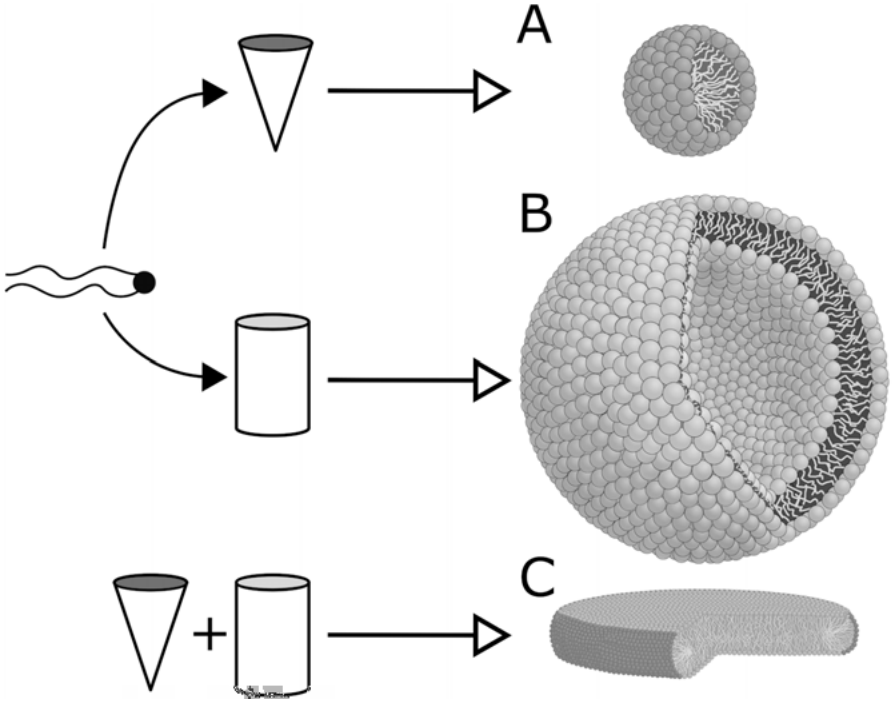
occur when the properties of lipid membranes are investigated using spectroscopic methods and the samples are placed in containers. These containers, named cuvettes, are composed of various materials with hydrophilic or hydrophobic (attracted to fat) properties. In the third publication of this thesis, Paper III, it was shown that the interactions between liposomes and different cuvette materials vary. These interactions and the resulting processes that occur at the cuvette surface can influence spectroscopic measurements, which is problematic when different compositions are being compared. Minimizing such interactions and thereby improving the comparability between experiments could be achieved by pre-treating quartz cuvettes with PEG. The adhesive property of PEG, as observed in Paper II, turned out to be useful for the research in the third publication, Paper III.

However, the method presented in the third publication – namely, pre-treating cuvettes with a polymer – had some limitations and was found to be unsuitable for certain types of liposomes. Instead of using an adsorbed polymer to passivate the surfaces of the cuvettes an SLB was utilized in the fourth publication, Paper IV. The aim of this study was to improve the comparability of the measurements, similar to the results presented in the third publication.

Papers III and IV highlight the importance of considering the interactions between lipid particles and solid surfaces. Neglecting these interactions can result in large deviations between experiments, and may also lead to erroneous conclusions when interpreting experimental data.

6. Populärvetenskaplig sammanfattning

Lipider är naturens byggstenar, liknande Lego-klossar, och bildar bland annat dina cellväggar. Finfördelade i vatten så bildar lipider vanligtvis strukturer av sig själva och vilka typer av självassocierade strukturer som bildas beror på byggstenarnas geometrier. Formen på byggstenarna kan variera och geometrier som koniska eller cylindriska är vanliga. Exempel på självassocierade strukturer som är relevanta i denna avhandling är A) miceller, B) liposomer och C) lipodiskar (*Figur 35*).



Figur 35 Ett schematiskt exempel på självassocierade strukturer, bildade av lipider, med olika geometrisk form såsom koniska och cylindriska.

Speciellt det senare exemplifierar mångsidighet hos lipider som byggstenar och de strukturer som kan bildas på grund av kombinationen av olika lipider.

Lipodiskar är sammansatta av cylindriska och koniska, polymerkopplade lipider. Polymeren som används i detta sammanhang är vattenlöslig polyetylenglykol (PEG) och lipiderna kallas därför PEG-lipider. En otillräcklig andel PEG-lipider kan resultera i uppkomsten av liposomer medan stora mängder leder till bildandet av miceller. Det är därför viktigt att välja lämplig mängd PEG-lipid för att erhålla ett homogent prov innehållande lipodiskar med liknande egenskaper. Ett noggrant val av olika lipider som blandas i rätt sammansättning kan leda till specifikt utformade partiklar med utvalda egenskaper.

De strukturella effekter som orsakas genom kombination av olika lipider studerades i den första publikationen. Av särskilt intresse var att undersöka hur närvaro av PEG-lipider och/eller gangliosider påverkar den typ av strukturer som bildas. Gangliosider är lipider som är sammansatta av en huvudgrupp baserad på socker kopplad till två kolvätekedjor. Det påvisades att gangliosider kan inducera bildandet av diskformade lipidaggregat. Gangliosiderna ger alltså en strukturell effekt liknande de ovan nämnda PEG-lipiderna. Ytterligare undersökningar visade att närvaron av både gangliosider och PEG-lipider kan främja viktiga och tidigare odokumenterade strukturella effekter. Studien exemplifierar vikten av att noggrant analysera och karakterisera sina prover.

Resultaten i denna avhandling visar vidare att interaktioner mellan lipidpartiklar och deras omgivning, t.ex. fasta ytor i olika behållare, kan leda till avvikande beteende. En analys av interaktionen mellan lipidpartiklar och en hydrofil (vattenälskande) yta, i detta fall kiseldioxid, presenterades i den andra publikationen. Resultaten visar att PEG-polymeren kan påverka interaktionen mellan de självassocierade lipiderna och den hydrofila kiseldioxidytan. Proverna med tillsatt PEG-lipid fäster starkt på kiseldioxidytorna. En höjning av temperaturen leder till irreversibla, d.v.s. bestående, förändringar som är beroende på typen av lipidpartikel. De med en högre andel PEG-lipider, såsom lipodiskar, tappar en del av PEG-lipiderna medan liposomer, med lägre PEG-lipidhalt, tenderar att brista. Det senare observeras inte för PEG-fria prover. Skillnaderna i interaktion mellan lipidpartiklar och fasta ytor kan bli betydelsefulla om blandningar av partiklar analyseras. Dessa kan vara komplexa blandningar, ibland oavsiktliga, med liposomer, miceller och lipodiskar närvarande. Partiklarna kan ha olika affiniteter för ytan eller påverkas olika av interaktioner med ytan. Det är därför fördelaktigt att ha ett väl karaktäriserat prov med homogena partiklar. Dessutom är det viktigt att känna till och ta hänsyn till de processer som interaktionen mellan lipidpartiklar och fasta ytor kan resultera i.

Interaktionen mellan självassocierade lipider, såsom liposomer, och fasta ytor kan vara ett problem om kontakt dem emellan är oundviklig. Detta kan vara fallet om lipidmembranens egenskaper undersöks med spektroskopiska metoder och proverna behöver placeras i behållare. Dessa behållare, så kallade

kyvetter, kan vara sammansatta av olika material med hydrofila eller hydrofoba (fettälskande) egenskaper. I den tredje publikationen påvisades att interaktionen mellan liposomer och olika kyvettmaterial varierar. Interaktionen och de efterföljande processerna vid kyvettytan kan påverka de spektroskopiska mätningarna, vilket kan vara problematiskt om olika sammansättningar jämförs. Exempelvis kan man vilja undersöka effekterna vid tillsats av PEG-lipider eller gangliosider. Genom att förbehandla kvartskyvetter med en PEG-polymer kan man i vissa fall minimera interaktionerna och därigenom förbättras jämförbarheten mellan experimenten. Den vidhäftande egenskapen hos PEG som observerades i den andra publikationen visade sig vara användbar för den tredje publikationen.

Metoden som presenterades i den tredje publikationen hade dock vissa begränsningar och var olämplig för vissa typer av liposomer. I den fjärde publikationen undersöktes möjligheten att utnyttja lipidmembra, istället för att använda en adsorberad polymer, för att passivera kyvetternas ytor. Syftet var att utveckla en mer generell metod som fungerar oavsett vilken typ av liposomer som studeras. Publikationerna tre och fyra belyser vikten av att ta hänsyn till interaktioner mellan lipidpartiklar och fasta ytor. Att förbise dessa kan resultera i stora avvikelser mellan experimenten och kan även leda till felaktiga slutsatser vid tolkningen av experimentella data.

7. Acknowledgements

My time as a doctoral student was affected by several relocations, giving me the chance to meet a lot of different people. Ironically, my time as a doctoral student ended similar to how it started: with a relocation.

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Philipp Grad
Uppsala, July 2022

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