Interactions between anesthetics and lipid rafts

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Success is a science; if you have the conditions, you get the result.

Oscar Wilde
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Abstract

The exact mechanism by which anesthetics induce membrane-mediated modifications that lead to loss of sensation is still an open question. Lipid rafts are membrane microdomains that have been associated with cell signaling pathways, as well as specific interaction with drugs. However, knowledge about the interaction of lipid rafts with anesthetics is scarce.

The interactions of liposomal lipid raft models of an equimolar mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), sphingomyelin (SM) and cholesterol (Chol) with the anesthetics tetracaine (TTC), lidocaine (LDC) and propofol (PPF) were studied at 25 and 37°C, as well as at different anesthetic concentrations. The effect of cholesterol was investigated by studying interactions with POPC/SM 1:1 liposomes. The following experimental techniques were used: quartz crystal microbalance with dissipation (QCM-D), differential scanning calorimetry (DSC) and phosphorus nuclear magnetic resonance (P-NMR).

The three anesthetics interacted with liposomes of both compositions, inducing membrane fluidization, depression of phase transition temperatures, liposome swelling and/or viscosity changes of the adsorbed liposome layers. Tetracaine interacts more with raftlike domains, lidocaine induces stronger modifications on POPC/SM liposomes and the results for propofol are not fully conclusive.

In comparison with the interaction of these anesthetics with eukaryotic cell membrane models previously studied in our group, there is fluidization of membranes from both studies, but the mechanisms of interaction are different. Although a direct comparison of the two models is not possible, the results show that the effects of the anesthetics on lipid membranes are dependent on their composition and the existent lipid phases.

Keywords

Lipid rafts; Anesthetics; Liposomes; QCM-D; DSC; P-NMR.
Resumo

O mecanismo exacto pelo qual os anestésicos modificam as membranas celulares levando à perda da função sensorial é uma questão em aberto. Os *lipid rafts* são microdomínios membranares lipídicos associados a vias de sinalização celular e a interacções específicas com fármacos. Contudo, o conhecimento sobre a interacção de anestésicos com *lipid rafts* é escasso.

As interacções de modelos lipossomais de *lipid rafts* de uma mistura equimolar de 1-palmitoil-2-oleoil-sn-glicerol-3-fosfocholina (POPC), esfingomielina (SM) e colesterol (Chol) e os anestésicos tetracaína, lidoicaína e propofol foram estudadas a 25 e 37°C e a diferentes concentrações de anestésico. O efeito do colesterol foi avaliado pelo estudo das interacções com lipossomas de POPC/SM 1:1. As técnicas experimentais utilizadas foram: microbalança de cristais de quartzo com dissipação (QCM-D), calorimetria diferencial de varrimento (DSC) e ressonância magnética nuclear de fósforo (P-NMR).

Os três anestésicos interagiram com os lipossomas de ambas as composições, induzindo fluidificação das membranas com dilatação dos lipossomas e/ou alterações na viscosidade da camada de lipossomas adsorvidos. A tetracaína interage preferencialmente com domínios *raftlike*, a lidoicaína induz maiores alterações em lipossomas POPC/SM e os resultados para o propofol não são conclusivos.

Comparando a interacção destes anestésicos com modelos membranares de células eucarióticas estudados anteriormente no nosso grupo, ocorre fluidificação de membranas de ambos os estudos, mas os mecanismos de interacção são diferentes. Embora não seja possível estabelecer uma comparação directa entre os dois modelos, os resultados demonstram que os efeitos dos anestésicos em membranas lipídicas dependem da sua composição e das fases lipídicas presentes.

Palavras Chave

*Lipid rafts*; Anestésicos; Lipossomas; QCM-D; DSC; P-NMR.
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Abbreviations

AFM atomic force microscopy
Chol cholesterol
C-NMR carbon nuclear magnetic resonance
CSA chemical shift anisotropy
DLS dynamic light scattering
DMPC 1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMSO dimethyl sulfoxide
DOPC 1,2-dioleoyl-sn-glycero-3-phosphocholine
DPPC 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DRM detergent resistant membrane
DSC differential scanning calorimetry
FRET fluorescence resonance energy transfer
Gang ganglioside
GPI glycosylphosphatidylinositol
HEPES N-(2-Hydroxyethyl) piperazine-N'-ethanesulfonic acid
H-NMR proton nuclear magnetic resonance
LA local anesthetic
L_d lamellar liquid-disordered phase
LDC lidocaine
LMV large multilamellar vesicle
L_o lamellar liquid-ordered phase
LSCFM laser-scanning confocal fluorescence microscopy
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
</tr>
<tr>
<td>( L_{\alpha} )</td>
<td>lamellar liquid-crystalline phase</td>
</tr>
<tr>
<td>( L_{\beta} )</td>
<td>lamellar gel phase</td>
</tr>
<tr>
<td>N.E.</td>
<td>not evaluable</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PG</td>
<td>phosphatidylglycerol</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>P-NMR</td>
<td>phosphorus nuclear magnetic resonance</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PPF</td>
<td>propofol</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>QCM-D</td>
<td>quartz crystal microbalance with dissipation</td>
</tr>
<tr>
<td>S.D.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SAM</td>
<td>self-assembled monolayer</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SLB</td>
<td>supported lipid bilayer</td>
</tr>
<tr>
<td>SM</td>
<td>sphingomyelin</td>
</tr>
<tr>
<td>( s_o )</td>
<td>solid-ordered phase</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
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<tr>
<td>TTC</td>
<td>tetracaine</td>
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List of Symbols

\[ \begin{array}{ll}
T_m & \text{main transition temperature} \\
P & \text{partition coefficient} \\
D & \text{diffusion coefficient} \\
k_B & \text{Boltzmann constant} \\
\eta & \text{viscosity} \\
I & \text{scattering intensity} \\
q^2 & \text{second order autocorrelation function} \\
q & \text{wave vector} \\
\Delta & \text{frequency shift} \\
\Delta D & \text{dissipation shift} \\
E_{\text{dis}} & \text{dissipated energy} \\
E_{\text{st}} & \text{stored energy} \\
\Delta m & \text{adsorbed mass variation} \\
C_f & \text{mass sensitivity constant} \\
N & \text{overtone number} \\
d & \text{thickness} \\
G & \text{complex shear modulus} \\
\rho & \text{density} \\
\Delta H & \text{enthalpy of transition} \\
\Delta \sigma & \text{chemical shift anisotropy} \\
\sigma_{||} & \text{chemical shift parallel to the applied magnetic field} \\
\sigma_{\perp} & \text{chemical shift perpendicular to the applied magnetic field}
\end{array} \]
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1.1 Motivation

Despite the fact that anesthetics have been used for over a century, the exact mechanism of action is still unknown. The two main theories of the molecular mechanism of anesthesia are the direct binding of anesthetics to receptors associated with ion channels and the perturbation of membrane lipids surrounding functional proteins. Even though lately the protein interaction hypothesis has been favored, there is still enough evidence for interaction between anesthetics and membrane lipids, and the potential role of lipid rafts on these interactions has been reported [1-5].

Liposomes constitute good physical models for the study of interactions between membranes and drugs because their membrane organization is similar to that of native cells, with a phospholipid bilayer separating the intracellular liquid volume and the extracellular space [6].

In a previous research carried out in our group [7], the interaction between several anesthetics and plasma membrane models was studied. In general, all anesthetics led to an increase in the membrane fluidity and a correlation between the effect of the anesthetic and its lipophilicity was found. In that work, the membrane was modeled using supported layers of liposomes and liposomes in solution of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), DMPC/cholesterol (Chol) and ternary mixtures of DMPC, Chol and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), which mimic the composition of eukaryotic plasma membranes.

There is evidence that membrane domains designated by lipid rafts play a special role in cell signaling pathways in several cell types. These lipid rafts may be extracted as detergent resistant membrane (DRM) domains from cell membranes [8, 9]. One of the most used compositions to model lipid rafts is an equimolar mixture of of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), sphingomyelin (SM) and Chol, based on the amounts on these lipids found in detergent-resistant domains extracted from cell membranes.

The question that we tried to investigate is the existence of a special affinity between anesthetics (local and general) and the lipid rafts. In other words, our goal was to assess if the interaction between anesthetics and the lipid membrane occurs preferentially in the raftlike domains, in comparison with more fluid membranes without cholesterol. Lidocaine and tetracaine were the chosen local anesthetic representatives, while propofol was the general anesthetic used.

The techniques used were: a quartz crystal microbalance with dissipation (QCM-D) to detect changes in the adsorbed mass, as well as in the viscoelastic properties of the intact liposome layer absorbed on oxidized gold-coated quartz crystals, upon interaction with anesthetics; differential scanning calorimetry (DSC), which detects changes on the phase transition of the acyl lipid chains; phosphorus nuclear magnetic resonance (P-NMR), which is sensitive to the motion of the polar head groups of phospholipids, being also an indicator of the hydrocarbon chain phases present on the system and dynamic light scattering (DLS) for determination of the size distribution of the liposomes.
1.2 State of The Art

1.2.1 Interactions of lipid rafts with drugs

One very interesting study is the one carried out by Ausili et al [10] on the effect of the anti-cancer drug edelfosine on model and cell-isolated rafts. This group concluded by differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR), X-ray diffraction and fluorescence microscopy results that edelfosine colonizes into lipid rafts and suggested that this might be the pathway for molecular action of edelfosine in cells [10]. Other studies that have proved a preferential interaction of drugs with the lipids of these rafts were performed with antibiotics, anti-psychotics and other anti-cancer drugs [11–13]. There is also evidence that many drugs interact directly with lipid rafts through the proteins embedded in them [12, 13], but this is a deviation from the scope of this work and will not be described.

1.2.2 Interactions of lipid rafts with anesthetics

The interaction of lipid rafts with anesthetics was already studied by other groups using diverse techniques and methodologies but the results are scarce and contradictory. For the interaction with lidocaine, the group of Kamata [14, 15] reported that lidocaine (18.4 mM) reversibly disrupted lipid rafts in erythrocyte membranes without modifying the cholesterol content. Results using model membranes were provided by Tsuchiya et al [16], having studied the interactions of several local anesthetics (LA), including lidocaine, with raftlike membranes and models of membranes of cardiac myocyte’s mitochondria with no lipid rafts. They found that all the LA interacted in a more pronounced way with the non-raft membranes than with raft model membranes, questioning the interaction of local anesthetics with rafts, at least in what respects the alteration of liquid-ordered domains in vitro. To our knowledge, there are no studies concerning the interaction of tetracaine with lipid rafts, although Baezinger et al [17] have shown that the presence of phosphatidic acid (PA) increases the partitioning of tetracaine into phosphatidylcholine membranes with or without cholesterol. The lipid environment will influence the effect of tetracaine on the transmembrane pore of the nicotinic acetylcholine receptor (nAChR), which is a prototype of a family of neurotransmitter-gated ion channels. For the general anesthetic propofol, there are also, to our knowledge, no publications on the direct action of propofol on lipid rafts. However, the promotion of fluid-like domains in gel phases by propofol has been reported by Balasubramanian et al [18], by showing that propofol induced the formation of fluid-like domains from the gel-phase domains of pure DMPC and DPPC:DMPC mixtures.

1.3 Original Contributions

This work aims to contribute to the increase of knowledge in the mechanism of membrane modifications induced by anesthetics using simplified lipid models and to correlate the findings with possible in vivo phenomena for supression of action potentials by modification of the lipid organization, as well as to try to find a specific preference of the three anesthetics tested for lipid rafts. The information on
the topic of anesthetic interaction with lipid rafts is very scarce, at least in what concerns the three anesthetics studied, as stated in Section 1.2. However, the study of anesthetic-induced perturbation of lipid rafts is extremely important to evaluate the action of these anesthetics, since it is possible that some receptors and ion-gated channels (with the Na\(^+\)-channels in evidence, since its inhibition leads to inhibition of the action potentials) are localized preferentially in lipid rafts of nerve cells [19, 20].

With very simple analytical techniques and using a very simplified model for lipid raft composition, but complex enough to form the so called microdomains that are named lipid rafts with a specific lipid phase and composition, we aim to draw more attention to the possibility of tetracaine, lidocaine and propofol having a raft-mediated anesthetic mechanism. We also expect, with this work, to suggest more complex studies with new models, techniques, and even cell studies to test the assumptions made. In final terms, all the findings that arise from this study and from other consequent works aim to stimulate the medical, pharmaceutical and engineering community to find more efficient formulations and administration/delivery systems for the anesthetics studied, promoting faster anesthesia onset and reducing the possibility of neural damage and side effects in other tissues/organs.

1.4 Thesis Outline

The work is presented on the following 4 chapters. The first chapter, Theoretical Background, aims to provide an introduction to the following topics: Eukaryotic cell membranes, focusing on its lipids; Lipid rafts and their roles in cells; Models for study of biomembranes and raftlike compositions; Anesthetics, their mechanism of action, types, side effects and clinical formulations, focusing on the three anesthetics studied; Experimental techniques, to explain the principles behind the experimental techniques used.

The Materials and Methods chapter covers the materials and techniques used to evaluate the interactions between model membranes and anesthetics, that is, quartz crystal microbalance with dissipation (QCM-D), differential scanning calorimetry (DSC) and phosphorus nuclear magnetic resonance (P-NMR), as well as the liposome preparation techniques and its size distribution determination by dynamic light scattering (DLS).

The Results and Discussion section presents the results of the interaction of each anesthetic with both membrane models: lipid rafts and POPC/SM membranes. For each anesthetic, the effect temperature is discussed, while the effect of concentration is discussed for tetracaine and lidocaine. From the results obtained, some assumptions are made.

The Conclusions and Future Work chapter aims to summarize the main assumptions, compare the results with a previous study carried in our group and suggest some future research directions on this topic.
2

Theoretical Background

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2.5 Experimental techniques .................................................... 20
2.1 Eukaryotic cell membranes

Membranes define the external boundaries of cells and regulate the molecular traffic into and out of the cells. In eukaryotic cells, the membrane has also the function of dividing the internal space of the cell into certain compartments with specific functions (cellular organelles). Another important property is the cell energy conservation and cell-to-cell communication. The unique functions of membranes arise from their physical properties. They are flexible, allowing shape changes for cell growth and movement. They are also breakable and self-sealing, allowing the fusion with vesicles for exocytosis and formation of endocytic vesicles for intracellular trafficking, without important losses of cell components. Finally, they are selectively permeable in order to retain certain compounds and ions on proper concentrations for cell homeostasis [21].

The eukaryotic cell membranes are composed of a phospholipid bilayer about 5 to 8 nm thick [21, 22]. The phospholipid molecules are amphipathic because they have hydrophobic and hydrophilic regions. They form a stable bilayer in an aqueous medium as a result of interaction of the hydrophilic head groups with the extracellular and intracellular aqueous environments, and hydrophobic interactions between the fatty acids of the bilayer interior [22]. Proteins are embedded in this lipid bilayer and stabilized by hydrophobic interactions between the fatty acids and the hydrophobic domains of proteins. Some proteins extend to one or both outer sides of the bilayer, and its orientation is asymmetric, because the domains facing the outer leaflet are not the same as of the inner leaflet. So, the individual lipids and proteins form a fluid mosaic pattern that is free to change constantly, because most of the interactions between components are not covalent, allowing individual components to move in the lateral plane of the membrane [21]. The fluid mosaic model was proposed by Singer and Nicholson in 1972 [23] and is schematized in Figure 2.1. Although polar lipids and proteins account for almost all the total mass of biological membranes, carbohydrates are also present incorporated in glycolipids and glycoproteins. These are complex molecules distinguished by their functions as receptors on cell surfaces for signaling and recognition [21, 24].

![Fluid mosaic model of the cell membrane. Modified from [25].](image)

Each cell type has got a unique proportion between proteins and lipids, and also specific lipids to account for different functions and biological roles [21]. Since we are modeling anesthesia, human
myelinated neurons gain special relevance. They have a myelin sheath, which is an extended plasma membrane that acts as an electrical insulator. To accomplish this role, these neurons’ membranes are primarily composed of lipids, while other human cell types have a higher proportion in proteins.

The protein composition of different cell types is more variable than the lipid composition, reflecting functional specialization. Generically, the most important types of proteins in cell membranes are transporters, responsible for movement of organic solutes and ions across the membrane, receptors that sense extracellular signals, such as antigens, growth factors, hormones, light, mechanical stress and nutrients, and adhesion proteins that hold neighbor cells together [21]. In terms of their position and integration in the membrane, there are essentially two types of proteins: transmembrane proteins that are firmly associated with the membrane by hydrophobic interactions, and peripheral proteins that are stabilized by electrostatic interactions and hydrogen bonds with the hydrophilic head groups of the lipids [21].

The most representative classes of lipids in mammalian cell membranes are: glycerophospholipids, where the hydrophobic moiety is composed of two acyl chains covalently linked to glycerol; sphingolipids, with a single acyl chain covalently linked to sphingosine (a fatty amine) and sterols, which are characterized by a rigid system of four fused hydrocarbon rings [21–24]. In terms of the polar group, some lipids have the polar group linked to the hydrophobic moiety by a phosphodiester linkage, and in that case they are generically named phospholipids. Other lipids have no phosphate but a single sugar or complex oligosaccharide as a polar group [21].

Glycerophospholipids are derivatives of phosphatidic acid and are named according to the alcohol on the head group. The classes of glycerophospholipids according to the alcohol on the head group are: phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidylserines (PSs), phosphatidylglycerols (PGs) and phosphatidylinositols (PIs). For each type of glycerophospholipid there is a considerable variety of acyl chains leading to different molecular species with specific distributions for each organism and tissue [21]. As an example, the structure of the phospholipid used in this study, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), is shown in Figure 2.2.

Sphingolipids are characterized by a sphingosine, which is a long amine alcohol, comprising a single saturated acyl chain and a polar group linked by a glycosidic or phosphodiester bond. The fatty acid forms a linkage with the amine group on C-2 and the resulting compound is named ceramide. All the sphingolipids are derived from ceramide, but differ on the polar group composition. Sphingomyelins (SMs) have a phosphocholine or a phosphoethanolamine as the polar group and thus are phospholipids (Figure 2.3). They are particularly common on the myelin sheath of neurons [21–24, 27–29]. Compared to natural occurring phosphatidylcholines, they have a higher main transition temperature (Tm), are more hydrophobic and undergo tighter packing due to the saturated acyl chain [30]. Glycosphingolipids exist more frequently on the outer layer of cell membranes and
have sugar moieties as head groups. Some examples of this class are cerebrosides, globosides and gangliosides, which vary on the composition and complexity of the sugar head groups [21].

Figure 2.3: Chemical structure of sphingomyelin, the most relevant sphingolipid in nerve cells. Modified from [26].

Sterols are characterized by a rigid structure of four fused rings, three with six carbons and one with five. Cholesterol (Chol) is the main sterol in animal tissues and it is also an amphipathic lipid, with a hydroxyl polar head group and a hydrophobic backbone (Figure 2.4). Apart from the role on membrane composition, cholesterol and its derivatives are also important precursors for hormones and bile acids [21]. Inside the membranes, cholesterol interacts with phospholipids leading to the fluidization of their hydrocarbon chains at temperatures below their main transition temperature, or to their rigidification if the temperature is above their \( T_m \). Other important biological roles of cholesterol are the reduction of passive permeability to small molecules, increase of the mechanical strength of membranes and modulation of the activity of many membrane-associated enzymes [30, 31].

Figure 2.4: Chemical structure of cholesterol. Modified from [31].

In terms of relative proportions of the main three lipid classes, cholesterol is typically present at 30-40% mol, sphingolipids at 10-20% mol, and glycerophospholipids at 40-60% mol [24]. The membrane lipids are asymmetrically distributed between the two monolayers. Generally choline-containing lipids, like PCs and SMs, are typically on the outer leaflet of the membrane, while PSs, PGs and PE are more common on the inner leaflet. Sometimes this distribution is interchangeable and induces signaling for specific events, like cell death [21, 23, 24, 30, 32]. The transverse distribution of cholesterol is roughly equal between the two leaflets, but since it has higher affinity for SMs and PCs compared to PSs and PE, it might be somewhat higher on the outer leaflet [24].

The basis of the membrane dynamics is the fact that the acyl chains are not covalently linked, allowing lipid motions and the formation of transient structures. There are different degrees of ordering of the acyl chains of lipids that are responsible for differences in fluidity, structure and flexibility of the membrane. Although several phases have been identified, the most well studied lipid lamellar phases are the lamellar gel phase (\( L\alpha \)), where the acyl chains are well-packed, fully extended (all-trans configuration) and ordered, and the fluid-like lamellar liquid-crystalline phase (\( L\beta \)), where the acyl chains are conformationally disordered and undergo fast long axis rotation and lateral diffusion rates [21, 23, 24, 27-28, 31-36]. The gel phase does not occur in cell membranes, only for model systems.
The phase behavior depends on the temperature and the kind of lipids present and the phase transitions become more and more complex with different lipid types [21, 33]. In mixtures, examples of intermediate phases are the lamellar liquid-disordered phase (Ld) and the lamellar liquid-ordered phase (Lo) [37]. The liquid-ordered phase is an intermediate phase between the Lβ and Lα phases and is meant to possess free rotational and translational diffusion of lipid molecules (as found in Lα phase) with fully extended and well-packed acyl chains in an all-trans configuration (characteristic of the Lβ phase) [21–24, 27–34]. On the other hand, Ld arises as a more appropriate term to describe the Lα phase on these conditions [24].

As described above, the lipids in the membrane will move laterally by interchanging their positions on the layer with neighboring lipid molecules. Another type of motion that might happen, although slower and requiring catalization, is the "flip-flop" motion of lipids from one leaflet of the membrane to another. This motion is energetically unfavorable and is catalyzed by flipases, providing a faster and less energetically expensive path [21, 23, 24].

Other than the asymmetric distribution between the two leaflets, there is also evidence for the formation of microdomains on the membranes by specific interaction of certain lipids [21, 23, 24, 30–34]. There are domains that form morphologically distinct structures, such as caveolae (invaginations of the cell membrane enriched in SM and Chol) and clathrin-coated pits [30]. However, there are domains that are not associated with a distinct morphological feature [31]. For instance, glycosphingolipids, such as gangliosides (Gangs) and cerebrosides, will form domains in the outer leaflet comprised of saturated acyl chains and poor in mixed chain phospholipids. Other type of domain results from the association between sphingolipids that have long, saturated acyl chains and cholesterol through the favorable interaction of the acyl chains with the sterol ring [21]. These domains are called lipid rafts [9, 10, 14, 21–24, 27–36, 38–43], and the interaction of anesthetics with lipid rafts is the main topic of this Thesis, so these structures will be described with more detail on the following section.

2.2 Lipid Rafts

Evidence for lateral lipid segregation within the bilayer under proper conditions and formation of distinct domains with different characteristics (such as a different lipid phase) was suggested by Philips in 1970, having found non-ideal mixing of different PC species [44]. In 1976, Lentz proved the existence of domains of PCs with saturated and unsaturated acyl chains [45] and, in 1977, Schmidt stated that sphingomyelins are able to induce formation of microdomains in biological membranes [46]. Regarding these data, Jain and White proposed in 1977 an improvement to the fluid mosaic model, the plate model, postulating that the separation between ordered and less-ordered regions of the membrane is a consequence of intermolecular interactions and lattice deformation [47].

Another important contribution to the raft concept was the definition of the lamellar liquid-disordered phase (Ld) and the lamellar liquid-ordered phase (Lo) by Ipsen et al in 1987 [37]. It has been stated that the Lo phase can coexist in equilibrium with the Ld phase [9, 10, 24, 27, 34, 35, 39, 41, 48, 49].
There is also evidence for an increase in bilayer thickness of the more ordered regions, which could provide a different and specific environment for protein anchoring within the membrane [41]. The $L_0$ phase is formed by preferential interaction of cholesterol with the saturated acyl chains of saturated lipids. Sphingolipids have a special affinity for cholesterol because, apart from their saturated acyl chains, the amine on the sphingosine group will very likely form hydrogen bonds with the hydroxyl group of cholesterol [24, 36, 50, 51].

These observations gave rise to the concept of lipid rafts proposed by Simons and Ikonen in 1997 [52]. Their work suggested the existence of lipid rafts, defined as transient microdomains in the outer leaflet of the cell membranes enriched in sphingolipids and cholesterol that would be associated with specific proteins. The raft hypothesis assumes that the aggregation of those lipids in the plane of the membrane is driven by intermolecular interactions, such as van der Waals interactions between the fatty acyl chains of sphingomyelin and glycosphingolipids, as well as interactions between these lipids and cholesterol, hydrogen bonds between adjacent moieties of glycosphingolipids and by steric shielding of cholesterol from the aqueous medium [41, 50, 53]. The ability of lipids and proteins to integrate the rafts depends on how well they can diffuse into them without modifying lipid order [50]. These regions are more rigid than the phospholipid-enriched regions, where the $L_d$ phase is predominant [41]. Even though different studies for raft size determination show a considerable dispersion, their length usually ranges from 10 to 100 nm [54], probably due to the fact that rafts are dynamic structures and are thought to exist in vivo as stable entities for only a few minutes [38, 51].

Lipid rafts are an active field of research due to the potential role played by the rafts’ lipids or proteins on several cell events [35]. The peripheral proteins anchored by glycosylphosphatidylinositol (GPI) are very commonly associated with rafts, as well as signaling proteins for signal transduction across the plasma membrane [9, 49, 50]. Other peripheral proteins are the dually acylated (Acyl) proteins. There are also transmembrane (TM) proteins that are anchored to lipids by weak interactions, whose incorporation on the membrane depends on its specific affinity for raft lipids, as well as on the existence of hydrophobic domains that match the thickness of the lipid bilayer on the rafts [55]. The diversity of molecules present in lipid rafts is schematized in Figure 2.5.

Some functions that have been attributed to the rafts are the sorting and targeting of lipids and proteins, mediation of membrane trafficking and signal transduction [8, 9, 12, 23, 24, 30, 35, 41, 42, 48, 50, 55-58]. It is also known that they provide specific sites for interaction with toxins and pathogens [48], immune response, cell adhesion, chemotaxis and regulation of the cytoskeleton, besides being important in proliferative and apoptotic pathways [12, 57]. Specifically in neuronal cells, some of the important roles reported are the growth-factor signal transduction, cell adhesion, axon guidance, synaptic transmission, membrane-mediated proteolysis and binding of neurotrophic factors to tyrosine-kinase receptors [42, 57]. Therefore, lipid rafts are essential to ensure good neuronal communication [58]. Modifications on raft structure and composition play a crucial role in certain diseases, such as prion diseases, Alzheimer, Parkinson, bacterial and viral infections (HIV-1 infection is one of the most important examples) and cancer [12, 48, 49].

The first evidence for the existence of rafts in vivo was the detection of detergent resistant mem-
branes (DRMs). DRMs are membrane fractions which resist solubilization by Triton X-100 at 4°C and are enriched in cholesterol, sphingomyelin and raft proteins [48]. However, this approach is problematic because decreasing the membranes’ temperature will certainly modify the physical state of lipids, as well as remove some of the raft lipids and proteins [24, 42]. Other problem is the complexity of the method, it requires constant amounts of starting material, internal markers, control of cholesterol depletion and use of more than one detergent [50]. Therefore, DRMs will not resemble the native rafts in abundance, size or composition, so this technique only cannot be used to prove existence of rafts in vivo. However, they are a good starting point for identification of potential raft proteins [35, 50].

For rafts’ monitoring in living cells, several techniques have been used, like fluorescence microscopy, fluorescence quenching, single-particle tracking and fluorescence resonance energy transfer (FRET). An example of lipid raft monitoring using fluorescent methods is presented in Figure 2.6. The problems associated with these methods are the dependence of the results on the molecules traced [33], as well as the perturbation inherent to the introduction of fluorescent probes, the low spatial resolution of light microscopy and the transient character of lipid rafts [33, 55, 57]. Other non-imaging methods have been used, such as nuclear magnetic resonance (NMR), which is based on the change in the orientation of the lipid head group orientation caused by the formation of domains, and differential scanning calorimetry (DSC), where the construction of phase diagrams and the regions of phase coexistence may be related to domain existence [33].

Since natural rafts are difficult to isolate without modification of their biochemical environment, simple synthetic models have attracted the researcher’s attention to mimic their properties in vitro and hypothesize about their functional role in vivo, as well as to study their formation, structural integrity and microdomain characteristics [9, 24, 27, 32, 34, 38, 41, 48, 49, 53]. The different models used to mimic lipid rafts will be the scope of the next section.
2.3 Models for the study of biomembranes and lipid rafts

Biomimetic membranes are artificial mixtures composed of lipids that resemble the composition of a cell membrane and allow the in vitro modeling of processes happening in biological membranes, as well as construction of biosensors, bioreactors and artificial cell designs [60, 61]. There is a huge variety of compositions, supports for deposition of model systems and preparation methods:

- Planar Phospholipid Bilayers, also commonly referred to as Black Lipid Membranes. This was the first method developed. These membranes are deposited on a hydrophobic support, like polyethylene or teflon, with an aqueous media on both sides of the membrane (Figure 2.7). This method has the disadvantage of yielding unstable membranes with a short lifetime [61].

- Supported lipid bilayers (SLBs) are supported on a solid substrate from which the membrane is separated by a 10 - 20 Å thick aqueous layer, as shown in Figure 2.8. The surface substrates commonly used are silica, borosilicate glass, mica, oxidized silicon, titanium oxide, gold, silver and platinum. The easiest method to deposit lipids onto a substrate is the adsorption and fusion of small unilamellar vesicles (SUVs), and the fusion is controlled by vesicle composition and size, surface charge, pH, ionic strength and osmotic pressure of the vesicles. This method results in very stable bilayers and allows the use of surface analytical techniques to prove interactions [61].
• Self-Assembled Monolayers/Lipid monolayers are obtained by modifying the substrates with alkane thiols self-assembled monolayers (SAMs) to render the surface hydrophobic (Figure 2.9). These membranes are even more rigid than supported lipid bilayers (SLBs) because they are fully coupled to the substrate, but they cannot resemble the bilayer environment [61].

• Liposomes (unilamellar vesicles), in suspension or adsorbed on substrates (a scheme of adsorption is shown in Figure 2.10), are good models to study the membranes of real cells, despite their simplicity [62]. The advantages of this method are related to the possibility of using to a greater extent the properties of the phospholipid membrane itself as a functional boundary and mimic the native cell membrane’s fluidity [63, 64].

Even though SLBs have been very popular as model systems for cell membranes because of their simplicity and reproductibility [65], the use of liposomes as model systems has attracted attention because they are the most similar system to the native cells, being the phospholipid bilayer a shell of separation between the intracellular liquid volume and the extracellular space [6]. They are also an
excellent method to mimic cell adhesion in a protein-free environment [66]. Due to these properties, the membrane systems used in this study are liposomes.

There are both non-specific and specific pathways of adsorption of vesicles on surfaces. The tendency of adsorption and fusion of intact vesicles to form a bilayer is determined by surface properties, composition, size, osmotic pressure, bivalent cations concentration and by the temperature of the experiment [6]. In this study only neutral or zwitterionic lipids will be used, so considerations on the surface charge of the vesicles will not be made. It has been shown on several studies that vesicles from phosphatidylcholines tend to fuse spontaneously on highly hydrophilic surfaces, such as silica, glass or mica, to adsorb non-specifically as intact entities on oxidized metals or metal oxides such as gold, platinum and titanium oxide, and to form monolayers on highly hydrophobic surfaces, like SAMs of alkanethiols on gold [6, 66–68]. It is also known that high concentration of bivalent cations, like Ca$^{2+}$ and Mg$^{2+}$ [65, 68], high temperature, high osmotic stress and high surface charge will promote the fusion of vesicles [6, 69, 70]. Oxidized gold allows the adsorption of intact zwitterionic vesicles at the temperatures studied, so it will be the surface used for adsorption of vesicles in this study [67, 69].

In addition to non-specific adsorption driven by electrostatic interactions and van der Waals forces, there are specific tethering methods that rely on the modification of vesicles to have specific affinity for certain molecules immobilized on the surface. Some of the most used tethering methods are the biotin-streptavidin pair, complementarity between DNA single strands, lipophilic anchors, histidine tag, polyethylene glycol (PEG) and amino-coupling to the surface [63, 64, 66, 69–71]. The advantages of the tethering methods are the reduction of non-specific binding of ligands, the assembly of systems with multiple components and the modulation of diffusion on the surface plane [70].

Several lipid compositions have been used to mimic the lipid rafts’ phase behavior. One prerequisite for raft simulation is the phase separation between L_o and L_d phases in the presence of cholesterol [8]. Generally, ternary mixtures of a high main transition temperature lipid (with saturated acyl chains), a low main transition temperature lipid (unsaturated acyl chains) and cholesterol are used as raftlike mixtures. Although these mixtures have a much simpler lipid composition than that of cell membranes, their phase behavior yields an adequate simulation of the phase behavior in vivo [9]. It is common to represent the phase behavior of model mixtures as ternary phase diagrams at a fixed temperature, being the room temperature (25°C) and the physiological temperature (37°C) the most representative [8, 35–38]. The most common raftlike mixtures are composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), DPPC[Chol] and DOPC[SM][Chol] [48]. Sphingomyelin seems to be the most appropriate high T_m lipid because it allows hydrogen bonding with the hydroxyl group of cholesterol, and is present on native membranes of many cell types. For the low T_m lipid, a better option would be 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) since it is the most relevant phosphatidylcholine on cell membranes, as found by extraction from biological sources [8]. The so-called “canonical raft mixture” consisting of an equimolar mixture of glycerophospholipid/sphingolipid/cholesterol is an accurate model, based on the fact that [DRMs] extracted from cells had an approximately equimolar composition on the three types of lipids [23]. According to these observations and to the fact that nerve cells’ rafts will be modeled, the canonical mixture of POPC/brain
SM/Chol 1:1:1 will be used. The ternary phase diagram of the three components at some relevant temperatures is shown in Figure 2.11. Since mixtures of SM with POPC are also known to phase separate on more rigid SM-rich domains and more fluid POPC-rich domains [49], the binary mixture of POPC/brain SM 1:1 will also be studied to understand the influence of cholesterol on the interactions we aim to study.

Figure 2.11: Phase diagram of POPC, SM and Chol at three representative temperatures. Modified from [38].

Research on the calorimetric characterization of these mixtures using DSC has provided quite conflicting results. On the study of Ausili et al [10], no phase transition was detected for the ternary mixture and the binary mixture showed two broad transitions, one with $T_m$ of -0.2°C, presumably from a POPC-rich phase, and one with $T_m$ of 18°C, attributed to the SM-rich phase. However, the group of Nicolini et al [34] reported for the ternary mixture a broad transition between 5 and 50-65°C with a maximum at about 20°C. Data from complementary techniques attributed the large peak to a transition from the $L_d + L_o$ + solid-ordered phase (s_o) three phase region to $L_d + L_o$, with an overall $L_d$ phase achieved at approximately 55°C. For the binary mixture, Pokorny et al [38] studied several compositions and they estimated, for the equimolar mixture of POPC and brain SM, the existence of two phase transitions, one from s_o to s_o + $L_d$ at approximately 3°C and one from s_o + $L_d$ to $L_d$ expected to finish at about 33°C, which is slightly below the brain SM main transition temperature ($T_m = 38°C$) [10]. The binary phase diagram obtained by DSC is schematized in Figure 2.12. For the ternary mixture, this group did not find any phase transition for equimolar mixtures of brain SM and cholesterol independently of the POPC content.
Figure 2.12: Phase diagram from the binary mixture POPC/brain SM estimated from DSC plots of the dotted compositions. Modified from [38].

2.4 Anesthetics

Anesthesia is defined as the reversible loss of sensory and autonomic nerve function by blocking the transmission of action potentials to the central nervous system [72] and was discovered more than a century ago, but the exact mechanisms of action are still unknown and subject of intense research. There is a considerable variety of molecules capable of inducing anesthesia, but their sites of action and interaction with specific molecules on nerve cells are still object of research [1]. To know if lipid rafts are somehow involved with local or general anesthesia is the main motivation of this work.

The main theories for the molecular mechanism of anesthesia that have been proposed are the direct binding to proteinaceous receptors associated with ion channels and the perturbation of membrane lipids surrounding functional proteins [2]. Lately, the protein hypothesis has been preferred over the lipid hypothesis, because some data suggest that the charged forms of anesthetics are devoid of pharmacological activity due to membrane impermeability. Furthermore, anesthetics are known to interact in vitro with voltage-gated and ligand-gated ion channels, blocking their action [2, 3]. However, evidence for the lipid hypothesis resides on the fact that there is evident correlation between anesthetics’ potency and membrane solubility and fluidization, which has led many authors to hypothesize that pharmacological effects of anesthetics depend on their interactions with biological membranes, at least as an intermediate step to specific binding to a protein [4]. Other evidence is that, since lipid fluidization is reversed by increased pressure and, since pressure increase is well known to reverse anesthesia, this provides some support to the membrane-fluidizing hypothesis [3].

There are essentially two types of anesthesia: local anesthesia and general anesthesia. Local anesthesia involves loss of sensation in a discrete area of the body and does not involve loss of consciousness. On the other hand, general anesthesia involves loss of sensation with loss of consciousness. The causes for the loss of consciousness are still unknown because it is not certain how the brain generates consciousness [1, 3].
2.4.1 Local anesthesia

The first substance to be used as a local anesthetic (LA) was cocaine in 1884. Since then, due to the strong addictive properties of cocaine, other anesthetics have been developed. The first synthetic anesthetic, procaine, was presented in 1905. Due to its versatility, it became the most used local anesthetic, but the search of better anesthetics has continued up to date [1].

Many of the molecules used nowadays for anesthesia are amphiphilic. They have a hydrophobic moiety responsible for binding and diffusion to cell membranes, and a hydrophilic moiety responsible for water solubility and spreading into tissues. Among the compounds able to initiate local anesthesia, tertiary amines ("caine" group) have attracted much attention [1]. The "caine" family anesthetics have an aromatic ring as the hydrophobic region and the amine group as the hydrophilic group. The linkage between the two groups can be achieved by an ester linkage or an amide linkage. Ester - LAs are, for instance, procaine, tetracaine and benzocaine, and amide - LAs are lidocaine, dibucaine and bupivacaine, among others. In this work one representative of the ester group, tetracaine and another of the amide group, lidocaine, will be studied [4].

The effects of local anesthetics are influenced by factors such as hydrophobicity, pKₐ, molecular size and polar interactions. A method to estimate the degree of interaction between local anesthetics and lipid bilayers is based on the partition coefficient (P) in the lipid phase relative to the aqueous phase. Based on these observations, ester - LAs were found to be more potent that amide - LAs. This correlates well with the findings that the former have higher partition coefficients that the latter, so a higher partition into the membrane causes more physiological effects. The pKₐs of these local anesthetics on an aqueous media range from 7.6 to 9.2 so, at physiological pH and also at the pH induced by the N-(2-Hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES) buffer used in this work (pH = 7.4), the anesthetics are predominantly on their positively charged form. Some anesthetic properties, like the ones listed on this paragraph, are shown for the local anesthetics used on this work, as well as for the general anesthetic propofol, in Table 2.1.

Table 2.1: Molecular weight, partition coefficient, pKₐ and water solubility of the studied anesthetics.

<table>
<thead>
<tr>
<th>Anesthetic</th>
<th>Mₐ (g/mol)</th>
<th>log P</th>
<th>pKₐ</th>
<th>Water solubility (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracaine</td>
<td>300.8 [73]</td>
<td>3.73  [74]</td>
<td>8.39 [75]</td>
<td>50 [73]</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>288.81 [76]</td>
<td>2.26  [74]</td>
<td>7.9  [77]</td>
<td>6.8 [77]</td>
</tr>
<tr>
<td>Propofol</td>
<td>178.28 [78]</td>
<td>3.63  [74]</td>
<td>11 [79]</td>
<td>150 x 10⁻⁶ [79]</td>
</tr>
</tbody>
</table>

Local anesthetics act to inhibit sensory stimuli perception by blocking nerve conduction of afferent signals to the brain, inducing direct or indirect modifications on voltage-gated sodium channels, impairing the initiation of action potentials by inhibition of an intracellular domains of Na⁺ channels. Since most of the LAs are positively charged at physiologic pH, they must be converted to the non-ionized form to diffuse across the membrane or incorporated into inverted micelles. The rate of conversion depends on the anesthetics pKₐ and tissue pH [4, 80, 81].

Regarding the administration techniques, they can be performed with injection (infiltration, intravenous, peripheral nerve block and epidural block, among others), or by topical application (creams,
eye drops, suppositories, sprays, patches or other administration vehicles) [72]. These anesthesia-inducing agents are not devoid of complications. They may cause rare allergic reactions (reported for 1% of procedures), being the most common contact dermatitis [4], neurotoxicity at spinal cord nerves by cell membrane disruption [82, 83] and apoptosis [84], as well as side effects on non-neural cells, like cardiac muscle cells [85]. An active field of study is to detect the exact mechanisms for occurrence of side effects and minimize them.

There are several theories to explain how the "caine" anesthetics modify the structure of membranes. One hypothesis is that these anesthetics enter into the membrane leading to its fluidization. Even though the nature of this fluidization is not clear, it seems to be related to either a local expansion of the membrane or the disordering of the phospholipids in the outer monolayer [86].

2.4.1.A Tetracaine

Tetracaine (TTC), an ester local anesthetic (Figure 2.13), is a very useful anesthetic because of its high potency, long acting time and low therapeutic dose [87]. However, in high dosages it may cause nerve damage due to the fact that it is a known surfactant [88]. It is commonly used in its hydrochloride form on a diversity of intravenous and topical formulations [72]. Regarding the pharmacological specialities used in Portugal and the fact that some topical formulations and solutions for injection have a tetracaine concentration between 5 and 10 mg/ml, the working concentration of 7.5 mg/ml was chosen [72, 89]. In this study, the concentrations used were 7.5, 3.75 and 1.5 mg/ml (24.9, 12.5 and 5 mM respectively).

![Chemical structure of tetracaine. Modified from [90].](image)

2.4.1.B Lidocaine

Lidocaine (LDC) is an amide local anesthetic (Figure 2.14), also available as intravenous and topical formulations [72]. However, it has also been reported to have a certain degree of neurotoxicity due to poor mixing with the cerebrospinal fluid [83]. The injectable lidocaine solutions commonly have a large range of concentrations, being the 10 and 20 mg/ml (69.3 and 34.6 mM respectively) two of the most commonly used [89]. For this reason, these are the concentrations used in our work.
2.4.2 General anesthesia

General anesthesia was discovered before local anesthesia, being used for the first time for surgical purposes in 1846. Back then, the patient was put to sleep by breathing diethyl ether. Since then, several drugs with very diverse structures and physical properties have been found to induce general anesthesia. Even though many of these molecules are highly lipophilic and might be able to fluidize the membrane and modify the function of surrounding proteins, other anesthetics that are less lipophilic, such as barbiturates, are able to form hydrogen-bonds and seem to interact directly with proteins [91].

General anesthetics are available as inhalable and intravenous formulations. They affect axon ion conductance by modifying mostly ligand-gated channels on the central nervous system, consequently inhibiting synaptic transmission [3, 85, 92]. In this work, the representative general anesthetic is propofol.

2.4.2.A Propofol

Propofol (PPF) is a phenol with two substitute isopropyl groups (Figure 2.15). It is very lipophilic and, due to its high pKₐ of 11, the tendency to form salts in solution is very low. It is a potent anesthetic and it is popular because of its rapid onset, short duration of action, and relatively reduced side effects. Since its solubility in water is very low, a variety of formulations have been developed to allow its clinical delivery. The most effective and less harmful formulations are based on oil-in-water or lipid based emulsions [79]. The current commercial form of propofol, Diprivan®, is an intravenous formulation with 10 mg/ml of propofol formulated in Intralipid®, which is an emulsion containing soybean oil, egg yolk lecithin and glycerol [72, 79, 93].

Propofol is known to fluidize lipid membranes and reduce their phase transition temperature [18, 93]. However, the GABA_A receptor is recognized as a target for propofol action. Despite this ob-
servation, the lipid interaction theory for the propofol mechanism of action is not completely discarded [93]. Bahri et al [93] studied the effect of PPF on model, erythrocyte and nerve cell membranes. They found a pronounced decrease in microviscosity of erythrocyte membranes for PPF concentrations higher than 1 mM, and eventually partial hemolysis of their membranes. Taking into consideration that erythrocyte membranes contain lipid rafts and these are structures with higher rigidity, we decided to use the concentration of 1 mM (0.18 mg/ml) in our work. In our laboratory, the solubilization of propofol was achieved in dimethyl sulfoxide (DMSO) at a volume that yielded a final concentration of 0.5% (v/v) in HEPES buffer. This concentration of DMSO was used because for higher concentrations there is evidence of modification of lipid bilayer structure [2].

2.5 Experimental techniques

In this section, the principles behind the experimental techniques used are described.

2.5.1 Dynamic Light Scattering (DLS)

Dynamic Light Scattering, also known as Photon Correlation Microscopy, is one of the most popular techniques for particle size determination. This is based on the fact that a monochromatic beam of light, when crossing a solution with particles undergoing Brownian movement, suffers a frequency shift due to the interaction with the moving particles that act as scatterers, and the wavelength of the scattered light will be different than the incident one. The size distribution of sphere-like particles is determined by measuring the diffusion coefficient of the particle and the autocorrelation function [94, 95]. This method implies that the particles undergo Brownian motion, so that the particles’ movement can be described by a probability density function (Equation 2.1) [94, 95]:

$$P(r,t|0,0) = (4\pi D t)^{-3/2} \exp \left( -r^2 / 4Dt \right)$$

(2.1)

$D$ is the diffusion coefficient, which is related to the velocity of the particles, and their velocity depends on the temperature, viscosity and size of the particles assuming that they are spheres with a small diameter so that the Stokes-Einstein equation (Equation 2.2) holds [94, 95]:

$$D = \frac{k_B T}{6\pi \eta a}$$

(2.2)

Here, $a$ is the radius of the sphere, $k_B$ the Boltzmann constant, $T$ the temperature of the experiment and $\eta$ the viscosity of the sample. Since from the light scattering the position of the particles can be obtained, through these formulas it is straightforward to obtain the radius of the spheres.

The autocorrelation function is obtained from the recorded intensity ($I$) as follows in Equation 2.3 [95]:

$$g^2(q; t) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

(2.3)

$g^2$ is the second order autocorrelation for a particular wave vector, $q$, and time $t$. The correlation is high for short time delays, which means that the particles do not move a great extent. For longer
times, the correlation decays exponentially. The correlation is therefore related to the probability density function of the moving particles and their diffusion coefficient, and in this fashion the frequency counts of the particles with a certain radius can be inferred using numerical methods [95].

2.5.2 Quartz crystal microbalance with dissipation (QCM-D)

The quartz crystal microbalance with dissipation (QCM-D) is a simple and highly sensitive analytical tool for the analysis of the mass and mechanical properties of adsorbed biomolecules and cells [96, 97]. Its added value for the analysis of biological matter resides on the fact that it allows measurements in fluids, as well as the measurement of the properties of thin films by mechanical displacement associated with an acoustic wave sensitive to the properties of the material [66, 97]. This tool has successfully been used for measurements and determination of mechanical properties of adsorbed proteins, lipids, nucleic acids, polymers and cells [68].

The core component of a quartz-crystal microbalance is the AT-cut quartz plate with fundamental resonance frequencies in the range of 5-30 MHz [66]. For this work, the crystals used have a resonance frequency of 4.95 MHz and the third, fifth, seventh and ninth overtone of this frequency were also excited to obtain a more complete mechanical description of the adsorbed film, as well as to know the depth of the interfacial phenomena, since the different overtones have different depths of penetration, with the lowest-number overtones having a higher depth of penetration and being sensitive to phenomena farther from the crystal surface [98] (Figure 2.16).

QCM-D measures changes in the crystal resonance frequency and the dissipation of energy of shear oscillations. The frequency change is related to the adsorbed mass on the crystals, while the dissipation factor is related to the acoustic energy loss by the adsorbed film [66, 99]. The operation principle of the quartz crystal microbalance with dissipation is based on the fact that the quartz crystals are piezoelectric, that is, they will deform mechanically when exposed to an oscillating electrical field, and an acoustic shear wave will propagate across the film [66, 97, 99]. The changes in velocity are associated with a frequency change, while the loss of amplitude of the wave is related to the

Figure 2.16: Depth of penetration of several overtones of the fundamental resonance frequency of the quartz crystals. Modified from [98].
To measure these values, the driving power to the crystals is switched off, the voltage over the crystal decays and a damped oscillation signal will be recorded. The frequency shift, $\Delta f$, is recorded immediately before switching off the power, while the dissipation shift, $\Delta D$, is recorded right after $[66, 101]$. This procedure is repeated every second, so the shifts are virtually obtained in real time $[96, 97]$. The dissipation parameter is defined as follows in Equation 2.4:

$$D = \frac{E_{\text{dis}}}{2\pi E_{\text{st}}}$$ (2.4)

In Equation 2.4, $E_{\text{dis}}$ is the energy dissipated in one oscillation period and $E_{\text{st}}$ is the energy stored by the film during this oscillation $[102]$. Ideal rigid layers, fully coupled to the crystal oscillation and homogeneously distributed over the resonator surface, behave like an elastic film, the change in velocity and frequency is proportional to the adsorbed mass, and the dissipation factor is negligible $[97, 99, 103]$. For this assumption to be valid, the dissipation values should vary less than $1 \times 10^{-6}$ for each $5$ Hz of variation in frequency $[102]$. In 1959, Sauerbrey proposed a relationship between the frequency shift and the changes in adsorbed mass ($\Delta m$), the so-called Sauerbrey equation (Equation 2.5):

$$\Delta f = -N \frac{\Delta m}{C_f}$$ (2.5)

$C_f$ is the mass sensitivity constant ($17.7$ ng/cm$^2$ at $f = 5$ MHz) and $N$ the overtone number $[103]$. However, soft or viscoelastic films are not fully coupled with the crystal oscillation and they will have a dissipative behavior. So, the Sauerbrey equation will underestimate the adsorbed mass $[97, 99, 103, 104]$. This is the case for films of lipid vesicles $[103]$. For a complete description of the properties of...
viscoelastic films, a set of frequency overtones and their energy losses have to be taken into account \[103, 104\].

The parameters that allow the characterization of the viscoelastic film are the film thickness (d), complex shear modulus (G), shear viscosity (\(\eta\)) and film density (\(\rho\)). In our case, the film density is assumed to be constant throughout the experiments. For viscoelastic solids or films, the appropriated fittings are provided by the Voigt model, and for viscoelastic fluids, the model used is the Maxwell model \[104\]. The model used in the analysis carried on this work is the Voigt model because we are analyzing an adsorbed film. The equations that relate the frequency and dissipation shifts to the viscoelastic parameters using a continuum mechanics approach were developed by Voinova et al \[105\] used by the modeling software (QTools). From the fittings of the frequency and dissipation shifts the closest values of the viscoelastic parameters are extracted, assuming a single adsorbed layer and fixed film density, as well as constant fluid viscosity and density \[96, 105\].

### 2.5.3 Differential Scanning Calorimetry (DSC)

The differential scanning calorimetry allows the thermotropic characterization of the samples. It has been the standard technique for studying phase transitions of lipidic systems, as well as the interaction between lipid bilayer systems and other molecules \[33, 35, 106\]. It consists on the recording of the differential heat capacity, \(C_p\), between the content of the sample cell and the content of the reference cell over a range of temperatures at a given scanning rate. The main parameter extracted from the scans is the main transition temperature, \(T_m\), of the samples, corresponding to the mid-temperature of the phase transition peak. The enthalpy of transition, \(\Delta H\), is also determinable if a proper baseline can be traced between the onset and endpoint of transition. The scans are performed in both heating and cooling directions, which allows for evaluation of reversibility of the thermal behavior of the samples, as well as to avoid the loss of data at the extremes of the temperature range due to the time required to reach a steady state on the beginning of each scan \[33\]. An example of a thermogram is shown in Figure 2.18.

![Thermogram Example](image)

**Figure 2.18:** Example of a typical thermogram and the parameters extracted. Modified from \[107\].
2.5.4 Phosphorus Nuclear Magnetic Resonance

Phosphorus nuclear magnetic resonance (P-NMR) is an excellent method to study biological and model membranes. Because of the high natural abundance of the $^{31}$P isotope and its high gyromagnetic ratio, $^{31}$P-NMR is especially suited for the study of phospholipids [48, 108–111]. The linewidth and lineshape of the $^{31}$P-NMR spectra are sensitive to the motion of the phospholipid headgroup and to its orientation relative to the applied magnetic field [48, 108–113]. Comparatively to other methods, $^{31}$P-NMR is a very simple method for the study of liposomes because it is a non-invasive method, and since it is sensitive to headgroup dynamics, it provides information about the local motions characteristic of the phases present in lipid membranes [48].

The typical lineshape for lamellar vesicles arises from the phosphorus headgroup fast rotational motion about the bilayer normal, which causes the chemical shift tensor (measured in parts per million (ppm) from an external reference of phosphoric acid, with the high-fields corresponding to negative chemical shifts) to be averaged to an axially symmetric tensor with an anisotropic component defined as the chemical shift anisotropy (CSA) [108, 110, 114]:

\[
\Delta \sigma = \sigma_\parallel - \sigma_\perp
\]  

In Figure 2.6 $\Delta \sigma$ is the CSA, being the difference between the shift of the liposomes with their normals parallel ($\sigma_\parallel$) or perpendicular ($\sigma_\perp$) to the direction of the applied magnetic field [108, 110, 114]. $\sigma_\parallel$ represents the low-field shoulder while $\sigma_\perp$ represents the high-field shoulder [110]. The linewidths of these spectra, correspondent to the CSA, are conveniently characterized as the width at half height and are related to the allowed motion in the phosphate group, with more restricted motions associated with larger linewidths [115].

The characteristic lamellar lipid phase has a lineshape comprising a high-field peak and a low-field shoulder, however other characteristic spectra may arise from other lipid phases. The hexagonal phase has a spectrum with a reversed asymmetry and linewidth narrower by about one half relatively to the lamellar spectrum, and small bilayer vesicles, micelles, inverted micelles, cubic or rhombic phases lead to narrow symmetric signals that are indicative of isotropic motion [41, 81, 108, 111]. However, these different lineshapes may arise even if the lipids still retain the lamellar phase as a result of headgroup environments that replicate those typical of other phases with higher degree of symmetry (Figure 2.19) [48].
Even though it might be hard to conceive that some of the lipids that incorporate biological membranes prefer a non-bilayer configuration upon hydration at physiological temperatures, this property is fundamental for processes like vesicle fusion, flip-flop of lipids and transport of molecules across the membrane. For instance, one hypothesis for transport of anesthetics across the membrane states that the anesthetics form hydrogen bonds with the polar headgroups of the membrane lipids and promote the formation of inverted micelles to carry it through the bilayer [81].

Well defined spectra like the ones from Figure 2.19 are obtained for one-component systems. However, for more complex mixtures or biological membranes, the spectra are generally complex, with higher amplitude of the low-field shoulders and often lack definition of the tensors due to superposition of spectra due to the different lipids present and poorer signal-to-noise ratio, as well as the possibility of remarkable isotropic components due to more mobile phosphate compounds. With this feature, the quantitative determination of the CSA for these spectra is difficult [116]. A spectrum obtained from a biological membrane is shown in Figure 2.20.

Figure 2.19: $^{31}$P-NMR spectra typical from representative lipid phases. Modified from [110].

Figure 2.20: $^{31}$P-NMR spectra of a biological membrane. Modified from [116].
3 Materials and Methods

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3.1 Materials

The lipids sphingomyelin (brain SM, porcine) (reference 860062P) and 1-palmitoyl-2-oleoyl-snglycero-3-phosphocholine (POPC) (reference 850457P) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), while cholesterol (reference C8667) was obtained from Sigma - Aldrich, Co. (St. Louis, MO, USA). The anesthetics tetracaine hydrochloride (reference T7508), lidocaine hydrochloride monohydrate (reference L5647) and 2,6-diisopropylphenol (propofol) (reference D126608) were obtained from Sigma - Aldrich.

Other chemicals relevant to this work, namely N-(2-Hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES) hemisodium salt (reference H7637), chloroform (reference 528730), sodium dodecyl sulfate (SDS) (reference L3771), phosphorus standard solution (0.65 mM phosphorus, reference P3869), L-ascorbic acid (reference A5960), ammonium molibdate tetrahydrate (reference 09878) and dimethyl sulfoxide (DMSO) (reference D8779) were supplied by Sigma - Aldrich. Sodium chloride (reference 106404), methanol (reference 6009) and Extran® MA 01 (reference 107555) were obtained from Merck KGaA (Darmstadt, Germany). Hydrogen peroxide 30% solution (reference 121076.1211) was supplied from Panreac Quimica SA (Barcelona, Spain). Hellmanex® II (reference 320.002) was purchased from Hellma Gmbh & Co KG (Mühlheim, Germany).

For all the solutions, Milli-Q water was used. HEPES buffer solution was prepared with a final concentration of 10 mM of HEPES, 0.1 M of NaCl and pH of 7.4. To avoid contamination by dust and other small particles, the solution was filtered using a BD PlastipakTM (Franklin Lakes, NJ, USA) syringe and 0.45 μm GHP Acrodisc® syringe filters from Pall Life Sciences (Ann Arbor, MI, USA). All the solutions of tetracaine (7.5, 3.75 and 1.5 mg/ml) and lidocaine (20 and 10 mg/ml) were prepared by dissolving the adequate amounts of anesthetic in HEPES buffer. Propofol was first dissolved in DMSO and then this solution was dissolved in HEPES for a final volumetric concentration of DMSO of 0.5%. A control solution of HEPES with 0.5% DMSO was also prepared for the experiments with propofol.

The gold-coated quartz crystals (AT - cut, 4.95 MHz, 14 mm diameter) for QCM-D experiments were obtained from Q-Sense AB (Gothenburg, Sweden).

The extrusion device for liposome preparation was home-made. Polycarbonate filters of 600, 200 and 100 nm of diameter from Nuclepore, Whatman (Kent, United Kingdom) were used.

3.2 Preparation of the liposomes

The preparation of the liposomes was performed respecting the protocol provided by Avanti Polar Lipids, Inc. [117].

The amount of lipids necessary for a final concentration of 3.195 mM (50 mM for P-NMR experiments) in lipids were solubilized on a mixture of chloroform and methanol (4:1) and put on a round-bottom flask. The solvent was evaporated with nitrogen and the resulting lipid film was kept under vacuum for more than 3 hours to evaporate all the traces of the solvent.

The film was hydrated thereafter with HEPES buffer and kept inside a thermostatized water bath at...
65°C for 1 hour, interchanging heating with manual and vortex agitation for a complete dissolution of the lipid film. The bath was kept at 65°C because it is a temperature 10°C above the upper limit of the lipid raft mixture transition [34], and because several studies reported a temperature of extrusion of 65-70°C for the POPC/SM 1:1 liposomes, based on the fact that the sphingomyelin phase transition ranges from 37 to 55°C, depending on the molecular species present on the sample [49].

After this procedure, the resulting large multilamellar vesicles (LMVs) solution was submitted to five freeze-thaw cycles with liquid nitrogen and in the water bath at the same temperature referred above to disrupt LMVs.

Finally, large unilamellar vesicles (LUVs) were obtained by extrusion of the solution, passing the sample 20 times through polycarbonate filters with pore diameters of 600, 200 and 100 nm sequentially. The number of passes through each membrane and the N₂ pressure used are shown in Table 3.1. This procedure will typically result in LUVs with a size distribution of 120-140 nm.

<table>
<thead>
<tr>
<th>Diameter of pores (nm)</th>
<th>Number of passes</th>
<th>N₂ pressure (bar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>600</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>200</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>12</td>
</tr>
</tbody>
</table>

The liposome solutions were kept at 4°C and were used preferentially within 7 days. This short duration time derives from the fact that lipids containing ester-linked hydrocarbon chains undergo spontaneous hydrolysis, and POPC has two ester-linked hydrocarbon chains [118].

### 3.3 DLS

The experiments for size determination were performed on a Brookhaven BI-200SM Goniometer Light Scattering System. The detection range of the instrument is from 2 nm to the micrometer range. The device is equipped with a BI-9000AT correlator, a He-Ne laser (632.8 nm, 35 mW, model 127, Spectra Physics) and a avalanche photodiode detector. The setup was thermostatted to 25°C. The samples used were liposome solutions of both compositions with a concentration of 1.12 mM. The reference cell was filled with HEPES buffer and the sample cell was filled with HEPES buffer and a small amount of the liposome solution. The measurements were repeated at least five times. Numerical modeling of the particle sizes to fit the autocorrelation function was performed using an inverted Laplace transform known as the CONTIN method. The resulting scatter plots of intensity vs. size were Gaussian-fitted to identify the peak and error of the population distributions.

### 3.4 Determination of the phosphorus content of the samples

To determine the phosphorus concentration of the samples, the following protocol [119] was used:

- The volume of the 3.195 mM lipid concentration solution equivalent to 0.100 µmol in phosphorus (47 µl from POPC/SM/Chol mixture and 31 µl from POPC/SM mixture) was placed on a Pyrex®
tube and evaporated with N₂ to eliminate the solvent. For each batch, three samples were obtained. Six test tubes were prepared with increasing content in phosphorus.

- The tubes (reference and samples) were filled with 0.45 ml of a H₂SO₄, 8.9 M.
- The tubes were heated to 200°C with an oil bath during 25 minutes, then cooled to room temperature, and filled with 0.15 ml of hydrogen peroxide 30%.
- After another heating period for 30 minutes at 200°C, all the tubes were cooled to room temperature.
- The tubes were filled with 3.9 ml Milli-Q water, 0.5 ml of ammonium molibdate tetrahydrate solution were submitted to by vortexing for 5 times, followed by addition of 0.5 ml ascorbic acid 10% solution again with vortexing for 5 times.
- Finally, they were heated to 100°C for 7 minutes.

The determination of the amount of phosphorus on each sample was made using a DU®-70 UV-Vis spectrophotometer from Beckman Coulter Inc. (Brea, CA, USA) and detecting the absorbance value for the wavelength of 820 nm.

### 3.5 QCM-D

The device used in this work was a Q-Sense E4 (Q-Sense AB, Gothenburg, Sweden), fitted with a 4-sensor chamber and connected to a flow pump (Ismatec IPC-N 4). Before every experiment, the gold-coated quartz crystals were submitted to two cycles of UV-Ozone exposition performed on a PSD Series UVOzone Cleaning & Sterilization device from Novascan (Ames, IA, USA) during 10 minutes, intercalated by washing with Milli-Q water and blow-drying with nitrogen. This procedure has the purpose of removing contaminations on the crystal surface and rendering it more hydrophilic.

The frequency and dissipation changes for the fundamental resonance frequency and up to the 9th overtone were monitored using QSoft 401 software. The frequency shifts for each overtone are normalized with respect to the overtone number. In the beginning of each experiment, it was checked that the crystals’ dissipation for the fundamental frequency in air was close to the expected value of 35 x 10⁻⁶. The baseline for all experiments was established with HEPES buffer at a steady flow of 0.100 ml/min, flowing for approximately 7 minutes, followed by 5 minutes of stabilization. It was checked that the dissipation was within 20% of deviation of the nominal dissipation for the fundamental frequency value for aqueous media, 350 x 10⁻⁶.

For all the anesthetics and different concentrations, the experiments were performed at 37°C, and additional experiments at 25°C were performed for the highest concentrations of each anesthetic. The flow rate for every experiment was also 0.100 ml/min. For every experiment, after the period of baseline stabilization, an influx of approximately 2 ml of the raftlike liposome solution and 2.8 ml of the POPC/SM liposome solution was performed. The samples had a concentration of 1.12 mM in lipids. After stabilization of the normalized frequency and dissipation shifts, a 10 minute rinsing with HEPES,
or HEPES + 0.5% DMSO for the propofol experiments was performed to eliminate the loosely bound liposomes and to obtain a single layer of irreversibly adsorbed liposomes. After stabilization, 2 ml of the anesthetic solution flowed into the system, and a final rising was performed after to eliminate the loosely bound particles (whether anesthetics or disrupted liposomes). For each experiment, the frequency and dissipation shifts for the 3rd to the 9th overtones were collected at relevant data points and the mean ± standard deviation (S.D.) of the frequency and dissipation shifts of different experiments of the same liposome composition, temperature and anesthetic were calculated. The data from the fundamental resonance frequency generally resulted in poor signals. After every experiment, the balance was first rinsed, still with the crystals inside, with Milli-Q water and then air, followed by a more aggressive cleaning with old crystals with influx of Hellmanex 2% solution, followed by rinsing with Milli-Q water and air. The crystals used in the experiments were submitted to 5-minute washing in the ultrasonic cleaner with a solution of sodium dodecyl sulfate (SLS) in Milli-Q water, rinsed with Milli-Q water and blow-drying with nitrogen.

All the crystals used in the experiments were checked for impedance to ensure that they were well-suited for adsorption. The impedance measurements were performed on a KSV QCM-Z500 device. Before every experiment, the balance parts were cleaned with ethanol 96% and the balance was calibrated. Crystals with impedance below 30 Ω were considered suited for experimental purposes.

For viscoelastic modeling, the recorded data were fitted on QTools 3 to the Voigt model considering a film with a single layer and using the frequency and dissipation shifts from the 3rd to the 9th overtones. The viscosity and density of the fluid were considered the same as the values for water (0.001 Pa.s and 1000 Kg/m³, respectively), and the density of the film was set as 1060 Kg/m³, as stated by Viitala et al [103]. The boundary conditions that resulted on minimization of the Chi-Square error and appropriated values for the estimated parameters are referred on Table 3.2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Min. value</th>
<th>Max. value</th>
<th>Steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (Pa.s)</td>
<td>0.0005</td>
<td>0.01</td>
<td>50</td>
</tr>
<tr>
<td>Shear Modulus (Pa)</td>
<td>1000</td>
<td>1E9</td>
<td>50</td>
</tr>
<tr>
<td>Thickness (m)</td>
<td>1E-10</td>
<td>1E-6</td>
<td>50</td>
</tr>
</tbody>
</table>

The best fits for each concentration were chosen regarding the minimum Chi-Square values, as well as the outputs that make physical sense. The relevant points were collected and averaged, and displayed as mean ± S.D. values.

### 3.6 DSC

The measurements were carried out in a VP-DSC Micro-Calorimeter from MicroCal® (Northampton, WA). Both cells have a volume of 0.5105 ml and are pressurized to 26 psi. The scans were performed in a temperature range from 10 to 50°C at a scanning rate of 60°C/h, both in heating and cooling directions. Before each scan, the samples underwent a period of pre-scan thermostatization of 15 minutes. For each sample, at least three heating/cooling cycles were performed as a check for
thermodynamic stabilization and reproducibility.

The reference cell was filled with HEPES for the tetracaine and lidocaine measurements and with HEPES + 0.5% DMSO for the propofol measurements. Before the measurements, reference scans were performed with the sample cell filled with buffer. The samples were prepared right before injection by diluting the appropriate amount of the liposome solution with the buffer for experiments without anesthetic or with the proper anesthetic concentrations, for a final nominal lipid concentration of 1.12 mM and the working anesthetic concentrations. Since liposomes are too sensitive for degassing and are disrupted upon this procedure, only the buffers for dilution and the anesthetic solutions were degassed in order to minimize the occurrence of bubbles inside the cells.

After the experiments with each lipid mixture/anesthetic pair, the device was cleaned by two cycles of passing 200 ml of Milli-Q water through the reference cell and 50 ml of methanol + 400 ml of Milli-Q water on the sample cell.

The thermograms (heat capacity, \( C_p \), versus temperature) were analyzed using the software Origin 7.0 (OriginLab corporation, Northampton, WA). After subtraction of the reference scan, the samples scans were normalized with respect to the exact phosphorus concentration of the samples (determined as described in Section 3.4). It was not possible to draw a realistic baseline for enthalpy calculations because the onset of the transitions was lower than 10°C and the device did not allow experiments at lower temperatures. Therefore, the presented results will be the subtracted and normalized scans and the temperature of the maximum of the transition will be the only parameter extracted.

3.7 Phosphorus Nuclear Magnetic Resonance

\(^{31}\text{P}-\text{NMR} \) spectra were obtained using a Bruker Avance II+ 500 MHz (UltraShield Plus Magnet) (Karlsruhe, Germany) operating at a magnetic field of 11.746 T, a \(^{31}\text{P} \) frequency of 202.457 MHz and a BBO probe. All the chemical shift values are quoted in parts per million (ppm) with reference to phosphoric acid 85%, with positive values associated to low-field shifts.

All spectra were obtained with gated broadband proton decoupling. The spectral width was of 80 KHz (400 ppm), interpulse time was 2s and a 30° radiofrequency pulse (11.3 µs) was applied. The spectra were submitted to exponential multiplication before Fourier transformation, resulting in a 50 Hz line broadening to improve the signal-to-noise ratio. The accumulation time for each experiment was 40 minutes.

The liposomes used had a final lipid concentration of 50 mM and, for each experiment, 360 µl of the liposome solution were mixed with 40 µl of the anesthetic solution ten times more concentrated, so that it results on a final concentration equal to the ones used in QCM-D and DSC. All the samples were tested at 12, 25, 37 and 60°C. The studies of the effect of the anesthetic concentration on the raftlike samples were performed at 37°C.

The spectra were processed on a personal computer running the TopSpin 2.1 software (Bruker Biospin GmbH, Germany) under Linux Red Hat 4.
# Results and Discussion

<table>
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4.1 DLS

The results of the dynamic scattering analysis of the samples of POPC/SM/Chol and POPC/SM liposomes are depicted in Table 4.1.

**Table 4.1:** Mean ± S.D. values of liposome sizes for the two compositions obtained from DLS.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC/SM/Chol 1:1:1</td>
<td>136 ± 23</td>
</tr>
<tr>
<td>POPC/SM 1:1</td>
<td>112 ± 25</td>
</tr>
</tbody>
</table>

The data shown have a considerable standard deviation. However, their average values are within expected from the extrusion method with 100 nm polycarbonate filters. These results are in good agreement with findings that the incorporation of cholesterol into lipid bilayers increases the size of phosphatidylcholine liposomes [120].

4.2 QCM-D

4.2.1 Adsorption of intact liposomes

First of all, it is important to check if the liposomes adsorbed intact on oxidized gold as expected. An example of the verified frequency and dissipation shifts for the 3rd overtone are shown in Figure 4.1.

The mean ± S.D. of adsorption of single layers of liposomes is shown, for both liposome compositions and experimental temperatures, on Figure 4.2. We chose to represent the 3rd overtones of frequency and dissipation shifts because they provided very good results, without significant noisy contributions from the external oscillations, and these are the overtones with the highest depth of penetration, that is, sensing farther from the crystal surface. This allowed to gain an overview of the characteristics of the adsorbed layer.

![Figure 4.1](image_url)

**Figure 4.1:** Example of frequency (blue) and dissipation (red) shifts for the 3rd overtone for a typical liposome adsorption experiment. The experiment shown was performed at 37°C with adsorption of POPC/SM/Chol 1:1:1 liposomes.

The adsorption behavior exemplified on Figure 4.1 was verified for the two compositions and temperatures. There is a considerable negative frequency shift and stabilization, and there is also a
considerable positive shift in dissipation. These observations are compatible with the formation of a film of liposomes on the oxidized gold surface since these values are of the same order of magnitude of the ones found in other studies that proved intact lipid adsorption on gold-coated crystals by complementary imaging techniques [6, 7, 65, 67, 69]. The shifts due to rinsing with buffer are variable between experiments but the final values for frequency and dissipation shifts remain quite high, being consistent with the existence of an irreversibly adsorbed liposome layer on the gold-coated surfaces. The high dissipation shift is characteristic of a viscoelastic layer, confirming the need of modeling the results with a viscoelastic model in opposition to the Sauerbrey model for rigid elastic films [97, 99, 103, 104].

Liposomes do not behave like rigid spheres and it is known that, upon adsorption on the gold surface, they flatten [103]. These data will be quantitatively described upon viscoelastic modeling and film thickness extraction, so the final thickness of the layer will be lower than the average diameter of the liposomes determined by DLS (Table 4.1). The less rigid liposomes are expected to flatten more, and this explains why the POPC/SM liposomes have a much lower average frequency shift than the POPC/SM/Chol liposomes at 37°C (Figure 4.2, left). In fact, the lipid membranes of the binary composition are, at this temperature, almost completely in a liquid-disordered phase and experience higher deformation of liposomes [38]. The area occupied by each flattened liposome on the surface is higher, and less liposomes can be adsorbed upon a single layer formation that in the case of less deformed liposomes. Regarding these phenomena, the adsorbed mass is lower for the binary liposomes, and this translates to a less negative frequency shift.

Comparing the adsorption at different temperatures, for the raftlike composition the adsorption behavior at 25 and 37°C is similar, both in terms of frequency and dissipation, with slightly higher frequency shifts at 25°C and higher dissipation shifts at 37°C. These observations are consistent with the reported phase behavior [34, 38]. At 25°C, the POPC/SM/Chol liposomes are reported to be in a L_o + L_d coexistence region, as well as at 37°C. However, at 25°C, the proportion of the L_o phase is higher, so the liposomes will be more rigid at this temperature and will deform less. This implies that the adsorbed mass density will be higher at 25°C. In contrast, the dissipation shift is slightly higher at 37°C as a result of reduced rigidity of the membranes, implying a more viscoelastic behavior.

For the binary mixture, there are marked differences on adsorption at the two temperatures stud-
ied, that show good correlation with the phase behavior reported for POPC/SM liposomes at 25 and 37°C, with coexistence of L₀ and Lᵈ domains. The liposomes at 25°C will be more rigid, and the adsorbed mass in a QCM-D experiment is higher, as expected, than at 37°C.

4.2.2 Interactions of adsorbed liposomes with tetracaine

4.2.2.A Effect of cholesterol

To assess the effect of a clinically administrated concentration (7.5 mg/ml) of tetracaine at 37°C on raftlike liposomes and the liposomes without cholesterol, a comparison of frequency and dissipation shifts, as well as of the modeled viscoelastic parameters, will be performed. First of all, examples of the frequency and dissipation variations with time will be shown for all the studied overtones on Figure 4.3 and Figure 4.4.

![Figure 4.3](image1)

**Figure 4.3:** Example of a QCM-D experiment of interaction of adsorbed POPC/SM/Chol liposomes with tetracaine 7.5 mg/ml at 37°C. Left: frequency shifts. Right: dissipation shifts. (1) 5-min liposome introduction; (2) 10-min rinsing; (3) 5-min tetracaine introduction; (4) 10-min rinsing.

![Figure 4.4](image2)

**Figure 4.4:** Example of a QCM-D experiment of interaction of adsorbed POPC/SM liposomes with tetracaine 7.5 mg/ml at 37°C. Left: frequency shifts. Right: dissipation shifts. (1) 5-min liposome introduction; (2) 10-min rinsing; (3) 5-min tetracaine introduction; (4) 10-min rinsing.

When tetracaine was introduced on the system with adsorbed raftlike liposomes, there was an initial sharp negative frequency shift, followed by a slow increase of frequency with time (Figure 4.3). This behavior was registered for all the frequency overtones. For the dissipation shifts, there was a...
marked increase upon tetracaine addition. For the 5th and higher overtones there is evidence of an initial sharp dissipation increase, followed by slow decrease. The peaks were observed on 75% of the experiments at this concentration, while the other 25% did not have a peak neither in frequency or dissipation shifts for the evaluated overtones. When interacting with adsorbed POPC/SM liposomes (Figure 4.4), it led to a fast frequency decrease and dissipation increase that remained approximately constant over the steady-state period.

**Figure 4.5:** Mean ± S.D. values for the frequency (left) and dissipation (right) shifts in the different overtones for the interaction of adsorbed POPC/SM/Chol (top) and POPC/SM (bottom) liposomes with tetracaine 7.5 mg/ml at 37°C.

The Figure 4.5 show the mean ± S.D. values obtained from several measurements of the interaction between POPC/SM/Chol liposomes and tetracaine 7.5 mg/ml at 37°C. On the initial step of interaction with tetracaine, there is a significant drop in the frequency shift, especially for the 3rd overtone, and also a positive shift in the dissipation values (15.5 x 10^-6 for the 3rd overtone). The increase in dissipation means that the film becomes more dissipative and viscous.

For the stabilization period of tetracaine (about 1h for all the experiments), the frequency undergoes a slow increase, as seen in Figure 4.3. Since, in average, for the 5th overtone and higher, the final frequency is higher than the one before tetracaine introduction, the possibility of partial rupture of smaller liposomes arises. However, for this hypothesis to be significant, one should notice an inverse pattern in dissipation for all overtones. For the 3rd overtone there is no decrease of dissipation between the tetracaine introduction and the end of the steady-state stabilization period, but only for the 5th and higher overtones. These observations for the higher overtones are quite consistent with
the hypothesis of partial rupture of smaller liposomes (also verified by Cho et al. [121] on their studies on POPC membrane rupture by the AH peptide). The modeling of the viscoelastic properties shown in Table 4.2 will provide more insight on these phenomena.

### Table 4.2: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM/Chol liposomes interacting with tetracaine 7.5 mg/ml at 37°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
<th>HEPES</th>
<th>TTC (initial)</th>
<th>TTC (final)</th>
<th>HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.15 ± 0.08</td>
<td>2.0 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Shear modulus (kPa)</td>
<td>9 ± 2</td>
<td>5.98 ± 0.06</td>
<td>4 ± 1</td>
<td>9 ± 3</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>117 ± 7</td>
<td>111 ± 8</td>
<td>141 ± 11</td>
<td>160 ± 22</td>
<td>138 ± 12</td>
</tr>
</tbody>
</table>

### Table 4.3: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM liposomes interacting with tetracaine 7.5 mg/ml at 37°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
<th>HEPES</th>
<th>TTC (initial)</th>
<th>TTC (final)</th>
<th>HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.2 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>2.16 ± 0.05</td>
<td>2.12 ± 0.05</td>
<td>1.90 ± 0.09</td>
</tr>
<tr>
<td>Shear modulus (kPa)</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>75 ± 5</td>
<td>76 ± 2</td>
<td>83 ± 5</td>
<td>86 ± 4</td>
<td>72 ± 8</td>
</tr>
</tbody>
</table>

The thickness of the layer increases initially with the addition of tetracaine, and increases with time. This is somewhat conflicting with the assumption of rupture of some liposomes. Our explanation is that, for all the liposomes, tetracaine initially induces swelling of liposomes by interacting with the polar groups of the phospholipids, since it is in its charged form. This change of headgroup environment will also increase the disordering of the hydrocarbon chains, so the lipids undergo higher electrostatic repulsions and the global volume of the liposomes will increase, resulting in higher thickness. Since the lipids become more disordered, the liposomes become less rigid and more deformable, and this is confirmed by the initial decrease of the shear modulus.

For the smaller liposomes, the increase of line tension and osmotic pressure will cause them to rupture and form supported lipid bilayer domains. For the larger liposomes, the line tension is not sufficient for rupture, so they continue swelling. However, the thickness of the swelled liposomes will dominate the ruptured portion, so the thickness curve does not provide any evident information about the ruptured domains, as it seems that the film, has a whole, has higher thickness after 1h of stabilization. On the end of tetracaine effect, the shear modulus has increased markedly, which is consistent with the formation of more rigid and elastic domains on the surface. The fluctuations on the modeled viscosity values are also a result of partial rupture, and there is a final slight decrease in viscosity relative to the values prior to tetracaine introduction.

After rinsing, the frequency shift undergoes further increase, as well as further decrease of the dissipation shift for all the overtones. This might be due to elimination of loosely bound rupture fragments.

For the binary mixture, the most important thing to state is that there is no evidence of rupture of liposomes. The dissipation increases with tetracaine addition and remains almost constant throughout the stabilization period of tetracaine. For the frequency shift there is not a pronounced peak for the third overtone, only for the higher ones and the frequency increase upon stabilization is not very significant.
The viscoelastic modeling shown in Table 4.3 supports this hypothesis, because there is tetracaine-induced swelling with a slight variation of thickness throughout the tetracaine stabilization. The slight increase in viscosity is stable over the tetracaine stabilization period and may be related to the increase of the adhesion force between the liposomes and the crystal, as well as an increased internal packing of the liposome film. Unfortunately, we could not obtain reasonable values for the shear modulus (shown as not evaluable (N.E.) so we cannot compare the rigidity of the two liposomes after tetracaine addition.

The main conclusions about tetracaine action in both systems are that there is partial rupture of smaller POPC/SM/Chol liposomes at 37°C, but this does not apply to the POPC/SM. Tetracaine induces swelling in both systems, and the swelling is more significant in the raftlike liposomes, consistent with fluidization of lipids, being specially perturbing when the system has cholesterol. Since, at 37°C, the POPC/SM liposomes are almost completely in a liquid-disordered phase [38], and for the raftlike system there are still cholesterol-sphingomyelin liquid-ordered domains at this temperature [38, 43, 122], it is likely that tetracaine interacts preferentially with raftlike liquid-ordered domains.

4.2.2.B Effect of temperature

![Graphs showing frequency and dissipation shifts](image)

**Figure 4.6:** Mean ± S.D. values for the frequency (left) and dissipation (right) shifts in the different overtones for the interaction of adsorbed POPC/SM/Chol (top) and POPC/SM (bottom) liposomes with tetracaine 7.5 mg/ml at 25°C.

For the raftlike liposomes at 25°C interacting with a tetracaine concentration of 7.5 mg/ml (Figure
the predominant behavior is the frequency decrease (average of 53 Hz for the 3rd overtone) with tetracaine adsorption and stabilization for the 3rd overtone, and with a small frequency increase for the higher overtones. The dissipation increases with tetracaine addition for all the overtones, with an average shift of $21 \times 10^{-6}$ for the 3rd overtone. This implies that the proportion of $L_o$ and $L_d$ phases influences the tetracaine interaction with the raftlike liposomes, since there is no evidence for the partial rupture seen at 37°C. These observations are consistent with swelling of the liposomes due to interaction of tetracaine with the polar headgroups. The film becomes thicker and more viscous, as confirmed by Voigt model-based simulation results shown in Table 4.4. The shear modulus increases with tetracaine addition. Since tetracaine is a known surfactant, it would be expected that the membranes would become more fluid and deformable, but the increase in the shear modulus might mean that there is some structural modification of membrane domains that we cannot explain with this technique only.

The introduction of tetracaine 7.5 mg/ml in the binary liposomes at 25°C promotes, as seen in Figure 4.6, for all the overtones, a stable frequency decrease of about 50 Hz and a positive dissipation shift of $8 \times 10^{-6}$. Viscoelastic modeling shown in Table 4.5 indicates thickening of the lipid film and a slight increase in viscosity. It is interesting to notice that the thickening of the adsorbed layer of POPC/SM liposomes is less expressive than for the raftlike liposomes, while the viscosity increase is similar.

Table 4.4: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM/Chol liposomes interacting with tetracaine 7.5 mg/ml at 25°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
<th>HEPES</th>
<th>TTC (initial)</th>
<th>TTC (final)</th>
<th>HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.3 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>2.5 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Shear modulus (kPa)</td>
<td>8 ± 2</td>
<td>5 ± 2</td>
<td>12 ± 4</td>
<td>14 ± 4</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>96 ± 2</td>
<td>93 ± 1</td>
<td>106 ± 6</td>
<td>122 ± 11</td>
<td>103 ± 12</td>
</tr>
</tbody>
</table>

Table 4.5: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM liposomes interacting with tetracaine 7.5 mg/ml at 25°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
<th>HEPES</th>
<th>TTC (initial)</th>
<th>TTC (final)</th>
<th>HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.4 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>2.6 ± 0.3</td>
<td>2.6 ± 0.3</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Shear modulus (kPa)</td>
<td>2.38 ± 0.08</td>
<td>3.3 ± 0.1</td>
<td>10 ± 2</td>
<td>10 ± 2</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>83 ± 4</td>
<td>83 ± 5</td>
<td>94 ± 1</td>
<td>96 ± 1</td>
<td>80 ± 2</td>
</tr>
</tbody>
</table>

To gain further insight on the observed phenomena, $\Delta D$ vs. $\Delta f$ plots were traced for both temperatures and compositions, as shown in Figure 4.7.

For the raftlike at both temperatures, one can see that, at the start of tetracaine influx, there is a fast period (depicted by the small amount of data points in this region) of linear decrease of frequency and with a small dissipation increase due to tetracaine-induced swelling. However, at some point, this trend is reversed and the frequency starts to increase with dissipation increase. Since, after rinsing, the frequency shift is much lower than before tetracaine introduction for the experiment at 37°C, the changes induced by tetracaine on the adsorbed amount are irreversible, as expected from partial rupture of liposomes. At 25°C, the effect of tetracaine is quasi-reversible upon rinsing and there was
probably rinsing of tetracaine that was attached to the liposomes.

For the binary composition, one can see that the dissipation changes with temperature are not very significant, but still slightly more significant at 25°C. Also, the tetracaine action for both temperatures at this composition is close to reversible.

With these observations, it is clear that the interaction of tetracaine is more significant for the raft-like liposomes and, in this case, is much more significant at 37°C, and the the induced modifications are irreversible. At 25°C, the changes are still significant, but the phenomenon is quasi-reversible after rinsing and there are no permanent structural modifications of the adsorbed liposomes. For the POPC/SM liposomes, we can conclude that tetracaine interacts with the liposomes at both temperatures, but the effects are stronger at 25°C, where there is still a considerable ratio of liquid-ordered SM-enriched domains [38], and tetracaine will presumably interact with the headgroups of the lipids in these domains and promote its fluidization and consequent liposome swelling.

4.2.2.C Effect of tetracaine concentration

Figure 4.8 shows the average values of frequency and dissipation shifts obtained for the 3rd overtone at several steps of the process of interaction of tetracaine with the two mixtures. We chose the third overtone but, with the exception of the concentration of 7.5 mg/ml (see Appendix A and Figure 4.1...
the results obtained with the other overtones are similar. For the raftlike mixture with a tetracaine concentration of 1.5 mg/ml, the interaction of tetracaine will cause a decrease in frequency, but the effect in dissipation is negligible. For the concentration of 3.75 mg/ml, the frequency decrease is higher than for 1.5 mg/ml, but the dissipation suffers a considerable increase. These results indicate swelling induced by tetracaine. These modifications in the membrane are quasi-reversible upon rinsing of tetracaine since the frequency and dissipation return approximately to its initial value. For the concentration of 7.5 mg/ml, after the initial frequency decrease, there is a significant increase during the steady-state with a concomitant increase in dissipation. This behavior was already described in Section 4.2.2.A and attributed to partial rupture of small liposomes, after an initial swelling. For the POPC/SM liposomes, the increase in dissipation and consequent swelling of liposomes only occurs for the highest concentration, and for the others there is a small decrease.

In conclusion, the fluidizing effect of tetracaine is concentration-dependent, and more expressive for the raftlike liposomes at physiological temperatures.
4.2.3 Interactions of adsorbed liposomes with lidocaine

4.2.3.A Effect of cholesterol

Figure 4.9: Mean ± S.D. values for the frequency (left) and dissipation (right) shifts in the different overtones for the interaction of adsorbed POPC/SM/Chol (top) and POPC/SM (bottom) liposomes with lidocaine 20 mg/ml at 37°C.

Table 4.6: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM/Chol liposomes interacting with lidocaine 20 mg/ml at 37°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
<th>HEPES</th>
<th>LDC (initial)</th>
<th>LDC (final)</th>
<th>HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.2 ± 0.3</td>
<td>2.0 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Shear modulus (kPa)</td>
<td>7 ± 4</td>
<td>3 ± 2</td>
<td>10 ± 6</td>
<td>8 ± 5</td>
<td>1.1 ± 0.8</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>98 ± 5</td>
<td>97 ± 4</td>
<td>88 ± 4</td>
<td>89 ± 4</td>
<td>96 ± 3</td>
</tr>
</tbody>
</table>

Table 4.7: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM liposomes interacting with lidocaine 20 mg/ml at 37°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
<th>HEPES</th>
<th>LDC (initial)</th>
<th>LDC (final)</th>
<th>HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.0 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Shear modulus (kPa)</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>96 ± 18</td>
<td>99 ± 17</td>
<td>116 ± 8</td>
<td>121 ± 24</td>
<td>95 ± 26</td>
</tr>
</tbody>
</table>

From the analysis of Figure 4.9 (top), the frequency shifts are negative (about 25 Hz for the 3rd overtone), but the effects on the dissipation of the film are negligible. Viscoelastic modeling results shown in Table 4.6 indicate that lidocaine increases slightly the viscosity of the film and the shear
modulus increases with a small thickness decrease. The observations are consistent with rigidification of the lipid film by lidocaine introduction and increase of the adhesion force between the crystal and the liposomes. After rinsing, the frequency and dissipation shift values return to approximately the same values as the initial ones, implying that the interaction of lidocaine with the liposomes is reversible upon rinsing.

For the binary mixture (Figure 4.9 (bottom)), the main feature comparatively to the raftlike liposomes is an increase of dissipation for all the overtones. This results in liposome swelling, confirmed by viscoelastic modeling summarized in Table 4.7. This result is much more important than the slight viscosity increase. The absence of coherent data for the shear modulus modeling leaves an open question about the effects of lidocaine on the structure of the lipid film.

Finally, the main conclusion is that the fluidizing effect of lidocaine is much more intense for the POPC/SM liposomes at 37°C.

4.2.3.B Effect of temperature

The effects of lidocaine 20 mg/ml on the raftlike liposomes are similar at both temperatures (Figure 4.10, top, and Tables 4.6 and 4.8), despite a slight increase in dissipation at 25°C upon lidocaine addition. From analysis of Figure 4.10, bottom and Tables 4.7 and 4.9 for the binary membrane liposomes and comparing these results with the results at 37°C, the dissipation increase is much lower at 25°C, while the frequency shifts and viscosity increase are similar. There is also evidence for reversibility of lidocaine action after rinsing. The swelling of liposomes is much more intense at 37°C as well, since there is a higher thickness increase at the highest temperature.
Figure 4.10: Mean ± S.D. values for the frequency (left) and dissipation (right) shifts in the different overtones for the interaction of adsorbed POPC/SM/Chol (top) and POPC/SM (bottom) liposomes with lidocaine 20 mg/ml at 25°C.

Table 4.8: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM/Chol liposomes interacting with lidocaine 20 mg/ml at 25°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>LDC (initial)</th>
<th>LDC (final)</th>
<th>HEPES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.3 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>1.88 ± 0.04</td>
</tr>
<tr>
<td>Shear modulus (kPa)</td>
<td>7 ± 2</td>
<td>2.1 ± 0.3</td>
<td>9.3 ± 0.3</td>
<td>8.6 ± 0.7</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>98 ± 3</td>
<td>91 ± 1</td>
<td>85 ± 2</td>
<td>88 ± 2</td>
<td>95 ± 4</td>
</tr>
</tbody>
</table>

Table 4.9: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM liposomes interacting with lidocaine 20 mg/ml at 25°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>HEPES</th>
<th>LDC (initial)</th>
<th>LDC (final)</th>
<th>HEPES</th>
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</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.0 ± 0.3</td>
<td>1.9 ± 0.3</td>
<td>2.0 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Shear modulus (kPa)</td>
<td>4 ± 2</td>
<td>5 ± 2</td>
<td>12 ± 4</td>
<td>11 ± 4</td>
<td>4.5 ± 0.9</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>78 ± 4</td>
<td>77 ± 3</td>
<td>79 ± 2</td>
<td>78 ± 2</td>
<td>79 ± 3</td>
</tr>
</tbody>
</table>

From the observation of Figure 4.11, one can see, in comparison with the same analysis performed for tetracaine (Figure 4.7), that the effects in frequency and dissipation, as expected, are much lower. The effect of tetracaine-induced swelling with frequency increase does not occur here, which correlates well with the fact that this kind of behavior happens for surface-active molecules [123], which is the case of tetracaine, but not of lidocaine [124]. These data provide further evidence that the fluidizing effects of lidocaine are stronger for the POPC/SM liposomes, particularly at 37°C.
Figure 4.11: $\Delta D$ vs $\Delta f$ plots (3rd overtone) of the interaction of lidocaine 20 mg/ml with POPC/SM/Chol (top) and POPC/SM (bottom) liposomes at 37 (left) and 25°C (right). (1) Start of tetracaine influx; (2) End of tetracaine influx; (3) Rinsing.

However, for the raftlike mixture, this trend is reversed and lidocaine interacts slightly more with the liposomes at 25°C, where the ratio of $L_o$ domains is higher [38].

4.2.3.C Effect of lidocaine concentration

The modifications due to the introduction of lidocaine 10 mg/ml on the system with adsorbed raftlike liposomes, as shown in Figure 4.12 top are negligible (see Appendix A), indicating that this concentration does not alter the properties of the liposomes. At this concentration, the amount of lidocaine is not enough to induce any kind of lipid ordering alteration. On the other hand, the concentration of 10 mg/ml (see also Appendix A) on lidocaine induces noticeable effects on frequency and dissipation of the POPC/SM adsorbed liposomes (Figure 4.12 bottom). In comparison with the concentration of 20 mg/ml, the frequency shifts induced by both concentrations are similar, but slightly higher for the highest concentration. The main difference between both concentrations is the dissipation change, which is considerably larger for the highest concentration. This indicates a significant liposome swelling, resulting from fluidization of the lipid membranes. This confirms that the POPC/SM liposomes are more vulnerable to lidocaine action.
4.2.4 Interaction of adsorbed liposomes with propofol

4.2.4.A Effect of cholesterol

The introduction of propofol 0.18 mg/ml on the raftlike liposomes resulted in a negative frequency shift of about 30 Hz, and the dissipation undergoes an initial decrease, but returns to the initial value upon stabilization (Figure 4.13, top). One curious effect is that rinsing does not promote any noticeable change in the frequency shift, meaning that propofol was irreversibly coupled to the liposomes. A small increase in dissipation indicates a slight thickening of the liposomes. These assumptions are confirmed by the viscoelastic parameters obtained from modeling and shown in Table 4.10. The pattern of the frequency shift for the binary mixture is the same than for the raftlike liposomes, but the frequency shift is about half. The dissipation shift decreases slightly upon propofol addition (Figure 4.13, bottom). The viscoelastic modeling confirms a less intense effect in viscosity in the binary composition, and the changes in thickness are negligible (Table 4.11).
Figure 4.13: Mean ± S.D. values for the frequency (left) and dissipation (right) shifts in the different overtones for the interaction of adsorbed POPC/SM/Chol (top) and POPC/SM (bottom) liposomes with propofol 0.18 mg/ml at 37°C.

Table 4.10: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM/Chol liposomes interacting with propofol 0.18 mg/ml at 37°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
<th>H + DMSO</th>
<th>PPF (initial)</th>
<th>PPF (final)</th>
<th>H + DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.02 ± 0.05</td>
<td>2.00 ± 0.08</td>
<td>2.25 ± 0.07</td>
<td>2.27 ± 0.06</td>
<td>2.21 ± 0.07</td>
</tr>
<tr>
<td>Shear modulus (kPa)</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>102 ± 3</td>
<td>99 ± 3</td>
<td>91 ± 1</td>
<td>99 ± 5</td>
<td>106 ± 6</td>
</tr>
</tbody>
</table>

Table 4.11: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM liposomes interaction with propofol 0.18 mg/ml at 37°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
<th>H + DMSO</th>
<th>PPF (initial)</th>
<th>PPF (final)</th>
<th>H + DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Shear modulus (kPa)</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>89 ± 4</td>
<td>91 ± 3</td>
<td>89 ± 6</td>
<td>87 ± 3</td>
<td>86 ± 6</td>
</tr>
</tbody>
</table>

In conclusion, the interaction of propofol at 37°C is slightly more intense for the raftlike liposomes, and the temperature effect analysis will allow to conclude if this is due to the existence of SM-Chol domains, or simply due to phase behavior.
4.2.4.B Effect of temperature

Figure 4.14: Mean ± S.D. values for the frequency (left) and dissipation (right) shifts in the different overtones for the interaction of adsorbed POPC/SM/Chol (top) and POPC/SM (bottom) liposomes with propofol 0.18 mg/ml at 25°C.

Table 4.12: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM/Chol liposomes interacting with propofol 0.18 mg/ml at 25°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
<th>H + DMSO</th>
<th>PPF (initial)</th>
<th>PPF (final)</th>
<th>H + DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Shear modulus (Pa)</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
<td>N.E.</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>104 ± 7</td>
<td>94 ± 2</td>
<td>97 ± 7</td>
<td>108 ± 2</td>
<td>112 ± 7</td>
</tr>
</tbody>
</table>

Table 4.13: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM liposomes interaction with propofol 0.18 mg/ml at 25°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
<th>H + DMSO</th>
<th>PPF (initial)</th>
<th>PPF (final)</th>
<th>H + DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (mPa.s)</td>
<td>2.8 ± 0.2</td>
<td>2.7 ± 0.2</td>
<td>2.5 ± 0.3</td>
<td>2.5 ± 0.3</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Shear modulus (lPa)</td>
<td>6 ± 2</td>
<td>8 ± 3</td>
<td>11 ± 4</td>
<td>9 ± 2</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>77 ± 5</td>
<td>78 ± 5</td>
<td>85 ± 4</td>
<td>87 ± 2</td>
<td>81 ± 2</td>
</tr>
</tbody>
</table>

At 25°C for the raftlike liposomes, the principal observation relatively to the experiment at 37°C is that there is an increase in dissipation upon propofol addition (Figure 4.14 and 4.15, top), instead of the decrease observed at 37°C. This indicates that, at this temperature, there are more favorable conditions for liposome swelling, confirmed by viscoelastic modeling (Table 4.12). On the other hand,
for the POPC/SM liposomes at 25°C, the effect of propofol in the frequency shift is less expressive, and there is a slight increase in frequency for the higher overtones (Figure 4.14 and 4.15 bottom).

![Figure 4.15: ΔD vs Δf plots (3rd overtone) of the interaction of propofol 0.18 mg/ml with POPC/SM/Chol (top) and POPC/SM (bottom) liposomes at 37 (left) and 25°C (right). (1) Start of tetracaine influx; (2) End of tetracaine influx; (3) Rinsing.](image)

4.2.5 Final remarks

- There is adsorption of intact liposomes for both compositions and temperatures, consisting of a viscoelastic film of adsorbed liposomes. The liposomes without cholesterol are more deformable under adsorption at 37°C, so the surface density is lower because each liposome occupies a higher surface area.

- There is a large scattering in the values of the thickness correspondent to the adsorbed layers of the same liposome composition. Although we are not able to fully understand this fact, we may attribute this to some irreproducibility of the adsorption process, as well as imperfections in the modeling.

- At physiological temperatures, tetracaine at a concentration of 7.5 mg/ml induces partial rupture of raftlike liposomes, and this rupture shall be dependent of liposome size. For the POPC/SM 1:1 liposomes, the rupture seems unlikely, and the main modification is liposome swelling due to membrane fluidization and a slight increase in viscosity due to increase of the adhesion force between the liposomes and the crystal.
- For the raftlike liposomes, the effects of tetracaine 7.5 mg/ml are more significant at 37°C (at 25°C there is no evidence of rupture), while for the POPC/SM liposomes the fluidizing effects are more pronounced at 25°C. There is also concentration dependence of tetracaine action for both mixtures at 37°C, with clearly more marked effects at 7.5 mg/ml.

- Lidocaine seems to interact with the raftlike liposomes promoting a slight increase in viscosity, but there is no swelling. However, the effects are much more expressive for the POPC/SM 1:1 liposomes, for which liposome swelling occurs, especially at 37°C. There is also evidence for reversibility of lidocaine action after rinsing. There are also concentration-dependent changes at 37°C for both compositions with the highest concentration inducing the more visible changes, and the POPC/SM liposomes are clearly more vulnerable to lidocaine action.

- Propofol interacts less expressively with both membrane systems. At physiological temperatures, the main modification is a change of liposome viscosity, more noticeable in the raftlike liposomes. However, the interactions are stronger at 25°C for both compositions, where a slight liposome swelling occurs.

- In conclusion, all the three anesthetics interact with both liposome compositions, inducing liposome swelling, structural changes in membrane rigidity and a slight viscosity increase of the liposome layers in most cases. Regarding these observations, tetracaine induces the more pronounced effects, in particular in raftlike membranes. Propofol also appears to have some preference for interaction with raftlike liposomes, while lidocaine induces more modifications on POPC/SM membranes.

4.3 DSC

4.3.1 Cholesterol effect

Figure 4.16 shows the thermograms of the liposomes with two lipid compositions studied so far.

![Figure 4.16: Thermograms for the POPC/SM/Chol (black) and POPC/SM (gray) liposomes in HEPES.](image)

For the ternary mixture liposomes, there is no clear sign of a phase transition on the range of temperatures studied. In the mixture without cholesterol, there is a broad transition peak that starts in
a temperature lower than 10°C, thus outside the possible temperature range of the calorimeter, and
finishes at about 40°C. The maximum of the transition is situated at about 29.5°C.

POPC is an unsaturated lipid with a very low main transition temperature of -3°C [10]. Brain SM
undergoes a gel to liquid-crystalline phase transition with an onset temperature of 32°C and a Tm of
38°C [10]. It is clear that the effect of mixing both lipids in an equimolar ratio will broaden the phase
transition, and this phase transition ends at a temperature close to the Tm of brain SM itself. Since
the onset temperature was not detectable by us (however, Pokorny et al [38] estimated a first phase
transition at 3°C), we cannot access the full temperature range where coexistence of liquid-ordered
and liquid-disordered phases occurs. With these observations, we can say that POPC destabilizes
the acyl chain ordering of sphingomyelin molecules and induces a system with more rigid domains
enriched in SM and fluid-like domains enriched in POPC.

When cholesterol is introduced, the phase transition disappears for the range of temperatures
studied. This correlates well with the observation that cholesterol stabilizes liquid-ordered domains
due to preferential interaction with the acyl chains of sphingomyelin [38, 43, 122].

4.3.2 Tetracaine

![Figure 4.17: Thermograms for the POPC/SM/Chol (left) and POPC/SM (right) liposomes with increasing concentrations of tetracaine. Black: no anesthetic; Dark gray: tetracaine 1.5 mg/ml; Medium gray: tetracaine 3.75 mg/ml; Light gray: tetracaine 7.5 mg/ml.](image)

The results of increasing tetracaine concentration for each lipid composition are shown in Figure
4.17 For the raftlike liposomes, the introduction of tetracaine with a concentration of 1.5 mg/ml does
not induce any effect in terms of phase transition. For the two highest tetracaine concentrations,
a broad phase transition seems to be induced, and the maximum of this transition decreases in a
concentration dependent manner (20.3°C for 3.75 mg/ml and 19.2°C for 7.5 mg/ml). Since there is a
very pronounced decrease of Cp after the maximum of the transition, a suspicion for lipid solubilization
arises, which correlates well with the known tetracaine surfactant properties. For the binary mixture,
there is a concentration-dependent depression of Tm, indicating fluidization of the membrane. The
endpoint of the transition is also achieved at lower temperatures. Other interesting phenomenon is
the increase of cooperativity of the transition with the increase of the anesthetics concentration, that
is, the width at half height of transition decreases and the range of temperatures at which the phase transition happens becomes narrower.

Other studies show that tetracaine promotes interdigitation of the acyl chains of phospholipids in a gel phase [88, 125]. In the binary mixture, if interdigitation exists, it would be due to sphingomyelin because it forms liquid-ordered domains in the range of temperatures studied. The formation of an interdigitated phase for the sphingomyelin acyl chains might be due to the fact that the charged tetracaine molecules (at pH = 7.4, the majority of the molecules will be in the protonated form) interact with the polar headgroups of the lipids and locate in the headgroup network between adjacent headgroups. With this conformation, tetracaine will induce defects between the acyl chains of sphingomyelin that are rigid at this temperature, inducing the formation of an interdigitated phase comprised of interdigitated acyl chains [125]. In one-component DSC of phospholipids, this phenomenon is noticeable by the increase of the enthalphy of transition and the absence of the gel to gel pre-transition, existing only one gel phase, the interdigitated phase [88]. Since we cannot identify, at least within the possible temperature range, these features, we cannot conclude anything about interdigitation of sphingomyelin acyl chains. For the mixture with cholesterol, it is very unlikely that interdigitation of acyl chains exists. Cholesterol has been stated to prevent interdigitation because the packing of cholesterol with the extended acyl chains of sphingomyelin creates a more favorable energy match between the head groups and the membrane core, and the tendency of interdigitation of acyl chains to provide an energetically favorable configuration for the membrane is avoided [125].

The main conclusion is that tetracaine has a more pronounced effect on raftlike liposomes, inducing a phase transition in the raftlike mixture for the two highest concentrations, and the correspondent broad peak may be a consequence of tetracaine association with cholesterol and inhibition of the cholesterol effect on membrane ordering.

4.3.3 Lidocaine

![Figure 4.18: Thermograms for the POPC/SM/Chol and POPC/SM 1:1 liposomes with increasing concentrations of lidocaine. Black: no anesthetic; Dark gray: lidocaine 10 mg/ml; Medium gray: lidocaine 20 mg/ml.](image)

For the raftlike liposomes, lidocaine at 10 mg/ml has induced a broad phase transition corresponding to the $L_0 + L_d$ to $L_d$ transition, with no identifiable onset and endpoint at this temperature range,
and with a maximum at 28.3°C. For the concentration of 20 mg/ml, the maximum of the transition has an average value of 25.7°C, indicating further lidocaine-induced lipid fluidization. Here, there is no evidence for lipid solubilization, so it seems that lidocaine will penetrate into the membrane, disorder the lipid acyl chains, and presumably interact with the liquid-ordered domains with the formation of complexes between lidocaine and lipids from these domains.

For the liposomes without cholesterol, the effect of the two concentrations on the decrease of the maximum of the transition is similar, but the highest concentration induces a more intense phenomenon. This suggests that lidocaine-induced fluidization does not depend on the concentration of anesthetic in the same manner as in the raftlike liposomes, since for both concentrations the $T_m$ is estimated to be around 27°C. Since the $C_p$ becomes negative after the peak for both concentrations, there is also some evidence supporting lipid solubilization on the binary mixture liposomes.

### 4.3.4 Propofol

First of all, the effect of DMSO on the phase transition of the liposomes is shown in Figure 4.19. For the raftlike mixture, DMSO at this concentration does not induce major modifications on the system. For the binary mixture, DMSO causes decrease of $T_m$ of the samples to an average of 25.9°C, inducing fluidization of the lipid domains to some extent.

![Figure 4.19: Thermograms for the DMSO effect on POPC/SM/Chol and POPC/SM liposomes. Black: HEPES; Gray: HEPES + 0.5 % DMSO](image)

Regarding the interactions with propofol, from the observation of Figure 4.20 one can see that propofol did not induce noticeable effects on the raftlike mixture phase behavior at this concentration. For the binary composition, propofol induces further $T_m$ decrease to an average of 22.9°C, as well as causing the endpoint of the broad peak to be localized at lower temperatures.
4.3.5 Final remarks

- The raftlike mixture in HEPES does not have a phase transition in the range of temperatures studied, while the mixture without cholesterol shows a broad peak with a maximum at 29.5°C, indicating a phase transition. It is evident that cholesterol stabilizes the liquid-ordered domains.

- Tetracaine induces fluidization of the lipid membranes for both compositions and probably solubilization of lipids with formation of small vesicles. Its effect is more important in the case of lipid rafts where a phase transition is induced for the higher concentrations.

- Lidocaine induces, at both concentrations studied, a broad phase transition in the raftlike mixture, and also decreases the $T_m$ of the binary mixture. The solubilization of lipids is more extensive for the mixture without cholesterol, suggesting a more intense interaction with the POPC/SM liposomes.

- DMSO, at the concentration used to solubilize propofol, does not induce major changes in the lipid ordering of the ternary mixture liposomes, but fluidizes the domains in the binary mixture to a small extent.

- The action of propofol at the concentration of 0.18 mg/ml on the raftlike liposomes is negligible, while in the binary mixture the fluidization of the liquid-ordered domains is evident, but to a small extent ($T_m$ decreases 3°C relatively to the liposomes with 0.5 % DMSO).

- Tetracaine has the most noticeable effect on the depression of phase transition temperature and disordering of the lipid domains, with induction of a phase transition in the raftlike liposomes and solubilization and/or formation of small vesicles for both compositions. Lidocaine interact more with the POPC/SM liposomes, but also induces a phase transition in the raftlike liposomes. Propofol has less fluidizing abilities than the other two anesthetics and these were only noticeable for the POPC/SM liposomes.
4.4 P-NMR

4.4.1 Cholesterol effect

We present in Figure 4.21 the spectra obtained with the raftlike and binary liposomes at four temperatures: 12, 25, 37 and 60°C.

![Figure 4.21: Comparison of the spectra of POPC/SM/Chol liposomes (black) and POPC/SM liposomes (gray) in HEPES.](image)

Significant differences between both liposome compositions are seen for all temperatures. The main differences in the lineshapes occur at 37°C. The headgroups of the binary liposomes undergo practically full isotropic motion at this temperature, implying that the system tends to be in a more disordered phase, allowing more degrees of motion for the phosphate headgroups. This is compatible with the thermograms for this mixture, where the endpoint of the phase transition of the POPC/SM 1:1 liposomes is close to 37°C. At 60°C, the linewidth is broader for the raftlike liposomes, and there is also a small high-field component due to liquid-ordered domains, which imply that cholesterol broadens significantly the phase coexistence region relatively to the one of an equimolar mixture of POPC and SM. This fact also correlates well with the DSC results for both compositions (see Section 4.3.1).

At 25°C, the spectrum for the POPC/SM liposomes appears with reversed asymmetry with respect to the POPC/SM/Chol spectrum, with a larger isotropic motion component. For the ternary membranes, the lamellar component due to liquid-ordered domains is the most significant phase. This also correlates well with the domain-stabilizing effect of cholesterol. At 12°C, the spectra for both mixtures indicate the predominance of a lamellar phase consistent with the existence of more ordered phases. However, for the binary mixture, there is a remarkable isotropic motion component that indicates that, for the binary mixture, the liquid-disordered phase is already relevant at this temperature.

From all these observations, we can conclude that cholesterol clearly stabilizes the more ordered domains and broadens the phase transition to a liquid-disordered phase of the mixture of sphingomyelin and POPC. For the binary mixture, POPC induces relevant disordering on the packing of the sphingomyelin acyl chains in a gel phase (that, as already stated, does not appear in mixtures) and broadens the L₀ + L₅ phase coexistence region, which is compatible with our DSC results. Comparing to previous NMR studies on both mixtures, the lineshapes found in this work are different from...
the lamellar lineshapes found for temperatures up to 45°C in other studies, with the narrowing of CSA of the lamellar spectra being indicative of membrane fluidization in that case [10, 27].

4.4.2 Tetracaine

The superposition of the spectra of the raftlike liposomes in HEPES at 12, 25, 37 and 60°C with the same liposome composition with tetracaine 7.5 mg/ml is shown below in Figure 4.22. A study of the effect of different tetracaine concentrations at 37°C was also performed (see Appendix B), but the results at 3.75 mg/ml were not conclusive. However, from the concentrations of 1.5 and 7.5 mg/ml, it is expected that the fluidization of lamellar liquid-ordered domains is concentration-dependent.

Figure 4.22: Comparison of the spectra of POPC/SM/Chol (left) and POPC/SM (right) liposomes in HEPES (black) and with tetracaine 7.5 mg/ml (gray).

For the mixture at 12°C, the main modification caused by tetracaine at 7.5 mg/ml is a slight narrowing of the spectrum linewidth, and this is compatible with fluidization by tetracaine. Another important feature is the appearance of a slight isotropic motion peak, indicating some degree of solubilization of liquid-ordered domains or the appearance of inverted micelles to incorporate the tetracaine molecules inside the membrane, or maybe the incipient formation of an interdigitated phase of sphingomyelin acyl chains [125]. However, data from the literature state that cholesterol avoids interdigitation of acyl chains caused by surface-active molecules like tetracaine [125], so this behavior seems unlikely.

At 25°C, the linewidth also appears to be decreasing slightly with tetracaine introduction. It is also interesting that the isotropic motion component is also more pronounced when tetracaine is added to the mixture at this temperature, where there was natively coexistence of liquid-ordered and liquid-disordered phases, so tetracaine induces further fluidization of the liquid-ordered domains of Chol and SM.

At 37°C, the main feature of adding tetracaine is the decrease of amplitude of the high-field shoulder, which implies that the liquid-ordered domains of SM and Chol are affected by the addition of tetracaine at physiological temperatures. Still, from the observation of the changes in the lineshape, the fluidization does not occur to a great extent, at least from what can be concluded from this technique. At 60°C, the changes induced by tetracaine are negligible.
Regarding these results, tetracaine at the concentration of 7.5 mg/ml induces fluidization of the lipid membranes (with a greater action at 25°C in particular), presumably due to solubilization of liquid-ordered domains or by incorporation of tetracaine by inverted micelles into the membranes.

For the binary composition, interesting findings arise when the system is in a L_o+ L_d coexistence region. At 12°C, it is clear that the isotropic component becomes preponderant, instead of a small shoulder seen in the liposomes without anesthetic. This indicates that tetracaine probably causes fluidization of the system and solubilization of liquid-ordered domains, formation of inverted micelles, as well as the possibility of induction of an interdigitated phase due to interdigitation of the acyl chains of sphingomyelin. Since data from DSC are inconclusive on the existence of interdigitation for the mixture without cholesterol (see Subsection 4.3.2), data from other techniques like proton nuclear magnetic resonance (H-NMR) would be necessary to conclude about the occurrence of interdigitation.

At 25°C, the spectrum follows the same lineshape, whilst with narrower linewidth, as for the liposomes without anesthetic. This preference for the isotropic motion of the polar headgroups indicates further fluidization of the lipid bilayer due to same phenomena stated for the membranes at 12°C.

At 37°C and 60°C, the system is in a fully isotropic motion configuration, and there is not an identifiable effect of tetracaine, at least from what can be concluded from this method, for a system on a fully lamellar liquid-disordered state.

For the binary membrane composition liposomes, there is even greater evidence of fluidization of liquid-ordered domains induced by tetracaine by modification of the lipid polar headgroup conformation, and the possibility of solubilization of membrane lipids due to tetracaine is stronger than in the system without cholesterol, as well as the formation of an interdigitated phase of sphingomyelin acyl chains at low temperatures. However, at physiological temperatures, the effect of tetracaine is more important on the raftlike liposomes, as shown by the other techniques (Sections 4.2.2 and 4.3.2).

### 4.4.3 Lidocaine

![Figure 4.23](image_url): Effect of increasing lidocaine concentration for the POPC/SM/Chol liposomes at 37°C. Black: liposomes in HEPES. Dark gray: lidocaine 10 mg/ml. Light gray: lidocaine 20 mg/ml.
At 37°C, a study of the effect of increasing lidocaine concentration on the raftlike liposomes was performed. The results are shown below in Figure 4.23. For the concentration of 10 mg/ml, the effect of lidocaine on the polar headgroups of lipids is negligible, and this correlates well with the observations from the other techniques that also reported a negligible effect of lidocaine at this concentration (see Figure A.3). At 20 mg/ml, the high-field components characteristic of lamellar phases (therefore, liquid-ordered domains) appear with reduced amplitude, suggesting a lidocaine-induced fluidization of liquid-ordered domains at 37°C. The effect of lidocaine 20 mg/ml at the four different temperatures studied is shown in Figure 4.24.

![Figure 4.23: Comparison of the spectra of POPC/SM/Chol (left) and POPC/SM (right) liposomes in HEPES (black) and with lidocaine 20 mg/ml (gray).](image)

At 12°C, lidocaine 20 mg/ml does not modify the lineshape of the raftlike mixture spectrum relatively to the liposomes in HEPES at this temperature. At 25°C, the system with lidocaine still holds the lamellar phase, but the shoulder due to isotropic motion has higher amplitude, implying that lidocaine induces fluidization of the system noticeable by a change in the lipid headgroup configuration that may correlate with fluidization of the membranes. At 37°C, as seen as well in Figure 4.23, the isotropic component is the main feature, and the shoulder due to the lamellar liquid-ordered phase is losing amplitude and relative importance, confirming that lidocaine at 20 mg/ml induces fluidization of raftlike systems at physiological temperatures, reducing the order of the liquid-ordered domains and changing the headgroup environment. This observation is consistent with the literature [14–16]. At 60°C, the action of lidocaine is shown by a slight narrowing of the isotropic peak and fluidization of the remaining liquid-ordered domains.

For the binary mixture at 12°C, the spectrum becomes slightly broader than for the liposomes without anesthetic. There is also the induction of a small isotropic peak. The reasons for the appearance of the small peak, even though they are also due to the incorporation of the anesthetic into the membrane, might not be exactly the same as tetracaine. Lidocaine has a lower pKₐ than tetracaine (Table 2.1), so the proportion of uncharged lidocaine molecules will be higher than for tetracaine at pH = 7.4. So, the uncharged lidocaine molecules will account significantly for the lidocaine effect on membranes, and will presumably penetrate directly inside the membrane due to their amphipathic nature. At 25°C, the modifications due to lidocaine introduction are not meaningful, as well as for
37°C and 60°C. However, one cannot conclude that lidocaine does not interact with the POPC/SM 1:1 membranes, what can be said is that the CSA tensor is not sensitive to lidocaine action. The same phenomenon was found by Castro et al \cite{126} when evaluating the lidocaine effect on the headgroup of DMPC molecules. They found by $^2$H-NMR and $^1$H-$^13$C separated local field experiments that lidocaine affected the lipid headgroup, but $^{31}$P-NMR experiments did not reveal modifications in the lineshape upon interaction of DMPC with lidocaine. This is explained by the fact that only one torsion angle of the choline headgroup is sensitive to lidocaine action, and the CSA tensor is not sensitive to this modification. Therefore, lidocaine seems to interact with the POPC/SM liposomes without inducing relevant changes on the polar headgroup mobility and conformation of phospholipids, probably because it penetrates deeply into the membranes, since data from other techniques confirm a noticeable interaction between lidocaine and POPC/SM liposomes (Sections \ref{sec:4.2.3} and \ref{sec:4.3.3}).

### 4.4.4 Propofol

First of all, the spectra obtained with the liposomes in HEPES + 0.5% DMSO will be compared with the spectra in HEPES to check if there is any degree of modification by DMSO on the ordering of the lipid membranes, as seen in Figure \ref{fig:4.25}.

**Figure 4.25:** Comparison of the spectra of POPC/SM/Chol (left) and POPC/SM (right) liposomes in HEPES (black) and in HEPES + 0.5% DMSO (gray).

From the spectra, for all the temperatures studied one can notice that DMSO at the concentration of 0.5% does not modify the lineshape of the raftlike mixture spectra, so it does not interfere with the properties of the lipid system with this composition. For the binary mixture, in the isotropic spectra at 37 and 60°C, the effects due to DMSO are not recognizable. At 12°C, there is a slight broadening of the linewidth, as well as the appearance of a small isotropic peak, while at 25°C the modifications are also not seen. So, there is a slight effect of DMSO on the polar headgroup orientation of the liquid-ordered domains. Since this effect was not seen in the system with cholesterol, one can state that cholesterol has a role of membrane stabilization against organic solvents.
From the spectra shown in Figure 4.26, propofol at the concentration of 0.18 mg/ml does not modify significantly the lineshapes of the raftlike and binary composition liposomes for all the temperatures. So, for both mixtures, it seems unlikely that propofol modifies the headgroup mobility of the phospholipids, at least within the range of sensitivity of this method.

4.4.5 Final remarks

On a final note, the main conclusions taken from the P-NMR analysis are:

- The cholesterol effect on the liposomes is a broadening of the linewidth, as well as the broadening of the $L_o + L_d$ phase coexistence region since the isotropic motion components seem to appear at higher temperatures.

- Tetracaine interacts more with binary mixtures than with raftlike liposomes at low temperatures. In contrast, at physiological temperatures, the interaction is stronger with the ternary mixture.

- Lidocaine, in the raftlike mixture, clearly changes the lipid headgroup conformation at physiological temperatures. However, for the binary composition, no disordering effects of lidocaine are noticeable through changes in the polar headgroup conformation, at least in the scope of this technique.

- The effect of the organic solvent used to increase propofol solubility, DMSO, is negligible at the concentration used when interacting with raftlike liposomes, but there is a small effect for the temperatures of phase coexistence on the binary mixture.

- Propofol seems to exert a negligible effect on the lipid headgroup motion for all the temperatures and the two compositions studied, at least on the headgroup parameters accessible by this technique.

- The results suggest that tetracaine is the anesthetic that exerts more modifications on the polar headgroups seen by this technique, which is expected due to the fact that it is ionized at physio-
logical pH and interacts with polar headgroups. Propofol does not exert noticeable modifications on the polar headgroups and seems to penetrate directly into the membrane core of the membranes. Lidocaine also seems to not modify the polar headgroups of POPC/SM liposomes and penetrate into the membrane core, while for the raftlike liposomes modifies the polar headgroup conformation at physiological temperatures.
Conclusions and Future Work
First of all, we must stress that the techniques used in this study — QCM-D, DSC, and P-NMR — yield complementary data needed to characterize the anesthetic-model membrane interactions but do not allow direct comparisons, since the experimental conditions cannot be exactly reproduced. Taking this into consideration, we may draw some unequivocal conclusions.

For the two local anesthetics studied, it is clear that tetracaine has a more pronounced effect on lipid organization than lidocaine, inducing fluidization in a concentration-dependent manner by affecting the polar headgroups of the phospholipids for both the raftlike composition and the POPC/SM liposomes. The effects are more noticeable for the raftlike liposomes. The solution techniques (DSC and P-NMR) indicate the possibility of solubilization and/or formation of inverted micelles (to carry the tetracaine) in both types of liposomes. For lidocaine, the effects on lipid fluidization are not so intense, and the P-NMR results suggest that lidocaine interacts deeply within the membrane core of the POPC/SM liposomes, while for the raftlike mixture the interaction is more superficial. The higher fluidizing effect of tetracaine correlates well with the higher partition coefficient of tetracaine, and with the observation that ester- [LA], like tetracaine, are more potent than amide- [LA], such as lidocaine.

At physiological temperatures, tetracaine at the concentration of 7.5 mg/ml induced partial rupture of adsorbed POPC/SM/Chol liposomes, which did not happen at lower temperatures and for the POPC/SM liposomes. This rupture must be dependent on liposome size, and the implied mechanisms can be further investigated with imaging techniques that allow the visualization of adsorbed liposomes, like atomic force microscopy (AFM) and laser-scanning confocal fluorescence microscopy (LSCFM). Apart from this behavior at this particular composition, temperature and tetracaine concentration, the general effects of tetracaine 7.5 mg/ml on the liposomes were their swelling and a slight increase in viscosity of the adsorbed layer. The fluidizing abilities of tetracaine were proven to increase with concentration at 37°C. Another remarkable effect of tetracaine on lipid organization was the induction of a phase transition on the raftlike liposomes by tetracaine at the concentratons of 3.75 and 7.5 mg/ml. This indicates that tetracaine partially abolishes the effect of cholesterol on stabilization of more ordered domains.

On the other hand, lidocaine seems to penetrate and interact more deeply in the POPC/SM membranes, promoting liposome swelling and a slight increased viscosity. The interaction with the raftlike membranes is less intense and is reflected on an increase in viscosity of the liposome layer, but no swelling. However, results from DSC show that lidocaine induces a phase transition on the POPC/SM/Chol liposomes for the anesthetic concentrations studied, providing evidence for interaction with raftlike domains, albeit less intense than with membranes without cholesterol. It is also important to say that these lidocaine-induced effects are quasi-reversible upon rinsing. With these observations, one could state that lidocaine interacts with both model membranes, but the interactions are preferential for non-raftlike membranes, which is in agreement with the findings of Tsuchiya et al [2]. However, since the mechanisms implied in the interaction of lidocaine with both compositions are different, further research on this topic has to be carried and we cannot discard any specific interaction of lidocaine with lipid rafts.

Propofol cannot be compared directly with the other two anesthetics because its concentration at
the target sites is one order of magnitude smaller than the lowest value used for local anesthetics. First of all, propofol effects are irreversible upon rinsing and seem to involve interaction with the membrane core of the membranes, as suggested by P-NMR, for both lipid compositions. Propofol induces swelling of adsorbed liposomes at lower temperatures, implying that it fluidizes the membranes by interacting with liquid-ordered domains. However, at physiological temperatures, the results of both raftlike and POPC/SM compositions in terms of lipid swelling are negligible, and only the viscosity of the liposome layers seems to be slightly modified. The modifications in viscosity are more noticeable for the raftlike composition, so this might support a certain preference of propofol for lipid rafts. Since it is commonly assumed that propofol initiates its anesthetic action by interacting with the $\text{GABA}_A$ receptor, and some studies have found that $\text{GABA}_A$ localizes in certain lipid rafts [19, 20], the interaction of propofol with lipophilic domains of this protein through modification of the surrounding lipid rafts structure shall be considered when studying the mechanism of action of propofol. However, DSC results show that propofol does not induce a phase transition on the raftlike liposomes like the other two anesthetics while it induces depression of the maximum temperature of transition for the POPC/SM liposomes. Regarding the combined results, we cannot draw an unequivocal conclusion on a preferential interaction of propofol with lipid raft models.

In short, the main conclusion of this work is that, at physiological temperatures, tetracaine seems to have a stronger interaction with raftlike liposomes, lidocaine has a deeper effect on POPC/SM liposomes, and that the mechanisms of interaction of propofol are not fully conclusive, despite the fact that QCM-D results suggest some preference for interaction with raftlike domains.

A comparison with the work of Paiva [7] for the same anesthetics and other models of eukaryotic membranes that do not constitute lipid rafts models show different results, although the general conclusions did not change. In fact, all anesthetics led to fluidization of lipid membranes of $\text{DMPC}$, $\text{DMPC} [\text{Chol}]$, and $\text{DMPC} [\text{DPPC}, \text{Chol}]$ liposomes, and tetracaine presented the strongest effect. However, the mechanisms of interaction were clearly different. For most concentrations of lidocaine, tetracaine and propofol used, there was an increase of the frequency shift. For tetracaine, there was rupture of DMPC liposomes, but the Chol-containing liposomes $[\text{DMPC}, \text{Chol}]$ and $[\text{DMPC}, \text{DPPC}, \text{Chol}]$ are resistant to rupture. In contrast, for the models studied in the present work the interaction with anesthetics leads to a decrease in the frequency shift. However, an evaluation on which model has a stronger interaction with these anesthetics is not possible at this point, because the frequency shifts follow opposite patterns and viscoelastic modeling was not performed in the work of Paiva. With this limitation, we cannot compare the alterations on the viscoelastic properties of the model membranes of the two studies. Finally, the results of the two studies show that the effects of anesthetics on lipid membranes depend on their composition and the existent lipid phases.

Some future directions on this study would be the evaluation of the interactions of these anesthetics with adsorbed liposomes by AFM and confocal microscopy, as referred above, as well as other nuclear magnetic resonance techniques generally used for lipid studies: H-NMR and carbon nuclear magnetic resonance (C-NMR). H-NMR would provide information of the coexistence of different lipid phases, and a good marker would be one of the acyl chains of POPC, since POPC alone does not
undergo any further phase transition alone above -3°C. C-NMR would provide, due to the sensitivity to carbon environment on a specific position, information of modification of the configuration of acyl chains, as well as of carbon-containing headgroups, to assess a specific site of interaction of anesthetics with membrane lipids.

In terms of model composition, it would be interesting to perform QCM-D and $^{31}$P-NMR experiments on POPC liposomes, to assess the effect of the absence of more rigid lipid domains. For the raftlike models, more sophisticated preparations, containing small percentages of other lipids present in nerve cell membranes (such as phosphatidylethanolamines, phosphatidylserines and gangliosides), or asymmetric vesicles that also account for the asymmetry of the lipid bilayers, would be less simplistic models.

A simulation of the interaction of anesthetics with the models studied so far, but changing the pH of the experiment would also be an interesting way to evaluate the interactions on different tissues, since the pH of the tissues will affect the state of ionization of the anesthetics and its penetrability into the membranes [80].

Other interesting direction in the following of this study and Paiva’s study would be the study on the effect of anesthetics on models of cardiac cell membranes and mitochondrial cell membranes to gain further insight on anesthetic-induced toxicity in cardiac cells, which is a proven side-effect of anesthesia.
Bibliography


Appendix A - Supplementary information for the QCM-D analysis
A.1 Tetracaine

Figure A.1: Mean ± S.D. values for the frequency (left) and dissipation (right) shifts in the different overtones for the interaction of adsorbed POPC/SM/Chol (top) and POPC/SM (bottom) liposomes with tetracaine 1.5 mg/ml at 37°C.

Table A.1: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM/Chol liposomes interacting with tetracaine 1.5 mg/ml at 37°C, obtained with QTools modeling.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Liposomes</th>
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<th>TTC (initial)</th>
<th>TTC (final)</th>
<th>HEPES</th>
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<tr>
<td>Viscosity (mPa.s)</td>
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<td>2.1 ± 0.2</td>
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<td>Shear modulus (kPa)</td>
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<td>6 ± 2</td>
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<td>139 ± 6</td>
<td>151 ± 17</td>
<td>138 ± 13</td>
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Table A.2: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM liposomes interacting with tetracaine 1.5 mg/ml at 37°C, obtained with QTools modeling.

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<tr>
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<th>TTC (final)</th>
<th>HEPES</th>
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<td>Viscosity (mPa.s)</td>
<td>1.9 ± 0.1</td>
<td>1.79 ± 0.08</td>
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<td>2.2 ± 0.1</td>
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Figure A.2: Mean ± S.D. values for the frequency (left) and dissipation (right) shifts in the different overtones for the interaction of adsorbed POPC/SM/Chol (top) and POPC/SM (bottom) liposomes with tetracaine 3.75 mg/ml at 37°C.

Table A.3: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM/Chol liposomes interacting with tetracaine 3.75 mg/ml at 37°C, obtained with QTools modeling.

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Table A.4: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM liposomes interacting with tetracaine 3.75 mg/ml at 37°C, obtained with QTools modeling.

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<td>65 ± 4</td>
<td>85 ± 7</td>
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A.2 Lidocaine

Figure A.3: Mean ± S.D. values for the frequency (left) and dissipation (right) shifts in the different overtones for the interaction of adsorbed POPC/SM/Chol (top) and POPC/SM (bottom) liposomes with lidocaine 10 mg/ml at 37°C.

Table A.5: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM/Chol liposomes interacting with lidocaine 10 mg/ml at 37°C, obtained with QTools modeling.

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<td>4 ± 3</td>
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Table A.6: Mean ± S.D. values of the viscoelastic properties of adsorbed POPC/SM liposomes interacting with lidocaine 10 mg/ml at 37°C, obtained with QTools modeling.

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Appendix B - Supplementary information for the P-NMR analysis
B.1 Study of tetracaine concentration on raftlike mixture spectra

![Figure B.1](image)

**Figure B.1:** Effect of increasing lidocaine concentration for the POPC/SM/Chol liposomes at 37°C. Black: liposomes in HEPES. Dark gray: 1.5 mg/ml. Medium gray: 3.75 mg/ml. Dark gray: 7.5 mg/ml.