Interactions between anesthetics and lipid rafts

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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 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.**

***Index Terms*—Lipid rafts, Anesthetics, Liposomes, QCM-D, DSC, P-NMR.**

I. Introduction

A considerable variety of molecules are known to establish anesthesia, but their site of interaction with the cell membranes of nerve cells is still object of research [1]. The two main theories proposed for interaction with membranes are the direct binding to proteins and the perturbation of membrane lipids that surround functional proteins [1], [2]. There are evidences that support both hypothesis. One of the evidences that supports the lipid interaction hypothesis is the fact that the anesthetic potency is well correlated with induction of membrane fluidization [3].

When inducing fluidization, it is not known if the anesthetics show any preference for specific membrane domains. A type of domains that has attracted much attention lately is lipid rafts. Lipid rafts are transient microdomains with higher rigidity than the rest of the membrane [4], [5]. They are enriched in cholesterol and sphingomyelin and are associated with specific proteins [4], [5]. These microdomains are in a liquid-ordered phase (L_o), with intermediate characteristics between a gel (L_β) and a liquid-crystalline (L_α) phase, in coexistence with the bulk of the membrane that is in a fluid-like liquid-disordered (L_d) phase [6], [7], [8]. It has been shown that lipid rafts play a key role in cell signaling, membrane trafficking and signal transduction [7], [9], [10], as well as preferred entry sites for toxins and pathogens [10]. Modifications of lipid rafts may lead to diseases like Alzheimer, Parkinson, prion diseases and cancer [10], [11], [12]. Also, some drugs initiate their action in cells by interacting preferentially with lipid rafts [12], [13], [14].

In this work, the interaction between anesthetics and lipid rafts will be investigated. To model the lipid composition of these rafts, liposomes composed of the canonical equimolar raft mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), sphingomyelin (SM) and cholesterol (Chol) were prepared. Liposomes are simple but accurate models of cell membranes, since their lipid bilayer is a functional boundary like in cells [15]. Liposomes of an equimolar mixture of POPC and SM were also prepared to evaluate the effect of cholesterol on the interactions. The three anesthetics used were: tetracaine, a local anesthetic of the ester type; lidocaine, a local anesthetic of the amide type, and propofol, a general anesthetic.

In order to evaluate the interactions of the anesthetics with the model systems described, the techniques used were a quartz crystal microbalance with dissipation (QCM-D), differential scanning calorimetry (DSC) and phosphorus nuclear magnetic resonance (P-NMR). QCM-D consists on the measurement of the shifts in resonance frequency and dissipation of an acoustic shear wave that propagates across a piezoelectric quartz crystal coated with gold in our case [16], [17], [18]. The shifts in frequency and dissipation may be due to adsorption/desorption of molecules on the gold-coated surface or changes in the viscoelastic properties of the adsorbed layer [17], [18]. Oxizided gold-coated quartz crystals were used in our study because they are a good surface for adsorption of intact zwitterionic liposomes without spontaneous rupture into a solid supported lipid bilayer [19], [20]. DSC allows the thermal characterization of the samples by providing information on the phase transitions occurring within a certain temperature range [9], [21], [22]. P-NMR is very useful for the characterization of the headgroup mobility of the phospholipids, providing evidence for lipid packing and phase coexistence [8], [10].

II. Materials and Methods

A. Materials

Sphingomyelin (brain SM, porcine) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesterol (Chol), tetracaine hydrochloride, lidocaine hydrochlo-

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ride monohydrate, 2,6-diisopropylphenol (propofol), N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) hemisodium salt, chloroform , sodium dodecyl sulfate (SDS) and dimethyl sulfoxide (DMSO) were supplied by Sigma - Aldrich (St. Louis, MO, USA). Sodium chloride, methanol and Extran® MA 01 were obtained from Merck KGaA (Darmstadt, Germany). Hellmanex® II was purchased from Hellma Gmbh & Co KG (Müllheim, Germany). The gold-coated quartz crystals (AT - cut, 4,95 MHz, 14 mm diameter) for QCM-D experiments were obtained from QSense AB (Gothenburg, Sweden). Polycarbonate filters of 600, 200 and 100 nm of diameter from Nuclepore, Whatman (Kent, United Kingdom) were used for extrusion of the liposomes.

B. Preparation of liposomes

The preparation of the liposomes was performed according to the protocol provided by Avanti Polar Lipids, Inc. [23]. The amount of lipids necessary for a final concentration upon dilution of 1.12 mM (50 mM for ^{31}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 (10 mM, 0.1 M NaCl, pH = 7.4) 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. After this procedure, the resulting large multilamellar vesicle (LMV) solution was submitted to five freeze-thaw cycles. 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 (5, 5 and 10 times respectively). The liposome solutions were kept at 4°C and were used preferentially within 7 days.

C. Preparation of anesthetic solutions

Tetracaine and lidocaine solutions were prepared disolving the appropriate amounts of each anesthetic in HEPES buffer, to obtain final tetracaine concentrations of 7.5, 3.75 and 1.5 mg/ml, and 20 and 10 mg/ml for lidocaine. Since propofol is fairly insoluble in HEPES, propofol was first solubilized on DMSO and then in HEPES, to yield a final propofol concentration of 0.18 mg/ml and 0.5 % (v/v) in DMSO. This concentration in DMSO was chosen because it has been stated to not modify the properties of lipid membranes [2].

D. Dynamic Light Scattering

Dynamic light scattering (DLS) was employed to determine the size distribution of the extruded liposomes. The experiments were performed on a Brookhaven BI-200SM Goniometer Light Scattering System. 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 thermostatized to 25°C. 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.

E. 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. The normalized frequency (Δf) and dissipation (ΔD) shifts were monitored for the fundamental and up to the 9th overtone. For all the anesthetics and different concentrations, the experiments were performed at 37°C. Additional experiments at 25°C were performed for the highest concentrations of each anesthetic. For comparative purposes, the mean $\pm$ standard deviation of Δf and ΔD for each overtone were calculated. Viscoelastic modeling of the data was performed on the software QTools 3 using the Voigt model for a single layer of adsorbed liposomes. The boundary conditions for modeling are shown in Appendix A.

F. DSC

The measurements were carried out in a VP-DSC Micro-Calorimeter from MicroCal (Northampton, WA, USA). 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. The reference cell was filled with HEPES for the tetracaine and lidocaine measuments and with HEPES + 0.5% DMSO for the propofol measurements. Before the measurements, reference scans were performed with the sample cell filled with buffer. Since liposomes are disrupted upon degassing, only the buffer and anesthetic solutions were degassed prior to mixing with liposomes to avoid bubbles. The thermograms 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 effective phosphorus concentration, determined using the procotol in [24]. 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. With this limitation, the only extractable parameter is the main transition temperature of the samples.

G. P-NMR

^{31}P-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}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. All the spectra were acquired at 12, 25, 37 and 60°C for the highest concentration of each anesthetic, while the concentration effect experiments were carried at 37°C only.

III. Results and Discussion

A. DLS

The size distribution for the POPC/SM/Chol 1:1:1 liposomes was 136 $\pm$ 23 nm, while for the POPC/SM 1:1 liposomes was 112 $\pm$ 25 nm. The data are in good agreement with expected values from the extrusion through 100 nm-diameter pore membranes, with a predicted liposome size distribution between 120 and 140 nm.

B. QCM-D

First of all, it is important to ensure that the liposomes adsorbed intact. The mean frequency and dissipation values for both concentrations and temperatures (Tables I and II) are in the same range of values found in a previous study in our group for adsorption of intact liposomes in gold-coated crystals (proven by atomic force microscopy and confocal microscopy in that case) [19]. This observation supports the assumption of adsorption of intact liposomes of the compositions studied here. The high dissipation values are typical of viscoelastic films that are not fully coupled with the crystal oscillation and dissipate energy [25]. The frequency shifts are clearly dependent on the composition and temperature, which is related to the lipid phase behavior, and to the fact that liposomes do not behave like rigid spheres upon adsorption. Liposomes flatten to increase the contact points with the gold surface [25]. The less rigid liposomes deform more and this is why, at 37°C, the frequency shift for the POPC/SM liposomes is considerably less expressive than for the POPC/SM/Chol liposomes. Since the liposomes without cholesterol are less rigid and their membranes are almost completely in a liquid-disordered phase at this temperature [26], they will be more deformable, and each liposome will occupy a higher surface area. This results in less adsorbed mass per area, expressed in a lower frequency shift. At 25°C, the frequency and dissipation shifts for both compositions are quite similar, since in both types of liposomes L_o and L_d phases coexist [26].

1) Tetracaine: From Figure 1 for an example of an experiment on the action of tetracaine 7.5 mg/ml on POPC/SM/Chol liposomes at 37°C, one can see that there is a sharp frequency decrease and dissipation increase with tetracaine addition. The dissipation increase indicates tetracaine-induced swelling. However, during the stabilization period, there is a slow increase of frequency for all overtones and a decrease of dissipation for the 5th and higher overtones. This suggests partial rupture of smaller liposomes, but the larger liposomes continue to swell [27]. The hypothesis of swelling is confirmed by viscoelastic modeling, where the thickness of the layer increased approximately 50 nm in average, while the partial rupture induced fluctuations on the viscosity, with a final decrease of 0.2 mPa.s relatively to the viscosity before tetracaine addition. The determined shear modulus is also compatible with the observations, since an inital decrease of 2 kPa indicates that the liposomes became more deformable and it is compatible with the structural changes that lead to fluidization of the membrane and its swelling. From the initial and end of tetracaine action, there was an increase of approximately 5 kPa on this modulus, which can be explained by the rupture of liposomes and the formation of more rigid supported bilayer fragments strongly coupled to the crystal.

Table I
Mean $\pm$ S.E. of frequency and dissipation shifts for the 3rd overtone of adsorption experiments of POPC/SM/Chol liposomes at 25 and 37°C.

T (°C)	Δf (Hz)	ΔD (1 x 10^{-6})
25	-201 $\pm$ 16	27 $\pm$ 2
37	-204 $\pm$ 9	31 $\pm$ 2

Table II
Mean $\pm$ S.E. of frequency and dissipation shifts for the 3rd overtone of adsorption experiments of POPC/SM liposomes at 25 and 37°C.

T (°C)	Δf (Hz)	ΔD (1 x 10^{-6})
25	-199 $\pm$ 32	23 $\pm$ 1
37	-150 $\pm$ 2	24 $\pm$ 2

At the same temperature, the behavior of the POPC/SM liposomes upon tetracaine addition is clearly different (Figure 2). There is an initial sharp decrease in frequency and increase in dissipation, and these values are stable over time. Viscoelastic modeling results yield an increase of film thickness of about 10 nm and increased viscosity by 0.3 mPa.s upon tetracaine addition. The results for shear modulus modeling were, unfortunately, not evaluable. The increase in thickness and dissipation points towards tetracaine-induced swelling, while the decrease in frequency and viscosity increase may be due to a stronger adhesion force between the liposomes and the crystal (due to decrease of electrostatic repulsions between the crystal and the liposomes) or increased packing of the lipid film [29], [28].

The effect of the two temperatures used (37 and 25°C) on the interaction of tetracaine 7.5 mg/ml on the liposomes with the two compositions is shown by ΔD vs Δf (3rd overtone values) plots of Figure 3. An initial linear increase of dissipation when the frequency decreases means that the adsorbed layer swells. However, this trend is reversed and there is increase of dissipation with some extent of frequency increase, meaning that the liposomes continue to expand but there is a structural modification. This is relevant at both temperatures for the raftlike mixture. At 37°C (Figure 3), there is an additional increase of frequency with constant dissipation.

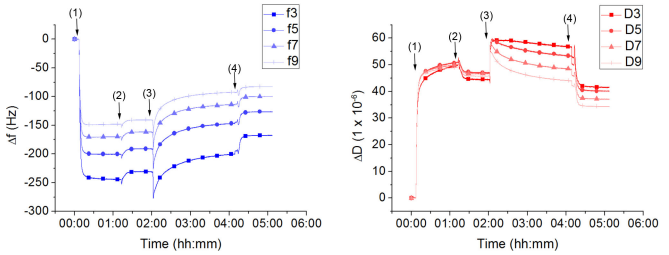

Figure 1. Example of a QCM-D experiment of interaction of adsorbed POPC/SM/Chol liposomes with tetracaine 7.5 mg/ml at 37°C. Left: frequency shifts. Right: dissipation shifts. (1) 5-min liposome introduction; (2) 10-min rinsing; (3) 5-min tetracaine introduction; (4) 10-min rinsing.

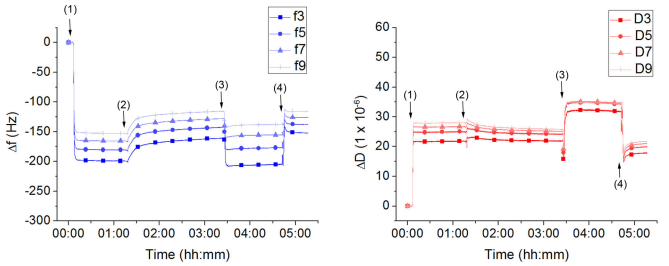

Figure 2. Example of a QCM-D experiment of interaction of adsorbed POPC/SM liposomes with tetracaine 7.5 mg/ml at 37°C. Left: frequency shifts. Right: dissipation shifts. (1) 5-min liposome introduction; (2) 10-min rinsing; (3) 5-min tetracaine introduction; (4) 10-min rinsing.

In conclusion, it is clear that the effects of tetracaine on the raftlike liposomes are more expressive at 37°C and they are fairly irreversible, while at 25°C the effects are reversible upon rinsing. For POPC/SM liposomes, it seems that tetracaine interacts slightly more at 25°C.

When studying the effect of tetracaine concentration (Figure 4), the observations for the frequency and dissipation shifts for the 3rd overtone clearly indicate that the effect of tetracaine is concentration-dependent, and that the main modifications are noticeable at 7.5 mg/ml, with a considerable increase in dissipation, indicative of liposome swelling. At 3.75 mg/ml, there is a more modest swelling on the raftlike liposomes, but there is a slight dissipation decrease for the binary mixture, indicative of a more rigid adsorbed layer. This also provides further evidence for a preferential interaction of tetracaine with raftlike domains at 37°C.

2) Lidocaine: In terms of frequency and dissipation shifts evolution with time (data not shown), lidocaine at the concentration of 20 mg/ml promotes frequency decrease for all the overtones and both compositions, but the modifications in dissipation are negligible for the raftlike mixture. Viscoelastic modeling confirms the absence of swelling, but a 0.3 mPa.s increase in viscosity and 5 kPa in the shear modulus at 37°C show effects of lidocaine on the structure of the membranes, particularly decrease of deformability. For the POPC/SM liposomes, especially at 37°C, there is dissipation increase and evidence of liposome swelling, as confirmed by viscoelastic modeling with a thickness increase of 32 nm and a 0.1 mPa.s increase in viscosity. The temperature effects on both compositions upon interaction with lidocaine are shown in Figure 5. The data confirm that the most relevant changes

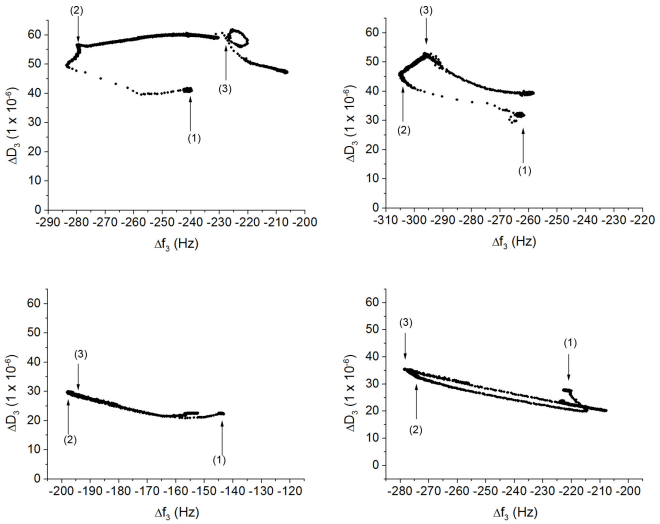

Figure 3. ΔD vs Δf plots (3rd overtone) of the interaction of tetracaine 7.5 mg/ml with POPC/SM/Chol (top) and POPC/SM (bottom) liposomes at 37 (left) and 25°C (right). (1) Start of tetracaine influx; (2) End of tetracaine influx; (3) Rinsing.

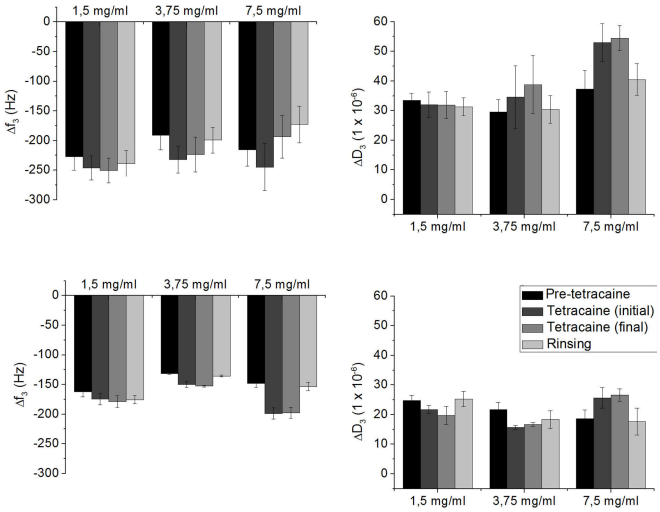

Figure 4. Values of frequency (left) and dissipation (right) shifts for the 3rd overtone upon tetracaine addition and rinsing at the three studied concentrations for POPC/SM/Chol (top) and POPC/SM (bottom) liposomes at 37°C.

are found for the POPC/SM liposomes at 37°C (Figure 5 c)), suggesting a preferential interaction of lidocaine with membranes without cholesterol.

The concentration effect study of Figure 6 provides further evidence for this hypothesis, since the concentration of 10 mg/ml does not induce any changes in the raftlike liposomes at 37°C, while it promotes frequency decrease and dissipation increase in the binary membranes, with the dissipation increase being related to swelling induced by the anesthetic.

3) Propofol: Propofol has interacted with the two membrane compositions irreversibly, since there are no major changes caused by rinsing on the frequency and dissipation shifts. Propofol induces negative frequency shifts for the two compositions and temperatures, resulting in viscosity increase, except for the POPC/SM, 25°C experiment. For the

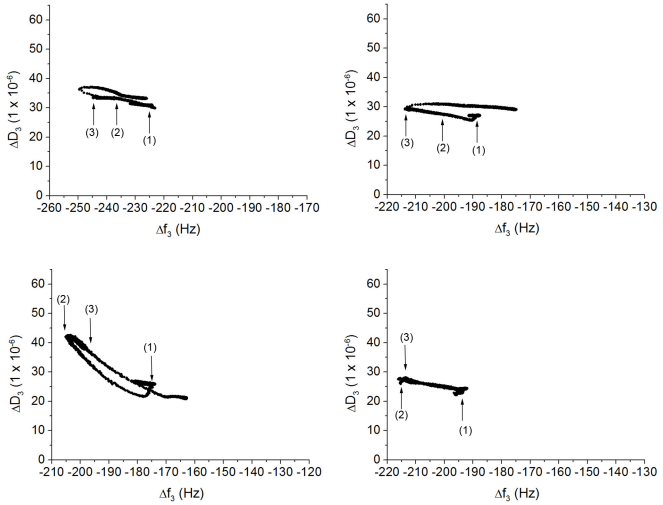

Figure 5. ΔD vs Δf plots (3rd) of the interaction of lidocaine 20 mg/ml with POPC/SM/Chol (top) and POPC/SM (bottom) liposomes at 37 (left) and 25°C (right). (1) Start of lidocaine influx; (2) End of lidocaine influx; (3) Rinsing.

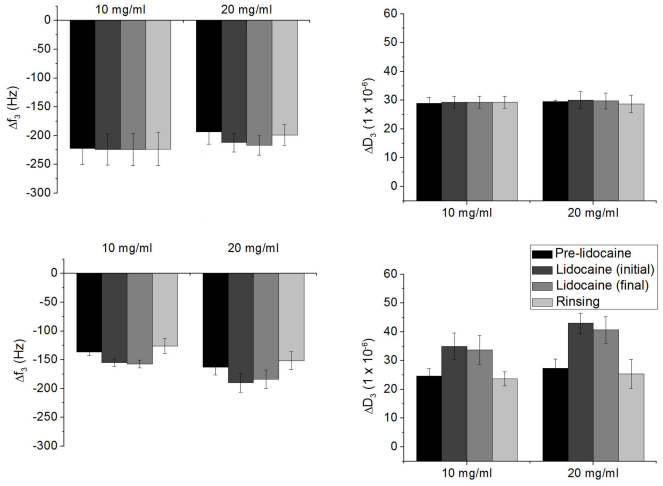

Figure 6. Values of frequency (left) and dissipation (right) shifts for the 3rd overtone upon lidocaine addition and rinsing at the two studied concentrations for POPC/SM/Chol (top) and POPC/SM (bottom) liposomes at 37°C.

experiments at 25°C there is a slight dissipation increase that explains the slight increase of the modeled thickness, and this swelling is more significant for the raftlike liposomes, where the thickness increases 14 nm. At 37°C for both compositions, the changes in dissipation are quite negligible and results in a slight decrease of the liposome layer thickness. Figure 7 shows that the effects are more intense, at both temperatures, for the raftlike liposomes. The fact that the interactions are more intense at 25°C for both mixtures implies that propofol interacts with these model membranes essentially through the liquid-ordered domains that are more frequent at this temperature than at 37°C in both mixtures [13], [26], with a preference for the raftlike domains.

C. DSC

The thermograms for the two liposome compositions in HEPES are shown in Figure 8. For the raftlike composition,

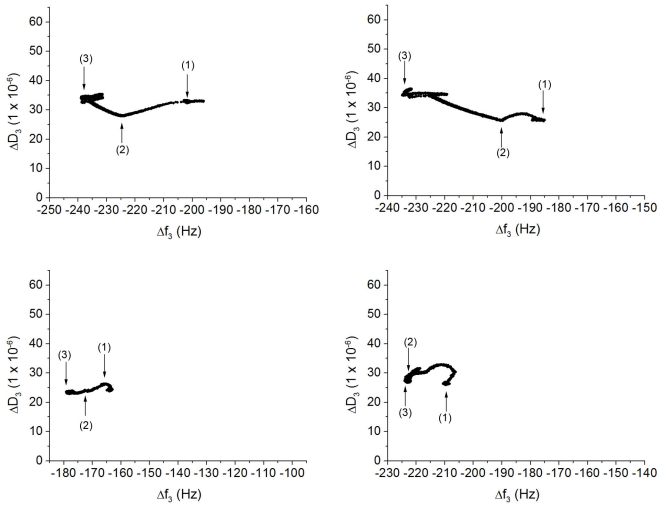

Figure 7. ΔD vs Δf plots (3rd overtone) of the interaction of propofol 0.18 mg/ml with POPC/SM/Chol (top) and POPC/SM (bottom) liposomes at 37 (left) and 25°C (right). (1) Start of propofol influx; (2) End of propofol influx; (3) Rinsing.

there is no evidence for a phase transition in the range of temperatures evaluated. However, in the binary mixture without cholesterol, a broad transition that begins below 10°C and is finished at about 40°C was observed, with a maximum of the curve at approximately 30°C. The peak corresponds to the coexistence region of L_o and L_d phases, with the L_o domains enriched in sphingomyelin [26]. At 40°C, a temperature slightly above the main transition temperature of brain SM (38°C [13]), both lipids are fully miscible on a fluid-like L_d phase.

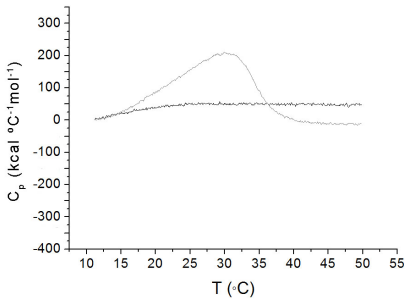

Figure 8. Thermograms of POPC/SM/Chol liposomes (black) and POPC/SM liposomes (gray) in HEPES.

The effect of the anesthetics studied on the thermotropic behavior of the mixtures is shown in Figures 9 to 11. It is noticeable that tetracaine at 1.5 mg/ml does not have any remarkable effect on the raftlike mixture thermotropic behavior, while the two highest concentrations induce a broad phase transition (Figure 9). For the binary mixture, all the concentrations induced decrease of T_m and the endpoint of the transition in a concentration-dependent manner. It is clear that tetracaine induces fluidization of both mixtures and that, for the two higest concentrations, tetracaine inhibits partially the cholesterol effect on the membrane organization. Furthermore, the large negative C_p values indicate that tetracaine may sol-

ubilize the lipids with formation of small vesicles or micelles in the two studied compositions.

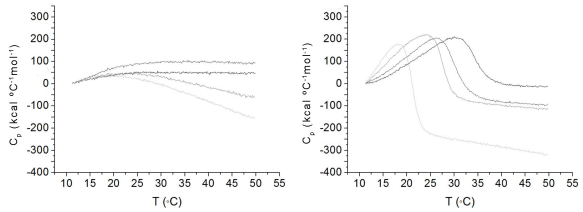

Figure 9. Effect of increasing concentrations of tetracaine on the thermotropic behavior of POPC/SM/Chol (left) and POPC/SM (right) liposomes. Black: no anesthetic; Dark gray: tetracaine 1.5 mg/ml; Medium gray: 3.75 mg/ml; Light gray: 7.5 mg/ml.

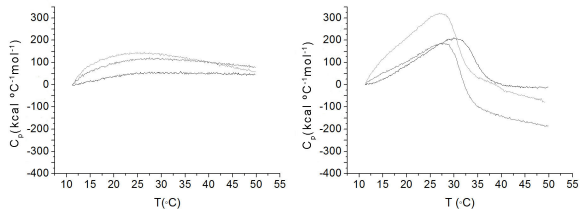

Figure 10. Effect of increasing concentrations of lidocaine on the thermotropic behavior of POPC/SM/Chol (left) and POPC/SM (right) liposomes. Black: no anesthetic; Dark gray: lidocaine 10 mg/ml; Medium gray: 20 mg/ml.

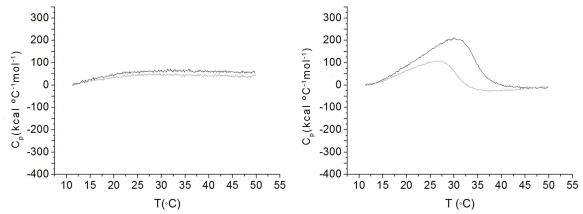

Figure 11. Effect of propofol on the thermotropic behavior of POPC/SM/Chol (left) and POPC/SM (right) liposomes. Black: HEPES + 0.5% DMSO; Gray: propofol 0.18 mg/ml.

When the raftlike liposomes are exposed to lidocaine (Figure 10), the induction of a phase transition with a broad peak revealed increased fluidization with increased lidocaine concentration. For the binary mixture, the fluidizing effect is similar for both concentrations. There is evidence for lipid solubilization for the POPC/SM liposomes, while for the raftlike mixture lidocaine might form complexes with the lipids and stay in the membrane.

Propofol (Figure 11) does not induce any remarkable effect on the phase behavior of raftlike liposomes. For the binary mixture, it induces a decrease of about 3°C on the main transition temperature. It is important to notice that 0.5% DMSO had induced some fluidization of the liposomes without cholesterol.

D. P-NMR

The cholesterol effect was assessed by comparing the spectra of both liposome compositions at the four representative temperatures, as shown in Figure 12. Significant differences between the liposomes with raftlike and binary POPC/SM

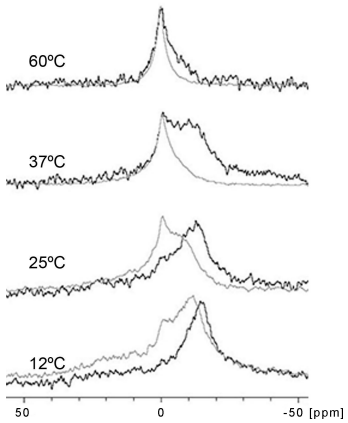

Figure 12. Spectra for the POPC/SM/Chol (black) and POPC/SM liposomes (gray) in HEPES.

composition are seen for all temperatures. In particular, the lineshapes at 37°C are quite different. The headgroups of the binary liposomes undergo pratically full isotropic motion at these temperatures, implying that the system is fully in a liquid-disordered phase. This is consistent with the thermograms for this mixture, where the endpoint of the phase transition of the POPC/SM 1:1 liposomes is of 40°C. For the ternary mixture, at 60°C the linewidth is higher, and there is also a small high-field component due to liquid-ordered domains. At 25°C, the spectrum for the POPC/SM liposomes appears with reversed assymetry 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 [26]. At 12°C, the spectra for both mixtures indicate the predominance of a lamellar phase in agreement 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. All these observations correlate well with the cholesterol-stabilizing effect of more ordered domains in a lamellar phase [8].

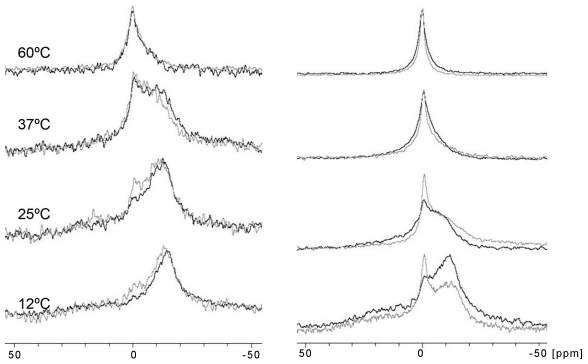

Figure 13. Effect of tetracaine 7.5 mg/ml (gray) on POPC/SM/Chol (left) and POPC/SM (right) liposomes

For the raftlike mixture, tetracaine at the concentration of 7.5 mg/ml induces fluidization of the lipid membranes,

preasumably due to solubilization of liquid-ordered domains or by incorporation of tetracaine inside inverted micelles into the membranes (Figure 13) [30]. This fluidizing effect is specially noticeable by the appearance of isotropic shoulders at 12 and 25°C, as well as a slight decrease in amplitude of the lamellar components at 37°C. For the binary mixture, there is even greater evidence of fluidization of liquid-ordered domains induced by tetracaine through the induction of isotropic peaks at the lower temperatures, which seems to be in contradiction with the results from DSC and QCM-D. The possibility of solubilization of membrane lipids due to tetracaine is stronger than in the system without cholesterol, since the appearance of isotropic components induced by tetracaine is more pronounced for the binary mixture.

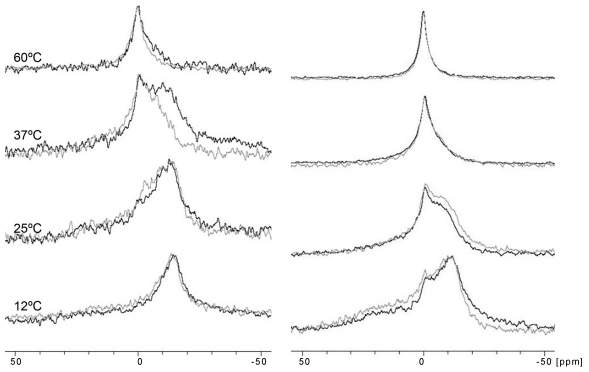

Figure 14. Effect of lidocaine 20 mg/ml (gray) on POPC/SM/Chol (left) and POPC/SM (right) liposomes

For lidocaine at the concentration of 20 mg/ml (Figure 14), there is also evidence of interaction with raftlike mixtures by fluidizing liquid-ordered domains in raftlike mixtures from the observed reduction of the contribution of the lamellar liquid-ordered phase to the lineshape at 37°C. For the binary mixture, the modifications induced in the polar headgroup motion of the phospholipids are negligible. However, this does not imply that lidocaine does not interact with the polar headgroups of these liposomes, as shown in other studies [31]. One explanation is that, in this case, lidocaine penetrates deeply into the membrane and its influence on the polar headgroup motion is not sensed by this method.

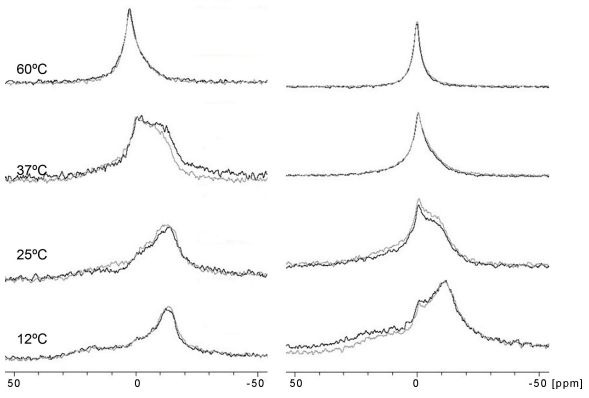

Figure 15. Effect of propofol 0.18 mg/ml (gray) on POPC/SM/Chol (left) and POPC/SM (right) liposomes

From the spectra shown in Figure 15, propofol at the concentration of 0.18 mg/ml does not modify significantly the lineshapes of the raftlike liposomes for all the temperatures, with only a slight decrease of amplitude of the high-field components indicative of a lamellar liquid-ordered phase at 37°C. For the binary mixture, no relevant modifications of the polar headgroups are observed as well. So, in the scope of these observations, propofol does not induce detectable changes in the lipid headgroup motion by this method.

IV. Conclusion

For the two local anesthetics studied, it is clear that tetracaine has a more pronounced effect on lipid organization, inducing fluidization in a concentration-dependent manner for both compositions. These effects are stronger for the raftlike liposomes, with a especially marked difference between both compositions at 37°C. At physiological temperatures, tetracaine at the concentration of 7.5 mg/ml seems to induce partial rupture of adsorbed POPC/SM/Chol 1:1:1 liposomes. Another remarkable effect of tetracaine in the raftlike liposomes is the induction of a phase transition at the concentrations of 3.75 and 7.5 mg/ml. There is also evidence, from the P-NMR technique, that tetracaine interacts with the liposomes through perturbation of the mobility of the polar headgroups. For lidocaine, the effects on lipid fluidization are not so intense, but there is a marked difference between both compositions, with swelling effects for the POPC/SM liposomes that do not occur for the raftlike mixture. There is also evidence for a different mechanism of interaction in both mixtures, since lidocaine seems to interact with the membrane core of the POPC/SM liposomes while, for the raftlike mixture, the interaction is more superficial. The higher potency of tetracaine correlates well with the higher partition coefficient of tetracaine found in literature [32].

Propofol is not directly comparable with the other anesthetics, since its concentration at the target sites is one order of magnitude smaller than the lowest value used for local anesthetics. The main observations are that propofol interacts irreversibly with the liposomes for both compositions through the membrane core of the liposomes, and the effects shown by the QCM-D technique are more expressive for the liquid-ordered domains of the raftlike composition, despite the fact that it does not induce a phase transition in raftlike liposomes. With these results, there is not a clear preference of propofol for lipid rafts. However, since propofol is known to exert its action on nerve cells through interaction with the $GABA_A$ receptor [33] and it is likely that this receptor localizes in lipid rafts [34], the possibility of interaction with this protein by modifying the lipid environment surrounding this protein is reinforced by the results of this work, that show that propofol interacts with lipid rafts to some extent.

The general conclusion is that all the three anesthetics interact with both raftlike and non-raftlike liposomes. Tetracaine seems to interact more with raftlike domains, while lidocaine interacts more with membranes without cholesterol at physiological temperatures. The results on a preference on a given liposome composition are not fully clear for propofol, but

QCM-D suggests some preference for interaction with raftlike liposomes. The use of fluorescent imaging techniques, like laser scanning confocal fluorescence microscopy (LSCFM), atomic force microscopy (AFM), and other nuclear magnetic resonance experiments may help to elucidate the mechanisms behind lipid perturbation by these anesthetics.

As a final remark, a comparison with the work of Paiva [35] 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 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), DMPC/Chol and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/DMPC/Chol liposomes, and tetracaine presented the strongest effect. However, the mechanisms of interaction were different than for the models studied on the present work. For most concentrations of lidocaine, tetracaine and propofol used, there was an increase of the frequency shift for the three compositions. In particular, tetracaine had induced rupture of DMPC liposomes. 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 on the work of Paiva. These results show that that the effects of anesthetics on lipid membranes depend on their composition and the existent lipid phases.

APPENDIX
CONDITIONS FOR VISCOELASTIC MODELING OF QCM-D DATA

The boundary conditions for estimation of film viscosity, shear modulus and thickness from the raw QCM-D data from the 3th to the 9th overtone that yielded the best fits are shown in Table III. In some cases, due to failure of convergence of the method, it was not possible to find a realistic value for the shear modulus.

Table III
BOUNDARY CONDITIONS FOR ESTIMATION OF FILM VISCOSITY, SHEAR MODULUS AND THICKNESS ON QTOOLS.

Parameter	Min.	Max.	Steps
η (Pa.s)	0.0005	0.01	50
G (Pa)	1000	1E9	50
d (m)	1E-10	1E-6	50

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