# Effect of Tetracaine on DMPC and DMPC+Cholesterol Biomembrane Models: Liposomes and Monolayers

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**Abstract**

Different types of lipid bilayers/monolayers have been used to simulate the cellular membranes in the investigation of the interactions between drugs and cells. However, to our knowledge, very few studies focused on the influence of the chosen membrane model upon the obtained results. The main objective of this work is to understand how do the nature and immobilization state of the biomembrane models influence the effect of the local anaesthetic tetracaine (TTC) upon the lipid membranes. The interaction of TTC with different biomembrane models of dimyristoylphosphatidylcholine (DMPC) with and without cholesterol (CHOL) was investigated through several techniques. A quartz crystal microbalance with dissipation (QCM-D) was used to study the effect on immobilized liposomes, while phosphorus nuclear magnetic resonance ($^{31}$P-NMR) and differential scanning calorimetry (DSC) were applied to liposomes in suspension. The effect of TTC on Langmuir monolayers of lipids was also investigated through surface pressure-area measurements at the air-water interface. The general conclusion was that TTC has a fluidizing effect on the lipid membranes and, above certain concentrations, induced membrane swelling or even solubilization. However, different models led to variable responses to the TTC action. The intensity of the disordering effect caused by TTC increased in the following order: supported liposomes < liposomes in solution < Langmuir monolayers. This means that extrapolation of the results obtain in *in vitro* studies of the lipid/anaesthetic interactions to *in vivo* conditions should be done carefully.

**Keywords:** Tetracaine; biomembrane models; liposomes; Langmuir monolayers; DMPC; cholesterol.

## Introduction

Although the molecular mechanisms of local anaesthetics action are still not completely understood, three main hypotheses are generally considered to explain them: direct interaction with voltage-gated sodium channels, induction of structural alterations in the lipid matrix of the biomembranes and action on the lipid-protein interfaces [1, 2]. In the latter two cases, the mechanisms involve nonspecific interactions of the anaesthetics with the lipid bilayers. Even the direct interaction with the sodium channels implies diffusion of the anaesthetic molecules across the phospholipid bilayers of the cell membranes, leading to structural perturbations on the lipid micro-environment of those channels activity which may cause the blockade of sodium transport [3]. Thus, further understanding of the interaction between anaesthetics and lipid structures still constitutes an important goal, which many authors have been looking for.

The majority of studies on the interaction of drugs with biomembrane models have been carried out with supported lipid bilayers (SLBs) and liposome suspensions, although some investigation has been done using monolayers. Monolayers and SLBs have been used due to their simplicity and reproducibility [4, 5]. However, liposomes are recognized to reproduce better the native cell membrane's fluidity and mobility due to their three-dimensional spherically closed structure [6, 7]. Immobilization of liposomes onto surfaces is an added value for the use of vesicles as cellular tissue mimics, proving to be very useful to detect and analyse the interactions between cell membranes and biomacromolecules or other compounds [8, 9].

Phosphatidylcholine-cholesterol biomembrane models have been widely used to study the interaction with anaesthetics, because these are the major constituents of eukaryotic plasma membranes [10-13]. Dimyristoylphosphatidylcholine (DMPC), a zwitterionic glycerophospholipid with two acyl chains, containing 14 carbon saturated bonds each, which is present in varied mammalian membranes, has probably been the most investigated lipid in this type of studies [14]. CHOL is the main sterol component in the biological membranes, being its content rather high (30 - 50 mol %). CHOL molecules are known to distribute in the regions with saturated hydrocarbon chains, forming separate liquid ordered phases which coexist with liquid-disordered phases [15], and cause a reduction in the average cross-sectional area of phosphatidylcholines in the liquid crystalline membranes (known as condensing effect)[16].

In a previous work [17], we compared the effect of several local and general anaesthetics upon different types of model membranes containing phosphatidylcholines and found that tetracaine (TTC) was the local anaesthetic that induced the strongest perturbation on DMPC and DMPC+CHOL

membranes. The interaction of TTC with an adsorbed layer of liposomes is known to be a very complex process which may alter the packing and water content of these lipid structures [18]. The effect of TTC onto the lipid bilayers depends on several factors, such as the anaesthetic concentration, the solution pH, the ionic strength, the molecular structure and physical state of the lipid. Like other local anaesthetics, TTC is known to cause destabilisation of the lipid structure, inducing an increase in its surface area and fluidity, and influencing the lipid phase transition [19]. TTC interacts in a different way with phosphatidylcholines (PC), phosphatidylethanolamines (PE), and phosphatidylserines (PS), depending on the charge of the anaesthetic and on the charge and shape of the lipid molecules [20],[21]. Structural and thermodynamic aspects of the interaction of TTC with phospholipids have been previously investigated. In particular, for membranes involving PC, questions like the anaesthetic location within the membrane, its buffer:membrane partition coefficient and apparent pKa when bound to the membrane, as well as changes in the fluidity of the bilayer, were studied using various techniques, namely, fluorescence spectrometry [13, 22] nuclear magnetic resonance (NMR) [20, 23] potentiometry [24], calorimetry and dynamic light scattering (DLS) [25].

Despite the large number of studies on the interaction of TTC with lipids, to our knowledge, a comparison of the results obtained with different types of model membranes is still missing. The objective of this work is to understand how the nature and immobilization state of the membrane models (bilayer or monolayer, supported or in suspension) influence the interaction of TTC with lipids.

In the present work, we investigated the changes induced by TTC on the mass and viscoelastic properties of the adsorbed liposome layer using a quartz crystal microbalance with dissipation (QCM-D). The results were complemented with information on the phase behavior and headgroup mobility of the phospholipids of the same liposomes in suspension, using differential scanning calorimetry (DSC) and $^{31}$P-NMR, respectively. The effect of the anaesthetic on the structure of the phospholipid monolayers was also investigated through surface pressure - area measurements using the Langmuir technique.

## Experimental

### *Materials*

The phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and cholesterol (CHOL) were supplied by Avanti Polar Lipids. Chloroform, N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), sodium chloride, and the anaesthetic tetracaine hydrochloride (TTC) were obtained from Sigma-Aldrich. HEPES buffer (10 mM HEPES and 0.1 M NaCl in Milli-Q water; pH = 7.4) was

used in the preparation of liposomes and anaesthetic solutions. TTC solutions were prepared with concentrations ranging from 2.5 mM up to 25 mM, which is the concentration of the pharmaceutical formulations (e.g. Tetracaine B. Braun Medical).

Solutions of Hellmanex II 2% (Hellma GmbH) and sodium dodecyl sulfate (SDS, Sigma-Aldrich) 3% were used to clean the QCM-D parts and the quartz crystals, respectively.

Sensors for QCM-D experiments were gold-coated AT-cut 5 MHz piezoelectric quartz crystals (diameter 14 mm) from Q-Sense.

*Methods*

*Preparation of the liposomes*

Unilamellar liposomes were prepared by the extrusion method. Defined amounts of DMPC and CHOL (when was the case) were weighed to obtain liposomes suspensions of DMPC and DMPC+CHOL (70:30 mol%) with a concentration of lipids of 0.7 mg/mL (for QCM-D and DSC) and 50 mg/mL (for $^{31}$P-NMR). The lipids were dissolved in chloroform and dried, under rotation, with a nitrogen stream. The lipid film was then dried in a vacuum oven, during a minimum of 3 h, to ensure the complete removal of the solvent. Hydration of the film was done with HEPES buffer at a temperature ≈10 ºC above the temperature of the gel to liquid crystalline phase transition ($T_m$ = 23.7 ºC for DMPC , $T_m \approx$ 30 ºC for DMPC+CHOL[17]), alternating with vortex agitation. This procedure took about 1 h. The obtained suspension was subjected to 5 cycles of freezing in liquid nitrogen and thawing in a water bath at the temperature referred above. After that, the suspension was extruded several times at the hydration temperature through polycarbonate membranes (Nucleopore, Whatman) of decreasing pore size (5 times 600 nm, 5 times 200 nm and 10 times 100 nm), under pressurized nitrogen. Liposome suspensions were stored at 5 ºC and used within 2 weeks. Liposome size was checked by dynamic light scattering (DLS) using a Spectra Physics model 127 He-Ne laser (632.8 nm, 35mW) and a Brookhaven instrument with a BI-200SM goniometer, a BI-2030AT autocorrelator and a APD detector. The mean diameter of the liposomes determined by this method was 102 ± 5 nm.

*Quartz crystal microbalance measurements*

A quartz crystal microbalance with dissipation (QCM-D, Q-Sense model E4) was used to investigate the interaction of the anaesthetic with the adsorbed liposomes, by measuring the normalised frequency change of the gold coated quartz crystals ($\Delta f/n$, where $n$ = 1, 3, 5, 7, 9, 11, 13 corresponds to the

number of the overtone of the fundamental frequency, 5 MHz) and their dissipation change ($\Delta D$), upon sequential addition of HEPES (baseline), liposome suspension, HEPES (rinsing), TTC solution and HEPES (rinsing). All injections were done during approximately 10 minutes, at a flow rate of 0.1 mL/min, and after that, the flux was stopped and frequency and dissipation signals were left to stabilize. The experiments were done at 25ºC and the results are averages of, at least, four independent measurements.

After each experiment, the sensors were sonicated 5 min in SDS solution, abundantly rinsed with Milli-Q water and sonicated 2x5min in Milli-Q water, and finally blown dried with nitrogen. Immediately before use, the crystals were submitted to two successive UV/ozone treatments (10 min each), separated by rinsing with Milli-Q water and drying with nitrogen flow. Microbalance parts were washed with Hellmanex solution and then abundantly with Milli-Q water.

QCM-D data were processed, using the software from Q-Sense QTools 3, for viscoelastic modeling. The Voigt model was applied to estimate the thickness and viscosity of the adsorbed film, considering a single layer and using the frequency and dissipation shifts of all overtones. The viscosity and density of the fluids were considered equal to the values of water (0.001 Pa.s and 1000 $Kg/m^3$, respectively), and the density of the film was kept constant and equal to 1060 $Kg/m^3$, as assumed by Viitala *et al*. for anionic phospholipid films [26]. The minimum and maximum values attributed to viscosity, shear modulus and thickness of the adsorbed film, which resulted on minimization of the Chi-Square error for the fittings, were respectively 0.001 - 0.01 Pa.s, $1x10^3$-$1x10^6$ Pa and $1x10^{-8}$-$2x10^{-7}$ m. The values presented for the thickness and viscosity of the films are the average of the results of four experiments.

*Nuclear magnetic resonance ($^{31}$P-NMR)*

$^{31}$P-NMR spectra of both liposomes suspensions were obtained in the absence and in the presence of TTC, using an equipment that comprises a double bay console Bruker Avance II+ with a BBO probe and a 500 MHz UltraShield Plus Magnet, operating at a magnetic field of 11.746 T and a $^{31}$P frequency of 202.457 MHz. In the experiments with anaesthetic, the thermostated liposomes suspensions were mixed with appropriate amounts of a concentrated TTC solution, immediately before running the $^{31}$P-NMR spectra, in order to obtain the desired final concentration. The accumulation time for each experiment was 40 minutes. All the spectra were acquired at 12, 25 and 37 ºC and obtained with gated broadband proton decoupling. The chemical shift values were quoted in parts per million (ppm) with reference to phosphoric acid 85%, being positive values associated to low-field shifts. The spectral width was of 80 KHz (400 ppm), interpulse time was 2s and a 30º radiofrequency pulse (11.3 μs) was

applied. The spectra, processed running the TopSpin 2.1 software (Bruker Biospin GmbH, Germany) under Linux Red Hat 4, were submitted to exponential multiplication before Fourier transformation, resulting in a 50 Hz line broadening to improve the signal-to-noise ratio.

*Differential scanning calorimetry (DSC)*

DSC experiments were performed in a MicroCal VP-DSC microcalorimeter. A scan rate of 1 ºC $min^{-1}$ was used in both heating and cooling cycles, covering the temperature range of 10–50 ºC. At least three heating/cooling cycles were performed in each experiment and three independent experiments were done for each system. The mixtures of TTC solutions and liposomes suspensions (0.7 $mg \cdot mL^{-1}$) were prepared immediately before conducting the tests. The Origin 7.0 software was used to subtract the baselines from the thermograms and to convert the raw data into data of molar heat capacity. The thermodynamic parameters were obtained by numerical integration of the peaks and/or by using the non-two-state model provided by the software.

*Surface Pressure–Area measurements*

Surface pressure-area ($\pi$–$A$) isotherms were carried out on a KSV 2000 Langmuir-Blodgett system (KSV instruments, Helsinki) installed inside a protective box. The homemade mini trough has a total surface area of 84 $cm^2$ and the volume of the subphase was 40 mL. The TTC-containing subphase, from 0 to 12.5 mM, was prepared in the HEPES buffer solution (pH 7.4) with ultra pure water purified by means of a Milli-Q plus water purification system. The spreading solutions (0.3-0.4 mM) were prepared in a chloroform:methanol mixture (4:1, v/v). Procedures for $\pi$–$A$ measurements and cleaning were described elsewhere [27]. In each measurement, the lipid solution was spread on the subphase with a SGE gastight micro syringe. The spread monolayer was compressed 10, 30 or 60 min after spreading, by one moving barrier at the compression rate of 0.2 $Å^2$ $molecule^{-1}$ $min^{-1}$. All the $\pi$–$A$ measurements were carried out at the room temperature (~25 ºC) and were repeated at least twice to ensure reproducibility.

**Results and Discussion**

*QCM-D experiments*

Figure1 shows typical QCM-D curves obtained for DMPC and DMPC+CHOL liposomes interacting with TTC solution with a concentration 25 mM. The addition of the liposome suspensions to the quartz

crystals immersed in HEPES (step 1) leads to a large decrease in all the harmonics of the crystal resonance frequency and to a significant increase of energy dissipation. The non-overlapping of the $\Delta f/n$ responses for the different overtones and the high values of $\Delta D$ are indicative of the formation of viscoelastic films. According to our previous study [28], the values observed for the frequency and dissipation shifts are consistent with the presence of monolayers of deformed, but intact, vesicles. The larger shifts obtained for the cholesterol containing liposomes will be commented later, together with the results of the viscoelastic modelling. The small changes in frequency and dissipation observed after rinsing with HEPES (step 2) for both types of liposomes mean that the majority of the vesicles remain irreversibly adsorbed on the surface. The contact with the 25 mM anaesthetic solution (step 3) led to different results, depending on the liposomes composition.

For DMPC liposomes (Figures 1 A and B), an initial sharp negative frequency shift took place for all overtones followed by a drastic increase in frequency, while dissipation presented a marked increase upon TTC addition and then an accentuated decrease for all overtones. Furthermore, the overlapping degree of $\Delta f/n$ for the different harmonics increases, indicating an increase in rigidity. This behaviour suggests rupture and eventual removal of liposomes from the crystal surface, being the final values of $\Delta f/n$ and $\Delta D$ consistent with the presence of supported bilayer patches [29, 30]. Experimental results reported in the literature confirm that at high concentrations, TTC originates significant changes in the membrane structure: it may form TTC-lipid mixed micelles or dimers and ultimately dissolve the membranes, due to its surfactant properties [13].

In the case of DMPC+CHOL liposomes (Figures 1C and D), the increase in frequency was not so intense meaning that these liposomes are less susceptible to the anaesthetic action. The loss of adsorbed mass may be attributed to water release from interstices or from the liposome cores and/or to the rupture of some vesicles. The higher resistance of the cholesterol-containing liposomes at 25°C should be due to the fact that the lipid mixture is below the transition temperature (≈ 30°C), thus in the gel state. Similar conclusions about the effect of CHOL were reached by us in a previous study, involving various local anaesthetics [17]. In contrast with frequency, dissipation increased for the lower harmonics and decreased for the higher ones, upon TTC addition. Taking into account that higher harmonics probe closer to the surface of the crystals than lower harmonics, which probe the region next to the liquid [31], these results suggest that the liposome layer must be heterogeneous and that its fluidity increases from the solid to the outer liquid interface. This is in agreement with the results of Cheng *et al.*[32], which evidenced differential effects of CHOL on the conformational dynamics of DMPC acyl chains at different depths of the membranes. Moreover, the increased fluidity at the liquid

interface caused by TTC on the CHOL-containing liposomes may be associated with the formation of finger-like structures at the outer surface of the liposomes, as reported by other authors [25].

In order to investigate the effect of TTC concentration, QCM-D experiments were also carried out with more diluted TTC solutions (figures not shown). Although the dispersion of the results is large, the effect of the addition of more dilute TTC solutions (12.5 mM and 5 mM) was distinct from that observed with TTC 25 mM. In the case of DMPC, the peaks in $\Delta f/n$ and $\Delta D$ following the addition of the anaesthetic solutions were rare, and, if present, they were much less pronounced than those produced by TTC 25 mM. The subsequent strong increase of frequency and decrease of dissipation observed with the concentrated TTC solution, which was attributed to rupture and eventual removal of liposomes from the crystals surface, was not observed with the diluted anaesthetic solutions. Instead, a small decrease of frequency and increase in dissipation occurred, indicating that the anaesthetic causes some swelling of the adsorbed vesicles. The same effect, but less intense, was observed with DMPC+CHOL liposomes, which is consistent with the higher resistance of the CHOL-containing membranes. A similar behaviour concerning the effect of TTC concentration on the lipid membranes was reported by Takeda *et al.*[25].

Further insight into the effect of TTC on the lipid membranes may be obtained by plotting $\Delta f/n$ *vs* $\Delta D$ which eliminates time as a variable. We focus on the most concentrated TTC solution because it corresponds to that generally used in the pharmaceutical formulations. Figure 2 presents the graphics $\Delta f/n$ *vs* $\Delta D$, for the 3$^{rd}$ and 11$^{th}$ overtones, correspondent to the addition of TTC 25 mM to DMPC and DMPC+CHOL liposomes, and subsequent rinsing.

The behaviour of the two types of liposomes under the anaesthetic action is clearly different. Upon addition of TTC to the DMPC liposomes, there is an initial decrease in frequency along with an increase in the dissipation, which reflects the adsorption of the anaesthetic and its swelling effect. This trend changes after a certain point: the sensed mass starts decreasing (frequency increases), while the dissipation still increases. This means that TTC fluidizes the membrane and simultaneously leads to the progressive loss of the vesicles content. When rupture and eventual removal of liposomes starts, both the frequency and the dissipation decrease even after stopping the TTC influx or rinsing with the buffer. The final values are consistent with the formation of "islands" of a rigid adsorbed layer. The shape of the curves for the 3$^{rd}$ and 11$^{th}$ overtones is similar, indicating that the described changes are both sensed at the membrane/liquid interface and near the crystal surface. Contrarily, for DMPC+CHOL liposomes, the effect of TTC depends on the depth. In the outer zone of the adsorbed layer (see 3$^{rd}$ harmonic curve), TTC leads to an increase of the fluidity of the membrane, although much less pronounced than that observed for DMPC liposomes, accompanied by a significant loss of mass. Rinsing with the buffer

allows the initial rigidity to be practically recovered. In the inner part of the membrane ($11^{th}$ harmonic), the loss of mass is always accompanied by an increase of rigidity.

The viscoelastic modulation, carried out using the Voigt model, for the three TTC concentrations studied, led to the values of viscosity and thickness of the adsorbed layers presented in Figure 3. Comparison of the results obtained with DMPC and DMPC+CHOL liposomes shows that the later form thicker and more viscous layers, as a result of their lower deformability. Rinsing with buffer caused negligible changes on the thickness and viscosity of the adsorbed liposome layers.

The contact with TTC 25 mM led in both cases to an initial increase of thickness and decrease of viscosity. These effects were more significant for DMPC liposomes, especially in the case of the thickness, which increased above 120%. The instability induced by TTC on the DMPC liposomes is evidenced by the large scattering of the data correspondent to the period of stabilization (TTC final, not shown in Figure 3): in about half of the experiments, the liposomes layer thickness remained high (average value 165 nm), but in the remaining cases an abrupt decrease was observed, reaching an average thickness of 77 nm. Rinsing with HEPES led to a significant decrease of the adsorbed layer thickness, which reflects the formation of the bilayer patches referred above, while the viscosity remained constant. For the DMPC+CHOL liposomes layer, the thickness and viscosity did not change further after the first contact with TTC. After rinsing, the thickness decreased, although remained slightly higher than the initial one, while the viscosity almost did not changed, assuming a value lower than the initial.

As expected, the effect of TTC 12.5 mM on the thickness and viscosity of the lipid membranes was less pronounced. The decrease of viscosity caused by the anaesthetic was negligible, and the increase of thickness was smaller than that observed with TTC 25mM, especially in the case of DMPC. Rinsing with HEPES produced different effects on DMPC and DMPC+CHOL liposomes layers thicknesses: while for DMPC a substantial decrease was observed, being the final average thickness relatively close to the initial one, for DMPC+CHOL the decrease was not significant.

For the lowest TTC concentration studied (5 mM), the thickness and viscosity of both DMPC and DMPC+CHOL liposomes layers almost did not changed, indicating that at this concentration, the effect of the anaesthetic on the immobilized liposomes membranes is negligible.

*$^{31}$P-NMR experiments*

The structural changes induced by TTC on the liposome membranes were investigated by $^{31}$P- NMR as a function of the temperature and of the anaesthetic concentration. The spectra were obtained at 12, 25 and 37ºC but the results presented in Figure 4 refer only to the temperatures 12 and 37ºC.

In the absence of tetracaine, the spectra of DMPC liposomes show that the isotropic component of the motion of the lipid headgroups increases with the temperature, suggesting that the number of degrees of freedom is larger at higher temperatures because the system tends to be more disordered. Addition of CHOL to the DMPC led to a different temperature dependence of the spectral line shape. At 37ºC (above $T_m \approx 30$ ºC), the anisotropic component becomes dominant as evidenced by the broadening of the peak which means an increase of the membrane order. However, below $T_m$ (at 12 or 25ºC), CHOL induces disorder in the liposomes which yields sharper lines. A similar behaviour was reported previously by other authors [20]: above $T_m$, CHOL induces the conformational ordering of the lipid chains, leading to an increase of the membrane mechanical strength and a reduction of passive permeability, while maintaining membrane fluidity [33, 34]; below $T_m$, it was found to have a disordering effect. In the present work, the determination of the exact values of the anisotropic parameters was not possible, due to a rather complex shape of the $^{31}$P-NMR signal, but a rough estimation based on the comparison of the half-height peak widths is in agreement with the conclusions of Auger *et al.* [20].

The effect of TTC on DMPC liposomes depends strongly on the temperature. At 12ºC, below $T_m$, the DMPC membrane is in the solid-gel state. Addition of TTC leads to sharper $^{31}$P-NMR signals that result from the fluidization of the membrane and consequent raise of the disorder. This effect becomes more accentuated with the increase of the anaesthetic concentration. At 37 ºC, above $T_m$, the DMPC membrane is in a liquid-crystalline phase and the addition of TTC shows almost no effect at all studied concentrations of the anaesthetic. This seems contradictory with data reported in the literature, which show that TTC has a higher binding affinity to lipids in the liquid-crystalline phase than in the solid-gel phase [13, 22, 35]. However, we must stress that $^{31}$P-NMR yields information only about the ordering state of the membrane, and not about the affinity between the drug and the lipids.

For the DMPC+CHOL membranes mixtures, at 12ºC (below $T_m$) the addition of TTC does not affect the line shape of the spectra, meaning that it does not interfere with the properties of the lipid system which already presents some disorder. At 37ºC, the addition of low concentrations of the anaesthetic has no significant effect on the highly ordered liposome membrane. However, at the highest studied concentration, a sharpening of the signal indicates that the anaesthetic changes the properties of the membrane, increasing its fluidity.

*DSC analysis*

The effect of TTC on the thermotropic behaviour of DMPC and DMPC+CHOL liposomes was studied by DSC, as a function of the anaesthetic concentration. The obtained thermograms are shown in Figure

5. In the experiments with DMPC+CHOL liposomes no baseline could be defined, and the normalized peaks could only be compared in a qualitative way.

The gel-liquid crystalline phase transition temperature ($T_m$) of DMPC is 23.7 ºC, in agreement with the literature [36, 37]. The new experiments confirm the continuous decrease of $T_m$ as the anaesthetic concentration increases, suggesting a chain disordering effect in the lipid membrane. The analysis of $\Delta H_{cal,}$ given by the peak area, may give information about the effect (location) of the anaesthetic molecules in the liposomes. At low concentration (2.5 mM), TTC does not affect $\Delta H_{cal,}$ but causes a decrease in $T_m$. This fact may reflect a superficial interaction between the anaesthetic and the lipid molecules [38] and/or the intercalation of TTC molecules between the chains of the lipids without lowering the stability of the membrane [39]. At 5 mM, a local minimum in $\Delta H_{cal}$ was observed. Increasing the concentration to 8.3 mM gives rise to a $\Delta H_{cal}$ increase, despite the continuous $T_m$ decrease. This behavior points to the formation of an interdigitated phase induced by TTC. According to some authors [25] , TTC has the ability to induce the formation of an interdigitated gel phase, only if it is in the charged form. In this work, at neutral pH, TTC is partially in the charged form. A similar behaviour was observed by Takeda *et al*. [25] with multilamellar DMPC liposomes, for TTC concentrations between 6 and 22 mM and was attributed to bilayer interdigitation.

One can also observe that a small shoulder emerges in the high temperature side of the peak. This shoulder increases with TTC concentration and suggests the formation of two phases, one rich and the other poor in TTC. Increasing TTC concentration to 12.5 mM gives rise to a decrease in $\Delta H_{cal}$, which is probably associated with the onset of a vesicle-to-micelle transformation. It was not possible to test with DSC the effect of higher TTC concentrations on this type of liposomes, due to equipment limitations in the low temperature range ($T \geq 10$ºC). However, these results suggest a growing tendency of the anaesthetic to induce phase separation that may lead to liposome solubilization, as observed in QCM-D, for a TTC concentration of 25 mM. This hypothesis is also supported by the study of Takeda *et al*. [25], who observed that above 22 mM, the anaesthetic induced the appearance of new phases, resultant from the solubilization of the membranes.

The presence of CHOL in the liposomes increases $T_m$, broadens the peaks, and causes a huge decrease in the energy associated with the transition. For the pure DMPC+CHOL liposomes, the main transition temperature becomes ≈ 30ºC, and a small sharper peak, appears superimposed with the main peak, at around 22 ºC. The existence of two superimposed peaks that tend to merge as the number of heating/cooling scans increases, suggests domains with different CHOL contents [40, 41]. According to the literature, at low temperatures, the mixture of DMPC with 30 mol% CHOL is mainly in a liquid

ordered phase, $l_0$, coexisting with a small amount of solid-gel phase [42]. At higher temperatures, the solid phase disappears and, between ≈ 30-37ºC, part of the liquid ordered phase gives rise to a liquid disordered phase, $l_d$. This $l_d$ phase is associated with low CHOL concentrations, while the $l_0$ phase is associated with high CHOL concentrations [43]. The obtained results point to the initial presence of two phases that merge in one phase, probably the $l_0$ phase. The broad and small enthalpic peak at ≈ 30ºC may be related with the transition from $l_0$ to the $l_d$ phase [42].

Concerning the effect of TTC on DMPC+CHOL liposomes, although quantification of $\Delta H_{cal}$ was not possible, it is not very much affected upon addition of solutions up to 5 mM of TTC, but apparently, increases slightly for 12.5 mM. We may speculate that at the concentration of 12.5 mM, TTC assists cholesterol in its condensation effect of lipids, inducing interdigitation, and/or increasing the headgroups interactions. The results presented in the literature relatively to the effect of CHOL in these processes are contradictory. Mostly CHOL, at a high concentration, seems to inhibit the interdigitation process [44], **Error! Bookmark not defined.**], but at least one study reports rigidification of a DMPC liposome membrane containing 40% of cholesterol through the action of capsaicin [45]. At 25 mM, the DSC profiles (Figure 5B) show that TTC induces an abrupt decrease of the baseline at the upper temperature side of the transition peak, suggesting that solubilization of the liposomes may occur.

Comparison of the variation of $T_m$ as a function of the anaesthetic concentration, for DMPC and DMPC+CHOL liposomes, shows that the decrease in $T_m$, as the concentration of the anaesthetic increases, is slightly less pronounced in CHOL containing liposomes. This means that the existence of CHOL not only stabilizes liposomes but also diminishes their permeability to the anaesthetic. The higher resistance of DMPC+CHOL membrane to the TTC action is in agreement with the $^{31}$P-NMR results, despite the difference in the ratios lipid:anaesthetic used in both techniques (ten times larger for the $^{31}$P-NMR experiments than for DSC). Several other authors reached similar conclusions, as, e.g., Zhang *et al.* [13], who studied TTC-DMPC interactions, and attributed to cholesterol a condensing effect of the bilayer and the responsibility for the solubility decrease of TTC in the liposome membrane.

*$\pi$–A isotherms*

Figure 6 shows the effect of the TTC-containing subphase (0, 0.01, 0.1, 2.5, 5 and 12.5 mM) on the DMPC and DMPC+CHOL monolayers. For all the $\pi$ –*A* isotherms presented, 10 min was the waiting time adopted, for the solvent evaporation, before starting the compression run. However, for 0.1 mM and 2.5 mM content of TTC, the compression isotherms were repeated at 30 and 60 min after spreading

in order to see the influence of time on the interaction of TTC with DMPC. It was found that the complete compression isotherms (omitted) do not change significantly within the interval of 10–60 min. Pure DMPC (panel on the left) behaves as a liquid-expanded monolayer with the lift off at ≈ 104 $Å^2$/molecule and the collapse at ≈ 42 mN/m. The isotherm of DMPC mixed with CHOL (panel on the right) shifts to smaller areas than expected for an ideal mixture of both components and collapses at ≈ 45 mN/m. In fact, the lift-off of the mixed monolayer (≈ 72 $Å^2$/molecule) occurs at areas lower than the averaged area calculated based on the area of single monolayers of both components (≈ 85 $Å^2$/molecule). This behaviour is consistent with the condensing effect of cholesterol on lipid monolayers, as previously referred [46]. The studies reported in the literature, involving DMPC, CHOL and DMPC:CHOL (7:3) mixture show slight differences that can be assigned to different experimental conditions such as buffer solution and temperature [47-50] The results obtained in this work remain within the dispersion found for the reported studies.

As the concentration of TTC in the subphase increases, the monolayer of DMPC becomes progressively more expanded: the area at the lift-off deviates to larger areas, while the collapse surface pressure decreases. This indicates that TTC disrupts the DMPC organization at the air–water interface.

Low contents of the anaesthetic (0.01 and 0.1 mM) do not affect significantly the lipid monolayer at high surface pressures. TTC penetrates in the lipid expanded monolayer at low surface pressures being progressively excluded upon compression. At higher contents of TTC (2.5, 5 and 12.5 mM) the lipid monolayer changes drastically, TTC dissolves the lipid monolayer and the collapse surface pressure decreases significantly with the TTC content in the subphase.

The effect of TTC on the binary mixture DMPC+CHOL (panel on the right), follows a similar trend to that observed with DMPC.

Common features to both systems DMPC and DMPC+CHOL, are the drastic decrease of the collapse surface pressure of the monolayer with the increase of TTC content in the subphase and the nearly invariance of $\pi$-A isotherms at $\pi$ < 15 mM/m for TTC contents higher than 2.5 mM. The main difference remains in the swelling extension of the monolayer induced by TTC, which is much lower in the presence of CHOL.

The dashed lines in Figure 6 allow comparison of the expansion effect per lipid molecule ($\Delta A$) induced by TTC for both DMPC (A) and DMPC+CHOL (B) monolayers at 5 mN/m and 10 mN/m, as a function of the TTC content in the subphase. The area increase per lipid molecule is very pronounced at the lower surface pressure, and decreases with the increase of $\pi$ in both systems. However, at the same surface pressure, the monolayer swelling becomes less pronounced (~1/2) in the mixed monolayer than

for the pure DMPC, which is consistent with the condensing effect and stronger interactions induced by the presence of CHOL in the monolayer. This is in agreement with the QCM-D data previously described. In fact, TTC gives rise to a larger increase in the liposome layer thickness in the absence of CHOL for [TTC] ≥ 12.5 mM. It is interesting to note that, while in the Langmuir monolayers the swelling effect of TTC remains nearly constant for 2.5 ≤ [TTC] ≤ 12.5 mM in both systems, the QCM-D technique only allows the detection of the swelling effect at [TTC] ≥ 12.5 mM.

**Conclusions**

The effect of the local anaesthetic TTC on the lipid bilayer of DMPC and DMPC+CHOL unilamellar vesicles was investigated using supported liposomes by QCM-D, and liposomes in solution by $^{31}$P-NMR and DSC. The results were compared with those obtained with Langmuir monolayers of the same lipid compositions, through surface pressure-area measurements.

QCM-D shows that the interaction of TTC at a concentration ≥ 12.5 mM, with immobilized liposomes of DMPC and DMPC+CHOL, results in the membrane fluidization and swelling of the vesicles, with the consequent decrease of viscosity and increase of thickness of the adsorbed layer. These effects were more significant for the highest studied TTC concentration (25 mM). For this concentration, pure DMPC liposomes suffered solubilization of the lipid membrane, while DMPC+CHOL liposomes were more resistant: rupture was never observed and the increase of fluidity was less pronounced. In the CHOL-containing liposomes, the anaesthetic action on the membrane fluidity varies with depth: it is more important at the liquid interface which may be explained by the formation of finger-like structures at the outer surface of the liposomes.

DSC results confirmed the disordering effect produced by TTC on the lipid membrane of the liposomes in suspension. For the pure DMPC liposomes, TTC, at a concentration > 5mM, induces membrane interdigitation and phase separation in regions with different contents of TTC. The presence of CHOL was found to stabilize the liposomes through the reduction of their permeability to TTC. However, in contrast with the QCM-D, the DSC results suggest that solubilization of both types of liposomes may occur in the presence of TTC (25 mM). This difference may be attributed to the experimental conditions used in both techniques. Besides the different immobilization state of the liposomes, which may influence their interaction with the anaesthetic, different temperature regimes were used in both techniques. While in the DSC, the systems were submitted to heating/cooling cycles between 10 and 50ºC, in QCM-D the experiments were performed at a constant temperature of 25ºC.

$^{31}$P-NMR analyses were carried out for several TTC concentrations, at two different temperatures: below and above the gel-liquid crystalline phase transition temperatures ($T_m$) of the lipids which constitute the membranes. Below $T_m$, increasing TTC concentration favours fluidization of the membrane of pure DMPC liposomes, but does not affect the DMPC+CHOL liposomes whose membrane is already in a higher disordered state. Above $T_m$, the opposite behaviour is observed: only DMPC+CHOL membranes are affected, suffering an increase of fluidity in the presence of TTC (25mM).

The study of the Langmuir monolayers showed that TTC disrupts the lipid organization of both lipid membranes, leading to its expansion. This effect is much more pronounced for pure DMPC than for the mixed monolayer and is in agreement with the QCM-D results, where TTC gives rise to a larger increase in the liposome layer thickness in the absence of CHOL (for [TTC] ≥ 12.5 mM). However, the swelling effect of TTC on the Langmuir monolayers remains nearly constant for [TTC] ≥ 2.5 mM both with DMPC and DMPC+CHOL, while the QCM-D technique detects swelling of the immobilized liposomes only at [TTC] ≥ 12.5 mM.

Overall, the results obtained with different techniques and various model membranes converge into the conclusion that TTC has a fluidizing effect on the lipid membranes and, above certain concentrations, induces swelling or even solubilization. It affects more deeply the DMPC than the DMPC+CHOL membrane. The stability of the lipid membrane towards TTC action depends on the physical model adopted for the cellular membrane, on the the ratio of anaesthetic/lipid(s) concentrations and on the characteristics/limitations of the experimental techniques. The intensity of the disordering effect caused by TTC increases in the following order: supported liposomes < liposomes in solution < Langmuir monolayers. This is consistent with the fact that the fraction of lipid molecules which are exposed to TTC increases in the same order: part of the external layer in the supported liposomes, the whole external layer for the liposomes in solution and all the molecules in the monolayer.

The conclusions of this study show that extrapolation of the results obtained in *in vitro* studies to *in vivo* conditions should be done with great care.

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**Figure captions**

**Figure 1:** Variation of the normalized frequency $\Delta f/n$ and dissipation $\Delta D$ for all harmonics of the fundamental frequency n =3, 5, 7, 9, 11 and 13 (from black to light grey) in typical experiments carried out with DMPC (A and B) and DMPC+CHOL (C and D) liposomes, consisting in: 1) contact of the gold-coated quartz crystals with the liposomes suspension, 2) rinsing with HEPES, 3) addition of the TTC solution (25 mM). The origin corresponds to the quartz crystals in contact with HEPES. Figs 1C and 1D reproduced with permission from reference [17]. Copyright 2012 Elsevier.

**Figure 2:** Typical $\Delta f/n$ vs $\Delta D$ curves for the interaction of TTC 25 mM with DMPC (A) and DMPC+CHOL (B) liposomes. The results are presented for the $3^{rd}$ and $11^{th}$ overtones.

**Figure 3:** Viscosity (A) and thickness (B) of adsorbed DMPC and DMPC+CHOL liposomes interacting with TTC.

**Figure 4:** Effect of TTC concentration on $^{31}$P-NMR spectra of DMPC (A and C) and DMPC+CHOL liposomes (B and D) at 12 and 37ºC, respectively.

**Figure 5:** DSC thermograms of DMPC (A) and DMPC+CHOL (B) liposomes, in the presence of different TTC concentrations.

**Figure 6:** $\pi$-$A$ isotherms of DMPC (A) and DMPC+CHOL mixture (B) on buffered subphases containing TTC with different concentrations. Dashed lines allow comparison of the expansion effect per lipid molecule ($\Delta$A) induced by TTC at surface pressures 5 mN/m and 10 mN/m.

**Figure 1**

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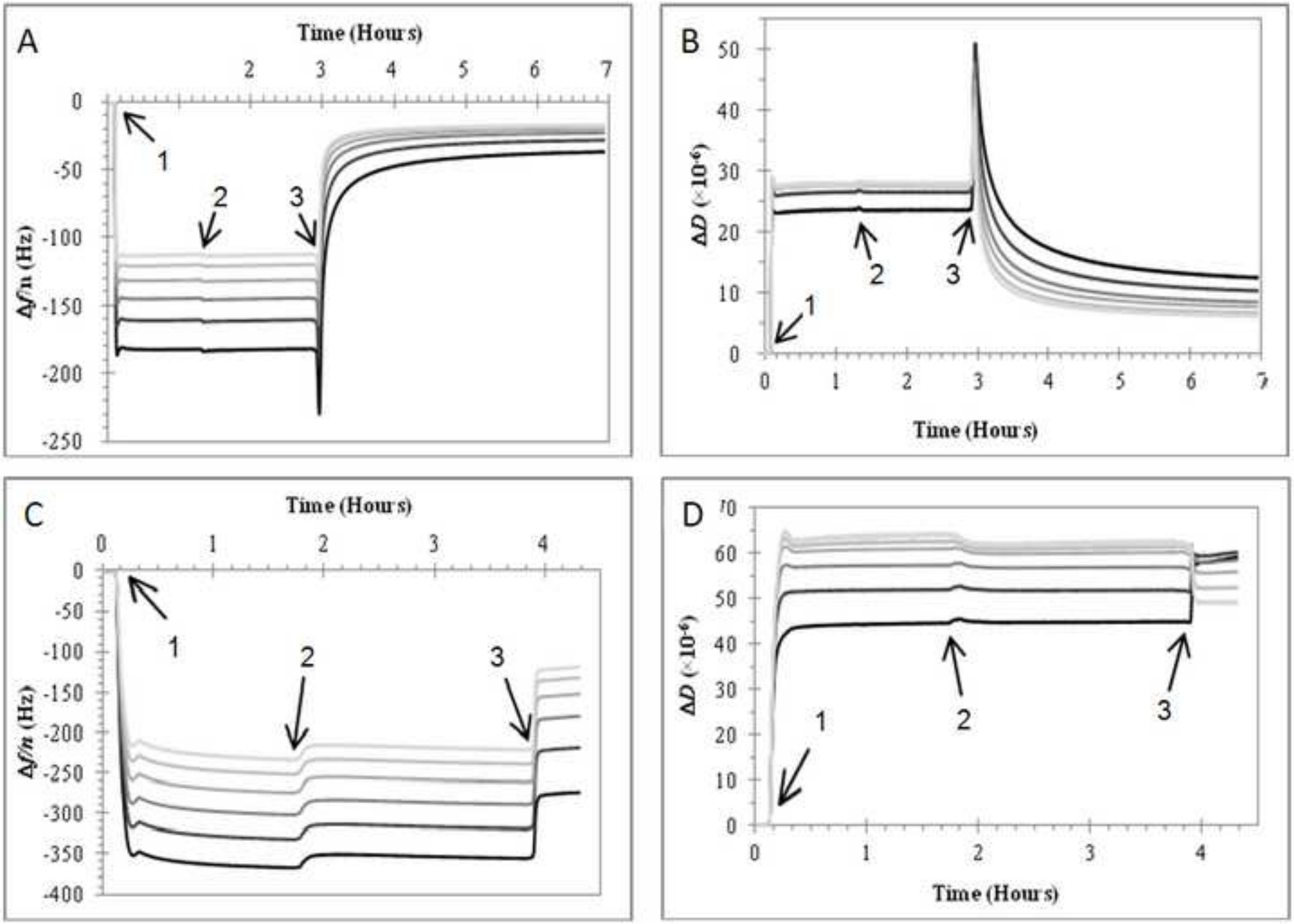

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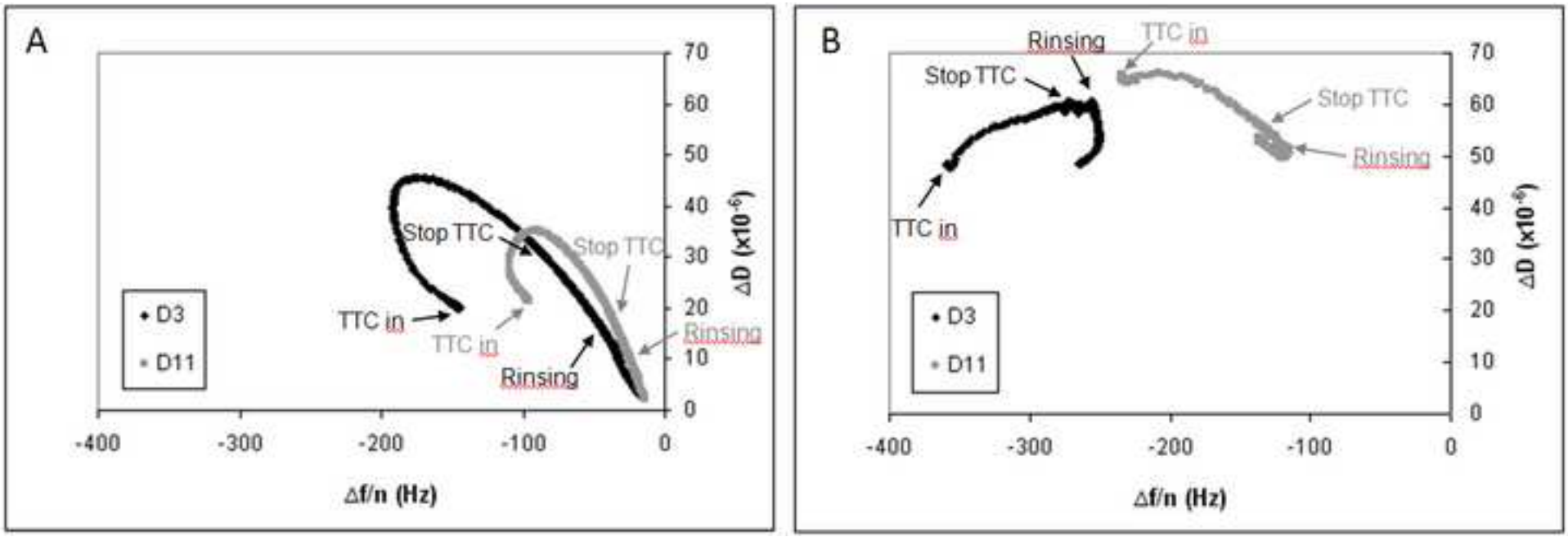

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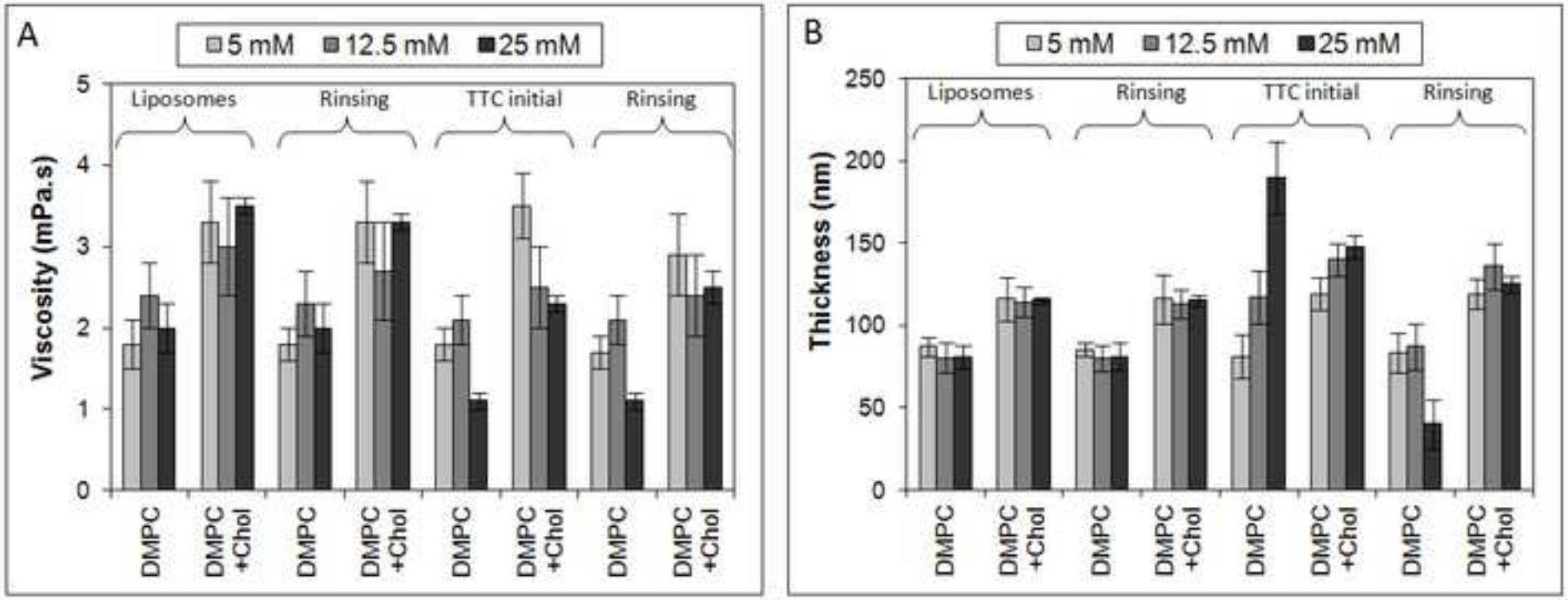

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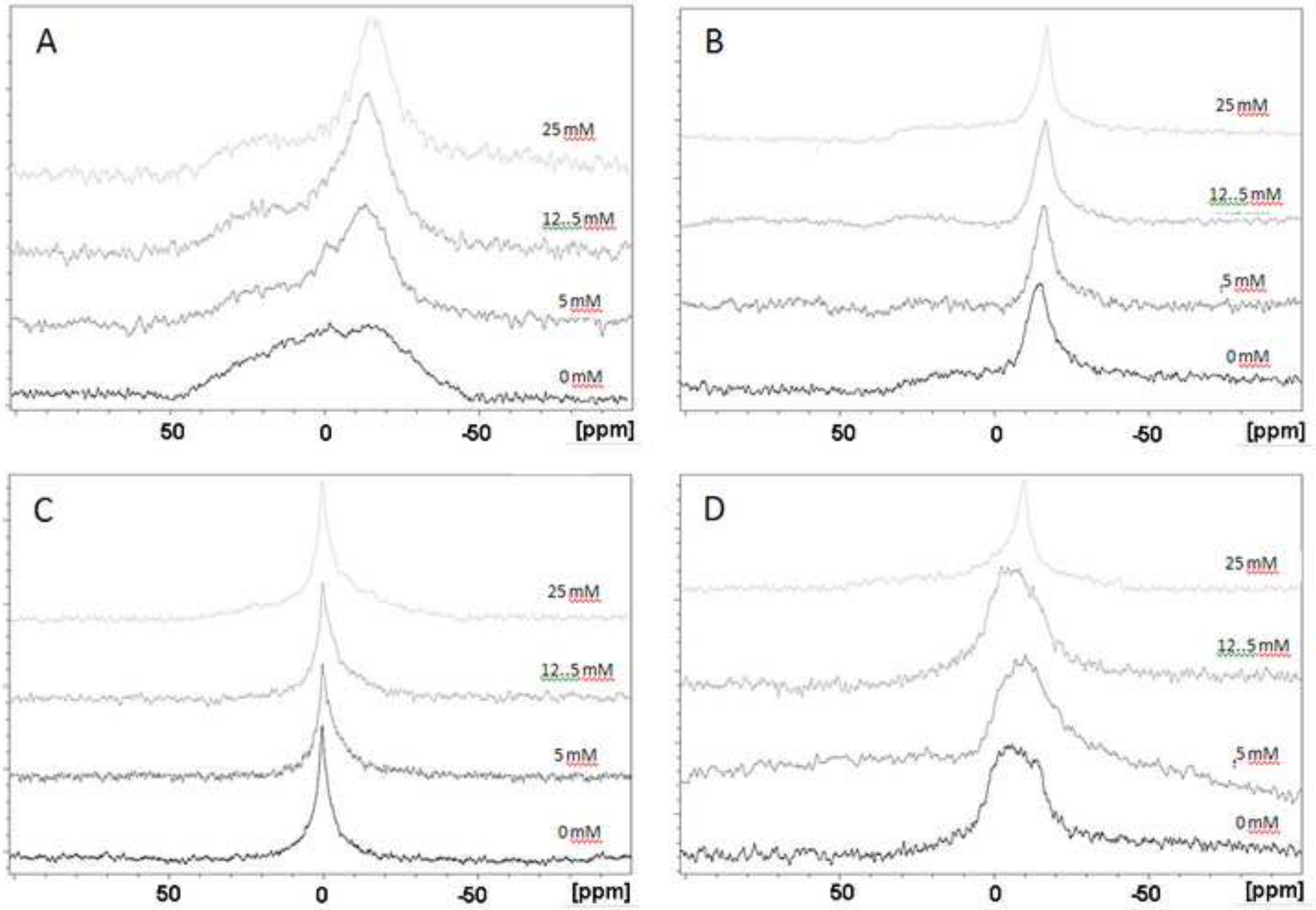

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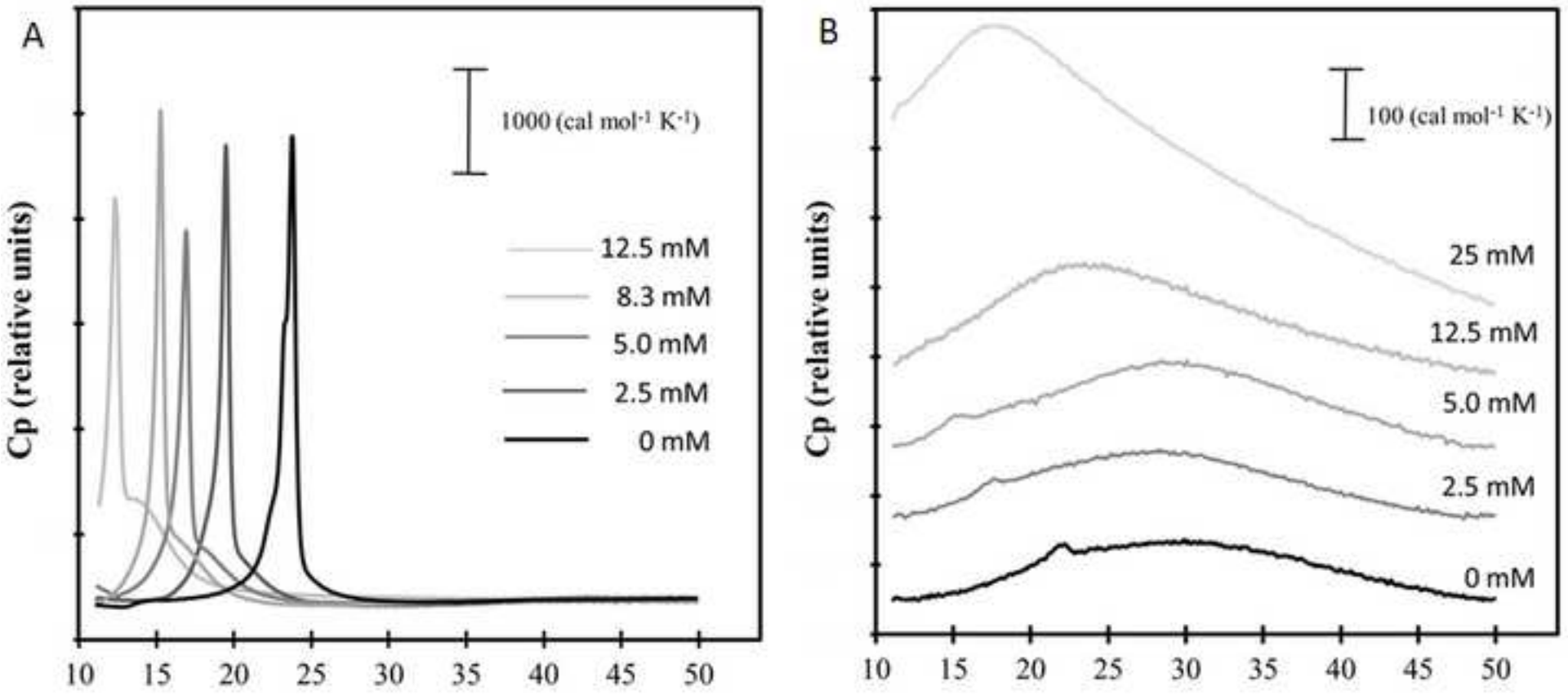

Figure 6

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