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Importance of polyunsaturated acyl chains in chlorpromazine interaction with phosphatidylserines: A ^{13}C and ^{31}P solid-state NMR study☆

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Abstract

The polyunsaturated fatty acid docosahexaenoic acid (DHA, c22:6, n-3) is found at a level of about 50% in the phospholipids of neuronal tissue membranes and appears to be crucial to human health. Dipalmitoyl phosphatidylcholine (DPPC, 16:0/16:0 PC), 1-palmitoyl-2-oleoyl phosphatidylserine (POPS) and the DHA containing 1-stearaoyl-2-docosahexenoyl phosphatidylserine (SDPS) were used to make DPPC (60%)/POPS (29%)/SDPS (11%) bilayers with and without 10 mol% chlorpromazine (CPZ), a cationic, amphiphilic phenothiazine. The T_1 relaxation measurements make it clear that the saturated acyl chains carbons (palmitic, stearic and most of the oleic chain) and the choline head group are not affected by CPZ addition. The observed increased signal intensity and T_1-values of DHA indicate reduced mobility of C_4 and C_5 due to CPZ binding. ^{31}P NMR spectra confirm that CPZ binding to the phosphatidylserines in the bilayer enhances phospholipid head group mobility.

Keywords: ^{13}C NMR; ^{31}P NMR; DPPC/SDPS/POPS; DPPC/SDPS/POPS bilayers; Chlorpromazine HCl interaction

1. Introduction

The effects of phospholipid acyl chain length and degree of unsaturation on bilayer thickness is well documented [1] and so is the effect of bilayer thickness on membrane enzyme activity [2]. The polyunsaturated fatty acid docosahexaenoic acid (DHA, c22:6, n-3) is found at a level of about 50% in the phospholipids of neuronal tissue membranes and appears to be crucial to human health [3,4]. Despite this cruciality, only sparse information has been gathered on DHA's physical function(s) in the membrane. Findings on the conformational changes of rhodopsin (the MI-to-MII transition) suggest that phospholipid membranes with polyunsaturated acyl chains promote these conformational changes of rhodopsin [5]. DHA has been modelled by molecular mechanics methods and suggested to have a rigid and ordered structure [6–8]. Contrary to the results of these modelling studies, DHA with its long run of double-bonded carbons separated by a single methylene group has been found in a compressibility study [9] to have high flexibility and minimal sensitivity to temperature in that DHA showed to be the most easily compressed acyl chain, when compared with saturated (stearoyl) and monounsaturated (oleic) acyl chains in phospholipids with choline head group.

The importance of the specific phospholipid head group is illustrated by the membrane protein topology and activity-

Abbreviations: CPZ, Chlorpromazine; CSA, Chemical Shift Anisotropy; DMPC, Dimyristoyl phosphatidylcholine (14:0/14:0 PC); DMPE, Dimyristoyl phosphatidylethanolamine (16:0/16:0 PE); DPPC, Dipalmitoyl phosphatidylcholine(16:0/16:0 PC); HPLC, High pressure liquid chromatography; PA, Phosphatidic acid; PBPS, Bovine brain phosphatidylserine; POPS, 1-Palmitoyl-2-oleoyl phosphatidylserine (16:0/18:1 (n-9) PS); SDPS, 1-Stearaoyl-2-docosahexenoyl phosphatidylserine (18:0/22:6 (n-3) PS); PC, Phosphatidylcholine; PI, Phosphatidylinositol; PKC, Protein kinase C; PLA2, Phospholipase A_2; PS, Phosphatidylserine; Tc, Transition temperature.

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determining properties of glycerophospholipids with anionic head groups [10], these phospholipids alter the structure of human recombinant prion protein associated with membranes in living cells [11]. Influence of lipid composition on membrane protein activities has recently been reviewed by Lee [12].

The above observations indicate that the activity of membrane-bound proteins can be influenced by the lipid composition of the membranes. Thus, it is possible that perturbation of lipid organization in a bilayer by amphiphilic molecules will influence the activity of such proteins even without direct interaction between the protein and the amphiphile. Chlorpromazine, a cationic, amphiphilic phenothiazine, has been found to interact preferentially with bilayers containing phospholipids with a high proportion of phosphatidylserines and highly unsaturated acyl chains [13]. Furthermore, CPZ has been found to slightly increase lipid order when the bilayer is above the gel to liquid crystalline phase transition temperature, Tc, and decrease lipid order when the bilayer is below Tc [14].

Membrane perturbation with CPZ and other amphiphils induces a host of genes in both bacteria and mammalian cells (reviewed in [15]). It is, thus, possible that CPZ's reported/claimed antagonistic effect on the D_2-receptor is partially due to perturbation by CPZ of the membrane that contains the receptor. In micromolar concentration CPZ causes large increases in the mean molecular areas in monolayers of acidic phospholipids, whereas no such molecular area increase is found for the neutral glycerophospholipids in monolayers [16]. Similar findings by us [17], using magic angle spinning solid state ^{13}C NMR on bilayer samples with partial hydration (12 H_2O per phospholipid), showed that CPZ had low or no interaction on the acyl packing of liposomes made of phospholipids without a net negative head group charge and with saturated acyl chains, such as palmitoyl (DPPC) and myristoyl (DMPC), while it caused a large (5–15 ppm) shift to higher ppm values of ~30% of the acyl chain carbon resonances in liposomes composed of pig brain PS (PBPS) and DPPC. PS is a major anionic phospholipid in mammalian cell membranes like peripheral and central nervous system myelin and PBPS was subjected to CPZ interaction studies as PBPS bilayer and in a mixture with DPPC as a DPPC (60 mol%)/PBPS (40 mol%) bilayer. This pig brain PS contained molecular species of phospholipids with the following acyl chains: two major molecular species 18:0–18:1 (49%) and 18:0–22:6 (28%), and five minor molecular species each in the 3–7% range, of which two are known 16:0–22:6 (6%) and 18:0–20:4 (3%).

Recently [13], we have studied the interaction on fully hydrated (30 H_2O per phospholipid) DPPC (60%)/PBPS (40%) bilayers above the gel to liquid crystalline phase transition temperature, Tc. In this recent study on a DPPC(60%)/PBPS(40%) bilayer and with CPZ added (DPPC(54%)/PBPS(36%)/CPZ(10%)), the Tcs were found to be about the same, 303.5 and 305.8 K, respectively. With this acyl chain composition of pig brain PS (18:0–18:1 (49%), 18:0–22:6 (28%), 16:0–22:6 (6%) and 18:0–20:4 (3%)), the sample composition can be outlined as a DPPC (60%)/SOPS (20%)/SDPS (11%)/OTHER (9%). Compared with the sample of this work DPPC (60%)/POPS (29%)/SDPS (11%), the samples differ in the amounts of polyunsaturated PS (11% SDPS and 9% OTHER versus 11% SDPS of this study) and monounsaturated PS (20% SOPS versus 29% POPS of this work). (We have carried out solid-state NMR experiments on pure SOPS and pure POPS bilayers with CPZ added and found the CPZ interaction to be negligible for both of these monounsaturated phosphatidylserines.) On the basis of the amount of unsaturated acyl chains, it is reasonable to expect the Tcs of samples used in the work presented here to be comparable with the Tcs of 303.5–305.8 K of the previous study.

A general feature of the phosphatidylserine ^{31}P static NMR spectra is a large chemical shielding anisotropy (CSA) (the CSA is generally larger for serine than for choline and ehanolamine head groups). The CSA appears to be influenced by the chemical nature of the fatty acyl chains [13]. Furthermore, the similarities of the static shielding tensor of phosphatidylserine and -choline taken together with the somewhat larger CSA for phosphatidylserines, suggest that the phosphatidylserine phosphate moiety differs conformationally or motionally from the phosphatidylcholine phosphate moiety [18,19]. This can be accounted for by greater rigidity of the phosphatidylserine head group than the phosphatidylcholine head group. This rigidity supposedly results from electrostatic interactions and/or hydrogen bonding between or within the phosphatidylserine head groups. Thus, dilution of negatively charged PBPS with neutral DPPC removes some of this interaction and will allow greater freedom of motion of the phosphatidylserine head group. The gel to liquid crystalline phase transition of a phospholipid bilayer upon increase in temperature is accompanied by several structural changes in the lipid molecules. The principal change is the *trans*-gauche isomerization in the saturated carbons in the acyl chains and the average number of gauche conformers can be related to bilayer thickness.

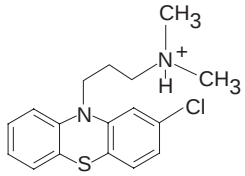
In our previous study [13] we deduced from our analysis of the composition of molecular species in PBPS that it must have been SDPS in the PBPS that caused the main, strong interaction with CPZ since POPS and SOPS showed negligible interaction with CPZ. In the present study, we have investigated phospholipid acyl chain unsaturation effect on CPZ bilayer interaction further by employing fully hydrated (30 H_2O per phospholipid) and authentic DPPC(60%)/POPS(29%)/SDPS(11%) and DPPC(54%)/POPS(26%)/SDPS(10%)/CPZ(10%) bilayers both below and above the gel to liquid crystalline phase transition temperature, Tc. The biologically abundant phosphatidylserines, POPS and SDPS, where the POPS species has its unsaturated *sn*-2 acyl chain bond at C_9-C_{10}, and the DHA containing SDPS species, with the 6 unsaturated acyl chain

bonds at C_4-C_5, C_7-C_8, $C_{10}-C_{11}$, $C_{13}-C_{14}$, $C_{16}-C_{17}$ and at $C_{19}-C_{20}$. With this distribution of unsaturated acyl chain bonds and the chosen molar percentages, the contribution to the NMR spectra from the C=C resonances other than C_9-C_{10} of POPS will be due to the DHA acyl chain of the SDPS phospholipid. In this way specific phospholipid bilayer interaction of CPZ can be detected. This DPPC (56 mol%)/POPS (29 mol%)/SDPS (11 mol%) phospholipid bilayer was studied without and with 10 mol% of CPZ added. Samples were pH adjusted to 7.4 in order to ensure that the serine head group carboxyl group was deprotonated (pK_a of ~4.4). ^{13}C [20] and ^{31}P [19] solid-state NMR techniques were employed to obtain structural and dynamic information of this phospholipid bilayer when interacting with the CPZ amphiphile.

2. Materials and methods

2.1. Liposome preparation

Chlorpromazine HCl (CPZ) and synthetic 1, 2-dipalmitoyl phosphatidylcholine (DPPC, powder) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Synthetic 1-palmitoyl-2-oleoyl phosphatidylserine (POPS, dissolved in chloroform), and 1-stearaoyl-2-docosahexenoyl phosphatidylserine (SDPS, dissolved in chloroform) were purchased from Avanti Polar Lipids Inc. (Birmingham, Alabaster, AL, USA). Phospholipid bilayers containing choline and serine head groups were made in a molar composition of 60% PC and 40% PS (29% POPS, 11% SDPS) and dissolved in *t*-butanol and then lyophilized to dryness. The PC/PS and the PC/PS/CPZ bilayers were kept under an argon atmosphere and not exposed to air and light. Each sample of dry powder was then suspended in H_2O. These suspensions contained multilamellar liposomes and unilamellar systems were obtained by freeze-thawing 7 times. At the freeze-thawing stage all samples were adjusted to a pH of 7.4 by adding a small amount of 0.05 M NaOH. Subsequently, the lipid suspension was divided into two equal parts and to one part was added an amount of CPZ HCl (dissolved in H_2O) to obtain a 10% molar ratio. Thus a sample of 54% PC, 36% PS (26% POPS and 10% SDPS) and 10% CPZ was obtained as well as the corresponding sample without CPZ. The samples with added CPZ HCl were then incubated on a waterbath for 24 h at 317 K. Subsequently, the samples were subjected to 24 h of lyophilization giving partially hydrated liposomes with a hydration level of ~12 water molecules per lipid molecule (determined by ^1H-MAS NMR). Then, water was added to the samples to obtain fully hydrated bilayers (~30 water molecules per lipid molecule) [21,22] and the samples were equilibrated at 315 K for 48 h (above the samples gel to liquid crystalline transition temperature(s)) and packed in NMR rotors (Scheme 1).



Scheme 1. Chlorpromazine (CPZ).

2.2. CP-MAS-^{13}C NMR spectroscopy

The ^{13}C-MAS NMR experiments were obtained at 100.62 MHz with the Bruker AVANCE DMX 400 instrument equipped with *magic angle spinning* (MAS) hardware and used ZrO_2 spinning rotors with a diameter of 4 mm. Experiments were done at sample temperature of 310 K with sample spinning rate of 1500 Hz. Calibration of the MAS probe temperature has been done by the manufacturer (Bruker, Germany) upon delivery of the solid state equipment. Confirmation of the MAS probe temperature calibration in the temperature range with relevance to phospholipids bilayer phase transitions was carried out on a pure DPPC sample. ^{13}C NMR spectra were recorded from 293 to 317 K, and the DPPC phase transition was found to occur between 313.6 and 315.6 K. These experiments were carried out with high-power proton decoupling during the acquisition, i.e. without Nuclear Overhauser Effect (NOE). In this study, experiments of the two DPPC/POPS/SDPS and DPPC/POPS/SDPS/CPZ bilayer systems were carried out with a relaxation delay of 5 s between transients, unless otherwise stated. Typically, a total of 16,000 transients were acquired. The spectra were multiplied with an exponential window function increasing the line-width by 2 Hz to reduce noise prior to Fourier transformation.

^{13}C spin-lattice relaxation times were obtained by a modified inversion-recovery pulse sequence using a composite 180° pulse [23] to counteract potential problems associated with non-uniform excitation across the range of ^{13}C chemical shifts. A recycling delay of 10 s between transients were used between the 256 and 512 transients accumulated a sample temperature of 310±0.5 K. In order to obtain accurate relaxation data on the palmitic acyl chain methyl group, relaxation experiments using a pulse program with broadband ^1H-decoupling and a 50 s relaxation delay were also carried out with 128 transients.

2.3. ^{31}P NMR spectroscopy

Static ^{31}P spectra were acquired on these two fully hydrated bilayer samples at the various temperatures ranging from 296 to 318 K at 161.98 MHz and high-power decoupling during acquisition, i.e. without Nuclear Overhauser Effect (NOE). Typically, 512 transients were collected for each experiment with a relaxation delay of 5 s between transients. These fids were multiplied with an exponential window function increasing the line-width by 50 Hz to reduce noise prior to Fourier transformation. Magic

angle spinning ^{31}P experiments (T_1 measurements) were carried out with a rotor spinning speed of 2 KHz. These fids of 64 transients were Fourier transformed without apodization in order to keep spectral resolution. ^{31}P relaxation data were obtained with ^{1}H-cross-polarization at temperatures from 296 K to 318 K, and with rotor spinning speed of 2 kHz. Typically 512 transients were accumulated.

3. Results

The ^{13}C magic angle spinning (MAS) spectra of bilayer samples DPPC/POPS/SDPS and DPPC/POPS/SDPS/CPZ in the liquid crystalline phase were recorded at a temperature of 310 K and are presented as spectral regions in Figs. 1–4 where the top spectrum shows the phospholipid sample with 10% CPZ and the bottom spectrum the corresponding sample without CPZ. Fig. 1 shows the DPPC, POPS and SDPS acyl chain sp^3 carbon resonances in the 12–38 ppm region. The two spectra (Fig. 1, top and bottom) are dominated by the palmitic (DPPC and POPS) as well as the oleic (POPS) molecular species. The molar composition of the samples cause the palmitic (16:0) acyl chain resonances to give ~75% of the peak intensities in this spectral region, whereas the contribution from the SDPS species in this spectral region is 10% (5% from each of the 18:0 and 22:6 acyl chains). Thus, only the palmitic carbon resonances are assigned, see Fig. 1 (some of these peaks contain contribution from carbon resonances of other acyl chains than palmitoyl chains). The phospholipid choline and serine head group carbon resonances as well as the glycerol moiety resonances appear in the 52–73 ppm spectral region—see Fig. 2. Of these resonances, only the choline head group resonances come from a single molecular species, the DPPC molecule. The serine resonances come from two PS species, POPS and SDPS, and the molar composition gives a PC/PS peak ratio of 1.5. The three glycerol resonances will be composed of the three phospholipid species in the two samples, DPPC, POPS and SDPS.

From the T_1 data presented in Table 1, one finds that the carbon T_1 values of the choline head group are not affected

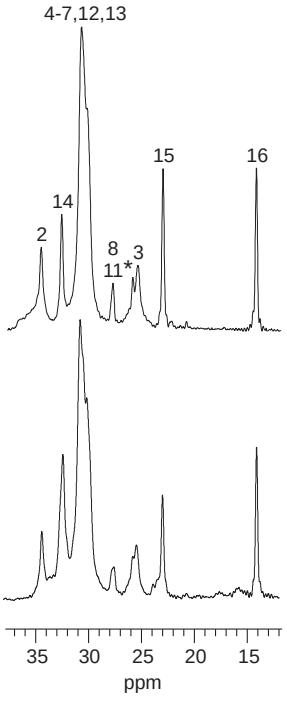


Fig. 1. Methylene and methyl carbon resonance region (12–38 ppm) of samples DPPC(60%)/POPS(29%)/SDPS(11%) (bottom spectrum) and DPPC(54%)/POPS(26%)/SDPS(10%)/CPZ(10%) (top spectrum). Spectra are acquired at 310 K (samples are in liquid crystalline phase). The samples molar composition cause the palmitic (16:0) acyl chain resonances to dominate (~ 75% of the peak intensities) in this spectral region. Thus, only the palmitic carbon resonances are assigned in the two spectra. An asterisk "*" indicate a possible DHA resonance. See the text for details.

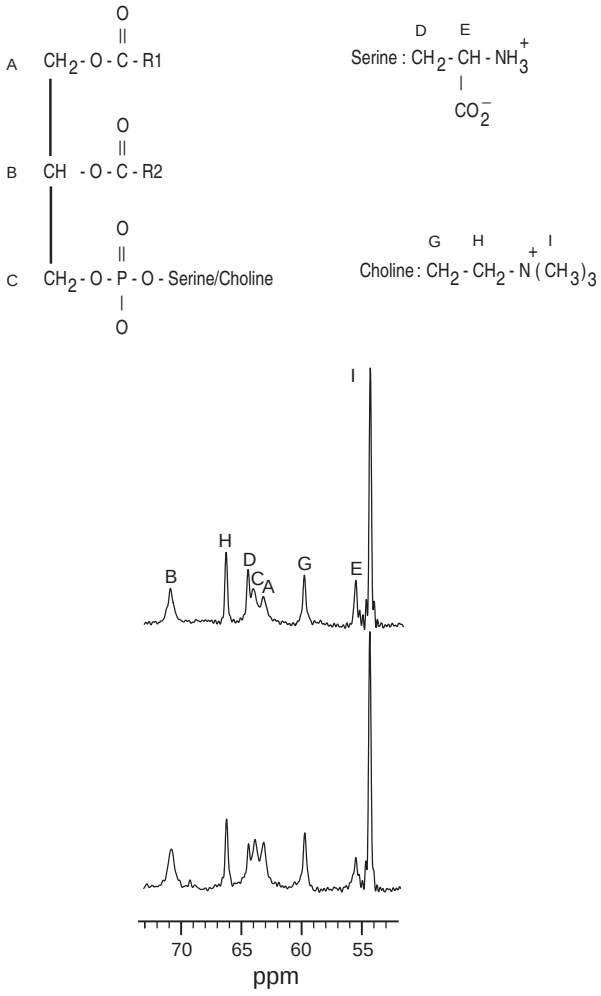


Fig. 2. Top: Structural formula of the glycerol moiety and the two phospholipids head groups, serine and choline, with the corresponding assignment letters used in the spectra. Bottom: Phospholipid head group and glycerol carbon resonance region (52–73 ppm) of samples DPPC(60%)/POPS(29%)/SDPS(11%) (bottom spectrum) and DPPC(54%)/POPS(26%)/SDPS(10%)/CPZ(10%) (top spectrum). Spectra are acquired at 310 K (samples are in liquid crystalline phase). The molar composition makes a PC/PS ratio of 1.5. See the text for details.

Table 1
^{13}C spin-lattice relaxation times T_1 (s) at 310 K DPPC/POPS/SDPS and DPPC/POPS/SDPS/CPZ bilayers

Carbon	DPPC/POPS/SDPS	DPPC/POPS/SDPS/CPZ
C=O	2.04	2.15
CO_2^-	1.51	0.61
POPS C=C:		
C9 (C^a)	0.75	1.83
C10 (B^a)	1.32	1.03
DHA C=C:		
(E^a)	0.34	1.16
(F^a)	1.04	0.99
(G^a)	0.75	2.67
Glycerol carbon:		
sn-1	0.12	0.31
sn-2	0.28	0.33
sn-3	0.13	0.15
Serine carbon:		
α	0.27	0.07
β	1.44	0.77
Choline carbon:		
α	0.34	0.30
β	0.26	0.22
CH_3	0.32	0.29
Palmitic carbon:		
2	0.55	0.36
3	0.68	0.52
4–14	0.72	0.60
15	3.51	2.12
16	5.03	5.33
Oleic carbon:		
8, 11	0.55	0.61

[a] Peaks labeled in Fig. 3.

by the addition of CPZ, whereas the serine head group T_1 values show a reduction in presence of CPZ. The glycerol carbon T_1 values, on the other hand, demonstrate a diverse effect of CPZ. The *sn*-1 glycerol carbon display an increased T_1 value due to CPZ in contrast to both the *sn*-2 and *sn*-3 glycerol carbons (where the POPS and SDPS unsaturated acyl chain and the phosphate and head group are attached, respectively) that have T_1 values unaffected by CPZ.

Fig. 3 shows the 125–135 ppm region where the C=C resonances of the acyl chains of samples DPPC/POPS/SDPS and DPPC/POPS/SDPS/CPZ are found. The molar composition of the samples makes the oleic(18:1)/DHA(22:6) acyl chain ratio 2.5. The oleic(18:1) acyl chain of POPS double bond (at C_9-C_{10}) and the six double bonds of DHA of SDPS (double bonds at C_4-C_5, C_7-C_8, $C_{10}-C_{11}$, $C_{13}-C_{14}$, $C_{16}-C_{17}$,$C_{19}-C_{20}$) makes the carbon–carbon double bond ratio between POPS and SDPS to be 1/6. Consequently, the observed sp^2 carbon resonances in the ^{13}C NMR spectra can be expected to be close to the described 1/6 ratio multiplied by the species percentages of the samples. Thus, samples with and without CPZ has a POPS/SDPS acyl chain C=C ratio of $(0.29\times 1$ double bond)/$(0.11\times 6$ double bonds) or approximately 0.4.

Comparison of the C=C resonances with/without CPZ (see Fig. 3) shows a pronounced intensity change of some of these, the peaks at 127–129 ppm, upon CPZ interaction.

The crowded spectral region displayed in Fig. 3 pose an obstacle to a complete resonance assignment. However, in a recent solid-state NMR where $^1H-^{13}C$ two-dimensional cross-polarization experiments were employed [24], the investigators managed to firmly assign DHA's C_{19} and C_{20} to 126.8 and 131.3 ppm, respectively. Thus, peak A is assigned to resonance C_{20} and peak H to resonance C_{19}—see Fig. 3. The remaining C=C resonances of DHA (C_4-C_5, C_7-C_8, $C_{10}-C_{11}$, $C_{13}-C_{14}$, $C_{16}-C_{17}$) are located between 127.4 and 128.4 ppm and could not be individually assigned. In an early study on C=C resonance assignment and estimation of chemical shifts Gunstone et al. [25] showed that in monoenoic acyl chains, like the oleic chain of POPS, the C_{10} resonance would come at a higher chemical shift than the C_9, they found 130.02 and 129.78 ppm, respectively. Based on our own previous work, on the signal intensities of these resonances (Fig. 3) and on POPS and the described higher chemical shift of C_{10} of the C_9=C_{10} pair, peaks B and C in Fig. 3 can be assigned to the oleic acyl chain of POPS where resonance B corresponds to C_{10} and resonance C to C_9 of POPS. These two C=C resonances from the middle of POPS's acyl chain, display almost no changes in intensity and T_1 values (Table 1) when CPZ is added (T_1 values for peaks B, C, E, F and G in Fig. 3 could be determined).

As evident in Fig. 3 there is no intensity change of DHA's resonances C_{19} and C_{20} upon addition of CPZ (peaks A and H, respectively). Furthermore, peaks E and G (Fig. 3 and Table 1) display a marked increase in T_1 value when CPZ is present (peak F has approximately similar T_1 values without and with CPZ). Thus, the part of SDPS's DHA acyl chain that are affected by the presence of CPZ is the part close to the polar region of the bilayer, as demonstrated by the intensity and T_1 value increase of these resonances.

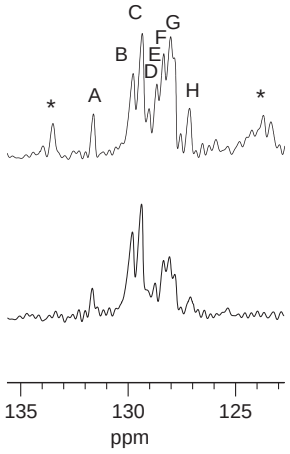


Fig. 3. Double bonded acyl chain carbon resonance region (125–135 ppm) of samples DPPC(60%)/POPS(29%)/SDPS(11%) (bottom spectrum) and DPPC(54%)/POPS(26%)/SDPS(10%)/CPZ(10%) (top spectrum). Spectra are acquired at 310 K (samples are in liquid crystalline phase). The molar composition of the samples makes the oleic(18:1)/DHA(22:6) ratio of 2.5. This causes the total oleic(C=C)/DHA(C=C) peak ratio to be 0.4. See the text for details.

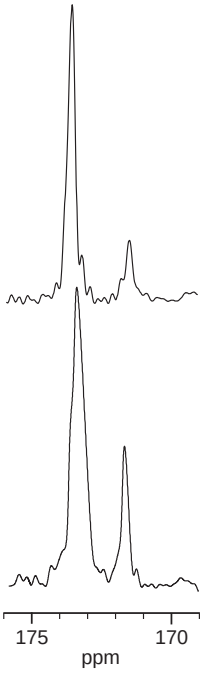


Fig. 4. Carbonyl and carboxyl carbon resonance region (170–176 ppm) of samples DPPC(60%)/POPS(29%)/SDPS(11%) (bottom spectrum) and DPPC(54%)/POPS(26%)/SDPS(10%)/CPZ(10%) (top spectrum). Spectra are acquired at 310 K (samples are in liquid crystalline phase). The molar composition (PC/PS ratio) makes the theoretical ratio between the carbonyl and carboxyl resonances to be 2.5. See the text for details.

All these assignments are further supported by the molar composition of the samples. The spectrum of the sample without CPZ demonstrates DHA double bonded carbon resonances with smaller signal intensity than necessary for a good correspondence with the molecular ratio of the sample. On the other hand, the sample containing CPZ show these resonances with larger signal intensities and some with increased T_1 values (Table 1), when compared with the sample without CPZ. The appearance of some broad peaks around 124 ppm labeled with an asterisk "*" (Fig. 3, top spectrum) when CPZ is present correspond to double bonded carbon resonances of the CPZ molecule, as does the peak at ~133.5 ppm labeled with an asterisk "*" (Fig. 3, top spectrum). Another interesting feature in the carbon T_1 data (Table 1) is the ~240% increase in the T_1 value of carbon C_9 of the unsaturated acyl in the POPS molecule and the ~28% T_1 value reduction of C_9's acyl chain neighbor, the C_{10} carbon, in presence of CPZ.

Fig. 4 shows the carbonyl resonance (~173 ppm) and the serine head group carboxyl (~171 ppm) [26] resonance of samples DPPC/POPS/SDPS (bottom spectrum) and DPPC/POPS/SDPS/CPZ (top spectrum). The molar composition (PC/PS ratio) makes the theoretical peak ratio of 2.5 between the carbonyl and carboxyl resonances. From the two spectra shown in Fig. 4, it is apparent that the carbonyl resonance (~173 ppm) of the bilayer is not affected by

Table 2
^{31}P chemical shift anisotropy (CSA, in ppm) from 296 K to 318 K DPPC/POPS/SDPS and DPPC/POPS/SDPS/CPZ bilayers

Temperature (K)	DPPC/POPS/SDPS	DPPC/POPS/SDPS/CPZ
296	108	88
297	103	87
298	104	84
299	104	83
300	101	84
301	103	86
302	99	82
303	96	79
304	96	79
305	92	80
306	94	67
307	91	67
308	88	70
309	89	69
310	87	69
311	88	67
312	82	70
313	81	71
314	79	69
315	79	67
316	77	69
317	75	73
318	76	66

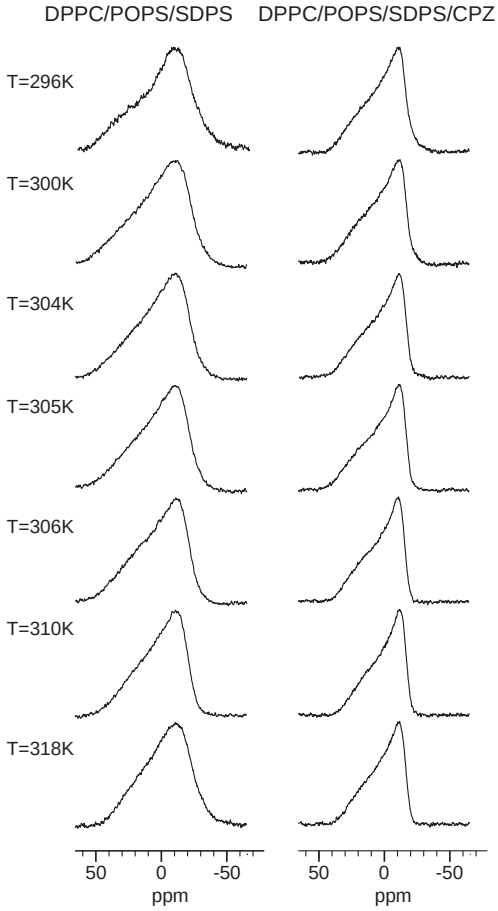


Fig. 5. Static ^{31}P NMR spectra of samples DPPC(60%)/POPS(29%)/SDPS(11%) (left column) and DPPC(54%)/POPS(26%)/SDPS(10%)/CPZ(10%) (right column). Sample temperatures from 296 K (top spectra) to 318 K (bottom spectra). See the text for details.

Table 3
^{31}P T_1 values (s) from 296 K to 318 K DPPC/POPS/SDPS and DPPC/POPS/SDPS/CPZ bilayers

Temperature (K)	DPPC/POPS/SDPS		DPPC/POPS/SDPS/CPZ		
	PC	PS	PC	PS	CPZ–PS
296	0.57	0.53	0.77	0.64	0.60
298	0.54	0.52	0.65	0.64	0.60
300	0.57	0.54	0.64	0.58	0.62
302	0.51	0.50	0.57	0.51	0.51
304	0.55	0.51	0.61	0.52	0.49
305	0.51	0.49	0.52	0.50	0.51
306	0.54	0.49	0.51	0.53	0.53
308	0.53	0.50	0.52	0.52	0.49
310	0.57	0.47	0.49	0.49	0.42
312	0.55	0.51	0.51	0.48	0.42
314	0.55	0.51	0.49	0.47	0.41
316	0.57	0.57	0.46	0.46	0.45
318	0.58	0.52	0.50	0.48	0.46

addition of CPZ. A corresponding comparison of the serine head group carboxyl resonance (~ 171 ppm), on the other hand, makes it evident that the 10% CPZ reduces the carboxyl resonance intensity by about 2/3 and the T_1 (Table 1) value by 40% (from 1.15 to 0.61 s). The corresponding T_1 values for the carbonyl resonance is about unchanged in presence of CPZ.

In general, the ^{31}P CSA data presented in Table 2 and Fig. 5 show that the sample without CPZ has a higher CSA than when CPZ is added over the whole temperature range measured (296–310 K). The CSA of the sample without CPZ (the DPPC/POPS/SDPS sample) displays a fairly steady decrease in CSA value as temperature increases. In addition to a general decrease in CSA value upon temperature increase, the CPZ containing sample (the DPPC/POPS/SDPS/CPZ sample) displays a sudden drop in CSA of 13 ppm from 305 to 306 K. Thus, the CPZ containing sample displays this sudden reduction in CSA at a sample temperature of about 305.5 K, in correspondence with the main melting (transition) temperature displayed by this kind of phospholipid sample. The ^{31}P T_1 values are measured at the central band of the MAS spectra, and presented in Table 3. In Fig. 6 the three central band peaks are displayed at several of the temperatures investigated. They can be assigned [13,27] to the three molecular species: PC, PS and CPZ–PS complex. Both the PC and the PS species show similar T_1 values with and without CPZ and the CPZ–PS complex shows a T_1 similar to the PC and the PS species—see Table 3.

4. Discussion

The observed intensity decrease of the glycerol carbon resonances of the DPPC/SDPS/CPZ sample (Fig. 2) when compared with the DPPC/SDPS sample is most pronounced for the *sn*-3 carbon, i.e. the glycerol carbon where the phosphorus and head group are attached. A similar signal intensity decrease/line broadening is observed for the serine carboxyl resonance of the DPPC/SDPS/CPZ sample when compared with the DPPC/SDPS sample. An explanation for these observations can be found in the possibility of an altered transverse relaxation of dipolar coupled spins under radiofrequency irradiation (decoupling) [28]. In such a case destructive interference effects cause line broadening due to (molecular) motion interfering with the coherent modulation from radiofrequency decoupling. Even carbons without directly attached protons (such as carbonyl and carboxyl carbons) can to some extent experience these effects when coupled to other nearby protons. Furthermore, dipolar interactions are expected to be weak for nonprotonated sp^2 carbons and the main line broadening mechanism will be the chemical shift anisotropy (CSA). (Protonated sp^2 carbons of the acyl chains' olefinic double bonds will experience both the described line broadening mechanisms [28].)

With the possibility of such effects (as described above) complicating the spectral interpretations the ^{13}C T_1 data obtained on the DDPC/POPS/SDPS and DDPC/POPS/

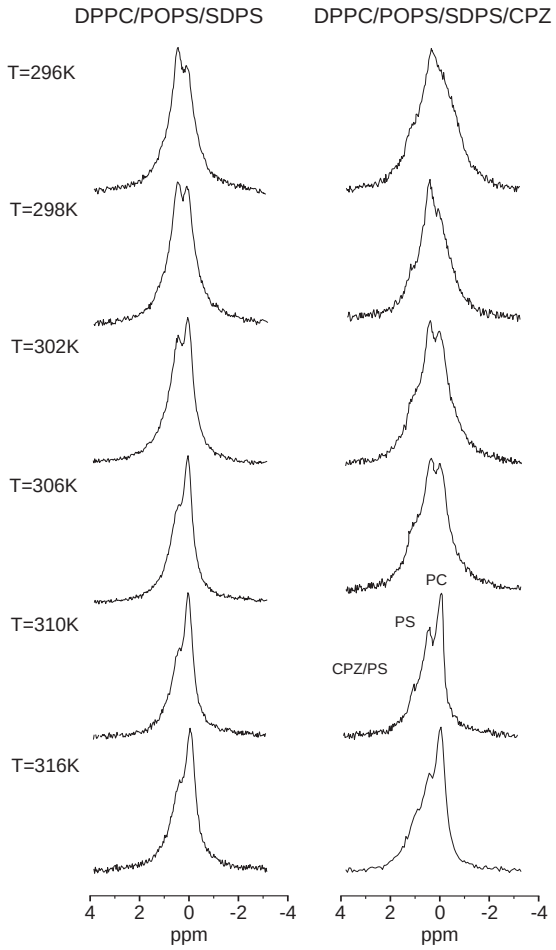


Fig. 6. ^{31}P MAS spectra of samples DPPC(60%)/POPS(29%)/SDPS(11%) (right column) and DPPC(54%)/POPS(26%)/SDPS(10%)/CPZ(10%) (left column) between sample temperatures 296 and 318 K. Note sudden decrease in CSA of CPZ containing sample at 305 K. See the text for details.

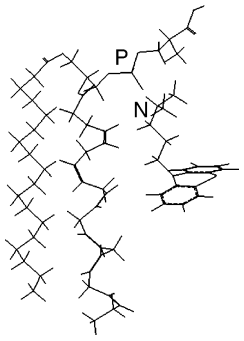
SDPS/CPZ samples (of this work) are of great value. This is even more so due to the simpler molecular species makeup of the DDPC/POPS/SDPS sample of this work when compared with the higher molecular species complexity of our previous work [13], where we employed pig brain PS (PBPS). For example, the C=C region of the ^{13}C spectra displayed in Fig. 3, the C_9 and C_{10} resonances of POPS could be assigned, so that the remaining C=C resonances are known to belong to SDPS's *sn*-2 attached DHA acyl chain. Of these latter C=C resonances, the C_{19} and C_{20} of DHA could be firmly assigned, and all the remaining unassigned C=C resonances are then known to belong to the DHA acyl chain, namely the C_4, C_5, C_7, C_8, C_{10}, C_{11}, C_{13}, C_{14}, C_{16} and C_{17} carbon resonances.

Binding of CPZ to phospholipids can be followed in the ^{13}C spectra where serine head group carbon resonances show increased intensity when CPZ is added to the DPPC/POPS/SDPS bilayer. This result is contrary to the results found in our previous work [13] on a PC/PS/CPZ sample where the PS component is an extract from pig brain and composed as follows: The PS composition of PBPS used has been determined to mainly contain these components: 18:0–18:1 (49%), 18:0–22:6 (28%), 16:0–22:6 (6%) and 18:0–20:4 (3%). Thus, this can be described as a DPPC(60%)/SOPS(20%)/SDPS(11%)/OTHER (9%) sample. Effects of an altered transverse relaxation of dipolar coupled spins under radiofrequency irradiation (decoupling) has been described earlier [28] and the possibility of such effects producing potentially confusing changes in signal intensities when CPZ is present, make the ^{13}C T_1 measurements on both DPPC/POPS/SDPS and DPPC/POPS/SDPS/CPZ samples important. (Unfortunately, we did not carry out ^{13}C T_1 experiments on the pig brain PS samples (PBPS) of our previous work, this kind of sample has a higher molecular species complexity than the samples of this work.)

In a previous study [17] addition of CPZ to partially hydrated DPPC/PBPS bilayer (~ 12 H_2O/phospholipid) ~ 30% of the main acyl chain carbon resonances in the ^{13}C NMR spectra were shifted down field by 5–15 ppm, demonstrating CPZ interdigitation among the phospholipid acyl chains. The fully hydrated DPPC/POPS/SDPS bilayer of this study showed no such down field shift of acyl chain resonances in the ^{13}C NMR spectra when CPZ was present. The lower mobility of the phospholipids in partially hydrated bilayers when compared with the fully hydrated bilayers of this study is evident from the broader line shapes in the ^{13}C NMR spectra of the partially hydrated bilayers [17]. Thus, a less dense molecular packing of the phospholipids in fully hydrated bilayers would presumably not make interdigitated CPZ molecules come in close enough contact with acyl chain carbons to perturb the p-orbitals of these carbons, and consequently, a 5–15 ppm shift to higher ppm values of these acyl chain carbon resonances is not observed in the fully hydrated bilayers of this study.

Phospholipid head group (and phosphate) motion and an altered motion caused by an interacting amphiphile like CPZ in a bilayer will give static ^{31}P NMR spectra that differ in chemical shift anisotropy (CSA). In the static ^{31}P NMR spectra of DPPC/POPS/SDPS/and DPPC/POPS/SDPS/CPZ (Fig. 5 and Table 2), the former demonstrate a CSA that is 10–17 ppm larger than the latter over a quite large temperature range covering the gel to liquid crystalline phase transition temperature. Thus, the presence of CPZ causes an enhancement of the phospholipid head group mobility. A separate (new) ^{31}P chemical shift for the CPZ-phosphate is observed when CPZ binds to the phosphate of phosphatidylserine bilayers as demonstrated in the ^{31}P NMR spectra of the DPPC/POPS/SDPS and DPPC/POPS/SDPS/CPZ bilayers. In the previous study of 60%/40% DPPC/PBPS bilayer the bulky choline head groups imposes conformational restrictions [13,29] on the CPZ-phosphate complex and also promote CPZ-carboxyl binding which was not observed for the "all serine" head group samples and therefore seem less favoured.

The T_1 relaxation measurements make it clear that the unsaturated acyl chain carbons (palmitic, stearic and most of the oleic chain) do not change in mobilities upon CPZ addition (these carbons have similar T_1 values (Table 1) without and with CPZ in the bilayer). The unsaturated carbons of the DHA acyl chain, on the other hand, display a 2–3 times increase in T_1 value with CPZ present, i.e. these unsaturated carbons experience a decreased mobility when CPZ is present in the bilayer. The choline head group carbon resonances, two of the glycerol carbons (the *sn*-2 and *sn*-3 carbons) and the carbonyl resonance display no change in T_1 upon CPZ addition. The serine head group carbon resonances (the Cα, Cβ and CO_2^-) display a 2–3 times reduction in the T_1 value upon CPZ addition, possibly due to an increased mobility of these carbons (the phospholipids are in the slow motion regime at the relaxation measurement temperature). The ^{31}P relaxation measurements show that all three head group components (PC, PS and CPZ–PS) do not



Scheme 2. Molecular model of chlorpromazine (CPZ) interaction with a 1-stearoyl-2-docosahexanoylserine (SDPS) molecule. The CPZ molecule (right) is positioned with its positive charge on the nitrogen atom (labelled N) on the end of CPS's acyl chain. This positive charge is in the vicinity of the phosphate's (labelled P) negative charge in the SDPS molecule (left). Both acyl chains of the SDPS molecule have sp^3 carbon dihedral angles of 60° (liquid crystalline state). The molecular model suggests that the carbons C_4 and C_5 of DHA (*sn*-2 position) will be affected by an interdigitating CPZ molecule.

vary by any significant amount as function of sample temperature, only the CPZ–PS component is to some degree temperature sensitive below the main phase transition temperature. The PC and the PS components display very similar ^{31}P relaxation in both bilayer samples, i.e. with and without CPZ present.

In addition to the importance of the phospholipid head group, also the degree of phospholipid acyl chain unsaturation will determine [30] part of the CPZ interaction with the bilayer. The observed increased signal intensity of C=C SDPS's DHA acyl chain carbon resonances and an increase in the corresponding T_1-values for two (out of three that could be measured) of the C=C peaks where the C_4 and C_5 resonances reside and, thus, the reduced mobility of C_4 and C_5 appear to be due to CPZ binding. A molecular model of the CPZ interaction with a 1-stearoyl-2-docosahexanoylserine (SDPS) molecule generated by the Titan software (Wavefunction, Irvine, CA) is presented in Scheme 2. In this model a CPZ is located with its positive charge (acyl chain nitrogen) in the vicinity of a SDPS's phosphate group negative charge. SDPS's acyl chains have the sp^3 carbon dihedral angles of 60° (liquid crystalline state). Even though the actual conformation(s) of the DHA's acyl chain (in the *sn*-2 position) may differ somewhat from the conformation displayed in Scheme 2, the model suggests that CPZ interdigitated in such a bilayer will have an effect on both carbons C_4 and C_5 of the DHA.

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