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Existence of lipid microdomains in bilayer of dipalmitoyl phosphatidylcholine (DPPC) and 1-stearoyl-2-docosahexenoyl phosphatidylserine (SDPS) and their perturbation by chlorpromazine: A ^{13}C and ^{31}P solid-state NMR study☆

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Abstract

The polyunsaturated fatty acid docosahexaenoic acid (DHA, 22 : 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) and the DHA containing 1-stearoyl-2-docosahexenoyl phosphatidylserine (SDPS) were used to make DPPC (60%)/SDPS (40%) bilayers with and without 10 mol% chlorpromazine (CPZ), a cationic, amphiphilic phenothiazine.

Resonances that are present in ^{13}C NMR spectrum of the DPPC (60%)/SDPS (40%) sample and that disappear in presence of 10% CPZ most probably are due to the special interface environment, e.g. the hydrophobic mismatch, at the interface of DPPC and SDPS microdomains in the DPPC/SDPS bilayer. In itself the appearance of resonances at novel chemical shift values is a clear demonstration of a unique chemical environment in the DPPC (60%)/SDPS (40%) bilayer. The findings of the study presented here suggest CPZ bound to the phosphate of SDPS will slow down and partially inhibit such a DHA acyl chain movement in the DPPC/SDPS bilayer. This would affect the area occupied by a SDPS molecule (in the bilayer) and probably the thickness of the bilayer where SDPS molecules reside as well. It is quite likely that such CPZ caused changes can affect the function of proteins embedded in the bilayer.

Keywords: ^{13}C NMR; ^{31}P NMR; DPPC/SDPS; DPPC/SDPS; Lipid microdomains; Bilayers; ChlorpromazineHCl interaction

1. Introduction

Cellular membranes like those of retinal rod outer segments [1], mitochondria [2], spermatozoa [3] and cerebral grey matter [4] have a high percentage of phospholipids with polyunsaturated acyl chains, i.e. the ω-3 and ω-6 fatty acids [5]. Low levels of these polyunsaturated fatty acids are related to a variety of pathological conditions like cancer [6], visual disorder [7] and cardiovascular disease [8]. Thus, these apparently unrelated diseases, indicate a fundamental role played by the polyunsaturated fatty acids in the cells. The ω-3 fatty acid, docosahexaenoic acid (DHA) and the ω-6 fatty acid, arachidonic acid (AA), differ in that the latter serves as a precursor for prostaglandins [9] in platelet function, whereas the former does not appear to be metabolized [1].

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, Pig brain phosphatidylserine; POPS, 1-palmitoyl-2-oleoyl phosphatidylserine (16 : 0/18 : 1 (n-9) PS); SDPS, 1-stearoyl-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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DHA has a function as ligand for retinoid X receptor [10], influences the Cl^- channels [11] (cystic fibrosis) and the neuronal K^+ channels [12], is released in response to serotonin [13], has an essential role in brain maturation and neuronal function and is here found in bilayer phospholipids at a 30–50% level. Bilayers with such high DHA levels have distinct properties, probably related to the functioning of integral membrane proteins. When compared with less unsaturated phospholipid acyl chains, DHA-containing bilayers have reduced thickness in the fluid state and consequently an increased area occupied per phospholipid at the aqueous interface of the bilayer [14]. A substantial amount of work has been done on the phase behavior of binary lipid mixtures to get a better understanding of lipid interactions in cell membranes. In bilayers composed of two or more phospholipids with different gel to liquid crystalline phase transition temperatures, different acyl chain lengths and different degrees of acyl chain unsaturation, it is conceivable that one can find segregation of bilayer components in the plane of the bilayer [15]. In such a case, one important driving force would be the hydrophobic mismatch due to differences in thickness of the phospholipid species in the bilayer [16]. Effects of adding an amphiphile like chlorpromazine (see later) to such a bilayer could possibly provide valuable information on both the modes of action by the drug (amphiphile) and the robustness of the lipid microdomains in the bilayer.

Experimental evidence of lipid microdomain formation in lipid bilayers has been presented from various techniques [17–23]. In an early study, Cullis and Hope [17] detected a bilayer stabilizing effect of cholesterol on a sphingomyelin–phosphatidylethanolamine lipid mixture by employing ^{31}P NMR. ^{13}C NMR has been used to study cholesterol and DPPC mixtures where cholesterol is found to induce fluid-phase immiscibility in this kind of bilayer [18]. More recently, ^{2}H NMR experiments have determined that ceramide in phosphatidylcholine bilayers cause microdomain formation of different composition and phase state in POPC/ceramide bilayers at physiological temperature [19].

Several investigators studying the role of phospholipid acyl chain unsaturation and stability of lipid rafts composed of sphingomyelin and cholesterol [20,22] support the notion that such lipid rafts are promoted by polyunsaturated acyl chains. The docosahexenoyl (22:6, DHA) acyl chain is found to enhance membrane lipid raft formation by lipid phase separation [24–26].

Molecular mechanics modelling of DHA suggests a rigid and ordered DHA structure [27–29]. Contrasting these modelling studies, DHA has been found in a compressibility study [30] 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 (oleoyl) acyl chains in phospholipids with choline head group. Furthermore, findings in a ^{2}H NMR and X-ray diffraction study, suggest that the *sn-2* attached (22:6) acyl chain in a (16:0–22:6) PC bilayer has a distribution of mass that is shifted toward the bilayer aqueous interface. The (16:0) acyl chain, on the other hand, is found to be displaced toward the membrane center [31]. The activity of membrane-bound proteins is influenced by the lipid composition of the membranes [32]. Consequently, perturbation of lipid organization in a bilayer by amphiphilic molecules can influence the activity of such proteins even without direct interaction between the protein and the amphiphile.

We have previously investigated [33], by the use of solid-state NMR [34], chlorpromazine interaction with partially hydrated phospholipid bilayers with choline head group and saturated acyl chains (DPPC (60%)/DMPC (40%)) and mixture of choline and serine head groups (DPPC (60%)/PS (40%)). These PS phospholipids extracted from pig brain (PBPS) have the following acyl chain composition: 49% of (18:0–18:1), 28% of (18:0–22:6), and five minor molecular species where two are known: 6% of (16:0–22:6) and 3% of (18:0–20:4). This study showed that CPZ has low or no interaction with the DPPC (60%)/DMPC (40%) bilayer. Conversely, CPZ causes a 5–15 ppm low field shift of ~30% of the saturated carbon acyl chain resonances in the DPPC (54%)/PS (36%)/CPZ (10%) bilayer.

It is important to note that phosphatidylcholine and phosphatidylserine species of like acyl chain composition [35] and samples of mixed bovine brain phosphatidylserine (about 8% of the 22:6 acyl chain) and DPPC [36] have been found to be completely miscible.

Supported by these findings [33], we investigated further [37] by ^{13}C and ^{31}P NMR [38] on 1,2-dipalmitoyl phosphatidycholine (DPPC, 16:0–16:0 PC) in mixture with the previously described PS extract from pig brain: DPPC (60%)/PBPS (40%) as well as pure 1-palmitoyl-2-oleoylphosphatidylserine (POPS) and pure PBPS bilayers with and without CPZ. The data showed that CPZ prefers binding to the phosphate of phosphatidylserine and that also carboxyl binding of CPZ is present in the DPPC/PBPS/CPZ bilayer and that CPZ interaction depends on acyl chain unsaturation. Recently, we followed up these findings by a ^{13}C and ^{31}P solid-state NMR study [39] on a bilayer composed of 1,2-dipalmitoyl phosphatidycholine (DPPC, 16:0–16:0 PC), 1-palmitoyl-2-oleoylphosphatidylserine (POPS) and the DHA containing 1-stearoyl-2-docosahexenoyl phosphatidylserine (SDPS) in a DPPC (60%)/POPS (29%)/SDPS (11%) bilayer with and without 10% CPZ. The T_1 relaxation data showed that the palmitic, the stearic and most of the oleic acyl chains (as well as the choline head group) were not affected by CPZ. The data indicated reduced mobility of DHA's c4 and c5 carbons upon CPZ binding as well as a clear increase in serine head group mobility. This recent study [39] showed that chlorpromazine interacts preferentially with bilayers containing phospholipids with a high proportion of phosphatidylserines with highly unsaturated acyl chains.

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 ethanolamine head groups). The CSA appears to be influenced by the chemical nature of the fatty acyl chains [37]. 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

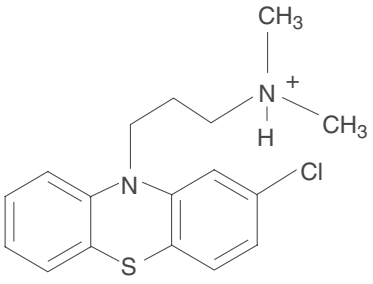
the phosphatidylcholine phosphate moiety [38,40]. This can be accounted for by greater rigidity of the phosphatidylserine head group than the phosphatidylcholine head group. This rigidity supposedly results from electrostatic interactions and/or hydrogen bonding between or within the phosphatidylserine 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 studies [33,37,39] we deduced from the composition of molecular species in PBPS that it must have been SDPS of the pig brain PS extract (PBPS) that caused the main, strong interaction with CPZ. (We found that POPS and SOPS had only negligible interaction with CPZ). In the study presented here, we employ bilayer samples of DPPC (60%) and the DHA containing synthetic/pure SDPS (40%) as well as the CPZ containing counterpart DPPC (54%)/SDPS (36%)/CPZ (10%) to enhance the possibility of obtaining information on DHA's role in CPZ interaction.

2. Materials and methods

2.1. Liposome preparation

ChlorpromazineHCl (CPZ) (Scheme 1) and synthetic 1,2-dipalmitoyl phosphatidylcholine (DPPC, powder) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Synthetic 1-stearoyl-2-docosahexenoyl phosphatidylserine (SDPS, dissolved in chloroform) was 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 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 CPZHCl (dissolved in H_2O) to obtain a 10% molar ratio. Thus a sample of 54% PC, 36% PS and 10% CPZ was obtained as well as the corresponding sample without CPZ. The samples with added CPZHCl 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 ^{1}H–MAS–NMR). Then, water was added to the samples to obtain fully hydrated bilayers (~30 water molecules per lipid molecule) [41,42] 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. 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/SDPS and DPPC/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 was 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 [43] 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 which were accumulated at 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 ^{1}H-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 to 318 K, and typically 512 transients were accumulated.

3. Results

The ^{13}C Magic Angle Spinning (MAS) spectra of DPPC/SDPS and DPPC/SDPS/CPZ bilayer samples were recorded at a temperature of 310 K and are presented as spectral regions in Figs. 1–3 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 and SDPS acyl chain sp^3 carbon resonances in the 12–38 ppm region as well as the phospholipid choline and serine head group carbon resonances and the glycerol moiety resonances

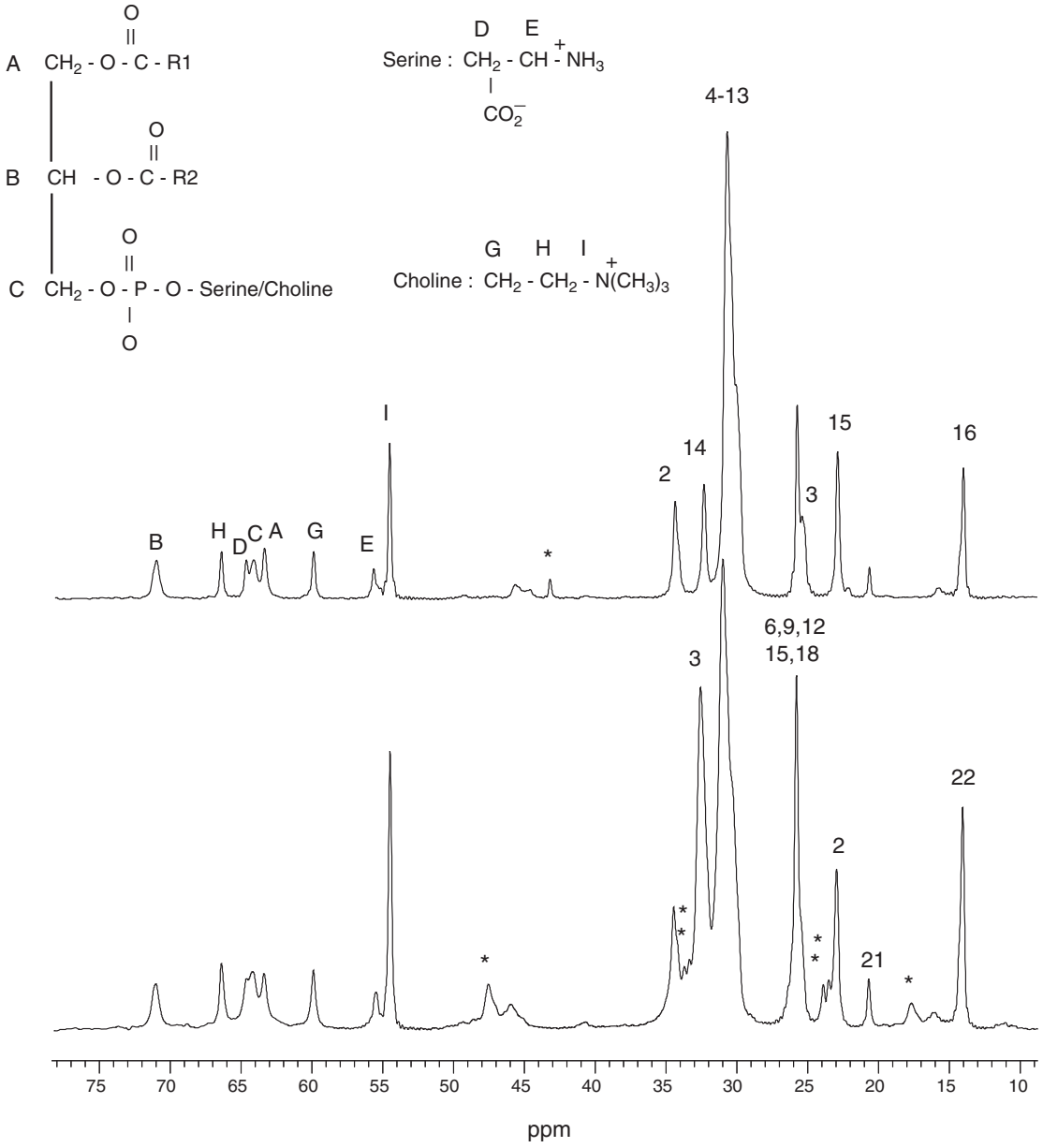


Fig. 1. ^{13}C NMR spectra, acquired at 310 K, displaying spectral region of phospholipid head group, glycerol and saturated acyl chain carbons (10–75 ppm). On top of the figure the structural formula of the glycerol moiety and the two phospholipids head groups, serine and choline, with corresponding assignment letters used in the spectra. Bottom spectrum: resonances of DPPC (60%)/SDPS (40%) sample. Resonances in the 10–35 ppm region of the bottom spectrum are labeled with the DHA (22:6) acyl chain carbon numbers. Resonances labeled with an asterisk "*" denote peaks that change chemical shifts when CPZ is present—compare with top spectrum. Top spectrum: corresponding spectrum of DPPC (54%)/SDPS (36%)/CPZ (10%). Glycerol and phospholipid head group resonances are labeled A–I in top spectrum where the 10–35 ppm region is labeled with the palmitic (16:0) acyl chain resonances of DPPC. Peak labeled with an asterisk "*" denotes a CPZ resonance. See the text for details.

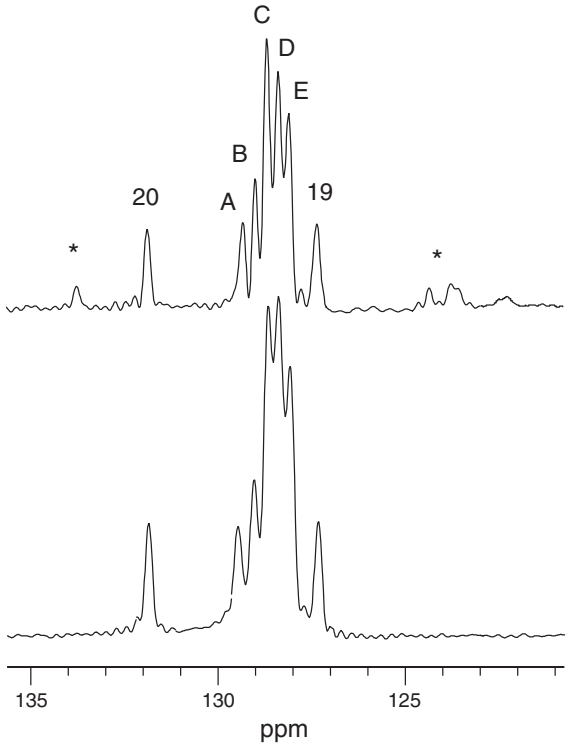


Fig. 2. Double bonded acyl chain carbon resonance region (125–135 ppm) of samples DPPC (60%)/SDPS (40%) (bottom spectrum) and DPPC (54%)/SDPS (36%)/CPZ (10%) (top spectrum). Spectra were acquired at 310 K (samples are in liquid crystalline phase). Assigned DHA resonances are C_{20} and C_{19}. Peaks labeled A–E correspond to carbons 4, 5, 7, 8, 10, 11, 13, 14, 16 and 17 of DHA, these resonances could not be individually assigned. See the text for details.

(in the 52–73 ppm spectral region). Fig. 1 bottom spectrum shows resonances of DPPC (60%)/SDPS (40%) sample and spectrum on top of Fig. 1 shows the corresponding spectrum of DPPC (54%)/SDPS (36%)/CPZ (10%) sample. The two spectra (Fig. 1, top and bottom) are dominated by the palmitic (DPPC) molecular specie. The molar composition of the samples cause the palmitic (16:0) acyl chain resonances to give ~60% of the peak intensities in this spectral region, whereas the contribution from the SDPS species in this spectral region is 40% (20% from each of the 18:0 and 22:6 acyl chains). Fig. 1, bottom spectrum, displays resonances in the 10–35 ppm region labeled with the DHA (22:6) acyl chain carbon numbers of DPPC (60%)/SDPS (40%) sample. Resonances labeled with an asterisk "*" denote peaks that change chemical shifts when CPZ is present—compare with top spectrum. The corresponding top spectrum of Fig. 1 displays resonances in the 10–35 ppm region labeled with the palmitic (16:0) acyl chain resonances of DPPC and glycerol and phospholipid head group resonances are labeled A–I (DPPC (54%)/SDPS (36%)/CPZ (10%) sample). Peak labeled with an asterisk "*" denotes a CPZ resonance. Additionally, Fig. 1 also shows the structural formulas of the glycerol moiety and the two phospholipids head groups, serine and choline, with the corresponding assignment letters used in the spectra. The three glycerol resonances will be composed of the two phospholipid species in the two samples, DPPC and SDPS.

From the T_1 data presented in Table 1, one finds that the carbon T_1 values of the choline head group β carbon and carboxyl carbon both show reduced T_1 values by the addition of CPZ. The glycerol carbon T_1 values, on the other hand, demonstrate a diverse effect of CPZ. The *sn-1* and *sn-3* glycerol carbons display an increased T_1 value due to CPZ in contrast to the *sn-2* glycerol carbon that shows a reduced T_1 value in presence of CPZ.

Resonances in Fig. 1 (bottom spectrum) at 46–48 and 16–18 ppm in the DPPC (60%)/SDPS (40%) spectrum appear at chemical shift values usually not found/reported in ^{13}C NMR spectra of phospholipid bilayers. The largest of these peaks, for example the one at 48 ppm displayed in bottom spectrum of Fig. 1, disappears in presence of CPZ (see Fig. 1, top spectrum). Similarly, the peak at 18 ppm, Fig. 1, bottom spectrum, disappears when CPZ is added to the sample (see Fig. 1, top spectrum). This suggests that the special chemical shifts of these resonances are due to the molecular organization in the DPPC (60%)/SDPS (40%) bilayer, i.e. the peaks at these unusual chemical shifts are due to microdomain formation/molecular segregation of DPPC and SDPS phospholipids. Furthermore, these domains are perturbed by the 10% CPZ present in the DPPC (54%)/SDPS (36%)/CPZ (10%) sample,

Fig. 3. Carbonyl and carboxyl carbon resonance region (170–176 ppm) of samples DPPC (60%)/SDPS (40%) (bottom spectrum) and DPPC (54%)/SDPS (36%)/CPZ (10%) (top spectrum). Spectra are acquired at 310 K. 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.

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

Carbon	DPPC/SDPS	DPPC/SDPS/CPZ
C=O	2.73	2.47
CO_2^-	2.03	0.72
DHA C=C:		
C_{19}	0.83	0.80
C_{20}	0.53	0.61
(A*)	0.88	0.65
(B*)	0.75	0.55
(C*)	1.31	1.55
(D*)	1.69	1.35
(E*)	1.53	1.78
Glycerol carbon:		
sn-1	0.21	0.32
sn-2	0.37	0.27
sn-3	0.16	0.28
Serine carbon:		
α	0.22	0.33
β	1.53	0.73
Choline carbon :		
α	0.17	0.15
β	0.18	0.16
CH_3	0.20	0.24
Palmitic carbon:		
2	0.39	0.46
4–13	0.71	0.82
14	1.50	1.24
15	1.52	1.69
16	4.23	4.46
DHA carbon:		
6, 9, 12, 15, 18	0.56	1.24
21	1.82	1.53

Peaks present in Fig. 1—bottom spectrum that disappear or are reduced when CPZ is added, Fig. 1—top spectrum.

At chemical shift (ppm):		
47.56	1.08	No peak
45.95	0.99	0.80
33.71	1.77	No peak
33.40	1.29	No peak
23.94	0.99	No peak
23.57	0.95	No peak

* Peaks labeled in Fig. 2.

see Fig. 1—top spectrum, where the above-mentioned resonances at 48 and 18 ppm are not present in contrast to the remaining smaller resonances at 46 and 16 ppm. Furthemore, spectral changes of saturated carbon resonances and glycerol/head group resonances, without and with CPZ, shown in Fig. 1, bottom and top spectrum, respectively, indicate that these are due to saturated acyl chain carbons. Hydrophobic mismatch of DPPC domains alongside SDPS domains appears to be the origin of peaks at 48 and 18 ppm (Fig. 1, bottom spectrum). Peak intensity changes (as well as changes in T_1 values—see later) indicate that palmitic carbons 2 and 15 are responsible for the unexpected resonances at 48 and 18 ppm in spectrum of the DPPC (60%)/SDPS (40%) sample. Presumably, SDPS acyl chains experience the microdomain formation as well, as evidenced by the peaks labeled with an asterisk in bottom spectrum of Fig. 1. These assignments, except the assignment of the DHA C6–C18 peak that can be confirmed by comparison with spectra of samples with less DHA content [39], are tentative. The CPZ induced perturbation of the DPPC and the SDPS microdomains cause changes in the above-described resonances—see Fig. 1 top spectrum. Intensities of the assigned resonances displayed in Fig. 1 are in accordance with the molar ratio of SDPS's docosahexenoyl and stearoyl acyl chains at 20% each. The gel to liquid crystalline phase transition temperature of SDPs is not reported in the literature; however, it is likely to be below 0° C. Thus, at a sample temperature of 310 K the SDPS phospholipids would be in the liquid crystalline phase and DPPC just below its transition temperature of 314–315 K. However, the carbon resonances in spectra of DPPC/SDPS and DPPC/SDPS/CPZ have the narrow appearance that corresponds to phospholipids in the liquid (crystalline) state.

Fig. 2 shows the 125–135 ppm region where the C=C resonances of the acyl chains of samples DPPC/SDPS and DPPC/SDPS/CPZ are found. Comparison of the C=C resonances with/without CPZ (see Fig. 2) 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. 2 poses an obstacle to a complete resonance assignment. However, in a recent solid-state NMR study, where $^1H-^{13}C$ two dimensional cross-polarization experiments were employed [44], the investigators managed to firmly assign DHA's C_{19} and C_{20}. Thus, the peak at 131.3 ppm is assigned to resonance C_{20} and the peak at 126.8 ppm to resonance C_{19}. The remaining C=C resonances of DHA (C_4-C_5, C_7-C_8, $C_{10}-C_{11}$, $C_{13}-C_{14}$, $C_{16}-C_{17}$) located between 127.4–130.5 ppm (peaks labeled A–E in Fig. 2 top spectrum) could not be individually assigned. As evident in Fig. 2 there is no significant intensity change of DHA's resonances C_{19} and

Table 2
^{13}P chemical shift anisotropy (CSA, in ppm) from 296 to 318 K DPPC/SDPS and DPPC/SDPS/CPZ bilayers

Temperature (K)	DPPC/SDPS	DPPC/SDPS/CPZ
296	99	85
297	99	86
298	97	86
299	98	87
300	95	86
301	95	84
302	94	83
303	94	83
304	91	80
305	90	79
306	88	78
307	88	76
308	86	76
309	86	73
310	84	70
311	82	70
312	82	66
313	80	66
314	79	65
315	78	63
316	76	66
317	73	63
318	71	64

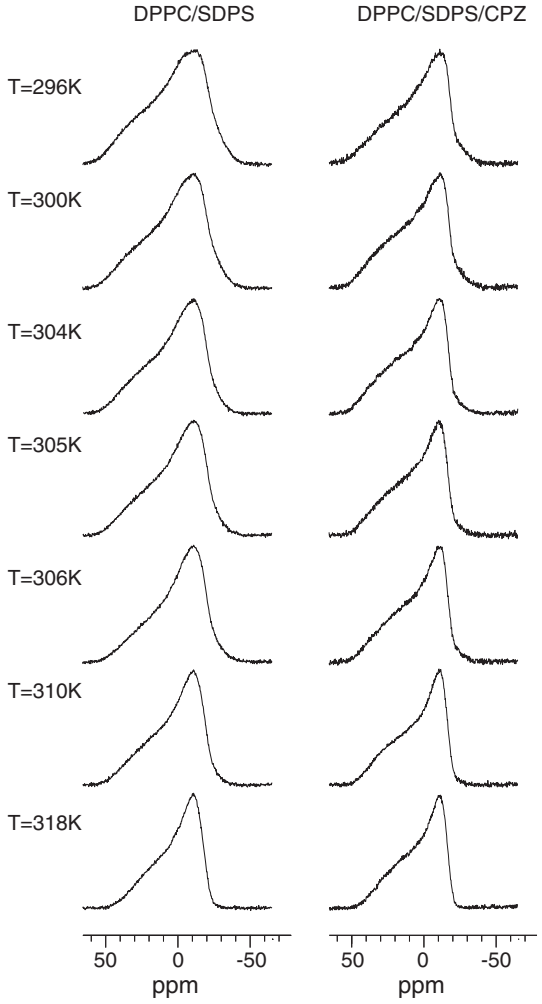


Fig. 4. Static ^{31}P NMR spectra of samples DPPC (60%)/SDPS (40%) (left column) and DPPC (54%)/SDPS (36%)/CPZ (10%) (right column). Sample temperatures from 296 K (top spectra) to 318 K (bottom spectra). See the text for details.

C_{20} upon addition of CPZ. Furthermore, the C=C resonances of DHA (Fig. 2 and Table 1) all display similar T_1 values with and without CPZ. In contrast to this, the DHA's sp^3 carbons in between sp^2 carbons, i.e. carbon 6, 9, 12, 15, and 18 have a combined peak with a T_1 value that more than doubles when CPZ is present. The single resonance peak of DHA's carbon 21 (see Fig. 1 and Table 1), on the other hand, shows a 16% reduction in presence of 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 T_1 value increase of these resonances. The appearance of some broad peaks around 124 ppm labeled with an asterisk "*" (Fig. 2, top spectrum) when CPZ is present corresponds to double bonded carbon resonances of the CPZ molecule, as does the peak at ~133.5 ppm also labeled with an asterisk "*".

Fig. 3 shows the carbonyl resonance (~173 ppm) and the serine head group carboxyl (~171 ppm) [45] resonance of samples DPPC/SDPS (bottom spectrum) and DPPC/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. 3, it is apparent that resonance intensities are not affected by addition of CPZ. A comparison of both the carbonyl resonance (~173 ppm) the serine head group carboxyl resonance (~171 ppm), without and with CPZ, makes it evident that the 10% CPZ reduces the SDPS's carboxyl resonance T_1 value (Table 1) by 65% (from 2.03 to 0.72 s). The corresponding T_1 values for the combined PC/PS carbonyl resonance show a 10% reduction in presence of CPZ.

In general, the ^{31}P CSA data presented in Table 2 and Fig. 4 show that the sample without CPZ has a higher CSA, than with CPZ, over the whole temperature range measured (296–318 K). The CSA of the sample without CPZ (the DPPC/SDPS sample) displays a fairly steady decrease as temperature increases. In addition to a general decrease in CSA value upon temperature increase, the CPZ-containing sample (the DPPC/SDPS/CPZ sample) displays a sudden drop in CSA of 13 ppm from 304 to 310 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 were measured at the central band of the MAS spectra, and presented in Table 3. The decrease in T_1 value as sample temperature increases demonstrates that the bilayer phospholipids are in "slow motion" motional regime. In Fig. 5 the three central band peaks are displayed at several of the temperatures investigated. They can be assigned [37,46] 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, slight decrease of the glycerol carbon resonances of the DPPC/SDPS/CPZ sample (Fig. 1) as compared to 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. An explanation for this observation can be found in the possibility of an altered transverse relaxation of dipolar coupled spins under radio-

Table 3
^{13}P T_1 values (seconds) from 296 to 318 K DPPC/SDPS and DPPC/SDPS/CPZ bilayers

Temperature (K)	DPPC/SDPS		DPPC/SDPS/CPZ		
	PC	PS	PC	PS	CPZ–PS
296	1.29	1.03	1.39	1.18	0.99
298	1.03	0.97	1.19	0.99	0.84
300	1.02	1.00	1.02	0.95	0.89
302	0.96	0.85	0.99	0.89	0.74
304	0.96	0.83	0.91	0.88	0.70
306	0.95	0.87	0.87	0.83	1.00
308	0.88	0.80	0.87	0.81	0.71
310	0.93	0.84	0.84	0.76	0.65
312	0.84	0.79	0.84	0.79	0.68
314	0.91	0.84	0.88	0.83	0.64
316	0.82	0.88	0.82	0.79	0.59
318	0.75	0.76	0.79	0.67	0.55

frequency irradiation (decoupling) [47]. In such a case destructive interference effects can cause line broadening due to (molecular) motion interfere 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 non-protonated 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 [44]).

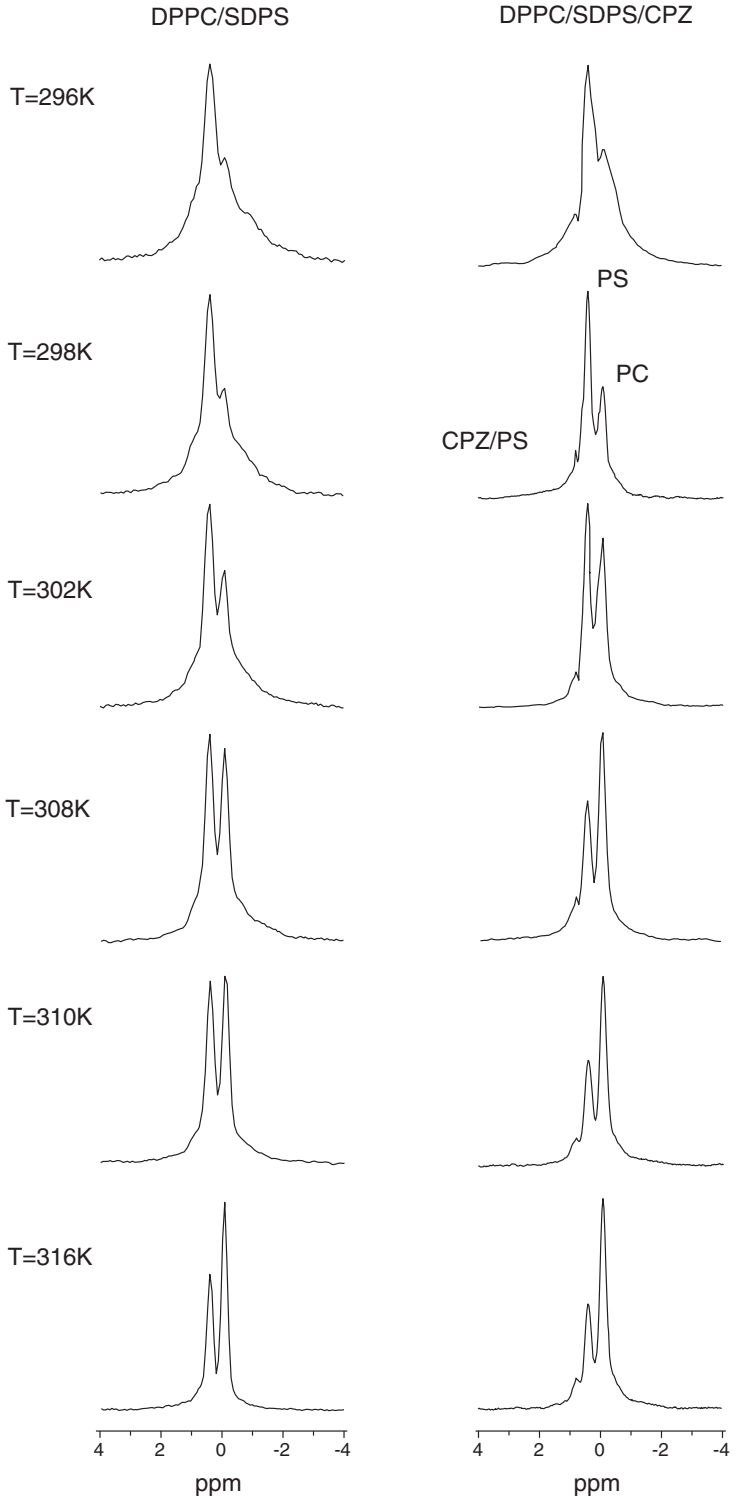


Fig. 5. ^{31}P MAS spectra of samples DPPC (60%)/SDPS (40%) (left column) and DPPC (54%)/SDPS (36%)/CPZ (10%) (right column) between sample temperatures 296 and 318 K. Note sudden decrease in CSA of CPZ-containing sample at 305 K. See the text for details.

Both the choline head group β carbon and the serine head group carboxyl carbon show reduced T_1 values by the addition of CPZ and presumably this is caused by an increased mobility. The glycerol carbon T_1 values, on the other hand, demonstrate a diverse effect of CPZ. The *sn-1* and *sn-3* glycerol carbons display lower mobility (an increased T_1 value) due to CPZ, whereas the *sn-2* glycerol carbon shows higher mobility (a reduced T_1 value) in presence of CPZ. This increased mobility of the *sn-2* moiety is not evident in the C=C resonances of DHA (Fig. 2 and Table 1). These resonances all display similar T_1 values with and without CPZ. However, the DHA's sp^3 carbons in between sp^2 carbons, i.e. carbon 6, 9, 12, 15, and 18 demonstrate reduced mobility in that these resonances have a combined peak with a T_1 value that more than double when CPZ is present. The single resonance peak of DHA's carbon 21 (see Fig. 1 and Table 1), on the other hand, shows a T_1 value with 16% reduction in presence of CPZ. Thus, the part of SDPS's DHA acyl chain that demonstrate a reduced mobility when CPZ is bound to the bilayer is the part close to the polar region of the bilayer, as demonstrated by an increase in the T_1 values of these resonances.

A comparison of both the carbonyl resonance (~173 ppm) the serine head group carboxyl resonance (~171 ppm), without and with CPZ, makes it evident that the 10% CPZ reduces the SDPS's carboxyl resonance T_1 value by 65% (from 2.03 to 0.72 s, see Table 1) indicating an increased mobility of the PS head group when CPZ is bound. The corresponding T_1 values for the combined PC/PS carbonyl resonance show a 10% reduction in presence of CPZ.

The ^{31}P CSA [48] data (Table 2 and Fig. 4) show that the sample without CPZ has a higher CSA, than when CPZ is present, over the whole temperature range measured (296–318 K). Thus, the CSA data support the ^{13}C T_1 data in that they both demonstrate increased mobility of the head groups when CPZ is bound to the bilayer. Resonances that are present in the spectrum of the DPPC (60%)/SDPS (40%) sample (Fig. 1, bottom spectrum) and that disappear in presence of 10% CPZ (Fig. 1, top spectrum) most probably are due to the special interface environment, e.g. the hydrophobic mismatch, at the interface of DPPC and SDPS microdomains. In itself the appearance of resonances at novel chemical shift values is a clear demonstration of a unique chemical environment in the DPPC (60%)/SDPS (40%) bilayer. Rationalizing the effects of microdomains in the DPPC/SDPS sample (see Fig. 1) it is reasonable to expect the existence of special local environments at the interface of microdomains where one predominantly consists of DPPC molecules and a neighboring domain contains SDPS molecules. Taking, for example, the two peaks at 48 and 18 ppm displayed in bottom spectrum of Fig. 1, and assuming that these two resonances are due to acyl chain CH_2 groups of the palmitoyl, stearoyl or C_3/C_{21} of the 22:6 acyl chain. Such a CH_2 group is in a special environment of the interface of two DPPC and SDPS microdomains. In such a case, the 48 ppm peak would come as a result of a CH_2 group that experienced an increased local field due to a neighboring electronegative nucleus, such as oxygen, or the deshielding region of a C=C group with strong magnetic suceptibility. The peak in Fig. 1 bottom spectrum, at 18 ppm, on the other hand, could be due to the special microdomain interface, where the CH_2 spends most of its time in the shielding region of a 22:6 acyl chain C=C group, i.e. this acyl chain resonance is shifted to a lower parts per million value by the environment of the microdomain interface. The demonstrated disappearance of bilayer microdomain ^{13}C peaks in presence of CPZ together with the found CPZ effects on DHA mobility [49] point at the crucial role DHA in *sn-2* position of SDPS has in formation and perseverance of phospholipid microdomains in a bilayer like the DPPC (60%)/SDPS (40%) of this study.

The found tendency of DHA, determined by ^2H NMR and X-ray diffraction [31], that the *sn-2* attached (22:6) acyl chain in a (16:0–22:6) PC bilayer has a distribution of mass that is shifted toward the bilayer aqueous interface would imply bending of the DHA acyl chain. In such a process, it is possible that the terminal methyl group can be located somewhere in vicinity of the polar region of the bilayer. The findings of the study presented here suggest CPZ bound to the phosphate of 1-stearoyl-2-docosahexenoyl phosphatidylserine will slow down and partially inhibit such a DHA acyl chain movement in a bilayer. This would affect the area occupied by a SDPS molecule (in the bilayer) and probably the thickness of the bilayer where SDPS molecules reside as well. It is quite likely that such CPZ caused changes can affect the function of proteins embedded in the bilayer.

References

[1] D.B. Jump, The biochemistry of n-3 polyunsaturated fatty acids, J. Biol. Chem. 277 (2002) 8755–8758.
[2] L.A. Witting, C.C. Harvey, B. Century, M.K. Horwitt, Dietary alterations of fatty acids of erythrocytes and mitochondria of brain and liver, J. Lipid Res. 2 (1961) 412–418.
[3] A. Lenzi, L. Gandini, V. Maresca, R. Rago, P. Sgrò, F. Dondero, M. Picardo, Fatty acid composition of spermatozoa and immature germ cells, Mol. Hum. Reprod. 6 (2000) 226–231.
[4] J.S. O'Brien, E.L. Sampson, Fatty acid and fatty aldehyde composition of the major brain lipids in normal human gray matter, white matter, and myelin, J. Lipid Res. 6 (1965) 545–551.
[5] W. Stillwell, S.R. Wassall, Docosahexaenoic acid: membrane properties of a unique fatty acid, Chem. Phys. Lipids 126 (2003) 1–27.
[6] C.E. Borgeson, L. Pardini, R.S. Pardini, R.C. Reitz, Effects of dietary fish oil on human mammary carcinoma and on lipid-metabolizing enzymes, Lipids 4 (1989) 290–295.
[7] M. Neuringer, W.E. Connor, D.S. Lin, L. Barstad, S. Luck, Biochemical and functional effects of prenatal and postnatal ω-3 fatty acid deficiency on retina and brain in rhesus monkeys, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 4021–4025.
[8] J.A. Glomset, Fish, fatty acids, and human health, N. Engl. J. Med. 312 (1985) 1253–1254.
[9] M. Lagarde, N. Gualde, M. Rigaud, Metabolic interactions between eicosanoids in blood and vascular cells, Biochem. J. 257 (1989) 313–320.
[10] A.M. de Urquiza, S. Liu, M. Sjöberg, R.H. Zetterström, W. Griffiths, J. Sjövall, T. Perlmann, Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain, Science 290 (2000) 2140–2144.
[11] S.D. Freedman, H.M. Katz, E.M. Parker, M. Laposata, M.Y. Urman, J.G. Alvarez, A membrane lipid imbalance plays a role in the phenotypic expression of cystic fibrosis in cftr−/− mice, PNAS 96 (1999) 13995–14000.

[12] J.S. Poling, S. Vicini, M.A. Rogawski, N. Salem Jr., Docosahexaenoic acid block of neuronal voltage-gated K+ channels: subunit selective antagonism by zinc, Neuropharmacology 35 (1996) 969–982.
[13] M.C. Garcia, H.Y. Kim, Mobilization of arachidonate and docosahexaenoate by stimulation of the 5-HT2A receptor in rat C6 glioma cells, Brain Res. 768 (1997) 43–48.
[14] A. Salmon, S.W. Dodd, G.D. Williams, J.M. Beach, M.F. Brown, Configurational statistics of acyl chains in polyunsaturated lipid bilayers from deuterium NMR, J. Am. Chem. Soc. 109 (1987) 2600–2609.
[15] C. Leidy, K. Gousset, J. Ricker, W.F. Wolkers, N.M. Tsvetkova, F. Tablin, J.H. Crowe, Lipid phase behavior and stabilization of domains in membranes of platelets, Cell Biochem. Biophys. 40 (2004) 123–148.
[16] M.Ø. Jensen, O.G. Mouritsen, Lipids do influence protein function—the hydrophobic matching hypothesis revisited, Biochim. Biophys. Acta 1666 (2004) 205–226.
[17] P.R. Cullis, M.J. Hope, The bilayer stabilizing role of sphingomyelin in the presence of cholesterol. A ^{31}P NMR study, Biochim. Biophys. Acta 597 (1980) 5335–5542.
[18] B. Mantripragada, B. Sankaram, T.E. Thomson, Cholesterol-induced fluid-phase immiscibility in membranes, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 8686–8690.
[19] Y.-W. Hsueh, R. Giles, N. Kitson, J. Thewalt, The effect of ceramide on phosphateidylcholine membranes: a deuterium NMR study, Biophys. J. 82 (2002) 3089–3095.
[20] S.R. Shaikh, M.R. Brzustowicz, N. Gustafson, W. Stillwell, S.R. Wassall, Monounsaturated PE does not phase-separate from the lipid raft molecules sphingomyelin and cholesterol: role for polyunsaturation? Biochem. 41 (2002) 10593–10602.
[21] M.R. Brzustowicz, V. Cherezov, M. Caffrey, W. Stillwell, S.R. Wassall, Molecular organization of cholesterol in polyunsaturated membranes: microdomain formation, Biophys. J. 82 (2002) 285–298.
[22] S.R. Shaikh, A.C. Dumaual, D. LoCascio, R.A. Siddiqui, W. Stillwell, Acyl chain unsaturation in PE modulates phase separation from lipid raft molecules, Biochem. Biophys. Res. Commun. 311 (2003) 793–796.
[23] S. Moffett, D.A. Brown, M.E. Linder, Lipid-dependent targeting of G proteins into rafts, J. Biol. Chem. 275 (2000) 2191–2198.
[24] D. Huster, K. Arnold, K. Gawrisch, Influence of docosahexaenoic acid and cholesterol on lateral lipid organization in phospholipid mixtures, Biochem. 37 (1998) 17299–17308.
[25] M.R. Brzustowicz, V. Cherezov, M. Zerouga, M. Caffrey, W. Stillwell, S.R. Wassall, Controlling membrane cholesterol content. A role for polyunsaturated (docosahexaenoate) phospholipids, Biochem. 41 (2002) 12509–12519.
[26] S.R. Shaikh, A.C. Dumaual, A. Castillo, D. LoCascio, R.A. Siddiqui, W. Stillwell, S.R. Wassall, Oleic and docosahexaenoic acid differentially phase separate from lipid raft molecules: a comparative NMR, DSC, AFM, and detergent extraction study, Biophys. J. 87 (2004) 1752–1766.
[27] K.R. Applegate, J.A. Glomset, Computer-based modeling of the conformation and packing properties of docosahexaenoic acid, J. Lipid Res. 27 (1986) 658–680.
[28] K.R. Applegate, J.A. Glomset, Effect of acyl chain unsaturation on the conformation of model diacylglycerols: a computer modeling study, J. Lipid Res. 32 (1991) 635–644.
[29] K.R. Applegate, J.A. Glomset, Effect of acyl chain unsaturation on the packing of model diacylglycerols in simulated monolayers, J. Lipid Res. 32 (1991) 645–655.
[30] B.W. Koening, H.H. Strey, K. Gawrisch, Membrane lateral compressibility determined by NMR and X-ray diffraction. Effect of acyl chain polyunsaturation, Biophys. J. 73 (1997) 1954–1966.
[31] K. Rajamoorthi, H.I. Petrache, T.J. McIntosh, M.F. Brown, Packing and viscoelasticity of polyunsaturated w-3 and w-6 lipid bilayers as seen by 2H NMR and X-ray diffraction, J. Am. Chem. Soc. 127 (2005) 1576–1588.
[32] F.D. Gunstone, M.R. Pollard, C.M. Scrimgeour, H.S. Vedanayagam, Fatty acids. Part 50. ^{13}C nuclear magnetic resonance studies of olefinic fatty acids and esters, Chem. Phys. Lipids 18 (1977) 115–129.
[33] W. Nerdal, S.A. Gundersen, V. Thorsen, H. Høiland, H. Holmsen, Chlorpromazine interaction with glycerophospholipid liposomes studied by magic angle spinning solid state ^{13}C NMR and differential scanning calorimetry, Biochim. Biophys. Acta 1464 (2000) 165–175.
[34] W.-g. Wu, L.-M. Chi, Comparisons of lipid dynamics and packing in fully interdigitated monoarachidoylphosphatidylcholine and non-interdigitated dipalmitoylphosphatidylcholine bilayers: cross polarization/-magic angle spinning ^{13}C-NMR studies, Biochim. Biophys. Acta 1026 (1990) 225–235.
[35] J.R. Silvius, Gagné, Calcium-induced fusion and lateral phase separations in phosphatidylcholine–phosphatidylserine vesicles. Correlation by calorimetric and fusion measurements, Biochem. 23 (1984) 3241–3247.
[36] T.P. Stewart, S.W. Hui, A.R. Portis Jr., D. Papahadjopoulos, Complex phase mixing of phosphatidylcholine and phosphatidylserine in multilamellar membrane vesicles, Biochim. Biophys. Acta 556 (1979) 1–16.
[37] A.U. Gjerde, H. Holmsen, W. Nerdal, Chlorpromazine interaction with phosphatidylserines: a ^{13}C and ^{31}P solid-state NMR study, Biochim. Biophys. Acta 1682 (2004) 28–37.
[38] J.L. Browning, J. Seelig, Bilayers of phosphatidylserine: a deuterium and phosphorus nuclear magnetic resonance study, Biochem. 19 (1980) 1262–1270.
[39] S. Chen, A.U. Gjerde, H. Holmsen, W. Nerdal, Importance of polyunsaturated acyl chains in chlorpromazine interaction with phosphatidylserines: a ^{13}C and ^{31}P solid-state NMR study, Biophys. Chemist. 117 (2005) 101–109.
[40] S.J. Kohler, M.P. Klein, Orientation and dynamics of phospholipid head groups in bilayers and membranes determined from nuclear magnetic resonance chemical shielding tensors, Biochem. 16 (1977) 519–526.
[41] M.J. Janiak, D.M. Small, G.G. Shipley, Temperature and compositional dependence of the structure of hydrated dimyristoyl lecithin, J. Biol. Chem. 254 (1979) 6068–6078.
[42] D.M. Small, Observations on lecithin. Phase equilibria and structure of dry and hydrated egg lecithin, J. Lipid Res. 8 (1967) 551–557.
[43] M. Levitt, Symmetrical composite pulse sequences for NMR population inversion. I. Compensation of radiofrequency field inhomogeneity, J. Magn. Reson. 48 (1982) 234–264.
[44] S. Everts, J.H. davis, 1H and ^{13}C NMR of multilamellar dispersions of polyunsaturated (22:6) phospholipids, Biophys. J. 79 (2000) 885–897.
[45] D.L. Holwerda, P.D. Ellis, R.E. Wuthier, Carbon-13 and phosphorus-31 nuclear magnetic resonance studies on interaction of calcium with phosphatidylserine, Biochem. 20 (1981) 418–428.
[46] T.J.T. Pinheiro, A. Watts, Resolution of individual lipids in mixed phospholipid membranes and specific lipid–cytochrome *c* interactions by magic-angle spinning solid-state phosphorus-31 NMR, Biochem. 33 (1994) 2459–2467.
[47] F. Adebodun, J. Chung, B. Montez, E. Oldfield, X. Shan, Spectroscopic studies of lipids and biological membranes: carbon-13 and proton magic-angle sample sample-spinning nuclear magnetic resonance study of glycolipid-water systems, Biochem. 31 (1992) 4502–4509.
[48] P.R. Cullis, B. de Kruyff, R.E. Richards, Factors affecting the motion of the polar head group in phospholipid bilayers. A ^{31}P NMR study of unsonicated phosphatidylcholine liposomes, Biochim. Biophys. Acta 426 (1976) 433–446.
[49] L.L. Holte, F. Separovic, K. Gawrisch, Nuclear magnetic resonance investigation of hydrocarbon chain packing in bilayers of polyunsaturated phospholipids, Lipids 31 (1996) 199–203.