Chapter 3

Dynamics of Model Membranes by NMR

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Additional information is available at the end of the chapter

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Abstract

Amphiphilic molecules can create various aggregates in water. Concern about exploring such structures has been unabated for several decades due to the wide range of possible applications of lipid aggregates, from food technology to the pharmaceutical industry. The form of self-assembled structures depends on many factors, such as the type of amphiphilic molecule, the concentration, the level of hydration, the temperature, and the pH. Liposomes and micelles are the most widely known types of closed structures. Liposomes are more often used in the fields of medicine and pharmacy because they consist of nontoxic compounds and their composition and size can be controlled. Nuclear magnetic resonance (NMR) is one of the methods, which is most commonly used to study liposome properties. It can be used to observe changes in the structure, dynamics, and phase transition of lipid membranes. The membrane properties are changed under the influence of external factors, such as temperature, pH, and the presence of ions or drugs. The chapter aims to introduce and discuss the possibilities of the most useful NMR methods, ^{31}P and ^{1}H, to study the liposome properties. It also aims to show how various changes in the structure or dynamics of lipid molecules are visible in the NMR spectra.

Keywords: lipid aggregates, model membrane, liposome, dynamics, splitting, half-width of signal, ^{1}H NMR, ^{31}P NMR

1. Introduction

The phenomenon of creating various types of aggregates by lipids in water is of particular interest to professionals in the fields of biophysics, biochemistry, medicine, and pharmacy. The reason for this broad interest is the similarity between formed aggregates and subcellular structures, such as lysosomes and biological membranes. Thus, the structures, particularly those such as bilayers, have been used as models of biological membranes for many years.

The main advantage of this model is its ability to decide the composition, both in terms of lipid and protein content and the environment in which they are located (one in which various types of ions are present). Model membranes enable the study of their thermotropic properties, the transport of ions through them, and the phenomenon of vesicle fusion. Currently, model biological membranes (liposomes) are widely used as drug delivery systems and in various kinds of therapies.

The use of nuclear magnetic resonance (NMR) to study these structures poses several challenges. For instance, the technique uses a method of sample preparation that differs from the standard methods used for measurement in a liquid. An additional problem is the formation of lipid aggregates in water, which exhibits differ in the NMR time scale. This is due to the fact that NMR spectrometer "sees" the hydrated surface of the lipid aggregate as a substance similar to liquid, and the hydrophobic core of the aggregate as something similar to a solid. In fact, biological membranes are in the liquid crystalline phase (L_α); therefore, model membranes are usually studied in this phase. This shows that, in the case of the dispersion of lipids in water, other parameters typical for this type of structure will have a significant impact on the appearance of the NMR spectrum. The main features of lipid aggregates affecting the NMR spectrum include the type of lipid aggregate, its size, the degree of lipid hydration, and the thermodynamic state of the membrane. These four parameters determine the dynamics of lipid molecules and individual chemical groups trapped in a complex structure.

1.1. Amphiphilic molecules self-assembly and critical micelle concentration

Amphiphilic molecules (e.g., phospholipids) have lipophilic parts (hydrophobic) and polar parts (hydrophilic). In aqueous environments, these kinds of molecules undergo two basic effects [1]: the adsorption of water molecules on the surface of lipids and self-association. The result of both effects is that when dispersed in water, amphiphiles spontaneously aggregate. It is precisely this property of phospholipids that makes them the basic material from which the cell membrane is formed. Lipids, due to their ability to self-assemble, are divided by the properties of their polar headgroups; thus, the characteristics of the lipids' headgroups (i.e., non-ionic, zwitterionic, anionic, cationic, and catatonic) are emphasized, while the hydrophobic parts of lipids differ in their number of hydrocarbon chains, in their length (number of carbons in the chain), and in their degree of saturation [1–3]. Therefore, the self-assembly phenomenon occurs as the result of two opposite forces: first, that connected with the hydrophobic effect (the energetically unfavorable contact between fatty acid chains and water) and second, that connected to hydrophilic interactions with water molecules [1]. The result of these effects is the formation of micelles, bilayers, or other aggregates, because only the hydrophilic headgroups of lipids can be exposed to water.

Micelles are the simplest structures that can form amphiphilic lipids in water. The Gibbs phase rule says that at a certain temperature and under certain pressure, lipid molecules and micelles can be in equilibrium only at a fixed value of the mole fraction of hydrocarbon in water [4]. This value of lipid concentration in water is known as critical micelle concentration (CMC). Below CMC value, lipid molecules exist as monomers dispersed in water, whereas above CMC value lipid molecules tend to self-associate, forming micelles, bilayers, or other aggregates. The CMC, which is greater for charged molecules than for uncharged molecules [1], decreases

significantly in conjunction with decreases in the length of the fatty acid chains [5]. Lipids with relatively weak headgroups (i.e., with weak opposing forces) form bilayer-like aggregates, such as vesicles, and disc-like micelles [6].

Formed aggregates are usually large in size, mainly because they must take forms in which the fatty acid chains are not exposed to direct contact with water. Moreover, the type of lipid structure depends on the energetic of the lipid-water interface and on the shape of the lipid molecule [2, 3, 6]. Further consideration of this issue must take into account factors that influence the formation of the free energy of micellization and the micelle size. The formation of the free energy of micellization and its dependence on the aggregate size involves the bulk term, surface term, curvature term, and packing term [2–4]. The major driving force in the formation of aggregates is its hydrophobic effect and the contribution to the bulk term; however, it is not associated with the size of the formation, which depends on the free energy of micellization [4]. The hydrophobic interaction between fatty acid chains exposed to water and the different repulsive interactions between headgroups (steric, electrostatic, and hydration) contribute to the surface term. The repulsive interactions increase the surface area, whereas the hydrophobic interactions decrease the surface area [4, 7]; these effects are known as opposite interactions. The molecular conformation and motional properties of polar headgroups and the formed membrane surface, which identify the lipid type, are well known. However, information about the structure of lipid molecules packed at aggregates, especially if more than one kind of lipid is present, is very limited. The study of lipid-lipid interactions at the membrane polar-apolar interface is important because the membrane surface is the most probable site of selective electrostatic or steric associations [2–4]. The existing opposite forces have an influence on the curvature of formed agglomerates and restrict the packing of lipid molecules, contribute to the curvature and packing terms, and result in an optimum aggregate size [4]. All terms involved in the free energy of micellization determine favorable molecular packing, which is directly connected to the formed favorable aggregate structures of a specific type of lipid. Moreover, in the bilayer-like structures, the lipid molecules manifest an asymmetric transmembrane lipid-packing geometry [2, 3]. It follows, then, that the average area per lipid headgroup and the effective length of the lipid molecule are greater in the membrane's outer layer than in its inner layer [4]. Therefore, the packing and curvature terms are closely connected to the lipids' molecular shape and configuration. As a consequence, the micelles of single-chain lipids may be formed favorably as a result of weak packing restrictions, whereas those of double-chain lipids, due to stronger packing restrictions, are favorably formed bilayer-like structures [2].

1.2. Lipid hydration

Amphiphilic lipid self-assembly is a specific equilibrium between hydrophobic and hydrophilic interactions, but the bilayer hydration is determined mainly by interactions between the hydrophilic headgroups and the solvent [2, 3]. The level of hydration affects the self-assembly, curvature, shape and size of the aggregate, and the phase behavior. The hydration of the lipid aggregate depends on the specifics of the lipids (i.e., their headgroups). The hydration of hydrocarbon chains is much smaller and is restricted by the hydrophobic interactions [6]. The hydration process is also connected to the thermodynamic state of the lipid membrane. When the fluidity of fatty acid chains increases, the lipid molecules occupy a larger area, which

increases hydration as a result of the increase of exposure of the headgroups and hydrocarbon chains to the water molecules [1]. The properties of interbilayer water differ from those of free bulk water. Thus, each lipid molecule, with its water of hydration, should be treated as a separate thermodynamic and physicochemical entity [3, 4]. In general, it seems that the steric density fluctuations have only a slight influence on hydration parameters; however, they play an extremely important role in the surface hydration [1].

Of particular interest in this area is the use of lipids which build, in nature, biological membranes. The most common amphiphilic glycerophospholipids contained in their polar headgroups include phosphate, carboxyl, carbonyl, and choline residues, all of which take part in creating the hydrogen bonds [1, 2]. The availability of the headgroups for hydrogen bonding with the water molecules is the most important factor in the hydration of the bilayer. The coulombic charges of the lipid molecules participate less in the hydration process, probably because of an insufficient concentration of water molecules [1, 3].

1.2.1. Influence the hydration on lipid dynamics

The motion of phospholipid molecules within the lipid bilayer has been characterized as lateral diffusion, axis rotational Brownian motion of the headgroup, or glycerol backbone, wobbling, and flip-flopping [7–9]. The collective properties are different from those at the local molecular level. The phenomena accompanying the local molecular motion in the lipid aggregate include phase transitions, a morphological change of the lipid membrane as a whole (e.g., fusion/fission), pore formation, and the formation of heterogeneous structures, such as phase separation/domain formation [10]. Thus, the motion of the lipid aggregate as a whole cannot be explained on the basis of the lipid molecule motions (i.e., at the molecular level).

Thus, studies of membrane dynamics are concerned with the molecular motion of lipids. As mentioned previously, the rotational motion of the headgroups relates to hydrophobic interactions, hydrogen bonding, and the curvature of the lipid membrane; in this way, an increase in the curvature of, for example, liposomes, induce the level of hydration [11–16]. The reorientation of the headgroups is also restricted by the intermolecular force between them [1, 19]. Thus, the reorientation of phospholipid headgroups is restricted by breaking the intermolecular bonds (hydrogen bond and/or $PO_4^-/N^+(CH_3)_3$ bond) [10, 16–18]. Thus, the hydration of the polar headgroups weakens the strength of the $PO_4^-/N^+(CH_3)_3$ bond [10, 16, 19]. The dynamics of the lipid membrane interface is also connected with the mobility of the glycerol backbone of phospholipid (PO_4^-–$(CH_2)_2$–$N^+(CH_3)_3$) [7, 16, 20]. The mobility of the glycerol backbone is associated with the lateral diffusion of lipid molecules because it promotes hydration and, consequently, the reorientation of the headgroups. The reorientation of the headgroups (i.e., high hydration) causes the hydrocarbon chains to be more greatly exposed to water molecules, which indicates that the increased mobility of the phospholipid headgroups make the membrane polar-apolar surface more hydrophobic [6, 10, 16, 19].

1.3. Model membrane phase transitions

Membranes composed of one type of phospholipid have a clearly defined phase transition, which is caused by temperature variations. The phase transition temperature is primarily

dependent upon the type of phospholipid (number and length of hydrocarbon chains) and the level of lipid molecule hydration. The most frequently described phase of self-assembling aggregates is the liquid crystalline phase as a characteristic of cell membranes. Therefore, the most commonly studied phases are lamellar and nonlamellar, such as the hexagonal and cubic phases (normal and inversed).

The typical phase for lipid membranes at low temperatures is the lamellar crystalline phase L_C. As the temperature increases, the van der Waals' interactions decrease, which maintains the order of the hydrocarbon chains in the crystalline phase. The rotational motion of the hydrocarbon chains is then activated by the temperature. The phase transition between crystalline L_C and gel L_β phase occurs at T_s temperature [4]. In the lamellar L_β phase, the lipid molecules take up a larger area and are more hydrated than in the L_C phase [21]. The correlation time of the rotational motion of the acyl chains is about 10^{-5} s, which is about 100 times slower than the isomerization of the hydrocarbon chains [22]. Further increases in temperature cause a rise in the quasilamellar segments within the membrane in the intermediate periodic phase P_β. In this phase, the surface of the membrane is usually rippled, which occurs at T_p temperature [23]. After the intermediate phase, above T_p temperature, the hydrocarbon chains start to melt and form *trans-gauche* isomers. Internal reorientations, which have stochastic characteristics, are transferred along the acyl chains at times ranging from microseconds to milliseconds [24]. The presence of *trans-gauche* isomers determines an increase of distance between the lipid molecules in the membrane and decrease van der Waals' interactions. The increase of movement in the hydrophobic and hydrophilic parts of the lipid bilayers is characteristic of the melting state (i.e., the liquid crystalline phase) L_α [25]. The temperature of transition from the intermediate pleated phase to the liquid crystalline phase is called the melting temperature or the temperature of main phase transition T_t. The value of T_t thus depends on the length and level of the saturation of the hydrocarbon chains as well as on the level of hydration of the lipid molecules. The most important factor in the determination of this value is a hydration level between 0 and 30%. Hydration levels in this range have a major impact on decreases in the temperature T_t. However, because a 30% hydration level mainly increases the amount of water molecules not associated with the membrane (bulk water), changes of the hydration level between 30 and 50% are less likely to influence decreases in the temperature T_t [4].

When CMC values are extremely high, which results in low volumes of water, some kinds of lipids form nonlamellar phases, such as hexagonal (H_I) and cubic (C_I). Inversed hexagonal (H_{II}) and cubic (C_{II}) phases may be formed in trace amounts of water. Not all lipids can form these phases: their ability to form hexagonal and cubic phases depends on the stereochemical structure of the molecules. Lysophospholipids tend to form normal nonlamellar phases, whereas phosphatidylethanolamine (PE), cholesterol (Ch), cardiolipin (CL), and phosphatidic acid (PA) tend to form inversed nonlamellar phases [26].

However, preferred aggregation structures depend not only on the type of lipid, temperature, and hydration level but also on the pH. Under neutral pH conditions, the phosphatidylcholine (PC) and PE headgroups are electrically neutral, whereas the phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI) headgroups have net negative charges. The mixture of the lipids in the membrane transmits a surface charge density that

has an effect on the membrane permeability to ions and charged molecules, on the membrane protein function, and on the thermodynamic phase of the membrane [2–4]. For example, in the case of PE bilayers, which interact with fewer water molecules, the main gel-to-liquid crystalline phase transition (L_β/L_α) temperature increases by as much as 30°C compared to their counterpart PC bilayers (about 20–25°C) [4].

2. NMR spectra of model membranes

Amphiphilic lipids form aggregates of many different shapes and sizes; these aggregates can be at different phases. Moreover, minor changes in the concentration, temperature, or chemical structure of the lipid molecules may induce phase transitions between states. Additionally, the effects of molecular interactions and dynamics on macroscopic properties are evident in self-assembly systems [27]. NMR studies of self-assembly systems therefore begin by observing the dynamic parameters, which results in a better understanding of the static properties of the system [1]. Certainly, NMR is the most powerful technique with which to quantify the molecular dynamic in solution; however, in the case of lipid aggregates, it has some limitations.

NMR relaxation studies provide information about local dynamics and the conformational state of lipid hydrocarbon chains. This method is used to study aggregate properties (e.g., the size of micelles) [1]. The reorientation dynamics of aggregated lipid molecules is characterized by a locally preferred orientation; that is, lipid molecules undergo rapid internal motions, such as *trans-gauche* isomerizations, which are slightly anisotropic. NMR spectra from the lipids in micelles and bilayers are generally in the motional narrowing regime, which is caused by a time scale of lipid reorientation of 10^{-9} s or less [1–3, 7]. Thus, at conventional magnetic field strengths, essential information about the lipid aggregates is stored in the transverse relaxation rate T_2.

Polar headgroups and hydrocarbon chains (typically 12–16 carbons) can be studied using ^{1}H, ^{13}C, and ^{31}P NMR (e.g., dipole-dipole interactions, scalar interactions, and chemical shift anisotropy (CSA)) [27]. The ^{31}P has relatively large CSA. A complication revealed during the analysis is a change in the degree of the solvent's protonation, which is caused by direct dependency of CSA from the pH. In fact, the relaxation time T_2 is also sensitive to changes in micelle/liposome size because the rotational correlation time is proportional to the cube of the radius [1, 27]. Electrostatic and hydrodynamic intermolecular interactions are independent on the rotational diffusion of lipid molecules.

2.1. NMR time scale

The motional model of aggregated lipid molecules concerns the time scale separation of fast local and slow overall motion. The reorientational motion of lipid molecules divides into [4]

-fast local motion, which is slightly anisotropic, and

-slow isotropic motion (i.e., aggregate tumbling and the lateral diffusion of lipid molecules within the membrane surface).

These two motions occur on different time scales. Therefore, the special density can be written as the sum of the fast and slow components [28] as follows:

$$j(\omega_0) = \left(1 - S^2\right) j_f(\omega_0) + S^2 j_s(\omega_0) \tag{1}$$

where $j_f(\omega_0)$ and $j_s(\omega_0)$ are reduced spectral density functions that describe the fast and slow motions, respectively; ω_0 is the resonance frequency; and S is the order parameter.

The order parameter can be described as the average [28]

$$S = \frac{1}{2}\left(3\cos^2\theta - 1_f\right) \tag{2}$$

where θ is the angle between the axis of the maximum component of the electric field gradient tensor and the director axis.

For spherical aggregates such as micelles and liposomes, the slow motion (tumbling and lateral diffusion) is described by the Lorentzian spectral density function [1]:

$$j_{s(\omega_0)} = \frac{2\tau_s}{1 + (\omega_0\tau_s)^2} \tag{3}$$

where τ_s is the correlation time, and the correlation function is [1]

$$g_s = g_t g_d = e^{\frac{-t}{\tau_t}} e^{\frac{-t}{\tau_d}} = e^{\frac{-t}{\tau_s}}, \tag{4}$$

where subscripts t and d correspond to the tumbling and lateral diffusion motions, respectively.

The correlation time of tumbling and lateral diffusion of the sphere of radius R can be written as [1]

$$\tau_t = \frac{4\pi\eta R^3}{3k_B T} \wedge \tau_d = \frac{R^2}{6D} \tag{5}$$

where D is the lateral diffusion coefficient.

Taking into consideration the above equations, it is possible to write the relations for relaxation times [28] as follows:

$$T_1 = \frac{3\pi^2}{40}\chi^2\left[\left(1 - S^2\right)20\tau_f + S^2\left(\frac{4\tau_s}{1 + (\omega_0\tau_s)^2} + \frac{16\tau_s}{1 + (2\omega_0\tau_s)^2}\right)\right] \tag{6}$$

and

$$T_2 = \frac{3\pi^2}{40}\chi^2\left[\left(1 - S^2\right)20\tau_f + S^2\left(6\tau_s + \frac{10\tau_s}{1 + (\omega_0\tau_s)^2} + \frac{4\tau_s}{1 + (2\omega_0\tau_s)^2}\right)\right] \tag{7}$$

where subscripts *s* and *f* correspond to slow and fast motions, respectively; χ is the gyromagnetic ratio.

2.2. Preparation of the NMR sample

Liposomes are most often used in NMR studies as models of biological membranes. Liposomes, spherical structures consisting of one (large LUV or small SUV unilamellar vesicle) or more (multilamellar vesicles (MLV)) lipid bilayers, are divided by the number of bilayers as well as by size (**Figure 1**).

The classical preparation MLV method consists of hydrating the thin lipid film. Suitable amounts and types of lipids are dissolved in organic solvent (e.g., chloroform) and are predried under a stream of dry nitrogen. After the formation of a thin lipid film, the sample is allowed to continue drying in a vacuum evaporator for 1–12 h, after which it is hydrated [16]. In the preparation of the NMR sample, deuterated solvents (deuterated water) must be used. An appropriate amount of water should be added to the sample to obtain a final lipid concentration of not less than 20 mg/ml. The sample is then gently mixed, often in a water bath, at a temperature close to the main phase transition. After removing the thin lipid film from the glass walls, it is vortexed for 5–7 min. After completion of the procedure, a sample containing MLV is obtained [16]. When are need LUV or SUV, other methods (most commonly ultrasound disintegration and extrusion) are used. The sonication is carried out in an ice-water bath for 15–45 min, depending on the unit capacity and the expected size of the liposomes [16]. In the case of extrusion, special filters with a proper pore size and pressurized MLV are forced through the pores, thus depriving them of the unwanted bilayers. Combined methods are frequently used (e.g., sonication combined with extrusion). Extrusion is then used to calibrate the liposomes (i.e., to reduce deviations in the size distribution) [29]. Sonication is used more frequently than other methods due to the procedure's lower cost.

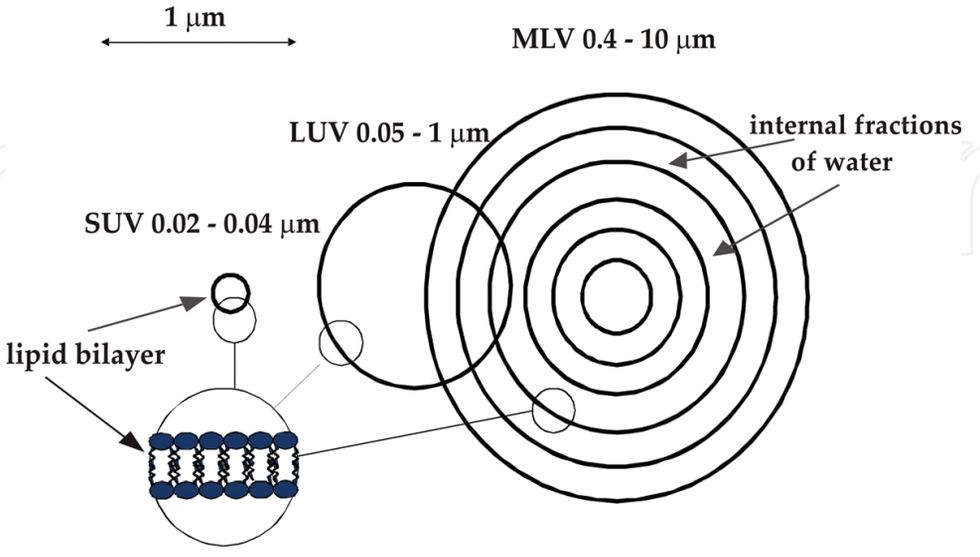

Figure 1. Types of the liposomes.

2.3. Lipid membrane dynamic studies

The ^{31}P NMR technique provides information about the local order, the mobility of the phosphate part of the lipid head, and the overall structure of the lipid aggregate [30]. The lineshape of ^{31}P NMR spectra is closely related with the CSA tensor and to the orientation of the lipid molecules relative to the applied magnetic field [31]. Thus, the CSA $\Delta\sigma$ depends on the phosphate group motion and on the temperature. The ^{31}P NMR spectrum shows the characteristic lamellar lineshape ($\sigma_\perp$—high-field maximum) and low-field shoulder ($\sigma_\parallel$). $\Delta\sigma$ can be calculated from the following expression [30]:

$$\Delta\sigma = 3\left(\sigma_\parallel + \sigma_\perp\right), \tag{8}$$

where $\sigma_\parallel$ and $\sigma_\perp$ are the values of ^{31}P shielding of the lipid molecules, oriented parallel or perpendicular relative to the magnetic field.

The spectral second moment is a measure of the shape of the ^{31}P signal related to the various motions of the headgroup. The second moment S_2 can be calculated from the following formula [30]:

$$S_2 = \frac{\int_{-\infty}^{+\infty} \nu^2 f(\nu) d\nu}{\int_{-\infty}^{+\infty} f(\nu) d\nu}, \tag{9}$$

where ν is frequency and $f(\nu)$ is the lineshape.

The CSA for lipid molecules ordered in water is about 100 ppm; for lipid aggregates, it decreases to about 50 ppm due to lateral diffusion of the lipid molecules, leading to further averaging; for liposomes larger than 200 nm, the CSA is reduced to 40 ppm [31]. Thus, the ^{31}P lineshape depends on the size of the lipid aggregate. The CSA value decreases as the liposome size decreases, that is, the liposome curvature increases. In the case of small aggregates (e.g., SUV), the CSA can be reduced to 10 ppm.

Analysis methods of ^{31}P NMR spectra most often are used to test functionalized liposomes that are used as drug carriers, the best known being PEGylated liposomes. The polyethylene glycol (PEG) caps the liposome, which gives it a longer circulation time in the blood, meaning that it decreases the uptake of the liposomes by the reticulum endothelium system (RES) and allows the drug to be released gradually [32]. In addition, PEG is biocompatible, which enables the possibility of further functionalization of the liposomes by attaching antibodies or ligands [33, 34]. The ^{31}P spectra analysis of PEGylated liposomes obtained using the thin film method revealed information about the amount of free lipids or building micelles (narrow line) and lipids building MLV (broad shoulder) [31, 35]. It is also possible to obtain more than one narrow signal (**Figure 2**).

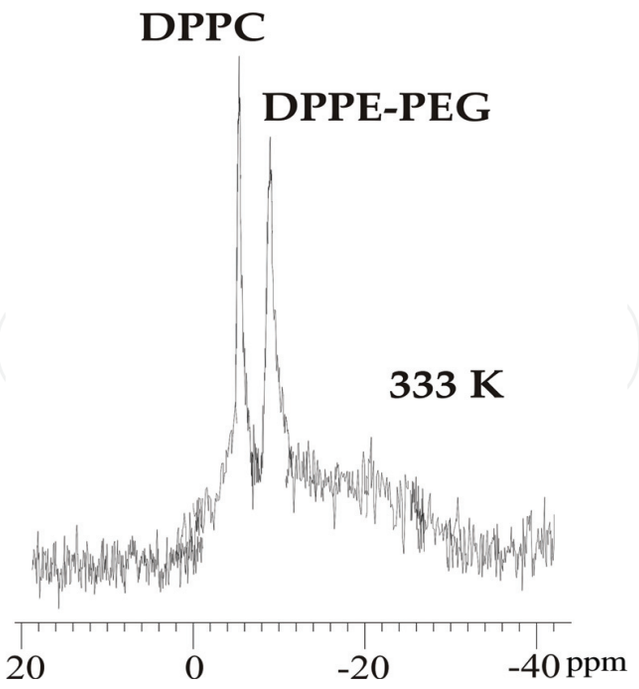

Figure 2. The ^{31}P NMR spectrum of the PEGylated DPPE/DPPC/Ch (phosphatidylethanolamine/dipalmitoylphosphocholine/cholesterol) multilamellar vesicles obtained at 333 K.

The number of narrow peaks depends on the number of phospholipid types used in the experiment. Then, each phosphate group is in a different chemical surrounding, which causes different chemical shifts. Splitting (δ) between narrow signals also depends on motional averaged CSA rather than on an isotropic chemical shift [31]. Moreover, it may affect the value of the chemical shift and cause difficulties in the assignment of peaks. As mentioned previously, the lineshape depends on the orientation and motion of the lipid molecules and all aggregates. Thus, the temperature of measure is very important. Additionally, in the case of lipid aggregates, a change in temperature causes a change in the phase/physical state. Temperature changes have a slight impact on narrow signals but a significant impact on the broad shoulder [31]. The ^{31}P NMR spectrum of MLV in the low-field shoulder is extremely broad below the temperature during the major phase transition.

The ^{31}P NMR studies of the influence of drugs on the organization and fluidity of a liposome membrane as a function of temperature have been previously reported [30]. The presence of the antibiotic azithromycin affects the thermotropic behavior of DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) and DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) membranes. Moreover, temperatures between 35 and 45°C affect the ^{31}P lineshape of DOPC and DPPC liposomes, as follows: the position of the narrow peak and CSA values does not change; however, the lineshape in a part of the broad shoulder does. The monitoring of the lineshape at the same range of temperatures of DPPC or DOPC liposomes with azithromycin revealed the following new information: the presence of the antibiotic causes smoothing of the line and a decrease in CSA. In fact, only above 40°C did the narrow line stay within the spectrum, and the CSA value averaged zero. The azithromycin molecules increased the membrane fluidity below the main phase transition temperature [30].

The example of hydrophobic molecules contained in the lipid membrane and its influence on the dynamics of the lipid bilayer were presented in Ref. [36]. β-carotene, a well-known hydrocarbon carotenoid, appeared in photosynthetic membranes. The presence of β-carotene in a DPPC liposome membrane fulfilled opposite roles in different membrane states. The ^{31}P spectra of β-carotene/DPPC MLV as a function of β-carotene concentration and temperature showed changes in the ^{31}P resonance lineshape [36]. In both cases, the CSA decreased and the spectral line smoothed. Thus, at temperatures above those in the main phase transition for DPPC, the β-carotene caused a fluidization effect on the membrane (in the liquid crystalline state). The effect is also connected to a decreased temperature in the main phase transition. However, at temperatures below those in the main phase transition, β-carotene rigidified the membrane (in the fluid state) [36]. This effect manifested itself as a broadening of the ^{31}P signal. The similar effect can be observed in the case of the PC/octadecylamine MLV contained lycopene (**Figure 3**).

In the ^{1}H NMR spectra of LUV/SUV, the most useful parameter for analyzing the spectrum is the half-width ($\Delta\nu_{1/2}$) of the signals, because the $\Delta\nu_{1/2}$ of the resonance signals is directly connected to the motional freedom of particular chemical groups. The increase of the $\Delta\nu_{1/2}$ of

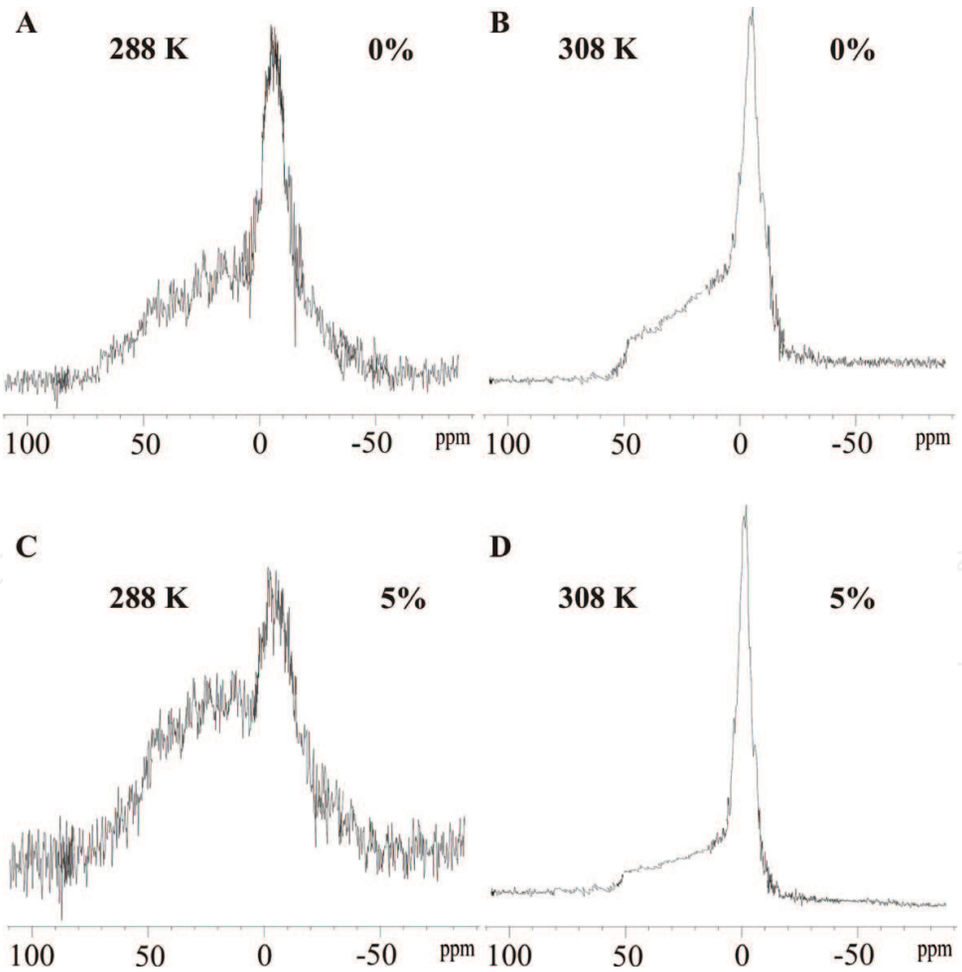

Figure 3. The ^{31}P NMR spectra of the positively charged PC/octadecylamine MLVs (containing 5 mol% of octadecylamine) obtained at 288 K (A and C) and at 308 K (B and D) with 5% of the lycopene (C and D).

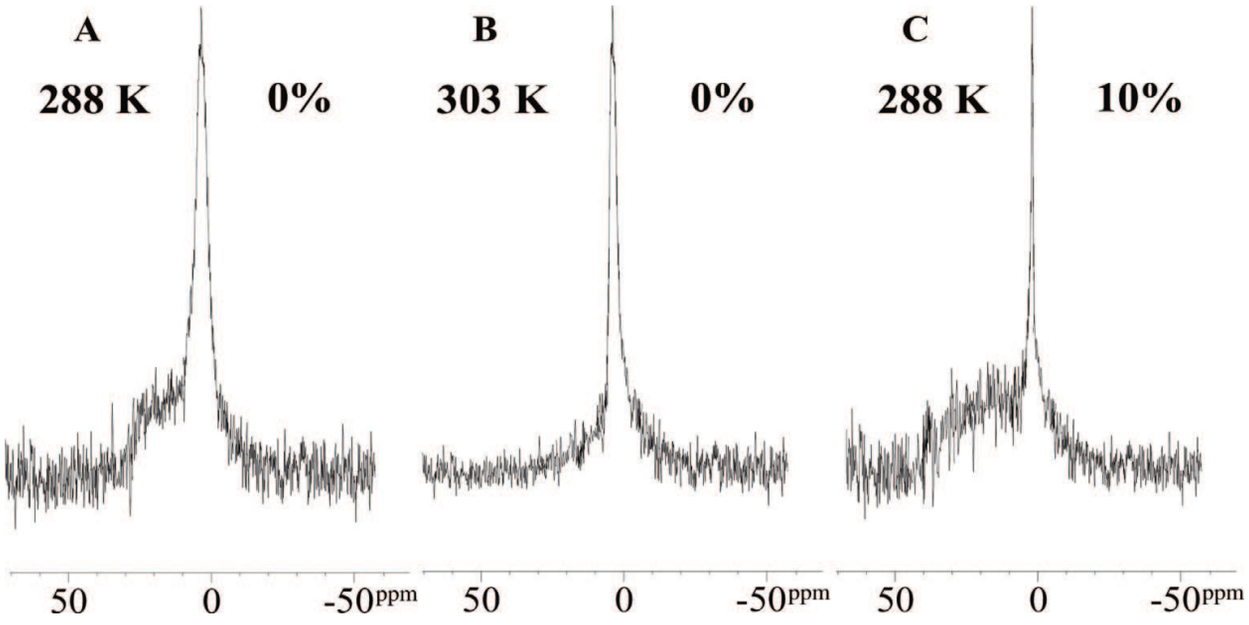

Figure 4. The ^{31}P NMR spectra of the PC MLVs (A). The temperature (B) and polysialic acid (C) effect of narrowing the ^{31}P NMR spectra of PC MLVs.

the ^{1}H resonance signal indicates a restriction of the motion. A change in the $\Delta\nu_{1/2}$ signal assigned to a choline group from the outer layer indicates that the studied drug interacts only with the liposome surface; in other words, it does not penetrate the hydrophobic core of the membrane. This effect was observed in the case of amphotericin B [37], which rigidified the hydrophilic surface of the PC membrane; this effect increased the fluidity of the hydrophobic part of the lipid bilayer. It is manifested as a decrease in the $\Delta\nu_{1/2}$ signals assigned to protons from the $-(CH_2)_n$ and $-CH_3$ groups of hydrocarbon chains [37].

Quite the opposite effect was observed in the case of polysialic acid (**Figure 4**). The ^{31}P NMR spectra of PC MLVs showed a slight narrowing of the isotropic part and a broadening of the anisotropic part of the resonance line at a temperature range of 10–30°C [38].

However, when the polysialic acid was involved in several cell processes in the external environment of the liposome, the effect was even more noticeable. The interaction between well-hydrated and anionic polysialic acid and the polar headgroups of PC increased the fluidity of the hydrophilic part (decrease of $\Delta\nu_{1/2}$) and simultaneously rigidified the hydrophobic core (increase broad shoulder) of the membrane [38].

While the soy isoflavone, genistein, reduces the hydration level of the phosphate groups (hydrophilic part), i.e. decreases its mobility, and rigidified the hydrophobic part of the asolectine liposomes [35]. It also was found that isoflavone prevents lipid molecules from peroxidation [35].

2.4. Phase transitions of lipid membrane studies

Phase transition studies via the use of NMR are based on the fact that residual couplings depend on the extension of the anisotropic domains in combination with the rate of molecular

diffusion [39]. The isotropic phases are perfectly visible in ^{1}H and ^{13}C NMR spectra, but anisotropic phases from liquid crystals are much more difficult to observe. The broadening of ^{1}H and ^{13}C spectra can provide some information about non-isotropic phases but do not bring detailed information about phase transition. In this case, the ^{31}P NMR technique is the one that is most useful. If the phase is isotropic on the NMR time scale, static dipolar, quadrupolar, and shift anisotropy interactions are averaged to zero by molecular motion. When the phase is anisotropic, however, a static interaction effect should be seen on the spectrum [39].

Some of the first and most comprehensive studies on the physical properties of liposomes have been presented in Ref. [40]. The authors analyzed the changes of the ^{31}P spectra of DPPC and DPPE MLV membranes with the addition of a different concentration of piracetam. An additional narrow peak appeared on the spectrum assigned to piracetam. The intensity of the signal was dependent on the piracetam-to-lipid molar ratio [40]. Temperature had a significant influence on the lipid bilayer's physical properties. Fortunately, the possible effect of temperature on the main phase transition of the lipid membrane caused by the drug could be observed in the ^{31}P spectra. The presence of piracetam decreased CSA and increased the mobility of the lipid polar headgroup, manifested as a narrowing ^{31}P line and suggesting that piracetam molecules are inserted between lipid molecules at the hydrophilic part of the membrane [40]. Thus, the presence of hydrophilic piracetam molecules decreased the temperature of the main phase transition. In the H_{II} phase, which can be the transient form in the fusion process, the piracetam resonance signal was no longer distinguishable. Finally, it can be concluded that piracetam combines with lipid molecules, which is exhibited as one signal due to the isotropic motion of the entire complex [40].

The ^{31}P method, therefore, also may be used to fix the temperature of the MLV phase transition [41–44]. The temperature studies of PC/Ch MLV revealed the changes of lineshape ranging from 10 to 40°C (**Figure 5**). Temperature changes could be observed during the phase transition between L_{α} and H_{II} [41].

The monitoring of the ^{31}P lineshape every 2°C led to observations of intermediate lineshapes between those characteristic of L_{α} and H_{II} and allowed us to precisely fix the temperature of the phase transition [41].

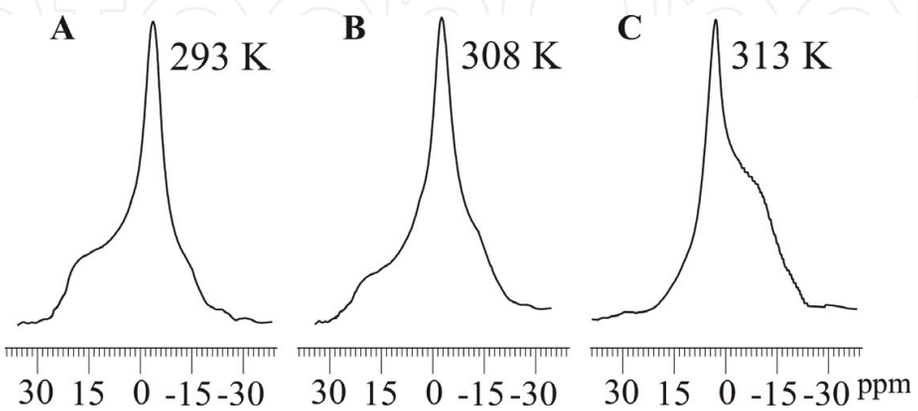

Figure 5. The ^{31}P NMR spectra of PC/Ch MLVs obtained in different temperatures. The characteristic ^{31}P lineshape for lamellar phase at 293 K (A), transient form at 308 K (B) and nonlamellar phase (inversed hexagonal) at 313 K (C).

2.5. Usage of paramagnetic ions as a chemical shift reagent and transport of ions through lipid bilayer

Paramagnetic ions are used to distinguish signals. The most frequently used are praseodymium (Pr^{3+}) ions or other ions from the lanthanides group [37]. The concentration of paramagnetic ions added to the external environment of the liposomes is important. The shift reagent could distinguish the signals within a few ppms; however, if the concentration is too great, it could drastically broaden the studied signal or even broaden the entire spectrum. In this case, the paramagnetic effect is dominant, and paramagnetic interactions may destroy the membrane structure [45]. It may be observed in the ^1H NMR spectra of DOPC SUV as broadened signals due to Eu^{3+} ions. In fact, this effect is associated with the properties of Eu^{3+} ions, which interact to the same extent with the hydrophobic and hydrophilic parts of the lipid bilayer. Moreover, the $\Delta\nu_{1/2}$ of the signal assigned to water also increased, indicating that the Eu^{3+} ions also interact with water molecules from the hydration shell of the liposome [45].

The Pr^{3+} ions enabled the distinguishing of signals assigned to the polar headgroups of lipids from the inner and outer layers of liposomes in the ^1H spectra. The splitting of choline signals of PC/Ch SUVs is showed in **Figure 6**. The signal corresponding to the protons from the $-N^+(CH_3)_3$ groups split due to interaction with paramagnetic ions in the external environment of LUV/SUV [46].

The signal shifted toward lower magnetic field values was assigned to protons from the outer layer, and the signal shifted to higher magnetic field values was assigned to protons from the inner layer [37]. The splitting of the $-N^+(CH_3)_3$ signal is strongly dependent on geometric

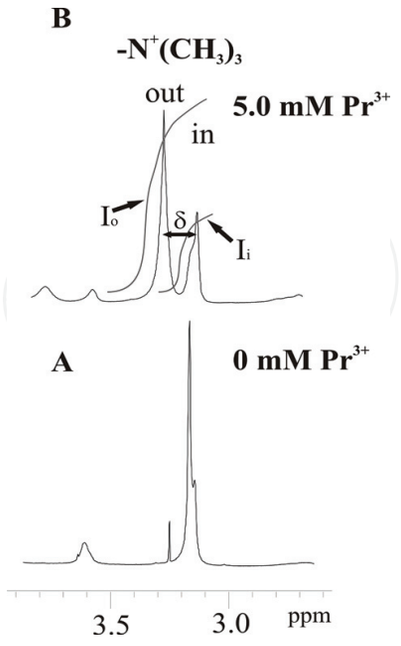

Figure 6. The ^1H NMR spectrum of choline groups of PC/Ch SUVs in the absence (A) and presence (B) of Pr^{3+} ions (5 mM).

conditions and axial symmetry at the lipid-lanthanide binding site [37, 46]. The ratio of the area under the signal corresponded to the outer layer and the area under the signal corresponded to the inner layer (I_o/I_i), thereby providing information about the size of the liposome [46]. Along with decreases in liposome size, the number of lipid molecules in the inner layer decreased. Thus, the areas under the signals from the inner and outer layers differed. The distinguishing of signals from the choline groups in the inner and outer layers of the membrane presents new possibilities for interpreting the results. For example, the addition of the amphotericin B to the external environment of the PC SUV liposome (after hydration of the lipid bilayer) did not have an effect on the size of the liposomes because there was no change in the I_o/I_i ratio. However, the Pr^{3+} ions could interact with the carboxylic group of amphotericin B due to increased splitting of the choline group signals [37].

The ^{31}P NMR spectra can reveal information about the transport through membrane or about ion competing binding sites in lipids and in other biomolecules. This is very important in the assay of biological membranes, since ion transport defects may cause various illnesses, such as manic depressive and neurodegenerative diseases [47]. The adenosine triphosphate (ATP) could be used as a model membrane ligand of metal cations [47]. In these types of studies, it is very important to keep a constant pH and temperature during the experiment. It is well known that the binding of metal cations is dependent on both parameters. The narrowing/broadening of the ^{31}P signal or changes in the distance (splitting) between the three phosphate signals could be the effect of complexes created by ATP and Mg^{2+}/Li^{+} ions [47]. The analysis of ^{31}P spectra led to the conclusion that the biochemical action of Li^{+} ions may be explained as their ability to compete with Mg^{2+} binding sites. Thus, the therapeutic role of Li^{+} in maniac-depressive illness is enabled by modulating the activity of G proteins in signal transduction [47]. It has been suspected that the Mn^{2+} ions also can complete Mg^{2+} binding sites. This ability of Mn^{2+} ions probably pays a significant role in course of neurodegenerative illnesses [48]. Thus, the same effect can be observed in the case of Mn^{2+} ions (**Figure 7**).

The ^{1}H and ^{31}P NMR may also be used to study the macroscopic rearrangement of liposome membrane as, for example, a fusion process [41]. A number of authors have suggested that the fusion process is associated with the development of transient forms related to the appearance of the H_{II} phase [41, 49, 50]. To induce the fusion process of PE/PS/PC liposomes, Ca^{2+} ions should be used as a fusogenic agent [41]. The ^{31}P spectra of MLV showed changes in resonance lineshapes with an increased molar ratio of Ca^{2+} ions to PS (**Figure 8**).

The characteristic lineshape for the nonlamellar phase, H_{II}, was revealed when the molar ratio of Ca^{2+}/PS was 2.0 [41]. The ^{1}H and ^{31}P NMR spectra of SUV, after the addition of Pr^{3+} ions, demonstrated splitting of the resonance signals. In both cases, there was an overall decrease in splitting and even in the intensity of the split signals [41]. Only the Pr^{3+} ions are associated with the outer layer of the membrane. During the fusion process, because the lipid molecules translocation from the outer layer to the inner layer due to transient form (inversed micelle) formation, the Pr^{3+} ions with the translocated lipids moved to the internal water portion of the liposome [41]. This effect determined the decrease in the splitting. In fact, during the fusion process, the internal and external chemical environments of the polar heads became identical, and the size of the liposomes increased [41].

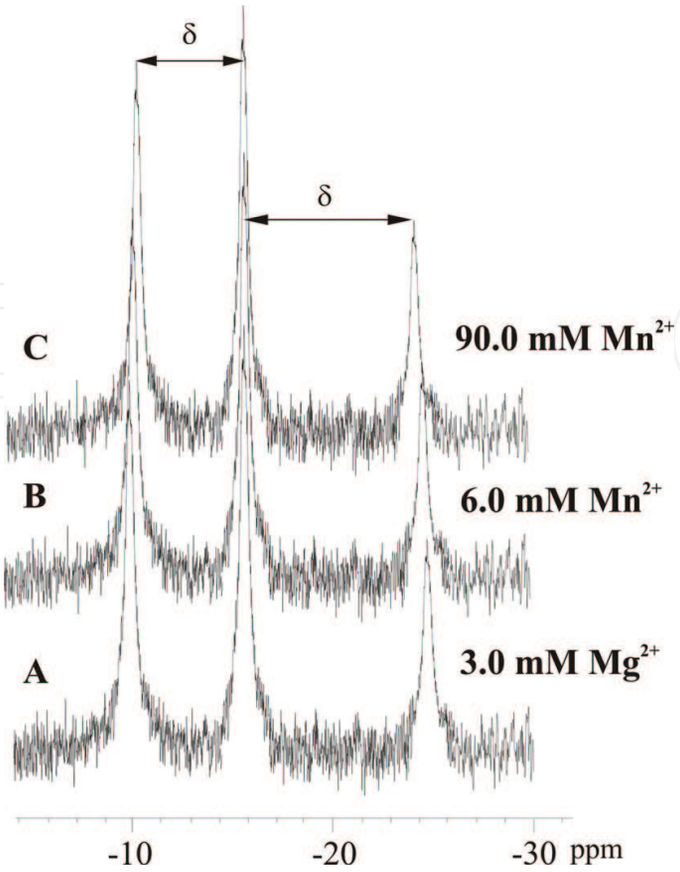

Figure 7. The ^{31}P NMR spectra of ATP in the presence of Mg^{2+} ions (A) and Mn^{2+} ions (B, C).

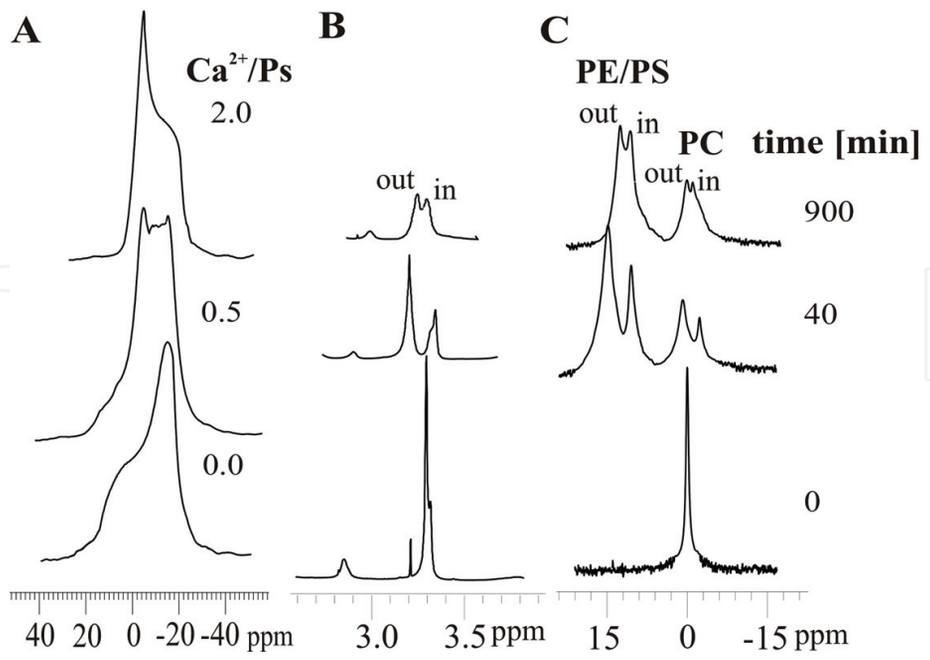

Figure 8. The changes of the ^{31}P resonance lineshape of PC/Ch MLVs caused by fusogenic reagent (Ca^{2+} ions) (A); the time changes of the ^{1}H NMR spectra (B) and the ^{31}P NMR spectra (C) of PE/PS/PC SUVs in the presence of Ca^{2+}/PS molar ratio 2.0 after addition of 0.5 mM Pr^{3+} ions.

2.6. Drug delivery study

The potential use of LUV/SUV as a drug carrier to cells is attractive as a therapy for increasing therapeutic effects and for reducing drug toxicity in normal cells. The concept of the application of temperature-sensitive liposomes is based on an increase in the permeability of the lipid bilayer at a proper temperature, due to rearrangement of the molecules from one stable state to another [51–53]. Temperature-sensitive liposomes may be used with local hyperthermia for the treatment of cancer via chemotherapy [51, 54, 55]. The release rate of the drug depends on the rate of change in temperature and by the serum compounds (i.e., lipoproteins) [56]. Thus, as drug carriers, the liposomes should be stable in the serum and release the drug slowly at a proper temperature [34, 57]. The ^{1}H spectra of PC and PC/octadecylamine (positively charged) LUV showed changes of the chemical shift of signals with increased temperatures ranging from 5 to 50°C (**Figure 9**).

For both types of liposomes, the values of chemical shift increased; that is, the spectral lines shifted toward the direction of the lower magnetic field [51, 57]. This effect is typical for lipid membranes. The ^{1}H spectra also revealed the narrowing of spectral lines (decrease in $\Delta\nu_{1/2}$) assigned to $-N^{+}(CH_3)_3$, $-(CH_2)_n$, and $-CH_3$. Moreover, the largest changes were observed in the $-(CH_2)_n$ signal from the hydrophobic core of the membrane [51]. Studies of the splitting of signals from the choline groups have demonstrated decreased splitting with increased temperatures, the result of increased liposome size. The PC liposome size changes from 20–30 nm to 1 μm. In fact, between temperatures 35 and 40°C, the structure of the PC liposomes becomes damaged and unstable. On the other hand, the size of the PC/octadecylamine liposomes changes slightly from 20 to 60 nm and seems to be stable at temperatures of 40–50°C [51]. Additionally, temperature-sensitive liposomes at higher temperatures may aggregate or fuse [49]. It is possible for temperature-sensitive PC/octadecylamine liposomes to transfer drug to cells by fusion or via an endocytosis process in moderate hyperthermia [51].

The ^{1}H NMR can also be used to study the permeability of lipid membrane. As mentioned before, the controlled release of drugs is very important. The PA/Ch/PEG-Ch (palmitic acid/cholesterol/PEGylated cholesterol) liposomes exhibit no permeability of drugs (calcein and doxorubicin) up to 20 mol% PEGylated cholesterol concentration, but in 10 mol% PEG-Ch concentration the permeability of membrane limited and can be controlled [34]. Moreover, the PA/Ch/PEG-Ch liposomes are stable in various pHs [34].

The release of drugs (cytosine 1-β-D-arabinofuranoside and 5-fluorouracil) from the DPPC liposomes was studied [57]. The ^{1}H spectra showed the shifting signals and the changes in splitting of signals dependent from temperature. The temperature-dependent controlled release of 5-fluorouracil was successfully provided [57].

The ^{31}P NMR technique also can be used to study liposomes used as drug carriers, such as in the case of cisplatin-loaded PEGylated LUV. The ^{31}P NMR technique has been used to measure the chemical shift of placebo (control) liposomes and cisplatin-loaded liposomes at room temperature and at 60°C [58]. The results revealed a characteristically broadened signal at temperatures below those in L_β to L_α phase transition (52.5°C for PC). At a temperature of 60°C, sharp signals were obtained in both cases. The analysis of spectra revealed some asymmetry in peaks on the

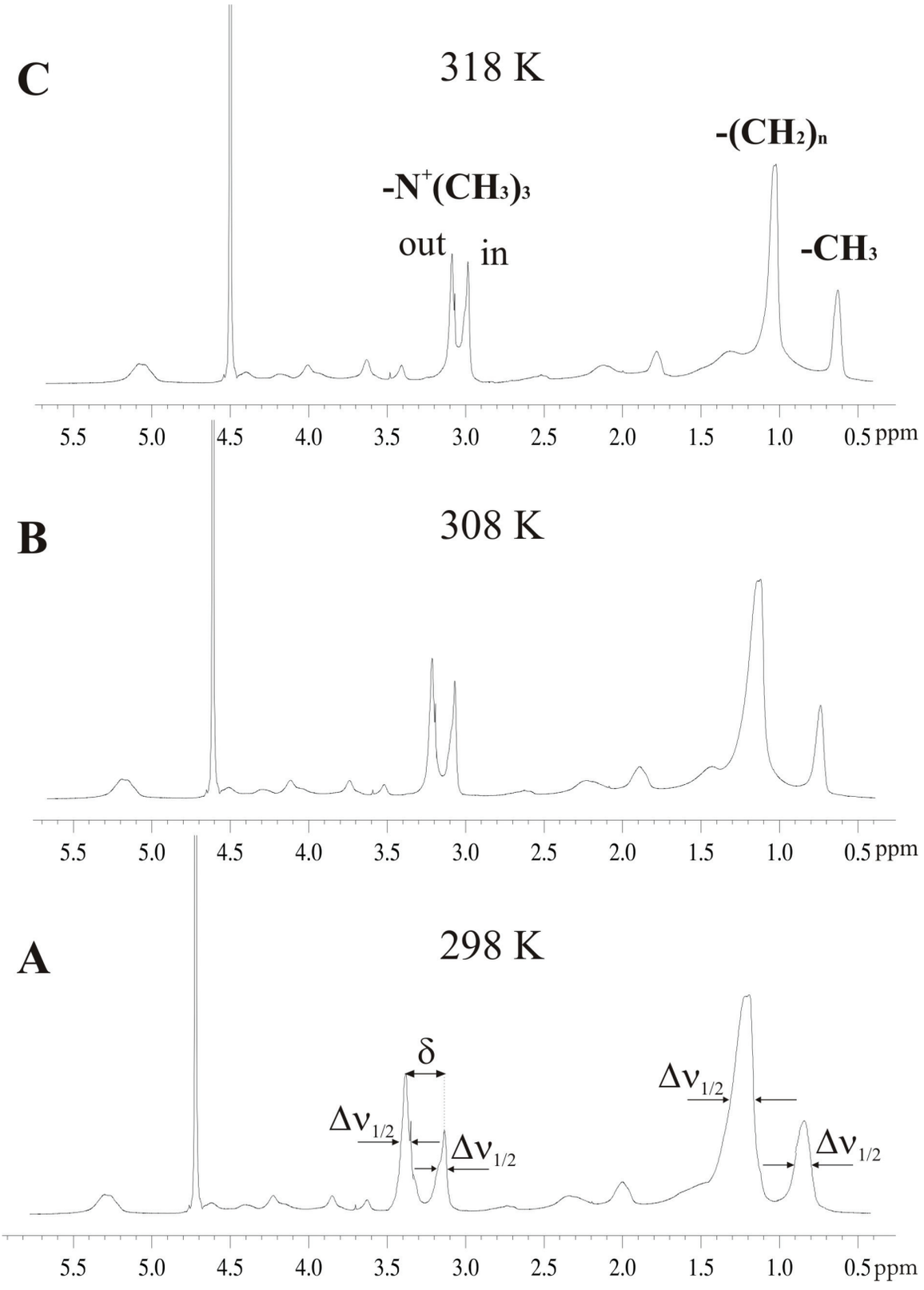

Figure 9. The effect of temperature on half-width and splitting of the ^{1}H resonance signals of PC/octadecylamine LUVs at 298 K (A), at 308 K (B), and at 318 K (C).

right side. This effect is expected in the case of phospholipid LUV. The monitoring of the $\Delta\nu_{1/2}$ signal can reveal information about interactions between cisplatin and phospholipid molecules. The $\Delta\nu_{1/2}$ signal has a lower value for cisplatin-loaded liposomes than that for control liposomes [58]. Because both types of LUV have the same lipid composition and concentration and similar size distributions, the implication is that differences in $\Delta\nu_{1/2}$ are caused by interactions between cisplatin and phospholipid molecules. This effect is probably a result of the hydration process [58].

3. Conclusions

The NMR techniques usually are used to determine the molecular structure but, in the case of lipid aggregates, are more important to know a nature of interactions between the molecules and their dynamics. Thus, the most often are used the ^{1}H and ^{31}P NMR techniques. The ^{1}H and ^{31}P spectra of liposomes led to observe the dynamics changes in the hydrophilic and hydrophobic part of membrane (half-width of signal). The changes of molecules/chemical groups dynamics can be caused by various substances added to liposome membrane, loaded to liposome, or coated a liposome. Moreover, the changes in splitting of signals can revealed the information about permeability of liposome membrane. These parameters are important to characterize the properties of liposome membrane. Additionally, the measurement can be provided in various physicochemical conditions. The pH, temperature, and concentration of added substances have significant influence on the physical state of membrane, the dynamics of molecules, the interactions between molecules, and the processes occurred on the membrane surface. Thus, the NMR technique is a proper tool to study the phase behavior, the transport of ions, the diffusion of drugs through the membrane, the membrane permeability, and the stability of membrane in various conditions.

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