# Formation of wormlike micelle structure in phosphatidylcholine aqueous mixture

**Doctoral Dissertation**

# FORMATION OF WORMLIKE MICELLE STRUCTURE IN PHOSPHATIDYLCHOLINE AQUEOUS MIXTURE

**September 2014**

**Graduate School of Marine Science and Technology**

**Tokyo University of Marine Science and Technology**

**Doctoral Course of Applied Marine Biosciences**

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博士学位論文内容要旨

# Abstract

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| 論文題目<br>Title | Formation of wormlike micelle structure in phosphatidylcholine aqueous mixture | | |

In past decade, wormlike micelles have drawn intention in basic research and application due to their distinctive characteristics. Wormlike micelles are defined as flexible, long cylindrical, and polymer-like structure showing viscoelastic properties. These wormlike micelles are formed by the self-assembly of the surfactant molecules in both aqueous media; called normal wormlike micelle, and non-polar organic solvents; called reverse wormlike micelle. Due to the formation of wormlike micelles in either aqueous or non-polar media, they can be applied in various field including oil field applications, personal care products, biosensors, tissue engineering and drug delivery systems.

Normal wormlike micelle can be formed by several types of surfactants including nonionic, cationic, zwitterionic surfactants. On the other hands, reverse wormlike micelle has been reported in systems containing lecithin and organic solvent or oil with small amount of water.

Lecithin or phosphatidylcholine (PC) is one of phospholipids containing choline as a head hydrophilic group. Phosphatidylcholine composes of two fatty acids in molecule; while, lysophosphatidylcholine (LPC) has one fatty acid at sn-1 position of glycerol backbone giving LPC has more hydrophilicity than PC

We observed the gelation of mixed phosphatidylcholine in aqueous solution was found at high temperature. We assumed that gelation caused by wormlike micelle structures of two different self-assembly structures of phosphatidylcholine; therefore, the objective of this study is to elucidate how

wormlike micelles of phosphatidycholine aqueous mixtures are formed. In this study, pulsed-field-gradient nuclear magnetic resonance (PFG-NMR), differential scanning calorimetry (DSC) and rheological experiment were perform to evaluate the effect of temperature and total concentration of phosphatidylcholine as well as the ratio between phosphatidylcholine and lysophosphatidylcholine on the formation of wormlike micelles.

In first experiment, the viscosity of individual phospholipids; LPC or PC, were lower than 2 mPa·s which close to that of water. The mixtures of two phospholipids at total concentrations ≥56 mM showed a marked maximum in viscosity around LPC molar fraction 0.5 – 0.7, which was ascribed to wormlike micelle formation. In addition, the results indicated that a minimum concentration of both LPC and PC were prerequisite for formation of phosphatidylcholine wormlike micelles in aqueous solutions. The diffusion coefficient of the phosphatidylcholine, as measured using pulsed-field-gradient stimulated spin echo NMR, suggested that LPC and PC form spherical micelles and vesicle structures, respectively. Individual spherical micelles of LPC and a vesicle structure of PC were found at low temperature in the mixtures of LPC and PC. The formation of wormlike micelle is reflected in a steep decrease of the diffusion coefficient at temperatures above 55°C.

In second experiment, more details on thermally induced phosphatidylcholine wormlike micelle were given. Wormlike micelle can be formed after the melting of crystalline like structure in both LPC and PC at higher 40°C detected by DSC. Line width at half maximum in NMR showed the steep increased indicated the restricted in alkyl chain after wormlike micelle formation. The diffusion coefficient of mixtures, as measured by PFG-NMR, also showed the decrease after wormlike micelle structure was formed. For rheological response, the formation of wormlike micelle causes the mixtures switch from low viscosity, Newtonian fluid to viscoelastic, shear-thinning fluid.

Overall, this finding proposed the method in creating wormlike micelle structure by mixture of two surfactants, more hydrophilicity with *CPP* less than 1/2 and the other with *CPP* higher than 1/2. The better understanding in self-assembly on the formation of phosphatidylcholine wormlike micelles could help fabricating the structure for food matrix, delivery systems or biomimetic materials.

# Chapter 1: General introduction

In past decade, wormlike micelles have drawn intention in basic research and application due to their distinctive characteristics. Wormlike micelles are defined as flexible, long cylindrical, and polymer-like structure with radii of a few nanometers and length up to 10 micrometers (Cates and Candau, 1990; Hoffmann, 1994). They show viscoelastic properties similar to polymers. These wormlike micelles are formed by the self-assembly of the surfactant molecules in both aqueous media, that is normal wormlike micelle, and non-polar organic solvents, called reverse wormlike micelle. Due to the formation of wormlike micelles in either aqueous or non-polar media, they can be applied in various field including oil field applications, personal care products, biosensors, tissue engineering and drug delivery systems (Blin *et al.*, 2005; Lawrence and Rees, 2000; Vintiloiu and Leroux, 2008; Wang *et al.*, 2007; Yang, 2002).

Normal wormlike micelle can be formed by several types of surfactants including nonionic, cationic, zwitterionic surfactants. Polyoxyethylene alkyl ether ($C_nEO_m$) and cyltrimethylammonium bromide (CTAB) have been widely studied on wormlike micelle formation (Acharya and Kunieda, 2003; Davies *et al.*, 2006; Raghavan *et al.*, 2002). On the other hands, reverse wormlike micelle has been reported in systems containing lecithin and organic solvent or oil with small amount of water (Shchipunov, 2001; Tung *et al.*, 2007).

Lecithin or phosphatidylcholine (PC) is one of phospholipids containing choline as a head hydrophilic group. Phosphatidylcholine composes of two fatty acids in molecule; while, lysophosphatidylcholine (LPC) has one fatty acid at *sn*-1 position of glycerol

backbone giving LPC has more hydrophilicity than PC (Faergemand and Krog, 2003; Hanahan, 1997; Krog, 1997).

It has been reported that temperature plays an important role on the formation of wormlike micelles. By hindrance electrostatic repulsions between surfactant head-group or hydration between surfactant head-group and water, thereafter, it induces a molecular geometry to be optimal for packing into cylindrical shape (Chu *et al.*, 2010; Davies *et al.*, 2006; Sharma *et al.*, 2010).

In our observation, the gelation of mixed phosphatidylcholine in aqueous solution was found at high temperature. We assumed that gelation caused by wormlike micelle structures of two different self-assembly structures of phosphatidylcholine. In addition, the wormlike micelle formation by phosphatidylcholine in aqueous solution has been only reported in system containing bile salt (Arleth *et al.*, 2003; Leng *et al.*, 2003; Pedersen *et al.*, 1995). Thus, the objective of this present study is to elucidate how wormlike micelles of phosphatidycholine aqueous mixtures are formed. In this study, pulsed-field-gradient nuclear magnetic resonance (PFG-NMR), differential scanning calorimetry (DSC) and rheological experiment were undertaken to evaluate the effect of temperature and total concentration of phosphatidylcholine as well as the ratio between phosphatidylcholine and lysophosphatidylcholine on the formation of wormlike micelles.

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# Chapter 2: Literature reviews

## 2.1 Self-assembly structure of surfactant

Surfactants molecule compose of both hydrophobic and hydrophilic moieties on same molecule; therefore, they have ability to aggregate in various structures such as spherical micelle, cylindrical micelle, ellipsoidal micelle, vesicle, bilayer or reverse micelle (Myers, 2005). At low concentration, surfactant exists as monomer in solution because the entropy of mixing overcomes the attractive force between surfactant molecules (Jonsson *et al.*, 1998). As concentration increases; however, they can aggregate into various thermodynamically stable structures depending on their nature and environmental conditions (Jonsson *et al.*, 1998; Krog, 1997; Myers, 2005). The geometry of self-assembly structures can be predicted on the basis of critical packing parameter (*CPP*; *p*) as followed: $CPP = v/a_0 l$, where v is volume of hydrophobic tail, l is the effective maximum length of hydrophobic part and l $a_0$ is the cross-sectional area of the hydrophilic head group. When surfactants aggregate with each other, they tend to form monolayers that have curvature allowing the most efficient packing of the molecules. Spherical micelles are formed when *CPP* is less than 1/3, wormlike micelles when *CPP* is between 1/3 and 1/2, vesicles when *CPP* is between 1/2 and 1, and lamellar or bilayer structure when *CPP* is 1 (Fig. 1) (Chu *et al.*, 2013; Israelachvili, 2011).

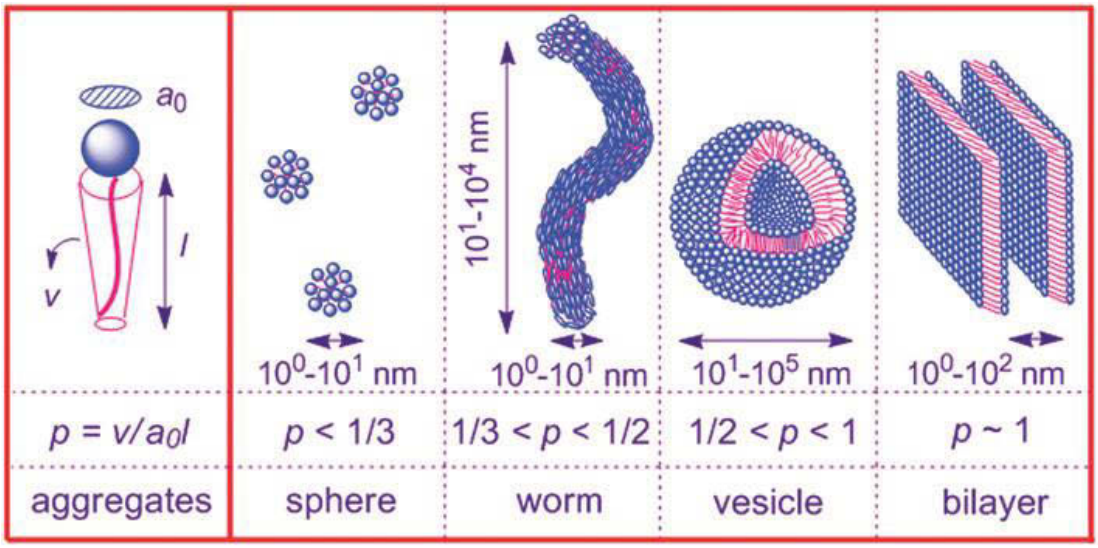


**Fig. 1** Relationship between critical packing parameter; *CPP* and geometry of self-assembly surfactants

Source: Chu *et al.*, Chem. Soc. Rev., 2013, 42, 7174

### 2.2 Wormlike micelle structure

Wormlike micelles are long and flexible cylindrical micelles with contour lengths of a few micrometers and radii of nanometers. Due to the entanglement of wormlike micelle into network, their formation can exhibit viscoelastic properties even in dilute solutions (Cates and Candau, 1990; Hoffmann, 1994; Kumar *et al.*, 2009; Tung *et al.*, 2007). Wormlike micelles can be formed either in water; where normal wormlike micelles are observed, or in non-polar organic solvents, where they are called as reverse wormlike micelles. Normal wormlike micelles have been studied in various systems; for example, cationic, anionic, and nonionic surfactants including mixed formulation such as mixed cationic-anionic surfactants, mixed nonionic surfactants, and even in mixed nonionic-ionic surfactants system. Table 1 shows example of surfactants system containing wormlike

micelle structures. Among these, cationic surfactant such as cetyltrimethylammonium bromide (CTAB) is reported the formation of wormlike micelle in the addition of salt or other cosurfactants. On the other hand, only lecithin in organic solvents is found to form reverse wormlike micelles with an addition of a small amount of water or polar solvents (Shchipunov, 2001, Tung *et al.*, 2007).

**Table 1** Example of normal wormlike micelle structure from various surfactants

| Surfactant | Type of surfactants | Systems | References |
|---|---|---|---|
| Sodium 4-(8-methacryloyloxyoctyl)oxybenzene sulfonate (MOBS) | Anionic | Surfactant with aromatic salts | Zhu *et al.*, 2006 |
| sodium dodecyl sulfate (SDS) and alkanoyl-*N*-methylethanolamide (NMEA) | anionic and nonionic | Mixed ionic and nonionic surfactants | Rodriguez *et al.*, 2003 |
| Cetyltrimethylammonium bromide (CTAB) | Cationic | Surfactant with aromatic salt | Davies *et al.*, 2006 |
| Alkyltrimethylammonium bromide ($C_nTAB$) and sodium oleoate (NaOA) | cationic and anionic | Mixed surfactants | Raghavan *et al.*, 2002 |
| Alkyltrimethylammonium bromide (CTAB) and alkanoyl-*N*-methylethanolamide (NMEA) | cationic and nonionic | Mixed ionic and nonionic surfactants | Acharya *et al.*, 2003 |

**Table 1** (continue) Example of normal wormlike micelle structure from various surfactants

| Surfactant | Type of surfactants | Systems | References |
|---|---|---|---|
| Pentaethylene glycol monododecyl ether ($C_{12}E_5$) | Nonionic | Surfactant with increasing temperature | Bernheim-Groswasser et al., 2000 |
| Polyoxyethylene dodecyl ether ($C_{12}EO_n$) and polyoxyethylene cholesteryl ether ($ChEO_m$) | Nonionic | Mixed nonionic surfactants | Acharya and Kunieda, 2003 |
| 3-(*N*-erucamidopropyl-*N*,*N*-dimethyl ammonium) propanesulfonate (EDAS) | Zwitterionic | Surfactant with temperature | Chu *et al.*, 2010 |
| Erucyl dimethyl amidopropyl betaine (EDAB) | Zwitterionic | Surfactant with temperature | Kumar *et al.*, 2007 |

From a rheological viewpoint, the dynamics of wormlike micelle suspensions can be described using a single (reptational) relaxation process. Consequently, the rheology of these suspensions can be described using a simple Maxwell fluid model (Cates and Candau, 1990; Hoffmann, 1994).

## 2.3 Formation of wormlike micelle structure

Wormlike micelles can be formed by various paths depend on nature of surfactants, such as the addition of salts, application of ultraviolet/visible light and changes in temperature or pH. In this section, we summarized environmental factor affecting formation of wormlike micelle structure.

### 2.3.1 Salts

Salts affect on the formation and growth of wormlike micelle especially in ionic surfactants. The addition of salts has an influence on alteration of charged surfactant head group; therefore, varying in surfactant geometry suitable for wormlike micelle formation. Davies *et al.* (2006) studied on the effect of 5-methyl salicylic acid (5mS) on the CTAB micelle structure. At concentration of 5mS lower than 5M, CTAB was found to be spherical micelle; while, CTAB formed vesicle structure with 5mS concentration higher than 15 mM. CTAB can form the wormlike micelle structure with the addition of 5mS in the range of 5 – 15 mM. The increase of 5mS in system caused the decrease in hydrophilic area and increase of hydrophobic volume due to the adsorption of 5mS at hydrophilic surface; thus, CTAB changed from spherical micelle to wormlike micelle and then to vesicle structure.

### 2.3.2 Ultraviolet/visible light

Ultraviolet/visible (UV/Vis) light has been used as stimulator for wormlike micelle formation due to the change of *cis*-*trans* isomerization, dimerization of surfactants or the additives. A $C_{22}$-zwitterion surfactant, Erucyl dimethyl amidopropyl betaine (EDAB) and *ortho*-methoxy-cinnamic acid (OMCA) was used in the study by Kumar *et al.*, (2009). OMCA are recognized as photoisomerization aromatic salt. Small spherical micelles or short cylindrical micelles are formed, in system of EDAB–*trans*-OMCA. When *trans*-OMCA is isomerized to *cis*-OMCA under UV radiation, sample showed the increase of viscosity and elongation of wormlike micelle because *cis*-isomer has a weaker interaction with EDAB. Thereafter, wormlike micelle can be formed.

### 2.3.3 Temperature

It has been reported that temperature plays an important role on the formation of wormlike micelles. For nonionic surfactant; Pentaethylene glycol monododecyl ether ($C_{12}E_5$) was researched by Bernheim-Groswasser and coworkers in 2000. They found that at low temperature, $C_{12}E_5$ formed spherical and short (<50 nm) cylindrical micelle. With temperature was raised to 18°C, spherical micelle and cylindrical micelle with the length of 50 – 100 nm were found. At 29°C, it formed long wormlike micelle (length > 100 nm) with branching.

For cationic surfactant, Hassan *et al.* (1996) reported vesicle-to-wormlike micelle transition of cetyltrimethylammonium hydroxynaphthalene carboxylate (CTAHNC). At temperature <40°C, vesicle with low viscosity was formed. When temperature increased to 50°C; however, wormlike micelle was formed as evidenced by rheological measurement.

In case of zwitterionic surfactant, on the other hands, a 50 mM EDAB exhibited a gel-like response at 25°C. Storage modulus (G') increased and elastic modulus (G'') decreased with increasing temperature to 40°C. At higher temperature (>60°C); however, it switched from gel-like to viscoelastic response of Maxwell model (Kumar *et al.*, 2007).

### 2.3.4 Electricity

Tsuchiya *et al.* (2004) reported a viscoelastic wormlike micelle from (11-ferrocenylundecyl)trimethylammonium bromide (FTMA) with sodium salicylate by redox reaction. In reduced form of FTMA, system contained wormlike micelle with high viscoelasticity. In contrast, ellipsoidal micelles with low viscoelasticity were found in oxidized form of FTMA.

### 2.3.5 pH

Wormlike micelle can be obtained by controlling pH. pH regulates ionization of additives. Lin *et al.* (2009) reported that 60 mM CTAB with 40 mM potassium phthalic acid (PPA) formed wormlike micelle at pH 3.9. At pH 5.35, on the other hands, system showed Newtonian behavior. This was due to the ionization of PPA. Having two carboxylic groups in structure, pKa of PPA is 2.89 and around 4.60-5.51. Therefore, at pH 3.9, PPA ionized one carboxylic group and loosely bound to CTAB resulting in proper aggregation of CTAB to form wormlike micelle. While, all carboxylic groups ionized at pH 5.35 resulting in strongly bound of PPA to CTAB, then rod-like micelle was formed instead of long wormlike micelle.

## 2.4 Phosphtidylcholine or lecithin

Phosphatidylcholine, commonly known as lecithin, is usually the most abundant phospholipid in animals and plants such as egg yolk, soybean and rapeseed. Phospholipids are complex lipids composed of phosphate-containing polar head-group and non-polar hydrocarbon chains. Phospholipids can be mainly divided into 4 types; phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylserine (PS) depending on functional groups attached to phosphate groups. The hydrophilic head groups of these molecules are either anionic (Phosphatidylinositol) or zwtterionic (phosphatidylcholine and phosphatidylethanolamine) while the hydrophobic tail groups consist of two fatty acids. The type and contain of fatty acid chain are various depending on sources and extraction method (Faergemand and Krog, 2003; Hanahan, 1997; Krog, 1997; Pichot *et al.*, 2013).

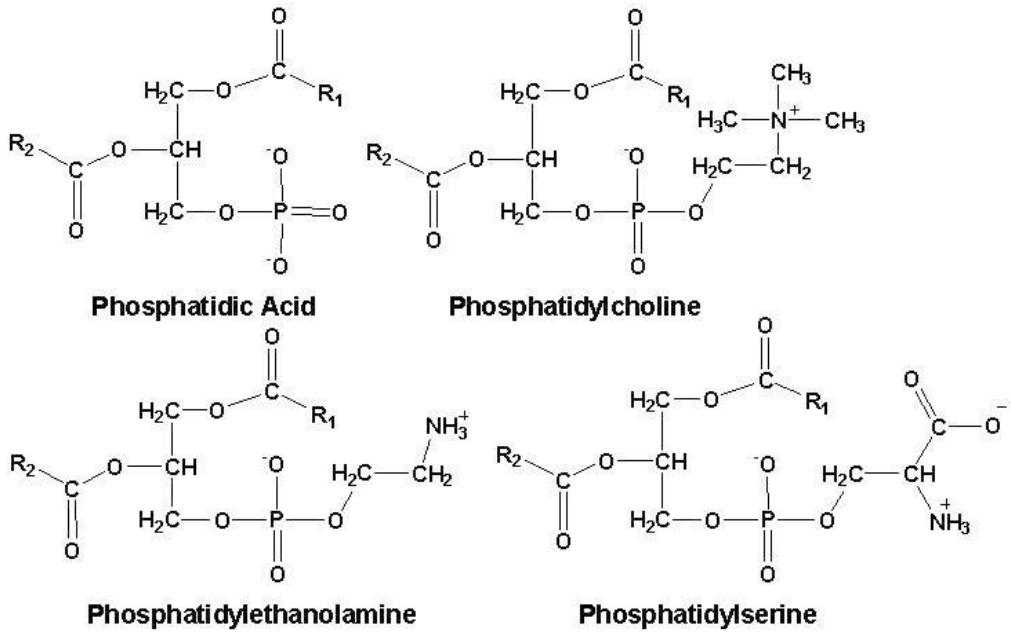


**Fig. 2** Chemical structures of phospholipids

As a natural amphiphilic molecule, lecithin is widely used for emulsion systems in food or pharmaceutical industries as emulsifier/stabilizer. Soy lecithin is the most widely used surfactant ingredients in food industry because it can be extracted during the processing of soybean oil. While, Egg lecithin is found in egg yolk play an important role in stabilizing mayonnaise and salad dressing. Lysophosphatidylcholine (LPC) obtained by hydrolysis of phosphatidylcholine by enzyme phospholipase $A_2$, has one mole of fatty acid per mole of phospholipid in position *sn*-1. This cause LPC shows more hydrophilicity than PC (Faergemand and Krog, 2003; Hanahan, 1997; Krog, 1997; Pichot *et al.*, 2013).

## 2.5 Molecular mobility observed by NMR

In this section, we describe some fundamental theories and techniques using nuclear magnetic resonance (NMR) to evaluate the molecular mobility. There are three types of mobility in molecule; that is, translation, rotation and vibration. Translational motion is mobility that whole molecule changes its location in three dimensional space. Rotational motion corresponds to motion of molecule spinning around axis in three dimensional space. Vibrational motion is motion that changes shape of the molecule including stretching and bending.

NMR measurement is a powerful tool for investigating the structural change and give information about molecular mobility. NMR uses magnetic field gradients to measure the molecular diffusion coefficient. The sample is located in a magnetic field whose magnitude varies linearly in space as

$$B(r) = B_0 + Gr \quad (1)$$

where G is magnetic field gradient. The effect of gradient is a linearly varying resonance frequency along the direction of G that is parallel to $B_0$.

Spin-lattice relaxation time ($T_1$) and spin-spin relaxation time ($T_2$) measurements provide information about local mobility and flexibility of molecules. $T_1$ relaxation time is the longitudinal relaxation time. It is also known as relaxation in the z-direction. $T_1$ indicates the time required for any molecule become magnetized after first being placed in magnetic field, in other words, the time required to regain longitudinal magnetization following an r.f. pulse. $T_1$ is determined by the thermal interactions between the resonating protons and other protons and other magnetic nuclei in the magnetic environment. $T_1$ quantifies the rate of transfer of energy from the nuclear spin system to the neighbouring molecules. $T_2$ relaxation time is the transverse relaxation time. It is also known as relaxation in xy plane. $T_2$ is a measure of how long transverse magnetization would last in a perfectly uniform external magnetic field. After a 90° pulse the nuclear spins are aligned in one direction (phase coherence), but this arrangement is gradually lost. Unlike $T_1$ interactions, $T_2$ interactions do not involve a transfer of energy. $T_2$ relaxation does not affect the total amount of z-magnetization, but the degree of synchronization of the transverse magnetization components. $T_2$ is related to the line width at half-height of the NMR signal. The spin-spin relaxation is related to spin-lattice relaxation, since an increase in z-magnetization without a decrease in the magnetization in the xy plane is not possible (Furó, 2005; Pavia *et al.*, 2008).

Pulsed-field-gradient spin-echo (PFG-SE) NMR is used to observe the diffusion coefficient of molecules. Fig. 3 shows the schematic depiction of pulse sequence. The magnetic field gradients are turn on during the defocusing and 180° refocusing periods of

an echo sequence of r.f. pulses but not during the signal detection. In contrast, if the refocusing is achieved with two separate 90° pulses with delay between the experiment; this technique is called pulsed-field-gradient stimulated spin echo (PFG-STE) NMR. PFG-NMR experiments record the variation of signal intensity

$$I(g) = I(0) \exp[-\gamma^2 \delta^2 g^2 (\Delta - \delta/3) D] \quad (2)$$

with increasing gradient strength G, thereafter diffusion coefficient can be obtained. The advantage of PFG-SE is that the maximum possible signal is recovered in the absence of relaxation effect. On the other hands, the disadvantage comes from the long period that the magnetization is in the xy plane. A severe loss of signal can be found for short $T_2$ sample. Consequently, PFG-SE is suitable for sample whose $T_2$ is not much shorter than $T_1$ relaxation time. In case of macromolecules, $T_2$ is mostly much shorter than $T_1$. Hence, PFG-STE is applied for getting diffusion coefficient for macromolecules because the decay of the signal by spin relaxation during most of Δ is governed by $T_1$ instead of $T_2$ in PFG-STE (Furó, 2005; Price, 2009; Stejskal and Tanner, 1965; Tanner, 1970).

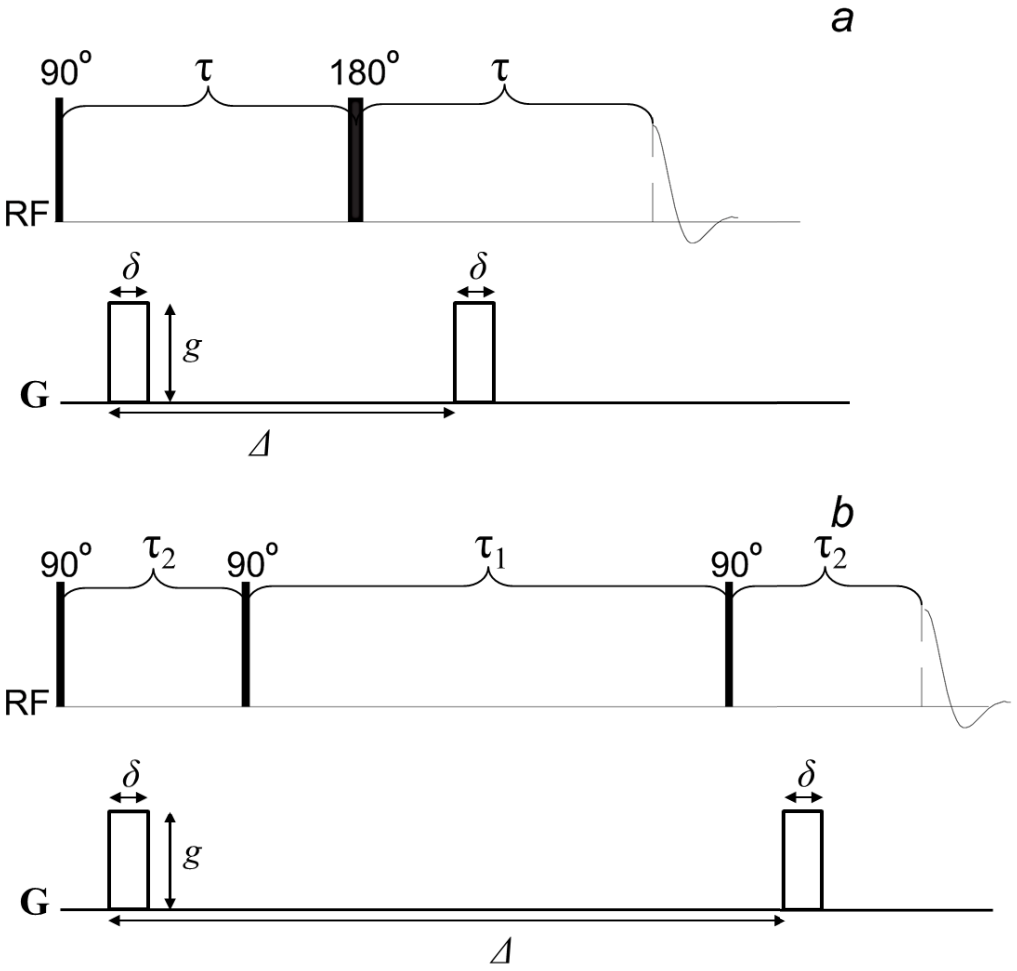


**Fig. 3** The pulse sequences for the pulsed-field-gradient spin echo (PFG-SE, a) and stimulated spin echo (PFG-STE, b) NMR experiments which can measure molecular self-diffusion coefficients. The experiments apply pulses of magnetic-field gradient (G), embedded in the sequence of radiofrequency pulses (r.f.). The time domain NMR signal is detected after the echo top. Usually, both experiments record the decrease of this signal (Eq. (2)) on increasing the strength G of the gradient pulses of duration $\delta$ and spacing $\Delta$.

Adapted: Furó, J. Mol. Liq., 2005, 117, 117

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# Chapter 3: Thermally induced gelation and formation of wormlike micelle of mixed phosphatidylcholine in aqueous solution


## Abstract

We report for the first time on wormlike micelle formation in aqueous mixtures of lysophosphatidylcholine (LPC) and phosphatidylcholine (PC). The viscosity of mixtures of these two phospholipids at total concentrations ≥56 mM showed a marked maximum around LPC molar fraction 0.5 – 0.7, which was ascribed to wormlike micelle formation. The diffusion coefficient of the lecithin, as measured using pulsed-field-gradient stimulated spin echo NMR, suggests that LPC and PC form spherical micelles and vesicle structures, respectively. In mixtures of LPC and PC, individual spherical micelles of LPC and a vesicle structure of PC were found at low temperature. Wormlike micelle formation was manifested in a steep decrease of the diffusion coefficient at temperatures above 55°C. The results indicate that a minimum concentration of both LPC and PC, as well as a molar ratio of LPC to PC close to 0.5-0.6, are prerequisite for thermally induced formation of lecithin wormlike micelles in aqueous solutions. Following formation on heating, the wormlike micelles remained stable on cooling, and no population of smaller spherical micelles could be detected.

## 3.1 Introduction

An increasing amount of research effort is being directed towards wormlike micelles. Wormlike micelles are one structure formed by surfactant self-assembly. This structure is characterized by long flexible chains. The dynamics of wormlike micelle suspensions can be described using a single (reptational) relaxation process (Cates and Candau, 1990). Consequently, the rheology of these suspensions can be described using a simple Maxwell fluid model (Cates and Candau, 1990; Hoffmann, 1994). Characteristic relaxation times and zero shear-rate viscosities reported for dilute or semi-dilute (<10% w/w) wormlike micelle aqueous suspensions are in the range 0.1-100 s and 0.1-$10^3$ Pa·s, respectively (Cates and Candau, 1990; Kumar *et al.*, 2009, Tung *et al.*, 2007).

Wormlike micelles can be formed in either non-polar organic solvents, where reverse wormlike micelles are observed, or in water, where they can also be called normal wormlike micelles. Wormlike micelles can be formed using different routes, such as application of ultraviolet/visible light (Kumar *et al.*, 2009) and changes in temperature (Davies *et al.*, 2006) or pH (Lin *et al.*, 2009). Wormlike micelles have been considered for applications in different fields, including drug delivery systems (Lawrence and Rees, 2000; Vintiloiu and Leroux, 2008), personal care products (Yang, 2002) and biosensors (Blin *et al.*, 2005; Wang *et al.*, 2007). In water, most studies have concentrated on ionic surfactants such as cetyltrimethylammonium bromide (CTAB) and cetyltrimethylammonium 3-hydroxynaphthalene 2-carboxylate (CTAHNC), which form wormlike micelles on addition of salt (Davies *et al.*, 2006; Hassan *et al.*, 1996; Horbaschek *et al.*, 1998; Lin *et al.*, 2009). In non-polar solvents, on the other hand, formation of reverse lecithin wormlike

micelles has been observed on addition of water (Kumar *et al.*, 2009; Tung *et al.*, 2007; Shchipunov, 2001; Shumilina *et al.*, 2006).

Lecithin is the common name applied either to a mixture of phospholipids found in egg yolk and soybean in high concentrations, or strictly to the main member of this mixture, phosphatidylcholine. Phosphatidylcholine, hereafter PC, is a glycerol esterified with two moles of fatty acid, while the third glycerol oxygen is phosphorylated with a choline residue. The length and double-bond content of the fatty acids vary depending on the source. It is one of the major components of the cell membrane. Bilayer structure has therefore been studied as a model of cell membranes. Lysophosphatidylcholine, hereafter LPC, obtained through hydrolysis of phosphatidylcholine by enzyme phospholipase $A_2$, has one mole of fatty acid per mole of phospholipid in position *sn*-1 (Chiba and Tada, 1989; Faergemand and Krog, 2003; Hanahan, 1997; Krog, 1997). The use of lecithin as a stabilizer of w/o and o/w emulsions and its possible applications in the food, pharmaceutical and medical care industries have been reviewed recently (Pichot *et al.*, 2013).

Surfactant self-assemblies are usually related to the critical packing parameter (*CPP*): $CPP = v/a_0 l$, where, where $v$ is the volume of the hydrophobic chain, $l$ is the effective length of the hydrophobic chain and $a_0$ is the effective surface area of the head-group (Israelachvili, 2011). The *CPP* of LPC is about 0.34, and LPC tends to form spherical micelles (Kumar, 1991). As noted above, PC has two alkyl chains and therefore a higher *CPP* (around 0.74). As a result, it tends to form planar or vesicle structures (Kumar, 1991).

Our hypothesis was that mixtures of PC and LPC could display a different self-assembly than the individual phospholipids. Therefore, we focused on the phase behavior of LPC/PC mixtures in aqueous solution. The effect on the bulk properties was studied with falling-ball measurements of the viscosity. The self-assembly behavior was studied using pulsed field gradient nuclear magnetic resonance, hereafter PFG-NMR, which is used to measure the self-diffusion coefficient of molecules and macromolecular aggregates. Application of PFG-NMR to surfactant solutions (Gröger *et al.*, 2004; Price *et al.*, 1999) and other multicomponent systems (Lamanna, 2005) yields weight-averaged information on the distribution of diffusion coefficients. Dynamic light scattering (DLS), on the other hand, yields z-averaged distributions of diffusion coefficients. PFG-NMR can therefore be more suitable than DLS in characterization of multi-component samples, especially when the main interest is in the behavior of the smaller macromolecules (Gröger *et al.*, 2004). In this study, we elucidated the effect of temperature and content of PC and LPC on the formation of wormlike micelles. A better understanding of the factors determining formation of lecithin wormlike micelles could contribute to the design of suitable self-assembly structures for applications in food, drug delivery or biomimetic materials. Mixtures of uncharged phospholipids could be an especially suitable choice in some emulsified formulations, as they have been shown to confer higher stability against droplet flocculation compared with charged phospholipids in the presence of small amounts of NaCl or $CaCl_2$ (Han and Washington, 2005; Han *et al.*, 2001; Washington *et al.*, 1990).

## 3.2 Materials and methods

### 3.2.1 Materials

Egg yolk PC and LPC (Fig. 1) were kindly provided by Kewpie Company (Tokyo, Japan). According to information from the manufacturer, majority of alkyl chains were C-18 (57.5%) and C-16 (33.2%). Deionized water was obtained by a Water Purifier (Autostill WA500, Yamato Scientific Co., LTD., Tokyo, Japan). $D_2O$ (deuterium isotopic content: 99.0%) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

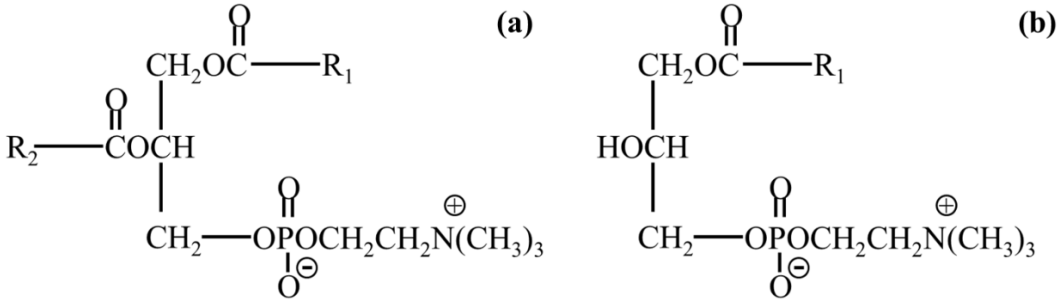


**Fig. 1** Chemical structure of phosphatidylcholine (PC; a) and lysophosphatidylcholine (LPC; b); $R_1$, $R_2$ = alkyl chains.

PC and LPC were dispersed in deionized water with a sonicator (Qsonica Q125, Connecticut, United States) for 5 min. An ice bath was used to prevent overheating of the sample. The phospholipid dispersions were mixed to obtain mixtures of $C_T$ (total lecithin concentration; $C_T = C_{LPC} + C_{PC}$ = molar concentration of LPC + molar concentration of PC) in the range 8-72 mM, and sonicated for 1 min. Sonication was performed at 20 kHz,

amplitude 60%, and a time interval of 5 s. Solutions used in NMR measurements were prepared with $D_2O$ instead of deionized water.

### 3.2.2 Methods

#### *2.2.1 Light microscopy*

The structure of individual LPC and PC dispersions was observed at room temperature using a light microscope (Olympus model BX 53; Olympus, US).

#### *2.2.2. Viscosity measurements*

The viscosity of lecithin mixtures was measured with the falling ball method (Brizard *et al.*, 2005). A coated steel ball was placed in a 20 cm glass tube filled with sample. Sample tubes were placed in a temperature-controlled water bath. The time ($t$) required for the coated steel ball to fall through the glass tube was measured, and the viscosity was calculated using deionized water as a reference:

$$\eta = \eta_0 \frac{t}{t_0} \qquad (1)$$

where $\eta$ is the viscosity, $\eta_0$ is the viscosity of $H_2O$ and $t_0$ is the falling time in $H_2O$.

#### *2.2.3. Diffusion measurements*

Diffusion coefficients were measured using the pulsed-field-gradient stimulated spin-echo (PFG-STE) pulse sequence on a Bruker Avance II 400WB spectrometer (Bruker Corp., US) equipped with a gradient probe (Price, 2009; Stejskal and Tanner, 1965). In the PFG-STE pulse sequence, the intervals between the first and second ($\tau_1$) and the second and third ($\tau_2$) r.f. pulses were 1 ms and 10 ms, respectively. The gradient pulse duration $\delta$ and the interval time of the field-gradient-pules $\Delta$ were 1 ms and 10 ms, respectively. The

gradient field strength $g$ was varied in 8 steps in the range 100-1200 or 100-1500 G/cm. The diffusion coefficient was determined using the following relation between the echo signal intensity and field-gradient parameters:

$$I(g) = I(0) \exp[-\gamma^2 \delta^2 g^2 \langle z^2 \rangle / 2] \quad (2)$$

where $I(g)$ and $I(0)$ are echo signal intensities at $t=2\tau_2+\tau_1$ with and without the field gradient pulse, respectively. $\gamma$ is the gyromagnetic ratio of $^1H$. $\langle z^2 \rangle$ is the mean square displacement in the z direction (the direction of PFG) and is equal to $2DT_d$ for normal diffusion, where $D$ is the diffusion coefficient and the diffusion time $T_d$ for a square-shaped gradient in the PFG-STE pulse is $\Delta - \delta/3$. Therefore, eq. (2) is rewritten as:

$$I(g) = I(0) \exp[-\gamma^2 \delta^2 g^2 (\Delta - \delta/3) D] \quad (3)$$

The temperature was controlled using a Bruker BVT-3200 temperature unit and varied from 25-75°C. The temperature in the sample tube was measured with an optical fiber thermometer (Takaoka Electric Mfg. Co., Tokyo, Japan).

## 3.3 Results and Discussion

### 3.3.1 Phase diagram

The viscosity of lecithin mixtures was used to construct phase diagrams at different temperatures. The concentration of LPC and PC was varied in the range 0-48 mM. At 25°C, mixtures of LPC and PC showed low viscosity and turbidity at all tested concentrations. LPC solutions were transparent, and their viscosity was below 2 mPa·s. Conversely, PC solutions were turbid, but their viscosity was also below 2 mPa·s. We expected the turbidity of PC solutions to reflect the presence of large self-assembled structures, either vesicle or

planar micelles. In Fig. 2, we present the results of light microscopy. As seen from Fig. 2a, no features could be identified for the individual LPC dispersion. As seen from Fig. 2b, however, large roughly micelles with a diameter of about 2 µm were observed in the individual PC dispersion.

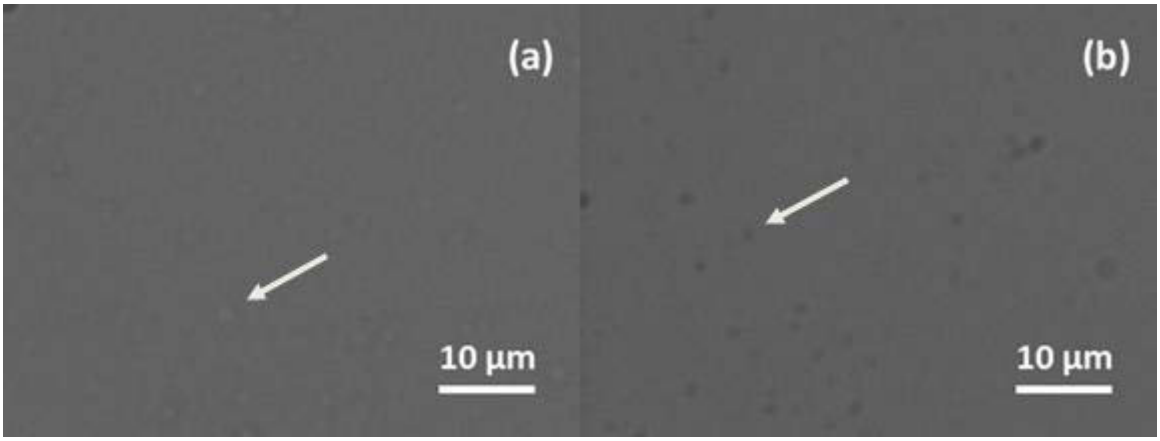


**Fig. 2** Micrograph of LPC (a) and PC (b) at room temperature. Bright circles, indicated with an arrow in 2a, are artifacts from entrapped air bubbles. Dark circles, indicated with an arrow in 2b, are large surfactant assemblies.

Our preliminary tests revealed a marked increase in the apparent viscosity and reduced turbidity of many solutions on heating above ≈50°C. For this reason, more detailed studied were carried out at 55°C and 75°C. The phase diagrams of the lecithin mixtures at 55°C and 75°C are shown in Fig. 3. An arbitrary threshold of 50 mPa·s was chosen to represent (apparent) gelation. This apparent gelation is closely related to formation of very large mixed micelles, as will be shown later in section 3.2. At 55°C, the viscosity of individual solutions of LPC and PC remained low for all concentrations tested (Fig. 3a). Mixtures of the two phospholipids, however, showed a markedly increased viscosity,

especially mixtures where the concentration of both LPC and PC was above 24 mM. In addition, the turbidity of mixtures decreased markedly around 55°C.

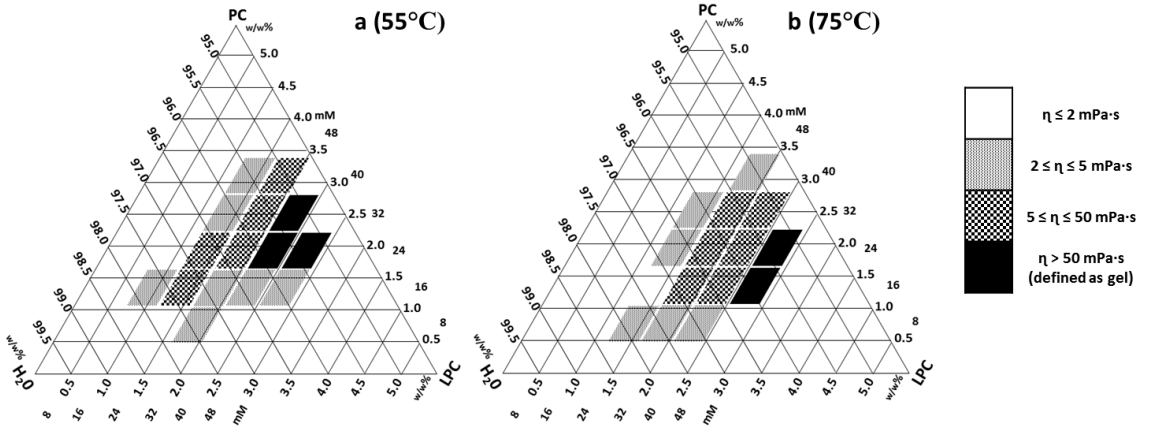


**Fig. 3** Partial phase diagram of lecithin mixtures, defined according to their viscosity, at 55°C (a) and 75°C (b).

Fig. 4 shows the viscosity of PC/LPC mixtures at 55°C as a function of the LPC molar fraction, $f_{LPC}$ ($f_{LPC} = C_{LPC}/C_T$). For $f_{LPC} \approx 0.5$-$0.7$, mixtures with $C_T \leq 48$ mM showed a maximum, and for $C_T \geq 56$ mM we observed gelation for the same values of $f_{LPC}$. These results suggest formation of wormlike micellar structures. Davies *et al.* (2006), who studied mixtures of CTAB and 5-methyl salicylic acid, formation of wormlike micelles leads to a decrease in turbidity and an increase of the viscosity. This is the first time, to our knowledge, that wormlike micelle formation is reported in aqueous solutions of lecithin without addition of salts. Formation of wormlike micelles has been reported by Arleth *et al.* (2003) for a mixture of PC with the bile salt sodium glycochenodeoxycholate. They noted formation of wormlike micelles only at low concentrations of the bile salt close to its critical micellization concentration, noting that this trend is opposite to the usual trend in micellar systems, where the extent of aggregation is enhanced by increasing the surfactant

concentration. Our findings are different, as a minimum content of both LPC and PC is required for wormlike micelle formation. Shumilina *et al.* (2006) reported formation of reverse PC wormlike micelles in *n*-decane mixtures on addition of a small amount of water. The reverse wormlike micelles disintegrated on addition of LPC. Our findings are, as expected, opposite to theirs. We find that the LPC is essential for formation of the normal lecithin wormlike micelles in aqueous solutions.

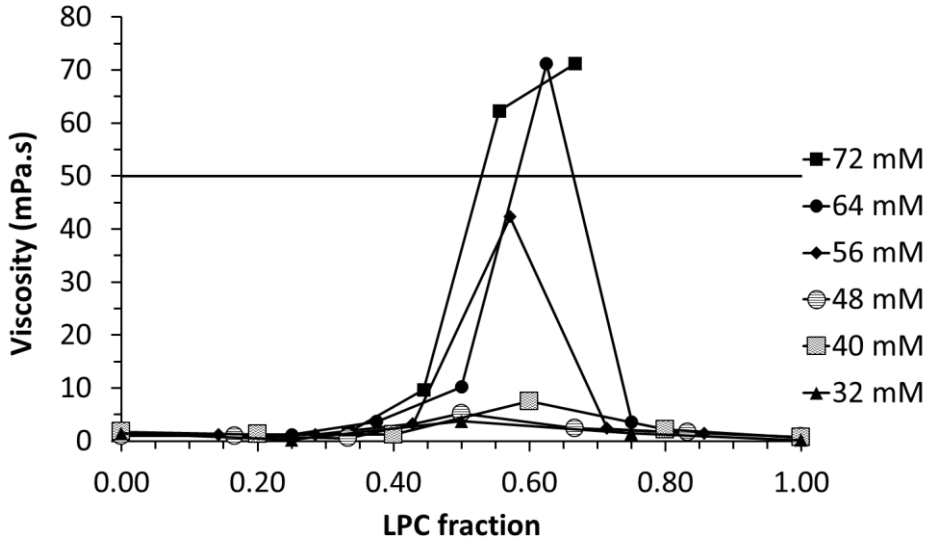


**Fig. 4** Viscosity of lecithin mixtures as a function of $f_{LPC}$ at 55°C. The horizontal line indicates apparent gelation (50 mPa·s). The total lecithin content $C_T$ is indicated in the figure.

The viscosity of lecithin mixtures is plotted as a function of temperature in Fig. 5. For mixtures $C_T$ = 48 mM (Fig. 5a-c), all samples had viscosities below 10 mPa·s throughout the heating process. At these concentrations, we propose that the mixtures form relatively short wormlike micelles. As explained by Cates and Candau (1990), the equilibrium length of the wormlike micelle is expected to increase with increasing

surfactant concentration. On subsequent cooling, however, the viscosity of mixtures with $f_{LPC}$ above 0.5 increased on below about 50°C. This increase probably reflected an increase in the equilibrium length of the wormlike micelle, as explained by Cates and Candau (1990), due to the effect of temperature on scission reactions of the chains. Tung et al. (2007) studied reverse wormlike micelles formed by lecithin in n-decane/water system and normal wormlike micelles formed by cetylpyridinium bromide and sodium salicylate in water, and studied the effect of temperature only following extensive heating, i.e., after formation of the wormlike micelles was complete. They reported in both cases a marked increase in zero-shear rate viscosity with decreasing temperature, and ascribed it to the increase in equilibrium length of the wormlike micelles with decreasing temperature. For mixtures with $C_T$ = 64 mM (Fig. 5d-f), the viscosity for $f_{LPC}$ = 0.5 increased slightly and for $f_{LPC}$ = 0.63 the viscosity increased steeply on heating above 50°C, suggesting the formation of short and long wormlike micelles, respectively. Because the initial state of the mixtures is obviously not an equlibrium one, we observe first the increase in viscosity due to overcoming of a kinetic barrier. However, once this kinetic barrier has been passed, increasing temperature is expected to decrease the viscosity, as explained above. Indeed, further heating above about 55°C led to a decrease of the viscosity, which we can assign to the effect of temperature on the equilibrium length of the wormlike micelles. Indeed, the viscosity of mixtures with $C_T$ = 64 mM also showed a marked increase on subsequent cooling below 60°C.

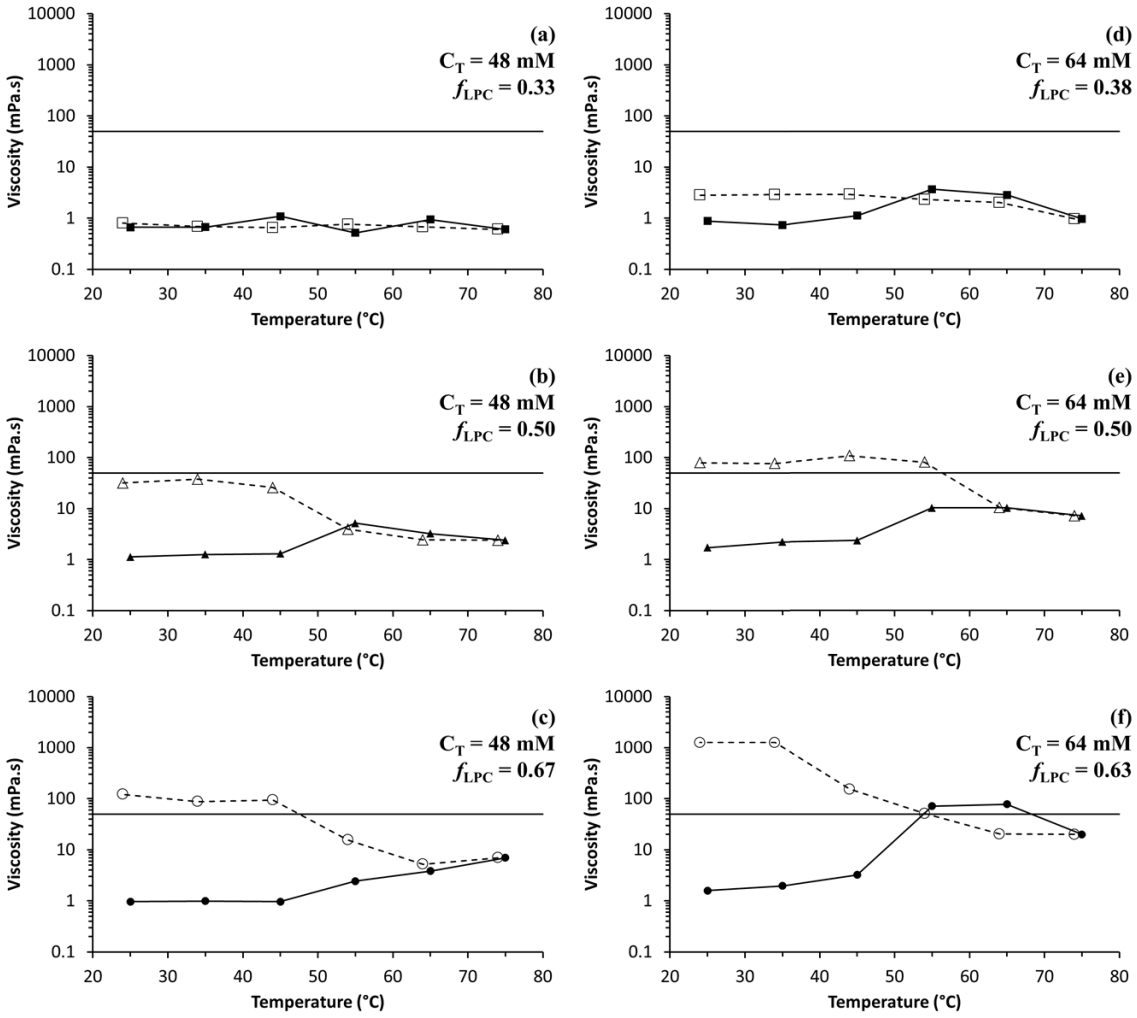


**Fig. 5** Viscosity of 48 mM (a-c) and 64 mM (d-f) of lecithin mixtures ($f_{LPC}$) as a function of temperature. The molar fraction of LPC ($f_{LPC}$) is indicated in the figure. Filled symbols, heating; open symbols, cooling. Plots for cooling process were shifted by -1°C for clarity.

Fig. 5 shows a clear hysteresis between the heating and cooling processes. It seems that heating is needed to overcome a certain kinetic barrier for formation of the wormlike micelles, but this structure is more stable than the mixture of individual planar and spherical micelles. The wormlike micelle is the dominant form at low temperature at equilibrium, which can be reached much more quickly by first heating the mixture to above

50°C. Reorganization of the micelles probably requires enhanced mobility, which is attained at elevated temperatures. We do not wish to speculate on the exact nature of the kinetic barrier that needs to be overcome, but in comparison with single-surfactant micelles, it is clear that micelles formed by two types of surfactants should have a higher degree of internal order. The exact approach of the chains could therefore require a higher kinetic energy compared with that needed for formation of the native single-surfactant micelles. We note that formation of wormlike micelles on heating has also been reported by Tung *et al.* (2007) and Shrestha *et al.* (2007), who proposed that surfactant exchange was increased at elevated temperature, and this increased mobility facilitated formation of intermicellar interaction, resulting in formation of the wormlike micelles.

### 3.3.2 Molecular mobility studied by NMR

The diffusion coefficient *D* of LPC and PC was measured using PFG-STE NMR. In spectra with $g > 100$ G/cm, several peaks were found between 0-6 ppm and assigned to protons on the glycerol backbone and the alkyl chain of fatty acids in LPC (Fig. 6), cf. peak assignment by Haque *et al.* (1972). The total peak area over this range (0-6 ppm, without the water peak) was used in the diffusion analysis.

Figure 7a shows the relative echo intensity ($I/I_0$) as a function of $\gamma^2\delta^2g^2(\Delta-\delta/3)$ of solutions of pure PC and LPC. In these samples, $I/I_0$ shows a single exponential decay. Therefore, the diffusion coefficient *D* could be calculated from eq. (3) for all concentrations and temperatures tested of pure LPC or PC. This finding means that a narrow size distribution of micelles was present in all single-surfactant solutions.

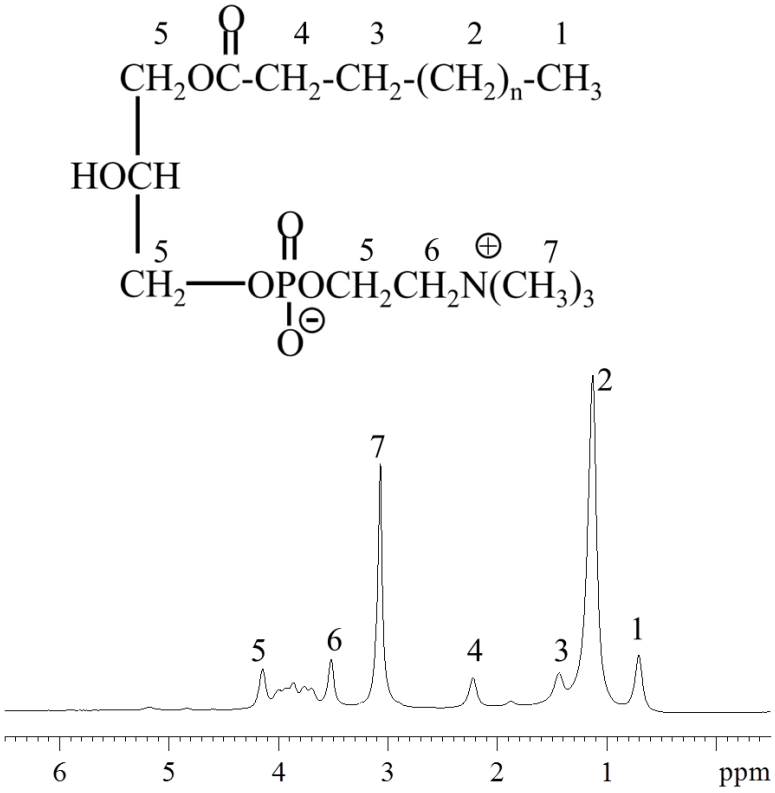


**Fig. 6** Proton NMR Spectrum of 92.5 mM lysophosphatidylcholine (LPC) in $D_2O$ at 25°C

The relative echo intensity ($I/I_0$) in the PFG-STE experiment for $C_T$ = 64 mM and $f_{LPC}$ = 0.6 as a function of temperature is shown in Fig. 7b. At 25°C and 35°C, a clear curvature is seen, which indicates the presence of diffusion of more than one component. If we assume the presence of two populations, we can rewrite equation (3) as follows:

$$I(g) = I(0)(f_1 \exp[-\gamma^2\delta^2 g^2 (\Delta - \delta/3)D_1] + f_2 \exp[-\gamma^2\delta^2 g^2 (\Delta - \delta/3)D_2]) \quad (4)$$

where subscripts 1 and 2 refer to the first (faster) and second (slower) components, respectively, and $f_1$ and $f_2$ are their respective fractions. We assumed that the fast component ($f_1$) corresponded in all cases to LPC micelles. We therefore fixed in the fitting procedure $D_1$ to $D$ measured for solutions of LPC. Thus, we used only 2 fit parameters: the diffusion coefficient and fraction of the slower component, $D_2$ and $f_2$, respectively (note

that $f_1 + f_2 = 1$). The slower component is assumed to reflect diffusion of both PC micelles and the wormlike micelles formed by co-micellization of LPC and PC. Good fits to the data at 25, 35 and 45°C could be obtained using eq. (4). As the temperature is increased, the wormlike micelle is formed, as evidenced by the disappearance of the faster diffusing component, which started at around 45°C, in agreement with the viscosity data in Fig. 5. The plots for 55, 65 and 75°C were almost linear indicating that the first component became negligibly small. For these temperatures, fits were obtained using eq. (3). At these temperature, only the slow component is present, so that $f_2 = 1$ and $D_2 = D$.

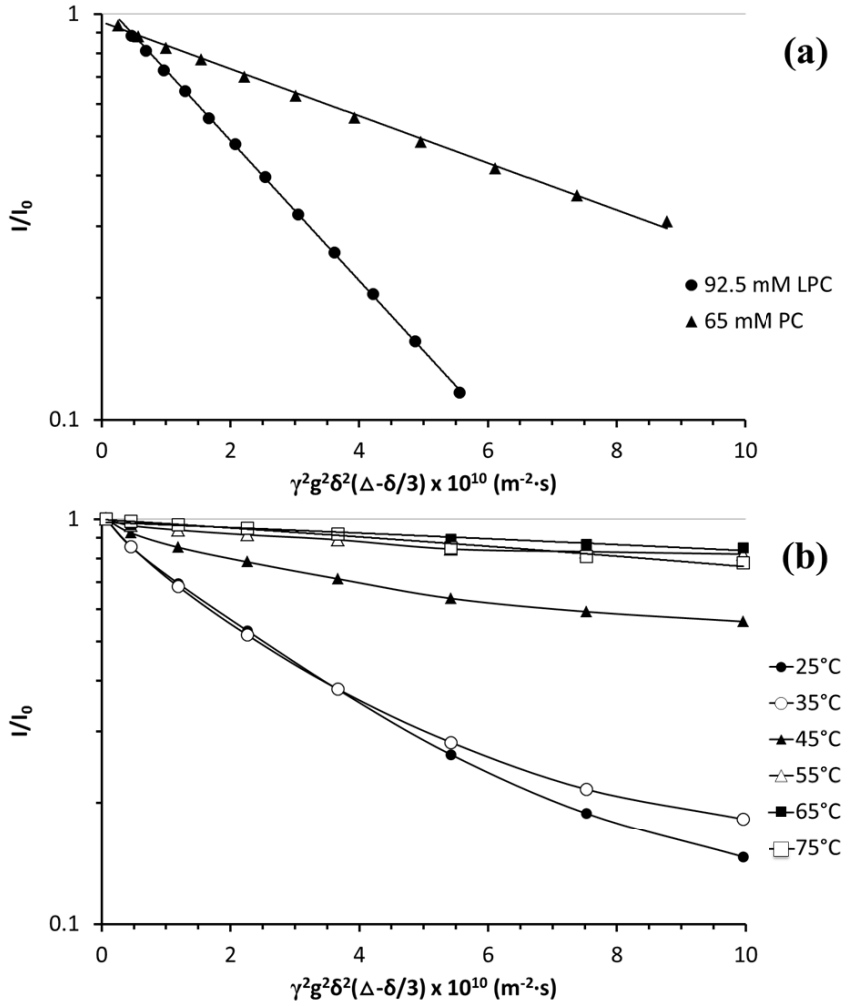


**Fig. 7** Semi-logarithmic representation of the relative NMR echo intensity ($I/I_0$) as a function of $\gamma^2g^2\delta^2(\Delta-\delta/3)$ for (a) individual phospholipids at 25°C (a) and (b) a lecithin mixture ($C_T$ = 78.75 mM, $f_{LPC}$ = 0.6) different temperatures indicated in the figure. Solid lines indicate least square fits to eq. (3) or eq. (4).

Fig. 8 shows the *D* values of LPC (7a) and PC (7b). For 3.75 mM LPC, *D* at 25°C was $4.93 \times 10^{-11} m^2 s^{-1}$, which corresponds to a displacement of 1 μm during the diffusion time of 10 ms. There are two possible modes of diffusion of surfactant chains. One is micelle translation. The other is intermicellar displacements of individual surfactant chains

(see, e.g. Myers, 2005). LPC is assumed to form a spherical micelle with a diameter close to the combined length of two C-18 alkyl chains (the most common alkyl chain in our LPC sample). The projection of a C-C bond along the axis of alkyl chains is approximately 0.12 nm. The length of a C-18 alkyl chain is therefore 17 × 0.12 ≈ 2.0 nm. Because the displacement of $^1$H (1 μm) is much larger than the length of two C-18 chains (4 nm), it reflects translation of the spherical micelle, rather than intermicellar movements.

We can further analyze data using the Einstein-Stokes relation:

$$D = \frac{k_B T}{6\pi\eta R_H} \qquad (5)$$

where $k_B$, $T$ and $\eta$ are Boltzmann constant, absolute temperature and viscosity of the solvent, respectively. The hydrodynamic radius $R_H$ is defined as the radius of a hard sphere yielding $D$. Assuming that the LPC micelle is a hard sphere, we obtain $R_H$ = 4.98 nm for the 92.5 mM solution at 25°C. This value is a bit larger than the length of two alkyl chains. We expect this difference to reflect hydration of the LPC spheres. With increasing temperature, the $D$ value was increased, and the increase of $D$ seems to be steeper than that estimated from the increase of temperature. The diffusion coefficient of the spherical micelles formed by 3.75 mM LPC solutions (calculated assuming a constant $R_H$ value) is indicated with a dashed line in Fig. 8a. The $D$ value showed an upward deviation from this line at temperatures above 65°C. As the temperature increases, the charge on phosphate choline head group decreases and some hydrogen bonding between the head groups and water is lost. As a result, the micelle size can decrease, as has been reported for SDS micelles in water (Hammouda, 2013). The $D$ value of 2.6 mM PC at 25°C was 1.63 × $10^{-11}$ $m^2s^{-1}$, which corresponds to a displacement of 560 nm during the diffusion time of 10 ms. Fig. 2

showed the formation of very large spherical PC micelles, which we assume to be vesicles with internal planar structure. Although it is probably only the largest vesicles which are observed, we still expect most PC assemblies to have diameters of at least several hundred nm. A displacement of 560 nm could therefore reflect not only micelle displacement, but also intermicellar mobility of individual PC chains. PFG-NMR measures the self-diffusion coefficient. The self-diffusion coefficient of macromolecules in binary mixtures with a solvent always decreases with increasing macromolecule concentration, due to excluded-volume interactions and increased friction between the macromolecule and the solvent (Le Bon *et al.*, 1999). The micelle displacement should therefore be concentration dependent. On the other hand, the structure of most micelles is virtually independent of the surfactant concentration, and we therefore expect the intermicellar mobility to be independent of concentration of PC. Because we observe a decrease in $D$ with increasing concentration of PC, we can conclude that intermicellar displacements do not contribute significantly to the observed displacement of 560 nm. Therefore, $D$ describes the movement of the PC micelle itself, and not (to any significant extent) that of individual PC molecules inside the micelle.

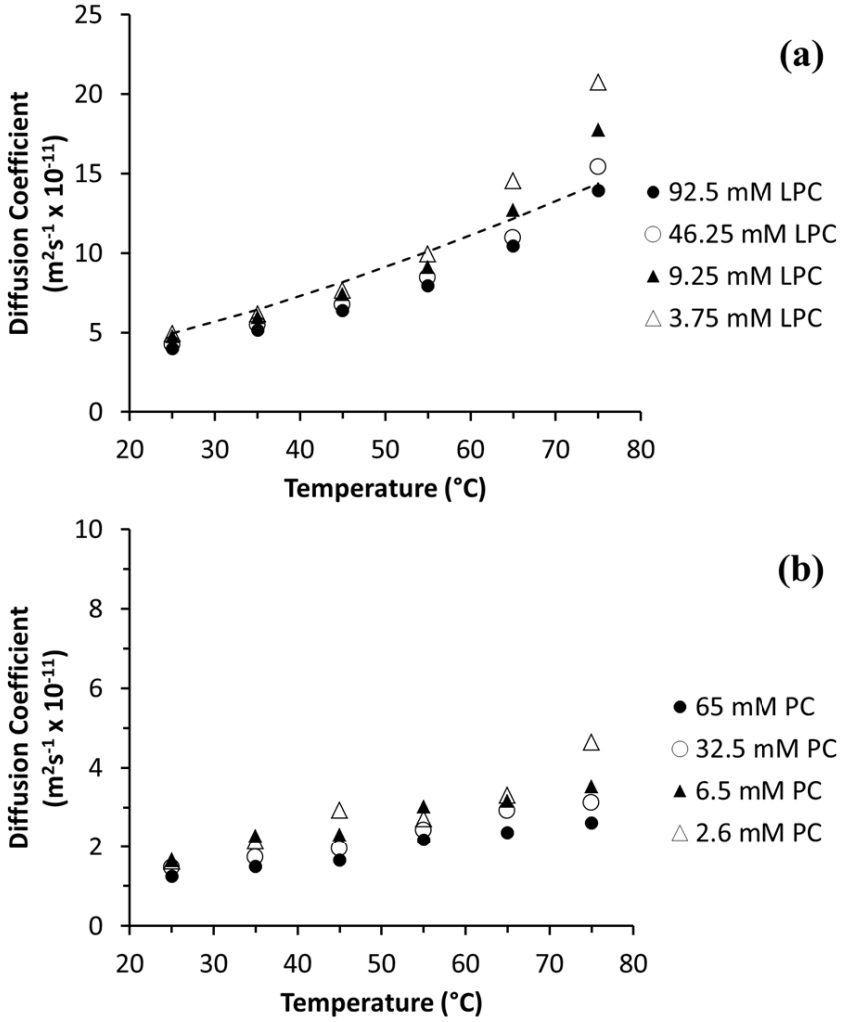


**Fig. 8** Diffusion coefficient *D* of lysophosphatidylcholine (LPC; a) and phosphatidylcholine (PC; b) in $D_2O$ as a function of temperature. The dashed line shows *D* calculated using the Einstein-Stokes relation (eq. 5) assuming $R_H$ = 4 nm.

Figure 9 shows the slower component fraction ($f_2$) and diffusion coefficient ($D_2$) of a LPC/PC mixture ($f_{LPC}$ = 0.6) at various temperatures. The results obtained for all samples with $f_{LPC}$ = 0.6 at $C_T \geq 39$ mM suggest the increase of wormlike micelle content with increasing temperature. This is reflected in the increase of $f_2$ and the decrease of $D_2$ values with increasing temperature up to 65°C. Gokhale *et al.* (2005) found a similar decrease in *D*

with increasing temperature due to the formation of wormlike micelle in CTAB with addition of *p*-nitrophenolate. Above 75°C, $D_2$ increased with increasing temperature. This increase is similar to the increase seen for solutions of the individual surfactants as shown in Fig. 8. Similarly to our explanation of the viscosity data of Fig. 6, we can ascribe the reversing of the trend at high temperature to a decrease in the equilibrium length of the wormlike micelles. This effect can only be observed after the initial kinetic barrier to wormlike micelles formation has been overcome.

It is clear from Fig. 9 that the increase in $f_2$ on heating of mixtures with $f_{LPC} = 0.6$ is also observed for mixtures at much lower total lecithin contents $C_T$ (3-8 mM). We assume that this increase also reflects formation of wormlike micelles. However, for these mixtures, $D_2$ shows unexpected behavior. We first note again that the equilibrium length of wormlike micelles is expected to decrease strongly with decreasing surfactant concentration (Cates and Candau, 1990). It is therefore possible that the diffusion coefficient of the PC micelles, present in the initial non-equilibrium state, and the wormlike micelles which are formed on heating, is not so different. In addition, the diffusion of any PC micelles still present will be restricted in a non-trivial manner by the emerging wormlike structure. There is probably some compensation between the increase in restriction due to wormlike micelle formation and the increase in kinetic energy due to elevated temperature. It seems that these contrasting effects, along with the smaller difference between the size of the wormlike micelles and the PC micelles, can lead to either a decrease or increase in $D_2$ on heating of solutions with low $C_T$.

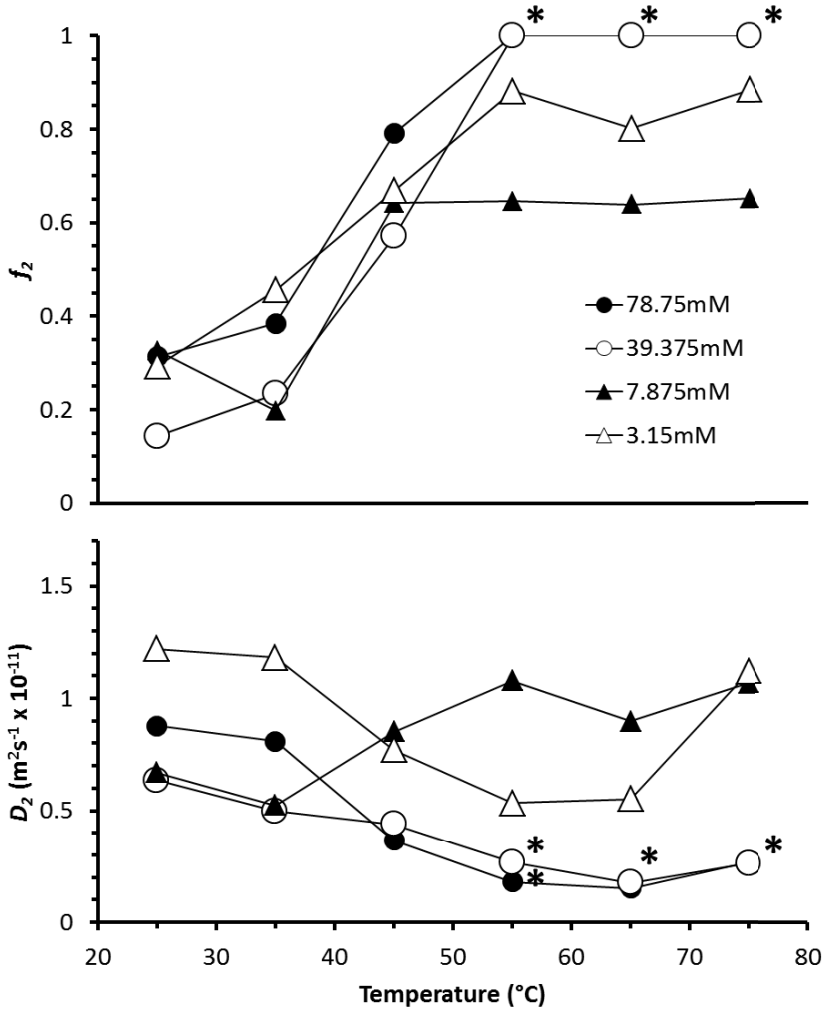


**Fig. 9** $f_2$ (Top) and $D_2$ (bottom) of lecithin mixtures with $f_{LPC}$ = 0.6 at various $C_T$ in $D_2O$ as a function of temperature. Measurements where a single diffusion component was found are indicated with an asterisk.

It should be emphasized that the results showing two distinct populations with very different diffusion coefficients reflect a non-equilibrium state. As was already evidenced by the viscosity data shown in Fig. 5, the starting state at low temperature is not an equilibrium state. As the temperature increases, the kinetic barrier can be overcome, and the wormlike micelles are formed, as evidenced by the huge increase in viscosity. On cooling, the

viscosity either remained close to its value at high temperature or increased. This finding indicates that the wormlike micelle is the thermodynamically stable state at low temperatures as well. This rheological hysteresis is a clear indication that the starting state at 25°C is not an equilibrium one. Therefore, the results shown in Fig. 9 should depend on the thermal history of the sample, as they reflect a non-equilibrium state during the first heating step. We note that several studies on formation of wormlike micelles from mixtures of cationic and anionic surfactants (Davies *et al.*, 2006; Lin *et al.*, 2009) employed a methodology of initial extensive heating at temperatures ranging between 50-65°C, and all characterization was done following this initial heating and assumed to be at an equilibrium state. Our investigation is different as we observed the changes in both surfactant diffusion and viscosity taking place on initial heating and wormlike micelle formation.

The information on phospholipid diffusion supports the rheological findings, namely, at $f_{LPC} \approx 0.5$-$0.7$, wormlike micelles are formed on heating above 55°C. In addition, it allows us to estimate the distribution of the two phospholipids between the small spherical micelles, which according to our results may very well contain a small amount of PC chains, and the larger planar and wormlike micelles. More importantly, the distribution indicates that at the highest temperatures tested, no fast diffusion component is left. This finding confirms that the wormlike micelles are formed by co-micellization of all the PC and LPC chains, and also means that no spherical micelles remain at the end of the co-micellization process.

## 3.4 Conclusion

Our research has demonstrated that a mixture of LPC and PC can form wormlike micelles in aqueous solution. Reverse wormlike micelle formation has been reported in the literature for phospholipids in w/o emulsions (Kumar *et al.*, 2009; Shchipunov, 2001; Shumilina *et al.*, 2006), but this is the first report to our knowledge on formation of normal phospholipid wormlike micelles in water without the addition of salts. The formation of the wormlike micelle requires a minimum concentration of both PC and LPC. The data indicate that the wormlike micelle is more stable than the micelles formed by each phospholipid individually for all temperatures tested, but its formation requires heating in order to overcome kinetic barrier. Because uncharged phospholipids confer higher stability on o/w emulsions compared to charged phospholipids when small amounts of NaCl or $CaCl_2$ are present (Han and Washington, 2005; Han *et al.*, 200; Washington *et al.*, 1990), the wormlike micelles reported herein could be used as both stabilizers and thickeners of such systems. We finally note that further research should be carried out to elucidate how general the formation of wormlike micelles is when two surfactants, one with $CPP < 0.5$ and the other with $CPP > 0.5$, are mixed.

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# Chapter 4: Elucidation of the formation of mixed phosphatidylcholine wormlike micelle structure by nuclear magnetic resonance (NMR)


## Abstract

We demonstrate the formation of wormlike micelle from mixture of lysophophatidylcholine (LPC) with *CPP* <1/3 and phophatidylcholine (PC) with *CPP* >1/2 in aqueous solution. At low temperature less than 40°C, the mixtures contained individual structure of spherical micellar LPC and vesicle PC. They transformed into wormlike micelle after alkyl chain in both of LPC and PC melted at temperature higher than 40°C which result from DSC and peak width in NMR measurement. From pulse field gradient stimulated spin echo NMR, LPC and PC formed spherical micelles with faster molecular mobility and vesicle structures with slower molecular mobility, respectively. The diffusion coefficient of lecithin mixtures exhibited the decrease after wormlike micelle structure was formed. Overall, this finding proposed the method in creating wormlike micelle structure by mixture of two surfactants, more hydrophilicity with *CPP* less than 1/3 and the other with *CPP* higher than 1/2.

## 4.1 Introduction

Surfactants have the ability to self-assemble into various structures such as spherical micelle, rod-like or wormlike micelle, vesicle or bilayer structure (Myers, 2005). Among these, wormlike micelle structure has drawn many attentions in this research field due to the wide range of applications such as biosensors (Blin *et al.*, 2005; Wang *et al.*, 2007), personal care products (Yang, 2002) or drug delivery systems (Lawrence and Rees, 2000; Vintiloiu and Leroux, 2008). Wormlike micelle is a long, flexible, polymer-like structure showing viscoelastic properties. The dynamics of wormlike micelle suspensions can be described using a single (reptational) relaxation process (Cates and Candau, 1990). Consequently, the rheology of these suspensions can be described using a simple Maxwell fluid model (Cates and Candau, 1990; Hoffmann, 1994). The self-assembly geometry can be predicted by the basis of packing of surfactant expressed as critical packing parameter (*CPP*) which is defined as $v/a_0l$, where $v$ is the volume of the hydrophobic chain, $l$ is the effective length of the hydrophobic chain and $a_0$ is the effective surface of the head-group. For $CPP < 1/3$, spherical micelles are expected; when $1/3 < CPP < 1/2$, wormlike micelles are expected and $CPP > 1/2$, vesicle are expected (Israelachvili, 2011).

Wormlike micelle can be formed in both aqueous media; called normal wormlike micelle, and non-polar solvent; called reverse wormlike micelle. For normal wormlike micelle, several systems had been reported on the viscoelastic solution containing wormlike micelle structure such as cationic surfactant in the presence of salt, mixed nonionic-zwitterionic surfactants, and mixed cationic-anionic surfactant (Acharya and Kunieda, 2003; Davies *et al.*, 2006; Kumar *et al.*, 2007; Raghavan *et al.*, 2002). On the

other hand, phosphatidylcholine or lecithin is known to form reverse wormlike micelle in organic solvent or oil (Shchipunov, 2001; Tung *et al.*, 2007).

Lecithin or phosphatidylcholine (PC) is one of phospholipids containing choline as a head hydrophilic group. Phosphatidylcholine composes of two fatty acids in molecule; while, lysophosphatidylcholine (LPC) has one fatty acid at *sn*-1 position of glycerol backbone giving LPC has more hydrophilicity than PC (Faergemand and Krog, 2003; Hanahan, 1997; Krog, 1997).

However, in our study, we noticed that the mixture of phosphatidylcholine with different geometry, that is, LPC and PC, caused the gelation in aqueous media resulting from wormlike micelle structure. Therefore, our objectives were to clarify the mechanism of formation of wormlike micelle structure from mixed phosphatidylcholine with different hydrophobicity; that is LPC and PC, in an aqueous solution. Pulsed-field-gradient nuclear magnetic resonance (PFG-NMR) was used to clarify the motion of both functional groups for example; methyl group in choline phosphate head group or alkyl group in hydrophobic fatty acid chains, and surfactant molecule itself during the formation of mixed phosphatidylcholine wormlike micelle over the temperature range (Gröger *et al.*, 2004; Price *et al.*, 1999).

## 4.2 Materials and methods

### 4.2.1 Materials

Egg yolk PC and LPC (Fig. 1) were kindly provided by Kewpie Company (Tokyo, Japan). Deionized water was obtained by a Water Purifier (Autostill WA500, Yamato

Scientific Co., LTD., Tokyo, Japan). $D_2O$ (deuterium isotopic content: 99.0%) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

PC and LPC were dispersed in deionized water with a sonicator (Qsonica Q125, Connecticut, United States) for 5 min. An ice bath was used to prevent overheating of the sample. The lecithin mixtures were mixed to obtain mixtures of $C_T$ (total lecithin concentration; $C_T = C_{LPC} + C_{PC}$ = molar concentration of LPC + molar concentration of PC) 64 mM with various ratio of LPC to PC, and sonicated for 1 min. Sonication was performed at 20 kHz, amplitude 60%, and a time interval of 5 s. Solutions used in NMR measurements were prepared with $D_2O$ was instead of deionized water.

Samples were 64 mM lecithin mixtures with various LPC/PC ratio from 0/64, 8/56, 16/48, 24/40, 32/32, 40/24, 48/16, 56/8, and 64/0 which equal to LPC molar fraction ($f_{LPC}$) of 0, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 0.875, and 1, respectively.

### 4.2.2 Methods

#### *4.2.2.1. DSC measurements*

The DSC measurements were carried out in nitrogen atmosphere with temperature range of 5 – 65°C at heating rate of 0.6°C/min by using a micro DSCVII CS Evol. (Setaram-France) instrument equipped with a cooling apparatus. Temperature and enthalpy calibrations were carried out using deionized water.

#### *4.2.2.2. Diffusion coefficient measurements*

Diffusion coefficients using the pulsed-field-gradient stimulated spin-echo (PFG-STE) pulse sequence were performed on a Bruker Avance II 400WB spectrometer

equipped with a gradient probe (Price, 2009; Stejskal and Tanner, 1965). The temperature was varied by using a Bruker BVT-3200 temperature unit and varied in the range of 25-75°C. An optical fiber thermometer (Takaoka Electric Mfg. Co., Tokyo, Japan) was used to measure the temperature in the sample tube.

The diffusion coefficient was determined using the following relation between the echo signal intensity and field-gradient parameters:

$$I(g) = I(0) \exp[-\gamma^2 \delta^2 g^2 (\Delta - \delta/3) D] \quad (1)$$

where $I(g)$ and $I(0)$ are echo signal intensities at $t=2\tau_2+\tau_1$ with and without the field gradient pulse, respectively. $\gamma$ is the gyromagnetic ratio of $^1H$. $\delta$ is the gradient pulse duration and $\Delta$ is the intervals time the field-gradients that correspond to the interval between two gradient pulses.

For experiment, $\delta$ and $\Delta$ were 1 ms and 10 ms, respectively. The gradient field strength $g$ was varied in 16 steps in the range 100-1500 G/cm.

#### *4.2.2.3. Rheological measurements*

Steady rheological experiments were performed on Haake (MARS II) rotational rheometer. Samples were studied at temperature range 25 – 75°C on couette apparatus (inner diameter of cup = 27.208 mm and diameter of bob = 25.080 mm). Samples were kept at each temperature for 15 min before each measurement for equilibration. For experiments, shear rate was 0.1 – 100 $s^{-1}$.

## 4.3 Results and Discussion

In order to understand the irreversible gelation behavior shown in Fig.4 in Chapter 3, the change of flexibility by heating in alkyl chain of LPC and PC in micelles was considered by measuring differential scanning calorimetry (DSC) and half line width in the first spectrum in PFG-STE measurements on 64 mM lecithin mixtures at various LPC/PC ratios.

In DSC measurements (Fig.1), the 64 mM PC solution showed a small and broad endothermic peak at around 20°C on heating process; meanwhile, the 64 mM LPC solution showed two major endothermic peaks at around 20°C and 30-40°C. Endothermic peaks can be assigned to melting of crystalline like structure. On the consequent cooling process, any peaks were not detected indicating that the crystalline like structure needs a long setting time to be restored after the melting. Lecithin mixture showed complicated behavior probably because of the competitive melting of spherical micelle and formation and melting of wormlike micelle lower than 40°C, and showed no peak at temperatures higher than 40°C and in the consequent cooling process as seen for individual solution of LPC and PC. These results suggest that the formation of wormlike structure by co-micellization of PC and LPC requires the melting of crystalline like structure at temperature more than 40°C to give higher flexibility and molecular mobility in the alkyl part.

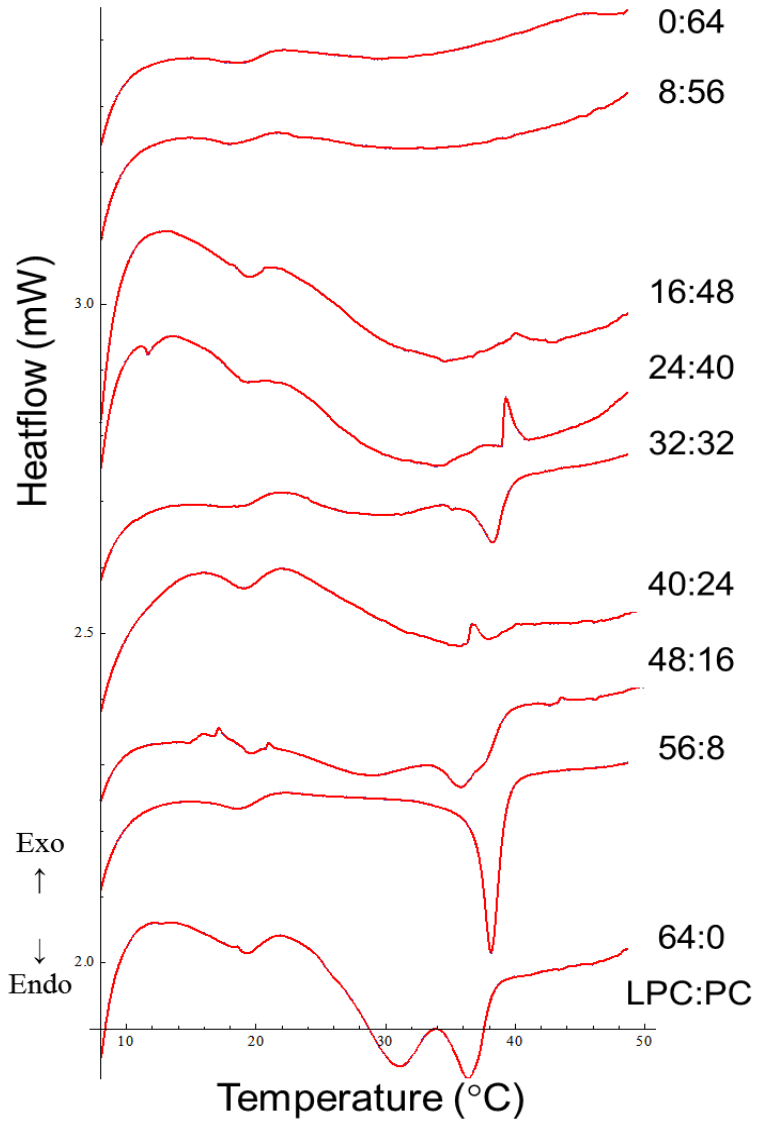


**Fig. 1** DSC curves of 64 mM lecithin mixtures at various LPC/PC ratio.

The half line-width in spectra is inversely proportional to the apparent $T_2$ relaxation time ($T_2$*) and can be used as an indicator for the molecular mobility, that is, the increase of half line-width indicates the decrease of molecular mobility. The half line-width for the peak of alkyl group in fatty acids in the first spectra (g=100G/cm) in PFG-STE measurements varying temperature were calculated for samples with various

LPC:PC ratio and shown in Fig. 2. For mixtures with LPC:PC ratio of 0:64, 8:56 and 16:48, the half line-width were around 60 Hz and showed small temperature dependences on heating and cooling (Fig. 2a). The mixtures mainly contain planer micelle of PC, and therefore, the results show the small change of flexibility of alkyl chain in the micelles.

For mixtures with LPC:PC ratio of 56:8 and 64:0, the half line-width were less than 50 Hz at temperatures lower than 45°C, increased gradually with increasing temperature up to around 55°C and increased steeply more than 55°C (Fig. 2c). The mixtures mainly contain spherical micelle of LPC. As shown below, the diffusion coefficient of LPC was steeply increased with increasing temperature at around 60°C indicating the decrease of micelle size, which is caused by the contraction of micelle or rearrangement to form smaller micelle with increasing the hydrophobic interaction at higher temperatures. The increase of the hydrophobic interaction is thought to induce tightness in micelle, therefore, the increases of the half line-width were considered to be caused by the restriction in the molecular mobility of alkyl group. On cooling process, the half line-width were decreased down to ca. 50 Hz at 45°C (open symbols) showing the increase of the molecular mobility of alkyl group with decreasing hydrophobic interaction.

For mixtures with LPC/PC ratio of 32/32, 40/24 and 48/16, the half line-width were less than 55 Hz at lower temperatures and start to increase at temperatures from 35 to 40°C (Fig. 2b), which is corresponding the temperature range for the endothermic peaks showing the melting of crystalline like structure in micelles, and increased further in the cooling process (open symbols). There should be two components of $T_2$* in the peak for alkyl group in mixers corresponding to LPC and PC, nevertheless, the half

line-width were just determined from the line-width at the half height of peak with no consideration of the distribution in molecular mobility. As shown below, the mixture with LPC/PC ratio of 32/32 had two diffusional components at lower temperature than 45°C and became one component at temperatures higher than 50°C indicating the co-micellization to from the worm like micelle. Therefore, it is considered that the steep increases of the half line-width are corresponding to the restriction of molecular mobility of alkyl group in the wormlike micelle and that the further increases of the half line-width indicate the decrease of molecular mobility with decreasing temperature in the wormlike micelle.

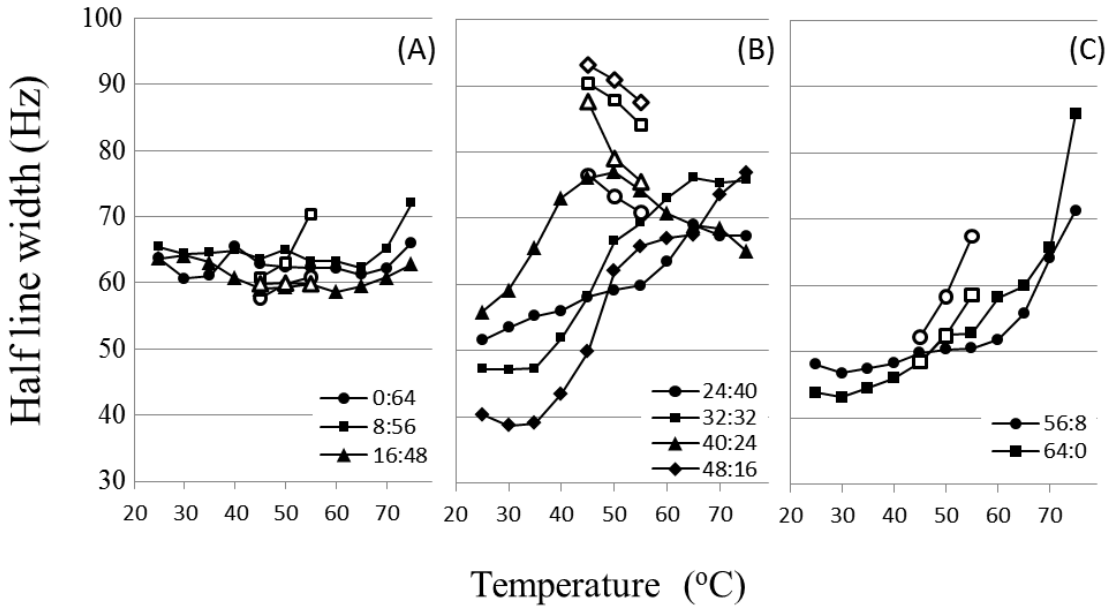


**Fig. 2** Half-line width of alkyl peak in hydrophobic fatty acids chain of 64 mM lecithin mixtures at different at various LPC:PC ratios (A = 0:64, 8:56, 16:48; B = 24:40, 32:32, 40:24, 48:16; C = 56:8, 64:0) as a function of temperature.

Pulsed-field-gradient stimulated spin echo (PFG-STE) NMR was used for determining diffusion coefficient of 64 mM lecithin mixtures and the results are shown in

Fig. 3. *D* value of LPC was 4.35 × $10^{-11}$ $m^2s^{-1}$ at 25°C sugesting the formation of spherical micelle. With increasing the temperature, *D* vaule steeply increased at around 70°C. This was due to the increasing in brownian movement of spherical micelle and also the decreasing in micelle size. Not only the lost of hydrogen bonding between choline head groups and the water as reported for SDS in water, but also the restrcited of alkyl group in hydrophobid fatty acids moeity as was already pointed out are resposible for the reducing in micelle size (Hammouda, 2013).

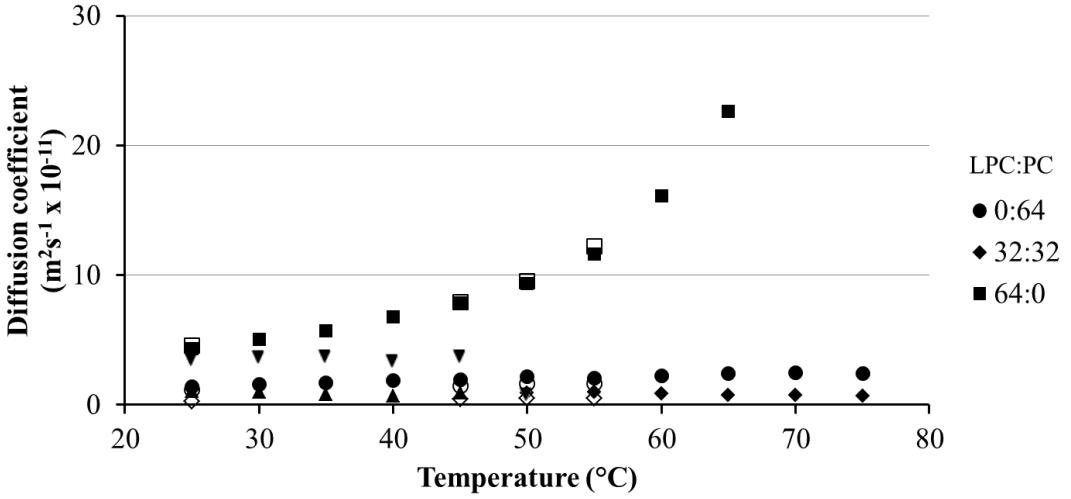


**Fig. 3** Diffusion coefficient *D* of 64 mM lecithin in $D_2O$ at various LPC:PC ratios as a function of temperature. Filled symbol represents heating process; opened symbol represents cooling process.

For PC solution, *D* values were relatively low in the range of 1 – 3 × $10^{-11}$ $m^2s^{-1}$ throughout heating process. As seen in the temperature dependence of half line-width in Fig. 2, PC forms a restricted planar structure at any temperature.

For LPC/PC mixture with LPC/PC ratio of 32/32, the echo intensity in the PFG-STE experiment at temperatures lower 45°C showed a clear curvature in the plot of decay against $\gamma^2\delta^2g^2$ ($\Delta - \delta/3$) in Eq.(1) indicating the presence of diffusional components of more than one component (data was not shown). We assume the presence of 2 populations; then equation (1) was rewritten as follows:

$$I(g) = I(0)(f_1 \exp[-\gamma^2\delta^2g^2 (\Delta - \delta/3)D_1] + f_2 \exp[-\gamma^2\delta^2g^2 (\Delta - \delta/3)D_2]) \quad (2)$$

where subscripts 1 and 2 refer to the first (faster) and second (slower) component, respectively, and $f_1$ and $f_2$ are their respective fractions ($f_1 + f_2 = 1$).

It is apparently seen that first component in LPC/PC mixture is spherical micelle with faster diffusion; while, second component was rod-like or wormlike micelle with slower diffusion coefficient which is less than $1 \times 10^{-11}$ $m^2s^{-1}$. As mention earlier in Chapter 3, the increase of $f_2$ and the decrease of $D$ values with increasing temperature indicated the formation of wormlike micelle. On cooling step, $D$ value of LPC/PC mixture remained low indicating that the wormlike micelle is the thermodynamically stable state after forming.

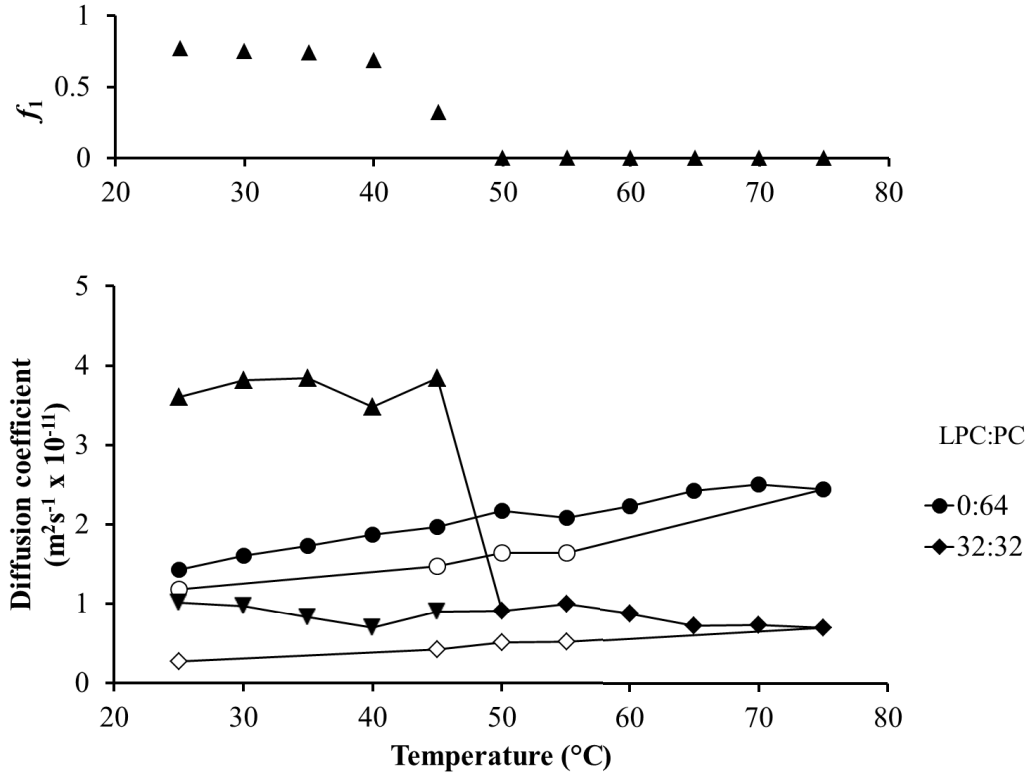


**Fig. 4** $f_1$ (top) and diffusion coefficient (bottom) of 64 mM lecithin mixtures in $D_2O$ at different LPC:PC ratio as a function of temperature. Filled symbol represents heating step; opened symbol represents cooling step.

The diffusion coefficient (*D*) of 64 mM lecithin mixtures with LPC:PC ratio of 32:32 measured at different temperatures is shown in Fig. 5. Diffusion coefficient showing a single component, calculated by eq. (1), is chosen to plot against diffusion time (*Δ*). *D* value decreases with increasing temperature. This is due to the formation of wormlike micelle of lecithin mixture as discussed previously. In addition, *D* value all decreases as a function of Δ. This suggests that the observed *D* is a combination of wormlike micelle self-diffusion with intermicellar diffusion of lecithin and/or lecithin molecule self-diffusion inside the wormlike micelle. *D* value decreases on subsequent cooling to 25°C. This result

indicates the decrease of molecular mobility with decreasing temperature in the wormlike micelle as well as to the result of half-line width of alkyl peak in Fig. 2. *D* value, however, shows an independence on Δ during cooling step. As a consequence, measured diffusion coefficient is responsible for only diffusion of wormlike micelles themselves.

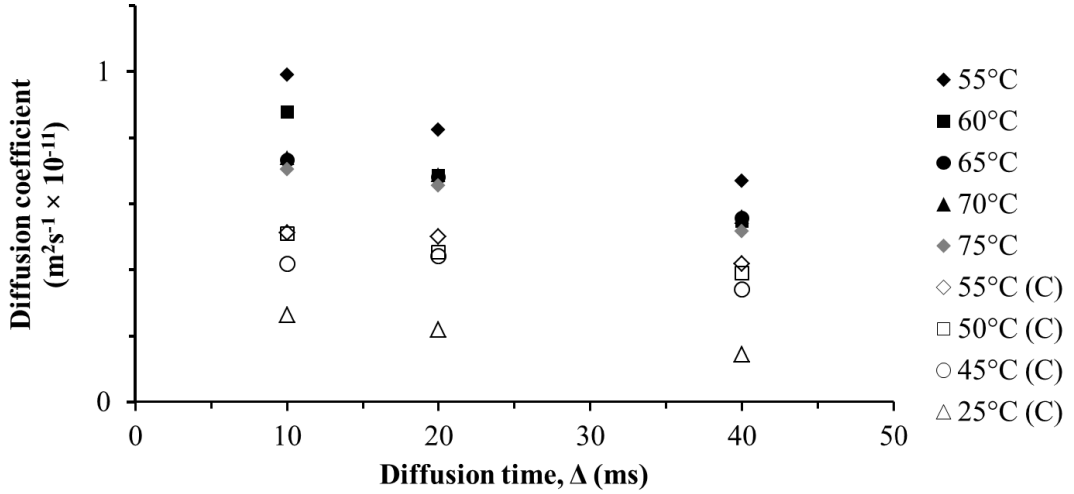


**Fig. 5** Diffusion coefficient (*D*) of 64 mM lecithin mixtures with LPC/PC ratio 32:32 in $D_2O$ as a function of diffusion time. Measuring temperatures are indicated in figure. Filled symbol represents heating step; opened symbol represents cooling step.

The rheological response of 64 mM lecithin mixtures with different LP:/PC ratio; which were 32:32, 40:24, 48:16, at various temperature is shown in Fig. 5. Mixtures show Newtonian behavior; that are their viscosities are independent to shear rate, with the viscosity are quite low at 25°C. This response is corresponding to the presence of mix spherical micelle and small content of short rod-like or wormlike micelle. When temperature increases, mixtures switch to a shear-thinning behavior; that is a plateau of viscosity at low shear rate, then followed by the decrease of viscosity at higher shear rate.

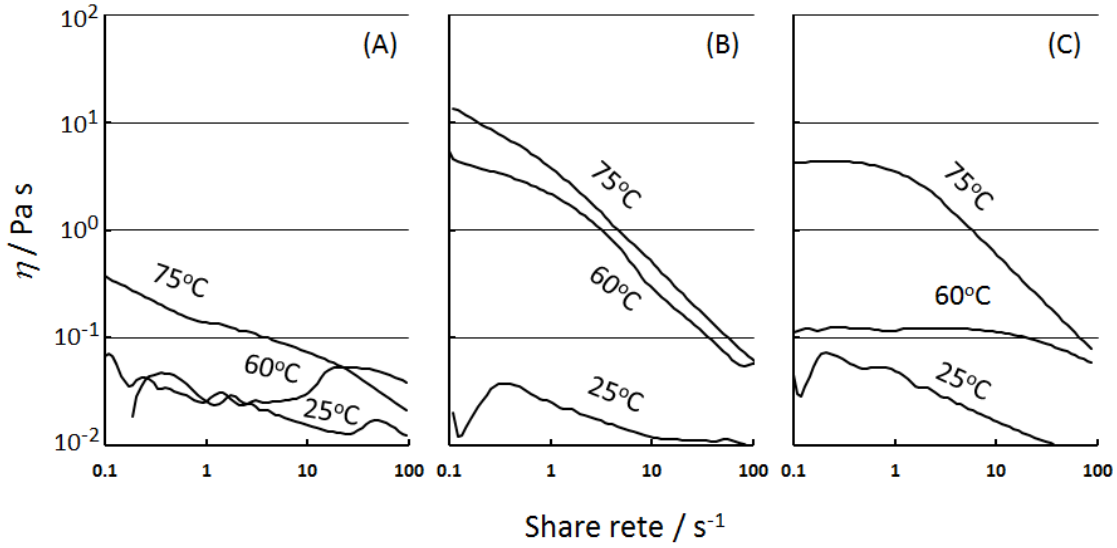


**Fig. 6** The steady-shear rheological response of 64mM lecithin mixtures with LPC/PC ratio of 32/32 (A), 40/24 (B) and 48/16 (C) at 25°C, 60°C and 75°C.

### 4.4 Conclusions

Our research has demonstrated that a mixture of LPC and PC can form wormlike micelles in aqueous solution without the addition of salts. A suitable LPC:PC ratio for the formation of wormlike micelle is 32:32, 40:24 and 48:16 which is equal to $f_{LPC}$ around 0.5 – 0.75. However, wormlike micelle can be formed after the melting of fatty acids chains in both PC and LPC showing an endothermic peak around 20°C and 40°C in DSC. In PFG-NMR experiment, half-line width of alkyl peak from fatty acid chains and diffusion coefficient shows that alkyl chains become restricted after wormlike micelle is formed with slow molecular mobility. Wormlike micelle structure is more stable state of mixed individual phospholipids which wormlike micelle can still maintain its structure after cooling. The formation of wormlike micelle causes the mixtures switch from low viscosity, Newtonian fluid to viscoelastic, shear-thinning fluid.

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# Chapter 5: General conclusions

Phosphtidylcholine, a natural zwitterionic surfactant, has been previously reported in the formation of wormlike micelle in non-polar media or in water with addition of bile salts. Our research demonstrates that mixture of phosphatitylcholine with different in number of fatty acid chains can form wormlike micelle structure in aqueous media without adding any salts.

The aqueous mixtures of lysophosphatidylcholine (LPC) with *CPP* <1/3 and phosphatidylcholine with *CPP* >1/2 show the formation of wormlike micelles. However, minimum concentration of both LPC and PC is required for wormlike micelle formation. Total concentration of lecithin mixture controls the length of wormlike micelle; meanwhile, ratio between LPC and PC indicates the self-assembly structures, such as spherical micelle, vesicle or wormlike micelle. It should be kept in mind that the results showing two populations with different diffusion coefficient reflect a non-equilibrium state.

Differential scanning calorimetry (DSC) is used to evaluate phrase transition of wormlike micelle structure. This reveals that wormlike micelle structure can be formed after the fatty acid chains in both PC and LPC melts resulting in the mobility of alkyl chains which can be also detected by NMR.

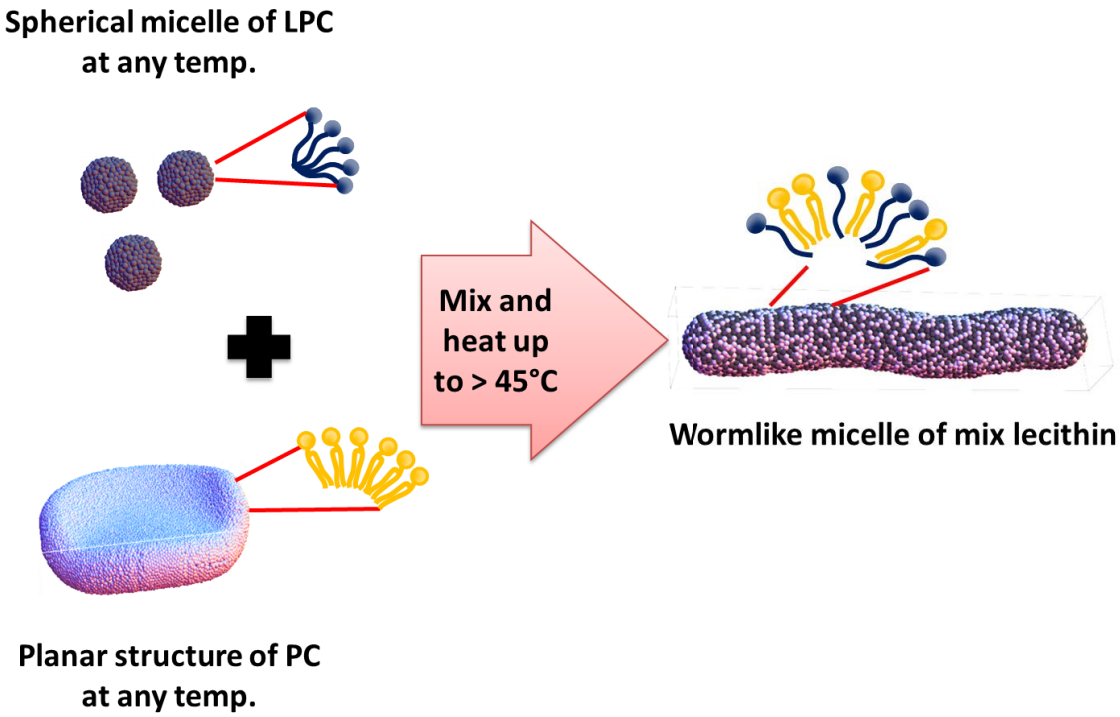


**Fig. 1** Schematic diagram of phosphatidylcholine wormlike micelle with PC and LPC

Pulsed-field-gradient nuclear magnetic resonance (PFG-NMR) is a powerful tool to obtain molecular mobility of molecules. With increasing of temperature, *D* value of spherical micelle steeply increases; while, that of planar shows a little increase. *D* value of wormlike micelle is very low compared to those of spherical micelle and planar due to the restricted mobility in wormlike micelle.

In the rheological aspect, the formation of wormlike micelle causes the mixtures switch from low viscosity, Newtonian fluid to viscoelastic, shear-thinning fluid.

A better understanding of the factors determining formation of phosphatidylcholine wormlike micelles could contribute to the design of suitable self-assembly structures for various applications such as food, drug delivery or biomimetic materials. For example, the wormlike micelles could be used as both stabilizers and thickeners of o/w emulsion containing salts systems. In encapsulation system, amphiphilic nutracueticals can be merged with surfactant for suitable self-assembly structure in each application.However, this study still needs further investigation on details in rheological behavior for the specific application.

## Acknowledgements

First, I would like to extend my gratitude to the Government of Japan and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) who gave me an opportunity to come to Japan and financially supported me so that I could finish my doctoral study successfully.

This dissertation would not be complete without valuable assistances and supports. I am sincerely thankful to my advisor Associate Professor Dr. Shingo Matsukawa, for his knowledge, invaluable advice, and encouragement. I also would like to thank to my committee members, Professor Toshiaki Ohshima, Professor Toru Suzuki, as well as to Associate Professor Dr. Tomoaki Hagiwara for their valuable comments and guidance on this dissertation including Professor Hiroo Ogawa and Assistant Professor Dr. Yuri Tashiro from Laboratory of Physico-chemical properties of Foods.

It is a pleasure to thank Dr. Tom Brenner for his help and suggestion during my research work as well as to Dr. Qiuhua Zhao. My profound thank is given to all my lab mates in the Laboratory of Physico-chemical properties of Foods. I wish to thank all my friends both in Japan and Thailand especially Thai friends at TUMSAT whose names are not given for their love and support.

Last but not least, my heartfelt gratitude goes to my family for their valuable support and warm encouragement in a number of ways to motivate me to perform best as I can. Without them this could not be happened.