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Thermotropic phase behavior of membranes made from *Erwinia carotovora* rough form lipopolysaccharide

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Abstract

The rough lipopolysaccharide (LPS) from *Erwinia carotovora* was found to form several μm diameter vesicles in aqueous solution. Spontaneous formation of spherical particles at ambient temperature was observed on addition of ethanol or a multivalent metal chloride to the solution. The LPS altered the gel–liquid crystalline phase transitions of the hydrocarbon chains in the temperature range 13–32°C. The vesicle formation indicated a marked increase of phase transition enthalpy ΔH up to three times greater than that of the LPS dispersed in pure water. The thermotropic phase behavior of the LPS was affected by metal ions, ionic strength and pH of the solution. Protonation of the LPS in acidic solution caused the transition temperature t_m and ΔH to increase, whereas deprotonation in alkaline solution led to the opposite effect. Multivalent metal ions such as Mg^{2+} and Ca^{2+} raised the t_m and increased the ΔH value. The LPS was organized to a greater degree in saline, acidic solution or divalent metal chloride solution than in pure water. The phase behavior varied according to the solvent conditions or metal chlorides that affect the phosphate and carboxyl groups, indicating that these charged moieties have an influence on the molecular organization within the membrane. Approximately two molar equivalents or more of Mg^{2+} ion induced a prominent thermotropic peak at ≈ 13°C. This ionotropic phase property of the natural LPS is supposedly directed by cross-linkage through divalent metal ions and negatively charged moieties such as phosphate bound to the LPS molecule. The functions of the LPS membrane are thus susceptible to change according to the chemical and physical conditions of the environment.

Keywords: Erwinia carotovora; Lipopolysaccharide; LPS membrane; DSC; Thermotropic phase behavior

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1. Introduction

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria. As LPS contains polysaccharide, fatty acids and phosphates, the formation of vesicle-like particles in water can be expected for this type of amphiphile [1]. LPS forms an asymmetric bilayer with phospholipids such as phosphatidylethanolamine on the cell surface [2]. The outer membrane acts as an impermeable barrier against large hydrophobic molecules, owing to its highly ordered molecular assembly, and protects the cell from or adapts it to environmental changes [3,4]. LPS exhibits many biological functions: mitogen activity, Shwartzman reaction, endotoxicity and others [5]. Many of these activities are characterized by the accumulation of LPS molecules [6]. The accumulated LPS can undergo thermally driven transitions due to changes in molecular packing of the fatty acid groups [7].

The investigation of the organization of LPS is important from the physiological and physicochemical points of view [8]. Research on the thermal properties of LPS aggregates has revealed thermotropic phase behavior with a gel–liquid crystalline phase transition when dealing with smooth or rough form LPS [9,10] and lipid A related compounds [11]. The phase transition property depends on the LPS molecular structure, which differs not only according to the origin of the organism, but also within the same strain of bacteria [12–14]. Divalent and monovalent metal cations are bound to the cell surface of Gram-negative bacteria [4,15]. These metal ions have been postulated to alter the physiological properties and membrane functions of LPS aggregates [16,17]. In this respect, research on the effects of chemical components on the phase properties and thin film formation is important for understanding the biological and chemical functions of LPS. However, the effects of metal ions and solution conditions on the phase properties of LPS aggregates remains obscure. An investigation into the phase transition properties is necessary in order to understand the functions of this supramolecular aggregate. In this work, phase behavior and thin film formation properties of the rough form LPS from *Erwinia carotovora* have been studied by high sensitivity differential scanning calorimetry.

2. Materials and methods

2.1. LPS preparation

Erwinia carotovora FERM P-7576 was cultured in a liquid medium by a two-stage cultivation method, and extracellularly produced rough LPS (R-LPS) was isolated from the cell wall blebs by phenol/chloroform/petroleum ether extraction and purified by ultracentrifugation as reported previously [18,19].

2.2. Fluorescence microscopy

The dried LPS was dispersed in water with the aid of vortex mixing and sonication. Spherical particles were formed by the addition of up to 25% ethanol to the aqueous

LPS solution at ambient temperature. Fluorescence microphotographs were obtained by using 3,6-Bis(dimethylamino)-10-dodecylacridinium bromide as the chromophore.

2.3. Preparation of dephosphorylated LPS

Dephosphorylated LPS was prepared by the method described previously [20]. LPS (200 mg) was suspended in 46% aqueous hydrofluoric acid and agitated for 120 h at 4°C. The solution was dialyzed against distilled water in a dialysis tube and lyophilized. This hydrolysis technique enables the elimination of phosphate groups without cleavage of the ketosidic linkage of 3-deoxy-D-*manno*-octulosonic acid (KDO) and lipid A. The phosphorus content of the dephosphorylated preparation was 0.5% (20% of the starting material [18] by the colorimetric assay method [21].

2.4. Differential scanning calorimetry

Calorimetric scans were performed with an MC-2 differential scanning calorimeter (Microcal Inc., Northampton, USA) with a sample cell volume of 1.258 cm^3. An appropriate amount of the metal chloride solution or water was added to aliquots of the LPS solution. The concentration of LPS was adjusted to 5.0 mg cm^{-3}. The sample solutions were agitated and sonicated, then degassed using an aspirator for 10 min at ambient temperature. DSC curves were obtained at a heating scan rate of 0.75°C min^{-1} from 5 to 50°C under an applied pressure of 1.5×10^5 Pa of nitrogen. The scans were repeated 10 times with each sample after the sample had been cooled to a particular temperature in the calorimeter. The reference cell was filled with the same solution without the LPS. The pH of the LPS solution was controlled by addition of HCl or NaOH solution. Measurement of pH was achieved under vigorous agitation of the LPS solution using a pH electrode. The pH of the *E. carotovora* LPS dispersed in pure water was pH 9.3.

3. Results

3.1. Fluorescence microscopy

The LPS when dispersed in the water gave opalescent solutions; because the LPS was not thoroughly dissolved, flake-like objects were observed in the photomicrograph (Fig. 1A). When ethanol or a divalent metal chloride was added, a precipitate appeared in the solution. The LPS solution formed a turbid suspension on the addition of HCl or NaCl. As shown in Fig. 1B, the addition of ethanol to the LPS solution followed by agitation altered the physical shape of the aggregate, resulting in the formation of vesicle-like spherical particles a few to several μm in diameter. Such spherical particles were not visible in the dispersed LPS solution.

3.2. Effects of solution condition on phase properties of LPS

The excess heat capacity function of the LPS at different pH values is illustrated in Fig. 2. All data were obtained from the final scan of each series of 10 measurements. The

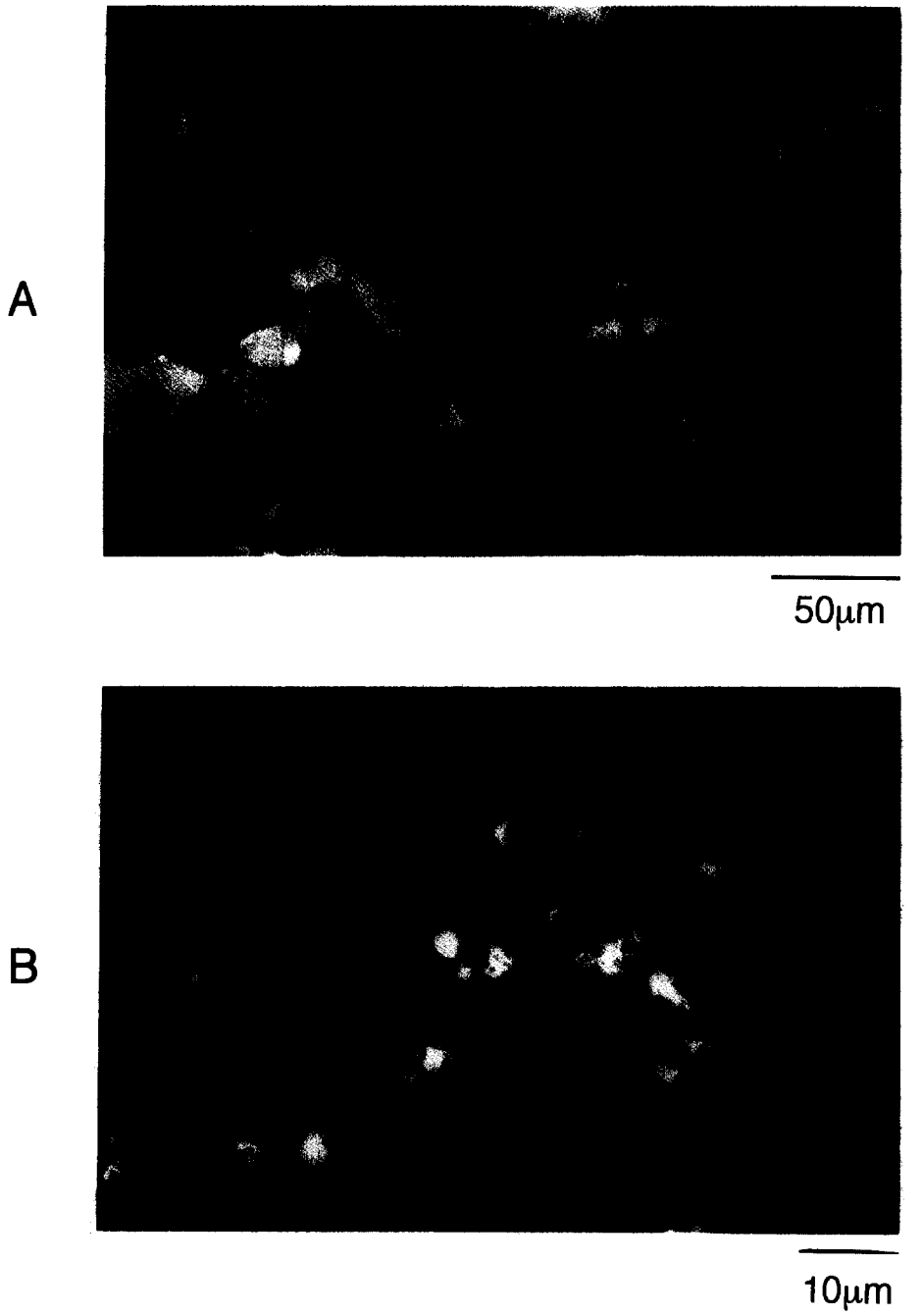

Fig. 1. Fluorescence microscopy of *Erwinia carotovora* R-LPS. A, LPS dispersed in pure water; B, 25% ethanol solution.

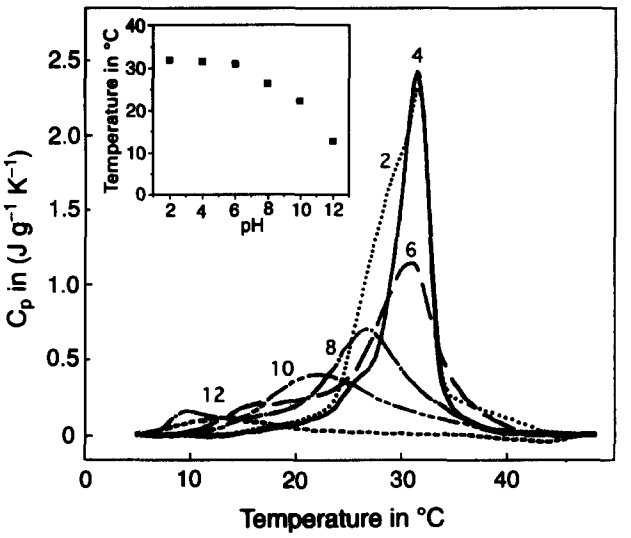

Fig. 2. DSC thermograms for phase properties of LPS as a function of pH value of the aqueous solution. Inset: t_m difference against pH value.

enthalpy change ΔH was associated with the phase transition from the gel to the liquid crystal phase. The ΔH value decreased with increasing pH value. In a pH 2 solution the LPS showed ΔH up to 15.4 J g^{-1} with a transition temperature t_m of 31.5°C. At pH 4 the ΔH value was reduced to 11.3 J g^{-1}, but the t_m value was similar to that at pH 2. In an alkaline solution at pH 12, the ΔH was reduced markedly to 1.2 J g^{-1}. As the phosphate and carboxylic groups are dissociated in alkaline solution, electrostatic repulsion of these charged groups between the LPS molecules reduced molecular aggregation and thus decreased phase transition energy.

As listed in Table 1, in NaCl solution the ΔH value increased with increasing concentration. The ΔH value was increased up to 2.5 times in 1.0 M NaCl solution

Table 1
Effect of NaCl concentration on enthalpy change ΔH associated with the gel-to-liquid crystal phase transition for *E. carotovora* R-LPS

NaCl/mM	t_m/°C	Peak width at half height/°C	ΔH/J g^{-1}
10	26.3	8.6	5.5
30	25.8	8.0	6.1
50	26.7	7.7	7.5
100	26.4	7.1	8.2
200	25.7	6.1	9.2
500	26.0	3.3	10.8
1000	27.0	3.1	12.5

compared with pure water. On the other hand, NaCl indicated little effect on the t_m value. The peak width at half height of the phase transition curves was decreased by the addition of NaCl to increase the ionic strength. In 1.0 M NaCl solution the peak width was 3.1°C, whereas in pure water the peak width was 8.6 °C. The high peak width in pure water is due mainly to structural heterogeneity and electrostatic repulsion of the phosphate groups. By increasing the ionic strength of the solution, Na^+ ion is accumulated around the negatively charged phosphate and carboxylic acid, then the negative charge in neutralized, increasing ΔH and improving molecular organization.

3.3. *Effects of multivalent metal chloride on phase properties of the natural LPS*

The thermotropic behavior of the LPS dispersions (5.0 mg cm^{-3}) in $MgCl_2$ solutions of different concentrations is plotted in Fig. 3. In the presence of $MgCl_2$, the ΔH value increased markedly: in 2.0 mM solution ΔH was approximately twice that in pure water, and in 4.0 mM solution the ΔH was 14.1 J g^{-1}. The t_m value varied much more in the presence of divalent metal ions than in the NaCl solution. Above 3.0 mM concentration two major peaks were observed around 13 and 23°C, and in the 4.0 mM solution the ΔH values of the peaks were 6.5 and 7.6 J g^{-1}, respectively. In 2.0 mM $CaCl_2$ solution, the LPS showed an increase of ΔH to 13.6 J g^{-1} and an upward shift of t_m (Fig. 4). The thermograms in $CaCl_2$ solution at 3.0 mM concentration showed variable multiple transition peaks. In solutions of 4.0 mM or higher concentration, broad thermotropic peaks were observed, and ΔH fell to 4.9 J g^{-1} in the 5.0 mM $CaCl_2$ solution. This value was lower than that in pure water. In heavy metal chloride solutions, such as manganese chloride, LPS exhibited peak separation in a manner different from that in the presence of alkaline earths (data not shown). The organization

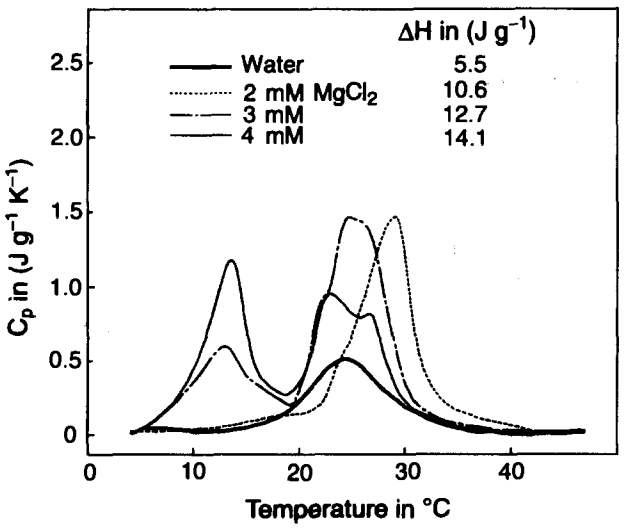

Fig. 3. Phase properties of LPS as a function of $MgCl_2$ concentration.

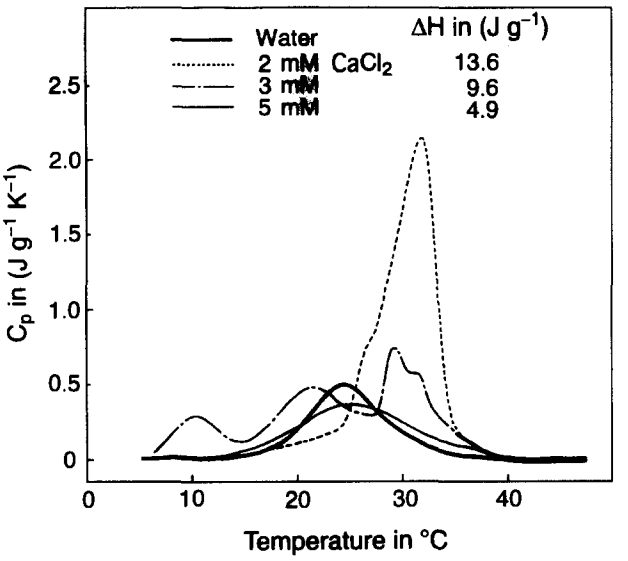

Fig. 4. Phase properties of LPS as a function of $CaCl_2$ concentration.

of LPS to vary the phase properties was directed by various metal ions. This difference can be interpreted as being due to the electrostatic repulsion of the phosphate and carboxyl groups.

3.4. *Phase properties of dephosphorylated LPS*

The heat capacity as a function of temperature for hydrofluoric acid treated LPS studied is shown in Fig. 5. Dephosphorylation caused the DSC curve of the LPS to

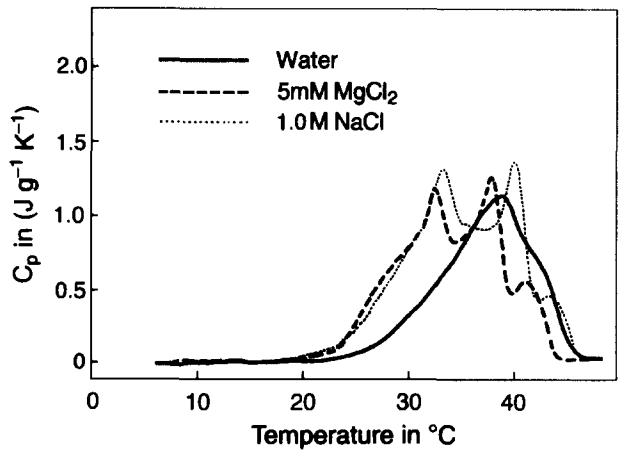

Fig. 5. Phase properties of dephosphorylated LPS in pure water, 1.0 M NaCl and 5.0 mM $MgCl_2$ solution.

vary; the t_m was shifted to 40°C and ΔH was up to 15 J g^{-1} in pure water. In the NaCl solution no multiple peaks were observed for the natural LPS. Monovalent and divalent metal chloride solutions produced similar DSC curves for the dephosphorylated LPS preparation. In such solutions the ΔH value was improved compared with pure water, however, the value was not so great. The shapes of the DSC curves in 1.0 M NaCl and 5.0 mM $MgCl_2$ solutions resembled one another, and the ΔH value was no larger than that of the natural LPS. The induced membrane behavior was different from that of the natural LPS, in which multiple peaks are supposedly caused by the carboxyl moieties of KDO and the phosphate groups not removed by hydrofluoric acid.

4. Discussion

Organization of the rough form LPS from *E. carotovora* provided spherical particles a few to several μm in diameter in the aqueous solution. Addition of ethanol or divalent metal chloride markedly altered the physical shape of the LPS aggregates. The vesicles made visible by fluorescence microscopy were larger than either the cell wall blebs excreted by *E. carotovora* [19] or the LPS aggregates from other Gram-negative bacteria [22–24]. *Salmonella minnesota* Rd LPS forms round-shaped vesicles in the presence of $MgCl_2$ [25]. The diameter of those vesicles is up to 10 μm, similar to that of *E. carotovora* R-LPS. As the size of the hydrophobic and the hydrophilic moiety of the amphiphile affect the shape of supramolecular organization [26], the molecular shape of *E. carotovora* R-LPS must be close to that of *S. minnesota* Rd-LPS.

The phase transition temperature of *E. carotovora* LPS in pure water was 24.5°C, lower than that of rough mutants of the other enterobacteria [10, 27, 28]. The enthalpy change is considered to indicate the $\beta \rightarrow \alpha$ acyl chain melting of the hydrocarbon chains. On addition of up to 2.0 mM $MgCl_2$ the LPS solution showed marked upward shifts of t_m and ΔH. The electrostatic interaction of LPS aggregates with cationic protein is reduced in the presence of Mg^{2+} or Ca^{2+} [29]. This tendency indicates that the negative charge of the LPS is masked by these divalent metal ions at 2 molar equivalent concentration. The effect of divalent metal ions on the phase property of the LPS was dependent on its concentration; different properties were observed below or above 3.0 mM ($\approx$ 2 molar equivalent per LPS molecule). In 4.0 mM $MgCl_2$ solution the LPS showed two major peaks of approximately equal ΔH value. This suggests that the new peak appearing at $\approx$ 13°C in the presence of $MgCl_2$ is caused by the excess of divalent metal ions. An increase of $MgCl_2$ concentration provided the same endothermic curves. These phenomena could be attributed to the chemical heterogeneity frequently observed for LPS samples, e.g. varying phosphate substitution. In $CaCl_2$ solution different phase behavior was observed. Multiple peaks were induced in 3.0 mM $CaCl_2$ by repeating measurements, but LPS provided a broad single peak of low ΔH value in 4.0 mM or more $CaCl_2$ solution. These ionotropic phase properties could be ascribed to the different nature of Mg^{2+} and Ca^{2+} in formation of a divalent metal ion/LPS complex in the LPS organization.

As is seen in Fig. 2, an increase in pH up to 6 causes a reduction of ΔH values, but the t_m value remains at much the same level. In alkaline solution both t_m and the ΔH value are reduced. This trend is attributable to the electrostatic repulsion of the negatively charged phosphate and carboxyl groups in the alkaline solution, in accordance with the results obtained using lipid A [10,28]. In acidic solution the peak width at half height became small: in the pH 4 solution the value was 3.4°C but the value rose to 5.9°C in the pH 2 solution. In the alkaline solution (pH 12), electrostatic repulsion suppressed the organization of the LPS to $\approx 10\%$ of the ΔH value in pure water. These data suggest the existence of a relationship between the membrane structure and the degree of ionization. The phase behavior of the LPS in solutions at various pH values was quite different from that found in a previous study [30]. In that report, *Escherichia coli* LPS showed its maximum ΔH at pH ≈ 7.5. The difference from this study could be attributed to the difference in molecular structure and chemical heterogeneity.

The phosphate groups are linked at the 1 and 4′ positions of the lipid A component, and KDO is linked at the 6′ position [31,32]. The major part of the divalent cations is postulated as being incorporated into lipid A and carbohydrate binding regions that have an influence on the three-dimensional structure of the lipid assembly within the membrane. As the dephosphorylated LPS exhibited similar thermotropic curves in NaCl or $MgCl_2$ solution, the new peak of natural LPS at ≈ 13°C in the presence of excess of divalent metal ions may result from not only the effect of masking from the negative charge of the phosphate and carboxylic acid groups but also such other effects as intermolecular cross-linkage through metal ions.

These phase transition properties of natural LPS at different pH values and in NaCl solution resemble those of phosphatidylglycerol, which is acidic because of the phosphate group ($pK_a \approx 2.9$) [33]. The protonation of negatively charged moieties enhanced the packing of the LPS molecules. However, the phase properties in the presence of divalent cations such as Mg^{2+} and Ca^{2+} were different [34].

After reducing the electrostatic repulsion of the negative charge of the phosphate groups of the LPS by treatment with hydrofluoric acid, the modified LPS preparation showed a shift of ΔH to $15\,J\,g^{-1}$ in water, a value roughly equivalent to or greater than that obtained for the natural LPS in 5.0 mM $MgCl_2$, 1.0 M NaCl and pH 4 solutions. This indicates that the reduction of the electrostatic repulsion by dephosphorylation has a phase transition energy level close to that obtained through masking of the charged groups by a divalent metal ion, protonation of the negative groups in acidic solution, or the ionic effect of high NaCl concentrations on natural LPS.

Bacterial cell surface bound metal ions such as Mg^{2+} and Ca^{2+}, and also protonation, markedly increased the ΔH value and t_m of the LPS aggregates, and excessive levels of Mg^{2+} ion induced peak separation. These features are considered to be mediated by the interaction of the divalent metal ions with the phosphate and carboxyl groups of LPS. On the basis of the phase properties, the LPS membrane is more susceptible to the chemical and physical conditions of the environment than are other amphiphiles.

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