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DISTORTION OF THE LAMELLAR ARRANGEMENT OF PHOSPHOLIPIDS BY DEEP ROUGH MUTANT LIPOPOLYSACCHARIDE FROM *SALMONELLA MINNESOTA*

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The concentration dependent effects of deep rough mutant lipopolysaccharide (LPS) from *Salmonella minnesota* (R595) on two different phospholipid model membranes was investigated by differential scanning calorimetry and small-angle X-ray scattering (SAXS). At low concentrations of LPS the well ordered multilamellar arrangement of dipalmitoylphosphatidylcholine (DPPC) vesicles is strongly distorted resulting in a loss of positional correlation of the lipid lamellae and smaller domain sizes within the lamellae. The pre-transition of DPPC was abolished at a LPS/DPPC molar ratio of 0.1:1 and the main or chain melting transition was strongly broadened. Moreover, the enthalpy was significantly decreased and a transition was hardly detected at an equimolar mixture of LPS/DPPC. LPS also affected the lamellar arrangement of a mixture of dipalmitoylphosphatidylethanolamine (DPPE) and dipalmitoylphosphatidylglycerol (DPPG). Furthermore, a phase separation was observed for this phospholipid mixture resulting in DPPE enriched and depleted domains. Similarly to DPPC, only a weak phase transition was observed at the highest LPS concentration used (LPS/DPPE-DPPG 1:1 mol/mol). SAXS measurements showed that for both systems increasing the concentration of LPS resulted in a concomitant increase of the formation of cubic structures, which are predominant at an equimolar mixture of LPS/phospholipid. However, because of the small number of peaks it was not possible to unambiguously identify the space group of the cubic structure, complicated by the coexistence with a lamellar phase, which was particularly detectable for the LPS/DPPC mixture.

Keywords: *calorimetry, cubic structures, lipid mixtures, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, X-ray scattering*

Introduction

Lipopolysaccharides (LPS) are major lipid components of the outer membrane of Gram-negative bacteria, located only in the outer leaflet of this membrane [1]. Chemically the LPS molecule consists of two covalently linked main parts, a poly- or oligosaccharide moiety with various length in different bacteria and a hydrophobic lipid-A segment referred to as the 'endotoxin principle' [2, 3]. LPS molecules, in particular their sugar moieties, are responsible for the effective permeability barrier function against invading molecules and their lipid moiety (lipid A) for a variety of biological functions [4]. LPS may be released from the bacteria into the surrounding medium (e.g. blood circulation) and their aggregates have been shown to have relevant dose-dependent biological effects in the human host organism. While at low concentration this may be beneficial, high concentrations of LPS are pathophysiological and may lead to sepsis, septic shock, and consequently to multi-organ failure [5]. LPS molecules can modify the immune response either by binding to specific membrane receptors or in a non-specific way by interacting with the host cell membrane [6]. These toxic effects are supposed to originate from their intercalation into the phospholipid bilayers of these membranes.

The lyotropic behaviour and aggregate structure of LPS itself is complex. It depends on the number and composition of the fatty acyl chains as well as on their amphipatic nature resulting from the chemical structure of the sugar molecule [7, 8]. The physico-chemical properties of natural LPS molecules as well as lipid-A analogs in the absence and presence of divalent cations as a function of temperature and/or water content have been investigated [9, 10]. These studies showed that minimal structural changes in the LPS molecule or any changes in the ionic strength of the medium may cause drastic changes in their biological effects. In the present work, the effects of LPS prepared from *Salmonella minnesota* R595 upon the thermotropic and structural behaviour of phospholipid model membranes were investigated. Thus liposomes are widely used as model systems for the more complex biological cell membrane,

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made from different lipids and water molecules [11–14]. Thereby, dipalmitoylphosphatidylcholine (DPPC) was used as model for mammalian cytoplasmic cell membranes, whereas a mixture of dipalmitoylphosphatidylethanolamine and dipalmitoylphosphatidylglycerol (DPPE/DPPG) was used as bacterial cytoplasmic model membrane according to their main phospholipid components [15–17].

Pure DPPC liposomes exhibit two-phase transitions; the pre-transition (~ 35°C) characteristic for the transformation from the lamellar ($L_{\beta'}$) gel to the rippled ($P_{\beta'}$) gel phase and the main transition (~ 41°C), between the rippled gel and the liquid-crystalline (L_α) phase [18, 19]. The pre-transition exhibits a weak first order character. Therefore a strong perturbation can be expected by addition of any foreign molecule [20, 21]. The thermotropic behaviour of the DPPE/DPPG system was already studied thoroughly in the whole composition range as a function of pH and ionic strength [16, 22]. A mixture of DPPE/DPPG (80/20 mol/mol) was used in this study, mimicking the composition of the inner membrane of e.g. *Escherichia coli*, a typical Gram-negative bacteria. This phospholipid mixture undergoes a main transition around 61°C. Depending on the ionic strength of the aqueous medium this mixture exhibits a diffuse scattering in the SAXS pattern in the gel phase owing to the low number of positionally correlated bilayers. Moreover, this system can transform upon moving through the main transition into a state, where there are no positional correlations anymore between the adjacent bilayers. This is called a thermal unbinding transition and has been described theoretically [23, 24] and experimentally for the present system [25]. We show that addition of LPS drastically changes the heat capacity profile as well as the SAXS pattern of both DPPC and the DPPE/DPPG mixture. However, the complex scattering pattern observed for the LPS/phospholipid mixtures did not allow an unambiguous phase assignment, but indicate the coexistence of lamellar and cubic structures over a wide composition range.

Experimental

Materials and methods

Synthetic 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG, Na-salt) (purity>99%) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Ala, USA). Deep rough mutant LPS (Re-LPS) from *Salmonella minnesota* strain R595 was extracted from the bacteria by the phenol/chloroform/petrolether method, purified and used in its natural salt form [26]. The purity of LPS was checked by gas chromatography (GC) applying inositol standard procedures [27–29].

Appropriate amounts of phospholipid and lipopolysaccharide were dissolved in a mixture of chloroform and methanol (2:1 *v*:*v*). The solution was then evaporated at 40°C and the resulting lipid film was kept in vacuum to remove residual traces of solvent. Sodium phosphate buffer (10 mM, pH 7.4), prepared from de-ionized, triple-quartz-distilled water, was added to the dried lipid film to yield a lipid concentration of 20 mass%. The sample was heated above T_m (main phase transition temperature) of the respective phospholipids, cooled to 5°C, reheated again above T_m and vortexed intensively. This procedure was repeated 20 times to achieve homogeneous dispersions. In order to calculate the LPS/phospholipid molar ratio an approximated average LPS molar mass of 2500 was used. The studied LPS/phospholipid molar ratios was in the range from 0.01:1 to 1:1 mol/mol.

DSC measurements were performed with a DSC 2920 instrument (TA Instruments, US) with a heating rate of 1°C min^{-1}. The reference cell was empty. The calibration of the calorimeter was carried out by using a pure indium sample (T_{onset}=156.6°C). The individual asymmetric components of the complex DSC curves were calculated by using the commercial mathematical program Origin 7.5 applying the following equation:

$$y = y_0 + A\left(1+e^{-\frac{x-x_c+w_1/2}{w_2}}\right)^{-1}\left[1-\left(1+e^{-\frac{x-x_c+w_1/2}{w_3}}\right)^{-1}\right]$$

where A is the amplitude, x_c is the center, w_1 is the distance of the two terms and w_2, w_3 are the width of each term.

SAXS measurements were performed using a Kratky Compact camera (Anton Paar, Graz, Austria) supplied with a position sensitive detector (M. Braun, Garching, Germany). The scattering of Ni-filtered CuK_α radiation (λ=1.542 Å) was recorded in the 10^{-3} to 10^{-1} Å^{-1} range of the scattering variable, defined as $s=2\sin\Theta/\lambda$, where 2Θ is the scattering angle. The primary beam was line focused. Thus the intensity curves were corrected considering the geometry of the beam profile [30]. For the X-ray measurements the samples were transferred into thin walled capillaries (Hilderberg, Germany) with a diameter of 1 mm. After centrifugation to remove air bubbles the capillaries were sealed with a two-component synthetic resin and transferred into metal capillary holders placed into an aluminium block. This block was positioned directly into the beamline and was used as a thermal incubator for controlled annealing at different temperatures. The SAXS measurements were performed at selected temperatures.

Results and discussion

Effects of Re-LPS on DPPC liposomes

The deep rough mutant LPS induces concentration dependent changes in the curve of DPPC multilamellar vesicles as shown in Fig. 1, while the thermodynamic data are summarized in Table 1. Both the pre- (35.3°C for pure DPPC) and main transition (40.7°C for pure DPPC) are affected by the addition of LPS. At low concentrations of Re-LPS both transitions are broadened and the pre-transition is abolished at a LPS/DPPC molar ratio of 0.1:1. Increasing the concentration of LPS results in a continuous broadening of the main transition or chain melting transition with a strong tailing towards the low temperature side indicating the onset of chain melting of LPS, the low melting component. Furthermore, a strong decrease of the enthalpy was also observed without affecting the main transition temperature (T_m). Only at the highest concentration of LPS studied (LPS/DPPC 1:1 mol/mol) T_m is slightly, but significantly shifted to higher values (41.3°C). Moreover, the transition enthalpy is reduced drastically.

The small angle X-ray scattering patterns of the systems are also significantly affected depending on the LPS content. In Fig. 2, a series of measurements in the temperature range of the gel and liquid-crystalline phase (26 and 45°C, respectively) is presented. The SAXS patterns of the pure DPPC system exhibit sharp Bragg peaks typical for the multilamellar arrangement in both phases [31, 32]. The first order Bragg peak appears at 0.0156 and at 0.0147 $Å^{-1}$ for the gel and the liquid-crystalline phase, respectively. These characteristic Bragg peaks are abundant also upon addition of LPS (0.1:1 LPS/phospholipid ratio) indicating that the multilamellar arrangement is still present in both phases. Notably, the Bragg peaks shifted towards lower scattering angles, indicating larger periodicities of the multilamellar lattice. Moreover, they are slightly broadened and exhibit a lower scattering peak intensity. While the decrease of the intensity of the Bragg peaks

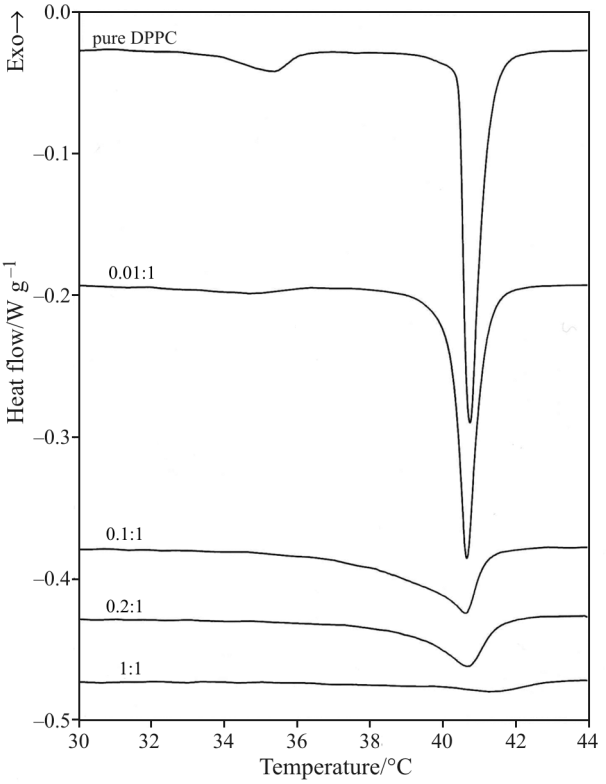

Fig. 1 DSC curves of the LPS/DPPC model systems in 10 mM sodium-phosphate buffer, pH 7.4 (molar ratio indicated in the panel)

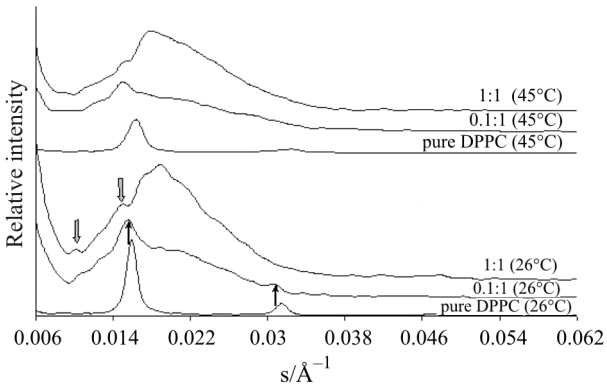

Fig. 2 SAXS patterns of the LPS/DPPC model systems in 10 mM sodium-phosphate buffer, pH 7.4, at 26 and 45°C (molar ratio indicated in the panel). The first and second order of the Bragg reflection are marked by bold arrows for the LPS/DPPC 0.1:1 molar ratio at 26°C. The dashed arrows indicate two reflections in the LPS/DPPC 1:1 mol/mol model system that may be related to a cubic phase

Table 1 Thermodynamic parameters of the pre- and main transition of the LPS/DPPC model systems

	LPS/DPPC (LPS/lipid mol/mol)	T_m/°C±0.2	T_{os}/°C±0.2	ΔH/kJ mol^{-1} lipid±10%
Pre-transition	pure DPPC	35.3	33.5	0.97
	0.01:1	34.7	32.0	0.45
Main transition	pure DPPC	40.7	40.4	6.9
	0.01:1	40.6	40.2	6.4
	0.1:1	40.6	38.8	5.2
	0.2:1	40.7	38.9	3.8
	1:1	41.3	38.3	1.15

originates from the reduction of the total volume of the domains with multilamellar structure of high positional correlation, their broadened profiles are the consequence of the smaller domain-size. In addition, the Bragg peaks are superimposed on an intense broad scattering background extending from about 0.01 to 0.03 $Å^{-1}$. This scattering background is complex and suggests to be composed of a superposition of a number of Bragg peaks. At a 1:1 molar ratio of LPS/DPPC the Bragg peak arising from the multilamellar arrangement is hardly detectable being further shifted to lower scattering angle that indicates larger lamellar repeat distances. This can be explained by the incorporation of the charged LPS molecule into the DPPC bilayer resulting in electrostatic repulsion between the bilayers. In addition, a peak located at 0.0098 $Å^{-1}$ can be clearly discerned from the scattering profile. Closer inspection of the pattern reveals higher scattering harmonics of this Bragg peak that may be related to the existence of a cubic phase. Interestingly, this complex pattern does not depend strongly on the temperature as can be deduced from the similar SAXS pattern at 26 and 45°C. Although the detailed identification of the structure cannot be determined owing to the low resolution, it is obvious that the formation of the multilamellar structure is hindered by the incorporation of high amounts of LPS owing to the electrostatic repulsion caused by the charged LPS molecule as outlined above. The changes observed in the SAXS profiles are in good agreement with the DSC data. Thus the decrease of the main transition enthalpy upon increasing amounts of LPS reflects the reduced fraction of the multilamellar structure undergoing the gel to liquid-crystalline phase transition.

LPS effects on DPPE-DPPG liposomes

The pure DPPE-DPPG system shows only a main transition (Fig. 3). The characteristic data of the chain melting process, summarized in the Table 2, are in accordance with reported data in the case of the pure phospholipid system [16]. Again LPS exhibits significant effects depending on its concentration as can be deduced from the curves and their analysis (Tables 2 and 3). Already at a molar ratio of 0.01:1 LPS/DPPE-DPPG the transition is broadened and the peak profile indicates a splitting into two transitions. This splitting of the main transition into two components is evident at higher LPS content (LPS/DPPE-DPPG 0.02:1 as well as 0.1:1 mol/mol, respectively) with one component appearing at higher temperature. In addition both components are broadened with increasing LPS content. The DPPE/DPPG molar ratio (0.8) of the model system investigated is close to the critical ratio, where phase separation occurs resulting in domains being highly enriched in DPPE and in domains having a lower DPPE content. Therefore, the presence of small amounts of LPS may induce such a phase separation. With increasing concentration of LPS the two peaks become more clearly separated being due to a decrease of the phase transition temperature of the low melting component and an increase of the phase transition temperature of the high melting component, respectively.

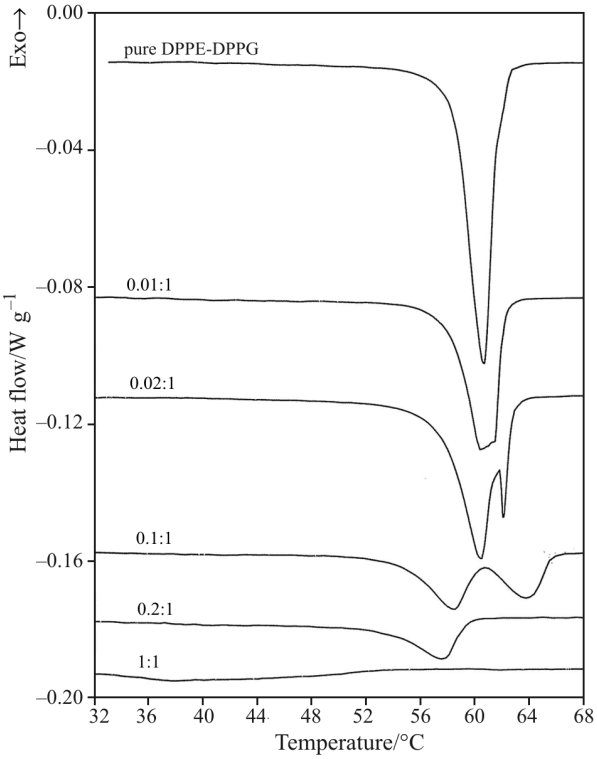

Fig. 3 DSC curves of the LPS/DPPE-DPPG model systems in 10 mM sodium-phosphate buffer, pH 7.4 (molar ratio indicated in the panel)

Table 2 Thermodynamic parameters of the main transition of the LPS/DPPE-DPPG model systems

LPS/DPPE-DPPG (LPS/lipid mol/mol)	T_m/°C±0.2	T_{os}/°C±0.2	ΔH/kJ mol^{-1} lipid±10%
pure DPPE-DPPG	60.5	58.2	39.5
0.01:1	60.5, 61.5*	58.4	27.1
0.02:1	60.4, 62.0*	57.4	33.2
0.1:1	58.0, 63.5*	54.4	11.0, 8.1**
0.2:1	57.6	25.6	10.2
1:1	38.0	32.0	1.9

Table 3 Calculated transition temperatures and enthalpies for the complex curves of LPS/DPPE-DPPG mixtures

LPS/DPPE-DPPG (LPS/lipid mol/mol)	1. peak		2. peak	
	T_m/°C±0.2	ΔH/kJ mol^{-1} lipid±10%	T_m/°C±0.2	ΔH/kJ mol^{-1} lipid±10%
0.01:1	60.4	23.7	61.5	3.4
0.02:1	60.4	24.7	62.1	7.5
0.1:1	58.6	10.0	63.3	7.0

Finally, at a molar ratio of 0.2:1 LPS/DPPE-DPPG only a single phase transition (T_m=57.6°C) can be observed. Generally, a decrease of enthalpy is observed with increasing LPS content, whereby at an equimolar ratio of LPS and DPPE-DPPG a drastic reduction of the transition enthalpy as well as a marked decrease of the transition temperature is observed (Table 2). It should be noted that the value of 38°C observed for this mixture is in the range of pure-LPS that undergoes a gel to liquid-crystalline phase transition around physiological temperature.

The complex heat capacity profiles at lower LPS content were considered as the sum of two peaks and analyzed as described in the Method section. The best fit was obtained by using asymmetric peak profiles as shown in Fig. 4. The result of the data analysis for the LPS/DPPE-DPPG system ranging from 0.01:1 to 0.1:1 mol/mol are summarized in Table 3. The temperature of the first transition decreased from 60.4 to 58.6°C, while the temperature of the second transition was shifted from 61.5 to 63.3°C. Latter is close to the value of pure DPPE. Concomitantly, the enthalpy of the first transition decreased from about 24 to ~10 kJ mol^{-1}, while that of the second transition increased from 3 to 7 kJ mol^{-1}. Thus increasing the amount of LPS resulted in a larger fraction of highly enriched DPPE domains.

The scattering profiles of these systems were measured at two characteristic temperatures at 40°C (30°C in the case of LPS/DPPE-DPPG 1:1 mol/mol) and at 70°C corresponding to the gel and liquid-crystalline phase of the pure DPPE-DPPG system. The characteristic patterns detected in the temperature region of the gel phase are shown in Fig. 5. Thereby, DPPE-DPPG without LPS exhibits a scattering profile composed of several broad Bragg reflections that can be related to an oligolamellar structure [25]. The shape of the SAXS pattern changes continuously with increasing LPS concentration. At a molar ratio of 0.1:1 LPS/DPPE-DPPG a characteristic Bragg peak with a shoulder at higher scattering angle appears, which is more expressed at the highest LPS concentration investigated, i.e. at 1:1 LPS/DPPE-DPPG molar ratio. Closer inspection of the complex SAXS patterns reveals that besides of this Bragg peak (0.0174 Å^{-1}) and its shoulder (0.0247 Å^{-1}) additional peaks can be detected, e.g. at 0.0358 and 0.0427 Å^{-1} (inset Fig. 5). The displacements of these peaks can be indexed as √2/a- , √4/a- , √9/a- and √10/a-fold of a periodicity of 81.3±0.8 Å suggesting the existence of a cubic

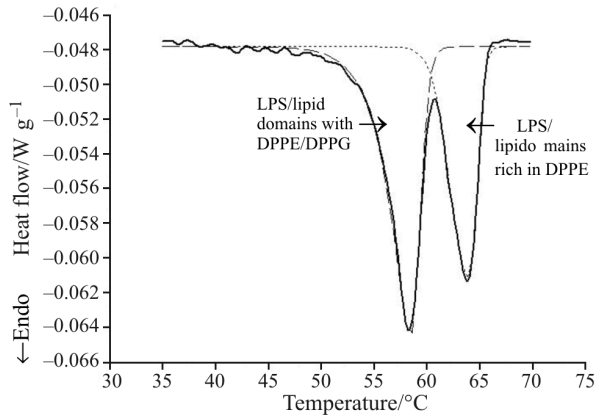

Fig. 4 Presentation of the separated DSC peaks for LPS/DPPE-DPPG 0.1:1 mol/mol; — – experimental curve and ···, --- – peak fits

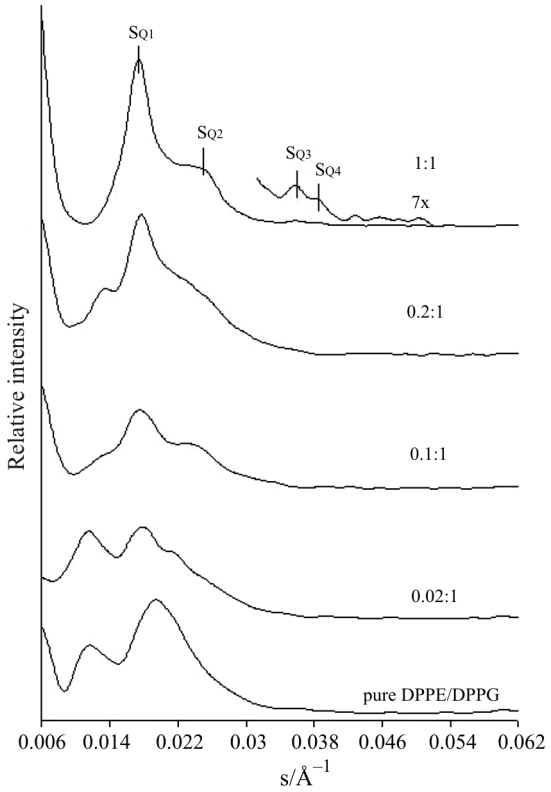

Fig. 5 SAXS patterns of the LPS/DPPE-DPPG model systems in 10 mM sodium-phosphate buffer, pH 7.4, at 40°C (molar ratio indicated in the panel). SAXS patterns for the equimolar lipid mixture was recorded at 30°C

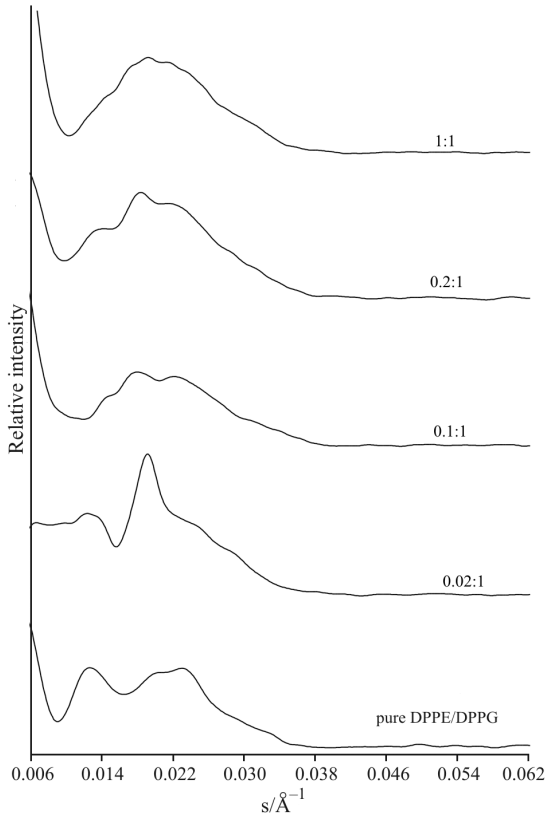

Fig. 6 SAXS patterns of the LPS/DPPE-DPPG model systems in 10 mM sodium-phosphate buffer, pH 7.4, at 70°C (molar ratio indicated in the panel)

phase. This lattice spacing is relatively small for a cubic phase. However, a range extending from about 80 to 140 Å was given for systems containing the same LPS [10, 33]. Latter become predominant upon increasing the LPS concentration, which is consistent with the observed strong decrease of the chain melting transition. At 70°C the lipid mixtures even exhibit more diffuse scattering patterns as can be seen in Fig. 6. This suggest that the oligolamellar structure being detected at temperatures below the phase transition becomes uncorrelated in the liquid-crystalline phase as reported earlier for DPPE/DPPG mixtures [25] and thus contributes to the diffuse scattering background. In contrast to the gel phase the appearance of a number of low intensity broad Bragg peaks do not allow an unambiguous determination of the existing structures. Nevertheless, there is strong evidence from these complex profiles that over a wide composition range lamellar and cubic structures coexist.

Conclusions

Addition of Re-LPS to either DPPC or DPPE/DPPG liposomes results in a distortion of the lamellar structure depending strongly on the LPS/phospholipid molar ratio, whereby in the low concentration regime the LPS molecules can be considered as a guest molecule. In the case of DPPC already small amounts of LPS prevent the formation of the rippled gel phase, as can be deduced from the loss of the pre-transition. Concomitantly, the marked broadening of the chain melting transition indicates a strong decrease of the cooperativity of this transition. This disordering effect is supported by the SAXS data, which also show a broadening of those Bragg peaks that correspond to the lamellar phase. In the same LPS concentration regime the main transition of the DPPE/DPPG mixture is also characterized by a loss of cooperativity, but more importantly LPS induces a phase separation resulting in domains being enriched and depleted in DPPE. However, at high concentrations of LPS, where this lipid cannot be considered anymore as a guest molecule, the structural arrangement seems to be dominated by the phase preference of this molecule. In both systems only a weak thermal transition was observed and accordingly the SAXS data indicate the predominant formation of a cubic structure over the whole temperature range investigated. Owing to the small number of peaks an unambiguous identification of the space group was not possible. Further investigations using Synchrotron radiation and rotating X-ray capillaries may be successful in the determination of this aggregational state. In this respect it should be noted that LPS (R595) by itself exhibits already a complex phase behaviour [10]. A coexistence of the lamellar gel and liquid-crystalline states with cubic phases was observed over a wide temperature range (30–50°C) also at high water content (80 mass%) and the presence of another cubic phase was suggested at higher temperatures.

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