Eur Biophys J (2003) 31: 633–636
DOI 10.1007/s00249-002-0256-9

BIOPHYSICS LETTER

Marcus Larsson · Kåre Larsson · Tommy Nylander
Per Wollmer

The bilayer melting transition in lung surfactant bilayers: the role of cholesterol

Received: 6 May 2002 / Revised: 15 August 2002 / Accepted: 6 September 2002 / Published online: 1 November 2002

Abstract Aqueous dispersions of a porcine lung surfactant (PLS) extract with and without cholesterol supplementation were analyzed by X-ray scattering. Lamellar liquid-crystalline and gel-type bilayer phases are formed, as in pure phosphatidylcholine (PC)-cholesterol systems. This PLS extract, developed for clinical applications, has a cholesterol content of less than 1% (w/w). Above the limit of swelling, the bilayer structure shows a melting (main) transition during heating at about 34 °C. When 13 mol% cholesterol was added to PLS, so that the cholesterol content of natural lung surfactant was reached, the X-ray scattering pattern showed pronounced changes. The main transition temperature was reduced to the range 20–25 °C, whereas according to earlier studies of disaturated PC-cholesterol bilayers in water the main transition remains almost constant when the amount of solubilized cholesterol is increased. Furthermore, the changes in scattering pattern at passing this transition in PLS-cholesterol samples were much smaller than at the same transition in PLS samples. These effects of cholesterol solubilization can be related to phase segregation within the bilayers, known from pure PC-cholesterol systems. One phase, solubilizing about 8 mol% cholesterol, exhibits a melting transition, whereas the other bilayer phase, with a liquid-crystalline disordered conformation, has a cholesterol content in the range 20–30 mol% and this phase shows no thermal transition. The relative amount of bilayer lipids that is transformed at the main transition in the PLS-cholesterol sample is therefore only half compared to that in PLS samples. The reduction in transition temperature in the segregated bilayer of lung surfactant lipids is probably an effect of enrichment of disaturated PC species in the phase, which is poor in cholesterol. This work indicates that cholesterol in lung surfactant regulates the crystallization behavior.

Keywords Lung surfactant · Alveolar surface lipids · Bilayer phase transition · Lipid bilayer crystallization · Cholesterol segregation

K. Larsson (✉) · T. Nylander
Department of Physical Chemistry 1 and
Camurus Lipid Research Foundation, Chemical Center,
Lund University, PO Box 124, 221 00 Lund, Sweden
E-mail: k.larsson@klchem.se
Fax: +46-46-294811

M. Larsson · P. Wollmer
Department of Clinical Physiology,
Lund University, 205 02 Malmö, Sweden

Introduction

The alveolar surfactant system covers the alveoli with a film of submicron thickness, with the main function to reduce the surface tension in the lung. Fast lateral transport into a uniform layer is required to account for the surface area changes induced by breathing. Lung surfactant is sometimes also transported upwards along the bronchial tree. The flow properties of the surfactant are therefore important, and a relevant question is whether thermal phase transitions, that can influence the fluidity, may take place under extreme physiological conditions. We have therefore performed X-ray scattering studies of a lung surfactant extract with and without cholesterol at temperatures between 5 and 42 °C. This porcine lung surfactant (PLS) extract was developed for applications in surfactant therapy and has recently been characterized with regard to aqueous interaction (Larsson et al. 2002a).

The alveolar surface film consists of lipid bilayers forming lamellar bodies (LBs) and tubular myelin (TM). The weight proportion of lipids to proteins in alveolar surfactant is about 10:1, with two hydrophilic proteins (SP-A and SP-D) and two hydrophobic proteins (SP-B and SP-C) embedded in the bilayer (cf. van Iwarden and van Golde 1995). About 80% of the phospholipids in lung surfactant are phosphatidylcholines (PCs), half of which is dipalmitoylphosphatidylcholine (DPPC) (Kahn et al. 1995).

Since the 1950s, the traditional model of the alveolar surface layer involves a surfactant monolayer on a water phase. Recent ultrastructural (Larsson et al. 2002b) and theoretical work (Kashchiev and Exerowa 2001) have challenged this view in favor of an organized surfactant bilayer in continuum with a monolayer towards air, forming a functional unit at the alveolar surface.

Cholesterol is known to strongly effect PC bilayer crystallization, and its role in the alveolar surface bilayer has been examined in this work. Lung surfactant lipids in humans have been found to contain 7.3% (w/w) cholesterol (Veldhuizen et al. 1998); another value reported earlier was 9% (Post et al. 1982). The concentration of 7.3% (w/w) cholesterol corresponds to 13.3 mol%, assuming that the rest of the lipids have an average molecular weight of 750. Orgeig and Daniels (2001) in a recent paper report that most mammals have 14–20 mol% cholesterol in their surfactant.

There are numerous reports on aqueous PC-cholesterol ternary systems, and their complex characters were mainly revealed by studies reported in the 1980s. An immiscibility gap between 8 and 20 mol% cholesterol in bilayers of PC-cholesterol in excess of water was found (Knoll et al. 1985). Furthermore, it was reported that the melting transition (the main transition) remained constant when up to 20 mol% cholesterol was solubilized in the bilayers (Recktenwald and McConnel 1981). An extensive neutron scattering study of bilayers with varying cholesterol content in dimyristoylphosphatidylcholine (DMPC) later showed the molecular organization of the segregating bilayer regions in the ripple bilayer phases formed at equilibrium (Mortensen et al. 1988). The structure was found to consist of cholesterol-poor stripes of crystalline bilayers alternating with stripes of disordered cholesterol-rich bilayers. When the total cholesterol concentration reached that of the cholesterol-rich stripes, the ripple character was observed to disappear.

Bilayers of DPPC-cholesterol in water were studied also by deuterium NMR spectroscopy by Vist and Davies (1990) and by ESR spectroscopy by Sankaram and Thompson (1991). Similar phase equilibria were reported in these studies, although different nomenclature was used. Sankaram and Thompson described a "cholesterol-induced fluid-phase immiscibility" above the main transition, the co-existence of two fluid (i.e. liquid-crystalline) lamellar phases. The cholesterol-rich phase was later termed Lα(O), as the hydrocarbon chains are considered to be more ordered than in the ordinary lamellar liquid-crystalline phase (cf. Sparr 2001).

The present work indicates that a similar phase separation is involved in the bilayer organization of lung surfactant. Ringer solution was used in the aqueous swelling experiments, to correspond to the physiological situation in the lung where calcium ions in particular play an important role in the organization of surfactant bilayers. The question of whether or not the rippled superstructures of the bilayer phases occur has not been considered in this work, as this would require equilibration of the samples for very long times with very small temperature variations (cf. Mortensen et al. 1988). In lung surfactant lipids it is mainly of interest to study transitions with kinetics corresponding to that in the breathing cycle.

Materials and methods

PLS obtained from Leo Pharmaceutical (Ballerup, Denmark) has been characterized earlier with regard to its aqueous swelling behavior (Larsson et al. 2002a). PLS containing about 2% (w/w) proteins (SP-B and SP-C) and 0.5–1% cholesterol was examined alone and mixed with cholesterol. The PLS-cholesterol sample was prepared by mixing PLS with 7% (w/w) cholesterol in chloroform solution in order to obtain molecular mixing. The PLS-cholesterol sample obtained after evaporation of the chloroform was then mixed with Ringer solution (40:60 in weight ratio). The corresponding PLS-Ringer solution sample without cholesterol was also examined (treated in the same way by suspension in a chloroform solution followed by chloroform evaporation).

X-ray data were recorded by a Kratky compact system equipped with a two-position sensitive detector, with 1024 channels of width 53.6 μm (OED 50 M from Mbraun, Graz, Austria). The small-angle detector was placed at distance of 27.7 cm from the sample, while the wide-angle detector was placed at an angle of 20.2° with a sample-to-detector distance of 29.7 cm. The Cu Kα nickel-filtered radiation of wavelength 1.542 Å was provided by a Seifert IF 300 X-ray generator operating at 50 kV and 40 mA. Temperature control of the sample within 0.1 °C was achieved by using a Peltier element.

The diffraction in the wide-angle and small-angle regions were recorded during heating, every 5 °C from 5 to 30 °C, then every 2 °C up to 42 °C.

Results

We have reported some X-ray data for PLS earlier (Larsson et al. 2002a). The X-ray scattering curves of the wide-angle and small-angle regions of PLS in Ringer solution (40:60) are shown in Figs. 1 and 2, respectively. The diffraction peak at 4.2 Å disappeared at about 34 °C.

The scattering patterns recorded from samples of PLS-cholesterol swollen in Ringer solution (40:60) are shown in Figs. 3 and 4. This cholesterol concentration corresponds to that in the alveolar surface bilayers. There are striking differences compared to the curves from PLS without cholesterol (Figs. 1 and 2), discussed below.

The reproducibility of the X-ray curves from different preparation batches was also checked.

Discussion

As shown in Fig. 1, the wide-angle scattering of PLS is dominated by a peak at 4.2 Å at 30 °C, whereas at 38 °C this peak has disappeared and only diffuse scattering is seen, characteristic for the liquid-crystalline conformation of the bilayer. It can therefore be concluded that PLS exhibits a melting transition, evident from the disappearance of the 4.2 Å peak (corresponding to the

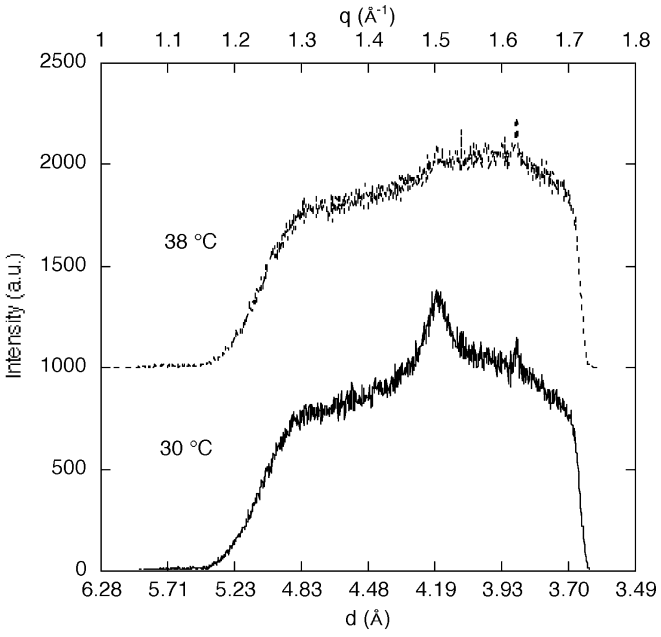

Fig. 1 X-ray wide-angle scattering curves of PLS-Ringer solution (40:60) at 30 °C (*bottom*) and 38 °C (*top*). The relative intensity is given versus spacing *d* (Å)

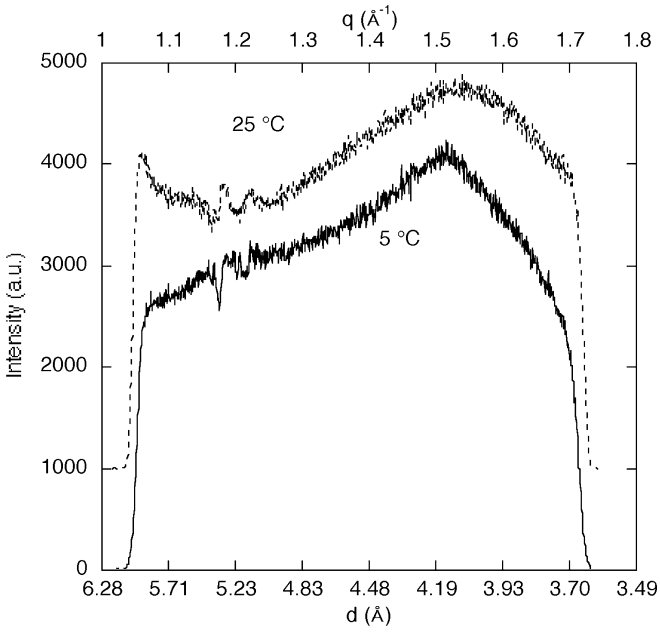

Fig. 3 X-ray wide-angle scattering curves of PLS/cholesterol-Ringer solution (40:60) at 5 °C (*bottom*) and 25 °C (*top*)

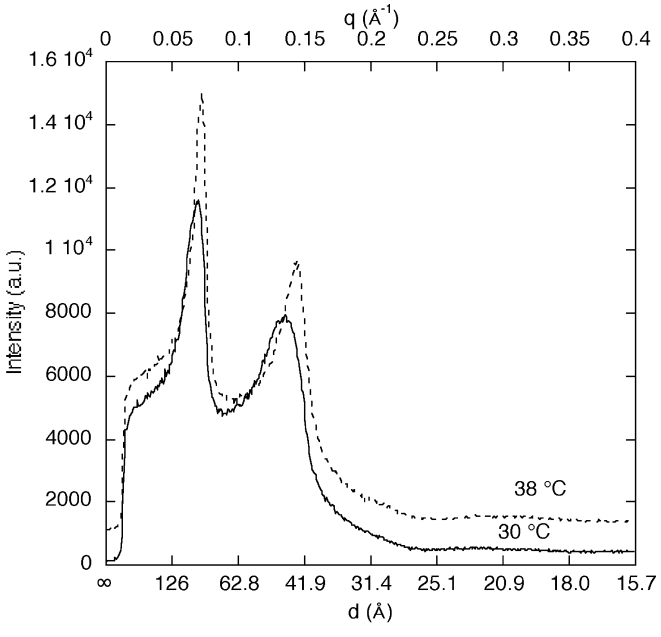

Fig. 2 X-ray small-angle diffraction of PLS-Ringer solution (40:60) at 30 °C (*full line*) and 38 °C (*dashed line*)

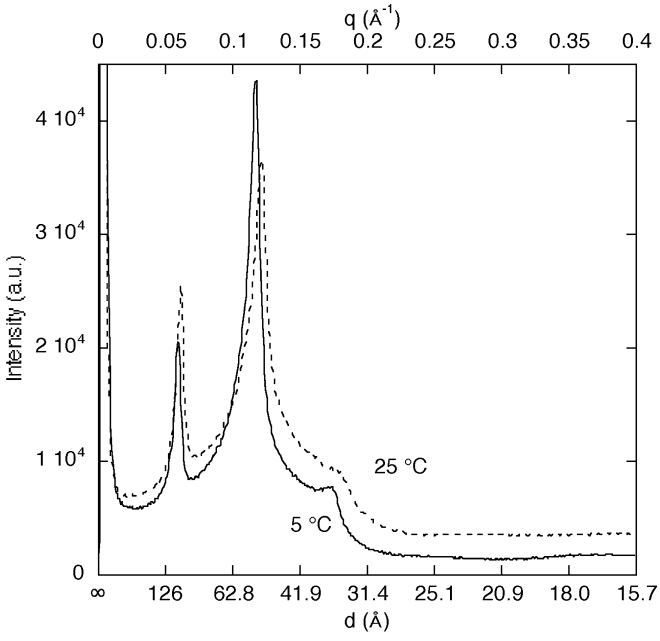

Fig. 4 X-ray small-angle diffraction of PLS/cholesterol-Ringer solution (40:60) at 5 °C (*full line*) and 25 °C (*dashed line*)

hexagonal hydrocarbon chain packing). The small-angle region seen in Fig. 2 shows diffraction peaks due to the lamellar repetition distance of the bilayers, with a small decrease at the main transition (87 Å at 38 °C and 92 Å at 30 °C). The intensity of the second-order peak in relation to the first-order peak is higher when the bilayer is crystalline, as should be expected as a result of a smaller temperature fall-off of diffraction intensities in the more ordered state of the bilayer.

X-ray scattering curves from PLS-cholesterol (with a cholesterol concentration corresponding to that in lung lavage) swollen in Ringer solution to a concentration of 40:60, the same as in the PLS samples, are shown in Figs. 3 and 4. Curves recorded every 5 °C from +5 °C indicate that the interval 20–25 °C is the melting transition interval, and scattering curves below and at the end of this transition range are shown.

In the wide-angle region (Fig. 3) a broad scattering maximum centered at 4.2 Å can be seen at 5 °C, and at higher temperatures the dominating scattering is further broadened. The intensity distribution in the wide-angle region results in a slightly concave curve (in relation to increasing intensity) at and below 20 °C and a convex curve at and above 25 °C. This probably reflects an increased contribution around 4.5 Å due to a transition into a liquid conformation of parts of the chains. The small-angle region (Fig. 4) shows a minor decrease in bilayer repetition distance at the transition: 107 Å at 5 °C (105 Å at 20 °C, not shown in Fig. 4) and 103 Å at 25 °C.

The most important differences between these scattering curves and the PLS curves (Figs. 1 and 2) is a pronounced reduction in the main transition temperature with cholesterol, which is contrary to prior knowledge from pure PC systems (Recktenwald and McConnel 1981).

The diffraction curves of PLS-cholesterol are consistent with phase segregation within the bilayer known from pure PC-cholesterol systems. In the DMPC-cholesterol system (Mortensen et al. 1988) the cholesterol-poor bilayer phase contains 7 mol% cholesterol and the cholesterol-rich phase 20 mol% cholesterol, whereas the corresponding values reported in DPPC-cholesterol bilayers are 7 and 30 mol% respectively (Sankaram and Thompson 1991). The PLS-cholesterol sample below the main transition should therefore be expected to contain about equal amounts (assuming that the cholesterol-rich phase contains 20 mol% cholesterol) of the crystalline bilayer phase and the liquid-crystalline bilayer phase. Therefore the disordered character of the X-ray scattering curves is not surprising.

The reduction in main transition temperature when cholesterol is solubilized in PLS bilayers is probably an effect of segregation of disaturated PC species to the cholesterol-poor bilayer phase. This will result in a reduction in transition temperature, as unsaturated PC species with lower transition temperature have become enriched.

References

Kahn MC, Anderson GJ, Anyan WR, Hall SB (1995) Phosphatidylcholine molecular species of calf lung surfactant. Am J Physiol 269:L567–L573

Kashchiev D, Exerowa D (2001) Structure and surface energy of the surfactant layer on the alveolar surface. Eur Biophys J 30:34–41

Knoll W, Schmidt G, Ibel K, Sackmann E (1985) Small-angle neutron scattering study of lateral phase separation in dimyristoylphosphatidylcholine-cholesterol mixed membranes. Biochemistry 24:5240–5246

Larsson M, Haitsma JJ, Lachmann B, Larsson K, Nylander T, Wollmer P (2002a) Enhanced efficacy of porcine lung surfactant extract by utilization of its aqueous swelling dynamics. Clin Physiol Funct Im 22:39–48

Larsson M, Larsson K, Wollmer P (2002b) The alveolar surface is lined by a coherent liquid-crystalline phase. Prog Colloid Polym Sci 120:28–34

Mortensen K, Pfeiffer W, Sackmann E, Knoll W (1988) Structural properties of a phosphatidylcholine-cholesterol system studied by small-angle neutron scattering: ripple structure and phase diagram. Biochim Biophys Acta 945:221–245

Orgeig S, Daniels CB (2001) The role of cholesterol in pulmonary surfactant: insight from comparative and evolutionary studies. Comp Biochem Physiol Mol Integr Physiol 129:75–89

Post M, Batenburg JJ, Schuurmans EA, Laros CD, van Golde LM (1982) Lamellar bodies isolated from human lung tissues. Exp Lung Res 3:17–28

Recktenwald DJ, McConnel HM (1981) Phase equilibria in binary mixtures of phosphatidylcholine and cholesterol. Biochemistry 20:4505–4510

Sankaram MB, Thompson TE (1991) Cholesterol-induced fluid-phase immiscibility in membranes. Proc Natl Acad Sci USA 88:8686–8690

Sparr E (2001) Responding model membranes: lipid phase behavior, domain formation and permeability. Thesis, University of Lund, Sweden

van Iwarden JF, van Golde LM (1995) Pulmonary surfactant and lung defense. In: Robertsson B, Taeusch HW (eds) Surfactant therapy for lung disease. Dekker, New York, pp 75–92

Veldhuizen R, Nag K, Orgeig S, Possmayer F (1998) The role of lipids in pulmonary surfactant. Biochim Biophys Acta 1408:90–108

Vist MR, Davies JH (1990) Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: ^{2}H nuclear magnetic resonance and differential scanning calorimetry. Biochemistry 29:451–464