Eur Biophys J (1996) 25: 61–65

BIOPHYSICS LETTER

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Solutes in small amounts provide for lipid-bilayer softness: cholesterol, short-chain lipids, and bola lipids

Received: 28 May 1996 / Accepted: 27 July 1996

Abstract The effect of the incorporation of small amounts (~1 mole%) of amphiphilic solutes, such as cholesterol, a short-chain lipid ($DC_{10}PC$), and a bola lipid, into multilamellar DMPC bilayers is studied by small-angle neutron scattering and differential-scanning calorimetry. The anomalous swelling behavior observed in the transition region of pure DMPC bilayers is interpreted as an indication of bilayer softening and thermally reduced bending rigidity. Small amounts of the solutes are found to maintain or even enhance the bilayer softness. In the case of cholesterol, a systematic study shows that the well-known rigidification effect is observed only for cholesterol concentrations above 3–4 mole%. The results are discussed in relation to the physical properties of internal cell membranes.

Key words Lipid bilayer · Softness · Swelling · Cholesterol · Short-chain lipid · Bola lipid · Small-angle neutron scattering · Calorimetry

The mechanical softness of lipid bilayers is determined not only by the thermodynamic state of the bilayer but also by its composition. A quantification of the softness (or rigidity) of a lipid bilayer is obtained by considering the elastic moduli (Evans et al. 1991; Bloom et al. 1991; Sackmann 1994), i.e. the area compressibility modulus, K_A, and the bending rigidity, κ, which are the only relevant elastic constants for a fluid lipid bilayer. In a simple continuum mechanical description based on a shell model (Evans and Skalak 1980) these two moduli are not independent but are connected by the relation,

$$\kappa \propto K_A d_B^2 \tag{1}$$

where d_B is the bilayer thickness. In the case of many-component bilayers, particularly when various solutes interact with and/or partition into the bilayer, the relationship between K_A and κ is more complex (Duwe et al. 1990; Evans et al. 1994). In that case, the simple reciprocal relation between the (solute-induced) change in the bilayer area, A, and the bilayer thickness, d_B, which is anticipated in the derivation of Eq. (1), is not necessarily a good approximation. Furthermore, lateral heterogeneity of the membrane structure may couple to the bilayer curvature and effectively lower the bending rigidity (Leibler 1986).

The interaction of specific endogenous solutes with the fluid-lipid bilayer component of cell membranes is of paramount importance for the structure and molecular organization of the membrane on the one hand and for the various functions of the membrane on the other hand. Furthermore, different externally added solutes are of importance for regulating membrane functions, e.g. in relation to drug action and the influence of pesticides. In order to fully understand the effect of solutes on membrane function it is essential, as a first step, to come to grips with the influence of these solutes on the thermodynamics and thermomechanics of lipid bilayers. There are two limiting classes of solutes in relation to lipid membranes: (i) solutes that display a significant partitioning between the bilayer and the aqueous phase (e.g. short-chain alcohols, bile salts, and various drugs) and (ii) solutes that have very low solubility in water and therefore selectively position themselves in the bilayer (e.g. peptides and proteins with a large hydrophobic domain, other lipids different from the host lipid, such as cholesterol and bipolar lipids, as well as long-chain alkanes and alcohols, cf. insert in Fig. 1). It is generally found (Evans et al. 1994) that solutes with high wa-

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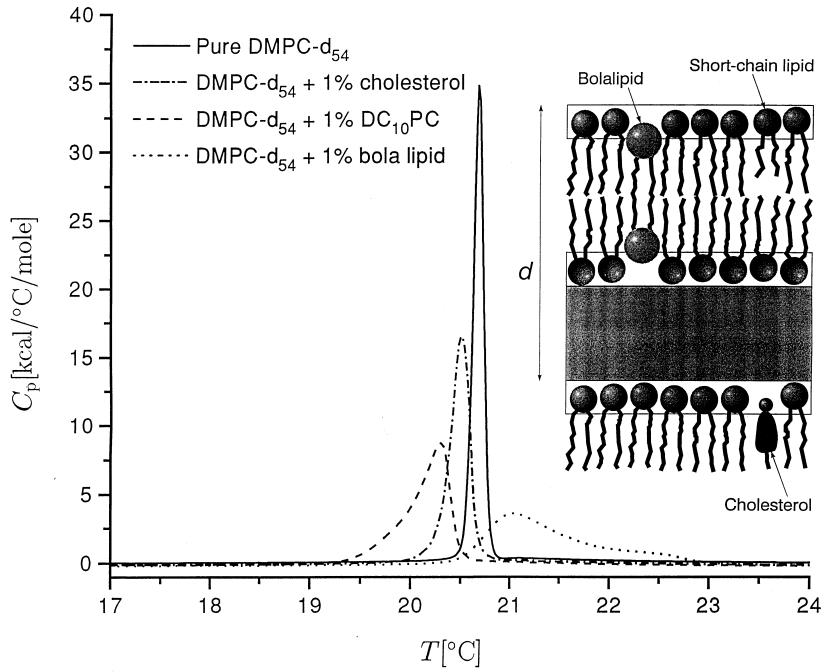

Fig. 1 Specific-heat, $C_P(T)$, as a function of temperature for multilamellar DMPC-d_{54} bilayers with 1 mole% of either cholesterol, $DC_{10}PC$, or bola lipid. The $C_P(T)$ curve for pure DMPC-d_{54} bilayers is shown for comparison. Scan rates were 20 °C/h, except for the pure DMPC-d_{54} sample, where the scan rate was 12 °C/h. The insert shows a schematic illustration of a repeat unit of a multilamellar phospholipid bilayer with solutes such as cholesterol, short-chain phospholipid, and bola lipid

ter solubility belonging to class (i) universally reduce the area compressibility modulus, K_A, and often reduce the bending rigidity, κ (Safinya et al. 1989; Duwe et al. 1990) whereas solutes in class (ii) can either reduce or increase the stiffness moduli, depending on the molecular structure and possibly on the solute concentration (Bloom et al. 1991). Obviously, large bulky solutes incorporated in the lipid bilayer can change the bilayer area and the bilayer thickness in very different ways, e.g. depending on the hydrophobic matching of the solute to the bilayer.

In this Letter we shall address the question of the relative ability of a series of different solutes preferentially positioned in the lipid bilayer (class (ii)) to modulate the bilayer stiffness of multilamellar bilayers of DMPC[1]. We have focussed on a temperature region around the main gel-to-fluid phase transition for the reason that the bilayer softness in this region, due to strong fluctuations, is very sensitive to addition of solutes that may change the underlying bilayer heterogeneity (Mouritsen and Kinnunen 1996). The bending rigidity is gauged indirectly from a measurement of the swelling behavior (Hønger et al. 1994) as described below. We shall focus on the regime of rather low contents of solutes, in the range of 1 mole%, a range which is usually not considered in biophysical studies of membranes. We shall provide evidence that the series of solutes studied either enhance or maintain the bilayer softness. Specifically we find that cholesterol in amounts up to about 3 mole% softens the lipid bilayer and only in larger amounts, above 3–4 mole%, leads to the well-known stiffening effect which is known to be of ultimate importance for the mechanical coherence of eucaryotic plasma membranes (Yeagle 1988; Needham and Nunn 1990).

Deuterated DMPC-d_{54}, cholesterol, and $DC_{10}PC$ were purchased from Avanti Polar Lipids Inc. (Birmingham, AL, USA). Bipolar lipid (bola lipid)[2] consisting of two hydrophilic head groups, connected by two short (supposedly membrane-spanning, as indicated in the insert to Fig. 1) hydrocarbon chains, $-(CH_2)_{12}-$, was a generous gift from Prof. Dr. Erich Sackmann (Technical University of Munich). All materials were used without further purification. Mixtures of DMPC-d_{54} with the different solutes were prepared by co-solubilization in chloroform. The solvent was subsequently evaporated under dry nitrogen atmosphere and further dried under vacuum to remove traces of the solvent. Samples of multilamellar bilayers of the mixtures were formed by hydrating the dry film in a D_2O buffer solution under excess water conditions. The phase transition properties of the multilamellar samples were studied by DSC using an MC-2 Ultrasensitive Scanning Calorimeter from Microcal Inc. (Northampton, MA, USA). Heating and cooling experiments were performed with a typical scan rate of 20 °C/h. The swelling properties were investigated by measuring the lamellar repeat distance using the SANS facility at Risø. A detailed account of the SANS experiments and the data analysis of pure DMPC-d_{54} has previously been published (Lemmich et al. 1996). The same procedures were applied for DMPC-d_{54} with the different solutes. Briefly, scattering data were corrected for background and analyzed in terms of a one-dimensional paracrystalline theory, identifying the repeat distance, *d*, as one of the key parameters. The instrumental smearing was taken into account in the analysis. It should be noted that

[1] *Abbreviations used:* $DC_{10}PC$, didecanoyl phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; DMPC-d_{54}, DMPC with fully deuterated acyl chains; DPPC, dipalmitoyl phosphatidylcholine; DSC, Differenting Scanning Calorimetry; SANS, small-angle neutron scattering

[2] For the exact chemical structure, see Duwe et al. (1990)

it is also possible to estimate d directly from the position of the Bragg reflections.

In Fig. 1 are shown the specific-heat, C_p, vs. temperature for mixtures of DMPC-d_{54} with 1 mole% of cholesterol, $DC_{10}PC$, and bola lipid, respectively, compared to that of pure DMPC-d_{54}. The transition temperature of pure DMPC-d_{54} is $T_m = 20.7\,°C$ which is below that of protiated DMPC due to the deuteration. It is observed, that each solute has a distinct effect on the position and width of the specific-heat peak. Cholesterol and the short-chain lipid $DC_{10}PC$ induce freezing-point depression, $\Delta T_m = -0.2\,°C$ and $-0.4\,°C$, respectively, whereas the bola lipid increases the transition point ($\Delta T_m = 0.35\,°C$) and broadens the transition considerably. Evidently there is a "shoulder" on the high-temperature side in the case of the bola lipid. Owing to the very low concentrations we shall not discuss details of the phase-separation phenomena which the solutes invariably introduce, but only describe the solute effect in terms of the broadening of the specific-heat peak. The width of the transition varies in the order: bola lipid > $DC_{10}PC$ > cholesterol > pure DMPC-d_{54}.

The results obtained from the SANS measurements in terms of the lamellar repeat distance, d, are shown in Figs. 2 and 3. In the case of pure DMPC-d_{54}, the data show that the multilamellar sample displays anomalous swelling behavior in the transition region (Hønger et al. 1994) in the sense that the repeat distance has a peak at the transition and that the increase of the repeat distance upon lowering the temperature from the fluid phase towards the transition exhibits an effective power-law behavior (Kirschner and Cevc 1993; Lemmich et al. 1995). It has been shown, that the strong variation of d in the fluid phase is due to a variation in the hydrophobic lipid bilayer thickness (Zhang et al. 1995; Lemmich et al. 1995; 1996) together with an increase in the thickness of the aqueous layer between the lamellae, and that it is the aqueous layer that has a non-monotonus temperature variation and therefore leads to the peak in the repeat distance (Lemmich et al. 1995). In this sense our use of the term "anomalous swelling" is different from that proposed by Zhang et al. (1995) who use the term to describe the increase in the hydrophobic lipid bilayer thickness, as the temperature is reduced towards T_m. The anomalous swelling behavior has been explained (Hønger et al. 1994) in terms of a thermal renormalization of the bending rigidity, $\kappa \rightarrow \kappa - r^2/K_A$, due to a coupling (of strength r^2) between the lateral density fluctuations and the bilayer curvature. Therefore, close to the transition, where the compressibility modulus, K_A, is decreased (Evans and Kwok 1982), the effective bending rigidity is reduced. Such a thermal reduction has been observed directly by flicker-noise analysis of large unilamellar vesicles (Fernandez-Puente et al. 1994). The reduction in κ leads to an increase in the entropic repulsion between the bilayers. In the simplest case, where the membranes are of negligible thickness and unbound, this effect can be explained in terms of Helfrich's theory for undulating membranes (Helfrich 1978) as due to a repulsive force or interaction free energy per unit area, f_s, which acts between the bilayers in a multi-lamellar stack and which scales with the repeat distance as $f_s \sim (k_B T)^2/(\kappa d^2)$. Thus the repulsion and the repeat distance increase as κ is reduced. Anomalous swelling behavior at the phase transition can therefore be interpreted as a manifestation of a thermal renormalization of the bending rigidity and hence a softening of the lipid bilayer (Hønger et al. 1994).

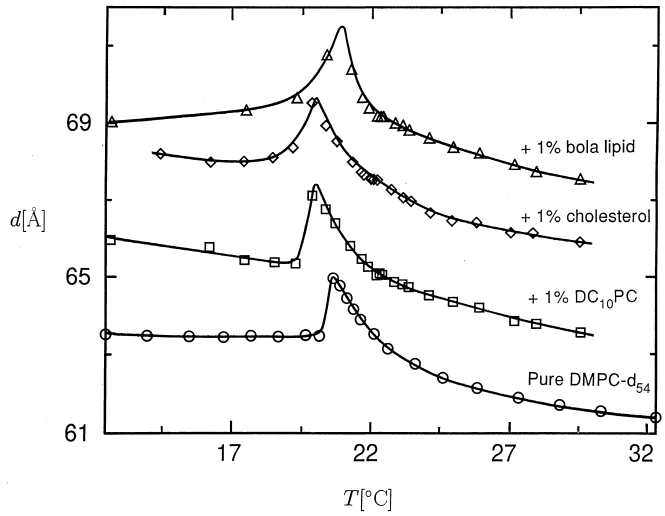

Fig. 2 Temperature dependence of the lamellar repeat distance, d, as obtained from SANS measurements in a region around the phase transition temperature of multilamellar bilayers of pure DMPC-d_{54} (○), and DMPC-d_{54} with 1 mole% of $DC_{10}PC$ (□), cholesterol (◇), or bola lipid (△). For the sake of clarity, each data set above that for pure DMPC-d_{54} has been displaced by 2 Å relative to that below. The solid lines are guides to the eye

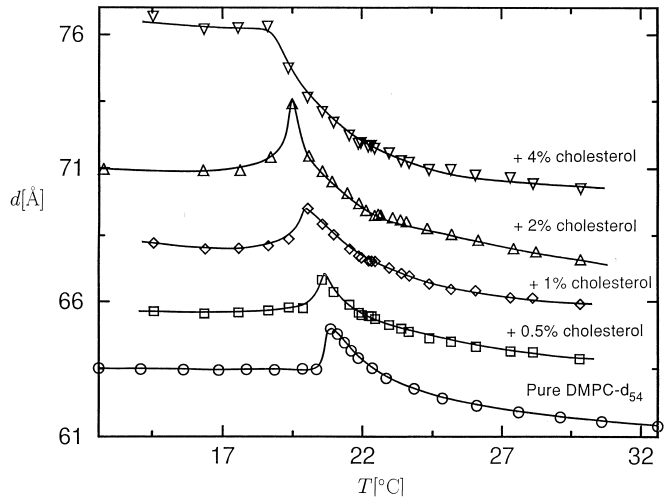

Fig. 3 Temperature dependence of the lamellar repeat distance, d, as obtained from SANS measurements in a region around the phase transition temperature of multilamellar bilayers of DMPC-d_{54} with cholesterol in concentrations 0 (○), 0.5 (□), 1 (◇), 2 (△), or 4 (▽) mole%. For the sake of clarity, each data set above that for pure DMPC-d_{54} has been displaced by 2 Å relative to that below. The solid lines are guides to the eye

Figure 2 provides a comparison between the effects of the three different solutes, all in a concentration of 1 mole%, on *d* in the region around the transition. It is seen that upon addition of the solutes, the anomalous swelling behavior remains and the range of temperatures over which the anomaly extends is enlarged corresponding to the broadening of the specific-heat peak in Fig. 1. It is not possible from the data shown in Fig. 2 to put forward any definite conclusions when comparing the heights of the swelling peaks. Clearly, the broadening of the peaks, induced by the addition of the solutes, makes it difficult to detect the maximum value of *d* with the present temperature resolution. In the case of the short-chain lipid $DC_{10}PC$, there is however reasonable evidence that the addition of 1 mole% increases the height of the swelling peak, indicating a softening effect on the bilayer. This is in good agreement with a recent theoretical study (May and Ben-Shaul 1995) where a molecular chain-packing model was used to predict that adding short-chain amphiphiles to a layer of long-chain amphiphiles has the effect of lowering its bending rigidity. The effect may be of relevance for understanding the ability of $DC_{10}PC$ to function as an enhancer for transport of various substances, e.g. hormones, across membranes (Risbo et al. 1995; and unpublished).

There are also indications of an increase in the height of the swelling peak for the system containing 1 mole% bola lipid (as illustrated by the solid line in Fig. 2) compared to the one observed for pure DMPC-d_{54}. More data points would however be required in order to decide, whether this increase is significant. The actual configuration of the bola lipid in the bilayer is not known with certainty. Some bacteria are known to have a certain amount of membrane-spanning bola lipids in their cell membranes, which can help in stabilizing the cell under extreme physical conditions, such as very high temperature and/or very low pH (Duwe 1989). However in the present case, the hydrophobic length of the bola lipid is comparable to the hydrophobic length of one DMPC molecule only, but on the other hand, the bola lipid has relatively large head groups. Thus, it is not clear, whether the membrane-spanning configuration, indicated in Fig. 1, is the most favorable. It has alternatively been suggested (Duwe 1989) that the bola lipid in this case might choose to place both its head groups in the same monolayer, i.e. it is adsorbed at the hydrophobic-hydrophilic interface. This would give rise to a pronounced local curvature, which in turn would lead to an effective lowering of the bending rigidity (Leibler 1986). Our data seem to indicate that this is plausible. On the other hand, this configuration of the bola lipids is not likely to stabilize the ordered gel phase to such an extent that the phase transition temperature is increased as shown by the DSC data in Fig. 1. It is known from analysis of vesicle-shape fluctuations (Duwe et al. 1990) that bola lipids of the type considered here in concentrations of 2–5 mole% in DMPC lead to hyperelastic bilayers in the fluid phase. Furthermore, the vesicles exhibit violent fluctuations close to the phase transition, but becomes more stiff, once the gel phase is reached, possibly due to expulsion of the bola lipid via blebbing transitions (Duwe 1989). One could also imagine that the phase transition from the fluid phase to the gel phase involves a rearrangement of the bola lipids from the adsorbed state into the membrane-spanning state as described above.

Turning next to the effect of varying the solute concentration, Fig. 3 shows the repeat distance for varying cholesterol concentrations up to 4 mole%. This data set shows clearly that the anomalous swelling peak is enhanced upon adding cholesterol up to about 3 mole% and that the peak is completely removed for 4 mole% cholesterol. This is an important observation, since it shows that in small amounts, cholesterol leads to a progressive softening of the lipid bilayer whereas in contents beyond about 3–4 mole%, cholesterol has the opposite effect leading to the well-known rigidification of the lipid bilayer. It has previously been proposed (Cruzeiro-Hansson et al. 1989), based on a theoretical model for the phase equilibria in the lecithin-cholesterol system, that the cholesterol molecule, owing to its different ways of interacting with the conformational and positional degrees of freedom of the lipid molecules, acts as an interfacially active molecule which positions itself in the boundaries between the gel and fluid domains which are dynamically created in the transition region by the strong fluctuations (Mouritsen and Jørgensen 1994). This was predicted to lead to an enhancement of the fluctuations and as a consequence bilayer softening and increased passive permeability (Corvera et al. 1992). Enhanced permeability of Na^+ and K^+ has indeed been observed experimentally upon addition of a small amount of cholesterol (de Gier et al. 1979; Corvera et al. 1992). However, a direct determination of the elastic moduli for lipid vesicles with small amounts of cholesterol still needs to be done.

In fact, only little work has been done on the physical properties of lipid bilayer membranes with low levels of cholesterol. The reason for this probably lies in biochemists' and physiologists' preoccupation with lipid bilayers containing large amounts of cholesterol, typically 20–30 mole%, which correspond to the actual levels of cholesterol and related sterols in eucaryotic plasma membranes (Yeagle 1988). However, the internal membranes of eucaryotes, e.g. those of the Golgi apparatus, contain much less cholesterol and some membranes, e.g. endoplasmatic reticulum (ER), contain hardly any cholesterol at all (Bretscher and Munro 1993). It has been suggested that the specific gradient of cholesterol concentration found in relation to Golgi and ER acts as to facilitate protein sorting in the secretory pathway (Pelham and Munro 1993) via cholesterol's ability to modify the membrane thickness. It is possible that the conspicuous membrane softening caused by low levels of cholesterol discussed in the present work may play a role for the special morphology and frequent vesicle-budding processes associated with the Golgi and ER membranes.

Acknowledgements This work was supported by the Danish Natural Science Research Council, the Danish Technical Research Council, and the Carlsberg Foundation.

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