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Effect of cholesterol on the phase state and permeability of mixed liposomes composed of anionic diphosphatidylglycerol and zwitterionic dipalmitoylphosphatidylcholine

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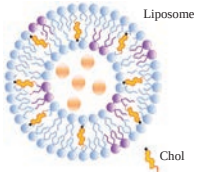
The incorporation of cholesterol into a solid liposomal membrane makes the membrane liquid with a higher lipid mobility (a lower microviscosity) and suppresses the membrane permeability toward inorganic ions.



Bilayer lipid vesicles (liposomes) have been used as nanocontainers for the encapsulation and delivery of biologically active substances.[1–5] 'Liquid' liposomes are typically applied to encapsulation with lipid bilayers in a liquid-crystalline (fluid) state that allows the migration of lipid molecules to occur within each lipid leaflet and between them. Cholesterol, a modified steroid, is commonly embedded into the bilayer for controlling the fluidity and permeability of liposomal membranes[6,7] and the circulation time of liposomal containers.[8–10]

'Solid' liposomes with lipid bilayers in a gel (solid) state are characterized by a much slower mobility of lipid molecules within the bilayer.[11] Drugs based on solid liposomes show antitumor and antiviral activity;[12–16] therefore, solid liposomes can be used as potential drug delivery agents.[17–19] However, structural defects are often formed in the solid liposomal membranes, which initiate a leakage of the content from liposomes to surrounding solution[20,21] and thus restrict the use of solid liposomal containers. The incorporation of cholesterol into a solid lipid membrane increases the membrane fluidity;[22] this is expected to heal defects in the lipid bilayer and prevent an early release of encapsulated drugs.

Here, we describe the preparation of mixed liposomes composed of zwitterionic dipalmitoylphosphatidylcholine (DPPC), doubly anionic diphosphatidylglycerol (cardiolipin, CL^{2-}) and cholesterol (Chol) and consider how an increase in the Chol content affects the phase state and microviscosity of an originally solid DPPC/CL^{2-} bilayer. Special attention is paid to a correlation between the structural organization of a DPPC/CL^{2-}/Chol ternary membrane and the membrane permeability toward inorganic ions.

DPPC, CL^{2-} and Chol from Sigma were used as received (Figure S1, Online Supplementary Materials). Small unilamellar liposomes, including those loaded by NaCl solution, were prepared by a standard sonication procedure (Procedure S1, Online Supplementary Materials). The molar ratio of CL^{2-} (ν_{CL}) was 0.05, while the molar ratio of cholesterol (ν_{Chol}) was varied from 0 to 0.5.

The phase state of the liposomal membrane was controlled by differential scanning calorimetry (DSC), which has been widely used for assaying structural reorganization in mixed lipid bilayers.[23–25] A calorimetric curve for DPPC/CL^{2-} binary liposomes with ν_{CL} = 0.05 represented in Figure 1 (curve *1*) shows one peak at 37.5 °C. The incorporation of Chol in the liposomal membrane leads to a gradual decrease in the melting peak at 37.5 °C (curves *2* and *3*) and its disappearance at ν_{Chol} = 0.2 (curve *4*). In the DPPC/CL^{2-} binary liposomes, anionic CL^{2-} molecules are nearly uniformly distributed among zwitterionic DPPC molecules.[26] Following the above definition, the liposomes are solid below 37.5 °C and liquid above 37.5 °C. The addition of an increasing amount of Chol causes the size of melting clusters to reduce and the melting peak to decrease, but the peak position on the temperature scale (at 37.5 °C) does not change. At ν_{Chol} = 0.2, the size of clusters is so small that the melting peak disappears and the liposomes become liquid in the entire temperature interval from 12 to 60 °C.

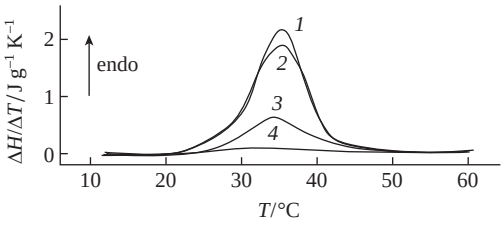


Figure 1 Calorimetric curves of (*1*) DPPC/CL^{2-} and (*2*)–(*4*) DPPC/CL^{2-}/Chol liposomes; ν_{CL} is 0.05; ν_{Chol} is (*2*) 0.05, (*3*) 0.1 and (*4*) 0.2; total lipid concentration, 1 mg ml^{-1}; 10^{-2} M Tris buffer, pH 7.

Thus, at 20–22 °C (room temperature), we observe the transfer of the liposomal membrane from solid to liquid when increasing the Chol content up to 20 mol%. This result is in good agreement with published data.[27] The phase transfer is accompanied by a change in the liposomal membrane microviscosity, which was detected by the fluorescence anisotropy monitoring of diphenylhexatriene (DPHT) incorporated into the lipid bilayer.[28] Briefly, 1 ml of a 2 mg ml^{-1} suspension of liposomes was mixed with 1 ml of a 2×10^{-7} M suspension of DPHT. The mixture was incubated for 1 h at room temperature, and the fluorescence

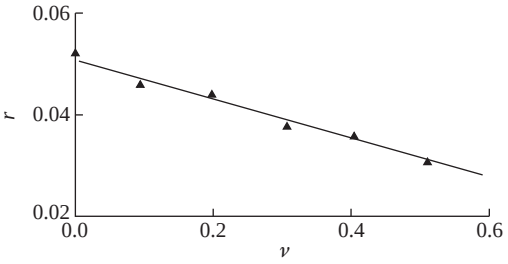


Figure 2 Fluorescence anisotropy of DPHT *vs.* the molar fraction of cholesterol; ν_{CL} = 0.1; total lipid concentration, 1 mg ml^{-1}; 10^{-2} M Tris buffer, pH 7.

intensity of DPHT was measured at 433 nm (λ_{ex} = 366 nm) using parallel and crossed polarizers ($I_{||}$ and $I_{\perp}$, respectively). The fluorescence anisotropy was determined as $r = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$. The lower the fluorescence anisotropy, the lower the lipid bilayer microviscosity. To avoid light scattering from liposomes, the fluorescence anisotropy was measured at various sample dilutions, and the characteristic anisotropy r_0 was found by the linear approximation of the obtained dependence to a zero liposome concentration.

Figure 2 shows that an increase in the Chol content leads to a progressive decrease in r_0, that is, a decrease in the membrane microviscosity. This result is consistent with published data, and it reflects the ability of cholesterol to reduce the packing density of the alkyl chains of lipid molecules.[29]

The effect of Chol on the permeability of a liposomal membrane was examined by conductometry. A series of NaCl-loaded liposomes with various Chol concentrations was prepared. The leakage of NaCl solution from liposomes was accompanied by an increase in the electrical conductivity of the suspension and compared with the maximum increase in the conductivity induced by an irreversible destruction of liposomes after addition of a tenfold excess of Triton X-100, taken as 100%. Figure 3 represents the time dependence of the relative conductivities of liposome suspensions. For solid DPPC/CL^{2-} binary liposomes deprive of Chol a 20% increase of the conductivity was observed within 100 min (curve *1*) thus showing the leakage of NaCl into surrounding solution. The incorporation of Chol into the lipid membrane resulted in slowing the kinetics of salt leakage (curves *2* and *3*). No salt leakage was detected from liposomes with $\nu_{Chol} \geqslant 0.3$ (curves *4*–*6*). Thus, by increasing the Chol content, the leakage of salt from liposomes can be completely suppressed.

Therefore, we observed a correlation between the structural organization and permeability of the DPPC/CL^{2-}/Chol ternary membrane. The solid membrane is permeable toward inorganic ions due to permanent structural defects in the low-flowing bilayer. The incorporation of cholesterol makes the membrane liquid and

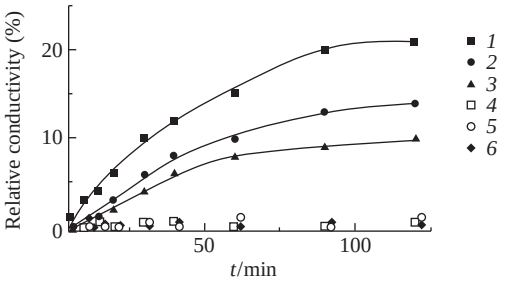


Figure 3 Time dependence of the conductivity of (*1*) DPPC/CL^{2-} liposomal suspension and (*2*)–(*6*) DPPC/CL^{2-}/Chol liposomal suspension loaded with a 1 M NaCl solution; ν_{CL} is 0.05; ν_{Chol} is (*2*) 0.1, (*3*) 0.2, (*4*) 0.3, (*5*) 0.4 and (*6*) 0.5; total lipid concentration, 1 mg ml^{-1}; 10^{-2} M Tris buffer, pH 7.

suppresses the ion permeation due to a healing effect of the fluid bilayer with a lower microviscosity (a higher mobility of lipid molecules). This result is of interest for the preparation of liposomal containers for drug encapsulation.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2016.03.003.

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