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Size and permeability of liposomes extruded through polycarbonate membranes

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Summary

The effects of extrusion of multilamellar liposomes (MLV) and reverse-phase evaporation liposomes (REV) on their sizes and permeabilities were studied. Extrusion through polycarbonate membranes decreases the mean size of both liposomes with improved homogeneity of the size distribution. Extrusion increases moderately the water permeability ($\bar{k}_W$) of lecithin-only MLV and REV. But, the increase in $\bar{k}_W$ is small for the cholesterol-containing REV. The increase in $\bar{k}_W$ is not due to the decrease in size or the lamellar number, but to the increased disorder of the bilayer. The permeabilities of amino acids ($\bar{k}_A$) are increased by extrusion as well as by sonication. The increase in $\bar{k}_A$ is considered to be due to the decrease in the lamellar number. The addition of digitonin into lecithin-cholesterol MLV eliminates the condensing effect of cholesterol, i.e. the $\bar{k}_W$ of lecithin-cholesterol MLV increased to the level of lecithin-only MLV. This suggests that digitonin–cholesterol complex forms separated domains in the PC bilayer.

Introduction

When liposomes are used as drug delivery in vivo, the liposome size affects the tissue distribution and the half-time of circulation (Juliano and Stamp, 1975; Kimelberg, 1976). Generally, the size distribution of liposomes is heterogeneous and the mean size depends upon the preparation method (Szoka and Papahadjopoulos, 1980). An improved method to prepare liposomes with a more homogeneous distribution and high encapsulation efficiency has been proposed, in which method

liposomes were sequentially extruded through polycarbonate membranes (Olson et al., 1979, Szoka et al., 1980). The extrusion may alter the structure of liposomal membrane, e.g. the number of lamellar, possibly accompanying the change in permeabilities of drugs across the liposomal membrane. Therefore, in this report, we study whether or not the extrusion changes the permeabilities of water and hydrophilic and hydrophobic amino acids. Furthermore, the effect of extrusion is compared with the effect of addition of digitonin into cholesterol-including liposomes.

Materials and Methods

Materials

Lecithin (PC) was obtained from egg yolk following the Faure method (1950). The purity of lecithin was 88% (mole ratio) as determined by thin-layer chromatography. Other components were 9% lysolecithin, 1% phosphatidyl ethanolamine and 2% mixture of lysophosphatidyl ethanolamine and sphingomyelin. We used this fraction as lecithin without further purification. Chloroform solutions of lecithin were stored in sealed ampules under nitrogen gas at $-80°C$ until use. Cholesterol (Chol; reagent grade) was purchased from Sigma and used without further purification. Digitonin (reagent grade) was purchased from Wako Pure Chemical. Polycarbonate membranes (Nuclepore) were purchased from Nuclepore Corp. Water was filtered with 0.1 μm nitrocellulose membrane to remove dust.

Preparation of liposomes

Multilamellar liposome (MLV)

MLV were prepared by following the technique of Bangham et al. (1965). Four different types of MLV were prepared: PC; PC-Chol (9 : 1, in mole ratio); PC-Chol (7 : 3); and PC-Chol (1 : 1). The lipid mixture in chloroform was deposited on the wall of a round flask by removal of chloroform on a rotary evaporator. After adding a necessary amount of 20 mM sucrose solution (buffered with 4 mM Pipes-Tris buffer, pH 7.4; final concentration 60 μmol lipid/ml) into the flask, the liposome suspension was shaken gently by hand for 1 h under nitrogen gas at 25°C. Then, the liposome suspension was diluted to 3.6 μmol lipid/ml with 20 mM sucrose solution and filtered with 3.0 μm polycarbonate membrane to remove large aggregated liposomes.

Reverse-phase evaporation liposome (REV)

REV were prepared by the technique of Szoka and Papahadjopoulos (1978). Three types of REV were prepared PC; PC-Chol (9 : 1); and PC-Chol (7 : 3). The lipid mixture in chloroform (60 μmol total lipid) was deposited on the wall of a round flask and dissolved in 3 ml of ether which had been freshly distilled in the presence of $NaHSO_3$. Then 1 ml of 20 mM sucrose solution (buffered with 4 mM Pipes-Tris, pH 7.4) was added to the lipid solution and sonicated in a bath type sonicator (Branson B-42, 240 W) under nitrogen gas at 20°C for 5 min. Then, ether

was removed in two stages on a rotary evaporator. Ether was removed under vacuum at about 400 mm Hg until the suspension became a gel. Following a brief vortex mixing, evaporation was continued under about 730 mm Hg until a homogeneous suspension was obtained. The liposome suspension was diluted to 3.6 μmol/ml with 20 mM sucrose solution and filtered with 3.0 μm polycarbonate membrane to remove large aggregated liposomes.

Extrusion of liposomes

The liposomes were sequentially passed through polycarbonate membranes with pore diameters of 1.0, 0.8, 0.6, 0.4, 0.2 and 0.08 μm at a pressure of 2–5 kg/cm^2. Polycarbonate membranes are suitable for sizing liposomes because they have straight-through pores with a uniform pore size (+0%, −20% of nominal diameter) and no lipid is retained on the surface of the membranes in the process of the extrusion (Olson et al., 1979).

Water permeability

Water permeability of liposomal membranes was measured by the light scattering method as previously described (Takeguchi et al., 1981). The liposome suspension, equilibrated with 20 mM sucrose (buffered to pH 7.4 with 4 mM Pipes-Tris), was mixed with a hypertonic 100 mM sucrose solution in a 1 : 1 volume ratio by a Durrum stop-flow spectrophotometer. The intensity of 90° light scattering was monitored by a storage oscilloscope and recorded by a Polaroid camera. The scattering intensity does not follow a single exponential curve because of the heterogeneity of water permeability. We used the cumulative expansion method to obtain the mean rate constant of water efflux across liposomal membranes (Takeguchi et al., 1981; Koppel, 1972). The scattering intensity, Y, is expanded as follows:

$$Y = A\left\{1 - \left(1 + \mu_{2W}t^2/2\right)\exp\left(-\bar{k}_W t\right)\right\} \quad (1)$$

where $\bar{k}_W$ is the mean rate constant of water efflux and $\mu_{2W}/\bar{k}_W^2$ is the polydispersity index of water permeability. Parameters in Eqn. 1 were determined by the non-linear least-squares curve fitting.

Amino acid permeability

Amino acid permeabilities of liposomal membranes were also determined by the light scattering method as described elsewhere (Takeguchi et al., 1981; Rabon et al., 1980). The liposome suspension, equilibrated with 20 mM sucrose (buffered to pH 7.4 with 4 mM Pipes-Tris), was mixed with a 100 mM amino acid + 20 mM sucrose solution (buffered to pH 7.4 with 4 mM Pipes-Tris) in a 1 : 1 volume ratio. The mixing induced the change of the liposome volume in two stages. Initially, the liposomes shrink to maintain an equi-osmolar condition, then, the liposomes swell due to the water influx associated with the amino acid influx. The change in the scattering intensity was able to be simulated by cumulative expansion terms

$$Y = A\left\{1 - \left(1 + \mu_{2W}t^2/2\right)\exp\left(-\bar{k}_W t\right)\right\} - 1.2A\left\{1 - \left(1 + \mu_{2A}t^2/2\right)\exp\left(-\bar{k}_A t\right)\right\} \quad (2)$$

where $\bar{k}_W$ and $\bar{k}_A$ are the mean rate constants of water efflux and amino acid influx,

respectively. The value of 1.2 was introduced for the swelling process in Eqn. 2, because the volume of maximally-swelled liposomes was about 1.2 times the initial unshrunken size (Takeguchi et al., 1981). The value of $\mu_{2W}/\bar{k}_W^2$ and $\mu_{2A}/\bar{k}_A^2$ are the polydispersity index of $\bar{k}_W$ and $\bar{k}_A$, respectively (Koppel, 1972). In the simulation, values of $\bar{k}_W$ and μ_{2W} were taken as equal to the corresponding values in Eqn. 1.

Size of liposome

The mean size of unextruded and extruded liposomes was measured by the quasi-elastic light scattering (QELS) method as described elsewhere (Morii et al., 1981; Dubin, 1972; Bloomfield, 1981). The intensity of laser light scattering fluctuates due to the thermal motion of liposomes in the solution. We used a single clipping method to calculate the auto-correlation coefficient, $g^{(1)}(\tau)$, of the fluctuation (Selser et al., 1976). The auto-correlation curve does not decay with a single exponential curve because of the polydispersity of size distribution. Therefore, we used the cumulative expansion method (Koppel, 1972) to obtain the mean decay constant, $\bar{\Gamma}$. That is,

$$|g^{(1)}(\tau)| = (1 + \mu_2\tau^2/2)\exp(-\bar{\Gamma}\tau) \tag{3}$$

where τ is the lag time and $\mu_2/\bar{\Gamma}^2$ is the polydispersity index of the size distribution. The mean decay constant is related to the mean translational diffusion coefficient, $\bar{D}_{trans}$:

$$\bar{D}_{trans} = \bar{\Gamma}/K^2, \tag{4}$$

and

$$K = (4\pi n/\lambda)\sin(\theta/2) \tag{5}$$

where λ, n and θ are the wavelength of incident light, the refractive index of solvent and the scattered angle, respectively. In our experiments, $\theta = 90°$. The mean radius of liposome, $\bar{R}$, is calculated from Stokes-Einstein equation:

$$\bar{R} = kT/6\pi\eta\bar{D}_{trans} \tag{6}$$

where k, η and T are the Boltzman constant, the viscosity of solvent and absolute temperature, respectively. The QELS measurements were carried out at 298°K. The QELS apparatus consisted of a He-Ne laser (5 mW, GLG2033, NEC), an optical cell (Union), a photomultiplier (R649, Hamamatsu TV), a photon counter (C-1230, Hamamatsu TV) and a microcomputer (TRS-80, Tandy).

Results

Size of liposome

Here, the effects of extrusion on the size of MLV and REV were studied. The results on MLV are in accord with previous results (Morii et al., 1981). Above 0.4

μm extrusion, MLV liposomes are destroyed and reformed into smaller sizes by the sequential extrusion through polycarbonate membranes. Furthermore, the polydispersity index and skewness of the size distribution were improved by the extrusion. But, below 0.2 μm extrusion, liposomes maintain their size, i.e. they pass through the pores by changing their shapes.

The mean diameter of unextruded REV (0.35–0.5 μm) is smaller than that of unextruded MLV (0.8–1.2 μm). The polydispersity index of unextruded REV is also smaller than that of MLV. The extrusion of REV decreases the liposome size and improves the homogeneity of the size distribution as shown in Figs. 1 and 2. The skewness for unextruded and extruded REV is almost zero, that is, the size distribution is symmetrical. Incorporation of cholesterol into liposomes increases the liposome size (Fig. 1) and heterogeneity of the size distribution (Fig. 2), which agree with the previous findings by Szoka et al. (1980).

Water permeability

The extrusion of MLV and REV induces the increase in the water permeability as shown in Figs. 3 and 4. For this reason, the decrease in the lamellar number induced by the extrusion cannot be considered, because $\bar{k}_w$ does not depend on the liposome size, e.g. $\bar{k}_w$ of unextruded MLV (0.8 μm) is nearly the same as that of REV (0.38 μm). $\bar{k}_w$ is sensitive to the mobility of the acyl chains of phospholipids and the disorder of the lipid bilayer structure (Träuble, 1971). Therefore, present increase of $\bar{k}_w$ induced by the extrusion would suggest the increased disorder of lipid bilayer.

Incorporation of cholesterol into MLV and REV decreases $\bar{k}_w$ as shown in Figs. 3 and 4. This may be due to the Chol-induced decrease of the acyl chain mobility (a condensing effect) (Demel, 1967; Oldfield and Chapman, 1972; Shimshick and

Fig. 1.

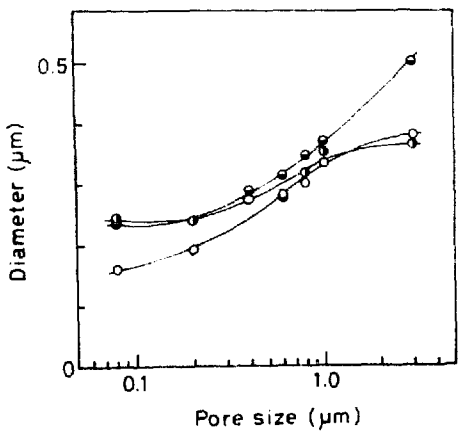


Fig. 2.

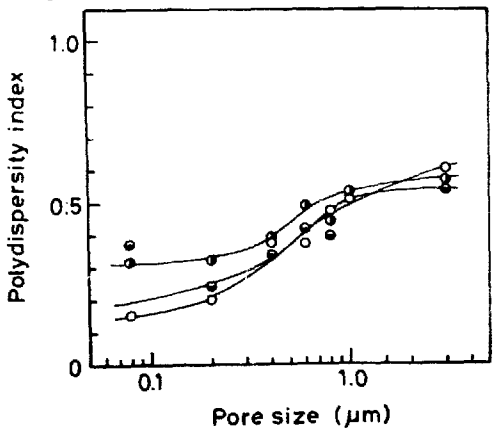


Fig. 1. Mean diameter of reverse-phase evaporation liposomes (REV) determined from the method of quasi-elastic light scattering (QELS method). REV were sequentially extruded through polycarbonate membranes until to the indicated pore size. ○, PC REV; ◑, PC + 10 mol% cholesterol REV; and ◒, PC + 30 mol% cholesterol REV.

Fig. 2. Polydispersity index of the size distribution of REV. The index is defined as $\mu_2/\bar{\Gamma}^2$, where $\bar{\Gamma}$ = constant × (1/mean diameter). ○, PC REV; ◑, PC + 10 mol% cholesterol REV; and ◒, PC + 30 mol% cholesterol REV.

Fig. 3.

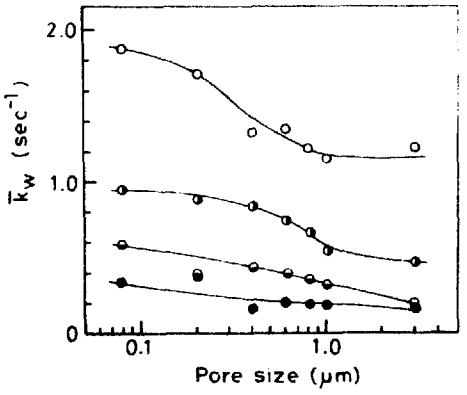


Fig. 4.

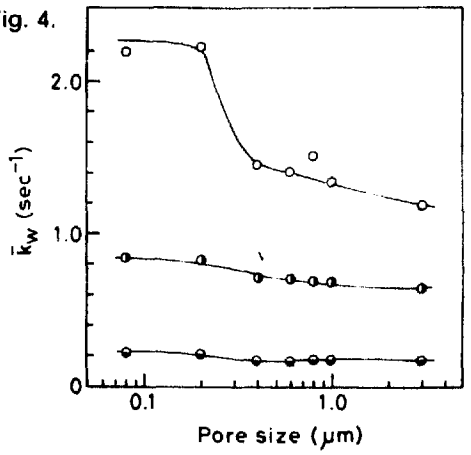


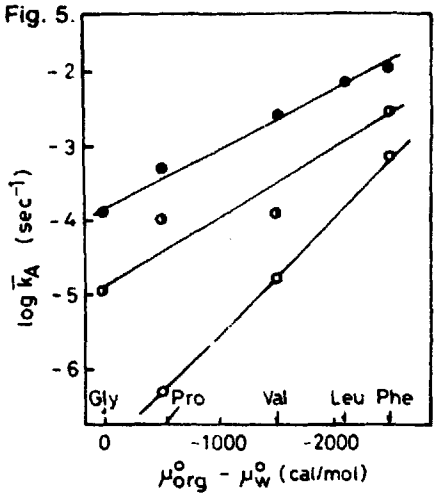
Fig. 3. Effects of extrusion on water permeability, $\bar{k}_w$, of multilamellar liposomes (MLV) determined from the light scattering method. MLV were sequentially extruded through polycarbonate membranes until to the indicated pore size. ○, PC MLV; ◑, PC + 10 mol% cholesterol MLV; ◒, PC + 30 mol% cholesterol MLV; and ●, PC + 50 mol% cholesterol MLV.

Fig. 4. Effects of extrusion on water permeability, $\bar{k}_w$, of reverse-phase evaporation liposomes (REV). ○, PC REV; ◑, PC + 10 mol% cholesterol REV; and ◒, PC + 30 mol% cholesterol REV.

McConnell, 1973). The extrusion of Chol-including MLV increased $\bar{k}_w$ moderately, but their values of $\bar{k}_w$ were much smaller than those of PC-only MLV even after the extrusion. In the case of Chol-including REV, the extrusion effect was negligibly small.

Amino acid permeability

In our previous paper, the permeability of amino acids across the liposomal membrane has been shown to correlate linearly with a hydrophobicity scale (Takeguchi et al., 1981), in which experiments MLV gently sonicated for 1 h were used. As the scale of hydrophobicity, the additional free energy of transfer from water to organic solvent ($\mu^0_{org} - \mu^0_w$) reported by Nozaki and Tanford (1971) was used. Here, we measured amino acid permeabilities of 3 different types of liposomal membranes: PC-MLV; the sonicated PC-MLV; and the PC-MLV sequentially extruded through until 0.08 μm. The rate constant, $\bar{k}_A$, was obtained on 5 amino acids; Gly, Pro, Val, Leu and Phe. The magnitude of $\bar{k}_A$ for all 3 types of liposomes is in order of Gly < Pro < Val < Leu < Phe. Amino acid permeabilities of MLV membrane are increased for all 5 amino acids by the sequential extrusion until 0.08 μm as well as by the sonication (Fig. 5). The permeability of Phe through the PC-MLV membrane was increased 4.2-fold by the extrusion and the permeability of Gly was increased more than 200-fold. These results indicate that the extrusion decreases the hydrophobic barrier as well as the hydrophilic barrier. Similarly, the sonication which decreases the liposome size induced the increase in the amino acid permeabilities. These increases in amino acid permeabilities would mostly reflect the decrease in the lamellar number of lipid bilayer. This is supported by the following thermodynamic consideration. A 10-fold increase in the rate constant accompanied a change in ($\mu^0_{org} - \mu^0_w$); −1.3 kcal/mol for the sonicated MLV, −1.0 kcal/mol for the ex-



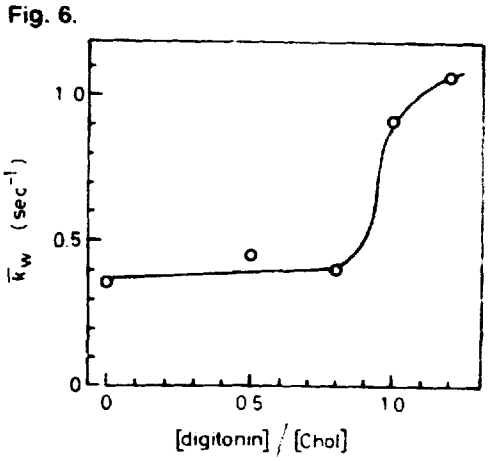


Fig. 5. Relationship between the rate constant of amino acid permeability, $\bar{k}_A$, and $(\mu^0_{org} - \mu^0_w)$. The value of $(\mu^0_{org} - \mu^0_w)$ were taken from the paper by Nozaki and Tanford (1971) (we used -2.1 kcal/mol for Leu instead of original -1.8 kcal/mol). Linear lines were drawn by the least-squares method. ○, PC-MLV; ○, PC-MLV sequentially extruded through until 0.08 μm; and ●, sonicated PC liposome.

Fig. 6. Water permeability, $\bar{k}_w$, of Chol-including MLV as a function of digitonin/cholesterol mole ratio. The content of cholesterol is 17 mol%.

truded MLV and -0.61 kcal/mol for the unextruded MLV. The values for the sonicated and the extruded liposomes approximately agree with the value calculated from the theory of absolute rate process, -1.4 kcal/mol (Takeguchi et al., 1981). This indicates that the partition coefficient between water and lipid bilayer is the major determinant of relative magnitude of $\bar{k}_A$ for the sonicated and the extruded

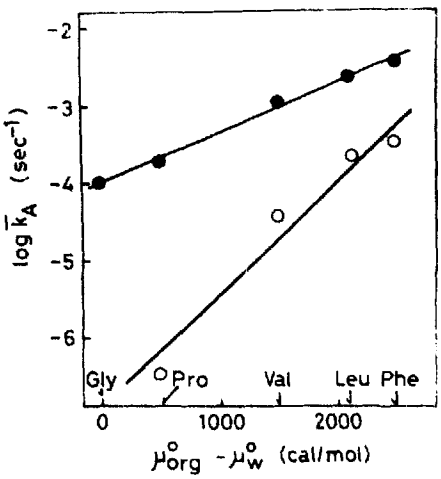


Fig. 7. Relationship between the rate constant of amino acid permeability, $\bar{k}_A$, and $(\mu^0_{org} - \mu^0_w)$ of Chol-including MLV (10 mol%, without extrusion). Linear lines were drawn by the least-squares method. ○, without digitonin; and ●, with equimolar digitonin.

liposomes which have smaller lamellar numbers, in comparison with the unextruded MLV.

Digitonin effect

Addition of digitonin into PC-Chol liposomes increases glucose permeability (Nakamura et al., 1979, 1980). The addition of digitonin causes an abrupt increase of water permeability at the ratio of [digitonin]/[Chol] = 1 (Fig. 6), and the water permeability increases nearly to the level of PC-only MLV, i.e. the condensing effect of cholesterol disappears. This suggests that Chol–digitonin complex forms separated phases in PC bilayer. Similarly, the amino acid permeabilities are increased by the addition of digitonin (Fig. 7). Especially, the relative increase is large in the case of hydrophilic amino acids. The addition of digitonin would not change the lamellar number, but the disappearance of the condensing effect explains the increase of $\bar{k}_A$ for the hydrophobic amino acids. Furthermore, the phase separation induces an additional pathway for hydrophilic amino acids through the boundary space around the domains of Chol–digitonin complex (Takeguchi et al., 1981).

Discussion

Diameters of liposomes are decreased by the extrusion through polycarbonate membranes, accompanying the more homogeneous size distribution as previously reported (Olson et al., 1979). We have demonstrated here that water permeability across the PC-MLV and PC-REV membranes is increased by the extrusion (Figs. 3 and 4). Furthermore, permeability for hydrophilic amino acids such as glycine and proline in PC-MLV is also greatly increased by the extrusion. In the case of Chol-including liposomes, the water permeability is smaller than PC liposomes even after the extrusion (Figs. 3 and 4). These results suggest that Chol-including liposomes may be superior to PC liposomes in view of drug retention power when extruded liposomes are used as a drug carrier for hydrophilic compounds.

It seems reasonable to speculate that the water permeability of liposomal membranes is influenced by the packing structure of lipid bilayers and the mobility of the acyl chains of phospholipids. Chan et al. (1973) have shown that water permeabilities for small (about 0.03 μm) and large (about 0.08 μm) liposomes are different predominantly due to the packing constraint: the small liposomes become less permeable as the size decreases. On the contrary, the extrusion increased the water permeabilities of MLV (0.23–1.2 μm) and REV (0.16–0.5 μm). Apparently, the size of extruded liposomes is not so small as to induce the packing constraint. For a possible explanation, the extrusion increases the extent of disorder of the lipid bilayer structure.

Permeabilities of hydrophobic amino acids as well as hydrophilic amino acids are also increased by the extrusion. These results on amino acids would reflect the fact that the extrusion decreases the lamellar number.

Akiyama et al. (1980) have shown by NMR that the addition of equimolar digitonin to Chol-including phospholipid multibilayer causes the formation of the

rigid complex with cholesterol. Furthermore, the Chol–digitonin complex forms domains in the PC bilayer, that is, phase separation occurs. We have demonstrated that the addition of digitonin into Chol-including MLV increases the permeabilities of water and amino acids. Especially, the increase in permeability of hydrophilic amino acids is relatively large. This suggests that the phase boundary around the Chol–digitonin domain contributes to the increased permeability for hydrophilic compounds. This also explains the following fact that the addition of digitonin to mitochondria and endoplasmic reticulum of liver increases the permeabilities of ions (Becker, 1980).

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