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Ethanol-induced aggregation and fusion of small phosphatidylcholine liposome: participation of interdigitated membrane formation in their processes

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Abstract

The mechanism for the ethanol-induced aggregation/fusion of uniform-sized small liposomes comprised of dipalmitoyl (DPPC) or egg yolk (eggPC) phosphatidylcholines was studied by measuring the average size using a photon correlation spectroscopy, by observing directly the states in the liposomal solutions using freeze-fracture electron microscopy and by attempting resonance energy transfer using fluorophore-labeled phospholipids. Abrupt increases in the apparent size of DPPC liposomes were observed in the presence of above 44 mg/ml ethanol, where microscopically plateau membranes form interdigitated structure, in which the acyl chains fully interpenetrate the hydrocarbon chains of the apposing monolayer. On the contrary, in the eggPC liposome, where the membranes cannot form interdigitated structures even in the presence of high concentration of ethanol, such intense aggregation and fusion were not observed, suggesting their intimate relation to the interdigitated structure formation. The formation of interdigitated structures in the adhering region leads to an increase in the interfacial area and an exposure of hydrophobic acyl chain terminal on the surface area, and enhances hydrophobic interactions between two interdigitated bilayers. Thus, the resultant interdigitated structure makes the aggregated state stable and partially initiates the bilayer mixing between the two apposed membranes, leading to fusion.

Keywords: Fusion; Aggregation; Interdigitation; Liposome; Ethanol; Vesicle

1. Introduction

It was now well established that saturated-chain phosphatidylcholines (PCs) can form interdigitated structure phases, in which the terminal methyl group of the acyl chains extend beyond the bilayer midplane, effectively interpenetrating into the opposing monolayer, in the presence of short chain alcohols and some other amphiphilic ligands [1,2]. The induction of the interdigitated phase by alcohols have been studied in detail in our laboratories and others [3–16]. In our previous studies [12,17], the effects of unilamellar liposome sizes (curvature of liposomes) on the ethanol-induced interdigitation in dipalmitoyl phosphatidylcholine were investigated and it was shown that the microscopically plane membranes become interdigitated in the presence of ethanol, this result coinciding well with Boni and co-workers' finding reported at the same time as ours [18,19]. In these studies, it was also found that sonicated liposomes are not stable in the presence of interdigitating concentrations of ethanol; they form higher aggregates at all temperatures examined [12,17].

McConnell and Schullery [20], and Veiro and Rowe [21] indicated that ethanol stimulates the rate of aggregation and/or fusion of sonicated small unilamellar vesicles (SUVs). Recently, it was demonstrated that large liposomes having a vast trapped volume can be produced after exposure of small liposomes to high ethanol concentrations and warming slowly, suggesting the utilization of these liposomes for drug delivery systems [18,19,22]. However, the mechanism for ethanol-induced aggregation/fusion of small liposomes was not made clear.

Basic research into the molecular mechanisms of

Abbreviations: SUV, small unilamellar vesicle; PC, phosphatidylcholine; H_{II}, inverted hexagonal; Q_{II}, inverted cubic; PCS, photon correlation spectroscopy; RET, resonance energy transfer; DPPC, dipalmitoyl-L-α-phosphatidylcholine; MLV, multilamellar vesicle; EM, electron microscopy; GPC, gel permeation chromatography; T_c, gel/liquid-crystalline phase transition temperature; pyrene-PC, 1-palmitoyl-2-pyrenedecanoyl-*sn*-3-phosphocholine.

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biomembrane fusion has been most successful using liposomes [23–28]. Divalent cation ions, peptides such as alamethsine and melittin, proteins such as Influenza virus hemagglutinin and *N*-ethylmaleimide sensitive factor, and some amphiphiles such as polyethylene glycol are known as membrane fusion agents [24,25,29,30]. With respect to overall fusion reactions, it is likely that each fusing agent will display individual characteristics. However, that particular element of the fusion mechanism which describes the initiation of bilayer mixing between the two apposed membranes will be governed by the same basic physical forces which apply to the fusion of two liposomes [27]. It seems that the fusion agents create a local environment which promotes the formation of similar type of intermembrane intermediates which would cause liposome-liposome fusion. They may involve the inverted hexagonal (H_{II}) and inverted cubic (Q_{II}) phases [31].

In this study, the mechanism for ethanol-induced aggregation and/or fusion of uniform-sized SUVs was studied by measuring the apparent average size and the size distribution of liposomes using a photon correlation spectroscopy (PCS) as a function of incubation time. The direct observation of the states in the liposomal solutions using freeze-fracture electron microscopy (EM) and the measurement of resonance energy transfer (RET) using fluorophore-labeled lipids were also performed. A novel mechanism involving the participation of interdigitated structure formation in the stability of adjacent membranes and the initiation of bilayer mixing between the two apposed membranes will be proposed.

2. Materials and methods

2.1. Chemicals

L-α-Phosphatidylcholine, dipalmitoyl (DPPC) (99 + %, crystalline, P-6267) and L-α-phosphatidylcholine from egg yolk (eggPC) (99%, Type V-E, P-5763, lot# 11H8407) were obtained from Sigma (St. Louis, MO, USA). *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Ph-PE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA) and no further purifications were performed. Ethanol was purchased from Wako Pure Chemical Industries, Osaka, Japan. De-ionized and reverse-osmotic treated water was distilled with a quart still once.

2.2. Preparation

The stock solution of PCs was prepared in chloroform/ethanol (99:1, v/v) solution and it was kept in the freezer under a nitrogen gas atmosphere in the dark until ready to use.

Multilamellar vesicles (MLVs) were prepared as follows. The PC stock solution was dried in a rotary evaporator under reduced pressure to form a lipid film on the wall of a round-bottomed flask. This film was left in vacuo for at least 12 h to ensure complete removal of the solvent. Buffer solution, composed of 0.15 M NaCl, 1 mM Tris, 1 mM EDTA and 0.02% NaN_3, adjusted at pH 7.0 using HCl solution, was added to the thin film containing lipid. Nitrogen gas was bubbled to remove any dissolved oxygen and the lipid was hydrated at about 50° C (at room temperature, ca. 20° C in the case of eggPC). During this incubation, the sample was vortexed periodically.

Uniform-sized SUVs were obtained as follows: 4 ml of MLV solution of 75 mM DPPC or eggPC was sonicated at about 50° C (at 4° C in the case of eggPC) for 30 min under a stream of nitrogen gas with a Ultrasonic Generator Model US150 (Nihonseiki Kaisha, Tokyo, Japan) at a power of about 50 W by continuous mode. The samples were centrifuged for 10 min at 2500 rpm to remove the small amount of titanium particles and any large MLV. The size distribution of sonicated liposomes has two peaks typically at 31.5 nm and 68.5 nm, and the fractions of each peak are 91% and 9%, respectively, in the peak analysis by weight. This result coincided with Huang's finding that sonicated liposomes have two peaks in the size distributions but are relatively homodisperse [32]. In order to get uniform size liposomes, 1 ml of resulting suspension was gel-filtered through a Sepharose CL-4B column (24 mm I.D. × 24 cm) using buffer solution as an eluent at the flow rate of 0.72 ml/min at about 20° C. The fractions of liposomes in the eluted solution were detected by monitoring optical density at 402 nm using a BioUV Model AC-5000 monitor (ATTO, Tokyo, Japan). About 5 ml of the eluted solution from 75 ml of elution volume was collected (the typical elution pattern is shown as a dotted line in the left graph of Fig. 4). The average size of fractionated liposomes was about 25 nm in diameter, and the distribution was mono-dispersed (a typical size distribution of the DPPC SUV is represented as an incubation time of zero in the absence of ethanol in Fig. 2).

The aggregation/fusion experiments were carried out by mixing 4 ml of the fractionated liposomal solution and 2 ml of buffer solution containing desired ethanol (this procedure was required in order to avoid high local ethanol concentrations in the SUVs before the sample could be completely mixed) and then, continuously measuring the average size and the size distribution of liposomes by photon correlation spectroscopy at 25° C. The PC concentrations in the sample were kept within 0.8 mM to 1.2 mM.

In the RET measurements the preparations of liposomes were the same as that in the absent of fluorescence dyes except for the addition of *N*-NBD-PE and *N*-Rh-PE stock solutions (chloroform/methanol, 1:9 in v/v) to the DPPC or eggPC stock solution. In these cases, each fluorophore-labeled lipid contained 1 mol% of PCs.

The PC concentrations were determined as those of P_i

according to Bartlett's method [33], which was modified in our laboratory.

2.3. Photon correlation spectroscopy (PCS)

PCS measurements of liposomal solutions were carried out with a Zetasizer 4 photon-correlation spectrometer of Malvern Instruments (Worcs., UK) with temperature control by a Peltier heating/cooling unit. It uses a 632 nm He-Ne laser at a power of 5 mW and the light scattering was measured at 90°. The size was calculated with an NEC Power Mate SX/16i computer (Boxborough, MA, USA) connected with the photometer. The analysis of average size and distribution was performed using software ver. 1.1 supplied by Malvern Instruments, and it contained some automatic correction of the refractive index for layered particles. Average diameters and size distributions were evaluated as a *Z*-average using monomodal method (a cumulant analysis) and as a weight-weighted distribution in diameter using a multimodal method (exponential sampling algorithm), respectively [34,35]. An analysis range in a multimodal method was set from 5 nm to 3 μm in diameter at the first run of each measurement and then, it was reduced to 5–500 nm if there was no peak in the range from 500 nm to 3 μm. Further details are described in a previous paper [36].

Effects of ethanol and other chemicals containing the buffer solutions on refractive index were very small (blow 0.6%) and they were not taken into consideration. However, influences of ethanol on the viscosity of the sample solutions are significantly large, and the corrections were performed using the reported values [37].

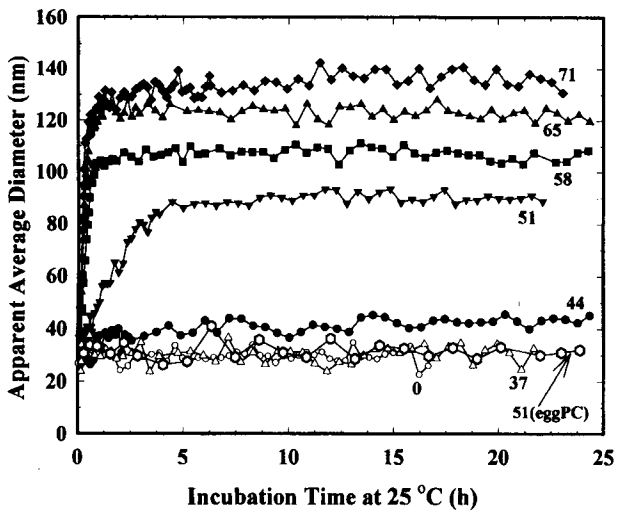

Fig. 1. Changes in apparent average diameters of DPPC and eggPC SUVs as a function of incubation time at a variety of ethanol concentrations. Numerals in the graph depict concentrations of ethanol (mg/ml). Average diameters were evaluated as a *Z*-average using monomodal method (a cumulant analysis) using the PCS measurement; see Materials and methods for details. The lipid concentrations in the sample were kept within 0.8 mM to 1.2 mM.

2.4. Freeze-fracture electron microscopy

Liposomes were frozen in liquid nitrogen from 25° C. Samples were fractured and replicated at a vacuum of $5 \cdot 10^{-7}$ mbar or better at −115° C employing a JEOL JFD-9010 Freeze-Etching Device. Replicas were floated off in chloroform and their micrographs were obtained using a JEOL JEM-1210 electron microscope (Tokyo, Japan) at magnifications of 40 000 ×.

2.5. Fluorescence measurements

In the RET measurements steady-state emission spectra were observed with an excitation at 475.0 nm at 25.0 ± 0.1° C by using a Hitachi F-650 spectrofluorometer equipped with a thermostated cuvette holder. Peak absorbance of samples was kept < 0.1 to reduce inner filter effects [38] and the total lipid concentrations were set in about 50 μM. The bandpass was 2 nm for both excitation and emission monochrometers. The sample was magnetically stirred well during the measurements.

3. Results

3.1. Apparent size changes

Fig. 1 shows changes in average diameters of DPPC or eggPC SUVs as a function of incubation time at a variety of ethanol concentrations. In the absence or presence of 8 to 37 mg/ml ethanol, the apparent size changes of SUVs were very slow and significant changes in the size could not be observed even after 24 h. Above 44 mg/ml ethanol, however, the increments in the apparent size of DPPC SUVs were large and the rate of increasing the size was accelerated. Above 58 mg/ml ethanol, especially, the rapid change in the size happened within 1 h. However, the changes were scarcely observed after about 7 h above 44 mg/ml ethanol. Furthermore, the resultant apparent sizes depended upon the ethanol concentrations and increased with increasing the concentration. On the other hand, in the presence of high concentration of ethanol the significant size changes of eggPC SUVs could not be observed even after 24 h.

Fig. 2 depicts weight-weighted size distribution of DPPC SUVs at various incubation times in the presence of a variety of ethanol concentrations. In the absence and presence of 37 mg/ml ethanol, only the distributions changed. On the other hand, in the presence of 51 or 71 mg/ml ethanol the distribution changes were large and rapid. At the initial step the distribution peaks shifted to the larger size. However, a new peak in the distribution appeared within 1 h and its intensity increased further with time.

3.2. EM photographs and fluorescence energy transfers

The PCS is a very convenient method for the determination of the size of colloidal particles but it has a disadvan-

tage such a difficulty of discriminating between aggregated and fused liposomes. In order to confirm whether the increase in the apparent size of DPPC SUVs ascribes to the aggregation or the fusion, measurements of freeze-fracture EM and RET by mixing fluorophore-labeled lipids were attempted in this study.

Fig. 3 shows the EM photographs of the DPPC liposomal solution in the absence (left) and presence of 65 mg/ml ethanol after 20 h incubation (center and right). The former clearly suggested the high homodispersity of SUVs, being comparable with the results estimated from the elution pattern of gel permeation chromatography (GPC), shown as a dotted line in the left graph of Fig. 4; on the contrary, the latter (center of the photographs) indicates that large aggregates (typically indicated by an arrow A in the photograph) are formed in the suspension containing 65 mg/ml ethanol. The photograph exhibiting large aggregates was very similar to that observed by Boni et al. [19]. It is worth noting that in this photograph (center) a fused-large liposome (indicated as an arrow B) coexist with large aggregated liposomes. Though fused-large liposomes could be find out as shown in a EM photograph (right), the population was little.

In eggPC SUVs solution containing 65 mg/ml ethanol almost liposomes kept small and aggregated liposomes were slightly observed but no fused-large liposome was found even after 20 h incubation.

A bar graph in Fig. 3 shows the size distribution of the same sample and at the same incubation time as that of which the EM photographs were taken in the presence of 65 mg/ml ethanol. The comparison of the photographs with the size distribution suggests that the smaller peak in the distribution corresponds to fused liposomes and the larger peak corresponds to aggregates of small liposomes.

The EM measurement is the most convincing method but it is scarce of a quantitative analysis. On the other hand, the RET method using fluorophore-labeled lipids has some advantages that it is superior to other methods in quantitative measurements, and liposomal fusion mechanisms can be understood in molecular level.

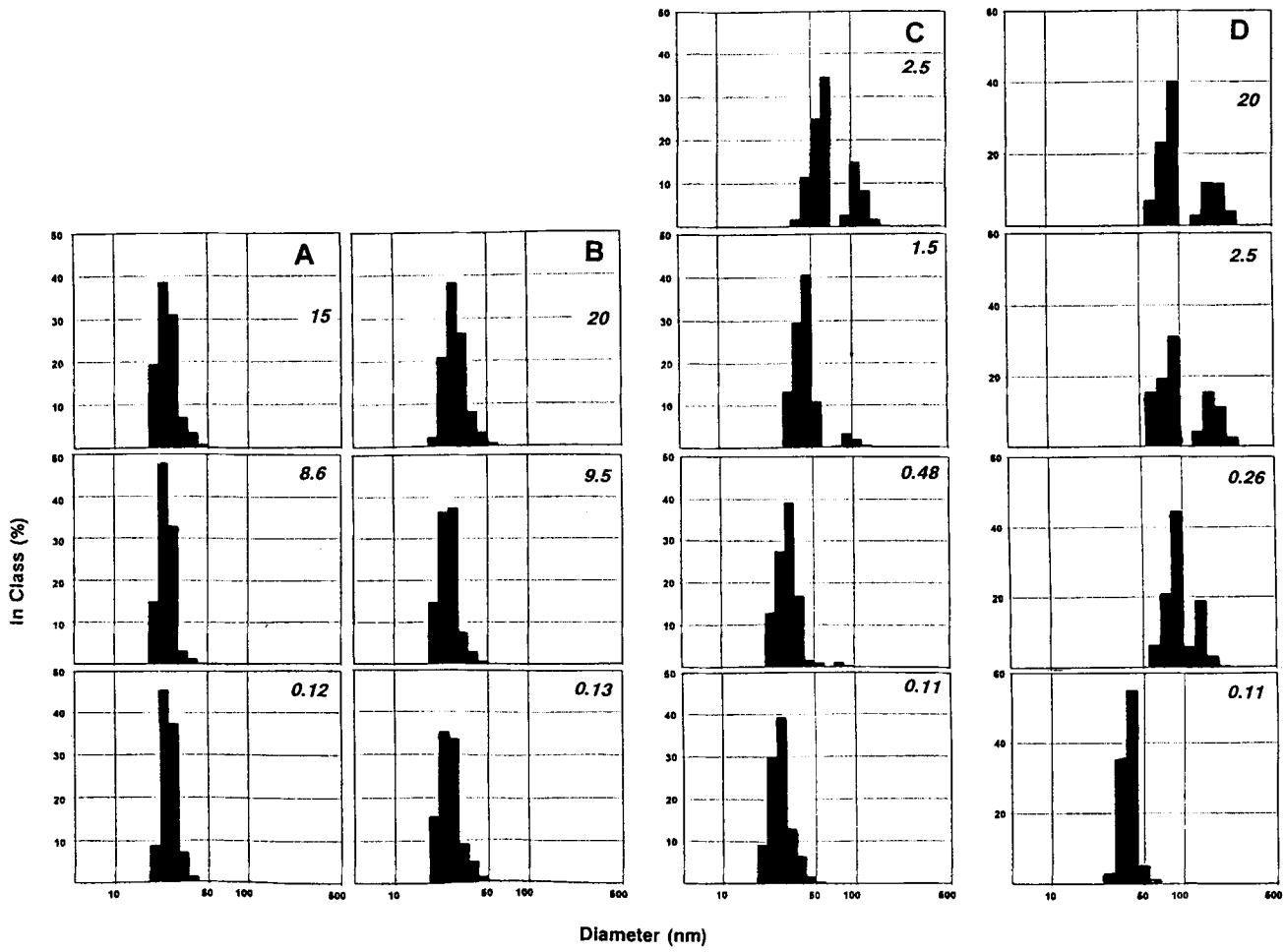

Fig. 2. The change in weigh-size distribution of DPPC SUVs at various incubation times in the presence of a variety of ethanol concentrations. (A) Without ethanol; (B) with 37 mg/ml ethanol; (C) with 51 mg/ml ethanol; and (D) with 71 mg/ml ethanol. A numeral in each graph shows incubation time (hour). Size distributions were evaluated as a weight-weighted distribution in diameter using a multimodal method (exponential sampling algorithm) using the PCS; see Materials and methods for details. The initial DPPC concentrations were kept within 0.8 mM to 1.2 mM.

The dilution technique of the RET method performed in this study is well established [38] and as follows [39]. One population of SUV contains both donor, *N*-NBD-PE, and acceptor, *N*-Rh-PE such that RET occurs. Illumination of light at the excitation wavelength (475 nm) of the donor leads to emission of fluorescence at the wavelengths (λ_{max} = 590 nm) characteristic of the acceptor. Fusion of these liposomes with liposomes not containing fluorophores leads to a dilution of the donor and acceptor such that collisional interactions between them are less frequent. This reduced RET so that acceptor fluorescence is reduced whilst donor fluorescence (λ_{max} = 530 nm) becomes more evident. The detail techniques were described elsewhere [38,39].

In the RET measurements of fluorophore-incorporated SUVs using DPPC or eggPC as a matrix phospholipid, ratios of intensities at 590 nm to those at 530 nm were evaluated as a measure of fusion. However, there was not a significant difference of the ratios between in the absence and presence of high concentration of ethanol even after 24 h in both cases of DPPC and eggPC SUVs, indicating, judging from the combination with the EM photograph measurements, that the addition of ethanol to the DPPC SUV solutions can lead to the aggregation of them but the population of fused liposomes is very small.

3.3. Gel permeation chromatography (GPC) of ethanol-induced aggregated SUVs

The reversibility of the aggregation of DPPC SUVs was investigated by removal of ethanol from the aggregated and/or fused liposomal solutions using GPC. Aggregated liposomes can run down a sizing column according to their disaggregated size rather than their aggregated size [40].

A typical elution pattern of DPPC liposomes after 24 h incubation with 58 mg/ml ethanol is shown as a solid line in the left graph of Fig. 4. Fig. 4 also includes a typical elution pattern of a sonicated DPPC liposome as a measure of size, shown in the bar-graphs A to D on the right side of Fig. 4, corresponding to the size distribution in the fraction of A to D on the elution curve of a sonicated liposome (as described in a dotted line). The incubated DPPC liposomal solution does not contain small particles below 50 nm in diameter at all, as shown in the size distribution of incubated liposomal solution before the removal procedure of ethanol (as depicted in an inserted bar graph in the EM photographs of Fig. 3) but the removal of ethanol by gel filtration resulted in appearance of the small fraction (< 50 nm). This indicates that the aggregation of small liposomes is reversible after removal of ethanol. The fraction of the

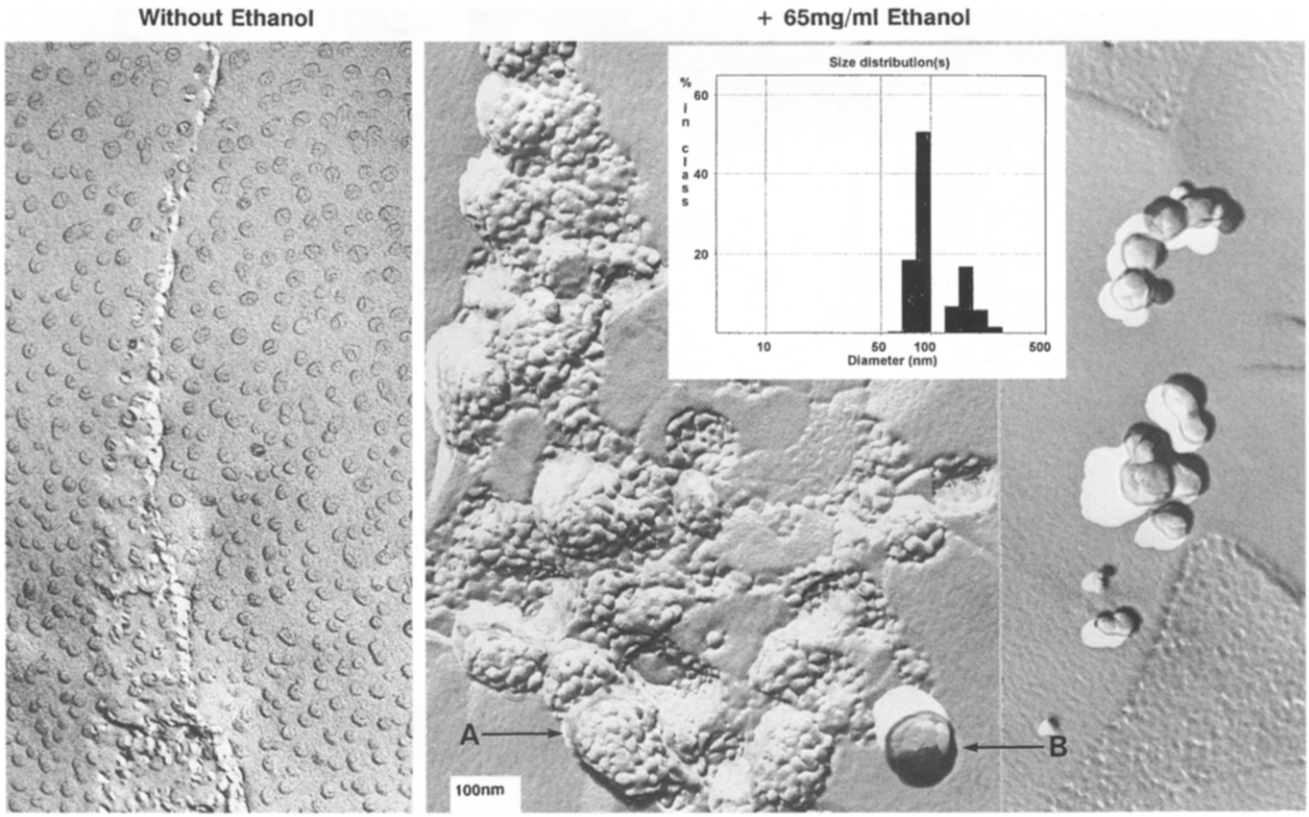

Fig. 3. Photographs of freeze-fracture electron microscope and a weighted-size distribution of DPPC liposomes. Left, before incubation; center and right, after incubation with 65 mg/ml ethanol for 20 h at 25° C (different views of the same sample). Arrows A and B in the center photograph indicate typical large aggregates (a part of aggregates composed of aggregated liposomes) and fused-large liposomes, respectively. A bar graph shows the size distribution of the same sample and at the same incubation time as that of which the EM photographs were taken. Micrographs were obtained at magnifications of 40 000 ×.

medium size still remained; the comparison of these results with the EM photographs suggests that the medium size fraction represents fused liposomes.

3.4. Relations of SUV aggregation / fusion with ethanol-induced interdigitated structure formation of the membranes

Fig. 5 shows the averages size as a function of ethanol concentrations after 14 h incubation of DPPC and eggPC SUVs, after the average sizes have stopped as shown in Fig. 1. Above 44 mg/ml ethanol, an abrupt increment in the apparent size was observed in the DPPC SUVs. It was previously shown that above 44 mg/ml ethanol, membranes of DPPC MLV form the interdigitated structure, as shown in upper place in this figure [3–13]; this concentration is coincident with the ethanol concentration above which the rate of increase in the apparent liposomal size was accelerated. Fig. 5 also shows the fluorescence intensity ratios of pyrene-labeled PC in DPPC MLV liposomes and they were quoted from our previous report [9]. The intensities of the fluorescence vibronic bands of 1-palmitoyl-2-pyrenedecanoyl-*sn*-3-phosphocholine (pyrene-PC), which is a PC analogue with a covalently coupled pyrene moiety at the end of one of its acyl chains, as well as their parent pyrene are known to be sensitive to medium polarity. The intensity ratios of band III (at 387.5 nm) to band I (at 376.5 nm) can serve as a measure of solvent polarity. By the comparison of the ratios of pyrene in a membrane with ratios of pyrene in solvents (alkanol) of known polarity, the polarity around the pyrene moiety in a membrane may be estimated. This measurement provides information about the location of the pyrene group in the bilayer. The decrease in the fluorescence ratio means the increase in the polarity around the pyrene moiety and indicates the formation of interdigitated membrane. The two lines in this figure overlap over this region of ethanol concentrations. On the other hand, little aggregation and

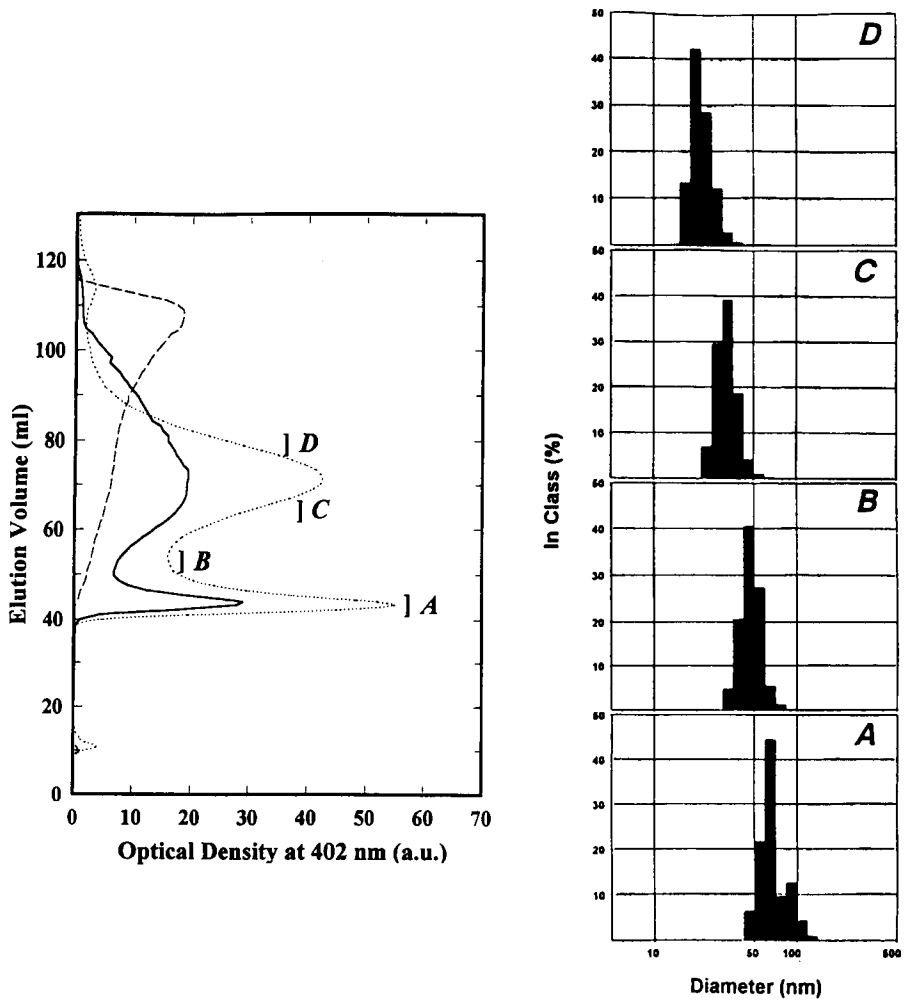

Fig. 4. Typical elution patterns of DPPC SUV incubated with ethanol and a sonicated DPPC liposome. Solid line in the left graph, liposome after 24 h incubation with 58 mg/ml ethanol; dotted line in the left graph, sonicated liposome; and dashed line in the left graph, buffer with 58 mg/ml ethanol. The solid line is subtracted the apparent optical density of the buffer in the presence of 58 mg/ml ethanol (the dashed line) from over all elution volume. Bar graphs A to D on the right side of this figure, the size distributions corresponding to the fractions of A to D on the elution curve of a sonicated liposome (as described in a dotted line). These bar graphs are shown as a measure of size in each fraction. Ethanol concentration (a dashed line) could be detected as an apparent change in absorbance due to the refractive index change of the eluting solution.

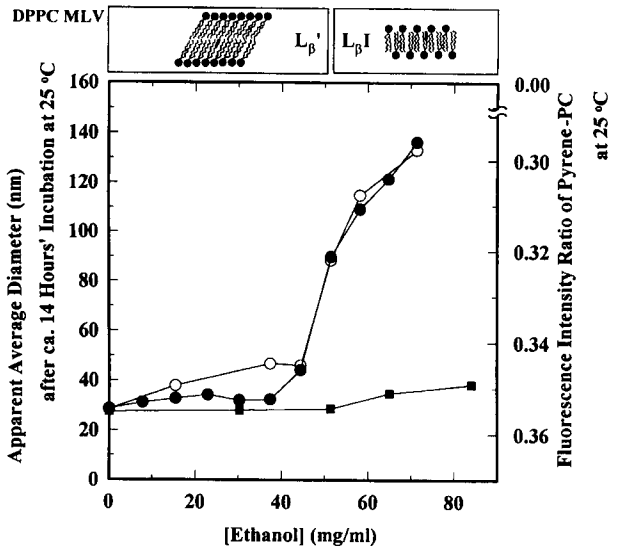

Fig. 5. Apparent sizes of DPPC and eggPC SUVs after 14 h incubation, fluorescence intensity ratios of pyrene-labeled PC (pyrene-PC) in DPPC MLVs, and schematic phase structures of DPPC MLV as a function of ethanol concentrations. Filled circles, average diameters of DPPC SUVs; filled squares, average diameters of eggPC SUVs; and open circles, fluorescence intensity ratios. Fluorescent intensity ratios were quoted from our previous report [9]. The intensities of the fluorescence vibronic bands of PC analogue with a covalently coupled pyrene moiety at the end of one of its acyl chains are sensitive to medium polarity. The intensity ratios of at 387.5 nm (band III) to that at 376.5 nm (band I) can serve as a measure of solvent polarity. The decrease in the fluorescence intensity ratio means the increase in the polarity around the pyrene moiety of pyrene-PC. See the text and Ref. [9] in detail.

fusion were observed in the eggPC SUVs. It was already shown that eggPC does not form interdigitated structures even in the presence of high concentration of ethanol [1,2].

These evidences clearly suggest that the aggregation/fusion induced by ethanol are intimately related to the interdigitated structure formation of the membranes.

4. Discussion

4.1. Forces between liposomal membranes

The distance between two liposomal surfaces is governed by a balance among several nonspecific long- and short-range interactions [41]. At long range, a repulsive electrostatic (double-layer) force balances an attractive van der Waals force, as described in the classic DLVO theory [42]. The former force, which arises from ion binding, was insignificant in monovalent salt solutions, e.g., NaCl up to 1 M [43]. Taking into account only attractive van der Waals and repulsive electrostatic forces, we would expect that in the absence of any strong electrostatic repulsion all bilayer surfaces should come into strong irreversible adhesive contact with no water remaining between them. That this does not occur is due to the existence of an additional strongly repulsive force at short range below 1–3 nm, commonly referenced to as a hydration force, a solvation force or a structural force [43], which has been observed using the osmotic pressure technique on PC multilayers [44]. It has now been found to occur in many systems, and in particular, this force occurs between zwitterionic lipid bilayers of PCs and phosphatidylethanolamines [45]. Their highly hydrated head groups ensure that their bilayers will not adhere or aggregate strongly, and will not fuse, even under conditions where there is no repulsive electrostatic force.

Repulsive hydration forces between two liposomal bilayers arise whenever there are hydrated (hydrophilic) surface groups. As the two surfaces approach each other, the water between them must be removed to the bulk solution, which is energetically unfavorable and appears as repulsive hydration forces between them [46]. The range of the forces so far measured between amphiphilic surfaces is 2–3.5 nm, below which the force rises steeply, dominating over the van der Waals and electrostatic forces [43].

4.2. Aggregations of small liposomes

In terms of bilayer structure unsonicated (multilamellar) or large (unilamellar) liposomes and small liposomes such as sonicated vesicles differ primarily in their surface curvatures [47]. It has been pointed out that liposomal curvature can have a profound influence on the molecular packing of the phospholipid molecules in a bilayer. More specifically, it has been argued that the packing arrangements of these molecules in flat bilayers are more regular than in bilayer liposomes – 30 nm in diameter.

Aggregation and/or fusion of liposomes take place in order to dissipate an excess of surface energy originating from the distortion of their molecular packing [48] and would undergo rearrangements in their molecular packing to attain a state of greater stability once this opportunity is rendered. In fact, evidence is accumulating to indicate that small liposomes have the tendency to undergo the fusion below the gel/liquid-crystalline phase transition temperature (T_c) for a long period (e.g., see [20,49]). However, in our case as shown in the absence of ethanol in Fig. 1, the size changes were very slow, and significant changes in the size could not be observed even after 24 h although the measurements were performed below T_c. This was also confirmed by the EM photographs and the RET technique. It is necessary for the achievement of aggregation and fusion that an excess energy due to the high curvature of small liposomes should be superior to the repulsive hydration force. This indicates that under our experimental conditions the excess surface energy of small liposomes is too small to overcome the barrier of dehydration of water between liposomal surfaces for 24 h.

Above 44 mg/ml ethanol, on the other hand, the increments in the apparent size of DPPC liposomes were large and the rate of increase in the size was accelerated.

The EM photographs of the liposomal solutions indicated that large aggregates were formed in the suspensions.

In general, water molecules associated with hydrophobic groups of PC molecules (as a repulsive hydration force) prevent aggregation between liposomal membranes, as already described. In the presence of high concentration of ethanol, the associated water molecules are displaced with ethanol molecules, which adsorb on the membrane surface or distribute into the hydrophobic region of membranes [50]. This displacement may induce DPPC liposomal aggregations due to the disappearance of highly hydrated water on the bilayer surfaces. The reversibility of aggregation was indicated by the ethanol-removal experiment, as shown in Fig. 4, supporting this mechanism. The aggregation of small liposomes by 'dehydrating' agents such as ethanol has been discussed by several researchers. For example, Massenburg and Lentz reported on the effects of poly(ethylene glycol) on DPPC large unilamellar vesicles [51]. In the eggPC SUVs, however, the aggregation were not observed in spite of the same surface structure as the DPPC SUVs. This indicates that only the disappearance of rehydration force by the displacement can not induce the aggregation in a short time, suggesting the participation of other inducing factors to the aggregation.

As shown in Results, the ethanol-induced aggregation is closely related to the interdigitated structure formation of the membranes. Above 45 mg/ml ethanol, however, interdigitated membranes are formed in the multilamellar or large unilamellar liposomes, where membranes are microscopically flat [12,17], while in the small liposomal membranes, having high curvature, the formation of interdigitated membrane can not be induced by ethanol [18,19,22]. Therefore, it may seem that in the DPPC SUVs having average diameter of about 25 nm the membranes can not form interdigitated structures. But assuming that the membrane come into contact over a small region and that the contact zone spreads out, the regions composed of adhesive bilayers may be formed in the contact region, where the interdigitated structure can be developed because of the formation of plateau membranes.

In the formation of interdigitated membranes, water molecules located at the interface region must be replaced by a bulkier solvent molecule such as ethanol which creates an increase in the interfacial area [1,2]. This surface-area increase allows the positioning of the acyl-chain terminal methyl groups at the bilayer interface region. This leads to the exposure of hydrophobic acyl chain terminal on the surface area of the membranes and increases the hydrophobic interaction between two interdigitated bilayers. Thus, the resulting interdigitated structure can induce the high stability of the aggregated state through the intense hydrophobic interaction.

Simon et al. [41] measured the hydration repulsive pressure between PC bilayers as a function of area per lipid molecule by a comparison of X-ray diffraction data from three different lipid structures: the gel, liquid-crystalline and interdigitated phases. They clearly demonstrated that the magnitude of the hydration pressure significantly decreases with increasing area per molecules and for the interdigitated phase, which is thinner than normal bilayer phases and has the largest area per lipid molecule among bilayer phases. It was suggested that the hydration pressure is the largest for the gel phase and smallest for the interdigitated phase. Thus, the small repulsive hydration force in the interdigitated structure also contributes the stabilization of the adjacent state of two bilayers.

4.3. Fusion

Several models of fusion have been suggested, often involving intermediate nonbilayer structures such as an H_{II} phase, based on EM photographs of small liposomes or membranes [31]. Other proposed models, such as the 'stalk' model, have been based on optical visualization and capacitance/conductance measurements of black lipid membranes [52].

The stalk model involves the stochastic exposure of a small hydrophobic group from within the membrane which protrudes from the surface as a 'stalk', leading to fusion [52]. Earlier work on calcium ion-mediated fusion [53] also suggested that the occurrence of fusion is due to an increased hydrophobicity arising from the condensation of head groups.

Helm et al. [54] found that while increasing the attractive or decreasing the repulsive component of the total interaction between two bilayers will always lead to increases adhesion, whether it also favors fusion depends on the origin of the attraction. In particular, Helm et al. [54], and Marra and Israelachvili [43] found that the existence of a strongly attractive van der Waals force is not sufficient to promote the fusion of bilayers. The reason for this appears to be that these attractive forces act mainly between the outer surface head-groups and not between the interior parts of the membranes. On the other hand, when the membranes are stressed so as to expose hydrophobic groups previously buried within the membranes, the resulting increase in adhesion also leads to fusion.

Helm and Israelachvili [55] proposed that fusion is likely to be caused by the bilayer thinning induced by the osmotic and electric field stresses, and by an increase in the hydrophobic area exposed between two adjacent bilayers. They have shown that an increase in the area by only a few percent above the equilibrium can initiate fusion.

As described above, many workers have suggested that the exposure of hydrophobic groups to the surface of membranes is necessary to induce the joining of the adhering two bilayers. In the formation of interdigitated membranes, as described above discussion, the positioning of the acyl-chain terminal methyl groups at the bilayer interface region [1,2] leads to the exposure of hydrophobic acyl chain terminal on the surface area of the membranes and

increases the hydrophobic interaction between two interdigitated bilayers. Thus, the resulting interdigitated structure can lead the initiation of bilayer mixing (joining of lipids) between the two apposed membranes.

MacConnell and Schullery [20] demonstrated that glycerol, ethylene glycol, propylene glycol and ethanol dramatically increase the fusion rate of sonicated small DPPC liposomes. It is well established that these solutes can induce the formation of the fully interdigitated structure in PC membranes [1,2,13,56,57]. This supports the possibility that the interdigitated structure formation contributes to the acceleration of the fusion process of small liposomes by ethanol. They also found that the incorporation of cholesterol to the small liposomal membranes results in the inhibitions of the fusion [20]. In our previous paper [9] it was suggested that the ethanol induction of interdigitated structure is prevented by the presence of cholesterol. This also supports the intervention of the interdigitated formation in the ethanol-induced aggregation and/or fusion process of small liposomes.

Our results on aggregation (adhesion) and fusion lead to the following suggested intermediate stages, as depicted in Fig. 6 as a schematic illustration: two small bilayers experience a weak initial van der Waals attraction, then a closer approach, a much stronger repulsion due to a hydration forces. However, due to the displacement of hydrated or binding water to head groups of lipids by ethanol distributing or adsorbing to the bilayers, the hydration force becomes weak, initiating the aggregation of liposomes. In the next step, the water binding and/or hydrated to head groups of PC molecules is removed to the bulk solution, and the head groups come into contact over a small region. The contact zone spreads out and the regions composed of adhesive bilayers are formed in the contact region, where the interdigitated structure can be developed because of the formation of microscopically flat membrane, leading the stabilization of aggregated (adhered) state of liposomes. The resultant interdigitated structure enhances the interaction of adhering bilayers (lipids), leading to the initiation of bilayer mixing (joining of lipids) between the two apposed membranes. Then the fission of the interdigitated membranes happens through the joining of lipid molecules.

Our important conclusion is that the attractive hydrophobic interaction between bilayers leads to bilayer-bilayer aggregation and/or fusion, as suggested by Helm and Israelachvili [55]. There have been many reports of fusion occurring because of the exposure of hydrophobic domains within protein or membranes [58,59]. The formation of interdigitated structure domain in biomembranes could be one of the triggers causing the membrane adhesion and/or fusion. The formation of interdigitated bilayers in biological membranes has not yet been observed but the possibilities have been suggested by many studies which have been carried out using model membranes [1,2].

Many studies [20,21,60] have suggested that ethanol stimulates the rate of aggregation and fusion of sonicated SUVs and this corresponds with our results. It was also demonstrated the application of ethanol-induced fusion of small liposomes to a novel preparation of large liposomes having a vast trapped volume [18,19,22]. On the other hand, Zeng et al. [61] recently argued that ethanol-induced size change is due to vesicle aggregation, not fusion. This discrepancy is perhaps due to the difference in vesicle size being used at the beginning of the experiment, where Zeng et al. used large unilamellar vesicles of which size is about 100 nm in diameter. They carried out size distribution measurements using the PCS measurements to confirm the aggregation/fusion of liposomes but the EM measurements were not attempted. They have both advantage and disadvantage for the measurements, as already mentioned. This may be one of factors for the different conclusion. The aspect of differences in the vesicle size warrants further study.

In summary, the interdigitated-membrane formation induced by ethanol allows the positioning of the hydropho-

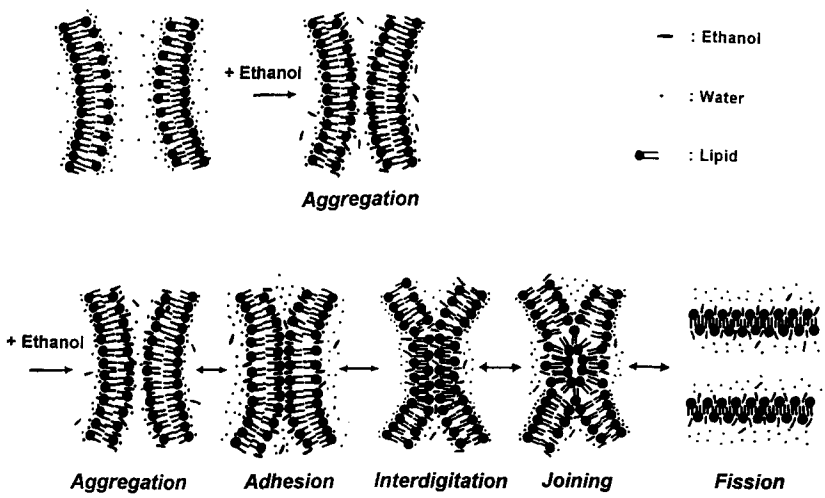

Fig. 6. Mechanism of the ethanol-induced DPPC SUV-SUV aggregation and fusion.

bic methyl groups of acyl chain terminal at the bilayer interface region causing both the thinning of membranes and the increasing surface area. The resultant interdigitated structure leads to the stabilization of DPPC SUV aggregates and the initiation of bilayer mixing due to the enhanced hydrophobic interaction between the two apposed membranes in the adhering area.

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