Relationship between the Emulsion Stability and Phospholipid Distribution in the Aqueous Phases Inside and Outside of an Emulsion Droplet

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The distribution of phospholipids between the inside and outside of an emulsion droplet was studied in relation to the emulsion stability. The emulsion was prepared with various mixtures of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC), *n*-decane and water containing HCl, NaCl or NaOH. ^{31}P -NMR spectra of the emulsion in the presence of lanthanide ion Pr^{3+} allowed quantitative measurement of the phospholipids in the internal and external aqueous phases, and in multilamellar vesicles. By a phospholipase A_2 treatment of PC facing the continuous aqueous phase, most of the internal phospholipids were suggested to be composed of PC. The ratio of the phospholipids inside and outside changed with the emulsifying conditions, and was well correlated with the emulsion stability. Thus, it is suggested that the phospholipid distribution in the emulsion of PC and LPC showed the change of their hydrophile-lipophile balance and of the interfacial absorption force.

The composition of phospholipids varies among species, organs, and even between the inside and outside of biomembranes.¹⁾ The asymmetrical distribution of membrane phospholipids allows a structural barrier to be formed such as cell membranes or liposomes.²⁻⁶⁾ A structural barrier like the water-oil-water type of emulsion has often been observed by emulsifying two or more kinds of emulsifiers of different hydrophile-lipophile balance.^{7,8)} Phosphatidylcholine (PC) is one of the most common phospholipids for constructing biomembranes. Lysophosphatidylcholine (LPC), although it exists as minor component in phospholipids, has higher water solubility and emulsifying properties, and interacts with PC by the wedge mechanism to change the structure and the fluidity of a membrane.^{9,10)} Thus, we have been studying the formation of emulsions with the diverse interaction of PC and LPC.

Previously, we investigated the headgroup motion of emulsified PC and LPC by the line widths of ^{31}P -NMR and the T_2^* relaxation time of ^{13}C -NMR spectra, and found that the

emulsion stability was well correlated with the headgroup motional properties.^{11,12)} Namely, in the emulsion system composed of PC, water and *n*-decane, the line width of the phosphorus signal was extremely broad, indicating that most of the PC had aggregated to form lamella. On the other hand, an equimolar mixture of PC and LPC gave a stabler emulsion than that of PC alone, and the phosphorus signal narrowed. This result suggested that the PC was located at the interface of the emulsion by fusion of the lamella structure with LPC; consequently, the headgroup of PC had motional freedom. Moreover, as PC and LPC change their hydrophile-lipophile balance depending on the aqueous phase conditions, it can be expected that some of the emulsifying conditions allow the formation of an internal aqueous phase, and phospholipids would be distributed partly at the internal interface of the oil particles.

In this paper, we discuss the relationship between the formation of the internal aqueous phase and the stability of an emulsion containing PC and LPC. In order to study the distri-

bution of phospholipids on the inside, outside, at the interfaces and in the lamella. ^{31}P -NMR was used in the presence of the lanthanide probe ion Pr^{3+} in the continuous aqueous phase of the emulsion.

Materials and Methods

Preparation of aqueous dispersions and emulsions of phospholipids. PC and LPC were obtained from egg lecithin as previously described,¹¹⁾ and were separately dispersed in deionized water prior to mixing. After they had been mixed and the aqueous phases conditioned by the addition of a 0.1 M HCl, NaCl or NaOH solution, they were agitated by a disperser (Physoctron fitted with an NS-10 generator shaft) at 20,000 rpm. These aqueous dispersions of the mixture of PC and LPC were submitted to ^{31}P -NMR measurements in the absence and presence of 5 mM PrCl_3 . The emulsion was obtained by the addition of *n*-decane (5 ml) dropwise to the aqueous dispersion of phospholipids (5 ml) with agitation by the disperser. The emulsion stability was evaluated by the change of particle size distribution, which was measured by a particle size counter (Coulter Counter, Model T_{411}), and the ratio of the separated aqueous layer after 24 hr standing at 25°C. The emulsion was also analyzed with a ^{31}P -NMR spectrometer in the presence and absence of Pr^{3+} .

Measurements of the ^{31}P -NMR spectra. ^{31}P -NMR spectra were recorded on a JEOL GX-270, employing a 45° pulse, with 32-k data points, a 4000 Hz spectral window and 1.5 sec pulse delay, the protons not being decoupled. The ionic concentration of Pr^{3+} (5 mM) was obtained by pipetting 10 μl of a D_2O stock solution of PrCl_3 into NMR tubes. It has been shown that these peak areas can be used reliably for a quantitative analysis of phospholipids.¹³⁾ The down-field shift is dependent on the concentration of Pr^{3+} because of the pseudo-contact shift effect of paramagnetic Pr^{3+} in rapid exchange between the phosphate sites on the headgroups.¹⁴⁾

Phospholipase A_2 treatment of the emulsified PC. The aqueous dispersion of 64 mM PC and of equimolar mixtures of PC and LPC in various concentrations was emulsified with *n*-decane and deionized water, and then treated with phospholipase A_2 . An aqueous solution of phospholipase A_2 and CaCl_2 was pipetted into the emulsion to give 200 units/ml and 2 mM Ca^{2+} in the aqueous phase, and this was allowed to stand at 25°C. The amount of unaltered PC was determined by HPLC¹¹⁾ after the emulsion had been acidified with 0.1 M-HCl and dispersed in acetonitrile. In addition, dioctanoyl-PC was prepared by the acylation of glycerophosphorylcholine,¹⁵⁾ and the emulsion of dioctanoyl-PC (64 mM) was also treated by phospholipase A_2 for use as a model emulsion in which

most of the phosphatidylcholine faced a continuous aqueous phase.

Results and Discussion

^{31}P -NMR spectra of the aqueous dispersion and the emulsion of phospholipids in the absence and presence of Pr^{3+}

The headgroup of LPC has motional freedom in the globular micelle to give a narrow ^{31}P -NMR spectrum of *c.* 50 Hz. The signal was shifted down-field with the addition of Pr^{3+} , indicating that most of the polar headgroups of LPC faced a continuous-aqueous phase. The phosphorus signal of PC in the aqueous dispersion was broadened to more than 2000 Hz, which was caused by tight restriction of the headgroup in the lamella, and the chemical shift of the signal scarcely changed even in the presence of Pr^{3+} . In the mixtures of PC and LPC, the phosphorus signals became broader with the increase of PC/LPC ratio. When the ratio was more than 1/2, ^{31}P -NMR spectrum in the presence of Pr^{3+} clearly showed the existence of phospholipids in the lamella state.

Figure 1 shows ^{31}P -NMR spectra of equimolar mixtures of PC (32 mM) and LPC (32 mM) emulsified with *n*-decane and an aqueous phase of 10 mM HCl, NaCl or NaOH. With HCl and NaCl, no lamella signal was observed, and the signal was split by the addition of Pr^{3+} , however, the phosphorus signal showed a complete down-field shift by the addition of Pr^{3+} before the emulsification. Thus, the split signal indicates the existence of discontinuous aqueous phases by forming a W/O/W emulsion, and a part of the phospholipids is presumed to face the internal aqueous phase, in which Pr^{3+} was not distributed by adding it into the continuous aqueous phase after emulsification. In the aqueous dispersion of PC and LPC, the amount of internal phospholipids was less than 10%; however, it was markedly increased with emulsification. The line width of the inside signal was about 150 Hz, and no broadening or down-field shift was observed despite the increase of Pr^{3+} in the continuous aqueous phase. The decrease of

Fig. 1. ^{31}P -NMR Spectra of Equimolar Mixtures of Emulsified PC and LPC in the Absence (a–c) Presence (a'–c') of 5 mM Pr^{3+} in the Aqueous Phase.

The emulsion was composed of PC (32 mM), LPC (32 mM), *n*-decane and water containing a, a') 10 mM HCl, b, b') 10 mM NaCl, c, c') 10 mM NaOH. The spectra show the phospholipid signal inside (I), outside (O) and in the lamellar state (L).

the phosphorus signals of lamella and the increase of the phospholipids distributed in the internal aqueous phase suggested that PC in multilamellar vesicles in the continuous aqueous phase had been promoted to pass into the *n*-decane phase, and that this PC had head-group motional freedom on the internal interface. The ratio of internal/external phospholipids in the emulsion varied with the acid concentration, and reached a maximum in the conditions of 5 mM to 50 mM HCl.

Conversion of PC and dioctanoyl-PC by phospholipase A_2

The emulsions with PC, dioctanoyl-PC and the mixture of PC and LPC were treated with phospholipase A_2 , and the amounts of un-

altered PC and dioctanoyl-PC according to time are shown in Fig. 2. The whole phosphorus signal of the dioctanoyl-PC in the emulsion showed a down-field shift; hence, their headgroups were confirmed to face the continuous aqueous phase, and they were hydrolysed to LPC (1-mono-octanoyl) rapidly by phospholipase A_2 . On the other hand, the conversion of PC in the emulsion was slow in comparison with that of dioctanoyl-PC. In the emulsion composed of PC, *n*-decane and water, the phosphorus signal shows that most of PC was in the lamellar state¹²⁾; accordingly, it can be presumed that most of the PC remained in multilamellar vesicles in the aqueous phase of the emulsion, and that it was prevented from being converted by phospholipase A_2 .¹⁶⁾

The emulsion of the equimolar mixtures of PC and LPC soon reached a plateau and the amount of unaltered PC gradually decreased. The result indicates that phospholipase A₂

Fig. 2. Amounts of Unaltered PC by the Phospholipase A₂ Treatment (mol% of the Total Phospholipids) in the Emulsion Composed of Equivolumes of *n*-Decane and Deionized Water Containing ● 32 mM PC and 32 mM LPC, ▲ 16 mM PC and 16 mM LPC, ■ 8 mM PC and 8 mM LPC, □ 64 mM DiOctanoyl PC and ○ 64 mM PC. The times on the abscissa indicate the periods of enzymatic treatment.

quickly attacked PC facing the continuous aqueous phase, and after that, the reaction proceeded slowly with the permeation of phospholipase A₂ or the exchange of phospholipids between the internal and external interfaces until all the PC had been converted to LPC. Accordingly, the amount of unaltered PC just after it reached a plateau can be regarded as the PC located inside the oil phase and in the multilamellar vesicles. The molar ratio of unaltered PC was increased by the dilution of the mixture of PC and LPC in the emulsion. In ³¹P-NMR spectra of their emulsified mixture, the ratio of phospholipids located on the internal interface increased as the concentration of phospholipids in the emulsified mixture decreased (Fig. 3a), and the amount of internal phospholipids agreed well with that of the unaltered PC after the phospholipase A₂ treatment. Moreover, as no phosphorus signal of lamella PC was observed, the result suggests that most of the internal phospholipids were composed of PC. PC has been known to form

Fig. 3. Proportion of Phospholipids in the Internal (I) and External (O) Aqueous Phase and in the Multilamellar Vesicle (L).

The emulsions were composed of equimolar mixtures of PC and LPC of various concentrations, *N*-decane and an aqueous phase (a, deionized water; b, 10 mM HCl). The molarity on the abscissa indicates the respective concentration of PC and LPC in the aqueous phase. The stability of the corresponding emulsions shown below was evaluated by the proportion of separated aqueous layer (Δ) and the change of mean diameter (●) of the emulsion droplets. The dotted line represents a phase inversion.

a water pool in non-polar solvents¹⁷); therefore, PC can be presumed to participate in the formation of an internal aqueous phase in the emulsified mixture of PC and LPC.

Relationship between the phosphorus signals in the presence of Pr^{3+} and the emulsion stability

In the emulsion under deionized aqueous phase conditions, the ratio of the separated aqueous phase was lower than 5% when the concentration of phospholipids was in the range of 16 mM to 32 mM (Fig. 3). When the concentration of phospholipids was less than 8 mM, the mean diameter of the oil particles increased markedly during standing. The ratio of the separated aqueous phase become higher and small amount of lamella could be observed as the concentration of phospholipids increased; however, the distribution of particle size was almost unchanged during 24 hr standing after emulsification. In acidic conditions, the amount of internal phospholipids was

large in comparison with the deionized conditions, and no lamella signal was observed even at a high concentration of phospholipids. The emulsion in the acidic condition showed a small change of particle size and low proportion of the separated aqueous layer. However, when the concentration of phospholipids was less than 16 mM, a phase inversion occurred to separate the aqueous layer during standing. These results suggest that, in the emulsion containing concentrated phospholipids in deionized water, excess phospholipids in the continuous aqueous phase promoted aggregation of the oil particles. As the particle size of the emulsion, however, was scarcely increased, it can be presumed that the oil particles were prevented from merging with each other by the phospholipids in the continuous phase. In addition, oil particles in the diluted phospholipid conditions merged to increase their size, and the proportion of the separated aqueous layer became larger as the amount of phospholipids decreased.

Fig. 4. Phospholipid Ratios of I/O/L (See Fig. 3) in the Emulsions Composed of 64 mM Phospholipids (PC and LPC in Various Compositions), *n*-Decane and an Aqueous Phase (a, 10 mM HCl; b, 10 mM NaCl; c, 10 mM NaOH).

Below is shown the ratio of the separated aqueous layer of the corresponding emulsions. The dotted line represents a phase inversion.

Emulsion stability with various PC/LPC ratios

The amount of internal phospholipids and the emulsion stability change with the ratio of PC/LPC. Figure 4 shows the ratio of phospholipids on the internal and external interfaces and in the lamella state. In the aqueous phase of 10 mM HCl, the ratio of phospholipids inside increased corresponding to the amount of PC. No lamella signal was observed by ^{31}P -NMR, and a phase inversion occurred when the PC/LPC ratio was more than 3. On the other hand, in the aqueous conditions of 10 mM NaCl or NaOH, the amount of the separated aqueous layer in the emulsion became markedly large with the existence of lamella signals. These results suggest that the oil particles became prone to aggregate in the presence of multilamellar PC. This was also confirmed by comparing the separation rates of the aqueous phase between the emulsions of LPC and those containing multilamellar PC which was added after emulsification. In the emulsion system composed of *n*-decane, deionized water and LPC in the range of 8 mM to 32 mM, no aqueous phase was observed after 1 hr; however, in the emulsions containing 32 mM PC, 2% to 15% of aqueous phase was separated.

Conclusion

The proportion of phospholipids located inside and outside the interfaces and in the lamella states was measured by ^{31}P -NMR spectra. It varied on the aqueous phase conditions, and on the composition or concentration of the phospholipids. In a stable emulsion, most of the phospholipids were shown to be located on inside and outside the interfaces; however, with increasing of lamellar PC, the ratio of PC inside decreased and separation of the aqueous layer was promoted.

PC and LPC are amphoteric emulsifiers, and hence their hydrophile-lipophile balance

changes depending on the degree of dissociation of the phosphate and the quarternary ammonium residues. In an emulsion system composed of phospholipids, *n*-decane and water, a change of phospholipids distribution was clearly observed by ^{31}P -NMR spectra, and was presumed to be correlated with the varied hydrophile-lipophile balance and the interfacial absorptive property of phospholipids.

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