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Studies on the Effect of Water-Soluble Additives and on the Encapsulation Mechanism in Liposome Preparation by the Freeze-Thawing Method

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In order to obtain information for a formulation study of liposomes prepared by the freezing-thawing (FT) method, the influence of various water-soluble additives and conditions of preparation (such as storage temperature during the freezing process) on the properties of the liposomes was investigated using L-asparaginase (A-ase) as a model drug. Turbidity ($A_{1\%}$), an index of the particle size, was decreased by NaCl, $CaCl_2$, $MgCl_2$ and glucose, but scarcely affected by KCl, bovine serum albumin or gelatin. The encapsulation efficiency ($EN\%$) was decreased by all additives, but to differing extents. By optical microscopic observation, the generation of large yolk phospholipid (YPL) aggregates was found during preparation without any additive, but was not observed in the presence of NaCl. In the latter case, the mixture visibly froze at below $-16\,^{\circ}C$, but both $EN\%$ and $A_{1\%}$ were low when it was stored at $-20\,^{\circ}C$, whereas they increased remarkably when the storage temperature was lowered to below the eutectic point ($-21\,^{\circ}C$) of NaCl. Both $EN\%$ and $A_{1\%}$ became maximum at a certain temperature and decreased at lower temperatures, and this suggests that the cooling velocity also affects them. Based on differential scanning calorimetry measurements, unfrozen water was presumed to be present around YPL particles and the amount of this water seems to affect $EN\%$ very significantly.

A schematic description of the encapsulation mechanism in the FT method is proposed.

Keywords——encapsulation mechanism; freeze-thaw; liposome; L-asparaginase; yolk phospholipid; water-soluble additive; particle size; optical microscopy; differential scanning calorimetry; eutectic point

Liposomes continue to prove useful as drug carriers, models of cell membranes, matrices for reconstitution of membrane proteins, and so on.[1] We have presented two novel methods for preparing liposomes, the freeze-thawing (FT) method[2] and the freeze-drying method,[3] which have various advantages.[4] In the FT method, improvement of the encapsulation efficiency ($EN\%$), which has been regarded as extremely important for the development of a liposomal dosage form,[5] was attained by controlling the preparation conditions adequately,[6] and the obtained liposomes were confirmed to act as good drug carriers when given by intramuscular administration.[7]

From the viewpoint of a practical formulation study for an injectable dosage form, which may be the preferred route for therapeutic use of liposomes, various additives are necessary to adjust the osmotic pressure and pH, or to increase the stability. In this paper, the influence of such materials as electrolytes, sugars and proteins on the properties of liposomes was investigated using L-asparaginase (A-ase) as a model drug. At the same time, the effect of the conditions of preparation, such as the storage temperature at which yolk phospholipid (YPL) suspension was kept frozen for 24 h before thawing, was examined.

These studies are interesting from not only practical but also fundamental physicochemical viewpoints with regard to the encapsulation mechanism. Liposomal vesicles are well known to consist of phospholipid concentric bilayers and water spaces.[8] Thus, the water

captured in the narrow spaces of vesicles may have different properties from bulk water,[9] and the nature of the water during the freezing process can affect with the properties of the generated liposomes.[10] Thus, additives may be presumed to influence the water structure, though no detailed studies have been performed so far. Thus, thermal analysis of YPL suspension in the temperature range below the freezing point and morphological observation were performed together with evaluation of the generated liposomes. A possible mechanism of encapsulation by the FT method is proposed on the basis of the results.

Experimental

Materials——Yolk phospholipid (YPL) was extracted from egg yolk as described previously.[2] L-Asparaginase (Leunase injection, denoted as A-ase), gelatin and bovine serum albumin (BSA) were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), Difco (U.S.A.) and Sigma (U.S.A.), respectively. All other chemicals (sodium chloride, potassium chloride, calcium chloride, magnesium chloride, glucose and D-mannitol) used were of the highest grade commercially available.

Preparation of Liposomes——Liposomes were prepared by the freeze-thawing (FT) method as described in the previous paper,[2] as follows: 1 ml of YPL suspension (10% (w/v) in 0.05 M Tris–HCl buffer (pH 7.4), this buffer is abbreviated as only Tris buffer hereafter) was mixed with 1 ml of Tris buffer containing A-ase (20 IU/ml) and a water-soluble additive in a 10 ml test tube and was frozen at -20 ± 1 °C in a controlled freezer and kept at this temperature for 24 h (this temperature is designated as the storage temperature hereafter). The frozen mixture was then thawed at room temperature, and subsequently shaken in a Vortex mixer (1000 rpm) for 20 min.

When the influence of storage temperature was investigated, the following apparatuses or coolants were used: -18 to -5 °C, a thermostat (using 80% (v/v) methanol as a refrigerant, Thermocool LCH-400F, Toyo Kagaku Sangyo, Ltd., Japan); -20 °C, a controlled freezer; -40 °C, a freeze-dryer (SER-50, Shimadzu, Japan); -75 °C, dry ice in ethanol; -196 °C, liquid nitrogen.

Calculation of Encapsulation Efficiency (EN%)——EN% was calculated from the difference of A-ase activities between liposomes solubilized with Triton X-100 and without solubilizing, as described in the previous paper.[2]

Evaluation of Particle Size Distribution——In the previous papers,[2,6] particle size distribution was evaluated by using electron micrography, but this method was very troublesome, so turbidity, $A_{1\%}$, was adopted as a convenient index of mean particle size of liposomes. The utility of $A_{1\%}$ was confirmed by comparison with a sedimentation method.

a) Turbidity ($A_{1\%}$): As described in the previous paper,[6] the liposome preparation was diluted with Tris buffer so that the concentration of YPL was 0.025% and allowed to stand at room temperature for 2 h. Subsequently, the absorbance (A) of the diluted liposome suspension was measured at 600 nm at 25 °C in a 1 cm cuvette by using a spectrophotometer. $A_{1\%}$ was calculated by multiplying A by 40 ($= 1/0.025$).

b) Sedimentation Method: The particle size distribution of liposomes was measured by using a centrifugal particle size analyzer (SA-CP2, Shimadzu, Japan) at 3600 rpm and calculated automatically with the attached data processor.

Thermal Analysis (DSC)——The energy changes occurring upon warming of the frozen YPL suspension or the mixture of YPL and water were determined by using a differential scanning calorimeter (DSC) (thermal analyzer DT-30 and SC-30, Shimadzu, Japan). About 5 mg of a sample was taken in an aluminum measuring cell, which was sealed, and cooled to approximately -70 °C by using liquid nitrogen. Dry nitrogen gas was used to prevent condensation of moisture, and the measurement was performed from -40 to 50 °C at the heating rate of 5 °C/min.

Results and Discussion

Correspondence between Turbidity and Mean Particle Size

In the previous paper,[6] based on a comparison of $A_{1\%}$ and the particle size distribution determined by electron micrography, it was suggested that $A_{1\%}$ can be used as an index of particle size of liposomes. To confirm this, a comparison was performed between the results of the sedimentation method and $A_{1\%}$ measurement for various liposomes.

In Fig. 1a, particle size distributions determined by the sedimentation method are shown for liposomes whose $A_{1\%}$ values were 1 and 24, as examples. Good log-normal distributions were observed in both cases, and the mean particle sizes (D_e) were estimated as 0.3 and 1.1 μm, respectively from the 50% frequency.

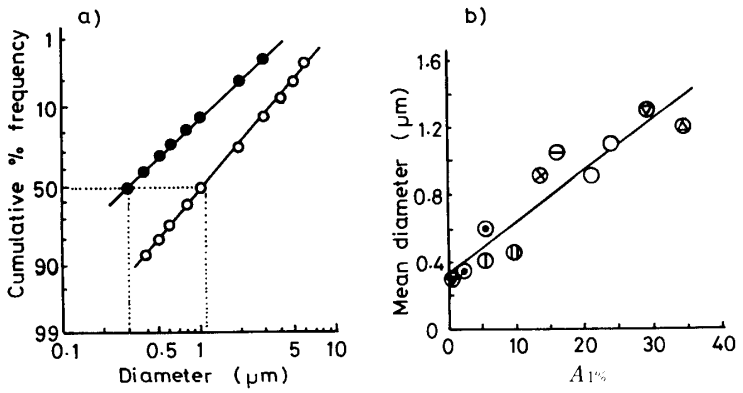

Fig. 1. Comparison of $A_{1\%}$ and the Particle Size of Liposomes Determined by the Sedimentation Method

a) An example of a log-probability plot for the particle size distribution determined by the sedimentation method for liposomes whose $A_{1\%}$ values were 1 (●) and 24 (○).
b) Relationship between mean diameter and $A_{1\%}$. Liposomes were prepared with various additives at the concentrations shown in parenthesis.
⊙, NaCl (0.05, 0.2 M); ⊕, $CaCl_2$ (0.1 M); ⊖, $MgCl_2$ (0.025 M); ⊗, mannitol (0.5 M); ◍, glucose (0.05 M); ◬, BSA (0.4%); ▽, gelatin (0.4%); ○, without any additives; ▼, without any additive and before being subjected to freezing.

Figure 1b shows the relation between $A_{1\%}$ and D_e determined as above for the eleven liposome suspensions prepared with various kinds of additives. There is a good linear relationship irrespective of the variety of additives and their concentrations. Thus, in this paper, $A_{1\%}$ is principally used as an index of particle size hereafter.

Effect of Additives on $EN\%$ and $A_{1\%}$

As described previously,[6] $EN\%$ of A-ase in liposomes was markedly affected by the addition of electrolytes. Thus, the influences of inorganic salts and sugars, used frequently for the preparation of injections, and BSA and gelatin, as examples of proteinous materials having analogous properties with A-ase, were examined further.

A mixture of YPL suspension and an equal volume of the solution containing A-ase and a water-soluble additive was frozen at −20 °C and stored at the same temperature for 24 h, then thawed and shaken in a Vortex mixer. The $EN\%$ and $A_{1\%}$ of liposomes thus obtained are shown for inorganic salts (Fig. 2), glucose and D-mannitol (Fig. 3a), and BSA and gelatin (Fig. 3b).

As shown in Fig. 2, both $EN\%$ and $A_{1\%}$ decreased with increase of the salt concentration in the cases of NaCl, $CaCl_2$ and $MgCl_2$; however, in the case of KCl, $A_{1\%}$ hardly changed and the extent of the decrease in $EN\%$ was less than those of the above three inorganic salts. The reason for the smaller effect of KCl is not clear at present, but the following fact may be relevant: the eutectic point of KCl (−11 °C)[11] is higher than the storage temperature (−20 °C), whereas the eutectic point of NaCl (−21 °C)[11] is lower than the storage temperature (−20 °C).

On the addition of mannitol or glucose, $EN\%$ and $A_{1\%}$ decreased with increase of the additive concentration, but the extents of decrease differed (Fig. 3a). On the other hand, upon addition of BSA and gelatin, $EN\%$ was decreased, but $A_{1\%}$ hardly changed, remaining similar to the value in the absence of any additive (Fig. 3b).

Morphological Observation during the Preparation Processes

The morphological changes of YPL particles during the preparation processes were observed by optical microscopy, and the results for the case without any additive are shown in Fig. 4. YPL particles in the sonicated suspension before freezing were so fine that they could

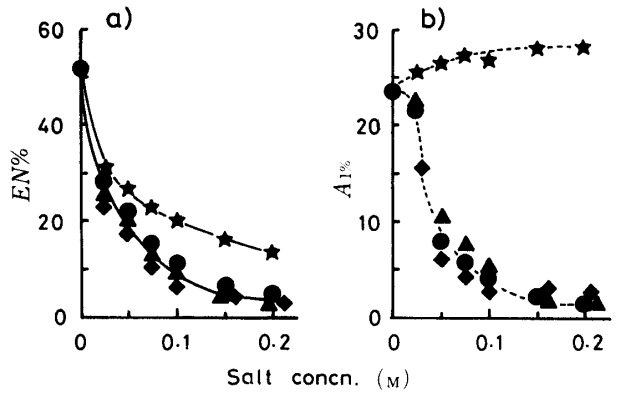

Fig. 2. Effect of the Addition of Inorganic Salt on $EN\%$ (a) and $A_{1\%}$ (b)

●, NaCl; ★, KCl; ▲, $CaCl_2$; ◆, $MgCl_2$. YPL 5% (w/v), A-ase 10 IU/ml.

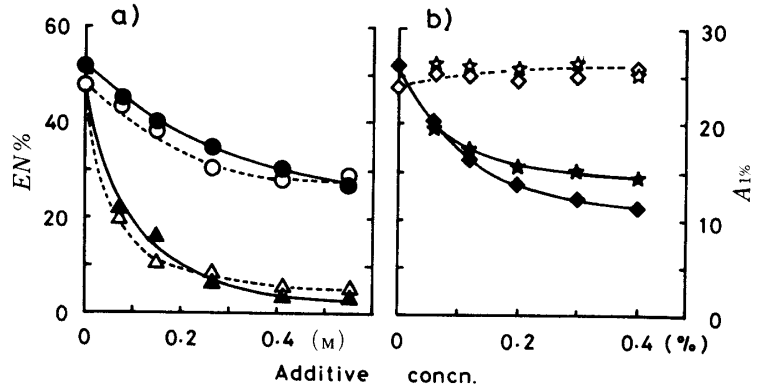

Fig. 3. Effect of Sugars (a) or Proteins (b) on $EN\%$ and $A_{1\%}$

●○, mannitol; ▲△, glucose. ★☆, BSA; ◆◇, gelatin.
Solid symbol, $EN\%$: open symbol, $A_{1\%}$.

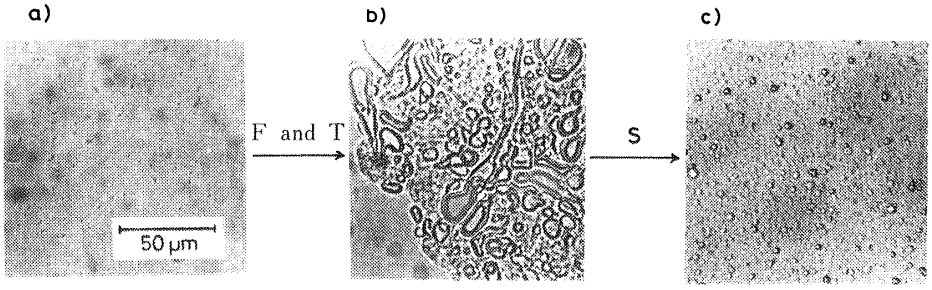

Fig. 4. Photomicrographs of YPL Particles Prepared without Any Additive during the Preparation Processes of FT Liposomes

F and T, freezing and thawing; S, shaking in a Vortex mixer.

not be clearly seen by optical microscopy (Fig. 4a). After freezing at −20°C and thawing at room temperature, large aggregates resulting from the fusion of YPL particles, as large as 50 μm, were observed (Fig. 4b). When these aggregates were dispersed by shaking in a Vortex mixer, liposome particles of a few μm were generated (Fig. 4c), with an $A_{1\%}$ of 24.0.

However, when 0.2 M NaCl was added to the A-ase solution before freezing, no large aggregates of YPL were observed even after freezing (−20°C) and thawing. Consequently, liposomes generated after shaking were very small and the $A_{1\%}$ remained almost at the initial level, about 1.0.

These morphological results quantitatively reflected the relationship between $A_{1\%}$ and particle size shown in Fig. 2. The large $A_{1\%}$ value of FT liposomes after the shaking process must be related to the formation of large YPL aggregates after the freeze-thawing process.

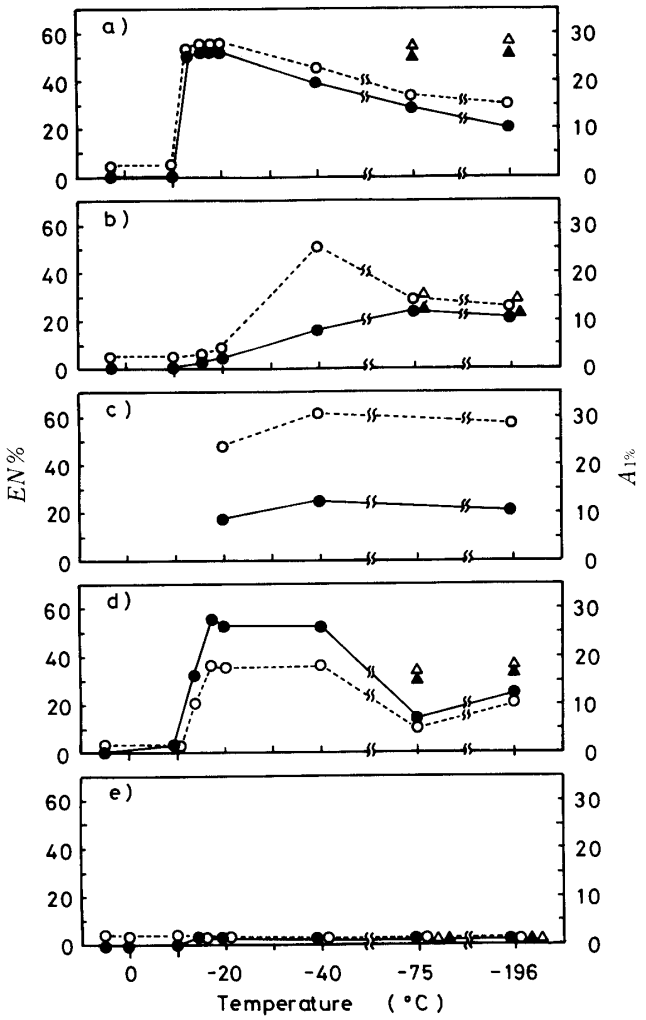

Fig. 5. Effect of Storage Temperature During the Freezing Process on $EN\%$ and $A_{1\%}$

a, without any additive; b, 0.2 M NaCl; c, 0.2 M KCl; d, 0.55 M mannitol; e, 0.55 M glucose.
●▲, $EN\%$; ○△, $A_{1\%}$; circles, one-step freezing; triangles, two-step freezing (see the text for details).

Effect of Storage Temperature During the Freezing Process

FT liposomes with various additives were prepared at various storage temperatures ranging from −196 to 5°C, and $EN\%$ and $A_{1\%}$ determined are shown in Fig. 5 (circles). The concentrations of these additives were close to the highest of those in Fig. 2 or 3.

Next, to investigate the influence of the thermal history in freezing, liposomes were prepared by two steps of freezing. That is, YPL mixture was kept at −20°C for 24 h at first, then kept at another temperature for another 24 h, then thawed at room temperature and shaken. These results are also shown in Fig. 5 (triangles).

It is interesting that $EN\%$ and $A_{1\%}$ showed similar behavior to each other in all cases. These results suggest that the formation of YPL aggregates (detectable in terms of $A_{1\%}$ increase) is essential to achieve a high $EN\%$.

Without any additive (Fig. 5a), YPL suspension visibly froze at below −10°C, but $EN\%$ and $A_{1\%}$ were scarcely increased by storage at −10°C. Both $EN\%$ and $A_{1\%}$ became higher on storage at below −13°C. $EN\%$ became the highest at around −20°C, but became a little lower at below −40°C.

Based on Fig. 5a, it is conceivable that in the freezing process, ice crystals of pure water are generated at first, then the solute is gradually concentrated in the remaining unfrozen region. The unfrozen regions still remain at −10°C and a rather lower temperature than the freezing point of the solvent (water, in this case) is necessary to allow all the suspension to freeze completely to form YPL aggregates. The formation of aggregates seems to be dependent on the cooling velocity in the freezing process, because higher $EN\%$ is attained

from slower freezing (*e.g.* storage at -20°C) rather than such rapid freezing (as at -196°C).

In the case of 0.2 M NaCl (Fig. 5b), YPL suspension visibly froze at below -16°C, but $EN\%$ and $A_{1\%}$ were very low even at -20°C and became the highest at -75 and -40°C, respectively. These results were markedly different from those without any additive. In the case of KCl (Fig. 5c), $A_{1\%}$ and $EN\%$ of liposomes prepared at -20°C were higher than those with NaCl.

From Fig. 5b and c, it seemed very important to cool the mixture below the eutectic point of the additive solution for the formation of YPL aggregates, as the eutectic points of NaCl and KCl are -21 and -11°C,[11] respectively. It is presumed that storage at -20°C was not cold enough to allow the NaCl mixture to freeze completely and achieve high $EN\%$.

In the case of mannitol (Fig. 5d), YPL suspension froze at below -13°C, and $EN\%$ and $A_{1\%}$ became higher. However, they bacame a little lower as the temperature was decreased below -40°C. This result may be related to the fact that the eutectic point of mannitol is near the freezing point of water, -1 to -2°C,[11] and consequently is shows a similar tendency to the case without any additive.

In the case of glucose (Fig. 5e), YPL suspension visibly froze below -16°C, but both $EN\%$ and $A_{1\%}$ scarcely increased at any storage temperature. The morphological observations also showed that YPL aggregates were not formed. This may be because glucose hardly shows eutectic freezing[12] and a large amount of unfrozen water remains. However, further research is necessary to clarify these phenomena in more detail.

As regards the liposomes prepared by the two-step freezing method (triangles in Fig. 5), both $EN\%$ and $A_{1\%}$ on storage at -75 or -196°C, after previous storage at -20°C for 24 h, were at almost the same level as those after single storage at -20°C in the cases of no additive and mannitol (Fig. 5a and d). The results suggest that no unfrozen region remains after the storage at -20°C in these cases.

On the other hand, with NaCl (Fig. 5b), the first cooling at -20°C had no effect of $EN\%$ and $A_{1\%}$, and they were almost the same as those after direct storage at -75 or -196°C (one-step freezing). This suggests that some fraction still remained unfrozen at -20°C, and the fraction froze entirely at a temperature lower than -20°C. For glucose, no effect was observed due to cooling at -20°C beforehand.

Thermal Analysis of the Mixture of YPL and Water

The results in Fig. 5 suggest that the formation of YPL aggregates is greatly affected by the storage temperature and has some correlation with the change of water structure in the freezing process. Thus, differential scanning calorimetry (DSC) was employed to evaluate the phase transition of the mixture containing YPL, water and water-soluble additives.

As shown in Fig. 6a, a sharp endothermic peak, corresponding to the melting point of water (Fig. 6b), was observed at around 0°C in the YPL–water mixture (weight ratio, 20 : 80). This peak became smaller as the ratio of YPL increased and disappeared as the YPL–water ratio became about 4 : 1 (Fig. 6c). Therefore, the free water ratio (R_{fw}) was calculated from this peak at around 0°C by using Eq. 1 and is shown in Fig. 7 against YPL weight percent in the mixture.

$$R_{fw} = (A_{mix}/A_w) \times 100 \quad (1)$$

Here, A_{mix} and A_w are the peak areas per 1 g of the YPL–water mixture and water, respectively.

As shown in Fig. 7, R_{fw} values decreased as YPL percent increased and became zero when the YPL content was about 80%. The result in Fig. 7 suggests that a certain amount of water does not show a phase transition (melting) phenomenon, whereas when the YPL content was more than 80%, all of the water added was restricted to YPL.

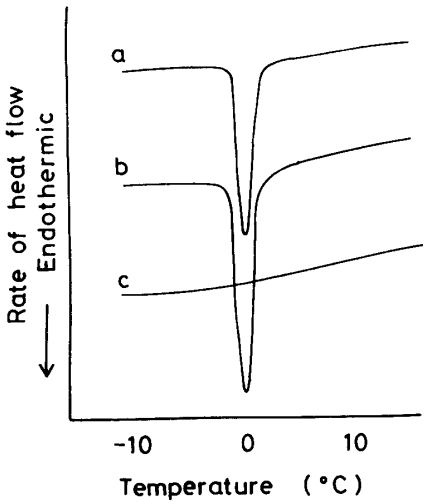

Fig. 6. DSC Thermograms for the Warming of YPL–Water Mixture

a, YPL–water mixture (20:80); b, water alone; c, YPL–water mixture (weight ratio, 79:21).

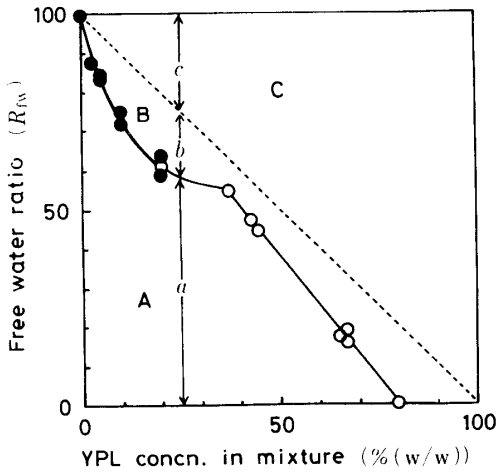

Fig. 7. Phase Diagram in YPL–Water Mixture

The regions A, B and C correspond to free water, restricted water and YPL phase, respectively.
●, R_{fw} value in the YPL suspension; ○, R_{fw} value in the mixture of YPL powder and water.

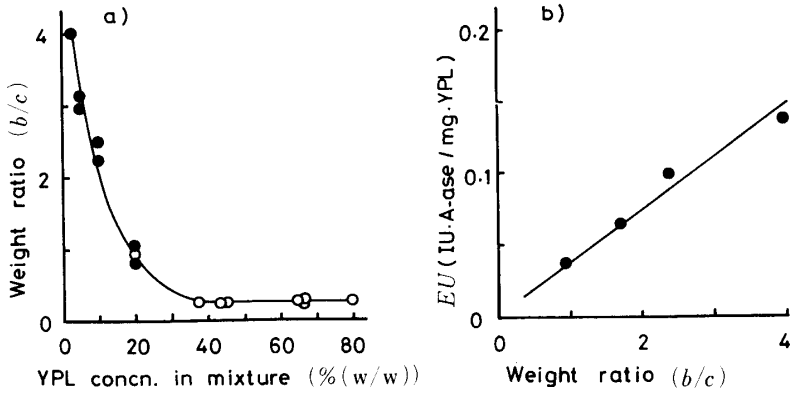

Fig. 8. Comparison of the Amount of Restricted Water and Encapsulation Efficiency

a) The weight ratio (●○) of restricted water against YPL concentration.
b) The relationship between the weight ratio of restricted water (b/c) and the amount of encapsulated A-ase per 1 g of YPL (EU).

The regions A, B and C in Fig. 7 seem to correspond to free water, restricted water and YPL phase, respectively. Here, each value is designated as a, b and c (%), respectively, as shown in the figure. Then, $(b+c)$ is regarded as the unfrozen fraction in the mixture and b/c corresponds to the amounts of restricted water per unit weight of YPL.

The b/c values are plotted in Fig. 8a against the weight fraction of YPL. The number of water molecules tied to one YPL molecule decreased rapidly as the YPL concentration increased and then remained almost the same when the YPL concentration was more than 40%. About ten water molecules were attached to one YPL molecule at this stage. This result agrees with that reported by Chapman *et al.*[13]

As this tied water seemed to be related to the encapsulation efficiency of A-ase, EN% of A-ase in liposomes prepared at various YPL concentrations was taken from the previous paper[6] and the relation between the amount of A-ase encapsulated per unit weight of YPL (EU) and the b/c value is plotted in Fig. 8b. There was a fairly good relationship. This suggests that the unfrozen water phase presumed to exist around YPL particles plays an important role

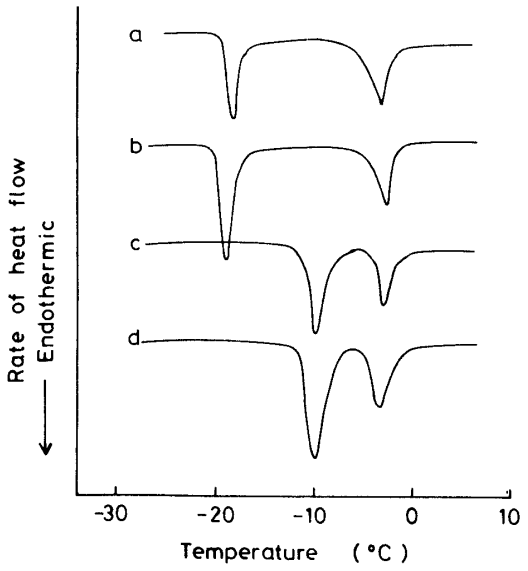

Fig. 9. DSC Thermograms for the Warming of YPL–Salt–Water Mixture

a, YPL–NaCl–water (weight ratio, 10:5:85); b, NaCl–water (5:90); c, YPL–KCl–water (10:10:80); d, KCl–water (10:90).

in the encapsulation of drugs.

As regards the mixture of YPL–NaCl–water, two peaks were observed in DSC measurement at −21 and −4 °C (Fig. 9a). By comparing this result with that for the mixture of NaCl–water (Fig. 9b), the former peak was attributable to the eutectic point of NaCl solution and the latter to the melting point of water, lowered from that of pure water by the addition of NaCl. As the DSC thermogram of the NaCl–water–YPL mxture showed a similar pattern to that of the NaCl–water system, eutectic crystals of NaCl and water were also presumed to be formed irrespective of the presence of YPL particles.

In the mixtures of YPL–KCl–water and KCl–water, the peak at −14 °C corresponding to the eutectic point of KCl and water was observed as shown in Fig. 9c and d, respectively.

From the results in Figs. 5 and 8, it is presumed that an ice phase occurs at first, when the mixture of YPL and additive solution is kept at temperature between the melting point of water and the eutectic point, but a fairly large amount of the unfrozen phase still remained, and thus YPL was not concentrated sufficiently to coagulate, resulting in low $A_{1\%}$ and $EN\%$.

Effect of the Addition of the Additives after Thawing

Previously,[6] we showed that when A-ase was added to YPL suspension after the thawing process, the $EN\%$ became about a half of that obtained when A-ase was added at the initial stage. Therefore, the effect of the addition of A-ase together with additives after the thawing process was investigated. That is, 1 ml of Tris buffer containing A-ase (20 IU/ml) and additives was added to 1 ml of the suspension containing YPL (10% w/v) aggregates, which was prepared without any additives by freezing at −20 °C and thawing at room temperature beforehand, and the mixture was shaken in a Vortex mixer. NaCl, glucose and gelatin were used as additives and both $EN\%$ and $A_{1\%}$ were determined for these liposomes (Fig. 10). In Fig. 10, $EN\%$ values of the liposomes prepared by the ordinary FT method are taken from Figs. 2 and 3 and shown for comparison.

$A_{1\%}$ values were nearly constant irrespective of the addition of these additives and were higher than those in Figs. 2 and 3, because the YPL aggregates had completely formed before the addition, and the generation of the aggregates might depend upon YPL concentration during the freezing process. On the other hand, $EN\%$ decreased with increasing additive concentration in all cases. These results seem to indicate that the addition of additives after thawing does not affect the dispersion of YPL aggregates by shaking, but does interfere with the encapsulation during the shaking process. Thus, the difference in $A_{1\%}$ due to the kinds of additives seems to be related more closely to the freezing process than to the shaking process.

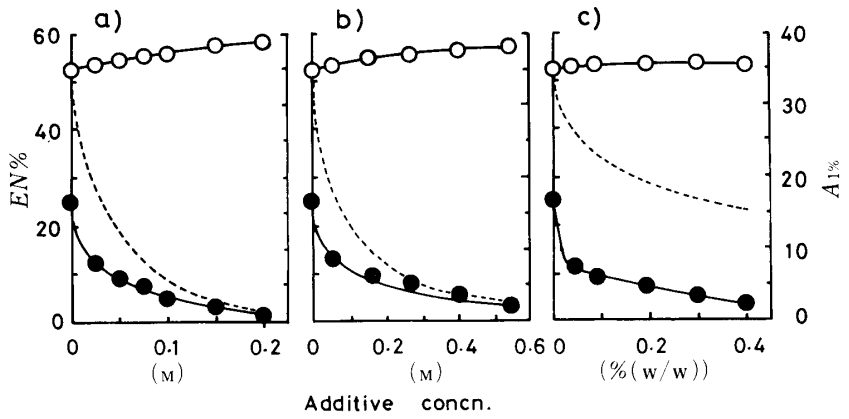

Fig. 10. Effect of the Addition of Additives to YPL Aggregates After the Thawing Process on $EN\%$ and $A_{1\%}$

a, NaCl; b, glucose; c, gelatin.
●, $EN\%$; ○, $A_{1\%}$; ------, $EN\%$ of liposomes prepared by the ordinary FT method.

From the results in Figs. 2, 3 and 10, encapsulation is presumed to be achieved at both the freezing process (denoted as EN_f step) and the shaking process (EN_s step). The difference in the influence of additives on $EN\%$ and $A_{1\%}$ may be attributable to the difference in the extents of their contributions to the EN_f and EN_s steps.

In the case of gelatin, where $A_{1\%}$ scarcely changed and $EN\%$ was reduced by half at higher additive concentrations in the ordinary FT preparation (Fig. 3), the EN_f step is probably similar to that without any additive, whereas the EN_s step is interfered with in a concentration-dependent manner (Fig. 10). $EN\%$ remaining at the higher additive concentrations in Fig. 3 is presumed to reflect the values encapsulated in the EN_f step, because A-ase once trapped in liposomes does not leak out easily.[6] The addition of KCl or BSA probably has the same effect as gelatin because they all showed similar tendencies in $EN\%$ and $A_{1\%}$ (Figs. 2 and 3).

On the other hand, NaCl and glucose seem to interfere with both the EN_f and EN_s steps, so both $EN\%$ and $A_{1\%}$ decreased to almost zero as the additive concentration increased (Figs. 2 and 3).

Discussion of the Encapsulation mechanism for FT Liposome

From the above results, the encapsulation mechanism for FT liposomes was presumed to be as shown schematically in Fig. 11. Without any additive, ice crystals are formed at the early stage of the freezing process when YPL suspension is frozen at −20°C, then YPL particles and A-ase are gradually dehydrated and concentrated in the unfrozen phase. At this stage, YPL particles are closely packed in contact with each other, forming large aggregates containing A-ase inside them. These aggregates change to liposomal particles upon being thawed and shaken in a Vortex mixer.

On the other hand, in the presence of NaCl (Fig. 11b), ice crystals are similarly formed at the early stage of the freezing process when the YPL suspension is kept at −20°C, so YPL particles, A-ase and NaCl are concentrated in the unfrozen phase as the ice crystals grow. However, as this process proceeds, the aqueous phase composition approaches the NaCl eutectic composition, 23% (w/v), and remains unfrozen, because the eutectic point (−21°C) is below this storage temperature (−20°C). Thus, YPL particles are dispersed in a larger space during the freezing process than in the case without any additive, and aggregates large enough to give high $EN\%$ do not occur. However, these unfrozen regions are eventually frozen if the storage temperature is taken below the eutectic point, and then YPL particles are packed more closely and become fused to each other (Fig. 5b).

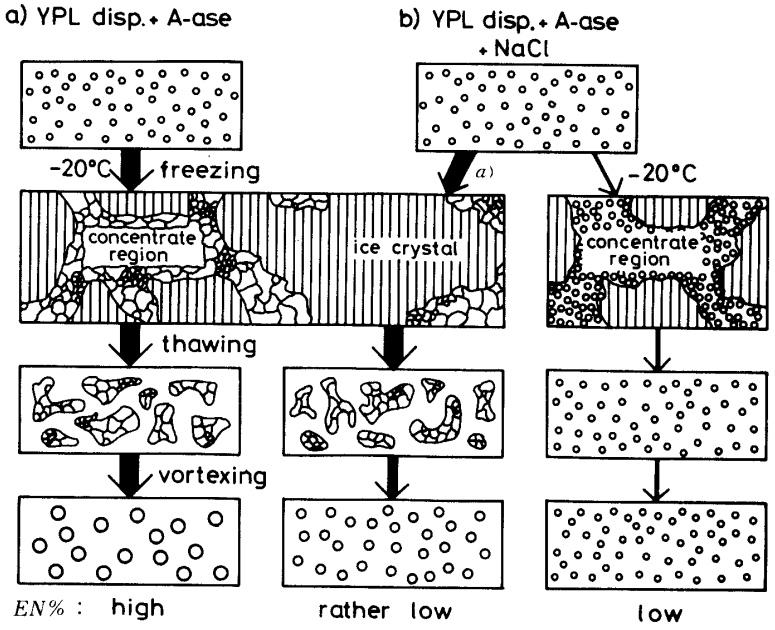

Fig. 11. Schematic Representation of the Proposed Encapsulation Mechanism for FT Liposomes

a, without any additive; b, with NaCl. *a*) Below eutectic point.

The finding that $EN\%$ and $A_{1\%}$ decreased with increasing concentration of NaCl (Fig. 2a) can be explained by presuming that the unfrozen region increases and the formation of YPL aggregates is increasingly prevented as the amount of NaCl increases. The phenomenon that both $EN\%$ and $A_{1\%}$ were lower on storage at -40°C than at -20°C without any additive suggests that the freezing velocity in the former case is too rapid for the solutes to be concentrated sufficiently in the unfrozen regions.

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