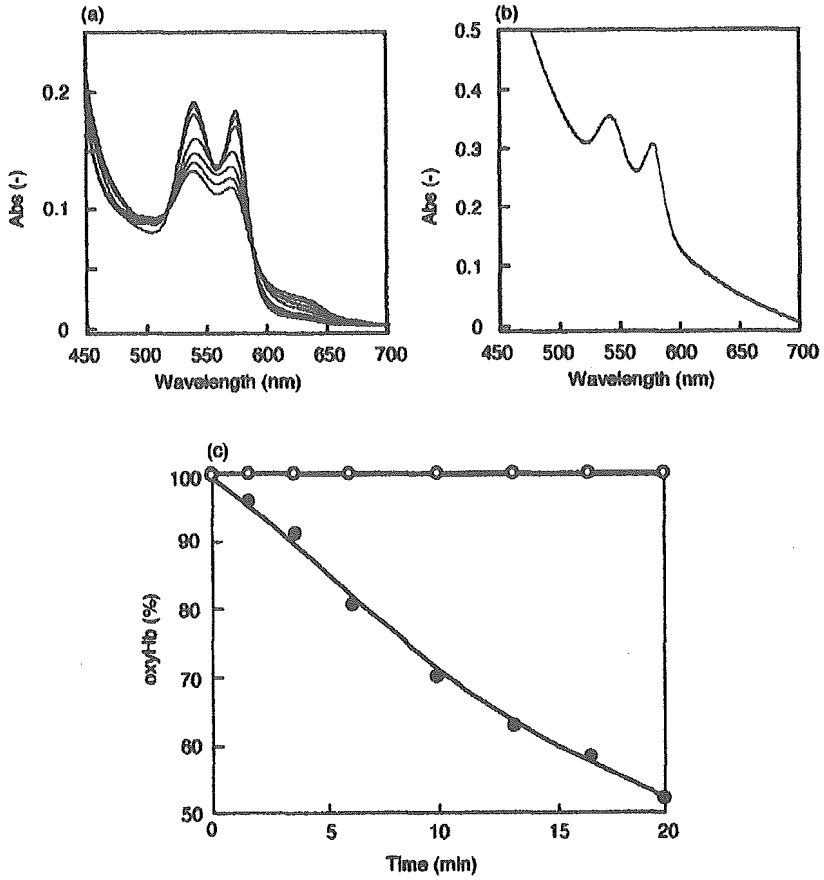

Figure 1. Spectral changes of (a) the Hb solution and (b) the Hb vesicle dispersion ([heme] = 12 μM) during the reaction with $O_2^{-\bullet}$ at 37 °C (0.9 μM s^{-1}). The repeatative scanning was started at a 2 min interval. (c) Changes of the oxyHb in the Hb solution (●) and the Hb vesicle dispersion (○) during the reaction with $O_2^{-\bullet}$.

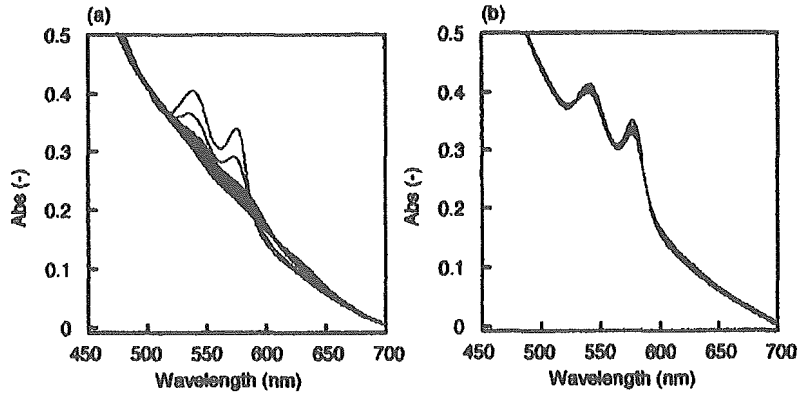

Figure 2. Spectral changes of (a) the Hb vesicle dispersion ([heme] = 12 μM) and (b) catalase-coencapsulating Hb vesicle dispersion ([heme] = 12 μM, $[catalase]_{fed}$ = 4.2 × 10^4 unit mL^{-1}) during the reaction with H_2O_2 at 37 °C. The repetitive scanning was started at a 2 min interval.

2b), a very small amount of oxyHb was converted to metHb without the formation of ferrylHb. This is because the reaction of Hb or catalase with H_2O_2 should be competitive in the vesicles. Most of the H_2O_2 should be eliminated by catalase, and a small amount of H_2O_2 should react with Hb to form metHb (the elimination rate of catalase is about a thousand times greater than that of Hb). We measured the time course of the oxyHb percentage after the addition of 10-fold excess H_2O_2 to oxyHb in the vesicles and calculated the initial decreasing rate of oxyHb (Figure 3). The Hb vesicles without catalase showed a decreasing rate of 42% min^{-1}, whereas the Hb vesicles with catalase showed a lower decreasing rate, depending on the concentration of catalase ($[catalase]_{fed}$). The rate decreased to 5.0% min^{-1} when more than 5.0 × 10^4 unit mL^{-1} was coencapsulated within the vesicle. In this case, the ratio of Hb to catalase was calculated to be 35. We confirmed that the metHb formation of the Hb vesicles by exogenous H_2O_2 could be effectively suppressed by catalase-coencapsulation.

We next examined the catalase encapsulation effect on the autoxidation-derived H_2O_2, namely endogenous H_2O_2. We measured the rate of metHb formation in the Hb vesicles at 40 Torr of oxygen partial pressure (PO_2) in

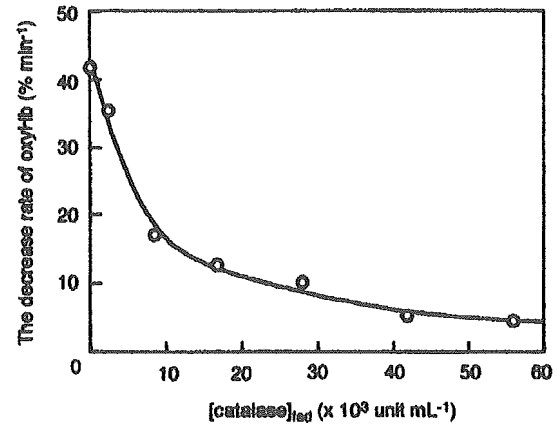

Figure 3. The relationship between the decrease rate of oxyHb in the vesicle ([heme] = 12 μM) and the coencapsulated catalase during the reaction with H_2O_2 ([H_2O_2] = 120 μM).

vitro. When the PO_2 is approximately the P_{50} of Hb, the rate of metHb formation tends to show a maximum (*32*, *33*), and the average oxygen partial pressure in mixed venous blood is estimated to be 40 Torr. Therefore, we used the constant oxygen partial pressure of 40 Torr to measure the rate of metHb formation of the Hb vesicle dispersion for estimation of the in vivo behavior. As shown in Figure 4a, the rate of metHb formation in the Hb vesicles was 3.5% h^{-1}, and in the case of the catalase-coencapsulating Hb vesicles, the rate of metHb formation was significantly reduced from 2.8 to 2.0% h^{-1} with an increase in the encapsulated catalase concentration from 2.8×10^3 to 1.7×10^4 unit mL^{-1}. However, the rate of metHb formation was not reduced even when 2.0×10^4 unit mL^{-1} catalase was coencapsulated (Figure 4b). Inside the vesicle, Hb is autoxidized to metHb with the production of $O_2^{-\bullet}$, and the resulting $O_2^{-\bullet}$ immediately caused a one-electron oxidation of Hb to metHb and the $O_2^{-\bullet}$ became H_2O_2. On the other hand, the $O_2^{-\bullet}$ was immediately dismutated to H_2O_2. As previously reported, the resulting endogenous H_2O_2 also autoxidizes Hb to metHb, or metHb via ferrylHb. Therefore, the autoxidation of Hb should trigger a further oxidation accompanied by the production of reactive oxygen species. In fact, in the group of catalase-coencapsulating Hb vesicles, the reduction of the metHb formation rate indicates that the metHb formation was suppressed by the elimination of the endogenous H_2O_2. The 2.0% h^{-1} rate of metHb formation of the Hb vesicles coencapsulating more than 1.7×10^4 unit mL^{-1} catalase showed that the metHb formation by H_2O_2, which was produced by the autoxidation of Hb in the vesicle, could be completely suppressed by catalase.

It is expected that SOD, which dismutates two $O_2^{-\bullet}$ and two H^+ to H_2O_2 and O_2, should contribute to the reduction of the metHb formation rate. In the Hb solution ([Hb] = 2.0 g dL^{-1}) containing SOD (2.0×10^3 unit mL^{-1}) and catalase (2.0×10^4 unit mL^{-1}), the rate of the metHb formation at 37 °C under atmospheric conditions was 1.3% h^{-1}, which was reduced to 74% compared with a bare Hb solution (1.8% h^{-1}). However, the rate was almost the same as that of the Hb solution containing catalase (1.4% h^{-1}, [catalase] = 2.0×10^4 unit mL^{-1}). From this experiment, we used only catalase as an effective prolongation tool of the Hb function in the Hb vesicles.

To study the rate of the metHb formation in vivo, the Hb vesicles were injected into the rat tail vein (20 mL kg^{-1}). It was confirmed that the LPS concentration in

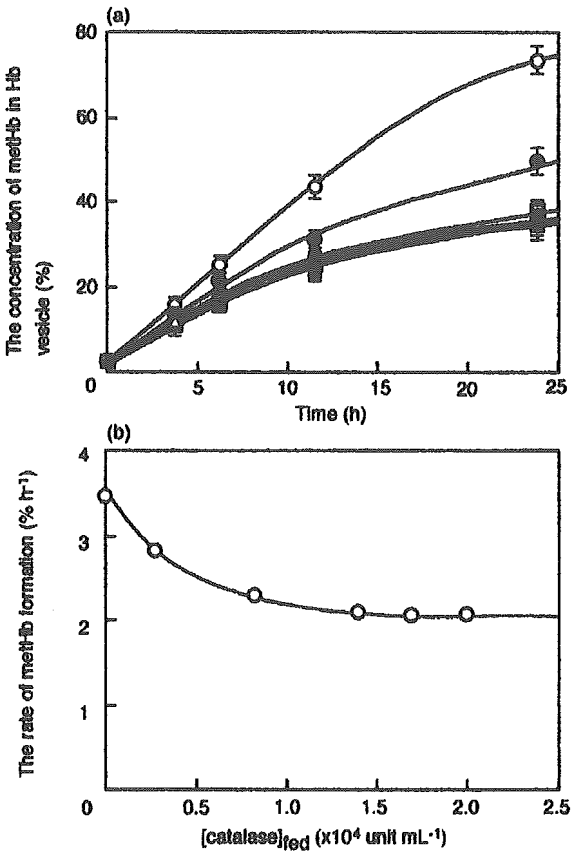

Figure 4. (a) Changes of metHb percentage in the Hb vesicles at 40 Torr of oxygen partial pressure ([Hb] = 2.0 g dL^{-1}). The control is catalase-free Hb vesicles (○). The encapsulated catalase in the Hb vesicle was 2.8×10^3 (●), 8.4×10^3 (□), 1.4×10^4 (■), 1.7×10^4 (△), and 2.0×10^4 unit mL^{-1} (▲). (b) The relationship between $[catalase]_{fed}$ and the rate of metHb formation.

these samples was below 0.1 EU mL^{-1}, approved for in vivo administration. If LPS-contaminated Hb vesicles were injected, the rate of metHb formation should dramatically increase. In the catalase-coencapsulated Hb vesicles ([catalase] = 2.8×10^4 unit mL^{-1}), where the LPS concentration was 10 EU mL^{-1}, the rate of metHb formation was 8.0% h^{-1} (dotted line in Figure 5a), whereas the rate was 2.9% h^{-1} in the catalase-coencapsulated sample of which the LPS concentration was 0.1 EU mL^{-1}. LPS is a major constituent of the outer envelope of Gram-negative bacteria; therefore, it is an extremely potent stimulator of the mammalian immune system and causes the activation of macrophages (*34*, *35*), which should increase the rate of metHb formation by elevation of the reactive oxygen species concentration such as H_2O_2. In this case, there was no effect of the encapsulation with catalase in the Hb vesicles. The effect of such inflammation should exceed the effect of catalase coencapsulation. Therefore, we used the samples where the LPS concentration was below 0.1 EU mL^{-1} in order to accurately measure the rate of metHb formation. In the catalase-free Hb vesicles, the rate of the metHb formation was 3.4% h^{-1}, and the time for 50% metHb formation ($T_{50\%metHb}$) was 14 h. On the other hand, in the groups of catalase-coencapsulated Hb vesicles ([catalase] = 2.8×10^3, 8.4×10^3, 1.7×10^4, 2.8×10^4, 4.2×10^4, and 5.6×10^4 unit mL^{-1}), the rates of metHb formation were 2.9, 2.3, 2.0, 1.8, 1.7, and 1.7% h^{-1}, respectively, and the $T_{50\%metHb}$ were 18, 22, 29, 36, 38, and 39 h, respectively. Figure 5a

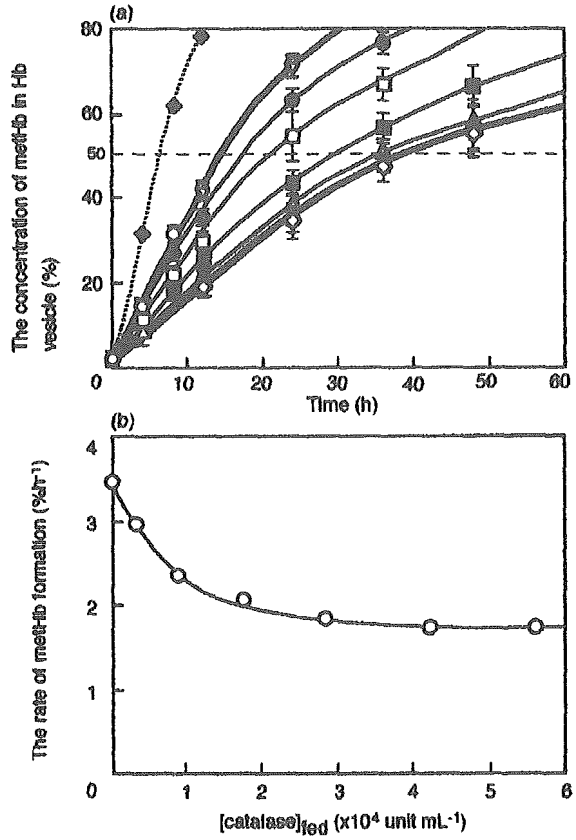

Figure 5. (a) Changes of metHb percentage in the Hb vesicles in vivo (20 mL kg^{-1}, Wistar rat). The control is catalase-free Hb vesicles (○) and the mixture of the Hb vesicle dispersion and the catalase solution (5.6×10^4 unit mL^{-1}, ▽). The encapsulated catalase in the Hb vesicle was 2.8×10^3 (●), 8.4×10^3 (□), 1.7×10^4 (■), 2.8×10^4 (△), 4.2×10^4 (▲), and 5.6×10^4 unit mL^{-1} (◇). The dotted line (◆) is the LPS-contaminated Hb vesicles ([catalase] = 2.8×10^4 unit mL^{-1}). (b) The relationship between $[catalase]_{fed}$ and the rate of metHb formation.

showed the changes in the metHb concentration, indicating that the rest of metHb in the Hb vesicles was the functional ferrous Hb (oxyHb). In the case of the administration of the mixture of the catalase-free Hb vesicle dispersion and catalase solution (5.6×10^4 unit mL^{-1}), there was no significant difference from the control groups (catalase-free Hb vesicles) as shown in Figure 5a. These results showed that the administered catalase rapidly disappeared from the blood stream and could not eliminate hydrogen peroxide.

It was confirmed that, by catalase-coencapsulation, the rate of metHb formation was reduced and the $T_{50\%metHb}$ was also prolonged. As shown in Figure 5b, the rate of metHb formation was saturated when the catalase concentration was over 4.2×10^4 unit mL^{-1} in comparison with 1.7×10^4 unit mL^{-1} in vitro as shown in Figure 4b. Such a high catalase concentration in vivo suggests that metHb formation should be caused by the exogenous H_2O_2 from the blood circulation in addition to the endogenous H_2O_2. It is indicated that the saturated metHb formation rate should be due to the substantial autoxidation of Hb because of the elimination of endogenous and exogenous H_2O_2.

CONCLUSION

Catalase coencapsulated in the Hb vesicle could eliminate H_2O_2, which was not only produced by the Hb autoxidation in the vesicle but also generated in vivo, and the rate of metHb formation in the Hb vesicle could thus be effectively suppressed by catalase coencapsulation. We succeeded in substantially prolonging the oxygen-carrying ability of the Hb vesicles in vivo by coencapsulating catalase.

ACKNOWLEDGMENT

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Effective Encapsulation of Proteins into Size-Controlled Phospholipid Vesicles Using Freeze–Thawing and Extrusion

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We are aiming to improve the encapsulation efficiency of proteins in a size-regulated phospholipid vesicle using an extrusion method. Mixed lipids (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, 1,5-dipalmitoyl-L-glutamate-*N*-succinic acid (DPEA), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5,000)] (PEG-DSPE) at a molar ratio of 5, 5, 1, and 0.033 were hydrated with a NaOH solution (7.6 mM) to obtain a polydispersed multilamellar vesicle dispersion (50 nm to 30 μm diameter). The polydispersed vesicles were converted to smaller vesicles having an average diameter of ca. 500 nm with a relatively narrow size distribution by freeze–thawing at a lipid concentration of 2 g dL^{-1} and cooling rate of −140 °C min^{-1}. The lyophilized powder of the freeze–thawed vesicles was rehydrated into a concentrated protein solution (carbonyl hemoglobin solution, 40 g dL^{-1}) and retained the size and size distribution of the original vesicles. The resulting vesicle dispersion smoothly permeated through the membrane filters during extrusion. The average permeation rate of the freeze–thawed vesicles was ca. 30 times faster than that of simple hydrated vesicles. During the extrusion process, proteins were encapsulated into the reconstructed vesicles with a diameter of 250 ± 20 nm.

Introduction

Vesicles with a lipid bilayer membrane have been vigorously studied as a mimetic model of the biological membrane (*1–3*). The vesicles also have a high potential for application as a carrier of drugs or bioactive macromolecules (such as anti-cancers, enzymes, and functional proteins) in the medical fields (*2, 4–7*). To apply the vesicles in clinical use, we studied the methodology or technology to prepare the vesicles on a large scale according to their purposed performances such as size distribution, encapsulation efficiency, preparation speed, and so on. Phospholipid vesicles encapsulating concentrated Hb (Hb-vesicles, HbV) can be a candidate for oxygen carriers such as red blood cells, having an excellent capability to concentrate atmospheric oxygen and release it in response to a lower partial oxygen pressure (*8–10*). The first HbV was prepared by a film hydration and sonication method in 1980 by Djordjevich et al. (*11*). Several kinds of vesicle preparation methods such as reverse phase evaporation (*12*), detergent removal (*13*), dehydration and rehydration (*14–16*), microfluidization (*17*) or high-speed blending (*18*), and freeze–thawing (*19*) have since been used to encapsulate Hb within vesicles. These preparation methods do not satisfy the precise size control, high encapsulation efficiency, and high yield without causing Hb oxidation and denaturation. Furthermore, the preparation methods using additives such as detergents or organic solvents are undesirable from the safety and environmental viewpoints. We have focused on the development of an extrusion method using isopore membrane filters to control the size of the vesicles without any additives (*20–24*). The drawbacks of the extrusion in a concentrated Hb solution were the slow permeation rate and clogging of the filter pores (*25, 26*). Cullis et al. reported the extrusion of freeze–thawed vesicles in order to more readily prepare the size-controlled vesicles (*27*). Unfortunately, the freeze–thawing process in a concentrated Hb solution had no advantage to improving the extrusion procedure, because the vesicles were cryoprotected by concentrated Hb molecules (40 g dL^{-1}) and the Hb became denatured during the freeze–thawing cycle. Recently, we clarified that the Hb dispersion of the lyophilized powder of the freeze–thawed and poly(ethylene glycol)-modified vesicles very smoothly permeated through a membrane filter. This method could prevent Hb denaturation during the freeze–thawing cycles because freeze–thawing was performed in an Hb-free solution and the lyophilized powder was mixed with the Hb solution before extrusion. In this paper, we report the basic understanding of the effect of the freeze–thawing conditions on size regulation of the vesicles and the effect of the freeze–thawed vesicles on filter permeability and water-soluble protein encapsulation (in this case, Hb) during extrusion in order to prepare size-controlled HbV.

Experimental Procedures

Materials. Powders of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) and cholesterol were purchased from Nippon Fine Chemical Co., Ltd., (Osaka, Japan) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5000)] (PEG-DSPE) was purchased from the NOF Co. (Tokyo, Japan). 1,5-Dipalmitoyl-L-glutamate-*N*-succinic acid (DPEA) was synthesized in our laboratory (*28*). An Hb solution was obtained from outdated donated blood (Japanese Red Cross) according to a previously described

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purification method (*29*). The final concentration of the Hb was adjusted to 40 g dL^{-1}.

Preparation of Vesicle Dispersion. DPPC, cholesterol, DPEA, and PEG-DSPE were dissolved in alcohol at a molar ratio of 5, 5, 1, and 0.033 and atomized and evaporated using a spray dryer (Cracks) to prepare a lipid powder at Nippon Fine Chemical Co., Ltd. The mixed lipid powder was dispersed in a NaOH solution (7.9 mM) at 5 g dL^{-1} and hydrated for 2 h to obtain a multilamellar vesicle dispersion. The NaOH concentration was adjusted to be equimolar with DPEA for neutralization. The obtained multilamellar vesicle dispersion was diluted with pure water to the experimental concentrations (1–5 g dL^{-1}). The extruded small vesicles were prepared by passing through the polycarbonate membrane filters in which the final pore size of the filter was 0.1 μm ϕ. The diameter of the resulting vesicles was determined to be 180 ± 15 nm (the size of the vesicles is represented as an average diameter ± standard deviation) using a COULTER submicron particle analyzer (N4SD, Coulter, Hialeah, FL). The extruded large vesicles were prepared by passage through the polycarbonate membrane filters in which the pore size of the filter was 10 μm ϕ. The resulting vesicle dispersion was centrifuged (300*g*, 15 min) to remove the small vesicles in the supernatant. The precipitated vesicles were collected and redispersed into pure water. The diameter of the resulting vesicles was determined to be 3–10 μm.

Freeze–Thawing Treatment. The cooling rate of freezing was strictly controlled using a differential scanning calorimeter (DSC) (Seiko 120 DSC) from −1 to −40 °C min^{-1}. A dispersion (60 μL) of the small or large extruded vesicles was sealed in a silver pan. The pans were placed on the sample stage. The cooling rate and final temperature (−120 °C) was programmed using the included software. After freezing, the pan was kept at 40 °C. The fast freezing (−140 ± 5 °C min^{-1} as an average) was performed by direct dipping of the sealed pan in liquid nitrogen. The thawing was performed by direct dipping of the pan in a 40 °C water bath. The freeze–thawed vesicles were centrifuged (300*g*, 15 min) to precipitate the large vesicles (>3 μm), and the amount of lipids in the supernatant and precipitate was determined by the molibuden blue method (Phospholipid Test Wako; Wako Pure Chem., Tokyo). To obtain the lyophilized powder of the freeze–thawed vesicles, the polydispersed vesicle dispersion (5–25 mL) was sealed in a glass vial, frozen in liquid nitrogen for 3 min, and then thawed in a water bath (40 °C) for 15 min. The resultant vesicle dispersion was frozen in liquid nitrogen for 3 min and dried *in vacuo* using a Bio freeze-dryer (BFD-2F2, Nihonfreezer, Inc., Tokyo, Japan) to obtain the lyophilized powder. The total surface area of the vesicles was measured using 6-*p*-toluidino-2-naphthalene-sulfonic acid (Tns), and the lamellarity of the vesicles was calculated according to our previous paper (*23*).

Sizing and Encapsulation of Hb into Vesicles with Extrusion. The lyophilized powder was dispersed in a concentrated Hb solution (40 g dL^{-1}). After being stirred for 2 h at 25 °C, the dispersion was introduced into an extruder (Lipex Biomembrane, Canada) and extruded through the acetylcellulose membrane filters [FUJI Film Micro Filter; filter pore sizes 3 (FM_3), 0.45 ($FM_{0.45}$), 0.3 ($FM_{0.3}$), and 0.22 μm ($FM_{0.22}$), Fuji Photo Film, Tokyo, Japan] by maintaining a nitrogen gas pressure of 20 kgf cm^{-2}. The permeated volume of the dispersion was recorded on videotape, and the rate of the permeation was calculated. The error in this method was estimated to be less than 2%. The unencapsulating Hb was removed by three ultracentrifugations ($10^5 g$, 90 min).

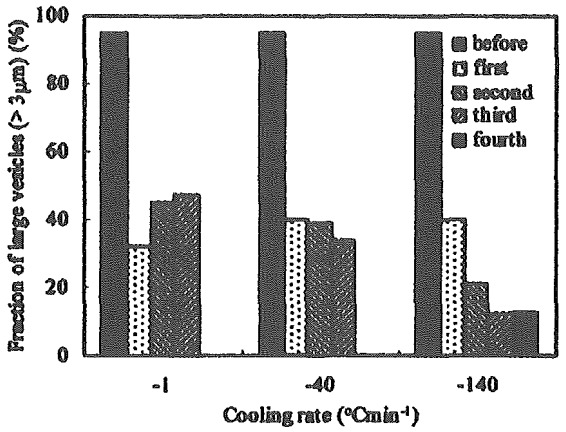

Figure 1. Influence of the cooling rate and number of freeze–thawing cycles on the fragmentation of large vesicles (>3 μm) during freeze–thawing for extruded large vesicles (3–10 μm). The initial vesicular dispersion was prepared by extrusion (10 μm membrane filter pore size), and the small vesicles (<3 μm) were removed by centrifugation. The concentration of the lipids was adjusted to 2 g dL^{-1}.

Determination of Hb and Phospholipid Concentration of the Hb-Vesicles (HbV). The concentrations of the Hb and the phospholipid were determined by a cyanomethemoglobin method (Hemoglobin Test Wako; Wako Pure Chem., Tokyo) and the molibuden blue method, respectively. The encapsulation efficiency of Hb was represented by the weight ratio [Hb]/[lipid].

Results and Discussion

Effect of Freeze–Thawing on Size of Vesicles. The hydrated phospholipid mixture spontaneously formed polydispersed multilamellar vesicles (50 nm to 30 μm diameter). We considered that it was difficult to quantitatively study the effect of freeze–thawing on the size of the vesicles. Therefore, we first studied the change in the size distribution by freeze–thawing treatment for the large (3–10 μm) and small (180 nm) vesicles. To quantify the size distribution of the polydispersed vesicles, we fractionated the large vesicles (>3 μm) by centrifugation (300*g*, 15 min) and determined the phospholipid amount of the fraction. More than 95% of the precipitated vesicles had >3μm diameter, and no small (180 nm) vesicles were precipitated. We roughly determined that the percentage of the precipitation fraction is the large vesicles fraction (>3μm) with less than 5% error. Figure 1 shows the change in the percent of the precipitated fraction (i.e., the large vesicles) after the freeze–thawing cycles. The fraction percent of the large vesicles was 95% before freeze–thawing. After the first freeze–thawing cycle, the percentage of the large vesicles became less than 40% independent of the cooling rate. However, it decreased with the freeze–thawing cycles for the cooling rate of −140 °C min^{-1}. This tendency decreased at −40 °C min^{-1}. When the cooling rate was −1 °C min^{-1}, the percentage was reversibly increased after the second and third freeze–thawing cycles.

The mechanism of the size reduction by freeze–thawing of the large vesicles would be based on breaking the bilayer membrane as a result of dehydration and mechanical pressure accompanied with the growth of ice crystals in the inner and the outer aqueous phases during freezing, and the rehydration of the broken membranes to reconstruct smaller vesicles during thawing (*30, 31*).

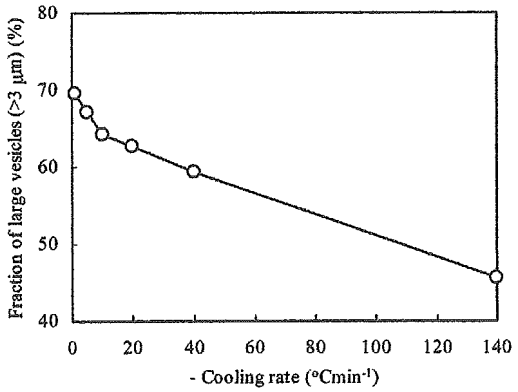

Figure 2. Influence of the cooling rate on the production of fused large vesicles (>3 μm) during freeze–thawing of extruded small vesicles (180 ± 15 nm). The initial vesicular dispersion was prepared by extrusion (0.1 μm membrane filter final pore size) and had no large vesicles (>3 μm).

We confirmed this when the cooling rates were −40 or −140 °C min^{-1}. However, the reverse phenomenon was observed at the cooling rate of −1 °C min^{-1} and could be explained in term of vesicle fusion by freeze–thawing. It is well-known that freeze–thawing is often used for small unilamellar vesicles (SUV) to convert them into large unilamellar vesicles (LUV) by vesicle fusion (*32*). The efficiency of fusion is determined by the average distance between the vesicles, namely, a high vesicle concentration and slow cooling rate result in a short average distance as described below.

We confirmed that the small vesicles (180 ± 15 nm) were fused into larger ones (>3 μm) after the first freeze–thawing cycle, as shown in Figure 2. The percentage of the large vesicles decreased with the increasing cooling rate. The slow cooling rate would result in the growth of large ice crystals, and the local concentration of the vesicles would be increased as a result of the exclusion by ice. Therefore, rehydration should cause the reconstitution of the inter- and intravesicles, namely, fusion. Such fusion should be predominant when small vesicles have been formed by previous preparation, i.e., freeze–thawing of the vesicles.

The mixed lipids formed polydispersed vesicles after hydration in pure water at 5 g dL^{-1}, as shown in Figure 3a. We fractionated the large vesicles (>3 μm) by centrifugation (300*g*, 15 min) and determined that the lipids forming large vesicles occupied 70% of the total lipids. To clarify the influence of the lipid concentration on the freeze–thawing of the polydispersed vesicles, we diluted the large vesicle dispersion (5 g dL^{-1}) with pure water until the concentration of the dispersion became 1, 2, or 3 g dL^{-1}. The diluted samples were frozen at −140 °C min^{-1} and then thawed at 40 °C, and this treatment was repeated three times. As has been estimated, the highly concentrated samples afforded the larger vesicles because fusion would be dominant because of the higher local concentration of the vesicles during freezing. The large vesicle fraction (>3 μm) was reduced to 14% and 13% for the 5 and 3 g dL^{-1} solutions after freeze–thawing, while they were 7% and 6% for the 2 and 1 g dL^{-1} solutions, respectively. The preferable concentration to effectively reduce the large vesicle fraction while considering feasibility in scale was 2 g dL^{-1}. At this condition, the main diameter of the freeze–thawed vesicles was uniformly around 500 nm, as shown in Figure 3b. The conversion of large vesicles to smaller ones and small ones to larger ones as described in Figures 1 and 2 should convert the large vesicles with a wide size distribution to smaller vesicles with a narrow size distribution. The average lamellarity (the number of bilayer membranes) is one parameter to determine the encapsulation efficiency. The efficiency tends to be low for the vesicles having a large lamellarity. Usually, the lamellarity of the vesicles spontaneously formed by conventional lipid hydration is up to 10 (*33*), whereas the average lamellarity of these freeze–thawed vesicles was found to be 2. Therefore, for large polydispersed vesicles, this method was effective not only to regulate the size of the vesicles to ca. 500 nm with a narrow size distribution but also to produce the vesicles with a large encapsulation volume.

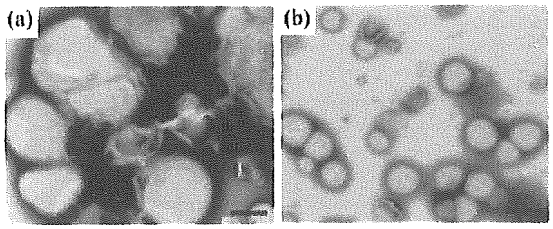

Figure 3. Transmission electron micrographs of the vesicles (a) just after hydration and (b) after three freeze–thawing cycles. Bars represent 500 nm.

Lyophilization of Freeze–Thawed Vesicles and Rehydration in a Hb Solution. The average diameter of the freeze–thawed vesicles as determined by a dynamic light scattering method was 519 ± 78 nm, as shown in Figure 4a. This value was also supported by the size of the vesicles obtained from the TEM observation. The freeze–thawed vesicles were lyophilized in order to replace the water with a concentrated Hb solution. The lyophilized powder was smoothly and homogeneously dispersed and almost restored to the original size of the freeze–thawed vesicles (from 519 ± 78 to 529 ± 100 nm) as compared in Figure 4 b. In general, the lyophilized powder from the vesicle dispersion roughly preserves the vesicle structure, namely, the orientation of the headgroup of the lipids directed toward the surface of the vesicles. However, the rehydrated vesicles tend to become larger and afford a wider size distribution due to fusion. Cryoprotective agents such as saccharides, glycerol, or dimethyl sulfoxide (DMSO) are used to retain the original size and size distribution of the vesicles during the freezing and lyophilizing procedures (*34, 35*). In this experiment, we established cryoprotection by (i) utilization of the freeze–thawed vesicles and (ii) surface modification with PEG. The vesicles prepared by the freeze–thawing repetition to form a steady state should have a resistance to the freezing process. In the lyophilizing process, we froze the sample dispersions under the same condition as the freeze–thawing treatment. In addition, the content of the PEG-lipid was used to reproduce the original size of the freeze–thawed vesicles in an Hb solution. We mixed 0.3 mol % PEG-lipid with the lipid components that were enough to prevent the aggregation of the vesicles (*36–39*). The excluded volume of the PEG chains should prevent the contact between vesicles, to not trigger the aggregation and fusion of vesicles during the lyophilization and rehydration processes. The further incorporation of the PEG-lipid resulted in a significant reduction of the encapsulation efficiency of Hb in the vesicles (*18, 40*). After rehydration with the Hb solution, the encapsulation efficiency of Hb in the resulting vesicles was very low (0.8) as the value of [Hb]/[lipids].

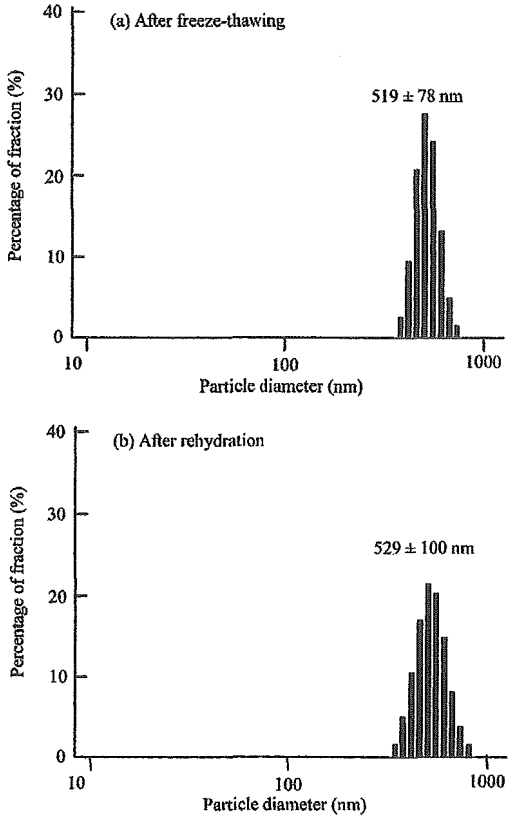

Figure 4. Size distribution of the vesicles measured with a COULTER submicron particle analyzer. The size of the vesicles is represented as the average diameter ± standard deviation: (a) vesicle dispersion just after freeze–thawing and (b) vesicle dispersion rehydrated in a Hb solution.

Extrusion of Rehydrated Vesicles. Further size control is necessary for the dehydrated vesicles (529 ± 100 nm mean diameter) to obtain the final targeting size of HbV (250 ± 20 nm mean diameter). We performed the extrusion for the rehydrated vesicles to regulate the size and encapsulate Hb into the rehydrated vesicles. In addition, the effect of the presizing of the vesicles on the filter permeability was compared with that of the conventional method. In a conventional extrusion method of polydispersed HbV, we used filters with six different pore sizes (FM_3, $FM_{0.8}$, $FM_{0.65}$, $FM_{0.45}$, $FM_{0.3}$, and $FM_{0.22}$) (*24*). One advantage of the present method is the omission of two filters ($FM_{0.8}$ and $FM_{0.65}$) because the size of the vesicles has already been controlled to 529 ± 100 nm. The filter permeability of the dispersion is compared in Figure 5 with the various concentrations of the lipids during the freeze–thawing. The concentration of the lipids during the extrusion was adjusted to 5 g dL^{-1} in all of the experiments. The solution viscosity of the Hb (40 g dL^{-1}) was 50 cP at 140 s^{-1} (25 °C). When the lipid mixture was dispersed into a Hb solution at 5 g dL^{-1}, the viscosity increased to 75 cP at 140 s^{-1} (25 °C). The rate of the filter permeation of HbV was remarkably improved for all filters using the lyophilized powder of the freeze–thawed vesicles compared with the conventional method. The average time required for extrusion was 30 times shorter when the freeze–thawing was carried out at the lipid concentration of 1 and 2 g dL^{-1}. The freeze–thawing at a higher lipid concentration decreased the filter permeability. The rate was twice as low when the lipid concentration was above 3 g dL^{-1} because of the higher content of large vesicles (>3μm).

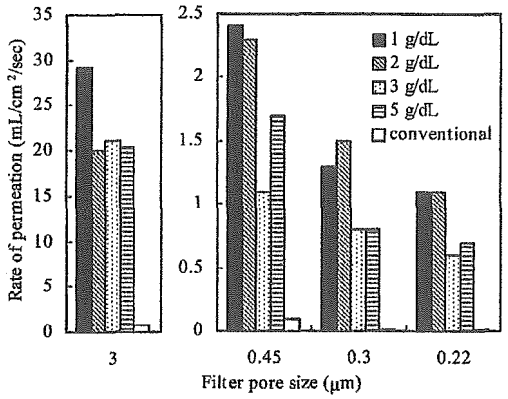

Figure 5. Effect of freeze–thawing on the rate of filter permeation of the HbV with concentration of the lipids during the freeze–thawing. The lyophilized powder of the freeze–thawed vesicles was rehydrated with a Hb solution (40 g dL^{-1}) at 5 g dL^{-1} and the dispersion was extruded through a membrane filter (4.9 cm^2) at a N_2 pressure (20 kg fcm^{-2}) at 14 °C.

The mechanism of the sizing with the filter extrusion is the disruption of the vesicles and the stripping of the bilayer membrane during the passage through the smaller pore size than that of vesicles (*41, 42*). The large multilamellar vesicles having an onion-like structure are unfavorable for filter extrusion because of the low deformability of the vesicles. The high filter permeability of the freeze–thawed vesicles would be due to the low number of bilayer membranes (the lamellarity is ca. 2) and the precontrolled size. The diameter of the vesicles was finalized to 250 ± 20 nm after passing through the $FM_{0.22}$, and Hb was encapsulated into the vesicles with a high efficiency (1.7–1.8 as the value of [Hb]/[lipids]). The reconstruction of the freeze–thawed vesicles by extrusion is necessary to produce HbV with a high encapsulation efficiency. The optimal size of the freeze–thawed vesicles is determined from the size of the final vesicles. If the freeze–thawed vesicles had a size similar to the final pore size during the filter extrusion, the vesicles could pass through the filter membrane without reconstruction of the vesicles for encapsulation. If the freeze–thawed vesicles were very large in comparison to the filter pore size at extrusion, the merit of this pretreatment would be lost. In this sense, the reasonable size of the freeze–thawed vesicles should be two or three times larger than that of the final size. Since the targeting size of HbV is a 250 nm diameter, the size of the freeze–thawed vesicles (ca. 500 nm) is reasonable to encapsulate Hb by extrusion. The metHb content of the final HbV was equal to that of the starting Hb solution (below 2%), meaning that the denaturation of the Hb did not occur during the extrusion process.

In conclusion, we have made great progress in the extrusion using the freeze–thawed vesicles. The desirable cooling rate and concentration of the lipids were −140 °C min^{-1} and 2 g dL^{-1}, respectively. The time required for extrusion of the HbV was 30 times shorter using the lyophilized powder of the freeze–thawed vesicles under these conditions. This finding would be available to manufacture size-controlled vesicles encapsulating concentrated proteins or unstable drugs.

Acknowledgment

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原著

酸素輸液ヘモグロビン小胞体に混在するリポポリサッカライドの定量法

Detection of Lipopolysaccharide Contaminating Hemoglobin-Vesicles as Artificial Oxygen Carriers

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和文抄録

菌体由来のリポポリサッカライド（LPS）は両親媒性分子であるため，疎水性相互作用によりリン脂質小胞体或いはリポソームの脂質二分子膜に取込まれ易く，一般的なリムルステスト（*Limulus* Amebocyte Lysate: LALゲル化反応試験）では正確に定量されないことが問題となる．リン脂質小胞体の内水相に高濃度ヘモグロビン（Hb）を内包したHb小胞体（HbV）の品質管理の面でも正確なLPS定量の確立は重要課題である．そこで本研究では，界面活性剤deca (oxyethylene) dodecyl ether ($C_{12}E_{10}$) でHbVを溶解してLPSを遊離させた後，LAL試薬と混合し，ゲル化反応を比濁時間分析法によって解析する方法を検討した（検出波長660 nm)．界面活性剤$C_{12}E_{10}$はゲル化反応を濃度依存的に阻害するので，$C_{12}E_{10}$濃度とHbV可溶化および阻害作用の相関を解析し，LPSの検出限界が0.1 EU/mLとなる最適測定法を確立した．

Abstract

A method to quantitatively measure the bacterial endotoxin content (lipopolysaccharide, LPS) in phospholipid vesicles or liposomes has been required because the conventional Limulus amebocyte lysate (LAL) test does not provide an accurate measurement due to the hydrophobic interaction of LPS and vesicles that shields the activity of LPS to clot the LAL coagulant. Hemoglobin-vesicles (HbV) are artificial oxygen carriers encapsulating a conc. Hb solution in phospholipid vesicles. To accurately measure the LPS content in the HbV for the quality control, we tested the solubilization of HbV with deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$) to release the LPS entrapped in the vesicles as a pretreatment for the succeeding LAL assay of the kinetic-turbidimetric gel clotting analysis (detecting wavelength, 660 nm). The $C_{12}E_{10}$ surfactant interferes with the gel clotting in a concentration dependent manner, and the optimal condition was determined in terms of minimizing the dilution factor and $C_{12}E_{10}$ concentration. We clarified the condition that allowed the measurement of LPS higher than 0.1 EU/mL in the HbV suspension.

Keywords

endotoxin, hemoglobin, lipopolysaccharide, oxygen carriers, *limulus* amebocyte lysate.

1．緒言

ヘモグロビン（Hb）を濃度高くリン脂質小胞体の内水相に内包したヘモグロビン小胞体（HbV）が人工酸素運搬体として開発され，赤血球と同等の酸素運搬機能と安全性が動物投与試験から明らかにされてきた[1-5]．生物製剤に分類されるHbVの製造工程はGMP（good manufacturing practice）の基準に沿って，不純物やウィルス／菌体の混入防御に関して厳格に対処しなければならない．エンドトキシンとして知られるグラム陰性菌体由来のリポポリサッカライド（LPS）は極く微量でも様々な毒性を示す[6]．例えばLPSの致死量（LD_{50}）は，ラットで3 mg/kg，犬で1 mg/kgである[7,8]．米国FDAはヒトに対する非経口的投与薬剤の場合，LPS許容投与量を5 EU/kgと規定している[9]．この規定はHbV静注液にも該当すると考えられる．LPS濃度はエンドトキシン活性　(Endotoxin Unit: EU; 1EU=100

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pg）として表され，カブトガニの血球抽出物（*Limulus* amebocyte lysate;LAL試薬）との反応によって測定する（リムルステスト）．LALはLPSに対し濃度依存的に凝固しゲルを形成する[10]．ウサギ発熱試験に比較して，リムルステストでは検体が少量でも繰返し測定できる利点を有する[11]．人工酸素運搬体の適応は主として出血ショック蘇生や血液希釈であり，投与量は20 mL/kg或いはそれ以上が想定され，この場合LPSの許容量は，0.25 EU/mL（= 5/20）となる．これは注射用水の許容量0.25 EU/mLと同じである．

菌体由来LPSは両親媒性の巨大分子であり，蛋白質や生体膜と疎水性相互作用をする[12]．HbもLPSと強く結合し様々な神経毒性を示すことが報告されている[13]．LAL試薬のゲル化を惹起するのはLPS分子中のLipid-Aとよばれる糖結合リン脂質の部分である[14]．数本の脂肪酸を有するLipid-Aは，リン脂質小胞体の二分子膜に容易に挿入されるので，LPS特有のLAL試薬のゲル化反応や，その他の反応性が阻害される[15,16]．従って，HbVの品質管理においてLPSの正確な定量法の確立が極めて重要と成る[17-20]．本研究では，界面活性剤を利用してHbVを容易に溶解しLPSを遊離する条件を見出すとともに（Fig. 1），トキシノメータ®によりLAL試薬のゲル化反応を比濁時間分析法によって解析し，阻害反応をできるだけ抑制する前処理条件を決定することを目的とした．

2．方法

ヘモグロビン小胞体（HbV）の調製

PEG修飾HbVは既報[21-23]に従い無菌雰囲気下にて調製した．高純度Hbは北海道赤十字血液センター（札幌）および日本赤十字社（東京）から提供された期限切れ献血血液から精製した．限外濾過膜によるストローマ除去と，加熱処理（60℃, 10時間）で変性沈澱した夾雑蛋白質の除去により，高純度Hbを得た（>99.9%）[24]．濃厚Hb溶液（38 g/dL）にアロステリック因子として14.7mMのpyridoxal-5`-phosphate（PLP，メルク社製，Darmsdart，Germany）を，モル比でPLP/Hb = 2.5となるように添加し小胞体に内包した．小胞体の脂質二分子膜の構成成分は，1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine（DPPC），コレステロール，1,5-di-*O*-hexadecyl-*N*-succinyl-L-glutamate（DPEA）（日本精化製，大阪）および，1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000}（PEG-DSPE，日本油脂社製，東京）である[25]．DPPC/コレステロール/DPEA/PEG-DSPEの混合比は5/5/1/0.033（モル比）とした．HbV粒子は生理食塩水中にHb濃度10 g/dLになるように分散した．HbVの物性パラメータは以下の通り:粒径，251±80 nm; [Hb]，10 g/dL; [metHb]，<3%; [lipids]，5.6 g/dL;酸素親和度（P_{50}），31Torr．分析法は全て既報[26]に従った．

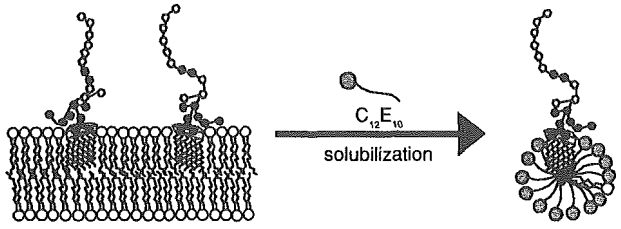

Figure 1. Solubilization of LPS-contaminated vesicles with a detergent to form mixed micelles.

界面活性剤によるHbVの可溶化条件の決定

HbV分散液を純水で2又は5倍希釈し（[Hb]=2 or 5 g/dL，200 μL），deca(oxyehylene) dodecyl ether（$C_{12}E_{10}$，Sigma社製，純水中に0～10vol%で溶解，800 μL）と混合して石英キュベット（2 mm）に入れた．混合液を42℃，2分加温して可溶化を促進させた．紫外可視分光光度計（V-560，東京電子，東京）により，660 nmの濁度を測定した．比較として，リポソームや生体膜の可溶化によく用いられるドデシル硫酸ナトリウム（SDS，関東化学（株）製，東京），Triton-X100，nona(oxyethylene) dodecyl ether（$C_{12}E_9$）（Sigma社製）についても比較検討した．

リムルステストにおける検量線と界面活性剤の影響

日本薬局方で規定された標準LPS粉末（*E. coli* UKT-B由来，マンニトールとグリシンを含む，和光純薬工業社製，東京）を注射用蒸留水（大塚製薬社製，大阪）に溶解して原液（[LPS] =500 EU/mL）を調製した．LPS標準溶液（[LPS]=0.01-20 EU/mL）は原液を注射用蒸留水で希釈して調製した．LPS標準溶液（200 μL）と$C_{12}E_{10}$溶液（0～5 vol%，800mL）を混合し，注射用蒸留水で8倍希釈し，等容量のLAL試薬（リムルスES-Ⅱテストワコー，和光純薬工業社製）と混合した．LAL試薬には，カブトガニ*Limulus Polyphemus*血球抽出物，トリス塩酸緩衝液（pH = 7.1），β-1,3-グルカン誘導体を含む．注射用蒸留水中のLPS含量は検出範囲外（<0.0001 EU/mL）であった．LPS含量は，LAL試薬とLPSのゲル化反応による濁度変化をトトキシノメータ®（ET-201，和光純薬工業社製）を用いた比濁時間分析法により定量した．ゲル化時間はマニュアルに従い660 nmの透過率が95%まで減少した時間と定義した．Hb溶液およびHbVの場合，Hbの強い光吸収が400～600 nmにあるため，キシノメータ®の測定波長660 nmは，Hbの吸収を避けているので適当と考える．

HbV分散液中のLPS定量法とその検証

Fig. 2のスキームに沿って，HbV分散液（[lipid] = 6.0 g/dL，[Hb] = 10 g/dL）をLPSフリーの試験管に分注して注射用蒸留水で希釈し，$C_{12}E_{10}$溶液と混合，42℃で2分加温した．次いで混合液を注射用蒸留水により8倍希釈しその後，溶液をLAL試薬に添加し，ゲル化による濁度変化をトキシノメーターET-201を用いた比濁時間分析法により解析した．

本法の妥当性を検証するため，添加LPSの回収率を測定した．

LPS標準溶液（[LPS] = 0.4～10.0 EU/mL）を脂質粉末に添加し，脂質濃度を6.0 g/dLに調整した．脂質分散液を攪拌し，注射用蒸留水により5倍希釈し，凍結融解を3回行ってLPS担持小胞体を調製した．回収率は，上述の方法によって定量したLPS濃度と添加LPS濃度の比較から算出した．

更にHbVでも同様の実験を行った．HbV分散液（[Hb]= 4 g/dL）を等容量のLPS標準液（[LPS] = 0.04～4.0 EU/mL）と混合してLPS担持HbV分散液（[Hb] = 2 g/dL）を調製し，$C_{12}E_{10}$溶液で溶解し上述の方法によりリムルステストを行った．Hb溶液については，同上のLPS標準溶液（[LPS] = 0.002～2.0 EU/mL）を等容量のHb溶液（[Hb] = 4 g/dL）と混合して，LPS含有Hb溶液（[Hb] = 2 g/dL）を調製し，直接LAL試薬と混合後LPS濃度を定量した．回収率は，定量したLPS濃度と添加LPS濃度の比較から算出した．

$C_{12}E_{10}$処理の有効性を確認するために，LPS担持小胞体を上述の方法に従って，$C_{12}E_{10}$を使う系と使わない系で比較検討した．先ず，LPS溶液（1.0 EU/mL）を脂質粉末に添加（1.2 g/dL）して小胞体を調製．脂質分散液に凍結融解を3度行って分散を高め，粒径を約500 nmに制御した．上述のように，$C_{12}E_{10}$を使う系と使わない系でLPS濃度を定量した．

3．結果

$C_{12}E_{10}$界面活性剤によるHbVの可溶化

波長660 nmにおけるHbV分散液の吸光度は1.13であり，HbV粒子による光散乱（濁度）のためHb溶液の吸光度（0.08）よりも極めて高い値となった．しかし$C_{12}E_{10}$溶液の添加につれ吸光度は減少し，HbVの可溶化が示唆された．完全溶解には2 vol%の$C_{12}E_{10}$溶液の添加を必要とした．この時の吸光度は小胞体が無いHb溶液のみの吸光度と等しくなった．可溶化処理後，溶液は赤色から褐色に変化し，metHbへの変化が示唆されたが，沈澱形成は認められなかった．結果的に溶液中の$C_{12}E_{10}$濃度とHbVの脂質濃度は各々1.6 g/dL，0.24 g/dLであった．$C_{12}E_{10}$は混合ミセルの87 wt%（86 mol%）を占めている．他の界面活性剤については，$C_{12}E_9$が$C_{12}E_{10}$と同様のHbV可溶化能を示した．一方，Triton-X100では吸光度は0.4までの低下，SDSでは0.6までの低下であり，HbVを充分に可溶化できなかった．

リムルステストに対する$C_{12}E_{10}$の影響

ゲル化時間とLPS終濃度の関係を対数プロットとして示し，これを検量線とした（Fig. 3）．幅広いLPS濃度範囲（LPS終濃度：0.000125～0.25 EU/mL）で直線関係が得られた．$C_{12}E_{10}$濃度を増加させるにつれ，ゲル化が妨げられる傾向（ゲル化時間の延長）が見られた．しかし，$C_{12}E_{10}$終濃度が0.1 vol%の時はゲル化の著しい阻害は見られなかった．LPS濃度0.01 EU/mLの場合，$C_{12}E_{10}$無添加のゲル化時間は約32分であったが，$C_{12}E_{10}$濃度0.1 vol%では39分，2.0 vol%では約107分に延長した．エチレンオキサイド鎖が1ユニット短い$C_{12}E_9$の場合，濃度0.1 vol%の場合はゲル化時間が50分に延長し，$C_{12}E_9$のゲル化阻害効果は$C_{12}E_{10}$よりも高かった．

HbV分散液中のLPS定量

LPS検出限界の向上のため，可溶化処理におけるHbV濃度の増加，又は希釈率の低減によって希釈倍率を変えてみた（Table1）．$C_{12}E_{10}$濃度0.1 vol%の条件（Entry-1）に，最小の検出限界（0.1 EU/mL）が得られた．Entry-2，3，4の条件では希釈倍率を減らすことができたが，Fig. 3に示すように，$C_{12}E_{10}$濃度が0.4 vol%以上では著しいゲル化の阻害起こるため，検出限界が悪くなった（0.23 EU/mL）．

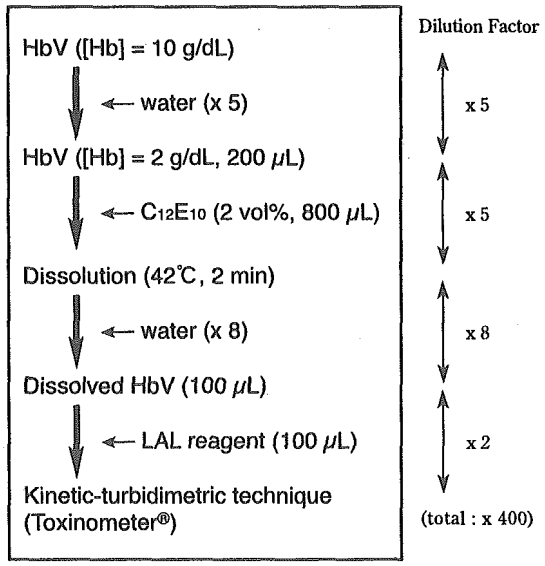

Figure 2. Procedures to quantitatively measure LPS in the HbV suspension using $C_{12}E_{10}$ for HbV solubilization and LAL assay, and dilution factors at every mixing of solutions. The experimental condition is for Entry 1 in Table 1.

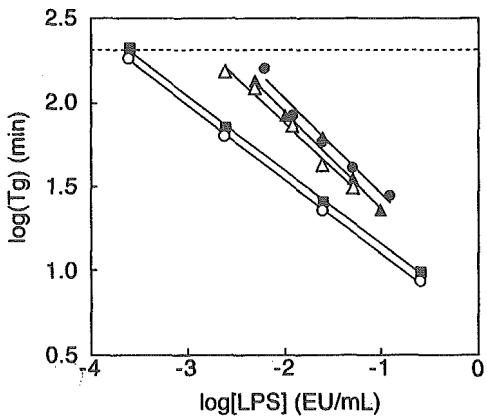

Figure 3. Calibration curves for the quantitative measurement of LPS in the presence of $C_{12}E_{10}$ at various concentrations (vol%); 2.0 (●), 0.8 (▲), 0.4 (△), 0.1 (■), 0 (○). [LPS] is the final concentration in the test tube for turbidimetry. Dilution factors should be multiplied to obtain [LPS] in the HbV suspension at [Hb] = 10 g/dL. For example, [LPS] should be multiplied with 400 at [$C_{12}E_{10}$] = 0.1 vol%. The broken line indicates the gelation time limit for Toxinometer® (200 min).

Table 1. Solubilization conditions of HbV with $C_{12}E_{10}$ and detection limit of LPS. The calibration curves are shown in Fig. 3.

Entry	Condition of HbV solubilization: HbV / 200 μL ([Hb] g/dL)	Condition of HbV solubilization: $C_{12}E_{10}$ / 800 μL ([$C_{12}E_{10}$] vol%)	dilution factor[a]	Final [$C_{12}E_{10}$] (vol%)	Detection limit of LPS (EU/mL HbV)[b]
1[c]	2	2	400	0.1	>0.1
2	2	2	100	0.4	>0.25
3	2	2	50	0.8	>0.25
4	5	5	20	2.0	>0.23

[a] see Fig. 2. from HbV([Hb]= 10 g/dL) to gel clotting assay.
[b] at [Hb]=10 g/dL.
[c] The optimal condition in this study

Table 2. Inhibition/enhancement testing to monitor the recovery of spiked LPS. LPS was spiked in the $C_{12}E_{10}$ -solubilized vesicles and HbV, and Hb solution without $C_{12}E_{10}$. *n = 3. All the data are converted to the conditions of [Hb]=10 g/dL, or [lipid]=6 g/dL.

Sample	Spiked LPS (EU/mL)	Recovery (%)
Vesicles (6 g/dL) with $C_{12}E_{10}$	0.4	108.6±4.6*
	1.25	92
	2.5	116
	5	103
	10	120
HbV (Hb, 10 g/dL; lipid, 6 g/dL) with $C_{12}E_{10}$	0.1	107
	0.4	111.3±3.6*
	1.0	114
	10.0	112
Hb solution (10 g/dL) without $C_{12}E_{10}$	0.005	121
	0.02	103.9±4.4*
	0.05	101
	0.5	124
	5	97

添加LPSの回収率は，低濃度から高濃度まで幅広いLPS濃度範囲で92%と124%の間におさまった（Table 2）．Hb溶液のLPS定量では，界面活性剤を必要としないためより低いLPS濃度（0.005 EU/mL）の検出が可能であり，$C_{12}E_{10}$を必要とするHbVの検出限界（0.1 EU/mL）と比べて検出感度が向上した．1987年に発表されたFDAガイドラインによると，検出限界濃度の4倍濃度でのLPS添加回収試験の実施を必要とし，回収率は100±25%以内でなければならない．そこでリン脂質小胞体に0.4 EU/mL（=4×0.1 EU/mL），Hb溶液に0.02 EU/mL（=4×0.005 EU/mL）のLPSを添加し回収率を測定したところ，全て100±25%以内であることが確認できた．

LPS担持小胞体中のLPS定量では，$C_{12}E_{10}$を用いた上記リムルステストにより1.03 EU/mLと判明した．一方，$C_{12}E_{10}$を添加せず小胞体を破壊しない状態でリムルステストをしたところ0.6 EU/mLとなった．この場合，回収率は60%に留まった．

4．考察

両親媒性LPSとリン脂質小胞体の疎水性相互作用がLAL試薬の活性に影響すること，またその他LPSの生理活性が抑制されることは多々報告されている[15,16,27,28]．他方，HbとLPSの相互作用についてはJurgensらが最近，Hb分子1個当たり3～5個のLPSが結合することを報告している[29]．従ってリムルステストによるHbVのLPS定量の前段階として，何らかのLPS遊離法が必要なことは明らかである．これまでにリン脂質小胞体の前処理として有機溶媒や界面活性剤を使用する方法が報告されているが[18,19,30]，HbVに多量に含まれる蛋白質Hbは有機溶媒に不溶なので，我々は界面活性剤を利用する方法を検討し，Hb濃度10 g/dLのときLPS濃度0.1 EU/mLまでの測定を可能とした．HbVの分散状態と形態の安定度は極めて高いが，界面活性剤として$C_{12}E_{10}$を添加し42℃で2分間インキュベーションすればHbVは完全に溶解でき，前処理法として利用できることが明らかとなった．

Fig. 3に示すように，$C_{12}E_{10}$は特に高濃度でLAL試薬のゲル化時間を延長した．しかし$C_{12}E_{10}$最終濃度0.1 vol%では阻害作用は低減される．他の界面活性剤を検討したところ，nona (oxyethylene) dodecyl ether（$C_{12}E_9$）はHbVの溶解能には優れるがより強いゲル化阻害作用を示し，Triton-X100はHbVの溶解能が不十分となった．イオン性界面活性剤SDSも十分に溶解できないし，更に文献によればSDSは僅か0.005 wt%以上でLAL試薬のゲル化を完全に抑制する[19]．対照的に非イオン性$C_{12}E_{10}$は，濃度依存的に穏和なゲル化阻害作用を示す．つまり，$C_{12}E_{10}$はHbVの溶解能に優れ，且つ，リムルステストにおいて十分に低いゲル化阻害作用を示すに留まる．HbVを$C_{12}E_{10}$で溶解すると混合ミセルが形成されるが（Fig. 1），このとき$C_{12}E_{10}$は構成成分全体の86 mol%を占め，PEG-DSPEの含量（0.04 mol%）を遥かに凌ぐ．$C_{12}E_{10}$ミセルの会合数は約100なので[31]，LAL試薬に含まれるLPS認識蛋白質（Factor C）はPEG-DSPEの立体障害の影響を殆ど受けずに容易に混合ミセルの親疎水界面付近に存在するLPSのLipid-A部位と結合することができると考えられる．

LPS検出限界の向上には，希釈率の低減と$C_{12}E_{10}$濃度の低下が要件と成る．しかし希釈率低減は逆に$C_{12}E_{10}$最終濃度の上昇をもたらす．検討の結果，$C_{12}E_{10}$濃度が0.1 vol%のとき，Fig. 3に示す通り阻害作用は極めて僅かであり，Table1のEntry-1に示す最適の前処理条件を決定できた．このとき総希釈倍率は400となった．FDAの指針には，最大投与量とLAL試薬の感度に応じた最大希釈倍率（Maximum Valid Dilution, MVD）が定義されている．HbVの許容LPS濃度 0.25 EU/mLと，Fig. 3のLPS検量線上の検出限界0.00025 EU/mL（log（0.00025）EU/mL=-3.60）より，HbVのMVDは1000（= 0.25/0.00025）と算出される．従って希釈率400はMVD範囲内にある．

小胞体に含有するLPSの定量を$C_{12}E_{10}$の有無で比較したところ，$C_{12}E_{10}$で溶解した方が高いLPS濃度を示したことから，脂質二分子膜内に挿入していたLPS或いは小胞体の内水相に取込まれていたLPSが遊離して検出されたことが明らかである．LPS添加回収試験の結果，Table 2に示す通り広範なLPS濃度において回収率は92～124%の範囲で再現性があった．FDA指針に従えば，回収率が100±25%のときに溶質の影響が無いと判断できる[9]．この基準値は1991年に100±50%に変更された[32]．Hb分子1個当たり3～5個のLPSが結合すると報告されているが[29]，我々のHb溶液での添加回収試験ではHbに拠る阻害作用を認めていない．LevinらはHbに結合したLPSはLAL試薬のゲル化を促進すると報告しているが[13,33]，逆にArchambaultらはLPSがHbに結合することでLAL活性が低下するとしている[34]．JurgensらはLAL活性はLPSとHbの濃度に依存することを観測している[29]．このような見解の相違は恐らく，LPSの濃度設定に関係すると考えられる．我々の実験条件では，LPS濃度を0.2 EU/mL（約20 pg/mL）以下に設定したが，他の研究グループは著しく高いLPS濃度（100 pg/mL～800 μg/mL）で測定を実施しており，場合によってはLPSの臨界凝集濃度（10～38 μg/mL）を越えている[35]．LPSが凝集するとLAL活性が著しく低下することが知られているので[36]，Hbが存在するとLPSは凝集せずにHbに結合して分散性が向上し，見かけ上LAL活性が上昇したと考えられる．他方，我々の測定条件では極めて希薄なLPS濃度に設定しているためLPSの凝集は無く，より自由にHb溶液中に分散し，Hb溶液の阻害作用は無いものと推測できる．

5．結論

本研究では，Hb小胞体を界面活性剤（$C_{12}E_{10}$）で前処理することによりHb小胞体に結合したLPSを遊離させた後に，LAL法にてLPS定量する方法を検討し，0.1 EU/mLまでの検出限界を得ることができた．また，添加回収法によりその妥当性を検証することができた．本法は現在，Hb小胞体の製造工程で日常的に使用されている．

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Detection of Lipopolysaccharide in Hemoglobin-Vesicles by *Limulus* Amebocyte Lysate Test with Kinetic–Turbidimetric Gel Clotting Analysis and Pretreatment of Surfactant

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ABSTRACT: A method to quantitatively measure the bacterial endotoxin content (lipopolysaccharide, LPS) in phospholipid vesicles or liposomes is necessary because the conventional *Limulus* amebocyte lysate (LAL) test does not provide an accurate measurement due to the hydrophobic interaction of LPS and vesicles that shields the activity of LPS to clot the LAL coagulant. This interference was evident from isothermal titration calorimetry results in our study that clearly demonstrated the insertion of the LPS molecule into the phospholipid bilayer membrane. Hemoglobin-vesicles (HbVs; particle diameter = 251 ± 80 nm; [Hb] = 10 g/dL) are artificial oxygen carriers encapsulating a conc. Hb solution in phospholipid vesicles, and their oxygen transporting ability has been extensively studied. To accurately measure the LPS content in the HbV suspension, we tested the solubilization of HbV with deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$), used to release the LPS entrapped in the vesicles, as a pretreatment for the succeeding LAL assay of the kinetic–turbidimetric gel clotting (detecting wavelength, 660 nm). The $C_{12}E_{10}$ surfactant interferes with the gel clotting in a concentration-dependent manner, and the optimal condition was determined in terms of minimizing the dilution factor and $C_{12}E_{10}$ concentration. We clarified the condition that allowed the measurement of LPS at >0.1 endotoxin units (EU)/mL in the HbV suspension. Moreover, the utilization of histidine-immobilized agarose gel effectively concentrated the trace amount of LPS from the $C_{12}E_{10}$-solubilized HbV solution and washed out $C_{12}E_{10}$ as an inhibitory element. The LAL assay with the LPS-adsorbed gel resulted in the detection limit of 0.0025 EU/mL. Pretreatment with $C_{12}E_{10}$ would be applicable not only to HbVs but also to other drug delivery systems using phospholipid vesicles encapsulating or incorporating functional molecules. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 93:310–321, 2004

Keywords: liposomes; calorimetry (ITC); surfactants; phospholipids; nanotechnology

INTRODUCTION

Phospholipid vesicles or liposomes have been extensively studied as a drug delivery system since the formation of a vesicular structure was discovered in the suspension of egg yolk phosphatidylcholine,[1] and some are now approved for clinical use as antifungal or anticancer therapies.[2] Vesicles encapsulating concentrated hemoglobin (Hb), so-called Hb-vesicles (HbVs) or liposome-encapsulated Hb (LEH), have been developed as oxygen carriers, and their sufficient ability to transport oxygen that is comparable to the ability of blood has been well clarified.[3–7] In comparison with other Hb-based oxygen carriers, such as polymerized Hb or crosslinked Hb, HbVs most closely reproduce the characteristics of natural red blood cells, such as the cell membrane function of physically preventing the direct contact of Hb

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with the components of blood and vasculature during circulation.[8–11] The advantages of artificial oxygen carriers are the absence of blood-type antigens and infectious viruses and stability for long-term storage.[12] In this sense, the infusion of oxygen carriers becomes superior to the conventional blood transfusion, which still has the potential of mismatching, the risk of infections secondary to infusion of contaminated blood, and the problem of only a few weeks storage life.[13]

The process of production of a HbV has to be guaranteed with a good manufacturing practice (GMP) standard so that the HbV is a biological product that adheres to the strict regulations of impurity and viral and bacterial contamination. It is strictly required to monitor the content of the lipopolysaccharide (LPS), known as an endotoxin, which is a component of the outer membrane of gram-negative bacteria that possesses a large variety of biological influences on numerous mammalian cells and tissues.[14] An endotoxin is an extremely potent toxin with lethal doses (LD_{50}) of 3 and 1 mg/kg in rats and dogs, respectively.[15,16] The U.S. Food and Drug Administration (FDA) has established a guideline for human maximal permissible endotoxin dose for parenteral products [5 endotoxin units (EU)/kg][17] that may include Hb-based oxygen carriers. This limit is based on the endotoxin activity (1 EU = 100 pg) and can be measured by the *Limulus* amebocyte lysate (LAL) assay, in which LAL clots and forms a gel in the presence of LPS.[18] In general, the LAL method has advantages over the rabbit pyrogen testing because the LAL method requires a lower amount of sample and the assays can easily be repeated.[19] Because the volume of oxygen carriers to be infused for shock resuscitation or acute hemodilution is estimated to be <20 mL/kg, the specific endotoxin limits should be 0.25 EU/mL (=5/20), which is similar to that for water for injection (0.25 EU/mL).

Bacterial LPS is an amphiphilic gigantic macromolecule, therefore, it hydrophobically interacts with protein and biomembranes.[20] Hb strongly interacts with LPS, showing synergistic toxicity.[21–23] The constituent of endotoxin that causes LAL gelation is a glycophospholipid that is designated lipid-A.[24] Lipid-A possesses several fatty acid constituents that are readily inserted into the bilayer membrane of the phospholipid vesicles. The inclusion of lipid-A in the phospholipid vesicles markedly reduces several functions of lipid-A, such as its LAL gelation activity.[25,26] As a consequence, the researchers who study HbVs or other phospholipid vesicles for delivering other functional molecules encountered a problem in measuring the LPS content for the quality control of these materials.[27–30] Considering this background information, we aimed to find the optimal condition for the pretreatment of HbVs using a surfactant to release LPS[28,29] with a minimal interference effect for the subsequent kinetic–turbidimetric LAL assay using a Toxinometer®. For a better detection limit, we tested the histidine-immobilized agarose gel that effectively adsorbs LPS to concentrate the trace amount of LPS and to eliminate the solutes that interfere with the LAL assay.[30–33]

EXPERIMENTAL

Preparation of Poly(ethylene glycol) (PEG)-Modified Hb-Vesicles (HbVs)

The PEG-modified HbVs were prepared under sterile conditions as previously reported.[8,34–36] Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and Japanese Red Cross (Tokyo, Japan). The purification process included ultrafiltration to remove the stromal components and pasteurization at 60°C for 10 h to denature the concomitant proteins. This process results in extremely high purity of Hb (>99.9%).[35,37] The encapsulated Hb (38 g/dL) contained 14.7 mM pyridoxal 5′-phosphate (PLP, Merck Company, Darmsdart, Germany) as an allosteric effector at a PLP/Hb molar ratio of 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate (DPEA) at a molar ratio of 5:5:1 (Nippon Fine Chemical Company, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (PEG–DSPE; NOF Company, Tokyo, Japan).[38] Thus, the vesicular surface is covered with PEG chains. The molar composition of DPPC/cholesterol/DPEA/PEG–DSPE was 5:5:1:0.033. The HbV particles were suspended in saline at an Hb concentration of 10 g/dL. The physicochemical parameters of the HbV were as follows: particle diameter, 251 ± 80 nm; [Hb], 10 g/dL; [metHb], <3%; [carbonylhemoglobin (HbCO)], <2%; lipids, 5.6 g/dL; and oxygen affinity (P_{50}), 31 Torr. All the analytical methods are described elsewhere.[12]

Thermodynamic Analysis of Insertion of LPS into Vesicles by Isothermal Titration Calorimetry (ITC)

An OMEGA titration microcalorimeter (MCS ITC, Microcal Inc., Northampton, MA) was used to analyze the interaction of LPS with the phospholipid bilayer membrane.[38,39] The model phospholipid vesicles of DPPC/cholesterol/DPEA/PEG–DSPE (5:5:1:0.033 by mol) were prepared under sterile conditions by simply dispersing the lipid powders in pure water (0.05 g/dL). The resulting suspension was freeze-thawed to enhance the dispersion and to regulate the particle diameter to 519 ± 78 nm. Twenty-five cumulative injections of an LPS solution (10 μL, 334 μM in pure water, from *Salmonella Minnesota* wild type, MW = ~20 kDa[40]; Sigma Chemical Company, St. Louis, MO) into the vesicle suspension (1.35 mL, [lipid] = 835 μM) were performed using a computer-controlled microsyringe while the suspension was stirred at 400 rpm and the temperature was strictly controlled at 37°C. Changes in the calorific values and the total calorific values were automatically measured. An identical injection of LPS into pure water in the absence of vesicles and injection of pure water into the vesicle suspension were performed as references.

Determination of the Solubilization Condition of HbV with Surfactants

An HbV suspension, diluted twice or five times with pure water ([Hb] = 2 or 5 g/dL, 200 μL), and deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$, 0–10 vol% dissolved in pure water, 800 μL; Sigma Chemical Company) were mixed in a quartz cuvette (2 mm thickness). The resulting suspension was heated at 42°C for 2 min to enhance the solubilization. The turbidity was measured at 660 nm with an ultraviolet–visible (UV–vis) spectrophotometer (V-560, Jasco, Tokyo). For comparison, other surfactants that are often used for the dissolution of liposomes or biomembranes,[28,29,41] [sodium dodecyl sulfate (SDS), Kanto Chemical Company, Tokyo, Japan; Triton-X100 and nona(oxyethylene) dodecyl ether ($C_{12}E_9$), Sigma Chemical Company] were also tested for the solubilization of HbV.

Calibration Curves for LAL Assay and Influence of a Surfactant

A powdered LPS (LPS purified from *Escherichia coli* UKT-B containing mannitol and glycine, Wako Pure Chemical Industries, Tokyo, Japan), a control standard LPS defined in Japanese Pharmacopoeia, was dissolved in pure water (Water for Injection, Otsuka Pharmaceutical Company, Tokyo, Japan) to prepare the stock solution ([LPS] = 500 EU/mL). The LPS standard solutions ([LPS] = 0.01~20 EU/mL) were prepared by dilution of the stock solution with pure water. The LPS solutions (200 μL) were mixed with a $C_{12}E_{10}$ solution (0–5 vol%, 800 μL), diluted eight times with pure water, and then mixed with an equal amount of LAL solution (*Limulus* ES-II Test Wako, Wako Pure Chemical Industries) containing a lysate from *Limulus polyphemus*, Tris-HCl buffer (pH = 7.1), and a derivative of β-1,3-glucan. The LPS content in the water for injection is below the detection limit (<0.0001 EU/mL). The LPS content was measured as the turbidity change during the gel clotting in the reaction of the LAL reagent and endotoxin reaction with a parallel turbidimetric time assay using a Toxinometer® (ET-201, Wako Pure Chemical Industries).[42,43] The gelation time was defined by the reduction of the transmittance at 660 nm to 95% of the initial value according to the instruction manual.

Measurement of LPS in an HbV Suspension (Method 1) and Confirmation of its Validity

The HbV suspension ([lipid] = 6.0 g/dL, [Hb] = 10 g/dL) was put in LPS-free glass tubes, diluted with water for injection, mixed with a $C_{12}E_{10}$ solution, and then heated at 42°C for 2 min, as shown in Figure 1. The solution was then diluted eight times with water for injection. The LAL reagent (Wako Pure Chemicals Industries) was then added to the solution, and the LPS content was measured as the turbidity change in gel-clotting in the LAL–endotoxin reaction with a parallel turbidimetric time assay using a Toxinometer® ET-201.

To evaluate the validity of the Method 1, the recovery of spiked LPS was measured. An LPS standard solution ([LPS] = 0.4, .25, 2.5, 5.0, and 10.0 EU/mL) was added to the powdered lipid to adjust the lipid concentration to 6.0 g/dL. The suspension was agitated, diluted five times with the water for injection, and then freeze-thawed three times to prepare the LPS-contaminated vesicles. The recovery ratio was calculated by comparing the LPS concentration measured by the method already described and the spiked LPS concentration. A similar experiment was performed with HbV. An HbV suspension ([Hb] = 4 g/dL)

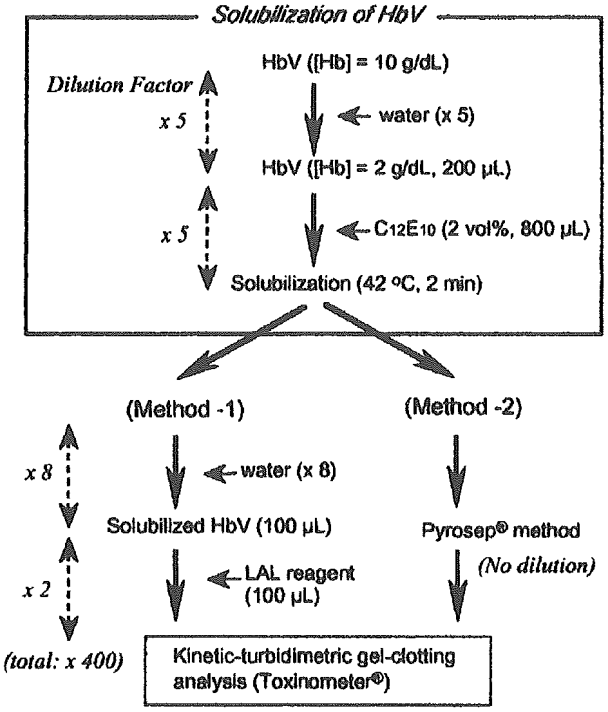

Figure 1. Procedures to quantitatively measure LPS in the HbV suspension using $C_{12}E_{10}$ for HbV solubilization and LAL assay, with dilution factors at every mixing of solutions. Method 1 is the condition for Entry 1 in Table 1. The Pyrosep® method (Method 2) does not have a dilution after solubilization of HbV.

was mixed with an equal volume of the LPS standard solution ([LPS] = 0.04, 0.16, 0.4, and 4.0 EU/mL) to prepare the LPS-spiked HbV ([Hb] = 2 g/dL), and solubilized by $C_{12}E_{10}$ for LAL assay as already described. As for the Hb solution, the same LPS standard solution ([LPS] = 0.002, 0.008, 0.02, 0.2, and 2.0 EU/mL) was mixed with an equal volume of an Hb solution ([Hb] = 4 g/dL) to prepare the LPS-spiked Hb solution ([Hb] = 2 g/dL), which was directly mixed with the LAL reagent to measure the LPS concentration. The recovery ratio was calculated by comparing it to the spiked LPS.

To confirm the effectiveness of the $C_{12}E_{10}$ treatment, LPS-contaminated vesicles were tested by Method 1 with or without $C_{12}E_{10}$. The vesicles were prepared by the addition of an LPS solution (1.0 EU/mL) to the powdered lipids (1.2 g/dL). The resulting suspension was freeze-thawed three times to enhance the dispersion of the lipids and to regulate the particle diameter to ~500 nm. The LPS concentration was measured, as already mentioned, using $C_{12}E_{10}$, and also without the addition of the $C_{12}E_{10}$ solution.

LPS Measurement in $C_{12}E_{10}$-Solubilized HbV using Histidine-Immobilized Agarose Gel (Pyrosep®, Method 2)

The *Limulus* PS Single Test Wako (Wako Pure Chemicals Industries) was used. A 5-mL aliquot of the $C_{12}E_{10}$-solubilized HbV solution ([Hb] = 0.4 g/dL, $[C_{12}E_{10}]$ = 1.6 vol%) was injected into a glass capillary column that contained 0.7 mL of histidine-immobilized agarose gel (Pyrosep®) to adsorb the LPS into the gel. The glass capillary column has a filter at the connected end of a silicone tube to retain the gel but allows permeation of the solution by aspirating air through the silicone tube. The gel was washed with 2 mL of LPS-free water to remove all the solutes except LPS, and then 0.3 mL of LAL-ES reagent (*Limulus polypherus* amebocyte lysate lyophilized, containing Tris-HCl buffer and β-1,3-glucan derivative) was injected into the capillary. All of the suspension was immediately pushed back into a glass vial, and the gelation time of the suspension was measured with a Toxinometer® (ET-201or ET-301 BL) with an extended MT-358 analysis module (Wako Pure Chemicals Industries). The time course of the transmittance change was detected at 660 nm, as already described. The inhibition/enhancement testing was performed to confirm the recovery ratio of the spiked LPS to the HbV in the same manner as already described.

RESULTS

Interaction of LPS with Phospholipid Vesicles Measured by Isothermal Titration Calorimetry (ITC)

ITC of the LPS injection showed exothermic peaks for every injection into the vesicles suspension, and the enthalpy change (ΔH) was calculated to be ~−80 kcal/mol (Fig. 2). As a reference experiment, the LPS solution was injected into pure water, and it was confirmed that the thermodynamic change was negligibly small (data not shown). The maximum amount of incorporation was 7.6 mol% into the outer surface of the vesicles under the assumption that the lamellarity of the vesicles was 2.

Solubilization of HbV with $C_{12}E_{10}$ Surfactant

The absorbance of the HbV suspension at 660 nm was 1.13, which is significantly higher than that of the Hb solution (0.08) due to the light scattering effect of the HbV particles. However,