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Journal:	*ACS Biomaterials Science & Engineering*
Manuscript ID	ab-2021-004243.R1
Manuscript Type:	Article
Date Submitted by the Author:	10-May-2021
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A revised manuscript submitted to
ACS Biomaterials Science & Engineering

Preparation of Artificial Red Blood Cells (Hemoglobin Vesicles) Using the Rotation-Revolution Mixer for High Encapsulation Efficiency

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ABSTRACT

Hemoglobin vesicle (Hb-V) is an artificial red blood cell encapsulating a highly concentrated hemoglobin (Hb) in a liposome comprising phospholipid, cholesterol, negatively charged lipid, and PEG-conjugated phospholipid. Safety and efficacy of Hb-V as a transfusion alternative have been extensively studied. For this study, we prepared Hb-V using the kneading method with a rotation-revolution mixer as an alternative to the conventional extrusion method. We optimized the kneading operation parameters to obtain Hb-V with a high yield. Results show that the Hb encapsulation efficiency was increased dramatically up to 74.2%, which is higher than the extrusion method (20%) because the kneading method enabled mixing of a highly concentrated carbonylhemoglobin (HbCO) solution (40 g/dL) and a considerably large amount of powdered lipids in only 10 min. The high viscosity of the Hb-lipid mixture paste (ca. $10^3 - 10^5$ cP) favorably induces frictional heat by kneading and increases the paste temperature (ca. 60°C), which facilitates lipid dispersion and liposome formation. During the kneading operation using a thermostable HbCO solution, Hb denaturation was prevented. The Hb-V prepared using this method showed no marked change of particle sizes, Hb denaturation, or Hb leakage from liposomes during two years of long-term storage stability tests. Collectively, these results demonstrate that the kneading method using a rotation-revolution mixer shows good potential as a new method to produce Hb-V.

Key words: Liposomes, Hemoglobin, Blood Substitutes, Homogenization, Planetary Mixer

1. INTRODUCTION

The current blood donation – blood transfusion system has been established as an indispensable technology for medical care. However, because donor blood also presents difficulties such as infectious risks, a blood-type mismatching, and a short shelf-life (3–6 weeks in a refrigerator), the realization of blood substitutes, especially an alternative to red blood cells (RBCs), has been desired as an urgent task.[1–4] An oxygen-binding protein, hemoglobin (Hb), is the most abundant protein in blood. It can be isolated from RBCs with no pathogen or blood-type antigen. Therefore, hemoglobin-based oxygen carriers (HBOCs) have been investigated worldwide for the purpose of their clinical use. The two main types of HBOCs in development are based on cell-free Hb and encapsulated Hb. Actually, Hb is a complex made up of four subunit polypeptide chains: two α and two β. Free Hb outside of RBCs in plasma rapidly dissociates to two αβ dimers and causes severe symptoms such as renal toxicity and vasoconstriction.[5,6] To avoid these toxicities with increase of the molecular weight,[7,8] chemically modified Hbs such as intramolecular crosslinked Hbs,[9–11] water-soluble polymer conjugated Hbs,[12–15] and polymerized Hbs[16–18] are known. On the other hand, encapsulation of Hbs such as liposome-encapsulated Hbs[19–21] and polymersome-encapsulated Hbs[22,23] are also effective to eliminate the toxicity of free Hb. We specifically examined the liposome-encapsulated Hb, which mimics the physiological importance of Hb compartmentalization in RBCs.

Historically, Bangham and Horne found in 1964 that the amphipathic molecule phospholipids self-assemble in water to form bilayer membranes,[24] so-called phospholipid vesicles or liposomes, which are known as comprising the most common alternative to biological membranes. After the first report of encapsulation of Hb in microcapsules using polymer membranes by Chang in 1964,[25] Djordjevici and Miller first reported the liposome-encapsulated Hb (LEH) in 1977, but problems such as particle size control and inhibition of aggregation because of interaction with plasma proteins were difficult to resolve.[20]

Subsequently Tsuchida's group established Hb-vesicle (Hb-V) encapsulating high concentrated Hb, which resolved the difficulty of particle size control.[21,26,27] The dispersion stability in the bloodstream was improved by arranging PEG on the particle surface.[28,29]

Liposomes are prepared easily using sonication and a reverse phase evaporation method using organic solvent, etc.[30–32] However, in the cases of LEH and Hb-V, which are assumed to be administered intravascularly, extrusion[21] and microfluidization[33] methods were used to avoid protein denaturation and solvent residues. In the extrusion method for example, a phospholipid mixture was dispersed in an aqueous phase and was extruded through filters of different pore size to regulate the particle size gradually. To enhance the high oxygen carrying capacity of Hb-V, the Hb solution concentration should be as high as possible (35–45 g/dL). As a result, the Hb solution viscosity increases with its concentration (ca. 10^2 cP at 40 g/dL Hb is equal to olive oil).[34] The viscosity increases further with lipid addition. This high viscosity of the mixture induces filter clogging in the extrusion method. Therefore, the amount of a lipid to be mixed was limited. To resolve this difficulty, a method of mixing freeze-dried liposome was proposed.[27] However, the amount of the lipid to be mixed with Hb remains limited. The freeze-drying process is time-consuming and costly. Because a large amount of Hb-V was necessary for a series of preclinical studies, a new method has been sought that enables production of Hb-V for a large quantity and in a short time.

As a result of thorough examination of the background and difficulties presented above, we specifically examined the kneading method using a rotation-revolution mixer for preparing Hb-V. Principles of this mixer are that a cylindrical container (vessel) sealing raw materials rotates around a central axis and simultaneously rotates around a second axis, using a planetary motion. Therefore, this mixer is also designated as a "blade free planetary mixer".[35,36] The technique is also known as "dual (asymmetric) centrifugation (DAC or DC)".[37] This mixer has been well known for purposes of mixing viscous materials and for pulverizing purposes as a ball mill since the 1970s. It is used widely in laboratories and

industries for pharmaceuticals, cosmetics, chemicals, etc.[38–41] Because of the convenience it lends to mixing of highly viscous products, this mixer has been used more and more to prepare functional liposomes and polymersomes since the first preparation was reported in 2008.[42] The usage of this mixer is reported for encapsulation of a water-soluble fluorescence dye calcein,[42] a short interfering RNA[43] and vancomycin,[44] and for entrapping a lipophilic drug: chloramphenicol.[45] This mixer can prepare liposomes rapidly and aseptically with fewer steps without contaminations. Therefore, it would be apparently useful for Hb-V preparation.

For this study, we attempted to prepare Hb-V using the kneading method with a rotation-revolution mixer. We optimized the kneading condition with testing of a set of parameters; lipid composition, Hb concentration, fed Hb/lipid ratio in raw materials, and kneading time. Additionally, we investigated the long-term storage stability for two years of the Hb-V prepared with an optimized condition.

2. MATERIALS AND METHODS

2.1. Preparation of lipid mixture

For this study, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) was purchased from H. Holstein Co., Ltd. (Tokyo, Japan). Cholesterol and 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG) were purchased from Nippon Fine Chemical Co., Ltd. (Osaka, Japan). 1,2-Distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (PEG_{5000}, PEG- DSPE) was purchased from NOF Corp. (Tokyo, Japan) (**Figure 1**). To prepare mixed lipids, specific molar ratios of DPPC, cholesterol, DHSG, and PEG-DSPE (5/4/0/0, 5/4/0.9/0, and 5/4/0.9/0.03) were dissolved in 2-methyl-2-propanol (500 mL; Fujifilm Wako Pure Chemical Corp., Osaka, Japan) by stirring in a 500 mL flask at 60°C. Then the lipid mixture solution was freeze-dried (EYELA FD-1000; Tokyo Rikakikai Co.,

Ltd., Tokyo, Japan) for 1 day to obtain a powdered lipid mixture.

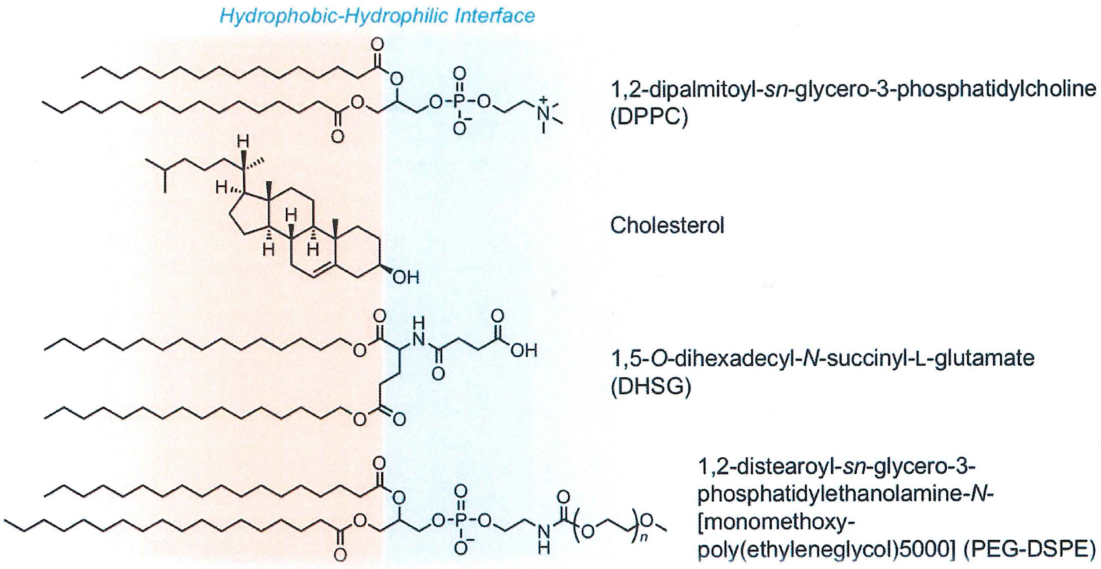

Figure 1. Four lipid components used to prepare Hb-V.

2.2. Preparation of HbCO solution

Human Hb was purified from outdated human RBCs provided by the Japanese Red Cross Society (Tokyo, Japan). Hb was stabilized by carbonylation to form carbonyl hemoglobin (HbCO) and was pasteurized (60°C for 12 hr) for virus inactivation. Then, the obtained HbCO solution was dialyzed, nanofiltered for virus removal, and then concentrated by ultrafiltration to 40 g/dL.[19,46] The 0.3, 5, 15, 25, and 35 g/dL HbCO solutions were prepared by diluting this HbCO solution with distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan).

2.3. Preparation of liposome encapsulating HbCO (HbCO-V)

HbCO solution (40 g/dL, 35 mL) and mixed lipid powder (10 g, DPPC/cholesterol/DHSG/ PEG- DSPE = 5/4/0.9/0.03 by mol) were filled in a cylindrical PTFE container (outer diameter, 93 mm; height, 110 mm; with multiple concave inner surfaces). Subsequently, they were kneaded using the rotation-revolution mixer (ARE-500; Thinky Corp., Tokyo, Japan) under a CO atmosphere at 1,000 rpm for clockwise revolution,

at 980 rpm for counterclockwise rotation, and for 4–22 min (**Figure 2**). After kneading, the surface temperature of the container was measured immediately using an infrared thermometer (SK-8940; Sato Keiryoki Mfg. Co., Ltd., Tokyo, Japan). Then the obtained Hb-lipid mixture paste was dispersed with saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). Then, the dispersion was centrifuged (himac CF 12RX; Hitachi Ltd., Tokyo, Japan) at 3,000 rpm for 60 min to remove undispersed lipids and gigantic liposomes as precipitates. Furthermore, the supernatant was filtered through 0.80 μm filters (Advantec cellulose acetate hydrophilic filter; Toyo Roshi Kaisha Ltd., Tokyo, Japan). After the unencapsulated Hb was separated by ultracentrifugation (himac CP80WX; Hitachi Ltd., Tokyo, Japan) at 19,000 rpm for 60 min, the precipitate was dispersed with saline again to obtain HbCO-V. The HbCO-Vs of different lipid composition (DPPC/cholesterol/DHSG/ PEG- DSPE = 5/0/0/0, 5/4/0/0, and 5/4/0.9/0 by mol) were prepared using the same method. The HbCO-Vs encapsulating different Hb concentration (0.3, 5, 15, 25, and 35 g/dL) were also prepared using the same method.

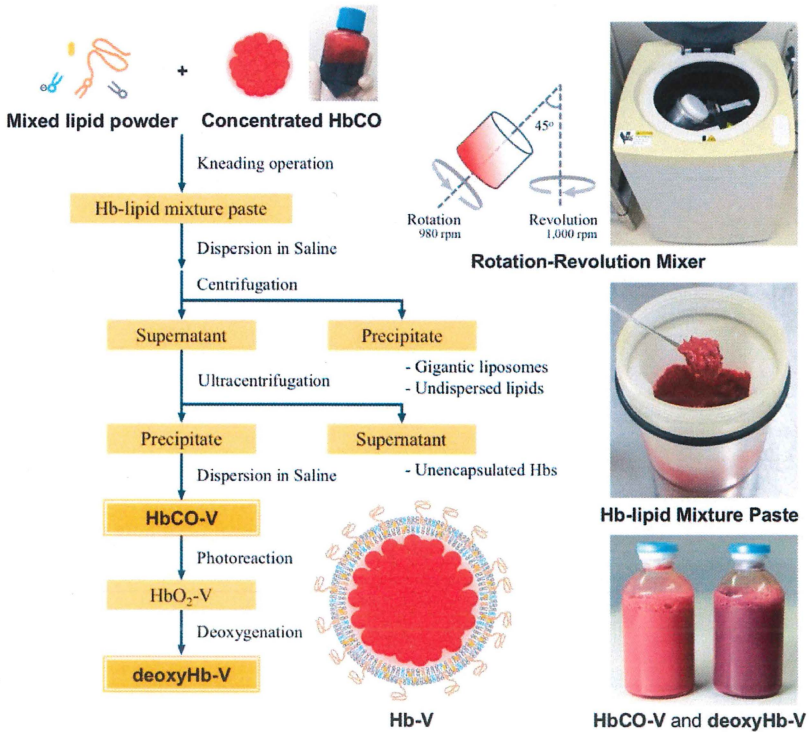

Figure 2. Schematic protocol of the preparation of Hb-Vs.

2.4. Physicochemical characteristics of Hb-Lipid mixture paste and HbCO-V

The Hb-lipid mixture paste viscosity immediately after kneading was measured using a rheometer (Physica MCR 301; Anton Paar GmbH, Graz, Austria) at 25°C at 1–1000 s^{-1}. The cone diameter was 50 mm. The gap angle between the cone and the plate was 1°. The average particle sizes (nm) of the Hb-lipid mixture pastes for optimization and the final HbCO-V products were measured using a light scattering method (nanoparticle analyzer, SZ-100; Horiba Ltd., Kyoto, Japan) after dilution with phosphate buffered saline (PBS, pH 7.4 1X; Gibco Life Technologies, Paisley, Scotland) as dispersant. The Hb concentration of HbCO-V was measured using the SLS-Hb method (Hemoglobin B-Test Wako; Fujifilm Wako Pure Chemical Corp., Tokyo, Japan). As a pretreatment, *N*-octyl-β-D-glucopyranoside (Dojindo Laboratories, Kumamoto, Japan) was used to dissolve the liposomal membrane of HbCO-V completely. Then the Hb encapsulation efficiency (Hb yield) was calculated using equation (1).

$$\text{Hb yield (\%)} = 100 \times \frac{C_2 \times V_2}{C_1 \times V_1} \qquad (1)$$

In that equation, C_1 and V_1 respectively denote the concentration and the volume of the fed HbCO solution. Also, C_2 and V_2 respectively denote the Hb concentration and the volume of HbCO-V.

The total lipid concentration of HbCO-V was estimated from the phospholipid concentration measured using a choline oxidase-DAOS method (Fujifilm Wako Pure Chemical Corp., Tokyo, Japan). As a pretreatment, decaethylene glycol monododecyl ether (Sigma-Aldrich Corp., MO, USA) was used to dissolve the liposomal membrane of HbCO-V completely. The Hb/lipid ratio was calculated using Hb and lipid concentrations in g/dL (Hb/lipid ratio = [Hb]/[lipid]).

2.5. Preparation of liposome encapsulating deoxy Hb (deoxyHb-V) for long-term storage

The HbCO-V dispersion was prepared with optimized condition by four-fold scaling up (Hb, 40 g/dL 135 g; mixed lipid, DPPC/cholesterol/DHSG/PED-DSPE = 5/4/0.9/0.03 by mol 45 g; kneading time, 10 min). The HbCO in the vesicle dispersed in saline was converted to oxyhemoglobin (HbO_2) by photoreaction, exposing to visible light (high-pressure sodium lamp, EYE Sunlux Ace 360 W; Iwasaki Electric Co., Ltd., Tokyo, Japan) under a flowing O_2 gas (> 99.5% purity, clinical grade).[19] Then the HbCO conversion was confirmed with disappearance of peak HbCO (λ_{max} 419 nm) using UV-visible spectrophotometer (V-660 with 60 mm Integral Sphere; Jasco Corp., Tokyo, Japan). The obtained HbO_2-V dispersion was filtered through 5.0 and 0.8 μm filters (Advantec cellulose acetate hydrophilic filter; Toyo Roshi Kaisha Ltd., Tokyo, Japan). The Hb concentration was adjusted to 10 g/dL. The filtrated HbO_2-V dispersion was deoxygenated with stirring under a flowing N_2 gas (> 99.95% purity, clinical grade) until confirmation of the oxygen partial pressure reached 0–0.05 Torr using a Fiber optic oxygen transmitter (Pre Sens Precision Sensing GmbH, Regensburg, Germany). The resulting deoxyHb-V dispersion was dispensed aseptically and anaerobically into sealable sterile vials (Mita Rika Kogyo Co., Ltd., Osaka, Japan). The vials were pouched with an aluminum bag along with an oxygen absorber, and were stored at 25°C for roughly 1, 3, 6, 9, 12, and 24 months. Four batches of deoxyHb-V were prepared (#1–4) using this method. The particle size distributions (mean±SD) of #1–4 were, 236.0±38.4 nm, 270.7±33.0 nm, 243.6±51.8 nm, and 259.0±27.7 nm, respectively.

2.6. Physicochemical characteristics of deoxyHb-V after long-term storage

The Hb concentration and the average particle size were measured using the methods described above. To measure the level of metHb (%), a small volume of Hb-V was suspended in PBS in a Thunberg cuvette and deoxygenated for 10 min by N_2 bubbling for spectrophotometric analysis (300–500 nm). Then, the level of metHb (%) was calculated using the ratios of the absorbance at 405 and 430 nm, which correspond respectively to λ_{max} of metHb and deoxyHb. The level of free Hb (%) was calculated from the Hb concentration and the volume of a supernatant, which was obtained by centrifugation (2000 × *g*, 15 min,

CHIBITAN-R; Hitachi Koki Co., Ltd., Tokyo, Japan) of the Hb-V dispersion in the presence of high molecular weight dextran (from *Leuconstoc* spp. Mr 450,000–650,000; Sigma-Aldrich Corp., MO, USA).

3. RESULTS

3.1. Influence of the lipid composition

Liposomes encapsulating Hb of different lipid compositions (DPPC/Cholesterol/DHSG/PEG-DSPE = 5/0/0/0, 5/4/0/0, 5/4/0.9/0, and 5/4/0.9/0.03 by mol) were prepared using the kneading method (kneading time was 10 min) to confirm the effect of each component on the Hb yield and size distribution. As a result, in all four samples, the surface temperatures of the containers were higher than 50°C after the kneading operation (50.0, 51.0, 61.7, 60.0°C, respectively). The average particle size of the liposomes in the Hb-lipid mixture paste became smaller as the number of lipid components increased (**Figure 3**). When using only the phospholipid DPPC as the lipid component of liposomes (5/0/0/0), the average particle size was extremely large (2107.0±560.8 nm). We did not obtain liposome in the supernatant after the first centrifugation. When cholesterol was added to DPPC (5/4/0/0), the average particle size was slightly smaller (1221.7±500.2 nm). In this condition, the small amount of liposome encapsulating Hb was obtained in the supernatant (Hb yield, 0.33%; lipid yield, 0.61%; Hb/lipid ratio, 0.75). When the negatively charged lipid, DHSG, was added further (5/4/0.9/0), the particle size became even smaller (461.0±184.2 nm). The Hb and lipid yields were higher (35.8 and 42.7%; Hb/lipid ratio, 1.18). The liposome prepared with lipid mixture including all four lipid components including PEG-DSPE (5/4/0.9/0.03) was the smallest in terms of the average particle size (276.9±99.2 nm), the highest in terms of the Hb and lipid yields (58.4 and 61.3%), and the highest in terms of the Hb/lipid ratio (1.33).

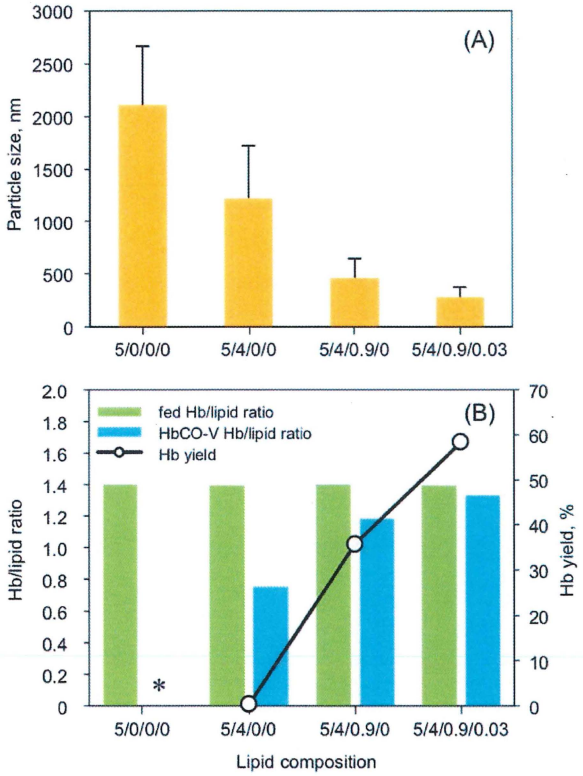

Figure 3. Influence of the lipid composition on the preparation of HbCO-V by the kneading method for 10 min. (A) Particle size (mean±SD, nm) of the Hb/lipid mixture paste after kneading operation, and (B) Hb yield and Hb/lipid ratio of the obtained HbCO-V when the lipid composition was changed. The lipid composition represents the molar ratio of mixed lipid components; DPPC/Cholesterol/DHSG/DSPE-PEG. *When the lipid composition was only DPPC (5/0/0/0), almost no Hb encapsulated liposomes were obtained.

3.2. Influence of the lipid amount

Liposomes were prepared using different amounts of the lipid mixture (2.5, 7.5, 10.0, 12.5, and 17.5 g; DPPC/Cholesterol/DHSG/PEG-DSPE = 5/4/0.9/0.03 by mol) and constant amount of HbCO solution (40 g/dL, 35 g). Results show that the surface temperatures of the container after 10 min of the kneading operation were 47.0, 54.8, 59.5, 61.1, and 60.1°C, respectively, when the amounts of lipid mixture were 2.5, 7.5, 10.0, 12.5, and 17.5 g. The Hb yield was low (9.3%, **Figure 4A**) in the batch with 2.5 g of lipid mixture added for 35 g of HbCO solution. Initially, we thought that the low Hb yield might result

from the limited amount of Hb that can be internalized with a small amount of lipid. However, the lipid yield was similarly low (35.9%) because most of the lipid was not dispersed well and removed by the first centrifugation. When the amount of added lipid mixture was increased, the Hb and lipid yields increased as well (Hb yields, 49.9, 61.2, and 69.9%; lipid yields, 69.2, 69.8, and 84.3%; when the amount of lipid mixtures, 7.5, 10.0, and 12.5 g, respectively). In the batches where the amount of added lipid mixture was increased further (lipid, 17.5 g), both Hb and lipid yields increased slightly (Hb yield, 74.2%; lipid yield, 84.6%). The Hb/lipid ratio was almost constant up to 10 g of the amount of lipid mixture added (1.37 to 1.22 when the amount of lipid added was 2.5 to 10 g, respectively), and decreased slightly to 0.71 when the lipid mixture 17.5 g was further added.

The viscosities of the Hb-lipid mixture paste immediately after kneading increased significantly with the amount of a lipid mixture added. According to information obtained from the mixer manufacturer (Thinky Corp.), the shear rate induced during the kneading operation is estimated as several hundred per second. The 40 g/dL Hb solution is a Newtonian liquid, as is apparent as the **Figure 4B** shows. The viscosity is modestly high 49.7 cP at a shear rate of 113 s^{-1}. The lipid addition to the Hb solution results in more highly viscous paste. The viscosities were 216, 3880, 5630, 24,300, and 39,600 cP at a shear rate of 113 s^{-1} when the amounts of lipid mixtures were, respectively, 2.5, 7.5, 10.0, 12.5, and 17.5 g. The pastes showed non-Newtonian shear-thinning profiles.

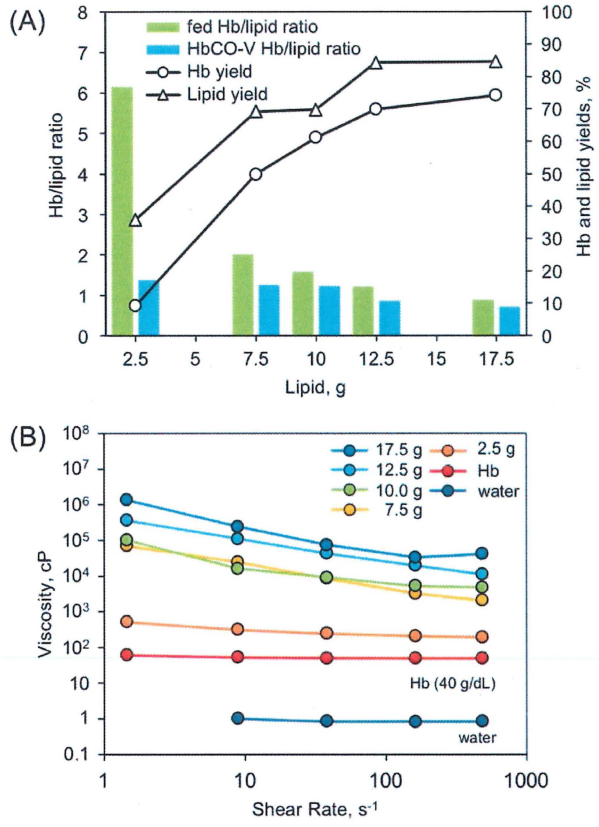

Figure 4. Influence of the amount of the lipid on the preparation of HbCO-V using the kneading method. (A) Hb and lipid yields (%) of the HbCO-V prepared using the kneading method and Hb/lipid ratio of the fed materials and HbCO-V when the amount of lipid was changed from 2.5 to 17.5 g at the constant amount of HbCO solution (40 g/dL, 35 mL). (B) Shear rate dependence of the viscosity (cP) of the Hb-lipid mixture paste after kneading operation when the amount of lipid was changed from 2.5 to 17.5 g. The pastes showed shear-thinning profiles. The viscosities of the 40 g/dL Hb solution and the water are also shown.

3.3. Influence of Hb concentration

Liposomes were prepared using different concentrations of 35 mL of HbCO solution (0.3–40 g/dL) for 10 g of the lipid mixture (DPPC/Cholesterol/DHSG/PEG-DSPE = 5/4/0.9/0.03 by mol). The total kneading time was fixed to 16 min. Results show that the surface temperatures of the container after the kneading operation were 53.8, 54.4, 57.4, 59.0,

59.8, and 60.0°C, respectively, when the HbCO concentrations were 0.3, 5, 15, 25, 35, and 40 g/dL. The average particle size was 449.3±178.9 nm (mean±SD) when the most dilute 0.3 g/dL of HbCO solution was used (**Figure 5**). The average particle size tended to become smaller (455.8±188.3 – 308.7±118.4 nm) and the Hb yield and the Hb/lipid ratio tended to become higher (37.9–55.2% and 0.12–0.93, respectively) as the HbCO concentration was increased from 5 to 35 g/dL. The most concentrated 40 g/dL of HbCO solution caused the smallest average particle size (276.9±99.2 nm), the highest Hb yield (58.4%), and the highest Hb/lipid ratio (1.33).

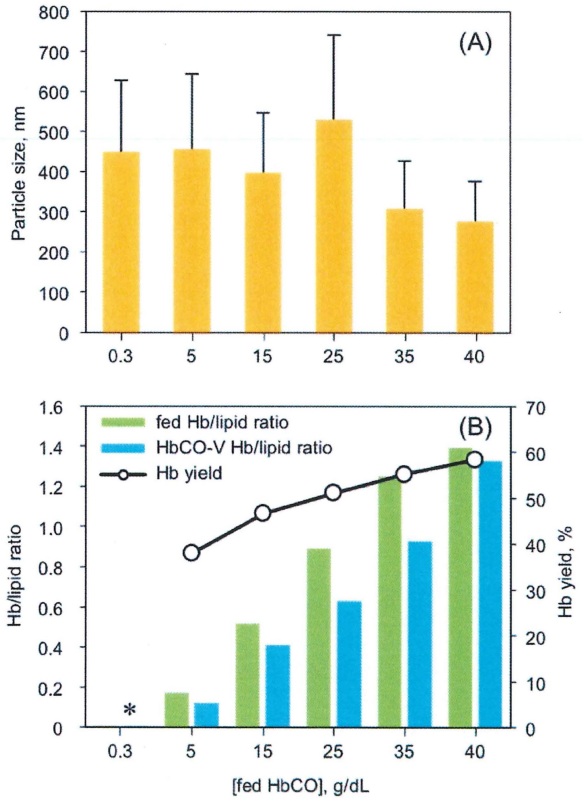

Figure 5. Influence of the concentration of the fed HbCO on the preparation of HbCO-V using the kneading method. (A) Particle size (mean±SD, nm) of the Hb-lipid mixture paste immediately after kneading and (B) Hb yield (%) and Hb/lipid ratio of HbCO-V dispersion prepared using the kneading method when the concentration of fed HbCO was changed from 0.3 to 40 g/dL. *No accurate yield was obtained because the Hb concentration of the obtained Hb-V was extremely low.

3.4. Influence of kneading time

Liposomes were prepared using different kneading times (4, 6, 8, 10, 12, 14, 18, and 22 min). As feed materials, 10 g of lipids (DPPC/Cholesterol/DHSG/PEG-DSPE = 5/4/0.9/0.03 by mol) and 35 mL of HbCO solution (40 g/dL) were used for each kneading operation. Results show that the surface temperatures of the container after the kneading operation were 48.7, 53.9, 57.8, 60.2, 61.5, 66.0, 68.1, and 69.2°C when the respective kneading times were 4, 6, 8, 10, 12, 14, 18, and 22 min. The particle size of the Hb-lipid mixture paste after the shortest 4 min kneading was 296.4±57.5 nm. Smaller particle size (217.8±42.8 – 179.7±33.6 nm) was obtained as the kneading time increased (10 to 22 min). The Hb yield and the Hb/lipid ratio tended to increase as the kneading time increased to 10 min (Hb yield, 47.2 to 55.2%; Hb/lipid ratio, 0.87 to 1.17; kneading time, 4 to 10 min, respectively). However, it remained constant after 10 min (Hb yield, 55.2–54.6%; Hb/lipid ratio, 1.17–1.15; kneading time, 10–22 min, respectively).

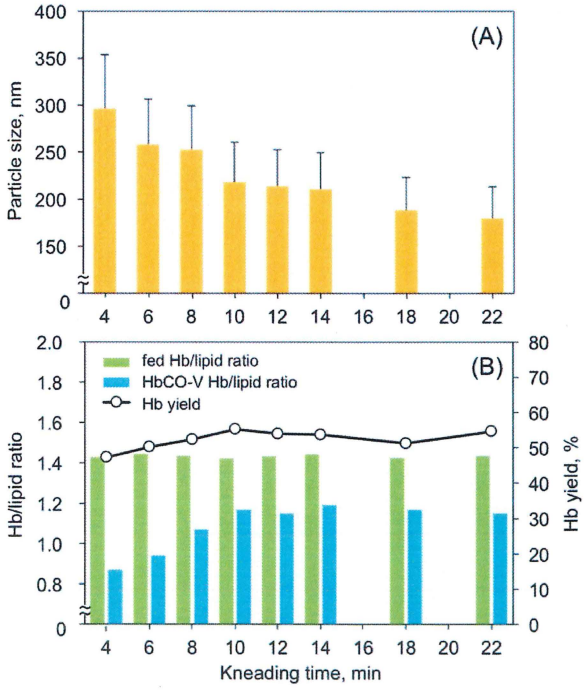

Figure 6. Effects of the kneading time on HbCO-V preparation using the kneading method. (A) Average particle size (mean±SD, nm) of the Hb-lipid mixture paste immediately after

kneading and (B) Hb yield and Hb/lipid ratio of the obtained HbCO-V dispersion prepared using the kneading method when the kneading time was changed from 4 to 22 min.

3.5. Long-term storage stability

The stability for long-term storage was tested on four batches of the deoxyHb-V dispersion at 25°C for roughly 1, 3, 6, 9, 12, and 24 months. As a result, the Hb concentration (9.9–11.0 g/dL) and the level of free Hb (0.8–1.7%) were nearly constant for 24 months. The average particle size shifted slightly during two years of storage, but the shift was within a reasonable range (about 230–300 nm). The level of metHb increased for some time after the start of storage (<3 months), but it began to decrease thereafter (3–12 months, <14.2%), eventually resulting in a lower value (<5.2%). No significant variation was shown in any parameters after long-term storage. Thereby, the structure and dispersibility of liposomes are maintained in a stable manner.

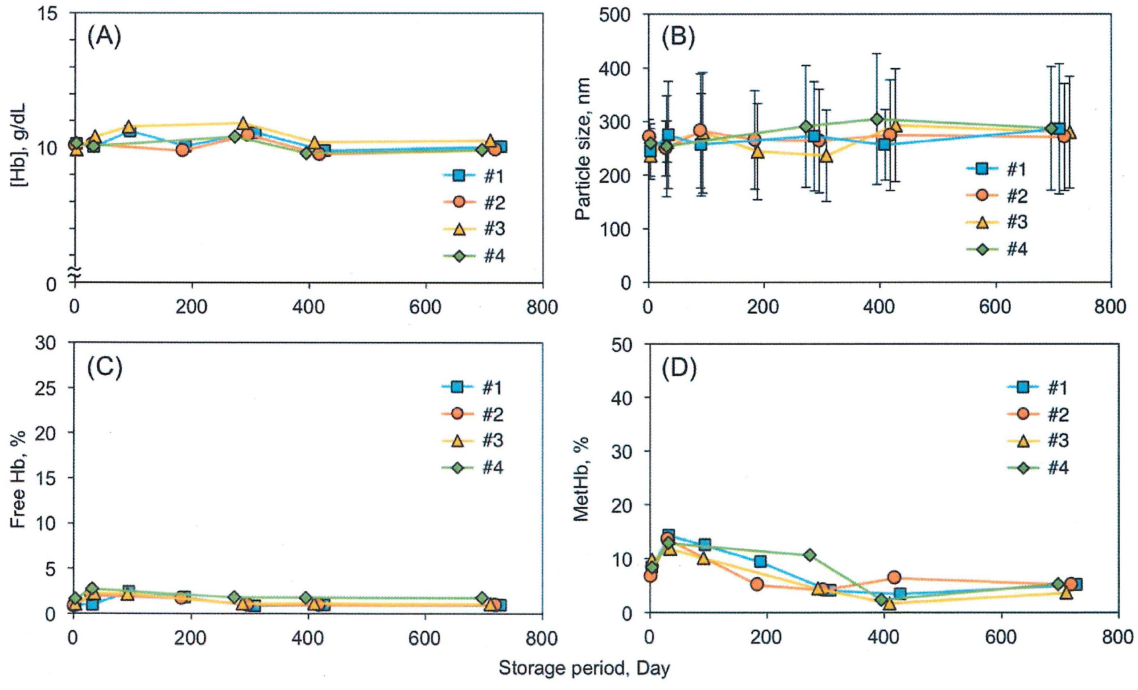

Figure 7. Stability of deoxyHb-V dispersion prepared using the kneading method during long-term storage (0, 3, 6, 9, 12, and 24 months roughly) was evaluated in terms of (A) Hb concentration (g/dL), (B) average particle size (mean±SD, nm), (C) free Hb level (%), and

(D) metHb level (%). The dispersing medium is a physiological saline solution. Each datum presents results obtained from four batches of the preparation of deoxyHb-V as #1–4.

4. DISCUSSION

Our primary finding obtained from this study is that the kneading method using the rotation-revolution mixer is extremely useful as a new method for preparing Hb-V. Advantages of this method are that the large amount of lipids can be mixed with a concentrated Hb in spite of the high viscosity of Hb-lipid mixture. Moreover, the process can be performed quickly and aseptically. The Hb yield was increased dramatically to the highest 74.2%. The obtained Hb-V was in no way inferior to Hb-V prepared using the conventional extrusion method. It was revealed to maintain its structure and dispersibility stably even after two years of storage at room temperature.

The rotation-revolution mixer has been used widely in laboratories and industrial fields since the 1970s as a tool for mixing and pulverizing purposes. In recent years, it has been applied gradually to the experimental preparation of functional liposomes[43-45,47] and polymersomes.[48] However, the preparation of liposomes encapsulating highly concentrated biological proteins such as a Hb (about 40 g/dL) as HBOCs have not been reported before. Conventionally, we prepared Hb-V by extrusion method.[26-28,34,46,49] The very highly viscous mixture of a lipid and a concentrated Hb solution is actually unsuitable for the extrusion method because the amount of lipid added is limited to about 5wt% of lipid to an Hb solution. Moreover, the filters made of cellulose acetate or nitrate with different pore sizes (3.0, 0.8, 0.65, 0.45, 0.3, and 0.22 μm) have to be replaced in a pressure-resistant filter holder for stepwise extrusions. The time required for the entire extrusion process was several hours for repeated filter exchange and extrusion of viscous fluids. In contrast, the present simple preparation method requiring only 10 min using a rotation-revolution mixer can take

advantage of high viscosity. During kneading, rotation of the cylindrical container pushes the paste in an outward direction by the centrifugal forces, while revolution of the container around its own axis pushes the paste in an opposite direction due to the adhesiveness of the paste to the wall of the container and induce swirling flow in the container for homogenization. The adhesiveness is higher for a viscous paste to induce stronger swirling flow, and more friction energy can be transferred to the paste for homogenization. Actually, the high viscosity of the Hb-lipid mixture was observed to cause frictional heat by the components themselves during kneading operations: not by the machine or the container. The temperature of the container's outer surface reached about 60°C, which is higher than the phase transition temperature of DPPC (T_c = 41°C). This temperature is assumed to increase the lipid membrane fluidity.[34] Therefore, the dispersion of lipids and the formation of liposomes are facilitated. Reportedly, HbO_2 autoxidizes at an ambient temperature. It is denatured thermally at temperatures higher than about 62°C, whereas HbCO is resistant of autoxidation and thermostable up to about 78°C (no progression to autoxidation in HbCO).[46] For this reason, using HbCO is important to perform the kneading operation without causing autoxidation or denaturation of Hb. The particle size distributions of Hb-V, prepared by the conventional extrusion method in our group, are reported as 250±20 nm,[27] 257±87 nm,[28] 280±50 nm,[34] 273±67 nm,[46] 279±95 nm,[49] etc. The kneading method using the rotation-revolution mixer can provide Hb-V with similar size distributions of nearly 230-300 nm on the average at the optimal conditions.

The components of the lipids are selected carefully for optimal structure and the biocompatibility of Hb-V. The addition of cholesterol to DPPC is known to reduce the curvature of unilamellar vesicles and moderate the considerable change in segmental motion of acyl chains at the phase transition temperature.[50] The addition of a negatively charged lipid, DHSG, is important for the preparation of large unilameller vesicles because it causes an electrostatic repulsion among lamellar structures and prevents the multilamellar formation. We confirmed the increase of a Hb yield and a Hb/lipid ratio by the addition of DHSG from

this study. A negatively charged lipid such as phosphatidyl glycerol and fatty acid is known to cause complement activation, but DHSG reduces that activation.[51,52] PEG modification to the surface of liposome is necessary for biocompatibility, dispersibility in blood plasma, and for extending the circulation half-life *in vivo*.[49,53,54] This study revealed that PEG modification is also important for lipid dispersibility, as judged from data indicating that Hb yield was increased by the addition of PEG-DSPE. Accordingly, all of these four components of the lipids are important for preparing Hb-V using the rotation-revolution mixer.

The Hb yield was also influenced by the amount of the lipid addition, the fed HbCO concentration, and the kneading time. The Hb yield is apparently related to the viscosity of the Hb-lipid mixture paste in this kneading method. This point is clear from the result of improved Hb yields as the viscosity of the Hb-lipid mixture pastes increased (**Figure 4**). It shows a remarkable progress in comparison to the extrusion method that resulted in only 20% yield because of the limitation of the amount of the lipids added (for example, only 5 g of lipids to 100 g of concentrated Hb solution). Actually, the maximum Hb yield 74.2% was obtained when 17.5 g of lipid was used for 35 g of concentrated Hb solution. Given that spherical Hb-V particles are closely packed in the paste, the highest filling rate should be 74%. This theoretically estimated value is coincident with the result obtained from this study (74.2%). However, the Hb/lipid ratio was slightly low, 0.71, when 17.5 g of lipids was used compared with the Hb/lipid ratio 1.22 when 10 g of lipids were used. Considering the use of Hb-V for an O_2 carrier, the Hb/lipid ratio should be as high as possible. Consequently, the optimal amount of the lipid is 10–12.5 g for 35 g of the Hb solution in view of the balance between the Hb yield and the Hb/lipid ratio.

The average particle size of the obtained Hb-V tended to become smaller with increased kneading time. The Hb yield tended to increase with the kneading time increase up to 10 min. It reached a plateau after 10 min. Generally, a longer mixing time and a higher mixing speed can be expected to provide efficient homogenization. Additionally, in the case

of preparation of a liposome dispersion, they provide efficient lipid dispersion and minimization of liposome size by kneading.[42] The large particle size and the slightly low Hb yield and Hb/lipid ratio for the first 10 min kneading in **Figure 6** imply that the multilamellar vesicles (MLV) are formed mainly by insufficient homogenization. MLVs were shifting to unilamellar vesicles by longer kneading, which increases the Hb yield and Hb/lipid ratio. Further kneading for more than 10 min did not increase the Hb yield and Hb/lipid ratio but reached a plateau at about 60% for the Hb yield in fact. The inner aqueous phase volume decreases as the particle size of the liposome becomes smaller if all obtained liposomes are unilamellar vesicles. That would result in a lower Hb/lipid ratio because the surface to volume ratio increases. A stable Hb yield and Hb/lipid ratio might be attributed to the simultaneous progress of the formation of unilamellar vesicles and liposome size minimization. For this reason, it is expected that an excess kneading of more than 22 min leads to a smaller liposome size and a lower Hb/lipid. For the efficient preparation of Hb-V, we concluded that the optimal kneading time is 10 min in this experimental setting.

Reportedly, several bead materials are used as a homogenization aid to prepare liposome or polymersome using a similar mixer, DAC.[37,47,48] During our preliminary experiments, we added 11% of the batch volume of 0.2–0.5 mm glass beads to the Hb-lipid mixture, but no remarkable difference was found in the particle size and the Hb/Lipid ratio (data not shown). In addition, contaminations of lipid fragments because of unwanted chemical reactions on the surface of glass beads[37] and the contamination of material fragments because of abrasion of beads and a container represent a concern. In our Hb-V preparation, the paste of lipid and Hb solution already ensures sufficient viscosity by lipids and concentrated Hb solution themselves that facilitates dispersion of lipids and formation of liposome. Therefore, we concluded that bead addition as a homogenization aid is not necessary to prepare of Hb-V using the rotation-revolution mixer.

Parameters in the operation of a rotation and revolution mixer must be regulated

depending on the liposome formulation purpose. If an encapsulated substance (Hb in the case of the Hb-V) is important for the liposome function (e.g., oxygen carrying capacity of the Hb), then it is necessary to maintain a high encapsulation efficiency rather than to reduce the liposome size. In this case, we infer from the data in **Figure 4A** that regulation of the amount of the lipid added is more important to maintain high encapsulation efficiency than the kneading time. If important functional molecules or lipids are inserted into the lipid membrane, or if the liposome size is more important than the function of the encapsulated substance, then the kneading time or speed must be increased from the data in **Figure 6**. In either case, it is necessary to consider that encapsulated substances and functional molecules should not be denatured by the shear stress and the resulting high temperature during the kneading process.

Long-term storage stability tests show that the structure of Hb-V is maintained for two years. The particle size barely changed throughout long-term storage. It is assumed that the PEG modification to the surface of liposome stabilizes the dispersion state and prevents the aggregation and fusion by their steric hindrance. The liposome structure was maintained stably without leakage of encapsulated Hb because the level of free Hb was almost unchanged. In addition, the Hb concentration did not change. Therefore, denaturation of Hb (except for autoxidation to metHb) rarely occurred during long-term storage at room temperature. Sakai *et al.* reported that Hb-V (using 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol instead of DHSG, and adding homocysteine as a reductant) prepared with the extrusion method can be stored for more than one year at ambient temperatures without any marked diameter change or leakage of the encapsulated Hb.[53] This study revealed that Hb-V prepared using the rotation-revolution mixer can be stored for two years at room temperature.

HbCO is used as a raw material to prepare Hb-V and thereby prevent autoxidation to metHb and denaturation. As described above, HbCO is thermostable up to 78°C.[46] We

confirmed that HbCO is stable against very high shear stress. We also confirmed that metHb is rarely formed even after the kneading operation (based on UV spectra, data not shown). In consideration of *in vivo* administration, HbCO encapsulated in liposome was converted into HbO_2 by photoreaction.[19] Additionally, for long-term storage of the Hb-V suspension, it is necessary to remove oxygen to convert it into deoxyHb-V because the completely deoxygenated Hb shows no autoxidation.[53] We assume that these additional steps (especially the photoreaction process) might be the reason why the level of metHb presented in this report is 5–10%, even immediately after preparation through all processing steps. Furthermore, an increased level of metHb was observed after about one month of storage at room temperature under a nitrogen atmosphere. This result is likely to be attributable to autoxidation of the remaining HbO_2 in the presence of a small amount of dissolved oxygen that was not removed completely during the oxygen removal process using nitrogen of 99.95% purity. In addition, oxygen contamination is a concern during the dispensing step. In erythrocytes, the low level of metHb, normally less than 0.5% of the total Hb,[55] is maintained by enzymatic metHb reduction system. However, the highly concentrated Hb used in Hb-V was purified by pasteurization and nanofiltration, removing the enzymatic reduction system and all other substances except Hb and water. No additives were included to reduce metHb. In spite of this, it was interesting to observe that the level of metHb did not increase, but rather decreased (approximately after 6 months). The Hb concentration decrease has not been observed in long-term storage, implying that the level of metHb decrease is attributable to autoreduction according to the literature.[56-58] Even though the details of the mechanism are not clarified, we speculate that the amino acid residues in the globin chains could be the potential electron donors especially in a highly concentrated Hb solution encapsulated in Hb-V.[59] A few approaches can be suggested to eliminate dissolved oxygen of Hb-V sufficiently: using higher-purity nitrogen in the oxygen removing process, adding a small amount of oxygen removers such as a thiol or a sulfate to Hb-V, and using an electrochemical method with adding hydrogen.[60]

The optimal condition to prepare a Hb-V using a rotation-revolution mixer is applied in the practical production of Hb-V for various preclinical studies to confirm its safety and efficacy.[61] We recently confirmed the efficacy of Hb-V as a substitute for blood transfusion in resuscitation therapy for a rabbit model with obstetric hemorrhage.[62] In addition, the efficacies of Hb-V as an organ perfusate,[63] a CO carrier for inflammatory disease,[64] and a photosensitizing agent for the port-wine stain model[65] were clarified by recent *in vivo* studies. The safety of Hb-V was also confirmed in good laboratory practice (GLP) preclinical studies in terms of the single-dose and repeated-dose toxicity studies, the safety pharmacology study, immunogenicity testing, etc. with intravenous administration using SD rats and beagle dogs. Currently, we are manufacturing Hb-V using the kneading method under good manufacturing practices (GMP). We have started Phase I clinical trials.

Adopting the kneading method using a rotation-revolution mixer for production of Hb-V has led to increased batch size and decreased process time compared with the conventional extrusion method. To scale up further, we can suggest simply repeating batch production with application of the optimal condition. In addition, a larger container (approx. 10 L) and a larger mixer are available from the same equipment manufacturer.

5. CONCLUSIONS

We developed and optimized a kneading method to prepare a Hb-V using a rotation-revolution mixer. Using this method, a large amount of lipid and a concentrated Hb solution were homogenized effectively because of the friction of the highly viscous Hb-lipid mixture paste. Therefore, the Hb encapsulating efficiency was increased considerably to the highest 74.2%. The liposomal structure of Hb-V was maintained stably without any Hb leakage or Hb denaturation after two years of storage at room temperature. This method seems to be the most suitable for preparation of the Hb-V made of the viscous paste of the Hb

and lipids. This method enables to scale up production of Hb-V. Therefore, it is expected that preclinical and clinical studies will be accelerated in our project.

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The manuscript was composed based on the contributions of both authors. Both authors have given approval to publication of the final version of the manuscript.

Funding Sources:

This research was supported partly by Health and Labour Science Grants (Health Science Research Including Drug Innovation) from the Ministry of Health, Labour and Welfare, Japan (H24-Soyakusougou-Ippan009), and the Project Promoting Clinical Trials for Development of New Drugs and Medical Devices (17lk0201034h0003, 20lK1403022h0003) from the Japan Agency for Medical Research and Development: AMED.

Notes:

The authors declare the following competing financial interests: H.S. is an inventor holding some patents related to the production and utilization of Hb-vesicles.

ACKNOWLEDGMENTS

The authors acknowledge Dr. Takashi Matsuhira, Dr. Keizo Yamamoto, and Mrs. Naoko Kobayashi (Nara Medical University), Mr. Zi Gui Lim and Mr. Ziyang Kuang (Temasek Polytechnic), Mr. Yong Jun Chen (National University of Singapore), and Ms. Bing Li (Waseda Bioscience Research Institute in Singapore) for their assistance with experiments.

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