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Characteristics of Bovine Hemoglobin as a Potential Source of Hemoglobin-Vesicles for an Artificial Oxygen Carrier[1]

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Hemoglobin-vesicles (HbV) have been developed for use as artificial O_2 carriers in which a purified Hb solution is encapsulated within a phospholipid bilayer membrane. In this study, bovine Hb (BHb) was tested as a source of HbV instead of human Hb (HHb). We compared the preparation process and characteristics of BHbV with those of HHbV. The purification of BHb was effectively performed simply with an ultrafiltration system including a process for removing virus and scrapie agent. The removal ratio of the phospholipid components of bovine red blood cells was over 99.99%, and the protein purity was over 99.9%. The deoxygenated and carbonylated BHb showed denaturation transition temperatures at 83 and 87°C, respectively, which are higher than those of HHb (80 and 78°C, respectively), and resistant to pasteurization (60°C, 10 h). The purified BHb was concentrated to over 40 g/dl, and encapsulated in a phospholipid bilayer membrane to form BHbV with a diameter of about 280 nm. The O_2 affinity (P_{50}) of the BHbV was regulated by coencapsulation of an appropriate amount of Cl^- (as NaCl), which binds to BHb as an allosteric effector, in the range 16–28 Torr, comparable to human blood (P_{50} = 28 Torr). This is quite simple in comparison with HHb which requires phosphate derivatives such as pyridoxal 5′-phosphate as a replacement for 2,3-diphoshoglyceric acid. The viscosity and colloid osmotic pressure of the BHbV when suspended in 5% human serum albumin are 3.5 cP and 20 Torr, respectively, comparable to those of human blood. In conclusion, BHb can be used as a source for the production of HbV, not only because of its abundance in the cattle industry, but also because of the physicochemical advantages of the purification process, thermal stability, and regulation of O_2 affinity in comparison with HHb.

Key words: blood substitutes, encapsulation, hemoglobin, liposome, purification.

Phospholipid vesicles encapsulating concentrated human Hb (Hb vesicles, HbV) can serve as an O_2 carrier with O_2 transporting ability comparable to that of blood (*1–6*). The advantages of Hb-based O_2 carriers are the absence of a blood type antigen and infectious viruses, small particle size for penetration through constricted vessels where red blood cells (RBC) cannot penetrate, and stability for long-term storage (*7*). In this sense, infusion of O_2 carriers appears superior to conventional blood transfusion, which still has the potential of mismatching, infections such as HIV and hepatitis virus, graft versus host disease, and the problem of a preservation period of only a few weeks. Even though acellular Hb modifications such as polymerized Hb and polymer-conjugated Hb are now in the final stages of clinical trials, the cellular structure of HbV (particle diameter, *ca.* 280 nm) most closely mimics the characteristics of natural RBC such as the cell membrane function of physically preventing the direct contact of Hb with the components of the blood and vasculature during circulation. In comparison with acellular Hb modifications, Hb encapsulation in vesicles suppresses hypertension induced by vasoconstriction due to the high affinity of Hb for nitric oxide and carbon monoxide as endogenous vasorelaxation factors (*8–10*).

As a source of O_2 carriers several groups have utilized bovine Hb (BHb) (*11–16*) since BHb is abundant in the cattle industry. The characteristics of BHb in comparison with human Hb (HHb), especially its O_2 binding properties, have been extensively studied (*17–19*), and the crystal structure of BHb has been reported by Perutz *et al.* (*20*). Quite recently, glutaraldehyde-polymerized BHb has been clinically tested (*11*), and one product is now approved for veterinary use in the US (1998) and for clinical use in South Africa (2001).

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Abbreviations BHb, bovine Hb, HHb, human Hb, HbV, Hb-vesicle, PS, phosphatidylserine, PE, phosphatidylethanolamine, PI, phosphatidylinositol, Sph, sphingomyelin, BMM, bemberg microporous membrane, DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine; MCV, mean corpuscular volume, DSC, differential scanning calorimetry; P_{50}, oxygen affinity; PLP, pyridoxal 5′-phosphate, IHP, inositolhexaphosphate, OTE, oxygen transporting efficiency, COP, colloid osmotic pressure

In this study, we aimed to evaluate BHb as a starting material for HbV and to characterize the resulting BHbV. Since the antigenecity of heterogeneic BHb in the human body is not well understood yet, we believe that the BHbV will be tested in *in vitro* and *in vivo* experiments for the future use mainly in veterinary medicine, organ preservation, tissue engineering, *etc.* We studied the purification and encapsulation procedures of BHb that were originally developed for HHb. By comparing the characteristics of the process and the final product, we evaluated the feasibility of using BHb as a starting material.

MATERIALS AND METHODS

Purification of BHb from Fresh Bovine Blood—The whole BHb purification procedure was performed under sterile conditions in a class 10,000 clean room with clean benches equipped with high-efficiency particulate air (HEPA) filters (*9*). Fresh blood from Japanese black cattle was purchased from Shibaura Zoki (Tokyo), and the washed RBC were obtained on the same day as sacrifice by three rounds of centrifugation (4,500 ×*g*, 20 min) and gentle mixing with an equal volume of saline. Hemolysis and the removal of stroma were performed simultaneously with a tangential flow ultrafiltration system of a Biomax V-screen (Millipore, Bedford, MA, cut off Mw.: 1000 kDa) by adding pure water to the washed RBC maintaining the whole volume of the retentate in the tank. The filtrate was concentrated to about 10 g/dl by ultrafiltration (Biomax V-screen, cut off Mw.: 8 kDa). The BHb solution (oxyhemoglobin) was converted to carbonylhemoglobin (HbCO) using an artificial lung with a hollow fiber module for medical use (CAPIOX-IIX, Terumo, Tokyo) (*21*). The HbCO solution was heated at 60°C for 10 h (*22*), the temperature maintained by a continuous flow of hot water in the jacket. The skin temperature was less than 65°C. After being cooled to around 15°C, the denatured and precipitated proteins were removed by filtration (Millistak+15 CE, Millipore). The filtrate was dialyzed and then concentrated to 40 g/dl using an ultrafiltration system (Biomax V-screen, Cut off Mw· 8 kDa).

HPLC of the Remaining Stromal Lipids in the Purified BHb Solution—To confirm the content of residual stromal lipid components in the purified BHb, lipid extraction was performed according to a modification of the method of Bligh and Dyer (*22, 23*). The extracted lipids were injected into an analytical column of a TSKgel Silica-60 (Tohsoh, Tokyo) and eluted with acetonitrile/methanol/phosphoric acid (900:95:5 by volume) at a flow rate of 1.1 ml/min with a liquid pump (LC-8A, Shimadzu, Tokyo). The absorbance was monitored at 210 nm with a UV-vis detector (SPD-10A, Shimadzu). All peaks were assigned by comparing their elution times to standard lipids including phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and sphingomyelin (Sph). The removal efficiency of each phospholipid in the purified Hb solution from that in RBC was calculated from the corresponding peak areas (*22*).

Thermal Stability of Hb Solutions—Thermograms (40–100°C) of Hb solutions were obtained with a differential scanning calorimeter (DSC, SSC 5200H, Seiko Instruments, Tokyo). The solutions (10 g/dl, 60 μl) were sealed with silver pans. The scanning rate was 1.0°C/min.

Purity of BHb Solution—The protein purity was confirmed by isoelectric focusing (IEF) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). PhastGel IEF 3-9 (pH 3–9) and PhastGel gradient 8-25 (Amersham Pharmacia Biotech, Uppsala, Sweden) were used for the protein separation The proteins on the gels were stained with a PhastGel Silver Stain Kit (Amersham Pharmacia Biotech), and the protein amounts, isoelectric points, and molecular weights were evaluated from the absorption intensities of the stained bands in the gels measured at 546 nm with PhastImage (Amersham Pharmacia Biotech). Native-PAGE was performed with a PhastGel gradient 8-25

Process of Virus Removal Using a Bemberg Microporous Membrane (BMM)—The filtration efficiency of the purified BHb solution to remove viruses was tested with PLANOVA™-15N made of BMM (Asahi Kasei, Tokyo, total membrane area, 1 m^2). This filter comprises hollow fiber membranes with a mean pore size of 15 nm. A purified BHb solution (concentration, 2, 5, 7, and 10.1 g/dl, 13°C) was permeated in a dead-end fashion through the PLANOVA™ at a constant pressure, 80 kPa, and the rate of permeation was measured (*24*)

Preparation of Poly(ethylene glycol) (PEG)-Modified BHb-Vesicles (BHbV)—PEG-modified BHbV was prepared under sterile conditions as previously reported (*9, 25*). The encapsulated carbonylhemoglobin (HbCO, 38 g/dl) contained NaCl (0–100 mM) to regulate O_2 affinity, and 5 mM homocysteine (Aldrich, Milwaukee) as a reductant The lipid bilayer was composed of Presome PPG-I, a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol at a molar ratio of 5.5:1 (Nippon Fine Chem., Osaka). The HbCO solution and lipids were mixed and stirred for 12 h at 10°C The resulting mutilamellar vesicles were extruded through membrane filters (Fuji Film, Tokyo) using Extruder™ (Lipex Biomembranes, Vancouver) with a final filter pore size of 0.22 μm. After rinsing with saline, the BHbV surface was modified with PEG (Mw 5 kDa, 0 3 mol% to the outer surface of lipids) using 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG (Sunbright DSPE-50H, acid type, NOF, Tokyo), where succinic acid is a crosslinker between PEG and DSPE. The hydrophobic alkyl chains of PEG-DSPE were inserted into the lipid bilayer of BHbV by mixing with a saline suspension of BHbV at 37°C for 2 h (*26*). After decarbonylation of HbCO to HbO_2 (*27*), the resulting PEG-modified BHbV was ultracentrifuged to remove the un-introduced PEG-lipid, and redispersed in saline at a BHb concentration of 10 g/dl. The suspension was then filtered through sterilizable filters (pore size: 0.45 μm; Toyo Roshi, Tokyo). Particle sizes were measured by a light scattering method (Coulter particle analyzer, model N4SD). The concentrations of BHb and phospholipids were measured by the cyanomethemoglobin method (Hemoglobin Test Wako, Wako Fine Chem., Tokyo) and the Molibuden blue method (Phospholipid Test Wako), respectively. To regulate the colloid osmotic pressure (COP) of the suspension, BHbV was suspended in 5 g/dl human serum albumin (Bayer, Leverkusen, Germany) (*6*). COP was measured with a colloid osmometer (model 4420, Wescor, Logan, UT). The viscosity of the resulting suspension was measured with a capillary viscometer (Oscillatory Capillary Rheometer and Density Meter, OCR-D, Anton Paar, Graz, Austria) at 37°C with a shear rate range 1–332 s^{-1}

(capillary diameter and length, 0.9948 and 100.2 mm, respectively).

O_2 Affinity of BHb and BHbV—The O_2 affinity (P_{50}) and Hill number were calculated from an O_2 equilibrium curve obtained by a Hemox Analyzer (TCS Medical Products, Southampton, PA). BHbV was suspended in a HEPES buffer solution (150 mM, pH 7.4). The purified and decarbonylated BHb solution as a reference was diluted with the HEPES buffer at different NaCl concentrations. The measurements were performed at 37°C.

RESULTS

Purification of BHb—A purified BHb solution was obtained from the blood of Japanese black cattle with a yield of about 80%. It was confirmed that the purification process for HHb comprising filtration, carbonylation, heating, and ultrafiltration procedures could be applied to BHb without noticeable difficulty. However, the initial step of blood centrifugation to sediment RBC and to remove plasma proteins required a centrifugal force of 4,500 ×*g*, which is higher than that required for human blood (2,500 ×*g*) since the mean corpuscular volume (MCV) of bovine RBC is about 40 μm³ (*28*), smaller than that of human RBC (*ca.* 90 μm³) However, fresh bovine blood showed less hemolysis than outdated human blood, and the yields of the washing procedures were comparable (85%). The yield after ultrafiltration to remove stromal components was 96%. The yield after heat treatment and removal of other proteins was 94%.

HPLC Profiles of Stromal Lipids—The elution time of the phospholipids extracted from bovine RBC were 9 min for PS, 12 min for PI, 13 min for PE, and 32–35 min for Sph (Fig 1). The peak at around 23 min are not lipid components but a heme because it shows an absorption corresponding to the Soret band. Heme has a hydrophobic nature so it can be extracted into the organic solvent by the method of Bligh and Dyer. Bovine RBC do not contain phosphatidylcholine according to the literature (*29*) In the case of purified BHb solution, on the other hand, no peak was detected except the heme. The removal efficiencies of phospholipids were greater than 99.99% (above the detection limit).

IEF and SDS- and Native-PAGE—The molecular weight of BHb, calculated from the amino acid sequence, is 64,442

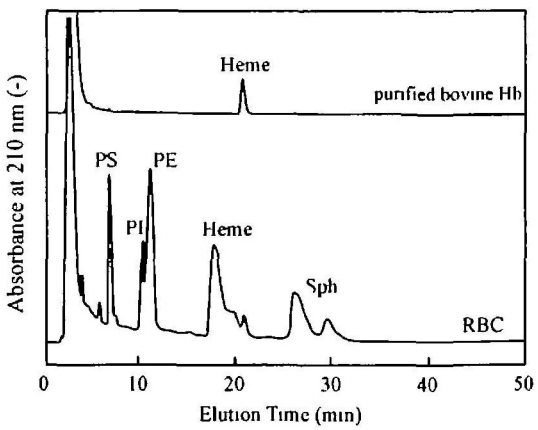

Fig. 1 **HPLC profiles of extracted lipid components from bovine RBC and purified BHb.** After ultrafiltration of BHb through a filter with a 1,000 kDa cut-off, no phospholipid peaks were observed The peaks at around 23 min are not lipid components but a heme because it has an absorption corresponding to the Soret band PS, phosphatidylserine, PE, phosphatidylethanolamine, PI, phosphatidylinositol, Sph, sphingomyelin.

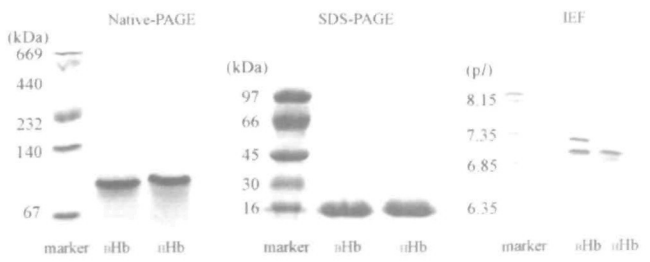

Fig 2 **Native-PAGE, SDS-PAGE, and IEF patterns of purified BHb in comparison with HHb.** Markers, HMW and LMW marker kits (Amersham Pharmacia) for Native-and SDS-PAGE, respectively, and a Broad p*I* marker kit for IEF

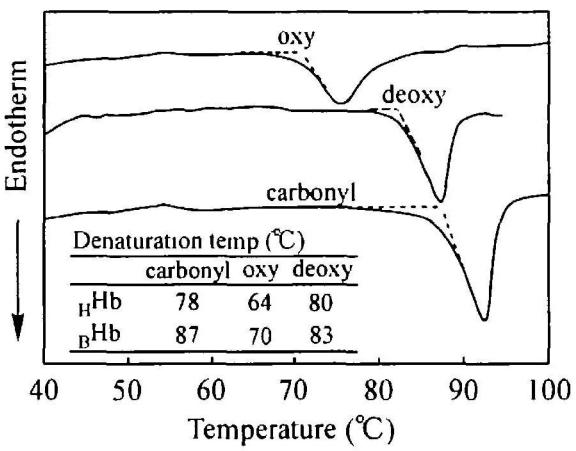

Fig 3 **Calorimetric thermograms of BHbs.** [Hb] = 10 g/dl, 60 μl, scanning rate = 1 0°C/min. Inset Comparison of the denaturation temperature with HHb BHbs show higher denaturation temperatures than HHbs, indicating the higher thermal stability of BHbs

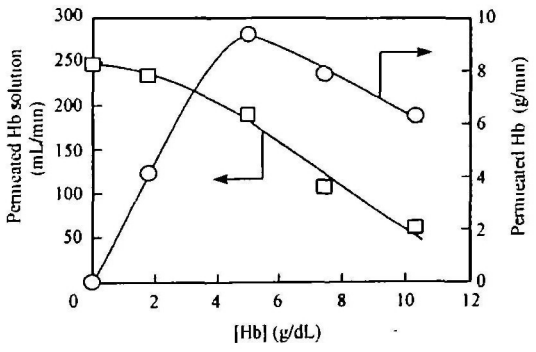

Fig. 4 **Permeation profiles of purified BHb solution through PLANOVA™-15N (total membrane area, 1 m²) at 13°C and 80 psi.** From the relationship between [BHb] and the permeation rate, and the relationship between [BHb] and the permeated amount of BHb, the most effective permeation can be performed at around 5 g/dl

TABLE I **Characteristics of BHbV prepared with different concentrations of chloride ion in the concentrated BHb solution.**

Parameter	Cl^- (mM)				HHbV	RBC
	0	10	50	100		
P_{50} (Torr)	16	17	22	28	33	28
Hill number (–)	1 7	1 9	1 9	1 9	2.1	2 8
OTE (%)*	11	14	19	30	37	28
[Hb]/[Lipid]	2 0	1 9	1 8	1 6	1 8	–
Diameter (nm)	284 ± 82	284 ± 78	283 ± 83	273 ± 74	273 ± 67	8,000
[metHb] (%)	4 0	5 0	3.8	3 7	3 1	< 0.5

*OTE is oxygen transporting efficiency Hb O_2 saturation difference between 40 and 110 Torr O_2 partial pressure

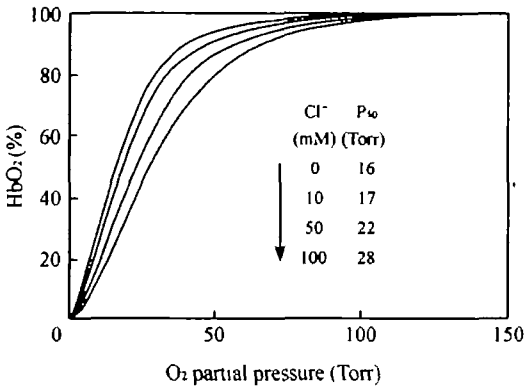

Fig. 5. **O_2 equilibrium curves of BHbV prepared with different concentrations of chloride ion in the encapsulated BHb solution.** Measured with a Hemox Analyzer (TCS Medical Products) A sample solution was diluted with a HEPES buffer (150 mM, pH 7 4), and measurements were performed at 37°C. The O_2 equilibrium curves shifted to the right with increasing Cl^- concentration

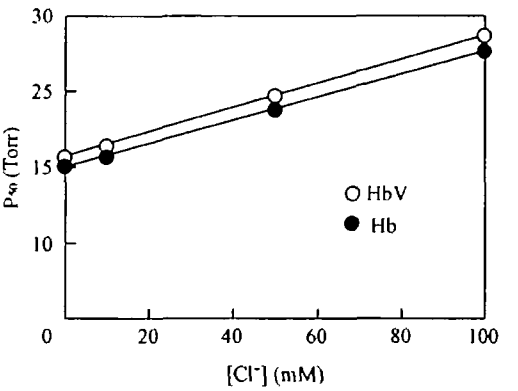

Fig 6 **Comparison of the O_2 affinity of a BHbV with that of a BHb solution at different Cl^- concentrations.** A sample solution was diluted with a HEPES buffer (150 mM, pH 7 4), and measurements were performed with a Hemox Analyzer at 37°C The values for BHbV are from the O_2 equilibrium curves in Fig 5

(*30*), which is slightly smaller than that of HHb (64,450). The slight difference in the positions of Hbs in Native-PAGE (Fig. 2) may reflect a difference in not only the molecular weight but also the surface properties of the proteins, because SDS-PAGE did not show any significant differences in the molecular weights of the subunits. IEF measurements revealed two main components of BHb with different p*I*, 7.0 and 7.3, which correspond to two different β-subunits, β^B and β^A, respectively (*30, 31*). The trace amount of BHbs at p*I* 7 4 should be the partially oxidized BHb with β^A. The protein purity of purified BHb was confirmed by SDS-PAGE and IEF to be greater than 99.9% (above the detection limit).

Thermal Stability of BHb—DSC thermograms of the Hb solutions showed the denaturation temperatures of BHbs in the oxy, deoxy, and carbonyl states to be, 70, 83, and 87°C, respectively (Fig. 3), significantly higher than those of HHbs (64, 78, and 80°C, respectively).

Process of Virus Removal Using PLANOVA™—The permeation rate of pure water through PLANOVA™-15N was 250 ml/min (Fig. 4). The permeation rate of the BHb solution at [BHb] = 5 g/dl was 190 ml/min, and this decreased to 70 ml/min at [BHb] = 11 g/dl. Multiplying the BHb concentration by the permeation rate gives the amount of BHb permeated per minute. This clearly demonstrates that the most effective permeation can be performed at [BHb] = 5 g/dl. More than 30 liters of the purified BHb was filtered without a change in the permeation rate

Characterization of BHbV—The physicochemical parameters of BHbV prepared by the same method but with different Cl^- concentrations in the concentrated BHb solution are summarized in Table I. The mean particle diameter is around 280 nm, and size distribution is around 80 nm for all samples. The [BHb]/[Lipid] weight ratio, which represents the encapsulation efficiency of BHb, was 2.0 without NaCl in the concentrated BHb solution. However, raising the NaCl concentration in the inner BHb solution to 100 mM decreased the ratio to 1.6 The metHb content for all samples was around 3 or 5%, within experimental error. The COP of HbV suspended in 5 g/dl human serum albumin was 20 Torr, equal to the suspending media, and the viscosity of the suspension was 3.5 cP, which is similar to that of human blood (3–4 cP).

Regulation of O_2 affinity of BHbV by Chloride Ion (Cl^-)—The O_2 affinity (P_{50}) of BHbV in the absence of Cl^- was 16 Torr (Fig. 5), which is almost the same as that of the BHb solution (Fig. 6). Raising the Cl^- concentration in the inner BHb solution of the BHbV to 100 mM increased the P_{50} value to 28 Torr, the same as the BHb solution. The O_2 transporting efficiency (OTE), which expresses the O_2 saturation difference (%) between the arterial and venous O_2 partial pressures (40 and 110 Torr, respectively), increased from 11 to 30% with the increase in P_{50} values. Hill numbers were in the range 1.7–1.9, and did not apperr to have any relationship with the chloride concentration.

DISCUSSION

Our study clarifies the intrinsic physicochemical characteristics of BHb, such as thermal stability, regulation of O_2 affinity in vesicles, and purification procedures that are

important for a main component of an artificial O_2 carrier.

The thermograms of the Hb solutions indicated that carbonylation and deoxygenation ensure thermal stability of BHb in the same manner as for HHb. Moreover, the denaturation temperatures of deoxy-, carbonyl-, and oxy-BHbs are all higher than those of HHbs. The higher resistance of BHb to denaturation at higher and lower pH in comparison with HHb has been confirmed spectrophotometrically in 1954 (*32*), however, our study is the first to demonstrate the higher thermal stability of BHb compared with HHb. This stability would be advantageous for pasteurization and long term storage. The pasteurization conditions for biological materials such as human albumin for infusion should be 10 h at 60°C (*22, 33*). Heating stroma-free BHb solution leads not only to virus inactivation but also to denaturation of concomitant proteins that can be removed as precipitates. As a result, the protein purity is greater than 99.9%.

One characteristic point of the bovine RBC biomembrane is that it does not contain PC, a main phospholipid in human RBC (*29*). Instead, the main phospholipid of bovine RBC is Sph. There is a difference between Sph and PC in the interface region of the RBC bilayer membrane. In PC, this region includes the glycerol backbone and the components of the two ester bonds linking the acyl groups to the backbone. In contrast, this region in Sph contains the amide bond between the acyl chain and the primary amino group and the hydroxyl group. The hydroxyl group and the amide bond should afford an important hydrogen bond donor capacity that is not found in PC (*34*). In addition, the presence of these groups in the belt region where a relatively low dielectric constant exists suggests that the hydrogen bonds formed by these groups with other phospholipids, cholesterol and proteins will be stronger than hydrogen bonds formed in an aqueous medium with a high dielectric constant. Since PC is easily dispersible in water to form micelles with other components, the removal ratio of PC from human RBC remains >99.7% after ultrafiltration using a filter with a 1,000 kDa cut-off, and further ultrafiltration is required through a 70-kDa filter for a removal ratio greater than 99.99%. On the other hand, in the case of BHb, Sph binds strongly to membrane proteins and other phospholipids so that the removal ratio of phospholipids is higher than 99.99% using the same ultrafiltration with a 1,000 kDa cut-off.

The use of BHb for the liposome-encapsulated Hb has already been reported by Rudolph and coworkers (*35*), however, the physicochemical advantages of BHb in terms of the purification process and thermal stability, and the relationship between Cl^- concentration and O_2 affinity in vesicles were not demonstrated. Moreover, the O_2 affinity remained at around 17 Torr, which is far smaller than the physiological value of human RBC (28 Torr). The O_2 affinity of both HHb and BHb, especially the latter, is strongly affected by the addition of Cl^- (*17, 20*). One characteristic of BHb is that its O_2 affinity is substantially lower than that of HHb (P_{50} is 37°C and pH 7.2; BHb: 12.8 Torr *vs.* HHb: 4.4 Torr) in the absence of an allosteric effector. Mammalian Hbs are classified into two groups: those with intrinsically high O_2 affinity, and those with intrinsically low O_2 affinity. HHb is an example of the former and BHb of the latter group (*19*). The O_2 affinity of BHb can be easily regulated to the appropriate value by simply adding Cl^- to around 28 Torr, similar to the values of human blood. Ueno *et al.* have shown that there are five Cl^--binding sites on the BHb molecule (*36, 37*) These include two amino groups in the α-chain (Val-1 and Lys-99) and three amino groups in the β-chain (Met-1, Lys-81, and Lys-103). It is proposed that the influence of Cl^- on O_2 affinity is due to these excess positive charges in the central cavity of BHb and its widening in the transition from the R to the T-structure (*20*). The widening would allow more Cl^- to enter and neutralize the positive charges.

In the case of HHb, the regulation of O_2 affinity requires an allosteric effector of a phosphate derivative such as pyridoxal 5′-phosphate (PLP), 2,3-diphosphoglyceric acid (2,3-DPG), or inositolhexaphosphate (IHP) (*38, 39*). The naturally present allosteric effector in human RBC, 2,3-DPG, is chemically unstable and expensive, thus its utilization is not practical. Mammalian Hbs that are classified as having low O_2 affinity, including BHb, show low sensitivity to DPG. BHb differs from HHb by 17 amino acid replacements in the α-chain and 23 in the β-chain. Most of the replacements occur in positions that would not affect O_2 affinity (*20*). The main difference is that His NA2(2)β is deleted in BHb and the N-terminal residue is Met. It is suggested that the hydrophobic side chains of the residues adhere to the hydrophobic interior of the β-chains and pull the two helices (A) towards the center of the molecule by 2.1 Å so that they become locked more tightly to neighboring segments of the polypeptide chain, thus mimicking the effect of DPG on deoxy HHb (*20*).

An analysis of the O_2 equilibrium curves showed the low intrinsic O_2 affinity of BHb to be due a larger O_2 dissociation constant from the T-structure. The thermal stability of BHb in comparison with HHb may be due to the tighter structure of the β-chains.

O_2 affinity of blood is a crucial factor in determining O_2 delivery and unloading to tissues. It has been assumed that the O_2 affinity of Hb-based O_2 carriers should be the same or lower than that of RBC to facilitate O_2 unloading by increasing the O_2 transporting efficiency (OTE). However, the determination of an appropriate O_2 affinity remains controversial (*40*). Although faster release of O_2 could be advantageous, it could also lead to autoregulatory vasoconstriction and/or decreased functional capillary density in the peripheral circulation (*41–43*). In spite of these controversies, the O_2 affinity of BHbV can be regulated quite easily without major changes in other physical parameters. In the case of other chemically modified Hb solutions, their chemical structures themselves determine their O_2 affinities; thus regulation is difficult. The appropriate O_2 affinities for O_2 carriers has not yet been completely determined, however, the easy regulation of O_2 affinity for BHbV may be useful for meeting the requirements of clinical indications.

During the encapsulation of concentrated HHb solution within a phospholipid bilayer membrane, electrostatic interactions between the components strongly affects the encapsulation efficiency (*25*). Negatively-charged lipid bilayer membranes repulse each other so that the lamellarity (number of bilayer membranes) decreases to one or two. However, in higher ionic strength suspension medium, the electrostatic repulsion is shielded so that the lamellarity increases. Accordingly, in order to enhance HHb encapsulation, we regulated the ionic strength of the concentrated HHb solution to be as low as possible (*25*). Thus the slightly

decreased encapsulation efficiency ([Hb]/[[lipid]] of BHbV in Table I, from 2.0 to 1.6 with increasing the NaCl concentration, is plausible and in accordance with our previous results.

The COP of a 10 g/dl Hb solution is 40 Torr, however, that of a HbV suspension at the same Hb concentration is essentially zero, because the concentrated Hb solution is encapsulated in vesicles. Accordingly, one plasma expander should be added to the HbV suspension to maintain homeostasis in maintaining the blood volume in circulation and avoiding tissue edema. When HbV is suspended in 5 or 8 g/dl albumin solution, then the COP should be 20 or 40 Torr, respectively. The viscosities of these suspensions are 3.5 and 4.0 cP, respectively (37°C, 450 s^{-1}), which are close to the conditions of human blood. It is advantageous that the COP and viscosity of the HbV suspension can be regulated to any value by selecting an appropriate plasma expander (*44*).

The source of Hb for the production of HbV should be outdated human RBC, bovine RBC, or recombinant technology depending on the usage (*e.g.*, human or veterinary use) Compared with HHb, BHb has the advantages of abundance and superior physicochemical characteristics, such as the thermal stability and the ability to regulate O_2 affinity. Because of the enormous stability of BHb under certain conditions, rigorous procedures for viral and bacterial inactivation would be possible. Additionally, the BMM filtration system (PLANOVA™-15N) showed a good permeation rate in this study indicating the possibility of large scale production. The high efficiency of virus removal from Hb solutions has been confirmed in other studies (*24, 45*). The high purity of the Hb solution guaranteed no plugging of the filter pores in our study. Thus the extreme diminution of the possibility of viral infection with a reduction factor of >13 $\log_{10}$ is established by the combination of pasteurization at 60°C (*46*) and the filtration procedure (*45*). As for the possible transmission of prion-mediated disease, which is threatening transfusion medicine, it has been reported that the same membrane, PLANOVA™-15N, can eliminate the pathogenic scrapie agent with a reduction factor of >5 87 $\log_{10}$ (*47, 48*). Moreover, safer BHb for further manufacture could be obtained exclusively from closed herds with well-documented health histories and controlled access, unlike human blood donors (*49*).

In conclusion, BHb has several advantages over HHb in its abundance and physicochemical characteristics such as (a) easier removal of membrane phospholipids, (b) higher thermal stability suitable for pasteurization, and (c) simple control of O_2 affinity by Cl^-, none of which can be achieved with HHb. The characteristics of the resulting BHbV are comparable to those of conventional HHbV for *in vivo* use; thus BHb can be used as an abundant source for the production of HbV, especially for veterinary use.

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