Physical Properties of Hemoglobin Vesicles as Red Cell Substitutes

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Hemoglobin vesicles (HbV) as red cell substitutes were prepared from a purified carbonylhemoglobin (HbCO) solution and a lipid mixture composed of phospholipids, cholesterol, and α-tocopherol. The diameter was controlled to 251 ± 87 nm using an extrusion method; the vesicles penetrated through the membrane filters with regulated pore sizes. After the ligand exchanging reaction (HbCO → HbO_2), the oxygen affinity (P_{50}) of HbV was 32 Torr, which was controlled with the coencapsulation of pyridoxal 5′-phosphate. The rate of metHb formation in HbV was nonenzymatically reduced with the coencapsulation of DL-homocysteine. The Hb concentration of the HbV suspension, which was dispersed in a phosphate buffered saline solution (pH 7.4), was controlled at 10 g/dL. At this concentration, the total lipid concentration was 6.2 g/dL and the viscosity, 2.6 cP (230 s^{-1}), was lower than that of the blood (4.4 cP). The HbV suspension showed a typical non-Newtonian flow for a particle dispersion and agreed well with the Casson model. The viscosity at shear rates lower than 23 s^{-1} showed a maximum with increasing the mixing ratio of human blood, plasma, or albumin, while no maximum was observed for the mixture with washed red blood cells. The aggregates of HbV are formed by interaction with plasma proteins, including albumin, while the aggregates reversibly dissociate at higher shear rate.

Introduction

Recent progress in red cell substitutes utilizing hemoglobin (Hb)[1] is remarkable and especially for acellular Hb solutions, *e.g.*, chemically-modified Hb (Winslow, 1992) and recombinant Hb (Looker *et al.*, 1992), which are now undergoing evaluation in clinical tests. The issues of acellular modified Hb solutions are sometimes discussed in relation to the physiological significance of the cellular structure of red blood cells (RBC). Liposome-encapsulated Hb or Hb vesicles (HbV), in which purified and concentrated Hb is encapsulated into phospholipid vesicles and which have a cellular structure similar to RBC, are also promising red cell substitutes (Djordjevich *et al.*, 1988; Hunt *et al*., 1985; Farmer *et al.*, 1988; Tsuchida, 1994). The encapsulation of Hb with chemical reagents or enzymes is the important advantage of vesicles in comparison with acellular Hb-type red cell substitutes. The biological activity or toxicity of Hb and Hb-related products such as methemoglobin (metHb) and released heme can also be shielded using bilayer membranes. We have been developing the quality of HbV for years from the viewpoint of molecular assembly (Tsuchida and Takeoka, 1995). Some groups (Djordjevich *et al.*, 1987; Rabinovici *et al.*, 1993; Zheng *et al.*, 1993) evaluated its physiological effects using *in vivo* experiments; however, they did not have sufficient quality such as Hb concentration, metHb content, size distribution and viscosity.

We have for the first time succeeded in preparing the HbV with the following high performances superior to previous reports for this type of red cell substitute: (1) The particle is small enough to pass through sterilizable filters, and (2) the HbV contains a high Hb concentration with a lower level of lipids. The high encapsulation efficiency was obtained with controlling the electrostatic interaction among the lipid bilayers and Hb molecules and the microviscosity of the membrane (Sakai *et al.*, 1992; Takeoka *et al.*, 1994). (3) The oxygen affinity is controlled with the coencapsulation of the allosteric effectors that are used to increase the oxygen transporting efficiency (Wang *et al.*, 1992). (4) The suppression of the metHb formation is improved with simply coencapsulating a reductant in the HbV without enzymes (Sakai *et al.*, 1994).

Our recent concept is to construct the HbV with high quality from plain and well-known components so as to meet the demands for a red cell substitute. The complexity will retard the clinical application of HbV. In our purification of Hb, the heat treatment of carbonylhemoglobin (HbCO) is completed for virus inactivation and the denaturation of the concomitant proteins (Sakai *et al.*, 1993). The metHb reducing systems such as NADH-cytochrome b_5 reductase, NADH-dehydrogenase, and NADPH-flavin reductase are also removed during this process. Preservation of the activity of these enzymes is one method for suppressing the metHb formation (Ogata *et al.*, 1994); however, it varies with the source of outdated blood. Moreover, it is difficult to inactivate virus using the heat treatment, and the complicated mechanism of metHb suppression is influenced by many unknown factors. In our system, an appropriate amount of reductant is simply added to the purified Hb solution to suppress the metHb formation. The rheological prop-

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[1] Abbreviations: Hb, hemoglobin; RBC, red blood cells; HbV, hemoglobin vesicles; metHb, methemoglobin; HbCO, carbonylhemoglobin; PLP, pyridoxal 5′-phosphate; Hcy, DL-homocysteine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol; PBS, phosphate-buffered saline; HbO_2, oxyhemoglobin; TEM, transmission electron microscopy; SDP, size distribution processor method; HbNO, nitrosylhemoglobin; P_{50}, oxygen affinity; OTE, oxygen transporting efficiency; pI, isoelectric point; γ, shear rate; τ, shear stress; f_c, Casson yield stress; η, Casson viscosity.

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erties of the HbV suspension are also important for the blood circulation. A drastic increase in viscosity would cause a problem.

In this paper are reported the physical characteristics of our plain but excellent HbV in terms of the size control, concentration of the components, oxygen affinity, suppression of metHb formation, and especially the detailed rheological properties which relate to the interaction of the HbV with blood components.

Materials and Methods

Preparation of HbV. HbV were prepared in a way similar to the method previously reported (Takeoka *et al.*, 1994). Hemoglobin was simply obtained with the purification of outdated RBC (Hokkaido Red Cross Blood Center) through the following steps (Sakai *et al.*, 1993): (1) stabilization of Hb using carbonylation, (2) solvent treatment (CH_2Cl_2) for hemolysis and removal of the stromata, (3) removal of the residual solvent with active carbon (Merck Co.), (4) heat treatment (60 °C, 10 h), (5) dialysis to remove the small molecules with a seamless cellulose tube, and (6) ultrafiltration (cut of MW. 30 000, Prep/Scale TFF 1 ft^2 cartridge, Millipore). The profile of the obtained HbCO solution is as follows: [Hb], 40 g/dL; [metHb], <1%; protein purity, 99.95%; residual phospholipids, <0.2%; residual organic solvent, <0.1 ppm; yield, *ca.* 80%. Pyridoxal 5′-phosphate (PLP, 18 mM, Merck Co.) as an allosteric effector and DL-homocysteine (Hcy, 15 mM, Aldrich Co.) as a reductant of metHb were added to the HbCO solution. The pH value was regulated with sodium carbonate to 7.0 at 37 °C.

The powder (15 g) of Presome PPG-I (Nippon Fine Chemical Co.), which contained 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol (DPPG), at a molar ratio of 5/5/1, was dissolved in $CHCl_3$, and α-tocopherol (0.1 g, Merck) was added to the mixture. A lipid film was prepared on the inner wall of a flask using a rotary evaporator and dried *in vacuo* for 48 h. After dispersing the lipid mixture into an Hb solution (330 mL; [Hb], 38 g/dL) for 24 h at 5 °C, the resulting multilamellar vesicles were sized using extrusion through membrane filters with a stepwise reduction of the pore size down to 0.22 μm ∅ (FM filter, filter size: 75.5 mm ∅, pore size (μm ∅): 3.0, 0.8, 0.65, 0.45, 0.3, and 0.22, Fuji Film Co.). Each filter was attached to a high pressure resistant chamber (Remolino, Millipore), and the suspension was extruded under an N_2 gas pressure below 25 kg/cm^2. After washing the resulting HbV using ultracentrifugation (50 000*g*, 30 min) and dispersing them in a phosphate-buffered saline (PBS, 20 mM phosphate, pH 7.4 (37 °C)), the HbCO in HbV was converted to oxyhemoglobin (HbO_2) by exposing the liquid membrane of HbV (thickness: <1 mm) to visible light (500 W halogen lamp) under an O_2 atmosphere (Chung *et al.*, 1995). The suspension was concentrated using ultracentrifugation, passed through sterilizable filters (pore size 0.45 μm ∅, Toyo Roshi Co.), and then finally adjusted to a 10 g/dL Hb concentration. The whole procedure was performed under sterilized conditions below 10 °C.

Characterization of HbV. The vesicular size was measured using a Coulter particle analyzer (N4-SD) with the size distribution processor method (SDP) and also confirmed with a transmission electron microscopy (TEM, JEM-100CX, JEOL, magnification: ×50 000) after the samples were negatively stained with uranyl acetate. The concentrations of phospholipid, cholesterol, and hemoglobin were determined using a molibuden blue method (Phospholipid test Wako, Wako Pure Chem. Co.), a cholesteroloxidase-*p*-chlorophenol method (Cholesterol test II Wako), and a cyanomethemoglobin method (Hemoglobin test Wako), respectively. In the case of cholesterol determination, sodium dodecyl sulfate (2.3 mM) was added to completely destroy the vesiclular structure (Hamada *et al.*, 1995). For the measurement of the Hb concentration, the temperature was raised to 50 °C for the turbidity disappearance. The concentration coincides well with the result of the atomic absorption spectrometry (AA-6400, Shimadzu Co.) of iron from the Hb. The ζ potential of the vesicles was measured using electrophoresis with LEZA-600 (Otsuka Electronics Co.).

Table 1. Characteristics of HbV

[Hb] (g/dL)	10.0
[lipid] (g/dL)	6.2
[Hb]/[lipid] (g/g)	1.61
diameter (nm)	251 ± 87
P_{50} (Torr)	32
Hill number	2.2
pH (at 37 °C)	7.4
viscosity (cP, at 230 s^{-1})	2.6
HbCO (%)	2
metHb (%)	3

The oxygen affinity and Hill number of the HbV were calculated from an oxygen dissociation curve measured with a Hemox analyzer (TCS-Medical Products) at 37 °C. The HbV was diluted with PBS (pH 7.4, 37 °C).

Measurement of metHb formation. The HbV suspension was incubated at pH 7.4, 37 °C, and oxygen partial pressure of 149 Torr. About 10 μL of the sample was periodically pipetted out and sealed in an UV cuvette with 3 mL of PBS. The deoxygenation of the sample using N_2 bubbling resulted in the presence of only deoxyHb and metHb. The percentage of metHb was calculated *in situ* from the ratio of absorbances at 405 nm (metHb) and 430 nm (deoxyHb) in the Soret band (Hamada *et al.*, 1995). The HbV sample of 0% metHb (100% deoxyHb) was prepared by incubation of the sample with sodium dithionite under an N_2 atmosphere at 25 °C for 2 h. The sample of 100% metHb was prepared via the metHb formation of nitrosylhemoglobin (HbNO) (Maeda *et al.*, 1987). An NO gas was bubbled in the deoxygenated HbV suspension to form 100% HbNO (λ_{max} in Soret band: 415 nm). An N_2 gas was then bubbled through the suspension to expel the excess NO. After the N_2 gas, an O_2 gas was bubbled through the sample to convert the HbNO to metHb (λ_{max}: 405 nm).

Rheological Properties of HbV Suspensions. The viscosities of HbV dispersed in PBS and its mixtures with human blood, human plasma, albumin (albumin(5%)−cutter, Bayer Co.), or washed RBC ([Hb] = 15 g/dL, in PBS) were measured with a cone−plate rotation viscosimeter (VS-AK, Shibaura System Co.) at 37 °C (Makino *et al.*, 1991). The human blood sample (50% hematocrit) was stored with 10 vol % of an ACD solution.

Results and Discussion

Characteristics of HbV. The HbV was observed by TEM (Figure 1). The size distribution was calculated to be 198 ± 44 nm (mean ± SD, n = 55). The Hb in the vesicles is blackened, and this is surrounded by the thin bilayer membrane. The particle diameter was also confirmed by the light scattering method using the SDP method with the distribution 251 ± 87 nm (mean ± SD) (Figure 2). This method can distinguish five different peaks in a wide range (1−10 000 nm); however, only one peak was detected. This one peak indicates that the size distribution is relatively narrow.

The characteristics of the HbV are summarized in Table 1. The weight ratio of the total amount of Hb to

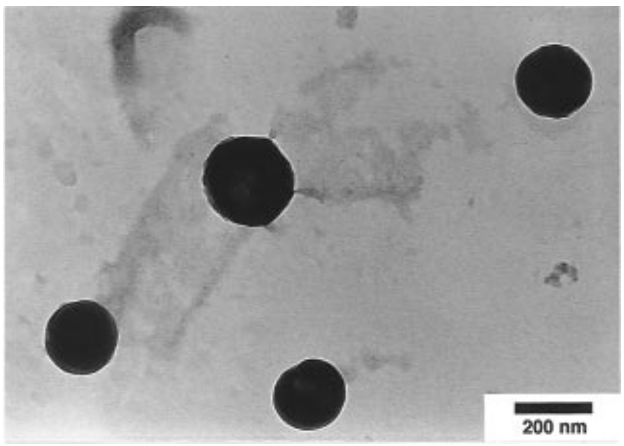

Figure 1. Transmission electron micrograph of HbV stained with uranyl acetate. The size distribution was calculated to be 198 ± 44 nm (mean ± SD, $n = 55$).

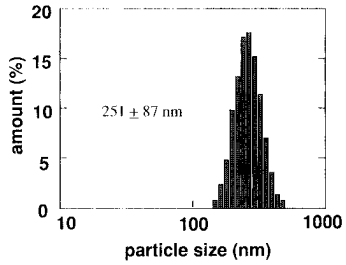

Figure 2. Size distribution of HbV measured by light scattering method using the coulter particle analyzer with a SDP method.

lipids, a parameter indicating the encapsulation efficiency, was as high as 1.61. This is the highest value that has been reported for vesicles with a regulated vesicular size (*e.g.,* [Hb]/[lipid] = 1.34, Takeoka *et al.,* 1994; *ca.* 0.9, Zheng *et al.*, 1994; *ca.* 1.3, Rabinovici *et al.,* 1990). When the Hb concentration of the suspension is controlled to 10 g/dL, the lipid concentration becomes 6.2 g/dL and the viscosity of the suspension is 2.6 cP at 230 s^{-1}, which is lower than that of blood (4.4 cP). The metHb and HbCO contents of the HbV after the ligand exchange reaction were less than 3% and 2%, respectively. Figure 3 shows the oxygen-dissociation curve of the HbV obtained using a Hemox analyzer. The oxygen affinity (P_{50}, O_2 partial pressure required for half-saturation of Hb with O_2) is controlled to 32 Torr with coencapsulating PLP. The oxygen transporting efficiency (OTE, the difference in O_2 saturation (%) between 40 and 110 Torr) was 37%.

The protein denaturation often generates insoluble components which lead to an increase in the viscosity and filtration difficulty. The extrusion method had not been generally considered viable for a large-scale production. However, our purified HbCO solution contains no concomitant proteins, which plug the pores of the membrane filters. The Hb denaturation is suppressed with carbonylation. Therefore, good filtration is maintained during the procedure, *e.g.*, less than a 10-min filtration of a 200-mL sample solution through a filter (pore size from 3.0 to 0.22 μm ∅). The extrusion method provides vesicles with well-regulated diameters in comparison with the other methods such as the Microfluidizer because of the penetration through the filters with regulated pore sizes. The Microfluidizer is often used for the large-scale production of vesicles. However, there are the problems of a wide size distribution, especially the formation of a large amount of small particles (<150 nm) with a small encapsulation volume and the denaturation of Hb

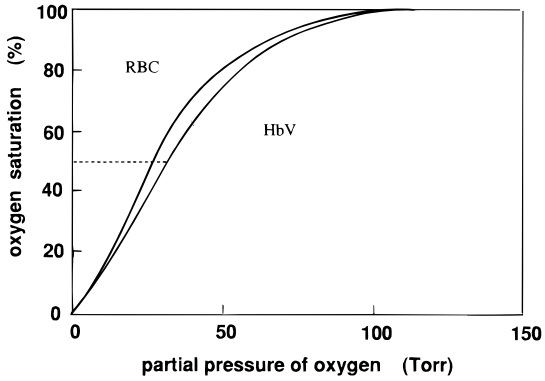

Figure 3. Oxygen dissociation curves of HbV coencapsulating PLP and human RBC measured using the Hemox analyzer (TCS-Medical Products Co.) at 37 °C. The HbV was diluted with PBS (pH 7.4, 37 °C).

([metHb] of *ca.* 10%) probably due to the direct exposure to a high shear stress (Beissinger *et al.*, 1986; Zheng *et al.*, 1994).

In order to efficiently transport oxygen using the HbV, it is important to encapsulate a larger amount of Hb with a reduced amount of lipids. During the preparation of the HbV, multilamellar vesicles are converted to vesicles with a smaller size and smaller number of bilayers by the shear stress during extrusion. We have clarified the relationship between the intermolecular interaction during extrusion and the resulting structure of the HbV (Takeoka *et al.,* 1994; Sakai *et al.,* 1992). The introduction of negatively charged lipids into the bilayer membrane is effective in reducing the number of bilayer membranes through the electrostatic repulsion between bilayers. The ζ potential of the vesicles is −21 mV, which is similar to RBC (−18 mV) and lower than the previously reported HbV composed of palmitic acid (−13 mV). The pKa of phosphatidylglycerol is reported to be 1.1 (Toccane *et al.*, 1990), so nearly 100% DPPG dissociates in the bilayer membrane. The ion concentration of an Hb solution for preparation must be controlled as low as possible because the electrostatic interaction is shielded by ionic strength (Takeoka *et al.*, 1994). The HbCO solution is dialyzed in advance against pure water to remove the small solutes, and necessary ions such as PLP, Hcy, and sodium carbonate for pH adjustment are added to the solution. The total osmolarity was *ca.* 80 mOsm, which is lower than the outer medium (300 mOsm). The lipid membrane is relatively strong against this level of osmotic pressure. The pressure difference should be gradually equilibrated during the removal of the extracellular Hb solution. Actually, both the size distribution and the oxygen affinity of the HbV did not change for months, which indicates that the concentration of the important small solutes which affect the oxygen affinity did not change.

The Hb concentration in the inner aqueous phase of vesicles is affected by the solution pH. The isoelectric point (pI) of Hb is 7.0 at 25 °C. The hemoglobin molecule is negatively charged when the pH is above 7.0, and the electrostatic repulsion between the Hb and the negatively-charged bilayer membrane results in a low encapsulation efficiency of the Hb within the vesicles. The degree of dissociation of the DPPG is constant, *i.e.*, 100% at around the pI; therefore, the solution pH is adjusted to be lower than the pI. However, the change in oxygen affinity, Hb denaturation by direct interaction with lipids and also metHb formation at the lower pH must be considered (Shviro *et al.*, 1982; Szebeni *et al.*, 1988; LaBrake *et al.*, 1992). The best solution pH for preparation is approximately 7.0 at 25 °C.

The number of bilayer membranes decreases with an increase in the microviscosity of the bilayer membrane (decrease in the mobility of lipids). During extrusion of the HbV, the multilamellar vesicles are converted to vesicles with a smaller size and lower number of bilayer membranes by the shear stress. When the molecular motion in the membrane is high, the deformation of vesicles during extrusion would occur more easily even for the lipid membrane with a larger number of bilayers, so the reduction of the number of bilayers is not so effective. Therefore, the utilization of lipids with a higher phase transition temperature is better (*e.g.*, 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine, hydrogenated soy phosphatidylcholine); however, this makes extrusion difficult because a higher shear stress (higher pressure for the extrusion) is required. On the basis of this point, mixed lipids containing DPPC and DPPG were selected.

Control of Oxygen Affinity of HbV. The oxygen affinity (P_{50}) was controlled to 32 Torr with the coencapsulation of PLP (PLP/Hb = 3 by mol). The OTE and Hill number were 37% and 2.2, respectively (Figure 3). The main binding sites of PLP are the N-terminal valine of the α- and β-chains and β-82-lysine within the β-cleft (Benesch *et al.*, 1972; Fishman *et al.*, 1990). They are the parts of the binding site of 2,3-diphosphoglyceric acid (2,3-DPG). The bound PLP retards the dissociation of the ionic linkage between the β chains at conversion of deoxyHb to HbO_2 in the same manner as 2,3-DPG, and the oxygen affinity of Hb is thus lowered using PLP. The amount of released oxygen calculated from the OTE and the Hb concentration of 10 g/dL was 6.2 mL for 100 mL of the HbV suspension, which is similar to the value of human blood: 7.0 mL for 100 mL of blood ([Hb] = 15g/dL, P_{50} = 28 Torr, OTE = 28%). The P_{50} value and Hill number can be adjusted with changing the concentration of PLP or the other allosteric effectors such as inositol hexaphosphate (Wang *et al.*, 1992). The oxygen affinity can thus be controlled so that it meets the demands from a clinical point of view; this is one of the advantages of HbV.

The chemical binding process of the allosteric effectors to Hb and its purification are necessary for modified Hb's in order to control the oxygen affinity (Winslow and Chapman, 1994). Recombinant Hb has a intramolecular cross-linkage between the α chains (Looker *et al.*, 1992). It exhibits a P_{50} of 33 Torr under physiological conditions. The further control of oxygen affinity seems to be difficult without changing the chemical structure.

Suppression of metHb Formation in HbV. Ferrous Hb reversibly binds oxygen; however, it is gradually oxidized to form ferric Hb, metHb, which does not bind oxygen. Hemoglobin in the HbV with no reduction system autoxidizes under physiological conditions. Hb autoxidation generates superoxide radicals and hydrogen peroxide. They not only accelerate Hb oxidation but also induce toxicological effects such as ischemia-reperfusion injury (D'Agnillo and Chang, 1993). HbV forms 35% metHb after incubation for 24 h at 37 °C with the initial rate of metHb formation at *ca.* 3.0%/h. This is higher than the metHb percentage (initial rate *ca.* 1.0%/h, 20% after 24 h) observed for the acellular Hb solution purified using our method. This indicates that the encapsulation of Hb enhances the metHb formation. It is reported that the negatively-charged membrane electrostatically at first and then hydrophobically interacts with Hb at a lower pH, *e.g.*, 5.6. This results in the metHb formation and the deformation of the globin chains (Shviro *et al.*, 1982; Szebeni *et al.*, 1988; LaBrake *et al.*, 1992). However, the HbV coencapsulating Hcy generates 22% metHb after 24 h of incubation (initial rate *ca.* 1.0%/h), indicating that Hcy effectively reduces the metHb formation. The metHb content is initially less than 3%, since HbCO is stable against oxidation and heating. The increase in the metHb percentage during the ligand exchange reaction from HbCO to HbO_2 is negligible because of the mild conditions using the HbV liquid membrane (Chung *et al.*, 1995).

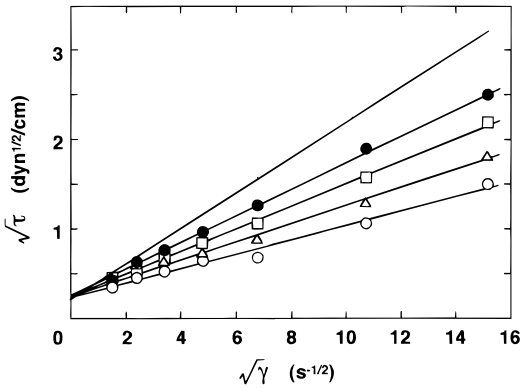

Figure 4. Casson plots for the HbV suspensions at various Hb concentrations and for human blood measured using a cone−plate rotation viscosimeter at 37 °C. [Hb] (g/dL): ●, 10; □, 8; △, 5; ○, 2; —, blood. γ : shear rate. τ : shear stress.

It is reported that Hb behaves as a pro-oxidant for vesicles composed of unsaturated phospholipids (Sarti *et al.*, 1994; Sebeni *et al.*, 1988). We clarified that it is also important to use saturated phospholipids rather than unsaturated ones to decrease lipid peroxidation and metHb formation (Yokohama *et al.*, 1994).

From our studies on the effect of various nonenzymatic reductants, especially thiols including glutathione, it has been determined that the active oxygen species generated during the oxidation of the reductants by O_2 adversely accelerate the metHb formation (Sakai *et al.*, 1994). Hcy has hitherto showed the best suppressive effect as a reductant due to its relatively slow oxidation rate and high metHb reduction rate.

It is also reported that the chemical modification of Hb, *e.g.* intramolecular cross-linking with bis(2,3-dibromosalicyl) fumarate, enhances the metHb formation from 50 to 60% after 24 h at pH 7.0 and 37 °C (Yang and Olsen, 1994). These values are higher than that in our system. One method which effectively restricts the metHb formation for acellular modified Hb solutions is the direct conjugation of superoxide dismutase and catalase to the modified Hb (D'Agnillo and Chang, 1993). The coencapsulation of a regulated amount of oxygen radical scavengers such as superoxidedismutase/catalase will be effective for the further suppression of the metHb formation. Further study is now continued in our laboratory to reduce the metHb level, and the results will be reported.

Rheological Properties of HbV Suspension. The viscosity of the HbV suspension was measured using a cone−plate viscosimeter. The suspensions showed a nonlinear decrease in viscosity with increasing the shear rate at all of the Hb concentrations. Such non-Newtonian flow is typical for particle suspensions. Casson plots for each suspension, $\sqrt{\gamma}$ versus $\sqrt{\tau}$ (γ , shear rate; τ, shear stress) are shown in Figure 4. All of the suspensions showed a linear relationship, indicating that the rheological property of HbV agrees well with the Casson model with the following equation (Oka, 1984):

$$\sqrt{\gamma} = \sqrt{\eta}(\sqrt{\tau} - \sqrt{f_c}) \quad (1)$$

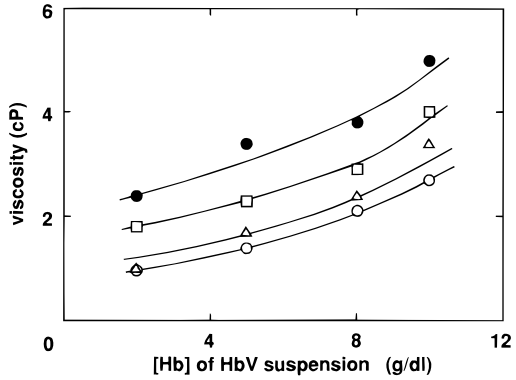

Figure 5. Viscosity of the HbV suspensions in PBS at various Hb concentrations, measured using a cone−plate rotation viscosimeter at 37 °C. Shear rate (s^{-1}): ●, 11.5; □, 23; △, 46; ○, 230.

where f_c is the Casson yield stress and η is the Casson viscosity. Therefore, the rheological property of the HbV is similar to that of blood and the suspension has an yield stress of *ca.* 0.063 dyn/cm^2.

The viscosity of the HbV suspension increases with the Hb concentration due to the increasing number of HbV particles (Figure 5). At 10 g/dL, the viscosity at 230 s^{-1} was 2.6 cP, lower than blood, due to the low lipid concentration (6.2 g/dL) and the small volume of total vesicles (crit of HbV *ca.* 35%), which was a result of the high encapsulation efficiency ([Hb]/[lipid] = 1.61). In comparison, the "synthetic erythrocyte" of Djordjevich (1988) showed 4−6 cP at the crit of 45−55%, and the "liposome encapsulated Hb" of Beissinger *et al.* (1986) showed *ca.* 10 cP at the crit of 57% with the same oxygen carrying capacity as our HbV.

The dispersibility of the HbV in human blood was studied with measuring the viscosity of the mixture of HbV and human blood. The viscosity increased with the volume of HbV especially at lower shear rates (Figure 6a), and the maximum (11 cP) was observed at 40% HbV at 11.5 s^{-1}. At the higher shear rate (230 s^{-1}), on the other hand, the viscosity linearly increased from 2.6 to 4.4 cP. It is indicated that reversible aggregation of the HbV occurs with the mixing of human blood. The mixture of the HbV with washed RBC, on the contrary, showed a linear decrease in viscosity with the amount of HbV even at a lower shear rate (Figure 6b), indicating no specific interaction between the HbV and RBC. The same viscometric measurements were completed for the mixtures of HbV with albumin and human plasma (Figure 6c,d). The maxima were also observed at around 60% volume of the HbV suspension in both systems. This value is different from that of the mixture with human blood (40%), and the viscosity increase is smaller (4.2 cP for albumin and 6.6 cP for human plasma). These results indicate that the aggregation of the HbV is induced by some specific interaction with plasma components. It is reported that the phospholipid vesicles, especially the negatively charged vesicles, adsorb the plasma proteins such as albumin and lipoproteins (Hernandez-Caselles *et al.*, 1993; Loughrey *et al.*, 1990), which leads to a decrease in the dispersibility of the vesicles. We confirmed that the adsorbed proteins on the vesicular surface were major plasma proteins such as albumin and globulin (Ohgushi *et al.*; 1994).

The optical microscopic observation in the static condition (shear rate = 0 s^{-1}) showed the flocculated formless HbV aggregates with a size around 10 μm in the mixtures with human blood, plasma, and albumin. In the case of the mixture with blood, the scope was filled with the flocculated HbV aggregates and dispersed RBC. The high viscosity of the mixture with human blood is due to a large volume of the HbV aggregate and RBC. The right shift of the HbV composition in Figure 6c,d, at which the maximum was observed, can be explained by the concentration effect of the HbV in the plasma of the human blood with 50% hematocrit. This also supports no interaction between the RBC and HbV but does suggest an interaction between the plasma and HbV.

The shear rate in the blood stream is reported to be higher than 30 s^{-1}, *e.g.* 30−60 s^{-1} for the vena cava, 200−600 s^{-1} for the aorta, and 530 s^{-1} for the capillary on the average (Whitmore, 1968; Shiga, 1990). It is known that

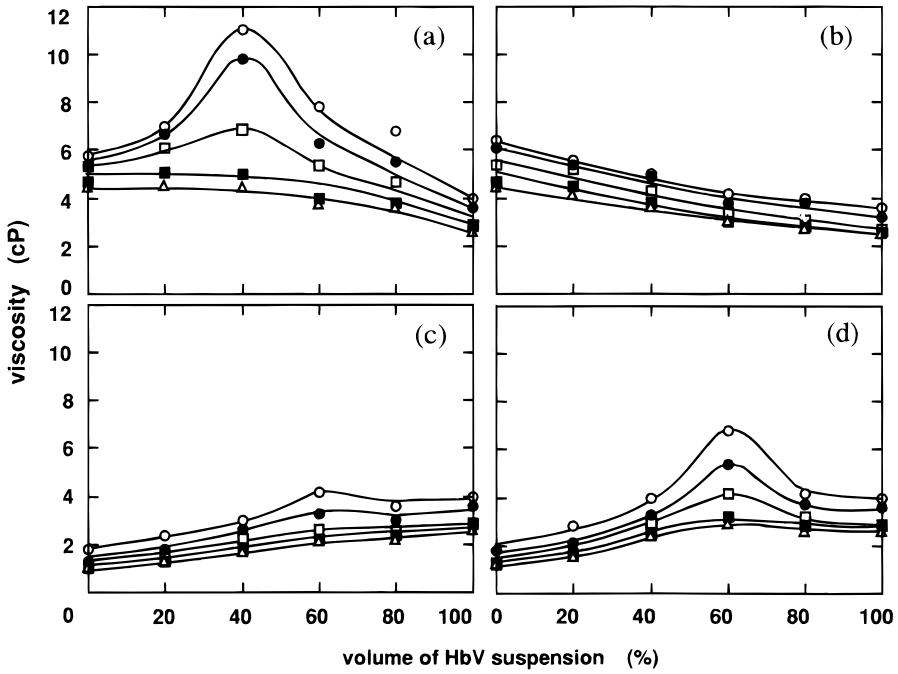

Figure 6. Shear-rate dependence of the viscosity of the mixture of the HbV suspension with (a) human blood, (b) washed RBC, (c) albumin, and (d) human plasma, measured using a cone−plate rotation viscosimeter at 37 °C. The composition of the HbV suspension varied from 0 to 100%. Shear rate (s^{-1}): ○, 11.5; ●, 23; □, 46; ■, 150; △, 230.

the blood stream is not always regular, and it sometimes stops partially on the condition of peripheral capillary level. Since the viscosity of the mixture reversibly changes at any shear rate, the HbV aggregates (ca. 10 μm ∅) would reversibly dissociate at higher shear rate. Therefore, this level of viscosity increase might not be serious. Actually, there was not a serious problem in peripheral oxygen tension observed during the 40% exchange-transfusion of the HbV into rats. These results will be reported elsewhere (Izumi *et al.*, 1996; Sakai *et al.*, 1995). We also confirmed that the oxygen transporting ability of the HbV to tissues was similar to that of RBC.

Conclusion

The HbV are well controlled in their size (*ca.* 250 nm) using an extrusion method. The concentration of Hb is 10 g/dl with a low lipid concentration with selecting the optimal preparation conditions. The oxygen affinity is controlled with the coencapsulation of PLP so that the amount of released oxygen is close to that of RBC. The rate of metHb formation of HbV is reduced to one-half its original value with the coencapsulation of Hcy. Additionally, the increase in the viscosity for the mixture of blood and HbV is especially observed at a lower shear rate; however, it would not be serious under the normal shear rate in the blood stream. We have been studying the rheological properties of the HbV suspension in the microcirculation in a living body.

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