Surface Modification of Hemoglobin Vesicles with Poly(ethylene glycol) and Effects on Aggregation, Viscosity, and Blood Flow during 90% Exchange Transfusion in Anesthetized Rats

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Poly(ethylene glycol) (PEG_{5000})-conjugated phosphatidylethanolamine was introduced onto the surface of hemoglobin vesicles (HbV); phospholipid vesicles encapsulating concentrated Hb ($d = 0.257 \pm 0.087$ μm; $P_{50} = 32$ Torr). The obtained PEG-modified HbV (HbV-PEG) was studied for use as a red cell substitute from the viewpoint of rheology, surface properties, and hemodynamics. The viscosity of the unmodified HbV suspended in saline ([Hb] = 10 g/dL) was 2.6 cP (shear rate = 358 s^{-1}, 37 °C), less than that of human blood (4 cP). However, when suspended in a 5 g/dL albumin solution (HbV/albumin), it increased to 8 cP due to the molecular interaction between albumin and vesicles, and the viscosity increased with decreasing shear rate, *e.g.*, 37 cP at 0.58 s^{-1}. As for the HbV-PEG/albumin, on the other hand, the viscosity was 3.5 cP at 358 s^{-1} and was comparable with that of human blood. Optical microscopy showed formless flocculated aggregates of the unmodified HbV, while no aggregates were confirmed for the HbV-PEG. The steric hindrance of PEG chains seemed to be effective in preventing intervesicular access and the resulting aggregation. To estimate the flow profiles in the capillaries, the suspensions were allowed to penetrate through isopore membrane filters (pore size = 0.4−8μm, *cf.* capillary diameter = 4−10 μm). The penetration rate of the HbV-PEG/albumin was higher than that of the unmodified HbV/albumin due to the suppression of aggregation, whereas both of them were significantly higher than that of human blood due to the smaller size of vesicles than RBC. Ninety percent exchange transfusion was performed with the HbV-PEG/albumin or HbV/albumin in anesthetized Wistar rats ($n = 6$). The blood flow in the abdominal aorta increased 1.5 times, and the total peripheral resistance decreased in the HbV-PEG/albumin-administered group in comparison with the HbV/albumin group. As for the blood gas parameters, the base excess and pH remained at higher levels in the HbV-PEG/albumin group, and the O_2 tension in mixed venous blood for the HbV-PEG/albumin group tended to be maintained at a higher level than that for the HbV/albumin group. Thus, the PEG modification of HbV reduced the viscosity by the suppression of aggregation and resulted in prompt blood circulation *in vivo*.

INTRODUCTION

To date, various efforts have been made to develop red cell substitutes, especially those utilizing Hb, to overcome the problems associated with blood transfusions such as the necessity for blood typing and cross-matching, blood-borne infections, and difficulties in storage (Chang, 1991; Winslow, 1995; Tsuchida, 1995). Hemoglobin vesicles (HbV)[1] or liposome-encapsulated hemoglobin (LEH) are candidates for red cell substitutes that have the cellular structure of phospholipid vesicles containing concentrated Hb (Djordjevich and Miller, 1980; Hunt *et al.*, 1985; Rudolph, 1995; Tsuchida and Takeoka, 1995). On the other hand, in the field of acellular Hb solutions such as chemically modified Hb and recombinant Hb, clinical trials are now underway (Winslow, 1995). However, vasoconstriction induced by acellular Hb solutions is reported. This is due to the trapping of endothelial-derived relaxation factor [EDRF; nitric oxide (NO)] by Hb with an intrinsic high affinity for NO (Vandegriff and Winslow, 1995; Tsai *et al.*, 1995). NO trapping also induces platelet activation (Olsen *et al.*, 1996). Complexation of acellular Hb with endotoxin synergistically enhances the endotoxin biological activities, and it would be dangerous to use acellular Hb in septic shock (Kaca *et al.*, 1995). These problems seem to be related to the lack of physiological significance of the cellular structure of red blood cells (RBC), and it is expected that they will be solved by the encapsulation of Hb.

In previous papers, we have succeeded in preparing HbV with excellent physicochemical properties in terms of Hb encapsulation, Hb concentration, diameter control, oxygen affinity, *etc.* (Sakai *et al.*, 1996; Takeoka *et al.*, 1996). Moreover, in a 40% exchange transfusion with

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[1] Abbreviations: Hb, hemoglobin; HbV, hemoglobin vesicles; RBC, red blood cell; PEG, poly(ethylene glycol); DDS, drug delivery system; HbV-PEG, poly(ethylene glycol)-modified hemoglobin vesicles; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol; PEG−DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine−*N*-[poly(ethylene glycol)]; HbV-PEG/albumin, HbV-PEG suspended in albumin; HbV/albumin, HbV suspended in albumin; PBS, phosphate-buffered saline; P_{50}, oxygen affinity; PLP, pyridoxal 5′-phosphate; MAP, mean arterial pressure; HR, heart rate; TPRI, total peripheral resistance index; PaO_2, arterial blood oxygen tension; $PaCO_2$, arterial blood carbon dioxide tension; PvO_2, mixed venous blood oxygen tension; $rPtO_2$, renal cortical tissue oxygen tension; $sPtO_2$, skeletal muscle tissue oxygen tension; τ, shear stress; γ, shear rate.

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the HbV suspended in saline, a high oxygen transporting capability of the HbV similar to that of the washed rat RBC was confirmed (Izumi *et al*., 1996). Ninety percent exchange transfusion with the HbV was also successful in the presence of albumin as a plasma expander using rats (Izumi *et al*., 1997).

Because the red cell substitutes are literally to be used as substitutes for a large amount of lost red blood cells, the rheological properties of HbV itself and the mixture with blood are important in relation to hemodynamics. Surface modification of phospholipid vesicles with some natural or synthetic glycolipids (Wu *et al*., 1981; Allen *et al*., 1987; Gabizon and Papahadjopoulos, 1988; Takeoka *et al*., 1992) or poly(ethylene glycol) (PEG)-conjugated lipids (Kilbanov *et al*., 1990; Woodle and Lasic, 1992; Woodle *et al*., 1994; Harasym *et al*., 1995; Zalipsky *et al*., 1995) is known to improve the dispersion state of the vesicles and prolong the circulation time *in vivo* for drug delivery systems (DDS). For HbV, the surface was also modified to improve the dispersion state of the vesicles in the presence of water-soluble polymers or blood components (Yoshioka, 1991; Tsuchida and Takeoka, 1995). The PEG-modified HbV has been studied, and its oxygen transporting ability has been evaluated (Usuba *et al*., 1993; Zheng *et al*., 1993). PEG has thus been widely used for vesicular materials including DDS and HbV. However, little attention has been paid to the effect of the PEG modification on the low solution viscosity in the field of DDS because of the significantly low lipid concentration and small dosage. Also, these effects of PEG-modified HbV have not been clearly demonstrated in comparison with the unmodified HbV.

Using our HbV with high Hb encapsulation efficiency and high Hb concentration (Sakai *et al*., 1996), we aimed to modify the surface with PEG chains by a simple method. The PEG-conjugated lipids with various molecular weights of PEG are currently commercially available, among which 2000 and 5000 seem to be most commonly used (Vertut-Do *et al*., 1996). The effects of PEG, *e.g*., longevity of circulation time and prevention of aggregation, increase with increasing chain length at the same molar composition. Our method enables PEG incorporation only on the outer surface of the vesicles; therefore, we thought that a smaller molar composition would be better not to disturb the lipid bilayer by the transbilayer asymmetry, and we selected a larger molecular weight of 5000. We tried to evaluate the HbV-PEG in comparison with the unmodified HbV, in terms of the dispersion state, rheological properties in a mixture with blood and the resulting oxygen transporting capabilities *in vivo* by observing the physiological responses to 90% exchange transfusion in anesthetized rats.

EXPERIMENTAL PROCEDURES

Preparation of HbV and Surface Modification with PEG−Phosphatidylethanolamine. HbV was prepared in the same manner as previously reported in the literature (Sakai *et al*., 1993, 1995). The encapsulated carbonylhemoglobin (*ca*. 38g/dL) contained pyridoxal 5′-phosphate (18 mM, Merck Co.) as an allosteric effector and DL-homocysteine (5 mM, Aldrich Chemical Co.) as a metHb reductant. The lipid bilayer was composed of Presome PPG-I [a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, (DPPC), cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phospatidylglycerol (DPPG), Nippon Fine Chemicals Co.] and α-tocopherol (Merck Co.) at a molar composition of 5/5/1/0.1. After sizing by an extrusion method and removal of unencapsulated Hb using ultracentrifugation (50000*g*, 40 min), the surface modification of HbV with PEG was performed by simply mixing the HbV suspension (lipid: 2.2 g/dL, 833 mL) with a saline solution of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine−*N*-[poly(ethylene glycol)] (PEG−DPPE, Avanti Co., 0.1 g/dL, 231mL) at 37 °C under a CO atmosphere and stirring for 30 min. The resulting HbV-PEG was diluted with saline to an Hb concentration of 0.5 g/dL, and carbonylhemoglobin was converted to oxyhemoglobin by illumination with visible light under an O_2 atmosphere (Chung *et al*., 1995). After the ultracentrifugation and redispersion of the HbV-PEG in 5 g/dL of albumin solution (albumin 5% cutter, Bayer Co.) were repeated, the suspension was filtered through sterilizable filters (Dismic, Toyo Roshi Co.; pore size = 0.45 μm). The free PEG−DPPE, which was not incorporated into the HbV, was removed during the process of ultracentrifugation (50000*g*, 40 min) and redispersion. Finally, the Hb concentration was regulated to 10 g/dL using the albumin solution to obtain HbV-PEG/albumin. The unmodified HbV suspended in the albumin solution (HbV/albumin) was prepared in the same manner.

Quantitative Analyses of PEG-DPPE. The freeze-dried powder of HbV-PEG was dispersed in $CDCl_3$, and the filtrate (filter paper, Toyo Roshi, 5B) was analyzed using ^{1}H-NMR spectroscopy (JEOL, EX-270). The PEG content was estimated from the intensity ratio of the PEG methylene proton peak (δ 3.63) to that of the choline methyl protons in DPPC (δ 3.39) (Yoshioka, 1991; Hamada *et al*., 1995).

Viscosity and Optical Microscopy of HbV-PEG/Albumin Mixed with Human Blood. Human venous blood was drawn into EDTA (1 mg/mL blood)-containing tubes. The hematocrit of the blood was 55%. The HbV-PEG or HbV in albumin was mixed with the human blood at volume compositions of 0, 15, 30, 60, 90, and 100%. The rheological properties and density at 37 °C were measured with a capillary viscosimeter [Oscillatory Capillary Rheometer and Density Meter (OCR-D, Anton Parr)], where the sample in the measuring capillary (0.9948 mm in diameter, 100.2 mm in length) was set into sinusoidal oscillations at a given frequency of 2 Hz to mimic the pulsatile flow *in vivo* (Schneditz *et al*., 1985). The shear rate ranged from 0.58 to 358 s^{-1}. The whole measurement was performed within 6 h after blood collection. The dispersion states of the vesicles and RBC were observed with optical microscopy (Olympus BH-2) at a magnification of 750.

Permeability through Isopore Membranes. To mimic the blood flow in narrow capillaries, the permeabilities of human blood and the HbV-PEG/albumin and the HbV/albumin through filters were observed. Each isopore membrane filter (Nuclepore, Coaster Co.; pore size = 0.4, 0.6, 1.0, 2.0, 3.0, 5.0, 8.0 μm; diameter = 25 mm) was attached to a high-pressure resistant chamber (Extruder, Lipex Biomembrane, Inc., Canada). The filtration area was regulated to 1 cm^2. After the suspension was prefiltered through disposable filters (Dismic, Toyo Roshi Co.; pore size = 0.45 μm), 5 mL of the suspension was injected and 5 min later the initial filtration rate of the outlet volume was measured at a pressure of 0.3, 0.4, or 4 kg/cm^2 at 37 °C.

Protein Adsorption onto the Vesicular Surface. The unmodified and PEG-modified vesicles, which did not encapsulate Hb, were prepared with the same lipid composition in the same manner except that phosphate-buffered saline (PBS, pH 7.4, 37 °C) was used in place of a concentrated Hb solution for lipid hydration. After regulation of the lipid concentration to 3.4 g/dL, the vesicles were suspended in a 5% albumin solution and incubated for 3 and 12 h at 37 °C. The vesicles were separated by gel permeation chromatography (Sepharose

CL-4B, Pharmacia; column size: height = 12 cm, diameter = 3 cm) with PBS as an eluent. It took about 5 min to obtain the fractions of HbV and HbV-PEG. The concentrations of total lipids in the fractions were calculated from the cholesterol concentration measured with a cholesterol oxidase−*p*-chlorophenol method (Cholesterol test II Wako, Wako Pure Chemicals Co.). The amount of adsorbed albumin was measured by *o*-phthalaldehyde (OPA) fluorescence assay (Peterson, 1983), in which the fluorescence (λ_{ex} = 340 nm, λ_{em} = 440−455 nm) induced by the reaction of thiol groups on albumin with OPA was measured with a fluorometer (Jasco FP-550).

Ninety Percent Exchange Transfusion Protocol. The procedure was almost the same as that previously reported (Izumi *et al*., 1996). Twelve Wistar rats (weight 348 ± 11 g, mean ± SD) were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Two catheters (PE-20 tubing, o.d. = 0.8 mm, i.d. = 0.5 mm), one of which was introduced into the right jugular vein for sample infusion and the other into the right common carotid artery, were used for blood withdrawal. The catheter in the common carotid artery was then connected to a pressure transducer (Polygraph System, Nihon Koden, Tokyo) for continuous monitoring of the mean arterial pressure (MAP) and the heart rate (HR). Next, a median abdominal incision was made and a miniaturized 20-MHz pulsed doppler probe (HDP 20 20R, 2 mm in diameter, Crystal Biotech, Holliston, MA) was implanted on the abdominal aorta to measure the blood flow. The probe was connected to a pulsed doppler flow module (Model PD-20, Crystal Biotech) (Gardiner *et al*., 1990; Haywood *et al*., 1981). Needle-type polarographic oxygen electrodes (Intermedical Co.) were placed in the cortex of the kidney and the cervical muscle for continuous renal cortical and skeletal muscle tissue oxygen tension measurements (Nelimarkka *et al*., 1982). Approximately 10 min was allowed for the parameters to stabilize. From the MAP, blood flow (*Q*), and body weight, the total peripheral resistance index (TPRI), which indicates the resistance to blood flow, was calculated using the following equation:

$$\text{TPRI} = (\text{MAP}/Q)/(\text{body weight}) \qquad (1)$$

Ninety percent of the estimated total blood volume of the rat (50 mL/kg) was exchanged with the HbV-PEG/albumin (HbV-PEG group, n = 6) or the HbV/albumin (HbV group, n = 6) at 2 mL of withdrawal (via the common carotid artery)/infusion (via the jugular vein) cycles at a rate of 1 mL/min. Blood samples for hematocrit measurements and arterial and venous blood gas analyses (Corning 170 pH/blood gas analyzer, Corning Medical, Medfield, MA) were taken with a heparinized 2 mL syringe on the first withdrawal as basal values at exchange rates of around 10, 40, 60, 70, 80, and 90%, and 30 min after the completion of the exchange transfusion. All animal studies were approved by the Animal Care and Use Committee of Keio University. The care and handling of the rats were in accordance with the National Institutes of Health guidelines.

Data Analysis. Data are expressed as the mean ± SD for the indicated number of animals. Data were evaluated using analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test. The level of confidence was placed at 95% for all experiments.

RESULTS AND DISCUSSION

1. Characteristics of HbV-PEG/Albumin as a Red Cell Substitute. The characteristics of the HbV-PEG/albumin are summarized in Table 1. The diameter was controlled to 0.25 ± 0.08 μm. By increasing the weight ratio of Hb to lipid to 1.74, the lipid concentration was reduced to 5.74 g/dL. This is the highest encapsulation efficiency ever reported, due to the control of intermolecular interactions during the assembling and sizing procedure of HbV (Takeoka *et al*., 1996). The surface modifier, PEG−DPPE, was easily introduced onto the surface of HbV by the addition of the PEG−DPPE solution. Diameter changes were observed within experimental error. After the free PEG−DPPE was removed by repeating the centrifugation and redispersion, the content of PEG−DPPE was measured by ^{1}H NMR and the composition of the lipids was clarified to be DPPC/cholesterol/DPPG/α-tocopherol/PEG−DPPE = 5/5/1/0.1/0.014 by molar ratio (PEG−DPPE = 0.13 mol %). More than 90% of the added PEG−DPPE was incorporated onto the outer surface of the HbV. The oxygen affinity, P_{50} (oxygen partial pressure required for the half-saturation of Hb with oxygen), was regulated to 32 Torr by coencapsulating PLP (18.6 mM). The amount of oxygen release was calculated to be 6.2 mL/100 mL, which was close to the 7.0 mL/100 mL of human blood due to the increased oxygen transporting efficiency (the difference in O_2 saturation between 40 and 110 Torr PO_2) of the HbV-PEG, 37%, compared to that of human red cells, 28%. No leakage of Hb was observed during and after the introduction of PEG−DPPE. The oxygen affinity of HbV-PEG was almost the same within experimental error, indicating that pH change in the inner aqueous phase and leakage of coencapsulated small molecules such as PLP, Na^+, and Cl^-, were negligibly small. The densities of the HbV-PEG/albumin and unmodified HbV/albumin suspensions were almost the same (1.0336 and 1.0335 g/cm^3, respectively), and they were smaller than that of human blood (1.0525 g/cm^3), mainly due to the fact that the concentration of albumin solution used is 5 g/dL, which is lower than the plasma protein concentration, *ca*. 7.5 g/dL. The concentration of albumin of the suspension is expressed as 3.2 g/dL due to 36% of the total volume being HbV particles.

Table 1. Characteristics of HbV-PEG/Albumin in Comparison with Human Blood (RBC)

parameter	HbV-PEG/albumin	human blood
diameter (μm)	0.25 ± 0.08	*ca*. 8
P_{50} (Torr)	34	28
[Hb] (g/dL)	10	12−17
[lipid] (g/dL)	5.71	1.8−2.5[a]
[Hb]/[lipid] (g/g)	1.75	*ca*. 31[b]
O_2 release (mL/dL)	6.1	5.5−7.7
metHb (%)	<3	<0.5
HbCO (%)	<2	<5
viscosity (cP at 230 s^{-1})	3.7	4−5
osmolarity (mOsm)	300	*ca*. 300
oncotic pressure (Torr)	20	*ca*. 25
pH	7.4	7.2−7.4
density (g/cm^3)	1.0336	1.05
hematocrit (%)	36[c]	40−54

[a] Total cell membrane components. [b] Weight ration of Hb to total cell membrane components. [c] Volume percentage of HbV particles.

2. Effect of PEG Conjugation on the Aggregation of HbV in Albumin Solution and Blood Mixture. *2.1. Optical Microscopic Observation of Dispersion States of HbV-PEG/Albumin.* The dispersion states of the HbV-PEG/albumin and HbV/albumin when mixed with human blood were observed by optical microscopy (Figure 1). In the case of HbV-PEG/albumin, only RBCs were confirmed because of the small diameter of HbV-PEG (0.25 ± 0.08 μm), and no aggregate of HbV-PEG was confirmed in the photograph. In the case of HbV/albumin, on the other

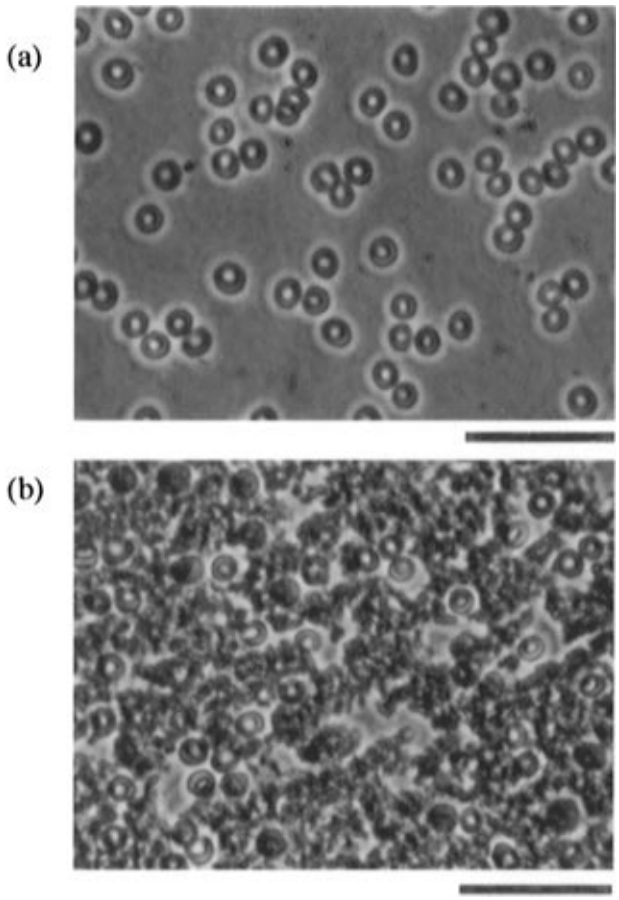

Figure 1. Optical microscopic observation of (a) HbV-PEG/albumin (30%) + blood (70%) and (b) HbV/albumin (30%) + blood (70%). The bars indicate 40 μm. Only the RBCs can be seen in (a), while the aggregates of HbV are seen between RBCs in (b).

hand, flocculated aggregates of the HbV were actually confirmed among the RBCs. The RBCs were neither aggregated nor deformed, indicating that both HbV and HbV-PEG did not interact with RBCs. The aggregates of HbV were actually observed before mixing with blood. It is quite obvious that PEG chains suppress the HbV aggregation.

2.2. Viscosities of HbV-PEG/Albumin and Mixtures with Blood. When the unmodified HbV was dispersed in PBS, the viscosity of the vesicular suspension became 2.6 cP (shear rate = 230 s^{-1}), which is lower than that of blood (Sakai *et al*., 1996). However, when dispersed in a 5 g/dL albumin solution to adjust the colloidal osmotic pressure, the HbV/albumin showed 8 cP viscosity (shear rate = 358 s^{-1}), which was substantially higher than that of blood (3.7 cP). In Figure 2, the HbV/albumin shows a non-Newtonian flow typical for particle suspensions and higher viscosities, especially at lower shear rates. In the case of the HbV-PEG/albumin, on the other hand, the viscosity was significantly reduced in comparison with that of the unmodified HbV/albumin and was almost the same as that of human blood at any shear rates (*e.g.*, 3.5 cP at 358 s^{-1}). These results indicate that the unmodified HbV aggregates due to the molecular interaction of albumin with the vesicular surface and increases the viscosity, while the surface modification of the HbV with PEG chains suppresses HbV aggregation and provides a low viscosity almost the same as that of human blood.

The effect of compositions of PEG−DPPE on viscosity was previously studied at 0.063, 0.13, and 0.25 mol %, for which the viscosities at 10.2 s^{-1} were 5.8, 5.2, and 7.2 cP, respectively. All of the compositions showed a

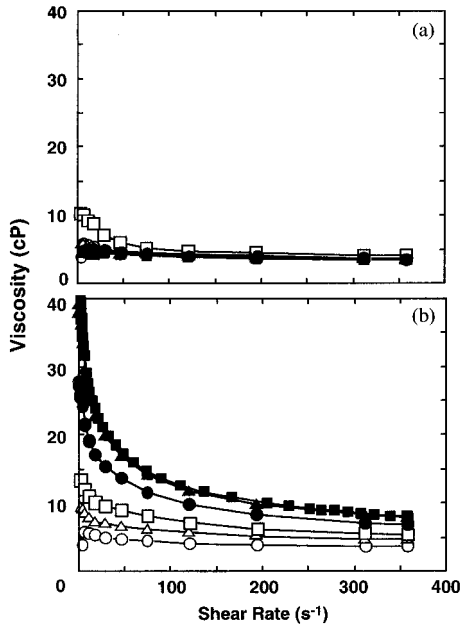

Figure 2. Shear rate dependence of the viscosity of (a) HbV-PEG/albumin and (b) HbV/albumin in the mixture with human blood measured with a capillary rheometer at 37 °C. Blood volume (%): 0 (■), 10 (▲), 40 (●), 70 (□), 85 (△), 100 (○).

significant decrease in viscosity (*cf*. 22.5 cP without PEG−DPPE); however, the viscosity began to increase at 0.31 mol %. We therefore selected the composition at around 0.13 mol %.

When human blood was mixed with the HbV/albumin, the viscosity proportionally increased with increasing volume ratio of HbV/albumin. This indicates that the unmodified HbV already aggregates in the albumin solution, and there is no detectable additional interaction between the HbV/albumin and blood components from the rheological point of view. In the case of HbV-PEG/albumin, the viscosity was not changed at any shear rate and any mixing ratio, except at 30% HbV-PEG/albumin, at which there was a slight increase in viscosity; however, it is negligible in comparison with the high viscosity of the unmodified HbV/albumin.

Figure 3 shows the Casson plots for the mixtures of HbV/albumin or HbV-PEG/albumin with human blood, $\sqrt{\gamma}$ versus $\sqrt{\tau}$ (γ, shear rate; τ, shear stress). In the case of the HbV-PEG/albumin, all of the mixtures with blood coincided well with that of blood. A linear relationship indicates that the rheological property of the HbV-PEG agrees well with the Casson equation for a non-Newtonian fluid ($\sqrt{\tau} = k_0 + k_1\sqrt{\gamma}$) (Oka, 1984), with constants, $k_0 = 0.19 \pm 0.07$ (mean ± SD) $(\text{dyn/cm}^2)^{1/2}$ and $k_1 = 0.18 \pm 0.002$ $s^{1/2}$. The stress yield (k_0^2), which indicates the change point from a solid to a fluid phase, seems to be similar to that of human blood. This linear relationship at all compositions indicates the absence of additional interaction with blood components. On the contrary, HbV/albumin showed a drastic increase in $\sqrt{\tau}$ at lower $\sqrt{\gamma}$ with increasing $\sqrt{\gamma}$ and then a linear relationship. The curves seem to fit the generalized Casson equation reported by Oka (1971). This equation is valid with the assumptions that the stress acts to break bonds between particles and that the shear rate acts independently in tending to prevent their reforming. Therefore, it can be easily surmised that the aggregates dissociate at the higher shear rates and that the sizes of HbV aggregates are different at each shear rate. The small stress yield for unmodified HbV/albumin may indicate that the aggregation is induced by the weak interaction between the albumin and vesicles.

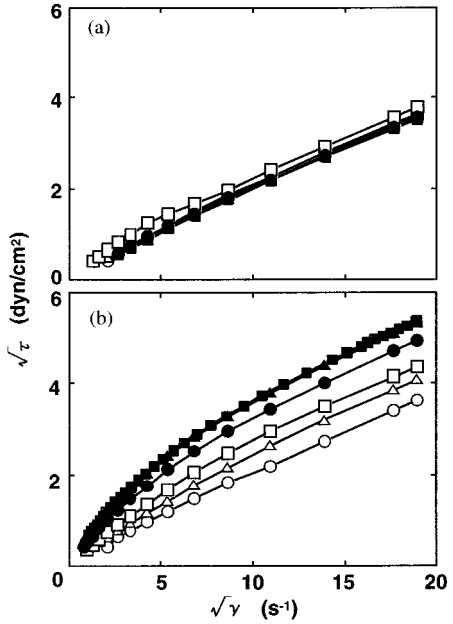

Figure 3. Casson plots for the (a) HbV-PEG/albumin and (b) HbV/albumin of the mixture with human blood. Casson equation: $\sqrt{\tau} = k_0 + k_1\sqrt{\gamma}$ (τ, shear stress; γ, shear rate; k_0, k_1, constants. Blood volume (%): 0 (■), 10 (▲), 40 (●), 70 (□), 85 (△), 100 (○).

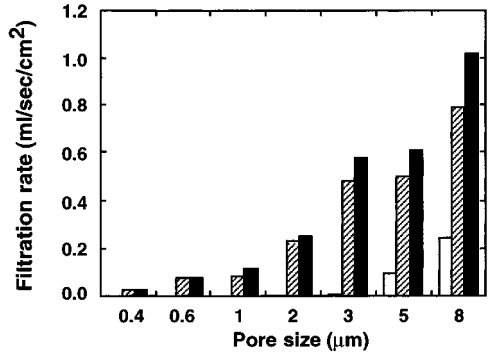

Figure 4. Comparison of the rates of penetration of the HbV/albumin (slashed bar), HbV-PEG/albumin (black bar), and human blood (white bar) through the Nuclepore isopore membrane filters at the applied pressure of 0.3 kg/cm^2 at 37 °C. For the membrane of 0.4 μm pore size, the applied pressure was 0.5 kg/cm^2.

2.3. Permeability of HbV-PEG/Albumin through Isopores. In Figure 4, the permeabilities of the HbV-PEG/albumin, HbV/albumin, and human blood through isopore membranes are shown as a model of the blood flow through capillaries. This method is conventionally used for the RBC deformability measurement (Kukuchi and Koyama, 1984). The capillary and precapillary diameter is usually 3−15 μm, and some narrow during circulatory failure such as hemorrhagic shock. A biconcave-shaped RBC with a diameter of 8 μm deforms to a parachute-like configuration and permeates through the narrow capillaries. However, with decreasing pore size of the membranes from 5 to 2 μm, the flow rate decreased for all suspensions, especially for human blood even though the applied pressure (0.3 kg/cm^2 ≈ 220 Torr) is about twice that of normal blood pressure. When the pore diameter is 3 μm, blood could hardly permeate, whereas both the unmodified HbV/albumin and HbV-PEG/albumin showed high permeability. The permeation through capillaries is very much influenced not only by viscosity but also by the capillary diameter. It was reported that the narrower the capillary is, the lower the viscosity of the blood is when the diameter of the artificial capillaries decreased from 500 to 50 μm with increased shear rate (Fahraeus and Lindqvist, 1931). However, when the diameter of the capillaries decreases to <4 μm, the viscosity increases and finally there should be a limitation for the RBC penetration (Pries *et al.*, 1992). Due to the small size of HbV (0.25 ± 0.08 μm), both the HbV-PEG/albumin and HbV/albumin suspensions promptly permeate through the membrane filters with pores of sizes down to 0.4 μm without changes in vesicular size or Hb leakage. Especially the HbV-PEG/albumin permeates more rapidly than the unmodified HbV/albumin because HbV-PEG did not aggregate. Even though the HbV/albumin showed aggregation in the optical microscopy, the aggregates dissociated at higher shear rates and permeated more promptly than expected. The difference in the permeation rate between the two suspensions becomes smaller, which should be related to the smaller difference in viscosity at higher shear rates in Figure 2. Both the HbV and HbV-PEG can penetrate through sterilizable filters of 0.45 μm in pore size for preparation. No Hb leakage was confirmed, supporting the belief that the aggregation is reversible and that there is no deformation of the bilayer membrane. After the permeation, the suspension was ultracentrifuged, and the amount of PEG−DPPE in the supernatant was analyzed. However, no PEG−DPPE was detected. Therefore, PEG−DPPE was not removed during permeation through the pores of the membranes used in this experiment.

In spite of the fact that the viscometric properties of the HbV-PEG/albumin and blood are almost the same by viscometric analysis (Figures 2 and 3), the flow patterns in the capillaries *in vivo* would be influenced not only by the viscosity but also by the relationship between the diameters of the particles and capillaries.

3. Quantitative Analysis of Adsorbed Albumin onto the Vesicular Surface. The amounts of adsorbed albumin onto the vesicles after 3 and 12 h of incubation at 37 °C were quantitatively analyzed under the assumption that adsorbed albumin on the surface would induce vesicular aggregation. After 3 h, the amounts of albumin were 7 and 10 μg/mg lipid for the unmodified and PEG-modified model vesicles, respectively. After 12 h, the amounts became 9 and 11 μg/mg, respectively. At 11 μg/mg, the surface of one vesicle (*ca.* 2.0 × 10^5 nm^2) was calculated to be covered with *ca.* 100 albumin molecules (size: 3.8 × 15 nm); *ca.* 3% of the surface was covered with albumin. Some of the adsorbed albumin would be removed from the surface during gel permeation chromatography as Chonn *et al.* (1991) reported. However, the time required to obtain the HbV or HbV-PEG fraction was only 5 min and the desorption must be minimal. Contrary to the assumption, there was no significant difference between the modified and unmodified vesicles. PEG chains should prevent intervesicular access and aggregation rather than albumin adsorption. The repulsive interaction between PEG-modified surfaces has been clarified by the distance between the membranes using X-ray diffraction (Needham *et al.*, 1992) and the steric hindrance calculated using computer analysis (Torchilin *et al.*, 1994; Hristova and Needham, 1995). Because PEG chains do not completely cover the surface of the vesicles at the molar composition employed, albumin would penetrate through the PEG chain layers to the vesicular surface to be adsorbed. Even so, the repulsion between the PEG-modified vesicles, which are much larger than albumin, would be effective and the adsorbed albumin on the surface could not induce vesicular aggregation. This hypothesis is also supported by the results of Harasym *et al.* (1995), who found that the coupling of proteins onto PEG-modified phospholipid vesicles was not

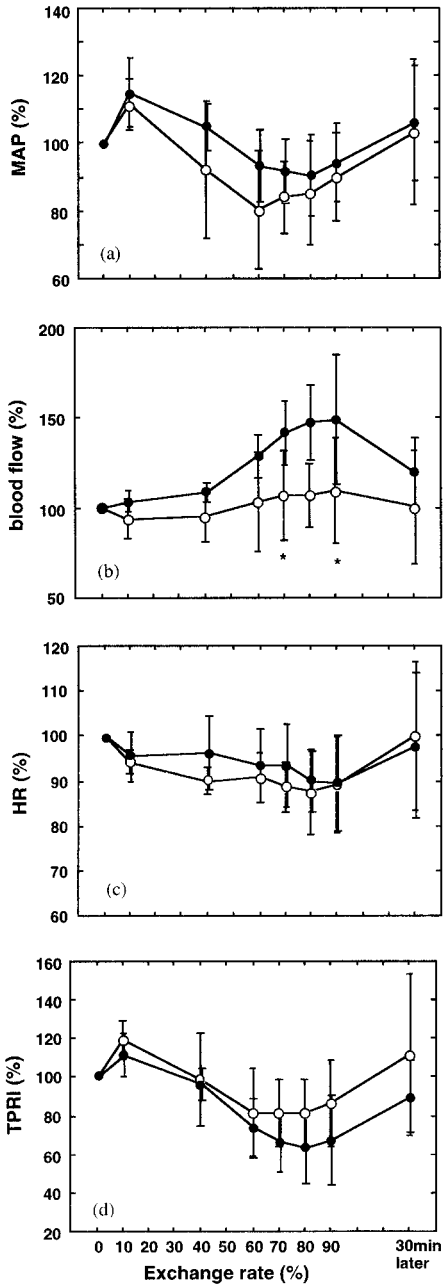

Figure 5. Changes in hemodynamic parameters during the 90% exchange transfusion with HbV/albumin (○, $n = 6$) and HbV-PEG/albumin (●, $n = 6$) and 30 min post-transfusion, shown as percentages of the basal values: (a) MAP, mean arterial pressure; (b) blood flow in abdominal aorta, significant differences at 70% ($p < 0.05$) and 90% ($p < 0.05$); (c) HR, heart rate; (d) TPRI, total peripheral resistance index.

retarded but that the aggregation during the coupling was suppressed by PEG chains.

4. Ninety Percent Exchange Transfusion. During the exchange transfusion, the hematocrit of both the HbV and HbV-PEG groups decreased from about 50 to 5%, indicating that almost 90% exchange transfusion was theoretically and actually performed. In the glass capillaries for hematocrit measurements, the HbV precipitated on the RBC layer, while the HbV-PEG did not, and it was still dispersed in the supernatant after centrifugation (12000*g*), indicating that the HbV-PEG is well dispersed even in the presence of blood components.

Figure 5 summarizes the changes in hemodynamic parameters, which are shown as percentages of the basal values. The HR remained normal in both groups. The MAP showed a slight transient increase for both groups

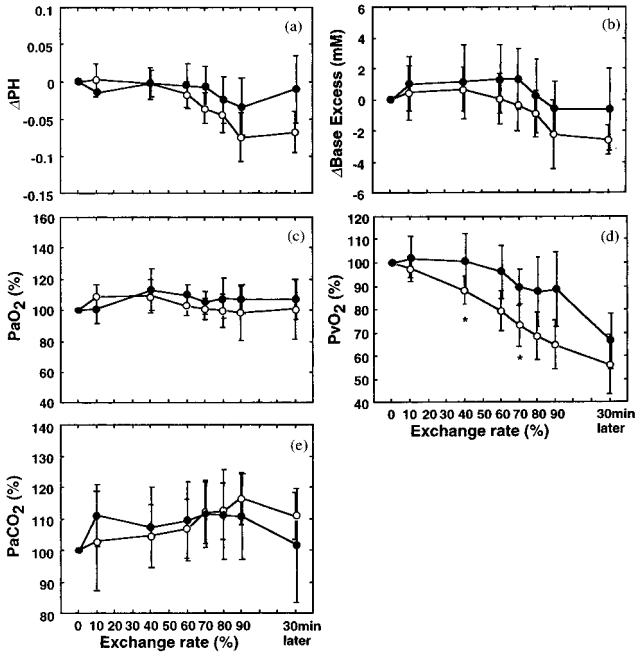

Figure 6. Changes in pH and blood gas parameters during the 90% exchange transfusion with HbV/albumin (○, $n = 6$) and HbV-PEG/albumin (●, $n = 6$) and 30 min post-transfusion: (a) pH, significant differences at 30 min post transfusion ($p < 0.05$); (b) base excess; (c) PaO_2, arterial blood partial oxygen tension; (d) PvO_2, mixed venous blood oxygen tension, significant differences at 40% ($p < 0.05$) and 70% ($p < 0.05$); (e) $PaCO_2$, arterial blood partial carbon dioxide tension. pH is shown as a difference from the basal value. Blood gas parameters are shown as percentages of the basal values.

and then decreased to 80−90% of the initial values. The transient increase at the first stage is maybe due to some form of immunological reaction accompanying the phospholipid vesicle injection as discussed later. The HbV-PEG group showed a significantly (*ca.* 50%) higher blood flow rate in the abdominal aorta with *ca.* 20% lower TPRI than the HbV group. This would be the result of the lower viscosity of the HbV-PEG/albumin, which promotes blood flow in the capillaries. The previous report of the 90% exchange transfusion with washed ratRBC/albumin (Hb = 10 g/dL) showed about a 30% increase in blood flow (Izumi *et al.*, 1997), less than that of HbV-PEG group, even though the HbV-PEG showed almost the same viscometric characteristics as mentioned above. This indicates that the rheological properties in narrow capillaries are not the same as those measured with the viscosimeter and that HbV-PEG would show prompt flow in the capillaries *in vivo* in just the same way as in the filter permeability measurements in Figure 4, because HbV-PEG is not aggregated and is smaller than RBC.

Parts a−d of Figure 6 show the differences in pH and the changes in blood gas parameters from the basal values. The pH began to decrease at an exchange ratio of around 60% for the HbV group. In spite of the fact that these levels of decreases are still small in comparison with the administration of albumin alone, as reported in the previous paper (Izumi *et al.*, 1997), the HbV-PEG group remained at a significantly higher level than the HbV group. The base excess values showed the same profiles. As for the arterial partial pressure of oxygen (PaO_2) and carbon dioxide ($PaCO_2$), both groups showed slight increases but remained in the normal range. The mixed venous partial oxygen pressure (PvO_2) of the HbV-PEG group was significantly higher than that of the HbV group. PvO_2 is a parameter for O_2 supply (Kobayashi *et al.*, 1995). Low PvO_2 indicates low oxygen saturation of

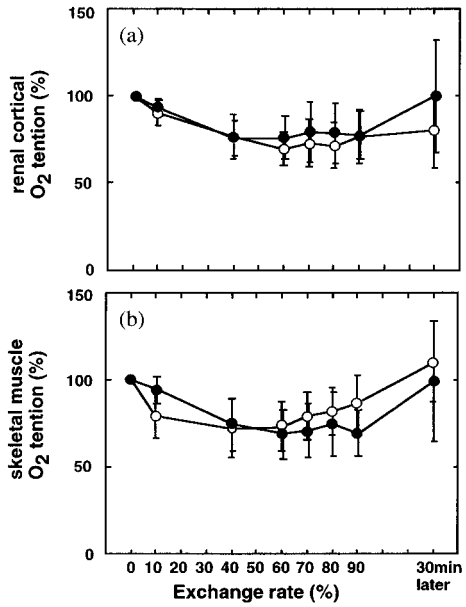

Figure 7. Changes in renal cortical (a) and skeletal muscle (b) tissue oxygen tensions measured by needle-type poplarographic oxygen electrodes during the 90% exchange transfusion with HbV/albumin (○, $n = 6$) and HbV-PEG/albumin (●, $n = 6$) and 30 min post-transfusion. Both are shown as percentages of the basal values.

Hb, and a fall in PvO_2 implies decreased O_2 transport (Kandel and Aberman, 1983). The graph indicates that the HbV group is slightly hypoxic and the HbV-PEG shows better O_2 supply.

In spite of the fact that the PvO_2 was improved by the surface modification with PEG, the oxygen tensions at the renal cortex ($rPtO_2$) and skeletal muscle ($sPtO_2$) for both groups decreased to *ca.* 70% of the initial values at 90% exchange and were significantly low (Figure 7) in comparison with the RBC group ($rPtO_2 = 99\%$; $sPtO_2 = 99\%$ at 90% exchange; Izumi *et al.*, 1997), though they are significantly high in comparison with those who received only albumin ($rPtO_2 = 22\%$; $sPtO_2 = 35\%$ at 90% exchange; Izumi *et al.*, 1997). We presume that both the HbV and HbV-PEG somewhat influence the vasomotion, *e.g.*, indirect induction of vasoconstriction, which results in the decreased density of the capillaries and oxygen diffusion from capillaries to tissues. There is no difference in the high affinity of Hb for the endothelial-derived relaxation factor (nitric oxide) between HbV-PEG and HbV. One reason seems to be related to the shear stress in the capillaries. Some endothelial relaxation factor is reported to be generated in response to the shear stress in the capillaries (Malek and Izumo, 1995). The prompt flow of small HbV results in a shear stress decrease and vasoconstriction. The high fluidity in the vessels would adversely influence the vasomotion, and it would not always be convenient for blood flow (Tsai *et al.*, 1996). The other reason seems to be related to the immunological reaction accompanied by transient hypertension (Figure 5a) and thrombocytopenia, in which complements such as C3a would be generated and they would activate platelets and increase the tromboxane A_2, which induces the vasoconstriction (Miyamoto *et al.*, 1988; Watanabe *et al.*, 1988; Loughley *et al.*, 1990; Rudolph, 1995). Anyway, both of the two groups recovered from the transient reaction and low tissue oxygen tensions at 30 min post exchange transfusion. All of the rats that received HbV/albumin or HbV-PEG/albumin survived for about 20 h, in spite of the fact that those which received only albumin died within 20 min post exchange transfusion.

In conclusion, the effects of the PEG modification of the HbV were observed as the suppression of intervesicular aggregation and prompt flow in vessels. This affects the hemodynamics, the increased blood flow, and the resulting stable blood gas parameters, indicating that HbV-PEG has the potential for efficient oxygen supply.

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