Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute

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Abbreviations: Hb, hemoglobin; HbV, hemoglobin-vesicles; EV, empty vesicles; ^{99m}Tc, technetium-99m; RBC, red blood cells; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPEA, 1,5-dihexadecyl-L-glutamate-*N*-succinic acid; PEG-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5000)]; PLP, pyridoxal-5' phosphate; HMPAO, hexamethylpropyleneamine oxime; %ID, percentage of infused dose; s.e.m., standard error of the means; MPS, mononuclear phagocyte system

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Abstract

Phospholipid vesicles encapsulating concentrated human hemoglobin (Hb-vesicles: HbV) also known as liposomes, have a membrane structure similar to that of red blood cells (RBC). These vesicles circulate in the bloodstream as an oxygen carrier, and their circulatory half-life times ($t_{1/2}$s) and biodistribution are fundamental characteristics required for representation of their efficacy and safety as a RBC substitute. Herein, we report the pharmacokinetics of HbV and empty vesicles (EV) that do not contain Hb, in rats and rabbits to evaluate the potential of HbV as a RBC substitute. The samples were labeled with technetium-99m (^{99m}Tc), and then intravenously infused into animals at 14 mL/kg to measure the kinetics of HbV elimination from blood and distribution to the organs. The $t_{1/2}$s were 34.8 and 62.6 h for HbV, and 29.3 and 57.3 h for EV in rats and rabbits, respectively. At 48 h after infusion, the liver, bone marrow, and spleen of both rats and rabbits had significant concentrations of HbV and EV, and the percentages of the infused dose (%ID) in these three organs were closely correlated to the circulatory half-life times in elimination phase ($t_{1/2\beta}$). Furthermore, the milligram of HbV per gram of tissue correlated well between rats and rabbits, suggesting that the balance between organ weight and body weight is a fundamental factor determining the pharmacokinetics of HbV. This factor could be used to estimate the biodistribution and the circulation time of HbV in humans, which is estimated to be equal to that in rabbit.

Introduction

Hemoglobin (Hb) isolated and purified from red blood cells (RBC) has been tested as a principal component of RBC substitutes for carrying oxygen. However, the plasma retention time of isolated Hb is particularly short (half-life: 0.5~1.5 h) because of the dissociation of the Hb tetramer into the dimeric form, which is subsequently filtered by the kidney and it is known that this dimeric form is nephrotoxic (Savitsky et al., 1978). The potential of phospholipid vesicles as effective carriers of proteins and other bioactive materials has previously been proposed, since the cellular structure of such vesicles can protect the entrapped material from degradation and improve the biodistribution of proteins and other bioactive materials (Gregoriadis and Neerunjun, 1974; Papahadjopoulos et al, 1991). Phospholipid vesicles encapsulating concentrated Hb (HbV) have been proposed as a promising candidate RBC substitute, because encapsulation of Hb within a lipid membrane decreases potential side effects and toxicity of Hb, thereby making vesicles more RBC-like (Djordjevich and Miller, 1980; Gaber and Farmer, 1984; Tsuchida, 1998). The study of the safety and efficacy of HbV formulations by our research group has led to the development of a HbV formulation as a promising candidate for introduction into clinical trials (Tsuchida, 1998, Sakai et al., 2000a, 2001, 2004b, Takeoka et al., 2002).

Determination of the circulation time (half-life) of vesicles has been an important research focus, especially in RBC substitute development, because prolonged oxygen delivery is a required property for an artificial oxygen carrier. There are many reports describing the pharmacokinetics of vesicles, especially in mice and rats; however, it is difficult to apply these published data to the quantitative simulation of a clinical

application. This is because of the lack of understanding of the species-dependence of relevant mechanisms and correlative factors related to the clearance kinetics of vesicles. Some reports suggest that the circulatory half-life of vesicles injected in small doses into small animals such as mice or rats empirically corresponds to half-lives that are two or three times longer in humans (Gabizon et al, 2003). In addition, the infusion dose of HbV as a RBC substitute, in terms of lipid content, is nearly a hundred times larger compared with other therapeutic uses of vesicles, even though HbV encapsulate a highly concentrated form of Hb (35-40 g/dL). Furthermore, there are many other factors such as the lipid formulation (Allen et al., 1989), vesicle size (Awasthi et al, 2003), and surface modification (Klibanov et al, 1990) that influence the circulation time and distribution of the infused vesicles. There are no clinical data available for using large infusion doses of vesicles such as those required for a RBC substitute. Therefore we focused this research on determining the correlation factors between data from different species to simulate the pharmacokinetics of HbV. In addition, empty vesicles (EV) that do not contain Hb were studied as a reference to clarify the specific influence of encapsulated Hb on the circulation properties of the vesicles.

Scintigraphic imaging is a particularly powerful tool that can be used to develop and evaluate the formulation of vesicles (Goins and Phillips 2001). Using imaging, Phillips et al. have reported on the pharmacokinetics of liposome-encapsulated Hb radiolabeled with technetium-99m (^{99m}Tc) (Rudolph et al., 1991; Phillips et al., 1992; Phillips et al., 1999) and achieved a formulation with long circulation times. These liposomes had a small size (< 200 nm), neutral surface, and PEG modification (10 mol%), and were regarded as long-circulating vesicles (so-called stealth liposomes) ($t_{1/2}$ was 65 h after 25%

intravenous top-load in rabbits) (Phillips et al., 1999). However, this particular liposome formulation had a low efficiency of Hb encapsulation, because the requisites for stealth liposomes, such as small size, neutral surface, and dense PEG modification were a disadvantage for efficient Hb encapsulation (Perkins et al., 1993; Nicolas et al., 2000). As mentioned above, the infused dose of RBC substitutes will be extremely high, so high encapsulation efficiency of Hb is essential for a successful oxygen-carrying RBC substitute. We have developed HbV with a lipid formulation and encapsulation conditions that have improved the encapsulation efficiency (Takeoka et al., 1996; Sou et al., 2003) and the present HbV formulation has an oxygen carrying capacity equal to RBC because of this higher encapsulation efficiency (1.7-2.0 g of Hb per gram of lipids). This paper is the first report on the detailed pharmacokinetics of this HbV formulation using scintigraphic imaging of ^{99m}Tc-HbV for monitoring the circulation properties and biodistribution. Factors that would permit estimation of human pharmacokinetics of large quantities of vesicles will be discussed.

Materials and methods

Materials: 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, and 1,5-dihexadecyl-L-glutamate-*N*-succinic acid (DPEA) were purchased from Nippon Fine Chemical Co. Ltd. (Osaka, Japan); 1,2-distearoyl-*sn*-glycero-3- phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5000)] (PEG-DSPE) was purchased from NOF Co. (Tokyo, Japan). DPPC, cholesterol, DPEA, and PEG-DSPE were dissolved in alcohol at a molar ratio of 5, 5, 1, and 0.033, respectively, atomized and evaporated using a spray dryer (Cracks) to prepare a lipid powder, at Nippon Fine Chemical Co., Ltd. The mixed lipid powder was hydrated with NaOH solution, submitted to three cycles of freeze-thawing, and the resultant dispersion was then lyophilized at Kanto Chemical Co. (Tokyo, Japan). The Hb solution was obtained from outdated donated blood (Japanese Red Cross) according to the purification method described previously (Sakai et al., 2002). The Hb solution (oxyhemoglobin) was converted to carbonylHb by purging the solution with 100% carbon monoxide until testing proved conversion (99% < HbCO). The final concentration of Hb was adjusted to 40 g/dL. Homocysteine, pyridoxal-5' phosphate (PLP), and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of HbV: HbV were prepared according to a method described previously (Takeoka et al., 1996; Tsuchida, 1998; Sakai et al., 2001; Sou et al., 2003). All HbV preparation work was performed under sterile conditions. The purified carbonylHb solution (40 g/dL) containing 5 mM homocysteine and pyridoxal-5' phosphate (PLP/Hb ratio of 2.5 (mol/mol)) was mixed with the lyophilized powder containing the mixed lipids (DPPC, cholesterol, DPEA, and PEG-DSPE). After controlling the size of the HbV with an extrusion method (final pore size of the filter**:** 0.22 μm, Fuji microfilter, Fuji

Photo Film Co., Tokyo, Japan), the unencapsulated Hb was removed by three ultracentrifugation steps (10^5g, 30 min each). CarbonylHb was converted to OxyHb by exposure to visible light in an atmosphere of O_2. HbV were suspended in a physiological salt solution and filtered through sterilized filters (pore size: 0.45 μm Dismic, Toyo Roshi, Tokyo, Japan) and deoxygenated by bubbling with N_2 prior to storage (Sakai et al., 2000b). The control empty vesicles (EV) encapsulating glutathione (30 mM) was prepared using the same extrusion method.

Characterization of HbV and EV: The characteristics of HbV and EV are summarized in Table 1. The concentrations of Hb and phospholipid were determined by a cyanomethemoglobin method (Hemoglobin Test Wako; Wako Pure Chem., Tokyo) and the cholineoxidase method (Phospholipid C Test Wako; Wako Pure Chem., Tokyo), respectively. The encapsulation efficiency of Hb was represented as a w/w ratio of [Hb]/[lipid]. Methemoglobin (MetHb) and CarbonylHb content were determined by spectrophotometry (Assendelft, 1970). The diameters of the resulting HbV (247±44 nm) and EV (259±32 nm) were determined using a COULTER submicron particle analyzer (N4SD, Coulter, Hialeah, FL). Endotoxin contamination was determined to be below 0.2 EU/mL by the *Limulus* assay test (Sakai et al., 2004a).

^{99m}Tc-labeling of HbV and EV: Radiolabeling of HbV was performed according to a method described previously (Phillips et al., 1992). A saline solution of sodium [^{99m}Tc]pertechnetate (5 mL, 75 mCi) (Nycomed Amersham, San Antonio, TX) was injected into a vial containing lyophilized hexamethylpropyleneamine oxime (HMPAO; 0.5 mg, $SnCl_2$; 7.6 μg) (Ceretec^{TM}; Amersham, Arlington, IL). The mixed solution was

incubated for 5 min at room temperature. The ^{99m}Tc-HMPAO solution (1 mL) was then added to the HbV suspension ([Hb]**:** 10 g/dL, 1 mL), and the resulting mixed solution was incubated for 1 h. After removing free ^{99m}Tc-HMPAO by gel filtration (Sephadex-G25 column), total radioactivity was measured in a dose calibrator (Radex, Mark 5 Model, Houston, TX) and the labeling efficiency (*E*) was calculated as the percentage of post-radioactivity in ^{99m}Tc-HbV to pre-radioactivity. The ^{99m}Tc-HbV suspension was mixed with unlabeled HbV suspension and the resultant HbV suspension ([Hb]: 9.5 g/dL, [lipid]: 4.75 g/dL) was used for the experiment. The ^{99m}Tc-EV were also prepared with same method and the lipid concentration was adjusted to the same lipid concentration as that of HbV suspension tested ([lipid]: 4.75 g/dL). The ^{99m}Tc labeled HbV and EV dispersion (0.5 mL) was mixed with rat plasma (1.5 mL) from a donor rat and incubated at 37 ^{o}C to check the labeling stability. A 100 μL aliquot of incubated sample at 48 h after mixing was passed through a Bio Gel A-15m (200-400 mesh) spin column. The sample was eluted by sequential addition of 100 μL Dulbecco's phosphate-buffer saline (pH 7.3) under the centrifugal force of 1000 rpm for 1 min. Each fraction was collected separately and counted in a scintillation well counter (Canberra Multichannel Analyzer, Meridan, CT). Another 100 μL aliquot of incubation sample was used as a standard. The sum total of activity eluted with HbV or EV fractions was compared with total radioactivity in the standard.

Animal Experiments: Animal experiments were performed under the National Institutes of Health Animal Use and Care guidelines and approved by the University of Texas Health Science Center at San Antonio Institutional Animal Care Committee. Male Sprague-Dawley rats (200-274 g) were anesthetized with 3% isoflurane (VedCo, St

Joseph, MO) in 100% oxygen gas. Rats were then placed in the supine position under a Picker (Cleveland, OH) large-field-of-view gamma camera using a low-energy all-purpose collimator and interfaced with a Pinnacle imaging computer (Medasys, Ann Arbor, MI). Image acquisition was begun as HbV or EV were infused into the tail vein at 1 mL/min. Each rat received a total dose of 0.17-0.37 mCi ^{99m}Tc-activity, Hb: 1.33 g/kg b.wt.; lipids: 0.67 g/kg b.wt. as an equivalent of 14 mL/kg for the HbV-group (n=5), and 0.48-0.55 mCi ^{99m}Tc-activity, lipids: 0.67 g/kg as 14 mL/kg for the EV-group (n=5). The infused dose (in volume) was estimated to be 25% of blood volume where the total blood volume was assumed to be 5.6% of body weight (Frank, 1976). The rabbit experiment was performed in the same manner. Male New Zealand White rabbits (2.2-2.9 kg) were anesthetized with an intramuscular injection of ketamine/xylazine (both from Phoenix Scientific, St. Joseph, MO) mixture (50 and 10 mg/kg body weight, respectively). One ear of a rabbit was catheterized with a venous line, and the other ear was catheterized with an arterial line. HbV or EV were infused in the venous line at 1 mL/min under the same gamma camera and the blood samples were drawn from the arterial line. Each rabbit received a total dose of 3.7-4.5 mCi ^{99m}Tc-activity, Hb: 1.36 g/kg b.wt.; lipids: 0.68 g/kg b.wt. as 14.25 mL/kg for the HbV-group (n=5), and 3.5-4.9 mCi, lipids: 0.68 g/kg as 14.25 mL/kg for the EV-group (n=4). The infused dose (in volume) was estimated to be 25% of blood volume where the total blood volume was assumed to be 5.7% of body weight (Kozma et al., 1974).

Image Analysis: One-minute dynamic 64x64 pixel scintigraphic images were acquired over a continuous period of 0.5 and 2 h for rats and rabbits after the infusion of HbV or EV, respectively. Static images were also acquired at 3, 6, 12, 24, 36, and 48 h post-infusion.

The image analysis was performed using a nuclear medicine analysis workstation (Pinnacle computer; Medasys, Ann Arbor, MI). The regions of interest were drawn over the whole body, liver, and spleen in images. The counts of radioactivity were decay-corrected at each time, and converted to a percentage of the whole body counts. Corrections were made for the blood pool contribution of the liver and spleen of the rat (17% and 6% respectively, of the total blood volume). For rabbit, the liver was corrected by 25.4% of the total blood volume, and the spleen was individually corrected by 1.047±0.076% for HbV, and 1.592±0.049% of the total blood volume for EV as %ID of just after infusion respectively.

Blood Persistence and Biodistribution: Blood was collected from the tail vein of the rat or arterial line of the rabbit (50 or 100 μl) at various times post-infusion. The radioactivity of blood samples was quantified in a scintillation well counter (Canberra Multichannel Analyzer, Meridan, CT) at the same time. The counts at each time were converted to the percentage of the counts of sample collected immediately after infusion. The elimination rate constants (k) were calculated by the least-squares method and half-life time ($t_{1/2}$) was calculated from equation (1).

$$t_{1/2} = \frac{0.693}{k} \qquad (1)$$

The animals were rapidly sacrificed at 48 h and the tissue samples were collected, weighed and counted for radioactivity in a scintillation well counter (Canberra Multichannel Analyzer, Meridan, CT) to calculate the biodistribution. To calculate the percentage of infused dose (%ID) per organ, total blood volume, muscle and skin mass were estimated as 5.6%, 40%, and 13% of total body weight for rat (Frank, 1976; Petty, 1982), and 5.7%, 45%, and 10% of total body weight for rabbit (Kozma et al., 1974;

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Kaplan et al., 1979), respectively. The bone was estimated as 10% of total body weight for rat (Frank, 1976; Petty, 1982) and 12 times the femur weight for rabbit (Dietz, 1944).

Estimation of the Biodistribution in Humans: The total Hb or lipids per organ (*Ws*) was calculated from the %ID and ID of Hb or lipids in terms of weight.

$$Ws(mg) = \frac{\%\mathrm{ID} \times \mathrm{ID}}{100} \qquad (2)$$

The organ weight (*Wo*) of experimental animals was measured by an electronic balance and the Hb per organ weight (*R*) was calculated.

$$R(mg/g) = \frac{Ws}{Wo} \qquad (3)$$

Ws was calculated from equation (3) for humans, where the weights of liver, spleen, and bone (*Wo*) were estimated as 1.8, 0.18, and 5.0 kg respectively for average humans (70 kg) (International Commission on Radiological Protection, 1984), and the *R* value was applied as an average value between rats and rabbits shown in Table 4 for each organ. The ID of HbV ([Hb]=9.5 g/dL, [lipids]=4.75 g/dL) was calculated to be 25% of the blood volume (4.9 L, 70mL/kg b.wt.), and the %ID was calculated from equation (2). The half-life times ($t_{1/2\beta}$) were estimated from equation (4), where, constant value (*C*) was determined as a slope of the fitting line in this study and $\%\mathrm{ID}_{\mathrm{total}}$ was sum values of %ID for liver, spleen, and bone.

$$t_{1/2\beta} = \frac{C}{\%\mathrm{ID}_{\mathrm{total}}} \qquad (4)$$

Statistical Methods: Values are reported as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed using Microsoft Excel for Windows. The image analysis and biodistribution data were compared using the Student's unpaired t-test. A *p*

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value <0.01 or 0.05 was considered statistically significant.

RESULTS

Labeling Efficiencies: The labeling efficiencies of ^{99m}Tc-HbV and ^{99m}Tc-EV were 69.1 ± 2.0% (n=2) and 75.6 ± 5.1% (n=3) for the rat studies, and 62.0 ± 4.8% (n=5) and 70.9 ± 2.1% (n=2) for the rabbit studies. Labeling efficiencies were similar for both ^{99m}Tc-HbV and ^{99m}Tc-EV even though ^{99m}Tc-HbV used homocysteine and ^{99m}Tc-EV used glutathione. The ^{99m}Tc would be located in the inner aqueous phase of vesicles, and both homocysteine and Hb of HbV, and glutathione of EV would possibly bind the ^{99m}Tc (Rudolph et al., 1991; Phillips et al., 1992). The incubation of labeled HbV and EV in serum for 48 h revealed that 5% and 4% of the ^{99m}Tc dissociated from HbV and EV, indicating that the labeling was very stable and the contents were stably encapsulated inside the vesicles.

Circulation Kinetics: To determine the circulation kinetics as shown in Fig. 1 (a) and (b), the radioactive counts of blood samples were plotted as a percentage of the counts for blood sample collected immediately at the end of the infusion with time. The elimination profiles of infused HbV showed two components with an initial fast clearance followed by a slower clearance phase which is regarded as a distribution (α) phase in the mononuclear phagocyte system (MPS) and an elimination (β) phase, respectively. The clearance rate constant in the distribution phase of HbV was equal to that of EV, and k_β was 1.3 times smaller than that of EV in rats as shown in Table 2. The circulation half-life times ($t_{1/2}$s) associated with both the distribution and elimination phases of HbV and EV in rats were 34.8 and 29.3 h, respectively. The clearance rates of HbV and EV were

slower in rabbits compared with those in rats, especially for the distribution phase. The k_α of HbV was 0.0226 h^{-1} in rabbit, which was a quarter of that in rats and 1.4 times larger than that of EV in rabbit. k_β for HbV was 1.3 times smaller than that of EV. The $t_{1/2}$s of HbV and EV were 62.6 and 57.3 h in rabbits, respectively.

Imaging Study: The gamma camera images of rats or rabbits receiving HbV were acquired at various times to determine the organ distribution profiles with time. As shown in Figs. 2 and 3, radioactivity was observed over the whole body of animals and in the heart, demonstrating that HbV were circulating. Immediately after infusion, the heart, liver, and spleen were identified, because these organs had a large blood pool volume and the relative intensities of the liver and spleen increased in comparison with the heart with time. The %ID in liver and spleen calculated from gamma camera images with decay correction and correction for blood pool contribution are shown in Figure 4. The %ID in liver was increased during the infusion and decreased after the infusion ended, especially in HbV as shown in Fig. 4 (a) and (c). This initial decrease was most likely due to the adjustment of blood volume after top-loading. The values of %ID in liver and spleen were quickly increased during the first 6 to 12 h after infusion and reached a plateau at 48 h. At 48 h, the liver had 10.9 ± 0.8% and 7.6 ± 1.0% of HbV in rats and rabbits respectively, while the spleen had 6.6 ± 0.3% and 0.98 ± 0.14% of HbV in rats and rabbits respectively.

Biodistribution: The detailed biodistribution data of HbV at 48 h are shown in Table 3. HbV could be precipitated easily by ultracentrifugation of blood sample and no Hb was detected in the supernatant serum in the blood sample for 48 h. In addition, no Hb was detected in urine for 48 h supporting that the Hb was not eluted from vesicles during

circulation. HbV and EV were mainly distributed in liver, bone marrow, and spleen, and the %ID values for HbV were smaller than those of EV in these organs. Relatively high values for the bowel, feces and urine were likely due to metabolism during excretion of HbV. The sum values of %ID for liver, spleen, and bone ($\%ID_{total}$), which are the main organs for MPS uptake, were 26.60% and 13.64% for HbV, and 36.36% and 17.84% for EV in rats and rabbit, respectively. The corresponding $t_{1/2\beta}$s given in Table 2 were 39.1, 79.2, 30.1, and 60.2 h respectively. These $t_{1/2\beta}$s are in proportion to the reciprocal of $\%ID_{total}$ as shown in Fig. 5 and the constant value (C) in equation (4) was determined to be 1074.1 as a slope of the fitting line.

The calculated total lipids and Hb doses (in mg) delivered to the liver, bone, and spleen are summarized in Table 4. These values are independent of the species-dependence of relative weight balances of organs in whole body and represent the amount of uptake of the HbV in a gram of each organ. The spleen had 14.43 ± 0.54 mg and 14.92 ±1.25 mg of Hb per gram in rat and rabbit, and the liver and bone also had similar values in rat and rabbit.

Discussion

The improvement in oxygen-carrying capacity of HbV as a RBC substitute requires longer circulation and a higher encapsulation efficiency of Hb. The HbV formulation described in this study has high encapsulation efficiency ([Hb]/[lipid]=2.0), and a circulatory half-life time of 34.8 h and 62.6 h in rats and rabbits, respectively. This value is equal to the 65 h circulation half-life time for a PEG-liposome encapsulated Hb formulation with a long circulation time in rabbits (Phillips et al., 1999). Other long-circulation vesicle formulations are successful for therapeutic uses such as cancer therapy or antibacterial treatment (Papahadjopoulos et al., 1991, Gabizon et al., 2003). However, the characteristics of small size (below 200 nm), neutral surface, and incorporation of significant amounts of PEG-lipid (5-10 mol%) of these formulations are ineffective in encapsulating Hb into vesicles (Perkins et al., 1993; Nicolas et al., 2000). The HbV formulation described in the present study is mainly composed of DPPC and cholesterol, only 0.3 mol% of PEG-lipid to prevent aggregation of the vesicles (Sakai et al., 2000b; Sou et al. 2000), and 9 mol% of anionic DPEA to reduce the lamellarity of the bilayer membrane (Sou et al. 2003). In general, anionic phospholipids such as phosphatidylglycerol (PG) or phosphatidylserine (PS) are used for the preparation of anionic vesicles; however, some side effects such as complement and platelet activations have been reported (Reinish et al., 1988). These immunological responses accelerate plasma protein adsorption on the surface of vesicles (opsonins) and then those vesicles are rapidly trapped into MPS. Our DPEA has a carboxylic group to negatively charge the surface of vesicles instead of a phosphate group of anionic phospholipids, and it does not have side effects like those reported for PG-containing vesicles (Wakamoto et al., 2001). The safety studies of HbV are underway and the initial results in rats suggest that the

DPEA vesicles have fewer side effects on immunological responses such as complement activation and thrombocytopenia compared with vesicles containing other anionic phospholipids. This bio-inactive surface imparted by DPEA contributes to the stable circulation of HbV.

The diameter of vesicles is also an important factor for circulation kinetics and encapsulation efficiency. Recently, Awasthi et al. (2003) reported that the maximum size to show long circulation characteristics of PEG vesicle was around 240 nm in rabbits. The larger size of HbV is advantageous for the encapsulation efficiency of Hb; however 250 nm HbV are of maximum and reasonable size to satisfy both long-circulation and high Hb content requirements. We satisfied both long-circulation and high encapsulation efficiency of Hb by developing the lipid formulation, and strictly regulating the diameter by the extrusion method. The clear effect of encapsulated Hb on the circulation time of vesicles was prolongation of the β phase for both animals. This is most likely due to greater saturation of the MPS by the encapsulated Hb.

Biodistribution data showed that HbV and EV were mainly distributed into liver, spleen, and bone. We have already clarified that Hb and phospholipid from HbV readily disappeared from the Kupffer cells in liver and macrophages in spleen in rats within a week after administration (Sakai et al., 2001). The trapping of HbV in MPS is regarded as a normal physiologic pathway for removal of aged RBC; therefore this should be a reasonable pathway for the elimination and metabolism of Hb-based RBC substitutes. The importance of the biodistribution of Hb-based RBC substitutes has been discussed and a vasoconstrictive effect of modified Hbs has been indicated (Sakai et al., 2000a).

These side effects are triggered by the unusual biodistribution of small sized modified Hb (< 100 nm) to smooth muscle across the endothelium, or the space of Disse in fenestrated endothelium of hepatic sinusoids, where the vasorelaxation factors NO and CO are bound to Hbs (Goda et al., 1998). The smaller vesicles might be effective for longer circulation of encapsulated Hb, but this would have the risk of causing similar or unusual side effects as those observed for modified Hbs.

As summarized in Table 3, the %ID of HbV and EV in biodistribution data at 48 h is significantly different between rats and rabbits (p<0.05 for many organs). The rat had more HbV and EV in liver, bone, and especially spleen, and less HbV and EV in blood. These data suggest that the biodistribution pattern of vesicles was not specifically changed by the encapsulation of Hb or the animal species tested; however the quantitative values of %ID were significantly affected by these factors. Image analysis showed that the %ID required for saturating the liver and spleen with time was as shown in Fig. 4. The former liposome encapsulated Hb, which had non-PEG, showed significantly greater %ID (liver: 15.4±2.1 %ID, spleen: 18.1±3.3 %ID) in rabbit (Rudolph et al; 1991). The “saturated” level observed at those infusion doses would be determined by the balance between rate of uptake from the circulation, which was strongly affected by the HbV formulation and the rate of metabolic processing. The full saturation of MPS by the increased infusion dose of HbV might diminish the difference of pharmacokinetics between HbV formulations because the metabolic processing should become dominant factor. At 48 h, the blood clearance was in the slower β phase (Fig 1), so that the inverse proportion between %ID and $t_{1/2\beta}$ is quite reasonable, and the determined constant C is available to estimate the $t_{1/2\beta}$ from the %ID. In addition, we have discovered that the most important factor for explaining the difference of %ID accumulating in the organs of the

MPS between species is due to the different ratio of organ weight to body weight between species. For example, the average spleen weights of the experimental animals for HbV were 0.65 ± 0.07g in rats (b.w. 216 ± 20 g, n=5), and 0.87 ± 0.21 g in rabbits (b.w. 2670 ± 97 g, n=5). Therefore, the ratio of organ weight to body weight of rats is 9 times larger than that of rabbits, which means that rats have a 9 times larger mass capacity in spleen at the same infusion dose based on body weight. When the uptake of HbV is calculated in terms of mg of lipid and Hb per gram of MPS organ, the values in rats and rabbits are very close to each other as summarized in Table 4 indicating that the concentration of HbV in these organs was species-independent in this case. These values can be used to quantitatively estimate biodistribution of HbV based on organ weight. By using these two factors of *C* values and mg of lipids per organ weight, we were able to roughly estimate the biodistribution and circulation time of HbV in humans (see Materials and Methods). Laverman et al. (1999) reported that the distribution pattern of PEG-liposomes in humans was similar to that of rats and rabbits, with high uptake in liver, spleen, and bone marrow. Other biodistribution studies of vesicles also suggested a high uptake in liver, spleen, and bone marrow in humans (Dams et al., 2000, Gabizon et al., 2003) and these reports support our estimation. Based on the MPS organ weights of average humans and the mg uptake of lipid and hemoglobin per gram MPS organs at 48 h (human liver weight 1.8 kg, human spleen 0.18 kg, and human bone 5.0 kg) (International Commission on Radiological Protection, 1984), we estimated that %IDs of HbV are 5.4% (liver), 4.5% (spleen), and 6.4% (bone), and a $t_{1/2\beta}$ of approximately 66 h in humans after a 25% top-loading ([Hb]: 9.5 g/dL, [lipid]: 4.75 g/dL). The normal range of human organ weight is relatively wide such as 1.4-1.8 kg (liver) and 0.08-0.3 kg (spleen), so the $t_{1/2\beta}$ would be varied around 3 days. This $t_{1/2\beta}$ is approximately 2 times larger than that of rat, and this

ratio almost follows that derived from empirical speculation (Gabizon et al., 2003). This method of estimating vesicle circulation kinetics and organ uptake in different animal species may be useful for all types of vesicle (liposome) formulations that are currently under development as drug delivery vehicles. More studies will be required to further validate this method of estimating circulation kinetics and organ uptake in different animal species.

The development of RBC substitutes is progressing, and some modified Hbs have been studied in clinical trials. The reported $t_{1/2}$ was 23 h for polymerized bovine Hb (Hughes et al., 1995), 16-20 h for o-raffinose-cross-linked and polymerized human Hb (Carmichael et al., 2000), and 24 h for glutaraldehyde-cross-linked and polymerized human Hb (Gould et al., 1998). Even though HbV have not yet been tested clinically, we have demonstrated in the present report that HbV have significantly improved properties, based on their circulation kinetics and biodistribution, suggesting their improved safety and efficacy as a RBC substitute. In addition, the successful application of vesicles as RBC substitutes at this large infusion dose suggests a promising future for vesicles (liposomes), and the present formulation would potentially be available not only as a RBC substitute, but also for various applications such as drug delivery systems.

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Footnotes

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Figure Legends

Figure 1: Circulation kinetics of HbV and EV after top-loading intravenous infusion (14 mL/kg) in rats and rabbits. The radioactivity was determined by scintillation counting of blood samples with time. The percentage of radioactivity is calculated as a percentage of baseline radioactivity in a blood sample withdrawn just after HbV or EV infusion.

Figure 2: Static gamma camera images of whole body of rats infused with HbV or EV acquired at 3 h, and 24 h after infusion. The images were acquired for 1 min at 3 h, and 2 min at 24 h. The arrows show heart (H), liver (L), and spleen (S).

Figure 3: Static gamma camera images of rabbits acquired at 2 h, 24 h, and 48 h after HbV or EV infusion. The images were acquired for 1 min at 2 h, 2 min at 24 h, and 5 min at 48 h. The arrows indicate heart (H), anterior liver (AL), posterior liver (PL), spleen (S), and kidney (K).

Figure 4: The percentage of infused dose (%ID) for liver and spleen calculated from the gamma camera image acquired at particular times and after decay correction. The blood pool contribution was corrected using values of 17 and 6 % of the total blood volume for liver and spleen in rats respectively. For rabbit, the liver was corrected by 25.4% of the total blood volume, and the spleen was individually corrected by 1.047±0.076% for HbV, and 1.592±0.049% of the total blood volume for EV as %ID of just after infusion respectively.

Figure 5: The proportional relationship between the circulation half-life time ($t_{1/2\beta}$) and

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the reciprocal of $\%ID_{total}$ in the elimination phase. The $\%ID_{total}$ was calculated as a sum value of %ID in liver, bone, and spleen at 48 h. The fitting line was determined by the regression analysis (Coefficient of determination; R^2=0.9985).

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TABLE 1 Characteristics of ^{99m}Tc-HbV and ^{99m}Tc-EV suspensions.

Parameters	^{99m}Tc-HbV	^{99m}Tc-EV
[Hb][a)] (g/dL)	9.5	0
[lipids] (g/dL)	4.75	4.75
particle diameter (nm)	247±44	259±32
endotoxin level (EU/mL)	< 0.2	< 0.2

a) MetHb: < 1%, carbonylHb < 2%

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TABLE 2 Kinetic parameters of HbV and EV clearance from blood in rats and rabbits (25% top-loading).

Animal	sample	distribution (α) phase		elimination (β) phase		$t_{1/2}$ (h)
		k_α (h^{-1})	$t_{1/2\alpha}$ (h)	k_β (h^{-1})	$t_{1/2\beta}$ (h)	
Rat	HbV	0.0894	7.8	0.0177	39.1	34.8
	EV	0.1004	6.9	0.0230	30.1	29.3
Rabbit	HbV	0.0226	30.7	0.0088	79.2	62.6
	EV	0.0159	43.6	0.0115	60.2	57.3

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TABLE 3 Biodistribution of HbV and EV as a percent of the infused dose per organ (%ID/organ) and percent of the infused dose per gram of organ (%ID/ g organ) at 48 h after 25% top-loading in rats or rabbits.

Organ	Rat		Rabbit	
	HbV	EV	HbV	EV
	%ID/organ ± s.e.m.			
Blood	33.27 ± 1.11*	24.13 ± 0.65	50.95 ± 2.02†	52.76 ± 4.80‡
Liver	10.04 ± 0.86*	14.13 ± 0.40	7.55 ± 0.46†	8.64 ± 0.34‡
Bone	10.06 ± 0.21*	13.05 ± 0.38	5.37 ± 0.33*†	7.36 ± 0.28‡
Spleen	6.50 ± 0.30*	9.18 ± 0.37	0.72 ± 0.10*†	1.84 ± 0.28‡
Bowels	7.30 ± 1.59	4.16 ± 0.35	9.61 ± 2.31	8.62 ± 4.42
Skin	2.37 ± 0.33	2.29 ± 0.12	0.88 ± 0.05†	1.09 ± 0.21‡
Kidney	2.40 ± 0.10*	3.35 ± 0.08	1.47 ± 0.13†	1.69 ± 0.21‡
Muscle	1.94 ± 0.28	1.98 ± 0.27	2.51 ± 0.31	2.62 ± 0.76
Lung	0.62 ± 0.03	0.54 ± 0.03	0.55 ± 0.02	0.43 ± 0.06
Heart	0.17 ± 0.01	0.16 ± 0.01	0.12 ± 0.01†	0.13 ± 0.02
Brain	0.16 ± 0.01*	0.09 ± 0.01	0.08 ± 0.01*†	0.05 ± 0.00‡
Testis	0.12 ± 0.01*	0.09 ± 0.01	0.06 ± 0.02†	0.07 ± 0.01
Feces	9.50 ± 1.17	6.95 ± 0.29	5.06 ± 2.56	2.02 ± 0.55‡
Urine	13.61 ± 0.31	12.87 ± 0.41	11.30 ± 1.22	7.81 ± 1.44‡
	%ID/ g organ ± s.e.m.			
Blood	2.919 ± 0.032	1.706 ± 0.044	0.356 ± 0.017	0.354 ± 0.030
Liver	1.244 ± 0.096	1.378 ± 0.045	0.093 ± 0.004	0.131 ± 0.019
Bone	0.497 ± 0.021	0.518 ± 0.020	0.043 ± 0.003	0.062 ± 0.002
Spleen	10.059 ± 0.072	10.790 ± 0.402	0.823 ± 0.072	1.483 ± 0.072
Bowels	0.390 ± 0.073	0.202 ± 0.008	0.031 ± 0.006	0.029 ± 0.014
Skin	0.091 ± 0.014	0.070 ± 0.004	0.004 ± 0.000	0.004 ± 0.001
Kidney	1.604 ± 0.057	1.839 ± 0.055	0.089 ± 0.005	0.110 ± 0.017
Muscle	0.024 ± 0.003	0.020 ± 0.003	0.002 ± 0.000	0.002 ± 0.001
Lung	0.619 ± 0.022	0.458 ± 0.014	0.068 ± 0.004	0.057 ± 0.012
Heart	0.264 ± 0.009	0.187 ± 0.012	0.026 ± 0.002	0.027 ± 0.006
Brain	0.111 ± 0.010	0.062 ± 0.003	0.011 ± 0.001	0.006 ± 0.001
Testis	0.042 ± 0.002	0.027 ± 0.001	0.013 ± 0.002	0.016 ± 0.003

* Difference is statistically significant from EV in same species at $p<0.01$

† Difference is statistically significant from HbV in rat at $p<0.05$

‡ Difference is statistically significant from EV in rat at $p<0.05$

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TABLE 4 Comparison of HbV and EV as mg lipids/g organ and mg Hb/g organ at 48 h after 25% top-loading in rats or rabbits.

Organ	HbV in rat		HbV in rabbit		EV in rat	EV in rabbit
	mg lipids/g organ[a)]	mg Hb/g organ[b)]	mg lipids/g organ[a)]	mg Hb/g organ[b)]	mg lipids/g organ[a)]	mg lipids/g organ[a)]
Blood	4.23 ± 0.20	8.40 ± 0.40	6.47 ± 0.24[†]	12.93 ± 0.48[†]	2.94 ± 0.06	6.55 ± 0.64[†]
Liver	1.79 ± 0.12	3.56 ± 0.23	1.68 ± 0.06	3.36 ± 0.12	2.38 ± 0.06	2.24 ± 0.18
Bone	0.72 ± 0.01	1.42 ± 0.02	0.78 ± 0.05	1.57 ± 0.09	0.89 ± 0.04	1.09 ± 0.08
Spleen	14.43 ± 0.54	28.63 ± 1.06	14.92 ± 1.25	29.85 ± 2.50	18.58 ± 0.51	25.83 ± 1.43[†]

[†] Difference is statistically significant from HbV in rat at $p<0.05$

a) calculated values from ID of lipids and % ID /g organ

b) calculated values from ID of Hb and % ID /g organ

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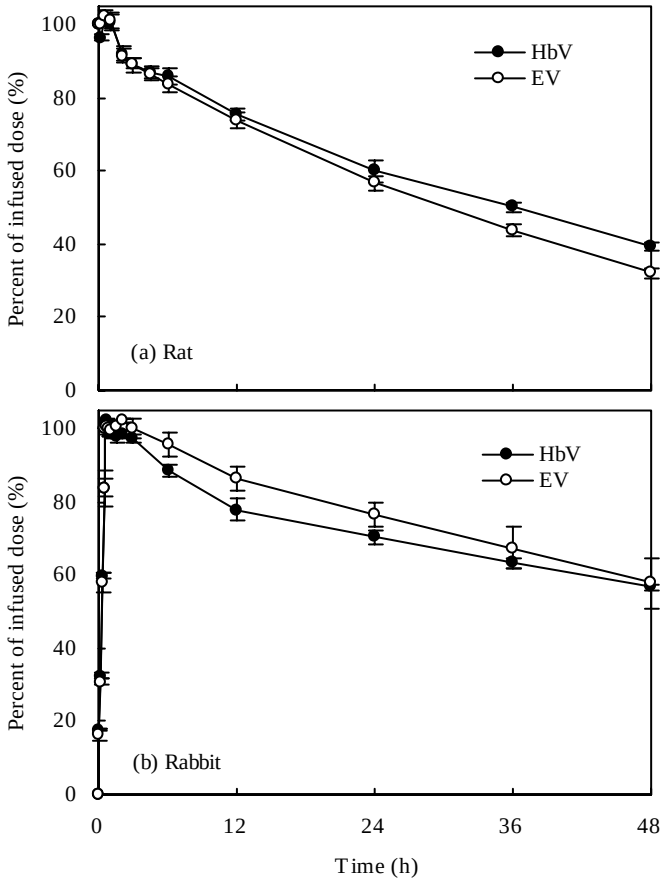

Figure 1

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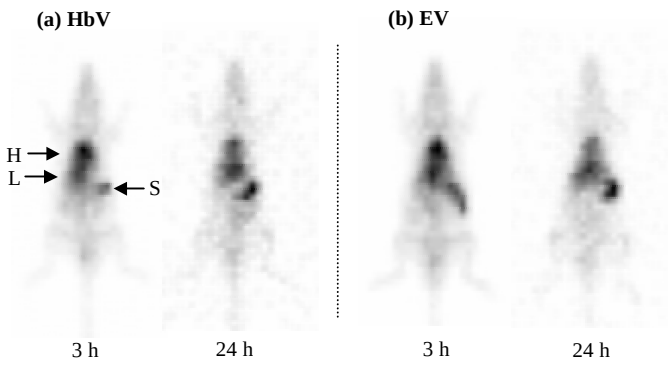

Figure 2

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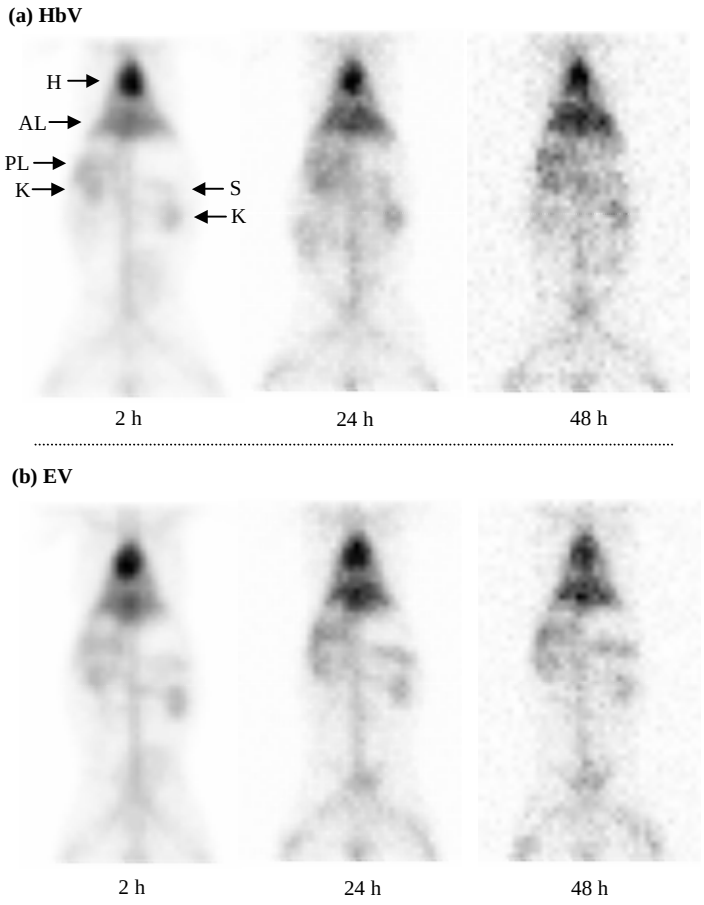

Figure 3

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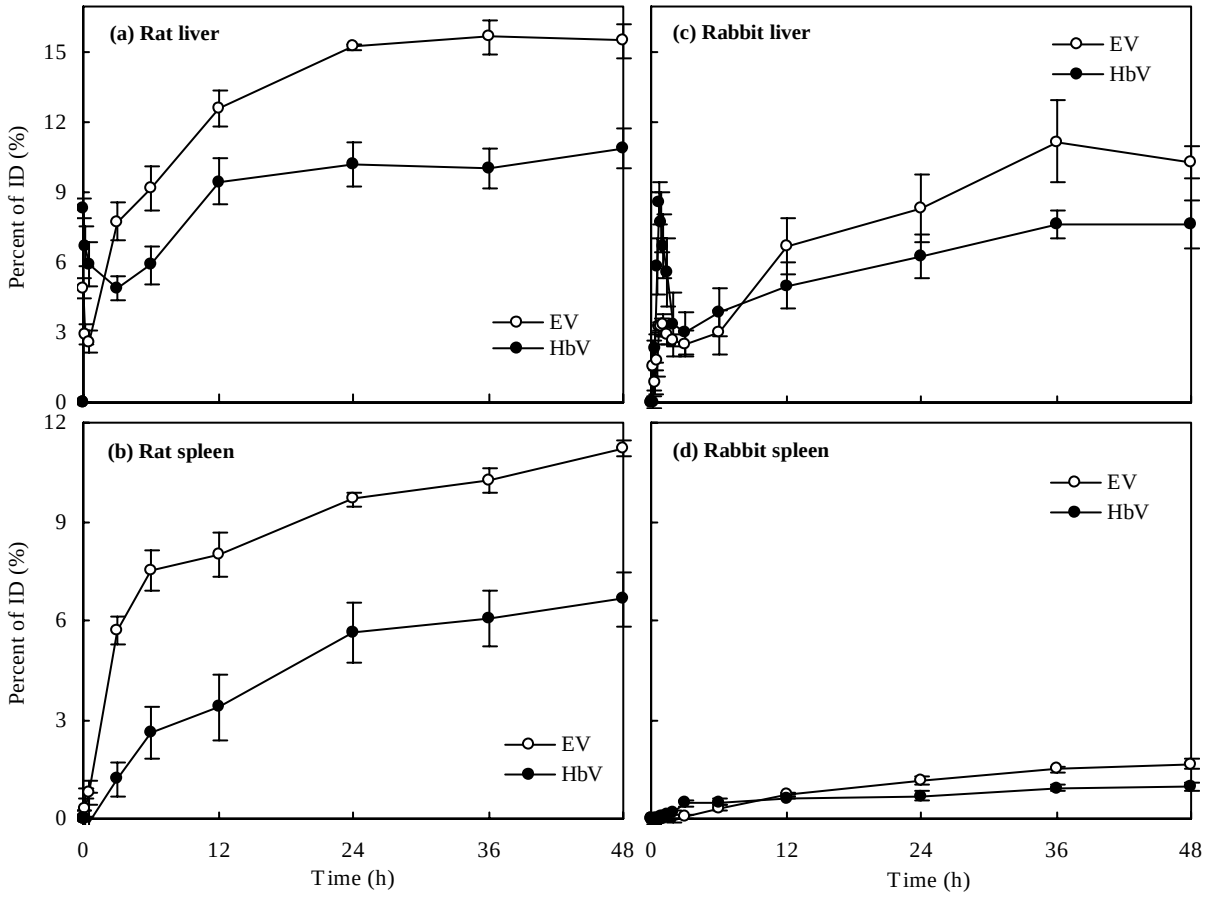

Figure 4

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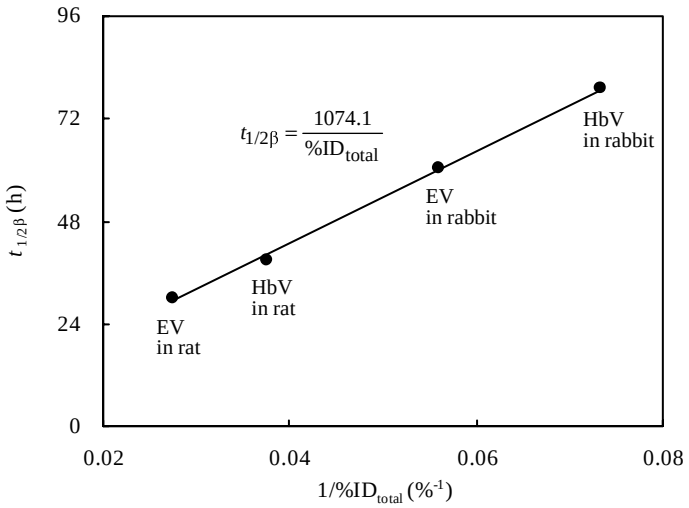

Figure 5