3. Results

3.1. Surface modification and radiolabeling

The average diameter of vesicles was controlled to 270 nm by the stepwise extrusion through cellulose acetate membrane filters with a final pore size of 0.22 μm as shown in Table 1. The surface of the vesicles were modified during spontaneous incorporation of PEG conjugated to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) into the lipid bilayer of preformed vesicles. The incorporation efficiency of PEG-DSPE was approximately 85%, independent of the added amount. Theoretically, the surface of PEG (0.3)-[SA-Ve] is not fully covered with PEG chains in mushroom conformation (theoretically calculated covered surface area: 85%), and surface coverage is completed with >0.6 mol% of PEG-DSPE. The ^{99m}Tc-labeling efficiency was approximately 84%, independent of the vesicular formulation. Since the ^{99m}Tc was located in the inner aqueous phase of vesicles encapsulating glutathione, the surface properties would not have been altered by the labeling procedure. The incubation of labeled ^{99m}Tc-vesicles in rabbit serum for 48 h revealed that more than 95% of the incorporated ^{99m}Tc remained in the prepared vesicles, regardless of the composition of the vesicles. Also in human plasma, 98% of incorporated ^{99m}Tc remained with PEG(0.6)-[SA-Ve] at 24 h. These data indicate that the labeling procedure results in a stably labeled vesicle preparation and maintains the ^{99m}Tc within vesicles, even during incubation in plasma at 37 °C.

3.2. Circulation kinetics and biodistribution

First, the circulation kinetics and organ distribution of several formulations were compared to determine the optimized component for targeting bone marrow. For this purpose, scintigraphy was superior to other methods because it was possible to quantitatively determine the organ distribution of the injected vesicles in whole body. The elimination rate of SA-Ve from circulating blood was much faster compared with that of control vesicles (Ve): the circulating half-life times ($t_{1/2}$s) of the SA-Ve and Ve were 0.6 and 9.4 h at injection dose of 15 mg/kg b.w. (Fig. 1(A)). Incorporation of as little as 0.3 mol% of PEG-DSPE did not affect the circulation time of SA-Ve. Incorporation of above 0.6 mol% of PEG-DSPE prolonged the circulation time of SA-Ve and the $t_{1/2}$ increased with increasing amounts of PEG-DSPE incorporation as summarized in Table 1. The incorporation of 2.6 mol% of PEG-DSPE also gave a remarkable improvement in circulation time for control Ve ($t_{1/2}$: 24.8 h). At 24 h post injection, the radioactivity of excised organs was counted using a scintillation counter. Major organs exhibiting the uptake of vesicles were bone marrow and liver for SA-Ve (Figs. 1(B) and (C)), while liver and spleen were the organs with the highest accumulation of control Ve (Figs. 1(C) and (D)). PEG modification clearly inhibited hepatic uptake of both SA-Ve and control Ve, and this effect became significant as the amount of PEG-DSPE incorporated increased (Fig. 1(C)). While a maximum amount of SA-Ve was observed in bone marrow when the SA-Ve contained 0.6 mol% PEG-DSPE, further incorporation of PEG-DSPE led to a decrease in the distribution of SA-Ve in bone marrow (Fig. 1(B)). Other organs apart from kidney and muscle for PEG(2.6)-[SA-Ve] exhibited only a small amount of activity (<1%ID, Supplementary Table 1 online). Injection in rabbits of a mixed solution of ^{99m}Tc-HMPAO and glutathione in a similar ratio as would be found within ^{99m}Tc-vesicles served as a control study of the radiolabeling agents without encapsulation within the vesicles. As shown in Fig. 2(A), injection of ^{99m}Tc-HMPAO/glutathione was rapidly eliminated from blood circulation ($t_{1/2}$: 3 min), and gamma camera images indicated that the labeling agents were rapidly excreted in urine through the kidney (Fig. 2(B)). Region of interest analysis showed that 67.1±0.8% of injected radioactivity was detected in bladder within 1 h after injection (Fig. 2(C)). At 6 h, biodistribution data also showed significant radioactivity in the urine (76.91±4.80%ID) and kidney (6.11±0.53%ID), but other organs including bone marrow had only minimal %ID dose uptake as summarized in Table 2. This control study shows that a mixture of ^{99m}Tc-HMPAO and glutathione is rapidly removed from the blood by renal excretion, which is

Table 1
Specification of prepared vesicles

Sample[a]	Mean diameter±SD (nm)	PEG-DSPE (mol%)	$t_{1/2}$ (h)[b]
SA-Ve	269±11	0	0.6
PEG(0.3)-[SA-Ve]	276±13	0.3	0.6
PEG(0.6)-[SA-Ve]	273±12	0.6	1.0
PEG(1.4)-[SA-Ve]	275±12	1.4	3.9
PEG(2.6)-[SA-Ve]	274±12	2.6	5.4
Ve	262±43	0	9.4
PEG(2.6)-Ve	259±74	2.6	24.8

[a]SA-Ve is based on DPPC/CH/SA (molar ratio, 1:1:0.2), and Ve is DPPC/CH (molar ratio, 1:1) as a control sample. PEG-modified samples were prepared using the spontaneous incorporation of PEG-DSPE into the prepared SA-Ve or Ve.
[b]The $t_{1/2}$ values were calculated from Fig. 1(A) data.

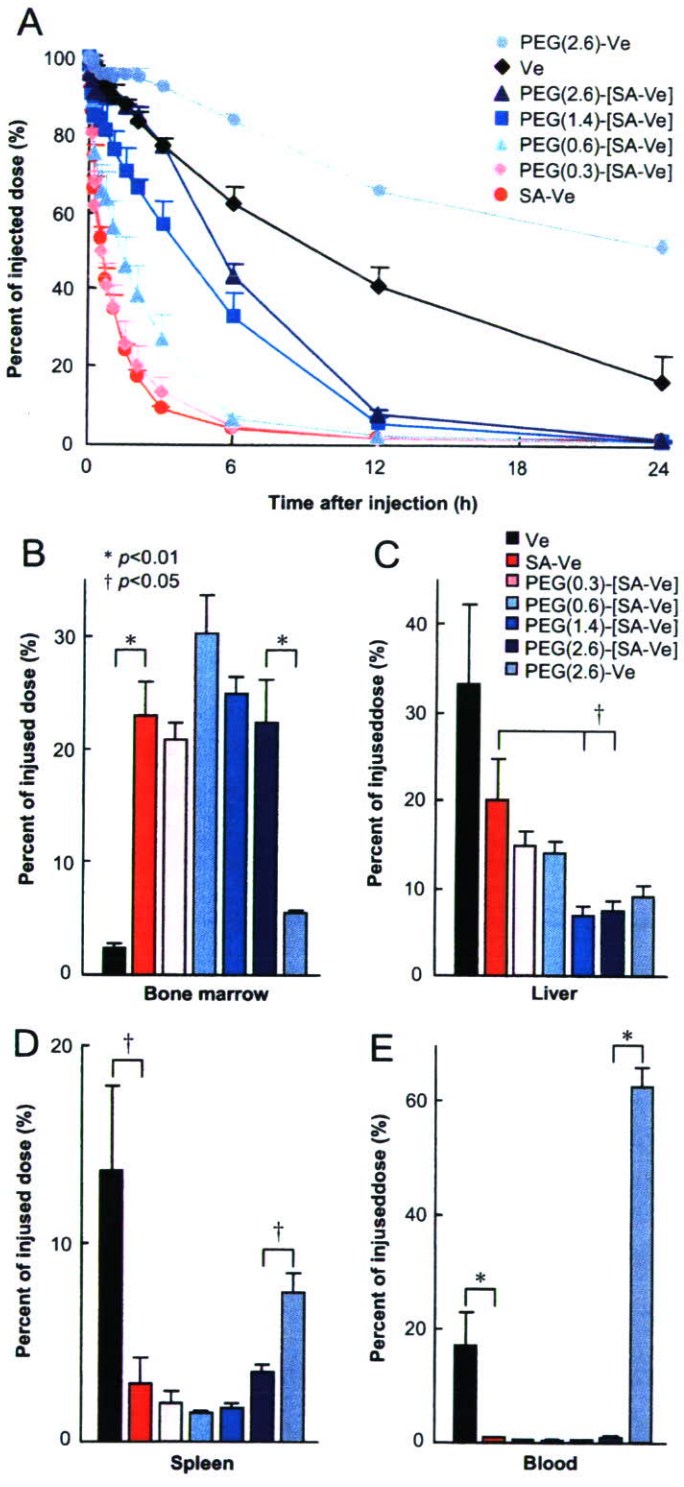

Fig. 1. Effect of surface modification with SA and PEG-DSPE on circulation kinetics and organ distribution of phospholipid vesicles. (A) Circulation kinetics of SA-vesicles (SA-Ve) and control vesicles (Ve) containing various amounts of PEG-DSPE after i.v. infusion (lipids: 15 mg/kg b.w.) in rabbits. ^{99m}Tc radioactivity was quantitated by scintillation counting of blood samples with time. The percentage of injected dose was calculated as a percentage of baseline radioactivity in a blood sample withdrawn just after injection. (B)–(E) Distribution of SA-vesicles (SA-Ve) and control vesicles (Ve) containing various amounts of PEG-DSPE as a percentage of the injected dose in bone marrow (B), liver (C), spleen (D), and blood (E) at 24 h after i.v. infusion in rabbits. *, Statistical significance ($p<0.01$), †, statistical significance ($p<0.05$).

typical of small molecules. These results indicate that the SA-Ve were clearly directed to bone marrow, and the process of accumulation of SA-Ve into bone marrow is correlated with competitive trapping by liver. Surface modification of SA-Ve with the proper amount of PEG-lipids inhibits the trapping of SA-Ve in liver and directs SA-Ve to bone marrow, a process which could be regarded as a combination of active and passive targeting. Conventional anionic vesicles containing phosphatidyl glycerol (PG) were inactive for targeting of bone marrow (Supplementary Table 2 online). The injected PEG(0.6)-[SA-Ve], which was the formulation showing the highest persistence in bone marrow at 24 h, were almost removed from circulation within 6 h (as little as 6.4±0.5%ID of PEG(0.6)-[SA-Ve] was circulating in blood at 6 h). Therefore, the initial distribution kinetics of PEG(0.6)-[SA-Ve] was studied in detail.

3.3. Distribution kinetics of PEG(0.6)-[SA-Ve]

Scintigraphic images clearly showed the injected radioactivity of PEG(0.6)-[SA-Ve] to be redirected from heart and liver, both organs having large blood pool contributions, and increasingly deposited in the bone marrow over time (Fig. 3(A)). The distribution kinetics in bone marrow, liver, and spleen, analyzed from the scintigraphic images, quantitatively indicated that significantly higher doses had accumulated in bone marrow, reaching 68.5±3.3%ID by 6 h after injection (Fig. 3(B)). The biodistribution data calculated from the radioactivity of excised organs also showed that 69.74±0.3%ID of PEG(0.6)-[SA-Ve] had accumulated in bone marrow, as shown in Table 2. At the same time point, liver and spleen had much smaller amounts of 11.51±2.88 and 5.00±1.19%ID, respectively. When ^{99m}Tc-HMPAO/glutathione was injected without encapsulation into PEG(0.6)-[SA-Ve], bone marrow, liver, and spleen had only 1.13±0.24, 1.52±0.14, and 0.01±0.00%ID, respectively. The isolated femur was further separated into soft bone marrow, joint bone (sponge bone), and skeleton and each separate tissue counted for radioactivity. As shown in Fig. 3(C), 66.5±1.1% of radioactivity in one femur was detected in soft bone marrow. The joint bone including soft bone marrow had 28.8±1.3% of radioactivity, and less radioactivity was detected in the separated skeleton (4.7±0.3%). These results indicate that the intravenously injected PEG(0.6)-[SA-Ve] mostly accumulates into soft bone marrow. The gamma camera images clearly show that the bone marrow uptake was evenly distributed over whole bone (Fig. 4), and the localization of radioactivity representing the distribution of PEG(0.6)-[SA-Ve] in these images was analyzed for separate regions. The spine and pelvis had 21.23±0.42% and 18.09±0.60%, values which were much higher than other regions. The right and left femurs had equal radioactivity of 7.97±0.05% and 8.34±0.18%; these values are in agreement with a report

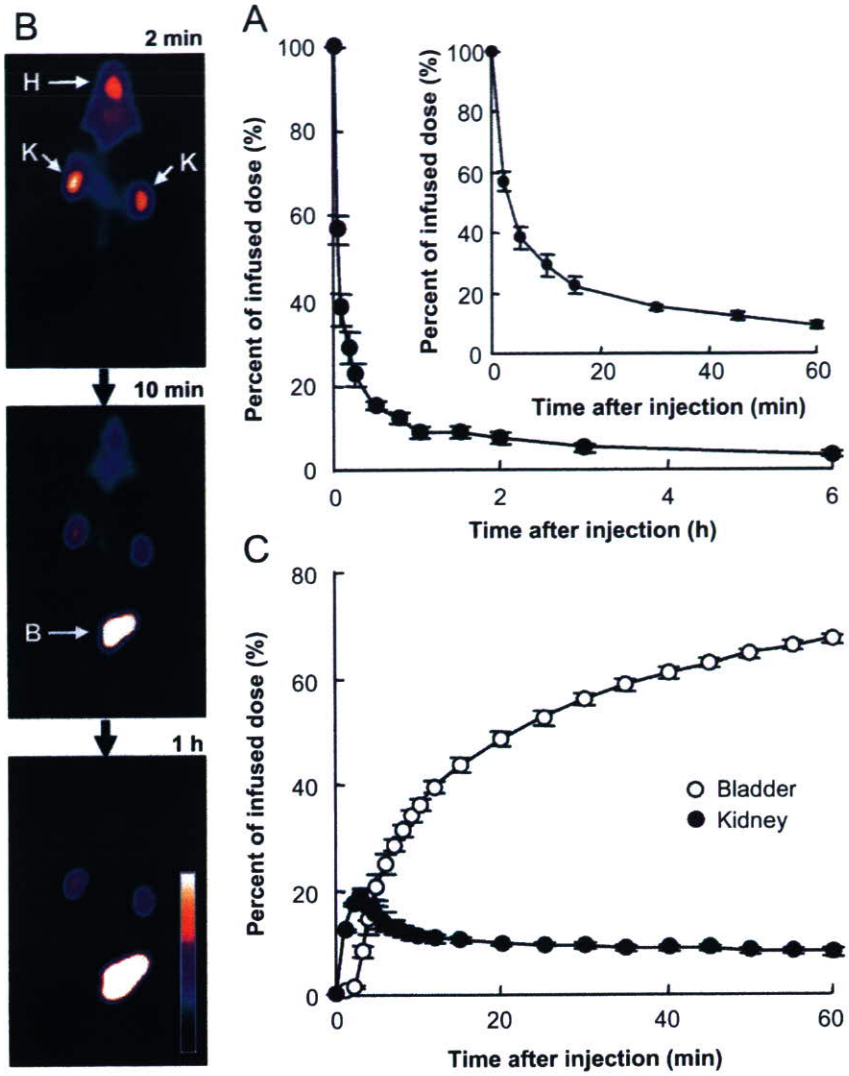

Fig. 2. Circulation and distribution kinetics of mixture of ^{99m}Tc-HMPAO and glutathione without encapsulation in vesicles after i.v. infusion in rabbits. (A) Circulation kinetics (B) Gamma camera images of rabbits acquired at various times after infusion. H: heart, K: kidney, B: bladder. (C) Distribution profiles as a percentage of the injected dose analyzed from the gamma camera images.

describing the relationship of 12 times that of a femur as being equivalent to whole bone in rabbits [28].

3.4. Microscopic localization of PEG(0.6)-[SA-Ve] in bone marrow

The initial studies were designed to demonstrate that PEG(0.6)-[SA-Ve] functions as a nanoparticulate carrier as well as identify their microscopic localization in tissues. We used PEG(0.6)-[SA-Ve] double-labeled by encapsulating water-soluble TR-SOD in an aqueous phase and embedding lipid-soluble C_1-BODIPY C_{12} in bilayer membrane (Fig. 5(A)). As shown in Fig. 5(B), the bone marrow sections have fluorescence from both the TR-SOD and C_1-BODIPY C_{12}. The fluorescence was locally concentrated, and larger fluorescent domain was 30 μm in size along the long axis. Fluorescent distribution in red pulp of spleen was dense, whereas it was sparse in liver. An important finding from this observation is that the fluorescence from membrane probes and encapsulated probes are co-localized in bone marrow. These images clearly indicate that PEG(0.6)-[SA-Ve] functions as a nanoparticle-carrier to deliver the encapsulated agents to bone marrow tissues. A second study was performed to identify the histological location of PEG(0.6)-[SA-Ve] in bone marrow. Femoral bone marrow tissue was taken from rabbit at 6 h after i.v. injection of PEG(0.6)-[SA-Ve] and examined using TEM. TEM observation clearly demonstrated the location of PEG(0.6)-[SA-Ve] in bone marrow (Figs. 6(A) and (B)). A massive number of vesicles were trapped in endosomes and lysosomes of BMMϕ, but no vesicles were observed in cytoplasm and cell nucleus (Fig. 6(B)). The diameter of these vesicles averaged 270 nm which was the original diameter of the intravenously administered PEG(0.6)-[SA-Ve]. Several similar BMMϕ with vesicles in endosomes and lysosomes were observed, while no vesicles were observed

Table 2
Biodistribution of PEG(0.6)-[SA-Ve] and ^{99m}Tc-HMPAO/glutathione as a percent of the injected dose (%ID) and %ID per gram of tissue at 6 h after i.v. infusion in rabbits

Organs	PEG(0.6)-[SA-Ve]		^{99m}Tc-HMPAO/glutathione	
	%ID ± SEM (%)	%ID/g tissue ± SEM (%/g)	%ID ± SEM (%)	%ID/g tissue ± SEM (%/g)
Blood	6.58 ± 2.91	0.065 ± 0.028	3.34 ± 1.68	0.025 ± 0.013
Bone marrow	69.74 ± 0.86	0.806 ± 0.048	1.13 ± 0.24	0.010 ± 0.001
Liver	11.51 ± 2.88	0.237 ± 0.067	1.52 ± 0.14	0.022 ± 0.001
Spleen	5.00 ± 1.19	5.387 ± 0.807	0.01 ± 0.00	0.011 ± 0.001
Bowel	5.85 ± 0.31	0.014 ± 0.000	4.41 ± 0.19	0.009 ± 0.000
Skin	1.57 ± 0.21	0.009 ± 0.001	2.34 ± 0.30	0.010 ± 0.001
Kidney	2.40 ± 0.10	0.148 ± 0.011	6.11 ± 0.53	0.440 ± 0.066
Muscle	1.86 ± 0.17	0.003 ± 0.000	2.60 ± 0.63	0.002 ± 0.001
Lung	0.19 ± 0.03	0.024 ± 0.006	0.12 ± 0.03	0.010 ± 0.001
Heart	0.03 ± 0.01	0.010 ± 0.002	0.03 ± 0.01	0.006 ± 0.001
Brain	0.01 ± 0.00	0.002 ± 0.000	0.01 ± 0.00	0.001 ± 0.000
Testis	0.03 ± 0.01	0.024 ± 0.005	0.02 ± 0.00	0.008 ± 0.002
Urine	3.57 ± 1.74	—	76.91 ± 4.80	—

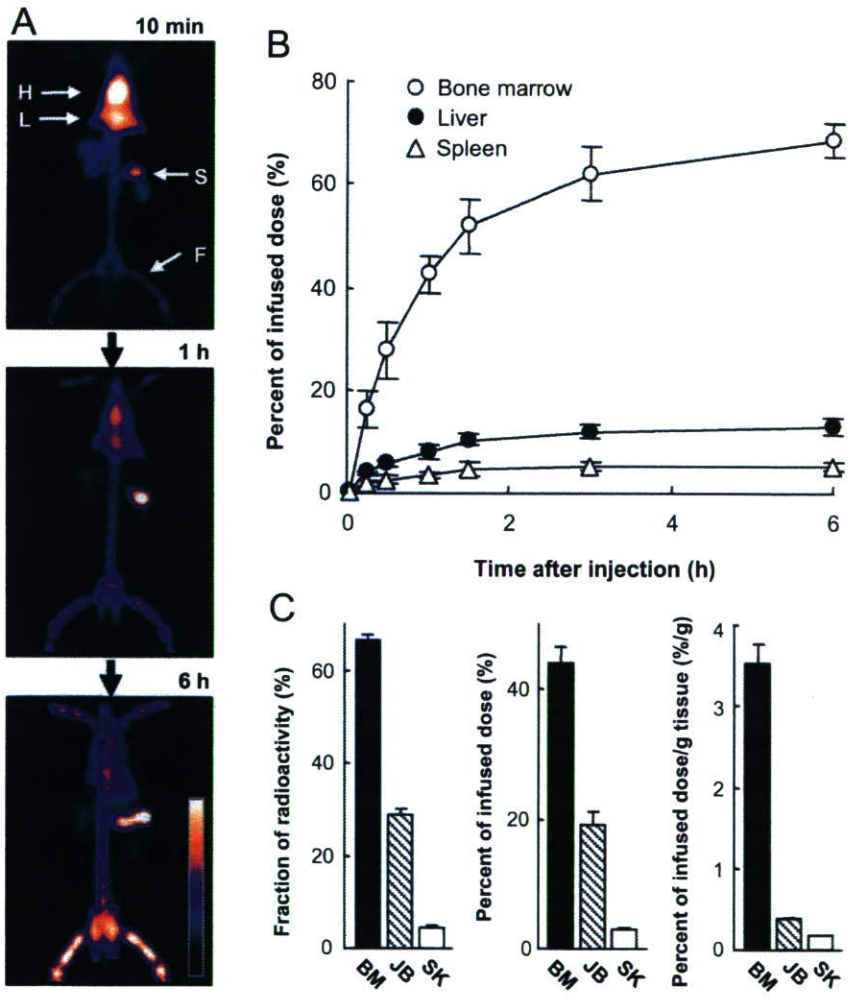

Fig. 3. Initial distribution kinetics of PEG(0.6)-[SA-Ve] after i.v. infusion (lipids: 15 mg/kg b.w.) in rabbits. (A) Gamma camera images of rabbits acquired at various times after infusion. H: heart, L: liver, S: spleen, F: femur. (B) Distribution profiles as a percentage of the injected dose analyzed from the gamma camera images. The total bone marrow was estimated to be 12 times that of one femur. (C) Distribution of radioactivity of PEG(0.6)-[SA-Ve] in separated soft bone marrow (BM), joint bone (sponge bone) (JB), and skeleton (SK) of one femur collected at 6 h after i.v. infusion. Three panels show the fraction of radioactivity, percent of injected dose (%ID), and %ID/g tissue, respectively.

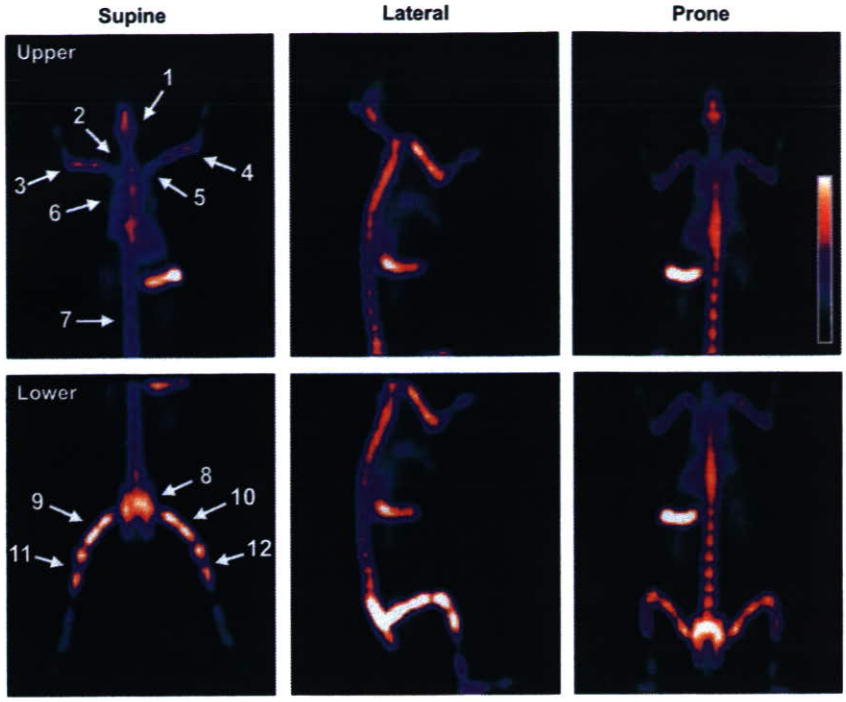

Fig. 4. Gamma camera images of rabbit receiving PEG(0.6)-[SA-Ve], acquired from various angles at 6 h after i.v. infusion. Bone marrow is clearly displayed in these images throughout the rabbit body. Relative radioactivity in separated bone parts were calculated to 1; head (8.41 ± 1.58%), 2; neck (1.10 ± 0.11%), 3; right arm (5.72 ± 0.33%), 4; left arm (5.54 ± 0.40%), 5; shoulder (3.62 ± 0.69%), 6; sternum (4.11 ± 1.35%), 7; spine (21.23 ± 0.42%), 8; pelvis (18.09 ± 0.60%), 9; right femur (7.97 ± 0.05%), 10; left femur (8.34 ± 0.18%), 11; distal right foot (7.88 ± 0.25%), and 12; distal left foot (7.98 ± 0.33%) as percentages to radioactivity of whole bone ± SEM.

in other types of cell such as granular leukocytes, erythroblasts, and endothelial cells in observed section. These microscopic localization studies demonstrate that BMMϕ are the cellular components responsible for clearance of vesicles from the circulation and their uptake by the bone marrow.

4. Discussion

These studies demonstrate that PEG-[SA-Ve] are efficient carriers for targeting the BMMϕ. These vesicles should be useful in the development of bone marrow targeted agents for therapeutic applications. Additionally, this *in vivo* model appears to be an ideal model with which to investigate the role of BMMϕ in the hematopoietic environment. The radiolabeling method for the vesicles encapsulating glutathione with ^{99m}Tc-HMPAO has previously been established for imaging studies [14,23,24]. In the present vesicle formulation, we confirmed the stability of the ^{99m}Tc radiolabeled-vesicles during incubation in serum and plasma at 37 °C for 48 h (more than 95% remaining with vesicles), and we also determined that the free labeling agent is not specifically distributed into organs such as bone marrow, liver, and spleen, but rapidly eliminated through renal excretion as shown in Fig. 2 and Table 2. This evidence provides strong support that the radioisotope distribution reflects the true biodistribution of vesicles. As shown in Fig. 1, comparative data showing the organ distribution of several formulations clearly demonstrated that the uptake of vesicles by bone marrow is induced by the incorporation of SA ($p < 0.01$); furthermore, the incorporation of a small amount of PEG-DSPE on the surface of SA-Ve prolongs its circulation time and tends to enhance the bone marrow selectivity by preventing hepatic uptake. Thus, maximum distribution to bone marrow was observed at 0.6 mol% PEG-DSPE (Fig. 1(B)). The degree of hepatic uptake was reduced as the PEG-DSPE content increased, and this effect became significant above 1.4 mol% ($p < 0.05$). Bone marrow uptake was also reduced above 1.4 mol%. In general, 5–10 mol% of PEG-lipids is incorporated into most of the long circulating vesicle formulations for passive targeting [8,9]. In the present study, prolonged circulation time of vesicles was observed above 0.6 mol% of PEG-DSPE, and the circulation times were prolonged more in vesicles with higher PEG-DSPE content. For the effective targeting of bone marrow, however, higher concentrations of PEG blocked the active targeting of the vesicles to bone marrow. These results indicate that the dense PEG layer on the vesicular surface covers the surface properties having the character of SA and depress uptake by BMMϕ. Therefore, the optimal amount of PEG incorporation was found to be 0.6 mol%, as this concentration passively enhances active targeting. Theoretically, approximately 0.4 mol% of PEG (Mw 5000)-lipids is estimated to be the critical content required to fully cover the vesicle surface which consists of DPPC and CH (1:1 molar ratio) with the mushroom conformation of PEG chains from Eqs. (1) to (3). Thus, it is

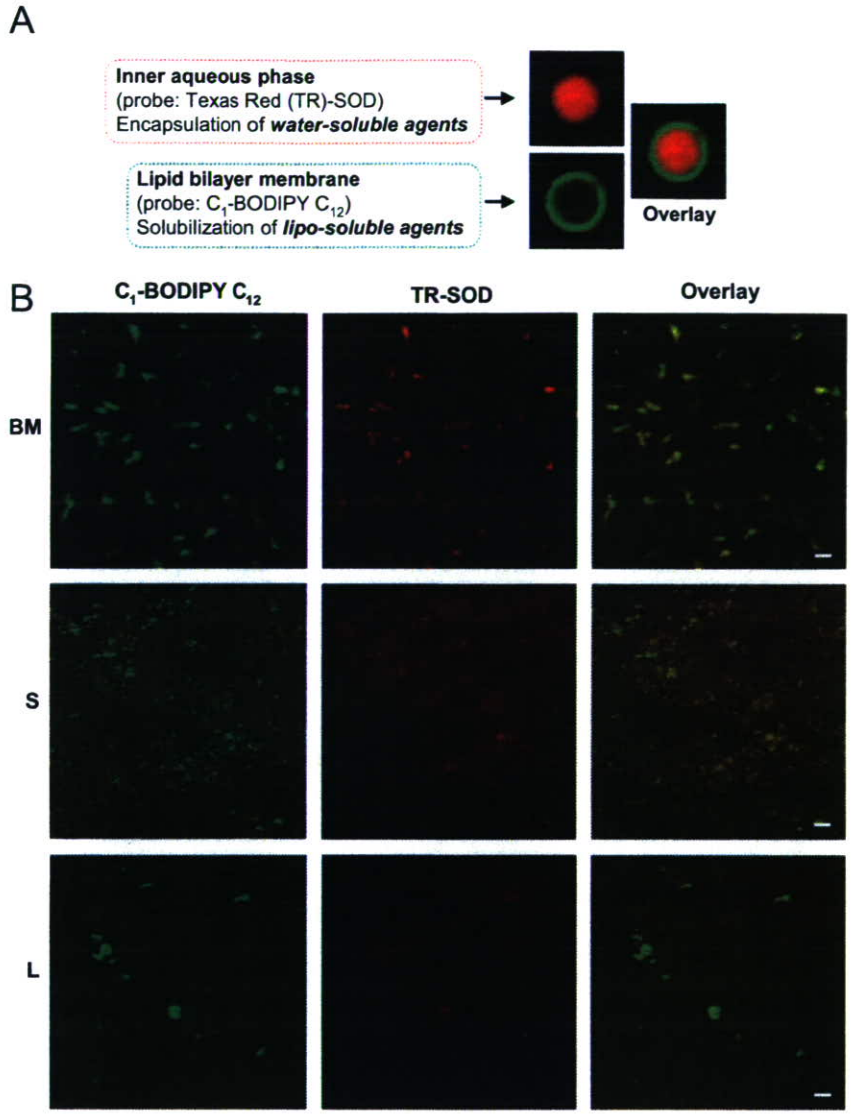

Fig. 5. Histological examination of fluorescence delivered into bone marrow tissues using PEG(0.6)-[SA-Ve] as carriers. (A) Fluorescence localization in double fluorescence-labeled large multilamellar PEG(0.6)-[SA-Ve] with diameter of ca. 10 μm. This observation was performed before extrusion to submicron size to enable observation of the structure within resolution of a confocal microscope. This image indicates that red fluorescence comes from TR-SOD which is encapsulated in inner aqueous phase and green fluorescence comes from C_1-BODIPY C_{12} which is embedded in bilayer membrane. (B) Confocal scanning images of femoral bone marrow (BM), spleen (S), and liver (L) taken from rabbit at 6 h after i.v. injection of double fluorescence-labeled PEG(0.6)-[SA-Ve] with size of 247 ± 22 nm in diameter (lipids: 15 mg/kg b.w.). The scale bars represent 20 μm.

estimated that the optimal incorporation amount of PEG-lipids is slightly higher than that required to fully cover the vesicular surface. This finding provides useful information for the design of vesicle surface to passively enhance the active targeting with PEG-modification *in vivo*.

To examine the participation of the anionic properties of vesicles in BMMϕ uptake, we investigated the organ distribution of conventional anionic vesicles containing PG with same protocol. These PG-vesicles do not distribute to the bone marrow (Supplementary Table 2 online, only 5.36 ± 0.65%ID of PG-vesicles were taken up by the bone marrow at 24 h after i.v. injection). Comparative data for Ve and SA-Ve are shown in Fig. 1(B) and Supplementary Table 1. Previous publications have also supported the observation that PG-vesicles do not distribute to the bone marrow [33], and neutral vesicles with various sizes in the range of 136.2–318 nm do not distribute to the bone marrow [34]. These results indicated that the targeting of bone marrow is not general for neutral vesicles and is achieved not only by the anionic surface of vesicles. The results suggest that SA is specifically responsible for the bone marrow targeting.

Histological observations showed that the vesicles and encapsulated agents are distributed at the same locations into bone marrow tissues, clearly indicating that the encapsulated agents were delivered to the bone marrow tissues by the vesicles (Fig. 5). Higher magnification TEM observations have demonstrated that a massive number of vesicles are trapped in the endosomes and lysosomes of the BMMϕ (Fig. 6). These observations indicated that the

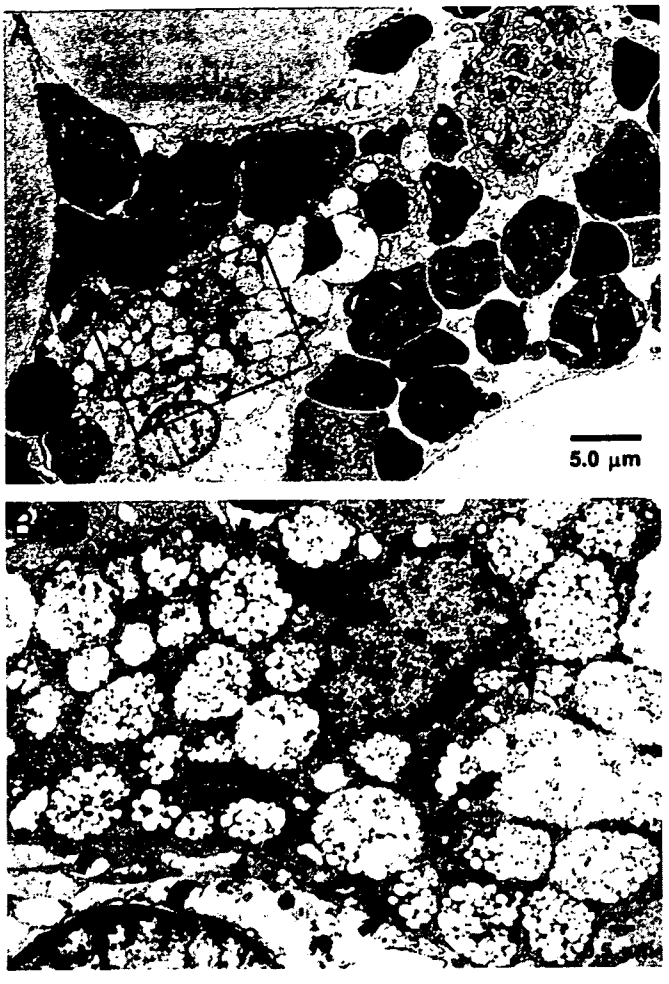

Fig. 6. Transmission electron micrographs of femoral bone marrow tissue section, taken from rabbit at 6 h after i.v. injection of PEG(0.6)-[SA-Ve] (lipids: 15 mg/kg b.w.). (A) Low magnified micrograph representing the bone marrow tissue including macrophage and various bone marrow cells. (B) High magnified micrograph of framed region in panel (A). A massive number of vesicles with original diameter (average 270 nm) are trapped in several endosomes or lysosomes of macrophage. Some are indicated by arrows, which shows same position in (A) and (B).

uptake of PEG(0.6)-[SA-Ve] by bone marrow was responded by the endocytosis of BMMϕ. One potent trigger to accelerate the cellular endocytosis for vesicles is an interaction with the receptors on the surface of cells, that are known as a receptor-mediated endocytosis which is investigated as a potent pathway for drug targeting to specific cell including macrophage [2,10,11]. Scavenger receptors are membrane glycoproteins that are present mainly on cells of the macrophage lineage [35,36]. Various polyanionic compounds such as dextran sulfate, polyinosinic acid, and acetylated low density lipoproteins have been reported as ligands for this receptor [37,38]. These compounds are taken at high levels by macrophages via a scavenger receptor-mediated mechanism. On the other hand, many polyanions such as chondroitin sulfate, poly(D-glutamic) acid, and polycytidylic acid are not ligands for scavenger receptors [37,38]. Previous investigations indicated that the scavenger receptors on macrophages contribute to the recognition of polyanionic structures, resulting in selective uptake. Enhanced uptake of succinylated proteins has been investigated in cultured brain microvessel endothelial cells. Endothelial cells also express the scavenger receptor on their surface. Large succinylated proteins such as catalase (Mw 227 kDa) and bovine serum albumin (Mw 70 kDa) were taken up by the cells via a scavenger receptor-mediated mechanism, whereas significant uptake was not observed for native proteins and small succinylated proteins such as SOD (Mw 34 kDa) and soybean trypsin inhibitor (Mw 21 kDa) [39]. This indicates that succinylation of large molecules is involved in the uptake via a scavenger receptor-mediated mechanism. Recently, Szabó et al. reported the uptake of branched polypeptides by bone marrow culture-derived murine macrophages. They indicated that the succinylation of branched polypeptides significantly enhanced the uptake by macrophages, and the uptake was inhibited by blocking of the class-A scavenger receptors [40]. Because the terminal hydrophilic head group of SA is corresponding to the succinylated structure, we speculate that the interaction between PEG-[SA-Ve] and the scavenger receptors on BMMϕ might participate in the selective uptake. However, further mechanistic investigation on uptake of PEG-[SA-Ve] by BMMϕ, splenic macrophages, and hepatic Kupffer cells is necessary to clarify the mechanism of organ selective macrophage uptake.

Previous pharmacokinetic studies have been performed using vesicles containing SA that have the same lipid composition as in the present study with PEG(0.3)-[SA-Ve], but a significantly higher dose was employed (lipids: 680 mg/kg b.w.) [17]. In these studies, the bone marrow-selective distribution was not observed, so it appears that the bone marrow selectivity is limited by the injection dosage in certain applications. As the vesicle dosage increases, the MPS in the bone marrow becomes saturated; as a result, liver and spleen uptake is increased. In our previous organ distribution study in rabbits, >50%ID of the vesicles were still in circulation at 48 h after infusion of a massive dose of vesicles, while the bone marrow had 7.36 ± 0.34% of 680 mg/kg b.w. at the same time point [17]. This value is equivalent to 50.0 mg/kg b.w., and it can be used to estimate the maximum uptake capacity of MPS for vesicles. When vesicle dosage increases above 50.0 mg/kg b.w., the bone marrow is the first organ to become saturated, and the accumulation of vesicles then increases in the liver and spleen. Such sequential saturation of the MPS eliminates organ selectivity. Therefore, the bone marrow targeting of SA-Ve becomes striking when the dose of vesicles is below the saturation dosage for bone marrow, as observed in the present study (15 mg/kg b.w.). The ability of vesicular nanoparticles to encapsulate a wide variety of agents provides significant opportunities for bone marrow delivery applications. In the present study, we have demonstrated the delivery of scintigraphic and fluorescence imaging agents to bone marrow by using the

SA-Ve vesicles. This method has advantages in delivering the therapeutic agents to treat bone marrow disorders.

5. Conclusion

This is the first report to show the organ distribution of PEG-[SA-Ve] at small dose injection. Organ distributions of several vesicular formulations were quantitatively compared to determine the component to induce the significant distribution into bone marrow. Our data have indicated that surface modification of phospholipid vesicles with two compounds, SA and PEG-DSPE, cooperatively induces the significant bone marrow targeting properties to vesicles. In this system, BMMϕ participated in the uptake of PEG-[SA-Ve], and the efficient delivery of the vesicles as encapsulating agents into the bone marrow was achieved within 6 h after injection. These results indicated that the PEG-[SA-Ve] is a potent carrier for drug delivery into BMMϕ *in vivo* and may be useful for delivering a wide range of therapeutic agents to bone marrow.

Acknowledgments

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Appendix A. Supplementary materials

The online version of this article contains additional supplementary data. Please visit doi:10.1016/j.biomaterials.2007.01.041.

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Solution to the problems of acellular hemoglobins by encapsulation and the intrinsic issues of hemoglobin vesicles as a molecular assembly

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SUMMARY

Hemoglobin vesicles (HbV), or liposome-encapsulated hemoglobins, are developed as artificial oxygen carriers for the use as a transfusion alternative. The safety and efficacy of HbV have been clarified in detail: HbV can overcome the side effects of hemoglobin (Hb) molecules (stroma-free, and intra- or intermolecularly crosslinked) such as vasoconstriction, hypertension and possible vascular damage induced by direct contact of the vascular surface with Hb. On the other hand, intrinsic issues related to the suspension of HbV as a molecular assembly have to be considered: blood compatibility, structural and dispersion stabilities of the vesicles, and the requirement of prompt degradation in the reticuloendothelial system. Having overcome these issues, the results make us confident in advancing further development of HbV. Easy manipulation of physicochemical parameters of HbV provides possibilities for various clinical applications in addition to their use as a transfusion alternative.

PHYSIOLOGICAL IMPORTANCE OF THE RED BLOOD CELL CELLULAR STRUCTURE FOR ENCAPSULATED HEMOGLOBIN DESIGN

It has been well documented during the long history of the development of hemoglobin (Hb)-based oxygen carriers (HBOCs, Figure 1) that many side effects of stroma-free Hb and chemically modified Hbs exist: renal toxicity; entrapment of gaseous messenger molecules [nitric oxide (NO) and carbon monoxide (CO)] inducing vasoconstriction, hypertension, reduced blood flow, and reduced tissue oxygenation at microcirculatory levels[1–4]; neurological disturbances; malfunction of esophageal motor function[5]; and myocardial lesions.[6,7] These side effects of Hb molecules imply the importance of the cellular structure of red blood cells (RBCs). From the retrospective and recent observations, the main justifications for Hb encapsulation in RBCs are: (i) a decreased high colloidal osmotic pressure[8]; (ii) prevention of the removal of Hb from blood circulation; (iii) prevention of direct contact of toxic Hb molecules and endothelial lining[9]; (iv) preservation of the chemical environment in

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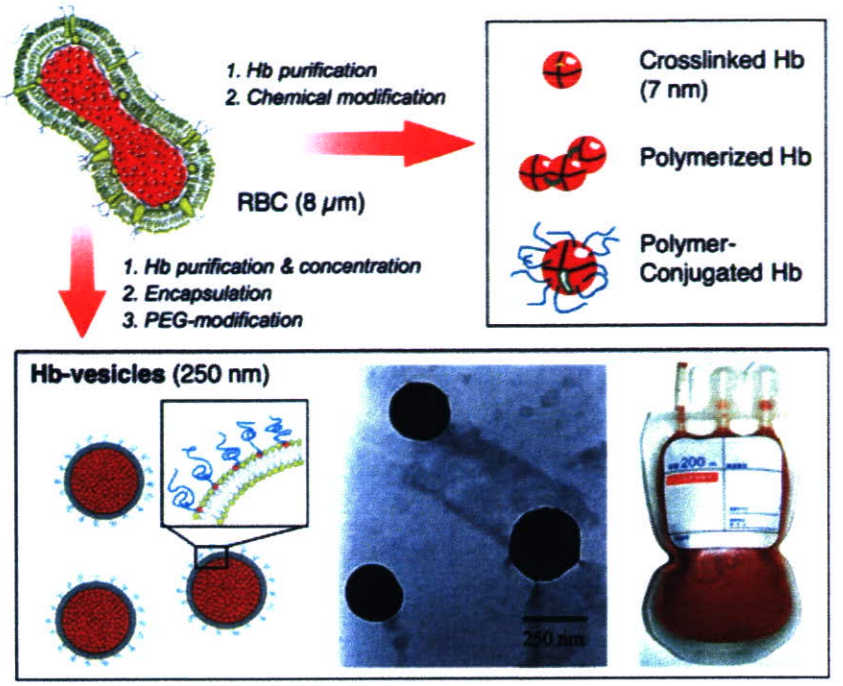

Figure 1. Schematic representation of a series of Hb-based oxygen carriers. In the case of Hb-vesicles (Hbv), the purified and concentrated Hb solution (35 g/dL) is encapsulated in phospholipid vesicles and the surface is modified using PEG chains. The particle size is well regulated at 250 nm, which is apparent from the transmission electron micrograph. The color of HbV is like a mixture of red wine and milk, due to the light scattering effect of small particles. Hb, hemoglobin; PEG, polyethyleneglycol; RBC, red blood cell.

cells, such as the concentration of phosphates [2,3-diphosphoglycerate (2,3-DPG), adenosine triphosphate, etc.] and other electrolytes; (v) RBCs are the major component that renders blood as non-Newtonian and viscous, which is necessary to pressurize the peripheral artery for homogeneous blood distribution and for maintenance of blood circulation[10]; and (vi) the cellular structure of RBCs retards oxygen release in comparison to acellular Hb solutions,[11,12] thereby retaining oxygen to peripheral tissues where oxygen is required.

For those reasons, the optimal structure of HBOCs might be to mimic the RBC cellular structure. The pioneering work of Hb encapsulation to mimic the cellular structure of RBCs was performed in 1957 by Chang,[13] who prepared microcapsules (5 μm) made of nylon, collodion and other materials. Toyoda[14] in 1965 and the Kambara-Kimoto group[15] in 1968 also investigated encapsulation of Hbs with gelatin, gum arabic, silicone, etc. Nevertheless, results emphasized the extreme difficulty in regulating the particle size to be appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne[16] reported in 1964 that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevich and Miller[17] in 1977 prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acids, etc. The US Naval Research Laboratories showed remarkable progress in the use of LEH.[18-20] Terumo Corporation (Tokyo) developed a different LEH called Neo Red Cells (Table 1).[21,22]

However, some intrinsic issues of encapsulated Hbs remained, which were mainly related to the nature of molecular assembly and particle dispersion. What we call HbV, with their high-efficiency production processes and improved properties, were established by our group based on technologies of molecular assembly in concert with precise analyses of pharmacological and physiological aspects (Table 2).[23-25] We use stable carbonylhemoglobin (HbCO) for purification with pasteurization at 60°C for 10 hours. The purity of the obtained Hb solution is extremely high.[26,27] Utilization of the stable and purified HbCO enables higher concentrations than 40 g/dL using ultrafiltration and easy handling of encapsulation by the extrusion method without causing protein denaturation. It has been confirmed that HbV encapsulates nearly 35 g/dL with a thin bilayer membrane. In the final process, HbCO in HbV is photodissociated by irradiation of visible light under an oxygen atmosphere to convert HbO_2.[28]

Table 1. A list of representative LEH extensively studied aiming at industrialization

Product Name	Group	Characteristics	Current status
Hb-vesicles (HbV)	Waseda University and Keio University	1. Pasteurization of HbCO at 60°C for virus inactivation, and high purity and concentration of encapsulated Hb 2. Lipid composition to improve blood compatibility 3. PEG modification and deoxygenation for 2 years storage 4. (Hb) = 10 g/dL	Preclinical
Neo Red Cells (NRC)	Terumo Corporation	1. Inositol hexaphosphate to regulate P_{50} (= 40–50 torr) 2. Lipids: HSPC/cholesterol/fatty acid/PEG-lipid 3. Storage in a refrigerator for 6 months 4. (Hb) = 6 g/dL	Preclinical
Artificial Red Cells (ARC)	NOF Corporation and Waseda University	1. Polymerized lipids (DODPC) for stabilization 2. Storage in powdered or frozen state 3. Difficulty in degradation in RES	Suspended
LEH	US Naval Research Laboratory	1. Freeze-dried powder with trehalose 2. Low Hb encapsulation efficiency 3. Thrombocytopenia, complement activation	Suspended
Synthetic erythrocytes	Rush-Presbyterian-St. Luke's Medical Center, University Illinois	1. The first attempt of LEH	Suspended

DODPC, 1,2-dioctadecadienoyl-*sn*-glycero-3-phosphatidylcholine; Hb, hemoglobin; HbCO, carbonylhemoglobin; HSPC, hydrogenated soy phosphatidylcholine; LEH, liposome-encapsulated Hbs.

Table 2. Characteristics of Hb-vesicles developed in Waseda University

Parameter	
Particle diameter	240–280 nm
P_{50}	25–28 torr
(Hb)	10 g/dL
Suspending medium	Physiologic saline solution (0.9% NaCl)
Colloid osmotic pressure	0 torr
Intracellular Hb concentration	ca. 35 g/dL
Lipid composition	DPPC/cholesterol/DHSG/DSPE-PEG_{5000}
Weight ratio of Hb to lipids	1.6–1.9 (w/w)
Stability for storage at room temperature	2 years
Circulation half-life	32 hours (rats)

DHSG, 1,5-*O*-dihexadecyl-N-succinyl-L-glutamate; DPPC, 1,2,dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphatidyethanolamine-*N*-polyethyleneglycol; Hb, hemoglobin.

The oxygen-bound HbV can provide oxygen-transport capacity that is both sufficient and comparable to that of RBCs in experiments related to extreme blood exchange[29-34] and fluid resuscitation from hemorrhagic shock.[35-38] A recent experiment of HbV as a priming solution for cardiopulmonary bypass in a rat model showed that HbV protects neurocognitive function by transporting oxygen to brain tissue even when the hematocrit is markedly reduced.[39] Other studies investigating HbV suspension as a possible perfusate for organ transplantation are also underway for the heart, liver, intestine, etc.

In fact, Hb encapsulation provides a unique opportunity to add new functions to particles. Other regulators, such as antioxidants and enzymes, can be embedded on the capsule or coencapsulated to reduce methemoglobin (metHb),[40-42] as can allosteric effectors to modulate oxygen affinity (P_{50}).[33,43] The P_{50} of HbV is regulated by coencapsulation of pyridoxal 5′-phosphate (PLP) in place of 2,3-DPG. The present HbV, being developed by Waseda University, contains PLP at PLP/Hb = 2.5 by mol; the resulting P_{50} is about 25–28 torr, which shows sufficient oxygen transporting capacity as a transfusion alternative. The P_{50} of HbV without PLP and Cl^- is 8–9 torr. This formulation is effective for targeted oxygen delivery to anoxic tissues caused by reduced blood flow.[34,44,45]

In addition to HbV, new encapsulated Hbs without liposomes have emerged with the use of recent advanced nanotechnologies, such as polymersome,[46] polyethyleneglycol (PEG)-poly(ε-caprolactone) copolymer nanoparticles,[47] and *in vivo* evaluation of oxygen-carrying capacities of these new materials is anticipated. Encapsulation of Hb can reduce the toxicity of cell-free Hbs. However, many hurdles must be surmounted to realize encapsulated Hbs because of the components of the capsules themselves and their structural complexity as a molecular assembly. It is also important to consider the larger dosage requirement of encapsulated Hb for blood substitution in comparison with those available with conventional drug delivery systems, which require no large dosage.

STRUCTURAL STABILIZATION OF ENCAPSULATED HEMOGLOBIN FOR STOCKPILING

Hb autoxidizes to form metHb and loses its oxygen-binding ability during storage, as well as during blood circulation. Therefore, prevention of metHb formation is necessary. A method exists to preserve deoxygenated Hbs in a liquid state using well-known intrinsic characteristics of Hb: the Hb oxidation rate in a solution is dependent on the oxygen partial pressure; also, deoxyHb is not autoxidized at ambient temperatures.[48] In the case of HbV, not only the encapsulated Hb but also the capsular structure (liposome) must be physically stabilized to prevent irreversible intervesicular aggregation, fusion and leakage of the encapsulated Hb.

Liposomes, as molecular assemblies, have been generally inferred to be structurally unstable. The US Naval Research Laboratory tested the addition of cryoprotectants and lyoprotectants, such as trehalose, to LEH for its preservation as a powder without causing hemolysis after rehydration.[49,50] In addition, many researchers have developed stabilization methods for liposomes that use polymer chains.[51-54] Polymerization of phospholipids that contain two dienoyl groups (DODPC) was studied extensively in our group. For example, gamma-ray irradiation induces radiolysis of water molecules and generates OH radicals that initiate intermolecular polymerization of dienoyl groups in DODPC. This method produces enormously stable liposomes, resembling rubber balls, which are resistant to freeze-thawing, freeze-drying and rehydration.[55,56] However, the polymerized liposomes were so stable that they were not degraded easily in the macrophages, even 30 days after injection.[57] It became widely believed that polymerized lipids are inappropriate for intravenous injection. Subsequently, it was clarified that the selection of appropriate lipids (phospholipid/cholesterol/negatively charged lipid/PEG-lipid) and their composition are important to enhance the stability of nonpolymerized liposomes.[31,58] Surface modification of liposomes with PEG chains is sufficient for dispersion stability.[32] In fact, in comparison to RBCs, HbV is highly resistant to hypotonic shock, freeze-thawing and enzymatic attack by phospholipase A_2.

We investigated the possibility of long-term preservation of HbV during storage for 2 years through a combination of two techniques: deoxygenation and PEG modification.[59] The PEG chains on the vesicular surface stabilize the dispersion state and prevent aggregation and fusion for 2 years because of their steric hindrance.[60] The original metHb content (ca. 3%) before preservation decreased gradually to less than 1% in all samples after 1 month because of the presence of a

reductant, such as homocysteine, inside the vesicles that consumed the residual oxygen and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the oxygen partial pressure: a lack of increase in the metHb formation was observed because of the intrinsic stability of the deoxygenated Hb. In fact, the metHb content did not increase for 2 years. These results indicate the possibility that the HbV suspension can be stored at room temperature for at least 2 years, which would enable stockpiling of HbV for any emergency.

BLOOD COMPATIBILITY OF LIPOSOMES AND HEMOGLOBIN VESICLES

Liposome is not a solute but a particle in a suspension. The surface of the particle may be recognized, interact with blood components, including complements. The so-called *injection reaction*, or pseudoallergy, is caused by complement activation, giving rise to anaphylatoxins that trigger various hypersensitivity reactions. This reaction is sometimes observed not only with liposomal products,[61] but also with fat emulsions[62] and a perfluorocarbon emulsion.[63] Therefore, the examination of blood compatibility of encapsulated Hbs is important for clinical use. Transient thrombocytopenia and pulmonary hypertension in relation to complement activation is an extremely important hematologic effect observed in rodent models after infusion of LEH (containing DPPG: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl glycerol) developed by the US Naval Research Laboratory[64,65] and of other products. In our group, exchange transfusion with the proto-type HbV (containing DPPG, no PEG modification) in anesthetized rats engendered transient thrombocytopenia and slight hypertension.[30] Similar effects were also observed for administration of negatively charged liposomes.[66,67] The transient reduction in platelet counts caused by complement-bound liposomes was also associated with sequestration of platelets in the lung and liver. Such nonphysiological platelet activation probably leads to initiation and modulation of inflammatory responses as platelets contain an array of potent proinflammatory substances. However, it must be emphasized that the present HbV formulation apparently does not induce thrombocytopenia in animal experiments, probably because the present HbV contains PEG-modification and a different type of negatively charged lipid (DHSG: 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate), not DPPG or a fatty acid.[68-70]

Detailed blood compatibility of HbV in relation to negatively charged lipid was examined by Dr H. Ikeda at the Hokkaido Red Cross Blood Center (Sapporo) and his colleagues.[69-72] The present PEG-modified HbV containing DHSG did not affect the extrinsic or intrinsic coagulation activities of human plasma, although HbV containing DPPG and no PEG modification tended to shorten the intrinsic coagulation time. The kallikrein-kinin cascade of the plasma was activated slightly by the proto-type DPPG-HbV, but not by the present PEG-DHSG-HbV. Moreover, the complement consumption in the plasma was detected by incubation with DPPG-HbV, but not with the present PEG-DHSG-HbV.[71] The exposure of human platelets to high concentrations of the present HbV (up to 40%) *in vitro* did not cause platelet activation and did not adversely affect the formation and secretion of prothrombotic substances or proinflammatory substances that are triggered by platelet agonists. These results imply that HbV, at concentrations of up to 40%, has no aberrant interactions with either unstimulated or agonist-induced platelets. It can be concluded that the present PEG-DHSG-HbV has a higher blood compatibility.

BIODISTRIBUTION, METABOLISM AND EXCRETION OF HEMOGLOBIN VESICLES

The dosage of blood substitutes should be considerably larger than those of other drugs, while their circulation time is considerably shorter than that of RBCs. Therefore, their biodistribution, metabolism, excretion and side effects must be characterized in detail, especially in relation to the reticuloendothelial system, RES (or termed the mononuclear phagocytic system).

Normally, free Hb released from RBC is bound rapidly to haptoglobin and is consequently removed from circulation by hepatocytes. However, when the Hb concentration is greater than the haptoglobin binding capacity, unbound Hb is filtered through the kidney, where it is actively absorbed. Hemoglobinuria and eventual renal failure occur when the reabsorption capacity of the kidney is exceeded. The encapsulation of Hb in vesicles completely suppresses renal excretion. However, HbV in the bloodstream is ultimately captured by phagocytes in the RES in much the same manner as senescent RBCs are, as confirmed by radioisotope ^{99m}Tc-labelled HbV

injection.[19,68] The HbV are finally distributed mainly in the liver, spleen and bone marrow. The circulation half-life is dose-dependent; when the dose rate was 14 mL/kg, the circulation half-life was 32 hours in rats. The circulation time in the case of the human body can be estimated as two or three times longer; or about 2 or 3 days at the same dose rate.

It is generally accepted that the liposome clearance by RES at a small dosage is accelerated by opsonization (absorption of plasma proteins such as antibodies and complements on the liposomal surface); PEG-modification prevents opsonization for prolonged circulation times.[73] However, considering the condition that the dosage of HbV is extremely high and requires a considerable amount of opsonins, and that HbV does not induce complement activation,[71] then the opsonin-dependent phagocytosis would not be a major component in the case of HbV with a large dosage. Actually, opsonin-independent phagocytosis, a direct recognition by macrophages, has been clarified in some studies.[74,75]

Transmission electron microscopic analysis of the spleen 1 day after infusion of HbV revealed the presence of HbV particles in the phagosomes of macrophages.[76] However, after 7 days, the HbV structure cannot be observed. We confirmed transient splenomegaly with no irreversible damage to the organs and complete metabolism within a week. Immunochemical staining with a polyclonal antihuman Hb antibody was used as the marker of Hb in the HbV, clarifying that HbV almost disappeared after 7 days in both the spleen and liver.

Bilirubin and iron are believed to be released during metabolism of Hb, but our animal experiments of topload infusion, daily repeated infusions and 40% blood exchange showed that neither of those products increased in the plasma within 14 days.[77-79] The released heme from Hb in HbV might be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages. Bilirubin would normally be excreted in the bile as a normal pathway; no obstruction or stasis of the bile is expected to occur in the biliary tree. Berlin blue staining revealed considerable deposition of hemosiderin in the liver and spleen, even after 14 days. Hemosiderosis often occurs in patients who have received repeated blood transfusions because of the shorter half-life of the stored RBCs. Moderate splenomegaly and hemosiderin deposition were also confirmed in the spleen after injection of stored RBCs, partly because of the accumulation and degradation of stored RBCs with lowered membrane deformability and shortened circulation half-life.[79]

As for membrane components of Hb-vesicles, phospholipids are metabolized and reused as a component of the cell membrane, or excreted in bile, especially as fatty acids and CO_2 in exhaled air. The plasma cholesterol level elevated transiently 3 days after injection, that was released from macrophages after degradation of HbV in the phagosomes.[77,79] However, the plasma phospholipid level did not increase significantly. It was recently clarified using ^{3}H-cholesterol that the cholesterol of HbV is released from macrophages to blood; it is ultimately excreted in the feces. The PEG chain is widely used for surface modification of liposomal products. The chemical crosslinker of PEG-lipid is susceptible to hydrolysis to release PEG chains during metabolism. The released PEG chains, which are known as inert macromolecules, should be excreted in urine through the kidneys.[80]

In order to know the physiological capacity of RES for degradation of HbV, we tested massive intravenous doses by daily repeated infusion of 10 mL/kg/day into Wistar rats for 14 days. The cumulative dosage was 140 mL/kg (Hb and lipids, 20,689 mg/kg). The total volume was equal to 2.5 times of whole blood volume (56 mL/kg).[78] Even though the splenohepatomegaly was significant, all rats tolerated the infusions, and the body weight increased until the intentional sacrifice for the succeeding 14 days. The phagocyted HbV disappeared though significant hemosiderin deposition and was confirmed in the spleen, liver, kidney, adrenal grand and bone marrow. We could not define a lethal dose of HbV in this experiment.

The profile of liposome clearance is species-dependent. More precise data are necessary to extrapolate the phenomena observed in animal experiments to humans. However, these results imply that the metabolism and excretion of HbV are within the physiological capacity that has been well characterized for the metabolism of senescent RBCs and conventional liposomal products.

UNIQUE RHEOLOGICAL PROPERTY OF HEMOGLOBIN VESICLES SUSPENSION

The extremely high concentration of the HbV suspension [(Hb) = 10 g/dL; (lipids) = 6 g/dL, volume fraction, ca. 40 vol%] imparts an oxygen-carrying capacity that is comparable to that of blood. The HbV suspension does

not possess a colloid osmotic pressure (COP) because one HbV particle (ca. 250 nm diameter) contains about 30,000 Hb molecules, and HbV acts as a particle, not as a solute. Therefore, HbV must be suspended in or coinjected with an aqueous solution of a plasma substitute. This requirement is identical to that for emulsified perfluorocarbon, which does not possess COP;[81,82] it contrasts to characteristics of other HBOCs, intramolecular crosslinked Hbs, polymerized Hbs and polymer conjugated Hbs, which all possess very high COP as protein solutions.[8,83]

Animal tests of HbV suspended in plasma-derived human serum albumin (HSA) or recombinant HSA (rHSA) showed an oxygen-transporting capacity that is comparable to that of blood.[36,39] We reported previously that HbV suspended in plasma-derived HSA or rHSA was almost Newtonian: no aggregation was detected microscopically.[31,32] In Japan, rHSA will be approved for clinical use in 2007,[84] but various plasma substitutes are used worldwide, such as hydroxylethyl starch (HES), dextran (DEX), and modified fluid gelatin (MFG). The selection among these plasma substitutes should be determined not only according to their safety and efficacy, but also by the related price, experience of clinicians and customs of respective countries. Water-soluble polymers generally interact with particles such as polystyrene beads, liposomes and RBCs to induce aggregation or flocculation.[85,86] For that reason, it is important to determine the compatibility of HbV with these plasma substitutes. With that background, we studied rheological properties of HbV suspended in these plasma substitute solutions using a complex rheometer and a microchannel array.[87] The rheological property of an HBOC is important because the infusion amount should be considerably large, which might affect the blood viscosity and hemodynamics.

The HbV suspended in rHSA was nearly Newtonian. Its viscosity was similar to that of blood, and the mixtures with RBCs at various mixing ratios showed viscosities of 3–4 cP. Other polymers, HES, DEX and MFG, induced flocculation of HbV, possibly by depletion interaction, and rendered the suspensions as non-Newtonian with the *shear-thinning* profile.[87] These HbV suspensions showed a high viscosity and a high storage modulus (G') because of the presence of flocculated HbV. On the other hand, HbV suspended in rHSA exhibited a very low G'. The viscosities of HbV suspended in DEX, MFG and high molecular weight HES solutions responded quickly to rapid step changes of shear rates of 0.1–100 s^{-1} and a return to 0.1 s^{-1}, indicating that flocculation formation is both rapid and reversible. Microscopically, the flow pattern of the flocculated HbV perfused through microchannels (4.5 μm deep, 7 μm wide, 20 cmH_2O applied pressure) showed no plugging. Furthermore, the time required for passage was simply proportional to the viscosity.

It has been regarded that lower blood viscosity after hemodilution is effective for tissue perfusion. However, microcirculatory observation shows that, in some cases, lower viscosity decreases shear stress on the vascular wall, causing vasoconstriction and reduced functional capillary density.[88] Therefore, an appropriate viscosity might exist, which maintains the normal tissue perfusion level. The large molecular dimension of HbV result in a transfusion fluid with high viscosity. A large molecular dimension is also effective to reduce vascular permeability and to minimize the reaction with NO and CO as vasorelaxation factors. These new concepts suggest reconsideration of the design of artificial oxygen carriers.[89] Actually, new products are appearing, although they are in the preclinical stage, not only HbV, but also zero-link polymerized Hb[90] and others with larger molecular dimensions and higher oxygen affinities.[91] Erni *et al.* clarified that HbV with a high O_2 affinity (low P_{50}, such as 8–15 torr) and high viscosity (such as 11 cP) suspended in a high-molecular-weight HES solution was effective for oxygenation of an ischemic skin flap.[45,92,93] That study showed that HbV would retain O_2 in the upper arterioles, then perfuse through collateral arteries and deliver oxygen to the targeted ischemic tissues, a concept of targeted oxygen delivery by an HBOC.[44] Some plasma substitutes cause flocculation of HbV and hyperviscosity. However, reports show that hyperviscosity would not necessarily be deteriorative and might be, in some cases, advantageous in the body.[10] The combination of HbV and plasma substitute solutions provides a unique opportunity to manipulate the suspension rheology, not only as a transfusion alternative, but also for other clinical applications, such as oxygenation of ischemic tissues and *ex vivo* perfusion systems.

CONCLUSION

Other related issues for HbV in a clinical situation include the interference effect of HbV on spectrophoto metric measurements in routine clinical laboratory tests

and noninvasive pulse oximetry monitoring of arterial blood oxygen saturation. Such interference is caused by strong light scattering resulting from the small HbV particles in blood.[94] We clarified that HbV can be removed easily from a blood specimen by the addition of high molecular weight dextran and centrifugation. Pulse oximetry can be improved by some modifications of the detection wavelength and software.

Encapsulation of Hb was initiated with the simple idea of duplicating the structure and function of RBCs. However, we are convinced that obstacles remain for the approach to realize the sophisticated function of RBCs; for example, it is impossible to mimic the flexibility of the unfilled biconcave structure of RBCs. The present HbV lacks ionophores in the bilayer membrane which facilitate the transport of small functional molecules from the outer medium, such as ascorbic acid or glutathione, to reduce metHb in HbV that does not contain enzymatic metHb reducing system, because the unstable enzymes are removed during the virus inactivation process of Hb purification.[26,27] On the other hand, clear advantages of simplified HBOCs exist, such as the absence of blood-type antigens and infectious viruses, along with stability for a long-term storage at room temperature for any emergency, all of which might overwhelm the functions of RBCs. The shorter half-life of the HBOCs in the bloodstream (2–3 days) limits their use, but they are applicable as a transfusion alternative for shorter periods of use. Easy manipulation of physicochemical properties of HbV such as P_{50} and viscosity supports the possible development of tailor-made oxygen carriers that suit various clinical indications. The achievements of ongoing HbV research described above give us confidence in advancing further development of HbV, with the expectation of its eventual realization.

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DISCLOSURE

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