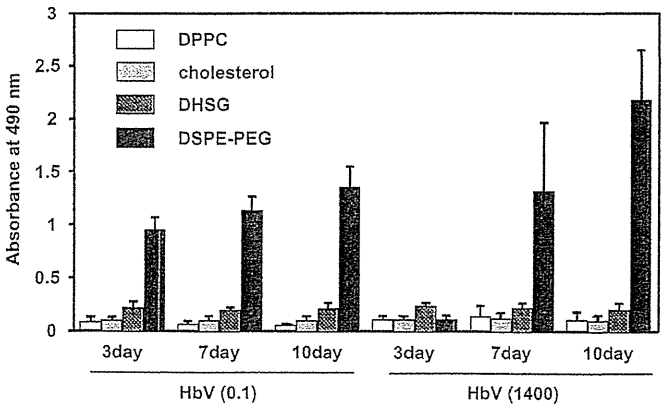

FIG. 5. Determination of the specific recognition site of IgM against HbV. A 10-nmol sample of DPPC (open bar), cholesterol (gray bar), DHSG (hatched bar), or DSPE-PEG (closed bar) in 50 μl of 100% ethanol was placed in a dish to completely remove the ethanol. IgM against each lipid component was detected by ELISA. Each point represents the mean ± S.D. (n = 4).

Therefore, such contradictory findings between the present and Dams' study could be the result of differences in the physicochemical properties of the liposomes used in the study, such as particle size or lipid composition. In fact, the diameter of the HbV liposomes used was approximately 250 nm, whereas the diameter of the PEGylated liposome used by Dams et al. (2000) was approximately 80 nm. In addition, our HbV was composed of DPPC, cholesterol, DPSH, and DSPE-PEG at a molar ratio of 5:5:1:0.3, whereas the PEGylated liposome used by Dams et al. (2000) was composed of partially hydrogenated egg-phosphatidylcholine, cholesterol, *N*-hydroxysuccinimidyl hydrazino nicotinate hydrochloride DSPE, and DSPE-PEG at a molar ratio of 1.85:1:0.07:0.15.

In addition, Ishida et al. (2006b) reported that the ABC phenomenon was induced by the selective binding of IgM to the second injected PEGylated liposome, and subsequent complement activation by IgM resulted in an accelerated clearance and enhanced hepatic uptake of the second injected PEGylated liposome. Wang et al. (2007) also reported that the hepatic clearance of liposomes was positively correlated with the plasma levels of IgM against the PEGylated liposome. In this study, we also found a substantial elevation in plasma IgM levels at both low (0.1 mg Hb/kg) and high doses (1400 mg Hb/kg contains more than 100 μmol of phospholipids/kg). Because the degree of IgM elevation at a high dose of HbV was significantly larger than that for a low dose, the IgM response against HbV might be dose-dependent.

To identify the recognition site of anti-HbV IgM, we also examined the production of IgM in the presence of each of the lipid components of HbV. Among these components, only anti-HbV IgM was found to react with DSPE-PEG (Fig. 5). In addition, the IgM level against HbV was well correlated with that against DSPE-PEG (data not shown, r = 0.611, $p < 0.01$). These data clearly indicate that the recognition site of anti-HbV IgM is mainly DSPE-PEG. It is also noteworthy that the synthetic negatively charged lipid (DHSG) in HbV does not seem to show a remarkable immunogenicity. It was previously reported that the spleen, especially the marginal zone, plays an important role in the induction of the ABC phenomenon, which elicits IgM against PEG, and the IgM responded in a T cell-independent manner in rats and mice (Ishida et al., 2006a, 2007). The fact that plasma IgM levels against HbV and DSPE-PEG were significantly enhanced at both doses (Figs. 4 and 5) suggests that HbV interacts directly with the marginal zone. Future studies will be needed to elucidate the details of the mechanism of the elicitation of IgM against HbV.

In contrast to the low-dose treatment, the injection of a high dose (1400 mg Hb/kg) of HbV did not appear to induce the ABC phenomenon in mice (Fig. 1B; Table 1). In fact, the hepatic uptake clearance for the second injection was only 1.5 times higher than that for the first injection (Table 2). Ishida et al. (2004) previously reported that the lipid dose of a prior injection of liposomes strongly affected the pharmacokinetic behavior of a subsequent injection at a dose of from 0.001 to 25 μmol of phospholipids/kg. They reported that liver accumulation in mice increased sigmoidally with increasing lipid dose, whereas the blood concentration sigmoidally decreased with increasing lipid dose. In general, the increased hepatic or splenic distributions of liposomes were accompanied by an increased scavenging of liposome by MPS, such as Kupffer cells and red pulp zone splenocytes (Goins et al., 1995). MPS or any other systems that are involved in the removal of liposomes are influenced by the injection dose (Laverman et al., 2000), and the uptake by MPS was saturated with increasing doses of liposomes. Our previous study showed that the distribution of HbV in the liver was saturated at 1400 mg Hb/kg (more than 100 μmol of phospholipids/kg) but not at 200 mg Hb/kg (approximately 25 μmol of phospholipids/kg) (Taguchi et al., 2009b). Consequently, HbV at a dose of 1400 mg Hb/kg did not appear to induce the ABC phenomenon, even though accompanied with remarkable IgM elicitation, because the hepatic uptake of HbV via MPS was saturated in the case of a high-dose injection. From these results, it was expected that ABC phenomenon might not be apparently induced at various intervals at proposed dose of HbV (1400 mg Hb/kg) because ABC phenomenon in mice was observed the most strongly at the 7- to 10-day interval (Ishida et al., 2003b). In fact, we previously reported that ABC phenomenon was not induced in hemorrhagic shock model rat, when HbV was injected at a dose of 1400 mg Hb/kg at hourly intervals (Taguchi et al., 2009a), at which the patients with massive hemorrhage are transfused.

However, our study has limitations with respect to explanation of full-length study of the ABC phenomenon of HbV. We have not examined the plasma IgM levels when multiple high doses of HbVs were administered. Dams et al. (2000) previously reported that weekly injections of *N*-hydroxysuccinimidyl hydrazino nicotinate hydrochloride PEG liposomes dramatically influenced the circulatory half-life at second injection, but the effect was almost normalized at fourth injection. Therefore, it seems that the higher levels of IgM elevations are not observed after multiple high-dose administration of HbV. On this point, further study could be needed for elucidating the effect of multiple high-dose administration of HbV on their pharmacokinetics.

In conclusion, the present study clearly shows that repeated injections of HbV induce the ABC phenomenon, when the first injection of HbV was a dose of 0.1 mg Hb/kg, but was not apparent at a dose of 1400 mg Hb/kg. These results suggest that, in a clinical situation, the repeated use of HbV at a dose of 1400 mg Hb/kg would not be expected to induce the ABC phenomenon. Thus, it is unlikely to be necessary to consider the ABC phenomenon in an administration schedule or regimen of HbV treatment as a red blood cell substitute.

References

Dams ET, Laverman P, Oyen WJ, Storm G, Scherphof GL, van Der Meer JW, Corstens FH, and Boerman OC (2000) Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. *J Pharmacol Exp Ther* 292:1071–1079.

Goins B, Klipper R, Sanders J, Cliff RO, Rudolph AS, and Phillips WT (1995) Physiological responses, organ distribution, and circulation kinetics in anesthetized rats after hypovolemic exchange transfusion with technetium-99m-labeled liposome-encapsulated hemoglobin. *Shock* 4:121–130.

Ishida T, Ichihara M, Wang X, and Kiwada H (2006a) Spleen plays an important role in the induction of accelerated blood clearance of PEGylated liposomes. *J Control Release* 115: 243–250.

Ishida T, Ichihara M, Wang X, Yamamoto K, Kimura J, Majima E, and Kiwada H (2006b) Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. *J Control Release* 112:15–25.

Ishida T, Ichikawa T, Ichihara M, Sadzuka Y, and Kiwada H (2004) Effect of the physicochemical properties of initially injected liposomes on the clearance of subsequently injected PEGylated liposomes in mice. *J Control Release* **95**:403–412.

Ishida T, Maeda R, Ichihara M, Irimura K, and Kiwada H (2003a) Accelerated clearance of PEGylated liposomes in rats after repeated injections. *J Control Release* **88**:35–42.

Ishida T, Masuda K, Ichikawa T, Ichihara M, Irimura K, and Kiwada H (2003b) Accelerated clearance of a second injection of PEGylated liposomes in mice. *Int J Pharm* **255**:167–174.

Ishida T, Wang X, Shimizu T, Nawata K, and Kiwada H (2007) PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. *J Control Release* **122**:349–355.

Koide H, Asai T, Hatanaka K, Urakami T, Ishii T, Kenjo E, Nishihara M, Yokoyama M, Ishida T, Kiwada H, et al. (2008) Particle size-dependent triggering of accelerated blood clearance phenomenon. *Int J Pharm* **362**:197–200.

Laverman P, Brouwers AH, Dams ET, Oyen WJ, Storm G, van Rooijen N, Corstens FH, and Boerman OC (2000) Preclinical and clinical evidence for disappearance of long-circulating characteristics of polyethylene glycol liposomes at low lipid dose. *J Pharmacol Exp Ther* **293**:996–1001.

Lee J, Lee J, Yoon S, and Nho K (2006) Pharmacokinetics of 125I-radiolabelled PEG-hemoglobin SB1. *Artif Cells Blood Substit Immobil Biotechnol* **34**:277–292.

Murata M, Tamai I, Sai Y, Nagata O, Kato H, Sugiyama Y, and Tsuji A (1998) Hepatobiliary transport kinetics of HSR-903, a new quinolone antibacterial agent. *Drug Metab Dispos* **26**:1113–1119.

Noble CO, Krauze MT, Drummond DC, Yamashita Y, Saito R, Berger MS, Kirpotin DB, Bankiewicz KS, and Park JW (2006) Novel nanoliposomal CPT-11 infused by convection-enhanced delivery in intracranial tumors: pharmacology and efficacy. *Cancer Res* **66**:2801–2806.

Okamura Y, Takeoka S, Eto K, Maekawa I, Fujie T, Maruyama H, Ikeda Y, and Handa M (2009) Development of fibrinogen gamma-chain peptide-coated, adenosine diphosphate-encapsulated liposomes as a synthetic platelet substitute. *J Thromb Haemost* **7**:470–477.

Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, Kobayashi K, and Tsuchida E (2001) Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am J Pathol* **159**:1079–1088.

Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, Takeoka S, Suematsu M, Takaori M, Kobayashi K, and Tsuchida E (2004a) Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J Pharmacol Exp Ther* **311**:874–884.

Sakai H, Masada Y, Horinouchi H, Yamamoto M, Ikeda E, Takeoka S, Kobayashi K, and Tsuchida E (2004b) Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med* **32**:539–545.

Sakai H, Masada Y, Takeoka S, and Tsuchida E (2002) Characteristics of bovine hemoglobin as a potential source of hemoglobin-vesicles for an artificial oxygen carrier. *J Biochem* **131**:611–617.

Sakai H, Seishi Y, Obata Y, Takeoka S, Horinouichi H, Tsuchida E, and Kobayashi K (2009) Fluid resuscitation with artificial oxygen carriers in hemorrhaged rats: profiles of hemoglobin-vesicle degradation and hematopoiesis for 14 days. *Shock* **31**:192–200.

Sakai H, Sou K, Horinouchi H, Kobayashi K, and Tsuchida E (2008) Haemoglobin-vesicles as artificial oxygen carriers: present situation and future visions. *J Intern Med* **263**:4–15.

Sakai H, Takeoka S, Park SI, Kose T, Nishide H, Izumi Y, Yoshizu A, Kobayashi K, and Tsuchida E (1997) Surface modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. *Bioconjug Chem* **8**:23–30.

Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, and Tsuchida E (2003) Pretreatment of serum containing hemoglobin vesicles (oxygen carriers) to prevent their interference in laboratory tests. *Clin Chem Lab Med* **41**:222–231.

Sakai H, Tsai AG, Kerger H, Park SI, Takeoka S, Nishide H, Tsuchida E, and Intaglietta M (1998) Subcutaneous microvascular responses to hemodilution with a red cell substitute consisting of polyethyleneglycol-modified vesicles encapsulating hemoglobin. *J Biomed Mater Res* **40**:66–78.

Sakai H and Tsuchida E (2007) Hemoglobin-vesicles for a transfusion alternative and targeted oxygen delivery. *J Liposome Res* **17**:227–235.

Samad A, Sultana Y, and Aqil M (2007) Liposomal drug delivery systems: an update review. *Curr Drug Deliv* **4**:297–305.

Sou K, Klipper R, Goins B, Tsuchida E, and Phillips WT (2005) Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute. *J Pharmacol Exp Ther* **312**:702–709.

Taguchi K, Maruyama T, Iwao Y, Sakai H, Kobayashi K, Horinouchi H, Tsuchida E, Kai T, and Otagiri M (2009a) Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model. *J Control Release* **136**:232–239.

Taguchi K, Urata Y, Anraku M, Maruyama T, Watanabe H, Sakai H, Horinouchi H, Kobayashi K, Tsuchida E, Kai T, et al. (2009b) Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. *Drug Metab Dispos* **37**:1456–1463.

Tuffin G, Waelti E, Huwyler J, Hammer C, and Marti HP (2005) Immunoliposome targeting to mesangial cells: a promising strategy for specific drug delivery to the kidney. *J Am Soc Nephrol* **16**:3295–3305.

Veronese FM and Pasut G (2005) PEGylation, successful approach to drug delivery. *Drug Discov Today* **10**:1451–1458.

Wang X, Ishida T, and Kiwada H (2007) Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. *J Control Release* **119**:236–244.

Wang XY, Ishida T, Ichihara M, and Kiwada H (2005) Influence of the physicochemical properties of liposomes on the accelerated blood clearance phenomenon in rats. *J Control Release* **104**:91–102.

Yamaoka K, Tanigawara Y, Nakagawa T, and Uno T (1981) A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobiodyn* **4**:879–885.

Address correspondence to: Masaki Otagiri, Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: otagirim@gpo.kumamoto-u.ac.jp

Hemoglobin-Vesicle, a Cellular Artificial Oxygen Carrier that Fulfils the Physiological Roles of the Red Blood Cell Structure

Hiromi Sakai, Keitaro Sou, Hirohisa Horinouchi, Koichi Kobayashi, and Eishun Tsuchida

Abstract Hb-vesicles (HbV) are artificial O_2 carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane (liposome). The concentration of the HbV suspension is extremely high ([Hb] = 10 g/dL) and it has an O_2 carrying capacity that is comparable to that of blood. HbV is much smaller than RBC (250 vs. 8000 nm), but it recreates the functions of RBCs; (i) the slower rate of O_2 unloading than Hb solution; (ii) colloid osmotic pressure is zero; (iii) the viscosity of a HbV suspension is adjustable to that of blood; (iv) HbV is finally captured by and degraded in RES; (v) co-encapsulation of an allosteric effector to regulate O_2 affinity; (vi) the lipid bilayer membrane prevents direct contact of Hb and vasculature; (vii) NO-binding is retarded to some extent by an intracellular diffusion barrier, and HbV does not induce vasoconstriction. (viii) Both RBC and HbV can be a carrier of not only O_2 but also exogenous CO. However, HbV has limitations such as a shorter functional half-life when compared with RBCs. On the other hand, the advantages of HbV are that it is pathogen-free and blood-type-antigen-free; moreover, it can withstand long-term storage of a few years, none of which can be achieved by the RBC transfusion systems.

1 Introduction

Biconcave RBCs deform to a parachute-like configuration to flow through a narrower capillary. This profile is believed to be effective to increase the surface-to-volume ratio and stir the intracellular viscous Hb solution to facilitate the gas exchange. On the other hand, physicochemical analyses have revealed that O_2 unloading of Hb is significantly retarded by compartmentalization in RBC. Why has nature selected such an inefficient cellular structure for gas transport? Interestingly, some of the answers to this question have been revised by the

H. Sakai (✉)
Research Institute for Science and Engineering, Waseda University, Tokyo, Japan
e-mail: hiromi@waseda.jp

E. Takahashi, D.F. Bruley (eds.), *Oxygen Transport to Tissue XXXI*,
Advances in Experimental Medicine and Biology 662,
DOI 10.1007/978-1-4419-1241-1_62,

research of blood substitutes. They are, (i) retardation and targeting of O_2 unloading at microcirculation to avoid autoregulatory vasoconstriction; (ii) reduction of a high colloidal osmotic pressure, COP, of an Hb solution to zero, to increase blood Hb concentration; (iii) rheology control of blood, a RBCs dispersion, to a non-Newtonian viscous fluid; (iv) prevention of extravasation or excretion through renal glomeruli; (v) preservation of the chemical environment in cells, such as the concentrations of electrolytes and enzymes; (vi) prevention of direct contact of toxic Hbs and endothelial cell lying, and (vii) modulation of reactions with NO as an endothelium derived relaxation factor, EDRF. These observations reassure the importance of the cellular structure of RBCs to design Hb-based oxygen carriers.

Hb-vesicles (HbV) are artificial O_2 carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane [1]. Concentration of the HbV suspension is extremely high ([Hb] = 10 g/dL, [lipids] = 6 g/dL, volume fraction, ca. 40 vol%) and it has an oxygen carrying capacity that is comparable to that of blood. In this review paper, we summarize the characteristics of HbV that can fulfill some of the physiological roles of the cellular RBC structure.

2 Structural Stability and Suspension Properties

Many people think liposomes are unstable capsules. However, it depends on the lipids and the composition. In the case of RBCs, the sophisticated cytoskeleton network structure stabilizes the cellular structure. However, hypotonic hemolysis easily occurs. We confirmed that HbV are more resistant than RBCs to hypotonic shock, freezing by liquid N_2 and thawing, enzymatic attack (phospholipase A_2) [2], and a shear stress (1500 s^{-1}). Moreover, it can be stored at room temperature over 2 years [3]. In spite of such high stability, we confirmed with animal experiments that HbV are eventually captured by reticuloendothelial system (RES) and degraded promptly within 2 weeks without decomposing (hemolysis) in blood circulation [4]. Phospholipid vesicles for the encapsulation of Hb would be beneficial for heme detoxification through their preferential delivery to the RES, a physiological compartment for degradation of senescent RBCs, even at doses greater than putative clinical doses [5].

Colloid osmotic pressure (COP) is an important factor to determine blood volume in the body. Hb solution (5 g/dL) showed the COP of 16 Torr [6]. Polymerization of Hb reduces COP depending on the resulting molecular weight. PEG-conjugated Hb shows the largest COP, which is about 3 times higher than blood (ca. 25 Torr) due to the highly hydrated PEG chains [7]. On the other hand, both HbV and washed RBCs showed 0 Torr because of the suspension of large particles: the number of total particles of HbV is less than 1/100 of the number of Hb molecules at the same Hb concentration [6]. COP acts in opposition to hydrostatic pressure to balance the distribution of fluid between blood and interstitial compartments [7]. COP is a colligative property,

depending proportionally on the concentration of protein exerting the force and specifically on the macromolecular properties of that protein. Solutions with high COP cause significant transcapillary filtration of water in the direction from the interstitial space into the vascular lumen. An increase in blood volume is advantageous to increase cardiac output for resuscitation, though the composition of other components of blood and tissue will also be compromised. HbV, on the other hand, does not have an oncotic effect, and the particle should be suspended in a plasma expander (plasma substitute, water-soluble polymer). The COP of the resulting suspension is identical to that of the suspending medium. When HbV is suspended in 5% rHSA, the suspension shows 20 Torr at any Hb concentration. HbV can create a suspension of [Hb] = 10 g/dL at the physiologic COP, that cannot be attained easily by other chemically modified Hb solutions.

The HbV suspended in rHSA ([Hb] = 10 g/dL) was nearly Newtonian [8]. Other plasma substitute polymers -hydroxylethyl starch (HES), dextran (DEX), and modified fluid gelatin (MFG)- induced HbV flocculation, possibly by depletion interaction, and rendered the suspensions as non-Newtonian with a shear-thinning profile. These HbV suspensions showed a high storage modulus (G') because of the presence of flocculated HbV. However, 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 in shear rates of $0.1-100\ s^{-1}$ and a return to $0.1\ s^{-1}$, indicating that flocculation is both rapid and reversible. The HbV suspension viscosity was influenced by the presence of plasma substitutes. The HbV suspension provides a unique opportunity to manipulate rheological properties for various clinical applications.

3 The Rate of O_2-Unloading, and NO- and CO-Bindings

The O_2-release from flowing HbVs was examined using an O_2-permeable, fluorinated ethylenepropylene copolymer tube (inner diameter, 28 μm) exposed to a deoxygenated environment [9]. Measurement of O_2 release was performed using an apparatus that consisted of an inverted microscope and a spectrophotometer, and the rate of O_2 release was determined based on the visible absorption spectrum in the Q band of Hb. HbVs and human RBCs were mixed in various volume ratios at [Hb] = 10 g/dl, and the suspension was perfused at the centerline flow velocity of 1 mm/s through the narrow tube. The mixtures of cell-free Hb solution and RBCs were also tested. Because HbVs were homogeneously dispersed, increasing the volume of the HbV suspension resulted in a thicker marginal RBC-free layer. Irrespective of the mixing ratio, the rate of O_2 release from the HbV/RBC mixtures was similar to that of RBCs alone. On the other hand, the addition of 50 vol% of acellular Hb solution to RBCs significantly enhanced the rate of deoxygenation. This difference between the HbV suspension and the acellular Hb solution should mainly be due to the difference in the particle size (250 vs. 7 nm) that affects their diffusion for the facilitated O_2

transport. It has been suggested that faster O_2 unloading from the HBOCs is advantageous for tissue oxygenation. However, this concept is controversial with regard to the recent findings, because an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders. We confirmed that HbVs do not induce vasoconstriction and hypertension. This is not only owing to the reduced inactivation of NO (see below) but also possibly due to the moderate O_2 release rate that is similar to RBCs.

One of the important roles of the RBC structure is the retardation of NO-binding. However, the mechanism has been controversial, whether, (i) an unstirred layer surrounding the cell should be the diffusion barrier, (ii) cytoskeletal cell membrane can be the diffusion barrier, or (iii) the highly concentrated Hb solution can be the barrier. To clarify the mechanism, we analyzed HbVs with different intracellular Hb concentrations, $[Hb]_{in}$, and different particle sizes using stopped-flow spectrophotometry [10]. In the case of different $[Hb]_{in}$ (1- 35 g/dl), NO-binding is retarded at a higher $[Hb]_{in}$, on the other hand, CO-binding did not show such retardation. In the case of different particle diameter of HbV at constant $[Hb]_{in}$, 35 g/dl, NO-binding is retarded with a larger particle, while the CO-binding did not show such changes. The computer simulations can recreate these tendencies. The two-dimensional concentration changes of free-NO and unbound free-hemes in one HbV at the $[Hb]_{in}$ was 1 g/dl showed that NO diffuses rapidly into HbV and the reaction proceeds quite homogeneously. On the other hand, HbV at $[Hb]_{in} = 35$ g/dl showed heterogeneous distribution. The concentration gradients of both NO and heme change from the interior surface to the core. The intrinsically fast NO-binding induces an intracellular diffusion barrier in a highly concentrated Hb solution, but not for the slow CO-binding. We can estimate the apparent binding rate constant of a particle encapsulating a 35-g/dl Hb with 8000-nm diameter, and they are similar to the reported values for RBCs. The mechanism of retardation of NO-binding is controversial, but from these data, we estimate that (i) rapid NO-binding reaction induces intracellular diffusion barrier, (ii) cellular membrane cannot be a barrier for gas diffusion, and (iii) a higher $[Hb]_{in}$ and larger size are the factors for retarding NO-binding. However, we have to admit that NO-binding of HbV is much faster than that of RBC. The absence of vasoconstriction for HbV cannot be explained with these data. We believe that small Hb would permeate across the endothelium to reach to the site where NO is produced and transferred to the smooth muscle. It was recently reported that dextran conjugated Hb permeates through the endothelium. However, much larger HbV would remain in the lumen and does not bind NO in the endothelium.

4 Resuscitation from Hemorrhagic Shock with HbV

The first attempt of HbV to restore the systemic condition after hemorrhagic shock was conducted using anesthetized Wistar rats. After 50% blood withdrawal, the rats showed hypotension and considerable metabolic acidosis and

hyperventilation. They received HbV suspended in rHSA, shed autologous blood (SAB), or rHSA alone. The HbV group restored mean arterial pressure, similar to the SAB group, which was significantly higher than the rHSA group. No remarkable difference was visible in the blood gas variables between the resuscitated groups. However, two of eight rats in the rHSA group died before 6 h [11]. After removing the catheters and awakening, the rats were housed in cages for upto 14 days. The HbV group gained body weight; the reduced Hct returned to the original level in 7 days, indicating elevated hematopoiesis. Both groups showed transient elevation of AST and ALT at 1 day. Splenomegaly was significant in the HbV group at 3 days because of the accumulation of HbV. However, it subsided within 14 days. Histopathological observation indicated that a significant amount of HbV accumulated in the spleen macrophages, and complete disappearance within 14 days. These results indicate that HbV is useful as a resuscitative fluid for hemorrhagic shock. Its performance is comparable to that of SAB. Similar experiments using beagles have shown 1-year survival after resuscitation with HbV.

The above elevations of AST and ALT after resuscitation with HbV or RBC indicate the systemic reperfusion injury. Recent reports on cytoprotective effects of exogenous CO urged us to test infusion of CO-bound HbV and RBC in hemorrhagic-shocked rats to improve tissue viability [12]. Using the similar model, hemorrhagic shocked Wistar rats received CO-HbV, CO-RBC, O_2-HbV, or O_2-RBC suspended in 5% rHSA. All groups showed prompt recovery of blood pressure and blood gas parameters, and survived for 6 h of observation period. Plasma AST, ALT and LDH levels were elevated at 6 h in the O_2-HbV and O_2-RBC groups. They were significantly lower in the CO-HbV and CO-RBC groups. Blood HbCO levels (26–39%) decreased to less than 3% at 6 h while CO was exhaled through the lung. Both HbV and RBC gradually gained the O_2 transport function. Collectively, both CO-HbV and CO-RBC showed a resuscitative effect and reduced oxidative damage to organs. Adverse and poisonous effects of CO gas were not evident for 6 h in this experimental model. Further study is necessary to clarify the neurological impact of a longer observation period, though the results suggest a possible new clinical indication.

In conclusion, HbV can mimic the functions of RBCs. However, the half-life of HbV is much shorter than that of RBCs, and limits their use. On the other hand, the advantages of HbV are that it is pathogen-free and blood-type-antigen-free; moreover, it can withstand long-term storage of a few years, none of which can be achieved by the RBC transfusion systems. We continue further development of HbV aiming at the eventual realization and contribution to the clinical medicine.

Acknowledgments This work is supported by Health and Labour Sciences Research Grants (Health Science Research Including Drug Innovation), Ministry of Health, Labour and Welfare, Japan (H.S. H.H, E.T, K.K.), and Grants in Aid for Scientific Research from the Japan Society for the Promotion of Science (B19300164) (H.S.).

References

1. Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E. (2008) Haemoglobin-vesicles as artificial oxygen carriers: present situation and future visions. J Intern Med 263:4–15.
2. Sakai H, Okamoto M, Ikeda E, Horinouchi H, Kobayashi K, Tsuchida E. (2009) J Biomed Mater Res A 90:1107–1119.
3. Sakai H, Tomiyama KI, Sou K, Takeoka S, Tsuchida E. (2000) Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. Bioconjug Chem 11:425–432
4. Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, Kobayashi K, Tsuchida E. (2001) Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. Am J Pathol 159:1079–1088.
5. Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, Takeoka S, Suematsu M, Takaori M, Kobayashi K, Tsuchida E. (2004) Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. J Pharmacol Exp Ther 311:874–884.
6. Sakai H, Yuasa M, Onuma H, Takeoka S, Tsuchida E. (2000) Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. Bioconjug Chem 11:56–64.
7. Vandegriff KD, McCarthy M, Rohlfs RJ, Winslow RM. (1997) Colloid osmotic properties of modified hemoglobins: chemically cross-linked versus polyethylene glycol surface-conjugated. Biophys Chem 69:23–30.
8. Sakai H, Sato A, Takeoka S, Tsuchida E. (2007) Rheological properties of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma-substitute solutions. Langmuir 23:8121–8128.
9. Sakai H, Suzuki Y, Kinoshita M, Takeoka S, Maeda N, Tsuchida E. (2003) O_2 release from Hb vesicles evaluated using an artificial, narrow O_2-permeable tube: comparison with RBCs and acellular Hbs. Am J Physiol Heart Circ Physiol 285:H2543–H2551
10. Sakai H, Sato A, Masuda K, Takeoka S, Tsuchida E. (2008) Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. J Biol Chem 283:1508–1517
11. Sakai H, Masada Y, Horinouchi H, Yamamoto M, Ikeda E, Takeoka S, Kobayashi K, Tsuchida E. (2004) Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. Crit Care Med 32:539–545.
12. Sakai H, Horinouchi H, Tsuchida E, Kobayashi K. (2009) Hemoglobin-vesicles and red blood cells as carriers of carbon monoxide prior to oxygen for resuscitation from hemorrhagic shock in a rat model. Shock 31(5):507–514.

原著

ヘモグロビン小胞体を含有する血液検体の臨床検査 -デキストラン添加による干渉作用の回避-

Clinical Laboratory Test of Blood Specimens Containing Hemoglobin-vesicles - Interference Avoidance by Addition of Dextran-

宗 慶太郎[1]，小峰 梨沙[2]，酒井 宏水[1]，小林 紘一[3]，土田 英俊[1]，村田 満[2]

Keitaro Sou [1], Risa Komine [2], Hiromi Sakai [1], Koichi Kobayashi [3], Eishun Tsuchida [1], Mitsuru Murata [2]

和文抄録

ヘモグロビン（Hb）小胞体（250 nm）は赤血球（8 μm）に比べ1/30程度の大きさの人工酸素運搬体である．Hb小胞体を含有する血液検体を遠心分離操作するとHb小胞体は血清に浮遊し，Hb小胞体による干渉作用が血液生化学検査を阻害することが解っているが，その他にも免疫学検査，凝固線溶検査，血糖検査などにおけるHb小胞体の干渉作用を検討する必要がある．本研究では，これらの検査におけるHb小胞体の干渉作用を明らかにすると共に，その回避法を明らかにすることを目的とした．遠心分離により血球と同時にHb小胞体を沈降分離させるため，各種分子量のデキストラン（Dex）を添加して血液中のHb小胞体を凝集させる条件を設定した．さらにヒト血液にHb小胞体を15vol％混合した試料についてこの分離条件を適用し，各検査項目について干渉の有無を検討した．Dexの分子量（487 kDa）および濃度（終濃度：2.6 g/dL）の設定により，通常の遠心分離（3000-5000 rpm, 10 min）でHb小胞体を沈降分離できることを確認した．Hb小胞体は生化学および凝固線溶検査の多くの項目で干渉作用を示したものの，Dexを添加して遠心除去することにより大部分の項目で干渉を回避できた．ただし，Dex添加により生化学検査でリポプロテインの低下，および凝固線溶検査でvon Willebrand factor（vWF）活性の低下，トータルplasminogen activator inhibitor type-1（PAI-1）の上昇を認め，これらについてはDex添加による干渉を受ける項目として注意を要する．血糖検査項目として検討したグルコースとグリコヘモグロビンは，Hb小胞体とDexの干渉なく測定できた．従って，Hb小胞体投与後の血液検体に本法を利用すれば，従来通り遠心分離で血清や血漿が得られ，生化学・免疫学検査，凝固線溶検査，血糖検査の多くの項目で干渉なく検査ができる．

Abstract

Hemoglobin-vesicle (HbV) is an artificial oxygen carrier of which the size (250 nm) is 30 times smaller than red blood cells (8 μm). HbVs remain in serum after centrifugation because of its small size and as a result, HbV interferes with the clinical laboratory test. Here we examine the interference of HbV in other clinical laboratory tests such as immunological test, coagulation fibrinolysis examination, and blood sugar test. To precipitate the HbV by conventional centrifugal separation of blood, we determined an appropriate condition to aggregate HbV by an addition of Dextran (Dex). The obtained plasma or serum was evaluated by common clinical laboratory test. HbV could be precipitated by addition of Dex (Mw. 489 kDa, final concentration: 2.6 g/dL in blood) with conventional centrifugation (3000-5000 rpm, 10 min). Though the presence of HbV interfered with the measurement of many analytes, the interference could be removed by the addition of Dex. However, it should be cautioned that this method underestimates lipoprotein concentration and von Willebrand factor (vWF) activity, and overestimates total plasminogen activator inhibitor type-1 (PAI-1). Blood sugar tests for glucose and glycated hemoglobin (Hb_{A1C})

(1) 早稲田大学 理工学術院 理工学研究所　Research Institute for Science and Engineering, Waseda University

(2) 慶應義塾大学 医学部 臨床検査医学　〒160-8582 東京都新宿区信濃町35　Department of Laboratory Medicine, School of Medicine, Keio University, 35 Shinano-cho Shinjuku-ku Tokyo 160-8582, Japan

(3) 慶應義塾大学 医学部 呼吸器外科　Department of Thoracic Surgery, School of Medicine, Keio University

論文受付 2009年1月13日　論文受理 2009年1月30日

could be performed without interference effect of HbV and Dex. Taken together, the present method will be useful to separate serum and plasma from the blood specimens containing HbV for accurate clinical laboratory tests.

Keywords
Hemoglobin-vesicles, oxygen carrier, blood specimen, clinical laboratory test, interference, dextran

1．はじめに

輸血代替物として人工酸素運搬体の使用が想定される救命救急や大手術では，各種の臨床検査やモニタリングにより患者の容態の管理が行われる．大量に投与される人工酸素運搬体の臨床応用を円滑に進めるには，人工酸素運搬体の投与が周辺の医療技術や機器にどのような影響を及ぼすか把握しておく必要がある．これまでに，血液検体検査のほか，Co-oximetry，pulse oximeter，あるいはmagnetic resonance（MR）oximetryによる酸素飽和度のモニタリングなどにおいて人工酸素運搬体の影響が調べられてきている[1-19]．Hbベース，パーフルオロカーボン（PFC）ベースを含め，人工酸素運搬体は血球成分に比較して小粒径のため，遠心分離を経てなお血漿層に浮遊する．このため，人工酸素運搬体の特性吸収や濁度が血液生化学検査の多くの項目で測定値に影響を与える．血漿タンパク質と同程度の大きさの修飾Hbは実験的にも血漿から分離するのが極めて困難であり，干渉作用の少ない機器の選定や修飾Hb濃度の関数として干渉分の補正係数を算出することで対処せざるを得ない[5,10,12,13]．ただし，干渉は濃度だけでなく，経時的に変化するMetHb含量などの影響を受けるため[11]，実際にはこれらの因子を複合的に考慮した補正が必要となる．特定の測定機種について独自の補正を含めた解析手法は臨床試験データの詳細解析には有効と思われるが，補正係数が測定機種に依存するため一般に普及する方法としては適していない．

一方，リン脂質二分子膜でHb溶液を被覆したHb小胞体（250 nm）は，赤血球（8 μm）に比較すれば1/30程度の大きさであるが，血漿タンパク質や修飾Hb（数nm）に比較すれば数十倍の大きさがある．この大きさになると，超遠心分離機を使用すれば比較的短時間で沈殿させることができ，また，デキストランなど高分子凝集剤の添加によりHb小胞体を凝集させれば，遠心分離操作で血清からHb小胞体を分離できる．このような分離法の工夫により血液生化学検査における干渉を回避できることがわかっている[8,9]．本研究では，デキストラン添加によるHb小胞体の凝集現象を利用し，血液から一段階の遠心分離操作により血漿や血清を分離する条件を検討した．更にこの条件で得られる血清や血漿について生化学・免疫検査，凝固線溶検査，血糖検査を実施し，Hb小胞体による干渉作用を回避できる項目，およびデキストラン添加による干渉作用のある項目を明らかすることを目的とした．

2．実験方法

2.1．デキストラン（Dex）添加によるHb小胞体の凝集

市販の分子量の異なるDex粉末（分子量；11, 19,6, 40.2, 72.2, 124, および487 kDa, SIGMA）を生理食塩水に溶解させて高分子凝集剤として使用した．Hb小胞体分散液（Hb; 0.05 g/dL, 3 mL）とDex溶液（20 g/dL, 0.3 mL）を混合し（Dex終濃度：1.8 g/dL），25℃に静置して溶液濁度の変化（λ＝700 nm）から凝集を検出し，その経時変化を観測した．この結果からHb小胞体の凝集生起に要するDex分子量および時間に関する知見を得た．

2.2．血清分離条件の検討

Hb小胞体が浮遊する血液から血清を分離する実験では，出血蘇生試験のためHb小胞体を投与したビーグル犬から採取した血液を使用した[20]．循環血液量の50％を脱血した後に，同量のHb小胞体分散液（[Hb]=8.6 g/dL, 5％ リコンビナントアルブミンに浮遊）を投与し，4時間経過した時点での採血液を利用した．採血液（5 mL）とDex溶液（20 g/dL, 0.5 ～0.88 mL）を混合し，25℃で10分間静置した．この混合液を遠心分離（5000 rpm, 10分）して上澄み液からのHb小胞体の除去を観測した．これらの結果から，Dexの分子量と濃度についてHb小胞体を完全に除去できる条件を決定した．

2.3．ヒト血液検体検査

健康成人から採血した新鮮血8.5容に対しHb小胞体を1.5容の割合で混合し各種採血管（ベノジェクトⅡ，テルモ）に分注した．さらに予めDex（分子量400～500 kDa, SIGMA）を20 g/dLになるよう生理食塩水に溶解した溶液を終濃度2.6 g/dLで添加混合した．10分間室温放置後3000 rpm，10分遠心分離し血清または血漿を得た．得られた血清の生化学検査［総タンパク，アルブミン，総ビリルビン，アスパラギン酸アミノトランスフェラーゼ（AST），アラニンアミノトランスフェラーゼ（ALT），γグルタミルトランスペプチダーゼ（γ-GTP），乳酸脱水素酵素（LDH），ロイシンアミノペプチダーゼ（LAP），クレアチニンキナーゼ（CK），コリンエステラーゼ（ChE），尿素窒素，尿酸，総コレステロール，エステル型コレステロール，遊離型コレステロール，リポプロテイン（A），トリグリセライド，リン脂質，遊離脂肪酸，高密度リポタンパク質-コレステロール（HDL-C）定量，カリウム，カルシウム，無機リン，クレアチニン，C反応性蛋白（CRP）定量，フェリチン，ハプトグロビン］，免疫学検査［免疫グロブリンG（IgG），免疫グロブリンM（IgM），B型肝炎ウィルス表面（HBs）抗原，B型肝炎ウィルス表面（HBs）抗体，B型肝炎ウィルス（HCV）抗体）］，血漿の凝固線溶検査［活性化部分トロンボプラスチン時間（APTT），プロトロンビン時間（PT），アンチトロンビンIII（ATIII），トータルプラスミノゲンアクチベーターインヒビター-1（PAI-1），フィブリノゲン，フォン・ヴィレブラン

ド因子（vWF）抗原，vWF活性，D-ダイマー，フィブリン分解産物（FDP）]，糖尿病関連検査［血中グルコース，グリコヘモグロビン（Hb_{A1C}）は血液検体で測定］をそれぞれ実施した．これら全ての検査を株式会社エスアールエル（東京）に依頼した．比較はHb小胞体添加血液に生理食塩水を添加し得られた血清または血漿と，生理食塩水添加血液にDexまたは生理食塩水を添加し得られた血清または血漿とした．また血清採取用真空採血管は凝固促進剤，血清分離剤が収容されているものなど幾つか種類があるので（Fig. 3の採血管収容物を参照），採血管の違いによる影響も調べた．干渉作用の有無の判定では，米国FDAのClinical Laboratory Improvement Amendments（CLIA）の規定する臨床試験に関する測定誤差の許容範囲（CLIA limit）を判断基準とし[5,8]，基準値との較差（誤差）が許容範囲を正に超える項目を（↑），負に超える項目を（↓）と表記した．また，基準値との較差が許容範囲内である場合には干渉作用無（none）と判断した．CLIA limitが規定されていない項目に関しては，較差の許容範囲を20％として判定した．今回はコントロールとの比較による干渉の有無の判定を目的としたため，血液にHb小胞体，Dex溶液ないし生理食塩水を添加して希釈率を統一（1.35倍希釈）して比較した．希釈率の補正は行っていない．

3．結果および考察

3.1．Dex添加によるHb小胞体の凝集

まず，Hb小胞体に各種分子量のDexを添加し，溶液濁度変化（Δ O.D.）を経時的にモニターした結果をFig. 1に示す．分子量72.1 kDa以下のDexをHb小胞体に添加してもほとんど溶液濁の変化を認めないが，分子量124 kDaのDexでは溶液濁度の上昇が観測され，さらに分子量487 kDaのDex添加により著しい溶液濁度の増大が観測された．粒子による光散乱強度は粒子径の6乗に比例するため，凝集による粒子径の増大は溶液濁度の増大（光散乱強度の増大）として検出される．高分子添加により懸濁粒子が凝集する現象はよく知られ，リン脂質小胞体は共存する高分子との相互作用が比較的詳しく調べられている[21-24]．高分子による小胞体凝集の主要な作用として，小胞体表面の電荷（ゼータ電位）の中和あるいは遮蔽による粒子の不安定化，小胞体間の架橋，高分子による粒子の排除（枯乾効果）などが知られ，一般に同一の繰り返し単位を有する高分子であれば高分子量体ほど凝集能が高く，凝集生起の臨界分子量の存在が認められる[23]．今回の結果より，分子量487 kDa程度のDexがHb小胞体の凝集に適していると考えられる．Hb小胞体の凝集は数分間で進行するため，Dexを添加してHb小胞体を凝集させる条件を室温（25℃）で10分間静置に設定した．

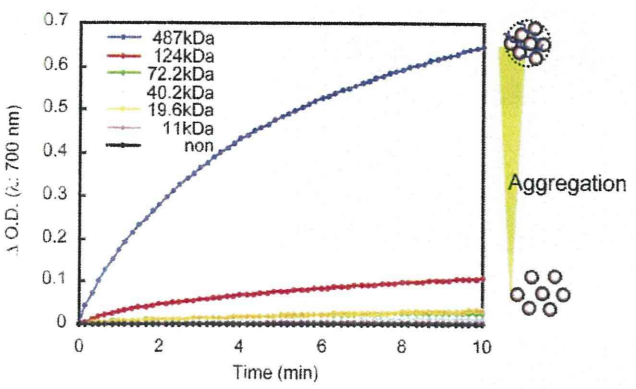

Fig. 1 Kinetics of aggregation of HbV in the presence of 1.8 % dextran at 25 ℃. Dex solution was added to the HbV dispersion. Increase of the ΔO.D. indicates the formation of larger aggregate of HbV by the addition of Dex.

3.2．血清分離条件の検討

Hb小胞体の浮遊する採血液に対し分子量の異なるDex溶液を添加し，10分間静置した後に遠心分離した採血管をFig. 2aに示す（Dex終濃度：1.8 g/dL）．Dex分子量の効果は明確で，487 kDaのDexを添加した系のみ透明な血漿が得られた．124 kDaではHb小胞体の沈殿を認めるものの不十分であり，それ以下の分子量では沈殿を認めなかった．遠心加速度（$r\omega^2$）による粒子の沈降速度（v）は次の式（1）で表すことができる．

$$v = \frac{d^2}{1.8} \times \frac{(\sigma - \rho)}{\eta} \times r\omega^2 \qquad (1)$$

ここで，d（cm）：粒子の直径，σ（g/cm^3）：粒子の密度，ρ（g/cm^3）：溶液の密度，η（g・cm^{-1}・s^{-1}）：溶液の粘度．
沈降速度は粒子直径の2乗に比例するため，凝集により見かけ上の粒子の直径を大きくすることで沈降速度が増大する．Fig. 1の結果との対応から，Dex 487 kDa の添加によるHb小胞体の著しい凝集により遠心で沈降できる大凝集体が生起するものと考えられる．この結果より，Dexの分子量を487 kDaに設定した．

次に，Dex 487 kDaの濃度条件を検討した．7 mL採血管（プレーン）に高分子凝集剤としてDex 487kDaの0.3～0.75 mLを添加した採血管を作成し，採血液（5 mL）を各採血管に採取して，25℃で10分間静置した．この混合液を遠心分離（5000 rpm，10分）して血漿層からのHb小胞体の除去の有無を観測した．結果として，透明な血漿層が得られるのはDex 487kDaを終濃度で1.8 g/dL以上添加した場合であり，更に血漿層を超遠心分離して分析すると，2.6 g/dL以上でほぼ完全にHb小胞体が除去されることを確認した（Fig. 2b）．血漿層にHb由来の吸収はなくDex 487kDa添加による赤血球やHb小胞体の溶血もない．沈殿物は下層の血球層と上層のHb小胞体で明らかな界面を認め，容易に各々の沈降占有容積率（クリット値）を計測できる．またHb小胞体の沈殿と血漿の界面も明確であるため，血漿採取は比較的容易である．以上より，Hb小胞体投与後には，Dex 487kDaが終濃度2.6 g/dLとなるように封入された採血管を使用すれば，従来通りの遠心分離にて濁度やHb吸収の干渉作用のない血漿ないし血清を採取できることが示された．

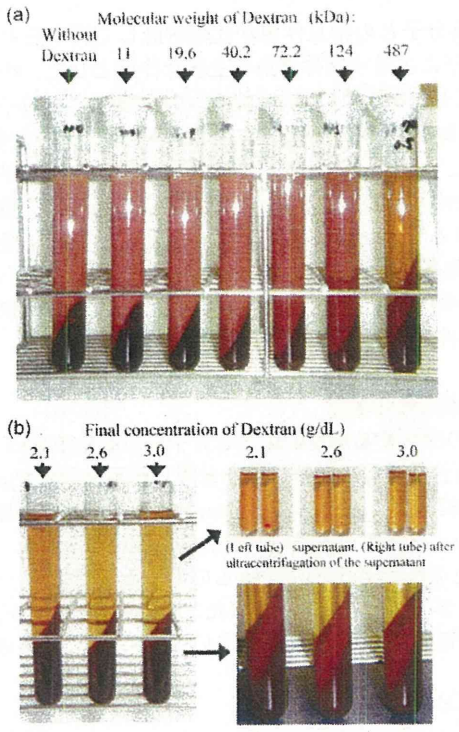

Fig. 2. Precipitation of the aggregated HbV by centrifugation. (a) Effect of the molecular weight of the Dex. (b) Effect of the concentration of the Dextran. HbV could be precipitated in centrifugation of blood sample in presence of 2.6 g/dL Dex (Mw. 487 kDa). Aggregated HbV forms pellet on a red blood cell pellet after centrifugation.

3.3. ヒト血液検体検査

高分子量Dex添加によりHb小胞体を除去する方法について，血液検体検査への適合性をヒト血清，血漿の外観と検査結果で評価した．Hb小胞体添加血液に生理食塩水を加えて遠心分離した血清，血漿はHb小胞体が浮遊しているため赤色であった．しかしDex添加によりHb小胞体を除去した血清と血漿（Fig. 3）は一部の血清採取用採血管（Fig. 3c, d）で少し赤みが帯びていたもののほぼ黄色であった．赤みが帯びていた血清はDexと血液の混和が不十分であったためHb小胞体を除去しきれなかったことが考えられる．Hb小胞体の除去効果は血清，血漿の外観から肉眼で確認可能であった．各検査においてHb小胞体浮遊血清，血漿は以下のような干渉作用を示した．生化学検査（Table 1～3）においては総タンパク，アルブミン，LDH，CK，クレアチニン，CRP，ハプトグロビンの上昇が見られた．また，総コレステロール，エステル型コレステロール，遊離型コレステロール，リン脂質ではHb小胞体の脂質膜が影響し上昇した．尿素窒素とIgMは低下し，ChEは低下傾向を示した．総ビリルビン，AST，ALT，γ GTPにおいては検査不能と判断された．免疫学検査項目であるHBs抗原・抗体，HCV抗体いずれもHb小胞体による影響は認められなかった．これらの結果は4種すべての血清採取用採血管で同様であった．生化学・免疫学検査の全32項目中，Hb小胞体の干渉作用により適切な測定値が得られない項目は17ないし18項目に上った（Table 1～3）．一方，Dexの添加によりHb小胞体を除去することで，3ないし4項目（遊離脂肪酸，リポプロテイン，遊離コレステロール，フェリチン，ALT，AST）を除き適切

採血管収容物	検査項目
(a) プレイン（収容物なし）	＜生化学・免疫学検査＞ 総タンパク，アルブミン，総ビリルビン，AST，ALT，g-GTP，LDH，LAP，CK，ChE，尿素窒素，尿酸，総コレステロール，エステル型コレステロール，遊離型コレステロール，リポプロテイン(A)，トリグリセライド，リン脂質，遊離脂肪酸，HDL-C定量，カリウム，カルシウム，無機リン，クレアチニン，CRP定量，フェリチン，ハプトグロビン，IgG，IgM，HBs抗原，HBs抗体，HCV抗体
(b) 凝固促進フィルム	
(c) 血清分離剤 凝固促進フィルム	
(d) 血清分離剤 凝固促進フィルム トロンビン	
(e) 3.8% クエン酸ナトリウム	＜凝固線溶系検査＞ APTT，PT，ATIII，トータルPAI-1，フィブリノゲン，vWF抗原，vWF活性
(f) トロンビン アプロチニン 蛇毒	＜凝固線溶系検査＞ D-ダイマー，FDP
(g) フッ化ナトリウム	＜糖尿病関連検査＞ 血中グルコース

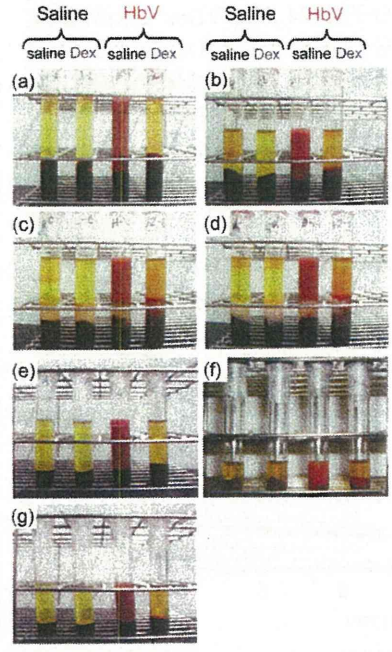

Fig. 3. Blood collecting tubes and analytes of clinical laboratory tests. (a)-(g) Blood samples after centrifugation. The blood collecting tube contain blood+saline+saline (control), blood+saline+Dex (interference effect of Dex), blood+HbV+saline (interference effect of HbV), or blood+HbV+Dex (this method).

Table 1. Clinical chemistry and immunological tests (Blood collection tube without contents)

Analytes	Units	CLIA limits	Blood+Saline			Blood+HbV			
			+Saline (Control)	+Dex	IF*	+Saline	IF*	+Dex	IF*
Total protein	g/dL	± 10%	5.1	4.8	none	14	↑	4.8	none
Albumin	g/dL	± 10%	3.3	3.2	none	4.3	↑	3.2	none
Total bilirubin	mg/dL	±0.4mg/dL or ± 20%	0.2	0.1	none	impossible	×	0.1	none
AST	IU/L	± 20%	11	10	none	impossible	×	10	none
ALT	IU/L	± 20%	7	7	none	impossible	×	7	none
γ-GTP	IU/L	± 20%	8	8	none	impossible	×	7	none
LDH	IU/L	± 20%	134	118	none	270	↑	127	none
LAP	IU/L	ND, ± 20%	63	62	none	58	none	64	none
CK	IU/L	± 30%	46	46	none	89	↑	47	none
ChE	IU/L	ND, ± 20%	243	242	none	215	none	247	none
Urea nitrogen	mg/dL	± 2 mg/dL or ± 9%	6.1	6.0	none	1.2	↓	6.3	none
Creatinine	mg/dL	±0.3 mg/dL or ± 15%	0.39	0.43	none	2.03	↑	0.47	none
Uric acid	mg/dL	± 17%	2.6	2.5	none	2.5	none	2.7	none
Total cholesterol	mg/dL	± 10%	129	119	none	334	↑	113	none
Cholesterol ester	mg/dL	ND, ± 20%	99	92	none	238	↑	92	none
Free cholesterol	mg/dL	ND, ± 20%	30	27	none	96	↑	21	↓
Triglyceride	mg/dL	± 25%	90	73	none	86	none	69	none
phospholipid	mg/dL	ND, ± 20%	163	156	none	235	↑	153	none
Free fatty acid	mEQ/L	ND, ± 20%	0.08	0.08	none	0.09	none	0.14	↑
HDL-C	mg/dL	ND, ± 20%	43	43	none	39	none	45	none
Lipoproteins	mg/dL	± 30%	12	5	↓	15	none	6	↓
K^+	mEQ/L	±0.5 mmol/L	2.4	2.5	none	2.5	none	2.6	none
Ca^{2+}	mg/dL	±0.25 mmol/L	6.2	6.2	none	5.9	none	6.2	none
Inorganic phosphate	mg/dL	ND, ± 20%	2.1	2.0	none	2.2	none	2.2	none
CRP	mg/dL	ND, ± 20%	≤ 0.02	≤ 0.02	none	0.1	↑	≤ 0.02	none
Ferritin	ng/mL	ND, ± 20%	3.5	3.0	none	3.4	none	2.8	↓
Haptoglobin	mg/dL	ND, ± 20%	80	74	none	117	↑	80	none
IgG	mg/dL	± 25%	747	734	none	655	none	744	none
IgM	mg/dL	ND, ± 20%	93	86	none	60	↓	88	none
HBs antigen	IU/mL	positive or negative	< 0.05	< 0.05	none	<0.05	none	< 0.05	none
HBs antibody	mIU/mL	positive or negative	< 10.0	< 10.0	none	< 10.0	none	< 10.0	none
HCV antibody		positive or negative	0.0	0.0	none	0.0	none	0.0	none

AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GTP: γ—glutamyltranspeptitase, LDH: lactate dehydrogenase, LAP: leucine aminopeptidase, CK: creatine kinase, ChE: cholinesterase, HDL-C: high density lipoprotein cholesterol, CRP: C-reactive protein, HBs: hepatitis B surface, HCV: hepatitis C virus. * IF means interference. (↑) overestimation, (↓) underestimation, (none) no interference.

Table 2. Clinical chemistry and immunological tests (Blood collection tube containing clot activator)

Analytes	Units	CLIA limits	Blood+Saline			Blood+HbV			
			+Saline (Control)	+Dex	IF*	+Saline	IF*	+Dex	IF*
Total protein	g/dL	± 10%	5.2	4.8	none	14.3	↑	4.9	none
Albumin	g/dL	± 10%	3.3	3.2	none	4.2	↑	3.2	none
Total bilirubin	mg/dL	±0.4mg/dL or ± 20%	0.1	0.1	none	Impossible	×	0.1	none
AST	IU/L	± 20%	11	11	none	Impossible	×	12	none
ALT	IU/L	± 20%	5	8	↑	Impossible	×	8	↑
γ-GTP	IU/L	± 20%	7	7	none	Impossible	×	7	none
LDH	IU/L	± 20%	131	114	none	265	↑	115	none
LAP	IU/L	ND, ± 20%	61	62	none	58	none	62	none
CK	IU/L	± 30%	48	47	none	73	↑	48	none
ChE	IU/L	ND, ± 20%	239	236	none	220	none	241	none
Urea nitrogen	mg/dL	± 2 mg/dL or ± 9%	6.1	6.3	none	Impossible	×	5.9	none
Creatinine	mg/dL	±0.3 mg/dL or ± 15%	0.45	0.41	none	1.80	↑	0.42	none
Uric acid	mg/dL	± 17%	2.5	2.5	none	2.3	none	2.7	none
Total cholesterol	mg/dL	± 10%	126	126	none	333	↑	119	none
Cholesterol ester	mg/dL	ND, ± 20%	95	96	none	239	↑	91	none
Free cholesterol	mg/dL	ND, ± 20%	31	30	none	94	↑	28	none
Triglyceride	mg/dL	± 25%	88	80	none	82	none	69	none
phospholipid	mg/dL	ND, ± 20%	162	157	none	229	↑	157	none
Free fatty acid	mEQ/L	ND, ± 20%	0.08	0.08	none	0.09	none	0.15	↑
HDL-C	mg/dL	ND, ± 20%	41	42	none	39	none	43	none
Lipoproteins	mg/dL	± 30%	14	11	none	14	none	4	↓
K^+	mEQ/L	±0.5 mmol/L	2.4	2.4	none	2.5	none	2.6	none
Ca^{2+}	mg/dL	±0.25 mmol/L	6.0	6.1	none	5.7	none	6.0	none
Inorganic phosphate	mg/dL	ND, ± 20%	2.1	2.0	none	2.8	none	2.1	none
CRP	mg/dL	ND, ± 20%	≤ 0.02	≤ 0.02	none	0.08	↑	≤ 0.02	none
Ferritin	ng/mL	ND, ± 20%	2.8	2.5	none	2.8	none	2.6	none
Haptoglobin	mg/dL	ND, ± 20%	78	78	none	118	↑	82	none
IgG	mg/dL	± 25%	736	721	none	640	none	742	none
IgM	mg/dL	ND, ± 20%	90	84	none	55	↓	85	none
HBs antigen	IU/mL	positive or negative	< 0.05	< 0.05	none	< 0.05	none	< 0.05	none
HBs antibody	mIU/mL	positive or negative	< 10.0	< 10.0	none	< 10.0	none	< 10.0	none
HCV antibody		positive or negative	0.0	0.0	none	0.0	none	0.0	none

AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GTP: γ—glutamyltranspeptitase, LDH: lactate dehydrogenase, LAP: leucine aminopeptidase, CK: creatine kinase, ChE: cholinesterase, HDL-C: high density lipoprotein cholesterol, CRP: C-reactive protein, HBs: hepatitis B surface, HCV: hepatitis C virus. * IF means interference. (↑) overestimation, (↓) underestimation, (none) no interference.

Table 3. Clinical chemistry and immunological tests (Blood collection tube containing clot activator, inert barrier material, and thrombin)

Analytes	Units	CLIA limits	Blood+Saline			Blood+HbV			
			+Saline (Control)	+Dex	IF*	+Saline	IF*	+Dex	IF*
Total protein	g/dL	± 10%	5.0	4.8	none	14.0	↑	5.0	none
Albumin	g/dL	± 10%	3.3	3.2	none	4.3	↑	3.2	none
Total bilirubin	mg/dL	±0.4mg/dL or ± 20%	0.1	0.1	none	Impossible	×	0.1	none
AST	IU/L	± 20%	9	11	↑	Impossible	×	11	↑
ALT	IU/L	± 20%	6	8	↑	Impossible	×	7	none
γ-GTP	IU/L	± 20%	8	7	none	Impossible	×	7	none
LDH	IU/L	± 20%	113	110	none	260	↑	117	none
LAP	IU/L	ND, ± 20%	61	62	none	59	none	63	none
CK	IU/L	± 30%	48	47	none	55	none	49	none
ChE	IU/L	ND, ± 20%	239	240	none	225	none	244	none
Urea nitrogen	mg/dL	± 2 mg/dL or ± 9%	5.9	6.1	none	0.2	↓	5.8	none
Creatinine	mg/dL	±0.3 mg/dL or ± 15%	0.39	0.40	none	1.90	↑	0.5	none
Uric acid	mg/dL	± 17%	2.6	2.6	none	2.6	none	2.7	none
Total cholesterol	mg/dL	± 10%	127	123	none	337	↑	121	none
Cholesterol ester	mg/dL	ND, ± 20%	95	93	none	245	↑	92	none
Free cholesterol	mg/dL	ND, ± 20%	32	30	none	92	↑	29	none
Triglyceride	mg/dL	± 25%	89	79	none	84	none	70	none
phospholipid	mg/dL	ND, ± 20%	161	160	none	232	↑	157	none
Free fatty acid	mEQ/L	ND, ± 20%	0.07	0.08	none	0.09	↑	0.14	↑
HDL-C	mg/dL	ND, ± 20%	40	41	none	40	none	43	none
Lipoproteins	mg/dL	± 30%	14	9	↓	13	none	4	↓
K^+	mEQ/L	±0.5 mmol/L	2.4	2.4	none	2.5	none	2.7	none
Ca^{2+}	mg/dL	±0.25 mmol/L	6.1	6.1	none	6.0	none	6.1	none
Inorganic phosphate	mg/dL	ND, ± 20%	1.9	1.9	none	2.3	↑	2.2	none
CRP	mg/dL	ND, ± 20%	≤ 0.02	≤ 0.02	none	0.08	↑	≤ 0.02	none
Ferritin	ng/mL	ND, ± 20%	3.1	2.6	none	2.7	none	2.6	none
Haptoglobin	mg/dL	ND, ± 20%	77	82	none	121	↑	83	none
IgG	mg/dL	± 25%	736	729	none	670	none	748	none
IgM	mg/dL	ND, ± 20%	89	85	none	60	↓	87	none
HBs antigen	IU/mL	positive or negative	< 0.05	< 0.05	none	< 0.05	none	< 0.05	none
HBs antibody	mIU/mL	positive or negative	< 10.0	< 10.0	none	< 10.0	none	< 10.0	none
HCV antibody		positive or negative	0.0	0.0	none	0.0	none	0.0	none

AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GTP: γ—glutamyltranspeptitase, LDH: lactate dehydrogenase, LAP: leucine aminopeptidase, CK: creatine kinase, ChE: cholinesterase, HDL-C: high density lipoprotein cholesterol, CRP: C-reactive protein, HBs: hepatitis B surface, HCV: hepatitis C virus. * IF means interference. (↑) overestimation, (↓) underestimation, (none) no interference.

Table 4. Coagulation fibrinolysis examination

Analytes	Units	CLIA limits	Blood+Saline			Blood+HbV			
			+Saline (Control)	+Dex	IF*	+Saline	IF*	+Dex	IF*
APTT	second	±15%	38.5	41.2	none	Impossible	×	40.8	none
PT	second	± 15%	12.2	12.2	none	Impossible	×	11.9	none
fibrinogen	mg/dL	± 20%	157	149	none	Impossible	×	161	none
ATIII	%	ND, ± 20%	69	67	none	82	↑	70	none
vWF antigen	%	ND, ± 20%	48	34	↓	Impossible	×	43	none
vWF activity	%	ND, ± 20%	52	24	↓	Impossible	×	21	↓
Total PAI-1	ng/mL	ND, ± 20%	6	15	↑	3	↓	12	↑
FDP	μg/mL	ND, ± 20%	≤ 2	≤ 2	none	≤ 2	none	≤ 2	none
D-dimer	μg/mL	ND, ± 20%	0.22	0.2	none	≤ 0.10	↓	0.18	none

APTT: Activated partial thromboplastin time, PT: Prothrombin time, ATIII: AntithrombinⅢ, PAI-1: plasminogen activator inhibitor-1, FDP: fibrinogen degradation products. * IF means interference. (↑) overestimation, (↓) underestimation, (none) no interference.

Table 5. Blood sugar tests

Analytes	Units	Blood+Saline			Blood+HbV			
		+Saline (Control)	+Dex	IF*	+Saline	IF*	+Dex	IF*
Glucose	mg/dL	65	66	none	57	none	69	none

Analytes	Units	Blood+Saline	Blood+HbV	IF
Hb_{A1C}	%	4.8	4.8	none

Hb_{A1C} : glycated hemoglobin. * IF means interference. (↑) overestimation, (↓) underestimation, (none) no interference.

な測定値が得られた．遊離コレステロール，フェリチン，ALT，ASTの干渉作用は試験管によるため，検体数を増やして確認する必要がある．採血管の種類に依らず共通して干渉作用のある項目は，遊離脂肪酸とリポプロテインの２項目であった．血液にDexを添加した検体でもリポプロテインの低下を認めるため，脂質粒子として血漿中に存在するリポプロテインが，Dex添加により凝集して一部が沈降することで低値となったものと考えられる．遊離脂肪酸は血液にHb小胞体とDexを共存させた検体でのみ増大し，血液にHb小胞体あるいは血液にDexを添加した検体では干渉を認めなかった．このことから，Hb小胞体にDexを添加することにより遊離脂肪酸として検出される成分が増大したと考えられ，遊離脂肪酸についてはDexを添加しない方が正確な測定値が得られている．

凝固線溶検査ではHb小胞体の存在によりATIIIで上昇，トータルPAI-1で低下を示し，その他FDP，D-ダイマーを除くすべての検査で測定不能と判断された（Table 4）．一方，Hb小胞体浮遊血液にDexを添加し遠心分離で除去した血漿では，vWF活性の低下とトータルPAI-1の上昇を除き干渉作用を回避できた．この２項目では，Hb小胞体の有無に依らず血液にDexを添加した場合にも同程度のvWF活性の低下とトータルPAI-1の上昇を認めることから，Dexの添加が干渉作用の原因と考えられる．これらの項目については他の方法を模索する必要がある．糖尿病関連検査に関してはグルコース，Hb_{A1C}ともにHb小胞体の影響を受けずに測定可能であった（Table 5）．本研究では，一回の測定であるが多くの項目でHb小胞体の干渉作用を回避できる効果が示された．生化学・免疫学検査や凝固線溶検査は人工酸素運搬体の安全性を評価する上でも重要な検査項目であり[25,26]，今後はこの方法で測定検体を増やし，統計的に信頼性を解析すると共に，Hb小胞体の投与が想定される各種病態における血液検体における評価も必要に応じて実施する必要がある．

4．結論

Hb小胞体が混在する血液検体では，通常の遠心により血液検査に適した血漿を得ることができず，多くの項目で測定が干渉される．一方，Dex（分子量400～500 kDa）の添加（終濃度; 2.6g/dL）により，従来通りの遠心で血清または血漿が得られる．生化学・免疫学検査，凝固線溶検査，糖尿病関連検査の大部分

の検査項目について干渉作用なく測定できることが確認され，Hb小胞体投与後の血液検体検査が容易になると考える．ただし，Dex添加の影響により生化学検査ではリポプロテイン（A）の低下，凝固検査ではvWF活性の低下，トータルPAI-1の上昇が見られているので，これらの項目については注意を要す．

謝辞

本研究は，厚生労働科学研究費補助金（医薬品・医療機器等レギュラトリーサイエンス総合研究事業）によって行われた．記して謝意を表す．

参考文献

1. Ma Z, Monk TG, Goodnough LT, McClellan A, Gawryl M, Clark T, Moreira P, Keipert PE, Scott MG. Effect of hemoglobin- and Perflubron-based oxygen carriers on common clinical laboratory tests. Clin Chem 1997;43:1732-1737.
2. Callas DD, Clark TL, Moreira PL, Lansden C, Gawryl MS, Kahn S, Bermes EW Jr. In vitro effects of a novel hemoglobin-based oxygen carrier on routine chemistry, therapeutic drug, coagulation, hematology, and blood bank assays. Clin Chem 1997;43:1744-1748.
3. Ali AC, Campbell JA. Interference of o-raffinose cross-linked hemoglobin with routine Hitachi 717 assays. Clin Chem 1997;43:1794-1796.
4. Alonsozana GL, Elfath MD, Mackenzie C, Gregory LC, Duh SH, Trump B, Christenson RH. In vitro interference of the red cell substitute pyridoxalated hemoglobin-polyoxyethylene with blood compatibility, coagulation, and clinical chemistry testing. J Cardiothorac Vasc Anesth 1997;11:845-850.
5. Kazmierczak SC, Catrou PG, Best AE, Sullivan SW, Briley KP. Multiple regression analysis of interference effects from a hemoglobin-based oxygen carrier solution. Clin Chem Lab Med 1999;37:453-64.
6. Wolthuis A, Peek D, Scholten R, Moreira P, Gawryl M, Clark T, Westerhuis L. Effect of the hemoglobin-based oxygen carrier HBOC-201 on laboratory instrumentation: cobas integra, chiron blood gas analyzer 840, Sysmex SE-9000 and BCT. Clin Chem Lab Med 1999;37:71-6.
7. Chance JJ, Norris EJ, Kroll MH. Mechanism of interference of a polymerized hemoglobin blood substitute in an alkaline phosphatase method. Clin Chem. 2000;46:1331-1337.
8. 酒井宏水，富山賢一，政田陽平，武岡真司，堀之内宏久，小林紘一，土田英俊．酸素輸液（ヘモグロビン小胞体）を含有する血清の生化学的検査．人工血液 2002;10:47-53.
9. Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, Tsuchida E. Pretreatment of serum containing hemoglobin vesicles (oxygen carriers) to prevent their interference in laboratory tests. Clin Chem Lab Med 2003;41:222-231.
10. Jahr JS, Osgood S, Rothenberg SJ, Li QL, Butch AW, Gunther R, Cheung A, Driessen B. Lactate measurement interference by hemoglobin-based oxygen carriers (Oxyglobin, Hemopure, and Hemolink). Anesth Analg 2005;100:431-436.
11. Osgood SL, Jahr JS, Desai P, Tsukamoto J, Driessen B. Does methemoglobin from oxidized hemoglobin-based oxygen carrier (hemoglobin Glutamer-200) interfere with lactate measurement (YSI 2700 SELECT Biochemistry Analyzer)? Anesth Analg 2005;100:437-9.
12. Björkholm M, Fagrell B, Przybelski R, Winslow N, Young M, Winslow RM. A phase I single blind clinical trial of a new oxygen transport agent (MP4), human hemoglobin modified with maleimide-activated polyethylene glycol. Haematologica 2005;90:505-515.
13. Moon-Massat PF, Tierney JP, Hock KG, Scott MG. Hitachi Hemolytic Index correlates with HBOC-201 concentrations: impact on suppression of analyte results. Clin Biochem 2008;41:432-5.
14. Cameron SJ, Gerhardt G, Engelstad M, Young MA, Norris EJ, Sokoll LJ. Interference in clinical chemistry assays by the hemoglobin-based oxygen carrier, Hemospan ((R)). Clin Biochem 2009;42:221-224.
15. Ali AA, Ali GS, Steinke JM, Shepherd AP. Co-oximetry interference by hemoglobin-based blood substitutes. Anesth Analg 2001;92:863-9.
16. Shepherd AP, Steinke JM. CO-oximetry interference by perflubron emulsion: comparison of hemolyzing and nonhemolyzing instruments. Clin Chem 1998;44:2183-90.
17. Hughes GS, Francom SF, Antal EJ, Adams WJ, Locker PK, Yancey EP, Jacobs EE. Effects of a novel hemoglobin-based oxygen carrier on percent. oxygen saturation as determined with arterial blood gas analysis and pulse oximetry. Ann Emerg Med 1996;27:164-9.
18. Lurie F, Driessen B, Jahr JS, Reynoso R, Gunther RA. Validity of arterial and mixed venous oxygen saturation measurements in a canine hemorrhage model after resuscitation with varying concentrations of hemoglobin-based oxygen carrier. Anesth Analg 2003;96:46-50
19. Chan FP, Jahr JS, Driessen B, Daunt DA, Li KC. Validation of in vivo MR measurement of oxygen saturation after resuscitation with a hemoglobin-based oxygen carrier in a rabbit model. Acad Radiol 2001;8:583-90.
20. 池田達彦，堀之内宏久，井澤菜緒子，泉陽太郎，河野光智，渡辺真純，川村雅文，酒井宏水，土田英俊，小林紘一，Beagle犬

を用いた50％脱血ショックにおけるHb小胞体の蘇生効果および酸素運搬能の評価，第13回日本血液代替物学会アブストラクト，人工血液 2006;14:21.
21. Sunamoto J, Iwamoto K, Kondo H, Shinkai S. Liposomal membranes. VI. Polysaccharide-induced aggregation of multilamellar liposomes of egg lecithin. J Biochem 1980;88:1219-26.
22. Meyuhas D, Nir S, Lichtenberg D. Aggregation of phospholipid vesicles by water-soluble polymers. Biophys J 1996;71:2602-12.
23. Takeoka S, Sou K, Arase S, Ohgushi T, Tsuchida E. Critical molecular weight effects in the aggregation of phospholipid vesicles triggered by water-soluble polymers and an integrated glycolipid. Macromolecules 1996;29:8132-8136.
24. Sakai H, Sato A, Takeoka S, Tsuchida E. Rheological properties of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma-substitute solutions. Langmuir 2007;17;23:8121-8.
25. 高折益彦．人工血液としての条件liposome-encapsulated hemoglobinの有効性，安全性への検討．人工血液 2002;10:28-35.
26. 高折益彦．酸素運搬体の臨床治験へ．人工血液 2004;12:67-73.

AUGUST 2009
Volume 20, Number 8

Bioconjugate Chemistry

REVIEWS

Artificial Oxygen Carriers, Hemoglobin Vesicles and Albumin−Hemes, Based on Bioconjugate Chemistry

Eishun Tsuchida,*,† Keitaro Sou,† Akito Nakagawa,† Hiromi Sakai,† Teruyuki Komatsu,†,‡ and Koichi Kobayashi§

Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan, PRESTO, Japan Science and Technology Agency (JST), and Department of General Thoracic Surgery, School of Medicine, Keio University, Tokyo 160-8582, Japan. Received October 10, 2008; Revised Manuscript Received December 10, 2008

Hemoglobin (Hb, Mw: 64 500) and albumin (Mw: 66 500) are major protein components in our circulatory system. On the basis of bioconjugate chemistry of these proteins, we have synthesized artificial O_2 carriers of two types, which will be useful as transfusion alternatives in clinical situations. Along with sufficient O_2 transporting capability, they show no pathogen, no blood type antigen, biocompatibility, stability, capability for long-term storage, and prompt degradation in vivo. Herein, we present the latest results from our research on these artificial O_2 carriers, Hb-vesicles (HbV) and albumin−hemes. (i) HbV is a cellular type Hb-based O_2 carrier. Phospholipid vesicles (liposomes, 250 nm diameter) encapsulate highly purified and concentrated human Hb (35 g/dL) to mimic the red blood cell (RBC) structure and eliminate side effects of molecular Hb such as vasoconstriction. The particle surface is modified with PEG-conjugated phospholipids, thereby improving blood compatibility and dispersion stability. Manipulation of physicochemical parameters of HbV, such as O_2 binding affinity and suspension rheology, supports the use of HbV for versatile medical applications. (ii) Human serum albumin (HSA) incorporates synthetic Fe^{2+}porphyrin (FeP) to yield unique albumin-based O_2 carriers. Changing the chemical structure of incorporated FeP controls O_2 binding parameters. In fact, PEG-modified HSA-FeP showed good blood compatibility and O_2 transport in vivo. Furthermore, the genetically engineered heme pocket in HSA can confer O_2 binding ability to the incorporated natural Fe^{2+}protoporphyrin IX (heme). The O_2 binding affinity of the recombinant HSA (rHSA)-heme is adjusted to a similar value to that of RBC through optimization of the amino acid residues around the coordinated O_2.

1. INTRODUCTION

Transfusion of donor blood is currently an indispensable routine procedure in modern medical treatments because the risk of transmission of viral illness has became extremely low. Nevertheless, this level of safety has been achieved at great cost and hepatitis virus or unknown pathogens cannot be excluded completely, even by the nucleic acid amplification test (NAT) system. Furthermore, the transfusion of donor blood necessitates cross matching and compatibility tests to avoid a hemolytic reaction in the recipient, and the donated red blood cells (RBCs) must be refrigerated at 4 °C (up to 3 weeks in Japan). These requirements limit the availability of blood transfusion in disaster or emergency situations.

* To whom correspondence should be addressed. Eishun Tsuchida, Ph.D. Phone: +81-3-5286-3120. Fax: +81-3-3205-4740. E-mail: eishun@waseda.jp.
† Waseda University.
‡ JST.
§ Keio University.

10.1021/bc800431d CCC: $40.75
Published on Web 02/10/2009

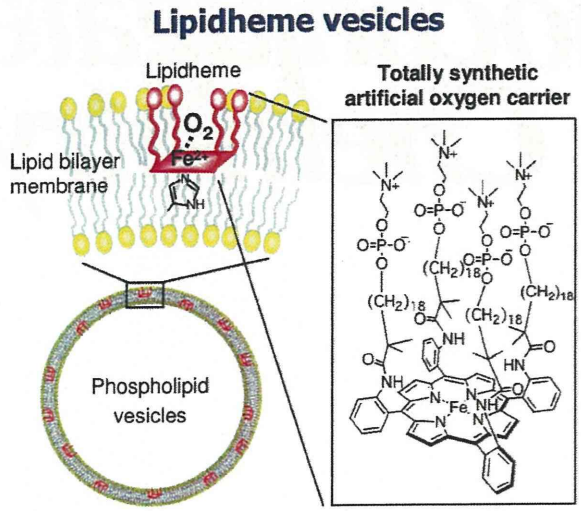

Figure 1. Lipidheme phospholipid vesicles as a totally synthetic artificial O_2 carrier (*7*).

During the past several decades, various artificial O_2 carriers have been synthesized and studied for as RBC substitutes by many scientists in the fields of organic chemistry, inorganic chemistry, biochemistry, and polymer chemistry. These O_2 carriers are classified as perfluorocarbon-based materials, synthetic Fe^{2+}porphyrin-based materials, and Hb-based materials. In this review, we highlight recent developments of our research related to RBC substitutes of the latter two types.

Actually, Hb consists of four polypeptide chains (globin proteins), each of which has an Fe^{2+}protoporphyrin IX (heme) as a prosthetic group. The globin chain forms a compact globular conformation; the heme group is incorporated into the hydrophobic pocket with an axial coordination of histidine (*1*). On exposure of the Hb solution to O_2, the heme forms a stable O_2 adduct complex. However, if the heme is eliminated from the globin wrapping, the heme complex is oxidized immediately and irreversibly to its Fe^{3+} state by proton-driven oxidation or μ-oxo dimer formation (*2*). In the 1970s and 1980s, much research was directed to mimic the O_2 carrier by synthesizing substituted porphyrin derivatives. In aprotic solvents, the proton-driven oxidation is excluded completely. Therefore, the remaining problem is how to suppress irreversible oxidation via dimerization. One successful approach was steric modification of porphyrin. Some superstructured Fe^{2+}porphyrins were prepared using an elegant organic synthesis technique (*2*−*4*). In particular, Collman reported that the tetrakis(α,α,α,α-*o*-pivalamido)phenylporphinatoiron complex with 1-methylimidazole can reversibly bind O_2 in benzene at room temperature (*5, 6*). Nevertheless, these synthetic porphyrins were all oxidized irreversibly in aqueous media.

To create a hydrophobic environment in water, it is possible to use a bilayer membrane of a phospholipid vesicle instead of globin protein. In 1983, we synthesized an amphiphilic Fe^{2+}porphyrin having four alkylphosphocholine groups (lipidheme), which is efficiently embedded into the bilayer of the phospholipid vesicle to yield a homogeneous hybrid. This lipidheme/phospholipid vesicle can bind and release O_2 under physiological conditions (Figure 1) (*7*−*9*). The 10 mM lipidheme/phospholipid vesicle solution dissolves 29 mL O_2/dL compared to 27 mL/dL of human blood. Subsequent to that finding, we synthesized over 60 lipidheme molecules. A new lipidheme having four dialkyl-*sn*-glycerophosphocholine groups is self-organized in water to form self-assembled porphyrin bilayer vesicles without phospholipid (*10*). Furthermore, in 1995, we found that synthetic Fe^{2+} porphyrin bearing a covalently linked proximal base (FeP**1**) is incorporated into human serum albumin (HSA) and the obtained HSA-FeP**1** hybrid coordinates O_2 in aqueous medium (*11*).

In 1985, we began the study of Hb-based O_2 carriers using purified human Hb aiming at the beneficial utilization of outdated RBC to support the present blood donation−transfusion system. This project has been supported for a long time by Japanese Red Cross Society and Ministry of Health and Welfare, Japan. On the basis of a fundamental concept that the cellular structure of RBC is necessary for O_2 transport in the bloodstream, we designed the phospholipid vesicle encapsulating Hb: the so-called Hb-vesicle. It has to be emphasized that the Hb-vesicle comprises a concentrated Hb solution and four kinds of natural and synthetic lipids that assemble to form a hierarchical corpuscle structure (molecular assembly) through the well-regulated secondary interactions, such as hydrophobic and electrostatic interactions. To date, chemically modified Hb of several types have been developed as RBC substitutes or O_2 therapeutic reagents. Herein, we review the latest developments of our research into Hb-vesicles and albumin−hemes.

2. HEMOGLOBIN VESICLES THAT MIMIC THE RBC CELLULAR STRUCTURE

2.1. Physiological Importance of Cellular Structure of RBC for Encapsulated Hb Design. Historically, stroma-free Hb isolated from RBCs were tested as a principal material for carrying O_2. However, the plasma retention time of stroma-free Hb is particularly short (half-life of 0.5−1.5 h) because of the dissociation of the Hb tetramer ($\alpha_2\beta_2$; Mw, 64 500; 6.5 nm diameter) into dimers ($2\alpha\beta$), which are subsequently filtered by the kidney (*12*). Cell-free Hb-based O_2 carriers have been developed to overcome the problems of stroma-free Hb through chemical modification, "bioconjugation", of Hb molecules (Figure 2). They include intramolecularly cross-linked Hb (DCLHb) to prevent dimerization, recombinant cross-linked Hb produced by *E. coli*, polymerized Hb using glutaraldehyde or other cross-linkers, and polymer-conjugated Hb such as PEG-conjugated Hb and polysaccharide-conjugated Hb (*13*−*21*). During the long history of the development of cell-free Hb-based O_2 carriers (HBOCs), the many side effects of stroma-free Hb and chemically modified Hbs have been well-documented: renal toxicity; entrapment of gaseous messenger molecules (NO and CO) inducing vasoconstriction, hypertension, reduced blood flow, and reduced tissue oxygenation at microcirculatory levels (*22*−*25*); neurological disturbances; malfunction of esophageal motor function (*18*); myocardial lesions (*26, 27*); and death (*28*). These side effects of Hb molecules underscore the importance of the large dimension of HBOCs or the RBC cellular structure. Retrospective and recent observations have indicated the main justifications for Hb encapsulation in RBCs: (i) a decreased high colloidal osmotic pressure (*15*); (ii) prevention of the removal of Hb from blood circulation; (iii) prevention of direct contact of toxic Hb molecules and the endothelial lining (*29*); (iv) retardation of reactions with endogenous NO and CO (*24, 25, 30, 31*) (Figure 3); (v) preservation of the chemical environment in cells, such as the concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes; (vi) 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 (*32*); (vii) the RBC cellular structure retards O_2-release in comparison to acellular Hb solutions (*33, 34*), thereby retaining O_2 to peripheral tissues where O_2 is required. For those reasons, the optimal structure of Hb-based O_2 carriers might be to mimic the RBC cellular structure.