Potential Clinical Application of Hemoglobin Vesicles as an Artificial Oxygen Carrier and Carbon Monoxide Carrier

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Introduction

Blood donation and transfusion systems have contributed considerably to human healthcare. However, even in these modern days, blood is unavailable for patients in some situations, for example, in a prehospital situation after a traffic accident or obstetric hemorrhage, in remote rural areas, after natural disasters, and after terrorist attacks. It is therefore necessary to consider blood supply systems for such emergency situations. Moreover, the progressing aging society and the present COVID-19 epidemic strongly influence the collection of sufficient numbers of blood donors [1]. To resolve such blood-related problems, research and development of artificial red cells (hemoglobin vesicles, Hb-V) is going on in Japan, aimed at the realization of a transfusion alternative for clinical use [2]. Outdated donated RBCs can be regenerated as storable artificial red cells that are free from contamination by pathogens and blood type antigens. Immediate injection of Hb-V on site is expected to be able to save lives when blood is unavailable. Our academic consortium has clarified the safety and efficacy of HbV as a transfusion alternative. Moreover, some benefits of HbV compared to RBCs such as small size, stability, and handling suggest other potential clinical applications such as organ preservation fluid, photosensitizer, and CO carriers [3].

Why is encapsulation of Hb necessary? Actually, although Hb is the most abundant protein in blood, Hb is compartmentalized in RBCs with intracellular concentration of about 35 g/dL. In spite of such abundance in blood as a binding site of oxygen, Hb induces various toxicities once it is released from RBCs during blood circulation, such as dissociation into dimers for extravasation [4], renal and neurological toxicities, and vasoconstriction because of high reactivity of Hb with NO as endothelium derived vasorelaxation factor [5]. Moreover, the degraded compounds of heme can be expected to facilitate Fenton reactions to induce peroxidation of unsaturated lipids in cell membranes [6]. These potential toxicities imply the physiological importance of cellular membranes of RBCs for encapsulation. They also show a capability of mimicking the cellular structure for producing Hb-based oxygen carriers (HBOCs). Ultrathin membranes of synthetic polymer and cross-linked protein membrane artificial red blood cells containing Hb and enzymes were prepared in 1950s by Thomas Chang of McGill Univ., and by other groups. Studies of encapsulation of functional molecules with phospholipids started after the discovery of liposomes by Bangham in the 1960s. Djordjevici and Miller in 1977 first reported liposome encapsulated Hb (LEH). Many research groups have since used different lipid species and compositions as attempts to encapsulate Hb using liposomes and to improve their encapsulation efficiency, biocompatibility, stability during storage, and oxygen-carrying capacity [7]. Because of the difficulty in resolving the issues presented above, most groups eventually terminated their development. However, we have continued the research and development of hemoglobin vesicles (HbV) and have clarified their safety and efficacy through abundant preclinical studies. After having full consultation with Pharmaceuticals and Medical Devices Agency (PMDA) and achieving GLP preclinical studies, our academic consortium initiated a phase 1 (first-in-human) study of HbV in 2020.

Preparation of Encapsulated Hb Using Liposomes

The research and development of HBOCs in Japan began in the 1980s with the concept of recycling of unused donated blood. Pyridoxalated Hb polyoxyethylene conjugate (PHP) produced by Ajinomoto Co. Inc. and Neo Red Cells (NRC) produced by Terumo Corp. were developed at that time.

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Because the intraerythrocytic components are not only Hb but also glycolytic enzymes, carbonic anhydrase, metHb reducing enzymes, catalase, superoxide dismutase, etc., some groups tried to preserve such enzymes. However, these enzymes are generally unstable. The enzymatic activities cannot be preserved during an Hb purification process and during a long storage period. Our present concept is to eliminate such unstable enzymes during virus inactivation/removal processes for the utmost safety from infection, even though the donated blood was confirmed as virus-free through specific nucleic acid amplification tests [7]. The processes of Hb purification from outdated donated human blood includes procedures of pasteurization (60 °C, 12 h) and nanofiltration [8], respectively, for virus inactivation and removal. For such purposes, carbonylation of Hb in advance to produce carbonylhemoglobin (HbCO) is effective. HbCO is thermally stable for pasteurization. It can be stored for a long time as a starting material of HBOC production.

We have so many selections of lipids as amphiphiles for Hb encapsulation, such as phospholipids (with various polar head groups, and esterified with various length of saturated or unsaturated fatty acids), cholesterol, and surface modifiers (polymers, charges), as natural or synthetic products, but the lipids should be selected carefully in terms of the encapsulation efficiency, stability during storage and blood circulation, and biocompatibility after intravenous administration. Even though encapsulation can shield the toxicity of molecular Hb, one must be careful about the biocompatibility of the materials for encapsulation. Actually, encapsulated Hb using polymerized phospholipid showed enormous stability during storage, but it did not decompose in reticuloendothelial system (RES) for a long time. Encapsulated Hbs with liposomes containing negatively charged phosphatidyl glycerol or fatty acid induced complement and platelet activation [7]. For Hb encapsulation, we selected a mixture of four lipids: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, 1,5-*O*-dihexadecyl-L-glutamate, and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} [6]. The oxygen affinity (P_{50}) of HbV is adjusted, if necessary, by co-encapsulation of an allosteric effector, pyridoxal 5′-phosphate (PLP), to 9–30 Torr, depending on the usage. Currently, we produce HbV with P_{50} = 15 mmHg by co-encapsulating equimolar PLP to Hb. Therefore, the oxygen dissociation curve is left-shifted in comparison to human RBCs. This formulation is effective for targeted oxygen delivery to tissues where oxygen is needed. The particle size is adjusted to 250–280 nm by extrusion or kneading [9]. The spherical unilamellar structure encapsulating a concentrated Hb solution is confirmed by small-angle X-ray scattering [10]. Actually, HbCO can be converted to HbO_2 by illumination with visible light under an aerobic atmosphere [11]. Finally, the deoxygenated HbV is purged with nitrogen in vials or in plastic bags sealed in aluminum bags for long-term storage. Recent studies have clarified that HbV is useful as a CO carrier for anti-inflammatory and anti-oxidative effects in some pathological conditions. For such uses, carbonyl state CO-HbV without the processes of decarbonylation and deoxygenation is purged with CO gas (Fig. 22.1). A considerable difference exists in colloid osmotic pressure (COP), across the liposomal membrane, between outer saline medium (COP = 0 Torr) and inner concentrated Hb solution (COP = ca. 254 Torr). That pressure difference slightly affects the liposomal membrane fluidity. However, the spherical structure is preserved for a long time without causing hemolysis [12]. The Hb concentration is adjusted to 10 g/dL, which is slightly lower than that of human blood (12–15 g/dL), but much higher than the transfusion threshold, which is known to be 6–7 g/dL in critical patient blood [13]. Usually, HbV is suspended in a physiological saline solution. The outstanding difference of HbV in comparison to chemically modified Hb solutions is the absence of colloid osmotic pressure. These are the same characteristics of RBCs. Some chemically modified Hb solutions exceed the physiological colloid osmotic pressure (20–25 Torr), limiting their Hb concentration. HbV can be suspended in or co-injected with colloidal solutions such as human serum albumin, HES, or modified fluid gelatin solutions to adjust the colloid osmotic pressure [14]. The percentage of the occupied volume of the HbV particles corresponds to about 40–45% (cf., hematocrit of blood is about 40–55%). Therefore, the suspension is a concentrated particle dispersion, similar to RBCs in blood. A certain level of viscosity is expected to be important for inducing shear stress on the vascular wall to facilitate vasorelaxation and blood flow [15].

Potential Clinical Applications of HbV as a Transfusion Alternative

The first distinguished animal experiment of HbV was testing extreme hemodilution up to 90% blood exchange (hematocrit reduction from 50% to 5%) using rats by repeated 1 mL blood withdrawal and 1 mL injection of HbV suspended in 5% albumin, which showed stable hemodynamic and blood gas parameters and tissue oxygenation [16, 17]. These results assured the sufficient oxygen carrying capacity of HbV and became the driving force for HbV R&D.

The ultimate usage of HbV will be to save lives of patients suffering from massive hemorrhage where blood is not available. We conducted various studies of resuscitation from hemorrhagic shock using rodents, rabbits, and beagle dogs by injection of HbV suspended in 5% albumin [3]. The beagle dogs survived for over 1 year and the rats for over 14 days after resuscitation from hemorrhagic shock without major

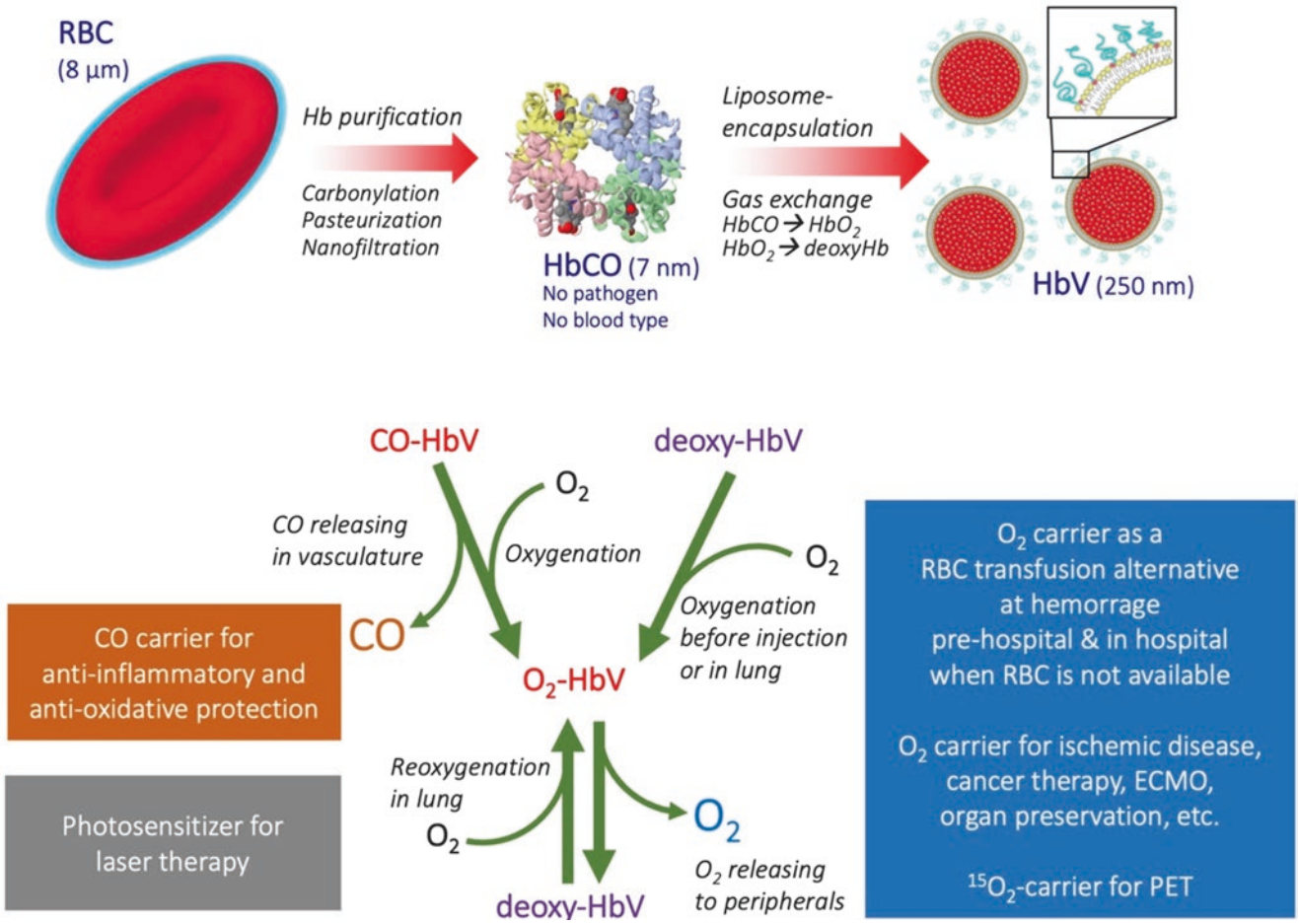

Fig. 22.1 Preparation of hemoglobin vesicles (HbVs) as oxygen and carbon monoxide carriers and a photosensitizer for various clinical applications

side effects except the transient splenomegaly, with an increase in plasma cholesterol levels attributable to the RES trap of HbV and succeeding degradation [18].

Recent studies showed that one-year-stored HbV showed effective resuscitation without showing acute lung injury [19]. Continuous injection of HbV into rats with hemorrhagic shock and uncontrolled hemorrhage, mimicking a condition of prehospital treatment, showed survival even after the hematocrit decreased to less than 2% [20]. Hemorrhagic-shocked rabbits with thrombocytopenic coagulopathy were resuscitated using HbV, with transfusion of platelet rich plasma or artificial platelets. The combination of the oxygen-carrying fluid and hemostatic agent showed effective resuscitative effects without disturbing hemostasis [21, 22]. The small HbV (250 nm) has been proven effective for urgent resuscitation through intraosseous injection when peripheral vessels are collapsed and inaccessible [23]. In addition, HbV infusion has been confirmed as effective for the initial management of massive obstetric hemorrhage in a pregnant rabbit model [24].

Assuming a peri-operational usage of HbV, a pneumonectomy model with hemorrhage of about 30–40% blood volume was prepared using mice and rats; HbV with 5% albumin was injected intravenously [25, 26]. The oxygen-carrying capability of HbV was comparable to that of rodent RBCs, even under impaired lung function after pneumonectomy. In fact, HbV with a high oxygen affinity might have more beneficial effects on oxygenation. Results show that HbV infusion did not interfere with the recovery from surgical injury. Following these promising results, a similar study of pneumonectomy model using beagle dogs is underway by Kohno et al. of Tokai University.

Prolonged hypotension after hemorrhagic shock causes irreversible heart dysfunction called "shock heart syndrome," which is associated with lethal arrhythmias. The standard protocol of hemorrhagic shock resuscitation is to inject crystalloid first, followed by colloid injection, such as HES, gelatin, and dextran, and finally by packed RBC transfusion. This protocol follows a strategy to avoid allogeneic transfusion. However, Takase et al. clarified that the initial resuscitation

with simply crystalloids or colloids, without O_2 carrying capacity, shows higher incidence of lethal arrhythmias than either RBC transfusion or HbV injection. The results indicate that resuscitation with O_2-carrying HbV injection initially should be of primary importance for avoiding shock heart syndrome and for improving the survival rate by preventing electrical remodeling while preserving myocardial structures [27].

Potential Clinical Applications of HbV for Oxygen and Carbon Monoxide Therapeutics

The COVID-19 epidemic has caused grievous damage to pulmonary alveolar structure and subsequent respiratory failure in many patients. Extracorporeal membrane oxygenation (ECMO) has been proven to be an effective therapy for such condition [28]. ECMO requires a fluid to prime the circuit, resulting in dilution of blood. The hemodilution effect increases with smaller body weight. In the case of cardiac surgery using ECMO, the effect on the neurological function of newborn patients is considerable because the decreased oxygen supply during surgery affects brain function. Damage appears after the newborns are grown up. However, observation of a rat model confirmed that ECMO primed with HbV suspended in 5% HSA showed sustained oxygenation and prevented neurocognitive decline, as confirmed with water-maze testing [29]. Moreover, HbV is proven to be effective as an extracorporeal perfusion fluid to carry oxygen for organs for *ex vivo* experimental purpose to observe intestinal peristaltic motion [30]. It is effective for preservation of an amputated rat leg by perfusion with HbV for several hours and then re-implantation to the rat [31]. It is also effective for subnormothermic machine perfusion of pig livers for preservation [32]. HbV is useful as a carrier of $^{15}O_2$ for positron emission tomography (PET). Actually, injection of $^{15}O_2$-HbV into a stroke rat model visualized the lowered oxygen metabolism in the infarcted area in PET images [33].

Because of the smaller size of HbV than of RBCs, the injected HbV particles are distributed homogeneously in the plasma phase. In the microscopic view of microcirculation, plasma skimming is readily observed at a branch of a small artery or arterioles where the hematocrit values of daughter branches differ because of the different blood flow rate in each branch. This condition induces plasma skimming. The branch of a slower blood flow rate shows a lower hematocrit. In such a condition, HbV distributes homogeneously in the plasma phase and distributes more in the branch of a slower blood flow rate. HbV can carry oxygen where RBC flow is limited in the ischemic tissues. It has been demonstrated that intravenous administration of HbV after occlusion of the middle cerebral artery can attenuate the infarction volume [34]. Moreover, HbV can rescue placental hypoxia in a rat pre-eclampsia model [35]. Because of the same mechanism HbV increases the oxygen tension of tumor tissue where the capillary structure is abnormal and RBC flow is considerably limited [36].

HbV is useful not only as an oxygen carrier, but also as a photosensitizer for laser irradiation therapy. Rikihisa et al. reported the utilization of HbV as a photosensitizer, a target of laser treatment of port-wine stain (capillary malformation) [37, 38] because injection of small HbV distributes between the RBCs in capillaries and increases capillary Hb levels effectively, thereby producing more heat and photocoagulation.

Various kinds of CO-releasing molecules (CORM) have been reported: not only metal complexes but also organic compounds [39–41]. They show beneficial cytoprotective effects in animal models of septic shock and ischemia reperfusion injury, and inflammatory diseases. These results were the driving force to test the CO-bound HbV (CO-HbV) as another type of CORM. In fact, CO-HbV was first tested for resuscitation from hemorrhagic shock in a rat model [42]. The blood HbCO increased to about 30% immediately after injection, but it decreased in 3 h. Also, the dissociated CO appeared in the exhaled air simultaneously. Plasma enzyme levels, AST and ALT, were lower than those associated with O_2-HbV injection, indicating that CO ameliorated reperfusion injury. Taguchi et al. applied CO-HbV for models of bleomycin-induced pulmonary fibrosis [43], dextran sulfate sodium-induced colitis [44], and acute pancreatitis [45]. Liberated CO showed marked anti-inflammatory and anti-oxidative properties, probably because of the interaction of CO with hemeproteins related to the production of reactive oxygen and nitrogen species in the body under pathological conditions.

From the viewpoint of production, CO-bound HbV can be produced more easily than deoxygenated HbV because the processes of decarbonylation and deoxygenation are not necessary. After releasing CO in blood circulation, HbV reversibly binds O_2. It thereby becomes an oxygen carrier. CO-HbV is expected to provide unique opportunities for the clinical treatment of various pathological conditions. However, additional studies must be conducted to confirm the absence of the chronic neurological toxicity of CO because CO is known fundamentally as a toxic gas. Its neurological effects are known to manifest later.

Safety Evaluations of HbV

Encapsulation of Hb can shield the toxic effects of Hb. Nevertheless, one must be careful about the toxicity of the capsules and of the liposomal lipid composition. One outstanding advantage of HbV in comparison to conventional

liposome-encapsulated Hb is the absence of complement activation and anaphylactoid reaction, as confirmed through a pig study [46]. Even though the conventional liposomes showed a considerable increase in the pulmonary vascular resistance at repeated injections, HbV with optimized lipid composition showed no such reaction. Preclinical studies of HbV show no significant effect on the complement system, immunological response, blood coagulation, platelet function, kallikrein–kinin, hematopoiesis, etc. [47]. The HbV particles are finally captured by the RES or mononuclear phagocytic system (MPS). Therefore, transient hepatosplenomegaly is observed depending on the HbV dosage [18]. However, the HbV components are degraded completely in RES, and are eliminated and excreted through urine and feces [48]. Our studies using rodent models showed that the macrophages capturing the liposomes become similar to myeloid-derived suppressor cells (MDSCs) which cause suppression of splenic T cell proliferation [49, 50], though the response is transiently observed and its effect is minimal. Reportedly recent studies of PEGylated liposomes for cancer therapy and other PEGylated materials clarified that the presence of anti-PEG antibody is frequently observed in humans. It might change the pharmacokinetics of the agents (ABC phenomenon) [48] or induce immunological reactions [51]. This point is expected to demand some attention in the ongoing clinical research and development of HbV.

Summary

Including liposome-encapsulated Hb, recent trends in the development of HBOCs are to make them larger [52–60] to prevent extravasation, to retard reactions with NO [61], and to lower the colloid osmotic pressure [62]. However, not only the particle design, but also the design of its suspension in terms of physicochemical properties such as Hb concentration, colloid osmotic pressure, and viscosity are important factors that must be examined to make the fluid function as a blood substitute. As described in this chapter, we summarized potential clinical applications of HbV evidenced using various animal experimental models. Of course, other HBOCs have some potential to be used in the same manner.

An ultimate and optimal usage of HBOCs, taking the advantages of their characteristics superior to packed RBCs, should be for saving lives of lethal patients of massive bleeding and trauma when blood for transfusion is not available. However, it is difficult to obtain an informed consent from a lethal patient. Accordingly, clinical studies in the next stage would be limited to injections to patients with perioperational bleeding at elective surgeries or patients with post-operational anemia. A success of such study will eventually enable various usage of HBOCs for any purposes.

Key Points

- Encapsulated Hbs, including HbV, have been designed to mimic the cellular structure of RBCs for eliminating the toxic effect of molecular Hbs.
- However, it is important to consider the capsule material safety as well, especially on the absence of complement activation, dispersibility in blood, and degradation in RES.
- Safety and efficacy of HbV as a transfusion alternative have been evaluated in hemorrhagic shock-resuscitation preclinical studies.
- HbV has been tested not only as a transfusion alternative, but also as a new agent for oxygen therapeutics, a perfusate for organ preservation, and a photosensitizer for laser therapy.
- CO-HbV liberates CO in blood circulation and shows anti-oxidative and anti-inflammatory effects. CO-HbV will be a new promising agent though its neurological effect has to be examined carefully.

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Conflict of Interest Of the authors, H.S. is a holder of the patents related to the production and utilization of HbV.

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