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STABILIZED HEMOGLOBIN VESICLES

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ABSTRACT

The Hb-vesicles which encapsulate the purified and concentrated Hb more than 40 g/dl with a uni- or bi-lamellar membrane are prepared by extruding the dispersion of mixed lipids through membrane filters (final pore size: 0.2 μmϕ). They transport large amount of oxygen with satisfying rheological properties such as oncotic pressure and solution viscosity. Oxygen affinity of the Hb-vesicles is adjusted so as to exceed the ability of oxygen transport of human blood by coencapsulating allosteric effectors in the Hb-vesicles. The solution is sterilizable because of the diameter of Hb vesicles less than 0.2 μmϕ. The Hb-vesicles are stabilized by using polyphospholipid or glycolipid as membrane components. No change in oxygen affinity and particle size was confirmed during long time storage at 4 °C. The stabilized Hb-vesicles can also be stored as frozen or dried state. The dried Hb-vesicles are regenerated by simply adding pure water. Simple *in vitro* test indicates that Hb-vesicles have the reduced inhibitory action of Hb to the EDRF-mediated vasorelaxation.

INTRODUCTION

Hb-vesicles are the concentrated SFHb encapsulated with thin lipid membrane like a red cell. They have many advantages which are expected to overcome the problems of the blood substitutes based on modified Hb.

Advantages of the Hb-vesicles come from the encapsulation of concentrated Hb with thin phospholipid membrane. Oncotic pressure is very low and

adjustable to human blood(ca. 25 Torr). Solution viscosity is also lower than blood (ca 4.5 cp) and adjustable. Oxygen affinity of Hb is controlled by coencapsulating allosteric effectors. The advantage of none chemically modified Hb is quite obvious. Furthermore, direct contact and actions of stripped Hb to tissues are reduced by this encapsulation.

However, the following issues have been remained to be unsolved in conventional Hb-vesicles. (i) Conventional Hb-vesicles have particle size of ca. 0.8 - 0.4 μmϕ and large size distribution [1-3]. Therefore, sterilization, for example, with 0.2 μmϕ pore size membrane filter is impossible. (ii) The phospholipid vesicles disappear from blood stream through fast RES clearance, which is relating to the particle size and dose amount. Particle size less than 0.2 μmϕ with maintaining the inside high Hb concentration is essential for Hb-vesicles. In addition, reduction of the administered lipid amount is an important factor to reduce the influence of vesicle on RES. The development of encapsulation techniques will be described in detail at another paper on this symposium. (iii) Aggregation and fusion of Hb-vesicles and leakage of Hb occur by long term standing or outer stimuli, since a phospholipid vesicle is an assembly of lipids by hydrophobic interaction. This instability of Hb-vesicles *in vitro* and *vivo* is expected to be overcome by using two kinds of special lipids. One is polyphospholipid obtained by polymerization of unsaturated phospholipid as Hb-vesicles [4-6]. The phospholipid molecules are connected by covalent bond to promise high structural stability of the vesicle. The other is glycolipid having oligosaccharide chain [7, 8]. The saccharide chains extend upon the surface of Hb-vesicles and prevent the aggregation of vesicles. The vesicles covered with oligosaccharide chains show high stability even in dry state by hydrogen bond between saccharide chain and vesicular surface.

This paper describes the preparation and performances of stabilized Hb-vesicles from engineering side.

MATERIALS AND METHODS

Purification and adjustment of Hb

Firstly, HbO_2 was converted to HbCO in RBC prior to purification procedure because HbCO is stable against oxidation and denaturation of Hb during solvent treatment and heating [9]. Then, hemolysis was carried out by mixing RBC with organic solvents such as dichloromethane or hexane without dilution of the Hb

solution. The organic solvent layer with stromata was separated easily by mild centrifugation (1,900g, 15 min). The resulting hemolysate was heated at 60 °C for more than 1 hour in order to denature water-soluble proteins other than HbCO. At the same time, pasteurization is also performed if necessary. After separation of the denaturated proteins by centrifugation (1,900g, 20 min), purified Hb of which concentration about 25 g/dl was obtained. Water-soluble low molecular weight compounds were removed by dialysis, and then osmolarity, pH and other properties of the Hb solution were adjusted by additives.

The HbCO was concentrated to ca. 40 g/dl with a hollow fiber module of which cut off molecular weight was less than 30,000, and allosteric effector was added to the Hb solution.

Preparation of Hb-vesicles

Phospholipid, cholesterol, and fatty acid are mixed in an organic solvent at a mixing molar ratio of 7/7/2 and freeze-dried from benzene. For the stabilized Hb-vesicle systems, polymerizable unsaturated phospholipid was used, or glycolipid was added to the lipid mixture in organic solvents [8]. The powder was added to a HbCO solution (ca. 40 g/dl) and dispersed by mechanical mixing at 4 °C. The resulting large multilamellar vesicles were converted to uni- or oligolamellar vesicles as changing the pore size of polycarbonate membrane filter until 0.2 μmϕ [10]. Hb molecules which were not encapsulated were removed by dialysis with a hollow fiber module. Decarbonylation of HbCO was carried out after the preparation of Hb-vesicles. HbCO is easily and completely converted to HbO_2 in the Hb-vesicle by irradiating visible light onto the hollow fiber module, and sterilized air passes through outer side of the fiber and the Hb-vesicle dispersion through the inner side. The procedure was completed within 20 min. Concentration of the Hb-vesicles was performed by using the same hollow fiber module. The Hb-vesicles with unsaturated phospholipid were polymerized by γ-ray irradiation (total dose: 0.75 Mrad, 4 °C) [4, 5].

Characterization of Hb-vesicles

The size distribution was measured with a particle analyzer (COULTER N4-SD) and transmission electron micrograph (negative stain method). The oxygen-binding curves were obtained with a Hemox-analyzer (TCS-Medical Products) in a Hemox solution (pH7.4, 37 °C). Leakage of Hb from the Hb-vesi-

cles was calculated by Hb concentrations before and after subjecting a Hb-vesicle dispersion to gel permeation chromatography.

Inhibitory Activity of EDRF-mediated Vasorelaxation [11]

Thoracic aortic strips of 3 mm in width were prepared from New Zealand albino rabbits of either sex, 2.0-2.5 kg, and mounted in an organ bath under 1 g of tension in a Tyrode solution (37 °C) of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 2.1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.6 mM, pH7.4 aerated with room air. Changes in isomeric force were recorded on a polygraph. The tissues were precontracted with phenylephrine (PE, 1 μM), and acetylcholine (ACh, 1 μM) was added to elicit a steady-state relaxation. Concentration-response curves to Hb were then constructed by adding cumulative concentrations of Hb solutions (from 10 ng/ml to 1 mg/ml). The response to each concentration was expressed as a percentage of the maximal relaxation induced by ACh.

RESULTS AND DISCUSSION

Purification of Concentrated Hb

Protein purity of our purified Hb was 99.95 %, and the percentages of residual phospholipids were less than 0.2 % [9]. The metHb was less than 1.0 %. No change was observed in the oxygen-binding curves before and after purification.

The Hb solution concentrated above 40 g/dl shows the excellent passing ability through membrane filters with pore size of 0.2 μmϕ, that proves the high purity and is very important for the preparation of Hb-vesicles with the following extrusion procedure.

Properties of Hb-vesicles

The size of Hb-vesicles was reduced to 0.2 μmϕ by extruding the dispersion of lipid mixture in a concentrated Hb solution through 0.2 μmϕ pore size polycarbonate membrane filter. The resulting vesicles had a mean diameter of 195 ± 37 nmϕ (ca. 0.2 μmϕ) with narrow size distribution. This Hb-vesicle dispersion is sterilizable by filtrating it through 0.2 μmϕ pore size filter before use. Concentration of endotoxin in the final product measured by chromogenic assay

with a TOXICOLOUR system (Seikagaku Co., Japan) was negligible (< 0.01 EU/ml). The HbCO was less than 1.0 % after decarbonylation.

Oxygen affinity of the encapsulated Hb is easily controlled by coencapsulating an allosteric effector such as pyridoxal 5'-phosphate and ions such as chloride ion and proton. On the assumption of oxygen-transporting efficiency (OTE) as the difference in oxygen-binding percentages between the oxygen partial pressure of 110 Torr at lung and 40 Torr at mixed vein, this value is estimated from oxygen saturation curve, namely P_{50} and Hill coefficient. Normal red cells with P_{50} (28 Torr) and Hill coefficient (2.8) to yield OTE 28 %. In our Hb-vesicles, P_{50} and OTE could be adjusted not only exactly the same as those of RBC, but also superior to RBC. For example, P_{50} values were from 35 to 40 Torr, and Hill coefficient around 2.2. OTE was more than 40 % as shown in FIG. 1.

Oxygen transporting amount of Hb-vesicles (ml/100ml) is determined from the equation (1).

O_2 Transport of Hb-vesicles (ml/100ml) = [bound O_2 (ml)/Hb(g)]
x oxygen transporting efficiency (%) x ([Hb]/[Lipid]) x [Lipid] - - - (1)

The unit of concentration is (g/100 ml). Oxygen transporting efficiency was optimized by coencapsulating allosteric effectors. The [Hb]/[Lipid] ratio is determined from the Hb concentration in the interior of Hb-vesicle and the number of bilayer membrane. The Hb concentration used in the experiment is as high as possible. Therefore, the [Hb]/[Lipid] ratio is owing to the number of bilayer membranes. Unilamellar Hb-vesicle is an ideal structure, which enables to reduce the dose amount of lipids significantly. The number of bilayers was controlled by the conditions of the feed Hb solution such as ionic strength, pH, temperature, *etc.*, because Hb and lipids are kinds of electrolytes. The Hb-vesicle having the [Hb]/[Lipid] ratio of 2.4 was obtained. This means the encapsulation of about 40 g/dl of Hb within the unilamellar vesicles.

Solution Properties

Oncotic pressure of a SFHb solution is relatively high and exceeds that of human blood (ca. 25 Torr) when the concentration of Hb is above 8 g/dl. On the other hand, that of Hb-vesicles themselves is less than 1 Torr because about 14,000 Hb molecules are incorporated into the inside of one vesicle. Additionally,

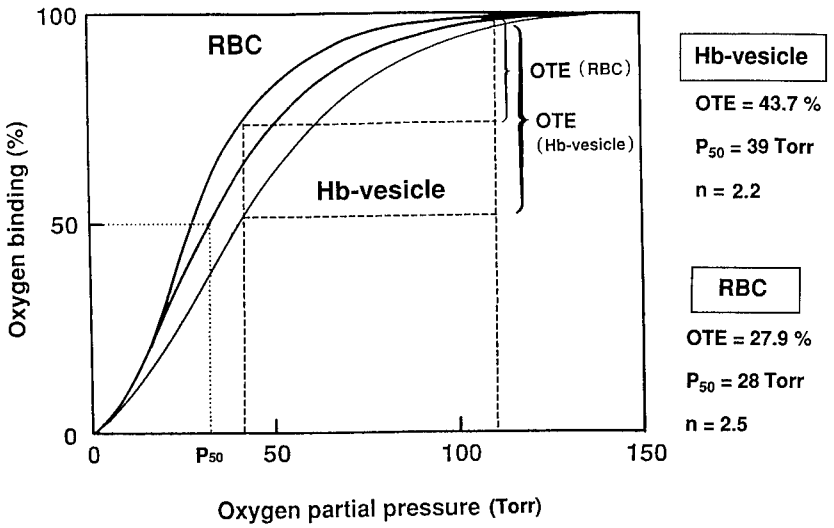

FIGURE 1
Oxygen binding properties (37 °C, pH 7.4) of Hb-vesicles controlled by coencapsulation of allosteric effectors.

oncotic pressure of Hb-vesicles is easily adjustable to that of human blood by additives such as albumin (5.6 wt%) or dextran with average molecular weight of 40,000 (2.2wt%).

Solution viscosity of human blood is about 4.5 cp. While, the viscosity of a Hb-vesicle dispersion is very low and increases slightly from 1 to 2 cp with the concentration of vesicles. Even after albumin, for example, was added to adjust the oncotic pressure to 25 Torr, the solution viscosity of Hb-vesicles is still lower than that of human blood. These solution properties are advantages of the Hb-vesicles.

Stability of the Stabilized Hb-vesicles

Storage at 4 °C or in frozen state: Oxygen binding properties of Hb-vesicles (P_{50}, n, and OTE) were maintained during long term storage at 4 °C over 2 months [4]. That indicates no leakage of allosteric effectors from the Hb-vesicle. However, 10 % of Hb was converted to met-Hb. But this metHb formation could be avoided by storing Hb-vesicles in the frozen state for such long term storage.

Excellent stability of our stabilized Hb-vesicles against freeze-thawing repetition guarantees storage in a freezer [4, 6]. No leakage of Hb and no change in the vesicular size was observed even after 10 freeze-thawing repetition, whereas the vesicular size and the leakage amount of Hb significantly changed after only one repetition for conventional Hb-vesicles.

Storage as freeze-dried powder: The dispersion of Hb-vesicles stabilized with polyphospholipid could also be freeze-dried [5], that had been estimated to be impossible for the conventional Hb-vesicles without the addition of disaccharides [12]. The powder was then dissolved into water as shown in FIG. 2. In the absence of sucrose, the particle size measured with a particle analyzer changed without leakage of Hb. It means that the polyphospholipid vesicles are stable even in the absence of sucrose but do not disperse spontaneously and completely. Therefore, a small amount of sucrose (50 mM) was added to the dispersion of polyphospholipid vesicles before freeze-drying in order to enhance the dispersion of the freeze-dried powder. In the case of conventional Hb-vesicles, more than 80 % of Hb leaked out with the particle size distribution becoming broader.

Storage as desiccated flakes: To the dispersion of Hb-vesicles modified with oligosaccharide by glycolipid, 100 mM of sucrose was added. It was desiccated over P_2O_5 and then dried *in vacuo* to yield flakes of Hb-vesicles. The Hb-vesicles without glycolipid leaked 11 % of Hb after redispersion of the flakes. While the incorporation of 8 mol% of glycolipid reduces Hb-leakage less than 3 %. Size distribution was also preserved effectively by glycolipid. The stability is probably caused by effective covering of the vesicular surface with the oligosaccharide chains through hydrogen bond.

Suppression of the aggregation of Hb-vesicles by glycolipid: The mixtures of phospholipid/cholesterol/negatively-charged lipid are usually used to provide stable vesicles. However, this kind of vesicles easily aggregates in the presence of Ca^{2+} ion because Ca^{2+} binds on the negatively-charged surface and crosslinks vesicles [8]. As shown in FIG. 3, an increase in the viscosity at low shear rates in the presence of Ca^{2+} means the aggregation of vesicles. The Hb-vesicles with glycolipid showed little increase in the viscosity. The same profiles were also observed after the addition of dextran. The Hb-vesicles containing

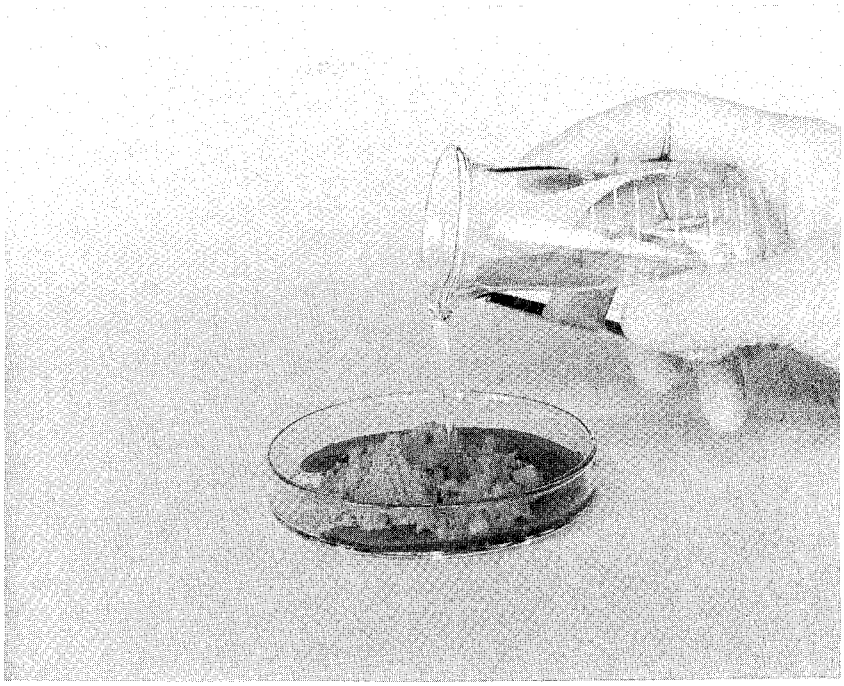

FIGURE 2
A picture of the powder of the stabilized Hb-vesicles(polyphospholipid).

glycolipids maintained the low viscosity in comparison with the vesicles without glycolipids. This suppression of vesicular aggregation is considered to be that the oligosaccharide chains extending form the surface exclude access of the vesicles.

Reduction of EDRF-mediated Vasorelaxation [11]

Endothelium-derived relaxing factor (EDRF) is considered to be nitric oxide (NO), and the abnormal elevation of blood pressure after the administration of modified Hb attributes to the high reactivity of stripped Hb with NO released from endothelium cells.

On the other hand, RBCs which also contain high concentrated Hb do not cause such a problem. The difference seems to be due to the encapsulation of Hb

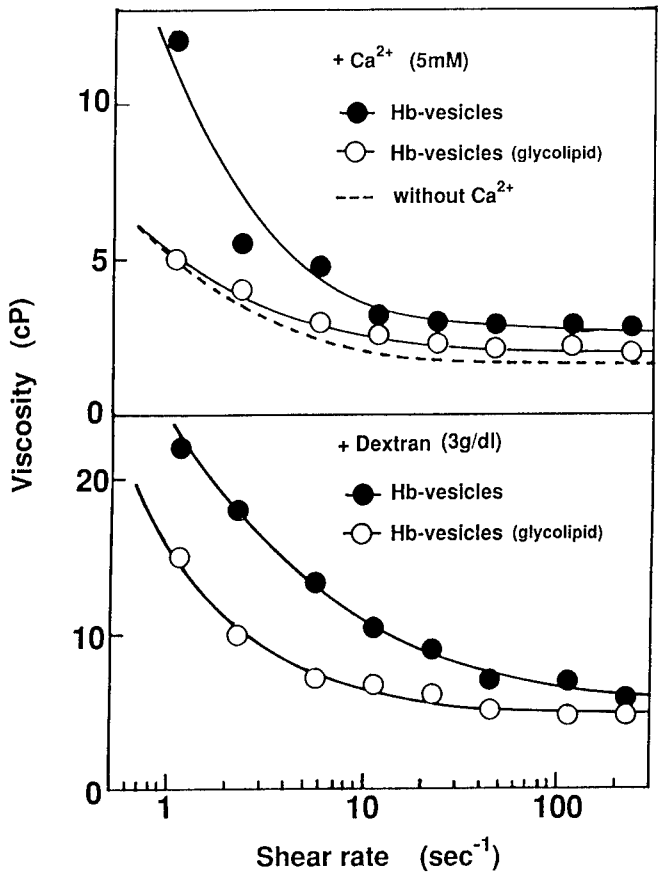

FIGURE 3
Stability of Hb-vesicles (glycolipid) against the aggregation of vesicles after the addition of Ca^{2+} or dextran ($\bar{M}n.$ = 40,000).

within a vesicle. The *in vitro* inhibitory activity of Hb-vesicles to EDRF-induced vasorelaxation in comparison with stripped Hb and RBCs was studied under the same Hb concentration. In all cases, the inhibitory action of vasorelaxation appeared, but the Hb concentration to yield the action was different in each sample. FIGURE 4 clearly indicates the effect of Hb encapsulation. Encapsulated Hbs, namely both RBCs and the Hb-vesicles, show nearly hundred times less inhibitory activity to ACh-induced relaxation.

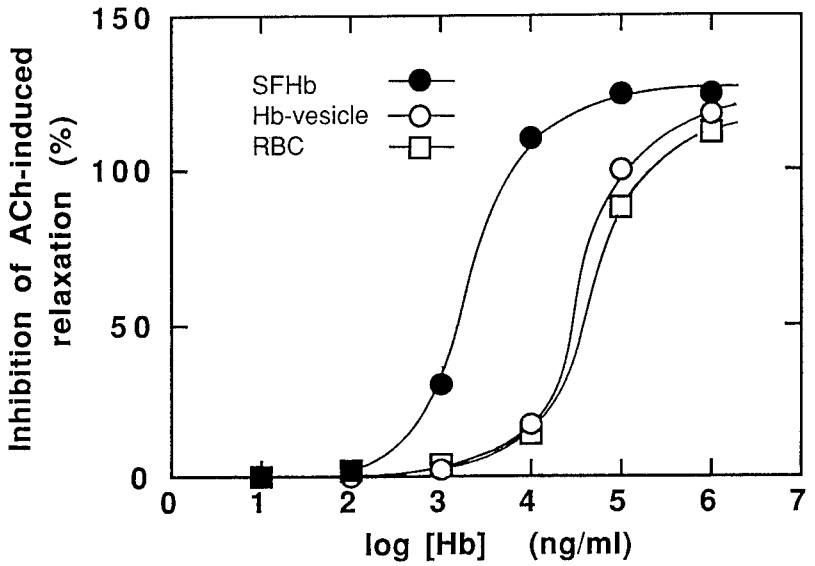

FIGURE 4
Inhibition of ACh-induced relaxation by the addition of solutions containing Hb.

CONCLUSION

The Hb-vesicles with high quality are prepared by extrusion. The properties of Hb-vesicles are: (i) oncotic pressure and solution viscosity of the Hb-vesicle solution are low and adjustable to human blood, (ii) the Hb-vesicles using polyphospholipids or glycolipids show excellent stability against freezing or drying, (iii) the Hb-vesicles show less inhibitory activity to EDRF-mediated vasorelaxation to the same value as RBC.

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