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Preparation of hemoglobin-containing liposomes using octyl glucoside and octyltetraoxyethylene

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Hemoglobin (Hb) was encapsulated in phosphatidylcholine vesicles by removal of the detergent *n*-octyl β-D-glucoside (OG) or *n*-octyltetraoxyethylene (C_8E_4) out of mixed detergent-lipid micelles in Hb solution. Three types of apparatus were used for dialysis. Dialysis buffer flow rates, the surface area of the dialysis membranes, and detergent–lipid interactions determined the rate of dialysis, which influenced liposome size and lamellarity. Slow dialysis led to the formation of multilamellar liposomes, at increased dialysis rates Hb liposomes became smaller and unilamellar. Hb was enclosed at highest concentrations in larger liposomes, which included the negatively charged lipid phosphatidylserine or phosphatidic acid as a membrane component. Co-encapsulation of the allosteric factor inositol hexaphosphate led to oxygen dissociation curve values almost identical to those of whole blood. The oxygen-release capacity of Hb liposome suspensions in the physiological partial pressure range was comparable to whole blood. Storage of Hb liposomes for 2 months leaves oxygen-carrying characteristics virtually unchanged, with met-Hb levels increasing to only 11% of total Hb. Preparation of Hb liposomes by dialysis of octyl glucoside or C_8E_4 is a mild and efficient method for encapsulation of Hb. Since these Hb liposomes can be produced in scale-up batch sizes, they are a candidate for use as an oxygen-carrying blood surrogate.

Introduction

There is need for an oxygen-carrying blood substitute, to be available in emergency situations, that meets the following specifications. (1) The blood surrogate should have no toxic or antigenic properties, and should be biodegradable; (2) disease transmission should be excluded; (3) storage stability should exceed 12 months; (4) the blood surrogate should contain high amounts of functional hemoglobin, with an oxygen-release capacity comparable to that of human erythrocytes, to ensure adequate supply of oxygen at normal oxygen partial pressures. For this purpose, stroma-free hemoglobin must be re-encapsulated together with an allosteric factor that regulates oxygen dissociation. Furthermore, in order to prevent degradation of hemoglobin (Hb) to its physiologically ineffective met-Hb form, a mild method must be employed for encapsulation.

With these goals, Hb has been enclosed in lipid vesicles by several methods [1–6]. Hb-containing liposomes do not have the antigenic properties of erythrocytes, and their components, lipids and hemoglobin are biodegradable. Isolation of Hb minimizes the risk of disease transmission. However, degradation of Hb to its physiologically ineffective met-Hb form and storage stability represent major problems for previous methods, as well as the production of scale-up sized batches under sterile conditions.

Detergent dialysis has proved to be a method of encapsulation that is very gentle to the hemoglobin when nonionic detergents are used [7]. The detergent *n*-octyl β-D-glucoside has been repeatedly used as a solubilizing agent for membrane proteins [8–11], and for membrane reconstitution experiments, such as the production of large unilamellar vesicles with incorporated glycophorin [12]. According to our own observations, the detergent *n*-octyltetraoxyethylene also has no adverse effects on tetrameric structure and function of Hb.

Abbreviations: OG, *n*-octyl β-D-glucoside; ODC, oxygen-dissociation curve; ORC, oxygen-release capacity; PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine, IHP, inositol hexaphosphoric acid.

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Using detergent dialysis, unilamellar liposomes of controllable dimensions can be formed [13]. However, the presence of Hb during dialysis influences the dialysis process, and consequently liposome characteristics. In this study, different dialysis methods for preparation of Hb liposomes from mixed micellar suspensions are presented and compared. Oxygen-dissociation curves and shelf lives of Hb liposome suspensions are characterized. A scale-up method is described.

Materials and Methods

Phosphatidylcholine from fresh egg yolk (PC), cholesterol, phosphatidylserine from bovine brain (PS), and phosphatidic acid from egg yolk phosphatidylcholine (PA) were purchased from Sigma Chemical Co., St. Louis, MO; *n*-octyl β-D-glucoside (OG) from Calbiochem, Frankfurt, F.R.G.; *n*-octyltetraoxyethylene (C_8E_4) from Bachem Feinchemikalien, Bubendorf, Switzerland; α-tocopherol from Serva, Heidelberg. Radioactively labeled substances were obtained from NEN Chemicals, Dreieich, F.R.G.

Lipids and detergent (OG or C_8E_4) were dissolved together with α-tocopherol (1–5 wt.% of the lipid component) in chloroform/methanol. The mixture was dried in a round-bottom flask on a rotary evaporator, and last traces of the solvent were removed under high vacuum.

Stroma-free hemoglobin (Hb) was prepared from outdated human blood according to the method of DeVenuto et al. [14]. The resulting solutions, containing 10–15 g Hb/dl, could be adjusted to the desired Hb concentration by ultrafiltration in membrane cones (Amicon, Danvers, MA) or dilution with isotonic phosphate buffer (0.01 M phosphate/0.15 M NaCl (pH 7.4)). All Hb solutions were filtered through a 0.22 μm sterile filter (Millipore, Molsheim, France) before use. Gentamycin (Biochrom, Berlin) was added at a concentration of 1 mg/10 ml to retard bacterial growth. Final hemoglobin solutions contained 2–3% met-Hb.

Hb concentrations and the percentage of met-Hb were determined by spectrophotometric analysis (Spektralphotometer DMR 10, Zeiss), with the help of the equations of Benesch et al. [15]. Absorbance measurements at 540, 560 and 576 nm allowed the determination of the total molar Hb concentrations. Met-Hb proportions were assayed by absorbance measurements at 560, 576 and 630 nm.

Hb was encapsulated following the method of fast and controlled dialysis of mixed detergent/lipid micelles for preparation of liposomes [16]. Three types of dialysis apparatus were used: Lipoprep (Diachema, Langnau, Switzerland), Liposomat (Diachema), and hollow fiber cartridges (Nippon Medical, Japan). Lipoprep and Liposomat were equipped with highly permeable dialysis membranes with a cutoff of 10 kDa (Diachema).

Hb solutions were added to the dry lipid/detergent mixture to yield a lipid concentration of 17 mmol/l, with a lipid/detergent ratio of 0.2 mol/mol. The mixed micelle solutions were dialyzed for at least 14 h against phosphate buffer at room temperature. OG concentrations during dialysis were determined with radioactively labeled OG ([*glucose*-U-^{14}C]OG) by the radioactivity in the dialysis buffer. C_8E_4 was extracted out of the mixed micelle or liposome solutions following the first step of extraction according to Bligh and Dyer [17], separated from the lipids by thin-layer chromatography, and quantified photometrically using Dragendorff's reagent for polyethyleneglycols.

After dialysis, nonencapsulated hemoglobin was separated from the finished liposomes on a Sepharose 4B-CL column (Pharmacia, Uppsala, Sweden), equilibrated with phosphate buffer. The presence of two separate Hb fractions on the Sepharose 4B-CL column served as the first criterion for Hb liposome formation, since the first fraction, the void volume, also contained all of the lipid.

Hemoglobin concentrations in liposome solutions were measured after reconversion of the liposomal membranes to micelles with OG, in order to avoid vesicle light-scattering. Starting lipid concentrations (without Hb) were calculated by phosphate measurement [18]. Radioactive compounds ([^{14}C]methylated met-Hb, L-α-dipalmitoyl[2-*palmitoyl*-9,10(*n*)-^{3}H]phosphatidylcholine) were co-encapsulated to determine Hb/lipid ratios.

In order to modify the oxygen affinity of the stroma-free Hb to that in erythrocytes, inositol hexaphosphoric acid (IHP; BDH, Poole, U.K.) was added in equimolar amounts to the hemoglobin solution and in the same concentration to the first 3 l of dialysis buffer, during preparation of the liposomes. Oxygen-dissociation curves were generated by measurements in the Erythrox (Technische Universität Aachen, F.R.G.).

Liposome size was determined by laser autocorrelation spectroscopy at a scattering angle of 90° (Nanosizer, Coulter Electronics, Harpenden, U.K.).

Electron micrographs were used for determination of size distribution and lamellarity of the liposomes. The liposome samples were prepared for electron microscopy by freeze-fracture and negative staining, as well as the thin-section method of Hamilton et al. [19].

Results

Hb liposome preparation using the Lipoprep

PC-OG mixed micelles in Hb solution were dialyzed against phosphate buffer with a buffer flow rate of 100 ml/h. As determined by measurement of radioactive OG in the dialysis buffer, OG removal during dialysis was slow in comparison to the other methods. Approx. 3 h were needed for liposome formation.

The Hb liposomes were multilamellar, as electron micrographs revealed, with an average size of 200 nm. Nanosizer polydispersity values ranged from 1 to 3, indicating the liposomes were homogeneous. The molar Hb/lipid ratio was approx. 1 : 270.

Hb encapsulation was reduced when cholesterol was included in the lipid component. With increasing cholesterol content the amount of Hb encapsulated became less. At molar contents of 12% cholesterol or more, multilamellar aggregates were formed that contained no Hb.

Hb liposome preparation using the Liposomat

In compliance with the Lipoprep method, PC liposomes were prepared by dialysis of OG. The buffer flow rate was 150 ml/h. Due to the larger dialysis membrane surface area of the Liposomat and the increased buffer flow rate, OG removal during dialysis was quicker. Liposomes were formed after approx. 1 h of dialysis.

The Hb liposomes thus prepared were homogeneous in size and mostly unilamellar (Fig. 1), with a polydispersity index of 1–2 and an average size of 180 nm. The molar Hb/lipid ratio for these liposomes was approx. 1 : 280.

Hb trapping was compared for different starting Hb concentration. The results are presented in Table I.

Slight variations of the starting lipid concentration had no measurable effect on trapping efficiency. Nevertheless, very low lipid concentrations (below 10 mM) in the starting micelle solution led to reduced entrapment of Hb. At starting lipid concentrations above 25–30 mM, lamellarity of the liposomes increased, also resulting in reduced trapping efficiency.

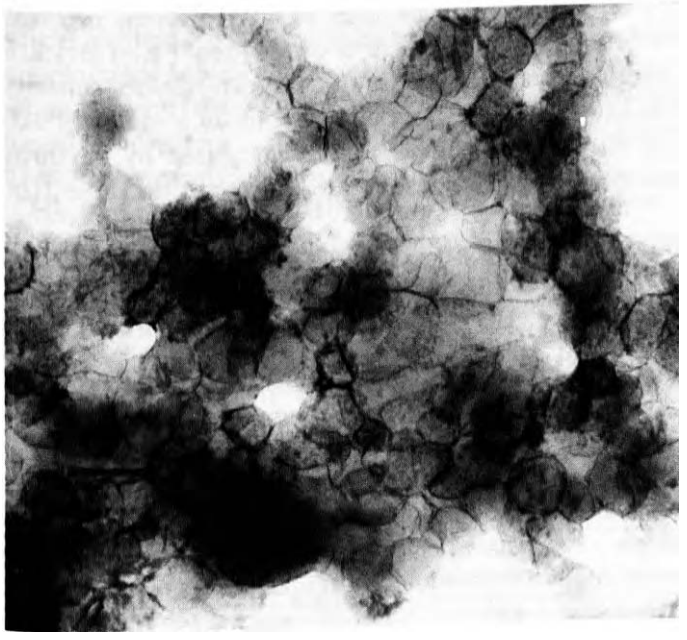

Fig. 1. Hb liposomes prepared by the Liposomat method, using OG. Electron micrograph: negative staining. Bar represents 100 nm.

TABLE I

Effect of starting Hb concentrations on the amount of Hb entrapped in the liposomes (Liposomat method)

Starting Hb solution (g Hb/dl)	Diluted liposome suspension (mg Hb/ml)
5.2	0.4
7.5	0.8
11.1	1.0
13.2	1.1
15.5	1.2
18.3	0.9
21.1	0.8
24.5	0.7

Hb liposome preparation with hollow fiber cartridges

This method permits scaling up of Hb liposome batch sizes to 100 ml or more. PC/OG liposomes prepared by this method were similar to those prepared in the Liposomat, also unilamellar, but a little smaller, with an average size of 160 nm (polydispersity 1–2). The molar Hb/lipid ratio was approx. 1 : 280.

In the same way, vesicles were prepared including 10% of negatively charged lipid (PS or PA) in their lipid component, using C_8E_4 as a detergent. For characterization of this dialysis process, C_8E_4 in the Hb/mixed micelle or Hb/liposome solutions during dialysis was quantified. The results are presented in Fig. 2, indicating that hollow fiber dialysis is very effective, with the detergent being removed very quickly. Liposomes were formed during the first passage through the hollow fiber cartridge. Hb liposomes prepared by this method were uni- to multilamellar and less than homogeneous in size (polydispersity 3–4), with an average size of 450 nm (Fig. 3). The molar Hb/lipid ratio, however, was

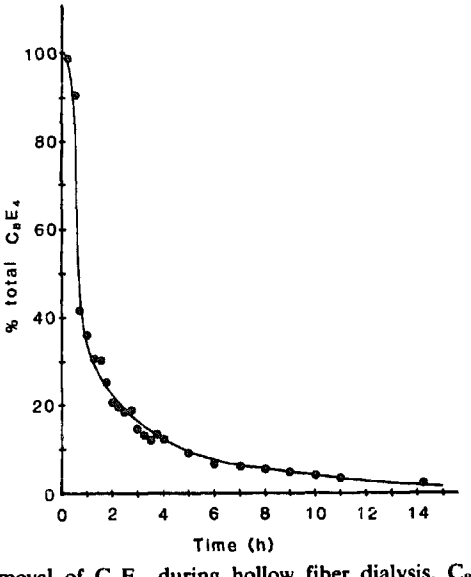

Fig. 2. Removal of C_8E_4 during hollow fiber dialysis. C_8E_4 concentrations were determined in the Hb/mixed micelle solution. The buffer flow rate was 0.7 l/h.

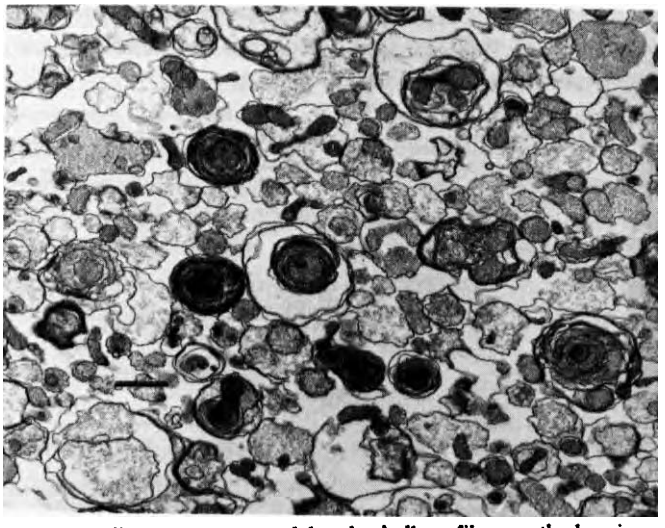

Fig. 3. Hb liposomes prepared by the hollow fiber method, using C_8E_4. Electron micrograph of thin sections of liposome. Bar represents 500 nm.

markedly increased to approx. 1:50. Hb adsorbed to the outer surface of the liposomes amounted to less than 1% of the total Hb encapsulated, as determined by incubation of empty liposomes in the starting Hb solution.

The different types of Hb liposome prepared by different methods are compared in Table II.

After separation from nonencapsulated Hb, no residues of OG or C_8E_4 in the finished Hb liposomes were detectable.

Hb encapsulation efficiencies ranged from 2 to 25% of the starting solution, depending on the dialysis method used. The highest amount of Hb was encapsulated when starting solutions contained between 13 and 15 g Hb/dl. At higher starting Hb concentrations, not only Hb encapsulation efficiency, but also the total

TABLE II

Comparison of Hb liposomes prepared by different dialysis methods

Method	Lipid	Lamellarity	Average size (nm)	Hb/lipid (mol/mol)	Residual detergent
OG					
Lipoprep	PC	MLV [a]	200	1:270	n.d. [d]
Liposomat	PC	ULV [b], OLV [c]	180	1:280	n.d. [d]
Hollow fiber	PC	ULV	160	1:280	n.d. [d]
C_8E_4					
Hollow fiber	PC/PS	MLV	450	1:50	n.d.
	PC/PA	MLV	450	1:50	n.d.

[a] Multilamellar vesicles.
[b] Unilamellar vesicles.
[c] Oligolamellar vesicles.
[d] Not detectable.

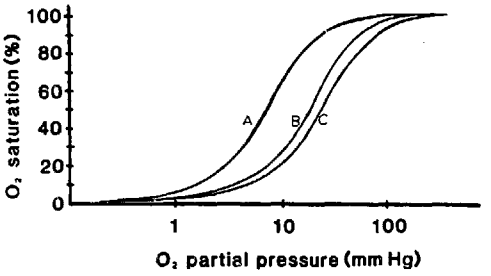

Fig. 4. Oxygen-dissociation curves. (A), Hb solution; (B), erythrocytes; (C), Hb liposomes (PC/OG).

amount of Hb encapsulated, decreased. Recycling of nonencapsulated Hb is possible.

Lipid recovery was 95–98% in the OG method, and 91% in the C_8E_4 method.

Concentrated Hb liposome suspensions

Hb liposome suspensions could be easily concentrated by ultracentrifugation and resuspension of the liposomes. The liposomes remained intact. A concentrated Hb liposome suspension ('hematocrit = 50%) of the C_8E_4 type contained 4–5 g Hb/dl. Met-Hb levels were similar for the different types of Hb liposome, reaching 7–8% of total Hb in concentrated Hb liposome suspensions.

Oxygen-dissociation curves

Oxygen-dissociation curves (ODC) of the stroma-free Hb solution and of concentrated Hb liposome suspensions prepared with IHP were compared with those of normal human blood (Fig. 4). Addition of IHP to the stroma-free Hb solution during encapsulation led to a shift to the right of the ODC, to values nearly identical with those of whole blood.

The Hill coefficient, the oxygen affinity p_{50} (expressed as partial pressure of oxygen (p_{O_2}) at 50% oxygen saturation), and the oxygen-release capacity ORC (assuming arterial and venous p_{O_2} values of 100 and 40 torr, respectively) were calculated by Erythrox measurements. The results are presented in Table III.

Shelf life

Storage stability at 4°C under nitrogen atmosphere is very good. Hb liposome suspensions of the PC/OG type could be stored up to 60 days, with the met-Hb

TABLE III

Oxygen-carrying characteristics

Oxygen carrier	Hill coefficient	p_{50} (mmHg)	ORC (%)	Met-Hb (% total Hb)
Erythrocytes	2.8	27.1	24.4	1
Hb solution	2.4	14.2	11.7	3–4
Hb liposomes	2.4	32.1	24.8	7–8

level reaching a value of only 11% of total Hb, and the ORC being only slightly reduced to 23%. Even after 300 days the met-Hb content was 14%, and ORC 22%.

Discussion

As the results of this study show, removal of nonionic detergents from mixed detergent lipid micelles in Hb solution is an efficient method for encapsulation of Hb in liposomes. Unfortunately, it is not easy to compare our Hb encapsulation values to those of other groups. Hb/lipid ratios in liposome suspensions are usually omitted, and Hb encapsulation efficiency alone says little when lipid recovery in liposomes is not given. However, encapsulation of 1.8 l of a 15 g Hb/dl starting solution per mol of total lipid is stated by Gaber et al. [20], and Hunt et al. [4] report encapsulation of 5 l Hb solution per mol of total lipid for the same starting Hb concentration. By comparison, up to 8.5 l starting solution containing 15 gHb/dl could be encapsulated per mol of total lipid, using our detergent dialysis method.

Including lipids that bear a negative charge in the liposomal membrane increased Hb encapsulation, as has also been observed by other authors [3,5], but decreased liposome size (results not shown). Therefore, in addition, C_8E_4, a detergent which generally leads to the formation of larger lipid vesicles, was applied for Hb liposome preparation. Hb liposomes thus produced were less homogeneous and partly multilamellar, but contained Hb at high concentrations, with only small amounts of Hb adsorbed to the outer surface.

Two effects seem to be involved. Firstly, interactions of Hb with negatively charged erythrocyte membrane lipids due to electrostatic binding have been described [21,22]. Secondly, encapsulation of high-molecular-weight proteins is much increased in liposomes with large internal volumes, not only due to the more favorable general surface/volume ratio in the liposomes, but also due to steric hindrance and bound water layer effects on encapsulated protein concentrations [23].

Hb/lipid ratios and Hb liposome size depend not only upon starting Hb concentrations, detergent, and lipid composition, but also upon the dialysis method used. The rate of dialysis seems to play an important role. Among other factors, the dialysis rate in our experiments was influenced by the buffer flow rate and the membrane surface area, with hollow fiber cartridges having the largest surface area, and Lipoprep the smallest. Slow dialysis of OG led to multilamellar liposomes, and at increased dialysis rates the liposomes became smaller and unilamellar.

Since hemoglobin, especially isolated hemoglobin, is easily denatured or converted to met-Hb when outer conditions are altered, changes of pH, temperature, and medium composition during preparation were kept to a minimum as far as possible. On the one hand, it was our goal to mimic the characteristics of human whole blood (pH, medium composition), on the other, to conserve the Hb molecule in its functional form (preparation at room temperature, storage at 4°C).

OG and C_8E_4 had no adverse effects on Hb structure and function at those concentrations used for liposome production. Slight increases in met-Hb content during liposome preparation were most likely due to mechanical factors. The cooperative effects of the Hb molecule are intact in the liposomes, as displayed by the sigmoidal character of the oxygen-dissociation curve.

It could be demonstrated that our Hb liposomes are able to undergo reversible oxygenation at physiological oxygen partial pressure values. The ORC of our Hb liposome suspensions is identical to the ORC of whole blood. Even after months of storage, ORC decreases only slightly to values around 22%. However, it must be noted that ORC is hardly affected by met-Hb levels up to 20% of total Hb, although met-Hb is ineffective in oxygen transport. Met-Hb levels are a more exact criterion for actual storage stability than are the in vitro oxygen-carrying characteristics of a blood surrogate.

With the nonionic detergent method, contamination is a problem that can be solved, since liposome preparation takes place in a closed system, and hollow fiber cartridges are sterile. Presently, scale-up sized batches need to be produced under sterile conditions. These Hb liposome suspensions will be tested in animal model systems for their in vivo characteristics as a blood surrogate.

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