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Biochimica et Biophysica Acta 1196 (1994) 123–130

BBA Biochimica et Biophysica Acta

Microencapsulation of hemoglobin in liposomes using a double emulsion, film dehydration/rehydration approach

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Received 31 May 1994

Abstract

A double emulsion, film dehydration/rehydration approach was developed for encapsulation of hemoglobin (Hb) at high concentration in liposomes. The liposome-encapsulated Hb (LEH) membrane was formulated to contain either phosphatidylinositol (PI) or polyethyleneglycol phosphatidylethanolamine (PEG-PE) along with partially hydrogenated egg-PC, cholesterol, and α-tocopherol in a molar ratio of 0.1:1:1:0.02, respectively. The methods introduced in this study followed a multi-step procedure. First, a primary emulsion of Hb in organic solvent containing dissolved lipids was formed. Next, the emulsion was dispersed into an aqueous continuous phase to form a water-in-oil-in-water type double emulsion. Other than the lipids noted above, no surfactants were used in this system. The double emulsion was then converted to LEH by the following steps: evaporating the organic solvent; dehydrating the water to form a dry, thin Hb-lipid film; rehydrating the film in Hb solution to form the LEH; reducing the size of the LEH using 'microfluidization' i.e., high pressure/hydrodynamic shear; and lastly washing the down-sized LEH in buffer. Physico-chemical properties of the model LEH were measured, including oxygen content, encapsulated Hb concentration, oxygen affinity and cooperativity, vesicular size distribution, viscosity, and stability. The suitability of LEH prepared in this manner as a red blood cell substitute was shown using continuous isovolemic exchange transfusion techniques in a small animal model: clearance, efficacy and acute toxicity were evaluated.

Keywords: Double emulsion; Red blood cell; Liposome-encapsulated hemoglobin; Viscosity; Microfluidization; Clearance; Efficacy

1. Introduction

Double emulsions are used widely in food, pharmaceutical and other industries. A large number of investigations have been made with oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) systems from the different standpoints of emulsion science and emulsion technology. W/O/W double emulsions have found a number of uses, such as an antigen adjuvant (incorporating an antigen in the inner aqueous phase to induce prolonged antibody response) [1], as a prolonged drug delivery system [2], as a liquid membrane in various separation systems [3–7] and in many biochemical and biomedical applications [8,9], which include use of double emulsion as an efficient oxygen-carrying fluid.

An important aspect of this study was the development of an approach for forming liposomes that may be suitable as an effective and safe red blood cell (RBC) substitute. Djordjevich and Miller [10] were the first to use liposome methods to form synthetic red blood cells by sonicating concentrated, stroma-free hemoglobin (Hb) solution and phospholipid. Hunt developed 'reverse phase evaporation' techniques to form LEH [11]. Other methods of preparing liposome-encapsulated Hb (LEH) involved detergent dialysis [12] and lipid polymerization [13]. Recent studies involved gentler methods to make LEH [14–17]. Hemoglobin encapsulated in more conventional liposomes have been shown to provide an effective means of oxygen delivery in vitro and in experimental animals [7,16,43]. Following film hydration processing Beissinger et al. [17] used a Microfluidizer™ (Microfluidics, Newton, MA) to better control the formation of LEH. This approach, although overcoming many of the problems encountered with respect to the other LEH-based RBC substitute methods, still resulted in LEH with low encapsulated Hb solution concentration [17], and therefore low oxygen content. Other recent studies using microfluidization include those involving LEH hemodynamics [18] and LEH organ biodistribution [19].

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SSDI 0005-2736(94)00212-6

The main objective of this study was the development of a double emulsion-based approach for forming liposomes with the potential for high encapsulated Hb concentration. In addition, our methodology included solvent removal and film dehydration and rehydration steps. This approach was validated for the encapsulation of Hb in liposomes to form a product that may be suitable as an effective and safe RBC substitute.

2. Materials

Human stroma-free Hb solutions were prepared at 4°C following aseptic techniques as described elsewhere [9]. All equipment used in processing Hb and LEH was depyrogenated and all water used (for example in the washing and lysing steps and in the preparation of phosphate-buffered saline (PBS)) was sterile and pyrogen-free. Briefly, water was depyrogenated by passing it through Hollow Fiber cartridges of MW 10000 (Amicon). Glassware was washed with Terg-A-Zyme (Fisher), and rinsed with pyrogen-free water. All took place in our 'sterile' biohazard cabinet (Baker). The Microfluidizer was depyrogenated chemically by circulation of 0.1 M NaOH through the equipment for 4 h at 40°C followed by rinsing with pyrogen-free water. If necessary 6% sodium azide was used for further cleaning.

To maximize oxygen-carrying capacity, high concentrations of Hb solution were used and the cryopreservative glucose was added in a ratio of 0.35 g per g of Hb to the liposomes [20,21]. Pyridoxal 5-phosphate (P-5-*P*) (Sigma, St. Louis, MO) was added to the Hb solutions to control oxygen affinity of the LEH to a value similar to that of fresh RBC [17]. The antioxidant catalase, which acts as a scavenger of free radicals, was added to the Hb solution [16]; glutathione and NADH are being evaluated [22] in our current study.

The liposome membrane lipids used to encapsulate the Hb solution included partially hydrogenated egg phosphatidylcholine (Egg-PC), cholesterol (CHOL), phosphatidylinositol (PI) or polyethyleneglycol phosphatidylethanolamine (PEG-PE) and α-tocopherol (α-T). Either PI or PEG-PE were chosen as the negatively charged phospholipid because others have found that their inclusion in certain formulations results in a liposome membrane with significantly enhanced circulation time [23]. Egg-PC and PEG-PE were obtained as gifts from Liposome Technology (Menlo Park, CA). The rest of the lipids were obtained from Sigma (St. Louis, MO). The liposome membrane was formulated to contain the lipid molar ratios for PC/CHOL/PI/α-T of 1.0:1.0:0.1:0.02. Formulations were prepared at a lipid to Hb loading of about 150 μmol per ml of precursor Hb solution. The organic solvents diethyl ether and trichlorotrifluoroethane (Aldrich, Milwaukee, WI) were used to dissolve the lipids. The volume ratio of the two solvents used depended on the density of the Hb solution, i.e., the density of the mixed solvent was similar to that of the Hb solution so as to have excellent Hb encapsulation efficiency.

3. Methods

3.1. Primary emulsion

A W_1/O primary emulsion was prepared in which Hb solution was considered as the W_1 phase and organic solvent with lipids as the oil phase. Cholesterol is known as an effective natural surfactant of W/O emulsions and egg-PC as an effective natural surfactant in O/W emulsions [24]. The LEH were prepared at a lipid to Hb loading of about 150 μmol per ml of precursor Hb solution (from 20 to 30 g%) and at a 1.0 to 1.0 volume ratio of Hb solution to organic solvent. First 30 ml of Hb solution was emulsified in 30 ml of oil phase by slowing adding Hb solution (5 ml of Hb solution per min) to the oil phase, while stirring vigorously on a magnetic stirring table (Fisher, Itasca, IL) placed in a hood at 5°C. After all the Hb solution was added to the oil phase, the Hb/O primary emulsion was stirred for about an hour.

3.2. Secondary emulsion

The primary emulsion was then dispersed in 50 ml of PBS via mixing for 30 min by magnetic stirring to form a Hb/O/W double emulsion. It should be noted that although no surfactant was added in the PBS, the Hb/O primary emulsion dispersed well into the PBS. This may be a result of the contribution made by egg-PC, since egg-PC acts as O/W natural surfactant [24].

3.3. Solvent removal, dehydration and rehydration

A rotary evaporator (Rotovapor, Fisher) operating under partial vacuum at room temperature was used to remove the organic solvents and the water from the Hb/O/W double emulsion. As the organic solvents were removed, LEH spontaneously formed. The evaporation procedure was continued until dryness to maximize removal of all organic solvents and (at least 95%) of the water so that the Hb concentration within the LEH was as high as possible. This resulted in the deposition of an apparently dry Hb/lipid-membrane film on the walls of the round bottom flask. Hb solution (50 ml) was then added under agitation to rehydrate and resuspend the LEH. Then microfluidization was used to reduce the LEH size. The down-sized LEH were washed in isotonic PBS and centrifuged at $30000 \times g$ for 30 min to remove all unencapsulated Hb solution and any residual organic solvent. This LEH washing procedure was repeated as many times as necessary (usually no more than three times) to obtain a clear supernatant. Since the evaporation procedure used to con-

centrate the encapsulated Hb solution may also increase the concentration of the encapsulated PBS, the washed LEH solution was then dialyzed against isotonic PBS to ensure normal crystal osmotic pressure in the encapsulated Hb phase. Neither Hb leakage nor a significant change in the encapsulated Hb concentration was found following dialysis. LEH were then filtered under vacuum through 3 μm CF filters (Nucleopore). In the last step LEH were resuspended in isotonic PBS containing 7.5 g% egg albumin to make them isooncotic with respect to blood plasma. For comparison purposes LEH were also made using reverse evaporation and film hydration techniques [11,17].

3.4. Physico-chemical properties of LEH

Oxygen content in terms of ml of O_2 per ml of the LEH suspension sample was determined using a Lex-O_2-Con (Hospex Fiberoptics, Chestnut Hill, MA). The oxy-hemoglobin equilibrium dissociation and association curves for the two LEH preparations were obtained using a Hemox-Analyzer (TCS Medical Products, Huntingdon Valley, PA). Oxygen affinity (P_{50}) and cooperativity (Hill exponent n) were determined from the generated curves. Encapsulated Hb concentration was determined by dissolving the liposome membrane with n-octyl β-D-glucopyranoside detergent solution [15]. Briefly, 0.1 ml of LEH (at a lipocrit of about 30%) was dissolved in 5 ml of concentrated detergent solution (30 mg/ml). The mixture was stirred for 40–45 min at room temperature, until the liposome membrane dissolved (or formed micelles) and the solution lost all its turbidity. Also, precursor Hb solution was mixed with 5 ml of concentrated detergent solution (30 mg/ml) and only negligible metHb was generated during the 45 min incubation period, suggesting the viability of this method. The solution was then filtered through a 0.1 micron filter and centrifuged at $30\,000 \times g$ for 30 min to separate any residual membrane material. The resulting Hb solution concentration was measured for oxy, reduced and met-Hb components by the method of Benesch et al. [25], with the extinction coefficient values provided by Van Assendelft and Zijlstra [26].

The procedure used for preparing negatively stained whole mount preparations of LEH samples for transmission electron microscopy was based on standard techniques used for biological specimens. Briefly, a primary fixation with glutaraldehyde followed by secondary fixation with osmium tetroxide was used to preserve the shape and size of the lipid vesicles [27,28]. Thin-section samples were prepared by embedding the specimen for 24 h in epoxy plastic and then sectioning using an ultramicrotome. Thin section samples were observed using a Zeiss model 900 electron microscope with an accelerating voltage of 50 or 80 kV. In order to obtain a good estimate of average particle size, photomicrographs of several fields of the thin section samples were taken. The images from the negatives were projected onto a digitizing pad (Scriptel) and the diameters of the particles were input into a computer to obtain a histogram for the size distribution. One example of LEH formed using film hydration techniques was recently given in a study by Despande and Beissinger [29].

Steady shear viscosity of the suspension samples was measured in a uniform shear field with a Wells-Brookfield Syncro-Lectric Microviscometer (Model LVT) equipped with a 0.80° cone (Model CP-40, Stoughton, MA). Shear rates from 45 to 450 s^{-1} at 37°C were employed. The cone-and-plate geometry is very useful as it gives a good approximation of viscometric flow with constant shear rate throughout the flow field [30]. The stability of the LEH to shear, i.e., leakage of Hb, was evaluated as a function of shear rate by shearing the LEH samples for 30 min in the viscometer. The effect of shear rate on leakage of encapsulated Hb was obtained for freshly prepared LEH (lipocrit = 30%) incubated at 37° for up to 24 h in either 7.5 g% egg albumin/PBS or human plasma. Following centrifugation ($13\,600 \times g$, Microcentrifuge Model 235C, Fisher Scientific) the concentration of total Hb in the supernatant of the sheared and incubated sample was compared with that of the unsheared sample. The benzidine method [31], which is known to be accurate to concentrations as low as 1 mg/dl, was used for determining plasma Hb concentration at low concentration levels (i.e., in the mg/dl range) as a result of leakage of Hb from LEH.

3.5. In vivo studies

Clearance and efficacy of LEH were evaluated on unconscious rats in Illinois Institute of Technology's Small Animal Lab. Female rats (Harlan Sprague, Indianapolis, IN) weighing 225 to 275 g (8 to 12 weeks of age) were used. One day prior to the exchange, rats were anesthesized with ketamine and surgically implanted with an intraatrial silastic cannula via the jugular vein. Immediately prior to the exchange transfusion, rats were reanesthesized and a second cannula was implanted into the femoral vein. Isovolemic exchange transfusions were initiated immediately after implantation of the second cannula [32]. Briefly, a peristaltic pump (Manostat, New York, NY) was then used to perform exchange transfusions by removing blood at a constant rate of about 0.2 ml/min, while simultaneously injecting an equal volume of either LEH suspension or control.

The decrease in hematocrit level was recorded during the exchange transfusion. Termination of life in the rats was assessed by blood pressure measurement. Small volumes of LEH were tested in animals to determine circulation half-life. The method developed took advantage of the difference in density between the LEH and RBC. 50 μl blood samples were withdrawn at intervals and centrifuged in microhematocrit tubes. The LEH forms a layer, which packed above the RBC and the buffy coat. The size of this layer decreased as the LEH was removed by the reticuloendothelial system, and the circulation half-life was calcu-

lated following a 50% exchange transfusion. Normalized volume % of LEH was normalized to the 50% exchange transfusion value.

3.6. Statistics

The experimental data were analyzed using the *t*-test. The experimental results obtained (for about 4 to 7 rats) are represented by the mean along with the sample standard deviation.

4. Results and discussion

The LEH used in this work for the in vivo studies contained about 0.9 μmol of encapsulated Hb per 82 μmoles of total lipid. Optimization of liposome encapsulation efficiency for Hb may be desirable from a processing standpoint. We have obtained preliminary results with two different surfactants: sucrose distearate (Crodesta F-10, Croda, New York, NY) and polyoxypropylene-polyoxyethylene block copolymer (Pluronic L101, BASF, Parsippany, NJ), which were found to give liposome encapsulation efficiencies for Hb of about 90% (unpublished results). Similar results using other surfactants for encapsulating anhydrous glucose in liposomes have been found in an earlier study [33]. Table 1 compares the resulting encapsulated Hb concentrations obtained using our double emulsion, film hydration [17] and reverse evaporation [34] techniques. In our hands, for a precursor Hb solution concentration of 10.5 wt%, the double emulsion method gave the highest results: about 20 wt%.

In our studies here we have attempted to maximize oxygen content for a given LEH suspension sample by encapsulating the highest concentration of Hb possible; our methodology described below reflects that goal. Table 2 shows the resulting encapsulated Hb concentrations as a function of precursor Hb solution concentration. Encapsulated Hb concentrations as high as 24.1 wt% were obtained for a precursor Hb concentration of 25 wt%. However, for a precursor Hb concentration of 10.5 wt%, the encapsulated Hb concentration was nearly as high, i.e., about 20.0 wt%. These results indicate that there is a limit to encapsulated Hb concentration; in this study the limit was about 25 wt%. The limit may depend on colloid osmotic pressure differences between the inner Hb solution phase and the outer PBS-albumin solution phase of the LEH system, since an osmotic pressure difference due to colloidal material (i.e., Hb and albumin) would result in water movement to balance the system. This effect has been experimentally investigated in our previous study on Hb-in-oil-in-water multiple emulsions [9] and is being evaluated in our current liposome studies.

Table 1
Encapsulated Hb concentration in freshly prepared LEH samples using three different methods

Method	Precursor Hb solution concentration (wt%)	Average of encapsulated Hb concentration (wt%)
Film hydration	10.5	4.5 ± 1.0
Reverse	10.5	5.0 ± 1.0
Double emulsion	10.5	20.0 ± 1.5

The experimental data for three to five runs are represented for each method.

Table 2
Encapsulated Hb concentration in freshly prepared LEH samples using the double emulsion method

Method	Precursor Hb solution concentration (wt%)	Average of encapsulated Hb concentration (wt%)
Double emulsion	5.0	11.3 ± 1.5 ($n = 3$) [a]
Double emulsion	10.5	20.0 ± 1.5 ($n = 3$)
Double emulsion	20.0	23.6 ± 2.0 ($n = 3$)
Double emulsion	25.0	24.1 ± 1.5 ($n = 5$)

The experimental data for three to five runs are represented for each method.
[a] Average value ± 1 standard deviation. The number of separate runs (n) for each precursor solution concentration are shown in parentheses.

Trifluorotrichloroethane [34] and dichlorofluoromethane [35] have been successfully used in related methodologies and have demonstrated that the enzymatic activity of alkaline phosphatase and horseradish peroxidase can be maintained. Solvents, e.g., dichlorofluoromethane (Freon-21) at a normal boiling point of about 9°C, that are prepared using this method at a temperature of 21°C, are expected to be rapidly vaporized and probably would be removed as contaminants. Although, the amount of solvent remaining in reverse-phase-vaporization after overnight dialysis was not determined, Deamer [36] has investigated this in vesicles prepared by the ether infusion technique and could not detect any ether in the vesicles after dialysis.

The amount of Met-Hb generation accompanying LEH processing appeared to be small, with only a 5% increase during the encapsulation process. The oxygen content for the resulting LEH suspension sample (50% by volume of LEH, containing an average encapsulated Hb concentration of 25 g%) was 13 to 14 volume% oxygen (compared to 20% for whole blood). It was reported in a previous study that Microfluidizer™ processing at temperatures below 9°C had little or no effect on met-Hb generation; also no oxygen content reduction in precursor Hb solution or in the encapsulated Hb solution was found for either material that had been passed up to 15 times through the Microfluidizer™ [37]. The changes in percentage met-Hb and total concentration of entrapped Hb solution in LEH during storage at −15°C were determined for up to 30 days (data not shown). Very little reduction in total encapsulated Hb concentration was found; percentage met-Hb increased to a

Table 3
The surface tension measurement of various solutions

Substance	Temperature (°C)	Contact phase	Surface tension (dyne/cm)
Deionized water	20	wet air	72.8
PBS	20	wet air	72.8
PBS + 1.5 wt% PC	20	wet air	26.3
Solvent [a]	20	wet air	18.5
Solvent + 2.5 wt% cholesterol	20	wet air	18.5
Solvent + 5.0 wt% PC	20	wet air	18.5
Solvent + 2.5 wt% cholesterol + 5.0 wt% PC	20	wet air	18.5

[a] Solvent is 37.5% trichlorotriflanaroethane and 62.5% diethyl ether.

little greater than 7%. Addition of various components to the lipid phase of LEH systems appeared to reduce the oxidative interactions between Hb and membrane lipid. Some of these included the addition of cholesterol to the membrane phase to protect Hb from oxidation [38,39]. Also oxidation of Hb to met-Hb may have been inhibited by using partially hydrogenated PC instead of natural unsaturated egg-PC. Partial hydrogenation of egg-PC to an iodine value of 40 as used here is known to convert the polyunsaturated fatty acids to monosaturated fatty acids, which are far less susceptible to oxidation [40].

Egg-PC has been noted to be an effective natural surfactant in O/W emulsions [24]. This claim seems to be supported as applied to our system. The surface tension of PBS was reduced dramatically from 72.8 to 26.3 dyne/cm by addition of 1.5 wt% egg-PC (see Table 3). However, the claim that cholesterol is an effective surfactant of W/O emulsions seems not to be borne out as applied to our system because the surface tension of the 'oil phase' did not change after addition of cholesterol (see Table 3).

A thin-section electron micrograph of the LEH passed 10 times through the Microfluidizer™ is shown in Fig. 1 at a magnification of 30 000-times using a Zeiss model 900 electron microscope. The particle size range of the 3-micron-filtered sample appears to be from as small as 40–50 nm to a little larger than 1 μm. However, the average particle size in the micrograph is about 250–300 nm.

Oxygen affinity curves for LEH suspensions were obtained using a Hemox-Analyzer (TCS Medical Products, Huntington Valley, PA). Although the curves generated were continuous, discreet points are shown to allow better comparison between the different types of sample tested. The P_{50} for the LEH, precursor Hb solution (both containing P-5-*P*) and human whole blood samples are 22, 19 and 25 mmHg, respectively (see Fig. 2). Oxygen cooperativity results for LEH appear to be near the normal values expected for whole blood with a Hill exponent of about 2.2.

Steady shear viscosity results were obtained (see Fig. 3) for LEH suspension samples (in PBS containing 7.5 g% albumin). The viscosity of the LEH sample (30% by

Fig. 1. Thin-section electron micrograph of PI-LEH passed ten times through a Microfluidizer™ is shown. Original magnification: 30000×. Bar = 0.25 μm.

volume LEH) was a bit higher than that of either the human or rat whole blood, at hematocrits of 45 and 46%, respectively. Viscosity was measured for mixtures of LEH suspension and whole blood (rat) to show the effect of different volume-to-volume ratios of LEH (50% by volume LEH) and rat whole blood (hematocrit 46%). The results indicated that no interactions between LEH and rat whole blood had occurred (data not shown).

Release of Hb from LEH into the surrounding aqueous phase was measured as a function of shear rate and incubation time. The effect of shear rate on leakage of

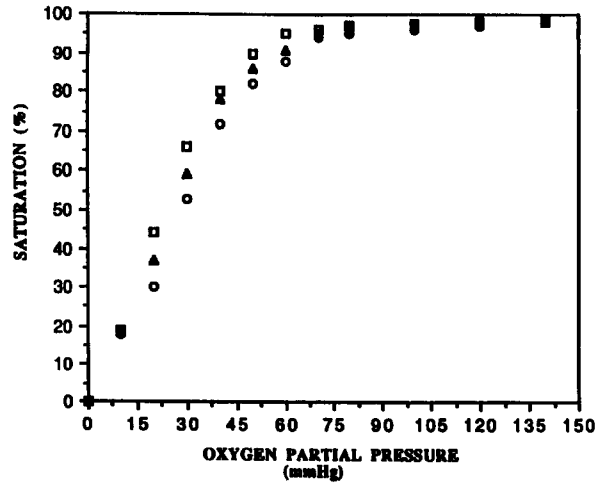

Fig. 2. Hemoglobin-oxygen saturation curve of precursor hemoglobin solution ▲, human whole blood ○, and PI-LEH □ for a typical system is shown.

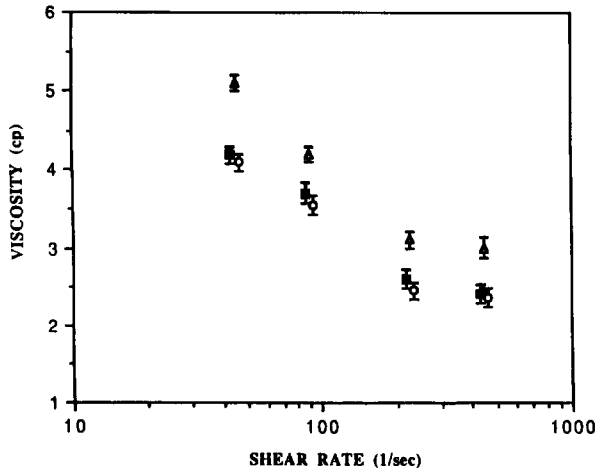

Fig. 3. Steady shear viscosity of PI-LEH (30% by volume) △, rat whole blood (46% by volume) ■, and human whole blood (46% hematocrit) ○. The mean ± one standard deviation is shown.

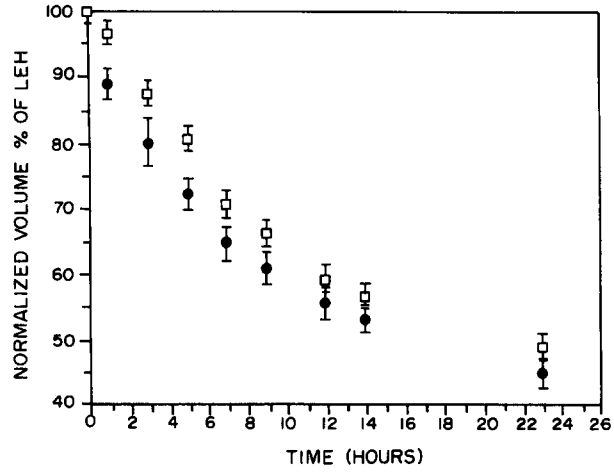

Fig. 4. Clearance of PI-LEH ($n = 7$) ● and PEG-PE-LEH ($n = 4$) □ in rats following 50% isovolemic exchange transfusion. The average along with ± one standard deviation is shown.

encapsulated Hb after incubations of 2, 8 and 24 h at 37°C was obtained for LEH samples in either 7.5 g% egg albumin/PBS or human plasma. The results showed that the leakage of encapsulated Hb from LEH for both human plasma and egg albumin (7.5 wt%)/PBS samples was small, i.e., 0.6% or less (data not shown). High cholesterol content in the membrane is known to reduce membrane permeability in serum and plasma [41].

Clearance and efficacy were evaluated in rats using exchange-transfusion of blood with LEH prepared using the double emulsion approach. The decrease in hematocrit levels was recorded during the exchange-transfusion. Circulation half-life following 50% isovolemic exchange-transfusion with LEH was about 16 h (see Fig. 4). Studies with PEG-PE and PI LEH ($n = 4$ and 7 rats, respectively) were used to demonstrate this feature and confirmed studies attempted previously with empty liposomes, which included either PI or PEG-PE as the negatively-charged membrane component [42]. High cholesterol content in the membrane also appears to be an important factor helping to prolong liposome stability in circulation. These times are desirably long and compare very favorably to results reported in another study for LEH containing dimyristoyl phosphatidylglycerol (PG) [17]. However, previous studies with up to 10% toploading or 50% exchange made with distearoyl phosphatidylcholine (PC), dimyristoyl phosphatidylglycerol (PG) and cholesterol have shown that these LEH demonstrated toxic responses, which included thrombocytopenia and elevation of plasma thromboxane B_2 [18].

The efficacy of LEH was demonstrated in that rats survived the isovolemic exchange transfusion of either PI or PEG-PE LEH to reduced hematocrit levels of about 2.0 to 3.0% ($n = 7$ for rats receiving PI LEH and $n = 4$ for rats receiving PEG-PE LEH), while the control rats transfused with PBS containing 7.5 g% albumin survived to hematocrit levels of only about 6% ($n = 7$). Typical results

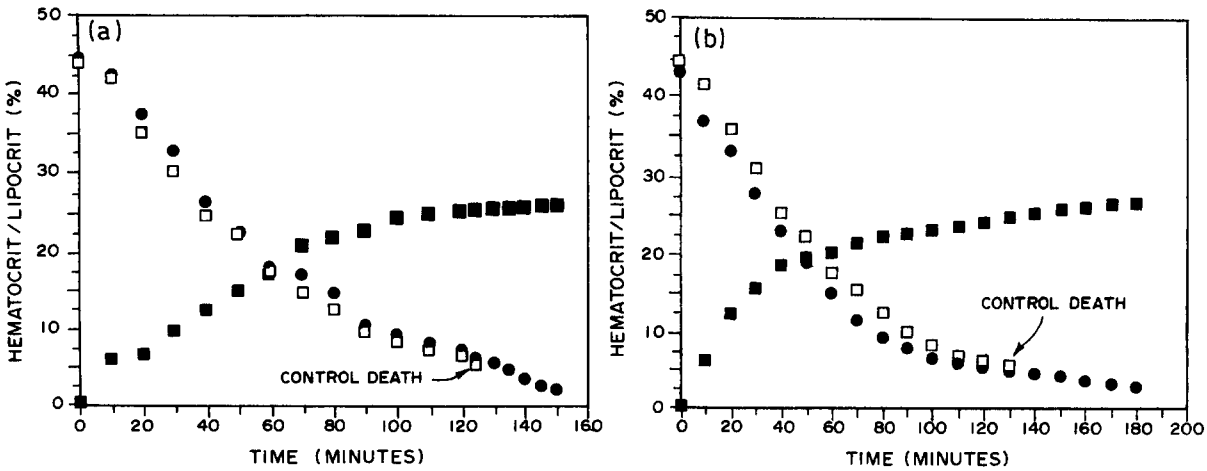

Fig. 5. (a) Hematocrit in rats during isovolemic exchange transfusion with 7.5 wt% albumin-PBS (□) and PI-LEH (●) for a typical exchange-transfusion. The lipocrit of PI-LEH during the exchange is also shown (■). (b) Hematocrit in rats during isovolemic exchange transfusion with 7.5 wt% albumin-PBS (□) and PEG-PE-LEH (●) for a typical exchange-transfusion. The lipocrit of PEG-PE-LEH (■) during the exchange is also shown.

are shown in Fig. 5. These results confirming the efficacy of PI and PEG-PE are consistent with those found in other recent studies for terminal hematocrit obtained for control and LEH-exchanged animals [11,43].

It was recently found that PG (from egg) liposomes bound to rat platelets, which was mediated by complement [44]. 'Stealth' liposomes (liposomes containing PI or PEG-PE) that are designed to evade recognition and rapid uptake by the (reticuloendothelial system, however, more recently referred to as the mononuclear phagocytic system) MPS [45–47] were used. Although LEH containing either PI or PEG-PE have been shown by the data herein to be efficacious, recent experiments [48,49] suggested that such systems, by overloading the MPS [50,51], caused alterations in phagocytic activity and increased host susceptibility to infectious challenge [52]. The inability to differentiate LEH formulations based on in vitro phagocytic activity suggests that the in vivo Listeria infection model may be more relevant in discerning the immunotoxicity of the LEH formulations tested [48].

5. Conclusions

An approach for the encapsulation of hemoglobin in liposomes that uses double emulsion techniques was developed in this work. The method produced LEH with several desirable physico-chemical properties. Coupled with the satisfactory results for efficacy and long circulation half-life obtained in our small animal model, our work suggests that LEH produced using this approach may be suitable as a red blood cell substitute.

Acknowledgements

We would like to thank the Microfluidics Corporation for aid in adapting the Microfluidizer™ M110 equipment for our purpose and to Ms. Loyda Vida of the University of Illinois at Chicago for her assistance in using the Hemox Analyzer to obtain oxygen saturation and dissociation curves. This work was supported by a grant from the Army Medical Research and Development Command (DAMD17-89-Z-9007).

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