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HEMOGLOBIN-LIPOSOMES AS BLOOD REPLACEMENT FLUID

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ABSTRACT

Human hemoglobin is encapsulated in unilamellar liposomes by French press extrusion in order to form hemoglobin liposomes (HBL). The mean diameter of the HBL is 50 nm by TEM. Therefore the preparation can be passed through microbial retentive filters (200 nm). The encapsulation efficiency is 11.5% and the hemoglobin content 0.52 g/100 ml for a 5.75% (v/v) dispersion. The HBL are able to bind 3.8% (v/v) O_2 (32% (v/v) dispersion) and show the same oxygen dissociation characteristics as the hemoglobin solution. The lipid mixture consists of hydrogenated soy lecithin, which is inexpensive and stable against oxidation, and cholesterol in a molar ratio 1:1. The French press extrusion process is suitable for a scaling up.

INTRODUCTION

In medicine there is an increasing need for an oxygen supplying blood replacement fluid, when enough blood of a suitable

type is not available. Here we report the encapsulation of hemoglobin (Hb) in unilamellar liposomes. HBL are expected to avoid the risks of antigenic reactions and infections with hepatitis NANB, B or with HIV. Both the lipid membrane and the content of the liposomes consist of metabolizable material. The oxygen affinity of the Hb molecule can be controlled by modulators like 2,3-DPG (2,3-diphosphoglycerate) in the same way as in the natural red blood cells (RBC). In comparison to free hemoglobin, circulation in the blood as well as the shelf lifes of HBL are prolonged.

In the last decade, various methods of preparation for encapsulation of Hb have been proposed[1], e.g. reverse phase evaporation[2], hand shaken method[3,4], detergent dialysis[5], filter extrusion[6,7], cell disruption bomb (Parr[A] bomb)[8]. With a view to an economic large scale production in the future, we preferred as preparation method the extrusion by a French pressure cell[B], and as the lipid mixture hydrogenated soy lecithin and cholesterol.

MATERIALS AND METHODS

Hemoglobin

Hb solutions were prepared from outdated human blood. RBC were packed by centrifugation at 1000 g for 10 min and washed three times with 0.9% NaCl solution. They were then hemolysed by a Parr cell disruption bomb[A,8]. We used CO_2 at pressures of 510 MPa. To remove the stroma, the solutions were acidified to a pH of 5.5 by addition of 1 N HCL . Although the isoelectric point of the membrane proteins of RBC is lower (pI 4-5)[9] they are sufficiently precipitated and can be removed by centrifugation at 50,000 g for 30 min at 6^0 C. The supernatant was adjusted to pH 7.4 with 1 N NaOH. Sometimes this procedure led to a precipitation,which was centrifuged again as above. The stroma-free Hb solution was

filtered through microbial retentive filters (0.2 μm) into sterile vials using a clean air bench and stored at 4–6° C. The Hb solution was characterized by the following six criteria: total Hb content, MetHb content, VIS-Spectra, oxygen content, oxygen dissociation curve (ODC) and isoelectric focussing (IEF). The Hb content was determined by the standard CNMetHb method. MetHb was determined as suggested by van Assendelft[10]. The photometric measurements were carried out by a diode array spectrophotometer[C]. The oxygen content was measured by a Lex-O_2-Con[D] oxygen analyser[11]. The oxygen dissociation curve (ODC) was recorded by a Hem-O-Scan[E]. For isoelectric focussing, electrophoresis unit FBE 3000[F], Servalyt[G] precoats pH 3–10 and protein test mixture 9[G] were used.

Lipids

The lipid mixture consisted of hydrogenated soy lecithin (E 200 H)[H] and cholesterol[I] at molar ratio of 1:1. Impurities such as lysolecithin were detected by TLC[1,K]. The peaks were scanned using a densitometer[L]. The composition of fatty acids was determined by FID-monitored GC[M] analysis of the methyl-esters prepared by previous treatment of the phophatidylcholine with boron trifluoride-methanol[1]. The separation was performed by means of GC column[M] with 15% DEGS on Chromosorb WAW 80/100 mesh. Heptadecanoic acid methyl ester[N] was used as inner standard. The determination of the phase transition temperature T_m and enthalpy ΔH of the hydrogenated soy lecithin and various mixtures with cholesterol was carried out with a differential scanning calorimeter[O] at heating rates of 1K/min.

Liposomes

The film method is used to prepare multilamellar vesicles (MLV) of Hb solution (2.67 μmol/ml) and lipid (180 μmol/ml). This liposome dispersion is forced 4 to 5 times through the orifice of a French pressure cell[B] at 120 MPa (18,000 psi) to form uni- or

TABLE 1

Hemoglobin Data

	Hb-content		MetHb	O_2	P_{50}
	mmol/l	g/100 ml	%	%	hPa
human blood	2.3	15.0	<1	20	36
citrated blood	1.86	12.0	1	15	
Hb solution	2.82	18.2	14	22	36

oligolamellar vesicles. They are separated from free Hb by gel chromatography on sepharose 6B CL[F] with 154 mmol NaCl solution. After filtration through 0.2 μm filters into sterile vials the preparation is stored at 4° C. The size distribution is determined from TEM micrographs[P] after negative staining with 2% uranyl-acetate solution as well as from static and dynamic laser light scattering[Q]. Details of the technique are given by W. Burchard[12]. The instrument is described in [13]. The lipid content is determined by phosphate analysis[1]. The Hb content was calculated from determination of the Fe by AAS[R]. The oxygen content of the HBL is measured under normal atmospheric conditions. The oxygen dissociation curves ODC are measured as mentioned above.

RESULTS AND DISCUSSION

Characterization of the Hb solution

Results of the Hb- and MetHb content are shown in Table 1 together with the oxygen content and ODC data.

Five patterns of Hb derivates are typically obtained by IEF: HbA ($\alpha_2 \beta_2$), HbA_2 ($\alpha_2 \delta_2$), two intermediate MetHbs` ($\alpha_2^+ \beta_2$) and

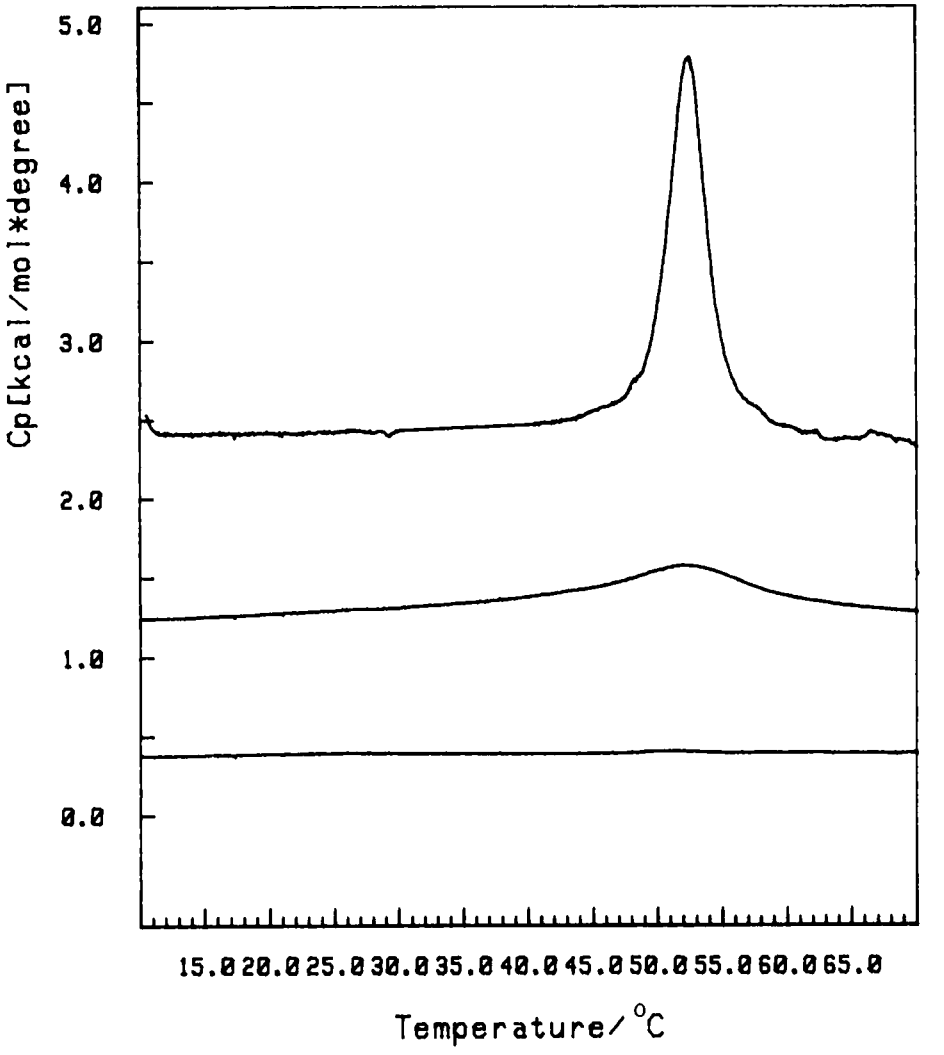

FIGURE 1

Apparent molar heat capacities C_p of aqueous dispersions of hydrogenated soy lecithin E 200H / cholesterol as a function of temperature

lipid	T_m (° C)	ΔH (kcal/mol)
a) hydrogenated soy lecithin	51.9	11.10
b) - with 30% cholesterol	50.5	7.8
c) - with 50% cholesterol	49.8	0.4

($\alpha_2\ \beta_2^+$) and MetHb ($\alpha_2^+\ \beta_2^+$). Plasma proteins were not found. Hemolysis by a cell disruption bomb avoids the use of organic solvents, which might be incorporated in hydrophobic pockets of the Hb molecule and lead to denaturation. As a result the Hb content is high enough for HBL preparation without further concentration steps. Oxidative degradation to MetHb, the Fe^{3+} derivative, which no longer is able to bind oxygen reversibly, is still a serious problem.

Characterization of the hydrogenated soy lecithin

The lecithin was characterized by TLC, GC and DSC. E 200H contained 2% of lysolecithin, other impurities were not detectable. The fatty acids were about 75% stearic acid and 25% palmitic acid with little variation between different batches. Other fatty acids, particularly unsaturated ones, were not found. Lipid peroxidation, which is known to be catalysed by Hb, is impossible. The phase transition temperature of 52^0 C was comparable with that of distearoylphosphatidylcholine DSPC (54.3^0 C)[14]. As expected, the phase transition peak disappeared in mixtures with cholesterol of above 50% (*mol/mol*) (Figure 1).

HBL Shape and Size Distribution

Figure 2 shows a TEM micrograph of negative stained HBL with a diameter of about 250 nm. Two bilayers of neighboured vesicles can be recognized. Most of the vesicles are unilamellar, but smaller. The thickness of the bilayer measured under a 200,000 fold magnification is 5.3 nm. The diameters of at least 200 liposomes are used to calculate the size distribution, which is given in Figure 3 Most of the HBL show diameters in the range of 30 to 100 nm.

The particle weight can be assessed by dynamic and static laser light scattering. Conclusions can be drawn on the geometric and hydrodynamic radius R_g, R_h and on ρ as an indicator of the

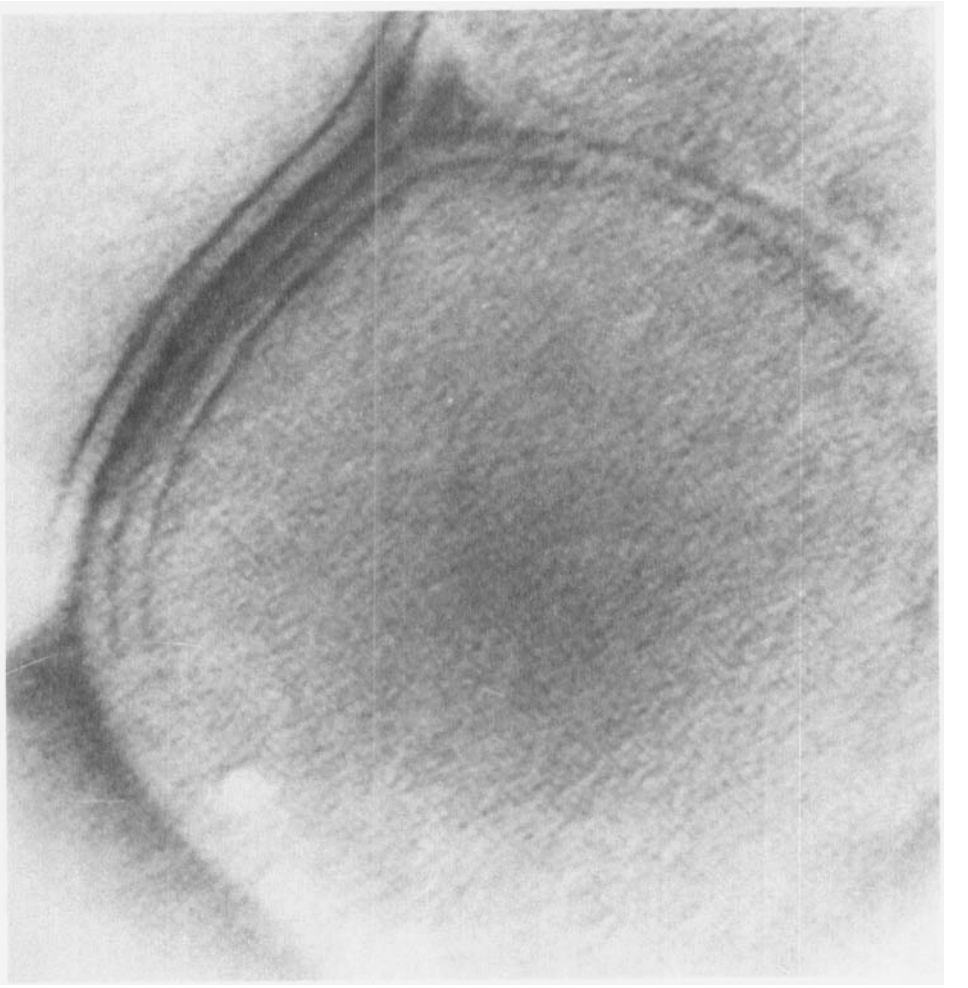

FIGURE 2

TEM micrograph of a negative stained HBL, magnification 85,000x, 1 cm = 50 nm

shape of the particles (solid sphere = 0.778, hollow sphere = 1.00, random coil = 1.50)

The HBL mean diameter obtained by this method is too large when compared with that from TEM. This arises from the fact that in TEM the number average diameter is determined, while in dynamic

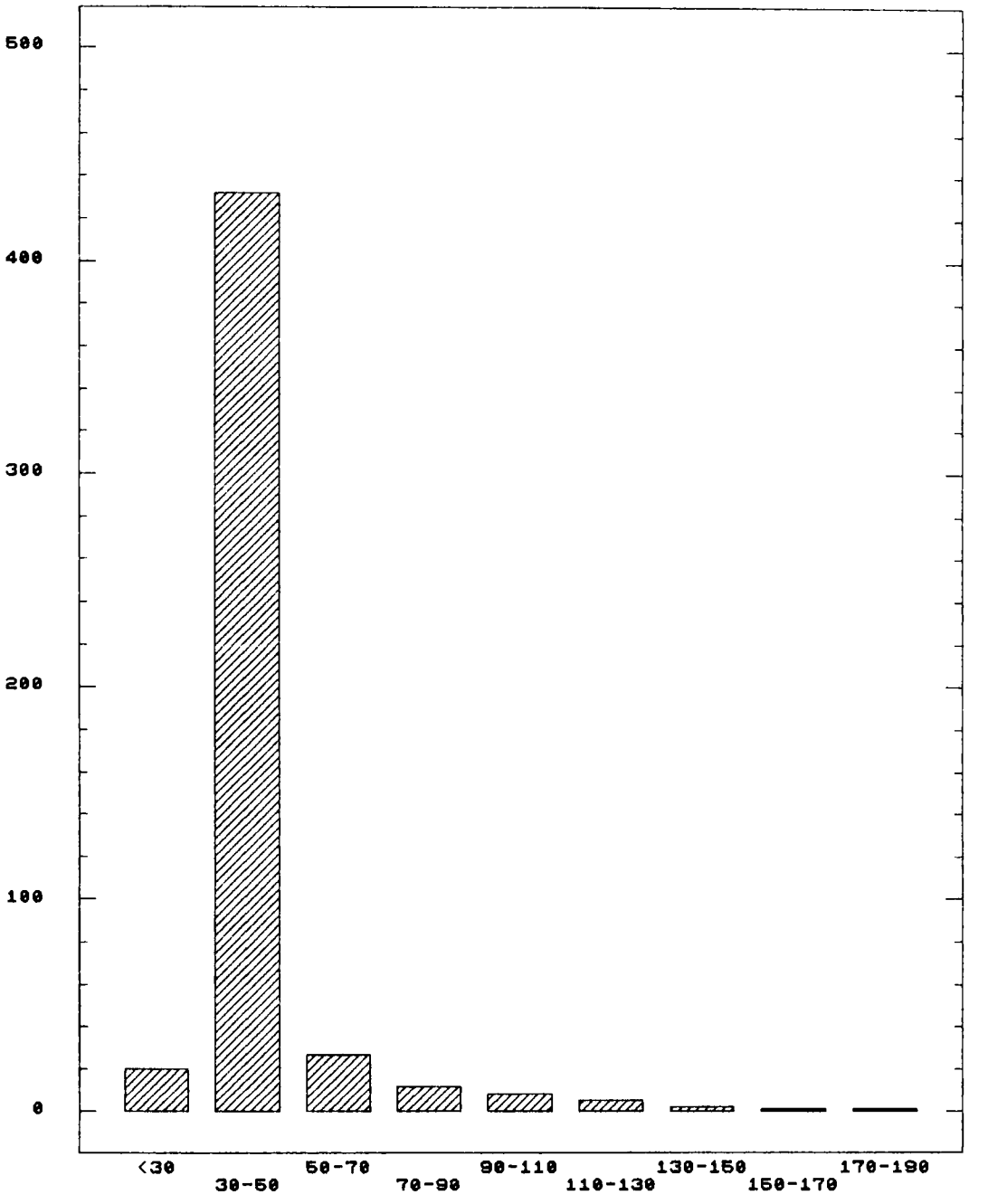

FIGURE 3
Size distribution of HBL from TEM.

TABLE 2

Results of Photon Correlation Spectroscopy

	1. day	16. day
M_W	164 x 10^6	149 x 10^6
R_G	61.9 nm	62.1 nm
R_H	58.2 nm	58.2 nm
ρ	1.06	1.07

light scattering the Z-average is measured (i.e. the particles are weighted by M^2). Our preparation covers too wide a range.

HBL Composition

The determination of the phosphatidylcholine content of HBL was carried out by phosphate analysis, as the phosphate content of the Hb solution was less than 0.08% of the total phosphate content of the preparation.

In Figure 4, spectra of the Hb solution and HBL are shown. The difference, an exponential increase of the absorbance with small wavelengths, results from light scattering with the liposomes. Because of the poor solubility of E 200H, the turbidity could not be removed by tensides like Triton X 100, sulfobetains or n-octylglucopyranoside. Corrections by adding empty liposomes to the blank decreased the accuracy of the photometric measurements. Hb content was determined via AAS of the Fe of the Hb. Encapsulation efficiency was calculated from the recovery of the Hb, the encapsulated volume from the inner volume of the vesicle and the lipid content.

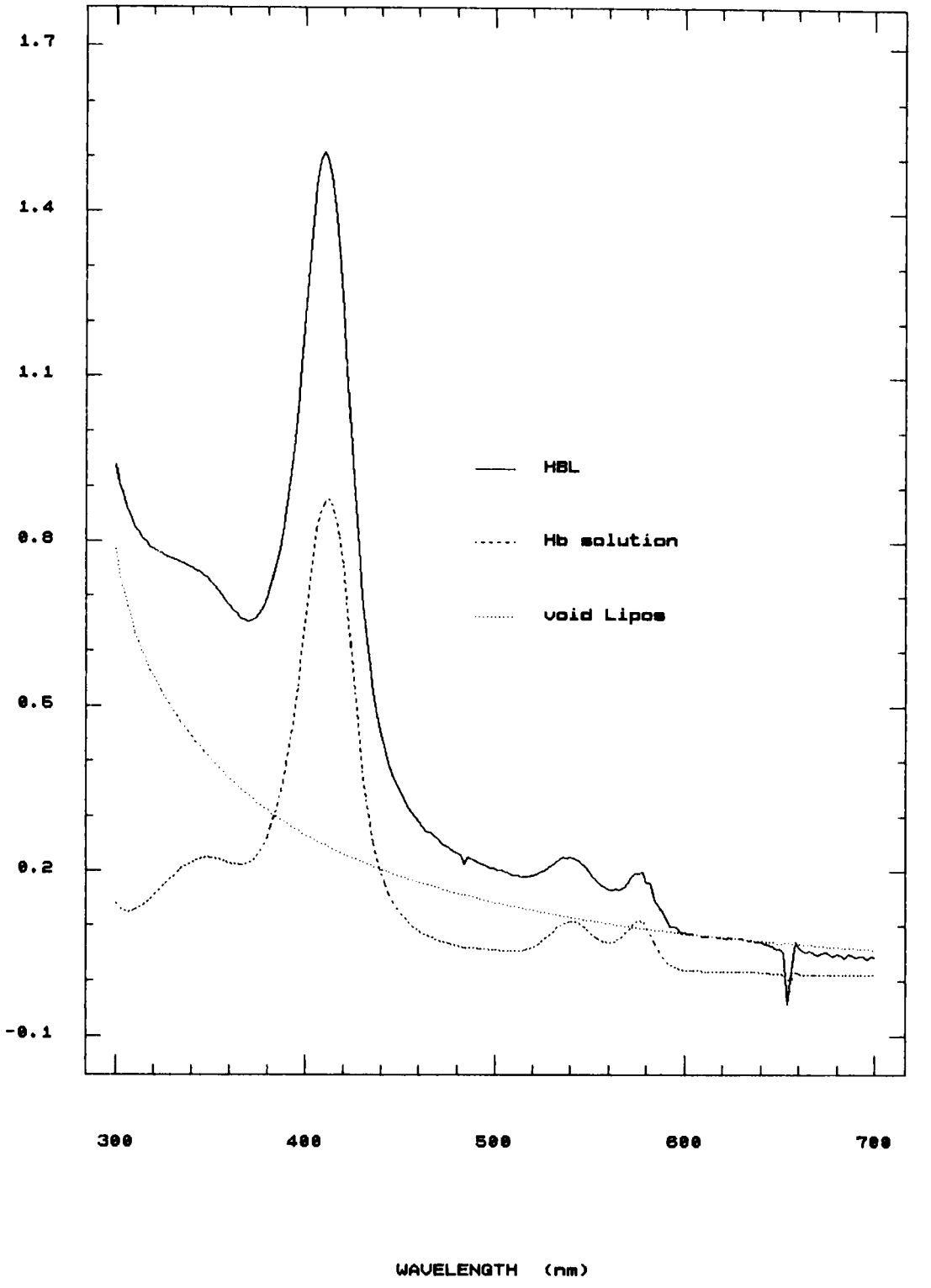

FIGURE 4

VIS-Spectra of Hb solution, HBL and void liposomes.

TABLE 3

HBL Data

mean Hb content	0.52 g/100 ml (n=4; ± 7%)
lipid recovery	35 %
encapsulation efficiency	11.5 %
encapsulated volume	0.7 µl/µmol lipid

O_2 transport behaviour

By measuring the oxygen content of air-saturated HBL, the total oxygen carrying capacity of the preparation was determined to be up to 3.8% (v/v) O_2 for a 32% (v/v) dispersion. From this parameter and from the Hb content it can be concluded that the functionality of the oxygen carrier is unaffected by the preparation procedure. Oxygen dissociation curves of the HBL are sigmoid, but shifted to smaller O_2 presssures, which is caused by the loss of the physiological modulator 2,3-DPG during isolation and storage of the Hb. In future preparations 2,3-DPG or other modulators should therefore be coencapsulated with the Hb.

CONCLUSIONS

Hemoglobin can be encapsulated in unilamellar liposomes using French press extrusion. The oxygen carrying function of the Hb is not affected by this procedure. In our HBL the Hb/lipid ratio is higher than in MLVs` and the size ranges below the limit of 200 nm, which is important for microbial retentive filtration as well as for long shelf life of the liposomes. It contains neither negative nor positve lipids, which are known to improve the encapsultion efficiency but also recognition of liposomes by the RES thus causing

rapid elimination from circulation. The results of the reported experiments show that there is a good basis for developping blood replacement fluids with sufficient oxygen transport properties. The availability of such fluids would be very useful in times of catastrophes when lack of blood conserves occurs. The RBCs of outdated blood (stored for more than 35 days) are easily available, as in most cases only plasma is used for the isolation of various blood components. The isolated Hb can be stored for a long time in order to be transfered in HBL when necessary. The question, whether HBL could also be stored, is still to be answered. In comparison to the isolated components, the Hb-lipid-system shows accelerated oxidative degradation[3]. When using hydrogenated lecithin, fat autoxidation, however, is no longer a problem. Before testing the system in animals, MetHb formation should be diminished. With hydrogenated lecithin, some of the preparation methods like detergent dialysis or reverse phase evaporation do not work because of the poor solubility of the lecithin. In addition, our method is suitable for scaling up and the used hydrogenated lecithin is less expensive than synthetic ones

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