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Biochimica et Biophysica Acta 1196 (1994) 65–75

BBA Biochimica et Biophysica Acta

Entrapment of haemoglobin into liposomes by the dehydration-rehydration method: vesicle characterization and in vivo behaviour

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Received 10 December 1993; revised 16 June 1994

Abstract

Haemoglobin (Hb) was isolated from human erythrocytes under conditions which maintained NADH–cytochrome-b_5 reductase activity and suppressed oxidation of Hb during storage at 4°C (methaemoglobin values < 3% after 29 days). Hb was entrapped into liposomes composed of hydrogenated egg phosphatidylcholine and equimolar cholesterol according to the dehydration/rehydration procedure of Kirby and Gregoriadis ((1984) Biotechnology 2, 979). However, encapsulation of Hb in its intact form was poor (< 5%) as a result of its oxidation and denaturation during freeze-drying. The addition of cryoprotectants and the use of both, higher initial concentrations of Hb and very small void vesicles resulted in Hb-rich dehydration/rehydration vesicles (phospholipid/Hb molar ratio of about 200:1) of the preferred size of 110 nm (mean). Highly homogeneous and small void vesicles as starting material were prepared using the one-step method of Brandl et al. ((1990) Drug Dev. Ind. Pharm. 16, 2167). The cryoprotectants were chosen with respect to their sufficient protection of Hb without affecting its loading into vesicles during freeze-drying and rehydration. ^{51}Cr-labelling of Hb was used for the in vivo monitoring of the fate of Hb-containing vesicles rather than ^{125}I-labelling since the latter induced strong interactions of Hb with liposomes. Upon intravenous administration into rats, liposomal ^{51}Cr-Hb showed greater blood levels and prolonged circulation times in the blood compared to free Hb. The present approach provides high yield entrapment of labile molecules into vesicles of small size known to exhibit long circulation time.

Keywords: Hemoglobin; Liposome; Blood surrogate; Freeze-drying; NADH–cytochrome-b_5 reductase; Radiolabel

1. Introduction

Haemoglobin-containing liposomes (HbL) are currently under investigation as a potential non-antigenic, universal and biodegradable substitute for red blood cells (for a review see Ref. [1]). The main clinical indication in using HbL is resuscitation of trauma victims from severe shock, especially if suitable donor blood is not available. One of the objectives with the formulation of Hb-based blood substitutes is to provide Hb in a form that is stable under storage. In this study we show that the activity of the enzyme NADH–cytochrome-b_5 reductase (NADH diaphorase) can be maintained in stroma-free Hb solution. The enzyme is able to protect Hb against oxidation during storage upon admixture of glucose.

Recent studies with Hb-containing liposomes have been directed towards improved efficiency of Hb entrapment and HbL formulations that exhibit optimal behaviour in vivo [2–7]. For sufficiently long circulation times in blood it seems to be a prerequisite to generate small and homogeneous vesicle sizes. Our previous work focused on the preparation of HbL by using high-pressure homogenizers which resulted in very homogeneous and small HbLs in the size range of around 60 nm [8–11]. These vesicles, however, had insufficient Hb contents, i.e., Phospho-

Abbreviations: Chol, cholesterol; DPPC, L-α-dipalmitoylphosphatidylcholine; DRV, dehydration/rehydration vesicle; DSPG, L-α-distearoylphosphatidylglycerol; Hb, haemoglobin; HbL, haemoglobin-containing liposome; HEPC, hydrogenated egg PC; IHP, inositol hexaphosphate; metHb, methaemoglobin; NADH, β-nicotinamide-adenine dinucleotide reduced form; NADPH, β-nicotinamide-adenine dinucleotide phosphate reduced form; PC, L-α-phosphatidylcholine; PEG, polyethyleneglycol; RES, reticulo-endothelial system; SD, standard deviation; SUV, small unilamellar vesicle; TCA, trichloroacetic acid.

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SSDI 0005-2736(94)00225-8

lipid/Hb molar ratios of 700:1 only. Therefore, one of the key objectives in the present study was to develop a method for the preparation of Hb-containing liposomes with a high yield of Hb entrapment and vesicle size preferably below 200 nm in diameter. The dehydration/rehydration method [12] is known for its high yield entrapment of a variety of drugs in the absence of direct contact of drugs (such as haemoglobin) with organic solvents or exposure to ultrasonication. Dehydration/rehydration vesicles, however, tend to be large and oligo- to multilamellar. A previous approach [13] using the DRV technique had revealed efficient entrapment of haemoglobin in such large vesicles without addressing the problem of integrity of entrapped haemoglobin. In this study, a modified dehydration/rehydration technique for the encapsulation of Hb has been developed yielding high entrapment of intact Hb in vesicles of a size compatible with a longer circulation time. Parts of this study have been already presented [14,15].

2. Materials and methods

2.1. Lipids

Lipids used were hydrogenated egg phosphatidylcholine (HEPC) type E-PC-3 from Lipoid (Ludwigshafen, Germany), distearoylphosphatidylglycerol (DSPG) from Sygena (Liestal, Switzerland), and cholesterol (Chol, biochemical grade) from British Drug Houses (Poole, Dorset, UK). E-PC-3 has recently been reported to contain 40.2% palmitic acid, 55.9% stearic acid and less than 0.5% unsaturated fatty acids [16]. ^{14}C-labelled dipalmitoylphosphatidylcholine (^{14}C-DPPC, specific activity: 1.85 GBq/mmol) was from Amersham (Amersham, UK). Fructose, inositol hexaphosphate (phytic acid, dodecasodium salt), β-nicotinamide-adenine dinucleotide (NADH) and β-nicotinamide-adenine dinucleotide phosphate (NADPH) (both dinucleotides in the reduced form) were from Sigma (Poole, Dorset, UK). Sucrose, glucose, mannitol, and all other reagents were AnalaR grade from British Drug Houses.

2.2. Haemoglobin solution

Stroma-free Hb was prepared as described elsewhere [9,17]. In brief, HIV and hepatitis B antibody-free outdated blood or erythrocyte concentrates were obtained from North London Blood Transfusion Centre (London, UK) and used within two weeks after the expiry date. For the rupturing of red blood cells a homogenizer Mini Lab 8.30H of APV Rannie AS (Albertslund, Denmark) was used. Stroma was removed upon acidic precipitation and centrifugation. The final steps of the preparation were carried out under aseptic conditions in a class I sterile room. They comprised separation of Hb solution from stroma and filtration through microbe retentive Minisart filters (0.2 μm pore size; Sartorius, Göttingen, Germany) into sterile containers.

2.3. Analysis of haemoglobin solution

Total Hb was determined photometrically at 540 nm after conversion into cyanometHb using Drabkin's reagent as described [18]. The degree of oxidation, expressed as relative metHb content, was monitored photometrically at 630 nm before and after conversion into cyanometHb as described [18]. Enzymatic activities were determined according to Beutler [19].

2.4. Radiolabelling of haemoglobin

Hb was radiolabelled with the gamma emitters iodine-125 or chromium-51. Radioiodination of the Hb was carried out by the chloramine T method [20] using sodium iodide (^{125}I, activity: 3.7 GBq/ml, Amersham). Hb was separated from the reaction mixture by gel chromatography on Sephadex G-25 PD10 minicolumns (Pharmacia, Uppsala, Sweden). To determine any free ^{125}I left in the ^{125}I-Hb fraction, a small amount of the labelled protein was mixed with 0.05 ml 1% bovine serum albumin solution and precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) solution. Upon centrifugation at 3000 rpm for 10 min both pellet and supernatant were counted for ^{125}I. Labelling with chromium was carried out by mixing the Hb solution with sodium [^{51}Cr]chromate (specific activity: 9.25–18.5 GBq/mg chromium, Amersham). After 4 h, unbound chromate was removed via excessive dialysis in a cuprophane bag. Both isotopes were counted in a Wallac Clinigamma 1272 (LKB, Bromma Sweden).

2.5. Entrapment of haemoglobin in liposomes

Dispersions of 'empty' small unilamellar vesicles (SUVs) were either prepared by the film method (followed by ultrasonication) [12] or by the one-step method using a homogenizer as previously described [11,21]. Experiences with the type of homogenizer used (Mini Lab 8.30 H) in preparing liposomes are summarized elsewhere [22]. 'Empty' SUVs were used to generate large vesicles in the presence of Hb by the dehydration/rehydration method, initially according to the original protocol of Kirby and Gregoriadis [12], and subsequently with slight modifications. Unentrapped Hb was removed by size exclusion chromatography on Ultrogel columns (LKB, Bromma Sweden). When needed, vesicles were concentrated by centrifugation at $50\,000 \times g$ for 30 min.

2.6. Analysis of haemoglobin-containing liposomes

Unentrapped Hb was determined photometrically as above. Vesicle Hb content was assayed photometrically

and corrected for turbidity as described [9]. In brief, straight baselines were drawn between the absorption minima at $\lambda \approx 340$ nm and at $\lambda \approx 510$ nm and subtracted from the spectra. The extinction at the Soret peak ($\lambda \approx 415$ nm) was then used for quantitation of Hb on the basis of extinction coefficients given elsewhere [18]. This procedure was found necessary since attempts to destroy liposomes with tensides or solvents led to incomplete removal of turbidity and/or denaturation of Hb with the lipids used here (data not shown). Photometric data were checked for accuracy using radiolabelled Hb. MetHb content in the fraction of unentrapped Hb was measured photometrically as described above. It was assumed that metHb concentrations inside and outside the liposomes would be similar. The above mentioned turbidity correction for Hb did not appear applicable in this case. For instance at the relatively low absorption peaks of metHb (545 and 576 nm) but not at the oxyHb peak (415 nm) the relative contribution of turbidity is high and proper correction therefore would be more difficult.

Liposomal lipid content was estimated on the basis of ^{14}C radioactivity from ^{14}C-DPPC incorporated in the vesicles during preparation: samples of Hb-containing liposomes (500 μl) were digested with 200 μl of a 1:1 mixture of concentrated nitric acid and 33% hydrogen peroxide solution by heating overnight at about 90°C until dry and then redissolved in Aquasol (NEN Research Products, Boston, USA). ^{14}C Radioactivity was measured in a LKB 1216 Rackbeta liquid scintillation counter (LKB, Bromma Sweden).

Vesicle sizes were determined in a Malvern Master sizer, or Malvern Autosizer. Vesicles were observed morphologically by negative staining with uranyl acetate in a Philips EM 400 transmission electron microscope. Diameters on micrographs of final magnification (60 000 × to 100 000 ×) were used for the determination of vesicle size distributions (by number). Vesicle volume based size distributions were derived using a PASCAL programme and presented as relative volume frequency histogram.

2.7. *In vivo behaviour of haemoglobin-containing vesicles*

Randomized groups of Wistar rats (both sexes, 125 to 150 g body weight), four animals per group, were injected into the tail vein with Hb-containing vesicles or free Hb, both containing ^{51}Cr-labelled Hb. Doses of 0.8 ml were applied containing a total of 17.5 mg of Hb (about 10^5 cpm) and, when applicable, 68 mg of liposomal phospholipid. 100 μl blood samples were drawn from the tail veins at time intervals and assayed for ^{51}Cr. The same animals were used for all time points. The animals were killed after blood radioactivity levels had fallen below detection limits (at 4 h for the free Hb group and 22 h for the HbL group) and their tissues removed, washed and analysed for ^{51}Cr radioactivity.

3. Results and discussion

3.1. *Characteristics of haemoglobin solution*

Current trends in Hb-based blood substitutes include the preparation of highly purified Hb solutions [23,24]. The reason for this is that after intravenous infusion of free Hb impurities such as traces of cell membrane lipids (especially phosphatidylserine) or proteins have been found responsible for unwanted side effects such as coagulative activity [25] or coronary vasoconstriction [26]. However, no such side effects for Hb entrapped in liposomes have been reported to date. Phosphatidylserine, if present in Hb solutions used for entrapment, would be incorporated in the liposomal bilayer. Although phosphatidylserine-containing liposomes have shown procoagulant activity [27], this should be negligible, if at all present, as only a trace of the phospholipid would be present in our preparations. Purification steps, other than precipitating the stroma by acid, were therefore omitted during Hb isolation in the present study.

Hb solutions obtained as described above contained 18.2 ± 2.5 g/100 ml (mean ± S.D., $n = 4$) of Hb in the absence of protein concentration steps. MetHb values in the Hb solutions, determined immediately after preparation, were $3.4 \pm 0.5\%$ of total Hb. In order to evaluate whether the natural mechanism of preventing accumulation of metHb in erythrocytes is maintained in Hb solution, tests on relevant enzyme activities were performed. One of these mechanisms involves NADH-dependent reductase and cytochrome b_5 as a mediator [28]. A membrane-bound and two cytosolic forms of the NADH–cytochrome-b_5 reductase in erythrocytes have been described in the literature [29,30]. In our Hb solutions, NADH--cytochrome-b_5 reductase activity amounted to 1.52 ± 0.43 IU. In comparison, the human red cells used for isolation of Hb showed activities of 3.77 ± 0.50 IU. NADPH metHb reductase activity, another antioxidative mechanism, was not detectable in our Hb solutions. The metHb-reducing function of this enzyme has been questioned recently [31].

3.2. *Stability of haemoglobin solutions under storage*

In stroma-free Hb, 3–4% metHb is usually present after isolation and a small, but consistent accumulation of metHb during storage is known to occur (e.g., [2]). Several attempts have been made to compensate for the lack of antioxidative activity in stoma-free Hb by the addition of ascorbic acid and desferal. These however turned out to act as prooxidants rather than antioxidants at least at the concentrations used [32]. On the other hand catalase showed minor effects whereas NADH and glutathione were shown to provide good antioxidative protection [32]. The suitability of such agents, however, for the long-term stabilization of stroma-free Hb is questionable in view of their own instability in aqueous solution. Thus, in order to evaluate

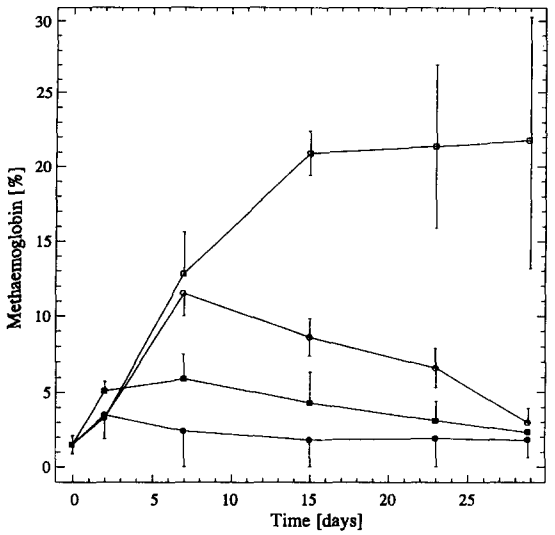

Fig. 1. Oxidative degradation (measured as % methaemoglobin formed) of Hb in solution during storage under different conditions. □, in the presence of air (control); ○, in the presence of air and glucose; ■, in the presence of nitrogen; ●, in the presence of nitrogen and glucose. Bars denote S.D. of three experiments.

whether NADH–cytochrome-b_5 reductase can provide another means of protection against metHb formation during storage, the Hb solutions were incubated aseptically under a variety of conditions. To a number of vials, glucose (2.64 mg/ml Hb solution) was added and/or air expelled by flushing with sterile nitrogen. MetHb values were determined at time intervals (up to 29 days) at 4–8°C as an indicator of oxidative degradation of Hb. Fig. 1 shows that whereas in the control sample (air atmosphere, no glucose added), metHb values (and S.D.) increased constantly to more than 20% of the Hb content within 15 days, in the sample stored in the presence of air and glucose metHb increased to a little more than 11% but declined subsequently to 2.8 ± 1.0% (29 days). With Hb samples kept under nitrogen with or without added glucose there was a slight increase in metHb content during the first few days but this was subsequently reduced to 1.7 ± 1.1 and 2.1 ± 1.6% respectively after 29 days.

These results suggest that, in the absence of additional purification steps (other than the removal of stroma by acidic precipitation) during the isolation of Hb from red blood cells, a considerable proportion of the activity of at least one protective enzyme (NADH–cytochrome-b_5 reductase) is maintained in the Hb solution. A reduction of Hb stability after chromatographic purification has been observed by other authors as well [33], which may now be explained by loss of NADH–cytochrome-b_5 reductase. The enzyme can thus be utilized not only to protect Hb against oxidation but, as shown in Fig. 1, also to reduce previously formed metHb.

The antioxidative action of NADH–cytochrome-b_5 reductase was found to improve significantly by adding glucose. This finding differs from observations by Stratton et al. [32], who could not find any antioxidative effect of glucose in their Hb solutions. However, it is known that NADH–cytochrome-b_5 reductase cannot utilize glucose as a substrate directly, a fact that has given rise to speculations about glycolytic enzymes being present in Hb solutions (as prepared here) as well.

3.3. Radiolabelling of haemoglobin

Circulation times of Hb-containing liposomes in vivo have previously been determined by two methods. Farmer et al. [34] used the 'haemocrit' approach by measuring the volume of packed Hb-containing vesicles after centrifugation of blood samples. Phillips et al. [35] on the other hand, employed technetium-99m to label preformed Hb-containing vesicles. After extraction of liposomes, the label was found associated not only with Hb but also with the aqueous and the lipid phase of the Hb-containing liposomes [36]. Therefore, in order to monitor the fate of free as well as liposomal Hb in vivo an alternative labelling method was deemed necessary. Radiolabelling of the Hb molecule, preferably by introducing a gamma emitter would also allow its detection in biological samples even at low levels without the need of any separation. Two methods of radiolabelling were compared, namely radioiodination and radiochromation.

Table 1
Influence of initial Hb and sucrose concentration on entrapment and oxidative degradation of Hb (% of methaemoglobin formed) and the size of formed vesicles during preparation of DRVs

Initial Hb (mg/ml)	Sucrose (mmol/l)	Phospholipid/haemoglobin ratio (mol/mol)	Methaemoglobin formed (% of total Hb)	z-average vesicle diameter (nm)
10	–	9797 ± 1218	≈ 100	2628 ± 1215
10	10	9320 ± 401	≈ 100	2151 ± 535
10	50	6902 ± 431	≈ 100	1480 ± 235
20	–	1521 ± 164	96.6 ± 4.1	1750 ± 303
20	50	1121 ± 9	87.2 ± 3.1	1303 ± 81
20	150	1215 ± 388	69.6 ± 3.5	674 ± 134
60	–	656 ± 241	59.5 ± 4.2	788 ± 139
60	150	1155 ± 168	13.3 ± 3.9	645 ± 197

Hb was entrapped in vesicles by freeze-drying and subsequent rehydration.
Values denote mean ± S.D.; $n = 3$.

Table 2
Effect of cryoprotectants on the entrapment and oxidative degradation of Hb (% of methaemoglobin formed) and the size of formed vesicles during preparation of DRVs

Cryoprotectants		Inositol hexaphosphate	Phospholipid/ haemoglobin ratio (mol/mol)	P	Methaemoglobin formed (% of total Hb)	P	z-average vesicle (nm)
composition	concentration (mmol/l)						
Sucrose	150	equimolar [a]	260 ± 19		27.1 ± 1.8		198 ± 9
Glucose, fructose, mannitol	100, 100, 100	equimolar [a]	321 ± 75	n.s. [b]	22.4 ± 2.0	0.05 [b]	213 ± 7
Glucose, fructose, mannitol	100, 100, 100	none	323 ± 9	0.05 [b]	30.5 ± 1.6	0.05 [c]	216 ± 4
Glucose, fructose, mannitol, dextran	50, 50, 50, 0.6	equimolar [a]	653 ± 64	0.001 [b]	20.9 ± 2.5	0.05	370 ± 13

Hb (60 mg/ml) was entrapped in vesicles in the presence of various saccharides by freeze-drying and subsequent rehydration.
Values denote mean ± S.D.; $n = 3$. P denotes significance level; n.s. not significant.
[a] Equimolar to haemoglobin.
[b] Comparison between sucrose and other saccharide mixtures.
[c] Comparison between equimolar inositol hexaphosphate and none.

For radioiodination, Hb (7 mg) was incubated with 18.5 MBq ^{125}I-sodium iodide for 20 min. There was no evident turbidity or precipitation in the sample during incubation. ^{125}I binding to Hb was 650 kBq/mg. Upon TCA precipitation, more than 95% of the radioactivity was found in the pellet. Further UV/VIS-spectra of labelled Hb did not show any significant differences compared to those of native Hb. However, when assaying the Hb content of vesicles by gamma counting, surprisingly high values of encapsulation were obtained (75.3 ± 5.0% of the amount used; not shown). These values also differed significantly from those obtained by the photometric assay (16.6 ± 1.4% entrapped; same samples). This discrepancy might be explained in at least two ways: firstly, attachment of ^{125}I to the Hb molecule might be unstable in the presence of phospholipid the isotope becoming somehow associated with liposomes. Secondly, ^{125}I-Hb, in contrast to native Hb, may exhibit high affinity for phospholipid vesicles and therefore adsorb onto their surface. The former possibility was investigated by extracting the phospholipid from liposomes with chloroform/methanol (3:1). Aliquots of the aqueous phase, which contained (denatured) Hb, and the lipid phase were assayed for ^{125}I. Almost 100% of the radioactivity was recovered in the aqueous phase indicating that the ^{125}I label was not associated with lipid but with Hb (data not shown). To resolve the question as to whether ^{125}I-Hb shows unusual affinity for phospholipid bilayers, 'void' small unilamellar vesicles of the same lipid composition used to entrap Hb (total lipids: 56 mg/ml), were incubated for one hour in a mixture of native and iodinated Hb (177 mg/ml and 0.5 mg/ml respectively). After separation of the vesicles from the Hb by gel chromatography, the two sets of fractions containing eluted liposomes and Hb respectively were assayed photometrically and by gamma counting for Hb content: 42.4 ± 3.8% of the radioactivity but only 1.6 ± 0.3% of the specific absorption corresponding to native Hb were found associated with the vesicles ($n = 6$, mean ± S.D.) (results not shown). It thus can be concluded that iodinated Hb is adsorbed to a great extent to vesicles on incubation. In addition, it was observed that a considerable portion of the native Hb and ^{125}I-Hb mixture was retained on the top of the column during chromatography, probably in the form of ^{125}I-Hb aggregates. These findings agree with the results of Bleeker et al. [37] who found different chromatographic patterns for ^{125}I-Hb and unlabelled Hb when size exclusion gels and anion exchange material were used.

For chromium-51 labelling, 18.5 MBq ^{51}Cr-sodium chromate were mixed with 40 ml of Hb solution. After extensive dialysis, binding efficiency was 780 kBq of

Table 3
Influence of preparation conditions and lipid composition on the entrapment and oxidative degradation of Hb (% of methaemoglobin formed) and the size of formed vesicles during preparation of DRVs

Liposomal lipid composition	Method of void vesicle preparation	Rehydration conditions		Molar phospholipid to haemoglobin ratio (mol/mol)	Methaemoglobin formed (% of total Hb)	z-average vesicle size (nm)
		temperature (°C)	buffer medium			
HEPC/DPPG/Ch	film method, sonication	20	isoosmotic	1155 ± 168	13.3 ± 3.9	645 ± 162
HEPC/DPPG/Ch	film method, sonication	50	isoosmotic	485 ± 104	15.2 ± 4.0	655 ± 179
HEPC/DPPG/Ch	one-step method	50	isoosmotic	176 ± 15	15.8 ± 7.1	147 ± 15
HEPC/Ch	one-step method	50	isoosmotic	206 ± 3	19.4 ± 5.6	153 ± 18
HEPC/Ch	one-step method	50	hypoosmotic	326 ± 81	26.4 ± 11.6	176 ± 38

Hb was entrapped in vesicles by freeze-drying and subsequent rehydration.
Values denote mean S.D.; $n = 3$.

chromium-51 per g Hb with more than 95% of the total radioactivity found in the pellet upon TCA precipitation (data not shown). UV/VIS-spectra of ^{51}Cr-Hb showed slightly increased absorption in the metHb region compared to native Hb. There was no unusual affinity of ^{51}Cr-Hb to phospholipid bilayers.

3.4. Haemoglobin-containing DRVs

Preparation of Hb-containing liposomes by the dehydration/rehydration method was carried out initially by using 49.5 mmol/l of both of HEPC and Chol and 10 mg/ml of Hb. Hb entrapment was 0.5 mg/ml, representing an encapsulation efficiency of 5%. Total recovery of Hb (by photometric assay) was 25% of that used. Moreover, relative metHb values were almost 100% of total Hb found in all samples (results not shown). It can thus be concluded that substantial oxidation and denaturation of Hb had occurred during the procedure leading to the formation of precipitates of oxidized Hb which could not be detected photometrically. It is known that Hb is oxidized as a result of water removal during freeze-drying [38]. Oxidation can however be diminished in the presence of carbohydrates. For instance, Labrude et al. [38] reported almost complete protection of Hb (metHb < 5%) by the addition of 250 mmol/l glucose during freeze-drying whereas Rudolph reported considerable oxidation (metHb > 45%) at this glucose level [39]. In our hands, freeze-drying and rehydration of the Hb solution in the presence of increasing amounts of sucrose (0, 10, 50 and 100 mmol/l) led to metHb values of 31.7, 3.7, 1.5 and 1.8% respectively (results not shown). Thus, 10 mmol/l of sucrose seemed to be enough to prevent significant oxidation of Hb. Sucrose however may also act as a cryoprotectant for the vesicles, and therefore prevent their rupture during dehydration, a process deemed necessary for the loading of Hb into liposomes generated on rehydration [12]. In this respect it is of interest that Hauser and Strauss [41] reported retention of ions by small unilamellar vesicles during freeze-drying in the presence of 150–300 mmol/l sucrose and that 'void' vesicle sizes remained unaffected after freeze drying and rehydration in presence of 250 mmol/l sucrose [39] and that 60% or more of Hb was retained by vesicles on freeze-drying and rehydration when trehalose was present in concentrations as low as 10 mmol/l [40].

In subsequent experiments the effect of increasing amounts of sucrose on metHb formation and Hb entrapment was studied. Resulting vesicles were analysed for their Hb content (given as molar ratio of phospholipid/Hb), oxidative deterioration of Hb (given as metHb values) and size (Table 1). It appears that sucrose at the 10 mmol/l level in the presence of vesicles fails to prevent oxidation of Hb (100% metHb formed). With increasing concentrations of sucrose (and Hb), metHb values are lower. Furthermore, elevated initial concentrations of Hb (no sucrose added) also promote a marked antioxidative effect, the protein apparently acting in a self-protecting manner. In addition, as metHb formation and, consequently, precipitation is limited, Hb entrapment is improved. However, with higher sucrose concentrations mean sizes of the formed vesicles are smaller, probably because sucrose is limiting the extent of vesicle fusion during dehydration and rehydration. At 60 mg/ml Hb and 150 mmol/l sucrose, a reasonable compromise between sufficient protection of Hb against oxidation and effective loading into vesicles seems to have been achieved (Table 1).

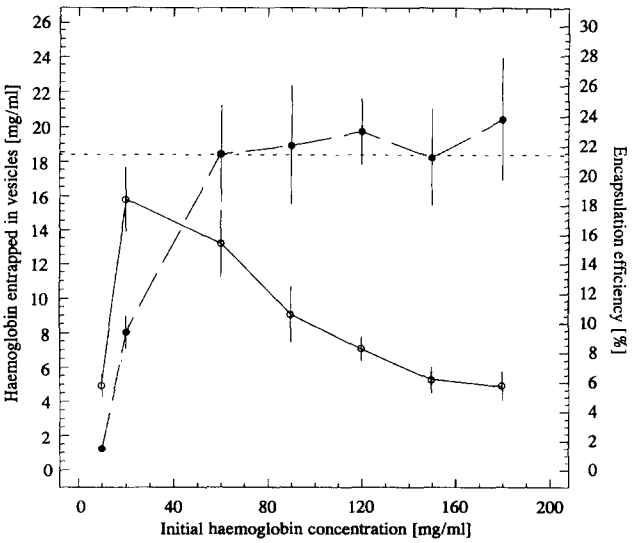

Fig. 2. Comparison of Hb total content and % encapsulation in DRVs as a function of initial haemoglobin concentration. Sucrose (150 mmol/l) was used as a cryoprotectant in all DRV preparations. ●, amount of Hb entrapped (mg/ml liposome dispersion); ○, % of Hb entrapped.

Following the observation of a beneficial effect of increasing Hb concentration on its entrapment, further studies with even higher concentrations of Hb but with constant concentrations of sucrose (150 mmol/l) were performed. Fig. 2 shows that above 60 mg/ml Hb, a plateau is achieved in terms of absolute amount of Hb entrapped. However, efficiency of Hb encapsulation (% entrapment) reaches its optimum at lower concentration of Hb (20 mg/ml). Thus, between 20 and 60 mg Hb/ml, the absolute amount of entrapped Hb is still significantly rising whilst the relative entrapped fraction (% of total Hb employed) is falling. The observed plateau of absolute entrapment can be explained by the tenfold increase in Hb and vesicle concentrations during the first rehydration step in which 10% of original amount of water is added to the powder obtained on dehydration [12]. Above the initial Hb concentrations of 60 mg/ml, the resulting vesicle-Hb-mixture during the first step of rehydration (600 mg/ml) is so viscous that complete rehydration and thorough mixing by mechanical agitation is impossible.

Having established optimum sucrose and Hb concentrations in terms of Hb entrapment, attempts were made to substitute sucrose by a cryoprotectant which is readily

metabolizable. Thus, an equimolar mixture of glucose, fructose and mannitol and a mixture of glucose, fructose, mannitol and dextran (not metabolizable) amounting to the same (total) concentration in terms of monosaccharide units as that of sucrose were tested (Table 2). Results show that use of monosaccharides led to phospholipid/Hb molar ratios that were similar to those obtained with sucrose ($1:321 \pm 75$ compared to $1:260 \pm 19$) and to a slightly lower level of metHb formation. With the mixture containing dextran however, entrapment values were reduced (Table 2). It may be that the macromolecular sugar competes with Hb for the aqueous space inside the vesicles.

Human red blood cells contain an allosteric effector, 2,3-diphosphoglycerate, which adjusts the oxygen affinity of Hb to physiological values. In Hb-containing liposomes the effector is usually substituted by inositol hexaphosphate (IHP) [1]. Results in Table 2 show that when IHP is mixed with equimolar Hb before dehydration/rehydration, a significant reduction (by 32%) of oxidative degradation of Hb is observed. This result could be explained either by the known [42] ability of IHP to combine with metHb and lower its redox potential or by its activation of NADH–cytochrome-b_5 reductase [43].

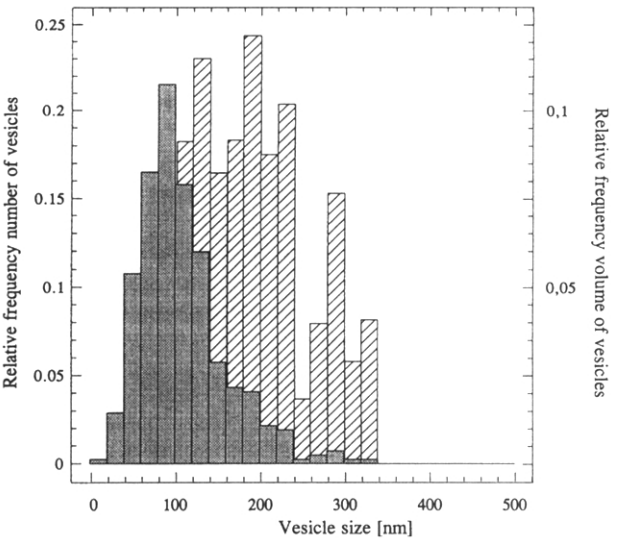

Fig. 4. Relative frequency distribution of diameters (fraction of total number of vesicles measured) and corresponding relative volumes (fraction of total vesicle volumes) of haemoglobin-containing DRVs. Dotted bars, relative distribution of vesicle diameters; hatched bars, corresponding relative distribution of vesicle volumes. Vesicle diameters of more than 400 vesicles as measured on negative staining electron micrographs were used for calculation. Lipid composition: hydrogenated egg PC and cholesterol (1:1 mol/mol). Cryoprotectants were glucose, fructose and mannitol, 100 μmol each.

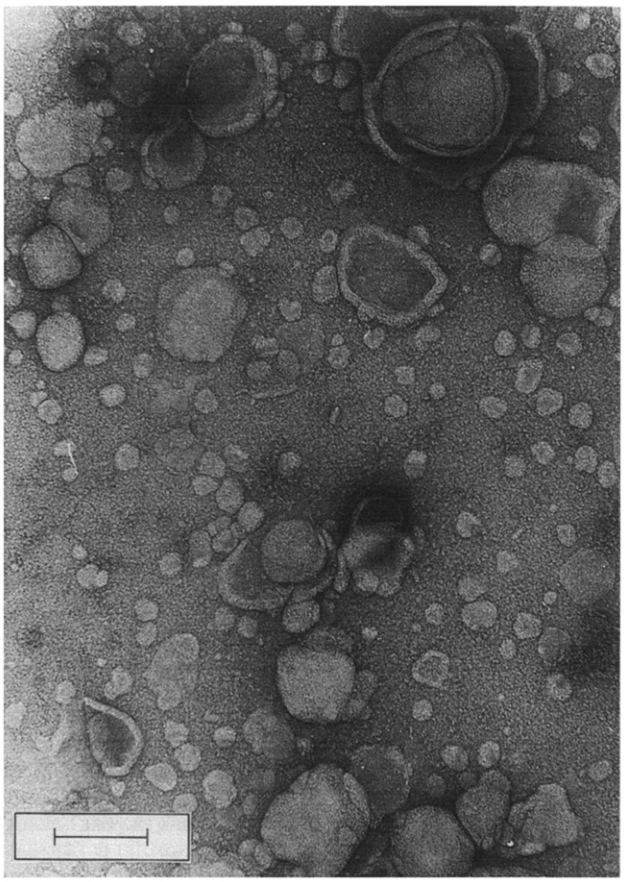

Fig. 3. Electron micrograph of haemoglobin-containing DRVs. Sample was prepared by negative staining using 2% uranyl acetate solution. Bar denotes 200 nm.

The influence of some variations of preparation process parameters on the characteristics of the Hb-containing liposomes is illustrated in Table 3. First, the effect of temperature during the rehydration step was examined. From previous work it was known that the onset of heat-induced irreversible Hb denaturation in the presence of phospholipid vesicles commences above 50°C [17]. Therefore, rehydration temperatures of up to 45–50°C were applied. The resulting Hb-containing liposomes contained more (about 2.4-fold) Hb than those rehydrated at room temperature (20°C) without a significant increase in metHb formation (Table 3). In contrast, Szebeni et al. [44] have reported a decrease in Hb-entrapment at 45°C with handshaken liposomes compared to that obtained at room temperature.

When Hb was loaded into vesicles prepared by the one-step method [11], entrapment was more than doubled (phospholipid/Hb ratio 176 ± 15) and mean vesicle size was much smaller (z-average 145–175 nm) compared to corresponding values obtained with Hb loading into vesicles prepared by sonication (Table 3). This could be attributed to the very homogeneous population of small unilamellar void vesicles achieved by the former method [21,22]. It appears that addition of DPPG in liposomes improves Hb entrapment (ratio of 1:176 compared with a value of 1:206 for DPPG-free liposomes; Table 3), possibly because of charge-induced interactions between Hb and the lipid. Indeed, interaction of Hb with DPPG vesicles but not with isoelectric PC vesicles could be eluci-

dated by differential scanning calorimetry [17] and its effect on Hb entrapment, already observed by others [44], has led to the adoption of these lipids (e.g., DMPG) in most recent Hb-liposomal formulations. Finally, the influence of the osmolarity of the rehydration medium on Hb-entrapment was examined. It is evident that isoosmolarity of the rehydration medium and the medium containing the vesicles before dehydration contributes to higher Hb entrapment values (isoosmotic and hypoosmotic media; Table 3) probably because of reduced osmotic stress and solute leakage [12].

Electron microscopic observations (Fig. 3) of Hb containing liposomes made of HEPC and cholesterol reveal vesicles as round, negatively stained dark shadowed structures. When the diameters of more than 400 of such vesicles were measured and used to calculate the size distribution (shown as a relative frequency histogram in Fig. 4), the arithmetic mean of the diameters was 110 nm. Almost 95% of the analysed vesicles had diameters in the range of 40 to 240 nm with very few larger vesicles being apparent. In contrast to conventional DRV preparations [45,46], the present vesicles are much smaller and more homogeneous in size, probably because of the use of cryoprotectants in their preparation. When the size distribution by number is converted into a volume-based size distribution (Fig. 4), it becomes evident that larger vesicles contribute a great portion to the total entrapped volume and there is a distinct tendency towards bimodal distributions present. Together with data from photon correlation spectroscopy (PCS), present electron microscopy data rule out a bimodal distribution of vesicles resulting from aggregation phenomena: volume distributions obtained by the latter technique (Fig. 4) are in good agreement with *z*-averages of 140 to 200 nm and a distribution range of up to 350 nm as observed by PCS (results not shown). In early studies with Hb-containing liposomes, a vesicle size that was small enough to allow unrestricted circulation in normal capillaries (< 4 μm) was considered a prerequisite [47]. Lateron, the need to sterilize Hb-containing vesicles required a size of less than 0.2 μm [34]. More importantly, small unilamellar liposomes are the vesicles of choice when extended presence in the blood circulation is required [48–51]. These are also known to impair the reticuloendothelial system only mildly [52] even when given in large doses [53]. Hb-containing vesicles as prepared here appear to satisfy such criteria for in vivo use.

Taking into consideration findings in Fig. 2 and Tables 1–3, the final optimal Hb-containing preparations (3 batches) were made as follows: HEPC/Ch (49.5 mmol/l each) void vesicles, prepared by the one-step method, were mixed with Hb (60 mg/ml), glucose, fructose and mannitol (100 mmol/l of each) as cryoprotectants and IHP (equimolar to Hb). After rehydration at 45–50°C using isoosmotic medium, the resulting HbL exhibited the following characteristics: A total of 25.9 ± 0.3 mg of Hb was entrapped representing a phospholipid/Hb molar ratio of 206 ± 3 :1, an encapsulation efficiency of 21.6 ± 0.3% and recovery values for Hb and phospholipid of 88.8 ± 4.8% and 84.3 ± 7.9%, respectively. The metHb content at the end of the procedure was 19.4 ± 5.6%. The vast majority of vesicles had diameters of 40 to 240 nm (EM) and displayed *z*-averages of 140 to 200 nm (PCS).

Comparison of the present values of Hb entrapment with those of others is difficult as entrapment efficiency says little if lipid contents, lipid recovery and phospholipid/Hb ratios are not given. Jopski et al. [54] have described the preparation of Hb-containing liposomes by detergent dialysis with phospholipid/Hb molar ratios of 270–280:1 and average vesicle diameters of 160–200 nm. Better phospholipid/Hb ratios (50:1) were also described in the same work [54] for 450 nm vesicles consisting of egg PC. However, the detergent dialysis technique is not suitable for vesicles composed of long-chain saturated phospholipids and equimolar cholesterol [54]. HbL made of hydrogenated soy PC and cholesterol by high-pressure homogenization showed phospholipid/Hb ratios of 700:1 and mean vesicle diameters of 60 nm [17]. Thus, the phospholipid/Hb ratios (about 210:1) of HEPC/cholesterol vesicles of 110 nm diameter as obtained in the present study are quite good in comparison. It is assumed that such ratios cannot be improved further for vesicles of this size range. The phospholipid/Hb molar ratio is primarily determined by the Hb content present during vesicle formation. In this study the Hb concentrations used

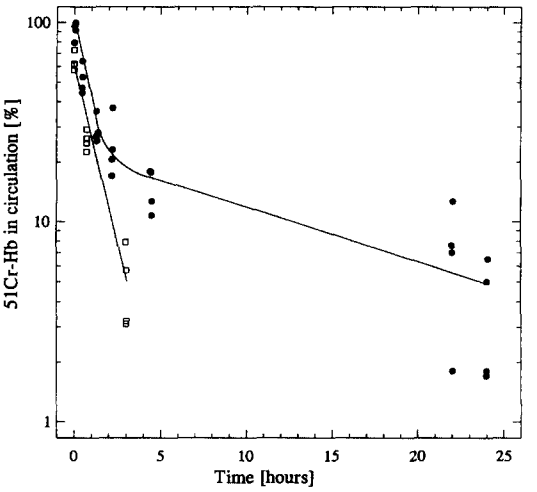

Fig. 5. Clearance of liposome-entrapped and free haemoglobin from the blood of injected rats. Rats were injected intravenously with a total of 17.5 ± 0.2 mg of Hb either in the free form or entrapped in DRVs composed of hydrogenated egg PC and cholesterol (1:1 mol/mol) containing 42.9 mg of PC. The total injected volume was 0.8 ml (corresponding to about 10% of the blood volume of the animals which was estimated as 7% of their body weight). Values at time intervals denote % of the injected dose present in the circulation. □, free Hb; ●, liposomal Hb. Blood level data for liposomal Hb are from two separate experiments: (a) time points 2, 30, 135 min and 22 h and (b) time points at 5, 80, 270 min and 24 h. Values were not corrected for the increase of the blood volume caused by the injection.

were higher than in any known previous attempts. This was achieved as a result of a 10-fold increase in the concentration of Hb during vesicle formation [12]. Indeed, the DRV technique [12] should theoretically allow to employ even higher Hb concentrations. We found however, that maximum entrapment was achieved with initial concentrations of 60 mg Hb/ml (i.e., 600 mg/ml during vesicle formation) (Fig. 2).

As a last step prior to in vivo experiments, the stability of ^{51}Cr-Hb-containing vesicles was tested in the presence of mouse plasma at 37°C for 30 min. Released ^{51}Cr-Hb, separated from the medium via gel chromatography, accounted for only 3.1% of total liposomal Hb (results not shown).

3.5. Intravascular circulation and organ distribution of haemoglobin-containing liposomes and free haemoglobin

Liposomes and other colloidal drug carriers are eliminated rapidly from the blood circulation by mononuclear phagocytic cells of the RES (reticulo-endothelial system) mainly of the liver and spleen. In the case of massive liposome transfusions (e.g., top load transfusion of 25% of blood volume) however, phagocytic activity is largely saturated and a considerable fraction of liposomes stays in the circulation for longer periods [34,35]. This RES saturation may give rise to opportunistic infections [52]. Injection of volumes which correspond to 10% of the total blood volume were therefore chosen in the present study. Furthermore, liposomes made of hydrogenated egg PC (and cholesterol) were employed for the in vivo studies with HbL as they are believed to be less immunosuppressive than those made of soy-PC [49].

Fig. 5 shows that in animals injected with free Hb there is a rapid exponential loss of ^{51}Cr-radioactivity in the circulation with values reaching low levels after 3 h (8.1 ± 4.3% of injected). In contrast, the elimination of liposomal ^{51}Cr-Hb is biphasic and similar to that observed (and described on the basis of a mathematical model) by Juliano and Stamp [48] for small liposomes. The fitted curve in Fig. 5 is based on the equation [48]:

$$A = A_1 \cdot e^{-0.693 \cdot t/T_1} + A_2 \cdot e^{-0.693 \cdot t/T_2}$$

where A is the actual plasma level in % of the (theoretical) plasma level A_0 at the time of injection; A_0 is the sum of A_1 and A_2; and T_1 and T_2 are half times for the two phases of clearance. A_1 and A_2 were 80% and 20% respectively and T_1 and T_2 were 40 min and 11 h, respectively. After 22 h a small portion of the dose (7.3 ± 4.4% of injected) can still be detected in blood. Thus, levels of ^{51}Cr-HbL remaining in circulation are higher than those of free Hb over the whole period of observation. This is to our knowledge the first kinetic study on Hb-containing liposomes based on the injection of volumes corresponding to 10% of the estimated blood volume.

Although injected volume, total injected lipid dose, lipid composition and vesicle size differ from those used in previous kinetic studies on haemoglobin-containing liposomes, the biphasic elimination kinetics, the increased blood levels and the prolonged blood circulation times observed here are in good agreement with published data (e.g., [34,35,55]). No attempts were made however to fit a single (exponential) curve to the liposomal Hb blood levels and therefore a single half life value representing the whole elimination process cannot be given. One possible explanation for the observed biphasic elimination kinetics could be an initially rapid uptake of HbL followed by partial saturation of the phagocytic activity of the RES and a subsequently delayed clearance of the remaining vesicles. Previous work has shown that void small unilamellar vesicles made of the same lipids and injected in a similar dose depress phagocytic activity moderately (by about 30%) 3 h after injection [53].

In order to determine sites of deposition or excretion of free liposomal Hb, animals were killed at the end of the study and their tissues (liver, spleen, kidneys, heart and

Table 4
Tissue distribution of ^{51}Cr radioactivity in rats after injection of free or liposome-entrapped ^{51}Cr-Hb

Tissue	Free ^{51}Cr-Hb (4 h after injection)		Liposomal ^{51}Cr-Hb (22 h after injection)	
	% in organ	% per g of organ	% in organ	% per g of organ
Liver	10.27 ± 0.84	1.37 ± 0.12	47.34 ± 4.42	6.57 ± 1.38
Spleen	2.67 ± 0.67	3.53 ± 0.43	26.80 ± 9.33	35.10 ± 8.89
Kidneys	8.57 ± 0.25	6.07 ± 0.52	0.90 ± 0.15	0.56 ± 0.09
Lungs	1.26 ± 0.28	0.55 ± 0.11	0.76 ± 0.38	0.62 ± 0.25
Heart	0.41 ± 0.08	0.61 ± 0.08	0.09 ± 0.03	0.16 ± 0.04
Blood [a]	5.01 ± 2.29	0.55 ± 0.25	7.28 ± 4.40	0.77 ± 0.47
Total recovered	28.18 ± 3.04		83.18 ± 1.67	

Rats were injected as in Fig. 5 and killed at 4 (free Hb) and 22 h (HbL group). Values (mean ± S.D., $n = 3$) denote % of injected radioactivity recovered in whole tissue or per g of tissue.

Values denote mean ± S.D.; $n = 3$.

[a] Values based on the assumption that the blood pool represents 7% of body weight.

lungs) analysed for radioactivity. Results in Table 4 show that in the free Hb group a total of only 28% of administered radioactivity was detectable in the tissues examined after 4 h. Highest levels were found in the kidneys, the probable site of excretion. Although urine and bladder were not examined in this study these may well account for a great portion of the label which was no longer detectable after 4 h. In contrast, total radioactivity level in the tissues of the HbL group after a much longer time interval (22 h) was 83% of the dose. Liposomal ^{51}Cr-Hb was found to accumulate mostly in liver and spleen the latter showing a higher level of radioactivity per g of tissue. Thus, the mode of elimination of liposome-entrapped ^{51}Cr-Hb is determined by its carrier.

In conclusion, our results show that stabilization of Hb during storage against oxidation can be achieved by ensuring that, in the protocol used, levels of the enzyme NADH–cytochrome-b_5 reductase in solutions of Hb are maintained. Moreover, Hb can be entrapped in dehydration/rehydration vesicles effectively if cryoprotectants are added in concentrations sufficiently high so as not only to inhibit degradation of Hb during freeze-drying, but also to allow the rupture or destabilization of vesicles, a process deemed essential for the efficient entrapment of Hb by the DRV method [12]. Under optimal conditions small unilamellar Hb-containing DRVs are formed mainly in the size range of 40 to 240 nm diameter which is considered [56] close to ideal for vesicle longevity in the blood circulation. Such liposomes contain Hb in a molar ratio of 1:200 relative to the phospholipid content, which is regarded high for vesicles of this size range.

The elimination of Hb from the blood circulation of rats after infusion of volumes corresponding to 10% of the blood volume is substantially retarded when entrapped in vesicles made of hydrogenated egg PC and cholesterol, as judged from ^{51}Cr radiolabelled Hb. However, the circulation time of HbL as observed in this study is still far from ideal. Lipids which are known to further prolong circulation times of vesicles [56] perhaps in conjunction with a highly hydrophilic surface (e.g., by using PEG [57] or polysialic acids [58]) should be employed for the preparation of HbL by the DRV-method.

Acknowledgements

We are grateful to APV Rannie Albertslund, Denmark for lending us a Mini Lab 8.30 H homogenizer. We wish to thank Dr. Zanelli, CRC, Northwick Park, London, and Mr. D. Bachmann, Pharm. Institute, University of Freiburg, Germany, for their valuable help with the radiolabelling experiments and with electron microscopic analyses respectively. The help of Miss H. Da Silva, Mr. A. Rogers, Miss N. Skalko, Dr. Z. Wang, and other members of the School of Pharmacy is greatly appreciated. Financial support by the ARC-programme of Deutscher Akademischer Austauschdienst and a post-doctoral grant of Deutsche Forschungsgemeinschaft to M.B. are gratefully acknowledged.

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