Biochimica et Biophysica Acta, 1146 (1993) 157–168

BBAMEM 75894

Molecular mechanism of the lipid vesicle longevity in vivo

Gabriele Blume and Gregor Cevc

Medizinische Biophysik-Forschungslaboratorien, Urologische Klinik und Poliklinik der Technischen Universität München, Klinikum r.d.I., München (Germany)

(Received 9 July 1992)
(Revised manuscript received 25 November 1992)

Key words: Drug carrier; Lipid vesicle; Cell uptake; Model membrane; Biodistribution

An important, if not the chief, condition for the prolongation of the circulation times of lipid vesicles in vivo is the suppression of macromolecular adsorption onto the surface of such vesicles. This adsorption can be prevented very efficiently by a zone of suitably designed and mobile steric hindrances near the lipid layer surface. Lipid vesicles with such a surface coat, cryptosomes, thus circulate in blood for very long periods of time after systemic applications. Lipid vesicles composed of phosphatidylcholine molecules and of suitable polyoxyethylene (PEG) derivatives of phosphatidylethanolamine, for example, remain in the blood circulation 8–10-times longer than standard liposomes made of phosphatidylcholine only: in mice the half-lifes of the former and latter vesicles, after an i.v. administration, are approx. 0.6 h and between 5.9 and 13.8 h, respectively. Vesicle longevity is not destroyed by the phosphatidylcholine chains fluidity. Vesicles consisting of a mixture of distearoylphosphatidylethanolamine-PEG (DSPE-PEG) with distearoylphosphatidylcholine or cryptosomes made of DSPE-PEG and soy-bean phosphatidylcholine, consequently, have a very similar fate in vivo. Furthermore, the cryptosome longevity is not affected directly by the presence of the net charges on the lipid membranes and is little sensitive to the details of the group coupling the PEG-headgroups and the lipidic (hydrophobic) anchor. However, the life-time and the distribution of the stabilized lipid vesicles in vivo depend quite sensitively on the surface density of the sterically active headgroups; often (if not always) the resistance to plasma components adsorption as well as the resulting longevity in vivo both show a maximum near the same lipid/stabilizer molar ratio. Optimum bilayer composition may differ for the different combinations of the main and sterically active membrane components. Its position is probably determined by the variations in the molecular mobility and the effective surface-coverage effects: both must be sufficiently high for the vesicle phagocytosis and accumulation in the reticulohystiocytic system to be suppressed. On the contrary, the bilayer surface hydrophilicity, which hitherto has been believed to be of paramount importance for the liposome longevity in vivo, is per se not relevant for the biological fate of the lipid vesicles, provided that this hydrophilicity exceeds some minimum value.

Introduction

Lipid vesicles, liposomes, are generally believed to be nearly universal carriers of the therapeutic and other substances [1]. This is owing to the amphiphatic structure of the basic liposome components; lipid amphiphily causes each lipid vesicle to consist of at least one internal aqueous compartment and at least one surrounding lipid double layer with a hydrophobic, fatty interior, the former and the latter acting as potential reservoirs for the water- and fat-soluble agents, respectively.

In the last few years, the popularity of liposomes as a form of drug carriers has increased further by the discovery of vesicle compositions which prolong vesicle circulation in the blood stream for many hours [2–4]. Equally important was the observation that such vesicles have a propensity to accumulate in tumorous [6,7] or inflamed tissues.

To optimize the properties of such long-lived liposomes, which we call cryptosomes (based on the greek words 'cryptos', hidden and 'soma', body), it is crucial, however, to understand how and why the injected particles are eliminated from the blood. It is also important to comprehend the mechanisms which can be used to evade such elimination. In our previous paper we have shown that liposome longevity is related to the diminished capability of the phagocytic cells to take-up 'hidden vesicles', cryptosomes [8,9]. In this paper we wish to discuss, based on our new experimental results, the origin of vesicle 'invisibility' to the phagocytes and thus discuss the mechanism of carrier longevity in vivo.

Correspondence to: G. Cevc, Medizinische Biophysik-Forschungslaboratorien, Urologische Klinik und Poliklinik der Technischen Universität München, Klinikum r.d.I., Ismaningerstr. 22, D-8000 München 8, Germany.

Our basic assumption is that in order to be eliminated by the cells in the body each lipid vesicle must first be recognized as a desired target and then phagocytosized. The first step in this process involves adsorption and denaturation of the serum proteins at the bilayer/serum interface. The interaction of the serum derived macromolecules with the lipid layer depends on the bilayer packing density, on the relative surface hydrophobicity and on the net charges at the bilayer surface as well as on the protein itself: imperfections in the lipid chain packing, high number of the exposable hydrophobic residues near or at the protein surface, complementary charges on the lipid and protein molecules as well as low lipid/protein molar ratios all tend to increase the macromolecular adsorption and denaturation at the surfaces of lipid vesicles and other foreign bodies. (Final stages of vesicle adsorption and digestion as well as the intra-cellular processing of their basic and associated materials also depend on the vesicle size, type and composition.)

Materials and Methods

Chemicals. 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) were obtained from Boehringer Mannheim (Germany). Soy-bean phosphatidylcholine (SPC) was from Lipoid (Ludwigshafen, Germany) or Natterman Phospholipids (Köln, Germany). Lipid purity prior to the experiments was controlled by the thin-layer chromatography (TLC) and was typically found to be higher than 99% for the synthetic and approx. 98% for the biological lipids. ^{3}H-labelled dipalmitoylphosphatidylcholine ([^{3}H]DPPC) was from Amersham Buchler (UK). Methoxy-polyethyleneglycol 5000 (methoxy-PEG110, with 110 repetitive units) as well as polyoxyethylene-bis(acetic acid) (COOH-PEG77-COOH; with 77 repetitive units), were purchased from Sigma (USA).

Lipid synthesis. DSPE-triazine-PEG110 (DSPE-PEG110) was synthesized as described previously [8]. DSPE-PEG77-COOH, differing from DSPE-PEG110 in the length of the polyoxyethylene headgroup, in the presence of an amide- instead of a triazine-group at the coupling site, and at the headgroup terminus was prepared by the method of Kung and Redemann [32]. This was originally developed to couple aliphatic dicarboxylic acids to lipids.

To obtain DSPE-PEG77-COOH, PEG-dicarboxylic-acid (0.57 mM) was first converted into an anhydride by the reaction in 50 ml chloroform with *N,N*-dicyclohexylcarbodiimide (DCDI, 5.7 mM) for 4 h at room temperature. A solution of DSPE (0.3 mM) in 10 ml chloroform and triethylamine (1 mM) was then added to the PEG-dicarboxylic-acid-anhydride/DCDI solution and the mixture was stirred for 7 days at room temperature. Conversion yield was approx. 70%. The lipid product was purified by silica-gel column chromatography with chloroform/methanol/water (50:20:1, v/v) as an eluent; ninhydrin and Molybdenum blue followed by sulphuric acid charring were used to identify the spots on silicic acid thin-layer chromatography plates. (DSPE-PEG77-COOH in aqueous suspensions under our experimental conditions is largely present in the ionized form, so that abbreviation DSPE-PEG77-COO$^-$ would be more appropriate than DSPE-PEG77-COOH; we prefer to use the latter, however, for the sake of convenience.)

Vesicle preparation. To ensure the reproducibility and homogeneity of the vesicle size distribution a 5 wt% suspension of DSPC or DSPC/DSPE-PEG multilamellar vesicles, prepared from a dry lipid film, was sonicated by a tip-sonicator in 30 mM carboxyfluorescein solution (pH 7.2) until the average vesicle diameter was approx. 80 nm. After three days of annealing at room temperature the un-encapsulated carboxyfluorescein (CF), which at the given concentration is self-quenched, was removed by enforced gel-filtration; a Sephacryl S400 mini-column and centrifugation at approx. $1000 \times g$ for 3 min was typically used for this purpose.

Vesicle size determination. Vesicle size determination was based on the dynamic light scattering measurements with a ZetaSizer 2C (Malvern, UK) instrument used in the AutoSizer mode. This permitted identification of the monomodal as well as bimodal vesicle size distributions within a 15% confidence limit. The quoted values give the mean value of the vesicle number distribution.

Encapsulation and permeability studies. In each drug-release assay, liposomes (50 μg total lipid) were mixed with 1 ml of prewarmed human plasma containing 0.02% sodium azide and incubated at 37°C. Release of the model-drug CF increases the dye fluorescence because of the dilution-dependent de-quenching. The change of CF-fluorescence, indicative of the model-drug loss through the vesicle membranes, was monitored spectrometrically (in a LS-5 instrument; Perkin-Elmer, UK) with excitation at 490 nm and emission wave-length of 520 nm. (A 500 nm cut-off filter in the emission path was used to reduce the signal from the light scattered on liposomes.) Total amount of the entrapped carboxyfluorescein and the CF loss were calculated after terminal lysis of the liposomes with Triton X100 (1.2 vol% final concentration). To compensate for the vesicle size variability all encapsulation values are given in relative units (per unit volume).

Calorimetric studies. To assess the ternary phase diagram of DSPC and DSPE-PEG in aqueous suspensions as well as to test the degree of mixing of these two lipids in a complex system, differential scanning calorimetry with a MC-2 instrument (MicroCal,

Northampton, MA) was done. Typically, a 1–5 mM suspension of multilamellar vesicles was used for the measurements, with DSPE/DSPE-PEG molar ratios ranging between 0 and 100%. In each experiment at least two heating-cooling cycles were done at a scanning rate of 0.5 degrees per min. Measured data were analyzed with the software routines supplied by the manufacturer. The calorimetric transition temperature data were found to be reproducible to within 0.25 degrees.

Alternatively, lipid chain-melting phase transition temperature was monitored by measuring depolarization of the diphenylhexatriene molecules incorporated into lipid bilayers, as described in Ref. 10.

Protein adsorption. In each protein adsorption assay, an aliquot of the liposome suspension containing 1 mg of lipids was mixed with 0.5 ml of prewarmed human plasma and then incubated at 37°C. Vesicle size in these experiments varied between 300 and 350 nm. At different times thereafter samples were taken from this bulk suspension and washed twice with 0.9% saline by centrifugation at $20\,000 \times g$ for 30 min. Depending on the amount of DSPE-PEG added, 70% or more of vesicles were found in the pellet after centrifugation. The adsorbed protein quantity was then determined spectrophotometrically by using a modified Bradford [15] assay (Bio-Rad, München). This is based on Coomassie-blue staining and determination of the extinction maximum for the protein-dye complex at 595 nm, as compared to the absorption intensity of simple dye at 465 nm.

In vivo experiments. Small unilamellar liposomes were prepared, as for the stability experiments, in 100 mM Hepes buffer (pH 7.2) from DSPC and SPC, or from these lipids with the addition of different molar concentrations of DSPE-PEG; a small amount of [^{3}H]DPPC (2 μCi/mg total lipid) was included as a marker. In each test, four female, 2-months-old NMRI mice (with a weight of approx. 33 g) were injected with 1.5 mg phospholipid in 0.15 ml of a liposomes-suspension through the tail vein. This corresponds to a specific dose of approx. 45 mg lipid/kg body weight. In control experiments, [^{3}H]cholesterylhexadecylether was used as a non-exchangable marker with essentially identical results.

At selected times, blood samples (40 μl) were collected from the freshly cut tail-ends. At 8 or 24 h after the lipid application mice were killed and their organs were excised. Blood specimens and 100 mg of each tissue sample were discolored with 0.4 ml H_2O_2 and 0.2 ml $HClO_4$ at 80°C overnight. Subsequently, all probes were neutralized with 0.2 ml CH_3COOH. After the addition of 10 ml Aquasol-2 (NEN-DuPont, Dreieich, Germany), total activity of the samples was measured in a beta-scintillation counter (Berthold, Wildbad, Germany).

Results

We have studied the interdependence between the lipid vesicle composition, bilayer affinity to protein adsorption, and vesicle elimination from the murine bloodstream. But first of all the physico-chemical properties of all vesicles were determined.

Liposome characterization and stability.

Immediately after they have been made by sonication, nearly all liposomes investigated in this study had a diameter of less than 80 nm, as concluded from the dynamic light scattering experiments. One day later, the size of the DSPC vesicles was greater by a factor of 2.14 or 1.75 in the buffered saline or 30 mM CF solution, respectively. Diameters of the sterically stabilized vesicles, in the majority of cases, did not change appreciably during this period of time (not shown); only occasionally, when the lipid mixture contained more than 50 mol% DSPE-PEG110, after a while, large particles tended to appear in the suspension. In our experience, even pure 5% DSPE-PEG110 forms turbid suspensions with a mean particle size, as determined by dynamic light scattering, of approx. 400 nm.

Similar concentration dependence was also observed in our studies of the drug encapsulation efficacy, in the zeta-potential measurements and in the corresponding measurements of the drug permeation loss in serum, as studied for the model drug 5,6-carboxyfluorescein (Fig. 1). The encapsulation efficiency was measured to first increase to 9.74% and then decrease to approx. 2% with increasing relative concentration of DSPE-PEG110 in the host matrix consisting of DSPC (Fig. 1A).

The concentration dependence of transbilayer release of carboxyfluorescein is slightly more complex (Fig. 1B). The initial daily loss (24%) measured for the DSPC bilayers falls by some 50% (to 12%) if only a few percent of DSPE-PEG110 are added into phosphatidylcholine host bilayers. Subsequently, however, the drug permeation gradually increases to just above 15% for the 9:1 DSPC/DSPE-PEG110-mixtures.

Likewise, the zeta-potential of phosphatidylcholine in the presence and absence of proteins (-1 and -2.8 mV, respectively) first attains a relatively high negative value of -4 to -5 mV upon the addition of small amounts of the negatively charged DSPE-PEG110. But this potential then decreases and ultimately increases again, in a quasi-parabolic manner, upon further rising the concentration of the ethoxylated phospholipid in the host bilayer (Fig. 2).

The smallness of measured zeta-potentials is due to the fact that electric charges are burried under a lengthy polar head [11]. The concentration dependence of this variability, however becomes more easily understandable by looking at the phase diagram of the

distearoylphosphatidylcholine/ distearoylphosphatidylethanolamine-PEG mixed bilayers (Fig. 3). This diagram is suggestive of the phase separation of both lipid components in the fluid phase near a molar ratio of 1:1. Below this concentration ratio, DSPC and DSPE-PEG110 appear to mix nearly ideally, the chain-melting phase transition temperature increasing quasi-linearly from 55.5°C for the pure DSPC component to 68°C for the DSPC/DSPE-PEG110 (1:1) mixture. (The defects responsible for the permeability increase illustrated in Fig. 1B are of course to few to show up in such a phase diagram.) The transition enthalpy simultaneously decreases from 46 kJ/mol for the pure DSPC compound to 38 and 28 kJ/mol for the DSPC/DSPE-PEG110 (4:1) and (1:1) mixtures. The relatively high value of the chain-melting phase transition enthalpy for the latter mixture support the view that in spite of their long heads DSPE-PEG110 molecules can form quite stable lipid lamellae. This has also been confirmed independently by X-ray diffractometry and light scattering as well as by the light microscopy for the pure DSPE-PEG110 suspensions.

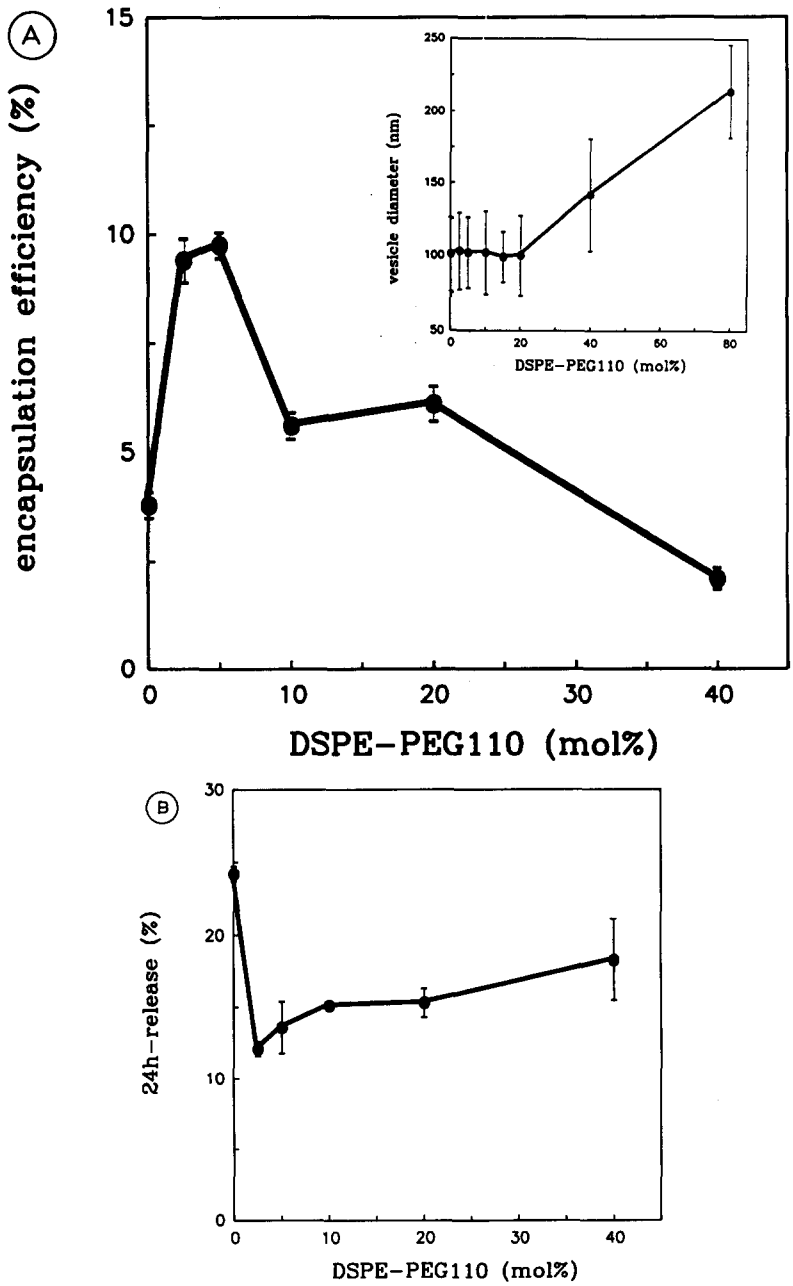

Fig. 1. Encapsulation efficiency (A) and permeability loss (B) of the distearoylphosphatidylcholine/distearoylphosphatidylethanolamine-PEG mixed vesicles (DSPC/DSPE-PEG110) containing 30 mM (5,6)-carboxyfluorescein (CF) as a function of the molar concentration of the headgroup-modified lipids in the system. Error bars in this an all following figures represent standard errors.

Interactions with serum proteins. Total protein mass that has adsorbed irreversibly to the vesicle surface upon an 8 h exposition to serum changes non-monotonously with the increasing DSPE-PEG110 concentration (Fig. 4A). From an initial value of approx. 225 μg per mg lipid the adsorbed protein amount decreases, by nearly 70%, to just about 75 μg protein/mg lipid. (This would correspond to a molar ratio of approx. 1:15 to 1:45, if serum albumin was the only protein species bound.)

To characterize vesicle-protein association in more detail we have also calculated the zeta-potential difference caused by the addition of serum to various cryptosomes (cf. Fig. 4B). (This was done by subtracting both sets of data from Fig. 2.) With the exception of the result pertaining to standard liposomes, made of pure DSPC, this difference was decreasing monotonously with increasing DSPE-PEG 110 concentration in the bilayers.

Comparison of the vesicle zeta-potential variations and in the amount of surface-adsorbed proteins gives a rough impression about the state of such proteins. Any change in the surface potential value of the non-ionic membranes can only be a consequence of lipid–protein interactions.

From the discrepancy between the observed concentration dependence of the vesicle potential change and the adsorbed protein mass, we infer that some of the proteins must have undergone conformational changes upon the adsorption to the lipid bilayers. From the fact that the protein-dependent change in zeta-potential and the adsorbed protein mass are only proportional in the membranes with a high DSPE-PEG110 content (see the straight line in inset to Fig. 4) we conclude that the zeta-potential values of vesicles with low

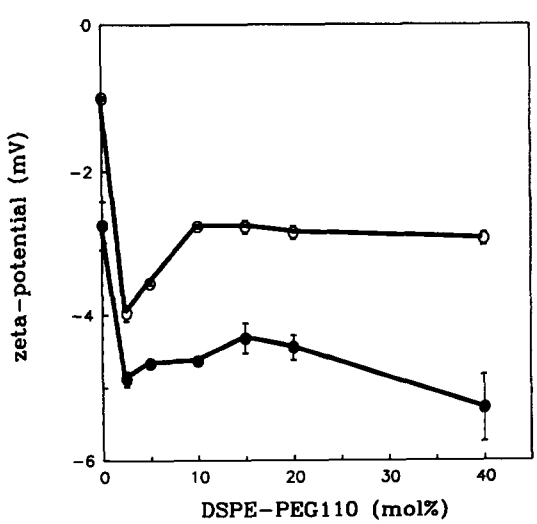

Fig. 2. Zeta-potential of the DSPC/DSPE-PEG110 mixed vesicles in pH 7.2 buffer before (open symbols) and after incubation in serum for 8 h (full symbols) as a function of the nominal DSPE-PEG 110 concentration.

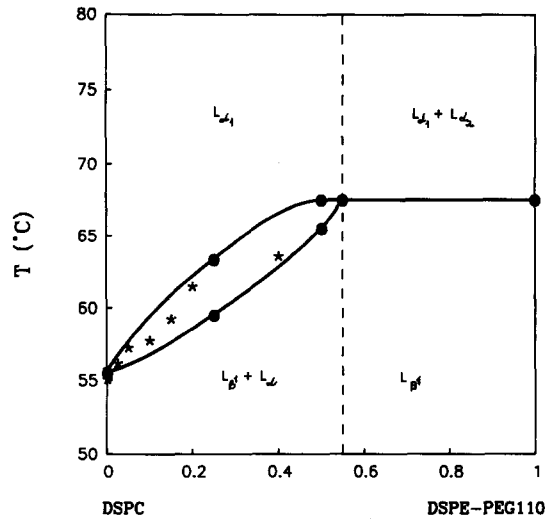

Fig. 3. Phase diagram of the DSPC/DSPE-PEG mixed vesicles as determined by the scanning differential calorimetry (bullets) or diphenylhexatriene polarization measurements (crosses). Vertical line delineates the position of a tentative lyotropic phase boundary.

DSPE-PEG110 concentrations are anomalously high. This could be a sign of protein denaturation on the poorly stabilized vesicles with a low DSPE-PEG110 content. Our ongoing FTIR experiments should help us to quantify this interdependence better.

Uptake in vivo

The clearance and biodistribution of DSPE-PEG110 containing liposomes are illustrated in Figs. 5 and 6. These pictures show that as few as 10 mol% of DSPE-PEG110 in each lipid bilayer may prolong the circulation time of the resulting small lipid vesicles in the bloodstream drastically, by a factor of 3.3 for the DSPC- and by a factor of 2.6 for the SPC-containing vesicles. Simultaneously, the uptake of such vesicles by the resident macrophages in the liver and spleen is reduced by a factor of 6.9 for the DSPC- and by a factor of 5.8 for the SPC-derived vesicles, respectively.

Vesicle interactions with body fluids and organs are only little affected by the lipid bilayer fluidity. Vesicles made from lipids which have fluid chains under the experimental conditions of this study (SPC) are eliminated similarly rapidly as liposomes made from the ordered-phase phospholipids (DSPC; cf. Figs. 7 and 8). This also pertains to the standard liposomes made from DSPC or SPC (cf. Fig. 5).

This conclusion is corroborated by the biodistribution data shown in Figs. 7B and 8B. Vesicles consisting of DSPC/DSPE-PEG110 as well as SPC/DSPE-PEG110 mixtures are still present in the blood at relatively high concentrations even 24 h after a systemic application. Accumulation of such vesicles in the liver of the experimental animals is by a factor of approx. 5 lower than for the ordinary liposomes, however. The uptake of the surface-modified lipid vesicles

by the spleen macrophages is also suppressed quite successfully by the surface-attached PEG-groups on the fluid- as well as gel-phase lipid vesicles. Such vesicles thus may be called cryptosomes irrespective of the state of their hydrocarbon chains.

Liposome life-time in vivo is known to depend on the absolute amount of lipid applied. Based on the published saturation-quantity data for the simple [12–14] and stabilized [21] liposomes we infer that our experiments were probably done above the saturation limit for the former and below this limit for the latter type of liposomes. All relative comparisons, consequently, are to be considered as the lower limit difference.

Circulation time of the sterically stabilized liposomes also depends on the concentration of the steri-

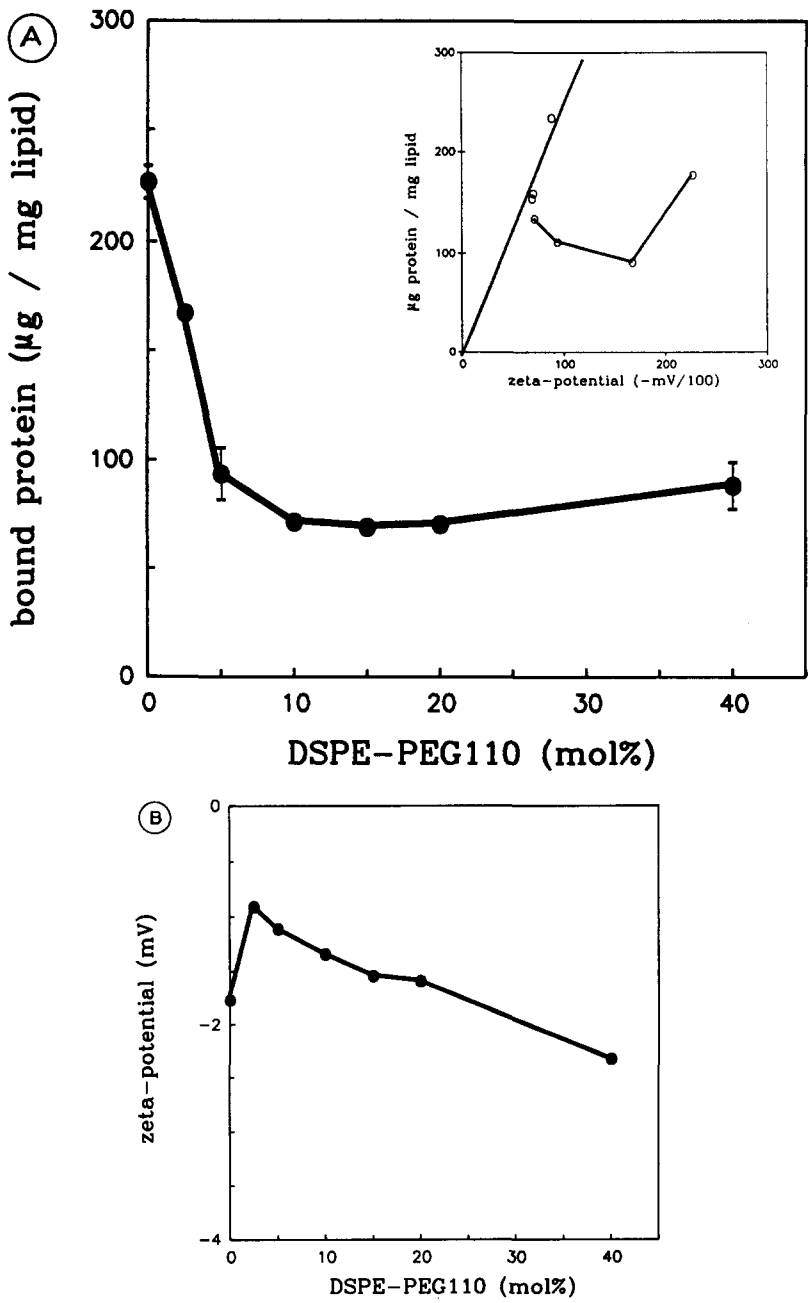

Fig. 4. Protein adsorption from the serum of normal humans to the surface of the unilamellar lipid vesicles as a function of bilayer composition. (a) As determined by means of a modified Bradford assay; (B) as given by the difference of zeta-potentials from Fig. 2. All measurements were done at 37°C for 8 h, as described in the text. (Inset) Interdependence between the amount of the vesicle adsorbed protein and the resulting change in the bilayer surface potential (zeta-potential). This inset was constructed by combining the data from the main bodies of Figs. 4A and 4B. The straight line connects the only two points for which the protein-induced the zeta-potential change is proportional to the liposome-associated protein amount.

cally active component in each lipid bilayer (cf. Figs. 7 and 8). In our experience, vesicle longevity first increases and then decreases with the amount of the DSPE-PEG110 component in the DSPE or SPC matrix (Figs. 7 and 8).

Taken separately the observed maxima are relatively flat. With all data presented in this work considered together, however, there is no doubt that all biologically relevant concentration dependences go through a maximum with increasing DSPE-PEG110 concentration.

These conclusions depend crucially on the (quasi-) quantitative incorporation of the DSPE-PEG110 molecules into phosphatidylcholine bilayers. Based on the critical micelle concentration, or better to say, on the critical concentration for the self-aggregation of DSPE-PEG110, which we have measured (to be published), we have calculated the relative concentration of this lipid in our 10% lipid suspensions (approx. 140 mM for pure DSPC) to be always higher than 90% and in most cases higher than 95%. Indeed, from the 'CMC'-data for DSPE-PEG110 we conclude that the propensity of this lipid for the vesicle formation is similarly high as that of dilauroylphosphatidylcholine. The fear of the DSPE-PEG110 micellization or high monomer concentration thus appears to be exagerated except for quite dilute lipid suspensions such as are used, for example during chromatographic liposome characterization.

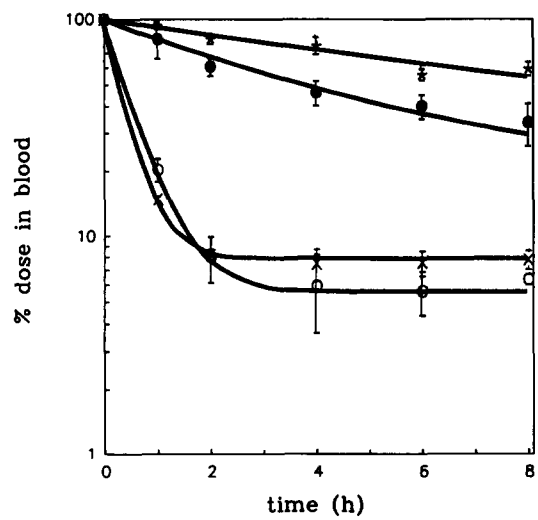

Fig. 5. Clearance of various lipid vesicles from the blood-circulation in mice ($n = 4$) as a function of the time after an intravenous material administration. Data points give the relative amount of the liposome-associated dose in standard rigid DSPC (×) and fluid-phase SPC (○) liposomes or in the corresponding cryptosomes stabilized with 10 mol% of a PEG110-derivative (DSPC/DSPE-PEG: ⋆ or SPC/DSPE-PEG: •). Liposome elimination from the blood is approximately mono-exponential with a characteristic life-time of $\tau_1 = 0.38$ h and $\tau_2 = 0.53$ h for the standard or $\tau_1 = 9.52$ h and $\tau_2 = 4.09$ h for the cryptosomes; the corresponding limiting vesicle concentrations ($t \to \infty$ are 7.8% and 5.5% for the standard liposomes or 19.9% and 18.2% for cryptosomes. Life-times were calculated by using a bi-exponential clearence model, as described previously [8]. (Note the different time scale in this and following graphs.)

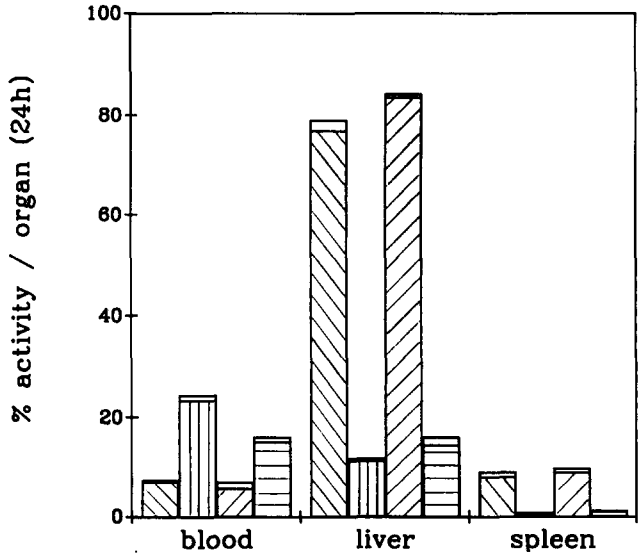

Fig. 6. Biodistribution of the standard gel-phase (DSPC, left dashed) and fluid-phase (SPC, right dashed) liposomes or of the long-lived cryptosomes consisting of the same phospholipids but supplemented with 10 mol% DSPE-PEG110 (vertically and horizontally dashed, respectively) after an intravenous administration in mice. Tissue distribution stems from the radioactivity counts for the tritium labelled vesicles measured 24 h after an application of 1.5 mg phospholipid per mouse ($n = 4$).

The position of the resulting maximum is slightly different for the ordered- and fluid-phase vesicles: for membranes in the gel phase, optimal results are obtained with DSPC/DSPE-PEG mixtures with a molar ratio of approx. 7:1 (cf. Fig. 7); for the fluid-phase SPC/DSPE-PEG mixtures the corresponding optimum is shifted to approx. 4:1, however. Fig. 8 demonstrates this.

This compares nicely with the total amount of proteins adsorption onto the surface of lipid vesicles as a function of headgroup-modified phospholipid concentration (cf. Fig. 4). It also indicates that the surface hydrophilicity is not the chief factor for determining whether or not a given lipid vesicle will be long-lived in vivo: if this was the case, the life-time of each lipid vesicle should increase monotonously with the content of PE-PEG in each lipid bilayer.

Supporting evidence comes from the complementary data obtained with other types of lipid vesicles applied in vivo. Our bio-distribution studies carried out with the vesicles containing different amounts of the synthetic neo-glycolipids show that such sugar-containing carriers are eliminated from the murine bloodstream nearly as rapidly as ordinary phosphatidylcholine liposomes. This elimination, moreover, is largely independent of the concentration of the headgroup-modified molecules in each lipid bilayer. High density of the OH-groups near the surfaces of such vesicles, conse-

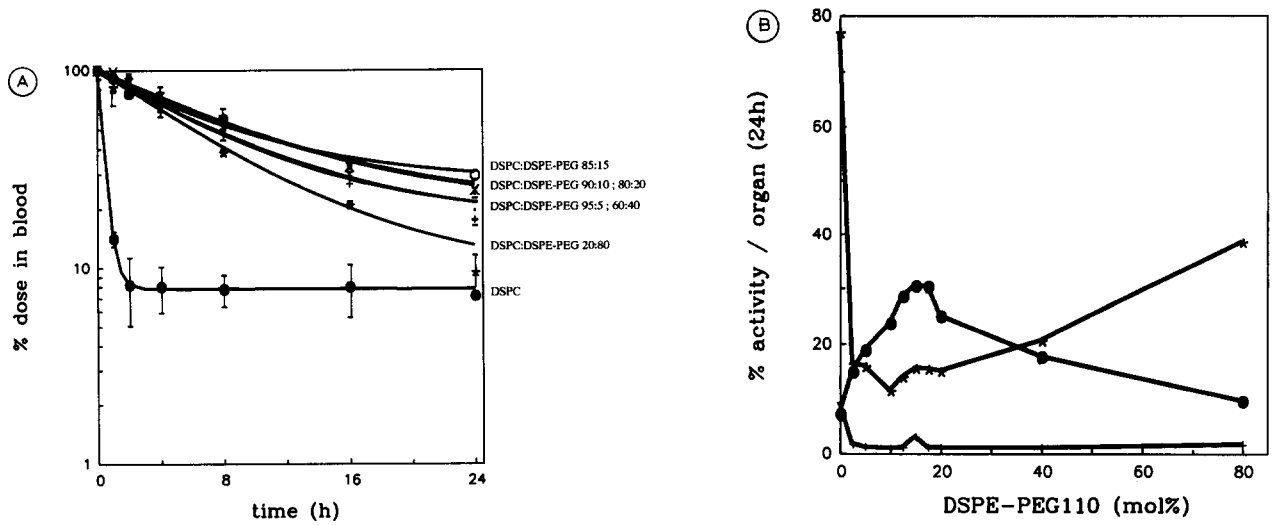

Fig. 7. Effect of the nominal concentration of DSPE-PEG in mixed gel-phase membranes containing DSPC on: (A) vesicle elimination from blood and (B) vesicle biodistribution in mice 24 h after an intravenous administration. The radioactivity associated with tritiurated DPPC was measured for blood (●), liver (⋆) and spleen (+) 24 h after a single-dose injection of 1.5 mg of phospholipid per mouse ($n = 4$).

quently, is not sufficient to prevent the protein adsorption to the carrier surface and strong phagocytosis of such carriers in vivo.

The fact that the hydrophilicity of glycolipid-containing vesicles is much higher than that of DSPC liposomes and, in sum, is not much different from the hydrophilicity of the DSPE-PEG110 molecules in our cryptosomes indicates that factors other than bilayer surface hydrophilicity govern the interactions between the corresponding lipid vesicles and phagocytic cells in vivo.

We think that chemical differences between both mentioned systems are unimportant in this respect. The fact that even relatively large changes in the chemical nature of the lipophilic PEG-derivatives do not impede the vesicle longevity vindicates this conclusion. Liposomes composed of DSPC and lipophilic PEG-derivatives with terminal carboxylic groups (DSPE-PEG77-COOH), for example, are chemically different from our preferred DSPE-PEG additives both at the headgroup termini and at the headgroup attachment sites. In spite of this, they both belong to the class of cryptosomes: in comparison with the conventional DSPC liposomes the DSPC/DSPE-PEG77-COOH cryptosomes have a long life-time in blood (an increase by a factor of 3.3 relatively to τ_{DSPC}). Their

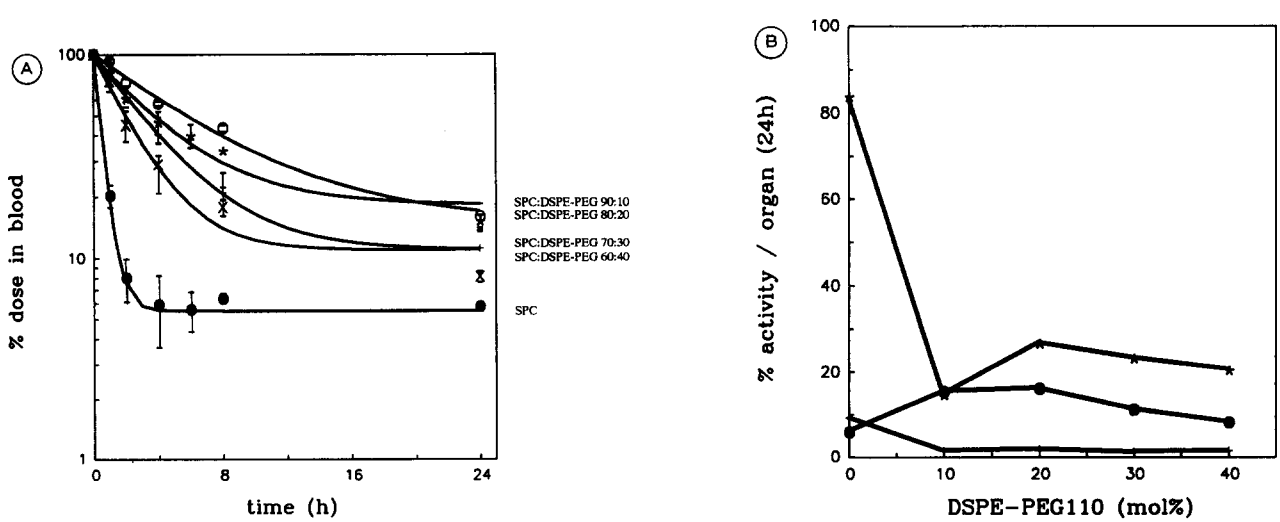

Fig. 8. Effect of the nominal DSPE-PEG concentration in the fluid-phase SPC/DSPE-PEG mixed membranes on: (A) vesicle elimination from blood and (B) on the distribution of corresponding lipid vesicles in mice after intravenous administrations. Symbols and other details are the same as in Fig. 7.

uptake by the reticulo-endothelial system also is correspondingly slow (decrease by a factor of 2.6 relatively to the results obtained with the DSPC liposomes).

Discussion

To avoid excessive liposome uptake by the phagocyte-rich organs – and thus to prolong the vesicle life-time in blood – diverse 'stabilized' vesicles have been introduced. The first really long-lived liposomes were described by T. Allen and colleagues [2,3,17], soon to be followed by others [4,5]. This formulation contained a special glycolipid, ganglioside GM1, as the decisive, uptake-suppressing liposome component. Further improvement and appreciable cost-reduction was achieved by the invention of the second generation of long-lived liposomes which contained the polyoxyethylene derivatives of phosphatidyethanolamine as the longevity mediating ingredient. The earliest report dealing with such liposomes was submitted by our group to this journal [8]; nearly simultaneously, however, related reports by other groups were published [18–21]. Several patents have also been applied for in this field [22,23].

Initial explanation for the longevity of surface-modified liposomes was based on the postulate that it is the combination of high lipid bilayer rigidity [24,25], absence or shielding of the net surface charges [3,17,26], and a high surface hydrophilicity [17,18,27–29] which prevents or slows-down the liposome uptake by the phagocytic cells. In our original publication on cryptosomes [8] we have drawn attention to the significance of the steric forces and of the surface mobility effects in this respect. The former aspect has also been reiterated in a recent publication in this journal by Lasic and colleagues [30]. All this notwithstanding, no complete description and identification of the carrier properties which render liposomes long-lived has been published to date. In this article we thus attempt to summarize our current vistas on the mechanism of the particle longevity in blood. We also report new experimental data which highlight some of the aspects of carrier-body interactions and shed some light on the final carrier elimination from the blood circulation.

Most relevant in this respect are our data obtained with vesicles containing increasing amounts of DSPE-PEG. This variation in the basic DSPC matrix makes vesicle surfaces increasingly hydrophilic. The lack of any correlation between the vesicle surface hydrophilicity and longevity, to us, signifies that current rationale for the prevention of the drug-carrier elimination from the bloodstream must be revised.

To strengthen this point we have synthesized a series of neo-glycolipids containing different number of the hydrophilic segments on each lipid headgroup. This was achieved by attaching a series of well defined oligosaccharides to the same type of a hydrophobic anchor, which in the present case consisted of one distearoylphosphatidylethanolamine molecule. (The longest such substance prepared in our laboratory to date is DSPE-maltopentaose. This has a length of the hydrophilic headgroup of approx. 2 nm in the fully extended state and is estimated to be 2 times more hydrophilic than each individual PEG-headgroup with 110 residues, based on semi-empirical molecular-modelling calculations.) From the fact that vesicles containing such neo-glycolipids are very short-lived in mice (Blume and Cevc, to be published) we conclude that, indeed, it is not enough to render lipid bilayers highly hydrophilic if one is to make the resulting lipid vesicles inattractive for the phagocytic cells and thus long-lived.

The fact that some of the illustrated concentration dependences in the DSPE-PEG110 reach region are rather weak does not affect this conclusion. The reason for the relatively small concentration sensitivity observed in marker-release studies, for example, is trivial and originates in the strong sensitivity of the lipid bilayer permeability to membrane defects. The relatively low overall sensitivity of the zeta-potential measurements is due to the fact that only charges in the vicinity of the lipid bilayer surface contribute markedly to the electrophoretic liposome mobility. The most relevant, biological assays, however, always show a strong and well defined dependence on the DSPE-PEG110-concentration. A difference by a factor of 2 between the sub-optimal and optimal cryptosomes albeit small at the first glance, represents a nearly 100% efficiency improvement in comparison with previously known compositions.

Results described in the previous sections of this work demonstrate that the rigidity of hydrocarbon chains is not a prerequisite for the long vesicle life-times in vivo. Suggestion that lipid chain rigidity is not inevitable for the preparation of long-lived vesicles has also been made recently by Lasic and colleagues [26]. To date, however, the direct evidence for such a conclusion was lacking, all previous experiments with the unsaturated lipids having been performed with cholesterol containing vesicles, cholesterol being known rigidifier of the lipid chains in a membrane [24].

Data presented in Figs. 5 and 6 thus prove for the first time that the fluidity of lipid bilayer interior is of little importance for the fate and distribution of lipid vesicles in vivo, provided that each lipid bilayer is adequately shielded from the interactions with serum. (This does not pertain to the membrane permeability, of course.) After 8 h the difference between the fluid- and gel-phase cryptosomes is still significant (61% and 38%, respectively). After 24 h, however, the residual cryptosomes have essentially the same blood concentration in both cases (18.2% and 19.9%, respectively).

This conclusion has far reaching practical implica-

tions. Cholesterol in the lipid bilayers may interfere severely with the incorporation of large amounts of the lipophilic drugs into each bilayer. This is owing to the competition between the fat-soluble and cholesterol molecules for similar locations in the hydrophobic membrane interior.

On the one hand, many lipophilic drugs thus tend to phase separate from the phosphatidylcholine/cholesterol mixed vesicles, often in the form of microcrystals. This restricts the applications range of phosphatidylcholine/cholesterol/PEG-PE mixed cryptosomes as compared to that of simple phosphatidylcholine/PEG-PE long-lived liposomes. On the other hand, the presence of cholesterol in some long-lived liposomes may partly explain why different authors could not incorporate high amounts of the PEG-derivatives in their vesicles [21]. (Another reason for this is the variable total lipid concentration. This causes a large proportion of the PEG-modified molecules to partition into the aqueous subphase in the case that the total lipid concentration is too low.)

Our finding that vesicles containing merely DSPC and DSPE-PEG110 (and probably also SPC and SPE-PEG110 mixtures) can be made highly resistant to the uptake by the liver and spleen macrophages should thus be of some relevance for the future design of long-lived drug delivery systems. We believe that suitably stabilized, and thus long-lived, liposomes prepared with unsaturated, biological lipids are the carriers of choice for the delivery of lipophilic (pro)drugs in vivo, especially in cancer therapy.

If neither the hydrophilicity of lipid vesicle surface nor the tight packing of hydrocarbon chains are sufficient for making a particulate drug carrier long-lived, new explanations for this phenomenon must be sought. We believe to have identified the most important of these in the mobility of the polar lipid headgroups or, better to say, in the steric *and* dynamic forces arising from the polar headgroups sweeping the interfacial region between the hydrophobic membrane interior and the surrounding protein solution.

How can this be understood at the molecular level? Each polar-apolar interface provides an ideal site for the hydrophobic adsorption and denaturation of the macromolecular serum components. This is also true for the ordinary lipid bilayer surface, especially when the lipid chains are in the fluid, and thus relatively expanded state [31]. Standard lipid vesicles, therefore, bind the serum components quite rapidly, avidly and irreversibly and also cause most of the adsorbed protein molecules to denature. Owing to such opsonization, the protein-coated vesicles have a very short lifetime in vivo.

Lipids with long, tightly packed polar heads form a new, well defined surface which is off-set from the original interface by the thickness of the headgroup region. Hydrophobic protein residues are now denied access to the hydrophobic membrane regions. This hampers strongly the *hydrophobic* binding of the protein molecules to the lipid vesicles. But the interactions between the *hydrophilic* protein residues and the corresponding polar residues at the 'new' bilayer surface are not prevented. Relatively rigid polar lipid headgroups or headgroup arrays thus can act as novel protein binding sites, owing to the numerosity and fixed positions of the polar segments on the headgroups. Lipid-protein hydrogen bonds as well as dispersion (van der Waals) forces are both relevant in this respect. (Charge–charge interactions could also play some rôle. In our case, however, this is unlikely to be the case, as can be seen from the comparison of data shown in both panels of Fig. 4.)

Strong and/or irreversible cohesion between the polar residues of the lipid and protein molecules inevitably will result in protein denaturation, different in origin but similar in consequences to the denaturation caused by the protein adsorption at the polar-apolar interface ('hydrophobic adsorption'). Therefore, strong *hydrophobic* or *hydrophilic* lipid–protein interactions both may promote the phagocytosis of lipid vesicles and thus shorten the vesicle circulation time in vivo.

A different situation is encountered when surfaces are covered by the layer of polar lipid headgroups which are sufficiently mobile. Such surfaces are unlikely to bind proteins persistently due to the permanent rupturing of the protein-lipid bonds by the thermal surface excitations. Protein adsorption to the sterically stabilized vesicles is thus seldom associated with extensive and rapid protein denaturation. Surface modified vesicles, consequently, are removed less readily from the bloodstream than standard lipid vesicles. It takes time before gradual degradation and, even far more importantly, dilution of the protective surface coat in serum causes the protein molecules to accumulate and/or denature at the lipid bilayer surface in appreciable amounts. Once this has happened, however, the plentiness of the irreversibly bound, denatured proteins on each vesicle makes the vesicle attractive to phagocytes. This process culminates in the clearance of all opsonized vesicles from the bloodstream.

Mobility and suitable size of the lipid polar headgroups therefore appear to be of paramount importance for the prevention of protein adsorption and denaturation at the lipid bilayer surface. Optimization of these two factors may suffice for achieving long vesicle circulation and life-times, as well as long lifetimes of other similar particles, in the blood.

We trust that it is difficult if not impossible to create sufficiently dense, thick and yet mobile hydrophilic regions between the hydrophobic membrane interior and the surrounding biological medium by using short

headgroups; lipid molecules with such headgroups, such as DSPE-PEG20, thus do not give rise to the long-lived liposomes. The same is true for the carriers which consist of lipids with a strong tendency for the headgroup–headgroup interactions. Many glycolipids fall in this category: such molecules tend to cluster and form rigid, rather than mobile, plaques at the vesicle surface. (This may explain why GM1 but not other gangliosides can be used for making cryptosomes [2–5,17].) It is also inadvisable, however, to increase the concentration of the lipids with long-heads in carriers beyond a certain limit: molecular crowding is prone to suppress the segmental headgroup mobility. We believe this to be the reason why vesicles with a high molar PEG-concentration are relatively short-lived.

To demonstrate this effect the data from Figs. 7 and 8 are replotted in Fig. 9 in a slightly different manner. The carrier life-time in vivo is now represented as a function of the surface density of the polar residues, rather than as a function of the nominal PC/PE-PEG molar ratio. This makes the two sets of the data which pertain to the fluid- and gel-phase lipid bilayers, respectively, quite similar. This strengthens our conclusion that the hydrophobic bilayer interior of any surface-shielded lipid membrane is likely to act merely as a simple host-matrix *.

Last but not least we would like to briefly comment the published liposome half-life times. These vary widely between different publications [2–4,8,18–21]. We believe that this is not due to the different coupling procedures which in our experience do not affect the experimental results appreciably. One source of the observed variability are the deviant procedures used by different authors for the evaluation of $t_{1/2}$ values. An even more important source of variations are the different lipid concentrations used; these can affect the results of experiments even prior to the onset of RHS saturation owing to their effect on the distribution of PEG-lipids between the lipid and aqueous sub-compartments. The last, and probably most important, factor is the wide variability in the choice of experimental animals. It can be anticipated that cryptosome life-time will increase with the increasing body-weight of test animals or, better to say, with the increasing time which the blood takes to pass once through the liver and other RHS organs. This may result in 50-fold different $t_{1/2}$ values if mice and humans are compared, and to a difference of at least by a factor of 2 between mice and rats.

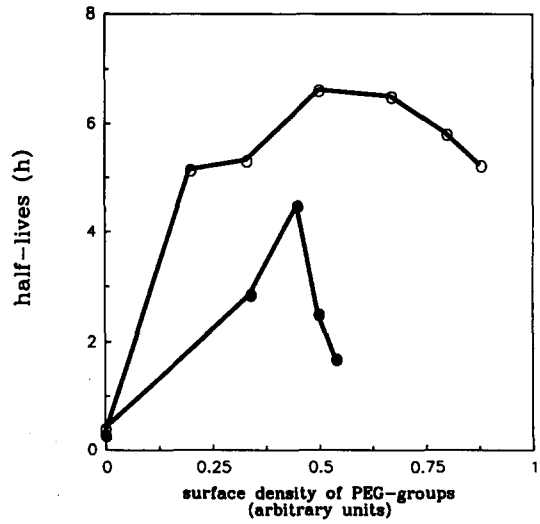

Fig. 9. Half-times, $t_{1/2}$, for various cryptosomes as a function of the effective surface density of the headgroup modified lipids in the bilayer. Open and filled symbols pertain to the gel- and fluid-phase bilayers, respectively. To obtain this picture data points from Figs. 6 and 7 were transformed by assuming that each lipid molecule in a gel- and fluid-phase occupies 0.55 and 0.75 nm^2, respectively; moreover, a Langmuir binding isotherm with $K = 0.5$ was used to estimate the relative amount of added DSPE-PEG110 molecules in the mixed lipid bilayers.

In summary, we have shown that the differences between liposomes and cryptosomes are more complex than has been believed before. The fluidity of the apolar lipid chains and lipid charges have been found to be of little importance in this respect. Hydrophilicity or presence of the 'steric barriers' at the lipid bilayer surface were concluded to be parameters of secondary importance, when a distinction is to be made between the basic types of drug carriers. We suggest that it is the *mobility* of polar surface barriers in combination with a sufficiently, but not excessively, large thickness of this barrier which prevents or slows down the adsorption of the macromolecules from the blood. We argue, moreover, that denaturation rather than simple adsorption of proteins acts as the primary trigger for the vesicle uptake by phagocytes in vivo.

We suggest that all polymers with similar physicochemical properties and a low biological reactivity might prove useful for the generation of good cryptosomes. It is even likely that by using different headgroup lengths, diverse headgroup types, or combinations of more than one sterically active component lipid vesicles will be prepared which will be superior to those described in this and previous articles.

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft through grants Ce 19/5–1 and SFB 266/C9. The use of the Central Animal Facilities

* Qualitatively similar picture is obtained if the data from Fig. 8 are rescaled directly, by multiplication with a factor of 0.73 to allow for the different molecular areas in the gel- and fluid-lipid phases, and then compared to the data from Fig. 7.

of Gesellschaft für Strahlenforschung, Neu-Herberg b. M., is also gratefully appreciated.

References

1 Gregoriadis, G., ed. (1983) Liposome Technology, CRC Press, Boca Raton, Fl.
2 Allen, T.M., Ryan, J.L. and Papahadjopoulos, D. (1985) Biochim. Biophys. Acta 818, 205–210.
3 Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223, 42–46.
4 Haga, V., Kato, F., Yoshida, M., Kohara, F. and Kato, Y. (1986) Chem. Pharm. Bull. 34, 2979–2988.
5 Hwang, K.J. and Beaumier, P.L. (1988) in Liposomes as drug carriers. (Gregoriadis G., ed.), pp. 19–35, John Wiley & Sons New York.
6 Gabizon, A. and Papahadjopoulos, D. (1988) Proc. Natl. Acad. Sci. USA 85, 6949–6953.
7 Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K., Lee, K.D., Woodle, M.C., Lasic, D.D., Redemann, C. and Martin, F.J. (1991) Proc. Natl. Acad. Sci. USA 88, 11460–11464.
8 Blume and G. Cevc, G. (1990) Biochim. Biophys. Acta 1029, 91–97.
9 Blume, G. and Cevc, G. (1992) J. Liposome Res. 2, 355–368.
10 Van Blitterwijk, W.J., Van Hoeven, R.P. and Van der Meer, B.W. (1981) Biochim. Biophys. Acta 644, 323–332.
11 Cevc, G. (1990) Biochim. Biophys. Acta 1031, 311–382.
12 Kao, Y.J. and Juliano, R.L. (1981) Biochim. Biophys. Acta 677, 453–461.
13 Sato, Y., Kiwada, H. and Kato, Y. (1986) Chem. Pharm. Bull. 34, 4244–4252.
14 Ellens, H., Mayhew, E. and Rustum, Y.M. (1982) Biochim. Biophys. Acta 714, 479–485.
15 Bradford, M.A. (1978) Anal. Biochem. 72, 248–253.
16 Senior, J. (1987) CRC Crit. Rev. Ther. Drug. Carrier Syst. Syst. 3, 123–193.
17 Allen, T.M., Hansen, C. and Rutledge, J. (1989) Biochim. Biophys. Acta 981, 27–35.
18 Klibanov, A. Maruyama, K., Torchilin, V.P. and Huang, L. (1990) FEBS Lett. 268, 235–237.
19 Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P. and Huang, L. (1991) Biochim. Biophys. Acta 1062, 142–148.
20 Allen, T.M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) Biochim. Biophys. Acta 1066, 29–36.
21 Allen, T.M. and Hansen, C. (1991) Biochim. Biophys. Acta 1068,133–141.
22 Fisher, D. (1988) Liposomes PCT/GB89/01262.
23 Woodle, M.C., Martin, F.J., Yau-Young, A. and Redemann, C.T. (1991) Liposomes with enhanced circulation time, US Patent, 5,013,556.
24 Kirby, C., Clarke, J. and Gregoriadis, G. (1980) Biochem. J. 186, 591–598.
25 Senior, J., Crawley, J. and Gregoriadis, G. (1985) Biochim. Biophys. Acta 839, 1–8.
26 Woodle, M.C., Lasic, D.D., Redemann, C., Newman, M., Babbar, S., Martin, F.J. (1991) Period. Biolog. 93, 349–352.
27 Maruyama, K., Yuda, T. Okamoto, A. Ishikura, C., Kojinia, S., Iwatsuru, M. (1991) Chem. Pharm. Bull. 39, 1600–1622.
28 Mori, A., Klibanov, A.L., Torchilin, V.P. and Huang, L. (1991) FEBS Lett. 284, 263–266.
29 Senior, J., Delgado, C., Fisher, D., Tilcock, C. and Gregoriadis, G. (1991) Biochim. Biophys. Acta 1062, 77–82.
30 Lasic, D.D., Martin, F.J., Gabizon, A., Huang S.K. and Papahadjopoulos, D. (1991) Biochim. Biophys. Acta 1070,187–192.
31 Cevc, G., Strohmaier, L., Berkholz J. and Blume, G. (1990) Stud. Biophys. 138, 57–70.
32 Kung, V.T. and Redemann, C.T. (1986) Biochim. Biophys. Acta 862, 435–439.