DRUG-CARRIER AND STABILITY PROPERTIES OF THE LONG-LIVED LIPID VESICLES, CRYPTOSOMES, IN VITRO AND IN VIVO

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ABSTRACT

Lipid vesicles composed of phosphatidylcholine and suitable polyoxyethylene-derivatives of phosphatidylethanolamine (cryptosomes) remain in circulation 8-10 times longer than standard liposomes after an i.v. administration in mice. In contrast to previous belief, this longevity is not destroyed by the net charges on the lipid vesicle surface and is not a direct consequence of the high surface hydrophilicity; also bilayer fluidity is not an obstacle for the attainment of long circulation times. All these three factors, however, can affect the effectiveness of the drug encapsulation into lipid vesicles and the stability of the resulting carrier suspensions. Terminal headgroup modifications, moreover, can affect the final carrier and drug distribution after vesicle applications *in vivo* and lead to accumulation in certain body subsites, such as the gut.

INTRODUCTION

Over the last years a variety of lipid vesicles (liposomes) has been studied in a hope to ultimately be able to use such vesicles for the drug delivery in human and animal medication [1]. Many corresponding experiments have failed to fulfill the expectations, however. Two main reasons were normally given for this: 1) the fact that most lipid vesicles are often incapable to transfer drugs efficiently to the targets outside the blood vessels, with exception of the organs of the reticulohystiocytic system (RHS); 2) the loss of small, water soluble substances from the vesicle interior in the presence of body fluids.

To eliminate the latter problem, vesicles consisting of long-chain diacyl-phospholipids (chiefly distearoylphosphatidylcholin) and cholesterol were introduced. Such 'rigid vesicles', indeed, have improved retention characteristics [2]. We believe that cholesterol is not a prerequisite for this, however. Indeed, this substance can seal the defects in the inadequately aged vesicles and affect the kinetics of the lipid exchange between the phospholipid vesicles and lipoproteins; but this seems not be

essential since correctly made simple phospholipid vesicles can be made comparably impermeable as phosphatidylcholine/cholesterol mixed vesicles [3].

In order to avoid excessive liposome uptake by the phagocyte-rich organs — and thus to prolong the vesicle lifetime in the blood stream — diverse formulations of the 'stabilized' vesicles containing certain glycolipids [4, 5, 6] and polyoxyethylene-ethers [3, 7, 8, 9, 10, 12, 13] were introduced. These are often called 'stealth-liposomes'.[1]

The first really long-lived liposome formulation was described by T. Allen and colleagues [4]. It contained ganglioside GM1 as the phagocytosis and liposome-uptake suppressing component. Further improvement and appreciable cost reduction was achieved by the invention of the second generation long-lived liposomes which contained polyoxyethylene derivatives of the phosphatidylethanolamine as the active component. The first paper describing such liposomes (cryptosomes) was submitted for publication by our group [3] but at essentially simultaneously related reports from other groups were prepared and published [7, 8].

Initial explanation for the longevity of certain liposomes was the high lipid bilayer rigidity, absence or shielding of net charges and high surface hydrophilicity are necessary to keep vesicles in the blood-stream [5, 13]. In our original publication on cryptosomes we have then drawn attention to the significance of steric forces and of surface mobility [3], the former aspect having been reiterated also in the recent publication by Lasic and colleages [10]. All this notwithstanding, no complete description and identification of the carrier properties which make liposomes long-lived was published to date. In this contribution we therefore present some results which shed new and additional light on these issues. In particular, we show that the membrane rigidity and absence of charge are not necessary for the liposome life-time prolongation; we also argue that strong vesicle-surface hydrophilicity alone is not sufficient for this effect. A more detailed account on this will be given separately.

MATERIALS AND METHODS

<u>Chemicals</u>   1,2-Distearoyl-*sn*-glycero-3-phosphocholin (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) were obtained from Boehringer Mannheim (FRG). Soy-bean phosphatidylcholine (SPC) was from Lipoid KG (Ludwigshafen, FRG) or Natterman Phospholipids (Köln, FRG). Lipid purity was controlled by TLC and was found to be higher than 99% for the synthetic and appr. 95% for the biological lipids. 3H-labelled dipalmitoyl-phosphatidylcholin (3H-DPPC) was from Amersham Buchler (xxx, UK). 5(6)-Carboxy-fluorescein (CF) was purchased from Eastman Kodak (Rochester, NY, USA) and used without any purification; carboplatin and tuftsin

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1. 'Stealth' liposome is a trademark of LTI (Palo Alto, California); L. Huang has wittily invented the word 'ninjasomes' and our group fancies the construct 'cryptosomes' — from the greek words 'cryptos' for hidden and 'soma' for body — for the description of long-lived drug carriers.

were obtained from Sigma (Deisenhofen, FRG). Activated methoxy-polyethyleneglycol 5000 (methoxy-PEG110, with 110 repetitive units) as well as polyoxyethylene-bis-(acetic acid) (COOH-PEG77-COOH[2]; with 77 repetitive units)were also supplied by Sigma.

Lipid Synthesis   DSPE-triazine-PEG110 (DSPE-PEG110) was synthesized as described previously. DSPE-PEG77-COOH, which differs from DSPE-PEG110 in the length of the polyoxyethylene headgroups and in the presence the amide- instead of the triazine-groups at the coupling sites and at the headgroups termini, was prepared by the method of Kung and Redemann. (This was originally developed to couple aliphatic dicarboxylic acids to lipids.) In brief, 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 15 minutes at room temperature. A solution of DSPE (0.3 mM) in 10 ml chloroform and triethylamine (1mM) 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 appr. 70%. The product was purified by silica-gel column chromatography with chloroform/methanol/water (50:20:1) as an eluent; ninhydrin and molibdenum blue followed by sulphuric acid charring were used to identify individual spots on the silicic acid thin-layer chromatography plates.

Bilayer Characterization   To assess the packing density of the lipid bilayers containing pure DSPC or various DSPE/DSPE-PEG mixtures, small-angle x-ray diffraction (SAXS) was used. Typically, a 5-10% lipid suspension of multilamellar vesicles was used for the measurements in a home-built SAXS-diffractometer equipped with a Cu-cathode (Phillips, Eidhoven, Netherlands), a Ge-monochromator (Huber, Rimsting, FRG), and a position sensitive detector (MBraun, Garching, FRG). All measured diffractogramms were corrected for the contributions from the background scattering and trivial line-shape effect by special software routines developed in our laboratory.

Stability experiments   DSPC or a 9:1 molar mixture of DSPC and DSPE-PEG (dried in vacuo from a CHCl$_3$ solution) were suspended in a phosphate buffer (pH 7.0) or agent solutions to a final concentration of 5 weight-%. The resulting crude suspension was then sonicated by a titanium tip at 70°C until the average vesicle diameter was approximately 100 nm, as determined by dynamic light scattering on a Zetasizer 2 (Malvern, U.K.) using multimodal data analysis. Depending on the experiment, carboxyfluorescein (30 mM), a macrophage activating agent dansyl-tuftsin (60 mM)

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2. Under our experimental conditions most of the terminal COOH-groups are ionized so that the denotation DSPE − PEG77 − COO$^-$ would be more appropriate.

or the anti-neoplastic drug carboplatin (27 mM) were used as (model) drugs. To avoid drug leakage through the lipid membrane imperfections originating from the sonication-defects all vesicle suspensions were aged for at least one day prior to the measurements. Unencapsulated substances were removed by gel-filtration through a sephacryl S400 mini-column (1ml) by centrifugation at 2000-g for three minutes.

In a typical drug release assay, liposomes (500 μg of lipid) were mixed with 1 ml of prewarmed human plasma and incubated at 37°C . The leakage of CF and dansyl-tuftsin was determined by fluorescence spectroscopy (LS-5; Perkin-Elmer, UK); carboplatin content in the probes was measured by atomic absorption spectroscopy. (For more details see Ref. [11].)

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 10 mo% DSPE-PEG; a small amount of 3H-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 appr. a specific dose of 45 mg lipid/kg body weight and is thus below the saturation limit [14].

At selected times, blood samples (40 μl) were collected from the freshly cut tail-ends. 8 or 24 hours after the lipid application mice were killed and their organs excised. Blood specimens and 100 mg of each tissue sample were decoloured with 0.4 ml of 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 (DuPont, USA), total activity of the samples was measured in a beta-scintillation counter (Berthold, Wildbad, FRG).

RESULTS AN DISCUSSION

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 100 nm, as concluded from the dynamic light scattering measurements. One day later, the size of 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 dansyl-tuftsin or carboplatin containing DSPC vesicles did not change appreciably over the same period of time (cf. Figure 1a); the resulting small liposomes were even found to be colloidally stable for more than two years.

In contrast to this, suspensions of DSPC/DSPE-PEG110 in a 60 mM solution of dansyl-tuftsin underwent dramatic structural transformations, suggestive of oligopeptide-DSPE-PEG110 interactions. These showed-up, for example, in the vesicle size increase documented in Figure 2. Conversely, the compounds CF or carboplatin did
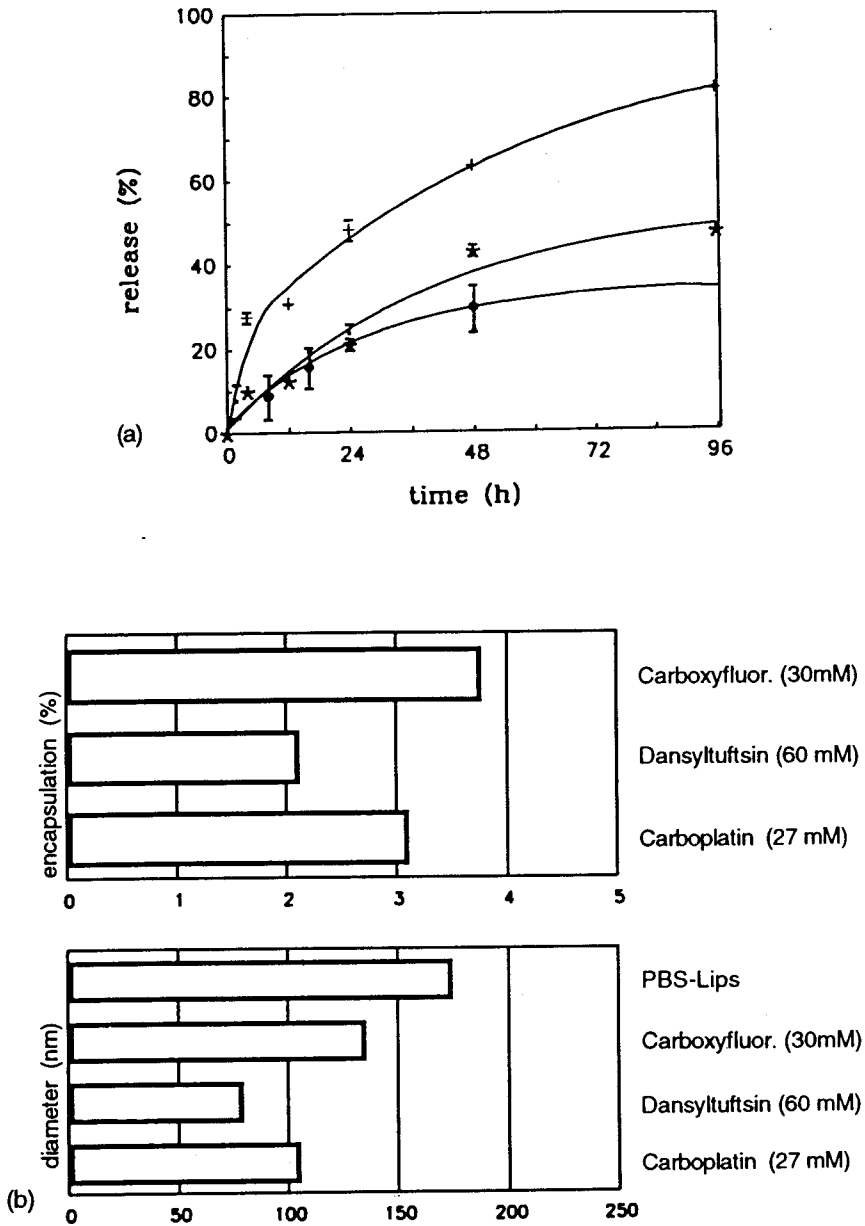
Fig. 1:   (a) — Time-course of the plasma-mediated release of the model-drug carboxyfluorescein (∗), of the macrophage activating agent dansyl-tuftsin (+), and of the cytostaticum carboplatin (•) from DSPC vesicles at 37°C . By a non-linear fitting procedure this release was found to be mono-exponential with decay-times of $\tau = 39.7$, 58.4 and 27.5 h, for carboxyfluorescein, dansyl-tuftsin and carboplatin, respectively, the corresponding limiting values ($t \rightarrow \infty$) being 53.9, 96.4 and 35.7%, respectively.   (b) — Effectiveness of encapsulation (%) and vesicle diameter (nm) 24 h after the corresponding vesicle preparation.
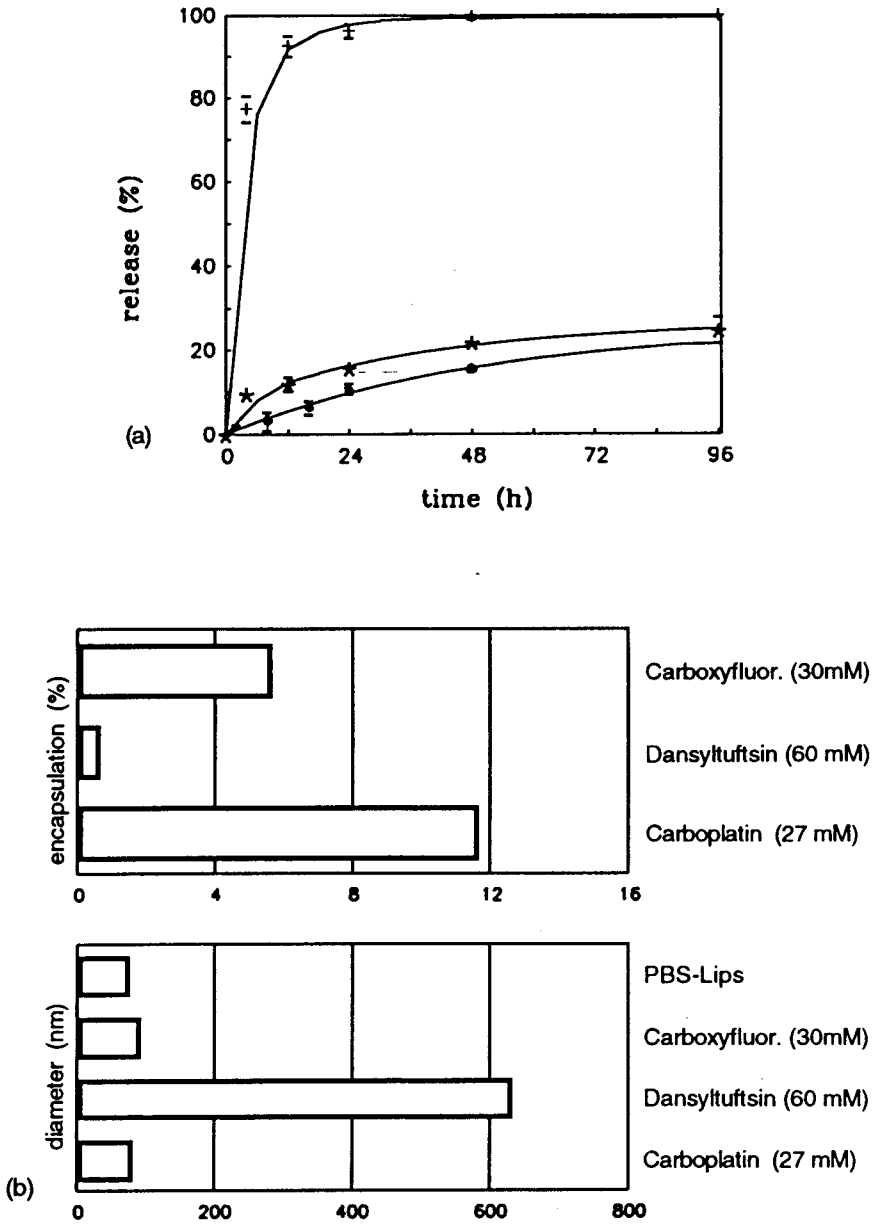
Fig. 2:   (a) — Release of carboxyfluorescein (∗), dansyl-tuftsin (+), and carbo-
platin (•) from DSPC-DSPE-PEG110 (9:1) vesicles at 37°C as a function of time,
t, after incubation with human plasma. Characteristic parameters for the mono-
exponential leakeage-process were calculated to be $\tau = 38.8$, 7.6, and 51.2 h for
carboxyfluorescein, dansyl-tuftsin and carboplatin, respectively, the corresponding re-
lease values being 26.6, 99.4 and 25.7%, respectively.   (b) — Effectiveness
of the drug encapsulation (%) and vesicle diameter (nm) for the corresponding lipid
vesicles at $t = 24$ h.

not interact comparably strongly with the 'sterically active' DSPE-PEG derivatives in the lipid membranes as concluded from the the colloidal stability of the corresponding cryptosomes in solutions of CF and carboplatin (cf. Figure 2).

This implies that the positively charged tetrapeptide dansyl-tuftsin interacts specifically with the bulky, polar headgroup of the PE-PEG molecules. By doing so it prevents efficient drug encapsulation while also diminishing the colloidal vesicle stability. For certain long-lived carriers of the complex molecules, such as PEG-modified lipid vesicles in combination with oligopeptides and polypeptides, the use of chemical drug modifications thus may prove necessary.

From a comparison of data in figures 1 and 2 it can be seen that cryptosomes have a higher capacity for the incorporation of the investigated non-ionic or negatively charged hydrophilic substances than standard phosphatidylcholine vesicles. The high specific encapsulation efficiency values of 12.05 and 4.43×10^5 mol/litre, in the case of carboplatin and CF, respectively, show this, being by a factor of 9 and 4 higher than for conventional DSPC vesicles (Figures 1b and 2b). These results thus are indicative of some attraction between the surface of lipid vesicles and the drug molecules; an interaction, however, which is weak enough not to interfere with the colloidal vesicle stability. It is unlikely, that such attraction involves simple charge-charge interactions, since CF is negative and carboplatinum is neutral with DSPE-PEG being negatively charged. We thus think that the local polarity effects and hydrogen bonding may play some rôle in this.

In vivo results   Clearance and biodistribution of DSPE-PEG110 containing liposomes are illustrated in figures 3 and 4. Even as little as 10 mol% of DSPE-PEG110 in the lipid bilayers drastically prolong the circulation time of the corresponding small lipid vesicles in blood (by a factor of 3.3 for DSPC and by a factor of 2.6 for SPC); simultaneously such modified membranes reduce the vesicle uptake by the resident macrophages in the liver and spleen by a factor of 6.9 for the DSPC-containing vesicles and by a factor of 5.8 for the SPC-containing carriers, respectively.

This PEG-activity is relatively little affected by the membrane fluidity. Vesicles with a completely fluid major lipid component under our experimental conditions (SPC) have a similar temporal dependence of the elimination as the ordered-phase, rigid liposomes (DSPC). (Interestingly, standard liposomes made from the ordered DSPC or fluid SPC also have similarly short life-times in vivo.)

Such conclusions are also corroborated by our biodistribution data pertaining to the sterically stabilized carriers. DSPC/DSPE-PEG110 as well as SPC/DSPE-PEG110 vesicles from figure 4 both are seen to give rise to relatively high blood-levels and to accumulate in the liver by a factor of appr. 5 less avidly than ordinary, non-stabilized liposomes; the same is true for the uptake of the corresponding lipid vesicles by the spleen macrophages. Phosphatidylcholine vesicles with a surface layer of PEG-groups thus deserve the name: cryptosomes, independent of the type and/or state of their chains.
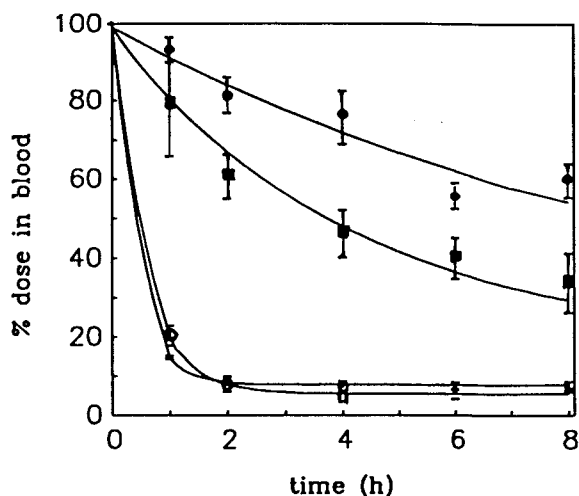
Fig. 3: Clearance of various formulations of lipid vesicles from the blood-circulation in mice as a function of the time after intravenous administrations. Data points give the relative amount of the carrier-associated dose in the standard rigid DSPC (o) and fluid-phase SPC (□) liposomes, the corresponding cryptosomes being stabilized with 10 mol-% of a PEG110-derivative of phosphatidylethanolamine (DSPC/DSPE-PEG: ● or SPC/DSPE-PEG: □).
Liposome elimination from the blood is approximately mono-exponential with a characteristic life-time of $\tau = 0.38$ h and $\tau = 0.53$ h for the standard or $\tau = 9.52$ h and $\tau = 4.09$ 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 the cryptosomes (from [11]).
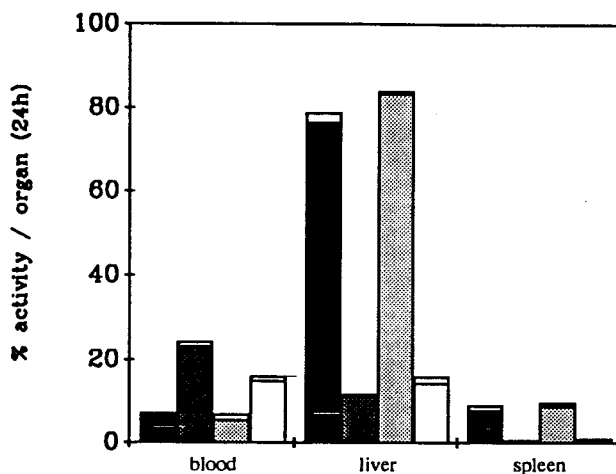

Fig. 4: Biodistribution of standard ordered- (DSPC, black) and fluid-phase (SPC, light grey) liposomes or of the corresponding long-lived cryptosomes containing 10 mol-% DSPE-PEG110 (dark grey and white, respectively) after intravenous administrations in mice.
Tissue distribution corresponds to the radioactivity counts for the tritium labelled vesicles measured 24 h after a single application of 1.5 mg phospholipid per mouse (i.e. 45 mg/kg) (from [11]).

This finding is of extreme practical interest. To date, lipid vesicles made from
the biological or unsaturated lipids were routinely doped with a large quantitity of
cholesterol ($\sim$ 50%) in order to render such carriers long-lived. This prevented in-
corporation of therapeutically significant amounts of lipophilic drugs in such vesicles
and their lipid bilayers and has narrowed drastically the scope of potential crypto-
some applications. We find, however, that even simple fluid liposomes (e.g. made
of soy-bean phosphatidylcholine) have a low propensity for the accumulation in the
liver and spleen and long circulation times provided that they are sterically stabi-
lized, with 10 mol% DSPE-PEG110, for example. This permits efficient cryptosome
loading with the lipophilic substances; we thus suggest that SPC/DSPE-PEG110 or
even SPC/SPE-PEG110 with suitable lipophilic (pro)drugs included can find broad
applications, especially in the cancer therapy [15].

To fully exploit this fact it should be realized that the circulation time of sterically
stabilized liposomes depends on the amount of the sterically active component in the
lipid bilayer membranes. We observe, for example, that initially the vesicle longevity
increases with the DSPE-PEG110 concentration. Depite this observation we do not
believe that this dependence is indicative of direct importance of the surface polarity
(hydrophilicity) for the liposome resistance to phagocytosis and elimination from the
bloodstream.

The first evidence for this conclusion comes from the measured repeat distance of
the DSPC/DSPE-PEG110 mixed multibilayers (Figure 5). This shows that even small
amounts (5%) of DSPE-PEG110 with long headgroups in lipid membranes already
suffice for pushing the adjacent surfaces quite far apart, beyond the separation of 5-6
nm at least, this being the limiting separation that can still be measured with our
experimental set-up.

This implies that, *on the average*, lipid vesicles with minute quantity of DSPE-
PEG110 added repel each other nearly as efficiently as vesicles with a 5-10 times
higher DSPE-PEG110 concentration. (It is also possible that some of this effect is
due to the manipulation of the elastic membrane properties which would give rise to
the stronger fluctuation-mediated interbilayer repulsion. The absence or weakness of
higher-order reflections in our diffractograms could be indicative of this.)

Additional testimony in favor of such assumption comes from our study of the
life-time and bio-distribution of vesicles containing large amounts of oligosaccharide-
lipid derivatives. Such lipid vesicles are eliminated rapidly from the murine blood
stream even when the surface density of the OH-groups, and thus the vesicle surface
hydrophilicity, is comparable to or higher than that of our long-lived DSPC/DSPE-
PEG110 cryptosomes with 2.5$\cdots$10 % DSPE-PEG (to be published).

The chemical differences between these two investigated systems are unlikely to
be the cause for so widely different behaviour since even relatively large changes in
the chemical nature of the lipophilic PEG-derivatives have been shown not to impede
the vesicle longevity. Liposomes composed of DSPC and lipophilic PEG-derivatives
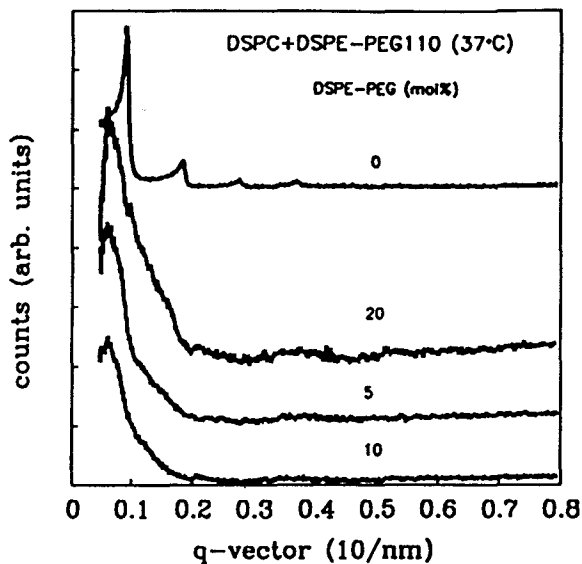
Fig. 5: X-ray small angle diffractograms of DSPC/DSPE-PEG110 mixed multibilayers as a function of the concentration of DSPE-PEG110.
All experiments were performed in the gel-phase at 37°C with a suspension of appr. 0.5 M lipid in water. Individual curves have been shifted relatively to each other to improve the presentation clarity.
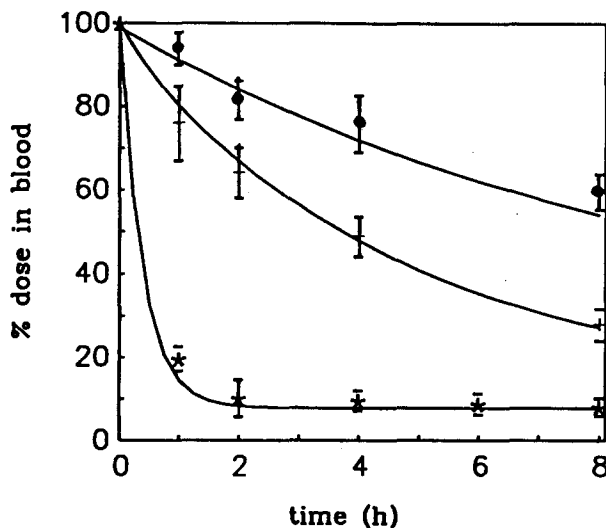

Fig. 6: Clearance of standard (DSPC: *) and sterically stabilized (DSPC/DSPE-PEG77-COOH (9/1): +) or (DSPC/DSPE-triazin-PEG110 (9/1): •) vesicles from the murine blood-stream as a function of time after an i.v. administration.
Liposome elimination is quasi mono-exponential with a characteristic time-constant of $\tau = 0.38$ h for the standard or $\tau = 4.62$ h and $\tau = 9.52$ h for the stabilized cryptosomes, respectively; the corresponding limiting vesicle concentrations ($t \rightarrow \infty$) are 7.8% for the standard liposomes and 12.4% and 19.9% for cryptosomes.
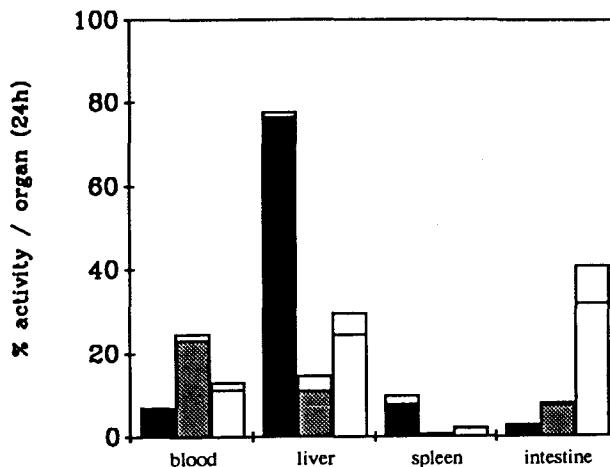
Fig. 7: The effect of various types of DSPE-PEG in lipid vesicles on the carrier biodistribution in mice after intravenous administrations in mice.
Illustrated tissue distributions give the distribution pattern of the radioactivity counts corresponding to vesicles labelled with tritium-DPPC 24 h after an application of 45 mg phospholipid per kg body weight; DSPC: black; DSPC/DSPE-PEG110 (9/1): white; DSPC/DSPE-PEG-COO⁻ (9/1): grey.

with a terminal carboxylic group (DSPE-PEG77-COOH), for example, have distinctly cryptosomal properties, despite the fact that both the headgroup termini and the coupling sites in the two systems differ: DSPC/DSPE-PEG77-COOH vesicles have a lifetime in blood of 4.62 hours, which is by a factor of 3.3 greater than for conventional DSPC vesicles. Also the uptake of DSPC/DSPE-PEG77-COOH vesicles by RHS is relatively slow (decrease by a factor of 2.6 relatively to the results obtained with DSPC liposomes) (cf. figures 6 and 7).

While the terminal carboxylic group seems not to interfere strongly with the vesicle longevity this segment may induce significant and interesting variations in the tissue distribution of the corresponding drug carriers after intravenous applications in mice (figure 7). When DSPE-PEG77-COOH is included into cryptosome membranes, for example, a high amount of the starting vesicle material is found in the intestine, 24 hours after the administration i.v.. The radioactivity distribution is essentially constant over the whole intestinal tract, as concluded from the radiography counts for the extracted individual segments of the gut (not shown). It remains to be seen whether or not the DSPE-PEG77-COOH containing vesicles with terminally attached antibodies in the form of immuno-cryptosomes will show a yet different distribution pattern *in vivo*.

CONCLUSIONS

We have shown that different sterically stabilized vesicles containing DSPE-triazin-PEG110 or DSPE- PEG77-COOH behave as cryptosomes, this is, they remain in the blood circulation for long periods of time and avoid the phagocyte-reach organs of the reticulohystiocytic systems. This potential of the surface-modified lipid vesicles was demonstrated to be essentially inaffected by the lipid bilayer fluidity and is also not to be a consequence of the high surface hydrophilicity of lipid vesicles.

These conclusions are relatively new. Indeed, in a recent paper by Lasic, which reiterates our previous analysis of the basic reasons for the liposome longevity, the suggestion was advocated that lipid chain rigidity is not a prerequisite for the long vesicle life-times in vivo. This argument was based on the data obtained for phosphatidyl-choline/cholesterol mixtures, however. To date, direct evidence for this conclusion was thus lacking, all previous experiments with unsaturated lipids having been performed with rigid or rigidified vesicles. Our data presented in figures 3 and 4 thus, for the first time, directly prove that the state of the membrane bilayer interior is not an important determinant of the vesicle life-time and distribution in vivo, provided that the lipid bilayer surfaces adequately shield this interior from the interactions with the blood components. (This does not pertain to the membrane permeability, of course.)

In previous publications on long-lived liposomes a combination of the surface hydrophilicity and the absence of charged groups on the liposome surface were suggested to reduce the likelihood of vesicle interactions with the body proteins and macrophages. Based on our results obtained with strongly hydrophilic vesicles consisting of phosphatidylcholine and a neo-glycolipid DSPE-maltopentaose we conclude that the former requirement is not generally true. From data shown in figures 6 and 7 we, furthermore, infer that the significance of the negative charges on the bilayer surface hencetoforth has been overemphasized. Negative charges on the DSPE-PEG77-COO$^-$ termini, for example, do not promote the accumulation of the corresponding vesicles in the liver or spleen of the test animals to an appreciable extent. (Slightly different circulation times and biodistribution patterns found in this study for the DSPC/DSPE-PEG110 and DSPC/DSPE-PEG77-COOH vesicles are probably due to the different lengths of the polyethyleneglycol segments rather then being a consequence of the terminal lipid charges.)

Modified lipid-headgroup termini may change the biodistribution of vesicles on which they reside, however. As much as 30% of the total applied lipid mass may end-up in the gastrointestinal tract, for example, if the number of carboxylic groups on very long spacers on each liposomal surface is sufficiently high.

Surface charges as well as local polarity effects and the intermolecular hydrogen bonding also may be involved in the vesicle-drug interactions. In this study we have shown, for example, that fluorescently labelled molecules of dansyl-tuftsin, a macrophage activating agent, can not be incorporated efficiently into the lipid vesicles stabilized with a sterically active lipid derivative DSPE-triazine-PEG110. Con-

versely, carboxyfluorescein and carboplatinum can be incorporated better into the DSPE-PEG-stabilized cryptosomes than into ordinary DSPC vesicles.

We postulate that optimized long-lived carrier vesicles for the hydrophilic drugs should be composed of bilayers which under physiological conditions are in the ordered, gel-phase, with a small vesicle diameter and a high encapsulation efficiency, the latter being improved by the suitable choice of the charge density and polarity at the bilayer surface. As expected, the permeation-mediated loss into plasma of CF and carboplatin from our DSPC vesicles is less than 25% and 24%, respectively, over a period of 24 hours (figure 1a). Addition of 10 mol% DSPE-PEG110 to DSPC reduces this loss to even lower values below 15% and 11%, respectively (figure 2a). The rate of permeation of the encapsulated drug carboplatin from thee DSPE-PEG110 liposomes is constant within the first 24 hours, some 0.5% being released per hour. This corresponds to a total release of 5 μ g carboplatin per millilitre of liposome suspension.

ACKNOWLEDGEMENT

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