# On the mechanism of hepatic transendothelial passage of large liposomes

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**Abstract Liposomes of 400 nm in diameter can cross the 100-nm fenestrations in the endothelium of the hepatic sinusoid, provided they contain phosphatidylserine (PS) but not phosphatidylglycerol (PG) [Daemen et al. (1997) Hepatology 26, 416]. We present evidence indicating that (i) the PS effect does not involve a pharmacological action of this lipid on the size of the fenestrations, (ii) fluid-type but not solid-type PS liposomes have access to the hepatocytes and (iii) the lack of uptake of PG liposomes by hepatocytes is not due to a lack of affinity of the hepatocytes for PG surfaces. We conclude that the mechanism responsible for the uptake of large PS-containing liposomes by hepatocytes in vivo involves a mechanical deformation of these liposomes during their passage across the endothelial fenestrations.**



*Key words:* Liposome; Liver; Endothelial fenestration; Membrane rigidity; Phosphatidylserine; Hepatocyte

## 1. Introduction

The use of particulate drug carriers such as liposomes to optimize the payload for the cell-specific delivery of drugs offers the advantage that one such carrier unit may contain a large number of active drug molecules. This approach may be particularly favorable for readily accessible cells such as circulating blood cells, endothelial cells of the vascular system or tissue macrophages such as Kupffer cells in the liver. It may, however, present a serious problem for target cells outside the circulation because of anatomical constraints. In this respect, the liver is an exceptional organ because of the numerous open fenestrations in the endothelial lining of the sinusoid. This condition provides an almost unrestricted accessibility of trans-endothelially located cell populations such as the hepatocytes and the stellate cells for particles with sizes well below the diameter of the fenestrations, i.e. approximately 100 nm [1,2]. In the past, the usefulness of liposomes for the delivery of large quantities of drugs to Kupffer cells has been demonstrated extensively by us [3] as well as by several others [4,5]. Recently, we showed that liposomes can also be delivered effectively to the sinusoidal endothelial cells, when targeted with a proper ligand for the scavenger receptor on these cells [6,7] but also that liposomes with diameters of up to 400 nm may gain access to the hepatocytes, provided they have the proper lipid composition, i.e. they should contain the negatively charged component phosphatidylserine [8]. We tentatively explained this observation by assuming that either the phosphatidylserine was acting as a pharmacologically active agent causing an increase in the average diameter of the fenestrations or the liposomes were forcefully squeezed or 'extruded' through the fenestrations, possibly mediated by a process of forced sieving involving blood cells, as proposed earlier by Wisse et al. [9]. We now present evidence arguing against the pharmacological option and in favor of the extrusion concept.

## 2. Materials and methods

### 2.1. Animals

Pathogen-free male Wag/Rij rats (Harlan CPB, Zeist, The Netherlands), weighing 200–250 g, were used throughout the experiments. The animals received care in accordance with the institution's guidelines.

### 2.2. Materials

All phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol was obtained from Sigma Co. [$^3$H]Cholesteryl-oleylether (specific activity 1.7 MBq/mmol) was a product of Amersham (Buckinghamshire, UK). Collagenase A was obtained from Boehringer Mannheim (Almere, The Netherlands). All other chemicals were obtained from Merck or Sigma and of the highest purity available.

### 2.3. Preparation of liposomes

Liposomes were prepared from phosphatidylcholine (PC), cholesterol (CHOL) and either phosphatidylserine (PS) or phosphatidylglycerol (PG) in a 5:4:1 molar ratio. PC was either from egg yolk or synthetic distearoyl-PC and PS was either from bovine brain or synthetic distearoyl-PS. PG was in all cases PG derivatized from egg-PC. The lipid mixture, labeled, when required, with [$^3$H]cholesteryl-oleylether (3.6 kBq/μmol total lipid), was dried down from chloroform/methanol 9:1 stock solutions under reduced nitrogen pressure. The dried lipids were then hydrated with buffer (135 mM NaCl/10 mM HEPES (4-[2-hydroxyethyl]-L-piperazine ethanesulfonic acid), pH 7.4) and shaken for 10 min either at room temperature, or, for the distearoyl lipid mixture, at 60°C. The liposomes thus obtained were extruded repeatedly either through two polycarbonate membranes (Costar, Nuclepore, Cambridge, MA, USA) of 400 nm pore diameter alone, in order to obtain the larger size liposomes, or three times through membranes of 400 and 200 nm pore size consecutively and 10 times through 50 nm pore size to obtain the small-size liposomes. Extrusion was carried out at room temperature except for the solid-type liposomes which were extruded at 60°C. The average size and size distribution of the two types of liposomes thus obtained was determined by dynamic light scattering employing a Nicomp particle analyzer, model 370 (Nicomp Particle sizing systems, Santa Barbara, CA, USA). The average size of the large fluid liposomes was 336.6 nm with a mean S.D. of 134.8 ($n=5$), and of the large solid liposomes $297.7 \pm 114.1$ nm ($n=3$), both measured in the solid particle mode. Average size of the small fluid liposomes was 77.9 nm with a mean

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S.D. of 25.3 ($n=3$) and of the small solid liposomes 157.1 ± 57.2 nm ($n=3$), both measured in the vesicle mode. The lipid content of final preparations was determined by lipid phosphorus assay [10], which also served to calculate the specific radioactivity of the preparations (Bq/μmol of lipid). Liposome preparations were stored under nitrogen at 4°C and used within 2 weeks of preparation.

*2.4. Intrahepatic distribution*

Rats were injected intravenously via the vena penis with 5 or 10 μmol liposomal lipid. Two or 24 h after injection, hepatocytes and hepatic non-parenchymal cells were isolated as described before [8,11]. Briefly, livers were pre-perfused for 5 min with perfusion buffer containing EGTA to remove blood. After another brief perfusion with $Ca^{2+}$-containing perfusion medium, the liver was connected to a recirculating perfusion system and perfusion was continued for another 10 min with perfusion medium containing collagenase and $Ca^{2+}$. From the liver cell suspension thus obtained parenchymal cells were isolated by low-speed sedimentation; non-parenchymal cells were isolated from the supernatant by means of centrifugation for 15 min at 1500×*g* on a Nycodenz concentration gradient (Nycomed, Oslo, Norway). Cells were counted and aliquots were taken for radioactivity measurements.

It should be appreciated that cholesteryl-oleyl-ether (CE) is widely acknowledged as a metabolically inert compound that remains firmly associated with cells after internalization [12]. In accordance, we demonstrated earlier that liposomal [$^3$H]CE is released from Kupffer cells in vitro at a rate of less than 0.5% $h^{-1}$ [13]. We also observed recently that the encapsulated liposomal markers [$^{14}$C]sucrose and colloidal gold produce essentially the same intrahepatic distribution patterns as the lipid bilayer label [$^3$H]CE [8]. It is therefore justified to exclude any involvement of a selective [$^3$H]CE label transfer from Kupffer cells to hepatocytes in the delivery of liposomal [$^3$H]CE to the latter cell type. Furthermore, label transfer from endothelial cells can be discarded because these cells in vivo do not engage in the uptake of non-targeted liposomes [7,14].

## 3. Results

In our previous paper [8] we suggested as one of the possible explanations for the drastically different intrahepatic distribution of PS and PG liposomes, that the PS might have a pharmacological effect on the fenestrations, causing an increase of their size. The results presented in Fig. 1 demonstrate, however, that the simultaneous presence of large unlabeled PS-containing liposomes has no effect on the intrahepatic distribution of large (350 nm) PG-containing liposomes. Both with PG liposomes alone and with PG liposomes combined with PS liposomes not more than 3% of the total liver-associated PG liposomes distribute to the hepatocytes. For comparison we included an experiment with labeled PS liposomes. The result confirmed our previous observation, i.e. these liposomes distribute approximately equally between parenchymal and non-parenchymal cells, indicating as observed before, that the PS liposomes efficiently reach the hepatocytes. From these results we conclude that the difference in hepatic distribution between PS and PG liposomes is unlikely to be due to a pharmacological effect of the PS on the size of the fenestrations.

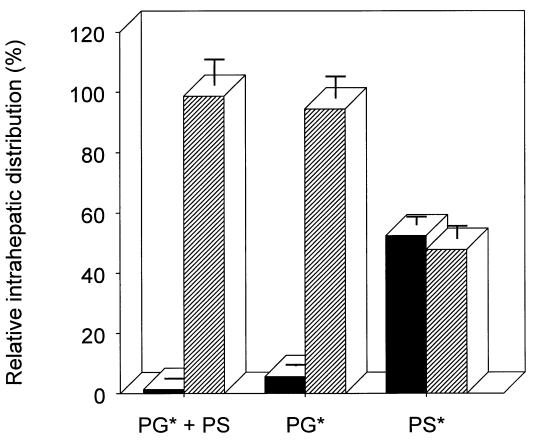


Fig. 1. The presence of PS liposomes does not influence the intrahepatic distribution of PG liposomes. Large radiolabeled PG liposomes were injected either as such or mixed with an equal amount of unlabeled PS liposomes. For comparison, radiolabeled PS liposomes were injected. Intrahepatic distribution after 4 h is given as percent of total liver-associated radioactivity ± S.D. Filled bars, hepatocytes; open bars, non-parenchymal cells. * indicates the liposome type that was labeled.

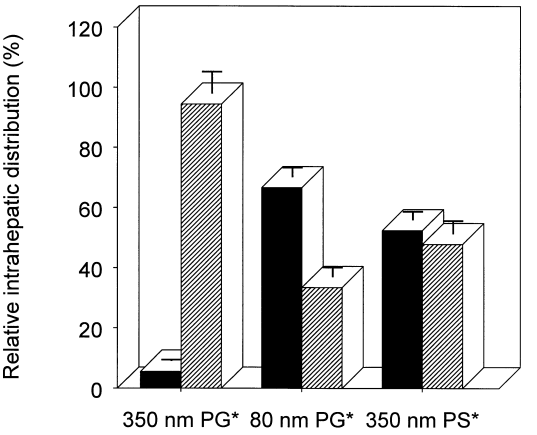


Fig. 2. Hepatocytes are able to take up small PG liposomes. Small radiolabeled PG liposomes were injected and after 24 h the distribution of radioactivity between parenchymal and non-parenchymal cells of the liver was determined. For comparison, intrahepatic distribution of large PG liposomes as well as of large PS liposomes is included. Filled bars, hepatocytes; open bars, non-parenchymal cells; error bar, S.D.

It might be argued that the lack of uptake of PG liposomes by hepatocytes, as presented in Fig. 1 is due an intrinsic lack of affinity of the hepatocyte for the PG. Therefore, we carried out an experiment with PG liposomes of sufficiently small size (approximately 80 nm) to allow easy passage through the 100-nm fenestrations. Fig. 2 shows the result. As much as two thirds of the total liver-associated amount of small PG liposomes were taken up by the hepatocytes. For comparison we again included the intrahepatic distribution of the large PG liposomes, with only 2–3% in the hepatocytes, and of the large PS liposomes with a fifty/fifty distribution. Clearly, the hepatocytes are able to recognize, bind and internalize PG-containing liposomes once they have gained access to these cells by virtue of their small size.

If it is not a PS-induced widening of the diameter of the fenestrations that is responsible for the trans-endothelial passage of the large PS liposomes, the alternative explanation we offered in our previous paper should be considered, i.e. a mechanical effect by which the liposomes are forced across the fenestrae, conceivably mediated by mechanical action of white blood cells being squeezed along the narrow sinusoid. This option was taken from an idea proposed earlier by Wisse, who introduced the concept of forced sieving and endothelial massage in order to explain the movement of fluid into and out of the space of Disse [9]. If such a forced extrusion of liposomes were to take place, one would have to

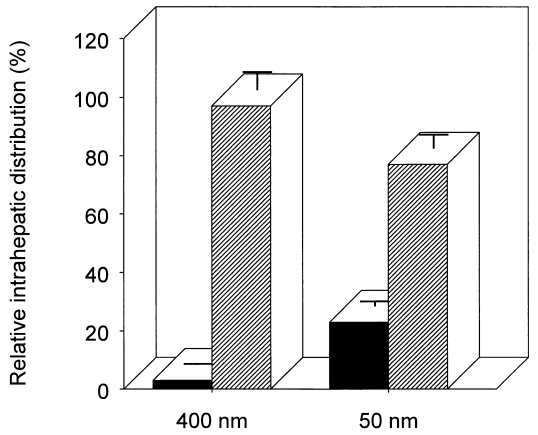


Fig. 3. Large rigid-bilayer PS liposomes do not have access to hepatocytes. Small and large radiolabeled liposomes consisting of DSPC, DSPS and CHOL (5:1:4) were injected and intrahepatic distribution was determined. Filled bars, hepatocytes; open bars, non-parenchymal cells; error bar, S.D.

assume that these liposomes have to be deformable. For a liposome to be deformable its bilayers should be in a relatively fluid (liquid-crystalline) state [15], as is likely to be the case for egg PC/brain PS/CHOL liposomes with a high content of (poly)unsaturated hydrocarbon chains. The bilayers of DSPC/DSPS/CHOL liposomes, on the other hand, with only saturated long chain hydrocarbon moieties, are much more rigid and thus less flexible. Therefore, we repeated our previous experiment on PS liposomes, but now composed of the rigid mixture DSPC/DSPS/CHOL and both small and large in size. Fig. 3 demonstrates that in contrast to our findings on large 350-nm egg PC/PS/CHOL liposomes in Figs. 1 and 2, the large DSPC/DSPS/CHOL liposomes almost only associate with the non-parenchymal cells. The hepatocytes take up negligible quantities of these liposomes. This lack of uptake of rigid PS liposomes by hepatocytes is not due to an intrinsic lack of affinity of these cells for rigid PS surfaces. This is demonstrated by the right panel of Fig. 3, which shows that a substantial proportion of small liposomes of this rigid composition associates with hepatocytes and therefore does gain access to these cells.

## 4. Discussion

Widely varying compounds like ethanol and serotonin have been shown to affect the diameter of endothelial fenestrae [16,17]. Furthermore, PS and/or its metabolites have been reported to possess a variety of pharmacological activities [18,19]. PS was also shown to stimulate phosphorylation of actin by protein kinase C in a strongly concentration-dependent way [20], while cytoskeletal elements have been reported to be closely associated with the fenestrae [21]. Thus, we considered it conceivable that PS-containing liposomes might exert a dilating effect on the fenestrae. However, the lack of effect of PS liposomes on the intrahepatic distribution of PG liposomes, as presented in Fig. 1, strongly argues against a specific pharmacological effect of the PS causing a dilation of the fenestrations. Nor can an intrinsic lack of affinity of the hepatocyte for PG-containing liposomes account for the difference between the two liposome types, as was clearly shown by the results presented in Fig. 2. If there is no increase in the size of the fenestrations and if the PS liposomes do not lose significant amounts of an encapsulated marker during their passage across the endothelium, as we demonstrated in our previous paper [8], the large PS liposomes must pass through the fenestrations intact. The results presented in Fig. 3 suggest that such passage involves the deformation of the liposomal bilayer so as to adjust the shape of the liposome to the much smaller size of the fenestrae, because the non-flexible rigid-bilayer liposomes containing PS apparently do not have access to the hepatocytes.

An important question that remains to be answered is why fluid-type PS liposomes apparently do and fluid-type PG liposomes do not reach the hepatocytes. At present we can only address this question in a speculative way. For a PS liposome to be squeezed into and through a fenestra it probably has to experience in the vascular compartment some degree of retardation in the vicinity of the fenestra, conceivably achieved by a reversible interaction between the liposome and the endothelium. Such an interaction might be mediated by scavenger receptors which we showed to be present on these cells and which we hold responsible for the massive in vivo uptake of liposomes carrying covalently coupled poly-aconitylated (and thus strongly anionic) albumins on their surface [6,7]. Despite their multiple negative charges, liposomes containing up to 100% of PS do not irreversibly interact with these receptors in vivo, as revealed by their complete lack of association with endothelial cells isolated following intravenous injection of such liposomes, but these liposomes do interact strongly with these cells in vitro, provided no serum is present (Kamps et al., unpublished observations). In addition, we observed that PS-containing liposomes, during the first 5 min following their intravenous administration, transiently accumulate in the liver (Kamps et al., unpublished observations). On the basis of these observations we speculate that the negative charges of bilayer-associated PS are able to transiently associate with the endothelial cells, while PG liposomes with their more concealed negative charges do not or do so to a lesser extent.

In conclusion, the experiments described in this paper give reason to believe that the specific ability of large PS-containing liposomes to penetrate the hepatic sinusoidal endothelium so as to gain access to the liver parenchymal cells lying across the endothelial lining involves a possibly blood cell-mediated [9], forced extrusion of the liposomes through the endothelial fenestrae, by virtue of a weak and transient interaction with sites on the endothelium conceivably represented by scavenger receptors. The notion that even relatively large liposomes may thus reach trans-endothelial areas in the liver opens up possibilities of using large liposomes to efficiently deliver even large molecules such as proteins and DNA, which are difficult to encapsulate in small vesicles, to cells located there, such as hepatocytes and stellate cells.

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