Lymphatic uptake and biodistribution of liposomes after subcutaneous injection.
II. Influence of liposomal size, lipid composition and lipid dose

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Abstract

The present paper reports on the results of a systematic study on liposome variables potentially affecting lymphatic disposition and biodistribution of liposomes after sc injection. Liposomal size was found to be the most important factor influencing lymphatic uptake and lymph node localization of sc administered liposomes. Lymphatic uptake from the sc injection site of small liposomes (about 0.04 μm) was relatively high (76% of the injected dose (%ID)) as compared to large, non-sized liposomes, which remained almost completely at the site of injection. Small liposomes were less efficiently retained by regional lymph nodes than larger liposomes. Liposomal lipid composition did not influence lymphatic uptake with one exception: Lymphatic uptake was decreased in case of neutral liposomes composed of (DPPC). Lymph node localization was substantially enhanced by inclusion of phosphatidylserine (PS) into the liposomal bilayers. Saturation of lymphatic uptake and lymph node localization did not occur over a large liposomal lipid dose range, illustrating the efficient performance of lymph nodes in capturing sc administered particles. © 1997 Elsevier Science B.V.

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1. Introduction

Targeting of particulate carrier systems to the lymphatic system has a number of applications including diagnosis and treatment of diseases with lymphatic involvement, such as tumor metastases, viral and bacterial infections, and immunization. The relatively high absorption of high molecular weight substances and particulates into the lymphatics after local administration, such as subcutaneous (sc), intramuscular (im) and intraperitoneal (ip) injection, has stimulated

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research on the use of colloidal carriers for lymphatic drug delivery. Relatively high lymph node localization of colloidal particles, such as liposomes, after local administration has been reported by several researchers [1–4]. The sc route of administration has been most extensively investigated for the lymphatic targeting of liposomes.

Detailed information on factors influencing lymphatic uptake and lymph node localization of sc administered liposomes is not readily available. Although several reports have indicated lymphatic uptake and lymph node localization of sc administered liposomes, results are often not comparable as different liposome labels, lipid compositions and animals were used. Moreover, most studies monitored the fate of the drug rather than that of the particles. Several reports point to a decreasing lymphatic uptake with increasing liposome size [5–8]. Only smaller liposomes (i.e., roughly less than ca. 0.15 μm) appear to enter the lymphatic capillaries, whereas larger liposomes remain at the site of injection. It has also been observed that the use of small liposomes is beneficial for achieving a relatively high lymph node localization. A few reports deal with the effect of liposome charge on lymph node localization of sc administered liposomes. Experiments utilizing liposomes labeled with [99m]Technetium showed that positively charged and neutral liposomes localize to a higher extent in regional lymph nodes than negatively charged liposomes [9]. Similar results were reported by the same group when using these liposomes for the detection of lymphatic metastases [10]. However, these results were invalidated later by showing that the technetium marker did not represent intact liposomes in lymph nodes [11]. The latter study showed that negatively charged liposomes localized to a greater extent in the lymph nodes compared to positive liposomes, which in turn localized better than neutral liposomes. In contrast, a more recent study on lymph node localization of small methotrexate-containing liposomes after im administration revealed that positive liposomes localized more in regional lymph nodes as compared to neutral and negatively charged ones [12].

In general, literature on lymphatic absorption and lymph node localization of sc administered liposomes is limited and incomplete. Therefore, a systematic study on liposomal variables potentially affecting the in vivo fate of sc administered liposomes was per-

formed. The present paper reports on the effects of liposomal size, lipid composition and lipid dose on lymphatic uptake, lymph node localization and disposition in blood, liver and spleen of sc administered liposomes.

2. Materials and methods

2.1. Chemicals

Egg-phosphatidylcholine (EPC), egg-phosphatidylglycerol (EPG), dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were donated by Lipoid (Ludwigshafen). Phosphatidylserine (PS) was obtained from Avanti Polar Lipids (Alabaster, AL). $1\alpha,2\alpha(n)$-[^{3}H]-Cholesteryloleylether (spec. act. 1.71 TBq mmol^{-1}) was supplied by Amersham (Buckinghamshire, UK). Cholesterol (Chol) and 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid (Hepes) were obtained from Sigma (St. Louis, MO). Fetal calf serum (FCS) was supplied by Bocknek (Canada). Hionic Fluor, Soluene-350 and Plasmasol were purchased from Packard (Downers Grove, IL). All other reagents were of analytical grade.

2.2. Preparation of radiolabeled liposomes

Liposomes were prepared by the thin film-extrusion method [13]. [^{3}H]-Cholesteryloleylether was added as a marker of the lipid phase. A mixture of the appropriate amounts of lipids, including the [^{3}H]-label, was dissolved in a mixture of chloroform/methanol (4:1 v/v) and evaporated to dryness by rotation under reduced pressure at 40°C. After flushing the lipid film with nitrogen for at least 20 min, the film was hydrated in a sterile Hepes/glucose-buffer (10 mM Hepes, 1 mM EDTA, 270 mM glucose, pH 7.4). Liposomes were non-sized or sized by extruding the liposome dispersion through an appropriate combination of single or stacked 0.6, 0.2, 0.1 or 0.05 μm polycarbonate membrane filters (Nuclepore; Costar, Cambridge, MA) under nitrogen pressure. Liposomes with a mean size of 0.04 μm were prepared by sonication of small (0.07 μm) liposomes in a bath sonicator (Bransonic 5, Branson, Tamson, Zoetermeer, The Netherlands) for 45 min.

2.3. Liposome characterization

Radioactivity of the liposomal dispersion was assayed in Hionic Fluor as scintillation mixture and counted in a Philips PW 4700 liquid scintillation counter. Mean particle size was determined by dynamic light scattering at 25°C with a Malvern 4700 system using a 25 mW He–Ne laser (NEC, Tokyo) and the automeasure version 3.2 software (Malvern, Malvern, UK). For viscosity and refractive index, values of the Hepes/glucose buffer were used. As a measure of particle size distribution of the dispersion, the system reports a polydispersity index. This index ranges from 0.0 for an entirely monodisperse up to 1.0 for a polydisperse dispersion.

2.4. Animal experiments

Male Wistar rats (U:WU, CPB) from the animal facility of Utrecht University with an approximate body weight of 220 g were used. Animals received standard laboratory chow and water ad libitum. Rats were injected sc with a single dose of [^{3}H]-labeled liposomes into the dorsal side (i.e., the upper side) of the right foot. At various time-points post-injection a blood sample was drawn from the tail vein. At the end of the observation period the site of sc injection, regional lymph nodes (popliteal and iliac) [14], blood, liver and spleen were collected and assayed for radioactivity.

2.5. Radioactivity measurements

Radioactivity in blood samples (120 μl) was determined by adding 120 μl of Plasmasol, 500 μl of water and 500 μl of 35% hydrogenperoxide. The samples were decolorized overnight at 40°C. Radioactivity was assayed in Plasmasol as scintillation fluid. A total blood volume per rat of 75 ml kg^{-1} body weight was used for calculation of the percentage dose in the blood circulation [15]. Lymph nodes, spleen and the right foot were solubilized completely in an appropriate amount of Soluene-350 at 40°C. Solubilized lymph nodes and samples (500 μl) of solubilized foot and spleen were decolorized with 200 μl of 35% hydrogenperoxide overnight at 40°C. Livers were homogenized in 12 ml of water with a Potter-S homogenizer (Braun, Melsungen, Germany).

Samples (200 μl) of liver homogenate were dissolved in 1 ml of Soluene-350 and decolorized with 35% hydrogenperoxide overnight at 40°C. Decolorization of samples was repeated until the samples were only slightly colored. Radioactivity of the decolorized samples was assayed in Hionic Fluor as scintillation fluid.

Lymphatic uptake is defined as the percentage injected dose radioactivity (i.e., 100%) minus the percentage of dose radioactivity recovered from the injection site. Lymph node localization is expressed as the percentage injected dose radioactivity per gram lymph node tissue (popliteal and iliac). Results represent the mean of 4 rats ± standard deviation (sd).

2.6. Pharmacokinetics

All pharmacokinetic parameters were calculated using the curve fitting program KINFIT (MEDI\WARE, Groningen, The Netherlands) for each animal individually. Mean and sd were calculated for each treatment group.

2.7. Statistics

The effect of different treatments was evaluated by ANOVA with 95% confidence interval. Differences were considered significant when the p-value was less than 0.05.

3. Results

3.1. Reference liposomes

A single dose of small liposomes (mean size about 0.07 μm) with the composition EPC:EPG:Chol (10:1:4 molar ratio) (referred to as 'reference liposomes'), was sc administered to rats at a dose of 2.5 μmol total lipid (TL). Liposomes were radiolabeled with a tracer amount of [^{3}H]-cholesteryloleylether which has proven to be a reliable label to monitor the fate of liposomes in vivo [16,17]. The dorsal side of the foot was chosen as site of sc injection as injection of liposomes into this site of rats results in relatively high lymphatic uptake as compared to sc administration into the flank [18]. The in vivo fate of the liposomes was studied over a period of 52 h post-in-

jection. For comparative reasons, quantitative data obtained with the reference liposomes, serve as a reference for data obtained with liposomes of different size and composition.

Judging from the results presented in Fig. 1A, lymphatic uptake (i.e., the percentage of injected dose taken up from the sc injection site by the draining lymphatic capillaries) of the reference lipo-
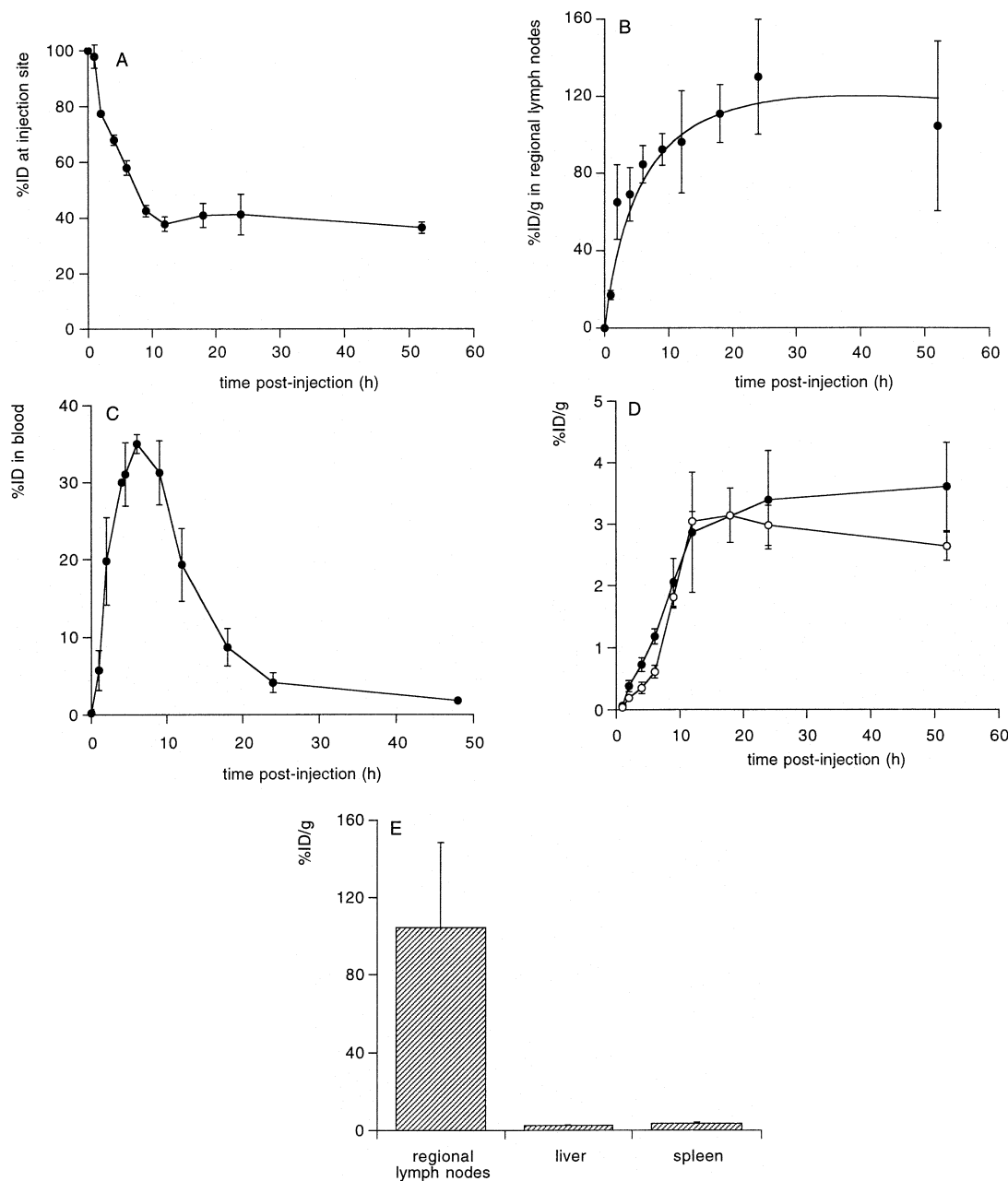

Fig. 1. Biodistribution of sc administered reference liposomes. A single dose of reference liposomes (EPC:EPG:Chol, 10:1:4 molar ratio, mean diameter 0.07 μm, 2.5 μm TL) was injected sc into the dorsal side of the foot of rats. Recovery of liposomal label was determined at several time-points post-injection. (A) Percentage of injected dose recovered from the sc injection site. (B) Percentage of injected dose per g tissue recovered from the regional lymph nodes. (C) Percentage of injected dose circulating in the total blood volume. (D) Percentage of injected dose per g tissue recovered from liver (○) and spleen (●). (E) Comparison of percentage of injected dose per g tissue in regional lymph nodes, liver and spleen. Values represent the mean percentage ± sd of 4 animals.

somes occurred over the first 12 h after injection. After this initial period, the absorption process was completed with about 40% of the injected dose (%ID) remaining at the injection site. Consistent with the time frame over which lymphatic uptake occurred, lymph node localization (i.e., the percentage of injected dose radioactivity per gram lymph node tissue (%ID g^{-1})) reached a maximum value of about 120%ID g^{-1} (i.e., 1.2%ID) over a period of 12 h after injection (Fig. 1B). Levels of radioactivity did not change significantly over the subsequent 40 h. Liposome localization was negligible in lymph nodes not involved in drainage of the injection site and in control lymph nodes on the left, non-treated side of the body.

Blood levels of radiolabeled liposomes are presented in Fig. 1C. The peak blood level (about 35%ID) was reached about 6 h post-injection. At the end of the observation period, i.e., 52 h after injection, liposomal radioactivity in the blood had declined to levels less than 5%ID. When plasma and the cellular fraction were separated, all radioactivity in blood was associated with the plasma fraction. Liposomal label recovery from liver and spleen is presented in Fig. 1D. In line with the observations on the kinetics of lymphatic uptake and lymph node localization, the majority of the recovered liposomal label localized in liver and spleen (about 30 and 2%ID, respectively, i.e., 3%ID g^{-1} for both organs) during the initial 12 h after injection. The biodistribution pattern did not change significantly over the subsequent period of 52 to 96 h (results not shown). A comparison of the degree of localization in regional lymph nodes, liver and spleen at the end of the observation period (i.e., 52 h post-injection) is shown in Fig. 1E. Clearly, when the data are expressed as the percentage injected dose per g tissue, lymph node localization was much higher than localization in liver and spleen (40- and 31-fold higher, respectively).

3.2. Influence of dose

To study if lymphatic uptake and lymph node localization depend on lipid dose, liposomes with the same composition as reference liposomes but somewhat larger (mean size 0.10 μm), were administered sc into the foot of rats in escalating lipid doses (i.e., 10, 10^2, 10^3 and 10^4 nmol TL). For each of the liposome doses investigated, about 60%ID was re-

covered at the site of injection 52 h post-injection (results not shown). The absolute amount of lipid recovered from the injection site is presented in Fig. 2A. The straight line indicates that no saturation of the lymphatic absorption process was observed over the dose range investigated. The same observation applies for lymph node localization (Fig. 2B). The absolute amount of liposomal label recovered from lymph nodes 52 h after injection increased almost linearly with increasing liposome dose. The same linear relationship was found for large, non-sized
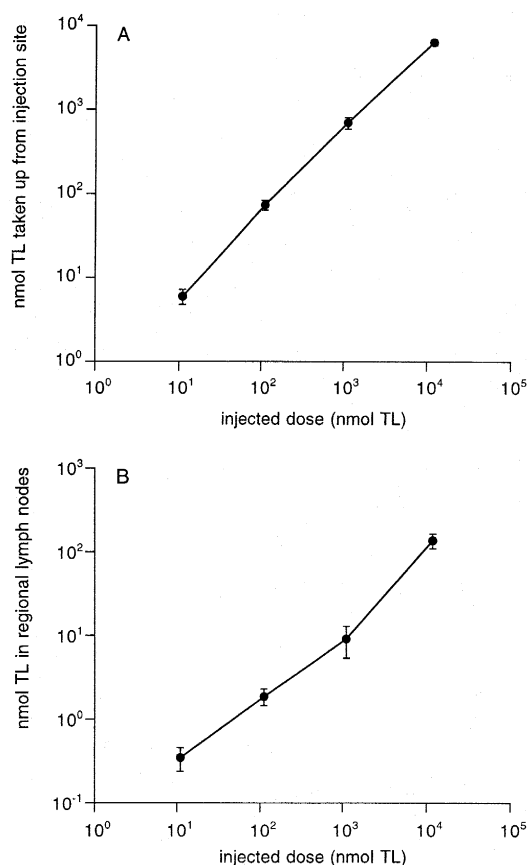

Fig. 2. Influence of dose on lymphatic uptake and lymph node localization of small liposomes. A single dose of small (0.10 μm) liposomes (EPC:EPG:Chol, 10:1:4 molar ratio) was injected sc into the dorsal side of the foot of rats in escalating lipid dose (i.e., 10, 10^2, 10^3, 10^4 nmol TL). Levels of radioactivity at the site of injection and in regional lymph nodes were determined 52 h post-injection. (A) Absolute amount of total lipid taken up from the sc injection site. (B) Absolute amount of total lipid recovered from regional lymph nodes. Values represent the mean percentage $\pm$ sd of 4 animals.

liposomes at the same dose range (results not shown). Moreover, repeated injections of small liposomes (1 injection of 3.5 μmol TL given daily over 4 subse-quent days), also resulted in a linear dose-dependency of the accumulation of liposomes in regional lymph nodes (results not shown).
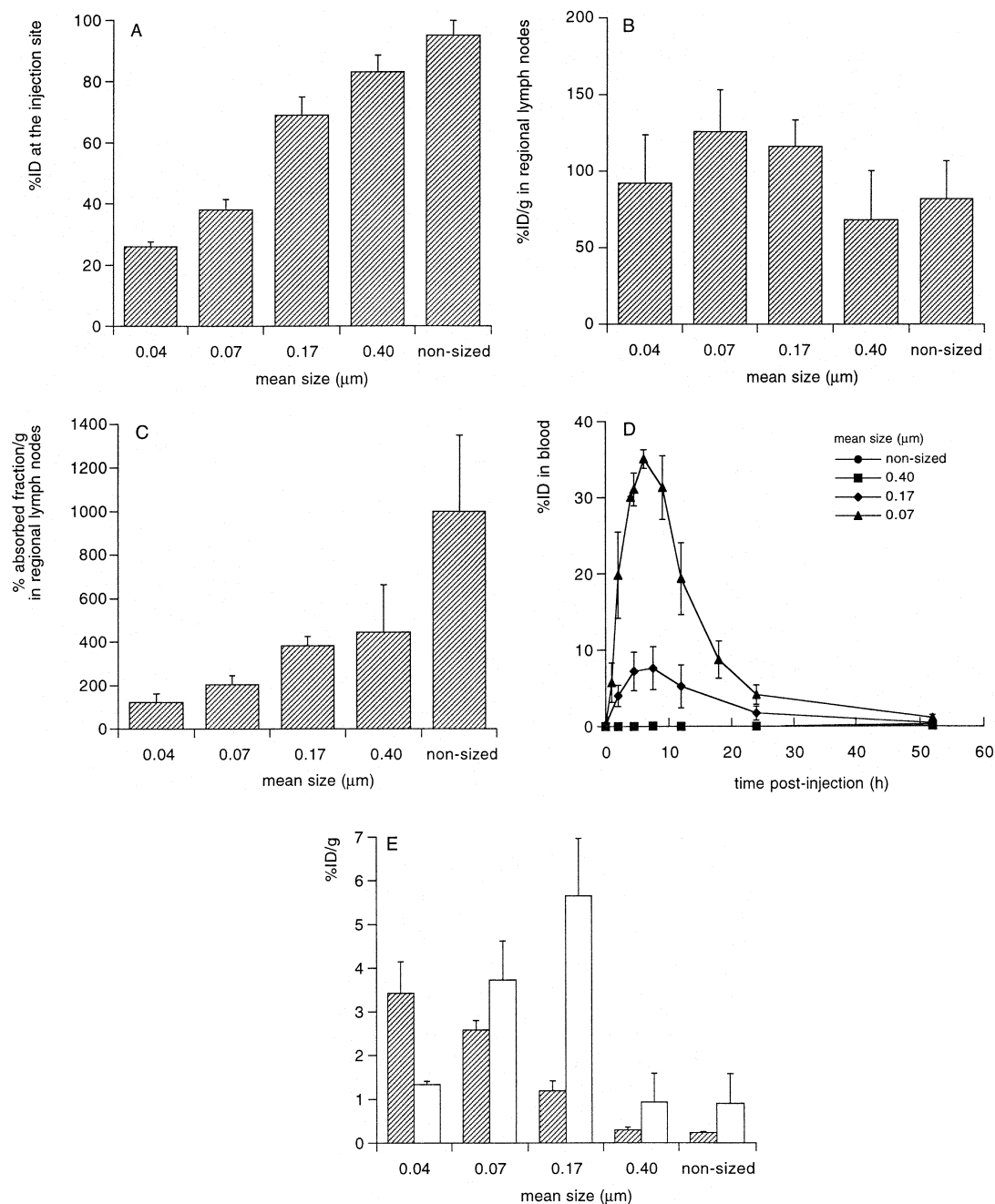

Fig. 3. Influence of size on the pharmacokinetics and biodistribution of sc administered liposomes. A single dose of liposomes (EPC:EPG:Chol, 10:1:4 molar ratio, 2.5 μmol TL) of varying size was injected sc into the dorsal side of the foot of rats. Levels of radioactivity were determined 52 h post-injection. (A) Percentage of injected dose recovered from the sc injection site. (B) Percentage of injected dose per g tissue recovered from the regional lymph nodes. (C) Percentage of the lymphatically absorbed fraction per g recovered from the regional lymph nodes. (D) Percentage of injected dose circulating in the total blood volume. (E) Percentage of injected dose per g tissue recovered from liver (▨) and spleen (□). Values represent the mean percentage ± sd of 4 animals.

3.3. Influence of size

Liposomes with the same lipid composition as reference liposomes but with varying mean size were injected sc at a dose of 2.5 μmol TL. Liposomes were either non-sized, or had a mean diameter of 0.40, 0.17, 0.07 or 0.04 μm (referred to as 0.40, 0.17, 0.07 or 0.04 μm liposomes). The degree of lymphatic uptake was found to be negatively correlated with liposome size (Fig. 3A). Over a time period of 52 h after injection, 0.04 μm liposomes were taken up by the lymphatics to a relatively high extent (74%ID), whereas large, non-sized liposomes remained almost completely localized at the site of injection.

Remarkably, lymph node localization depended only slightly on liposome size. Lymph node localization was found to be between 70 and 130%ID g^{-1} for all sizes investigated (Fig. 3B) and was slightly lower for larger liposomes. It should be realized, however, that, as lymphatic uptake decreases with increasing liposome size (Fig. 3A), the fraction of injected liposomes reaching regional lymph nodes is much higher for smaller liposomes than for larger liposomes. Consequently, lymph node localization should be corrected for the lymphatically absorbed fraction (i.e., the difference between administered dose and amount recovered at the injection site) to establish the true efficiency of lymph node localization of the different liposome types. Therefore, the 'relative lymph node localization' was calculated by correcting the percentage of dose recovered in the lymph nodes for the lymphatically absorbed fraction (Fig. 3C). It was found that the relative lymph node localization of small liposomes was much less as compared to the relative lymph node localization of larger liposomes. Only 120%ID g^{-1} of the absorbed fraction of 0.04 μm liposomes localized in regional lymph nodes, whereas more than 1000%ID g^{-1} of the absorbed fraction of large, non-sized liposomes localized in regional lymph nodes (Fig. 3C). Interestingly, determination of lymph node localization at several time-points post-injection over a time-period of 52 h, revealed that the maximum lymph node concentration of the large, non-sized liposomes was reached within 1 h without any significant change thereafter (results not shown).

Concentrations of liposomes in blood and liver are in agreement with the observed pattern of size dependency of lymphatic absorption, as increased lymphatic absorption translated into increased blood levels (Fig. 3D) and higher liver uptake (Fig. 3E). In contrast to localization in the liver, the extent of splenic localization was not positively correlated with the extent of lymphatic absorption (Fig. 3E).

3.4. Influence of lipid composition

The effects of liposomal charge (negative charge conferred by PG or PS) and bilayer fluidity (as a result of the presence of cholesterol or the use of phospholipids with different degree of saturation) on lymphatic delivery of sc administered liposomes was studied utilizing liposomes with the lipid compositions listed in Table 1.

Lymphatic uptake from the injection site was independent of the presence of charged lipid, the presence of cholesterol, and bilayer fluidity as compared to lymphatic uptake of reference liposomes (Fig. 4A). DPPC-liposomes showed a different behavior as compared to reference liposomes; lymphatic uptake of these liposomes was decreased to less than half of the uptake of reference liposomes. Lymph node localization was found to be independent of cholesterol content, the presence of EPG and bilayer fluidity. DPPC-liposomes and PS-containing liposomes localized in lymph nodes to a higher extent (ca. 3-fold) than reference liposomes (Fig. 4B).

The time course of blood levels induced by the sc injection of the various types of liposomes is shown

Table 1
Liposomal lipid compositions studied

Lipid composition	Molar ratio
EPC:EPG:Chol (reference liposomes)	10:1:4
EPC:EPG	10:1
EPC:Chol	10:4
EPC	
EPC:PS:Chol	10:1:4
EPC:PS	10:1
DPPC:DPPG:Chol	10:1:4
DPPC	

Compositions of small (0.07 μm) liposomes used to study the influence of the effect of liposomal charge and bilayer fluidity on the lymphatic uptake, lymph node localization and biodistribution after sc injection.
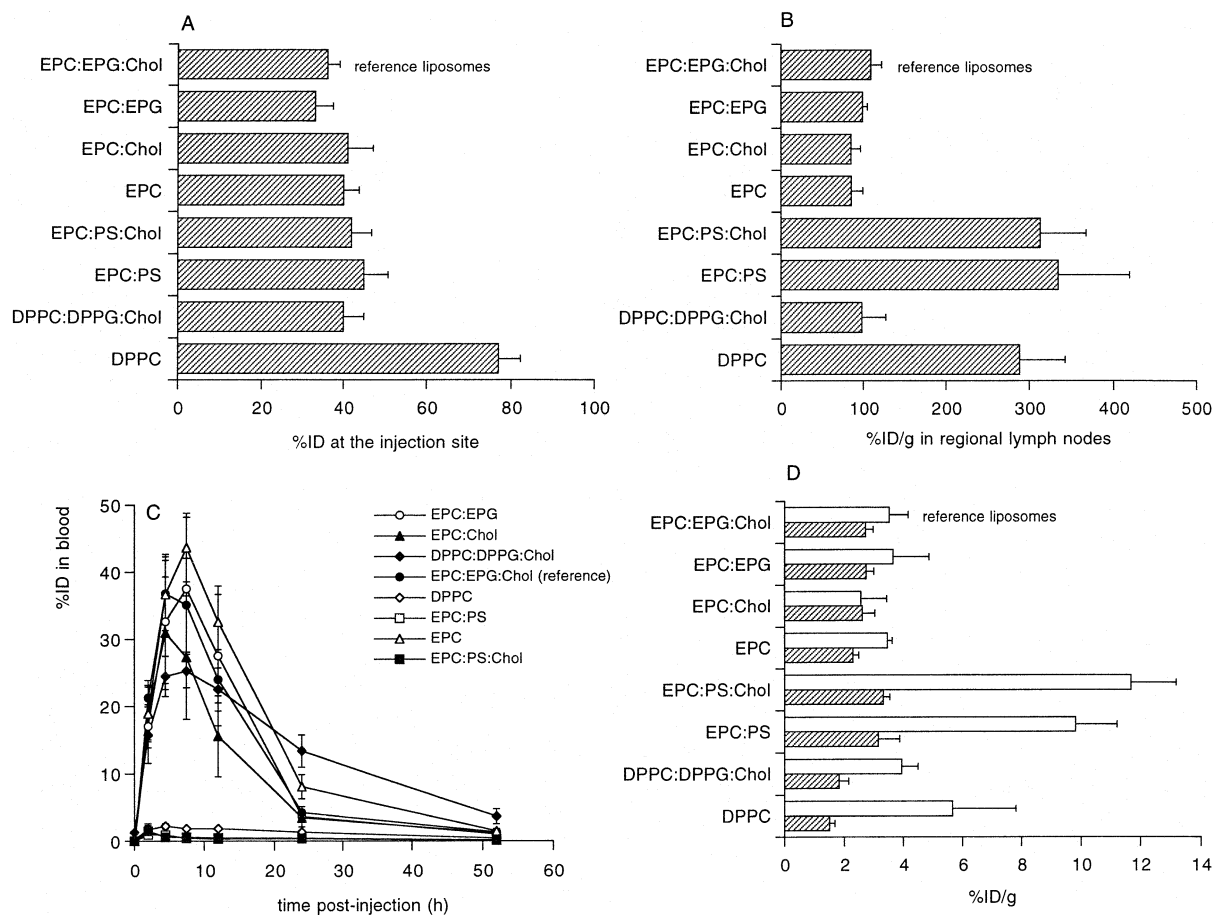
Fig. 4. Influence of composition on the pharmacokinetics and biodistribution of sc administered liposomes. A single dose of small liposomes (mean diameter 0.07 μm, 2.5 μmol TL) of varying composition was injected sc into the dorsal side of the foot of rats. Levels of radioactivity were determined 52 h post-injection. (A) Percentage of injected dose recovered from the sc injection site. (B) Percentage of injected dose per g tissue recovered from the regional lymph nodes. (C) Percentage of injected dose circulating in the total blood volume at several time-points after injection. (D) Percentage of injected dose per g tissue recovered from liver (▨) and spleen (□). Values represent the mean percentage ± sd of 4 animals.

Table 2
Effect of FCS on liposomal size in vitro

Lipid composition (molar ratio)	Mean size (nm)		Polydispersity index	
	Before	After	Before	After
EPC:EPG:Chol (10:1:4) reference liposomes	71 ± 1	65 ± 1	0.22 ± 0.02	0.20 ± 0.02
EPC	70 ± 0	70 ± 1	0.20 ± 0.03	0.20 ± 0.02
DPPC:DPPG:Chol (10:1:4)	75 ± 2	73 ± 2	0.15 ± 0.06	0.22 ± 0.03
DPPC	67 ± 1	115 [a] ± 6	0.25 ± 0.03	0.44 [a] ± 0.02

Small liposomes with a mean size of about 0.07 μm and a polydispersity index < 0.1 were incubated in 80% FCS (15 mM TL) for 24 h at 37°C. Data represent the mean size and polydispersity index ± sd of 3 dispersions; before: immediately after mixing with the incubation medium and after: 24 h after incubation.

[a] p < 0.01.

in Fig. 4C. The liposome levels in blood after administration of PS-containing liposomes and DPPC-liposomes were strikingly low as compared to those of the other liposome types, which all showed a blood concentration-time profile with a clear absorption and distribution–elimination phase. Uptake in liver and spleen is presented in Fig. 4D. Liver uptake for all liposome types was about the same. Regarding splenic uptake, concentrations of PS-containing liposomes were 3-fold higher as compared to reference liposomes. Splenic uptake of DPPC-liposomes was increased slightly (1.5-fold). The other liposome types did not differ significantly from the reference liposomes with regard to the degree of splenic localization.

As DPPC-liposomes showed a substantially decreased lymphatic uptake as compared to reference liposomes (Fig. 4A), it was hypothesized that DPPC-liposomes might aggregate at the site of injection. To approach this hypothesis experimentally, the aggregation behavior of DPPC-liposomes incubated with FCS in vitro was investigated. An increased particle size and polydispersity was seen only with DPPC-liposomes (Table 2).

4. Discussion

Several studies have demonstrated the potential of liposomes as 'lymphotropic' drug delivery systems for targeting to regional lymph nodes after sc administration [2,3,7]. The majority of the published data were obtained by following the fate of liposome-encapsulated drugs after sc administration. It should be realized however, that liposome-encapsulated drugs may influence the fate of liposomes in vivo and may be distributed differently than the liposomal carrier after release from the liposomes. Also, the influence of physicochemical properties of the liposomal carrier, such as liposome size and lipid composition, on 'lymphotropic' behavior has not been described in detail yet. With greater understanding of the biodistribution of the drug carrier, the fate of the encapsulated drug may be anticipated more easily. The present study deals with a systematic investigation of factors potentially influencing lymphatic disposition of sc administered liposomes.

Sc injection of reference liposomes into the dorsal side of the foot of rats, resulted in an initial 12 h period of lymphatic uptake from the site of injection. After this 12 h time-period lymphatic uptake appeared to be finished with about 40%ID remaining at the injection site (Fig. 1A). A possible explanation for the incomplete uptake might be the heterogeneous size distribution of the liposome dispersion with liposomes substantially larger than the mean size of 0.07 μm being retained at the site of injection. Another explanation relates to an alteration of the interstitial pressure during the initial period of uptake. As lymphatic absorption from the injection site may be the result of an elevated interstitial pressure caused by the injection itself [18], lymphatic absorption may stop when the interstitial pressure is normalized. A third possibility is aggregation of liposomes at the site of injection resulting in the formation of large aggregates that are not taken up by the lymphatic capillaries.

Once liposomes have traversed the interstitium and entered the lymphatic capillaries, they pass through the lymphatic system where they can be captured in regional lymph nodes. The time frame over which lymph node localization occurs is in line with the observed time frame of occurrence of lymphatic uptake, i.e., the initial 12 h after administration (Fig. 1B). Lymph node localization of reference liposomes in regional lymph nodes was about 30- to 40-fold higher than uptake in spleen and liver, the natural target organs for circulating liposomes (Fig. 1E). Considering the fact that liposomes will encounter lymph nodes only once when passing through on their way to the blood circulation and do not have, as in the case of liver and spleen, the possibility of multiple passage, lymph node localization of liposomes is apparently an efficient process. It was of interest to determine at which lipid dose saturation of lymph nodes occurs. Therefore, lymphatic uptake and lymph node uptake was studied over a large liposomal lipid dose range (10–10^4 nmol TL). Saturation of lymphatic uptake and lymph node localization did not occur over the dose range investigated (Fig. 2). Together with the observation of efficient capture process, this observation of high capacity to retain particulate matter illustrates the suitability of the sc route for lymph node targeting of particulates.

Obviously, if lymphatic drug delivery is the main goal, lymphatic uptake is an important parameter

determining the absolute amount of liposomes arriving in the regional lymph nodes. In line with findings of others, it appears that size is the most crucial factor influencing lymphatic uptake of liposomes [6–8,10]. Small liposomes, with a mean size smaller than 0.1 μm, were taken up into the lymphatic capillaries to a high extent, whereas lymphatic uptake clearly declined with an increasing mean liposome size (Fig. 3A). The size-dependent uptake is likely to be related to the process of particle transport through the interstitium. The structural organization of the interstitium dictates that the diameter of administered particles should be small enough to allow migration through the aqueous channels in the interstitium. Therefore, larger particles will have more difficulty to traverse the interstitium and will remain at the site of injection to a large, almost complete extent.

Following lymphatic uptake, liposomes pass through a system of lymphatic vessels and will encounter one or more lymph nodes where a fraction will be retained. Liposomes may accumulate in regional lymph nodes as a result of simple mechanical filtration in the intranodal meshwork of the lymph node or by phagocytosis by lymph node macrophages [19]. When expressed as %ID g^{-1}, lymph node localization was about the same for all liposome sizes evaluated (Fig. 3B). However, this result should not lead to the conclusion that liposome size does not affect lymph node localization. When expressed as the percentage of the lymphatically absorbed fraction (relative lymph node localization), lymph node localization is much higher for larger liposomes than for smaller liposomes (Fig. 3C). Evidently, larger liposomes are retained more efficiently by lymph nodes than smaller liposomes, most probably because larger liposomes are likely to be filtered out more efficiently in lymph nodes than smaller liposomes. Also, in view of the outcome of studies on the interaction between liposomes and macrophages, larger particles may be phagocytosed more efficiently by macrophages than smaller particles [20]. Both mechanisms may have contributed to the enhanced lymph node localization of large liposomes as compared to small liposomes. The observation of relative high lymph node localization of large particles as compared to small liposomes is in line with results obtained after ip injection of liposomes as reported by Hirano and Hunt [1].

The extent of lymphatic uptake and lymph node localization of particles after sc administration has been shown to be influenced by surface characteristics of injected particles and components of the interstitium and lymph [21]. Therefore, lipid composition should be considered as another factor influencing lymphatic uptake and lymph node localization of sc administered liposomes. The present results, however, do not point to lipid composition as a factor of importance for lymphatic uptake, with one exception: DPPC-liposomes were absorbed from the injection site to a much lesser extent than the other compositions investigated (Fig. 4A). The low lymphatic uptake of DPPC-liposomes is probably related to a strong tendency of these liposomes to aggregate. The observed tendency of DPPC-liposomes to grow in size during incubation with FCS (Table 2) supports the view that aggregation at the injection site may have limited lymphatic uptake of liposomes after sc administration.

Also lymph node localization was not strongly affected by the liposomal lipid composition. Only PS-containing liposomes and DPPC-liposomes behaved differently as compared to reference liposomes (Fig. 4B). It was observed that PS-containing liposomes localized to a much higher extent in regional lymph nodes than reference liposomes. It has been shown that PS-exposure serves as a signal for triggering recognition by macrophages [22]. Presumably, the substantially increased lymph node localization of PS-containing liposomes may be attributed to the same mechanism. Remarkably, also lymph node localization of DPPC-liposomes was increased as compared to reference liposomes. This observation is probably not related to preferential uptake by lymph node macrophages, as liposomes with rigid bilayers are known to be less susceptible to uptake by macrophages [20]. As hypothesized above, DPPC-liposomes may have aggregated at the site of injection as well as during the process of lymphatic absorption and transport to regional lymph nodes. Aggregation may have yielded a bigger fraction of larger particles in lymph which may explain the observed higher lymph node localization (Fig. 4B).

Once liposomes have been taken up by the lymphatic capillaries and passed through regional lymph nodes, they reach the general circulation where they behave as if administered by the intravenous (iv)

route. Administration of reference liposomes resulted in a peak blood level of about 35%ID at about 6 h after injection (Fig. 1C). When [^{3}H]-labeled reference liposomes labeled with an additional hydrophilic marker ([^{125}I]-tyraminylinulin) were injected sc, the hydrophilic label followed the same pattern of distribution as the lipid marker, suggesting that the liposomes reach the blood circulation intact and are capable of transporting hydrophilic drugs from the sc injection site into the general circulation (results not shown). Following administration of the large, non-sized liposomes, radioactivity in blood was not detectable (Fig. 3D), which is in line with the negligible lymphatic uptake of these liposomes (Fig. 3A). Also after administration of 0.40 μm liposomes with the same composition as reference liposomes, 0.07 μm DPPC- and PS-containing liposomes blood levels of radioactivity were negligible. However, considerable lymphatic uptake (17, 23 and 55–60%ID, respectively) of the latter liposome types was observed (Fig. 3AFig. 4A). Apparently, for these liposomes the rate of elimination from the blood is faster than the rate of entry via the lymphatics.

In summary, liposomal size is the most important factor influencing lymphatic uptake and lymph node localization of sc administered liposomes. Small liposomes were taken up to a high extent, whereas large, non-sized liposomes remained almost completely at the site of injection. The opposite holds true for the degree of lymph node localization of absorbed liposomes as large liposomes are more efficiently retained by lymph nodes than small liposomes. Liposomal lipid composition did not appear to influence lymphatic uptake significantly, except in the case of neutral liposomes composed of DPPC. Decreased lymphatic uptake of DPPC-liposomes is possibly the result of spontaneous aggregation at the injection site. Lymph node localization was substantially enhanced by inclusion of PS into the liposomal bilayers, suggesting an important role of macrophages in lymph node localization of sc administered liposomes. Furthermore, saturation of lymphatic uptake and lymph node localization did not occur over a large liposomal lipid dose range, illustrating the efficient performance of lymph nodes in capturing sc administered particles. The results presented in this paper establish the significance of sc administration of liposomes in achieving high liposome levels in regional lymph nodes, and are relevant for the design of liposomes with optimal lymphotropic characteristics for drug targeting, diagnostic and immunization purposes.

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