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Optimization of Technetium-99m-Labeled PEG Liposomes to Image Focal Infection: Effects of Particle Size and Circulation Time

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In previous studies we have shown that liposomes sterically stabilized with polyethylene glycol (PEG), preferentially localize in infectious and inflammatory foci. In this study, we further optimized the formulation of PEG liposomes for infection imaging in a rat model. **Methods:** The biodistribution and imaging characteristics of different liposomal formulations labeled with ^{99m}Tc were determined in rats with *S. aureus* infection of the left calf muscle. The influence of liposomal size (mean diameter varying from 90 nm to 220 nm) as well as circulation time (modulated by inclusion of 0-10 mole% phosphatidylserine) were studied. **Results:** The smallest liposomes displayed improved characteristics for infection imaging: 90-nm liposomes revealed the highest abscess uptake (1.6% ± 0.4% ID/g, 24 hr postinjection) in combination with the lowest splenic accumulation (6.9% ± 0.7% ID/g, 24 hr postinjection) as compared to the larger sized preparations. Enhanced abscess-to-blood ratios (4.0 versus 1.3 at 24 hr postinjection) were obtained by including 1.0 mole% phosphatidylserine in the lipid bilayer of the PEG liposomes. However, enhanced blood clearance of these liposomes reduced their absolute abscess uptake. **Conclusion:** These results indicate that the in vivo behavior of PEG liposomes can be modulated to optimize their characteristics for infection imaging.

Key Words: PEGylated liposomes; sterically stabilized liposomes; *S. aureus* infection

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Liposomes are microscopic lipid vesicles consisting of one or more concentric lipid bilayers enclosing discrete aqueous spaces. Liposomes have been investigated extensively as carriers for drugs in attempts to achieve selective deposition and/or controlled release of the encapsulated contents (1-5). In addition, liposomes have been tested as vehicles to image infection and inflammation (6,7). However, conventional liposomes are rapidly taken up by cells of the mononuclear phagocyte system (MPS), which are primarily located in the liver and spleen (8,9). A decade ago, one of the major goals in liposome research was

to enhance their circulatory residence time to allow enhanced targeting to non-MPS tissues. It has been demonstrated that small, neutral, cholesterol-rich liposomes composed of rigid phospholipids of high-phase transition temperature show prolonged circulation times at relatively high lipid doses (10-12). More recently, it was demonstrated that inclusion of polyethyleneglycol (PEG), conjugated to phosphatidylethanolamine in the bilayer increased the blood circulation time as well (13,14). This increment was at least as large as that observed with the rigid lipid composition but without the requirements of specific lipid composition, particle size and lipid dose (15-17). The prolonged circulation time of PEG liposomes, also referred to as sterically stabilized or Stealth[®] liposomes (Sequus Pharmaceuticals Inc., Menlo Park, CA), is caused by reduced recognition by the MPS, as reflected by delayed and diminished hepatic and splenic accumulation. The development of long-circulating liposomal formulations has offered several new applications for liposomes such as: (a) long-term controlled release of drugs in the circulation; (b) improved antibody-guided delivery of liposomes; and (c) enhanced targeting to non-MPS-related pathological sites such as tumors and inflammatory foci (18,19).

Our previous studies in rats have shown that PEG liposomes labeled with either ¹¹¹In or ^{99m}Tc may be excellent radiopharmaceuticals for imaging infectious and inflammatory foci (1,2). The aim of this study was to tailor the PEG-liposomal formulation for scintigraphic application in rats with focal *S. aureus* infection. The PEG-liposomal formulation we used in our previous studies was originally developed for controlled delivery of chemotherapeutics (15,20,21). In this study, we modified the size and lipid composition of the liposomes to optimize their in vivo behavior for imaging infection. Different liposome dispersions with a narrow size distribution were produced (mean size: 90, 120, 160 and 220 nm) and evaluated in vivo. In addition, the effects of enhanced blood clearance were investigated by incorporating increasing amounts of phosphatidylserine (PS) (0, 1 and 10 mole%) in the lipid bilayer. It has been shown that PS exposure strongly increases the recognition of PEG liposomes by macrophages, thereby causing enhanced blood clearance (22,23).

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TABLE 1

Biodistribution of Technetium-99m-PEG Liposomes of Different Sizes in Rats with *S. aureus* Infection in the Left Calf Muscle 24 Hours Postinjection (%ID/g \pm s.d.)

	90 nm	120 nm	160 nm	220 nm
Blood	1.51 \pm 0.17	1.27 \pm 0.07	1.43 \pm 0.24	0.85 \pm 0.03
Muscle	0.05 \pm 0.02	0.04 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01
Bone marrow	0.86 \pm 0.12	0.95 \pm 0.21	1.27 \pm 0.38	0.59 \pm 0.08
Bone	0.14 \pm 0.02	0.16 \pm 0.04	0.19 \pm 0.04	0.10 \pm 0.02
Abscess	1.59 \pm 0.36	1.57 \pm 0.23	1.72 \pm 0.39	1.37 \pm 0.28
Lung	0.46 \pm 0.07	0.39 \pm 0.09	0.49 \pm 0.09	0.30 \pm 0.02
Spleen	6.86 \pm 0.68	8.98 \pm 0.63	24.6 \pm 2.7	39.7 \pm 5.2
Kidney	2.77 \pm 0.21	3.12 \pm 0.19	3.01 \pm 0.41	3.06 \pm 0.17
Liver	0.67 \pm 0.06	0.86 \pm 0.06	0.85 \pm 0.09	0.75 \pm 0.10
Intestine	0.54 \pm 0.13	0.73 \pm 0.18	0.75 \pm 0.21	0.39 \pm 0.02
Abscess-to-muscle ratio	34.8 \pm 8.4	38.7 \pm 12.2	57.0 \pm 13.8	48.5 \pm 8.4

These studies allow the selection of an optimal PEG-liposomal formulation for scintigraphic detection of infection.

MATERIALS AND METHODS

Preparation of Liposomes

Partially hydrogenated egg-phosphatidylcholine with an iodine value of 40 (PHEPC) was obtained from Asahi Chemical Industry Co. Ltd. (Ibarakiken, Japan) (17). The PEG 1900 derivative of distearoylphosphatidylethanolamine (PEG-DSPE) was a gift from Sequus Pharmaceuticals Inc. (Menlo Park, CA) and prepared as described previously (18). Cholesterol and phosphatidylserine were from Sigma Chemical Co. (St. Louis, MO). Glutathione was from E. Merck (Darmstadt, Germany).

A mixture of lipids containing PEG-DSPE/PHEPC/cholesterol in a molar ratio of 0.15:1.85:1 was prepared in chloroform/methanol (10/1, v/v). A lipid film was formed by rotary evaporation followed by high vacuum to remove residual organic solvent. The film was hydrated at room temperature in buffer (10 mM HEPES, 135 mM NaCl, 50 mM glutathione, pH 7.4) at a phospholipid concentration of 120 mM. Untrapped glutathione was removed by gel filtration on an Econo Pac 10DG column (Bio-Rad, Richmond, CA) eluted with 5% glucose.

Liposome preparations were sized by multiple extrusion of the suspension through polycarbonate screen membranes. With the present lipid composition, 90 nm was the smallest liposomal diameter that could be prepared. Mean particle size was determined by dynamic light scattering with a Malvern 4700 system using a 25 mW He-Ne laser and the automeasure 3.2 software (Malvern, Malvern, UK). For viscosity and the refractive index, the values of pure water were used. As a measure of the particle size distribution of the dispersion, the system reports a polydispersity index. This index ranges from 0.0 for an entirely monodisperse dispersion up to 1.0 for a completely polydisperse dispersion. The liposome preparations tested had a mean diameter of 90, 120, 160 and 220 nm with a polydispersity index of 0.2.

PEG liposomes with increasing PS content (0, 1 and 10 mole%) were produced by substituting the appropriate amount of PHEPC in the initial organic lipid mixture with PS. The suspensions were processed in an extruder as described above. The PS-containing liposome preparations had a mean size of 120 nm with a polydispersity index of 0.2.

Labeling Procedures

Preformed glutathione-containing liposomes were labeled with ^{99m}Tc essentially as described previously (24). Technetium-99m was transported through the bilayer by *d,l*-hexamethylpropylene amine oxime (HMPAO) and trapped irreversibly in the internal aqueous phase by reduction of the lipophilic complex with glutathione (25). Briefly, the glutathione-containing liposomes (75

mmole phospholipid/ml) were incubated for 15 min at room temperature with ^{99m}Tc -HMPAO (10 MBq/mmmole phospholipid). Unencapsulated ^{99m}Tc -HMPAO was removed by gel filtration on a 10DG Econo Pac column. Typically, 70%–85% of the added ^{99m}Tc label was entrapped within the liposomes.

Animal Model

A calf muscle abscess was induced in young, male, randomly bred Wistar rats (weight 200–220 g). After ether anesthesia, approximately 4×10^8 colony forming units of *S. aureus* in 0.1 ml 50:50% suspension of autologous blood and normal saline was injected in the left calf muscle (26). Twenty-four hours after the inoculation, when swelling of the muscle was apparent, the ^{99m}Tc -labeled liposomes were injected through the tail vein.

Biodistribution Studies

Twenty-four hours after *S. aureus* inoculation, rats were injected with 4 MBq of ^{99m}Tc -liposomes through the tail vein. Groups of at least five rats per liposomal preparation were used. Twenty-four hours after the injection, the rats were killed with 30 mg intraperitoneally injected phenobarbital. Blood was obtained by cardiac puncture. Subsequently, tissues (infected left calf muscle, right calf muscle, liver, spleen, kidney, intestine, right femur and bone marrow from the right femur) were dissected, weighed and their activity was measured in a shielded well scintillation gamma counter. To correct for physical decay and to calculate radiopharmaceutical uptake in each organ as a fraction of the injected dose, aliquots of the injectate, containing 1% of the injected dose, were counted simultaneously.

Imaging Studies

Twenty-four hours after *S. aureus* inoculation, rats received 10 MBq ^{99m}Tc -liposomes through the tail vein (three rats per liposomal formulation). Rats were anaesthetized (halothane/nitrous oxide) and were placed prone on a single-head gamma camera equipped with a parallel-hole, low-energy collimator. Rats were imaged at 5 min and 1, 2, 4, 8, 12 and 24 hr after injection. A symmetric 20% window was used for the 140-keV energy peak. Images (300,000 counts per image) were obtained and stored in a 256×256 matrix.

The scintigraphic results were analyzed by drawing ROIs over the abscess, the heart to represent blood-pool activity, the normal contralateral calf muscle for use as a background region and the whole animal. Abscess-to-background ratios and percentage residual activity in the abscess (abscess-to-whole body ratio) were calculated.

Statistical Analysis

All mean values are given \pm s.d. The tissue uptake levels obtained with the various liposomal preparations were compared by one-way analysis of variance (ANOVA). Multiple comparisons

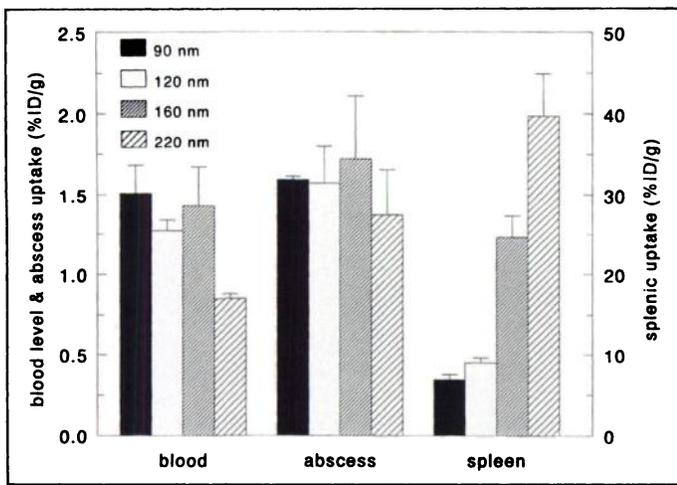


FIGURE 1. Effect of liposome diameter on blood level, abscess uptake and spleen uptake of ^{99m}Tc -labeled PEG liposomes in rats with *S. aureus* infection of the left calf muscle 24 hr postinjection. Five rats per group were used. Error bars represent s.d.

were made between the experimental groups. To correct for these multiple comparisons, p values were corrected by the Bonferroni method, and only Bonferroni-corrected p values are given.

RESULTS

Effect of Particle Size

The biodistributions of different sized ^{99m}Tc -labeled PEG liposomes in rats with *S. aureus* infections are shown in Table 1. The 24-hr postinjection blood levels of the smaller (90, 120 and 160 nm) liposomal preparations were significantly higher than those of the larger (220 nm) PEG liposomes ($p < 0.01$) (Fig. 1), indicating that the diameter of the PEG liposomes is one of the factors influencing circulation time. Label uptake values in the abscess obtained with the four liposomal preparations amounted to approximately 1.5%ID/g and were not significantly different. Uptake in the contralateral calf muscle was low and not significantly different for each of the four liposomal preparations (0.03%–0.05%ID/g). Marked differences in splenic uptake were observed (Fig. 1). The larger the liposomes, the higher the splenic uptake. Splenic uptake of the 90-nm PEG liposomes was relatively low (6.86% \pm 0.68%ID/g, corresponding to a total splenic uptake of 4.0%ID) and significantly lower than the splenic uptake of the other liposomal preparations ($p < 0.05$). The 220-nm PEG liposomes displayed a sixfold higher splenic uptake as compared to the 90-nm PEG liposomes (39.7%ID/g, corresponding to a total splenic uptake of more than 22%ID). At 24 hr postinjection, total activity in the blood, abscess, kidneys, lungs and liver was 17.9, 3.1, 6.0, 0.7 and 7.4%ID per organ, respectively.

Abscess-to-muscle ratios obtained with the sized PEG liposomes were high: 24-hr postinjection values as high as 35, 39, 57 and 49 were obtained with the 90-, 120-, 160- and 220-nm PEG liposomes, respectively (Table 1). Abscess-to-blood ratios for each of these PEG-liposomal formulations exceeded 1.2, 24 hr postinjection. Thus, the biodistribution data presented in Table 1 indicate that the most optimal characteristics for abscess imaging were obtained with the 90-nm PEG liposomes: high abscess uptake combined with relatively low uptake in background tissues, especially the spleen.

The images obtained with the 90-nm PEG liposomes are shown in Figure 2. The abscess was clearly visualized at 1 hr postinjection. Quantitative analysis of the images revealed that the abscess-to-background ratio increased with time and exceeded 6 at 24 hr postinjection. Whole-body clearance of the radiolabel for each of these liposomal formulations amounted to 26%–39% after 24 hr

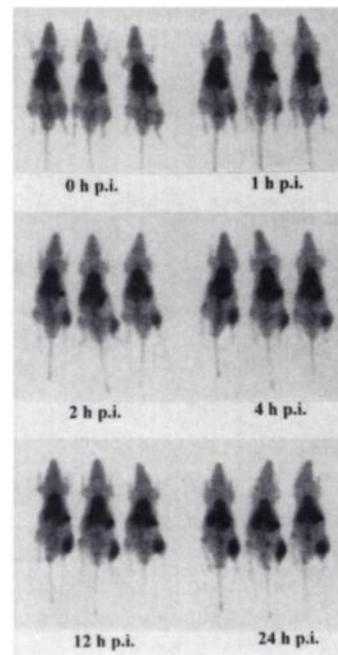


FIGURE 2. Scintigrams of rats with unilateral *S. aureus* infection in calf muscle imaged 0 (5 min), 1, 2, 4, 12 and 24 hr postinjection of ^{99m}Tc -labeled PEG liposomes with a mean diameter of 90 nm.

postinjection (data not shown). Figure 3 shows the effect of the diameter of the PEG liposomes on the blood residence time as reflected by the clearance of the radiolabel from the heart region. The 90-nm and 120-nm PEG liposomes were eliminated from the blood at a slower rate (initial $t_{1/2} \geq 12$ hr) than both other liposome formulations studied (initial $t_{1/2} < 8$ hr).

Effect of Circulation Time

PS was incorporated to modulate the circulation time of the PEG liposomes. Biodistribution data obtained at 24 hr postinjection of three PEG-liposomal preparations (mean size 120 nm) with increasing PS content (0, 1, 10 mole%) are shown in Table 2. Incorporation of 1.0 mole% PS in the lipid bilayer of the PEG liposomes already markedly decreased their blood residence time, as evidenced by the ninefold lower blood level 24 hr postinjection: abscess uptake was threefold lower. As a result, the abscess-to-blood ratio of the 1.0 mole% PS-containing liposomes was threefold higher (4.0 versus 1.3), despite their decreased absolute uptake

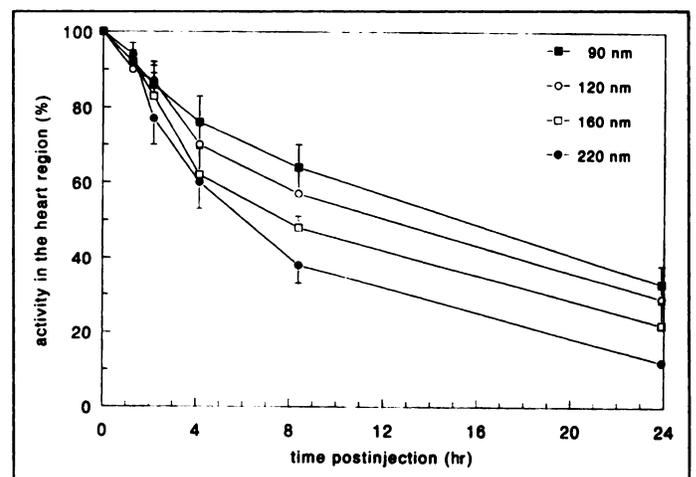


FIGURE 3. Quantitative analysis of the scintigraphic images of rats (three rats per group) injected with ^{99m}Tc -labeled PEG liposomes of different diameter. Blood-pool activity measured 5 min postinjection was set at 100%. Error bars represent s.d.

TABLE 2

Biodistribution of Technetium-99m-PEG Liposomes (120 nm) with Increasing PS Content in Rats with *S. aureus* Infection in Left Calf Muscle 24 Hours Postinjection (%ID/g \pm s.d.)

	0% PS	1 mole% PS	10 mole% PS
Blood	0.84 \pm 0.05	0.09 \pm 0.01	0.03 \pm 0.01
Muscle	0.04 \pm 0.01	0.01 \pm 0.002	0.01 \pm 0.01
Bone marrow	0.92 \pm 0.23	0.93 \pm 0.26	1.26 \pm 0.72
Bone	0.16 \pm 0.05	0.10 \pm 0.04	0.57 \pm 0.87
Abscess	1.10 \pm 0.14	0.34 \pm 0.21	0.16 \pm 0.05
Lung	0.28 \pm 0.03	0.08 \pm 0.01	0.04 \pm 0.01
Spleen	7.95 \pm 1.14	17.8 \pm 4.1	20.4 \pm 4.4
Kidney	3.36 \pm 0.32	4.33 \pm 0.49	4.17 \pm 0.57
Liver	0.81 \pm 0.12	1.04 \pm 0.22	1.31 \pm 0.21
Intestine	0.34 \pm 0.02	0.19 \pm 0.04	0.09 \pm 0.03

in the abscess. PS incorporation induced an enhanced deposition of PEG liposomes in tissues rich in mononuclear phagocytes, particularly enhanced uptake in the spleen was observed.

Quantification of the scintigraphic images clearly shows the drastic effect of PS incorporation in the lipid bilayer on the circulation kinetics of the PEG liposomes (Fig. 4). The $t_{1/2}$ PS-containing PEG liposomes were cleared much faster (initial $t_{1/2} \leq 3$ hr) than the non-PS-containing PEG liposomes. Whole-body clearance of the PS-containing liposomal preparations was slightly enhanced as compared to that of the non-PS-containing preparation (whole-body retention at 24 hr postinjection: 59% \pm 2% versus 50% \pm 3%, respectively).

The effect of PS incorporation on the abscess-to-background ratios is shown in Figure 5. The highest abscess-to-background ratios were obtained with the PEG liposomes without PS and with 1.0 mole% PS (6.7 \pm 0.4 and 6.6 \pm 1.7, respectively). These ratios were significantly higher than those obtained with the 10 mole% PS-containing PEG liposomes from 6 hr onwards ($p < 0.05$).

DISCUSSION

In previous preclinical studies, we have shown that PEG liposomes labeled with ^{111}In or $^{99\text{m}}\text{Tc}$ preferentially localize in infectious and inflammatory foci (1,2). The mechanism of abscess uptake of PEG liposomes remains to be elucidated. Studies have shown that uptake of PEG liposomes by monocytes is insignificant (14,15). Most likely, PEG liposomes

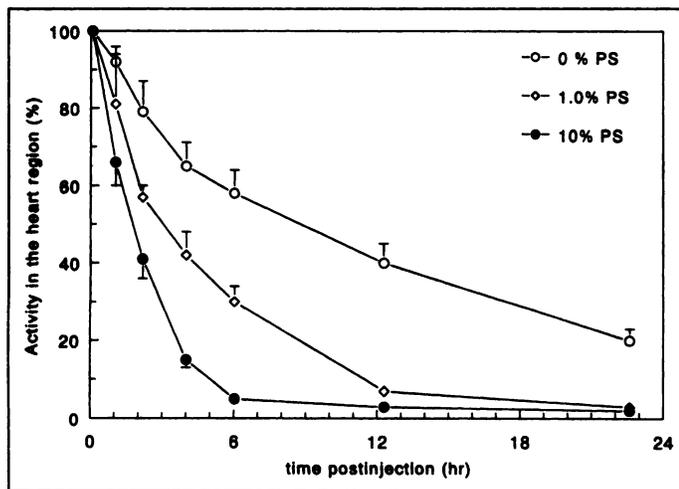


FIGURE 4. Quantitative analysis of the scintigraphic images of rats (three rats per group) injected with $^{99\text{m}}\text{Tc}$ -labeled PEG liposomes with different PS content. Blood-pool activity measured 5 min postinjection was set at 100%. Error bars represent s.d.

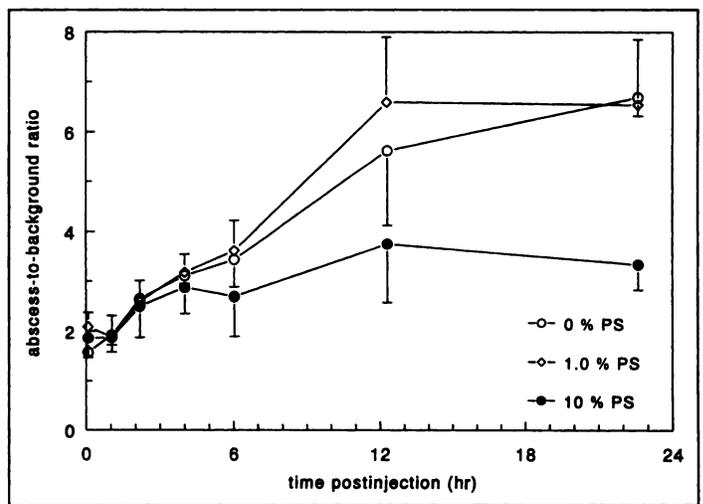


FIGURE 5. Quantitative analysis of the scintigraphic images of rats (three rats per group) injected with $^{99\text{m}}\text{Tc}$ -labeled PEG liposomes with different PS content. The region in the uninfected contralateral right calf muscle was taken as the background region. Error bars represent s.d.

extravasate in areas of inflammation as a result of locally enhanced capillary permeability.

In our previous experiments, we used a PEG liposome formulation developed for delivery of chemotherapeutics to tumors and infectious foci (10,27). In these liposomal drug targeting applications, one aims to maximize the localization of the drug at the pathological site. When using radiolabeled PEG liposomes to image infectious or inflammatory foci, one aims to achieve an optimal compromise between sufficient target uptake and maximal target-to-background ratios relatively early after injection. In this study, we investigated whether the PEG liposome formulation can be optimized for the latter purpose.

Carefully tuning the size of the PEG liposomes had a marked effect on their in vivo distribution after intravenous administration. Blood clearance of the larger PEG liposomes was faster than that of smaller liposomes (Fig. 3), which has also been observed for non-PEGylated liposomes (28). In addition, the splenic uptake of these liposomes was markedly higher than that of the smaller PEG liposomes (Table 1). PEG liposomes with a mean diameter of 90 nm combined two favorable characteristics for imaging: optimal uptake in the infectious focus and relatively low splenic uptake (Fig. 1). The abscess was visualized as early as 1 hr postinjection, and target-to-background ratios improved with time (Fig. 2). In the rat model used in this study, the abscess uptake of these 90-nm PEG liposomes was higher than the abscess uptake obtained with the PEG-liposomal preparation we used in our previous studies (1.6%ID/g versus 1.0%ID/g) (2). In addition, the splenic uptake of the 90-nm PEG liposomes was considerably lower (6.9%ID/g versus 12.5%ID/g). The liposomal formulation used in our previous studies was prepared using a microfluidizer, yielding a preparation with a similar mean size, but with a much wider size distribution (polydispersity index: 0.4 versus 0.2). Most likely, the earlier formulation contained a relatively higher proportion of larger liposomes with consequently reduced abscess uptake and increased splenic uptake. As compared to the ^{111}In -labeled PEG liposomes used in our previous study (1) the 90-nm $^{99\text{m}}\text{Tc}$ -labeled PEG liposomes described here displayed a somewhat reduced abscess uptake (1.9%ID/g versus 1.6%ID/g). However, abscess-to-muscle ratios obtained with the 90-nm PEG liposomes were higher (20 versus 35).

The biodistribution as well as imaging data indicate that the

larger the liposomes the higher their splenic uptake. The remarkably high and rapid splenic uptake of the 220-nm liposomes is most likely due to physical filtration rather than phagocytosis by spleen macrophages (19,29). Using fluorescence microscopy, it has been shown that larger PEG liposomes localize in the red pulp and marginal zone without being internalized by macrophages (30).

Imaging cannot only be improved by enhancing uptake in the target, but also by reducing background activity. We studied the effect of enhanced blood (and thus background) clearance by using PS inclusion as a tool to modulate circulation time. PS is asymmetrically distributed in mammalian cell membranes, being preferentially localized in the inner leaflet. Studies have shown that PS exposure in the outer leaflet of the cell membrane serves as a signal for triggering their recognition by macrophages (31). Presumably, PS-containing liposomes are cleared from the blood by the same mechanism. The reduced circulatory half-life of the PS-containing PEG liposomes (Fig. 4), however, caused a substantial reduction of the absolute abscess uptake as compared to PEG liposomes without PS. Still, abscess-to-blood ratios were at least three times higher for the PEG liposomes with 1 mole% PS incorporated in the lipid bilayers. The enhanced accumulation of the PS-containing liposomes in liver and spleen confirmed that their enhanced clearance from the blood was most likely mediated by cells of the mononuclear phagocytotic system. This enhanced splenic and hepatic uptake indicates that the radiolabel was retained in the cells rather than excreted after phagocytosis of the PS-containing PEG liposomes by MPS cells. Consequently, while abscess-to-blood ratios improved on incorporation of PS, abscess-to-background ratios for liver and spleen decreased. Enhanced blood clearance will lead to improved abscess-to-background ratios only when the radiolabel is excreted from the body after uptake of the liposomes by MPS cells. In principle, this might be achieved by using chelated radionuclides that can be excreted after being released from the liposomes.

The circulatory half-life of liposomes can also be enhanced without the use of PEG, by preparing small liposomes composed of bilayers with a rigid nature (14,15). We have chosen to use sterically stabilized liposomes, as such liposomes allow fine-tuning for a particular application, while the formulation of the small and rigid liposomes mostly cannot be modified without compromising their long circulating characteristic. An additional advantage of the use of PEG liposomes is that the circulation time of PEG liposomes is relatively independent of the lipid dose administered (18).

CONCLUSION

PEG liposomes can be fine-tuned to allow optimal imaging of focal infection. PEG liposomes with a mean size of 90 nm showed superior imaging characteristics as compared to larger-sized PEG liposomes. Enhanced blood clearance improved the preparation only partially, mainly because the radiolabel was not excreted from the body after being eliminated from the circulation.

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