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Intravenous administration of superoxide dismutase entrapped in long circulating liposomes
II. In vivo fate in a rat model of adjuvant arthritis

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Abstract

Rheumatoid arthritis (RA) is a prevalent and debilitating autoimmune disease that affects the joints. RA is characterized by an infiltration of the affected joint by blood-derived cells. In response to activation, these cells generate reactive oxygen species, resulting in an oxidative stress situation. One approach to counteract this oxidative stress situation is the use of antioxidants as therapeutic agents. The free radical scavenger enzyme superoxide dismutase (SOD) may be used as a therapeutic agent in rheumatoid arthritis, but its rapid elimination from the circulation is a major limitation. Targeted delivery of SOD may overcome this limitation. In this study, the utility of PEGylated liposomes (PEG-liposomes) for targeting SOD to arthritic sites was explored. The targeting of SOD to arthritic sites following intravenous administration of both PEG-liposomes and positively charged liposomes lacking PEG but containing stearylamine (SA-liposomes) in rats with adjuvant arthritis was studied. At 24 h post injection, the blood levels of long circulating liposomes with a mean size of 0.11 μm and 0.20 μm were 8- and 3-fold higher, respectively, as compared to the SA-liposomes. The majority of SOD administered in liposomal form remains within the liposomes when they circulate in the bloodstream. The highest target uptake was observed with PEG-liposomes with a mean size of 0.11 μm and the lowest uptake with the SA-liposomes. These results demonstrate that SOD can be targeted to inflamed sites most efficiently via small-sized PEG-liposomes. Small-sized PEG-coated liposomes are to be preferred if prolonged circulation and enhanced localization of SOD at arthritic sites are desired.

Keywords: Superoxide dismutase; Rheumatoid arthritis; Long circulating liposome; Pharmacokinetics; Biodistribution

1. Introduction

Rheumatoid arthritis, an autoimmune inflammatory disease of unknown origin, is the most common disease of connective tissues [1–3]. Since a cure for this disease with unknown etiology is not available [1,4], there is still an urgent need for a therapeutic agent capable of preventing disease progression or reversing joint destruction.

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Rheumatoid arthritis is characterized by an infiltration of the affected articulations by blood-derived cells, mainly neutrophils [5], macrophages and dendritic cells [6]. In response to activation, these cells are responsible for the generation of reactive oxygen species (ROS) [6,7], which are released in vast amounts into the surrounding tissue. If the endogenous antioxidant defense is overcome, the resulting oxidative stress can induce destruction of the affected joint constituents such as synovial fluid, cartilages and lipids [7]. One approach to decrease the oxidative stress is the use of antioxidants as therapeutic agents [8–11]. The most abundant ROS produced during the inflammatory process is the superoxide radical [7]. Given the important role of the superoxide radical there is a major interest in the free radical scavenger enzyme superoxide dismutase (SOD) as a therapeutic agent in rheumatoid arthritis. However, a major limitation of the therapeutic use of SOD is its rapid elimination from the circulation via the kidneys, with a plasma half-life of 6 min [12,13]. In order to improve its therapeutic activity attempts have been made to prolong the half-life of SOD. For example, it has been covalently conjugated to poly-(ethyleneglycol) (PEG) [14], dextran and albumin [15]. Another approach to increase blood half-life is the incorporation of SOD in liposomes [16,17]. Recently we reported pharmacokinetic studies indicating that liposomal encapsulation of the enzyme increased the plasma terminal half-life of the enzyme after i.v. administration 5–10-fold (depending on liposome type used) [18]. The SOD-liposome formulations tested induced a reduction of arthritis indices in a rat model of adjuvant arthritis. In particular, positively charged liposomes containing stearylamine (SA) appeared to be promising in this regard.

At present, a leading liposome-based strategy to target therapeutic agents to sites of pathology like tumors and sites of infection and inflammation is to utilize liposomes with prolonged circulation times [19–21]. Most popular in this regard is the inclusion of PEG linked to the phospholipid distearoylphosphatidylethanolamine. Such PEG-liposomes are referred to as long circulating liposomes (LCL), sterically stabilized liposomes or Stealth liposomes [22,23]. The addition of PEG to the surface of the liposomes gives the liposomes a hydrophilic 'sterically stabilized' surface, a property that contributes to a lower affinity of macrophages of the mononuclear phagocyte system (MPS) for the circulating liposome particles and consequently to a prolonged blood circulation [19–21]. In a variety of experimental models, these long-circulating PEG-liposomes have been shown to localize preferentially at sites of infection and inflammation [13,23–25], provided that they are relatively small in size (<150 nm). The crucial aspect behind this beneficial phenomenon is that the prolonged residence time in the blood enables the PEG-liposomes to extravasate to a relatively high degree at sites of enhanced vascular permeability [26–28].

The valuable property of PEG-liposomes to localize preferentially at sites characterized by an increased capillary permeability may be exploited to further improve the application of liposomes as carriers of SOD. In this study, we compared the utility of PEG-liposomes for targeting SOD to arthritic sites with that of the previously used non-PEG-liposomes containing SA in rats with adjuvant arthritis. The results demonstrate that PEG-liposomes are superior with respect to circulation time and extent of localization at arthritic sites.

2. Materials and methods

2.1. Chemicals

Egg phosphatidylcholine (E-PC) was obtained from Lipoid (Ludwigshafen, Germany). Distearoylphosphatidylethanolamine-poly(ethyleneglycol) 2000 (PEG-DSPE) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol), SA, diethylenetriaminepentaacetic acid (DTPA) and bovine erythrocyte Cu-Zn SOD were purchased from Sigma (St. Louis, MO, USA). ^{111}In-8-Hydroxyquinoline (^{111}In-oxine) was obtained from Mallinckrodt (Petten, The Netherlands). Na^{131}I was purchased from Medgenix (Fleurus, Belgium; spec. act. >5 mCi/mg). 1,3,4,6-Tetrachloro-3α,6α-diphenylglucouril was from Pierce (Rockford, IL, USA). All other chemicals were of reagent grade.

2.2. Animals

Male Wistar rats older than 2.5 months and

weighing 200–250 g were obtained from the Central Animal Facility of the Medical Faculty of the University of Nijmegen, The Netherlands. Animals were fed with standard laboratory food and water ad libitum.

2.3. Synthesis of ^{131}I-SOD

SOD was radiolabeled using the Iodogen method [29]. Briefly, 300 μg SOD (10 μg/μl) and 10 μl 0.5 M phosphate buffer, pH 7.4 were mixed with 5 mCi $Na^{131}I$ in a glass vial, precoated with 25 μg 1,3,4,6-tetrachloro-3α,6α-diphenylglucouril. The reaction mixture was incubated for 10 min at room temperature. Subsequently, the reaction mixture was eluted with PBS on a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden) to separate labeled SOD from free ^{131}I. The protein-containing fractions were pooled. The specific activity of the preparation was adjusted to 1 mCi/mg by adding non-labeled SOD to the ^{131}I-labeled product. The labeling efficiency was approx. 30%. The radiochemical purity of the ^{131}I-SOD was determined by instant thin-layer chromatography (ITLC) on Gelman ITLC-SG strips (Gelman Laboratories, Ann Arbor, MI, USA) with 0.15 M citrate buffer pH 5.0 as the mobile phase. More than 95% of the ^{131}I was associated with the SOD.

2.4. Liposome preparation

2.4.1. ^{111}In-DTPA-SOD liposomes

Multilamellar liposomes were prepared by the dehydration-rehydration method followed by extrusion as previously described [18]. Briefly, mixtures of the appropriate amounts of lipids in chloroform were dried under a nitrogen stream until a homogeneous film was formed. This film was dispersed (32 or 16 μmol lipid per ml hydration medium) in a solution of SOD in 6 mM DTPA (0.5, 2.0 or 5 mg/ml), frozen in liquid nitrogen and lyophilized overnight. Then, buffer (0.28 M mannitol/10 mM citrate buffer pH 5.6) was added to the lyophilized powder in a volume amounting to 1/10 of the volume of the original dispersion. This hydration step lasted 30 min, and, subsequently, 0.145 M NaCl/10 mM citrate buffer pH 5.6 was added to adjust the final volume to the starting volume. After 30 min, liposomes were extruded sequentially through polycarbonate filters ranging from 0.6 to 0.2 μm or 0.05 μm in pore size. Non-encapsulated protein was separated from the liposome dispersion by ultracentrifugation, at $300\,000 \times g$ for 120 min at 4°C in a Beckman LM-80 ultracentrifuge. Finally, liposomes were dispersed in 0.145 M NaCl/10 mM citrate buffer pH 5.6 in 1/5 of the initial volume. To remove the non-encapsulated DTPA, liposomes were passed through an Econo-PAC 10DG column (Bio-Rad).

^{111}In labeling: preformed SOD-liposomes containing DTPA were labeled with ^{111}In, which was transported through the bilayer in the form of ^{111}In-oxine and trapped irreversibly in the internal aqueous phase, due to the chelation by intraliposomal DTPA. Briefly, 0.3 mCi (1.4 ml) of ^{111}In-oxine in 0.5 M Tris-HCl, pH 7.0 was added to 2.0 ml of each liposomal preparation (20 μmol total lipid/ml) and incubated for 1 h. The non-encapsulated ^{111}In-oxine was removed by gel filtration on a Econo-PAC 10DG column. The encapsulation efficiency of the remote labeling procedure was always higher than 85%.

2.4.2. ^{131}I-SOD liposomes

Multilamellar liposomes were prepared by the dehydration-rehydration method followed by extrusion as described above, but with the following changes: (1) the lipid film was dispersed in a solution of ^{131}I-SOD (0.5–1 mg/ml, 1 mCi/mg), and (2) non-encapsulated protein was removed by gel filtration on a Sephadex G-200 column (18 cm × 1 cm) and eluted with 0.145 M NaCl/10 mM citrate buffer pH 5.6.

2.5. Liposome characterization

Mean liposome size was determined by dynamic light scattering with a Malvern 4700 system. As measure of particle size distribution the polydispersity index (PI) was used. PI can range from 0 (monodisperse) to 1.0 (polydisperse).

Phospholipid concentration was determined according to Fiske and Subbarow [30] as modified by King [31].

2.6. Animal experiments

2.6.1. Induction of the inflammation

Wistar rats were injected with a single intradermal injection of 0.10–0.15 ml of a suspension of *Mycobacterium butiricum* killed and dried (Difco) in incomplete Freund's adjuvant at 10 mg/ml, into the subplantar area of the right hind paw [18]. The parameter of interest of adjuvant-induced arthritis is the swelling of the paw which typically is established after day 7 post induction.

2.6.2. Imaging protocol

Seven days after the induction of the inflammation, the various liposomal preparations under investigation were injected i.v., three rats per group (divided randomly). Rats were anesthetized (halothane/nitrous oxide/oxygen) and placed prone on a single-head γ-camera equipped with a parallel-hole medium (^{111}In)- or high (^{131}I)-energy collimator (Orbiter, Siemens, Hoffmann Estates, IL, USA). The groups of rats were imaged synchronously at selected time points after injection. The scintigraphic results were analyzed by drawing regions of interest over the heart region and over the whole animal.

2.6.3. Biodistribution protocol

To study the biodistribution of the radiolabeled liposomes, groups of five rats each were injected i.v. 7 days after induction of inflammation with a single dose (10 μmol total lipid) of the different liposomal preparations. 24 h or 48 h post injection rats were killed by intraperitoneal injection of 30 mg phenobarbital. Blood was obtained by cardiac puncture. Following cervical dislocation, different organs and tissues (inflamed foot (foot+), non-inflamed contralateral normal foot (foot−), liver, spleen, blood, lung, kidney and muscle) were dissected, weighed and assayed for radioactivity in a shielded well-type γ-counter.

2.7. Statistical analysis

Results are presented as mean ± standard deviation (S.D.). Statistical analysis was performed using the ANOVA test.

3. Results

3.1. In vivo fate of the liposomal carrier: liposomes labeled with ^{111}In-DTPA

To study the in vivo behavior of SOD-liposomes in rats with a chronic arthritic inflammation, the SOD-liposomes were labeled by the coencapsulation of the complex ^{111}In-DTPA in the aqueous internal space of the liposomes. This complex is a high affinity complex at physiological pH [32] and has a very short half-life due to efficient renal excretion when present as free complex in the blood circulation (half-life in the order of minutes) [33]. These characteristics make the ^{111}In-DTPA complex an appropriate label to monitor the in vivo fate of liposomes after i.v. injection.

The in vivo fate of three different SOD-liposome types was studied in a rat adjuvant arthritis model. Major liposome characteristics are shown in Table 1. Two PEG-liposome formulations differing in size (mean sizes of 110 nm and 200 nm) were studied and compared with a SOD-liposome containing SA. The latter formulation containing SA (further referred to as SA-liposomes) was included in this study as it has been shown earlier [18] to have antiarthritic activity in this model. All three SOD-liposome dispersions had similar SOD loading char-

Table 1
Characteristics of ^{111}In-labeled SOD-liposomes studied in vivo

	Lipid composition	Molar ratio	EE (%)	Mean size (μm)	PI
110 nm PEG-liposomes	E-PC:Chol:DSPE-PEG	1.85:1:0.15	7	0.11 ± 0.06	< 0.13
200 nm PEG-liposomes	E-PC:Chol:DSPE-PEG	1.85:1:0.15	20	0.20 ± 0.02	< 0.24
SA-liposomes	E-PC:Chol:SA	7:2:1	30	0.21 ± 0.03	< 0.23

All formulations contained 12–15 μg of SOD per μmol of total lipid.
EE, initial protein/lipid ratio divided by final protein/lipid ratio.

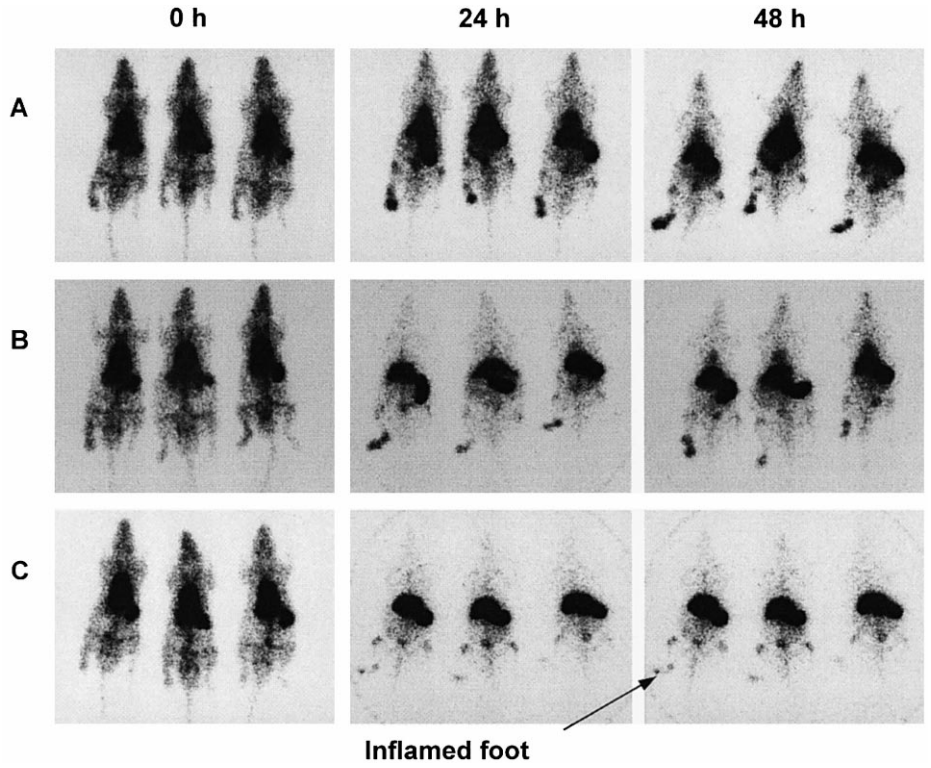

Fig. 1. Scintigrams of rats with adjuvant arthritis imaged at selected time points post injection for: (A) 110 nm PEG-liposomes, (B) 200 nm PEG-liposomes and (C) SA-liposomes, labeled with ^{111}In-DTPA.

acteristics as reflected by similar protein-to-lipid ratios.

3.1.1. Imaging studies

Whole body images obtained at different time points post injection of the three SOD liposomal formulations are shown in Fig. 1. The arthritic area was visualized with each of the three liposome types. The uptake in the inflamed foot was clearly higher for the 110 nm PEG-SOD-liposomes as compared to the other two liposome types (200 nm PEG-liposomes > SA-liposomes). In addition, uptake by the liver, spleen and kidneys was observed. The whole-body retention of radiolabel derived from quantitative analysis of the images is shown in Fig. 2. At 46 h post injection, label excretion amounted to approx. 35% in case of the SA-liposomes and approx. 20% in case of both PEG-liposome types.

Fig. 3 shows heart activity data derived from quantitative analysis of the images. The activity in the heart region represents the activity in the blood pool. The three SOD-liposome types under study exhibit bicompartmental behavior regarding blood

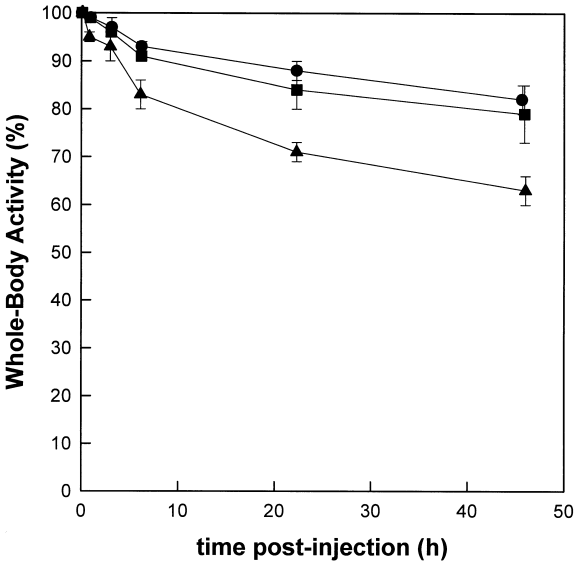

Fig. 2. Whole-body activity profiles derived from quantitative analysis of scintigraphic images of rats with adjuvant arthritis (three rats per group), injected with ^{111}In-DTPA-labeled liposomes: 110 nm PEG-liposomes (●), 200 nm PEG-liposomes (■), and SA-liposomes (▲). The whole-body activity at 5 min post injection was set at 100% ID. Values are mean ± S.D.

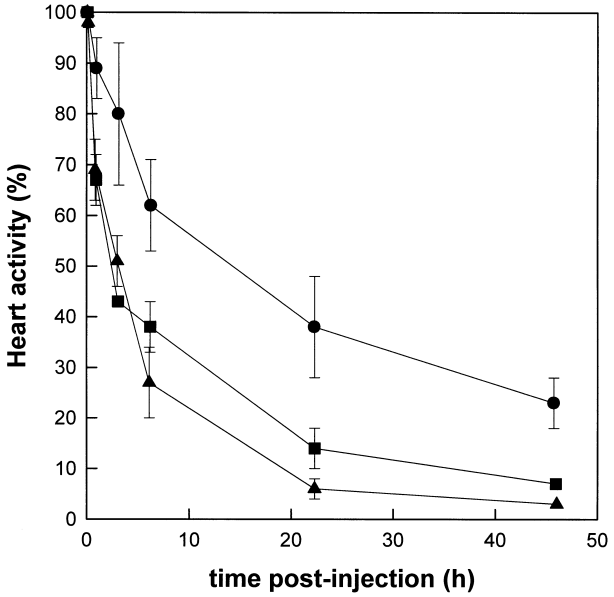

Fig. 3. Heart activity profiles derived from quantitative analysis of scintigraphic images of rats with adjuvant arthritis (three rats per group), injected with ^{111}In-DTPA-labeled liposomes: 110 nm PEG-liposomes (●), 200 nm PEG-liposomes (■) and SA-liposomes (▲). The heart activity at 5 min post injection was set at 100% ID. Values are mean ± S.D.

clearance. The smaller sized PEG-liposomes (110 nm PEG-liposomes) showed longer residence time in the blood compartment as compared to the other two formulations studied: at the end of the observation period (46 h post injection) 23 ± 5% of the injected dose (ID) was still present in the circulation, while values of 7 ± 1% and 3 ± 1% were observed for 200 nm PEG-liposomes and 200 nm SA-liposomes, respectively.

3.1.2. Biodistribution studies

The biodistribution data obtained at 24 h post injection are shown in Fig. 4. These data clearly indicate that the spleen is the main organ of accumulation. Splenic uptake in terms of the percentage of the injected dose per gram of tissue (ID/g) was highest for the 200 nm PEG-liposomes formulation: mean uptake amounted to 37 ± 10% ID/g, indicating that almost 20% of the injected dose accumulated in the spleen. At the 24 h observation time point, splenic uptake values for the 110 nm PEG-liposomes and SA-liposomes were 19 ± 3% ID/g and 21 ± 7% ID/g, respectively. The liver showed less pronounced accumulation; hepatic uptake was highest for the SA-liposomes: mean uptake amounted to 1.6 ± 0.6% ID/g. Hepatic uptake values for the 200 nm PEG-liposomes and 110 nm PEG-liposomes were 0.9 ± 0.2% ID/g and 0.6 ± 0.2% ID/g, respectively. The blood levels were highest for the 110 nm PEG-liposomes (1.3 ± 0.5% ID/g), followed by the 200 nm PEG-liposomes (0.6 ± 0.1% ID/g) and SA-liposomes (0.05 ± 0.05% ID/g). The three SOD-liposome types also showed significant differences in uptake by the inflamed foot (foot+, Fig. 4). The highest uptake was seen with the 110 nm PEG-liposomes (0.6 ± 0.2% ID/g), while uptake of the 200 nm PEG-liposomes and uptake of the SA-liposomes were significantly lower (0.3 ± 0.1% ID/g and

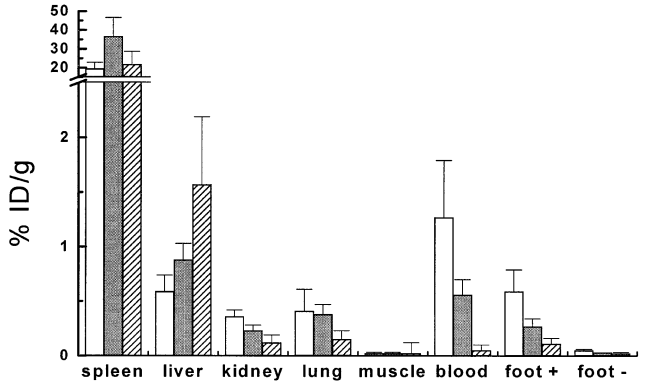

Fig. 4. Biodistribution of ^{111}In-DTPA-labeled liposomes: 110 nm PEG-liposomes (white), 200 nm PEG-liposomes (grey) and SA-liposomes (hatched), in rats with adjuvant arthritis, 24 h post injection (five rats per group). Values are mean ± S.D. % ID/g−% of the injected dose/g of tissue; foot+, inflamed foot; foot−, non-inflamed foot.

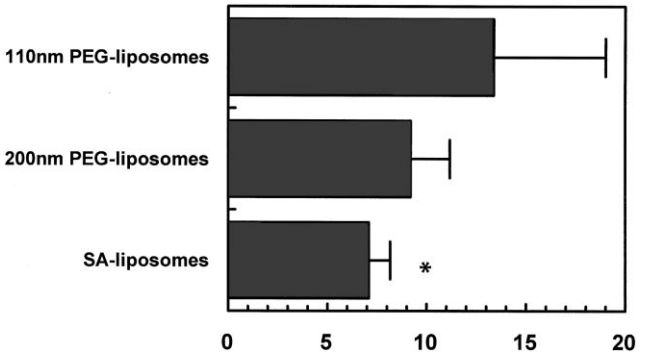

Fig. 5. Inflamed foot-to-non-inflamed foot ratio obtained with 110 nm PEG-liposomes, 200 nm PEG-liposomes, and SA-liposomes, 24 h post injection. Values represent mean ± S.D., five rats per group. *Significantly different from the other two ratios.

Table 2
Characteristics and injected doses of the ^{131}I-SOD-liposomes studied in vivo

	Lipid composition	Molar ratio	Mean size (μm)	EE (%)	SOD dose (μg)	Lipid dose (μmol)
200 nm PEG-liposomes	E-PC:Chol:DSPE-PEG	1.85:1:0.15	0.21 ± 0.01	20	133	11
SA-liposomes	E-PC:Chol:SA	7:2:1	0.20 ± 0.01	25	66	8
SOD	–	–	–	–	75	–

EE, initial protein/lipid ratio divided by final protein/lipid ratio.

0.1 ± 0.1% ID/g, respectively). The inflamed foot-to-non-inflamed foot ratios are shown in Fig. 5. The ratios obtained with the PEG-liposomes were significantly higher than the ratio obtained with the SA-liposome formulation ($P < 0.05$), with the highest ratio (13 ± 6) obtained in case of the 110 nm PEG-liposomes.

3.2. *In vivo fate of the encapsulated drug: liposomes labeled with ^{131}I-SOD*

To study the possible release of SOD from circulating liposomes, the enzyme was labeled with ^{131}I. Characteristics of the PEG-liposomes and SA-liposomes as well as the SOD, and lipid doses used in the in vivo experiments are presented in Table 2.

3.2.1. *Imaging studies*

Fig. 6 shows the whole-body activity results derived from quantitative analysis of the images. For the free enzyme (^{131}I-SOD), a rapid label excretion up to 25% in the first minutes post injection was observed. At 46 h post injection, the excreted amount of the injected label dose was about 95%. In case of injection of SOD-liposomes, label excretion during the first 10 h post injection was much slower. How-

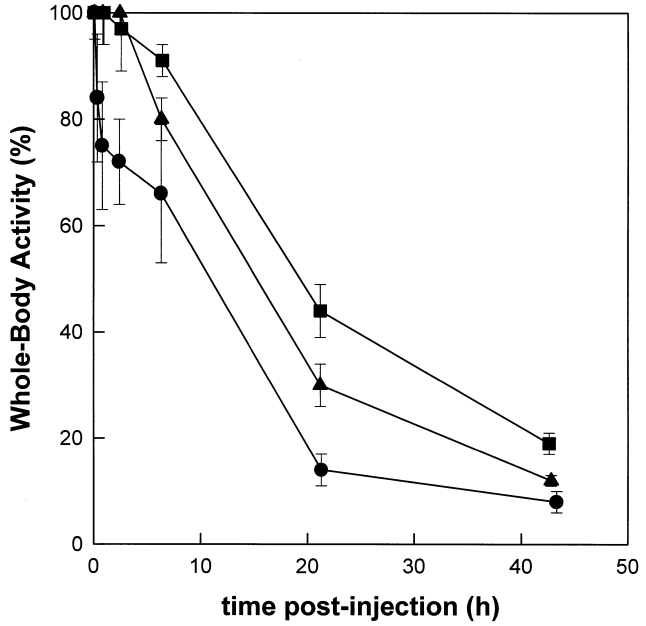

Fig. 6. Whole-body activity profiles derived from quantitative analysis of scintigraphic images of rats with adjuvant arthritis (three rats per group), injected with ^{131}I-labeled 200 nm PEG-liposomes (■), ^{131}I-labeled SA-liposomes (▲) and ^{131}I-SOD (●). The whole-body activity at 5 min post injection was set at 100% ID. Values represent mean ± S.D.

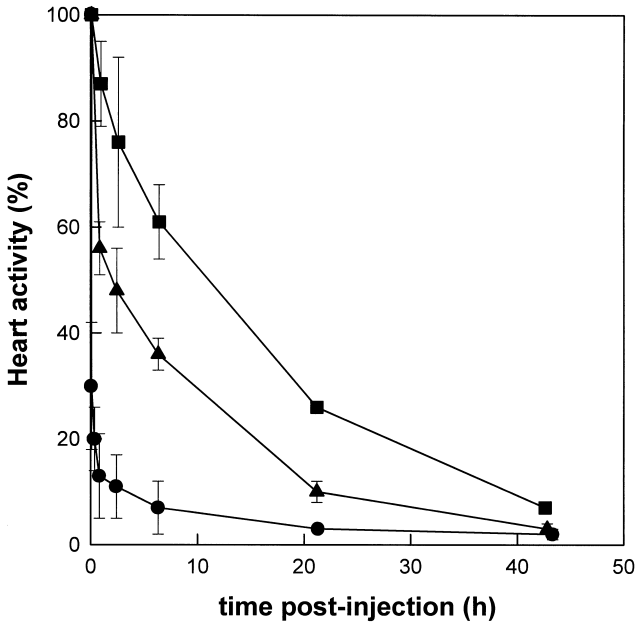

Fig. 7. Heart activity profiles derived from quantitative analysis of scintigraphic images of rats with adjuvant arthritis (three rats per group), injected with ^{131}I-labeled 200 nm PEG-liposomes (■), ^{131}I-labeled SA-liposomes (▲) and ^{131}I-SOD (●). The heart activity at 5 min post injection was set at 100% ID. Values represent mean ± S.D.

ever, at 46 h post injection, label excretion was extensive and amounted to about 80% and 90% for 200 nm PEG-liposomes and SA-liposomes, respectively. Comparison of the whole-body activity profiles obtained with the ^{131}I-SOD-liposomes (Fig. 6) and with the ^{111}In-labeled SOD-liposomes (Fig. 2) reveals a much higher rate and extent of label excretion in case of ^{131}I-SOD-liposomes. Importantly, the blood pool activity time course profiles shown in Fig. 3 (^{111}In-labeled SOD-liposomes) and Fig. 7 (^{131}I-SOD-liposomes) are similar which indicates that the majority of encapsulated SOD is not released from the circulating SOD-liposomes within the blood stream. Free ^{131}I-SOD was rapidly cleared; only 20% of the injected dose was in the blood compartment at 20 min after injection (Fig. 7).

3.2.2. Biodistribution studies

The 48 h biodistribution data are summarized in Fig. 8. Strikingly, the ^{131}I levels observed in all tissues studied were very low as compared with the ^{111}In levels shown in Fig. 4. For the free enzyme, the main organ of accumulation was the kidney with an uptake of 1.4 ± 0.7% ID/g. Label recovery from all the other organs studied was very low (in all tissues less than 0.05% ID/g). In case of the two ^{131}I-labeled SOD-liposome types, the highest uptake was observed in the spleen. The second organ of uptake was the kidney, likely reflecting label excretion. Label accumulation in all other tissues studied (including the inflamed foot) was low (< 0.2% ID/g).

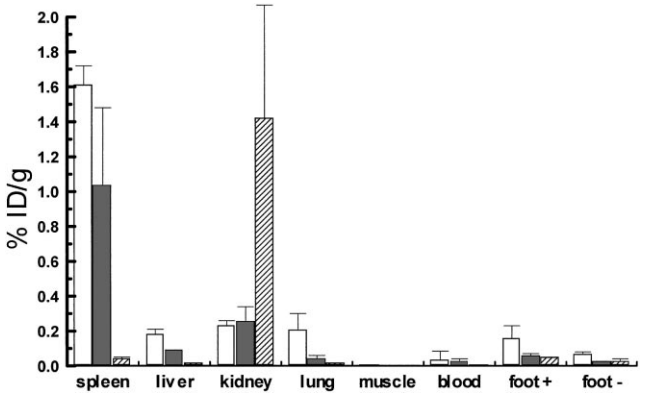

Fig. 8. Biodistribution of ^{131}I-labeled 110 nm PEG-liposomes (white), ^{131}I-labeled SA-liposomes (grey) and ^{131}I-SOD (hatched), in rats with adjuvant arthritis, 48 h post infection (five rats per group). Values are mean ± S.D. % ID/g—% of the injected dose/g of tissue; foot+, inflamed foot; foot−, non-inflamed foot.

4. Discussion

In any study dealing with the monitoring of the in vivo fate of liposomes, the selection of reliable radiolabels should be based on considerations relating to the objectives of the study, the physico-chemical nature of the liposomes, and the physico-chemical nature of the molecules to be incorporated [34]. The molecule of interest in this study, SOD, is a water soluble enzyme. Accordingly, we have chosen a water soluble complex (^{111}In-DTPA) which was co-encapsulated in the internal aqueous space of the liposomes. The In-111 radionuclide is an appropriate label as it emits γ-radiation of a suitable energy and its physical half-life of 67 h allows monitoring of the in vivo fate of liposomes during long periods of time after administration [32]. When ^{111}In-DTPA is released from liposomes circulating in the blood, it is rapidly cleared from the circulation and leaves the body through renal excretion [33]. When the ^{111}In-DTPA complex is released from the liposomes in tissues other than blood, the liberated label remains associated with the site, primarily due to translocation of radiolabel to iron-binding proteins [35–37]. Consequently, the ^{111}In label can only accumulate in tissues for long periods of time after being delivered via liposomes [38]. Therefore, we consider that ^{111}In-DTPA is an appropriate label to study the disposition of liposomes in vivo.

After i.v. administration of various ^{111}In-labeled SOD-liposome types, a small degree of leakage of the radiolabel in the blood circulation was observed (Fig. 2). For the formulations studied, the lipid composition seems to be more important than the liposome size in terms of whole body excretion (Fig. 2). Liposomes coated with PEG showed less leakage and therefore less label excretion than the SA-liposomes, suggesting a protective effect of the PEG coating against plasma protein-mediated leakage. At 24 h post injection, the blood levels of 110 nm and 200 nm PEG-liposomes were 8- and 3-fold higher, respectively, as compared with the SA-liposomes lacking PEG (Fig. 3). In addition to the PEG coating, size appears to be an important factor influenc-

ing the circulation time of PEG-liposomes as the larger 200 nm PEG-liposomes were cleared more rapidly than the 110 nm PEG-liposomes (Fig. 3). Thus, the presence of a PEG coating as well as a small size are favorable factors for achieving long circulation half-lives of SOD-liposomes, which is in line with other literature reports [20,21,24,25,27].

The biodistribution data show that the spleen is the main organ of uptake for the three SOD-liposome types under investigation. The splenic uptake of the 200 nm PEG coated SOD-liposomes was about 2-fold higher than the uptake of the smaller sized 110 nm PEG-liposomes. The markedly high splenic uptake of the 200 nm PEG-liposomes has been shown to be likely due to physical filtration rather than phagocytosis by spleen macrophages [22,25,39]. Using fluorescent microscopy, it was demonstrated that larger PEG-liposomes localize in the red pulp and marginal zone without being internalized by macrophages [40].

The present study shows that PEG-liposomes, in particular small-sized ones, are to be preferred over SA-liposomes for the targeting of SOD to the arthritic lesions in rats with adjuvant arthritis (Figs. 4 and 5). Most probably, the longer residence time of the PEG-liposomes in the bloodstream explains why localization of the PEG-liposomes in the inflamed area is higher than the localization of the SA-liposomes: the persistently higher blood concentrations of PEG-liposomes facilitate the continued extravasation of the PEG-liposomes at the inflammatory focus.

The mechanisms of retention of liposomes in the inflammatory lesion remain to be elucidated. The increased blood volume in the inflamed foot can only partly explain the enhanced uptake of PEG-liposomes in inflamed foot, as the ^{111}In levels at the arthritic site increase as a function of time (Fig. 1) while the ^{111}In levels in the blood decrease in time.

Obviously, successful targeting of SOD to sites of arthritis can only be achieved when the liposomal SOD contents is not lost during transport in the bloodstream. From the present study, it can be concluded that the majority of SOD administered in liposomal form remains within the liposomes when they circulate in the bloodstream. This conclusion is based on the similar blood pool activity profiles obtained in case of the ^{111}In-labeled liposomes (Fig. 3) and the ^{131}I-SOD liposomes (Fig. 7). In contrast, however, it should be noted that ^{131}I levels in tissues (Fig. 8) were much lower than ^{111}In levels in tissues (Fig. 4). A possible explanation for the lower levels of the ^{131}I label in tissues is the dehalogenation of the protein after release from liposomes, possibly mediated by the presence of multiple deiodinases in many tissues [41]. The use of oxidants, such as iodogen for the direct radioiodination of proteins, results primarily in the formation of iodinated tyrosine residues. Proteins labeled by this approach have been observed to undergo extensive loss of label in vivo presumably due to recognition of these iodoresidues by the deiodinases [42]. This explanation for the much lower tissue accumulation of the ^{131}I label is supported by the higher degree of whole body excretion (Figs. 2 and 6) of the ^{131}I label as compared to the ^{111}In label.

In conclusion, this study shows the superiority of small-sized PEG-liposomes for the targeting of SOD to inflamed sites. The present data warrant our hope that PEG-liposomes may become effective vehicles for the treatment of rheumatoid arthritis.

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