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# Superoxide dismutase entrapped in long-circulating liposomes: formulation design and therapeutic activity in rat adjuvant arthritis

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**Abstract**

The aim of this study was to investigate whether long-circulating liposomes can improve the anti-inflammatory activity of superoxide dismutase (SOD). Small-sized poly(ethyleneglycol) (PEG)-liposomes containing SOD were prepared via different preparation protocols and characterized in terms of encapsulation efficiency (EE), size, enzymatic activity and protein structure, to establish conditions where high EE can be combined with preservation of enzyme activity and structure. It was observed that structural information from circular dichroism analyses does not correlate with data on enzyme activity. SOD-containing PEG-liposomes prepared by the dehydration–rehydration method appeared to represent the most attractive formulation for in vivo evaluation. The therapeutic potential of selected SOD-containing PEG-liposomes was established and compared with SOD entrapped in stearylamine (SA)-liposomes and 'free' SOD upon intravenous (i.v.) injection in an arthritic rat model. Both small PEG-liposomes and SA-liposomes showed a superior therapeutic activity compared to 'free' SOD, with PEG-liposomes inducing stronger anti-inflammatory effects than SA-liposomes. 

*Keywords:* Superoxide dismutase; Long-circulating liposome; Rheumatoid arthritis; Therapeutic activity; Rat adjuvant arthritis

## 1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that leads to damage of the articular cartilage and subchondral bone [1]. Although the number of drugs used in the treatment of RA has increased over the past 10–20 years, there is still an urgent need for more effective drugs with reduced side effects [1–3]. At present, there is a revival of interest in the use of the antioxidant enzyme superoxide dismutase (SOD) for clinical applications, in particular for the therapy of RA [4].

SOD has been proposed as a promising NSAID, as this enzyme can protect against the damaging effects of reactive oxygen species involved in this inflammatory joint disease [3,5]. SOD is a cytoplasmic enzyme that dismutases the superoxide radical in molecular oxygen and hydrogen peroxide, thus helping to protect cells against the toxic byproducts of aerobic metabolism. SOD has been studied for use in the treatment of several diseases in which the superoxide radical is involved [3]. Because of its poor pharmacokinetic profile [6], controlled delivery strategies are desired. Among those, intravenously administered SOD-containing liposomes were reported to be therapeutically superior to the 'free' enzyme [7–9]. A maximum therapeutic benefit of SOD is expected, if one would be able to target SOD to its target sites, that is, inflamed tissues. A certain

*Abbreviations:* CD, circular dichroism; Chol, cholesterol; DSPE-PEG, distearoylphosphatidylethanolamine-poly(ethyleneglycol) 2000; EE, encapsulation efficiency; E-PC, egg-phosphatidylcholine; F/T, multilamellar liposomes obtained by the freeze–thawing method; IEP, isoelectric point; Lip, total lipid; MLV, multilamellar liposomes; PEG, poly(ethyleneglycol); PI, polydispersity index; Prot, protein; (Prot/Lip)i, initial protein-to-lipid ratio; (Prot/Lip)f, final protein-to-lipid ratio; RA, rheumatoid arthritis; Ret. Act., retained enzymatic activity; S.D., standard deviation; sDRV, multilamellar liposomes prepared by the dehydration–rehydration method; SOD, superoxide dismutase, [EC 1.15.1.1]

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degree of target site accumulation can be achieved by utilizing small (< 0.15 μm), long-circulating liposomes that are known to be able to escape from the circulation at inflammation sites [10–12]. In rats, small liposomes bearing poly(ethyleneglycol) (PEG) at their surface have circulation half lives of over 24 h and indeed localize preferentially in inflamed tissues [13,14]. Therefore, small-sized PEG-liposomes were chosen in this study as preferred liposome type for SOD targeting.

Up until now, hardly any efforts were made to optimize the pharmaceutical characteristics of SOD-containing PEG-liposomes. Therefore, we prepared small-sized SOD-containing PEG-liposomes via different methods. Specific attention was paid to issues of critical importance such as maximizing encapsulation efficiency (EE) and preservation of enzyme activity. Proper selection of the experimental conditions could indeed substantially increase the EE without loss of enzyme activity. These optimized SOD-containing PEG-liposome formulations were used for therapeutic activity studies in vivo. A rat model of adjuvant arthritis was used to investigate whether long-circulating liposomes offer advantages over 'conventional' liposomes regarding the anti-inflammatory activity of SOD. The results show that long-circulating PEG-liposomes are superior to conventional liposomes in enhancing SOD therapeutic activity in rats with adjuvant arthritis.

## 2. Materials and methods

### 2.1. Chemicals

Egg-phosphatidylcholine (E-PC) was obtained from Lipoid, Ludwigshafen, FRG. Distearoylphosphatidylethanolamine-poly(ethyleneglycol) 2000 (DSPE-PEG) was obtained from Avanti Polar Lipids, Alabaster, AL, USA. Cholesterol (Chol), and bovine erythrocytes Cu,Zn–SOD were purchased from Sigma, St. Louis, MO, USA. All other chemicals were of reagent grade.

### 2.2. Animals

Male Wistar rats of more than 3 months in age and weighing 450–500 g were obtained from Instituto Gulbenkian de Ciência, Oeiras, Portugal. Animals were fed with standard laboratory food and water ad libitum. All animal experiments were carried out with the permission of the local animal ethical committee, and in accordance with the Declaration of Helsinki.

### 2.3. Liposome preparation

Three different liposome preparation methods for the encapsulation of SOD in long-circulating liposomes were used. The aqueous phase consisted of an isotonic NaCl/citrate buffer pH 5.6, unless otherwise stated.

#### 2.3.1. Film hydration method, pH 5.6 [multilamellar liposomes (MLV)]

MLV were prepared as follows: a mixture of the appropriate amounts of E-PC:Chol:DSPE-PEG in a molar ratio of 1.85:1:0.15 in chloroform was dried under a nitrogen stream until a homogeneous film was formed. This film was dispersed in a solution of 0.50 mg/ml SOD in 0.145 M NaCl/10 mM citrate buffer pH 5.6 (lipid concentration = 16 μmol/ml).

#### 2.3.2. Freeze–thawing method, pH 5.6 [multilamellar liposomes obtained by the freeze-thawing method (F/T)]

F/T were prepared as follows: a mixture of the appropriate amounts of E-PC:Chol:DSPE-PEG in a molar ratio of 1.85:1:0.15 in chloroform was dried under a nitrogen stream until a homogeneous film was formed. This film was dispersed in a solution of 0.50 mg/ml SOD in 0.145 M NaCl/10 mM citrate buffer pH 5.6 (lipid concentration = 16 μmol/ml). Five cycles of freezing in liquid nitrogen (5 min) followed by thawing at 37 °C (5 min) concluded this protocol.

#### 2.3.3. Dehydration–rehydration method, pH 5.6 [multilamellar liposomes prepared by the dehydration–rehydration method (sDRV)]

Multilamellar liposomes were prepared by the dehydration–rehydration method as previously described [9]. Briefly, a mixture of the appropriate amounts of E-PC:Chol:DSPE-PEG in a molar ratio of 1.85:1:0.15 in chloroform was dried under a nitrogen stream until a homogeneous film was formed. This film was dispersed in a solution of SOD (0.05–20 mg/ml) in water (lipid concentration: 16, 32 or 48 μmol/ml), frozen in liquid nitrogen and lyophilized overnight. Then, a 0.28 M mannitol/10 mM citrate buffer pH 5.6 was added to the lyophilized powder up 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 reach the starting volume. Except for the dispersions that were extruded (see below), nonencapsulated protein was separated from the liposome dispersion by dilution (26 times) and ultracentrifugation at 300,000 × *g* for 20 min at 4 °C in a Beckman LM-80 ultracentrifuge. Finally, liposomes were dispersed in a 0.145 M NaCl/10 mM citrate buffer pH 5.6.

#### 2.3.4. Extrusion method (Extrusion), pH 5.6

As a rule, liposomes obtained by the three previously described methods were extruded sequentially through polycarbonate filters with pore sizes of 0.6, 0.2, 0.10 or 0.05 μm. Nonencapsulated protein was separated from the liposome dispersion by dilution (26 times) and ultracentrifugation at 300,000 × *g* for 120 min at 4 °C in a Beckman LM-80 ultracentrifuge. Finally, liposomes were dispersed in a 0.145 M NaCl/10 mM citrate buffer pH 5.6.

*2.3.5. Dehydration–rehydration followed by extrusion; pH 4.0 or 3.3 (sDRV→Extrusion)*

Liposomes were prepared as described above with the following modifications: (1) for hydration of the lyophilized powder to form liposomes, a 0.28 M mannitol/4 mM acetate buffer with pH 4.0 or 3.3 was used. The volume of the hydration buffer was 1/10 of the original dispersion volume; (2) to reach initial volume of the dispersion, a buffer consisting of 0.145 M NaCl/4 mM acetate buffer pH 4.0 or 3.3 was utilized; (3) after the separation of the non-encapsulated protein by ultracentrifugation, liposomes were dispersed in a 0.145 M NaCl/10 mM citrate buffer pH 5.6, independently of the pH of the hydration buffer.

*2.4. Preparation of SOD-liposomes for in vivo studies*

For these studies, SOD-liposomes were prepared by the dehydration–rehydration method, pH 5.6, followed by sequential extrusion through polycarbonate filters ranging from 0.6 to 0.2 μm or 0.05 μm in pore size. The lipid concentration was 16 or 32 μmol lipid per milliliter hydration medium and the film was dispersed in a solution of SOD (0.5–5 mg/ml). Nonencapsulated protein was separated from the liposome dispersion by ultracentrifugation, at 300,000 × *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.

*2.5. Circular dichroism (CD) studies*

To monitor the secondary and tertiary structure of SOD in aqueous media, CD spectra were run with a Jasco J-600 CD spectropolarimeter (Japan Spectroscopic, Tokyo, Japan).

*2.5.1. Secondary structure*

Two milligrams per milliliter SOD solutions were prepared in 10 mM phosphate buffer at pH 5.6 or 3.3 and in 33 mM $H_3PO_4$ at pH 1.5. When necessary, the pH was raised to 5.6 with 1 M NaOH. The reading conditions were: wavelength range—260–190 nm, step resolution—0.1 nm, scan speed—50 nm/min, time constant—0.125 s, band width—1 nm.

*2.5.2. Tertiary structure*

Two milligrams per milliliter SOD solutions were prepared in 10 mM phosphate buffer at pH 5.6 and 3.3. When necessary, the pH was raised to 5.6 with 1 M NaOH. The reading conditions were: wavelength range—350–250 nm, step resolution—0.2 nm, scan speed—100 nm/min, time constant—0.064 s, band width—1 nm.

*2.6. Liposome characterization*

Mean liposome size was determined by dynamic light scattering with a Malvern 4700 system. As a measure of the 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 [15] as modified by King [16], or with the Test-Combination Phospholipid Kit (Boehringer Mannheim).

*2.7. Protein determination*

Protein was determined with a modified Lowry method [17] with prior disruption of liposomes with Triton X-100 and sodium dodecylsulphate [18]; "final protein" represents total protein associated with liposomes after the removal of the nonencapsulated protein.

*2.8. SOD activity assay*

The SOD enzymatic activity assay was based on the ability of the enzyme to inhibit autoxidation of epinephrine to adrenochrome at pH 10.2 [19,20]. In the case of determination of liposome-encapsulated enzyme, the enzyme was first released from the liposomes by the addition of 20% Triton X-100 (yielding a dispersion containing 10% Triton X-100). All activity measurements were performed after dilution of the enzyme to a final concentration of 6 μg/ml at pH 5.6, 4.0 or 3.3 (yielding a dispersion containing 0.3% Triton X-100). Control experiments showed that this procedure did not affect SOD activity.

*Encapsulation efficiency*

The EE was calculated as follows:

$$\mathrm{EE} = 100[(\mathrm{Prot/Lip})\mathrm{f}/(\mathrm{Prot/Lip})\mathrm{i}]\ (\%)$$

where (Prot/Lip)i—initial protein-to-lipid ratio; (Prot/Lip)f—protein-to-lipid ratio in the final liposomal dispersion; Prot—protein; Lip—lipid.

The retained enzymatic activity was defined as:

$$\text{Ret. Act.} = (\text{Final activity}/\text{Initial Activity})100\ (\%)$$

*2.9. Animal experiments*

*2.9.1. 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 [9]. The parameter of interest of adjuvant-induced arthritis is the swelling of the paw, which typically is established 7 days after the induction.

*2.9.2. Treatment schedules*

Treatments started at day 7 post-induction according to one of the following schemes: (a) *single dose–response studies*: intravenous (i.v.) tail-vein injection of a single dose

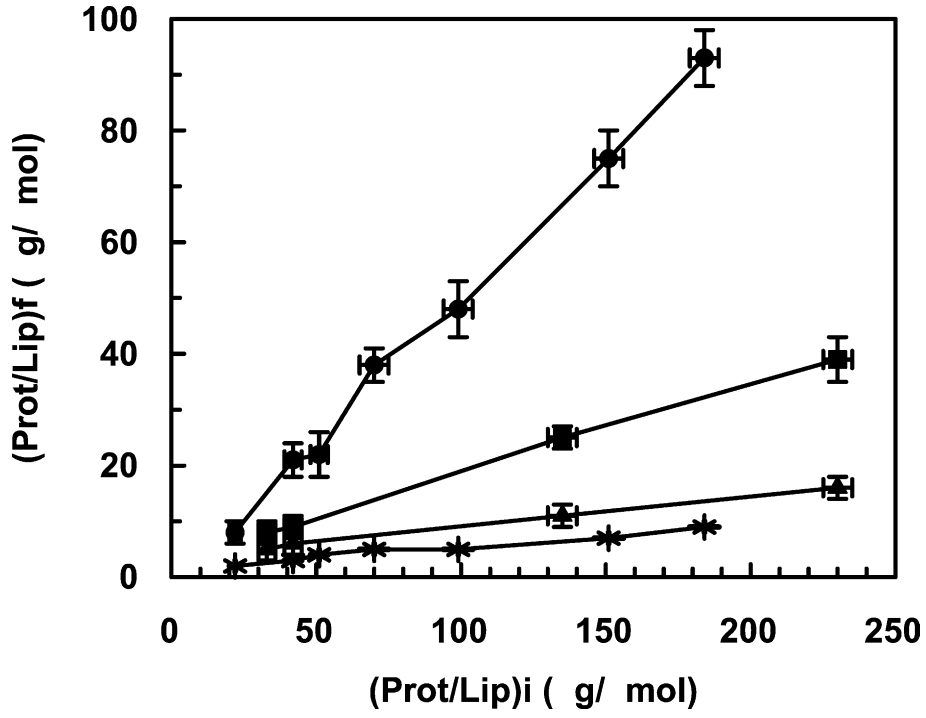


Fig. 1. Effect of (Prot/Lip)i on the (Prot/Lip)f during the extrusion process for the SOD PEG-liposomes. sDRV–non-extruded PEG-liposomes (●); sDRV–PEG-liposomes extruded through a final pore size of 200 nm (■), sDRV–PEG-liposomes extruded through a final pore size of 100 nm (▲), sDRV–PEG-liposomes extruded through a final pore size of 50 nm (*). [Lip]i = 32 μmol/ml. Error bars represent standard deviation; $n = 4$.

of 33, 198 or 363 μg SOD per rat on day 7 after the induction; (b) *multiple dose–response studies/injection frequency effect*: a dose of 33 μg SOD per rat was given as (1) one injection on day 7 (1 injection in total); (2) one injection on days 7, 11 and 15 (3 injections in total); (3) one injection on days 7, 9, 11, 13, 15 and 17 (6 injections in total); and (4) one injection on days 7–17 (11 injections in total); (c) *multiple dose–response studies/dose effect*: a daily i.v. tail-vein dose was given for 11 days (days 7–17), the daily dose was 66 μg SOD per rat in the case of free SOD and 16, 33 or 66 μg SOD per rat in the case of PEG-liposomes. Each treatment group contained at least five rats. The paw oedema was assessed by measurement of paw circumference [21]. SOD therapeutic activity was expressed as ''percentage oedema regression'' calculated using the formula:

$$\%\ \text{oedema regression} = (\text{Cat} - \text{Cbt})/(\text{Cbi} - \text{Cbt})100\ (\%)$$

where Cat = ankle *c*ircumference *a*fter the 11-day *t*reatment period (day 18); Cbt = ankle *c*ircumference *b*efore *t*reatment, 7 days after induction (day 7); Cbi = ankle *c*ircumference *b*efore the *i*nduction (day 0).

Table 1
Initial lipid concentration [Lip]i and pH effect on the encapsulation parameters of SOD in 110 nm PEG-liposomes

| | [Lip]i (μmol/ml) | [Prot/Lip]i (μg/μmol) | [Prot/Lip]f (μg/μmol) | EE (%) | Ret. Act. (%) |
|---|---|---|---|---|---|
| pH 5.6 | 16 | 48 ± 2 | 4 ± 1 | 8 ± 2 | >95 |
| | 32 | 24 ± 2 | 3 ± 1 | 13 ± 2 | >95 |
| | 48 | 15 ± 2 | 3 ± 1 | 21 ± 2 | >95 |
| pH 4.0 | 16 | 40 ± 2 | 5 ± 1 | 12 ± 1 | 70–75 |
| pH 3.3 | 16 | 46 ± 2 | 14 ± 2 | 30 ± 2 | 50–55 |
| | 32 | 24 ± 2 | 9 ± 2 | 36 ± 2 | 50–55 |

sDRV liposomes were prepared at the pH as indicated in the first column; after extrusion, the pH of the aqueous phase was adjusted to pH 5.6. [SOD] = 1.0 mg/ml. $n = 4$.

Table 2
pH effect on the SOD activity in the absence of liposomes and encapsulation parameters for extruded SOD–PEGylated liposomes exposed to two different pH conditions

| pH | Time (h) | (Prot/Lip)i (μg/μmol) | EE (%) | Ret. Act. (%) | Size (μm) |
|---|---|---|---|---|---|
| 5.6 | 4, 20 or 24 | – | – | 95–100 | – |
| 3.3 | 4, 20 or 24 | – | – | 25–30 | – |
| 3.3 → 5.6 | 20 (pH 3.3) + 4 (pH 5.6) | – | – | 90–95 | – |
| 1.5 | 4, 20 or 24 | – | – | <1 | – |
| 1.5 → 5.6 | 20 (pH 1.5) + 4 (pH 5.6) | – | – | 40–45 | – |
| 5.6 → 3.3 → 5.6 | – | 42 ± 2 | 15 ± 1 | 90–95 | 0.11–0.12 |
| 5.6 | – | 42 ± 2 | 7 ± 1 | 90–95 | 0.11–0.12 |

$n = 3$. [SOD] = 1.0 mg/ml.

### 2.10. Statistical analysis

All mean values are given ± standard deviation (S.D.). Statistical analysis was performed using ANOVA test.

## 3. Results

### 3.1. Effect of preparation method on EE

SOD-liposomes were prepared at pH 5.6, as at this pH, no loss of enzymatic activity and no structural changes of the protein as detected by CD were observed. Three different liposome preparation methods for the encapsulation of SOD in long-circulating liposomes were studied: (1) the method of film hydration followed by extrusion (MLV → Extrusion), (2) the method of freeze–thawing followed by extrusion (F/T → Extrusion) and (3) the method of dehydration–rehydration followed by extrusion (sDRV → Extrusion). For the sDRV → Extrusion, the EE observed was more than twice the EE observed for the MLV → Extrusion and the F/T → Extrusion (9 ± 1% vs. 3 ± 1% and 4 ± 1%, respectively). Similar results were obtained when calculating the final protein-to-lipid ratio [(Prot/Lip)f] (14 ± 3 vs. 6 ± 1 and 6 ± 1 μg/μmol, respectively). Thus, with the sDRV → Extrusion method, a larger fraction of added SOD can become

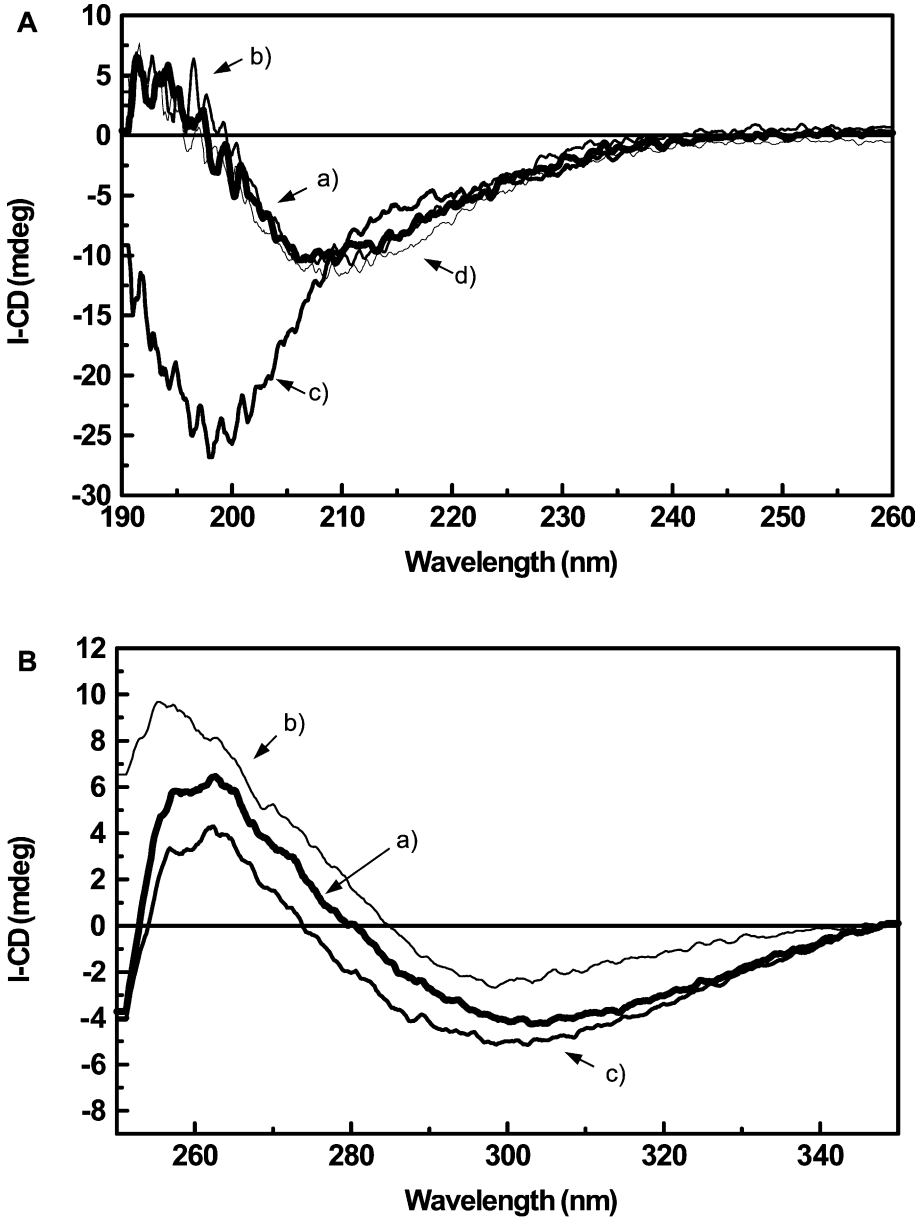


Fig. 2. CD spectra of Cu,Zn–SOD at different pH. (A) Secondary structure information: (a) [SOD] = 2.0 mg/ml, pH 5.6, (b) [SOD] = 2.0 mg/ml, pH 3.3, (c) [SOD] = 2.0 mg/ml, pH 1.5, (d) [SOD] = 2.0 mg/ml, pH 1.5 → 5.6 (solution c titrated from pH 1.5 to 5.6). (B) Tertiary structure information: (a) [SOD] = 2.0 mg/ml, pH 5.6, (b) [SOD] = 2.0 mg/ml, pH 3.3, (c) [SOD] = 1.5 mg/ml, pH 3.3 → 5.6 (solution b titrated from pH 3.3 to 5.6).

Table 3
Characteristics of the SOD-liposomes studied

| | Lipid composition | Molar ratio | Mean size[a] (μm) | PI[b] | Prot/Lip[c] (μg/μmol) |
|---|---|---|---|---|---|
| SA-liposomes | E-PC:Chol:SA | 7:2:1 | 0.21 ± 0.01 | <0.20 | 12–15 |
| PEG-liposomes | E-PC:Chol:DSPE-PEG | 1.85:1:0.15 | 0.11 ± 0.01 | <0.15 | 12–14 |

[a] Mean ± S.D. of 25 dispersions.
[b] PI—polydispersity index.
[c] Prot/Lip—protein-to-lipid ratio.

liposome "associated". For this reason, the sDRV → Extrusion method was the liposome preparation method of first choice for further studies.

### 3.2. Effect of extrusion on size and final protein-to-lipid ratio

As particle size affects the degree of localization of long-circulating liposomes in inflamed tissue [10,13,14,22], the effect of extrusion through membrane filters with decreasing pore size on the final protein-to-lipid ratio was studied (Fig. 1). Extrusion through a final pore size of 0.2, 0.1 or 0.05 μm resulted in liposomes with average sizes of 0.2, 0.14 or 0.11 μm, respectively. During the extrusion process, the content of SOD in long-circulating liposomes dropped dramatically. When the initial lipid concentration was increased, the final protein-to-lipid ratio showed a slight tendency to drop, but the EE increased almost linearly (Table 1).

### 3.3. Effect of pH on degree of SOD encapsulation, enzymatic activity and protein structure

To prevent loss of SOD during the extrusion process (Fig. 1), the effect of pH on SOD EE was studied. Decreasing the pH below the isoelectric point (IEP) of the protein (4.95), yielding a positively charged protein, was expected to enhance the EE by increased interactions between SOD and the liposome bilayers. By decreasing the pH from 5.6 to 3.0, the EE indeed increased from 8% to 30–35%, but, unfortunately, the enzymatic activity dropped from 90% to 50% of the original activity (Table 1).

To find conditions where high EE is obtained while enzyme activity and structure are preserved, a limited number of studies were performed on the effect of pH on the SOD activity in an aqueous milieu in the absence of liposomes (Table 2). A pH drop from 5.6 to 3.3 resulted in a loss of activity; enzyme activity could be fully recovered by increasing the pH. However, in case of a pH drop to 1.5, no full recovery of the SOD-activity was observed upon readjusting the pH at pH 5.6.

The intention of the CD analyses was to provide guidance in the selection process of the preferred SOD-liposome formulation conditions. CD spectra were obtained to monitor possible pH-dependent changes in the secondary and tertiary structure of SOD in the absence of liposomes. Structural changes might explain the observation of non-recoverable activity of the enzyme at low pH. Secondary structure data are shown in Fig. 2A. No differences were observed in the secondary structure of the enzyme at pH 3.3 and 5.6. But, at pH 5.6, the enzymatic activity was 95%, and

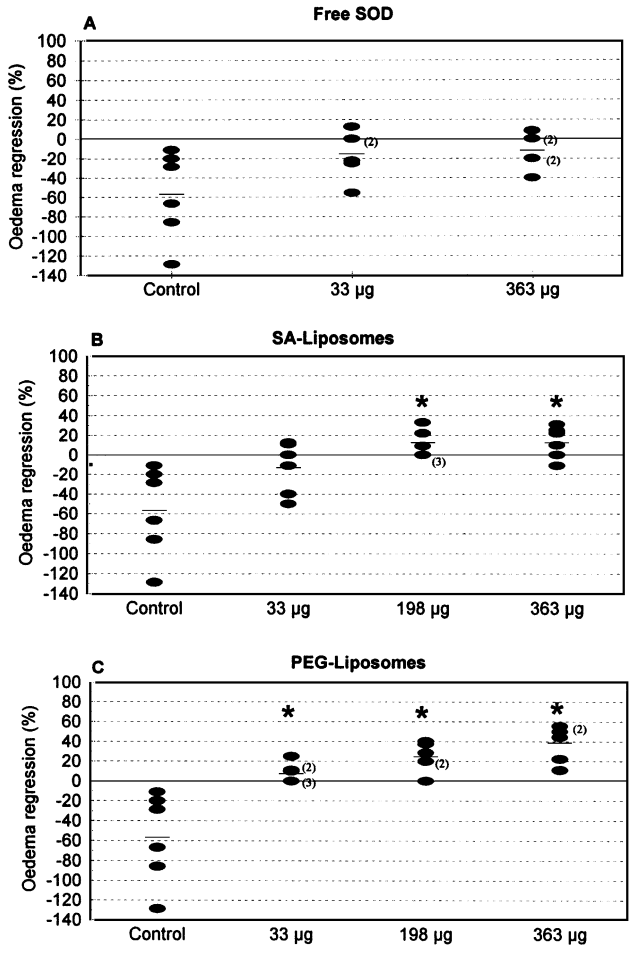


Fig. 3. Anti-arthritic activity of free and liposomal SOD in a rat adjuvant arthritis model: single dose–response relationship. Single dose tail vein i.v. treatment was given on day 7 after arthritis induction. Control animals did not receive any treatment. Treatment consisted of single i.v. doses (33, 198 and 363 μg SOD per rat) of free SOD (A), SOD in SA-liposomes (B) and SOD in PEG-liposomes (C). The number of animals per group was six; dots represent the change in swelling of the inflamed paw induced by the treatment (expressed as percentage oedema regression) assessed at day 18. [—: mean value; (*x*): number of animals with the same oedema regression]. Negative values point to swelling increase during the observation period starting on the first day of treatment (day 7) and ending at the day of assessment (day 18). Positive values indicate swelling decrease during the same observation period. * —Significantly different from the control group ($P<0.05$).

at pH 3.3, it was only 25–30% of the reference (initial) activity. At pH 1.5, a dramatic change was observed in CD spectrum and the enzymatic activity was less than 1%. However, when the same protein solution was titrated up to pH 5.6, a complete recovery to the original secondary structure was observed. Remarkably, SOD activity only came back to 40–45% of the original level.

At the tertiary structure level, some changes in CD spectra occurred at pH 3.3 as compared to SOD at pH 5.6 (Fig. 2B). These may be indicative for the loss of activity upon pH decrease (see Table 2). When the pH of the protein environment was readjusted at pH 5.6, a full recovery of the enzymatic activity was obtained. Nevertheless, a change in the CD spectrum compared to the original spectrum at pH 5.6 was observed.

Tables 1 and 2 provide conflicting results regarding recovery of SOD activity upon pH changes. In Table 1, there was no full recovery of SOD activity for SOD-liposomes prepared by hydrating the sandwich SOD–lipid structure at pH 3.3 and subsequently raising the pH to 5.6, while under exactly the same conditions, but in the absence of liposomes, the protein fully recovered its activity. This finding was disappointing, because the higher SOD EE at low pH was off set by the inactivation of the SOD due to a change in the SOD structure. One might hypothesize that this damage to the SOD structure might be the result of a close, pH-dependent interaction between SOD and the lipid bilayer during the hydration process. No structural damage or loss of enzymatic activity might occur when lipid hydration is performed at pH 5.6. To test this, sDRV liposomes were hydrated at pH 5.6. Then, the pH was lowered to pH 3.3 before the extrusion step and brought back to pH 5.6 after extrusion. SOD EE and SOD activity were compared with liposomes prepared at pH 5.6 where the pH was kept constant during extrusion. Table 2 shows the results. SOD EE was twice as high (15%) for the pH $5.6 \rightarrow 3.3 \rightarrow 5.6$ liposomes as for the pH 5.6-liposomes (7%) while the enzymatic activity level was still higher than 90%. The protein was kept at pH 3.3 for 4 h; this period of time is sufficiently long to reduce its activity (Table 2). Readjusting the pH to 5.6 fully restores the original intrinsic SOD activity. Thus, it is possible to prepare small-sized SOD PEG-liposomes (0.11–0.12 μm) with relatively high EE and full preservation of SOD enzymatic activity.

### 3.4. Therapeutic activity in vivo

Two different SOD-liposome formulations were selected for therapeutic activity studies. They are referred to as stearylamine (SA)-liposomes (conventional liposomes) and PEG-liposomes. The SA-liposomes were selected as they already have been shown to be therapeutically active in the rat model of adjuvant arthritis [9]. Lipid composition, size and SOD loading characteristics are shown in Table 3. Both liposome formulations show the same protein-to-lipid ratio, to enable comparative in vivo studies at the same liposomal lipid dose.

#### 3.4.1. Single dose–response studies

Fig. 3 shows the anti-inflammatory activity of free SOD and SOD encapsulated in both liposome types at escalating single SOD doses (i.e. 33, 198 and 363 μg of SOD per rat). I.v. administration of the free enzyme did not result in significant oedema regression, even at the highest dose

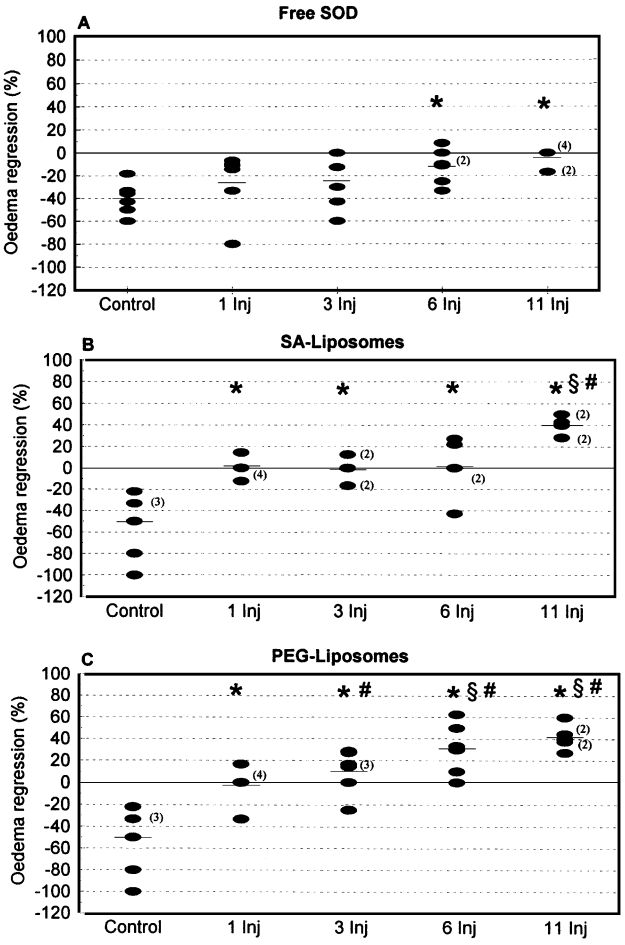


Fig. 4. Anti-arthritic activity of free and liposomal SOD in a rat adjuvant arthritis model: effect of injection frequency. Treatment involved different frequencies (four different schedules) of i.v. doses of 33 μg SOD (per injection) per rat of free SOD (A), SOD in SA-liposomes (B) or SOD in PEG-liposomes (C): (1) one injection on day 7 (1 injection in total) (1 Inj); (2) one injection on days 7, 11 and 15 (3 injections in total) (3 Inj), (3) one injection on days 7, 9, 11, 13, 15 and 17 (6 injections in total) (6 Inj), and (4) one injection on days 7–17 (11 injections in total) (11 Inj). Control animals did not receive any treatment. The number of animals per group was six; dots represent the change in swelling of the inflamed paw induced by the treatment (expressed as percentage oedema regression) assessed at day 18. [—: mean value; (*x*): number of animals with the same oedema regression]. Negative values point to swelling increase during the observation period starting on the first day of treatment (day 7) and ending at the day of assessment (day 18). Positive values indicate swelling decrease during the same observation period. *—Significantly different from the control group ($P<0.05$). §—Significantly different from the 1 Inj group ($P<0.05$). #—Significantly different from free SOD ($P<0.05$).

tested. In contrast, liposomally encapsulated SOD displayed significant anti-inflammatory activity. At the lowest dose level (i.e. 33 μg SOD per rat), only the PEG-liposomes showed significant activity ($P<0.05$). At the two higher dose levels (i.e. 198 and 363 μg SOD per rat), both SOD-liposome types showed significant activity ($P<0.05$). Increasing the SOD dose resulted in a significant improvement of the anti-inflammatory activity only when SOD was administered in the form of PEG-liposomes ($P<0.05$) (Fig. 3). The mean oedema regression obtained at the 363-μg dose was 39% for the PEG-liposomes and 13% for the SA-liposomes.

*3.4.2. Multiple dose–response studies*

To study the effect of injection frequency (Fig. 4), the anti-inflammatory activity of free SOD and SOD in both liposome types was evaluated at the dose level of 33 μg SOD (per injection) per rat in four different i.v. treatment schedules: (1) one injection on day 7 (1 injection in total); (2) one injection on days 7, 11 and 15 (3 injections in total); (3) one injection on days 7, 9, 11, 13, 15 and 17 (6 injections in total); and (4) one injection on days 7–17 (11 injections in total).

For free SOD treatment (Fig. 4A), a slight but significant activity was achieved only with the dose schedules involving 6 and 11 injections ($P<0.05$). All treatment schedules involving liposomal SOD (Fig. 4B and C) induced significant anti-inflammatory activities ($P<0.05$). In the case of SOD in SA-liposomes (Fig. 4B), the 11-injection schedule yielded a significantly improved therapeutic effect as compared to the 1-injection schedule ($P<0.05$). Remarkably, in the case of SOD in PEG-liposomes (Fig. 4C), the 6-injection schedule gave already a significant improvement as compared to the 1-injection schedule ($P<0.05$). When compared to free SOD treatment, SA-liposomes yielded superior anti-inflammatory effects only at the 11-injection schedule while PEG-liposomes were more effective already at the 3-injection schedule ($P<0.05$). Whatever the treatment schedule and SOD formulation, the swelling of the inflamed paw did not increase during a 15-day period after termination of treatment.

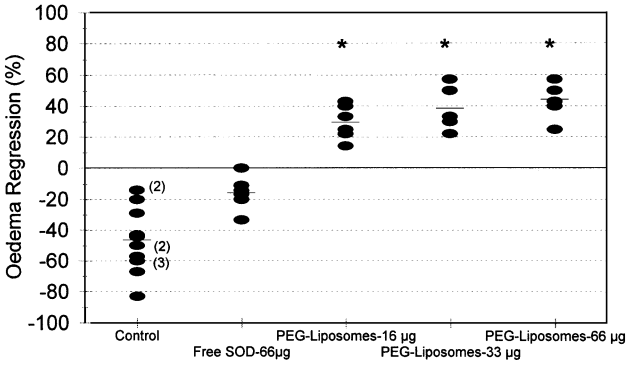


Fig. 5. Anti-arthritic activity of free SOD and SOD in PEG-liposomes in a rat adjuvant arthritis model: effect of dose. Treatment involved a daily i.v. dose given for 11 days (days 7–17). The daily dose was 66 μg SOD per rat for free SOD and 16, 33 or 66 μg SOD per rat for PEG-liposomes. Control animals did not receive any treatment. The number of animals per group was 6–14; points represent the change in swelling of the inflamed paw induced by the treatment (expressed as percentage oedema regression) assessed at day 18. [—: mean value; (*x*): number of animals with the same oedema regression]. *—Significantly different from the control and free SOD groups ($P<0.05$).

Fig. 5 presents the anti-inflammatory effects of different PEG-liposomal SOD doses (i.e. 16, 33 and 66 μg SOD per rat) at the 11-injection schedule. The results reveal that the therapeutic activity of SOD in PEG-liposomes lacks dose dependency over the dose range studied. These results are in line with previous results obtained for the SA-liposomes suggesting that further enhancement of the therapeutic activity cannot be accomplished in this model by increasing the liposomal SOD dose.

## 4. Discussion

Liposomes have attracted considerable attention for increasing the short half-life of SOD and to target the enzyme to the inflamed sites and thereby to increase its anti-inflammatory activity [7,23–25]. It is striking that studies reported up to now employed positively charged liposomes containing SA as carriers of SOD. However, the exact reason why SA was used is obscure. In fact, the use of positively charged SA-liposomes seems disadvantageous if targeting of SOD to the site of inflammation is the goal, as is the case in the present study. SA-liposomes can be expected to be rapidly opsonized after i.v. administration with rapid blood clearance and low uptake by the inflamed target as a consequence. In recent years, it has been shown that attaching PEG, conjugated to PE in the lipid bilayers, considerably delayed and decreased the recognition of liposomes by cells of the mononuclear phagocyte system (MPS), mainly those located in the liver and spleen. As a result, PEG-liposomes show an increased residence time in the blood [26–28]. Previous studies demonstrate that the prolonged residence time in the blood enables the PEG-liposomes to localize preferentially at sites of enhanced vascular permeability, for example, sites of arthritis [10,13,14,22]. Therefore, PEG-liposomes "loaded" with drugs can provide site-selective delivery of anti-rheumatic agents. Previous studies did not show antigenicity of bovine SOD at the doses used in rats [9]. No changes in biodistribution and pharmacokinetics were observed as compared to empty liposomes [13].

A variety of methods for the preparation of liposomes has been described in the literature. The three preparation approaches used in this paper to obtain SOD-liposomes have a common denominator: the small chance of protein damage because no organic solvents or detergent are in contact with the protein and no sonication is used. Methods involving organic solvents and/or sonication cannot be applied to entrap SOD since they can inactivate the enzyme

[29]. The extrusion-sDRV method yielded the best results in terms of EE. Indeed, this method has been described in the literature to yield high EE with hydrophilic proteins [9,30]. This can be explained by the two-step procedure involving (1) hydration of the protein–lipid sandwich lyophilized mass obtained after dehydration with a relatively small volume of buffer (without salts) followed by (2) adjustment to the final volume in the second step [31].

However, after liposome formation, extrusion of the hydrated sDRV is necessary to decrease liposome size, as only small-sized long-circulating liposomes are known to accumulate in inflamed sites. Unfortunately, extrusion leads to a large decrease in EE (Fig. 1), the reason being that the sDRV liposomes fracture during extrusion allowing SOD to escape from the liposomal internal aqueous space (high protein concentration) to the external phase (low protein concentration). Table 1 shows that the protein concentration gradient is still partly retained during extrusion as the EE for SOD still exceeds the EE for the encapsulated water phase [32–35]. A standard approach to prevent protein loss from the liposomes is to charge the protein with a charge opposite to the liposome bilayer. In this case, the DSPE-PEG provides the bilayer with a negative charge (on the phosphate group). As the presence of PEG at the surface of liposomes interferes with charge–charge interactions [36], it was expected that this approach would fail to establish sufficiently strong interactions between the protein and the lipid bilayer. However, at pH 3.3, SOD is positively charged (IEP = 4.95) and indeed appears to bind to the negatively charged PEG-bilayer surface. Additional evidence for this interaction was obtained by incubation of empty PEG-liposomes with SOD at pH 3.3. Substantial binding of SOD to the PEG bilayers was seen. The SOD–PEG bilayer interaction is pH dependent and decreases when the pH is increased from 3.3 to 5.6 resulting in desorption of the protein from the bilayer (data not shown). This pH effect on SOD binding to liposomes was utilized to reduce SOD loss during the extrusion process. Indeed, a higher EE of SOD was obtained in the case of extrusion at pH 3.3, but, unfortunately, a loss of enzymatic activity was also observed (Table 1). In the absence of liposomes, there is a loss of enzymatic activity at pH 3.3 and 1.5 (Table 2) compared to pH 5.6. After increasing the pH from pH 1.5 or 3.3 to pH 5.6, this loss of SOD activity was reversible when starting the pH increase at pH 3.3, whereas it turned out to be irreversible when starting from pH 1.5. CD spectra did not show permanent changes in the SOD secondary structure when going through the pH cycle: 5.6 to 3.3 and back to 5.6 (Fig. 2) Interestingly, as far as tertiary structure is concerned, some permanent changes were observed (Fig. 2). Nevertheless, the enzymatic activity was completely restored upon increasing the pH. This demonstrates that information obtained by CD-spectroscopy does not necessarily correlate with the enzyme activity data. An explanation for this discrepancy (CD vs. activity) may be that the tertiary structure changes are not immediately influencing the active center of SOD. It should be kept in mind that the tertiary structure changes may have an impact on other characteristics of the molecule such as pharmacokinetic profile or its immunogenicity (Table 2). Recovery of enzyme activity upon pH increase does not happen in the presence of liposomes prepared at pH 3.3 (Table 1). This irreversible inactivation is probably due to changes in SOD at the secondary level and/or loss of copper from the active center due to lipid–protein interactions during hydration of the SOD–lipid cake. This hypothesis is supported by the observation that no loss of SOD activity occurs in the presence of fully hydrated liposomes at pH 5.6 when the pH is decreased to 3.3 only during the extrusion step.

The process for the preparation of SOD-containing PEG-liposomes developed in this work yielded a relatively high EE without loss of enzymatic activity. Therefore, we moved forward toward therapeutic evaluation studies in the rat adjuvant arthritis model. Rat adjuvant-induced arthritis is a model of chronic polyarthritis with features that resemble RA [21,37]. Previously, we have reported on the pharmacokinetics and biodistribution of PEG-liposomes in the rat adjuvant arthritis model [14]. PEG-liposomes were clearly superior to SA-liposomes in terms of localization at the inflamed site, most likely as a result of their longer residence time in the bloodstream. The studies described herein were designed to investigate whether the stronger targeting capability of PEG-liposomes system can be translated into an enhanced anti-inflammatory effect as compared to SA-liposomes. The present results show that PEG-liposomes indeed are superior to SA-liposomes in terms of anti-inflammatory activity. In fact, at the single dose escalation studies, improved activity due to liposome encapsulation was observed only in the case of PEG-liposomes (Fig. 3). Also, in the multiple dose–response studies, involving the 3-injection and 6-injection schedules, PEG-liposomes induced stronger anti-inflammatory effects than SA-liposomes (Fig. 4). Apparently, the stronger targeting capability of PEG-liposomes as compared to SA-liposomes after i.v. administration results in an enhanced anti-inflammatory effect of the enzyme.

In conclusion, this report clearly shows how manipulation of the preparation conditions can lead to increased EE of active SOD. The process for the preparation of SOD containing PEG-liposomes developed in this work yielded a relatively high EE without loss of enzymatic activity. PEG-liposomes are superior to SA-liposomes regarding enhancement of the anti-inflammatory effect of SOD in rats with adjuvant arthritis. We conclude that PEG-liposomes represent an attractive SOD delivery system, which should be considered for clinical evaluation.

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