Pharm Res (2015) 32:91–102
DOI 10.1007/s11095-014-1447-7

RESEARCH PAPER

Superoxide Dismutase Enzymosomes: Carrier Capacity Optimization, *in Vivo* Behaviour and Therapeutic Activity

M. Luísa Corvo • H. Susana Marinho • Paulo Marcelino • Rui M. Lopes • Carlos A. Vale • Claúdia R. Marques • Luísa C.D. Martins • Peter Laverman • Gert Storm • M. Bárbara A.F. Martins

Received: 29 April 2014 / Accepted: 2 July 2014 / Published online: 19 July 2014

ABSTRACT

Purpose A strategy not usually used to improve carrier-mediated delivery of therapeutic enzymes is the attachment of the enzymes to the outer surface of liposomes. The aim of our work was to design a new type of enzymosomes with a sufficient surface-exposed enzyme load while preserving the structural integrity of the liposomal particles and activity of the enzyme.

Methods The therapeutic antioxidant enzyme superoxide dismutase (SOD) was covalently attached to the distal terminus of polyethylene glycol (PEG) polymer chains, located at the surface of lipid vesicles, to obtain SOD-enzymosomes.

Results The *in vivo* fate of the optimized SOD-enzymosomes showed that SOD attachment at the end of the activated PEG slightly reduced the residence time of the liposome particles in the bloodstream after IV administration. The biodistribution studies showed that SOD-enzymosomes had a similar organ distribution profile to liposomes with SOD encapsulated in their aqueous interior (SOD-liposomes). SOD-enzymosomes showed earlier therapeutic activity than both SOD-liposomes and free SOD in rat adjuvant arthritis. SOD-enzymosomes, unlike SOD-liposomes, have a therapeutic effect, decreasing liver damage in a rat liver ischemia/reperfusion model.

Conclusions SOD-enzymosomes were shown to be a new and successful therapeutic approach to oxidative stress-associated inflammatory situations/diseases.

KEY WORDS antioxidant therapy · liver ischemia/reperfusion · PEGylated liposomes · rat adjuvant arthritis · superoxide dismutase enzymosomes

ABBREVIATIONS

ID/g	Injected dose per gram of tissue
maleimide-PEG-PE	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000] (ammonium salt)
Conjugation/encapsulation efficiency (%)	[final (protein/lipid)/initial (protein/lipid)] × 100
PEG	Polyethylene glycol

M. L. Corvo (✉) • M. B. A. Martins
Intracellular Trafficking Modulation for Advanced Drug Delivery
iMED.ULisboa, Faculdade de Farmácia, Universidade de Lisboa
Campus do Lumiar Estrada do Paço do Lumiar, 22, 1649-038 Lisbon, Portugal
e-mail: lcorvo@ff.ul.pt

M. B. A. Martins
e-mail: barbaramartins@ff.ul.pt

H. S. Marinho
Departamento de Química e Bioquímica and Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal
e-mail: smarinho@fc.ul.pt

P. Marcelino
Chronic Disease Research Center, Faculdade de Ciências Médicas Universidade Nova de Lisboa, Campo dos Mártires da Pátria
Lisbon, Portugal
e-mail: p.marcelino@netcabo.pt

R. M. Lopes • C. A. Vale • C. R. Marques • L. C. Martins
iMED.ULisboa, Faculdade de Farmácia, Universidade de Lisboa, Campus do Lumiar Estrada do Paço do Lumiar, 22, Lisbon 1649-038, Portugal

R. M. Lopes
e-mail: rlopes@campus.ul.pt

P. Laverman
Department of Radiology and Nuclear Medicine Radboud University Medical Center, Nijmegen, The Netherlands
e-mail: Peter.Laverman@radboudumc.nl

G. Storm
Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS) Utrecht University, Utrecht, The Netherlands
e-mail: g.storm@uu.nl

SATA	N-succinimidyl S-acethylthioacetate
SOD-ATA	Thiolated Superoroxide dismutase
SOD-AT	Deacetylated SOD-ATA

INTRODUCTION

The clinical use of therapeutic enzymes is expected to attain in incoming years a global market of more than 5,000 million dollars [1]. Oxidative stress, i. e. "an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" [2] has been associated with inflammation, with ischemia/reperfusion injury and also with the development of several diseases [3]. Superoxide dismutases (SODs) were first shown to have anti-inflammatory activity more than 40 years ago [4–6], and protective effects have been observed in several oxidative stress-related diseases [7]. However, the therapeutic success of SODs has been hindered by rapid clearance from blood by glomerular filtration and, consequently, low accumulation in the pathological areas. Therefore, the therapeutic potential of systemically administered SOD can be better exploited if its plasma half-life is increased which could result in an increased enzymatic activity at the target site as well [8, 9].

Several strategies have been used to prolong the circulation time and improve the therapeutic action of SODs. Among them are SOD conjugation or chemical modification with polymers [10] or with acyl chains [11], SOD incorporation into delivery systems [9, 12], and the development of recombinant SOD [13]. In previous work, we showed that the administration of SOD associated to carrier systems minimizes tissue damage in oxidative stress-related disease models [14, 15]. Also, we developed and optimized the following SOD-containing liposomal formulations: I. For parenteral use: Long-circulating PEG-liposomes with a) SOD encapsulated in their aqueous interior SOD-liposomes, and b) Acyl-SOD incorporated in the lipidic inner and outer bilayers of liposomes (Acyl-SOD-enzymosomes) (Acyl-SOD was SOD chemically modified by covalent linkage of fatty acid chains to the accessible ε-amino groups of the enzyme, is a different enzyme entity as evidenced by its affinity to hydrophobic regions of the liposomal membranes and its different physicochemical properties such as octanol/water partition coefficient) [16]. Both liposomal SOD formulations demonstrated long circulation times in the blood in order to accumulate at inflamed target sites by enhanced permeability and retention effect [8, 17]. II. For topical use: c) SOD Transfersomes® (mixed lipid deformable vesicles especially designed for transdermal delivery) [12].

With this work we aimed at developing and optimizing distal surface-exposed SOD enzymosomes (SOD-enzymosomes) with long circulation times in the blood, in order to allow their accumulation at inflamed target sites and rapid display of enzymatic activity. Since for these SOD-enzymosomes release from the liposome particles is not needed and the distal location from the carrier surface obviates steric hindrance to the accessibility of substrate to the catalytic site of the enzyme, SOD is immediately available for therapeutic action once the particles enter the inflamed area [18]. In fact, the improvement of carrier-mediated delivery of therapeutic enzymes through attachment of the enzyme to the outer surface of liposomes, is an unusual strategy since only a few publications [11, 16, 19–22] report on the preparation of carriers with hydrophobized enzymes that become partially exposed at the surface of liposomes, while many publications report on the attachment of antibodies to the liposome surface for active targeting.

With this aim in mind we designed a new 'brand' of SOD-enzymosomes, by covalently attaching SOD to the distal terminus of polyethylene glycol (PEG) chemically bounded to head groups of liposome particles. The presence of PEG is expected to shield the exposed enzyme at the liposomal surface to the medium while not blocking the access of the enzyme to its substrate. After proper design and characterisation, *in vivo* fate and therapeutic activity studies were carried out, comparing the newly developed PEG-enzymosomes with surface-exposed SOD (SOD-enzymosomes) with the PEG-liposomes with encapsulated SOD in the internal aqueous space (SOD-liposomes). Two pathological models were used: a rat adjuvant arthritis model and a liver ischemia/reperfusion model.

MATERIALS AND METHODS

Materials

Chemicals

1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000] (ammonium salt), (maleimide-PEG-PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (PEG-PE) were purchased from Avanti Polar Lipids, Inc. Egg phosphatidylcholine (PC) was obtained from Lipoid GmbH. ^{111}In-8-hydroxyquinoline (^{111}In-oxine) was obtained from Mallinckrodt, Petten, The Netherlands. *Mycobacterium butyricum* (killed and dried) and the Incomplete Freund Adjuvant were purchased from Difco Laboratories (Detroit, MI, USA). Bovine Cu,Zn-superoxide dismutase (SOD) was from Sigma-Aldrich. N-succinimidyl S-acethylthioacetate (SATA) was from Thermo Scientific Pierce.

Animals

For all experiments male Wistar rats older than 2.5 months and weighing 200–300 g were used. For pharmacokinetic and biodistribution studies rats were obtained from the Central Animal Facility of the Medical Faculty of the University of

Nijmegen, The Netherlands. For the therapeutic activity experiments rats were obtained from Charles Rivers, Barcelona, Spain (rat adjuvant arthritis model) and from Instituto Bento da Rocha Cabral, Lisbon, Portugal (ischemia/reperfusion model). Animals were fed with standard laboratory food and water *ad libitum*. All animal experiments were carried with the permission of the local animal ethical committee, and in accordance with the Declaration of Helsinki, the EEC Directive (86/609/EEC) and the Portuguese laws D.R. n° 31/92, D.R. 153 I-A 67/92, and all following legislations.

Methods

Construction and Optimization of SOD-Enzymosomes

Preparation of Maleimide-PEG-PE Liposomes. Multilamellar maleimide-PEG-PE liposomes were prepared as follows: mixtures of the appropriate amounts of the lipids PC:Chol:maleimide-PEG-PE:PEG-PE (molar ratios ranging from 68.25:30.5:1.25:0 to 68.25:30.5:0:1.25) were dissolved in chloroform and dried under a nitrogen stream until a homogeneous film was formed, which was then dispersed, under gentle stirring, in 137 mM NaCl/10 mM citrate buffer with 1.0 mM EDTA, pH 6.0. These dispersions were extruded sequentially through polycarbonate membrane filters with pores of 0.6 μm, 0.4 μm, 0.2 μm and 0.1 μm using an extruder (model T.001 Lipex Biomembranes). The liposome dispersions were then diluted, with the same buffer, to a total lipid concentration of 10 mM.

Conjugation of SOD to Maleimide-PEG-PE Liposomes. The conjugation of SOD to liposomes was performed after the attachment of SH-anchor groups to the enzyme (thiolation). The thiolated enzyme was then covalently attached *via* thioether bonds to the lipid anchor maleimide-PEG-PE located at the distal functional end of polymer (PEG) chains bounded to the surface of the preformed liposomes. The procedure was based on the work of [23, 24] with some modifications in order to minimize the loss of catalytic activity of the enzyme and to maximize the conjugation efficiency.

Thiolation of SOD with SATA. In brief, the thioacetylation reagent, N-succinimidyl S-acethylthioacetate (SATA), was dissolved in dimethylformamide and added to a 1 mg/ml SOD buffer solution (HEPES, pH 7.5, 135 mM NaCl and 1.0 mM EDTA): 5 μl of SATA solution was added per mg of SOD. The degree of sulfhydryl incorporation was manipulated by using different molar ratios of SATA in relation to the protein. SATA:SOD molar ratios in the range 1:1, 4:1 and 8:1 were used. After 30 min incubation at room temperature under rotary shaking, the thioacetylated enzyme, SOD-ATA, was separated from unreacted SATA on an Econo-Pac 10DG column (Bio-Rad), eluted in the appropriate buffer. HEPES buffer was replaced by citrate buffer (13.7 mM NaCl/10 mM citrate buffer with 1.0 mM EDTA, pH 6.0) during elution. The elution of SOD-ATA and of other reaction components was monitored at 238 nm and 280 nm in a Shimadzu UV-1603 spectrophotometer.

The effect of the composition of the eluent on the retention of activity of the modified enzyme was studied. A standard SOD solution was allowed to react with SATA, the mixture divided in three aliquots and further separated by size exclusion, each one eluted with a different buffer: a) 13.7 mM NaCl/10 mM citrate buffer with 1.0 mM EDTA, pH 6.0, b) 10 mM HEPES buffer, pH 7.5, containing 135 mM NaCl and 1.0 mM EDTA, c) 13.7 mM NaCl/10 mM citrate buffer, pH 6.0. The enzymatic activity was then performed using the Bioxytech SOD-525 Kit (Oxis Research). The results showed a 30% decrease in modified SOD activity when the reaction mixture was eluted with a buffer solution containing EDTA. But when the same mixture was eluted with a buffer solution lacking EDTA in its composition the enzymatic activity was only reduced by 15%. As a consequence, the use of EDTA was restricted to the modification reaction. The thioacetylated enzyme was stored at −30°C until use.

Determination of the Degree of Thioacetylation. The degree of SOD thioacetylation was assayed with the method described by Böhlen *et al.* (1973) [25]. Briefly, the unblocked ε-NH_2 groups of either the thioacetylated or the native enzyme were bound to fluorescamine. The intensity of fluorescence emission of bound fluorescamine at 475 nm was measured for excitation at 390 nm in a spectrofluorimeter, model F-3000 (Hitachi). The degree of modification is [1 - (emission per microgram of modified enzyme/emission per microgram of native enzyme)×100]. Since fluorescamine can react with trace amines present in the reagents and solvents, a reagent blank was run routinely.

Conjugation of the Thiolated Enzyme to Liposomes. SOD-ATA was deacetylated in the presence of hydroxylamine and the thiolated enzyme, SOD-AT, was added to liposomes containing maleimide-PEG-PE. In brief, the deacethylation was carried out by adding 0.1 ml of hydroxylamine·HCl (0.5 M hydroxylamine, 0.5 M citrate, 25 mM EDTA, pH 6.0) for each mg of protein to be deacetylated. This protein solution was then diluted to 12.5 μM with buffer (13.7 mM NaCl/10 mM citrate buffer with 1.0 mM EDTA, pH 6.0). For the coupling reaction, 0.4 mg of SOD-AT was added to 1.0 ml of freshly prepared liposomes (10 mM lipid) at a maleimide:protein molar ratio ranging from 0:1 to 10:1 and allowed to react overnight at room temperature under gentle, but constant, rotary shaking. After this reaction, the SOD-enzymosomes were separated from the unconjugated enzyme by dilution and ultracentrifugation at 176 000×g for 1.5 h at 4°C on a L8-60 M ultracentrifuge (Beckman). The final pellet was dispersed in 2 ml of buffer (13.7 mM NaCl/10 mM citrate

buffer with 1.0 mM EDTA, pH 6.0) and stored at 4°C when required.

Characterization of the SOD-Enzymosomes and SOD-Liposomes

Mean particle size was measured by dynamic light scattering with a Zetasizer, model 1000 HSA (Malvern). The protein coupled to liposomes was determined according to Lowry at al [26] with prior disruption of liposomes with Triton X-100 and sodium dodecyl sulphate [9]. Phospholipids were quantified according to the method of Rouser and co-workers [27].

The catalytic activity of SOD was measured according to Misra and Fridovich [28], which is based on the ability of the enzyme to inhibit the autoxidation of epinephrine at pH 10.2.

In the case of determination of liposome-encapsulated enzyme, the enzyme was first released from the SOD-liposomes by the addition of 20% (v/v) Triton X-100 (yielding a dispersion containing 10% (v/v) Triton X-100). In the case of SOD-enzymosomes, when required, vesicles were also destroyed by the addition of 20% (v/v) Triton X-100. In all activity measurements (either the enzyme solution or the SOD-enzymosome or SOD-liposome) suspensions were diluted to a concentration of 6 μg/ml [yielding a dispersion containing 0.3% Triton X-100 (v/v)]. The retention of enzyme activity was defined as:

Ret. Act. = (Final activity/Initial activity) × 100 (%), taking the activity of native SOD as 100%.

This ratio normalizes the activity of the incorporated enzyme in relation to the activity of native enzyme. The decrease of activity of incorporated enzyme was a consequence of random alterations of the enzyme molecule, due to the covalent linkage of the enzyme to the surface of liposomes. These alterations were not dependent on the amount of enzyme linked consequently the retention of the enzymatic activity it was not dependent on the efficiency of enzyme incorporation.

Stability Studies

Two different stability tests were performed: storage stability and stability in the presence of albumin. SOD-enzymosomes stability evaluation was performed as a suspension at two different temperatures (4°C and 37°C). In the first study (storage stability), after the conjugation of the thiolated enzyme to liposomes, the pellets obtained after ultracentrifugation (at 176 000×g for 1.5 h at 4°C on a L8-60 M ultracentrifuge (Beckman)) were suspended in citrate buffer (citrate 10 mM/NaCl 145 mM, pH 6.0) and kept at 4°C during 32 days. In the second study (stability in the presence of albumin) the pellets obtained after ultracentrifugation were suspended in the same buffer with 2% (w/v) BSA and incubated at 37°C during 96 h. Comparison was made with the same conditions without albumin. At predetermined time points, the SOD-enzymosomes were separated from unconjugated enzyme by 1/26 ml dilution and ultracentrifugation at the same conditions as described above and the pellets were suspended in the same volume. In these studies, stability evaluation was performed in terms of conjugated SOD to total lipid ratio variation with time, liposome size and polydispersity index.

In Vivo Pharmacokinetics and Tissue Distribution

SOD-Enzymosomes for Pharmacokinetics and Biodistribution Studies. SOD-enzymosomes were prepared as described above with the encapsulation of DTPA in the internal aqueous phase. Briefly, the lipidic film of PC:Chol:Maleimide-PEG-PE:PEG-PE at the molar ratio 68.25:30.5:0.75:0.5 was dispersed (20 μmol lipid per ml hydration medium), under gentle stirring, in 140 mM NaCl/10 mM citrate buffer with 6.0 mM DTPA, pH 6.0 and extruded sequentially through polycarbonate membrane filters to a final pore size of 0.05 μm. The conjugation was performed as described previously with a ratio molar SATA:SOD of 4:1 and a SOD concentration of 1 mg/ml.

SOD-Liposomes for Pharmacokinetics and Biodistribution Studies. SOD-liposomes were prepared by the dehydration-rehydration method followed by extrusion [8, 14]. Briefly, mixtures of the appropriate amounts of Egg PC:Chol:DSPE-PEG (1.85:1:0.15 molar ratio) were used to form a lipidic film and dispersed (32 μmol lipid per ml hydration medium) in a solution of SOD in 6 mM DTPA (5 mg/ml). Liposomes were extruded sequentially through polycarbonate to a final pore size of 0.05 μm. The nonencapsulated protein was removed by ultracentrifugation and liposomes were dispersed in 0.145 M NaCl/10 mM citrate buffer pH 5.6.

^{111}In Labelling for Pharmacokinetics and Biodistribution Studies. ^{111}In labelling: preformed SOD liposomes or enzymosomes containing DTPA were labelled with ^{111}In, as described in [8]. The encapsulation efficiency of the remote labelling procedure was always higher than 90%.

Imaging Protocol. Radiolabeled SOD-enzymosomes and SOD-liposomes were injected i.v. in three rats per group (divided randomly) as described in [8]. Rats were placed prone on a single-head gamma camera equipped with a parallel-hole medium (^{111}In) (Orbiter, Siemens Inc., Hoffmann Estates, IL). The rat groups were imaged synchronously at selected time points after injection. The scintigraphic results were analysed by drawing regions of interest over the heart region and over the whole animal.

Biodistribution Protocol. To study the biodistribution of the radiolabeled SOD-enzymosomes and SOD-liposomes, groups of 5 rats each were injected i.v 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 (liver, spleen, blood, lung, kidney and muscle) were dissected, weighed and assayed for radioactivity in a shielded well-type gamma counter.[8].

In Vivo Therapeutics

SOD-Enzymosomes for Therapeutic Activity Studies. SOD-enzymosomes were prepared as described above. Briefly, PC:Chol:Maleimide-PEG-PE:PEG-PE at the molar ratio of 68.25:30.5:0.75:0.5 and dispersed in 145 mM NaCl/10 mM citrate buffer, pH 6.0 (20 μmol lipid/ml), dimensioned through a final polycarbonate membrane pore size of 0.05 μm. The conjugation was performed as described previously with a SATA:SOD molar ratio of 4:1 and a SOD concentration of 1 mg/ml. The non-conjugated enzyme was separated from the enzymosome dispersion by ultracentrifugation and liposomes were dispersed in 0.145 M NaCl/10 mM citrate buffer pH 5.6.

SOD-Liposomes for Therapeutic Activity Studies. SOD-liposomes were prepared by the dehydration-rehydration method described above, followed by sequential extrusion through polycarbonate filters up to a final pore size of 0.05 μm. The lipid concentration was 32 μmol lipid per ml hydration medium and the film was dispersed in a solution of SOD (5 mg/ml). Nonencapsulated enzyme was separated from the liposome dispersion by ultracentrifugation and liposomes were dispersed in 0.145 M NaCl/10 mM citrate buffer pH 5.6.

Animal Models

Inflammation Induction (Rat Adjuvant Arthritis). 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 [14]. The parameter of interest of adjuvant-induced arthritis is the swelling of the paw measured by the ankle circumference.

Liver Ischemia/Reperfusion Model. Wistar rats were anaesthetized and liver ischemia was induced for 30 min as described in [29]. After 24 hours animals were sacrificed and blood was collected by cardiac puncture. Ischemia/reperfusion-induced liver injury was followed through the determination of aspartate aminotransferase (AST) activity in serum.

Treatment Schedules in the Rheumatoid Arthritis Model. SOD therapeutic treatment: administration starting at day 1 after induction and therapy evaluation at day 12 after induction [12]. Treatments started at day 1 post-induction and injections were repeated at day 5 and 9 at a dose of 66 μg SOD per rat according to the regimen followed in [9]. Each treatment group contained at least 5 rats. The paw oedema was assessed by measurement of the ankle circumference in cm.

Treatment Schedules in the Liver Ischemia/Reperfusion Model. SOD therapeutic treatment was administered in the tail vein at a dose of 66 μg SOD per rat a few minutes before the cut of sutures and re-establishment of liver irrigation. Each treatment group contained at least 5 rats.

Determination of Total Thiols in Plasma. Total thiols were quantified using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) [30]. Briefly 0.07 ml of fresh plasma was collected into 1.12 ml methanol and 0.21 ml of 0.4 M Tris–HCl buffer pH = 8.9, plus 0.07 ml of 0.02 M DTNB in methanol was then added. After 15 min, the mixture was centrifuged for 15 min at 3 000 g (4°C). Absorbance was read at 412 nm against methanol. Haemoglobin was measured to check for haemolysis. Samples with haemoglobin values higher than 130 mg/dl were not considered in the final data analysis.

Aspartate Aminotransferase (AST) Activity. Serum AST activity was assayed using an ADVIA® Chemistry System (Bayer HealthCare). The assay [31] was based on the measurement of oxaloacetate formed by using a NADH-dependent coupled reaction catalysed by malate dehydrogenase. Oxidation of NADH by oxaloacetate was followed spectrophotometrically by the decrease of absorbance at 340 nm after the addition of 2-oxoglutarate. The analytical range is 0–1,000 U/L, and 1 U is defined as the quantity of enzyme that catalyses the formation of 1 μmol of NAD^+ per minute at pH 7.6 and 37°C.

Statistical Analysis

Results are given as the mean ± standard deviation of at least 3 independent experiments made in triplicate, except where otherwise specified. For animal experiments, data is represented as the mean ± standard deviation and statistical significance was tested using one-way ANOVA test.

RESULTS

Development of SOD-Enzymosomes

The process of covalent linking SOD-AT to preformed liposomes containing the reactive lipid linkers maleimide-PEG-PE was optimized. With that purpose we evaluated the effect of the SATA:SOD molar ratio (and, consequently, the degree

of thioacetylation of SOD-ATA), the deacetylation procedure with formation of SOD-AT and the effect of the molar percentage of the reactive lipid linkers on the conjugation of SOD-AT to liposomes (Table I).

In Table I, we show the results obtained for the effect of SATA:SOD molar ratio on the performance of the process of covalent linkage of SOD-AT to the reactive linker maleimide located at the distal terminus of PEG chains attached to the surface of liposomes. These results were obtained for liposomes bearing either a constant molar ratio of the lipid linker (maleimide-PEG-PE) or a partial substitution of this linker by the non-reactive PEG-PE.

The effect of the molar percentage of the lipid linker maleimide-PEG-PE into the preformed liposomes was studied in the range 0 to 1.25, with increments of 0.25, keeping constant the total percentage of PEG lipid at the surface of liposomes (Fig. 1). The decrease in the molar percentage of the reactive pegylated chains was compensated by a correspondent increase on PEG-PE. So, the total fraction of pegylated lipid was kept invariable at 1.25%. The percentages of PC and cholesterol were kept constant at, respectively, 68.25 and 30.5. The different liposomal formulations had a mean liposome size of 0.10 μm and were incubated with SOD-ATA, as described in the Methods section. When the amount of maleimide- PEG-PE increased the amount of SOD covalently linked also increased up to a value of approximately 50 μg/μmol of total lipid. At this conjugation efficiency, the IE appeared to have reached a maximum of around 90% when the % maleimide PEG-PE was higher than 0.75%. For all the formulations no significant differences in the retention of enzymatic activity was observed.

It has to be noted that, as expected, when no maleimide-PEG-PE was incorporated in the bilayer, no SOD was attached to the membrane. This indicates that the coupling of SOD is specific and due to its covalent bond with the functional PEG end. Furthermore, no SOD was noncovalently adsorbed at the outer surface of the liposomal membrane. For further evaluation, we have chosen a molar level of maleimide-PEG-PE of 0.75%.

Stability of SOD-Enzymosomes

The ability of the liposomes to retain the SOD linked by a covalent bond to their outer surface was studied by testing the stability of the covalent bond at two different temperatures, 4°C during 32 days and 37°C during 96 h, respectively. The conjugated SOD to total lipid concentration ratio remained stable (31.2±1.9 μg/μmol) at 4°C during the observation period (32 days). Both the size of liposomes and the polydispersity index did not change during the observation period at 4°C (0.23±0.01 μm; 0.150–0.190). No considerable loss of enzymatic activity was observed since the retention of enzymatic activity was higher than 85% as compared to the initial enzymatic activity (t=0 day).

Since the 1980s, studies of the interaction of lipid vesicles with serum components have been described, and mainly concluded that serum components like albumin induce vesicle leakage [32]. To test the stability of the developed formulation in the presence of albumin, SOD-enzymosomes stability studies were performed at 37°C and in the presence of 2% (w/v) BSA. The presence of BSA did not cause any effect up to 96 h. In fact, the conjugated SOD to lipid ratio observed was 36.1±3.2 μg/μmol and 38.1±3.2 μg/μmol in the absence and presence of 2% (w/v) BSA, respectively. The average of the retention of enzymatic activity was 85% of the initial enzymatic activity. No significant changes were found for the size distribution and polydispersity index in the presence of BSA.

Table I Effect of the SATA:SOD Molar Ratio on the Conjugation of SOD-AT to Long Circulating Liposomes of PC:CHOL:maleimide-PEG-PE and PC:CHOL:maleimide-PEG-PE: PEG-PE

Liposomal composition (molar ratio)	SATA:SOD (molar ratio)	Conjugation efficiency (%)	Conjugated protein (μg protein/μmol lipid)	Retention of activity (%)
PC:CHOL:maleimide-PEG-PE (68.25:30.5:1.25)	8:1	88–96	44–50	46–60
PC:CHOL:maleimide-PEG-PE (68.25:30.5:1.25)	4:1	88–95	45	55–60
PC:CHOL:maleimide-PEG-PE (68.25:30.5:1.25)	1:1	36–44	16–22	60–75
PC:CHOL:maleimide-PEG-PE:PEG-PE (68.25:30.5:0.75:0.5)	8:1	92–98	40–50	50–75
PC:CHOL:maleimide-PEG-PE:PEG-PE (68.25:30.5:0.75:0.5)	4:1	90–95	42–50	55–60
PC:CHOL:maleimide-PEG-PE:PEG-PE (68.25:30.5:0.75:0.5)	1:1	30–42	14–20	62–70

SATA:SOD molar ratio 1:1, degree of modification 3–5%; SATA:SOD molar ratio 4:1, degree of modification 8–10%; SATA:SOD molar ratio 8:1, degree of modification 15–20%; incubation time 1 h.

Lipid concentration of liposomes suspension: 10 μmol/ml

Incubated SOD-AT/Lipid ratio = 45

Conjugation efficiency: $[(\text{Protein/Lipid})_{\text{conjugated}}/(\text{Protein/Lipid})_{\text{incubated}}] \times 100\%$

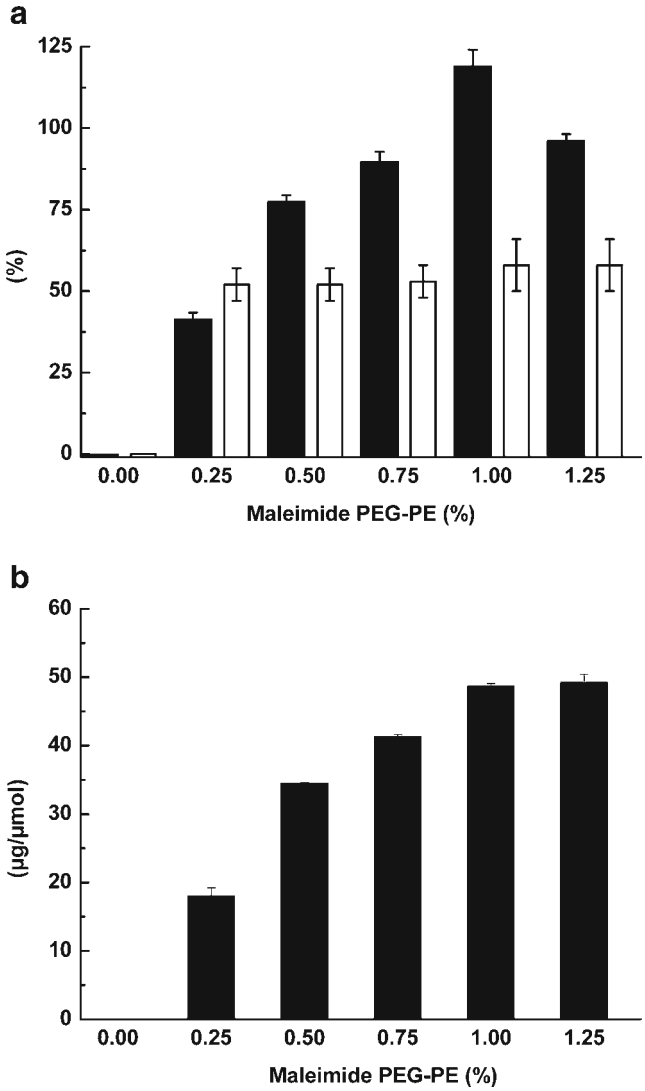

Fig. 1 Characteristics of maleimide-PEG-PE enzymosomes as a function of the percentage of reactive lipid linker. The percentage of reactive lipid linker was complemented by a percentage of PEG-PE in order to keep the total PEGylated fraction at 1.25%. The percentages of Chol and PC were kept constant. **a** Full bars: incorporation efficiency (%); Empty bars, retention of enzymatic activity (%) **b** conjugated SOD to lipid ratio ($\mu g/\mu$mol). SATA:SOD molar ratio 8:1. Maleimide-PEG-PE:protein molar ratio (0–10):1.

In Vivo Fate of SOD-Enzymosomes

To study the effect of the location of the enzyme SOD in the liposomes, *i.e.* surface-exposed on the terminal PEG ends (SOD-enzymosomes) or entrapped in the aqueous interior (SOD-liposomes), the *in vivo* behaviour of both SOD liposomal formulations regarding circulation kinetics and tissue distribution was monitored in rats. Both formulations were labelled by coencapsulation of the complex ^{111}In-DTPA in the aqueous internal space of the particle. This complex has been widely used to follow liposome behaviour *in vivo* [8, 15, 33] by virtue of its favourable characteristics: a high affinity complex at physiological pH and 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) [34].

Both SOD liposomal formulations used for the *in vivo* studies had a particle size of 0.11-0.12 µm and the same protein-to-lipid ratio (12–15 µg of SOD per µmol of total lipid), in order to enable comparative *in vivo* studies at the same liposomal lipid dose.

Imaging Studies

In Fig. 2 we show whole body images obtained immediately post-injection and 24 h post-injection for the two formulations studied (SOD-enzymosomes; SOD-liposomes). It is clear from the images that the main organs of uptake are the liver and spleen.

The whole-body retention of ^{111}In-DTPA radiolabel derived from the quantitative analysis of scintigraphic images taken as function of time for some selected time points is shown in Fig. 3. Both radiolabelled liposomal formulations showed similar profiles but for SOD-enzymosomes ^{111}In-DTPA was excreted at a higher level during the first 4 h. From 4 h onwards, both curves displayed the same clearance rate showing parallel profile. Actually, 46 h post-injection, the percentage of ^{111}In-DTPA excretion amounted to approximately 45% for SOD-enzymosomes and approximately 55% for SOD-liposomes. This fact can be explained by a higher release profile for the label observed during the first 4 h post-injection for SOD-enzymosomes.

Figure 4 shows the heart activity derived from the quantitative analysis of scintigraphic images taken as function of time for some selected time points. The activity in the heart region represents the activity in the blood pool. The two SOD

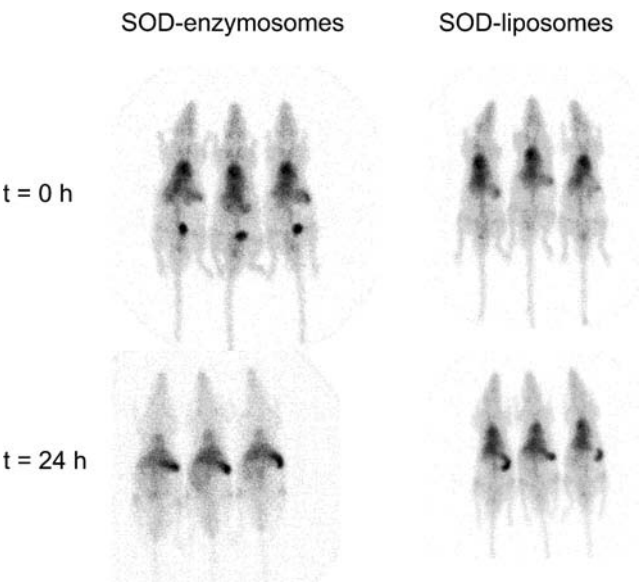

Fig. 2 Scintigrams of rats obtained immediately and 24 h post-injection with either SOD-enzymosomes or SOD-liposomes, labelled with ^{111}In-DTPA.

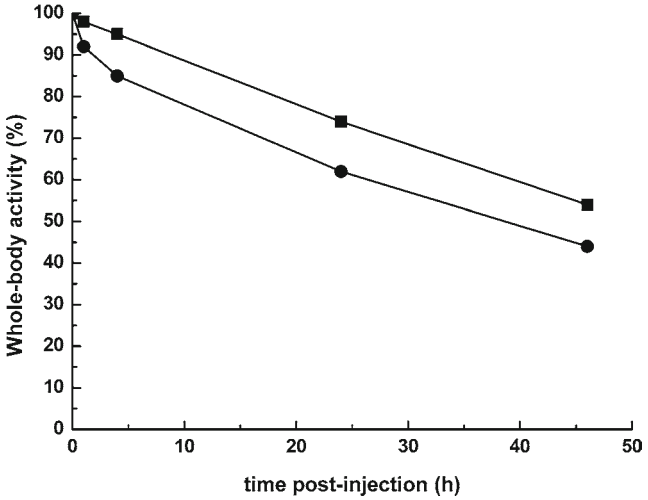

Fig. 3 Whole-body activity profiles derived from the quantitative analysis of scintigraphic images of rats injected with ^{111}In-DTPA labeled particles. Three animals were used in each group: SOD-enzymosomes (●), SOD-liposomes (■). The whole-body activity at 5 min post-injection was set at 100% injection dose (ID). Values are the mean ± SD.

liposomal formulations under study (SOD-liposomes and SOD-enzymosomes) exhibit biphasic behaviour regarding blood clearance. As expected, the SOD Liposomes formulation showed a somewhat longer residence time in the blood [8, 35]. At the end of the observation period (48 h post-injection) more than 40% of the injected dose of the encapsulated label was still present in the circulation. The presence of SOD in the outer surface of the enzymosomes appears to affect negatively the blood circulation kinetics, with about 20% of the injected dose still circulating at 24 h after administration. The area under the curve (AUC) also decreased around 30%. Nevertheless, these findings show that the long circulating properties of SOD-enzymosomes are not severely compromised by the presence of the enzyme on the outer surface.

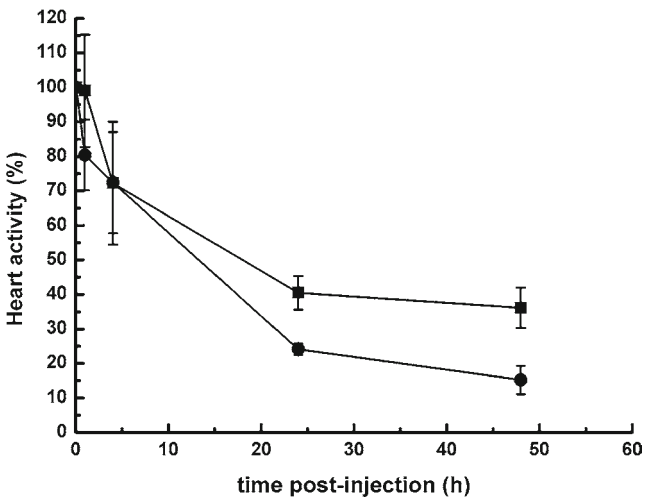

Fig. 4 Heart activity profiles derived from quantitative analysis of scintigraphic images of rats with adjuvant arthritis injected with ^{111}In-DTPA-labelled liposomes. Three animals were used in each group: SOD-liposomes (■), SOD-enzymosomes (●). The heart activity at 5 min post-injection was set at 100% of the injected dose. Values are the mean ± SD and $p < 0.01$ vs SOD-liposomes at 24 h and 48 h.

Biodistribution Studies

The biodistribution data obtained by dissection of selected organs and measured for radioactivity presence at 24 h and 48 h post-injection are shown in Fig. 5. Region of interest (ROI) analysis of the scintigrams results (Fig. 3) are in line with the results obtained by the tissue dissection distribution data (Fig. 5). The main organs showing accumulation of both formulations are the spleen and the liver with a higher splenic tissue uptake in terms of the percentage of the injected dose per gram of tissue (ID/g). At 24 h, the mean values for splenic uptake were around 11 ± 2% ID/g and for hepatic uptake around 1.12 ± 0.21% ID/g and 1.05 ± 0.11% ID/g for SOD-enzymosomes and SOD-liposomes, respectively. The blood levels were higher for the SOD-liposomes (1.51 ± 0.20% ID/g), than for SOD-enzymosomes (1.14 ± 0.15% ID/g) formulation. The accumulation profile observed at 48 h was similar to that observed at 24 h, with lower values for the amount detected in blood but higher in the two main organs of accumulation (liver and spleen). No statistically significant differences ($p > 0.5$) were observed for both formulations studied except for blood values ($p < 0.05$).

Therapeutic Activity

For the therapeutic activity studies, two different SOD liposomal formulations (SOD-liposomes and SOD-enzymosomes) were compared. The lipid composition, size and SOD loading characteristics of the two formulations are shown in Table II. Both liposomal formulations had the same protein-to-lipid ratio, in order to enable comparative *in vivo* studies at the same liposomal lipid dose. The effects of both formulations were tested in two different animal models for pathological situations involving oxidative stress: rat adjuvant arthritis, which is a good model of inflammation, and liver ischemia/reperfusion which includes both inflammatory and non-inflammatory processes [36].

Rat Adjuvant Arthritis Model

In the rat adjuvant arthritis model, the circumference around the ankle was used to measure the degree of inflammation, *i.e.* the severity of the disease. The anti-inflammatory effects of the two different PEG-liposomal formulations (SOD-liposomes and SOD-enzymosomes) using a dosing schedule involving 3 injections in total (given on days 1, 5 and 9), at a dose of 66 μg SOD per rat are shown in Fig. 6. At day 1 (before the first treatment), all animals showed an increase of the circumference around the ankle, from 2.9 ± 0.0 cm to 4.0 ± 0.1 cm. The results reveal that the therapeutic activity of SOD-enzymosomes was faster than that of SOD-liposomes. In fact,

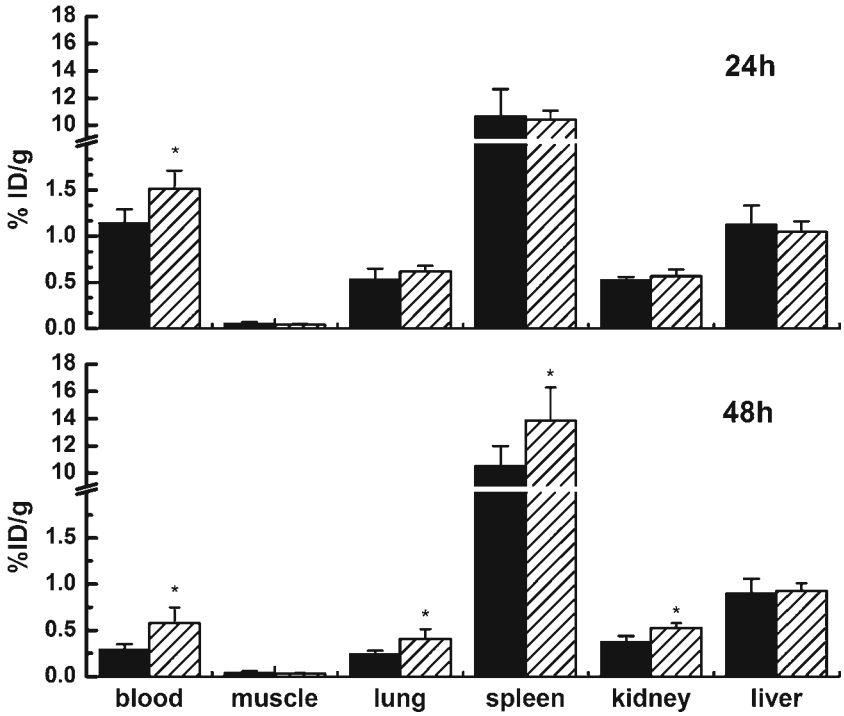

Fig. 5 Biodistribution of ^{111}In-DTPA-labeled liposomes in rats. SOD-enzymosomes (black bars) and SOD-liposomes (dashed bars). % ID/g, % of the injected dose/g of tissue. Values are the mean ± SD 24 h and 48 h post-injection (five rats per group). * $p < 0.05$ vs SOD-liposomes.

after just one dose, rats treated with SOD-enzymosomes already showed a decreased circumference around the ankle of 3.4 ± 0.1 cm ($p < 0.01$ vs control and vs SOD-liposomes), whereas values of 4.0 ± 0.1 cm and 3.8 ± 0.2 cm were observed for the SOD-liposomes and control groups, respectively. The same differences in the circumference around the ankle was also observed at day 3 ($p < 0.05$) and day 4 ($p < 0.01$) of treatment with SOD-enzymosomes when compared to treatment with SOD-liposomes. The values for the circumference around the ankle for the animals treated with SOD-liposomes only reached the values obtained with SOD-enzymosomes after 5 days of induction. At the end of the observation period, both formulations presented similar therapeutic effects significantly different from the control group ($p < 0.05$).

We have previously shown that the levels of total thiols in plasma decrease during rat adjuvant arthritis [12]. As can be seen in Fig. 7, the concentration of total thiols in plasma at the end of the observation period (day 11) is about 50% of that in negative control (naïve) rats (440 μM). Moreover, the disease severity, measured by the ankle circumference, showed a positive correlation with the levels of total thiols in plasma. As shown in Fig. 7, treatment with SOD-liposomes and SOD-enzymosomes led to smaller decreases of total thiols in plasma (about 37% and 32%, respectively). Results shown in Fig. 7 would suggest that levels of total thiols in plasma may be used as a biomarker of the severity of the inflammatory process.

Liver Ischemia/Reperfusion Model

Aspartate aminotransferase activity in serum is a biomarker of liver cell damage due to ischemia/reperfusion [37]. The levels of AST activity in serum increased in rats when liver ischemia was induced (Fig. 8). This result was expected since, using the same ischemia/reperfusion model, we had previously found increased levels of both AST and alanine aminotransferase (ALT) activities 24 h after ischemia which correlated with liver damage as shown by histological analysis and by magnetic resonance imaging [29]. Treatment with SOD-enzymosomes

Table II Characteristics of SOD-enzymosomes and SOD-liposomes used for the *In Vivo* Studies

Formulation	Particle size (μm)	Initial SOD/lipid (μg/μmol)	Encapsulated or Conjugated SOD/lipid (μg/μmol)	Encapsulation/Conjugation Efficiency (%)	Ret. Enz. act. (%)
SOD-liposomes	0.11 ± 0.02	150–160	50–60	5–10	>95
SOD-enzymosomes	0.12 ± 0.02	50–55	50–55	90–100	60–70

All formulations contained 12–15 μg of SOD per μmol of total lipid

SOD-liposomes: SOD long circulating liposomes with SOD encapsulated in the internal aqueous space of liposomes. SOD-enzymosomes: SOD covalently attached to the outer surface of long circulating liposomes (PEG end). Ret. Enz. act.: Retention of enzymatic activity.

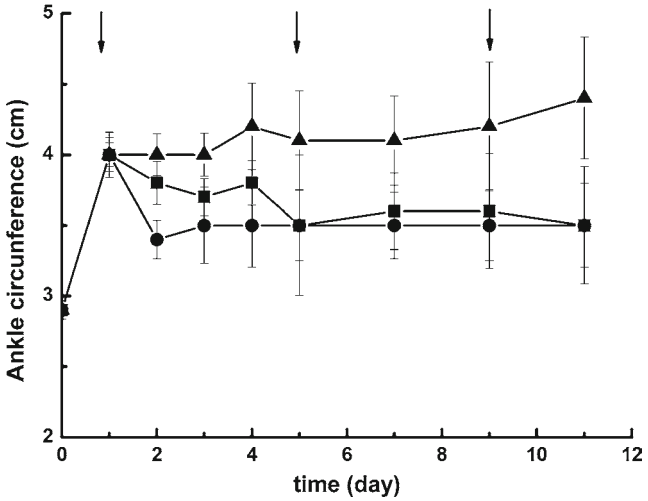

Fig. 6 Anti-inflammatory activities of SOD-enzymosomes and SOD-liposomes in the rat adjuvant arthritis model. SOD-enzymosomes (●), SOD-liposomes (■) and control (▲) using a 3 injection frequency schedule (day 1, 5 and 9) at a dose of 66 μg/kg of bodyweight (arrows). Ankle circumference values are the mean ± SD obtained from 6 animals.

decreased significantly the levels of AST activity in serum indicating that this therapeutic approach gives less liver cell damage due to ischemia/reperfusion. However, treatments either with SOD-liposomes or free SOD (in saline) had no effect on the levels of AST activity.

DISCUSSION

Drug targeting has the potential to improve therapeutic interventions in many diseases. However, most studies have focused on cancer with only limited attention being given to the application of targeted drug delivery in other diseases like chronic inflammatory disorders, such as rheumatoid arthritis or liver ischemia/reperfusion situations [38]. Yet, site-specific drug targeting using liposomal carriers can improve the therapeutic performance of anti-inflammatory agents [38, 39]. This is due to fact that at inflammatory sites, such as arthritic joints, there is an increased vascular permeability allowing the enhanced permeability and retention (EPR) effect to occur [38], and thus facilitate the continued extravasation of long circulating liposomes with a small size at the inflammatory sites.

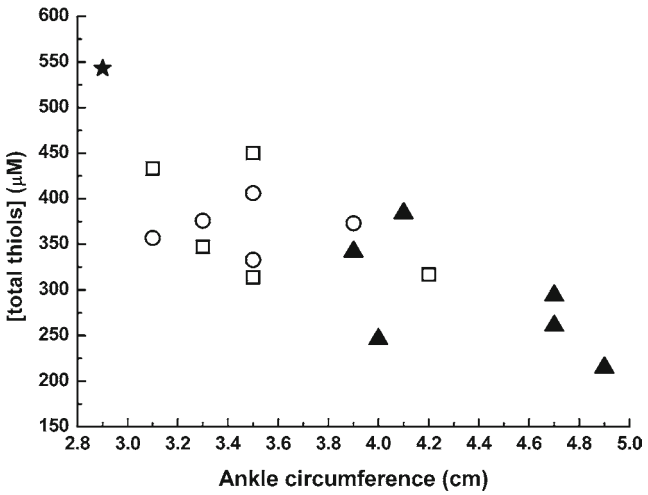

Fig. 7 Levels of total thiols in the plasma of rats positively correlates with adjuvant arthritis severity. The circumference around the ankle was measured at the end of the treatment period (day 11). Negative control (normal rat) (star), SOD-enzymosomes (○), SOD-liposomes (□) and control (non-treated rat) (▲) using a 3 injection frequency schedule (day 1, 5 and 9). $R^2 = 0.5671$; $Y = -100 \times + 731$.

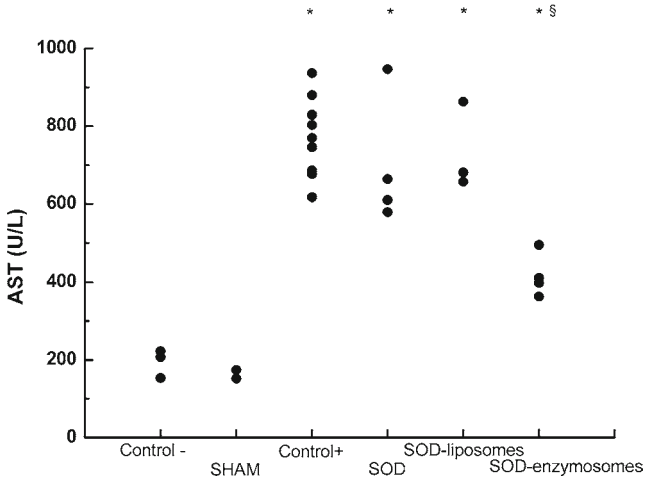

Fig. 8 Aspartate aminotransferase activity in rat serum after liver ischemia/reperfusion and treatment with different SOD formulations. Liver ischemia was induced in rats for 30 min and blood was collected after 24 h. Control -, normal rats; SHAM, rats sham-operated; Control +, rats with induced liver ischemia; SOD, rats with induced liver ischemia treated with SOD; SOD-liposomes, rats with induced liver ischemia treated with SOD-liposomes; SOD-enzymosomes, rats with induced liver ischemia treated with SOD-enzymosomes. .* $p < 0.05$ vs Control - or vs SHAM. § $p < 0.05$ vs Control +, vs SOD, or vs SOD-liposomes.

The use of liposomal SOD avoids rapid SOD clearance and yields a strongly improved accumulation in the inflamed areas in rat arthritis [8, 35]. However, SOD-liposomes have shown only a limited therapeutic efficacy since they are not able to completely reduce joint swelling. Gaspar *et al.* [11] suggested that this limited therapeutic efficacy of liposomal SOD could be due to an inadequate extent and/or rate of release in inflamed areas. This latter hypothesis was confirmed by using liposomes containing a hydrophobized SOD (Acyl-SOD) which was localized in the lipid bilayers of the liposomes, partially buried into the outer surface and exposed to the external medium. These Ac-SOD-enzymosomes showed a faster onset of anti-inflammatory activity when compared to SOD-liposomes [11]. However, Ac-SOD enzymosomes only had around 40% of the total enzymatic activity in an intact form while for SOD-enzymosomes all the enzymatic activity is available as all the enzyme is linked at the outer liposomal membrane.

In this work we were able to further improve the therapeutic action of SOD-liposomes with passive targeting effects by EPR to inflammation sites, by developing a new liposomal formulation where SOD was attached as a water soluble enzyme to the surface of liposomes. This allows a faster action of SOD since there is no need to have a liposomal disruption to release the therapeutic enzyme. Moreover, this availability may be fundamental in inflammatory situations where a faster therapeutic intervention may be needed such as in liver ischemia/reperfusion situations.

The process of covalent linkage of SOD to the distal PEG ends at the surface of PEG-liposomes was designed and optimized, to minimise alterations in the enzyme activity and also to maintain the long circulating properties of the liposomes. By controlling the ratio of functionalized and non-functionalized PEG at the surface of enzymosomes, we achieved a sufficient enzyme load at the surface of the long circulating enzymosomes suitable for the animal studies. Furthermore, the vesicles structural integrity was maintained as well as the enzyme activity indicating a preserved enzyme structure.

The therapeutic activity of our SOD-enzymosomes was tested in two different inflammatory situations. In the rat adjuvant arthritis model our new SOD-enzymosomes showed a faster onset of therapeutic activity than SOD-liposomes (encapsulated in the inner aqueous space), which is in accordance with previous studies made with Ac-SOD Enzymosomes [11]. The main difference between the SOD-enzymosomes developed in the current work and the Acyl-SOD Enzymosomes used in the study of Gaspar *et al.* [11] is the localization of SOD in the liposome structure in the latter. Since this Ac-SOD has palmitate acyl chains covalently linked to the ε-NH_3 of the lysine residue, the physical properties of this new identity of SOD changed showing a higher affinity for the hydrophobic region of the liposomal bilayers as compared to SOD. It is expected for Ac-SOD to be localized in the liposomal bilayers with only around 40% of the total activity in the outer surface. Therefore, for the SOD-enzymosomes developed in this work the interaction of the enzyme with nearby membranes (e.g. erythrocytes) should not be expected to occur and the SOD biodistribution is only based on the biodistribution of the liposomal nanoparticle and not mediated by release from the nanoparticles while they circulate in the bloodstream.

In the ischemia/reperfusion induced liver injury model, SOD-enzymosomes showed therapeutic activity (Fig. 8). However, SOD-liposomes (or free SOD) showed no therapeutic activity. The reason for this difference is not clear since the biodistribution studies, showed that SOD-enzymosomes and SOD-liposomes have a similar accumulation in liver at 24 h post-administration (Fig. 5). However, as discussed above, SOD availability in enzymosomes is faster than in liposomes. Also, we recently showed using magnetic resonance imaging (MRI) that a liposomal formulation similar to our SOD-enzymosomes but loaded with superparamagnetic iron oxide nanoparticles (SPION), accumulates at liver damage sites induced by ischemia/reperfusion [29]. Therefore, our results with SOD-enzymosomes are encouraging and support further testing of this formulation, not only in this particular pathological model, but also in other inflammation models. The organ distribution and drug half-life must be considered when doing these tests. In this way the potential applications of SOD-enzymosomes can be expanded, limiting the injury occurring during inflammatory and other pathological situations.

CONCLUSION

The design and characterization of SOD-enzymosomes with a suitable enzyme load, keeping the vesicles structural integrity and preserving the enzyme activity was achieved, as can be concluded from *in vivo* studies.

Results clearly demonstrate that SOD-enzymosomes with SOD attached at the distal PEG end showed prolonged circulation and a similar biodistribution profile to that of long circulating liposomes. In an animal model of arthritis, SOD-enzymosomes showed an earlier therapeutic activity than both SOD-liposomes and free SOD. This work also shows, for the first time, that SOD-enzymosomes, unlike SOD-liposomes, have a therapeutic effect in a rat liver ischemia/reperfusion model suggesting that they are able to decrease liver injury.

ACKNOWLEDGEMENTS AND DISCLOSURES

The authors gratefully acknowledge the support of: Fundação para a Ciência e Tecnologia, Portugal (grants POCTI/42259/FCB/2001; SFRH/BD/12264/2003; PEst-OE/QUI/UI0612/2013; PEst-OE/SAU/UI4013/2011); Sociedade Portuguesa de Transplantação - Bolsa de Investigação SPT/Astellas and Instituto de Investigação Bento da Rocha Cabral for animal supply (ischemia/reperfusion model); we thank Filipa Fontes for laboratory assistance in the preparation of liposomal formulation (ischemia/reperfusion model).

REFERENCES

1. Pisal DS, Kosloski MP, Balu-Iyer SV. Delivery of therapeutic proteins. J Pharm Sci. 2010;99(6):2557–75.
2. Sies H, Jones DP. Oxidative stress. In: Fink G, editor. Encyclopaedia of stress. London: Elsevier; 2007. p. 45–8.
3. Saeidnia S, Abdollahi M. Toxicological and pharmacological concerns on oxidative stress and related diseases. Toxicol Appl Pharmacol. 2013;273(3):442–55.

4. Cushing LS, Decker WE, Santos FK, Schulte TL, Huber W. Orgotein therapy for inflammation in horses. Mod Vet Pract. 1973;54(7):17–21.
5. Marberger H, Bartsch G, Huber W, Menander KB, Schulte TL. Orgotein: a new drug for the treatment of radiation cystitis. Curr Ther Res Clin Exp. 1975;18(3):466–75.
6. Huber W, Schulte TL, Carson S, Golddhame RE, Vogin EE. Some chemical and pharmacologic properties of a novel anti-inflammatory protein. Toxicol Appl Pharmacol. 1968;12(2):308.
7. Carillon J, Rouanet JM, Cristol JP, Brion R. Superoxide dismutase administration, a potential therapy against oxidative stress related diseases: several routes of supplementation and proposal of an original mechanism of action. Pharmaceut Res. 2013;30(11):2718–28.
8. Corvo ML, Boerman OC, Oyen WJ, van Bloois L, Cruz MEM, Crommelin DJA, *et al.* Intravenous administration of superoxide dismutase entrapped in long circulating liposomes. II In vivo fate in a rat model of adjuvant arthritis. Biochim Biophys Acta. 1999;1419(2):325–34.
9. Corvo ML, Jorge JCS, van't Hof R, Cruz MEM, Crommelin DJA, Storm G. Superoxide dismutase entrapped in long-circulating liposomes: formulation design and therapeutic activity in rat adjuvant arthritis. Biochim Biophys Acta. 2002;1564(1):227–36.
10. Veronese FM, Caliceti P, Schiavon O, Sergi M. Polyethylene glycol-superoxide dismutase, a conjugate in search of exploitation. Adv Drug Deliv Rev. 2002;54(4):587–606.
11. Gaspar MM, Boerman OC, Laverman P, Corvo ML, Storm G, Cruz MEM. Enzymosomes with surface-exposed superoxide dismutase: in vivo behaviour and therapeutic activity in a model of adjuvant arthritis. J Control Release. 2007;117(2):186–95.
12. Simões SID, Delgado TC, Lopes RM, Jesus S, Ferreira AA, Morais JA, *et al.* Developments in the rat adjuvant arthritis model and its use in therapeutic evaluation of novel non-invasive treatment by SOD in transfersomes. J Control Release. 2005;103(2):419–34.
13. Riedl CR, Sternig P, Galle G, Langmann F, Vcelar B, Vorauer K, *et al.* Liposomal recombinant human superoxide dismutase for the treatment of Peyronie's disease: a randomized placebo-controlled double-blind prospective clinical study. Eur Urol. 2005;48(4):656–61.
14. Corvo ML, Martins MBF, Francisco AP, Morais JG, Cruz MEM. Liposomal formulations of Cu, Zn-superoxide dismutase: physicochemical characterization and activity assessment in an inflammation model. J Control Release. 1997;43(1):1–8.
15. Corvo ML, Boerman OC, Oyen WJ, Jorge JC, Cruz MEM, Crommelin DJA, *et al.* Subcutaneous administration of superoxide dismutase entrapped in long circulating liposomes: in vivo fate and therapeutic activity in an inflammation model. Pharm Res. 2000;17(5):600–6.
16. Gaspar MM, Martins MBF, Corvo ML, Cruz MEM. Design and characterization of enzymosomes with surface-exposed superoxide dismutase. Biochim Biophys Acta. 2003;1609(2):211–7.
17. Cruz MEM, Gaspar MM, Martins MBF, Corvo ML. Liposomal superoxide dismutases and their use in the treatment of experimental arthritis. Methods Enzymol. 2005;391:395–413.
18. Vale C, Corvo ML, Martins LCD, Marques CR, Storm G, Cruz MEM, Martins MBF Construction of enzymosomes: optimization of coupling parameters. 2 NSTI-Nanotech 2006, 2006, 396–397.
19. Martins MBF, Jorge JCS, Cruz MEM. Acylation of L-asparaginase with total retention of enzymatic activity. Biochimie. 1990;72(9):671–5.
20. Cruz MEM, Jorge JCS, Martins MBF, Gaspar MM, Simões ACE, Perez-Soler R. Liposomal compsn. contg. L-Asparaginase - used for effective treatment of tumours, partic. human acute lymphoblastic leukaemia. Patent Number: EP485143-A1; 1992.
21. Martins MBF, Goncalves APV, Cruz MEM. Biochemical characterization of an L-asparaginase bioconjugate. Bioconjug Chem. 1996;7(4):430–5.
22. Solaro R. Targeted delivery of proteins by nanosized carriers. J Polym Sci, Part A: Polym Chem. 2008;46(1):1–11.
23. Heeremans JLM, Kraaijenga JJ, Los P, Kluft C, Crommelin DJA. Development of a procedure for coupling the homing device Gluplasminogen to liposomes. Biochim Biophys Acta. 1992;1117(3):258–64.
24. Derksen JTP, Scherphof GL. An improved method for the covalent coupling of proteins to liposomes. Biochim Biophys Acta. 1985;814(1):151–5.
25. Bohlen P, Stein S, Dairman W, Udenfriend S. Fluorimetric assay of proteins in the nanogram range. Arch Biochem Biophys. 1973;155(1):213–20.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951;193(1):265–75.
27. Rouser G, Fleischer S, Yamamoto A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids. 1970;5(5):494–6.
28. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. J Biol Chem. 1972;247(10):3170–5.
29. Martins MBF, Corvo ML, Marcelino P, Marinho HS, Feio G, Carvalho A. New long circulating magnetoliposomes as contrast agents for detection of ischemia-reperfusion injuries by MRI. Nanomedicine: NBM. 2014;10(1):207–14.
30. Marinho HS, Baptista M, Pinto RE. Glutathione metabolism in hepatomous liver of rats treated with diethylnitrosamine. Biochim Biophys Acta. 1997;1360(2):157–68.
31. Bergmeyer HU, Scheibe P, Wahlefeld AW. Optimization of methods for aspartate aminotransferase and alanine aminotransferase. Clin Chem. 1978;24(1):58–73.
32. Kirby C, Clarke J, Gregoriadis G. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. Biochem J. 1980;186(2):591–8.
33. Boerman OC, Laverman P, Oyen WJ, Corstens FH, Storm G. Radiolabeled liposomes for scintigraphic imaging. Prog Lipid Res. 2000;39(5):461–75.
34. Kranenborg MH, Oyen WJG, Corstens FHM, Oosterwijk E, vander Meer JWM, Boerman OC. Rapid imaging of experimental infection with technetium-99 m-DTPA after anti-DTPA monoclonal antibody priming. J Nucl Med. 1997;38(6):901–6.
35. Boerman OC, Oyen WJG, Storm G, Corvo ML, van Bloois L, vander Meer JWM, *et al.* Tc-99 m labeled liposomes to image experimental arthritis. Ann Rheum Dis. 1997;56(6):369–73.
36. Abu-Amara M, Yang SY, Tapuria N, Fuller B, Davidson B, Seifalian A. Liver Ischemia/Reperfusion Injury: Processes in Inflammatory Networks-A Review. Liver Transpl. 2010;16(9):1016–32.
37. Gonzalez-Flecha B, Cutrin JC, Boveris A. Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. J Clin Invest. 1993;91(2):456–64.
38. Crielaard BJ, Lammers T, Schiffelers RM, Storm G. Drug targeting systems for inflammatory disease: One for all, all for one. J Control Release. 2012;161(2):225–34.
39. van den Hoven JM, Van Tomme SR, Metselaar JM, Nuijen B, Beijnen JH, Storm G. Liposomal Drug Formulations in the Treatment of Rheumatoid Arthritis. Mol Pharmaceutics. 2011;8(4):1002–15.