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Design and characterization of enzymosomes with surface-exposed superoxide dismutase

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Abstract

Superoxide dismutase (SOD) was chemically modified by covalent linkage of fatty acid chains to the accessible ε-amino groups of the enzyme. This acylation method gave rise to a different enzyme entity (Ac-SOD) as evidenced by different physicochemical properties such as octanol/water partition coefficient and isoelectric point (p*I*) as compared to SOD. Ac-SOD was incorporated in conventional and long-circulating liposomes (LCL) and characterized in terms of incorporation efficiency, protein to lipid ratio (Prot/Lip), enzymatic activity retention and zeta potential. The observation that Ac-SOD liposomes present enzymatic activity on their external surface indicates that these formulations can act independent of rate and extent of enzyme release as required in case of SOD liposomes. The decrease of superficial charge of liposomal formulations containing Ac-SOD, as compared to SOD liposomes, may be related to the negatively charged enzyme molecules localized on the liposome surface. The comparative characterization of Ac-SOD and SOD liposomal formulations evidenced that the two enzyme forms differ substantially regarding their intraliposomal location: SOD tends to be localized in the internal aqueous spaces, whereas Ac-SOD is expected to be localized in the lipid bilayers of the liposomes, partially buried into the outer surface and exposed to the external medium. These liposomal structures with surface-exposed SOD were designated as Ac-SOD enzymosomes. The properties of these enzymosomes may influence the therapeutic effect, as the release of the enzyme from extravasated vesicles is no longer a necessary requirement for achieving dismutating activity within the inflamed target site.

Keywords: Enzymosome; Superoxide dismutase; Enzymatic activity

1. Introduction

The enzyme Cu,Zn-superoxide dismutase (SOD) was defined by McCord and Fridovich [1] in 1969 as a natural defence system that limits the toxic effects of oxygen-derived free radicals. It catalyses the dismutation of the toxic superoxide radical anion ($O_2^{\cdot -}$) to O_2 plus H_2O_2, disrupting the sequence of biochemical inflammatory processes induced by the free radical [2]. Treatment of inflammatory pathologies using SOD seems a promising alternative to conventional anti-inflammatory therapies based on the use of nonsteroidal anti-inflammatory drugs avoiding their side effects in particular those involving the gastrointestinal (GI) tract [3]. However, the clinical application of this enzyme is limited by its short half-life time in the bloodstream and poor penetration into cells. Many attempts have been made to overcome these limitations such as covalent attachment of SOD to polyethylene glycol (PEG) [4,5], dextran [6], albumin [7], modification with Ficoll [8] and incorporation in liposomes [9–11]. Nakaoka et al. [4] observed that the coupling of PEG to SOD did not change the SOD distribution pattern in spleen, lung, heart and GI tract 24 h post i.v. injection [4]. Turrens et al. [10] have demonstrated an increased half-life of SOD when administered in liposome-incorporated form. Unfortunately, low incorporation efficiencies were obtained due to the method selected for liposome preparation [10]. Recently, higher liposomal incorporation efficiencies were achieved [9,12,13]. The therapeutic activity of these SOD liposomes may be limited by the rate and degree of release of the enzyme from liposomes localized at the inflammation site. The focus of this paper is the preparation of liposomes with surface-exposed SOD (hereafter referred to as enzymo-

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somes). The presentation of SOD on the liposome surface is expected to be more beneficial than encapsulation of SOD in liposomes, as release of enzyme from the liposomes at the site of inflammation may not be needed to achieve therapeutic activity. To attain surface localization of SOD on the enzymosomes, we used the strategy of acylation of the enzyme by covalent linkage of palmitic acid to ε-NH_2 groups of SOD (referred to as Ac-SOD). We hypothesized that the acylation procedure will result in a more hydrophobic enzyme with increased affinity for the liposomal bilayers [14–16]. Besides the goal of exposing SOD on the external liposome surface, we also aimed for SOD targeting to inflammation sites. To realize the second aim, we needed to prepare liposomes with a circulation time long enough to obtain localization at the inflammation sites. A number of studies have convincingly demonstrated that so-called long-circulating liposomes (LCL) tend to localize preferentially at sites of inflammation after i.v. administration [12,17–19]. The preferential localization appears to be the result of the inflammatory response provoking a locally increased capillary permeability allowing liposome extravasation [20]. Therefore, LCL have attractive prospects for the targeted delivery of Ac-SOD.

In the present work, specific attention was paid to a comparative physicochemical characterization of SOD and Ac-SOD and to the corresponding liposomal formulations. SOD and Ac-SOD were incorporated in two types of liposomes: PEG-coated LCL (hereafter referred to as PEG-liposomes) and non-PEG-liposomes containing stearylamine (further referred to as SA-liposomes). Both liposomal formulations were characterized with regard to incorporation efficiency, zeta potential, retention of enzymatic activity and externally exposed enzyme activity.

2. Materials and methods

2.1. Chemicals

Egg phosphatidylcholine (PC) was obtained from Lipoid (Ludwigshafen, Germany), distearoylphosphatidylethanolamine-poly (ethylene glycol) 2000 (DSPE-PEG) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol), stearylamine (SA), dimiristoylphosphatidylcholine (DMPC) and bovine erythrocyte Cu,Zn-SOD was purchased from Sigma (St. Louis, MO, USA). Sephadex G-200 was purchased from Pharmacia Biotech, Uppsala, Sweden. All other chemicals were of analytical grade.

2.2. Chemical modification of SOD

The Ac-SOD was prepared as previously described [21,22]. Briefly, palmitoyl chloride was added to a 50-mM carbonate buffer (pH 9.4) containing 1% sodium cholate. The mixture was sonicated for 90 s and immediately added to the enzyme and incubated with gentle stirring at room temperature for 2 h. To promote the separation of the excess of the palmitoyl chloride, hydrolysed to palmitic acid and remaining in suspension by the presence of the sodium cholate micelles, the preparation was diluted with equal volume of cold water and placed in a refrigerator to achieve a temperature of 5 °C. The palmitic acid was then removed by centrifugation at 20,000 × g for 30 min at 6 °C (Sigma 202 MK, Laboratory centrifuge, Osterode, Germany). The remaining compounds were removed by dialysis against water for 72 h. A final centrifugation at 20,000 × g for 1 h at 6 °C (Sigma 202 MK, Laboratory centrifuge) was carried out and the supernatant containing Ac-SOD was lyophilised either for incorporation in liposomes or for physicochemical characterization. Native enzyme was used as a reference in all modification steps.

The modification degree of Ac-SOD was determined by a fluorimetric assay. Briefly, unblocked ε-NH_2 groups were bound to fluorescamine according to the method of Böhlen et al. [23]. The fluorescence intensity was measured in all samples (λ_{ex} = 390 nm; λ_{em} = 475 nm) in a fluorescence spectrophotometer F3000, Hitachi, Japan. The modification degree, representing the percentage of accessible ε-NH_2 groups covalently linked to hydrophobic palmitoyl chains, was defined as:

$$(1 - (\text{emission of 20 } \mu\text{g modified enzyme}) / (\text{emission of 20 } \mu\text{g native enzyme}))100.$$

2.3. Liposome preparation and characterization

2.3.1. Incorporation methods

Multilamellar liposomes were prepared by the simple dehydration–rehydration method (sDRV) as previously described [15]. Briefly, the selected lipid mixtures were dried under a nitrogen stream. The obtained homogeneous film was dispersed in water (16 or 32 μmol/ml) to form empty multilamellar vesicles. The lipid compositions were PC, Chol and SA at a molar ratio of 7:2:1 for conventional liposomes (SA-liposomes), PC, Chol and DSPE-PEG at a molar ratio of 1.85:1:0.15 for pegylated liposomes (PEG-liposomes) and DMPC for thermotropic behaviour studies. To prepare Ac-SOD liposomes, 1 ml of empty liposomes was added to the lyophilised protein, frozen and lyophilised overnight. The lyophilised powder was rehydrated in the presence of 0.1 ml of 280 mM mannitol in 10 mM citrate buffer (pH 5.6). The hydration step lasted 30 min, and after that, 145 mM NaCl in 10 mM citrate buffer (pH 5.6) was added to adjust up to 1 ml. The liposomes were sequentially extruded through polycarbonate membranes of different porosities. Gel filtration was the technique selected for removing the non-incorporated Ac-SOD from Ac-SOD liposome preparations as it showed higher lipid recovery (81% vs. 69%) and was easier than the discontinuous sucrose gradient centrifugation.

SOD liposomes were prepared according to Corvo et al. [9]. Briefly, liposomes were prepared as described for Ac-SOD liposomes with the following modifications. The film was dispersed in an aqueous solution of SOD, the non-incorporated SOD was separated from liposome suspension, after extrusion steps, by ultracentrifugation at 250,000 × *g* for 3 h at 10 °C (LM-80 ultracentrifuge, Beckman).

Two different liposome types were used in this study: PEG-liposomes (composed of PC/Chol/DSPE-PEG) and SA-liposomes (composed of PC/Chol/SA). To study the effect of particle size on the IE, SA- and PEG-liposomes were extruded until reaching a mean diameter of about 0.2 or 0.1 μm. According to size (0.2 or 0.1 μm), SA-liposomes are designated as SA (0.2) or SA (0.1) and PEG-liposomes as PEG (0.2) or PEG (0.1).

2.3.2. Incorporation parameters

Liposomes were characterized by lipid composition, size and by the following incorporation parameters: incorporation efficiency, protein recovery, protein-to-lipid ratio, enzymatic activity and zeta potential.

Abbreviations and equations used to determine incorporation parameters: Prot—protein; Lip—lipid; (Prot/Lip)i—initial (Prot/Lip); (Prot/Lip)f—final (Prot/Lip); initial (Prot/Lip)—initial Prot to Lip ratio (μg/μmol of Lip); final (Prot/Lip)—final Prot to Lip ratio (μg/μmol of Lip); incorporation efficiency (%)=({final (Prot/Lip)}/{initial (Prot/Lip)}) 100—(IE).

The protein was determined using the method described by Lowry et al. [24] after disruption of the liposomes with Triton X-100 and sodium dodecyl sulfate (SDS) [25]. Lipid determinations were performed using the method described by Rouser et al. [26].

The enzymatic activity of Ac-SOD and SOD formulations was determined according to Misra and Fridovich [27] and Sun and Zigman [28]. The ability of the enzyme to decrease the rate of autoxidation of epinephrine to adrenochrome was measured at pH 10.2 (Abs = 480 nm) [27,28]. For total enzymatic activity determination of SOD or Ac-SOD incorporated in liposomes, appropriated dilutions must be done to prepare a final protein concentration of 6 μg/ml with a maximum of 0.3% Triton X-100. For the determination of the enzymatic activity exposed to the external surface (Exp. Act.), dilutions of SOD or Ac-SOD liposomes with 0.145 M NaCl/10 mM citrate buffer (pH 5.6) must be done to prepare a final protein concentration of 12 μg/ml. A curve of the inhibition (%) of the autoxidation of epinephrine as a function of amount of SOD or Ac-SOD is plotted. The amount (μg) of SOD or Ac-SOD required to inhibit 50% of the epinephrine autoxidation is calculated. The retention of enzymatic activity is calculated as follows:

$$[((\mu g\ SOD\ or\ Ac-SOD\ (control\ solution))/(\mu g\ SOD\ or\ Ac-SOD\ (final\ preparation))]\ 100$$

The total retention of enzymatic activity (Ret. Act.) of Ac-SOD in the studied formulations was defined as the ratio, in percentage, of the enzymatic activity quantified on the final liposomes and on the initial rehydrated liposomes, after disruption of liposomes in both samples with Triton X-100 at 5%. The enzymatic activity exposed to the external surface was quantified in intact liposomes (without disruption with Triton X-100). This parameter, exposed activity (Exp. Act.) corresponds to the ratio, in percentage, of the enzymatic activity of Ac-SOD exposed to the external surface in relation to the total enzymatic activity of the final liposomes when disrupted with detergent.

Liposome mean diameter (∅) was determined by dynamic light scattering in a ZetaSizer 1000 HS_A (Malvern, UK). As a measure of particle size distribution of the dispersion, the system reports the polydispersity index (PI) ranging from 0.0 for an entirely monodisperse sample up to 1.0 for a polydisperse suspension.

Zeta potential was calculated by using Smoluchowsk's equation in a ZetaSizer 2000 (Malvern). The scattering angle was 12° and the electric field intensity ranged from 18.5 to 19.6 V/cm.

Statistical analysis was performed by the unpaired two-tailed Student's *t* test, and differences with P values <0.05 were considered significant.

2.4. Thermotropic behaviour of phospholipid membranes

Phase-transition temperature of liposomal formulations was determined as previously described [29] by light scattering in a fluorescence spectrophotometer (F3000, Hitachi), with an excitation and emission wavelength set at 450 nm, connected to a thermostatically controlled bath. Liposomes made with DMPC either unloaded or with Ac-SOD incorporated were prepared. Aliquots of the liposomal formulations under study were added to a fluorescence cell and light scattering recorded. The temperature of the samples was changed by increments of 0.5–1 °C in the range between 19 and 30 °C.

3. Results

3.1. Physicochemical characterization: Ac-SOD vs. SOD

A comparative physicochemical characterization of Ac-SOD and SOD is presented in Table 1. The acylated enzyme presents a modification degree of 30%, meaning that 30% of the accessible ε-NH_2 are covalently linked to hydrophobic palmitoyl chains. The enzymatic activity of Ac-SOD was only reduced 10% as compared to SOD. The octanol/water partition coefficient was 3.13 for Ac-SOD and 0.15 for SOD. The 20-fold increase in this value evidences the hydrophobic character of the acylated enzyme.

A slight decrease on the p*I* was observed upon acylation. The zeta potential of Ac-SOD had a higher negative value as

Table 1
Physicochemical characterization of SOD and Ac-SOD

Enzyme	Ac-SOD	SOD
Modification degree (%)	30[a]	0[a]
Activity retention (%)	90[a]	100[a]
Partition coefficient[b]	3.13[a]	0.15[a]
Isoelectric point (p*I*)	4.83[a]	4.96[a]
Molecular weight (kDa)	36[a]	36[a]
Zeta potential (mV)	− 16.7[c]	− 5.7[c]

[a] Data previously published [22].
[b] Octanol/water partition coefficient.
[c] In citrate buffer, pH 5.6, 28 mosM.

compared to SOD. The decrease either of the p*I* or of the zeta potential may be related to blockage of some ε-NH_2 groups of SOD by palmitic chains.

3.2. Incorporation of Ac-SOD in liposomes

3.2.1. Effect of lipid concentration and particle size on incorporation parameters

For liposome preparation, it is desirable to obtain high protein loading efficiencies. The effect of the lipid concentration on the drug loading of the final liposomal formulations was studied. The increase of the initial lipid concentration for SA-liposomes, from 16 to 32 μmol/ml, resulted in a 2-fold increase of the IE (from 31% to 63%) and in an increase of the protein recovery from 25% to 38%. Therefore, the initial lipid concentration used for subsequent studies was set at 32 μmol/ml.

Fig. 1 shows that the Ac-SOD IE was dependent from lipid composition rather than on the particle size. A higher IE and final (Prot/Lip) were obtained for SA-liposomes (58 ± 4%–63 ± 7% and 8 ± 1 μg/μmol, respectively) as compared to PEG-liposomes (43 ± 5% and 6 ± 1 μg/μmol,

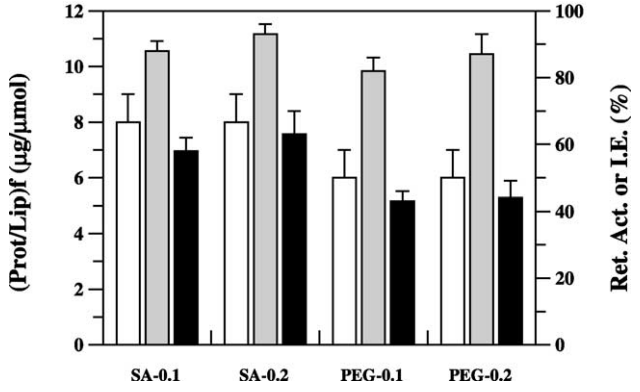

Fig. 1. Incorporation parameters of Ac-SOD in SA- and PEG-liposomes. Influence of the lipid composition and mean diameter of liposomes on: final (Prot/Lip)—white columns; total enzymatic activity retention (Ret. Act. %)—grey columns; IE (%)—black columns. SA-0.1 and SA-0.2—conventional liposomes with mean diameters of 0.1 or 0.2 μm, respectively. PEG-0.1 and PEG-0.2—LCL with mean diameters of 0.1 or 0.2 μm, respectively. The initial lipid concentration was 32 μmol/ml and the initial protein concentration was 500 μg/ml. The results represent means ± S.D. of at least three preparations.

Table 2
Effect of initial (Prot/Lip) on liposome properties of SA-liposomes incorporating Ac-SOD

(Prot/Lip)i (μg/μmol)	(Prot/Lip)f (μg/μmol)	IE (%)	Enzymatic activity (%)	Exposed enzymatic activity (%)	Zeta potential (mV)
13 ± 2	8 ± 1	60 ± 3	93 ± 1	30 ± 2	31 ± 1
36 ± 2	15 ± 1	43 ± 2	92 ± 2	37 ± 3	28 ± 1
57 ± 5	20 ± 1	36 ± 3	94 ± 2	49 ± 3	26 ± 1

Initial protein concentration—500–2500 μg/ml.
Initial lipid concentration—32 μmol/ml.
SA-liposomes incorporating Ac-SOD with a mean size of 0.2 μm.
Liposomes were rehydrated with citrate buffer, pH 5.6, 280 mosM.
Values are means ± S.D. of at least three independent preparations.

respectively). The preservation of enzymatic activity of Ac-SOD liposomes is an important parameter to evaluate the effect of suitability of the incorporation method and to characterize the obtained formulations.

The total retention of enzymatic activity (Ret. Act.) of Ac-SOD ranged from 82% to 94% for all liposomal formulations tested (Fig. 1), attesting that the incorporation method could preserve the activity of the enzyme.

3.3. Effect of protein-to-lipid ratio on properties of SA-liposomes containing Ac-SOD

Table 2 shows the effect of the initial (Prot/Lip) on the IE, final (Prot/Lip), enzymatic activity exposed on the liposome surface and zeta potential of SA-liposomes containing Ac-SOD. The effect of the initial (Prot/Lip) on the IE of Ac-SOD was roughly as follows: a 4- to 5-fold increase of the initial (Prot/Lip) resulted in a 2- to 3-fold increase of the final (Prot/Lip) and a 2-fold decrease of the IE. Apparently, saturation was not achieved over the initial (Prot/Lip) range studied. In previous work, concerning the incorporation of SOD in SA-liposomes, similar results were obtained [9,12].

At an initial (Prot/Lip) of 13 μg/μmol, 30% of the total liposome-bound enzymatic activity is exposed on the liposome exterior, evidencing that surface-bound enzyme molecules are able to convert substrate molecules in the surrounding medium. A 4- to 5-fold increase of the initial

Table 3
Ac-SOD and SOD liposomal formulations: physicochemical characterization

Liposomes (enzyme)	(Prot/Lip)i (μg/μmol)	(Prot/Lip)f (μg/μmol)	IE (%)	∅ (μm) (PI)
PEG-Ac-SOD	24 ± 4	13 ± 2	54 ± 4	0.10 ± 0.02 (<0.12)
PEG-SOD	100 ± 5	18 ± 1	18 ± 3	0.10 ± 0.01 (<0.12)
SA-Ac-SOD	20 ± 3	12 ± 2	60 ± 5	0.12 ± 0.02 (<0.15)
SA-SOD	44 ± 2	17 ± 3	39 ± 2	0.11 ± 0.02 (<0.12)

PEG-Ac-SOD—LCL incorporating chemically modified SOD.
SA-Ac-SOD—conventional liposomes incorporating chemically modified SOD.
PEG-SOD—LCL incorporating native SOD.
SA-SOD—conventional liposomes incorporating native SOD.
Values are means ± S.D. of at least three independent preparations.

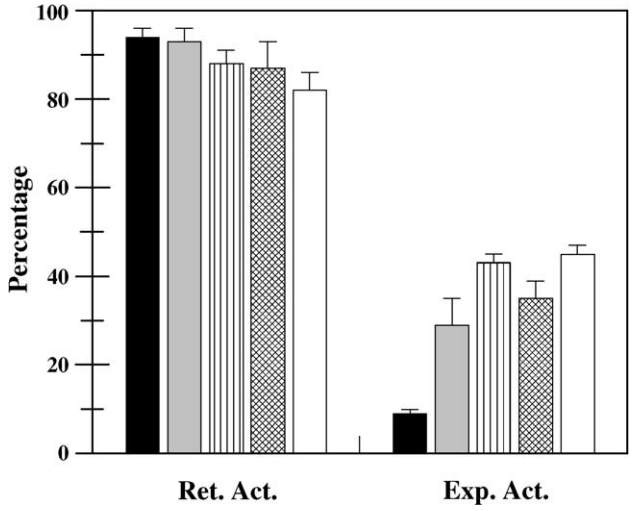

Fig. 2. Enzymatic activity retention of Ac-SOD and SOD incorporated in SA- and PEG-liposomes. Influence of the mean diameter (0.1 and 0.2 μm) on the total retention of enzymatic activity (Ret. Act.), and on the exposed enzymatic activity (Exp. Act.) to the liposome surface: SA-Ac-SOD-0.1 (▥) and SA-Ac-SOD-0.2 (▩): SA-liposomes with a mean diameter of 0.1 or 0.2 μm incorporating Ac-SOD. SA-SOD-0.2—SA-liposomes (■) with a mean diameter of 0.2 μm incorporating native SOD. PEG-Ac-SOD-0.1 (□) and PEG-Ac-SOD-0.2 (▨): PEG-liposomes with a mean diameter of 0.1 or 0.2 μm incorporating Ac-SOD. The initial lipid concentration was 32 μmol/ml and the initial protein concentration was 500 μg/ml. The results represent means ± S.D. of at least three preparations.

(Prot/Lip) yielded a 1.5-fold increase of the Exp. Act. This success in designing enzymosomes with 30–50% of the enzymatic activity exposed on the surface is critical for the evaluation of the basic hypothesis behind the present work: surface-located enzyme is to be preferred over release of enzyme for achieving therapeutic activity.

Liposome surface charge is a crucial determinant factor of the biological behaviour of liposome particles. The inclusion of SA in the liposomes confers a positive zeta potential. The incorporation of Ac-SOD, enzyme with a negative zeta potential (− 16.7 mV) affects the zeta potential of SA-liposomes incorporating this enzyme (Table 2). An increase of final Prot/Lip corresponds to a decrease of the Ac-SOD liposome zeta potential from 31 to 26 mV.

3.4. Effect of Ac-SOD on thermotropic behaviour of phospholipid bilayers

To determine to what extent the Ac-SOD interacts with the lipid bilayers, we measured the effect of Ac-SOD on the thermotropic behaviour of DMPC bilayers. We found that the phase-transition pattern of such liposomes changed from a sharp peak at 23.5 °C in control vesicles to a wide peak ranging from 22 to 25 °C, with the same overall heat content, after incorporation of the Ac-SOD.

3.5. Comparison of Ac-SOD enzymosomes and SOD liposomes

3.5.1. Physicochemical characterization, enzymatic activity and zeta potential

The IE of Ac-SOD either in SA-liposomes or PEG-liposomes was substantially higher in comparison with SOD (Table 3). For enzymosomes containing Ac-SOD, the IE was 1.5-fold higher in SA-liposomes and 3-fold higher in PEG-liposomes compared to the corresponding values for liposomes containing SOD. From a drug loading capacity point of view, this difference is translated to the need for using two to four times more SOD than Ac-SOD to reach the same final (Prot/Lip): 12–18 μg of protein to micromoles of lipid.

The enzymatic activity of Ac-SOD enzymosomes was compared with SOD liposomes (Fig. 2). The total retention of enzymatic activity associated with the liposome dispersions ranged from 82% to 94%, irrespective of the form of the enzyme, liposome type or size. This confirms that the experimental conditions used in this work did not affect the enzymatic activity. The Exp. Act. of Ac-SOD enzymosomes is 3- to 4-fold higher than that of SOD liposomes, which is

Table 4
Effect of nature of the incorporated enzyme (Ac-SOD or SOD) on liposomes zeta potential

Liposome	SA (Unloaded lip.)	SA-SOD	SA-Ac-SOD	PEG (Unloaded lip.)	PEG-SOD	PEG-Ac-SOD
Zeta potential (mV)	20 ± 2	22 ± 1	14 ± 1	− 3 ± 1	− 4 ± 1	− 5 ± 1
∅ (μm) (PI)	0.16 ± 0.01 (< 0.15)	0.16 ± 0.02 (< 0.17)	0.19 ± 0.02 (< 0.2)	0.17 ± 0.01 (< 0.10)	0.17 ± 0.01 (< 0.10)	0.16 ± 0.01 (< 0.12)

Initial protein concentration—500–700 μg/ml.
Initial lipid concentration—32 μmol/ml.
PEG-Ac-SOD—LCL incorporating chemically modified SOD.
SA-Ac-SOD—conventional liposomes incorporating chemically modified SOD.
PEG-SOD—LCL incorporating native SOD.
SA-SOD—conventional liposomes incorporating native SOD.
Unloaded lip.—liposomes devoid of protein.
Liposomes were rehydrated with citrate buffer, pH 5.6, 28 mosM.
Liposomes were sequentially extruded through polycarbonate membranes of different porosities. The last filter had a porosity of 0.1 μm.
Values are means ± S.D. of at least three independent preparations.

less than 10%. For vesicles containing Ac-SOD, the Exp. Act. was similar for both SA-enzymosomes and for PEG-enzymosomes with the same size. Larger liposomes with a mean diameter of 0.2 μm, had a lower Exp. Act. than 0.1-μm liposomes: 29% and 35% for SA-enzymosomes and for PEG-enzymosomes, respectively.

Table 4 compares the zeta potential of SA and PEG unloaded liposomes with the corresponding liposomes incorporating either Ac-SOD or SOD. SA-liposomes containing SOD showed a similar zeta potential as compared to unloaded SA-liposomes (20–22 mV). In contrast, the incorporation of negatively charged Ac-SOD molecules in SA-liposomes decreased the positive zeta potential by 8 mV. For PEG-liposomes, no statistically significant differences ($P>0.05$) in zeta potential values were observed between unloaded and Ac-SOD loaded liposomes.

4. Discussion

The chemical modification of SOD gave rise to a different enzyme entity [22] and so new methodologies to incorporate and characterize Ac-SOD were developed in this work. To reveal whether the use of Ac-SOD may offer advantages over SOD, galenic and pharmaceutical properties of respective liposomal forms were compared. The higher incorporation parameters obtained for Ac-SOD liposome formulations evidence the higher affinity of Ac-SOD for the hydrophobic region of the liposomal bilayers as compared to SOD. The higher IE of Ac-SOD in SA-liposomes compared to PEG-liposomes observed for lower initial (Prot/Lip) ratios (Fig. 1) may be due to the competition of Ac-SOD with Chol for the insertion in the liposomal bilayers. This competition is dependent on the molar amount of the sterol: 20 mol% in SA-liposomes and 33 mol% in PEG-liposomes and on the ratio of Protein to Chol being this effect negligible when the initial (Prot/Lip) increases (Table 3). This finding is in agreement with literature [30,31] where the competition of hydrophobic drugs with Chol is described. Another possible explanation could be the different electrostatic interactions occurring between the negatively charged Ac-SOD and the bilayers. The positively charged SA-liposomes can favour these interactions while the presence of the PEG at LCL surface may reduce the protein–lipid charge–charge interactions [32]. While in vitro toxicity against L1210 cells was observed, it was also shown that liposomes containing SA are less toxic than free SA [33]. Other experiments using SA-liposomes incorporating L-asparaginase showed less toxicity than free enzyme against Chinese hamster ovarian cells [34]. The observation that Ac-SOD enzymosomes present considerable enzymatic activity on their surface indicates that they can act independently of the rate and extent of enzyme release, as required in the case of SOD liposomes and so they can have a different mechanism and therapeutic action. The proportion of exposed Ac-SOD is somewhat larger for the smaller liposomes than for the larger. This effect may be explained by the larger exposed surface area of the smaller liposomes, which have a lower degree of lamellarity [35–37].

The influence of the negative charge of Ac-SOD molecules on the zeta potential of Ac-SOD liposomes was observed for SA-liposomes but not for PEG-liposomes. This is in according to Zeisig et al. [38] as the presence of PEG causes a masking effect on the surface charge of liposomes.

The widening of the thermotropic phase-transition peak by incorporation of Ac-SOD into DMPC vesicles indicates a decrease in cooperativity of the phase transition. This would be compatible with a perturbation of the acyl chain packing in the bilayers and thus with a hydrophobic interaction of the palmitoyl chains of the Ac-SOD with DMPC acyl chains. Native SOD encapsulated in DPPC liposomes was shown not to have such an effect [39].

Based on the above results, we propose that the two enzyme forms differ substantially regarding their intraliposomal location: SOD tends to be localized in the internal aqueous space with the additional possibility of electrostatic interaction with the cationic bilayers of SA-liposomes. The hydrophobic Ac-SOD is expected to interact with the acyl chain region of the bilayers, although electrostatic interactions between the protein and the interface of the bilayers are also likely to occur in the case of positively charged SA-liposomes.

The present results allow us to conclude that it is possible to design enzymosomes that display significant enzymatic activity on their surface via the use of acylated enzyme. Another important finding is that the surface-exposed enzyme is still able to convert substrate despite the presence of PEG chains on the surface of the PEG-enzymosomes. The PEG coating utilized was apparently not a barrier for the substrate to reach the exposed enzyme molecules. This point is crucial for the intended therapeutic application of PEG-enzymosomes containing Ac-SOD, as release of enzyme from extravasated liposomes is no longer a prerequisite for the dismutating activity within the inflamed target site. Ac-SOD enzymosomes made with PEG are circulating microreservoirs joining together the advantages of expressing activity before disruption and a sustained release pattern of the enzyme.

We envisage that Ac-SOD enzymosomes can be therapeutically used in the following ways: (1) for passive targeting to inflammation sites with a subsequent rapid and intense effect in rheumatoid arthritis, and (2) as long-circulating enzymatically active particles for the treatment of other diseases such as ischemia/reperfusion pathologies.

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