CONSTRUCTION OF ENZYMOSOMES: OPTIMIZATION OF COUPLING PARAMETERS

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ABSTRACT

A strategy not usually used to improve carrier mediated delivery of therapeutic enzymes is its attachment to the outer surface of liposomes. Few publications report the construction of carriers with surface attached enzymes. In contrast, many publications report the attachment of antibodies to liposome surface for active targeting.
In this work our goal and concept is to build enzymosomes**,** attaching covalently the therapeutic enzyme either directly to the outer surface of the phospholipids bilayer or bounding the enzyme to the terminus of polyetylenoglycol polymer chains, located at the surface of lipid vesicles.
For both cases we optimized the process of covalent link of enzyme in order to minimize alterations of the activity of the enzyme. A suitable enzyme load, keeping the vesicles structural integrity and preserving the enzyme activity were achieved, as can be concluded from the *in vitro* and *in vivo* experiments with superoxide dismutase enzymosomes.

Keywords: enzymosome, enzyme conjugation, PEGylated vesicles, antioxidant therapy, superoxide dismutase.

1 INTRODUCTION

Different strategies can be used to improve carrier mediated delivery of therapeutic proteins as [1]: its incorporation into polymeric carriers, into aqueous space of lipid, detergent or lipid-detergent vesicles or incorporation of hydrophobized ones into lipid bilayer of vesicles.
A strategy, not usually used for therapeutic enzymes, is its attachment to the outer surface of liposomes, using technologies developed for antibodies [2, 3, 4]. Few publications report the construction of carriers with surface attached enzymes in contrast with a huge number of publications with antibodies attached to carriers, a concept widely used for the active targeting of nanocarriers.
Superoxide Dismutase (SOD) is used as a therapeutic agent for oxidative stress related diseases such as Rheumatoid Arthritis and Ischaemia/Reperfusion situations. Our objective was to develop and optimize SOD enzymosomes, with long circulation times in the blood in order to accumulate at inflamed target sites, while maintaining enzymatic activity in its intact form.

2. MATERIALS AND METHODS

2.1 Materials

N-[4-(p-maleimidophenyl)butyrate]-phosphatidyl-ethanolamine (MPB-PE); 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Maleimide(Polyethylene Glycol) 2000] (Maleimide-PEG-PE); Egg phosphatidylcholine (PC) and 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (PEG-PE) from Avanti Polar Lipids. N-succinimidyl S-acethylthioacetate (SATA) and *N*-hydroxysulfosuccinimide (NHS) from Pierce. Superoxide dismutase (SOD) from Sigma.

2.2 Preparation of liposomes

Multilamelar liposomes were prepared with mixtures of lipids, dissolved in chloroform, dried until formation of an homogeneous film, which was then dispersed in citrate buffer, pH 6.0. The dispersions extruded through polycarbonate membrane filters in an extruder (Lipex Biomembranes). The liposome dispersions were diluted, with buffer, to a total lipid concentration of 10 mM.

2.3 Conjugation of SOD to liposomes

The conjugation of SOD to liposomes containing the lipid anchors MPB-PE or Maleimide-PEG-PE was performed according to [2, 3], with some modifications [4]. In brief the thioacetylation reagent, SATA, in dimethylformamide was mixed with the enzyme in a buffer solution. The thioacetylated enzyme, SOD-ATA, after separation from unreacted SATA on a PD-10 column, was de-acetylated with hydroxylamine. The thiolated enzyme, SOD-AT was added to the liposomes containing the reactive groups MPB or maleimide. Enzymosomes were separated by ultracentrifugation and suspended in buffer.

2.4 Characterization of the enzymosomes

Mean particle size was measured by dynamic light scattering with a Malvern Zeta III. The protein coupled to liposomes was determined according to a modification of the method described by Lowry at al. Phospholipids were assessed by the colorymetric assay of Fiske and Subbarow. Free amino groups were assayed with the method described by Bohlen et al. The catalytic activity of SOD was measured according to Misra and Fridovich method.

3. RESULTS

Conjugation of SOD directly to MPB reactive groups located at the liposome outer surface was performed using different liposome compositions. The results are in Table 1.

Table 1: Performances of the conjugation of SOD to the lipid linker MPB-PE as a function of SOD-enzymosomes lipid composition and SATA:SOD molar ratio.

Liposome composition (molar percentage)	Conjugated SOD	
	μg SOD/ μmol lipid	activity of SOD / μmol lipid
SATA:SOD (molar ratio) 8:1		
PC:Chol:PG:MPB-PE (64 : 6.7 : 28 : 1.25)	20	85
PC:Chol:MPB-PE (68.25:30.5:1.25)	37	230
PC:Chol: MPB-PE (67 : 30 : 2.5)	20	215
SATA:SOD (molar ratio) 4:1		
PC:Chol:MPB-PE (68.25:30.5:1.25)	17	173

SOD-AT / Lipid molar ratio: in the range 40 to 50.
Lipid concentration (liposome suspension):10 μmol/ml.

Using the selected parameters (in bold, Table 1) a study of the effect of the percentage of maleimide-PEG-PE in the conjugation parameters was performed for percentages of this lipid linker in the range of 0.25 to 1.25%. The total PEG chains (reactive and non-reactive) was 1.25% for all the cases. The percentage of maleimide-PEG-PE was corrected by an equivalent percentage of PEG-PE, in order to keep constant the total number of PEGylated chains. The results are in Figure 1.

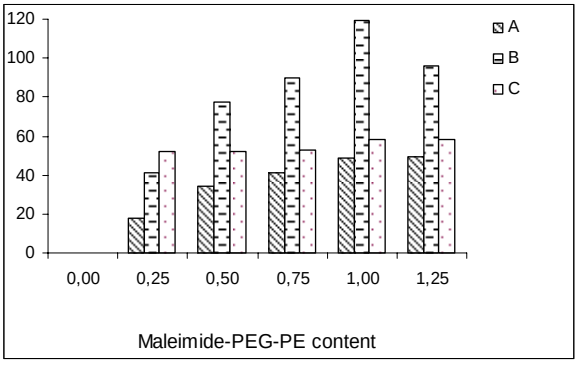

Figure 1 Effect of maleimide-PEG-PE content in the characteristics of SOD-PEG-enzymosomes.
[A] Conjugated protein (μg SOD/μmol lipid);
[B] Conjugation Efficiency:
$(((Protein/Lipid)_{conjugated}/(Protein/Lipid)_{incubated})\times 100)$;
[C] Retained enzymatic activity (%).

A comparison of the in vivo fate, blood clearance, of ^{111}Indium labeled long-circulating SOD carriers, SOD-PEG-enzymosomes (SOD-Enzymosomes) and SOD-PEG-liposomes containing SOD in the aqueous space (SOD Long-Circulating Liposomes), was performed in Wistar rats. The profiles obtained are presented in Figure 2.

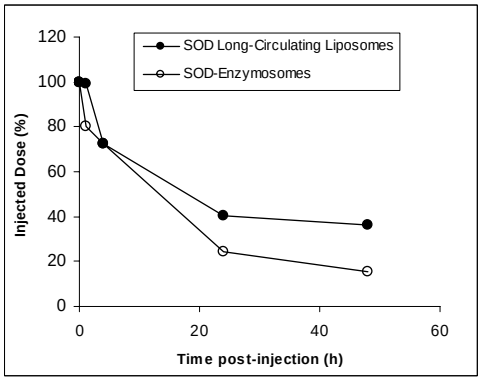

Figure 3. Comparison of in vivo fate of *iv.* injected (in Wistar rats): SOD-PEG-enzymosomes and SOD-PEG-liposomes.

4. CONCLUSIONS

According with the results of the studies performed to optimize the conjugation of SOD to the surface of vesicles (PEGylated and non PEGylated vesicles) a formulation was selected for in vivo studies: SOD derivative prepared from a SATA:SOD molar ratio of 8:1 and a lipid composition EPC:Chol:Maleimido-PEG-PE:PEG-PE with a molar ratio of 68,25:30,5:0,75:0,50.
From the enzymatic activity results and also from imaging and biodistribution studies the selected SOD-PEG-enzymosome formulation is adequate to be used as therapeutic agents.

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