

## **SUPEROXIDE DISMUTASE LOADED LIPOSOMES. THE INFLUENCE OF FORMULATION FACTORS ON ENZYME ENCAPSULATION AND RELEASE**

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### **Abstract**

The objective of this paper was to prepare superoxide dismutase (SOD) loaded conventional and PEG-coated (polyethylene glycol) liposomes and to study the influence of some formulation factors on their characteristics in terms of facilitating antioxidant potential and delivery. An experimental design with 3 factors and 2 levels was used, in order to optimize SOD encapsulation efficiency and to determine the activity of released SOD in certain experimental conditions. The studied variables were: the molar ratio phospholipids:cholesterol, the type of phospholipids used and the concentration of SOD solution used for liposomes preparation. The liposomes were characterized through SOD concentration, encapsulation efficiency, % of SOD released after 24h at 4°C and % of SOD released after 24h at 37°C. The results showed that the concentration of SOD solution used in the preparation process had a significant effect on all studied responses, making this drug delivery systems promising for reliable and safe therapies.

### **Rezumat**

Obiectivul acestei lucrări a fost prepararea unor lipozomi convenționali și PEG-ilați (polietilen glicol) cu superoxid dismutază (SOD) și studiul influenței unor factori de formulare asupra proprietăților lor, în ceea ce privește potențialul lor antioxidant și de transport. S-a folosit un plan experimental cu 3 factori și 2 nivele, cu scopul de a optimiza eficiența încorporării SOD și de a determina activitatea SOD după cedare din lipozomi în anumite condiții experimentale. Variabilele studiate au fost: raportul molar fosfolipidă:colesterol, tipul fosfolipidei utilizate și concentrația soluției de SOD folosită la prepararea lipozomilor. Lipozomii au fost caracterizați prin concentrația de SOD, eficiența încorporării SOD, % de SOD cedat după 24h la 4°C și % de SOD cedat după 24h la 37°C. Rezultatele au arătat că factorul care a influențat semnificativ toate caracteristicile lipozomilor a fost concentrația soluției de SOD folosită în procesul de preparare a lipozomilor, făcând acest sistem de transport pentru SOD unul potrivit pentru o terapie sigură și eficientă.

**Keywords:** superoxide dismutase (SOD), liposomes, formulation, encapsulating efficiency.

## Introduction

Superoxide dismutase (SOD) is an antioxidant enzyme and potential anti-inflammatory agent. Its therapeutic applications are however limited due to very short plasma half-life and poor cellular penetration [6]. Therefore, appropriate delivery systems are searched for SOD and liposomal formulations are one of the promising approaches, because liposomes are highly efficient in terms of facilitating antioxidant delivery and achieving prophylactic and therapeutic efficacies against oxidative stress-induced damage [13].

Conventional liposomes have the major drawback that are rapidly cleared by the reticular endothelial system and this can be avoided by utilizing sterically stabilized liposomes. The sterical stabilization is achieved mainly by modifying the surface of the liposomes with hydrophilic polymers such as polyethylene glycol (PEG) [18].

The activity of liposomes as carrier for drugs depends upon various factors such as charge, rigidity, composition of the liposomal membrane, encapsulating efficiency, stability, release rates and body distribution after administration [7]. In order to meet particular needs, liposomes can be formulated using a variety of lipids and lipid mixtures or some of their physicochemical properties such as size, structure and surface charge can be modified. One of the most important goals in formulation of liposomes is to encapsulate a sufficient amount of therapeutic agent, so that they can be therapeutically effective. The encapsulation efficiency depends on factors related to properties of both drug and liposomes [10,14]. Encapsulation of hydrophilic molecules such as SOD is difficult, due to their own affinity with the outer aqueous phase which determines increased diffusion of molecules to be entrapped.

There is considerable interest towards the development of drug-delivery systems that would result in the selective delivery of antioxidants to tissues in sufficient concentrations in order to ameliorate oxidant-induced tissue injuries. The ability for precise adjustments of liposome parameters such as size, charge, lipid composition, and the conjugation of ligands coupled with the more efficient and safer loading techniques offers the flexibility to make liposomes an effective platform for the delivery of antioxidants [13]. Antioxidants have yet to be rendered into reliable and safe therapies because of their poor solubility, inability to cross membrane barriers, extensive first-pass metabolism, and rapid clearance from cells. Antioxidant liposomes should be particularly useful for treating diseases or conditions in which oxidative stress plays a significant pathophysiological role because this technology has been shown to suppress oxidative stress.

These diseases and conditions include cancer, trauma, irradiation, retinotherapy or prematurity, respiratory distress syndrome, chemical weapon exposure, and pulmonary infections [12].

In the current paper we have studied the influence of some formulation factors on the encapsulation of SOD in liposomes and on its release in active form. An experimental design has been used, with the purpose of optimizing the encapsulation efficiency of SOD. The experimental design and optimization protocols are well described in the literature for the development of different pharmaceutical formulations. In our group of research, these techniques have particularly been applied for pellets [17], minitables [3,16] and tablets [15,5] formulation, as well as for formulation of nano- and microparticles [11,1].

### Materials and Methods

**Chemicals.** Superoxide Dismutase from bovine erythrocytes (3200U/mg protein), cytochrome C from horse heart, Xanthine, Xanthine Oxidase from bovine milk, L- $\alpha$ -phosphatidylcholine (PC) (Egg Chicken,  $\geq 50\%$  TLC) were purchased from Sigma-Aldrich Chime (Germany). Cholesterol (ovine wool,  $>98\%$ ) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG-DSPE) were from Avanti Polar Lipids (USA). All other chemicals used were of reagent grade.

**Experimental design.** An experimental design with 3 factors and 2 levels was developed using Modde 9 software (Umetrics, Sweden) [8], in order to study the influence of some formulation factors on SOD encapsulation in liposomes and on its release 24 hours after preparation, at 4°C and 37°C. The selected formulation factors (independent variables) were: the molar ratio phospholipids: cholesterol, the type of phospholipids used and the concentration of SOD solution (mg/mL) used for liposomes preparation. These factors and their levels of variation are presented in Table I. The effect of independent variables was investigated on SOD concentration in liposomes (U/mL), encapsulation efficiency (%), % of SOD released after 24h at 4°C and % of SOD released after 24h at 37°C (Table II). Table III presents the matrix of the performed experiments.

**Table I**  
Independent variables and their level of variation

Formulation variables	Symbol	Levels	
Molar ratio phospholipids: cholesterol	X <sub>1</sub>	2	5
Type of phospholipid	X <sub>2</sub>	PC	PC+PEG-DSPE
Concentration of SOD solution (mg/mL)	X <sub>3</sub>	0.1	0.4

**Table II**  
Dependent variables (responses)

No.	Response	Symbol
1	SOD concentration in liposomes (U/mL)	Y <sub>1</sub>
2	Encapsulation efficiency (%)	Y <sub>2</sub>
3	% SOD released at 24h, 4°C	Y <sub>3</sub>
4	% SOD released at 24h, 37°C	Y <sub>4</sub>

**Table III**  
Matrix of experimental design

Exp. name	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>
N1	2	PC	0.4
N2	5	PC+PEG-DSPE	0.1
N3	2	PC	0.1
N4	5	PC+PEG-DSPE	0.4
N5	2	PC	0.25
N6	2	PC	0.25

In order to determine a statistical correlation between the formulation factors studied and the observed responses, statistical parameters calculations were performed with the statistical module from Modde 9 software (Umetrics, Sweden).

**Preparation of liposomes.** The liposomes were prepared by lipid film hydration method, essentially according to the technique first described by Bangham *et al.* [2]. Briefly, phospholipids and cholesterol were dissolved in 2 mL ethanol in a 300 mL round-bottomed flask. After complete dissolution, the solvent was evaporated under reduced pressure at 50°C in a rotary evaporator, leading to the formation of a thin and homogeneous film of lipids on the surface of the flask. In order to completely remove the residual solvent, the film was maintained under a nitrogen gas flow for 1h. Then the film of lipids was hydrated with 5 mL SOD aqueous solution in phosphate buffered saline (PBS, pH=7.8) for 15 minutes at 37°C. The resulted liposomes dispersion was maintained overnight at 4°C and then the excess unloaded SOD was removed by centrifugation (30 minutes, 8000×g).

**Determination of SOD content and encapsulation efficiency.** The activity of liposome-encapsulated SOD was determined in samples obtained after liposomes were lysed with Triton-X-100 in a final concentration of 0.5% (w/v). SOD activity was measured by the xanthine/xanthine-oxidase/cytochrome c method according to McCord and Fridovich [9]. In this method, superoxide radical produced by the xanthine/xanthine-oxidase

system reduces the cytochrome c and its reduced form can be assessed spectrophotometrically at 550 nm. SOD competes with cytochrome c for the dismutation of superoxide radical and SOD activity in the sample is measured as the inhibition of the rate of reduction of cytochrome c by the superoxide radical. One SOD unit is equivalent to the SOD activity that inhibits the rate of reduction of cytochrome c by 50%. All activity measurements were performed using a blank containing the same Triton-X 100 concentration as the liposome sample.

SOD content (U/mL) represents the activity of SOD in the lysed liposomes per 1 mL of liposomal dispersion. Encapsulation efficiency (%) is calculated from the ratio of the encapsulated SOD activity and the total SOD activity used in the preparation of liposomes.

**Determination of released SOD activity.** The activity of released SOD from liposomes was assessed 24 hours after their preparation and purification. For this purpose, liposomes samples (1 mL) were maintained at 4°C and 37°C for 24 hours. After this period, the samples were centrifuged (30 minutes, 8000×g), supernatants were withdrawn and SOD activity was assessed according to the method of McCord and Fridovich. Results were expressed as the ratio between SOD concentration in the supernatants and initial SOD concentration in liposomes.

## Results and Discussion

**Liposomes' preparation and characterization.** SOD was encapsulated into two types of liposomes, conventional and PEG-coated liposomes, by film hydration method, and the influence of three formulation factors on liposomes' characteristics was studied according to an experimental design. Liposomes were prepared using different molar ratios between phospholipids and cholesterol. The phospholipid was PC in the case of conventional liposomes and a mixture of PC and PEG-DSPE (molar ratio 1.85:0.15) in the case of PEG-coated liposomes. The main characteristics (responses) of liposomes prepared are presented in Table IV.

**Table IV**  
Matrix of responses

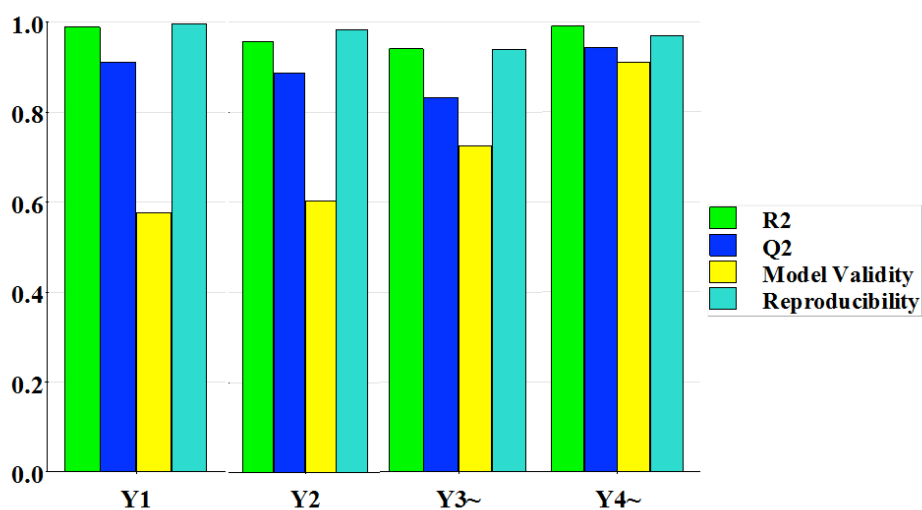
Exp. name	Y <sub>1</sub>	Y <sub>2</sub>	Y <sub>3</sub>	Y <sub>4</sub>
N1	194.6	9.9	62.5	55.4
N2	69.7	14.1	36.9	32.7
N3	101.3	20.5	20.9	5.2
N4	283.5	14.4	48.8	39.0
N5	152.3	15.4	71.7	20.2
N6	145.3	14.7	87.2	16.2

Y<sub>1</sub>: SOD concentration in liposomes (U/mL);

Y<sub>2</sub>: Encapsulation efficiency (%); Y<sub>3</sub>: % SOD released at 24h, 4°C; Y<sub>4</sub>: % SOD released at 24h, 37°C.

These responses were chosen based on two main purposes. First we aimed to study the influence of the formulation factors on SOD encapsulation and encapsulation efficiency with the objective of optimizing the encapsulation efficiency. Second, we tested the influence of formulation parameters on SOD release in active form in certain experimental conditions designed for testing its biological activity.

**Experimental Design Analysis. Goodness of Fit.** The matrix of the results is shown in Table IV. The statistical module from Modde 6 software was used in order to fit the experimental data with the chosen experimental design and to calculate the statistical parameters. To check the validity of the experimental design, the following statistical parameters were determined:  $R^2$ ,  $Q^2$  and ANOVA (analysis of variance) test.



**Figure 1.**

Summary of fit for the experimental design

Y<sub>1</sub>: SOD concentration in liposomes (U/mL); Y<sub>2</sub>: Encapsulation efficiency (%); Y<sub>3</sub>: % SOD released at 24h, 4°C; Y<sub>4</sub>: % SOD released at 24h, 37°C.

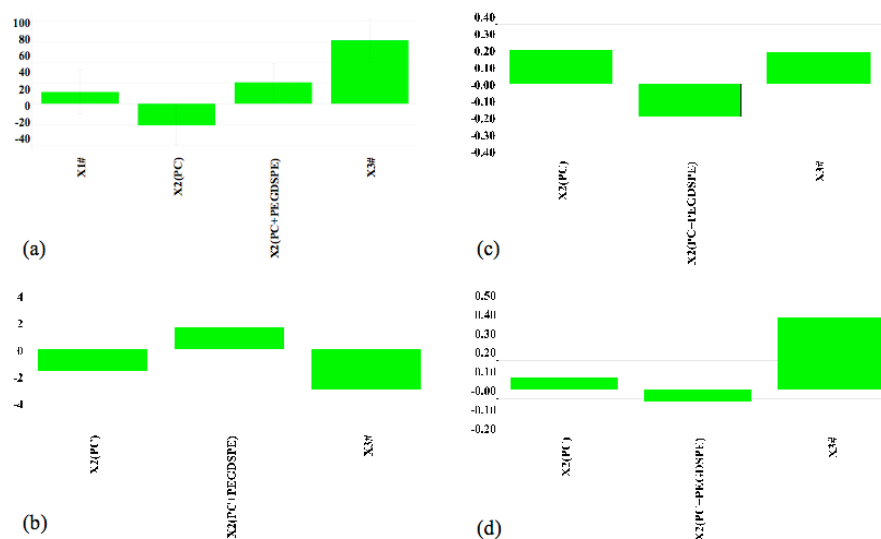
$R^2$  and  $Q^2$  provide the best summary of fitting the model. The results obtained after fitting and the statistical parameters calculation, using data obtained from the experimental design, are shown in Figure 1. The results fit well for all responses. The ANOVA test shows if the variance of the results is determined by the modifications of the formulation factors or represents a variance determined by experimental errors [16,3]. The results of the ANOVA test have shown that the experimental data obtained for all responses were good ( $p$  for model was lower than 0.05 and  $p$  for residual was higher than 0.05) for all responses.

**Experimental Design Analysis. The influence of formulation factors on liposomes' properties.** The influence of the formulation factors on SOD concentration in liposomes, encapsulation efficiency, % of SOD released after 24h at 4°C and % of SOD released after 24h at 37°C is presented in Figure 2. As shown in this figure, all the studied responses were influenced by the concentration of SOD solution used for liposomes preparation, while the molar ratio phospholipids: cholesterol had no significant influence on the studied responses.

SOD concentration in liposomes (Y1) was significantly influenced by two formulation factors: the type of phospholipid and concentration of SOD solution used in the preparation process. SOD concentration in liposomes was higher in the case of PEG-coated liposomes than in the case of conventional liposomes. It appears that the use of a lipid composition with higher hydrophilic properties increase the encapsulation of the hydrophilic SOD molecule. The amount of SOD encapsulated in liposomes increased when a higher concentration of SOD was used in the preparation process.

SOD encapsulation efficiency (Y2) was influenced by the initial SOD concentration. The encapsulation efficiency increased when lower SOD concentrations were used in the preparation process. Previously, Galovic et al. (2002) [4] showed an increase of entrapment efficiency by increasing the initial SOD concentration from 0.1 to 0.7 mg/mL and a decrease of encapsulation when higher SOD concentrations were used, when preparing SOD liposomes using proliposome method. The differences may be due to the different preparation methods and lipid composition used.

The % of SOD released after 24h at 4°C (Y<sub>3</sub>) and 37°C (Y<sub>4</sub>) significantly increased when the concentration of SOD used in the preparation process increased. The percentage of active SOD outside the liposomes 24h after preparation was higher when the liposomes were maintained at 4°C than in the case of keeping them at 37°C, maybe due to partial loss of activity of released SOD at 37°C. Thus, at 4°C the highest activity of released SOD was almost about 87% of initial SOD content while at 37°C the highest activity was about 55%. Concerning the influence of lipid film composition on these two responses, the % of released SOD after 24h, whatever the temperature, was higher in the case of conventional liposomes than in the case of PEG-coated liposomes.



**Figure 2.**

The influence of the formulation factors on:  $Y_1$ , SOD concentration in liposomes (U/mL) (a);  $Y_2$ , encapsulation efficiency (%) (b);  $Y_3$ , % of SOD released at 24h, 4°C (c); and  $Y_4$ , % of SOD released at 24h, 37°C.

**Optimum formula determination.** The optimization module from Modde 6 software permits the determination of the optimum formula. In this case, we searched the optimum conditions for SOD encapsulation in liposomes. The optimum formulation was obtained using the mixture of PC and PEG-DSPE (molar ratio 1.85:0.15) and a concentration of 0.1 mg/mL for SOD solution, for any of the studied molar ratios phospholipids:cholesterol.

### Conclusions

This work presents the possibility to encapsulate SOD in conventional or PEG-coated liposomes by film hydration method, and a study concerning the influence of some formulation factors such as the molar ratio phospholipids:cholesterol, the type of phospholipids and the concentration of SOD solution on SOD encapsulation and release. The most important factor, that influenced both studied liposomes' characteristics, was the concentration of SOD solution used in the preparation process.

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