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# Biodegradable Microspheres as Carriers for Native Superoxide Dismutase and Catalase Delivery

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## ABSTRACT

The purpose of this research was to encapsulate superoxide dismutase (SOD) and catalase (CAT) in biodegradable microspheres (MS) to obtain suitable sustained protein delivery. A modified water/oil/water double emulsion method was used for poly(D,L-lactide-co-glycolide) (PLGA) and poly(D,L-lactide) PLA MS preparation co-encapsulating mannitol, trehalose, and PEG400 for protein stabilization. Size, morphology, porosity, mass loss, mass balance, in vitro release and in vitro activity were assessed by using BCA protein assay, scanning electron microscopy, BET surface area, and particle-sizing techniques. In vitro activity retention within MS was evaluated by nicotinammide adenine dinucleotide oxidation and $H_2O_2$ consumption assays. SOD encapsulation efficiency resulted in 30% to 34% for PLA MS and up to 51% for PLGA MS, whereas CAT encapsulation was 34% and 45% for PLGA and PLA MS, respectively. All MS were spherical with a smooth surface and low porosity. Particle mean diameters ranged from 10 to 17 μm. CAT release was prolonged, but the results were incomplete for both PLA and PLGA MS, whereas SOD was completely released from PLGA MS in a sustained manner after 2 months. CAT results were less stable and showed a stronger interaction than SOD with the polymers. Mass loss and mass balance correlated well with the release profiles. SOD and CAT in vitro activity was preserved in all the preparations, and SOD was better stabilized in PLGA MS. PLGA MS can be useful for SOD delivery in its native form and is promising as a new depot system.

**KEYWORDS:** superoxide dismutase, catalase, microspheres, protein delivery, in vitro activity.

## INTRODUCTION

Protein delivery has been intensively investigated during recent years.[1,2] The main issue is related to protein complex architectures that determine the high biological activity of such macromolecules. Therefore, it is mandatory protein-polymer compatibility when formulating polymeric controlled delivery systems for protein molecules. Poly(D,L-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) are biodegradable polymers widely used for microencapsulation of biological and nonbiological molecules. Although many previous works demonstrated the effectiveness of PLA and PLGA as encapsulating agents and their biocompatibility,[3-5] they present some drawbacks when employed for protein delivery.[6] The main reason is that these polymers show low affinity with very hydrophilic molecules, such as proteins, owing to their intrinsic hydrophobicity. Therefore, protein and peptide entrapment in biodegradable PLA and PLGA microspheres (MS) is not an easy task since any possible interaction during the preparation process and within MS may lead to protein denaturation with high loss of activity.[7,8] Several strategies have been tested to guarantee, to a certain extent, native protein encapsulation, and good results have been obtained by co-encapsulation of stabilizers (eg, sugars, hydrophilic polymers).[9-11] Composite MS were successfully prepared by PLGA coating of starch MS able to entrap and protect the proteins and protect them from interaction with the surrounding environment.[12] However, preparation of such systems is often laborious, and many parameters must be maintained under control. This is particularly evident when attempts are made for enzyme delivery. Superoxide dismutase (SOD) and catalase (CAT) are among the most potent antioxidants known in nature. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, and it is widespread in nature in eukaryotic and prokaryotic organisms.[13] There are at least 3 forms of SOD in nature. Human and bovine erythrocytes contain SOD enzyme with divalent copper and divalent zinc. Chicken liver mitochondria and *Escherichia coli (E coli)* contain a form with trivalent manganese.[14,15] *E coli* also contains a form of the enzyme with trivalent iron. The Cu-Zn enzyme is a dimer of molecular weight (MW) 32 500 and the 2 subunits are joined by a disulfide bond. Although the enzyme is not particularly fast in spontaneous dismutation of superoxide radicals, the ability of the enzyme to provide protection to organisms is demonstrated by the existence of a motor neuron disease in individuals who have point mutations in SOD and by the finding that the lack of SOD can lead to a form of anemia.[16]

CAT is among the most efficient antioxidants known so far. It is present in the peroxisomes (membrane-bound microbody organelles that house various oxidation reactions in which toxic peroxides are generated as side products) of nearly all aerobic

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cells and serves to protect the cell from the toxic hydrogen peroxide effects by catalyzing its decomposition into molecular oxygen and water without the production of free radicals. In addition, CAT acts on toxic compounds by peroxidative reaction, and this group of compounds includes phenols, formic acid, formaldehyde, and alcohols. The protein exists as a dumbbell-shaped tetramer of 4 identical subunits (220 to 350 kd). Each monomer contains a heme prosthetic group at the catalytic center. CAT monomers from certain species (eg, cow) also contain one tightly bound nicotinammide adenine dinucleotide (NADP) per subunit. This NADP may serve to protect the enzyme from oxidation by its $H_2O_2$ substrate.[17,18]

It is well known that one of the main issues in using protein as drugs is their inability to cross the cell membrane as they are highly hydrophilic. For this reason liposomes have been investigated as SOD and CAT carriers.[19-23] When entrapped in liposomes, SOD proved to be effective against several forms of inflammatory manifestations (ie, rheumatoid arthritis,[24] ischemia, and liver necrosis).[25,26] CAT liposomes were used to treat alveolar type II cell injury caused by hydrogen peroxide. In spite of their efficacy in delivering SOD and CAT, liposomes offer several shortcomings related to their well-known instability and their inability to ensure long-term sustained release. In turn, polymeric MS have proved to be stable and able to release drugs gradually in a sustained manner. Moreover, SOD MS have been formulated using colyophilization technique for polyethylene glycol (PEG)-SOD MS formation and further coating with a PLGA polymer.[27] Recently, water/oil/water (w/o/w) method was employed for SOD encapsulation in PLGA MS.[28] CAT gel MS were instead used for its delivery to the joints.[29]

In light of these considerations, SOD and CAT are good candidates for microencapsulation in biodegradable PLA and PLGA MS aimed to treat inflammatory manifestations as depot systems. Obviously, all precautions have to be taken to avoid their denaturation and consequent loss of activity. Thus, the aim of this study was the preparation of SOD- and CAT-loaded PLA and PLGA MS and their characterization in terms of loading, morphology, size, porosity, in vitro release, mass balance, mass loss, and in vitro activity of the entrapped enzymes. The validity of such formulations was evaluated on the basis of their capacity to release native enzymes. For this purpose, stabilizers (ie, mannitol, trehalose, and PEG400) were co-encapsulated with the proteins.

## Materials and Methods

### *Materials*

CAT and SOD from bovine liver, PLA (MW 106 000 d) and PLGA (50:50, MW 40 000-70 000 d) polymers, micro-BCA protein test kit and bovine serum albumin (BSA) standards, hydrogen peroxide ($H_2O_2$), mannitol, trehalose, PEG400, NADH reduced, and poly(vinyl alcohol) (PVA, MW 30-70 kd) were purchased from Sigma Aldrich Chemical (Milan, Italy). Diethanolamine, triethanolamine, and mercaptoethanol were provided by Fluka (Milan, Italy). Sodium hydrogen orthophosphate, concentrated hydrochloric acid, and sodium hydroxide were from Farmitalia Carlo Erba (Milan, Italy), and methylene chloride was from J. T. Baker (Milan, Italy). Ethylenediaminetetraacetic acid (EDTA) and manganese chloride were from Galeno (Milan, Italy). Ultra pure water was obtained by reverse osmosis through a Milli-Q system (Millipore, Rome, Italy). All other reagents and solvents were of the highest purity available.

### *Microsphere Preparation*

Enzyme-loaded PLA and PLGA MS were prepared by modification of a w/o/w double emulsion method elsewhere proposed.[30] Briefly, an amount of SOD and CAT corresponding to 10% loading was dissolved in different volumes (300-70 µL) of 88 mM mannitol, trehalose, and PEG400 solutions (pH 7.4), respectively. The protein solutions were added upon stirring to a small volume of 15% to 25% polymer-methylene chloride solution to form a w/o emulsion. After proper emulsification, the emulsion was injected into 50 mL of 6% PVA solution under stirring (1500 rpm, at 4°C) to form a primary w/o/w double emulsion. Then the double emulsion was poured into 500 mL of deionized water and maintained at 4°C. In order to evaporate the organic solvent, the temperature was slowly increased up to 15°C to 20°C over 2 hours. The resulting MS were filtered by a Millipore 5-µm nitrocellulose filter, washed with deionized water, and vacuum dried at room temperature overnight.

### *Evaluation of Enzyme Encapsulation*

In order to extract the proteins from the MS, enzyme-loaded MS samples (5 mg) were dissolved in 0.5 mL 1M NaOH by overnight rotation; then the solution was neutralized with 0.5 mL 1M HCl. Protein content in the samples was determined by micro-BCA protein assay[31] by using an ultraviolet and visible absorption (UV/VIS) Jasco N-520 spectrophotometer (Jasco Inc, Easton, MD). All data are the result of 3 measurements, and the error was calculated as SD.

### *Enzyme-microsphere Interaction and Stability*

Enzyme-polymer interaction and stability were evaluated by dissolving SOD and CAT in 0.1 M phosphate buffered saline (PBS) (pH 7.4) in the presence of blank PLA and PLGA MS and by incubating the samples at 37°C. Control samples made of protein 0.1 M PBS solutions were also incubated in the same conditions. Aliquots were periodically withdrawn and submitted to micro-BCA assay. All data are the average of 3 measurements, and the error was expressed as SD.

### *Size Distribution, Morphology, and Porosity*

Size distributions of SOD and CAT containing PLA and PLGA MS were determined by Accusizer 770 Optical Particle Sizer (PSS Inc, Santa Barbara, CA). Morphology was investigated by scanning electron microscopy (SEM) using a Philips XL30 microscope (Philips Electron Optics, Heindoven, NL). Porosity was evaluated by using a Micromeritics ASAP 2010 apparatus (Micromeritics, Norcross, GA) and expressed as BET surface area. The error was calculated as SD on triplicate samples.

### *In Vitro Release Study*

The in vitro SOD and CAT release was determined by suspending PLA and PLGA MS in 10 mL of 0.1 M PBS (pH 7.4) and by incubating them at 37°C. At predetermined intervals, samples were centrifuged (2000 rpm, 1 minute, room temperature) and 1 mL of the supernatant was removed for micro-BCA protein assay and replaced with an equal volume of fresh medium. The analysis was performed in triplicate. Mass balance analysis was also performed at different time points in order to establish the protein remaining in the MS and to highlight possible degradation during the release experiment. In detail, release samples were taken and supernatants were separated by centrifugation. The pellets were then dissolved in 0.5 mL 1 M NaOH by overnight rotation, and the solution was neutralized with 0.5 mL 1 M HCl. Enzyme content was established by micro-BCA protein assay. Analyses were performed in triplicate and the error was calculated as SD.

### *Microsphere Mass Loss and Degradation*

Mass loss and degradation of enzyme-loaded PLA and PLGA MS during the release experiment were investigated upon suspension of 20 to 30 mg of MS in 10 mL of 0.1 M PBS (pH 7.4) and incubation at 37°C. Samples were taken at 0, 30, 45, and 90 days, and the MS were recovered by filtration through a 5-μm nitrocellulose filter (Lida Manufacturers Inc, London, UK) and vacuum dried for 48 hours. Mass loss was determined by the difference between the initial weight and the weight of the recovered MS after drying. Analyses were performed in triplicate, and the error was calculated as SD. Correlation between changes in morphology due to degradation and mass loss was investigated by SEM at the same time intervals on the recovered MS samples.

### *In Vitro Activity*

Entrapped SOD and CAT activity retention was evaluated in vitro according to the methods proposed elsewhere.[32,33] Briefly, SOD activity study was performed by reading the absorbance decrease at 340 nm during 10 minutes at 25°C due to NADH oxidation induced by mercaptoethanol. Slope increment owing to SOD addition was calculated, and the relative activity of the enzyme extracted from the MS was determined using a standard curve created by plotting the logarithm of SOD amount versus the percentage slope increment. SOD activity retention was expressed as percentage ratio between the active SOD amount resulting from the standard curve and the theoretical SOD amount added and measured by micro-BCA protein assay. All samples and standards were allowed to equilibrate at 25°C before running each experiment. Similarly, CAT activity was measured at 25°C by directly reading $H_2O_2$ absorbance decrease at 240 nm during 10 minutes after enzyme addition. In order to calculate the activity of the CAT extracted from the MS, a standard curve obtained by plotting $H_2O_2$ concentration versus CAT amount was employed. CAT activity retention was expressed as percentage ratio between the active CAT amount resulting from the standard curve and the theoretical CAT amount added, measured by micro-BCA protein assay. All samples and standards were allowed to equilibrate at 25°C before running each experiment. Enzyme extractions were performed by suspending 10 mg of MS in 0.1 M PBS, and the samples were incubated for 4 hours at 37°C. Analyses were performed in triplicate, and the error was calculated as SD.

## Results and Discussion

The effect of the preparation parameters on the MS properties was evaluated in order to assess the optimal conditions for SOD and CAT inclusion in PLA and PLGA MS (Table 1). Generally, the loading was increased when the volume of the protein solution adopted for the w/o emulsion was reduced from 300 to 100 μL. Volumes less than 100 μL resulted in a lower protein encapsulation either for SOD or CAT MS. In fact, too small aqueous volumes could cause increase of the interface surface available for protein diffusion. The same effect was observed when too large aqueous volumes (≥300 μL) were employed. Thus, 150 to 100 μL was established as the optimal volume range for SOD- and CAT-loaded MS preparation. The highest protein content in PLA MS was observed when mannitol and trehalose were co-encapsulated with CAT and SOD, respectively. Moreover, PLGA MS showed higher SOD entrapment efficiency than PLA MS, and a 51% encapsulation efficiency was achieved when using mannitol. In contrast, CAT-loaded PLGA MS did not show remarkable improvement in comparison to CAT-loaded PLA MS. The highest CAT encapsulation efficiency (45%) was obtained when co-encapsulating mannitol in PLA MS. These findings imply that PLGA is probably more efficient for SOD entrapment, whereas PLA is more efficient for CAT. Moreover, the SOD loading in PLGA MS resulted almost independently from the type of stabilizer used for the preparation. On the contrary, CAT

**Table 1.** Dependence of MS Characteristics on Process Parameters*

| Preparations | Excipients | Volume of Protein Solution (µL) | Polymer | Drug Content (%) | Encapsulation Efficiency (%) | Size (µm) |
|---|---|---|---|---|---|---|
| Catalase MS | Mannitol | 300 | 15% PLA | 3.7 | 37 | 12 |
| | | 150 | 15% PLA | 4.5 | 45 | 14 |
| | | 100 | 15% PLA | 4.5 | 45 | 10 |
| | | 70 | 15% PLA | 2.1 | 21 | 17 |
| | | 100 | 25% PLA | 3.9 | 39 | 12 |
| | | 150 | 15% PLGA | 2.9 | 29 | 13 |
| | Trehalose | 300 | 15% PLA | 3.1 | 31 | 10 |
| | | 100 | 15% PLA | 3 | 30 | 10 |
| | | 150 | 15% PLGA | 3.2 | 32 | 12 |
| | PEG400 | 300 | 15% PLA | 2.4 | 24 | 13 |
| | | 100 | 15% PLA | 3.4 | 34 | 9 |
| | | 70 | 15% PLA | 3.2 | 32 | 17 |
| | | 150 | 15% PLGA | 3.4 | 34 | 14 |
| SOD MS | Mannitol | 300 | 15% PLA | 3.3 | 33 | 14 |
| | | 100 | 15% PLA | 3.9 | 39 | 15 |
| | | 100 | 25% PLA | 3.2 | 32 | 12 |
| | | 150 | 15% PLGA | 5.1 | 51 | 12 |
| | Trehalose | 300 | 15% PLA | 4.4 | 44 | 8 |
| | | 100 | 15% PLA | 4.1 | 41 | 12 |
| | | 150 | 15% PLGA | 4.9 | 49 | 13 |
| | PEG400 | 300 | 15% PLA | 2.4 | 24 | 13 |
| | | 150 | 15% PLA | 3.1 | 31 | 14 |
| | | 100 | 15% PLA | 3.1 | 31 | 12 |
| | | 150 | 15% PLGA | 4.7 | 47 | 15 |

*MS indicates microspheres; PLA, poly(D,L-lactide); PLGA, poly(D,L-lactide-co-glycolide); PEG, polyethylene glycol; and SOD, superoxide dismutase.

loading was less dependent on the polymer and more dependent on the kind of excipient co-encapsulated with the protein. In addition, particle size did not seem to be affected by the process parameters investigated. In fact, all mean diameters ranged from 10 to 17 µm, and these values were reproducible. The best SOD and CAT preparations are reported in Table 2. The MS showed similar mean diameters for all the preparations, and porosity, expressed as BET surface area, was approximately 4.5 $m^2/g$ for PLGA MS and 0.95 to 0.98 $m^2/g$ for PLA MS. In addition, blank PLA and PLGA MS porosity did not show changes in BET surface area: 0.95 ± 0.03 $m^2/g$ for PLA MS and 4.4 ± 0.2 $m^2/g$ for PLGA MS. Moreover, mean diameters were practically unchanged compared with the loaded MS: 12.5 ± 2 µm and 13.8 ± 3 µm for blank PLA and PLGA MS, respectively. These findings imply that protein entrapment does not modify the MS morphology. In addition, the kind of stabilizer employed did not show any effect on the MS characteristics. SEM of blank and loaded MS correlated well with these observations (Figure 1). In fact, all SOD and CAT PLA MS were spherical with a smooth surface and without visible pores. Instead, PLGA MS showed isolated small pores that probably are responsible for their slightly higher porosity.

In vitro SOD release from PLA MS was mainly incomplete with 40% to 50% initial burst and only approximately 60% of total SOD released after 53 days; the pattern was similar for all preparations. In contrast, PLGA MS showed a SOD sustained release with only 5% to 30% initial burst, and the release was complete after 60 days (Figure 2). The linear extrapolation of SOD release profiles from PLGA MS provided good correlation coefficients between 0.9730 and 0.9870 (Figure 3). In addition, SOD release from PLGA MS with mannitol showed a good linearity ($r$ = 0.9738) over almost the total time range investigated, as the initial burst effect was remarkably lower with respect to PLGA MS with trehalose and PEG400. CAT release was incomplete for both PLA and PLGA MS with 30% to 40% and 40% to 60% initial burst from PLA and PLGA MS, respectively (Figure 4). Nevertheless, more than 80% of release from PLGA MS was achieved after 25 days when using mannitol. Unfortunately, immediately after day 25, CAT concentration started to decrease and reached approximately 60% at day 70. A similar pattern was observed for all the CAT-containing MS preparations. This trend may be owing to either CAT adsorption on the polymer or protein degradation. The initial burst difference between CAT containing

**Table 2.** SOD and CAT Containing PLA and PLGA MS Characterization*

| Microspheres | Excipients | Drug Content % ± SD | Encapsulation Efficiency % ± SD | Mean Diameter µm ± SD | BET Surface Area $m^2/g$ ± SD |
|---|---|---|---|---|---|
| SOD PLA MS | Mannitol | 4.0 ± 0.9 | 40 ± 9 | 14 ± 1 | 0.95 ± 0.03 |
| | Trehalose | 4.2 ± 0.3 | 42 ± 3 | 12 ± 5 | |
| | PEG400 | 3.1 ± 0.1 | 30.9 ± 0.2 | 14 ± 3 | |
| CAT PLA MS | Mannitol | 4.5 ± 0.2 | 45 ± 2 | 14 ± 3 | 0.98 ± 0.04 |
| | Trehalose | 3.1 ± 0.1 | 30.5 ± 0.7 | 10 ± 4 | |
| | PEG400 | 3.4 ± 0.1 | 34 ± 1 | 13 ± 4 | |
| SOD PLGA MS | Mannitol | 5.1 ± 0.1 | 50.5 ± 0.1 | 12 ± 2 | 4.5 ± 0.3 |
| | Trehalose | 4.9 ± 0.2 | 49.2 ± 0.2 | 13 ± 3 | |
| | PEG 400 | 4.7 ± 0.9 | 47 ± 9 | 15 ± 1 | |
| Catalase PLGA MS | Mannitol | 3.0 ± 0.1 | 29.5 ± 0.7 | 13 ± 2 | 4.5 ± 0.2 |
| | Trehalose | 3.2 ± 0.1 | 32 ± 1 | 12 ± 3 | |
| | PEG400 | 3.4 ± 0.3 | 34 ± 3 | 14 ± 2 | |

*SOD indicates superoxide dismutase; CAT, catalase; PLA, poly(D,L-lactide); PLGA, poly(D,L-lactide-co-glycolide); MS, microspheres; and PEG, polyethylene glycol.

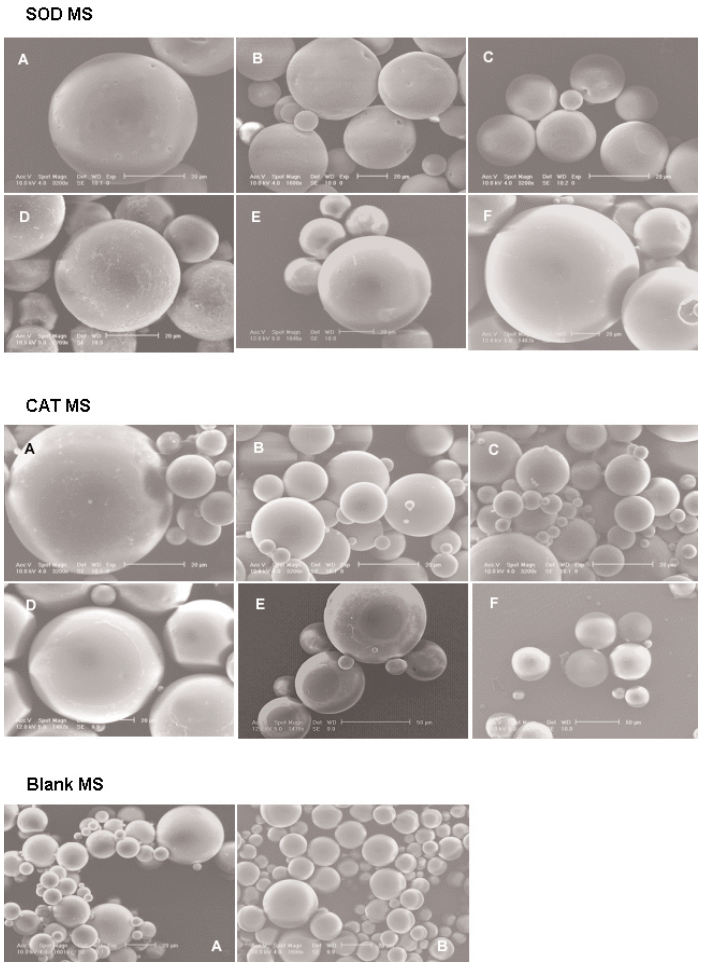


**Figure 1.** SEM photomicrographs of SOD and CAT MS with mannitol, trehalose, and PEG400 respectively, with PLGA (A, B, C) and PLA (D, E, F). Blank MS are with PLGA (A) and PLA (B).

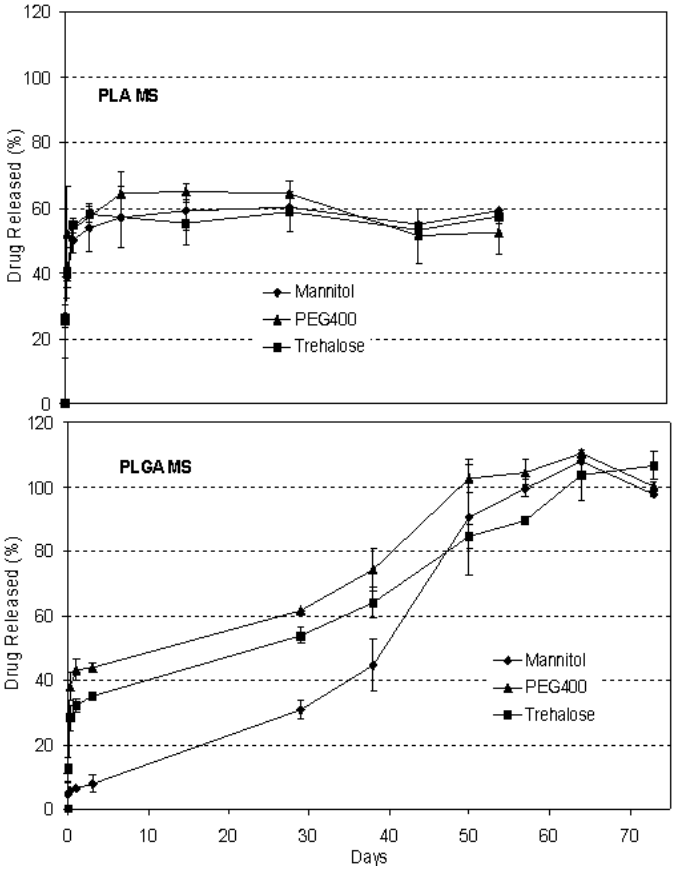


**Figure 2.** In vitro release of SOD from PLA and PLGA MS at 37°C in 0.1 M PBS (pH 7.4), in triplicate.

PLA and CAT containing PLGA MS correlated well with their different BET surface areas.

Mass balance revealed that SOD was not degraded either in PLGA MS after 73 days or in PLA MS until day 54 (Figure 5). The same results were obtained from CAT PLGA MS. In fact, the mass balance after 70 days did not show any protein

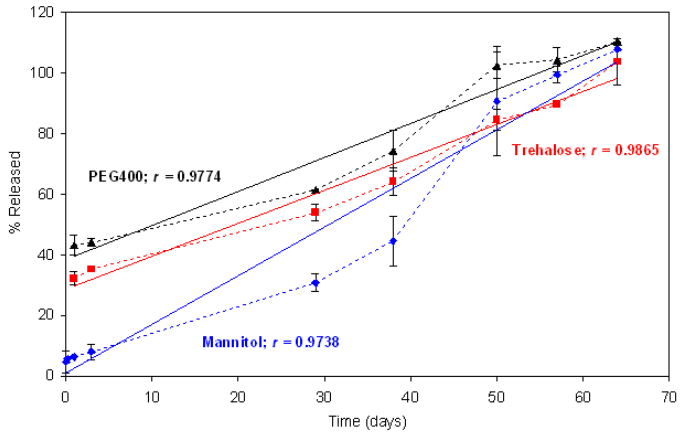


**Figure 3.** Linear extrapolation of SOD release patterns from PLGA MS.

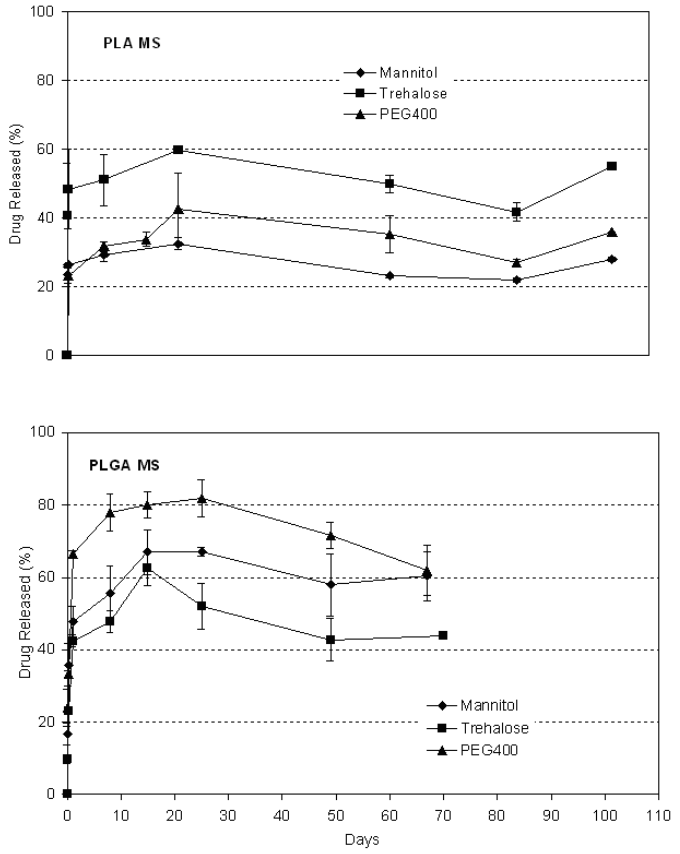


**Figure 4.** In vitro release of CAT from PLA and PLGA MS at 37°C in 0.1M PBS (pH 7.4), in triplicate.

loss during the release study. However, CAT behavior was different when encapsulated in PLA MS. Mass balance at day 44 showed, already, a certain decrease in protein concentration with respect to the total loaded protein. In light of this observation, mass balance of PLA MS was monitored up to 103 days to evaluate possible gradual protein degradation. The results pointed out the progressive reduction of CAT concentration with time (in particular when PEG400 and, partially, trehalose were employed), while MS with mannitol did not show remarkable modifications after a slight initial

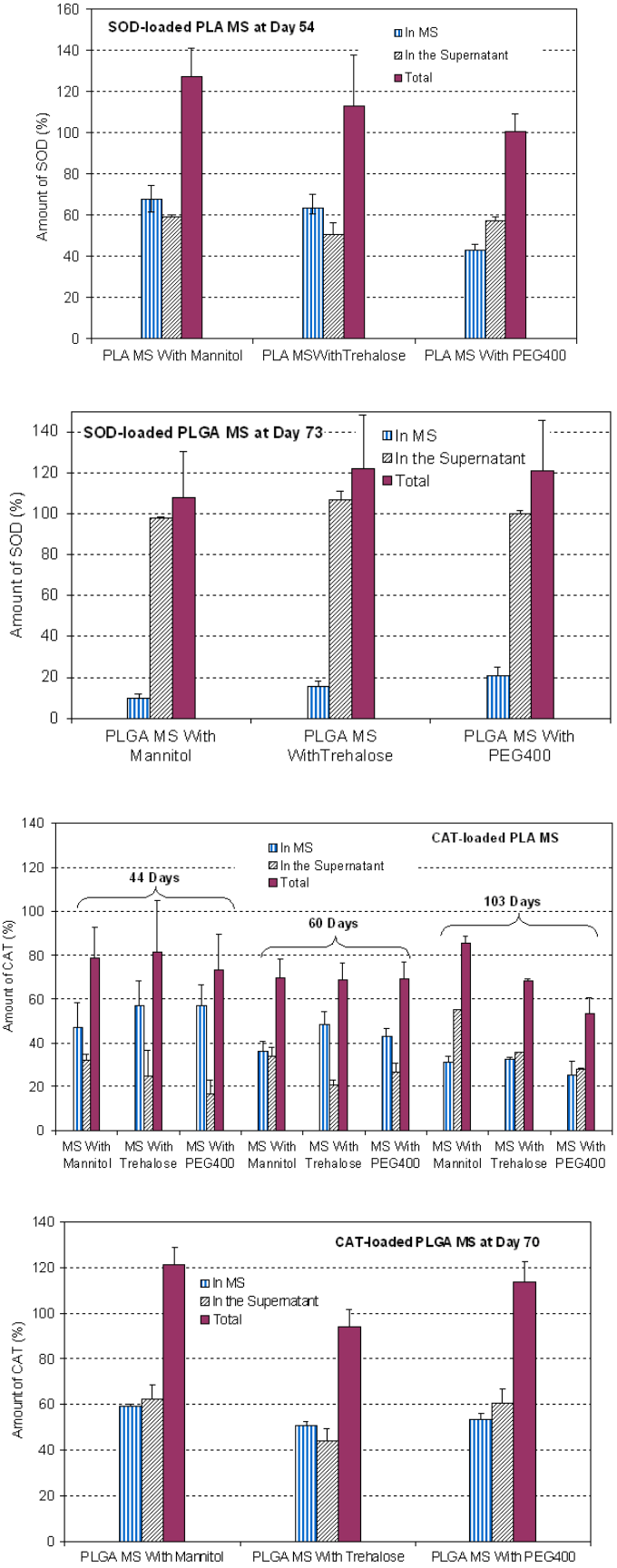


**Figure 5.** Mass balances of SOD- and CAT-loaded PLA and PLGA MS. CAT PLA MS were monitored during 103 days to evaluate possible protein degradation during the release study.

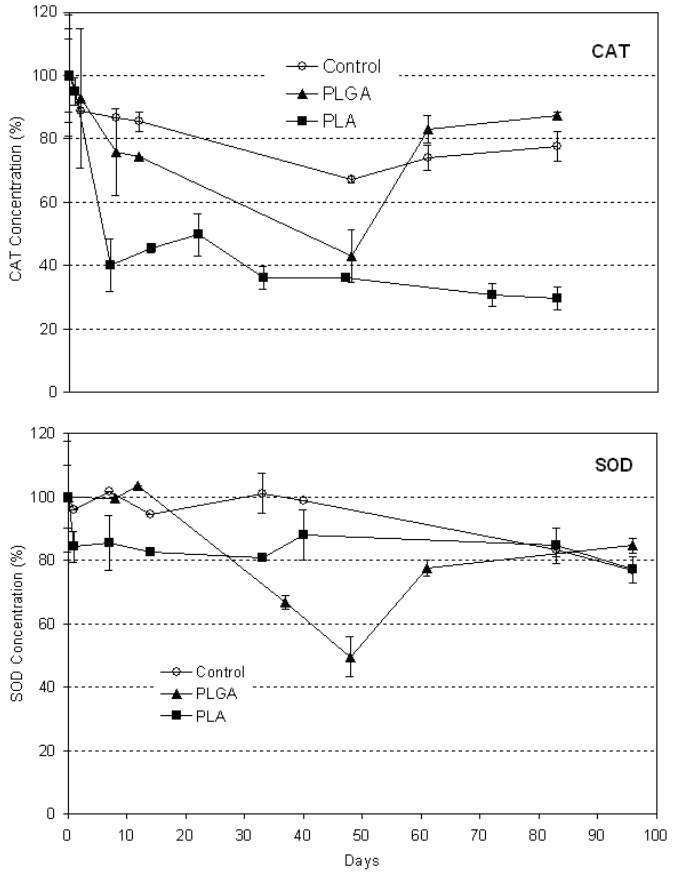


**Figure 6.** SOD and CAT interaction with blank PLA and PLGA MS at 37°C in 0.1M PBS (pH 7.4).

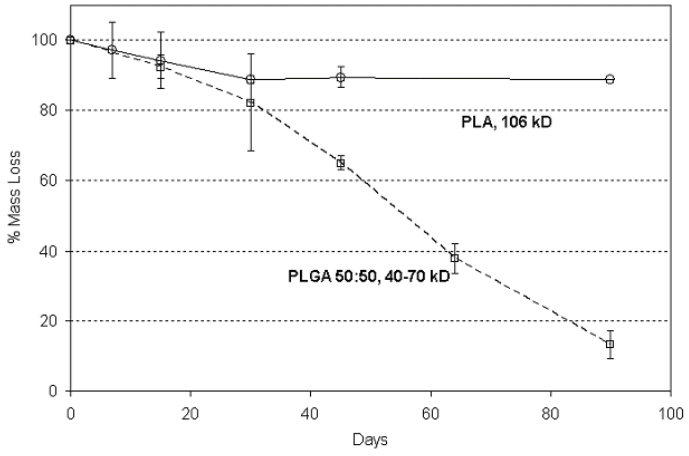


**Figure 7.** Mass loss of PLGA and PLA MS performed in 0.1M PBS (pH 7.4) at 37°C.

decrease. These findings may suggest that CAT is less stable than SOD under these operating conditions and that CAT is better stabilized in PLGA MS than in PLA MS.

In order to explain the behavior previously observed, CAT and SOD interactions with blank PLA and PLGA MS were investigated. Enzyme concentration profiles, recorded in 0.1 M PBS (pH 7.4) at 37°C, showed that CAT is less stable than SOD in solution under these operating conditions. In fact, SOD control remained quite stable, decreasing slowly to 80% only after day 40, while CAT concentration dropped already at day 3 (Figure 6). In the presence of blank PLA MS, CAT showed sudden decrease to 40% and then stable values until day 83. On the other hand, with blank PLGA MS, CAT concentration decreased slowly to approximately 45%, and after day 48 it started to increase again. CAT concentration decrease may be ascribed to protein adsorption to the polymer and degradation, while the observed increment after day 48, in the presence of PLGA MS, may be due to polymer erosion. On the other hand, in presence of PLA MS, SOD concentration, after an initial drop to 80%, remained constant throughout the experiment. On the contrary, with PLGA MS, the concentration decreased linearly to 45%, starting from day 12 to day 48, and then increased back to control levels at day 60. Adsorption to the polymer may be the reason for SOD concentration decrease, whereas polymer erosion may be responsible for SOD diffusion back into solution. This behavior suggests that CAT strongly interacts with both PLA and PLGA polymers. These findings correlated well with the observed release patterns. On the other hand, SOD seems to interact preferentially with PLGA polymer, although its release from PLGA MS was complete. This behavior may be addressed to a faster PLGA polymer erosion with respect to PLA.

In this regard, the mass loss study performed on the MS correlated with the observations coming from the release and interaction analysis. In fact, polymer degradation, monitored over 90 days (Figure 7), demonstrated the higher stability of PLA MS, which lost only approximately 10% to 15% of the total mass, whereas PLGA MS showed a progressive decrease of weight up to day 90, when the remaining mass was approximately 15% of the initial. The faster PLGA degradation along with the complete SOD release observed from PLGA MS indicates that erosion is the main mechanism involved in protein release, and this finding explains the CAT and SOD concentration increase after day 48 observed during the interaction study (Figure 6). In addition, the higher PLA resistance to erosion justifies the incomplete release for both CAT and SOD PLA MS. In turn, the incomplete CAT release from PLGA MS is probably the result of a combination of protein-polymer adsorption and CAT degradation. The morphology studies performed on the incubated MS at day 0, 30, 45, and 90 (Figure 8) confirmed a visible higher PLGA erosion with respect to PLA MS.

All preparations provided very satisfactory results in terms of activity retention. In fact, both CAT and SOD were, to a great extent, still active after their release from either PLA or PLGA MS. However, SOD showed a slightly higher activity retention when entrapped in PLGA MS, whereas CAT activity appeared almost unchanged in the 2 systems (Figure 9). PEG400 resulted in the best SOD stabilizer with 85% and 100% of retention in PLA and PLGA MS, respectively, whereas CAT was equally stabilized by all 3 excipients with

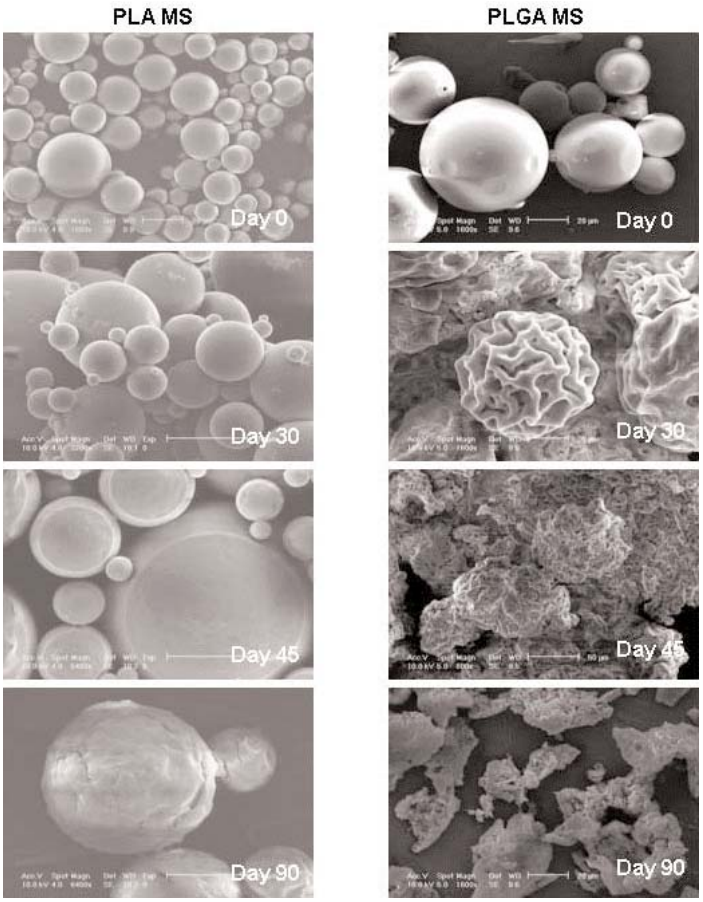


**Figure 8.** SEM photomicrographs of PLA and PLGA MS at 0, 30, 45, and 90 days.

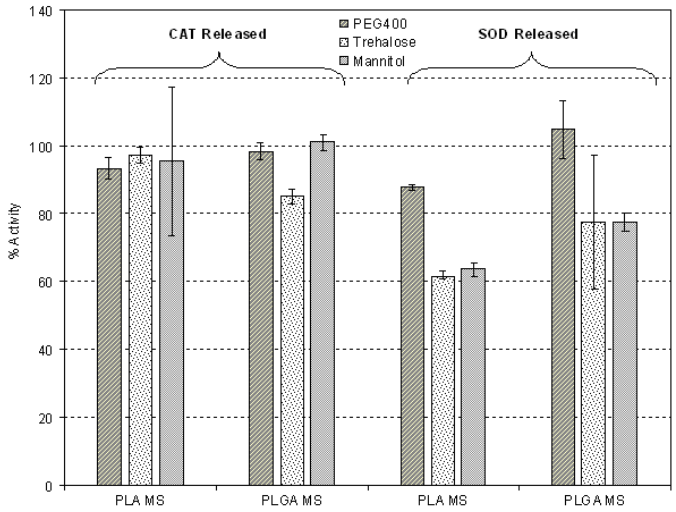


**Figure 9.** Activity retention of CAT and SOD released from PLA and PLGA MS depending on type of polymer and stabilizer employed for the preparation.

an activity retention close to 90% to 100%. The effect of protein aqueous volume was also investigated (Figure 10). The best CAT activity retention was obtained using 100-µL protein volume for the w/o emulsion. On the contrary, SOD retention was not strongly affected by the volume used, although trehalose seemed to stabilize the protein better when a 300-µL volume was employed.

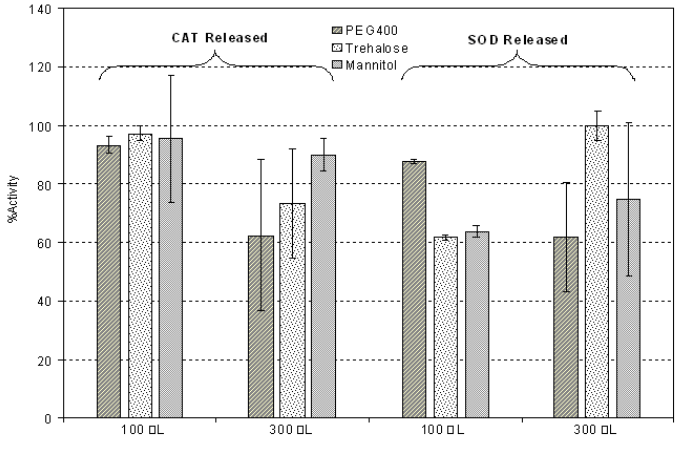


**Figure 10.** Activity retention of CAT and SOD released from PLA and PLGA MS depending on protein aqueous volume employed for the preparation.

## CONCLUSION

The enzymes were stabilized by co-encapsulation with excipients, and their properties in terms of size, morphology, and protein content make these formulations suitable for native SOD and CAT delivery. Moreover, the 2-month SOD release from PLGA MS may be potentially useful for long-term sustained release of the enzyme for the treatment of inflammatory manifestations, such as rheumatoid arthritis or other intra-articular and joint diseases.

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