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Research paper

Preparation of superoxide dismutase loaded chitosan microspheres: Characterization and release studies

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Abstract

Superoxide dismutase (SOD) is the most potent antioxidant enzyme. In this study, SOD was encapsulated in chitosan microspheres to obtain suitable sustained protein delivery. Protein-loaded chitosan microspheres with various formulations were prepared based on complex coacervation process. Due to the inherent characteristic of SOD, high encapsulation efficiency could not be obtained with simple preparation method. The pH of chitosan solution is 3.0; when the chitosan microspheres were prepared with this solution, encapsulation was low. Therefore, several strategies have been tested to increase the encapsulation efficiency and good results have been obtained. 70–80% protein encapsulation efficiency was obtained. The addition of PEG to the protein solution enhanced the encapsulation efficiency also. Mean sizes of microspheres were between 1.38 and 1.94 μm. Factors affecting the release behaviour of SOD from microspheres have been studied. They included pH values of chitosan solution (the pH of chitosan solution is 3.0), addition of PEG to the protein solution and the use of adsorption technique. In general, biphasic release profiles were obtained with these formulations. The protein activity changed between 70 and 100% during the release. In general, the protein activity remained in acceptable limits. The SOD encapsulated chitosan microspheres can be prepared by changing the pH or addition of PEG, allowing the safe incorporation of protein for controlled release.

Keywords: Chitosan; Superoxide dismutase; Microspheres; PEG; Controlled release; Protein

1. Introduction

Superoxide dismutase is an antioxidant enzyme, which protects cells against the detrimental effects of oxidative stress. The clinical application of this enzyme is ranged from the treatment of rheumatoid arthritis, aging, and cancer to respiratory distress syndrome [1].

SOD has been investigated for use in the treatment of several diseases in which the superoxide radical is involved [2]. However, a major limitation of the therapeutic use of SOD is its short plasma half life of 6 min and poor cellular penetration [3–5]. Therefore, an appropriate drug delivery system is needed. Liposomal forms were used for SOD delivery [3,5]. Liposome encapsulated SOD has been extensively studied [6], and chitosan-coated liposome has been evaluated in order to increase its releasing period and to facilitate its cellular penetration [7]. High efficiency entrapment of superoxide dismutase into cationic liposomes containing synthetic aminoaminoglycolipid was obtained [4]. Nevertheless, certain types of liposomes could be toxic to the cells [8]. There are a few studies about encapsulation of SOD into PLA and PLGA microspheres to obtain suitable sustained delivery [9–11]. Morita et al. [9] prepared SOD-loaded biodegradable PLA and PLGA microspheres using PEG as protein micronization adjuvant. By this technology, the initial release of bSOD was efficiently reduced and *in vivo* release performance of the microspheres (size

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<5 μm) was examined in SCID mice [10]. PLGA microspheres were reported as a useful system for SOD delivery [11]. However, the elaborations of these polymers requires the use of aggressive conditions (organic solvents, sonication, etc.) that affect the stability of the encapsulated molecules [12].

The development of delivery systems for protein drugs with short biological half lives using biodegradable polymers is of current interest [10,13]. However, limited information is available on the microsphere systems of SOD [10]. Poly(ε-capralactone) [14] PLA, PLGA and poly (D-,L-lactide) microspheres were used as a carrier for SOD [9–11]. An alternative to these hydrophobic particles are carriers made of hydrophilic polymers such as chitosan.

Bovine SOD (bSOD) is a dimer with a molecular weight of 31 kDa and an isoelectric point of 4.95. Thus the SOD molecule is negatively charged at physiological pH [15].

Because of the particularly mild conditions (not organic solvent and high stirring rate) required for particular system formation, chitosan microparticles appeared to be very promising systems for the delivery of sensitive macromolecules such as proteins. Chitosan is a hydrophilic, biocompatible, and biodegradable polymer at low toxicity. It is a promising material for drug and more specifically protein delivery. Recently a number of chitosan based formulations (microspheres and nanoparticles) were developed for the delivery of peptides and proteins. Chitosan has the ability to form particles with specific polyanions. These microparticles are formed immediately on mixing the two aqueous phases (one containing chitosan and the other containing sodium sulphate or tripolyphosphate) through inter or intramolecular linkages created between phosphate and chitosan amino group [16–19].

The main objective of this study was to prepare chitosan microspheres that were able to highly encapsulate and deliver SOD. However, because SOD was positively charged, it is difficult to obtain high SOD encapsulation into chitosan microspheres; therefore, different modifications (such as changing the pH of chitosan solution, and addition of PEG to the solution) were applied on the chitosan microsphere preparation technique.

2. Materials and methods

2.1. Materials

Chitosan (Medium, mol wt ca 400,000 Da, viscosity 200 mPa in 1% acetic acid at 20 °C with a deacetylation grade of about 87%) was purchased from Fluka (Germany). bSOD (Sigma, USA), sodium sulphate (Carlo Erba, Italy), PEGs 400 and 6000 (Sigma, USA) were used. Other chemicals were of pharmaceutical grade.

2.2. Preparation of microspheres

SOD-loaded chitosan microspheres were prepared according to our earlier report (17). bSOD was added to sodium sulphate solution (20%w/v) immediately before the pouring stage. Then sodium sulphate solution (50 ml) containing SOD was poured into the acidic chitosan solution from a beaker while stirring (IKa-Werk, Germany). The microspheres were separated by centrifugation (Sigma, Germany) for 10 min at 15,000 rpm., freeze-dried (Lyovac, Leybold Heraeus, Germany), and stored at 4 °C. Table 1 reports the code and composition of chitosan microspheres. Each formulation was prepared three times ($n = 3$).

In formulation studies, different modifications were tried in order to enhance the encapsulation efficiencies. Chitosan concentrations and pH of chitosan solutions were changed, and SOD was treated with sodium alginate or PEG. In this formulation, 300 μl of PEG was mixed to the protein

Table 1
Codes, formulation and characterization of SOD-loaded chitosan microspheres

CODE	Chitosan (%)	SOD (1 mg/ml) (μL)	pH	Sodium Alginate (1 mg/ml) (μL)	PEG (μL/Type)	Encapsulation efficiency (%)	Microsphere size (μm)	Activity (%)
CS.1	1	300	3.0	–	–	35.83 ± 0.69	1.55 ± 0.08	84.03 ± 0.43
CS.2	1	300	5.0	–	–	88.33 ± 0.83	1.47 ± 0.12	82.63 ± 0.45
CS.3*	1	300	3.0	–	–	84.00 ± 0.64	1.47 ± 0.06	99.72 ± 0.13
CS.4***	1	300	3.0	250	–	56.87 ± 0.53	1.62 ± 0.03	98.04 ± 0.27
CS.5	1	300	3.0	–	100/PEG-6000	77.81 ± 0.76	1.61 ± 0.03	99.44 ± 0.39
CS.6	0.1	300	3.0	–	–	27.89 ± 0.86	1.38 ± 0.03	83.47 ± 0.28
CS.7**	1	300	3.0	–	–	58.53 ± 0.68	1.53 ± 0.05	84.03 ± 0.43
CS.8	1	300	4.0	–	–	81.06 ± 0.84	1.40 ± 0.07	70.03 ± 0.29
CS.9***	1	100	3.0	–	100/PEG-400	74.40 ± .0.59	1.57 ± 0.03	98.04 ± 0.38
CS.10	1	300	3.0	–	300/PEG-400	92.51 ± 0.37	1.86 ± 0.07	100.00 ± 0.54
CS.11***	1	300	3.0	–	300/PEG-400	81.77 ± 0.68	1.94 ± 0.05	99.86 ± 0.32
CS.12***	1	100	3.0	–	300/PEG-400	75.09 ± 0.76	1.84 ± 0.06	99.72 ± 0.54
CS.13***	1	300	3.0	–	100/PEG-400	79.61 ± 0.46	1.75 ± 0.09	98.04 ± 0.37

Each batch was prepared in 3 times ($n = 3$).
* In this formulation, SOD adsorbed to the chitosan microspheres.
** Sodium sulphate solution including SOD, added as two portions.
*** Volume completed to 1 ml with distilled water.

solution and this mixture was added to chitosan solution as previously mentioned. During the preparation, the pH of the chitosan solutions were adjusted to 4 or 5 (CS.1, CS.2, and CS.8) by adding 1 N NaOH. PEG 400 or PEG 6000 (dissolved in distilled water) solutions were added to internal sodium sulphate solution (CS.9–CS.13).

2.3. Modifications of microspheres

PEG modified chitosan microspheres were formed spontaneously upon incorporation of 50 ml of sodium sulphate solution containing a mixture of bSOD and various amounts of PEG (100–300 μl) and chitosan solution.

Alginate modified microspheres were formed upon incorporation of 50 ml of sodium sulphate solution containing a mixture of bSOD and sodium alginate and 50 ml of the chitosan solution.

The pH of chitosan solution is three unless otherwise stated, therefore the pH of chitosan solution was adjusted to pH 4 or 5 and mixed with sodium sulphate solution for microsphere formation at formulations CS.2 and CS.8.

2.4. Determination of size distribution of microspheres

Particle sizes of microspheres were determined in an Olympus BH.2 microscope equipped with a camera and computer controlled image analysis system (Spot Advanced, Japan). At least 1000 particles (spherical shaped) were measured in each lot.

2.5. Determination of SOD content

The amount of SOD entrapped in microspheres was determined by measuring the difference between the total amount of protein incorporated in the particle preparation medium and the amount of nonentrapped protein remaining in aqueous medium after the encapsulation process. Protein content in the supernatant was spectrophotometrically (Shimadzu UV-Biospec 1601, Japan) determined at 595 nm by Bradford's method (21). BSA was used as a reference. The mean of three experiments is given ($n = 3$).

2.6. SOD activity measurement

SOD activity was carried out with the RANSOD kit (Randox, USA) [20]. This method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl) -3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) and superoxide radical, which is assayed in a spectrophotometer at 505 nm. The inhibition of the produced chromogen is proportional to the activity of the SOD present in the sample ($n = 3$).

2.7. Protein integrity

In order to determine the effect of preparation technique and release conditions on protein structure, the integrity of the encapsulated SOD after release study was analyzed by SDS–PAGE. The released samples were removed and lyophilized for concentrating of protein. Then the protein samples were dissolved in PBS and loaded into SDS–PAGE. Electrophoresis was performed at 20 mA by using a vertical gel electrophoresis system (Sigma, USA). The gel was stained with Coomassie blue R-250 solution (Merck, Germany) to visualize and the protein was determined using a gel image system (Kodak, DC 40, Camera and ID Image Analysis Software, USA).

2.8. In vitro release of SOD

Ten milligrams of the chitosan microspheres suspended in 2 mL of PBS buffer (pH 7.4) was shaken in a water bath at 37 ± 0.5 °C. At predetermined time intervals the samples were withdrawn and fresh medium was added to the microspheres. Concentration of SOD in the supernatant was spectrophotometrically determined at 595 nm according to Bradford [21]. Analyses were performed in triplicate.

2.9. Statistical analysis

All mean values are given ± standard deviation (S.D.). Statistical analysis was performed using Kruskal–Wallis one-way ANOVA followed by Mann–Whitney two-tailed test when indicated.

3. Results and discussion

As seen in Table 1, protein encapsulation studies carried out at acidic pH value of chitosan solution (pH 3) indicated the lower SOD encapsulation (35.8%), because protein is dissolved at a pH below its isoelectric point (4.95), so that the SOD molecule is predominantly positively charged. Chitosan is dissolved in aqueous acidic solution at pH 3 and at this pH value chitosan is strongly protonated. Therefore, two cationic molecules (SOD and chitosan) are present in the encapsulation medium and the cationic chitosan poorly encapsulated the cationic SOD. A similar result was reported previously for insulin encapsulation [16,18]. 15–30% of insulin associated to chitosan nanoparticles at a pH where insulin was predominantly positive charged [16].

In order to increase the entrapment of SOD into chitosan microspheres, different modifications such as increasing the pH of chitosan solution, addition of sodium alginate or PEG were used in further studies.

Firstly, the effect of pH on SOD encapsulation efficiency was studied. By increasing the pH value of chitosan solution from 3.0 to 4.0 and 5.0, encapsulation efficiencies of SOD into chitosan microspheres increased from 35.8% to 81.0–88.0% (Table 1). The effect of pH on SOD encapsulation efficiency is remarkably high ($p < 0.001$). This may be due to yielding different charged proteins as increasing the pH of the solution above the isoelectric point (IEP 4.95). Ma et al. (2000) found that the

association of insulin was strongly influenced by the pH of medium.

Secondly, at acidic pH (3.0), the effect of PEG addition on protein encapsulation was investigated. As seen in Table 1, addition of PEG into protein solution increased the SOD encapsulation efficiency of chitosan microspheres ($p < 0.001$). Encapsulation efficiency increased from 35.8% (CS.1) to 70.0–90.0% as dependent on formulations. On the contrary of the report of Xu and Du (2003), protein encapsulation increased with the addition of PEG into the SOD solution in our study; however, they mixed the PEG solution with chitosan solution [22]. Higher SOD entrapment was obtained with PEG 6000 than that obtained with PEG 400. Instead of PEG, the addition of sodium alginate to the protein increased the SOD encapsulation percentage of microspheres from 35.8% to 56.0% ($p < 0.001$).

Another approach is to change the addition method of sodium sulphate solution to chitosan solution. This solution was added as two steps. Firstly half of the sodium sulphate solution (25 ml) was added to chitosan solution while stirring, then second part of the sodium sulphate solution (25 ml) containing SOD was added to the chitosan solution. During the microsphere formation, after the addition of the first part, a high portion of chitosan charge is closed with sulphate ion and the addition of the second part of sodium sulphate solution containing SOD yielded higher protein entrapment such as 58.5% (Table 1).

In order to obtain high SOD encapsulation, the protein was adsorbed onto empty chitosan microspheres. As seen in Table 1, 84% of SOD encapsulation was obtained by this method (CS.3).

The highest SOD encapsulation (92.5%) was obtained with the formulation containing PEG 6000 (CS.10).

Particle sizes of SOD microspheres are given in Table 1. Formulation factors such as PEG addition and chitosan concentration affected the size of chitosan microspheres ($p < 0.001$).

In vitro release profiles of SOD loaded chitosan microspheres are given in Figs. 1–4. In general, biphasic profiles were obtained with these microspheres except for some formulations (CS.4, CS.5, and CS.7).

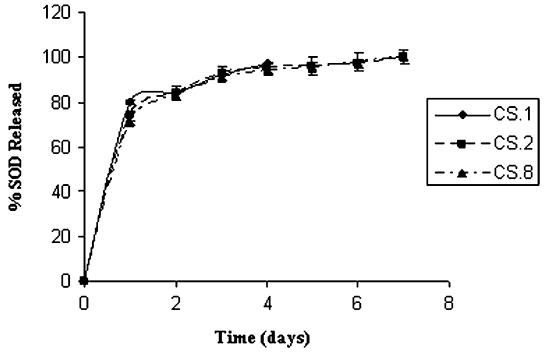

Fig. 1. *In vitro* SOD release from chitosan microspheres that were prepared at different pH; pH 3.0 (CS.1), pH 5.0 (CS.2), and pH 4.0 (CS.8).

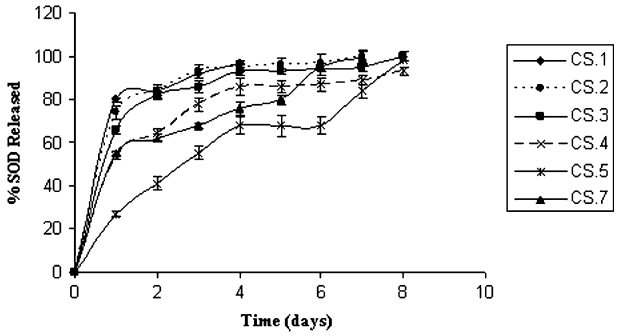

Fig. 2. Release profiles of chitosan microspheres prepared by adopting different techniques; at different pH values (CS.1 and 2), protein adsorption (CS.3), sodium alginate (CS.4), PEG addition (CS.5), and addition of sodium sulphate in two portions (CS.7).

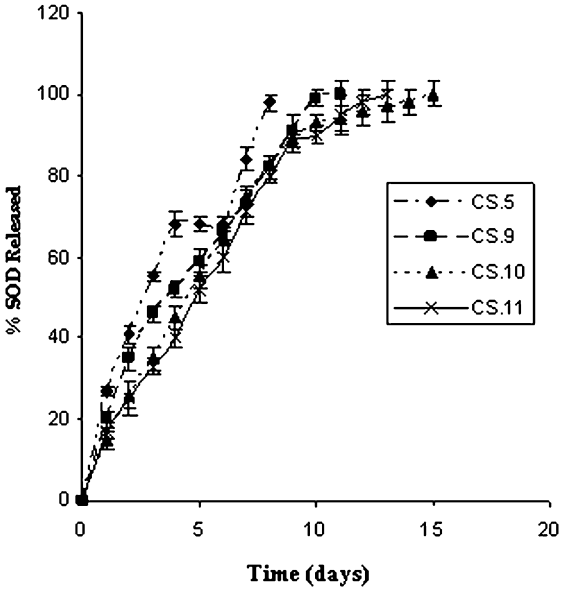

Fig. 3. Release profiles of SOD-loaded chitosan microspheres prepared with PEG addition; 100 μl PEG-6000 (CS.5), 100 μl PEG-400 with sodium alginate and water (CS.9), 300 μl PEG-400 (CS.10), and 300 μl PEG-400 with water (CS.11).

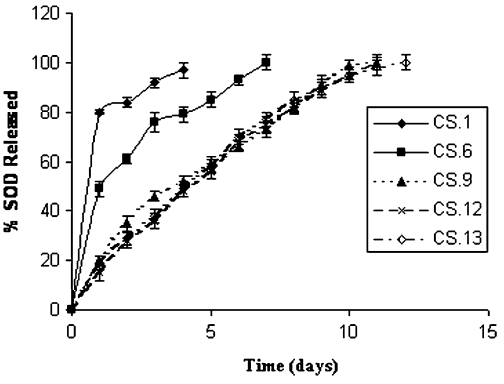

Fig. 4. Effect of chitosan and SOD concentration on protein release from chitosan microspheres; 300 μl SOD (CS.1), 0.1% chitosan solution (CS.6), 100 μl SOD with 100 μl PEG-400 (CS.9), 100 μl SOD with 300 μl PEG-400 (CS.12), and 300 μl SOD with 100 μl PEG-400 (CS.13).

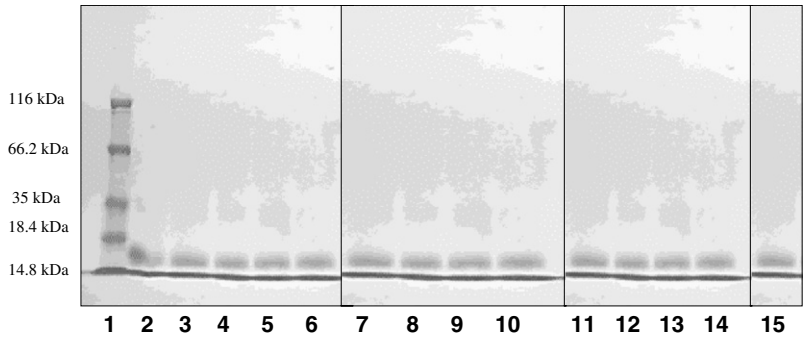

Fig. 5. SOD's structural control that was released from chitosan microspheres at 7.5% SDS–PAGE. **1**, Fermentas Protein Weight Marker; **2**, bSOD; **3**, CS.1; **4**, CS.2; **5**, CS.3; **6**, CS.4; **7**, CS.5; **8**, CS.6; **9**, CS.7; **10**, CS.8; **11**, CS.9; **12**, CS.10; **13**, CS.11; **14**, CS.12; **15**, CS.13.

Chitosan microspheres formed at different pH values (CS.1, 2, and 8) indicated a burst effect in the first day of release experiment (Fig. 1). Approximately 70–80% of the protein was released from microspheres within the first day and SOD release completed during the first 4–8 days (Fig. 1).Although adsorption promotes the SOD encapsulation, high protein release was observed within the first day release of this formulation (CS.2) (Fig. 1).

The effect of sodium sulphate addition to chitosan solution in two steps and the release behaviour of microspheres are shown in Fig. 2. Significantly different release patterns were obtained by changing the sodium sulphate addition technique ($p < 0.001$).

A summary of different release profiles of different formulations is given in Fig. 2. In general, burst release was gradually decreased in the formulations containing sodium alginate (CS.4) and PEG (CS.5) ($p < 0.001$). The much lower burst release from modified microspheres suggests that the main SOD are encapsulated inside the matrix.

The burst release of SOD from chitosan microspheres prepared with PEG addition (CS.5, 10, and 11) was decreased to 15–20% within the first day (Fig. 3). Except PEG 400 containing formulation (CS.5), SOD release was completed during 11–15 days as dependent on formulations.

As seen in Fig. 4, the amount of protein has no significant effect on SOD release from microspheres ($p > 0.001$), but chitosan concentration has an importance in the release behaviour of SOD.

Enzyme activities of SOD released in the dissolution medium were assayed. Table 1 shows the activity of the released protein. Activity loss of SOD microspheres prepared under different acidic pH values was about 16–18 and 30%, respectively.

The highest activity loss was found in the formulation coded as CS.8. Chitosan microspheres formed by different sodium sulphate addition methods indicated 85% protein activity (Table 1). However, very low activity loss was observed at PEG addition microspheres (CS.5, 9, and 13); no activity loss was observed in formulation CS.10.

The process for the preparation of SOD containing PEG chitosan microspheres developed in this study yielded relatively high encapsulation efficiency without loss of enzymatic activity. This effect may be due to the formation of the intermolecular hydrogen bonding between the electronegative oxygen atom of PEG and the amino groups of chitosan in the solution as previously reported by Kim and Lee (1995) [23].

To determine whether the release procedure affected the integrity of SOD, SDS–PAGE was applied to release samples after the *in vitro* release studies of SOD microspheres. As seen in the gel photograph (Fig. 5), the structure of SOD remained stable during the release experiment.

A general approach to prevent protein loss from the microspheres is to charge the protein with a charge opposite to that of the polymer; however, chitosan is a cationic polymer and has acid-solubility. SOD bears a positive charge at pH 3.0 which chitosan solved at this pH. By adjusting the pH of chitosan solution to 4 or 5, SOD encapsulation efficiency was highly increased.

4. Conclusions

In this study, positive charged SOD could be encapsulated into chitosan microspheres at acidic pH values. Besides adjusting the pH to 5, the addition of PEG to the protein solution increased the SOD encapsulation efficiency. Thus, long term SOD release can be obtained with chitosan microspheres. On the other hand, poor cell permeability of SOD can be overcome by SOD loaded chitosan microspheres because chitosan has an increasing effect on cell permeability [24]. Moreover, superoxide anion scavenging activity of chitosan derivatives was reported previously [25]. Proper selection of the experimental conditions could indeed substantially increase the encapsulation efficiency without loss of enzyme activity and could obtain long term release. This kind of chitosan microspheres may be a promising delivery system for water soluble superoxide dismutase.

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