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A controlled release system of superoxide dismutase by electrospun fiber and its antioxidant activity in vitro

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Abstract In this paper, a new controlled release system of superoxide dismutase was developed by electrospun composite fibers. Highly loading efficacy of sod from 85.6 to 98.0% was achieved. The superoxide dismutase can be released from the system for 234 h, and obvious initial burst release of superoxide dismutase in vitro was not observed. In vitro release rate of superoxide dismutase in the first 66 h basically is faster than the corresponding rate at a later stage. Antioxidant activity of the released superoxide dismutase was still high, and it remained stable during the preparation by electrospinning and release experiment. We hope this composite system be used as an implanted form, in the treatment for several disease involved with the superoxide radical in the future.

1 Introduction

In the past several decade years, superoxide dismutase (SOD) has been extensively investigated [1–3]. It was reported that SOD can protect cells against the harmful effects of oxidative stress, and has clinical application in the treatment of several diseases in which the superoxide radical is involve, such as from the treatment of rheumatoid arthritis, aging, and cancer to respiratory distress syndrome [4]. Now, it has become the most potent antioxidant enzyme. However, its use is associated with a major disadvantage of short plasma half life of only 6 min and poor cellular penetration [5, 6]. Therefore an appropriate drug delivery system is of great importance to improve it. In the past several years, there have been many reports on how to prolong half life of SOD in the body. Some researches of liposome encapsulated SOD have been reported [6]. Aoki et al. has reported high efficiency entrapment of superoxide dismutase into cationic liposomes containing synthetic aminoaminoglycolipid [5]. But, Dokka et al. found that certain types of liposomes could be toxic to the cells. The development of delivery systems for protein drugs with short biological half lives using biodegradable polymers is of current interest [7]. Morita et al. developed PLGA microspheres as a useful system for SOD delivery. However, the preparation requires aggressive conditions and these affect the stability of the encapsulated SOD [8]. Celik and Akbuga reported the preparation of superoxide dismutase loaded chitosan microspheres by different modifications, such as changing the pH of chitosan solution and addition of PEG to the solution [9].

Electrospinning is a remarkably simple and versatile technique to generate continuous fibers directly from a variety of polymers and composite materials. It was reported that the fiber diameters can be controlled from tens of nanometers to micrometers, and the fibers have large surface areas, high porosities, and ease of construction into different shapes [10–13]. Over the past decade, the use of electrospun nanofibers in biomedical fields has been drastically increased, because the fibers have many

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advantages, such as extended the half life in the body, reduced toxicity, improved therapeutic effect and convenient operation [14, 15]. Kenawy et al. first researched the release of tetracycline hydrochloride from electrospun poly(ethylene-*co*-vinyl acetate), poly(lactic acid) and their blends [16]. Jing et al. investigated the poly(ethylene glycol)-poly(L-lactic acid) diblock copolymer fibers for the controlled release of 1,3-bis(2-chloroethyl)-1-nitrosourea and found that its short half-life in human body can be extended e to certain extent by incorporating it into the fibers [17]. Recently electrospun fibers for the controlled delivery of BSA have been investigated by several researchers [18–20].

In this paper, we developed a controlled release system of SOD by electrospun fibers and investigated its antioxidant activity in vitro. The SOD can be released from the system for a long time, and the antioxidant activity of the released SOD was almost not reduced. The composite fibers may be useful as an implanted form, in the treatment for several disease involved with the superoxide radical in the future.

2 Experiments

2.1 Materials

SOD was purchased from Cixi Coregene Biology Technology Co. Ltd. (Zhejiang, China). PLLA (MW = 100,000) was purchased from Shandong Jianbao Biomaterials Ltd. (Shandong, China). Dichloromethane was obtained from Chemical Reagent Co. Ltd. (Shanghai, China). Coomassie brilliant blue G-250 and pyrogallol were used. PBS buffer solution (pH 7.4) and 50 mmol/l Tris–HCl buffer solution (pH 8.20, including 0.1 mmol/l EDTA) was used. All other materials and reagents used were of analytical grade.

2.2 Fabrication of the products

In the experiment, the PLLA (5 g) was first dissolved in dichloromethane (50 ml) in a concentration to form a solution by using a bath sonicator (KQ-100, China). Then a predetermined amount of SOD powders was dispersed in dichloromethane. The PLLA was added to the solution by continuous stirring.

In a typical procedure of electrospinning, first, the polymer solution was transferred to a glass syringe. Then, the syringe pump (model WZ-50C2, Zhejiang University Medical Instrument Co., Ltd.) was used to deliver solution through a tube connecting the syringe with the needles. A high voltage DC generator (Beijing Machinery & Electricity Institute) was used to produce a 14.0–20.5 kV voltage to spin solution through inner needle. An aluminium foil was used to collect the random fibers. The distance from the spinneret to the collector was fixed at 14 cm. All the experiments were performed at room temperature. Finally, the fibers were taken out, dried for 48 h and stored in a refrigeratory. The blank fibers without SOD were fabricated by the same method.

2.3 Characterization of the products

A field emission scanning electron microscopy (FE-SEM, Hitachi S-4800, Japan) was used to observe the morphology of collected fibers. The samples for SEM observation were sputter coated with gold. The fiber diameter of the electrospun fibers was measured with software Image J.

2.4 Determination of SOD content in the fibers and SOD encapsulation efficiency

To determine SOD content in the fibers, 10 mg of the electrospun fibers were dissolved in the 3 ml dichloromethane. Subsequently, SOD was extracted with 10 ml PBS solution. The resulting solution was analyzed by 722 spectrophotometer at 595 nm by Bradford's method [21]. The measured SOD content (MC) was defined as follows: MC (%) = (measured amount of SOD/fiber sample weight) * 100%. The theoretical SOD content (TC) was also defined as follows: TC (%) = (added amount of SOD/added amount of SOD and PLLA) * 100%. Therefore, SOD encapsulation efficiency (EE) could be determined: EE (%) = (MC/TC) * 100%. SOD content in the fibers and encapsulation efficiency for each sample were the average of triplicate samples.

2.5 Release profiles of SOD in vitro

About forty milligrams of the fibers suspended in 10 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 fibers. Concentration of SOD was spectrophotometrically determined at 595 nm according to Bradford method. Each formulation was prepared three times ($n = 3$).

2.6 Determination of SOD activity in vitro

SOD activity was tested by Marklund's method [22]. This method is based on the inhibition of pyrogallol autoxidation, which is assayed in a spectrophotometer at 420 nm. The inhibition of the pyrogallol autoxidation is proportional to the activity of the SOD present in the sample ($n = 3$). The SOD sample was added to the 50 mmol/l Tris–HCl buffer solution (pH 8.20, including 0.1 mmol/l EDTA) and stirred. Then 6 mmol/l pyrogallol was added to

the solution and immediately stirred. The absorption of the mixture was recorded.

2.7 SOD integrity

In order to investigate the effect of preparation technique and release conditions on integrity of the protein structure, different SOD samples released from the fibers were analyzed by denaturating SDS–PAGE. Electrophoresis was performed at 20 mA on 12% polyacrylamide gel using a Mini Protein system and the gels were stained with coomassie brilliant blue R-250. SOD bands were scanned with a gel image system (MultiImage^{TM} Light Cabinet Filter Positions, Alpha Innotech, USA).

3 Results and discussion

3.1 Characterization of the electrospun fibers

The effects of key processing parameters of electrospinning such as polymer concentration, polymer solution flow rates, voltage, etc. were investigated. Experiments indicate the fiber can be prepared when polymer concentration is 5% and voltage is from 14.0 to 20.5 kV. The sample 1, 2, 3 and 4 were prepared. Fiber diameter, SOD content and encapsulation efficiency of the products were shown in the Table 1. The Table 1 shows the SOD loading efficacy from 85.6 to 98.0%. It indicates that highly loading efficacy can be achieved by electrospinning technology. The SOD content (%) in the fibers from 3.3 to 9.1. That is to say a relatively wide range of SOD content in the fibers can be gained. The Table 1 also indicates the fibers of the products were micro-nanofibers and average diameter of the fibers could be fine tuned by adjusting the processing parameters.

SEM images of the sample 1, 2, 3 and 4 are shown in Fig. 1 (S1, S2, S3 and S4). The fiber morphology shows the surface is smooth and no SOD crystals are detected. The fibers of S1, S2 and S3 look uniform. But the fibers of S4 are not uniform and the big size fibers in the S4 sample are porous probably because of highly SOD content (98.0%).

Table 1 Characterization of the composite fibers

Samples	PLLA/SOD (w/w)	Fiber diameter (μm)	Encapsulation efficiency (%)	SOD content (%)
S1	50.0/1.7	0.2–1.5	98.0	3.3
S2	50.0/2.7	0.6–2.2	95.3	5.0
S3	50.0/3.7	0.8–2.4	86.7	6.0
S4	50.0/5.7	0.3–6.4	85.6	9.1

Electrospinning condition: voltage 18.3 kV, flow rate 4.0 ml/h, SOD content in the fibers and encapsulation efficiency for each sample were the average of triplicate samples

3.2 Release profiles of SOD in vitro

Figure 2 showed the release profiles of SOD from the composite fibers. The curve S1, S2, S3 and S4 in Fig. 2 showed the release profiles of SOD from the fiber S1, S2, S3 and S4, in the PBS at 37.0°C, respectively. From Fig. 2, in the first 6 h, around 6.6%, 8.2%, 13.1% and 16.2% of SOD was released from S1, S2, S3 and S4 samples, respectively. Obvious initial burst release of SOD was not observed in the experiments. It was reported that initial release happens mainly because of the diffusion of drug near the fiber surfaces. So, the results indicate SOD was finely incorporated into the fibers. From the whole curve, in vitro release rate of SOD in the first 66 h basically is faster than the corresponding rate at a later stage. Miyajima et al. reported that the release profile of drug from PEG–PLLA matrix is mainly controlled by not only diffusion of the drug but also degradation of the matrix [23, 24]. PLLA biodegrades slowly. Ping and coworkers reported that less than 1% of PLLA (MW = 100,000) fiber weight was lost within 28 days and the pH value of the buffer solution just slightly changed from 7.40 to 7.37 because of the acid release from the degradation of PLLA [25]. We tested the weight loss of the blank PLLA (MW = 100,000) electrospun fibers and the SOD-loaded PLLA (MW = 100,000) electrospun fibers (S4 sample) in the PBS within 234 h. The results showed that 3.5% of the weight loss of the blank PLLA fibers was observed, which was little higher than that Ping reported. The results also showed that 12.0% of the weight loss of the SOD-loaded PLLA fibers (S4 sample) was observed in the PBS within 234 h. For the S4 sample, SOD content in the fibers was 9.1% and accumulated SOD release (%) was 92.9% within 234 h. Therefore, we believe that 12.0% of the weight loss of the S4 sample mainly is due to the diffusion of the SOD from the PLLA fibers to the PBS. These results demonstrate that SOD release mainly depends on the diffusion, instead of the biodegradability. In the first 66 h, probably because of the fast diffusion of SOD near the surface of fibers, release rate of SOD is faster. Then the slower release rate was due to the slower diffusion of SOD located far away from the surface of fibers and increase in diffusion length. In this study, the sustained release for the fiber samples could last for 234 h. In the 234 h, around 75.5%, 78.3%, 80.3% and 92.9% of SOD was released from S1, S2, and S3 samples, respectively. A controlled release system of SOD by electrospun fibers can be achieved. The delivery of proteins is a particularly demanding application of controlled drug delivery. This system perhaps can prolong half-life in human body and improve efficacy of SOD, and hope this composite system be used as an implanted form, in the treatment for several disease involved with the superoxide radical in the future.

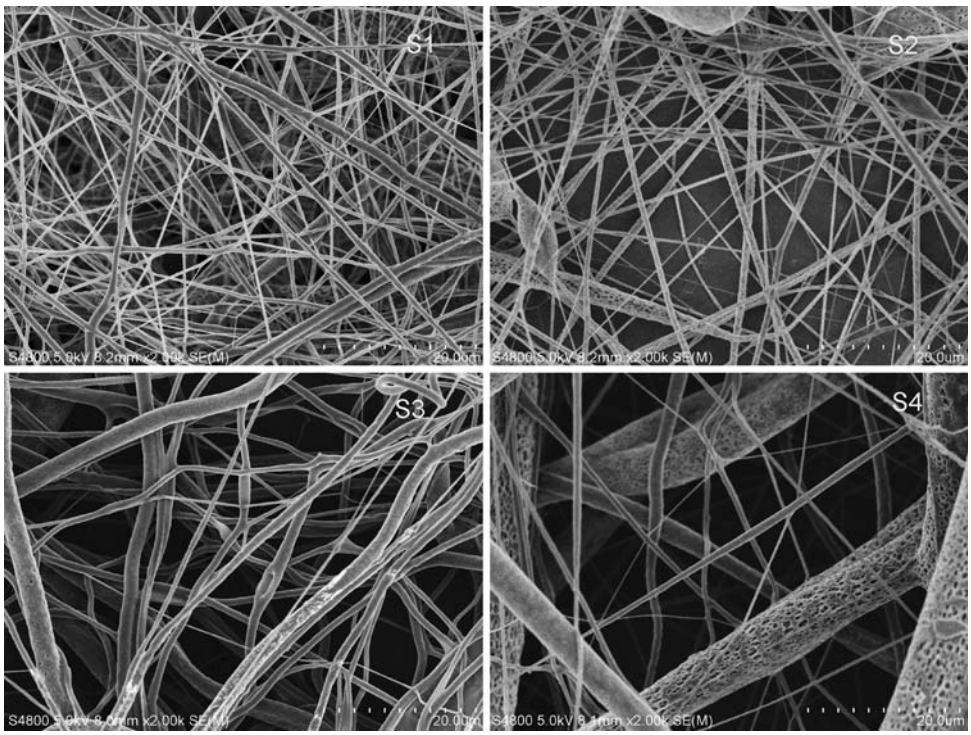

Fig. 1 Scanning electron microscope images. S1: 3.3% SOD content (S1 sample); S2: 5.0% SOD content (S2 sample); 6.0% SOD content (S3 sample); 9.1% SOD content (S4 sample)

3.3 Antioxidant activity in vitro

In order to investigate antioxidant activity in vitro of the SOD released from the composite fibers, the method of pyrogallol autoxidation was used. In the experiment, we investigated antioxidant activity of the SOD released from the fibers S3 and S4. The results were shown in Fig. 3. From Fig. 3, in the 114 h, for the fibers S3, highest activity and lowest activity are 95.6% and 71.8%, respectively. That is to say, the SOD released from the fiber S3 has very low activity loss. The same trend can be found from the fiber S4. These data indicate that SOD still has the antioxidant activity during the preparation by electrospinning and release process.

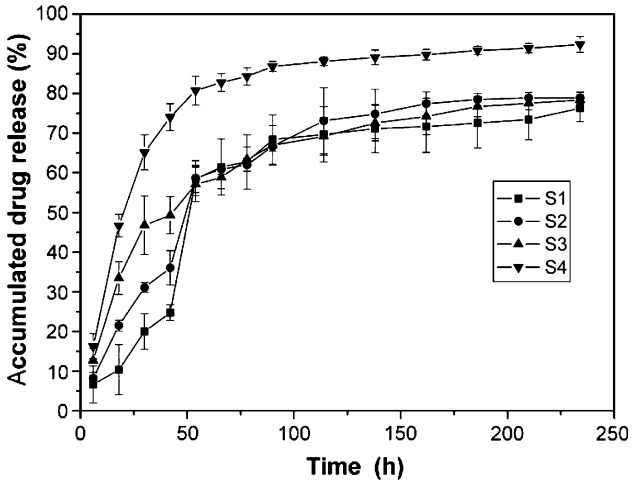

Fig. 2 In vitro release profiles of SOD from the composition electrospun fibers. Each data point represents the average of $n = 3$ samples, error bars represent standard deviations (curve S1: S1 sample, curve S2: S2 sample, curve S3: S3 sample, curve S4: S4 sample). Release condition: pH 7.4 phosphate buffer solution, 37.0°C

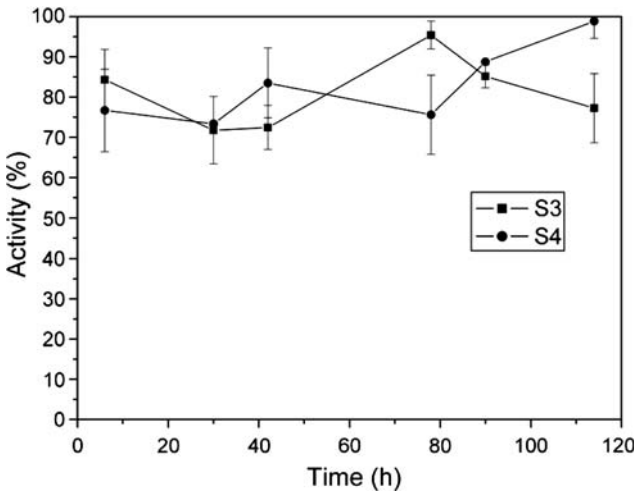

Fig. 3 In vitro antioxidant activity of the SOD from the samples S3 abd S4. Each data point represents the average of $n = 3$ samples, *error bars* represent standard deviations (curve S3: S3 sample, curve S4: S4 sample)

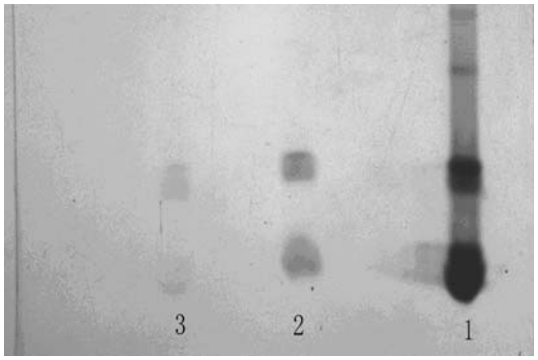

Fig. 4 Gel photographes of the SOD by SDS–PAGE. 1: virgin SOD, 2: SOD released from S4 sample, 3: SOD released from S3 sample

3.4 Integrity of SOD

SDS–PAGE was carried out to determine whether the electrospinning and release procedure affected the integrity of SOD. The gel photographes of the virgin SOD, the SOD released from the fiber S3 and the SOD released from the fiber S4 were shown in Fig. 4. The size of SOD samples released from different fibers were in accord with the virgin SOD, so we can draw a conclusion that the SOD remained stable during the preparation by electrospinning and release experiment.

4 Conclusions

In this paper, we develop a controlled release system of SOD by electrospun fibers. Highly loading efficacy of SOD from 93.8 to 98.0% was achieved. Obvious initial burst release of SOD in vitro was not observed. The SOD can be released from the system for a long time. Antioxidant activity of the released SOD still was high. The SOD remained stable during the preparation by electrospinning and release experiment. We hope this system be useful as an implanted form, in the treatment for several disease involved with the superoxide radical in the future.

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