The Preparation of Silk Fibroin Drug-loading Microspheres

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Abstract: This paper deals with the development of a kind of water-in-oil-in-water (w/o/w) multiple-emulsification method to fabricate the SF drug-loading microspheres. The silk fibroin (SF) microspheres were prepared by using SF as vehicle and dexamethasone sodium phosphate (DSP) as drug model. The surface morphology was observed by SEM, the particle diameter and its distribution were observed with Laser particle sizer. The structure was studied by X-ray diffraction and Fourier transform infrared. The properties of drug release were assessed in vitro. The results showed the average size of microspheres varied from 6.53 μm to 68.60 μm. The structure of SF drug-loading microspheres had an obviously change compared with pure SF; and the change is the appearance of silk I and silk II structures; and its molecular conformation was β-sheet. The average drug-loading varied from 5.32 % to 9.01 %, and the average loading varied from 56.43 % to 99.07 %. The drug-loading and loading efficiency increased with the increase of SF concentration. The drug-loading and loading efficiency differed when the treated organic solvent differed, their order is: isopropanol<acetone<ethanol. Drug release of SF drug-loading microspheres: there was a slow release effect when the concentration of SF was 3% or 6%. Moreover, in the experimental condition, there was an obvious burst release of the drug when the SF concentration was 9 % or 12 % or when the treated solvent was ethanol.

Keywords: silk fibroin, microspheres, preparation, structure, drug-loading, controlled release

1. Introduction

In recent years, the encapsulated technology on the polymer drug-loading microspheres was actively applied in the medical field; especially natural macromolecule materials received much more attentions for their unique biocompatibility [1-6]. Among them, silk fibroin (SF) had received many researchers' interesting for its good biocompatibility, convenient preparation and rich sources. Former preparation of drug-loading microspheres all used chemical cross-linking agent, SF, SF and chitosan or sodium alginate as vehicle [7-10]. Chemical cross-linking agent had a potential threat to human. There are researches on the immobilized enzyme using SF as vehicle; due to the small quantity of the immobilized enzyme, the encapsulation was easy [8]. In general composite vehicles were used to raise the drug loading [9,10]. In this paper no chemical cross-linking agents were used, and the vehicle had only one component, i.e. SF; using dexamethasone sodium phosphate (DSP) as drug model. The silk fibroin drug-loading microspheres were prepared by improved water-in-oil-in-water (w/o/w) multiple-emulsification to enhance the drug loading and loading efficiency. The surface morphology, particle size and distribution of the size were observed by SEM and Laser particle sizer. The structure was studied by X-ray diffraction and Fourier transform infrared methods. The properties of drug release were also studied by in vitro.

2. Materials and methods

2.1 Materials

Cocoon shells of Bombyx mori, isopropanol (Changzhou wuwei reagent Co., Ltd. AR), acetone and ethanol (Shanghai reagent Co., AR), DSP (Tianjin Tian yao Pharmaceuticals Co., Ltd.), CaCl2 (Shanghai Meixing Chemical Engineering CO., LTD., AR), Disodium hydrogen phosphate (Sinopharm Chemical Reagent Co. Ltds, AR), Potassium dihydrogen phosphate (Shanghai chemical reagent Co., Ltd., AR), Span-80 and liquid paraffin (Tianjin damao chemical reagent Co., CP) et al.

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2.2 Preparation of SF drug-loading microspheres

After degumming, the Cocoon shells of Bombyx mori were dissolved in the mixed solution of C_aCl_2: H_2O: C_2H_5OH=1:8:2 (molar ratio) at 72±2°C, SF solution was obtained by dialyzing and filtering [7]. Liquid paraffin which included a certain amount of Span-80 was heated to 37°C. Then a mixture of SF and DSP was dropped into oil phase slowly (the mass ratio of DSP-to-SF was 1:10), the mixture emulsion was emulsified for 20 min with stirring at 720±20 rpm. After adding organic solvent (the volume ratio of organic solvent to-SF was 4:1) and stirring for a certain time, the mixture emulsion was centrifuged at 3000 rpm; and then removed the supernatant. Adding a certain amount of organic solvent, then the mixture was stored at 4°C for a certain time, and then centrifuged. The mixture emulsion was washed twice with isopropanol and once with deionized water. Then the SF microspheres were obtained under vacuum freeze-drying (USA, VIRTIS GENESIS 25-LE freeze-drier) at -40°C.

The effects of the SF concentration and organic solvent on the properties of drug-loading microspheres were investigated. The concentration of SF was 3 %, 6 %, 9 % and 12 % respectively. The organic solvent was isopropanol, acetone and ethanol respectively.

2.3 Morphology

Morphology of the microspheres was observed by using SEM (Japan, S-570). The microspheres particle size and its distribution were measured by Laser particle sizer (Zhuhai, LS800).

2.4 Structure of the SF drug-loading microspheres

X-Ray Diffraction (XRD): X-ray diffraction was performed by using X'Pert PRO MRD polycrytalling diffractometer (Holland, PANalytical Company) with CuK_α radiation from a source operated at 40 kV and 40 mA. The diffraction intensity curves with 2θ from 5° to 45° were obtained.

Fourier Transform Infrared (FTIR): Samples were prepared with KBr disk. FTIR spectra were obtained with NICOLET-5700 FT-IR (USA, Thermo Electron Corporation) and the wave number ranged from 400 cm^{-1} to 4000 cm^{-1}.

2.5 Drug-loading and loading efficiency of microspheres

Samples of 50 mg dried drug-loading microspheres were added into the tube of phosphate buffer saline (PBS, pH 7.4); let the volume of the buffer solution be V. Oscillated the tube at 37°C for 12 h, and then centrifuged the mixture at 3000 rmp for 20 min. Then took a certain amount of supernatant to test its absorbance by using ultraviolet spectrophotometer (UV-2550, Japan Shimadzu), and absorbance A_1 was detected. Moreover, the residual solution was carefully removed. And then the same volume of PBS was added, and centrifuged the tube after oscillating it at 37°C for 6h, and absorbance A_2 was detected by the same method. By measurement the absorbances A of different DSP concentrations C (μg/ml) could be obtained, and then the regressive equation between A and C could be established as follows:

$$A=0.02704C+0.01263. \quad (1)$$

And the weight of the drug in the microspheres W_1 could be calculated from the DSP concentration C_1 and C_2 in the supernatant according to the following equation:

$$W_1=(C_1\times n_1 + C_2\times n_2)\times V, \quad (2)$$

Where n_1, n_2 were respectively the diluted times of the samples that were used to test the absorbance. Thus from equation (1) concentration C_1 and C_2 could be calculated according to A_1 and A_2 respectively; W_1 could then be obtained by equation (2). Thus the Drug-loading and loading efficiency could be obtained as follows:

$$\text{Drug - loading}\,(\%)=\frac{W_1}{W_2}\times 100\%\cdot$$

$$\text{Loading efficiency}\,(\%)=\frac{W_1}{W_3}\times 100\%\cdot$$

Where W_2 is the total weight of microspheres, W_3 is the input weight of drug (DSP).

2.6 Drug release of the microspheres

50 mg dried microspheres were put into the bag filter and the microspheres were suspended in 2 ml of PBS, pH 7.4. After putting the bag filter in the conical flask which contained 50ml PBS, the microspheres were oscillated at 37±1°C. Took samples at 1h, 4h, 8h, 12h, 24h, 48h and 72h respectively. Took 5 ml released solution at designated times, and then 5 ml PBS was added to conical flask to keep the same volume. Absorbance was detected at 242nm by using ultraviolet spectrophotometer, the drug release was calculated according to the absorbance (n=3).

$$\text{Drug release } (\%) = \frac{\text{Drug released } (\mu g)}{\text{Drug in microspheres } (\mu g)} \times 100\%$$

3. Results and discussion

3.1 Particle size of microspheres

3.1.1 Effect of the SF concentration on size of the mircrospheres

From Figure 1 it can be seen that the particle size of SF drug-loading microspheres increased with the concentration of SF, especially when the concentration of SF was above 6 %.The reason was that the droplets coming into being during the emulsification were of the same size, the SF particle would be bigger during the crystallization of the SF when the concentration of the SF solution increased. Moreover, during preparation of SF microspheres volume of the added organic solvent was determined according to the SF solution volume. In this research work, when the volume ratio of the organic solvent to SF solution was constant, with the increase of the SF concentration, SF solution volume decreased in the equal weight of SF. Thus the volume ratio of the SF per mg to organic solvent decreased, and the remained oil in the microspheres increased, and the SF particles tended to aggregate.

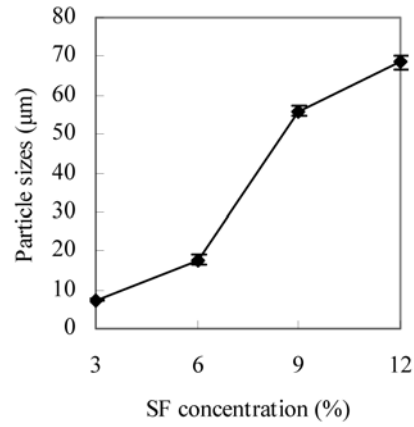


Figure 1 Effect of the SF concentration on size of the mircrospheres (n=3). The organic solvent that was used during the preparation of the SF microspheres was isopropanol.

3.1.2 Effect of different organic solvent on size of the mircrospheres

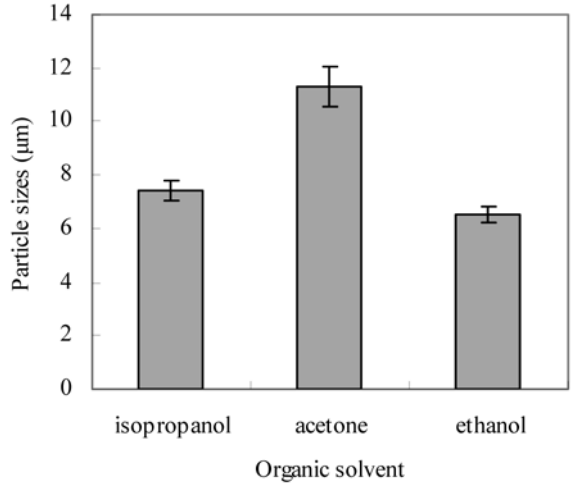


Figure 2 Effect of different organic solvent on size of the microspheres (n=3). The SF concentration used is 3%.

Figure 2 shows the average particle size of the SF drug-loading microspheres when the SF concentration was 3%. From Figure 2 it is seen that the average size varied from 6.53 μm to 11.30 μm. SF drug-loading microspheres prepared with different organic solvent had no obvious differences.

3.2 Morphology

According to Figure 3, the shapes of the particles were subglobular. The microspheres treated by

ethanol conglutinated seriously, which suggested that there was more oil remained in the microspheres treated by ethanol.

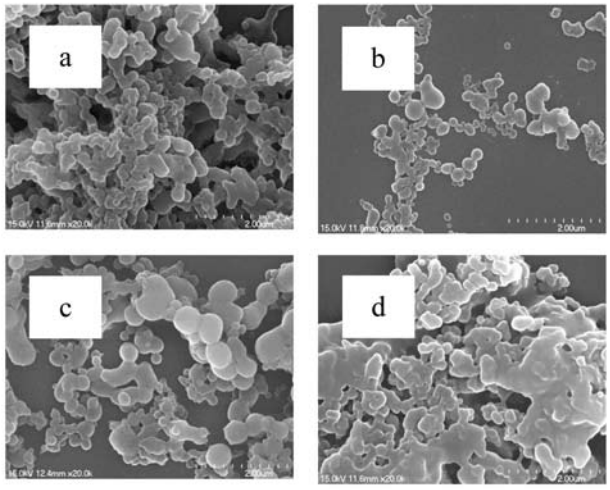


Figure 3 SEM photographs of the microspheres. (a) The organic solvent used was isopropanol; the concentration of the SF solution was 3%. (b) The organic solvent used was acetone; the concentration of the SF solution was 3%. (c) The organic solvent used was isopropanol; the concentration of the SF solution was 6%. (d) The organic solvent used was ethanol; the concentration of the SF solution was 3%.

3.3 Structure of the microspheres

3.3.1 XRD of SF drug-loading microspheres

Bombyx mori SF has crystalline state and random coil in different conditions, crystalline state includes silkIand silkII. On the XRD curves, the main diffraction peaks of silkI were 12.2°, 19.7°, 24.7°, 28.2° etc, and peaks of silkII were 9.1°, 18.9°, 20.7° etc [11]. Figure 5 and Figure 6 showed XRD curves of SF drug-loading microspheres prepared with different SF concentration and different organic solvent. It was shown in Figure 5 (curve e) that pure SF had a minor peak near 9.1°, which indicated the structure of pure SF was mainly amorphism. A silkII peak appeared around 20.6°, but the peaks of silk fibroin solvent with a concentration of 9% and 12% are less obvious than the other cases. A minor silkII peak appeared around 9.1° and silkI peak appeared around 24.6° under different experiment conditions. The structure of SF microspheres had an obvious change comparing with that of the pure SF due to the appearance of the silkI and silkII. Thus it was suggested that the change of organic solvent quantity has significant affects on the structure of the microspheres. The organic solvent seized H_2O from the hydrate layer of the protein molecule when the solvent dissolved in water, and by this way the water film of the protein molecule was destroyed, and the protein molecule precipitated, and its structure changed into β-sheet. But there were many X-ray diffraction peaks in DSP XRD curves (Figure 4), a number of drug crystallization peaks disappeared in SF drug-loading microspheres XRD curves. The reason may be that the drug was encapsulated in microspheres or within the range of this experiment, the amount of added DSP had no obviously effect on the structure of drug-loading microspheres prepared under different conditions and with different organic solvent.

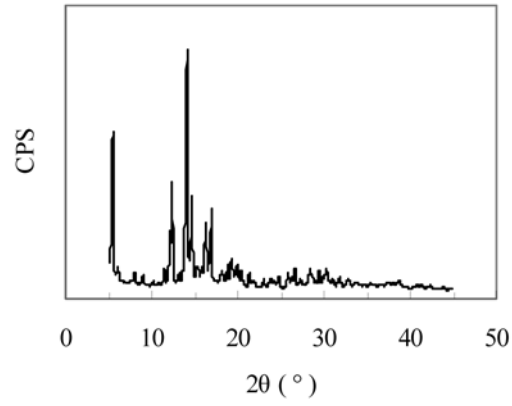


Figure 4 XRD curves of DSP.

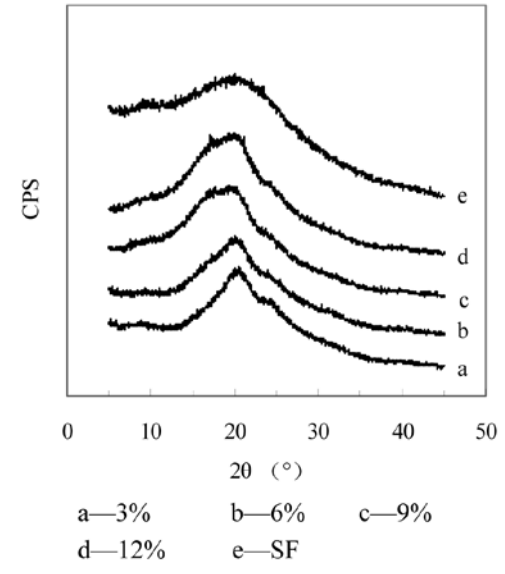
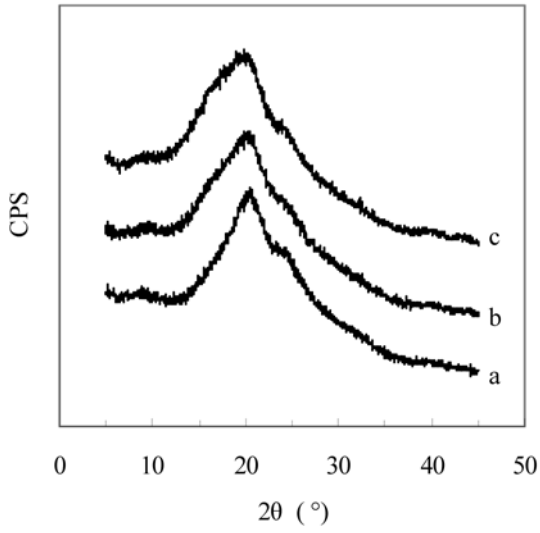


Figure 5 XRD curves of SF drug-loading

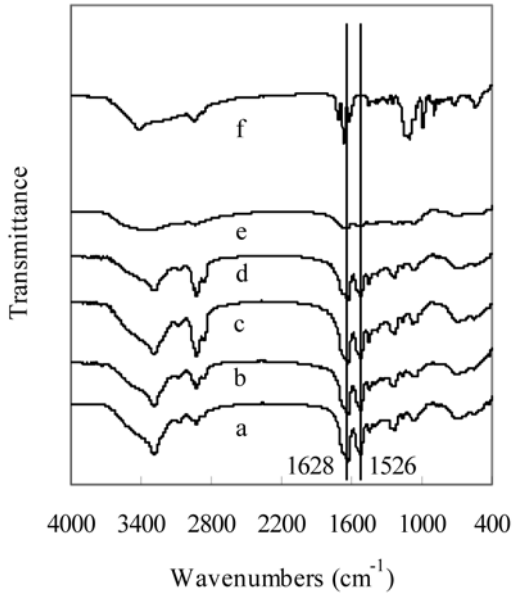
microspheres prepared with different SF concentrations.



a—isopropanol b—acetone
c—ethanol

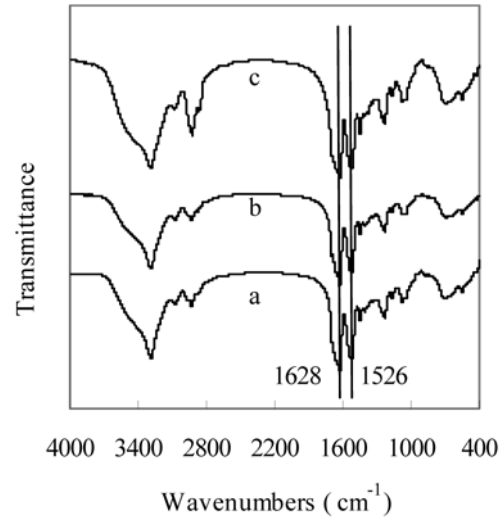
Figure 6 XRD curves of SF drug-loading microspheres treated with different organic solvent.

3.3.2 FTIR of SF drug-loading microspheres



a—3% b—6% c—9%
d—12% e—pure SF f—DSP

Figure 7 FTIR spectra of SF drug-loading microspheres prepared with different SF concentrations.



a—isopropanol b—acetone
c—ethanol

Figure 8 FTIR spectra of SF drug-loading microspheres treated with different organic solvent.

Figure 7 and Figure 8 shows FTIR spectra of SF drug-loading microspheres prepared with different concentrations of SF, different organic solvent. It can be seen in Figure 7 (curve e) that characteristic peaks of amideI, amideII of pure SF appeared at 1648 cm^{-1}, 1536 cm^{-1}, essentially belonged to the amide characteristic peaks of random coil conformation. The FTIR spectra of SF drug-loading microspheres prepared with different experimental conditions were essentially the same, their amideI, amideII moved to low wavenumbers respectively, and there are obvious β-sheet characteristic peaks around 1628cm^{-1}, 1526 cm^{-1}. It was indicated that the structure of SF was obviously changed in the preparation of microspheres, the molecular conformation of SF had transformed from random coil to β-sheet. There were also no DSP diffraction peaks in FTIR spectra. The results accorded with that of the XRD.

3.4 Drug-loading and loading efficiency of the microspheres

3.4.1 Effect of SF concentration on the drug-loading and loading efficiency of the microspheres

Table 1
The drug-loading and loading efficiency of microspheres prepared by different SF concentrations

SF concentration [%]	drug-loading [%]	loading efficiency [%]
3	5.13±0.14	56.43±1.54
6	8.36±0.06	92.00±0.64
9	8.76±0.17	96.30±1.87
12	9.01±0.05	99.07±0.60

The organic solvent that are used during the preparation of the SF microspheres was isopropanol (n=3).

From Table 1 it is clear that the drug-loading and loading efficiency increased with SF concentration, especially when the SF concentration was 3% or 6%. The reason is that the volume ratio of the SF per mg to organic solvent decreased, and the remained oil in the microspheres increased. Thus the drugs were difficult to be washed by deionized water.

3.4.2 Effect of different organic solvent on the drug-loading and loading efficiency of the microspheres

organic solvent	drug-loading [%]	loading efficiency [%]
isopropanol	5.13±0.14	56.43±1.54
acetone	6.87±0.11	75.61±1.21
ethanol	7.85±0.13	86.46±1.47

The SF concentration used is 3% (n=3).

From Table 2, it can be seen that the drug-loading and loading efficiency differed when the treated organic solvent differed, their order is: isopropanol<acetone<ethanol. All the three solvents were polarity solvents. But their polarity was different; the order of their polarity was: isopropanol<acetone<ethanol. It's probably due to their different polarity the changing speed of the protein structure induced by the solvent differed, and the ability to remove the oil differed, thus the drug-loading and the loading efficiency differed.

3.5 Drug release of SF drug-loading microspheres

3.5.1 Effect of the SF concentration on drug release of the microspheres

Figure 9 showed drug release of different SF concentration. All of DSP released out within 72h from the SF drug-loading microspheres in the case of SF concentration was 3% or 6%, which showed that the microspheres had a slow release effect. When SF concentration was 9% or 12%, DSP released out within 12h, which showed burst release obviously. The reason may be that when the SF concentration was high, most of drugs encapsulated or adhered in the surface of microspheres, so the drugs had an obvious burst release. As DSP was water-solubility drug, the drug could only release slowly by passive diffusion and degradation of the SF when the drug was encapsulated in the microspheres.

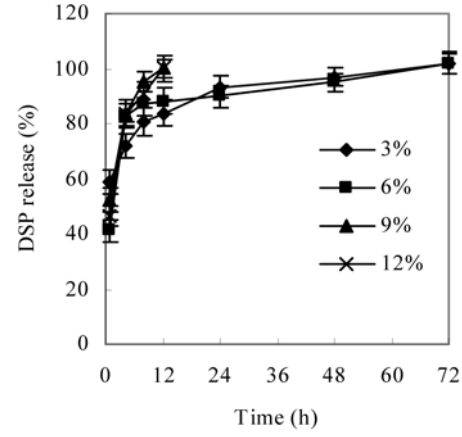


Figure 9 Effect of the SF concentration on drug release of the microspheres (n=3).The organic solvent that was used during the preparation of the SF microspheres was isopropanol.

3.5.2 Effect of different organic solvent on drug release of the microspheres

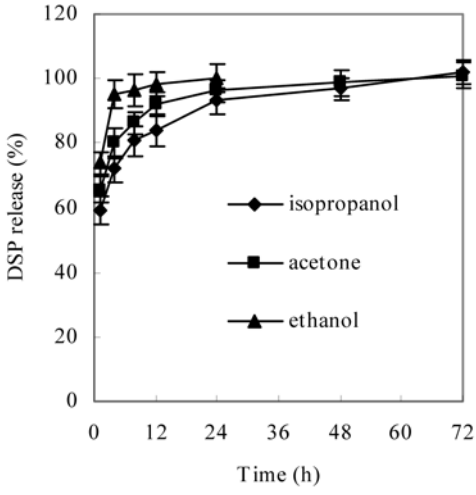


Figure 10 Effect of different organic solvent on drug release of the microspheres (n=3). The SF concentration used is 3%.

From Figure 10, it could be seen that the drug release speed of the microspheres had the following order: isopropanol<acetone<ethanol. Especially the SF drug-loading microspheres treated by ethanol had an obvious burst release of the drug, which suggested that the drug adhered more on the microsphere surface, and encapsulated less in the microspheres when treated by ethanol. The result showed that ethanol had the same function of inducing the crystallization of the SF, and fabricating the microspheres of the same structure as isopropanol and acetone, except the low drug loading of the SF mirospheres.

4. Conclusions

A kind of water-in-oil-in-water (w/o/w) multiple emulsifications method to fabricate the SF drug-loading microspheres was developed in this work. The drug-loading and loading efficiency increased with the increase of SF concentration. The average drug-loading varied from 5.32 % to 9.01 %, and the average loading efficiency varied from 56.43 % to 99.07 %.The drug-loading and loading efficiency differed when the treated organic solvent differed, their order was: isopropanol<acetone<ethanol. The drug-loading in this research was higher than previous study that took enzyme as the drug model [8]. The drug-loading and drug efficiency of the microspheres treated by ethanol were comparatively higher, but the burst release of the drug was obvious. Using acetone as the organic solvent, there was a slow release effect . Using isopropanol as the organic solvent; there was a slow release effect when the concentration of SF was 3% or 6%. There is an obvious burst release of the drug when the SF concentration was 9% or 12%. SF drug-loading microspheres' average size varied from 6.53 μm to 68.60 μm, and the shapes of the particles were subglobular. The structure of SF drug-loading microspheres had an obvious change compared with pure SF, the change was the appearance of the silkI and silkII structures, its crystallinity enhanced, and its molecular conformation was β-sheet.

Acknowledgement

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References:

[1] Hickey T, Kreutzer D, Burgess DJ, Moussy F. Dexamethasone/PLGA microspheres for continuous delivery of an anti-inflammatory drug for implantable medical devices. Biomaterials 2002;23:649-1656.

[2] Denkbas EB, Seyyal M, Piskin E. 5-Fluorouracil loaded chitosan microspheres for chemoembolization. Microencapsule 1999;16(6):741.

[3] Berthold A, Cremer K, Kreuter J. Preparation and characterization of chitosan microspheres as drug carrier for prednisolone sodium phosphate as model for anti-inflammatory drugs. J Control Release 1996;39:17-25.

[4] Wang SB, Chen AZ, Weng LJ, Chen MY, Xie XL. Effect of drug-loading methods on drug load, encapsulation efficiency and release properties of alginate/poly-L-arginine/chitosan ternary complex microcapsules. Macromol Biosci 2004;4:27-30.

[5] Brown KE, Leong K, Huang CH, Dalal R, Green GD, Haimes H B, Jimenze P A, Bathon J. Gelatin/ chondroitin 6-sulfate microspheres for the edeliver of therapeutic proteins to the joint. Arthritis Rheum 1998;41:2185-2195.

[6] Schlapp M, Friess W. Collagen/PLGA microparticle composites for local controlled delivery of gentamicin. Pharm Sci 2003;92:2145-2151.

[7] Xie RJ, Li MZ, Wu HY, Feng YX, Yang JF. The 6th China international textile forum. Chemical Industry Press, 2007. p.341.

[8] Wang XQ, Wenk E, Matsumoto A, Meinel L, Li C, Kaplan DL. Silk microspheres for encapsulation and controlled release. J Control Release 2007;117:360-370.

[9] Peng XH, Zhang LN. Studies on the structure and controlled release of chitosan/fibroin microsphere inclusion drug. Acta Polymerica Sinica 2000;4:502-505.

[10] Han LL, Zhang YZ, Yin GB. Structure and controlled release of medicine microcapsule wrapped with fibroin-alginate. Fine Chemicals 2004;21(7):251-254.

[11] Magoshi J, Magoshi Y. Physical properties and structure of silk. Physical properties and structure of silk. J Polym Sci Polym Phys 1977;15:1675-1683.

Title of Your Paper

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$$c2 = a2 + b2. \quad (1)$$

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T3	5	Bad
T4	7	Very bad

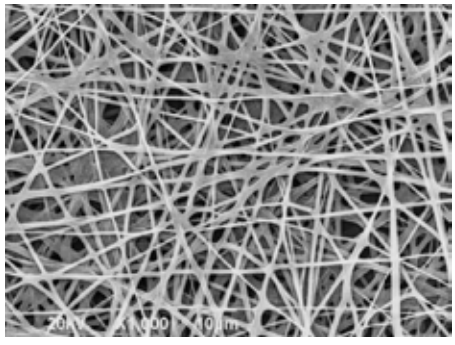

Figure 1 SEM of sample

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References:

[1] Driessens FCM, Boltong MG, Bermudez O, Planell JA. Formulation and setting times of some calcium orthophosphate cements: a pilot study. J Mater Sci: Mater Med 1993;4:503-508.

[2] Nancollas H. In vitro studies of calcium phosphate crystallisation. In: Mann S, Webb J, Williams RJP, editors. Biomineralization. Chemical and biochemical perspectives. New York: VCH, 1989. p. 157-182.

[3] Brown W, Chow LC. Combinations of sparingly soluble calcium phosphates in slurries and paste as mineralizers and cements. US Patent No. 4612053, 1986.

[4] Information on http://www.weld.labs.gov.cn