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The synthesis, characterization and properties of bovine serum albumin microspheres carrying 8-phenylamine-1-naphthalene-sulfonic acid.

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Abstract

As a novel drug carrier, protein microsphere has advantages in high efficiency, low toxicity, clear target direction, controllable release, easy degradation etc. In the paper, the Bovine Serum Albumin microspheres carrying 8-phenylamine-1-naphthalenesulfonic acid (ANS) as drug alternatives were synthesized by emulsifying-crosslinking method. Its shape and size were characterized through scanning electron microscope. And the recovery of ANS, encapsulation efficiency of microspheres, ANS loading rate and the cumulative release percentage were measured by ultraviolet spectrophotometer. Smooth surface of microsphere particle with 2-10 μm in size for empty microspheres and 2-14 μm for carrying ANS microspheres were observed under the scanning electron microscopy (SEM). Encapsulation efficiency of microspheres is 88.34%, and the ANS loading rate is 5.49%. ANS release kinetics *in vitro* was also detected and it was found that the release curve is more in line with Korsemyer-Peppas Model: lnQ=0.115lnt ± 3.857 (R2=0.99067).

Keywords: Bovine serum albumin microspheres, Swelling capacity, Encapsulation efficiency, Loading rate, Release kinetics.

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Introduction

With the development of modern biotechnology and various chemical synthesis methods, more and more active drug preparation have been produced for the treatment of various human diseases. However, when these drugs are used, there are many imperfection, such as short half-life, poor stability in the body, and low bioavailability. Thus, the drug delivery becames one of the major challenges in treatment strategy. It is now widely adopted that the drug is hidden in macromolecules (such as sugars, gelatin, proteins, etc.) by enclosure technique for delivery and slow release in the body to maintain effective active substance levels over long periods of time [1-4]. Therefore, the use of nano and microspheres as carriers in the targeted transmission of drugs has attracted the attention of many scholars. A carrier of drugs requires suitable particle size, no toxicity, good biocompatibility, certain stability, biodegradable properties and no chemical reaction with drugs. The nature macromolecules of protein, as a material meets these requirements, so it has been used in a large number of studies [5-8]. In this paper, bovine serum protein (BSA), as a material for microspheres, was used to enclose 8-phenylamine-1-naphthalenesulfonic acid (ANS), as a model drug, to reveal the properties of protein microspheres. And the shape and size of the microspheres are characterized. ANS loading rate, encapsulation efficiency, and ANS release rate of microspheres *in vitro* were studied to explore its application in the biological and medical targeted drug. All could provide some references for the development of protein microspheres.

Methods

Reagents and apparatus

Apparatus: KQ-400KDE type of high power ultrasonic cleaner; low-speed centrifuges; UV-2550 UV visible spectrophotometer(Shimadzu, Japan); Scanning electron microscopy (Czech company).

Reagents: 8-aniline-1-naphthalene sulfonic acid (ANS)Bovine serum albumin (BSA)and glutaraldehyde (50%) are sigma reagents; Protease was obtained from Aladdin Co.

Experimental methods

Preparation of BSA protein microspheres: 20 mg ANS and 250 mg BSA were added in 4 mL phosphate buffer at pH 6.6 for one hour of ultrasonic dissolution to be used as water phase. Then 40 mL castor oil was taken as an oil phase. When the oil phase was stirred on the electric agitator, the water phase is slowly added into it. Stirring for 30 min and then homogenization for 15 min in the ultrasonic container, w/o colostrums were obtained. Next, 0.8 ml glutaraldehyde was added into it. After 4 hours of lasting stirring for curing, 30 mL acetate ethyl Ester were mixed in it. Stirring was done for 20 min again. Centrifuge, suction filtration, and washing precipitation 3 times with ethyl acetate (take 10 mL each time) were followed. Light yellow microspheres powder was then

obtained. The blank microspheres were obtained in the same way [9,10].

Establishment of a standard curve: 30 mg ANS was dissolved in 250 ml anhydrous ethanol. Then the diluent with the concentration of 0.07, 0.13, 0.20, 0.27, 0.33 mol·mL^{-1} was obtained, respectively and the absorbance was measured at 374 nm. The standard curve for the concentration of ANS is based on the absorbance (A) and the equation is A=0.3632 C+0.0084 (R2=0.9990). It shows a good linearity in the experimental concentration range from 0.07 to 0.33 mol·mL^{-1}.

Similarly, the standard curve of ANS dissolved in phosphate buffer at pH 6.6 was obtained. The equation is A=0.3679 C +0.0127(R2=0.9999). It also shows a good linearity within the range from 0.07 to 0.33 mol·mL^{-1}.

Purification of ANS-carrying microspheres: The microspheres were purified by lasting 20 min of washing impurities in anhydrous ethanol and then filtering with 0.45 μm microfiltration membrane.

Size of the microsphere: A small amount of the blank microspheres and the drug loaded microspheres was ground and then characterized by SEM.

Swelling capacity of the microsphere in water: The ANS loaded microspheres were dispersed in water. After full swelling was achieved, the solution was centrifuged. After residual water on the surface of the microsphere was bloted up, the microspheres were weighed. The swelling capacity is calculated.

Swelling capacity=[(the weight of wet microsphere-the weight of dry one)/the weight of dry microsphere] × 100%.

The recovery of ANS: The solution of mixing 10 mg blank microspheres and 20 mg of proteases in 10 mL of phosphate buffer was prepared for three copies and settled into an ultrasonic transmitter. ANS with the mass of 0.6, 1.4 and 3.5 mg were added into them, respectively. 15 mL anhydrous ethanol was added to dilute the solution. Then they were kept in a constant warm water bath at 37°C for one hour. The following is centrifugation and filtration. The filtrate was detected in absorbance and the measured concentration of ANS was calculated according to the standard curve equation.

Measuring the precision of ANS: The standard solution with the concentration of 0.13, 0.20 mol·mL^{-1} ANS in anhydrous ethanol was measured separately at 2-hour intervals, measured 6 times a day, and measured for 3 consecutive days. Calculate its relative standard deviation (RSD) during a day and during the intervals of days, respectively.

ANS loading rate and encapsulation efficiency of microspheres: 0.05 g ANS loaded microspheres were added in 0.5% of the protease solution (5 mL), stirring for 30 min at 37°C in constant warm water bath. It was followed by adding 20 mL anhydrous ethanol in it, continuously stirring for 5 min. Then, the solution was filtered with a 0.45 μm microporous membrane, and the filtrate in absorbance was measured. ANS loading rate and encapsulation efficiency were calculated according to the standard curve equation. (ANS loading rate=the mass of ANS in microspheres/the mass of ANS-carrying microspheres × 100%, encapsulation rate=the mass of ANS in microspheres/the mass of total ANS × 100%).

ANS release rate of microspheres ***in vitro*****:** The purified 0.0600 g ANS loaded microspheres were added into 30 mL of phosphate buffer (pH 7.0), being stirred under a water bath at 37°C on a magnetic stirrer. Every 1, 3, 5, 7, 12, 20, 32, 44 h, 10 mL of the solution was taken out and centrifuged (800 r·min^{-1}). After taking 5 mL of supernatant for detecting, 5 mL of phosphate buffer was replenished and poured into the cone bottle. The solution absorbance from different periods was measured to calculate the cumulative release percentage. The operation above was repeated.

Results and Discussion

Characterization of microspheres by SEM

It can be seen from Figures 1 and 2 that both blank protein microspheres and ANS loaded microspheres are spheroids with a smooth and uniform surface through SEM [11]. The size of the blank microspheres is range from 2 to 10 microns, while ANS loaded microspheres is from 4 to 14 microns. The prepared BSA microspheres met the size requirements of drug loaded microspheres.

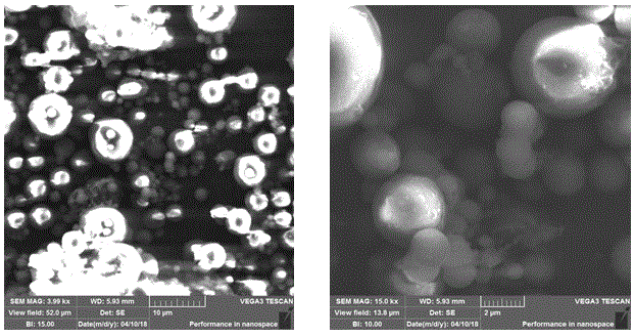

Figure 1. *Blank protein microspheres through SEM.*

Swelling capacity of microspheres

The ANS loaded microspheres were dispersed in water and observed under a polarized microscope. The microspheres were found to swell with time and their shapes became more and more irregular. It was detected that the average swelling capacity of ANS loaded microspheres is (85.72 ± 0.9) % when fully expanding at room temperature. The polymeric matrix absorb liquid to result in swelling and the transport of drug outside the matrix is accelerated. Thus, the drug release rate is controlled by the degree of swelling of the polymeric matrix [12]. High swelling capacity of microspheres indicates its potential in applications as drug carrier.

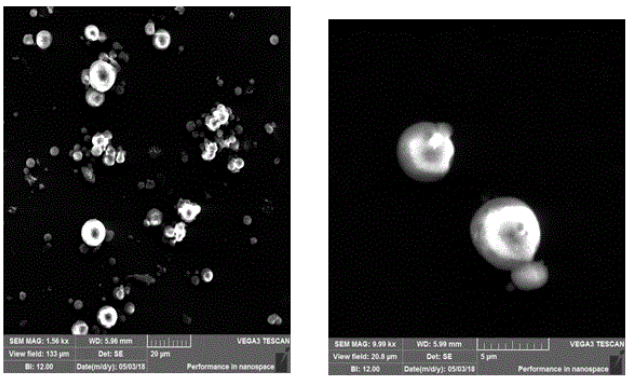

Figure 2. *Protein microspheres loading ANS (× 1500) through SEM. A × 1500; B × 10000.*

Table 1. *The results of recovery of ANS.*

Number	Quality of blank microspheres (mg)	Quality of adding ANS (mg)	Measured quality of ANS (mg)	The recovery of ANS (%)
1	10	0.6	0.53	88.33
2	10	0.6	0.52	86.24
3	10	1.4	1.23	85.71
4	10	1.4	1.19	85.45
5	10	3.5	3.16	86.75
6	10	3.5	3.04	91.43
The average recovery RSD		0.8732	_	_
		0.0259	_	_

The recovery rate of ANS

The recovery rate can check the approximation degree between the measured results and the true value or the recognized reference value. In order to identify the accuracy of the experiment, the average recovery rate of ANS was detected in blank microsphere environment. From Table 1, the average recovery rate come to 96.11%, which shows that the method has higher accuracy, better extraction rate.

The precision of the experiments

It can be seen in Figure 3 that the absorbance of ANS during a day and during the intervals of days is relatively stable and the error caused by the absorbance change is negligible. The detection absorbance of ANS provided the accuracy for experiments. Thus, the method has little influence on the subsequent determination of ANS loading rate, encapsulation efficiency, and ANS release percentage *in vitro*.

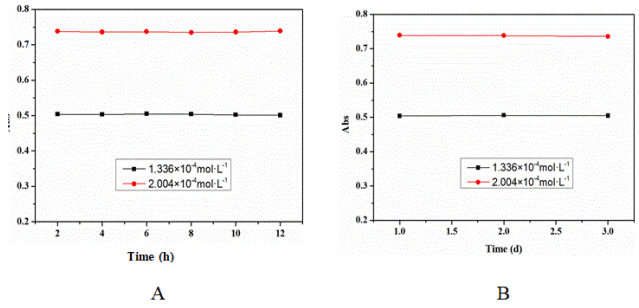

Figure 3. *(A) The precision of ANS during a day and (B) During the intervals of days.*

ANS loading rate and encapsulation efficiency of microspheres

Drug loading rate refers to the drug content for a particle as a whole, and the encapsulation ratio refers to the availability of the drug after being given. The experimental result shows that the ANS loading rate in microspheres is greater than 85%, indicating the feasibility of encapsulating drug by BSA and broad and bright prospect. While the encapsulation ratio is about 5.5%, which means only a small amount of ANS were encapsulated in microspheres among all given (Figure 4). The great amount of ANS must be given when encapsulating in progress, so further exploration should be done for it.

ANS release rate of microspheres in vitro

Drug release is a key process in which the drug is subjected to absorption, distribution, metabolism, and excretion [13]. The drug slow-release rule [14] has a great effect on its efficacy. Delaying the drug release time can maintain the drug effect for a long time, enhance the curative effect and reduce the frequency of drug delivery [15-17]. The release of ANS from encapsulation microspheres was shown in Figure 5, in which a very rapid release up to more 55% was displayed in the first three hours and then the release gradually slow down. The release rate of ANS reached 69.5% in 44 hours, from which

there was no significant change. Different mathematical models including first-order, zero-order, Higuchi, and Korsemyer-Peppas model were used to fit the percentage change of free ANS in ANS-loaded microspheres over time, shown in Figure 6. The fitting result according to the linear equation is presented in Table 2. It was found that the ANS release behavior from encapsulation microspheres is close to the Higuchi model and Korsemyer-Peppas model, R2 of 0.93599 and 0.99067, respectively. Compared with the Higuchi model, the Korsemyer-Peppas model is more linear.

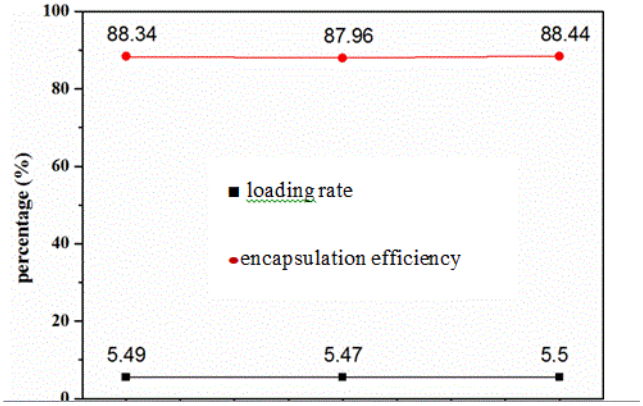

Figure 4. *ANS loading rate and encapsulation efficiency of microspheres.*

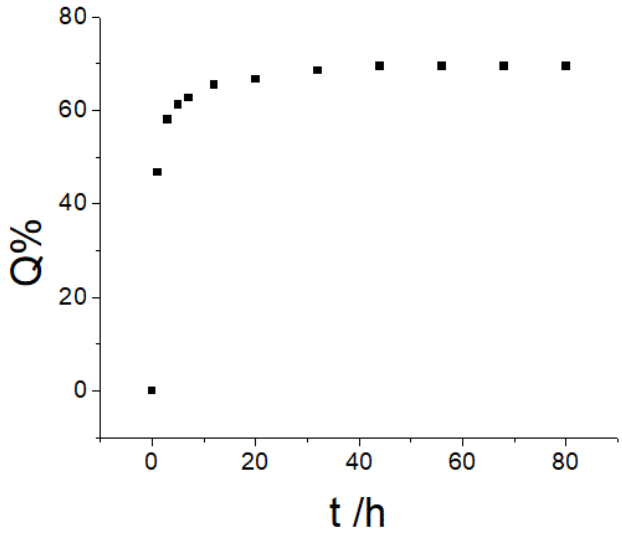

Figure 5. *Cumulative release curves of ANS in encapsulation microspheres.*

The basic equation of Higuchi model is C=[D(2qt-Cs)Cst] 1/2. Where C=total amount of drug release per unit area of the matrix, D=diffusion coefficient for the drug in the matrix, qt=total amount of drug in a unit volume of a matrix, Cs=dimensional solubility of the drug in the polymer matrix, t=time. Higuchi model was simplified to $C=Kt_{1/2}$. Where K is the Higuchi dissolution constant. Higuchi described the drug release as a diffusion process based on Fick's law i.e. square root time dependent [18].

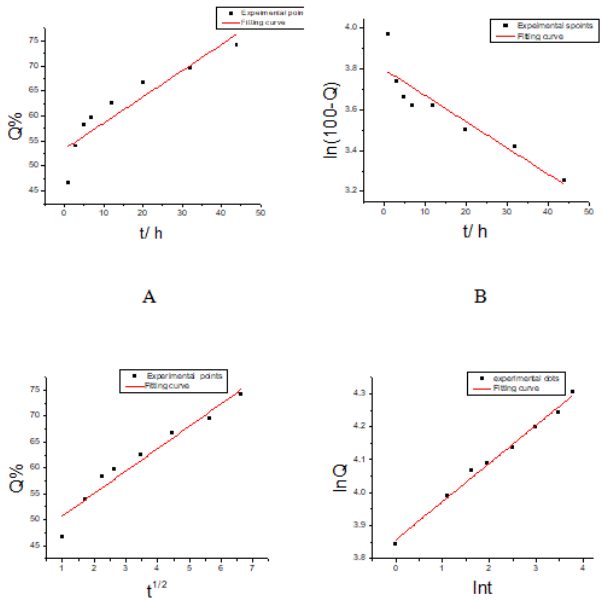

Figure 6. *(A) Cumulative release fitting curve according to zero order equation (B) To first order equation (C) To Higuchi equation (D) To Korsemyet-Peppas Model.*

Table 2. *Result according to the different fitting equation.*

Tapies of fitting equation	Equation	correlation coefficient
Zero order equation	Q=0.522t+53.358	0.81162
First order equation	ln(100-Q)=-0.0128t+3.796	0.81599
Higuchie equation	$Q=4.3456t_{1/2}+46.333$	0.93599
Korsemyet-Peppas Model	lnQ=0.115lnt+3.857	0.99067

Korsmeyer-Peppas model is represented by the equation: C_t/C_∞ =ktn where C_t/C_∞ is a fraction of drug released at time t, k is the release rate constant and n is an exponent for drug release [19]. A modified form of this equation was developed to C_t/C_∞ =ktn+b. There may be several simultaneous processes in the release model: diffusion of water into it, it is swelling as water enters formation of gel, diffusion of drug, and dissolution of microcapsules. The high swelling capacity of protein microspheres looks to give a good conformation.

Conclusion

The drug-loaded microspheres require maintaining the efficacy for a long time to achieve the purpose of longer interval delivery. The protein microspheres obtained in the experiments are small enough in size of microns sphere, high in swelling capacity and in drug loading rate. The drug efficiency can last for more than 40 hours, and about 70% of drug can be utilized. Its release follows the Korsmeyer-Peppas model. For hydrophobic molecule of ANS, it takes up more than 80% content in total protein microspheres. All is an important basis for its utilization in drug slow-release.

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