

Preparation Poly(lactide-co-glycolide) Microsphere of Bone Sialoprotein

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Abstract

OBJECTIVE: To prepare PLGA microsphere of BSP. **METHODS:** emulsion solvent extraction-evaporation process was used to prepare microsphere. Technology of preparation was optimized by the orthogonal design. The surface morphology of the microsphere was observed by EM and SEM. The drug loading, the encapsulation efficiency and release rate in vitro were measured using micro-BCA kit. **RESULTS:** The mean particle size of the microsphere was $50 \pm 0.5 \mu\text{m}$, and its mean span was 0.58 ± 0.10 , the drug loading and the encapsulation efficiency were $(0.725 \pm 0.075) \%$ and $(64.876 \pm 0.680) \%$ respectively. The reappearance of pharmaceutical technology was good. **CONCLUSION:** this research described the optimized preparation technology of PLGA microspheres of BSP.

Keywords: Bone sialoprotein; Poly(lactide-co-glycolide); Orthogonal design

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1. Introduction

Bone sialoprotein(BSP for short) is a non-collagen protein of glycosylation, phosphorylation and sulfation mainly secreted by osteoblasts and osteoclasts, which was extracted from cattle bone for the first time in 1972, belongs to proteins family of SIBLING(small integrin-binding ligand, N-linked glycoproteins) [1]. BSP is rich in sialic acid(named N-acetyl neuraminidase of the end of sugar chain) which is an acyl derivative of neuraminidase [13]. Molecular weight of BSP is 70~80 kD, core protein is 33~34 kD.

Since BSP can start mineralization crystal growth when bone comes into being, it also mediates the combination between osteoblasts and bone mineral as well as having effects of inducing bone differentiation and remodeling bone shape. However, BSP is applied systemically or locally, effective concentration in position of bone defect can not be maintained, it affects bone recovering, and therefore consider preparing slow-release microspheres of BSP to release BSP stably in vivo and promote bone repair. PLGA(lactide-co-glycolide) is a polymer copolymer which is polymerized by lactide(LA) and glycolic acid(GA) under the catalyst, PLGA has been approved by the U.S. FDA for

carrier of slow-release drug. Slow-release microspheres of BSP has not been reported at home and abroad.

This research is reported as follow: optimized technique parameters and prepared the PLGA slow-release microspheres of BSP which can sustainedly release BSP.

2. Materials and instruments

BSP [2], purity>95%; PLGA, LA:GA=50/50, Chengdu Organic Chemistry Co.Ltd.,Chinese Academy of Sciences; methylene chloride, analytical grade, Guangzhou Xincheng Fine Chemical Plant; polyvinyl alcohol, analytical grade, Tianjin Kermel Chemical Reagent Co.,Ltd.; Micro BCA Protein Assay Kit, Pierce Company; Kubota 5220 centrifuge, Kubota Corporation, Japan; nikon E600, Nikon Corporation, Japan; Hitachi S-3000N scanning electron microscope, Hitachi, Japan; sigma1-15 centrifuge, Sigma, Germany; Biocell ht2 Reader, Biocell Company, Australia; JY92-2D ultrasonic cell crusher, Ningbo Xinzhi Biology and Science Co.,Ltd.; 85-2 type homiothermous and magnetic stirrer, Jiangsu Ronghua Instrument Manufacturing Co.,Ltd.; LGJ-12 vacuum freeze dryer, Beijing Songyuan Huaxing Science and Technology Development Co.,Ltd.

Table 1 Factor-level in orthogonal design

level	factors			
	A	B	C	D
	concentration of PLGA A%(w/v)	volume ratio of internal and external phase (v/v)	concentration of PVA A%(w/v)	ultrasonic power (W)
1	6%	1:8	2%	200
2	9%	1:12	4%	300
3	12%	1:16	6%	500

Drug loading, encapsulation efficiency and span were different under different experimental condition(**Table 2**).

Table 2 Results of orthogonal experiment($\bar{x} \pm S, n=3$)

Number of experiments	A	B	C	D	encapsulation efficiency (%)	drug loading(%)	span
1	1	1	1	1	25.20±0.60	0.552±0.002	0.75±0.015
2	2	2	2	2	34.64±0.80	0.596±0.018	0.65±0.010
3	1	3	3	3	36.00±1.00	0.564±0.009	1.15±0.030
4	2	1	2	3	34.00±0.90	0.650±0.025	0.80±0.008
5	2	2	3	1	51.31±1.30	0.644±0.015	1.25±0.020
6	2	3	1	2	45.00±1.25	0.624±0.024	1.00±0.011
7	3	1	3	2	73.33±1.66	0.750±0.040	1.31±0.013
8	3	2	1	3	54.24±1.48	0.618±0.018	0.83±0.005
9	3	3	2	1	63.46±2.30	0.736±0.036	0.53±0.008

2.1 Preparing microspheres with double emulsion evaporation

Prepared microspheres by double emulsion solvent evaporation method using a model protein such as BSA [3]: moderate PLGA was dissolved in dichloromethane and then appropriate BSA solution was taken to disperse in it to form colostrum under ultrasound, the colostrum was added slowly into the PVA solution to form a double emulsion which was mixed continuously to volatilize organic solvents, then the microspheres were centrifuged for 6 min at 1500 rpm and washed with distilled water, finally, they were freeze-dried and preserved at -20 °C. Optimized process parameters of PLGA microspheres of BSP using orthogonal design were shown in 3.1 results.

2.2 Microsphere morphology, particle size and distribution

Microspheres morphology was observed using optical microscope and scanning electron microscopy. Particle size and span were detected by microscopic counting microsphere at least 200 for each count. Span was calculated as follows: $\text{span} = (\text{D}_{90} - \text{D}_{10}) / \text{D}_{50}$, D_{90} , D_{10} , D_{50} is particle size when particle size of 90%, 10%, 50% microspheres is less than the value respectively [4].

2.3 Measuring BSP concentration in microspheres

2.3.1 Establishing the standard curve

Using micro-BCA method [5], the operational steps according to the kit instructions: (1), prepared accurately standard solutions of different protein concentration: 0.0 µg/ml, 0.50 µg/ml, 1.0 µg/ml, 2.5 µg/ml, 5.0 µg/ml, 10.0 µg/ml, 20.0 µg/ml, 40.0 µg/ml. (2), prepared

working solution, A solution:B solution:C solution=50:48:2. (3), standard solution of different concentration was added to microtiter plate per hole 150 µl, each concentration repeated twice, and then added 150 µl working fluid to each well, shaking a little so as to mix well. (4), incubated for 2 h at 37 °C. (5), the microtiter plate was then cooled to room temperature and read OD values at 562 nm. (6), made the measured data correspond to the standard concentration and used linear regression analysis, the regression equation was $A = 0.006 + 0.009 \times c (\mu\text{g/ml})$, the correlation coefficient $r = 0.997$, linear relationship was good.

Table 3 Results analysis of orthogonal design

	encapsulation efficiency				drug loading				span			
	A	B	C	D	A	B	C	D	A	B	C	D
K ₁	31.947	44.177	41.480	46.657	0.571	0.651	0.598	0.644	0.883	0.987	0.860	0.860
K ₂	43.437	46.730	44.033	50.990	0.639	0.619	0.661	0.657	1.033	0.927	0.660	1.020
K ₃	63.677	48.153	53.547	41.413	0.701	0.641	0.653	0.611	0.923	0.927	1.320	0.960
R	31.730	3.976	12.067	9.577	0.130	0.032	0.063	0.046	0.150	0.060	0.660	0.160
P	*										*	

* $P < 0.05$

2.3.2 Measuring BSP concentration in microspheres

Weighed 10 mg dried microspheres which then were dissolved in 4 ml 0.1 mol/L NaOH solution (containing 5 % SDS), shaking for 24 h at 37 °C, centrifuged to obtain supernatant and read OD values with micro-BCA method which were substituted in an equation of standard curve to calculate the concentration of protein: microsphere drug loading=(weight of BSP in microspheres/weight of microspheres)×100 %; encapsulation efficiency=(weight of BSP in microspheres/inventory rating)×100 %.

2.4 Releasing test of BSP microspheres in vitro

Weighed 10 mg microspheres and put in 15 ml centrifuge tubes, 0.02 mol/L PBS buffer(pH7.4, 0.02 % NaN_3) of 4 ml was added into, shake at 50 rpm, 37 °C, centrifuge tube was centrifuged for 5 min at 2100 rpm on 1 d, 3 d, 7 d, 12 d, 17 d, 21 d, 25 d, 30 d, obtained supernatant 0.8 ml(added to the centrifuge tube 0.8 ml PBS buffer at the same time) which was then centrifuged for 1 min at 12000 rpm, took 0.6 ml to measure with micro BCA method. The release time was horizontal and the percentage of cumulative release was vertical axis, drew a chart. Percentage of cumulative release=(total protein releasing from microspheres/total protein in microspheres)×100 %. Repeated testing percentage of cumulative release three times, said data was shown as $\bar{x} \pm \text{SEM}$.

3. Results

3.1 Orthogonal experiment and analysis

3.1.1 Single factor of preparation PLGA microspheres of BSP

Different single factor was considered to effect on-quality of microspheres: selected PLGA concentration range from 3 % to 15 %, internal and external phase volume ratio 1:6~1:18, PVA concentration 0.5 %~8 %, ultrasonic power and time 100 W~500 W, 30 s~120 s, stirring speed 800 rpm~2400 rpm, the ratio of inventory rating was 1:2 to 1:8 and so on. Make sure other factors were constant so as to investigate the influence of single factor on encapsulation efficiency and drug loading.

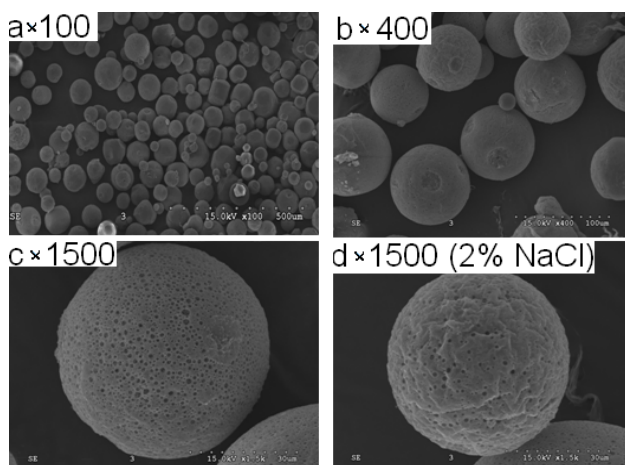


Figure 1. Scanning electron microscope of PLGA microspheres of BSP

The encapsulation efficiency and drug loading of microspheres increased as concentration of PLGA increased from 3 % to 12 %, from 12 % to 15 %, the encapsulation efficiency increased unconspicuously, but drug loading decreased. Therefore, this experiment used 6 % to 12 % concentration of PLGA. When internal and external phase volume ratio is 1:18, output rate of microspheres was low relatively; 1:6, the reaction system was so viscous not to diffuse microspheres equably. For this reason, we confirmed that the internal and external phase volume ratio was 1:8~1:16. Low ultrasonic power and short ultrasonic time resulted in incomplete emulsification of colostrum, contrariwise, it led to protein inactivation easily, finally the power range was 200 W~300 W, ultrasonic time was 30 s. When stirring rate was low, no regular microspheres formed, having severe adhesion each other, the higher the stirring rate, the smaller particle size of microspheres. Because of utility of microspheres in this research is for tissue embedding not for injection, having no strict requirements on the size, consequently, stirring rate was 1500 rpm for 10 min when the colostrum was put into the external phase, and then adjusted to 1000 rpm to volatilize organic solvents. Microspheres dispersed well at low concentration of PVA, but it was so low to burst release seriously while so high to form a block easily in the mixing process. Determined the concentration of PVA was 2 %~6 %. With increasing

feed ratio, the encapsulation efficiency and the drug loading of microspheres increased gradually, but when it increased to 1:2, the encapsulation efficiency and drug loading did not increase, indicating drug loading capacity of microspheres was limited, range from 1:4 to 1:8 was selected.

3.1.2 Design and analysis of orthogonal experiment

Selected four factors which effected the quality of microspheres more significantly: concentration of PLGA in the organic phase, volume ratio of internal and external phase (solution volume ratio of PLGA and PVA), concentration of PVA, ultrasonic power (table 1). Optimized process conditions according to L₉(3⁴) orthogonal design, repeated three times each level, drug loading, encapsulation efficiency and span (evenness) were assessment criteria.

The results of the orthogonal design were for visual analysis and variance analysis, the R value from table 3 can be judged, PLGA concentration had a great impact on drug loading and encapsulation efficiency, effect of PVA concentration on span was obvious. The larger the K value, the smaller the level of span, the better drug loading and encapsulation efficiency of microspheres. Variance analysis of the data in table 2 suggested that the influence of the concentration of PLGA on encapsulation efficiency of BSP microspheres were significantly different ($P < 0.05$), while the influence of volume ratio of internal and external phase, PVA concentration and ultrasonic power on the drug loading and encapsulation efficiency had not significant difference ($P > 0.05$); PVA concentration on particle size and span of microspheres had significant influence ($P < 0.05$).

The best experimental combination was A₃-B₃-C₂-D₂, the PLGA concentration of 120 mg/ml, internal and external phase volume ratio of 1:16, PVA concentration in the dispersed phase of 4%, ultrasonic power is 300 W. Repeated preparation of the 3 batches of BSP microspheres, average diameter was $50 \pm 0.5 \mu\text{m}$, the average span was 0.58 ± 0.10 , the average drug loading was $(0.725 \pm 0.075)\%$, the average encapsulation efficiency was $(64.876 \pm 0.680)\%$, optimized condition of preparing microsphere had good reproducibility withing the multiple emulsion solvent evaporation method.

3.2 Microsphere morphology and particle size

Shown in figure 1, BSP microspheres were observed under scanning electron microscope with different magnification multiple, they were round, uniformly dense surface porosity, average particle size was $50 \mu\text{m}$ almost, diameter of more than 90 % microspheres was between $40 \mu\text{m}$ and $70 \mu\text{m}$. Added 2 % NaCl aqueous solution into external phase when prepared BSP microspheres and found that the number of surface void on microspheres decrease and aperture became smaller, it suggested that adding NaCl to the external phase can

mitigate microsphere burst and make microsphere release relatively flat.

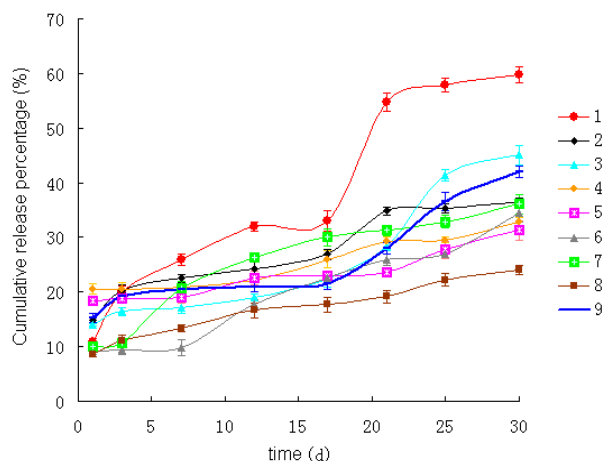


Figure 2. Cumulative release in vitro of BSP from PLGA microspheres

3.3 Results of microspheres release in vitro

The results of nine groups microspheres in vitro release for 30 days with orthogonal design were shown in Figure 2. There was certain different release behavior of microspheres under different preparation condition, good release curve should have relatively low burst release within 24 h, the whole process of release was flat, such as the 9th group release curve. Difference of preparation process between the 9th group microspheres and the optimal microspheres was only ultrasonic power, both of their release curves were very similar.

4. Discussion

We have done experiments using PLGA of different proportions (LA/GA, 50/50, 75/25) before, their viscosity and molecular weight were different, so were degradation rate in vitro, because the viscosity of 75/25 was higher than 50/50', preparation PLGA microspheres at a lower concentration of 75/25 can achieve, but the time of degradation was up to three months. From the application point of view, experiments in this research used the former (50/50). On the other hand, microsphere suspension needed to be precooled at 4 °C, -20 °C for 2~4 h respectively before freeze-drying for taking into account the integrity of microspheres.

The concentration of PLGA microsphere had great influence on drug loading and encapsulation efficiency, in a certain range, the encapsulation efficiency and drug loading will increased as the concentration increased, but the concentrations increase can not be unlimited, encapsulation efficiency didn't increase any more when PLGA concentration reached to a high level, while the drug loading was slow to decline and they cost more. PVA concentration and volume ratio of internal and external phase had influence on microsphere

size and span, generally, increasing concentration of PVA resulted in minishing the microsphere size, the smaller the volume ratio of internal and external phase, the better the microspheres dispersed, the shorter the span. Ultrasonic power was too low to emulsify completely and so high to make a great impact on protein and affect its activity. While ultrasonic time should be as short as possible to avoid adverse impact on protein activity between water interface and oil interface.

There were two factors affecting the release of protein drugs, one was correlative with the surface morphology of microspheres, LA/GA ratio and molecular weight (viscosity) of the polymer, large holes on the surface of microsphere and small size, burst release seriously [6]; the other was drug the structural characteristics itself and molecular weight. Release of BSP protein is incomplete in figure 2, that may result of interaction between protein with polymer microspheres [7].

In this research, we prepared the PLGA microspheres of BSP, optimized process and obtained good results using orthogonal design. The next step is to measure the activity of BSP in the microspheres so as to confirm whether BSP activity will be affected by the process of preparation and in vitro release, on the other hand, we should promote research of integration between osteoblast and bone mineral for creating the conditions in clinical application of BSP.

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