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Preparation and Characterization of Chitosan Microsphere Loading Bovine Serum Albumin

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Abstract: To optimize the preparation process of chitosan microspheres and study its loading capacity, chitosan microsphere was prepared by crosslinking with glutaraldehyde, and bovine serum albumin (BSA) was absorbed onto chitosan microsphere. Scanning electron microscope (SEM), Fourier transform infrared spectroscopy (FTIR), TA instruments and zeta potentiometer analyzer were used to characterize the parameters with respect to size, thermal characters, morphology, and zeta potential of the microspheres. The loading capability and *in vitro* release tests were carried out. The results showed that chitosan microsphere with particle size less than 10 μm and positively charged ($+25.97 \pm 0.56$ mV) can be obtained under the aldehyde group to amino group ratio at 1:1. A loading capacity of BSA at 28.63 ± 0.15 g/100 g with corresponding loading efficiency at $72.01 \pm 1.44\%$ was obtained for chitosan microsphere. *In vitro* test revealed a burst release followed by sustained-release profile.

Key words: chitosan microsphere; bovine serum albumin; crosslinking

1 Introduction

In encapsulating processes, the drugs may release through different mechanisms, such as diffusion. The residue drugs on the surface of these microcapsules may account for the burst-release stages. Another type of sustained-release drug delivery system (DDS) is that the objective molecules were absorbed onto the surfaces of the microcapsules by electrostatic interactions or hydrogen bonds. The common used polymers in this system include poly(lactic acid) (PLA), poly(D,L-lactic-co-glycolic acid) (PLGA)^[1], zein^[2], chitosan, and so on. All these substances have the advantages of good biodegradability and biocompatibilities. However, PLA or PLGA may undergo bulk erosion *in vivo* that produces a very low pH environment within

these matrices, which may adversely affect sensitive therapeutic agents such as proteins. Zein is a natural polymer, which has many advantages as carrier for DDS^[2], but these protein suffers from its sources, as the extraction of zein from corn is a laborious process^[3].

Chitosan is the only natural alkaline polysaccharide containing many free amino groups. This polysaccharide has such features as non-toxic, biodegradable, and biocompatibility, which is obtained from deacetylation of chitin, and is a linear chain polysaccharide formed through the linkage of α -amino-D-glucosamine by β -1,4-glucosidic bond. Chitosan with high molecular weight is insoluble in water and organic solvents, but can dissolve in weak acids by converting the glucosamine units into R-NH_3^+ ^[4,5]. As many amino and hydroxyl groups distributed on the chains of chitosan molecules, the inner- or inter-hydrogen bonds can be formed. Chitosan has many bioactivities such as immunity, antibiosis, and wound healing, and has been used in waste water disposal^[6-8], food industry, spinning, chemical industry, bioengineering, drug delivery system^[4,9-11] and tissue engineering^[12] as a kind of functional polymer. Chitosan has also been used in agriculture as sustained-release carrier for fertilizer in the form of microparticles^[13]. Chitosan has been used not only in the natural form, but also in the form of

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microcapsule, as microcapsule can be used as carriers for sustained release of drugs or other active molecules. The aim of this paper is to use chitosan microsphere adsorbing BSA as model in DDS.

Chitosan microspheres/nanoparticles have been prepared by different methods such as ion-crosslinking, ionic/covalent crosslinking^[14-16], chemical crosslinking^[17], membrane emulsification technique^[18], spray-drying technique^[19], inverse phase emulsion dispersion method^[20], compounding strategies such as modified ionotropic gelation method combined with a high voltage electrostatic field^[21] and there are many reports on the uses of chitosan microspheres or nanoparticles as carriers as protein^[15,16,21-28], and DNA delivery systems^[24]. In addition, the modified chitosan hydrogel with pH-sensitive characteristics has also been developed for controlling the release of protein molecules^[23,29,30]. In the protein delivery system, bovine serum albumin (BSA) is the most frequently used model protein molecules^[19, 30,31]. Recently, the chitosan-BSA system has been designed as model for isolation of protein from the solution as this system does not change the secondary or tertiary structure of the protein molecules^[32]. The sustained- or targeted-drug delivery system based on chitosan and other polymers such as alginate and pectin has also been attempted^[22,25,28, 33-36]. However, less study has been conducted on the relationship between the combining ways of the BSA on chitosan microspheres and their relationship to the loading capacity and *in vitro* release profile. Therefore, the purpose of this study is to elucidate the relationship between the surface features of chitosan microspheres and their loading capacity to BSA. The possible absorptive mechanisms of chitosan microsphere were indicated.

2 Materials and methods

2.1 Materials

Chitosan with $\geq 90\%$ deacetylation degree was purchased from Jinan Haibeide Co., Ltd. (Shandong, China), and the viscosity-average molecular weight is 466 KD. BSA was purchased from Shanghai Pufei Bio-Technology Co., Ltd. Bradford reagent was supplied by Applygen Technologies Inc.

2.2 Preparation and characterization of chitosan microsphere

Chitosan microspheres were prepared by chemical crosslinking method. Briefly, a 1% chitosan solution (w/v) was dissolved thoroughly into an aqueous solution

of acetic acid (2% v/v) under stirring at 12 000 rpm. 2 g span 80 was added into 100 mL liquid paraffin to form 2% solution (w/v). Then 200 mL chitosan solution was added drop wise (about 1 mL/min) into the liquid paraffin under magnetic stirring to form water-in-oil (w/o) emulsion. After adding the chitosan solution, the stirring was continued for another 10 min. Different amount of glutaraldehyde was added into the solution as crosslinker with the final ratio of aldehyde group to amino group at 1:1, and the solution was stirred for another 3 h. The microparticle suspension was subsequently centrifuged at 4 000 rpm for 25 min. The pellet was collected and washed in turn with 10 mL petroleum, isopropyl alcohol and water, respectively. This washing procedure was repeated triplicate before freeze-drying of the pellet overnight. Lyophilization of the samples was performed using a Christ freeze-dryer.

For the preparation of chitosan microsphere containing BSA, the adsorption process was employed as reported previously^[31], in which the blank microsphere and 0.5 mg/mL BSA in pH 5.8 PBS were prepared. The chitosan microsphere was added into the BSA solution with chitosan/BSA ratio at 2:1 (w/w) and incubated at 4 °C for 24 h. The solution was centrifuged at 4 000 rpm for 20 min and washed with water for three times. The pellets were lyophilized.

Microstructure analysis of the microspheres prepared by different protocols were carried out by mounting the microsphere onto SEM stubs, and sputter coated with gold for 3 min for SEM analysis (S-3400, Hitachi science systems, Ltd) at an accelerating voltage of 10 kV. FTIR analysis was conducted on an FT-IR spectrometer (spectrum one, Perkin Elmer, Ltd, America) to characterize chemical functional groups of chitosan microsphere with different preparation conditions and the effect of BSA adsorption on the changes of chitosan surfaces. 5-10 mg powder samples were compressed into KBr disks for the FT-IR measurement. The spectrum was collected in a range between 4 000 and 370 cm^{-1} with a resolution of 1 cm^{-1} (100 scans per sample). The dried chitosan microsphere was suspended into deionized water at a certain concentration. The size distribution was determined by measuring the size of microspheres from SEM images, 10 microspheres were selected randomly from each image and the sizes were averaged. The zeta potential of the chitosan microspheres was measured in demineralized water at neutral pH by dynamic light scattering (DLS) with a Zetasizer 3000 HS instrument (Malvern Instruments, Malvern, UK).

Thermal properties of the different materials were analyzed by thermogravimetric analysis (TGA) by DSC-TGA Standard Module. 5-10 mg samples were measured on a Perkin Elmer Pyris Diamond TG/DTA analyzer at a heating rate of $10\text{ }^{\circ}\text{C min}^{-1}$ under nitrogen atmosphere over the temperature range of $37\text{--}650\text{ }^{\circ}\text{C}$. Samples of approximately 10-20 mg were used for the TGA measurements.

2.3 Estimation of BSA content in the chitosan microspheres prepared by different process

BCA protein assay kit was used to determine the loading efficiency of BSA in chitosan microsphere according to the instructions of manufacturer. In the adsorption process, the free BSA was determined after centrifugation at 4 000 rpm for 20 min, the loading efficiency (*LE*) and loading capacity (*LC*) were calculated according to the following formula:

$$LC(g/100\text{ g}) = \frac{\text{total amount BSA-free BSA}}{\text{microparticle weight}} \times 100 \quad (1)$$

$$LE/\% = \frac{\text{total amount BSA-free BSA}}{\text{total BSA}} \times 100\% \quad (2)$$

2.4 *In vitro* release

In vitro release experiments for drug-loaded microparticles were performed in pH7.4 PBS buffer. Before the experiments, the release medium was prepared and placed for equilibrium at $37\text{ }^{\circ}\text{C}$ overnight. The 10 mg microparticles were then placed in different beakers containing 50 mL release medium at $37 \pm 0.5\text{ }^{\circ}\text{C}$. 3 mL aliquots were withdrawn at 0.5, 1, 2, 4, 8, 24 hours post incubation for the first 24 hours, and then at 24 h intervals for the following periods and replaced with an equal volume of fresh medium throughout the study. The BSA release amount were assayed by measuring absorbance value at 280 nm. The accumulative release rate of BSA was calculated. The release experiments were performed triplicate and the results were expressed as $\bar{X} \pm \text{Std}$.

2.5 Data analysis

All the tests were repeated at least in triplicates, and the results were expressed as $\bar{X} \pm \text{Std}$.

3 Result and discussion

3.1 Morphological characterization of chitosan microspheres

In the drug or protein delivery systems, the microspheres prepared from biodegradable polymers

such as chitosan, PLA have been used extensively^[36]. The possible adsorption ways of BSA on the surfaces of chitosan microspheres is studied in this work. Chemical crosslinking method was used for the preparation of chitosan microsphere in this study. The hypothesis was that the BSA molecules adhered to different locus of the chitosan microsphere surface, as the surface was not absolutely smooth, or the amino groups were not crosslinked by glutaraldehyde molecules completely, the BSA molecules combined at these loci with different interactions, that is, physical adhesion or electrostatic interactions.

Fig.1 SEM images of the chitosan microsphere crosslinked by glutaraldehyde with aldehyde group and amino group (ratio at 1:1)

The morphology of the presented chitosan microspheres was analyzed by SEM as shown in Fig.1. The chitosan microspheres with spherical appearance can be obtained. The zeta potential was positive ($25.97 \pm 0.56\text{ mV}$) when the aldehyde to amino ratio at 1:1, which suggested the positive charges of the chitosan microsphere. Higher zeta potential also means good dispersibility of these microspheres.

The blank chitosan microsphere crosslinked by glutaraldehyde with different aldehyde group and amino group ratio at 1:1; 3:1; 5:1; 10:1 and 20:1 for as many as five batches were prepared. The result showed that the morphology and uniform of chitosan microspheres changed significantly with the increasing value of glutaraldehyde to amino ratio (data not shown). The optimal aldehyde to amino ratio is 1:1 for the preparation of blank microsphere in terms of appearance and uniformity (Fig.1). When the aldehyde/amino ratio increased to 20:1, the chitosan microspheres stuck to each other, which is not expected as it is assumed that higher content of aldehydes may cause the amino groups to crosslink

more thoroughly, thus become more regular in the appearance of the microsphere. High aldehyde/amino ratio need less time to form microspheres due to the increased cross-linking rate. So under the same cross-linking time, the whole microspheres which had formed in the high aldehyde/amino ratio reaction system were broken into pieces under the shearing force of solution.

3.2 FT-IR analysis of the group changes

Fig.2 FTIR spectra of chitosan (a), cross-linking chitosan microspheres (b), BSA (c) and the BSA-loaded chitosan microspheres by adsorption method (d)

Fig.2 is the FTIR spectrum of chitosan microspheres. In this study dried drug-loaded and blank chitosan microspheres were analyzed by FTIR to observe the possible interaction of the functional groups of all the molecules. Chitosan powder (a) exhibits main characteristic bands of hydroxide group (-OH) at 3435.02 cm^{-1} . The broad peaks at 1646 cm^{-1} and 1539 cm^{-1} is stretching vibration of amide I and II bands, respectively, which indicated significant deacetylation of the chitosan molecule. After cross-linking between chitosan and glutaraldehyde (b), the two peaks were located at 1557 cm^{-1} and 1654 cm^{-1} , respectively. The significant decreased transmittance at 1638 cm^{-1} as shown in Fig.2(d), which is assigned to the vibrations of amide linkages, confirmed the formation of new chemical bonds between the residue amino groups of chitosan microspheres and the carbonyl groups of BSA molecules, which may cause the increase of TG_{50} as shown below. The wider peak appeared at 3435.02 cm^{-1} may be explained by the accumulative effects of the -OH vibrations from both chitosan and BSA molecules as shown in Fig.2(d).

3.3 TG analysis

TG analysis results were shown in Fig.3. The temperature at which 50% weight loss (TG_{50}) was 318.55° , 364.92° , 391.65° and 394.81° , respectively for chitosan powder, chitosan microsphere, BSA and chitosan microsphere bearing BSA, respectively. The significant higher TG_{50} of chitosan microspheres

compared with that of chitosan powder indicates the amino groups of chitosan have been crosslinked by the aldehyde groups. In addition, chitosan microsphere bearing BSA has highest TG_{50} , which also indicated that in the adsorption process, the BSA molecules acts not only as the objective molecules, but also act as partial crosslinking effects.

Fig.3 TG analysis of the chitosan microspheres: (a) chitosan powder; (b) chitosan microsphere; (c) BSA; (d) chitosan microsphere bearing BSA

3.4 Loading capacity and *in vitro* release study of BSA-loaded chitosan microspheres

The results of UV/Vis spectroscopy showed that the concentrations (X) and the absorbance (Y) of BSA had good linear relationship. The linear equation is $y=1.6229x-0.0035$, $R=0.9999$. To ensure that the absorbed BSA released completely from the chitosan microsphere, the loaded microspheres were dissolved in hydrochloric acid at pH 2. After 2 h of incubation, the chitosan microparticles were found to be completely disintegrated and the BSA in the suspension could be determined for loading efficiency and loading capacity. A loading capacity $28.63\pm0.15\text{ g/100 g}$ with corresponding loading efficiency at $72.01\pm1.44\%$ was obtained for chitosan microsphere, which reached the highest encapsulation efficiency of chitosan/TPP nanoparticles as prepared by Yang *et al*^[15]. However, this value is lower than the protein-loaded chitosan microspheres which is prepared by a modified ionotropic gelation method combined with a high voltage electrostatic field which showed prospective method for the preparation of protein-delivery system^[21].

Fig.4 showed an obvious burst release was observed for chitosan microspheres which is common to many microparticles-based drug delivery systems^[26,31]. For the first 4 hours, about $(32.4\pm3.81)\%$

Fig.4 *In vitro* release profiles of BSA from chitosan microsphere

BSA released from chitosan microspheres. At 240 h, about 70% BSA released for the microspheres. The release profiles may be closely related to the ways of the interactions between BSA molecules and chitosan microspheres. BSA molecules, when absorbed onto the surfaces of chitosan microspheres may released quickly, which constitutes the burst effects of the delivery systems, when the BSA molecules entered into the valleys of the coarse surfaces of the chitosan microspheres, the BSA molecules may acted as partial crosslinkers and released very slowly until the microspheres disintegrated, these may explain the sustained release profile of chitosan microspheres. In our another study^[17], the chitosan microspheres showed significantly higher loading capacity for bovine coronavirus N protein than that for BSA. It is supposed that this may be caused by different isoelectric points of these proteins. As the pH 5.8 solution was used for adsorption of the different proteins by chitosan microspheres, that is, the larger distance between pH 5.8 and isoelectric points of the protein, the more electric charges carried by the protein molecules, and thus the more attractive interactions between chitosan microspheres and protein molecules. Ma and Liu^[21] prepared the BSA-loaded chitosan microspheres by ionotropic gelation using TPP and ethanol as solvents under a high voltage, the microspheres developed from different concentrations of coagulation solution showed pH-dependent release profiles.

4 Conclusions

The uniform-sized chitosan microspheres with size less than 10 μm can be prepared under a certain amount of crosslinkers. The chitosan microspheres containing BSA by absorptive method changed the characteristics of the blank chitosan microspheres

as evidenced by FTIR and TG analysis. The obvious sustained process of the chitosan microspheres to BSA can be viewed.

References

- [1] O Karal-Yılmaz, M Serhatlı, K Baysal, *et al.* Preparation and *in Vitro* Characterization of Vascular Endothelial Growth Factor (VEGF)-Loaded Poly(D,L-Lactic-Co-Glycolic Acid) Microspheres Using a Double Emulsion/Solvent Evaporation Technique [J]. *J. Microencapsul.*, 2011, 28(1): 46-54
- [2] X M Liu, Q S Sun, H J Wang, *et al.* Microspheres of Corn Protein, Zein, for an Ivermectin Drug Delivery System[J]. *Biomaterials*, 2005, 26: 109-115
- [3] R Shukla, C Munir. Zein: the Industrial Protein from Corn[J]. *Ind. Crop Prod.*, 2001, 13: 171-192
- [4] V R Sinha, A K Singla, S Wadhawan, *et al.* Chitosan Microspheres as a Potential Carrier for Drugs[J]. *Int. J. Pharm.*, 2004, 274(1-2): 1-33
- [5] W Yue, P J Yao, Y N Wei, *et al.* An Innovative Method for Preparation of Acid-Free-Water-Soluble Low-Molecular-Weight Chitosan (AFWSLMWC) [J]. *Food Chem.*, 2008, 108: 1 082-1 087
- [6] M A Di, M Sittiger, M VRisbud. Chitosan: a Versatile Biopolymer for Orthopaedic Tissue-Engineering[J]. *Biomaterials*, 2005, 26(30): 5 983-5 990
- [7] X D Zhang, D Z Yang, Nie J. Chitosan/Polyethylene Glycol Diacrylate Films as Potential Wound Dressing Material[J]. *Int. J. Biol. Macromol.*, 2008, 43(5): 456-462
- [8] L F Qi, Z R Xu, Jiang X, *et al.* Preparation and Antibacterial Activity of Chitosan Nanoparticles[J]. *Carbohydr. Res.*, 2004, 339: 2 693-2 700
- [9] A S Pedro, E Cabral-Albuquerque, D Ferreira, *et al.* Chitosan: An Option for Development of Essential Oil Delivery Systems for Oral Cavity Care [J]? *Carbohydr. Polym.*, 2009, 76: 501-508
- [10] Q S Sun, X Q Che, Zhao K. Chitosan Matrix and the Release of Protein-derived Drugs[J]. *Chinese Polym. Bull.*, 2008, 6: 46-49
- [11] Y B Yang, Q S Sun, X Q Che, *et al.* Application of Chitosan in Pharmaceutical Dosage Forms[J]. *J. Clin. Rehab. Tissue Eng. Res.*, 2008, 12(41): 8 131-8 134
- [12] S E Kim, J H Park, Y W Cho, *et al.* Porous Chitosan Scaffold Containing Microspheres Loaded With Transforming Growth Factor-B1: Implications for Cartilage Tissue Engineering[J]. *J. Control. Rel.*, 2003, 91: 365-374
- [13] L Wu, M Z Liu. Preparation and Properties of Chitosan-Coated NPK Compound Fertilizer with Controlled-Release and Water-Retention[J]. *Carbohydr. Polym.*, 2008, 72: 240-247
- [14] E S Dragan, M V Dinu, D Timpu. Preparation and Characterization of Novel Composites Based on Chitosan and Clinoptilolite with Enhanced Adsorption Properties for Cu^{2+} [J]. *Bioresour. Technol.*, 2010, 101: 812-817
- [15] W Yang, J Fu, T Wang, *et al.* Chitosan/Sodium Tripoly-Phosphate Nanoparticles: Preparation, Characterization and Application as Drug Carrier[J]. *J. Biomed. Nanotechnol.*, 2009, 5(5): 591-595
- [16] M Vandana, S K Sahoo. Optimization of Physicochemical

- Parameters Influencing the Fabrication of Protein-Loaded Chitosan Nanoparticles[J]. *Nanomedicine (Lond)*, 2009, 4(7): 773-785
- [17] Q S Sun, Li X, X Q Che, et al. Evaluation on the Immune Efficiency of Bovine Coronavirus N Protein-Loaded Chitosan Microsphere[J]. *Chinese J. Prev. Vet. Med.*, 2009, 31: 882-886
- [18] W Wei, G H Ma, L Y Wang, et al. Hollow Quaternized Chitosan Microspheres Increase the Therapeutic Effect of Orally Administered Insulin[J]. *Acta Biomater.*, 2010, 6(1): 205-209
- [19] C Kusonwiriawong, W Pichayakorn, V Lipipun, et al. Retained Integrity of Protein Encapsulated in Spray-Dried Chitosan Microparticles[J]. *J. Microencapsul.*, 2009, 26(2): 111-121
- [20] L Zhou, J Liu, Z Liu. Adsorption of Platinum(IV) and Palladium(II) from Aqueous Solution by Thiourea-Modified Chitosan Microspheres[J]. *J. Hazard. Mater.*, 2009, 172(1): 439-446
- [21] L Ma, C Liu. Preparation of Chitosan Microspheres by Ionotropic Gelation Under a High Voltage Electrostatic Field for Protein Delivery[J]. *Colloids Surf. B: Biointerfaces*, 2010, 75(2): 448-453
- [22] S Takka, A Gürel. Evaluation of Chitosan/Alginate Beads Using Experimental Design: Formulation and *in vitro* Characterization[J]. *AAPS Pharm. Sci. Tech.*, 2010, 11(1): 460-466
- [23] H Hu, L Yu, S Tan, et al. Novel Complex Hydrogels Based on N-Carboxyethyl Chitosan and Quaternized Chitosan and Their Controlled *in vitro* Protein Release Property[J]. *Carbohydr. Res.*, 2009, 345(4): 462-468
- [24] N Csaba, M Köping-Höggård, M J Alonso. Ionically Crosslinked Chitosan/Tripolyphosphate Nanoparticles for Oligo-nucleotide and Plasmid DNA Delivery[J]. *Int. J. Pharm.*, 2009, 382(1-2): 205-214
- [25] C Y Yu, L H Jia, S X Cheng, et al. Fabrication of Microparticle Protein Delivery Systems Based on Calcium Alginate[J]. *J. Microencapsul.*, 2010, 27(2): 171-177
- [26] S Shu, X Zhang, D Teng, et al. Polyelectrolyte Nanoparticles Based on Water-Soluble Chitosan-Poly(L-Aspartic Acid)-Polyethylene Glycol for Controlled Protein Release[J]. *Carbohydr. Res.*, 2009, 344(10): 1 197-1 204
- [27] Z M Wu, X G Zhang, C Zheng, et al. Disulfide-crosslinked Chitosan Hydrogel for Cell Viability and Controlled Protein Release[J]. *Eur. J. Pharm. Sci.*, 2009, 37(3-4): 198-206
- [28] C Y Yu, B C Yin, W Zhang, et al. Composite Microparticle Drug Delivery Systems Based on Chitosan, Alginate and Pectin with Improved pH-sensitive Drug Release Property[J]. *Colloids Surf B Biointerfaces*, 2009, 68(2): 245-249
- [29] J S Quan, H L Jiang, E M Kim, et al. pH-sensitive and Mucoadhesive Thiolated Eudragit-coated Chitosan Microspheres[J]. *Int. J. Pharm.*, 2008, 359(1-2): 205-210
- [30] J Wu, W Wei, L Y Wang, et al. Preparation of Uniform-Sized pH-Sensitive Quaternized Chitosan Microsphere by Combining Membrane Emulsification Technique and Thermal-Gelation Method[J]. *Colloids Surf. B: Biointerfaces*, 2008, 63(2): 164-175
- [31] S R Jameela, A Misra, A Jayakrishnan. Cross-linked Chitosan Microspheres as Carriers for Prolonged Delivery of Macromolecular Drugs[J]. *J. Biomater. Sci. Polym. Ed.*, 1994, 6(7): 621-632
- [32] V Boeris, B Farruggia, G Picó. Chitosan-bovine Serum Albumin Complex Formation: A Model to Design an Enzyme Isolation Method by Polyelectrolyte Precipitation[J]. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.*, 2010, 878(19): 1 543-1 548
- [33] X Li, X Kong, S Shi, et al. Preparation of Alginate Coated Chitosan Microparticles for Vaccine Delivery[J]. *BMC Biotechnol.*, 2008, 8: 89-93
- [34] C Remuñán-López, M L Lorenzo-Lamosa, J L Vila-Jato, et al. Development of New Chitosan-Cellulose Multicore Micro-Particles for Controlled Drug Delivery[J]. *Eur. J. Pharm. Biopharm.*, 1998, 45(1): 49-56
- [35] G Coppi, V Iannuccelli, E Leo, et al. Chitosan-alginate Microparticles as a Protein Carrier[J]. *Drug Dev. Ind. Pharm.*, 2001, 27(5): 393-400
- [36] G W Vandenberg, C Drolet, S L Scott, et al. Factors Affecting Protein Release from Alginate-Chitosan Coacervate Micro-Capsules During Production and Gastric/Intestinal Simulation[J]. *J. Control. Rel.*, 2001, 77(3): 297-307