JPP Journal of Pharmacy and Pharmacology

JPP 2002, 54: 1287–1292

Received March 19, 2002
Accepted June 4, 2002
ISSN 0022-3573

Study on biodegradable microspheres containing recombinant interferon-α-2a

Shaobing Zhou, Xianmo Deng, Shuiying He, Xiaohong Li, Wenxiang Jia, Dapeng Wei, Zairong Zhang and Jihui Ma

Abstract

In this work, a new microsphere delivery system comprising calcium alginate microcores surrounded by a biodegradable poly-DL-lactide-poly(ethylene glycol) (PELA) coat was designed to improve the loading efficiency and stability of peptide drugs. Recombinant interferon (IFN)-α-2a, used as a model peptide drug, was efficiently entrapped within the alginate microcores using a high-speed stirrer and then microencapsulated into PELA copolymer using a water-in-oil-in-water solvent extraction method. Microspheres were characterized in terms of morphology, size and distribution, encapsulation efficiency, IFN biological activity retention and in-vitro peptide release. The IFN potency test showed that IFN entrapped in the core-coated microspheres could retain its biological activity during the encapsulation and release procedure. The release profiles were determined by the measurement of peptide presenting in the release medium at various intervals. The IFN potency, calculated by the Wish cells/vesicular stomatitis virus system, was used to determine IFN biological activity. The results showed that the core-coated microspheres could stabilize IFN in the PELA matrix. We compared the new delivery system with conventional microsphere delivery systems based on biodegradable poly-DL-lactide and poly-DL-lactide-poly(ethylene glycol). The core-coated microspheres had the highest amount of entrapment, encapsulation efficiency and biological activity retention. The extent of burst release (14%) from the core-coated microspheres in the initial protein release was much lower than the 31% burst release from the conventional microspheres. In conclusion, this work presents a new approach for water-soluble macromolecular drugs delivery (e.g. protein, peptide drugs, vaccines).

Introduction

Peptide and protein drugs are a rapidly growing class of pharmaceutics in a number of clinical disciplines, such as cancer therapy and the treatment of infectious diseases. Despite the rapid development of these peptide drugs, they retain severe pharmacological deficiencies owing to their rapid degradation and elimination from the body. This results in the multiple administration of superpharmacological doses of peptides and subsequent clinical toxicity (Cohen et al 1995). In recent years, the preparation of protein or peptide-loaded microspheres has attracted much attention. Microsphere drug delivery systems based on polylactide (PLA), polylactide-co-glycolide (PLGA) and poly-DL-lactide-poly(ethylene glycol) (PELA) have been extensively investigated because of their many advantages (Cohen et al 1991; Deng et al 1999). Compared with commonly used PLA and PLGA, PELA shows much potential in protein delivery systems (Li et al 1999). Examples of the advantages of the drug delivery system include biocompatibility, controllable biodegradability, absorbability and low toxicity of the degradation end products, sustained release potential and ease of administration (Wang & Wu 1998).

The delivery of peptide and/or protein pharmaceuticals to patients is not easy, largely owing to their inherent physical and chemical instability (Schöneich et al 1997). Problems with polymeric drug delivery systems include structural or conformational changes, or the loss of peptide and/or protein activity during preparation and storage of, and release from, this type of system (Lu & Park 1995a). The reason for the structural/conformational changes or decreased activity may be the harsh preparation

Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences, Chengdu 610041, P. R. China

Shaobing Zhou, Xianmo Deng, Shuiying He, Xiaohong Li

Department of Microbiology and Immunology, Hua Xi Medical Center, Sichuan University, 610041, P. R. China

Wenxiang Jia, Dapeng Wei, Zairong Zhang, Jihui Ma

Correspondence: S. Zhou, Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences, PO Box 415, Chengdu 610041, P. R. China. E-mail: shaobingzhou@163.net

Funding: This work was supported by the National Science Foundation of China (grant no. 20004009) and National 973 project.

or formulation conditions (Lu & Park 1995a; Uchida et al 1996). Another reason may be owing to the acidic microenvironment created inside the microspheres during polymer degradation and the reactivity of the peptide and/or protein with the polymer and/or its degradation products (Tobio et al 2000). It has become increasingly apparent that the most commonly used protein encapsulation methods often lead to unwanted protein aggregation (Alonso et al 1994; Lu & Park 1995a, b), especially for the formation of microspheres by the water-in-oil-in-water (w/o/w) technique. In particular, the formation of the first emulsion, in which an aqueous peptide and/or protein solution is emulsified into a polymer solution in methylene chloride, leads to protein aggregation at the aqueous–organic interface (Sah 1999).

Maintenance of peptide and/or protein activity during microsphere preparation and release is a further complicating factor. Several research groups have started developing new encapsulation strategies that avoid exposure of hydrated proteins to physical stress factors (Griebenow et al 1999; Carrasquillo et al 2001). In this work, our strategy was to develop a system comprising a stabilizing core, which contains the peptides, coated by a biodegradable polymer wall. The rationale behind this system was that the peptide would be stabilized in the core and would be released following erosion of the polymer coat (Tobio et al 2000). Our previous study confirmed that the new microsphere delivery system composed of alginate microcores surrounded by PELA polymer could improve the loading efficiency and stability of proteins (Zhou et al 2001). In this work, we used alginate cross-linked with calcium chloride to prepare peptide-loaded microcores. A solution of sodium alginate upon contact with calcium ions in aqueous solution forms a hydrogel. This is a complex coacervation process that occurs as a result of the physical cross-linking between carboxylate anions of guluronate and calcium ions. Alginate together with other polysaccharides is usually found in biotechnological and pharmaceutical applications. Microcapsules of alginate and chitosan or carboxymethylcellulose have been prepared for encapsulating cells, proteins and enzymes (Carlos & Waldo 2001). Reports indicate that the biological activity is very much retained during the calcium alginate encapsulating process (Hori et al 1996).

Alginate particulates can improve protein loading efficiency and the preservation of protein stability, but their degradation rate cannot be efficiently controlled, and a sustained and gradual protein release rate from alginate particulates cannot be maintained. In order to solve these problems, we designed a new microsphere delivery system consisting of alginate complex microcores surrounded by a PELA coat. Our previous study confirmed that the microspheres prepared from PELA (10% polyethylene glycol (PEG) content, MW 6 kDa) achieved the best spherical surface structure and the highest loading efficiency among PELA copolymers containing the same content (10%) and different molecular weights of PEG (MW 6, 4, 2, 1.54 and 0.8 kDa) (Deng et al 2001). The biodegradable and biocompatible nature of PELA (10% PEG content) makes it a suitable candidate polymer for the development of control delivery systems of water-soluble drugs, peptides and vaccines. The hydrophilic domains of PELA copolymers could increase the affinity between the hydrophilic alginate complex microcores and the outer coat in the preparation process. It was indicated that the core-coated microspheres have higher peptide efficiency than that of conventional microspheres. Attempts to strengthen the alginate microcores by covering with a polymer coat have had varying degrees of success. The core-coated microspheres can stabilize protein in the PELA matrix, which is the major advantage of the novel protein delivery system over conventional microspheres. This delivery system may have potential applications for hydrophilic drugs, peptides, proteins and antigens.

With the success of biotechnology and recombinant technology, proteins are being looked on as future therapeutic agents. The interferon (INF) peptide has a wide spectrum of antivirus, antitumour and immunomodulation functions and is suitable for the treatment of chronic hepatitis B and hepatitis C, chronic granulocytic leukaemia, hairy cell leukaemia, condylomata acuminata, herpes keratitis and other infectious viral diseases and some tumours. In this work, INF-α-2a, prepared by recombinant DNA technology, was used as a model peptide drug. We used the IFN potency, calculated by the Wish cells/vesicular stomatitis virus system (VSV) system, to determine the activity of IFN entrapped in microspheres.

Materials and Methods

Chemicals

IFN-α-2a (1×10^6 IU per vial) was purchased from Hainan New Continent Pharmaceutical Limited Corporation, China. PEG (MW 6 kDa) and polyvinyl alcohol (PVA, 88% hydrolysed, MW 130 kDa) were purchased from Guangzhou Chemical Reagents Department, China. DL-Lactide (85%) was produced by Chemical Factory of Hubei University, China. PLA and PELA block copolymer containing 10% PEG (MW 60 kDa) were synthesized by ring-opening polymerization as described previously (Deng et al 1990). The molecular weight of PLA and PELA was obtained by gel permeation chromatography (Waters ALC/GPC244, USA), using polystyrene as standard. Sodium alginate (3500 cps for a 2% solution at 25°C) was obtained from Sigma Chemical Co. A standard IFN sample (12 000 IU/vial) was purchased from Sigma Chemical Co. Wish cells, VSV and recombinant IFN were provided by the National Institute for the Control of Pharmaceutical and Biological products, Beijing, China. Tissue culture plates (96 wells) and bottles were purchased from Nunc, Denmark. All other chemicals and solvents were of reagent grade or better.

Preparation of microspheres

Conventional IFN-α-2a/PLA microspheres (MS-0)
The conventional IFN-α-2a/PLA microspheres were prepared by the solvent extraction based on the formation of

a modified double emulsion w/o/w reported by Ogawa et al (1988). After the complete removal of organic solvent, the microspheres were collected by centrifugation (Tomy Seiko Co., Japan). The resultant microspheres were rinsed with distilled water and centrifuged three more times, then lyophilized overnight and stored at 4°C in a desiccator.

Conventional IFN-α-2a/PELA microspheres (MS-1)
The procedure was essentially the same as that described above for MS-0.

IFN-α-2a-loaded calcium alginate microcores coated with PELA polymer (MS-2)
The method used to prepare sodium alginate microcores was adapted from Bodmeier & Paeratakul (1989). First, a solution of sodium alginate (1.5%, w/v) was prepared in double distilled water. IFN-α-2a solution was dispersed in the alginate solution using a high-speed stirrer. Then, a solution of calcium chloride (3%, w/v) was added dropwise using a disposable syringe (21 gauge) under high-speed stirring. The gelation process began almost instantaneously and formed IFN-loaded calcium alginate microcores. The microcores were used as the w_1 phase, and finally the PELA-coated microspheres (MS-2) were prepared by the w/o/w solvent extraction method as described previously (Ogawa et al 1988).

Characterization of core-coated microspheres

Scanning electron microscopy (Amray, USA) was used to observe the surface characteristics and the morphology of microcores and core-coated microspheres. Microsphere size and distribution were determined with a laser diffraction particle size analyser (Malven, Mastersizer 2000, UK).

The amount of IFN entrapment was measured by placing 100 mg of microspheres in 1.5 mL dichloromethane and extracting the IFN three times with 1.5 mL double distilled water. The IFN content in the extraction solution was determined using the method of Bradford (1976) and compared with a standard curve of data obtained by assaying known concentrations of IFN solutions. The amount of encapsulated IFN in microspheres, given as a percentage, indicates the amount (mg) of IFN encapsulated per 100 mg of microspheres. The encapsulation efficiency of the process indicates the percentage of IFN encapsulated with respect to the total amount used for the preparation of microspheres. The biological activity retention (%) of INF-α-2a in microspheres was calculated as: $(A_A/A_T)\times 100$, where A_A is the actual activity and A_T is the theoretical activity of INF-α-2a in microspheres. The biological activity of INF-α-2a entrapped in microspheres was determined by calculating the result from the IFN potency test (Lu et al 1998). In brief, the IFN potency was calculated by the Wish cells/VSV system. First, the dose–response standard curve of IFN was established in the Wish/VSV system. Second, extracted IFN potency from these microspheres was determined by the TCID50 method. That is to say, 3500 Wish cells were put into each well and cultured with a series dilution of test IFN samples for 24 h at 37°C, in a 5% CO_2 incubator. Then, 100 TCID50 mL^{-1} VSV were put into each well for 24 h cultivation. The cytopathic effect was judged and the TCID50 determined. Finally, the result was compared with that of the standard curve, and the IFN (IU) could be calculated.

In-vitro peptide release test

The in-vitro IFN release profiles of the microspheres were determined as follows. Pre-weighed microspheres containing IFN-α-2a were incubated in a test tube containing 15.0 mL phosphate-buffered saline (PBS; 154 mM, pH 7.41). The tubes were stored in the same air bath as that mentioned in the degradation test. At appropriate intervals, 1.0 mL of the release medium was collected by centrifugation and 1.0 mL fresh PBS was added back to the test tube. The amount of IFN was measured by the method of Bradford (1976) as described above. The percentage release against incubation time indicates the percentage of IFN released during each investigated interval with respect to the total IFN released over the entire incubation time. At the same time, in order to investigate the biological activity retention of different IFN formulations, these microspheres were incubated under the same conditions as mentioned in the degradation test. The biological activity retention was detected by the IFN potency as described above.

Statistical analysis

All samples were run in triplicate and expressed as the mean ± s.d. All analyses were performed using one-way analysis of variance. Values of $P < 0.01$ were considered significant.

Results and Discussion

Characteristics of microspheres

The conventional microspheres (MS-0, MS-1) and core-coated microspheres (MS-2) containing IFN-α-2a were prepared by a modified double emulsion w/o/w technique based on the solvent evaporation method. Our previous study revealed the efficient encapsulation of the alginate microcores, and that the microcores were dispersed within the PELA matrix of the microspheres (Zhou et al 2001). A scanning electron micrograph of the core-coated PELA microspheres is shown in Figure 1.The core-coated PELA microspheres containing IFN-α-2a had a smooth, spherical surface, with no evidence of collapsing (Figure 1). The particle size distribution, determined using a laser diffraction particle size analyser, of the alginate complex microcores with a mean diameter of 0.186 μm and size distribution of 0.1–0.38 μm was obtained. IFN-loaded core-coated PELA microspheres, 1–7 μm in size, could be taken up by Peyer's patches, liver and spleen tissues (Eldridge et al 1990).

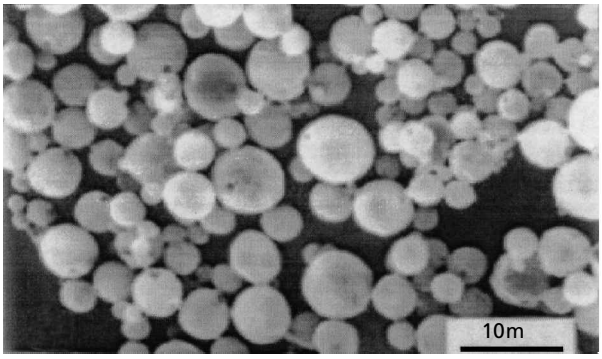

Figure 1 The dispersion pattern and morphology of core-coated poly-DL-lactide-poly(ethylene glycol) microspheres (MS-2) determined by scanning electron microscopy.

In order to successfully develop a peptide/PELA delivery system, it is essential that the biological activity of the peptide be retained throughout the microsphere preparation process. The influence of the matrix polymer on the characteristics of peptide entrapped within microspheres, such as the amount of entrapment, the encapsulation efficiency and the biological activity retention, were evaluated. The core-coated microspheres showed remarkable improvement in the amount of entrapment, encapsulation efficiency and biological activity retention (Table 1) compared with the conventional PLGA microspheres and PELA microspheres ($P < 0.001$). As shown in Table 1, lower entrapment (ca 0.23%) and biological activity retention (ca 6.5%) were obtained for microspheres prepared from PLA (MS-0). PLA polymers have certain drawbacks because of their hydrophobic nature. For hydrophilic peptides, the difference in physicochemical properties with the hydrophobic matrix had a bad effect on the peptide stability. Greater biological activity retention was obtained for microspheres prepared from PELA copolymer (MS-1) (ca 28.6%) than from PLA with similar molecular weight. The hydrophilic domains of PELA copolymers, acting as a peptide stabilizer or surface modifier of hydrophobic PLA networks, may promote the stability of peptides, increasing their loading efficiency. As shown in Table 1, the highest biological activity (ca 48%), amount of entrapment (ca 1.21%) and encapsulation efficiency (ca 68.7%) were achieved for core-coated PELA microspheres (MS-2). It may be the presence of certain amounts of hydrophilic alginate complex microcores, prepared by cross-linking between sodium alginate (anionic) and the cationic internal aqueous phase, that caused a high transfer resistance for peptide expelling from the internal aqueous phase to the external aqueous phase during the second emulsification. In addition, the direct contact between the hydrophilic alginate complex and the water-soluble peptide may be beneficial in maintaining peptide biological activity. The presence of hydrophilic PEG segments in the polymer chains improved the affinity of polymer with IFN-loaded alginate complex microcores.

In-vitro peptide release profile from microspheres

Figure 2 shows the percentage release of peptide from all samples of microspheres against incubation time. The IFN release profiles of all samples consist of a burst release followed by a gradual release phase. The extent of IFN burst release from the MS-0 microspheres during the initial phase was the greatest (ca 31%) among the three types of

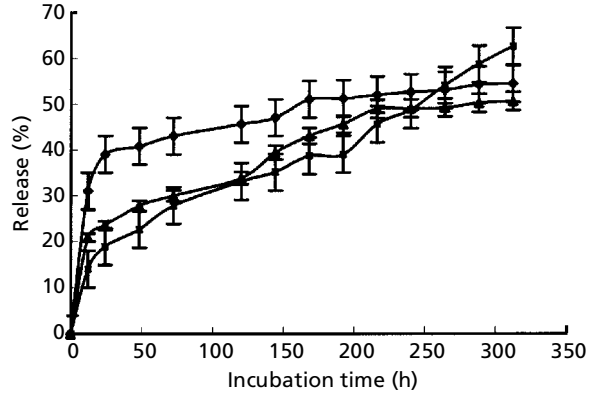

Figure 2 Percentage release of interferon (IFN)-α-2a, from MS-0 (◆), MS-1 (▲) and MS-2 (■) microspheres incubated in phosphate-buffered saline (pH 7.4) at 37°C.

Table 1 Characteristics of the microspheres studied.

Polymer microspheres	MW (kDa)	Diameter (μm)	Size range (μm)	s.d.	Amount of entrapment (%)	Encapsulation efficiency (%)	Biological activity retention (%)
PLGA (MS-0) (LA/GA: 75/25)	8.8	2.81	0.5–10.0	0.251	0.23±0.01	10.5±0.11	6.5±0.05
PELA (MS-1)	9.0	2.62	0.5–7.0	0.210	0.62±0.01	33.1±0.21	28.6±0.15
Core-coated PELA (MS-2)	9.0	3.10	1.0–7.0	0.248	1.21±0.02[a,b]	68.7±0.25[a,b]	48.0±0.23[a,b]

PLGA, polylactide-co-glycolide; PELA, poly-DL-lactide-poly(ethylene glycol). Data represent the mean±s.d., n = 3. [a]Statistically significant difference between MS-2 and MS-1; [b]Statistically significant difference between MS-2 and MS-0 ($P < 0.001$).

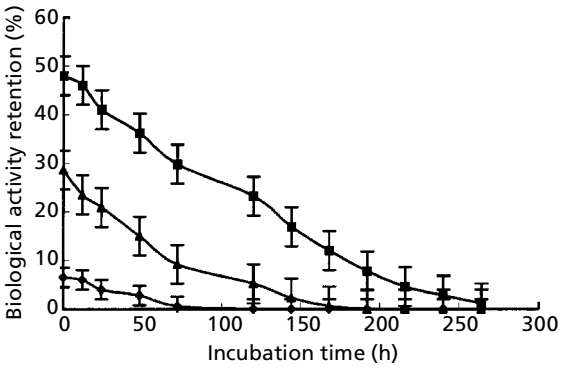

Figure 3 The biological activity retention of interferon-α-2a encapsulated in MS-0 (◆), MS-1 (▲) and MS-2 (■) microspheres against incubation time in phosphate-buffered saline (pH 7.4) at 37°C.

microspheres. The extent of IFN burst release from the MS-1 microspheres during the initial phase was approximately 21%, which was greater than the 14% burst release of IFN from the MS-2 microspheres. As shown in Figure 2, MS-0 microspheres showed 51% IFN release within 168 h; the release profile plateaued after 168 h, which indicates that little IFN was being released from the microspheres. MS-1 microspheres showed about 49% IFN release during 216 h, and after 216 h the release profile displayed a similar plateau as that for MS-0. However, MS-2 microspheres showed 39% IFN release within 8 days, and after 8 days, the release profile displayed a slightly faster release rate. It was concluded that IFN burst release could be reduced and sustained, gradual release profiles could be achieved by using the core-coated microsphere delivery system.

Figure 3 shows the biological activity retention of IFN-α-2a encapsulated in the three types of microspheres against incubation time in PBS at 37°C. IFN-α-2a encapsulated in the MS-0 microspheres had lost nearly all its biological activity after 3 days. After 7 days, IFN-α-2a encapsulated in the MS-1 microspheres had also lost nearly all its biological activity. However, the encapsulation of IFN in MS-2 microspheres was effective in reducing the loss of biological activity, and its biological activity could be maintained for up to 11 days. This suggests that encapsulation of IFN in core-coated microspheres can efficiently stabilize the biological activity.

The release involved two different mechanisms, namely diffusion of the peptide molecules and degradation of the polymer matrix. The burst release of peptide is associated with those peptide molecules dispersed close to the microsphere surface, which diffuse out during the initial incubation period. Thus, the small burst effect of the core-coated microspheres is owing to the preferential location of peptide molecules within the deep sections of the microsphere matrix because of the alginate complex microcores within the PELA matrix. In contrast, the large burst effect of MS-0 and MS-1 microspheres resulted from the preferential location of IFN within the shallow sections of the microsphere matrix. The gradual release of peptides from the microsphere matrix showed some similarities with the diffusion of macromolecules through a hydrogel-like structure after immersion in water (Li et al 2000).

IFN is a low molecular weight protein and its in-vivo half-life is very short (< 24 h). Proteins are highly organized, complex structures that must maintain structural and chemical integrity in order to function properly. Many manufacturing methods can jeopardize this by exposing proteins to potentially damaging conditions, such as aqueous–organic interfaces, elevated temperatures, vigorous agitation, hydrophobic surfaces and detergents (Klibanov & Langer 2000). In our core-coated system, the peptide was entrapped within alginate complex microcores, thus avoiding direct contact with organic solvents and preserving biological activity. During the in-vitro release test, the IFN encapsulated in MS-2 microspheres may avoid an acidic microenvironment generated from polymer degradation because of the alginate microcores; the encapsulated protein was severely hydrolysed within the fast degrading PLGA due to the acidic microenvironment in PLGA microspheres (Park et al 1995). Moreover, the hydrophobicity of the polymers may induce unfolding of protein/peptide molecules. So the protein/peptide drugs may lose their biological activity after being loaded in and released from the hydrophobic PLA microsphere systems (Sluzky et al 1991). However, the biological activity retention of IFN-α-2a entrapped within the core-coated microspheres could be maintained for longer than with the conventional microspheres.

Conclusion

A new microsphere delivery system consisting of alginate complex microcores surrounded by a PELA coat was prepared by a double emulsion w/o/w technique based on the solvent extraction method. IFNα-2a was used as a model peptide drug to examine the loading efficiency and biological activity retention of the peptide. The system made use of the advantages of natural and synthetic polymers. As a delivery system, it shows some advantages over the conventional PLA/PELA microsphere delivery systems, especially for hydrophilic drugs, peptides, proteins and vaccines. The initial IFN burst release from the core-coated microspheres was the least among the three types of microspheres studied. The novel delivery system achieved a longer in-vitro release rate and preserved the biological activity of the peptide for longer than the conventional delivery systems. More detailed investigations are necessary to clarify the effect of manufacturing parameters on microsphere characteristics, the effect of matrix polymer on peptide stability during microsphere preparation and the release profile, and the biological activity retention of peptide encapsulated in core-coated microspheres.

References

Alonso, M. J., Gupta, R. K., Min, C., Siber, G. R., Langer, R. (1994) Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. *Vaccine* **12**: 299–306

Bodmeier, R., Paeratakul, O. (1989) Spherical agglomerates of water-insoluble drugs. *J. Pharm. Sci.* **78**: 964–967

Braford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254

Carlos, P., Waldo, A. M. (2001) Chitosan based polyelectrolyte complexes. *Macromol. Symp.* **168**: 103–116

Carrasquillo, K. G., Aponte Carro, J. C., Alejandro, A., Diaz Toro, D., Griebenow, K. (2001) Reduction of structural perturbations in BSA by non-aqueous microencapsulation. *J. Pharm. Pharmacol.* **53**: 115–120

Cohen, S., Yoshioka, T., Lucarelli, M. (1991) Controlled delivery systems for proteins based on poly(lactic glycolic acid) microspheres. *Pharm. Res.* **8**: 713–720

Cohen, S., Chen, L., Apte, R. N. (1995) Controlled release of peptides and proteins from biodegradable polyester microspheres: an approach for treating infectious diseases and malignancies. *React. Polym.* **25**: 177–187

Deng, X. M., Xiong, C. D., Cheng, L. M. (1990) Synthesis and characterization of block copolymer from lactide and poly(ethylene glycol). *J. Polym. Lett.* **28**: 411–416

Deng, X. M., Li, X. H., Yuan, M. L., Xiong, C. D., Huang, Z. T., Jia, W. X., Zhang, Y. H. (1999) Optimization of preparative conditions for poly-DL-lactide-polyethylene glycol microspheres with entrapped Vibrio Cholera antigens. *J. Control. Release* **58**: 123–131

Deng, X., Zhou, S., Li, X, Zhao, J., Yuan, M. (2001) In vitro degradation and release profiles for poly-dl-lactide-poly(ethylene glycol) microspheres containing human serum albumin. *J. Control. Release* **71**: 165–173

Eldridge, J. H., Hammond, C., Meulbroek, J. A., Staas, J. K., Gilley, R. M., Tice, T. R. (1990) Controlled vaccine release in the gut-associated lymphoid tissues: orally administrated biodegradable microspheres target the Peyer's patches. *J. Control. Release* **11**: 205–214

Griebenow, K., Castellanos, I. J., Carrasquillo, K. G. (1999) Application of FTIR spectroscopy to probe and improve protein structure in sustained release devices. *Internet J. Vib. Spect.* **3** (5): 2 (www.ijvs.com)

Hori, P. R., Chandy, T., Sharma, C. P. (1996) Chitosan/calcium-alginate beads for oral delivery of insulin. *J. Appl. Polym. Sci.* **59**: 1795–1801

Klibanov, K., Langer, R. (2000) Protein stability in controlled-release systems. *Nat. Biotechnol.* **18**: 24–25

Li, X. H., Yuan, M. L., Xiong, C. D., Deng, X. M. (1999) Preparation of poly-dl-lactide-poly(ethylene glycol) microspheres encapsulating human serum albumin. *Acta Polym. Sin.* **2**: 156–161

Li, X. H., Deng, X. M., Yuan, M. L., Xiong, C. D., Huang, Z. T., Zhang, Y. H., Jia, W. X. (2000) In vitro degradation and release profiles of poly-dl-lactide-poly(ethylene glycol) microspheres with entrapped proteins. *J. Appl. Polym. Sci.* **78**: 140–148

Lu, J. H., Zhang, Y. H., Zhao, K. (1998) Medical and biological products (version no. 1). Beijing, Peoples Medical Publishing House, pp 884–899

Lu, W., Park, T. G. (1995a) Protein release from poly(lactic-co-glycolic acid) microspheres: protein stability problems. *PDA J. Pharm. Sci. Tech.* **49**: 13–19

Lu, W., Park, T. G. (1995b) In vitro release profiles of eristostatin from biodegradable polymeric microspheres: protein aggregation problems. *Biotechnol. Prog.* **11**: 224–227

Ogawa, Y., Yamamoto, M., Okada, H., Yashiki, T. (1988) A new technique to efficiently entrap leuprolide acetate into microcapsules of polylactic acid or copoly(lactic/glycolic) acid. *Chem. Pharm. Bull.* **36**: 1095–1103

Park, T. G., Lu, W., Crotts, G. (1995) Importance of in vitro experimental conditions on protein release kinetics, stability, and polymer degradation in protein encapsulated poly(lactic acid-co-glycolic acid) microspheres. *J. Control. Release* **33**: 211–222

Sah, H. (1999) Protein behavior at the water/methylene chloride interface. *J. Pharm. Sci.* **88**: 1320–1325

Schöneich, C., Hageman, M. J., Borchardt, R. T. (1997) Stability of peptides and proteins. In: Park, K. (ed.) *Controlled Drug Delivery, Challenges and Strategies.* ACS Professional Reference Book. Washington DC, American Chemical Society, pp 205–228

Sluzky, V., Tamada, J. A., Klibanov, A. M., Langer, R. (1991) Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. *Proc. Natl Acad. Sci. USA* **88**: 9377–9381

Tobio, M., Schwendeman, S. P., Guo, Y., McIver, J., Langer, R., Alonso, M. J. (2000) Improved immunogenicity of a core-coated tetanus toxoid delivery system. *Vaccine* **18**: 618–622

Uchida, T., Yagi, A., Oda, Y. (1996) Instability of bovine insulin in poly(lactic-co-glycolicacid) microspheres. *Chem. Pharm. Bull.* **44**: 235–236

Wang, N., Wu, X. S. (1998) A novel approach to stabilization of protein drugs in poly(lactic-co-glycolic acid) microspheres using agarose hydrogel. *J. Int. Pharm.* **166**: 1–14

Zhou, S. B., Deng, X. M., Li, X. H. (2001) Investigation on a novel core-coated micro-spheres protein delivery system. *J. Control. Release* **75**: 27–36