Chin. J. Chem. Eng., **15**(4) 566—572 (2007)

In vitro Evaluation of Lysozyme-loaded Microspheres in Thermosensitive Methylcellulose-based Hydrogel*

LIN Ying(林莹)**, SUN Jiali**(孙佳丽)**, JIANG Guoqiang**(蒋国强)**, ZAN Jia**(咎佳) **and DING Fuxin**(丁富新)**
Department of Chemical Engineering, Tsinghua University, Beijing 100084, China

Abstract Long-term injectable microspheres have some inherent disadvantages such as migration of microspheres from the original site and the burst effect. In order to avoid these problems, microsphere-loaded thermosensitive hydrogel system was designed and expected to achieve a zero-order release of biomolecular drugs in relative high initial drug loadings. Lysozyme, an antibacterial protein usually used to reduce prosthetic valve endocarditis, was selected as the model drug. Poly (DL-lactide-co-glycolide) (PLGA) microspheres, prepared by solvent evaporation method, were employed to encapsulate lysozyme and dispersed into thermosensitive pre-gel solution containing methylcellulose (MC), polyethylene glycol (PEG), sodium citrate (SC), and sodium alginate (SA). The mixture could act as a drug reservoir by performing sol-gel transition rapidly if the temperature was raised from room temperature to 37℃. The *in vitro* release results showed that the burst effect was avoided due to strengthening of diffusion resistance in the gel. The formulation was able to deliver lysozyme for over 30 days in a nearly zero-order release profile with a rate of 32.8μg·d^{-1} which exhibits its remarkable potential for effective application in long-term drug delivery.
Keywords microsphere, thermosensitive hydrogel, drug release, lysozyme, methylcellulose

1 INTRODUCTION

With the rapid development of biotechnology, biomacromolecules such as proteins, peptides, and antigens are increasingly used in the pharmaceutical fields[1,2]. Most of these drugs cannot be orally administered due to gastro- and/or intestinal degradation. Intravenous injection is the most common method of administration, but frequent and painful injection leads to patients' low compliance[3]. So encapsulation of biomacromolecules in long-term controlled release formulations becomes a promising way to overcome these obstacles.

Injectable microspheres prepared with biodegradable polymers such as poly (DL-lactide-co-glycolide) (PLGA) show excellent sustained release profiles over weeks[4—7]. However, there are some inherent disadvantages such as aggregation of microspheres due to hydrophobic interactions that occurs during injection[8], migration from the injection site[8,9], and the burst effect in the first few hours or days after administration[10].

Hydrogels, formed by temperature-triggered phase transitions, *in situ* imbibe a considerable amount of water while maintaining their shape[11,12]. Methylcellulose (MC) is one such polymer whose aqueous solution undergoes sol-gel transition due to hydrophobic interactions when heated above a particular temperature. Salts are capable of bringing down the gelation temperature of MC to around 37℃ by the dehydration effects. MC together with polyethylene glycol (PEG) and sodium citrate (SC) exhibits sol-gel transition property when heated above body temperature[13], but poor gel strength and stability restrain hydrogel's further application as an injectable implant[14]. In our previous study, sodium alginate (SA) is added into the MC-PEG-SC ternary reverse thermosensitive system. And it turns out that the new system has quicker gelation rate, higher gel strength, and lower swelling and erosion rate. The mutual entanglement of MC, PEG, and SA molecules plays an important role in the semi-interpenetrating network structure of hydrogel and consequently influences gel properties[15]. But the hydrogel system is not able to control drug release in an ideal manner because of its limited pore size.

The aforementioned hydrogel system consists of biocompatible components. Methylcellulose has been used extensively as a drug vehicle in pharmaceutical and topical application and is generally recognized as safe by the Food and Drug Administration. It is known as a promising biocompatible injectable scaffold for the repair of defects in the brain, nerve gap injuries, and spinal cord injuries[16—18]. The *in vitro* and *in vivo* results indicate that methylcellulose does not present toxicity problem. Although methylcellulose gel is not readily metabolized *in vivo*, it does undergo swelling and erosion as time goes by. So normally, removal of gel from the injection site is not necessary after drug is completely released. Alginate is also widely used to prepare hydrogel implants or injectable microspheres[19,20]. PEG and sodium citrate both have good biocompatibility.

In this study, microspheres are introduced into the thermosensitive hydrogel implant for sustained delivery of biomacromolecules. Drug-loaded microspheres are dispersed in the sol state polymer solution based on MC, and the mixture is incubated at 37℃ to form a gel which could act as drug reservoir. In this

Received 2006-08-18, accepted 2007-03-14.
* Supported by the National Natural Science Foundation of China (No.20576057) and Fundamental Research Foundation of Tsinghua University (JCqn2005033).
** To whom correspondence should be addressed. E-mail: dingfx@tsinghua.edu.cn

case, aggregation of microspheres will be prevented because they are dispersed in viscous sol solution. Thus, by combining microsphere and hydrogel, biomacromolecular drug could be released in a controlled manner for a long period of time.

In this study, lysozyme is selected as a model biomacromolecular drug because of its biostability and well-known characterization method. Lysozyme is a monomeric globular protein with a molecular weight of 14300. This antibacterial protein is able to reduce prosthetic valve endocarditis which is an infrequent but serious complication of cardiac valve replacement[21].

2 EXPERIMENTAL

2.1 Preparation of microspheres

Lysozyme (Sigma) loaded microspheres were prepared using a w/o/w solvent evaporation method which can be found in related literatures[22]. Briefly, 180mg of PLGA (D, L-lactide/glycotide molar ratio: 75/25, 50/50; M_w: 20000—200000; Jinan Yanxiang Chemical Fiber Textile Limited Company) was dissolved in 3ml of methylene chloride containing 1% (mass concentration) Span-80. Then, 300μl of lysozyme solution (concentration varies from 30 to 100mg·ml^{-1} in citrate buffer, pH 5.1) was added and the mixture was emulsified by sonication (JY92-Ⅱ Ultrasonic Disruptor, 16s, 100W, SCIENTZ BIOTECHNOLOGY CO., LTD.). The w/o emulsion was transferred into 30ml of 1.5% (mass concentration) poly vinylalcohol (PVA) (88% hydrolyzed, M_w 22000, Sigma) solution, and stirred at 600r·min^{-1} for 10min to create the second emulsion. The resulting w/o/w emulsion was poured into 400ml of 0.1% (mass concentration) PVA solution containing 3% (mass concentration) NaCl, and continuously stirred at room temperature (25℃) for another 4h to allow complete evaporation of methylene chloride. Microspheres were collected by filtration, washed 3 times with distilled water, and lyophilized.

2.2 Characterization of microspheres

2.2.1 *Lysozyme loading and encapsulation ratio*

The overall amount of lysozyme in microsphere was determined according to the method of Pérez and Griebenow[23]. Briefly, 20mg of microspheres were suspended in 2ml of methylene chloride for 1h to dissolve the outer PLGA layer completely. After centrifugation and removal of supernatant, the remaining protein powders were dried and put into 2ml of PBS (phosphate buffer solution 20mmol·L^{-1}, pH 7.4) for another 3h stirring. The insoluble aggregates were then collected and dissolved completely in 2ml of 6mol·L^{-1} urea solution. The protein content in supernatant and aggregates were determined by measuring the absorbance at 280nm from a lysozyme curve and a calibration curve of lysozyme in urea, respectively. The total content of lysozyme in microspheres equals to the summation of these two parts, where L_D and E_D refer to drug loading and encapsulation ratio, respectively.

$$L_D = \frac{\text{drug recovered from the microspheres(mg)}}{\text{microspheres(mg)}} \times 100$$

$$E_D = \frac{\text{drug recovered in the microspheres(mg)}}{\text{drug introduced in the microspheres(mg)}} \times 100$$

2.2.2 *Morphology and particle size distribution*

The morphologies of microspheres were examined by a scanning electron microscope (SEM) (JSM7401, Japan). The particle size distribution of microspheres was determined using a laser particle size analyzer (MASTERSIZER 2000, Malvern Instruments, England).

2.3 Preparation of microsphere and pre-gel mixture

Hot slurry was prepared by adding 0.4g of MC (viscosity of 2% solution in H_2O at 20℃ approximate 300—560mPa·s, Fluka) and 0.2g of SA (viscosity of 2% solution at 25℃ approximate 250mPa·s, Sigma) into 12ml of distilled water heated to 90℃ with stirring till the solids were completely dissolved. The slurry was cooled to 4℃ and mixed with 4ml of PEG (solute content 1.6g, M_w 11000, Sigma) solution. Then, 2.8ml of SC (solute content 0.7g) solution was added dropwise while stirring at 4℃. The pH value of the solution was adjusted to 7.4 with 3mol·L^{-1} hydrochloric acid. Then, the mixture was compensated with distilled water to the final volume of 20ml. Finally, the drug powder or drug-loaded microspheres were introduced into the pre-gel solution to form a homogeneous solution or suspension which can be gelled at 37℃ to act as a drug reservoir.

2.4 Lysozyme activity

Lysozyme is capable of digesting bacterial cell walls. *Micrococcus lysodeikticus* (Sigma) was used as the substrate to determine the activity of the enzyme. The substrate was prepared in sodium phosphate buffer (0.1mol·L^{-1}, pH 6.24) with an initial absorbance of 1.30 at 450nm. Then, 0.5ml of an appropriately diluted lysozyme solution was added into 2.5ml of substrate solution and the absorbance at 450nm was recorded every 15s during a total incubation period of 3min at 25℃. The lysozyme activity and the bioactive lysozyme concentration were determined by the following equation,

$$I = \frac{\Delta E_{450}}{0.001 \times E_w}$$

$$C = 0.0693 \times \Delta E_{450} + 0.0018$$

where ΔE_{450} is the reduction in the absorbance at 450nm per minute, E_w is the quantity of lysozyme in the reaction system, I is the enzymatic activity of the sample (U·mg^{-1}), and C is the bioactive lysozyme concentration (mg·ml^{-1}).

2.5 *In vitro* release

For lysozyme *in vitro* release from microspheres, 20mg of microspheres were sealed in a thermostated

cell with 2ml of PBS (20mmol·L^{-1}, pH 7.4) solution and incubated in water bath oscillator (100r·min^{-1}) at 37℃. Every two days, the suspension was centrifuged and supernatant was used for determination of bioactive protein concentration. The cell was refilled with 2ml of fresh buffer.

For *in vitro* release of lysozyme from microsphere-hydrogel formulation, 20mg of microspheres were suspended in 2ml of polymer pre-sol solution, and then the suspension was injected into the thermostated cell to form a hybrid gel. Then, 2ml of PBS was added into the cell and incubated in water bath oscillator (100r·min^{-1}) at 37℃. The content of active lysozyme in the release media was examined every two days followed by a buffer replacement.

2.6 Polymer erosion and depolymerization

At preset period during the incubation of microspheres or the mixture formulation, microspheres were collected and rewashed with distilled water, and then dried and dissolved into tetrahydrofuran to a concentration of 1mg·ml^{-1}. The solution was filtrated through a 0.45μm filter and analyzed by gel permeation chromatography (Viscotek TriSEC Model302, USA) with a GMHHR-H,M mixed bed column and a differential refractometer detector. The samples were eluted at a flow rate of 1.0ml·min^{-1} at 30℃ using tetrahydrofuran as mobile phase. The system was calibrated with a series of monodisperse polystyrene standards.

3 RESULTS AND DISCUSSION

3.1 Characterization of lysozyme-loaded microspheres

Microspheres of each formulation were made for three batches and characterized using the methods described. The optimized operation parameters are listed in section dealing with the preparation of microspheres. There are several influences on the characteristics of microspheres such as type and concentration of PLGA or additives applied during the preparation, the mass ratio between drug and PLGA and other experimental conditions[22]. If the concentration of the emulsifier PVA increased from 1.5% to 2% in the outer aqueous phase, the first w/o emulsion would be split into smaller droplets. The resulting larger oil-water interface provided more opportunities to drug molecules to diffuse into the outer aqueous phase and/or partially remain on the surface of microspheres during the solvent evaporation period. The drug loading was consequently decreased and/or the burst release became higher (data not shown).

PLGA with a molecular weight smaller than or close to lysozyme was not able to encapsulate the drug, since the encapsulation ratio was found to be less than 10%. That could be explained by the inability of short polymer chains to wrap well relatively big biomolecules[8]. PLGA with a molecular weight between 100000 to 200000 and a latide/glycolide molar ratio of 50/50 or 75/25 was found to be efficient.

The drug loading of high aqueous soluble biomolecules are usually restricted by w/o/w method, mostly less than 1%[10,23]. In order to sustain drug release for a certain period of time, a precise dose must be incorporated into the formulation. If the drug loading is getting higher, fewer excipients will be needed, and the total weight of the formulation will be decreased. So high drug loading is of great advantages in long-term drug delivery. Since lysozyme is a water-soluble protein, water introduced in microsphere preparation makes the drug loading not as high as that of water-insoluble drugs[24]. For the purpose of this study, by increasing the mass ratio of lysozyme to PLGA (50/50, M_w 200000) from 1∶20 to 1∶6, a minor decrease of encapsulation ratio was obtained (Table 1). The average particle sizes were near 130μm with narrow spans. Similar results were found by using PLGA(75/25, M_w 100000) except for a smaller size of around 97μm. Table 1 shows that at least sevenfold enhancement of drug loading up to 7.0% compared with the literature was achieved.

Figure 1 shows *in vitro* release profiles of microspheres in Table 1. The incomplete drug release from PLGA matrix was found, especially when the drug loading was low. This might be caused by the adsorption of lysozyme on the hydrophobic area of PLGA structure[25]. Since only bioactive proteins were taken into account, denaturation of lysozyme during the incubation period also contributed to the release incompleteness[10].

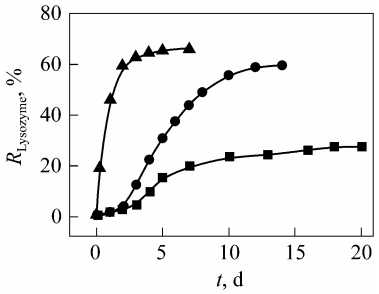


Figure 1 *In vitro* cumulative release profiles of lysozyme from microspheres (n=3)
mass ratio of lysozyme to PLGA (50/50, M_w 200000):
▲1∶6; ●1∶13; ■1∶20

Table 1 Physical properties for the microspheres

Lysozyme/PLGA①	E_D, %	L_D, %	Recovery, %	Average particle size, μm	Span
1:6	48.3	7.0	99.1	131.1	0.91
1:13	52.2	3.8	90.8	129.4	1.02
1:20	57.0	2.8	96.9	127.6	0.95

① Refers to the mass ratio; PLGA (50/50), M_w 200000.

Apparently, higher drug loading led to more drugs on the particle surface which resulted in higher initial burst release. Therefore, achieving both high drug loading and sustained release profile seems to be contradictive by using only the microsphere system.

3.2 Characterization of lysozyme-loaded thermosensitive hydrogel

2mg of lysozyme was introduced into 2ml of thermosensitive sol based on MC-PEG-SC-SA at room temperature. The sol rapidly formed a hydrogel at 37℃ within 6min with a shearing modulus of 24000Pa[15]. The hydrogel was freezed by liquid nitrogen and examined by SEM (Fig.2). A loose structure was observed in the hydrogel with large pores which could be the main drug penetration channels. Fig.3 pointed out that lysozyme could completely release from the hydrogel within 15 days, which revealed certain controlled release property as well as the ability of the gel to maintain bioactivity. But there was still nearly 30% burst release on the first day no matter how varied the composition of the gel was, due to the apparent pores observed.

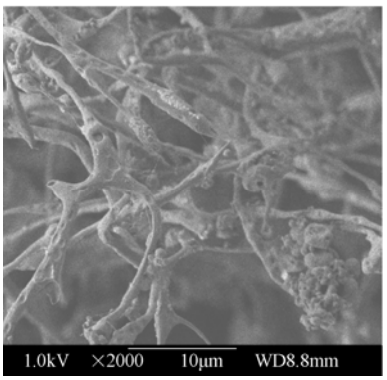


Figure 2 SEM image of the hydrogel

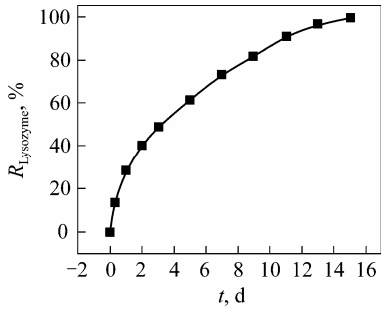


Figure 3 *In vitro* cumulative release profile of lysozyme from hydrogel (n=3)

3.3 Characterization of microsphere-hydrogel mixture formulation

Microsphere and hydrogel drug delivery systems both had certain capacity to control lysozyme release, but they were not good enough to obtain a sustained profile individually. Combination of these two systems can avoid some inherent disadvantages of each single ones, as discussed earlier in introduction. Here we aim at preparing a one-month sustained release formulation for lysozyme.

Microspheres, with a mass ratio of lysozyme to PLGA equal to 1∶6, were prepared by using various types of PLGA. The addition of 20mg of microspheres to 2ml of thermosensitive sol only had a neglectable influence on the gelation rate and gel strength (data not shown). PLGA (75/25, M_w 100000) was found to be the most efficient for the mixture formulation. The overall drug loading turned out to be 7.7%. And the morphology of the mixture formulation revealed that microspheres were well-encapsulated within the hydrogel matrix even after exposure in the release medium for 30 days (Fig.4).

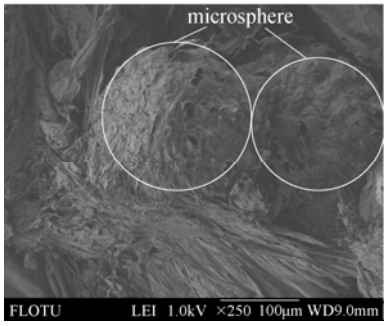


Figure 4 SEM image of the mixture formulation after 30 days' release of lysozyme

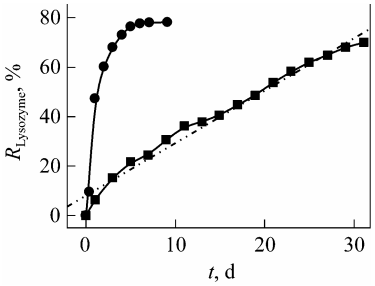


Figure 5 Comparison of *in vitro* cumulative release profiles of lysozyme from the microsphere system and the mixture formulation (n=3)
[mass ratio of lysozyme to PLGA (75/25, M_w 100000)=1∶6]
● microsphere; ■ mixture formulation; ---- linear fit, r=0.99

Figure 5 compared *in vitro* release property of the microsphere system to the mixture formulation. A burst release of 50% during the first day of incubation was noticed for the microsphere system and the release of drug lasted no longer than 10 days. On the other hand, microsphere-hydrogel mixture formulation dramatically performed a nearly zero-order release of lysozyme for over 30 days with no obvious burst release. The release rate calculated from the linear fit curve was 32.8μg·d^{-1}.

The hydrogel matrix built a release barrier between microsphere and environmental media. There might be two main explanations for the restrain on the release of lysozyme by the barrier. First, it was more difficult for water molecules to penetrate through the

PLGA outer shell, which limited the erosion and/or degradation rate of PLGA and the dissolution of lysozyme as well as its penetration[26]. Second, the diffusion of lysozyme was retarded by physical or chemical interactions between protein and the hydrogel matrix. The second hypothesis proved to be right since the hydrogel matrix alone has certain ability to control the release of lysozyme for 15 days. However, the first assumption is not preferred in terms of the degradation properties of PLGA within the hydrogel (Fig.6).

A steep drop of molecular weight of PLGA down to 36% was observed in the first 10 days of incubation for the mixture formulation. But for the microsphere system 88% of the molecular weight remained at the same time, while the most noticeable molecular weight loss occurred between 10 and 20 days. Therefore, the degradation of PLGA inside the hydrogel was obviously accelerated. The degradation of PLGA is attributed to surface and bulk hydroxylation and

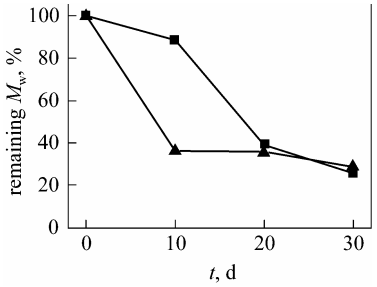
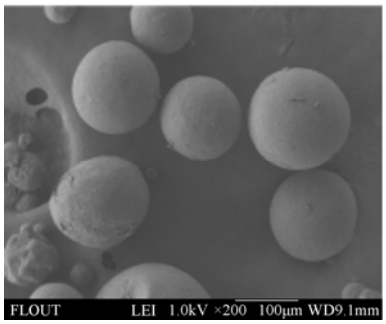
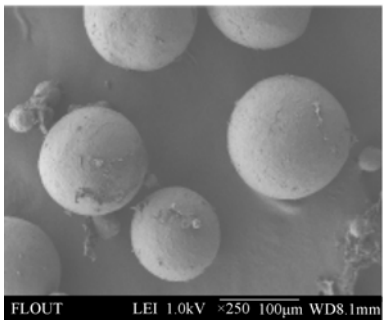


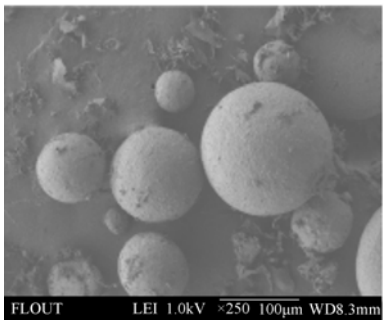
Figure 6 Remaining amount of molecular weight of degraded PLGA in the microsphere system and the mixture formulation (n=3)
[mass ratio of lysozyme to PLGA (75/25, M_w 100000)=1∶6]
■ microsphere; ▲ mixture formulation



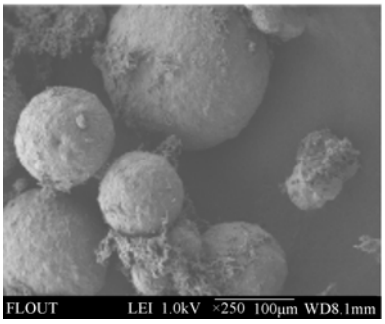
(a) before incubation



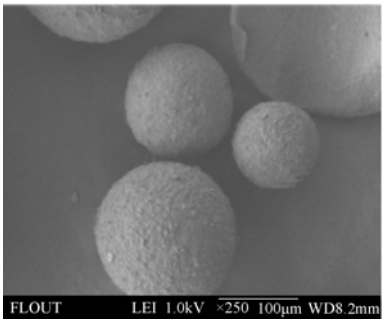
(b) microsphere system, after 10 days' incubation



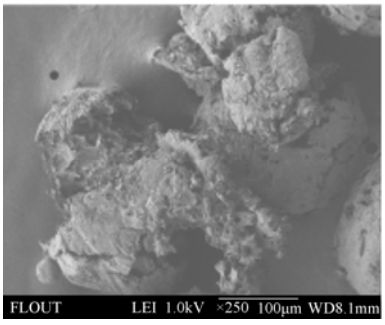
(c) microspheres in the mixture formulation, after 10 days' incubation



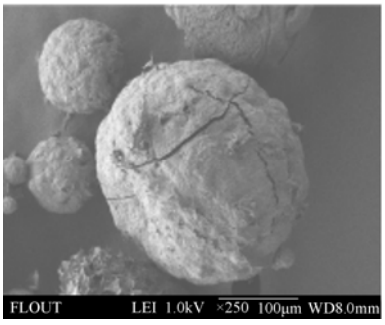
(d) microsphere system, after 20 days' incubation



(e) microspheres in the mixture formulation, after 20 days' incubation



(f) microsphere system, after 30 days' incubation



(g) microspheres in the mixture formulation, after 30 days' incubation

Figure 7 SEM images of microspheres during incubation
[mass ratio of lysozyme to PLGA (75/25, M_w 100000)=1∶6]

accelerated by the acidic degradation products[27]. Methylcellulose-based hydrogel carries certain amount of water which creates hydrophilic environment for polymer degradation. Furthermore, diffusion resistance from the hydrogel may cause the occurrence of small acidic regions where PLGA could be degraded much more quickly.

SEM analysis of microspheres during the incubation period is shown in Fig.7. An initial drop of molecular weight followed by mass loss has been reported elsewhere for degradation studies of PLGA. Only at longer degradation times, when polymer chains become short enough and microspheres become more porous, degradation products could diffuse out of the matrix and cause mass loss[28]. Thus, the surface morphology of microspheres begins to change which can be observed by SEM. It is reported that the beginning of mass loss took place 20 days later than the beginning of molecular weight decrease of PLGA(75/25) with the initial molecular weight of 65400[29]. In degradation studies of PLGA films, the mass loss also occurred when the molecular weight decreased to below 10000[30]. In our study, after 30 days' incubation, PLGA in the mixture formulation still had a molecular weight of 29000, which was considered to be too large to trigger mass loss. That might be the reason why spherical microspheres were observed. Surfaces of the microspheres entrapped in the hydrogel took much lower impact from water molecules as compared with those in direct exposure to the release buffer. Spherical structure of microspheres alone collapsed after 30 days' incubation in PBS, while those in the mixture formulation still remained in shape, although the remaining molecular weight for both systems appeared to be consistent.

Generally, the semisolid nature of the hydrogel or association of the PLGA microspheres in the gel might efficiently increase the effective distance of diffusion pathway for lysozyme molecules before they were released into the medium. As a result, the initial burst release was avoided. The acceleration of PLGA degradation inside the hydrogel also contributed to the linear zero-order release behavior of lysozyme for 30 days. Elucidation of the exact mechanism of lysozyme release from the microsphere-hydrogel mixture formulation needs further study.

4 CONCLUSIONS

In this study, characterization and application of microsphere-loaded thermosensitive hydrogel mixture formulation for the purpose of long-term delivery of high loading biomolecules have been discussed. The *in vitro* release study has shown that drug release profiles from microspheres strongly depend on the initial drug loading. Lysozyme encapsulated in PLGA (75/25, M_w 100000) microspheres could reach a relative high drug loading up to 7.7%, but serious burst release (over 50%) has been found in the first 24h. The methylcellulose-based thermosensitive hydrogel exhibits instantaneous gellation property in response to the change in environmental temperature from room temperature to 37℃. When microspheres are suspended in the gel, the burst effect can be effectively decreased due to diffusion resistance from the gel. PLGA degrades more quickly inside the gel, indicating that the flow of water entering into the polymer matrix could not be the major influence on polymer degradation and resulting protein release. The release rate of lysozyme can be adjusted by changing the formulations of both the microsphere and the hydrogel. The microsphere-hydrogel mixture formulation is able to deliver lysozyme for 30 days in a nearly zero-order release profile with a rate of 32.8μg·d^{-1}. And it has remarkable potential for application in effective and long-term drug delivery.

NOMENCLATURE

C	concentration of active lysozyme, mg·ml^{-1}
E	encapsulation efficiency, %
E_w	quantity of lysozyme in the reaction system, mg·ml^{-1}
ΔE_{450}	reduction in the absorbance at 450nm per minute
I	enzymatic activity, U·mg^{-1}
L	drug loading,%
M_w	molecular weight
n	batches of experiment
$R_{Lysozyme}$	cumulative release amount of lysozyme,%
r	correlation coefficient
t	time, day

REFERENCES

1 Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L.H., Langer, R., "Controlled delivery systems for protein based on poly(lactic/glycolic acid) microspheres", *Pharm. Res*., **8**, 713—720(1991).

2 Sinha, V.R., Trehan, A., "Biodegradable microspheres for protein delivery", *J. Control. Release*, **90**, 261—280(2003).

3 Dai, C.Y., Wang, B.C., Zhao, H.M., "Microencapsulation peptide and protein drugs delivery system", *Colloids Surf. B*: *Biointerf*., **41**, 117—120(2005).

4 Morlock, M., Kissel, T., Li, Y.X., Koll, H., Winter, G., "Erythropoietin loaded microspheres prepared from biodegradable LPLG-PEO-LPLG triblock copolymers: Protein stabilization and *in-vitro* release properties", *J. Control. Release*, **56**, 105—115(1998).

5 Zambaux, M.F., Bonneaux, F., Gref, R., Dellacherie, E., Vigneron, C., "Preparation and characterization of protein C loaded PLA nanoparticles", *J. Control. Release*, **60**, 179—188(1999).

6 Jackson, J., Liang, L., Hunter, W., Reynolds, M., Sandberg, J., Springate, C., Burt, H., "The encapsulation of ribozymes in biodegradable polymeric matrices", *Int. J. Pharm*., **243**, 43—47(2002).

7 Kostanski, J.W., Thanoo, B.C., DeLuca, P.P., "Preparation, characterization, and *in vitro* evaluation of 1- and 4-month controlled release orntide PLA and PLGA microspheres", *Pharm. Dev. Technol*., **5**, 585—596(2000).

8 Duvvuri, S., Janoria, K.G., Mitra, A.K., "Development of a novel formulation containing poly(D,L-lactide-co-glycolide) microspheres dispersed in PLGA-PEG-PLGA gel for sustained delivery of ganciclovir", *J. Control. Release*, **108**, 282—293(2005).

9 Plourde, F., Motulsky, A., Couffin-Hoarau, A.C., Hoarau, D., Ong, H., Leroux, J.C., "First report on the efficacy of L-alanine-based *in situ*-forming implants for the long-term parenteral delivery of drugs", *J. Control. Release*, **108**, 433—441(2005).

10 Aubert-Pouëssel, A., Bibby, D.C., Venier-Julienne, M.C., Hindré, F., Benoît1, J.P., "A novel *in vitro* delivery system for assessing the biological integrity of protein upon release from PLGA microspheres", *Pharm. Res*., **19**,

1046—1051(2002).
11 Kim, B.S.W.K., Bee, Y.H., "Thermosensitive sol-gel reversible hydrogels", *Adv. Dru. Delivery Res*., **54**, 37—51 (2002).
12 Zan, J., Zhu, D.Q., Tan, F.P., Jiang, G.Q., Lin, Y., Ding, F.X., "Prepararion of thermosensitive chitosan formulations containing 5-fluorouracil/poly-3-hydroxybutyrate microparticles used as injectable drug delivery system", *Chin. J. Chem. Eng*., **14**(2), 235—241(2006).
13 Takeuchi, M., Kageyama, S., Suzuki, H., Wada, T., Notsu, Y., Ishii, F., "Rheological properties of reversible thermo-setting *in situ* gelling solutions with the methylcellulose-polyethylene glycol- citric acid ternary system", *Colloid Polym. Sci*., **281**, 1178—1183(2003).
14 Lin, Y., Jiang, G.Q., Zan, J., Ding, F.X., "Gelation and *in vitro* release properties of methylcellulose-based thermo-sensitive hydrogels", *J. Tsinghua Univ. (Sci. & Technol.)*, **46**(6), 836—838,842(2006). (in Chinese)
15 Lin, Y., Zhu, D.Q., Zan, J., Jiang, G.Q., Ding, F.X., "Influence of sodium alginate on the properties of methylcellulose-based thermo-sensitive hydrogels", *J. Tsinghua Univ. (Sci. & Technol.)*, **46**(6), 839—842(2006). (in Chinese)
16 Wells, M.R., Kraus, K., Batter, D.K., Blunt, D.G., Weremowwitz, J., Lynch, S.E., Antoniades, H.N., Hansson, H.A., "Gel Matrix vehicles for growth Factor application in nerve gap injuries repaired with tubes: A comparison of biomatrix, collagen, and methylcellulose", *Exp. Neurol*, **146**(2), 395—402(1997).
17 Tate, M.C., Shear, D.A., Hoffman, S.W., Stein, D.G., LaPlaca, M.C., "Biocompatibility of methylcellulose-based constructs designed for intracerebral gelation following experimental traumatic brain injury", *Biomaterials*, **22**,1113—1123(2001).
18 Gupta, D., Tator, C.H., Shoichet, M.S., "Fast-gelling injectable blend of hyaluronan and methylcellulose for intrathecal, localized delivery to the injured spinal cord", *Biomaterials*, **27**, 2370—2379(2006).
19 Stockley, T.L., Robinson, K.E., Delaney, K., Ofosu, F.A., Chang, P.L., "Delivery of recombinant product from subcutaneous implants of encapsulated recombinant cells in canines", *J. Lab. Clin. Med*., **135**(6), 484—492(2000).
20 Jeon, Y., Kwak, K., Kim, S., Kim, Y., Lim, J., Baek, W., "Intrathecal implants of microencapsulated xenogenic chromaffin cells provide a long-term source of analgesic substances", *Transplant P*., **38**(9), 3061—3065(2006).
21 Jeong, B., van Wachem, P.B., van Luyn, M.J.A., Engbers, G.H.M., Krijgsveld, J., Zaat, S.A.J., Dankert, J., Feijen, J., "*In vivo* and *in vitro* release of lysozyme from cross-linked gelatin hydrogels: A model system for the delivery of antibacterial proteins from prosthetic heart valves", *J. Control. Release*, **67**, 323—336(2000).
22 Freiberg, S., Zhu, X.X., "Polymer microspheres for controlled drug release", *Int. J. Pharm*., **282**, 1—18(2004).
23 Pérez, C., Griebenow, K., "Effect of salts on lysozyme stability at the water-oil interface and upon encapsulation in poly(lactic-co-glycolic) acid microspheres", *Biotechnol. Bioeng*., **82**, 825-832(2003).
24 Singh, U.V., Udupa, N., "*In vitro* characterization of methotrexate loaded poly(lactide-co-glycolic) acid microspheres and antitumor efficacy in Sarcoma-180 mice bearing tumor", *Pharmaceutica Acta Helvetiae*, **72**, 165—173(1997).
25 Park, T.G., Lee, H.Y., Nam, Y.S., "A new preparation method for protein loaded poly(lactide-co-glycolic acid) microspheres and protein release mechamism study", *J. Control. Release*, **55**, 181—191(1998).
26 Lagarce, F., Faisant, N., Desfontis, J.C., Marescaux, L., Gautier, F., Richard, J., Menei, P., Benoit, J.P., "Baclofen-loaded microspheres in gel suspensions for intrathecal drug delivery: *In vitro* and *in vivo* evaluation", *Eur. J. Pharm. Biopharm*., **61**, 171—180(2005).
27 Zhao, G., Ping, Q.N., Liu, Y.J., "Study on the degradation of poly(d,l-lactide-co-glycolide) and poly(lactide acid) microspheres *in vitro*", *J. China Pharm. Univ*., **35**, 24—27(2004).
28 Grayson, A.C.R., Cima, M.J., Langer, R., "Size and temperature effects on poly(lactic-co-glycolic acid) degradation and microreservoir device performance", *Biomaterials*, **26**, 2137—2145(2005).
29 Blanco, M.D., Sastre, R.L., Teijón, C., Olmo, R., Teijón, J.M., "Degradation behaviour of microspheres prepared by spray-drying poly(D,L-lactide) and poly(D,L-lactide-co-glycolide) polymers", *Int. J. Pharm*., **326**, 139—147(2006).
30 Cai, Q., Shi, G., Bei, J., Wang, S., "Enzymatic degradation behaviour and mechanism of poly (lactide-co-glycolide) foams by trypsin", *Biomaterials*, **24**, 629—638(2003).