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Insulin nanoparticle preparation and encapsulation into poly(lactic-co-glycolic acid) microspheres by using an anhydrous system

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ABSTRACT

Insulin has been encapsulated in poly(lactic-co-glycolic acid) (PLGA) microspheres by solid-in-oil-in-oil (S/O/O) emulsion technique using DMF/corn oil as new solvent pairs. To get better encapsulation efficiency, insulin nanoparticles were prepared by the modified isoelectric point precipitation method so that it had good dispersion in the inner oil phase. The resulting microspheres had drug loading of 10% (w/w), while the encapsulation efficiency could be up to 90–100%. And the insulin release from the microspheres could last for 60 days. Microspheres encapsulated original insulin with the same method had lower encapsulation efficiency, and shorter release period. Laser scanning confocal microscopy indicated the insulin nanoparticle and original insulin had different distribution in microspheres. The results suggested that using insulin nanoparticle was better than original insulin for microsphere preparation by S/O/O method. Study about the secondary structure of insulin by Fourier transform infrared spectroscopy (FTIR) indicated high insulin structural integrity during the process. *In vivo* test showed insulin in microspheres retained its bioactivity. In addition, cytotoxicity evaluation by the MTT assay has proved that no extra toxicity was introduced into the microspheres during the emulsion process.

1. Introduction

A growing number of studies have been devoted to encapsulating protein drugs into microspheres (Duvvuri et al., 2005; Mok et al., 2007; Ubaidulla et al., 2007; Wang et al., 2007). To pursue this goal, many excellent emulsion systems have been developed. Since there are some common drawbacks caused by the outer water phase in the emulsion process, we turned to solve the problems by using an anhydrous system, typically a solid-in-oil-in-oil (S/O/O) emulsion system (Pradhan and Vasavada, 1994; O'Donnell and McGinity, 1998; Viswanathan et al., 1999; Kim et al., 2005; Leach and Schmidt, 2005; Li et al., 2005; Rao et al., 2005; Yamamoto et al., 2005). In this way, high encapsulation efficiency could be achieved due to the minimal solubility of protein in the outer oil phase (Carrasquillo et al., 2001a). Additionally, the solid state in process benefits the protein stability. It was reported that protein in solid state was kinetically trapped in the initial conformation, thus the conformational change could be largely prohibited (Tobio et al., 1999; Leach et al., 2005a).

For an S/O/O emulsion system, it is essential to employ a protein powder with small particle size in the first S/O suspension step. There are two primary methods to get smaller protein particles, and they both showed positive effect on the drug encapsulation. One is the spray-freezing into liquid (SFL) method, which can produce micro-sized protein particles with nanostructure (Mi et al., 2002; Yu et al., 2004; Engstrom et al., 2007). Williams reported that using this submicron protein particles in an S/O/O system could reduce the burst release of microspheres (Leach et al., 2005a). Similar results were observed by Griebenow (Al-Azzam et al., 2002). Another way to obtain protein microparticles is the seed zone method, which can produce insulin microcrystal (Lee et al., 2000; Kwon and Kim, 2004). Using these microcrystal in S/O/O system, Kim achieved better insulin encapsulation efficiency (Park et al., 2007). Moreover, several experimental results showed a reduction in burst release along with a decrease in protein particle size. However, most of the above-mentioned protein particles are in micron size, protein nanoparticles are seldom reported in this S/O/O method. It is reasonable to hypothesize that the encapsulation will benefit from the nano-sized protein particle.

In this study, insulin nanoparticles will be used in the S/O/O method, and we will test if it might be good to the encapsulation. The nanoparticle was produced by isoelectric point deposition method. Our objective was to investigate that if this excipient-free insulin nanoparticle could be well encapsulated into PLGA microspheres to get minimum burst, and to preserve insulin conformation at the same time. We also tested whether this "smaller-better" effect for good encapsulation and low burst would hold true until the insulin particle size was dropped to nano scale.

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2. Materials and methods

Polymer materials were synthesized in our laboratory by ring-opening polymerization (Zhu et al., 1991). Poly(lactic-co-glycolic acid) (PLGA) had a lactide/glycolide ratio of 75/25, and the polymer molecular weights were 10 kDa, 25 kDa, and 90 kDa. The insulin powder was purchased from Greenbird China, and the BCA protein assay kit came from Pierce; fluorescein isothiocyanate (FITC) and sorbitan sesquioleate (span 83) were purchased from Sigma. Corn oil was from commercial food suppliers. All other chemicals were of analytical grade.

2.1. Insulin nanoparticle preparation

Insulin nanoparticles were prepared by the modified isoelectric point precipitation method (Zhang et al., 2005). Briefly, 10 mg of insulin was dissolved in 10 mL of acidic water (pH 2.6; adjusted by HCl), then the solution was slowly adjusted to the isoelectric point of insulin (pH 5.3) under stirring. After the nanoparticle formation, the suspension was allowed to stand still for nanoparticles to precipitate. The supernate was recycled. Meanwhile, the precipitation was quickly frozen by liquid nitrogen and then lyophilized to get a dehydrated powder. The process was free of excipient.

2.2. PLGA microsphere preparation

Microspheres were prepared by following the S/O/O technique; the process was illustrated in Fig. 1. Dehydrated protein powder was suspended in a polymer solution (inner oil phase), and then the suspension was emulsified into an oil continuous phase (outer oil phase), which was a completely water-free emulsion process that can be used as an encapsulation procedure (Herrmann and Bodmeier, 1998; Marinina et al., 2000; Jiang and Schwendeman, 2001; Sanchez et al., 2003). Typically, PLGA was dissolved in N,N-dimethylformamide (DMF) at a concentration of 2% (w/v), and the insulin powder was suspended in 1 mL of the polymer solution by stirring. The weight ratio of insulin and PLGA was 1:9. Then the suspension was added into 10 mL of corn oil with 1% (w/v) span 83 and homogenized at 3000 rpm for 5 min to form an oil-in-oil (O/O) emulsion (Lee et al., 2000; Kim et al., 2005). After that, ethyl ether was slowly added into the emulsion to extract DMF. Then the microspheres were collected by centrifugation, washed in turn by ethyl ether, ethanol and water, and lyophilized.

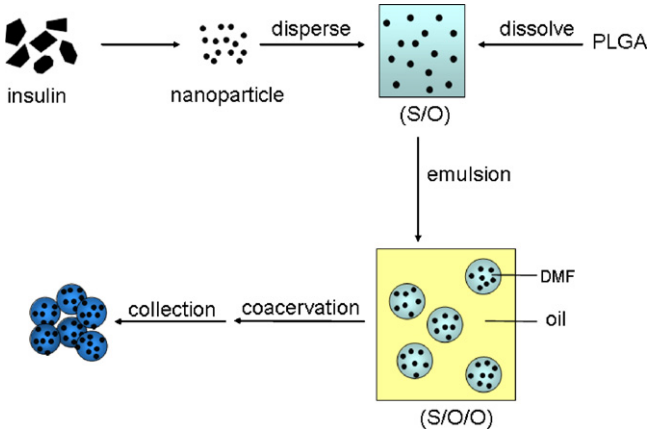

Fig. 1. Schematic presentation of the methods used to prepare insulin-loaded microspheres.

2.3. Morphologies of nanoparticles and microspheres

The surface morphologies of nanolized insulin and polymer microspheres were studied by a scanning electron microscope (SEM, Micron FEI Philips). The SEM samples of insulin particles and polymer microspheres were prepared from water suspension. A drop of suspension was deposited onto a silicon chip mounted on an aluminum stub, and it was air dried before measurements. To illustrate insulin morphology and drug dispersion in the S/O step, the nanolized insulin nanoparticle powder was suspended in DMF, which was the inner oil phase, and the suspension was deposited on the chip and measured in the same way as mentioned above.

2.4. Insulin crystallinity

Crystallinity of the nanolized insulin was examined by X-ray diffractometry (XRD). Wide-angle X-ray diffraction was performed using a powder X-ray Diffractometer (Rigaku D, max 2500kVPC) with a Cu tube anode. The angle was between 5°and 80°.

2.5. Drug loading and encapsulation efficiency

The amount of insulin loaded by microspheres was determined by the NaOH-SDS method (De Rosa et al., 2005). The appropriate quantity of microspheres was hydrolyzed overnight in 3 mL of 5 wt% SDS in 0.1 M NaOH. The insulin content was determined by using the BCA micro-assay kit (Pierce, BCA), using 3 replicate measurements of each point. The PLGA segment did not affect the protein test, since no protein signal was detected from hydrolyzed blank microspheres (with no protein encapsulated). The encapsulation efficiency (%, w/w) of insulin was calculated according to the following equation:$\text{Encapsulation efficiency}\,(\%) = \frac{\text{actual drug loading}}{\text{theoretical drug loading}} \times 100$.

2.6. Drug distribution

The dispersing ability of insulin powder in the inner oil phase was represented by the turbidity of powder suspension. The transmittance (*T*%) of the powder suspension at 500 nm in DMF was measured by using a UV–vis spectrophotometer (Shimadzu, UV-2401PC).

Insulin distribution inside microspheres was observed by Laser Scanning Confocal microscopy (CLSM, Leica TCS SP2). Insulin was first labeled by FITC, a commonly used tool for tracking protein distribution (Rojas and Papadopoulos, 2007), and then encapsulated by PLGA microspheres using the above-mentioned method. Samples on glass slides were using glycerin to wet and examined with the microscope at 20× magnification. The shown images were from a slice through the midsection of the microspheres.

2.7. In vitro release

In vitro release of insulin from microspheres was tested in a Tris–HCl buffer solution. In brief, approximately 50 mg microspheres were added to each centrifugal tube, which was filled with 5 mL Tris–HCl buffer solution (pH 8.2); then the tube was placed on a shaker at 37 °C. At various time points, 1 mL of supernatant was removed from the insulin concentration measurement after centrifugation, and the equivalent fresh buffer solution was added to the mixture. The insulin concentration measurement was performed using the BCA protein assay kit in triplicate. To avoid artificial insulin release behavior caused by saturation of insulin in the buffer solution, the buffer was adjusted to a little alkaline (pH 8.2) for better drug solubility since insulin has poor solubility in neutral media.

2.8. Insulin secondary structure analysis by FTIR

FTIR spectra were collected using a Vacuum FTIR spectrophotometer (Bruker, IF S66 V/S). Samples were prepared by mixing sample powder with KBr and scanned at 2 cm^{-1} resolution. All samples were prepared in a dry state, and tested in triplicate. Spectrum analysis was performed using the OPUS 5.0 software by following the reported method (Griebenow and Klibanov, 1996; Fu et al., 1999; Leach et al., 2005b). Background-corrected spectra were analyzed by second derivatization in the amide I band region for components peak frequencies. Gaussian curve-fitting was performed on the Fourier self-deconvoluted amide I band region. The secondary structure content was calculated from the areas of the individually assigned peaks and their fractions of the total area in the region.

2.9. Activity of insulin in vivo

To test the activity of insulin encapsulated in microspheres, the insulin was extracted from microspheres by immerging microspheres in Tris–HCl buffer for a few days. The insulin concentration in the microsphere extraction was determined by BCA. Meanwhile, a solution of original insulin with the same concentration was prepared as control.

Female Wistar rats of 200–220 g weight were injected with alloxan monohydrate 30 mg/kg, and hyperglycemia being induced about 3 days later. Microsphere extraction was injected subcutaneously (Hinds et al., 2005) in the back of rats (n = 10/group), at a dose of 8 U/kg body weight. As positive control, a solution of insulin was administered to rats under the same conditions. Buffer solution was injected as negative control. Serum samples were obtained from the tail vein of rats at 0–8 h after injection. Results were shown as the mean plasma glucose levels, values from different groups were compared with the control groups using a t-test.

2.10. Cytotoxicity evaluation by the MTT assay

Cytotoxicity of the blank microspheres (without drug) was tested using the MTT assay to evaluate the residual toxicity after the microsphere preparation process. It was performed as described by Fischer et al. (1999). Briefly, Vero cell lines were seeded in 96 well plates at 12,000 cells/well and incubated for 24 h, and the microsphere extract of various concentrations was then added to the well. After incubating for 24 h, 20 μL of the MTT solution in pH 7.4 PBS (5 mg/mL) was added into each well and the final concentration of MTT was 0.5 mg/mL. The plate was then incubated at 37 °C in 5% CO_2 for 4 h. The medium was removed and 200 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The plate was read spectrophotometrically at 492 nm in a Microplate Spectrophotometer (Multiskan MK3, Thermo Electron Corporation). Untreated cells were taken as the control with 100% viability. The relative cell viability (%) compared to control cells was calculated as follow:Relative cell viability (%) $= \frac{[\mathrm{Abs}]_{\mathrm{sample}}}{[\mathrm{Abs}]_{\mathrm{control}}} \times 100$.

3. Results and discussion

3.1. Characterization of insulin nanoparticles

Before nanolization, original commercial insulin in the size range of a few microns to 20 μm (Fig. 2a), which was consistent with Kim's observation (Kwon et al., 2004; Park et al., 2007). After nanolization, insulin exhibited monodisperse particles with a size around 40 nm (Fig. 2b). The two kinds of insulin particles were analyzed by XRD to estimate their crystalline structure and the results are shown in Fig. 3. The commercial insulin showed weak diffraction peaks, indicating that it was semi-crystalline (Park et al., 2007). The nanolized insulin displayed identical peaks with the original insulin. The coincidence of XRD peaks demonstrated that these two kinds of insulin particles had the same crystal structure, which means that the insulin crystal structure was not remarkably changed during the nanolization process.

Once the protein powder is produced, the next step is dispersing the protein particles in the inner phase of emulsion. In our system the inner phase was DMF. After suspended in DMF, commercial insulin powder presented a turbid suspension, while insulin nanoparticles suspension was transparent (Fig. 2d), and the transmittance (T%) at 500 nm of these two suspensions were 20% and

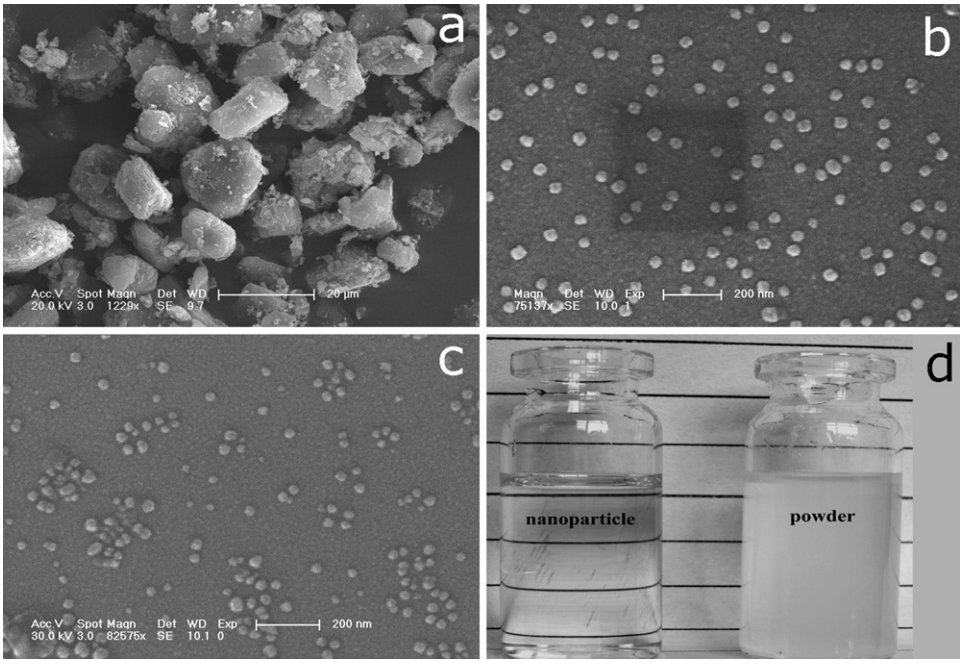

Fig. 2. The SEM images of (a) the commercial insulin, (b) the insulin nanoparticles, and (c) the insulin nanoparticles re-dispersed in DMF. Image (d) is the photograph of insulin nanoparticles and commercial insulin dispersed in DMF by stirring.

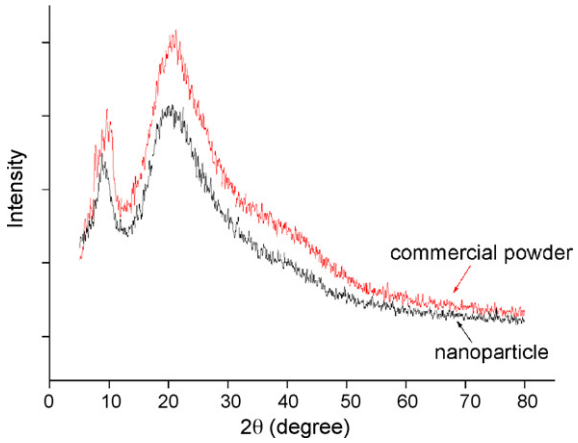

Fig. 3. Crystallinity of the commercial insulin and the insulin nanoparticles measured by X-ray diffractometry (XRD).

98% respectively. The results indicated that insulin nanoparticles could be well dispersed in DMF, giving a good chance to blend itself into polymer matrix. The morphology of the insulin nanoparticles dispersed in DMF (Fig. 2c) showed little difference from that of the water suspension (Fig. 2b). Insulin nanoparticles preserved its morphology in organic solvent DMF, and no noticeable aggregate was found. However, in the case of other organic solvents with lower polarity, such as dichloromethane, the insulin nanoparticles formed big aggregates (data not shown). It implies that both high solvent polarity and small particle size were important to the particle suspension state. On the other hand, the inner oil phase must have good solubility for polymer materials and low toxicity. Considering these aspects, we chose DMF as the inner oil phase.

3.2. Characterization of microspheres (drug loading, morphology, and drug distribution)

Microspheres were prepared from various kinds of PLGA materials and either commercial insulin or insulin nanoparticles were encapsulated in it (Table 1). The theoretical loadings of all the formulation were 10% insulin in the total microsphere. The encapsulation efficiency was only 50.1% as encapsulated the commercial insulin. In case of the insulin nanoparticles, the measured encapsulation efficiencies were significantly improved, which are from 90.5% to 100.8%. In commercial insulin big particles were likely precipitate during the emulsion process, and these precipitates would be eliminated by the following washing step leading to lower encapsulation efficiency. In contrast, insulin nanoparticles could be well dispersed in the inner oil phase, and was small enough to maintain in the emulsion droplet. So it could be well encapsulated by polymer microspheres. As reported, the small protein particle size increased encapsulation efficiency in coacervation encapsulation procedures (Al-Azzam et al., 2005). This "smaller-better" effect for encapsulation efficiency was proved true in our encapsulation of insulin nanoparticles. Another important factor for high encapsulation efficiency is the hydrophobic corn oil avoids possible drug dissolution in outer phase. That is why emulsion systems with outer oil phase often got high encapsulation efficiency for water-soluble drugs (Leach et al., 2005a; Li et al., 2005; Lopedota et al., 2007). However, the structure and molecular weight of polymer has relative weak effect on the encapsulation efficiency compared to the size of insulin particles.

To demonstrate microsphere formation, the microsphere morphology was observed by SEM. Both the microspheres had diameters ranged from 1 to 10 μm (Fig. 4), but they had different morphology. The microspheres prepared with the commercial insulin had holes on the surface (Fig. 4a) whereas microspheres encapsulated with insulin nanoparticles (Fig. 4b) exhibited a spherical shape and smooth surface. It seemed that big insulin particles disturbed the microsphere formation. Considering some of the commercial insulin particles had a dimension close to microspheres, it was reasonable to believe that larger particles of commercial insulin could not be encapsulated into the microspheres completely (Al-Azzam et al., 2002), and these insulin particles might stay on the surface of microspheres. Since no insulin crystals could be observed on the microsphere surface by SEM, they might be washed away during the process, leaving holes in microspheres. Some largest particles which were bigger than emulsion droplets probably were left unencapsulated, and these naked insulin particles could likely be lost during the washing step. This explained the low encapsulation efficiency in formulation 1.

Insulin particle size also had an effect on drug distribution in the microspheres. As shown in Fig. 5a, the labeled commercial insulin presented separate fluorescence intricately inside the microspheres, which means big bulks or aggregate. On the contrary, the nanoparticles in Fig. 5b showed continuous even fluorescence in shape of microspheres, indicating that it had been uniformly distributed in the microsphere matrix. The results were consistent with the dispersion experiment mentioned before. Apparently, the small dimension of nanoparticles dispersed well in the DMF droplets, and consequently caused uniform drug distribution in microspheres after the oil droplets coacervation.

3.3. In vitro release

A commonly reported problem of *in vitro* release behavior for most of drug loading systems was the burst release, especially within the initial 24 h. To reduce this unwanted phenomenon, a series of methods has been tried (Carrasquillo et al., 2001b; Hinds et al., 2005; Bouissou et al., 2006; Berkland et al., 2007; Hasan et al., 2007). A previous work reported that large protein particles contribute to burst release, thus utilizing submicron protein particles could help reduce it (Al-Azzam et al., 2002, 2005; Leach et al., 2005a). By using the insulin nanoparticles, a major reduction in burst was achieved. As listed in Table 1, the 24 h burst decreased to 7% for the formulation 2 (encapsulated nanoparticles). With appropriate polymer material, Microspheres loaded with the insulin nanoparticles could reduce the 24 h burst to 5% (formulation 4).

Table 1
Characterization of microspheres.

Formulations	Type of insulin	Material for microspheres	Drug loading[a] (w/w, %)	Encapsulation efficiency[b] (%)	24 h burst (%)	Insulin α-helix content[c] (%)
1	Commercial	PLGA 10k	5.01	50.1	44	41 ± 1
2	Nanoparticle	PLGA 10k	9.94	99.4	7	41 ± 0
3	Nanoparticle	PLGA 25k	9.05	90.5	6	40 ± 1
4	Nanoparticle	PLGA 90k	10.1	100.8	5	41 ± 1

[a] Determined by the NaOH-SDS method.
[b] Calculated by the equation mentioned in the text.
[c] Determined by FTIR as explained in the text.

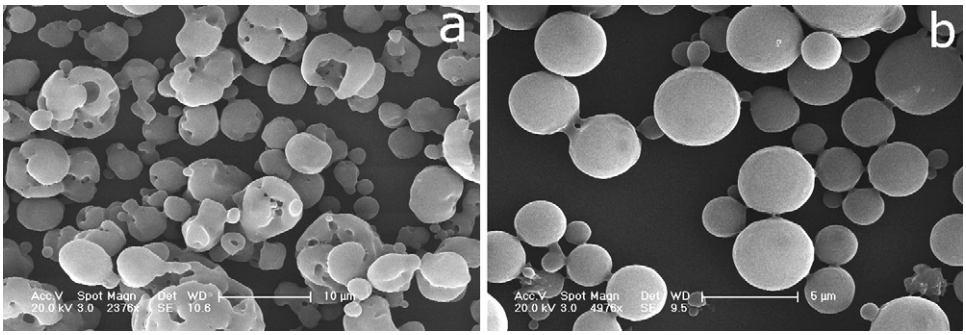

Fig. 4. The SEM images of (a) formulation 1: microspheres encapsulated with the commercial insulin and (b) formulation 2: microspheres encapsulated with insulin nanoparticles.

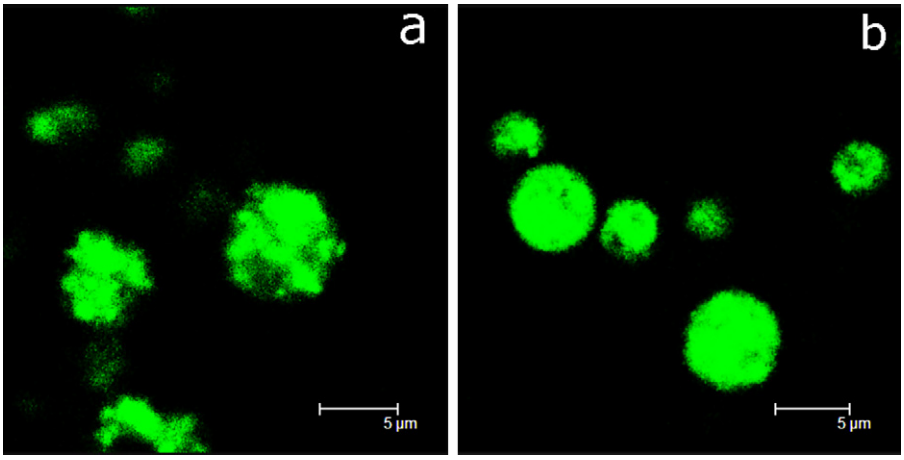

Fig. 5. The CLSM images of (a) formulation 1: microspheres encapsulated with the commercial insulin and (b) formulation 2: microspheres encapsulated with insulin nanoparticles.

The burst was significantly lower than using commercial insulin (formulation 1, 44%). Decreasing the size of insulin particle to nano scale had a strong effect on reducing burst release. This result is consistent with the works which using submicron drug particles to reduce burst release (Leach et al., 2005a). Microspheres loaded commercial insulin has holes in the microspheres, which enlarged the surface and accelerated the release. For nanoparticles, Surface insulin content (calculated by X-ray photoelectron spectroscopy, data not shown) was close to the drug loading. That is, the drug was not condensed on the surface. Since the burst is often caused by the surface drug, microsphere with nanoparticles had a reduced burst. Relatively, the molecular weight of polymer played a minor role in burst release. As in the formulations 2–4, when the material molecular weight increased from 10k to 90k, the 24 h burst slightly reduced from 7% to 5%. As our observation, the protein particle size was dominant to the burst effect relative to that of material molecular weight.

The *in vitro* release of insulin from microspheres is shown in Fig. 6. When commercial insulin was encapsulated in microspheres, the drug released fast and depleted in 10 days. On the contrary, for the formulations with nanoparticles, the release of insulin was prolonged for more than 60 days. Comparing the formulations 1 and 2, in which the same material and process were used, the distinct release behavior could merely be triggered by the difference in drug particle size. Rapid release for formulation 1 could be explained by the broken structure of microspheres (Fig. 4a). Holes on microspheres expanded the surface area and made it easier for the drug to be released. Also, it was hypothesized that the release was related to the distribution of protein in the microsphere (Azzam et al., 2005). In contrast, the insulin nanoparticles could be trapped in the polymer matrix separately, and insulin molecules only could be released when they diffused to the microsphere surface. On the other hand, microspheres made of PLGA with various molecular weights (formulations 2–4) showed slight difference by their release curves (Fig. 6), which means that a wide range of polymer molecular weights was compatible to this encapsulation method. The PLGA 90k has a little faster release speed, that maybe

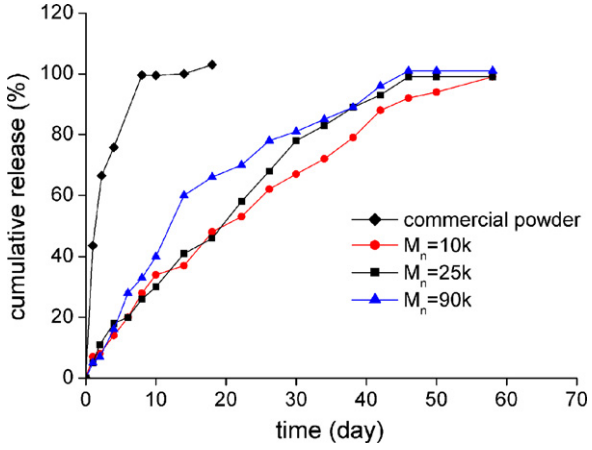

Fig. 6. Release profiles of microspheres encapsulated with the commercial insulin (formulation 1) and microspheres encapsulated with insulin nanoparticles (formulations 2–4).

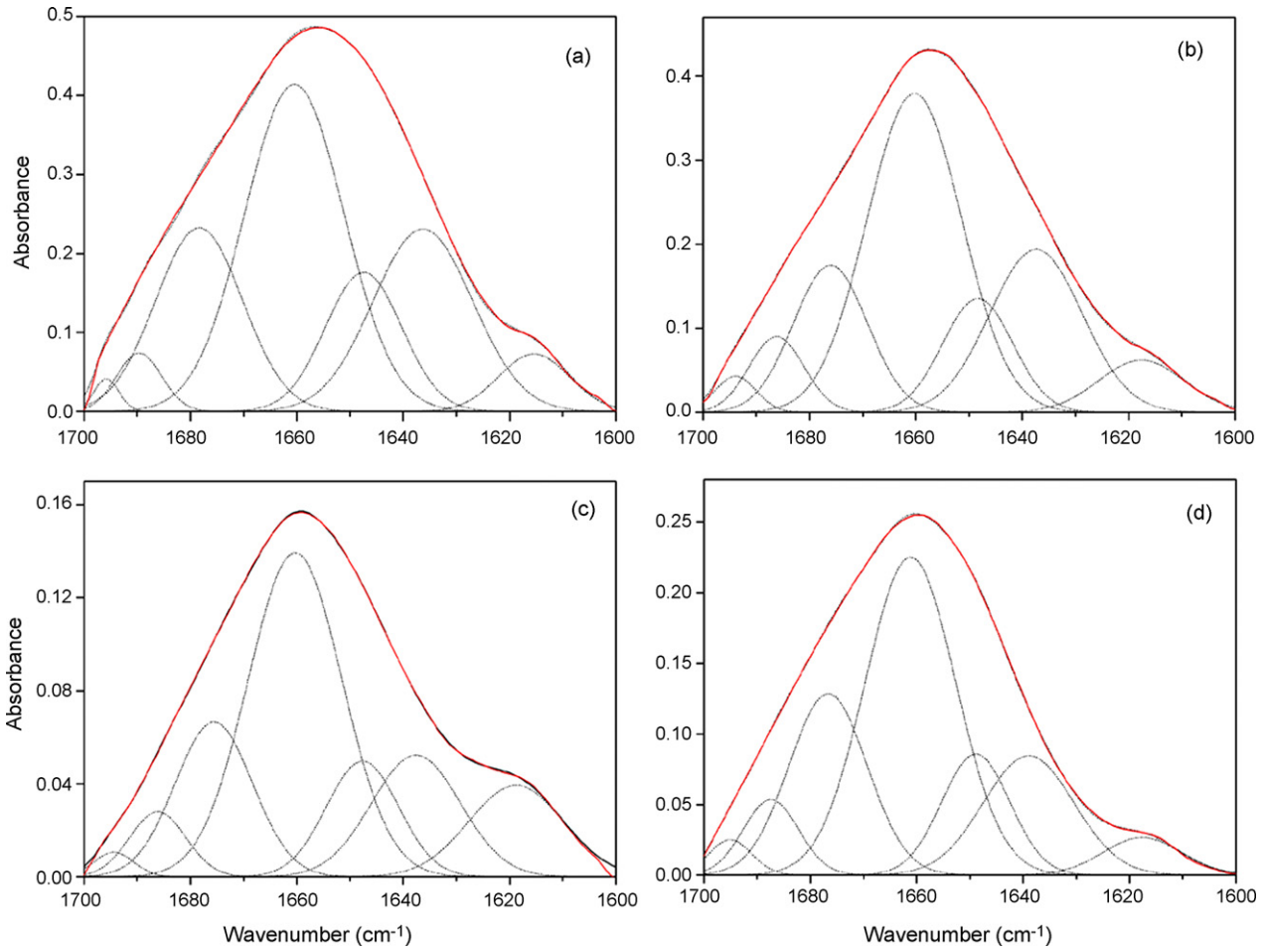

Fig. 7. Gaussian curve-fitted Fourier self-deconvolved FTIR amide I spectra: (a) commercial insulin; (b) insulin nanoparticles; (c) commercial insulin encapsulated in microspheres (formulation 1); (d) insulin nanoparticles encapsulated in microspheres (formulation 2). The individual Gaussian bands are shown as dotted lines.

caused by the relatively lower crystallinity of the material than the others.

3.4. Insulin secondary structure

The secondary structure is a reflection of protein conformation. In the present study, the amide I band (1600–1700 cm^{-1}) was employed to examine the secondary structure of insulin encapsulated inside the microspheres because the microsphere materials showed no absorption in this region (Al-Azzam et al., 2002). The content of α-helix was discussed as the criterion of the secondary structure since this structure is invariable when the sample is lyophilized (Griebenow and Klibanov, 1996). FTIR spectra of the amide I region of the commercial insulin and insulin nanoparticles, along with the curve fitting, are reported in Fig. 7. As can be seen, the spectra were suitably simulated by the sum of seven Gaussian peaks on the basis of second-derivative, and no significant spectral difference could be observed between the two insulin samples (Fig. 7a and b). The two samples had the same α-helix content of 41 ± 1%, which was consistent with previously reported work (Pikal and Rigsbee, 1997). From this result it can be seen that the insulin secondary structure was not affected by the nanoparticle preparing process, which might be because the denaturation of these nanoparticles was minimized due to ultra-rapid freezing by liquid nitrogen in the solid state. The spectra of microspheres are also presented, which containing the commercial insulin (Fig. 7c) and nanopaticles (Fig. 7d) respectively. The similarity of the spectra between these two kinds of samples and the spectrum of pure commercial insulin verifies the similar secondary structure of insulin. From Table 1 it can be seen that the α-helix of insulin in microspheres remained the same content as the commercial insulin before encapsulated (41 ± 1%). It indicated that insulin secondary structure was retained during the encapsulation process.

3.5. In vivo activity of insulin extracted from microspheres

The pharmacodynamic effect of treatment with insulin extracted from microspheres was similar to that with the original insulin solution (Fig. 8). Serum glucose level decreased in 1–4 h after injection, and there were no significant difference between the hypoglycemia effects of the two insulin doses. Though the two glucose curves were not identical, their area under the curve (AUC) was the same. It indicated that the biological activity of insulin in microspheres was mostly retained. In the encapsulation processing, protein was always in its solid state, thus the organic solvent only had a minor effect on protein conformation (Leach et al., 2005a). In addition, the non-aqueous environment gave the particles no chance to be absorbed on the water–oil interface, which was considered to be one of the most important factor for conformational changes (Griebenow and Klibanov, 1997). Kept in solid state will protect protein stability; meanwhile, small particles have better suspension and distribution ability. In this study, the formulation with insulin nanoparticles took both of the above-mentioned advantages to render a better result.

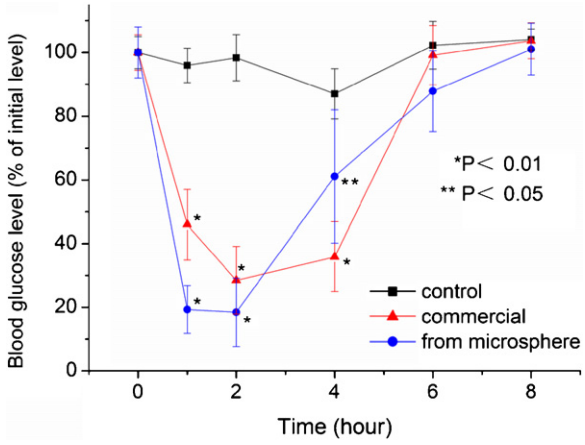

Fig. 8. Plasma glucose levels in diabetic rats (mean ± SD, $n = 10$/group) following subcutaneously administration of insulin extraction from microspheres or original insulin solution.

3.6. Cytotoxicity evaluation

From the pharmaceutics standpoint, the toxicity of formulation is fatal for a drug delivery system. In this study, the major probability of the toxicity of formulation came from the residual solvent. To examine the possible residue toxicity related to the drug-loading process, the cytotoxicity of microspheres was tested *in vitro* by the MTT assay. Cytotoxicity against vero cells was determined as a function of microsphere concentration, and untreated cells were used as the negative control. Because insulin would disturb cell growth, blank microspheres (undergoing the same drug-loading process but no protein encapsulated) were used. For comparison, original PLGA materials were used as the negative control. The cells incubated with the extract of microspheres and polymer materials both retained above 75% viability even when the concentration was up to 100 mg/mL (Fig. 9). The extract of microspheres showed no inhibition of the cell metabolism compared to the negative control over the concentration range tested. All the other tested materials with different molecular weights and corresponding microspheres showed similar results (data not shown), which means the residue toxicity induced during the microsphere preparation was so low that it would not affect the vero cell growth.

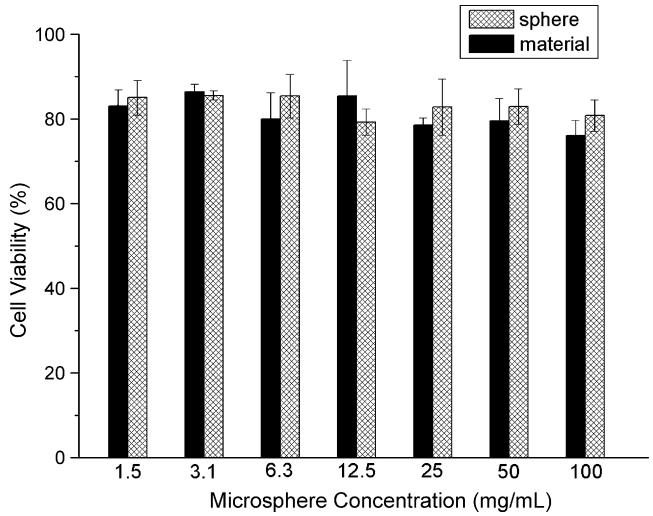

Fig. 9. Cytotoxicity evaluation by the MTT assay for the extract of polymer PLGA (4:1), $M_n = 10$k and corresponding blank microspheres.

4. Conclusion

A typical protein drug, insulin, was encapsulated in biodegradable PLGA microspheres successfully by using an anhydrous S/O/O emulsion system with new solvent pairs. The low cytotoxicity of microsphere extract promised the safety of this preparation technique. In the encapsulation process, both the solvent polarity and the particle size were important to the particle suspension state. To minimum the particle size, insulin was made into nanoparticles by isoelectric point precipitation method and it was proofed that the physical chemical property of the insulin was not changed. Utilizing insulin nanoparticles could improve the drug encapsulation efficiency and correspondingly enhance drug loading of microspheres. The small size of insulin nanoparticles contributed to the intact sphere shape and uniform drug distribution. Also the microspheres loaded with nanoparticles of insulin had the prolonged release period over 60 days. FTIR and *in vivo* test indicated insulin retained bioactivity during the encapsulation process. This S/O/O method offers another approach to high efficiency protein encapsulation.

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References

Al-Azzam, W., Pastrana, E.A., Griebenow, K., 2002. Co-lyophilization of bovine serum albumin (BSA) with poly(ethylene glycol) improves efficiency of BSA encapsulation and stability in polyester microspheres by a solid-in-oil-in-oil technique. Biotechnol. Lett. 24, 1367–1374.

Al-Azzam, W., Pastrana, E.A., King, B., Mendez, J., Griebenow, K., 2005. Effect of the covalent modification of horseradish peroxidase with poly(ethylene glycol) on the activity and stability upon encapsulation in polyester microspheres. J. Pharm. Sci. 94, 1808–1819.

Berkland, C., Pollauf, E., Raman, C., Silverman, R., Kim, K., Pack, D.W., 2007. Macromolecule release from monodisperse PLG microspheres: control of release rates and investigation of release mechanism. J. Pharm. Sci. 96, 1176–1191.

Bouissou, C., Rouse, J.J., Price, R., van der Walle, C.F., 2006. The influence of surfactant on PLGA microsphere glass transition and water sorption: remodeling the surface morphology to attenuate the burst release. Pharm. Res. 23, 1295–1305.

Carrasquillo, K.G., Carro, J.C.A., Alejandro, A., Toro, D.D., Griebenow, K., 2001a. Reduction of structural perturbations in bovine serum albumin by non-aqueous microencapsulation. J. Pharm. Pharmacol. 53, 115–120.

Carrasquillo, K.G., Stanley, A.M., Aponte-Carro, J.C., De Jesus, P., Costantino, H.R., Bosques, C.J., Griebenow, K., 2001b. Non-aqueous encapsulation of excipient-stabilized spray-freeze dried BSA into poly(lactide-co-glycolide) microspheres results in release of native protein. J. Control. Release 76, 199–208.

De Rosa, G., Larobina, D., La Rotonda, M.I., Musto, P., Quaglia, F., Ungaro, F., 2005. How cyclodextrin incorporation affects the properties of protein-loaded PLGA-based microspheres: the case of insulin/hydroxypropyl-beta-cyclodextrin system. J. Control. Release 102, 71–83.

Duvvuri, S., Janoria, K.G., Mitra, A.K., 2005. 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.

Engstrom, J.D., Simpson, D.T., Cloonan, C., Lai, E.S., Williams, R.O., Kitto, G.B., Johnston, K.P., 2007. Stable high surface area lactate dehydrogenase particles produced by spray freezing into liquid nitrogen. Eur. J. Pharm. Biopharm. 65, 163–174.

Fischer, D., Bieber, T., Li, Y.X., Elsasser, H.P., Kissel, T., 1999. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm. Res. 16, 1273–1279.

Fu, K., Griebenow, K., Hsieh, L., Klibanov, A.M., Langer, R., 1999. FTIR characterization of the secondary structure of proteins encapsulated within PLGA microspheres'. J. Control. Release 58, 357–366.

Griebenow, K., Klibanov, A.M., 1996. On protein denaturation in aqueous-organic mixtures but not in pure organic solvents. J. Am. Chem. Soc. 118, 11695–11700.

Griebenow, K., Klibanov, A.M., 1997. Can conformational changes be responsible for solvent and excipient effects on the catalytic behavior of subtilisin Carlsberg in organic solvents? Biotechnol. Bioeng. 53, 351–362.

Hasan, A.S., Socha, M., Lamprecht, A., El Ghazouani, F., Sapin, A., Hoffman, A., Maincent, P., Ubrich, N., 2007. Effect of the microencapsulation of nanoparticles on the reduction of burst release. Int. J. Pharm. 344, 53–61.

Herrmann, J., Bodmeier, R., 1998. Biodegradable, somatostatin acetate containing microspheres prepared by various aqueous and non-aqueous solvent evaporation methods. Eur. J. Pharm. Biopharm. 45, 75–82.

Hinds, K.D., Campbell, K.M., Holland, K.M., Lewis, D.H., Piche, C.A., Schmidt, P.G., 2005. PEGylated insulin in PLGA microparticles. In vivo and in vivo analysis. J. Control. Release 104, 447–460.

Jiang, W.L., Schwendeman, S.P., 2001. Stabilization and controlled release of bovine serum albumin encapsulated in poly(D,L-lactide) and poly(ethylene glycol) microsphere blends. Pharm. Res. 18, 878–885.

Kim, B.K., Hwang, S.J., Park, J.B., Park, H.J., 2005. Characteristics of felodipine-located poly(epsilon-caprolactone) microspheres. J. Microencapsul. 22, 193–203.

Kwon, J.H., Kim, C.W., 2004. A novel insulin microcrystals preparation using a seed zone method. J. Cryst. Growth 263, 536–543.

Kwon, J.H., Lee, B.H., Lee, J.J., Kim, C.W., 2004. Insulin microcrystal suspension as a long-acting formulation for pulmonary delivery. Eur. J. Pharm. Sci. 22, 107–116.

Leach, J.B., Schmidt, C.E., 2005. Characterization of protein release from photocrosslinkable hyaluronic acid-polyethylene glycol hydrogel tissue engineering scaffolds. Biomaterials 26, 125–135.

Leach, W.T., Simpson, D.T., Val, T.N., Anuta, E.C., Yu, Z.S., Williams, R.O., Johnston, K.P., 2005a. Uniform encapsulation of stable protein nanoparticles produced by spray freezing for the reduction of burst release. J. Pharm. Sci. 94, 56–69.

Leach, W.T., Simpson, D.T., Val, T.N., Yu, Z.S., Lim, K.T., Park, E.J., Williams, R.O., Johnston, K.P., 2005b. Encapsulation of protein nanoparticles into uniform-sized microspheres formed in a spinning oil film. AAPS Pharmscitech 6, 605–617.

Lee, J.H., Park, T.G., Choi, H.K., 2000. Effect of formulation and processing variables on the characteristics of microspheres for water-soluble drugs prepared by w/o/o double emulsion solvent diffusion method. Int. J. Pharm. 196, 75–83.

Li, Y., Jiang, H.L., Zhu, K.J., Liu, J.H., Hao, Y.L., 2005. Preparation, characterization and nasal delivery of alpha-cobrotoxin-loaded poly(lactide-co-glycolide)/polyanhydride microspheres. J. Control. Release 108, 10–20.

Lopedota, A., Trapani, A., Cutrignelli, A., Laquintana, V., Denora, N., Franco, M., Trapani, G., Liso, G., 2007. Effect of cyclodextrins on physico-chemical and release properties of Eudragit RS 100 microparticles containing glutathione. J. Incl. Phenom. Macro. 57, 425–432.

Marinina, J., Shenderova, A., Mallery, S.R., Schwendeman, S.P., 2000. Stabilization of vinca alkaloids encapsulated in poly(lactide-co-glycolide) microspheres. Pharm. Res. 17, 677–683.

Mi, F.L., Lin, Y.M., Wu, Y.B., Shyu, S.S., Tsai, Y.H., 2002. Chitin/PLGA blend microspheres as a biodegradable drug-delivery system: phase-separation, degradation and release behavior. Biomaterials 23, 3257–3267.

Mok, H., Park, J.W., Park, T.G., 2007. Microencapsulation of PEGylated adenovirus within PLGA microspheres for enhanced stability and gene transfection efficiency. Pharm. Res. 24, 2263–2269.

O'Donnell, P.B., McGinity, J.W., 1998. Influence of processing on the stability and release properties of biodegradable microspheres containing thioridazine hydrochloride. Eur. J. Pharm. Biopharm. 45, 83–94.

Park, S.H., Kwon, J.H., Lim, S.H., Park, H.W., Kim, C.W., 2007. Characterization of human insulin microcrystals and their absorption enhancement by protease inhibitors in rat lungs. Int. J. Pharm. 339, 205–212.

Pikal, M.J., Rigsbee, D.R., 1997. The stability of insulin in crystalline and amorphous solids: observation of greater stability for the amorphous form. Pharm. Res. 14, 1379–1387.

Pradhan, R.S., Vasavada, R.C., 1994. Formulation and in-vitro release study on poly(Dl-lactide) microspheres containing hydrophilic compounds—glycine homopeptides. J. Control. Release 30, 143–154.

Rao, K.R., Senapati, P., Das, M.K., 2005. Formulation and in vitro evaluation of ethyl cellulose microspheres containing zidovudine. J. Microencapsul. 22, 863–876.

Rojas, E.C., Papadopoulos, K.D., 2007. Induction of instability in water-in-oil-in-water double emulsions by freeze-thaw cycling. Langmuir 23, 6911–6917.

Sanchez, A., Tobio, M., Gonzalez, L., Fabra, A., Alonso, M.J., 2003. Biodegradable micro- and nanoparticles as long-term delivery vehicles for interferon-alpha. Eur. J. Pharm. Sci. 18, 221–229.

Tobio, M., Nolley, J., Guo, Y.Y., McIver, J., Alonso, M.J., 1999. A novel system based on a poloxamer PLGA blend as a tetanus toxoid delivery vehicle. Pharm. Res. 16, 682–688.

Ubaidulla, U., Khar, R.K., Ahmed, F.J., Panda, A.K., 2007. Development and in-vivo evaluation of insulin-loaded chitosan phthalate microspheres for oral delivery. J. Pharm. Pharmacol. 59, 1345–1351.

Viswanathan, N.B., Thomas, P.A., Pandit, J.K., Kulkarni, M.G., Mashelkar, R.A., 1999. Preparation of non-porous microspheres with high entrapment efficiency of proteins by a (water-in-oil)-in-oil emulsion technique. J. Control. Release 58, 9–20.

Wang, X.Q., Wenk, E., Matsumoto, A., Meinel, L., Li, C.M., Kaplan, D.L., 2007. Silk microspheres for encapsulation and controlled release. J. Control. Release 117, 360–370.

Yamamoto, H., Kuno, Y., Sugimoto, S., Takeuchi, H., Kawashima, Y., 2005. Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions. J. Control. Release 102, 373–381.

Yu, Z.S., Garcia, A.S., Johnston, K.P., Williams, R.O., 2004. Spray freezing into liquid nitrogen for highly stable protein nanostructured microparticles. Eur. J. Pharm. Biopharm. 58, 529–537.

Zhang, X.F., Hu, J.L., Chen, X.S., Jing, X.B., 2005. Preparation and characterization of biodegradable insulin-loaded microspheres. Chem. J. Chin. U 26, 554–557.

Zhu, J.H., Shen, Z.R., Wu, L.T., Yang, S.L., 1991. Invitro degradation of polylactide and poly(lactide-co-glycolide) microspheres. J. Appl. Polym. Sci. 43, 2099–2106.