Exenatide-loaded PLGA microspheres with improved glycemic control: *In vitro* bioactivity and *in vivo* pharmacokinetic profiles after subcutaneous administration to SD rats

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**A R T I C L E   I N F O**

Article history:
Received 16 January 2013
Received in revised form 5 June 2013
Accepted 5 June 2013
Available online 14 June 2013

Keywords:
Exenatide
PLGA microspheres
Glycemic control
Subcutaneous administration
Pharmacokinetic profile

**A B S T R A C T**

A subcutaneous exenatide delivery system was developed and characterized *in vitro* and *in vivo*. The results clearly showed that the exenatide loaded PLGA microspheres prepared by using a non-aqueous processing medium had low burst release and high drug encapsulation efficiency. Exenatide loaded in the microspheres preserved its bioactivity. The pharmacokinetics parameters were determined after subcutaneous administration of microspheres to SD rats. The plasma concentration of the single dose of the sustained-release microspheres attained $C_{\text{max}}$ of 108.19 ± 14.92 ng/ml at $t_{\text{max}}$ of 1.33 ± 0.58 h and the $t_{1/2}$ was 120.65 ± 44.18 h. There was a linear correlation between the *in vitro* and *in vivo* release behavior ($R^2 = 0.888$). Exenatide loaded microspheres may prove to have great potential for clinical use.

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1. Introduction

Diabetes mellitus (DM) is a metabolic disorder caused by a relative or absolute deficiency of insulin produced by β-cells of the pancreas. According to WHO estimates, more than 180 million people worldwide have DM, and this number is likely to be more than double by 2030 without intervention [21]. The number of diabetic patients in China is estimated to increase from 20.8 million in 2000 to 42.3 million in 2030, which means that China is one of the countries with largest number of people suffering from DM [15]. Etiologically, DM is classified as type 1 or insulin-dependent DM (IDDM), type 2 or non-insulin-dependent DM (NIDDM), and other specific type and gestational DM [10]. NIDDM is the most common disease among all DM types, accounting for approximately 90% of all diabetic patients.

Exenatide (synthetic exendin-4) is a 39-amino acid peptide isolated from salivary secretions of *Heloderma horridum* lizard venom and is mechanistically similar to glucagon-like peptide-1 (GLP-1). It shares a sequence homology of approximately 53% with the mammalian GLP-1 and is a highly potent GLP-1 receptor (GLP-1R) agonist. They have similar glucoregulatory effects [5,9].

Exenatide is currently available in the market (Byetta™) as twice-daily subcutaneous injection (5 or 10 µg/day). To reduce pain, it is necessary to develop a sustained released drug delivery system for exenatide. In addition, according to the report by Drucker et al. [4], exenatide once weekly provides better glycemic control and is more convenient than the current twice-daily regimen.

Yang et al. [17] prepared polysaccharide microspheres (PAMs) from acetylated pullulan using a water-in-oil-in-water (w/o/w) double emulsion method. Exenatide was utilized as a model peptide to demonstrate the potential of PAMs. As a control, PLGA microspheres (RG 502H, 8000 Da) were also prepared by the same method and compared with PAMs. As a result, the loading efficiency of PLGA microspheres was lower than that of PAMs, and the burst release was much higher. However, PA has not received FDA approval and could not be applied clinically at present.

This paper describes the study of preparation of sustained delivery of exenatide loaded microspheres by using a non-aqueous processing medium to ensure low burst release and high efficiency. Factors influencing the encapsulation efficiency were investigated to obtain an optimized prescription. The *in vitro* and *in vivo* release performance of the microspheres was also evaluated. The pharmacokinetics of exenatide loaded...
microspheres after subcutaneous administration was studied in SD rats [2,3,12].

2. Experiment

2.1. Materials

Exenatide was obtained from Shuangcheng Pharmaceutical Co. (Hainan, China). PLGA, 50/50 with Mw12 kDa (RG502H), Mw34 kDa (RG503H) and Mw65 kDa (RG504H) was purchased from Boehringer Ingelheim (Germany). PLGA, 75/25 with Mw12 kDa was purchased from Lakeshore Biomaterials (USA). Silicone oil was purchased from Dow corning (USA). HPLC grade acetonitrile was purchased from J&K Chemicals (China). Dichloromethane (DCM) and acetone were of analysis grade (Sinopharm Chemical Reagent Co., Ltd., China). HEK293 cell lines were purchased from NIH (USA).

2.2. Preparation of exenatide-loaded PLGA microspheres

Exenatide loaded PLGA microspheres were prepared using a water-in-oil-in-oil (w/o/o) emulsion solvent extraction method described by Deborah et al. [10] and Wu et al. [16] with minor modifications. The procedures are as follows: 120 µl (10 mg) exenatide solution was emulsified with 2 ml DCM solution by dissolving PLGA to produce primary emulsion (w/o). The primary emulsification was performed using a homogenizer (Polytron® PT-MR3100 Kinematica Switzerland) at 20,000 rpm for 60 s. Then, 4 ml silicon oil was added to the primary emulsion and homogenized at 20,000 rpm for 2 min to produce a w/o/o double emulsion. The resultant emulsion was immediately transferred into 46.6 mg heptanes or mixture of heptanes and ethanol (3:1). The formed microspheres were hardened by extracting additional DCM from them. Then, solid microspheres were harvested by centrifuging brachyly and washed with fresh heptanes 3 times before lyophilizing for 48 h.

2.3. Particle size and surface morphology

The particle size and size distribution of exenatide-loaded microspheres were analyzed using a Master-sizer 2000 laser particle analyzer (Malvern Instruments Ltd., Malvern, UK). The microspheres were dispersed by bath sonication for 1 min in deionized water containing a surfactant (0.1% liquefied detergent) to prevent suspension and aggregation before examination. Samples were analyzed in triplicate.

Polydispersity was determined by the SPAN factor expressed as follows:

\[
SPAN = \frac{D_{v(90)} - D_{v(10)}}{D_{v(50)}}
\]

where \(D_{v(90)}\), \(D_{v(10)}\) and \(D_{v(50)}\) are volume size diameters at 90%, 10% and 50% of the cumulative volume, respectively.

The surface morphology of microspheres containing exenatide was observed by scanning electron microscopy (TSEM136MM, Tescan, Czech Republic). For shape and surface analysis, the freeze-dried microspheres were mounted onto the aluminum stub using a double-sided adhesive tape and then sputter coated with a thin layer of gold under argon atmosphere (K750, Emitech, UK) before examination. The coated specimen was then examined under the microscope at an acceleration voltage of 2.0 kV and photographed.

2.4. Drug encapsulation efficiency

The amount of exenatide loaded into PLGA microspheres was determined by the method described by Yin et al. [19] as follows: 10 mg microspheres were dissolved in 1 ml mixed organic solvent (DCM/acetone = 7/3, v/v), vortexed for 30 s and then centrifuged at 15,000 rpm for 5 min. The supernatant was removed, and fresh mixed organic solvent (DCM/acetone = 3/1, v/v) was added, followed by vortex and centrifugation as before. This washing procedure was repeated three times. Finally, the residual organic solvent was dried in a vacuum drying oven (Binder Co., Germany) for 10 min. The residue was dissolved in 1 ml phosphate-buffered saline (pH 7.4) and the exenatide was quantitated using HPLC.

The L-2000 HPLC system (Hitachi, Japan) was comprised of a quaternary pump L-2130, an auto-sampler L-2200, a UV-vis detector L-2420 and a column oven L-2300. A reverse phase C18 column (Welch Materials, 250 mm × 4.6 mm, 5 μm, 300A) was used as stationary phase. The mobile phase was comprised of acetonitrile (A) (A, 0–20 min: 27% → 43%, 20–20.1 min: 43% → 27%, 20.1–28 min: 27%) and 0.05 M KH2PO4 solution (pH = 4.0, B) at the flow rate of 1.0 ml/min. The column temperature was maintained at 25 °C. The detection wavelength was 220 nm. Sample solution was injected at a volume of 20 μl. A calibration curve was prepared in the exenatide concentration range of 30–1000 μg/ml (\(R^2 = 1\)). The result was expressed as the mean ± SD of three experiments. The encapsulation efficiency (%) was calculated using the following formula: encapsulation efficiency (%) = actual loading/theoretical loading × 100%.

2.5. Drug release study

2.5.1. In vitro release study

The in vitro release of exenatide from the microspheres was studied as follows: 10 mg microspheres were placed in an Eppendorf tube containing 1 ml buffer (10 mM HEPES, 100 mM NaCl, pH 7.4) [13] and incubated under mild stirring (100 rpm) at 37 °C. The release medium was removed at each time point after centrifugation at 10,000 rpm for 5 min and the residue was washed with fresh release buffer. The residual microspheres were lyophilized for 24 h, the dried residual microspheres were processed, and the exenatide concentration was determined. The experiments were run in triplicate for each batch of microspheres.

2.5.2. In vivo release study

In vivo release study was performed in male SD rats weighing approximately 200 g (Siluake Co., Shanghai, China). The animals were housed (n = 3 per cage) and kept under controlled conditions of 12:12 h light: dark cycle, 22 ± 2 °C and 50 ± 15% RH. Food and water were provided ad libitum. The animal care and handling were conducted according to the guidelines issued by the Chinese Academy of Sciences.

Exenatide loaded microspheres were suspended in sterile PBS (the dose of peptide was 2 mg/kg) [11] and administered by subcutaneous injection. Blood samples were collected by retro-orbital bleeding into EDTA anticoagulated tubes at different time intervals for 20 days, transferred into 1.5 ml centrifuge tubes, and centrifuged at 15,000 rpm for 5 min. The supernatant was stored at −20 °C until use. The plasma concentration of exenatide was determined using the exendin-4 EIA kit (EK-070-94, Phoenix pharmaceuticals, USA). According to Ref. [20], the dose that we used in the control group of this study was 100 μg/kg.

2.5.3. IVIV-correlation

The data generated in the in vitro release study and in vivo evaluation of microspheres formulations were used to develop the IVIV-correlation (IVIVC). The level of correlation A was studied according to the FDA regulations. The relationship between
percent in vitro dissolution and the fraction of drug absorbed in vivo (Fv) was examined. The Fv was calculated according to Eq.:

$$\frac{C_t k \int_0^t C_i \cdot dt}{k \int_0^t C_i \cdot dt} \times 100$$

Linear regression analysis was applied to the IVIVC plots. The values of correlation coefficient ($R^2$), slope and intercept were calculated respectively.

2.6. Biological activity of encapsulated and not-released exenatide

The bioactivity of exenatide-loaded microspheres was evaluated. Exenatide was extracted from the original and released microspheres using the same method as described in the determination of drug loading efficiency.

FLAG–GLP-1R/CRE plasmid stably expressing HEK 293 cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with penicillin, streptomycin, 400 μg/μl G418 and 10% fetal bovine serum. Cells were plated in 96-well white, clear-bottom plates (Corning) at $2 \times 10^4$ cells/well in 200 μl medium and incubated at 37°C overnight, followed by removing the medium and adding 100 μl medium/well containing exenatide of different concentrations without blood serum. The specimens were incubated at 37°C for 4–5 h, followed by removing the medium. The luminescence signal was measured immediately using Luciferase Assay System (Promega, USA) to evaluate the effect of exenatide on cells. Z factor ($Z$ factor $= 1 - \frac{3 \cdot (SD_+ + SD_-)}{(M_+ - M_-)}$) was calculated according to the control. If $Z$ factor was > 0.5, the data of this plate were used. Softmax Xlfit was used to calculate LgeEC50 and it was requested that $R^2$ be $>$0.95.

2.7. Pharmacokinetic study

Pharmacokinetic profiling was conducted in normal SD rats as described in the in vivo release study. The relevant pharmacokinetic parameters, $C_{max}$, $t_{max}$, $AUC_{0-24}$, $AUC_{0-w}$, $k_{pl}$ and $t_{1/2}$ were calculated based on the reported method from Ref. [8].

2.8. Relative bioavailability [7]

The mean plasma level of exenatide after subcutaneous administration of a single dose of exenatide microspheres was determined using exendin-4 EIA kit and compared with that of the dried power commercially available. The relative bioavailability was calculated by comparing the SQ (subcutaneously) exenatide microspheres to the SQ exenatide of unencapsulated exenatide according to the following equation:

relative bioavailability $= \frac{[AUC]_A/dose_A}{[AUC]_B/dose_B} \times 100$

A represents the exenatide microspheres and B represents the unencapsulated exenatide.

![Fig. 1. Peptide absorbed on microspheres co-incubated with blank PLGA microspheres of different polymer molecular weight and composition. *P < 0.05, compared with the 65,000 (50:50) PLGA group.](image)

2.9. Statistical analysis

All analyses were carried out in triplicate ($n = 3$) and were presented as mean ± SD. Statistical analysis was performed using Student’s t-test and differences were judged to be significant at $P < 0.05$.

3. Results

3.1. Characterization of the microspheres

The data of loading efficiency and encapsulation efficiency are listed in Table 1, which presented that the microspheres prepared by w/o/w method had higher encapsulation efficiency than those prepared by w/o/w method. Here we studied the effects of polymer composition and molecular weight on microsphere encapsulation efficiency. The microsphere encapsulation efficiency increased from 72.9% ± 4.9% to 98.0% ± 9.4% with PLGA molecular weight increasing from 12 kDa to 65 kDa. As shown in Fig. 1, the amount of exenatide co-incubated with blank PLGA microspheres was significantly increased with the molecular weight increasing. Meanwhile, the encapsulation efficiency of PLGA 65 kDa (75:25) was significantly lower than that of PLGA 65 kDa (50:50).

The volume of external phase (silicon oil) was very important for encapsulation efficiency and particle size. From Table 2, we can see when the volume of silicon oil increased from 2 ml to 4 ml, the encapsulation efficiency increased from 64% ± 3% to 98% ± 9%, and the particle size also increased from 34 μm to 51 μm.

The hardening solvent played an important role in the morphology of microspheres. The microspheres shown in Fig. 2 looked not very round. The microspheres adhered together when the mixture of heptane and ethanol was used as the hardening solvent. Compared to Fig. 2, the microspheres in Fig. 3, which prepared with heptane as the only hardening solvent, were of a very round form with smooth surface and good dispersion.

<table>
<thead>
<tr>
<th>Molecular weight (Da)</th>
<th>Copolymer composition</th>
<th>$w/o/w$</th>
<th>$w/o/o$</th>
</tr>
</thead>
<tbody>
<tr>
<td>12,000</td>
<td>50:50</td>
<td>55.7 ± 5.9</td>
<td>2.58 ± 0.41</td>
</tr>
<tr>
<td>34,000</td>
<td>50:50</td>
<td>61.2 ± 6.7</td>
<td>2.84 ± 0.39</td>
</tr>
<tr>
<td>65,000</td>
<td>50:50</td>
<td>65.3 ± 3.8</td>
<td>3.03 ± 0.25</td>
</tr>
<tr>
<td>65,000</td>
<td>75:25</td>
<td>88.1 ± 3.8</td>
<td>4.08 ± 0.26</td>
</tr>
</tbody>
</table>
Table 2
Effect of silicon–oil volume on encapsulation efficiency and mean diameter of PLGA microspheres.

<table>
<thead>
<tr>
<th>Stirring rate (rpm)</th>
<th>Cexenatide (mg/ml)</th>
<th>CPLGA (mg/ml)</th>
<th>External volume (ml)</th>
<th>Mean particle size (μm)</th>
<th>Encapsulation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>83.3</td>
<td>100</td>
<td>2</td>
<td>34</td>
<td>64 ± 3</td>
</tr>
<tr>
<td>400</td>
<td>83.3</td>
<td>100</td>
<td>4</td>
<td>51</td>
<td>98 ± 9</td>
</tr>
</tbody>
</table>

Fig. 2. Morphology of exenatide loaded microspheres using heptane and ethanol as harden solvent observed by scanning electron microscope.

Fig. 3. Morphology of exenatide loaded microspheres using heptane only as harden solvent observed by scanning electron microscope.

Table 3
Effect of stirring rate on encapsulation efficiency and size distribution.

<table>
<thead>
<tr>
<th>No.</th>
<th>Stirring rate (rpm)</th>
<th>D [4,3]</th>
<th>d (0.1)</th>
<th>d (0.5)</th>
<th>d (0.9)</th>
<th>Encapsulation efficiency (%)</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>62</td>
<td>17</td>
<td>48</td>
<td>109</td>
<td>90 ± 4</td>
<td>1.90</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>51</td>
<td>16</td>
<td>42</td>
<td>87</td>
<td>98 ± 9</td>
<td>1.64</td>
</tr>
<tr>
<td>3</td>
<td>600</td>
<td>45</td>
<td>15</td>
<td>42</td>
<td>81</td>
<td>96 ± 8</td>
<td>1.56</td>
</tr>
</tbody>
</table>

Fig. 4. The particle size distribution of microspheres formed at 600 rpm.
We investigated the impacts of the stirring rate on encapsulation efficiency and size distribution. The span and encapsulation efficiency at a stirring rate of 200 rpm, 400 rpm or 600 rpm were shown in Table 3. When the stirring rate increased from 200 rpm to 400 rpm, the encapsulation efficiency increased from 90% ± 4% to 98% ± 9% and the span decreased from 1.90 to 1.64. When the stirring rate increased from 400 rpm to 600 rpm, the span decreased continuously from 1.64 to 1.56 while the encapsulation efficiency decreased slightly from 98% ± 9% to 96% ± 8%. We therefore used the stirring rate of 600 rpm to ensure high encapsulation efficiency and narrow size distribution. The particle size of the microspheres prepared under 600 rpm presented a normal distribution as shown in Fig. 4.

3.2. In vitro release and bioactivity assay

The in vitro release profiles of the exenatide loaded microspheres in 4 weeks were illustrated in Fig. 5. The PLGA molecular weight has great impacts on the exenatide release. Drug release from microspheres with PLGA 12 kDa was relatively rapid, with approximately 25% of the encapsulated drug released within 24 h. As the molecular weight increases from 12 kDa (50:50) to 65 kDa (50:50), the release amounts at each time point showed a downward trend. The hydrophilic/lipophilic ratio of the polymer could also affect the drug release behavior. The exenatide in microspheres prepared with PLGA (65 kDa, 75:25) released more slowly than that in the microspheres prepared with PLGA (65 kDa, 50:50).

![Fig. 5. In vitro release profiles of exenatide loaded PLGA microspheres made from different molecular weights and different copolymer compositions in pH 7.4 HEPES buffer.](image)

The bioactivity of exenatide in microspheres was evaluated to ensure the drug could keep glycemic control ability after being loaded in microspheres. Fig. 6 showed the lgEC50 value of the exenatide loaded in the microspheres, where the free exenatide was −9.473 and −9.473 respectively. The ratio of lgEC50 (loaded)/lgEC50 (free) was 0.99, indicating that exenatide in the microspheres still preserved its bioactivity.

The bioactivity values of exenatide released in the buffer at 1st, 4th, 7th, 14th, 21st and 28th day were also determined. The lgEC50
The pharmacokinetics parameters of exenatide aqueous solution and microspheres after subcutaneous injection in SD rats are listed in Table 4. The mean plasma levels of exenatide after subcutaneous administration of exenatide aqueous solution and microspheres were shown in Fig. 7. As shown in Table 4 and Fig. 7(A), the absorption and elimination of exenatide aqueous solution in vivo were rapid, and exenatide existed for 2 h with C_max of 633.92 ± 121.15 ng/ml at t_max of 0.28 ± 0.21 h. In contrast, the absorption and elimination of exenatide microspheres in vivo were very slow. The plasma concentration of the single dose of the sustained-release microspheres was 100.650 ± 44.184 h, which was about 120 fold that of exenatide aqueous solution injection (Fig. 8).

The mean relative bioavailability (RB) of the microspheres was calculated through determining the area under the plasma exenatide curves (AUC) using the trapezoidal rule. In our study, the estimated absolute bioavailability of exenatide microspheres was 37.861%.

### Table 4
Pharmacokinetics parameters of exenatide aqueous solution and microspheres after subcutaneous injection in SD rats.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Exenatide water solution</th>
<th>Exenatide microspheres</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{0-24} (ng h/ml)</td>
<td>468.863 ± 155.694</td>
<td>–</td>
</tr>
<tr>
<td>AUC_{0-∞} (ng h/ml)</td>
<td>469.950 ± 155.225</td>
<td>2814.727 ± 558.732</td>
</tr>
<tr>
<td>AUMC_{0-∞} (ng h/ml)</td>
<td>313.369 ± 116.481</td>
<td>947151.150 ± 649846.277</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>0.662 ± 0.039</td>
<td>248.867 ± 89.349</td>
</tr>
<tr>
<td>Cl or Cl/F (l/(h kg))</td>
<td>0.045 ± 0.013</td>
<td>0.463 ± 0.584</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>1.055 ± 0.042</td>
<td>120.650 ± 44.184</td>
</tr>
<tr>
<td>λ (h⁻¹)</td>
<td>0.657 ± 0.026</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>t_{max} (h)</td>
<td>0.278 ± 0.210</td>
<td>1.333 ± 0.577</td>
</tr>
<tr>
<td>C_{max} (ng/ml)</td>
<td>633.917 ± 121.148</td>
<td>108.193 ± 14.919</td>
</tr>
<tr>
<td>F</td>
<td>–</td>
<td>37.861</td>
</tr>
</tbody>
</table>

value was −9.801, −9.851, −8.786, −9.550, −9.384 and −9.798, and the corresponding ratio of IgEC50(loaed)/IgEC50/(free) was 0.995, 1.000, 0.892, 0.970, 0.953 and 0.995, respectively. The microspheres protected exenatide against degradation in the release buffer.

### 3.3. In vivo pharmacokinetic study

The pharmacokinetics parameters are listed in Table 4. The mean plasma levels of exenatide after subcutaneous administration of exenatide aqueous solution and microspheres were shown in Fig. 7. As shown in Table 4 and Fig. 7(A), the absorption and elimination of exenatide aqueous solution in vivo were rapid, and exenatide existed for 2 h with C_max of 633.92 ± 121.15 ng/ml at t_max of 0.28 ± 0.21 h. In contrast, the absorption and elimination of exenatide microspheres in vivo were very slow. The plasma concentration of the single dose of the sustained-release microspheres was 108.19 ± 44.184 h, which was about 120 fold that of exenatide aqueous solution injection (Fig. 8).

The mean relative bioavailability (RB) of the microspheres was calculated through determining the area under the plasma exenatide curves (AUC) using the trapezoidal rule. In our study, the estimated absolute bioavailability of exenatide microspheres was 37.861%.

### 3.4. IVIVC-correlation

The in vitro–in vivo correlation was assessed using the Wagner–Nelson method [1]. The fraction drug absorbed (Fa) is plotted against the fraction drug released (Fr). IVIVC for exenatide microspheres showed a good correlation coefficient (R² = 0.888).

### 4. Discussion

#### 4.1. The microspheres characteristics

Exenatide in the present study was encapsulated into PLGA using a non-aqueous processing medium as external oil phase to ensure low burst release and high encapsulation efficiency. The non-aqueous processing media are usually those polymer-immiscible solvents, such as petroleum ether and hexane, but they are miscible with organic solvents. The silicon oil used in this study could remove the dichloromethane from the microspheres [12], and could prevent water-soluble protein/peptide from diffusing into the external oil phase for immiscibility. Knowing that the more silicon oil, the larger the viscosity would be, less susceptible to the shearing force would increase the particle size.

The hydrophobicity of the copolymer increased with the PLA/PGA ratio increasing due to the hydrophobic nature of PLA, which reduced the interaction between polymer molecular chains and hydrophilic exenatide, resulting in a lower encapsulation efficiency of PLGA (75:25) as compared with PLGA (50:50) [16] when they were of the same molecular weight.

The difference between the microspheres in Figs. 2 and 3 may be due to the poor miscibility of ethanol and silicone oil. As a result, heptane containing alcohol cannot be completely extracted by silicon oil. As the density of silicon oil is larger than that of ethanol and heptane, the microspheres deposited in the down solvent and adhered to each other, or even into clusters. We therefore used heptane as the only hardening solvent in the subsequent studies.
4.2. In vitro release profiles

The relatively lower burst release shown in Fig. 5 contributes to the usage of the non-aqueous processing medium namely silicon oil. During the process for preparing microspheres using the w/o/w double emulsion method, the water soluble drug such as peptide could easily distribute to the external water phase, so the drug loss is inevitable [18]. Here the external water was replaced by non-aqueous processing media, which is polymer-immiscible but miscible with DCM. In this study, we prepared water-in-oil (w/o) emulsion as the primary emulsion, and then gradually added silicon oil into the primary emulsion to make w/o/w double emulsion. Due to the hydrophobicity of oil, the drug was retarded to diffuse to the external phase (non-aqueous processing media) [2,3,12]. Thus, there was very little drug staying on the surface of microspheres, which resulted in low initial burst release [18].

On the other hand, the solidification type and rate may affect the burst release. In the w/o/w double emulsion method, microspheres were solidified through the organic solvent volatilizing or diffusing to the external aqueous solution. The organic solvent was immiscible with the aqueous solution, so removal of the organic solvent was difficult. As a result, the solidification was very slow. During that process, the formed water channels connecting internal and external aqueous phases were solidified further [16]. The slow solidification rate led to the porous morphology of PLGA microspheres. However, in the modified w/o/o emulsion solvent removal method, the DCM was removed by extraction with adding heptane, and the heptane was miscible with DCM but immiscible with PLGA, which could fasten the extraction of DCM. The extraction of DCM was affected when heptane was added to the embryonic microspheres, and solidification occurred subsequently. As shown in Fig. 3, solidification was faster and the microspheres were with few pores on surface, which was benefit for the control of drug burst release.

Other than molecular weight, the degradation behavior of microspheres also depended on the hydrophilic/lipophilic ratio of the polymer. The more hydrophilic the polymer is, the more rapid its degradation would be. Lactic acid is more hydrophilic than glycolic acid, which makes lactide-rich PLGA copolymers more hydrophilic, and subsequently slows down the degradation process. Therefore, exenatide microspheres prepared with 75:25 PLGA showed slower release than 50:50 PLGA (80% vs. 90% release of the total drug in 28 days). We chose PLGA (65 kDa, 50:50) as the carrier in the following in vivo release study.

4.3. In vivo pharmacokinetics

The profile of the plasma exenatide concentration vs. time obtained from the in vivo study clearly showed that the microspheres were able to maintain a constant therapeutic drug level within therapeutic range over 20-day duration.

A comparison of the drug plasma concentration between Fig. 7(A) and (B) showed the feasibility of sustained-release microsphere formulation in achieving in vivo long-action by a single dose [6]. PLGA normally degrades faster in vivo due to non-specific enzymatic catalysis [14].

The mean relative bioavailability (RB) of the microspheres was a critical factor to evaluate a preparation. The RB of the microspheres in our study was not so good, and the reason may be that the exenatide loaded in microspheres had not released completely in vivo in 20 days.

4.4. IVIV-correlation

The good correlation coefficient ($R^2 = 0.888$) for exenatide microspheres indicated us that the microspheres presented similar release profile in vitro and in vivo. It also told us the in vitro release determination method was rational, which could be reused to evaluate the exenatide loading microspheres in future.

5. Conclusion

This modified w/o/o emulsion solvent removal method offers a novel approach to obtain microspheres with high protein encapsulation efficiency and low burst release, mainly by using oil as external phase instead of aqueous solution. The exenatide preserves its bioactivity in vitro and in vivo after the encapsulation process. Further pharmacodynamic study is needed in diabetic rats.

Acknowledgement

We thank for the financial support from the National Major Scientific and Technological Special Project for “Significant New Drugs Development” 2010ZX09009-001.

References


