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Effect of size on the *in vitro/in vivo* drug release and degradation of exenatide-loaded PLGA microspheres

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ABSTRACT

The aim of this study was to investigate the effect of particle size on the *in vitro/in vivo* drug release and degradation of macromolecule-loaded PLGA microspheres. The active pharmaceutical ingredient exenatide was encapsulated into PLGA microspheres with sizes of 3.80 μm and 18.15 μm, and the *in vitro/in vivo* drug release and degradation kinetics of microspheres were studied. Small microspheres (3.80 μm) exhibited a higher initial drug release followed by a slower long-term drug release rate ($Slope_{4–49day}$ = 0.81) compared with large microspheres (18.15 μm, $Slope_{4–49day}$ = 1.61). The rapid drug release rate of large microspheres from day 4 to day 28 was attributed to the rapid degradation of PLGA in large microspheres *in vitro*. After subcutaneous injection into rats, small microspheres released 72% of drug after 4-day administration and released the remaining drug completely after 21 days. Large microspheres showed a slower initial drug release followed by a more rapid drug release in comparison with small microspheres. The high burst release of small microspheres may induce side effects while slow release at late stage may be therapeutically ineffective. In conclusion, it was essential to control the fraction of small microspheres in microsphere formulations to obtain desired drug release behavior.

1. Introduction

Poly (D,L-lactide-coglycolide) (PLGA) microspheres have been widely used as sustained-release drug delivery systems for a variety of drugs, including peptides [1–3], proteins [4,5] and small drug molecules [6–8], due to their excellent biocompatibility and tailorable drug release profiles [9]. Drug release behavior is a key factor when developing PLGA microspheres, and it has been studied in many articles [6,7,10]. It was reported that many factors, such as particle size, molecular weight of PLGA, morphology, porosity of microspheres, additives, and the solubility of the active ingredient, can affect drug release from PLGA microspheres [11]. These factors have been shown to have a significant impact on the drug release behavior of PLGA microspheres via influencing the *in vitro* and *in vivo* processes of drug diffusion and polymer erosion.

A great number of studies have demonstrated that the particle size of PLGA microspheres had significant effects on the drug release from microspheres by affecting the surface area, porosity, and drug distribution. For example, Chen, et al. reported that gefitinib-loaded PLGA microspheres with a relatively small size (~20 μm and 20–50 μm) exhibited extremely rapid drug release, and drug release was completed within 7 days. Large microspheres (50–100 μm and > 100 μm) exhibited a much slower drug release during the first 13 days followed by an accelerated drug release up to 90 days [6]. In addition, the *in vitro* polymer degradation of the large microspheres was faster than that of the small microspheres. This remarkable difference of drug release performance between the small and large microspheres was attributed to the much shorter diffusion path and higher water uptake of small microspheres in comparison with large microspheres. In another report, small microspheres (10 and 20 μm) also showed faster drug release rate and shorter drug release time period compared with large microspheres (40 and 50 μm) [12]. Such influence of particle size on drug release from PLGA microspheres have been attributed to several factors including specific surface area, drug distribution of hydrophobic drug (i.e. piroxicam, < 100 mg/ml) in microspheres, and polymer degradation rate [13]. Interestingly, the *in vitro* drug release from 5-fluorouracil-loaded PLGA microspheres decreased with decreasing particle size in the range of 36–125 μm [14]. This was mainly due to large microspheres exhibiting a higher drug loading capacity, resulting in a more porous structure and higher drug diffusion rate compared with small microspheres. Similarly, a decreased drug release from the risperidone-loaded PLGA microspheres was also observed by reducing the particles

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size from ~19 μm to ~6 μm, which was caused by the decreased penetration of the medium into the interior of small microspheres due to the dense interior structure found with small microspheres [15]. These results suggested that the effect of particle size on the drug release from PLGA microspheres was diverse. Therefore, in order to achieve a desired drug release performance, it was important to control the particle size of PLGA microspheres.

In previous studies, the main focus has been on the relationship between particle size and the *in vitro* drug release or degradation of PLGA microspheres. *In vivo* drug release and degradation of PLGA microspheres with different particle size, however, have been rarely studied. The *in vivo* drug release and degradation of microspheres could be affected by many factors, such as injection sites, foreign response, properties of microspheres, and enzymes [16]. These important factors can easily be ignored during *in vitro* studies when assessing the effect of particle size on drug release from microspheres, possibly leading to inaccurate remarks regarding the particle size influence. For instance, the *in vivo* foreign body reaction can be strongly influenced by the particle size of microspheres, resulting in accelerated degradation of microspheres [17–19]. Therefore, the aim of the present study was to investigate the effect of particle size on the *in vitro/in vivo* drug release behavior and degradation of the drug-loaded PLGA microspheres.

In this study, exenatide, a peptide consisting of 39 amino acids was selected as the model macromolecule. Exenatide shares about 53% homology with mammalian gut hormone and has been approved for the treatment for Type II diabetes [20,21] by the FDA. Due to the short half-life time of the conventional subcutaneous exenatide injection, long-acting exenatide-loaded microspheres (Bydureon®) were developed and approved by the FDA for once-weekly treatment for type II diabetes [22]. However, since the particle size of Bydureon® is up to ~50 μm, a 23 gauge needle has to be used for the administration of Bydureon®, which can cause severe pain during the injection. Consequently, many studies have been carried out to prepare exenatide-loaded microspheres with particle sizes smaller than 50 μm [1,23,24]. Exenatide was selected to be used as the model drug not only because it is a typical macromolecule, but also the results of this paper may contribute to the formulation development of exenatide-loaded PLGA microspheres. In this paper, two types of exenatide-loaded microspheres with particle sizes of ~20 μm and ~5 μm were prepared using the SPG (Shirasu Porous Glass) membrane emulsification method combined with the double emulsion-solvent evaporation method [23]. In this study, we investigate the effect of particle size on the *in vitro/in vivo* drug release behavior and degradation of macromolecule loaded PLGA microspheres.

2. Material and methods

2.1. Materials

Poly (D,L-lactide-coglycolide) 50:50 copolymers (PLGA, RESOMER® RG 503 H, intrinsic viscosity (ηi) = 0.32–0.44 dl/g in chloroform at 25 °C) was obtained from Evonik Corporation (Birmingham, AL). Poly (vinyl alcohol) (PVA, 87–89% hydrolyzed, average Mw = 72.6–81.4 kDa) was provided by Kuraray CO., LTD. (Osaka, Japan). Exenatide was purchased from Shanxi Tiansen Pharmaceutical Co (Xi'an, China). Dichloromethane (DCM) and acetonitrile were purchased from Concord Technology CO., LTD. (Tianjin, China). All other reagents were of analytical grade.

2.2. Preparation of exenatide-microspheres with different size

Briefly, 400 mg PLGA was dissolved in 4 ml DCM to obtain the organic phase (O). The organic phase was then mixed with 0.4 ml of exenatide aqueous solution (5%, w/v, W_1) under high shearing (Ultra Turrax T18 basic, IKA, Germany) at 12000 rpm for 2 min to prepare W_1/O emulsion. The W_1/O emulsion was then mixed with 15 ml of external aqueous phase (W_2) containing 1% PVA (w/v) and 1% NaCl (w/v) under high shearing at 3000 rpm for 10 s to prepare a coarse $W_1/O/W_2$ emulsion. The obtained coarse emulsion was immediately poured into a membrane emulsification device ((Mini kit, SPG TECHNOLOGY CO., Ltd, Japan) and forced to pass through a Shirasu porous glass (SPG) membrane with the pore size of 10.0 μm and 40.1 μm, respectively, under the pressure of N_2 to obtain final emulsion A and B.

The obtained emulsion A and B were all diluted by 50 ml of 1% NaCl (w/v) aqueous solution instantly. The organic solvent was evaporated via vacuum-rotary evaporation at 40 °C for 15 min. The microspheres A (termed as small MS) and B (termed as large MS) were collected by centrifugation, and washed with distilled water for three times, and then freeze-dried to obtain the final microspheres.

2.3. Characterization of exenatide-microspheres

Characterization studies of exenatide-microspheres included the measurement of particle size distribution, morphology observation and determination of molecular weight of PLGA in microspheres.

2.3.1. The particle size distribution of exenatide-microspheres

Exenatide-microspheres were dispersed uniformly in distilled water. The particle size distribution was measured using a laser diffraction particle analyzer (BT-9300S, Dandong Baite Instrument Co., Ltd., China). Span was used to evaluate the uniformity of the microspheres.

$$\text{Span} = (d_{90} - d_{10}) / d_{50} \quad (1)$$

Where d_{90}, d_{50} and d_{10} represents the maximum particle diameters below 90%, 50% and 10% of the sample volume exists, respectively.

2.3.2. The surface and cross section morphology study of exenatide-microspheres

The surface and cross section morphology study of the exenatide-microspheres was carried out using scanning electron microscopy (SEM) (Hitachi SU8010, Japan). The cross-sections of microspheres were obtained after cutting with a razor blade. Exenatide-microspheres and the cross-sections were spread on a double-side conductive adhesive tape attached on the copper stub. The samples were covered with gold layer and observed using SEM.

2.3.3. Determination of loading efficiency (LE) and encapsulation efficiency (EE)

Exenatide-microspheres (10 mg) were added into 1 ml acetonitrile and vortexed for 30 min. The suspension was then centrifuged at 12000 rpm for 10 min. The supernatant was discarded. The residue was dried at 40 °C, and was reconstituted in 2 ml distilled water. The concentration of exenatide was determined by HPLC equipped with a UV detector at 214 nm. The analysis was performed on a TSK-Gel G2000SWXL column (300 mm × 7.8 mm, i.d., 5 μm) using 1.89% (w/v) Na_2SO_4 aqueous solution-acetonitrile-trifluoroacetic acid (15:5:0.02, v/v/v) as mobile phase at a flow rate of 0.8 ml min $^{< sup > -1 < /sup >}$. Loading efficiency (LE) was calculated from the mass of exenatide in microspheres versus the mass of microspheres. Encapsulation efficiency (EE) was calculated from the measured LE versus the theoretical LE.

2.4. In vitro drug release and degradation studies

2.4.1. In vitro drug release studies

About 20 mg exenatide-microspheres were dispersed in 2 ml 10 mM PBS solution (pH 7.4, containing 0.02% NaN_3 and 0.02% polysorbate 80) in EP tube. All samples were incubated at 37 °C in a reciprocal shaking water bath (ZWY-110X30, Zhicheng Inc., China). At each predetermined time point (1, 4, 7, 10, 14, 21, 28, 35, 42, 49, 56 and 63 days post-incubation), all microspheres were collected by centrifugation at 12000 rpm for 10 min. The collected microspheres were washed

0.2% exenatide loading

using distilled water for three times, and freeze-dried.

The remaining drug in the freeze-dried microspheres was analyzed using HPLC method which was described in the section of *2.3.3*. The *in vitro* cumulative drug release was calculated using the following equation:

$$\text{Cumulative drug release (\%)} = (M_0 - M_t)/M_0 \times 100\% \quad (2)$$

Where M_0 represents the mass of the drug encapsulated in the microspheres before incubation, and M_t represents the mass of the drug encapsulated in the microspheres at t days post-incubation.

2.4.2. In vitro drug degradation studies

2.4.2.1. Mass change of microspheres. During *in vitro* drug release, the weight of the freeze-dried microspheres (W_t) at each predetermined time point (1, 4, 7, 10, 14, 21 and 28 days post-incubation) was recorded. The mass remaining (%) was calculated as:

$$\text{Mass remaining (\%)} = W_t/W_0 \times 100\% \quad (3)$$

Where W_t is the weight of the remaining microspheres at t days post-incubation, W_0 is the initial weight of the microspheres before incubation.

2.4.2.2. Molecular weight (Mw) of PLGA in microspheres. During *in vitro* drug release, about 4 mg freeze-dried exenatide-microspheres at each predetermined time point (1, 4, 7, 10, 14, 21 and 28 days post-incubation) was dissolved in 2 ml N,*N*-dimethyl formamide (DMF). The molecular weight (Mw) of PLGA in the microspheres was analyzed using gel permeation chromatography (GPC, Waters 1515, Waters Corp., USA) equipped with Waters 2414 refractive index detector. The sample was eluted using DMF as mobile phase at a flow rate of 1.0 ml min $^{< sup > -1 < /sup >}$. The Mw of PLGA was calculated using polystyrene as standards.

2.4.2.3. Glass transition temperature (T_g) of PLGA in microspheres. During *in vitro* drug release, the glass transition temperature (T_g) of PLGA freeze-dried exenatide-microspheres at each predetermined time point (1, 4, 7, 10, 14, 21 and 28 days post-incubation) was analyzed using differential scanning calorimetry (DSC, Mettler-Toledo DSC822^e, Mettler Toledo Gmbh, Switzerland). The sample was heated from 30 °C to 100 °C at a rate of 20 °C/min to eliminate the thermal history of polymer and then reheated from 30 °C to 100 °C at 5 °C/min in a stream of N_2 gas.

2.4.2.4. Surface morphology of microspheres. During *in vitro* drug release, the surface morphology of the freeze-dried exenatide-microspheres at each predetermined time point (1, 4, 7, 10, 14, 21 and 28 days post-incubation) was observed using SEM.

2.5. In vivo drug release and degradation studies

2.5.1. In vivo drug release studies

The protocol of animal experiments in this study was approved by Animal Ethics Committee Shenyang Pharmaceutical University (approval number SYPU-IACUC-C2015-0428-102). The European Community guidelines as accepted principles for the care and use of experimental animals were also adhered. Forty-two female SD rats (180–220 g, Experimental Animal Center of Shenyang Pharmaceutical University, China) were divided into two groups randomly. All the animals were fasted for 12 h with access to water ad libitum prior to administration. The aqueous suspension of microspheres was obtained by dispersing 600 mg of exenatide-microspheres in 15 ml PBS aqueous solution containing 0.5% (w/v) sodium carboxymethyl cellulose and 0.1% (w/v) polysorbate 80. The rats in two groups received a subcutaneous injection of small MS and large MS at a single dose of 5 mg/kg (about 20 mg microsphere), respectively. Three rats in each group were sacrificed at each predetermined time point (1, 4, 7, 10, 14, 21 and 28 days post-injection). The remaining microspheres at the injection site were retrieved, carefully separated from the surrounding tissue, and freeze-dried.

The remaining drug encapsulated in the microspheres was analyzed using HPLC as described in the section of *2.3.3*. The *in vivo* cumulative drug release was calculated from equation (2).

2.5.2. In vivo degradation studies

During *in vivo* drug release studies, the Mw and T_g of PLGA in the remaining microspheres at the injection site were analyzed using GPC and DSC, respectively. The surface morphology of the microspheres at each time point was observed using SEM.

2.6. Pharmacokinetic studies

The pharmacokinetics of exenatide-microspheres with different sizes was assessed after subcutaneous injection. Twelve female SD rats (180–220 g, WeiTong LiHua animal center, Beijing, China) were divided into two groups randomly. All the animals were fasted for 12 h with access to water ad libitum prior to administration. Exenatide-microspheres were suspended in PBS aqueous solution containing 0.5% (w/v) sodium carboxymethyl cellulose and 0.1% (w/v) polysorbate 80. The rats in two groups received a subcutaneous injection of small MS and large MS suspensions at a single dose of 5 mg/kg, respectively. About 0.3 ml of blood was collected via retro-orbital puncture into heparinized tubes at each predetermined time point. Plasma samples were separated by centrifuging the blood samples at 5000 rpm for 10 min, and then stored at −20 °C until analysis. The plasma concentration of exenatide was analyzed using the exendin-4 EIA kit (EK-070-94, Phoenix pharmaceuticals, USA) [23,25]. The main pharmacokinetic parameters were calculated by a non-compartmental method using PK Solver software (version 2.0, China).

3. Results and discussion

3.1. Physicochemical properties of exenatide-microspheres with different particle sizes

In the present study, exenatide-microspheres with different sizes were prepared by a membrane emulsification method using 10.0 μm SPG membrane (small MS) and 40.1 μm SPG membrane (large MS), respectively. As shown in Table 1, the mean particle sizes of small MS and large MS were 3.80 μm and 18.15 μm, respectively. The spans of the two microspheres were both less than 1.5, indicating a uniform particle size distribution. The LE and EE of large MS were both higher than those of small MS. This was likely attributed to the decreased emulsion droplet surface area and increased diffusion path length between W_1 and W_2 in the preparation process of large MS, which allowed fewer exenatide to diffuse from W_1 phase into W_2 phase and thus leaving more drugs encapsulated in the microspheres [26].

As shown in Fig. 1A, two types of microspheres both presented smooth surface. However, the small MS showed a dense interior structure, while the large MS showed porous interior structure (Fig. 1B). This might be due to the different “hardening” kinetics of microspheres during preparation, which was dependent on the particle size [15]. For small MS (3.80 μm), the solvent can diffuse rapidly into

Table 1
Physicochemical properties of exenatide-microspheres with different particle size.

Formulation	Mean size (μm)	Span	LE (%)	EE (%)
Small MS	3.80	1.27	4.53	88.0
Large MS	18.15	1.28	4.89	97.5

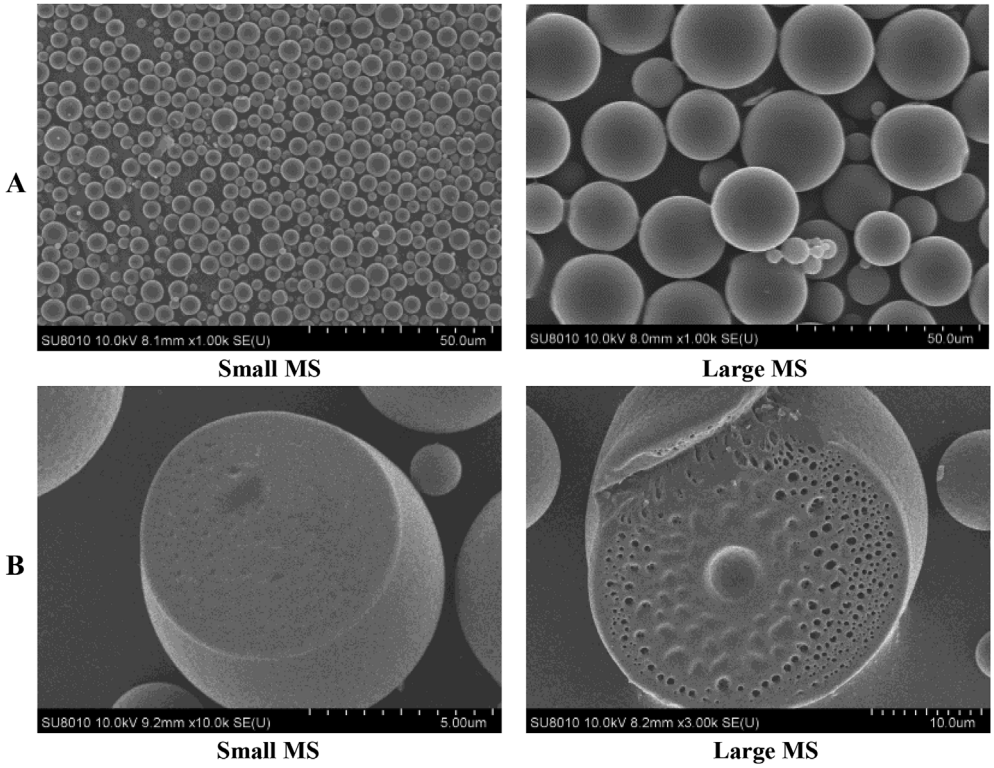

Fig. 1. SEM photographs of surface morphology (A) and SEM micrographs photographs cross sections (B) of exenatide-loaded PLGA microspheres prepared using different sizes of SPG membrane: 10.0 μm SPG membrane (small MS), and 40.1 μm SPG membrane (large MS).

the external aqueous phase, leading to a fast "hardening" procedure, and thus creating a dense interior structure. For large MS (18.15 μm), the organic solvent might diffuse with a longer time period due to the increased diffusion path length and decreased diffusion area compared with small MS, resulting in a slow interior "hardening" of the large MS. This may lead to the generation of pores in the inner part of the large MS.

3.2. *In vitro* drug release of exenatide-microspheres with different sizes

In vitro drug release profiles of small MS and large MS were presented in Fig. 2. As seen in Fig. 2, the two types of microspheres both showed a burst release (defined as the cumulative drug release after one day post-incubation, *ca.* 27% and 23%, respectively) followed by a slow drug release rate from day 1 to day 63. During the first 4 days, small MS exhibited a fast drug release rate in comparison with large MS. After 4 days, the drug release rate of large MS was more rapid than that of small MS. For instance, on day 28, the cumulative drug release from small MS was only 53.79%, whilst the cumulative drug release from large MS was 71.36%. Moreover, as seen in Fig. 2, the small MS showed a slightly longer drug release time period, approximately 63 days, than large MS which released drug continuously for only 49 days.

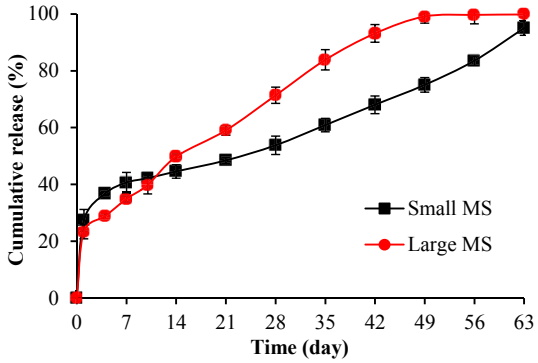

Fig. 2. The *in vitro* drug release profiles of exenatide-microspheres (small MS and large MS) in 10 mM PBS solution (pH 7.4, 0.02% NaN_3) at 37 °C. Each point represents the mean ± SD; n = 3.

In order to evaluate the drug release rate of the two types of microspheres after 4 days, the two release profiles from day 4 to day 49 were fitted using the zero-order model. The two types of microspheres both fitted well to the zero-order model as reflected by the acceptable regression coefficients ($R_{4–49day}$). The regression equations were as follows:

Small MS: $y = 0.81\ x + 33.28$ $R_{4–49day} = 0.9938$

Large MS: $y = 1.61\ x + 24.75$ $R_{4–49day} = 0.9958$

In addition, after 4 days the drug release rate of the small MS was slower than that of large MS, as reflected by the smaller $slope_{4–49day}$ of small MS in comparison with that of large MS.

3.3. *In vitro* degradation of exenatide-microspheres with different sizes

In order to understand the *in vitro* drug release mechanism of PLGA microspheres with different sizes, the effect of particle size on the degradation of PLGA microspheres was investigated. In this study, the remaining microsphere mass, T_g, and Mw of PLGA in microspheres, and the morphology of microspheres at each predetermined time point were studied to assess the degradation process of microspheres with different particle size after incubation in the PBS medium.

3.3.1. *The weight change of exenatide-microspheres*

The remaining mass of small and large MS during the *in vitro* drug release study was shown in Fig. 3A. As seen in Fig. 3A, during the first 7 days after incubation, the weight of small and large MS both showed little change, with the mass loss less than 4%. After 7 days, the mass remaining of small and large MS both decreased slowly. After 28 days,

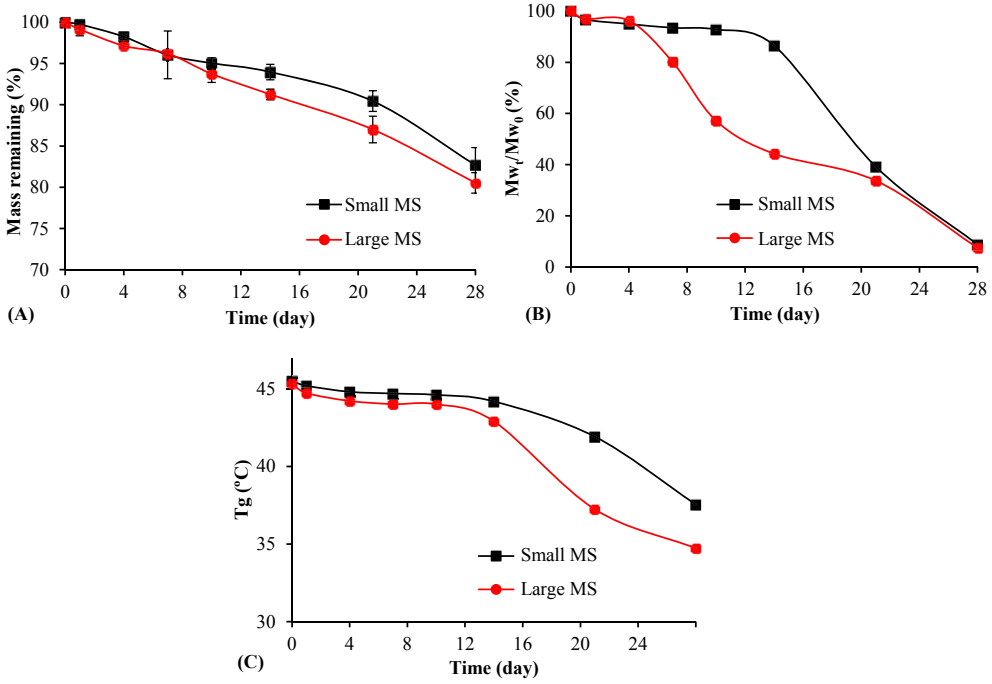

Fig. 3. The *in vitro* degradation profiles of exenatide-microspheres (small MS and large MS) incubated in 10 mM PBS solution (pH 7.4, 0.02% NaN_3) at 37 °C: (A) the mass remaining (%) vs time profiles; (B) the Mw_t/Mw_0 (%, the Mw at each determined time/the Mw at initial time) vs time profiles; (C) the T_g vs time profiles. Each point represents the mean ± SD; n = 3. The.

the remaining mass of small and large MS was 82.7% ± 2.1% and 80.5% ± 1.2%, respectively. During the whole 28 day study, the weight of large MS decreased slightly faster than that of small MS.

3.3.2. Polymer molecular weight (Mw) of exenatide-microspheres

The Mw of PLGA in the exenatide-microspheres at different *in vitro* release times was determined by GPC. Fig. 3B showed the plot of Mw_t/Mw_0 (%, the Mw of PLGA in the microspheres at each predetermined time/the Mw at initial time) against time. For small MS, the Mw of PLGA decreased slowly during the first 10 days, with a Mw_t/Mw_0 of 92.83% on day 10. From day 14 to day 28, PLGA degraded rapidly with a Mw_t/Mw_0 of 8.83% on day 28. Compared with small MS, the Mw of PLGA in large MS showed a greater decrease rate from day 4 to day 14 followed by a slower decrease from day 14 to day 28. These results indicated that the particle size had a significant influence on the degradation rate of microspheres, and the larger the particle size, the faster the microspheres degraded *in vitro*.

3.3.3. Glass transition temperature (Tg) of exenatide-microspheres

It has been reported that the T_g values of polymers are dependent on the molecular weight of polymers [10,27]. Therefore, the T_g of the microspheres were also measured during the *in vitro* test to further demonstrated the degradation of PLGA over the *in vitro* study. As seen in Fig. 3C, from day 1 to day 28, the T_g of small MS decreased from 45.5 °C to 37.5 °C, while T_g of large MS decreased from 45.3 °C to 34.7 °C. Within the first 14 days, the T_g values of small MS showed a slow decreasing rate (from 45.5 °C to 44.19 °C), suggesting a slow degradation rate of PLGA in small MS. After 14 days, small MS showed a fast reduction of T_g, indicating a rapid degradation process of PLGA in small MS. For large MS, the start of the sharp reduction of T_g values was observed on day 10. From day 14 to day 28, the T_g values of large MS decreased by 9.28 °C. These results may indicate that PLGA in large MS degraded faster than that in small MS, which was in consistent with the results of Mw of PLGA study.

3.3.4. Morphological changes of exenatide-microspheres

The degradation of microspheres was also studied by observing the morphological changes over time *in vitro* (Fig. 4). As shown in Fig. 4, pores could be observed on the surface of large MS after 4-days of incubation, whilst there was no evident change seen for small MS during the first 7-days of incubation. After 14-days of incubation, the surface of large MS became much more wrinkled and porous, compared with that of small MS. On day 21, large MS collapsed into fragments, whereas most of small MS stayed as intact. The collapsing of large MS might also be attributed to the reason that the large MS became too friable with degradation, and thus the large MS could be crushed during the centrifugation and drying process for sample preparation. After 60 days, both small and large MS collapsed into small fragments. These results directly confirmed that large MS displayed a more rapid *in vitro* degradation rate than small MS.

The above *in vitro* degradation studies indicated that the size of microspheres could significantly influence the polymer degradation of PLGA microspheres. The *in vitro* degradation rate of small MS (3.80 μm) was significantly slower than that of large MS (18.15 μm). During the first 10 day, small MS (3.80 μm) showed negligible polymer degradation, as reflected by the small change of microspheres weight, Mw and T_g values of PLGA and the surface morphology of microspheres over time. After 10 days, small MS (3.80 μm) degraded gradually with a slower rate in comparison with large MS (18.15 μm). By increasing the size of microspheres to 18.15 μm (large MS), an obvious polymer degradation was observed after 4-days of incubation, which was more rapid than small MS. Such size influence on the degradation rate of microspheres can be attributed to the following factors. Firstly, in comparison with small MS, the porous interior structure of large MS would allow more PBS medium penetrating into the interior of the microspheres, resulting in a more rapid degradation rate for large MS. On the contrary, small MS with dense interior structure could reduce the penetration of PBS medium into the microspheres, leading to a decreased degradation rate for small MS. Secondly, the autocatalysis effect also contributed to the fast degradation rate for large MS. It has been reported that the degradation mechanism of PLGA microspheres was mainly due to the hydrolysis of ester bonds of the PLGA chains [11,16,28,29]. Known as autocatalysis, the degradation of PLGA can be accelerated by the progressively decreased pH value within the microspheres, which was caused by the accumulation of acidic degradation products from PLGA, i.e. lactic acid and glycolic acid [30,31]. Therefore, large MS (18.15 μm) might entrap more acidic degradation products in the core due to its longer diffusion path and lower specific

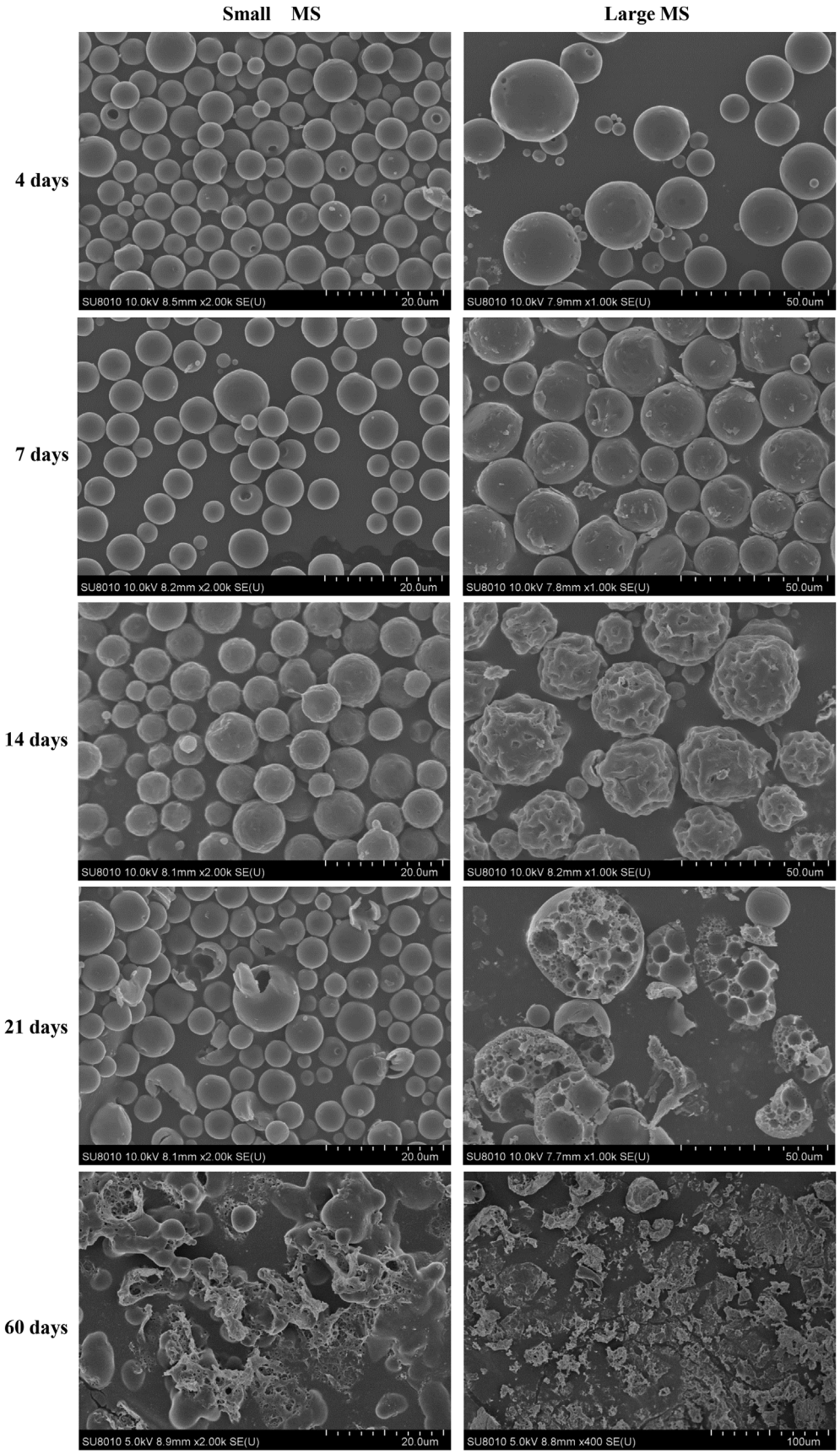

Fig. 4. SEM photographs of exenatide-microspheres (small MS and large MS) incubated in 10 mM PBS solution (pH 7.4, 0.02% NaN_3) at 37 °C.

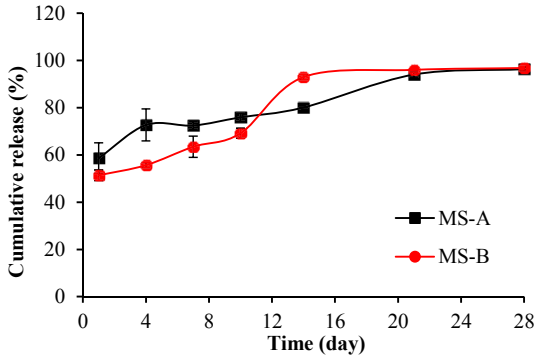

Fig. 5. The *in vivo* drug release profiles of exenatide-microspheres (small MS and large MS) after a subcutaneous injection to rats at a single dose of 5 mg/kg, respectively. Each point represents the mean ± SD; n = 3.

surface area, which would further accelerate the degradation in comparison with small MS (3.80 μm) [13,25,31,32]. Finally, the porous structure of large MS (Fig. 4) formed over the first 4 day allowed high penetration of PBS medium into the microspheres, which also contributed to the accelerated degradation of large MS.

According to the *in vitro* degradation results, the difference in initial release rate between small MS and large MS during the first 4 days should be attributed to the larger surface area of small MS as no evident erosion of microspheres was observed in the first 4 days for small MS. After 4 days, large MS showed a higher degradation rate than small MS and the erosion of microspheres became the dominant factor in controlling drug release as proved by the SEM study. As a result, the long-term *in vitro* drug release rate (day 4 to day 49) of large MS was significantly higher than that of small MS.

3.4. In vivo drug release of exenatide-microspheres with different sizes

In order to fully understand the effect of microsphere size on drug release, *in vivo* studies of drug release and degradation process were carried out in this study. As shown in Fig. 5, small MS and large MS both showed a high burst release (*ca.* 58.61% and 51.37%, respectively) *in vivo*, which was higher than the *in vitro* burst release. The high burst release *in vivo* for both small and large MS might be due to the increased drug concentration gradient in the *in vivo* drug release, since the released drug could be rapidly removed by the body fluid *in vivo*.

Despite the burst release, small and large MS exhibited slightly different *in vivo* drug release behavior. Small MS showed a rapid initial drug release with a cumulative drug release on day 4 up to 72.7%, while large MS released 55.7% of the drug after 4-day injection. The completion of drug release was achieved for large MS on day 14, whilst small MS finalized drug release on day 21. The faster drug release rate of small MS at early stage in comparison with large MS might be due to the large surface area and the short diffusion distance. After initial drug release, the amount of residual drug encapsulated in small MS was less than 30%, resulting in a low concentration gradient between the microspheres and surrounding body fluid and hence leading to a slow sustained release from day 4 to day 21.

3.5. In vivo degradation of exenatide-microspheres with different sizes

In previous sections, the *in vitro* degradation process of microspheres with different particle sizes has been fully assessed. The results showed that microspheres with a large size tended to degrade faster than small microspheres, which is in agreement with other reported results. As mentioned earlier, the effect of particle size on the *in vivo* degradation process of microspheres, had been little studied [6]. In this paper, the *in vivo* degradation of microspheres with different sizes was conducted to gain a full understanding on the degradation process and to possibly achieve a mechanism of drug release *in vivo*.

Since the microspheres after injection were surrounded by a biomembrane, it was difficult to completely separate the remaining microspheres from the surrounding tissue, and hence the total weight of the remaining microspheres over the *in vivo* study could not be achieved accurately. The determination of Mw and T_g of PLGA, however, did not require the total amount of microspheres from *in vivo* study (only circa 1 mg microspheres sample from *in vivo* was sufficient for Mw and Tg study). Therefore, the results of mass loss *in vivo* were not included in the manuscript. Only the Mw and T_g of PLGA in microspheres, and the morphology of microspheres at each predetermined time point were studied to assess the *in vivo* degradation process of microspheres.

3.5.1. Polymer Mw of exenatide-microspheres

As shown in Fig. 6A, after 1-day injection, the Mw of PLGA in the two formulations both dropped to half of the initial Mw of PLGA, which was much faster than that found with the *in vitro* study. The extremely rapid *in vivo* degradation of polymer was most likely due to the *in vivo* hydrolysis of ester bonds, which can be significantly accelerated by factors including the presence of hydrolytic enzymes [33], the inflammatory response [34,35], and the increase of chain mobility due to the absorption of lipids and other components [34]. In addition, small and large MS both exhibited a similar degradation rate *in vivo*. Previous literature reported that microspheres smaller than 5 μm could be phagocytosed by the inflammatory cells, whereas large microspheres (> 10 μm) can avoid phagocytosis [17–19]. Therefore, it was likely that the phagocytosis of small MS (3.80 μm) could accelerate the *in vivo* degradation rate for small MS. Such phagocytosis effects for small MS increased the degradation rate for small MS to the level that was comparable to the degradation rate of large MS *in vivo*.

3.5.2. Tg of exenatide-microspheres

T_g values of PLGA for small and large microspheres over the *in vivo* study time period are shown in Fig. 6B. As seen in Fig. 6B, T_g values of the small MS and large MS both decreased gradually with increasing

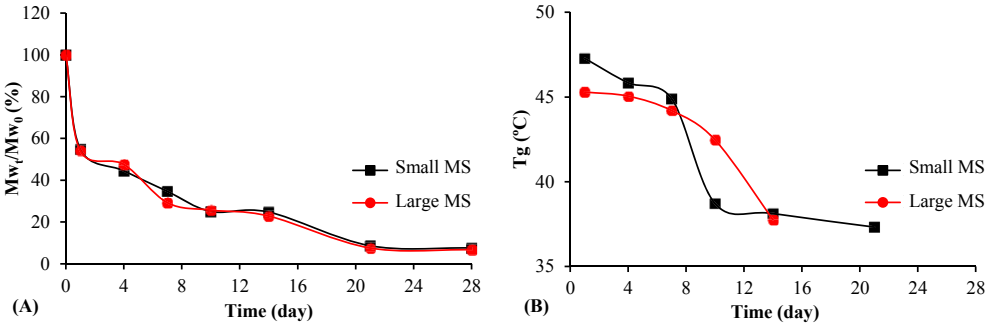

Fig. 6. The *in vivo* degradation profiles of exenatide-microspheres (small MS and large MS) after a subcutaneous injection to rats at a single dose of 5 mg/kg: (A) the Mw_t/Mw_0 (%) vs time profiles; (B) the T_g vs time profiles.

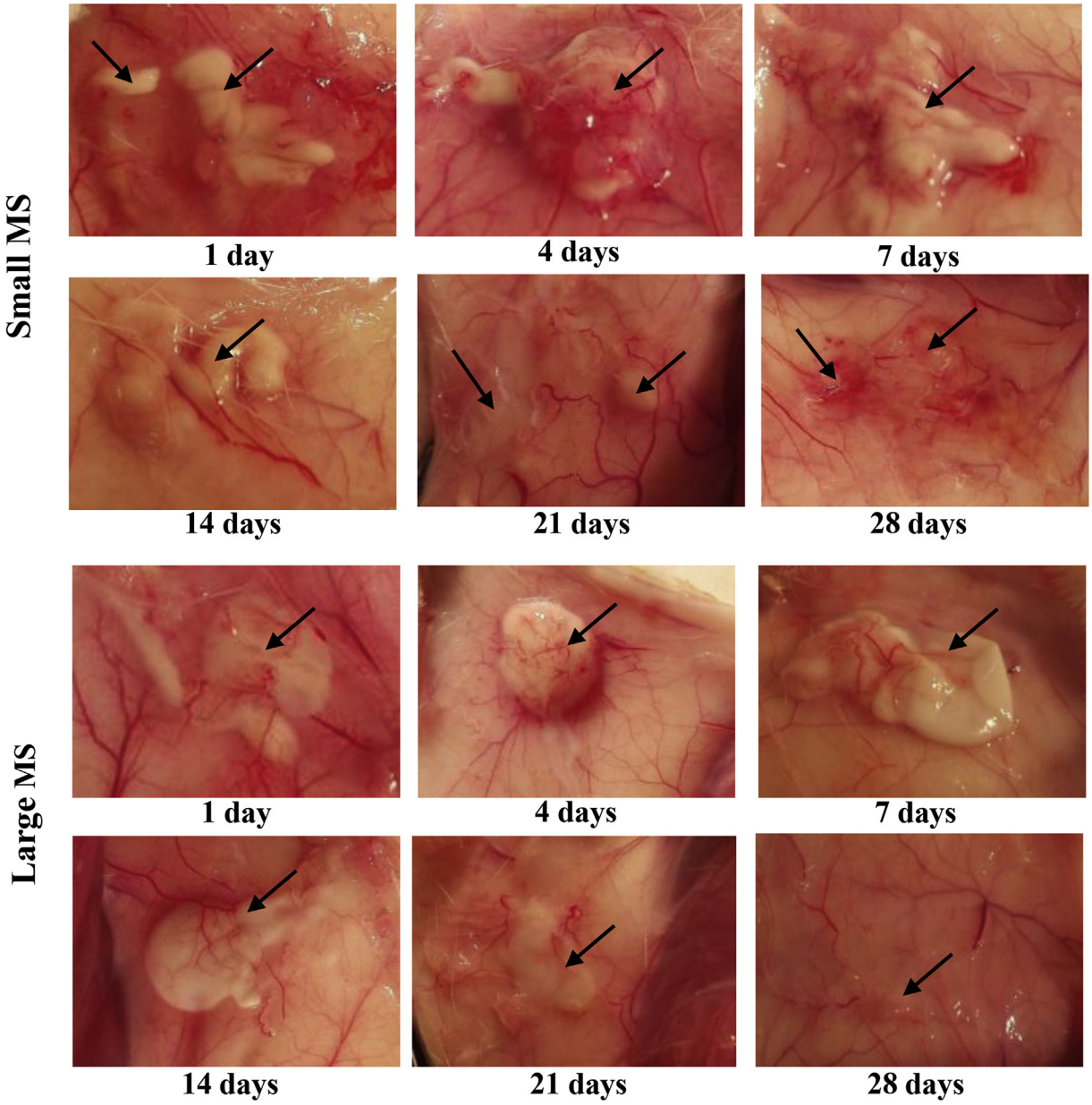

Fig. 7. Representative images of the injection sites after a subcutaneous injection of exenatide-microspheres to rats at a single dose of 5 mg/kg (about 20 mg microspheres).

time. Similar to the results of the PLGA Mw study *in vivo*, the T_g values of small and large microspheres showed a similar trend. After 7-day injection, the T_g of small MS was much lower than that of large MS, but at the same time point the two sizes of microspheres showed similar PLGA Mw. This was probably because the lipid and other small chemicals from *in vivo* were absorbed on the surface of microspheres, which may act as plasticizers for PLGA, leading to a decreased T_g value. After 28 days injection, T_g can not be detected for both small MS and large MS *in vivo*.

3.5.3. Morphological changes of exenatide-microspheres

In order to directly demonstrate the degradation of exenatide-microspheres after injection, the representative images of the injection sites were shown in Fig. 7. As shown in Fig. 7, over the entire 28 day time period of this study, the volume of the two types of microspheres both decreased gradually with time. At 28 days post-injection, the two types of microspheres both degraded completely. This indicated that the microspheres degraded more rapidly *in vivo* than *in vitro*. It should be noted that, during the first 4 days after injection of small MS, visible redness and swelling could be observed in the tissue at the site of injection. However, large MS did not cause any visible redness or swelling at the site of injection. This suggested that small MS may cause a more severe acute inflammatory response than large MS after injection, which may further accelerate the degradation of small MS.

To further understand the *in vivo* degradation process of microspheres, the morphological changes over time were also observed using SEM. As shown in Fig. 8, on day 7 post-injection, the small MS and large MS were both partially cracked, which also indicated that the *in vivo* degradation rate of microspheres was more rapid than the *in vitro* degradation rate. In addition, large MS were more porous and cracked than small MS after 7-day injection, despite that the two formulations exhibited similar PLGA Mw and T_g. After 21-day injection, most of the large MS had degraded into fragments, while some intact microspheres still can be observed for the small MS. This was probably because that large MS tended to collapse easily with degradation due to the porous interior structure, while the small MS could maintain the spherical

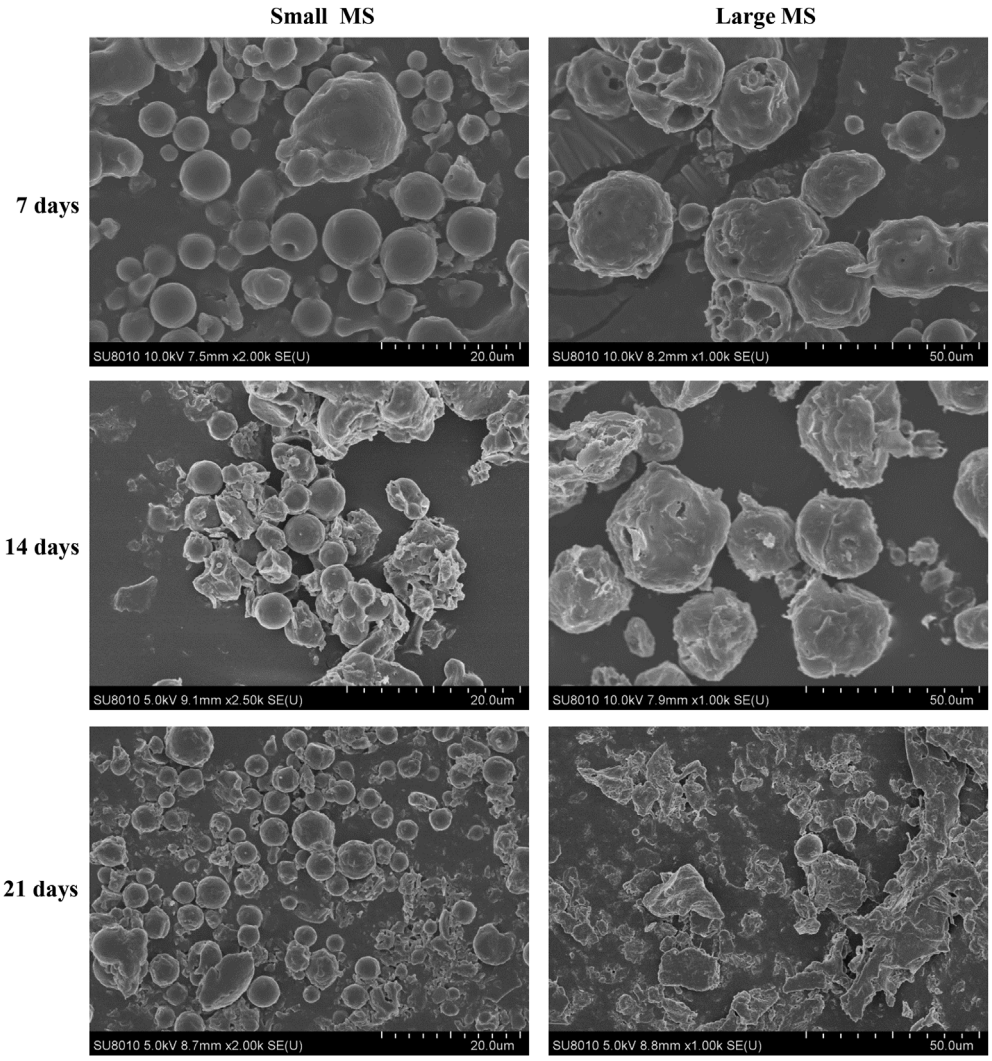

Fig. 8. SEM photographs of exenatide-microspheres (small MS and large MS) after a subcutaneous injection to rats at a single dose of 5 mg/kg, respectively.

shape and exhibited a surface erosion due to the dense interior structure.

The above *in vivo* degradation studies indicated that the two types of microspheres both degraded rapidly and showed similar degradation kinetics after subcutaneous injection into rats, which was different from the *in vitro* studies. Previous literature reported that the *in vivo* fate of microspheres was affected by many factors such as the properties of microspheres, the inflammatory response at the injection site, and the absorption of lipids [28]. Small microspheres (< 10 μm) may cause a greater inflammatory response compared to large microspheres [19]. Therefore during the inflammatory response, the inflammatory cells could produce more acidic products, free radicals and enzymes, leading to a lower local pH value and a higher local temperature compared with the injection of large microspheres [34]. In addition, with the particle size of 3.80 μm, small MS could be easily phagocytosed after injection [17–19]. The above factors may together contribute to the acceleration of the *in vivo* degradation rate for small MS.

Despite the similar *in vivo* degradation kinetics, small MS and large MS exhibited slightly different *in vivo* drug release behavior. Small MS showed a rapid initial drug release with a cumulative drug release on day 4 up to 72.7%, while large MS released 55.7% of the drug after 4-day injection. The completion of drug release was achieved for large MS on day 14, whilst small MS finalized drug release on day 21. The faster drug release rate of small MS at an early stage in comparison with large MS was mainly due to the larger surface area and the shorter diffusion length. After initial drug release, the residual drug encapsulated in small MS was less than 30%, resulting in a low drug concentration change from the microspheres to the surrounding body fluid. In addition, the small MS exhibited a more dense interior structure than large MS. The above two factors both contributed to the slow sustained drug release from small MS from day 4 to day 21.

3.6. Pharmacokinetics of exenatide-microspheres with different sizes

The pharmacokinetics of small MS and large MS were studied after subcutaneous injection into rats at a single dose of 5 mg/kg. The plasma concentration-time profiles of exenatide were shown in Fig. 9. As shown in Fig. 9, both types of microspheres showed high plasma concentration levels during the first 8 h, indicating a rapid initial drug release rate. In addition, small MS showed a significantly higher C_{max} (270.22 ± 74.13 ng/ml) than large MS (C_{max} = 200.98 ± 48.95 ng/ml, $p < 0.05$), indicating a more rapid initial drug release rate of small MS in comparison with large MS. These results were consistent with the *in vivo* drug release studies.

After 4 days post-injection, the exenatide plasma concentrations of small MS dropped to below 10 ng/ml and maintained at a low level (1.98–7.18 ng/ml) from 4 to 20 days post-injection. For large MS, the exenatide plasma concentrations could still be maintained at a high level (9.60–20.78 ng/ml) from 4 to 12 days, and after 14 days it dropped to below 5 ng/ml. In addition, it should be noted that the

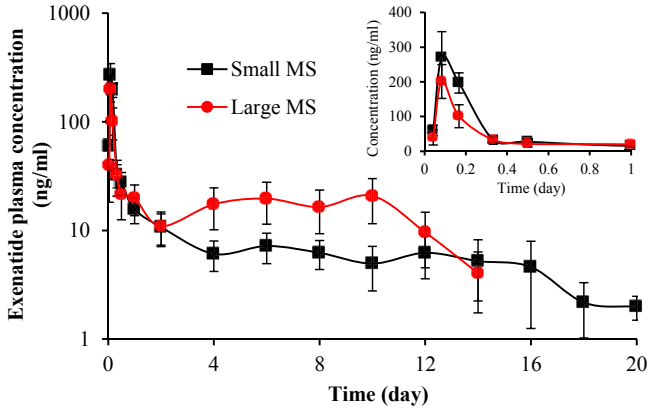

Fig. 9. The plasma concentration-time profiles of exenatide after intramuscular administration of exenatide-microspheres (small MS and large MS) to rats at dose of 5 mg/kg (mean ± S.D.; n = 6).

$AUC_{0-\infty}$ value of small MS (185.07 ± 52.85 ng d/ml) was significantly lower than that of large MS ($AUC_{0-\infty}$ = 266.09 ± 80.25 ng d/ml, $p < 0.05$). The lower exenatide plasma concentration levels and AUC value of small MS in comparison with large MS might be attributed not only to the low drug release rate of small MS but also to the phagocytosis of small particles (< 10 μm) by inflammatory cells.

The pharmacokinetic results indicated that the high initial drug release of small MS could lead to a substantially high plasma concentration which may cause undesirable side effects, while the slow release at the late stage usually lead to a low plasma concentration which may be therapeutically ineffective. Usually, microspheres prepared by the traditional emulsification solvent evaporation method contain a certain amount of microspheres smaller than 5 μm [36]. These small microspheres might contribute to the unexpected burst release. In addition, the drug exposure decreased with decreasing particle size in the range of 3–20 μm due to the phagocytosis of small microspheres (~5 μm) by macrophages [15]. Consequently, microsphere formulations that contain a large amount of microspheres of small particle size may possibly result in a low bioavailability. Therefore, it was essential to control the fraction of small microspheres in microsphere formulations to achieve a desirable drug release behavior and hence the aimed therapeutic effect.

4. Conclusions

The present study demonstrated that the particle size exhibited a significant influence on the *in vitro* drug release and degradation of the exenatide-loaded microspheres. By increasing the particle size from 3.80 μm to 18.15 μm, the initial drug release over the first 4 days decreased due to the reduced surface area, while the drug release rate from day 4 to day 28 increased since the large microspheres became more porous and degraded more rapidly than small microspheres. A similar *in vivo* degradation profile was observed for both types of microspheres after subcutaneous injection into rats, except that small microspheres could stay as intact microspheres for a longer time period compared to large microspheres. Small microspheres exhibited a higher initial drug release (72.7% on day 4) and slower long-term drug release rate up to 21 days *in vivo*, whereas large microspheres released about 50% of drug on day 4 and completed drug release after 14 days. Small microspheres showed a significant higher burst release *in vivo* compared with large microspheres, which could lead to a high plasma concentration that may cause side effects. Therefore, microspheres with a desired drug release performance should be formulated by controlling the fraction of small microspheres.

Conflicts of interest

The authors have declared no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jddst.2018.03.024.

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