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Effect of medium-chain triglycerides on the release behavior of Endostar® encapsulated PLGA microspheres

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ABSTRACT

The incomplete release of Endostar® from PLGA microspheres was observed in our previous study. In the present study, we focused on the effect of medium-chain triglycerides (MCT) on the *in vitro*/*in vivo* release behavior of Endostar® encapsulated PLGA microspheres, which were prepared by a water-in-oil-in-water (W/O/W) double-emulsion method with or without MCT. The *in vitro* accumulated release of Endostar® from microspheres co-encapsulated with 30% MCT was found to be 79.04% after a 30-day incubation period in PBS (pH 7.4) at 37 °C. However, the accumulated release of Endostar® from MCT-free microspheres was found to be only 32.22%. Pouches containing Endostar® encapsulated PLGA microspheres were implanted subcutaneously in rats. The effect of MCT on the *in vivo* release showed a similar trend to the *in vitro* release. After 30 days, only 9.87% of the total encapsulated Endostar® was retained in microspheres co-encapsulated with 30% MCT, while 42.25% of Endostar® was retained in MCT-free microspheres. The co-encapsulation of MCT provided the microspheres with a porous surface, which significantly improved the *in vitro*/*in vivo* release of Endostar® from PLGA microspheres. In addition, *in vitro* experiments showed that MCT co-encapsulated PLGA microspheres had more inter-connected pores, faster degradation of PLGA, and faster swelling of microspheres, which helped to explain the mechanism of the effect of MCT on improving the release of Endostar® from PLGA microspheres.

1. Introduction

Endostatin is a 20 kDa C-terminal fragment of collagen XVIII. It can inhibit angiogenesis, which is required for tumor growth in an endothelial cell (EC)-specific manner (O'Reilly et al., 1997). Endostatin induced EC apoptosis *in vivo* (Dhanabal et al., 1999), and inhibited EC proliferation and migration (Yamaguchi et al., 1999). Systemic administration of endostatin significantly inhibited the growth of animal tumors, human tumors, and metastases in various mouse xenograft tumor models (O'Reilly et al., 1997; Yamaguchi et al., 1999; Ye et al., 2002; Schuch et al., 2005; Yokoyama and Ramakrishnan, 2005). In 2005, Endostar® (recombinant human endostatin) was approved by the China State FDA as an angiogenesis inhibitor for treating non-small cell lung cancer (NSCLS) (Sun et al., 2005). Similar to most proteins, Endostar® is administered by multiple injections at a high dose in order to maintain its therapeutic level (Sim et al., 1999). Clinically, Endostar® is administered intravenously at a dose of 7.5 mg/m^2 per day during the first 2 weeks of treatment cycles. However, the Endostar® plasma concentration can fluctuate. In addition, the treatment causes poor patient compliance, which limits its clinical use. It has been reported that continuous subcutaneous administration of endostatin resulted in significantly greater tumor regression than intermittent administration in a tumor-bearing mouse model (Kisker et al., 2001; Kuroiwa et al., 2003).

PLGA (D,L-lactide-co-glycolide) microspheres have recently been widely investigated for protein delivery due to their biodegradable characteristics and they cause only mild tissue reaction (Visscher et al., 1985). Injectable PLGA microparticles, prepared for sustained release, stabilized the concentration of proteins in biological fluids (Kissel and Koneberg, 1996; Morita et al., 2000; Zhang et al., 2008). Typical protein release from PLGA microspheres includes two main phases: Phase I—a burst release in the first 24 h, followed by Phase II—a slow release. It was widely believed that a part of the protein was loosely associated with the surface and internal pores of the microspheres, whose solubilization and diffusion result in the burst release (Blanco and Alonso, 1998; van de Weert et al., 2000). The subsequent reduced rate of protein release (Phase II) was determined by the polymer degradation and protein diffusion through the water-filled channels (Cohen et al., 1991; O'Hagan et al., 1994). Toxicity problems caused by the burst release in Phase I and insufficient drug availability in Phase II are unable to maintain the therapeutic concentration and are undesirable in clinical therapies. Thus, in order to solve these problems, various additives including amphiphilic polymer (PEG, PVP, pluronic F68)

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(Morita et al., 2001) and fatty acid ester (Urata et al., 1999) have been applied to improve drug release by increasing the porosity of the polymer matrix. However, it was reported that the external porosity of PLGA microspheres was reduced after 5 h, and pores were almost completely closed after 24 h when the microspheres were immersed in water (Wang et al., 2002). Few studies have been performed on how these additives, especially hydrophobic substances, affect pore function. In our previous study, Endostar® encapsulated PLGA microspheres were designed to maintain the therapeutic concentration of Endostar® for 4 weeks following a single injection. Incomplete release of Endostar® from these microspheres was observed. After a 28-day incubation period, only 30% and 39% of Endostar® was released from microspheres prepared with 75:25 PLGA and 50:50 PLGA (Mw = 47,000), respectively (Wu et al., 2009). Hydrophobic medium-chain triglycerides (MCT, 6–12 carbon atoms), widely applied in the field of parenteral nutrition, were chosen as a pore-forming additive in our study. Considering the high hydrophilicity and remarkable acid resistance of Endostar® (Li et al., 2004; Wu et al., 2004), the W/O/W double-emulsion method was used to encapsulate Endostar® into PLGA microspheres with different amounts of MCT. The microspheres were characterized using gel permeation chromatography, optical microscopy, and scanning electron microscopy (SEM). The effects of MCT on the release behavior and release mechanism of Endostar® were studied. The effect of MCT on *in vivo* drug release was also investigated in order to substantiate the *in vitro* results.

2. Materials and methods

2.1. Materials

The following chemicals were used as received: Endostar® (Simcere Medgenn Company, China), poly(D,L-lactide-co-glycolide) (PLGA, 75:25), PURESORB 7502 (inherent viscosity of a 1.0 g/dl polymer solution in chloroform at 25 °C is 0.20 dl/g, PURAC Biochem Ltd, The Netherlands), medium-chain triglycerides (MCT, Lipoid GmbH, Germany), polyvinyl alcohol (PVA, 88 mol.% hydrolyzed, Mw = 30,000 Beijing Chemicals Co. Ltd., China), and the Micro-BCA assay kit (Pierce Biotechnology, Rockford, USA). All other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of microspheres

Endostar® encapsulated PLGA microspheres were prepared by the double-emulsion method as reported by Bouissou et al. (2004). Briefly, 200 μl Endostar solution (150 mg/ml) was injected into 1 ml of PLGA methylene chloride solution (20%, w/v), and homogenized at 17,500 rpm for 20 s. The resultant emulsion was emulsified in a small volume of PVA aqueous solution (0.3%, w/v) using a probe-type sonicator, further dispersed into 500 ml water containing 0.3% (w/v) PVA, and stirred at 500 rpm for 5 h at room temperature. The resultant microspheres were obtained as the lower layer by centrifuging the mixture at 3000 rpm for 15 min. They were washed with distilled water three times, and dried under reduced pressure at room temperature for 2 days. The microspheres were kept at 4 °C in a sealed container with silica gel. MCT co-encapsulated PLGA microspheres were prepared in the same procedure except that MCT was dissolved in methylene chloride in advance. Four batches of Endostar® encapsulated microspheres with different amounts of MCT co-encapsulated were prepared (Table 1).

2.3. Determination of the drug loading and encapsulation efficiency of the microspheres

The protein encapsulation efficiency of the microspheres was measured by the BCA assay (Sandor et al., 2001). For each batch, 7 mg of microspheres were added to 5 ml of a 0.1N NaOH solution containing 2% (w/v) sodium dodecyl sulfate (SDS) under stirring. All measurements were conducted in triplicate. After 24 h, 20 μl of the solution was added to 200 μl of bicinchoninic acid (BCA) working solution in a 96-well flat-bottomed plate (Biousing Biotech, China). The plate was covered and incubated at 37 °C for 30 min. Samples were measured by UV absorption at 562 nm. A standard curve was established in advance by plotting protein absorption against concentration. The drug loading and drug encapsulation efficiency were expressed as follows:

$$\text{Drug loading } E\%F = \frac{\text{the amount of protein embedded in microspheres}}{\text{the amount of microspheres}} \times 100\%$$

$$\text{Encapsulation loading } E\%F = \frac{\text{the amount of protein embedded in microspheres}}{\text{the theoretical amount of protein incorporated into microspheres}} \times 100\%$$

Table 1
Dosage formulation of Endostar®-loaded microspheres.

Formulation ID	A	B	C	D
Ratio of PLGA (w/w) %	90	85	70	60
Ratio of Endostar (w/w) %	10	10	10	10
Ratio of MCT (w/w) %	0	15	20	30

2.4. In vitro release

The *in vitro* release of Endostar® encapsulated PLGA microspheres was determined in PBS (pH 7.4, 0.01% sodium azide, 0.02% Tween 80). 20 mg of microspheres were dispersed in 5 ml of PBS, and then incubated at 37 °C under mild shaking. Aliquots of supernatant were withdrawn at predetermined time intervals. An equal amount of fresh PBS was added to maintain a constant volume of medium. The protein concentration was measured using the BCA assay kit mentioned in Section 2.3.

2.5. The morphology and size of microspheres

The morphology of the microspheres was observed using an optical microscope (Olympus BH2, Japan), and further analyzed by scanning electron microscopy (SEM) (JSM 5610LV, Tokyo, Japan). Dry samples were spray-coated with gold under reduced pressure for SEM examination. Endostar® encapsulated PLGA microspheres were suspended in 0.02% (w/w) Tween 80 solution under sonication for 1 min at room temperature, and the size was then analyzed using a laser light scattering detector on Sympatec HELOS Sucell (Sympatec GmbH, Germany).

2.6. Gel permeation chromatography (GPC)

The molecular weight of the PLGA microspheres was determined using GPC (Waters, USA) connected to an evaporative light scattering detector (Waters 515, USA). Three columns (Styragel®HR 3E, Styragel®HR 4E, and Styragel®HR 5E) were connected in series. Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 1 ml/min at 25 °C. 10 mg of microspheres were dissolved in 10 ml THF, and then filtered using a 0.22 μm Whatman filter. Clean glassware was used to avoid unnecessary contamination from plastic materials. 100 μl samples were injected into the column. Waters Millennium software was applied to collect and analyze the data. The weight–average molecular weight was calculated based on standard polystyrene with a molecular weight ranging from 900 to 1,740,000.

Table 2
Characteristics of Endostar®-loaded microspheres.

Formulation ID/MCT (%)	A/0%	B/15%	C/20%	D/30%
Calculated drug content (%)	10	10	10	10
Encapsulation Efficiency[a] (%)	95.07 ± 0.64	97.69 ± 2.58	93.27 ± 0.28	92.53 ± 4.47
Particle size[a] (μm)	12.14 ± 0.27	11.05 ± 0.88	16.09 ± 0.45	14.53 ± 0.37

[a] Each sample was assayed in triplets, and the value represents the mean ± SD.

2.7. In vitro degradation of PLGA

In vitro degradation of PLGA was determined in PBS (pH 7.4, 0.01% sodium azide, 0.02% Tween 80). 50 mg of PLGA microspheres were placed in each test tube containing 15 ml PBS and shaken at 37 °C. At predetermined time intervals, the pH in the upper clear solution was measured using a pH meter (Sartorius, Germany). The morphology and size of the dry microspheres were examined using the method described in Section 2.5. The molecular weight of PLGA was determined by GPC. All experiments were conducted in triplicate.

2.8. In vivo study

2.8.1. Determination of the concentration of Endostar® by HPLC

An Agilent 1100 liquid chromatography system, consisting of a binary solvent delivery system, an autosampling device and a UV detector was used to measure the concentration of Endostar®. A Diamonsil C18 reverse phase column (5 mm, 4.6 i.d. 250 mm; Dikma, China) was applied and maintained at 40 °C. UV absorption was measured at 203 nm. The flow rate of the mobile phase (acetonitrile–water, 25:75, v/v) was kept at 1 ml/min. 20 μl of filtered samples were injected into the Diamonsil column. A calibration curve was established in advance by plotting the UV absorption of Endostar® against concentration (0.01–0.2 mg/ml).

2.8.2. Preparation of polyethylene pouch devices

The conventional method of implanting microspheres subcutaneously in *in vivo* studies usually has undesirable effects, e.g. (a) microspheres stick to the tissue and cannot be removed; (b) microspheres move around. These effects make it difficult to collect retained drugs from the microspheres. Thus, the non-degradable polyethylene pouch introduced by Maryellen et al. (2002) was prepared for the *in vivo* study in order to collect all the microspheres. Briefly, a polyethylene membrane with a pore size of about 0.8 μm was heat-sealed on three sides to produce an open pouch with an opening of about 1 cm^2. 20 mg of microspheres were suspended in a suitable vehicle (1% carboxymethylcellulose and 2% mannitol, w/v), and loaded into the pouch. The pouch opening was then heal-sealed. UV irradiation was applied to sterilize the pouch before implantation in the *in vivo* studies.

2.8.3. In vivo release

Male Sprague–Dawley rats (weighing ~300 g; $n = 6$) were used to evaluate the *in vivo* performance of Endostar® encapsulated PLGA microsphere. The sealed pouches prepared in Section 2.8.2 were implanted subcutaneously at the back of the neck through incisions 2.0 cm in length. Each rat received a single implant, which was removed upon sacrifice in a CO_2 atmosphere at predetermined times: 1 day, 10 days, 20 days, and 30 days. The pouch was cut, and the microspheres inside the pouch were withdrawn. The microspheres were washed three times with distilled water, and then dried under reduced pressure at room temperature for 1 day. The dried microspheres were weighed, and added to 15 ml 30 mM sodium acetate buffer (pH 5.5). The mixture was then extracted with 5 ml dichloromethane. The concentration of Endostar® in the aqueous layer was determined by HPLC using the procedure described in Section 2.8.1. The *in vivo* Endostar® release was calculated based on the amount of remaining Endostar® in the microspheres following subcutaneous implantation. All experiments involving animals adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985).

3. Results and discussion

3.1. Effect of MCT on microsphere characteristics

Because of its large molecule size (Mw = 21,000) and high affinity for negatively charged polymer (Kisker et al., 2001), Endostar® was entrapped in PLGA microspheres with an encapsulation efficiency of over 90%. The physicochemical characteristics of Endostar® encapsulated PLGA microspheres are shown in Table 2. The size of the microspheres varied from 10 to 17 μm measured by the method described in Section 2.5, and there was no significant difference between the three formulations except that there was a slight increase in formulation B, which was attributed to the diversity of operation. Thus, it can be concluded that co-encapsulation of MCT did not influence microsphere particle size or the protein encapsulation efficacy.

However, the scanning electron photomicrographs showed a difference in appearance in the 20% MCT co-encapsulated microspheres and the MCT-free microspheres (Fig. 2). The MCT-free microspheres had a smooth surface, which was different from the porous surface of the MCT co-encapsulated microspheres. Medium-chain triglycerides are water insoluble and a poor solvent for PLGA. During the preparation of microspheres, it was shown that MCT was retained in the microspheres, and could not diffuse to the external aqueous phase (Traul et al., 2000). However, methylene chloride could rapidly diffuse into MCT-rich regions, resulting in the acceleration of PLGA precipitation to produce a porous surface.

3.2. In vitro release of Endostar

3.2.1. Effect of MCT on the in vitro release of Endostar® from PLGA microspheres

Fig. 1 shows the cumulative Endostar® release from Endostar® encapsulated PLGA microspheres with or without MCT. The release of Endostar® from the MCT-free microspheres was slow and incomplete. After 11 days, only 23.34% of Endostar was released. Thereafter, the release rate slowed down, and only 8.88% of drug was released in the subsequent 19 days. It is widely believed that the rate of protein release after the burst release depends on the molecular weight of the protein (Sandor et al., 2001), as well as the microsphere size and the erosion speed (Lemaire et al., 2003). The smooth surface of MCT-free microspheres makes water wicking difficult, resulting in slow erosion. Moreover, the large molecule size of Endostar® impeded its diffusion through the PLGA matrix. In general, the factors mentioned above resulted in the slow and incomplete release of Endostar® from MCT-free microspheres.

In contrast, the addition of MCT significantly enhanced Endostar® release during the first 0–11 days. Because the burst release is mainly related to the surface condition of pores (Blanco and Alonso, 1998; van de Weert et al., 2000), the rough surface of MCT co-encapsulated microspheres results in a greater burst

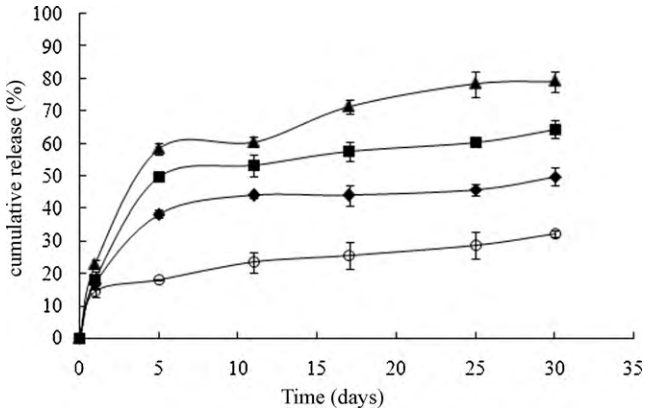

Fig. 1. Effect of MCT content on the release rates of Endostar® from PLGA microspheres in PBS at pH 7.4: (●) A: without MCT, (♦) B: with 15% MCT, (■) C: with 20% MCT, (▲) D: with 30% MCT.

release. In our study, the burst release on the first day increased slowly from 14.32% for MCT-free microspheres to 22.58% for 30% MCT co-encapsulated microspheres. The amount of Endostar® released from microspheres with MCT co-encapsulation from day 2 to day 11 was 9.02% (MCT-free), 27.13% (15% MCT), 35.21% (20% MCT), and 37.74% (30% MCT). This difference in Endostar® release can be attributed to the interconnecting channels on the pore surface caused by MCT. This porosity resulted in higher water accessibility and a pattern of diffusion-controlled release other than erosion-controlled release (Siegel and Langer, 1990). However, the effect of MCT on the late release phase varied. The co-encapsulation of 15% MCT in PLGA microspheres slowed down Endostar® release from the microspheres in this phase in contrast to that from MCT-free microspheres. The co-encapsulation of 20% and 30% MCT in PLGA microspheres accelerated Endostar® release. The release during day 11 to day 30 was 8.88% (MCT-free), 5.82% (15% MCT), 11.07% (20% MCT), and 18.72% (30% MCT). In contrast to 43.88–60.32% of Endostar® released from MCT co-encapsulated microspheres in the first 11 days, the drug release was relatively low in the late phase, when the effect of MCT on release enhancement was partly impaired. In the late phase, the degradation rate of PLGA dominated the release of the remaining protein, which was firmly attached to PLGA (Dong et al., 2005). The release of Endostar® increased gradually in the late phase with increased MCT co-encapsulated (>20%), which indicated that MCT might be involved in the process of PLGA degradation. The following experiments were carried out in order to validate this hypothesis.

3.2.2. Effect of MCT on degradation of PLGA and medium pH

PLGA degradation involves chain scission of ester bonds in the polymer backbone which are attacked by water molecules. Therefore, a higher rate of water infusion into the matrix may result in faster degradation. In addition, the medium pH and molecular weight of PLGA may affect PLGA degradation. The effect of MCT on PLGA microsphere degradation was evaluated after a 30-day incubation period in phosphate buffer (pH 7.4) at 37 °C. As shown in Table 3, the co-encapsulation of MCT resulted in a concentration-dependent degradation of PLGA. The average loss of PLGA molecular weight was 16.20% when 30% MCT was co-encapsulated, while the loss of PLGA in MCT-free microspheres was only 8.88%. Statistical analysis showed a significant difference regarding the effect of MCT on PLGA microsphere degradation.

Jiang et al. (2002) reported that a decrease in pH was observed during the degradation process due to small oligomers produced by PLGA degradation. The faster the degradation, the more small oligomers were observed. The decrease in pH was affected by these water-soluble oligomers, whose critical molecular weight was found to be around 1100 (Park, 1994). Moreover, pH affected the release rate of these proteins because they tended to aggregate at low pH (Lu et al., 2001). However, in our study, the decrease in pH was slight (7–9%) and no significant difference among the three formulations was observed (Table 3). Thus, the small pH difference among these formulations was attributed to the low degree of degradation (8.88–16.20%) of PLGA and insufficient release of oligomers into the dissolution medium, where the acids were artificially rapidly neutralized by the PBS buffer system.

3.2.3. In vitro morphology studies of different formulations with or without MCT

Bouissou et al. (2004) reported that the number of external pores on the microspheres played only a small role in affecting protein release because the external pores closed after microspheres were immersed in water for 24 h (Wang et al., 2002). However, our study showed different results. SEM and optical microscopic images of different microspheres incubated at different periods gave a clear illustration of the effect of MCT on the appearance of microspheres. Unlike the sparse pores caused by the evaporation of methylene chloride in the preparation of MCT-free PLGA microspheres, numerous large pores were observed by SEM on the surface of PLGA microspheres with 20% MCT co-encapsulated (Fig. 2). Moreover, after the microspheres were incubated for 15 days in PBS, the size of these pores increased, and the pores were inter-connected to each other. In addition, several large holes appeared. The detailed information on pore aggregation and interconnection on the surface of the microspheres was observed with high magnification (6000×, Fig. 3). This may explain why the results in our study are different from those reported by Wang et al. (2002). After a 30-day incubation period, these microspheres were severely eroded, and only fragments were left. The rapid disintegration of the microspheres could be attributed to both the porous surface and the quick degradation of MCT co-encapsulated PLGA microspheres. More-

Table 3
Variation of pH value in the release medium and molecular weight of PLGA with different amounts of MCT loaded after 30 days of *in vitro* release.

Release medium	Times	pH value	Molecular weight (Mw)
Microsphere A (0% MCT)	0 day	7.09 ± 0.17	28,100 ± 436
	30 days	6.44 ± 0.10	25,600 ± 173
	Decrease Percentage	9.15% ± 1.17%	8.88% ± 1.77%
Microsphere B (15% MCT)	0 day	7.00 ± 0.21	28,500 ± 624
	30 days	6.45 ± 0.22	25,400 ± 361
	Decrease Percentage	7.86% ± 0.85%	10.87% ± 0.70%
Microsphere D (30% MCT)	0 day	6.97 ± 0.14	28,400 ± 265
	30 days	6.46 ± 0.11	23,800 ± 361
	Decrease Percentage	7.31% ± 0.99%	16.20% ± 1.04%**

Each value represents the mean ± SD of 3 samples.
** $P < 0.01$, represent significant values between groups.

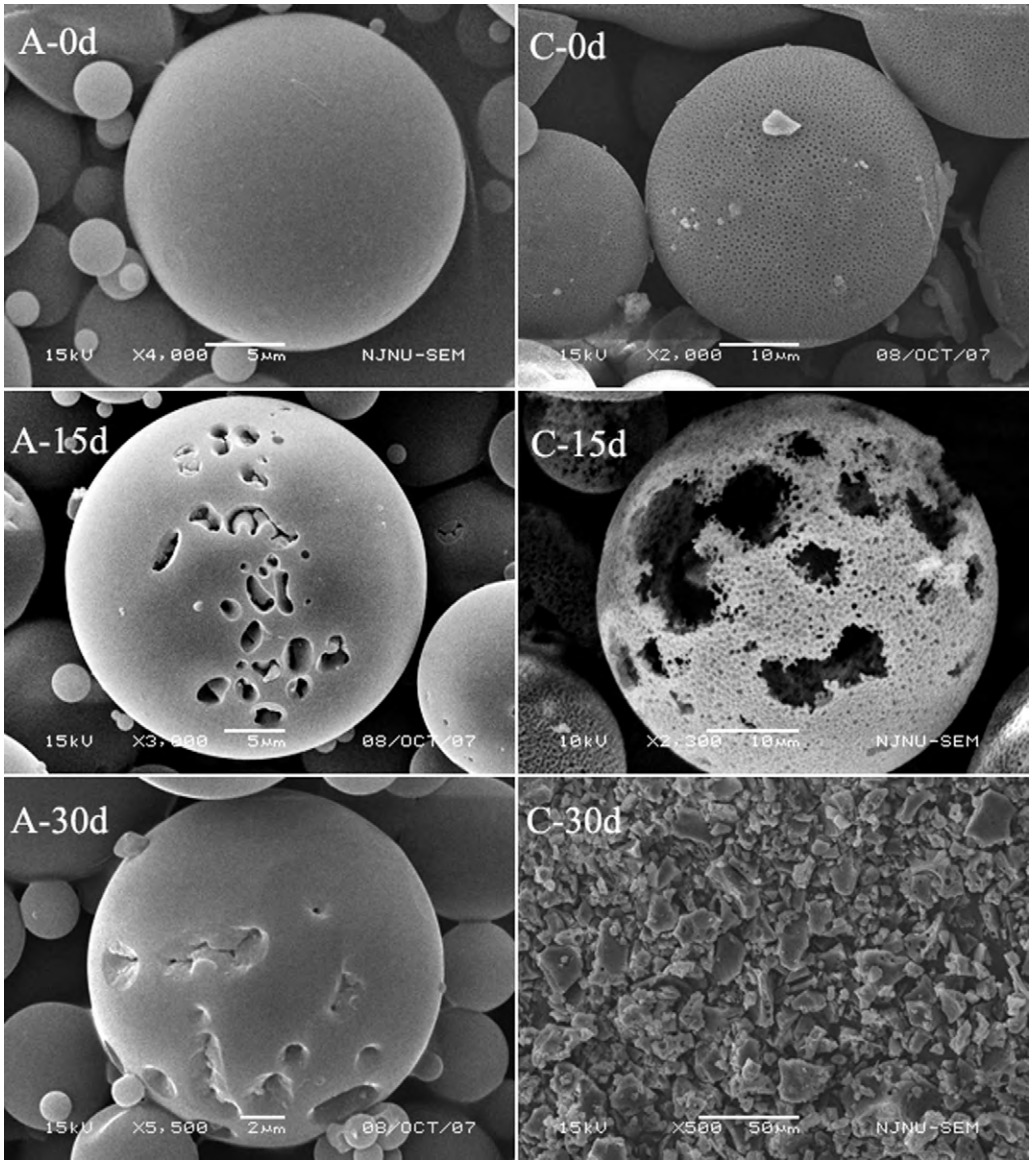

Fig. 2. Scanning electron micrographs of the surface of Endostar®-loaded microspheres with or without MCT. (A) Microspheres A incubated in PBS at 37 °C for 15 days and 30 days; (C) Microspheres C incubated in PBS at 37 °C for 15 days and 30 days.

over, the consequent increased release area may accelerate the release of Endostar®. In contrast, although incubated for 30 days, the MCT-free PLGA microspheres retained their smooth surface and spherical shape with only some etch marks on the surface.

The particle size of PLGA microspheres was determined after a predetermined incubation time. The volume–mean diameter of MCT co-encapsulated PLGA microspheres increased significantly after a 24-h incubation period, whereas that of the MCT-free micro-

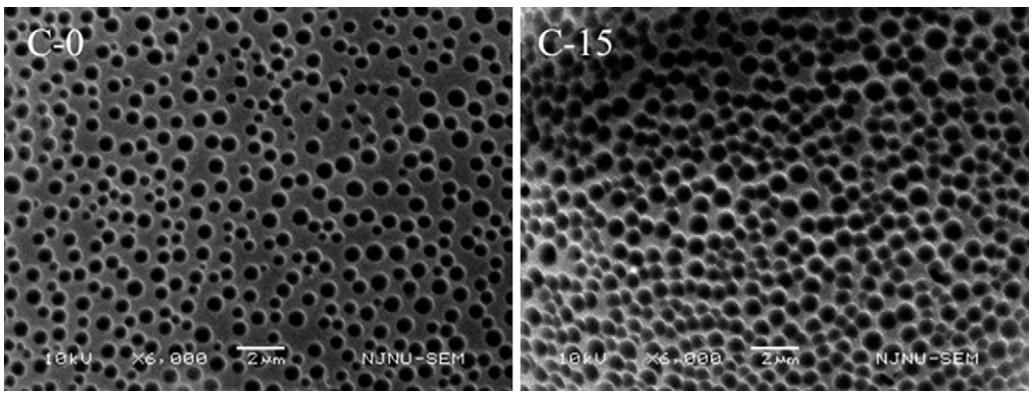

Fig. 3. External morphology of (C-0) microspheres C and (C-15) microspheres C incubated in PBS at 37 °C for 15 days at 6000× magnification using SEM.

Table 4
The effect of MCT on particle size of Endostar®-loaded microspheres incubated in PBS at 37 °C.

Formulation/MCT(%)	A/0%	B/15%	D/30%
Microsphere size (μm)[a]	12.14 ± 0.27	11.05 ± 0.88	14.10 ± 0.37
Microsphere size after 1 day incubation (μm)[a]	15.90 ± 0.44	22.18 ± 1.12	36.19 ± 2.47

[a] Each value represents the mean ± SD of 3 samples.

spheres showed only a small change (Table 4). In order to eliminate the interference caused by the sticky microspheres and the possible unreal size changes caused by the vacuum environment during SEM, optical microscopy was used to obtain photographs of fresh samples recovered from the release media (Fig. 4). Photographs taken using the optical microscope showed that MCT-free microspheres retained their original spherical shape and particle size after a 15-day incubation period, and only a small portion of these microspheres disintegrated after 30 days. However, microspheres containing 15% MCT swelled after a 24-h incubation period. After 15 days incubation, some microspheres disintegrated into fragments, which further broke down after 30 days incubation. In addition, microspheres co-encapsulated with 30% MCT showed a much greater change in shape and size, and could not be found following incubation for 15 days.

Our study showed that co-encapsulation of MCT resulted in a highly porous surface on the microspheres which made the microspheres more susceptible to water penetration through the interconnecting aqueous channels. Thus, the release of Endostar® was confirmed in a diffusion-controlled model. Moreover, swelling and destruction of the microspheres also increased PLGA degradation which affected the late slow release phase.

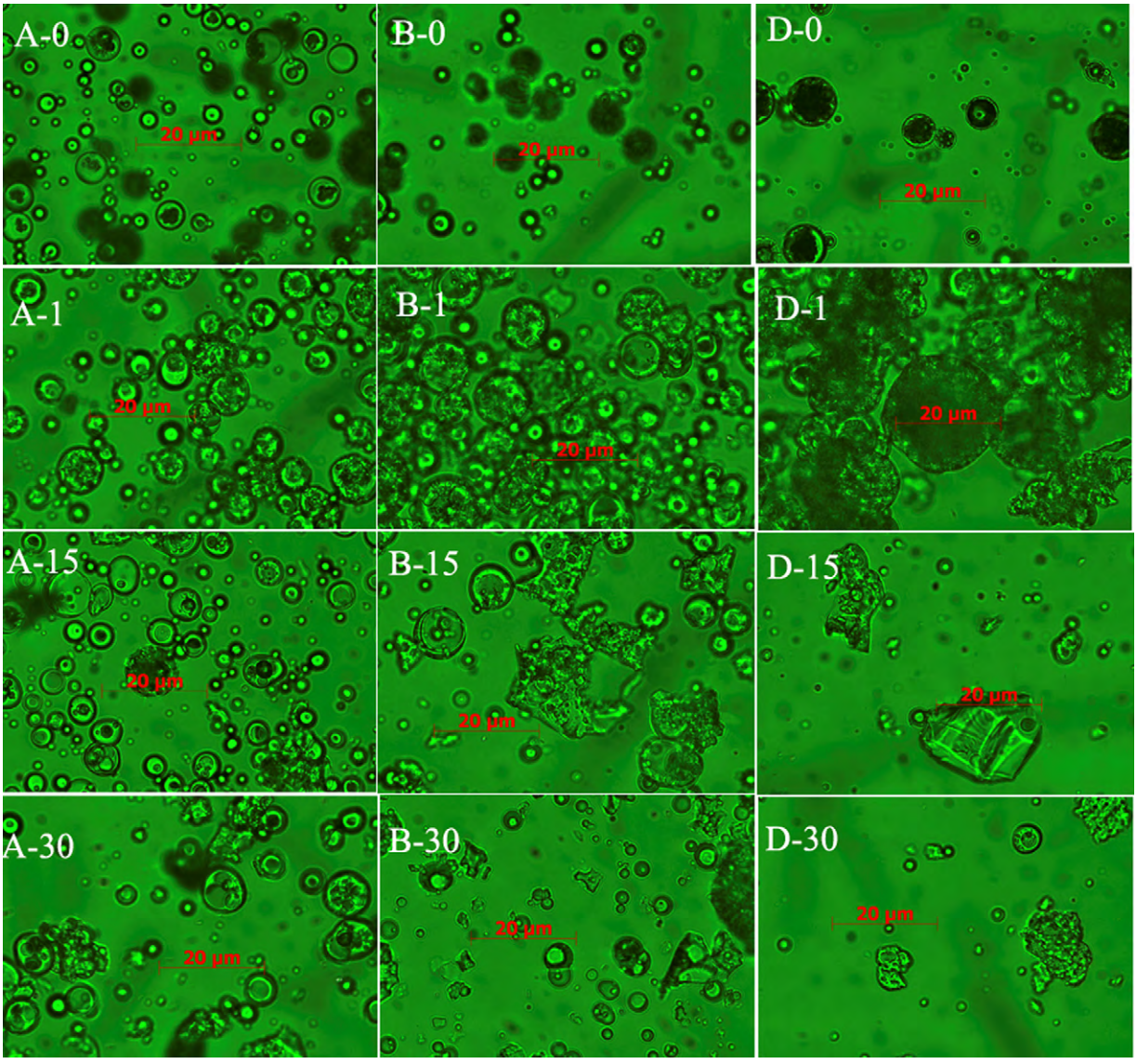

Fig. 4. Optical microscope photographs of the microspheres with different MCT content after incubation in PBS at 37 °C for 0 days, 1 days, 15 days, and 30 days: (A) without MCT; (B) with 15% MCT; (D) with 30% MCT.

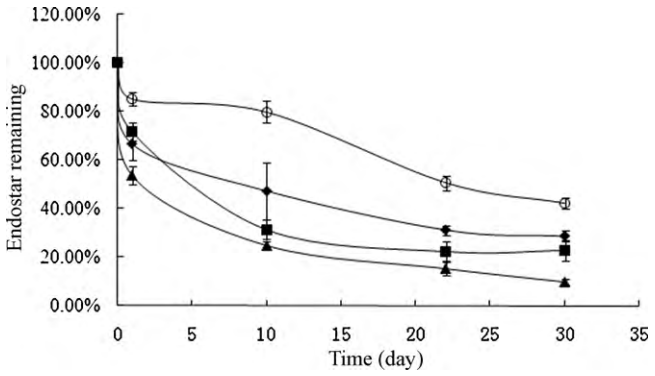

Fig. 5. Percentage of Endostar® remaining in the polyethylene pouch which was subcutaneously implanted in the neck of rats vs. time (days): (●) A: without MCT, (♦) B: with 15% MCT, (■) C: with 20% MCT, (▲) D: with 30% MCT. Each point represents the mean ± SD of 6 animals.

Table 5
PLGA polymer and protein remaining after incubation of microspheres within pouches *in vivo* for 30 days.

Particle size/MCT(%)	Percentage remaining at 30 days (%)	
	PLGA mass	Protein extracted
A/0%	80.63 ± 3.56	42.25 ± 2.24
B/15%	66.42 ± 3.51	28.67 ± 2.34
C/20%	52.21 ± 5.49	22.77 ± 4.28
D/30%	27.57 ± 0.77	9.87 ± 1.23

Each point represents the mean ± SD of 6 animals.

3.3. *In vivo* study

Endostar® was extracted from PLGA microspheres and analyzed using HPLC. Similar to the *in vitro* results, the *in vivo* data, shown in Fig. 5, illustrated the effect of MCT on Endostar® release, which was explained by the protein remaining in the PLGA microspheres. The more MCT were co-encapsulated, the more Endostar® was released after the 30-day *in vivo* experiment. The microspheres with 30% MCT co-encapsulated significantly increased the release of Endostar® at 24 h, where only 53.70% of Endostar® was retained compared to 85.19% of Endostar® retained in MCT-free microspheres. After 30 days, 42.25%, 28.67%, 22.77%, and 9.87% of the total loaded Endostar® was retained in the microspheres with 0%, 15%, 20%, and 30% MCT co-encapsulated, respectively (Table 5). Compared with the *in vitro* release, more Endostar® was released in the *in vivo* study, which can be attributed to widened pores and faster destruction of microspheres caused by enzymatic degradation. In addition, the more MCT co-encapsulated, the quicker PLGA microspheres degraded, which helps us to explain why only 27.57% of the microspheres with 30% MCT co-encapsulated were recovered from the implant sites after 30 days, compared with 80.63% of the MCT-free microspheres (Table 5). Thus, MCT had a significant effect on the degradation of PLGA *in vivo*, which was consistent with the *in vitro* results described in Section 3.2.2.

4. Conclusions

Endostar® co-encapsulated PLGA microspheres, prepared by the W/O/W double-emulsion method, had a high encapsulation efficiency and fine particle size. MCT was used in the formulation as an additive to prepare microspheres with a higher porous structure, which was susceptible to water penetration, resulting in swelling of the microparticles to more than twice the original size in the first 24 h. In addition, continuous swelling and breakdown of PLGA microspheres were observed after further incubation. This breakdown changed the release model of Endostar® co-encapsulated microspheres. The co-encapsulation of MCT enhanced the release of Endostar® from microspheres with a slight increase in the initial burst. Moreover, the rate of Endostar® release could be adjusted by changing the amount of MCT co-encapsulated. The effect of MCT on Endostar® release was further investigated in an animal experiment, which showed similar but more dramatic findings than the *in vitro* study.

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