International Journal of Pharmaceutics 384 (2010) 53–59

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Three-layered microcapsules as a long-term sustained release injection preparation

Yukako Ito*, Yuya Ochii, Keizo Fukushima, Nobuyuki Sugioka, Kanji Takada**

Department of Pharmacokinetics, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan

ARTICLE INFO

Article history:
Received 26 May 2009
Received in revised form
17 September 2009
Accepted 19 September 2009
Available online 25 September 2009

Keywords:
Three-layered milli-capsules
Long-term sustained release
Leuprolide acetate
Release experiment
Absorption experiment
Rats

ABSTRACT

Three-layered milli-capsules (3LMC), diameter of 1.85 ± 0.07 and 0.15 ± 0.09 mm thickness, were designed for the long-term subcutaneous (sc) administration of drugs. 3LMCs composed of (1) surface membrane (release rate control membrane), (2) drug-carrying layer and (3) base membrane were prepared by dispensing each solution in series. As surface membrane, poly-(ε-caprolactone) having MW of 70 kDa (PCL70) was used in combination with plasticizer, polysorbate 60 (Tween60). Base membrane was prepared with PCL70. Fluorescein isothiocyanate labeled dextrans (FD-4, MW = 4 kDa and FD-20, MW = 20 kDa) were used as model drug and *in vitro* release experiment was performed with PCL70 surface membrane containing Tween60 with 0.3, 1.0 and 3.0% (w/w). As the amount of Tween60 increased, release rate of FD-4 was increased. PCL70 + 0.3% Tween60 membrane showed a good sustained release property for 5 weeks; 50.3 ± 6.0% of FD-4 was released during 5 weeks. When FD-20 was encapsulated, long-term sustained release was not obtained, 10.7 ± 3.6% was released during 5 weeks. However, when lower MW drug, leuprolide acetate, was encapsulated, 3LMC composed of PCL70 + 0.3% Tween60 showed a good sustained release property, 63.0 ± 5.9% released for 5 weeks. Leuprolide acetate encapsulated 3LMC was evaluated in rat experiment. After sc administration to rats, 0.5 and 1.0 mg, plasma leuprolide concentration showed its maximum concentration at day 1, thereafter gradually decreased and maintained the effective concentration for 14 weeks. Plasma leuprolide concentration *vs*. time curve showed a good dose-dependency. When surface membrane prepared by blending PCL70 and poly(lactic acid) (PLA) in the molar ratio of 5:1 was used, long-term sustained release property was not obtained. Instead, lower MW PCL, PCL40, was blended with PLA (5:1) to prepare surface membrane, sustained release of leuprolide was observed for 5 weeks. Through those studies, 3LMC has been shown to be a long-term sustained release preparation by properly selecting the surface membrane.

1. Introduction

For the preparation of microcapsules as sustained release pharmaceutical preparation, batch system is used and the conventional preparing methods are classified into two categories, dispersion of the preformed polymers and polymerization of monomers (Mathiowitz and Kreitz, 1999; Arshady, 1999). Dispersion method is classified into three groups: (1) emulsion solvent extraction/evaporation method, (2) phase separation (coacervation) method and (3) spray-drying method (Freitas et al., 2005). In addition, many modified methods have been developed to improve the disadvantages of the three methods, i.e. low drug-loading efficiency and wide variation of the particle size (Li et al., 1988).

With the advance of microfabrication technology, microparticles including microcapsules and/or microspheres can be prepared individually (Yeo et al., 2003; Glangchai et al., 2008; George, 2007). Although drug molecules are universally distributed in the conventional microspheres, initial burst release cannot be avoided due to the drug molecules distributed on the surface of microspheres (Mathiowitz and Kreitz, 1999; Arshady, 1999; Freitas et al., 2005). To overcome this phenomenon, many scientists are struggling with the burst release problem (Hasan et al., 2007; Yeo and Park, 2004; Allison, 2008). Matsumoto et al. (2005) developed multi-reservoir type microsphere where drug molecules were localized at the center of microspheres. However, initial burst release was not completely overcome. We introduced ink-jet dispensing system to prepare three-layered microcapsules (3LMC) and developed a machine to produce 3LMC in large scale, where drug layer was sealed between two membranes (Takada, 2006). When microparticles are produced one by one, high drug-loading efficiency, theoretically 100%, is attained and each microparticle has the same shape and size. In addition, there is a possibility that

* Corresponding author. Tel.: +81 075 595 4626; fax: +81 075 595 6311.
** Co-corresponding author.
E-mail addresses: yukako@mb.kyotophu.ac.jp (Y. Ito), takada@mb.kyoto-phu.ac.jp (K. Takada).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.09.032

initial burst release phenomena can be escaped, because drug layer is completely closed with two membranes, surface and base membranes.

Our equipment was designed to produce three-layered microparticles as an oral delivery system of peptide/protein drugs, where enteric polymer membrane, drug-carrying layer and barrier membrane were formed. Those oral three-layered microcapsules were designated as a gastrointestinal mucoadhesive patch system (GI-MAPS) that was composed of three membranes, i.e. (1) water-insoluble basement membrane, (2) drug-carrying membrane and (3) pH sensitive bioadhesive surface membrane (Takada, 2006; Venkatesan et al., 2006a). These three membranes were consecutively produced by means of three jet nozzles to which three solutions, enteric polymer solution, drug solution and water-insoluble polymer solution were connected through tanks. The diameter of the obtained microparticles was approximately 700–900 μm. As pharmaceutical preparation, GI-MAPS is provided by filling into conventional capsules. After oral administration of the capsule, GI-MAPS is released in the stomach and thereafter enters into the small intestine where the enteric surface membrane dissolves at their targeted intestinal site based on its pH sensitiveness and adheres to the small intestinal wall (Takada, 2008). By adhering to the intestinal wall, closed space is created and as a result high drug concentration gradient is formed between inside the system and the enterocytes, which contributes to the enhanced absorption of a drug. The concept of GI-MAPS was ascertained by the feasibility studies with G-CSF, erythropoietin (EPO) and interferon (Venkatesan et al., 2006b; Ito et al., 2005; Eiamtrakarn et al., 2002).

3LMC is also applicable to injection preparation such as subcutaneous (sc) and intramuscular (im) injection preparations. In this case, three layers are (1) release rate controlling surface membrane, (2) drug-carrying layer and (3) base membrane. Different from GI-MAPS, two membranes, surface membrane and base membrane, must be biodegradable, because the preparation is injected into the subcutaneous tissue or muscle. The number of biodegradable polymer available for pharmaceutical purpose is limited because of the safety problem. In pharmaceutical preparations, polyesters such as polylactide (PLA), polyglycolide (PLG), poly(ε-caprolactone) (PCL) and their copolymer are a popular choice as carriers as they have good historical evidence as being approved for clinical use, because of relatively small range of available polymers and copolymers. In this feasibility study, PCL was used by itself or blended with PLA to prepare release rate control membrane of 3LMC and the obtained 3LMC was evaluated as a long-term sustained release preparation through both *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate labeled dextran (FD) having the mean molecular weight (MW) of 4 kDa (FD-4) and 20 kDa (FD-20) were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Leuprolide acetate (LA) was purchased from Technoscience Co. Ltd. (Chiba, Japan). D-Mannitol, ethyl acetate, poly(ε-caprolactone) of MW 70–100 and 40 kDa (PCL70 and PCL40) and polyvinyl alcohol (PVA) were obtained from Wako Pure Chemical Industries, Co. Ltd. (Osaka, Japan). Poly(lactic acid) (PLA) was obtained from Polysciences Inc. (Warrington, PA, USA). Polysorbate 60 (Tween60) was obtained from MP Biomedicals Inc. (Kayserberg, France). Male Wistar Hanover rats were obtained from Japan SLC Inc. (Hamamatsu, Japan). All other materials used were of reagent grade and were used as received.

2.2. Preparation of 3LMC containing FD-4 and/or FD-20

Three kinds of surface membrane, release rate controlling membrane, were prepared. For PCL70 membrane containing Tween60 as 0.3% (w/w) (designated as PCL70 + 0.3% Tween60), 249.25 mg of PCL70 and 0.75 mg of Tween60 were dissolved with 2.5 ml of ethyl acetate. Similarly, 247.50 mg of PCL70 and 2.50 mg of Tween60 were dissolved with 2.5 ml of ethyl acetate to make PCL70 membrane containing 1.0% (w/w) Tween60 (PCL70 + 1.0% Tween60). For PCL70 + 3.0% Tween60 membrane, 242.50 mg of PCL and 7.50 mg of Tween60 were dissolved with 2.5 ml of ethyl acetate. Thin PCL70 membranes were made by spreading the PCL70 solution on a Teflon® plate (30 cm × 40 cm) previously coated with 10% (w/v) PVA solution using Baker applicator (Imoto Seisakusho, Kyoto, Japan) having a clearance of 100 μm. The PCL70 membrane was allowed to dry at 30% humidity for 60 min at room temperature. The thickness of the dried membrane was measured to be 14.0 ± 0.9 μm by a digital micrometer, SONY μ-mate (Tokyo, Japan). Next, 8 mg of FD-4 or FD-20 was dissolved with 100 μl of phosphate buffered saline (PBS), pH 7.4, and 0.5 μl of the obtained solution was dispensed on the PCL70 film as a dot. After dry for 10 min at room temperature, 250 mg of PCL70 was dissolved with 2.5 ml of ethyl acetate and the obtained solution was used to make base membrane. Then, 20 μl of the PCL70 solution was dispensed three times onto the FD layer. After dried for 1 h at room temperature, 3LMCs were obtained by removing form the plate.

2.3. Preparation of 3LMC containing leuprolide acetate

To 249.25 mg of PCL70 and 2.5, 0.75 or 0.25 mg of Tween60 (1, 0.3 or 0.1%), 2.5 ml of ethyl acetate was added and dissolved well. Spreading this solution on a plate made the PCL membrane. The PCL membrane was allowed to dry for 60 min at room temperature. The each thickness of the dried membrane was 14.6 ± 0.9 μm (PCL70 + 1.0% Tween60), 14.5 ± 0.7 μm (PCL70 + 0.3% Tween60) and 14.5 ± 0.6 μm (PCL70 + 0.1% Tween60), measured by a digital micrometer. Another biodegradable membrane was prepared by blending PLA and PCL70 or PCL40. For PCL40/PLA membrane (molar ratio of 5:1), 28.60 mg of PCL40 and 100 mg of PLA were dissolved in 2.5 ml of methylene chloride. For PCL70/PLA membrane (molar ratio of 5:1), 60.70 mg of PCL70 and 100 mg of PLA were dissolved in 2.5 ml of methylene chloride. Thin membrane was prepared by spreading each solution on a Teflon® plate (30 cm × 40 cm). The thickness of the dried membrane was measured to be 8.33 ± 0.5 μm (PCL40/PLA) and 10.4 ± 0.6 μm (PCL70/PLA). Leuprolide acetate, 1.5 mg, and mannitol, 1.5 mg, were dissolved with 7.5 μl of PBS (pH 7.4) and 0.5 μl of the obtained solution was dispensed on the PCL film as a dot. After dried for 10 min at room temperature, 250 mg of PCL was dissolved with 2.5 ml of ethyl acetate and the obtained solution was used to make base membrane. PCL solution, 20 μl, was dispensed three times onto the drug layer. After dried for 1 h at room temperature, 3LMCs were obtained by removing form the plate. The obtained particles were dried for 2 h at room temperature. The mean diameter of the particles was 1.85 ± 0.07 mm as shown in Fig. 1.

2.4. Drug content in 3LMC

One 3LMC was cut and dissolved with 1.0 ml of PBS (pH 7.4). After centrifuging at 13,000 rpm for 10 min with high speed Kubota refrigerated microcentrifuge 3700 (Tokyo, Japan), the supernatant was obtained to measure drug content. FD concentration was measured spectrofluoromerically with Shimazdu RF-1500 (Kyoto, Japan) with an excitation wavelength of 494 nm and an emission wavelength of 512 nm. In the case of leuprolide, LC/MS/MS method was used. The LC/MS/MS system consisted of an API 3200 triple

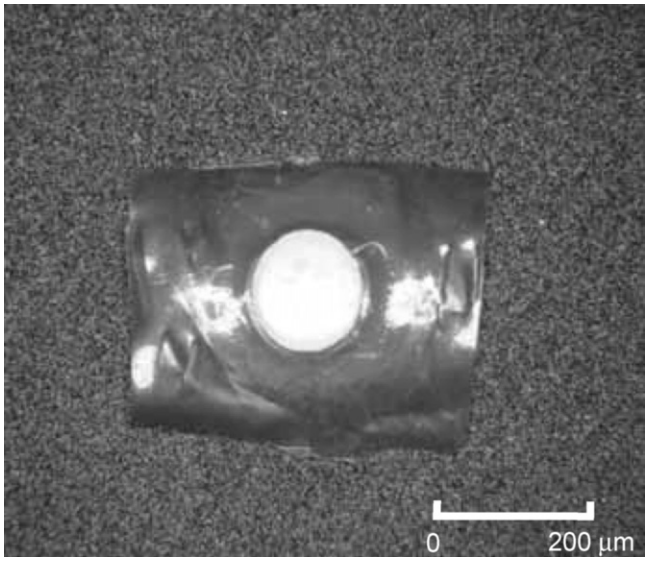

Fig. 1. Photograph of 3LMC containing leuprolide acetate, where surface membrane was made of PCL70.

quadrupole mass spectrometer equipped with turbo ion spray sample inlet as an interface for electrospray ionization (ESI), Analyst workstation (Applied Biosystems, CA, USA), LC-10AD micropump (Shimadzu, Kyoto, Japan) and AS8020 automatic sample injector (Tohso, Tokyo, Japan) by modifying the mass spectrometric method of Sofianos et al. (2008). The mobile phase, 1.0% formic acid:acetonitrile (70:30, v/v) was degassed and pumped through a Quicksorb ODS column (2.1 mm i.d. × 100 mm, 3 μm size, Chemco, Osaka, Japan) at a flow rate of 0.2 ml/min. Column temperature was maintained at 25 °C. For the detection of leuprolide (MW: 1209), the transitions of m/z 605.5 → 249.1 was optimized for the following conditions. The ionization was *via* the turbo ion spray inlet in the positive ion mode. The flow rates of nebulizer gas, curtain gas and collision gas were set at 8.0, 8.0 and 2.0 l/min, respectively. The ion spray voltage and temperature were set at 5000 V and 500 °C, respectively. The declustering potential, the entrance potential, the collision energy and the collision cell exit potential were set at 40.0, 7.0, 90.0 and 2.5 V, respectively.

2.5. Differential scanning calorimetry (DSC) analysis

A differential scanning calorimeter, PerkinElmer DSC7 (PerkinElmer Inc., USA) was used to obtain DSC thermal profiles of each polymer membrane. Samples were sealed into aluminum pans, and empty pans were used as references. Investigations were run at a scanning rate of 10 °C/min under nitrogen atmosphere. The temperature for the scan ranged from 30 to 200 °C.

2.6. In vitro release experiment

In vitro release experiments were carried out under perfect sink condition in a reduced scale with polypropylene tube. The 1.5 ml of degassed PBS (pH 7.4) was used as the dissolution medium. The tube was rotated with 13 rpm at room temperature by a Taitec rotator RT-50 (Tokyo, Japan). To determine the amount of FD or leuprolide released from 3LMC, the entire medium was removed and replaced with a fresh medium at each sampling time. Sampling time was at days 1, 2, 4, 7, 10, 14, 21, 28 and 35. All the obtained samples were stored at −80 °C till the assay. FDs or leuprolide contents were measured as described above. The cumulative released amount of FD or leuprolide from 3LMC was defined by the following equation:

$$\text{Cumulative amount released} = \left(\sum_{t=0}^{t} \frac{M_t}{M_{\text{theoretical}}} \right) \times 100\%$$

where M_t is the amount of FD or leuprolide released at time t, and $M_{\text{theoretical}}$ is the theoretical amount of encapsulated drug.

2.7. Stability experiment

3LMCs containing leuprolide acetate were kept under three different conditions, i.e. 4, 23 and 37 °C for 1, 2 and 3 months. Thereafter, 3LMC were dissolved with 1.5 ml of PBS (pH 7.4), and leuprolide contents were measured by LC/MS/MS method described above.

2.8. In vivo absorption experiment in rats

Male Wistar Hannover rats, 320–342 g, were anesthetized with an intraperitoneal (ip) injection of sodium pentobarbital, 50 mg/kg. After the hair of the dorsal skin was carefully removed, 3LMCs were implanted into the subcutaneous (sc) pocket. After administration, surgery was performed and each rat was kept in each cage. Water and food were given *ad libitum*. Blood samples, 300 μl, was obtained from the left jugular vein into a heparinized syringe at 0, 6, 12 and 18 h and at 1, 2, 3, 4, 5, 6, 7, 10, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91 and 98 day. After centrifuging at 12,000 rpm for 10 min, plasma samples were obtained. All the plasma samples were immediately frozen in a deep freezer at −80 °C until analysis. For iv injection study, leuprolide solution, 50 μg/ml, was injected into the right jugular vein, 50 μg/kg. After blank blood samples were obtained from the jugular vein, blood samples, 250 μl, were obtained at 5, 10 and 30 min and at 1, 1.5, 2, 3, and 4 h with heparinized syringe. By centrifuging at 12,000 rpm for 10 min, 100 μl of the plasma samples were obtained. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation, Kyoto Pharmaceutical University.

2.9. Pharmacokinetic analysis

The maximum drug concentration ($C_{\max}$) and the time to reach maximum concentration ($T_{\max}$) were determined from the authentic plasma drug concentration *vs.* time data. The area under the plasma drug concentration *vs.* time curve (AUC) after sc administration or iv injection was calculated using the linear trapezoidal rule up to the last measured drug concentration and the bioavailability (BA) of drug from 3LMC was calculated by the following equation.

$$\%\text{BA} = \left(\frac{\text{AUC}_{\text{sc}}}{\text{AUC}_{\text{iv}}} \right) \times \left(\frac{\text{Dose}_{\text{iv}}}{\text{Dose}_{\text{sc}}} \right) \times 100$$

2.10. Statistics

All values are expressed as their mean ± S.D. Statistical differences were assumed to be reproducible when $p < 0.05$ (Student's unpaired t-test).

3. Results

The thermal analysis of each membrane was carried out to investigate the effect of blending PCL and Tween60 or PLA. Fig. 2 shows the results of the DSC scans of each polymer membrane. The endothermal peaks appeared around 60 °C. In the case of PCL40/PLA membrane, the melting point was slightly shifted to lower temperature than the other membrane. Moreover, the shape of the

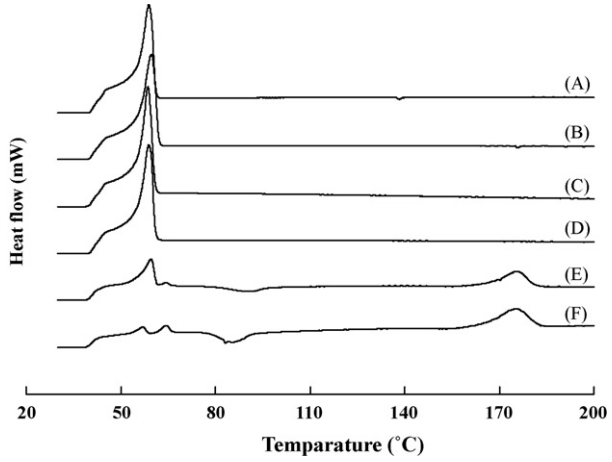

Fig. 2. DSC thermograms of surface membrane. (A) PCL70 + 0.1% Tween60, (B) PCL70 + 0.3% Tween60, (C) PCL70 + 1% Tween60, (D) PCL70 + 3% Tween60, (E) PCL70/PLA (5:1) and (F) PCL40/PLA (5:1).

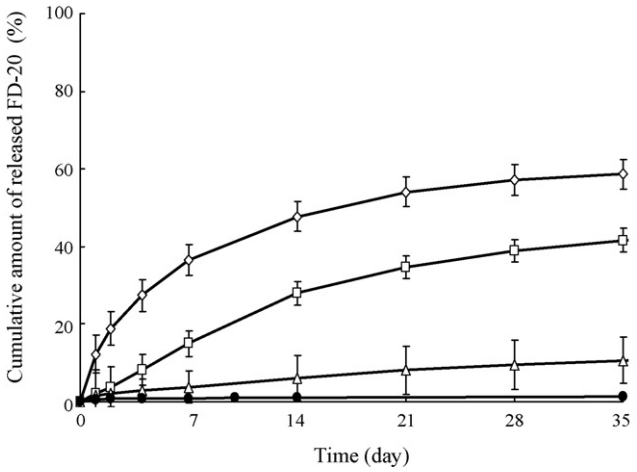

Fig. 4. *In vitro* release profiles of FD-20 (MW = 20 kDa) from 3LMC, where surface membranes were as follows: (◊) PCL70 + Tween60 (3.0%), (□) PCL70 + Tween60 (1.0%), (△) PCL70 + Tween60 (0.3%), and (●) PCL70. Each point represents the mean ± S.E. (n = 4–7).

melting peak was changed to broader. These results suggested that both of PCL40/PLA membrane and PCL70/PLA membrane existed in an amorphous or disordered crystalline phase compared with PCL70 + Tween60 membrane.

Two types of 3LMC made of PCL70 and FD-4 or FD-20 as a model drug was prepared. The mean diameter of 3LMC of FD-4 was 1.79 ± 0.05 mm and that of FD-20 was 1.80 ± 0.05 mm, respectively. There was not significant difference on the size between the two kinds of 3LMC. The FD contents were 25.1 ± 3.5 μg for FD-4 and 24.5 ± 1.7 μg for FD-20, respectively. With these 3LMCs, the release property of FD was studied in the *in vitro* release experiment and the results are shown in Fig. 3. When the surface membrane was made of 100% PCL70, FD-4 was not released from 3LMC. However, by formulating Tween60 as a plasticizer into PCL70 membrane, the release rate of FD-4 was increased. In the cases of PCL70 + 3.0% Tween60 and PCL70 + 1.0% Tween60 membranes, 91.3 ± 3.9% and 71.0 ± 3.3% of FD-4 were released during 5 weeks, respectively. When PCL70 + 0.3% Tween60 membrane was used, pseudo-zero-order release rate of FD-4 was observed and 50.3 ± 6.0% of FD-4 was released during 5 weeks after the start of release experiment. Thus, as the formulated amount of Tween60 to PCL70 membrane was increased from 0.3 to 1.0% and 3.0%, the release rate of FD-4 was considerably increased. To study the effect of MW of the encapsulated drug on the release rate from 3LMC, higher MW of FD, FD-20, was encapsulated and release experiment was also performed. The results are shown in Fig. 4. As the MW of FD increased, release rate of FD from 3LMC was decreased. Even in the case of PCL70 + 3.0% Tween60 membrane, less than 60% of FD-20, 58.6 ± 3.6%, was released from 3LMC during 5 weeks. As the Tween60 content in PCL70 membrane was decreased from 3.0% to 1.0%, release rate of FD-20 from 3LMC during 5 weeks was more decreased, i.e. 41.6 ± 5.2% for PCL70 + 1.0% Tween60 membrane and 10.7 ± 3.6% for PCL70 + 0.3% Tween60 membrane, respectively. When Tween60 was not formulated to PCL70, FD-20 was not released from 3LMC as in the case of FD-4. From these results, we may state that the release rate of drug from 3LMC was dependent on the MW of the encapsulated drug. In addition, it was revealed that 3LMC made of PCL70 was not useful for a long-term sustained release preparation of FD-20. However, with respect to the lower MW of FD, FD-4, 3LMC was found out to be a useful device. To ascertain the usefulness of 3LMC for lower MW drugs, leuprolide acetate (MW = 1269) was used as a model peptide drug and feasibility study was performed.

Fig. 5 shows the results of *in vitro* release experiment of leuprolide from 3LMCs. As the formulating amount of Tween60

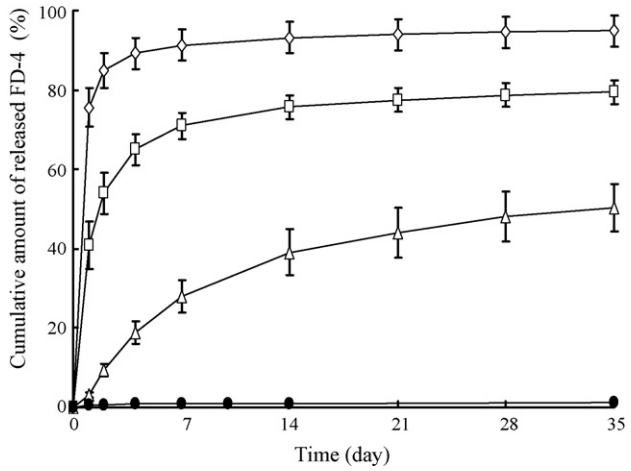

Fig. 3. *In vitro* release profiles of FD-4 (MW = 4.0 kDa) from 3LMC, where surface membranes were as follows: (◊) PCL70 + Tween60 (3.0%), (□) PCL70 + Tween60 (1.0%), (△) PCL70 + Tween60 (0.3%), and (●) PCL70. Each point represents the mean ± S.E. (n = 4–7).

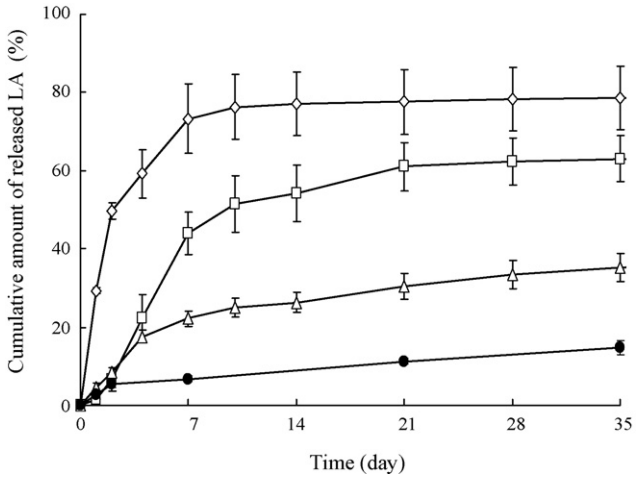

Fig. 5. Effect of Tween60 content on *in vitro* release profiles of leuprolide from 3LMC, where surface membranes were as follows: (◊) PCL70 + Tween60 (1.0%), (□) PCL70 + Tween60 (0.3%), (△) PCL70 + Tween60 (0.1%), and (●) PCL70.

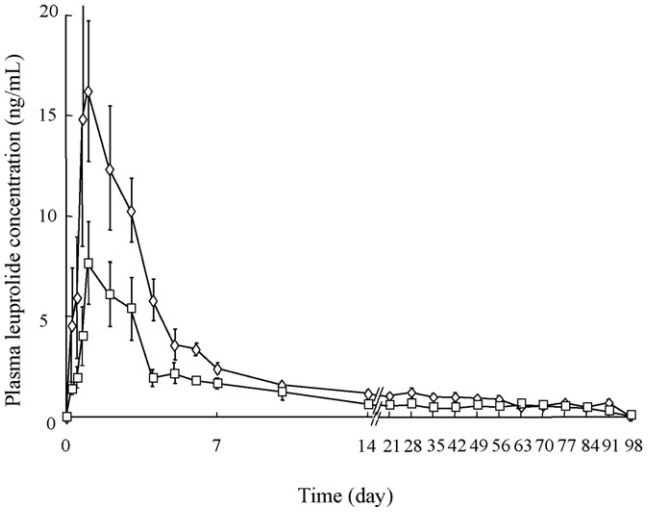

Fig. 6. Plasma leuprolide concentration vs. time curves after subcutaneous administration of 3LMC to rats, where release rate control membrane was PCL70 + Tween60 (0.3%). (◊) 1.0 mg/rat and (□) 0.5 mg/rat. Each point represents the mean ± S.E. (n = 4–6).

into the PCL70 surface membrane was increased, release rate of leuprolide from 3LMC was increased as in the case of FDs. When PCL70 + 1.0% Tween60 membrane was used, 73.3 ± 8.9% of leuprolide was released within 1 week. When the content of Tween60 in PCL70 membrane was decreased to 0.3%, 43.9 ± 5.5% of leuprolide was released during first week and thereafter slowly released till the end of 5 weeks, i.e. total released % was 63.0 ± 6.0%. On the other hand, when PCL70 + 0.1% Tween60 membrane was used, release rate of leuprolide was decreased, 35.2 ± 3.6% was released during 5 weeks. When 100% PCL membrane was used, the release rate of leuprolide was slow, i.e. 14.8 ± 1.8% of leuprolide during 5 weeks. However, as compared to the release rate of FDs, higher release rate was obtained.

Based on the results of the *in vitro* release experiments, 3LMC having PCL70 + 0.3% Tween60 surface membrane was evaluated *in vivo* rat experiment at two leuprolide dose levels, 0.5 and 1.0 mg. The results are shown in Fig. 6. After sc implantation of leuprolide 3LMC to rats, plasma leuprolide concentration reached to its peak level at 24 h after administration in both cases and C_{max} was 7.6 ± 2.1 ng/ml for 0.5 mg/rat study and 16.2 ± 3.5 ng/ml for 1.0 mg/rat study, respectively. To determine the bioavailability (BA) of leuprolide from 3LMCs, leuprolide solution was intravenously injected to another group of rats at 50 μg/kg. After iv injection, leuprolide disappeared from the systemic circulation within 3 h with an elimination half-life of 0.57 ± 0.02 h. The AUC was calculated to be 76.2 ± 6.4 ng h/ml. By comparing the AUCs obtained after iv injection of leuprolide solution and sc administration of 3LMC, BA of leuprolide from 3LMC was determined and the resulting pharmacokinetic parameter values are shown in Table 1. BA of leuprolide from 3LMCs were 63.3 ± 6.9% for 0.5 mg study and 57.0 ± 4.3% for 1.0 mg study, respectively. From those results, the concept of 3LMC as a long-term sustained release sc preparation has been confirmed.

Table 1
Pharmacokinetic parameters of leuprolide after administration of 3LMC to rats.

Dose (mg)	C_{max} (ng/ml)	T_{max} (day)	AUC_{0-98} (ng day/ml)	BA (%)
1.0	16.2 ± 3.5	1.0	120.4 ± 9.0	57.0 ± 4.3
0.5	7.6 ± 2.1*	1.0	66.9 ± 7.3*	63.3 ± 6.9

C_{max}: the maximum plasma leuprolide concentration; T_{max}: the time when plasma leuprolide concentration reaches to its maximum value; AUC_{0-98}: area under the plasma leuprolide concentration *vs.* time curve from day 0 to 98; BA: bioavailability. Each point shows the mean ± S.E. (n = 4–6).
* $p < 0.05$ compared to 1.0 mg study.

Table 2
Leuprolide acelate content in 3LMC.

Content of leuprolide (μg/capsule)	Encapsulation efficiency (%)
52.0 ± 2.9	99.4 ± 4.3

Each value shows the mean ± S.D. (n = 7).

Table 3
Long-term stability of leuprolide in 3LMC.

Period (months)	Drug contents (%)		
	4 °C	23 °C	37 °C
1	100.2 ± 1.3	100.0 ± 5.6	98.4 ± 10.1
2	99.3 ± 6.7	100.4 ± 6.6	98.6 ± 2.2
3	99.4 ± 5.3	100.4 ± 6.2	100.5 ± 3.5

Each value shows the mean ± S.D. (n = 5–7).

To make 3LMC as pharmaceutical preparation, we must confirm both the uniformity of leuprolide content and the stability of leuprolide in 3LMC. Therefore, *in vitro* studies on leuprolide content and stability were performed and the results are shown in Tables 2 and 3. Table 2 shows that the mean leuprolide content in 3LMC was 52.0 ± 2.9 μg. As the RSD was 5.6%, 3LMC will satisfy the standard of pharmaceutical preparation by preparing them with a sophisticated producing machine. In addition, a high encapsulation efficiency of leuprolide, 99.4 ± 4.3%, was obtained. Also, good stability of leuprolide in 3LMC was obtained. Namely, approximately 100% of leuprolide was recovered at 3 months after the start of the stability experiment at 4 and 23 °C. Even at 37 °C, 100.5 ± 3.5% of leuprolide was recovered from 3LMC at 3 months. From those studies, the concept of 3LMC as a long-term sustained release sc preparation has been ascertained.

In the above experiments, PCL70 membrane was used as the rate control surface membrane. Though PCL is classified to the category of biodegradable polymer, its biodegradation rate was reported to be very slow (Sinha et al., 2004; Sun et al., 2006). From the standpoint of safety, we must make the surface membrane more degradable. Therefore, surface membrane was prepared by blending poly(lactic acid) (PLA) to PCL70. In the case of the blend surface membrane of PCL70/PLA (5:1), 74.5 ± 3.5% of leuprolide was released in the *in vitro* release experiment within 2 days as shown in Fig. 7. Therefore, PCL40 was used instead of PCL70 and the *in*

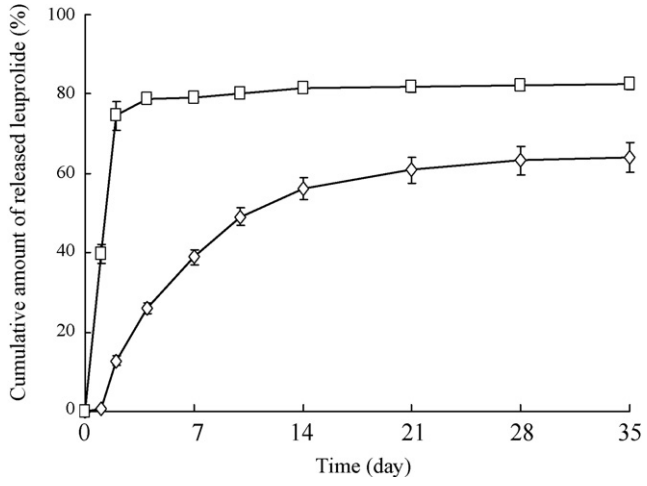

Fig. 7. Effect of PLA blend into PCL70 or PCL40 membrane on *in vitro* release profiles of leuprolide from 3LMC. (◊) PCL40/PLA (5:1) and (□) PCL70/PLA (5:1). Each point represents the mean ± S.E. (n = 4–7).

Table 4
Pharmacokinetic parameters of leuprolide after administration of 3LMC to rats, 1.0 mg.

Formulation	C_{max} (ng/ml)	T_{max} (day)	AUC (ng day/ml)	BA (%)
PCL70/PLA (5:1[a])	88.9 ± 18.7	0.25	164.2 ± 4.5[b]	77.3 ± 4.5
PCL40/PLA (5:1[a])	61.8 ± 3.0*	1.0	194.0 ± 2.4*,[c]	95.8 ± 1.5*

Each point shows the mean ± S.E. (n = 4–6).
* $p < 0.05$ compared to PCL70/PLA = 5:1.
[a] Molar ratio.
[b] From 0 to day 35.
[c] From 0 to day 42.

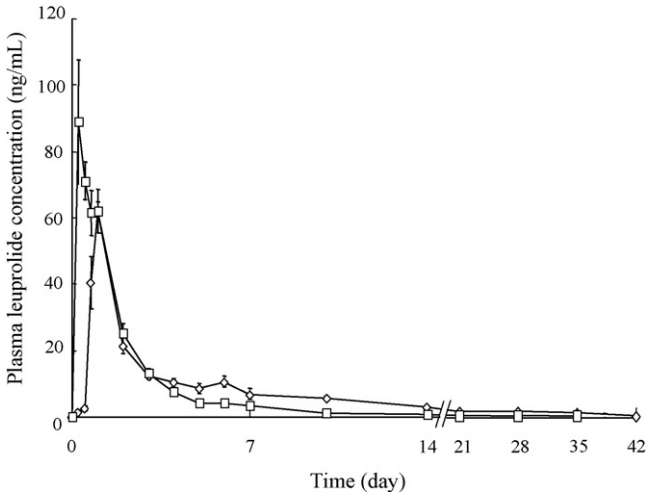

Fig. 8. Plasma leuprolide concentration *vs.* time curves after subcutaneous administration of 3LMC to rats, 1.0 mg. (◊) PCL40/PLA (5:1) and (□) PCL70/PLA (5:1). Each point represents the mean ± S.E. (n = 4–6).

vitro release experiment was also performed. In the case of PCL40, 64.1 ± 3.7% of leuprolide was released within 5 weeks (Fig. 7), and sustained release characteristics was obtained. To confirm the long-term sustained release characteristics of leuprolide, *in vivo* pharmacokinetic experiment was performed with 3LMC having two kinds of blend surface membrane, PCL70/PLA (5:1) membrane and PCL40/PLA (5:1) membrane, and the results are shown in Fig. 8. After the administration of 3LMC to rats, plasma leuprolide concentration reached C_{max} at 6 h for 3LMC having PCL70/PLA membrane and at 24 h for 3LMC having PCL40/PLA membrane, respectively. Pharmacokinetic parameter values are shown in Table 4. C_{max} of PCL40/PLA system was lower than that of PCL70/PLA system and AUC of PCL40/PLA system was higher than that of PCL70/PLA system. Therefore, PCL40/PLA system showed higher BA, 95.8 ± 1.5%, than PCL70/PLA system, 77.3 ± 4.5%. By comparing to the plasma leuprolide concentration *vs.* time curves obtained after sc injection of leuprolide acetate microspheres (Okada et al., 1991), 3LMC was revealed to have the same long-term sustained release property.

4. Discussion

The 3LMC was designed as a reserver type drug delivery system to diminish the burst release. At first, 3LMCs having different surface membrane were prepared where FDs were encapsulated and *in vitro* release profile was studied. As PCL membrane prevented the permeation of FDs, release rates of FD-4 and FD-20 were increased by formulating Tween60 into PCL membranes. In this case, by the addition of Tween60 to PCL membrane of hydrophobic polymer, the hydrophilicity of the obtained membrane was increased and the membrane permeability and water uptake were also increased (Ginty et al., 2008). However, the release rate of FD was dependent on its molecular weight by comparing FD-4 with FD-20 in the same blend membrane of PCL + Tween60. The release rate of higher molecular weight was found to be slower. Several models have been reported to explain the mechanism of drug release from biodegradable (erodible) system. However, they can be classified into two categories, i.e. surface-eroding system and bulk-eroding degradable system (Lao et al., 2008). On the other hand, drug release from 3LMC was thought to obey the subsequent three phases: (1) by absorbing water from the outside, drug layer swells and the pressure difference between the inside and outside the system accelerated the initial drug release, (2) drug was released with zero-order manner due to the concentration gradient between inside and outside of the system and (3) finally drug was released due to the degradation of the surface membrane. Initial burst release occurred at the initial release phase as shown above (1) and was affected with two factors. One was the hydrophilicity of the surface membrane which was controlled by properly selecting the formulated amount of Tween60. Second factor was the viscosity of the drug layer. As the main purpose of this study was to find out the optimum formulation of the surface layer, formulation of drug layer will be studied in the next step.

When leuprolide acetate was encapsulated into 3LMC, release rate was increased by increasing the amount of Tween60 to PCL70, though the initial burst release was not completely excluded in the *in vitro* experiment. To ascertain the efficiency of 3LMC system, leuprolide acetate encapsulated 3LMC was subcutaneously administered to rats where PCL70 + 0.3% Tween60 surface membrane was used. After administration, plasma leuprolide concentration transiently increased and thereafter decreased gradually. Finally, plasma concentration reached to the steady state at 2–13 weeks where the plasma leuprolide level was maintained over its minimum effective concentration, 0.4–1.0 ng/ml (Okada et al., 1991). These *in vivo* pharmacokinetic profile well corresponded to the result obtained in the *in vitro* release experiment. In addition, damages like irritation and/or inflammation were not observed at the administered site of the rat skin and 3LMC system was suggested to have a good biocompatibility. At 3 months and 1 year after administration of 3LMC having PCL surface membrane, 3LMCs were recovered from the rat skin and it was confirmed by a microscopic observation that 3LMCs were not degraded as reported by Sun et al. (2006) that the degradation rate of PCL was extremely lower than that of PLA, poly(glycolic)acid and their copolymer, PLG, poly(lactide-co-glycolide). Therefore, when long-term sustained release preparation is designed with 3LMC, we must properly select the biodegradable polymer. For example, in the case of 1 or 3 month sustained release preparation, the polymer must degrade within 1–2 months or 3–5 months in the body. Therefore, PLA was blended to PCL70 and PCL40 with the molar ratio of 1:5 and surface membranes were prepared. *In vitro* release experiment showed an initial burst release with PCL70 + PLA membrane. On the other hand, PCL40 + PLA membrane did not show big initial burst release and leuprolide was released almost the same pattern observed with PCL70 + 0.3% Tween60 membrane. Zhang and Feng (2006) reported that the *in vitro* drug release from nanoparticles was affected by drug diffusion, polymer swelling, polymer erosion or degradation. The faster drug release may be explained by the

higher hydrophilicity of the copolymers, which made the copolymer swell and degrade faster (Zhang and Feng, 2006). Indeed, in this experiment, as the hydrophilicity was increased by adding Tween60 or PLA into PCL70, the amount of drug released was increased. However, PCL40/PLA membrane was founded to have lower release profile and decreased the initial burst release. This suggested that the drug release was affected by the degree of the hydrophilic group rather than the crystallinity of the membrane measured by the thermal analysis. In other words, release rate was affected by these factors: (1) the crystallinity, (2) hydrophilicity of the surface membrane and (3) the hydrophilicity of the drug inside.

In vivo rat experiment showed high plasma leuprolide concentration due to burst release. Singh and Singh (2007) also studied the polymer delivery system for long-term sustained release of leuprolide, where DL-PLA was blended with benzyl benzoate and benzyl alcohol and successful plasma leuprolide concentration was maintained up to 12 weeks. However, in our experiment, sustained release of leuprolide did not continue more than 10 weeks by blending PLA to PCL. By blending PLA having shorter degradation half-life to PCL, the obtained surface membrane showed higher degradation rate than PCL70 + 0.3% Tween60 surface membrane and as a result enough plasma leuprolide did not continue more than 5 weeks. This higher degradation rate was ascribed to the biodegradable polymer, PLA. Therefore, long-term sustained release of leuprolide might be controlled by properly selecting the formulation of the surface membrane.

5. Conclusion

Three-layered milli-capsules (3LMC) were designed for the long-term sc administration of drugs. 3LMCs composed of (1) surface membrane (release rate control membrane), (2) drug-carrying layer and (3) base membrane were prepared by dispensing each solution in series. As surface membrane, poly(ε-caprolactone), PCL70, was used in combination with Tween60. Base membrane was prepared with PCL70. FD-4 and FD-20 were used as model drug. As Tween60 content increased, release rate of FDs was increased. PCL70 + 0.3% Tween60 membrane showed a good sustained release property for FD-4. When FD-20 was encapsulated, release rate was decreased. However, lower MW drug, leuprolide acetate was encapsulated, 3LMC composed of PCL70 + 0.3% Tween60 showed a good sustained release property, $63.0 \pm 5.9\%$ release for 5 weeks. After sc administration to rats, plasma leuprolide concentration showed its maximum concentration at day 1, thereafter gradually decreased and maintained the effective concentration, 0.4–1.0 ng/ml, for 14 weeks. Plasma leuprolide concentration *vs.* time curve showed a good dose-dependency. When surface membrane was made of the blend of PCL70 and PLA in the ratio of 5:1, long-term sustained release property was not obtained. Instead, PCL40 in combination with PLA (5:1) was used to prepare surface membrane, sustained release of leuprolide was observed for 5 weeks. PCL40/PLA surface membrane also showed better long-term sustained release property than PCL70/PLA membrane. Through those experiments, 3LMC has been shown to be a long-term sustained release preparation by properly selecting the surface membrane.

Acknowledgments

This study was supported by a strategic fund of MEXT (Ministry of Education, Culture, Sports, Science and Technology, MEXT) from 2008 to 2013 for establishing research foundations in Japanese private universities.

References

Allison, S.D., 2008. Analysis of initial burst in PLGA microparticles. Expert Opin. Drug Deliv. 5, 615–628.
Arshady, R., 1999. Microspheres, microcapsules and liposomes: general concepts and criteria. In: Microspheres, Microcapsules and Liposomes. Citus Books, London, pp. 11–45.
Eiamtrakarn, S., Ito, Y., Kishimoto, J., Yoshikawa, Y., Shibata, N., Murakami, M., Takada, K., 2002. Gastrointestinal mucoadhesive patch system (GI-MAPS) for oral administration of G-CSF, a model protein. Biomaterials 23, 145–152.
Freitas, S., Hans, P.M., Gander, B., 2005. Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology. J. Control. Release 102, 313–332.
George, S., 2007. Controlled patterning of peptide nanotubes and nanospheres using inkjet printing technology. J. Pept. Sci. 14, 217–223.
Ginty, P.J., Barry, J.J., White, L.J., Howdle, S.M., Shakesheff, K.M., 2008. Controlling protein release from scaffolds using polymer blends and composites. Eur. J. Pharm. Biopharm. 68, 82–89.
Glangchai, L.C., Caldorera-Moore, L., Shi, M., Roy, K., 2008. Nanoimprint lithography based fabrication of shape-specific, enzymatically-triggered smart nanoparticles. J. Control. Release 125, 263–272.
Hasan, A.S., Socha, M., Lamprecht, A., Ghazouani, F.E., Sapin, A., Hoffman, M., Maincent, P., Ubrich, N., 2007. Effect of the microencapsulation of nanoparticles on the reduction of burst release. Int. J. Pharm. 344, 53–61.
Ito, Y., Tosh, B., Togashi, Y., Amagase, K., Kishida, T., Sugioka, N., Shibata, N., Takada, K., 2005. Absorption of interferon alpha from patches in rats. J. Drug Target. 13, 383–390.
Lao, L.L., Venkatraman, S.S., Peppas, N.A., 2008. Modeling of drug release from biodegradable polymer blends. Eur. J. Pharm. Biopharm. 70, 796–803.
Li, S.P., Kowarski, C.R., Feld, K.M., Grim, W.M., 1988. Recent advances in microencapsulation technology and equipment. Drug Dev. Ind. Pharm. 14, 353–376.
Mathiowitz, E., Kreitz, M.R., 1999. Microencapsulation in Encyclopedia of Controlled Drug Delivery. John Wiley & Sons, Inc., New York, pp. 2, 493–546.
Matsumoto, A., Matsukawa, Y., Suzuki, T., Yoshino, H., 2005. Drug release characteristics of multi-reservoir type microspheres with poly(DL-lactide-co-glycolide) and poly(DL-lactide). J. Control. Release 106, 172–180.
Okada, H., Inoue, Y., Heya, T., Ueno, H., Ogawa, Y., Toguchi, H., 1991. Pharmacokinetics of once-a-month injectable microspheres of leuprolide acetate. Pharm. Res. 8, 787–791.
Singh, S., Singh, J., 2007. Phase-sensitive polymer-based controlled delivery systems of leuprolide acetate: in vitro release, biocompatibility, and in vivo absorption in rabbits. Int. J. Pharm. 328, 42–48.
Sinha, V.R., Bansal, K., Kaushik, R., Kumira, R., Trehan, A., 2004. Poly-ε-caprolacton microspheres and nanospheres: an overview. Int. J. Pharm. 278, 1–23.
Sofianos, Z.D., Katsila, T., Kostomitsopoulos, N., Balafas, V., Matsoukas, J., Tselios, T., Tamvakopoulos, C., 2008. In *vivo* evaluation and *in vitro* metabolism of leuprolide in mice—mass spectrometry-based biomarker measurement for efficacy and toxicity. J. Mass Spectrom. 43, 1381–1392.
Sun, H., Mei, L., Song, C., Cui, X., Wang, P., 2006. The *in vivo* degradation, absorption and excretion of PCL-based implant. Biomaterials 27, 1735–1740.
Takada, K., 2006. Oral delivery of haematopoietic factors: Progress with gastrointestinal mucoadhesive patches, microdevices and other microfabrication technologies. Am. J. Drug Deliv. 4, 65–77.
Takada, K., 2008. Microfabrication derived DDS: from batch to individual production. Drug Discov. Ther. 2, 140–155.
Venkatesan, N., Yoshimitsu, J., Ohashi, Y., Ito, Y., Sugioka, N., Shibata, N., Takada, K., 2006a. Pharmacokinetic and pharmacodynamic studies following oral administration of erythropoietin mucoadhesive tablets to beagle dogs. Int. J. Pharm. 310, 46–52.
Venkatesan, N., Uchino, K., Amagase, K., Ito, Y., Shibata, N., Takada, K., 2006b. Gastrointestinal patch system for the delivery of erythropoietin. J. Control. Release 111, 19–26.
Yeo, Y., Basaran, O.A., Park, K., 2003. A new process for making reservoir-type microcapsules using ink-jet technology and interfacial separation. J. Control. Release 93, 161–173.
Yeo, Y., Park, K., 2004. Control of encapsulation efficiency and initial burst in polymeric microparticle systems. Arch. Pharm. Res. 27, 1–12.
Zhang, Z., Feng, S.-S., 2006. *In vitro* investigation on poly(lactide)–Tween 80 copolymer nanoparticles fabricated by dialysis method for chemotherapy. Biomacromolecules 7, 1139–1146.