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Preparation and evaluation of biodegradable microspheres containing a new potent osteogenic compound and new synthetic polymers for sustained release

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ABSTRACT

In order to achieve the sustained release of 3-ethyl-4-(4-methylisoxazol-5-yl)-5-(methylthio) thiophene-2-carboxamide (BFB0261), a new potent osteogenic compound for the treatment of bone disorders, we prepared microspheres containing BFB0261 and newly synthesized three poly (D, L-lactic acid) (PLA), four poly (D, L-lactic acid–co-glycolic acid) (PLGA), and eight poly (D, L-lactic acid)-block-poly(ethylene glycol) (PLA-PEG) biodegradable polymers or copolymers, and evaluated the release pattern of BFB0261 from the microspheres *in vitro* and *in vivo*. The mean particle size of the microspheres, except for the microspheres constructed from PLA-PEG with a greater than 20% PEG component, was in the range of approximately 10–50 μm, and the preparations showed a spherical shape with a smooth surface. In an *in vitro* release study, the release of BFB0261 from PLA-1 (Mw: 36 kDa), PLAPEG9604H (PLA/PEG ratio: 96:4, Mw: 181 kDa), or PLAPEG8317 (PLA/PEG ratio: 83:17, Mw: 106 kDa) microspheres occurred in a zero-order manner with a slow release, and more than 50% of BFB0261 remained in each type of microsphere at 12 weeks after incubation. When the BFB0261 microspheres constructed from various polymers were intramuscularly administered to the rat femur, the microspheres constructed from PLA-1 or PLAPEG9604H were able to achieve a sustained release of BFB0261 at the injection site for 6 weeks. The present information indicates that microspheres constructed from PLA-1 or PLAPEG9604H may be feasible for bone engineering.

1. Introduction

Bone disorders, such as osteoporosis and its derived comminuted fractures are becoming increasingly prevalent as the aged population continues to grow. Serious bone defects are routinely treated by autogenic or allogeneic bone grafting (Stevens, 2008). Autogenic bone grafting is considered the golden standard procedure due to its immunocompatibility, and it generally results in a favorable clinical outcome, although several constraints remain such as the need for a second surgical procedure, possible morbidity, and a limited quantity of bone. Allogeneic bone grafting, the transplantation of bone obtained from human donors, is another option, but it has the potential to cause the transmission of donor pathogens and immunogenic responses, carries a high risk of infection, and involves high costs associated with a bone banking system. Due to the limitations associated with bone grafting, tissue engineering approaches have recently emerged as a potential alternative process for treating severely injured patients using a minimally invasive technique (Goulet et al., 1997; Sakou, 1998; Bishop and Einhorn, 2007; Fu et al., 2008).

Commonly, bone engineering strategies utilize a combination of biodegradable scaffolds and bioactive molecules such as growth factors and cytokines to recapitulate the natural processes of bone regeneration and development. Bone morphogenetic protein (BMP) is an effective growth factor that increases bone formation and recovery from diseases (Johnson et al., 1990; Ozkaynak et al., 1990; Cook and Rueger, 1996; Riley et al., 1996; Sakou, 1998). However, for enhancement of its effects, the sustained release of BMP is necessary at the damaged site. In addition, since very high doses of BMP are required, this results in high costs for BMP treatment. Therefore, in order to overcome these disadvantages of BMP, a new drug formulation of BMP combined with biodegradable and

Abbreviations: BFB0261, 3-ethyl-4-(4-methylisoxazol-5-yl)-5-(methylthio) thiophene-2-carboxamide; PLA, poly (D, L-lactic acid); PGA, poly (glycolic acid); PLGA, poly (D, L-lactic acid-co-glycolic acid); PLA-PEG, poly (D, L-lactic acid)-block-poly (ethylene glycol).

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biocompatible natural or synthetic polymers, which allows the sustained slow release of the active ingredient at the damaged site, has been developed.

Among the biodegradable and biocompatible natural polymers, type I collagen, which is a major component of bone and a suitable scaffold material, is one of the most widely used materials. Numerous studies have been conducted to examine the material properties of type I collagen in combination with BMP (Cook et al., 1995; Schimandle et al., 1995; Geiger et al., 2003). Furthermore, Takaoka et al. (1991) reported that a telopeptide, that elicited an immunogenic response when introduced into xenogenic hosts, depleted type I collagen may be an important carrier for the future clinical applications of BMP. However, the potential risk of collagen contamination with animal origin-derived pathogens persists. On the other hand, synthetic polymers pose little danger of eliciting immunogenicity or causing disease transmission in comparison to natural polymers. In addition, their characteristics such as strength, degradability, and adhesiveness can be altered to facilitate their clinical use. Their favorable characteristics for such uses as suture materials have prompted researchers to test their suitability as carriers for BMP. Biodegradable synthetic polymers such as polylactic acid (PLA), polyglycolic acid (PGA), and their copolymers (PLGA) are widely used as materials for growth factor delivery carriers in bone engineering. However, none has equaled collagen (Bostman, 1991; Hollinger and Leong, 1996; Jansen et al., 2005). Therefore, the development of new synthetic biodegradable polymers that are superior to collagen as BMP or other active ingredient delivery systems is required.

We recently determined that 3-ethyl-4-(4-methylisoxazol-5-yl)-5-(methylthio) thiophene-2-carboxamide (BFB0261), a novel compound, enhances bone formation. BFB0261 elevated the activities of alkaline phosphatase, an index of bone differentiation, in a concentration dependent manner in human osteoblastic cells. Moreover, treatment with BFB0261 also raised calcification in the cell. Based on these results, we expected that BFB0261would enhance bone regeneration instead of BMP. In the present study, we synthesized new synthetic biodegradable polymers, namely three PLA polymers of various molecular weights (PLA-1, PLA-2, PLA-3), four PLA-PGA (PLGA) copolymers of various molecular weights with various PLA/PGA molar ratios (PLGA7723, PLGA7030, PLGA6139, and PLGA6040), and eight PLA-PEG block copolymers of various molecular weights with various PLA/PEG molar ratios (PLAPEG9604H, PLAPEG9604L, PLAPEG8812, PLAPEG8515H, PLAPEG8515L, PLAPEG8317, PLAPEG8020 and PLAPEG7525), and prepared microspheres containing the various copolymers and BFB0261, as an active ingredient, to achieve a six-month sustained release. The surface morphology of the microspheres was assessed by scanning electron microscopy (SEM). We then determined the *in vitro* and *in vivo* release of BFB0261 from the microspheres constructed from the various copolymers.

2. Materials and methods

2.1. Materials

New synthetic polymers such as poly (D, L-lactic acid) (PLA) polymers: PLA-1 (weight average molecular weight (Mw): 36 kDa), PLA-2 (Mw: 37.3 kDa), and PLA-3 (Mw: 69.5 kDa); poly (D, L-lactic acid-co-glycolic acid) (PLGA) polymers: PLGA7723 (PLA/PGA ratio: 77: 23, Mw: 217 kDa), PLGA7030 (PLA/PGA ratio: 70:30, Mw: 21 kDa), PLGA6139 (PLA/PGA ratio: 61:39, Mw: 128 kDa), and PLGA6040 (PLA/PGA ratio: 60:40, Mw: 49.3 kDa); and poly (D, L-lactic acid)-block-poly(ethylene glycol) (PLAPEG) polymers: PLAPEG9604H (PLA/PEG ratio: 96:4, Mw: 181 kDa), PLAPEG9604L (PLA/PEG ratio: 96:4, Mw: 27 kDa), PLAPEG8812 (PLA/PEG ratio: 88:12, Mw: 32.5 kDa), PLAPEG8515H (PLA/PEG ratio: 85:15, Mw: 51.1 kDa), PLAPEG8515L (PLA/PEG ratio: 85:15, Mw: 8.76 kDa), PLAPEG8317 (PLA/PEG ratio: 83:17, Mw: 106 kDa), PLAPEG8020 (PLA/PEG ratio: 80:20, Mw: 33.7 kDa), PLAPEG7525 (PLA/PEG ratio: 75:25, Mw: 25.5 kDa), PLAPEG1113 (PLA/PEG ratio: 88:12, Mw: 41.4 kDa) and PLAPEG1115 (PLA/PEG ratio: 87:13, Mw: 36.5 kDa) were supplied by Taki Chemical Co. Ltd. (Hyogo, Japan). Their molecular characteristics are summarized in Table 1.

Table 1
Polymer composition and physical properties.

Name	Polymer molar ratio			Mw[a] (kDa)	Mn[b] (kDa)	Mw/Mn
	PLA (%)	PGA (%)	PEG (%)			
PLA0020	100	–	–	19.9	11.1	1.81
PLA-1	100	–	–	36	25	1.44
PLA-2	100	–	–	37.3	14.4	2.59
PLA-3	100	–	–	69.5	15.2	4.57
PLGA8515	85	15	–	15	–	–
PLGA7723	77	23	–	217	127	1.71
PLGA7515	75	25	–	15	–	–
PLGA7030	70	30	–	21	14	1.5
PLGA6139	61	39	–	128	72	1.78
PLGA6040	60	40	–	49.3	11.3	4.36
PLAPEG9604H	96	–	4	181	129	1.4
PLAPEG9604L	96	–	4	27	24	1.13
PLAPEG8812	88	–	12	32.5	29.3	1.11
PLAPEG8515H	85	–	15	51.1	36.8	1.39
PLAPEG8515L	85	–	15	8.76	7.07	1.24
PLAPEG8317	83	–	17	106	79.3	1.33
PLAPEG8020	80	–	20	33.7	27.2	1.24
PLAPEG7525	75	–	25	25.5	20.9	1.22
PLAPEG1113	88	–	12	41.4	35.9	1.15
PLAPEG1115	87	–	13	36.5	32.8	1.12

[a] Mw: weight average molecular weight.
[b] Mn: number average molecular weight.

BFB0261 was synthesized at Medicinal Chemistry Laboratories of Taisho Pharmaceutical Co. Ltd. (Saitama, Japan). PLA0020 (Mw: 19.9 kDa), PLGA8515 (PLA/PGA ratio: 85:15, Mw: 15 kDa), and PLGA7515 (PLA/PGA ratio: 75:25, Mw: 15 kDa) were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan); poly (vinyl alcohol) (PA-05GP) was obtained from the Shin-Etsu Chemical Co. Ltd. (Tokyo, Japan); and alpha minimum essential medium (α-MEM) was acquired from Irvine Scientific (CA, USA). All of the reagents used were of the highest grade available from commercial sources.

2.2. Preparation of microspheres

The microspheres constructed from various PLA, PLGA, or PLA-PEG polymers in the presence or absence of BFB0261 were prepared by an oil-in-water (o/w) emulsion solvent evaporation method (Gabor et al., 1999). The preparation method is illustrated in Fig. 1. Briefly, 10 mg of BFB0261 and 2 g of polymers (PLA, PLGA, or PLA-PEG) were dissolved in 10 ml of chloroform (trichloromethane). This organic solution was added to 100 ml of 0.1% (w/v) poly(vinyl

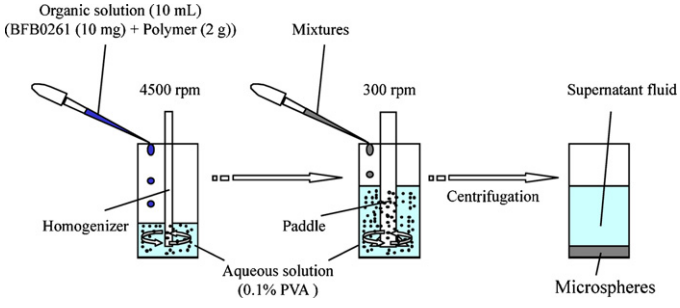

Fig. 1. Scheme of microsphere preparation method.

alcohol) (PVA) aqueous solution at 5 °C. The mixture was homogenized at 4500 rpm for 5 min at 5 °C to generate an oil-in water (o/w) emulsion. The obtained emulsions were added to 200 ml of 0.1% (w/v) PVA aqueous solution, and then the mixtures were aspirated at room temperature for 24 h to evaporate the chloroform under stirring conditions (300 rpm). After removing large particles by sieving (150 μm), the resulting microspheres were collected by centrifugation and washed 3 times with distilled water to remove the PVA. Microspheres, suspended in a small amount of distilled water, were immediately frozen at −40 °C for 3 h, before being freeze-dried for 38 h. The freeze-dried microspheres were stored at 5 °C until use.

2.3. Evaluation of the physicochemical properties of BFB0261 microspheres

2.3.1. Scanning electron microscopy

The surface structure of the microspheres was morphologically assessed using a scanning electron microscope (SEM) (Model: S-2500, Make: Hitachi, Tokyo, Japan). The samples were placed on double-sided adhesive tape, which had been previously applied to an aluminum stub. The excess samples were removed, and the samples were sputter coated with platinum/palladium under argon gas prior to imaging.

2.3.2. Particle size distribution

The particle sizes of the microspheres were measured using an optical microscope and were expressed as the mean volume diameter using a Luzex-F (Nireko, Japan).

2.3.3. Determination of BFB0261concetentration in the microspheres

The concentrations of BFB0261 in the microspheres were determined using an HPLC system (Hitachi, Tokyo, Japan), consisting of a pump and a UV detector set at 253 nm. The analytical column was a reverse phase C18 column (CAPCELL PAK C18 UG250, 5 μm, 4.6 mm × 150 mm, Shiseido, Tokyo, Japan), and the column temperature was maintained at 40 °C. The mobile phase was a mixture of acetonitrile: 0.01 M ammonium acetate (70:30 (v/v)), and the flow rate was 1.0 ml/min. Aliquots of 10 μl of supernatant were injected, and the concentration was calculated from a standard curve. Ten milligram of the freeze-dried microspheres was ultrasonically dissolved in 5 ml of acetonitrile for 10 min. The resulting solution was then centrifuged for 10 min at 15,000 rpm. The supernatant was injected into the HPLC system to determine the concentration of BFB0261. The recovery ratios of microspheres (%), encapsulation efficiency (%), and remaining drug in the outer solution (%) were obtained based on the following equations:

$$\text{Microsphere (MS) recovery (\%)} = \frac{\text{Microsphere (g)}}{\text{Drug (g)} + \text{ Polymer (g)}} \times 100$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Drug in microsphere (g)}}{\text{Total drug (g)}/(\text{Total drug (g)} + \text{Polymer (g)})} \times 100$$

$$\text{Drug in outer solution (\%)} = \frac{\text{Drug in outer solution(g)}}{\text{Total drug (g)}} \times 100$$

2.4. Sterilization of BFB0261 microspheres

Before using the BFB0261 microspheres for *in vitro* and *in vivo* studies, the microspheres were sterilized with UV light for 30 min. In order to compare the usefulness of UV light irradiation to that of other sterilization procedures, gamma-ray burst was performed on the microspheres constructed from PLAPEG1115 (PLA/PEG ratio: 87:13, Mw: 36.5 kDa).

2.5. In vitro drug release studies

The *in vitro* BFB0261 release studies were performed using a transwell (24-well) chamber without agitation. When we determined the biological effects of BFB0261 microspheres using the osteoblast cells, α-MEM containing 10 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) (pH 7.0) buffer was used as a standard culture medium (data not shown). Therefore, to reflect the results of *in vitro* biological assays in the results of *in vitro* drug release, α-MEM buffer containing 10 mM HEPES buffer was chosen as an optimal buffer to determine the *in vitro* BFB0261 release. BFB0261 microspheres (10 mg) were placed in the donor (upper) chamber, and 500 μl of α-MEM buffer was added to the donor and the receiver chambers, respectively, and incubated at 37 ± 1 °C. After incubation for the indicated times, the transwell chamber was centrifuged at 3000 rpm for 10 min, and the supernatant was collected from each side as a test sample. 500 μl of fresh release medium was then added to the donor and the receiver chambers to continue the drug release study. An aliquot (500 μl) of the supernatant was centrifuged at 15,000 rpm for 10 min. Thereafter, aliquots (40 μl) of the supernatant were added and mixed with 160 μl of acetonitrile. The mixture was centrifuged at 15,000 rpm for 10 min. The concentration of BFB0261 in the final supernatant was measured using a HPLC system as noted above (Section 2.3.3). All of the release tests were performed in triplicate.

2.6. In vivo release studies

2.6.1. Animals

Six-week-old female Wister rats were purchased from Charles River Japan Inc. (Atsugi, Japan). The animals were maintained under conventional housing conditions (23 ± 3 °C, 50 ± 20% relative humidity) and lighting (lights on, 7:00–19:00)), and were used after at least five days of acclimation. All the animal experiments reported here were reviewed and approved by the Taisho Pharmaceutical Animal Care Committee and conformed to the Japanese Experimental Animal Research Association Standards defined in the Guidelines for Animal Experiments (1987).

2.6.2. Experimental methods

The *in vivo* release of BFB0261 from the microspheres was evaluated by determining the residual concentration of BFB0261 at the injection sites. The microspheres constructed from PLA-1, PLGA6040, PLGA7030, PLGA7515, PLGA8515, PLAPEG8812, PLAPEG9604L, and PLAPEG9604H were intramuscularly administered to the femur or fibula of rats under anesthetized conditions. At the indicated times, the rats were sacrificed with a lethal dose of ether. The muscles were extirpated from the injection site, weighed, and frozen. The muscles were minced using scissors, soaked in 4 volumes of 90% (v/v) acetonitrile aqueous solution, and sonicated for 10 min. The minced muscles were homogenized and sonicated for 10 min. The homogenized muscle solutions were centrifuged at 15,000 rpm for 10 min. The amount of BFB0261 in the supernatant was measured using a HPLC system as noted above (Section 2.3.3). All of the release tests were performed four–eight times.

2.7. Statistics

Statistical analyses were performed using the Student *t*-test. A probability value of $p < 0.05$ was considered to indicate statistical significance.

Table 2
Characterization of BFB0261 microspheres.

Name	MS recovery (%)	Encapsulation efficiency (%)	Drug in outer solution (%)	Total recovery (%)	Particle size[a] (μm)
PLA0020	70.4	82.3	19.4	101.7	18.3 ± 8.2
PLA-1	68.2	78.1	18.6	96.7	8.8 ± 3.1
PLA-2	65.6	75.4	19.5	89.2	17.4 ± 14.1
PLA-3	60.0	73.0	16.2	94.9	20.8 ± 10.8
PLGA8515	50.4	78.3	19.7	98.0	22.3 ± 9.2
PLGA7723	61.5	69.8	19.7	89.5	54.9 ± 35.8
PLGA7515	63.2	81.9	16.5	98.4	21.2 ± 10.9
PLGA7030	70.0	74.7	20.2	94.9	24.9 ± 13.4
PLGA6139	67.5	71.8	22.2	94.0	35.3 ± 10.2
PLGA6040	67.6	75.2	17.9	93.1	33.3 ± 18.2
PLAPEG9604H	56.8	75.9	24.4	100.3	37.7
PLAPEG9604L	59.4	77.7	–	–	30.5 ± 17.6
PLAPEG8812	68.8	75.0	–	–	27.4 ± 10.9
PLAPEG8515H	69.6	75.5	22.2	102.2	43.8
PLAPEG8515L	41.2	80.0	–	–	–
PLAPEG8317	65.9	72.9	–	–	–
PLAPEG8020	–	–	–	–	–
PLAPEG7525	–	–	–	–	–

[a] Particle sizes are expressed as mean ± S.D. (n = 3–5).

3. Results and discussion

3.1. Preparation and evaluation of the physicochemical properties of BFB0261 microspheres constructed using various synthetic polymers

The composition and physical properties of the various PLA, PLGA, and PLA-PEG polymers used in this study are listed in Table 1, and the process parameters such as the recovery of the microspheres, drug encapsulation efficiency, and drug recovery in outer solution for each preparation are shown in Table 2. In all of the preparations, the drug encapsulation for each microsphere was approximately 70–80%, and the drug recovery in the outer solution (%), which was dissolved in the outer aqueous phase during liquid drying methods, was approximately 20–30%, thus showing complete recovery of the drug in each experiment. The recovery of microspheres was shown to be 50–70% for all preparations. For the mean particle sizes of the microspheres (Table 2), all of the microspheres constructed from PLA and PLGA polymers were in the range of approximately 10–50 μm, which is considered to be an adequate size (Hoshino et al., 2000). For the microspheres constructed from the PLA-PEG polymers, the mean particle sizes of microspheres constructed from PLAPEG9604H, PLAPEG9604L, PLAPEG8812, and PLAPEG8515H with a PEG proportion of between 4 and 15% were in the range of approximately 30–45 μm. However, when the proportion of the PEG component exceeded 20%, it became technically difficult to prepare these microspheres.

Representative SEM photographs of microspheres constructed from PLA and PLGA polymers are shown in Figs. 2 and 3, respectively. Both types of microsphere were spherical with a smooth surface, and no aggregation was observed. To date, numerous studies of biodegradable microspheres prepared with PLA or PLGA polymers have been conducted, and such microspheres have been reported to have a spherical shape and a smooth surface

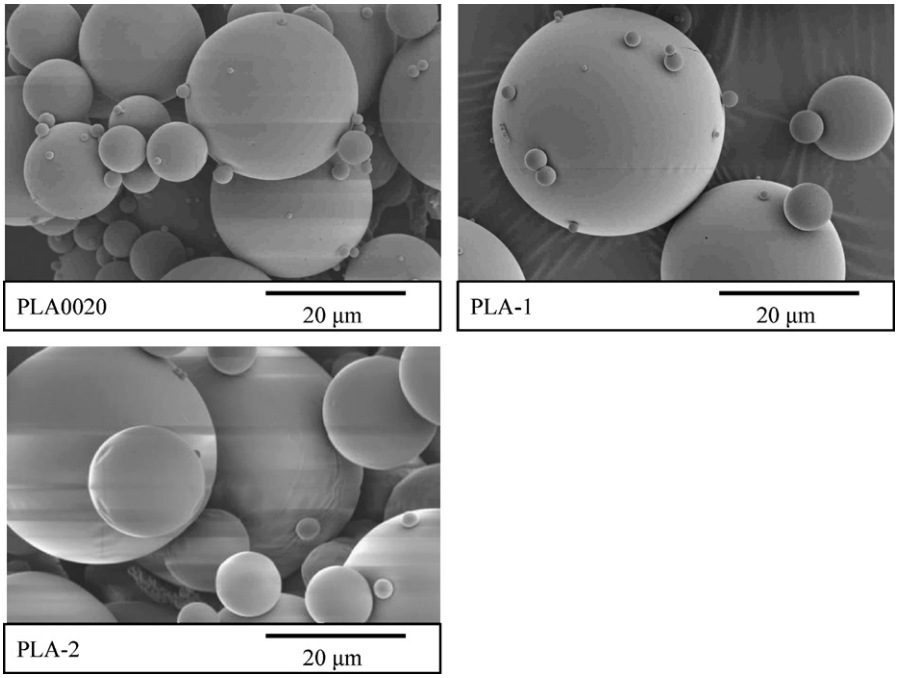

Fig. 2. Scanning electron microscope photographs of BFB0261 microspheres constructed from PLA polymers.

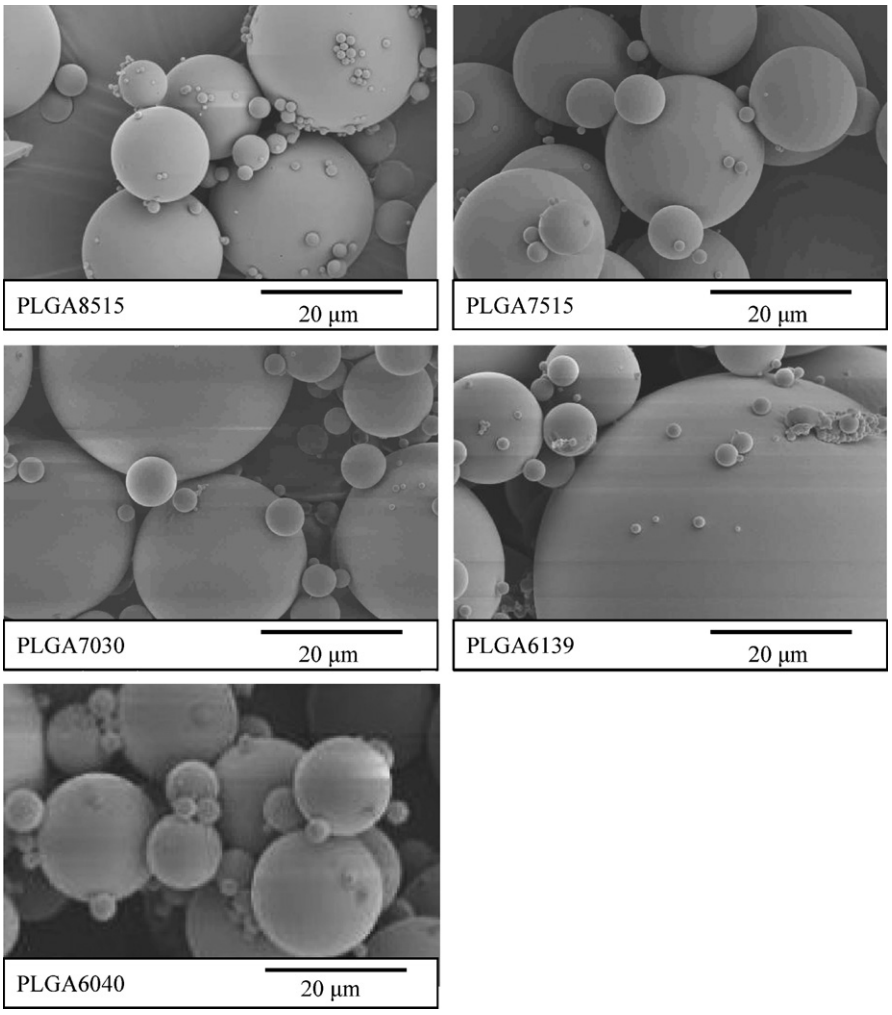

Fig. 3. Scanning electron microscope photographs of BFB0261 microspheres constructed from PLGA polymers.

(Shenderova et al., 1997; Matsumoto et al., 2008). Therefore, we thought that when preparing the microspheres using PLA or PLGA derivatives, spherical microspheres with a smooth surface would be obtained even if their mean molecular weight (Mw) and PLA/PEG ratio varied.

Fig. 4 shows SEM photographs of microspheres incorporating different proportions of PEG (4%, 12%, 15%, 17%, 20% and 25%). As the proportion of the PEG component increased, the porous region of the surface of the microspheres increased. The microspheres constructed of PLAPEG8020 (20% ratio of PEG) formed spheres packed with fibrous solids, which have poor dispersion properties in water, and aggregations were observed (Fig. 4E). In addition, although we tried to prepare microspheres constructed from PLAPEG7525 (25% ratio of PEG), we failed to obtain a spherical shape, and only fibrous aggregates tangled with each other were observed (Fig. 4F), suggesting that when the molar proportion of PEG in the polymer exceeds 20%, the system used in this study can no longer maintain a spherical form. This phenomenon is explained by the interaction between the strongly hydrophilic PEG and the water layer. During the preparation of the microspheres (Fig. 1), BFB0261 and polymers dissolved in the organic solvent were homogenized in the water layer to generate an emulsion state; subsequently, the solvent was evaporated under reducing pressure conditions, and consequently, the surface of the PEG polymer was in direct contact with the water layer. Therefore, as the proportion of the PEG component in the polymer increases, the affinity between the PEG and water layers becomes stronger such that it expands the contact surface area, resulting in the microsphere surface becoming 'frilly'.

3.2. Examination of the sterilization procedures when preparing the microspheres

For the clinical utilization of microspheres, an appropriate strategy for sterilizing the BFB0261/polymer composition is necessary. Recently, Moioli et al. (2006) reported that, in a comparison of sterilization procedures such as ethylene oxide (EO) gas, radio-frequency glow discharge (RFGD), and UV light, the release rate of TGF-β, the active ingredient from the microparticles, was significantly altered by UV irradiation while minimal effects were observed after EO gas or RFGD treatment, suggesting that the sterilization method should be carefully selected to achieve desirable drug release kinetics. Therefore, using microspheres constructed from PLAPEG1115 (PLA/PEG ratio: 87:13, Mw: 36.5 kDa, Mn: 32.8 kDa), which has almost the same characteristics as PLAPEG8812, the effects of gamma-ray burst (10 kGy) or UV light irradiation on the microspheres, particularly with respect to the *in vivo* release of BFB0261, were examined (Fig. 5). While the proportion of the residual drug remaining in the microspheres treated with gamma-ray burst was approximately 30% at 1 week after the intramuscular administration to the rat femur, that of the microspheres treated with UV light was shown to be about 55% ($p < 0.05$ versus that of gamma-ray burst). In addition, Table 3 shows

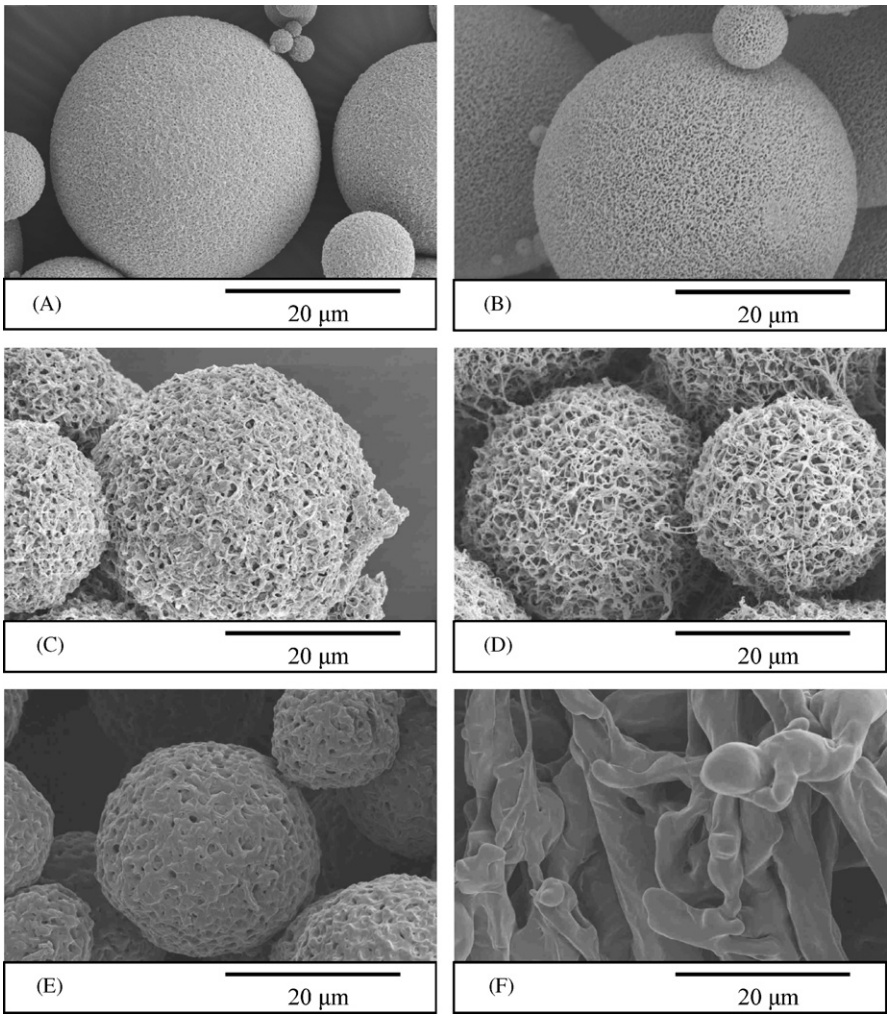

Fig. 4. Scanning electron microscope photographs of BFB0261 microspheres constructed from PLA-PEG polymers. (A) PLAPEG9604L (a molar ratio of 96:4), (B) PLAPEG8812 (a molar ratio of 88:12), (C) PLAPEG8515H (a molar ratio of 85:15), (D) PLAPEG8317 (a molar ratio of 83:17), (E) PLAPEG8020 (the molar ratio of 80:20), and (F) PLAPEG7525 (a molar ratio of 75:25).

the physical properties such as the Mw and the number average molecular weight (Mn) of PLAPEG1115 after UV light irradiation or gamma-ray burst (10 kGy). The UV light irradiation treatment did not affect these parameters, while the Mn of PLAPEG1115 treated with gamma-ray burst was significantly lower than that of the control or UV light, and the ratio of Mw/Mn of PLAPEG1115 was higher than that of the others. Therefore, we thought that since polymer degradation was promoted by gamma-ray sterilization, fast release from the microspheres would be observed after gamma-ray sterilization. Based on this result, UV light irradiation was thought to be useful for sterilizing the microspheres in this study, and all preparations used in both the *in vitro* and *in vivo* experiments thereafter were sterilized by UV light.

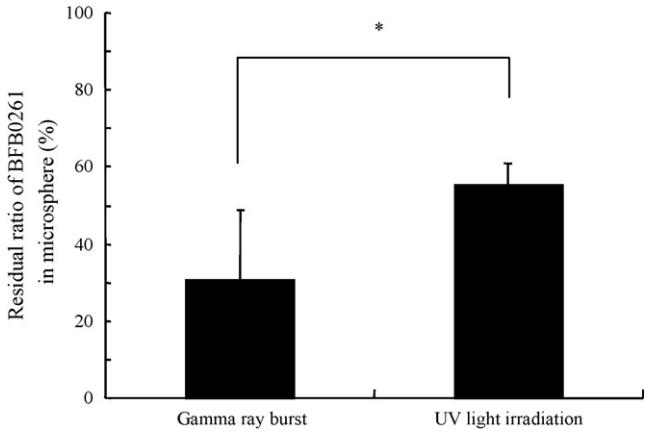

Fig. 5. Effect of sterilization procedures (gamma-ray burst and UV light irradiation) on the preparation of BFB0261 loaded microspheres. Each point represents the mean ± S.D. ($n = 4$). $^*p < 0.05$ versus gamma-ray burst.

3.3. *In vitro release properties of BFB0261 from the microspheres*

Fig. 6 shows the *in vitro* release of BFB0261 from the microspheres constructed from PLA polymers. As the Mw of PLA increased, the initial release of BFB0261 became more suppressed, and 12-weeks of sustainable release was observed for each microsphere. In particular, the release of BFB0261 from the microspheres constructed from PLA-1 and PLA-2 showed a zero-order manner,

Table 3
Effect of sterilization procedures on the physical properties of PLAPEG1115.

	Mw (kDa)	Mn (kDa)	Mw/Mn
PLAPEG1115 only	36.5	32.8	1.11
PLAPEG1115 + gamma-ray burst	34.5	29.1	1.19
PLAPEG1115 + UV light irradiation	36.5	32.7	1.12

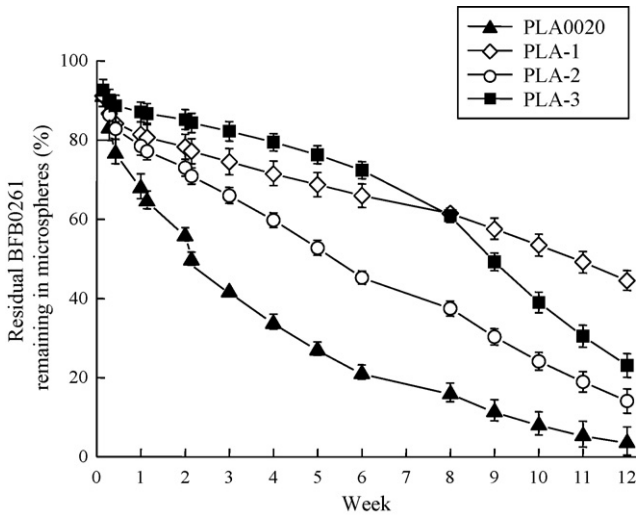

Fig. 6. Effect of the weight average molecular weight (Mw) of PLA polymers on the *in vitro* release of BFB0261 from PLA microspheres. Each point represents the mean ± S.D. (n = 3).

and the proportion of the residual drug remaining in the two microspheres at 12 weeks was shown to be approximately 45% and 15%, respectively. On the other hand, the microspheres constructed from a commercially used PLA polymer, PLA0020, showed a first-order manner, and the proportion of the residual drug remaining in the microspheres at 12 weeks was 4%. For the PLA-3 microspheres, two phases of release were observed before and after 8 weeks, and each phase obeyed a zero-order manner. The reason that PLA-3 showed a two-phase release may be explained by its wide molecular weight distribution. The Mw and Mn were approximately 69.5 and 15.2 kDa, respectively, thus showing a relatively wide molecular weight distribution (Mw/Mn = 4.57) (see Table 1). Since the Mw/Mn ratio of PLA-3 was so high in comparison to that of the other polymers, polymers with a smaller molecular weight may be initially decomposed, and subsequently the decomposition products may act as catalysts that accelerate the decomposition of large molecular weight polymers, and consequently, the PLA-3 microspheres may exhibit a two-phase release.

Fig. 7 shows the *in vitro* release of BFB0261 from the microspheres constructed from PLGA polymers. Unfortunately, each microsphere exhibited almost the same release pattern independently of the Mw of the polymers, and about 50% of BFB0261 had

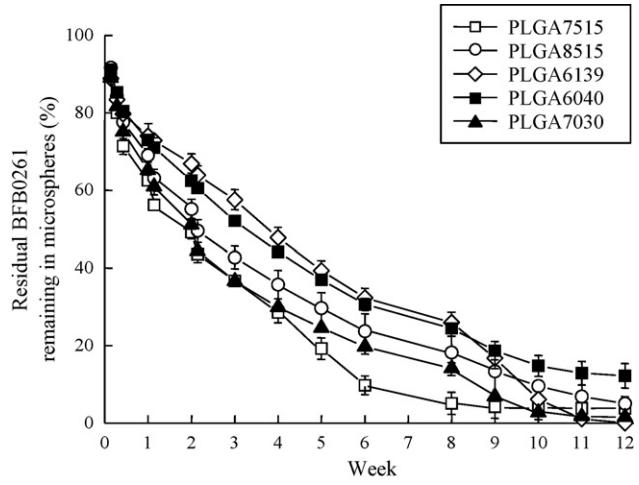

Fig. 7. Effect of the polymer composition of PLGA polymers on the *in vitro* release of BFB0261 from PLGA microspheres. Each point represents the mean ± S.D. (n = 3).

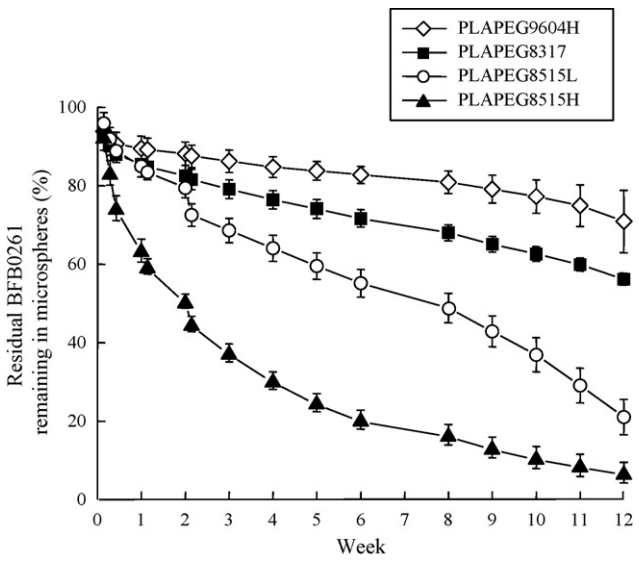

Fig. 8. Effect of the polymer composition of PLA-PEG polymers on the *in vitro* release of BFB0261 from PLA-PEG microspheres. Each point represents the mean ± S.D. (n = 3).

been released within 3 weeks. To date, it has been reported that acid substances, which are produced by the hydrolysis of microspheres constructed from PLGA polymers, accumulate in the interior or vicinity of the microsphere, and subsequently promote its degradation (Fu et al., 2000; Ding and Schwendeman, 2008). Therefore, we thought that even if the molecular weight or the molar proportion of PLGA were changed in the new synthetic polymers, then the decomposition of polymer would be promoted by the acid substances near to the microspheres, and consequently, would induce a quick release of BFB0261.

Fig. 8 shows the *in vitro* release of BFB0261 from the microspheres constructed from PLA-PEG polymers, and the sustained release of BFB0261 for 12 weeks was observed for all microspheres. The proportion of the residual drug remaining in the PLAPEG9604H microspheres at 12 weeks was approximately 70%. However, for the microspheres constructed from PLAPEG8515H, BFB0261 was quickly released from the microsphere (no less than 40% in 1 week). The release of the microspheres constructed from PLAPEG9604H, PLAPEG8317, or PLAPEG8515L followed a zero-order manner; whereas, that of PLAPEG8515H showed a first-order manner. In comparison to the ratio of PLA/PEG and the Mw of the preparations with a zero-order manner, as the molar proportion of PEG increased and the average molecular weight of the polymer decreased, the BFB0261 release rate increased. Based on these results, it was determined that the molar proportion of PEG and the mean molecular weight in PLA-PEG polymer should be adjusted to less than 15% and to more than 100 kDa, respectively, in order to achieve 12-weeks of sustainable release.

3.4. *In vivo BFB0261 release properties of the microspheres*

Fig. 9 shows the *in vivo* BFB0261 release patterns in the rat femur after the intramuscular administration of BFB0261 microspheres constructed from various polymers such as PLA-1, PLGA6040, PLGA7030, PLGA7515, PLGA8515, PLAPEG8812, PLAPEG9604L, and PLAPEG9604H. The microspheres containing the PLGA derivatives, PLGA6040, PLGA7030, PLGA7515, and PLGA8515, showed a fast and complete release of BFB0261 within 4 weeks. Since the proportion of residual drug remaining in their microspheres was also quite low after 3 weeks in the *in vitro* release study (Fig. 7), it was determined that these PLGA polymers cannot fully control the sus-

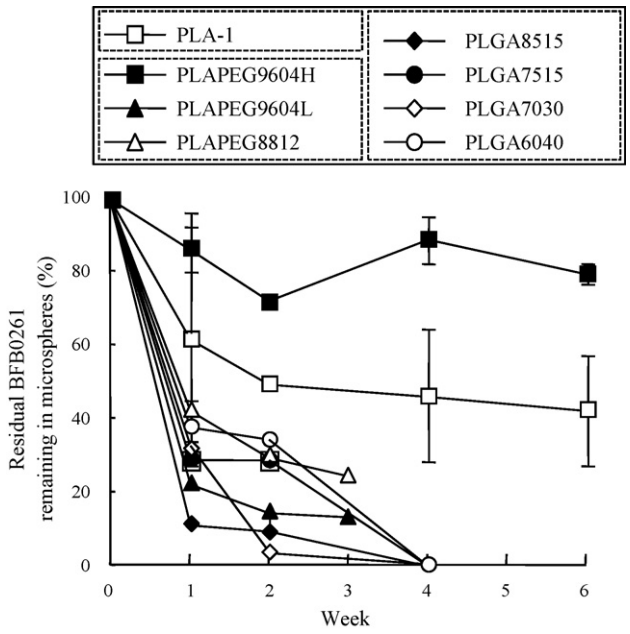

Fig. 9. *In vivo* BFB0261 release profiles of microspheres in the rat femur after their intramuscular administration. Microspheres containing 0.2 mg BFB0261 were constructed from various polymers such as PLA-1, PLGA6040, PLGA7030, PLGA7515, PLGA8515, PLAPEG8812, PLAPEG9604L, and PLAPEG9604H. Each point represents the mean ± S.E. (n = 2–4).

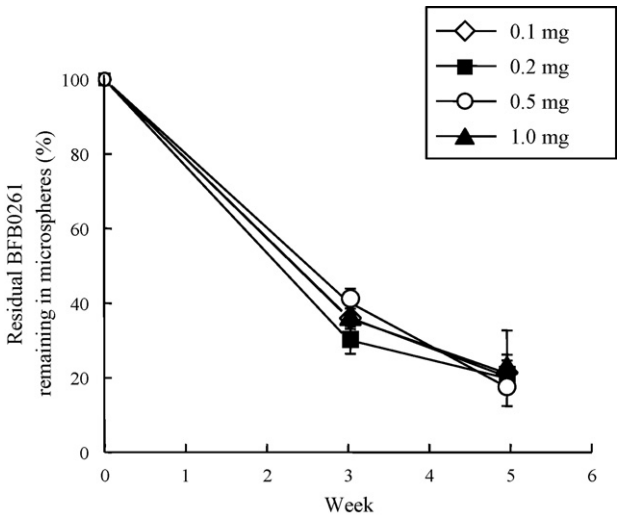

Fig. 10. *In vivo* BFB0261 release profiles of microspheres in the rat fibula after their intramuscular administration. Microspheres containing 0.1 mg, 0.2 mg, 0.5 mg, or 1 mg BFB0261 constructed from PLAPEG1113 (PLA/PEG: 88:12; Mw: 41.4 kDa; Mn: 35.9 kDa). Each point represents the mean ± S.E. (n = 8–10).

tained *in vivo* release of BFB0261. However, for the microspheres constructed from PLA-1 and PLAPEG9604H, the slow release of BFB0261 from the microspheres was observed, and the proportion of the residual BFB0261 remaining in the microsphere at 6 weeks was shown to be approximately 40% and 80%, respectively. The reason that PLAPEG9604H showed slow release in comparison to PLA-1 may be explained by its high molecular weight (Mw of PLAPEG9604H; 181 kDa versus that of PLA-1; 36 kDa). In addition, in the *in vitro* experiment (Figs. 6 and 8), the PLA-1 and PLAPEG9604H microspheres showed a zero-order release manner, and approximately 40% and 70% of the drug remained in the microspheres at 12 weeks, thus indicating that the *in vitro* result reflected the results of the *in vivo* experiment. Since the estimated release rates of BFB0261 calculated by the *in vivo* release study were approximately 6.6% per week for that of PLA-1 and 3.3% per week for that of PLAPEG9604H, it is expected that microspheres constructed from PLA-1 or PLAPEG9604H would be able to control the sustained release of BFB0261 for 10 weeks and 30 weeks, respectively, at the injected site.

Generally, for the driving force behind BFB0261 release from microspheres, there are two possibilities: one is the control of diffusion by new synthetic polymers, and the other one involves the concentration gradient between the microsphere and the outer phase (Batycky et al., 1997; Siepmann and Siepmann, 2008). However, it remains unclear which pattern was involved in the slow drug release from the microspheres in this study. Therefore, we examined the effect of varying the drug load in the microspheres to clarify the concentration gradient between the microsphere and the outer phase. Fig. 10 shows the *in vivo* release of BFB0261 in the rat fibula after the intramuscular administration of 0.1 mg, 0.2 mg, 0.5 mg, or 1 mg BFB0261 per 40 mg microspheres constructed from PLAPEG1113 (PLA/PEG ratio: 88:12; Mw: 41.4 kDa; Mn: 35.9 kDa), which has almost the same characteristics as PLAPEG8812. As a result, all of the microspheres containing 0.1–1.0 mg BFB0261 showed similar release patterns even if the drug contents of the microspheres varied. Therefore, it was thought that new synthetic polymers, such as PLA-1 or PLAPEG9604H, are involved in the slow drug diffusion from microspheres in which they serve as an important driving force.

4. Conclusions

For the treatment of bone disorders such as osteoporosis derived comminuted fractures and bone defect caused by resection of maxilla with sarcoma, a new drug formulation of bioactive molecules combined with biodegradable and biocompatible polymers to sustain the slow release of the active ingredient at the damaged site is required. In the present study, we prepared microspheres containing BFB0261, a new potent osteogenic compound, and various biodegradable PLA, PLGA, and PLA-PEG polymers. The mean particle sizes of each microsphere constructed from PLA or PLGA polymers were in the range of approximately 10–40 μm, and both preparations had a spherical shape with a smooth surface. For the microspheres constructed from the PLA-PEG polymers, when the proportion of the PEG component was 4–15%, spherical microspheres were obtained, and the mean particle size was in the range of approximately 30–40 μm. However, as the proportion of the PEG component increased to over 20%, it became technically difficult to prepare microspheres with a smooth surface due to the strong hydrophilic properties of PEG. In the *in vitro* release study, the release profile of BFB0261 from each microsphere constructed from PLA-1, PLAPEG9604H, or PLAPEG8317 showed a zero-order manner with a slow release, and subsequently, more than 50% of BFB0261 remained in each type of microsphere at 12 weeks after incubation. Furthermore, the microspheres constructed from PLA-1 or PLAPEG9604H were able to achieve a sustained release of BFB0261 for 6 weeks *in vivo*. Therefore, it was found that microspheres constructed from PLA-1 or PLAPEG9604H are feasible for bone engineering.

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