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The release profiles and bioactivity of parathyroid hormone from poly(lactic-co-glycolic acid) microspheres

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Abstract

Poly(lactic-co-glycolic acid) (PLGA) microspheres containing bovine serum albumin (BSA) or human parathyroid hormone (PTH)(1–34) were prepared using a double emulsion method with high encapsulation efficiency and controlled particle sizes. The microspheres were characterized with regard to their surface morphology, size, protein loading, degradation and release kinetics, and in vitro and in vivo assessments of biological activity of released PTH. PLGA5050 microspheres degraded rapidly after a 3-week lag time and were degraded completely within 4 months. In vitro BSA release kinetics from PLGA5050 microspheres were characterized by a burst effect followed by a slow release phase within 1–7 weeks and a second burst release at 8 weeks, which was consistent with the degradation study. The PTH incorporated PLGA5050 microspheres released detectable PTH in the initial 24 h, and the released PTH was biologically active as evidenced by the stimulated release of cAMP from ROS 17/2.8 osteosarcoma cells as well as increased serum calcium levels when injected subcutaneously into mice. Both in vitro and in vivo assays demonstrated that the bioactivity of PTH was maintained largely during the fabrication of PLGA microspheres and upon release. These studies illustrate the feasibility of achieving local delivery of PTH to induce a biologically active response in bone by a microsphere encapsulation technique.

Keywords: Microspheres; PLGA; Degradation; Controlled release; PTH; Bioactivity

1. Introduction

Proteins and peptides have received extensive interest for their therapeutic applications in diverse clinical settings [1]. Many recent tissue engineering studies have also focused on the use of growth factors to stimulate cellular activity in vivo, and regulate tissue regeneration [2–4]. However, protein and peptide drugs in general have short plasma half-lives, are unstable in the gastrointestinal tract and also have low bioavailabilities due to their relatively large molecular weight and high aqueous solubilities. These properties prevent them from being effectively used clinically [5]. In order to achieve high administration efficacy of proteins and peptides, polymeric particulate carriers (micro- and nanospheres) have been developed as an effective way to control the release profile of the contained substance and to protect unstable biologically active molecules from degradation. Both natural and synthetic biodegradable polymers have been investigated for controlled drug release [6–9]. Among these polymers, poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) were found to be remarkable for their application in drug delivery due to their excellent biocompatibility and biodegradability through natural pathways [10–13]. Injectable and in situ formed microspheres have also been developed for the treatment of human diseases and animal health [14,15].

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PTH is a peptide hormone that has both anabolic and catabolic effects on bone, depending on the dosage regimes and the delivery patterns [16]. It is well accepted that high levels of circulating PTH lead to a catabolic response whereas either high doses intermittently or low doses continuously may lead to an anabolic response [17,18]. PTH(1–34) exhibits full biological activity of the full-length PTH(1–84) and has been shown to be effective by intermittent subcutaneous (sc) injection for the treatment of osteoporosis in Phase III clinical trials [19]. Alternative PTH delivery methods have been investigated recently with various successes. A delivery developed by Leone-Bay et al. showed that PTH(1–34) administrated orally remained biologically active. However, the bioavailability of PTH is low, only 5% and 2.1%, relative to sc administration in rats and monkeys, respectively [20]. The pulmonary route has also been evaluated in rats by means of intratracheal instillation or inhalation of dry powders and the bioavailabilities were 40% and 34%, respectively [21,22]. Other methods for intermittent PTH delivery include programmed administration by osmotic pump [23] and pulsatile transdermal administration [24]. Both of them showed anabolic actions of PTH on bone, equivalent to sc administration. While PTH has been used extensively via systemic administration for treating osteoporosis, there is relatively little work focusing on local delivery of PTH. Notably, these few studies have investigated PTH administered locally via a direct gene delivery which was found to be beneficial in the treatment of bony defects [25,26]. Gene therapy strategies, although promising, have limitations and approaches to deliver PTH peptide merit investigation of initial feasibility.

The double emulsion technique is one of the most popular methods used to encapsulate hydrophilic drugs, particularly protein and peptide drugs, into microspheres. In this study, microspheres with different sizes were prepared by double emulsion techniques. Both degradation and BSA release kinetics were examined in vitro. A recombinant peptide hormone, PTH(1–34) was also encapsulated in PLGA5050 micropheres to examine in vitro and in vivo bioactivity after initial release.

2. Materials and methods

2.1. Materials

PLGA polymers (Medisorb®, PLGA5050, LA:GA = 50:50, $\eta_{inh} = 0.75$; PLGA7525, LA:GA = 75:25, $\eta_{inh} = 0.76$; PLGA8515, LA:GA = 85:15, $\eta_{inh} = 0.78$) were purchased from Alkermes Inc. (Wilmington, OH). Poly(lactic acid) with inherent viscosity of 1.6 dl/g was purchased from Boehringer Ingelheim (Ingelheim, Germany). Lyophilized parathyroid hormone PTH(1–34) was obtained from Bachem Bioscience Inc. (Torrance, CA). Other chemicals used were poly(vinyl alcohol) (PVA) (88 mol% hydrolyzed, MW = 25,000) obtained from Polysciences Inc. (Warrington, PA); bovine serum albumin (BSA, Fraction V) from Sigma; dichloromethane from Aldrich Chemical Company (Milwaukee, WI).

2.2. Microsphere preparation and characterization

Microspheres of various sizes were fabricated by a typical double emulsion technique [27] using optimized formulation parameters as shown in Table 1. Briefly, 100 μl BSA (3% w/v) solution or PTH buffer solution (PTH in 4 mM HCl/0.1% BSA solution) was emulsified in a 10% polymer solution in dichloromethane (DCM), using a probe sonicator at an output power of 15 W (Virsonic 100, Cardiner, NY) for 20 s over an ice bath to form primary water-in-oil (w/o) emulsion. The w/o emulsion was gradually added into 250 ml aqueous PVA solution under stirring or sonication to form a water-in-oil-in-water (w/o/w) double emulsion. The solution was stirred at room temperature for 3 h to evaporate dichloromethane and then centrifuged to collect solid microspheres. The resultant microspheres were washed with distilled water three times and freeze dried. The overall morphology of the microspheres was examined using scanning electron microscopy (SEM) (Hitachi S3200, Tokyo, Japan) after gold coating of the microsphere samples on a stub.

Table 1
Effects of PVA concentration and homogenization strength on particle size and BSA loading efficiency

Polymer type	PVA concentration (%)	Stirring condition	Average size (μm)	BSA loading (w/w%)	Encapsulation efficiency (%)
PLGA5050	1	Mechanical stirring at 700 rpm	20–50	2.48 ± 0.26	82.6 ± 2.6
PLGA5050	1	Sonication (15 W)	<1	2.23 ± 0.16	74.2 ± 1.6
PLGA5050	5	Mechanical stirring at 700 rpm	2–5	2.59 ± 0.64	86.4 ± 6.4
PLGA7525	1	Mechanical stirring at 700 rpm	20–50	2.29 ± 0.12	76.3 ± 0.12
PLGA8515	1	Mechanical stirring at 700 rpm	20–50	2.33 ± 0.21	77.5 ± 0.21
PLLA	1	Mechanical stirring at 700 rpm	20–50	2.26 ± 0.32	75.4 ± 0.32

2.3. Degradation study of PLGA5050 microspheres

Preweighed PLGA5050 microspheres (about 10 mg) were placed in individual test tubes containing 1.0 ml of phosphate buffered saline (PBS, 0.1 M, pH 7.4). The tubes were kept in an incubator that was maintained at 37°C. At predetermined degradation intervals, the microspheres were collected by centrifugation, washed with distilled water to remove residual buffer salts, and dried to constant weight in a vacuum desiccator. Mass loss of the microspheres was determined gravimetrically. The surface morphology of degraded PLGA5050 microspheres was analyzed using SEM at 15 kV.

2.4. Protein loading and encapsulation efficiency

Ten-milligram BSA-loaded microspheres were hydrolyzed in a mixture of 0.9 ml of 1 M NaOH and 0.1 ml PBS with vigorous shaking at room temperature for 2 h. BSA standard solutions (0.1 ml) were also hydrolyzed by adding 0.9 ml 1 M NaOH using the same procedures. After hydrolysis, 1 ml 0.9 M HCl was added to neutralize the sample solutions. Protein concentrations were determined by MicroBCA method (Pierce, Rockford, IL). Protein loading and encapsulation efficiency were determined by Eqs. (1) and (2), respectively:

$$\text{Protein loading (w/w\%)} = \frac{\text{Amount of protein in microspheres}}{\text{Amount of microspheres}}, \quad (1)$$

$$\text{Encapsulation efficiency} = \frac{\text{Actual protein loading}}{\text{Theoretical protein loading}} = \frac{\text{Retained protein amount}}{\text{Initially loaded protein amount}}. \quad (2)$$

2.5. In vitro BSA release

In vitro BSA release profiles from PLGA microspheres were determined as follows. Ten milligrams microspheres were suspended in 2 ml PBS (0.1 M, pH = 7.4) with 0.02% sodium azide as preservative. The microsphere suspensions were incubated at 37°C without shaking. At designated times, 1 ml release medium was collected by centrifugation and replaced with equal amount of fresh PBS. The amount of released BSA was measured using a MicroBCA protein assay (Pierce, Rockford, IL).

2.6. PTH(1–34) immunoassay

The concentration of PTH(1–34) in the release medium was measured using a PTH(1–34) ELISA kit with PTH antibody coated wells using the manufacturer's recommendations (Immutopics, San Clemente, CA). Triplicate wells were used for each time point (first 24 h). Absorbance measurements read at 450 nm recorded by a microtiter plate reader were used to calculate the PTH concentrations by the log-logit method using the GraphPad Prism® program (GraphPad Software, San Diego, CA) with a standard curve.

2.7. In vitro PTH bioactivity assay

The in vitro bioactivity of PTH released from PLGA5050 microspheres was determined by adenylate cyclase stimulation assay and cAMP binding protein assay. The adenylate cyclase stimulation assay was performed as previously described [28]. Briefly, rat osteosarcoma cells (ROS 17/2.8) were plated in triplicate into 24-well plates and cultured to confluence. To stimulate PTH receptor-mediated adenylate cyclase, the ROS cells were treated with known concentrations of PTH(1–34) or with microsphere eluent for designated times point in calcium- and magnesium-free hanks' balanced salt solution (Invitrogen, Carlsbad, CA) containing 0.1% bovine serum albumin (BSA) and 1 mM isobutylmethylxanthine (IBMX). The treated cells were incubated at 37°C for 10 min. The reaction was stopped by gently aspirating the media and the cAMP in cells was extracted by adding 250 μl/well cold 5% perchloric acid and incubating at −20°C until completely frozen. After thawing, the cAMP extracts were transferred to borosilicate glass tubes, and the pH was adjusted to 7.5 with 64 μl 4 N KOH/well. The extract was centrifuged to remove the precipitate.

The cAMP binding protein assay was performed as previously described to determine cAMP levels using known cAMP standards [28]. The cAMP extracts of samples were diluted 1:16 with cAMP binding buffer in order to be quantified within the standard curve. Before use, the [3H]-cAMP (ICN, Irvine, CA) was diluted to 10,000–15,000 cpm/25 μl with cAMP binding buffer. The [3H]-cAMP was incubated with standards or unknowns and cAMP binding protein for 90 min on ice. Dextran-coated charcoal was added to the reactants after the binding protein reaction to remove the unbound [3H]-cAMP. The samples were incubated on ice for 20 min and then centrifuged to pellet the dextran-coated charcoal. The supernatant of each tube was decanted carefully to a scintillation tube, 5 ml of scintillation fluid was added, and the radioactivity of the supernatants was determined using a liquid scintillation counter (Wallac® 1410; Wallac, Gaithersburg, MD) and cAMP levels were calculated by the log-logit method using the GraphPad Prism® program (GraphPad Software, San Diego, CA) with a standard curve.

2.8. In vivo PTH bioactivity assay

Five mice per group received sc injections of PTH or vehicle microspheres in 100 μl PBS. The PTH microspheres had a calculated theoretical PTH concentration of 1 μg PTH/1 mg microsphere. After 3 h, the mice were anesthesized with an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (5 mg/kg) and terminal serum samples were collected. Serum calcium levels were determined by colorimetric assay with the cresolphthalein complexone method (Sigma) as previously described [29].

3. Results

3.1. PLGA microspheres

PLGA5050 microspheres with three different particle sizes were obtained by adjusting the fabrication parameters of double emulsion processes (Fig. 1). Both the PVA concentration and emulsification strength employed in the second emulsion formation (mechanical stirring or sonication) greatly affected the size of the microspheres (Table 1). Under mechanical stirring, a higher PVA concentration (5%) produced microspheres with size of 2–5 μm while a lower PVA concentration (1%) resulted in microspheres having a size of 20–50 μm. When probe sonication was applied, nanospheres (<1 μm) were obtained even when a low concentration of PVA (1%) was used. As examined using SEM, all microspheres had spherical morphologies and smooth surface structures. The composition of the copolymer (ratio of LA to GA) had little effect on the size of the microspheres.

3.2. Degradation of PLGA5050 microspheres

The degradation curve (mass loss vs. time) of PLGA5050 microspheres is shown in Fig. 2. For the first 3 weeks, the microspheres maintained intact structure (Figs. 3a and b) and little mass loss was found. From the fifth week, the degradation rate increased and some microspheres became aggregated

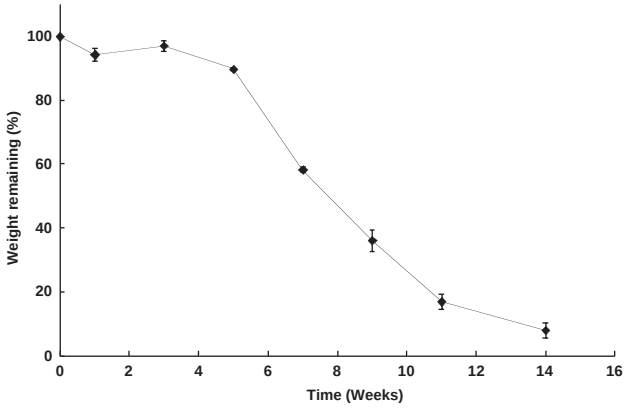

Fig. 2. Time dependence of mass loss profiles of PLGA5050 microspheres degrading in PBS at 37°C ($n = 3$).

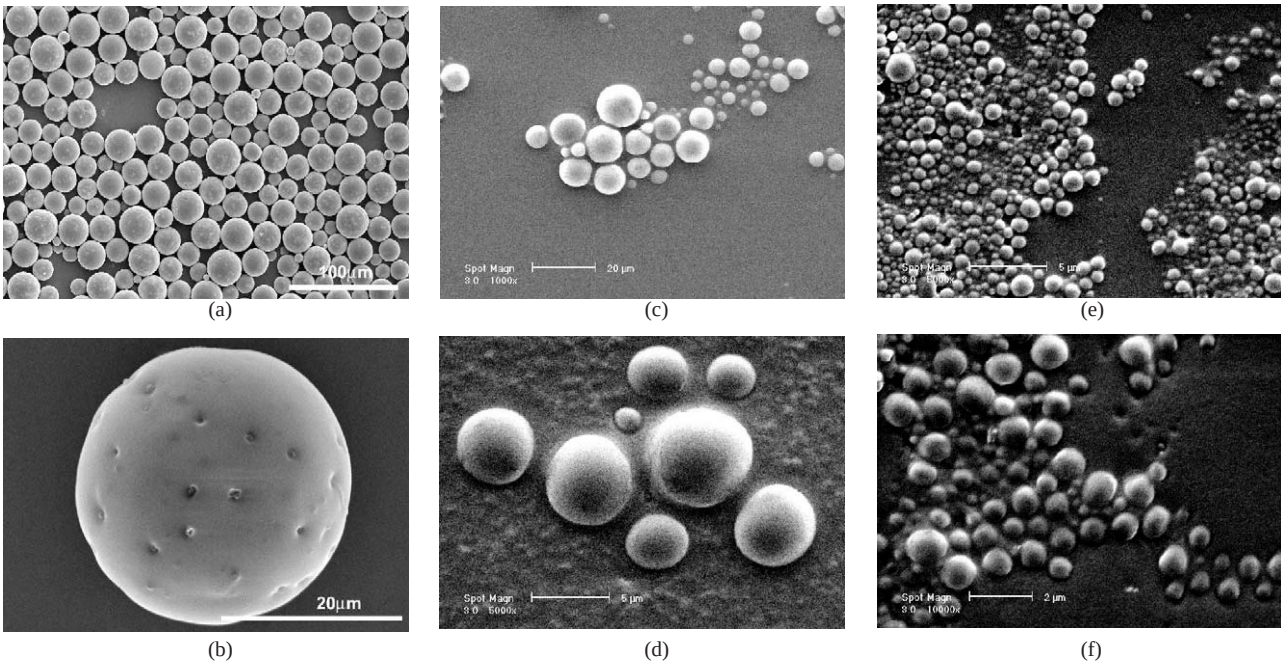

Fig. 1. SEM micrographs of PLGA5050 microspheres prepared under different conditions: (a, b) 1% PVA solution, mechanical stirring at 700 rpm; (c, d) 5% PVA solution, mechanical stirring at 700 rpm; and (e, f) 1% PVA solution, sonicating at 15 V.

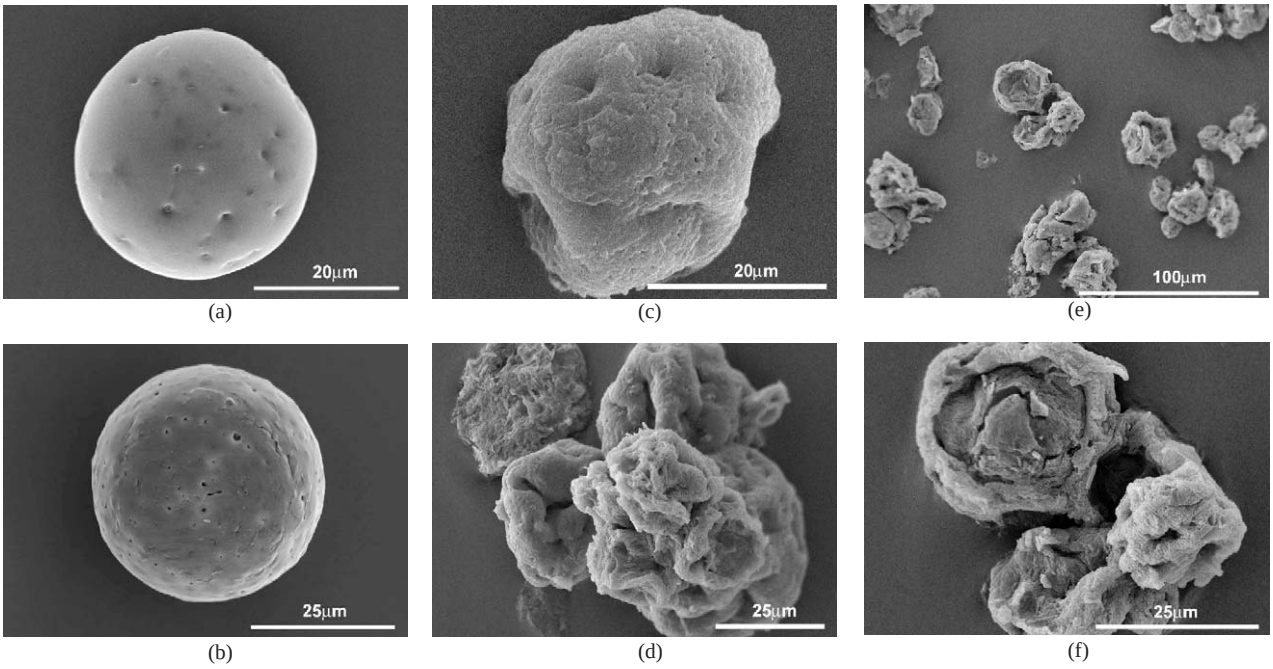

Fig. 3. SEM micrographs of PLGA50/50 microspheres at different degradation stages: (a) 24 h, (b) 3 weeks, (c) 5-weeks, (d) 7 weeks, and (e, f) 11 weeks.

(Figs. 3c–f). The microspheres deformed from original spherical shape into irregular shape with a rough surface texture. After 11–14 weeks of degradation, the microspheres had almost entirely degraded and the remaining mass was about 18% and 7%, respectively. The PLGA5050 microspheres were totally collapsed and disintegrated into irregular particles, and no intact spheres were observed.

3.3. In vitro BSA release

The release kinetics of PLGA microspheres was investigated using BSA as a model protein. Fig. 4a illustrates the cumulative release of BSA in a 4-week period from PLGA microspheres with different LA/GA composition. About 30% BSA was release from PLGA5050 microspheres, while only 10% was released from PLLA microspheres. According to the degradation study, the release of BSA in the first 4 weeks was mainly due to diffusion rather than degradation. Since PLLA is much more hydrophobic and also more densely packed (crystalline) than PLGA5050, it is more difficult for water to diffuse into and BSA to be released from PLLA microspheres. In a long release period of 20 weeks, there was another burst release for PLGA5050 between 10 and 12 weeks. The release of BSA was due to the degradation of PLGA5050 microspheres. From the degradation study, a great loss of mass began at 7 weeks while a sudden BSA release began at 12 weeks (Fig. 4b). The difference can be explained by the fact that PBS was not changed during the entire degradation study, whereas it was changed frequently during the

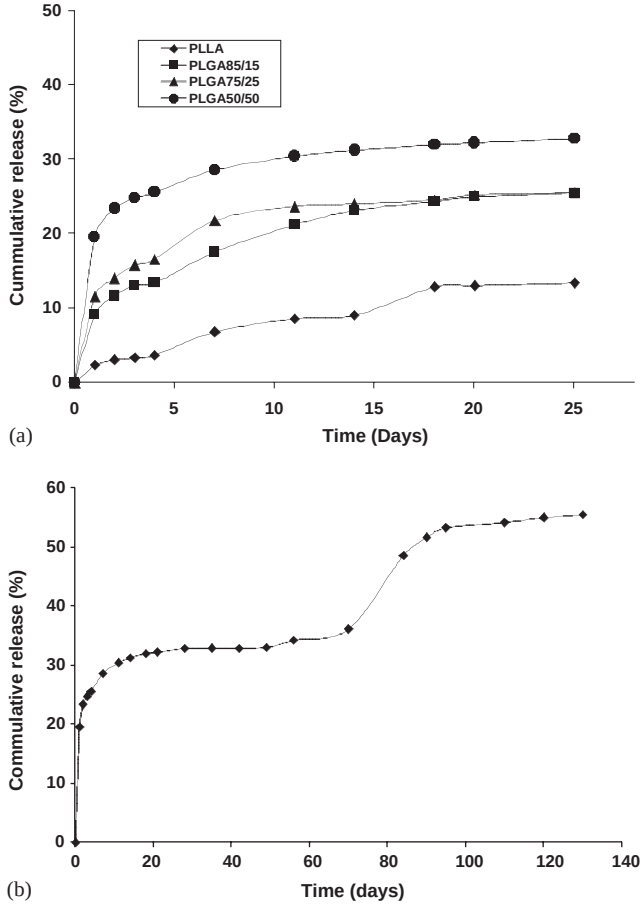

Fig. 4. Release of BSA from PLGA microspheres ($n = 3$): (a) 4-week BSA release from PLGA microspheres with varying LA/GA ratio; and (b) 20-week BSA release from PLGA5050 microspheres.

release study. In the degradation study, accumulated acidity accelerated the degradation of the PLGA5050 microspheres.

3.4. In vitro PTH release and bioactivity

Fig. 5 shows the absolute amount of PTH(1–34) released from PLGA 5050 microspheres in the initial 24 h. The corresponding bioactivity of released PTH in eluent is shown in Fig. 6. At 0 h, the microspheres were dissolved in dichloromethane and then extracted with distilled water. PTH levels in these samples were high (not shown in Fig. 5. since the amount was more than 100 pg/μl), but the bioactivity indicated by cAMP levels was lower than that of 6 h release group. This may largely be due to the harshness of the solvent, which likely diminished the bioactivity of PTH during the extraction. The highest levels of biologically active PTH in the initial 24 h were found at 6 h and levels decreased thereafter. However, there were still significant levels of PTH bioactivity in the 24 h group. The bioactivity of PTH at 6 h was equivalent to 10 nM PTH (a standard curve of PTH was used as a positive control). These data indicate that PTH retained its bioactivity after being incorporated into the microspheres.

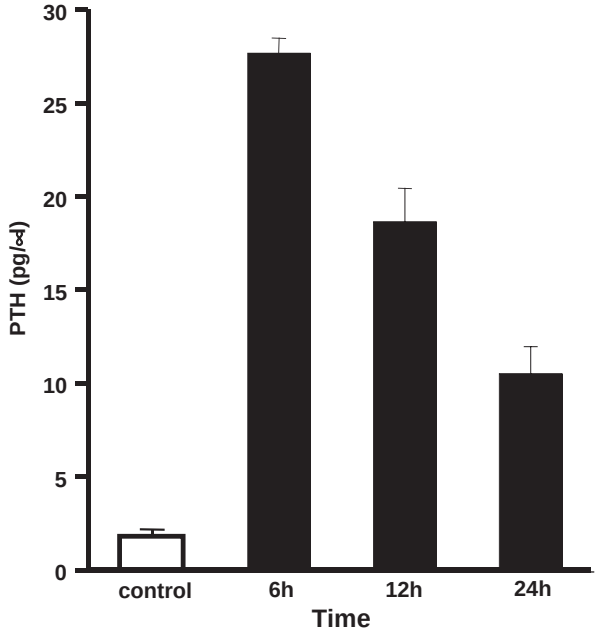

Fig. 5. PTH concentration in microsphere eluent. PTH microspheres were incubated at 37°C in PBS for 6, 12, and 24 h. The concentration of PTH released in the microsphere eluent at each time point was measured using a PTH(1–34) ELISA kit with PTH antibody coated wells. All values are the mean for triplicate samples. The concentration of PTH in the eluent of the control microspheres (BSA only) was less than 5 pg/μl. The concentration of PTH was the highest at the 6-h incubation time point and gradually decreased thereafter.

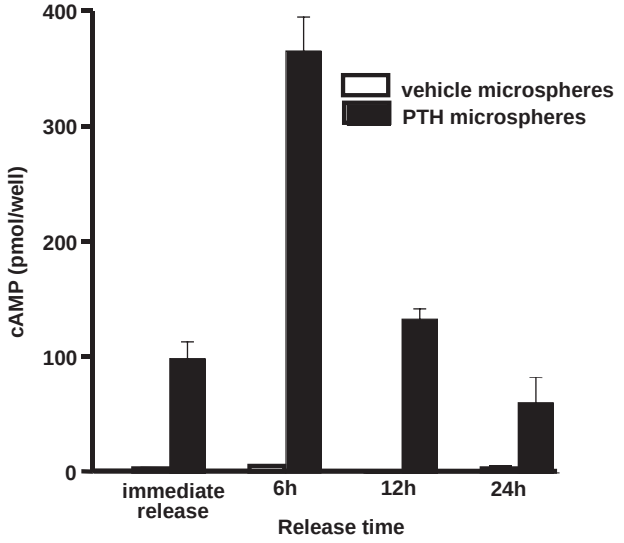

Fig. 6. Biologic activity of microsphere eluent. PTH receptor-mediated adenylate cyclase was stimulated by treating ROS cells with known concentrations of PTH(1–34) or with microsphere eluent (from vehicle controls or PTH microspheres) for each time point. The cAMP in the cells was extracted and a cAMP binding protein assay was performed to determine cAMP levels. All values are the mean for triplicate samples. The highest biologic activity of PTH was observed at the 6-h incubation time point and a significant decrease occurred at 12-h. Note the level of cAMP in all of the vehicle microspheres was less than 5 pmol/well.

3.5. In vivo PTH bioactivity

To further evaluate PTH microsphere bioactivity in vivo, PTH-incorporated PLGA5050 microspheres were injected into mice and serum calcium was analyzed via colorimetric assay. Fig. 7 shows that mice that received PTH microspheres had significantly elevated serum calcium levels, indicating effective delivery of biologically active PTH from the microspheres in vivo.

4. Discussion

Drugs formulated in polymeric devices are released by diffusion through the polymer barrier, by degradation of the polymer materials, or a combination of both diffusion and degradation mechanisms [30]. Therefore, the drug release kinetics from biodegradable carriers is closely related to degradation behavior of the materials. From this study, we found that the mass loss of degrading PLGA5050 microspheres had a lag time of 3 weeks. Following the lag time, there was a rapid mass loss. In addition, the PLGA5050 microspheres demonstrated an agglomeration tendency and lump formation before they disintegrated into small pieces or particles. The initial smooth surface of intact microspheres developed wrinkles and defects after 5 weeks of degradation. It was these wrinkles and defects that caused the microspheres to aggregate together and form

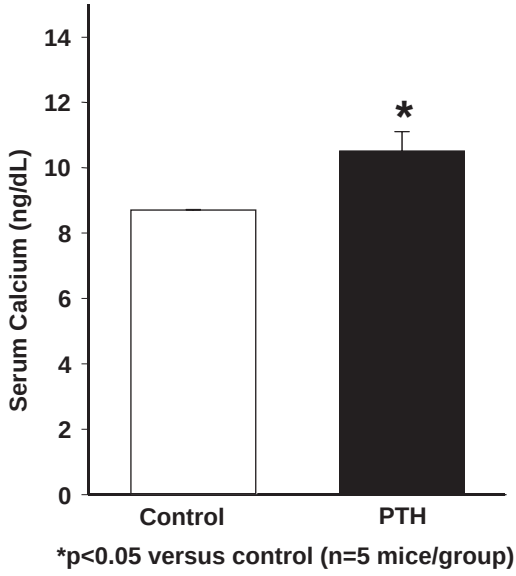

Fig. 7. Serum calcium levels in response to microsphere injection. Five mice per group were injected microspheres containing 10 mg PTH or vehicle microspheres in 100 μl PBS. The mice were anesthetized after 3 h and terminal serum samples were collected. The animals injected with PTH microspheres had significantly higher ($^{*}p<0.05$) serum calcium levels than the animals injected with vehicle microspheres.

lumps [31]. The aggregation tendency and the surface changes are an indication of the plasticization of the polymer, which may result from the reduction of glass transition temperature (T_g) below 37°C (the incubation temperature). The release of BSA from PLGA5050 microspheres exhibited corresponding patterns to the degradation profile. BSA was released from these systems in three phases: initial burst followed by a lag phase and final degradation-controlled release. The initial burst release of BSA was largely due to BSA being on the surface layer. The slow release after initial burst was the result of diffusion from the microspheres, which might be further slowed down due to the formation of intermolecular linkages and non-specific adsorption of the protein to the polymer. A second burst release at 8 weeks resulted from the rapid degradation and disintegration of the microspheres.

The size of microspheres was controlled by varying the fabrication parameters, mainly the PVA concentration and the emulsification strength. The prepared nanospheres had a protein encapsulation efficiency of 74% and diameter of several hundred nanometers. Nanospheres have been extensively used in drug delivery for cancer therapy as well as controlled delivery of vaccines [32–34].

The encapsulation efficiency for PTH was evidently lower than that of BSA. This might be due to the differences in molecular weights and structures between PTH and BSA. BSA has a globular structure with a high molecular weight of about 66 kDa while PTH(1–34) fragment is a linear small peptide with a low molecular weight of 4 kDa. Therefore, it was expected that more PTH than BSA would be lost during the formation of the final double emulsion.

Besides achieving desired release profiles, the main challenge of protein and peptides delivery is to maintain the bioactivity of the bioactive molecules during and after delivery. Intact PTH(1–84) and PTH(1–34) have plasma half lives of less than 3 and 11 min, respectively [21]. The rapid metabolic degradation of biologically active PTH may make multiple administrations of PTH necessary to maintain its effectiveness. This was also one of the main factors that has contributed to low bioavailability of PTH by oral administration [20]. In this study, PTH was resuspended in BSA/HCl solution and then encapsulated in PLGA microspheres. The double emulsion technique improved the encapsulation efficiency and minimized the loss of bioactivity both in vitro and in vivo. The polymer protected biologically active PTH from sensitive factors such as temperature and oxidation in vitro and enzymatic degradation in vivo. The solvent used has a critical effect on the bioactivity of PTH. Complete exposure of PTH molecules to some organic solvents, such as dichloromethane led to substantial loss of bioactivity. The fact that PTH remains active after encapsulation within, and release from microspheres clarifies an important initial step in tissue engineering strategies aimed at the use of PTH as an anabolic agent to improve bone regeneration.

5. Conclusion

PLGA microspheres with desired sizes were prepared by double emulsion technique in this study. Both a large protein (BSA) and a small peptide (PTH) were successfully incorporated into the micropheres. The long-term BSA release data demonstrated the potential of PLGA microspheres for controlled delivery of large proteins. PTH maintained its bioactivity both in vitro and in vivo after initial release, indicating that a local delivery of PTH to evoke a skeletal response can be accomplished by a microsphere encapsulation technique.

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References

[1] Langer R. New methods of drug delivery. Science 1990;249: 1527–33.

[2] Babensee JE, McIntire LV, Mikos AG. Growth factor delivery for tissue engineering. Pharm Res 2000;17(5):497–504.

[3] Reddi AH. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. Nat Biotechnol 1998;16(3):247–52.

[4] Lee H, Cusick RA, Browne F, Kim TH, Ma PX, Utsunomiya H, Langer R, Vacanti JP. Local delivery of basic fibroblast growth factor increases both angiogenesis and engraftment of hepatocytes in tissue-engineered polymer devices. Transplantation 2002;73:1589–93.

[5] Lee VH. Enzymatic barriers to peptide and protein absorption. CRC Crit Rev Ther Drug Carr 1988;5:69–87.

[6] Narayani R, Rao KP. Gelatin microsphere cocktails of different sizes for the controlled release of anticancer drugs. Int J Pharm 1996;143:255–8.

[7] Avgoustakis K, Beletsi A, Panagi Z, Klepetsanis P, Karydas AG, Ithakissios DS. PLGA-mPEG nanoparticles of cisplatin: in vitro nanoparticle degradation, in vitro drug release and in vivo drug residence in blood properties. J Controlled Rel 2002;79:123–35.

[8] Le Ray AM, Chiffoleau S, Iooss P, Grimandi G, Gouyette A, Daculsi G, Merle C. Vancomycin encapsulation in biodegradable poly(e-caprolactone) microparticles for bone implantation. Influence of the formulation process on size, drug loading, in vitro release and cytocompatibility. Biomaterials 2003;24:443–9.

[9] Carino GP, Jacob JS, Mathiowitz E. Nanosphere based oral insulin delivery. J Controlled Rel 2000;65:261–9.

[10] Wu XS. Synthesis, properties of biodegradable lactic/glycolic acid polymers. In: Wise, et al., editors. Encyclopedic handbook of biomaterials and bioengineering. New York: Marcel Dekker; 1995. p. 1015–54.

[11] Singh M, Shirley B, Bajwa K, Samara E, Hora M, O'hagan D. Controlled release of recombinant insulin-like growth factor from a novel formulation of polylactide-co-glycolide Microparticles. J Control Rel 2001;70:21–8.

[12] Oldham JB, Lu L, Zhu X, Poter BD, Hefferan TE, Larson DR, Currier BL, Mikos AG, Yaszemski MJ. Biological activity of rhBMP-2 released from PLGA microspheres. J Biomech Eng 2000;122:289–92.

[13] Lu L, Stamatas GN, Mikos AG. Controlled release of transforming growth factor beta1 from biodegradable polymer microparticles. J Biomed Mater Sci 2000;50:440–51.

[14] Tracy MA. Development and scale-up of a microsphere protein delivery system. Biotechnol Progr 1998;14:108–15.

[15] Jain RA, Rhodes CT, Railkar AM, Malick AW, Shah NH. Controlled delivery of drugs from a novel injectable in situ formed biodegradable PLGA microsphere system. J Microencapsul 2000;17(3):343–62.

[16] Morley P, Whitfield JF, Willick GE. Parathyroid hormone: an anabolic treatment for osteoporosis. Curr Pharm Des 2001;7(8):671–87.

[17] Stanislaus D, Yang X, Liang JD, Wolfe J, Cain RL, Onyia JE, Falla N, Marder P, Bidwell JP, Queener SW, Hock JM. In vivo regulation of apoptosis in metaphyseal trabecular bone of young rats by synthetic human parathyroid hormone (1–34) fragment. Bone 2000;27:209–18.

[18] Andreassen TT, Ejersted C, Oxlund H. Intermittent parathyroid hormone (1–34) treatment increases callus formation and mechanical strength of healing rat fractures. J Bone Miner Res 1999;14:960–8.

[19] Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster J-Y, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH. Effect of parathyroid hormone (1–34) on fractures and bone mineral density in postmenopausal women with osteoporosis. N Engl J Med 2001;344:1434–41.

[20] Leone-Bay A, Sato M, Paton D, Hunt AH, Sarubbi D, Carozza M, Chou J, McDonough J, Baughman RA. Oral delivery of biologically active parathyroid hormone. Pharm Res 2001;18(7):964–70.

[21] Codrons V, Vanderbist R, Verbeeck RK, Arras M, Lison D, Preat V, Vanbever R. Systemic delivery of parathyroid hormone (1–34) using inhalation dry powders in rats. J Pharm Sci 2003;92(5):938–50.

[22] Patton JS, Trinchero P, Platz RM. Bioavailability of pulmonary delivered peptides and proteins: a-interferon, calcitonins and parathyroid hormones. J Control Rel 1994;28:79–85.

[23] Dobnig H, Turner RT. The effects of programmed administration of human parathyroid hormone fragment (1–34) on bone histomorphometry and serum chemistry in rats. Endocrinology 1997;138:4607–12.

[24] Suzuki Y, Nagase Y, Iga K, Kawase M, Oka M, Yanai S, Matsumoto Y, Nakagawa S, Fukuda T, Adachi H, Higo N, Ogawa Y. Prevention of bone loss in ovariectomized rats by pulsatile transdermal iontophoretic administration of human PYH (1–34). J Pharm Sci 2002;91:350–61.

[25] Fang JM, Zhu YY, Smily E, Bonadio J, Rouleau JP, Goldstein SA, McCauley LK, Davidson BL, Roessler BJ. Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. Proc Nat Acad Sci USA 1996;93:5753–8.

[26] Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat Med 1999;5:753–9.

[27] Cohen S, Yoshioka T, Lucarelli M, Hwang LH, Langer R. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm Res 1991;8:713–20.

[28] Chen HL, McCauley LK, D'Silva NJ. CAMP binding protein assay for widespread use in cell signaling studies. Biotechniques 2002;33:1–5.

[29] Demiralp B, Chen HL, Koh AJ, Keller ET, McCauley LK. Anabolic actions of parathyroid hormone during bone growth are dependent on c-*fos*. Endocrinology 2002;10:4038–47.

[30] Wu XS. Preparation, characterization, and drug delivery applications of microspheres based on biodegradable lactic/glycolic acid polymers. In: Wise, et al., editors. Encyclopedic handbook of biomaterials and bioengineering. New York: Marcel Dekker, 1995; p. 1151–200.

[31] Viswanathan NB, Patil SS, Pandit JK, Lele AK, Kulkarni MG, Mashelkar RA. Morphological changes in degrading PLGA and $P(_{DL})$LA microspheres: implications for the design of controlled release systems. J Microencapsul 2001;18:783–800.

[32] Desai M, Hilfinger J, Amidon G, Levy RJ, Lavhasetwar V. Immune response with biodegradable nanospheres and alum: study in rabbits using staphylococcal entertoxin B-toxoid. J Microencapsul 1999;17:215–25.

[33] Lamprecht A, Ubrich N, Yamamoto H, Schafer U, Takeuchi H, Maincent P, Kawashima Y, Lehr CM. Biodegradable nanoparticles for targeted drug delivery in treatment of inflammatory bowel disease. J Pharmacol Exp Ther 2001; 299:775–81.

[34] Hans ML, Lowman AM. Biodegradable nanoparticles for drug delivery and targeting. Curr Opin Solid State Mater Sci 2002;6:319–27.