Preparation and Evaluation of β-TCP/Polylactide Microspheres as Osteogenesis Materials

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ABSTRACT: In the osteological field, the study of repairing and recovering processes for bone tissues has been one of the major clinical research subjects. To promote the bone healing, bioactive composite materials have been employed for the filling and supporting of defects, voids or diseased parts. In this study, a series of microsphere composites made from polylactide (PLA), a biodegradable polymer, and β-calcium phosphate (β-TCP), an osteogenesis material, were prepared and estimated their potential applications in participation to the growth of new bone tissues on bone defects. By employing the water/oil/water (w/o/w) emulsion technique to fabricate a series of bioactive and biodegradable microspheres materials, we have found that one type of the microspheres: with diameter at 177 ± 33 μm, and enclosure of β-TCP at 18% (wt/wt), exhibiting smooth surface by the optical microscopy and scanning electron microscopy (SEM) observations, and showing slow yet steady calcium ion release and PLA degradation by *in vitro* test, showed excellent repairing and recovery of the chondrocyte and osteocyte tissues on drilled defects on the condyle of New Zealand rabbits within 1 month of application. J Appl Polym Sci 108: 3210–3217, 2008

Key words: w/o/w emulsion; microspheres; polylactide; β-calcium phosphate; osteogenesis material

INTRODUCTION

The ideal bone generating materials should be biocompatible, osteoconductive and osteoinductive. The materials should also be degradable in concert with new bone growth lest it interferes with the new bone formation. Better yet, the materials that left behind could be served as constituents to the new bone generation. Of course, the materials should neither be immunogenic nor produce toxic by-products *in vivo*. However, many materials presently under study would only partially satisfy these requirements. In the past few years, efforts to investigate applications of the composite made of polymer and ceramic materials on bone tissue engineering have been increasing.[1–4] These composites have shown great potential as osteogenesis materials due to their multi-functional characteristics. For example, a composite system with the combination of collagen and hydroxyapatite (HAP) that possesses advantages of the biological function of collagen and the bioactive strengthening effects of HAP to the matrix to provide diverse functions for tissue repairing processes.[5,6] In general, synthetic polymers such as poly(α-hydroxyacids) and natural polymers such as collagen and chitosan have been employed in combining with a number of ceramics including HAP, β-TCP, and bioactive glasses.[7–9]

Synthetic biodegradable polymers have attracted growing attention for their applications on the tissue engineering in the past few years, particularly, for polylactic acid (PLA), polyglycolic acid (PGA), and their copolymers.[10–14] Because of their biodegradation properties, relatively good biocompatibility, and high mechanical strength, these polymers have frequently been used as implantable carriers, tissue repairing materials, and for drug delivery systems. Another attractive feature of these polymers is their ability to be manufactured or sculpted into a variety of shapes to fit to a given defect, and thus strongly enhances its potential applications in the biomedical field. However, the release of acidic by-products during the degradation could cause inflammatory response problems.[15,16] Another limitation of these polymers is that they are in lack of the bioactive function, especially in cases for bone tissue applications.[17,18]

Among many commercially available ceramics, HAP and β-TCP are frequently used as bone repairing and replacing materials because of their biocompatibility, bioactivity, and nontoxicity. Having the chemical composition close to the mineral composition of natural bone, calcium phosphate ceramics have been extensively employed as a bone substitute and revealed to be an invaluable osteo-integrative

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material.[19–22] In general, the resorption rate of HAP is slower than β-TCP under normal physiological environment. Consequently, this results to a longer-term ceramic persistency of HAP in the system. From clinical practice and experience, β-TCP ceramics possesses a more favorable resorption pattern and osteotransduction property, i.e., the ceramic material can be gradually absorbed followed by new bone formation and without compromising the intimacy of bone-implant contact.

A variety of approaches to the development of biodegradable and bioactive composites for tissue engineering applications are being studied, including combinations of PLA, collagen, and other biodegradable polymers such as HAP, β-TCP, and bioactive glasses in making of different scaffolds. Usually, HAP, β-TCP, and bioactive glass can be selected in combination with polymers to form bulk or porous biodegradable substrates to achieve specific applications.[23–27]

In this study, we utilized PLA and β-TCP as the components for the preparation of a series of microsphere composites. The microsphere systems based on PLA can be manufactured by various techniques, such as by phase separation and emulsion-evaporation. The preparative methods employed can affect the properties and performances of the microspheres as biomedical materials. In the present work, novel bioactive and biodegradable microspheres, composed of β-TCP and PLA, were fabricated by w/o/w emulsion technique. The materials are intended as scaffolds for bone tissue engineering applications. In addition to the assessing of physical properties of composite microspheres, the biological function of the composites was examined by the cell culture and animal test.

MATERIALS AND METHODS

Materials

PLA of average molecular weight 100,000 was purchased from Sigma (St. Louis, MO). β-TCP and polyvinyl alcohol (PVA) of average molecular weight 8000–10,000, 88% hydrolyzed, were purchased from Merck-Schuchardt (Germany). All other reagents used were of analytical grade.

Preparation of β-TCP/PLA microspheres

The microspheres were prepared by using a water-in-oil-in-water (w/o/w) emulsion system, according to the method described by Ogawa et al.[28] For determining the preparative condition of the microspheres with the size about 200 μm, we began with using only PLA as microsphere material and various process parameters were evaluated, such as PLA concentration, stirring rate, concentration of emulsion stabilizer, and composition of the inner aqueous phase and oil phase. The oil phase consisted of PLA dissolved in dichloromethane. The primary emulsion was obtained by dropping the inner aqueous phase into the oil phase with stirring for 9 min. The mixture was then poured into the outer aqueous phase containing PVA under vigorous stirring to prepare the second emulsion. The mixture solution was stirred for 6 h at room temperature. The microspheres, collected by centrifugation, were washed three times in distilled water and freeze-dried.

The β-TCP/PLA microspheres were fabricated by using the above-mentioned preparative parameters. Briefly, the inner water phase, consisted of β-TCP in aqueous PVA solution (3%, 0.5 mL), was emulsified into oil phase (12.5% PLA, 4 mL) by a mechanical stirrer. The mixture was then poured into outer aqueous PVA solution (3%, 100 mL) under vigorous stirring. The microspheres were collected by centrifugation, washed, freeze-dried and stored in a desiccator.

Particle size analysis of microspheres

Particle size distribution of the freeze-dried microspheres was determined using a Coulter 2600D Laser Scattering Particle Analyzer. Particle size was presented as mean diameter in micrometers and each sample was tested in triplicate.

Surface morphology of microspheres

The surface morphology of the prepared microspheres was examined by light microscope (Olympus, IX-70, Japan) and SEM (Hitachi, S-2700, Japan). SEM was performed on the dried microspheres coated with gold.

Determination of β-TCP entrapment in microspheres

About 100 mg of freeze-dried microspheres, precisely weighted, were dissolved in dichloromethane. The sample was filtrated and dried by vacuum. The dried β-TCP was accurately weight. Each sample was tested in triplicate. From the result, the loading capacity of PLA microspheres of β-TCP was determined. Loading capacity = (β-TCP weight/dried microsphere weight) × 100%.

Surface analysis

Information on the elementary composition at the surface of the microspheres was analyzed using field emission scanning electron microscopy (Philips XL40 FE-SEM, the Netherlands) combined with energy dispersive X-ray spectrometer (EDXS). The X-ray powder diffraction profiles of the microspheres were measured at room temperature with an X-ray dif-

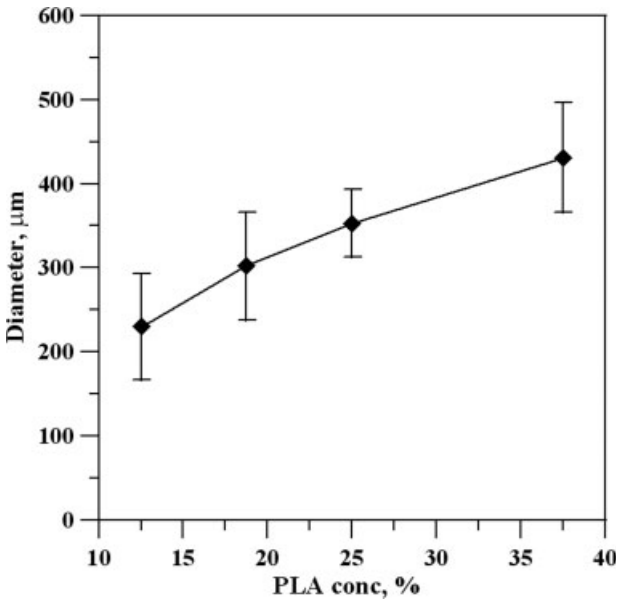

Figure 1 Effects of PLA concentration on diameter of PLA microspheres.

fractometer (SciTag X-400 X-ray diffractometer; 0.05° (2θ)/min).

In vitro degradation test

The *in vitro* degradation test of the prepared microspheres was conducted by incubating the microspheres in 3 mL of pH 7.4 phosphate buffer saline (PBS) on a shaker set at 40 rpm and 37°C. At predetermined time intervals, certain quantities of the microspheres were taken out of the incubation medium, washed with distilled water, dried, and the weight of these microspheres was measured. Another fresh 3 mL PBS was added into the vial for continuum degradation test. The degradation profiles were expressed as the accumulated weight losses of the microspheres.

In vitro release of calcium ion

Calcium release from the microspheres was studied in triplicate under aqueous conditions. The samples were placed in a flask with 3 mL of pH 7.4 PBS on a shaker set at 40 rpm and 37°C. At suitable time intervals, certain quantities of the solution was harvested and replaced with fresh buffer. Calcium levels were determined using an atomic absorption spectrophotometer (GBC 932AA, USA).

Animal study

Twelve New Zealand white rabbits between 1.5 and 2 kg were included in this randomized, blinded study. Each rabbit was anesthetized with Zoletil 50 (mixture of Tiletamine and Zolezepam, 1 : 1). Following the injection, the knees were shaved and the surfaces at surrounding sides of the joints were exposed via full-thickness incision. The defects (7 mm in diameter and 2 mm in height) were created in the interior of the joint and immediately grafted with β-TCP/PLA microspheres, PLA microspheres and no treatment as a control. The skin and soft tissue were sutured in layers with Dafilon sutures (France) and the surgical site was given antibiotics and allowed to recover. After 4 and 8 weeks of post surgery, the rabbits were euthanized and joints were harvested for further histological evaluation.

RESULTS AND DISCUSSIONS

Preparation of the microspheres

From the results of the particle size analysis, it revealed that the size of PLA microspheres increased with the increase of the concentration of PLA solution (Fig. 1). The increase in PLA concentration brought on an increase in the viscosity of oil phase that made it unfavorably to form small w/o/w emulsion droplets and led to an increase in particle size. These results were similar to that report Zhu et al.[29] In addition, the water phase was another key factor for particle size control. PVA with molecular weight of 8000–10,000 with 88% hydrolyzed was fit to the microsphere preparations. The size of PLA microspheres decreased with the increase of the PVA concentration as aqueous phase. The increase in PVA concentration resulted in the reduction of

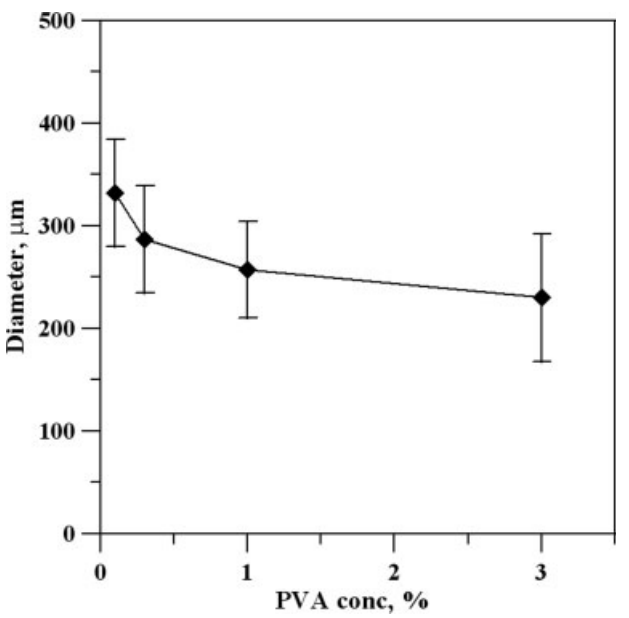

Figure 2 Effects of PVA concentration on diameter of PLA microspheres.

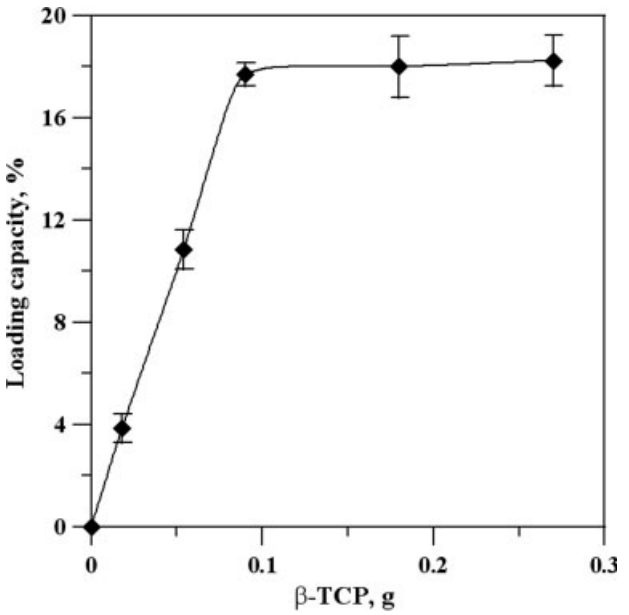

Figure 3 Effects of amount of β-TCP addition on loading capacity of composite microspheres for β-TCP.

the surface tension and led to a decrease in the particle size. If the concentration of PVA was too low, the effect of dispersion of PVA would be poor, and the resultant microspheres would be difficult of purification. Figure 2 showed the relationship between the concentration of PVA in dispersion solution medium and the sizes of the resultant PLA microspheres. On the basis of size distribution assays of PLA microspheres made from different preparative parameters, the condition of 12.5% PLA in dichloromethane, 3% PVA (emulsion stabilizer) and 600 rpm was chosen for the fabrication of β-TCP/PLA microspheres.

From the analysis of the particle size of the microspheres, it was found that the materials used as microsphere matrix also led to a significant effect on the size of microspheres. For comparison of the PLA microspheres and the β-TCP/PLA microspheres prepared at the same procedure conditions (stirring speed, solvent, and concentration of dispersion medium, etc.), β-TCP/PLA microspheres could reach a smaller size of 173 ± 33 μm with a narrower size distribution than that of PLA microspheres (230 ± 62 μm).

Loading capacity of PLA microspheres for β-TCP

In the final stage of microsphere preparation, the microspheres were washed with large quantity of distilled water to remove the emulsifier (PVA). As shown on Figure 3, the loading capacity of β-TCP/PLA microspheres for β-TCP could be affected by the content of β-TCP before the amount of β-TCP in inner water phase of 0.09 g.

Microspheres morphology and chemical composition

The microspheres prepared by w/o/w emulsion method, with and without β-TCP, both exhibited good sphericity, as shown in Figure 4. The incorporation of β-TCP did not make significant difference in the overall shape of the microspheres, and β-TCP

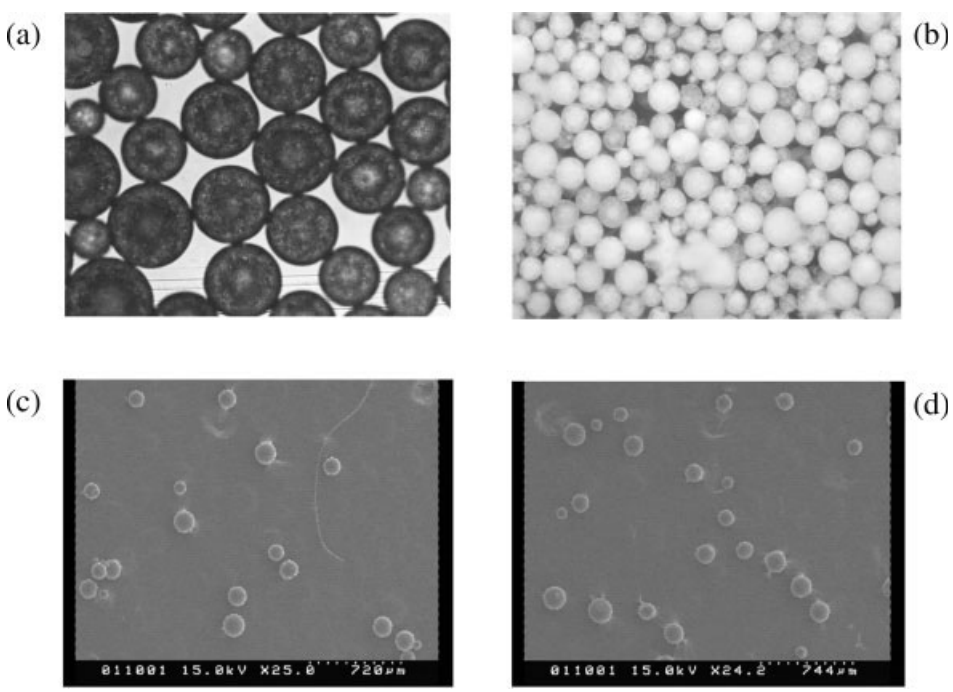

Figure 4 The surface morphology of the microspheres. (a,c) OM and SEM of PLA microspheres; (b,d) OM and SEM of β-TCP/PLA microspheres.

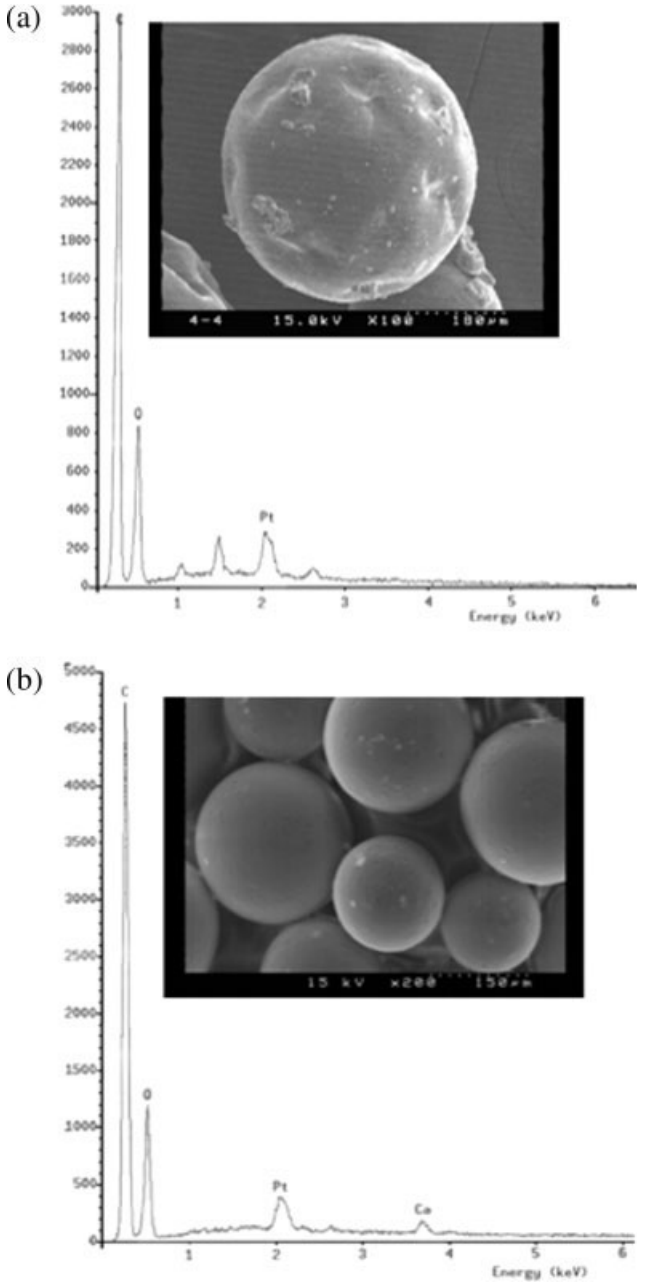

Figure 5 EDXA spectra of PLA microspheres (a) and β-TCP/PLA microspheres (b).

was distributed evenly throughout the microspheres. SEM examinations also revealed that the composite microspheres exhibited a smooth surface. The sunken marks of PLA microspheres are probably due to the evaporation of organic solvents during freeze-dried process. As for the substance on PLA microspheres, it might be the residuals of the elution reagent that failed to be rinsed away by distilled water. Furthermore, elemental analysis using EDXA [Fig. 5(a)] showed that the surface of the composite microspheres was made up of C, O, Ca, and P. In contrast, calcium could scarcely be detected on the surfaces of PLA microspheres [Fig. 5(b)]. Figure 6 showed the characteristic XRD patterns of the microspheres. The presence of β-TCP phase in the composite microspheres could be revealed by the distinct β-TCP diffraction peak at $2\theta = 32^\circ$. From these results, we could demonstrate that β-TCP could be entrapped inside of the PLA microspheres by w/o/w emulsion method.

Degradation and *in vitro* release of calcium ion

The microspheres degradation test result is shown in Figure 7. The composite microspheres showed a more rapid mass loss than the PLA microspheres. Detection of the calcium ion concentration of various β-TCP/PLA microspheres was carried out by atomic absorption spectrophotometer. For the composite microspheres, the calcium release showed a typical biphasic incubation-release profile (Fig. 8). There was an initial rush of calcium during the first 10 days of the study. This rapid release period probably represented the release of feebly entrapped and surface-associated β-TCP. After the first 10 days, the composite microspheres showed a slower release of calcium ions. The degradation rates were slightly different between the composite microspheres and PLA microspheres. For example, during 40 days there was a 31% reduction in microsphere mass for PLA microspheres and as high as 40% reduction in mass for the composite microspheres. However, the difference (9%) was quite consistent to the theoretical content of β-TCP for the 40% reduction in mass,

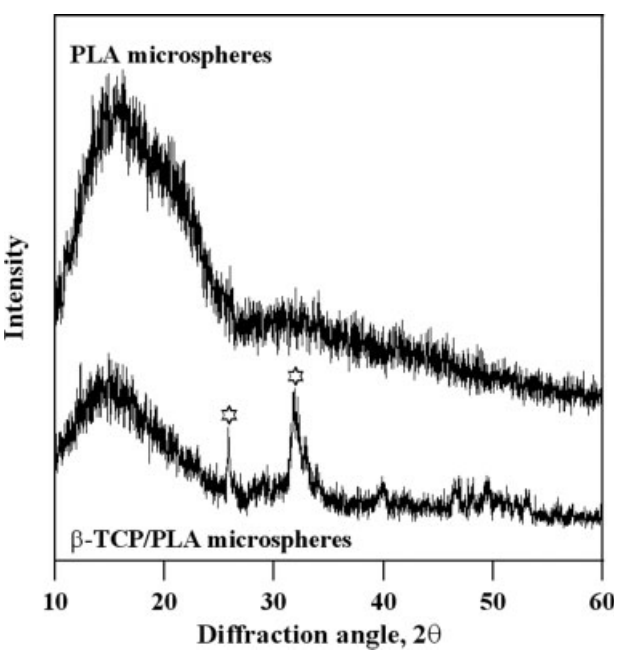

Figure 6 X-ray powder diffraction patterns of PLA microspheres and β-TCP/PLA microspheres (✡characteristic peaks for β-TCP).

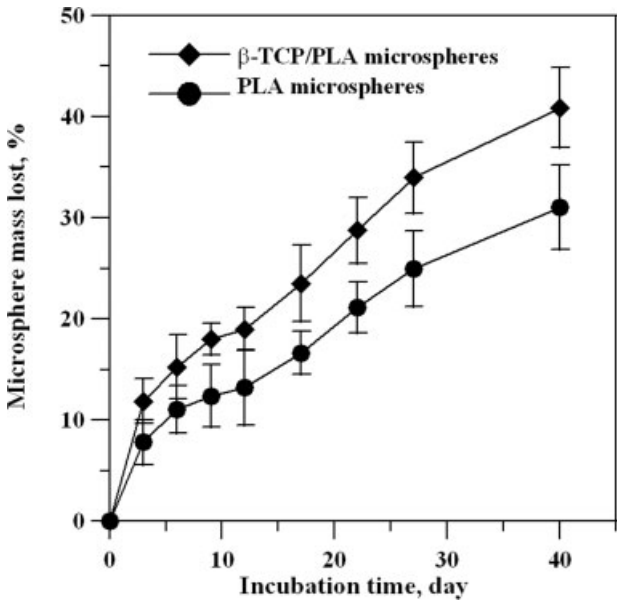

Figure 7 The degradation profile of PLA microspheres and β-TCP/PLA microspheres in pH7.4 PBS solution at 37°C for 40-day shaking.

and corresponded to the third of the released calcium mass based on the AA results. β-TCP, $Ca_3(PO4)_2$, can release three calcium ions per formula weight when totally dissolved. In other words, the degradation result corresponded to the AA analytical result. From the degradation and calcium release results, we could surmise that β-TCP was entrapped and spread in the whole microsphere, so the rate of calcium release from composite microspheres was thought to depend mainly on the rate of polymer degradation. Therefore, β-TCP would be steadily released and dissolved from the micropsheres when, concurrently, PLA was degraded gradually.

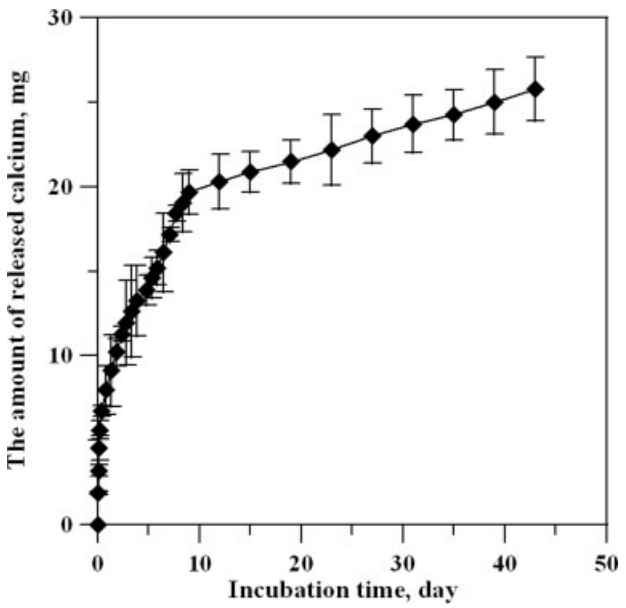

Figure 8 In vitro release of calcium from β-TCP/PLA microcspheres.

Histological examination in rabbit's condyle model

A 7-mm diameter, 2-mm in depth femur condyle defect was created on each rabbit front leg [Fig. 9(a)]. Consequently, the created condyle defects were tightly filled with PLA mircrospheres and β-TCP /PLA microspheres randomly as experimental group and empty defect as control group [Fig. 9(b)]. Figure 10 showed the transverse sections of tissues on the defect sites 4 weeks after surgery. After 4 weeks of implantation, the β-TCP/PLA microspheres degradation was faster compared with the PLA microspheres, which yielded smaller size and irregular shapes of microspheres at the same period. Varying stages of bone healing process were observed in the experimental groups. From the histological studies, there were apparent tissue recovery and dense bone structure observed. In addition, repairing of soft bone tissue was visibly present together with the mineralization. By examining the micrographs,

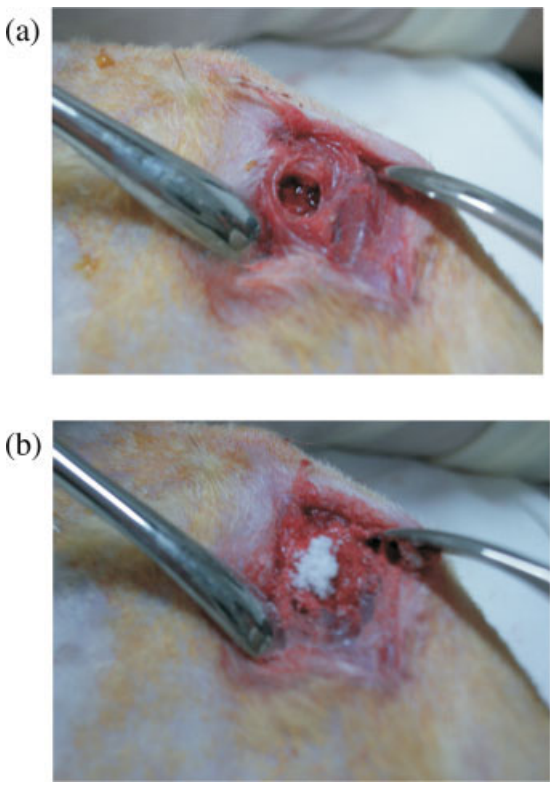

Figure 9 (a) Macroscopic appearance of an experimental site. A defect (7 mm in diameter) was created at joint of a rabbit, (b) A defect on the joint filled with microspheres. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

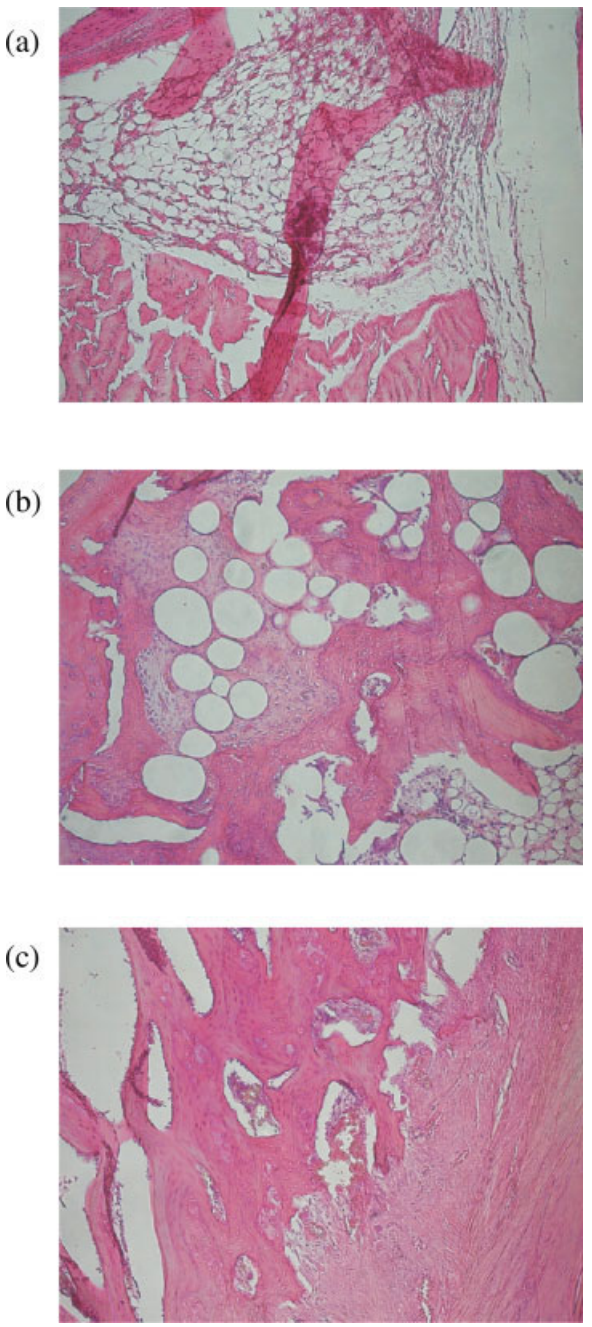

Figure 10 Histological evaluation of rabbit condyle defects at 4 weeks. (a) control, without treatment; (b) filled with PLA microspheres and (c) filled with β-TCP/PLA microspheres (100× H&E). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the structural characteristics of newly formed bone, numerous osteoblasts were found from the margins toward the center of the defect. When compared with the experimental groups, there was not obviously new bony proliferated inside the defect in the control even in the end of 8 weeks (Fig. 11).

Various approaches to the development of bioresorbable and bioactive composites materials for tissue engineering applications were being investigated extensively nowadays, and they included the combinations of PLA, PGA, and other resorbable polymers with HAP, β-TCP or bioactive glasses and glass–ceramics in different scaffold architectures. In most approaches, HAP, β-TCP, and bioactive glass in particulate or fibrous forms were combined with bulk or porous biodegradable polymer substrates to form sponge or film structures. However, these architectures of bulk scaffold could be unfavorably to fit tightly to the defect area. And, it could be necessary to undergo additional operation for the readjustment of implanted scaffold at the defect. Microspheres could not only fill to the voids of various topological profiles, but offer more intimacy to the surface areas. Most importantly, a microsphere provides extra dimensions to allow the cells and tissue to take hold onto its surface with smooth contour, Therefore, microspheres could be more favorably used as cell or tissue carrier, bone grafting and drug delivery encapsulating material in biomedical field. Consequently, there are strong interests in the fundamental and applicative researches of microspheres and their preparative methodology of producing even smaller microspheres.

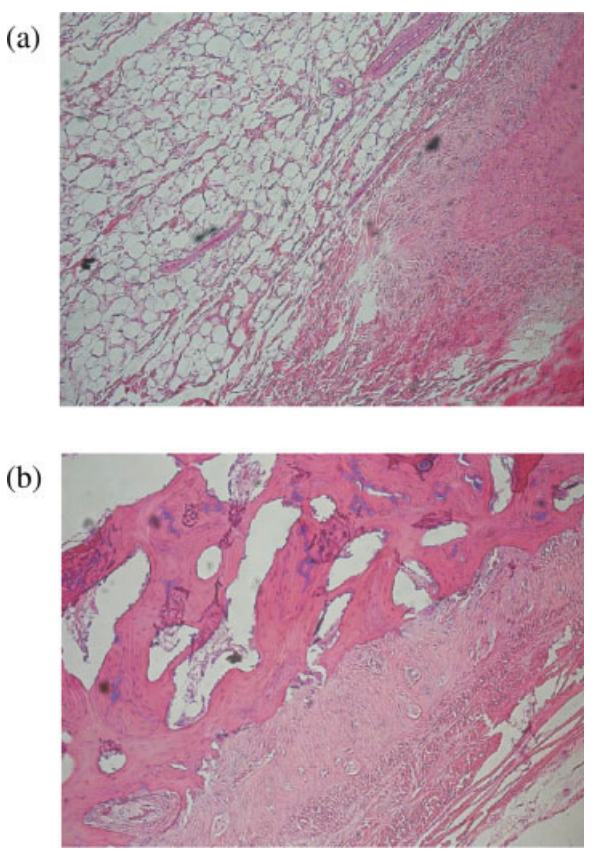

Figure 11 Histological evaluation of rabbit condyle defects at 8 weeks. (a) control, without treatment and (b) filled with β-TCP/PLA microspheres (100× H&E). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Generally speaking, w/o/w emulsion method was employed to prepare the PLA microspheres for entrapping hydrophilic drug, such as BSA.[30] In this work, we have attempted to use a w/o/w emulsion technique to encapsulate β-TCP into the PLA microspheres for preparing biodegradable β-TCP/PLA microspheres. From the results, we have demonstrated that the PLA microspheres containing the ceramics could be successfully prepared by this technique, and the size and surface morphology of these composite microspheres could be controlled by parameters, such as the polymer concentration, β-TCP content, and solvent used, etc.

CONCLUSION

In this study, we have successfully fabricated β-TCP/PLA microspheres by w/o/w emulsion system. The diameter of the microsphere was 177 ± 33 μm. Upon observing both PLA and β-TCP/PLA microspheres under optical microscope and SEM, the surface of β-TCP/PLA microspheres is smoother than neat PLA microspheres. The energy dispersive X-ray analysis (EDX) and X-ray diffractometer results confirm that β-TCP is packed inside of PLA microsphere and the loading capacity is 18% (wt/wt). Specifically, β-TCP/PLA microsphere degrades faster than the neat PLA microsphere but they both have similar degradation pattern, i.e., from outer layer to inner one. Apparently, during β-TCP/PLA microsphere degradation, the calcium ions were released rapidly at first and changed to a much slower yet steady rate after 10 days. From animal test, β-TCP/PLA microspheres showed excellent repairing and recovery of the osteocyte tissues on the wounded sites within 1 month of application.

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