Evaluating Chitosan/β-Tricalcium Phosphate/Poly(methyl methacrylate) Cement Composites as Bone-Repairing Materials

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Received 25 June 2002; accepted 8 January 2003

ABSTRACT: This study related to the preparation of chitosan microspheres by means of reacting chitosan with β-tricalcium phosphate (β-TCP) and glutaraldehyde by crosslinking reaction in the oil phase, followed by de-oil and purification processes to get the product. Three cement composites, Pure P, C1P1, and C2P1, were prepared by the polymerization of poly(methyl methacrylate) (PMMA) bone cement in the presence of 0, 50, and 66.7% chitosan/β-TCP microspheres, respectively. The result revealed the chitosan/β-TCP microspheres obtained was in the size range of 50–150 μm. The presence of chitosan/β-TCP microspheres in the prepared composites decreased the ultimate tensile strength, whereas the modulus remained the same as compared with the commercial PMMA bone cement. Addition of chitosan/β-TCP microspheres into commercial PMMA cement significantly improved the handling property of the cement paste—that is, the increased setting time and less stickiness behavior of this paste was beneficial, in manipulation, to the operation and easier fittings to the shape and gap of the bony defect and interface. The decreased curing temperature was also less harmful to the surrounding tissues. From scanning electron micrograph observations, chitosan/β-TCP microspheres can completely mix with bone cement powder and the prepared composites could provide scaffold for osteoblast cells growth and thus improve defects of commercial PMMA bone cement. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 89: 3897–3904, 2003

Key words: polysaccharide; poly(methyl methacrylate) bone cement; biodegradable

INTRODUCTION

Chitosan [poly(1,4),-β-D-glucopyranosamine] is a natural polysaccharide, which is widely present among marine and terrestrial invertebrates and lower forms of the plant kingdom. Chitosan can normally be obtained from crab or shrimp shells, by alkaline deacetylation of chitin. Recently, chitosan has shown great potential in the development of cheap and versatile drug delivery systems due to its biodegradability and good biocompatibility. [1–5] In medical applications, chitosan has been observed to accelerate wound healing and blood clotting.[6–8] Besides, chitosan is used in antacid and antiulcer activities, in immobilization of enzymes, and in artificial organ membranes for its gel-forming properties in the low pH range.[9,10] The unique chemical and biological properties of chitosan make it a very attractive material and it is used extensively in many types of applications. Furthermore, chitosan can be fabricated into film, fiber, bead, and powder shapes that increase its potential as a biomaterial. [11–13]

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Journal of Applied Polymer Science, Vol. 89, 3897–3904 (2003)

As a biomaterial, ceramic is generally used to repair or replace skeletal hard connective tissues. In cases of trauma damage, congenital deformities, disease, or tumor resection, bone losses or defects are so significant that bone cannot initiate the self-repairing process. When load bearing is not a primary requirement, porous ceramics can provide a suitable solution and act as a space-filling implant. When implanted, bone or tissues will grow into the interconnecting pores and maintain its long-term viability. That is, these implants serve as a structural bridge or scaffold for bone formation. With the aging of porous ceramics, their subsequent decrease in strength requires bone ingrowths to stabilize this structural implant. However, these porous ceramics cannot provide sufficient strength in weight-bearing applications. Materials such as calcium phosphate ceramics are inherently not suitable for weight-bearing implants, but they can be used for the repair and reconstruction of bone because of their osteotransduction—i.e., these materials can rapidly integrate into the bone structure in bone defects after implantation.[14,15]

For another orthopedic application, such as total hip arthroplasty, acrylic bone cement [poly(methyl methacrylate) (PMMA) bone cement] is often used to inject into the gap between implant and bone tissue for better fixation.[16] These cements have the advantage

that they can be *in situ* molded during the operation and provide sufficient strength as compared with calcium phosphate cements. But this acrylic bone cement will induce foreign body reaction and thus create flexible stress shielding. Besides, high temperature produced in the polymerization reaction necroses the surrounding bone tissues and thereafter causes mobility of the implant. When used as space-filling material, the monomer of cement will chemically bond with bone and thus provide better fixation with bone. But disintegration will happen in the interface after long term because the surrounding cells cannot grow on or into this PMMA bone cement matrix. Recently, new bioactive bone cements have been developed to overcome these disadvantages, such as PMMA bone cement modified with apatite and wollastonite containing glass-ceramic powder beads and hydroxyapatite powder (as an inorganic filler) to improve the osteoconductivity and mechanical properties.[17–19]

A good contact between bone tissue and implant is necessary for proper osteointegration. The use of bone replacement or repair materials in the form of cubes, wedges, or even granules provides a poor contour adaptation between bone and implant, thus reducing the contact area and resulting in less osseous ingrowth. This leads to a risk of instability and pseudoarthrosis. Moldable, *in situ* setting cements were then introduced and improved the above-mentioned problems. There are more than 25 different formulations of moldable cements, either in pure calcium phosphate or calcium phosphate in combination with other materials such as crosslinked gelatin, cellulose ether, fibrin glue, or polymers. Although these cements could harden *in situ* within minutes, the different resorption patterns and rates are not compatible with the bone-remodeling process and thus often result in secondary instability.

Among the most common cements, hydroxyapatite (HA) or tricalcium phosphate (TCP) is often incorporated as an additive because of its biocompatibility and nontoxicity. In general, the resorption rate of HA is slower than TCP in a physiological environment, thus resulting in a long-term persistence of HA ceramics. From clinical practice and experience, TCP ceramics have a better resorption pattern and osteotransduction property, i.e., ceramic can be gradually absorbed followed by new bone formation and without loss of bone-implant contact.

β-Tricalcium phosphate (β-TCP) is a biocompatible material; its structure and composition is similar to the inorganic part of natural bone, and thus is often used to act as bone-grafting and dental materials. Chitosan is another potential biopolymer that possesses biodegradable, biocompatible, and nontoxic properties. It can be manufactured into different shapes and is becoming a promising material in biomaterial applications.

The aim of this study was to prepare microspheres comprised of chitosan/β-TCP by using the emulsion technique. These microspheres were used to be an additive in PMMA bone cement to prepare chitosan/β-TCP/PMMA cement composites. The basic properties and degradation behavior of these composites were investigated. Furthermore, osteoblast cells culture was conducted to evaluate the biocompatibility of the materials and their potential application as bone-grafting materials. The applicability of these chitosan/β-TCP microspheres to improve the performance of commercial PMMA bone cement was also investigated.

EXPERIMENTAL

Materials

The bone cement used in this study was purchased from Howmedica Int. Ltd. (Ireland). Each package contains 40 g of prepolymerized powder that is composed of polymethyl methacrylate, methyl methacrylate–styrene copolymer, and barium sulfate, and a vial of 20 mL of liquid monomer. Chitosan was obtained from TCI, Tokyo, Japan, with the molecular weight of 300,000. Glutaraldehyde was used as the crosslinker (Acros, 50% w/v, Geel, Belgium). Acetic acid, mineral oil, span-80, and acetone were purchased from Sigma (St. Louis, MO). β-TCP was purchased from Merck-Schuchardt (Germany). All chemicals used in this study were of reagent grade.

Preparation of chitosan/β-TCP microspheres

The chitosan/β-TCP microspheres were prepared by the water/oil (w/o) emulsion method. To describe the procedure briefly: a 2% (2 g) solution of chitosan was dissolved in 5% acetic acid solution (100 mL). β-TCP powder (3.72 g) was then poured into this solution and stirred for 8 h to allow β-TCP powder to be well dispersed. A portion of this mixture (15 mL) was added to the mineral oil (120 mL), which contained surfactant span-80 (1.2 mL). During these processes, this dispersion medium was stirred with a mechanical stirrer at 1000 rpm at room temperature. About 10 min later, 4 mL of glutaraldehyde (50%) was added into the dispersion medium. Similarly, 1 h later, 4 mL more of glutaraldehyde was added into the medium and then stirred for extra 6 h. At the end of period, the chitosan/β-TCP microspheres were washed with acetone for several times and collected by centrifugation at 5000 rpm. Then, the microspheres were dried in vacuum and kept in a desiccator for further uses. The prepared chitosan/β-TCP microspheres were observed and examined by microscopy and scanning electron microscopy (SEM).

TABLE I
Compositions of Composites[a]

Item	Chitosan/β-TCP Microspheres	PMMA cement powder	Abbreviation
A	0	100	Pure P
B	50	50	C1P1
C	66.7	33.3	C2P1

[a] Number represents part (in weight percent) for each component.

Size determination of chitosan/β-TCP microspheres

The size and their distributions of the prepared microspheres were determined from micrographs taken from optical microscope (Olympus, IX-70, Japan). The β-TCP entrapment efficiency was also examined by these micrographs. The morphological characterization of the microspheres was examined by a scanning electron microscope (Hitachi, S-2700, Japan).

Preparation of chitosan/β-TCP/PMMA cement composites

PMMA bone cement powder was mixed with chitosan/β-TCP microspheres at various ratios (Table I). The powder mixture and liquid monomer were mixed at room temperature by keeping the ratio of the powder to liquid monomer constant at 2:1. After the mixing, the mixture cement was poured into a Teflon cylindrical mold. The setting time and the maximal curing temperature of this polymerization were determined.

Mechanical test

The mechanical properties of these composites were measured by using a MTS Systems (Eden Prairie, USA). The compression test and three-point bending test was determined on cylindrical specimens with a height of 25 mm and a diameter of 10 mm. The specimen was compressed or bended at a speed of 10 mm/min and the mechanical parameters were recorded automatically by the system.

Degradation measurement

These prepared samples were immersed in phosphate buffer saline (PBS) solution (pH 7.4) and then put into a 37°C shaker for slowly shaking. The degradation ratio was expressed by the weight loss of these samples after a particular period. The SEM micrographs were also used to verify this measurement.

Alkaline phosphatase activity measurement

As bone repair or grafting materials, two basic factors were considered in these materials, i.e., these materials should provide appropriate mechanical strength and be able to preserve the function of bone tissues (biocompatible). The phenotype and function of the osteoblast cells were characterized by the alkaline phosphatase activity. So, in addition to the basic physical properties, the alkaline phosphatase activity of osteoblast cells was also measured to verify the biocompatibility of these materials.

The osteoblast cells obtained from neonatal (less than 2 days old) Sprague-Dawley rats were used for the cell culture test because the composites were to be mainly used as bone-related materials.[20] Cells between the second and third passages were used in these experiments. Osteoblast cells were first grown in T-75 tissue culture flasks with Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS), 50 μg/mL ascorbic acid, and 10 m*M* β-glycerol phosphate. Cells (cell density of 5×10^5 cells/mL) were then transferred to the bacterial grade petri dishes with test samples (5 mm in diameter, 2 mm in height) immersed in the medium. After a particular period, samples were treated with 0.1% Triton X-100, pH 10.5 for one hour. Alkaline phosphatase enzyme was assayed by the method described in Lowry et al.[21] by using *p*-nitrophenol phosphate as substrate. Absorbance at 410 nm was read to determine the amount of *p*-nitrophenol produced (spectrophotometer, Agilent, UV8453, USA). The alkaline phosphatase activity of these composites were calculated according to the following equation:

$$\text{ALP activity (m}M\text{/h)} = 2 \times \text{absorbance of composite/absorbance of standard sample}$$

where the standard sample was 1 m*M* *p*-nitrophenol.

RESULTS AND DISCUSSION

Microspheres morphology

The microspheres prepared by w/o emulsion method with and without a mixture of β-TCP show mostly sphericity [Fig. 1(A) and Fig. 1(B)]. These microspheres are about 50–150 μm in diameter. The incorporation of β-TCP did not cause any obviously change in the overall size and shape. The β-TCP was almost entrapped in the chitosan microspheres under the investigation of micrographs and β-TCP weight loss before and after the preparation process. A SEM micrograph also revealed that these microspheres have a relatively smooth surface with wrinkles, as shown in Figure 1(C).

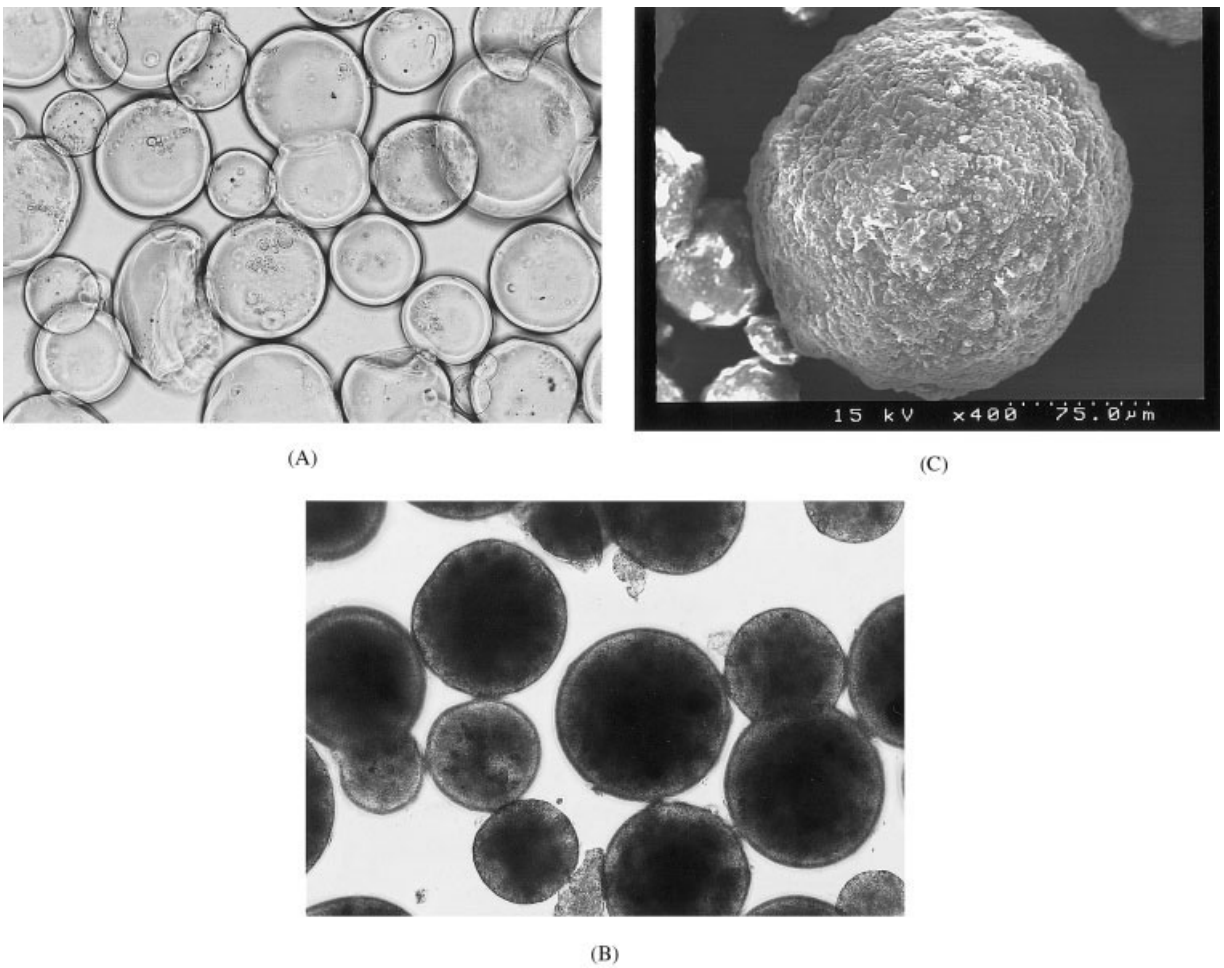

Figure 1 Photomicrographs of chitosan microspheres prepared by emulsion method: (A) without β-TCP added; (B) with β-TCP added; and (C) SEM of chitosan/β-TCP microsphere.

Morphological and physical characteristics of chitosan/β-TCP/PMMA cement composites

Figure 2 shows the SEM micrographs of surface and cross-section morphology of chitosan/β-TCP/PMMA cement composites. As can be seen, chitosan/β-TCP microspheres appearing in the surface and the incorporation of chitosan/β-TCP microspheres in these composites caused porous structures, whereas a more dense and smooth structure was observed in the pure PMMA cement. In other words, the presence of these microspheres altered the structure of the pure PMMA cement. These porous structures in chitosan/β-TCP/PMMA cement composites would be helpful to cell ingrowth, however, and would influence the overall mechanical strength.

Some physical characteristics of prepared chitosan/β-TCP/PMMA cement composites are given in Table II. As compared with pure PMMA cement, addition of chitosan/β-TCP microspheres decreased the ultimate compressive strength (UCS) and bending strength (UBS). The UCS decreased from 90.6±0.7 MPa (Pure P) to 83.5±0.3 and 73.5±1.4 MPa (C1P1 and C2P1, respectively), and the UBS decreased from 38±5.7 MPa (Pure P) to 23±1.4 and 22.5±0.7 MPa (C1P1 and C2P1, respectively). In addition, the compress modulus for Pure P, C1P1, and C2P1 almost remained unchanged, whereas the flexural modulus decreased about 11.5 and 11% for C1P1 and C2P1, respectively, as compared with pure PMMA cement (Table II). In general, the experimental results revealed that the modulus and mechanical strength decreased with increase in chitosan/β-TCP microspheres content.

In addition, the presence of chitosan/β-TCP microspheres also increased the setting time of the composites (Table II). The increased setting time was proportional to the weight ratios of chitosan/β-TCP microspheres. This increased tendency would be helpful to the manipulation of cement during the operation. Besides, addition of chitosan/β-TCP microspheres into commercial PMMA cement significantly improved the handling property of this cement paste; the paste became less sticky with an increase in chitosan/β-TCP microspheres content. These properties are desirable in surgical procedures, for bony defects can be easily

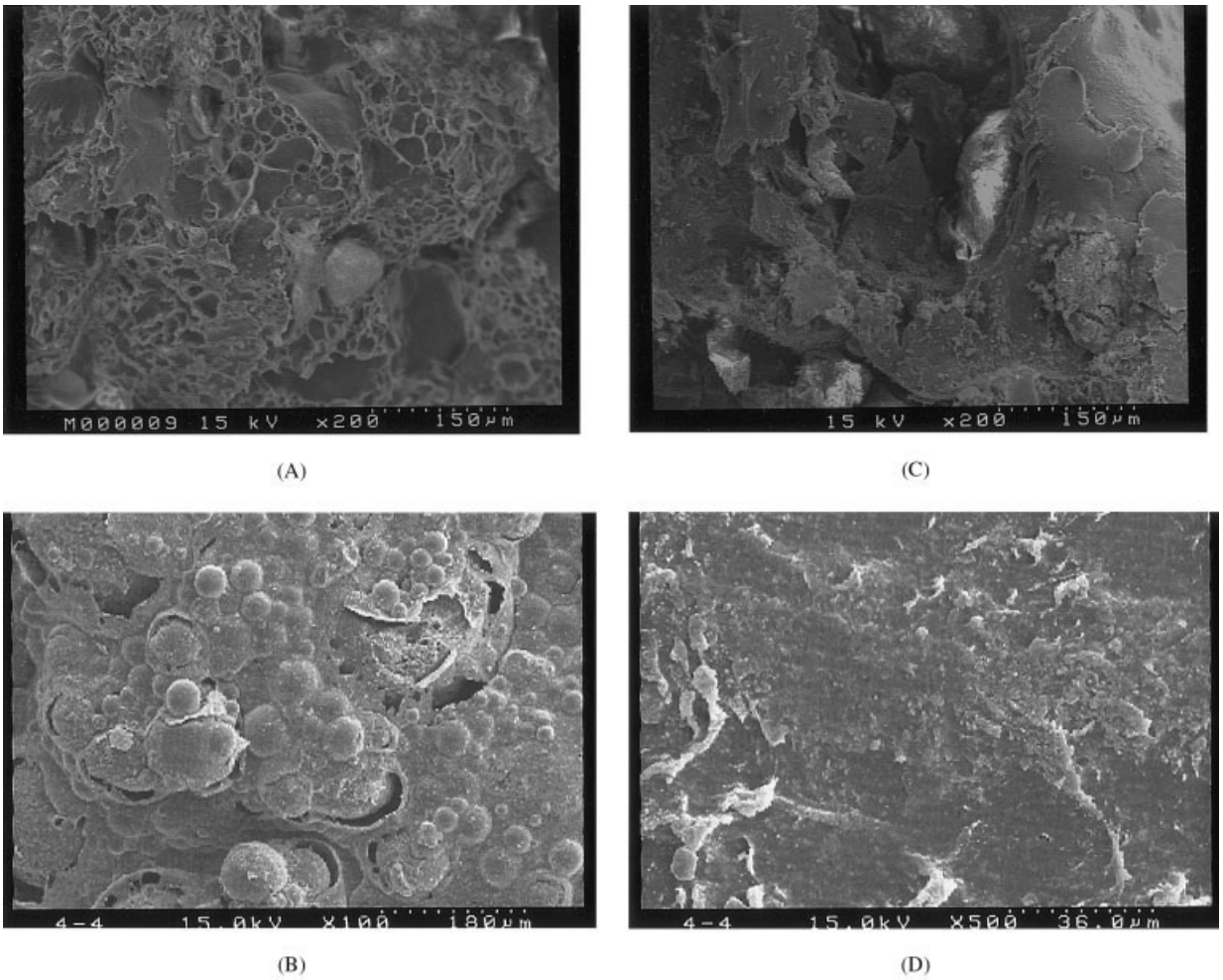

Figure 2 SEM of the composites: (A) Cross-sectional morphology of C2P1. (B) Surface morphology of C2P1. (C) Cross-sectional morphology of Pure P. (D) Surface morphology of Pure P.

filled and can reduce the interspace between bone and cement. The other important advantage of these prepared composites was substantial reduction of peak curing temperature to 55.6 and 42.6°C of C1P1 and C2P1, respectively, during the polymerization as compared the pure PMMA bone cement (78.7°C). This decreased curing temperature will not necrose all around bone tissues during *in situ* operation (especially for C2P1) and hence will increase the biocompatibility of this composite.

The materials degradation test result is shown in Figure 3. As can be seen, C2P1 had greater weight loss ratio (about 8.9%) than C1P1 and Pure P (5.7% and 1.2%, respectively) after 100 days of shaking. The SEM micrographs shows the surface morphologies of these materials (Fig. 4). After 100 days of shaking, the pure PMMA cement remained a dense morphology and the cement powders were obviously seen in this figure, whereas the C1P1 and C2P1 exhibited a rougher and porous morphology. The ruptured chitosan/β-TCP microsphere appearing in these composites revealed the degradation behavior was ongoing in this composite. From these observations, we concluded that the chitosan and β-TCP in the composites could gradually

TABLE II
Physical Properties of Chitosan/β-TCP/PMMA cement composites

Item	Ultimate compressive strength (MPa)	Modulus of compression (MPa)	Ultimate bending strength (MPa)	Modulus of bending (MPa)	Setting time (min)	Curing temperature (°C)
Pure P	90.6 ± 0.7	1163.1 ± 73.9	38 ± 5.7	628.4 ± 48.9	3.6 ± 0.6	78.7 ± 1.1
C1P1	83.5 ± 0.3	1099.7 ± 179.6	23 ± 1.4	555.7 ± 15.3	9 ± 1	55.6 ± 3.1
C2P1	73.5 ± 1.4	1136.3 ± 24.4	22.5 ± 0.7	558.9 ± 12.6	12.5 ± 0.7	42.6 ± 1.8

[a] Average ± *SD*, $n = 3$.

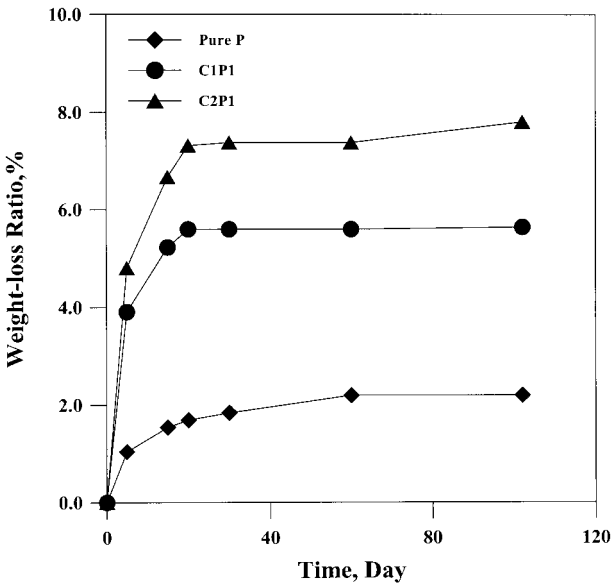

Figure 3 The degradation curve of Pure P, C1P1, and C2P1 composite in PBS, pH 7.4 solution.

dissolve in PBS solution, whereas PMMA powders could not—the dissolution produced rougher and porous structures. And this rough and porous surface could be beneficial for cell ingrowth and thereafter provide a better fixation between the implant or defect and surrounding bone tissues. In real applications, the resorption process could be facilitated by enzyme digestion. And we expect that one could utilize different weight ratios of chitosan/β-TCP microsphere and PMMA powder to prepare composites, and we expect this chitosan and β-TCP could gradually resorbed by bone tissues after new bone formation and thus improve the deficiency of commercial PMMA bone cement.

Alkaline phosphatase activity

One of the markers of the osteoblast cells phenotype is the expression of alkaline phosphatase activity. Figure 5 shows the alkaline phosphate activity in the rat osteoblast cell culture grown on these prepared materials. As shown in this figure, C1P1 composite pre-

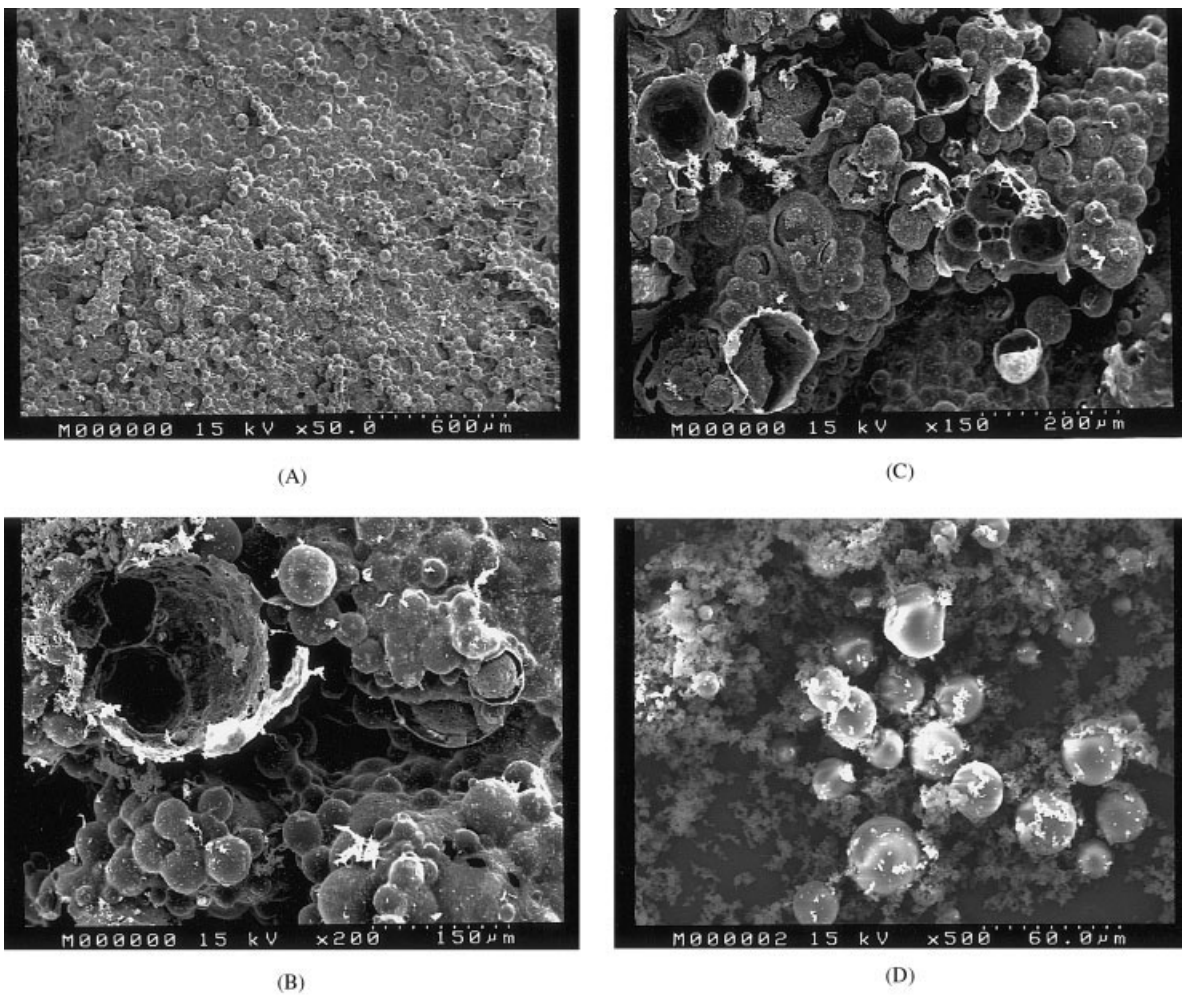

Figure 4 SEM of (A) Pure P, (B) C1P1, (C) C2P1 composite after 100-day shaking, and (D)PMMA cement powder.

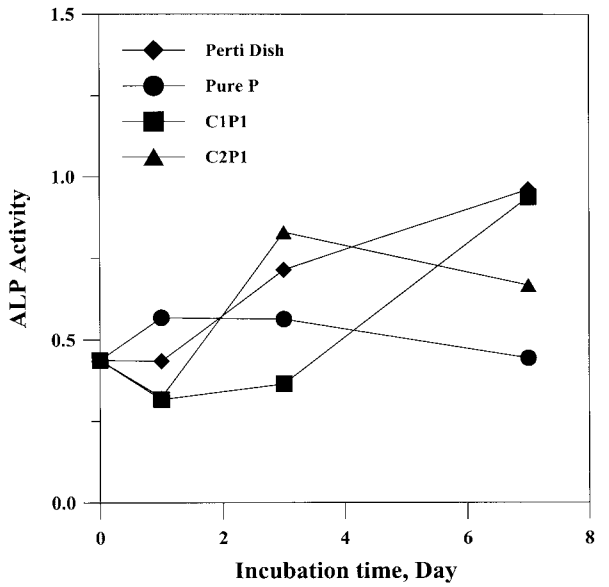

Figure 5 Alkaline phosphatase activities of osteoblast cells grown on Pure P, C1P1, and C2P1.

served about the same alkaline phosphatase activity with culture petri dish after 7 days of culture, though it exhibited a lag phase before 3 days of incubation time. The alkaline phosphatase activity of Pure P was smallest and decreased gradually after one day of culture; it revealed that this material was less biocompatible than C1P1 and C2P1. We observed equivalent cell growth and the same tendency of alkaline phosphatase activity detected by SEM observation (Fig. 6) and biochemical assay. As shown in Figure 6, osteoblast cells wrapped over the Pure P and C1P1 composite, whereas cells grown on the C1P1 exhibited rougher and denser cell surface than Pure P.

CONCLUSIONS

As a candidate to bone repair or grafting materials, two factors should be considered. First, the materials should provide appropriate mechanical strength. Second, and the most important feature, we expect, in addition to being biocompatible, is the induction of bone synthesis or bone ingrowth in defects. The latter could be obtained by stimulation of osteoblast cells activity. In this study, we tried to utilize biocompatible and biodegradable chitosan and β-TCP to prepare chitosan/β-TCP microspheres. These prepared microspheres were able to mix well with PMMA cement powder to prepare a homogeneous composite; in addition, this mixture was able to inject into the gap between the bone tissues and implant or into bone defects without the traditional surgical operation. Besides, the weight ratio of chitosan and β-TCP was kept constant at 35:65, in order to simulate the natural composition of bone (ratio of organic and inorganic component). We can postulate that these composites might provide appropriate mechanical strength by PMMA cement, and biocompatibility, osteoinduction and osteoconduction by chitosan/β-TCP materials.

From the preliminary results, the presence of the microspheres would decrease the mechanical strength; however, the compression strength was still close to femoral cortical bone (84 MPa)[22] and the modulus still remained the same range. These composites could provide a solution for one of the inherent problems with the existing bone repair materials—that is, a modulus mismatch between the prosthesis/cement/bone interfaces, which results in the loosening or failure in the long term. The applicability of these microspheres to improve the defects of commercial bone cement powder and obtain appropriate modulus and good osteogenesity in different applications is still under investigated in our lab.

Besides, increased setting time during polymerization was also helpful to manipulation during operation, and decreased peak curing temperature was less harmful to the surrounding bone tissues. As postulated earlier and, if this modified chitosan/β-TCP/PMMA bone cement can show good and consistent

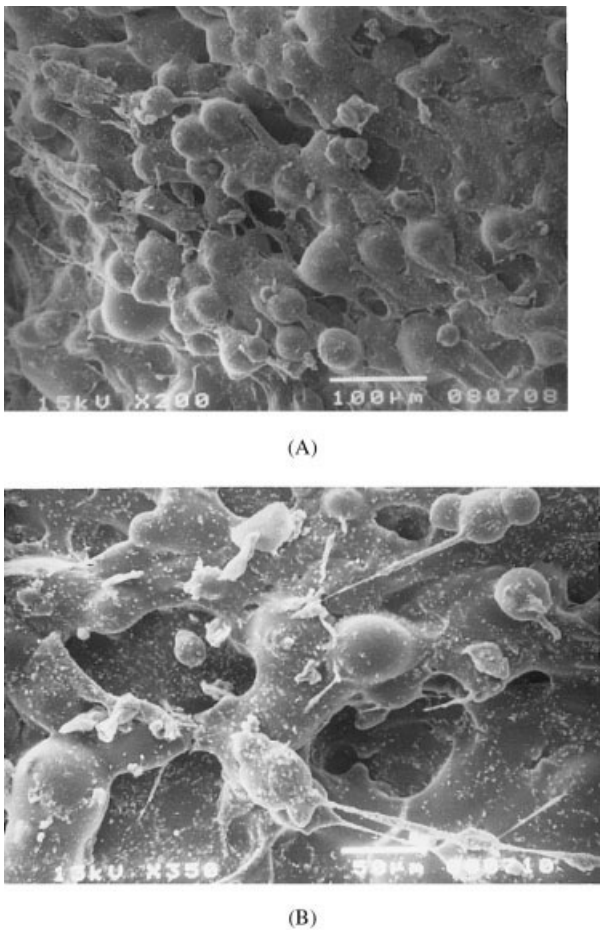

Figure 6 SEM of osteoblast cells grown on (A) Pure P and (B) C1P1 (after 7 days incubation).

osteogenesity in an animal study (currently under investigation) model, then eventually this type of material could be an improvement to the commercial cement being used in the medical community now.

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