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# Effects of adding resorbable chitosan microspheres to calcium phosphate cements for bone regeneration

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ABSTRACT

Calcium phosphate cements (CPCs) have been widely used as bone graft substitutes. However, the undesirable osteoinductivity and slow degradability of CPCs greatly hamper their clinical application. The aim of this study was to synthesize a type of injectable, bioactive cement. This was accomplished by incorporating chitosan microspheres into CPC. CPC containing chitosan microspheres was analyzed by X-ray diffraction (XRD) and scanning electron microscope (SEM). XRD showed that the hardened chitosan microsphere/CPC with different proportions of microspheres contained diffraction peaks of hydroxyapatite and chitosan. Compressive strength and dissolution in simulated body fluid were measured. The chitosan microsphere/CPC containing 10% (w/w) chitosan microspheres had a compressive strength of 14.78 ± 0.67 MPa. Cavity defects were created in both femoral condylar regions of New Zealand White rabbits. Chitosan microsphere/CPC (composite group) and α-TCP/CPC (control group) were implanted separately into the bone defects of both femurs. X-ray analysis was performed to observe the filling of these bone defects 3 days after surgery. The extent of bone substitute degradation and new bone formation were evaluated by SEM and histological examination at 8, 16, and 24 weeks after implantation. These results showed far more new bone formation and degradation of the chitosan microsphere/CPC composite in the bone defects. These data indicate that a chitosan microsphere/CPC composite might be considered as a promising injectable material for the generation of new bone tissue.



## 1. Introduction

Calcium phosphate cements (CPCs) have been widely used as bone substitutes in clinical applications such as trauma, tumor resection and congenital malformations. It is well recognized that an ideal scaffold should possess good osteoconductivity and osteoinductivity. These properties are favorable for attachment, proliferation and differentiation of the osteoblasts or osteoprogenitor cells. In addition effective osteoconductivity and osteoinductivity provide an interconnected porous network that facilitates cellular infiltration and nutrient and waste transport [1]. CPCs have good biocompatibility, are self-setting and possess osteoconduction properties [2–5]. Specifically, CPCs can be molded or injected and set in situ to closely adapt to complex shapes caused by bone defects [6–8]. In 1986, the first calcium phosphate cement was developed and consisted of tetracalcium phosphate and dicalcium phosphate anhydrous. CPCs were approved in 1996 by the Food and Drug Administration (FDA) for repairing craniofacial defects [9]. Since then, several other CPCs and injectable cements have been developed. Unfortunately, despite numerous CPC formulations, the inherent poor ability to be resorbed and scarcely any osteoinductivity undermine the clinical therapeutic efficacy of CPCs to some extent [10]. With these limitations in mind, an alternative CPC-based scaffold with suitable degradability and osteoactivity is worth further study to potentiate the performance of CPCs for bone repair.

Chitin is a natural biological material, which is present in the shells of crustaceans, insect cuticles and spiders [11,12]. Chitosan (CTS) is obtained from the total or partial deacetylation of chitin and has biocompatible, biodegradable and virtually non-allergic properties. The biggest advantage of chitosan is the fact that degradation products of chitosan are neutral or slightly alkaline [13]. Degradation products of some polymers, such as poly(lactic acid) are acidic and often cause a poor response and local metabolic disorder in the implant area [14,15]. In addition, the surface of CTS is hydrophilic, and this is conducive for cells to adhere, proliferate and differentiate [16]. Because of these properties chitosan has been recently used to improve currently used CPCs [17–19]. However, previous studies have mainly focused on adding chitosan to liquid components of CPCs or preparing chitosan fibers for CPC scaffolds [20,21]. A role for chitosan in improving the osteoconductive and degradable properties and pore size distribution of bone cement was not desired. Our chitosan microsphere/CPC composite is one type of injectable composite bone cement material made

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by the incorporation of chitosan microspheres into CPC powder components produced by Beijing Key Laboratory of Fine Ceramics, Institute of Nuclear and New Energy Technology, Tsinghua University (Beijing, China).

We found no other reports on the use of chitosan microsphere/CPC. The purpose of the present study was to synthesize a type of self-setting bioactive cement. The degradation, osteoconduction and biocompatibility of this chitosan microsphere/CPC were investigated in vivo.

## 2. Materials and methods

### 2.1. Fabrication and characterization of chitosan microsphere/CPC composite

CPCs generally consisted of both powder and liquid phases. The control CPC powder was composed of α-tricalcium phosphate [α-$Ca_3(PO_4)_2$], calcium dihydrogen phosphate monohydrate [$Ca(H_2PO_4)_2H_2O$] and calcium carbonate ($CaCO_3$) in a 10:3.5:1.5 molar ratio. CPC liquid was sodium dihydrogen phosphate ($NaH_2PO_4$)/sodium hydrogen phosphate ($Na_2HPO_4$) buffer solution in an equal molar ratio. The solid-to-liquid ratio was 1 g/ml. The above powders were mixed with chitosan microspheres (10%, w/w) to form a chitosan microsphere/CPC powder. The chitosan (DD > 90%, MW = 57,000) microspheres were prepared by a liquid-phase suspension process [22,23]. The dark brown microspheres were 100–400 μm in diameter and the average particle size was mainly affected by the stirring speed during the preparation process. The particle density was about 1.12 g/$cm^3$. The chitosan microsphere/CPC liquid was composed of the above liquid to which sodium bicarbonate ($NaHCO_3$) solution was added to allow pore forming and sodium alginate [$(C_6H_7O_6Na)n$] solution (1.5%, w/v) to prevent the CPC paste from collapse. The solid-to-liquid ratio was maintained at 1 g/ml. All materials were provided by Beijing Key Laboratory of Fine Ceramics, Institute of Nuclear and New Energy Technology, Tsinghua University (Beijing, China).

The phase composition of chitosan microsphere/CPC composites with different proportions of microsphere was characterized by X-ray diffraction (XRD, D8 Advance, Bruker, Germany). The surface morphology and the microstructure of the scaffold were analyzed by scanning electron microscope (SEM, Quanta 200 FEG, FEI, Netherlands).

The chitosan microsphere/CPC paste was first mixed at a powder to liquid ratio of 1:1. It was poured into a cylindrical mold (Ø 10 mm, height 10 mm) and immersed in simulated body fluid (SBF) at 37 °C. Every 2 days SBF was replaced. After immersion in SBF at 37 °C for 72 h, samples were dehydrated through a series of graded ethanol and freeze-dried. Compressive strengths were measured using a Universal Testing Machine (AG-IC20KN, Shimadzu, Kyoto, Japan). A constant displacement speed of 0.5 mm/min was used. The maximum load required to fracture each specimen was determined. The compressive strength was subsequently calculated from the maximum load and the diameter of each specimen (n = 5). The morphology of chitosan microsphere/CPC samples, which contained 10% (w/w) microspheres, was observed and photographed in 60 days.

### 2.2. Implantation of the bone substitute material into a rabbit model

All surgeries were performed under a protocol approved by the Animal Welfare Committee of Peking University Health Science Center. Twelve New Zealand White rabbits (male, 4 weeks old, with an average weight of 2.5 kg) were used in this study. All animals (Center of Experimental Animals, Peking University Health Science Center) were fed for 1 week prior to the experiments.

After anesthesia, the rabbits were placed in a supine position and the condyles of both femurs were exposed. A cylindrical bone defect, perpendicular to the surface of the bone from the medial to the lateral aspect, was created in each femur using a dental drill cooled with normal saline. The final dimensions of the bone defects were 4 mm in diameter and 6 mm deep. The powder and liquid forms of chitosan microsphere/CPC (composite group) and α-TCP/CPC (control group) were mixed in a volumetric proportion of 1:1 and were injected into the bone defects of the bilateral femurs, one on either side. The bone substitute materials solidified in approximately 15 min. Finally, the wounds were sutured in two layers and penicillin (240,000 IU) was injected into the rabbits for 3 days. Twenty-four femoral defects (n = 4 per group) were created in twelve rabbits. The procedure was performed under sterile conditions. After surgery, New Zealand White rabbits were kept in sterile conditions with freely available water and food. Animals were anesthetized and sacrificed by air embolism 8, 16, and 24 weeks after the surgery.

### 2.3. X-ray, SEM, and histological examination

Three days after surgery, all animals were anesthetized and X-ray examination was performed to observe the extent of filling in each of the bone defects.

At 8, 16, and 24 weeks after surgery, the animals were euthanized and tissue samples from the bone defect areas were harvested. Half of each sample was used for SEM. The samples were washed in running water for 24 h, fixed, dehydrated in a graded alcohol series and air dried. Sections (1–2 μm) of each the samples were observed under a scanning electron microscope (SEM, Quanta 200 FEG, FEI, Netherlands) and digitally photographed.

The other half of each sample was used for histological examination. The samples were fixed, decalcified, washed, dehydrated (the same as above), and then embedded in paraffin. Sections (5 μm) of each of the samples were prepared and stained with hematoxylin and eosin (H&E). The stained sections were viewed under a microscope (BX51, Olympus, Tokyo, Japan) and photographed digitally.

## 3. Results

### 3.1. Characterization of chitosan microsphere/CPC

After setting for 24 h in a 100% relative humidity chamber at 37 °C, XRD showed that the hardened chitosan microsphere/CPC with different proportions of microspheres contained diffraction peaks similar to hydroxyapatite (HA) (Fig. 1). HA is a naturally occurring mineral form of calcium apatite with the formula $Ca_5(PO_4)_3(OH)$. However, the chemical name is usually written $Ca_{10}(PO_4)_6(OH)_2$ to denote that the

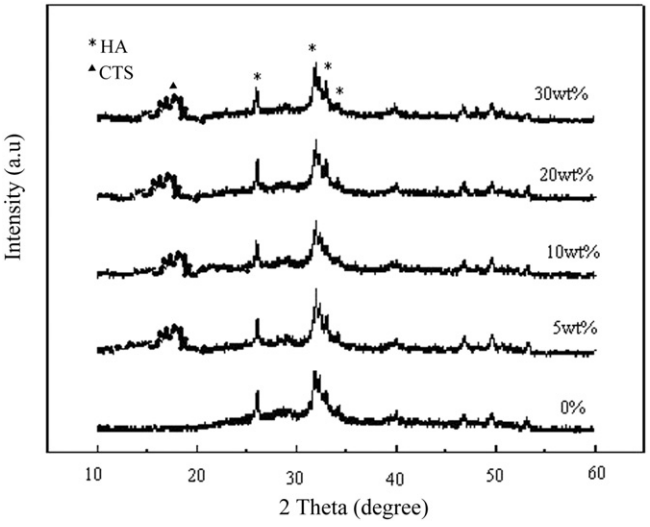


**Fig. 1.** XRD patterns of chitosan microsphere/CPC scaffolds containing different proportions of microspheres (*for peaks of HA, ▲for peaks of chitosan).