

REINFORCEMENT OF CALCIUM PHOSPHATE CEMENTS WITH PHOSPHORYLATED CHITIN*

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Abstract Phosphorylated chitins (P-chitins) as the additives of calcium phosphate cements (CPCs) were prepared by the phosphorylation of chitin with phosphorus pentoxide in methanesulfonic acid. Their physical properties and effects on CPCs from monocalcium phosphate monohydrate (MCPM) and calcium oxide (CaO) or dicalcium phosphate dihydrate (DCPD) and calcium hydroxide [$\text{Ca}(\text{OH})_2$] were investigated. Addition of P-chitin ($M_w = 2.60 \times 10^4$; degree of substitution, $DS = 0.68$) to the liquid phase in amounts up to 3 wt% for MCPM and CaO cements or 1.5 wt% for DCPD and $\text{Ca}(\text{OH})_2$ cements could enhance the mechanical strength considerably, while little influence on the setting time was observed. However, further addition of P-chitin will cause no setting.

Keywords Phosphorylated chitins, Calcium phosphate cements, Mechanical strength, Setting time

INTRODUCTION

Calcium phosphate cements (CPCs) as a class of viable biomaterials have many merits in their use as artificial hard tissues, such as osteotransductive, biodegradable, immediately moldable during the operation, *etc.* They could be alternatives to autogeneous bone for repair, substitution or augmentation purposes^[1]. Most of the CPCs have hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA] as their only or main end product, which is similar to the mineral phase of natural bone. As is known, bone is mainly composed of organic collagen and inorganic HA. The lack of organic substrate may lead to the decay or disintegration of CPCs upon early contact with blood^[2] or other body fluids and the materials fatigue easily relative to bone.

Different kinds of additives have been introduced into the cement powder or liquid before setting to improve or achieve certain properties of CPCs. Among them, some polymers, such as sodium alginate, hydroxypropyl methylcellulose and carboxymethylcellulose^[3] were used to improve the handling properties with respect to the cohesion of the cement paste through preventing liquid from penetrating into the cement paste. But the setting was retarded considerably.

Chitin is a abundant natural polymer and exhibits many useful biological, physiological and pharmacological properties. It is nontoxic, bacteriostatic, homeostatic, osteotransductive, and can be used as hemostat, wound-healing accelerator, cell growth regulator, *etc.* But the poor solubility of chitin in common solvents has greatly limited its utilization^[4]. Much attention has recently been paid to its chemical modifications (Fig. 1^[5]). Acid soluble chitosans and their derivatives including chitosan acetate and chitosan lactate have been tried as additives for CPCs consisting of tetracalcium phosphate (TTCP) and dicalcium phosphate (DCP) by Kawakami *et al.*^[6], with corresponding delayed setting time. Water insoluble phosphorylated chitins (P-chitins) and chitosans (P-chitosans) prepared by the procedures similar to those for cellulose phosphate were reported by

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Sakaguchi *et al.*^[7] and were later used as bone repair materials by Yokogawa *et al.*^[8], but their mechanical strengths were not reported. In this study, water-soluble P-chitins were synthesized by a procedure similar to that of Nishi *et al.*^[9] and their physical properties were characterized in order to find a P-chitin with good properties for the application in CPC materials. One kind of P-chitins was used as an additive in the preparation of CPCs consisting of monocalcium phosphate monohydrate [$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$, MCPM] and calcium oxide (CaO) or dicalcium phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, DCPD) and calcium hydroxide [$\text{Ca}(\text{OH})_2$]. Its effects on the physical properties of the CPCs were also investigated.

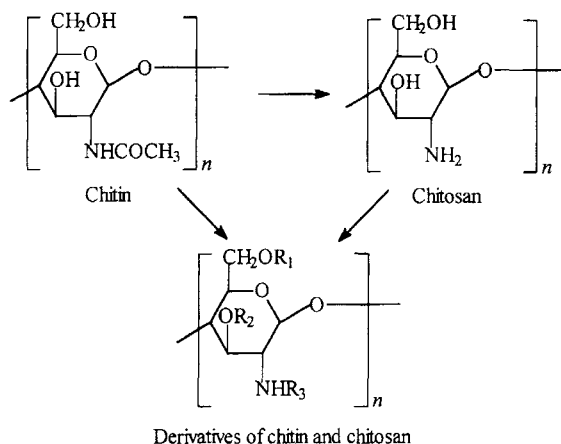


Fig. 1 The structures of chitin, chitosan and their derivatives R_1 , R_2 and $\text{R}_3 = -\text{SO}_3\text{H}$, $-\text{COCH}_3$, $-\text{CH}_2\text{COOH}$, $-\text{PO}(\text{OH})_2$, etc.

EXPERIMENTAL

Materials

Chitin was obtained from Tianjin Zhengtiancheng Company, China. Phosphorus pentoxide and methanesulfonic acid (analytical grade) were purchased from Beijing Chemical Co., Ltd. China. DCPD, MCPM, CaO, $\text{Ca}(\text{OH})_2$ and other chemicals (from Tianjin Chemical Co. Ltd. China) were all analytical reagents. Commercial CaO was heated at 900°C for 2 h to remove H_2O and CO_2 and then kept in a vacuum desiccator before use.

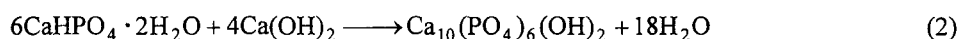
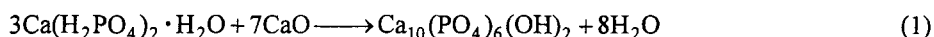
Preparation and Characterization of P-Chitins

Phosphorylated chitins were prepared using the procedure similar to that of Nishi *et al.*^[9]. The reaction mixtures were filtered and the filtrates were dialysed against distilled water (pH = 6) to remove small molecules completely. Phosphorus contents were measured by elemental analysis on an ICP-9000 (N+M) (JJA USA) instrument and degrees of substitution (DSs) were calculated accordingly. ³¹P-NMR spectra were measured on a Varian Unity plus 400 spectrometer in D_2O solution. Infrared (IR) spectra were recorded with KBr pellets on a Bio-Red FIS135 Fourier transform infrared spectrometer, with a resolution of 8 cm^{-1} and 32 scans. The particle diameter of the P-chitin was measured by Dynamic Light Scattering (DLS) by an INNDVO 300/BI 9000 AT (Brookhaven Company Limited, USA) instrument with 0.01 mol/L NaCl as the solvent. The relative molecular weight of the P-chitins were recorded on a GPC equipment with a Shodex column, a Waters 510 pump and a Waters 410 differential refractometer. An aqueous solution containing 0.25% NaNO_3 and 0.1% NaCl was used as an eluant at a flow rate of 0.8 mL/min with polyethylene glycol-polyethylene oxide (PEG-PEO) as the standards.

Preparation and Measurements of CPCs

DCPD, MCPM, $\text{Ca}(\text{OH})_2$ and CaO were respectively milled for 8 h in a rotating micromill (Model WL-1, Tianjin) to obtain a mean particle size around 6.88, 74.3, 3.32 or 1.95 μm . The particle size distribution was measured in absolute ethanol for CaO, $\text{Ca}(\text{OH})_2$ and DCPD or in ethylene glycol for MCPM by a sedimentation method based on Stokes' law using a Micron Photosizer (Model SKC-2000, Japan).

Cement powders were prepared by mixing calcium phosphate (DCPD or MCPM) and basic calcium compound [$\text{Ca}(\text{OH})_2$ or CaO] in a stoichiometric ratio according to Eqs. (1)^[10] or (2)^[11] in an agate mortar. The liquid phase was 1 mol/L phosphate buffers ($\text{pH} = 7.4$) prepared from $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ or 1 mol/L $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ aqueous solution. Different amounts of P-chitin were added to the liquid phase before mixing. A cement powder was mixed with a liquid phase using powder-to-liquid (P/L) ratios ranging from 0.8 to 2.6 g/mL.



The setting time of the CPC mixtures was measured according to the method recommended in International Standard ISO 1566 for dental zinc phosphate cements at the temperature of 37°C and relative humidity (RH) of 80%. After mixing for 2 min, the paste was loaded into 6 mm (diameter) $\times$ 8 mm (high) moulds and stored in a 37°C , 80% RH box for 24 h. The compressive strength was determined on a M500-10AX testing machine (Testometric Universal Tester, England) at a loading rate of $10 \text{ mm} \cdot \text{min}^{-1}$. The average compressive strength was taken from averaging the values of about five specimens. X-ray diffraction (XRD) patterns were recorded on a powder X-ray diffractometer using graphite-monochromatized copper $\text{K}\alpha$ radiation ($\lambda = 0.154 \text{ nm}$), generated at 40 kV and 100 mA. Morphology of the sample was observed from a S-3500N scanning electron microscope (Hitachi, Japan).

RESULTS AND DISCUSSION

Solubility of the P-Chitins

The dissolution of chitin in methanesulfonic acid before reaction was essential to the solubility of P-chitins. If the chitin has not been dissolved entirely, the reaction product will only partly dissolve in water no matter whether the degree of substitution (DS) was high or low. This was because the polymer chains were not free enough to take part in the phosphorylation. There were other factors that could affect the solubility of the P-chitins such as the reaction time and the DS . The longer the reaction time, the higher the DS was and the better the corresponding solubility of the products will be. Several of the P-chitins with different DS were obtained by controlling the amount of acylating reagent P_2O_5 and the reaction time. The P-chitin used in the CPCs with molecular weight $M_w = 2.60 \times 10^4$, $DS = 0.68$ was readily soluble in water and in the liquid phases of the CPCs.

IR Spectra of P-Chitins

The IR spectra of two P-chitins ($DS = 0.68$ and 1.71) are shown in Fig. 2 together with that of chitin for comparison. Because of the acylation reaction, the absorption band of hydroxyl group in chitin at 1318 cm^{-1} disappeared while a new peak at 1258 cm^{-1} displayed owing to the phosphate groups. In addition, $\text{C}-\text{OH}$ absorption at 1156 cm^{-1} and 1114 cm^{-1} were not observed either. The result suggested that most of the hydroxyl groups were phosphorylated.

NMR Spectra of P-Chitins

The proton decoupled ^{31}P -NMR spectra of three P-chitins ($DS = 0.53$, 0.68 and 1.71) were measured in D_2O at $\text{pH} 7.0$ and are shown in Fig. 3. The patterns of different peaks referred to the different phosphorylated chitin products formed from different reaction conditions including the amount of phosphorus pentoxide (P_2O_5) used and the reaction time. According to the space resistance and migration situations of ^{13}C -NMR^[12], the peaks at $\delta = 238$, 227 and 216 could represent the phosphorylation positions on C-6, C-3 and C-1, respectively. Because one peak meant that only the hydroxyl group on C-6 has been substituted, two peaks meant both hydroxyl groups on C-3 and C-6 have taken part in the reactions, and three peaks implied that breakage of glycoside linkage has occurred at C-1 or C-4. By the way, the small peaks around $\delta = 236$ (Figs. 3a, 3b and 3c) arose from ammonium phosphates.

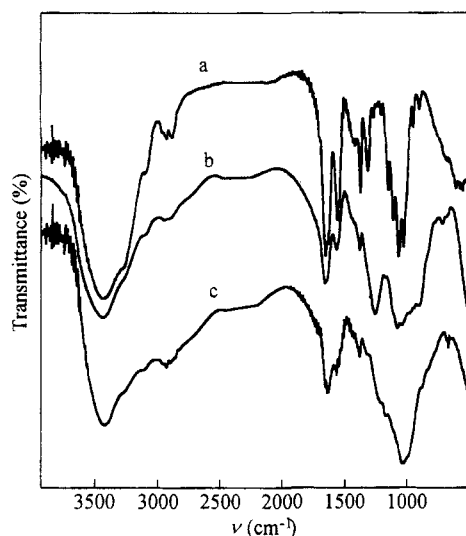


Fig. 2 IR spectra of chitin phosphates
DS: (a) 0 (chitin); (b) 0.68; (c) 1.71

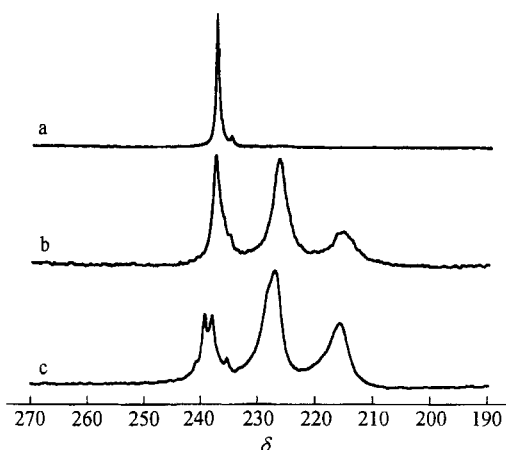


Fig. 3 ^{31}P -NMR spectra of P-chitins
DS: (a) 0.53, (b) 0.68, (c) 1.71

Molecular Weight of P-Chitins

Laser light scattering (LLS) measurements are extremely sensitive to even traces of incompletely dissolved or partly aggregated material (*i.e.* supermolecule or large extraneous contaminants). To avoid the aggregation of P-chitins, 0.01 mol/L NaCl was used as the solvent and the refractive index increment (dn/dc) of one kind of P-chitin ($DS = 0.68$) solution was given as 0.1309 g/L. However, the molecular weight could not be determined by static light scattering (SLS) because the light intensity was highly dependent on the measuring angles and had a negative relationship with the concentration. This indicates the existence of either aggregation or link breakage or both. Dynamic light scattering (DLS) measurement has proved the existence of aggregation. Figure 4 is the $G(d)$ distribution of a P-chitin with DS of 0.68 and concentration of 1.90×10^{-3} g/L at a scattering angle of 90° . Most of the particle diameters distribute within 50–206 nm, but a few lie within 2766–4648 nm. Because the pore size of filtration membrane used just before measurement is only 450 nm, the non-continuous and much larger particles in the final solution could only have arisen by aggregation. The abnormal phenomenon that the scattering intensity decreased with the increasing concentration could be explained as follows: the higher the concentration, the more aggregation, so that fewer molecules pass the filter. In very dilute solution (*e.g.* 1.0×10^{-5} g/L) especially in NaCl solution, the chance for polyions' aggregation was rather few. So the final real concentration obtained through filtration decreased with the increase of concentration before filtration. Adjusting the pH of the solution from 6 to 7 had little effect on the results.

Table 1 shows the GPC results of three P-chitins with $DS = 0.15$, 0.21 and 0.68 and $M_w = 3.93 \times 10^4$, 3.88×10^4 and 2.60×10^4 , respectively. Different molecular weight distributions could be obtained by adjusting the reaction conditions such as the amount of reagents and the reaction time.

Setting Mechanism of the CPCs

For convenience, cements prepared according to Eqs. (1) and (2) are designated as CPCI and CPCII, while cements containing P-chitin as PCPCI and PCPCII. According to Boudeville *et al.*^[10] the setting reaction of CPCI was a biphasic one. In the first reaction step, MCPM reacted with CaO immediately according to Eq. (3) to give DCPD, which was the main reactant in Eq. (2). Then in the second reaction step, DCPD reacted slowly with the remaining CaO to give hydroxyapatite according to Eq. (4). The hydration of CaO from Ca(OH)_2 was less than 10%. This means that the setting reaction of CPCI is complex as compared with that of CPCII. This coincided with the process of preparing CPCI and CPCII. At the same Ca/P molar ratio and similar reaction conditions, the

setting time of the pure CPCI (10 min) was longer than that of the pure CPCII (7 min), while the compressive strength was 1.32 MPa for pure CPCI and 2.86 MPa for pure CPCII after setting for 24 h.

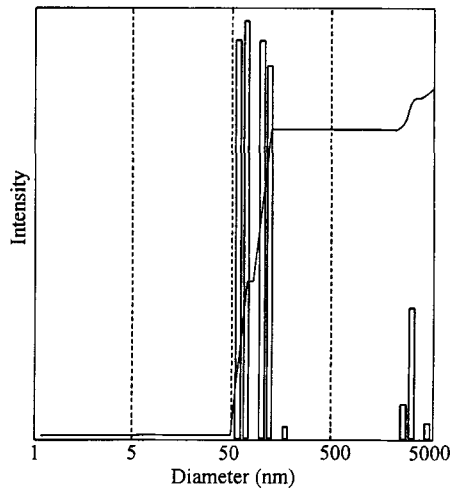
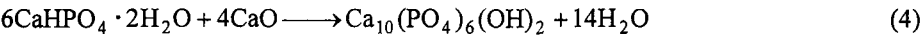
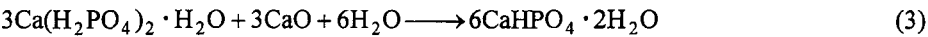


Fig. 4 DLS result of a P-chitin with $DS = 0.68$ and concentration = 1.90×10^{-3} g/L

Table 1. GPC results of three P-chitin samples

Sample no ^a	Retention time (min)	Area (%)	M_n	M_p	M_w	M_z	M_{z+1}	M_{z+1}/M_w	M_z/M_w	Polydispersity
1	29.57	100.00	22003	18442	39304	13555	352210	8.961	3.449	1.786
2	28.33	100.00	24267	27927	38755	80449	162607	4.196	2.076	1.597
3	29.22	100.00	20967	20340	26009	37567	60776	2.337	1.444	1.240

^a Sample 1: P-chitin with DS of 0.15; Sample 2: P-chitin with DS of 0.21; Sample 3: P-chitin with DS of 0.68



The XRD patterns of the CPC systems after setting for 24 h are shown in Fig. 5. The diffraction peaks were attributed to HA, intermediates and starting materials^[10, 11]. P-chitin could not be easily reflected by the peaks, because the amount of P-chitin added was too little to be detected by this technique. The obvious differences between the patterns of CPCI and CPCII are that there are more peaks of DCPD existing in the CPCI and PCPCI patterns (Figs. 5a and 5b), and there are octacalcium phosphate (OCP) peaks in CPCII and PCPCII patterns (Figs. 5c and 5d). While DCPD is the intermediate of the CPCI systems, OCP is formed competitively with HA in the supersaturated solution of calcium phosphate. During the setting process of the CPCII, especially the PCPCII, the mixture will become liquid in a short period, resulting in the formation of supersaturated solution.

The pH measurements were conducted with the advance of the setting reaction. The pH of the CPCII system declined from 14 to 7.4 after setting for 3 h, while the pH of the CPCI system kept around 7.4 throughout the whole reaction. The high pH of the system could increase the solubility of the calcium phosphate salts (e.g. DCPD) which have HPO_4^{2-} ions in their structure^[11]. Above the pH value of 12.7, the dominant phosphate species in the slurries (or pastes) is PO_4^{3-} ions which easily take part in the formation of HA crystals, and only a small fraction of the total phosphate will be in the form of HPO_4^{2-} .

Influence of P-Chitin on the CPCs

The data in Table 2 and 3 show the effects of P-chitin on the properties of CPCI and CPCII. The properties of both PCPCI and PCPCII could be distinctly improved to certain extent by the addition of P-chitin ($M_w = 2.60 \times$

10^4 ; $DS = 0.68$). When the added amount of P-chitin lies within a certain range, the mechanical strengths of PCPCI and PCPCII (especially PCPCII) are greatly increased while the setting time changes only slightly. Another apparent improvement is that there is no decay (wash off) in both PCPCs when the P-chitin containing cements are immersed immediately in running water after setting. When the amount of P-chitin introduced in the liquid phase exceeded the limit of 3.6 wt% for CPCI and 1.2 wt% for CPCII, the setting tends to be retarded and the compressive strength tends to be reduced. On addition of P-chitin in higher amounts, setting will no longer occur.

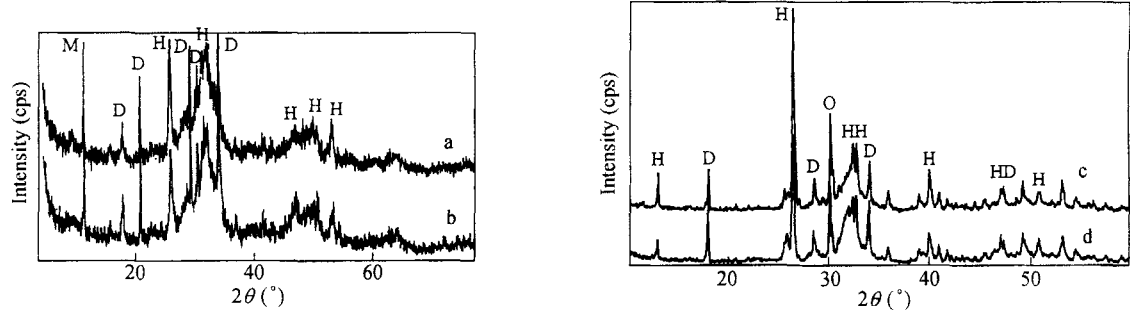


Fig. 5 XRD patterns of the CPCs and PCPCs
a) PCPCI; b) CPCI; c) PCPCII; d) CPCII
D = DCPD, H = HA, M = MCPM, O = OCP

Table 2. The effects of P-chitin on the setting time and mechanical properties of the CPCI

Cement no.	Powder (MCPM + CaO) (g)	Liquid (1 mol/L Phosphate buffers) (mL)	P-chitin* (g)	Setting time (min)	Compressive strength (MPa)	Young's modulus (Mpa)
1	2.78	2.6	0.00	10±0.7	1.32±0.09	27.86±11.62
2	2.78	2.6	0.03	10±1.0	6.70±1.18	238.07±68.30
3	2.78	2.8	0.09	10±0.2	8.02±1.61	294.05±48.25
4	2.78	3.0	0.15	10±0.8	8.40±2.28	309.13±54.31
5	2.78	3.0	0.21	10±0.3	7.25±0.24	277.50±32.87
6	2.78	3.2	0.27	10±0.7	5.49±0.92	179.66±57.95
7	2.78	3.4	0.33	11±0.2	5.04±1.08	205.22±92.74
8	2.78	3.4	0.39	15±0.9	2.68±0.24	99.32±17.05

* P-chitin with DS of 0.68

Table 3. The effects of P-chitin on the setting time and mechanical properties of the CPCII

Cement no.	Powder (DCPD + Ca(OH) ₂) (g)	Liquid (1 mol/L Na ₂ HPO ₄) (mL)	P-chitin* (g)	Setting time (min)	Compressive strength (MPa)	Youngs modulus (MPa)
1	6.64	2.6	0.00	7±1.0	2.86±1.10	184.54±51.80
2	6.64	2.6	0.01	7±0.7	6.47±0.69	239.39±102.44
3	6.64	2.6	0.03	8±0.9	10.36±0.78	408.63±79.02
4	6.64	2.6	0.06	10±0.3	13.08±4.21	368.80±96.12
5	6.64	2.6	0.09	10±0.5	11.79±0.31	269.02±22.30
6	6.64	2.6	0.12	12±0.2	13.08±1.63	414.30±173.64
7	6.64	2.6	0.15	17±0.1	12.20±2.48	370.79±152.69
8	6.64	2.6	0.18	20±0.8	16.57±3.08	508.59±50.95
9	6.64	2.6	0.21	30±1.3	13.27±4.20	473.56±113.94
10	6.64	2.6	0.25	60±1.5	6.76±1.90	336.82±83.54

* P-chitin with DS of 0.68

The reasons why P-chitin can improve the mechanical properties of both CPCs may be explained as follows: (1) P-chitin increases the solubility of the calcium phosphate powder. When the amount of P-chitin added to CPCs exceeds 0.06 g/mL of the liquid phase, the mixture becomes fluid before hardening. The duration of the fluid state change with the amount of P-chitin added. Normally, the more P-chitin added the longer the fluid state will persist, accompanied by a drastically declined mechanical strength. Above the limit of 0.15 g/mL, setting was not observed. It was noted that the rate of HA formation was dependent on the solubility of the starting materials in the interstitial liquid^[10]. The good solubility of the P-chitin contained in the starting materials may be responsible for the higher mechanical strength of the PCPCs. (2) The Ca^{2+} -binding property of the P-chitin becomes operative. Owing to the large contribution of the phosphoryl groups and the synergic actions of other multifunctional groups including the acetylamino group in the P-chitin molecule, it becomes possible for the P-chitin molecules to bind or chelate calcium ions and thus accelerate the deposition of HA over the surface of P-chitin. Nichi *et al.*^[13] has reported that the Ca^{2+} -binding ability of P-chitin is far stronger than its starting material chitin over a wide pH range and the amount of bound Ca^{2+} ion increased with a rise in pH. The strong binding force might further accumulate ions in the vicinity, triggering the nucleation or deposition of HA nearby.

But on the other hand, much more addition of P-chitin to the systems might destroy the stoichiometric balances of Eqs. (1) and (2), forming obstacles to the growth of HA crystals so that no setting took place.

Furthermore, the solubility of the starting materials and the Ca^{2+} -binding ability of the P-chitin depend on the pH of the slurry or paste. During the first 3 h of setting, the pH values of the PCPCII and CPCII (from 14 to 7.4) are higher than those of CPCI and PCPCI (about 7.4). That is one of the reasons why the mechanical properties in Table 3 are higher than those in Table 2.

Morphology of CPCs and PCPCs

Figure 6 shows the SEM micrographs of CPCI without P-chitin (a) and with 0.13 g/mL P-chitin (b), CPCII without P-chitin (c) and with 0.27 g/mL P-chitin (d) after setting for 24 h. The open pores could be seen in all micrographs. The pores in PCPCs with P-chitin look much denser than their original CPCs under the same magnification. As discussed before, the denser structure of the PCPCs might derive from the greater dissolution of the starting materials and the binding ability of P-chitin among HA crystals. That might be the reason why the compression strength could be improved by the addition of P-chitin.

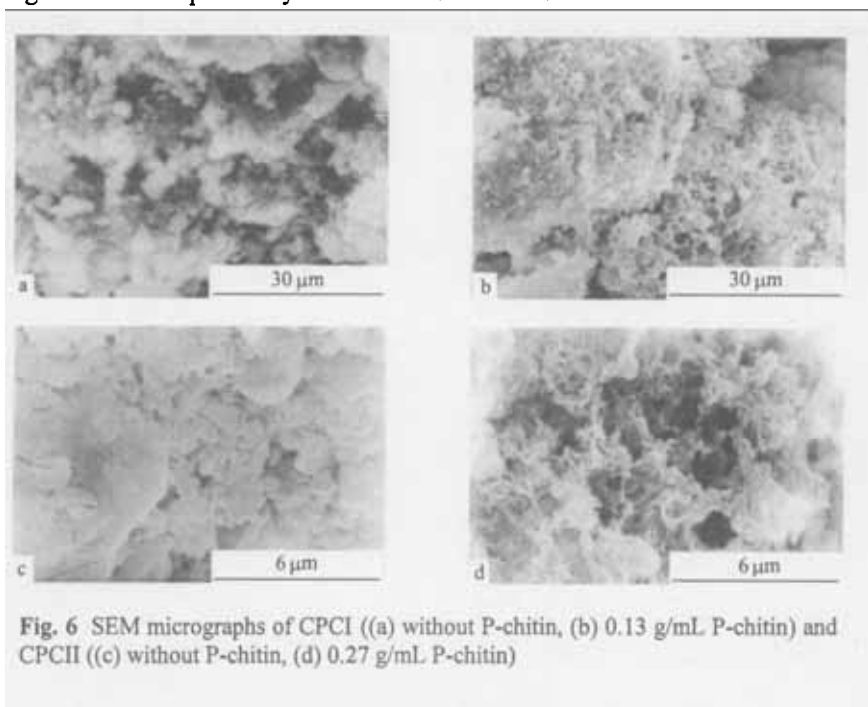


Fig. 6 SEM micrographs of CPCI ((a) without P-chitin, (b) 0.13 g/mL P-chitin) and CPCII ((c) without P-chitin, (d) 0.27 g/mL P-chitin)

CONCLUSION

Using medial value of mechanical strength for trabecular bone as a guide, the compressive strengths of CPCs could attain above 5 MPa. The mechanical properties of PCPCs studied seem neither too high nor too low and have the potentiality to be increased through optimizing the methods of preparation, including reducing the particle sizes, changing the mixing procedures, *etc.* Except for the appearance of 3 h apparently high pH values (from 14 to 7.4) at the beginning of the formation of CPCsII, the pH values of all CPCs could fulfil the requirement for physiological uses (*i.e.* about 7.4). Thus CPCII is more suitable for use in the prefabricated forms while CPCsI could be used directly for the purpose of filling the bone cavities. The setting time of both the CPCsI and CPCsII are reasonable. In general, water-soluble P-chitin had good effects on the CPCs and therefore their corresponding PCPCs might be useful potentially as a material for surgical and clinical applications.

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