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Fabrication of calcium phosphate-calcium sulfate injectable bone substitute using chitosan and citric acid

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Abstract In this study, an injectable bone substitute (IBS) consisting of citric acid, chitosan solution as the liquid phase and tetra calcium phosphate (TTCP), dicalcium phosphate anhydrous (DCPA) and calcium sulfate hemihydrate (CSH) powders as the solid phase was prepared. Four groups containing different percentages (0–30%) of calcium sulfate hemihydrate (CSH, $CaSO_4 \cdot 0.5H_2O$) were investigated. Initial setting times for IBS with CSH were longer than those without CSH. The setting times for all compositions were in the range of 25–45 min. The injectability was improved by the addition of CSH in the present system. Scanning electron microscopy images showed that fiber-like crystallization appeared in the cements. The enhancement of crystallinity was confirmed by XRD profiles where the peak intensity of HAp increased with incubation time and the addition of CSH. Also, the compressive strength increased with the addition of CSH. The maximum compressive strength obtained for IBS was with 20% CSH after 28-day incubation in 100% humidity at 37°C.

1 Introduction

The demand for biomaterials is increasing as the world population ages. Fortunately, calcium phosphate based ceramics have received clinical acceptance for bone substitution and augmentation [1–5] because of their similarities to natural bones. Calcium phosphate cements (CPC) are highly attractive for many clinical uses because they consist of the same ions as the mineral in natural bone, they have excellent osteo-conductivity, a self-setting ability, and the capability of being replaced by new bone. The present orthopedic implants have a major disadvantage in that they exist in hardened forms. The surgeon has to fit the surgical site around the implant due to a lack of available implants of the desired shape, which can lead to increased bone loss, trauma to the surrounding tissue, and a longer time for the surgery. [6]. HAp block shows necrosis and perforation of the mucosa covering the block [7]. On the other hand, it is difficult to prevent the dispersion of HAp granules and to mold the granules into the desired shape [8].

One of the major improvements in orthopedics in recent years is the development of an injectable system, a minimally invasive technique that is becoming popular. The injectable systems can mold to the shape of the bone cavity and set in situ when injected. Such systems should shorten the surgical operation time, reduce the damaging effects of large muscle retraction, decrease the size of the scars and diminish post-operative pain. It also allows the patient to achieve rapid recovery in a cost-effective manner.

The first CPC consisted of tetracalcium phosphate (TTCP, $Ca_4(PO_4)_2O$) and dicalcium phosphate anhydrous (DCPA, $CaHPO_4$) and was introduced in 1986 [9]. In an aqueous environment at 37°C, CPC transformed to hydroxyapatite (HAp) which is more similar to biological apatite than sintered HAp formed at high temperatures [10]. It has been reported that calcium phosphate cement consisting of TTCP and dicalcium phosphate dihydrate (DCPD, $CaHPO_4 \cdot 2H_2O$) or DCPA demonstrated good osteo-conductivity [11–13].

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Chitosan, a natural component of shrimps or crab shells, is biocompatible and biodegradable [14], and osteoconductive [15]. It is an amino-polysaccharide obtained by alkaline deacetylation of chitin. It has been reported that the aqueous solution of chitosan could be used as the liquid phase of CPC [16, 17].

Calcium sulfate hemihydrate (CSH, $CaSO_4 \cdot 0.5H_2O$) has a long history of clinical application because of its properties of rapid setting and good biocompatibility [18–20]. The transformation of CSH into calcium sulfate dihydrate (CSD, $CaSO_4 \cdot 2H_2O$) via an aqueous reaction has been used for a long time to produce materials for bone augmentation. However, CSD has some weak points, such as that the solidified CSD paste has low mechanical strength, CSD cement cannot form a chemical bond with tissue at the early stage of therapy and the in vivo resorption rate is too fast [21–23], all of which significantly limit its clinical applications. However, the fast resorption rate of this cement allows the introduction of porosity into the bone cement while being incorporated with the calcium phosphate cement [24].

In this study, the effect of CSH on the calcium phosphate based bone cement was investigated. The solution of citric acid and chitosan was used as the liquid phase and the powder component consisted of TTCP-DCPA-CSH.

The setting behavior and compressive strength were measured depending on the amount of CSH which was added. The surface morphology, and phase detection were performed using scanning electron microscopy (SEM) and X-ray diffraction. The injectability of this bone filling paste was also investigated.

2 Experimental procedure

2.1 Materials

In this investigation, the cement consisted of both powder and liquid phases. The liquid phase was an aqueous solution of chitosan and citric acid. The mass fractions of chitosan and citric acid were 2 and 20%, respectively. Chitosan was purchased from Sigma (Iceland) and the degree of deacetylation was a minimum of 85%. Citric acid was purchased from Samchun Pure Chemical Co. Ltd. (Pyongtack, Korea). The powder component was a mixture of TTCP, DCPA and CSH. TTCP was synthesized by the solid state reaction method. An equimolar amount of DCPA (Sigma, USA) and $CaCO_3$ (DC chemical Co. Ltd) were mixed and heated to 1500°C for 6 h in a box furnace. This calcined mixture was ground to particles with sizes ranging from approximately 1–50 μm. TTCP and DCPA powders were mixed at a molar ratio of 1:1 to obtain the CPC powder. Four different percentages of CSH powders were added to this CPC powder ranging from 0 to 30 wt.%. Depending on the CSH content, four types of cement were named as IBS-0 to IBS-30. The compositions of the four different cements are shown in Table 1. The liquid and solid components were mixed manually with a spatula and the liquid-to-powder (L/P) ratio was 0.41 ml/g.

2.2 Setting time measurement and injectability of the paste

Setting times of these cements were measured according to the ISO 9917 standard for dental silico-phosphate cement [25]. Setting times of the samples were measured by using a Gilmore apparatus. Any sample was considered set when a 400 g mass loaded to a needle with a tip diameter of 1 mm did not make any visible impression on the surface of the sample kept in 100% relative humidity at 37°C.

Injectability of the composite cement was evaluated using a disposable syringe and taking out the paste through the syringe by hand pressure. The syringes had a capacity of 3 ml with an opening nozzle size of 2 mm in diameter. As mixed paste of 4 g was added into the syringe and extruded after certain pre-set time periods. The paste was gently extruded from the syringe by hand until it was unable to inject entirely. The weight of the injected paste was then measured and injectability was calculated using the following equation [26, 27]:

$$\text{Inj}\% = (\text{Paste weight expelled from thesyringe})/(\text{total paste weight before injecting}) \quad (1)$$

2.3 X-ray diffraction analysis

The X-ray diffraction profiles (XRD, D/MAX-250, Rigaku, Tokyo, Japan) of different cements were recorded by X-ray

Table 1 Composition of injectable bone substitute

Sample name	Liquid components		Powder components		L/P ratio (ml/g)
	Chitosan (wt.%)	Citric acid (wt.%)	TTCP DCPA (wt.%)	TTCP DCPA (wt.%)	
IBS-0	2	20	100	0	0.41
IBS-10	2	20	90	10	
IBS-20	2	20	80	20	
IBS-30	2	20	70	30	

powder diffraction analysis with a scanning speed of 2°/min.

2.4 Morphology observation of cements

The morphology of the cements was observed under scanning electron microscopy (SEM, JSM-635, JEOL, Tokyo, Japan) after setting.

2.5 Elastic modulus and apparent density of the paste

Elastic modulus of the set cement was measured by the echo method using bar type set samples. The equation used for this was,

$$E_R = 0.9465 \times \left[\left(M \cdot f^2\right)/w\right] \times (L_r/t)^3 \times \left[1 + 6.59(t/L_R)^2\right]$$

where, M = mass, f = resonance frequency, L_r = length of bar, t = thickness of bar and W = width of the bar.

The density was measured by the weight-dimension method. Average of five samples was taken for each measurement data.

2.6 Compressive strength measurement

The bone cements were molded into cylindrical columns of 9.5 mm in diameter and 18 mm in length. The samples were incubated in 100% relative humidity at 37°C for 1, 3, 7, 14 and 38 days. At these time intervals the compressive strengths were measured using a computer controlled Universal Testing Machine (R & B, Korea). The cross-head speed was 1 mm/min. For each measurement, an average of five samples was taken.

3 Results

3.1 Setting time and injectability

After mixing the liquid and powder components together, all types of cement showed a paste consistency. The cements could be easily molded into desired shapes. The variation of setting times with CSH content is shown in Fig. 1. Setting time without CSH was 25 min. After the addition of 10 wt.% of CSH, the setting time was increased to 45 min, and decreased gradually with the increase of CSH content. At 20 and 30 wt.% CSH, the setting times were 39 and 35 min, respectively.

IBS-0 lost its injectability after 20 min of mixing while it retained almost 100% injectability after 10 min. On the other hand, IBS with CSH retained almost 100% injectability up to 15 min after mixing and lost it fully after 25 min as shown in Fig. 2.

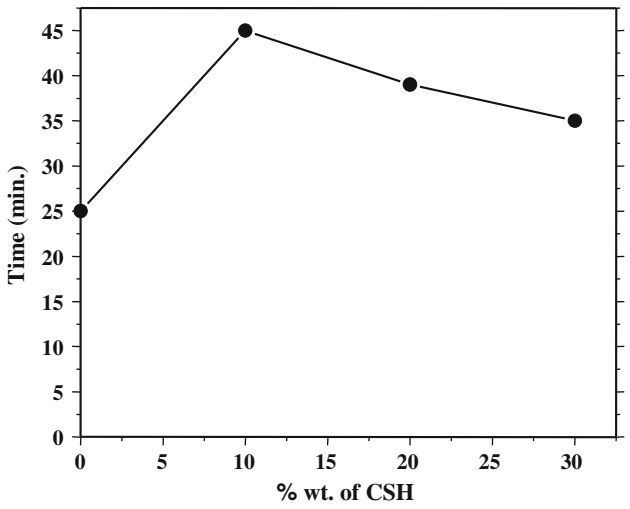

Fig. 1 Setting time versus percentage of calcium sulfate hemihydrate (CSH)

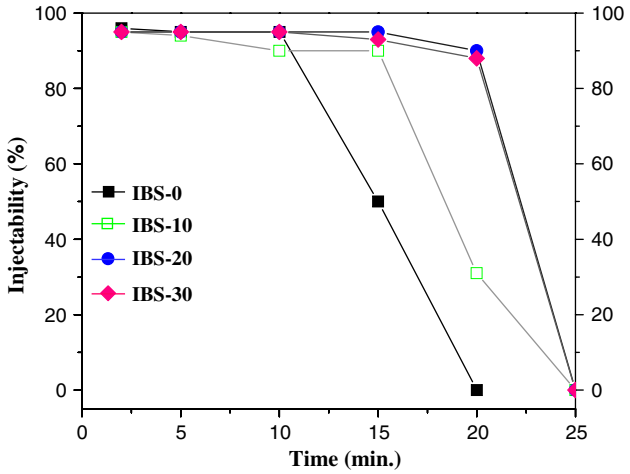

Fig. 2 Injectability of the paste depending on times after mixing

3.2 Morphology observation

Figure 3 shows the fracture surfaces of IBS-0 (a, b) and IBS-20 (c, d) after setting. The surface of IBS-20 was smoother than that of IBS-0 as can be observed from low magnification images. There also remained some residual pores. The surface of the IBS-0 comprised with micro-cracks, few needle like crystallites and course microstructures. On the other hand, the surface of IBS-20 was more homogeneous and comprised fine microstructures. Figure 4 shows the fracture surfaces of IBS-0 (a, b) and IBS-20 (c, d) after 14d incubation (a, c) and after 28d incubation (b, d). After 14 days of incubation, IBS-0 was covered with fine microstructures but after 28 days of incubation, fiber-like crystallizations appeared. In IBS-20, fiber-like crystallizations appeared after 14 days of incubation, and they increased and became finer as the time of incubation increased.

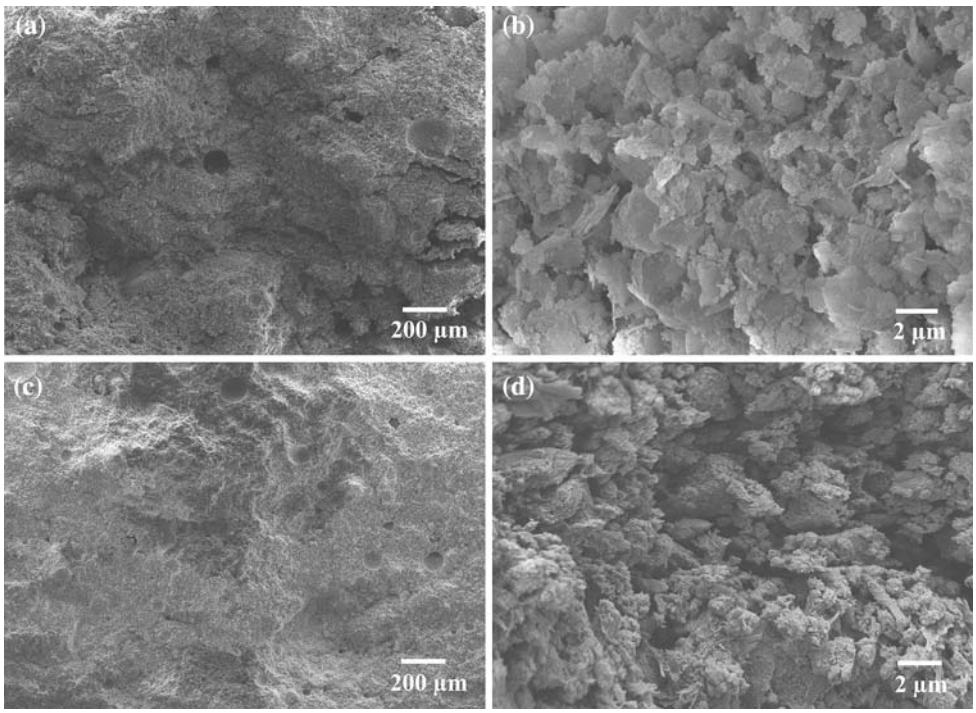

Fig. 3 SEM images of fracture surfaces of **a** IBS-0 and **b** its enlarged image, **c** IBS-20 and **d** its enlarged image

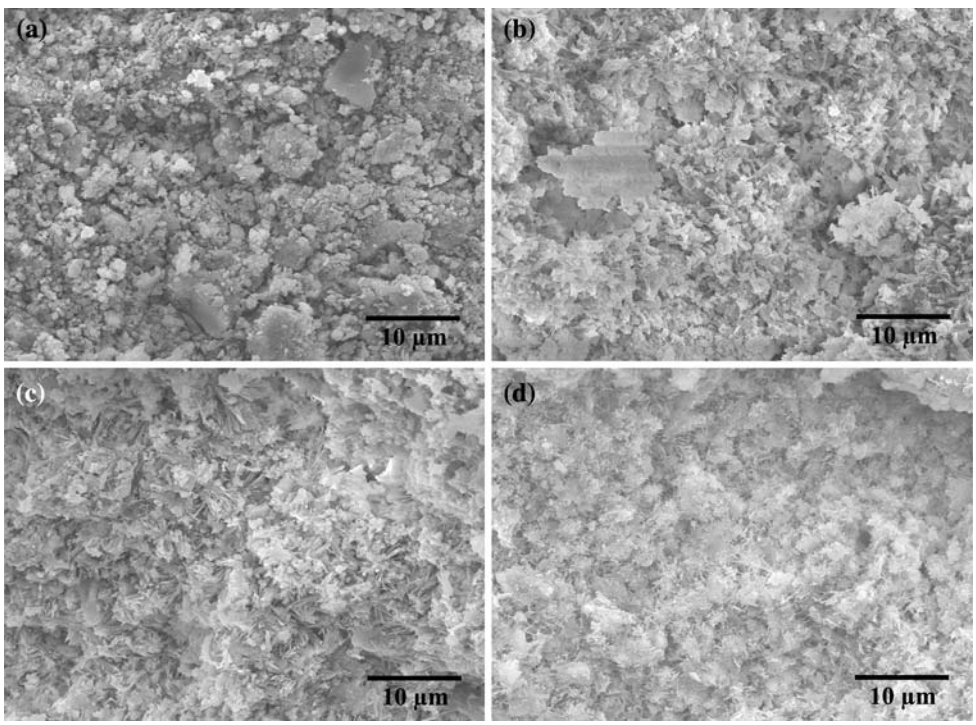

Fig. 4 SEM images of fracture surfaces of **a** IBS-0 incubated 14d, **b** IBS-0 incubated 28d, **c** IBS-20 incubated 14d and **d** IBS-20 incubated 28d

3.3 XRD analysis

Figure 5 shows XRD profiles of (a) $CaSO_4 \cdot 0.5H_2O$ (CSH), (b) TTCP, (c) IBS-0, (d) IBS-10, (e) IBS-20, and (f) IBS-30. TTCP partially transformed into HAp as HAp peaks appeared in all four types of cements. As the amount of CSH in the cement increased, the transformation of TTCP into HAp also increased. CSH did not convert into CSD in the cement. DCPA peaks appeared in all four types of cements. The intensity of these peaks was the highest for IBS-10 and lowest for IBS-30. Figure 6 shows the XRD profiles of IBS-20 (a) after mixing, (b) after 14 days and (c)

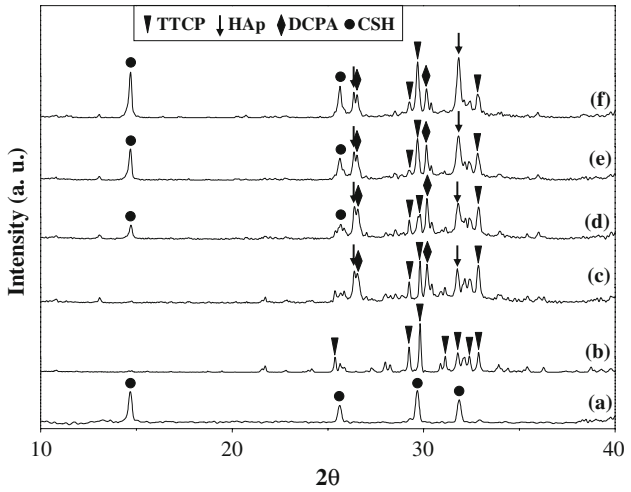

Fig. 5 XRD profiles of (*a*) $CaSO_4 \cdot 0.5H_2O$ (CSH), (*b*) TTCP, (*c*) IBS-0, (*d*) IBS-10, (*e*) IBS-20, and (*f*) IBS-30

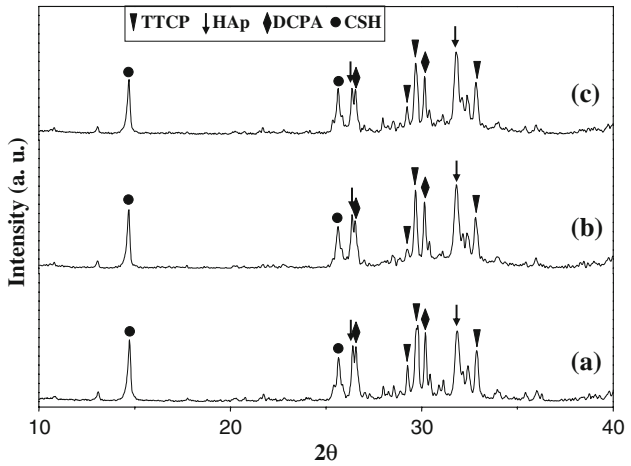

Fig. 6 XRD profiles of IBS-20 (*a*) after mixing and setting, (*b*) 14d and (*c*) 28d of incubation in 100% humidity at 37°C

after 28 days of incubation in 100% humidity at 37°C. As the incubation period increased, the intensity of the HAp peaks also increased.

3.4 Elastic modulus

Figure 7 shows the apparent density and elastic modulus of the cements. The density of the cements with CSH was lower than without CSH. The elastic modulus of the cement decreased with the addition of CSH. However, at 10 wt.% CSH, the cement showed the highest value of elastic modulus.

3.5 Compressive strength

The compressive strength (Fig. 8) of each cement increased with the time of incubation in 100% humidity at

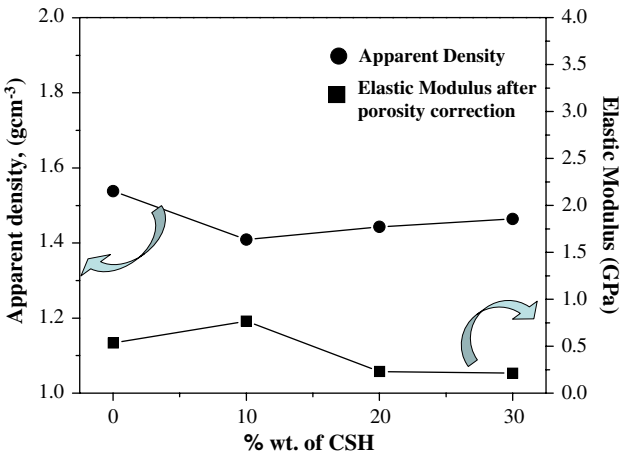

Fig. 7 Apparent density, elastic modulus depending on CSH content

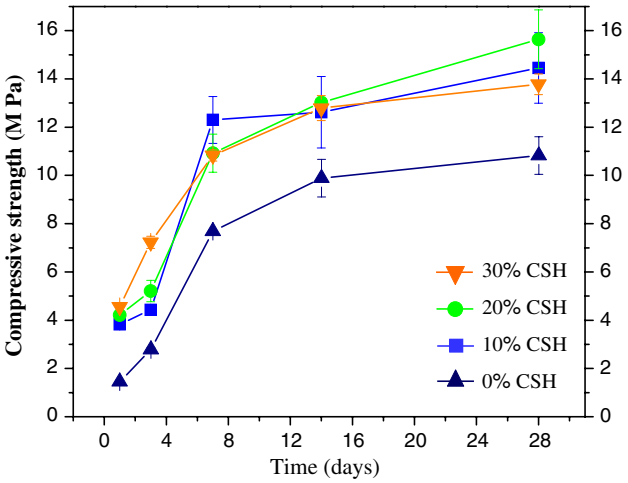

Fig. 8 Compressive strength in air at 37°C and 100% humidity

37°C. The increase of compressive strength after one week was very high and after that the rate was relatively slow. The cements with CSH showed higher strength than that without CSH. However, after 28 days of incubation, IBS-20 showed comparatively higher strength.

4 Discussion

In this study, TTCP-DCPA was used as the main component and a solution of chitosan and citric acid was used as the liquid component. CSH was added, from 0% to 30%, and its effects on setting time, mechanical properties and phases formed were investigated. Chitosan was cross-linked by citric acid and the viscosity of the liquid phase increased. Usually, calcium phosphate cement containing carboxylic acid hardens rapidly, with an insoluble matrix by chelation. The hardening of the present cement occurred as a result of several reactions, such as the chelate reaction

between citric acid and calcium in the powder component and the transformation reaction of the components of the cements to HAp. TTCP and DCPA were transformed according to the following reaction:

$$6CaHPO_4 + 6Ca_4(PO_4)_2O \rightarrow 3Ca_{10}(PO_4)_6(OH)_2 \quad (2)$$

If CPC is mixed with water, after a certain period, the mixing solution becomes supersaturated towards biological apatite and the apatite begins precipitating. The addition of chemicals can modify the setting reaction; for example, at neutral and slightly basic condition CSD is more soluble and can increase the rate of super-saturation of the solution [26]. In this investigation, the setting time increased significantly at 10 wt.% CSH addition and decreased successively at 20 and 30 wt.% CSH. It is hypothesized that two factors affected the setting time when CSH was added. One was the chelate reaction and the other was the super-saturation of the solution. At 10 wt.% CSH, the chelate reaction was retarded by the CSH due to interaction with the citric acid, which in turn increased the setting time. On the other hand, at 20 or 30 wt.% CSH, the cement became supersaturated, which accelerated the setting reaction and promoted the reaction as mentioned in Eq. 2. Retardation was dominant in the former (10% CSH) while super-saturation was dominant in the latter (20 and 30% CSH). In the present system, CSH did not undergo a hydration reaction according to XRD profiles, which was also responsible for lengthening the setting time.

Injectability is crucial for an injectable bone substitute. Considering the clinical application, the cement must be applied before it sets. The clinician should be allowed approximately 15 min working time including mixing and injection. The injectability of the present systems depended on setting time as well as CSH content. As the CSH content increased, the injectability also increased. Khairoun et al. [27] reported that the addition of calcium sulfate can improve the injectability of calcium phosphate cement. No cement had shown a filter-pressing phenomenon in which the liquid alone was pushed out, keeping the major portion of powder inside the syringe.

The addition of CSH to TTCP-DCPA cement affected the microstructure. $CaSO_4$ is a Ca rich and relatively soluble phase. When a fast-releasing calcium salt is present in cement, it decreases the time required for the paste to become super-saturated toward calcium deficient apatite. However, even though the setting time did not decrease, the SEM image of IBS-20 supported the XRD profiles where the peak of HAp increased. The apatite formation reaction continued and even after 28 days, it was not finished.

According to the XRD profiles (Fig. 5), HAp conversion was enhanced with the addition of CSH, and as the amount of CSH increased, the intensity of the HAp peak also increased. In contrast, the relative intensity of the TTCP peaks decreased. However, at 10% CSH, the conversion to HAp was the least but the peak intensity of DCPA was the highest. This phenomenon might be attributed to the setting behavior which has been discussed earlier. An anomalous behavior was observed regarding the CSH which remained unchanged in the cement. Usually, CSH in cement undergoes a hydration reaction and forms CSD. Citric acid might have obstructed this hydration reaction. The XRD profiles of IBS-20 (Fig. 6) indicated that the conversion reaction was enhanced as the incubation time increased as well as the intensity of the HAp.

The compressive strength of this system improved with the addition of CSH and after 28 days of incubation, IBS-20 showed maximum strength. For all four types of cement, the strength increased as the incubation period increased. This was due to the fiber-like crystallization of Hap, which appeared more in CSH added cements. Generally, the addition of calcium sulfate does not affect the compressive strength or sometimes decreases it [24].

The effect of CSH on the present systems appeared to be unconventional. The setting time increased initially and then decreased. Bohner and Baroud [28] reported that the addition of CSD to α-tri calcium phosphate cement did not affect the mechanical strength appreciably, whereas in this investigation, the compressive strength increased appreciably. The conversion reaction to biological apatite also enhanced while in other investigations, it was found to be the opposite. The in vivo resorption rate of calcium sulfate is too fast, which may negatively affect bone regeneration [1–23]. However, this drawback of calcium sulfate can be utilized being used as a porogen in injectable bone paste [24]. Fortunately, the present system showed better effects in terms of mechanical properties and microstructures as well as the apatite-forming ability.

5 Conclusion

Injectable bone cement has been synthesized using TTCP-DCPA as the powder component and a solution of chitosan and citric acid as the liquid component. With this cement, 0 to 30 wt.% CSH was added and the effect on mechanical properties and microstructures was reported. The setting time anomalously increased at 10 wt.% CSH and began to gradually decrease as the content of CSH increased. The injectability remained almost 100% up to 20 min for IBS-20 and IBS-30. In terms of microstructure, fiber-like apatite formed in all four types of cements after incubation. This apatite possibly increased the compressive strength of the cements. Addition of CSH to the present system facilitated the apatite formation.

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