J Mater Sci: Mater Med (2007) 18:1195–1201
DOI 10.1007/s10856-007-0139-9

ORIGINAL PAPER

Increase of the final setting time of brushite cements by using chondroitin 4-sulfate and silica gel

F. Tamimi-Mariño · J. Mastio · C. Rueda · L. Blanco · E. López-Cabarcos

Received: 24 October 2005 / Accepted: 14 March 2006 / Published online: 3 February 2007
© Springer Science+Business Media, LLC 2007

Abstract Chondroitin 4-sulfate (C4S) is a bioactive glycosaminoglycan with inductive properties in bone and tissue regeneration. Dicalcium phosphate dehydrate cements (known as brushite) are biocompatible and resorbable materials used in bone and dental surgery. In this study we analyzed the effect of C4S on the setting of a calcium phosphate cement and the properties of the resulting material. Brushite based cement powder was synthesised by mixing monocalcium phosphate with β-tricalcium phosphate and sodium pyrophosphate. When the concentration of C4S, in the liquid added to the cement powder, was between 1 and 8% the cement final setting time increases. Furthermore, the cement diametral tensile strength remains unaffected when solutions with concentrations of C4S below 5% were used, but decreases at higher C4S concentrations. Calorimetric analysis showed that the cements prepared with C4S alone and in combination with silica gel have a greater content of hydrated water. We concluded from our study that the addition of small amounts of C4S increases the cement setting time without affecting its diametral tensile strength and at the same time improves the cement's hydrophilicity.

F. Tamimi-Mariño · J. Mastio · C. Rueda ·
E. López-Cabarcos (✉)
Departamento de Física Química II, Facultad de Farmacia,
Universidad Complutense de Madrid,
Pza Ramón y Cajal s/n, Madrid 28040, Spain
e-mail: cabarcos@farm.ucm.es

F. Tamimi-Mariño · L. Blanco
Facultad de Odontología,
Universidad Complutense de Madrid,
Pza Ramón y Cajal s/n, Madrid 28040, Spain

Introduction

Calcium phosphate cements based on brushite are considered resorbable and biocompatible. The cement is usually presented as a liquid phase and solid phase that are mixed together in order to form a workable paste that would set into a hard material. The setting reaction occurs through an acid–base reaction between a basic calcium phosphate, usually β-tricalcium phosphate (β-TCP), and an acidic phosphate such as orthophosphoric acid or monocalcium phosphate [1].

In vivo, brushite cements are used in the form of blocks or as a setting paste that would act as a scaffold for bone regeneration. However this material has some inherent drawbacks that need to be dealt with, such as the short setting time, low injectability, lack of cohesion and poor mechanical properties. The addition of macromolecules or gels to the cement liquid phase opens new perspectives to overcome these problems.

The rationale for using macromolecules as nucleating agents in mineral-based materials is controlling their properties through the nucleation and growth of mineral microstructures. Thus, proteins, polysaccharides and gels have been used to manipulate the mechanical properties and biofunctionality of cements used in bone regeneration [2]. The addition in the liquid phase of glycosaminoglycans such as hyaluronic acid has been attempted as a way to improve the cohesion of brushite, although when added at concentrations higher than 1.5% of the liquid phase a dramatic decrease of the cement cohesion occurs [3, 4]. There are commercialized hydroxyapatite (HAP) calcium phosphate cements that contain glycosaminoglycans, such as C4S, in their composition, [3]. The

addition of C4S in HAP cements setting-reaction improves its biocompatibility when employed in subcutis and in periapical bone reconstruction as well as its physical chemical properties [5, 6]. Nevertheless, the effect of C4S has not yet been studied in brushite cements.

Chondroitin sulfate is a glycosaminoglycan with two isomeric forms depending on the sulfate group position; chondroitin 4 sulfate and chondroitin 6 sulfate. Both variants have bone regenerative capacity which was first investigated in rat and primate mandibles [7–10]. Combinations of chondroitin sulfate (CS) with other biomaterials have been employed to control the release of therapeutic molecules such as growth factors and bone morphogenic proteins [11]. Recently, chitosan sponges with C4S were used for delivering platelet derived growth factors (PDGF-BB) improving osteoblastic proliferation and increasing the osteoblastic migration when compared with chitosan alone [12]. More recently, poli(lactic-co-glycolic acid) combined with CS was used as a controlled release system for bone morphogenic proteins (BMP2), obtaining an optimal biocompatibility and osteogenic stimulation [13].

Nowadays, materials combining CS with collagen and HAP are being commercialized as bone substitute for bone regeneration. Recent studies [14] demonstrated that these materials have bone regenerative capacity equivalent to that of bovine HAP xenografts (Bio-oss®) and are well tolerated by the bone. Materials containing CS have some osteoinductive capacity and accelerate osteoblast activity as well as the production of extracellular matrix on which bone HAP will be deposited [15, 16].

It is now well known that CS has the capacity to be adsorbed on calcium phosphates such as HAP. For example, CS adheres to coral HAP in a ratio of 0.72 ± 0.06 mg/20 mg of coral HAP, and the interaction is influenced neither by the polysaccharide molecular mass [17] nor by the sulfate group position [18]. The adsorption ratio depends mainly on the electric charge density, being the sulfate groups more relevant for this phenomenon than the more pH sensitive carboxy groups. At pH 7 these glycosaminoglycans have negative electrical charges due to both the sulfate and carboxy groups since C4S pKa is 3.97 [17]. Furthermore, C4S adsorption to HAP increases at lower pH levels because as pH decreases C4S forms a more compacted structure that better interacts with the free surface calcium ions [19].

Regarding C4S possible effects on bone regeneration gels, it has been observed that C4S disrupts collagen I gels. Addition of CS enhances the crystallization of HAP resulting in larger volumes of gel filled with crystalline clusters. The effect of C4S on collagen gels is explained by its large size that disrupts the gel and lowers its viscosity. As a result, the mobility of diffusing species increases accelerating supersaturation, leading to both homogeneous and heterogeneous nucleation in the solution bulk and on collagen fibres, respectively [20]. This effect could influence the mechanical properties of the resulting mineral, for it is known that different populations of crystals provide different mechanical properties for the materials [21].

In addition to the above properties, C4S also has a direct effect on crystallization of several calcium phosphate species. Thus, it can prevent crystal growth and aggregation of calcium oxalate monohydrate and retards seeded HAP growth in vitro [22]. It is believed that the large hydrodynamic size and high charge density of these macromolecules are associated with the ability of these glycosaminoglycans to inhibit HAP formation and growth [23]. This phenomenon is of special interest when intending the production of calcium phosphate cement with an acceptable setting time and a small crystal size that would increase its mechanical properties.

Another group of macromolecules that has been used for improving calcium phosphate cements are the silica based polymers. Silica is a heavily charged molecule with hydrophilic properties that when added to calcium phosphate cements produces a fluid paste capable of setting in physiological environments. Silicate containing ceramics are bioactive materials that bind spontaneously to living bone, and are clinically used as bone substitutes [24]. Silica gels and silica solutions are biocompatible, osteoconductive promote HAP deposition, and improve the bioactivity, injectability and handling of HAP cements [25–27]. However, silica based polymers had only been tested in HAP cements, and it is expected to have a similar effect on brushite cements.

The aim of this work is to examine the feasibility of combining silica gels and C4S with brushite cements. We will use a liquid phase containing a solution of C4S alone, or combined with silica gel, in brushite cement setting reaction and analyze its effect on the structure and mechanical properties of the resulting material.

Materials and methods

The reagents, calcium carbonate (CC), dicalcium phosphate dihydrate (DCPD), monocalcium phosphate (MCP), sodium pyrophosphate and C4S (from Sigma Aldrich), citric acid and aerosil (from Panreac)

were of the highest purity and used without further purification.

Beta-tricalcium phosphate (β-TCP) was made by heating a stoichiometric mixture of CC and DCPD at 900 °C for 14 h:

$$CaCO_3 + 2CaHPO_4 \cdot 2H_2O \rightarrow Ca_3(PO_4)_2 + 5H_2O + CO_2 \quad (1)$$

The product of the reaction was analyzed by X-ray diffraction to assure the purity of the β-TCP formed.

The cement powder was made by mixing the reactants, β-TCP (1.428 g) and MCP (0.8 g), with the chemical retardant sodium pyrophosphate (0.012 g). The cement liquid was made of 0.5 M citric acid and different concentrations of C4S. The pH of the solution was always kept at 4.1 by using either NaOH or phosphoric acid. In order to decrease the repulsive negative charges of the C4S in the solution, silica gel was added to C4S at a concentration of 15 g/L

Silica gels were prepared by fusing weighed amounts of Aerosil with 2 M NaOH. The liquid was heated for 2 h at 60 °C to accomplish complete dissolution. Then, silica solution pH was adjusted with citric acid obtaining a final SiO_2 concentration of 15 g/L in a 0.5 M citric acid solution. The cement solution pH was always fixed to a value of 4.1 (measured with pH Meter Mettler Toledo MP230) using phosphoric acid and NaOH. Then, different concentrations of C4S were added to the solution in order to evaluate its effect on the cement setting reaction. Whenever C4S was added to the silica solution, gelification was retarded for 4 days up to 1 week. Solutions with a higher C4S concentration have the longer gelification time. Thus, to assure a complete gelification before use, the silica-C4S solution was prepared 1 week before to be mixtured with the cement.

Cement samples were made by mixing the different chondroitin gels or solutions with the cement powder according to the reaction:

$$Ca_3(PO_4)_2 + Ca(H_2PO_4)_2 + 8H_2O \rightarrow 4(CaHPO_4 \cdot 2H_2O) \quad (2)$$

To study the mechanical properties, cement samples were prepared by mixing the cement powder with the different liquids in a powder/liquid ratio of 2.56 for 30 s. The mixture was left to set in cylindrical moulds obtaining tablets with 8 mm diameter and 6 mm thickness. At least 10 tablets were made out for each cement sample. The cement tablets were maintained at 100 % humidity at 37 °C environment for 24 h before being tested. The mechanical properties were measured with a tablet hardness machine Pharma Test PTB 311E. The diametral tensile strength (DTS) was calculated from the formula:

$$\mathrm{DTS\,(MPa)} = 2F_{\max}/(\pi \times d \times l) \quad (3)$$

where $F_{\max}$ is the failure load, d is the sample diameter and l is the length of the probe.

The samples of the set cements were powdered and analyzed by X-ray diffraction using a Philips X-pert PW3050 diffractometer. For initial phase recognition diffractograms were recorded covering an angular interval between 2θ = 5 and 80°, and using a step size of 0.04° with time per step of 1 s. The main intensity peaks of β-TCP and brushite were separated in the diffraction patterns. Diffractograms used for Ritveld analysis were recorded covering an angular interval between 2θ = 10 and 90°, and using a step size of 0.03° with time per step of 3 s. Rietveld analysis was performed using the Xpert plus software, referring to the ICSD 016132 brushite and ICSD 06191 β-TCP files.

The cement sample setting time was measured according to the international standard ISO1566 for dental zinc phosphate cement [2]. According with this method, the cement is considered set when a 400 g weight loaded on to a Vicat needle with a tip diameter of 1 mm fails to make a perceptible circular indentation on the surface of the cement.

Differential scanning calorimetry measurements were carried out in the different set cement samples using a Metler Toledo DSC820. All samples were heated in a nitrogen gas atmosphere from 25 °C up to 500 °C at a rate of 10 C/min.

Results

As is illustrated in Fig. 1 the X-ray diffraction analysis indicates that the composition of the cement after completing the setting reaction is brushite and β-TCP. Intensity peaks related to other components were not detected. Rietveld analysis of the cement samples reveals that the percentual weight of β-TCP and brushite in the cement was around 25 and 75% respectively (see Table 1). There is a small reduction in brushite and a subsequent increase in β-TCP weight percentage when C4S and silica gels were added to the reaction.

In Fig. 2 we present the cement final setting time as a function of the C4S concentration for samples

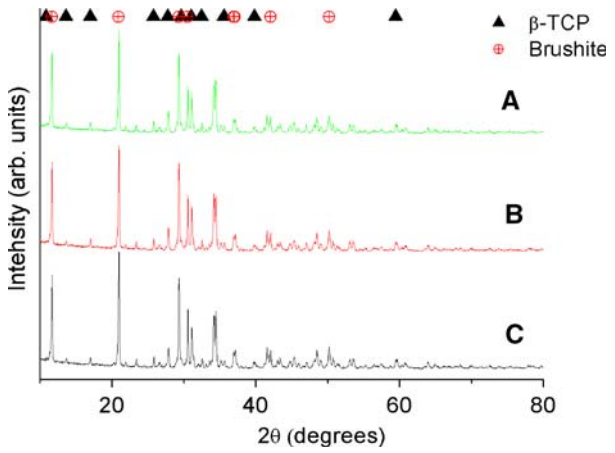

Fig. 1 X-ray diffraction patterns corresponding to the cement samples using the following liquids in the setting reaction: A) 0.5M citric acid, B) 0.5 M citric acid + 2% C4S, and C) 0.5 M citric acid + 2% C4S + 15 g/L silica gel. Black triangles correspond to β-TCP peaks and crossed circles to brushite peaks

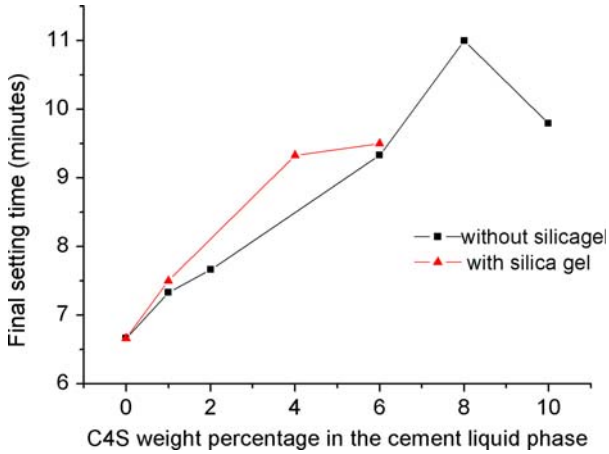

Fig. 2 Cement setting time as a function of the chondroitin 4-sulfate content for samples prepared with silica gel (triangles) and without silica gel (squares)

prepared with and without silica gel. As shown in Fig. 2 small concentrations of C4S (between 1–8%) prolong the cement setting time, whereas for C4S concentrations higher than 10% the setting time decreases. Regarding the addition of silica gel it appears that its effect on the cement final setting time is small or negligible as is illustrated in Fig. 2

The dependence of the cement diametral tensile strength as a function of the C4S concentration is shown in Fig. 3 . The points in Fig. 3 represent the average value of 10 different samples measured for each C4S concentration. As is illustrated in Fig. 3a, the diametral tensile strength slightly increases by the addition of C4S at concentrations up to 5%, and beyond this point the DTS of the cement suffered a conspicuous decrease of around 20%. The effect of silica gel is shown in Fig. 3b. When silica gel was added to the C4S solution the DTS remains constant up to 6% of C4S concentration.

The maximum C4S concentration was 10% in the cements prepared without silica gel and 6% in cements prepared with silica gel (15 g/L) because beyond these concentrations we were not able to produce a proper cement paste with an adequate setting reaction.

Table 1 Cement composition as obtained from Rietveld analysis

	Setting liquid composition		
	0.5 M citric acid	0.5 M citric acid + 2% C4S	0.5 M citric acid + 2% C4S + 15 g/L SiO_2
Brushite percentage	75%	73%	70%
β-TCP percentage	25%	27%	30%

The amount of water absorbed by the cements prepared with and without silica gel was measured by DSC. As can be seen in Fig. 4a the thermograms of the samples after cement setting show two endothermic peaks, a broad one at around 140 °C corresponding to the onset of evaporation of the hydration water and a well define peak at 180 °C associated with a phase transition in the mineral. It has been reported [28] that 25% of the hydration water of brushite is lost between 85 and 140 °C, 50% between 115 and 170 °C and total dehydration is achieved between 130 and 240 °C, when the phase transition from brushite to monetite occurs. Therefore, the peak at 140 °C is related to the partial dehydration of brushite (equation 4) whereas the peak at 180 °C corresponds to total dehydration and monetite formation (Eq. 5). The partial dehydration peak around 140 °C was more pronounced in the cement samples prepared with C4S (see Fig. 4b) and even steeper in the ones set with C4S and silica gel (Fig. 4c, Table 2).

$$Ca(HPO_4)\cdot 2H_2O \longrightarrow^{\text{(heated between 85 and 170 °C)}} Ca(HPO_4)\cdot H_2O \quad (4)$$

$$Ca(HPO_4)\cdot H_2O \longrightarrow^{\text{(heated between 130 and 240 °C)}} Ca(HPO_4)$$

Discussion

It has been reported that the addition of C4S to HAP calcium phosphate cements contributes to ameliorate the biocompatibility and bioactivity of the cements [5].

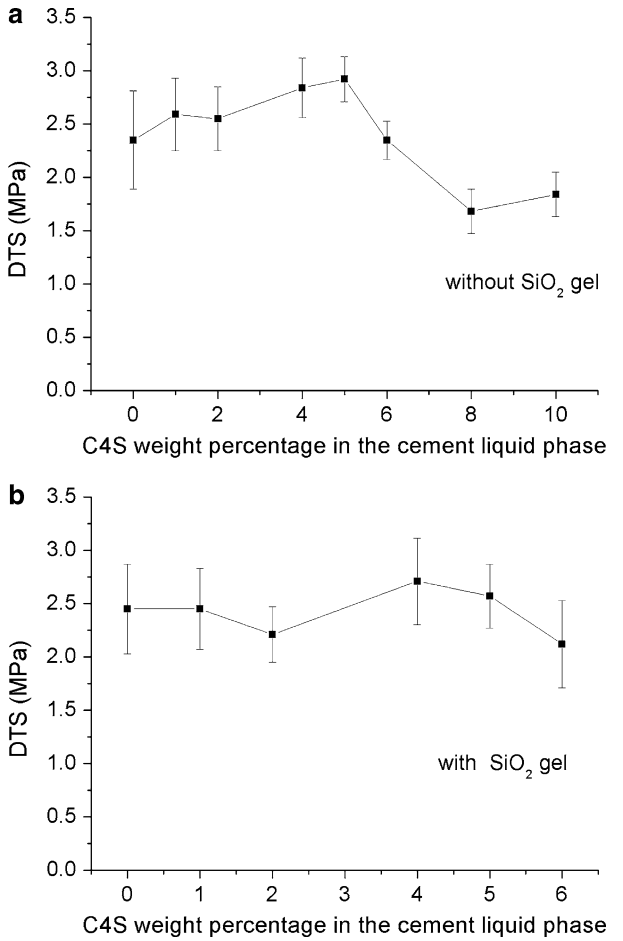

Fig. 3 (**a**) Dependence of the cement diametral tensile strength with C4S concentration. (**b**) Cement diametral tensile strength as a function of C4S concentration for samples prepared using 15 g/L silica in the liquid

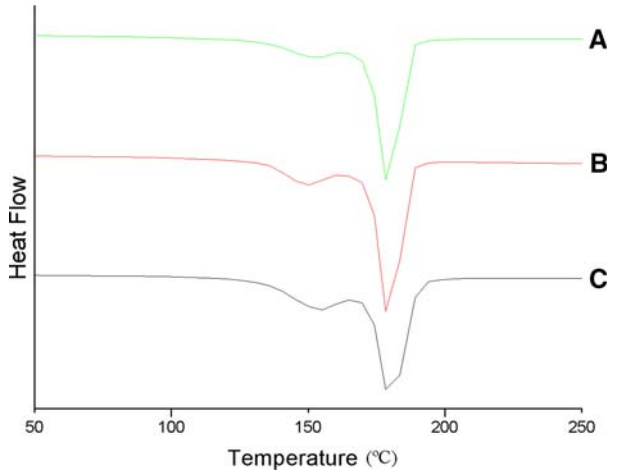

Fig. 4 DSC curves of cement samples after setting: A) Sample set using 0.5 M citric acid solution; B) Cement sample set with 2% C4S in 0.5 M citric acid solution, and C) Samples set with a gel made up of 2% C4S and 15 g/L silica in 0.5 M citric acid solution

In cements containing 2% C4S in the liquid solution used for setting the number of multinucleated giant cells which appear around the material decrease, and the amount of fibroblasts, collagen fibres and small vessels infiltrated into the internal porous structure of the cement increases. Furthermore, when less or non C4S is included in the cement setting reaction, cements are encapsulated with a dense fibrous connective tissue layer [5].

We have observed that moderate concentrations of C4S prolong the cement setting time into acceptable periods of 9 min when C4S concentration was 6%, reaching a maximum of 11 min at 8 % C4S. Beyond this point no further increase of the setting time was obtained. It has been reported that C4S has a direct effect on the crystallization process of calcium oxalate monohydrate [22]. It is believed that due to the large hydrodynamic size and high charge density, glycosaminoglycans inhibit HAP nucleation and growth [23]. The increase of the final setting time of the brushite cement is consistent with this interpretation and is probably provoked by the same causes.

The Rietveld analysis shows that there is a small decrease of brushite percentage in the cement when C4S or silica gel was added. However, a DTS value around 2.5 MPa was measured in all cases indicating that the small differences in composition slightly influence the mechanical properties of the set cement.

Yoshikawa et al (2004) proved that C4S addition to HAP calcium phosphate cements improves the properties of the material in periapical bone reconstructions [6], inducing osteoblast and osteoblast-like cell growth. Our study demonstrates that the highest C4S concentration that can be added to a brushite based calcium phosphate cement without decreasing its mechanical properties stands below 5 %. Thus, the bioactivity reported by Yoshikawa et al [6] in HAP cements with 2% C4S in the liquid phase could be increased in brushite cements by adding C4S in concentrations up to 5%. The C4S molecule has negative charges that facilitate its adhesion to the brushite crystals, contributing in this way to formation of crystal aggregates that maintain the diametral tensile strength of the material. Nevertheless, if excessive amounts of C4S are added to the cement, the repulsion between negatively charged C4S molecules will worsen the mechanical properties of the cement.

C4S is a polysaccharide containing sulfate and carboxy ionizable groups. The C4S solutions were prepared at fixed pH 4.1 assuring that the sulfonate groups were fully ionized and a considerable fraction of the carboxy groups were also ionized [29]. It is

Table 2 Transition enthalpies and temperatures of the cements derived from integration and the maximum of the DSC peaks respectively

Composition of the cement liquid phase	Enthalpy of first step in the brushite dehydration (J/g)	Enthalpy of second step in the brushite dehydration and monetite formation (J/g)	T_m of the first endothermic peak (°C)	T_m of the second endothermic peak (°C)
0.5 M citric	26.2	428.9	153.7	179.50
0.5 M citric acid + 2% C4S	36.0	382.7	148.5	179.69
0.5 M citric acid + 2% C4S + 15 g/L silica gel	46.8	389.7	152.1	180.37

known that in linear and flexible polyelectrolytes multivalent ions can lead to phase separation and precipitation because of electrostatic bridging between monomers along the polymer chain helped by the multivalent ions [30]. Carboxy groups in the C4S molecule interact with Ca^{+2} ions in the cement forming salt bridges during the cement setting reaction. This interaction proceeds in two steps; firstly the ionized sulfate groups decrease the negative charge density attracting the calcium ions towards the C4S chains, and secondly, the Ca ions would react with the carboxy group of two C4S chains. The weakening of this later interaction as the setting reaction advances results in dissociation of Ca ions which then react with phosphate ions to form brushite. This interaction inhibits partially the setting reaction of the cement and, therefore, prolongs the setting time. Nevertheless, when high amounts of C4S are added, the reaction equilibrium would be shifted to the left side of equation 6 leaving more free Ca^{+2} ions and the effect on the setting time would disappear.

$$n(\mathrm{C4S}) + n\mathrm{Ca}^{2+} \downarrow\leftrightarrow -[\mathrm{COO}\cdots\mathrm{Ca}\cdots\mathrm{COO}]_{n^-} \quad (6)$$

$$\mathrm{H}^{+}\mathrm{Ca}^{2+} + \mathrm{PO}_4^{3-}\mathrm{H}_2\mathrm{O} \rightarrow \mathrm{Ca}(\mathrm{HPO}_4)2\mathrm{H}_2\mathrm{O} \quad (7)$$

Recently, brushite cements have raised great interest due to their high resorption rate. However, in vivo, brushite can either be dissolved and quickly eliminated from the organism or be hydrolyzed to HAP that has a much slower resorption rate [31]. Brushite transformation into HAP is undesirable, and many studies have been dedicated to prevent this phenomenon. During storage, brushite can eventually dehydrate into monetite at room temperature [32]. Monetite is also an undesirable mineral for bone regeneration because it would transform into HAP when placed in a physiological environment [33–34]. It has been reported that two kinds of water molecules exist in brushite crystals; water molecules absorbed on the surface of the brushite grains and water molecules incorporated into the brushite crystal structure. Dehydration of brushite occurs in two steps: (i) A surface reaction, giving an intermediate state, with water molecules liberated from the surface of the brushite crystals. (ii) Thermolysis involving the lattice water molecules with the complete decomposition of the brushite into monetite [35]. The DSC study confirms this interpretation and permits a quantitative estimation of the two types of water molecules from the transition enthalpies.

The first dehydration process is more endothermic when C4S and silica gel were added to the cement, because these molecules prevent dehydration by interacting and protecting the brushite crystal surface. Nevertheless, the enthalpy of the second dehydration process and the phase transition to monetite slightly decreases, indicating that the inner part of the brushite crystals is probably distorted by the incorporation of some C4S molecules that will facilitate the final transformation to monetite. This could mean that C4S and silica gel protect the brushite crystal surface from dehydration but, once it was accomplished, the phase transformation into monetite will be easier. The addition of silica gel has an interesting consequence in this feature because it screened the effect of C4S on the brushite transformation into monetite, resulting in a more stable material.

Conclusions

The use of small percentages of C4S improves some of the properties of the brushite based calcium phosphate cements, such as the final setting time, without altering the mechanical properties of the material. We have found that the optimum concentration of C4S that can be added to brushite cement can be extended up to 5% of the liquid phase without decreasing the diametral tensile strength of the cement.

Acknowledgments The authors acknowledge financial support from the Spanish Science and Technology Ministry (MAT2006–13646–C03–01) and Comunidad de Madrid (GR/MAT/0501/2004).

References

1. U. GBURECK, O. GROLMS, J. E. BARRALET, L. M. GROVER and R. THULL, *Biomaterials* **24**(23) (2003) 4123
2. Y. YIN, F. YE, S. CAI, K. YAO, J. CUI and X. SONG, *J. Mater. Sci. Mater. Med.* **14** (2003) 255
3. M . BOHNER, U. GBURECK and J. E. BARRALET, *Biomaterials* **26**(33) (2005) 6423
4. M. BOHNER, S. MATTER. pat (WO0141824 (2002–11–06)
5. M. YOSHIKAWA, S. HAYAMI and T. TODA, *Mater. Sci. Eng: C.* **20**(1–2) (2002) 135
6. M. YOSHIKAWA and T. TODA, *J. Eur. Ceram. Soc.* **24**(2) (2004) 521
7. H.Z. HEROLD and A. TADMOR, *Isr. J. Med.Sci.* **5**(3) (1969) 425
8. M. MOSS, G. O. KRUGER and D. C. REYNOLDS, *Oral Surg. Oral Med. Oral Pathol.* **20**(6) (1965) 795
9. R.A. SKINNER, P. D. TOTO and A. W. GARGIULO, *J. Periodontol.* **47**(4) (1976) 196
10. B. AZAZ, D. GORDON and A. SHTEYER, *Refuat Hapeh Vehashinayim* **23** (1974) 75
11. X.H. ZOU, W. C. FOONG, T. CAO, B. H. BAY, H. W. OUYANG and G. W. YIP, *J. Dent. Res.* **83**(11) (2004) 880
12. Y.J. PARK, Y. M. LEE, J. Y. LEE, Y. J. SEOL, C. P. CHUNG and S. J. LEE, *J. Control Release* **67**(2–3) (2000) 385
13. D.S. KESKIN, A. TEZCANER, P. KORKUSUZ, F. KORKUSUZ and V. HASIRCI, *Biomaterials* **26**(18) (2005) 4023
14. A. SCABBIA, L. TROMBELLI, *J. Clin. Periodontol.* **31**(5) (2004) 348
15. C.M. SERRE, M. PAPILLARD, P. CHAVASSIEUX and G. BOIVIN, *Biomaterials* **14**(2) (1993) 97
16. A. REBAUDI, P. SILVESTRINI and P. TRISI, *Int. J. Periodontics Restorative Dent.* **23**(4) (2003) 371
17. N. VOLPI, *Biomaterials* **20**(15) (1999) 1359
18. N. VOLPI, *Biomaterials* **23**(14) (2002) 3015
19. SG REES, D. T. HUGHES WASSELL, R.J. WADDINGTON and G. EMBERY, *Biochim. Biophys. Acta.* **1568**(2) (2001) 118
20. G. GAFNI, D. SEPTIERAND and M. GOLDBERG, *J. Cryst. Growth***205**(4) (1999) 618
21. M. P. GINEBRA, F. C. M. DRIESSENS and J. A. PLANELL, *Biomaterials* **25**(17) (2004) 3453
22. J. OUYANG, S. DENG and J. ZHOUNG, *J. Cryst. Growth* **270** (2004) 646
23. C. C. CHEN and A. L. BOSKEY, *Calcif. Tissue Int.* **37**(4) (1985) 395
24. T. KOKUBO H. M. KIM and M. KAWASHITA, *Biomaterials* **24**(13) (2003) 2161
25. D. DELANEY and B. CONSTANTZ. pat (WO2004110316. (2004–12–23)
26. A.L. OLIVEIRA, P. B. MALAFAYA and R. L. Reis, *Biomaterials* **24**(15) (2003) 2575
27. A.E. PORTER, N. PATEL, J. N. SKEPPER, S. M. BEST and W. BONFIELD, *Biomaterials* **24**(25) (2003) 4609
28. G. BEREND and A. J. HEGEDÜS, *Thermochim. Acta* **11** (1975) 367
29. C. RUEDA, C. ARIAS, P. GALERA, E. LOPEZ-CABARCOS and A. YAGÜE, *Il Farmaco* **56** (2001) 527
30. M. MASUI, M. SUZUKI, Y. FUJISE and N. KANAYAMA, *Biochim. Biophys. Acta* **1546**(2) (2001) 261
31. L. M. GROVER, J. C. KNOWLES, G. J. P. FLEMING and J. E. BARRALET, *Biomaterials* **24**(23) (2003) 4133
32. U. Gbureck, S. Dembski, R. Thull and J. E. Barralet, *Biomaterials,* **26**(17) (2005) 3691
33. M. BOHNER, F. THEISS, D. APELT, W. HIRSIGER, R. HOURIET, G. RIZZOLI, E. GNOS, C. FREI, J. A. AUER and B. VON RECHENBERG, *Biomaterials* **24**(20) (2003) 3463
34. M. H. PRADO DA SILVA, J. H. C. LIMA, G. A. SOARES, C. N. ELIAS, M. C. ANDRADE, S. M. BEST and I. R. GIBSON, *Surf. Coatings Technol.* **137**(2–3) (2001) 270
35. L. TORTET, J. R. GAVARRI, G. NIHOUL and A. J. DIANOUX, *Solid State Ionics* 97 (1997) 253