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Investigation of Macroporous Calcium Phosphate Cement Obtained by Foamed Gelatin Polymer

Z. Orshesh, S. Hesaraki*, A. Khanlarkhani

Department of Nanotechnology and Advanced Materials, Materials and Energy Research Center, Karaj, Iran

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ABSTRACT

This study deals with the effect of gelatin on physical and mechanical properties of calcium phosphate bone cements. The mixture of tetracalcium phosphate (TTCP) and dicalcium phosphate (DCPA) as the cement powder was mixed with 6 wt% Na_2HPO_4 solution containing different amount (0, 2, 5 and 8% in w/w) of foamed gelatin as liquid phase. The physical properties were determined in the terms of setting time and macroporosity. The compressive strength was also checked before and after soaking the cements in simulated body fluid (SBF). The phase composition, microstructure and chemical groups were respectively determined using X-ray diffraction (XRD), Scanning electron microscopy (SEM) and Fourier transformation Infrared Spectroscopy (FTIR). The results showed that gelatin accelerated hydroxyapatite (HA) precipitation during setting and reduced the initial setting time from 35 min for cement without gelatin to 23 min for cement with the most amount of gelatin. Moreover, 17% (in v/v) macroporosity was induced in the cement structure using 8% solution of gelatin as the cement liquid. Gelatin addition promoted compressive strength of the set cement from 1.13 MPa (for gelatin-free cement) to 5.8 MPa.

1. INTRODUCTION

Calcium Phosphate materials are considered as one of the best bone substitute materials for certain applications, such as bone grafting, bone fillers in trauma, fracture repair, and dental applications [1]. Among calcium phosphate materials, calcium phosphate cements (CPCs) have introduced an upthrow in the field of since more than 2 decades ago, because they bioceramics have several desired characteristics which make them suitable to alleviate bone imperfections [2-4].

The favorable properties of these bone chemichally similar materials, are injectability, which allows them to mold the shape of imperfection completely, and discards the need for surgery, compatibility, bioactivity, osteoconductivity, and osteotransductivity, which means after implantation in bone defects they are rapidly integrated into the bone structure [5-9].

This group of biomaterials constitutes of calcium phosphate salts as powder phase (precursors) and an appropriate aqueous solution. Upon hydrolysis reaction, the powder phase of CPC dissolute in liquid phase and a new phase precipitate. In the case of exposure of CPC to the blood, hydrolysis reaction accelerates. Depending on the chemical composition of CPC precursors the final product of the reaction can be hydroxyapatite (HA) or brushite, which are the most stable calcium phosphates at PH>4.2 and PH<4.2 respectively. Both of these products considered as biocompatible phases and can help bone regeneration [10].

Precipitated HA which is similar to the mineral phase constituted natural bones, are inherently porous biomaterials, since they are formed by physical interlocking of precipitated needle like hydroxyapatite crystals which finally leads to setting the CPCs.

In spite of many intriguing properties that CPCs have, there are some shortcomings such as low mechanical strength and washout resistance of these kind of materials [5, 11]. Also, they are not osteogenic, causing long time for complete bone regeneration and very low resorption rate of the cements [7, 8, 12]. For instance, some CPCs can remain as long as 78 weeks after implantation [13]. In fact, lack of macropores in CPCs decrease their applicability as a regenerative medicine. As macroporosity supports tissue ingrowth and colonization, creating macropores in CPCs resulting in better penetration of bone tissue and better flowability of body fluids. It encourages the bone regeneration process and fastens bioresorption of the used cement [10, 14].

In this respect different strategies have been utilized to create macroporosities in the cements while rendering

*Corresponding Author's Email: s-hesaraki@merc.ac.ir (S. Hesaraki)

their favorable properties. In summary adapted methods for macroporosity creation in CPCs include leaching or degradation of second phase [15-18] foaming either in the form of gas foaming or adding foaming agent [19,10].

In leaching method, small particles, as porogen, were introduced to the cement paste. These porogens are dissolved before injection or degraded in vivo. Concerning with this method, many researchers employed different soluble particulates like sucrose, mannitol, $NaHCO_3$,$CaSO_4$-$2H_2O$, Na_2HPO_4, poly(DL-lactic-co-glycolic acid) (PLGA) microspheres or, gelatin microspheres to create macroporosity [15, 20-28]. Although macroporosities can be created through this route, but the need of adding large amounts of porogenic agents to ensure the interconnectivity of the pores is the main limitation of this method.

In the gas-foaming method, gas bubbles are created as a result of a chemical reaction between the cement reactants. Almirall et al. gained porous cement by the addition of hydrogen peroxide. Hesaraki et al. achieved porous cement through the formation of CO_2 bubbles produced via acid-base reaction of Na_2HPO_4 and $NaHCO_3$ [19, 29, 30]. An important drawback of the gas foaming method is the threaten of gas diffusion to the surrounding tissues and creating gas pockets even producing gas embolism problems [31]. Another route to obtain injectable macroporous CPCs, is liquid phase foaming. To gain this goal, a biocompatible and water soluble surface active molecule incorporated as a foaming agent. Protein-based foaming agents such as albumen meet these requirements. Del Valle and Ginebra showed albumin can form a stable liquid foam, which act as a template during cement setting. They showed that these foamed cements increased cement resorption rate compared the unfoamed cement with similar formulation [1, 32]. Another studied natural foaming agent is gelatin. Gelatin is a protein-based polymer, which is similar to the bone extracellular matrix, has an inherent foaming capacity, because it has amphiphilic nature that makes it a surface active compound [2, 14, 33]. Due to the good foamability of this protein, air bubbles entrapped in gelatin solution are stable and act as a porous template for cement. Other advantages (derived from gelatin origin and specific properties) make it not only a good candidate as foaming agent, but also multifunctional additive for enhancing other characteristics of CPCs, including paste injectability, cohesiveness, cell attachment (through integrin recognition of the RGD peptide sequence contained in gelatin [11]), and the resorption rate [12]. There are a few researches based on CPC/ foamed gelatin, [34, 35]. In these studies alpha-tricalcium phosphate was selected as powder phase and gelatin introduced in the liquid phase. They studied macroporosity, setting reaction, and in vivo properties of the CPC-gelatin cements.

In this study, we utilized gelatin as a foaming agent and studied the effect of this additive on the hydrolysis reaction of the cement and pore formation, especially when tetraclcium phosphate (TTCP)/dicalcium phosphate anhydrate (DCPA) mixture was selected as the powder phase composition.

In this respect, various foamed solutions produced from different concentrations of gelatin, were used as liquid phase of the cements. The effect of the foamed gelatin on apatite formation, porosity, setting time, and compressive strength (CS) of the cements (before and after soaking in simulated body solution, SBF) were investigated.

2. MATERIALS METHODE

Tetracalcium phosphate (TTCP) was synthesized by high-temperature solid-state reaction of an equimolar mixture of calcium carbonate ($CaCO_3$) and dicalcium phosphate anhydrate (DCPA) powders (both from Merck, Germany). It was heated to 1500˚C for 6 h. The product was manually ground in an agate mortar with a pestle and then was further ground in a planetary ball mill in ethanol for 1 h.

Afterward, the powder phase of the CPC was prepared by mixing synthesized TTCP, $Ca_4(PO_4)_2OH$ (with mean particle size of 12 μm), and commercially available DCPA, CaHPO4 (with mean particle size of 6.5 μm) at a molecular ratio of 1:1.

Solution of 6wt% Na_2HPO_4 with different amounts of gelatin (2, 5, 8% in w/w) were prepared as liquid phases. In order to dissolve gelatin in the solution of 6wt% Na_2HPO_4, the solution was heated to about 50˚C while it was mixing. Then, the solution was foamed by stirring for 5 minutes at a rate of 2000 rpm, using an internal mixer.

To prepare cement paste, powder and liquid phases were mixed. Since in foaming process, a cement paste with a low viscosity is needed [14, 34], a liquid to powder ratio (L/P) of 0.65 ml/g was selected to guarantee good foamability. As the foamed liquid phase supposed to act as a porous substrate, mixing these phases were carefully accomplished with a spatula.

Cement notation containing 2, 5 and 8 wt% of foamed gelatin are 2F, 5F, and 8F, respectively.

The control group (cement without any gelatin) was notated as N (no gelatin).

2.1. Characterization

The initial setting time was measured by a Gillmore needle according to the ASTM-C266-89 standard.

For compressive strength (CS) test, the cement paste was placed in a split cylindrical Teflon mold with 12 mm height and 6 mm diameter and kept in an incubator for 24 hours. Afterward, the test was performed on samples before and after 7 days immersion in SBF.

A universal testing machine (STM 20, Santam) with a crosshead speed of 1 mm/min was used for mechanical measurements.

To evaluate phase conversion of the cements, after setting and after 7 days immersion in SBF, the XRD analysis was performed. The data were acquired from 10 to 50 degrees with a scan rate of 0.02, 2θ/s using a Philips PW 3710 X-ray diffractometer with Cu-Kα radiation. To determine chemical bonds groups and monitoring phase evolution, FTIR performed on set cements. In more details, two milligrams of each composition were ground, then the prepared cement powder was mixed with 800 mg of the ground spectroscopic grade of KBr and pressed to make transparent KBr pellets. The Infrared spectra between 4000 and 400 cm^{-1} were measured at the resolution of 2 cm^{-1} using FTIR (BRUKER VECTOR 33).

The microstructure of the cements was also observed with scanning electron microscopy (SEM, S360 Cambridge) at an operating voltage of 15 kV. The surfaces of the samples were coated with a thin layer of gold before the SEM analysis.

Bulk density (D_b) of the CPCs, the volume of the macropores (P_{ma}), i.e. the large pores produced by the presence of gelatin, the volume of the micropores (P_{mi}) and total porosity (P_t) of the cements were also recorded using the following equations [20]:

$$D_b = M/V \quad (1)$$

$$P_t = (1 - D_b/D_p) \times 100 \quad (2)$$

$$P_{ma} = (1 - D_b/D_{control}) \times 100 \quad (3)$$

$$P_{mi} = P_t - P_{ma} \quad (4)$$

where M is mass and V is volume of the samples. D_p, powder density of the samples was obtained by picnometry and D_{cotrol} is the bulk density of control group (CPC without any gelatin).

2.2. Statistical Analysis The data were processed using software Excel 2010. The results were produced as mean ± standard deviation of at least four experiments. The statistical significance between mean values was determined by a one-way analysis of variance, and significance in differences of the mean values was evaluated by Tukey's post hoc test (SPSS v10.0, Chicago, IL, USA). The $p \leq 0.05$ was considered significant.

3. RESULT AND DISCUSSION

Figs. 1 and 2 shows the XRD patterns of the samples with different amount of foamed gelatin before and after immersion in SBF, respectively. The XRD pattern of control sample also included for comparison. The first interesting point is that in gelatin containing samples, even before immersion in SBF, the predominant detected phase is hydroxyapatite. However, in the control group (cement without gelatin) the most detected peaks are relevant to precursors (TTCP and DCPA). The second interesting point is that in sample 2F, all of the precursors are converted to HA while by the gelatin content increment in samples 5F and 8F, some peaks of TTCP and DCPA are detected before the immersion. However, the numbers and intensity of these peaks in 5F and 8F, are weaker than in sample N. In samples immersed in SBF for 7 days (even without gelatin) the dominant phase is HA. To study the microstructure of the samples and evaluate HA formation, SEM images with high magnification (× 10000) were prepared (Fig. 3).

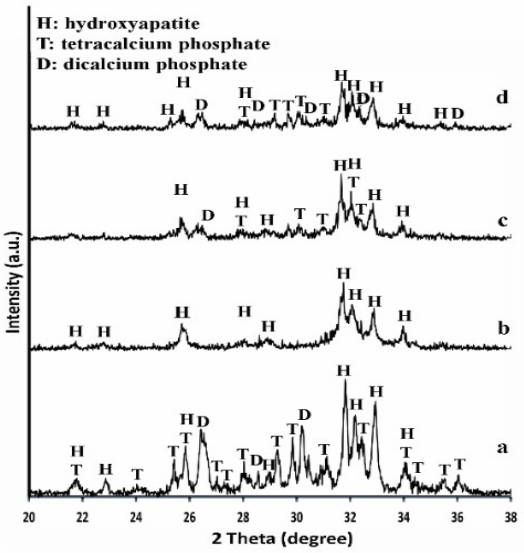

Figure 1. XRD pattern of samples (a) N, (b) 2F, (c) 5F, (d) 8F, before immersion in the SBF

As it is observed in Fig. 3, in sample N, before immersion, smooth particles are observed and there is no rough phase (that is usually assigned to HA) in the microstructure [36].

In contrast, in samples containing gelatin (2F, 5F, and 8F), the rough needle like crystals are obviously seen even before the immersing process. After immersion in SBF for 7 days, the microstructures of all samples are similar and precipitates are observed. Based on the XRD results these precipitates are HA phase.

Therefore, it can be concluded that, introducing gelatin to the CPCs, accelerates the hydrolysis process of the cements and formation of HA. This can be as a result of some chemical interactions between gelatin and calcium phosphate cement.

The ionizable groups found in a gelatin molecule are the carboxyl groups of aspartic acid, and carboxylic acids, the ε-amino group of lysine, the guanidinium group of arginine, the imidazolium group of histidine, and the terminal α-carboxyl and α-amino groups [33].

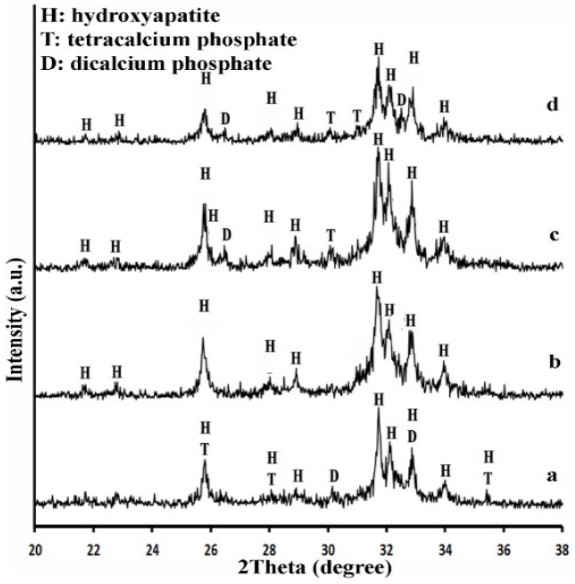

Figure 2. XRD pattern of samples (a) N, (b) 2F, (c) 5F, (d) 8F, after 7 days immersion in the SBF

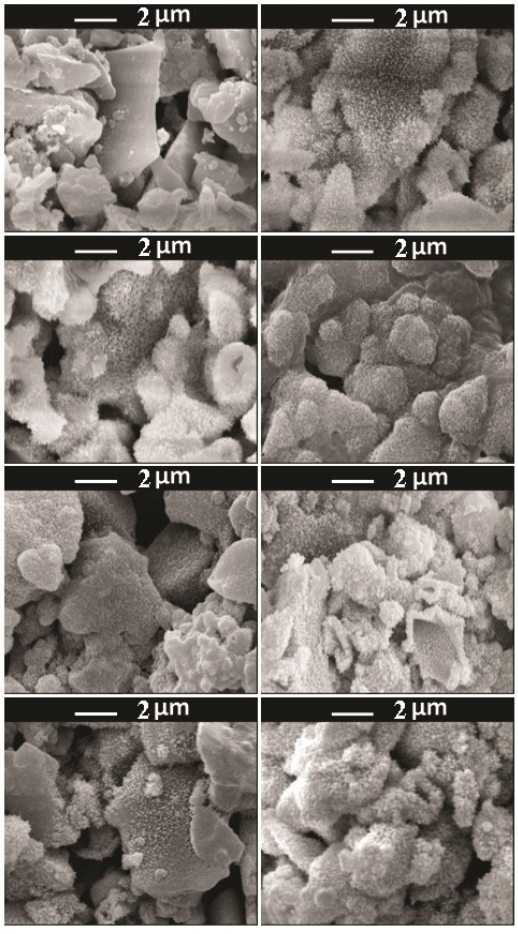

Figure 3. SEM images of respectively (a, b, c, d) N, 2F, 5F, 8F, before and (e, f, g, h) after 7 days soaking in SBF

According to the literature, existing carboxyl groups in the gelatin molecules can interact with calcium ions released as calcium phosphate salts solute, and form a chemical complex [21]. These complexes act as nucleation sites, which lower the activation energy for HA nucleation and accelerate this process. The complex formation along with the apatite precipitation would aid to decrease initial setting time. As mentioned above, in the sample 2F this interaction leads to complete phase conversion to HA. However, when higher amounts of gelatin are used (e.g. 5F and 8F), the higher carboxyl concentration captures more calcium ions by the complex formation. Thus, this causes depletion of calcium ions near the nucleation sites (complexes), and insufficient concentration of calcium ions results in an incomplete conversion of the cement to HA.

This claim also can be authenticated by investigating chemical groups of the samples. In this respect, FTIR of the samples before immersion in SBF are compared (Fig. 4). As it is obvious, the FTIR spectra of all gelatin-containing cements are nearly similar. In these samples, the bands at around 1650 and 3420 cm^{-1} are assigned to the hydroxyl group of adsorbed water, while the band appeared in 3600 is assigned to OH in apatite lattice. Moreover the bands at 1415 and 1450 are attributed to carbonate group in apatite lattice, which reflect the formation of carbonated apatite. The band at 950 cm^{-1} is also related to HPO_4^{2-} group. It confirms that the formed apatite is a calcium-deficient phase. The bands associated with amide II, CH and carbonyl groups are clearly observed in the spectra of gelatin, which are also found in the patterns of gelatin-containing cements.

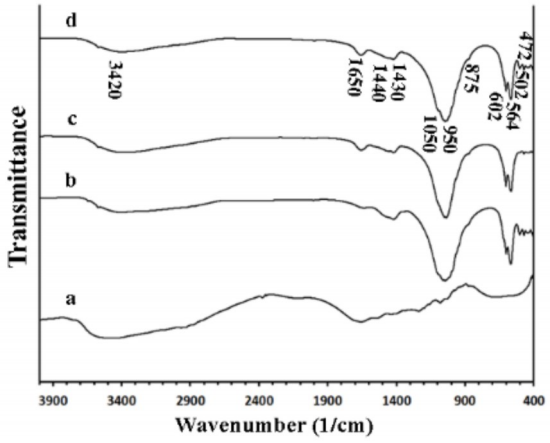

Figure 4. FTIR spectra of (a) gelatin, and samples (b) N, (c) 5F, (d) 8F before immersion in SBF

The bands at around 1650 cm^{-1}can also be related to the carbonyl groups of gelatin, which may interact with Ca^{2+} ions (to form e.g. calcium carboxylate complex) [29], [30]. The complex help to improve setting time and mechanical strength of the cement.

Table 1, shows the initial setting time of the cements, which were measured by the Gilmore needle method at room temperature. The result was interesting, the more

gelatin content, the shorter initial setting time. As mentioned elsewhere, gelatin can decrease the setting time by three approaches. First, it can cause the faster HA formation and so interlocking these nanocrystalswould result in of cements setting. Second, the gelation process of gelatin itself leads to decrement of initial setting time of the cement. Third, the complex formed through the interaction of carboxyl ions with Ca cations decreases the initial setting time.

TABLE 1. Setting time of different CPCs

Code	Setting time (min)
N	35 ± 2
2F	32 ± 3
5F	25 ± 4
8F	23 ± 3

Fig. 5 shows low magnification SEM images of samples N, 2F, 5F, 8F. It is obvious that the addition of foamed gelatin to the cement formulation would result in the macroporosity creation. These pores are the air bubbles, which were entrapped within the cement liquid phase during stirring (preparing) the gelatin and Na_2HPO_4 solution. As gelatin has amphiphilic characteristics, many of these air bubbles stabilized even after mixing the powder phase to the liquid phase [14, 33, 37]. As the viscosity of the liquid phase elevates by the presence of gelatin, samples with higher gelatin content have bigger stable air bubbles, which results in the formation of bigger pores in the cements. It is confirmed by the SEM images of samples 5F and 8F. Figs. 1(b&c) Additionally, by increasing the gelatin content, higher amounts of macropores are formed in the cement. Comparison of SEM images in Fig. 5 (a to c) clears that the macropore size increases as the gelatin amount increased. Table 1, summerizes the pore content of the samples.

According to the CS results (Table 3), for the unsoaked samples, generally samples containing gelatin have greater CS values than the control group (sample N) and by increasing in amount of gelatin up to 5 %, the CS increases from 1.13 MPa to 5.5 MPa. However, for sample 8F, the CS decreases to 2.4 MPa. The same trend is also found in the samples after soaking in SBF for 7 days.

The CS is usually influenced by two opposite factors. The formation of HA in the set cements, and the percentage of macroporosity. The former causes increment of CS of the cement and the second makes the cement weaker. However, porosity acts as a stress concentrator. By increasing porosity, the CS decreases. In the cements containing 2 and 5 wt% gelatin, HA formation is accelerated because of gelatin addition. It can overcome the negative effect of porosity.

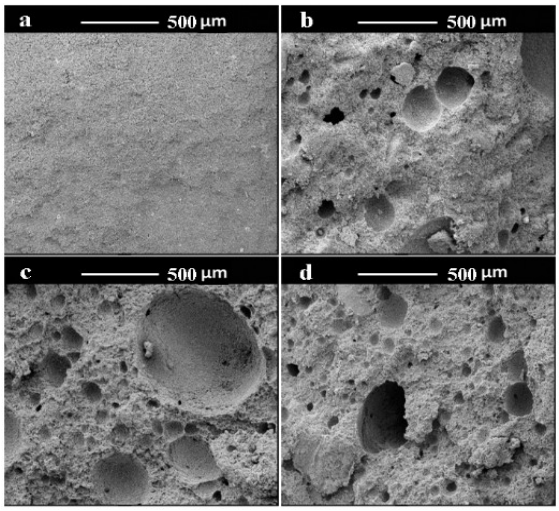

Figure 5. SEM images of (a) N, (b) 2F,(c) 5F, (D) 8F, before immersion in SBF

TABLE 2. Porosity of samples with different gelatin content

Code	Macroporosity (%)	Microporosity (%)	Total porosity (%)
N	0	29 ± 1	29 ±1
2F	7 ± 1	30 ± 3	37 ± 4
5F	11 ± 2	27 ± 4	38 ± 3
8F	17 ± 2	28 ± 4	45 ± 5

From Table 3, it can be seen that the CS of sample N is in the order of sample 5F, after 7 days soaking.

TABLE 3. Compressive strength of cements before and after immersion in SBF

Code	Compresive Strength before soaking in SBF (MPa)	Compresive Strength after soaking in SBF for 7 days (MPa)
N	1.13±0.2	5.12±1.05
2F	4.01±0.45	4.39±0.56
5F	5.5±1.76	5.82±1.95
8F	2.4±0.75	2.02±1.09

The HA formation in sample N can lead to the significant increase of CS. This is in accordance with the XRD patterns and SEM images (Figs. 2&3).

4. CONCLUSION

In this study the effect of gelatin addition to CPC was studied. Different amounts of gelatin were introduced into the cement liquid phase, and microstructure, phase evolution, setting time, and compressive strength were evaluated. The following conclusions were obtained:

1. Gelatin, a natural polymer which is similar to the organic part of natural bone, can accelerate the hydrolysis process of calcium phosphate cements and favors apatite precipitation in a way that in cements containing gelatin the predominant phase in as-set cement (before immersion in SBF) is HA.
2. Gelatin as an amphiphilic natural polymer creates macropores in the structure of final calcium phosphate cements. The amount of macroporosity in CPC containing 8wt% gelatin increases to about 17% (in v/v) comparing to the control group which has no macroporosity.
3. Gelatin decreases the initial setting time of the cements by about 35% for the cement with 8% gelatin solution.
4. Gelatin affects CS of cements in two different ways. On one hand, it can increase the CS as it accelerates HA formation and also as cohesive phase by increasing integrity of the cements, on the other hand it may decrease the CS as it creates macropores in the cement structure and weaken the mechanical strength. The final CS of the cements are defined by these two opposite concepts. The results showed that CS increased from 1.13 MPa for control cement to 5.5 MPa for 8F cement before soaking in SBF.

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