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Hydrolysis of monetite/chitosan composites in α-MEM and SBF solutions

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Abstract There are two objectives of this work. The first objective is to study the hydrolysis behavior of monetite cements formed in the presence and absence of the chitosan in cell culture media (α-MEM) and simulated body fluid (SBF) solutions at 37°C. During hydrolysis, monetite transformed to carbonated apatite. Therefore, the second objective is to examine how addition of chitosan affects on the formation of carbonated apatite phases. The changes in the phase structure of monetite after hydrolysis reactions were characterized using XRD, FTIR and SEM. Pure monetite and monetite/chitosan composite were soaked in α-MEM and SBF solution for 4 and 7 days. In α-MEM solution, the monetite particles started to transform into carbonated apatite with a slow rate. However, in SBF, the rate of monetite transformation to carbonated apatite was more rapid. The presence of the chitosan had no significant effect on the precipitation of carbonated apatite on the monetite particles.

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1 Introduction

Formation of carbonated apatite (CHA) at the physiological temperature from calcium phosphate precursors has a potential for healing complex bone defects. Precipitation and hydrolysis are two techniques to prepare carbonate apatite. The second technique is suitable for clinical application. Several calcium phosphate precursors could be hydrolyzed in body fluid for growing new bones. These include α-tricalcium phosphate (TCP), dicalcium phosphate dihydrate (DCPD) and dicalcium phosphates anhydrous (DCPA). All of these calcium phosphate precursors are biocompatible. However, their degradation rates are different from one another and depend on the solubility product. The solubility product of these precursors is in the order of (at pH 7): α-TCP > DCPD > DCPA > β-TCP > HA [1]. Because of the low value of the solubility product of HA and β-TCP at physiological pH, their degradation process in vivo is very slow.

This work focuses on the hydrolysis behavior of calcium phosphates in SBF and α-MEM solutions at 37°C. Being brittle in nature, calcium phosphates need the addition of a polymer to enhance the mechanical properties. Chitosan was chosen as the candidate polymer, which was mixed with calcium phosphate to improve the strength of the calcium phosphate materials. Chitosan–calcium phosphate composites have a great deal of use in orthopedic/periodontal applications [2–4]. Chitosan is a biodegradable linear polysaccharide and is composed of randomly distributed β (1-4) linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit). It is an abundant biopolymer and mainly found in invertebrates, insects, marine diatoms, algae, fungi, and yeasts. It has many commercial uses in various disciplines such as cosmetics, photography, water engineering, textile industry,

paper finishing, food and nutrition, solid-state batteries. Applications of chitosan in the biomedical field have grown even more, ranging from wound dressings, contact lenses, sorbents and enzyme supports, drug delivery systems, and tissue engineering. The unique properties of chitosan such as bioactivity, antimicrobial activity and enzymatic biodegradability led to their increased biomedical uses.

Many papers have been published in the literature about calcium phosphate–chitosan composites. However, the most extensive studies focused on the influence of the chitosan on the setting time, microstructure and mechanical properties of the calcium phosphate cements. Dawkins et al. [5] found that the addition of chitosan to monetite improved the mechanical properties and increased the setting time of the composites. Also it was found that the presence of chitosan enhanced resistance to washout property of the monetite chitosan composites as will be shown in the results part.

The research about the impact of chitosan on the hydrolysis reaction of the calcium phosphates is relatively minimal. In a previous study, Varma et al. [6] reported that the phosphorylated chitosan films treated with calcium hydroxide solution were highly susceptible to calcium phosphate growth upon immersion in a simulated body fluid (SBF) solution. On the other hand, untreated films did not exhibit any calcium phosphate growth upon immersion in SBF. The study indicated that the treatment with calcium hydroxide is the only reason for the growth of the calcium phosphate on the phosphorylated chitosan films. The present work will compare the effect of chitosan addition on the hydrolysis of calcium phosphates in two different solutions, SBF and α-MEM.

For rapid healing of bone fracture, calcium phosphate precursors with the ability to hydrolyze with an optimum kinetics of degradation are preferable. DCPA (Monetite) is an interesting candidate calcium phosphate that may be used for that purpose. It is biocompatible and it is rapidly resorbed and transformed into bone apatite [1, 7–9]. Monetite is currently being used as a precursor of hydroxyapatite cements for orthopedic applications [10–13]. It is found in hard tissues such as urinary and dental stones [14]. Also, its potential as bone regeneration material has been known for a long time [15]. It is similar to brushite in chemical composition, but their in vivo behaviors are quite different, mainly due to the differences in the solubility at physiological pH [16].

Despite the fact that the synthesis and characterization of monetite have been established in a substantial number of studies, little has been reported and discussed about its hydrolysis. In water at 37°C, DCPA undergoes a limited extent of hydrolysis, wherein a thin film of apatite deposits on the monetite surfaces. The solubility product of this coated layer slows down the kinetics of the degradation process of monetite [17]. Based on the literature, the possible way to enhance the hydrolysis of monetite to apatite is to add some ions to water [18]. The full conversion of monetite to hydroxyapatite was achieved by immersion of the monetite sample into a 0.1 M NaOH solution at 60°C for 48 h. The influence of the immersion time on the apatite transformation was reported and 4 h were enough for monetite to be completely converted to hydroxyapatite [19]. In another study, a series of Na^+ and CO_3^{2-}-containing calcium hydroxyapatite were produced under homogeneous precipitation conditions by the hydrolysis of $CaHPO_4$ in sodium carbonate (Na_2CO_3) solutions at 95°C [18]. When additives such as NaOH, NaF or NaCl were added to water, monetite is quickly transformed into the corresponding apatite (hydroxyapatite (HAP), flouroapatite (FAP) or chloroapatite (ClAP)) [20].

The hydrolysis reaction of monetite in calcium containing saline solution also was studied quantitatively. The results showed that apatite precipitation was almost completed within 6 days. The presence of Ca^{2+} and Cl^- ions in the solutions provided the driving force for apatite precipitation reaction [21]. Although, many studies focused on the hydrolysis of monetite in different media, its hydrolysis in SBF was not investigated. It is also noted that SBF solutions cannot be used as a cell culture media since they lack the necessary nutrients, such as amino acids, vitamins and glucose, to allow and sustain the proliferation of living organisms. So it is interesting to study the hydrolysis reaction of monetite in cell culture media as well. In this study, the α-MEM is used for studying monetite hydrolysis. The phenomenon of hydrolysis of monetite in α-MEM will be compared to that of monetite immersed in SBF solution. Accordingly, the specific objective here is to examine the hydrolysis behavior of the monetite cements formed in the presence and absence of chitosan in SBF and α-MEM. The results should provide information if chitosan has any effect on the hydrolysis reaction of monetite.

2 Materials and methods

2.1 Composite cement preparation

In this study, monetite was formed by mixing of calcium hydroxide ($Ca(OH)_2$)) (BHD laboratory, England) and 1 ml of sitting solution in the presence and absence of chitosan [Sigma-Aldrich, St Louis, Missouri] at 37°C for 24 h. The chitosan used in this study is a practical grade, 75–85 deacetylated chitin. It has a low molecular weight that range from 50,000 to 190,000. The setting solution was prepared by mixing 12 ml of orthophosphoric acid [85% v/v density, Fisher Scientific, Fair Lawn, New Jersey] and

3 g of sodium bicarbonate solution (>99.7%, $NaHCO_3$, Acros-Fisher Chemicals, Fair Lawn, NJ). In this process, 1.5 g of the calcium hydroxide powder was mixed with 5% by weight of chitosan powder until the constituency was a homogeneous powder. Afterwards, 1.5 ml of setting solution was added to the homogenous mixture powder of chitosan/$Ca(OH)_2$ to form homogeneous pastes. The pastes were cured in oven for 24 h at 37°C before SBF and α-MEM immersion, excepting for the washout evaluation experiments.

2.2 Washout resistance measurement

Washout resistance evaluation of monetite formed in the presence and absence of chitosan was tested using saliva-like solution (SLS). The chemical composition of SLS has been reported by Takagi et al. [22]. It contained 1.2 mmol/l $CaCl_2$, 0.72 mmol/l KH_2PO_4, 30 mmol/l KCl, 50 mmol/l HEPES buffer (*N*-2-hydroxyethyl-piperazine-*N*′-2′-ethane sulfonic acid) and its pH was adjusted to 7 using 0.1 mol/l NaOH. All the chemicals used were purchased form Fisher Scientific. As-mixed pastes of monetite formed in the presence and absence of chitosan were loaded into syringe without waiting for curing to occur and injected immediately into SLS at 37°C for washout resistance evaluation. This provides a very harsh condition for washout resistance evaluation and also simulates the actual application in treating an orthopedic emergency.

2.3 Monetite in α-MEM

0.5 g of monetite powder formed in the presence and absence of chitosan powder was soaked in a 5 ml of α-MEM solution in a sealed 15 ml centrifuge tube. The composition of MEM is mainly amino acids, salts (such as calcium chloride, potassium chloride, magnesium sulphate, sodium chloride and monosodium sulphate), glucose, vitamins (such as folic acid, nicotinic acid, riboflavin, and B_{12}). The MEM was supplemented with 10% FBS, 1 mM sodium pyruvate and 100 U/ml penicillin–streptomycin–amphotericin,

The centrifuge tubes were placed in a shaker water bath for 4 and 7 days at 37°C. The α-MEM solution was replenished with a fresh solution every 48 h. After 4 or 7 days, the solutions were filtered using a porcelain Buchner funnel, with vacuum filtration. The precipitated solids were washed with de-ionized water, left to dry overnight at 65°C.

2.4 Monetite in SBF

0.5 g of monetite powder formed in the presence and absence of chitosan powder was soaked in a 5 ml of 1.5× t-SBF in a sealed 15 ml centrifuge tube. The 1.5× t-SBF was prepared as described previously [23] by dissolving reagent grade NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, CaCl2, and Na_2SO_4 in deionized water. Using 1.5× t-SBF, referred to as SBF henceforth, has become our standard protocol and reduces the coating times. Previous researches have proved that the SBF solution, which has similar ionic composition to human body fluid, results in a ion-substituted carbonated apatite coating layer on material's surfaces, including glass, metals and polymers [16, 17, 21, 23, 24].

The centrifuge tubes were placed in a shaker water bath for 4 and 7 days at 37°C. The SBF solution was replenished with a fresh solution every 48 h. After the planned required time of the study, the solutions were filtered using a porcelain Buchner funnel, with vacuum filtration. The precipitated solids were washed with de-ionized water, left to dry overnight at 65°C.

2.5 Composite characterization

The changes in the crystallographic structures of the monetite produced with and without chitosan and before and after soaking in α-MEM and SBF were examined using X-ray diffraction (XRD) [Rigaku Ultima III Woodlands, TX] using a speed of 1° per minute in continuous scan mode with 40 kV and 44 mA. The XRD data was collected for a 2θ range between 10° and 40°.

The morphological developments of the monetite formed in the presence and absence of chitosan and soaked either in α-MEM and SBF were monitored using scanning electron microscope (SEM) [Hitachi S-4800, Pleasanton, CA]. The samples were mounted on conducting carbon tape, coated with gold and were visualized with accelerating voltage of 5 kV.

3 Results

3.1 Washout resistance measurement

Monetite formed in the presence and absence of chitosan were both injectable (Fig. 1a). Monetite with chitosan maintained its shape and showed no significant disintegration after 5 min of immersion in SLS (Fig. 1b). This shape was maintained even after 1 h. On the other hand, neat monetite samples crumbled into powders immediately after injection (Fig. 1b).

3.2 Hydrolysis in SBF

To verify the impact of the SBF solution on the hydrolysis and phase changes of monetite, XRD, FTIR and SEM of

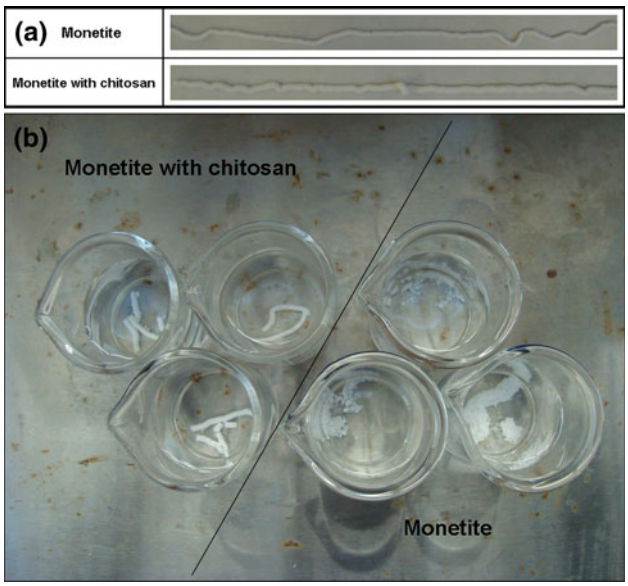

Fig. 1 Washout resistance test: **a** the shape of injected monetite formed in the presence and absence of chitosan; **b** the results of injected monetite formed in the presence and absence of chitosan in SLS after 5 min

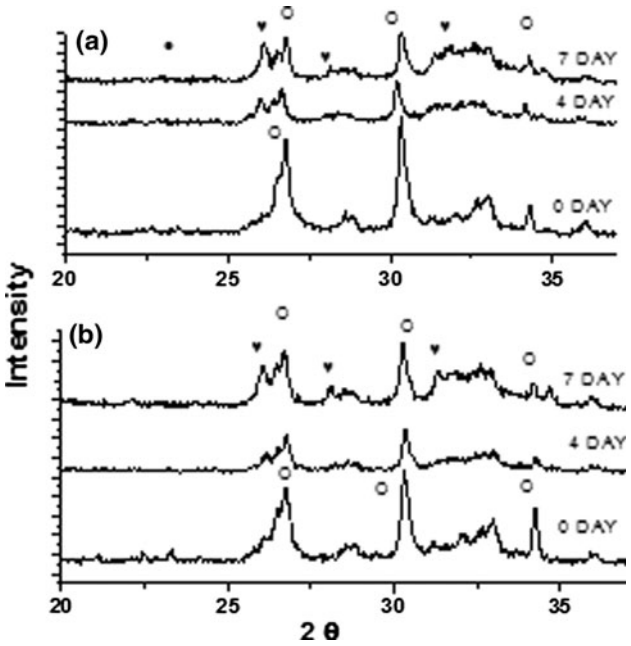

Fig. 2 XRD patterns of the phases produced after hydrolysis reaction in SBF of **a** monetite cement at different times, and **b** monetite chitosan composites at different times. These symbols for (*circle*) monetite and (*black heart*) apatite peaks

the monetite formed in the presence and absence of chitosan were studied. The XRD patterns for the pure monetite after soaking in SBF after 0, 4 and 7 days were shown in Fig. 2a. Likewise Fig. 2b shows the XRD patterns for the hydrolysis of monetite formed in the presence of chitosan in SBF for the same time. The pure monetite is identified by the presence of four main peaks at 2θ of 27°, 28°, 31° and 33°. When chitosan is incorporated during the monetite synthesis, new peaks appeared in the XRD patterns in addition to those of monetite, as seen in XRD patterns. They appeared at 2θ of 21.2°, 23.4°, and 29.5° and corresponded to the formation of brushite [5]. The brushite peaks disappeared when the composite soaked in SBF solution because its conversion to apatite. Both Fig. 2a, b depict the appearance of the apatite peaks with low intensity in XRD patterns of the monetite after soaking in SBF for 4 days regardless of the presence or absence of the chitosan. These peaks appear the 2θ of 26° and 32.1°. The intensities of these peaks got enhanced and became more intense after 7 days. The figures (a, b) show that even though after 7 days of soaking in SBF, the monetite peaks still persisted. This means the monetite particles did not completely convert into apatite after 7 days of soaking in SBF at 37°C. After 7 days of hydrolysis reaction, the approximately calculated conversion into carbonated apatite under different conditions was not statistically significant.

The FTIR spectra of monetite powder formed in the presence and absence of chitosan before and after hydrolysis are shown in Fig. 3. The spectrum of Fig. 3a which represents the formation of carbonated apatite is similar to what was previously reported [24]. The spectra exhibited bands at 1020, assigned to the stretching mode of the P–O bond. The shoulders at 962–964 and 1099–1100 cm^{-1} can be attributed to the presence of PO_4^{-3} and/or HPO_4^{-2} groups. The band at 873, 1410 and 1610 cm^{-1} can be attributed to the presence of CO_3^{2-} ion. These results suggest that the monetite formed in the presence and absence of chitosan form carbonated apatite.

To investigate the possible changes in the morphological structure of monetite during the hydrolysis in SBF solution, the morphological analysis results were correlated to the soaking time for the monetite formed in the presence and absence of chitosan. Fig. 4 shows the morphologies of these samples before and after hydrolysis in SBF solution, respectively. Before hydrolysis, monetite has well-defined platelet-shaped morphology with irregular trapezoid structure. Their thickness varied from 50 to 200 nm. A smooth surface was observed on these plate shapes crystals. Most of these plates-like shapes stuck together and formed an assembly or bundle of platelets as observed in Fig. 4. In the presence of chitosan, these monetite platelets show a high degree of symmetry in terms of the ordered layer structure compared to those formed in the pure monetite. After the end of the hydrolysis reaction, needle-shaped crystals of apatite were formed on the surface of these monetite platelets. The density of the apatite crystals enhanced with soaking time, and the surface of the

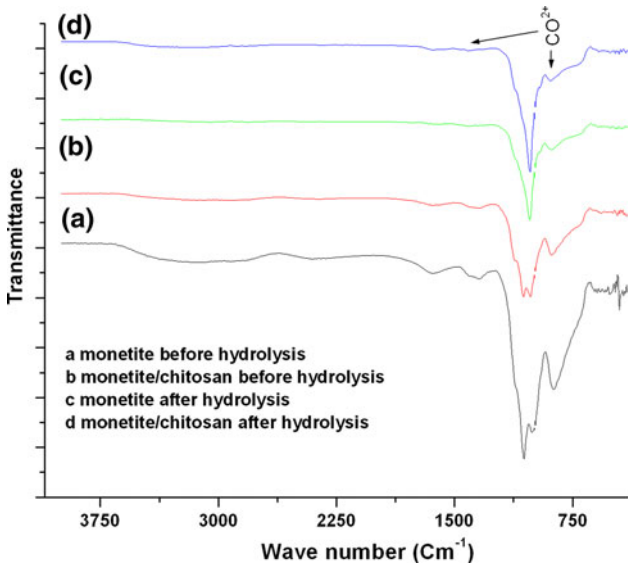

Fig. 3 Infra red spectrum for the phases produced after hydrolysis reaction in SBF for monetite *a* before hydrolysis, *c* after hydrolysis, and monetite chitosan composites *b* before hydrolysis, *d* after hydrolysis

monetite crystals were almost completely covered after 7 days. In the presence of chitosan, these needles were more agglomerated compared to those formed in the neat monetite.

3.3 Hydrolysis in complete media

To gain a better knowledge about the bioactivity of monetite in terms of the practical application of the monetite cement into the body, it is important to analyze its hydrolysis and phase development in α-MEM solution. Fig. 5a shows the XRD patterns of the neat monetite before and after the hydrolysis reactions in α-MEM for 4 days, 7 days and 4 weeks at 37°C. Similarly Fig. 5b displays the XRD patterns before and after hydrolysis for the monetite formed in the presence of chitosan. Both figures show that there is a little change in the XRD patterns of the monetite after 4 days and 7 days and 4 weeks of the hydrolysis reactions in culture media (α-MEM). These changes occurred due to the formation of apatite peaks in the patterns. However, after 4 weeks, these apatite peaks became more observable. Based on the results of the XRD data shown in Fig. 4a, b, the rate of the apatite formation during the hydrolysis reaction of monetite in complete α-MEM is very slow. It is also observed that most of the monetite particles are still un-reacted even after 4 weeks of hydrolysis process (Fig. 5a, b). After 4 weeks of hydrolysis reaction, the approximately calculated conversion into carbonated apatite under different conditions was not statistically significant (Fig. 6).

The data obtained from the FTIR spectra for monetite hydrolyzed in α-MEM support the results observed in XRD patterns. The changes in the FTIR spectra from 6a to 6b or 6c to 6d are minimal. This indicates the formation rate of apatite from monetite during the hydrolysis in α-MEM is very slow. The FTIR spectra could not determine if the formed apatite from the hydrolysis of monetite in α-MEM solution is a carbonated one or not.

SEM morphological structures of the monetite platelets, formed in the presence and absence of chitosan after soaking in α-MEM solution are shown in Fig. 7. After hydrolysis reaction in α-MEM, the monetite platelets lost their specific shapes and their dimensions decreased with time of soaking as be seen in Fig. 7. Additionally, needle-like apatite was formed on the surfaces of monetite platelets and its rate of formation increased with time, resulting in a rough surface feature on these platelets. As seen in Fig. 7, the presence of chitosan has no significant effect on the hydrolysis of monetite, where the monetite platelets were completely covered with apatite in presence or absence of chitosan. Even though the surfaces of these monetite platelets were completely covered with apatite crystals, the XRD and FTIR data showed that the monetite still remained unreacted after 7 days of soaking in α-MEM. This means that only a thin film of apatite was formed on the monetite surfaces regardless of the presence or absence of chitosan. Therefore, this is the reason why weak peaks of carbonated apatite were found in the XRD patterns.

4 Discussion

The washout resistance measurement indicated the presence of chitosan enhanced resistance to washout property of the monetite, confirming moneitite–chitosan a good candidate for application in treating an orthopedic emergency. The results matched the reported washout resistance measurement of calcium phosphate cement–chitosan [22].

It is well known that the hydrolysis reaction of calcium phosphates depends on many parameters such as temperature, pH, composition of solvents, and surface morphology of the calcium phosphate materials [17, 25]. The type of solvents is one of the significant parameters affecting the hydrolysis reaction. The insertion of monetite cements as an implant in the human body triggers interactions between the surfaces of the cement and body fluids. At the interface between calcium phosphate and body fluids, resorption or transformation from one phase of the calcium phosphates to other can occur. The phase, which is more thermodynamically stable at the pH of the fluid is precipitated. The fluid pH is not the only factor that determines the stability or the solubility of the calcium phosphate. There are other factors such as the fluid composition with respects to the concentrations of ions and the type of proteins added. Since the body fluid contains water and other ions and

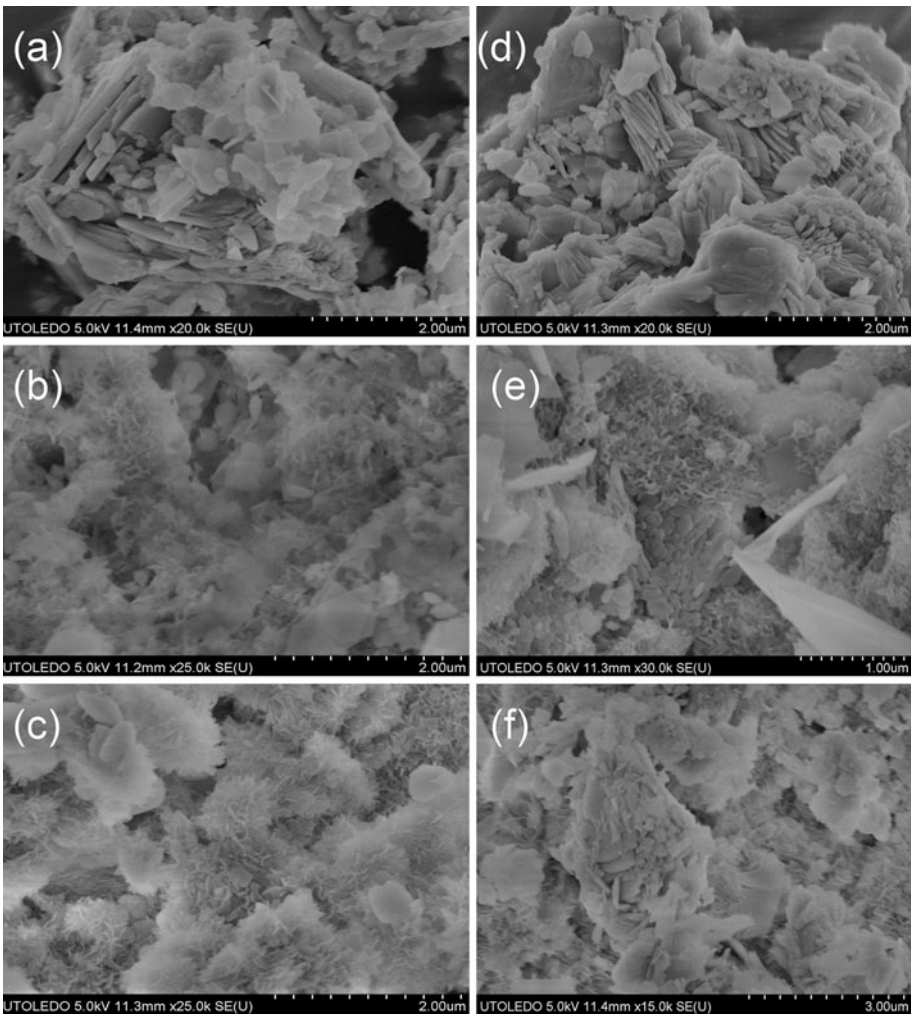

Fig. 4 Morphological structure for the phases produced after hydrolysis reaction in SBF for monetite after **a** 0 **b** 4, and **c** 7 days. And for monetite composites after **d** 0, **e** 4, **f** 7 days

components, it is essential to determine the stability of monetite in aqueous solution like SBF and others that contains protein such as α-MEM.

The degradation behavior of the monetite platelets formed in the presence and absence of the chitosan were evaluated by soaking in α-MEM and SBF for different periods of times at 37°C. During the hydrolysis process, different phases are produced. During in vitro studies, the presence of these phases has an impact on the growth and attachment of the cells on monetite surfaces in α-MEM solution.

The transformation mechanism of monetite to apatite during hydrolysis reactions occurs via two stages, the dissolution and precipitation reactions. The carbonated apatite was the main phase formed on the surfaces of monetite particles when they were soaked in SBF solutions at 37°C. Once the monetite is immersed in SBF, it starts to dissociate to calcium and phosphate ions. Because the solubility product of apatite is lesser than that of monetite, the solution becomes supersaturated with carbonated apatite. Consequently these calcium and phosphates ions precipitate on the surface of the monetite cement particles on the form of carbonate apatite. However, 7 days of soaking time was not enough for monetite to be completely transformed to carbonated apatite at 37°C. The reason for not completing the reaction within 7 days is attributed to the slow rate of the dissolution reaction of monetite particles in SBF solution. It is recognized that the monetite dissolution is not congruent. The incongruent dissolution reaction of monetite is the main reason for the decrease of the calcium concentrations in the solution and consequently the solution does not reach to the supersaturating level with monetite. Also it was reported that the formation of apatite layer around the monetite particles delayed the dissolution reaction rate of the interior particles of the monetite. Monetite is thermodynamically stable in an acidic environment. The pH of the solution played an important role in the hydrolysis of the monetite. In SBF solution, the pH value of 7.4 is not enough to drive the complete hydrolysis of monetite within 7 days. The same results were reported when the hydrolysis of monetite was previously studied as a function of time, in deionized water at 37°C, with a high liquid/solid ratio (2500 ml/g) in order to

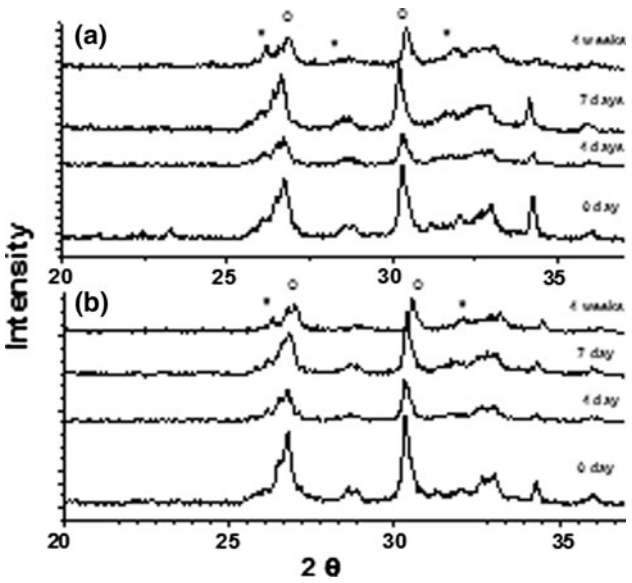

Fig. 5 XRD patterns of the phases produced after hydrolysis reaction in α-MEM of **a** monetite cement at different times, and **b** monetite chitosan composites at different times. These symbols for (*circle*) monetite and (*black heart*) apatite peaks

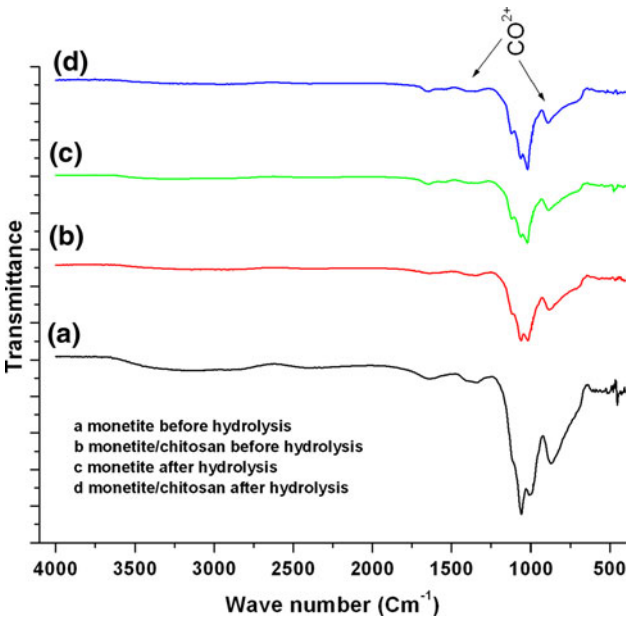

Fig. 6 Infra red spectrum for the phase produced after hydrolysis reaction in α-MEM for monetite *a* before hydrolysis, *c* after hydrolysis, and monetite chitosan composites *b* before hydrolysis, *d* after hydrolysis

avoid solubility limits being reached [17]. Although, the solutions were not saturated with respect to the solubility of monetite, dissolution stopped after 4 days and a very thin film was formed. The same conclusion was also obtained by Martin et al. [20]. They reported that the hydrolysis of monetite in water is limited, irrespective of the solid to liquid ratio. The incongruent dissolution of monetite and the formation of a thin film of apatite on the surfaces of the monetite particles were believed to be the reasons. With the increase in the pH value to 10 by adding alkali ions, the monetite rapidly transforms to appetite. It was reported that when the hydrolysis took place in the presence of alkali ions and at higher temperature, 4 h were enough to achieve full conversion from monetite to apatite. In another interesting study, when monetite was soaked in calcium containing saline solutions (i.e., 142 mM Na^{+1}, 5 mM K^{+1}, and 50 mM Ca^{+2} in water), it was completely transformed to apatite at 37°C after 6 days [21]. These results indicated the presence of alkali ions enhanced the dissolution rate of the monetite particles in the solution which led to the increase of the calcium ions in solution. This also helped the apatite precipitation.

Even though the pH value of α-MEM solution was approximately similar to that of SBF solution, the hydrolysis behavior in α-MEM was slower than that in SBF. Two different components affected the rate of dissolution of monetite in α-MEM solution, the inorganic ions and proteins. These calcium ions decrease the solubility of monetite in the solution. Other authors [26] have recognized that proteins can play an important function in mineralization by acting as inducers or inhibitors of HA formation depending on the types and levels of present. Furthermore, it appeared that the protein constituent could delay the rate of apatite precipitation. In vitro, it has been demonstrated that albumin retarded the transformation of apatite [27–29].

The incorporation of chitosan in the composites has no any significance on the hydrolysis of the monetite in both solutions, SBF and α-MEM. So based on the results of this study the presence of chitosan is beneficial in the monetite based composites. It did not inhibit the hydrolysis of the monetite in both solutions and while promoting the strength of the calcium phosphate and resistance to washout behavior.

5 Conclusions

Monetite formed in the presence and absence of chitosan was soaked in cell culture media (α-MEM) and SBF to examine the hydrolysis behavior of monetite in these solutions at 37°C. Carbonated apatite was formed on the surfaces of monetite platelets in both solutions. The formation rate of carbonated apatite on the monetite surfaces was faster in SBF solution compared to α-MEM. 7 days was not enough time for monetite to be transformed completely. In α-MEM solution, the monetite platelets still remained intact even after 4 weeks. The presence of the chitosan had no significant effect on the precipitation of carbonated apatite on the monetite particles.

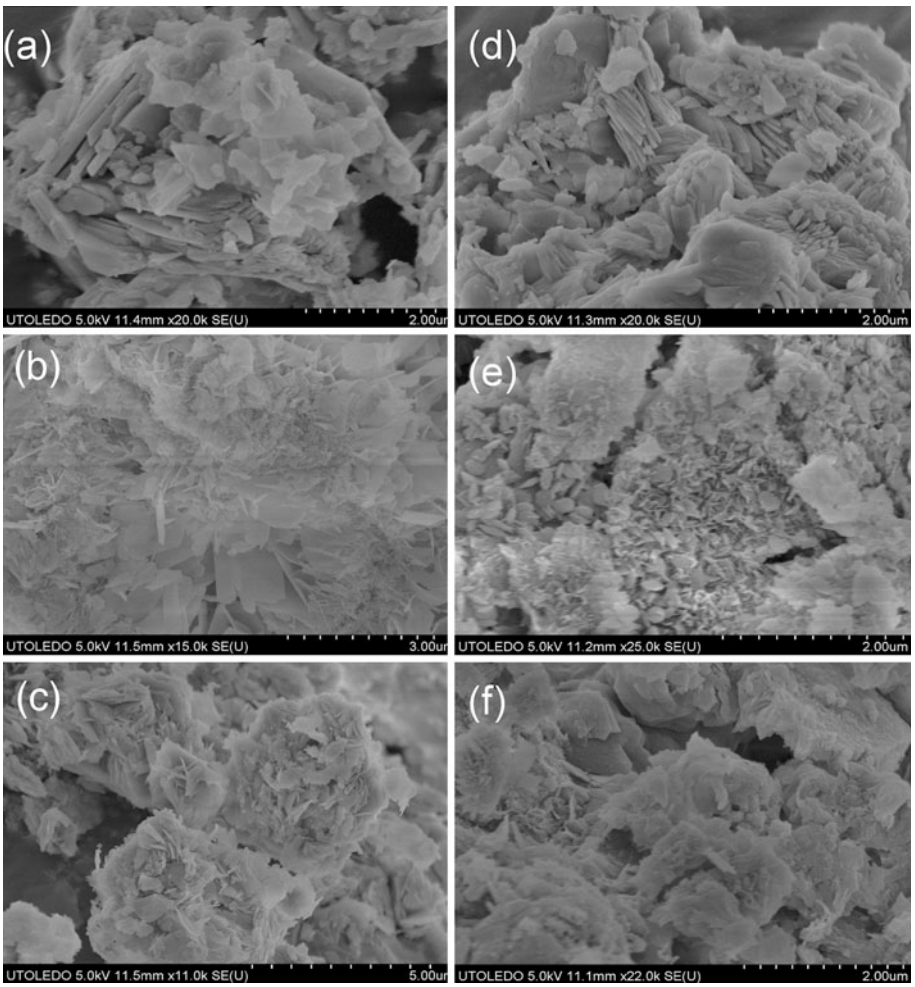

Fig. 7 Morphological structure for the phases produced after hydrolysis reaction in α-MEM for monetite after **a** 0 **b** 4, and **c** 7 days, and for monetite chitosan composites after **d** 0, **e** 4, **f** 7 days

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