An Alternative Synthesis Method for Di Calcium Phosphate (Monetite) Powders from Mediterranean Mussel (Mytilus galloprovincialis) Shells

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Abstract
Marine species, such as corals, sea shells and nacres, attract special interest in bioceramics field for bone graft, bone cements and drug delivery applications. Most of the marine structures are made up of pure calcium carbonate (calcite or aragonite) with a very small amount of an organic matrix. In the past the most common way to transform these structures to hydroxyapatite was hydrothermal transformation method. This current work introduces a new approach for producing fine powders of calcium phosphates from Mediterranean mussel (*Mytilus galloprovincialis*) shells. A comparative study was carried out to investigate the differences of these powders under only hot plate heating and hot plate heating together with ultrasonic agitation while H_3PO_4 was added. The temperature of the hotplate was kept constant at 80 ^oC and then, H_3PO_4 was added drop wise into the solution for 2 hrs. The mixture was then placed into an oven at 100 ^oC for 24 hrs. They were further calcined at 800 ^oC for 3 hrs. XRD, FTIR and ICP-MS were used to identify the structure and composition. It was found that the final powders were predominantly monetite, with some tricalcium phosphate as a secondary phase. This relatively simple and efficient method can be easily applied to produce calcium phosphate precursor powders for a range of biomedical applications.

Keywords: Biomimetic, calcium phosphate powders, mechano-chemical, ultrasound, Mediterranean mussel Mytilus galloprovincialis.

1. Introduction

Calcium phosphate-based bioceramics have been used successfully as one of the most efficient biomaterials for well over 40 years. Considerable attention has been given to the synthesis of hydroxyapatite and tri calcium phosphates for use in orthopedic and dental bone regeneration and repair materials. Generally these synthetic, highly porous substrate materials have relatively poor mechanical properties due to their interconnected porous structures. Calcium phosphate powders and monolithic structures can be produced either synthetically using quite a different number of methods or from naturally derived resources [1].

Natural materials such as marine structures can be easily overlooked for its potential applications due to the environmental concerns. While it is true that a wide range of marine structures are limited and protected, similarly there are also a variety of materials that are readily available and abundant and have yet to be exploited for their possible use [2]. Amongst these, Mediterranean Mussel (*Mytilus galloprovincialis*) shells can be commonly found in many places and often the shells are discarded after consumption and can therefore be acquired with minimal economic incentives. Their purity of the product will depend on the environment they have harvested and artificial growth of marine structures under controlled environments is becoming quite feasible.

Most marine structures are composed of calcium carbonate (aragonite or calcite) and can be easily converted into calcium phosphate materials by chemical exchange. The most common method of conversion is by hydrothermal exchange, which requires specialized equipment and long processing

times [1]. A number of investigations were directed using biological origin materials due to their chemical composition and/or the unique architectural structure for biomedical applications by applying a number of different synthesis routes. A number of investigations include; bones [3], dentine [4], enamel [5], and natural waste materials, such as eggshells [6], shells of some marine mollusks such as sea urchins [7], coral [8], nacre [9], sea [10] and land snails [11], cuttlefish bones [12] and the Brazilian river fish [13] just to name a few.

In this current study, it was aimed to evaluate an alternative conversion method for Mediterranean Mussel (*Mytilus galloprovincialis*) shells by using hot-plate and ultrasonic agitation. A comparative study was carried out to identify the different phases under individual heating and as combined heating and ultrasonification synthesis methods. By using this method one would only require simple equipment commonly found in laboratory providing an ease-of-use protocol.

2. Materials and Methods

2.1 Conversion and characterisation

The conversion of the *Mytilus* samples to calcium phosphate were carried out by two different synthesis methods: the first heating the natural powders containing solution on a hot plate without agitation (HP), while adding H_3PO_4 and a second one; by heating on a hot plate with ultrasonic stirring with the addition of H_3PO_4 (HPUS). *Mytilus* shells, or commonly known as black mussel shells were obtained in the Marmara Sea, Istanbul, Turkey. TG-DTA, SDT2960SDTV3.0FTA was used to determine the exact CaO content of *Mytilus* during dissociation. Sample weight of 30 - 40 mg was used during analysis under circulating air environment with a heating rate of 10^{o}C/min from room temperature to 1100^{o}C. The samples were first cleaned by removing the surface contaminants and then ground within a ball mill. Samples than were sieved through 75-100µm sieve and the organic matter were removed by using 5% NaClO before the conversion process. H_3PO_4 acid was introduced drop wise into the raw powders suspension in distilled water.

In the first method as a procedure the beaker containing the suspension was heated on a hot plate (HP) and the temperature was kept 80 °C. Stoichiometric molar ratio for the calcium phosphate was calculated using the CaO content and H_3PO_4 was added drop wise to solution for 2 hrs. In the second method the beaker and the suspension was ultrasonicated while heating on a hot plate and the acid was added drop wise (HPUS). After conversion the solution was filtered and the powders were dried in an oven at 100 ^{o}C for 24 hours, and then calcined at 800 ^{o}C for 3 hrs.

Structural characteristics of the synthesized powders were performed in Fourier Transform Infrared Spectroscopy (FT-IR). The synthesized calcium phosphate powders were further ground in an agate mortar and thoroughly mixed with KBr (FT-IR Grade, Sigma Aldrich, Australia). Three milligrams of powder sample was mixed with 300 mg of KBr powder (1% w/w). The FTIR spectra were collected using a Nicolet, Magna-IR 6700 Spectrometer FT-IR in the range 4000-400 cm^{-1}. The structural analysis was carried out using X-ray diffraction analysis (XRD) [Seimens D5000, Germany] employing CuK_{α} radiation (λ=1.5418 Å). The diffractometer was operated at 45 kV and 40 mA at a 2θ range of 20-80° employing a step size of 0.01°/s and a 4 sec exposure. The crystalline phases of the powders were identified by matching the XRD patterns with the Joint Committee on Powder Diffraction Standards (JCPDS) database.

2.2 Inductively coupled plasma-mass spectroscopy (ICP-MS)

Powders were digested to quantify calcium, phosphate, magnesium and strontium ions by inductively coupled plasma-mass spectroscopy (ICP-MS). An Agilent Technologies 7500ce series ICP-MS was used with sample introduction via a micromist concentric nebuliser (Glass expansion). The ICP operating parameters and the lens conditions were selected to maximize the sensitivity of a 1% HNO_3: HCl solution containing 1ng/ml of Li, Co, Y, Ce and Tl. Calibration standards were prepared in 1% nitric acid. Approximately 0.005g of sample was digested with 0.25 mL of HNO_3. The samples underwent a further 1:100 dilution with a 1% nitric acid solution before ICP-MS analysis.

3. Results

3.1 Thermal analysis (DTA/TGA)

Figure 1 shows the thermal analysis results from TGA-DTA. The weight loss due to the organic matter was approximately 2-3%. As the *Mytilus* sample predominantly consist of $CaCO_3$ at ~778 °C, a weight loss of about 42.2% following the decomposition reaction is observed.

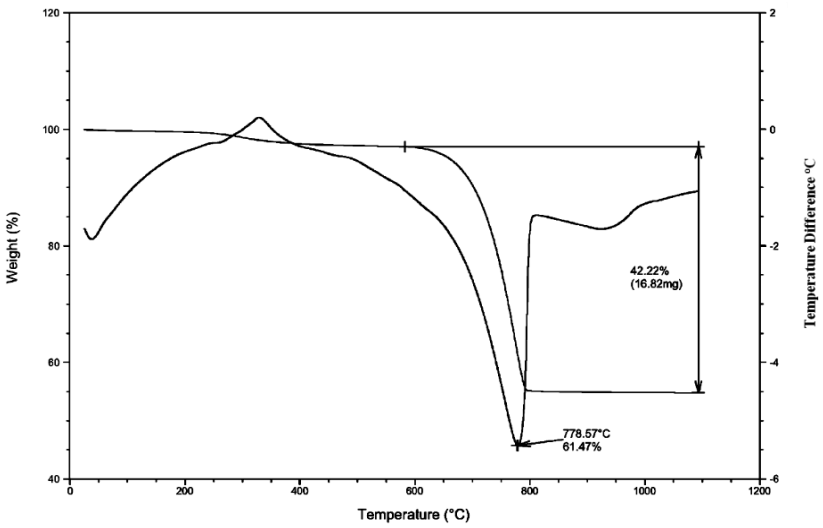


Figure 1: Themogravimetric curve for mussel shell (Mytilus Gallovincipialis)

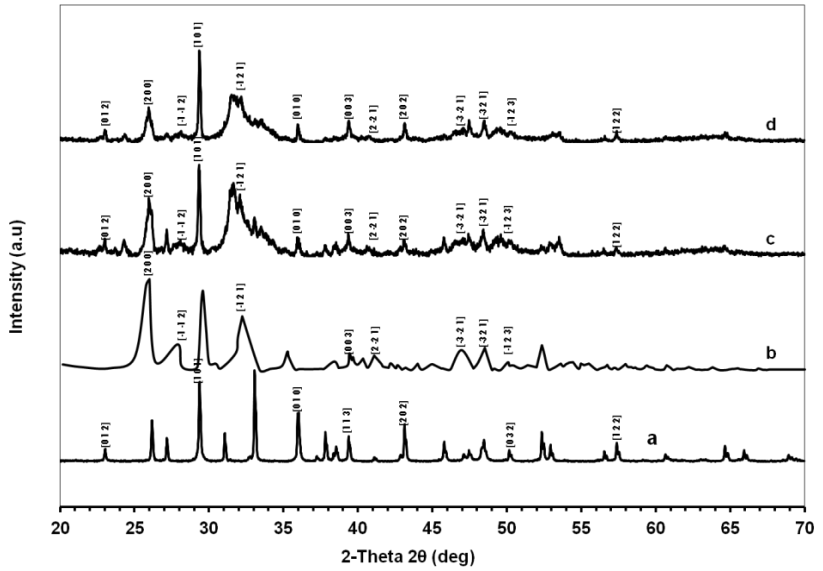


Figure 2: XRD patterns (a) Mussel shell (b) Monetite (c) Monetite formed by HP method (d) Monetite formed by HPUS method. Additional peaks show some retained other mixed $CaCO_3$ phases.

3.2 Physico-chemical characterization

Figure 2 shows the X-ray diffractogram of the raw mussel *mytilus* showing consistent peaks matching calcium carbonates. These peaks compared to the converted samples by hot-plating method (HP) and hot-plating under ultrasonication (HPUS) showed no distinct differences. The figure shows peak spacing consistent with well-ordered crystalline di-calcium phosphate anhydrous (monetite; $CaHPO_4$) (JCPDS 71-7159).

Figure 3 displays the FT-IR spectra of the converted *mytilus* samples by both HP and HPUS methods. The results here reinforce the presence calcium phosphate evident by the characteristic vibrations of the PO_4 tetrahedral ($v3$, 1042 and 1088 cm^{-1}; $v4$, 601 and 566 cm^{-1}; $v1$ 960cm^{-1} and $v2$ 470 cm^{-1}). With regards to the presence of carbonates, the spectra indicate its presence as suggested by the vibrational frequencies for CO_3 at 832 cm^{-1} for $v2$ and 1632, 1547, 1458and 1412 cm^{-1} for $v3$. Generally there are two types of carbonate substitution between CO_3 and the OH site (A-type) and at the PO_4 site (B-type) [1]. Typical peaks corresponding to A-type are centered on 1540cm^{-1} [14] and the regions around 870, 1430 and 1450 cm^{-1} correspond to B-Type. The occupancy of *v2* sites occurs competitively between OH^- and CO_3^{2-} groups, whereas the $v3$ site is competed between PO_4^{3-} and CO_3^{2-} ions [15].

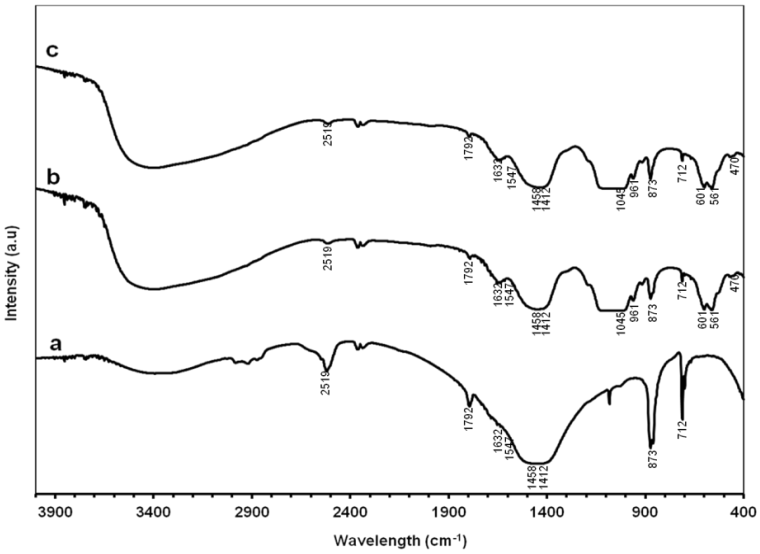


Figure 3: FT-IR spectra (a) Mussel shell (b) Monetite from mussel by HP method and (c) Monetite from mussel by HPUS method.

Table 1: The concentration of calcium, phosphate, magnesium and strontium present in the samples before and after conversion by ultrasonic (HPUS) and hot-plate (HP) methods quantified by ICP-MS

.	**Calcium (µg/L)**	**Phosphate (µg/L)**	**Magnesium (µg/L)**	**Strontium (µg/L)**
Untreated	5859 ± 20	54.79 ± 3	216 ± 13	248 ± 21
HPUS	4475 ± 13	19390 ± 0	135 ± 9	202 ± 3
HP	3447 ± 43	14800 ± 120	113 ± 18	162 ± 12

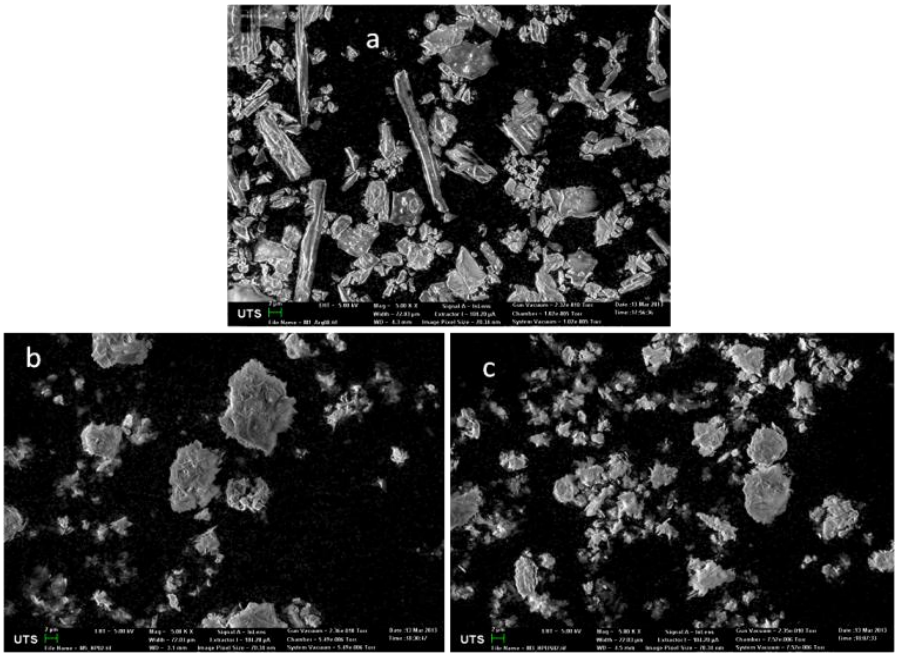


Figure 4: SEM images (a) original mussel shell aragonite particles (b) Monetite particles from HP method, and (c) Monetite particles from mussel by HPUS method

The FTIR spectra contain retained calcite (characteristic peaks at 875 cm-1), and other calcium phosphates after conversion. The XRD data also suggests that multiple phases are present. However as both XRD and FT-IR results showed evident formation of monetites by all two production methods, a more detailed examination of the chemical composition of the monetite samples were carried out by using ICP-MS looking specifically for the concentration of calcium, phosphate, magnesium and strontium amounts.

The presence and concentration of each respective ion is presented in **Table 1** showing level of phosphate concentration to be higher in HPUS method. Although statistically not significant both magnesium and strontium concentrations decreased marginally after conversion by both methods.

SEM was carried out in LEO Supra55VP which showed angular particle shape for all powders although aragonite particles were clear crystalline faceted appearance while the HPUS samples showed slightly smaller particle sizes than HP approximately 1-8 micron size (**Figure 4**).

4. Discussion

As well documented a range of calcium phosphates exist dependent of their Ca/P ratios and have different chemical constituents and dissolution rates (Table2). Depending on the type of application and environment, monetite, has been reported to have a very high aqueous solubility at physiological conditions [16]. Since its synthesis by Aoki et al. [17], Monetite's have been well investigated as precursors for a range of calcium phosphate bioceramics and can be used as a main constituent for β-TCP and HAp sintered products and bone cements [16-18]. The dicalcium phosphate cements were developed two decades ago and ever since there has been a substantial growth in research into improving their properties in order to satisfy the requirements needed for several clinical applications. In vivo behavior of the cements, including their ability to promote bone formation, biodegradation and potential clinical applications in drug delivery, orthopedics, craniofacial surgery, cancer therapy and biosensors are well documented [18].

Table 2 Different calcium phosphate phases and relevant Ca/P ratios.

Table 2: Different calcium phosphate phases and relevant Ca/P ratios.

	Mineral	Empirical formulas	Ca/P
Dicalcium phosphate dihydrate	Brushite	$CaHPO_4 \cdot 2H_2O$	1.00
Dicalcium phosphate	Monetite	$CaHPO_4$	1.00
Octacalcium phosphate		$Ca_8H_2(PO_4)_6 \cdot 5H_2O$	1.33
β-Tricalcium phosphate	Whitlockite	β-$Ca_3(PO_4)_2$	1.50
Hydroxyapatite		$Ca_{10}(PO_4)_6(OH)_2$	1.67
Tetracalcium phosphate monoxyde		$Ca_4(PO_4)_2O$	2.0
Defect apatites		$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ $0 < x < 2$	(10-x):6

Current methods for producing calcium phosphates from calcite and aragonite based marine structures, using hydrothermal exchange requires specialized equipment and hence there is a need for developing alternative conversion methods that allows ease-of-use protocol utilizing conventional laboratory equipment and chemicals. Both ultrasonication and hot-plate conversion methods match these requirements as supported by the presented results. The experimental results presented in this study show that monetite powders can be easily produced from mussel *mytilus* by either ultrasonication and heating or just hot-plate methods. Monetite presents an interesting class of biomaterial that can be used as a precursor material for other calcium phosphates. Monetite's potential as a biomaterial has recently been highlighted by a series of in vitro, and in vivo studies in which monetite-based biomaterials have shown promising results as bone substitutes. Monetite has a higher solubility than hydroxyapatite, octacalcium phosphate, and tricalcium phosphate, in physiological environments and is considered to exhibit a high in vivo resorbability in comparison to the apatitic cements [19].

From the XRD and FT-IR results it is evident that successful conversion can be achieved by both methods, moreover, the presence of natural trace elements and the ion-substitutions such as Mg and Sr will influence the biological performance of the powders. The presence of Mg has been extensively investigated and shown that increase in mechanical properties of apatites [1]. Strontium within calcium phosphates have gained considerable attention in

recent years due to its ability to promote osteoblast proliferation while inhibiting osteoclast formations [20].

One of the key highlights behind the current study lies in the fact that marine structures such as mussel *mytilus* naturally possess these elements. A number of studies investigated seasonal variability of trace metal concentrations and antioxidant enzymes observed in gills and digestive gland of the Mediterranean mussel Mytilus galloprovincialis from both polluted and nonpolluted populations. Trace metals (Cu, Fe, Mn, Pb, Mg, Sr and Zn) exhibited, in both organs, maximum values in later winter–early spring followed by a progressive decrease during the summer. Three trace elements Mg and Sr and Zn have positive influence in the HAp properties if they are substituted within the structure.

It is envisaged that combined with the high resorbability of the monetite with the release of magnesium and strontium, this can be an effective strategy in enhancing localized biological response. Further examination currently in process, will examine the morphological differences and *in-vitro* cell culture studies with osteoblasts and macrophages that will provide a clearer understanding prior to in vivo studies. The novelty of this study is shown by the successful conversion of mussel *mytilus* by a simple method for producing better healthcare products.

5. Conclusion

In this study, monetite powders were successfully produced from mussel *mytilus* by simple hotplate and ultrasonication method while preserving magnesium and strontium ions. The presented methods not only provide an alternative strategy for conversion, but it is safe and inexpensive utilizing conventional laboratory equipment. As a general principle if the marine structures are intended to be used as biomaterials it is strongly suggested that these materials should be collected from non-polluted waters or artificially grown in a specific clean and controlled environments. The present results suggest these after appropriate cleaning process, *monetite* powders produced by this relatively simple method have the potential to be used as a precursor in a range of calcium phosphate based biomedical materials.

6. References

1. Zapanta LeGeros R., Apatites in biological systems, Progress in Crystal Growth and Characterization, Vol. [4], 1–2, (1981), 1-45.
2. Green D. W. and B. Ben-Nissan. Biomimetic Applications in Regenerative Medicine: Scaffolds, Transplantation Modules, Tissue Homing Device and Stem Cells. In *Handbook of Materials for Nanomedicine*, Vol., Tochilin V. and M. Amiji (Ed.), Pan Stanford Publishing Pte Ltd, Place, (2010), 821-850.
3. Goller G., F. N. Oktar, S. Agathopoulos, D. U. Tulyaganov, J. M. F. Ferreira, E. S. Kayali and I. Peker, Effect of sintering temperature on mechanical and microstructural properties of bovine hydroxyapatite (BHA), J Sol-Gel Sci Techn, Vol. [37], 2, (2006), 111-115.
4. Göller G. and F. N. Oktar, Sintering effects on mechanical properties of biologically derived dentine hydroxyapatite, Materials Letters, Vol. [56], 3, (2002), 142-147.
5. Oktar F. N., Microstructure and mechanical properties of sintered enamel hydroxyapatite, Ceram Int, Vol. [33], 7, (2007), 1309-1314.
6. Kumar G. S., A. Thamizhavel and E. K. Girija, Microwave conversion of eggshells into flower-like hydroxyapatite nanostructure for biomedical applications, Materials Letters, Vol. [76], 0, (2012), 198-200.
7. Agaogullaria D., D. Kelb, H. Gökçea, I. Dumana, M. L. Öveçoglua, A. T. Akarsubasic, D. Bilgiçb and F. N. Oktard;e, Bioceramic Production from Sea Urchins, Acta Phys Pol A, Vol. [121], 1, (2012), 23-26.
8. Ben-Nissan B., Natural bioceramics: from coral to bone and beyond, Curr Opin Solid St M, Vol. [7], 4-5, (2003), 283-288
9. Oktar F., U. Tuyel, N. Demirkol, O. Gunduz, R. Samur, S. Kannan and S. Agathopoulos. A new safe method to produce bioceramic nano-powders from nacre venus verrucosa. Artificial Organs Conference, FYROM, 2010: International Journal of Artificial Organs, (Special Issue); 2010. p. 467-467.
10. Ozyegin L., F. Sima, C. Istoscu, I. A. Kiyici, I. N. Mihailescu, O. Meydanoglu, S. Agathopoulos and F. N. Oktar, Sea Snail: An Alternative Source for Nano-Bioceramic Production, Key Engineering Materials Vol. [493], (2012), 781-786.
11. Kel D., H. Gökçe, D. Bilgiç, D. Ağaoğulları, I. Duman, M. L. Öveçoğlu, E. S. Kayalı, I. AKıyıcı, S. Agathopoulos and F. N. Oktar, Production of Natural HA from Land Snails, Key Eng Materials Vol. [493], (2012), 287-292.
12. Rocha J. H. G., A. F. Lemos, S. Agathopoulos, P. Valério, S. Kannan, F. N. Oktar and J. M. F. Ferreira, Scaffolds for bone restoration from cuttlefish, Bone, Vol. [37], 6, (2005), 850-857.
13. Coelho T. M., E. S. Nogueira, A. Steimacher, A. N. Medina, W. R. Weinand, W. M. Lima, M. L.

Baesso and A. C. Bento, Characterization of natural nanostructured hydroxyapatite obtained from the bones of Brazilian river fish, J Appl Phys, Vol. [100], 9, (2006), 094312-094316.

14. Landi E., G. Celotti, G. Logroscino and A. Tampieri, Carbonated hydroxyapatite as bone substitute, Journal of the European Ceramic Society, Vol. [23], 15, (2003), 2931-2937.
15. Rau J. V., S. N. Cesaro, D. Ferro, S. M. Barinov and I. V. Fadeeva, FTIR study of carbonate loss from carbonated apatites in the wide temperature range, Journal of Biomedical Materials Research Part B: Applied Biomaterials, Vol. [71B], 2, (2004), 441-447.
16. C L. and C. S. Takagi, inventors; American Dental Association Health Foundation, assignee. Self-Setting Calcium Phosphate Cements and Methods for Preparing and using Them. U.S.A1996 06/11/1996
17. Aoki H., M. Hata, M. Akao, S. Iwai and K. Kato, The crystal structure of hydroxyapatite, Tokyo Ika Shika Daigaku Iyo Kizai Kenkyusho Hokoku, Vol. [12], (1978), 9-17.
18. Tamimi F., Z. Sheikh and J. Barralet, Dicalcium phosphate cements: brushite and monetite, Acta Biomaterialia, Vol. [8], 2, (2012), 474-487.
19. Gbureck U., T. Hölzel, U. Klammert, K. Würzler, F. A. Müller and J. E. Barralet, Resorbable dicalcium phosphate bone substitutes prepared by 3D powder printing, Advanced Functional Materials, Vol. [17], 18, (2007), 3940–3945.
20. Bonnelye E., A. Chabadel, F. Saltel and P. Jurdic, Dual effect of strontium ranelate: Stimulation of osteoblast differentiation and inhibition of osteoclast formation and resorption in vitro, Bone, Vol. [42], (2008), 128-138.