In Vitro Bioactivity of Barium Doped Biphasic Calcium Phosphate

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ABSTRACT
Background: Biphasic calcium phosphate (BCP), a combination of (HA+ βTCP), widely utilized as bone grafting material, however BCP lacks trace ions, which are present in a mineral component of human hard tissue. Objective: to enhance bioactivity of (BCP) through doping of Ba2+ ions in HA lattice. Results: XRD results showed reduction in Crystallinity and crystallite size on increasing Ba2+ amount in HA lattice, in addition an increase in lattice parameters along the a and c axis was observed. Furthermore, increased Ba2+concentration led to increase in βTCP phase. FTIR spectra showed presence of main functional group of HA, in vitro study carried out in SBF for 21 days showed the formation of apatite layer with acceptable Ca/P ratio Conclusion: Ba-BCP series have been prepared through microwave assisted wet precipitation method, Ba2+ was successfully doped inside HA, presence of Ba showed an enhancement in the in vitro bioactivity of BCP.


INTRODUCTION

Human bones are composed of 30% organic phase (i.e. collagen) and 70% inorganic phase (i.e. calcium phosphate). Synthetic Calcium phosphates (CP) such as hydroxyapatite (HA) and β-Tricalcium phosphate (βTCP) have received a lot of attention from scientists and researchers due to their excellent bio-compatibility when embedded inside human tissues. Biphasic calcium phosphate (BCP) is defined as combination of HA and βTCP, this composite shows unique behavior towards bone growth compared with single phase of HA or βTCP. HA has the chemical formula [Ca_{10}(PO_4)_{6}(OH)_{2}] with Ca/P ratio equal to 1.67, which is in good agreement with the ratio in mineral phase of human bone. HA has the ability to create direct bonding with the human bone. Highly crystalline structure of HA make it as nonresorbable materials (Kivrik and Taş, 1998), leading to slower healing bone growth. βTCP is regarded as a biodegradable material, in vivo result have shown faster bone growth replacing the implanted βTCP (Sanosh et al., 2010, Viswanath et al., 2008).

Properties of HA such as physicochemical properties, dissolution rate and bioactivity can be improve through ion substitution with cation at Ca^{2+} site of HA (Suchanek et al., 2004), whereas small quantities of cation (i.e. K^{+}, Mg^{2+}, Sr^{2+}, Al^{3+}) in HA structure play a vital role in its overall biological performance (Gozalian et al., 2011).

Barium ions (Ba^{2+}) are present as minor ion in human enamel (125 ppm) (Boanini et al., 2010, McConnell, 1973). Incorporation of Ba^{2+} ions in HA lattice is expected to increase lattice parameters along the a and c axis (Yasukawa et al., 2005). Previous studies (Yasukawa et al., 1997) have focused on the synthesis and structural characterization of HA. According to our best knowledge no study yet has been reported on the effect of barium ion on biologic behavior of BCP.

Methodology:
1.1. Preparation of BCP and Ba-BCP:

Chemicals used in synthesizing BCP and Ba-BCP samples were purchased from (Qerc.New zeland) and were used as received. The preparation have been done by a microwave assisted wet precipitation method. Calcium nitrate (Ca(NO_3)_2.4H_2O), barium nitrate (Ba(NO_3)_2), and di-ammonium hydrogen orthophosphate ((NH_4)_2HPO_4) were used as a source of Ca^{2+}, Ba^{2+}, and PO_4^{3-} ions respectively. Calcium nitrate and appropriate amount of barium nitrate were dissolved in 200 ml of deionized water (solution-D). Di-ammonium hydrogen

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orthophosphate was dissolved in 200ml of deionized water (solution-II). Solution-II was added dropwise to solution-I under stirring over 30 min. throughout synthesis, the pH was kept above 10 by the addition of NH₃OH. Subsequently, the solution was refluxed in a microwave oven (SHARP, model R-218LS) at 800W for 10 minutes (30 sec ON and 30 sec OFF), the white precipitate was filtered and washed with deionized water several times, dried at 80°C for 24 h followed by calcination at 1000 °C for 2 h to produce BCP and Ba-BCP series. The heat treated powders were characterized by using X-ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR). Different samples prepared in this study are listed in Table 1.

For the in vitro bioactivity analysis the samples were pressed into circular discs and immersed in SBF solution for 21 days.

### Table 1: Amount of reactant used in preparation.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Reactant concentrations (mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca⁺⁺</td>
</tr>
<tr>
<td>BCP</td>
<td>1.00</td>
</tr>
<tr>
<td>0.4Ba-BCP</td>
<td>0.96</td>
</tr>
<tr>
<td>1.0Ba-BCP</td>
<td>0.900</td>
</tr>
</tbody>
</table>

**Results:**
Phases purity of Ba-BCP series was determined by using X-Ray Diffractometer (XRD, Bruker D8) operated at 40kV and 30mA utilizing CuKα radiation, at a step size of 0.02° and step time of 1 sec, all the diffractogram were recorded at range 20-80 of 2θ angles.

The average crystallite size was calculated using Scherrer’s equation (Eq. 1); $D = \frac{0.9\lambda}{β\cos θ}$ Eq. 1
Where $D$= size of the crystallite size (nm), $λ$ = wavelength of the X-ray used (nm), $β$ = full width of the line at half of its maximum intensity in radians (FWHM), $θ$ = diffraction angle (X.L. Tang, 2005, Aminian et al., 2011).

The degree of crystallinity BCP and Ba doped BCP samples were calculated according to the fraction of crystalline phase available in the analysed volume from X-ray diffraction data by using Eq. 2

$$X_C = 1 - \left(\frac{I_{112}}{I_{300}} / \frac{I_{300}}{I_{300}}\right) \times 100\%$$ Eq. 2
where $X_C$ is the degree of crystallinity, $I_{300}$ is the intensity of (3 0 0) diffraction peak and $I_{112}$ is the intensity of the hallow between (1 1 2) and (3 0 0) diffraction peaks.

The percentage presence of βTCP as a secondary phase in Ba-BCP samples was determined from relative intensity ratio of the corresponding major phases by using Eq. 3a and b (Kumar et al., 2001, Dhal et al., 2013, Tarafder et al., 2013).

Percent of phase to be determined = Relative Intensity ratio of the phase x 100 Eq. 3a
Relative Intensity ratio = Intensity of the major peak of the phase / $\sum$ Intensity of major peaks of all phases Eq. 3b

The FTIR spectra were recorded using a Fourier Transformer Infrared spectrophotometer (PerkinElmer, model FT/IR-6100 type A), by using KBr disc method. All the spectra were recorded in the scanning range of 4000-400 cm⁻¹. FTIR spectra Figure 2, shows the characteristic peaks of crystalline HA.

**In vitro** test were made in SBF solution which was prepared according to Kokub receipt (Kokubo and Takada, 2006), whereas BCP and Ba-BCP samples were pressed into pellet by using universal testing machine through applying 35KN load. The pellets were immersed in 50 ml of SBF solution with pH 7.40 at 36.5°C for 21 days, morphology ofapatite was investigated by using the low vacuum scanning electron microscope (LVSEM JSM - 6390), elemental analysis of formed apatite layer was evaluated by using Energy Dispersive X-ray Spectrometer (EDX, Hitachi, SwiftED3000)

**Discussion:**
XRD results shown in (Figure 1) revealed that the powders were composed of HA (91%) and βTCP (9%), however the major component was HA (09-0432). Furthermore, the prepared BCP were free of contaminants such as CaO and CaCO₃, etc. The incorporation of Ba⁺⁺ ions in the HA structure which further showed serious effect on HA lattice. On increasing the amount of Ba⁺⁺ ions, a shift in the pattern towards low angle was observed, boiling of BCP peaks was increased, and peaks intensity was decreased, further more increased in the growth rate of βTCP phase. These dramatic changes in the HA lattice were ascribed to the bigger ionic radii of Ba⁺⁺ (0.135 nm) that replaced smaller radii Ca⁺⁺ (0.099 nm), which destabilized HA structure and inhibited the growth of HA, thus led to increase in the lattice parameter along with a and c axis (Table 2). Substitution of Ba⁺⁺ ions revealed a reduction in the degree of crystallinity and crystallite size of HA (Table 2), and increase in the growth rate of βTCP phase.
Table 2: Lattice parameters and crystallite size of BCP substituted with Ba\textsuperscript{2+} ions.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Lattice parameter</th>
<th>Crystallite size (nm)</th>
<th>Degree of crystallinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a-axis (Å)</td>
<td>c-axis (Å)</td>
<td>Cell Vol (Å\textsuperscript{3})</td>
</tr>
<tr>
<td>BCP</td>
<td>9.421</td>
<td>6.885</td>
<td>529.21</td>
</tr>
<tr>
<td>0.4Ba-BCP</td>
<td>9.429</td>
<td>6.887</td>
<td>530.26</td>
</tr>
<tr>
<td>1.0Ba-BCP</td>
<td>9.435</td>
<td>9.890</td>
<td>531.17</td>
</tr>
</tbody>
</table>

Fig. 1: XRD pattern of Ba-BCP series.

FTIR spectra showed a peak at 3571 cm\textsuperscript{-1} which was assigned to the O-H stretching mode of the hydroxyl group. A broad band observed at 3450 cm\textsuperscript{-1} was attributed to the absorbed water. Major absorption peaks found around 1016 cm\textsuperscript{-1} to 1114 cm\textsuperscript{-1} were due to the asymmetric stretching of P–O bond of phosphate group in HA. The peak at 963 cm\textsuperscript{-1} was assigned to the P–O symmetric stretching. The peak at 632 cm\textsuperscript{-1} corresponded to the vibration mode of the O-H bending mode. Peaks at 602 and 572 cm\textsuperscript{-1} were assigned to the O–P–O bending modes. The sharpness of the bands at 632, 602 and 572 cm\textsuperscript{-1} indicated the formation of a crystalline HA. As the barium concentration increased this peak became broader and wider which indicated the formation of material with reduced degree of crystallinity. In addition an inhibition in the growth of O-H peak as Ba ions increased inside lattice confirmed the growth of βTCP phase which is consistent with XRD result (Fig.1.)

Fig. 2: FTIR spectra of Ba-BCP series.
SEM images of BCP, 0.4Ba-BCP and 1.0Ba-BCP after soaking in the SBF for 21 days (Figure. 3) showed the formation of spherulite agglomerates synonymous with apatite like structure on the surface of Ba-BCP. Particles grew more closely packed as Ba*+* amount increased into BCP structure, proposing that increased Ba*+* ions assisted the formation of apatite layer on the surface of Ba-BCP. EDX analysis of apatite layer showed that it is mainly consisted of calcium, phosphorus, and oxygen, in addition minor amount of harium and carbon was also observed, whereas Ca+Ba/P ratio of apatite layer increased from 1.40 to 1.48 for BCP and 1.0Ba-BCP respectively. Existence of Ba*+* ions in the deposited apatite layer was attributed to the release of Ba ions from the Ba-BCP structure and then their re-deposition on the surface of apatite layer. This phenomenon of Ba ions re-deposition onto the apatite layer helps to prevent the Ba ions concentration from reaching toxic level inside human tissue.

**Fig. 3:** SEM micrographs of (a) BCP, (b) 0.4Ba-BCP and (c) 1.0Ba-BCP after 21 days in SBF solution.

**Conclusion:**

In summary, BCP, 0.4Ba-BCP, and 1.0Ba-BCP were successfully synthesized via microwave assisted wet precipitation method. The samples were analyzed via XRD to examine the phase purity. FTIR spectra showed characteristic peaks of crystalline HA and βTCP in Ba-BCP series. SEM images of BCP, 0.4Ba- BCP, and 1.0Ba- BCP after soaking in SBF solution for 21 days showed the formation of spherulite agglomerates synonymous with apatite like structure on the surface of BaHA confirming the ability of BaHA to accelerate bone formation process. EDX analysis showed that the Ca/P ratio in the range 1.40-1.49.

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