CHAPTER 4

NANOCRYSTALLINE BIPHASIC CALCIUM PHOSPHATE (HAp/β-TCP) THIN FILM PREPARED BY ELECTRON BEAM EVAPORATION TECHNIQUE

4.1 INTRODUCTION

Calcium phosphate bioceramics have excellent biocompatibility and bioactivity, hence it is widely used to fabricate bone and dental implants in a variety of forms (Hench 1991). Bulk form of calcium phosphate has poor mechanical property and hence it could not be used as implant material to replace large bone affected by cancer or fracture (Lopatin et al 1996). However, this problem was overcome by coating a thin layer of calcium phosphate on metallic implants which have excellent biocompatibility as well as good mechanical properties (Espana et al 2010). The most important clinical applications of calcium phosphates are in the form of coatings, particularly, for dental and orthopedic related biomaterials. The plasma spraying technique is commercially used for the deposition of HAp coating on metallic implant (Yang et al 2005). This has several disadvantages like the low adherence, non-stoichiometric compositions, and non-uniformity of thickness on implant material. Because of these drawbacks, several techniques have been proposed to produce the calcium phosphate coating on metallic substrates, such as the ion beam sputtering (Ananda Sagari et al 2007), sol-gel (Liu et al 2002), electrochemical deposition (Manso et al 2006), biomimetic deposition (Zhang et al 2009), hot isostatic pressing (Tadic and
Epple 2003), electrophoretic deposition (Stoch et al 2001), pulsed laser deposition (Bao et al 2008), RF magnetron sputtering (Yang et al 2005) and electron beam evaporation (Hamdi and Ektessabi 2001). Pure phase of HAp coated metallic implant possesses low bonding ability compared to the biphasic calcium phosphate. Due to this reason, the current research is focused on biphasic coatings on metallic substrate (Daculsi et al 2003). The partial dissolution of the bio-resorbable phase (β-TCP) and non-resorbable phase of HAp in biphasic calcium phosphate coatings is considered to be beneficial because it can aid bone apatite formation at the interface between the implant and bone (Daculsi 1998). Biphasic calcium phosphate ceramics consisting of HAp/TCP and HAp/tetra calcium phosphate showed better biological activity. In recent years, techniques are being investigated to produce biphasic calcium phosphate thin films (Kim et al 2005). Electron beam deposition is a familiar method for thin film preparation. It is used to produce the coating of superconducting, magnetic and optical materials (Tsunoda et al 2001). The present work, describes about the deposition of biphasic calcium phosphate thin film made of non-resorbable HAp and resorbable β-TCP on silicon substrate by electron beam evaporation and its biological performance.

4.2 EXPERIMENTAL METHODS

4.2.1 Material Preparation

Stoichiometric amount of calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$.4H$_2$O, Merck) and diammonium hydrogen phosphate ((NH$_4$)$_2$HPO$_4$, Merck) were dissolved separately in 250 mL of deionized water. Before mixing, the pH of the precursor solutions was adjusted to 10 by adding ammonia (AR grade, Merck). The calcium nitrate solution was added to the phosphate containing solution at constant flow rate with continuous
stirring. After mixing, the solution was vigorously stirred for 5 h. The pH of the solution was maintained at 10 during the reaction. Subsequently, the solution was autoclaved at 150 °C for 6 h at a pressure of 120 psi. The precipitate was collected, washed using deionized water and dried at 90 °C, and subjected to a 750 °C for 2 h sintering. Then the sample was crushed into fine powder and made into a pellet (diameter 10 mm and thickness 10 mm) by hydraulic pressing (Lawrence and Mayo) at 3 tons. Subsequently, the pellet was kept at 750 °C for 2 h in air with the ramping rate of 5 °C/min. This pellet was used as target for coating by electron beam evaporation.

4.2.2 Coating Procedure

The pure silicon (001) substrates (10 mm x 10 mm x 0.25 mm) were cleaned by ultrasonic method with ethanol, acetone and deionized water for 30 min. Calcium phosphate layer was deposited on silicon (001) substrate at room temperature by electron beam evaporation bombardment techniques. The evaporation was performed in three different coating units at different levels of vacuum.

Several attempts were made using diffusion pump based coating units (systems equipped with 2 kW and 3 kW electron gun) with vacuum in the range of $10^{-5}$ to $10^{-6}$ Torr. No crystalline phase was formed (Figure 4.1(a)). Finally few attempts were carried out in a cryo pump based coating unit having 6 kW electron gun set up which was made by turbo pump, cryo pump and scroll pump. Its major advantage is oil free vacuum coating unit (Shidling et al 2008). Silicon substrates were kept inside the vacuum chamber at a distance of 19 cm away from the source (target). The vacuum chamber was evacuated to a pressure of $4\times10^{-8}$ Torr before initiating the deposition process. Initially the BCP target was heated by e-beam with a fixed current at 65 nA for out-gassing of material. When stabilized to the target material, the current
was increased to 100 nA and the vapor flux was generated. The manually operated shutter was removed for the uninterrupted travel of vapor from source to substrate. The deposition rate was 1.6 Å per second. During the deposition, the pressure created inside the chamber was $1.6 \times 10^{-6}$ Torr. The thickness of the film was 500 nm. The samples were annealed at 500 and 700 °C for 2 h with a heating and cooling rate at 4 °C/min. These films were used for further analysis. At high vacuum, there might be vaporization of HAp at the single molecular state. Hence, deposition of such molecules gives BCP layer. Here after, as-deposited, annealed at 500 and 700 °C thin films were named ASBCP, BCP500 and BCP700 respectively.

![Figure 4.1 Images of (a) Diffusion pump based coating unit and (b) Cryo pump based coating unit of electron beam evaporator](image)
4.2.3 Characterization

The crystalline phase of the target material was analyzed by X-ray diffraction (Bruker AXS Diffractometer) using CuKα radiation (λ = 1.5406 Å) at 40 kV and 40 mA in the two theta range from 20° to 50° and pattern was recorded in locked coupled (powder) mode. The thin films were subjected to X-ray diffraction at a scan speed of 0.02°/s and the pattern was recorded by detector scan (thin film) mode. The X-ray tube was fixed at the incident angle of 2°. Fourier transform infrared (FTIR, Perkin-Elmer spectrum RXI FTIR system) spectrum was recorded for BCP target by KBr pellet technique in the range 400 to 4000 cm⁻¹. BCP700 film was subjected to the FTIR analysis. The surface morphology of the ASBCP and BCP700 thin films was studied using scanning electron microscope (SEM, LEO 440 STEREOSCAN Leica) and its elemental analysis was done by energy dispersive X-ray analysis (EDX, JEOL-3010 electron microscope). The particle size and surface roughness of ASBCP and BCP700 thin films were analyzed by atomic force microscope (AFM, Digital Instruments Nanoscope) in non-contact mode.

SBF solution was prepared as prescribed by Kokubo et al (2006), was used for the study of in vitro bioactivity. ASBCP, BCP500 and BCP700 thin films were immersed in 20 ml of SBF in plastic containers and it was maintained at 37 °C. SBF solution was renewed once in two days for a period of four weeks. The phosphate buffer solution (PBS) was prepared by dissolving NaCl, KCl, KH₂PO₄, Na₂HPO₄ in deionized water and pH 7.4 adjusted using HCl (Dulbecco and Vogt 1954). The static contact angles of sessile drop of water, PBS and SBF at a constant volume of 10 µl on BCP thin films were measured using an optical microscope setup equipped with color charge coupled device (CCD) camera at room temperature (30 °C). The contact angles were analyzed with the help of “imageJ” software.
The cell viability test was performed using MTT assay. MDAMB231 cell was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 °C. The cells were seeded on BCP700 film using polystyrene 24 well plates and compared with the induced toxic agent (TritonX) into triplicate of culture polystyrene plate. It was incubated at 37 °C for 24 h to allow cell adherence. Then, 10 µL of MTT reagent (10 µg/mL) was added to each well plate and incubated for 30 min at similar conditions. Finally, dimethylsulfoxide was added for dissolving the formasan crystals and absorbance was measured at 595 nm in an ELISA reader. The cell culture medium was used as a negative control and a TritonX toxic agent as a positive control.

50 mg of amoxicillin (AMX) drug was dissolved in 1 mL of deionized water. BCP700 sample was immersed in a drug containing solution at 37 °C with shaking speed of 100 rpm for 24 h and subsequently dried at 37°C. Antimicrobial activity of BCP700 and drug loaded BCP700 samples were evaluated by the disc diffusion method. For the determination of antimicrobial activity 20 mL sterile Muller-Hinton (MH) Agar was poured into 14 cm pertidish and it was solidified at UV sterile condition. After solidification 10^5 Colon Forming Units (CFUs) of Staphylococcus aureus (S. aureus, MTCC 3381) was spread in the entire region of the MH agar plate. After spreading, same size of with and without antibiotic loaded BCP700 samples were placed on the MH agar plate and incubated at 37 °C for 24 h. The microbial inhibition zone was measured every 3 h interval using standard scale and its image was documented.
4.3 RESULTS AND DISCUSSION

4.3.1 XRD Analysis

X-ray diffraction patterns of target and thin films of ASBCP, BCP500 and BCP700 were as shown in Figure 4.2 (a-d). All the crystalline planes of target (sintered at 750 °C) were matching with the standard JCPDS values of HAp (09-0432) and β-TCP (09-0169) phase, confirming their biphasic nature (Figure 4.2(a)). The ASBCP and BCP500 films revealed amorphous surface (Figure 4.2(b) and (c)). The annealed samples at 700 °C, HAp and β-TCP peaks showed high intensity which suggested that BCP phase was crystallized well on silicon substrate (Figure 4.2(d)). The appearance of low intensity peak (0210) and high intensity peak (300) compared to the target may be due to the preferential orientation of β-TCP (Boyd et al 2008). The quantification of HAp and β-TCP phases of the target were determined using the normalized intensity peaks of (211) and (0210) of HAp and β-TCP respectively from the relative intensity ratio (RIR) = \( \frac{I_{\beta-TCP}}{I_{\beta-TCP} + I_{HAp}} \) (Sampath Kumar et al 2000, Caroline Victoria and Gnanam 2002). The phase composition of HAp and β-TCP was found to be 39:61 wt %. Similarly for the BCP700 the ratio was found to be 34:64 wt %. The crystallite size (L) of target and thin film was calculated from XRD spectrum using Scherrer formula, \( L = \frac{K \lambda}{\beta \cos \theta} \), Where, \( \lambda \) is the X-ray wavelength (1.5406 Å) and \( \theta \) is the diffraction angle. K is a constant varying with crystal habit and chosen as 0.9 and \( \beta \) is the full width at half maximum (Landi et al 2000).

All the planes of target and thin film of XRD patterns were indexed and its full width at half maximum was analyzed by XRDA software (Desgreniers and Lagarec 1994). The average crystallite size was obtained as 50 and 60 nm for BCP target and BCP700 thin films, respectively. The crystallinity was determined by \( \beta_z \times (X_c)^{1/3} = K_\lambda \) Where \( X_c \) is the degree of crystallinity, \( \beta_z \) the full width of the peak at half intensity of (Z) reflection in degree, \( K_\lambda \) is a constant set at 0.24 (Landi et al 2000). The crystallinity was
95 %, 91 % of HAp and 93 %, 92 % of β-TCP phase in target and BCP700 respectively. Crystallinity of the target and thin film was found to be less than 5 %. There was no appreciable difference in crystallinity between the target and the thin films (Table 4.1).

Figure 4.2  XRD patterns of (a) Target (b) ASBCP (c) BCP500 and (d) BCP700

Table 4.1  Crystallite size and crystallinity values of target and BCP700 thin film

<table>
<thead>
<tr>
<th>Name of the sample</th>
<th>Average crystallite size (L) (± 1 nm)</th>
<th>HAp phase $X_c = (0.24/\beta_{002})^3$ (± 0.01)</th>
<th>β-TCP phase $X_c = (0.24/\beta_{214})^3$ (± 0.02 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target</td>
<td>50</td>
<td>0.95</td>
<td>0.93</td>
</tr>
<tr>
<td>BCP700</td>
<td>60</td>
<td>0.90</td>
<td>0.92</td>
</tr>
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</table>
4.3.2 FTIR Analysis

FTIR spectrum of target and BCP700 were as shown in Figure 4.3(a) and (b). A low intensity peak of hydroxyl stretch was observed at 3571 cm\(^{-1}\) and 633 cm\(^{-1}\) for the target. The low intensity of hydroxyl stretch (633 cm\(^{-1}\)) indicated the presence of \(\beta\)-TCP (Manjubala and Sivakumar 2001). The band at 1637 cm\(^{-1}\) corresponds to the bending mode of water. The peaks at 1093 cm\(^{-1}\) and 1051 cm\(^{-1}\) were due to the triply degenerate \(\nu_3\) asymmetric P-O stretching mode. The peak at 961 cm\(^{-1}\) was assigned to \(\nu_1\) non-degenerate P-O symmetric stretching mode. The bands at 601 and 562 cm\(^{-1}\) correspond to the triply degenerate \(\nu_4\) O-P-O bending mode and the band at 461 cm\(^{-1}\) was attributed to the doubly degenerate \(\nu_2\), O-P-O bending mode (Figure 4.3(a)).

![FTIR spectrum of (a) Target and (b) BCP700](image)

**Figure 4.3 FTIR spectrum of (a) Target and (b) BCP700**

In the case of BCP700 thin film, the hydroxyl stretch at 3571 cm\(^{-1}\) and 633 cm\(^{-1}\) peaks disappeared due to calcium phosphate layer containing \(\beta\)-TCP phase. The peaks at 600 and 570 cm\(^{-1}\) were attributed to the phosphate group in calcium phosphate layer. The peaks at 461 and 491 cm\(^{-1}\) were due to
the doubly degenerate $\nu_2$, phosphate bending mode and it was well resolved compared to the target. This might be due to the preferential orientation of plane of growth on silicon substrate (Figure 4.2(d)). The bands between 1100 to 1000 cm$^{-1}$ appear as a triplet in pure phase of HAp with peaks well resolved at 1096, 1085 and 1056 cm$^{-1}$, where as in the case of BCP bordering was observed (Manjubala and Sivakumar 2001). Similarly, the peak at 1050 cm$^{-1}$ for the BCP700, indicated the presence of triply degenerate $\nu_3$ asymmetric P-O stretching band. All the phosphate peaks of the films matched with that of the BCP target (Table 4.2). The peak at 757 cm$^{-1}$ belongs to Si-O-Si bonding of Si substrate (Figure 4.3(b)) (Pichugin et al 2008).

Table 4.2 FTIR functional group assignments of target and BCP700 thin film

<table>
<thead>
<tr>
<th>Wave numbers (cm$^{-1}$)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target</td>
<td>BCP700</td>
</tr>
<tr>
<td>3571, 633</td>
<td>-</td>
</tr>
<tr>
<td>1637</td>
<td>-</td>
</tr>
<tr>
<td>1093, 1051</td>
<td>1050</td>
</tr>
<tr>
<td>961</td>
<td>961</td>
</tr>
<tr>
<td>-</td>
<td>757</td>
</tr>
<tr>
<td>601, 562</td>
<td>600, 570</td>
</tr>
<tr>
<td>461</td>
<td>491, 461</td>
</tr>
</tbody>
</table>

**Assignments**

- Hydroxyl stretch
- Bending mode of water molecules
- P-O asymmetric stretching
- P-O symmetric stretching
- Si-O-Si bonding
- O-P-O bending modes
- O-P-O bending modes

4.3.3 SEM and EDX Analysis

Surface micrographs of ASBCP and BCP700 thin films were as shown in Figure 4.4(a) and (b). ASBCP surface exhibited non-uniform dense layer of coating along with irregularly shaped particles randomly distributed on the surface (Figure 4.4(a)). BCP700 films had cracks on the surface, due to the difference in coefficient of thermal expansion between the coating and the substrate (Figure 4.4(b)) (Hamdi and Ektessabi 2001, Lee et al 2005).
Figure 4.4 SEM micrographs of (a) ASBCP and (b) BCP700 (Insert EDX spectrum)

The elemental analysis of ASBCP and BCP700 were investigated and Ca/P ratio 1.54 and 1.62 was derived by EDX (Insert of Figure 4.4 (a) and (b)) respectively. The increase in Ca/P ratio in the case of BCP700 may be due to the evaporation of phosphate during thermal treatment (Hamdi and Ektessabi 2001). Elemental mapping analysis of the thin films revealed uniform distribution of calcium and phosphate on their surface (Figure 4.5).

Figure 4.5 Elemental mapping of BCP700 thin film (Silicon-Si, Calcium-Ca and Phosphate-P)
4.3.4 AFM Studies

The AFM images of ASBCP and BCP700 samples were as shown in Figure 4.6(a-d). ASBCP image reveal that the surface containing non-uniform nano sized spheres, were dense and rough (27±1 nm) (Figure 4.6(a) and (b)). The BCP700 exhibited smooth and uniform surface layers containing cracks. As expected the annealing seems to reduce the surface roughness (4±1 nm) (Figure 4.6(c) and (d)).

![AFM images of BCP thin film surface](image)

Figure 4.6 AFM images of BCP thin film surface of (a) ASBCP-2D (b) ASBCP-3D (c) BCP700-2D and (d) BCP700-3D

4.3.4 Wettability Studies

The wettability of a flat surface expressed by the contact angle $\theta$ between a liquid drop and solid surface due to intermolecular interactions as
described by Young’s equation (Aronov and Rosenman 2007) ie. 
\[ \cos \theta = (\gamma_{SV} - \gamma_{SL})/\gamma_{LV} \]
Where, \( \gamma_{SV} \), \( \gamma_{SL} \) and \( \gamma_{LV} \) are interfacial surface tensions of S, L and V as solid, liquid and gas respectively. Depending on the value of the contact angle, a surface is classified as hydrophobic (\( \theta > 90^\circ \)) or hydrophilic (\( \theta < 90^\circ \)). Here, the wettability characteristic of ASBCP and BCP700 thin films were analyzed by sessile drop method (Monkawa et al 2006). For both films, contact angle (\( \theta \)) for water, PBS and SBF was found to be less than 90°, revealing the hydrophilic nature of the films. The contact angle between the substrate and water (Figure 4.7(a)), PBS and SBF was measured to be 55, 29 and 30 ± 1° respectively for the ASBCP film. In the case of BCP700, the contact angle was 18, 20 and 19 ± 1° for water (Figure 4.7(b)), PBS and SBF solutions respectively. The contact angle was reduced by 68 %, 32 % and 37 % for water, PBS and SBF respectively compared to ASBCP. Wetting on the surface depends on the surface roughness, polar component of the surface energy and oxygen content of the surface (Lahann et al 2003). Here, reduction of surface roughness played an important role in enhancing the hydrophilic nature of the BCP700 thin film. In addition, AFM also confirmed the reduction in surface roughness of BCP700 (Figure 4.6(c) and (d)).

![Figure 4.7 Wettability states of (a) ASBCP and (b) BCP700](image-url)
4.3.6 In vitro Bioactivity Study

In vitro bioactivity study was carried out on the ASBCP, BCP500 and BCP700 by immersing them in SBF. The ASBCP and BCP500 films were found to dissolve on immersion in SBF. On the other hand, immersion in SBF the apatite formation was observed on the surface of BCP700 (Figure 4.8). It was suggested that the crystalline BCP layer, reduced the dissolution rate compared to other samples and assisted the apatite layer deposition.

![Figure 4.8 In vitro bioactivity tested on BCP700 (Four weeks soaked in SBF)](image)

4.3.7 Cell Viability

The cells seed on BCP700 sample showed almost same value of viable cells when compared with negative control (Figure 4.9). It was suggested that there was no toxicity due to BCP thin films coated on silicon substrate.
4.3.8 Antimicrobial Activity

Antibiotic loaded biomedical implants are used for the treatment of orthopedic disease, where it prevents infections or the inflammatory reaction after the surgical implantation. The antimicrobial activity was tested with \textit{S. aureus} (Figure 4.10).

![Figure 4.9 Cell viability on BCP700](image)

![Figure 4.10 Inhibition zone of pure and AMX loaded BCP700](image)
The diameter of inhibition zone for the AMX loaded BCP700 was 23 ± 0.5 mm against the S. aureus. There was no bacterial resistance around the native BCP700. This result indicated that the AMX loaded thin films could be used as promising bactericidal candidates for implant applications.

4.4 CONCLUSION

In this report, BCP thin films were produced on Si (001) substrate using the e-beam deposition technique. The ASBCP and BCP500 were found to be amorphous. The post annealing (> 500 °C) produced crystalline BCP films. The ASBCP and BCP500 coating dissolved within a week when immersed in simulated body fluid (SBF). The surface roughness and dissolution of the calcium phosphate layer was decreased by thermal treatment process. The crystalline BCP700 showed better bioactivity. BCP700 enhanced hydrophilic nature of the surface helps the cell attachment on the biomedical implants. No toxicity was observed on BCP deposited on silicon substrate. AMX loaded BCP film showed very good bacterial resistance against S. aureus. Here, we conclude that the electron beam evaporation method is a suitable and cost effective technique for the preparation of BCP films. Biphasic calcium phosphate thin film having resorbable β-TCP and non-resorbable HAp phases revealed enhanced bioactivity. It is a promising material for bone tissue regeneration and repair which is difficult to achieve as a single phase on the metal substrate.