Physical and Mechanical Characterization of Crystalline Pure β-Tri Calcium Phosphate & Its Dopants as Bone Substitutes

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Abstract. β-Tri calcium phosphate ceramics play a significant role in several biomedical application for their marked resorbability and bioactivity. One of them is in bone grafting, where it is used for treating bone defects caused by wounds or osteoporosis. In the present work an in-depth and systematic study of pure and different doped variants (Zinc, Magnesium and Titanium) of β-Tri calcium phosphate was done. We have prepared pure β-Tri calcium phosphate and its different dopants of different strength. All together seven different composition were studied. We have tried to investigate some important features which are the prerequisite for their application. These include lattice parameter study, mechanical properties, contacts behaviour with SBF, haemolytic characteristics, and its cytotoxic nature. The capability of new apatite development on the surface of pure and doped β-TCP samples were also considered and compared by using Simulated Body Fluid (SBF) to observe their interaction with human body fluid.

1. Introduction

Calcium phosphate ceramics correspond to a wide class of materials with a variety of chemical composition and structures. These material have been successfully applied in many fields of science and technology. Numerous formulations have been developed, and some of them like Tri Calcium Phosphates [TCP, Ca₃(PO₄)₂, Hydroxyapatite [HAP, Ca₁₀(PO₄)₆(OH)₂]], and their mixtures have proven to be very competent for various applications [1–3]. They are getting more attention due to their high biocompatibility and bioactivity.

The most important quality of these materials is that they can make direct chemical bond with bone tissues [4] and this interaction is related with and ruled by the physical and chemical properties of the materials [5]. Both HAP and β-TCP are widely used as bone replacement material. HAP is bioactive and non-biodegradable bone substitute material along with outstanding mechanical properties. Whereas, β-TCP is resorbable and bioresorption occurs in the course of osteoclastic activity [6]. Though, β-TCP ceramic is very favourable bone replacement material, but its weak mechanical properties and very rapid resorption rate is some unfavourable factors. To overcome these problems we think of some of its dopants with zinc, magnesium and titanium. Zinc is found in all human tissues and bones as a trace element and it is closely related to the bone metabolism [7]. Studies also confirm that zinc can encourage osteoblasts cell proliferation and differentiation as well as bone development. Similarly Magnesium is also crucial for numerous biochemical reactions in the body. It helps to maintain usual nerve and muscle function, and helps bones remain strong.

Titanium is good for osseointegration, they can form chemical and physical bonds with bone cells.
It does not form the unwanted fibrous tissue. Considering their (Zinc, Magnesium and Titanium) important role, β-TCP ceramic containing zinc, magnesium and titanium were developed in the present work. The main objective of this study is to compare the properties of its doped variants.

2. Experimental procedure

2.1. Preparation of sample
A.R. grade Calcium Carbonate [CaCO$_3$] and orthophosphoric acid [H$_3$PO$_4$] were used to prepare pure β-TCP powder and its three doped variants (Zn, Mg, and Ti) by a wet chemical precipitation method. Both reactants were procured from MERCK, INDIA Ltd., India. The procedure was done as elucidated by S. K. Samanta and A. Chanda [8]. Measured quantities of zinc oxide, magnesium chloride and titanium oxide were incorporated into the Calcium carbonate before its addition to H$_3$PO$_4$ solution, to synthesize doped β-TCP powder (Zinc, Magnesium and Titanium doping). In this work, 3 wt. % and 5 wt. % substitution of calcium carbonate were used.

2.2. Preparation of Pellets
The powder form pure and doped crystalline β-TCP materials were hard-pressed [8] uniaxially to form circular pellets. These (green ceramic pellets) were then sintered in a furnace at 1100 °C for 2 hrs. at a continual heating rate of 5°C/minute.

2.3. Characterization.
2.3.1. Lattice parameter study: The lattice parameter of the β-TCP and its doped variants were studied by using XRD. Described by Dickens et al. [9, 10], the beta-TCP crystallizes in the rhombohedral space group R3c with unit-cell parameters, where $a = 10.439$ Å and $c=37.375$ Å. There are always complexities of determining the lattice parameters $a$, and $c$ of the rhombohedral unit cell. But the measurements of the rhombohedral cell can be determined from the dimensions of the hexagonal cell, and this is an easier process than solving the rather complicated plane-spacing equation for the rhombohedral system [11]. In this study the unit cell parameters of all the variants are calculated in hexagonal set up.

2.3.2. Mechanical properties. Vickers Hardness Testing. The indenter was pitched into the sample by an accurately measured test force. The force was preserved for a precise stay time of 30 seconds. After the reside time was completed, the indenter was detached leaving a mark in the sample that looks square formed on the surface. The size of the indent was calculated optically by determining the two diagonals of the tetragonal indent. The average hardness of each of these composition was calculated as a function of an indentation load 300mN [12].

2.3.3. Contact angle study. This is the angle, predictably measured through the liquid, where a liquid–vapour edge meets a solid exterior. This angle is a guidance of the wettability of solid surface. A lesser contact angle means that the material is having water loving and high-energy surface characteristics. But an upper contact angle means that the material possesses water repellent and low-energy surface properties. This study was done by using contact angle analyser Phoenix 300.

2.3.4. Haemolysis study. A hemocompatibility profile study of all the developed samples were done by following the ASTM guidelines [13]. Three pellets of individual sample as test material (3 gm around), were taken. The sharp edges are smoothened by rubbing on soft muslin cloth. The average result is expressed in Table no.3.

2.3.5. Bactericidal study. Nutrient agar media was mixed with distilled water in a conical flask. This media was then autoclaved at 121°C for 20 mints to make it sterile. Once this media was cooled down to 40-45°C, the inoculated culture of Staphylococcus aureus was mixed with it and distributed to petri dishes (about 22 to 25 ml in each petri dish) and wait for some times to solidify the media. Then the porcelain bit were dripped into the sample under testing. Due to capillary action the sample will
come into the bit and they were placed on the Petri dish. Two bits for two types of concentration is taken. In one bit the higher concentration of 2 mg/10ml and in another the lower concentration of 1 mg/10ml were taken and placed on the Petri dish. After complete the sampling all the Petri dishes were kept into the incubator at 30-35°C for 24 hour.

2.3.6. MTT Assay. Peripheral blood mono nuclear cells (PBMC) cells were distributed on 48 well plates for 1 day. Then they were treated with β - tri calcium phosphate and its dopants and incubated for 24 hours. After that MTT was added to each well plate, shaken for 15 minutes and then incubated for four hours. Addition of MTT kill the cells and crystals were formed. Then the media was removed and DMSO was added to dissolve the crystals and a purple coloured solution was formed. The intensity of the colour depends on the amount of crystal formed. When cells died, OD was taken at 545 nm against a blank. The experiment was performed in duplicate and the mean data was recorded.

2.3.7. SBF study. Simulated body fluid (SBF) was prepared according to Kokubo’s method [14]. SBF was stored at 37 °C in an incubator and used within 2 days. The pH was checked and adjusted to 7.4 before use. Three pellets from each category were taken and immersed in the SBF solution. Every three days the SBF is discarded and fresh SBF is added to promote the apatite growth. The weight of the sample was noted from the day one. The optical density of the discarded SBF was also noted to get an idea about the dissolution of the pellets. The whole observation was done for 30 days.

3. Results and Discussion

3.1. Lattice parameter study: The XRD of different composition are shown below, Figure 1. The major peak was found at 31.025 which is well matched with the standard JCPDS card no 09-0169.
The lattice parameter (a-axis and c-axis) of the different composition are presented at Table 1.

**Table 1.** Lattice parameters of β-TCP & its dopants

| Sl no | Composition               | a axis (Å) | c axis (Å) |
|-------|---------------------------|------------|------------|
| 1     | Pure β-TCP                | 10.48      | 37.38      |
| 2     | 3% ZnO doped β-TCP        | 10.41      | 36.25      |
| 3     | 3% MgCl₂ doped β-TCP      | 10.42      | 37.17      |
| 4     | 3% TiO₂ doped β-TCP       | 10.45      | 36.97      |

The values of a and c axis are decreased in the case of all doped material. This may be due to the atomic radius of calcium (197 pm) which is higher than Zinc (134 pm), Magnesium (160 pm), and Titanium (147 pm). These results confirmed the substitution of calcium ion by zinc, magnesium and titanium.

**3.2. Mechanical properties**

Ideally, the mechanical properties have to be as similar as possible to the surrounding tissue avoiding possible implants failures because of stress forces in the material-tissue inter-face. Similar to other ceramics, CPCs are brittle, which is attributed to high strength ionic bonds [15]. Due to their high brittleness [16], these are focused on non-load-bearing application. The result of Mechanical Properties of β-TCP and its dopants sintered at 1100°C, applied load 300 mN is shown below, Table 2.

**Table 2.** Average data of Hardness

| Sl no | Composition               | Hardness (GPa) |
|-------|---------------------------|----------------|
| 1     | Pure β-TCP                | 0.652±0.04     |
| 2     | 3% ZnO doped β-TCP        | 0.693±0.02     |
| 3     | 5% ZnO doped β-TCP        | 0.704±0.03     |
| 4     | 3% MgCl₂ doped β-TCP      | 0.681±0.07     |
| 5     | 5% MgCl₂ doped β-TCP      | 0.687±0.09     |
| 6     | 3% TiO₂ doped β-TCP       | 0.665±0.04     |
| 7     | 5% TiO₂ doped β-TCP       | 0.672±0.08     |

Our study revealed that the hardness value of β-TCP can be improved by incorporation of dopants to some extent. This may be due to the densification of the ceramic matrix. But there is no proportional relation between the dopant amount and hardness.

**3.3. Contact angle study**

The observation by using contact angle analyser Phoenix 300 using glycerine as probe material showed that all the specimen are having a contact angle in between 40±4 °. Glycerine is hydrophilic in nature. This means that all the samples are truly hydrophilic with SBF. The result is expressed in the following table no 3.

**Table 3.** Average data of contact angle measurement

| Sl no | Composition               | CA(M) [deg.] | CA(L) [deg.] | CA(R) [deg.] |
|-------|---------------------------|--------------|--------------|--------------|
| 1     | Pure β-TCP                | 42.40        | 43.10        | 41.70        |
| 2     | 3% ZnO doped β-TCP        | 44.92        | 45.71        | 44.13        |
| 3     | 5% ZnO doped β-TCP        | 43.81        | 42.55        | 45.08        |
| 4     | 3% MgCl₂ doped β-TCP      | 40.28        | 39.47        | 41.09        |
| 5     | 5% MgCl₂ doped β-TCP      | 38.80        | 38.32        | 39.28        |
| 6     | 3% TiO₂ doped β-TCP       | 43.13        | 42.79        | 43.47        |
| 7     | 5% TiO₂ doped β-TCP       | 43.99        | 43.80        | 44.18        |

Here, CA (M) - Contact Angle Mean (in degree); CA (L) - Contact Angle (Left) (in degree); CA(R) - Contact Angle (Right). As they are highly hydrophilic in nature, body fluid will easily adhere to them.
3.4. Haemolysis study

Hemocompatibility is an important parameter to decide for an implant. In our study we have followed ASTM Standards to perform hemocompatibility test. For each test material four pellets were taken and their average OD is taken for the calculation.

The percentage of hemolysis was determined from the following equation:

\[
\% \text{hemolysis} = \left( \frac{O.D.(\text{Test}) - O.D.(\text{Neg.})}{O.D.(\text{Positive}) - O.D.(\text{Neg.})} \right) \times 100
\]

The hemolysis analysis data is presented in the following Table no.4

**Table 4.** Data for hemolysis analysis.

| SL no | Composition          | Wave length (nm) | Avg. O.D. of Test sample | O.D. (+) ve control | O.D. (-) ve control | % Haemolysis |
|-------|---------------------|------------------|--------------------------|---------------------|---------------------|--------------|
| 1     | Pure β-TCP          |                  | 0.071                    |                     |                     | 2.50         |
| 2     | 3% ZnO doped β-TCP  |                  | 0.074                    |                     |                     | 3.25         |
| 3     | 5% ZnO doped β-TCP  |                  | 0.075                    |                     |                     | 3.50         |
| 4     | 3% MgCl₂ doped β-TCP| 545              | 0.078                    | 0.46                | 0.061               | 4.26         |
| 5     | 5% MgCl₂ doped β-TCP|                  | 0.075                    |                     |                     | 3.50         |
| 6     | 3% TiO₂ doped β-TCP |                  | 0.072                    |                     |                     | 2.75         |
| 7     | 5% TiO₂ doped β-TCP |                  | 0.073                    |                     |                     | 3.00         |

These results show that all the compositions are highly hemocompatible (as show less than 5% hemolysis. Due to their high hemocompatibility they can be undoubtedly used in blood interface.

3.5. Bactericidal study: Antimicrobial activities of any material are understood by its degree of growth inhibition of microorganisms. We observed no zone of inhibition in either concentration in any petri dish after 24 hour. This suggest that in this concentration (1mg/10ml & 2 mg/10 ml) TCP and its dopants possesses no antibacterial property. The pictures of the studied Petri dish are shown in the figure 2.

![Figure 2a. β-TCP.](image1)
![Figure 2b. 5% Zn-TCP.](image2)
![Figure 2c. 3% Zn-TCP.](image3)
![Figure 2d. 5% Mg-TCP.](image4)
![Figure 2e. 3% Mg-TCP.](image5)
![Figure 2f. 5% Ti-TCP.](image6)
![Figure 2g. 3% Ti-TCP.](image7)

Red and yellow arrow indicate the porcelain bit of having 2mg/10 ml & 1 mg/10ml of composition respectively.
3.6. MTT Assay. The % cell viability is calculated by using the following formulae.

\[
\text{% cell viability} = \left[ \frac{\text{Sample OD}}{\text{Control OD}} \right] \times 100
\]

The average of % cell Viability as recorded are expressed in the below block diagram with standard deviation.

![Figure 3. % cell viability as observed in MTT assay.](image)

This assay result clearly reveals that there is no composition which yield a poor result. All the samples are showing more than 80% cell viability. This conclude that all these samples are not cytotoxic in nature.

3.7. SBF study

3.7.1. SEM images. Scanning Electron microscopy images show formation of apatite layer on each pellet of different grade after 30 days. We observed on immersion in the SBF solution, the porous apatite layers formed after 7 days. The samples started to form these porous layers after 7 days. The SEM images (shown below) after 30 days of immersion for all samples showed highly dense apatite layers. The size and thickness of calcium phosphate layers increased with increasing SBF immersion time.

![Figure 4a. TCP.](image)

![Figure 4b. 5% Zn-TCP.](image)

![Figure 4c. 3% Zn-TCP.](image)

![Figure 4d. 5% Mg-TCP.](image)

![Figure 4e. 3% Mg-TCP.](image)

![Figure 4f. 5% Ti-TCP.](image)

![Figure 4g. 3% Ti-TCP.](image)

The red arrow shows the zone of apatite layer.
The Ti doped samples showed maximum apatite formation. This may be due to the high dielectric constant of its oxide which is highly favorable to SBF [17]. The properties of a material having a high dielectric constant play an important role in determining response with SBF (like adhesion). Titanium oxide becomes heterogeneous and polarized as a function of exposure time to SBF environments. This leads to the increased adsorption of hydroxyl groups and others over time.

3.7.2. Weight degradation. Due to dissolution in the SBF the pellets were gradually losing its weight. We observed a study for 30 days. In each three alternate day we took out the sample from the SBF and gently dry them in air and weighed. The % of weight degradation is expressed in the below figure no.5. We noticed a rapid loosening of weight in the beginning days up to days 12-15, after that the grade of the dissolution becoming slow. The slow degradation of the pellets may be due to the onset of apatite layer on the pellets.

![Figure 5](image_url)  
*Figure 5. Plot of % weight degradation in SBF with days.*

3.7.3. %Transmittance.

The following bar diagram shows the %transmittance of SBF fluid in different days.

![Figure 6](image_url)  
*Figure 6. Plot of % transmittance of β-TCP and its dopants of SBF solution.*

The dissolution of the samples in the SBF were fast at the beginning and gradually became slow with time, which is also confirmed by the % weight degradation of pellets in the SBF. Up to 15 days there was a gradual and maximum change for all samples. After this the transmittance became almost stable. This also confirms that as the apatite layer is in the formation process it stops the leaching out of the materials in to the solution as days go.
4. Conclusion

The results clearly revealed that there is little bit change of hardness due the addition of dopants. But there is no direct proportionate relation between the hardness and dopants amount. The lattice parameters show there is a direct shortening of length of the axis due to the reduction of atomic radius in different composition. Contact angle of all sample clearly indicate that all the samples are truly hydrophilic. All the samples are hemocompatible as well as they are all nontoxic. SBF study indicates that the titanium doped variant is better than other varieties as it gives maximum apatite layer formation.

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