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Design and structural investigations of Yb^{3+} substituted β-$Ca_3(PO_4)_2$ contrast agents for bimodal NIR luminescence and X-ray CT imaging

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ABSTRACT

The quest for the development of bone substitutes with contrast agents for diagnostic imaging subsists to distinguish synthetic bone from native human tissue. To this aim, ytterbium (Yb^{3+}) substitutions in β-tricalcium phosphate (β-$Ca_3(PO_4)_2$, β-TCP) as contrast agents has been developed to differentiate implant materials thereby, facilitating as host for bimodal imaging application by means of NIR luminescence and X-ray computed tomography techniques. A facile aqueous chemical precipitation route with the aid of surfactant is used for the synthesis of Yb^{3+} substitutions in β-$Ca_3(PO_4)_2$. The characterization results affirms the ability of β-$Ca_3(PO_4)_2$ to host 4.36 mol% of Yb^{3+} while the excess Yb^{3+} crystallizes as $YbPO_4$. The structure refinement results favour the occupancy of Yb^{3+} at the Ca^{2+}(5) site of β-$Ca_3(PO_4)_2$. The absorption and photoluminescence spectra in the near infrared region with emission intensity ~1024 nm in the second biological window correspond to $^2F_{5/2} \rightarrow {}^2F_{7/2}$ transitions of Yb^{3+}. The designed Yb^{3+} substituted β-$Ca_3(PO_4)_2$ does not exhibit any toxicity in human osteosarcoma cell lines and delivers an excellent *in vitro* CT contrast ability allied by the enhanced signal intensity and high X-ray absorption coefficient.

1. Introduction

Globally, there is an exceeding demand to design functional bone fillers for applications in bone repair and tissue regeneration. Calcium phosphate cements are the most commonly used bone fillers that aids osteoinductivity and bone regeneration due to their close proximity with natural bone in terms of structure and composition [1, 2]. Due to the compositional similarities, it is thus a demanding task to differentiate the implant material from the natural bone tissue, even with the aid of clinical imaging technologies. In this context, the present work aims to develop a bimodal contrast agent, perceptible with both NIR luminescence and X-ray computed tomography (CT) as enhancers of calcium phosphate bone fillers.

β-Tricalcium phosphate [β-$Ca_3(PO_4)_2$, β-TCP] is a well-recognized potential bone filler owing to their salient features of biocompatibility, osteoinductivity and non-toxic characteristics. Recent reports emphasize the incorporation of cationic species (Na^+, Mg^{2+}, Sr^{2+}, Zn^{2+}) in tuning synthetic apatites for various biomedical applications with enhanced physical, chemical and physiological properties of the calcified tissues [3–7]. Ytterbium (Yb^{3+}) is of substantial interest as it offers the combination of near-infrared luminescence and CT contrast imaging. The high K-edge energy promotes Yb^{3+} to induce enhanced X-ray attenuation thereby obtaining excellent image quality at a minimal dose of contrast. Moreover, Yb^{3+} is among the most abundant rare earth element in the earth's crust and has a potential for commercial production of clinical contrast agents [8]. The presence of Yb^{3+} is ~3 mg/kg and it is recovered commercially from monazite sand that constitute 0.03% ytterbium and from other minerals like gadolinite and xenotime [9]. The five different Ca^{2+} sites of rhombohedral β-$Ca_3(PO_4)_2$ offers a wide range of cationic substitutions inclusive of lanthanides. Recent literature reports site occupancy and photoluminescence properties of Eu^{3+} in β-$Ca_3(PO_4)_2$ [10]. Yb^{3+}/Ho^{3+} co-doped apatites with stable upconversion luminescence and bone-bonding capacity were investigated to study material-tissue interaction [11]. The upconversion luminescence is reported by Orlova et al. in β-$Ca_3(PO_4)_2$ system with co-doping of Yb^{3+} and Er^{3+} aiming at its use in biosystems [12]. Herein, the lone substitution of Yb^{3+} in β-$Ca_3(PO_4)_2$ is expected to impart NIR luminescence and X-ray CT imaging features to the system, wherein a single moiety could be used as prospective candidate for bimodal imaging applications. The previous report of the authors also demonstrate the site occupancies of trivalent lanthanides in β-$Ca_3(PO_4)_2$ for various bio applications [13–16].

The present study is an attempt to synthesize a series of Yb^{3+} substitutions in β-$Ca_3(PO_4)_2$. The major focus is laid on the detailed structural elucidation of β-$Ca_3(PO_4)_2$ influenced by Yb^{3+} additions. Further, UV–Vis-NIR absorption, NIR luminescence, morphological

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Table 1
Elemental and phase fractions determined from ICP-AES and Rietveld refinement respectively, at 900 °C.

Sample code	Elemental fractions from ICP-AES		Phase fractions from Refinement	
	Ca^{2+} (mol.%)	Yb^{3+} (mol.%)	β-$Ca_3(PO_4)_2$ (wt %)	$YbPO_4$ (wt %)
Pure-TCP	50.14	–	100	–
1YbCP	47.84	2.17	100	–
*2*YbCP	45.63	4.36	100	–
*3*YbCP	43.45	6.55	92.10	7.90
*4*YbCP	41.31	8.70	85.30	14.70

features and the feasibility of Yb^{3+} doped β-$Ca_3(PO_4)_2$ as a CT contrast agent is evaluated *in vitro*.

2. Materials and methods

2.1. Synthesis of Yb^{3+} substitutions in β-$Ca_3(PO_4)_2$

In this study, assorted level of Yb^{3+} substitutions in β-$Ca_3(PO_4)_2$ were attempted through co-precipitation technique. $Ca(NO_3)_2.4H_2O$, $Yb(NO_3)_3$, $(NH_4)_2HPO_4$ and cetyltetramethyl ammonium bromide (CTAB) were used as precursors. All the compositions were synthesized at the nominal ratio of $(Ca^{2+}+Yb^{3+})/P$ at 1.5. The brief description of the synthesis is explained as follows. $(NH_4)_2HPO_4$ solution was slowly introduced into the solution of cationic mixtures comprising Ca^{2+} + Yb^{3+} under constant stirring conditions at 120 °C to obtain four different concentrations. Sample codes are mentioned in Table 1 and the same will be used throughout the manuscript. This was followed by the subsequent addition of aqueous NH_3 to raise the pH to 8 and this result to yield a white precipitate. Apparently, 2.5% CTAB that corresponds to the concentration of the $(Ca^{2+} + Yb^{3+})$ was added to the reaction mixture to improve the dispersion properties of the synthesized powders. The resultant mixture was allowed to stir continuously for the next 2 h. The obtained powder samples were heat treated at predetermined temperatures and utilized for characteristic studies.

2.2. Material characterization

The heat treatment of the powder compositions were performed at selective temperatures at a dwell time of 2 h by using high temperature muffle furnace and used for material characterization. XRD measurements were performed using a powder X-ray diffractometer (RIGAKU, ULTIMA IV, JAPAN) by using CuKα radiation (λ = 1.5406 Å) at 40 kV and 30 mA in the range of 5–90° with a step rate of 0.01° 2θ per second. Standard International Centre for Diffraction Data (ICDD) card Nos. of 00-009-0169 for β-$Ca_3(PO_4)_2$ and 00-045-0530 for $YbPO_4$ were used to index the XRD reflections. The quantitative analysis of diffraction patterns were performed by the Rietveld method and the crystal structure was refined with the GSAS-EXPGUI software in analogy with the previous description by the authors [13, 14]. The standard crystallographic data for the refinement of β-$Ca_3(PO_4)_2$ and $YbPO_4$ were respectively obtained from Yashima et al. [17] and Herrmann et al. [18]. Raman spectra was acquired from Raman spectrometer (Renishaw, United Kingdom) over the range between 100 and 1100 cm^{-1} using 785 nm laser with data acquisition time of 30 s and 0.5% of power. The morphology and size of the samples were analysed by Scanning electron microscope (FEI QUANTA, FEG 200) and the lattice fringes and SAED patterns were determined using high resolution Transmission electron microscope (JEOL TEM-2100). The absorption spectra were recorded with UV-VIS-NIR spectrometer (SHIMADZU 3600) in the range of 900–1000 nm. The emission spectra in the NIR range were accounted with a Spectrofluorometer (FLUOROLOG - FL3-11) equipped with Xenon lamp source. The emission spectra of Yb^{3+} substitutions in β-$Ca_3(PO_4)_2$ were recorded in the wavelength region 950–1100 nm upon excitation at 950 nm with slit width of 5 nm.

2.3. In vitro cytotoxicity studies

A characteristic MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay was carried out to appraise the cytotoxicity of Yb^{3+} doped β-$Ca_3(PO_4)$ on human osteosarcoma, MG-63 cell lines procured from the National Centre for Cells Science (NCCS), Pune, India. Cells were cultured in Dulbecco's modified Eagle's medium with standard culture conditions at 37 °C in 5% CO_2, supplemented with 10% fetal calf serum. The procedure is as follows: 10^4 cells were seeded in 96 well plates as triplicate at the different concentrations of 10, 50,100 and 200 μg and then incubated for 96 h at 37 °C. All *in vitro* experiments were compared with positive control, MG-132 (Sigma-Aldrich) drug, at a concentration of 0.48 μg. The assay was performed in accordance with EZcount™ MTT Cell Assay Kit. (Himedia, Cat. No. CCK003) and the absorbance were measured on ELISA reader [19].

2.4. Hounsfield units (HU) value measurement and CT imaging in vitro

To execute CT phantom test, various concentrations of Yb^{3+} doped β-$Ca_3(PO_4)$ and Omnipaque™ in the range of 0 to 10 mg/mL were dispersed in 1.5 mL Eppendorf tubes that contains phosphate buffer saline (PBS) buffer. A multi slice spiral CT system (GE HISPEED CT/e) was employed to generate CT phantom images. The experiments were performed with scanning parameters of 120 kVp and 160 mA. A field of view (FOV) of 54.07 mm × 146.00 mm and thickness of 0.9 mm was set at an exposure time of 800 ms/rotation. The attenuation values (HU) were measured and the images were resolved by kodak molecular imaging software. HU value of the material with an X-ray attenuation coefficient μ is calculated by the Eq. (1): [20].

$$HU = \frac{\mu - \mu_{water}}{\mu_{water}} \times 1000 \tag{1}$$

where, μ_{water} = X-ray attenuation coefficient of water.

3. Results

3.1. Structural investigations of Yb^{3+} substituted β-$Ca_3(PO_4)_2$

The previous reports by the authors emphasize the crystallization of β-$Ca_3(PO_4)_2$ at 900 °C with possibility to accommodate rare earth elements at its lattice [21]. In accordance with the same, all the Yb^{3+} doped calcium phosphate powders ensure the crystallization of β-$Ca_3(PO_4)_2$ at 900 °C. The powder XRD patterns of all the Yb^{3+} doped calcium phosphate powders after heat treatment at 900 °C (Fig. 1) yields highly crystalline β-$Ca_3(PO_4)$ that exhibit good matches with its respective ICDD standard. The compositions with Yb^{3+} substitution beyond 4.36 mol% unveils the crystallization of $YbPO_4$ as an additional product and its respective reflections enunciates more prominence in *4*YbCP. The results from the elemental analysis (Table 1) also affirms the progressive presence of Yb^{3+} as a function its enhanced additions during the synthesis.

The quantitative effect of Yb^{3+} influence on the structure of β-$Ca_3(PO_4)_2$ is determined through Rietveld refinement. The refinement results are graphically illustrated in Fig. 2. The refinement results affirms the attainment of discrete β-$Ca_3(PO_4)_2$ for *1*YbCP and *2*YbCP devoid of any secondary products, while *3*YbCP and *4*YbCP ensures the presence of $YbPO_4$ as additional product. The phase fractions derived from refinement also indicates the presence of $YbPO_4$ in an enhanced trend in the order of *3*YbCP and *4*YbCP (Table 1). The refinement results insists the respective crystallization of β-$Ca_3(PO_4)_2$ and $YbPO_4$ in rhombohedral (*R3c* space setting) and tetragonal (*I41* space setting). Rietveld agreement factors and the goodness of fit values indexed in