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Original Research Paper

Development of cerium and silicon co-doped hydroxyapatite nanopowder and its *in vitro* biological studies for bone regeneration applications

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ABSTRACT

Multi-ion, co-substituted bioactive glass ceramics play a significant role in the stimulation of physical and biological properties for outstanding effects in biomedical application. The following work attempts to develop HAP as a parent material doped with a combination of cerium (Ce^{4+} @1.25 wt%) and silicon (Si^{4+} @1, 3 and 5 wt%) by refluxing based sol-gel technique. The anti-bacterial tests exhibit *E. coli* showing higher inhibition efficiency, *in vitro* hemolytic test exhibit good compatible nature of dual doped HAP with erythrocytes (<5% of hemolytic). *In vitro* bioactivity assay confirms that the developed dual doped HAP possesses excellent bone-like apatite layer formation on their surfaces. *In vitro* cellular study was performed for Ce/Si-HAP@5% powder against MG-63 cells, which demonstrated the good cell viability at higher concentrations (up to 800 μg/ml). Further, dual doped HAP powders were characterized by various analytical techniques such as ATR-FTIR, Powder-XRD, TGA-DTA, SEM-EDS, TEM and XPS analysis. The studies confirm that the synthesized dual doped HAP will act as better bioactive glass ceramics for potential orthopedic and dentistry applications.

1. Introduction

Natural bone is a nanocomposite material with the combination of inorganic apatite and organic collagen which is embedded into the extracellular matrix (ECM) for rigid structural maintenance of bone [1]. The biological apatite consists of the major amount of calcium phosphate and a minor amount of anionic, cationic metal ions such as Cl^{-}, F^{-}, SiO_4^{4-}, CO_3^{2-}, Mg^{2+}, Na^{+}, Al^{3+}, K^{+}, Zn^{2+}, and Sr^{2+}, etc. respectively [2,3]. Nowadays, synthetic hydroxyapatite [HAP: $Ca_{10}(PO_4)_6(OH)_2$] is widely used as an alternative bone graft in various clinical surgeries due to its physical and chemical structural similarities with natural apatite mineral [4,5]. HAP has extraordinary properties such as biocompatibility, non-toxicity, osteoinduction, osteogenesis, osteointegrity and direct active chemical bond formation with hard tissues [6]. However, pure phase synthetic HAP exhibits lower biomineralization when compared with the other bioactive glass ceramics due to rather minimal bioresorption properties, lack of microbial restriction behaviour and lower fracture toughness with higher modulus strength [7,8]. These challenges can be overcome by the doping of bioactive metal ions in pure HAP crystal structure to improve the biological and mechanical properties for outstanding clinical applications [9]. In HAP crystal lattice, Ca^{2+} ions can be substituted or doped by various biofunctional metal ions such as Ag^{+}, Cu^{2+}, Zn^{2+}, Ti^{4+}, Al^{3+}, Mg^{2+}, Na^{+}, Co^{2+}, $Fe^{2/3+}$, Si^{4+}, Sr^{2+} and $Ce^{3+/4+}$, etc. [10,11]. However, these ions lead to changes in the physical properties of pure HAP, in terms of solubility, thermal stability, surface charge with surface area, the degree of crystallinity and slight modification of lattice parameters [12,13]. In recent years, many researchers have been focusing on the development of a customized approach to tailor the characteristics of synthetic bioceramics with the doping of dual bioactive metal ions. Hence, these metal ions play a crucial role in the enhancement of biological properties of pure HAP for improved clinical applications [14].

For the past few decades, pathogen restriction behaviour of pure HAP has been created by the addition of transition and rare-earth elements [15,16]. Nevertheless, among these elements, Cerium (Ce) was selected to include into the crystal lattice of HAP due to its similar nature as Calcium ions and also accumulation in a natural bone at the lower level of concentrations [17,18]. Generally, Ce has been widely used as an antibacterial agent for long-term medical applications owing to its thermal stability, low cost and excellent antimicrobial efficacy [19]. The

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ceramic materials with Ce ions were tremendously used in cancer treatments, Alzheimer diseases, drug delivery, dental fillings, catheters, and burn wound healings [20]. It is well known that Ce exists as +3 and +4 oxidation states because of its partial occupancy of 4f and 5d subshells of electrons and lead to excitation state by a change in the chemical environment [21]. Yingguang et al. have evaluated the antibacterial activity of Ce^{3+} substituted HAP using *S. aureus, E. coli,* and *L. casei* pathogens and found the excellent inhibition efficiency by the release of Ce^{3+} ions [22]. Ciobanu et al. have synthesized Ce^{4+} substituted HAP using co-precipitation method and examined its antibacterial activity against *E. coli* and *S. aureus* [23]. Gopi et al. have developed the Ce^{3+}/Eu^{3+} dual substituted HAP coatings on 316L SS by electrodeposition and studied the antibacterial activity, cytotoxicity, and anti-corrosion property. These studies have proved that coated implants play a vital role in biomedical applications [24]. Sanyal et al. employed the sol-gel technique to synthesize Zr^{4+}/Ce^{3+} co substituted HAP and fluorohydroxyapatite also evaluated its structural formation and bacterial restriction behaviour for clinical applications [25]. Gopi et al. prepared Sr^{2+}/Ce^{3+} co substituted HAP by microwave irradiation technique and confirmed the enhancement of bioactivity and antibacterial activity for better tissue engineering applications [26].

Silicon (Si) is the important trace element that plays a crucial role in the osteoblast differentiation by increasing metabolic activity of human osteoblast cells and is also involved in the development of connective tissues with the production of collagen type I [27,28]. Si has the ability to enhance the bioactivity of biological apatite and accelerates the bio-mineralization of natural bone and cartilage to repair the injuries in human bone [29]. Incorporation of Si ions into HAP crystal lattice improves the physicochemical properties of HAP with enhancement of the proteins-cell interactions to improve the bone regeneration and osteointegration properties [30]. It has been reported that Si-HAP coatings increase the biological activity and human osteoblast cell adhesion [31]. Gibson et al. synthesized Si-substituted HAP and examined its cellular response which demonstrated the prominent activity of osteoblast cells on Si-HAP surface [32]. Bang et al. employed the precipitation method to prepare the Si-HAP with different concentrations such as 0.4, 0.8 and 1.6 wt% and found slight changes in the crystal structure of HAP by incorporation of Si in terms of increase in the lattice parameters with reduction of grain size [33]. Friederichs et al. have synthesized Zn and SiO_4^{4-} co substituted HAP using wet precipitation method and performed the experimental chemistry, atomic remodeling studies to investigate the presence of Zn and Si atoms [34]. Jianyong et al. have developed the individual and combination of Si and Sr co-doped HAP by the adoption of the hydrothermal method and found to increase in the biological activity of osteoblast cells when compared with the undoped HAP [35]. Kim et al. reported Si and Mg co-substituted HAP using the precipitation method and observed as single phase HAP formation with improved biocompatibility behaviour for implantation and bone augmentation applications [36]. Zhang et al. studied the preparation of Si and Sr co-substituted HAP nanowires using Sr containing calcium silicate and trisodium phosphate sources by hydrothermal treatment and proposed them as multifunctional bioactive ceramics towards regeneration of hard tissue applications [37]. Hence, these findings suggest that dual ion incorporated HAP can act as a promising biomaterial for biomedical applications.

Therefore, in the present investigation, we have attempted to synthesize 1.25% of Ce along with 1, 3, and 5% of Si co-doped HAP by sol-gel method. The Ce and Si co-doped HAP was characterized by ATR-FTIR, XRD, TGA/DTA, SEM-EDS, HR-TEM and XPS analysis. In addition to this, we have performed the *in vitro* biological studies such as antibacterial activity, hemocompatibility, bioactivity and cytocompatibility to prove them as the suitable bioactive glass material for bone and dental applications.

2. Materials and method

Ceric (IV) ammonium nitrate [$(NH_4)_2$ Ce $(NO_3)_6$-SDFCL 98.5%], Calcium nitrate tetrahydrate [$Ca(NO_3)_2{\cdot}4H_2O$-SDFCL 99%], Triethyl phosphite [$P(OC_2H_5)_3$-Sigma Aldrich 98%], Tetraethyl orthosilicate ($Si(OC_2H_5)_4$ Sigma Aldrich 98%), Double distilled water [DD·H_2O], Phosphate buffer solution (PBS) and Dimethyl sulfoxide (DMSO).

2.1. Synthesis of Ce and Si-doped HAP

Based on the previous report, we found that 1.25% of Ce doped HAP exhibits excellent antibacterial activity, hemocompatibility and cytocompatibility properties [38]. Hence, the following work attempts to synthesize 1.25% of Ce doped HAP co-substituted with Silica using different concentrations such as 1, 3 and 5% by sol-gel method via refluxing process. Firstly, we have maintained the Ca (0.9875 M) to Ce (0.0125 M) ratio as "1 M" and mixed these sources in DD H_2O to obtain the clear solution. On the hand, the ratios of P to Si were maintained as "0.6 M" and precursors were dissolved in DD H_2O. The mixture of Ca/Ce solution was added dropwise to the P/Si solution and stirred for 1 h followed by aging at room temperature for 24 h. Further, the aged solution was refluxed at 85 °C for 16 h to induce the interaction between the ions. After refluxing process, the mixture of the solution was kept for evaporation at 80 °C for 8 h to obtain the gel formation by polycondensation. The formed gel was dried in an oven for 8 h at 100 °C followed by sintering at 900 °C for 2 h. In addition, Si-doped HAP was synthesized by following the above-mentioned procedure using various concentrations of Si such as 1, 3 and 5% respectively. Further, heat treated pure phase and dual doped Ce/Si-doped HAP powders were characterized by various techniques. Hereafter, 1, 3 and 5% of Si content incorporation into Ce doped HAP are mentioned as Ce/Si-HAP@1%, Ce/Si-HAP@3% and Ce/Si-HAP@5% respectively.

2.2. In vitro hemocompatibility

In vitro hemocompatibility study is done to evaluate the biocompatibility of prepared material and examined to evaluate the blood compatible behaviour with 1.25% of Ce-HAP, 5% of Si-HAP, Ce/Si-HAP@1%, Ce/Si-HAP@3%, and Ce/Si-HAP@5% powders. To perform the hemocompatibility assay, we collected 5 ml of fresh human blood from healthy volunteers with age of 26 years (Health Center, VIT University: Ref. No. VIT/IECH/01/Aug.2016) and transferred to the falcon tube containing an anticoagulant agent. The obtained blood sample was centrifuged at 4 °C for 2 min using 10,000 rpm to separate the plasma from erythrocytes. After separation of erythrocytes from plasma, for three repetitive times, it was diluted with 5 ml of PBS solution followed by centrifugation for 2 min at 10,000. The settled erythrocytes were mixed with 15 ml of freshly prepared PBS solution and kept in the refrigerator. From 15 ml of the blood sample, 0.2 ml was taken and separately mixed with 5 mg of Ce-HAP, Si-HAP, and Ce/Si-HAP powders which were dispersed in 0.8 ml of PBS solution. On the other hand, 0.2 ml of blood with 0.8 ml of DD H_2O was treated as a positive control whereas with 0.8 ml of PBS acts as negative control respectively. These mixtures of blood samples were incubated for 1 h at room temperature in shaking conditions followed by centrifugation for 3 min using 10,000 rpm. After this step, the obtained supernatant was used for readings at 570/655 nm by Biorad Elisa.

The following formula was used to determine the hemolytic ratio of Ce-HAP, Si-HAP, and Ce/Si-HAP powders.

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