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Drug and ion releasing tetracalcium phosphate based dual action cement for regenerative treatment of infected bone defects

R. Jayasree[a], T.S. Sampath Kumar[a,*], Govindaraj Perumal[b], Mukesh Doble[b]

[a] Medical Materials Laboratory, Department of Metallurgical and Materials Engineering, Indian Institute of Technology Madras, Chennai 600036, India
[b] Department of Biotechnology, Indian Institute of Technology Madras, Chennai 600036, India

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ABSTRACT

Calcium phosphate cements (CPCs) are ideally suited for the local delivery of antibiotics in infected bone defects as they have multiple binding sites for loading various drugs. CPCs can also be substituted with ions such as Ag^{+}, Zn^{2+}, Mg^{2+}, Sr^{2+}, etc., to exhibit extended broad-spectrum antimicrobial activity. Strontium (Sr) in particular is known to enhance the new bone formation and decrease bone resorption. The current work aims to develop a dual action tetracalcium phosphate (TTCP) based cement which releases both the Sr^{2+} ion and ornidazole antibiotic drug for the treatment of bone infections. The TTCP with Sr^{2+} ion substitution was prepared by the solid state reaction method and it was used to form ornidazole loaded CPC. The ornidazole loaded cement prepared using 8 at% Sr substituted TTCP (8SCPC-O) showed complete hydroxyapatite (HA) formation in phosphate buffered solution at the end of 1 week. Fine needle-shaped HA crystals were observed in 8SCPC-O cement. *In vitro* drug release studies showed an accelerated ornidazole release from the 8SCPC-O sample when compared to samples without Sr substitution. Ornidazole releasing cements were found to be biocompatible with skeletal myoblast (L6) cells. Antibacterial activity of ornidazole releasing cement was evident from day 1 onwards against *E. coli*. The above results suggest 8SCPC-O as a good candidate for treating local bone infections.

1. Introduction

Post-operative infections are one of the biggest problems in orthopedic surgery as these infections often lead to failure of the surgical procedure, loss of bone tissue and possible removal of implants requiring a second surgery [1]. Drug loaded poly(methyl methacrylate) (PMMA) cements and drug encapsulated PMMA beads with calcium phosphate cements (CPCs) are currently used for the localized delivery of antibiotics in infected bone defects [1]. However, PMMA beads are not resorbable and require removal by a second surgery [2]. Hence, current research is driven towards the integration of antibiotics with the degradable CPCs for the local delivery of antibiotics at the surgical site [3–7].

CPCs are formed when one or more reactive calcium phosphate powder is mixed with water or phosphate containing liquid to form a moldable paste that eventually hardens in a clinically acceptable time (~15 min) under *in vivo* conditions [8,9]. Advantages of CPCs include clinically ideal setting time, moldability, microporous structure [10] and biological properties which mimic hydroxyapatite (HA), the mineral component of human bone [8,10]. The possibility of using CPCs as local drug carrier seems to be appropriate in various bone pathologies requiring extended painful treatments to accelerate bone healing. Additionally, the drugs can be incorporated either in the liquid or solid component of the CPC compared to calcium phosphate particulate drug delivery systems, where the drugs are mostly adsorbed on the surface.

The addition of drugs into CPC may influence the overall physicochemical properties of the cement such as setting reaction kinetics, rheological properties and the microstructure [7]. For example, tetracycline group of drugs has a tendency to chelate with Ca^{2+} ions which interfere with mineral precipitation, nucleation and hence prolong the setting reaction [7,11,12]. Drugs such as chlorhexidine has been reported to accelerate the chemical setting process in brushite cement [13]. On contrary, drugs such as gentamicin sulphate can significantly lengthen the setting time and also lead to the formation of thinner and smaller brushite crystals in brushite cements [14]. The addition of cephalexin, a beta-lactam antibiotic drug into CPC resulted in the formation of HA crystals of smaller size [15]. Similarly, the pH changes during the setting reaction or interaction with the components of the CPC may cause partial denaturation of the incorporated drugs. Hence, the selection of a suitable drug which remains stable during the dynamic cement setting reaction is of vital importance. Ornidazole is a

* Corresponding author.
E-mail address: tssk@iitm.ac.in (T.S. Sampath Kumar).

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member of the nitroimidazole family widely used in the treatment of peri-implant infections, anaerobic bone infections and periodontal infections with good success rate [16–19]. Ornidazole has been delivered locally from degradable polymeric gels and films in periodontitis [19–21].

Tetracalcium phosphate ($Ca_4(PO_4)_2O$, TTCP, Hilgenstockite), the most basic of calcium phosphates was first found by Hilgenstock in 1883. Unlike other calcium phosphates, TTCP is highly soluble and it is not possible to synthesize TTCP in an aqueous medium due to the oxygen atom in its chemical structure. Hence it is generally synthesized at high temperatures by solid-state reaction [22]. Ionic substitution such as Ag^+, Zn^{2+}, Mg^{2+}, Sr^{2+}etc., in calcium phosphates has been found to be beneficial as it influences the structural properties, drug loading and release efficiency, antimicrobial activity and host tissue response [23,24]. Of all the biologically relevant ions reported, the Sr^{2+} ion is of interest in bone tissue engineering because of its potential to reduce bone resorption and stimulate bone formation through increased osteoclast apoptosis, enhanced pre-osteoblastic cell proliferation and collagen synthesis [25,26].

Generally, CPC systems, leading to the formation of HA can be classified as monocomponent and multicomponent systems. In monocomponent CPC system, a single calcium phosphate compound undergoes hydrolysis reaction to form HA; whereas, in the multicomponent CPC system, two or more calcium phosphate compounds undergo acid-base reaction [7]. In our earlier study, monocomponent CPC was prepared using Sr^{2+} ion substituted TTCP which undergoes hydrolysis reaction to form HA with the release of calcium hydroxide and Sr^{2+} ion [27]. This cement was found to have antibacterial activity and dentinal tissue remineralization ability due to its highly alkaline pH and Sr^{2+} ion release [27]. In the present work, a dual action multicomponent CPC system which releases both the strontium ions and ornidazole antibiotic drug has been developed for the regenerative treatment of infected bone defects. Ornidazole was incorporated in the CPC for the first time and studied for its effect on the physical and biological properties. *In vitro* studies to evaluate the drug release, antibacterial activity and cell viability were also performed.

2. Materials and methods

2.1. Synthesis of Sr substituted TTCP powder

Pure tetracalcium phosphate (PTTCP) was synthesized as reported earlier according to Eq. (1) [28,29]. Calcium carbonate, $CaCO_3$ (Merck, India) and dicalcium phosphate anhydrous, DCPA, $CaHPO_4$ (Central Drug House, India) taken in equimolar ratios were mixed thoroughly in an agate mortar and pestle for about 30 min. The powder was then heated to 1500 °C for 6 h followed by rapid quenching.

$$2CaHPO_4 + 2CaCO_3 \rightarrow Ca_4P_2O_9 + 2CO_2 + H_2O \quad (1)$$

To synthesize Sr substituted TTCPs, strontium carbonate ($SrCO_3$, Merck, India) was added to the above-said mixture, replacing $CaCO_3$ at the atomic percentages of 3, 5, 8 and 10 [27]. The synthesized powders were denoted as 3STTCP, 5STTCP, 8STTCP and 10STTCP respectively.

2.2. X-ray diffraction

The phase and crystallinity of the synthesized TTCP's were assessed by X-ray diffraction (XRD) analysis. X-ray diffraction data were collected using Bruker D8 Discover diffractometer equipped with Cu Kα radiation (λ = 1.54 A°) in the 2θ range of 20–60° and increments of 0.1°/step.

2.3. Cement fabrication

The liquid component of the cement contains 1 M disodium hydrogen phosphate (Na_2HPO_4) and 10 wt% citric acid, which acted as an accelerator and hardener respectively [28–31]. The powder component contains an equimolar concentration of TTCP and dicalcium phosphate anhydrous (DCPA) as basic and acidic reactants respectively. Cement fabricated using PTTCP, 3STTCP, 5STTCP, 8STTCP and 10STTCP as one of the powder components are coded as PCPC, 3SCPC, 5SCPC, 8SCPC and 10SCPC respectively. For fabricating the cement, powder component was mixed carefully with the liquid component in a liquid to powder (L/P) ratio of 0.5 ml/g to form a moldable paste. The cement was allowed to set at 37 °C in phosphate buffered solution (PBS) of pH 7.4. To prepare the drug-loaded CPCs, ornidazole drug (Sigma Aldrich, India) was added to the powder component (TTCP/DCPA powder) at 10 wt% concentration and mixed using an agate mortar and pestle. The same procedure mentioned earlier was followed to fabricate ornidazole loaded CPCs.

For further characterization, all the cement samples were stored in PBS of pH 7.4 at 37 °C and retrieved at definite time intervals of 3 h, 24 h, 7 days and 1 month, dried and powdered using an agate mortar and pestle. XRD study was performed to quantify the formation of HA at different time periods. The peaks obtained from the XRD results were indexed following Joint Committee on Powder Diffraction Standards (TTCP- JCPDS No. 25-1137, DCPA- JCPDS No. 70-1425 and HA- JCPDS No. 9-432).

The percentage of HA phase formation in various cement samples was quantified as reported earlier using the following equation

$$\%\text{Conversion} = \left\{ \frac{A_t}{A_\infty} + \frac{\left[1 - \left(\frac{D_t}{D_0}\right) + 1 - \left(\frac{T_t}{T_0}\right)\right]}{2} \right\} / 2 \times 100 \quad (2)$$

where D_0 and T_0 indicate the peak intensities of DCPA and TTCP in the unreacted cement powder [27,29]. A_∞ denotes the peak intensity of HA in a sample completely converted to apatite. D_t, T_t and A_t are the peak intensities of DCPA, TTCP and HA in the samples at time 't' (3 h, 24 h, 7 days and 1 month) after mixing. Peaks at 29.8° (0 4 0) for TTCP, 26.4° (0 2 0) for DCPA and 25.9° (0 0 2) for HA were chosen for the phase quantification. These peak intensities are determined using a commercial software X'pert Highscore plus.

2.4. Setting time

ASTM C191-08 standard test method was used to measure the setting time of the cement by means of a fabricated Vicat needle apparatus (Indian standard 5513:1996) [32]. Freshly prepared cement paste (n = 5) was packed in a polypropylene cylindrical mould. The needle was allowed to penetrate into the paste at regular intervals to assess the initial and final setting time.

2.5. Compressive strength measurements

Cement samples of dimension 12 × 6 mm were made as mentioned above and stored in PBS at 37 °C of pH 7.4. Compressive testing was performed as per ASTM F-451–08 standard on samples (n = 5) stored at time intervals of 1 day, 7 days and 1 month [33]. A screw-driven mechanical testing machine (Model 4467, Instron Corp., MA, United States) was used to apply a compressive load with a crosshead speed of 0.5 mm/min. Compressive strength (MPa) was calculated using Eq. (3)

$$\text{Compressive Strength} = 4F/\pi \text{dia}^2 \quad (3)$$

where F is the maximum load (N) up to fracture and dia is the diameter (mm) of cylindrical cement sample.

2.6. pH study

Cement samples of uniform cylindrical shape weighing 25 mg each

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