Materials Science and Engineering C 62 (2016) 692–701

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Deposition, structure, physical and *invitro* characteristics of Ag-doped β-$Ca_3(PO_4)_2$/chitosan hybrid composite coatings on Titanium metal

Ram Kishore Singh [a], Sharad Awasthi [b], Arunkumar Dhayalan [b], J.M.F. Ferreira [c], S. Kannan [a,*]

[a] *Centre for Nanoscience and Technology, Pondicherry University, Puducherry605 014, India*
[b] *Department of Biotechnology, Pondicherry University, Puducherry605 014, India*
[c] *Department of Materials and Ceramics Engineering, University of Aveiro, CICECO, Aveiro3810 193, Portugal*

ARTICLE INFO

Article history:
Received 9 October 2015
Received in revised form 28 January 2016
Accepted 3 February 2016
Available online 5 February 2016

Keywords:
Silver
β-$Ca_3(PO_4)_2$
Chitosan
Composite
Ti metal

ABSTRACT

Pure and five silver-doped (0-5Ag) β-tricalcium phosphate [β-TCP, β-$Ca_3(PO_4)_2$]/chitosan composite coatings were deposited on Titanium (Ti) substrates and their properties that are relevant for applications in hard tissue replacements were assessed. Silver, β-TCP and chitosan were combined to profit from their salient and complementary antibacterial and biocompatible features.The β-$Ca_3(PO_4)_2$ powders were synthesized by co-precipitation. The characterization results confirmed the Ag^+ occupancy at the crystal lattice of β-$Ca_3(PO_4)_2$. The Ag-dopedβ-$Ca_3(PO_4)_2$/chitosan composite coatings deposited by electrophoresis showed good antibacterial activity and exhibited negative cytotoxic effects towards the human osteosarcoma cell line MG-63. The morphology of the coatings was observed by SEM and their efficiency against corrosion of metallic substrates was determined through potentiodynamic polarization tests.

1. Introduction

Calcium phosphates and natural polymers have been widely used over the years in reconstructive surgery, to replace autografts or bone material of animal origin [1,2]. Chemical and crystallographic similarities with natural bone and tooth material [3], favourable features like solubility [4,5], excellent biocompatibility, osteoinductivity and resorbability [6,7] fostered a privileged acceptance of β-TCP based materials. Bacterial infection is a predicament issue for orthopaedic implants that has caused their premature removal and in many cases has resulted in the surgical revisions [8,9]. Hence, countering the bacterial infections at the implant site has been a major task for many researchers and in this context, successful attempts were made by developing bioceramics with cationic dopants that possess antibacterial properties. *In vitro* and *invivo* studies on the ionic (Ag^+, Cu^{2+} and Zn^{2+}) doped bioceramics revealed that these materials exhibit good inhibitory activities against the investigated microbes [10–12]. In particular, silver in either ionic or particulate forms exhibited effective bactericidal properties over a wide range of bacteria, preventing the biofilm growth and bacterial colonization on the various surfaces that are commonly employed in medical applications [13,14]. Silver also helped in increasing the toughness and strength of Ag-doped calcium phosphate ceramic materials [15]. In spite of all these positive aspects and of an enhanced antimicrobial efficiency with increasing silver concentrations, the cytotoxicity could not be avoided in the presence of relatively high amounts of silver[16].

Calcium phosphates in bulk forms are not preferred for hard tissue replacements due to poor mechanical features, making the coatings on metallic implants more attractive [17]. Moreover, there is a generalizedconsensus in that bioceramic coatings on metallic implants protect them against corrosion and deterioration. Several methods have been attempted for coatings on metallic implants [18–20]. Among the methods proposed for coating metallic implants, electrophoretic deposition (EPD) offers advantages of uniform surface deposition, cost-effectiveness and maintenance of phase purity of the ceramic after deposition. However, the generally obtained poor adhesion strength between the ceramic and the metallic surface has prevented using such methods for coating implants for biomedical applications. Certain studies have suggested that composite coatings of chitosan and bioceramic on metallic implant enhance the bonding strength between the ceramic and metallic interface. Chitosan is a natural cationic polymer obtained by deacetylation of chitin which possesses reactive amine group characteristic of a weak base [21] thus enabling this biopolymer to be protonated in weak acidic electrolytes. The protonated chitosan is likely adsorbed at the surface of the negatively charged calcium phosphate particles, contributing to their EPco-deposition onto the surface of the metallic substrate [22]. In addition, chitosan possesses the salient features of low toxicity, biocompatibility, biodegradability, wound healing ability [23] and antimicrobial property [24]. The various chemical modifications of chitosan and its high metal binding efficacy,

---

* Corresponding author.
E-mail address: para_kanna@yahoo.com (S. Kannan).

http://dx.doi.org/10.1016/j.msec.2016.02.013
0928-4931/

especially with silver, [25] also facilitate tailoring some desired specific properties [26].

The present study aims at developing Ag-dopedβ-TCP powders and combine them with chitosan to deposit composite coatings on Ti metal surface through the EPD process. The structural changes due to Ag^+ incorporation in the β-TCP powders were investigated. The antimicrobial properties of the coatings against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli*, and their potential cytoxicity towards human osteosarcoma cell line (MG-63) were evaluated. The effect of Ag-dopingβ-$Ca_3(PO_4)_2$ on the expression of the marker gene, alkaline phosphatase (ALP) in MG-63 cell line was also analysed. The morphological features of the composite coatings, their swelling behaviour, and protecting efficacy against corrosion of the metallic substrates are reported.

2. Materials and methods

2.1. Powder preparation

Undoped and five Ag-doped powders were synthesized by chemical precipitation technique. For this purpose, analytical grade calcium nitrite tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$], silver nitrate [$AgNO_3$] and diammonium hydrogen orthophosphate [$(NH_4)_2HPO_4$] were taken as precursors for Ca^{2+}, Ag^+ and PO_4^{3-} ions respectively. The concentrations of the precursors were set in order to have a fixed (Ca + Ag)/P molar ratio = 1.5 for all the compositions. The planned Ag molar concentrations in the synthesized powders and their sample codes are reported in Table1. The experimental data obtained by ICP-AES analysis (ARCOS, SPECTRO, GERMANY) is also given for comparison. In brief, two stock solutions were separately prepared using standard measuring flasks, one containing the cationic ($Ca^{2+} + Ag^+$) precursors and other the PO_4^{3-} containing reagent. The cationic solution was transferred to a beaker and subjected to magnetic stirring (TARSONS, India) of ~250 rpm at 90 °C. The $(NH_4)_2HPO_4$ solution was then drop wisely added. After the completion of addition, a white gelatinous slurry with a pH value of ~4.75 was obtained. The pH was then raised to ~8.0 by using NH_4OH solution under the same temperature and stirring conditions for 2 h. The resultant mixture was thereafter let to cool up to room temperature under rest conditions to enable the precipitate to settle down for 24 h. The supernatant solution was then decanted and the precipitate was transferred to a hot air oven and dried at 120 °C overnight. Each as obtained dried powder was fine grounded and considered as starting powder.

2.2. Powder characterization

The as prepared powder samples were subjected to heat treatment at different temperatures namely 300, 500, 700 and 1000 °C (MATRI-MC 2265 A, INDIA) for a dwelling period of 2 h. The changes in phase composition and crystallinity for all samples were determined using a high resolution X-ray diffraction (XRD, RIGAKU,ULTIMA IV) diffractometer with CuKα radiation ($\lambda = 1.5406$ Å) produced at 40 kV and 30 mA to scan the diffraction angles (2θ) between 10° and 70° with a step size of 0.02° 2θ per second. Slow scans for Rietveld refinement were done with a step size of 0.01° 2θ per second. Phase determinations were made using Standard ICDD (International Centre for Diffraction Data) card no. 00-009-0432 for HAP and 00-009-0169 for β-TCP. The Fourier transform infrared (FT-IR) spectra for the powders (NICOLET iS10, FT-IR spectrophotometer, USA) were recorded using KBr method. Raman spectra for the powders were recorded using a confocal Raman microscope (RENISHAW, United Kingdom). All the powder samples were excited at a wavelength of 785 nm by semiconductor diode laser with a data acquiring time of 30 s. The surface morphology of the powders was observed by Scanning Electron Microscope (SEM, HITACHI, JAPAN) equipped with an EDX instrument (Thermo Super Dry II, USA).

Table1
Comparison between the planned Ag-doping levels and the results of elemental concentrations obtained by ICP analysis.

Sample code	Concentration of Ag (mol%)	
	Planned	Experimental (from ICP analysis)
0Agβ-TCP	–	–
1Agβ-TCP	0.410	0.497
2Agβ-TCP	0.811	0.875
3Agβ-TCP	1.230	1.420
4Agβ-TCP	1.658	1.774
5Agβ-TCP	2.096	2.367

2.3. Quantitative analysis by Rietveld refinement

Data on quantitative phase analysis were gathered for pure and Ag-doped powders at 1000 °Cby the Rietveld refinement method using the GSAS-EXPGUI software applied to three independent X-ray scans per sample. The initial refinement of stoichiometric β-$Ca_3(PO_4)_2$ was done using the standard CIF (crystallography information file) from Yashima etal., [27]. The structural parameters taken for refinement were given as the space group *R3c*, Z = 21, $a = b = 10.4352$ Å and $c = 37.4029$ Å. The CIF of pure β-$Ca_3(PO_4)_2$ refined by Yashima etal., [27] was taken as a standard for refining substitutions of Ag-dopedβ-$Ca_3(PO_4)_2$. In the first step of the refinement, all the structural parameters were set to the literature values. Then, during the successive refinement cycles, numerous parameters were allowed to vary according to the relative amounts of the identified phases. The following refinement sequence was used as a standard for all the structures: scale factor, zero shift, background as a Chebyshev polynomial of fifth grade, peak profile and lattice parameters. Fittings were performed using pseudo-Voigt peak profile functions and a preferred orientation along [001] was taken into account with the Marsh model. The atomic parameters such as position, isotropic temperature and occupancy factors were also refined during the final step.

2.4. In vitro tests

2.4.1. Antimicrobial activity assays

In vitro tests involving antibacterial activity, cytotoxicity and quantitative RT-PCR were performed for the selected powder compositions after heat treatment at 1000 °C.Antimicrobial activity of the powder samples was tested against both *S.aureus*(*S.aureus*, ATCC-29213) and *E.coli*(*E.coli*, ATCC-25922) in a quantitative manner. Prior to antimicrobial tests, the samples were sterilized in an autoclave at 121 °C for 20 min. Fresh inoculums of *S.aureus* and *E.coli* were prepared in Müller–Hinton broth (Hi Media, India), kept at 37 °C for overnight incubation, and then used for further tests. The antibacterial tests were performed with slight modifications of the procedure reported by Stanic etal.[28]. The details included a series of test tubes, each containing 0.1 g sterilized test samples suspended in 9.9 ml sterile potassium hydrogen phosphate buffer solution (pH 7.4). The mixtures were subjected to thorough mixing for 5 min by using vortex. Then, 0.1 ml of the overnight incubated test culture was added to each tube and transferred to shaker incubator at 37 °C. For viable bacterial cell count determination, 1 ml aliquots were taken from each test tube at the time points of 0, 1, 2 and 4 h. Fresh Müller–Hinton agar (Hi Media, India) Petri plates with 0.1 ml aliquots spread over wereincubatedat 37 °C for 24 h, the CFU counts for each sample were determined in triplicate at time points of 0, 1, 2, and 4 h, and averaged. The percentage of microorganism reduction (R)was calculated according to the Eq.(1):

$$R = \frac{C_O - C}{C_O} \times 100\ \% \tag{1}$$