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In vitro characterisation of a sol–gel derived *in situ* silica-coated silicate and carbonate co-doped hydroxyapatite nanopowder for bone grafting

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ABSTRACT

Design and synthesis of materials with better properties and performance are essential requirements in the field of biomaterials science that would directly improve patient quality of life. For this purpose, *in situ* silica-coated silicate and carbonate co-doped hydroxyapatite (Sc/S.C.HA) nanopowder was synthesized *via* the sol–gel method. Characterisation of the prepared nanopowder was carried out by XRD, FTIR, TEM, SEM, EDX, ICP, zeta potential, acid dissolution test, and cell culture test. The substitution of the silicate and carbonate ions into hydroxyapatite structure was confirmed by FTIR analysis. XRD analysis showed that silica is an amorphous phase, which played a role in covering the surface of the S.C.HA nanoparticles as confirmed by acid dissolution test. Low thickness and low integrity of the amorphous silica surface layer facilitated ions release from S.C.HA nanoparticles into physiological saline solution. Zeta potential of the prepared nanopowder suspended in physiological saline solution was -27.3 ± 0.2 mV at pH 7.4. This negatively charged surface, due to the presence of amorphous silica layer upon the S.C.HA nanoparticles, not only had an accelerating effect on *in vitro* biomineralization of apatite, but also had a positive effect on cell attachment.

1. Introduction

Bone deficiencies, especially those resulting from tumors, trauma, or birth deficiencies, are common [1–2]. Bone grafting is a surgical intervention for restoration and reconstruction of bone deficiencies [1]. Although autografts and allografts are common for bone grafting procedures, the limitations and weaknesses of these kinds of grafts [2] requires development of artificial bone grafts with improved performance and properties [1].

Although hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) is commonly employed as an artificial bone graft due to its biocompatibility and osteoconductivity [3], it has some limitations. Firstly, the synthetic pure HA has a very slow dissolution rate [2], which limits the bioactivity and the osteoconductive properties of HA [4–6]. In fact, the synthetic pure HA has low reactivity with the natural bone [7]. This property leads to a relatively slow rate of osseointegration [3] that lengthens the time needed for patient rehabilitation [8,9]. Secondly, the apatite mineral present in bone differs from synthetic pure HA because of several ionic substitutions in its structure [10]. Finally, under acidic conditions, phase purity of HA could be changed, and when pH value is below 4.8, HA is not in a stable phase [11]. This property could limit the range of biomedical applications for HA.

Ionic substitution in the synthetic HA structure could not only help to mimic the structure of biological apatite, but also change the dissolution rate of HA [12]. It has been reported that the presence of carbonate (CO_3^{2-}) in the HA structure (carbonated HA) increases *in vitro* [13] and *in vivo* [14] HA dissolution rate. As a result, compared to HA, carbonated HA is replaced with the new bone at a higher rate [15]. On the other hand, there is more evidence on the importance of silicon in bone formation, the growth and development of bone, teeth and some invertebrate skeletons [16–20]. It has been reported that the presence of silicate (SiO_4^{4-}) in the HA structure (silicated HA) increases *in vitro* [13] and *in vivo* [21] dissolution rate of HA.

Liu et al. showed that the formation of negatively charged surface on bioceramics for *in vitro* biomineralization of apatite (*in vitro* bioactivity) is more important than the increase of the calcium concentration in the simulated body fluid (SBF) [22]. In this case, it is reported that formation of amorphous silica on the surface of $CaSiO_3$ ceramics in SBF solution has an enhancing effect on its *in vitro* bioactivity [23]. Likewise, the formation of surface amorphous silica with a suitable rate is important for fast *in vitro* bioactivity [24]. The mechanism presented for this fast apatite formation is the existence of silanol groups (Si—OH) on the surface of amorphous silica accelerating the nucleation and growth of apatite [25]. On the other hand, Borum et al. applied an amorphous silica

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coating layer on the surface-modified nanophase HA with dodecyl alcohol groups and showed that it could protect HA particles against an acidic environment [26].

On this basis, the purpose of the presented work was to improve nanophase HA dissolution rate *via* its structural changes by carbonate and silicate groups along with its surface modification by amorphous silica layer for enhancing the bioactivity, cell interaction, and the stability of it in acidic environments.

2. Materials and methods

2.1. Synthesis of silica-coated silicate and carbonate co-doped hydroxyapatite nanopowder

Calcium nitrate tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$, Merck] and phosphoric pentoxide [P_2O_5, Merck] were dissolved in separate beakers containing absolute ethanol [C_2H_5OH, Merck]. Both of the prepared solutions were mixed at a Ca/P molar ratio of 5:3. The resultant solution was then stirred for ~3 h at 30 °C, and was aged for ~6 h at 30 °C under static conditions. Following this process, tetraethylorthosilicate (TEOS, $C_8H_{20}O_4Si$; Merck) was added to the aged mixture under continuous stirring to form a final product. After ~1 h of stirring, 0.1 N HNO_3 and acetic acid were added as the catalyst. In our previous work [27], we demonstrated that acid catalyst is a better candidate for synthesis of silica coated apatite composite nanopowder compared with base catalyst. The molar ratio of H_2O/TEOS and water/acetic acid was 2:1 and 7:1, respectively. The final mixture was constantly stirred for ~3 h at 30 °C and was then aged for 24 h at room temperature under static conditions. The resulting gel was then dried at 80 °C for 24 h. Dry powder was then crushed using a ball mill (Fretch Pulverisette 5) with a 125 ml zirconia vial and four, 20 mm diameter zirconia balls at room temperature. The process was performed by a ball/powder mass ratio of 20:1 and a rotation speed of 150 rpm for 90 min. Finally, the calcination of the crushed dry powder was carried out at 600 °C for 30 min (heating rate: 5 °C min^{-1}), after which, the prepared powder was kept at room temperature to be cooled. The prepared silica-coated silicate and carbonate co-doped hydroxyapatite nanopowder is referred as Sc/S.C.HA nanopowder. For comparison purposes, HA nanopowder was also prepared.

2.2. Characterisation of Sc/S.C.HA nanopowder

Phase analysis of the prepared nanopowder was carried out by an X-ray diffractometer (XRD, Philips X'Pert-MPD). The diffraction spectrum was recorded in the 2θ range from 20 to 70° using Cu Kα (λ = 0.154186 nm, 40 kV and 30 mA) radiation with step size 0.05° and step duration 1 s.

Functional groups of the prepared nanopowder was identified using a Fourier transformed infrared (FTIR, Bomem MB 100) spectroscopy by a KBr disk method in the range of 4000–400 cm^{-1}.

The microstructure and nanostructure of the prepared nanopowder were studied by scanning electron microscopy (SEM, Phillips XL30) and transmission electron microscopy (TEM, Philips CM120) techniques, respectively. Moreover, the elemental composition of the prepared nanopowder was evaluated using energy dispersive X-ray spectroscopy (EDX), which was conducted on the Seron Technology AIS 2300C scanning electron microscopy.

In order to evaluate dissolution behavior and *in vitro* bioactivity of Sc/S.C.HA nanopowder, powder samples were compressed using a hydraulic press at room temperature and 250 MPa for 1 min, which led to disks with 5 mm in diameter and weighing approximately 70 mg. The prepared disks were immersed in physiological saline solution (0.9 wt% NaCl) [28] contained in plastic bottles for up to 4 days at 37 °C in a water bath. The surface area (mm^2) of the disk sample to physiological saline solution (ml) ratio was fixed at 10 to 1 [29]. After the predetermined immersion time, the disk samples were removed from the plastic bottles and the filtered solutions were analyzed with inductively coupled plasma optical emission spectroscopy (ICP-OES, Perkin-Elmer Optima 7300DV). Moreover, the removed disk samples were rinsed with deionized water and dried at 80 °C for 24 h. Following this, their surfaces were investigated by scanning electron microscopy (SEM, Phillips XL30).

Zeta potential of Sc/S.C.HA nanopowder was measured with a Zetasizer Nano-ZS (Malvern Instruments, UK). For this purpose, 2 mg of Sc/S.C.HA nanopowder was suspended in 10 ml physiological saline solution (0.9 wt% NaCl) with a pH value of 7.4 [30]. The measurement was done three times (each measurement was the average of 10 runs) and the mean value and standard deviation were calculated.

An Acid dissolution test was carried out by dispersing the powder samples with a ratio of 75 mg/ml in 2 M HCl. After sonication for 30 min in an ultrasonic bath, the apparent color of the solution or suspension was observed and recorded by camera [31].

In vitro cytotoxicity test by direct contact method was done in order to evaluate biocompatibility. Briefly, L929 fibroblast cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin streptomycin and were then incubated at 37 °C in a 5% CO_2 atmosphere for 5 days. Furthermore, the medium was refreshed after 3 days. After sterilization of the prepared Sc/S.C.HA disk samples, they were kept in contact with

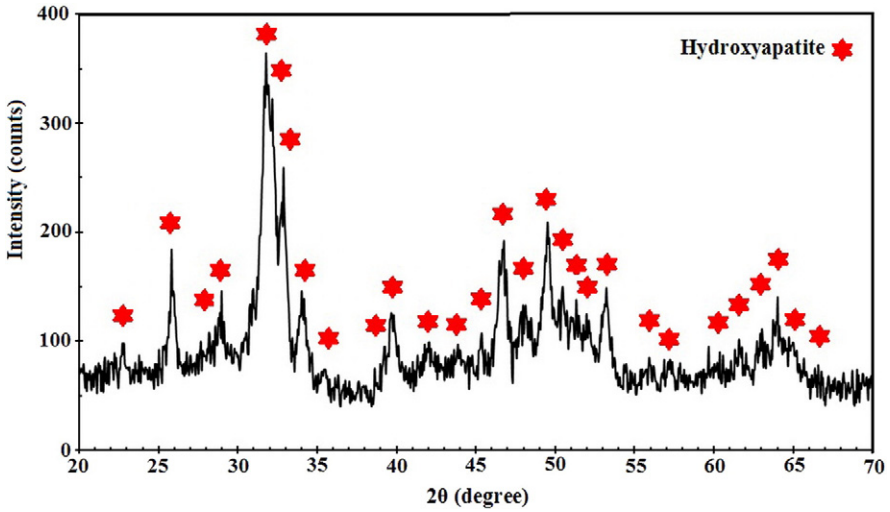

Fig. 1. XRD pattern of Sc/S.C.HA nanopowder.