Journal of the European Ceramic Society 37 (2017) 2943–2952

Contents lists available at www.sciencedirect.com

Journal of the European Ceramic Society

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

In vitro behaviour of Nurse's A_{ss}-phase: A new calcium silicophosphate ceramic

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ARTICLE INFO

Article history:
Received 2 November 2016
Received in revised form 24 February 2017
Accepted 4 March 2017
Available online 15 March 2017

Keywords:
Bioceramics
Calcium silicophosphates
Bioactivity
Biocompatibility
Biomedical applications

ABSTRACT

In the present study, a new single phase Si–Ca–P-based ceramic (called Nurse's A_{ss}) was obtained and its *in vitro* behaviour was explored for potential bone tissue regeneration. A porous Si–Ca–P single phase ceramic was obtained from high-temperature sintering of previously synthesised γ-dicalcium silicate and β-tricalcium phosphate. Apatite-mineralisation ability and the dissolution rate were systematically studied by immersing the material in simulated body fluid (SBF) for several time points. Massive new dense calcium deficient hydroxyapatite (CDHA) layer formation was observed at the SBF-sample interface. Adjacent to the dense CDHA layer, a porous structure developed parallel to the interface, formed by the pseudomorphic transformation of Si–Ca–P (Nurse's A_{ss}) into CDHA. The cell attachment test showed that the new material supported adult human bone marrow-derived mesenchymal stem cells (*h*MSCs) adhesion and spreading, and cells came into close contact with the ceramic surface during an extended 28-day culture. These findings indicate that the new calcium silicophosphate ceramic possesses good bioactivity and biocompatibility, and might be a promising bone graft substitute.

1. Introduction

With the world's increasing population age, the need for new bone implant materials has simultaneously increased. A successful bone implant requisite is rapid osseointegration and long-term mechanical stability. The new generation of tissue engineering scaffolds for bone regeneration should be highly bioactive, degradable and mechanically strong. There are many essential factors for tissue engineering scaffolds, among which bioactive composition [1,2], roughness [3,4] and macroporous morphology [5,6] are assumed critical for influencing the cell response.

The bone mineral fraction is the calcium–phosphate (Ca–P)-based apatite phase. For this reason, Ca–P ceramics, typically hydroxyapatite (HA) and tricalcium phosphate (TCP) ceramics, are widely used for bone tissue replacement and regeneration because of their generally good biocompatibility and similar chemical composition with biological apatite in bone tissue [7–10]. Regarding Ca–P materials that contain silicon, the role of Si is well-known in the development of healthy bone and connective tissues [11–15]. Silicon also modifies material properties and improves the biological activity of Ca–P materials that contains silicon. Thus they have been widely studied as biomaterials *per se* or associated with cells (cell–ceramic construct) for osseous repairs [16–21].

Interest in Si–Ca–P biomaterial is presently increasing because of its good bioactivity response and low cytotoxicity. In this context, Si–Ca–P (Nurse's A_{ss}) is a solid solution with an approximate composition of $7CaOP_2O_52SiO_2$, but should not be confused with the mineral of the same composition identified by Nagelshmidt in 1937 [22]. We recently synthesised a new single Si–Ca–P (Nurse's A_{ss}) phase material with a $Ca_2SiO_4/Ca_3(PO_4)_2$ molar ratio equal to 2:1 in the binary system dicalcium silicate (Ca_2SiO_4 = C2S)-tricalcium phosphate ($Ca_3(PO_4)_2$ = TCP) [23–25] by a solid-state reaction method [26,27]. However to date, studies into Nurse's A_{ss}-phase ceramic have been limited to establishing the simplest and most economical route of synthesis, and there are no reports on the *in vitro* bioactivity and biocompatibility of ceramic. So it would be

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http://dx.doi.org/10.1016/j.jeurceramsoc.2017.03.014
0955-2219/

very interesting to know the *in vitro* behaviour of this new ceramic to use a scaffolds for bone tissue engineering applications.

A method to estimate the bone-bonding potential ability of material is the simulated body fluid (SBF) method, which involves immersing materials into SBF for bone-like apatite formation on its surface. According to Kokubo et al. [28], bone-like apatite seems to activate signalling proteins and cells to start a series of events (attachment, proliferation and differentiation), which finally leads to bone formation. In other words, *in vivo* behaviour could be predicted by using the SBF method *in vitro*. Therefore, the aim of this study is to prepare a Si–Ca–P single phase ceramic by an economical route of synthesis, characterise its *in vitro* bioactivity and biocompatibility in SBF, and evaluate its ability to provide a suitable microenvironment to enhance the attachment and proliferation of adult human bone marrow-derived mesenchymal stem cells (*h*MSCs) for its use as a bone substitute or a supportive scaffold in bone tissue engineering.

2. Materials and methods

2.1. Material processing and characterisation

An Si–Ca–P (Nurse's A_{ss}) single-phase ceramic material was prepared from tricalcium phosphate and dicalcium silicate ceramics, which were used as starting materials. Details of the technique and the characterisation of the starting materials can be found in previous publications [26,27]. Briefly, a mixture of 47.39 wt% TCP and 52.61 wt% C2S was prepared. First, TCP and C2S powders were ground to an average particle size of ~30 μm, and the desired proportions of each component were weighed on an analytical balance and thoroughly mixed with PSZ-zirconia balls in a liquid medium. After drying, samples were isostatically pressed in bars at 200 MPa. The pellets obtained from the bars were put into small platinum foil crucibles, which were suspended from a platinum wire in the hot zone of an electrical furnace with an electronic temperature controller (±1 °C). Pellets were heated to 1500 °C for 3 h and were then liquid/nitrogen-quenched, ground and pressed. Second, heating at 1300 °C lasted 3 h with subsequent annealing at 1200 °C for 24 h. This combined heat treatment procedure was required to ensure that equilibrium conditions were achieved. The chemical analysis of the synthesised material as well as the X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and μ-Raman results, confirmed that the obtained material contained Nurse's A_{ss} as a single phase.

2.2. *SBF* in vitro *test*

The *in vitro* bioactivity of Nurse's A_{ss} ceramic disc was assessed by soaking samples in SBF solution, which was prepared according to the procedure described by Kokubo et al. [28]. Nurse's A_{ss} ceramic discs, which measured 10 mm in diameter and 5 mm in thickness, were cut from the bars, and then washed with pure acetone before being hanged with a nylon thread in polystyrene tubes that contained 100 ml of SBF (pH 7.25). The solution was refreshed with 25% of fresh SBF every 24 h. The solid/liquid weight ratio was equal to 0.5. The tubes with SBF and samples were incubated at 37 ± 0.5 °C in a shaking water bath for predetermined intervals. After different soaking periods that lasted from 1 day to 15 days, discs were removed from the SBF solution, gently washed 3 times with double-distilled water and dried for 24 h at room temperature.

To evaluate the dissolution rate, Nurse's A_{ss} ceramics were soaked in 100 ml Tris–HCl solution (pH 7.40) at 37 °C for 3, 5, 7, 10 and 15 days, and the solution was refreshed with 25% of fresh Tris–HCl every 24 h. Tris–HCl was selected because it does not contain inorganic ions (*e.g.*, Ca, P and Si). The weight of the disc before soaking was 0.18 g. After the set soaking time, ceramics were dried at 120 °C for 1 day, and the final weight of each sample was accurately recorded. Weight loss was expressed as the percentage of initial weight. Ten samples were used for this test.

After exposure to SBF, the surfaces and cross-sections of the samples were examined at 20 keV. Cross-sections were previously embedded in epoxy resin under vacuum and polished to 1 μm finish using a diamond paste, gently cleaned in an ultrasonic bath, and palladium-coated for SEM (SEM-Hitachi S-3500N, Ibaraki, Japan) examination and the EDS microanalysis (EDS-INCA-Oxford). The evolution of the samples was established by measuring the thickness of the layer formed on the ceramic–SBF interface by SEM.

SBF was removed after several immersion periods, and silicon, calcium, and phosphorus were determined in the removed SBF by inductively coupled plasma optical emission spectrometry (ICP-OES Perkin-Elmer Optima 2000™). For the TEM study (JEM-2010 JEOL), samples were prepared by carefully removing the reaction layer from the sample surfaces with a razor blade, and dispersing the powder on the surface of ethanol in a Petri dish. After drying, powder specimens were then collected on carbon-coated TEM copper grids (300 mesh). Electron beam transparent particles were chosen for the TEM examination by selected area diffraction (SAD), and also by EDS.

The atomic force microscopy (AFM) characterisation of samples' surface was performed in the tapping mode by a Cervantes Full Mode AFM System, equipped with a Dulcinea Control System (Nanotec Electrónica, Spain). Silicon cantilevers, with a 42 N/m force constant and a 330 kHz resonance frequency, were used. The structural characterisation of the samples' surface was also performed by confocal Raman (Witec ALPHA 300RA) with Nd:YAG laser excitation at 532 nm and a 100× objective (NA = 0.9).

2.3. In vitro *cell tests*

Adult human bone marrow-derived mesenchymal stem cells (*h*MSCs) were isolated from the bone marrow biopsy (40 ml) obtained through direct aspirations of the iliac crest from three healthy donors aged 25–40 years. They all provided informed consent and all the procedures were approved by the Institutional Ethical and Clinical Trials Committee (the V. Arrixaca University Hospital of Murcia, Spain). The isolation and culture procedures of the *h*MSCs and subcultures, and the characterisation of *h*MSCs, were processed by previously described methods [29]. For this study, only the *h*MSCs from the third passage (P3) were employed for all the experiments.

2.3.1. *Biofunctionality test*

The surface morphology of samples was analysed by SEM-EDS to evaluate the biofunctionality of the Nurse's A discs by studying cell adherence, morphology and growth on the ceramic surface. Briefly, discs were rinsed in an ultrasonic cleaner and dried a 100 °C prior to use. A tissue culture plate (Bechton Dickinson, Franklin Lakes, NJ, USA), was used as a control (hereinafter referred to as "plastic"). Prior to cell seeding, the material was sterilised by gas-plasma and conditioned by placing in DMEM with 10% FBS and incubating at 37 °C for 1 h.

After detaching *h*MSCs, viability was quantified by the trypan blue dye (Sigma–Aldrich) exclusion test. *h*MSCs were seeded onto the tops of the prepared disc-shaped Nurse's A_{ss} phase (6 mm in diameter, 3 mm high) at a density of 5×10^3 cells cm^{-2}, and were cultured for 1, 3, 7, 14, 21 and 28 days at 37 °C, 7.5% CO_2 in a humidified incubator. The medium was replaced every 3 days. After the incubation period, the cell-cultured discs or construct were/was rinsed with PBS for 10 min and fixed for 1 h with 3% glutaraldehyde in 0.1 M cacodylate buffer to then be postfixed with 1% osmium tetroxide. Next the cell-cultured construct was dehy-