A simple sol-gel route to the construction of hydroxyapatite inverted colloidal crystals for bone tissue engineering

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Abstract

Hydroxyapatite (HAp) scaffolds with uniform pore size and interconnected pore network were constructed based on the inverted colloidal crystal (ICC) geometry and a simple sol-gel formulation. Monodisperse polystyrene microspheres were self-assembled and annealed into a hexagonal close packed structure. HAp sol-gel was infiltrated in this template followed by thermal treatment for simultaneous HAp matrix sintering and polymeric colloidal crystal calcination. The resultant ICC scaffolds exhibit an ordered architecture that was able to offer a favourable environment for human osteoblasts adhesion and proliferation, an essential feature for bone ingrowth in tissue engineering applications.

Highlights

- Infiltration of a colloidal crystal template with a hydroxyapatite sol-gel system.
- Production of ceramic inverted colloidal crystal.
- Construction of ceramic structures with uniform and interconnected pore network.

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- Scaffolds exhibit suitable *in-vitro* environment for osteoblast adhesion and proliferation.

Keywords: Hydroxyapatite, sol-gel, inverted colloidal crystals, bone tissue engineering

1. Introduction

Bone tissue engineering requires the development of 3D scaffolds that provide the necessary support for cell adhesion, proliferation and differentiation, and whose architecture contributes to cell migration and regenerative activity through an adequate supply of oxygen and nutrients [1]. As biocompatible scaffolding materials, calcium phosphates, in particular hydroxyapatite, are frequently used due to their chemical similarity with the inorganic phase of human's bone and their bioactivity, noninflammatory and osteoinductive properties [2]. Although a number of scaffold fabrication technologies have been employed, non-uniform pore structures with limited interconnectivity are shortfalls recurrently reported [3]. Inverted colloidal crystal (ICC) systems have been recently proposed for the production of isotropic 3D structures with uniform pore size and high interconnectivity, higher diffusion rates and uniform cell distribution [4, 5].

In this work, a simple method for the design of 3D HAp scaffolds exhibiting the ICC architecture is suggested. Combining a polymeric colloidal crystal (CC) template with a ceramic sol-gel route, we present a procedure for the production of scaffolds with high porosity and controllable pore network along with the morphological, chemical and in-vitro characterization of the resultant products.

2. Materials and Methods

The CC template was prepared by annealing at 130°C for 4 hours the (270 $\pm$ 8) μ m diameter polystyrene (PS) microspheres, produced using a microfluidic apparatus, placed in a cylindrical mould and self-assembled into a hcp structure

under orbital agitation. The CC template was infiltrated with the HAp + binder gel mixture under vacuum and heated for drying (at 75°C), template removal (450°C and 550°C) and sintering (600°C, 800°C or 1100°C). HAp was produced through a sol-gel route using ethanolic solutions of calcium nitrate 4-hydrate and phosphorous pentoxide as precursors [6]. The binder consisted of a 25 wt% poly(vinyl acetate) solution in acetone. A schematic representation of ICC production is shown in Fig. 1. The ICC scaffold was characterized by scanning electron microscopy (SEM) for morphological assessment, Archimedes method for porosity evaluation, X-ray diffraction (XRD) for calcium phosphate phase composition, crystal size and crystallinity calculation, Fourier-transform infrared spectroscopy (FTIR) for chemical analysis and in vitro culture and fluorescence microscopy of human osteoblasts for cell adhesion and proliferation. Further details of the experimental procedure can be found in the supplementary material.

Fig. 1: Production of HAp ICC scaffolds: 1) Microspheres and colloidal crystal template construction; 2) Sol-gel synthesis and template infiltration by vacuum; 3) ICC production in a single step of spheres calcination and ceramic matrix formation .

3. Results and Discussion

3.1. Ceramic ICC scaffold

Fabrication of the ceramic ICC implied a single-step procedure of sphere removal and sintering, composed of 4 temperature stages. Gel drying occurs during the first stage with the loss of solvent and physically adsorbed water leading to a solid structure able to maintain its shape. In stage 2, the temperature above PS and PVAc glass transitions (supplementary material Fig. S2 and S3) caused the PS template softening. Above 250°C, pyrolysis of the dried gel molecular network began. Pyrolysis created tiny "holes" in the skeletal network that contributed to pore volume and surface area increase with no associated shrinkage. At the hold temperature the pyrolysis of organics was complete and HAp crystallization began. At stage 3, the PS template was completely calcined. The ICC became a porous structure composed by an assembly of ceramic particles.

The thermal treatment's last stage ensured the sintering, during which the majority of the densification and microstructure development took place. The plateau temperature was chosen to ensure that crystalline HAp was present (Fig. S1). At the lower temperatures (600°C and 800°C) the densification was not effective. At the higher temperature (1100°C), the ICC's present a fully interconnected structure with 80 vol.% porosity and pore sizes of $(196 \pm 13)\mu\text{m}$ (Fig. 2A). The scaffolds also present sub-micrometer porosity and roughness on their surface (Fig. 2C).

3.2. Matrix characterization

In order to characterize the ceramic matrix that composes the ICC scaffolds, DRX and FTIR analysis were conducted in the samples sintered at different temperatures (Fig.3). From the diffractograms it is possible to infer that temperature plays an important role on the formation of crystalline hydroxyapatite. As the sintering temperature is increased from 600°C to 1100°C, several of the HAp peaks become more distinct and narrower which suggests an increase in

Fig. 2: Images of HAp ICC scaffolds before (SEM) and after (Fluorescence) 10 days cell culture incubation. SEM images of the ICC showing its macroporosity (A), pore walls (B) and microporosity (C). Fluorescent micrographs of human osteoblasts (SaOs-2) on the HAp ICC (D and E); F - Proliferation assay with corrected absorbance.

crystallinity. Crystal size growth is verified from (25 ± 3) nm to (47 ± 5) nm and (62 ± 3) nm for $T=600^{\circ}\text{C}$, 800°C and 1100°C , respectively. Moreover, the crystallinity degree augments from 31% to 51% and 98% for the same temperature range.

Using stoichiometric hydroxyapatite it should be possible to sinter, without phase purity problems, at temperatures up to 1300°C [7]. However, a minor misbalance in the calcium/phosphorus ratio (from the standard molar ratio of 1.67) can lead to the appearance of additional crystalline phases during heat treatment. It can be seen that β -TCP ($\beta\text{-Ca}_3(\text{PO}_4)_2$) appeared at all the sintering temperatures. As the temperature rises to 1100°C a fraction of β -TCP phase transforms into α -TCP ($\alpha\text{-Ca}_3(\text{PO}_4)_2$). The CaO phase was observed after sintering at 800°C and above. This data indicates that in this experiment, as the sintering temperature increased, HAp partly decomposed into β -TCP/ α -TCP and CaO, following the reaction proposed by Fahti et al. [8]:

These calcium phosphates have different solubilities (HAp being the least soluble) [7], which in the case of ICC matrix biphasic composition can influence the scaffold dissolution/bioresorption rate in *in vivo* applications.

In Fig.3B the FTIR spectra of HAp ICC's sintered at different temperatures is presented. The most characteristic chemical groups in a HAp FTIR spectra are PO_4^- , OH^- and CO_3^{2-} . The first indication of the formation of HAp is in the form of broad band centered at about 1000 - 1100 cm^{-1} . The bands at 960 - 965 cm^{-1} and at 560 - 601 cm^{-1} correspond to the symmetric P-O stretching vibration of the PO_4^{3-} ion [9]. CO_3^{2-} group forms a weak peak at 877 cm^{-1} and a wider band between 1459 and 1546 cm^{-1} that suggest the presence of a type A carbonated hydroxyapatite (hydroxyl ions are replaced by carbonate ions)[8]. At 1100 °C the vibrational assignment of CO_3^{2-} is absent from the FTIR spectra evidencing that carbonate substitution no longer exists. The bands assigned to the stretching modes of hydroxyl groups of HAp (3571 cm^{-1} and 632 cm^{-1}) can also be observed in the spectra [9].

3.3. Biocompatibility evaluation

In vitro tests demonstrated the nontoxic nature of the produced HAp ICC scaffolds. One day after cell seeding, cells showed (56±7)% of adhesion. The osteoblasts adhesion onto the ICC surface is a necessary step before proliferation and further differentiation. Throughout the 10 days of culture, an increase in the resazurin test absorbance values was observed, corresponding to a proportional increase of the cell number (Fig. 2F). This shows that the samples were able to promote cellular proliferation. Fluorescence microscopy confirmed that osteoblasts were able to attach, proliferate and inhabit the scaffolds. The abundance of stained cells (bright spots corresponding to cell's nucleus) indicates a good viability (Fig. 2D and E). .

4. Conclusions

Ceramic porous scaffolds with ICC geometry were successfully produced based on a simple sol-gel route. The fabricated scaffold composed of HAp/ β -TCP showed an ordered pore structure that inversely replicated the microspheres' template, exhibiting macropores sizes of 196 μm in diameter and a porosity of about 80 vol.%. Osteoblasts ability to attach and proliferate within the scaffold reveals a favorable environment for bone ingrowth. Thus, the structures designed present interesting features that upon further development can lead to new products for bone tissue regeneration.

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Fig. 3: Diffractograms (A) and FTIR spectra (B) of HAp ICCs sintered at 600°C, 800°C and 1100°C. The diffractograms show the increase of crystallinity of the ceramic but also the evolution of the composition towards a HAp/ β -TCP biphasic ceramic with increasing sintering temperature.