Research Networking Programmes

Short Visit Grant ☐ or Exchange Visit Grant ☒

(please tick the relevant box)

Scientific Report

The scientific report (WORD or PDF file – maximum of eight A4 pages) should be submitted online within one month of the event. It will be published on the ESF website.

***Proposal Title**:* A novel bilayer silk/silk-nanoCaP scaffold for osteochondral pathology.

Application Reference N°: 5059

1) Purpose of the visit

Articular cartilage disorders, are painful, debilitating and disabling clinical problems affecting millions of people worldwide. These lesions are currently treated sub-optimally in the clinics due to the inherent biology of cartilage tissue. Several studies had demonstrated that the culture in 3D systems has an important chondrogenic and osteogenic potential, and allows to construct a solid structure for the implantation of cells in the lesions. The use of biopolymers from natural origin, such as silk fibroin (SF) has increasing interest for bone and cartilage tissue engineering owing their similarities with the extracellular matrix, chemical versatility, and good biological performance without toxicity or immunological reactions. On the other hand, calcium phosphates, namely β-tricalcium phosphate (β-TCP), are generally used as bioresorbable fillers due to their favourable osteoconductivity, resorbability and biocompatibility. Benefits are expected from doping β-TCP with trace elements existing in bone tissues, known to improve cell-material interactions and the strengthening of the mechanical properties of the biomimetic materials.

The goal of the 3B's Research Group is to develop novel biomaterials based on natural origin biodegradable polymers for applications in tissue engineering of bone and cartilage. This is accomplished by developing several isolation/purification tools and a wide range of nonconventional processing methods, allowing the fabrication of integrated biomaterials.
The aim of this project is to study the physicochemical properties of doped powders and the combination of doped powders with SF for create a scaffolds; moreover evaluate their

biological performance for osteochondral regeneration. It has been a good opportunity to participate in a complete process of fabrication and evaluation of scaffold and acquire knowledge about this area.

2) Description of the work carried out during the visit

a) Preparation and characterization of β-TCP powders doped with Zn, and/or Sr, and Mn.

Zn and/or Sr, and Mn doping b-TCP powders will be prepared by aqueous precipitation from using chemical precursors. (Ca+Sr)/P molar ratio equal to 1.48 for all the compositions to ensure no formation of hydroxyapatite (HAp). The precipitated suspensions will be kept for 24 h under constant stirring conditions and ripened for a further 24 h under resting conditions, at room temperature, and the pH must be 7. The resulting precipitates will be vacuum filtered and dry at 100 ºC, for 48 h. The as precipitated powders will be characterized before and after heat treatment at 1100ºC, for 2 h. Finally, the heat treated powders will be grounded to fine powders and sieve. Powders characterization will be evaluated by: (i) X-ray diffraction (XRD) to analyse the crystalline phases composition, and, (ii) Fourier Transform Infrared Spectrometry (FTIR) to obtain the infrared spectra of powders.

b)Fabrication and characterization of SF / doped TCP.

SF will be extracted from Bombyx mori cocoons by boiling the cocoons in a 0.02 M Na2CO3 solution for 1 h, and then rinsing with distilled water. The resulting SF will be dissolved in 9.3 M LiBr at 70 ºC for 1 h, and then dialyzed against distilled water by using an benzoylated dialysis tubing for 48 h to remove residual LiBr. Afterwards, the SF solution will be concentrated by dialysis in a 20 wt.% of PEG solution, to yield a solution of 16 wt.%.
SF/doped β-TCP scaffolds will be prepared using a solvent casting and particulate leaching technique followed by freeze-drying, as reported by Yan et al. [Nanomedicine, vol. 8, 2012; JTERM, vol.6, 2012]. Briefly, doped β-TCP will be mixed into NaCl particles and pouring the SF solution over the mixture and allow it to solidify at room temperature. After, the salt will be extracted by immersion in distilled water for 2 days. Finally, the scaffolds will be frozen at -80 ºC followed by lyophilization in a freeze-drier for 24 h to obtain porous scaffolds.
The characterization of the scaffolds will involve: (i) FT-IR (ii) morphological and morphometric analysis using SEM and Micro-Computed Tomography (µ-CT), (ii) degradation behaviour of the scaffolds after immersion in PBS.

c) Citotoxicity assay.

Human adipose stem cells (ASCs) were expanded using low-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% v/v Antibiotic/Antimycotic at 37°C under humidified atmosphere of 5% v/v CO2 in air. Culture mediums will be changed twice a week until day 7. At days 0, 7, scaffolds will be recovered . Cell studies: (i) DNA content, (ii) metabolic activity.

All graphed data presented as mean +/-SD from at least three experiments used two tailed Student´s T test. P-values <0.05 (*) and <0.01 (**) were considered statistically significant in all cases.

3) Description of the main results obtained

a) Characterization of β-TCP powders doped with Zn, and/or Sr, and Mn.
Zn and/or Sr, and Mn doping βTCP powders were prepared by aqueous precipitation from the starting chemical precursors. Undoped βTCP powder was prepared by the same procedure as a control.
The X-ray diffraction patterns (XRD, high-resolution Rigaku Geigerflex D/Mac, C Series diffractometer, Tokyo, Japan) were analyzed to observe the sample purity and phase identification of a crystalline material. Crystallographic identification of the phases of synthesized powders was accomplished by comparing the experimental XRD patterns with standards ICDD PDF 01-072-7587of βTCP and 01-081-2257 of βCCP. Undoped and doped powders heat-treated at 1100°C (Fig.1) consist of pure βTCP phase without any detected amorphous phase. It was observed that all the powders presents a typical distribution of intensity peaks of βTCP between 25 - 34°, with the highest peak in 31°, the second and third in 34.7° and 27.7° respectively.

In order to identify the molecular arrangement of the powders, Fourier transform infrared spectroscopy (FT-IR) under an attenuated total reflectance (ATR) model (IRPrestige-21, Shimadzu, Kyoto, Japan) analysis was performed. For this purpose, each powder was mixed with KBr in the proportion of 1/150 (by weight) and pressed into a pellet using a hand press. The FT-IR spectra of the powders (Fig. 2) showed the characteristic vibrational modes of PO4 tetrahedra, the presence of two vibrational PO4 bands at around 500 and 600cm−1, and another vibrational band in the range of 1000–1100 cm−1 in all the cases, confirming the formation of βTCP as the predominant crystalline phase. Also, we could see that after the calcination process at 1100°C, disappear vibrational bands characteristic of the presence of water (1600 and 3100 cm−1) and nitrate (1380 cm−1), which was present in the starting chemical precursors, and OH- group (3570 cm-1) and HPO42- (875 cm-1) bands completely disappear, which are characteristical for hydroxyapatite.

b) Characterization of SF / doped TCP.
Doped β-TCP/SF scaffolds were prepared using a solvent casting and particulate leaching technique followed by freeze-drying, and were designed to have 70 wt.% of SF and 30 wt.% of doped powders. Undoped β-TCP/SF (70:30 wt.%) and SF (100 wt.%) scaffolds were prepared as control.

The structural and chemical conformation of scaffolds was analyzed by XRD pattern (Fig.3). Characteristic peaks of silk-II structure located at 20.5 were detected for all the scaffolds, and the broad peak width and low intensity indicate that the SF was of low crystallinity, comprising an uncertain amount of random coil. It was observed that the powders were successfully incorporated in the SF scaffolds, like show the XRD intensity peaks between 25 - 34°, and this fact did not change its structure of β-sheet conformation, which is critical for the maintenance of the mechanical properties and structural stability of the scaffolds.

The morphological features patterns of the scaffolds were analyzed by scanning electron microscopy (SEM, Nova NanoSEM 200; FEI, Hillsboro, OR, USA). The results showed macro and micro porous structure, with macro-pores highly interconnected with a size around 500 μm, while the trabeculae of micro-pores have a size range of 1- 10 μm. It can be also observed a homogeneous distribution of TCP into the SF scaffold (Fig. 4).

Micro-computed tomography (Micro-CT) was used to qualitatively and quantitatively evaluate porosity and powders distribution profile in the scaffolds. The scanning of the scaffolds was conducted under 49.6 keV and 200.8 μA in the micro-CT (1072 scanner; SkyScan, Kontich, Belgium). The integration time was fixed at 1120 ms. Qualitative visualization of the 3-D morphology and the different phases in the scaffolds were performed using CTvox software (Skyscan). The porosity and powder content distribution profiles were processed in standardized software (CT Analyser, version 1.5., Skyscan). The 3D images showed high and homogeneous porosity and macro-pores interconnected, in concordance with the results of SEM. Moreover, it was found that the powder distribution was homogeneous in the scaffold (Fig.5).

The quantitative results of micro-CT (Table 1) showed that the scaffolds presents an adequate porosity index (total porosity >50%) and pore size (200-300nm), which is favorable for cell proliferation, nutrients exchange and new tissue formation in bone regeneration. The open porosity (interconnected pores) is high, desirable feature in the scaffolds structure because is necessary that pores connect with other pores, creating a three dimensional network through which cells can migrate. Trabecular thickness is less than 500 nm, which makes that the scaffold has a similar structure to the extracellular matrix, where the collagen fibers form structures of 200-500 nm.

Degradation behavior of the scaffolds was assessed after immersion in PBS (pH 7.4) at 37°C during 30 days. Samples were recollected at 3, 7, 14 and 30 days after immersion, rinsed with distillated water several times and dried in an oven at 37°C (2 days). The weight loss ratio was determined using the following equation:

Weight loss ratio: [(minitial - mfinal) / minitial] x 100%

The results showed that since the first point at day 3 the scaffolds that combine doped and undoped powders with silk exhibit less degradation compared with the scaffolds composed only by silk (P-value <0.05). After 30 days of incubation, the weight loss value of all the SF/powders scaffolds presented no significant differences (P-value >0.05) among them, however, presented significant differences in comparison with SF scaffolds (P-value <0.01). While SF scaffolds suffer weight loss of 20%, the SF/powders scaffolds just lost between 1-2%, so that, the presence of doped and undoped TCP into the scaffolds lead to a significant decrease of their weight loss. The study of the degradation behaviour of scaffolds is of great importance to predict their in vivo stability.

c) Citotoxicity assay.
Cell viability can be monitored by doublestranded DNA (dsDNA) synthesis. To obtain DNA, cells were lysed by osmotic shock and DNA was measured using the Quant-iT PicoGreen dsDNA kit (Molecular Probes, Invitrogen) accordingly with manufacturer's instructions. DNA content significantly increased from day 3 to day 7 (P-value <0.01), however, we could not find differences in DNA content between the differents scaffolds

along the time. Metabolic activity was measured using AlamarBlue® Cell Viability Reagent (Invitrogen) accordingly with manufacturer´s instructions, and normalized by their corresponding DNA contents. At day 7, TCP/SF and ZnSrTCP/SF scaffolds showed higher metabolic activity (P-value <0.01) than MnTCP/SF scaffolds, probably, due to a more suitable characteristics for cell growth. Note: Because of time problems, only it has been able to do the cytotoxicity assay in TCP/SF, ZnSrTCP/SF and MnTCP/SF scaffolds.

Conclusions

Doped and undoped β-TCP/SF scaffolds prepared using a solvent casting and particulate leaching technique followed by freeze-drying, have a suitable pore size, high porosity, high interconnectivity and good degradation behavior, which are important for supporting cell ingrowth, migration, proliferation and nutrient transportation. Moreover, the scaffolds supported the attachment, viability and proliferation of ASC in vitro, data clearly demonstrated that the scaffolds not presented cytotoxicity.

To conclude this work, it is necessary to perform the cytotoxicity assay with SrTCP/SF, ZnTCP/SF and SF scaffolds, and seed cell in the scaffolds for long time periods for to induce differentiation into bone and cartilage.

4) Future collaboration with host institution (if applicable)

It is necessary to perform some experiments for to finish the work and publish a paper, I will be in constant contact with the host institution for to participate in all the process and in the elaboration of the paper. We want to establish a long time collaboration and work together in future works.

5)* Projected publications / articles resulting or to result from the grant *(ESF must be acknowledged in publications resulting from the grantee's work in relation with the grant)

Approved oral communication in congress:

S. Pina, G. Jiménez, R.F. Canadas, A.P. Marques, J. M. Oliveiraand Rui L. Reis. "Calcium Phosphates-based Biomaterials with Sr- and Zn-Dopants for Osteochondral Tissue Engineering ". 4º TERMIS world Congress: Boston, MA, USA, 8-11 Sept 2015.

Expectations to publish a paper when all the experiments are finished.

6) Other comments (if any)

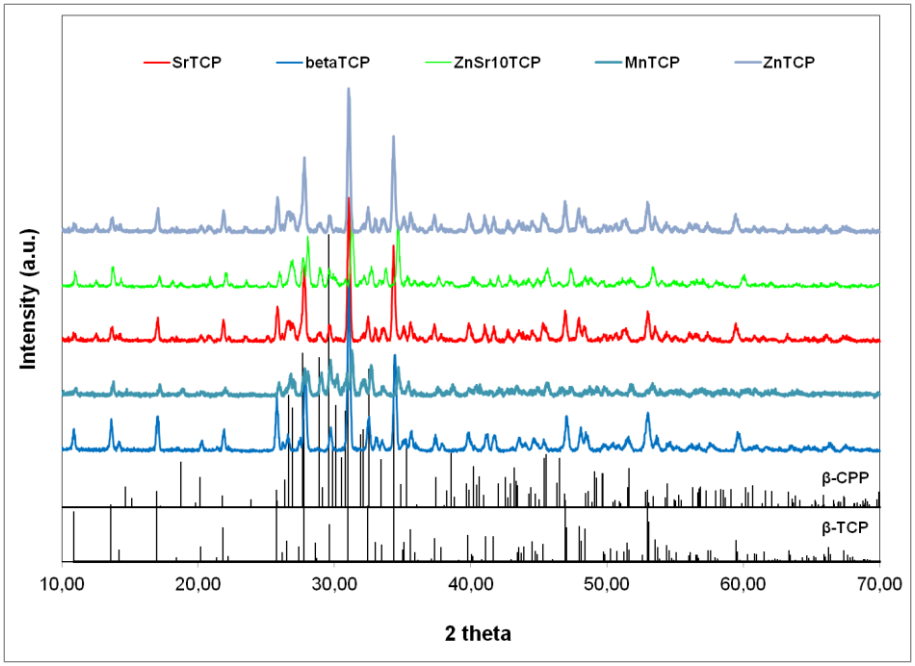

Fig.1. XRD patters for doped and undoped powders obtained after heat treatment at 1100°C.

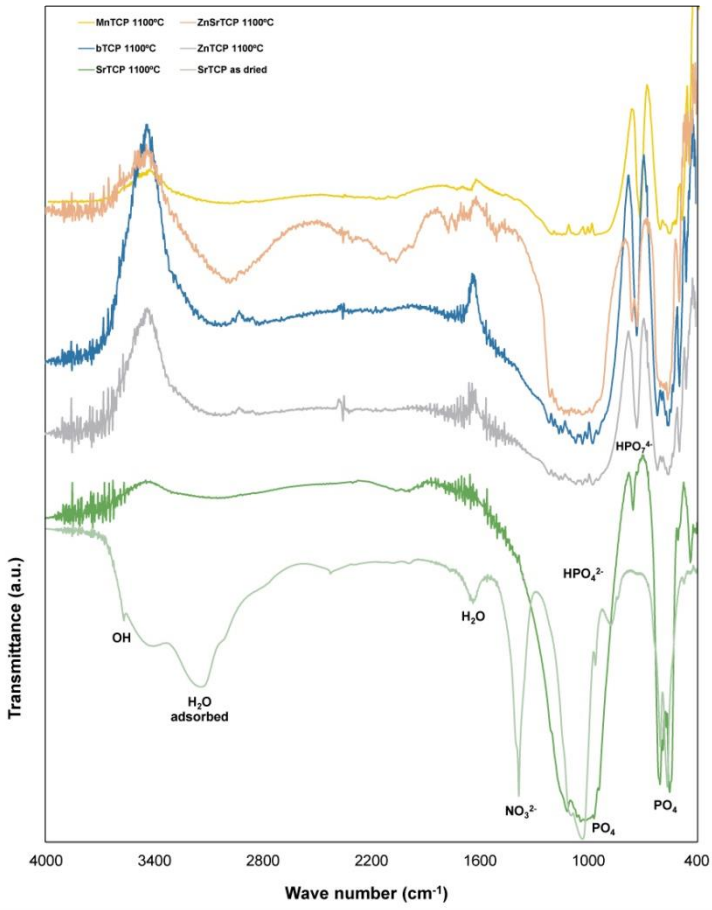

Fig.2 . FT-IR for SrTCP powder, before and after heat treatment at 1100ºC

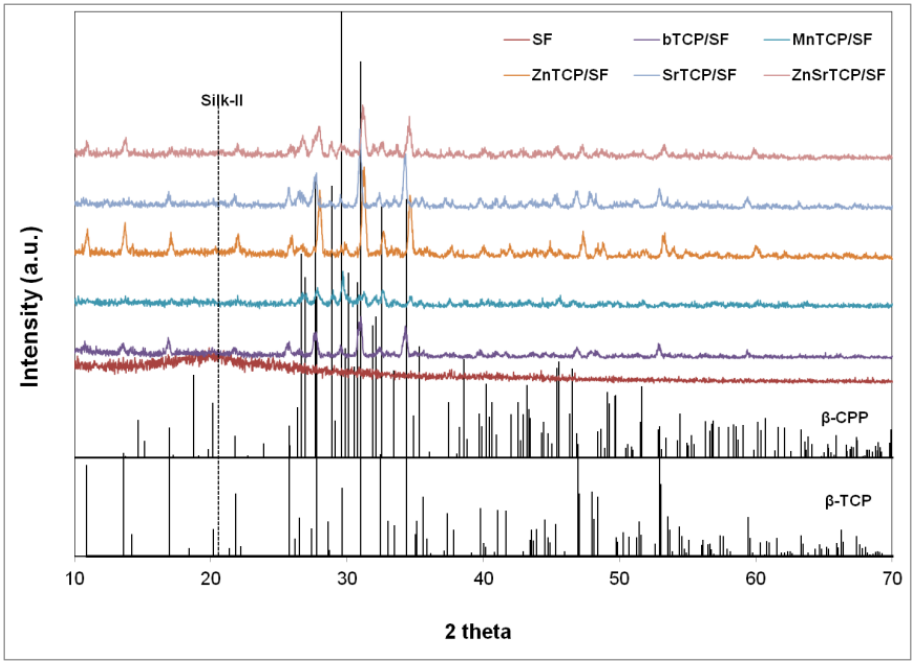

Fig.3. XRD patters for doped β-TCP/SF, undoped β-TCP/SF and SF scaffolds.

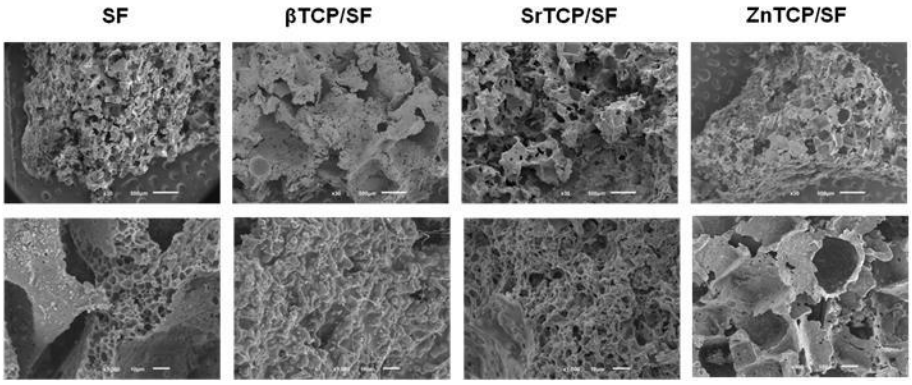

Fig.4. Morphology of SF, doped-βTCP/SF and undoped-βTCP/SF scaffolds determined by SEM.

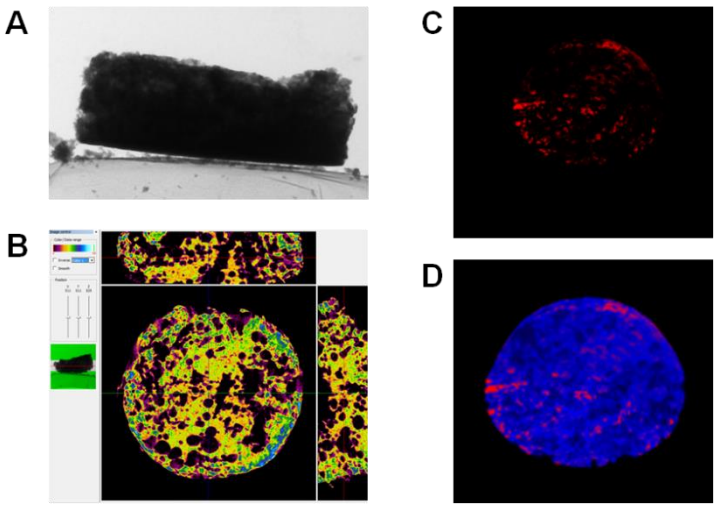

Fig.5. Micro-CT three-dimensional images of SrTCP/SF scaffold. (A) Acquisition, (B) Transversal view in three different axis, (C) 3D reconstruction of SrTCP powder distribution, and (D) SrTCP powder distribution in SF. The results are similar for all powders, we have chosen SRTCP powder as representative model.

Structure	Porosity (%)	Open porosity (%)	Mean pore size (µm)	Mean trabecular thickness (µm)
SF	64.7±2.05	55.6±7.3	236.7±9.9	105.2±5
TCP/SF	39.6±8.7	75.3±3.3	277.3±43.8	180.4±27.2
MnTCP/SF	65.8±9.3	79±2.9	299.7±58.5	130.9±25.7
SrTCP/SF	50.9±5.9	48.6±6	292.5±12.6	222.4±51.1
ZnTCP/SF	46.2±8.9	76.8±3.2	267.1±35.4	183,9±25,8
ZnSrTCP/SF	74.8± 1.85	86.5±0.57	356.5±41	110.5±7.4

Table 1. Microstructure of the scaffolds analyzed by micro-CT.

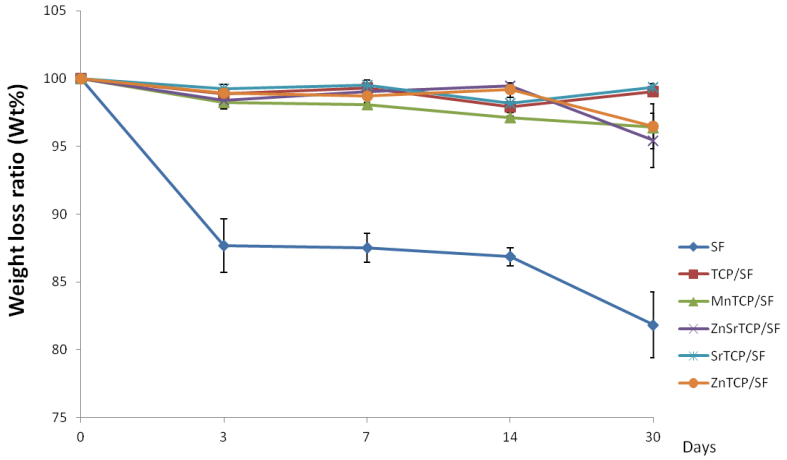

Fig.6. Degradation profile of SF, doped-βTCP/SF and undoped-βTCP/SF scaffolds.

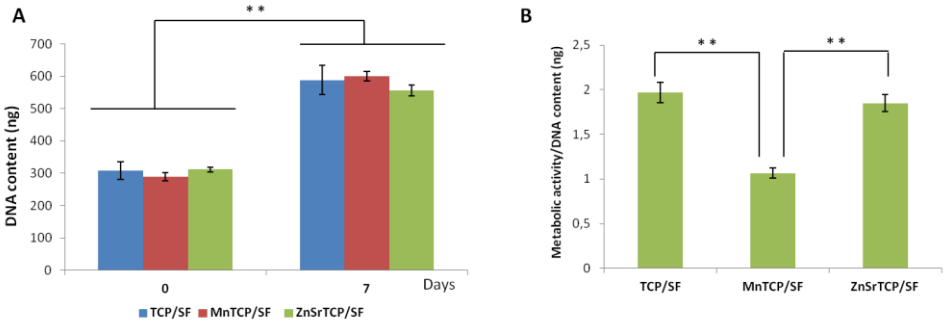

Fig.7. Citotoxicity assay. (A) DNA content, (B) Metabolic activity at 7 days.