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Quantitative analysis of vascular colonisation and angio-conduction in porous silicon-substituted hydroxyapatite with various pore shapes in a chick chorioallantoic membrane (CAM) model

Amandine Magnaudeix [a,b,1], Julie Usseglio [c,1], Marie Lasgorceix [a], Fabrice Lalloue [b], Chantal Damia [a], Joël Brie [a,c], Patricia Pascaud-Mathieu [a], Eric Champion [a,*]

[a] Univ. Limoges, CNRS, ENSCI, SPCTS UMR 7315, F-87000 Limoges, France
[b] Univ. Limoges, EA3842 Homéostasie cellulaire et pathologies, F-87000 Limoges, France
[c] CHU Limoges, Service de Chirurgie Maxillo-Faciale, F-87000 Limoges, France

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ABSTRACT

The development of scaffolds for bone filling of large defects requires an understanding of angiogenesis and vascular guidance, which are crucial processes for bone formation and healing. There are few investigations on the ability of a scaffold to support blood vessel guidance and it this is of great importance because it relates to the quality and dispersion of the blood vessel network. This work reports an analysis of vascularisation of porous silicon-substituted hydroxyapatite (SiHA) bioceramics and the effects of pore shape on vascular guidance using an expedient *ex ovo* model, the chick embryo chorioallantoic membrane (CAM) assay. Image analysis of vascularised implants assessed the vascular density, fractal dimension and diameter of blood vessels at two different scales (the whole ceramic and pores alone) and was performed on model SiHA ceramics harbouring pores of various cross-sectional geometries (circles, square, rhombus, triangles and stars). SiHA is a biocompatible material which allows the conduction of blood vessels on its surface. The presence of pores did not influence angiogenesis related-parameters (arborisation, fractal dimension) but pore geometry affected the blood vessel guidance and angio-conductive potential (diameter and number of the blood vessels converging toward the pores). The measured angles of pore cross-section modulated the number and diameter of blood vessels converging to pores, with triangular pores appearing of particular interest. This result will be used for shaping ceramic scaffolds with specific porous architecture to promote vascular colonisation and osteointegration.

Statement of Significance

An expedient and efficient method, using chick embryo chorioallantoic membrane (CAM) assays, has been set up to characterise quantitatively the angiogenesis and the vascular conduction in scaffolds. This approach complements the usual cell culture assays and could replace to a certain extent *in vivo* experiments. It was applied to silicon-substituted hydroxyapatite porous bioceramics with various pore shapes. The material was found to be biocompatible, allowing the conduction of blood vessels on its surface. The presence of pores does not influence the angiogenesis but the pore shape affects the blood vessel guidance and angio-conductive potential. Pores with triangular cross-section appear particularly attractive for the further design of scaffolds in order to promote their vascular colonisation and osteointegration and improve their performances.

1. Introduction

The dynamic process of angiogenesis is driven by molecular and cellular mechanisms and corresponds to the growth of new blood vessels from the existing vasculature, requiring the proliferation and migration of constituent endothelial cells [1]. Angiogenesis is crucial for bone formation and repair processes [2] since the

* Corresponding author at: Université de Limoges, CNRS, SPCTS, UMR CNRS 7315, Centre Européen de la Céramique, 12 rue Atlantis, 87068 Limoges Cedex, France.
E-mail addresses: amandine.magnaudeix@unilim.fr (A. Magnaudeix), julieusseglio@hotmail.com (J. Usseglio), marie.lasgorceix@etu.unilim.fr (M. Lasgorceix), fabrice.lalloue@unilim.fr (F. Lalloue), chantal.damia@unilim.fr (C. Damia), joel.brie@unilim.fr (J. Brie), patricia.pascaud-mathieu@unilim.fr (P. Pascaud-Mathieu), eric.champion@unilim.fr (E. Champion).
[1] These authors contributed equally to the work.

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administration of an anti-angiogenic molecule (TNP-740) in a rat model of femoral fracture prevents bone healing [3]. The new blood vessels are of prime importance for the supply of growth factors and cytokines involved in cell signalling, chemo-attraction and guidance of progenitor cells, as well as, the transport of phosphate and calcium for mineralisation [4]. Through its communicative functions, the vascular network is required for osteogenesis and it is thus spatially and temporally linked to bone formation [5]. This mechanism is involved in the success of the bone healing in the clinical context of critical bone defects requiring the implantation of porous bone substitutes, considering their biointegration by driving the different actors playing role in bone healing diminishing the inflammatory response [6]. In this context, another crucial but underestimated parameter is the angio-conductive properties of scaffolds to promote the angiogenesis process and improve the vascular supply [7]. Consequently, the concept of these vascularisation parameters must be taken into account in models describing the properties of ideal bone substitutes [1,8].

Among biomaterials with the highest biocompatibility, and owing to its chemical composition close to that of the bone mineral, hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is the material of choice for the development of bone substitutes [9] that has been used in bone surgery since the 1980's. HA and its related compounds have been chosen for their osteoconductivity, which has been promoted by the development of scaffold allowing bone cell ingrowth facilitating their osteointegration. For example, custom-made HA implants are used for the repair of large and complex craniofacial bone defects in maxillo-facial and neuro-oncology surgery [10]. Silicon has also been identified as a crucial element in the early stages of bone formation [11]. Subsequently, phosphorus substitution by silicon in HA (silicon-substituted hydroxyapatite, SiHA, $Ca_{10}(PO_4)_{6-x}(SiO_4)_x(OH)_{2-x}$) has been studied *in vitro* and *in vivo* [12,13]. SiHA appears to have a better ability to adsorb proteins [14] and, while controversial [15], it may improve the bioactivity of HA [16]. In addition to studies suggesting its positive role in bone cell proliferation and differentiation [17,18], other investigators have put forward potential roles for silicon ions in angiogenesis [19–21].

Furthermore, through measures of osteogenic gene expression, Kumar et al. [22], have shown that human mesenchymal stem cells are more sensitive to the scaffold architecture than its chemical composition. Indeed, beyond the effects due to the size and the architecture of scaffolds [23,24], cell colonisation of biomaterials is influenced by numerous parameters [25] including chemical composition, (mentioned above concerning differences between HA and SiHA), but also by physical properties [25] such as topography [26,27], surface energy [28], microstructure and porosity [29–31]. Besides these parameters, a number of investigations have highlighted the importance of pore geometry i.e., their size and their shape (for examples, see [32]). Thus, at the micrometric scale, due to the cell size and other constraints owing to their physiology, the minimal required size of pores would be 100 μm. However, larger pores with a size greater than 300 μm are suitable to promote vascularisation [32,33]. Concerning pore shape, theoretical models have been established to relate tissue growth with the geometrical parameters of pores with the significant parameters of influence being the radius of curvature [24,34,35], the convexity [24,36] and the presence and size of angles [34,36].

The chick chorioallantoic membrane or CAM is a highly vascularised extra-embryonic membrane that allows gas exchange for the avian embryo and that starts to develop at embryonic day 4, enlarging to reach a maximum at embryonic day 12 before rearranging until day 18 [37]. It is a biological model mainly developed to test the pro- or anti-angiogenic properties of chemical compounds [38]. The accuracy and feasibility of this model was validated several years ago in the biomaterials field, validating the biocompatibility of materials (i.e., influence of biomaterial implantation on inflammation) and their capacity to be vascularised [39]. To our knowledge, very few investigations have been published using hydroxyapatite based materials onto CAM [40–42]. None of them have focused on angiogenesis, whereas the first of two reports were focused on giant multinucleated cells corresponding to osteoclasts on the implanted materials [40,41], whilst the other was concerned with implant biocompatibility [42]. Consequently, the present study aimed to determine if the geometry of pores in silicon-substituted hydroxyapatite ceramics might influence angiogenesis and material colonisation by endothelial cells contributing to vascular growth. We therefore used the CAM model to evaluate and quantitatively analyse blood vessel colonisation of porous SiHA samples. This paper also describes a two-scale method to quantitatively assess and discriminate angiogenesis and angio-conduction parameters in biomaterial scaffolds using an *ex ovo* model.

2. Material and methods

2.1. Preparation and characterisation of porous SiHA ceramics

A raw powder of hypothetical chemical composition $Ca_{10}(PO_4)_{5.6}(SiO_4)_{0.4}(OH)_{1.6}$ was synthesised by an aqueous precipitation method as described previously [43].

Porous pellets were shaped by additive manufacturing using microstereolithography. Details concerning the elaboration and characterisation of SiHA parts can be found elsewhere [44,45]. Briefly, a UV curable slurry was produced by dispersing the SiHA attrition milled powder in a photopolymerisable organic mixture containing an amine modified polyester acrylate resin reactive at 365 nm (wavelength of the UV source of the equipment), a reactive diluent, a photoinitiator (ethanone, 2,2-dimethoxy-1,2-diphenyl-; EDMD) also absorbing in the range of wavelength of the UV source and a phosphate ester used as an electrosteric dispersing agent. Green multilayer parts were shaped layer-by-layer with an integral projection microstereolithography apparatus developed in the SPCTS Laboratory [46].

Processing parameters, i.e. UV light exposure energy and spread layer thickness, were set at 71.5 mJ cm^{-2} and 250 μm, respectively. The ceramic parts were designed according to various 2D patterns. A first set of designs was produced which consisted of ceramics with three pore cross-sectional sizes and various geometries: circle, rhombus, star, triangle and square with two densities of pores (for each shape of big and small sizes, the cross-sections were, respectively: 0.78 and 0.24 mm^2, 0.62 and 0.14 mm^2, 0.54 and 0.32 mm^2, 0.43 and 0.24 mm^2, 1.12 and 0.37 mm^2) hereafter referred to as low and high densities: resulting implants are presented Fig. 1A and B). For a second set of experiments designed to test the influence of each pore shape on vascular parameters, implants were designed with a unique pore geometry. For all the shapes, the pore size was calculated to have the same surface of available SiHA, the same individual pore surface (cross-section = 0.21 $mm^2 \pm 0.01$) and a homogeneous repartition of pores. This pore surface of 0.21 mm^2 corresponded to an equivalent diameter of 500 μm, i.e., within the range of suited size (300–600 μm) for bone cell colonisation of scaffolds (Fig. 1C and D, see also Fig. 5). Green samples were produced using the same batch of powder. Samples were cleaned post-construction in an ultrasonic solvent bath and then air-dried overnight at room temperature.

The green samples were debinded at 400 °C to remove the organics and further sintered at 1200 °C for 2 h in air atmosphere (Super Kanthal furnace). The final ceramic parts had a densification ratio of 77 ± 1%. The phase purity of SiHA sintered parts was checked by X-ray diffraction and Fourier transform infrared spectroscopy. No other chemical phase was detected other than apatite

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