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Production, characterisation, and cytocompatibility of porous titanium-based particulate scaffolds

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ABSTRACT

Despite its non matching mechanical properties titanium remains the preferred metal implant material in orthopaedics. As a consequence in some cases stress shielding effect occurs, leading to implant loosening, osteopenia, and finally revision surgery. Porous metal scaffolds to allow easier specialised cells ingrowth with mechanical properties closer to the ones of bone can overcome this problem. This should improve healing processes, implant integration, and dynamic strength of implants retaining. Three Ti-6Al-4V materials were metal injection moulded and tailored porosities were effectively achieved. After microstructural and mechanical characterisation, two different primary cells of mesenchymal origin (human umbilical cord perivascular cells (HUCPVC) and human bone derived cells (HBDC) which revealed to be two pertinent models) as well as one cell line originated from primary osteogenic sarcoma, Saos-2, were bestowed to investigate cell-material interaction on genomic and proteome levels. Biological examinations disclosed that no material has negative impact on early adhesion, proliferation or cell viability. An efficient cell ingrowth into material with an average porosity of 25 to 50 µm was proved.

Keywords: Ti-6Al-4V; metal injection moulding; porous; mesenchymal stem cells.

INTRODUCTION

No world-wide statistic is available to monitor global orthopaedic implant epidemiology. However, meaningful data can be gathered from country surveys. In 2009, 676,000 cases of total knee replacement and 327,000 cases of total hip replacements have been listed in United States, of which 45% and 52%, respectively, are concerning less than 64 years old patients [1]. From 54,200 planned chirurgical hips replacement (from 1990 to 2008 in Belgium) 3%, 8.5%, 16%, and 20% underwent revision surgery after 5, 10, 15, and 19 years, respectively [2]. Similar statistics were published for England and Wales for primary hip replacement between 2003 and 2011 (402,051 cases): 12% of patients were above 55 and 5% were revised within 7 years [3]. Additionally, the Finnish Arthroplasty Register reports that only 60% in patients younger than 55 years have a 15-year-implant survival [4].

The number of implant revision replacement procedures, which cost more than primary arthroplasty (~ +35% for a knee implant revision surgery [5]), will go crescendo with population ageing. However, not only the economic point of view should be taken into account but also the patient's well-being and the risk of infections or complications of such invasive procedures.

The two main reasons of implants revision are (1) aseptic loosening (osteolysis secondary to wear) [6] and (2) dislocation (irregularity within the implant's crystal structure influencing its properties) [7]. To overcome osteopenia, one strategy is to strengthen the intimate contact between the implant and the osseous tissue. Titanium and its alloys remain the principal choice of inert metal for load-bearing orthopaedic and

dental applications. The inherent titanium dioxide layer on its surface provides superior biocompatibility, cell adhesion and propagation of specific bone cells [8, 9]. However, compared to the surrounding bone (10-30 GPa) the material has a high Young's modulus (or E-modulus: measure of the stiffness of an elastic material; 110GPa), which leads to stress shielding effect and therefore implant loosening [10, 11]. The adaptation of the mechanical properties therefore seems desirable. Metal injection moulding (MIM) is a powder metallurgical manufacturing technique providing the capability of producing complex shaped parts in high numbers at low costs. During the last decade the processing of oxygen sensitive titanium powders became possible and today even permanent implants are available commercially. Although usually a nearly dense part is targeted the use of powders and a sintering process offer the possibility of porous parts, too. For this, space holders can be used which are removed during further processing (*e.g.,* [12, 13]) or coarse powder combined with an incomplete sintering process ([14]). However, MIM application is rather novel in this field and not well developed, yet.

In this study a titanium-based (Ti-6Al-4V) isotropic porous material produced by MIM of coarse powders was developed, exhibiting mechanical properties closer to bone than compact titanium materials. In addition the scaffold is mimicking a structure close to the one of bones, and therefore believed to be optimal for bone-cells colonisation and biological anchorage.

Titanium scaffolds for bone regeneration are already successfully employed in preclinical ([15-17]) and clinical studies ([18-20]). However the application of MIM using coarse powders remains rare compared to the other more classical metalworking processes (*e.g.,* 3D fibre (3DF) deposition [16], space holder method [15], and selective

electron beam melting [17]). MIM process allows on the one hand to produce complex parts in a single operation and in high volume and on the other hand to tailor the desired material porosity (and therefore the E-modulus) by adjusting (among other factors) the size and the shape of the metal powder particles [21].

Herein, 3 materials, consisting of different particle sizes combinations leading to different porosities (namely: Fine, Medium, and Large), successfully produced by MIM, are presented. Their mechanical properties were investigated by tensile testing. Subsequently, their cytocompatibility relevance was explored with two different types of human primary mesenchymal stem cells: human umbilical cord perivascular cells (HUCPVC) and human bone derived cells (HBDC). Additionally, the ability of Medium scaffold to serve as possible “bone graft” was examined via Saos-2 cell ingrowth.

MATERIALS AND METHODS

Sample processing and characterisation

Sample preparation

Due to its widespread biomedical application, the alpha-beta alloy Ti-6Al-4V ELI (American society for testing and materials standard (ASTM) B348 Grade 23[22]) was chosen. Spherical gas atomised powders of different sizes were used, purchased either from TLS Technik GmbH & Co (Bitterfeld, Germany; particle size ≤ 45 µm and ranging from 45 µm to 63 µm) or obtained from HZG (Helmholtz-Zentrum Geesthacht;

Germany; 125 to 180 µm powder diameters). In the coarser size fraction some cylindrically shaped particles were found which are residual from the atomising process. Because of their small number compared to the spherical particles they appear to have no significant effect on the mechanical and biological results. Beyond the different manufacturer origin, the powder production differed in the melting process: EIGA (*i.e.,* melting using a watercooled copper crucible) for the TLS powders and PIGA (*i.e.,* melting without any crucible but using pre-alloyed electrode feedstock material) for the latter one.

Three different materials (or porosities) were obtained, namely “Fine”, “Medium”, and “Large” by varying the powder composition (45-63 µm, mixed 125-180 µm + 10 weight percent (wt%) < 45 µm or 125-180 µm powder, respectively - see table 1). From each of those materials two types of specimen were prepared corresponding to the different investigations: (1) “dog-bone shape” (according to ISO 2740 [23]) for mechanical tests and (2) disc-shaped samples (Ø 10 mm; cut from larger discs by means of a punch in the chemical debound state of the samples) for biological tests and microscopic structural analysis.

All powder handling and feedstock production were performed under controlled argon (Ar) atmosphere. As main binder components paraffin wax (Merck KGaA, Darmstadt, Germany) and ethylene-vinyl-acetate (EVA; Basell Polyolefine GmbH, Frankfurt, Germany) were chosen but in various proportions according to the powder used (see table 1). Binder proportions in feedstocks were determined by measuring the tap density of the different powders used (according to DIN ISO 3953 [24]).

The injection moulding step was performed with an Arburg 320S injection moulding machine (Formplast GmbH, Mülheim an der Ruhr, Germany). In order to avoid homogeneity impairment, mass and tool temperature, and injection pressure were optimized for each material. Subsequently, removal of binder was performed by solvent (in heptane, at 40°C for 20 hours) and thermal (around 480°C under protective Ar atmosphere; heating rate of 2 K per minute) debinding. Finally, samples were sintered at 1,300°C for 2 h under high vacuum of about 10^{-4} mbar.

Sample characterisation: microstructure and porosity

The microstructure of the samples was examined using light optical microscopy (LOM; Olympus PMG 3; Olympus, Hamburg, Germany) before scanning electron microscopy (SEM; Zeiss DSM 962, Carl Zeiss, Jena, Germany) analyses. Disc-shaped samples were mounted in an epoxy-base material (Demotec 30, Demotec Siegfried Demel, Nidderau, Germany) and their surfaces were subsequently ground (sander Tegrapol-35 [Struers, Hannover-Garbsen, Germany] under a pressure of 60 N for 1 min at 300 rotations per minute) and finely polished with different silicon carbide wet sandpapers granulations (320 to 2,400 grit sheets [Struers GmbH, Hannover-Garbsen, Germany]).

Photographs obtained by LOM (cross section) were first processed in Adobe-Photoshop CS5 software (Adobe Systems Incorporated; version 12.0.5x32) in order to obtain black (pores) and white (material) images. Derived from methods normally employed to study material grains, porosity was studied with two different modes. First, a line intercept method [25] conducted on LOM micrographs was applied to obtain pore

chord arithmetic mean ($\overline{L}$) and its standard deviation ($\overline{\sigma_L}$). Then, derived from the planimetric method, LOM photographs were analysed by AnalySIS software (Olympus Soft Imaging Solution; version 3.2 build 819). Briefly, from a monochrome image the software is able to determine a continuous area (*i.e.*, connected pore) and by counting the pixels (with calibration factors in X and Y directions) to group the surface areas in different clusters (pore sizes distribution). Here also pore volumes' arithmetic mean ($\overline{\sqrt[2]{F}}$) and its standard deviation ($\overline{\sigma_{\sqrt[2]{F}}}$) were calculated. Moreover porosity was assessed not only via image analysis techniques but also density method. Total porosity - *P(%)* - of each material (see equations below) was obtained via a direct method (*i.e.*, density method): by dividing the density of the sintered part (ρ; ratio of its mass - m – to its geometric volume – V_{geom} –) by the theoretical bulk density of the alloy (ρ_{theo}).

$$\rho = \left(\frac{m}{V_{geom}} \right) \qquad P(\%) = \left(1 - \frac{\rho}{\rho_{theo}} \right) \times 100 \tag{1}$$

Sample characterisation: mechanical tests

Tensile tests were performed on a servohydraulic structural test machine (Zwick-50; Zwick GmbH & Co. KG, Ulm, Germany) equipped with a 50 kN load cell. The tensile tests were carried out at room temperature (RT) and at a strain rate of 1.2 x 10^{-5} s^{-1}. At least three samples of each material (dog-bone shape) were tested.

Sample characterisation: oxygen, nitrogen, and carbon content analysis

The determination of the oxygen and nitrogen contents was performed using a TC-436 analyser (LECO Instrumente GmbH, Mönchengladbach, Germany). Carbon was detected with a CS-444 analyser (LECO Instrumente GmbH, Mönchengladbach, Germany). To get an average, the measurements were performed at three different parts of each sample.

Sample characterisation: solidification shrinkage

The relative shrinkage (S) of the materials was obtained (see equation below) by measuring the length changes of a dog-bone specimen after moulding (L_1) and after sintering (L_2).

$$S(\%) = \left(\frac{L_1 - L_2}{L_1} \right) \times 100 \qquad (2)$$

Biological evaluation

Biological material

All biological tests were performed with primary cells, either human umbilical cord perivascular cells (HUCPVC) or human bone derived cells (HBDC). HUCPVC and HBDC isolations were approved by the local ethical committee and adapted from Sarugaser *et al.* [26] and Gartland *et al.* [27], respectively.

HUCPVC were derived from Wharton's jelly of umbilical cords. The cords were cut into pieces of about 5 cm, the vessels pieces were isolated, and tied together at the

ends with sutures, leading to a vessel loop. These were placed in T-175 cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) and cultured for 10 days without medium change in αMEM (Life Sciences, Karlsruhe, Germany) with 10% FBS for mesenchymal stem cells (Stem Cell Technologies, Vancouver, Canada). After visible outgrowth from the loops, medium was changed every 2-3 days. At about 60% confluency, the cells were harvested with a cell scraper and subcultivated in a density of 1,000 cells.cm^{-2}. For the experiments cells of the third to fifth passage were used.

HBDC were grown out of bone chips obtained from patients undergoing total hip arthroplasty. In brief, cancellous bone was cut into pieces of about 5 mm. After removal of bone marrow and adjacent cells by vigorous vortexing, the pieces were cultured in DMEM Glutamax-I (Life Sciences, Karlsruhe, Germany) with 10% foetal bovine serum (FBS, PAA Laboratories GmbH, Linz, Austria), 1% penicillin, and 100 mg.mL^{-1} streptomycin (Life Sciences, Karlsruhe, Germany) for about 10 days without medium change. At visibility of outgrowing HBDC, medium was changed every 3 days. Passaging was done at 70–80% confluence. Experiments were performed with cells in the 1st passage.

Cell ingrowth into porous titanium-based material was studied with Saos-2 cells purchased from the European Collection of Cell Cultures (ECACC, Salisbury, United Kingdom). This cell line, derived from a human primary osteogenic sarcoma, is known to be a relevant osteoblast cell model in particular for its osteoblastic phenotype during differentiation [28, 29]. Additionally, Saos-2 cells are more robust and, indeed, proliferate faster, thus are a suitable model for ingrowth study purpose. Saos-2 cells were

culture with McCoy's 5a modified medium (Life Sciences, Karlsruhe, Germany) supplemented with 10% FBS. Cells in the third passage were used for the experiment.

All cells were cultivated at 37°C under 5% CO_2 and 95% humidity controlled atmosphere.

Cell biology assessments: initial cell adhesion and proliferation assays

To compare the initial adhesion, 75,000 cells (HBDC and HUCPVC) in 50 µL medium were seeded on the different materials in 12-well plates and left adhered for 30 min. Thereafter the samples were incubated in 3 mL medium for the remaining time (*i.e.*, 30 min, 90 min, and 210 min). After the complete incubation time (*i.e.*, 1 h, 2 h, and 4 h) the samples were transferred into new wells and either MTT ((3-(4,5-Dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich Chemie GmbH, Munich, Germany) tests or DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich Chemie GmbH) stainings were performed. Each material was tested in four replicates and the experiment was repeated 3 times. For cell proliferation assays the methodology remains the same as described previously but the cultures were maintained for 5, 10, or 15 days (with media exchange every 2-3 days), followed by MTT tests.

In each well, 150 µL (1:10) of the MTT solution (5 $mg.mL^{-1}$ MTT in phosphate buffered saline (PBS; prepared as described in [30] with chemicals supplied by Sigma-Aldrich Chemie GmbH, Munich, Germany) was added to the cell supernatant. After an incubation period of 4 h in the incubator the cells were lysed and the purple formazan crystals solubilised by adding 1.5 mL solubilisation solution (10% sodium dodecyl

sulphate in 0.01 M HCl; Sigma-Aldrich Chemie GmbH, Munich, Germany) and overnight incubation. The solubilised formazan product was photometrically quantified using an enzyme-linked immunosorbent assay (ELISA) reader (Tecan Sunrise, TECAN Deutschland GmbH, Crailsheim, Germany) at 570 nm with a reference wavelength of 655 nm.

To perform DAPI staining, the carriers were first washed with PBS. Then, 3 mL of a DAPI solution (1 µg/mL in methanol; Sigma-Aldrich Chemie GmbH, Munich, Germany) was added per well and let incubate for 15 min in cell incubator prior to fluorescent microscopy evaluation (Eclipse Ti-S; Nikon, Düsseldorf, Germany).

Cell biology assessments: differentiation analyses

In these sets of experiments, 50,000 cells (HBDC or HUCPVC; in 50 µL media) were let adhere on each type of samples (n=4; experiment was repeated 3 times) for 30-45 min before adding fresh media (3 mL). After 10 days of culture, the respective culture media were supplemented with factors promoting osteogenic differentiation [31]: 10^{-8} M dexamethasone, 5 mg/mL L-ascorbic acid 2-Phosphat und 10^{-8} M 1α,25-Dihydroxyvitamin D3 (Sigma-Aldrich Chemie GmbH, Munich, Germany). After another week, 5 mM β-glycerolphosphate (Sigma-Aldrich Chemie GmbH, Munich, Germany) was added. The cultures were then maintained for 2, 3, or 4 weeks and media was refreshed every 3-4 days.

Once osteoprogenitors start to differentiate into osteoblasts, a range of genetic markers are transcribed and translated including collagen 1A1 (COL1A1), alkaline

phosphatase (ALP), and osteocalcin (OC; also known as bone gamma-carboxyglutamate (gla) protein [BGLAP]). In order to study the influence of the material and its porosities on cell differentiation, two different approaches were chosen to follow target molecules: on gene and protein levels.

Reverse transcription real-time polymerase chain reaction (RT-qPCR) was chosen to amplify and simultaneously quantify selected genes of both HBDC and HUCPVC (table 2) in order to monitor cell reaction to the different substrates. Prior to total ribonucleic acids (RNA) extraction with RNeasy Mini Kit (Qiagen, Hilden, Germany; a typical RNA purification method using silicon-gel column and centrifugation steps) the samples were washed twice with PBS. The procedures were followed as outlined in the manufacturer's protocol. Shortly, carriers were directly put in contact with a buffer containing guanidine isothiocyanate, a non-ionic detergent, in order that all cells (even the ones in the inner part of the material) were lysed. Homogenisation was performed via centrifugation through a QIAshredder column (Qiagen, Hilden, Germany). Ethanol (96-100%; Sigma-Aldrich Chemie GmbH, Munich, Germany) was added to the flow-through which was in turn applied to the silica-gel membrane (provided in the RNeasy Mini Kit). After several washing steps, total RNA was eluted. No additional deoxyribonuclease (DNase) digestion or ribonuclease (RNase) inactivation treatments were performed. RNA concentrations (optical density – OD – at 260 nm) and purity ($OD_{260/280}$) were measured by a NanoDrop 2000c (Thermo Scientific, Bonn, Germany). RNA integrity and genomic DNA contamination were subsequently verified on 1% agarose gel (2 µg RNA per sample and lane). More stable complementary desoxyribonucleic acid (cDNA) was directly synthesised (2 µg total RNA per sample) using the Sensiscript reverse

transcription kit from Qiagen (Hilden, Germany) without modification of the manufacturer protocol and then stored at -20°C until use. Primers (table 2) and amplicon designs were carefully chosen to ensure specific and efficient amplification and purchased from Eurofins MWG Operon (Ebersberg, Germany). Each qPCR was also assessed and validated (*e.g.*, optimal primer annealing temperature, reaction efficiency (cDNA tenfold dilution series over eight points), and specificity (PCR product checked on 1% agarose gel)). Reaction mixes (20 µL) consisted of 10 µL of SsoFast EvaGreen supermix (Bio-Rad, Munich, Germany), 7.5 µL of RNase free water, 0.25 µL of 1:4 prediluted cDNA, and 1 µL of each primers (250 nM). PCR and amplification monitoring was run in triplicate for each sample using a CFX96 Touch real-time PCR detection system with CFX Manager software (Bio-Rad , Munich, Germany; version 2.1). The thermal cycling conditions were composed of an enzyme activation step performed at 95°C for 3 min then each cycle (40 in total) was composed of a 95°C for 20 sec denaturation step, a 60°C for 20 sec annealing step, and a 75°C for 30 sec elongation step. A melting curve step was inserted to each run to confirm melting temperature (Tm) of PCR products, *i.e.*, qPCR specificity, (65°C to 95°C, increment of 0.5°C for 0.05 sec). As additional control, a non-template control (NTC) was also included in 96-PCR plates. Normalised gene expression (ΔΔC(t)) [32] method was chosen. In this relative quantification approach, gene expression of a target gene (*COL1A1*, *OC*, osteopontin (*OPN*, also known as phosphoprotein 1 or SPP1), osteoprotegerin (*OPG*, *i.e.*, tumour necrosis factor receptor superfamily, member 11b [*TNFRSF11B*]), integrin-binding sialoprotein [*IBSP* or BSP, bone sialoprotein 2], receptor activator of nuclear factor kappa B ligand (*RANKL*, synonym of tumour necrosis factor (ligand) superfamily, member 11

[*TNFSF11*]) is normalised by the one of reference genes (glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*], actin beta [*ACTB*], and ribosomal protein L10 [*RPL10*]) in the same sample and therefore allows comparison of expression of a gene of interest among different samples. Reference genes (table 2) were carefully selected based on the geNorm algorithm method [33] automatically calculated with the CFX Manager software. Due to inherent biological variability between cell culture samples four biological and three technical replicates were performed.

In a following step, OC ELISA, ALP activity measurement, and OC/OPN immunocytochemistry were carried out. The first performed tests refer to OC and ALP measurement assays, both proteins being involved in extracellular matrix mineralisation process. OC release in the cell culture media was measured by an ELISA assay kit from eBioscience (Frankfurt, Germany; Human Osteocalcin Instant ELISA). Briefly, 25 μL of cell culture supernatant (measured in duplicate) was added to microwells containing the pre-adsorbed anti-human osteocalcin monoclonal (murine) and a horse radish peroxidase (HRP) conjugated monoclonal anti-human osteocalcin antibody (murine; capture ELISA) and incubated at RT for 2 h. After several washings steps, a HRP substrate (tetramethyl-benzidine) was added to the wells. The catalytic reaction was then stopped by addition of acid (1 M phosphoric acid) and the absorbance of the formed coloured products was measured at 450 nm (620 nm reference wave length; with a Tecan Sunrise (TECAN Deutschland GmbH, Crailsheim, Germany).

Similarly, ALP activity was assessed in cell culture supernatants (QuantiChrome™ Alkaline Phosphatase Assay Kit; BioAssay Systems, Hayward, CA). This assay is based on the inherent catalytic and kinetic activity of ALP, *i.e.*, removing

phosphate group from ρ-nitrophenyl phosphate resulting in the formation of a coloured product (ρ-nitrophenol; yellow) easily spectrophotometrically measurable (Tecan Sunrise, TECAN Deutschland GmbH, Crailsheim, Germany). The kinetic activity of the ALP enzymes contained in each material cell culture supernatant (measured in triplicate) was normalised to a reference (tartrazine, provided in the kit). Three independent experiments were performed (n=3).

Additionally, immunocytochemistry was performed to target secreted HBDC and HUCPVC OC and OPN on the different materials. Here, cells were fixed in 3.7% formaldehyde (Sigma-Aldrich Chemie GmbH, Munich, Germany) prior to their permeabilisation (0.5% Triton-X [Sigma-Aldrich Chemie GmbH, Munich, Germany] in PBS). After non-specific binding sites blockage (1% Triton-X and 2% bovine serum albumin (BSA; Sigma-Aldrich Chemie GmbH, Munich, Germany) in PBS solution for 2 h at 37°C), the samples were immersed in primary antibody solutions (1:200 in Tris buffer saline or TBS solution [prepared as described in [30]with chemicals supplied by Sigma-Aldrich Chemie GmbH, Munich, Germany] containing 3% BSA), *i.e.*, monoclonal anti-OC and anti-OPN (dianova GmbH, Hamburg, Germany; raised in mouse and in rabbit, respectively) for 1 h at 37°C. Subsequently, the carriers were washed twice with PBS (5 min) and incubated with the secondary antibody solution (goat anti-mouse FITC and goat anti-rabbit Texas Red - dianova GmbH, Hamburg, Germany) again for 1 h at 37°C. Finally a DAPI nuclear counterstain was performed as described above. Labeled samples were observed with a Nikon Eclipse Ti (Nikon, Düsseldorf, Germany) fluorescent microscope.

Cell biology assessments: cell colonisation of porous scaffold

Here, Saos-2 cell ingrowth in porous carrier was studied only with Medium material. Twenty thousand cells in 50 µL medium were seeded on its surface in 12-well plates and left adhere for 30 min before addition of fresh medium (up to 3 mL). After one or two weeks of culture (with media change every 3 days) samples were critical point dried previous to scanning electronic microscope (SEM) evaluation (Auriga; Carl Zeiss, Jena, Germany). In brief, after a glutaraldehyde fixation step, carriers were stained in osmium tetroxide prior to an alcoholic dehydration row. Subsequently, samples were critical point dried in 2-propanol (Sigma-Aldrich Chemie GmbH, Munich, Germany) to preserve cell morphology by a Leica EM CPD300 (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). Cells on carriers were then visualised by low voltage mode in charge contrast, using the SEM in lens detector. Samples were also broken in the middle in order to appreciate cell-sample colonisation along the cross section.

Statistical Analysis.

Statistics were performed using the SigmaStat package (Systat software GmbH, Erkrath, Germany; version 11.0). Standard analysis comparing more than two treatments was done by using the one-way ANOVA (analysis of variance). Depending on the data distribution either a one-way ANOVA or an ANOVA on ranks was performed. Post-hoc tests were Holm–Sidak, Turkey, or Dunn's versus the control group, respectively.

RESULTS

Sample characterisation

Microstructure and porosity

To investigate the material's surface and to be able to estimate their pore diameters, LOM and SEM photographs were taken (Fig. 1).

As directly observable on the pictures for all samples, the shape of the original powder particles is still visible after the sintering process but formation of necks between particles is also evident on SEM micrographs. However, with smaller and more homogeneous particle size the connection between particles is tighter. As expected, the total porosity is increasing with the diameter of the spherical particles employed.

Porosity was assessed via different means: image analysis techniques and density method. In addition various geometrical values were obtained (table 3). Consistent with the previous observations, chord arithmetic mean ($\overline{L}$), pore area's arithmetic mean ($\overline{\sqrt[2]{F}}$), are correlated to the original powder particle sizes used. Thus, Fine exhibits a chord arithmetic mean of about 18 µm and pore volumes' arithmetic mean of 25 µm while these values rise considerably for Medium and Large. However, while for Medium and Large $\overline{L}$ values are alike (45 µm and 47 µm, respectively). With materials' pore size distribution examination (Fig. 2) three observations can be made. First, pores with diameters smaller than 75 µm are the most numerous in all materials with a clear

prevalence in material composed with smallest particle size (*i.e.,* representing 93%, 74%, and 64% of the total pores sizes of Fine, Medium, and Large, respectively). The second trend concerns the distribution of the pores ranging from 75 to 175 µm; here the contrary is observed: there are more numerous in bigger size particle materials (*i.e.,* 7 total percent for Fine, 18 for Medium, and 23 for Large). The last observation is for the pores ranging from 175 to 840 µm. While none are observed in Fine, they reach 11% and 17% for Medium and Large, respectively.

Logically, porosity calculated via density method presents similar results in terms of total porosity. Fine encompasses a porosity (P(%), expressed in volume percent – see table 3) of 15 ± 2, Medium 26 ± 3, and Large 31 ± 2.

Mechanical tests

In order to check if the new produced materials have mechanical properties closer to bone ones, tensile tests were carried out (table 4).

A good correlation between the materials porosities and their ultimate tensile strength (UTS), tensile Young's modulus (E), and elongation to fracture (ε_f) can be found. The maximum tensile stress (UTS) that the Fine material can withstand is decreased of about 41% compared to the standard MIM and HIP MIM processed materials (about 65% and 82%, for Medium and Large respectively). These reduced features are similar for the three materials Young's moduli: diminution of 36%, 58% and even 81%, for Fine, Medium, and Large, respectively. Additionally, yield strength (at 0.2% offset; 0.2 YS) of the materials drops off more considerably: of about 56% for Fine, 72% for Medium, and

was even not measurable for Large because the specimens broke before 0.2% elongation was reached. However, these dramatically decreases have the appreciable advantage to render the materials properties closer to the ones of bones, especially for the most porous material (Large) which presents an UTS of 143 ± 10 MPa (bone UTS 50–100 MPa - 17–20 GPa for trabecular and cortical bone, respectively [34]) and an elastic modulus of 21GPa (bone: 17 to 26 GPa[35]). Similarly, the elongation fracture decreased with sample porosity.

Fracture surfaces of the different materials were also investigated by SEM (Fig.3). On Fine's break plane, ductile breaks are clearly recognised on the area of wide sinter necks. Fracture areas are also observable on Medium and Large surfaces and more sinter necks are apparent with addition of smaller powder size fraction reinforcing the material stability.

Oxygen, nitrogen, and carbon content analyses

Impurity contents are presented in table 5. After MIM processing, O exceeds the ASTM B348 grade 23 which cannot be avoided in the case of MIM processing. However, Medium and Large exhibit extraordinarily high contents of oxygen and especially carbon compared to Fine. Probably the rather brittle tensile behaviour of the specimens observed in this study is partly caused by this high content of interstitials.

Solidification shrinkage

Thermal shrinkages were calculated as the percentage of length loss of the dog-bone specimen after sintering. Fine, composed of the finer particle size is the most subjected to contraction with a loss of size of about 8% (S% = 8.7 ± 0.2). Medium and Large are less affected with a dwindling of 4.5% (± 0.3) and 4% (± 0.3), respectively.

Biological evaluation

Initial cell adhesion and proliferation assays

As initial cell adhesion can have an influence on further cell fate, such as migration, proliferation, differentiation, appropriate functioning or long-term viability, early attachment of HBDC and HUCPVC on the different materials was investigated via MTT test and DAPI staining. In a subsequent step, proliferation was also studied after 5, 10, and 15 days by MTT measurements. In order to provide a better figure readability, statistical analyses are provides as tables (6-9).

Even if some significant differences on the initial adhesion of HBDC cultured on different carriers were determined (please see table 6), mainly MTT absorbances of cells grown on Medium after 2 h are higher than the ones obtained for all materials after 1 h adhesion, no real great disparities can be observed (fig. 4a). Based on DAPI stainings images (Fig. 4b) cells are alive and homogeneously adhered which provide even more support on the fact that HBDC seem to be affected neither by the different porosities nor by the initial adhesion times. Results are slightly different for HUCPVC adhesion assay (Fig. 5a; statistical analyses table 7). Taking the results all together, two main features

can be extracted. First, an increase absorbance, *i.e.,* an augmented cell metabolic activity *i.q.*, a higher cell number, is observable with longer incubation time. Secondly, a fine preference of the cells for the increased materials porosities can be noticed. Additionally, based on the fact that the same amount of cells was loaded, HUCPVC, which also present a homogeneous distribution throughout all material surfaces, seem to adhere better. This is not only supported by the MTT results (enhancement of about 3, 2.5, and 20% after 1 h, 2 h, and 4 h respectively – regardless material types), but also by DAPI staining pictures (Fig. 5b).

Initial proliferation of HBDC (Fig. 6a; table 8) and HUCPVC (Fig. 6b; table 9) was further studied after 5, 10, and 15 days (MTT assays). For HBDC, no common trend can be observed for the different materials and roughly, compared to the initial adhesion measured after 1h, no (or rather small) proliferation was observed (statistical analyses in table 8). For the Fine material, an increased proliferation after 10 days was measured (18% compared to the 1h early adhesion) but decreased again after 15 days (both being statistically significant). A diminished cell activity or growth was even observed for Medium over the 15 days to reach an increased metabolic activity of about 2% compared to the 1 h initial adhesion activity (decrease between Medium 5 days and 15 days is significant). Same tendency is even more apparent for Large with a cell activity inferior after 15 days than the one measured after 1 h. Besides, a preference of HBDC for larger porosities is also statistically significant after 5 days culture. Basically, here as well, HBDC do not seem to be affected by the different materials. The traits are completely different for HUCPVC. For all materials, a graded proliferation ascent over 15 days is discernable (increased cell activity of 26%, 38%, and 42%, after 15 days, compared to the

1 h initial adhesion values, for Fine, Medium, and Large, respectively) which is generally statistically proved (see Table 9). In addition, improved metabolic activity goes along with material increased porosity, *i.e.,* Large exhibits the highest proliferation rate.

Differentiation analyses on gene level (RT-qPCR.

Osteoblastic genotype of HBDC and HUCPVC (Fig. 7) during differentiation was studied by measuring *COL1A1*, *OC*, *OPN*, *OPG*, *IBSP*, and *RANKL* expression levels (qPCR), after 2, 3, and 4 weeks. The expressions of the above named messengers were normalized with reference genes expression (*GAPDH*, *ACTB*, and *RPL10*), therefore both primary cells can be compared on transcriptomic level. Roughly, only the expression levels of *COL1A1* and *OPG* are higher for HBDC and *COL1A1* RNA level increase over weeks for all materials. *OPG* and *RANKL* expressions are in both cell types oppositely fluctuating, especially in the first week differentiation of HUCPVC and in the third of HBDC. Expression of *IBSP* is particularly high in the first week HUCPVC differenciation. Two different tendencies can be observed for *OPN*. First, for HBDC, expression increase over time for all materials and is especially high on the fourth week for Fine and Medium. Conversely, a decrease is observable for HUCPVC (except for Fine material which remains stable).

Differentiation analyses on protein level: OC ELISA, ALP activity measurement, and OC/OPN immunocytochemistry.

In order to study the effect of the materials on the primary cells differenciation, ALP activity and OC release were assessed in cell culture supernatants while productions of OC and OPN were appraised via immunocytochemistry.

The activity of the hydrolases (*i.e.,* ALP) produced either by HBDC (Fig. 8a) or by HUCPVC (Fig. 8b) cultured on the various carriers was measured after 2, 3, and 4 weeks. Initial proliferation of both cell types after 15 days is also reminded on both graphs. For the bone derived cells, statistically difference in ALP catalytic activity is observable between "Fine 2 weeks" and "Medium 5 weeks" and "Large 3 weeks". However, for HUCPVC no (statistically proven) variation in the catalytic activity is observable, neither on time-scale nor on material-type.

A common tendency, *i.e.,* lower OC release in cell culture supernatants in the third week differenciation, appears for both HBDC and HUCPVC (Fig. 9; statistically relevant, please refer to tables 10 and 11). For the HBDC, OC expression, which is similar for all carriers, remains the highest in the second week measurements. Additionally, taking the initial proliferation (after 15 days culture) into account, higher porosity can be linked with elevated OC production. For HUCPVC, OC release is rather different for all materials but its concentration appears the highest after 4 weeks differenciation (statistically proved for Fine carriers). Due to initial adhesion values, OC expression is the highest for the Fine material but the release is still high for Medium and Large.

Finally, parallel stainings *i.e.,* OC (green), OPN (red), and nuclei (blue), were performed with both primary cells on all carriers after four weeks osteogenic differentiation promotion (Fig. 10). As expected, OC and OPN are much more translated

by HUCPVC than by HBDC. For HBDC OC, appearing yellow on the overlayed pictures, is more viewable on Medium immunocytochemistry images. OPN is also present on all materials. For HUCPVC however, yellow areas are much more present (especially for Medium & Large) and all stainings appear more reddish.

Cell colonisation

In order to study cell ingrowth into porous scaffold, Saos-2 cells were seeded on Medium. This sample was chosen according to several studies dealing with optimal pore sizes of a porous material to allow blood and nutrient supply in its inner part [36, 37] and also because it seems the most promising materials. After 1 and 2 weeks culture, photographs (Fig. 11) were taken first on the top of the carriers (where the cells were seeded; “Top view”) and samples were subsequently split in half in order to document cell ingrowth into the materials (“Inside view”). As expected, cells are proliferating on the top of the sample however, also on the inside view, cell proliferation and progression in the inner part of the porous scaffold is observable. Additionally, after one week culture, cells are lining the metal particles and few cytoplasmic projections at neighbour particles are visible. After 2 weeks, these phenomena increased and cells are able to bridge gaps between spheres.

DISCUSSION

Various parameters play an important role in material mechanical properties such as production process and parameters, and for MIM powders particle size, solidification shrinkage, and porosity. During sintering process, changes in the system free energy appear from the decrease in surface area leading to formation of necks between particles, porosity reduction, and material densification. The finer a powder is, the higher is its surface area: it will thus sinter faster (with larger solidification shrinkage) and at lower temperature than a coarser one [38]. Due to its packing density and surface energy, Fine material exhibits the highest sintering activity, the maximal solidification shrinkage (8.7%), and the lowest porosity. Medium, with its addition of fine powder, sinters better than Large, exhibits an intermediate porosity and the lowest solidification shrinkage (4%). Medium and Large pores are less isolated and heterogeneously distributed (rationally as starting powder sizes are rather disparate) than Fine. Nevertheless, the three MIM produced materials exhibit significantly reduced mechanical properties compared to titanium-based denser material. The decrease of tensile strength, Young's modulus, strain-to-failure, and fatigue strength with increase porosity is well documented in the literature [39, 40, 12] and is therefore rather different from the one of the wrought material (ASTM F136 [41] – see table 4 for comparison). One of the porosity enhancement drawbacks is the decrease of interconnected sintered necks leading to the localisation of strain on less numerous bridging areas thus reducing the structure stability. Similarly, pore occurrence and distribution (porosity morphology) can lead to deformation focalisation on pore cluster area..

Interstitial elements can influence the materials mechanical properties. A high content leads to strength increase but loss of ductility. Fine exhibits relatively low levels

of impurities compared to the other ones although all sintering processes took place in the same oven and under the same atmosphere. It can be assumed that at high temperature, Fine has a faster sintering activity and rapid shrinkage leading to a smaller total surface and therefore minor interstitials incorporation (and reciprocally for higher porosities). Additionally, O, C, and N integrations may be explained by the rather high binder proportions, the insufficient binder removal prior to sintering, or the high temperature during debinding (as at these temperatures titanium has a stronger affinity with oxygen and carbon). It has to be notified that typically 0-45 µm sized powders are used in order to have a minimal amount of binder and to ensure complete mould filling with the feedstock [42]. With higher particle size, homogeneity of the feedstock is critical and may lead to material imperfections. Nevertheless, MIM processing allows Fine and especially Medium and Large reduced mechanical properties to be closer to bone ones.

Fine, Medium, and Large exhibit a porosity of about 15, 26, and 31% of their total volume. Pore size distribution of Medium is again a median between Large and Fine. More than 90% of Fine pore size is between 2- and 75 µm with few up to 175 µm. Distribution shifted to larger sizes is observed for Medium and Large (26% and 36% of 75 µm up to 840 µm, for Medium and Large, respectively). It can be assumed as it is described in literature [14],[43-45] and due to the porosity morphologies, that at least Medium and Large are constituted of a network of connected pores.

Such open porosity and interconnection dimensions are essential to promote bone ingrowth, vascularisation, rapid bone regeneration, and osteointegration [46, 47]. While a consensus is difficult to find, pore size between 100 and 400 µm seems necessary to allow blood and nutrient supply within the graft [36, 37]. All of the materials presented

here possess the suitable characteristics and bone-cell ingrowth was proved with Medium carrier. As phenotypic expression of bone cells is mostly influence by the general structure and surface roughness than the surface reactivity [48, 49] this material seems particularly suitable for cell colonisation. Spheres act like a topographic guidance: first the cell are lining the material (cells appear flat and form focal contacts), are subsequently progressing into the material, and are bridging the gaps. Haptotaxis is thus stronger than cell-cell gathering and prove the material compatibility. Another crucial event for efficient further migration, proliferation, and differentiation (*i.e.,* osteointegration) is the cell initial adhesion behaviour [50]. Choice of the cell is a really important issue [51, 28]. As *in vivo* mesenchymal stem cells are the first to migrate to the implant site, HUCPVC and HBDC are both excellent models. Their drawback, as primary cells, is their high, but normal, phenotypic and genotypic variability. For Fine, Medium, and Large initial adhesion is good but no great difference neither between cells nor between materials is observed. Maybe a slight preference for highly porous materials over time can be notified. HUCPVC may adhere faster and better than HBDC. Moreover, HUCPVC and HBDC appear flat and not round, sign of direct surface adhesion. Penchant for large porosity is even more pronounced on further proliferation assays, especially for HUCPVC. However, variation between HUCPVC and HBDC proliferation has to be moderate and should be linked to their different cell metabolisms. Indeed, for HBDC the estimated population doubling time is 7 days [28] against 60 h for HUCPVC [26]. In this case, HUCPVC may be a better model to study initial adhesion and proliferation. Nonetheless, the most important message is that Fine, Medium, and Large exhibit no cytotoxicity and can provide an open-pore microcellular structures highly suitable to

bestow a more intimate contact between the implant and the surrounding osseous tissue. Such materials can be applied to correct small bone defect, non-load bearing indications in orthopaedic or in the field of bone regeneration (especially in dental application, *e.g.*, socket preservation, periodontal regeneration and sinus lift). Additionally, after refining processing parameters in order to get better material mechanical properties another application field can be artificial joints. Due to tailoring porosity possibilities, such materials can be adapted to any joint replacement needs from the knee requiring flexibility and toughness to finger joint which have to be capable of bending easily. Even some porosity gradient can be created in order to fit hip replacement requirements (*e.g.*, sturdiness and ability to bear the body's weight). Additionally, as titanium chemistry is rather well established, more osteoconductive and osteoinductive coating can be combined to create a hybrid cement (*i.e.*, calcium phosphate, hydroxyl apatite or BMP-2 based [52, 53]) / cementless (*i.e.*, porous titanium) implant. Furthermore, its economic efficiency makes MIM a very attractive metallurgical process. The main counterargument may be the difficulty to remove such so-"imbricate" implant however as 42.2% of revision surgeries are caused by instability, dislocation, and mechanical loosening [54] such titanium-based customised implant would definitively reduce this number.

The second batches of biological investigations refer to the analyses of differentiated cells genotypes and protein levels grown on different carriers. These experiments highlighted interesting dissimilarities between the primary cells employed, even if it was not the principal focus of this study. Target genes can be classified in two different categories: (1) genes of proteins implicated in osseous tissue organic matrix

(*COL1A1*, *OC*, *OPN*, and *IBSP*) and (2) genes producing factors for bone homeostasis (*OPG* and *RANKL*). During new bone formation, osteoblasts are first synthesising the organic component of the matrix which is mainly composed of type 1 collagen and in a following step, non-collagenous organic proteins such as OPN, sialoprotein, and OC which colonise the collagenous structure. Subsequently, matrix mineralisation can occur [55]. *COL1A1* expression is increasing during the three observed weeks for both cell types but is higher for HBDC, which can reveal a differentiation delay of HBDC compared to HUCPVC. This assumption takes more weight with the other gene expressions analyses. IBSP constitutes ≈ 12% of the non-collagenous proteins (major structural protein). It is an early marker of osteoblast differentiation. IBSP is having a role in extracellular matrix organization (implicated in hydroxyapatite (HA) nucleation) during *de novo* bone formation [56] and in cell adhesion. HBDC *IBSP* level remains low regardless the material. For HUCPVC, the level is high during the 2nd week (except for Large and especially for Medium) and falls again. Another observation in favour of fast HUCPVC differentiation is the high level of *OC*. OC represents 1 to 2% of the total bone proteins, binds strongly to HA and is a rather late maker of osteoblast maturation [51, 57]. Concomitantly, HUCPVC *RANKL* level on the second week is also high, which is again proof of bone turnover stimulation [58]. RANKL mediates osteoclast lineage commitment. The balance between *RANKL*/*OPG* is highly important [59] as OPG (decoy RANKL/ RANKL receptor) is inhibiting osteoclatogenesis [60]. Therefore, the expression ratio *RANKL*/*OPG* represents an index of osteoclastogenic stimulus. HBDC *OPG* levels are high, with the exception of Large after 4 weeks differenciation induction the latter exhibiting also the highest RANKL level. OPN acts as a cytokine and has

chemotactic properties. As it may have a role in anchoring osteoclast [61], it is also a rather late differentiation marker. Regardless of the material, *OPN* level is quite high for HUCPVC and increase on the third week for HBDC (especially for Fine and Medium).

Even if the direct correlation between mRNA expression and the direct protein-translation level is not always straight forward [62] in general, here, the same conclusions can be drawn on protein expression. OC release reveals the same patterns as observed on gene expression: OC release is higher in the second and the fourth week for differentiated HUCPVC on all materials and Medium and Large for HBDC (which are also higher than Fine). For ALP measurements, taking into account the huge error bar, no difference can be seen between materials and over weeks. ALP is associated with calcification and as its activity is high just before mineralisation, it is described as an early marker of osteoblast differentiation [51]. Thus, here, it is either too early for HBDC or too late for HUCPVC. Immunocytochemistry strengthen the trends already visualised: OC levels are higher for HBDC and HUCPVC, after 4 weeks differenciation induction on Medium. Even if this was not the main goal of this study, some findings regarding the two primary cells used here can be stated. First, HUCPVC can be a good model to replace the use of HBDC. Then, HUCPVC may be more suitable to study cell proliferation. Similarly, their different genotype patterns can orientate the choice of the cell according to the period (or the speed) of osteoblastic differentiation chosen to be studied.

CONCLUSION

Metal injection moulding of Ti-6Al-4V was successfully applied to tailor porosity. The mechanical properties of the new materials were significantly reduced and closer to bone. From the biological point of view, none of the three materials exhibits bio-incompatibility. Additionally materials (especially Medium) reveal themselves to be osteoconductive. Such porous titanium-based materials can be highly valuable, not only in an economic point of view of the metallurgical process but also due to the possibility to customise the material porosity/mechanical properties and its high biocompatibility.

DISCLOSURES

The authors declare no conflict of interest.

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FIGURE CAPTIONS

Fig. 1 Sample characterisation: microstructure and porosity. Microstructure of each samples (Fine, Medium, and Large) were examined using light optical microscopy (LOM; scale bars 500 µm) before scanning electron microscopy (SEM) analyses (scale bars 200 µm).

Fig. 2 Pore size distribution by LOM analysis. For each material (Fine = light grey, Medium = dark grey, Large = black) the continuous area (*i.e.*, connected pore) were clustered in different categories.

Fig. 3 Fracture surfaces. After mechanical test, SEM was used to analyse the fracture surfaces of the broken tensile specimens. Microphotographs on the right column present surface overviews while the ones on the left column allow more detailed visualisation. White and black arrows point ductile breaks and sinter necks, respectively.

Fig. 4 HBDC initial adhesion: (a) MTT measurements (after 1 h [light grey], 2 h [dark grey], and 4 h [black]) and (b) DAPI stainings. To facilitate result lecture, statistical analyses are presented in table 6.

Fig. 5 HUCPVC initial adhesion: (a) MTT measurements (after 1 h [light grey], 2 h [dark grey], and 4 h [black]) and (b) DAPI stainings. To facilitate result lecture, statistical analyses are presented in table 7.

Fig. 6 Proliferation of HBDC (a) and of HUCPVC (b) after 5 (light grey), 10 (dark grey), 15 days (black) (MTT measurements). As point of comparison MTT absorbances after 1 h initial adhesion is remembered (scales on the right, grey points) Statistical analyses are presented in tables 8 and 9 for HBDC and HUCPVC, respectively.

Fig. 7 Real-time PCR. Osteogenic differentiation of HBDC and HUCPVC (grown on Fine, Medium, and Large) was promoted for up to 4 weeks. Expression levels of *IBSP*, *OC*, *OPN*, *COL1A1*, *OPG*, *RANKL*, *ACTB*, *GAPDH*, and *RPL10* were studied after 2 (light grey), 3 (dark grey), and 4 weeks (black). Expression of each gene was normalised with reference genes ones and are presented as normalised fold expression.

Fig. 8 ALP content measurements. Osteogenic differentiation of HBDC and HUCPVC (grown on Fine, Medium, and Large; n=4) was promoted for 2 (light grey), 3 (dark grey), 4 weeks (black) and ALP activity was assessed in cell culture supernatants. MTT absorbances after 15 days proliferation is remembered (left scale, grey points). Stars indicate statistically significant difference between two groups (P = <0.05).

Fig. 9 OC content measurements. OC release in HBDC and HUCPVC culture supernatants was measured after 2 (light grey), 3 (dark grey), 4 weeks (black) osteogenic differentiation (for Fine, Medium, and Large). MTT absorbances after 15 days proliferation is remembered (left scale, grey points). Statistical analyses are presented in tables 10 and 11 for HBDC and HUCPVC, respectively.

Fig. 10 Immunocytochemistry. OC (green) and OPN (red) were targeted in HBDC and HUCPVC on the different materials thanks to specific labelled antibodies. Due to merged pictures, OC appears yellow. Nuclei were DAPI-stained and arise blue.

Fig. 11 Material colonisation. Saos-2 cell ingrowth into the Medium scaffold was investigated by SEM, after 1 and 2 weeks. Microphotographs were either taken on apical side of carriers (“Top view”) or after the latter ones being broken into two pieces to appreciate cell colonisation (“Inside view”, from apical to distal part, white arrows). On “Detail” pictures cytoplasmic projections are pointed out (black arrows). Scale bars 200 μm.