Titanium Scaffolds by Direct Ink Writing: Fabrication and Functionalization to Guide Osteoblast Behavior

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Abstract: Titanium (Ti) and Ti alloys have been used for decades for bone prostheses due to its mechanical reliability and good biocompatibility. However, the high stiffness of Ti implants and the lack of bioactivity are pending issues that should be improved to minimize implant failure. The stress shielding effect, a result of the stiffness mismatch between titanium and bone, can be reduced by introducing a tailored structural porosity in the implant. In this work, porous titanium structures were produced by direct ink writing (DIW), using a new Ti ink formulation containing a thermoresponsive hydrogel. A thermal treatment was optimized to ensure the complete elimination of the binder before the sintering process, in order to avoid contamination of the titanium structures. The samples were sintered in argon atmosphere at 1200 °C, 1300 °C or 1400 °C, resulting in total porosities ranging between 72.3% and 77.7%. A correlation was found between the total porosity and the elastic modulus of the scaffolds. The stiffness and yield strength were similar to those of cancellous bone. The functionalization of the scaffold surface with a cell adhesion fibronectin recombinant fragment resulted in enhanced adhesion and spreading of osteoblastic-like cells, together with increased alkaline phosphatase expression and mineralization.

Keywords: titanium; direct ink writing; titanium scaffold; thermoresponsive binder; osseointegration; recombinant protein

1. Introduction

The rise in the life expectancy of the population is leading to an increasing need for musculoskeletal surgical procedures involving bone substitutes and bone implants [1]. Titanium (Ti) and Ti alloys are the biomaterials of choice in load-bearing situations due to their excellent mechanical properties, corrosion resistance, nontoxicity and biocompatibility [2]. Despite this, the mismatch in the modulus of elasticity between bone and metallic implants is still an unsolved challenge leading to stress shielding, which can result in several complications, such as bone resorption and implant loosening. Although some Ti alloys with low elastic modulus have been developed to minimize this difference, they exhibit Young’s modulus values over 50 GPa, still too high to avoid stress shielding [2–4]. To solve this problem, efforts have been focused on the fabrication of porous structures to further reduce stiffness and closely match that of bone [5].
Furthermore, from a biological perspective, porosity facilitates cell and tissue colonization, and ensures optimum osseointegration. The development of porous metallic structures is of major interest for different orthopedic applications, such as intervertebral cages for vertebral fusion, femoral stems for total hip arthroplasty or metallic plates used for fixation after the removal of tumors [6–9].

In this context, additive manufacturing methods, based on the layer-by-layer fabrication of complex structures following a predefined digital model, have attracted much attention. The main advantage is that they allow building complex 3D structures with precise control of both the external geometry and the internal porosity [10–12]. Previous works have reported the use of different additive manufacturing methods for metallic materials, comprising: (i) direct energy deposition methods, like laser-engineered net shaping (LENS), selective laser melting (SLM), selective laser sintering (SLS) and electron beam melting (EBM), or (ii) microextrusion-based systems like direct ink writing (DIW) [13–16]. Among the aforementioned techniques, SLM and EBM are the most extensively used in the orthopedic field, and basically consist of using a thermal energy source, produced by a laser or an electron beam respectively, to selectively fuse zones of a powder bed [12]. However, these additive manufacturing technologies still have some drawbacks. They work with high heat sources that produce temperature gradients that can result in inhomogeneous microstructures and the formation of cracks. Moreover, these methodologies can produce the formation of lack-of-fusion defects that need a depowdering step after fabrication [17]. Particles that are fused but no sintered may difficult the control of the roughness that is of utmost interest for biomedical applications. For intricate structures, it is also practically impossible to completely remove the unmelted powder [18]. The presence of undesirable residual particles may also increase the risk of adverse inflammatory responses. Additionally, these technologies generate the waste of used powder that must be recycled [19].

In this context, direct ink writing (DIW) emerges as an alternative technique that, although has been applied mostly to polymers and ceramics, can also have some advantages for the development of porous metallic structures [20]. DIW is a low-cost technology that can be used to manufacture complex shapes with controlled geometries at the micrometer scale without wasting metal powder [16]. This technique enables the fabrication of scaffolds by extruding a pseudoplastic ink through a nozzle until the required geometry is created. Subsequently, a postprinting sintering treatment may be applied to consolidate the printed structure. Furthermore, the process is versatile, since it allows to change the printing material easily by using a different syringe. Besides, it avoids the use of a powder bed and its waste. DIW also offers the possibility to work with multimaterial inks [21]. In case that support material is needed, this can be easily removed from the green part.

One of the main challenges in DIW of metals is the formulation of printable metallic inks with a high load of metallic particles and optimum shear-thinning properties. Jakus et al. reported a formulation consisting of polylactic-co-glycolic acid copolymer (PLGA) as a binder, mixed with three solvents, dichloromethane (DCM), dibutyl-phthalate and 2-butoxyethanol [22]. Once the inks were prepared, they were left in a fume hood in order to eliminate the excess of DCM and to adjust the viscosity to 30–35 Pa·s, a step which can take several hours, and additionally required a sintering process in a reducing atmosphere. Another approach was proposed by Li et al., where Ti6Al4V powders were mixed with an aqueous solution of methylcellulose and stearic acid as a binder [23]. The slurry was stirred for 1 h to achieve the required homogeneity [24]. In this case, the removal of the binder mix required 8 h of treatment at 500 °C.

In this study, we focused on the design of a new ink formulation using noncytotoxic compounds as binder materials. We also aimed to simplify the steps reported in previous DIW processes. With these objectives in mind, we used a Pluronic F-127® hydrogel as the binder of a titanium powder paste. Pluronic F-127® is a copolymer of poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol), which has an inverse thermoresponsive behavior, with a gelling transition near-physiological temperature [25]. Taking advantage of these characteristics, Pluronic F-127® has been used as a binder for DIW of glass and ceramic pastes [26,27]. The use of Pluronic has several advantages, including the ease of use at room temperature, tunable viscosity, excellent
printability and high solubility. To the best of our knowledge, it has not been used in titanium-based inks. In this work, we intend to exploit the high potential of Pluronic as a binder of printable titanium inks, and explore different postprinting treatments in order to obtain tunable multiscale porosities.

When aiming at orthopedic or dental applications, however, the geometrical or mechanical properties are only one of the main issues to consider. The bioactivity of the surface is also a critical issue to ensure optimal osseointegration and full bone colonization of the 3D structure. In this regard, the functionalization of the surface of the 3D scaffold with specific bioactive motifs that can foster cell adhesion is a promising strategy. This has been attempted, for instance, by immobilizing full-length proteins from the extracellular matrix (ECM), e.g., fibronectin [28]. However, its clinical use is hindered mainly due to proteolytic degradation sensitivity and possible immune response induction [29]. Alternatively, short active peptides derived from ECM proteins, such as the well-known RGD (arginine-glycine-aspartic acid) sequence [30], have been used, although they are less active due to the lack of complementary domains. An intermediate strategy consists of functionalizing the surface with ECM protein fragments obtained by DNA recombinant techniques. In this regard, the cell attachment fragment (CAS) from fibronectin spanning the type III<sub>8-10</sub> domains has demonstrated higher cell adhesion and differentiation compared to RGD or fibronectin coating on smooth Ti surfaces [31]. The response on 3D-printed scaffolds, however, has not been evaluated yet.

Therefore, the novelty of the present study focuses on two issues. First, a new formulation of printable titanium paste using Pluronic F-127<sup>®</sup> as a binder is proposed and optimized to obtain 3D titanium scaffolds for orthopedic applications with tuned multiscale porosity. Subsequently, CAS fragments are covalently immobilized onto the Ti 3D structures to foster adhesion, proliferation and differentiation of human osteoblast SaOS-2 cells seeded onto the scaffolds.

2. Experimental Method

2.1. Ink Fabrication and Characterization

Ti powder (~325 mesh, 99.5% purity, density: 4.5 g/cm<sup>3</sup>, Alfa Aesar, MA, USA) was used in this study. The morphological characterization of the Ti powder was carried out by scanning electron microscopy (SEM; Carl Zeiss NTS GmbH, Jena, Germany) at a working distance of 11.3 mm and a potential of 20 kV. The size distribution and mean size of Ti particles were characterized by laser diffraction (Beckman Coulter, Brea, CA, USA).

Pluronic F-127<sup>®</sup> (CAS Number 9003-11-6, density: 1.1 g/cm<sup>3</sup>, Sigma-Aldrich, St. Louis, MO, USA) was used as a binder in the form of a hydrogel. It was prepared by dissolving the Pluronic powder in distilled water at a concentration of 30% (w/v) and subsequently blended using an asymmetric centrifugal mixer system (SpeedMixer DAC 150.1 FVZ, Hamm, Germany) at 3500 rpm for 5 min.

Ti-based inks were prepared by mixing Ti powder with different amounts of Pluronic hydrogel, as detailed in Table 1. The solid volume fractions were calculated with the aforementioned densities listed in the supplier datasheet for Ti powder (Alfa Aesar) and Pluronic F-127<sup>®</sup> (Sigma-Aldrich) respectively.

| Ink Reference | Titanium Powder (% w/w) | Hydrogel (% w/v) | Solid Volume Fraction (% vol) |
|---------------|--------------------------|------------------|-------------------------------|
| I<sub>1</sub>  | 72.4                     | 27.6             | 38                           |
| I<sub>2</sub>  | 69.0                     | 31.0             | 34                           |
| I<sub>3</sub>  | 65.6                     | 34.4             | 31                           |
| I<sub>4</sub>  | 62.5                     | 37.5             | 28                           |

The injectability of the different inks was determined by assessing the force needed to extrude the inks at a constant strain rate of 0.025 mm/s through a 410 μm diameter nozzle, using a servo-hydraulic
testing system (MTS Bionix 358, Warren, MI, USA), equipped with a 2.5 kN load cell. Moreover, the stability of the selected ink was tested after being stored for three weeks at room temperature.

2.2. Scaffolds Fabrication

2.2.1. Direct Ink Writing Process

Cylindrical scaffolds with a 0°/90° pattern were designed using CAD software (SolidWorks Corp., Waltham, MA, USA). Slic3r software (Slic3r v. 3.1.0, A. Ranellucci) was used to define the printing parameters and generate a customized G-code. Samples were printed using a customized DIW device (BCN3D Paste Caster, Fundació CIM, Spain). The nozzle size was 410 µm (Smooth Flow Tapered Tips, Nordson EFD, Westlake, OH, USA) and the layer height was set to 350 µm. The infill density was 45% and the infill speed 15 mm/s. The ink was introduced in a 3 cm³ syringe (Optimum, Syringe Barrels, Nordson EFD, East Providence, RI, USA), with the nozzle mentioned above and the whole assembly was installed into the DIW device.

2.2.2. Postprinting Processes

Thermal characterization of the ink was performed by thermogravimetric analysis (TGA, Mettler Toledo DSC 1 Star System, OH, USA), in order to determine the optimal protocol for binder removal. For this purpose, 15 mg of 30% w/v Pluronic F-127 were analyzed by Dynamic TGA between 25 °C and 600 °C at a 10 °C/min rate, under air and nitrogen atmospheres. Furthermore, an isothermal TGA of the binder was carried out at 275 °C to confirm if the binder elimination is possible below 300 °C, the temperature at which Ti oxidation occurs. In order to confirm the elimination of the binder, the carbon content of sintered samples was analyzed by infrared absorption by using a LECO CS-200 carbon analyzer (LECO, St Joseph, MI, USA).

Following the binder removal treatment, the scaffolds were sintered at different temperatures, namely 1200 °C, 1300 °C and 1400 °C, in a tubular furnace (Hobersal, Barcelona, Spain), under argon atmosphere at a flow rate of 2 L/min, in order to determine the optimum sintering temperature.

2.3. Scaffold Characterization

2.3.1. Physicochemical Characterization

The surface morphology of the Ti scaffolds was analyzed by scanning electron microscopy (Zeiss Neon 40, Carl Zeiss, Oberkochen, Germany), using a working distance of 9.1 mm and an acceleration voltage of 20 kV.

Sample shrinkage was evaluated by measuring the width and height of the samples before and after the sintering process using a digital caliper with 0.01 mm accuracy.

The architecture of 3D-printed scaffolds was analyzed by X-ray computed microtomography (µCT, micro-CT Skyscan 1272, Bruker, Kontich, Belgium) at a voltage of 100 kV, a current of 100 µA and an isotropic pixel size of 5 µm. Three-dimensional (3D) reconstruction and images analysis were performed using Nrecon and CTAn software, respectively (both from Bruker).

Pore entrance size distribution and open porosity were analyzed by mercury intrusion porosimetry (MIP, Autopore IV, Micromeritics, GA, USA) in the range between 0.01 and 45 µm. The strut porosity was calculated with Equation (1), where V is the volume of strut porosity normalized per unit of mass obtained from the sum of the incremental mercury intrusion in the pores smaller than 20 µm, and ρ_app is the apparent density of the scaffold which was determined by dividing the scaffold mass by the scaffold equivalent cylinder volume [26].

\[ \text{Strut porosity (\%) } = \left( \frac{V \times \rho_{\text{app}}}{\rho_{\text{app}}} \right) \times 100 \]  

Equation (1)

The scaffold porosity was estimated by micro-CT as the % of pores larger than 20 µm by the maximal spheres’ algorithm [32]. Mean pore size of the scaffolds sintered at 1200 °C, 1300 °C and
1400 °C was determined from SEM images using Fiji/ImageJ (NIH, Bethesda, MD, USA) processing software [33].

The surface roughness of the filaments was analyzed by white light interferometry (Wyko NT9300, Veeco Instruments, Plainview, NY, USA) in vertical scanning interferometry mode (VSI). Three measurements of the average surface roughness (Ra) were carried out at three different zones of the scaffold, using a 10× objective lens and a scanning area of 316 µm × 237 µm. Roughness data was analyzed with Wyko Vision 4.10 software (Veeco Instruments, Plainview, NY, USA).

2.3.2. Mechanical Characterization

The scaffolds were subjected to a compression test, in accordance with the ISO standard 13314:2011 [34]. Cylindrical specimens (10 mm in diameter and 12 mm in height) sintered at three different temperatures (1200 °C, 1300 °C and 1400 °C) were tested in a universal testing machine (Microtest machine EM1/20/FR, Microtest, Madrid, Spain) applying a cross-head speed of 2.5 mm/min. Load versus extension was continuously monitored and recorded. The yield strength and the elastic modulus, calculated as the slope of the linear portion of the stress–strain curve upon loading, were determined for each temperature.

2.4. Synthesis of Fibronectin Recombinant Protein Fragment

The CAS fragment of human fibronectin spanning the type III8-10 domains was produced by recombinant DNA techniques. This functionalization has been previously studied in our group, resulting in the covalent binding of CAS fragments to the surface (analyzed by X-Ray Photoelectron Spectroscopy, D8 advance, SPECS Surface Nano Analysis GmbH, Berlin, Germany), thus the same protocol was followed [35]. Briefly, cDNA from SaOS-2 cells was inserted into a pGEX-6P-1 plasmid (GE Healthcare, Hatfield, UK) and amplified in DH5α cells (Invitrogen, Waltham, MA, USA). Constructs were then isolated, purified and sequenced. BL21 E. coli cells (New England BioLabs, Cambridge, UK) were transformed with the proper insert and the resulting colonies were dynamically cultured in LB broth at 37 °C with 100 µg/mL ampicillin. Protein production was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) during 4 h. Bacterial suspensions were centrifuged, resuspended and sonicated. Lysed bacteria were centrifuged and the CAS fragments were purified from the resulting supernatant using a GSTrap affinity column (GE Healthcare). GST-tag was removed on-column and the purified CAS fragments were resolved by electrophoresis. The protein concentration was quantified by the BCA method.

2.5. Surface Functionalization

Ti scaffolds were silanized with 0.5 M of 3-chloropropyltriethoxysilane (CPTES, Sigma-Aldrich) and 0.05 M of diisopropylethylamine (DIEA) in anhydrous toluene for 1 h at 70 °C under agitation in a nitrogen atmosphere. After silanization, the samples were ultrasonically cleaned with anhydrous toluene for 10 min, washed with toluene, ethanol, isopropanol, distilled water and acetone, and dried with nitrogen. Then, the samples were incubated overnight with 100 µL of CAS solution prepared at a concentration of 100 µg/mL in phosphate-buffered solution (PBS). After incubation, the samples were rinsed with PBS (×3) and blocked with 1% w/v bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 30 min. Before cellular assays, the samples were sterilized in ethanol for 30 min and rinsed thrice in PBS.

2.6. Cellular Characterization

2.6.1. Cell Culture

Human osteoblast-like SaOS-2 cells (ATCC, USA) were cultured in McCoy’s 5A medium (Sigma-Aldrich) supplemented with 10% v/v fetal bovine serum (FBS), 50 U/mL penicillin, 50 µg/mL streptomycin, 20 mM HEPES and 2 mM glutamine, all from Invitrogen. Cells were maintained at
37 °C, 5% CO₂ and in a humidified environment, changing the culture medium thrice a week upon 70% confluence. The cells were harvested for the experiments using TrypLE solution (Invitrogen). Osteogenic factors were not added in any experiment.

2.6.2. Cell Adhesion

The cells were seeded on pristine and CAS-functionalized Ti scaffolds sintered at 1400 °C at a density of 5 × 10⁴ cells/sample using a serum-free medium, and incubated for 6 h. After incubation, cells were lysed with 300 µL of mammalian protein extraction reagent (M-PER, Thermo Fisher). The number of attached cells was quantified by measuring the lactate dehydrogenase (LDH) activity using the Cytotoxicity Detection Kit LDH (Roche Applied Science, Germany). Measurements were acquired spectrophotometrically at 492 nm using a Synergy HTX multimode microplate reader (Bio-Tek Instruments, VT, USA). Cell numbers were obtained using a calibration curve with an increasing number of cells.

Moreover, cell spreading and morphology was analyzed by SEM. For this purpose, the cells were fixed with 2.5% glutaraldehyde in PBS for 1 h at 4 °C and the samples were subsequently immersed in osmium tetroxide solution for 1 h and cleaned with distilled water. Afterwards, the specimens were progressively dehydrated in ethanol aqueous solution series (50%, 70%, 90% and 96%) and completely dehydrated in hexamethyldisiloxane (HDMSO, Sigma-Aldrich). Dried samples were mounted on aluminum stubs and observed using a Zeiss Neon 40 (Carl Zeiss) as described above.

2.6.3. Cell Proliferation

Cells were seeded on functionalized and pristine Ti scaffolds as described above, using a serum-free medium. After 6 h, the medium was supplemented with 10% v/v FBS and the medium exchanged every two days. At Days 3, 7, 14 and 21, the number of cells was evaluated by measuring the LDH activity as previously described.

2.6.4. Cell Differentiation

The osteoblastic cell differentiation was assessed by measuring the alkaline phosphatase (ALP) activity. To this end, extracts obtained in the proliferation study were used for quantifying the ALP activity using the SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnanSpec Inc., Fremont, CA, USA) following the manufacturer’s instructions. Enzyme activity was measured spectrophotometrically at 405 nm at each time point (3, 7, 14 and 21 days) using a Synergy HTX multimode microplate reader (Bio-Tek, Winooski, VT, USA), and the results were normalized to their corresponding number of cells.

2.6.5. Cell Mineralization

The presence of calcium deposits was analyzed at day 21 using Alizarin Red S (ARS) staining method (Sigma-Aldrich). Six hours after seeding 5 × 10⁴ cells/well in serum-free medium, the medium was replaced with a serum-supplemented medium and changed every two days. At the end of the incubation time, samples were washed with PBS and the adhered cells were fixed with 4% v/v paraformaldehyde at room temperature (RT) for 20 min. Ti scaffolds were then washed twice with Milli-Q water and 500 µL/well of 40 mM ARS (pH 4.2) was added. The plates were incubated with the dye at RT for 20 min while gently shaking. Prior to quantification, the excess dye was washed off using copious washings with Milli-Q water. Cetylpyridinium chloride (CPC) buffer (10% v/v in 10 mM NaH₂PO₄, pH 7) was added (300 µL/well) at RT for 30 min to elute stain. The supernatant was then collected, diluted 1:2 with CPC buffer and 100 µL were plated to measure absorbance at 570 nm using a Synergy HTX multimode microplate reader (Bio-Tek).
2.7. Statistical Analysis

All data are presented as mean values ± standard deviations of at least \( n = 3 \) samples. A nonparametric Kruskal–Wallis test followed by a Mann–Whitney test with Bonferroni correction was used to determine the statistically significant differences \((p < 0.05)\) between group means.

3. Results and Discussion

3.1. Titanium Powder Characterization

The Ti powder used for this study consisted of irregular particles (Figure 1a), with a unimodal particle size distribution, with a mean particle size of 22.45 \(\mu m\) ± 15.74 \(\mu m\) and 95% of the particles smaller than 55.60 \(\mu m\) (Figure 1b). Although the irregular powder morphology can be detrimental for the extrusion of the paste, it also can improve the green printed parts stability and manipulation after the removal of the binder, since angular shapes can promote mechanical interlocking between particles [36].

![Figure 1. Titanium powder: (a) SEM micrograph and (b) particle size distribution measured by laser diffraction.](image_url)

3.2. Ink Characterization

Regarding the development of the ink formulation, the efforts focused on obtaining a printable ink with a higher fraction of metal powder, to minimize shrinkage upon binder removal and to improve the mechanical properties. Based on these objectives, an injectability test was performed and the load needed to extrude each ink formulation was monitored as a function of the plunger displacement (Figure 2). \(I_1\) was the tested formulation with a higher fraction of metallic powder, but it was discarded because the load required to extrude it was higher than 90 N, which is the maximum loading capacity of the 3D printing machine.

The injectability study revealed an initial force increase followed by a zone of stable flow (which corresponds to the 3D printing regime), characteristic of a pseudoplastic material exhibiting shear-thinning properties. As expected, increasing the metal fraction increased the load necessary for printing.

The selected formulation was 69\% \(w/w\) of Ti powder, as it was injectable with the DIW equipment, showed a stable extrusion pattern in the test (starting at 4 mm displacement) and contained the highest amount of metal powder. Moreover, the stability of the ink was demonstrated by comparing the maximum load before (83.8 N ± 0.7 N) and after three weeks of storage (82.9 N ± 1.1 N).
3.3. Optimization of Binder Removal

Thermogravimetric analyses were performed under air and nitrogen atmosphere to study the decomposition of the binder. The TGA curves showed a two-step weight loss process (Figure 3a). The first step can be attributed to the removal of water and the second one to the decomposition of the polymer [37]. Under nitrogen atmosphere, higher temperatures were needed and the binder was not totally eliminated since a remnant of 1% was observed. In contrast, under air conditions, the binder was removed at lower temperatures and was totally eliminated above 384 °C. For this reason, it was decided to perform the binder removal process in the air atmosphere.

However, in order to prevent Ti oxidation, temperatures above 300 °C should be avoided. This is why an isothermal TGA of the ink was performed in air at 275 °C to check if it was possible to fully remove the binder at this temperature. As shown in Figure 3b, all the binder was removed after 1 h.

Since the amount of Ti ink used in the TGA study (41.31 mg) was smaller than the amount present in the printed scaffolds (1.7 g), the time required for removing the binder had to be adjusted. Green Ti scaffolds were subjected to 275 °C for 24 h and the weight evolution was monitored over time until stabilization, which was reached after 12 h. Therefore, this was the protocol used for binder removal in the DIW scaffolds (Figure 3c).

After binder removal, the scaffolds had enough strength to allow manipulation. They were sintered in a tubular furnace under Ar atmosphere at three different temperatures (1200 °C, 1300 °C and 1400 °C) for 2 h (Figure 3c) [38]. The carbon content of the sintered scaffold was found to be 0.089 ± 0.010%, lower than the maximum content of carbon for Titanium Grade 4 (0.1%).

The ease of binder removal and lack of residues remaining in the titanium in this study contrast to other studies with different binders, which measured a noticeable effect of the binder in volume reduction and mechanical properties [22]. Xu et al. compared the effect of using chitosan or PLA as a binder, and their results showed a significant reduction in ductility of the titanium prepared with chitosan, a result attributed to the increased difficulty to remove the chitosan without leaving traces in the final material [39].
Figure 3. Thermogravimetric analysis: (a) Dynamic TGA of the binder, Pluronic F-127, and (b) isothermal TGA of the ink at 275 °C. (c) Heat treatment profile: scaffolds are first introduced in an oven under air atmosphere to eliminate the binder and then sintered in a tubular furnace under Ar atmosphere.

3.4. Scaffolds Characterization

SEM images before and after the complete thermal treatment, comprising debinding at 275 °C and sintering at 1200 °C, are shown in Figure 4. The microstructure of the scaffolds sintered at 1200 °C showed good sintering between particles, with the formation of necks between adjacent particles due to solid-state diffusion (Figure 4c).

The global shrinkage of the scaffolds was determined by measuring the diameter and height before and after the thermal treatment, including the debinding and sintering steps. The calculated shrinkages after sintering at 1200 °C, 1300 °C and 1400 °C were higher in the axial direction compared with the contraction in the transversal direction (Table 2). This is in agreement with previous results reported in the literature, with the larger contraction in the vertical direction being associated with the effect of the gravity force during sintering [40]. The global shrinkage increased with the sintering temperature due to the enhanced solid-state diffusion between powder grains, also in agreement with other studies with similar sintering conditions of time and temperature [14,23,36,38–42].
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Figure 4. Images of 3D-printed scaffolds: (a) optical image of the scaffold; (b) SEM micrographs of the green body and (c) SEM micrographs after the complete thermal treatment, including the debinding and subsequent sintering at 1200 °C.

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All the scaffolds presented a bimodal porosity when observed in SEM, with the scaffold pores resulting from the filament pattern design, and the pores in the struts (Figure 5a) produced during the elimination of the binder and the sintering process [43]. Interestingly, the percentage of scaffold porosity did not change with the different sintering temperatures. However, a significant reduction in scaffold pore size was observed when the sintering temperature raised from 1200 °C to 1300 °C.
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(Table 2), due to the sample shrinkage during sintering. In all cases, the scaffold pore size was within the acceptable range to allow bone ingrowth, vascularization and transport of metabolic products, according to the literature [8,9,44–46].

Raising the sintering temperature resulted in a reduction of strut porosity (Table 2), although the differences were not statistically significant. The measured mean roughness values were within the optimal range to enhance bone osseointegration and implant interlocking [33,47,48].

Moreover, both the strut porosity and the pore entrance size decreased with increasing sintering temperature (Table 2), as shown in Figure 5a,b, which led to the consequent decrease of total porosity (Table 2 and Figure 5c). This is a consequence of the enhanced solid-state diffusion between titanium powder grains at higher sintering temperatures [42].

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**3.5. Mechanical Characterization**

The compressive Young’s modulus and the yield strength at the studied sintering temperatures are displayed in Figure 6. All the samples tested behaved plastically, with smooth curves without serrations, indicating deformation without microfracturing (data not shown). The Young’s modulus gradually increased with sintering temperature, which is associated with the reduction in porosity [42]. This result was expected, since other studies found a correlation of increased stiffness with increased sintering temperatures [14,38,41,42,49]. As shown in Figure 6c, there is a linear correlation between the total porosity and the stiffness of titanium porous structures. The Young’s modulus values obtained in this work, between 0.31 and 0.63 GPa, are of the same order as those published in the literature. For instance, 3D printing titanium scaffolds developed by Maleksaeedi et al. [41], with a scaffold...
porosity of 70%, had a Young’s modulus of 0.33 GPa. In the same way, El-Hajje et al. [50] measured values in the range of 0.86–2.48 GPa for titanium structures with a porosity of 32.2–53.4%.

Similar behavior was observed for the yield strength, even though there are no statistically significant differences between the values measured at different sintering temperatures. This result is also in agreement with other studies that measured an increase of the yield strength with the sintering temperature and the reduction in porosity [14,38,41,42,50]. The high dispersion of the yield strength values for the scaffolds treated at 1200 °C was attributed to the imperfect sintering of the powder particles at this temperature in comparison to higher temperatures, as observed by SEM.

Modeling the measured elastic modulus and yield strength with the Gibson–Ashby models for cellular solids [51] have not achieved a good agreement with the results. This is probably because the structures created in this study do not bend the horizontal struts when compressed, but transfer the vertical load from one layer to the next through the strut contact volumes, creating a linear path within the structure [52]. The excellent correlation shown in Figure 6c between porosity and Young’s modulus supports this hypothesis, as a more compact structure results in an increased number of supports per area. It has been shown that changes in the structural parameters of scaffolds built by selective laser melting modulate the mechanical properties of the scaffold (e.g., elastic modulus or Poisson’s ratio), effectively allowing the control of the stress concentration in the manufactured material [53]. An analysis by finite element modeling would allow testing this concept on our scaffolds, but it is out of the scope of the present work.

![Figure 6](image-url)

**Figure 6.** Mechanical properties of scaffolds at different sintering temperatures: (a) Young’s modulus; Samples with different symbols (*, #, $) present statistically significant differences ($p < 0.05$); (b) Yield strength at 1200 °C, 1300 °C and 1400 °C (different symbols indicate significant differences ($p < 0.05$) between conditions); (c) Porosity effect on the Young’s modulus; (d) Representative stress–strain curve for Ti scaffold sintered at 1400 °C.

| Type of bone | Direction and Type of Load | Elastic Modulus (GPa) | Reference |
|--------------|----------------------------|-----------------------|-----------|
| Cortical bone | Midfemoral Longitudinal compression | 17 | [54] |
| Cortical bone | Midfemoral Transverse compression | 11.5 | [54] |
| Cortical bone | - | 14.1–27.6 | [55] |
| Trabecular bone | Proximal femoral Longitudinal | 0.441 | [55] |
| Trabecular bone | Proximal femoral Transverse | 0.100–0.400 | [55] |
| Trabecular bone | Femoral bone | 0.0657–0.873 | [55] |
| Trabecular bone | Proximal tibia Longitudinal | 0.004–0.430 | [55] |
Overall, the mechanical properties of the prepared DIW Ti scaffolds are in the range of human trabecular bone. Obtained yield strengths are between 6.6 and 36.2 MPa [2,9]. Moreover, since a range of elastic modulus was obtained by DIW, the Young’s modulus of the scaffold can be adjusted in order to meet the requirements of the trabecular bone to be replaced, as shown in Table 3. Taking into consideration these results, as well as the higher mechanical properties of the scaffolds sintered at 1400 °C, these were selected for the surface functionalization and in vitro cell assessment.

**Table 3. Elastic modulus of human cortical and trabecular bone.**

| Type of Bone | Direction and Type of Load | Elastic Modulus (GPa) | Reference |
|--------------|---------------------------|----------------------|-----------|
| **Cortical bone** | Midfemoral | Longitudinal compression | 17 | [54] |
| | | Transverse compression | 11.5 | [54] |
| | | - | - | 14.1–27.6 | [55] |
| **Trabecular** | Proximal femoral | Longitudinal | 0.441 | [55] |
| | Femoral bone | Longitudinal | 0.0657–0.873 | [55] |
| | Proximal tibia | Longitudinal | 0.004–0.430 | [55] |

3.6. In Vitro Cell Response

The in vitro cell culture tests were performed with Ti scaffolds sintered at 1400 °C, either untreated or functionalized with CAS fragments, using SaOS-2 osteoblastic cells. Cell adhesion after 6 h was significantly increased by the presence of CAS fragments compared to bare Ti scaffolds (Figure 7a). Moreover, the cells attached to CAS-functionalized scaffolds were more spread than those attached to pristine Ti scaffolds, where the cells were less flattened and some of them even presented a rounded shape morphology (Figure 7). The improved adhesion and spreading should be attributed to the adhesive capacities of the CAS fragment, which contains the RGD sequence along with the synergistic PHSRN sequence, both mediating the integrin interaction and the creation of focal adhesions [46,56].

The proliferation of SaOS-2 cells was evaluated after 3, 7, 14 and 21 days. The number of cells onto bare Ti and CAS-functionalized Ti surfaces increased during the first 14 days of the proliferation assay, and decreased compared to bare Ti at 21 days (Figure 8b). The CAS fragment has been described to promote cell proliferation in smooth Ti surfaces [31]. Moreover, Guillem-Martí et al. have studied the adhesion and proliferation of SaOS-2 cells on smooth Ti, obtaining similar results on adhesion and proliferation than the Ti scaffolds used as a control in this work, which demonstrates the biocompatibility of uncoated Ti [46,57]. In this study, however, the Ti surface presents pores that have been shown to affect protein adsorption processes through capillary forces and increased specific surface area [58]. Besides, the three-dimensional pore structure of the samples allows for higher availability of nutrients at the edge of the scaffolds compared to the center. Due to the high number of cells on the surface of the samples after 21 days, the lack of available space may lead to the measured reduction of viable cells in the studied scaffolds [59].
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The differentiation assay was performed at the same time points than the proliferation assay (Figure 8d). ALP activity increased within the first seven days of incubation, with a higher increase for the CAS-functionalized Ti scaffolds compared to bare scaffolds. Afterwards, the ALP values decreased in both conditions. Noteworthy, the mineralization at 21 days was also higher when the Ti scaffolds were functionalized with CAS compared to the nonfunctionalized scaffolds (Figure 8c). These results are in agreement with previous studies, notwithstanding that it was found that the CAS fragments did not stimulate cell differentiation nor mineralization per se [35,60], when the CAS fragments were present in a three-dimensional matrix synergistic effects on cell differentiation were found between the CAS fragments, the increased roughness and the three-dimensional architecture [46]. Similarly, in this case, we hypothesize that the three-dimensional architecture of the scaffolds along with the filament roughness and the presence of CAS synergistically enhanced the differentiation and mineralization of SaOS-2 cells [54,61].

Considering possible applications based on the results of this study, the capability of the DIW process to strike a balance between porosity and mechanical properties of the manufactured structures must be highlighted as a step towards the development of optimized Ti scaffolds for orthopedic applications without stress shielding issues.
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Considering possible applications based on the results of this study, the capability of the DIW process to strike a balance between porosity and mechanical properties of the manufactured structures must be highlighted as a step toward the development of optimized Ti scaffolds for orthopedic applications without stress shielding issues.

Figure 8. Cell behavior on CAS-functionalized and nonfunctionalized Ti scaffolds. (a) Cell adhesion of SaOS-2 cells after 6 h of incubation and (b) cell proliferation after 3, 7, 14 and 21 days measured by LDH activity. (c) Mineralization after 21 days of incubation by Alizarin Red staining. O.D. stands for optical density. (d) Cell differentiation measured by ALP activity. At each time point, the symbol "*" means statistically significant differences between bare Ti and CAS-functionalized-Ti scaffolds (p < 0.05).

4. Conclusions

Porous tridimensional scaffolds with controlled geometry and interconnected porosity were successfully manufactured by using a direct ink writing process. Complete removal of the binder before sintering was achieved at lower temperatures than other DIW processes by optimizing the debinding thermal treatment. Adjustment of the sintering temperature allowed tuning the porosity of the titanium structures, and consequently the mechanical behavior. The sintered scaffolds presented an elastic modulus and yield strength in the range of human cancellous bone.

Additionally, adjustment of the strut porosity and surface functionalization with cell attachment-inducing fibronectin fragments resulted in a significant increase in SaOS-2 cell adhesion and spreading, together with an enhanced proliferation, differentiation and mineralization.

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