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Decoupling the role of chemistry and microstructure in hMSCs response to an osteoinductive calcium phosphate ceramic

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ABSTRACT

Calcium phosphates (CaP) are widely used synthetic bone graft substitutes, having bioactivity that is regulated by a set of intertwined physico-chemical and structural properties. While some CaPs have shown to be as effective in regenerating large bone defects as autologous bone, there is still the need to understand the role of individual material properties in CaP performance. Here, we aimed to decouple the effects of chemical composition and surface-microstructure of a beta-tricalcium phosphate (TCP) ceramic, with proven osteoinductive potential, on human mesenchymal stromal cells (hMSCs) differentiation. To this end, we replicated the surface structure of the TCP ceramic into polylactic acid without inorganic additives, or containing the chemical constituents of the ceramic, i.e., a calcium salt, a phosphate salt, or TCP powder. The microstructure of the different materials was characterized by confocal laser profilometry. hMSCs were cultured on the materials, and the expression of a set of osteogenic genes was determined. The cell culture medium was collected and the levels of calcium and phosphate ions were quantified by inductively-coupled plasma mass spectrometry. The results revealed that none of the tested combinations of properties in polymer/composite replicas was as potent in supporting the osteogenic differentiation of hMSCs as the original ceramic. Nevertheless, we observed some effects of the surface structure in the absence of inorganics, as well as combined effects of surface structure and the added salts, in particular calcium, on osteogenic differentiation. The approach presented here can be used to study the role of independent properties in other CaP-based biomaterials.

1. Introduction

Currently a few million bone graft procedures are performed worldwide each year [1], as a treatment of bone defects caused by trauma or tumor removal, or in spinal fusion. Synthetic biomaterials are widely used for repair and regeneration of damaged bone, because they can be produced in large quantities against acceptable costs, they have a long shelf-life and are relatively easily certified for clinical applications. These are important advantages, as ageing and related increase of accidents and diseases [2,3], such as osteoporosis, cause an important societal and economic burden on the healthcare systems. Nevertheless, the bone regenerative performance of the majority of synthetic bone graft substitutes is generally still considered inferior to that of autologous bone [1]. Therefore, synthetic bone graft substitutes deserve further improvements to successfully replace treatments based of natural bone grafts and biologics.

Calcium phosphate (CaP)-based ceramics are among the most widely used synthetic bone graft substitutes. Some CaPs, with specific properties, have been shown to possess the bone regenerative potential *in vivo* comparable to that of autologous bone [4,5]. From a chemical perspective, CaPs, comprising mainly calcium (Ca) and inorganic phosphate (P), resemble the natural bone mineral, which makes them biocompatible and simplifies the regulatory path leading to the approval for clinical use [2]. The chemical phase is, however, not the only factor determining the bioactivity of CaPs. Several studies have shown the effects on bioactivity of structural parameters including microstructure [6–8], particle size and morphology [9–11], porosity [12,13], mechanical properties such as stiffness [14–16], and physico-chemical parameters such as phase composition [17], ion substitution [18–21], and crystallinity [22,23]. While according to some studies chemistry is

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the determinant factor in the bone regenerative potential of CaPs [24–26], others point to the predominant importance of structural parameters [6,27,28]. Elucidating the role of each property in the biological response to a CaP is critical for the development of more efficient bone graft substitutes. Besides, this knowledge can also be applied to other biomaterials, such as polymers and composites, the properties of which are often easier to tune [2].

In our previous work, we have developed strategies to study the role of individual material properties in the bioactivity of CaPs. Danoux et al. created composite materials based on polylactic acid (PLA) and used, instead of a CaP, Ca and P salts as fillers, to test the individual effects of Ca and P ions on the growth and osteogenic differentiation of human mesenchymal stromal cells (hMSCs) [29]. The results showed that Ca ions had a dose-dependent effect on cell proliferation and differentiation. Similarly, the addition of P ions, within a certain concentration range, increased the expression levels of the osteogenic markers bone morphogenetic protein-2 (BMP-2) and osteopontin (OP). At higher concentrations, cell apoptosis was observed. In a different study, structural replicas in cyclic oleofin copolymer (COC) of CaP crystals were produced using a soft embossing technique [24]. This method allowed to reliably reproduce the microstructure of the CaPs down to the submicrometer level into COC, thus obtaining a material with the same surface topography but with different surface chemistry. Using the CaP crystals and their polymer replicas, we demonstrated that the presence of CaP had a more pronounced effect on the osteogenic differentiation of hMSCs than the microstructure. In the follow-up studies using the similar replication technique, transcriptomics and proteomics analyses were performed to obtain deeper insights in the individual effects of chemistry and topography on the behavior of hMSCs and osteoblastic cell lines [30,31]. In another study, in which microstructured PLA and its composite with nanosized hydroxyapatite (HA) was used, it was shown that the structural features mainly affected the hMSCs attachment, whereas the chemistry, i.e., the presence of HA, was determinant for cell proliferation and osteogenic differentiation [32]. Other researchers have also attempted to discriminate between individuals properties of CaPs. In a study by Dos Santos et al. [26], the surface of tricalcium phosphate (TCP) and HA ceramics was coated with a 20-nm gold layer in order to mask the effect of surface chemistry on cell behavior. They concluded that the nanoroughness of the materials had a negligible effect on the adhesion of human osteoblastic cells SaOs2, but it significantly affected cell proliferation and differentiation. In a similar study, Pegg et al. [33] produced titanium alloy surfaces coated with HA, without or with an additional thin gold layer. The authors observed that osteoblast-like cells proliferation and alkaline phosphatase (ALP) and osteocalcin (OC) gene expression levels were higher on the HA-coated than on the uncoated titanium, and that the gold sputtering did not have a significant effect. These results led to the conclusion that the osteogenic differentiation was mainly influenced by the topography of HA.

While the studies described so far delivered important information about the individual effects of chemical and/or structural properties of CaPs on their bioactivity, no conclusive evidence was delivered for a predominant effect of a property or set of properties. In the present study, we applied a combination of the previously developed tools [27,34] to decouple the roles of chemical and structural properties of a CaP ceramic with proven intrinsic osteoinductive potential, on the osteogenic differentiation of hMSCs. More specifically, we used discs of a beta-TCP ceramic with submicrometer-size structural features (grains and pores), annotated TCPs. These structural features of the ceramic have been suggested to be determinant for its ability to induce *de novo* bone formation upon intramuscular implantation in canine and rabbit models [27,35]. *In vitro*, the TCPs was shown to direct actin organization and increase primary cilia prevalence and length of hMSCs, as well as to increase the ALP enzymatic activity and osteogenic gene expression [36]. Moreover, it was shown that the ALP activity of hMSCs cultured in the medium that was conditioned by osteoclasts cultured on TCPs, was amplified compared to hMSCs cultured in osteoclast-conditioned medium from another TCP with a different microstructure [27]. Taken together, the rationale for choosing TCPs for the current study is its proven effect on the osteogenic differentiation *in vitro* and osteoinduction *in vivo* [27,35,36], as well as its bone regenerative potential that was shown to be comparable to that of autologous bone [5].

To achieve the aim of the study, i.e., understanding the roles of chemical and structural features of a CaP osteoinductive ceramic on the osteogenic differentiation of hMSCs, the soft-embossing technique was used to replicate the surface morphology of TCPs discs down to the submicrometer scale in a low molecular weight PLA, without, or containing a Ca salt, a P salt, or TCPs powder as a filler, using flat discs as controls. Additional controls were flat and microstructured tissue culture polystyrene (PS) discs. hMSCs were cultured on the different materials, and the ion content in cell culture medium and the levels of markers of osteogenic differentiation were studied. The results point at a combined effect of surface morphology and chemistry in the expression of osteogenic genes.

2. Materials and methods

2.1. Preparation of the polymer, ceramic and composite materials

Poly-D,L-lactic acid (PLA, Purasorb PDL05) with a molecular weight of 61 kg/mol relative to PS standards was obtained from Corbion Purac. Calcium carbonate (BioXtra $CaCO_3$ >99.0%) was purchased from Sigma-Aldrich. Disodium phosphate (Na_2HPO_4, sodium phosphate dibasic dihydrate) and sodium phosphate monobasic (NaH_2PO_4) were obtained from Fluka Analytical and Sigma-Aldrich, respectively. The two salts were mixed at a ratio 3:1 (w/w) to obtain a pH neutral source of inorganic phosphate ions [29]. TCPs powder was obtained from Kuros Biosciences.

The Ca and P salts, as well as the TCPs powder, were ground in a mill (Fritsch Rotor Mill Pulverisette 14) to reduce their particle size below 10 μm. Composite materials (PLA + Ca, PLA + P and PLA + TCP) were produced by mixing 15 wt% of the Ca salt, P salt, and TCP powder, respectively, with as-purchased PLA particles. The materials were then extruded into filaments with a cross-section of 1.5 × 1.6 mm^2 using a twin screw compounder (HAAKE MiniCTW) at a temperature of 85 °C and a rotation speed of 50 rpm (Fig. 1A). PLA without salts was extruded using the same procedure. The materials were subsequently ground again and reduced to flakes, after being immersed in liquid nitrogen to prevent melting during the grinding process. Finally, the flakes were inserted in a 1 mm thick Teflon mold and compacted to discs with a diameter of 9 mm and a height of 1 mm using a hot press (Atlas Manual Hydraulic Press GS15011) that kept a constant pressure of 9800 Pa at 85 °C for 10 min (Fig. 1A).

The TCPs ceramic discs with a diameter of 9 mm and a thickness of 1 mm, produced as described previously [27], were kindly supplied by Kuros Biosciences.

2.2. Surface structure replication procedure

To provide polymer and composite discs with TCPs microstructure, the previously developed replication procedure by soft embossing [24], with some modifications, was used (Fig. 1B). In short, PDMS (Sylgard 184, DowSil) was mixed with 10 wt% curing agent (Sylgard 184, Dow Corning, USA), defoamed and poured on top of TCPs discs. After 2 h of curing in an oven at 80 °C, the elastomer was carefully removed from the discs and the surplus of PDMS was removed with a scalpel. The PDMS molds were then placed in contact with the PLA or PLA-based composite discs, thin polytetrafluorethylene (PTFE) sheets were placed on both sides of the paired discs and then pressure was applied for 1 h at a temperature of 85 °C. Finally, the microstructured polymeric/composite discs were removed from the PDMS molds. Tissue culture polystyrene (Cell culture dish 100 mm, Nunclon Delta, tissue culture-treated) was

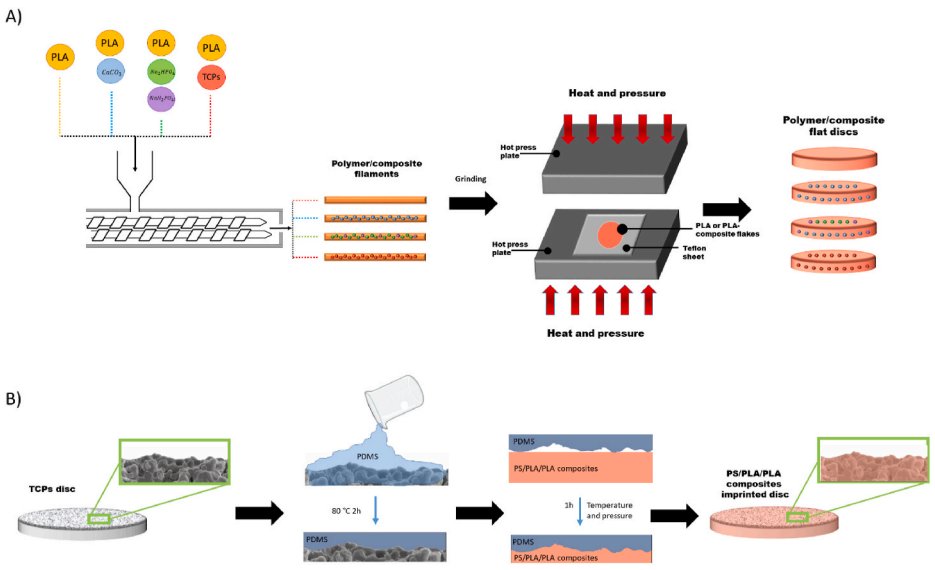

Fig. 1. Schematic representation of the disc preparation and microstructure replication process using soft embossing. **A)** Preparation of the polymer and composite discs. The raw materials for each individual composition (PLA, PLA + Ca, PLA + P, PLA + TCPs) were introduced in the twin-screw compounder, mixed homogeneously and extruded in the shape of filaments. The filaments were then ground and reduced to flakes, which were used to fill a Teflon mold, and inserted in a hot press that was kept at a constant pressure of 9800 Pa at 85 °C for 10 min to produce the discs. **B)** Microstructure replication procedure. The TCPs discs were covered with PDMS, which was cured to produce a mold. The ceramic discs were carefully removed from the PDMS mold, and the polymer/composite discs were placed in contact with the mold, heated and exposed to pressure. The effect of heat and pressure resulted in discs having the surface microstructure of the original ceramic surface.

cut in square pieces with an area of 1 × 1 cm^2 and a thickness of 1.5 mm. The pieces were microstructured using the same procedure as used for PLA at a temperature of 150 °C, and served as a control.

2.3. Materials characterization

The TCPs powder was characterized using x-ray diffraction (XRD) in the range 2θ 6°–70° with the time step of 0.5 s, angular separation of 2θ 0.02° and knife separation of 1 mm. The dataset was analyzed using Profex v.4.1 [37], and Rietveld refinement was performed. The powder was also analyzed by Fourier transform infrared spectroscopy (FTIR, Nicolet iS50, Thermo Fisher) in the attenuated total reflection mode, with 2 nm resolution, averaged over 32 measurements.

The surface structural properties of the TCPs ceramic discs and polymer- and composite replicas were observed using scanning electron microscopy (SEM, XL30, Philips) and quantitatively analyzed by confocal laser scanning optical profilometry (Keyence VKX-200, Keyence, Japan) using a 50x objective and XY stacks. Per condition, three discs were used, and per disc, 16 fields of view per disc (1 mm^2 in total) were quantified.

The profilometry data was processed using the Keyence Multifile Analyzer software, correcting for plane tilting on the surface of the discs. The arithmetical mean height (Sa), which expresses the root mean square of the difference of height of each point on the surface compared to the arithmetic mean height of the surface, was quantified. Besides the Sa, other parameters recommended in the ISO 25178 standard for Geometrical Products Specifications were used to characterize the surfaces of both the ceramic discs and their replicas. These parameters were the density of peaks, the texture aspect ratio (comparison of texture uniformity), and the upper and lower material ratio, which physically represent the areal material ratio that divides the areas that are removed by initial abrasion from the core surface and the percentage of the measurement area that contain valley structures able to hold liquid on the surface to improve lubricity. Energy-dispersive x-ray spectroscopy (EDS) analysis was performed to evaluate the presence of Ca and P atoms on the surface of the materials.

2.4. Cell culture

Prior to cell culture experiments, the materials were sterilized by washing three times in 70% isopropanol for 5 min, left to dry overnight, and then incubated in basic cell culture medium (αMEM with GlutaMax, 10% FBS, 0.2 mM ascorbic acid to phosphate (ASAP), 100 U/ml penicillin and 100 mg/ml streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2 for 2 h. HMSCs, obtained from bone marrow aspirates of one healthy human donor after informed consent, were expanded in basic cell culture medium at 37 °C in a humidified atmosphere with 5% CO_2. After a confluency of approximately 70% was reached, the cells of passage 3 were trypsinized and seeded on the discs at a density of 25,000 cells/cm^2. The cells were cultured for 14 or 21 days, with medium refreshment every two days.

The cell culture medium was collected at days 2, 7 and 14 and inductively-coupled plasma mass spectrometry (ICP-MS) analysis was performed to determine the concentration of Ca and P ions in the medium. To this end, 50 μL of medium per sample was diluted in a ratio 1:20 in a matrix of 1% HNO_3 containing 20 ppb of scandium as internal standard. Standard curves of Ca ions in the range 150–4800 ppb and of P ions in the range 100–3200 ppb, were used. The ion levels were calculated as the average of three measurements performed on three different samples per condition.

2.5. Cell attachment

After 3 days of culture, one sample per condition was washed three times with PBS and fixed using 4% paraformaldehyde (PFA) in PBS for 30 min, washed again with PBS and finally stored in PBS at 4 °C until staining. The cell nuclei (DAPI 1:60, Sigma-Aldrich) and actin cytoskeleton (phalloidin 568, 1:100 in 1% bovine serum albumin in PBS) were stained and imaged using a confocal microscope (Leica TCS SP8) with a 25X water objective, in stacks of 5x5 fields of view.

2.6. Gene expression analysis of markers of osteogenic differentiation

For real-time quantitative polymerase chain reaction (qPCR) analysis, the discs with cells were washed three times with PBS, the PBS was removed and the samples were stored at −80 °C. Prior to the RNA isolation, the samples were subjected to three freeze-thaw cycles. The isolation was done by adding Trizol (Trizol Reagent, Thermo Fisher Scientific) and processed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, measuring the concentration using a spectrophotometer (Biodrop, Thermo Fisher). Reverse transcription to create cDNA was carried out using the iScript™ cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed using the iQ™ SYBR® Green Supermix (Bio-Rad) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's protocol. The software qBase+ was used to calculate the relative expression using the ΔΔCt

method, normalized to the GAPDH housekeeping gene. The data was plotted as a heatmap, in which each cell represents the average value of three samples per condition for the respective microstructured material at a specific timepoint relative to the flat control made from the same material. The primer sequences used are summarized in Table 1.

2.7. Statistical analysis

The results of Ca and P ion quantification in cell culture medium were analyzed in Graphpad v.9.0.0 (Prism). Significant differences between materials were examined using two-way ANOVA with Sidak's correction. Statistically significant differences within a given timepoint are denoted as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

3. Results and discussion

The aim of the current study was to better understand the individual effects of chemical and surface structural properties of an osteoinductive CaP ceramic on the osteogenic differentiation of hMSCs. To this end, surface-structural replicas of the TCPs discs were produced in PLA without additives and in PLA containing only Ca salt, only P salts or TCPs powder to separately reintroduce the original chemistry factors. Flat PLA discs, as well as flat PS discs and PS discs with TCPs microstructure served as controls.

3.1. Characterization of TCPs discs

The XRD pattern of the TCPs powder used to produce the ceramic discs and PLA-TCPs composite (Fig. 2A) shows peaks characteristic of β-TCP, as well as peaks belonging to HA. The Rietveld analysis of the XRD pattern (Supplementary Fig. 1) revealed a phase composition of 91 ± 1 wt% β-TCP and 9 ± 1 wt% HA. β-TCP has been shown to have a higher degradation rate than HA *in vivo* [38], suggesting that the TCPs used here has an intermediate degradation rate. The FTIR spectrum (Fig. 2B) shows bands at 1018, 602 and 542 cm^{-1}, corresponding to phosphate groups [39,40]. The SEM and laser profilometer images of the TCPs disc surface (Fig. 3) show a microstructure with grains and micropores in the range of 0.6–1.0 μm and a roughness of 1.2 ± 0.2 μm, which is in accordance with the previously reported characterization of the ceramic [27].

3.2. Characterization of flat and microstructured polymer and composite discs

The structural properties of CaP bone graft substitutes have been suggested to play an important role in their bioactivity. In an *in vivo* study in dogs, in which TCPs was compared to another TCP with similar chemistry but different structural features (larger grain size and pore diameter, and rougher surface), it was suggested that the submicrometer-scale structural features of TCPs were determinant for its ability to be resorbed by osteoclasts and to induce ectopic bone formation [27]. Moreover, it has been suggested that CaP ceramics with surface features in the range of those of TCPs, promote protein adsorption to a higher extent than those with larger features [41]. Protein adsorption is relevant as it mediates cell adhesion and influences the long-term performance of implanted materials [42,43].

To better understand the importance of TCPs microstructure on hMSCs differentiation, discs made of PLA, PLA + Ca, PLA + P, PLA + TCPs and PS discs were provided with the TCPs surface morphology using a replication technique. While PS provides a non-degradable biocompatible substrate that is conventionally used for cell culture, the low molecular weight PLA degrades relatively fast in cell culture medium [29], which in turn should lead to a release of Ca and/or P ions from PLA composites with a Ca salt, P salt or TCP powder. In this way, the role of Ca and P ions can be evaluated separately as well as combined, on discs that are either flat or contain the TCPs surface morphology. Comparative analysis of the SEM images of the ceramic disc and the microstructured polymers and composite discs showed that the microstructures of both sets of materials were visually similar (Fig. 3A).

In parallel with the SEM imaging, EDS was performed to evaluate the elemental composition of the surface of the materials. The results (Supplementary Fig. 2) showed that the Ca and P salts and the TCPs powder were not present on the surface of the composite discs. EDS can typically detect atoms in the upper micrometer of the surface with a sensitivity of up to a part per thousand. The absence of Ca and phosphorus on the surface suggests that during the process of composite preparation and/or soft embossing, the inorganics were covered by the polymer matrix.

The microstructured polymer and composite discs were also imaged by confocal laser profilometry (Fig. 3B) and quantitatively compared to the original ceramic (Fig. 3C). The Sa values, often used as a measure of surface roughness, were similar for all replicas, without significant differences with the value of the TCPs discs. Spd, accounting for the number of peaks per unit area, and Str, representing surface textures uniformity, were also comparable among the ceramic and the microstructured polymer and composite discs. The values for Smr1 and Smr2, which are related to the areas that will suffer initial abrasion in contact with another material, and the percentage of material able to hold liquids, respectively were also similar for all materials, without significant differences with the TCPs. It should be noted that we observed the formation of very smooth, spherical features, reflecting light like a liquid, on the surface of the microstructured PLA + P discs (Fig. 3B and Supplementary Fig. 3). Since the P salts used for the preparation of the PLA + P discs are hygroscopic, we hypothesize that they may have attracted water that was trapped in the composite structure during the fabrication of the discs. The size of the features, plausibly water bubbles, was in the range of 30–60 μm, possibly causing higher, though not statistically significant, values for Sa and Smr1 for this material compared to the others.

In general, the quantitative surface characterization proved that we successfully reproduced the surface microstructure of TCPs discs in both PS and PLA, as well as in PLA-based composites with Ca and P salts and TCPs powder, which is in accordance with previous studies [24,32]. This set of microstructured materials, along with the corresponding flat controls, was then used to study the ions exchange dynamics in the cell culture medium and the osteogenic differentiation of hMSCs.

3.3. Quantification of ion content in the cell culture medium

When in contact with an aqueous physiological environment in the body, or with a cell culture medium *in vitro*, CaPs undergo a process of ions exchange with the environment, which is dependent on the properties of the ceramic, including its chemical phase and structural features. The local ionic concentration of the medium as well as the chemical composition of the material surface may, in turn, affect the cell response to the material. To investigate ions exchange dynamics, the medium was collected at day 2, 7 and 14 of the hMSCs culture on the different materials, and the Ca and P concentration was determined using ICP-MS. Medium from cells cultured on tissue culture plastic in the

Table 1
Sequences of primers used for RT-qPCR analysis.

Gene target	Primer sequence	
	Forward	Reverse
GAPDH	CGCTCTCTGCTCCTCCTGTT	CCATGGTGTCTGAGCGATGT
ENPP-1	CAGCGACCTTTGCAACTTTT	CCAAGGACCCCAACACCTA
OC	TGAGAGCCCTCACACTCCTC	CGCCTGGGTCTCTTCACTAC
OPN	GGTGATGTCCTCGTCTGTA	CCAAGTAAGTCCAACGAAAG
ALP	ACAAGCACTCCCACTTCATC	TTCAGCTCGTACTGCATGTC
RunX2	CCGCCTCAGTGATTTAGGGC	GGGTCTGTAATCTGACTCTGTCC

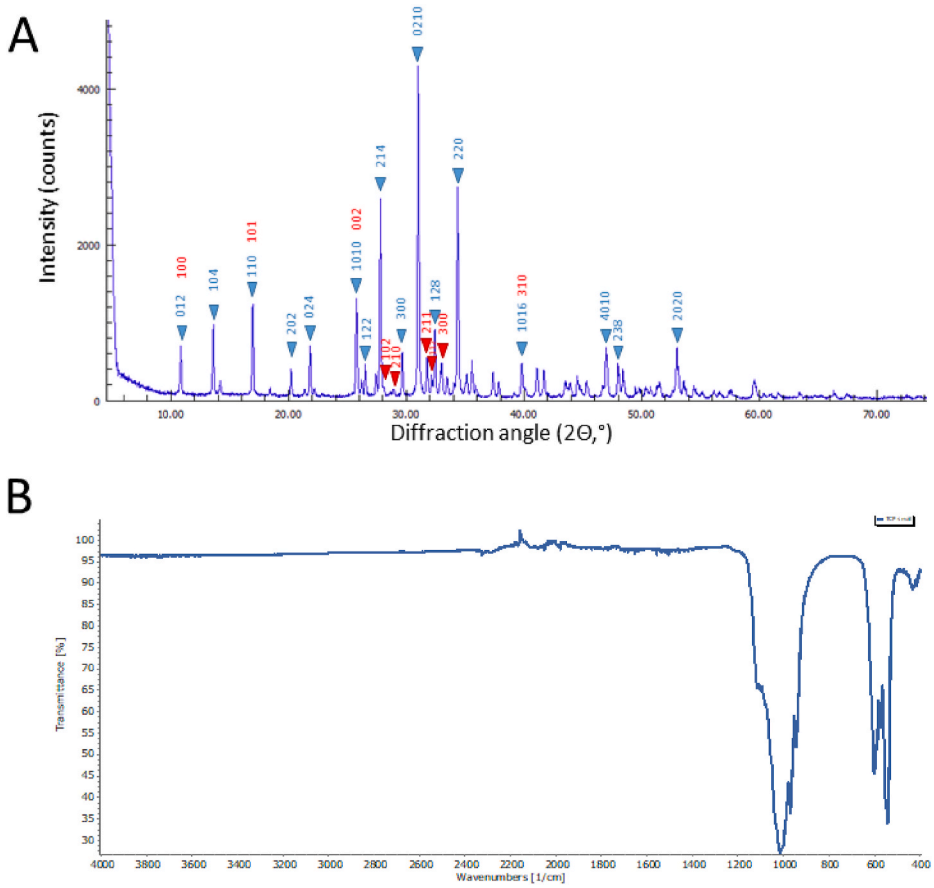

Fig. 2. A) XRD pattern of the TCPs powder used to produce the ceramic discs and as a filler of the PLA + TCPs composite. The pattern shows peaks characteristic of beta-TCP (blue) and HA (red). The content of each phase was quantified by Rietveld analysis (Supplementary Fig. 1). **B)** FTIR spectrum of the powder with bands at 1018, 602 and 542 cm^{-1}, corresponding to phosphate groups.

absence of any material served as a control. It is important to note that the Ca and P ion concentration of as-prepared cell culture medium was estimated to be ~78 ppm and 36 ppm, respectively [17].

The results showed lower Ca and P ion concentration of the medium in which hMSCs were cultured on TCPs discs, compared to the control, with differences being significant for Ca ion concentration at day 2 and 7 and for P concentration at day 2, 7 and 14 (Fig. 4A). Between day 7 and day 14, an increase in concentration of both ions was observed in the TCPs condition, although it remained somewhat lower than in the control sample. These results suggest an initial depletion of both ions from the cell culture medium by the TCPs discs, an effect that was observed previously for CaPs [31,44].

In Fig. 4B, a comparison is made between flat PLA discs without, and containing Ca or P salt. The Ca and P ion concentrations of flat PLA discs without salts were comparable to the concentrations of the control, at all time-points. The presence of the Ca salt in PLA did not seem to affect the Ca ion concentration of the medium. In contrast, the PLA + P showed a significantly higher P ion concentration at day 2, suggesting a burst release between the start of the experiment and day 2. At the later time points, the P ion concentration in the PLA + P condition was comparable to that of the PLA condition and the control.

The microstructured PLA discs without and containing Ca or P salt showed a similar trend to the flat discs, with significant difference between PLA + P and PLA and the control only for P ion concentration at day 2 (Fig. 4C).

No significant differences in Ca or P ion concentration were observed at any time point between PLA without and PLA containing TCPs powder or the control, for either flat (Fig. 4D) or microstructured (Fig. 5A) discs.

Polymers, in the form of particles or films have, among other applications, been widely used as delivery vehicles for biologics, including growth factors and antimicrobial agents, as well as for bioinorganics. PLA, an aliphatic polyester, is a good candidate for this application, since its degradation rate, and consequently, the release profile of the compounds it carries, can be tuned by modifying its molecular weight [45]. In this study, we used PLA with a relatively low molecular weight, which was expected to degrade in cell culture medium and release the incorporated ions. This was observed in an earlier study, where a PLA with a molecular weight of 59 kg/mol (thus similar to the polymer used in the current study), containing a Ca or a P salt, exhibited sustained release of Ca and P ions over a period of 14 days [29]. This result is in contrast to the results of the current study, where no release of the ions was observed. A potential reason for this may be the fact that, during the process of composite preparation and microstructuring, the inorganic filler was not exposed on the composite surface, but rather covered by a layer of polymer, as was shown by the EDS analysis (Supplementary Fig. 2). The period of time of 14 days may not have been long enough for sufficient polymer degradation to allow ion release. Another reason may be the difference in the form of the materials used in the two studies; while in the previous study, porous composite granules were used, in the current study, we used dense discs, with plausibly a smaller material-to-medium interface. The high P ion concentration that was measured at day 2 in the composites containing the P salt (Fig. 4C) was possibly a result of small water bubbles that formed on the surface of the PLA + P discs (Fig. 3B, Supplementary Fig. 3), which may have created pores in the material surface during disc sterilization in isopropanol, which in turn caused a burst release of the Pi ions during the first 2 days.

Ca and P ion concentrations of the medium containing PLA + TCPs

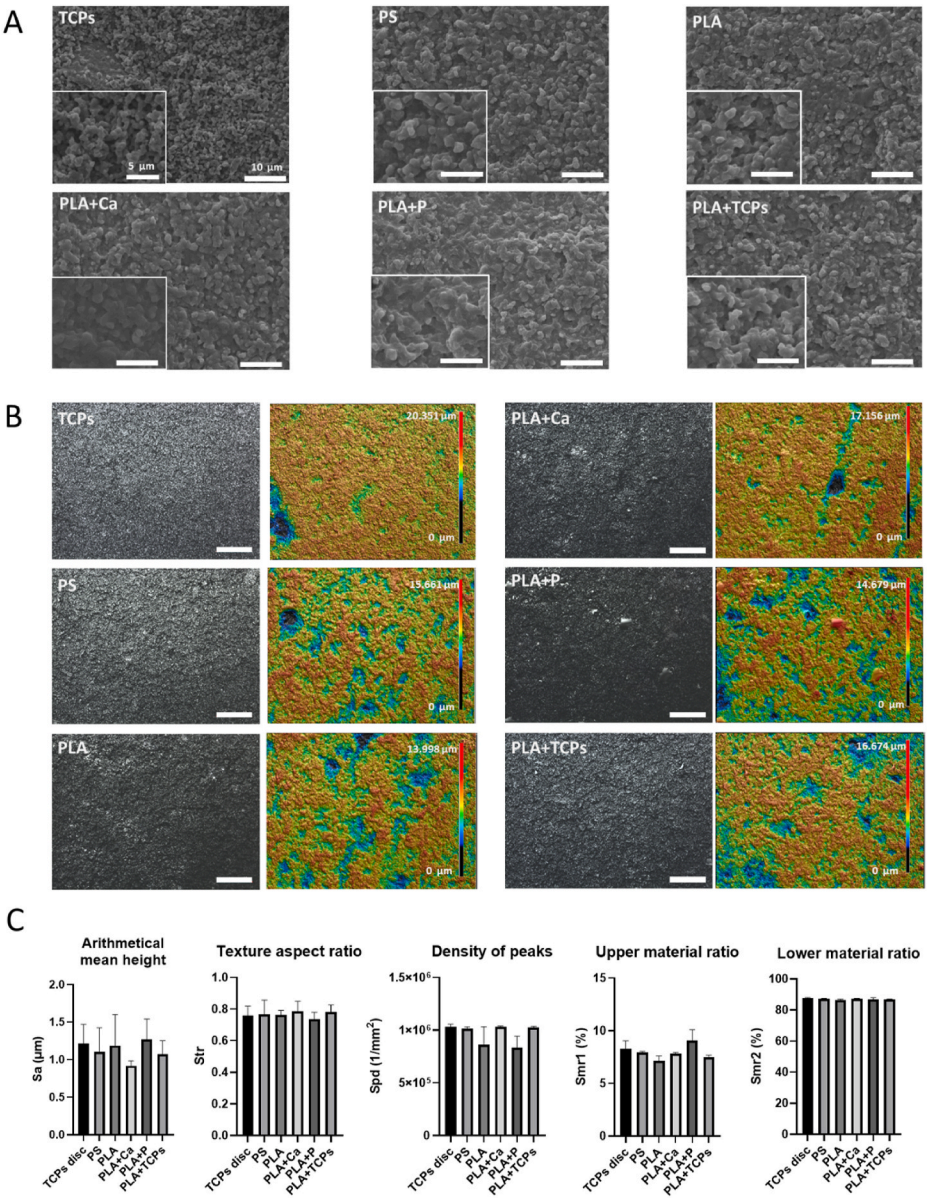

Fig. 3. A) SEM micrographs of the surface of the TCPs disc and the different microstructured polymer and composite discs. **B)** Confocal laser profilometry images and heatmaps of the surface of the different materials. Scale bars: 50 µm. **C)** Quantification of the microstructure parameters of the surface, measured over 16 fields of view covering an area of 1 mm^2 per sample (n = 3). No significant differences among the different materials was observed for any of the parameters.

discs were comparable to the PLA condition and the control. In a previous study, it was observed that the concentrations of Ca and P ions in the cell culture medium in the presence of a composite of low molecular weight PLA with HA, were lower than of the medium in the presence of PLA and of the tissue culture plastic control, i.e. medium not containing any material [32]. This ions depletion was suggested to be due to adsorption of ions and/or reprecipitation of CaP on the composite surface. In the current study, we observed the depletion in the case of TCPs itself, but not for the composite containing TCPs powder. This may due to several reasons: difference in the ceramic content between the current and the previous study (15 wt% *versus* 50 wt%); difference in the CaP phase between the current and the previous study (TCP *versus* HA); and the fact that in the current study, the TCPs powder was not exposed on the composite surface, in contrast to the previous study.

Concerning the effect of P salts-containing composite on the Ca ion concentration of the medium, a significant depletion of Ca ions was observed for both flat and microstructured discs (Fig. 5B), which was in accordance with an earlier study [29], and was attributed to either the spontaneous precipitation of a CaP layer on the surface of the discs [45], the mineralization of the extracellular matrix produced by the cells [46], or both phenomena. In contrast, the Ca salt-containing flat or microstructured composite discs did not significantly change the P ion concentration of the medium, for which we propose two different explanations. First, as the collection of cell culture medium for ICP-MS analysis was done at three timepoints (days 2, 7 and 14) with medium refreshment every two days, it is possible that the release occurred between the timepoints of the analysis and was not detected due to the medium refreshment. Second, the pores in the PLA + P material could have facilitated additional release of Pi ions as discussed above, compensating for the depletion due to CaP precipitation or extracellular matrix mineralization.

Finally, the surface structural properties did not significantly affect the ions concentration in the medium, as comparable values were measured for flat and microstructured materials (Fig. 5D), despite the significant differences in the surface roughness (Supplementary Fig. 4). This is again plausibly due to the fact that the inorganics were covered

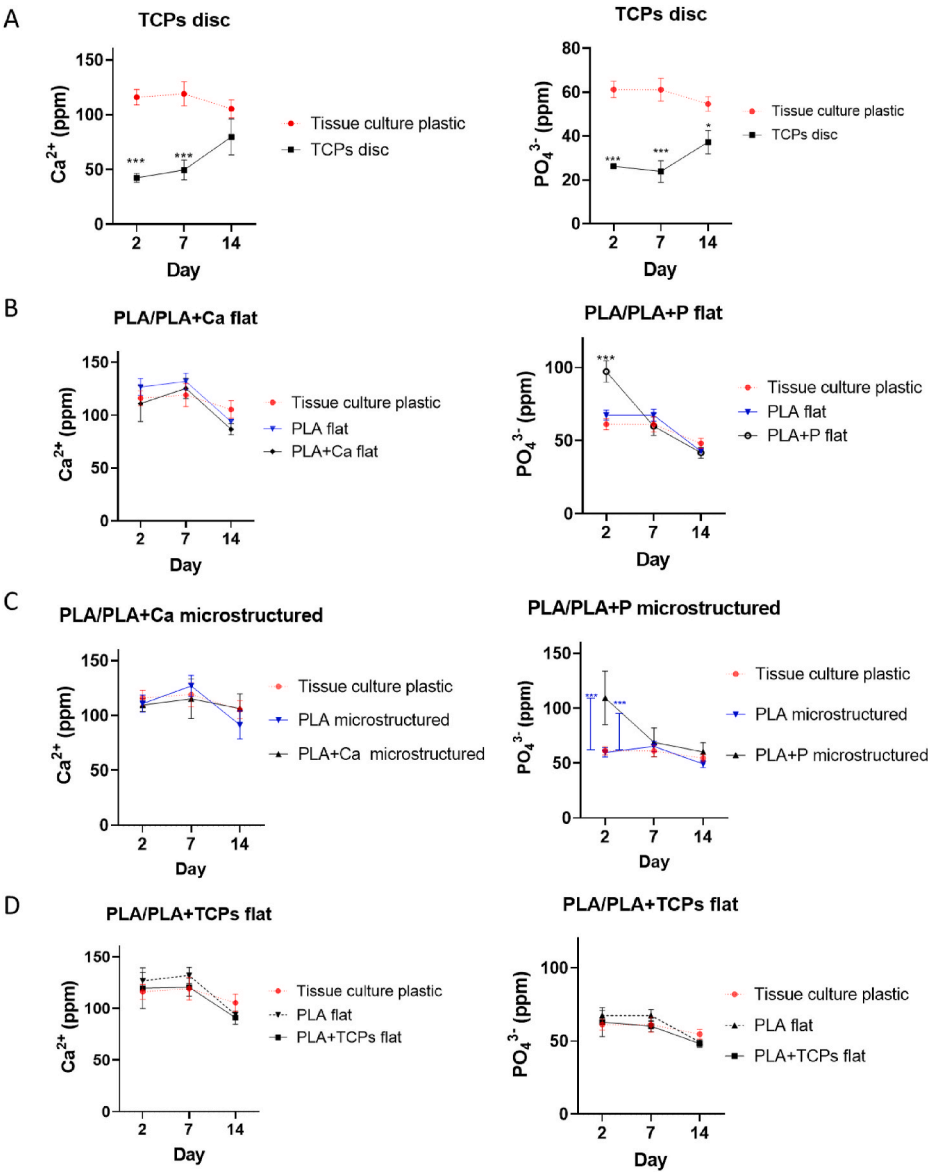

Fig. 4. Quantification of calcium and phosphate ions of cell culture medium upon hMSCs culture on the different materials for up to 14 days, determined by ICP-MS. Part 1. **A)** Levels of Ca and P ions in the medium with TCPs discs. **B)** Levels of Ca and P ions in the medium with flat PLA and composite discs. The addition of $CaCO_3$ to PLA did not significantly affect the levels of Ca ions in the medium. The addition of P salt to PLA increased significantly ($p < 0.001$) the levels of P ions in the medium at day 2, but not at day 7 or 14. **C)** Levels of Ca and P ions in the medium with microstructured PLA and composite discs. Again, only the addition of P salt modified the P ion levels at day 2. **D)** Ca and P ion levels in the medium with flat PLA-TCPs discs.

by the polymer matrix and that the duration of the study was not long enough to allow significant degradation of the polymer.

It is also important to note that the ions concentrations measured cannot be considered cumulative, as intermediate medium changes were performed every 2 days, while the measurements were only performed at days 2, 7 and 14.

3.4. Cell attachment and morphology

The confocal images are shown in Fig. 6, with the nuclei in blue (DAPI) and the actin fibers in red (phalloidin). In general, a higher cell density was observed on the flat than on the microstructured surfaces. Rough surfaces have been shown to enhance cell attachment [47], but also to decrease osteoblasts proliferation [48,49], which may explain the lower cell density. In areas with high cell density, the cells appeared more elongated and aligned in one direction, as compared with cells present in low cell density areas.

3.5. Gene expression analysis of markers of osteogenic differentiation

Upon fabrication and characterization of the materials and analysis of their effect on the ions concentration of cell culture medium, an analysis of the gene expression of a set of osteogenic markers was performed to identify potential changes in the expression profiles, as a function of chemical and/or structural characteristics of the materials (Fig. 7).

We selected a set of genes that is commonly used to evaluate the osteogenic differentiation of hMSCs on different biomaterials, including CaPs. ALP is an early marker of osteogenesis [50], which, in its enzymatic form, is known to convert extracellular pyrophosphate, a mineralization inhibitor, into inorganic phosphate, a mineralization promoter, and is therefore considered a good predictor of tissue mineralization [51,52]. BMP-2, another early marker, has been intrinsically related with the osteogenic differentiation of MSCs [53], alike Runt-related transcription factor 2 (Runx2), known to regulate osteoblast differentiation and function [54,55]. Ectonucleotide pyrophosphatase (ENPP-1), the gene that encodes the production of the plasma cell glycoprotein 1 (PC-1), has been previously shown to play a role in the induction of osteogenic differentiation of hMSCs by CaPs, and to be regulated by both Ca and P ions [31]. OPN and OCN are both late markers related with the formation and growth of apatite crystals [56,57], and are also affected by the presence of Ca and Pi ions [29]. It should be noted that, due to

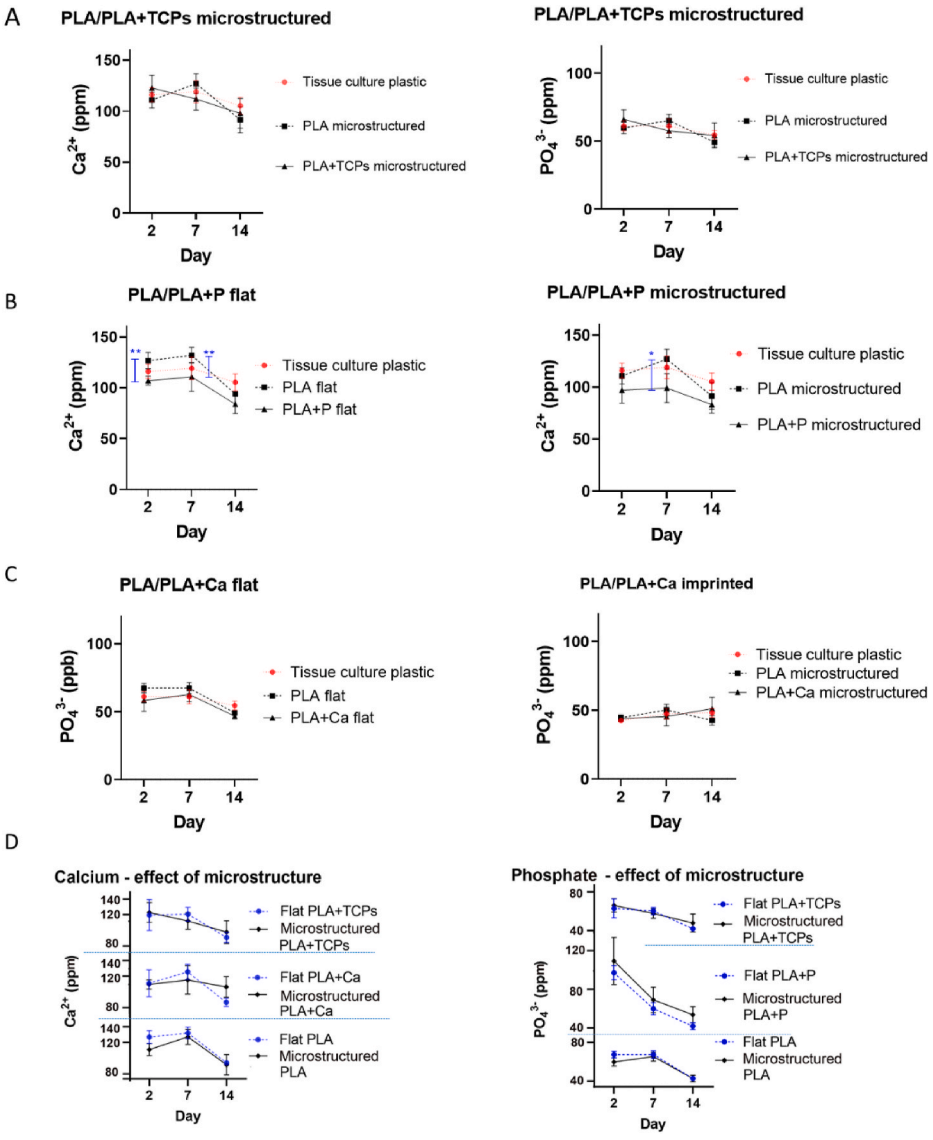

Fig. 5. Quantification of Ca and P ions of cell culture medium upon hMSCs culture on the different materials for up to 14 days, determined by ICP-MS. Part 2. **A)** Ca and P ion levels in the medium with microstructured PLA-TCPs discs. **B)** Levels of Ca ions in the medium with flat and microstructured PLA + P composites. **C)** Levels of P ions in the medium with flat and microstructured PLA + Ca composites. **D)** Effect of surface microstructure on ion concentration. Imprinting the PLA and composites surface with TCPs microstructure did not affect the ion content of cell culture medium.

technical issues, no data could be obtained for the BMP-2 and OCN expression at day 21.

Among the tested materials, TCPs showed the highest expression levels for all osteogenic markers, an effect that was in particular obvious at day 14. A range of CaPs has shown the ability to induce osteogenic differentiation of hMSCs and (pre)osteoblasts, even in the absence of other (soluble) stimulators of osteogenic differentiation [58–63]. However, since the properties of CaPs are largely intertwined, it is difficult to pinpoint which property or combination of properties is predominantly responsible for this effect. By producing replicas of the ceramic surface morphology in PLA, we aimed to separate the effect of the surface morphology from the effect of chemistry, i.e., the presence of CaP. By then introducing the Ca salt, the P salt or the TCPs powder into the (microstructured) PLA, we stepwise reintroduced the constituents of CaP. The results showed that none of the combinations, including the microstructured PLA + TCPs, which possesses both chemical and structural 'ingredients' of the original ceramic, was as potent as the ceramic itself in triggering the osteogenic differentiation of hMSCs. This result points at the importance of other properties that we were not able to replicate by the techniques used here. For example, the stiffness, surface energy and surface charge of PLA are different from those of the ceramic itself, and it is plausible that these properties are not mimicked by making structural replicas in the polymer or associated composites. The effects of substrate stiffness *versus* chemistry have been investigated before in the context of CaPs [14], however, the method of crosslinking the carrier polymer to vary stiffness that was used in that study, could not be applied to our system. Finally, we cannot exclude the potential negative effect of the polymer itself on the osteogenic differentiation of hMSCs, as by the techniques used here, we not only separated the individual properties of the CaP ceramic but also introduced a new material into the system.

Zooming in into the effects of surface morphology at different scale levels in the absence of inorganics, on the PS controls, we observed that all markers, with the exception of ENPP-1, were upregulated on the microstructured relative to the flat PS discs. This effect was more pronounced at day 14 than at day 21 (Fig. 7A and C). In particular the expression of ALP and OPN were upregulated on microstructured PS at day 14. No effect of microstructure was observed on the ENPP-1 expression, which was previously shown to be affected by Ca and P ion levels [31].

Similar to what was observed for the PS, microstructured PLA discs showed a higher expression of BMP-2, OCN and Runx2 as compared to flat PLA discs at day 14 (Fig. 7B), while a small upregulation of ALP was observed at day 21 (Fig. 7D). Collectively, these results showed that imprinting PS and PLA with TCPs topography in the absence of inorganic additives had a positive effect on the expression of several markers

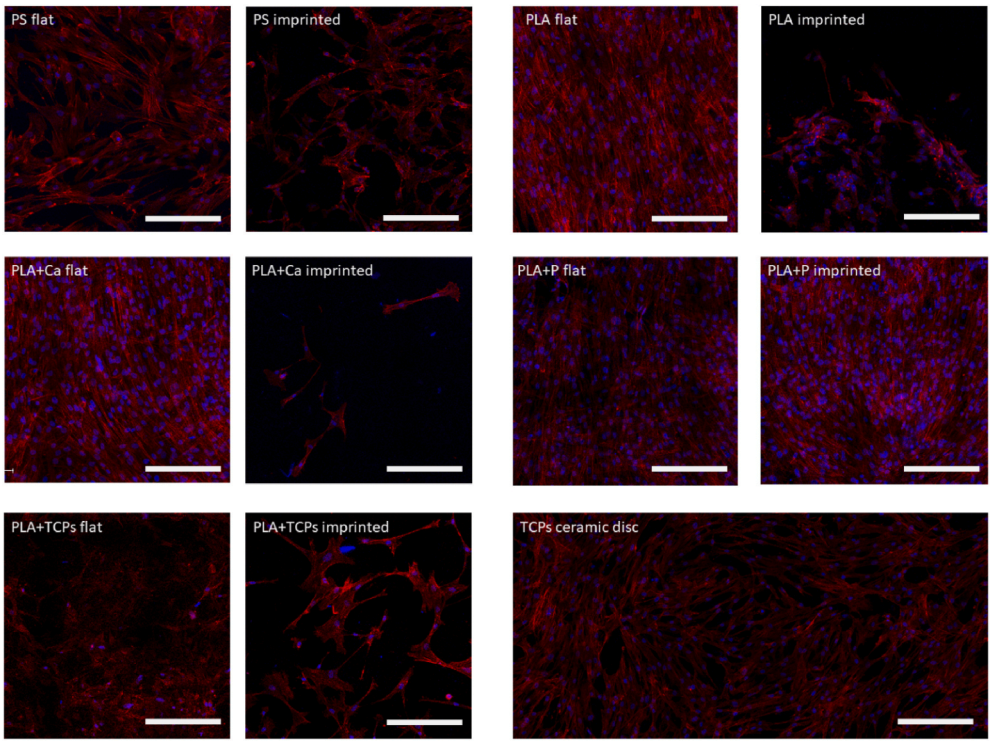

Fig. 6. Immunofluorescence assessment of cell attachment of hMSCs cultured on the different materials for 3 days. The confocal microscopy images show the nuclei in blue (DAPI) and the actin fibers in red (phalloidin). In general, a higher cell density was observed on the flat compared to the microstructured materials.

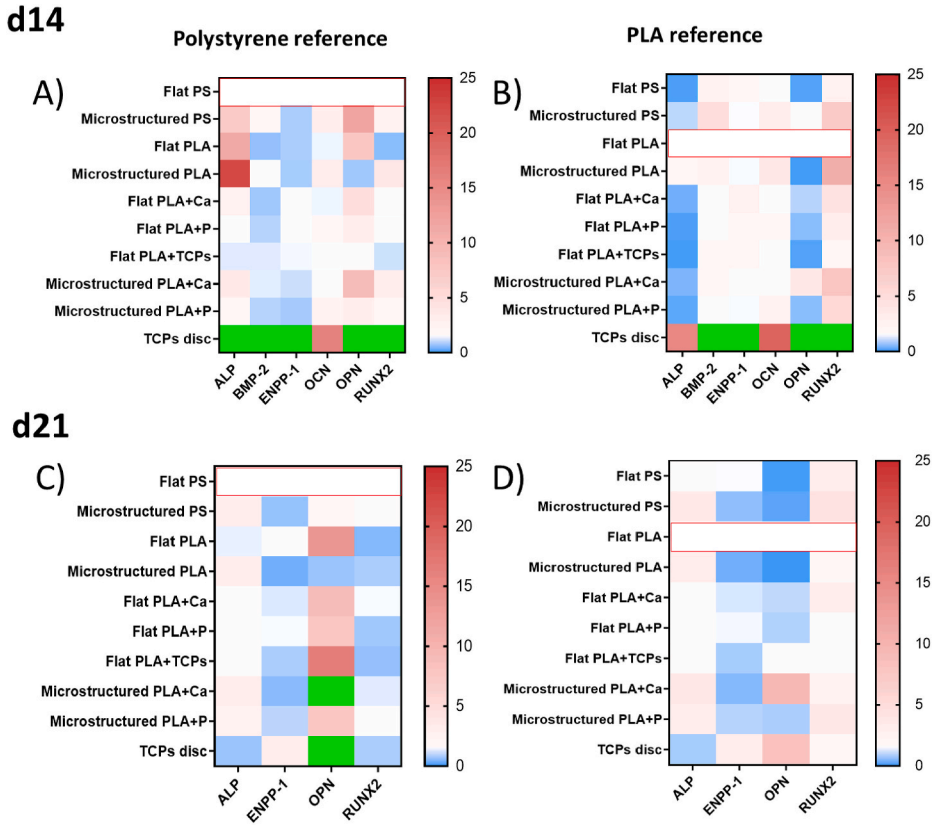

Fig. 7. Gene expression of osteogenic markers. The color codes represent: no changes with respect to the reference in white, downregulation in blue, and upregulation in red. Values beyond 25-fold changes are represented in green.

of osteogenic differentiation, in particular at the earlier time point of 14 days.

It is also remarkable that in general, the expression of osteogenic markers, in particular BMP-2, ENPP-1, OCN and Runx2 at day 14, as well as of ALP at day 21 was lower in hMSCs cultured on PLA than on PS. This effect was observed on both flat and microstructured discs. Difference is stiffness between the two materials (2.28–3.28 GPa for tissue culture PS [64] and 1–3.45 for poly(D,L lactic acid) [65] depending on its molecular weight; ~1.4 GPa for the molecular weight comparable to what was used here [66]) may have been a reason for this, as increasingly more studies show that substrate stiffness may affect cell attachment, proliferation and fate [67–69]. Obviously, differences in chemistry between the two polymers cannot be fully excluded either, although to the best of our knowledge, PLA has not been reported to have an intrinsic effect on the expression of the osteogenic genes tested here [70,71].

Concerning the effects of the individual ions, it was observed that the addition of Ca salt to flat PLA slightly increased the expression of BMP-2, ENPP-1 and OCN by day 14, while ALP and OPN were downregulated. Previous studies have shown that BMP-2 expression is dependent on the levels of Ca ions present in the medium [29,72], and a similar observation was made for the expression of ENPP-1 [31], OCN and OPN [73–75]. While the materials were designed in such a way that Ca ions would be released into the cell culture medium, the ICP-MS results showed that this was not the case (Figs. 4 and 5), which may explain limited to no effect on the expression of the markers of osteogenesis on PLA + Ca composites. In general, similar observations were made when comparing flat PLA with flat PLA + Ca and microstructured PLA with microstructured PLA + Ca, i.e., limited to no effect on the majority of osteogenic markers. An exception was the expression of OPN, that was significantly upregulated on microstructured PLA + Ca relative to microstructured PLA at both 14 and 21 days. The expression of OPN on microstructured PLA + Ca was comparable to the expression on TCPs at day 21. ICP-MS results showed a higher Ca ion concentration at day 14 for the microstructured PLA + Ca than for the microstructured PLA (Fig. 4C) (though the difference was not significant), which, in combination with the already observed positive effect of microstructure on PLA + Ca composites may have been a reason for the observed upregulation of OPN.

Similar to composites with Ca salt, PLA composites containing P salt did not elicit a significant effect on the osteogenic differentiation of hMSCs relative to PLA, on either flat or microstructured discs. The only remarkable effect was a downregulation of ALP on microstructured PLA + P *versus* microstructured PLA at day 14. PLA + P had a similar effect on the osteogenic differentiation as PLA + Ca; however, the effect on the OPN expression was not observed in the composites with Ca salt.

Finally, the addition of TCPs powder to flat PLA discs had a limited to no effect on the expression of osteogenic markers at either time point, whereas no results could be obtained from microstructured PLA + TCPs discs due to technical issues.

Taken together, the results of this study showed that none of the combinations of the properties tested, i.e., PLA discs with ceramic microstructure containing Ca salt, P salt or TCPs powder were able to recapitulate the effect the original TCPs ceramic has on the osteogenic differentiation of hMSCs. We observed some effects of the surface morphology in the absence of CaP and showed its synergy in combination with, in particular, Ca. A limitation of this study is the fact that the designed release of Ca and P ions from the composites did not occur to an extent that was expected, making it difficult to study the sole or combined chemical effects. In an earlier study, CaP was added to the polymeric replicas by the means of a thin coating, which ensured direct exposure of the ceramic to the cells [24].

In the follow up studies, it is important to obtain a closer control over the ions release/concentration in the medium. Moreover, other properties, such as substrate stiffness and surface energy should be more closely controlled. Besides, the microstructure replication technology we used, despite its accuracy, is strictly speaking a 2.5D method, as it only reproduces the features that are on the surface of the biomaterial. Other 3D elements, such as inner pores or interconnecting pores, which, in the context of bone graft substitutes are relevant for the processes of, e.g., vascularization, and more generally, bone tissue deposition [76,77], are thus not reproduced and require additional techniques such as those based on additive manufacturing [78]. Finally, in parallel with developing improved models both from a materials as well as from a biology/physiology perspective, it is important to invest in better characterization of materials and their interactions with biological systems, such as the local concentration of ions on the surface of CaP, which could not be measured in this study. An example of such an effort specifically for osteoinductive CaPs was recently presented in an elegant study by the Bohner group, which is important not only for understanding the phenomena such as osteoinduction [79], but also for developing bone graft substitutes with improved properties and performance.

4. Conclusions

In this study, we successfully replicated the surface morphology of an osteoinductive CaP ceramic in PLA and its composite containing a Ca, a P or a CaP filler to investigate the individual and combined effects of chemical and structural properties on the osteogenic differentiation of hMSCs. The original ceramic was more potent in triggering the osteogenic differentiation of hMSCs than any of the polymer/composite materials having individual or combined structural and chemical properties studied. PLA with the surface microstructure resembling that of the ceramic, but without any Ca or P present upregulated the expression of few osteogenic markers relative to flat PLA. This effect was enhanced when Ca was present in the polymer. Collectively, these results point at a combined effect of chemistry and surface morphology in regulating osteogenic differentiation of hMSCs. A limitation of this study was that the release of Ca and P ions from the composite was less controllable than intended, a challenge that should be addressed in future experiments. Nevertheless, the study delivered some valuable insights in how the response of the cells is guided by the different material properties. Moreover, this study showed that the properties decoupling 'tools' can be applied to a CaP ceramic with known biological performance, making it potentially interesting for other researchers working with CaP-based bone graft substitutes.

CRediT authorship contribution statement

V.P. Galván-Chacón: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **D. Pereira:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **S. Vermeulen:** Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. **H. Yuan:** Methodology, Resources. **J. Li:** Methodology, Validation, Investigation. **P. Habibovic:** Conceptualization, Methodology, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.03.030.

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