Microscopy to discern cells behaviour on different nano/microstructured calcium phosphates ceramics

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Background incl. aims
The formation bone tissue and the mineralization are positively influenced by the nanostructure or microstructure of calcium phosphate ceramics and by pore architecture of implants (1). Moreover, the release of Ca2+ was shown to induce cell growth, and PO43- released into medium induced osteogenic genes expression (2). The nano- or microstructure may significantly influence cell adhesion, proliferation and differentiation. The aim of this work was to study the effect of hydroxyapatite (HA) and beta tricalcium phosphate (β-TCP) of different structure on osteosarcoma cells adhesion, proliferation and osteogenic differentiation using microscopy techniques.

Methods
Porous ceramics from hydroxyapatite (HA), nanostructured HA (N-HA), calcium-depleted HA (CDHA), β-tricalcium phosphate (β-TCP), and nanostructured β-TCP (N-β-TCP) were prepared by 3D printing and sintering, and were characterized with SEM, XRD and tested in vitro with osteosarcoma SaOS-2 cells, seeded at a density of 368 x 103 cells/cm2 cultured in McCoy's 5A, 15% foetal bovine serum, 1 % of antibiotics (Penicilin/Streptomycin), and 40 μg/mL ascorbic acid. Cell proliferation was evaluated by dsDNA assay, and the metabolic activity was tested using MTS assay on days 7, 14, and 21. Focal adhesions were visualized using mouse monoclonal antibody Anti-Talin (T3287, Sigma Aldrich, 1:200), secondary Anti-mouse IgG Fab2-AlexaFluor488 (1:400, Molecular probes) and beta-actin was stained with Phalloidon-ATTO633 (1:1000, Sigma Aldrich) on day 1. Moreover, gene expressions of alkaline phosphatase (ALP) activity, Runx2, osteocalcin, collagen type I were tested. RNA was isolated from discs using RNeasy Mini Kit (74104, Qiagen, Hilden, Germany). The isolated mRNA was reverse transcribed to cDNA using qScript cDNA Synthesis Kit (QuantaBio, USA), following the manufacturer's protocol. Polymerase chain reaction was performed on Light Cycler 480 (Roche, Basel, Switzerlands), primers and probe were obtained from ThermoFisher Scientific. Data were evaluated using the 2−ΔCp method, for each gene relative to the housekeeping gene GAPDH.

Collagen type I, a midterm marker, was visualized using monoclonal antibody anti collagen I M-38c (1:50, DSHB, Iowa City, USA) and Anti-mouse IgG Fab2-AlexaFluor488 (1:400, Molecular probes) and propidium iodide (cell nuclei) on day 7 and 14. Osteocalcin, a late marker of osteogenic differentiation, was stained with Rabbit Anti-Osteocalcin (1:20, T4744, BMA Biomedicals) and Alexa Fluor 633 goat anti-Rabbit IgG (H+L) (1:200, Invitrogen) and Hoechst 34580 (2 μg/mL, H21486, Invitrogen) on day 21. All proteins were visualized using confocal microscopy and quantified using ImageJ. Statistical analysis was performed using GraphPad Prism 8.1.2. software, using One-way analysis of variance (ANOVA), according to normality test. The significance was set at $p < 0.05$.

Results
CDHA, N-HA and N-β-TCP had a nanostructure made of plate-like crystals. In contrast, HA and β-TCP had a microstructure of polyhedral grains. All nanostructured samples showed dot-like talin shape with low density of focal adhesions. Oppositely, both microstructured HA and β-TCP ceramics showed well visible and fibrous-like talin structures.