A microfluidics-based method for culturing osteoblasts on biomimetic hydroxyapatite

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Supplementary Material

A. Physico-chemical characterization of HA

A.1 Particle size distribution of $\alpha$-tricalcium phosphate

The particle size distribution of $\alpha$-tricalcium phosphate was analysed using laser diffraction. The $\alpha$-tricalcium phosphate powder was dispersed in isopropanol and sonicated for 5 minutes. The dispersion was loaded (obscuration of 10–20%) into a Mastersizer 3000, with a hydroEV wet dispersion unit (Malvern Instruments Nordic AB). Values of 1.63, 0.1, 1.39 and 2.866 were used for particle refractive index, absorption index, dispersant refractive index and particle density, respectively. Particle size analysis was conducted with software provided by the manufacturer (Mastersizer 3000, version 3.5). Figure S.M. 1 represent the average of 10 measurements.

Figure S.M. 1. Volume size distribution of $\alpha$-tricalcium phosphate particles
A.2 Setting time of the calcium phosphate cements

The initial and final setting times were determined with a Gilmore needle (#H-3150, Humboldt), using a 113 g load (needle tip diameter 2.12 mm) and a 453 g load (needle tip diameter 1.06 mm), as described by ASTM C266. Cylinder-shaped samples (\( \varnothing = 6 \text{ mm}, h = 3 \text{ mm} \)) comprised of 0.3 g of cement powder were tested in a controlled environment (37 ± 2 °C, 100% relative humidity). Preliminary samples were evaluated to identify the setting times, since repeatedly opening the sealed samples affected temperature, humidity and setting time. Final data was obtained on 5 independently prepared samples (each in duplicate, 10 in total), where the average setting time of each duplicate disc was recorded as a single independent value. Samples were evaluated every 5 minutes for initial setting time, and every 20 minutes for final setting time, based upon the expected setting time observed in preliminary samples. Samples were considered “set” when the Gilmore needle left a partial/ incomplete indentation.

Table S.M. 1. Initial and final setting times for calcium phosphate cement

|                | Initial setting time (t₁, min) | Final setting time (t₂, min) |
|----------------|--------------------------------|-----------------------------|
| CPC 0.65 mg/g  | 38.9 ± 1.1                     | 175.1 ± 14.2                |

A.3 Compressive strength of HA

Cylinder-shaped samples (\( \varnothing = 6 \text{ mm}, h = 12 \text{ mm} \)) were set for 7 days in an aqueous solution. Prior to testing, the cylinder surfaces were polished to 1200 grit fineness using silicon carbide polishing paper (Struers A/S). The compressive strength of the samples was tested in a universal testing machine (ref. n. 46-50639-32, Shimadzu) with a 5 kN load cell at a crosshead speed of 1 mm/min, with a spherically seated plate. The average compressive strength tested for the material was 24.9 ± 3.7 MPa.
A.4 Scanning Electron Microscopy (SEM) of HA

Micrographs of HA were obtained on a field emission SEM (Ziess LEO 1530, AB Carl Zeiss). A secondary electron in-lens detector was used with an acceleration voltage of 3 keV, and a working distance of 9 mm. Prior to SEM analysis, samples were sputtered with a thin coating of gold and palladium (Emitech SC7640, Quorum technologies), at 2 kV for 40 s. The characteristic plate-like structure of calcium-deficient hydroxyapatite was observed (Figure S.M. 2).

![Figure S.M. 2. Scanning Electron Microscopy (SEM) images of CPC in-chip at A) 500X and B) 10,000X magnification.](image)

A.5 X-ray Diffraction (XRD)

The crystalline phases of α-tricalcium phosphate, as well as the end products of the cementitious reaction were determined by XRD, with a Bruker D8 Advanced (Bruker Daltonics) using Cu Kα radiation ($\lambda = 1.5418$ Å). A scanning range of 3 to 60°, with a step size of 0.03° per step, and a dwell time of 0.2 seconds was performed. For phase identification, the diffraction patterns were compared with the Joint Committee on Powder Diffraction Standards for α-TCP (JCPDS #09-0348), β-TCP (JCPDS 70-2065) and HA (JCPDS #82-1943). The XRD spectra of the α-TCP powder as well as the one of the calcium phosphate cement after setting for 10 days (confirmed a complete transformation into hydroxyapatite) are shown in Figure S.M. 3.
Figure S.M. 3 XRD diffractogram of α-TCP and HA set for a 10-day period. Markers indicate peak matches to α-TCP and HA standards.
B. Cell adhesion studies

To decide on an ample incubation time prior to the application of flow, cell adhesion studies were performed. 50,000 MC3T3-E1 cells/cm² were seeded on pre-incubated HA discs (⌀ = 15 mm, h = 2 mm) in a 24-well plate containing supplemented cell culture medium. Prior to seeding, cells in suspension were stained with 5 µM of CellTracker Green CMFDA Dye (ref. n. C2925, Thermofischer Scientific) for 25 minutes. The cells were then imaged at 2, 4, 6 and 24 h using a fluorescent microscope (IX73 Inverted Microscope, Olympus) as shown in Figure S.M. 4. Based on the observed cell counts and morphologies, 2 h was chosen as a sufficient cell adhesion time prior to the start of flow.

**Figure S.M. 4.** Adhesion of MC3T3-E1 cells on CDHA at A) 2h, B) 4h, C) 6h and D) 24h.
C. Modification of HA in contact with cell culture medium

The modification of both HA-static and HA-on-chip in contact with cell culture medium over 10 days was evaluated. HA-on-chip samples were prepared and exposed to supplemented culture medium under 8 µl/min flow for 10 days. HA disc samples were also prepared and exposed to supplemented medium for 10 days, with medium being refreshed daily. After 10 days, HA samples were washed thrice in distilled water and then dehydrated by washing in ethanol solutions of increasing concentration (10%, 30%, 50%, 70%, 90% and 100% Ethanol) for 15 minutes each. The samples were then washed in acetone for 10 minutes and then dried overnight under vacuum.

Prior to SEM/EDS analysis, samples were coated with a thin layer of gold and palladium via sputtering at 2 kV for 40 s. The samples were then imaged using an acceleration voltage of 3 keV and a working distance of 8.5 mm. Characteristic HA crystals were observed in all samples imaged (Figure S.M. 5). In addition, EDS analysis were performed at an acceleration voltage of 20 KeV, maintaining the same working distance. The atomic levels of calcium and phosphorus were quantified and are represented as a Ca/P ratio in Figure S.M. 6. The EDS spectra were taken at least at six different spots of each sample.
Figure S.M. 5 SEM images of HA-on-chip taken A) before and B) after 10 days of flow in culture medium at 8 µl/min, as well as images of HA discs C) before and D) after 10 days of exposure to cell culture medium.

Figure S.M. 6 Ca/P ratio (obtained through EDS quantification) on the surface of HA discs and HA-on-chip samples, before and after exposure to culture medium for 10 days. In the case of HA-on-chip, a flow of 8 µl/min was perfused with a peristaltic pump. No significant differences were observed within each group of samples.
D. Ionic exchange study

D.1 Results of the PS-static samples

The [Ca$^{2+}$] and [P$^{5+}$] ionic exchange observed for PS-static is shown in Figure S.M. 7.

![Figure S.M. 7](image-url)  

**Figure S.M. 7.** Quantification of A) [Ca$^{2+}$] and B) [P$^{5+}$] in PS-static culture medium over the 10-day cell culture study period. The data represents separate values at each collection point, and is not cumulative. * indicates statistical difference between a sample time-point and fresh medium (p < 0.05, Dunett's test).
D.2 P-values of the ion exchange study

Below are included the p-values corresponding to the statistical evaluation of the 10-day ionic exchange assay (Figure 4) in the manuscript.

**Table S.M.2.** P-values corresponding to the data plotted in Figure 4A, where statistical differences in \([\text{Ca}^{2+}]\) between a sample (HA-on-chip, HA-static or PS-static) and fresh medium were evaluated (significance level of \(\alpha = 0.05\)).

| [Ca^{2+}] | HA-on-chip | HA-static | PS-static |
|-----------|------------|-----------|-----------|
| Day 1     | > 0.05     | < 0.0005  | > 0.05    |
| Day 2     | > 0.05     | < 0.0005  | > 0.05    |
| Day 3     | > 0.05     | < 0.0005  | > 0.05    |
| Day 4     | > 0.05     | < 0.0005  | > 0.05    |
| Day 5     | > 0.05     | < 0.0005  | > 0.05    |
| Day 6     | > 0.05     | < 0.0005  | > 0.05    |
| Day 7     | > 0.05     | < 0.0005  | 0.001     |
| Day 8     | > 0.05     | < 0.0005  | 0.02      |
| Day 9     | > 0.05     | < 0.0005  | 0.05      |
| Day 10    | > 0.05     | < 0.0005  | > 0.05    |
Table S.M. 3. P-values corresponding to the data plotted in Figure 4B, where statistical differences in [P$^{5+}$] between a sample (HA-on-chip, HA-static and PS-static) and fresh medium were evaluated (significance level of $\alpha = 0.05$).

| Day  | HA-on-chip  | HA-static   | PS-static  |
|------|-------------|-------------|------------|
| 1    | > 0.05      | < 0.0005    | > 0.05     |
| 2    | > 0.05      | < 0.0005    | > 0.05     |
| 3    | 0.002       | < 0.0005    | > 0.05     |
| 4    | 0.02        | < 0.0005    | > 0.05     |
| 5    | > 0.05      | < 0.0005    | > 0.05     |
| 6    | < 0.0005    | < 0.0005    | > 0.05     |
| 7    | > 0.05      | < 0.0005    | > 0.05     |
| 8    | 0.02        | < 0.0005    | > 0.05     |
| 9    | > 0.05      | < 0.0005    | > 0.05     |
| 10   | > 0.05      | < 0.0005    | > 0.05     |
**E. Flow rate characterisation of the syringe and peristaltic pump**

The flow rate of medium pumped by the peristaltic pump (LabV1-II, Shencheng) and syringe pump (PHD-2000 infusion, Harvard Apparatus) were measured using a flow sensor (MFS2, Elveflow) at 2, 8 and 14 µl/min for 100 s (Figure S.M. 8).

![Flow profiles for A-C) syringe and D-F) peristaltic flow at 2, 8, and 14 µl/min taken for 100 s.](image)

**Figure S.M. 8.** Flow profiles for A-C) syringe and D-F) peristaltic flow at 2, 8, and 14 µl/min taken for 100 s.