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ABSTRACT

Biocompatible and osteoconductive calcium carbonate (CC) and hydroxyapatite (HAP) have been studied as bone filling materials. In contrast to HAP, CC is highly resorbable, and to achieve optimal resorption rates, a functionalized calcium carbonate (FCC) consisting of CC particles with a HAP layer has been further developed. The aim of this study was to characterize the physicochemical and biological properties of FCC.

Physicochemical properties were studied with ATR-FTIR, XRD and SEM. pH change and ion release were studied in a static system in cell culture medium or water, and in a continuous dynamic system in Tris-buffer. Viability and morphology of pre-osteoblastic cells *in vitro* was studied with a WST method, as well as with SEM and TEM imaging.

There was a minor increase in pH in Tris-buffer and water dissolution systems at the beginning of FCC dissolution. In contrast, pH of the cell culture medium remained approximately unchanged during the dissolution. In Tris-buffer and water dissolution systems, increased levels of calcium release from FCC were detected, whereas in the cell culture medium FCC was shown to adsorb calcium from the medium, thus causing a decrease in the dissolved calcium concentration. This was associated with decreased viability of cells. Interestingly SEM and TEM imaging revealed internalization of FCC particles by the cells.

This study showed that FCC, similar to natural bone mineral, carbonated apatite has a capability to adsorb calcium from surrounding liquid. This needs to be carefully considered when cell culture assays are performed with these types of materials.

1. Introduction

Calcium phosphates, such as hydroxyapatite, have been widely investigated as bone substitutes for regenerative medicine since the 1970s [1]. Other interesting calcium-containing compounds are calcium carbonates, which have also been introduced for bone-filling applications [2]. Both hydroxyapatite and calcium carbonate have been shown to be biocompatible and osteoconductive but display a lack of osteoinduction [1]. Calcium carbonate is highly resorbable in contrast to hydroxyapatite, which has a lower rate of resorption [3,4]. These two materials, HAP and CC, have also been combined into functionalized calcium carbonate (FCC), which was first developed for industrial applications.

Hydroxyapatite (HAP) is usually written out as a hexagonal unit cell $Ca_{10}(PO_4)_6(OH)_2$, which has a low solubility but can provide nucleating sites for the precipitation of apatite crystals [5,6]. HAP coatings with a high degree of crystallinity are known to induce a higher degree of cell attachment and proliferation than less crystalline coatings [7]. Besides being biocompatible and osteoconductive, HAP bone can also form a strong bond to bone, *i.e.* HAP is a bioactive material by this definition of

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the term [6]. Due to its properties, HAP has been used in several product forms from scaffolds to particles, and in various clinical applications, such as in dental and orthopedic surgery as a bone filling material or as a coating material for metal and composite implants, as well as in drug delivery [6–9].

Natural calcium carbonates ($CaCO_3$, CC) have three crystalline anhydrous forms: calcite, aragonite and vaterite, which are stable, metastable and unstable, respectively [10]. In implantable materials made of coral, calcium carbonate is present as aragonite or the calcite form [11]. Natural coral has been widely investigated for its properties as a potential bone substitute. It is resorbable, biocompatible, osteoconductive and its porous structure is very close to the spongy structure of trabecular bone and furthermore resembles its mechanical properties [2]. Corals have also been tested for being used in maxillo-facial surgery and orthopaedics [2,11]. However, there are some limitations for using calcium carbonates of natural origin, for example the availability, biological variability, microbiological contamination, and ecological issues, such as obtaining calcium carbonate from endangered coral species [3,10,12]. In addition to using coral materials as a source, calcium carbonate from eggshells has been investigated as bone filling material [13,14]. For this reason, synthetic CC may provide an option for a larger scale use of CC in medical and dental applications.

In vivo, CC is resorbed rapidly by osteoclasts' carbonic anhydrase enzymatic activity, which releases its elemental components in an ionic form [2]. Therefore, coating CC with HAP, which has a lower resorption rate, has been studied to develop more stable structures for bone applications [15,16]. These types of implantable materials (CHACC and ProOsteon) have already been studied *in vivo* [15,17-19] and as substitutes for implantable metals [20]. Such material combinations of HAP and CC have showed improved bone formation and mineralization [14] and have also been studied against hypersensitivity of teeth [21].

Surface geometry and topography of the materials, such as macroporosity, microporosity and interconnection of pores are known to affect cell adhesion, proliferation and migration, as well as blood vessel and new bone formation [22]. The surface topography and dissolution rate of CC can be controlled by functionalizing the CC surface by HAP. Functionalized calcium carbonate (FCC) consists of a CC core and a HAP surface layer, where the HAP content varies from 13 to 85% in different FCC products [23]. FCC has been used in many different industrial fields, *e.g.* agriculture, cosmetics, and studied in the pharmaceutical industry as drug delivery materials [23,24]. As CC and HAP are well-established bone-filling materials, it could be expected that FCC could also have beneficial properties to be used as an implantable bone-filling material.

Thus, the aim of this study was to study the biologically significant properties of FCC on bone forming cells of the osteoblast lineage. FCC was first characterized physico-chemically and thereafter its effects on cell viability and morphology were studied by using a mouse pre-osteoblast cell line (MC3T3-E1).

2. Materials and methods

2.1. Materials

Hydroxylapatite (HAP) functionalized calcium carbonate (CC) particles (FCC) [24] were used in the study. FCC consisted of 51 wt% of HAP and 49 wt% of CC. The average diameter of the particles was 7 μm, surface area 55.4 m^2/g, and porosity 61.55% [23,24]. FCC was prepared by Omya International AG as described earlier [24] by etching calcium carbonate particles and re-precipitating a modified surface structure with in-situ or externally supplied CO_2 in the form of carbonic acid. Etching process produced morphology with recrystallized surfaces, consisting of incorporated hydroxyapatite (HAP). Surface structure and composition of FCC has been characterized in detail previously [24,25].

2.2. Cell cultures

A mouse cell line MC3T3-E1, subclone 4, (ATCC, CRL2593) was cultured in Minimum Essential Medium Eagle (M8042 Sigma Aldrich) with 10% FBS (Gibco, 10270–106) and 1% penicillin at 37°C in a humidified atmosphere containing 5% CO_2. At sub-confluency, the cells were trypsinized and plated on 96-well plates for viability assays. All experiments were performed with cells below 25 passages.

2.3. ATR-FTIR analysis

ATR-FTIR was used to analyze the vibrational features of FCC by using a PerkinElmer Spectrum (Version 10.4.2) spectrometer. The ATR-FTIR instrument averaged from 16 scans collected for wavelengths from 650 cm^{-1} to 4 000 cm^{-1} at 4 cm^{-1} resolution. The software was CPU32 Main 00.09.9934 22–4–2011 and a UATR crystal combination diamond/ZnSe at triplicate of bounces was used.

2.4. XRD analysis

Crystal structure of the FCC particles was analyzed by XRD with a Bruker D8 Discover instrument with Cu Kα radiation (λ = 1.54 Å). The FCC were measured using power XRD settings in the 2-theta range 5°−80°, with an increment of 0.04° and data collection of 0.2 s per step.

2.5. pH change and ion release in a continuous dynamic system

In vitro release of ions was measured in a continuous dynamic system in a fresh solution of Tris buffer (2-amino-2-hydroxymethyl-propane-1,3-diol), which was continuously fed (0.2 ml/min) through a bed of the sample particles at 40 °C. The pH of the (50 mM) Tris buffered solution (Trizma base, Sigma-Aldrich, pKa 8.06) was adjusted to 7.3 with 1 M HCl (J-T. Baker). The chamber was filled with particles (59 mg) as described previously [26]. The ion concentrations and pH were measured in solution samples (4 ml) collected at the time points of 20, 40, 60, 80, 100, 120, 240, 480, and 1440 min. pH was measured with a Mettle Toledo, Seven Easy electrode. For the ion analysis, 1 ml of the solution was diluted with ultrapure water (1:10) and acidified with concentrated HNO_3. The ion concentrations in the solutions were then measured with Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, PerkinElmer Optima 5300 DV, Waltham, MA, USA). The released ions were examined for silicon (LOQ 0.04 ppm, 251.611 nm), sodium (LOQ 0.2 ppm, 589.593 nm), calcium (LOQ 0.7 ppm, 393.366 nm), phosphorus (LOQ 0.03 ppm, 213.617 nm) and aluminum (LOQ 0.01 ppm, 396.153 nm).

2.6. pH change and calcium ion release in a static system

Since calcium was the main ion type to be dissolved from FCC and the phosphorus dissolved only slightly, only calcium release was measured in a static dissolution system. *In vitro* release of calcium and change in pH was measured for sterilized FCC particles (Autoclave, 121°C, 20 min) in the static system either in cell culture medium (M8042 Sigma Aldrich, 10% FBS, 1% penicillin) or in ultrapure water at 37°C in a humidified atmosphere containing 5% CO_2. The pH was measured with SDR SensorDish Reader (PreSence) with HD24 and software version SDR_v4.0.0. The measuring interval was adjusted to every 15 min. The pH was measured with five different concentrations of FCC, *i.e.* 12.5, 25, 50, 100 and 200 μg/ml and a blank control (0 μg/ml) in cell culture medium or in ultrapure water up to 14 days. During the 14-day dissolution test, cell culture medium or water was changed every 3–4 days by carefully pipetting just below the liquid surface and avoiding touching the bottom of the wells, similarly as when changing the culture medium for cells in the viability test. In another plate, cell culture medium or water was kept unchanged for the entire 14 days of dissolution. For each well, a sample for the calcium analysis was

collected after 14 days of dissolution. For calcium measurements was repeated second time without pH measurements.

All calcium samples were pipetted just below the liquid surfaces and then quickly spun. Before calcium analysis, samples from cell culture medium were diluted 1:20 and samples from water 1:10 with ultrapure water. Liquid was further mixed with 5% La-solution, centrifuged at 2800 rpm for 10 min and calcium concentrations were then measured with Atomic Absorption Spectroscopy (AAS) (PerkinElmer AAnalyst 400, with Ca-lamp).

2.7. Morphology of FCC particles

The morphology of the FCC particles was characterized from scanning electron micrographs (SEM) taken of the FCC particles as received and after a 14-day dissolution in the cell culture medium. After dissolution in the cell culture medium, the particles were washed with 70% ethanol before imaging. The electron beam was accelerated with voltages of 2.7 kV and the SEM used was a Leo Gemini 1530 (Carl Zeiss, Oberkochen, Germany).

2.8. Cell viability assay

To assess the *in vitro* viability of preosteoblasts in the presence of the FCC particles, MC3T3-E1 cells were seeded at 5 000 cells/well in 96-well plates. The total volume of each well was 200 μl. FCC particles were sterilized in an autoclave (121°C, 20 min), and FCC suspensions were made right before the viability experiment was started. Five different concentrations, *i.e.* FCC 12.5, 25, 50, 100 and 200 μg/ml were used, and a control (0 μg/ml) was included. Cell viability was determined by a WST method (Cell Counting Kit-8, CK04, Dojindo, 1:20 dilution) by measuring absorbance at 450 nm (Thermo Scientific, Multisckan FC with Skanlt software for microplate readers, UI version 4.1.0.43) at time points of 1, 3, 7 and 10 days. Medium was changed every 3–4 days. Background absorbance (FCC particles at each concentration in cell culture medium without any cells) was also measured and subtracted from sample values, when viability results were analyzed. Viability assay was repeated three times.

2.9. SEM and TEM imaging of cells in the presence of FCC particles

For the SEM and TEM imaging, MC3T3-E1 cells were cultured in the presence of 12.5 μg/ml and 25 μg/ml of FCC particles on glass coverslips for 10 days. The medium was changed every 3–4 days.

For the SEM images, the samples were fixed in a 5% glutaraldehyde in 0.16 M s-collidin buffer, pH 7.4, post-fixed by using 1% OsO_4 containing 1.5% potassium ferrocyanide and dehydrated with a series of increasing ethanol concentrations (30%, 50%, 70%, 80%, 90%, 96%, and twice 100%). The samples were then immersed in hexamethyldisilazane and left to dry by solvent evaporation. The samples were examined using a Leo Gemini 1530 (Carl Zeiss, Oberkochen, Germany) instrument operated at 2.7 kV acceleration voltages.

For TEM images, the samples were fixed with 5% glutaraldehyde in s-collidine buffer, post-fixed with 1% OsO_4 containing 1.5% potassium ferrocyanide, dehydrated with ethanol, and embedded in 45,359 Fluka Epoxy Embedding Medium kit. Thin sections were cut using an ultramicrotome to a thickness of 70 nm. The sections were stained using uranyl acetate and lead citrate. The sections were examined using a JEOL JEM-1400 Plus transmission electron microscope operated at 80 kV acceleration voltage.

2.10. Statistical analysis

Statistical analyses were performed by using the JMP pro 16.2.0 (570548). Cell viability in presence of different concentrations of FCC at each time point was compared to the respective control group. Statistical significance was analyzed by using the nonparametric multiple comparisons by Dunn methods for joint ranking. The same methods were used to compare calcium concentrations after incubating the samples with of different amounts of FCC particles. P-values < 0.05 were considered statistically significant.

3. Results

3.1. ATR-FTIR

Vibrational features of the molecular structure of FCC particles were characterized with ATR-FTIR. Peak assignments of the FCC particles (Fig. 1A) and ATR-FTIR spectra (Fig. 1B) showed strong absorbance at 712 cm^{-1}, 874 cm^{-1} and 1 427 cm^{-1}, which are from carbonate molecule, and strong absorbance at 1 023 cm^{-1}, which is from phosphate molecule from the hydroxyapatite part of FCC.

3.2. Phase composition of FCC

Crystal structure of FCC particles was characterized with XRD and the diffractogram is shown in Fig. 2. In comparison with reference data, it is clear that the FCC material consists of crystalline calcite (ICDD card 00–005–0586) and hydroxyapatite (ICDD card 00–009–0432). Furthermore, the XRD data also revealed a broad peak in the range of 9–18° 2Theta, which suggests that part of the FCC particles is of amorphous nature.

3.3. pH changes and ion release from FCC

pH changes in the presence of FCC and the corresponding ion dissolutions were studied in two different systems: in a continuous dynamic system with Tris buffer and in a static system with water or cell culture medium. In addition, the static dissolution system was performed by either changing the fluid (water or cell culture medium) during the experiment similarly as for cell viability experiments or without any liquid changes.

In the continuous dynamic system of Tris immersion, pH value was increased at the beginning of immersion, which was then followed by stabilization around eight hours (Fig. 3A). Dissolution during the continuous dynamic Tris immersion showed constant and low levels of phosphorous during the Tris immersion follow-up time (24 h) (Fig. 3B), while calcium concentration appeared high in the beginning of immersion but was then stabilized to the level of *ca*. 10 mg/ml at around 8 h of the immersion (Fig. 3B).

In static dissolution system with cell culture medium, pH remained approximately the same (7.3–7.4) during the follow-up time, irrespective of whether the medium was changed in-between or not (Fig. 4C-D). However, small differences were shown in the system, where the cell culture medium was changed every 3–4 days during the 14-day follow-up. In water, pH change differed from that of the cell culture medium. When the water was unchanged during the follow-up, the pH value increased in the beginning of immersion and, was then followed by stabilization at around day one with highest concentrations (200 μg/ml) of FCC (Fig. 4A). In the system, where water was changed every 3–4 days during the follow-up, the pH decreased gradually over time (Fig. 4B). All of the four systems showed minor peaks at the same time points, which were the times, when the plate was taken out from the incubator and the cell culture medium or water was changed in certain wells.

After 14 days of dissolution, calcium concentration of the cell culture medium was significantly decreased in the presence of FCC at 200 μg/ml and when the cell culture medium had not been changed during the follow-up period ($P < 0.05$, Fig. 5A). In the system, where the cell culture medium was changed every 3–4 days, no significant differences were observed in the calcium concentration (Fig. 5A). In the system, where water was not changed, the calcium levels were below the detection limits of the analysis method (< 1 mg/L) and were reliably measurable

A.

Peak assignments	Wavenumber [cm^{-1}]	Transmittance [%]
Carbonate v_4	713	89
Carbonate v_2	874	81
Phosphate v_3	1 023	68
Carbonate v_3	1 417	80

B.

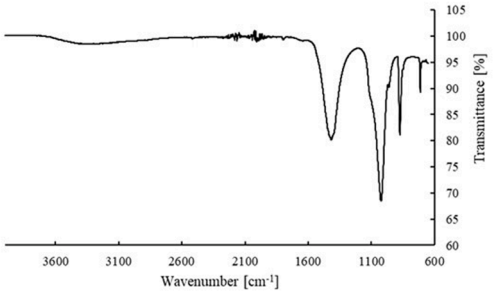


Fig. 1. Structural characterization of FCC with ATR-FTIR. **(A)** ATR-FTIR peak assignments of FCC particles. **(B)** ATR-FTIR spectra of FCC particles.

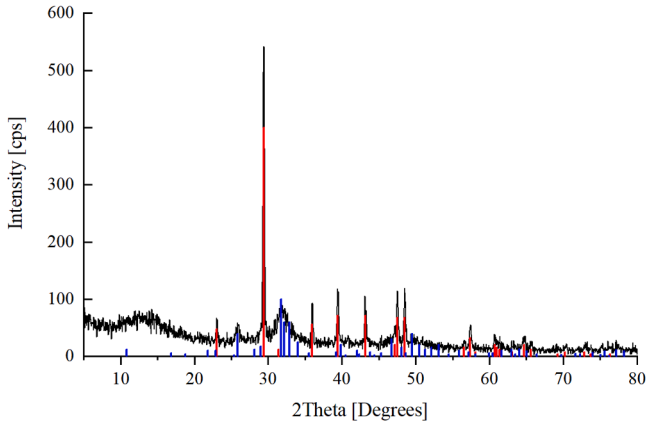


Fig. 2. Structural characterization of FCC with XRD (black) showing equivalent patterns as calcite (red) and hydroxyapatite (blue) reference data.

only when FCC concentration was higher than 50 μg/ml. However, when the liquid was changed, dissolved FCC was probably removed during changing of the water and no calcium was detectable (Fig. 5b).

SEM examination of FCC particles was made as received and after 14 days of cell culture medium dissolution. SEM images were obtained with 2.5 kX, 10 kX, 25 kX magnifications, the dimensions of the images being 46 × 36 μm, 12 × 9 μm and 4.8 × 3.6 μm, respectively. FCC particles without any dissolution had a fine porous surface structure and they varied in size (Fig. 6, left-hand panels). After 14 days of dissolution in the cell culture medium, the particles' surface porosity decreased (Fig. 6, right-hand panels), probably due to adhesion of proteins present in cell culture medium.

3.4. Cell proliferation and viability

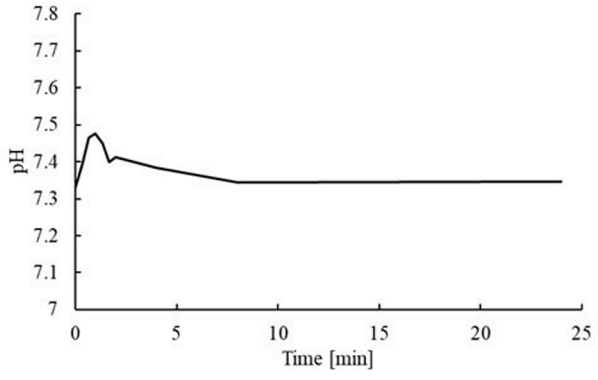
The viability of pre-osteoblastic MC3T3-E1 cells was significantly decreased ($P < 0.05$) in the presence of FCC particles (12.5, 25, 50, 100, 200 μg/ml) at all concentrations and time-points, except for day 10 time point with FCC at 12.5 μg/ml (Fig. 7). Pre-osteoblastic cells proliferated normally in control wells (*i.e.* without FCC particles) and visual inspection with a light microscope over the 14-day culture period confirmed normal cell proliferation and morphology in wells without FCC (data not shown). In addition, light microscopy implicated that FCC particles were located within MC3T3-E1 cells, which led us to closer investigations using SEM and TEM imaging.

SEM and TEM images were taken of MC3T3-E1 cells cultured in the presence of FCC particles (12.5 μg/ml and 25 μg/ml) on day 10 of cell culture. Both methods showed that MC3T3-E1 cells had internalized FCC particles, since FCC particles were observed both intracellularly (Fig. 8B,D-F), as well as on outside the cell and on the plasma membrane (Fig. 8A,C-D).

4. Discussion

Biomaterials' chemical properties and topographical features are known to affect cell adhesion and differentiation *in vitro* [5,27]. Therefore, in this study, we characterized the chemical properties, such as molecular bonding, phase composition, and solubility of FCC by FTIR, XRD and with dissolution tests, respectively. The molecular bonding of FCC showed absorption bands of carbonate at 713 cm^{-1} (v_4), 874 cm^{-1} (v_2) and 1417 cm^{-1} (v_4) [28]. These results indicated that CC was of the stable calcite form, as was also found in our XRD analysis. In addition, the carbonate peaks of ATR-FTIR showed strong absorption of phosphates at 1023 cm^{-1} (v_3), which is known to originate from the hydroxyapatite part of FCC [29]. Although CC is a stable calcite form of

A.



B.

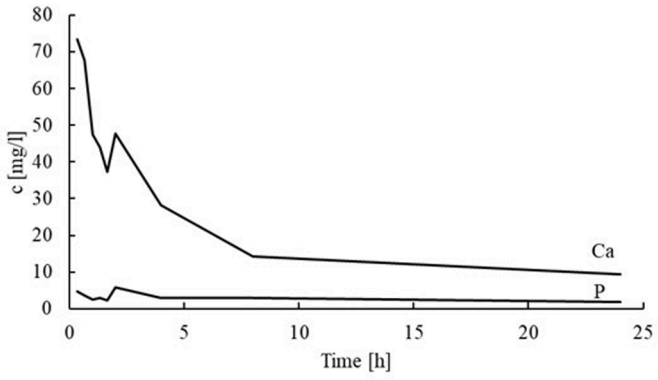


Fig. 3. FCC dissolution test in a continuous dynamic system. **(A)** pH changes caused by FCC dissolution in Tris-buffer immersion as a function of time (temperature was calculated to be at 37°C). **(B)** Dissolution of ions from FCC particles in Tris-buffer immersion as a function of time.

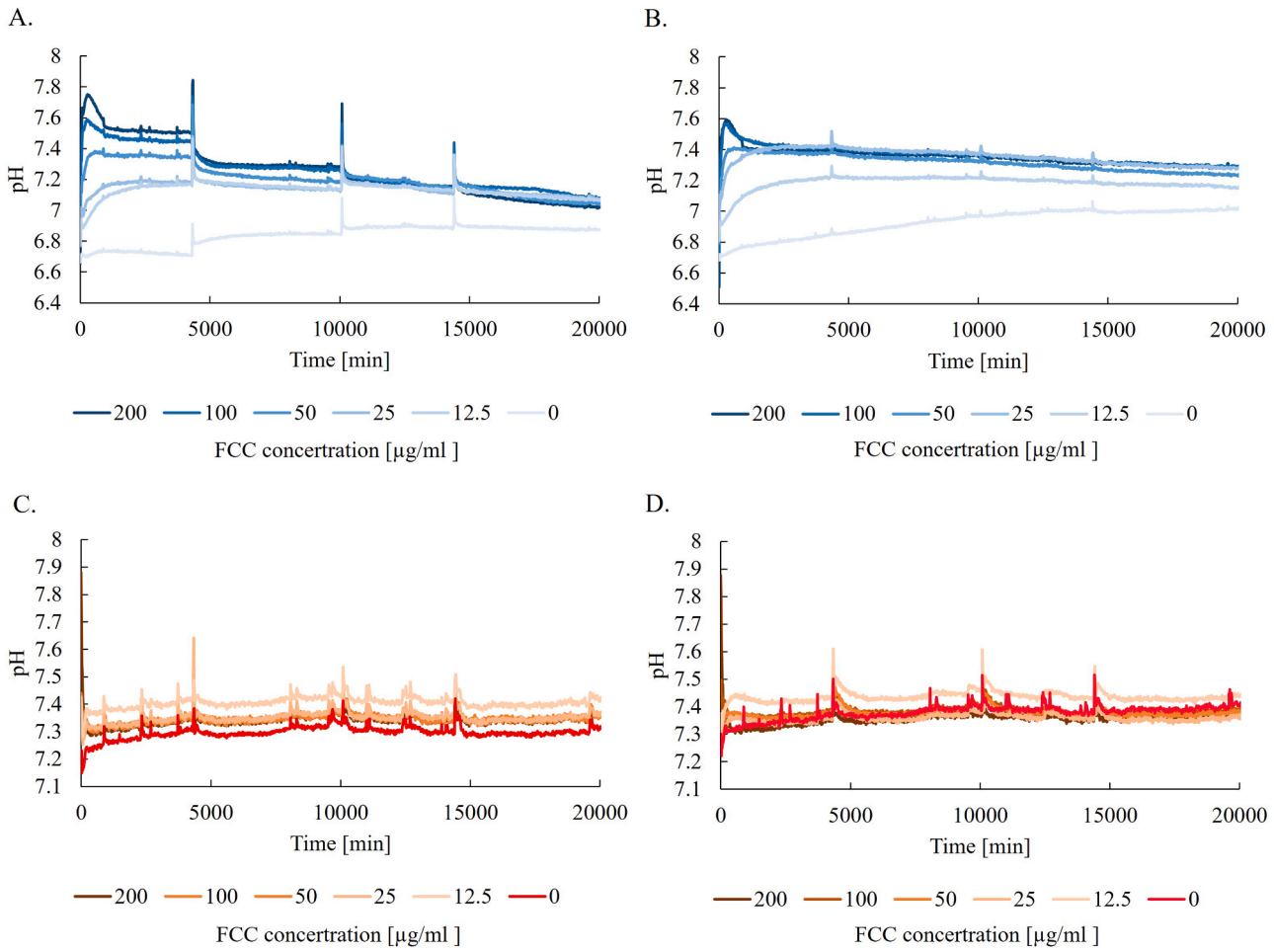


Fig. 4. pH changes caused by FCC in water (A, B) and cell culture medium (C, D) in a static system. **(A)** pH changes in water, when water was changed every 3–4 days and **(B)** when it was unchanged (same water throughout the period). **(C)** pH changes in cell culture medium, when medium was changed every 3–4 days and **(D)** when it was unchanged (same cell culture medium throughout the period) as a function of time (in a humidified atmosphere, 37 °C, 5% CO_2 (*g*)).

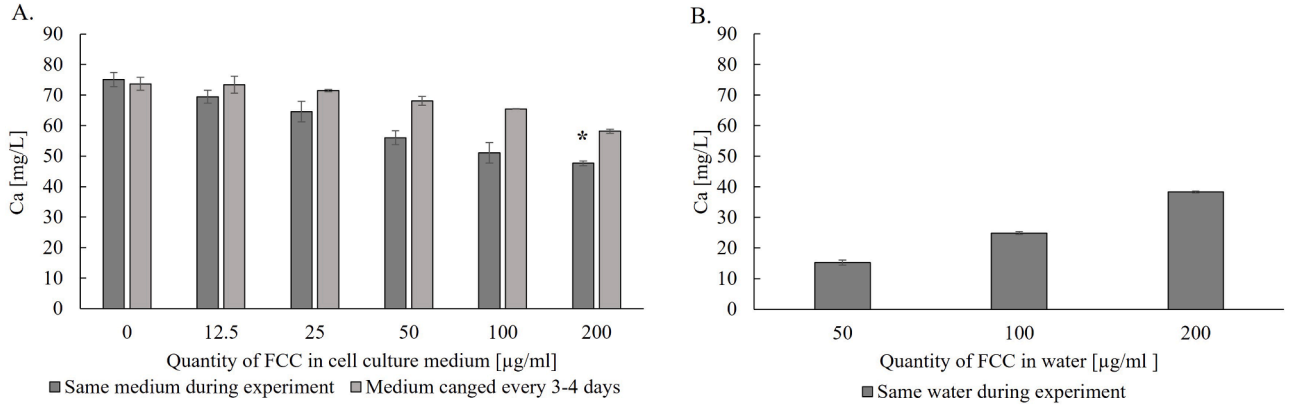


Fig. 5. Concentration of calcium in static dissolution system. **(A)** Concentration of calcium in cell culture medium and **(B)** in water in the static system after 14 days of FCC dissolution. Data are presented as an average ± SD ($n = 2$), *p < 0.05 *vs.* control group 0 µg/ml.

anhydrous CC's, its K_{sp} value is much higher than that of HAP [3], which indicated that high calcium level in our system originates from CC dissolution. To consider also that the atmosphere's carbon dioxide (g) impact calcium dissolution in the fluid and K_{sp} values.

The FCC particles in our study consisted of a CC core, which is first surrounded by a 100 nm porous meshwork of HAP and then by 1 µm thick lamellar structures of HAP [23]. The lamellar structure has been described to be responsible for FCC's mechanical interlocking and high mechanical stability [23]. In our study, the porous structure of FCC was visible in SEM images and the high porosity contributed to the ability of the material to adsorb calcium from the cell culture medium. Interestingly, nanometre-sized CC has previously been also shown to adsorb calcium from cell culture medium [30] and calcium phosphate ceramics with a high surface microporosity have been demonstrated to strongly adsorb proteins [5]. This property may limit the value of using *in vitro* assays in biological evaluation of such materials, since calcium is an

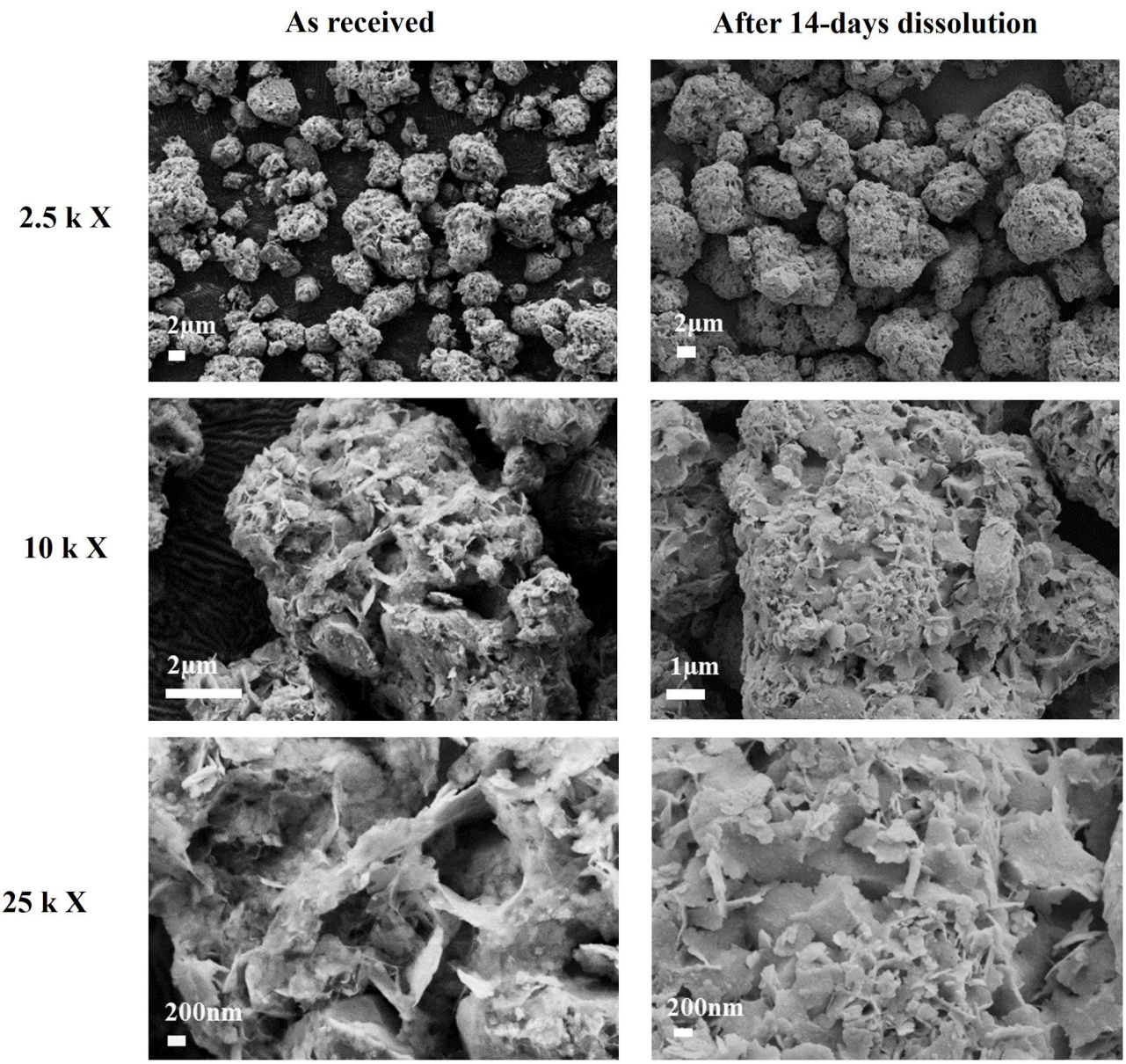


Fig. 6. FCC surfaces as examined by SEM. FCC surface is shown as received (left-hand panels) in original magnifications and scale bars are 2.5 k X/2 μm, 10 k X/2 μm and 25 k X/200 nm. FCC surface is shown after being immersed in cell culture medium up to 14 days (right-hand panels) in original magnifications and scale bars are 2.5 k X/2 μm, 10 k X/1 μm and 25 k X/200 nm.

essential element for cell viability and functionality.

We indeed observed that FCC particles adsorbed calcium from the cell culture medium, while calcium was in contrast released in the Tris buffer and water. In the buffered systems (Tris-buffer and cell culture medium) pH remained approximately the same during the follow-up time, while in the water, pH increased with increasing concentrations of FCC at the beginning of immersion. Surface charge, solubility and ion microenvironment are all known to influence protein adsorption [5] and could thereby also affect cell behavior *in vitro*. The ability of FCC to affect calcium concentrations led us to more closely study if the change of media could cause variation in the biological test system and thus influence the interpretation of data. However, the analysis demonstrated that medium changes did not affect calcium concentrations. For pH measurements, minor peaks were observed, when the plate was taken out from the incubator irrespective of if the media was changed or not, which indicates that it is rather the change in CO_2 atmosphere (known to affect the medium's buffering capacity) than the change of media, which caused this effect. According to our results, the solubility of FCC was however different depending on the type of dissolution fluids, indicating that the environment has a considerable influence on the physico-chemical and biological properties of biomaterial, which should be carefully considered when interpreting results from various *in vitro* studies.

We show here that FCC inhibited cell viability at all FCC concentrations, which were studied. This is in line with a previous study performed with human mesenchymal stromal cells and nanoscale CC particles [31]. In contrast, material's ability to adsorb calcium has been earlier proposed to have beneficial effects on cell proliferation and differentiation by activating different kinase pathways via ion channels [5]. Despite of negative effects on cell proliferation, Li et al. surprisingly reported that nanoscale CC at small doses stimulated osteogenic cell differentiation [31], which we however did not address in our study. In addition, there is also an early report showing negative effects of various sized HAP particles on osteoblast viability and growth factor secretion [32]. When a well-proven biocompatible HAP in certain *in vitro* conditions shows such negative effects on cell viability, the results of the

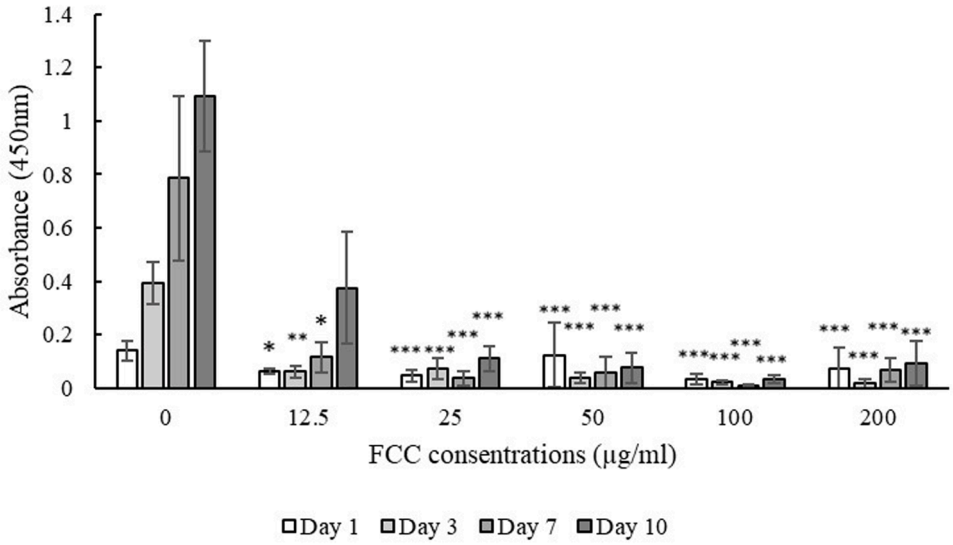


Fig. 7. Viability of pre-osteoblastic MC3T3-E1 cells in the presence of different concentrations of FCC. Data are presented as an average ± SD ($n = 3$), *p < 0.05, **p < 0.01, ***p < 0.001 *vs.* control group 0 µg/ml at respective time point.

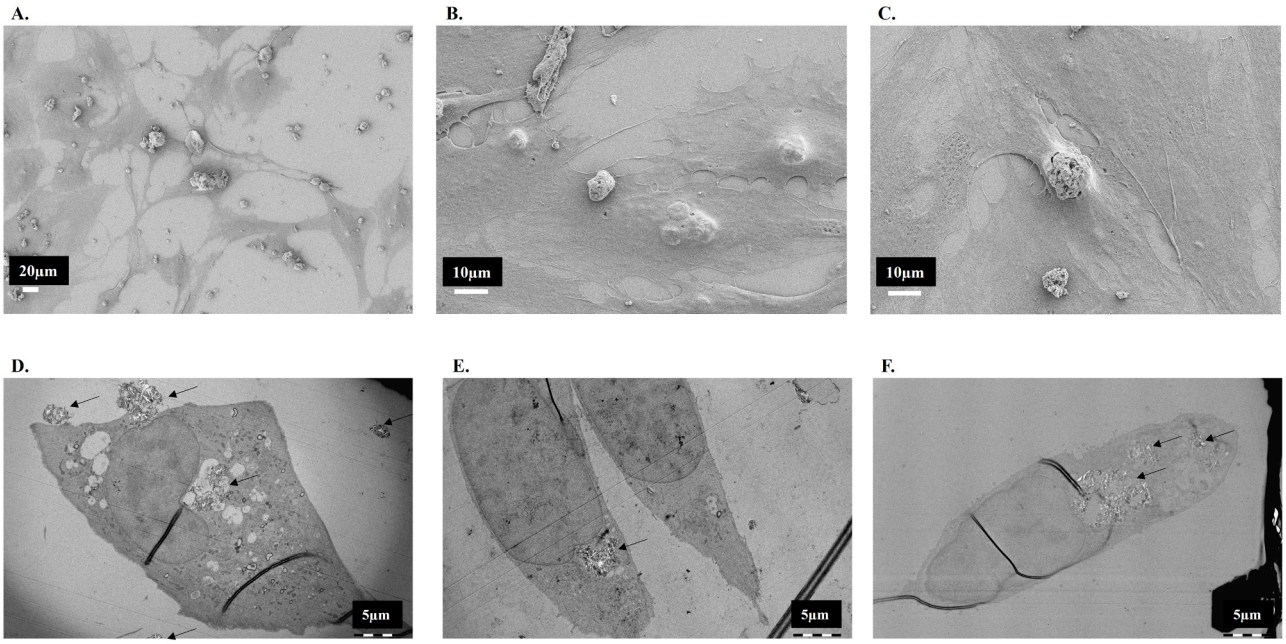


Fig. 8. (A.-C.) SEM and **(D.-F.)** TEM images from MC3T3-E1 cells cultured with FCC particles (12.5 µg/ml or 25 µg/ml) on day 10 of cell culture. In D-F, intracellular FCC particles are annotated with black arrows. Scale bars are 20 µm (A), 10 µm (B-C) and 5 µm (D-F).

present study must be as well interpreted with a caution. It should be noted that certain *in vivo* related factors, such as availability of calcium ions or growth factors in the extracellular fluid might contribute to a completely different cellular behavior. In addition, *in vitro* conditions are largely oversimplified compared to bone tissue *in vivo*, where the microenvironment is three-dimensional and composed of extracellular matrix, blood vessels, nerves, and various other cell types besides osteoblasts, known to act in a close contact with each other. Since no negative effects of FCC on bone cells *in vivo* have been reported so far, the effects observed *in vitro* might not always be fully replicated *in vivo*. For example, corallic CC and HAP compounds have indeed been used as implantable materials with good biological results both *in vivo* and *in vitro* [15–19]. Thus, based on the current knowledge, one cannot rule out the potential of FCC in bone applications in the future but more careful and in-depth studies are obviously needed.

Based on current knowledge, it is unclear if, and how, the mechanical properties and surface microporosity of the FCC particles could influence *in vitro* cell viability and differentiation. It is known that FCC is featured with an intraparticle porosity up to *ca.* 200 nm and that the fusion of HAP layer on CC core should keep the FCC particle cohesively as one particle. So far, nanomechanical properties of FCC particles have however not been investigated, which warrants further studies both from biological and biomechanical perspectives.

Interestingly, we observed that the osteoblastic cells internalized micrometer-scale particles of FCC, probably by phagocytosis (*i.e.* the process where cell is ingesting or engulfing large particles). Even though osteoblast are not traditionally considered as phagocytosing cells, similar results have been previously obtained *in vitro* with MC3T3-E1 cells and microscale HAP particles (<10 µm) [33], as well as with human osteoblast-like cells and MG-63 cells line [34,35]. Such a

behavior is something to be considered when surface-active microscale biomaterials are being tested in cell culture systems *in vitro* but whether such a phagocytic activity occurs or is relevant *in vivo* regarding cell viability or osteogenesis, still remains unknown.

5. Conclusion

The physicochemical characterization of functionalized calcium carbonate (FCC) showed that FCC adsorbed calcium ions from the cell culture medium. Such properties of FCC particle material were associated with a reduced osteoblastic cell viability *in vitro*, which was probably caused by adsorption of calcium and proteins from cell culture media, both of which are essential for cellular growth. Furthermore, FCC particles were shown to be internalized by osteoblast, which are not normally considered as phagocytosing cells. These data indicated that surface-active FCC can cause various cellular effects *in vitro*, which need to be considered, when the results obtained with this type of materials are being interpreted.

Declaration of Competing Interest

Authors except author TB and WH declare no conflicts of interests of the materials and devices used in the study. Main results of the study have led to a patent application by Omya.

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