

Biocompatibility and bioactivity of porous polymer-derived Ca-Mg silicate ceramics

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Abstract

Magnesium is a trace element in the human body, known to have important effects on cell differentiation and the mineralisation of calcified tissues. This study aimed to synthesise highly porous Ca-Mg silicate foamed scaffolds from preceramic polymers, with analysis of their biological response. Akermanite (Ak) and wollastonite-diopside (WD) ceramic foams were obtained from the pyrolysis of a liquid silicone mixed with reactive fillers. The porous structure was obtained by controlled water release from selected fillers (magnesium hydroxide and borax) at 350 °C. The homogeneous distribution of open pores, with interconnects of modal diameters of 160-180 µm was obtained and maintained after firing at 1100 °C. Foams, with porosity exceeding 80%, exhibited compressive strength values of 1-2 MPa. *In vitro* studies were conducted by immersion in SBF for 21 days, showing suitable dissolution rates, pH and ionic concentrations. Cytotoxicity analysis performed in accordance with ISO10993-5 and ISO10993-12 standards confirmed excellent biocompatibility of both Ak and WD foams. In addition, MC3T3-E1 cells cultured on the Mg-containing scaffolds demonstrated enhanced osteogenic differentiation and the expression of osteogenic markers including Collagen Type I, Osteopontin and Osteocalcin, in comparison to Mg-free counterparts. The results suggest that the addition of magnesium can further enhance the bioactivity and the potential for bone regeneration applications of Ca-silicate materials.

Keywords

Ca-Mg silicates; Bioactive; Preceramic Polymers; Porous; ceramic foams

1 Introduction

In tissue engineering and regenerative medicine, synthetic or natural porous scaffolds can be implanted into a defect to guide and stimulate the formation of bone *in situ* [1]. The scaffolds are used as templates for tissue growth and should be resorbed by dissolving at controlled rates as the new tissue forms [2,3]. The scaffolds should ideally not only provide a passive structural support for cells with well-defined mechanical and physical properties (in terms of compressive strength, porosity, pores interconnectivity), but they should also stimulate osteoblastic cell proliferation and differentiation [4].

Significant attention has been given to Ca-silicates, due to their excellent bioactivity and biodegradability [5]. In fact, previous studies have shown that, at a certain concentration range, Ca and Si ions stimulate cell proliferation [6,7]. In detail, soluble silica has a well-established role in the bioactivity mechanism proposed by Hench [8], in osteoblast proliferation [9,10,11] and bone mineralisation [12,13], while Ca is known to act in regulating osteogenesis [14,15], promoting osteoblast proliferation and differentiation [16,17].

Wollastonite (CaSiO_3) has been widely investigated, due to its ability to bond with living bone through the formation of an apatite interface layer [18,19]. Wu *et al.* even incorporated wollastonite powders in poly(lactide-co-glycolide) microspheres, used for drug delivery in regenerative medicine, to successfully modulate their bioactivity for bone repair application [20]. However, porous wollastonite has low mechanical strength and high degradation rates, which may not support new tissue growth or lead to high pH values, having detrimental effects on cells [21]. Wu *et al.* reported that a wollastonite scaffold of 82 % porosity, produced by polymer sponge method, exhibited a compressive strength of only 300 kPa [21].

Therefore, ion modifications of Ca-silicates have been proposed with the aim of tailoring both mechanical and biomedical properties. The CaO-MgO-SiO₂ system has received

growing interest in the field of bioactive materials. Zhai *et al.* have recently reported that this family of bioceramics possess a unique dual osteogenic/angiogenic stimulatory ability [22]. Mg was found to play an important role in osteoblast adhesion [23], differentiation and proliferation [24] and on the mineralisation of bone [25,26]. Further effects of the incorporation of Mg, specifically in Ca-silicates included reduced degradation rate and improved mechanical properties. These positive consequences might be due to the higher complexity of the crystal structure after Mg incorporation: in particular, Mg-O bonds are stronger than Ca-O bonds [27,28]. Ca-Mg silicates like akermanite ($\text{Ca}_2\text{MgSi}_2\text{O}_7$) and diopside ($\text{CaMgSi}_2\text{O}_6$) are known to possess good bioactivity, slower degradation rate than wollastonite (CaSiO_3) and great potential as bone tissue engineering materials [29,30].

Herein, two Ca-Mg-rich ceramics were made: one had akermanite as the only crystal phase, the other was diopside coupled with wollastonite. The novelty here is in the synthesis process, involving preceramic polymers, and in the shaping method, exploiting hydrated fillers as porogens. We have previously developed wollastonite coupled with an external layer of carbonate hydroxyapatite (HCA) [31], which has improved mechanical behaviour and promising osteogenic potential. The methodology applied the pre-ceramic polymer route, that uses the thermal decomposition of the precursors and the reactions between the ceramics left behind [32,33]. The technique uses low-cost, commonly available polymeric precursors (silicone resin), filled with powdered metal oxide precursors. The advantages of polymer-derived ceramics are: they can be easily shaped into highly porous bodies when the mixture is still in the polymeric form ($<350\text{ }^\circ\text{C}$) and subsequently ceramised; the firing is generally completed at low temperatures ($<1200\text{ }^\circ\text{C}$); the phase purity of the ceramised products is usually high.

The present study explored the cellular response of MC3T3 pre-osteoblast cells to the two Ca-Mg-rich ceramics. Cytotoxicity was tested in accordance to the ISO10993-5 and

ISO10993-12 standard guidelines. Cell attachment, viability and osteogenic differentiation were also assessed to investigate the role of Mg.

2 Experimental

2.1 Starting materials

A commercially available silicone resin (H62C, Wacker Chemie GmbH, Munich, Germany), which is liquid at room temperature, was chosen as a silica precursor, which provides a silica yield of 58 wt% when thermally treated [34]. CaO and MgO precursors consisted of CaCO₃ (Industrie Bitossi, Vinci, Italy) and Mg(OH)₂ (Industrie Bitossi, Vinci, Italy) in the form of microparticles (< 10 µm). Borax (sodium borate decahydrate microparticles, Na₂B₄O₇ · 10H₂O, Normapur Prolabo, France) was used as an additional foaming agent. All the other chemicals and reagents were purchased from Invitrogen/ Life Technologies Ltd. (Paisley, UK) and Sigma-Aldrich (Gillingham, UK).

The molar balance among the most important constituents (silicone:CaO precursor:MgO precursor) followed the stoichiometric SiO₂:CaO:MgO molar proportions of akermanite (i.e. CaO:MgO:SiO₂=2:1:2), or of a 50 mol% wollastonite:50 mol% diopside mixture (i.e. CaO:MgO:SiO₂=2:1:3). Samples will be referred to as “Ak” and “WD” respectively.

2.2 Foam preparation

H62C was first dissolved in isopropanol (10 g in 13 ml) and then mixed with micro-sized fillers, including sodium borate in the as-received hydrated form (the quantity of salt was 3 wt% of the theoretical ceramic yield of the other components). Stable and homogeneous dispersions in isopropanol were obtained by magnetic stirring and ultrasonication, then were left to dry overnight at 60 °C. After first drying, the mixtures were in the form of thick pastes, later manually transferred into cylindrical Al moulds and then subjected to a heat treatment for foaming at 350 °C in air for 30 min. Cylindrical samples, 10 mm in diameter and 10 mm

in height, were obtained from the foams. After polishing of the top surfaces, by means of abrasive paper, the samples were thermally treated at 1100 °C in air for 1 h at a heating rate of 2 °C min⁻¹.

2.3 Characterisation

The bulk density (ρ_b) of the foam, the quotient of the dry mass of a specimen divided by the exterior volume, including pores, was determined using a calliper and a digital balance. The skeletal density (ρ_s), the ratio of mass of discrete pieces of solid material to the sum of the volumes of the solid material in the pieces and closed or blind pores within the piece, was measured on foams, using a He gas pycnometer (Micromeritics AccuPyc 1330, Norcross, GA). Percentage porosity (%P) was then calculated using Eq.1, where ρ_b was the bulk density and ρ_s the skeletal density:

$$\%P = 1 - (\rho_b / \rho_s). \quad (\text{Eq.1})$$

Mercury intrusion porosimetry (PoreMaster 33, Quantachrome UK) was used to determine the pore interconnect size distribution. As the technique was destructive, it cannot be used to follow the change in pore sizes of the same scaffold as a function of sintering temperature. Optical stereomicroscopy (AxioCam ERc 5s Microscope Camera, Carl Zeiss Microscopy, Thornwood, New York, US) on foams and scanning electron microscopy (JSM 6010 L, JEOL USA; LEO GEMINI 1525 FESEM, LEO electron microscopy, Thornwood, New York, USA) on chromium-coated specimens were used to examine the morphological and textural features of the samples.

The phase identification was performed, on powdered samples obtained by manual grinding with mortar and pestle to below 100 μm , by X-ray diffraction (XRD; Bruker AXS D8 Advance, Bruker, Germany; CuK α radiation, 0.15418 nm, 40 kV–40 mA, $2\theta = 10^\circ$ – 70° , step size = 0.05°, 2 s counting time), supported by data from PDF-2 database (ICDD-

International Centre for Diffraction Data, Newtown Square, PA) and Match! programme package (Crystal Impact GbR, Bonn, Germany).

The compressive strength of foams was measured at room temperature, using an Instron 1121 UTM (Instron Danvers, MA), equipped with a 10 kN load cell, with a crosshead speed of 1 mm min⁻¹. The mean value of 5 to 10 samples was used for each data point.

2.4 Simulated body fluid testing

Foams with mass of 37.5 mg were immersed in 25 ml of simulated body fluid (SBF) at 37 °C and 120 rpm for 1, 2, 4, 8, 24, 72, 168, 336, 504 h [35]. At each time point, the pH was measured and samples of 1 ml of the media were taken and refreshed. After dilution with 9 ml of 2M HNO₃, the reacted medium was analysed by inductive coupled plasma (ICP) spectroscopy (Thermo Scientific ICP Spectrometer, Model iCAP 6300 Series Duo, Thermo Fisher Scientific, Waltham, MA, USA) for Si, Ca, Mg, P concentration in solution. All the experiments were done in triplicate. At the end of the test, the foams were washed in distilled water, dried and analysed by attenuated reflectance Fourier transform infrared spectroscopy (ATR-FTIR, Thermo Scientific Nicolet iS10) at room temperature, to monitor the growth of the hydroxycarbonate apatite layer, using a wavenumber resolution of 4 cm⁻¹ for 32 scans from 4000 to 600 cm⁻¹.

2.5 ISO 10993 tests for in vitro cytotoxicity

Potential cytotoxicity effects of Ak and WD samples were assessed in accordance to ISO 10993-5 and ISO 10993-12. Dissolution products released by the samples (0.2 g ml⁻¹ in α-MEM at 37 °C) over a 72 h period were prepared. Medical grade polyethylene (PE) was used as non-cytotoxic negative control and polyurethane (PU) containing 0.1% (w/w) zinc diethyldithiocarbamate (ZDEC) was used as toxic positive control. The dissolution products

were filter sterilised and dilution series (25%, 50%, 75% and 100%) were prepared and supplemented with 10% (v/v) foetal calf serum (FCS) prior to use.

Cell viability was assessed by a calorimetric cell metabolic activity assay based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into formazan. MC3T3-E1 cells were seeded on 96-well plates at 1×10^4 cells per well and left to grow in basal α -MEM for 24 h prior to incubation in (1) fresh basal α -MEM, (2) the ceramic dissolution products or (3) controls for further 24 h. Used culture media was removed and replaced with serum free MTT solution (1 mg ml^{-1} in α -MEM). Following an incubation period of 2 h, the MTT solution was removed and each well was filled with 100 μl isopropanol and shaken briefly to dissolve the formazan derivatives. The optical density was measured spectrophotometrically at 570 nm using a microplate reader (SpectraMax M5).

2.6 Cell seeding and culture on 3-D scaffolds

For cell attachment, proliferation and osteogenic differentiation studies, foams were cut to 37.5 mg of weight (the size was approximately $5 \times 5 \times 5 \text{ mm}^3$) and sterilised with 70% ethanol prior to washing in PBS. Upon confluence, MC3T3 cells were harvested and suspended in basal media at a concentration of 1×10^6 cells in 25 ml. 25 ml of cell suspension was added to each sterile 50 ml Falcon tube containing one foam 3-D scaffold. The tubes were placed in an incubator (humidified atmosphere at 37 °C, 5% CO₂ and 21% O₂) for 2 h with gentle agitation every 30 minutes to allow diffused cell adhesion. The solution was then replaced with osteogenic media (basal α -MEM supplemented with 100 μM ascorbate-2-phosphate, 10 nM dexamethasone and 10 mM of β -glycerophosphate). Cell-seeded foams were cultured in humidified atmosphere at 37 °C, 5% CO₂ and 21% O₂ for up to 21 days with media changes every 3-4 days. 1 ml of culture medium was collected prior to each media change for ICP analysis of Si, Ca, Mg and P concentrations. For gene expression analysis,

cells were also seeded on Mg-free wollastonite-hydroxycarbonate apatite samples (CaO:SiO₂=1.6), developed and discussed in a previous study [31], for comparison.

2.7 Analysis of cell viability on 3-D scaffolds

Viability of MC3T3 cells cultured on the 3-D foams at day 1, 3, 7 and 14 was determined by colorimetric WST-1 proliferation assay, in which WST-1 tetrazolium salts were cleaved to water-soluble formazan by cellular enzyme dehydrogenases. An increase in the number of metabolically active cells results in an increase in mitochondrial dehydrogenase activity, which in turn leads to an increase in the amount of formazan dye. At each time point, three cell-seeded foams were collected and each submerged in 5 ml of WST-1 reagent (1:10 dilution in α -MEM, Roche Diagnostic, West Sussex, UK). 100 μ l was aspirated in triplicate from each sample following 2 h incubation and, the optical density was measured spectrophotometrically at 440 nm using a SpectraMax M5 microplate reader.

2.8 Immunohistochemistry staining

Cell-seeded foams were fixed with 4% (w/v) paraformaldehyde (PFA) and used for immunohistochemical analysis of cell attachment and osteogenic differentiation. After permeabilisation with buffered 0.5% (v/v) Triton X-100 in PBS (300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, 20 mM HEPES and pH 7.2) and blocking with 10 mg ml⁻¹ BSA in PBS, samples were incubated with relevant primary antisera (diluted appropriately using 10 mg ml⁻¹ BSA in PBS) at 4 °C overnight. This was followed by 1 h incubation with Alexa Fluor[®] 488-conjugated secondary antibody.

The anti-Vimentin antibody (rabbit polyclonal, IgG, Abcam, Cambridge, UK), anti-Collagen Type I antibody (rabbit polyclonal, IgG, Abcam, Cambridge, UK), anti-Osteopontin antibody (rabbit polyclonal, IgG, Merck Millipore, Watford, UK) and anti-Osteocalcin antibody (rabbit polyclonal, IgG, Merck Millipore, Watford, UK) were used at dilutions of

1 1:500, 1:1000, 1:500 and 1:500 respectively. Alexa Fluor® 488-conjugated secondary
2 antibody (goat anti-rabbit, IgG, Abcam, Cambridge, UK, supplied at a concentration of 2
3 mg/ml) was used at a dilution of 1:1000. For analysis of cell attachment, F-actin was labelled
4 using CytoPainter F-actin staining kit (Abcam, Cambridge, UK) following the manufacture's
5 instruction. Briefly, Alexa Fluor® 568-conjugated phalloidin (1:1000 dilution in labelling
6 buffer) was added simultaneously with the secondary antibody (Alexa Fluor 488-conjugated)
7 during the incubation period. All samples were counter-stained with DAPI (0.1 µg ml⁻¹ in
8 PBS).

9 10 2.9 Optical, confocal and scanning electron microscopy

11 The samples were imaged under confocal microscopy (Leica SP5 MP laser scanning
12 confocal microscope and software, Leica Microsystems, Wetzlar, Germany). The same
13 samples were then dehydrated using an ascending series of ethanol and hexamethyldisilazane
14 (HDMS) prior to sputter coating with gold and observation under scanning electron
15 microscopy (JSM 6010 L, JEOL USA).

16 17 2.10 Analysis of gene expression

18 At day 21, two cell-seeded Ak or WD constructs were pooled and lysed for extraction of
19 total RNA using Qiagen RNeasy kit (Qiagen, Manchester, UK) following manufacturer's
20 instructions. The RNA samples were treated with DNase-1 reagent and reverse-transcribed
21 using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen, UK). SYBR green based
22 qPCR assays were carried out using the QuantStudio™ 6 Flex system (Thermo Fisher, UK)
23 for the analysis of osteogenic gene expression, including *Runx2* (F: 5'-
24 tcgcctcagtgatttagggcgca-3'; R: 5'-gtggcagtgatcatcatctgaaatac-3'), *Col1a1* (F: 5'-
25 gagaagtctcaagatggtggc-3'; R: 5'-gcgggggtcggagccctcgctt-3'), *Osteopontin* (F: 5'-
26 gatgaatctgacgaatctcacc-3'; R: 5'-ctcagaagctgggcaacagggat-3') and *Osteocalcin* (F: 5'-

ccctgagtctgacaaagccttca-3'; R: 5'-tactgggtctgatagctcgctc-3') [36] . The expression of genes of interest was normalised to the housekeeping gene/endogenous control, GAPDH (F: 5'-gggtggagccaaacgggtc-3'; R: 5'-ggagttgctgttgaagtcgca-3'). The relative transcript levels of genes of interest were analysed using the comparative C_T method ($\Delta\Delta C_T$ method). Bar graphs were plotted and statistical analysis was performed at the level of ΔC_T .

2.11 Statistical analysis

Mann-Whitney U tests (with Bonferroni correction if more than 3 groups) were performed. Results were deemed significant if the probability of occurrence by random chance was less than 5% (i.e. $p < 0.05$).

3 Results and Discussion

3.1 Scaffold characterisation

Fig. 1a,b show that the ceramised samples of both formulations exhibited extensive porosity. Pores were derived from the water released from the thermal decomposition of $Mg(OH)_2$ and borax. The simultaneous cross-linking of the polymer, which took place within the same temperature range as the dehydration of the fillers ($T < 350^\circ C$), allowed the porous shape to be maintained, without any viscous collapse. The foamed structure was maintained even after the subsequent ceramisation at $1100^\circ C$, implying the conversion of the silicone into silica and reaction with CaO and MgO from the fillers.

FIGURE 1 HERE

The pore shape and size were more uniform in WD samples (Fig. 1b,d), exhibiting pores with spherical morphology, in comparison to Ak foams (Fig. 1a,c). One likely reason is represented by the composition of silicone-based mixtures: the higher silica content of WD

1 samples resulted in a higher silicone:filler ratio and a consequently lower viscosity of pastes
2 upon the foaming step. Pore diameters were between 700 µm and 1 mm (Fig. 1c,d).

3 The results from Hg intrusion porosimetry allowed a comparison between foams of
4 different composition (Fig. 1e) in terms of the diameter of interconnections of adjacent pores.
5 An ideal scaffold should combine bioactive properties with a favourable structure, consisting
6 of pores with well-defined interconnections; in particular, interconnections with diameter
7 exceeding 100 µm allow effective tissue ingrowth and eventually vascularisation, which is
8 required for complete regeneration of bone [37]. The modal diameter of interconnects of
9 pores was 181 µm for Ak and 160 µm for WD foams, both above 100 µm.

10 **FIGURE 2 HERE**

11 Fig.2a confirmed that Ca-Mg silicates formed ~~by exploiting from the chemical~~ reactions
12 between the ceramic residue (~~derived from the~~ oxidative decomposition of H62C) and the
13 fillers. The XRD pattern for the unreacted Ak formulation features the distinctive peaks of
14 the desired phase ($\text{Ca}_2\text{MgSi}_2\text{O}_7$ - PDF#87-0046) with only minor peaks attributable to traces
15 of monticellite (CaMgSiO_4 - PDF#84-1319). The XRD pattern for the unreacted WD
16 formulation confirms the desired phases (CaSiO_3 - PDF#75-1396, and $\text{CaMgSi}_2\text{O}_6$ - PDF#72-
17 1497), with minor traces of akermanite. The presence of impurities was not considered a
18 problem, considering the reported bioactivity of the monticellite crystal phase. [38] ~~The~~
19 ~~unindexed peaks could contain Na and/or B, or these could be present in a glass phase around~~
20 ~~silicate crystals, although the amount of glass would be small (too small to analyse by EDX).~~
21 ~~Previous work [39], which applied quantitative XRD analysis to the polymer derived~~
22 ~~wollastonite-diopside system, showed crystallinity exceeding 83%, with the residual glass~~
23 ~~incorporating Na_2O and B_2O_3 , but also featuring some SiO_2 , CaO and MgO .~~

24 Fig. 2b shows FTIR spectra of Ak and WD samples. The FTIR spectrum of the unreacted
25 Ak samples exhibited vibrational bands in the same positions as reported in literature for

akermanite (1009, 972, 928, 906, 852, 683, 638, 586, 475 cm^{-1}) [40]. The FTIR spectrum of as-made WD samples showed a vibrational band at 460 cm^{-1} , attributed to Si-O bending [41]; vibrational bands at 960, 896, 564, 509, 470 cm^{-1} , which are known to be due to wollastonite [44]; and bands at 1066, 960, 858, 673, 635 cm^{-1} due to diopside [42]. These phases were confirmed by the XRD pattern (Fig. 2a), showing mainly wollastonite.

Table 1: Physical and mechanical properties of Ak and WD samples.

	ρ_b [g cm^{-3}]	ρ_s [g cm^{-3}]	P [%]	σ_{comp} [MPa]
Ak	0.51 ± 0.06	3.08 ± 0.01	82	1.0 ± 0.2
WD	0.53 ± 0.04	3.28 ± 0.02	83	1.8 ± 0.3

Mechanical and physical properties of the scaffolds are summarized in Tab. 1. Both Ak and WD scaffolds exhibited similar values of ρ_b and ρ_s . The %P for Ak and WD samples were 82 % and 83 % respectively. The mean compressive strength (σ_{comp}) for Ak samples was 1.0 ± 0.2 MPa, which is in the same range ~~which~~ as ~~that~~ reported in literature for akermanite foams obtained by polymer foam replication [21], ~~which had exhibiting~~ 82 % of porosity and 790 ± 210 kPa. WD samples, instead, reached a compressive strength of 1.8 ± 0.3 MPa, superior to that analogous foams reported in literature. For instance, Wu *et al.* reported diopside foams [28] with compressive strength of 500 ± 70 kPa and wollastonite foams [21] with a compressive strength of 300 ± 11 kPa, both obtained by polymer replication with porosities of 82%.

3.2 Surface/solution reactions, ion release and pH

Fig. 3 shows the dissolution profiles for the foam scaffolds, as a function of time, generated using both compositions. The dissolution profiles are the elemental concentrations in SBF as a function of immersion time. Si, Ca and Mg were released by the scaffolds,

although SBF already contained some Ca and Mg. P was not present in the materials but is in SBF ~~and it was recruited for the formation of hydroxycarbonate apatite (HCA) on the samples, as shown by the decrease in P content of the SBF over time.~~ A control of pure SBF with no foams immersed was also included in the ICP analysis. The error bars are standard deviations calculated from triplicates.

FIGURE 3 HERE

SBF did not contain Si (Fig. 3a) and, following immersion of Ak samples, the Si concentration increased to $62 \mu\text{g ml}^{-1}$ over the first 24 h. From 24 h to 72 h, the concentration of Si species increased to $92 \mu\text{g ml}^{-1}$, but with a slower rate of Si release. The amount of Si found in SBF was maintained at approximately $90 \mu\text{g ml}^{-1}$ from day 3 ~~up~~ to day 7, followed by stabilisation at $70 \mu\text{g ml}^{-1}$. Following the dissolution profile of WD samples, the Si concentration increased to $92 \mu\text{g ml}^{-1}$ over the first 72 h at an almost linear rate of release and then followed the same trend as Ak samples.

The Ca content of the control SBF was constant at approximately $90 \mu\text{g ml}^{-1}$ (Fig. 3b). Due to the release of Ca^{2+} ions from the Ak foams, the concentration of Ca in SBF reached $255 \mu\text{g ml}^{-1}$ after 7 days, which remained constant up to day 21. The concentration of Ca released from WD samples in SBF reached approximately $190 \mu\text{g ml}^{-1}$ after 3 days, followed by a slow increase to $200 \mu\text{g ml}^{-1}$ from day 3 ~~up~~ to 14. The Ak composition was slightly richer in Ca compared to WD and this could explain the higher amount of Ca ions released by Ak samples. The Ca concentration for SBF containing WD, however, slightly decreased as the immersion time increased from 14 days ~~up~~ to 21 days, reaching $180 \mu\text{g ml}^{-1}$. This was likely caused by calcium phosphate deposition on the scaffold surface, after 14 days of immersion.

The Ca release profiles of these two Ca-Mg silicates were also compared with those of the Mg-free wollastonite-hydroxycarbonate apatite bioceramics (WA) that we developed

previously [31]. WD samples exhibited a more controlled degradation, in terms of maximum level of Ca^{2+} released, especially when comparing them with the previous WA scaffolds derived from a higher CaO/SiO_2 ratio. In fact, for WA scaffolds the amount of Ca species reached $250 \mu\text{g ml}^{-1}$ after 1 week, which is comparable with the amount for Ak, while WD released only $190 \mu\text{g ml}^{-1}$, as mentioned above. After 2 weeks, WA released $320 \mu\text{g ml}^{-1}$ in the case of the scaffolds derived from a higher CaO/SiO_2 and the amount remained constant up to 3 weeks. This is considerably higher if compared with the Ca^{2+} release of Ak (maintained at approximately $250\text{-}260 \mu\text{g ml}^{-1}$) and of WD (approximately $190 \mu\text{g ml}^{-1}$).

Considering the release of Mg, Fig. 3c illustrates the release of Mg in SBF from Ak and WD samples. The control SBF showed levels of Mg between 32 and $38 \mu\text{g ml}^{-1}$ up to 21 days. After immersion of Ak samples in SBF for 24 h, the Mg species increased to $86 \mu\text{g ml}^{-1}$. From 24 h up to 14 days, the concentration of Mg ions kept increasing to $103 \mu\text{g ml}^{-1}$ at a slower rate of release and maintained at approximately $100 \mu\text{g ml}^{-1}$ up to 21 days. The release of Mg was much lower for WD samples. Mg concentration in SBF reached $50 \mu\text{g ml}^{-1}$ after the first 24 h, then decreased and maintained at approximately $45 \mu\text{g ml}^{-1}$ up to 14 days. There was a decrease to $40 \mu\text{g ml}^{-1}$ from 14 up to 21 days. Similar to Ca release, the slow release of Mg in WD could be due to the smaller amount of Mg included in the formulation of WD compared to Ak formulation.

Both compositions did not contain P, so that there was no release of P species from the foams (Fig. 3d). The control SBF, however, showed levels of P between 32 and $28 \mu\text{g ml}^{-1}$ up to 21 days immersion. Following the immersion of both Ak and WD samples, there was a noticeable decrease in concentration of P species in SBF. The P concentration rapidly decreased to $7 \mu\text{g ml}^{-1}$ after 72 h for Ak and to $0.5 \mu\text{g ml}^{-1}$ by day 14. The P concentration in SBF containing WD samples decreased to $12 \mu\text{g ml}^{-1}$ after 72 h and to $0.5 \mu\text{g ml}^{-1}$ by day 7. The formation of a HCA layer on the surface of both scaffolds by recruitment of P from SBF,

as evidenced by FTIR spectra (Fig. 2b), was likely the reason for decreased P concentration in SBF over time.

The dissolution of the foams caused the pH of the medium to increase (Fig. 3e). Values were comprised between 7.4 and 8.2 for Ak samples and from 7.4 to 7.9 for WD samples.

After immersion in SBF for 3 weeks, the XRD patterns (Fig. 2a) suggested the conversion of most of the akermanite into monticellite, which has the same Ca:Si molar ratio as akermanite, but lower Ca:Mg and Si:Mg molar ratio. This was confirmed by the FTIR spectra (Fig. 2b), showing only a few of the akermanite vibrational bands and bands at 942, 829 and 519 cm^{-1} , which can be attributed to monticellite [43]. A band at 896 cm^{-1} was likely due to wollastonite [44], as its presence was also confirmed by the XRD phase identification.

After immersion of WD samples in SBF for 3 weeks, the XRD pattern (Fig. 2a) of WD showed that the wollastonite was fully converted into diopside. Further evidence was given by the FTIR spectrum (Fig. 2b) where the vibrational bands of wollastonite were not present, only the vibrational bands related to diopside remained. Concerning the growth of hydroxycarbonate apatite over the 21day immersion in SBF, the FTIR spectra of both Ak and WD samples showed vibrational bands at 567 and 603 cm^{-1} , corresponding to the P-O ν_4 bending vibrations, and at 1050 cm^{-1} , corresponding to the P-O ν_3 stretching vibrations. Vibrational bands were observed as well for carbonate ions at approximately 1400-1500 cm^{-1} , corresponding to the carbonate ν_3 stretching vibrational mode, and, only for WD samples, at 870 cm^{-1} , corresponding to ν_2 . All of them can be associated with the presence of crystalline HCA [45].

3.3 Assessment of biocompatibility

MTT metabolic activity assay was performed in accordance to ISO 10993 to assess the potential cytotoxic effects of both Ak and WD materials. The ISO guidance states that if the viability of cells is reduced to < 70% of blank (i.e. non-cytotoxic controls), the test material

will be deemed to have cytotoxic potential. In addition, in a valid test, the 50% dilution of the test sample should result in at least the same or a higher viability in comparison to the 100% extract. As shown in Fig. 4a, the viability of MC3T3 cells cultured in neat dissolution products of both Ak and WD materials was comparable that of standard basal culture media and PE negative control, an indication of excellent biocompatibility.

Following cell seeding onto the foam scaffolds of Ak and WD, the WST-1 cell proliferation assay was used as a quantitative measure to monitor viability of cells on the scaffold for up to 14 days. The optical density of WST-1 solution, incubated with cell-seeded constructs for 2 h at each time point, indicated excellent gradual cell growth and on-going cell survival (Fig. 4b). There was no significant difference in optical density (i.e. cell viability) between the two materials at each time point.

FIGURE 4 HERE

The cell attachment of the foams of Ak and WD materials was examined by immunohistochemical staining for up to 21 days. The expression of two of the major cytoskeletal constituents Vimentin (green stain), intermediate filament proteins, and F-actin (red stain), microfilaments, was evident in MC3T3 cells on both Ak and WD foam scaffolds (Fig. 4c). More cell spreading was observed on WD foams in comparison to Ak foams.

3.4 Dissolution study in cell culture medium

Throughout the 21-day cell culture period, the cell culture media, after removal of cells by filtration, were analysed by ICP to determine the concentration of Si, Ca, Mg and P in the solution. Fig. 5 shows a graph of the elemental content found in the culture media as a function of time.

FIGURE 5 HERE

The samples did not contain P, but the culture medium was rich in P, due to the addition of β -glycerophosphate. Prior to the immersion of the foams ($t=0$), the level of P was 215 μg

ml⁻¹ and P content maintained between 195 and 230 µg ml⁻¹. For Ak samples (Fig. 5a), Ca concentration increased from 60 µg ml⁻¹, which was the Ca level of the medium, to 85 µg ml⁻¹ throughout 21 days ($p < 0.05$). Mg concentration observed was also stable between 20 and 30 µg ml⁻¹, with an initial concentration of 20 µg ml⁻¹ prior to the immersion of the samples. The culture medium contained Ca and Mg, but not Si. The Si concentration increase was due only to the dissolution of the foams and stabilised between 5 and 10 µg ml⁻¹ ($p < 0.05$).

For WD samples (Fig. 5b), the Ca levels were similar to the those of Ak samples, between 60 and 90 µg ml⁻¹ ($p < 0.05$). Concerning the Mg release, its concentration was lower than for Ak samples, reaching 22 µg ml⁻¹. The highest value for Si was reached at day 10 with 23 µg ml⁻¹, which was double the highest concentration of Si ions detected for Ak samples ($p < 0.05$), because the WD samples has higher silica content than Ak samples.

The fluctuation of the pH (data not shown for brevity) was between 7 and 8 for both the samples, within the range of normal cell culture pH, so the foams did not cause detrimental pH change in the culture medium.

The attachment of the cells to the scaffolds, the cell morphology and the formation of cell matrix were investigated by SEM imaging at different time points (Fig.6).

FIGURE 6 HERE

After 7 days (Fig.6 a-b), the cells seemed to have colonised the pores of the scaffolds and the cells exhibited elongated filopodia, which are used by the cells to probe the microenvironment and to promote their motility [46]. After 21 days (Fig.6 c-d) of culture, the cells appeared to form more condensations on WD samples (Fig. 6d) than on Ak samples (Fig. 6c). The EDS spectra reported in Fig.6e were taken for the apparent spheroid cell condensation as well as the scaffold surface adjacent to the cell groups. All of them were normalised on the peak of Si at 1.74 keV with the aim of highlighting the differences in Ca/Si and Ca/P ratios (even though the content of Si in the cell spheroids and in the adjacent

surfaces are expected to be much different). The spectra showed that Ca and P were present on the cellular deposits for both the samples Ak and WD, confirming that mineralisation occurred; while the spectra taken on the scaffold surfaces adjacent to the cell groups showed that relative P and Ca contents were much lower compared with Si content.

Concerning the growth of the cellular deposits mentioned above, SEM images of the samples seeded with cells after 21 days of cell culture were compared with those taken on the controls, that were the same Ak and WD samples immersed in the same culture medium, but not seeded with cells. The controls did not exhibit the formation of surface precipitates, that implies that the calcium phosphates observed on the seeded samples were not simply due to the reaction of the Ca ions released by the samples with the P provided by the culture medium, but they might be attributed to cellular osteogenic activities occurring throughout the cell culture test. A SEM micrograph of a WD control after 21 days in the cell culture medium, without cells, is reported in Fig. 6f as an example.

3.5 In vitro osteogenic differentiation

For analysis of osteogenic differentiation and matrix formation, ~~day-21~~ MC3T3 cell seeded Ak and WD constructs **after 21 days of cell culture** were first immunostained with antibodies against phenotypic osteogenic proteins (Fig. 7). The formation of bone-like matrix was evident. Osteoblastic activity was confirmed by the expression of Collagen Type I, a commonly used extracellular marker for early bone matrix formation. The expression of Collagen I [47] appeared particularly robust and well organised on WD foam scaffolds. In turn, more robust expression of Osteopontin [48], an extracellular protein that regulates matrix mineralisation, and Osteocalcin [49], a late marker for bone formation and matrix mineralisation, was observed in WD foam scaffolds in comparison to Ak foams.

FIGURE 7 HERE

We have previously demonstrated promising osteogenic differentiation of MC3T3 cells on Mg-free wollastonite-hydroxycarbonate apatite (WA) foam scaffolds [31]. Real-time qPCR gene expression analysis was used in the present study as a quantitative measure to demonstrate the positive effects of Mg that were incorporated in the newly developed Ak and WD foams (Fig. 8).

Levels of Runx2, a key osteoblast differentiation transcription factor, were significantly higher in Ca-Mg silicate ceramics Ak and WD, compared to Mg-free WA scaffolds. The level of Runx2 detected in day-21 WD constructs appeared higher than for Ak constructs, though this observation was not statistically proven. Interestingly, the transcription level of the three Runx target genes, Col1a1, Osteopontin and Osteocalcin, were all significantly higher in day-21 WD constructs compared to both Ak and Mg-free WA.

FIGURE 8 HERE

These observations confirmed that WD foams improved osteogenic cellular activities in comparison to Mg-free WA foams. However, it is not clear whether the stimulatory effects were due solely to Mg, as the AK contained Mg as well. Previous studies have demonstrated that Mg is a promising agent in skeletal tissue engineering applications due to its ability to stimulate new bone formation [23]. Mg was suggested to interact with integrins of osteoblasts that are responsible for cell adhesion and stability [23,50]. The importance of Mg in bone metabolism was underlined by studies that demonstrated Mg depletion can result in impaired bone growth, increased bone resorption and loss in trabecular bone volume in animal models [25,51,52]. In addition to Mg, Ca^{2+} and soluble silica were also released by the bioactive Ca-Mg silicate ceramics (Fig. 5). The stimulatory and dose-dependent osteogenic effects of these ionic products have been well-documented in a number of studies [10,53,54,55]. Although ICP analysis confirmed Ak foams released higher levels of Ca and Mg, the release of Si was significantly lower in comparison to WD foams. Si is a known essential element for

metabolic processes associated with the formation and calcification of bone tissue. High Si contents have been detected in early stages of bone matrix calcification [56] and aqueous Si has been shown to induce the precipitation of hydroxyapatite, the inorganic mineral phase that contributes to 70% of human bone mass [57]. The presence of Si in the form of orthosilicate acid has been shown to stimulate the osteoblastic differentiation of human osteoblast and bone marrow derived stromal cells [58,59]. Further, dietary Si intake has been shown to increase the bone mineral density in both men and premenopausal women [60,61]. Therefore, it is likely that the collective effects of Ca, Mg as well as Si released from WD foams played a significant role in the enhanced osteogenic differentiation and matrix formation observed in the present study. Further, the more consistent pore distribution in WD samples may also have contributed to the superior osteogenesis.

The present study completed the synthesis and a series of investigations whereby the porous polymer-derived Ca-Mg silicate scaffolds proved to be promising materials as an osteogenic alternative.

4 Conclusions

We conclude that:

- Ca-Mg-rich ceramics (based on akermanite and wollastonite-diopside) were successfully synthesised as highly porous foams, starting from silicone-based mixtures incorporating powdered oxide fillers. Some fillers provided an extensive foaming, at 350 °C, simply by water release in the polymer before its ceramic conversion; this conversion was completed at 1100 °C, with the formation of the desired phases;
- The use of a preceramic polymer, coupled with fillers, offers a distinctive opportunity to control the shaping (in the polymeric state) as well as the final ceramic phase compositions, with silicate crystals embedded in a boro-silicate matrix;

- After ceramisation, the foams exhibited an open porosity of 80 %, pores of 700 µm – 1 mm with interconnections of 160-180 µm and a compressive strength of 1-2 MPa;
- Throughout a 21 day SBF dissolution test in SBF, the glass-ceramics demonstrated suitable dissolution rates, pH and ionic concentrations. A cytotoxicity analysis, performed in accordance to ISO10993 standards, confirmed an excellent biocompatibility.
- MC3T3-E1 cells cultured on the WD foams demonstrated enhanced osteogenic differentiation and matrix formation in comparison to Mg-free counterparts.
- The addition of Mg can further enhance the bioactivity and the potential for bone regeneration applications of Ca-silicate materials.
- This work provides a platform from which up-scaled processes can be planned before eventual clinical translation.
- Future studies could provide indications of osteogenic differentiation as a function of time. Variation of gene expression could be investigated using PCR analysis and response of human skeletal stem cells to the materials and animal models will be the focus of further investigations.

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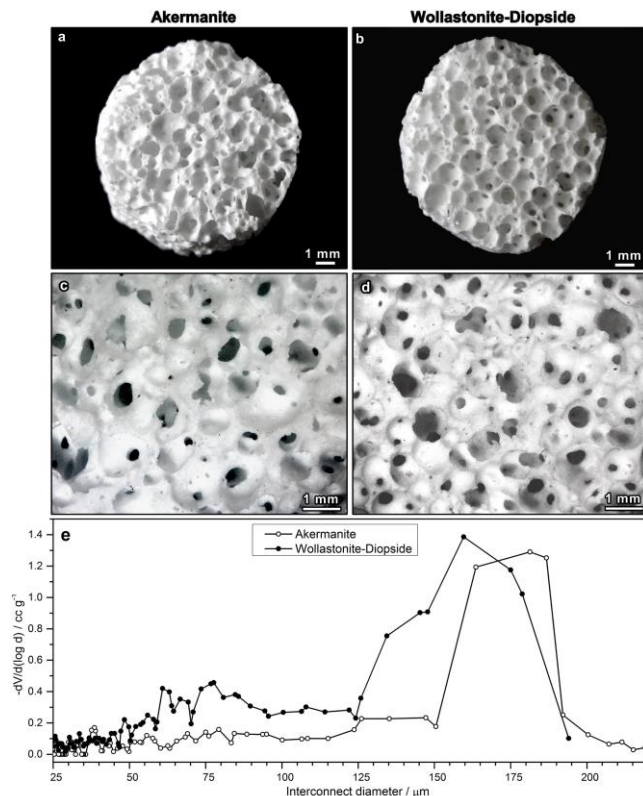


Fig.1: a-b) Stereomicroscopy of the ceramised foam scaffolds; c-d) higher magnification; e) interconnect size distribution obtained by Hg porosimetry

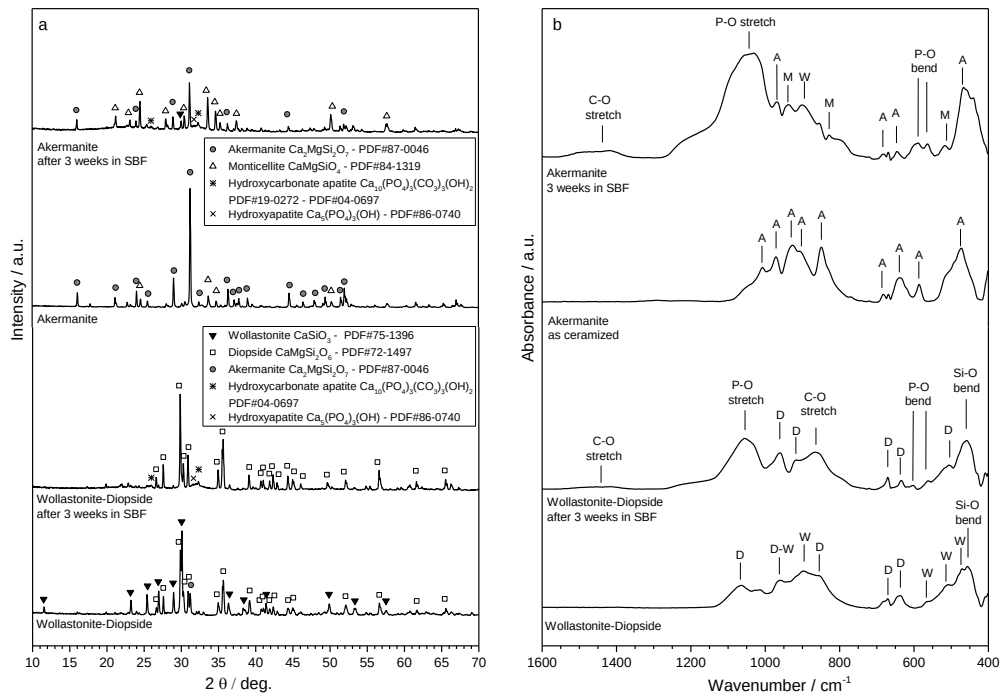


Fig.2 a) XRD patterns and b) FTIR spectra of Ak and WD foam scaffolds before and after soaking in SBF for 21 days

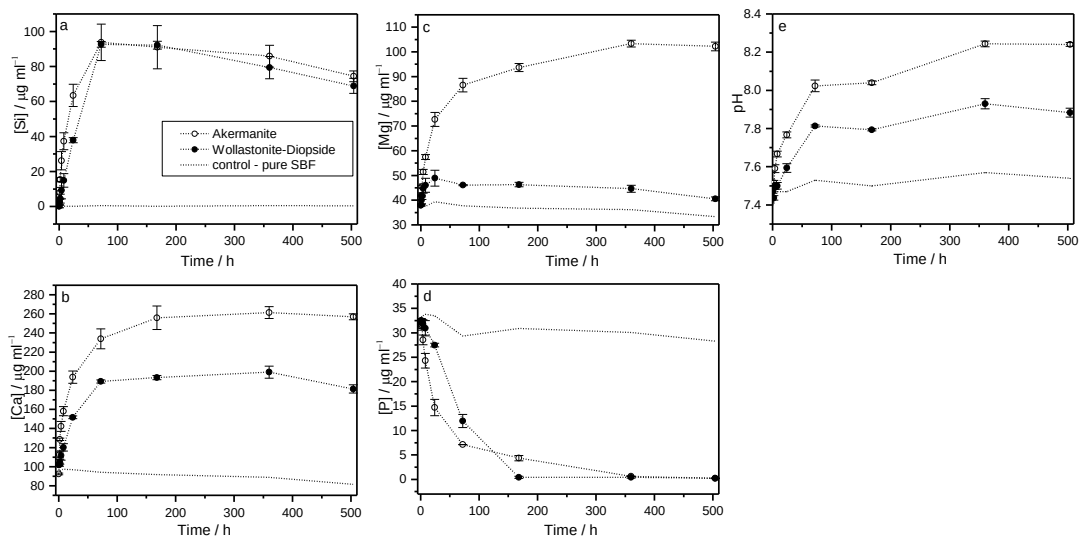


Fig.3 Dissolutions profiles for a) Si, b) Ca, c) Mg, d) P and e) pH trend of SBF as a function of time, for Ak and WD foams after soaking in SBF for 21 days. Control is SBF without any samples immersed. [Results are presented as mean $\pm$ SD, n=3 per each time point].

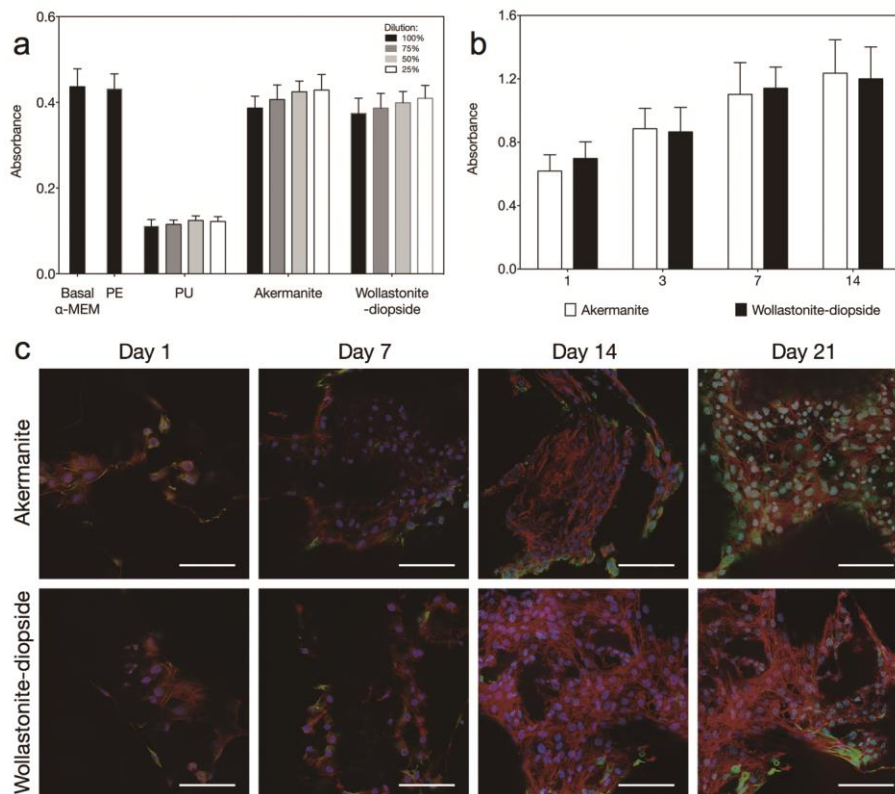


Fig.4: a) *In vitro* cytotoxicity analysis by MTT metabolic activity assay, in accordance to ISO 10993-5 and 10993-12 standards; b) WST-1 assay of MC3T3 cell seeded scaffolds up to 15 days. Mean optical densities/absorbance of WST-1 solution following 2 hours of incubation with cell-seeded constructs were plotted with standard deviation (n=3); c) Immunohistochemical staining of the cytoskeleton of MC3T3 cells cultured on scaffolds for up to 21 days. Vimentin (green fluorescence), F-actin (red fluorescence), DAPI nuclei counter stain (blue fluorescence). Scale bar = 100 μm .

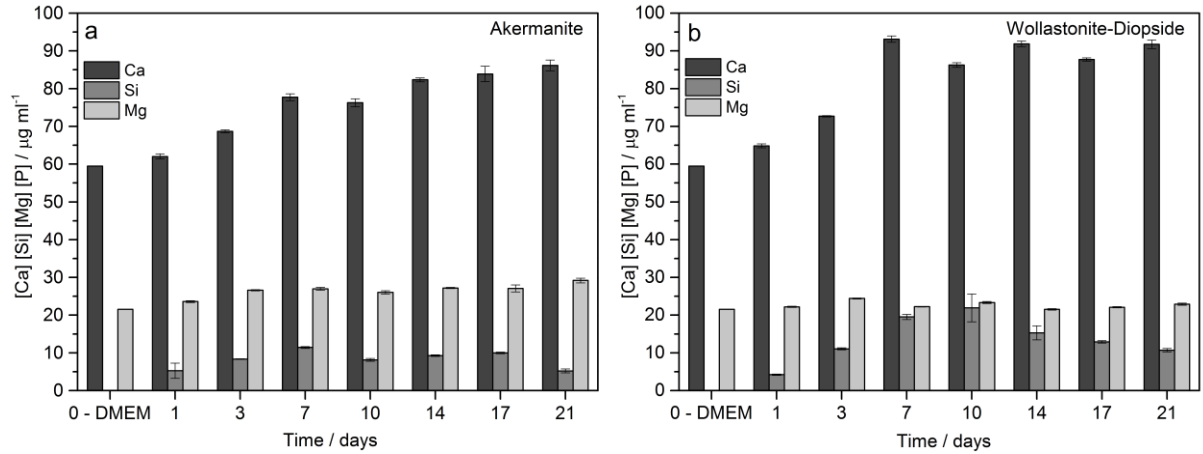


Fig.5: Elemental concentration of the cell culture medium after removal from the culture plate, dilution and filtration, measured by ICP.

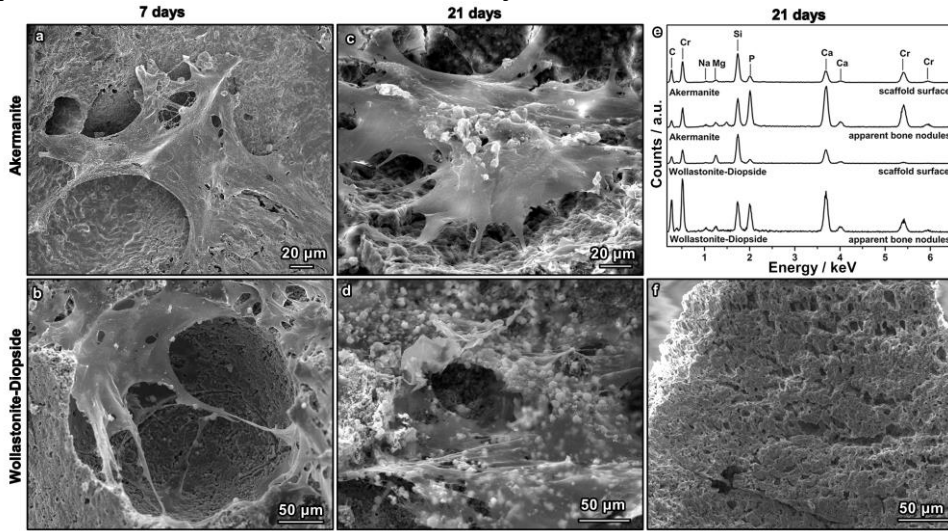


Fig.6: a-d) SEM micrographs at different time points of the MC3T3 cells cultures on the scaffolds Ak and WD; e) EDS spectra of the scaffold surface adjacent to the cells and of the spheroid condensations formed by the cells on Ak and WD foams after cell culture for 21 d; f) SEM micrograph of a WD sample without cells after immersion in culture medium for 21 d.

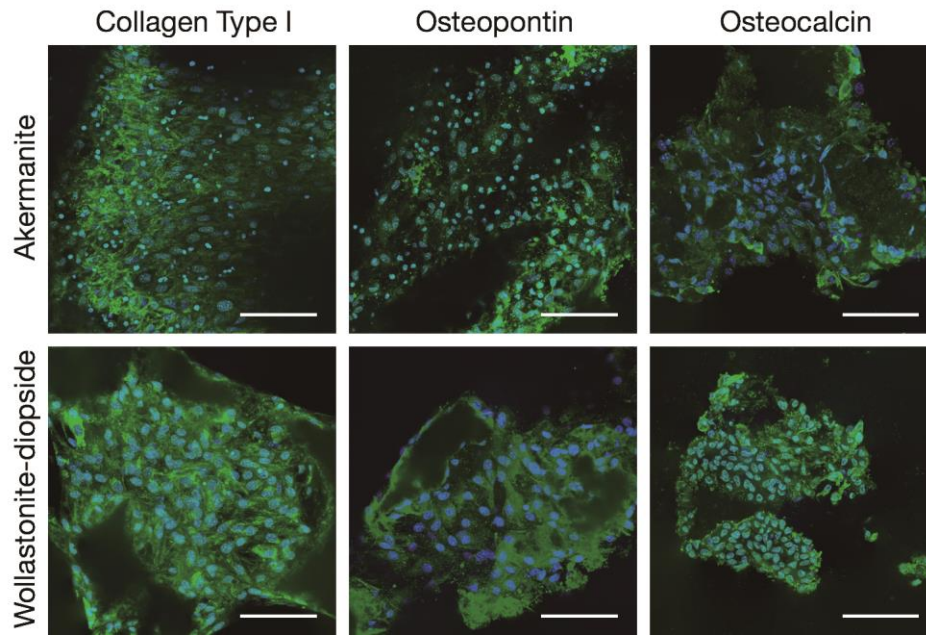


Fig. 7: Immunohistochemical examination of day 21 MC3T3 seeded Ak and WD scaffolds. Common osteogenic differentiation and bone formation markers including *Collagen Type I*, *Osteopontin* and *Osteocalcin* were immunolocalised (green fluorescence) in both AK and WD scaffolds. All samples were labelled with DAPI nuclear counter stain (blue fluorescence). Scale bar = 100 μ m.

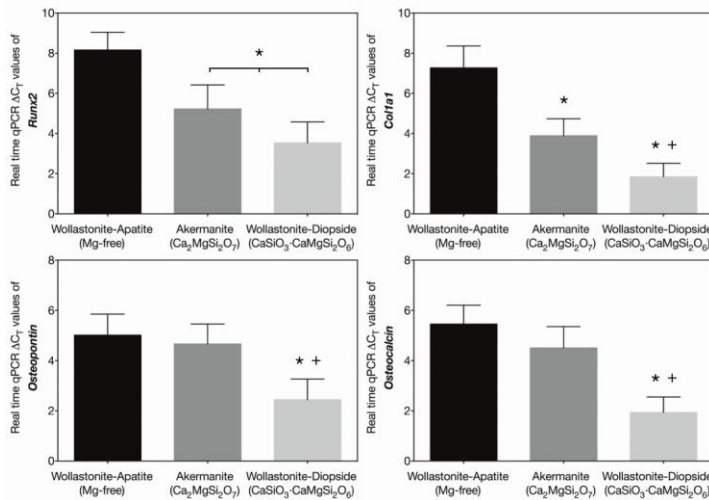


Fig. 8: Analysis of expression of *Runx2*, *Col1a1*, *Osteopontin* and *Osteocalcin* transcripts in day-21 MC3T3 cells cultured on AK and WD, compared to WA, scaffolds using real-time qPCR. Expression levels of the genes of interests were normalised to the expression of housekeeping gene GAPDH. $\Delta C_T = C_{T(target)} - C_{T(GAPDH)}$, hence ΔC_T values are inversely proportional to the expression of target gene. * $p < 0.05$ compared to Mg-free WA. + $p < 0.05$ compared to both Ak and Mg-free WA.