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Formation and properties of magnesium–ammonium–phosphate hexahydrate biocements in the Ca–Mg–PO_4 system

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Abstract Calcium substituted trimagnesium phosphate with the general formula $Ca_xMg_{(3-x)}(PO_4)_2$ ($0 < x < 1.5$) was synthesized by calcination of powder mixtures with the appropriate stoichiometry and reacted with 3.5 M diammonium hydrogenphosphate solution to form a cementitious matrix of magnesium ammonium phosphate hexahydrate (struvite). The degree of ionic substitution was shown to influence physical cement properties; clinically suitable cement formulations with setting times in the range 5–15 min and compressive strengths of >50 MPa were obtained for $x \leq 0.75$ together with a grinding time $\geq$1 h and a powder to liquid ratio $\geq$2.5 g/ml. The cement cytocompatibility was investigated by culturing human osteoblast cell line MG63 on cement surfaces demonstrating pronounced cell growth during 13 days cultivation.

1 Introduction

A major clinical demand for artificial bone substitutes is their ability to degrade in vivo followed by formation of new hard tissue. Many materials do not fulfil this criterion, e.g. sintered ceramics made of hydroxyapatite (HA) [1–4] or polymeric cements based on polymethylmethacrylate (PMMA) [5]. Degradable materials in clinical use are ceramics based on tricalcium phosphate [6] or self setting cements which form dicalcium phosphate dihydrate (brushite) [7]. The latter has the advantage that a mechanically stable implant is formed in situ at the application site which has been shown to degrade over a time period of 3–6 months [8–10]. Major concerns about a broad clinical use of brushite cements are their relatively poor mechanical properties compared to apatite cements and their strong acidic pH value during setting which can lead to the release of acid into the surrounding tissue around the implant [11].

An interesting alternative biocement is based upon the formation of magnesium ammonium phosphate hexahydrate (Struvite, $MgNH_4PO_4{\cdot}6H_2O$). Struvite is a natural mineral which occurs as pathological calcification in kidney stones [12]. This kind of cement has the advantage of a neutral pH value during setting since both the cement powder ($MgHPO_4$, $Mg_3(PO_4)_2$) as well as the cement liquid ($(NH_4)_2HPO_4$) are non-acidic compounds. Struvite cements are thought to be chemically degradable in vivo similar to brushite due to the relatively high solubility with a solubility product in the range of 5.21×10^{-15} ($pK_{(sp)} = 14.28$) to 2.12×10^{-13} ($pK_{(sp)} = 12.67$) over a pH range of 7.01–9.62 [13]. Few studies have investigated struvite forming cements for biomedical applications showing high strength of the materials (compressive strength > 50 MPa) and an appropriate setting time of 3–10 min which meets clinical demands [14–16].

Previous studies about struvite forming cements used multi-component mixtures of secondary ($MgHPO_4$) and tertiary ($Mg_3(PO_4)_2$) magnesium phosphates in conjunction with calcium orthophosphates (α-TCP, β-TCP, HA) as fillers [14]. This might be associated with some problems in manufacturing and handling of the materials due to their different particle sizes, therefore the preparation of

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homogenous powder mixtures is difficult and these may be de-mixed during transport or storage. The current study aimed to produce powders made of calcium magnesium phosphates to obtain single component cements to avoid these problems. Materials with the general formula $Ca_xMg_{(3-x)}(PO_4)_2$ with ($0 < x < 1.5$) were synthesized by sintering calcium and magnesium raw materials. These compounds were then ground for up to 4 h and their setting ability was tested using a 3.5 mol/l $(NH_4)_2HPO_4$ solution as liquid cement phase. Investigated parameters were the initial setting time ($t_{initial}$) according to the Gilmore needle test, the uncompacted wet compressive strength (CS) and their phase composition before and after the setting reaction. The in vitro cytotoxicity was evaluated by culturing the human osteoblastic cell line MG63 on the surface of the cement samples over a period of 13 days followed by analysis of cell proliferation and cell activity.

2 Materials and methods

Calcium magnesium phosphate powders with the general formula $Ca_xMg_{(3-x)}(PO_4)_2$ were synthesized by heating mixtures of monetite ($CaHPO_4$; Mallinckrodt Baker, Griesham, Germany), calcium carbonate ($CaCO_3$; Merck, Darmstadt, Germany), magnesium hydrogenphosphate trihydrate ($MgHPO_4{\cdot}3H_2O$, Riedel–de–Haen, Seelze, Germany) and magnesium hydroxide ($Mg(OH)_2$, Fluka, Steinheim, Germany) to 1100°C for 5 h followed by quenching to room temperature. The composition of the different powder mixtures is shown in Table 1. The sintered cake was crushed with a pestle and mortar and passed through a 355 μm sieve. Milling of the materials was performed in a planetary ball mill (PM400 Retsch, Germany) at 200 rpm with 500 ml agate jars, four agate balls (30 mm) and a load of 125 g powder per jar for up to 4 h. Particle size distributions were determined using laser particle size analysis (L300, Horiba, Kyoto, Japan). 100 mg of the powder particles were suspended in 200 ml isopropanol and dispersed by applying ultrasound for 15 min.

Cement mixtures were prepared by mixing the ground $Ca_xMg_{(3-x)}(PO_4)_2$ with a 3.5 mol/l $(NH_4)_2HPO_4$ solution at a powder to liquid ratio (PLR) of 3.0 g/ml for 30 s on a glass slab. For mechanical testing, these cement pastes were transferred into silicon rubber moulds to produce cuboids of 12 × 6 × 6 mm. The samples were hardened at 37°C and 100% humidity for 24 h followed by testing in compression with a universal testing machine Zwick 1440 (Zwick, Ulm, Germany) at a crosshead speed of 1 mm/min. The compressive strength was calculated by dividing the maximum load by the cross sectional area of the samples. The initial setting times of the cements were measured in a humidity chamber at 37°C and >90% humidity using the Gilmore needle test with a needle of 113.98 g and 2.117 mm diameter according to ASTM standard [17].

X-ray diffraction patterns of cement raw materials and set cements were recorded on a diffractometer D5005 (Siemens, Karlsruhe, Germany). Data were collected from $2\theta = 20–40°$ with a step size of 0.02° and a normalized count time of 1 s/step. The phase composition was checked by means of JCPDS reference patterns for farringtonite ($Mg_3(PO_4)_2$, PDF Ref. 33-0876) and struvite ($MgNH_4PO_4{\cdot}6H_2O$, PDF Ref. 15-0762). The microstructure of fracture surfaces was characterized by scanning electron microscopy (SEM) (LEO 440i, Leica, Germany). The solubility of the compounds in water was measured by stirring approx. 100 mg calcium magnesium phosphate in 10 ml double distilled water following filtering through a 0.4 μm pore size filter and ICP-MS analysis (Varian ICP-MS, Darmstadt, Germany) against standard solutions (Merck, Darmstadt, Germany).

2.1 Cell culture

Human osteoblast cell line MG63 (ATCC no. CRL-1427, Rockville, MD, USA) was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal calf serum, 1% penicillin and streptomycin, respectively (all from Invitrogen Life Technologies). The cells were incubated in a humidified 5% CO_2 incubator at 37°C. For biocompatibility the test samples were placed in quadruplicate into the wells of a 24-well plate (Nunc, Wiesbaden, Germany), cells were seeded onto the cement surfaces and on polystyrene with an initial density of 50,000 cells per well.

Table 1 Composition of powder mixtures for the preparation of $Ca_xMg_{(3-x)}(PO_4)_2$

$Mg_xCa_Y(PO_4)_2$	$CaHPO_4$ [mol]	$CaCO_3$ [mol]	$MgHPO_4{\cdot}3H_2O$ [mol]	$Mg(OH)_2$ [mol]
$Ca_{1.5}Mg_{1.5}(PO_4)_2$	1	0.5	1	0.5
$Ca_{0.75}Mg_{2.25}(PO_4)_2$	0.5	0.25	1.5	0.75
$Ca_{0.\ 5}Mg_{2.5}(PO_4)_2$	0.33	0.17	1.67	0.83
$Ca_{0.25}Mg_{2.75}(PO_4)_2$	0.167	0.083	1.83	0.92
$Mg_3(PO_4)_2$	–	–	2	1

2.2 Cytocompatibility testing

The cytocompatibility of the calcium and magnesium phosphate cements was determined by cell counting and a cell activity test after 3, 5, 7, 10, and 13 days of culture on the surfaces using the WST reagent as described earlier [18]. Briefly, cell proliferation was analyzed by electronic cell counting using a CASY 1 TTC cell analyzer (Schärfe System, Reutlingen, Germany). Cell activity was analyzed by using cell proliferation reagent WST 1 (Roche Diagnostics, Mannheim, Germany). After incubating the cells for 30 min with the WST reagent 1:10 in supplemented DMEM at 37°C the adsorption of the supernatant was quantified in a Tecan spectra fluor plus photometer (Tecan, Crailsheim, Germany). For each analysis the samples were examined in quadruplicate, the mean value and standard deviation were calculated.

3 Results

Ionic substitution of magnesium ions in trimagnesium phosphate resulted in the formation of compounds with the general stoichiometric formula $Ca_xMg_{(3-x)}(PO_4)_2$ ($0 < x < 1.5$). X-ray diffraction analysis revealed the presence of two major crystalline phases depending on the magnesium to calcium ratio. At low calcium content, the sintered powders were composed of farringtonite, whereas with increasing calcium substitution stanfieldite ($Ca_3Mg_3(PO_4)_4$) was identified as a further crystalline phase in agreement with the phase diagram of Ando [19] (Fig. 1). Solubility deceased with increasing the degree of ionic substitution such that the compound with an equimolar Ca:Mg ratio showed approximately only 50% of the solubility of pure farringtonite (Fig. 2). All materials formed low-temperature setting cement matrices in the presence of a highly concentrated diammonium phosphate solution. The setting products according to Eq. 1 were mainly magnesium ammonium phosphate hexahydrate (struvite) with minor by-products of newberyite (high magnesium content) and brushite (high calcium content):

$$\begin{aligned} &Ca_xMg_{(3-x)}(PO_4)_2 + (NH_4)_2HPO_4 \\ &\quad + (15-x)H_2O \rightarrow 2MgNH_4PO_4 \cdot 6H_2O \\ &\quad + (1-x)MgHPO_4 \cdot 3H_2O + xCaHPO_4 \cdot 2H_2O \end{aligned} \quad (1)$$

The setting times of the cements (Table 2) were influenced by the degree of ionic substitution, the grinding time and the PLR. Cements with clinically appropriate setting ($t_{initial}$ = 5–15 min) were obtained for $x \leq 0.75$ together with a grinding time ≥1 h and a PLR ≥ 2.5 g/ml. Particle sizes of cement reactants decreased with increasing milling time from approx. 24–48 μm (unground) to 6–7 μm after 4 h grinding (Table 3). The strengths of the struvite cements in this study were also strongly influenced by calcium for magnesium substitution in $Mg_3(PO_4)_2$ (Fig. 3). Maximum values of up to 80 MPa were obtained for a grinding time of 4 h and a Ca/(Ca + Mg) ratio of 0.25. At a high degree of ionic substitution (Ca/(Ca + Mg) ratio = 0.5), strength was found to decrease to values of less than 25 MPa independent of the grinding time. The mechanical performance of the cements was also decreasing with a smaller PLR (Fig. 4). However, even at a PLR of 2.0 g/ml with an initial liquid cement viscosity,

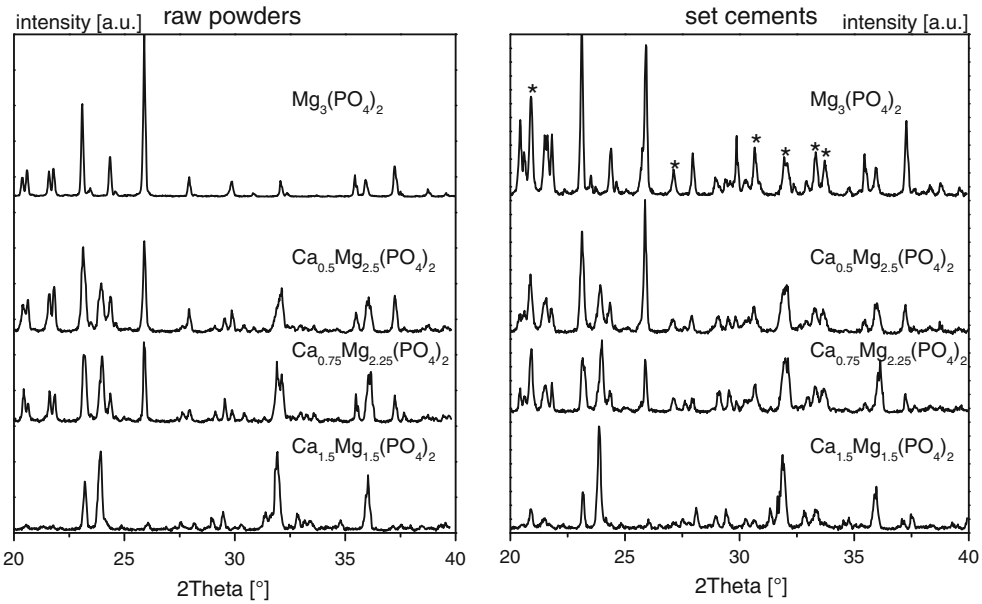

Fig. 1 X-ray diffraction patterns of cement raw materials and cements set for 24 h with 3.5 M $(NH_4)_2HPO_4$ at PLR = 3 g/ml. The diffraction patterns of the raw materials consist of pure farringtonite (PDF No. 33-0876) for $Mg_3(PO_4)_2$ and stanfieldite (PDF No. 11-0231) for $Ca_{1.5}Mg_{1.5}(PO_4)_2$ or mixtures of both phases for the other compounds. The most relevant peaks of struvite (PDF No. 15-0762) in the pattern of set cements are marked with *asterisk*

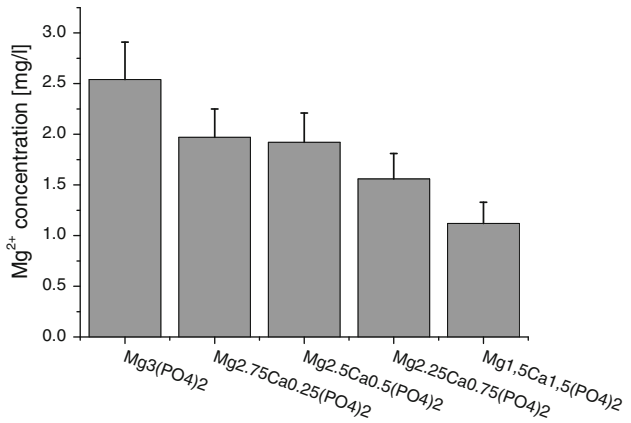

Fig. 2 Solubility of calcium magnesium phosphates in water measured with ICP-MS analysis

the CS was still above 40 MPa for 4 h ground cement raw materials. The morphology of set cements (Fig. 5) showed the appearance of small (struvite) crystals in a size range of approx. 1–5 μm for a Ca/(Ca + Mg) ratio up to 0.25. In contrast, cement made from $Ca_{1.5}Mg_{1.5}(PO_4)_2$ formed a matrix of needle-like crystals with a length of 20–30 μm and diameter of less than 2 μm.

For evaluation of the biocompatibility of the calcium magnesium phosphate cement, osteoblast-like cells were cultivated on prefabricated scaffolds, since in vitro cell testing of biomaterials is a well-established method to determine the cytocompatibility of a material [20–22]. Cell growth was continuously best on $Mg_{2.25}Ca_{0.75}(PO_4)_2$ based cements, where the cell population was constant over a period of 7 days (Fig. 6a). Cell activity determined by WST-test was higher on the $Mg_3(PO_4)_2$ surfaces. Here the cell activity increases to day 7 (Fig. 6b).

Table 3 Particle size in μm of $Ca_xMg_{(3-x)}(PO_4)_2$ cement powders depending on grinding time

Grinding time [h]	$Ca_{1.5}Mg_{1.5}(PO_4)_2$	$Ca_{0,75}Mg_{2,25}(PO_4)_2$	$Mg_3(PO_4)_2$
Not ground	41.4 ± 14.2	23.8 ± 2,9	47.8 ± 1.3
1 h	16.2 ± 1.6	7,7 ± 0.2	10.5 ± 2.6
4 h	7.3 ± 3.0	6.09 ± 0.1	7.5 ± 0.4

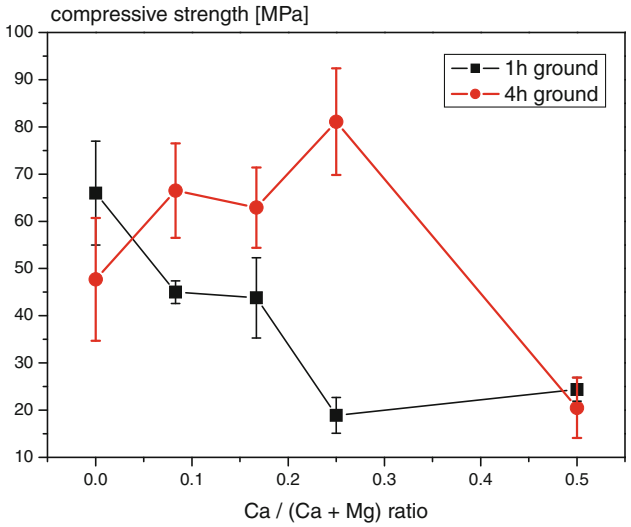

Fig. 3 Influence of Ca/Mg + Ca ratio in $Ca_xMg_{(3-x)}(PO_4)_2$ cements (liquid: 3.5 mol/l $(NH_4)_2HPO_4$ at PLR 3.0 g/ml) on wet compressive strength after 24 h setting at 37°C

4 Discussion

This study demonstrated that ionic substitution of farringtonite ($Mg_3PO_4)_2$) by introducing calcium ions alters the

Table 2 Initial setting times of $Ca_xMg_{(3-x)}(PO_4)_2$ cements depending on degree of ionic substitution, grinding time and PLR (cement liquid: 3.5 Mol/l $(NH_4)_2HPO_4$)

P/L [g/ml]	Setting time [min]			
	$Ca_{0.5}Mg_{2.5}(PO_4)_2$		$Ca_{0.25}Mg_{2.75}(PO_4)_2$	
	1 h ground	4 h ground	1 h ground	4 h ground
3.0	8	4	8	3.5
2.5	13	8	12	6.5
2.0	18	13	19	14
1.5	22	19	23	18
	$Ca_{1.5}Mg_{1.5}(PO_4)_2$		$Ca_{0.75}Mg_{2.25}(PO_4)_2$	$Mg_3(PO_4)_2$
Setting time [min] at PLR 3.0 g/ml; 1 h ground	32 min		14 min	4 min

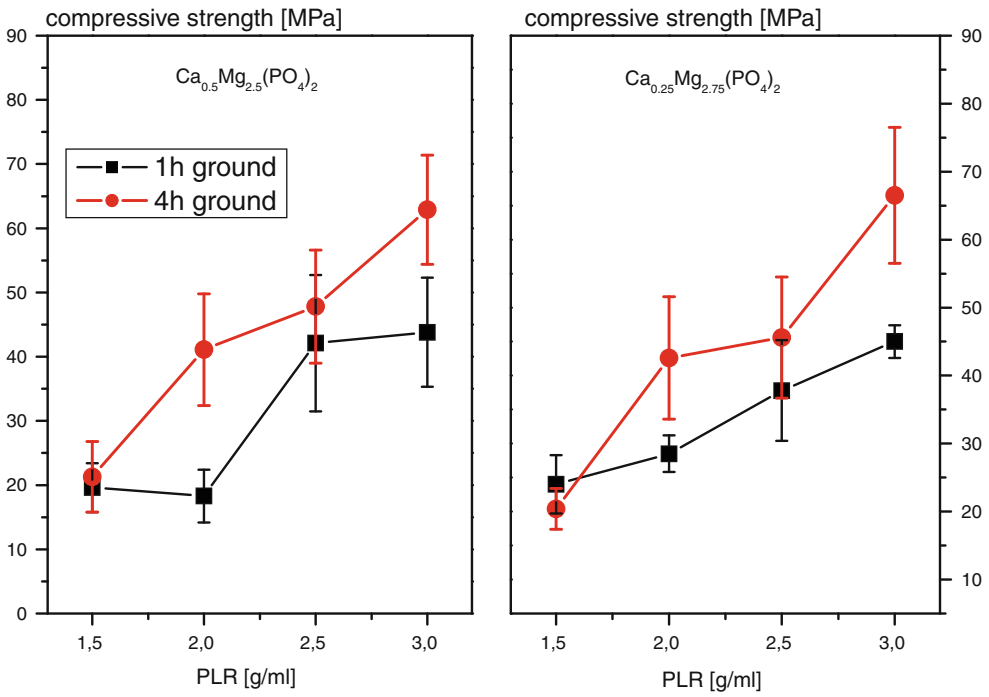

Fig. 4 Wet compressive strength of $Ca_xMg_{(3-x)}(PO_4)_2$ cements with 3.5 mol/l $(NH_4)_2HPO_4$ solution depending on the PLR ratio after 24 h setting at 37°C

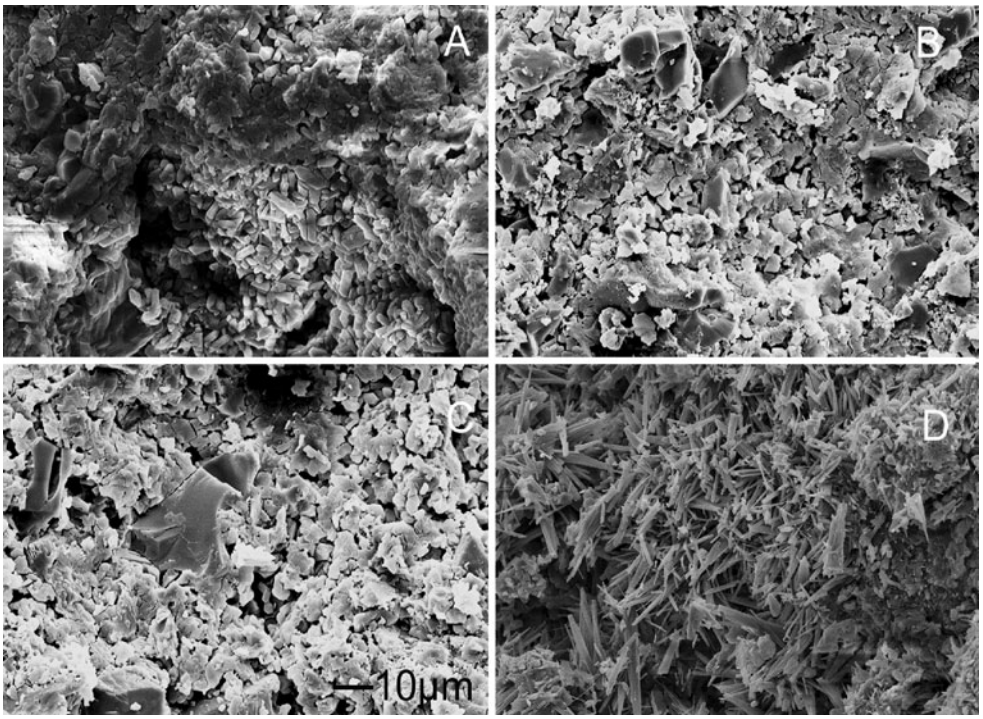

Fig. 5 SEM-micrographs of calcium magnesium phosphate cements set with 3.5 M $(NH_4)_2HPO_4$ at PLR = 3 g/ml for 24 h. **a** $Mg_3(PO_4)_2$, **b** $Mg_{2.5}Ca_{0.5}(PO_4)_2$, **c** $Mg_{2.25}Ca_{0.75}(PO_4)_2$ and **d** $Mg_{1.5}Ca_{1.5}(PO_4)_2$

physicochemical properties of struvite forming cements when mixed with a neutral $(NH_4)_2HPO_4$ solution as cement liquid. Two major effects were observable, firstly the setting time generally increases with a higher calcium content and secondly, a maximum of mechanical performance was found for a Ca/(Ca + Mg) ratio in the range between 0.16 and 0.25.

Setting times were measured by means of the Gilmore needle test. The initial setting time is the interval elapsed between the mixture of the components of the cement and

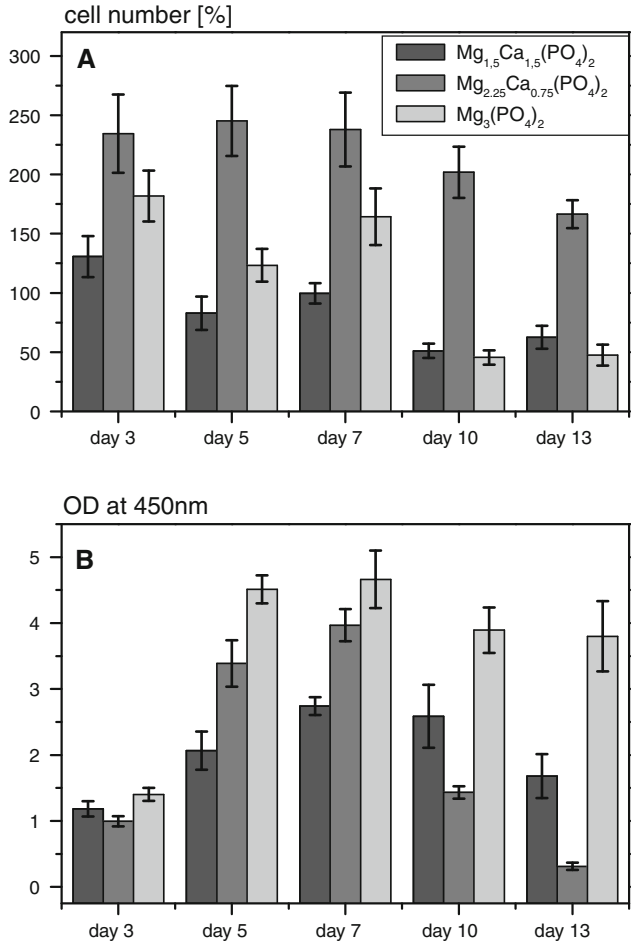

Fig. 6 Cytocompatibility assay. **a** Cell proliferation determined by cell counting. The cell numbers are normalized to the initial cell number (50000 ≈ 100%). **b** Cell activity determined by WST-test. Optical density (OD) is proportional to mitochondrial metabolism

the initial loss of plasticity, whereas the final setting time is the time required for the cement paste to acquire strength high enough to support a certain level of pressure. For dental and orthopaedic applications the following handling requirements have been formulated for calcium phosphate cements: 5 min $< t_{initial} <$ 10 min; $t_{final} \rightarrow t_{initial}$; CS as high as possible [23]. Cements in this study with clinically appropriate setting ($t_{initial}$ = 5–10 min) were obtained for $x \geq 0.75$ together with a grinding time ≥1 h and a PLR ≥2.5 g/ml. While the effect of grinding and PLR on the setting time is well documented in literature for calcium or magnesium phosphate cements [24–26], ionic substitution of magnesium phosphates is hardly investigated up to now. The setting mechanism of magnesium phosphate cement (similar to calcium phosphate cements or gypsum) is based on a continuous dissolution/precipitation reaction of cement reactants and setting product. Hence both the solubility product and the rate of dissolution determine the setting speed. The latter parameter is mainly influenced by the particle size/specific surface area of the compounds; this explains why the longer ground compounds exhibit a shorter setting time. The prolonging of the setting time with an increase of ionic substitution is likely not based on this parameter since the particle sizes of all compounds were in a comparable range after 4 h grinding (Table 3). A more likely explanation is based on a different solubility product while replacing magnesium by calcium ions. This was demonstrated by measuring the dissolution of the compounds at a fixed grinding regime (4 h ground) using ICP-MS analysis showing a subsequent decrease of solubility with increasing calcium content (Fig. 2).

The mechanical performance of the set cements was found to be in the range > 50 MPa for $x \leq 0.75$ together with a grinding time ≥1 h and a PLR ≥2.5 g/ml. At a high degree of ionic substitution (Ca/(Ca + Mg) ratio = 0.5), strength were found to decrease such that only values of less than 25 MPa were obtained independent of the grinding time. All strengths were measured after 24 h setting and incubation of the samples in PBS buffer without drying them before testing, thus strength values represented the clinical attainable mechanical properties. Strength data for other degradable cements (brushite-forming) reported in literature are in the range of 1–51 MPa for compressive strength [27]. However, caution must be exercised when comparing literature strength values because of differences in test methods and conditions. Many previous studies have used samples that were only stored in humid atmospheres or dried before testing. It is well known that the degree of hydration strongly affects the measured strength, e.g. Pittet and Lemaitre [7] showed that strengths were nearly double for dried samples than for samples stored in a high humidity atmosphere.

The key factor controlling the strength of the cement is porosity formed by the cement liquid since strength of brittle materials is inversely exponentially dependent on the level of porosity and inversely proportional to the size of the largest flaws [28] :

$$CS = (E_o R/(\pi c))^{0.5} \exp(-KP). \quad (2)$$

Here E_o is the modulus of a zero porosity material, c is the flaw size, R is the fracture surface energy and K is a constant. A correlation of cement porosity and ln(CS) of the cements of this study (Fig. 7) proofed this relationship after a linear curve fit. The mechanical performance was generally increasing with decreasing the amount of cement liquid (higher PLR, lower porosity). However, some strength values were found to be out of the area of validity, e.g. strength of $Ca_{0.75}Mg_{2.25}(PO_4)_2$ after 4 h grinding (81 MPa) was approximately 3–4 times as high than those of $Ca_{1.5}Mg_{1.5}(PO_4)_2$ (20–24 MPa) despite of having quite similar porosities. A feasible explanation is related to changes of the cement microstructure. While those with a

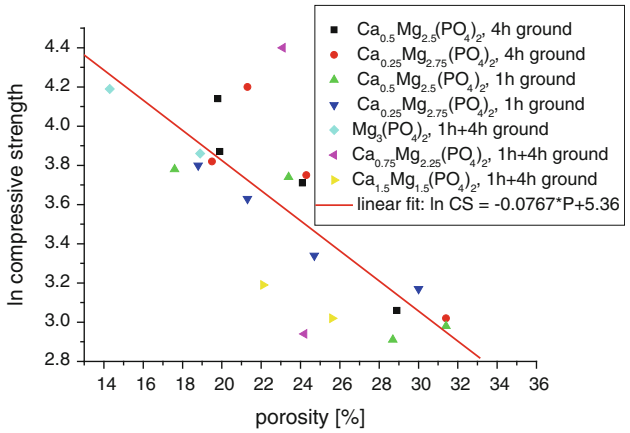

Fig. 7 Relationship between cement porosity and ln(compressive strength) for all cements of this study

Ca/(Mg + Ca) ratio ≤ 0.25 were formed by small and compact struvite crystals a size range of approx. 1–5 μm (Fig. 5a–c), an equimolar Ca:Mg ratio within the cement raw material formed struvite whiskers with a length of 20–30 μm and diameter of less than 2 μm.

Cultivation of osteoblastic cells on the farringtonite and the different magnesium cements containing calcium ions shows a good cytocompatibility of these materials (Fig. 6). Thereby the $Ca_{0.75}Mg_{2.25}(PO_4)_2$ based cement turned out to be charging the best cell proliferation in this system. The cell number is constant over a time period of 7 days, and then it decreases, probably due to limited space in the culture well. Cell number is lowest on the $Ca_{1.5}Mg_{1.5}(PO_4)_2$ formulation. Cell activity is rising continuously on all surfaces tested until day 7, than it decreases again. It is noticeable that the cell activity increase compared to cell number is higher on the $Mg_3(PO_4)_2$ surfaces than on the $Ca_{0.75}Mg_{2.25}(PO_4)_2$ cements. This may be due to the presence of larger cells showing high activity. Electron microscopic analysis of the cells showed larger cells on these samples confirming the assumption (data not shown).

5 Conclusion

Calcium substitution for magnesium in $Mg_3(PO_4)_2$ was successful to alter the setting and mechanical properties of struvite cements formed with diammonium phosphate solution. The high mechanical performance even at lower PLRs, controlled setting and the in vitro cytocompatibility of the set cements is promising for an application as biodegradable bone replacement material.

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