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Magnesium-substituted calcium phosphate bone cements containing MgO as a separate phase: synthesis and *in vitro* behavior

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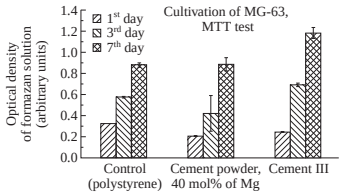
New magnesium-substituted bone cements containing MgO as a separate phase have been developed and demonstrated enhanced mechanical properties and cytological compatibility.



Calcium phosphate (CaP) bone cements (CPC) are widely used in orthopedics and stomatology to fill cavities, to insert implants, to connect tissue fragments, and as drug-delivery systems.[1] These materials possess an ability of self-setting *in vivo*, moldability, and injectability.[2,3] The introduction of magnesium ions into apatite CPCs increases the strength and biodegradation rate of such materials.[4] Magnesium participates in many physiological processes in the human body and, in particular, has an impact on the mineralization process of hydroxyapatite (HA) crystals formation and growth.[5]

The conventional apatite CPCs consist of a two powder components mixture: tetracalcium phosphate (TTCP) and dicalcium phosphate dihydrate (DCPD), which can be mixed with an acid cement liquid.[2] Our previous work[6] revealed a new magnesium-substituted CPC consisted of magnesium-substituted β-tricalcium phosphate (β-TCP) cement powder containing MgO as an additive. MgO was used in CaP ceramic materials as the dopant increasing mechanical and biological properties. In case of a low MgO content (<1 wt%), it was dissolved in CaP without any formation of separate phase.[7] The existence of MgO and hydroxyapatite HA nanopowders as the separate phases on the poly-L-lactide layers has been demonstrated as providing the essential improvement of biological properties due to the MgO introduction.[8] It has been also demonstrated that the both biocompatibility and mechanical properties were enhanced for the cements based on mixture of CPC and magnesium phosphate cement (MPC) with MgO.[9] Taking the above facts into account, the present work was aimed at the investigation of MgO influence on the mechanical properties and biological behavior of Mg-substituted CPC.

CaP–magnesium phosphate powders with the ratio (Ca + Mg):P of 2:1 and the degree of Mg for Ca substitution of 0, 5 and 40 mol% were used as the cement powders. The powders were prepared according to our previously reported procedure[6] using the following analytical grade reactants: $Mg(NO_3)_2$, $Ca(NO_3)_2$, $(NH_4)_2HPO_4$, and deionized water. The pH value of reaction mixture has been maintained at the level of 9–10 by addition of aqueous ammonia. A theoretical reaction (1) results in compounds with Mg:P and Ca:P ratios of 2:1 (and the expected formation of phases containing excess of cation) at the different weight ratios of 0:100, 5:95 and 40:60, to achieve the 0, 5 and 40 mol% of substitution, respectively.

$$4(1-x)\,Ca(NO_3)_2 + 4x\,Mg(NO_3)_2 + 4\,(NH_4)_2HPO_4 + 8\,NH_4OH \rightarrow$$
$$\rightarrow (1-x)\,Ca_4O(PO_4)_2 + x\,MgO + x\,Mg_3(PO_4)_2 + 16\,NH_4NO_3 + 6\,H_2O \quad (1)$$
$$x = 0,\ 0.05,\ \text{and}\ 0.4$$

The water was removed from precipitate by evaporation; the powders were air dried, heat-treated at 1500 °C in air for 1 h, and milled in a planetary ball mill for 1 h with the diethyl ketone medium. The aqueous NaH_2PO_4 solution (80% concentration) was used as the cement liquid. The liquid-to-powder ratio was varied over the range 0.5–0.8 ml g^{-1} (Table 1). The powders and liquid were mixed to give a cement paste, which was placed into a Teflon molds (d = 5 mm, h = 10 mm) and hardened for 3 days. Set cements were numbered as cement I (powder containing 0 mol% of Mg), II (5 mol% of Mg), and III (40 mol% of Mg).

The pH level was measured for the both powders and set cement materials.† The powder with 0 mol% of Mg had the pH value of 10 (typical of TTCP), while the Mg for Ca substitution resulted in a decrease of pH: powders with 5 and 40 mol% of Mg had the pH values of 7.5–8.0 and 7.0–7.5, respectively. All set

Table 1 Setting time, compressive strength and optimum liquid-to-powder (L/P) ratio for different cements.

Mg content (mol%)	Cement no.	Optimum L/P ratio/ml g^{-1}	Setting time/min	Compressive strength/MPa
0	I	0.8	6±0.5	18±1
5	II	0.5	37±5	3±1
40	III	0.5	5±0.5	40±2

† The cement powders and set cements were placed in deionized water, and the pH value was measured using an Econics Expert pH meter on the next day.

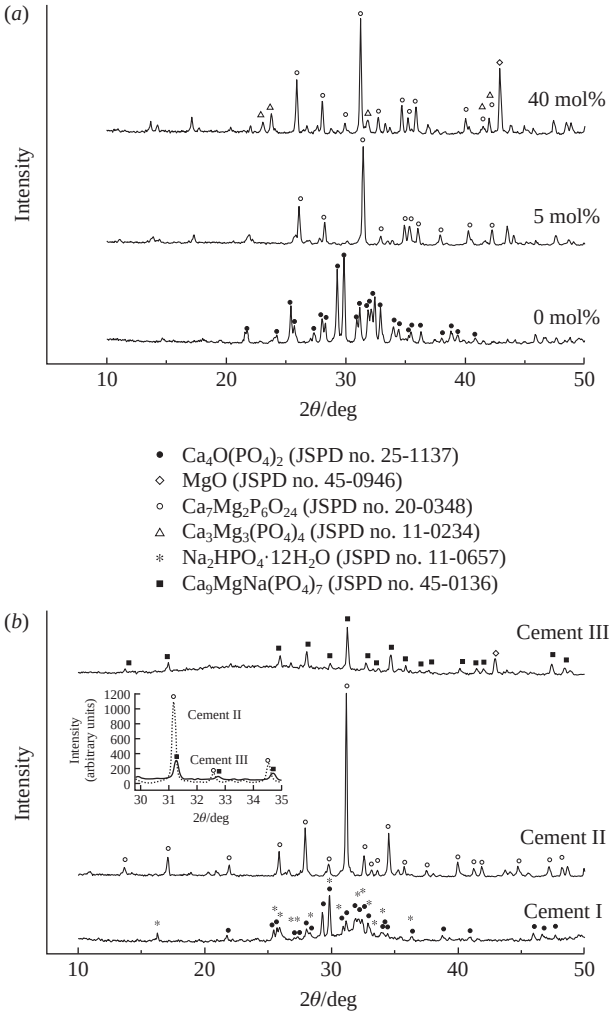


Figure 1 The XRD spectra of (*a*) cement powders and (*b*) set cements.

cements had neutral pH values close to 7.0–7.5, being independent on Mg content.

According to X-ray diffraction analysis (XRD), the Mg-free cement powder was a single-phase one and consisted of TTCP (JSPD no. 25-1137) [Figure 1(*a*)].‡ Introduction of Mg (5 mol%) resulted in the formation of the Mg-deficient substituted β-TCP phase $Ca_7Mg_2(PO_4)_6$ (JSPD no. 20-0348). The absence of any impurity phases was due to a formation of eutectic with melting of powders and low-crystalline solid solution. In the material containing 40 mol% of Mg, the major phases were two magnesium-substituted β-TCP ones: $Ca_7Mg_2(PO_4)_6$ and Mg-enriched $Ca_3Mg_3(PO_4)_4$ (JSPD no. 11-0234); and MgO as well (JSPD no. 45-0946).

The 3-days set cement I demonstrated a significant decrease in the degree of crystallization due to a formation of the amorphous phase as a result of powder interaction with the cement liquid [Figure 1(*b*)]. Additionally to the initial and amorphous phases, the traces of $Na_2HPO_4·12H_2O$ (JSPD no. 11-0657) were detected, which indicated a NaH_2PO_4 transformation due to alkaline media of the TTCP cement powder. There was no significant difference in the XRD pattern between the set cement II and cement powder, which indicated the weak interaction between powder and liquid due to the lower solubility in the acid cement liquid of neutral $Ca_7Mg_2(PO_4)_6$ as compared to alkaline TTCP. The set cement III contained an amorphous phase, crystalline MgO, and the Mg,Na-substituted β-TCP phase $Ca_9MgNa(PO_4)_7$ (JSPD no. 45-0136) resulting from the active interaction between the non-stoichiometric mixture of Mg-substituted β-TCP phases $Ca_7Mg_2(PO_4)_6$ and $Ca_3Mg_3(PO_4)_4$, MgO, and Na-containing cement liquid. The $Ca_9MgNa(PO_4)_7$ formation was confirmed by the main peaks position shifting to higher diffraction angles. Such significant activity in the chemical reaction between the cement powder containing 40 mol% of Mg and cement liquid can be caused by the presence of MgO, which interacts with NaH_2PO_4 with formation of an amorphous phase similar to that reported for MPC.[10]

The microstructure of the I and II cement materials (SEM, Tescan Vega II) were loose particle-like one with many micropores and an average crystal size of 6 μm. The cement III microstructure was dense, homogeneous and contained smaller particles with size of around 3 μm (Figure 2). The amorphous phase formed a matrix between crystals of MgO and substituted β-TCP.

Cements I and III demonstrated the satisfactory values of setting time in the range of 4–6 min (see Table 1). The introduction of Mg (5 mol%) in the cement powder (set cement II) led to the significant increase of the setting time (up to 37 min) due to the slow interaction between components.§ The compressive strength was 18±1 MPa for the Mg-free materials, but significantly decreased to 3–4 MPa for cement II (Table 1).¶ Such a value is too low, thus making these materials useless for biomedical applications. The highest strength of 40±2 MPa was demonstrated by set cement III due to the consolidated dense microstructure and presence of

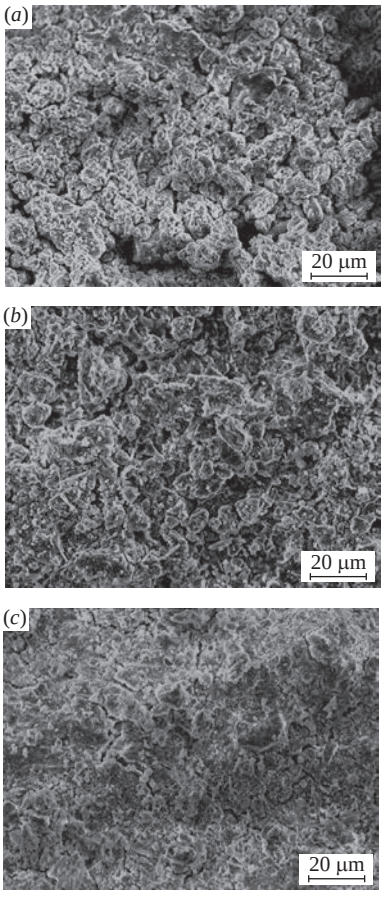


Figure 2 SEM microphotographs of set cements: (*a*) cement I, (*b*) cement II, and (*c*) cement III.

‡ Shimadzu XRD-6000, CuKα radiation at λ = 1,54184 Å, step = 0,02º, the phase composition was identified according to JCPDS database.

§ The setting time was determined by immersing a steel needle (diameter of 1 mm) into the sample until the needle could no longer penetrate more than 1 mm.[9]

¶ The compressive strength of cement samples was measured on an Instron 5581 machine under the uniaxial compression; the final statistical calculations were carried out using 5 samples.

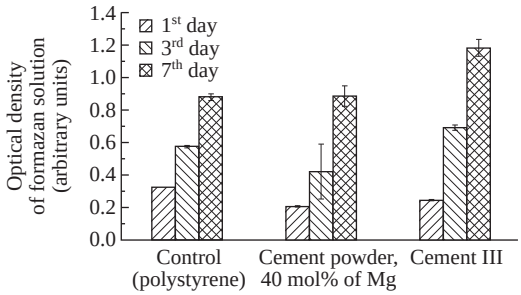


Figure 3 Optical density dynamics of formazan solutions (MTT test) for the cultivation of MG-63 cells on the cement powders, set cements, and polystyrene; $p < 0.05$ in comparison with the control (Student's *t*-test).

MgO excess.[10] To further improve the mechanical properties, the original technology of dispersion hardening was applied. Ceramic particles having the phase composition similar to that of cement powders were used as the dispersoid. The ceramic particles were obtained by heat treatment of the synthesized powders at 1500 °C with subsequent grinding in an agate mortar and separation of the 100–200 μm fraction. The dispersoid addition (15 wt%) resulted in reduced to 18±1 MPa strength of the cements III. However, the introduction of 30 wt% of the dispersoid led to a strengthening of more than 20% (up to 49±2 MPa). The further increase of dispersed particles amount resulted in the absence of cement setting.

To evaluate a cytocompatibility, the *in vitro* experiments were conducted on a model cell line of human osteosarcoma MG-63 (Russian Collection of Cell Cultures, Institute of Cytology, Russian Academy of Sciences, St. Petersburg) using the powder containing 40 mol% of Mg and cement III samples (Figure 3).[††] The powders and set cements III did not demonstrate any cytotoxicity towards the MG-63 cells and supported their adherence and proliferative activity, which indicated the strongly pronounced matrix characteristics of their surfaces. This led to the highest increase of the osteosarcoma cells population on set cements III in comparison with that in the control polystyrene media, reaching a value of 380% *vs.* 172 % (Table 2). The obtained results confirmed that the containing MgO as a separated phase cement materials III had cytocompatibility, *i.e.* non toxicity, and strongly pronounced matrix characteristics of the surface.

Table 2 Optical density [OD (arbitrary units), MTT test] dynamics of formazan solutions and pool of viable cells [PVC (%) compared to control] for the cultivation of human osteosarcoma MG-63 cells on the cement powders, set cements, and polystyrene (control).

Sample		Day of incubation			The cell population increase, 7th day *vs.* 1st day (%)
		1st	3rd	7th	
Control (polystyrene)	OD	0.323±0.001	0.574±0.006	0.879±0.020	172.1
	PVC	100	100	100	
Cement powder (40 mol% of Mg)	OD	0.207±0.003[a]	0.420±0.169	0.885±0.064[a]	327.5
	PVC	64.1	73.2	79.7	
Cement III	OD	0.246±0.003[a]	0.697±0.016[a]	1.182±0.054[a]	380.4
	PVC	76.2	121.4	131.5	

[a] $p < 0.05$ in comparison with the control (Student's *t*-test).

It can be also concluded, that the introduction of Mg in the cement powders caused the decrease of pH for the powders and cements. The 40 mol% Mg-substitution in the cement powders led to the formation of mixed substituted β-TCP and MgO phases. The treatment of this cement powder with cement liquid produced set cement having the dense homogeneous microstructure, the strength up to 40 MPa (49 MPa in the case of dispersion hardening), and setting time of 5.0±0.5 min. According to the *in vitro* tests, the containing MgO as a separated phase cement III demonstrated the cytocompatibility and, therefore, can be considered as a promising cement for *in vivo* investigations of biocompatibility, osteoconductivity, and for further biomedical applications in the bone surgery.

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[††] Sterile samples (the dry heat sterilization at 180 °C for 1.5 h) of cement powders and set cements were placed into 96 well plates for cultivation (Corning Costar, USA) into triplets with one plate per each incubation period and covered with complete growth medium (CGM) containing DMEM medium (PanEko, Russia), 10% fetal bovine serum (PAA, Austria), glutamine (0.65 mg ml^{-1}, PanEko, Russia), and gentamycin (50 μg ml^{-1}, PanEko, Russia). Once the neutral pH values were established, the plates with test samples and without them (control with a cultural plastic, polystyrene) were introduced into a cell suspension (the MG-63 culture with density of 15000 cells per well) at 200 μl of the CGM. The cells were incubated for 1, 3, and 7 days with regular replacements of the CGM. All the procedures were performed under sterile conditions in the humid air atmosphere containing 5% CO_2 at 37 °C. The MG-63 cell lines viability over time was assessed using an MTT test, which is based on the ability of dehydrogenase in the living cells to reduce 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, USA) into formazan. The amount of formed formazan is spectrophotometrically estimated and describes proliferative activity (viability and amount) of different human and animal cells.[11] The pool of viable cells (PVC) was evaluated at different experimental steps as a ratio between the optical densities of formazan solution (MTT reaction product) and control sample on a certain day of the experiment. A specimen was assumed to be cytocompatible in the case of cytotoxicity absence (PVC ⩾ 70%) on a certain day of cultivation. For cytocompatible samples, the increase in the cell population (with respect to the first day of cultivation) was also assessed.

The acquired data were processed by conventional methods of variationally statistics using Microsoft Excel 2000. The significance of differences was assessed using a parametric Student's *t*-test; differences were considered statistically significant at $p < 0.05$.