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INFLUENCE OF SODIUM ALGINATE ON PROPERTIES OF TETRACALCIUM PHOSPHATE/ NANOMONETITE BIOCEMENT

R. Štulajterová, L. Medvecký, M. Giretová, T. Sopčák, J. Briančin

Abstract

The tetracalcium phosphate/nanomonetite (TTCPMH) biocements with the addition of sodium alginate were prepared by mechanical homogenization of powder mixture with hardening liquid containing sodium alginate. The effect of various viscosity of different alginates on properties of TTCPMH cement mixture was investigated. The medium viscous (MED) alginate had a more negative effect on setting process and compressive strength than low viscous (LOW) alginate. An approx. 50% decrease in mechanical properties (compressive strengths, Young´s modulus, work of fracture (WOF)) was revealed after an addition of 0.25 wt % with rapid fall above 1 wt % of LOW alginate in biocement. A statistically significant difference in the WOF was found between of 0.25 and 0.5 LOW alginate biocements ($p<0.035$) whereas no statistical differences were revealed between WOF of 0.5 and 1 LOW alginate biocements ($p>0.357$). In the microstructure of composite cements, the increased amounts of granular or finer needle-like nanohydroxyapatite particles arranged into the form of more separated spherical agglomerates were observed. A low cytotoxicity of cement extracts based on measurement of cell proliferation was revealed.

Keywords: polymeric additive (sodium alginate), tetracalcium phosphate/nanomonetite biocement, mechanical properties, composites, bioactivity

INTRODUCTION

Hydrogels are three-dimensional, hydrophilic, polymeric networks capable of absorbing large amounts of water or biological fluids. They can be chemically stable or degrade with disintegration and dissolution. The calcium phosphate cements have been widely investigated in view of their favorable handling properties. Many calcium phosphate cements (CPC) pastes tend to disintegrate upon early contact with blood or other aqueous body fluids, which limit the use of these materials in clinical practice such as bone repair, reconstruction and regeneration. For prevention of insufficient cohesion, the cohesion promotors such as sodium alginate are added [1]. The free cement particles may leak into the tissues surrounding, causing side effects - nerve pain, venous and pulmonary embolism [2].

Radoslava Štulajterová, Ľubomír Medvecký, Mária Giretová, Tibor Sopčák: Institute of Materials Research of SAS, Watsonova 47, 040 01 Kosice, Slovak Republic
Jaroslav Briančín: Institute of Geotechnics of SAS, Watsonova 45, 040 01 Kosice, Slovak Republic

The sodium alginate belongs to the group of water-soluble polymers which are non-toxic and widely used in various pharmaceutical and biomedical applications and therefore do not require removal from the system [3]. The alginate scaffolds lack in mechanical strength and fast hydrolyse which diminish applicability in bone tissue engineering. The mechanical properties of alginate scaffolds have been improved by mixing with calcium phosphates like hydroxyapatite (HA). Besides, the addition of HA to the alginate polymer enhanced the cell adhesion and proliferation on the scaffolds with an average pore size of 150 μm and over 82% of porosity [4]. Alginates are ion-sensitive natural hydrogels and they gel through chelation with divalent cations [5]. A crosslinking reaction occurs between sodium alginate and calcium released by CPC during setting, resulting in the formation of water-insoluble calcium alginate gel [6]. It has been shown that the cohesion and cement toughness of CPC pastes (based on α-TCP and liquid phase NaH2PO4.2H2O) was improved by the addition of polymeric additives (i.e. carboxymethylcellulose (CMC) and hyaluronan (HA)) [7]. The carboxyl group of this polymer provides the possibility to form electrostatic interactions with calcium ions in the CPC matrix [8]. The biopolymers primarily include degradable components such as chitosan, gelatin, collagen, and synthetic polymer phases [9-15]. CPC composites with various biopolymers (gelatin, collagen, chitosan, alginate) were studied for modification of their properties. Incorporation of sodium alginate, as a liquid phase, to calcium phosphate cement (TTCP/DCPA) caused the increase of setting time [6]. Similar results were found in the case of alginate/CPC composites composed of MCPM-CaCO3 [16] or ACP-DCPD [17]. It was reported the reduction in mechanical properties when the amount of sodium alginate in TTCP/DCPA cement exceeded 10 wt % or alginate was added to cement in the form of microbeads and solid particles [18, 19]. The alginate microbeads helped the formation of macrochannels in the CPC and biodegradation of material [20]. Other authors [18] reported enhancement of ACP-DCPD compressive strength with the addition of 0.2 and 0.4 wt % sodium alginate ($p < 0.05$). With the further addition of sodium alginate, the compressive strength of the cement declined. The mechanism may involve the inhibition of diffusion of calcium and phosphate ions provided from the dissolution of ACP and DCPD.

The compressive and tensile strengths of the alginate/CPC based on α-TCP were greatly improved after soaking in medium and the formation of apatite crystalline phase was induced when immersed in simulated body fluid [21].

The aim of this study was to investigate the effect of the addition of two different types of sodium alginate (differing in viscosity) on properties of tetracalcium phosphate/nanomonetite (TTCPMH) cement mixture. The setting characteristics, phase composition, microstructure, compressive strength and in vitro cytotoxicity of composite extracts were evaluated.

MATERIALS AND METHODS

Preparation of cement mixtures with the addition of alginate

Tetracalcium phosphate ($Ca_4(PO_4)_2O$, TTCP) was prepared by annealing an equimolar mixture of calcium carbonate ($CaCO_3$, analytical grade, Sigma-Aldrich) and dicalcium phosphate anhydrous (DCPA) ($CaHPO_4$ (Ph.Eur.), Fluka) at 1450°C for 5h. The product was milled in a planetary ball mill (Fritch, 730 rpm) for 2 h and the phase purity was determined using the X-ray powder diffraction analysis (XRD, Philips X Pert Pro, CuKα). The TTCPMH was synthesized by in situ reaction between TTCP and diluted solution (1:4) of orthophosphoric acid (86%, analytical grade, Merck) in ethanol (96%). The H_3PO_4 was added at such amounts that the final Ca/P mole ratio in powder mixtures

equal to 1.67, which corresponds to the Ca/P mole ratio in stoichiometric hydroxyapatite. The sodium alginate/TTCP/nanomonetite composite system was prepared by mixing TTCPMH powder mixture with hardening liquid containing 2% NaH_2PO_4 (analytical grade, Sigma-Aldrich) and sodium alginate. The final content of sodium alginate in composites was 0.25; 0.50; 1.0; 2.0 wt % and 0.25, 0.5 wt % for low (LOW) (Alginic acid sodium salt from brown algae, BioReagent, plant cell culture tested, low viscosity, Sigma-Aldrich) and medium (MED) (Alginic acid sodium salt from brown algae, medium viscosity, Sigma-Aldrich) viscous sodium alginate respectively. The P/L ratio equals 2 for all biocements.

Mechanical testing and characterization methods

The cement paste was molded into pellets (6 mm D x 12 mm H) in stainless cylindrical molds and unaxially pressed at 6 MPa. After setting in 100% humidity at 37°C for 10 min, samples were hardened in 0.9% NaCl solution at 37°C for up to 7 days and, dried at 80°C for 2 hours. The compressive strength (mean of 5 samples) of samples was measured on a universal testing machine (LR5K Plus, Lloyd Instruments Ltd.) at crosshead speeds of 1 mm/min. The statistical evaluation of results (n = 5) was performed using ANOVA analysis at level $\alpha = 0.05$.

The phase compositions of 1 and 7 days hardened samples in 0.9% NaCl solution were analyzed by X-ray diffraction analysis (Philips X´ PertPro, using CuKα radiation) and Fourier transform infrared spectroscopy (FTIR) (Shimadzu, IRAffinity1, 400 mg KBr + 1 mg sample). The microstructures of fractured surfaces of samples were observed by field emission scanning electron microscopy (MIRA3 TESCAN). Relative densities of the samples were calculated from their masses and dimensions. The true density of powder composites was measured by the pycnometer according to EN ISO 3838. The setting times of cement pastes were evaluated according to ISO standard 1566 (Vicat method).

The concentrations of released calcium and phosphate ions after soaking (3 pellets/ 45 ml) of composites in 0.9% NaCl solution at 37°C for 7 days in a closed 50 ml polypropylene tube under dynamic conditions (MINIRotator, 6 rpm) were determined using ICP.

Swelling of alginates was determined from mass differences between origin dry alginate (drying at 100°C) and gel mass after soaking in 0.01 M $CaCl_2$. $2H_2O$ with P/L ratio equal to 1g alginate/200 mL solution. The 150 mg of alginate was uniaxially pressed (50 MPa) into pellet forms (6 mm in diameter) and immersed into Ca^{2+} solution at 25°C for 24 hours. The masses of gels were measured after filtration. Note that solution with Ca^{2+} ions was selected to mimic conditions during cement pastes preparation and setting process.

The amount of carboxylic groups in individual alginate type was evaluated after 24 hours of soaking powders in 0.01M solution of Ca^{2+} ions ($CaCl_2$ solution) at 25°C (pH was adjusted with NaOH solution to 7.4). The content of carboxylic groups in alginates was calculated from the amount of bonded Ca^{2+} ions into precipitates of calcium alginate. Precipitates were filtered and the concentration of calcium ions in supernatants was determined by ICP (Horiba Scientific/Activa optical emission spectrometer).

Cell cultivation and viability testing

Alginate/cement pastes were prepared by mixing powder calcium phosphate mixture with 2% NaH_2PO_4 alginate solution, immediatelly transfered to the form of cylinder shape and composite discs (Ø 7 mm, 1 mm height) were formed. After hardening in 100% humidity at 37°C for 20 minutes, the discs were sterilized in an autoclave at 121°C.

Samples were immersed separatelly into a sterile 50 ml polypropylene tube with complete culture medium composed from EMEM (Eagle´s Minimum Essential medium, Sigma), 10% FBS (fetal bovine serum, Biowest, France) and 1% ATB-ATM (antibiotic-antimycotic solution, Sigma) in a extraction ratio of 3 cm2/ml (according to EN ISO 10993-12:2012). The sample extracts were collected after 24 hours soaking at 37 ºC (incubator, Memmert), centrifuged (5000 RPM/5 min, centrifuge Hettich) to remove possible floating particles and the supernatant was used for cytotoxicity testing.

The cells of mouse preosteoblastic cell line MC3T3E1 (Sigma) were enzymatically released (0.25 % trypsin-EDTA; Sigma) after reaching subconfluence in culture flasks (SPLLife Sciences, Korea), counted (Neubauer hemacytometer) and the cell concentration was adjusted to 1.0x105 cells/ml. Into each well of a 96 well culture plate (Sarstedt, Germany), the 1.0x104 cells in 100 µl of culture medium carrefully were added and incubated at 37°C, 5% CO_2 and 95 % humidity for 24 hours. After 24 hours, the medium from the wells was discarded and exchanged for extracts. The cells in wells were cultivated for 24 hours. As a negative, controls were considered wells with cells cultivated in complete culture medium. After culture, the extracts were exchanged for fresh culture medium with Cell titer aqueus one solution cell proliferation assay (Promega, USA) and the formazan absorbance (produced by viable cells), were measured at 490 nm (Shimadzu UV1800).

RESULTS

XRD and FTIR analysis of cements and composites

The XRD analysis of TTCPMH and alginate composites verified the formation of calcium deficient hydroxyapatite CDHAP (JCPDS 24-0033) after 1 and 7 days of soaking in 0.9% NaCl solution (Fig.1). No differences between the XRD patterns of composites after 1 and 7 days of soaking were found.

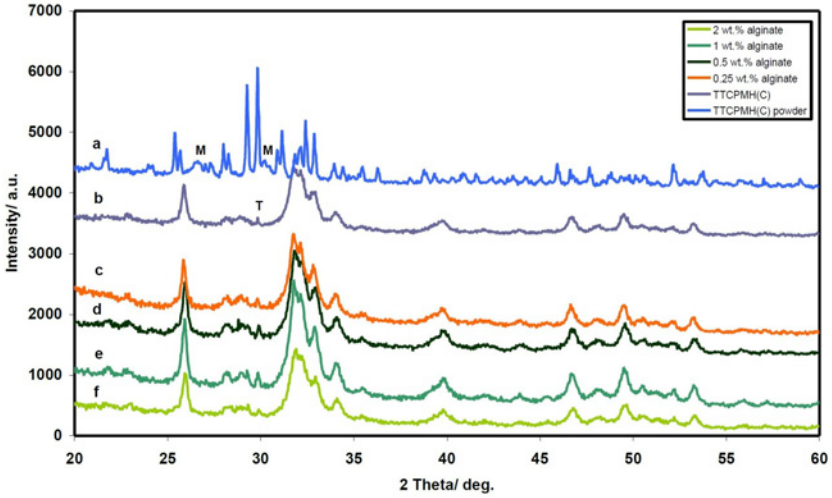


Fig.1. XRD patterns of cements and composites after hardening during 7 days in 0.9% NaCl: a) original TTCPMH powder, b) TTCPMH, c) 0.25 LOW, d) 0.50 LOW, e) 1 LOW, f) 2 LOW.

The FTIR spectra of composites with 0.5 and 2 wt % addition of alginic acid sodium salt (0.5LOW, 0.5MED and 2LOW) after 1 and 7 days hardening in 0.9% NaCl solution are shown in Fig. 2. Characteristic vibrations of the PO_4^{3-} groups in hydroxyapatite arise from antisymmetric (ν_3) and symmetric (ν_1) P-O stretching vibrations located at 1090, 1032 and 962 cm^{-1}, O-P-O bending (ν_4) vibrations at 565 and 602 cm^{-1}, and the librational mode of OH hydroxyapatite group at about 634 cm^{-1} were identified in spectra [22, 23]. Low intense peaks corresponding to ν_3 and ν_2 vibrations of CO_3^{2-} groups at 1479, 1425 and 874 cm^{-1} represent the AB-type of carbonated hydroxyapatite with simultaneously CO_3^{2-} substitution for PO_4^{3-} and OH^- groups [24]. The peak from stretching vibrations of the OH hydroxyapatite group was observed at 3560 cm^{-1}. Note that no additonal bands from vibrations of alginate were revealed in spectra probably due to the low intensity of vibrations of polysaccharide groups and lower alginate content in composites.

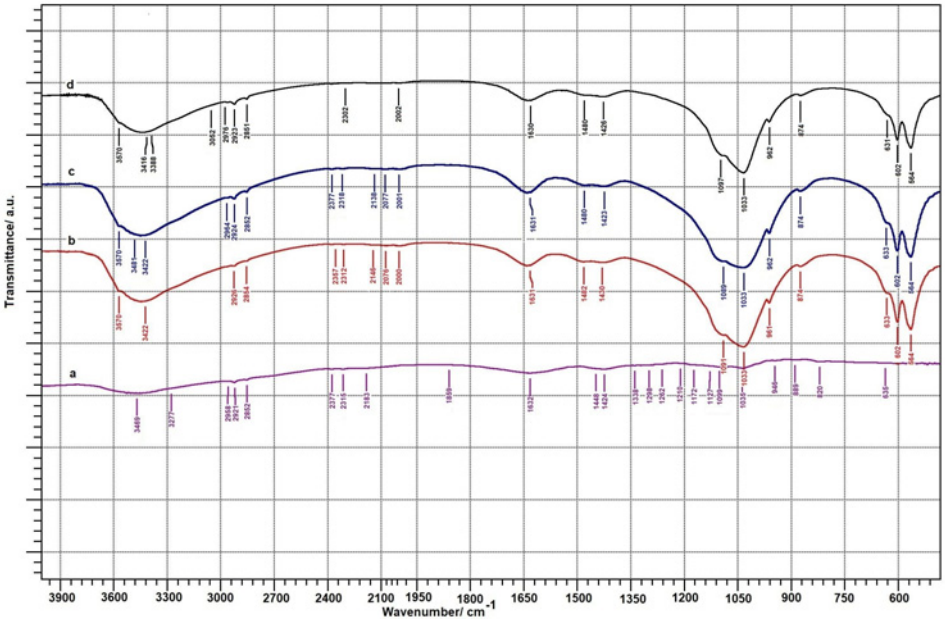


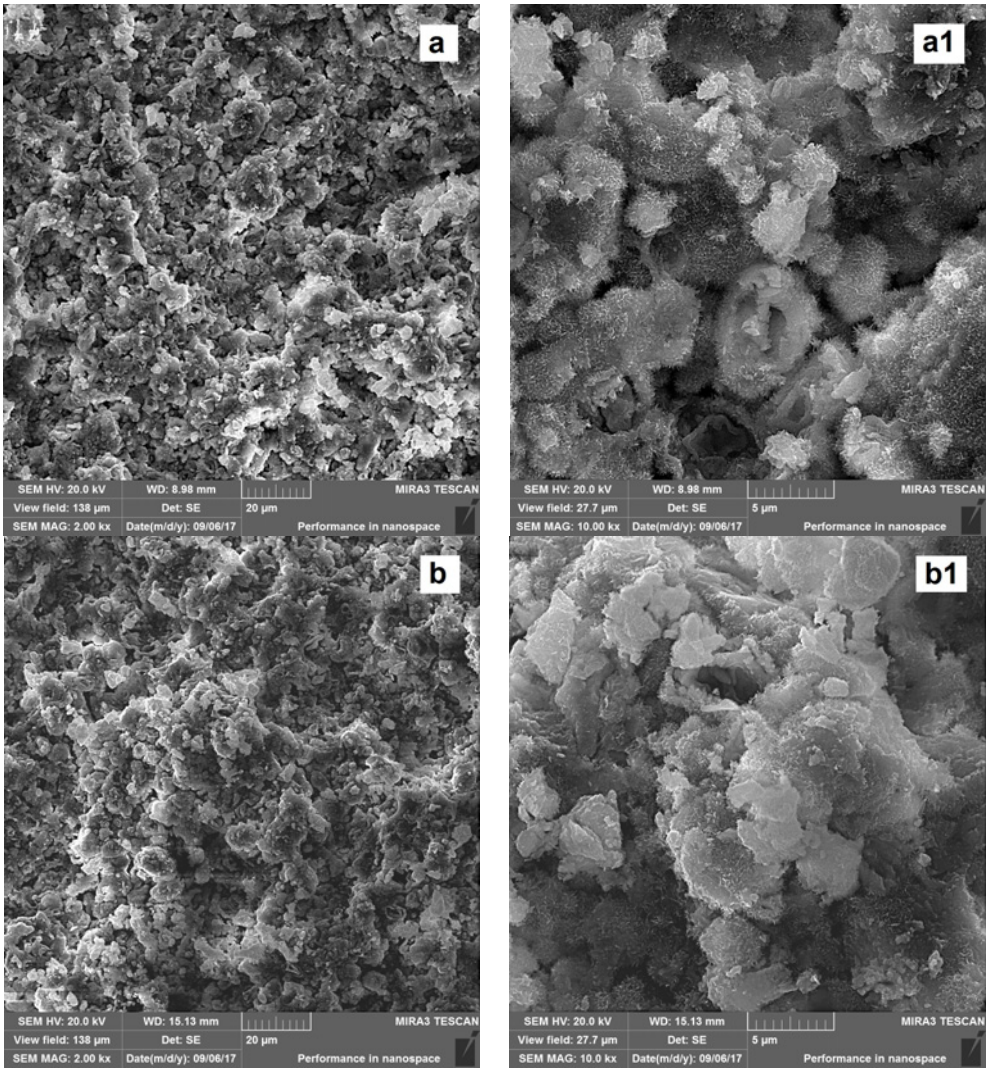
Fig.2. FTIR spectra of composites with 0.5 wt % and 2 wt % addition of sodium alginate after 1 day hardening in 0.9% NaCl: a) prepared calcium alginate, b) 0.5 LOW, c) 0.5 MED, d) 2 LOW.

Microstructure of hardened composites

The microstructure of fractured cements with 0.5 wt % and 2 wt % of sodium alginate salt after 1 day of hardening in 0.9% NaCl is shown in Fig. 3.

The coarse agglomerates of fine thin plate-like particles (size up to 500 nm) and cement matrix composed of globular particles were visible in the microstructure of pure cement. A high density of irregularly shaped micropores with size up to 2 μm and a small volume fraction of larger around 10 μm pores were observed in the microstructure. Fine thin needle-like nanohydroxyapatite particles with length up to 1 μm are arranged into the form of 5–10 μm agglomerates.

From a macroscopic point of view, the microstructures of all composite cements are very similar. A high number of larger irregularly shaped 2-10 μm micropores can be visible in microstructures. In LOW composites containing less than 2 wt % alginate the spherical particle agglomerates composed of fine needle-like hydroxyapatite particles with length does not exceed 100 nm. Note that individual agglomerates were not mutually tightly interconnected across boundaries via nanohydroxyapatite particles and agglomerates were separated from each other. In the case of cements with higher alginate content (LOW) and 0.5MED cements, a significantly lower amount of needle-like particles was created during the cement transformation and the granular fine nanoparticles connected to form irregularly shaped agglomerates of 5-10 μm size can be visible in microstructures.



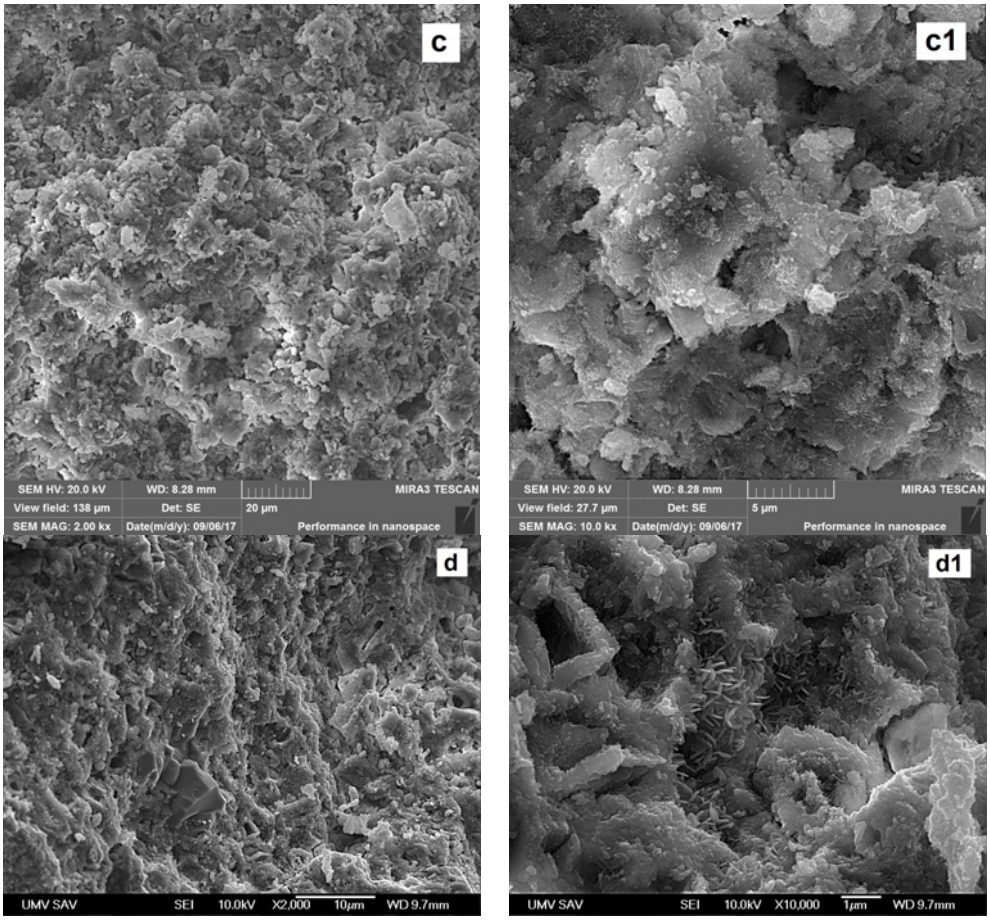


Fig.3. Microstructure of fractured cements with 0.5 wt % and 2 wt % of sodium alginate salt after 1 day of hardening in 0,9% NaCl: (a, a1) 0.5 LOW, (b, b1) 0.5 MED, (c, c1) 2 LOW, (d, d1) TTCPMH.

Cytotoxicity of extracts and evaluation of physico-chemical characteristics of alginates and powder composites

From cytotoxicity testing it resulted that the relative proliferations of osteoblasts cultured in sample extracts were not statistically different ($p>0.65$) and had a low cytotoxicity potential (no statistical difference from 70 % level for cell viability represents limits of the potential cytotoxicity of the sample (STN ISO 10993-5). The concentrations of calcium and phosphate ions released from 0.5 wt % alginate cement powder mixtures to 0.9% NaCl solution at 37°C and dynamic experimental conditions were evaluated after 2 and 24 hours of soaking (Table 1).

Relative standard deviations for determination of Ca^{2+} and PO_4^{3-} concentrations were 5% and 7% respectively.

Tab.1. Concentrations of Ca^{2+}, PO_4^{3-} ions after soaking of composites samples in 0,9% NaCl solution at 37°C for 2 and 24 hours.

Sample	Ca^{2+}/mM		PO_4^{3-}/mM	
	2h	24h	2h	24h
C + 0.5 wt % Alg. low	1.40	0.42	5.21	<0.05
C + 0.5 wt % Alg. med	1.43	0.76	0.49	<0.05

Results showed the rise in concentrations of both ions in solution after 2 h soaking with the following about 50% decrease in Ca^{2+} whereas the concentration of phosphate ions was very low irregardless of alginate type after 24 h soaking. An almost ten times higher concentration of phosphate ions was found in LOW than MED cement suspensions after 2 h soaking. The significant differences were revealed in swelling alginate cements where an about 56-fold rise in mass of MED alginate was found contrary to the 35-fold rise of LOW alginate after swelling. Similarly the MED and LOW alginates contained various amounts of carboxylates per gram, thus, 0.494±10 and 0.362±9 mmol carboxylates/1 g alginate respectively.

Compressive strength and setting time of composites

Results of the analysis of stress/strain curves are shown in Fig.4. The comparison of curves verifies the same character of dependence with a strong reduction of both parameters (around 70% and 30 % decrease in the work of fracture (WOF) and Young´s modulus respectively) after 0.25 wt % addition of low alginate. Statistically significant difference in the work of fracture was found between of 0.25 and 0.5 lowALG biocements ($p<0.035$) whereas no statistical differences were revealed between WOF of 0.5 and 1 lowALG biocements ($p>0.357$).

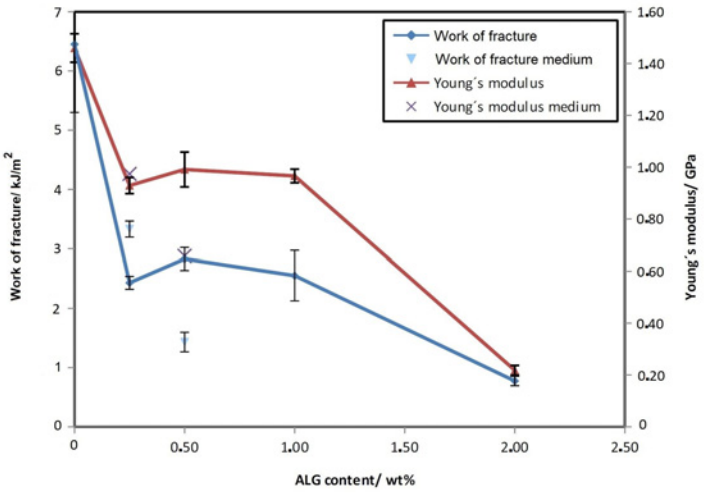


Fig.4. Dependence work of fracture and Young´s modulus on alginate content (wt %).

In the case of Young´s modulus, no statistical differences were identified between 0.25 and 0.5 ($p>0.221$) as well as 0.5 and 1 ($p>0.581$) lowALG biocements. Both the Young´s modulus and WOF of medALG samples significantly decreased after the addition of 0.25 and 0.5 alginate ($p>0.6$). Note that the rise in alginate content to 2 wt% caused rapid

decrease in WOF and Young´s modulus of LOW biocement. Very similar dependences of CS on content or alginate viscosities can be observed in Fig.5 which clearly demonstrate about a 50 % reduction of CS in 0.25 or 0.5 and 1 LOW biocements with a further approx. 50% reduction in 2 LOW cement.

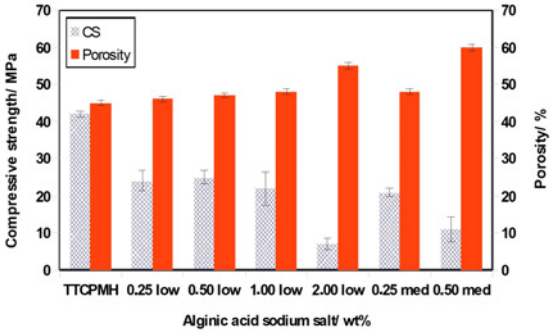


Fig.5. Effect of compressive strength and porosity of cements on addition of sodium alginate.

After 7 days of hardening, the porosities of LOW composites (up to 1 wt % of alginate) and 0.25 MED composite were around 47±1% of true density, which was almost the same as the porosity of pure cement (46±2%), and decreased in 2 LOW and 0.5 MED composites to 55±2 and 60±2% respectively.

The setting time of 0.5MED composite cements was 14 min contrary to 5 min of 0.5LOW and TTCPMH cements. Note that setting times of all LOW cement mixtures were close to 5 min.

DISCUSSION

Two types of alginates with different viscosity of gels were studied as additives in calcium phosphate biocement. The small addition of MED alginate (higher viscosity) caused strong changes (reduction) in mechanical properties, setting time and suppress workability of cement paste. On the other hand, a similar about 50% decrease in mechanical properties (CS, Young´s modulus, WOF) was revealed after an addition of 0.25 wt % of LOW alginate with their rapid fall above 1 wt % content of LOW alginate in biocement. It is known that dependence of mechanical properties on density of cements and the fast reduction of composite cement strength at higher contents of alginates could be related to an increase in cement porosity. Apart from this, enhanced amounts of granular or finer needle-like nanohydroxyapatite particles arranged to form of more separated spherical agglomerates were observed in microstructures of composite cement contrary to the large fraction of agglomerates composed of longer needle-like particles in the more compact microstructure of TTCPMH cement. The mentioned effects clearly show strengthening in its microstructure across boundary of agglomerates via nanohydroxyapatite particles whereas weak boundary strengthening can be only assumed in the case of composites. The large stresses are induced in microstructure after drying of cements due to contraction of swelled alginates which significantly helps to debond and aid separation of particle agglomerates in cement composites. Note that a similar decrease in mechanical properties was also measured in wet composite cements but the presence of gels in microstructures clearly enhanced creep with a reduction of friction between individual agglomerates.

About a 30% higher content of carboxylates in MED than LOW alginates may seem in contradiction with the above results because a higher amount of carboxylic groups indeed bound more calcium ions released during hydrolysis and transformation of cement components. It follows the possible strengthening structure via alginate chains, but the fast rise of setting time with MED alginate content in cements as well as the higher concentration of phosphate ions in the initial stage of setting verified inhibition of the cement transformation process. Also demonstrated was the nucleation of an enhanced number of hydroxyapatite nuclei with following refinement of final nanohydroxyapatite particles in microstructure due to the presence of a larger amount of calcium ions trapped on alginate chains. Despite the initial inhibition of setting process in composites, the full transformation of starting calcium phosphate phases was demonstrated after 24 hours hardening. Note that the LOW alginate amount (up to 2 wt %) only slightly affected by setting time of cement composites. It may be speculated that LOW alginate was more susceptable to degradation during soaking in NaCl solution, but analysis of the carbon content in cements revealed no changes in the carbon contents between freshly prepared composites and samples 7 days hardened.

It was shown that the addition of alginate to CPC or ACP-DCPD resulted in an increase of setting time [16]. The organic compounds with the charged functional groups (OH^-, NH_2^-, COO^-) had a great effect on the hydration reaction of calcium phosphates. Generally, the final setting times of cements with organic compounds were all increased but sodium alginate had the largest influence [17]. The other authors [6] reported that sodium alginate inhibits the CPC conversion to HAP during setting reaction. A strong decrease in the flexural strength from 8.4±1.8 MPa to 2.3±0.4 MPa was found in TTCP/DCPA cement and composite containing 50% microbeads respectively [19]. The mechanical strength (diametral tensile strength) of the cement (TTCP/DCPA) increased rapidly with the addition of sodium alginate up to 0.8 wt % (around 6 MPa) and soaking in distilled water at 37°C. The further addition of sodium alginate (SA) caused a decrease the mechanical strength and inhibition of the setting reaction. Setting time of our TTCPMH cements with LOW alginate was close to this one in non decayed fast setting TTCP/DCPA cement mixture which contained submicron particles of DCPA [25]. However, the content of origin TTCP phase was lower than in the above mixture, which verifies the faster transformation process of more active nanomonetite particles. In the case of α-TCP based cement, the initial hardened cement had a compressive strength of ~10 MPa and the value increased significantly up to ~60-70 MPa after 3-7 days of incubation in SBF [21]. It is clear from the above facts that generally an addition of alginate to composites mainly reduced mechanical properties of cements, but probably this change depends on the type of calcium phosphate cements. Note that the effect of different alginate viscosity on properties of alginate/CPC composites were not studied in literature, and it is hard to compare our results with other papers.

CONCLUSIONS

The small addition of two alginate types reduced compressive strength of TTCPMH cement. A gearter inhibition effect of MED alginate (more viscous, higher amount of carboxylic groups and stronger swelling) on setting process and the decrease in CS of composites was found, which demonstrates favorable properties of LOW alginate for utilization as the additive in TTCPMH cement. On the other hand, the prolongation of setting time at low content of MED alginate may be considered as a positive property in application of composite as the injectable cement paste, but the higher viscosity of composite paste significantly impairs its workability.

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