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Influence of several biodegradable components added to pure and nanosilver-doped PMMA bone cements on its biological and mechanical properties

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Abstract

Acrylic bone cements (BC) are widely used in medicine. Despite favorable mechanical properties, processability and inject capability, BC lack bioactivity. To overcome this, we investigated the effects of selected biodegradable additives to create a partially-degradable BC and also we evaluated its combination with nanosilver (AgNp). We hypothesized that using above strategies it would be possible to obtain bioactive BC.

The Cemex was used as the base material, modified at 2.5, 5 or 10 wt.% with either cellulose, chitosan, magnesium, polydioxanone or tricalcium-phosphate. The resulted modified BC were examined for surface morphology, wettability, porosity, mechanical and nanomechanical properties and cytocompatibility. The composite BC doped with AgNp were also examined for its release and antibacterial properties. The results showed that it is possible to create modified cement and all studied modifiers increased its porosity. Applying the additives slightly decreased BC wettability and mechanical properties, but the positive effect of the additives was observed in nanomechanical research. The relatively poor cytocompatibility of modified BC was attributed to the unreacted monomer release, except for polydioxanone modification which increased cells viability. Furthermore, all additives facilitated AgNp release and increased BC antibacterial effectiveness.

Our present studies suggest the optimal content of biodegradable component for BC is 5 wt.%. At this content, an improvement in BC porosity is achieved without significant deterioration of BC physical and mechanical properties. Polydioxanone and cellulose seem to be the most promising additives that improve porosity and antibacterial properties of antibiotic or nanosilver-loaded BC. Partially-degradable BC may be a good strategy to improve their antibacterial effectiveness, but some caution is still required regarding their cytocompatibility.

Keywords: composite bone cement, biodegradable additives, cytocompatibility, mechanical properties, antibacterial activity.

Abbreviations: AgNp Silver nanoparticles; ANOVA Analysis of variance; BC Bone cement; BC-A Antibiotic-loaded bone cement; BC-AgNp Nanosilver-loaded bone cement; BC-M Modified bone cement; CaP Calcium phosphate; Cell Cellulose; Chit Chitosan; DAPI 40,6-Diamidino-2-phenylindole; EDX Energy dispersive X-ray spectroscopy; GO Graphene oxide; Mg Magnesium; MMA Methyl methacrylate; PBS Phosphate-buffered saline; PDO Polydioxanone; PMMA Poly (methyl methacrylate); SEM Scanning electron microscopy; TCP Tri-calcium phosphate; TTC Tetrazolium chloride; TF Triphenylformazan

1. Introduction

Despite the body's natural ability to self-healing, some bone defects cannot be spontaneously repaired and biomaterials must be used to support tissues regeneration. A variety of synthetic bone substitutes have been developed and used for bone healing so far, including: calcium sulfate, calcium phosphate ceramics, bioactive glasses, poly(methylmethacrylate) cements, polylactic acid and poly(caprolactone) [1,2]. Acrylic bone cement (BC) based on polymethyl methacrylate (PMMA) is a biomaterial mainly used for implants' fixation, fractures' stabilization, antibiotic delivery, bone defect filling, coating of metallic implants, screw augmentation and treatment of bone metastases [3-8]. Wide applications of BC originate from its processability, fast polymerization, favorable mechanical properties, injectable capability and biostability in the human body [9-11]. Moreover, antibiotic-loaded BC (BC-A) is among the most suitable and widely studied local antibiotic delivery systems [12,13]. On the other hand, BC is bioinert, adheres insufficiently



to bone surfaces and it has relatively short fatigue life [14,15]. This may result in limited integration of BC with host bone tissue and in the formation of a fibrous connective membrane capsule on the cement-bone interface [16,17]. Additionally, incorrect chemical composition, especially liquid to powder ratio of BC components, and improper preparation may cause toxic effects due to the release of unreacted methyl methacrylate (MMA) monomer or certain additives [17-19]. Furthermore, BC displays porosity of up to 5-10% [20]. Despite some shortcomings, BC remains a gold standard in medicine, whereas practically none of the non-acrylic cements have been introduced into the medical market [21]. Numerous studies aimed at the improvement of BC biological or mechanical properties, including different modifiers such as carbon, stainless steel, titanium, graphene and zirconia for reinforcement [22,23]; calcium phosphate, chitosan, silica, magnesium, CaCl₂ and carbonate salts as bioactive fillers or partially degradable formulations [21,24,25]; BaSO₄, bismuth salicylate and iodine as radiopaque agents [26,27] as well as various types of antibiotics [28-31] and other bactericidal agents for antibacterial properties [32-35]. The future of BC research relies in multifunctional systems, that interact with body tissue and protect against infections [21,36]. In this study, we aimed to determine and compare the properties of the BC modified with different components for developing partially-degradable, bioactive and antibacterial biomaterial. We tested five commercially available components as potential biodegradable BC additives: cellulose, chitosan, magnesium, polydioxanone and tri-calcium phosphate. The additives were chosen based on their

properties and applications in medicine: cellulose is a natural biodegradable polymer that can be obtained at a low cost in different forms (i.e. particles, fibers, crystals, whiskers) from various sources; chitosan is natural amino-polysaccharide characterized by biodegradability, biocompatibility and osteoconductivity, and it is obtained by deacetylating the chitin; magnesium is the natural element in the human body and currently its alloy are used in orthopedic medicine because of their biodegradability and potential to support osseointegration and angiogenesis; polydioxanone is synthetic biodegradable poly(ester-ether), which is used for production of sutures or stents; and tricalcium phosphate is a resorbable and biocompatible ceramic material with characteristics similar to that of natural bone, favorable resorption pattern and osteogenic properties [37-42]. We also doping these selected biodegradable component with nanosilver. The idea of adding these additives to nanosilver-loaded BC is to form new pores and interconnecting channels, which should increase the AgNP release and improve the antibacterial effectiveness. The relations between the chemical compositions of BC and their structure, physical, mechanical and biological properties were characterized. The expected outcome was a new formula of modified BC with advantageous properties with respect to classical acrylic cements.

2. Materials and methods

2.1 Cement preparation

PMMA bone cement Cemex (Tecres Company, Italy) was used as the base material,

modified with one of five biodegradable components: cellulose – Cell (high purity powder 20 μm ; Merck KGaA, Germany), chitosan - Chit (powder, deacetylation 75-85%, medium molecular weight, viscosity 200-800 cps; Merck KGaA, Germany), magnesium – Mg (powder <250 μm , purity >99%; Merck KGaA, Germany), polydioxanone – PDO (powder; Merck KGaA, Germany) and tri-calcium phosphate – TCP (powder 4 μm , surface area > 80 m^2/g ; Merck KGaA, Germany). Three contents of modifiers were selected for the tests: 2.5, 5 and 10 wt.% based on preliminary research and literature review [45]. Usually, additives up to 20-30 wt.% are used to modify acrylic-based cements to improve porosity or increase physical and mechanical properties. However, in our preliminary studies revealed that concentrations above 10% result in deterioration of cement properties, such as: setting time, wettability and plasticity of cements. All BC specimens with/without modifications were prepared as described earlier [43,44]. The base BC material consisted of polymethyl methacrylate, barium sulfate, benzoyl peroxide (powder component) and methyl methacrylate, N-N-dimethyl-p-toluidine and hydroquinone (liquid component); its liquid-to-powder ratio was 0.33 [43]. The unmodified specimen was designated as BC and modified one as BC-M-x%, where M stands for the additives (BC-Cell, BC-Chit, BC-Mg, BC-PDO, BC-TCP) and x for its contents (2.5, 5 and 10%). For some experiments the BC-M were also doped with nanosilver (BC-AgNp; 50 nm average particle size, 99.9%; Mknano, Canada) or antibiotic - gentamicin sulfate (BC-A; Sigma Aldrich, Germany). The 1.5 wt.% content of AgNp and gentamicin was selected based on our previous studies [43,44]. The

applied modifiers and bone cement components are presented in Table 1.

Tab. 1. Chemical composition of bone cements and additives' specifications.

BC	BC-M:				
	BC-Cell	BC-Chit	BC-Mg	BC-PDO	BC-TCP
Powder composition (Tecres Company) (wt/wt)					
polymethyl methacrylate – 84.30 %					
barium sulfate – 13.00 %					
benzoyl peroxide – 2.70 %					
Liquid composition (Tecres Company)					
methyl methacrylate – 99.1 %w/w					
N,N-dimethyl-p-toluidine – 0.9 %w/w					
hydroquinone – 75 ppm					
Biodegradable additives specification (Merck kGaA)					
		chitosan			
	cellulose	particle size: 100-500 µm, petal shape, 75-85% deacetylation, medium molecular weight, viscosity 200-800 cps	magnesium		tricalcium phosphate
-----	particle size: 20 µm, cylindrical shape		particle size: 30-100 µm, cylindrical shape, >99% purity	polydioxanone	particle size: 2-10 µm, spherical shape, > 80 m ² /g surface area
				particle size: 50-150 µm, petal shape	
Antibacterial additives specification					
BC-M-AgNp (MkNano)					
nanosilver powder, particle size: ~50 nm, spherical shape, 99.9% purity					
BC-M-A (Sigma Aldrich)					
gentamicin sulfate powder, gentamicin C1A – 19%, gentamicin C2B+C1 – 33%, gentamicin C2+C2A – 49%					

2.2 Microscopic examinations

The microstructure of the BC was examined using a high-resolution scanning electron microscope SEM (JSM-7800F, Jeol, Japan). The chemical composition was

determined with the X-ray energy dispersive spectrometer EDS (Edax Inc., USA). The surface wettability was determined by water contact angle measurements with an optical tensiometer (Attention Theta Life, Biolin Scientific, Finland) based on the falling drop method. The volume of the drop was about 1 μL ; each measurement was carried out five times, immediately after the drop fall.

2.3. Setting properties

The curing time test was performed using the Vicat needle apparatus (ZI-1004, Zeal International, India) with a tip diameter of 1 mm and 400 g load. The BC curing time was considered as the length of time starting from mixing cement components to the moment the specimens were totally solidified (the indentation mark was not visible on the surface after the test). The polymerization temperature was monitored continuously using a thermocouple (Czah, Poland). The setting properties were evaluated for BC-M-10% (n=5).

2.4. Nanoindentation tests

The mechanical properties of the BC were determined using the nanoindentation technique with NanoTestTM Vantage equipment (Micro Materials, UK). A three-sided diamond, pyramidal Berkovich indenter was used. The experiments were performed on the BC in disk form (20x2 mm). The maximum force of indentation was 50 mN, the loading and unloading times were set up at 20 s and 15 s, and the holding time under maximum force was 5 s. During a single measurement, the load-displacement curve was determined. The surface hardness (H) and reduced Young's modulus (E_r) were calculated using

integrated software by Oliver and Pharr method [47]. The resistance of the material to elastic deformation, defined as H/E_r and the material's ability to dissipate energy at plastic deformation, i.e. H^3/E_r^2 , were calculated.

2.5 Compressive strength and micro-hardness

The compression tests were performed using a universal testing machine (HT-2401, Hung Ta Instrument, Taiwan) at 1 mm/min of strain rate. The BC specimens were of a cylindrical shape ($\phi 6 \times 12$ mm). The compressive strength was calculated by dividing the maximum strength by the cross-sectional area of the sample at 30% of strain. The hardness tests were carried out with the Vickers microhardness tester (FM-800, Future-Tech, Japan) at 10 s of the indentation press time and 10 N of press load. Before the test, the specimens were polished with 200 μm (P80) abrasive sandpaper in a Saphir 330 lapping machine (ATM GmbH, Mammelzen, Germany). Both tests were performed for five specimens of each type.

2.6 PBS exposure

The BC specimens ($n=3$) in disk form (20x2 mm) were immersed in a phosphate buffered saline (PBS; Merck, Germany) solution and stored at 37°C for 30 days. After the immersion, the specimens were removed from the solution, and dried at 37°C overnight. Next, the specimens were weighed (analytical balance accurate to 1.0 mg) and their relative changes in weight were calculated. To assess the porosity after exposure, the specimens

were polished with 58.5 μm (P240) abrasive sandpaper in a Saphir 330 lapping machine and their surface porosity was observed using SEM. The range of pore size was estimated using ImageJ software (National Institute of Mental Health, USA) and average pore size was determined as the median. Moreover, specimens in cylindrical shape were prepared as described above and mechanical tests were performed after 30-day exposure to PBS.

2.7 Cytocompatibility testing in Dental Pulp Stem Cells cultures

The MTS assay was used to assess the cytocompatibility of the BC, based on the procedure described previously [45,48]. The human dental pulp stem cells (DPSC) were obtained from the molar tooth of 31-43 years old donors, both genders, in agreement with the Polish Research Ethics Board; approval no. 1072.6120.253.2017. Before testing, the specimens were sterilized in 70% ethanol followed by 30 min exposure to UV light and then left overnight to dry. The cell culture tests were performed using three series of samples, first composed of BC modified with 10 wt.% of selected modifier and two others for BC modified with 5 wt.% of modifier. Each experimental series consisted of five specimens for each type of BC in disk form (20x2 mm). Following sterilization, the specimens were placed in the 24-well culture plates and covered by 2×10^4 DPSC suspended in 2 mL of culture medium. Cell viability was evaluated at day 7 culture using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS, Promega, Poland) and results were measured colorimetrically by the plate reader, taking absorbance at 490 nm (SpectraMax iD3, Molecular Devices, San Jose). The results were expressed as a percent

change in the number of live cells compared to those grown on the unmodified BC (100%).

2.8 Active substance release measurements

The active substance release for BC-M doped with AgNp was assessed using the UV-Vis spectrophotometry method [49,50]. The specimens (BC and BC-M-5%-AgNp-1.5%) in disk form (10x2 mm) were immersed in 5 mL of distilled water. After 3 and 28 days of immersion, solutions absorption was measured in the range from 200 to 600 nm by the UV-VIS spectrometer (Evolution 220, Thermo Fisher Scientific, USA) using deionized water as a blank. An additional verification procedure for AgNp release before the tests is described in the App. A. Furthermore, entire spectrum analysis was performed for the BC in which characteristic peaks were observed.

2.9 Assessment of antibacterial efficiency

To assess antibacterial efficiency of modified BC two different experiments were carried out. Beforehand, the specimens were sterilized in 70% ethanol and exposed to UV light for 20 min on each side. First experiment included the measurement of the turbidity of cultured bacteria broth according to the McFarland standard [51] and based on the procedure described previously [44]. The *Staphylococcus aureus* strain (ATCC 29213) was used for the tests and at initial concentration of 0.6×10^8 CFU/mL - 0.2 McFarland index (iMS). The experiment was performed using three specimens of BC, BC-A, BC-AgNp and BC-M-5%-AgNp in disk form (10x2 mm) and 2 mL of bacterial solution. The measurements were taken after 0.5, 1, 1.5, 2 and 3h using DensiChEK Plus equipment (BioMerieux,

Montreal, QC, Canada). The second experiment involved the measurements of dehydrogenase activity of formed biofilm, as described previously [52,53]. For this experiment, *Staphylococcus aureus* (ATCC 6538) bacteria were used with the initial concentration of 1.0 iMS. Five disc-shaped (10x2 mm) specimens were placed into the bacterial suspension and incubated for 4 days to allow for biofilm formation. Then, the specimens were washed carefully with PBS buffer and moved to a new clean 24-well plate. Each specimen was covered by a reaction mixture consisting of 375 μ L of Tris-HCl buffer (pH 8.4), 150 μ L of 2% glucose (Chempur, Poland), 150 μ L of 0.4% TTC (Alfa Aesar, Germany) and 75 μ L of 0.36% sodium sulfate (POCH, Poland) and incubated for 24 h at 37 °C. Afterward, triphenylformazan (TF) was extracted from the wells with n-butanol (Chempur, Poland) and its concentration was examined at 490 nm with a spectrophotometer (Hitachi U1900, Japan). The intensity of the developed color is proportional to the concentration of TF produced by dehydrogenase. As a control sample, dehydrogenase activity was measured on sterile BC specimens without antibacterial additives.

2.10. Statistical analysis

Statistical analysis of the data was performed using commercial software (SigmaPlot 14.0, Systat Software, San Jose, CA, USA). The Shapiro–Wilk test was used to assess the normal distribution of the data. All results were presented as means \pm standard deviations (SD) and statistically analyzed using one-way analysis of variance (one-way ANOVA).

Multiple comparisons versus the control group were performed using the Bonferroni t-test with the statistical significance set at $p < 0.05$.

3. Results

3.1. Microstructure

The surfaces of the BC specimens are shown in Fig. 1. For both BC and BC-M (2.5, 5 and 10 wt.%), a structure typical of acrylic cements was obtained, with PMMA chains and free spaces. The modifier's particles appeared on the surface of BC-M (Fig. 1: II-VI). The Cell and Mg particles were embedded in the pores between the chains, TCP particles covered the surfaces of the chains, while Chit and PDO particles, due to their large size, formed an integral structure with the PMMA.

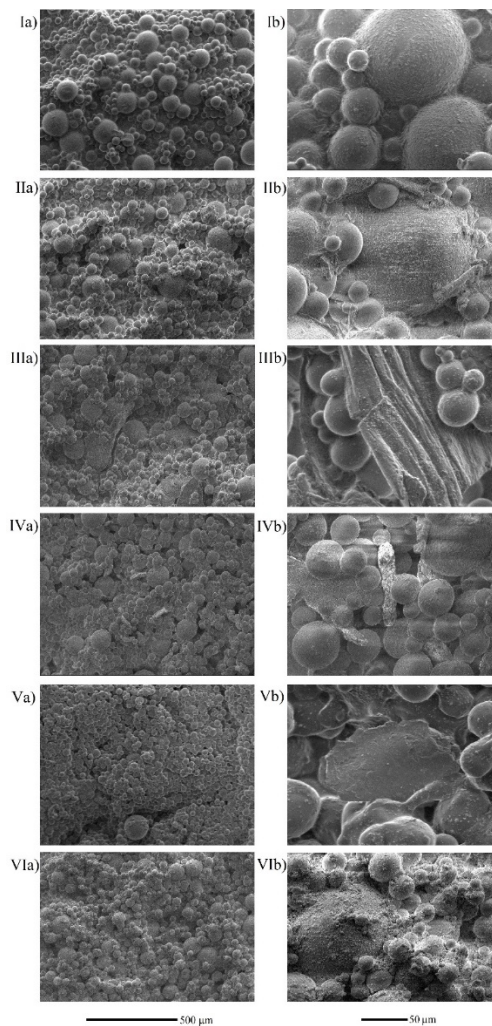


Fig. 1. SEM images of bone cement surfaces at two different magnifications (a -100x and b - 500x): I – BC, II – BC-Cell-5%, III – BC-Chit-5%, IV – BC-Mg-5%, V – BC-PDO-5%, VI – BC-TCP-5% (the pictures are representative for five specimens).

The particles of modifiers differed in shape and size. The Cell and Mg particles were cylindrical, resembling sticks, the Chit and PDO particles resembled petals, and the TCP particles were spherical. The particle size for Cell were in range of 40-80 μm , for Chit 100-500 μm , for Mg 30-100 μm , for PDO 50-150 μm and for TCP 2-10 μm .

3.2. Wettability

The wettability test results are shown in Fig. 2. All modifiers significantly increased

the values of BC contact angles. At the 5 wt.% of modifier, the PDO addition had the lowest impact on the contact angle (increase of 35%), Chit, Mg and Cell all showed more pronounced effect (67%) and TCP the most significantly influenced the contact angle (89%).

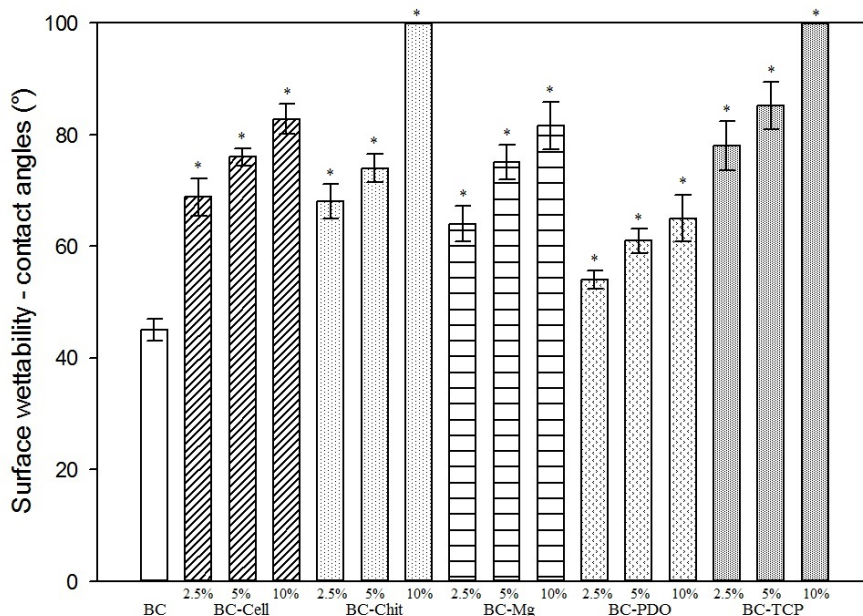


Fig. 2. Surface wettability of the tested bone cements determined by the measurements of the contact angle of distilled water (n=5; data are the expressed as mean \pm SD; * significantly different from control sample – BC ($p < 0.05$)).

3.3. Setting properties

The addition of modifiers did not significantly affect the setting properties of BC (Table 2). All modified BC had curing time in range of 13-17 min and maximum polymerization temperature in range of 34-42°C.

Tab. 2. Setting properties of the tested bone cements (n=3; data are expressed as the mean \pm SD; * significantly different from control – BC (p < 0.05)).

	Maximum polymerization temperature [°C]	Curing time [min]
BC	38.4 \pm 1.5	15:20 \pm 1:40
BC-Cell-10%	35.7 \pm 2.1	15:50 \pm 1:30
BC-Chit-10%	37.1 \pm 2.6	15:30 \pm 1:50
BC-Mg-10%	40.5 \pm 1.8	13:50 \pm 1:30
BC-PDO-10%	37.7 \pm 1.6	16:10 \pm 1:10
BC-TCP-10%	37.6 \pm 2.2	14:20 \pm 1:40

3.4. Nanoindentation mechanical properties

The nanoindentation tests results are shown in Table 3 and SFig. 4. The large standard deviation is due to small area of single indent and likely inhomogeneous structure of modified BC.

Tab. 3. Nanoindentation mechanical properties of the tested bone cements (n=10; * significantly different from control – BC (p < 0.05)).

Nanoindentation mechanical properties					
	Nanohardness (GPa)	Young's modulus (GPa)	H/Er	H ³ /Er ² (MPa)	
BC	0.169 \pm 0.029	4.232 \pm 0.632	0.039 \pm 0.001	0.273 \pm 0.065	
BC-Cell	2.5%	0.222 \pm 0.042	3.997 \pm 0.792	0.056 \pm 0.004*	0.689 \pm 0.153
	5%	0.329 \pm 0.074*	4.729 \pm 1.051	0.066 \pm 0.022*	1.751 \pm 1.408*
	10%	0.133 \pm 0.028	1.775 \pm 0.164*	0.074 \pm 0.001*	0.781 \pm 0.384*
BC-Chit	2.5%	0.286 \pm 0.104*	6.583 \pm 1.196*	0.042 \pm 0.012	0.609 \pm 0.419
	5%	0.135 \pm 0.034	1.797 \pm 0.527*	0.074 \pm 0.009*	0.799 \pm 0.412*
	10%	0.295 \pm 0.063*	3.476 \pm 0.453	0.084 \pm 0.011*	2.191 \pm 0.847*
BC-Mg	2.5%	0.537 \pm 0.099*	6.373 \pm 0.838*	0.084 \pm 0.006*	3.867 \pm 1.145*
	5%	0.436 \pm 0.112*	5.418 \pm 0.789*	0.079 \pm 0.012*	3.015 \pm 1.254*
	10%	0.602 \pm 0.098*	7.077 \pm 0.837*	0.085 \pm 0.005*	4.416 \pm 1.179*
	2.5%	0.227 \pm 0.047	5.176 \pm 0.419	0.044 \pm 0.005	0.458 \pm 0.207*

BC-PDO	5%	0.208 ± 0.119	5.745 ± 1.461*	0.033 ± 0.008	0.245 ± 0.172
	10%	0.540 ± 0.093*	7.097 ± 1.525*	0.077 ± 0.006*	3.179 ± 0.539*
BC-TCP	2.5%	0.508 ± 0.086*	6.732 ± 0.501*	0.075 ± 0.007*	2.967 ± 1.087*
	5%	0.171 ± 0.057	3.814 ± 0.171	0.044 ± 0.006	0.364 ± 0.231
	10%	0.145 ± 0.072	3.993 ± 1.463	0.035 ± 0.007	0.206 ± 0.139

The general trend associated with BC modifications was the improvement of their nanoindentation mechanical properties, however a decrease in the reduced Young's modulus was noted for BC-Cell 10% and BC-Chit 5% (decrease by ~ 60% in both cases). Only for BC-Mg, significant increase was observed for all tested additive contents. SFig. 4 presents the selected was also presented on load-displacements curves (curves for all tested BC before (control) and after exposure to PBS, with three characteristic parts (loading, dwelling and unloading). A significant change has been observed for BC-Mg and for this specimen, the maximum indentation depth decreased, contrary to the increases in this parameter for other modifications. After PBS exposure of all tested specimens, further increases in the above parameter were notified expect for Chit and PDO. It has been assumed that the increase of indentation depth is correlated with the decrease of the nanomechanical properties. None of modifiers negatively impacted the nanoindentation parameters – H/Er and H^3/Er^2 , and the most significant improvement of the above parameters was observed for the specimen with Mg addition.

3.5. Compressive strength and micro-hardness

The assessment of compressive strength is shown in Fig. 3. The addition of Mg and

TCP did not affect the compressive strength of the BC, whereas Chit, Cell and PDO significantly reduced compressive strength by 13%, 17% and 24%, respectively. Month-long exposure to the PBS solution resulted in a reduction in compressive strength for all tested BC, and the largest changes occurred for BC-Chit and BC-Cell.

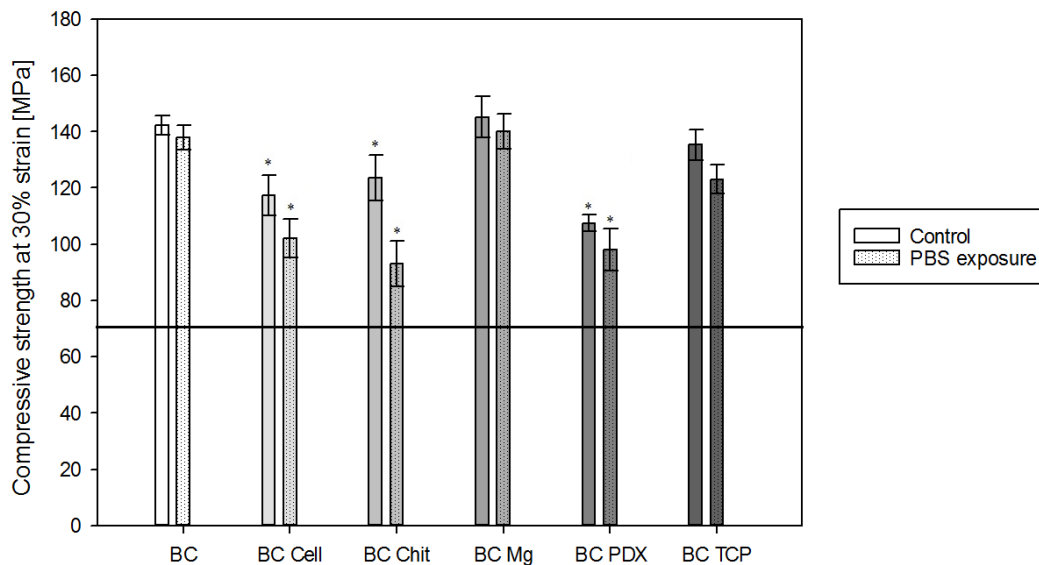


Fig. 3. Compressive strength of the tested bone cements determined at 30% strain as prepared (control) and after one month exposure to the PBS solution (PBS exposure) (n=5; data are expressed as the mean \pm SD; * significantly different from control – sample BC ($p < 0.05$)).

The microhardness of the modified BC is shown in Fig. 4. All modifiers significantly reduced the BC hardness. At 5 wt.% content, Mg decreased hardness by ~13.5% only, then Chit (~16.3%), TCP (~19.9%), Cell (~20.6%) and the highest impact had PDO (~27.7%).

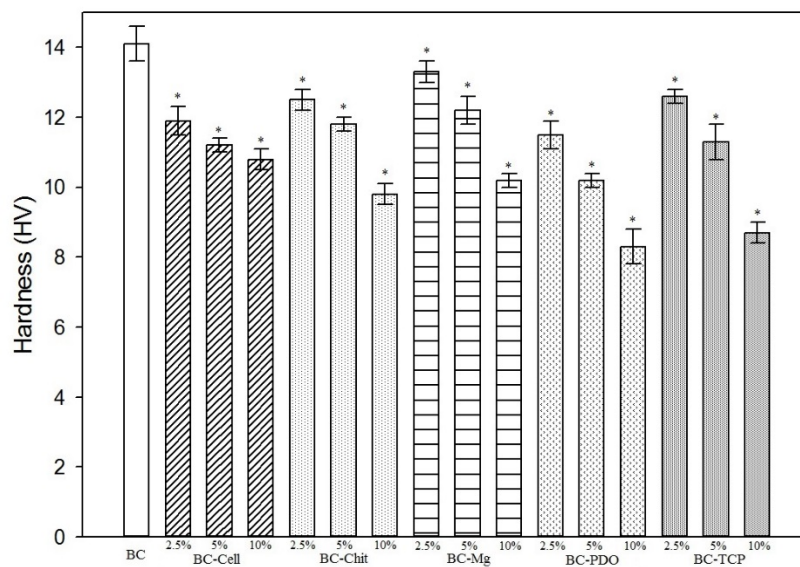


Fig. 4. Hardness of the tested bone cements (n=10; data are expressed as the mean \pm SD; *significantly different from control – BC ($p < 0.05$)).

3.6 Exposure to PBS

Exposure of the modified BC to the PBS solution for 30 days did not significantly affect their structure, but changes in porosity could be observed (SFig. 2). Some particles of modifiers appeared on the BC surface. For BC-Mg, the formation of crystalline flower-like structures could be observed on the surface of the chains. EDS analysis showed the presence of O, Na, Mg, P, Cl, K, and Ca. After one month of exposure to PBS solution, the slight weight losses for all specimens were observed (Fig. 5). The smallest loss of weight (~0.3%) was observed for the control specimen – BC, while for BC modified with Cell, PDO and TCP mass decreased by ~1-3.5%.

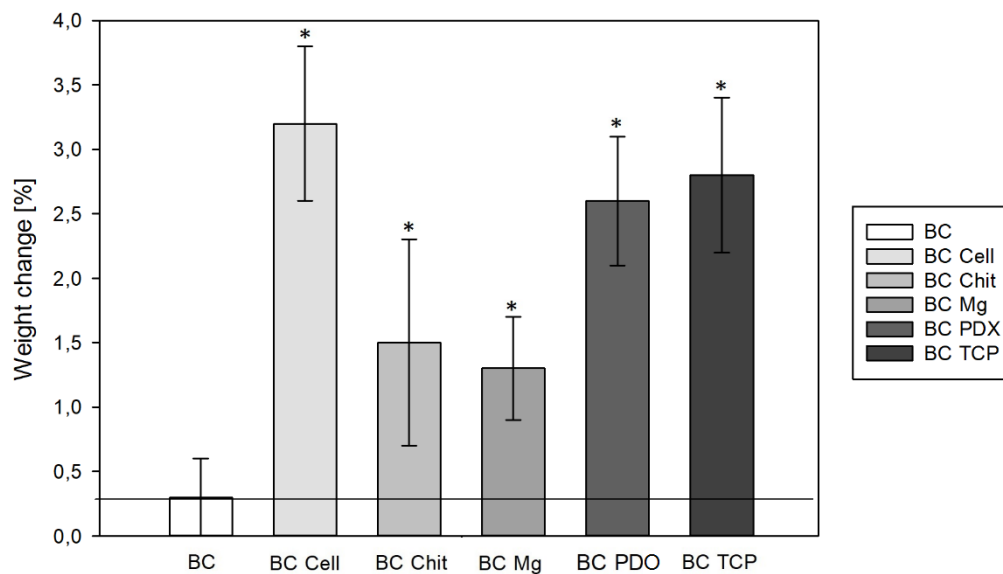


Fig. 5. Weight changes of tested bone cement (5 wt.% of modifier) after monthly ageing in PBS solution (n=3; data are expressed as the mean \pm SD * significantly different from control – sample BC ($p < 0.05$)).

The porosity of the modified BC and estimated average pore size ranges are shown in Fig. 6. Porosity increased for all modifiers while no significant changes in porosity were found in the control specimen - BC. Cell and PDO had the most considerable effect on increasing specimens porosity (Fig. 6: II,V).

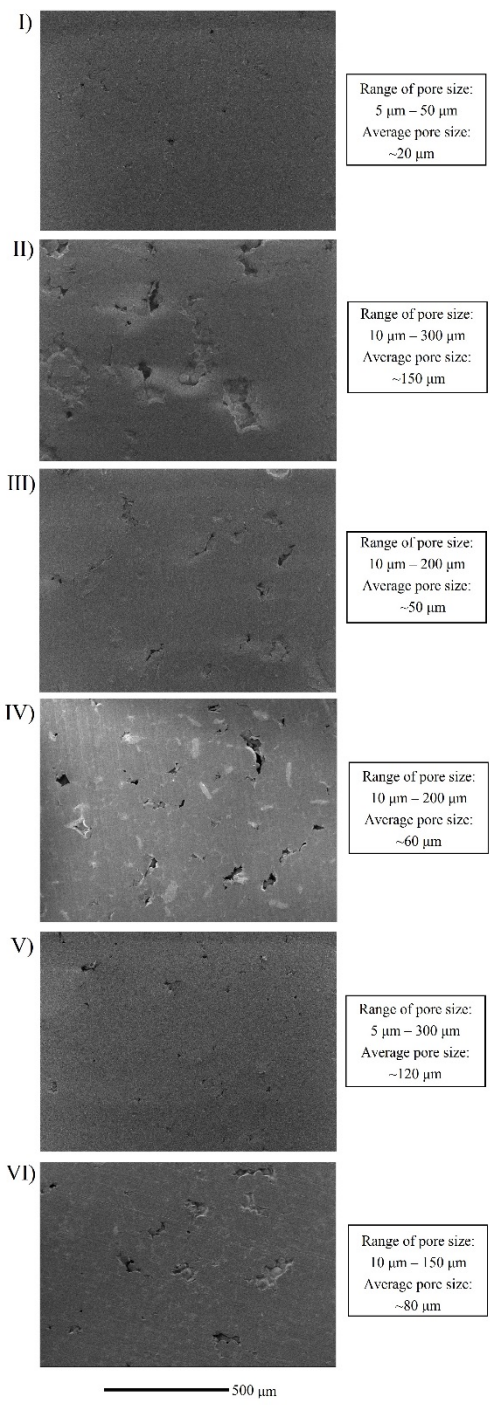


Fig. 6. SEM images of ground bone cement surfaces after monthly ageing in PBS solution (100x): I – BC, II – BC-Cell-5%, III – BC-Chit-5%, IV – BC-Mg-5%, V – BC-PDO-5%, VI – BC-TCP-5% (the presented pictures are representative for three specimens).

Exposure to the PBS solution had also an impact on the mechanical properties of tested specimens (Figs. 3 and S3). The mechanical properties of BC-Chit and BC-Cell

worsened the most, while BC-PDO and BC-Mg the least.

3.7 Cytocompatibility

The cytocompatibility after 7 days culture of DPSC on the tested BC is shown in Figs. 7 and 8. Except for PDO, all modifiers (at 5 and 10 wt.%) displayed the tendency to reduce the viability of DPSCs grown on the modified BC surfaces. Cell, Chit and TCP at 10 wt.% caused high cell mortality, while Mg reduced the viability of cells by about 70% (Fig. 8). Modifiers at 5 wt.% had smaller cytotoxicity compared to 10 wt.% content. Both factors (material preparation and cell donor) influenced the obtained results, but negative effects of BC modifications on cell viability could be observed regardless of the material prep and cell donor. In general, BC modifications with Cell, Chit, Mg or TCP had either none or negative effect on BC cytocompatibility (Figs. 7 and 8). However, doping BC with PDO (at either 5 or 10 wt.%) had either none or positive effect on BC cytocompatibility in DPSCs cultures (Figs. 7 and 8).

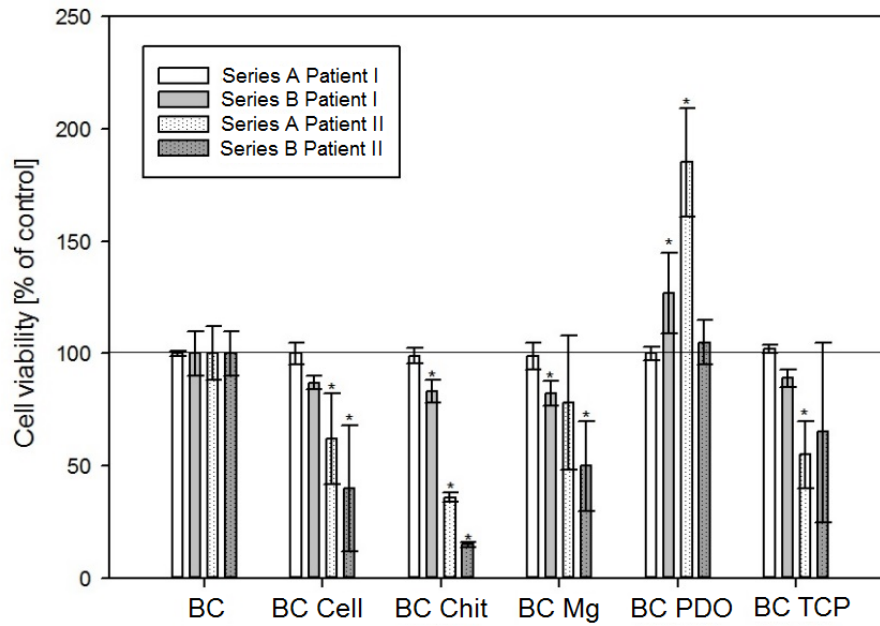


Fig. 7. Dental pulp stem cells (DPSC) viability on tested bone cements (5 wt. % of modifier) at 7 day of culture. Results are expressed as % change in cell viability compared to the unmodified bone cement (n=5; data are expressed as the mean \pm SD * significantly different from control – BC ($p < 0.05$)).

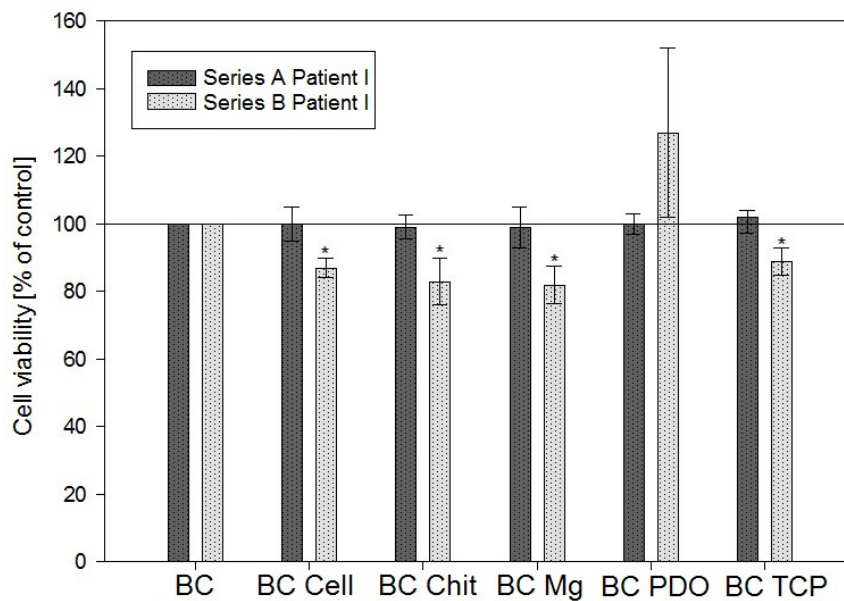


Fig. 8. Dental pulp stem cells (DPSC) viability on tested bone cements (10 wt. % of modifier) at day 7 culture. Results are expressed as % change in cell viability compared to the unmodified bone cement (n=5; data are expressed as the mean \pm SD * significantly different from control – BC ($p < 0.05$)).

3.8 Release of active substances

The UV-Vis spectra measured for BC and BC-M-5% solutions harvested after 3 and 28 days of incubation in deionized water are shown in Fig. 9: a and b, respectively. In all solutions a strong absorption band at 200-250 nm was observed. This could be attributed to PMMA (200 nm) and MMA (250 nm) [49]. Compared to control – BC, addition of Cell, TCP, Chit and Mg increased MMA release (band ~250 nm) after 3 as well as 28 days. The use of PDO reduced the amount of MMA monomer in solution.

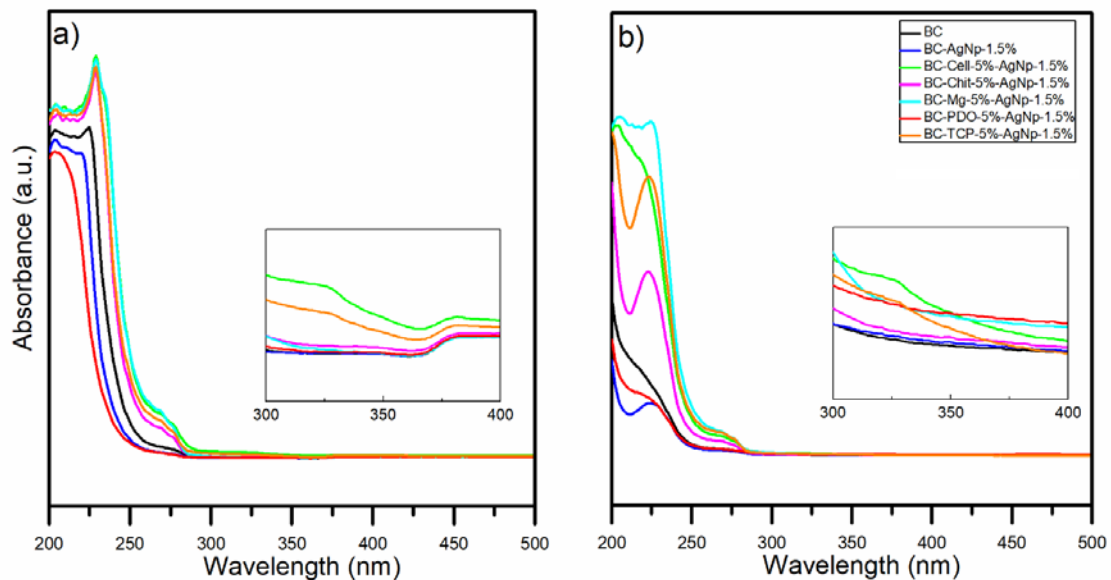


Fig. 9. UV-Vis spectra for the tested bone cements after 3 days (A) and 28 days (B) of incubation in distilled water (modifier content – 5 wt.%; the presented spectra are representative for three specimens).

The investigations of the AgNp release from the BC-M-5% are shown in Fig. 9. Typical absorption of AgNp is at 300-500 nm and the position of the absorbance maximum depends on size, shape and distribution of nanoparticles [50]. The absorption spectra of AgNp reference solutions had few bands (at. 270, 330 and 360 nm) as shown in SFig. 1 and

the same signals were detected in all spectra registered for BC-M-5%-AgNp, confirming the AgNp release. Analysis of the results after 3 days showed that the addition of Cell, TCP, Mg and Chit improved the AgNp release, while PDO addition had no effect. After 28 days (using 10x dilution of tested solution) improved release of AgNp was observed for Cell, PDO, Mg and TCP, but Chit addition reduced it.

3.9 Antibacterial properties

The inhibition of *Staphylococcus aureus* growth is shown in Tab. 4. Bacteria rapidly multiplied when incubated with the control BC specimen. The addition of bactericidal agents (antibiotic or AgNp) to the BC slowed down the bacterial growth. The use of a biodegradable component, especially Chit and Cell, improved the antibacterial effectiveness of the AgNp as observed by a greater inhibition of bacterial growth.

Tab. 4. Bacterial growth inhibition determined by McFarland standard values specifying the number of *Staphylococcus aureus* bacteria during incubation with the tested bone cements doped with antibiotic or nanosilver (n=5; data are expressed as the mean \pm SD; * significantly different from control – BC ($p < 0.05$); # significantly different from control – BC-AgNp ($p < 0.05$)).

Bacterial growth inhibition – McFarland index						
	0 h	0.5h	1h	1.5h	2h	3h
BC		0.39 \pm 0.01	0.42 \pm 0.01	0.83 \pm 0.02	1.97 \pm 0.02	3.55 \pm 0.05
BC-A		0.32 \pm 0.02*	0.37 \pm 0.02	0.62 \pm 0.02*	1.39 \pm 0.04*	2.32 \pm 0.04*
BC-AgNp-1.5%		0.30 \pm 0.01*	0.35 \pm 0.01*	0.67 \pm 0.01*	1.29 \pm 0.02*	2.80 \pm 0.02*
BC-Cell-5%-AgNp-1.5%	0.2 \pm 0.01	0.32 \pm 0.02*	0.39 \pm 0.04	0.70 \pm 0.04*	1.39 \pm 0.03*#	2.50 \pm 0.04*#
BC-Chit-5%-AgNp-1.5%		0.30 \pm 0.02*	0.39 \pm 0.02	0.71 \pm 0.03*	1.38 \pm 0.04*	2.44 \pm 0.04*#
BC-Mg-5%-AgNp-1.5%		0.36 \pm 0.01*#	0.45 \pm 0.02#	0.9 \pm 0.02*#	1.84 \pm 0.03*#	3.22 \pm 0.03*#
BC-PDO-5%-AgNp-1.5%		0.31 \pm 0.02*	0.44 \pm 0.02#	0.92 \pm 0.03*#	1.96 \pm 0.05#	3.65 \pm 0.05*#
BC-TCP-5%-AgNp-1.5%		0.30 \pm 0.03*	0.38 \pm 0.03	0.79 \pm 0.03#	1.58 \pm 0.04#	2.90 \pm 0.02*#

The assessment of the dehydrogenase activity of *Staphylococcus aureus* biofilm is shown in Fig. 10. The addition of bactericidal agents (antibiotic or AgNp) to the BC

significantly reduced biofilm dehydrogenase activity and the use of biodegradable components, especially Cell and Chit, in the BC-AgNp further improved its antibacterial effectiveness.

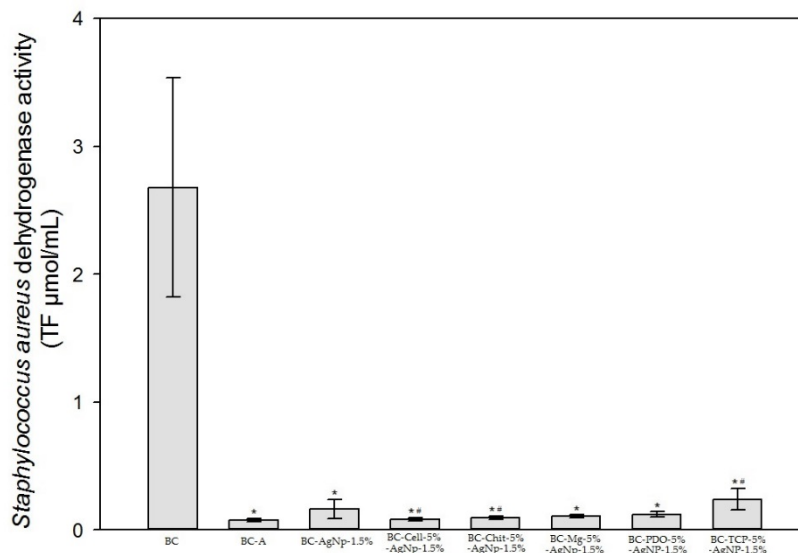


Fig. 10. *Staphylococcus aureus* biofilm activity determined by measurements of dehydrogenase activity after incubation with the tested bone cements doped with antibiotic or nanosilver (n=5; data are expressed as the mean \pm SD; * significantly different from control – BC ($p < 0.05$); # significantly different from control – BC-AgNp ($p < 0.05$)).

4. Discussion

Our results show that the studied modifiers are suitable for obtaining the partially-biodegradable antibacterial bone cement. We demonstrated that the selected modifiers influenced to a different extent the porosity, wettability, mechanical properties, cytocompatibility and antibacterial efficiency.

The addition of modifiers did not affect the polymerization process as no negative effects on structure, polymerization temperature and curing time of cement were observed.

All specimens possessed a structure typical for BC (Fig. 1) [54], but different microstructure.

The curing time for all modified BC was in range of 13-17 min, and the use of additives, except for Mg, resulted in a favorable decrease in the maximum polymerization temperature in range of 34-42°C. Hence all BC would be suitable for their clinical application [55]. Our results are in line with the literature regarding cement modifications, in which has been stated that it is possible to deposit additives particles in solid form into the pores of the cement matrix or between PMMA chains [56]. Moreover, this study discloses that, the modifications do not significantly affect the curing time, in opposition to other research, which have shown that, depending on the modifiers, this time may be accelerated [57,58] or reduced [59,60].

The addition of studied modifiers significantly improved porosity (Fig. 6 and SFig. 2) and reduced weight of modified BC (Fig. 5) after one month exposure to PBS. As a result of exposure, some particles could have been washed out from BC matrix or just dissolved, which caused a reduction in weight in modified BC and opened new pores in it. Cellulose solubility is very sensitive to the presence of ionic liquids, in particular phosphates [61], chitosan dissolves more quickly in the presence of chlorides [62], TCP dissolves very fast [63], Mg undergoes biodegradation [64] and PDO is a biodegradable polymer [65]. Our findings are in agreement with several other studies on the use of biodegradable additives to improve BC porosity. Current literature reports the following modifiers for BC to be effective: Chit [42,58,66], Mg [59,67], TCP [40] or combination like TCP-Chit [68]. Considering the BC weight loss, the Cell is the fast dissolving compound,

then TCP, PDO, Chit and Mg. Incorporating biodegradable components to the BC matrix allows for obtaining a time-varying porosity. The porosity, in turn, affect osteoconductive properties of specimens. Degradation of some specimens such as TCP or Mg-modified will results in the release of ions that may stimulate bone regeneration. Moreover, the gradual opening of new pores enables effective and long-term release of small doses of active substances from the BC. Thus, all these modifiers, and particularly cellulose, may be considered as applicable for creating the open porous structures of BC.

The use of modifiers at increasing content decreased BC wettability as measured by water contact angle. The optimum contact angle for cell adhesion is 55° and for bone regeneration in range of 35° - 80° [69,70]. All modifiers, at their low or middle content, demonstrated the contact angle values within the desired limits so that they remained hydrophilic. The modifiers affected wettability probably due to additive effect of the BC contact angle and the additives. The wettability of Cell powder was determined as of 56 - 72° , depending on water activity and moisture [71]. The Chit showed values of contact angle between 74 and 90° for the different basic solutions [72]. Mg alloys are superhydrophobic materials [73]. The TCP is hydrophilic material with the water contact angle at 66° [74] or 35° for microporous TCP [75]. The polymer PDO showed the water contact angle at 63° [76]. The decrease in hydrophilicity, as in present research, was observed for the Chit addition to the BC [15,57], but other additives like Mg surprisingly improved wettability of BC [67]. The present studies prove that all modifiers, especially

PDO, may be applied to form the BC with hydrophilic properties at contents up to 5 wt.%.

The addition of modifiers, except for Mg, reduced the mechanical properties of BC (Figs. 3 and 4). It is assumed that this is due to the weakening of the polymerization reaction and the increasing distance between MMA particles in the PMMA chain as well as the increasing the internal porosity of the cement. Mg exception is reasonable considering the compression strength of Mg, 297 MPa [77] and the studied BC, 142 MPa. The compression strength of other modifiers was lower, with Chit assessed at 10-30 MPa [78]. For sintered TCP the compression strength was below 150 MPa [79], but such manufacturing was not performed in this research. There is no data on compression strength for other additives; the tensile strength of 5-24 MPa was estimated for PDO, depending on its molecular weight [80], and 250-945 MPa for Cell nanofiber [81]. When two materials with significantly different mechanical properties are present in the composite or sandwich structure, it may lead to considerable stress discontinuity and the long term structural instability [82,83]. A month exposure to the PBS solution affected also mechanical properties of tested BC. The most significant decrease of compressive strength and microhardness occurred for BC-Cell and BC-Chit and the smallest changes were observed for BC-Mg and BC-PDO. This is due to the increase in porosity and weight change. In case of surface properties tested at the nanometric scale, the largest decreases in hardness were observed for BC-Mg and BC-TCP, and the smallest for BC-PDO. This may be related to the changes occurring on the surface of BC (SFig. 3). In general, doping solid PMMA materials with particles or fibers could

improve the mechanical properties of material by reinforcing effect. Such effects were observed in some previous studies with Cell (up to 3 wt.%) and Mg phosphate (up to 10 wt.%) [41,84,85]. However, increasing modifiers content caused an opposite effect due to inhomogeneous dispersion of particles and their agglomeration [86-88]. Similar trends were observed for PMMA resin and denture base, for which the addition of Cell particles improved its flexural strength and Young's modulus [89,90]. For the acrylic BC, which is a porous material created by the polymerization reaction, the effect of increasing mechanical properties may be different. The reduced mechanical properties of modified BC observed in this research are consistent with results of others, e.g. for an addition of Chit [15,57,91,92], TCP [93], MgO [56] or two components together such as TCP with Chit or HAp and Chit with GO [58,68,94]. On the other hand, some studies suggested that the addition of certain modifiers at small content could not affect or improve the mechanical properties of BC as the addition of Chit [56,66] and Mg [59,67]. However, increasing the Mg amount above 10 wt.% resulted in deterioration of mechanical properties [59]. The nanoindentation tests of unmodified BC were already reported, with results similar to ours. The modulus of elasticity for BC was about 4.0-6.0 GPa and its hardness was 0.2-0.3 GPa [95-97]. Best to our knowledge there are no literature reports presenting nanomechanical studies of modified BC as in this work. Generally, the decreased BC porosity improves the material stiffness and its hardness, because of increasing the connection between PMMA chains [95,96]. In our study, we added the modifiers to the BC structure and this addition

affected the distribution of the chains, but also caused filling the pores and free spaces in the matrix. Strengthening of the material resulted in improved nanomechanical properties (hardness and Young's modulus) of most modified BC. The additives also influenced the load-displacements curves (Fig. S4) of modified specimens and the results seem to partly correlate with their nanomechanical parameters. However, because only one representative curve was chosen for each BC, the exact correlation between the nanoindentation curves and the mechanical parameters could not be properly compared. The H/E ratio should be high, however, the high hardness and the H/E ratio also indicate the brittleness of the material. The H/E ratio is a reliable indicator of good wear resistance [98]. Coy et al. [99] reported that H^3/E^2 should be more than 0.1 for "hard" materials, but the 0.05 value is sufficient. In our study, all modified BC had higher values than 0.1, hence we assume their good wear resistance. The general trend in nanoindentation mechanical properties is opposite to the results obtained in typical mechanical tests, and except of Chit-modified BC, the composite cements become harder and resistant to plastic deformation, but they are more prone to brittle cracking. The opposite trends in nanohardness/microhardness vs. type of additive can be explained by e.g., emission and dislocation strengthening leading to the indentation size effect. We have observed the letter, but this cannot fully explain the positive effects of additives on nanoindentation and negative impacts on microhardness. Similar effects were reported previously for nanoindentation tests [100]. Due to the use of low force and small surface area contact of

Berkovich indenter ($\sim 55 \mu\text{m}^2$) with average maximum indentation depth at $\sim 1.5 \mu\text{m}$, nanomechanical tests provide information mostly on the material surface properties. Microhardness tests by the Vickers method assumes, the area of indentation is much larger and it is possible to determine the mechanical properties of the entire material matrix, not only single surface spots. The material structure itself is another factor that influences the results and their differences, as well as, deepens the indentation scale effect. The porosity impact, which is evaluated by microhardness tests, applies the appropriate high force, bigger size of the indenter and large area of tested surface. Furthermore, the "pile-ups" phenomenon in nanoindentation tests has been observed. It is caused by progressive dislocations below the indenter contact area with the tested material [101,102]. As a result, the value of the real indentation area may be different from this determined by the Oliver-Pharr method [47]. In the study of Cheng et al. [103] the materials with low H/E ratio value the showed smallest pile-up effect vs. materials with higher H/E ratio. In our work the H/E ratio (0.033-0.085) was quite high, so the pile-up effect could have occurred and this should be examined in future studies. Despite the decreases of compressive strength and hardness of modified BC, the modifications do not significantly deteriorate composite BC mechanical behaviors, even after a month exposure to the PBS solution, and all tested BC meet the ISO 5833 requirements [104] that recommend above 70 MPa compressive strength for acrylic cements.

BC cytotoxicity can be related to the released small amounts of unreacted MMA

monomer. Such undesirable effect may be a consequence of improper cement formulation after adding modifiers or its preparation. Generally, there are reports that small quantities of MMA can be released from cement and, in vivo they are progressively diluted in the body fluids and do not demonstrate negative effects on the surrounding tissues [17-19]. In our studies, the addition of all modifiers, except PDO, negatively and significantly affected cytocompatibility of the BC. All additives at 10 wt.% content, except PDO, deteriorated cytocompatibility (Fig. 8), but the reduction of their amounts weakened this effect (Fig. 7). Such negative effects on cytocompatibility cannot be attributed to the modifiers given that chitosan positively influence proliferation of MC3T3 mouse cells [105], Cell-based hydrogels were biocompatible in vivo [106], for PDO and its copolymer forming bicomponent the tissue reaction was minimal [107], TCP showed positive effect on cell proliferation [108], and Mg had a moderate positive effect on cell viability [109]. In our studies, only the polymer PDO increased viability of cells. This can be explained based on the results obtained by UV-Vis analysis (Fig. 9) that showed all modifiers, except the PDO, caused increasing release of MMA monomer. Thus the polymerization process may not be completed at the interface of PMMA resin modifier and the liberation of free monomer particles occurs at the interface. They are subsequently dispersed in solution. The PDO may enhance the polymerization reaction acting as a catalysator or bind loose monomer particles. Moreover, degradation of additives also had impact on the surface morphology and the surrounding material. The possible consequences of this process may be a release

of ions, molecules of additives, gas or even various radicals and they all may affect the cell response. It is to note that previous research demonstrated different results for BC with some modifiers: decrease of MG63 cell viability after TCP addition [109], or of L929 cells after Cell or TCP-Chit addition [68,90]; no adverse reactions for BC with Chit [91,92,110]; Mg [59,67] or MgO [56], and an enhancement of osteoconductivity and cell viability for BC with CaP, Chit-Hap, Chit-GO, HAp-TCP [40,42,58,111]. Despite lower cell viability on BC with Cell, Chit or Mg, we still consider their clinical applicability. *In vitro* tests do not fully reflect the complex conditions of human body and the circulation of body fluids may potentially eliminate the problem of the monomer toxicity. However, based on our present results, consideration should be taken to changing the powder-liquid ratio or reducing the modifiers size to reduce MMA release. The cytocompatibility studies showed that the addition of modifiers may affect the compatibility of cements and 5 wt.% contents is the upper limit value, except for PDO, which seems to be the most promising modifier in this aspect.

In our previous studies, we obtained bioactive BC doped with AgNp and confirmed its cytocompatibility as well as its sufficient mechanical properties. We also conducted a number of microbiological tests to confirm antibacterial effectiveness of such specimens and proposed their bactericidal activity mechanisms [43,44]. Our present goal was to improve the Ag release by addition of pore-forming soluble modifiers and thus increase its antibacterial efficacy. The UV-Vis spectral analysis indicated that the additions of studied

modifiers increased the release rate of AgNp following the enhanced dissolution rate of modifiers. The lower amounts of AgNp release from Chit-BC after 28 days vs. 3 days may be explained by the dissolved Chit could enhance an aggregation of AgNp and weakening the UV-Vis signal. The enhancement of AgNp release leads to increases in the antibacterial properties of BC-AgNp despite two experiments are slightly different. At the first experiment, the high inhibition of bacterial growth measured by McFarland index was observed for Cell and Chit (Tab. 4), which could be attributed to high dissolution rate of both modifiers together with AgNp. Regarding the inhibition of dehydrogenase activity of bacterial biofilm, its increase was observed for all modifiers except TCP (Fig. 10). Hence, the use of biodegradable components for BC-AgNp may result in antibacterial properties similar to cements enriched with gentamicin. In our previous studies, we reported that nanosilver provides favorable antibacterial protection of cement surface compared to studied antibiotics, but due to insufficient release rate, it is less effective in solution [44]. Additional modification of BC-AgNp may provide more effective infection control on contact with body fluids and tissue environment, especially upon antibiotic-resistant infection. In general, bacteria are not resistant to nanosilver thanks to three different nanosilver actions that lead to bacteria cell lysis and its mortality: 1) the release of free metals ions, 2) direct cell membrane damage and/or 3) generating reactive oxygen species [44]. To sum up, application of Cell or Chit to BC doped with AgNp allows for the most effective inhibition of *Staphylococcus aureus* growth.

Based on the current study, we postulate that it is possible to obtain a time-varying porosity of BC by incorporating a biodegradable component to its structure. The improvement of internal porosity allows for the increasing release of the active substance upon degradation of the specimen in the physiological environment. As a result the above processes improve the antibacterial properties of the cement doped with antibiotics or nanometals. However, higher porosity of the specimens affects their mechanical strength and increase toxic MMA monomer release. This is related to separation of PMMA chains and creation of free spaces in the BC structure, which causes the material to be more susceptible to compression. The presence of unreacted monomer is a normal phenomenon for PMMA cements, however, due to their low porosity, most of the monomer is permanently enclosed in the matrix. The use of biodegradable components causes the release of this monomer. In addition, we suspect that doping BC with relatively large additive components affects the polymerization process and may increase the amount of MMA monomer. Taken together, we assume the most promising additive to the BC is polydioxanone or its potential mixture with Cell at 5 wt.%. The choice of PDO as BC modifier is based on the following results: 1) no negative impact on setting properties, wettability, MMA release and cytocompatibility, 2) improvement of specimens porosity and weight change and 3) no significant deterioration in the mechanical properties of specimens that would prevent cement applicability. The choice of Cell as an additive is associated with its significant improvement of BC porosity and increased release of

bioactive substance (i.e. nanosilver) as well as improved antibacterial properties of BC after short incubation period. To best of our knowledge, such additives to cement have not been yet evaluated. The PDO has been proposed for drug carries, tissue engineering, biodegradable composite polymers together with Chit or TCP [112-115]. The Cell has been added as the drug carrier and in regenerative medicine [115,116], despite its possible cytotoxicity [117]. Our finding for PDO as a potential additive to BC are very promising, however, we assume that the addition of a small fraction of Cell may also extend cement applicability and enhance the antibacterial protection in the first period after surgery. It is particularly interesting that only the addition of PDO presumably prevents the appearance of the toxic MMA monomer from the cement. This may be due to a glass transformation of PDO [118] during the BC polymerization process and possible monomer binding by the PDO structure or an occurrence of chemical reaction of PDO and monomer resulting in blocking the cytotoxic functional groups in chemical molecule.

The authors recognize several limitations to the study. First, a constant liquid to powder ratio was used, and its change could likely affect the results. As it was previously observed, the liquid to powder ratio is one of the critical factors in the aspect of the mechanical properties of BC [119]. Hence, there is a concern that the addition of the modifiers could disturb the optimal liquid to powder ratio and also contribute to the deterioration of BC biological properties. Secondly, one method of cement preparation was selected and there is a potential possibility that the addition of modifiers into already pre-

mixed cement could contribute to the removal of the toxic monomer problem. Thirdly, mechanical tests were carried out on specimens without aging and it would be essential to evaluate these properties after or during additives biodegradation process. Lastly, in this study a qualitative method was used to assess the AgNp release, but an appropriate quantitative test would also be required. Therefore, additional tests to describe in details the properties of modified BC should be undertaken in the future.

5. Conclusion

The partially-degradable bone cements may be successfully prepared using different modifiers such as cellulose, chitosan, magnesium, polydioxanone, tri-calcium phosphate, and Ag nanoparticles. The addition of modifiers does not affect the polymerization process and influences the microstructure, physical, mechanical and biological properties of resulted composite cement. The modified cements gain additional bioactivity associated with time-varying porosity. This, in turn, affects their osteoconductivity, and leads to effective release of active substance (such as gentamicin or nanosilver) that enhance specimens antibacterial efficiency. However, the additives may deteriorate mechanical properties of bone cements and decrease their cytocompatibility, especially at higher content. The optimum modified bone cement may be obtained with the use of polydioxanone or cellulose at 5 wt.%, and results in specimens of increased porosity and improved antibacterial efficiency.

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Author Contributions

Conceptualization, M.W.; methodology, M.W., M.M-S., M.B., M.N., and A.M.O.; formal analysis, M.W., A.M.O., and A.Z.; investigation, M.W., M.M-S., M.B., M.N., K.Ł., and A.P.; data curation, M.W.; writing—original draft preparation, M.W.; writing—review and editing, M.W., A.Z., and A.M.O.; supervision, A.Z. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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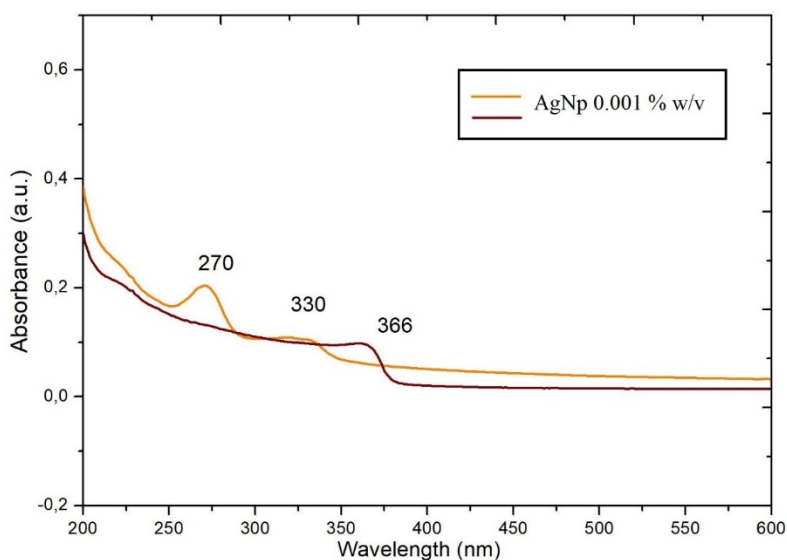
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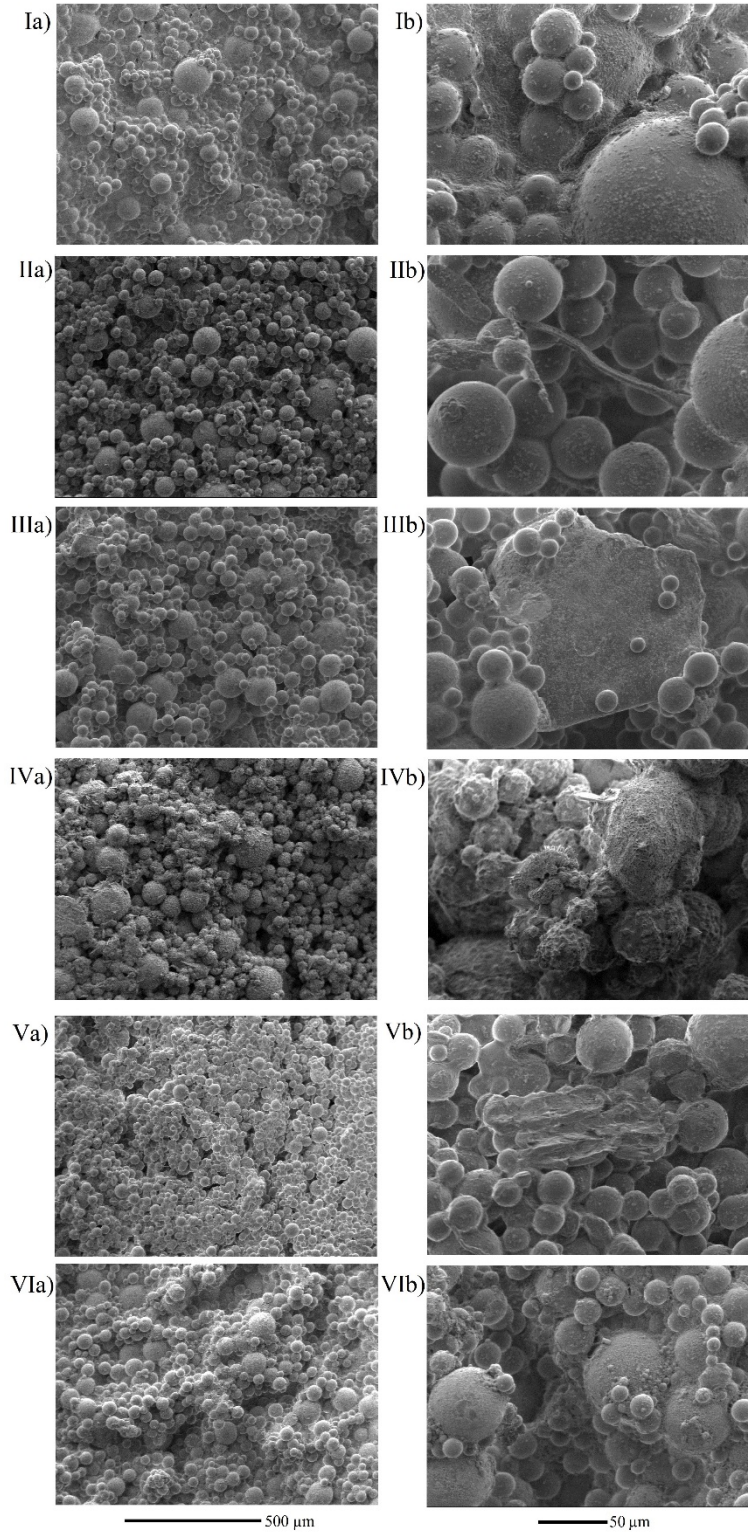
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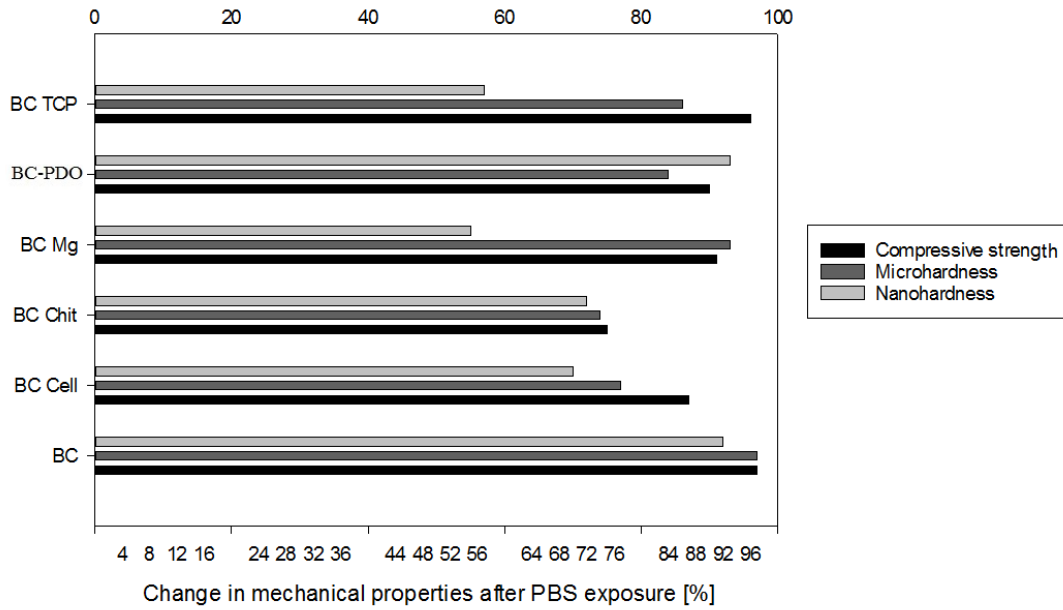


SFig. 1. UV-Vis spectra for nanosilver solutions (the presented spectra are representative for three specimens).



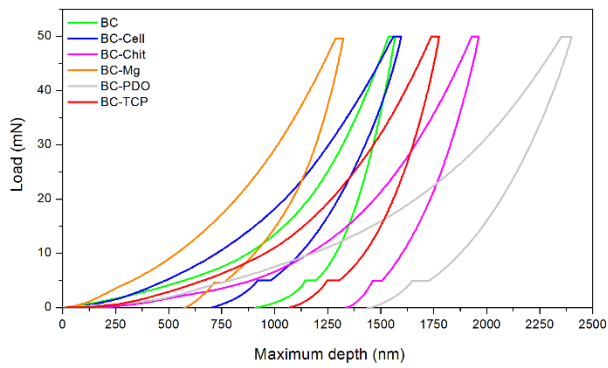


SFig. 2. SEM images of bone cement surfaces after a monthly exposure to PBS solution (a -100x and b - 500x): I – BC, II – BC-Cell-5%, III – BC-Chit-5%, IV – BC-Mg-5%, V – BC-PDO-5%, VI – BC-TCP-5% (the presented pictures are representative for five specimens).

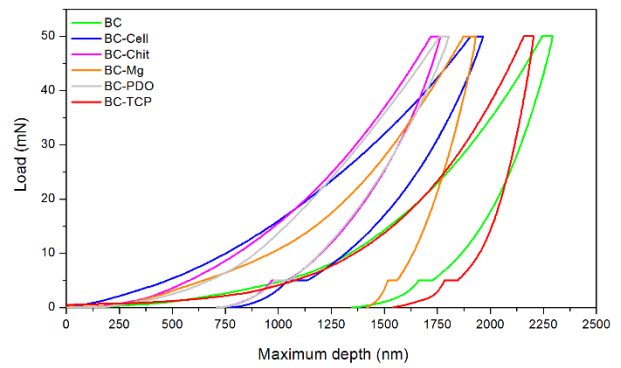


SFig. 3. Mechanical properties of testes bone cement after a month exposure to the PBS solution (the presented pictures are representative for five specimens).

nanoindentation curves / control



nanoindentation curves / exposure to PBS



SFig. 4. Load-displacement curves of the tested bone cements before and after a month exposure to the PBS solution (the presented curves are representative for 10 experiments).