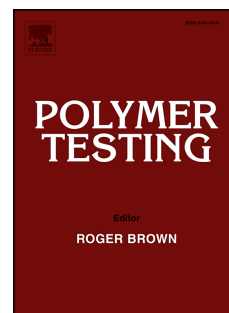


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Biomechanical testing of bioactive bone cements – a comparison of the impact of modifiers: antibiotics and nanometals

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Abstract

Apart from its bone filler and fracture stabilizing function, bone cement can be used as a carrier of bioactive substances, and such modified bone cement can protect the implant against microorganisms, treat local infections and combat bacteria introduced during the surgical procedure. In this paper, the effects of modifying antibiotics and nanosilver on the biomechanical properties of bone cement were examined. The following tests were carried out: curing time, wettability, microhardness, porosity, microstructure and mechanical tests. Additionally, preliminary tests on bactericidal properties in the form of bacterial growth inhibition zones were conducted. No negative impact of bioactive modifications on cement properties was observed, except for bending strength in bone cement with antibiotics. Unmodified bone cement and nanosilver-loaded cement fulfilled all of the requirements specified in the standards and assumptions regarding their biofunctionality. Antibiotic-loaded cement provided a greater range of bioactivity. Attention should be paid to the potential effects of nanosilver as regards the lack of bacterial resistance, prevention and destruction of biofilm structure and length of bioactivity. Bone cement containing nanometals can serve as an alternative to the bioactive bone cements that are currently in use.

Key words

bone cement; bioactive; nanoparticles, mechanical properties; antibacterial activity; biodegradation.

1. Introduction

Generally, bone cements are self-polymerizing biomaterials that are widely used in orthopedic treatment, traumatology and oncological, spinal or maxillofacial surgery. Their task is to stabilize complicated fractures, fix implants and repair bone defects [1-3]. Bone cement also transfers the load from the prosthesis to the bone and can be used as an implant coating or as a carrier matrix for a bioactive substance [4-6]. The following types of bone cement can be distinguished: polymeric, hydrogel, phosphate-calcium, bioactive acrylic and composite [6]. These materials must be biocompatible and must have sufficient mechanical properties [7]. In recent years, bioactive agents have been additionally used to improve the above properties. Bone cement should stimulate bone healing and lead to osteointegration, but it should also fight off any potential local infections and possess prophylactic activity [8-10]. Antibiotics are mainly used to give the cement bactericidal properties [11,12], although experimental tests on metal ions (e.g. Ag, Cu, Zn), xylitol, chitosan particles or nanoparticles of silver have also been conducted [13-15]. Yet modifications of bone cement with bioactive agents can result in poor biomechanical properties, e.g. the cement will not be able to fulfill its function, may be damaged due to mechanical stress and might succumb to aseptic loosening of the implant [16-18]. At present, only antibiotic-loaded bone cements are being



used commercially, although this is not an ideal solution due to the reduced mechanical properties of bone cement, short duration of the antibacterial effect and the non-negligible risk of ineffectiveness due to antibiotic-resistant bacteria [15,18]. There may also be distinct adverse reactions to high doses of antibiotic therapy, e.g. in the local aspect in a decrease in osteoblast replication (dose >400 ug/ml of tobramycin or >1000 ug/ml of vancomycin) or in the death of cells (at a dose of order 10 000 ug/ml), or in the systematic aspect, such as nephrotoxicity or ototoxicity [5,6,19]. On the other hand, nanosilver shows high activity against a wide range of microorganisms and a large therapeutic window [20].

This study aimed to compare modified polymethylmethacrylate (PMMA) bone cement with the local delivery of bioactive substances, either antibiotic or nanosilver, and their biomechanical properties. The search for new solutions for infection therapy seems particularly important, thus it was decided to continue research on the effects of nanometals on the biological and mechanical properties of bioactive bone cement.

2. Materials and methods

2.1 Cement preparation

In this study, three groups of PMMA bone cement specimens were prepared and examined. These were: 1) unmodified bone cement, 2) antibiotic-loaded bone cement and 3) bone cement modified with nanometals. As a starting point, the commercially available bone cements Cemex and Vancogenx (Tecres, Italy) were used. Silver nanoparticles (MkNano, Canada) were used for the modification with nanometals. Their size was 50 nm and purity was 99.9%. Unmodified and antibiotic-loaded cements were prepared following the manufacturer's instructions and according to international standards ASTM F451:99 and ISO 5833:2002 [21,22]. For the nanosilver-loaded cement the protocol was modified to include a further step. First, the modifier was added to the powder and mixed for 3 min by hand. The final composition of the tested specimens is presented in Tab. 1. The nanometals concentration was selected based on previous studies [23-25].

Tab. 1. Chemical composition of bone cements used for the research

	Unmodified Bone Cement	Antibiotic-loaded bone cement	Bone Cement modified with nanometals
Powder component:			
Polymethyl methacrylate	84.30% w/w	81.80% w/w	83.46% w/w
Barium sulphate	13.00% w/w	10.00% w/w	12.87% w/w
Benzoyl peroxide	2.70% w/w	1.50% w/w	2.67% w/w
Gentamicin sulphate	-----	4.20% w/w	-----
Vancomycin hydrochloride	-----	2.50% w/w	-----
Nanoparticles of silver	-----	-----	1.00% w/w
Liquid component:			
Methyl Methacrylate	99.10% w/w	98.20% w/w	99.10% w/w
N, N-dimethyl-p-toluidine	0.90% w/w	1.80% w/w	0.90% w/w

Hydroquinone	75 ppm	75 ppm	75 ppm
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The bone cements were prepared by combining the liquid component with powder in a bowl and hand mixing to a paste at an average speed of 2 revolutions per second. Then the paste was placed into molds to ensure that the required shape was formed and it was allowed to cure for 1 h in ambient conditions. The specimens were removed from the molds, wet ground to the required dimensions using 400 grit silicon carbide paper and left to cure for 24 h before testing. Specimens were prepared in different shapes depending on the test method, e.g. rectangular beams sized $2 \times 3 \times 20 \pm 0.1$ mm for bending, rectangular beams sized $4 \times 6 \times 8 \pm 0.1$ mm for compression, and cylindrical specimens 15 ± 0.1 mm in diameter and 3 ± 0.1 mm thick for the hardness test and bioactivity research. Specimens intended for testing the surface topography and measuring the contact angle were also wet ground using 2000 grit silicon carbide paper, polished using a $0.1 \mu\text{m}$ alumina suspension (AP-FF suspension, Struers A/S, Denmark) and cured for 24 h in ambient conditions. An example of the specimens is shown in Fig. 1.



Fig. 1. Sample specimens used in the research

2.2 Characterization of bioactivity

Measurement of both the contact angle and the bacterial growth inhibition zone was conducted to determine the bioactivity of bone cement. An optical tensiometer (Attention Theta Life, Biolin Scientific, USA) was used to examine surface hydrophilicity. The measurements were carried out using purified water (grade I) and the static sessile drop method. A combination of five bacterial strains was used for the antibacterial assays. These strains were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Enterobacter cloacae* and *Pseudomonas aeruginosa*, selected because they are the most common sources of orthopedic infections. Each strain of bacteria was incubated separately and then added to the bacterial suspension. A total of $100 \mu\text{l}$ of the suspension was seeded on Mueller-Hinton agar plates. The final concentration of bacteria, i.e. the inoculum, was 1.5×10^8 CFU ml^{-1} . Before testing, the specimens were sterilized in an autoclave at 120°C for 30 min. The experiment was performed using three specimens for each type of bone cement. The inhibition zone test consisted of placing the specimens, i.e. the cement disk (10 mm in diameter, 2 mm thick), in the bacteria plates and incubating them at 37°C . The entire experiment lasted 7 days, and measurement of the inhibition zone was carried out after 3, 24, 48 and 72 hours. The bacterial growth inhibition zone was determined as an area without bacterial growth. The area of bactericidal activity was assessed with the naked eye, and a

biological microscope (Axio Observer D1, ZEISS, Germany) was used to analyze the bacterial medium.

2.3 Physical characterization

The setting time was measured and topography analysis was performed in order to identify the bone cement's physical properties. The surfaces of the samples for this test were polished and covered by carbon using a carbon coater. A scanning electron microscope (Joel JSM-5500, Japan) was used for observations of the microstructure. The setting time test was performed using a Vicat needle apparatus (ZI-1004, India) with a tip diameter of 1 mm and a 400 g load. Setting of the bone cement was considered complete when the indentation mark on the surface was no longer visible.

2.4 Mechanical characterization

The following mechanical tests were carried out for each group of bone cements: three-point bending, compression and Vickers hardness. A minimum of five specimens was used for each mechanical test ($N=5+$). The compression tests and three-point bending were performed using a Universal Materials Testing Machine (LRX, Lloyd Instruments Ltd., UK). The following parameters were selected for testing: for three-point bending the span length was 15 mm and the loading rate was 1 mm/min; for compression the extension rate was 1 mm/min. Vickers hardness tests were conducted using a Vickers hardness tester (Duramin Hardness Tester, Struers, Denmark). The hardness press time was 15 s and the three press load values were 490.6 mN, 1.96 N and 2.94 N.

2.5 Statistical analysis

Statistical analysis of the data was performed using commercial software (SPSS Statistics 24, IBM Corporation, USA). The Kolmogorov-Smirnov test was used to assess normal distribution of the data. All of the results were presented as mean \pm standard deviation (SD) and were statistically analyzed using one-way analysis of variance (one-way ANOVA). A comparison between two means was performed using Tukey's test with statistical significance set at $P < 0.05$.

3. Results

3.1 Microstructure analysis

The topography of the studied bone cements is shown in the image in Fig. 2 and in the SEM images in Fig. 3. All of the obtained materials were characterized by high porosity. The antibiotic-loaded bone cement was the most porous. The pores are shown in the material by

filling them with alumina powder in the polishing step. The size of a single pore was estimated to be in the range of 10-220 μm .

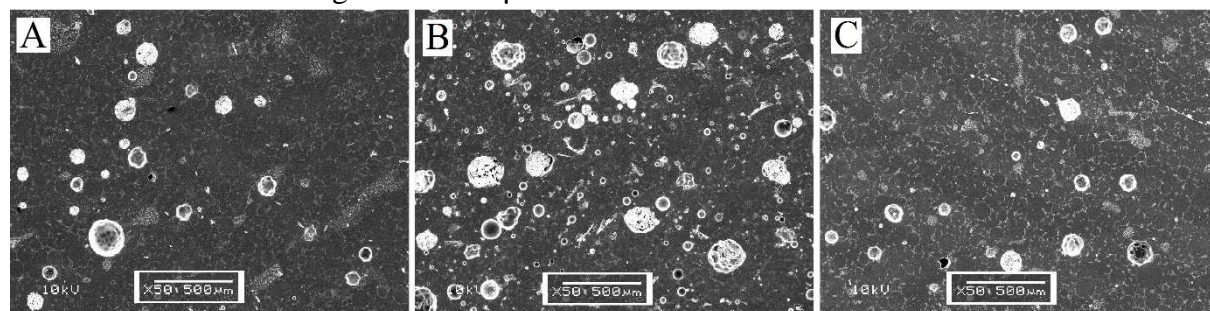


Fig. 2. Topography of the obtained specimens: A) unmodified bone cement, B) antibiotic-loaded bone cement and C) bone cement modified with nanometals (SEM – mag. 50x).

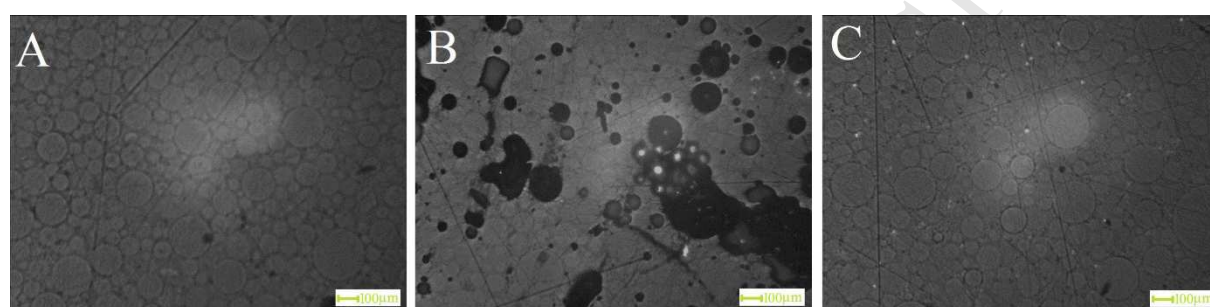


Fig. 3. Topography of the obtained specimens: A) unmodified bone cement, B) antibiotic-loaded bone cement and C) bone cement modified with nanometals (LM – mag. 10x).

3.2. Setting time

All groups of bone cements showed comparable setting times (Tab. 2, Fig. 4). The average values for bone cement were 15:36 (range: 14:56–15:47), 14:14 (range: 13:36–14:41) for antibiotic-loaded bone cement and 15:43 (range: 14:53–16:02) for bone cement with nanosilver.

Tab. 2. Setting time of the tested specimens (mean \pm SD; n=5)

Setting time [min]		
Unmodified Bone Cement	Antibiotic-loaded bone cement	Bone Cement modified with nanometals
15:36 \pm 0:28	14:14 \pm 0:26; ^a	15:43 \pm 0:32; ^b
^a significantly different from unmodified bone cement (ANOVA $p < 0.05$)		
^b significantly different from antibiotic-loaded bone cement (ANOVA $p < 0.05$)		

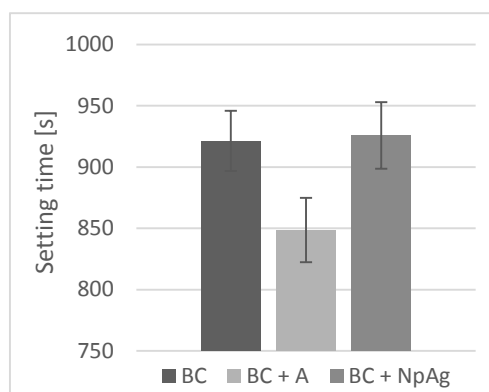


Fig. 4. Setting time of the tested specimens

3.3. Bioactive properties of bioactive bone cements

3.3.1. Determination of the contact angle

Similar results were obtained for all specimens in the range of the contact angle (Tab. 3, Fig. 7). The average value for bone cement was 41.8° , 43.0° for antibiotic-loaded bone cement and 40.4° for bone cement with nanosilver. The sample measurements are shown in Fig. 6.



Fig. 6. Sample measurements of the contact angle for the tested specimens: A) unmodified bone cement, B) antibiotic-loaded bone cement and C) bone cement modified with nanometals

Tab. 3. Values of the contact angle for the tested specimens (mean \pm SD; n=5)

Value of the contact angle [°]								
Unmodified Bone Cement			Antibiotic-loaded bone cement			Bone Cement modified with nanometals		
Time			Time			Time		
1s	5s	10s	1s	5s	10s	1s	5s	10s
41.9±2.7	41.7±2.8	41.5±2.8	43.9±2.3	43.2±2.9	42.0±1.2	40.9±2.9	41.0±2.9	39.9±3.1
41.8±2.5			43.0±2.2			40.4±2.6		
^a significantly different from unmodified bone cement (ANOVA p<0.05)								
^b significantly different from antibiotic-loaded bone cement (ANOVA p<0.05)								

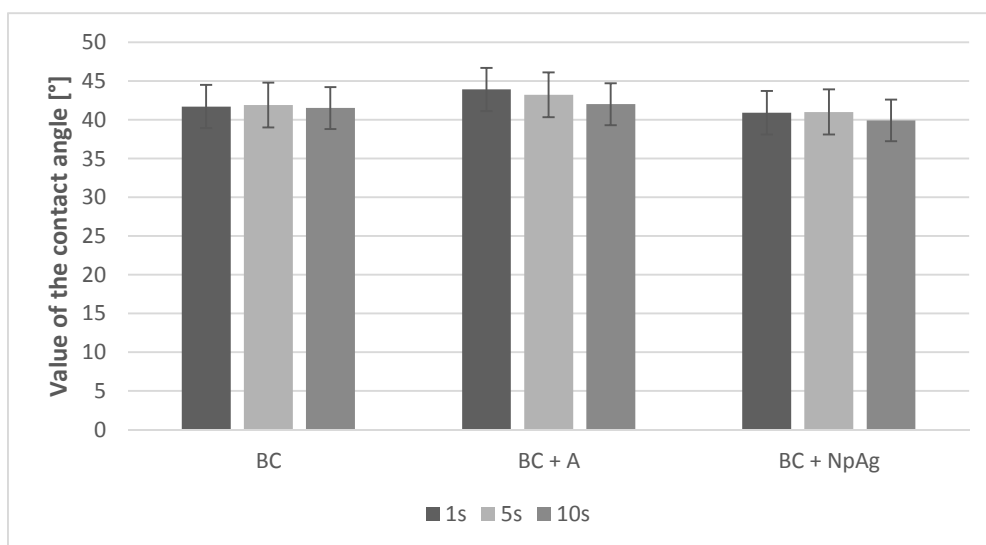


Fig. 7. Comparison of the contact angle results for the tested specimens

3.3.2. Determination of the bacterial growth inhibition zone

Vivid and live bacteria were found in the bone cement test and dead bacteria were found in the test on bioactive cements (red circles). The bacterial growth inhibition zones for the tested specimens are shown in Fig. 8.

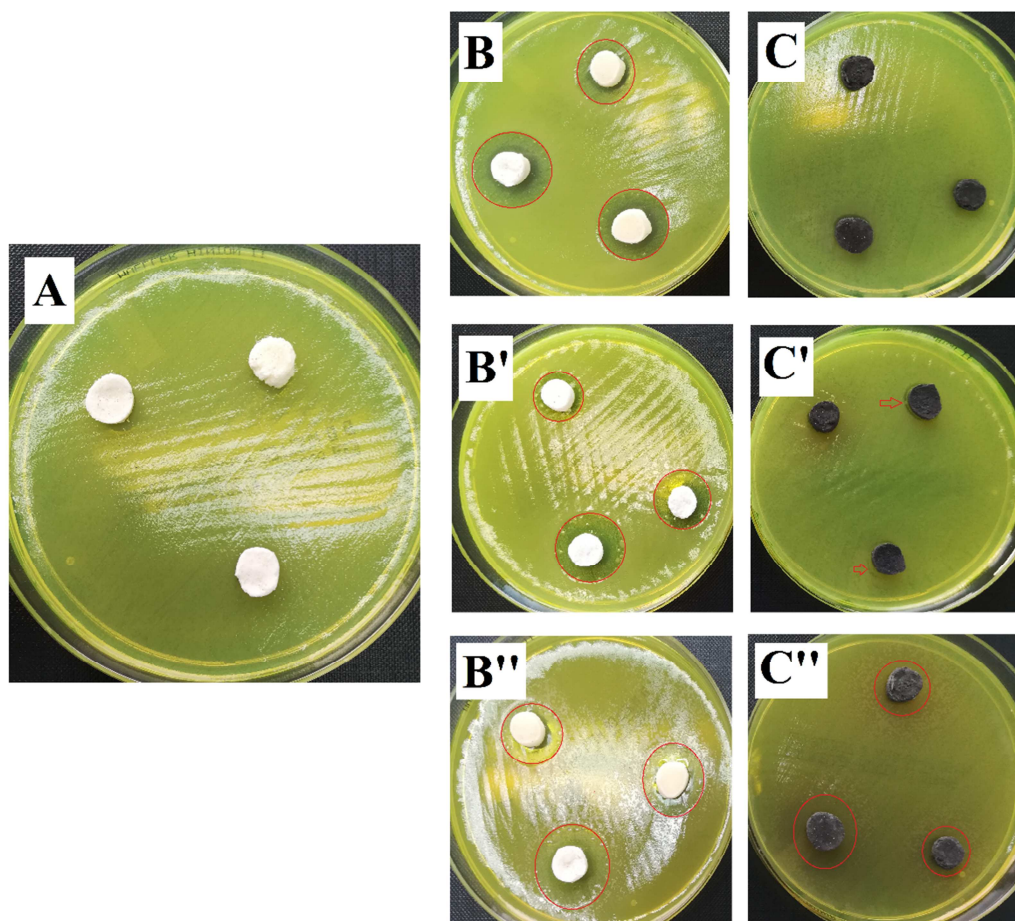


Fig. 8. Comparison of the bacterial growth inhibition zones for the tested specimens: A- unmodified bone cement after 72h, B) antibiotic-loaded bone cement and C) bone cement modified with nanometals; B,C - after 24h, B',C' – after 48h and B'',C'' – after 72 h; red circle – visible growth inhibition zone

3.4 Biomechanical tests

3.4.1. Compression test

A static compression test was carried out for various bone cement samples. The obtained results are presented in Tab. 4. The specified Young's modulus for all kinds of bone cements was ca. 1.37 GPa, and different values were obtained for stiffness and stress. The stiffness values were $40.43 \cdot 10^5$ N/m for bone cement, $40.08 \cdot 10^5$ N/m for antibiotic-loaded bone cement and $41.01 \cdot 10^5$ N/m for bone cement with nanosilver. Resistance to compression was ca. 101.76 MPa for bone cement, 102.35 MPa for antibiotic-loaded bone cement and 99.85 MPa for bone cement with nanosilver. Exemplary compression tests are shown in Fig. 9.

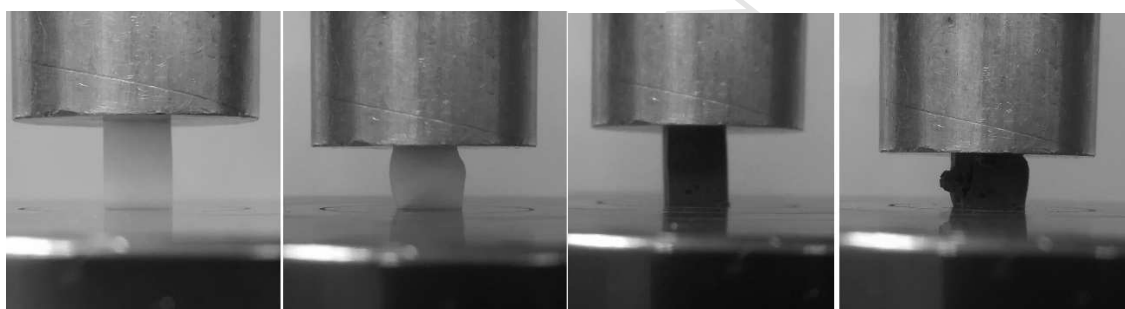


Fig. 9. Sample compressive strength test for the tested specimens: white – unmodified bone cement, black – bone cement modified with nanometals

Tab. 4. Compression test results for the tested specimens (mean \pm SD; n=5)

Compression test		
Unmodified Bone Cement	Antibiotic-loaded bone cement	Bone Cement modified with nanometals
Compression strength [MPa]		
101.76 ± 0.49	102.35 ± 1.11	$99.85 \pm 1.22^{a,b}$
Stiffness [$\cdot 10^5$ N/m]		
40.43 ± 0.16	40.08 ± 0.51	41.01 ± 0.41^{b}
Young Modulus [GPa]		
1.37 ± 0.01	1.37 ± 0.02	1.36 ± 0.02
^a significantly different from unmodified bone cement (ANOVA $p < 0.05$)		
^b significantly different from antibiotic-loaded bone cement (ANOVA $p < 0.05$)		

The average values of compression strength were calculated and are presented in Fig. 10.

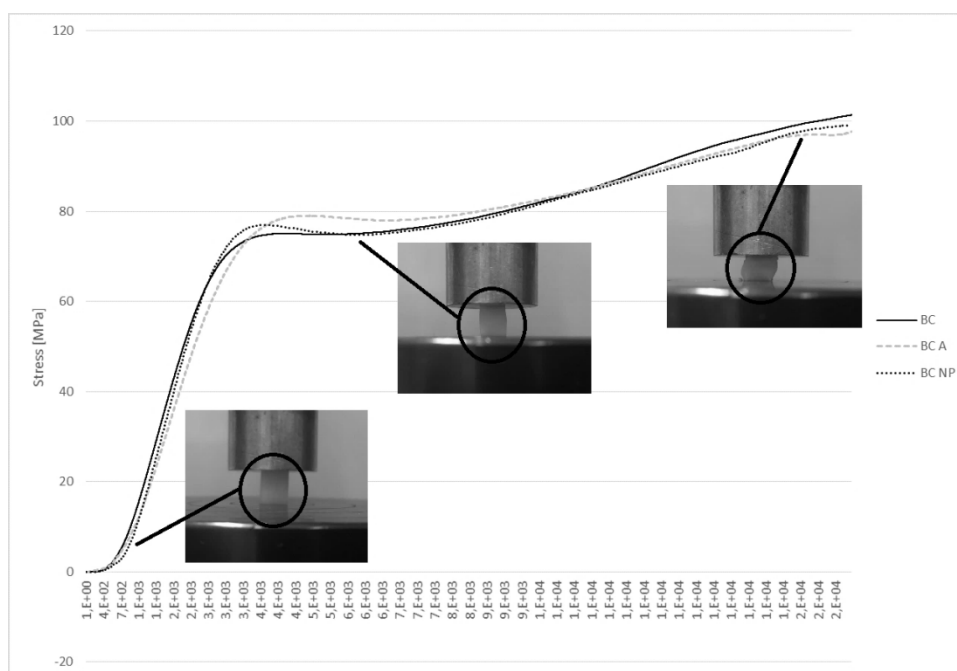


Fig. 10. Comparison of exemplary compressive strength diagrams for the tested specimens (in the image – unmodified bone cement specimen)

3.4.2. Three-point bending

A three-point bending test was carried out on the bone cements. The obtained results are presented in Tab. 5. The specified Young's modulus for all kinds of bone cements was statistically significantly different, i.e. 1.81 GPa for bone cement, 1.30 GPa for antibiotic-loaded bone cement and 2.12 GPa for bone cement with nanosilver.

Tab. 5. Three-point bending test results for the tested specimens (mean \pm SD; n=15)

Three-point bending		
Unmodified Bone Cement	Antibiotic-loaded bone cement	Bone Cement modified with nanometals
Bending Stress (MPa)		
51.56 \pm 12.05	35.23 \pm 6.54; ^a	60.13 \pm 8.61; ^{a,b}
Elongation at Fracture (mm)		
0.54 \pm 0.08	$\Sigma=0.58 \pm 0.08$	0.52 \pm 0.06; ^{a,b}
Stiffness (kN/m)		
52.43 \pm 7.52	38.57 \pm 10.55; ^a	57.47 \pm 10.79; ^b
Young's Modulus (GPa)		
1.81 \pm 0.28	1.30 \pm 0.35; ^a	2.12 \pm 0.29; ^{a,b}
^a significantly different from unmodified bone cement (ANOVA $p < 0.05$)		
^b significantly different from antibiotic-loaded bone cement (ANOVA $p < 0.05$)		

The average load data are shown in Fig. 11.

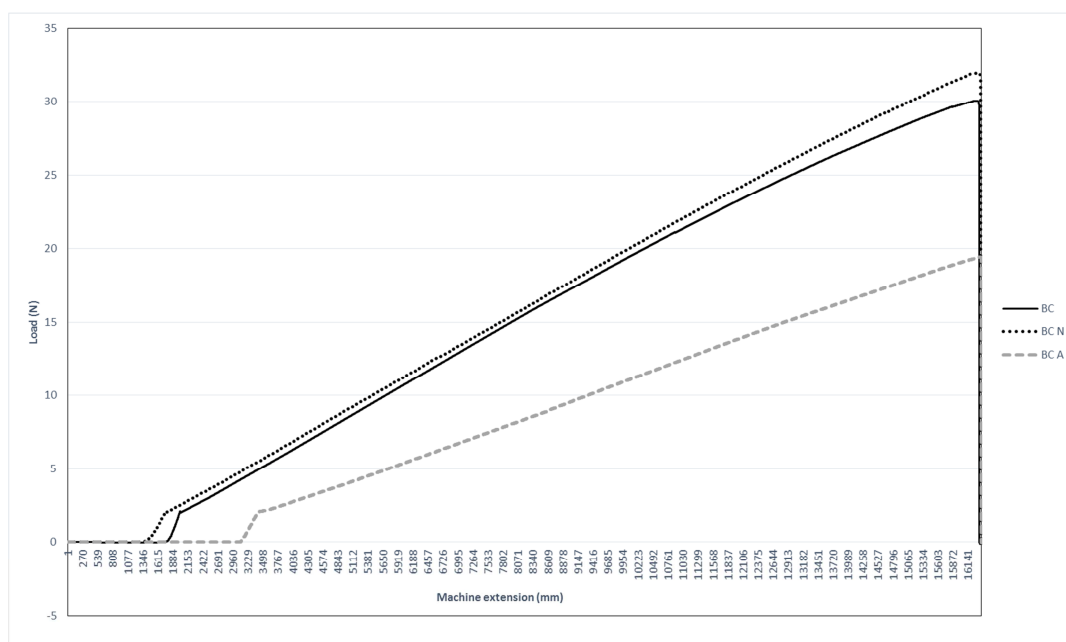


Fig. 11. Comparison of exemplary bending strength diagrams for the tested specimens

An analysis of the fractures obtained in the bending test was conducted. The images are shown in Figs. 12 (LM) and 13 (SEM). Microscopic analysis of the fractures allowed to classify them as fragile for all kinds of specimens. Plastic cracking areas were also noted. The occurrence of this phenomenon indicates that the polymerization process was not completely homogeneous, and it appears that the cracks started at many points along the circumference and spread towards the center.



Fig. 12. Sample images of the surface topography after fracture for the tested specimens: A) unmodified bone cement, B) antibiotic-loaded bone cement and C) bone cement modified with nanometals (LM - mag. 30x)

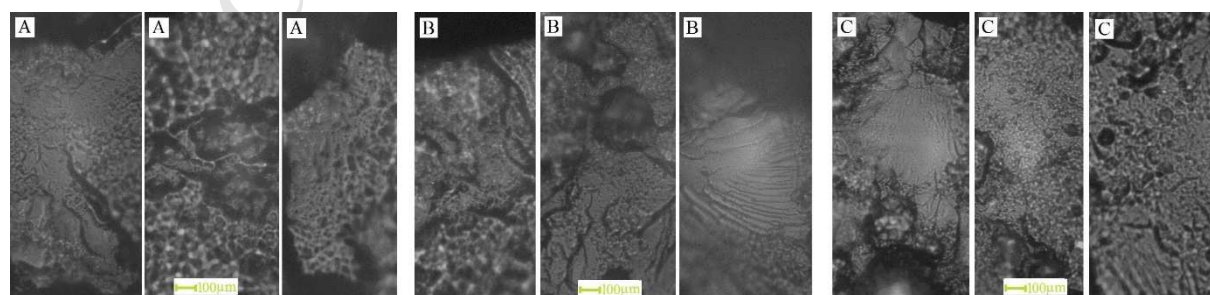


Fig. 13. Sample images of the surface topography after fracture for the tested specimens: A) unmodified bone cement, B) antibiotic-loaded bone cement and C) bone cement modified with nanometals (LM - mag. 10x)

3.4.3. Hardness test

Vickers hardness was measured ($n=5$) for the three kinds of bone cement. The results are presented in Fig. 14. This value was 21.8 HV for bone cement, 20.4 HV for antibiotic-loaded bone cement and 22.2 HV for bone cement with nanosilver. The images in Fig. 15 show the indenter's imprint.

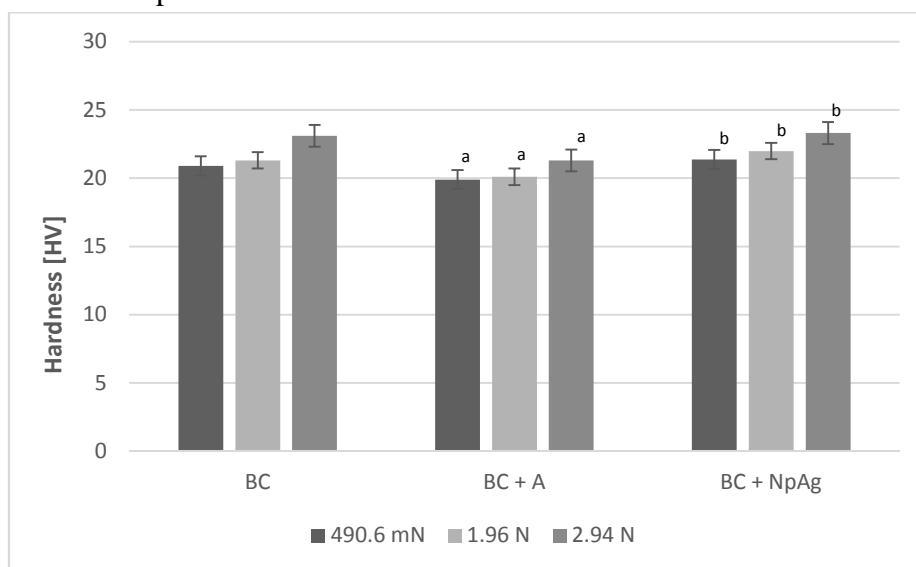


Fig. 14. Comparison of the hardness results for the tested specimens (^a means significantly different from unmodified bone cement (ANOVA $p<0.05$); ^b means significantly different from antibiotic-loaded bone cement (ANOVA $p<0.05$))

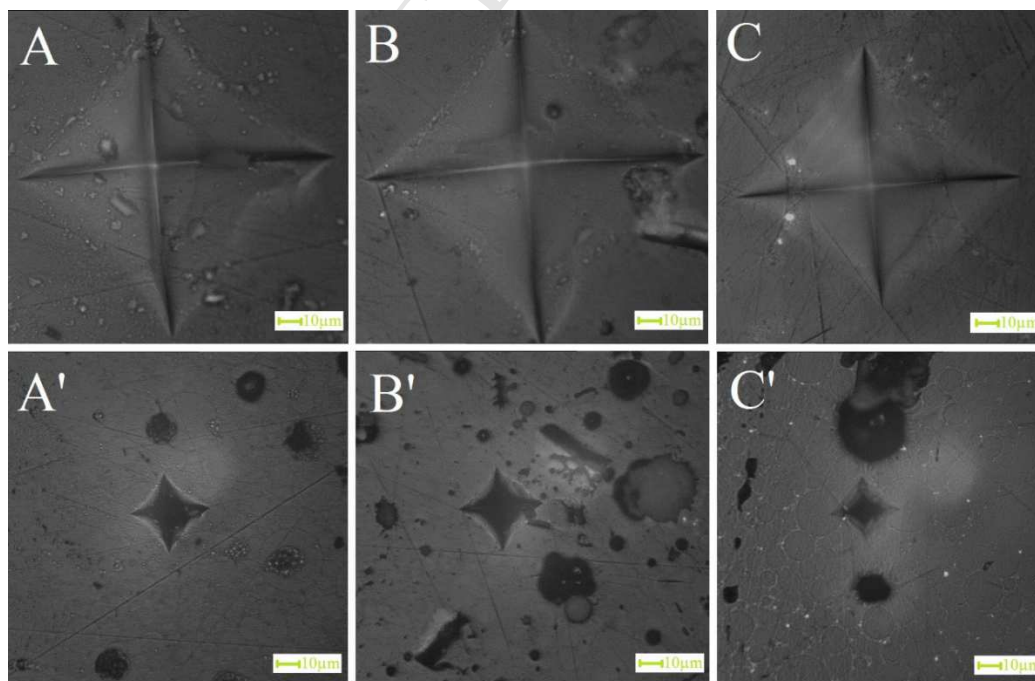


Fig. 15. Sample images of the imprints after the hardness tests for the tested specimens: A)

unmodified bone cement, B) antibiotic-loaded bone cement and C) bone cement modified with nanometals; A,B,C – 2.94N, 15s and A',B',C' – 490 mN, 15s

4. Discussion

The biocompatibility of a biomaterial, such as bone cement, is affected by its surface characteristics, namely by chemical composition, surface roughness and wettability [26]. In this study, in order to avoid any differences in chemical composition, bone cement based on PMMA from only one manufacturer was used. This cement was factory-modified through the addition of antibiotics (4.20% w/w gentamycin and 2.50% w/w vancomycin) and manually modified with nanosilver (1.00% w/w). Recently, bone cement containing double antibiotics has been the main choice among clinical specialists [27]. Hence it was decided to compare its biomechanical properties with pure, unmodified bone cement. The authors of this paper also replaced the antibiotics with other bioactive substances, in this case with nanosilver.

Bone cement is characterized by a porous structure with a system of channels and corridors [28,29]. All of the bone cements obtained in this study had such a structure, with pore size in the range of 10-220 μm , which was confirmed by the LM and SEM examination (Figs. 2 and 3). However, the biomaterials' porosity affected both their mechanical properties and bioactivity [12,13,14]. The bioactive property (depending on wettability or porosity) allows bone cells to deposit and proliferate, but it also allows the active substance to be applied to the material and its local release [11,15]. Some studies have shown that the active substance deposited in the pores is released mainly from the surface, yet due to bodily fluids and channels it is also eluted from inside. According to previous reports, the kinetics of release reaction correlated to a greater extent with porosity than with the amount of the active substance [11]. The release of the bioactive substance was also influenced by the type and quantity of this substance and by the process of how the bone cement had been prepared [30]. In the analysis of the bone cements' microstructures it was found that the antibiotic-loaded bone cement showed the most porous structure, and no effect of nanosilver on porosity was observed.

Another important feature of bone cement that allows for bone cells to be embedded is wettability [14,15], which is determined by measuring the bone cement's contact angle. The values of the contact angles for the bone cements were similar (40-43°), and such values are sufficient to ensure bioactivity as regards the osteointegrative process [31].

A key parameter in the application of bone cement is its setting time, i.e. the time when, after mixing the ingredients, the cement is formable and can be implanted in the body. This time period cannot be either too short or too long. All of the tested bone cements showed comparable values of their setting times. The average value for unmodified bone cement was 15:36 min (range 14:56–15:47), 14:14 min (range 13:36–14:41) for antibiotic-loaded bone cement and 15:43 min for bone cement with nanosilver (range 14:53–16:02).

As for the bactericidal aspect and bioactive properties of bone cement, the gold standard has been the addition of antibiotics [11,12]. However, bacteria are becoming increasingly more resistant to their effects [32]. Another issue is biofilm, which is a specific structure created by a bacterium that protects it and improves adhesion to any surface. Attempts to combat the infection via antibiotic therapy are then drastically weakened. In this

case, the required dose of antibiotics is 200-1000 times higher than for a normal bacterial colony [32,33,37]. Given the above, other solutions are currently being sought, such as nanosilver modification as presented in this study. Yet the aim of this study was not to check the modification's bioactivity but rather its impact on the biomechanical properties. In order to confirm their effectiveness only, the bacterial growth inhibition zone and that zone's microbiological analysis were tested. In the literature, only few studies were found in which the bactericidal activity of nanosilver in bone cement had been tested. Bactericidal activity was not detected in those studies and only bacteriostatic properties were found. Nevertheless, the effect of nanosilver on the formation of biofilm has been confirmed [34,35]. The growth inhibition zone was defined as 0 mm [34]. In vivo tests were also carried out, thus modified bone cement and bacterial colonies of *Staphylococcus aureus* were implanted in a rabbit's body. There was no impact on the prevention of infection [36]. In our research there was an inhibition zone and it oscillated between 3-10 mm (after 48 h). An analysis of the medium from this zone confirmed that the bacteria were dead. The differences may have been due to the type of nanosilver used, the method of its addition to the cement matrix, the porosity and combination of cement pores as well as the bacteria that were used for testing. Despite the differences in the above studies, nanometals are supposed to be an alternative for preventing both bacterial deposition on the surface and creation of the biofilm structure as well as for combating multi-resistance bacteria [20,34].

Unmodified bone cement and bone cement with nanosilver fulfilled the requirements of the standards for acrylic cements. The minimum values of the biomechanical test should be 70 MPa for compression strength, 50 MPa for flexural strength and 1.8 GPa for the specified Young's modulus [21]. The antibiotic-load bone cement did not fulfill the requirements for flexural strength and the Young's modulus. In the literature, the compressive strength of pure cement was estimated to be in the range of 60-100 MPa [12,14,37]; for cement containing an antibiotic it was approx. 60-70 MPa [11,14]. Bending strength for pure cement was 45-75 MPa and 40-50 MPa for antibiotic-loaded [12,37,38]. The determined Young's modulus for pure bone cement had a value of 2.8-3.5 GPa [34,37]. The strength values did not differ significantly in the case of the nanosilver modification and were as follows: flexural modulus 2.9 GPa and flexural strength 69 MPa [34]. Russo et al. (2017) put forward the hypothesis that nanometals improve the mechanical properties but there can also be 'weak points' [39]. The research presented in this paper does not allow us to refer to the above hypothesis, although a slight increase in the mechanical properties was observed.

The negative impact of both antibiotics and nanosilver is a constant topic of discussion in the literature, and it has been accepted that both the concentration and the released dose have an effect. In the case of bone cement, the insertion of a bioactive modifier in the polymer matrix reduces toxicity and allows for the application of a relatively constant dose in time [20,40]. However, this issue requires further research and was the main limitation of our study, as more bioactivity tests need to be carried out.

5. Conclusion

Apart from its task of filling bone defects or stabilizing fractures, bone cement can serve as a carrier for active substances. This paper examines the effects of bioactive



modifications (antibiotics and nanometals) on the biomechanical properties of bone cement. Significant differences were found in the setting time, compression strength, bending strength, Young's modulus and hardness, and there were no statistically significant differences in wettability (values of the contact angle). It was shown that bone cement with antibiotics did not fulfill the requirements specified in the standards.

The study presented here confirms that modifying bone cement with nanosilver does not interfere with its biomechanical properties, and such modified cement fulfills the requirements set out in the standards and assumptions required for its biofunctionality. Bone cement with nanosilver should be a better alternative as compared to antibiotic-loaded bone cement. Although this modification showed a much smaller bacterial growth inhibition zone. Attention should also be paid to nanosilver's potentially wide spectrum of activity, preventive effect on biofilm and long-term effectiveness.

Further research on the impact of bioactive modifications to and biomechanical properties of bone cement is necessary.

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Highlights

- This article examines the effect of bioactive modifications (biantibiotics and nanosilver) on the biomechanical properties of bone cement;
- There were significant differences in the aspect of: setting time, compression strength, bending strength, Young's modulus and hardness;
- There were no statistically significant differences in the aspect of: wettability (values of contact angle);
- Antibiotic-loaded bone cement showed a much bigger zone of inhibition of bacterial growth, but silver-loaded bone cement also has antibacterial activity, but in smaller range;
- Bone cement with antibiotics did not meet all the requirements specified in the standards.
- The modification of bone cement with nanosilver does not interfere with biomechanical properties,
- Bone cement containing nanometals is to be an alternative to currently used bioactive bone cements.