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To cite this article: F. Maciąg et al 2023 J. Electrochem. Soc. 170 082501

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Influence of Mesoporous Bioactive Glass Particles Doped with Cu and Mg on the Microstructure and Properties of Zein-Based Coatings Obtained by Electrophoretic Deposition

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Cu- and Mg-doped mesoporous sol-gel bioactive glasses (MSGG) were developed and their microstructure was investigated. Selected biological tests were also performed to assess their suitability for the functionalization of polymer zein coatings. The electrophoretic deposition (EPD) conditions to obtain composite coatings on Ti-13Nb-13Zr substrates were determined. The coatings with excellent adhesion to the substrates were macroscopically uniform, exhibiting open porosity and high roughness. The surfaces of the samples coated with MSGG/zein coatings became more hydrophilic than the titanium alloy substrates. However, with increase of the volume fraction of glass particles in the coatings, the contact angle increased. The coated alloy showed enhanced resistance to electrochemical corrosion in Ringer's solution. The chemical composition of the glass used in the coating affected the parameters of wettability and resistance to electrochemical corrosion. Both coating types showed an increase in the antibacterial properties against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria compared to pure zein. However, no significant differences were observed after a 24 h-long test.

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Manuscript submitted May 20, 2023; revised manuscript received July 20, 2023. Published August 4, 2023.

Supplementary material for this article is available online

Metallic biomaterials are required to exhibit biocompatibility and high electrochemical corrosion resistance.¹ The group of materials that meet these requirements includes titanium and its alloys.² Other notable properties of metals for orthopedic applications are low density, high mechanical strength, and relatively low Young's modulus.^{4,5} Particular attention is paid to β alloys, including near- β , due to their lower Young's modulus, excellent corrosion resistance, comparable strength, and better biocompatibility compared to other types of Ti alloys.⁶ The alloys from the Ti-Nb-Zr system rich in the β phase show a low modulus of elasticity and contain non-toxic alloying elements.⁷ The presence of Nb stabilizes the β phase and decreases the alloy's modulus of elasticity, while Zr increases the corrosion resistance due to the formation of a stable oxide layer.^{7,8} Recently, research interest in the Ti-13Nb-13Zr alloy has increased. The modulus of elasticity of conventionally used metallic biomaterials in biomedical engineering such as stainless steels (SS) (210 GPa), Co–Cr alloys (204–240 GPa) and $\alpha + \beta$ titanium alloys (100-120 GPa) are significantly higher than the modulus of human bone (5-30 GPa). However, the Ti-13Nb-13Zr alloy has a modulus of elasticity (66–92 GPa) close to bone.^{9,10} Despite the excellent properties of titanium biomaterials, making them a reasonable choice for bone implants, they are also inert to the human body.¹¹ In addition, biofilms can form on their surface, leading to serious infections and slowing of the osseointegration process between the bone tissue and the implant.¹² By applying coatings containing bioactive ingredients to the alloy surface, it is possible to influence the biological properties and improve the adhesion between the bone and the implant.¹³ For this purpose, composite coatings consisting of a biodegradable polymer matrix containing bioactive ceramic particles can be fabricated.^{14,15}

In the literature, many coatings fabricated from biodegradable polymers have been described for biomedical applications. Among these materials, interest in zein is increasing.^{16,17} It is a material of

natural origin, made of corn protein.¹⁸ It can be used, inter alia, for the transport of drugs and as the matrix for composite coatings.¹ The most important properties of zein are its biocompatibility and non-toxicity to the human body.²⁰ Zein is a class of prolamine proteins that are soluble in aqueous alcohols (70%-90%).² Generally, the classification of zein depends on its solubility and molecular weights: α (21–25 kDa), β (17–18 kDa), γ (18 kDa) and δ -zein (10 kDa),²² with the highest percentage being α -zein (around 70%) and the rest are respectively γ - (around 20%), β - (around 5%) and δ -zein (around 5%).²³ The structure of α -zein consists of α helices, which are made of repetitive homologous segments. Helices contain hydrophobic amino acids, such as proline, alanine and leucine, as well as hydrophilic residues, such as glutamine.^{24,25} The bioactive properties of zein, in terms of biomineralization capability, are poor.²⁶ Therefore, for this purpose, bioactive ceramics are introduced into zein. The addition of ceramics to zein also increases the mechanical strength of the coatings. The available literature includes information on the use of hydroxbioactive mesoporous glass.²⁷ hydroxyapatite,²⁸ silica,²⁹ or dense bioactive glass particles^{24,30} as bioactive components in zein coatings. In biomedical engineering, interest in silica-based bioactive glass is growing due to its bioactivity, osteoinductivity and antibacterial effects.

It is well known³² that long-term bone implants should have a permanent connection to bone tissue. For this reason, interest in porous materials, including coatings, in biomedical engineering has increased significantly. Pores also have a positive effect on bone tissue regeneration.¹³ Mesoporous materials are gaining great interest, due to their high specific surface area, high osteoinductivity and bioactivity.³³ Furthermore, the material may be doped with drugs or antimicrobial ingredients, and mesoporosity increases the interaction of implants with physiological fluids.^{31,34} Doping bioactive glass particles to give them additional unique properties is a growing practice. By adding ions of therapeutic elements, bioactive, osteogenic or antibacterial properties can be enhanced or inducted.^{35,36} This type of element includes, for instance, Cu, which



has antibacterial properties and a positive effect on bone development.³⁷ Furthermore, it supports angiogenesis and has limited cytotoxicity.³⁸ Another element that supports bone tissue is Mg, which stimulates osteoblast proliferation and regulates calcium transport in the body.^{39,40}

There are many methods of fabricating composite coatings for biomedical applications on metallic substrates, but electrophoretic deposition (EPD) has been attracting increasing interest recently.⁴¹ It is an electrochemical method that allows the co-deposition of ceramic and polymer materials and their combinations on the surface of electrically conductive materials.^{28,42} By adjusting the deposition time and the type and value of the current voltage during EPD, it is possible to control the thickness as well as influence the morphology of the coatings.⁴³ Furthermore, this method allows the coating to be deposited on complex-shaped components.⁴⁴ During the deposition process, particles dispersed in a colloidal solution or suspension and having a specific electric charge, under the influence of the applied electric field, move in the dispersing phase towards the oppositely charged electrode (substrate) and settle on its surface.^{45,46}

Among the available literature, there are not many publications reporting the EPD of zein coatings incorporating mesoporous sol-gel bioactive glasses. Batool et al.²⁷ reported a successful attempt at EPD of zein-based coatings containing mesoporous bioactive glass nanoparticles doped with Ag-Sr (Ag-Sr MBGNs) on 316 L stainless steel substrates. The suspension used consisted of zein (6 wt%), distilled water (20 wt%), ethanol (74 wt%), acetic acid (~10 ml to reduce the pH to \sim 3) and Ag-Sr MBGNs (3*10⁻³ kg dm⁻³) added to a 100 ml beaker. The voltage was in the range of 10-25 V, while the deposition time was 3 min. They obtained uniform composite coatings with homogeneously distributed Ag-Sr MBGN particles with their spherical agglomerates. The coatings after the bend test adhered well to the substrate surface and also showed wettability properties that favor the initial protein and subsequent osteoblast cell attachment. The coatings also improved the resistance of the substrate to electrochemical corrosion in the SBF solution and showed a high wear resistance. The available literature also includes publications that contain information on the deposition of zein composite coatings with bioactive ceramics.^{24,42} Meyer et al.²⁴ reported the EPD of copper-doped bioactive glass (CuBG)/zein coatings on a 316 L stainless steel substrate. They showed that hydroxyapatite formed easily on the coating surface and that the addition of Cu to the BG had an effect on the degradation process of zein, slowing it down. This is probably due to a cross-linking effect of the released Cu ions. Rivera et al.⁴² investigated the antibacterial, pro-angiogenic and pro-osteointegrative properties of CuBG/zein coatings. Their biological studies showed that coatings had osteoconductive and anti-infective properties. They also indicated that copper had pro-angiogenic properties and did not affect the proosteogenic activity of BG in-vitro. Both of the above-mentioned works revealed that it is possible to deposit composite zein-based coatings with bioactive glass, which enhances the biological properties of 316 L stainless steel substrates.

Our previous work⁴⁷ focused on EPD conditions to obtain homogeneous mesoporous sol-gel glass (MSGG)/zein coatings with high adhesion to titanium alloy substrates. The glass used had a reference chemical composition (70 SiO₂, 26 CaO, 4 P₂O₅, % mol) and did not contain Mg and Cu. The microstructure of the coating consisted of relatively uniformly distributed glass particles in a zein matrix and exhibited open porosity. The coatings improved the resistance of the titanium biomaterial substrates to electrochemical corrosion in Ringer's electrolyte at 37 °C and provided bioactivity. The composite coating showed a reduction of Grampositive S. aureus and Gram-negative E. coli bacteria compared to pure zein coatings. Following that previous study, the aim of the present work was to focus on developing the electrophoretic codeposition of zein with mesoporous sol-gel glass particles doped with Cu (MSGG-Cu) as well as with both Cu and Mg (MSGG-Cu-Mg). The adhesion strength was studied by using the standardized tape test method. The microstructure, surface topography, wettability

and surface free energy of the materials were also investigated. The effect of therapeutic elements in the mesoporous bioactive glasses on the resistance to electrochemical corrosion, antibacterial effect, bioactivity and cytocompatibility of the coated alloy was determined.

Material and Methods

The substrate material was near- β Ti-13Nb-13Zr titanium alloy, which was supplied in the form of a 30 mm diameter bar by Shaanxi Yunzhong Industry Development Co., Ltd., China. The bar was cut into discs about 2.5 mm thick, which were then subjected to mechanical treatment by grinding on 1200 grit sandpaper.

The two types of prepared MSGG doped with Cu as well as Cu and Mg simultaneously had the following chemical compositions (in mol %): 70 SiO₂, 25-x CaO, 5 P₂O₅ + x CuO (hereinafter referred to as MSGG-Cu) and 70 SiO₂, 25-x CaO, 5 P₂O₅ + x CuO + 5 MgO (x = 1-3) (hereinafter referred to as MSGG-Cu-Mg).

The sol-gel method with the supramolecular chemistry approach followed by the evaporation induced self-assembly (EISA) process was used to obtain the above-mentioned mesoporous bioactive glasses. The precursors used for synthesizing the bioactive glasses were as follows: Tetraethoxysilane (TEOS; $Si(OC_2H_5)_4$) acting as a source of silica, triethyl phosphate (TEP; $OP(OC_2H_5)_3$) as a source of P_2O_5 , calcium nitrate tetra-hydrate (Ca(NO₃)₂ * 4H₂O) as a source of CaO, copper (II) nitrate trihydrate $Cu(NO_3)_2 * 3H_2O$ as a source of CuO, and magnesium nitrate hexahydrate (Mg(NO₃)₂ * 6H₂O) as a source of MgO. Two types of non-ionic amphiphilic triblock copolymer: Pluronic[®] P123 (for MSGG-Cu) and Pluronic[®] F1279 (for MSGG-Cu-Mg), were used as the structure directing agent. The bioactive glass synthesis protocol was based on our previous work.⁴⁷ Firstly, the 5.5% (w/v) surfactant solution was prepared by completely dissolving the relevant Pluronic in ethanol. After obtaining a clear solution, HCl (catalyst for the hydrolysis and condensation reactions) was slowly added followed by 5 min of stirring before adding TEOS. The TEP was added after 30 min of stirring of the mixture. Then at every 30 min the precursors of CaO, CuO and MgO were subsequently added in two different ways: for MSGG-Cu the nitrates were added directly to the synthesis without prior dissolution in distilled water, while for MSGG-Cu-Mg respective salts were dissolved in H₂O (DI) to avoid precipitation of magnesium/copper salts. After 24 h of stirring, the solution was transferred to previously prepared Petri dishes made of polystyrene and covered with a lid. Dishes with the solution were homogenized under ambient conditions. The gelation process lasted 28 d. The obtained gel was then dried in an oven at 40 °C for 7 d followed by multi-stage drying to 120 °C. Thus, dried samples at 700 °C for 3 h at a heating rate of 2.5 °C min⁻¹ were calcined in an air atmosphere. The glass powders were then milled. Finally, particles with size d_{50} 1.9 μ m were gained.

The measurement of the specific surface area (S_{BET}) of the glass particles was carried out applying the N₂ adsorption method using BET analysis (Nova 1200e, Quantachrome). The morphology and microstructure of the particles were examined with a Nova NanoSEM450 scanning electron microscope (SEM) (Thermo Fisher Scientific, USA) and a JEM-2010 ARP transmission electron microscope (TEM) (JEOL, Japan). TEM samples of glasses were prepared using drops of ethyl alcohol with dispersed glass particles placed onto a copper grid covered with carbon and left to dry.

The powder form of zein was supplied by Merck (Poland). As a dispersing phase for EPD, anhydrous ethyl alcohol (EtOH) of 99.8% purity, glycerol (both from POCH, Poland) and distilled water were utilized.

To prepare the zein solution, a mixture of EtOH with the addition of glycerol (Table I) was first prepared by stirring it using a magnetic mixer (IKA, Germany). The next step was to gently pour the zein powder weighed on an OHAUS Corporation PA214CM/1 model (Switzerland) into the mixing solution (Table I). The solutions were left in the stirrer until the zein dissolved. Then, the beaker with the

Table 1	Table I. Data of suspensions employed for EPD.									
No	MSGG-Cu and MSGG–Cu–Mg [kg dm ⁻³]	Zein [kg dm ⁻³]	Ethyl alcohol [vol%]	Distilled water [vol%]	Glycerol [wt%]	pH of suspension with MSGG–Cu	pH of suspension with MSGG–Cu–Mg			
1	0.01	0.2	90	10	20	6.63	5.94			
2	0.04	0.2	90	10	20	7.10	6.68			
3	0.08	0.2	90	10	20	7.77	7.57			

zein solution was placed in an ultrasonic bath (POLSONIC Sonic-3, Poland) in order to break up the remaining zein agglomerates. Finally, a portion of the MSGG–Cu or MSGG–Cu–Mg powders was gently added to the zein solution (Table I). The solution was stirred and the beaker with the zein suspension with MSGG's particles was placed in an ultrasonic bath to remove any remaining glass agglomerates. The pH of the prepared suspensions was measured using a pH-meter, ELMETRON CPC-505 (Poland). Table II summarizes the conditions under which suspensions were prepared and the parameters used for electrophoretic deposition.

Measurement of zeta potential and conductivity of suspensions with MSGG–Cu and MSGG–Cu-Mg particles was carried out using a Malvern Zetasizer Nanodevice (Malvern Instruments Ltd., UK) applying a laser Doppler velocimetry (LDV) technique. The device was also equipped with a titrator. Standard solutions of hydrochloric acid (HCl) and sodium hydroxide (NaOH) of various concentrations were used to change the pH of the solutions studied during measurements. In order to not disturb the work of the laser, diluted suspensions consisting of $4*10^{-4}$ kg dm⁻³ MSGG-Cu or MSGG-Cu-Mg, $2*10^{-3}$ kg dm⁻³ of zein, $0.24*10^{-3}$ kg dm⁻³ of glycerol and ethyl alcohol - distilled water mixture in ratio of 9/1 were used for the measurements. The value of the potential was determined using the Debye–Hückel equation.

The EPD cell consisted of a two-electrode system. A 316L-SS plate was used as the counter electrode and the working electrode was a disc made of Ti-13Nb-13Zr alloy. The electrodes were spaced 10 mm apart. An EX752M multimode power supply (AIM-TTI, UK) was used to generate the constant DC voltage.

In order to assess the adhesion of the coatings to the substrates, the tape test was carried out, which was performed in accordance with the ASTM D3359–17B standard. Tape tests involved making a grid with a six-blade knife on the sample surface using two perpendicular cuts. Then a standardized tape was glued to the grid area and pressed against the pencil with an eraser, left for 60 s and torn off at an angle of 90°. The adhesion evaluation was based on observation of the grid by the unaided eye. The adhesion class was assigned according to the ASTM standard classification.

Microstructure of the coatings was investigated using SEM, TEM and scanning-transmission electron microscopy (STEM). A Thermo Fisher Scientific Tecnai TF20X-TWIN microscope was used for TEM and STEM investigation. The percentage of elements was analyzed using energy dispersive X-ray spectroscopy (EDS) in both SEM and TEM. Element distribution images of the coatings were obtained in STEM mode using a high angle annular dark-field (HAADF) detector in combination with an EDS microanalyzer. For the investigation of coatings by TEM, samples in the form of lamellas were prepared using the focused ion beam (FIB) method (QUANTA 3D200i, Thermo Fisher Scientific, USA).

The volume fraction of MSGG-Cu and MSGG-Cu-Mg in the coatings was determined. The coatings were peeled off, placed in ceramic crucibles and heated at a temperature of approximately 500 °C for 60 min, which led to the degradation of zein. The crucible was weighed before and after heating, assuming that only glass particles remained, and knowing the densities of MSGG-Cu and MSGG-Cu-Mg and zein (2.5 g cm⁻³, 2.5 g cm⁻³ and 1.23 g cm⁻³, respectively), the volume fraction of glasses in the coatings could be determined.

Surface roughness measurements of the coatings and substrates were made with a WYKO NT930 optical profilometer (Veeco,

USA). Wettability measurements of the samples were conducted with a Krüss DSA25E goniometer (Germany). The value of surface free energy (SFE) was determined using the Owens - Wendt - Rabel -Kaelble (OWRK) procedure.

Electrochemical investigations of corrosion resistance of the uncoated alloy along with the coated alloy were conducted in Ringer's electrolytes solution at 37 ± 0.5 °C using a multichannel electrochemical workstation, namely AUTOLAB PGSTAT128N potentiostat/galvanostat (by Metrohm Autolab, the Netherlands) with the use of the potentiodynamic polarization scan technique. The Ringer's solution was composed of 8.6 g l^{-1} of NaCl, 0.3 g l^{-1} of KCl, 0.25 g l^{-1} of CaCl₂, with pH adjusted to 7.4. A threeelectrode electrochemical cell arrangement was adopted, with a working electrode in the form of investigated sample, a saturated calomel electrode (SCE) acting as a reference electrode and a counter electrode in the form of platinum mesh. The open-circuit potential (OCP) as function of time was recorded, which was followed by the linear sweep voltammetry (LSV) experiment with the scan rate of 1 mV s⁻¹ at the potential window from -2.5 V to 2.2 V (vs SCE). For the EIS data, the amplitude of excitation signal was 10 mV and the frequency range was 10^5 Hz to 10^{-3} Hz. The EIS results were further processed using AUTOLAB NOVA software (by Metrohm Autolab, the Netherlands).

The bioactivity of the MSGG-Cu and MSGG-Cu-Mg particles was assessed by immersion and storage in simulated body fluid (SBF). A protocol developed by Kokubo⁴⁸ was used to prepare the artificial plasma. Powder samples were incubated for 1, 3, 7, 14 and 21 d at 37 °C in powder weight/SBF volume ratio of 1/500. After each measurement point, the samples were washed with anhydrous EtOH and dried at room temperature (RT) for FTIR examination. The changes of the concentration of elements (Ca, Si, P, Cu, Mg) contained in bioactive glasses storage in artificial plasma were evaluated by inductively coupled plasma atomic emission spectrometry (ICP-OES; Plasma 40, Perkin Elmer, USA).

Preliminary in-vitro cytotoxicity measurements of the coatings were performed using MG-63 cells (human osteosarcoma cell line). As the control, cell culture plate was used. The prepared samples were placed in a 48-well plate and sterilized using ultraviolet (UV) light on each side for 1 h, respectively. The cell culture process was performed in cell culture polystyrene flasks, and the medium was DMEM (Dulbecco's modified Eagle's medium, Gibco, Schwerte, Germany), supplemented with 10 vol% Fetal bovine serum (FBS, Sigma-Aldrich) and 1 vol% Penicillin/streptomycin (PS, Pen-Strep; Sigma-Aldrich). The cell monolayer was separated from the flask wall when it reached approx. 80% of confluency (after 2 d of incubation). A trypsin-EDTA solution (Life Technologies, Darmstadt, Germany) in phosphate buffer saline (PBS, Gibco, Germany) was used to separate the cells. To inhibit the effect of the trypsin solution, fresh medium was added to it. The detached cells were counted in a hemocytometer by the trypan blue exclusion method (Sigma-Aldrich, Taufkirchen, Germany). Then, the obtained cell suspension was used to cover the sterilized samples. The samples were placed in an incubator for 24 h and 72 h at 37 °C in 5% CO_2 atmosphere to allow the cells to grow.

The WST-8 assay was used to assess cell viability. After 24 and 72 h of incubation, the old medium was poured from the wells and the samples were washed with PBS and then supplemented with 0.7 ml of fresh DMEM containing 1 vol% WST-8 reagent. After 24 and 72 h, the medium was transferred to a 96-well plate in 3 aliquots

Tal	ole	II.	Conditions	of m	ixtures	preparation	n and	deposition	parameter	'S.
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No	Stirring time [min]	Dispersion time [min]	Stirring velocity [rpm]	Deposition voltage [V]	Deposition time [min]
1	$90^{a)} + 10^{b)}$	$30^{a)} + 10^{b)}$	1000	3, 5, 7, 10	5
2	$90^{a)} + 10^{b)}$	$30^{a)} + 10^{b)}$	1000	3, 5, 7, 10	5
3	$90^{a)} + 10^{b)}$	$30^{a)} + 10^{b)}$	1000	3, 5, 7, 10	5

a) time for the preparation solution of zein. b) time for the preparation suspensions with MSGG particles.

of $100 \ \mu$ l. Then, the absorbance at 450 nm was measured with a microplate reader. The viability of MG-63 cells was determined by the following equation:

$$Cell \ viability[\%] = \frac{OD_{sample} - OD_{blank}}{OD_{reference} - OD_{blank}} * 100$$

with:

 OD_{sample} = optical density of sample at 450 nm wavenumber,

 OD_{blank} = optical density of WST reactant at 450 nm wavenumber,

 $OD_{reference}$ = optical density of respective positive control at 450 nm wavenumber.

According to the supplier's protocols, live staining with DAPI (40,6-diamidin-2-phenylindole) and Calcein AM (Life Technologies, Darmstadt, Germany) was used to qualitatively assess cell morphology and viability. The images were made with a fluorescence microscope (FM) (Axio Scope A1, Carl Zeiss Microimaging GmbH, Jena, Germany). The antibacterial performance of MSGGs against E. coli and S. aureus was evaluated by using turbidity measurements. The bacteria were cultured in a lysogeny broth medium (LB) (Luria/Miller) at 37 °C and the samples were incubated in the LB at the concentration of 10 mg ml^{-1} for 24 h. The cultivated LB was then centrifuged at 4200 rpm for 10 min and the supernatants were collected. Each well of 24-well-plate was filled with 1 ml of MSGG particle/LB and 15 µl of Gram-positive bacterial suspension or 10 μ l of Gram-negative bacterial suspension and then incubated at 37 °C for 3 h, 6 h and 24 h. The wells were also filled with a pure medium and pure medium with bacteria as references. After certain times, 100 ml of each well was added in a 96-well plate. A microplate reader (PHOmo, Anthos Mikrosysteme GmbH, Germany) was used to determine the optical density (OD) at 600 nm. Three samples of each condition were tested. The viability of the bacteria was calculated as follow:

Bacteria viability[%] =
$$\frac{OD_{sample} - OD_{medium}}{OD_{control} - OD_{medium}} * 100$$

with:

 OD_{sample} = optical density of sample at 600 nm wavenumber,

 OD_{medium} = optical density of medium at 600 nm wavenumber, $OD_{control}$ = optical density of control bacteria at 600 nm wavenumber.

Alamar Blue assay by using Gram-positive *S. aureus* and Gramnegative *E. coli* was further performed to assess the antibacterial potential of the coatings. After sterilization under UV-light, the substrates were covered with 1 ml bacterial suspension (OD at 600 nm = 0.001) in 24-well-plate and incubated in agitation (90 rpm) at 37 °C. After 90 min the eluates were removed and 1 ml fresh LB was added. After 24 h of incubation at 37 °C the samples were moved to new well-plates in order to ensure that only the bacteria attached to the surface of the sample will be considered during the measurement. Following the protocol provided by the manufacturer, the reduction of Alamar Blue reagent [%] 1 was determined.

Reduction of Alamar reagent [%]
=
$$\frac{(E_{oxi,600 * A_{570}}) - (E_{oxi,570 * A_{600}})}{(E_{red,570 * C_{600}}) - (E_{red,600 * C_{570}})} * 100$$
 [1]

with:

 $E_{oxi,570}$ = molar extinction coefficient of oxidized AlamarBlue at 570 nm = 80586

 $E_{oxi, 600} =$ molar extinction coefficient of oxidized AlamarBlue at 600 nm = 117216

 $E_{red, 570} =$ molar extinction coefficient of reduced AlamarBlue at 570 nm = 155677

 $E_{red, 600} =$ molar extinction coefficient of reduced AlamarBlue at 600 nm = 14652

 A_{570} = absorbance of test wells at 570 nm

 A_{600} = absorbance of test wells at 600 nm

 C_{570} = absorbance of negative control well (media, AlamarBlue Reagent, no cells) at 570 nm

 C_{600} = absorbance of negative control well (media, AlamarBlue Reagent, no cells) at 600 nm

The metabolism of bacteria during growing involves the conversion of Alamar Blue from its non-fluorescent oxidized blue state to its fluorescent reduced pink state. Thus, the percentage of reduction of Alamar Blue reagent indicates the level of bacterial cell growth, meaning that antibacterial materials induce a low reduction of Alamar Blue reagent.

The results of the statistical analysis were expressed as mean \pm standard deviation (SD) of three replicates. The data were subjected to one-way analysis of variance (ANOVA) and mean comparisons were performed by Bonferroni correction. P values less than 0.05 were considered statistically significant. OriginPro 2021b was used for all analyses.

Results and Discussion

In the present work, electrophoretic deposition was employed for the development of zein coatings incorporated with Cu- and Mgdoped mesoporous sol-gel bioactive glasses to induce bioactive and antimicrobial properties of Ti-13Nb-13Zr alloy substrates. The first



Figure 1. SEM images of (a) MSGG-Cu and (b) MSGG-Cu-Mg particles.

	MSG	G-Cu	MSGG-Cu-Mg		
Element/X-ray line	wt%	at%	wt%	at%	
Ο Κα	44.6 ± 0.6	61.7 ± 0.5	42.7 ± 1.1	59.9 ± 1.0	
Si K α	35.0 ± 0.3	27.6 ± 0.4	33.9 ± 0.1	27.1 ± 0.4	
P K α	1.2 ± 0.1	0.8 ± 0.1	2.0 ± 0.1	1.5 ± 0.04	
Ca K α	15.3 ± 0.1	8.4 ± 0.1	13.7 ± 0.7	7.7 ± 0.5	
Cu K α	4.0 ± 0.2	1.4 ± 0.1	5.8 ± 0.3	2.0 ± 0.1	
Mg K α	_	_	1.9 ± 0.0	1.8 ± 0.0	

Table III. Quantitative data of SEM-EDS analysis for glass particles. Microanalysis was carried out for an area of $430 \times 550 \ \mu m^2$ (accelerating voltage 18 kV, spot size 2, WD 5 mm).

Table IV. Change in the content of elements in SBF through soaking	ng
of the MSGG particles after incubation for 21 d.	

Sample/Days	Ca	Р	Si [mg 1^{-1}]	Cu	Mg
SBF	84.2	22.8	0.6		38.1
MSGG-Cu/1d	199.5	< 0.5	44.9	47.6	_
MSGG-Cu/3d	177.4	< 0.5	49.4	48.7	_
MSGG-Cu/7d	201.1	< 0.5	44.5	48.8	
MSGG-Cu/14d	189.7	< 0.5	45.6	48.9	
MSGG-Cu/21d	197.2	< 0.5	43.0	48.7	
MSGG-Cu-Mg/1d	175.6	< 0.5	43.8	48.0	61.4
MSGG-Cu-Mg/3d	156.2	< 0.5	44.0	46.3	62.1
MSGG-Cu-Mg/7d	155.5	< 0.5	45.6	48.6	62.2
MSGG-Cu-Mg/14d	154.3	< 0.5	43.0	48.7	63.1
MSGG-Cu-Mg/21d	153.7	< 0.5	42.7	49.0	63.1

section will present the elaboration of the EPD conditions, including kinetics and mechanism, to obtain robust composite coatings. This allows for obtaining coatings with excellent adhesion strength for the characterization of microstructure, morphology, surface topography and surface properties. In the second section, titanium alloy substrates with the most promising coatings were investigated in terms of their effect on the resistance to electrochemical corrosion in Ringer's solution, antibacterial activity, bioactivity and cytocompatibility.

Doped mesoporous sol-gel glasses: microstructure and properties .- The MSGG-Cu and MSGG-Cu-Mg bioactive glasses had particles in irregular shape and size range of $0.3-3.4 \,\mu\text{m}$ (Fig. 1). The mean particle size determined with the laser diffractometer was 2.1 µm. Specific surface area measurements of glass particles showed high values: $324 \pm 2 \text{ m}^2 \text{ g}^{-1}$ for MSGG-Cu and 326 ± 2 m² g⁻¹ for MSGG-Cu-Mg. The SEM-EDS chemical microanalysis of the particles confirmed the occurrence of Si, Ca, P, O, Cu, and Mg (Table III). Mesoporous sol-gel bioactive glasses exhibit unique textural properties, high surface area, high bioactivity and biocompatibility.^{49,50} Therefore, they are very important materials for bone implants. In the present work, to reveal the mesoporous structure, both glasses, MSGG-Cu and MSGG-Cu-Mg, were characterized by TEM. They showed the presence of highly oriented hierarchical mesoporous channels. Exemplary bright-field (BF) TEM images of MSGG-Cu and MSGG-Cu-Mg particles are shown in Figures S1 and S2, respectively. Several images were taken using an electron beam (EB) perpendicular and parallel to the channels, in the [110] and [100] directions. The investigated particles of both glasses were in the range of 0.05 μ m to around 2 μ m and demonstrated a 2D hexagonal mesoporous structure with p6mm symmetry.

The mesopore size was investigated based on a fast Fourier transformation (FFT) of the experimental BF TEM images and a

reconstruction of inverse FFT (IFFT) images from streaks present in the FFT patterns. The mesopore size was determined in the IFFT images of several particles as the full width at half maximum of peaks measured on their intensity profiles along the line perpendicular to their walls. A typical example of determining the size of the mesoporous channels is shown in Fig. S3. The pore sizes of the investigated MSGG-Cu and MSGG-Cu-Mg particles are in the range 1.3–2.4 nm and 2.3–2.6 nm, respectively.

The bioactivity of the glasses was assessed by incubation in simulated body fluid for 21 d. In the case of both types of MSGG, the change in the concentration of their fundamental elements was similar. The SBF solution, in which the MSGG-Cu and MSGG-Cu-Mg particles were incubated, was subjected to ICP-OES analysis (Table IV). The study was performed with the distinction of several incubation times.

Calcium content in SBF increased rapidly after 1 d of incubation. Subsequent measurement points (3, 7, 14 and 21 d) showed slight decreases in content. On the other hand, the content of phosphorus already at the beginning (1 d) decreases below the detection level. The adsorption of P from SBF occurred as a result of the precipitation of calcium phosphate, which limited changes in the content of Ca ions. The concentration of silicon increased significantly after 1 d of incubation. Slight fluctuations in the concentration at subsequent time points were caused by the growing layer of CaP on the glass particles, which slowed the release of Si ions. The presence of the Ca/P layer on the particles was confirmed by FTIR (Fig. 2). Already after 1 d of incubation of the studied powders in SBF, the appearance of a significant amount of Cu ions and an increase in Mg were observed. FTIR spectra (Fig. 2) revealed some changes after incubation in SBF: new double bands in the range of 560–610 cm^{-1} characteristic for bending vibrations of PO_4^{3-} groups in HA appeared after just 1 d of immersion for MSGG-Cu. A new characteristic band of CO_3^{2-} bending vibrations at 873 cm^{-1} proved the crystallization of hydroxyapatite with carbonate substitution-CHA. For MSGG -Cu-Mg, the changes mentioned above occur over a longer period of time-3 d; probably this effect is related to the chemisorption of Ca and Mg and delayed crystallization of CHA. 51,52 The band at 570 cm⁻¹ after 1 d of incubation is related to P-O bonds in the amorphous phosphate structure.

In general, MSGG particles showed antibacterial properties against Gram-negative *E. coli* and Gram-positive *S. aureus* over time (Fig. 3). After the first three hours of incubation, the reference MSGG glass showed the highest antibacterial properties. This can be especially observed in the case of interaction with Gram-negative bacteria (p > 0.05). Presumably, doped particles dissolve more slowly, which also affects the pH changes in the experimental environment. After a lapse of time, the MSGG-Cu ($p \le 0.05$) and MSGG-Cu-Mg ($p \le 0.05$) glasses began to show increased antibacterial properties. This was due to the release of antibacterial ions over time. After 24 h, all glasses revealed similar activity against *E. coli*. In the case of Gram-positive bacteria, a much higher activity of glasses containing Cu and Mg is visible after 24 h compared to the reference glasses.



Figure 2. FTIR spectra of the MSGG-Cu (a) and MSGG-Cu-Mg (b) particles after incubation in SBF for various times.



Figure 3. Bacterial viability of *E. coli* (a) and *S. aureus* (b) after 6 h and 24 h of incubation. MSGG-Cu and MSGG-Cu-Mg particles in comparison with reference MSGG particles. Bacterial suspension was used as the control (100%) for the statistical analysis, * $p \leq 0.05$ (Bonferroni).

EPD conditions and their influence on adhesion of coatings to substrates.—The solubility of zein in the dispersive phase depends on the concentration of ethyl alcohol. Zein is dissolved in hydrated ethanol at a concentration in the dispersive phase in the range of 60%-90%.^{24,26,53} Additionally, the incorporation of glycerin into the solution increases the plasticity of the zein formed base of the coatings.⁵⁴ In our earlier work,⁴⁷ we showed that the solution of EtOH/H₂O in the ratio of 9/1 and 0.024 kg dm⁻³ glycerol, 0.2 kg dm⁻³ zein and 0.04 kg dm⁻³ reference MSGG is optimal for the fabrication of uniform MSGG/zein coatings with high adhesion to the substrates. Therefore, to study the effect of the incorporation of Cu and Mg to glasses on EPD in this study, zein solutions with MSGG-Cu and MSGG-Cu-Mg particles in various contents, but the same as in the case of reference MSGG,⁴⁷ 0.01 kg dm⁻³, 0.04 kg dm⁻³ and 0.08 kg dm⁻³, were used.

One of the requirements for the fabrication of coatings through EPD is a stable suspension. To evaluate the stability of the suspensions, their electrokinetic zeta potential and conductivity were measured. A 100-fold diluted suspension with the following chemical composition: EtOH/H₂O in the ratio of 9/1, with the addition of $0.24*10^{-3}$ kg dm⁻³ glycerol, $2*10^{-3}$ kg dm⁻³ zein and MSGG in three different contents ($0.1*10^{-3}$ kg dm⁻³, $0.4*10^{-3}$ kg dm⁻³), was prepared for measurements in

order to ensure free passage of the laser to the detector through the prepared suspension. In addition, the zeta potential of the MSGG was determined in the zein-free solution. Furthermore, measurements of the zeta potential of suspensions with MSGG particles were made with and without glycerol to examine the effects on the electrokinetic potential. The zeta potential and conductivity for the elaborated suspensions as a function of their pH are shown in Figs. S4 and S5 and in Tables V and VI.

The zeta potential of the MSGG containing suspensions was significantly dependent on their pH. Measurements made for the initial pH of the MSGG suspensions (zein-free) showed relatively low values of zeta potential. The stability rose significantly as the pH of the suspensions decreased or increased. The lowest zeta potential values of the suspension were at around 12 pH. In the range of 7–7.5 pH, an isoelectric point could be observed. In the case of the MSGG-Cu particles, the highest value of the zeta potential could be observed in the suspension with pH in the range of 3–3.5. On the other hand, the MSGG-Cu-Mg particles had the highest electro-kinetic potential in the suspension with the pH range of 5–6. No significant effect of the presence of glycerol in suspensions on their stability was detected.

In suspensions containing MSGG particles and zein, the limits of the zeta potential of suspensions changed slightly depending on pH

		MSGG-Cu			MSGG-Cu-Mg		
	Concentration of MSGG [kg dm ⁻¹]	pH	Zeta potential [mV]	Conductivity [mS cm ⁻¹]	pH	Zeta potential [mV]	Conductivity [mS cm ⁻¹]
glycerol non-glycerol	$\begin{array}{c} 0.4{}^{*}10^{-3} \\ 0.4{}^{*}10^{-3} \end{array}$	8.63 8.58	-3.3 -4.4	0.008 0.01	9.54 9.62	-9.1 -11.4	0.002 0.005

Table V. The zeta potential and conductivity for zein-free suspensions containing MSGG-Cu and MSGG-Cu-Mg particles.

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mS	cm^{-1}]	

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	MSGG-Cu			MSGG-Cu-Mg			
Concentration of MSGG [kg dm ⁻²]	pН	Zeta potential [mV]	Conductivity [mS cm ⁻¹]	pH	Zeta potential [mV]	Conductivity [mS cm ⁻¹	
0.1*10 ⁻³	6.63	6.1	0.10	5.94	5.2	0.5	
$0.4*10^{-3}$	7.10	4.3	0.2	6.68	4.2	0.6	
$0.8*10^{-3}$	7.77	1.5	0.2	7.57	3.2	0.2	

Table VI. The zeta potential and conductivity for suspensions with their initial pH depending on the concentration of MSGG-Cu and MSGG-Cu-Mg particles.

and the isoelectric point and was slightly shifted towards the alkaline reaction (Table V).

The values of zeta potential of all the zein-containing suspensions were relatively low, indicating their relatively low stability (Table VI). Despite the low stability of the suspensions, coatings were satisfactorily deposited on the cathode. As we described in a previous paper,⁴⁷ the mechanism of coating deposition, in which zein binds to MSGG particles, has a significant influence. A similar mechanism occurred in the co-deposition of zein with the MSGG-Cu and MSGG-Cu-Mg particles. The MSGG particles exhibiting a negative charge, bound by the deprotonated part of the zein chains, changed their surface charge to positive. Then, together with zein they migrated to the cathode and formed a coating. Figure 4 presents the scheme of electrophoretic deposition of glass particles and zein from the suspension.

On the basis of observing the as-deposited coatings with the unaided eye, it was possible to conclude that they were transparent and macroscopically homogeneous. During the EPD process, electrolysis of the water contained in the suspensions occurred, which led to the formation of open pores on the surface of the coatings.⁵⁵ The number of pores was also dependent on the concentration of MSGG-Cu or MSGG-Cu-Mg in the suspensions and the applied electrical voltage. Increase in the volume fraction of MSGG particles in the suspension, caused increase of concentration of ions released with dissolution of MSGG particles, which, in turn, resulted in a more intensive electrolysis. On the contrary, increasing the voltage intensified the process of electrolysis of the water contained in the suspension and increased the amount of gas bubbles formed. Analysis of the mass behavior of the coatings with the deposition time (Fig. S6) showed that the increase was almost linear for both types of coatings. At the beginning of the deposition, the weight gain increased rapidly and then gradually decreased until the process was complete.

Coating adhesion tests were carried out using the tape test method. The adhesion strength of zein coatings with MSGG-Cu and MSGG-Cu-Mg glasses was very high (Fig. 5). The classes of adhesion of both coating types depends on concentration of glasses particles in suspensions. The class adhesion of coatings deposited from suspension with the lowest glass concentration was 4B (less than 5% of the area of the coating removed) and 5B (0% of the area of the coating removed) for coatings obtained from suspensions with other glass concentrations.

Morphological and microstructural characterization of coatings.—Further investigations were carried out for the macroscopically homogeneous coatings obtained at a constant voltage of 5 V during 5 min (Fig. 6).

A grid of surface microcracks could be observed on the coatings surface with an increase in volume fraction of MSGG-Cu or MSGG-Cu-Mg particles in the suspensions (Figs. 6b, 6c, 6e, 6f). This is probably due to a shrinkage of zein during drying the coatings. The glass particles and their agglomerates up to 60 μ m large as well as open pores with diameters up to 25 μ m were evenly distributed on the surface of the coatings. The volume fractions of the MSGG-Cu and MSGG-Cu-Mg particles in the coatings obtained from suspensions with their various concentrations are presented in Table VII. The particles content grown with their concentration in the suspension used.

Coatings containing 6 ± 1 vol% (MSGG-Cu/zein) and 7 ± 1 vol% (MSGG-Cu-Mg/zein) were subjected to microstructure analysis on cross-sections using TEM. MSGG particles with their agglomerates embedded in the zein matrix were clearly noticeable in the dense coatings (Figs. 7 and 8).

Closed pores with diameters in the range of 0.1–0.3 μ m were observed mainly in the MSGG-Cu-Mg/zein coatings. These pores are most likely formed during the EPD process. However, the possibility of their formation during the lamella preparation using FIB cannot be excluded. The thickness of the coatings in the observed areas was 9.6 μ m and 7.3 μ m for the MSGG-Cu/zein and



Figure 4. Scheme of the electrophoretic co-deposition mechanism of MSGG-Cu or MSGG-Cu-Mg particles with zein.

MSGG-Cu-Mg/zein coatings, respectively. The STEM-EDS element distribution images revealed the occurrence of Si, Ca, P, O and Cu in the MSGG-Cu particles embedded in the coatings (Figs. 7 and 8). In addition, Mg was present in the MSGG-Cu-Mg/zein coating (Fig. 8).

Effect of the surface topography and composition of the coating on wettability .-- Both composite coatings incorporating MSGG-Cu and MSGG-Cu-Mg particles increased the roughness of the uncoated alloy (Fig. 9). The \hat{R}_a (arithmetic average height) and R_a (root mean square roughness) parameters of the coatings were increased by at least around 15 times for coatings with the lowest concentration of MSGG, and around 10 and 9 times, respectively, for the remaining concentrations of particles with coatings compared to the alloy substrate (Table VIII). On the other hand, R_{max} (maximum height of the profile) increased about 5 times for the coatings with a low MSGG content (4 or 5 vol%) and around 8 times for the coatings with a higher MSGG content (12 vol%) compared to that of the uncoated substrate. The increase in roughness was due to the presence of MSGG particles, their agglomerates and open pores. The decrease in the values of R_a and R_q , together with the increasing concentration of the MSGG particles, is most likely due to the fact that, the particles were more and more packed, which led to smoothing of the coating surface. The R_{max} parameter assumed increasing values due to the increase in coating thickness, which resulted from the larger content of MSGG particles.

The contact angle (CA) and free surface energy (SFE) parameters affect the adhesion of proteins to the material surface.^{56,57} The surfaces of materials with highly hydrophobic properties have high affinity for cells and reduce biocompatibility. In turn, surfaces that show high hydrophobicity block the interaction between cells, which is especially important in tissue engineering. Therefore, it is particularly important to maintain a certain balance in the hydrophilic and hydrophobic properties of the surface.⁵⁸ On the other hand, SFE is responsible for the initial interaction of the material surface with the biological environment, and the adhesion of cells and proteins.⁵⁹ The available literature^{59–62} reports on various contact angle ranges that are considered the most suitable for cell adhesion. The authors inform that the ranges of the contact angle oscillate between 40° – 70° .

In the present work, distilled water was used for the measurements. The CA of the MSGG-Cu/zein coatings compared to the CA of the alloy substrate was lower by approximately 20° (Fig. 10a), while the SFE was higher by approximately 10 mN m⁻¹ (Fig. 10b). The contact angle of the MSGG-Cu/zein coatings compared to the CA of the alloy substrate was lower by approximately 15° - 20° , while the SFE was higher by about 7–9 mN m⁻¹. In the case of



Figure 5. Macroscopic images of MSGG-Cu/zein and MSGG-Cu-Mg/zein coatings obtained from suspensions containing 0.01 kg dm⁻³ (a), (d), 0.04 kg dm⁻³ (b), (e) and 0.08 kg dm⁻³ (c), (f) MSGGs deposited at 5 V and a deposition time of 5 min and surface images of samples after the tape test.



Figure 6. SEM images of coatings obtained from suspensions with 0.01 kg dm⁻³ (a), (d), 0.04 kg dm⁻³ (b), (e) and 0.08 kg dm⁻³ (c), (f) MSGG-Cu or MSGG-Cu-Mg, respectively.

Table VII. Volume fractions of the MSGG-Cu and MSGG-Cu-Mg particles in the coatings.

$C_{\rm excentration} = f MSCC = \pi f(a) = \frac{1}{2} (a - b) = \frac{1}{2}$	Volume fraction of glass [vol%]			
Concentration of MSGG particles in suspension [kg dm]	MSGG-Cu/zein	MSGG-Cu-Mg/zein		
0.01	4 ± 1	5 ± 1		
0.04	6 ± 1	7 ± 1		
0.08	12 ± 1	12 ± 1		



Figure 7. STEM-HAADF image and element distribution images in the MSGG-Cu/zein coating.



Figure 8. STEM-HAADF image and element distribution images in the MSGG-Cu-Mg/zein coating.

MSGG-Cu-Mg/zein coatings, their CA compared to the substrate was lower by approximately 5° - 10° , and SFE was higher by approximately 4–6 mN m⁻¹. Therefore, these coatings have potential for use in orthopedic implants.

The contact angles of both coating types were within the range that is convenient for cell adhesion. Probably, the reduction in CA was influenced by the zein matrix. Although Muthuselvi and Dhathathreyan⁶³ reported that pure zein coatings showed higher CA values by around 70°, in our previous work⁶⁴ we showed that electrophoretically deposited zein coatings on titanium substrates showed lower CA values ($45.3^{\circ} \pm 3.8^{\circ}$). It is assumed that these differences in the CA of zein coatings may result from the presence of glycerol in the coatings. Muthuselvi and Dhathathreyan⁶³ showed

that the addition of a plasticizer in the form of glycerol or sorbitol can significantly reduce the value of the contact angle. They reported that this phenomenon occurs as a result of a two-stage reorientation of the change in the spatial arrangement of atoms of the surface. In the first stage, the macromolecular chains move, and in the next stage, the side chains move and orient themselves on the surface. They mentioned that such changes of orientation are especially occurred in well cross-linked solutions with strong hydrogen bonds. where the polarization is strong enough to diffuse or reorient the polar segments or the side chains, which leads to a change in the nature of the surface to a more hydrophilic.

Compared to zein coatings with undoped MSGG particles reported in our previous work,⁴⁷ the CA was lower, especially for



Figure 9. Surface topography of composite coatings containing 6 vol% MSGG-Cu (a) or 7 vol% MSGG-Cu-Mg (b) (U = 5 V, t = 5 min), observed by optical profilometry and images of droplets from the contact angle goniometer.



Figure 10. CA with distilled H_2O (a) and SFE (b) of the substrate material, MSGG-Cu/zein and MSGG-Cu-Mg coatings obtained from suspensions containing various concentrations of MSGG particles.

Table VIII. Parameters of surface roughness for the substrate and coatings.

	MSGG-Cu/zein coating				MSGG-Cu-Mg/zein coating			
Parameter	Substrate	4 vol% MSGG	6 vol% MSGG	12 vol% MSGG	5 vol% MSGG	7 vol% MSGG	12 vol% MSGG	
$R_a [\mu m]$	0.22 ± 0.02	3.28 ± 0.13	2.40 ± 0.12	1.71 ± 0.61	3.31 ± 0.15	2.68 ± 0.17	1.79 ± 0.12	
$R_q [\mu m]$	0.29 ± 0.02	3.89 ± 0.16	2.76 ± 0.11	2.64 ± 0.69	3.57 ± 0.17	2.88 ± 0.19	2.71 ± 0.16	
R_{max} [μ m]	5.8 ± 0.3	32 ± 4	34 ± 7	45 ± 12	29 ± 5	35 ± 5	49 ± 6	

coatings with higher concentrations of MSGG particles. Thus, it can be observed that the chemical composition of the MSGG particles also affects the wettability of the coating surface.

Shirazy et al.⁶⁵ showed that the wettability of a copper surface can be manipulated by a hydrogen reduction process, significantly reducing the Cu contact angle. However, in our case, we do not use hydrogen reduction of oxides on the surface of the coatings. Betlej et al.⁶⁶ implemented Cu, Zn and Ag ions on the surface of cellulose films. The contact angle of the films with metal ions was increased. The greatest change was observed when Cu was present on the surface of the film. The authors reported that the more hydrophobic surface of the cellulose coatings was significantly influenced by the implantation of metals, which disturbed the hydroxyl group and the inter- and intra-H bonding, increasing the contact angle. Therefore, it can be assumed that in our coatings, in which copper ions are present, a similar process may occur affecting the functional groups contained in the zein matrix. As a result, the contact angle increased when the volume fraction of the doped MSGG particles in the coating was also higher, because the ion releasing process was more intensive.

According to the literature, the surface of Mg is hydrophilic.^{67,68} However, Tabrizy et al.⁶⁹ showed that in some cases Mg ions can make the surface more hydrophobic. They reported that Mg could bridge with other organic ingredients to alter the wettability mechanism. It is supposed that, in the case of our coatings, a similar mechanism may occur, in which Mg ions interact with the organic components of zein, changing the nature of the coating to be more hydrophobic. Also taking into account the surface roughness of the coatings, a certain influence on CA values can be observed. With a decrease in the values of the roughness parameters, the values of CA increased. The decrease in the contact angle value at higher values of the roughness parameters may be related to the spread of droplets along the grooves or pores present on the surface of the material.⁷⁰

Coatings impact on the electrochemical properties of the alloy.—The graph in Fig. 11a represents an evolution of opencircuit potential (E_{ocp}) in time for bare Ti-13Nb-13Zr substrate as well as for coated samples immersed for 24 h in Ringer's solution at 37 °C. The lowest and most stable free corrosion potential values were registered for the virgin alloy, i.e. around -0.45 V. The E_{ocp} for both coatings exhibit a comparable tendency and stabilized after approx. 20 h reaching the values of 0.10 V and -0.02 V, respectively. The OCP value for the coating involving MSGG-Cu was slightly lower over time compared to the coating with MSGG-Cu-Mg and fluctuated between -0.07 V \div 0.07 V. Similarly, the OCP progress for the MSGG-Cu-Mg coating was characterized by slight oscillations in potential window $-0.13 \text{ V} \div 0.10 \text{ V}$. The superior corrosion resistance in testing environment of coated samples when compare to bare substrate was defined by the very high values of free corrosion potential.

The potentiodynamic polarization curves for each sample are shown in Fig. 11b. The analysis of LSV measurement results corroborate the conclusions from OCP experiments. It is widely accepted that a higher cathodic-anodic transition potential results in higher corrosion susceptibility and a lower corrosion current density is the indication of corrosion processes occurring with slow rate.⁷ Based on this statement and the results presented in Fig. 11b, it can be concluded that among the investigated samples the uncoated alloy exhibits the lowest corrosion resistance, what is expressed in terms of the relatively low value of corrosion potential (i.e. -0.43 V) and highest corrosion current density (20 μ A cm⁻²). For the MSGG-Cu/ zein and MSGG-Cu-Mg/zein coated alloys, the cathodic-anodic potential and current density equaled -0.60 V, -0.37 V and 0.06 μ A cm⁻², 0.04 μ A cm⁻², respectively. Despite the smallest value of cathodic-anodic potential for the MSGG-Cu-Mg/zein coated alloy, the lowest current density and a very wide plateau between -0.36 V ÷ 1.0 V indicated similar corrosion resistance to the MSGG-Cu-Mg/ zein coated alloy. A strong effect of Mg-containing glass on the coating manifests itself in the registered cathodic current densities (that is around 1.5 orders of magnitude higher than for MSGG-Cu/ zein coating) and in the shape of the cathodic branch of the polarization curve due to the higher rate of hydrogen evolution reaction, which is presumably accelerated by the short-circuit Cu and Mg particles present in the MSGG-Cu-Mg glass in the coating.

Figures 12a, 12b shows the EIS experiments data in form of Bode plot and Nyquist plot recorded for the uncoated and coated alloy in Ringer's electrolyte at 37 °C. For the medium and low frequency range, the modulus |Z| displayed rather high values of about 10⁶ $\Omega \cdot cm^2$ for all coated samples, Fig. 12a. For the virgin substrate, the modulus |Z| was relatively lower than that for the coated ones, which may suggest a higher corrosion susceptibility compared to that of the coated samples. From Fig. 12b, it may be inferred that the impedance spectrum for virgin alloy exhibits only one highfrequency arc, indicating that the corrosion processes took place at the metal-solution interface. The larger radius of medium- and lowfrequency semicircles observed for MSGG-Cu/zein and MSGG-Cu-Mg/zein coated samples is the evidence for significant enhancement in corrosion resistance when compared to bare metal. From the coatings employed in the present study, the MSGG-Cu/zein exhibited about two times higher impedance values than for MSGG-



Figure 11. Electrochemical measurements in Ringer's solution environment at 37 °C, (a) open circuit potential vs time and (b) potentiodynamic polarization curve.



Figure 12. Electrochemical impedance spectra for the virgin and coated alloy in Ringer's solution (a) Bode impedance and phase angle plot, (b) Nyquist impedance plot. Inserts depict the equivalent circuits for virgin substrate and substrate coated with MSGG-Cu/zein or MSGG-Cu-Mg/zein coating.

Cu-Mg/zein at medium- and low-frequency part of impedance spectra, making this coating the most corrosion resistant of the two.

The equivalent circuit for the titanium alloy (Fig. 12b) is composed of the electrolyte resistance (Rs), the charge transfer resistance (R_{ct}) and the constant phase element for double layer (CPE_{dl}) what reflects the existence of double layer and charge transfer processes (e.g. corrosion reactions) at the interface between passive film and metal substrate. A good fitting between the experimental and simulated results was obtained and the parameters are listed in Table IX. The equivalent model circuit for the MSGG-Cu/zein and MSGG-Cu-Mg/zein coated substrates (Fig. 12b) reflects a more complex nature of this system⁷² (cf. Figs. 7 and 8), namely the presence of outer more compact zone characterized by constant phase element CPE₁ (showing almost pure capacitive behavior) and resistance R1 placed on top of more porous one which electrochemical behavior can be expressed in terms of coating capacitance CPE_2 in parallel with the resistance on this layer R_2 .^{73,74} Moreover, the elements such as constant phase element CPE_{dl} and R_{ct} indicate the existence of double layer at the coating-metal boundary and, presumably, the occurrence of diffusion controlled (due to Warburg element W) electrochemical processes (e.g. corrosion reactions) taking place at this interface.⁷⁵ To evaluate the coated alloy system, the parameters obtained by fitting the equivalent circuit were used (Table IX).

This corresponds well with the morphology and microstructure of the surface of the coatings, with numerous boundaries separating particle clusters being observed. In the case of the MSGG-Cu-Mg/ zein coated alloy, there were many more boundaries that can cause easier and faster degradation of coatings in aggressive chloride ion environments.

Antibacterial assay on MSGG/zein coatings.—Both types of coatings with MSGG-Cu and MSGG-Cu-Mg particles showed antibacterial properties compared to pure zein coatings (Fig. 13). Antibacterial studies of MSGG particles showed a significant decrease in Gram-negative (p > 0.05) and Gram-positive ($p \le 0.05$) bacterial viability after 24 h. The results showed that doped MSGG-Cu and MSGG-Cu-Mg reduced bacterial viability to a greater extent over time compared to the reference MSGG particles (Fig. 3). However, in the case of coatings, the antibacterial properties were most likely influenced by the zein matrix, which surrounded the MSGG particles, thus inhibiting the free release of antibacterial Cu and Mg ions.

Cell studies.—The evaluations of cell viability by optical density measurement showed deterioration after 24 h of incubation for MSGG/zein coatings in comparison to the Ti substrate, which did not exhibit any cytotoxic response (data not shown). The comparison of cell viability for the different coating systems is shown in

Table IX. Parameters of equivalent circuit elements calculated for different samples immersed in Ringer's solution.

Elements of the electric equivalent circuits	Samples		
	Virgin substrate	MSGG-Cu/zein coated alloy	MSGG-Cu-Mg/zein coated alloy
$Rs[\Omega \cdot cm^2]$	39.99 ± 0.01	58.5 ± 8.78	58.5 ± 4.60
CPE1-T $[Fs^{n-1}cm^{-2}]$	$456.82 \times 10^{-7} \pm 0.01 \times 10^{-7}$	$381.97 \times 10^{-7} \pm 773.3 \times 10^{-7}$	$236.15 \times 10^{-7} \pm 14.97 \times 10^{-7}$
CPE1-P	0.87 ± 0.01	0.84 ± 0.02	0.88 ± 0.01
$R_1[\Omega \cdot cm^2]$	_	$50.57 \times 10^3 \pm 15.67 \times 10^3$	$29.01 \times 10^3 \pm 0.36 \times 10^3$
CPE2-T $[Fs^{n-1}cm^{-2}]$	_	$20.16 \times 10^{-7} \pm 5.11 \times 10^{-7}$	$1.57 \times 10^{-7} \pm 1.67 \times 10^{-7}$
CPE2-P	_	0.73 ± 0.06	0.94 ± 0.12
$R_2 [\Omega \cdot cm^2]$	_	18.65 ± 4.72	19.14 ± 4.93
$CPE_{dl}[Fs^{n-1}cm^{-2}]$	_	$9.69 \times 10^{-6} \pm 1.13 \times 10^{-6}$	$15.58 \times 10^{-6} \pm 0.52 \times 10^{-6}$
CPE _{dl} -P	_	0.97 ± 0.02	0.94 ± 0.01
$R_{ct}[\Omega \cdot cm^2]$	$0.69 \times 10^6 \pm 0.01 \times 10^6$	$1.74 \times 10^6 \pm 7.88 \times 10^6$	$0.71 \times 10^6 \pm 0.01 \times 10^6$
Zw	_	$5.02 \times 10^4 \pm 1.19 \times 10^4$	$3.06 \times 10^4 \pm 0.92 \times 10^4$
χ^2	7.84×10^{-9}	0.024	0.016



Figure 13. Alamar Blue assay against *E. coli* (a) and *S. aureus* (b) after 24 h of incubation. 1 - Zein (0.2 kg dm⁻³), 2 - Zein (0.2 kg dm⁻³) + MSGG (0.01 kg dm⁻³), 3 - Zein (0.2 kg dm⁻³) + MSGG (0.08 kg dm⁻³), 4 - Zein (0.2 kg dm⁻³) + MSGG-Cu (0.01 kg dm⁻³), 5 - Zein (0.2 kg dm⁻³) + MSGG-Cu (0.08 kg dm⁻³), 6 - Zein (0.2 kg dm⁻³) + MSGG-Cu-Mg (0.01 kg dm⁻³), 7 - Zein (0.2 kg dm⁻³) + MSGG-Cu-Mg (0.02 kg dm⁻³), 8 - Zein (0.2 kg dm⁻³) + MSGG-Cu (0.01 kg dm⁻³), 8 - Zein (0.2 kg dm⁻³) + MSGG-Cu (0.01 kg dm⁻³), 8 - Zein (0.2 kg dm⁻³) + MSGG-Cu (0.02 kg dm⁻³), 9 - Zein (0.2 kg dm⁻³) + MSGG-Cu (0.01 kg dm⁻³), 9 - Zein (0.2 kg dm⁻³) + MSGG-Cu (0.02 kg dm⁻³), 9 - Zein (0.2 kg dm⁻³) + MSGG-Cu (0.01 kg dm⁻³), 9 - Zein (0.2 kg dm⁻³) + MSGG-Cu (0.02 kg dm⁻³), 9 - Zein (0.2 kg dm⁻³) + MSGG-Cu (0.02 kg dm⁻³), 1 - Zein (0.2 kg dm⁻³) was used as a control for the statistical analysis, *p < = 0.05, **p < =0.01, ***p < =0.001 (Bonferroni).



Figure 14. Viability of MG-63 cells after 24 h and 72 h of incubation on the surface of MSGG/zein, MSGG-Cu/zein and MSGG-Cu-Mg/zein coatings. Cell culture plate was used as a control. Asterisks denote statistically significant differences, *p <=0.05, **p <=0.01, ***p <=0.001 (Bonferroni).

Fig. 14). Measurements were carried out on coatings containing 6 vol% MSGG or 6 vol% MSGG-Cu or 7 vol% MSGG-Cu-Mg. The cell viability was shown to decrease at day 3 of incubation ($p \le 0.05$) in all cases (Fig. 14). A pronounced cytotoxic effect was observed for all coatings under the experimental conditions investigated.

Cell viability measurements coincide with fluorescence microscopy observations (Fig. 15) in coatings that showed a low number of cells on their surfaces. This is especially visible in coatings containing glass particles doped with Cu, as well as Cu and Mg, in comparison with the Ti substrate or the control group.

In conclusion, MSGG/zein coatings reduce the initial cell viability compared to the control and the metallic substrate. One of the reasons for the decrease in the viability of MG-63 cells may be the excessively low level of degradation of zein, which has weak bioactive properties, and which surrounded the glass particles. For

this reason, MSGG may have reduced contact with cells, particularly during the relatively short time of the experiments. The presence of Cu and Mg ions additionally decreased cell viability. It is possible that antibacterial properties^{76,77} and the concentration of these elements in MSGG can affect the cell-material interaction, leading to reduced cell viability. Prabhu et al.⁷⁸ performed cell viability studies on bioactive glasses doped with antibacterial silver and neem. They showed a decrease in viability of the human adenogastric sarcoma cell line on doped glass compared to the base bioactive glass (SiO₂-Ca-P₂O₅) at a concentration of $0.1*10^{-3}$ to $0.5*10^{-3}$ kg dm⁻³.⁷⁸

Conclusions

The prepared MSGG particles showed antibacterial properties over time against Gram-negative E. coli and Gram-positive S. aureus and calcium phosphate formed on their surface after 1 d of incubation in SBF solution. The experimental conditions for the electrophoretic deposition of zein coatings on Ti-13Nb-13Zr alloy substrates were established. The resulting coatings showed very high adhesion strength. Single MSGG particles and their agglomerates with diameter of up to about 60 μ m could be observed on the coating surface. With the increase in the concentration of MSGG particles in the suspensions, the volume fraction in the coatings also increased and the volume fraction values were as follows: 4 or 6 or 12 vol% MSGG-Cu and 5 or 7 or 12 vol% MSGG-Cu-Mg for coatings deposited from suspensions containing 0.001 or 0.004 or 0.008 kg dm⁻³ of MSGG particles, respectively. Both on the surface and in the volume of the coatings, pores could be observed, which were the result of the hydrolysis reaction of water in the suspension during deposition. The surface roughness of all coatings increased several times compared to the substrate material. The coatings also showed a more hydrophilic nature than the uncoated substrate. Such coating properties are expected to be promising for cell attachment. The electrochemical studies indicated an increase in resistance to electrochemical corrosion of the alloy in Ringer's solution as a result of coatings deposition. Evaluation of cell viability on the MSGG/zein coatings showed a decrease in the number of cells on the surface of the coatings. However, since MSGG bioactive glass particles are embedded in biodegradable zein, advanced long-term studies are required to confirm the time-dependent effect of MSGG particles with different doping ions on osteoblast cell viability.



Figure 15. Fluorescence microscope images of Calcein-DAPI stained MG-63 cells after 72 h of culture on the surface of culture plate (control), Ti substrate and the coatings.

Acknowledgments

This work was supported by the National Science Centre, Poland (decision no DEC-2018/31/G/ST5/00429) and by the German Science Foundation (DFG) (project BO 1191/25–1). The authors appreciate the valuable contribution of Dr. Ł. Cieniek (AGH University of Krakow) to SEM investigation and Dr. M. Gajewska (AGH, ACMIN) for FIB lamella preparation and STEM-HAADF investigation.

Supplementary Material

BF-TEM images of highly ordered MSGG-Cu (Fig. S1) and MSGG-Cu-Mg particles, A typical example of determining the size of the mesoporous channels (Fig. S3), Dependence of the zeta potential and conductivity on the pH of suspensions (Fig. S4), Dependence of the zeta potential and conductivity on the pH of suspensions with different concentration of MSGG-Cu and MSGG-Cu-Mg (Fig. S5), Variation of deposit mass and deposition rate of the MSGG-Cu/zein and MSGG-Cu-Mg/zein coatings (Fig. S6).

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