THE ENCAPSULATION OF ANTIBACTERIAL DRUGS IN POLYMER NANOPARTICLES AND THEIR USE IN DRUG DELIVERY SYSTEMS ON ZrO_2 SCAFFOLD WITH BIOACTIVE COATING

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Abstract

Bone infections are a challenging problem as they may cause a permanent patient disability and even death. Additionally, their relapse rate is relatively high. The implantation of a local drug delivery system can be an effective way to fight bone infections. In this study, we present the process of surface bioactivation and immobilization of nanoparticles loaded with drugs. Our aim was to improve osseointegration of the ZrO_2 surface by coating it with a bioactive layer containing poly(L-lactide-co-glycolide)(PLGA) nanoparticles (NPs) loaded with antibacterial drugs (gentamicin and bacitracin) using a biomimetic precipitation method. The ZrO_2 substrates were prepared via pressing and sintering. The CaP-coating was obtained by immersing the substrates in ten-times concentrated simulated body fluid (10×SBF). NPs were prepared by the double emulsion method and the drug loading in NPs was assessed. Thus obtained NPs were applied on bioactivated ceramic substrates by the drop-casting method or by introducing them in the 10×SBF solution during the bioactivation process. The NPs were visualized using scanning electron microscopy (SEM). The NPs size and the Zeta potential were measured using dynamic light scattering (DLS) method. The microstructure of the coating and the efficiency of the NPs incorporation were tested by SEM. In this study, we proved the presented process to be an effective way to obtain biomaterials that could be used as drug delivery systems to treat bone infections in the future.

Keywords: *bioactivation, polymer nanoparticles, bioactive layer, biomimetic coating, bone tissue regeneration, bone implants*

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Introduction

Nowadays, bone infections are a widespread problem. Due to the limited ability of the antibiotics accumulation in the bone tissue, the infection treatment is rather difficult [1]. The increasing average age of the patients contributes to the reduction of immune response, more hip fractures and higher numbers of joint surgeries. The increasing number of joint infections is also a result of the overall population aging [2,3]. Bone infections are often extremely painful and lead to progressive destruction, abnormal bone formation and the body's systemic inflammatory response. As a consequence, they may cause permanent disability and even death [4,5]. Despite the availability of a large number of antibiotics, bone infections remain a challenge for clinicians and have a high relapse rate despite the seemingly effective treatment [6].

Bone and marrow infections can be caused by any pyogenic organism, as well as by some strains of fungi, but the most common pathogens are Gram-positive bacteria, including *Staphylococcus aureus* that causes 80% of osteomyelitis. Gentamicin sulfate is the most commonly used antibiotic to impair the bacterial protein synthesis and thus to prevent the infection from spreading [3,4].

S. aureus releases bacterial adhesins that help it stick to the host extracellular matrix proteins, which is the first step in the infection pathogenesis. Additionally, *S. aureus* shows the ability to effectively evade the human immune system, to penetrate mammalian cells and to persist intracellularly, which is one of the main reasons for the high recurrence rate of osteomyelitis [4,7].

The treatment of bone infections is based on a long-term and aggressive antibiotic therapy which is the last and the longest stage of the treatment and may last up to several weeks. The choice of active substance and the therapy duration depends on the patient risk factors, comorbidities or the presence of drug-resistant microorganisms. The chronic osteomyelitis treatment generally involves several steps. The first is to clean up the necrotic and infected tissue. Then, the defective area needs to be filled in to start the bone reparation or regeneration process. Only then the antibioitics are administered [4,8,9].

Antibiotics can be delivered in a variety of ways. The oral drug administration is very rare due to the low concentration of active substances that can reach the bones. That is why, intravenous antibiotics are commonly used, but such a therapy is not always effective and the disease recurs. The unsuccessful intravenous antibiotic delivery results from the fact that the infected bone fragment is often surrounded by sclerotic avascular bone, making it almost inaccessible to systemic antibiotics. For this reason, it is often necessary to remove the infected bone. The solution to this problem may be the implantation of a drug delivery system with an extended release time in order to ensure the appropriate concentration of antibiotics for a longer period of time. In general, the local drug delivery is regarded as more effective in fighting the disease than the systemic therapy [8,9].

Gentamicin, or rather gentamicin sulfate, is an aminoglycoside antibiotic that acts as an inhibitor of the protein synthesis process, binding the 30S subunit of the bacterial ribosome, thus preventing the appearance and spreading of infection. This antibiotic can be used when the use of potentially less toxic drugs is contraindicated. Gentamicin should be used in the treatment of infections caused by susceptible bacteria [3].

Bacitracin is a polypeptide antibiotic that is produced by bacterial strains of *Bacillus subtilis* and *Bacillus licheniformis*. It has an antibacterial effect against Gram-positive bacteria, and also inhibits the resistance to *S. aureus*. The most common side effect of the use of bacitracin is the renal dysfunction but it does not cause complications in the topical application. This side effect is sometimes observed after intravenous systemic administration, while bacitracin taken orally, is safe. In addition, it has been shown that bacitracin can support the osteogenic differentiation of bone marrow stem cells (hBMSC). The high bioactivity of bacitracin as well as its multifunctional properties make this antibiotic effective for the local treatment of bone infections and osteolysis [10-12].

Drug delivery systems (DDS) are considered an extremely valuable tool in modern medicine. They maintain the proper drug concentration in target tissues for a specified period of time, while preventing structural changes of the active substances. These systems are most often based on various types of drug carriers, in particular nanoparticles. The NPs use has a number of advantages. NPs are characterized by a high ability to encapsulate the drug. Their small size allows them to be administered in various ways: orally, intravenously, and even by inhalation. They can reach even the smallest capillaries. In addition, nanoparticles have the ability to penetrate the bone structure, which allows them to reach the infected bone areas [13,14].

In the treatment of bone infections supported by the implant, DDS systems based on biodegradable material can be successfully used. The use of poly(L-lactide-co-glycolide) (PLGA) nanoparticles as a layer allows the design of unconventional scaffolds that provide better functionality. Such an implant, in addition to its basic function, can also play the role of a local delivery system not only for drugs, but also for growth factors or other molecules accelerating the bone tissue regeneration [15].

Zirconium oxide is a material classified as non-bioactive bioceramics. It is characterized not only by high biocompatibility but also by good tribological properties, high strength, and hardness [16,17]. Hydroxyapatite (HAp) is a biocompatible bioceramic material with the molecular formula $Ca_{10}(PO_4)_6(OH)_2$, used in bone tissue engineering. This material is bioactive and has osteoconductive properties [16,18].

Bioinert materials can be coated with bioactive layers so as to improve their osseointegration. For this purpose, biocompatible materials are used which should also be osteoconductive and/or osteoinductive, as well as mechanically stable under physiological stress. They should also adhere to the implant. The coating materials are mainly calcium phosphates [16]. The presence of the ceramic bioactive coating increases the implant surface activity by promoting the adhesion and proliferation of osteoblasts and osteogenic cells, which accelerates the process of tissue repair and new bone formation [19,20].

The aim of this study was to bioactivate the bioinert surface of ZrO_2 substrate by coating it with a bioactive layer of calcium phosphate (CaP) that was doped with PLGA NPs loaded with antibacterial drugs using the biomimetic co-deposition method.

Materials and Methods

Preparation of ceramic substrates

The ceramic substrates were obtained via pressing and sintering. Briefly, 1.3 g of the ZrO_2 powder (TZ-3YS-E, Tosoh Corporation, Nanyo Manufacturing Comlex, Japan) was uniaxially pressed with a force of 15 kN for 1 min and then sintered for 2 h at the temperature of 1450°C. The produced samples (2 mm thick round lozenges of 1 cm in diameter) were grinded and polished with SiC abrasive papers until the 15 μm grit. Then they were cleaned 4 times by using ultrasounds: twice with acetone (CH_3COCH_3, Merck KGaA, Darmstadt, Germany), once with ethanol (C_2H_5OH, Merck KGaA, Darmstadt, Germany) and once with double distilled water. Having been cleaned, the substrates were immersed in the 5 M phosphoric acid for 3 days at 37°C.

Preparation of nanoparticles

The method of double emulsion with solvent evaporation was used to prepare the nanoparticles. A 2% solution of PLGA (La:Ga ratio 85:15, Mn = 100 kDa, d = 1.5, produced at the Center of Polymer and Carbon Materials, Polish Academy of Sciences, Zabrze, Poland) in dichloromethane (DCM, Avantor Performance Materials, Gliwice, Poland) and a 2% aqueous solution of polyvinyl alcohol (PVA, Sigma Aldrich, Germany) were prepared. Gentamicin (Gent, Sigma Aldrich, Germany) or bacitracin (Bct, Sigma Aldrich, Germany) respectively, in the amount of 6 mg, were added to 3 ml of the PLGA solution, and then homogenized with ultrasound for 3 min with an amplitude of 40% (Sonics, Vibra Cell VCX130, Newtown, CT, USA). The obtained emulsion was added to 20 ml of the PVA solution and stirred at 1000 rpm for 24 h at room temperature. In the next stage, the obtained nanoparticles were centrifuged five times (18000 rpm, 20 min, 4°C), and subsequently they were frozen at -80°C. The last step was the drying process that was carried out in the freeze-dryer (Christ Alpha 1-2 LDplus, Germany) for 24 h.

The morphology of obtained NPs was observed with scanning electron microscope (SEM, GeminiSEM 500, Zeiss, Jena, Germany). Their size and the Zeta potential were measured by dynamic light scattering method (DLS, Zetasizer nano-ZS, Malvern, UK). The encapsulation efficiency was checked by the fluorescence reader (FLUOstar Omega, BMG Labtech, Germany). We used the reaction with o-phthalaldehyde (OPA) and the phenomenon of fluorescence to quantify drugs.

TABLE 1. Reagents used to prepare 1 L of SBF-solution for each step.

	First step (1000 ml)		Second step (1000 ml)	
	Ca-solution (500 ml)	P-solution (500 ml)	Ca-solution (500 ml)	P-solution (500 ml)
Reagent	Mass [g]		Mass [g]	
NaCl	29.220	29.220	41.492	41.492
KCl	0.373	-	-	-
$CaCl_2 \cdot 2H_2O$	3.675	-	3.676	-
$MgCl_2 \cdot 6H_2O$	1.017	-	-	-
$NaH_2PO_4 \cdot 2H_2O$	-	1.560	-	-
$NaHCO_3$	-	0.840	-	-
K_2HPO_4	-	-	-	1.742

Bioactivation process

We used the biomimetic precipitation method to bioactivate the ZrO_2 surface. In general, the ceramic substrates were immersed in a 10×SBF solution to obtain a bioactive layer. The process was carried out in two stages and it was a combination of two different techniques already described. The first step was based on the paper of A. Tas *et al.,* while the second one on the study of D. Costa *et al.* [21,22]. TABLE 1 shows the reagents and their quantities that were used to prepare the SBF-solutions.

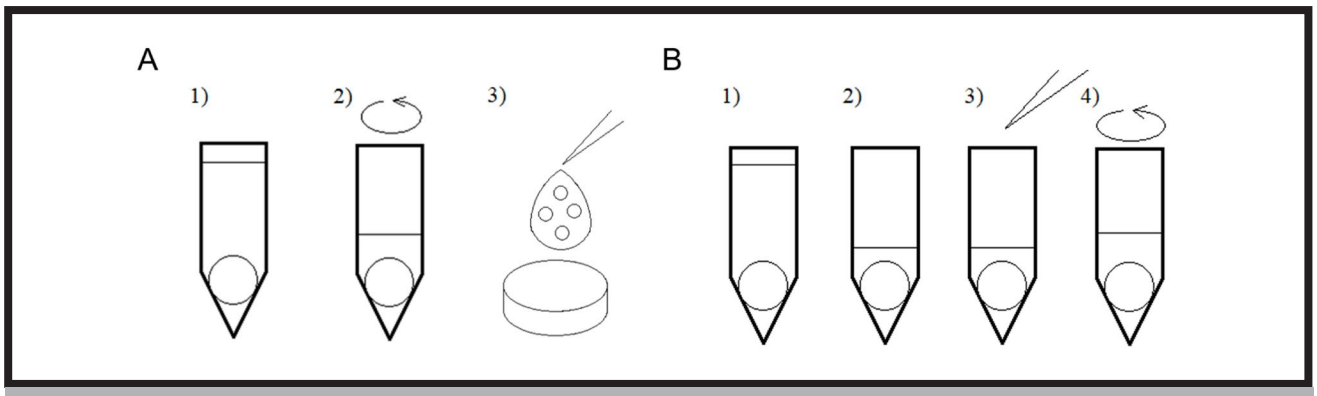

FIG. 1. Schematic representation of the drop casting method (A: 1 – first step of coating process (15 ml of the SBF-solution); 2 – second step of coating process (2.5 ml of the SBF-solution); 3 – adding a drop of dispersed NPs on the coated sample) and the precipitation process (B: 1 – first step of coating process (15 ml of solution); 2 – adding SBF-solution used in the second step; 3 – adding dispersed NPs; 4 – second step of coating process).

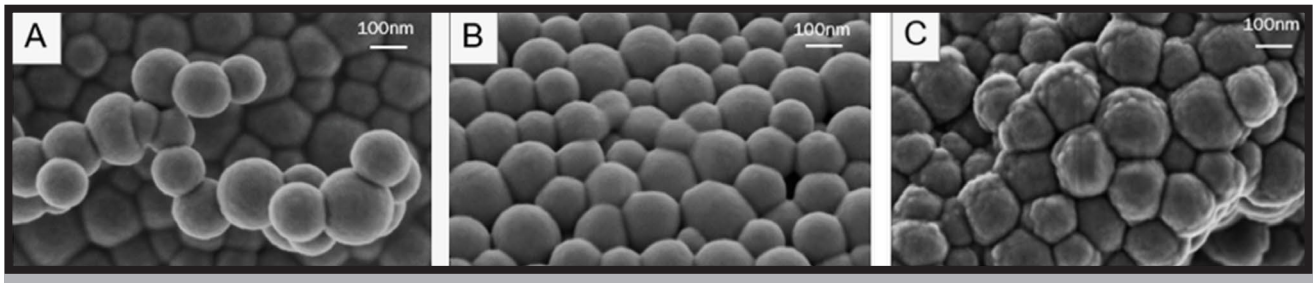

FIG. 2. SEM images of different types of nanoparticles: A – empty nanoparticles (PLGA); B – nanoparticles loaded with gentamicin (PLGA_Gent); C – nanoparticles loaded with bacitracin (PLGA_Bct).

In the first step, the samples were incubated in 15 ml of the SBF-solution for 24 h at 37°C under static conditions. During the second step, they were immersed in 2.5 ml of the SBF-solution for the same period of time but under dynamic conditions (shaking at a speed of 150 rpm) at room temperature. Then, the samples were rinsed three times with double distilled water (ddH_2O) and left to dry.

The microstructure and the quality of received coatings were observed using SEM.

Introducing NPs into the CaP-coating

To introduce NPs onto the ceramic substrates, we used two different methods whose schematic representations are shown in FIG. 1. The first one was the drop-casting method which is based on placing a drop of the NPs dispersed in water on the coated substrate. We applied 100 µl of dispersion with a NPs-concentration of 2 mg/ml. Then, the samples were dried at room temperature for 48 h. The second method included NPs in the SBF solution during the second step of the bioactivation process. We added 5 mg of NPs to 2.5 ml of the SBF solution to receive the same concentration (2 mg/ml) as in the first method.

To check the efficiency of the immobilization of NPs we analyzed the surface of substrates with SEM.

Results and Discussion

The results of the SEM observation of NPs are presented in FIG. 2. Picture A shows empty NPs while in the pictures B and C there are NPs loaded with gentamicin and bacitracin, respectively. The particles are agglomerated, yet their size is similar in every case, despite the NPs type. NPs are round and regular in pictures A and B, while bacitracin has an impact on the shape and morphology of NPs and makes them more irregular.

DLS results confirmed that the NPs size is similar in every case - approximately 200 nm. However, the size (FIG. 3) as well as the Zeta potential (FIG. 4) are dependent on the NPs type. As shown in TABLE 2, particles loaded with drugs are smaller (207.4 nm and 202.4 nm for particles loaded with gentamicin and bacitracin, respectively) and their Zeta potential increases (-23.9 mV for gentamicin and -13.4 mV for bacitracin) in comparison with the empty NPs whose size and Zeta potential are equal to 226.3 nm and -30.5 mV, respectively. This phenomenon results from the interaction of polymer with drugs whose positive nature changes the polymer surface charge. The encapsulation efficiency is higher for gentamicin (54.3%) in comparison with bacitracin (37.5%) but it is relatively high for both used drugs.

Coating the implant with a bioactive layer is a relatively common method to improve osseointegration. Different methods of the CaP-precipitation on the implant surface have been developed. A. Tas *et al.* created a layer of calcium phosphate on the surface of Ti6Al4V [21]. SBF with a ten times higher concentration (10×SBF) of calcium and phosphate ions was used. That solution did not require the use of buffering agents and the coating process itself was carried out at a linear speed. Before dipping in the 10×SBF, the Ti6Al4V surface was initially chemically etched in a 5 M KOH solution and then thermally treated at 600°C. The SBF solution made it possible to obtain a calcium phosphate coating not only on metals but also on ceramics or polymers. This method led to the formation of layers in as little as 2 - 6 hours at room temperature. The phosphate content in the coating and the Ca/P molar ratio made the coating classified as bone-like, which is additionally characterized by a relatively high adhesive force to the substrate surface.

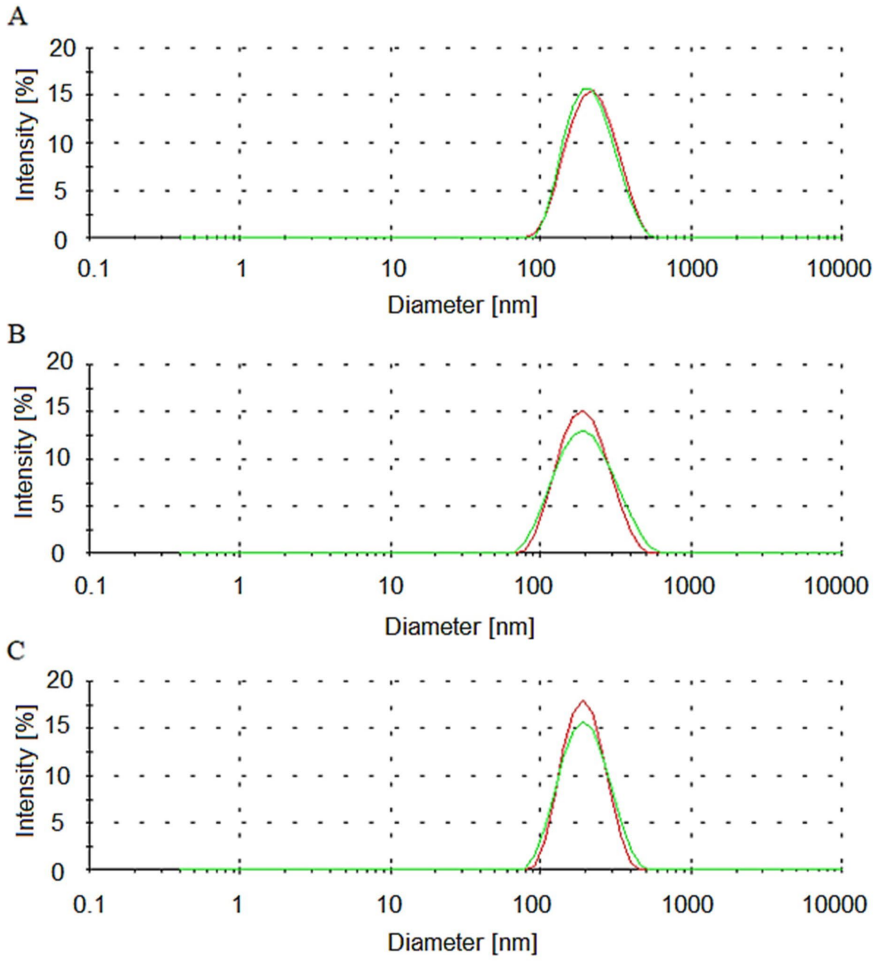

FIG. 3. Size distribution of nanoparticles determined by DLS method: A – PLGA, B – PLGA_Gent, C – PLGA_Bct.

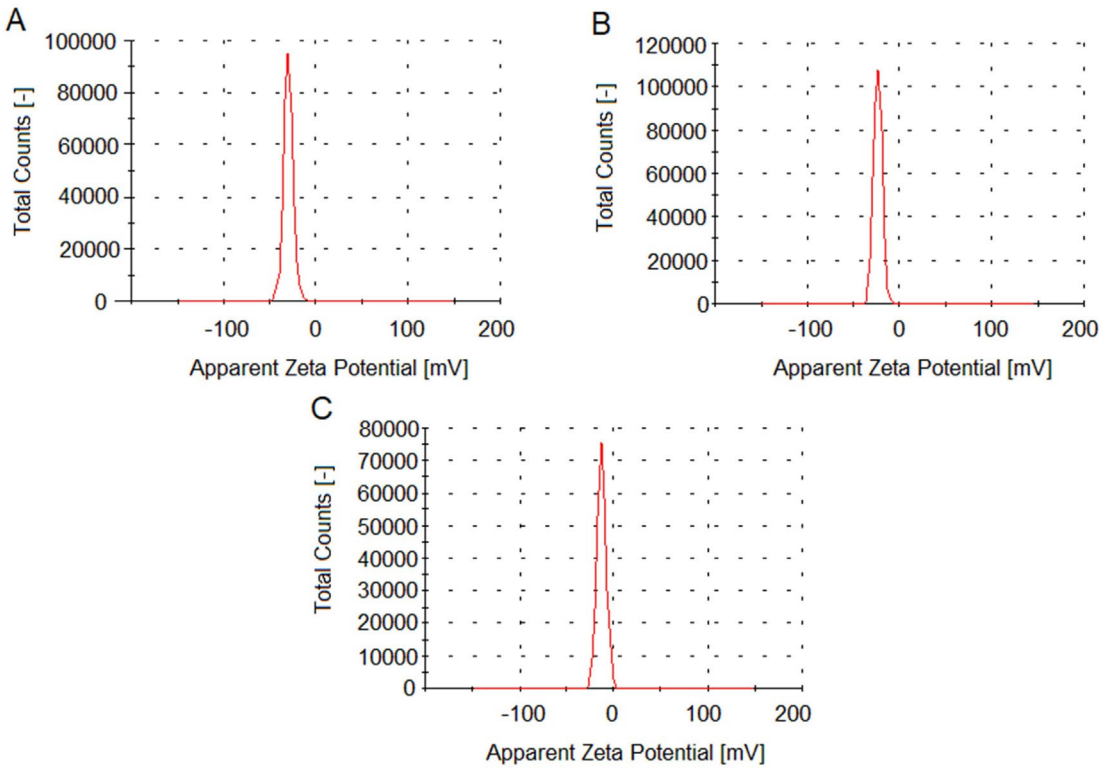

FIG. 4. Zeta potential distribution of nanoparticles: A – PLGA, B – PLGA_Gent, C – PLGA_Bct.

TABLE 2. Characteristics of NPs: average size, Zeta potential and encapsulation of drugs.

	Average size [nm]	Zeta potential [mV]	Encapsulation [%]
PLGA	226.3	-30.5	-
PLGA_Gent	207.4	-23.9	54.3
PLGA_Bct	202.4	-13.4	37.5

D. Costa *et al.* used simulated body fluid (SBF) to create a calcium phosphate (CaP) coating on polycaprolactone (PCL) foil [22]. Due to the presence of such a coating, the polymer substrates could be bioactive and osteoconductive, and their biocompatibility increased when compared to uncoated substrates. The hydroxyapatite (HAp) layer was obtained by dipping the substrate in SBF solution with a five times higher ion concentration (5×SBF) of the typical SBF solution, therefore the time needed to obtain the coating shortened from about 7 days to 24 hours. The coating process was based on the work of Tas *et al.* [21]. The bone-like layers with different surface topography were produced on the PCL substrates, depending on the concentration of Mg^{2+} and HCO^{3-} ions. The lower concentrations resulted in a fine surface topography, while high concentrations in a surface with roughness considered optimal for adherence and differentiation of osteoblasts. At the same time, the mechanical properties of CaP coatings were similar to those of natural bone tissue.

In our study, we combined both of the above-mentioned approaches. One method is based on the composition of the blood plasma while the other one contains only the most typical SBF ions. The absence of ions such as Mg^{2+} in the second step transforms the structure obtained in the first step into the more bone-like structure. The first step of our bioactivation process was based on the work of A. Tas *et al.,* while the second one was inspired by the research of D. Costa *et al.* [21,22].

In each stage, we used the Ca- and P-solutions that contained only appropriate ions. When adding the solutions to the tube we followed the order: first P- and then Ca-solution. We immersed the substrates for 24 h at 37°C under static conditions in 15 ml of the solution (7.5 ml of the P- and 7.5 ml of Ca-solution) prepared as shown in TABLE 1. During the second stage, the samples were incubated for the same period of time at room temperature under dynamic conditions (shaking at a speed of 150 rpm), in 2.5 ml of the 10×SBF solution (1.25 ml of the P- and 1.25 ml of Ca-solution) that was prepared as presented in TABLE 1. Then, the samples were rinsed three times with double distilled water (ddH_2O) and left to dry.

The bioactive layers we received correlated with those obtained in the above-mentioned papers, as presented in the SEM pictures A and B (FIG. 5).

Crystals obtained according to the first step of coating process (FIG. 5A) were thinner, while those after both steps (first and second) were thicker and the layer was denser (FIG. 5B).

In order to immobilize NPs on ceramic substrates we used two different methods. One of them was the drop-casting method (FIG. 5C). The other was based on the presence of NPs in the SBF solution during the second step of co-deposition process (FIG. 5D). In both methods the same concentration of dispersed NPs (2 mg/ml) was applied.

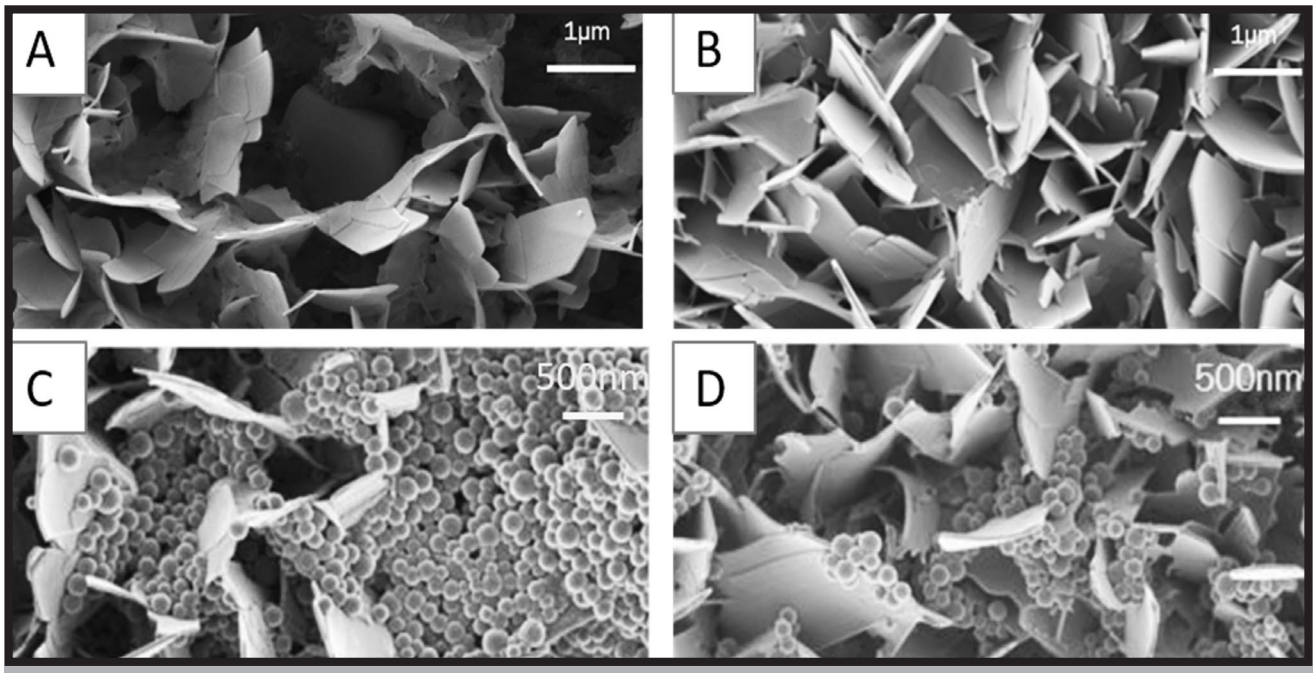

FIG. 5. SEM microphotographs of the bioactive layer on the ZrO_2 substrate after the first (A) and the second (B) step of the coating process and NPs deposited on the ZrO_2 substrates by drop-sitting method (C) and during the CaP layer preparation process (D).

As for the immobilization during co-deposition, the microstructure of bioactive layer did not differ from the one obtained without particles. That means that the NPs presence in the solution did not have a negative impact on the crystallization process. The NPs were evenly distributed on the surface and their adhesion to the surface was relatively strong.

Thanks to the drop-casting method, a larger number of NPs was immobilized so they nearly covered the whole surface. Although they were highly agglomerated and not homogeneously distributed, the NPs deposition was more precise in this method and the adhesion to the bioactive layer was also high.

Conclusions

In this paper we presented a method of producing ZrO_2 substrates coated with a bioactive layer containing polymer nanoparticles loaded with antibacterial drugs.

The biomimetic method of the bioactive layer deposition was stable and the obtained coatings consisted of flake-like crystals. In the first step of the process, the crystals were not fully developed. Therefore, it was important to perform the second step. During that stage, the microstructure transformed into a more stable form which was similar to hydroxyapatite. That explains the necessity of using the two-step process.

Both methods of the NPs immobilization were efficient. However, the number of deposited particles was higher in the drop-casting method. The NPs formed a layer that covered the surface almost completely. In both cases, NPs were agglomerated. Incorporating NPs in the solution during the coating process allowed for a more homogenous particles distribution on the surface of the ZrO_2 substrate. Yet, the immobilization process during co-deposition was less precise because the attached NPs could not be controlled in terms of their quantity.

It can be stated that the method used to prepare nanoparticles was characterized by high stability and efficiency and the obtained particles were of a spherical shape and a desirable size. The presented process of deposition of bioactive layers was an effective technique to bioactivate bioinert surfaces, the result of which was the layer consisting of flake-like crystals. Both methods of immobilizing nanoparticles allowed for the deposition of a sufficient number of particles that were relatively well attached to the substrate. The presented processes of the surface bioactivation and the NPs immobilization are a successful method of creating bioactive ceramic substrates. In further studies we are planning to test the drug release, antibacterial properties and biological properties of our biomaterials in contact with bone cells so as to confirm their potential for tissue engineering and the bone infections treatment.

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