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Biocompatibility and antimicrobial activity of zinc(II)-doped hydroxyapatite, synthesized by a hydrothermal method

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Abstract: In order to obtain multifunctional materials with good biocompatibility and antimicrobial effect, hydroxyapatite (HAp) doped with Zn²⁺ was synthesized by a hydrothermal method. Powders with different content of zinc ions were synthesized and compared with undoped HAp to investigate the influence of Zn²⁺ on the antimicrobial activity of HAp. Analyses of undoped and Zn²⁺-doped powders before and after thermal treatment at 1200 °C were performed by SEM and XRD. The antimicrobial effects of the powders were examined in relation to *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* in liquid medium. The results showed that the obtained powders had good antimicrobial activity, but higher antimicrobial activities of the powders doped with Zn²⁺ were observed after annealing at 1200 °C. For powders annealed at 1200 °C, *in vitro* biocompatibility tests: reduction of the tetrazolium salt 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT test) and Trypan Blue dye exclusion test (DET test) with MRC-5 fibroblast cells in liquid medium were performed. Based on the MTT and DET tests, it was shown that the powders do not have a significant cytotoxic effect, which was confirmed by SEM analysis of MRC-5 fibroblast cells after their *in vitro* contact with the powders.

Keywords: HAp; Zn; α -TCP; biocide; MTT assay; MRC-5.

INTRODUCTION

With the aging of the human population, the need for treatment of skeleton is more pronounced. Synthetic HAp [Ca₁₀(PO₄)₆(OH)₂] due to its composition is the most suitable for the mineral phase of human bone and teeth. Biocompatibility and osteoconductivity of HAp are well known and utilized in dentistry

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and orthopedic surgery to compensate of the damage of teeth and bones.^{1–4} HAp is also used for coating metal implants to improve their biocompatibility.^{4,5–12} However, during incorporation of filler HAp or HAp-covered implants in the living body, infection by pathogenic microorganisms can appear. To prevent this situation and the need for a second operation, biocidal metal ions or antibiotics are used.^{13–16} Use of antibiotics is not suitable because their effect is not last long-lasting and microorganisms can develop resistance. Certain metals, such as silver, copper and zinc, are known to be poisonous to microorganisms. On the other hand, copper and zinc are micronutrients that at low concentrations are necessary for the functioning of many processes in humans, but at large concentrations are toxic.^{17–19} The role of zinc in the formation and mineralization of bone is very important, as shown by data from the literature.^{20–22}

The synthesis of zinc-doped HAp powders by different methods and the antimicrobial activity and/or structural and morphological characteristics of the obtained powder were investigated in the last decade. Kim *et al.*²³ applied a wet-chemical method to synthesize Zn²⁺-substituted HAp. In their investigations, no antimicrobial activity of Zn²⁺ substituted HAp against *Escherichia coli* was observed. Chung *et al.*²⁴ applied a sol-gel method to synthesize Zn²⁺-doped HAp. In solid-state anti-microbial tests, their Zn²⁺-doped HAp demonstrated contact microbial inhibition against *Streptococcus mutans*. Ergun *et al.*²⁵ and Ming'Ou Li *et al.*²⁶ investigated only the structural and morphological characteristics of Zn²⁺-doped HAp. The synthesis of Zn²⁺ ion-substituted HAp using a neutralization method was performed by Stanić *et al.*²⁷ Their investigation confirmed that Zn²⁺-HAp had a viable cells reduction ability against *E. coli*, *Staphylococcus aureus* and *Candida albicans*.

In the present study, Zn²⁺ ion-doped HAp (0.2 and 0.4 mol % as compared to Ca) powders were hydrothermally synthesized. In order to investigate the influence of Zn²⁺ and phase composition on the antimicrobial activity, Zn-substituted powders were annealed at 1200 °C. The aim of the research was to examine the antimicrobial activity of zinc ions and simultaneously check the cytotoxic effect of zinc ions on MRC-5 fibroblast cells. The antimicrobial activity of Zn²⁺-doped HAp against different pathogens: *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27833) and *C. albicans* (ATCC 24433) was evaluated *in vitro*. The biocompatibility of the Zn-substituted powders was studied by reduction of the tetrazolium salt 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay) and Trypan Blue dye exclusion test (DET assay) *in vitro* using MRC-5 fibroblast cells.

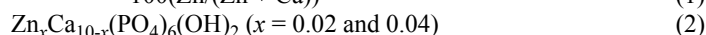
EXPERIMENTAL

Synthesis of HAp and Zn²⁺-doped HAp

Synthesis of HAp and Zn²⁺-doped HAp were performed according to the hydrothermal method described earlier.^{28–30} Appropriate quantities of the chemicals: Ca(NO₃)₂·2H₂O (Carlo

Erba Reagenti, *p.a.* grade), Na₂H₂EDTA·2H₂O (Zorka Šabac, *p.a.* grade), NaH₂PO₄·2H₂O (Analitika LTD, 99 % pure) and urea (Lach-Ner, 99.5 % pure) and Zn(NO₃)₂·6H₂O (Kemika Zagreb, 99 % pure) were dissolved in 1500 mL of distilled water. Doping was performed with 0.2 and 0.4 mol % according to formula (1), with a constant atomic ratio (Ca + Zn)/P = 1.67 to obtain a compound of approximate formula (2):

$$100(\text{Zn}/(\text{Zn} + \text{Ca})) \quad (1)$$



After dissolution of the chemicals, the dish with the solution was inserted in an autoclave, previously filled with 1500 mL of distilled water. The solution was heated at 160 °C for 3 h. After slow cooling, the obtained suspension (pH ~9.20) was filtered, and the residue was washed with distilled water and dried at 105 °C for 4 h. After drying, the powder was ground in a mortar.

The following dry powders were obtained in this way: HAp, Zn(0.2)HAp and Zn(0.4)HAp. These powders were heated at 1200 °C for 2 h (heating rate 10 °C/min) and ground to obtain HAp12, Zn(0.2)HAp12 and Zn(0.4)HAp12.

Characterization methods

A scanning electron microscope (SEM) JEOL JSM 5800 was used for the morphological characterization of the synthesized powders. Before the SEM analysis, the powders were coated with Au–Pd alloy. SEM analysis of MRC-5 fibroblast cells were performed on scanning microscope Tescan Mira 3 FEG. Before SEM observations, the MRC-5 cells were slowly vacuum dried and then coated with Au–Pd alloy.

X-Ray powder diffraction analysis of the powders was performed on an ITAL Structures APD 2000 instrument using a copper cathode as the X-ray source ($\lambda = 0.15406$ nm), in the 2θ angle range from 15 to 65° with a step size of 0.05° s⁻¹.

Antimicrobial test

Quantitative tests of the antimicrobial activity of all the obtained powders against *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27833) and *C. albicans* (ATCC 24433) were performed according to previously described the liquid challenge method in buffer solution.²² The incubation time was 1 h. The antimicrobial test was performed for both the dry and annealed powders. As a control, a blank sterile potassium hydrogen phosphate buffer solution without sample was used.²² The test was performed in two series, the one for dry powder and one for the annealed powders.

In vitro biocompatibility tests

Cell lines. Tests were performed on the cell line human fibroblasts of lung (MRC-5) that were grown attached to the surface of the flasks (Costar, 25 cm³) in Eagle's medium modified by Dulbecco (DMEM, Gibco BRL, England) with 4.5 g L⁻¹ glucose and 10 % FCS (fetal calf serum, Sigma). The medium contained the antibiotics: penicillin 100 IU mL⁻¹ and streptomycin 100 µg ml. The cell line was maintained under standard conditions at 37 °C in an atmosphere of saturated humidity with 5 % CO₂ (Heraeus). They were transplanted twice weekly, whereas in the experiments the logarithmic phase of growth was used between the third and tenth transplantation. Only viable cells were used in the experiments. The number of cells and their viability were determined by the color test rejection with 0.1 % Trypan Blue.³¹ The viability of cells used in the experiment was greater than 90 %.

Colorimetric assays with tetrazolium salts (MTT-test).³² The cells were collected during the logarithmic phase of growth, trypsinized, resuspended and counted in 0.1 % Trypan Blue.

Viable cells were sown at a concentration of $2 \cdot 10^5$ cells mL^{-1} in Petri dishes (50 mm, Center well, Falcon) in which the powders for the analysis were located. Control samples did not contain the investigated powders. Petri dishes with sown cells were thermostated at 37°C with 5 % CO_2 for 48 h. At the end of the incubation, of the cells were re-sown to fresh medium. Viable cells were sown (5×10^3 cells $100 \mu\text{L}^{-1}$) in microtiter plates with 96-well. The plates with sown cells were thermostated at 37°C with 5 % CO_2 for 48 h, 72 h or 96 h. The MTT solution, prepared just before addition, was added to all the wells of the plate, in a volume of $10 \mu\text{L}$ per well and incubation was continued for the next 3 h (in the incubator at 37°C with 5 % CO_2). Upon expiration of 3 h, $100 \mu\text{L}$ of HCl in 2-propanol (0.04 mol L^{-1}) was added to each well. The absorbance readings was performed immediately after incubation on a microtiter plate reader (Multiscan, MCC/340) at a wavelength of 540 nm with reference to 690 nm. Wells on a plate that contained only medium and MTT but no cells were used as a blank.

The fraction of surviving cells (% K) was expressed as a percentage of the control values according to formula (3):

$$\% K = 100N_s/N_k \quad (3)$$

where N_k is the number of cells in the control sample and N_s is the number of cells with the tested substance.

Trypan Blue dye exclusion test (DET)

The cells were collected during the logarithmic phase of growth, trypsinized, resuspended and counted in 0.1 % Trypan Blue. Viable cells were sown at a concentration of 2×10^5 cells mL^{-1} in Petri dishes (50 mm, Center well, Falcon) in which the powders for the analysis were located. Control samples did not contain the investigated powders. The Petri dishes with sown cells were thermostated at 37°C with 5 % CO_2 for the next 48 h. At the end of incubation, the cells were counted in the counting chambers after 48, 72 and 96 h using an inverted microscope. A Neubauer chamber was used for counting the cells in four squares. Each square was divided into 16 smaller squares so the total was 64. $100 \mu\text{L}$ of cells was taken and added to $100 \mu\text{L}$ of 0.1 % Trypan Blue. After intensive shaking, a few drops were placed on both counting fields of the chambers. Trypan Blue painted dead cells while living cells remain unpainted. The number of cells in 1 mL of suspension (X) was calculated using formula (4):

$$X = x \times 10 \times 2 \times 1000 \quad (4)$$

where x is the number of cells in 16 squares (the average number of cells in 4×16 squares); 10 is the depth of the chamber; 2 is the dilution factor and 1000 is the volumetric coefficient.

The fraction of surviving cells (% K) is expressed as a percentage of the control values, according to formula (3).

Cells preparation for painting

Preparation of the powdered substances. All substances were placed in a serum-free medium (DMEM) so that the concentration was 200 mg mL^{-1} . After 72 h, the medium was filtered (for sterility), 10 % serum was added, so that the medium was used in all the investigated samples except for the control.

The cells were collected during the logarithmic phase of growth, trypsinized, resuspended and counted in 0.1 % Trypan Blue. Viable cells were sown in a Petri dish (50 mm, Center well, Falcon) with a medium that has been tested 72 h with powder at a concentration 2×10^5 cells/ml. The control samples contained no test substance. Petri dishes with sown cells

were thermostated at 37 °C with 5 % CO₂ for 48 h. Painting was performed at the end of the incubation.

RESULTS AND DISCUSSION

The SEM micrographs of the obtained HAp and Zn-doped HAp powders are shown in Fig. 1. The photographs of HAp and Zn(0.2)HAp are similar. Both powders contain mainly spherical particles with a wide size distribution. However, spheres with a diameter of 1 to 2 μm prevail. It can be seen that these spherical particles consisted of a large number of aggregated rod like particles. This is particularly obvious in the case of the spherical particles of the sample Zn(0.4)HAp. Even for this sample, the prevailing sphere diameter is 1 to 2 μm.

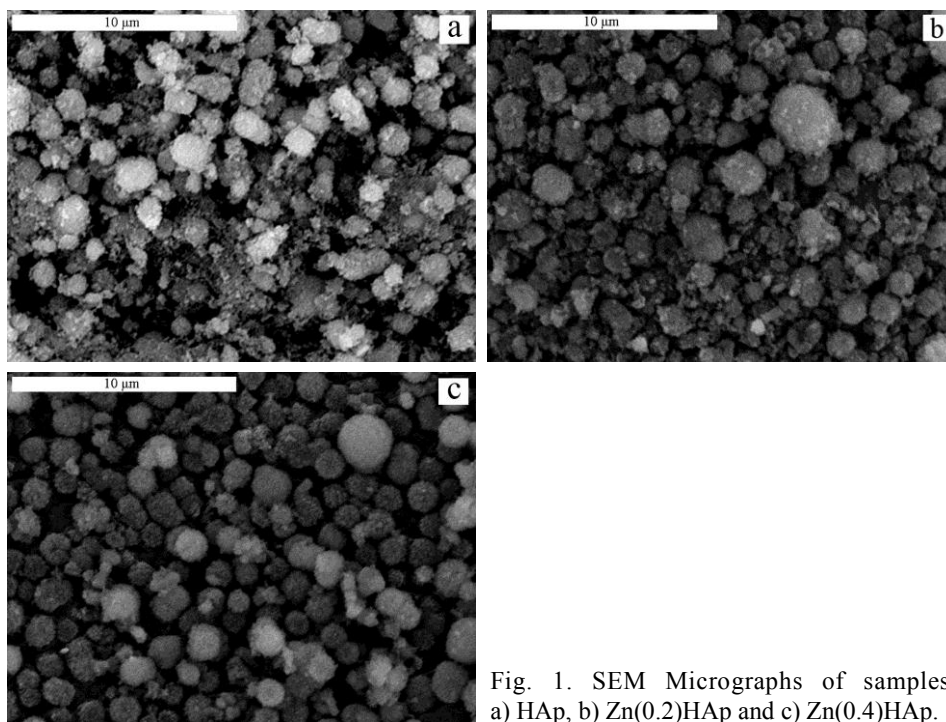


Fig. 1. SEM Micrographs of samples: a) HAp, b) Zn(0.2)HAp and c) Zn(0.4)HAp.

The micrographs of the powders at annealed 1200 °C for 2 h are shown in Fig. 2. Particles merged through the process of neck formation because of annealing can be seen in all cases. Particle size and size distribution were similar for all the samples and it could be concluded that doping of HAp with Zn²⁺, due to the small concentrations, had no effect on the annealing of the Zn-containing powders.

The XRD diffractograms of the synthesized dry and annealed powders are shown in Fig. 3. The dry HAp and Zn²⁺-doped HAp powders showed very low crys-

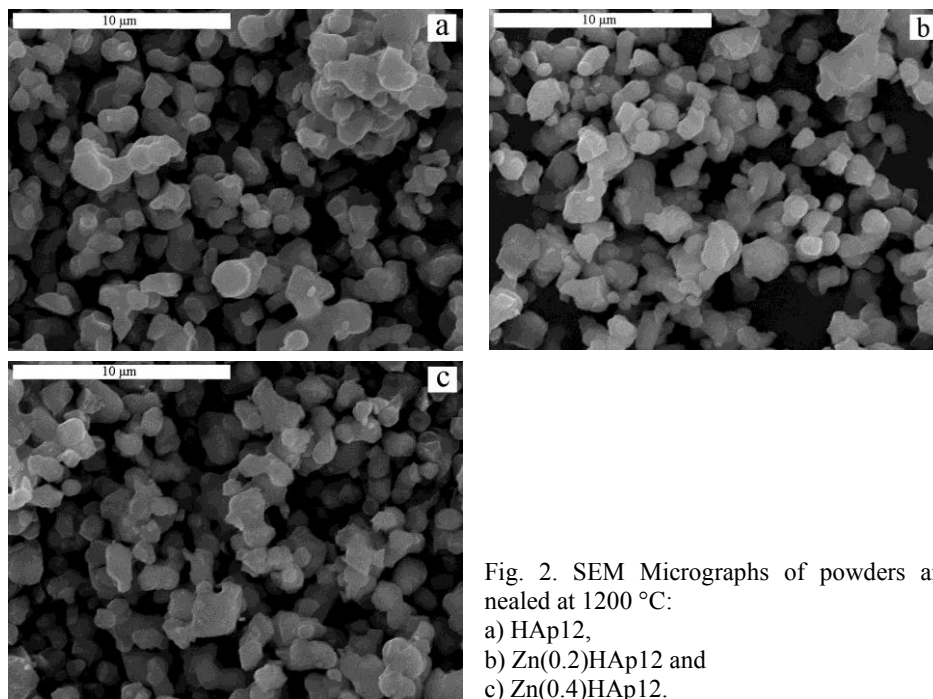


Fig. 2. SEM Micrographs of powders annealed at 1200 °C:
 a) HAp12,
 b) Zn(0.2)HAp12 and
 c) Zn(0.4)HAp12.

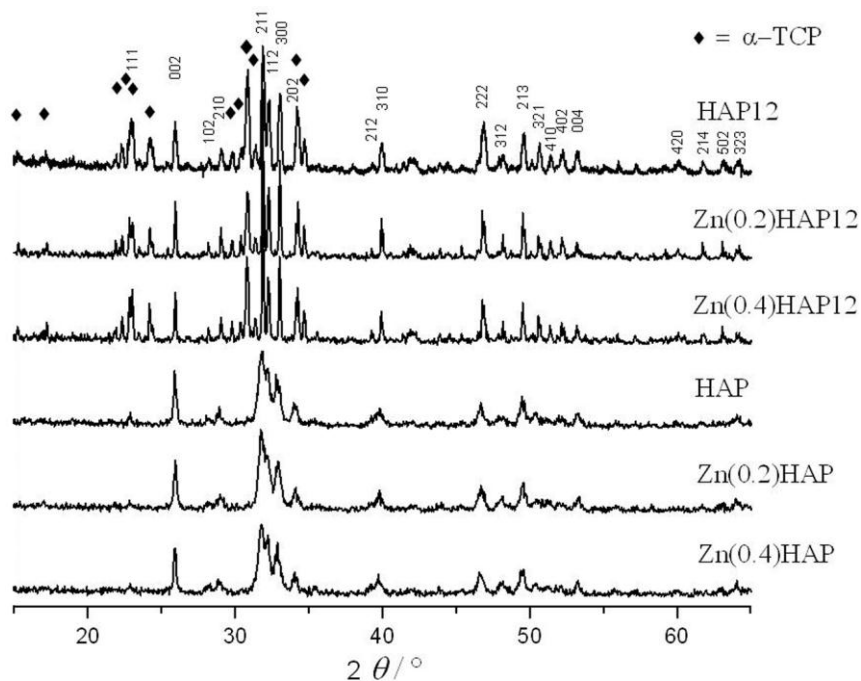


Fig. 3. XRD Patterns of undoped and doped HAP, annealed at 1200 °C and dry powders.

tallinity. Based on ASTM data (Card 9-432), all the peaks present were identified as peaks of hydroxyapatite. These diffraction patterns are very similar, with no clear effect of the doped ions on the diffractograms.

From diffractograms of the annealed powders, it can be seen that HAp was partially transformed to α -TCP. The peaks were identified based on ASTM data (Card 9-432 for HAp and card 09-348 for α -TCP). In Fig. 3, the HAp peaks are marked with the number of the crystal plane. All peaks are sharp, confirming that the annealed samples were well crystallized. Similar to the dry powders, there are not differences between diffractograms of the annealed powders.

The results of the antimicrobial activity of the undoped and Zn²⁺-doped HAp powders tested against *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27833) and *C. albicans* (ATCC 24433) are given in Table I. The degree of reduction, *R*, was calculated compared to HAp using Eq. (5):

$$R (\%) = 100(CFU_{\text{HAp}} - CFU_{\text{Zn-HAp}}) / CFU_{\text{HAp}} \quad (5)$$

Comparing the results of antimicrobial activity of dried, doped and undoped powders, it could be concluded that the activity increased with increasing content of Zn ions incorporated in the HAp powders. Undoped HAp powder exhibited the similar antimicrobial activity against all the tested species of microorganism, except against *S. aureus*, which was more susceptible. The most pronounced effects on reduction of all microorganism species were observed using Zn-doped powders annealed at 1200 °C. Probably, the reason for this higher activity lies in the fact that the TCP phase formed during annealing is more soluble than the HAp phase.^{3,4,28}

TABLE I. Antimicrobial effect of dry and annealed HAp and Zn²⁺-doped HAp powder

Sample	<i>S. aureus</i>		<i>E. coli</i>		<i>P. aeruginosa</i>		<i>C. albicans</i>	
	No. of colonies	<i>R</i> / %	No. of colonies	<i>R</i> / %	No. of colonies	<i>R</i> / %	No. of colonies	<i>R</i> / %
Control	6.0×10 ⁶	–	6.4×10 ⁶	–	1.0×10 ⁷	–	5.4×10 ⁶	–
HAp	2.2×10 ⁴	–	1.9×10 ⁵	–	6.4×10 ⁶	–	1.0×10 ⁶	–
Zn(0.2)HAp	2.2×10 ³	90.0	9.1×10 ⁴	52.10	5.3×10 ⁵	91.72	2.9×10 ⁵	71.00
Zn(0.4)HAp	9.6×10 ²	95.6	3.6×10 ⁴	81.05	2.0×10 ⁵	96.87	2.8×10 ⁵	72.00
Control	84×10 ²	–	636×10 ²	–	2.0×10 ⁶	–	1.4×10 ⁶	–
HAp12	253×10 ²	–	315×10 ²	–	7.0×10 ⁵	–	1.1×10 ⁵	–
Zn(0.2)HAp12	186	99.3	806	97.44	1.9×10 ³	99.73	7.2×10 ³	93.45
Zn(0.4)HAp12	261	100	1210	96.16	1.0×10 ²	99.98	5.7×10 ³	94.82

Determination of cells viability was conducted using two types of *in vitro* assays, MTT and DET. In both tests, the cells viability was explored after 48 h, 72 h and 96 h of contact with the powders.

The diagram of the MTT assay (Fig. 4) shows a real increase in the cell viability with the contact time. The numbers of the cells in contact with HAp and

Zn(0.4)HAp12 were about the same, while the cells in contact with powder Zn(0.2)HAp12 had a slightly lower survival rates. After 96 h, the cells showed the same viability as in the control sample, meaning that the powders exhibited no cytotoxic effect on the cells and thus their biocompatibility was demonstrated.

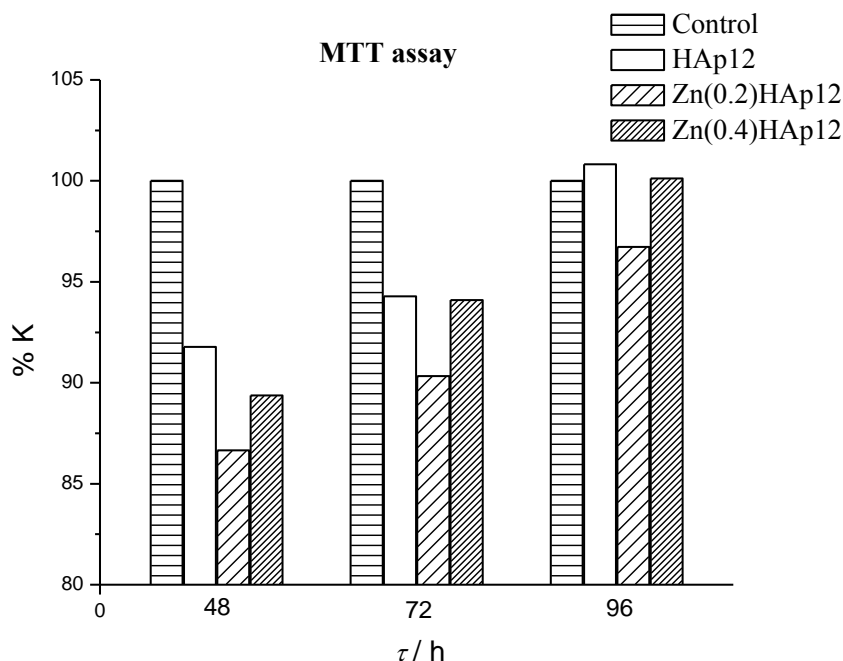


Fig. 4. Fractions of surviving cells in the MTT assay.

DET Test clearly confirmed the results of MTT assay (Fig. 5). The DET assay also showed a gradual increase in cell viability with increasing time of contact of the cells with the powder.

Better viability of cells that were in contact with powder Zn(0.4)HAp12 than with powder Zn(0.2)HAp12 can be explained by their exposure to slightly higher concentrations of Zn^{2+} ions that, as micronutrient, enhanced cells proliferation. Although, there is no significant difference in phase composition (Fig. 3) between undoped and Zn^{2+} -doped HAp, higher proliferation of MRC-5 cells after 96 h in powder extracts of Zn(0.2)HAp12 and Zn(0.4)HAp12 is evident.

Histological analysis of MRC-5 cells that clung to the surface of the Petri dish is shown in Fig. 6. The shape of cells is oval and elongated. It was noted that cytoplasmic extensions were spread from cells that connect to neighboring fibroblast cells. The appearance of the cells is an indication of their preserved viability. This confirmed the results obtained by the MTT and DET assays.

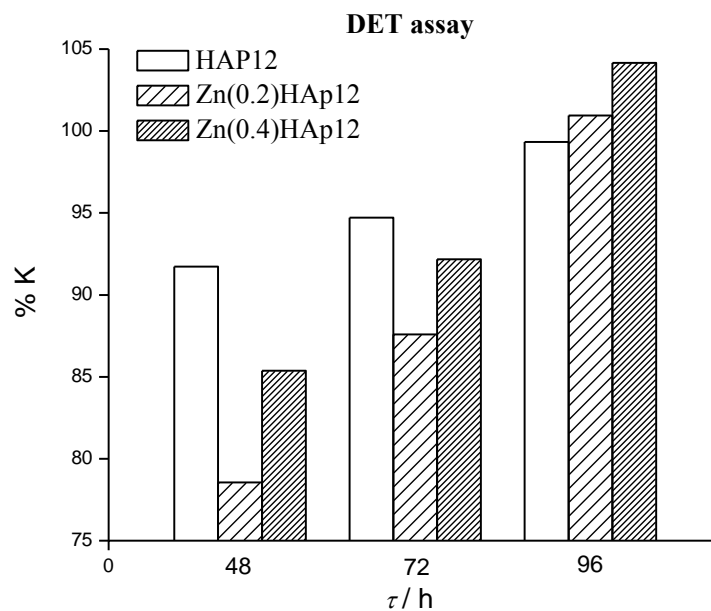


Fig. 5. Fractions of surviving cells in the DET assay.

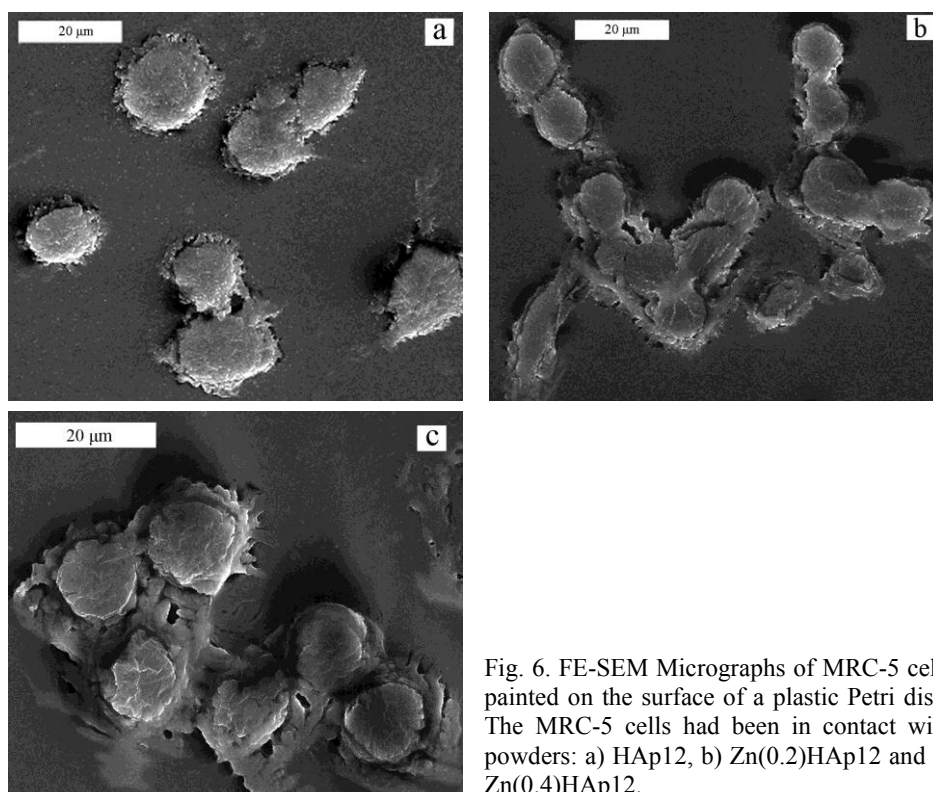


Fig. 6. FE-SEM Micrographs of MRC-5 cells painted on the surface of a plastic Petri dish. The MRC-5 cells had been in contact with powders: a) HAp12, b) Zn(0.2)HAp12 and c) Zn(0.4)HAp12.

CONCLUSIONS

The obtained Zn²⁺-doped Hap powders had good antimicrobial activities against the pathogenic microorganisms: *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans*. The HAp powders with a higher content of Zn²⁺ had a higher degree of reduction of the microorganisms. In addition, the annealed, Zn²⁺-doped HAp/ α -TCP powders showed better antimicrobial activity than the dry powders Zn²⁺-doped Hap powders. The results of the MTT and DET assays showed annealed Zn²⁺-doped Hap powder clearly exhibited good biocompatibility with fibroblast cells, MRC-5. The viability of the cells was better when in contact with the powder with the higher content of Zn²⁺. Histological analysis of SEM images of MRC-5 cells confirmed the results of the MTT and DET assays.

Based on previous results, it can be concluded that doping HAp with Zn²⁺, using a hydrothermal method, results in powders of good biocompatibility with fibroblast cells MRC-5 and antimicrobial activity to pathogenic microorganisms. By annealing of the obtained powders, these properties were enhanced, probably due to partial transformation of HAp to α -TCP, a more soluble phase. The higher content of Zn²⁺ has a pronounced effect against pathogens and as micronutrients improved the proliferation of the fibroblast cells.

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ИЗВОД

БИОКОМПАТИБИЛНОСТ И АНТИМИКРОБНА АКТИВНОСТ ХИДРОКСИАПАТИТА СА УГРАЂЕНИМ ЦИНКОМ СИНТЕТИСАНОГ ХИДРОТЕРМАЛНОМ МЕТОДОМ

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У циљу добијања мултифункционалних материјала са добром биокомпатибилношћу и добрим антимикробним дејством, хидроксиапатит (HAp) са уграђеним Zn²⁺ синтетисан је хидротермалном методом. За потребе истраживања утицаја Zn²⁺ на антимикробну активност, синтетисани су HAp прахови са различитим садржајем Zn²⁺ и употребљени са чистим HAp. Чисти прахови и они са уграђеним цинком, пре и после термичког третмана на 1200 °C, анализирани су скенирајућом електронском микроскопијом (SEM) и методом рендгенске дифракције. Антимикробни ефекат прахова испитиван је према сојевима *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* и *Candida albicans* у течном медијуму. Антимикробни тест је показао да добијени прахови имају добру антимикробну активност. Побољшана антимикробна активност прахова у које је уграђен Zn²⁺ постигнута је жарењем на 1200 °C. *In vitro* тестови биокомпатибилности, MTT и DET, са MRC-5 фибробластним ћелијама у течном медијуму урађени су за прахове жарене на 1200 °C. На основу MTT и DET тестова закључено је да прахови немају

значајан цитотоксични утицај што је потврђено SEM анализом MRC-5 фибробластних ћелија након њиховог *in vitro* контакта са праховима.

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