

INFLUENȚA CONDIȚIILOR DE SINTERIZARE ÎN PLASMĂ (SPS) ASUPRA Si_3N_4 UTILIZATĂ PENTRU IMPLANTURI OSOASE INFLUENCE OF SPARK PLASMA SINTERING CONDITIONS ON Si_3N_4 CERAMICS USED FOR BONE IMPLANTS

GABRIELA PURCEL, ADRIAN SURDU, ȘTEFANIA STOLERIU*

University POLITEHNICA Bucharest, 1, G. Polizu, 011061, sect. 1, Bucharest, Romania

Silicon nitride attracts the attention of many researchers for its use in orthopedic and dental applications, but is not yet established as a biomaterial, the research is ongoing. Present paper deals with the obtaining of Si_3N_4 ceramics unconventional heat treatment (spark plasma sintering).

The sintering process was pursued through the variation of four influencing parameters: sintering temperature, the heating rate, the pressure applied during sintering and soaking time. The obtained ceramics were characterized using X-ray diffraction for the phases determination and scanning electron microscopy for microstructure characterization. Also, were determined ceramic and mechanical properties..

By testing the biocompatibility of these ceramics in contact with the physiological environment by following the evolution of pH and ionic conductivity on immersion of ceramics in SBF is found that pH values and ionic conductivity samples varies widely in the first days but after stabilizes around SBF values.

Regarding the antibacterial effect, the tests indicate a triple value of biofilm inhibition area for the Si_3N_4 samples, as comparing with pure titanium, measured under the same condition. Also, was evaluated the MG-63 cell proliferation (by lactate dehydrogenase method - LDH) and morphology.

Nitruza de siliciu atrage atenția multor cercetători pentru utilizarea acesteia în aplicații ortopedice și dentare, dar nu este încă acceptată ca un biomaterial, cercetările fiind în curs de desfășurare. Lucrarea are ca scop obținerea ceramicilor de Si_3N_4 prin tratament termic neconvențional (sinterizare în spark plasma).

S-au urmărit patru parametri de influență ai procesului de sinterizare: temperatura de sinterizare, viteza de încălzire, presiunea aplicată în timpul procesului, respectiv palierul de menținere la temperatura maximă de tratament. Materialele ceramice obținute au fost caracterizate prin difracție cu raze X pentru determinarea compoziției fazale, prin microscopie electronică de baleiaj pentru caracterizarea microstructurii. De asemenea, au fost determinate proprietățile ceramice și mecanice.

Analiza biocompatibilității a acestor ceramici în contact cu mediul fiziologic, prin urmărirea evoluției pH-ului și conductivității ionice la imersarea ceramicilor în SBF, arată că valorile pH-ului și conductivității ionice variază foarte mult în primele zile, după care se stabilizează în jurul valorilor inițiale ale SBF-ului

În ceea ce privește efectul antibacterian, testele indică o zonă triplă de inhibiție a biofilmului pentru probele de Si_3N_4 , față de valoarea raportată pentru titan pur, măsurată în aceleași condiții. De asemenea, a fost evaluată proliferarea celulelor MG-63 prin metoda LDH, precum și morfologia acestora.

Keywords: Si_3N_4 , spark plasma sintering, biocompatibility

1. Introduction

Si_3N_4 represent a structural material characterized by high strength, fracture toughness, hardness, thermal shock resistance, wear resistance and low coefficient of friction. Due to these properties, silicon nitride attracts the attention of many researchers for its use in orthopedic and dental applications, but is not yet accepted as a biomaterial, the research is ongoing [1].

Among its applications, Si_3N_4 can be used as biomaterial for total joint replacements, micro-mechanical devices, intervertebral discs, and implants in traumatology. The absence of cytotoxicity and the biocompatibility were established during toxicity tests *in vitro* and *in vivo*. It was also found that the silicon nitride does not induce inflammation, but promotes the cells growth and proliferation [2].

Clinical data about Si_3N_4 usage in orthopedic surgery will be available in the future after will pass more time after implantation to create an overview of the use of such material. Intervertebral discs and spinal fusion devices made of Si_3N_4 are already in the medical use, with successful clinical results in the short time [3].

The sintering and densification of ceramic materials based on Si_3N_4 are quite complex and most often involves the use of sintering additives (generally, a mixture of rare earth oxides) and temperatures greater than 1700°C to promote liquid phase sintering [4-8]. $\alpha\text{Si}_3\text{N}_4 \rightarrow \beta\text{Si}_3\text{N}_4$ phase transformation and grain growth during the densification of Si_3N_4 usually occur in conventional sintering techniques, such as pressure sintering, hot pressing and hot isostatic pressing. Spark Plasma Sintering technique supposes the application of pressure, in addition to electric

* Autor corespondent/Corresponding author,
E-mail: stefania.stoleriu@upb.ro

current pulse, which significantly improves the sintering of Si₃N₄ and leads to obtaining of dense materials with a negligible increase of grain and with a low phase transformation [9].

The selection of Spark Plasma sintering technique is based on its advantages: fast sintering process; uniform sintering; low grain growth (can be prepared materials with nanograins); binders are not necessary; allow a better purification and activation of the powder particles surface; can be processed different materials (metals, ceramics, composites); high energy efficiency; easy manipulation although this sintering method allows obtaining for only a simple and symmetrical shapes and requires an expensive pulsed DC generator [10].

2. Experimental

The paper's aim is to achieve in terms of spark plasma sintering of Si₃N₄ ceramics with potential application in medical implantology.

As a precursor, a commercial silicon nitride powder (Hicol BV, Oud Beijerland, Netherlands) was used. This was characterized by X-ray diffraction, and in terms of grain size distribution.

As can be shown in Figure 1, the used silicon nitride contains two-phases, being identified the characteristic peaks both α-Si₃N₄ (according to ASTM sheet PDF 83-0700) and β-Si₃N₄ (according to ASTM sheet PDF 82-0709).

In terms of grain size, the powder has a multimodal spectrum (measured with a laser granulometer MASTERSIZER 2000), as shown in Figure 2.

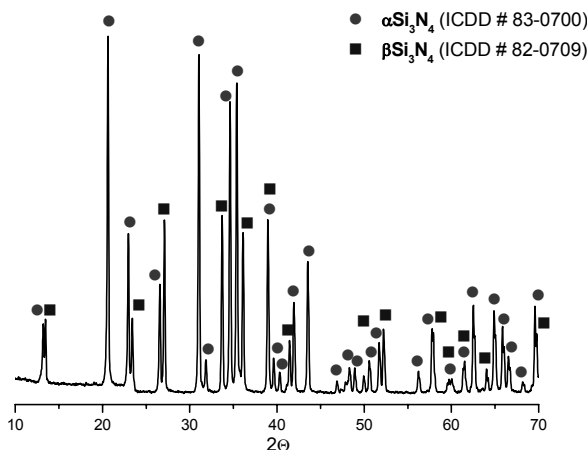


Fig. 1 - X-rays diffraction spectrum of the used silicon nitride powder / Spectrul de difracție a razelor X a pulberii de nitrură de siliciu.

The specific surface area was found to be 2390 cm²/g, and specific average diameters were: d(0,1) = 0,818 μm, d(0,5) = 4,245 μm and d(0,9) = 13,281 μm.

The ceramic samples were obtained by plasma sintering. The sintering temperature (varied from 1450 to 1600°C), holding time at maximum temperature treatment (was 0, 2, 5 and 8 minutes), the pressure applied during the sintering (varied 30 to 50 MPa) and the heating rate (varied from 100 to 200°C) were considered as factors that influence the sintering process (Table 1).

On the obtained ceramics there were made the followings:

- Determination of ceramic properties (relative density, open porosity);

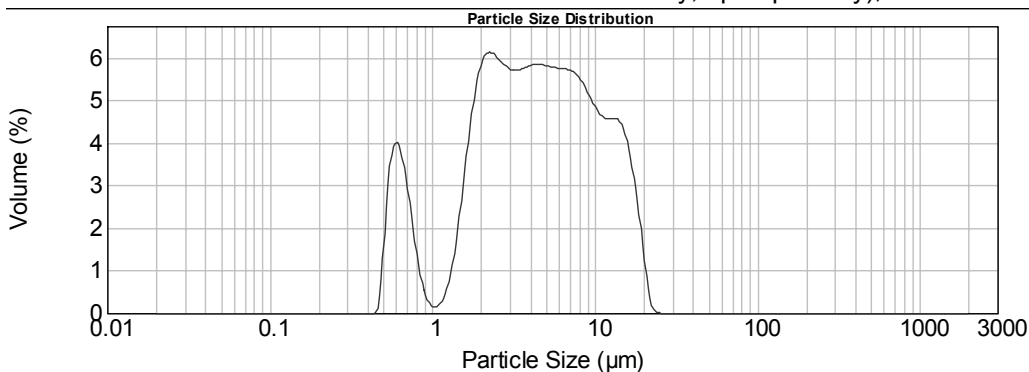


Fig. 2 - Grain size spectrum of used silicon nitride powder / Distribuția granulometrică a nitrurii de siliciu.

Parameters that influence the sintering process of Si₃N₄ samples / Parametrii de influență considerați pentru sinterizarea Si₃N₄

Table 1

Sintering temperature	1450°C	1500°C	1550°C	1600°C
Heating rate (°C/min)	150	150	150 100 200	150
Pressure (MPa)	40	40	40 30 50	40
Soaking time (min)	5	5	5 8 2	5

- Determination of mechanical properties (compressive strength);
- Phase determination (X-ray diffraction) and microstructures (scanning electron microscopy);
- Determination of biocompatibility properties (pH evolution to immersion in SBF, antibacterial properties and evaluation of cell proliferation and morphology).

3. Results and discussion

3.1. Determination of ceramic and mechanical properties

The results for ceramic and mechanical properties for samples obtained by SPS are shown graphically in Figures 3-7.

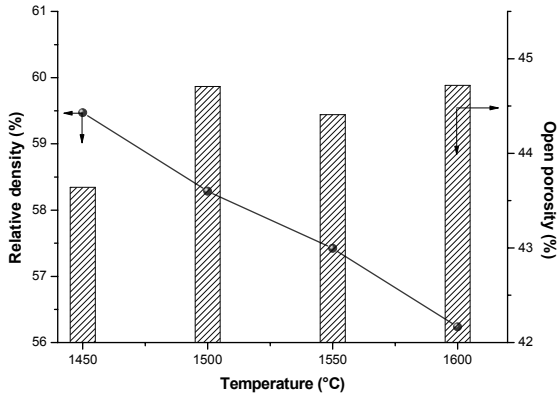


Fig. 3 - Influence of temperature on relative density and open porosity / *Influența temperaturii asupra densității relative și porozității deschise*

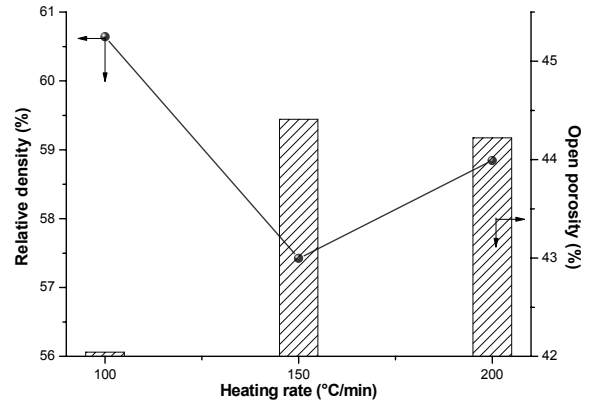


Fig. 4 - Influence of heating rate on relative density and open porosity / *Influența vitezei de încălzire asupra densității relative și porozității deschise*

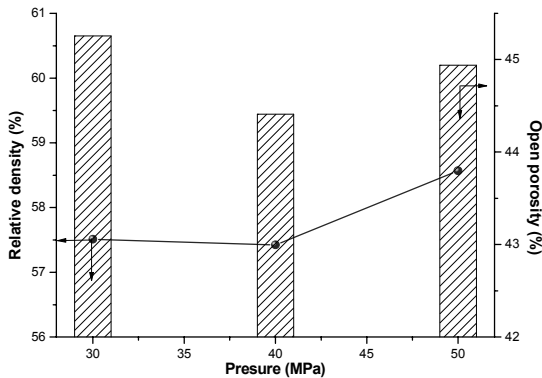


Fig. 5 - Influence of pressure on relative density and open porosity / *Influența presiunii asupra densității relative și porozității deschise*

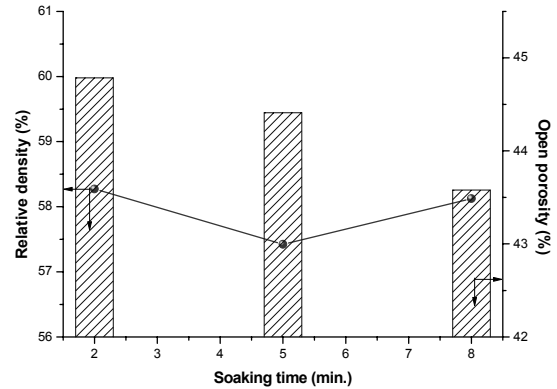


Fig. 6 - Influence of soaking time on relative density and open porosity / *Influența perioadei de menținere la temperatura maximă de tratament asupra densității relative și porozității deschise*

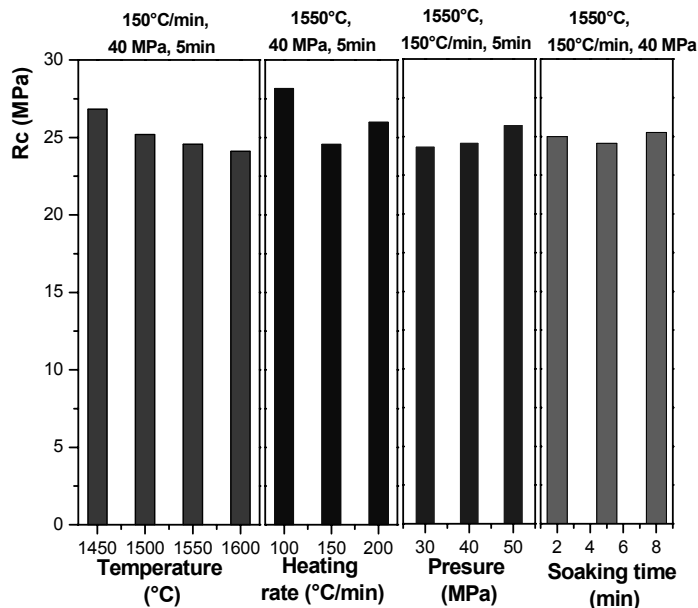


Fig. 7 - The influence of parameters on the compressive strength / *Influența parametrilor considerați asupra rezistenței mecanice la compresiune*

From presented results, the following aspects can be revealed:

- Increasing of sintering temperature causes a sharp decline of ceramic properties, probably due to initiation of reaction between graphite mold and silicon nitride powder.
- A low heating rate (100°C/min) allows the obtaining the highest relative density, probably due to a more pronounced sintering process.
- The pressure used to sintering and the soaking time does not appear to be relevant influencing factors for compositional systems studied.

- In terms of mechanical behavior it is noted again that the sample sintered at 1450°C and 1550°C, but with a low heating rate, shows the highest mechanical strength. The influence of the other factors considered, as ceramic properties, prove to be less important.

3.2. X-ray diffraction analysis

For samples sintered at different temperatures (the other parameters being considered constant: heating rate of 150°C/min, a pressure of 40 MPa, soaking time of 5 minutes), and the for sample sintered at 1550°C, but with low heating rate were carried out X-ray diffraction analysis to determine their phase composition. The obtained spectra are shown in Figure 8.

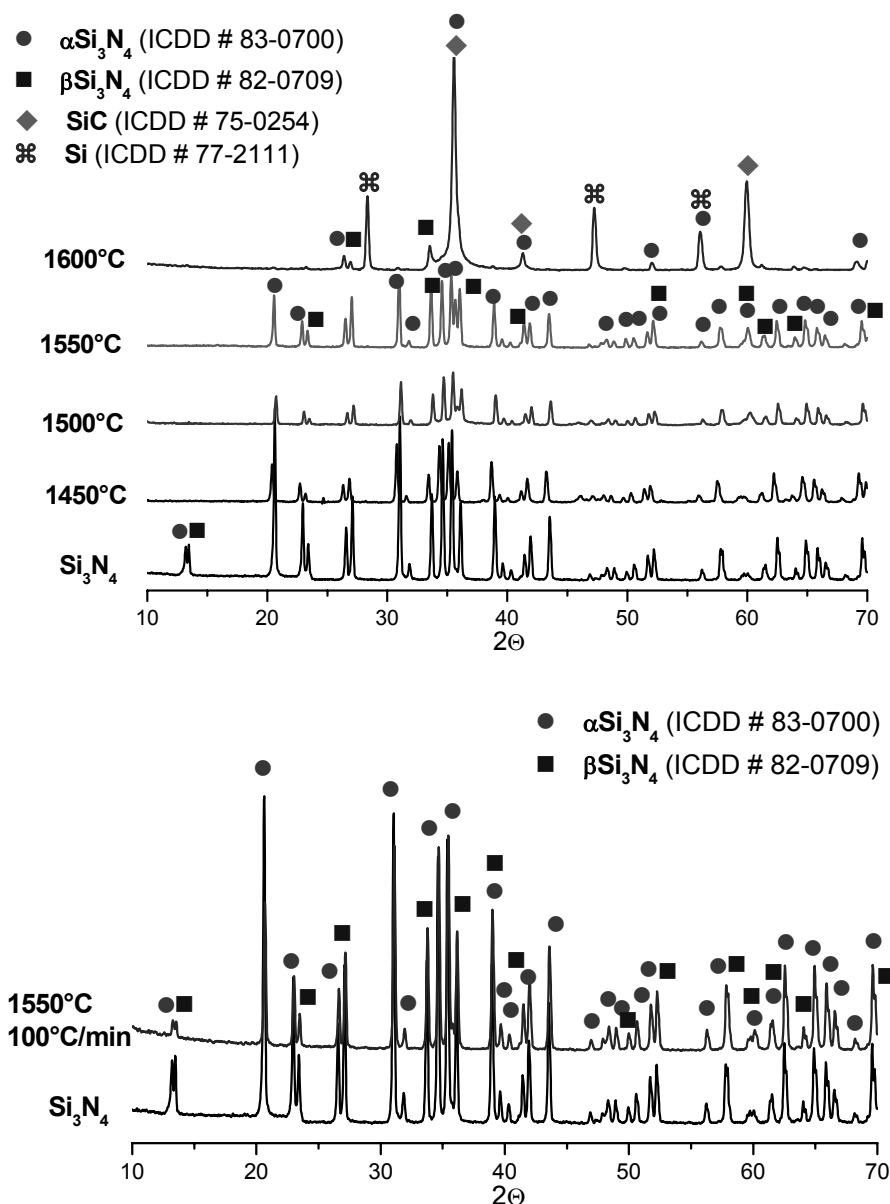
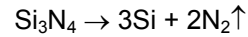
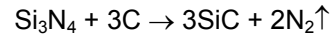


Fig. 8 – X rays diffraction spectra for the samples sintered / Spectrele de difracție a razelor X pentru probele sinterizate.

From X-rays patterns can be extracted the following statements:

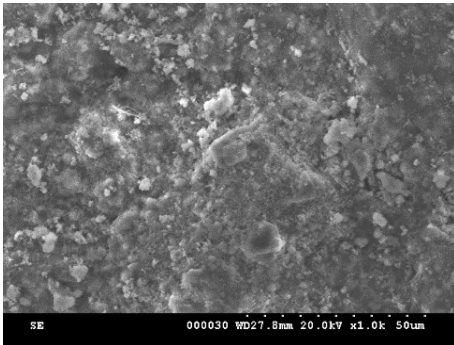
- Sintering temperature increasing affects the samples composition, observing that at 1600°C the sample contains mainly SiC (formed from reaction of Si_3N_4 with graphite mold used for sintering). The most important diffraction effect of SiC is detectable from 1500°C.
- The diffraction effects of specific metallic silicon occur only at the highest temperature as a result of decomposition of Si_3N_4 through the reaction of the Si_3N_4 with the carbon:



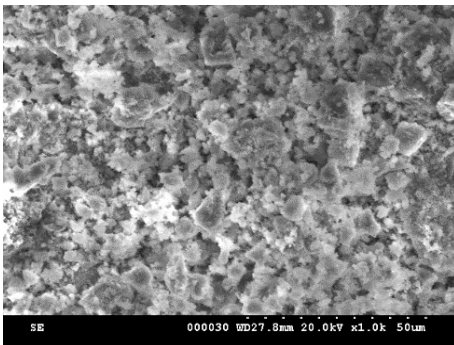
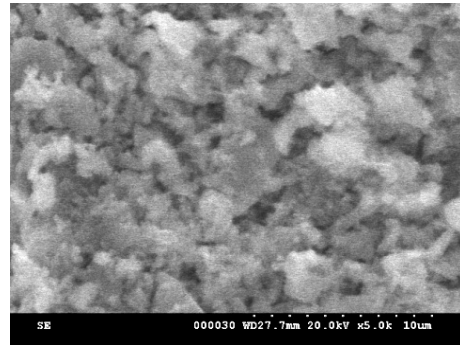
- For all studied samples, their cristallinity (measured by peak height) decreases when increasing temperature.
- It should be noted that the sample heated at 1450°C, and the sample heated at 1550°C, but with a low heating rate (100°C/min) are the only samples considered to be as monophasic samples.

3.3. Scanning electron microscopy

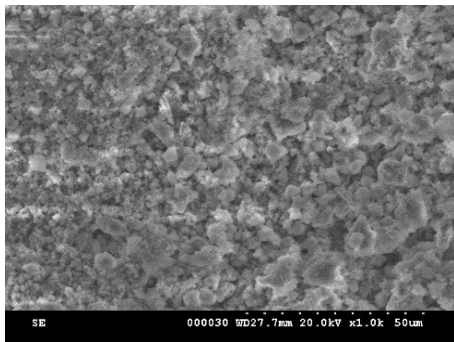
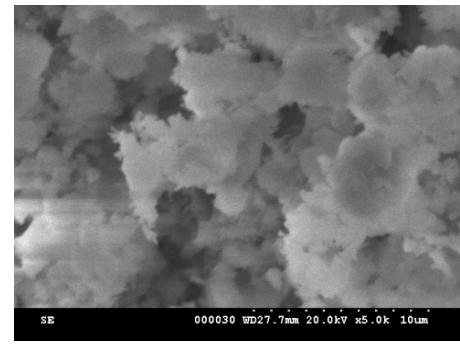
SEM images shown in Figure 9 clearly show that the samples is weak sintered, porous, regardless of the heat treatment.



1450°C; 150°C/min; 40 MPa; 5 min



1500°C; 100°C/min; 40 MPa; 5 min



1550°C; 100°C/min; 40 MPa; 5 min

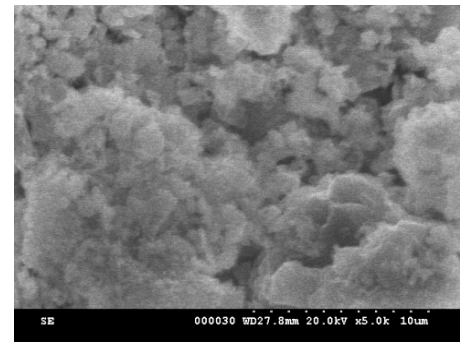
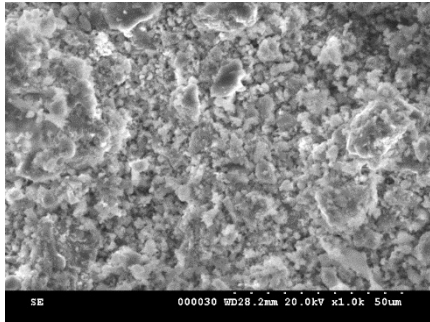
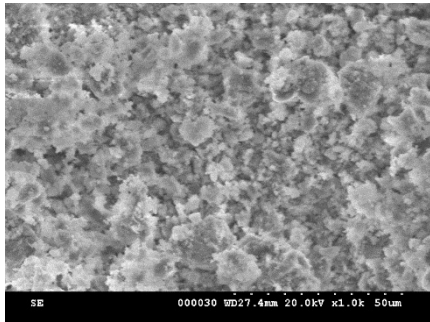
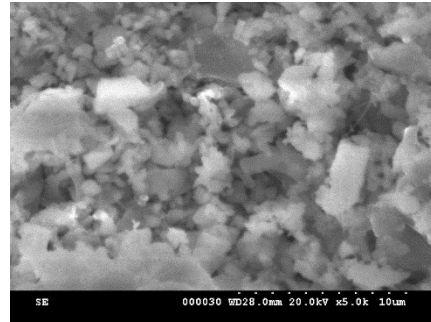


Fig. 9 - SEM images for sintered samples / Imagini SEM pentru probele sinterizate

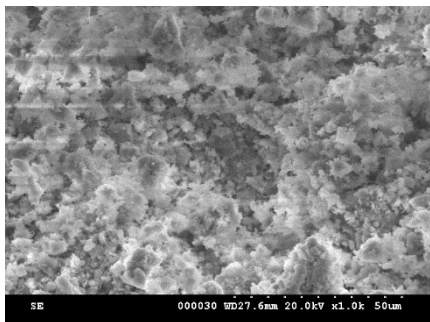
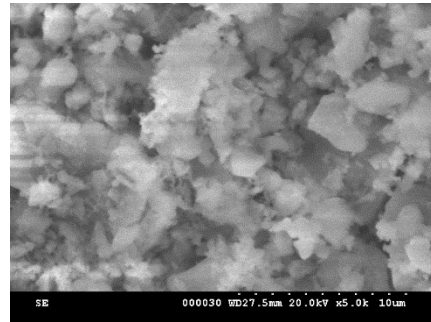
Fig. 9 continues



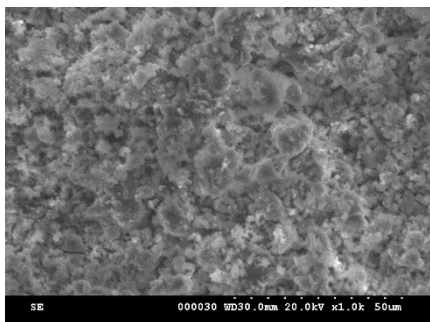
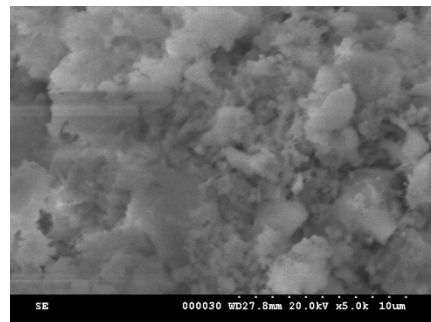
1550°C; 150°C/min; 30 MPa; 5 min



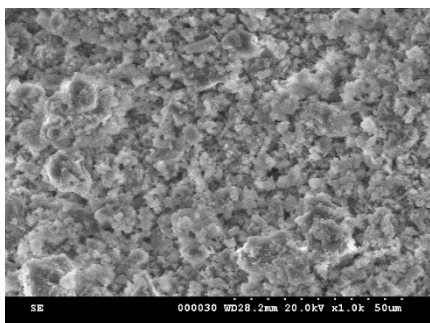
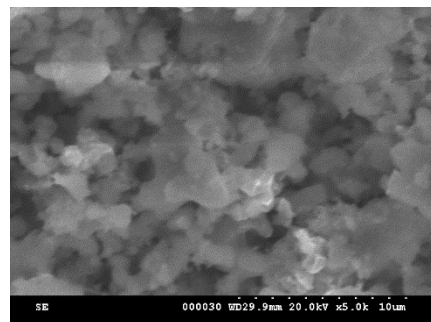
1550°C; 150°C/min; 40 MPa; 2 min



1550°C; 150°C/min; 40 MPa; 5 min



1550°C; 150°C/min; 40 MPa; 8 min



1550°C; 150°C/min; 50 MPa; 5 min

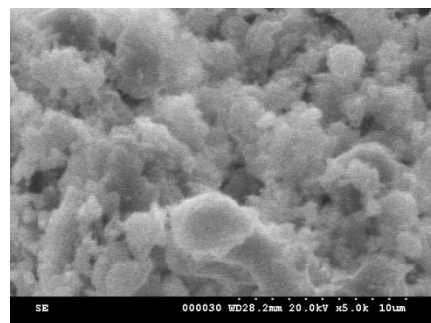


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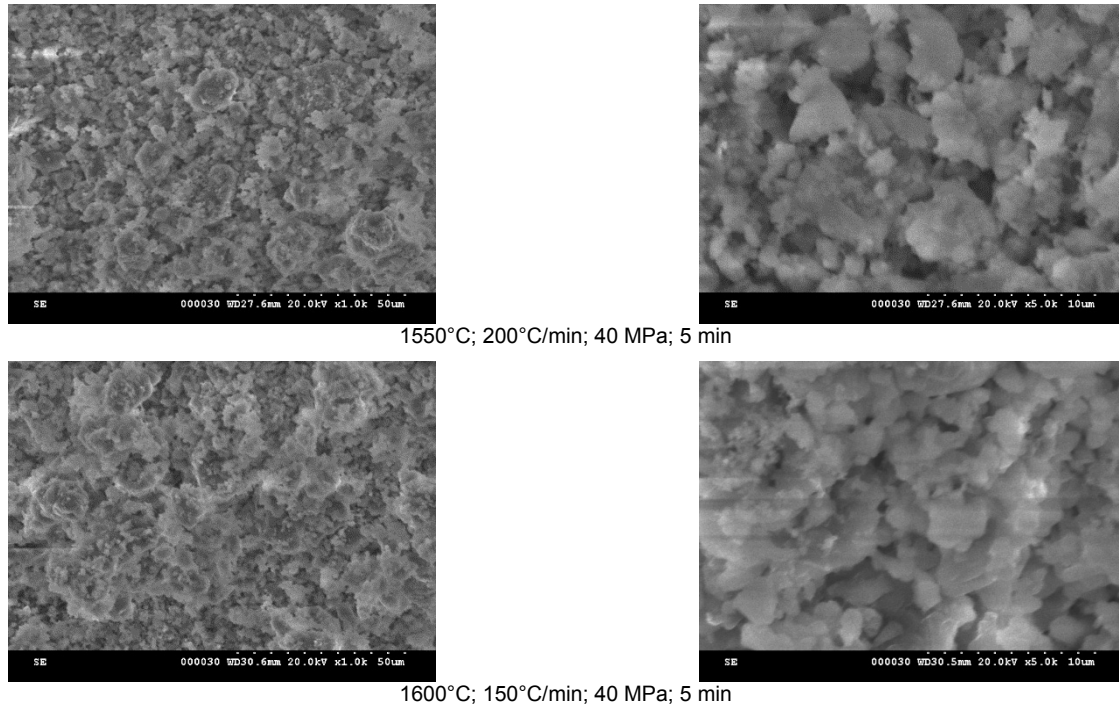


Fig. 9

4. Estimation of biocompatibility properties

Samples proven to be the best in terms of ceramic and mechanical properties were selected to estimate the biocompatibility properties.

4.1. Evolution of pH and ionic conductivity

Sintered samples were immersed in 10 cm³ of SBF (pH = 7.26, C = 9.148 mS/cm) and kept for 14 days. Changes in pH and ionic conductivity were measured for 7 days.

SBF (Simulated Body Fluid) or simulated biological fluids can be used not only to assess the *in vitro* bioactivity of materials, but also to cover different materials with hydroxyapatite [11].

The pH values and ionic conductivity for considered samples (Figure 10) show a short variation in the small times immersion in SBF,

which indicate an interaction between samples and SBF. For longer immersion times the pH do not vary up to a value, setting an equilibrium value close to SBF's pH.

The ionic conductivity indicates an ions exchange between sample and SBF up to 3 days, after which it is stopped and reaches an equilibrium value.

Regarding the comparison between the two samples, a stronger interaction with the SBF can be said to exist in the case of sample sintered at high temperature, but with a lower heating rate. The samples were further stored in SBF up to 14 days, after which they were immersed in distilled water for 12 hours to dissolve soluble salts, and then were slowly dried in an oven at 60°C. On the studied samples were carried out SEM analysis to observe the hydroxyapatite crystals deposited on the surface of the sample – Figure 11.

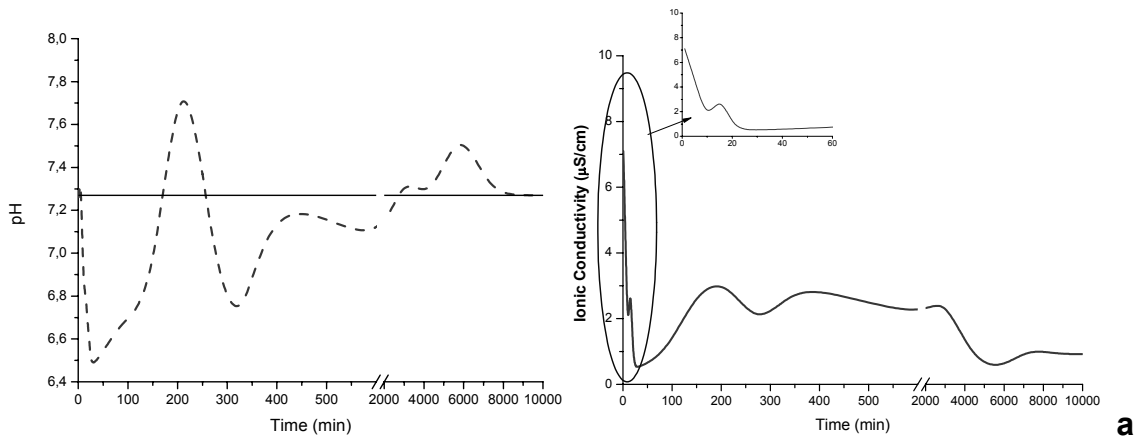


Fig. 10 continues

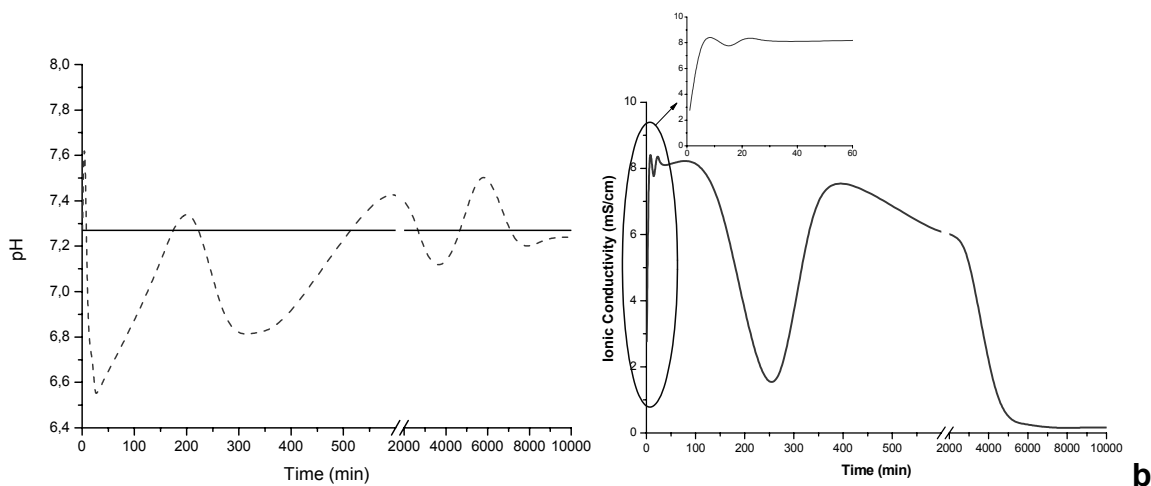


Fig. 10- Variation of pH (left) and ionic conductivity (right) for samples sintered at 1450°; 150°/ min, 40 MPa; 5 min (a) and 1550°; 100°/ min; 40 MPa; 5 min (b) / Variația pH-ului (stânga) și a conductivității ionice (dreapta) pentru probele sinterizate la 1450°; 150°/ min, 40 MPa; 5 min (a) and 1550°; 100° / min; 40 MPa; 5 min (b).

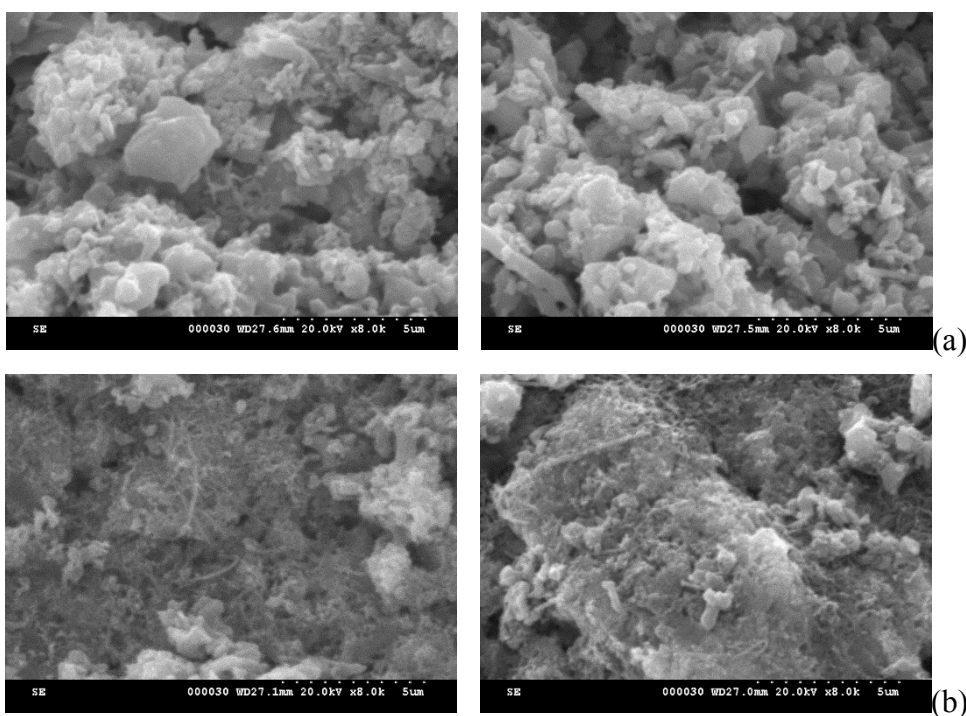


Fig. 11 - SEM images for the samples sintered at 1450°; 150° / min, 40 MPa; 5 min (a) and 1550°; 100°/ min; 40 MPa; 5 min (b), after 14 days immersion in SBF / Imagini SEM sinterizate la 1450°; 150° / min, 40 MPa; 5 min (a) and 1550°; 100°/ min; 40 MPa; 5 minutes (b) după 14 zile de imersare în SBF.

It can be easily noticed the different morphology of phosphate deposits on the surface samples. For the sample sintered at 1450°C can be seen that these deposits have a platelet morphology, and using semiquantitative EDAX analysis one can say that they are characterized by a Ca/P ratio of 0.86, lower than the 1.66 specific ratio of hydroxyapatite. This value allows the assumption that the analyzed deposits are calcium phosphates which then can be converted to hydroxyapatite.

Higher temperature sintering change the

morphology of the deposits which are layered uniformly. Also Ca/P ratio of these deposits is 0.79.

4.2. Determination of the antibacterial effect

Determination of the antibacterial effect *in vitro* of implantable materials required the use of pathogenic bacteria, Escherichia coli (ATCC 8738).

Escherichia coli is a gram-negative bacteria, oxidazo-negative. It is part of the enterbacteriaceae class which lives as epiphyte in the digestive tract. Escherichia coli is not always

limited to the intestine and has the ability to survive for a short period of time outside the body.

Escherichia coli is a bacteria conditionally pathogenic. Some strains produce enterotoxins and possess hemolytic activity. To the action of unfavorable physical factors is more resistant than other bacteria, surviving weeks and months. Contamination is produced from sick people by oro-fecale or by air, by droplets and dust and is one of the causes of infections in hospital. It was chosen for this study because it is one of the most infectious agents encountered in implantology [12].

Bacteria was cultured at 37°C on Luria Bertani culture medium which has the following composition: peptone 10 g/L; yeast extract 5 g/L, NaCl 5 g/L, agar 20 g/L. Bacterial cultures were stored at 4°C.

Quantitative evolution of antibacterial activity in vitro was performed after use of Kirby-Bauer method (halo method) [13].

Halo method is a qualitative method that determine the degree of inhibition of bacterial activity on solid medium and consists of measuring the diameter of the inhibition area of bacterial agent, due to the product's antibacterial effect (implantable material in this case).

The result was read at an interval of 18 hours. For the study on the Luria Bertani solid medium were used plates labeled „negative control” in which the pathogen has developed under normal conditions and „positive control” and plates which contain implantable samples.

Sterilized samples were placed in the middle of the Petri plates containing Luria Bertani culture medium, previously seeded with a culture area of *Escherichia coli* (0.1 ml bacterial suspension).

There were followed the evolution/involution of the pathogen agent culture to 18 hours by comparison with the control plates. Cultures were examined following macroscopic appearance (surface, topography, color, presence of pigmentation, consistency and growth rate). During development, the colonies change their

macroscopic appearance. The incubation temperature was 37°C in laboratory incubator Laboshake Gerhardt.

From Figure 12 and Table 2 one can conclude that the two samples considered, the sample heated at 1450°C, and the sample heated at 1550°C respectively, but with a low heating rate are characterized by a pronounced antibacterial activity, these having an inhibition of biofilm formation area of 226 mm² and 176 mm² respectively, which is triple values higher than the antibacterial activity determined under the same condition for pure titanium.

Table 2.

Antibacterial activity (area of zone of inhibition in mm²) of samples considered / *Activitatea antibacteriană (aria zonei de inhibare în mm²) pentru probele considerate*

Sample	Inhibition zone area (mm ²)
1450°; 150°/min; 40 MPa; 5 min	226
1550°; 100°/min; 40 MPa; 5 min	176
Titan pur	63

4.3. Evaluation of cell proliferation by lactate dehydrogenase (LDH) method

MG-63 cells, a line derived from osteoblasts, show similar morphology with fibroblasts and grows in monolayer. The culture of cells was carried out in Minimal Essential Medium Eagle (MEM), medium with 10% fetal bovine serum (FBS), 1% antibiotics (neomycin and penicillin-streptomycin, 1% L-glutamine and 1% nonessential aminoacids in standard condition such as 37±2°C, 5±1% CO₂, higher humidity than 90%.

The viability of the cell line MG-63 was determined using lactate dehydrogenase method (LDH). LDH is a cytosolic enzyme which is released into the culture medium due to loss of cell membrane integrity.

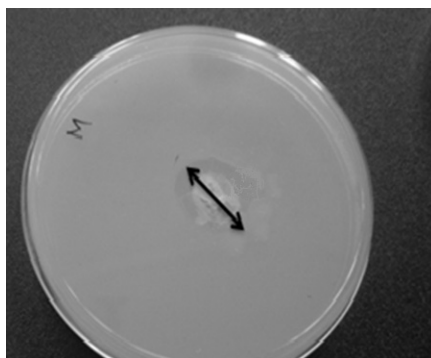


Fig. 12 - Inhibition area of bacterial biofilm: left-sample sintered at 1450°; 150°/min; 40 MPa; 5 min; Right-sample sintered at 1550°; 100°/min; 40 MPa; 5 min / *Zona de inhibare a filmului bacterial: stânga – proba sinterizată la 1450°; 150°/min; 40 MPa; 5 min, dreapta – proba sinterizată la 1550°; 100°/min; 40 MPa; 5 min*

The samples were sterilized for 72h in UV. After that, the samples were immersed in MEM culture medium for 30 min to fully cover the sample and allow filling pores. After 30 min. the culture medium was removed from Petri dish, then add a volume of 1ml medium with suspended cells (cellular stocking density 350000) on the ceramic samples, followed by incubation in standard conditions (37±2°C, 5±1% CO₂, humidity >90%) for 4h in order to ensure the adhesion of cells placed. For covering all samples, was added 2 ml culture medium and again incubated under standard conditions for 24 h. After incubation for 24 hours, supernatants were taken for LDH measurements. A 50 μl aliquot is transferred in 96 well plate and added reagents after following protocol: add 50 μl substrate followed by incubation in standard conditions, then add 50 μl stop solution and incubate for 1h. After that is measured with Mitra spectrophotometer at a wavelength of 490 nm (Figure 13). The positive control used was the cell from MG-63 cell line incubated in the same conditions as the cells is carried out testing of materials of interest. They were incubated in MEM culture medium, but treated with the lysis solution (killed 100%) for 24h.

The results demonstrate that exposure of MG-63 cell line to silicon nitride ceramics for 24h leads to a low activity of LDH and the viability percentage linked to this it was 87% and 86%, respectively for samples sintered at 1450°; 150°/min; 40 MPa; 5 min and at 1550°; 100°/min; 40 MPa; 5 min.

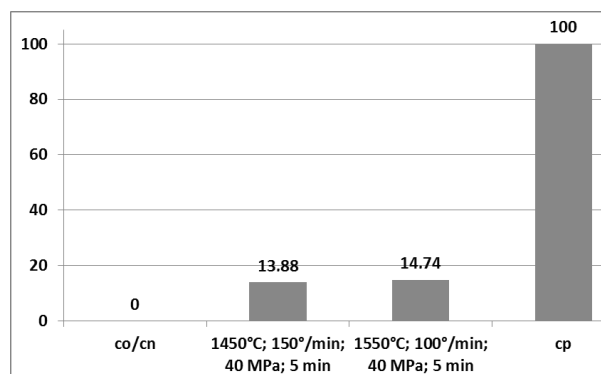


Fig.13 – The amount of LDH released into the culture medium for samples sintered at 1450°; 150°/min; 40 MPa; 5 min and at 1550°; 100°/min; 40 MPa; 5 min / Cantitatea de LDH eliberată în mediul de cultură pentru probele sinterizate la 1450°; 150°/min; 40 MPa; 5 min și la 1550°; 100°/min; 40 MPa; 5 min.

4.4. Evaluation of cell morphology by fluorescence

This test involves the use of Acridin Orange, a substance that marks cell cytoplasm and emits green fluorescence. The following steps were used: removal of the culture medium and washing with 1 ml PBS; adding 1 ml paraformaldehyde 3% for fixation; washing with 1ml PBS; adding 20 μL Acridin Orange per Petri dish; recording images.

After 24h one can see that the cells seeded on Si₃N₄ samples are fewer as comparing to control probably because the 4h time for cells adhering to a substrat is too low and when filling with culture medium, non-adherent cells were „washed” off from samples surface. Figure 14 shows that next to samples, cells are growing and proliferate under normal condition.

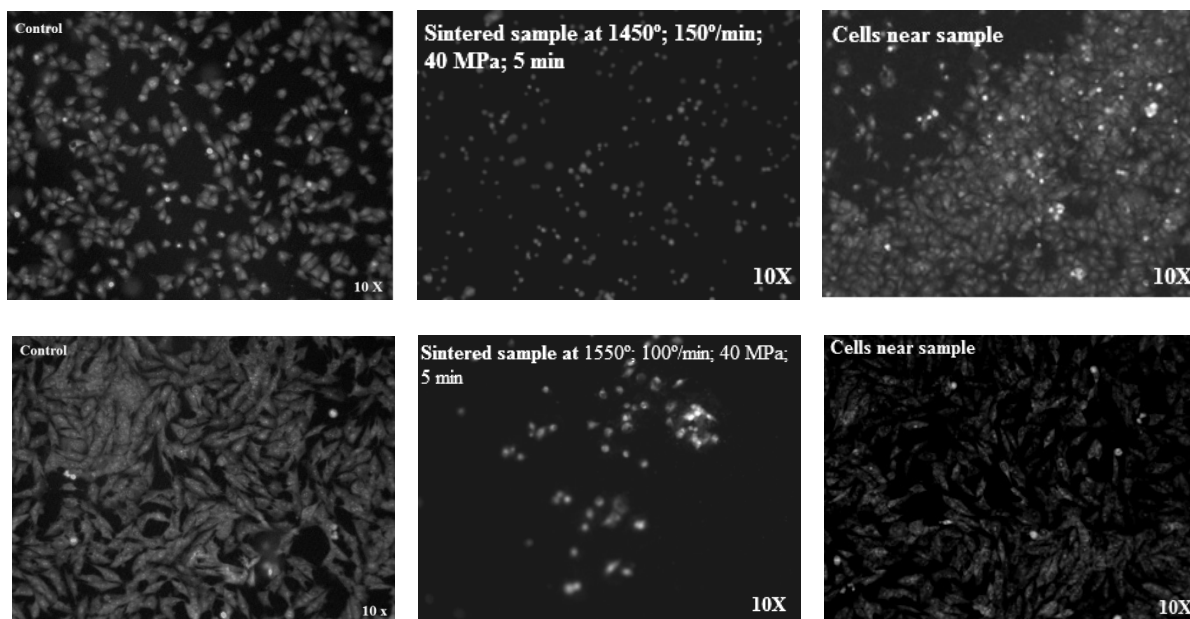


Fig.14 - Evaluation of cells morphology after 24h from sowing on sintered samples / Evaluarea morfologiei celulelor prin fluorescență după 24 ore de la însămânțare pe probele sinterizate.

5. Conclusion

The aim of present work was to obtain Si_3N_4 ceramics in terms of unconventional heat treatment (spark plasma sintering). The sintering process was followed through the variation of the four operating parameters: sintering temperature, the heating rate, the pressure applied during the process and soaking time.

X-ray diffraction used for the determination of phase composition indicated that with increasing temperature the samples are affected in terms of composition; at 1600°C the sample containing mostly SiC and silicon, due to the decomposition of Si_3N_4 . Also, as the temperature increases, decreases the crystallinity of the samples.

The electrono-microscopic analyses for microstructure characterization revealed that the samples are poorly sintered, porous, regardless of the heat treatment.

Testing for ceramic and mechanical determination (absolute density, relative density, the open porosity and compression strength) it was found that the properties of the ceramics decrease with increasing temperature; the pressure used for sintering and soaking time are not relevant influencing factors.

By testing the biocompatibility of Si_3N_4 ceramics in contact with the physiological environment by following the evolution of pH and ionic conductivity on immersion of ceramics in SBF it was found that pH values and ionic conductivity samples varies widely in the first days but after stabilizes around SBF value and in terms of phosphate deposits morphology, the sample obtained at 1450°C is characterized by platelet morphology and in the case of the other samples, the deposits are irregular distributed; Ca/P ratio reported for these is inferior than that reported for hydroxyapatite.

Regarding the antibacterial effect, the tests indicate a triple value biofilm inhibition area for the Si_3N_4 samples, comparing with inhibition area for pure titanium, measured under the same condition.

Exposure MG-63 cell line to silicon nitride ceramics for 24h leads to a low activity of LDH and therefore a good viability percentage. Also, the cells grows and proliferates next to samples, under normal condition.

Acknowledgment

This research was financially supported by Sectoral Operational Programme Human Resources Development, financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/156/1.2/G/135764 „Improvement and implementation of university master programs in the field of Applied Chemistry and Materials Science - ChimMaster”.

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