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Original Paper

Chemical and microstructural characterization of natural hydroxyapatite derived from pig bones

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Abstract. Hydroxyapatite (HAp) is the most popular bioceramic material and reknown for its excellent biocompatibility. It can be used for orthopedic replacements, bone reconstruction and also as dental implants. It stimulates osteoconduction being gradually replaced by the host bone after implantation. Three applications of HAp are of high importance: a) a porous or granulated material used in bone surgery, b) an additive to organic polymers improving their biofunctionality and c) plasma-sprayed or prepared by laser ablation coatings of metallic surfaces of bone implants. All these applications require a heat treatment of the material at elevated temperature. Natural HAp derived from animal bones has the advantage of inheriting a ''true'' chemical composition and structure of the raw material. Therefore, it seems to be an alternative solution for products based on the synthetic HAp. In this work pig bones were used as a source of the HAp material. Presented study was focused on chemical and microstructural characterization of natural HAp after heat treatment. HAp material was investigated by use of scanning electron microscopes with variable gas pressure. Ca/P ratio was determined by means of energy dispersive spectrometry. Reduced Ca/P ratio was recorded for HAp after heat treatment; however, in all cases it was higher than that of synthetic HAp. Biological tests revealed that the CAL-72 (human osteosarcoma) cells grow differently on the specimens of natural HAp depending on heat treatment conditions applied during sintering of HAp.

Keywords: Hydroxyapatite (HAp); E-SEM; heat treatment; biocompatibility; CAL-72 cell line

Hydroxyapatite (HAp – $Ca_{10}(PO_4)_6(OH)_2$) is the mineral component of natural bone used in medicine and dentistry for over 20 years [1–4]. HAp is biocompatible and osteoconductive and reveals excellent chemical and biological affinity with bony tissues [5, 6]. Studies on animals have proved the long-term biocompatibility of solid HAp and its interaction with soft tissue and bone without harmful side effects [7].

These properties make HAp a material of particular interest in guided tissue engineering. It is believed that HAp could be used for design of scaffold enabling growth of damaged bone. It has been determined that such scaffolds must exhibit porous structure [8, 9]. A common method for obtaining of porous ceramic scaffolds is a polymer sponge method. The last stage

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of this method comprises burning out of polymer material at high temperatures [10, 11].

The E-SEM has been successfully used to investigate HAp based biological materials [12, 13]. The main reason behind use of this technique is the possibility to record scanning electron micrographs of tested material without necessity to sputter coat conductive layer (C or Au). This feature enables observations of biological materials in electron microscope without the risk of damage [14].

This study was focused on determination of influence of heat treatment on the chemical composition and microstructure of natural hydroxyapatite. Literature gives few details on the behavior of natural HAp during heat treatment [15]. As sintering may affect material's biocompatibility, investigations are necessary to explain the nature of this process. The biocompatibility of the material was also studied by comparing the growth of CAL-72 cells on the HAp specimens.

E-SEM technique was chosen to carry out microstructural characterization of the investigated HAp material as well as a tool for observation of cells growing on the HAp surface.

Experimental

Materials

The natural HAp was obtained according to the following procedure: in the first step pig bones were boiled in distilled water for 24 h. Following the boiling, bones were cleaned off the spongy parts as well as marrow and soft tissue residues leading to the material consisted virtually of cortical part of pig bones. To remove organic matter, the material was leached with the use of 4 M sodium hydroxide water solution at 100 °C for 48 h. A product of leaching was rinsed with distilled water until a constant pH was achieved (Fig. 1). Finally, the material was ground and dried to a constant mass at 120 °C.

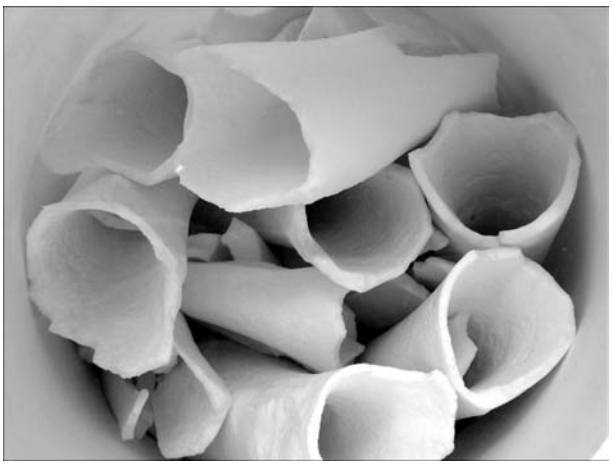

Fig. 1. Photography of pig bones after leaching out of organic matter (*cortical part* is all that remains)

For in vitro biocompatibility check, the CAL-72 cells were used. CAL-72 cell line is a stabilized human tumor cell line obtained from cartilaginous tissue.

Methods

The HAp powders were characterized by use of Environmental scanning electron microscope FEI Nova 200 NanoSEM operating under low vacuum conditions (water vapor pressure in the range of 70–90 Pa) with the use of LVD detector and operating at 18–19.9 kV, working distance 6.6–7.1 mm, spot 2.0.

The local chemical composition was determined for all elements of $Z > 5$ by use of an energy-dispersive X-ray spectrometer EDAX Genesis 4000. Quantitative analysis of elemental concentrations was achieved using ZAF correction programme with variable pressure correction overlay programme. The spectrometer was operating at 15 kV, working distance 10 mm.

To perform an XRD analysis the X'Pert Philips diffractometer with the Bragg Brentano camera geometry and CuKα incident radiation ($\lambda = 0.1540598$ nm) was used. Material was in the form of powder and the equipment was operating at 40 kV and 40 mA with a 2θ range of 10–80° with 0.02° steps.

Cell culture – CAL-72 cells were grown in DMEM standard medium (SIGMA) with the addition of 10% fetal calf serum (GIBCO) and with the addition of Insulin-transferrin-sodium selenite (SIGMA-Aldrich), at 37 °C in the presence of 5% of CO_2. The HAp specimens were formed as round-shape tablets fitting the wells of 24-multi-well plastic plates used for cell growth. Cells were seeded sparsely and grown for 9–10 days, enabling formation of the clones on the surfaces of HAp specimens. Then the cells were prepared for observations first by replacing the growth medium with phosphate buffered saline, and then by applying of methanol for few minutes. Giemsa stain was used for staining of the cells. The Giemsa staining was not necessary for ESEM observations, but for taken parallel control observations with the use of standard optical microscope (data not shown).

Results and discussion

Influence of heat treatment – chemical characterization

In order to remove residual organic matter, the HAp powder was sintered in air at 800 °C and 1200 °C. HAp powders resulting from sintering were characterized by means of XRD phase analysis. HAp powder sintered at 800 °C consisted entirely of HAp phase (Fig. 2). In XRD patterns obtained for HAp powder sintered at 1200 °C detected was peak corresponding to occurrence of CaO phase (Fig. 2). EDS analysis revealed that locally determined Ca/P ratio for the material sintered at 1200 °C was lower than that of non-sintered material and material sintered at 800 °C – see Table 1. This suggested that CaO origi-

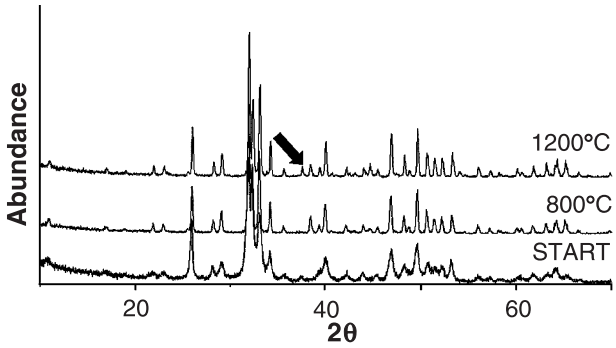

Fig. 2. XRD patterns of non-sintered HAp (*bottom line*) and HAp sintered at 800 °C (*middle line*) and 1200 °C (*top line*). Arrow indicates peak originating from CaO

Table 1. Ca/P ratio calculated basing on the results of EDS analysis

Sintering temperature	Ca/P ratio
Not sintered	1.718
800 °C	1.709
1200 °C	1.675
Synthetic HAp	1.670

nating from decomposition of HAp has tendency to migrate and agglomerate forming inclusions in the matrix. Formation of such CaO inclusions was detected within the framework of microstructural characterization – see below.

Influence of heat treatment – microstructural characterization

Microstructural characterization was performed for HAp powder sintered at 800 °C and 1000 °C in oxygen or in two step procedure, i.e. oxygen and carbon

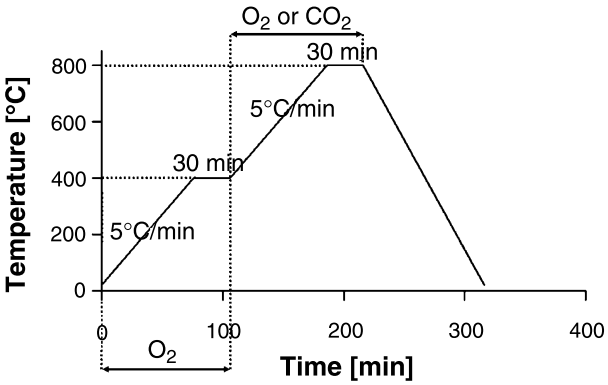

Fig. 3. Temperature pattern applied in investigations of temperature influence

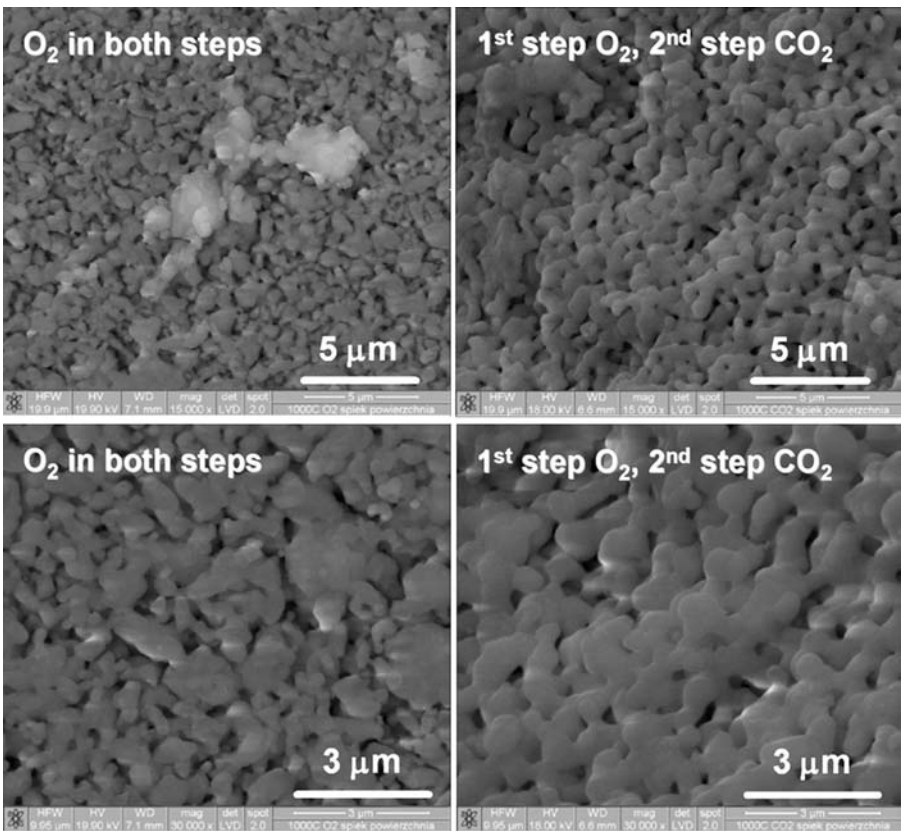

Fig. 4. E-SEM micrographs of HAp powder sintered in atmosphere of oxygen or oxygen and carbon dioxide at 1000 °C

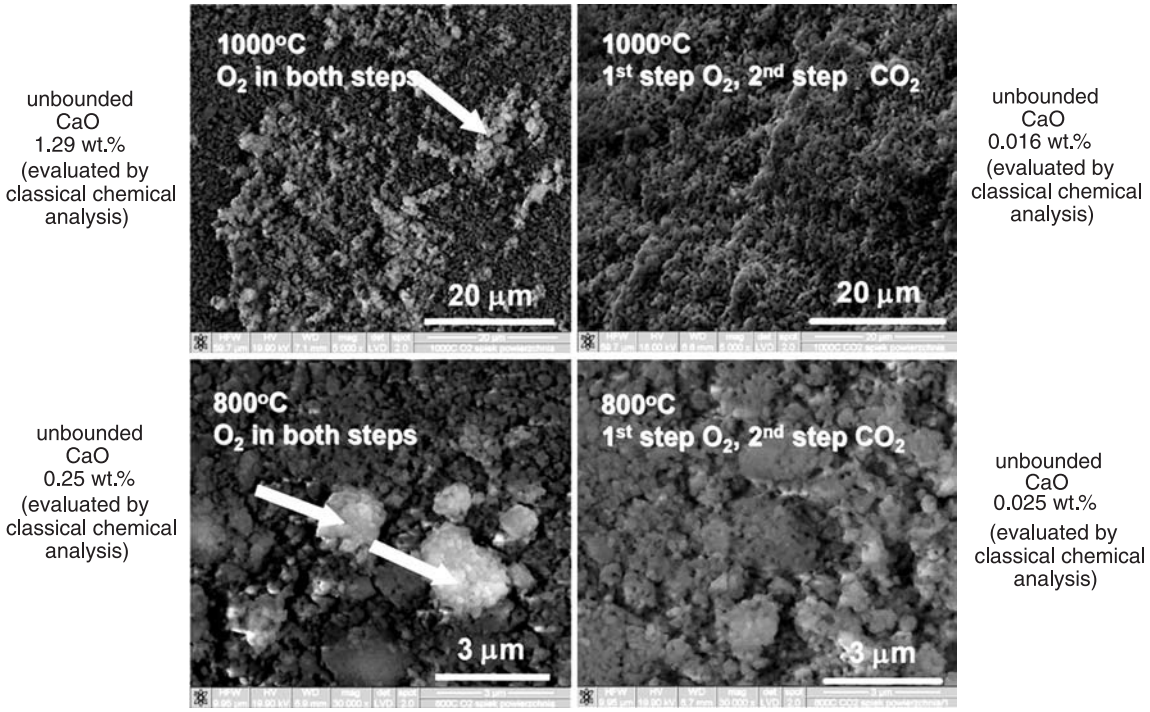

Fig. 5. E-SEM micrographs of HAp powder sintered in atmosphere of oxygen or oxygen and carbon dioxide at 800 °C or 1000 °C

dioxide atmospheres. Details of sintering procedure are given in the Fig. 3.

It was determined that sintering atmosphere has a significant influence on surface topography of the sample. Material sintered in carbon dioxide atmosphere reveals reduced number of pores and bigger crystallites when compared to material sintered in oxygen only – see Fig. 4. However, the main difference between samples sintered in oxygen only and the samples sintered in the oxygen and carbon dioxide were bright inclusions, protruding from the matrix – Fig. 5. It was assumed that the inclusions were composed of CaO and it was confirmed by EDS measurements.

The increase of crystallite size was observed in the samples sintered at higher temperature (regardless of the type of sintering atmosphere). Crystallites size varied for samples sintered in oxygen only in the range of 70–180 nm (800 °C) and 200–700 nm (1000 °C). For samples sintered in the oxygen and carbon dioxide crystallites size varied in the range of 70–140 nm (800 °C) and 700–1000 nm (1000 °C).

Cell growth on the HAp surface – biocompatibility check

CAL-72 cells were used for checking of the growth of cells on the surface of HAp specimens to exam-

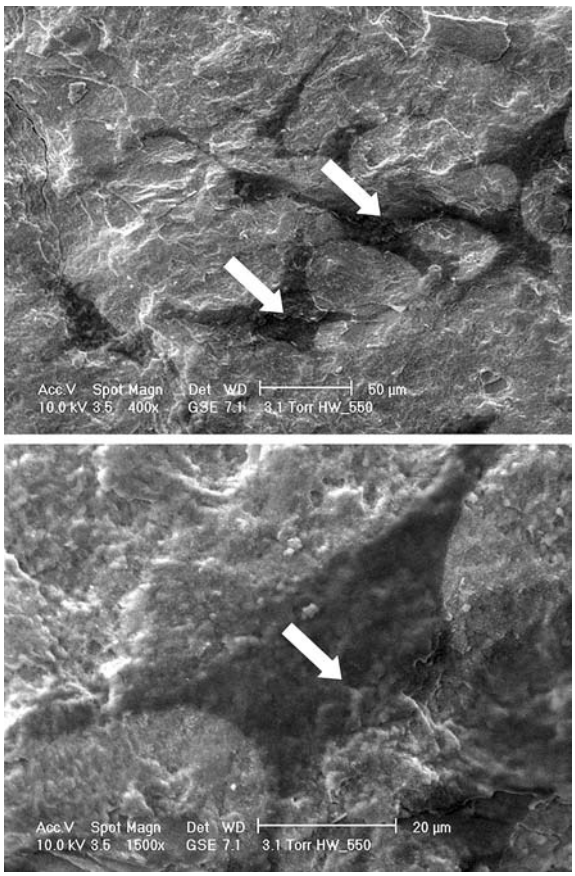

Fig. 6. CAL-72 cells on the surface of HAp

ine their biocompatibility. In general, CAL-72 cells were able to attach and form colonies on the HAp surface sintered at high temperatures (Fig. 6). This indicates that obtained material was biocompatible. The higher the heat treatment temperature was the more cell colonies were formed on the surface of the material. This can be attributed to the reduction of pore size occurring as a result of heat treatment. It cannot be excluded that cells growing on material are penetrating below the surface of the HAp to the pores.

Conclusions

It is possible to extract HAp phase from pig bones. Heat treatment of HAp at high temperatures (1200 °C) results in its decomposition and formation of inclusions comprising CaO phase. Careful considerations are needed when choosing heat treatment conditions in order to maintain a constant Ca/P ratio in the entirety of HAp material. Formation of CaO can be avoided if sintering of HAp material is carried out in atmosphere of carbon dioxide. Sintering in the atmosphere of carbon dioxide results in reduction of number of pores and increase of crystallites size. Biocompatibility of HAp material is expected to remain after the material was processed by means of techniques which employ heat treatment at high temperatures. E-SEM and EDS techniques enable carrying out of investigations of microstructural features of bioceramics used for bone reconstruction/implants.

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