Bull Mater Sci (2021) 44:17
https://doi.org/10.1007/s12034-020-02324-y

Making bioceramics from CaBiPO-apatite

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MS received 8 June 2020; accepted 14 September 2020

Abstract. To make ceramic antibacterial biocompatible material, we synthesized mixed calcium–bismuth oxophosphate (CaBiPO-apatite) with apatite structure. A mixture of CaBiPO-apatite and ammonium carbonate was used to prepare ceramic material in tablet form. Qualitative and quantitative phase analyses using powder XRD combined with IR spectroscopy shown the absence of any phase or chemical changes in the material during the process of making ceramics. *In vitro* experiment with human mesenchymal stromal cells showed high biocompatibility of the material caused by structural type and high porosity of material, despite the presence of bismuth in composition of the substance.

Keywords. Apatite; bismuth; ceramics; biocompatibility; mesenchymal stromal cells.

1. Introduction

Calcium phosphates of different structural types are usually used for making artificial bone implants [1–3]. It can be explained by the fact that the main mineral part of natural bones of mammals is $Ca_5(PO_4)_3OH$–hydroxyapatite [4]. When used in the form of bone cement [5], fine hydroxyapatite is placed in the bone fracture. When it comes to larger injuries or the replacement of entire bone sites, titanium implants with ceramic coatings are used [6]. Ceramics based on hydroxyapatite has received much less distribution due to unsatisfactory mechanical characteristics [7]. Studies aimed to obtain chemically modified hydroxyapatite to improve its mechanical and biological properties, are the most relevant at the moment [8,9].

Bismuth-contained compounds are well-known in medicine as antimicrobial agents. In the most part of such drugs, bismuth is in the form of organometallic compounds. But in inorganic crystal matrix, bismuth also saves its properties [10].

Using Bi-containing apatites, is the way to combine antimicrobial properties of bismuth with absolute biocompatibility of apatite crystal structure. Due to high isomorphic capacity of apatite structure type it allows to incorporate Bi in both cation positions of apatite structure (4*f* and 6*h*) in large enough quantity [11]. Antimicrobial and cytotoxicity of Bi-containing apatites were checked on *Escherichia coli* (gram negative), *Staphylococcus aureus* (gram positive) and *Candida albicans* (yeast) and their cytotoxicity was investigated *in vitro* using MTT assay method on fibroblast NIH 3T3 cell line [12,13]. These studies have confirmed the promise of using such compounds in medical practice.

In practical use of apatite as a material for bone implants, it is supposed to interact with mesenchymal series cells (MSC) *in vivo* [14]. In our previous work, we have successfully investigated the interaction of synthesized nanohydroxyapatite with such cells [15]. The expediency of studying the effect of apatite material on the functional characteristics of cells of this particular cell population is obvious. In this regard, the aim of this work is not only to make ceramic material based on the synthetic bismuth-containing apatite and its characterization, but also to check its compatibility with human mesenchymal stromal cells (MSC).

2. Experimental

The sample of Bi-apatite was synthesized via solid state reaction. Stoichiometric mixture of calcium nitrate tetrahydrate, bismuth nitrate pentahydrate and ammonium hydrophosphate was heated from 573 to 1223 K with 100° step during about 20 h and regular dispersion in an agate mortar. The process can be described by the reaction below:

$$Bi(NO_3)_3{\cdot}5H_2O + 4Ca(NO_3)_2{\cdot}4H_2O + 3(NH_4)_2HPO_4 \rightarrow Ca_4Bi(PO_4)_3O + 11NO_2 + 2.75O_2 + 6NH_3 + 25.5H_2O.$$

In comparison with previous attempts of synthesis of $Ca_4Bi(PO_4)_3O$ sample [12], we achieved lower values of

Published online: 05 February 2021

time and final synthesis temperature. The main features of crystal structure and stoichiometry of the compound were discussed in detail in our previous work [16].

Synthesized apatite was characterized on one hand by the powder XRD diffraction using Shimadzu XRD 6000 (CuKα radiation, geometry θ–2θ, in the 2θ range of 10°–120° with scan increment of 0.02°) and by energy dispersive X-ray fluorescence analysis in air on a Shimadzu EDX-900HS spectrometer equipped with a thermoelectrically cooled semiconductor detector (the elements that can be determined with this spectrometer range from Na to U).

Infra-red spectrum was collected on Shimadzu FTIR 8400S in the range of wavenumbers 4000–400 cm^{-1} with a resolution of 1 cm^{-1} and accumulation of a signal of 20 scans.

Thermal stability of the compound was checked using thermoanalyser Shimadzu DTG-60H (heating rate 10 K min^{-1}, nitrogen atmosphere, alundum crucible). It was shown that in the temperature range from room temperature to 1473 K, there were no processes.

To obtain ceramic samples, apatite powder was mixed with ammonium carbonate. Experimentally, it has been found that optimum content of $(NH_4)_2CO_3$ is 40 wt%. A higher content of ammonium carbonate leads to destruction of the ceramics during the sintering process. Apatite/ammonium carbonate mixture was pressed under 5 atm using hydraulic press and calcined in Snol oven with the rate of heating 1° min^{-1} till 873 K. The characteristics of obtained tablet samples are given in table 1.

Atomic scanning micro-images of sample powder and surface of ceramics were obtained using scanning electron microscope JEOL JSM-6490 LV.

To study the biological response of mammalian cells to the effect of the test material on the *in vitro* model, a direct contact method was used, since the material is intended for use in direct prolonged contact with blood and tissues.

As a test cell culture, human mesenchymal stromal cells (MSC) were used. The source of MSC were the biopsy specimens of adipose tissue of healthy volunteers, taken during plastic surgery. Each volunteer provided voluntary informed consent to participate in the study. The cells were isolated by thermal enzymatic treatment with type 1 collagenase (Sigma Aldrich) for an hour at 310 K and cultured in a complete growth medium at standard conditions (absolute humidity, 310 K, 5% CO_2). The complete growth medium had the following composition: medium α-MEM, 20% calf embryonic serum, glutamine, antibiotics penicillin/streptomycin. The environment and reagents of the firm 'Gibco' and the plastic from the company 'Costar' was used. Upon reaching the subconfluent monolayer (60–70%), the culture was transplanted. The medium was changed every 2–3 days. For experiments, the third passage cultures were used.

Prior to the experiment, the morphology of the cells, their viability with the use of the vital dye of trypan blue and the concentration of cells in the haemocytometer were fixed.

Sterility of the culture was determined by standard culture methods, mycoplasma and viruses by the PCR (polymerase chain reaction) method.

To characterize the immunophenotype of culture cells, a panel of monoclonal antibodies was used: CD 90 FITC, CD 44 FITC, CD 105 PE, CD 73 PE, CD 45 PC5, CD 14 PC5, HLA-DR PC7, CD 34 PC7 with the corresponding isotypic controls (Bacman Coulter, USA) on the flow cytofluorimeter BD FACS CANTO II (Becton Dickinson, USA).

The cells of the culture had a characteristic fibroblast-like morphology with a typical spindle-shaped form, were clearly delineated with pronounced processes, dense nuclei.

The viability of cells before sowing on the sample was 98–99%, the immunophenotype of cells was characteristic for MSC: CD 90+, CD 105+, CD 73+, CD 44+, CD 45−, CD 14−, HLA DR−, CD 34− [17]. The concentration of cells before sowing on the samples was 2×10^5. The

Table 1. CaBiPO tablets characterization ($\rho_{XRD} = 4.1748 \times 10^3$ kg m^{-3}).

Sample number	V (m^3) × 10^9	ρ (kg m^{-3}) × 10^{-3}	Porosity (%)*
Before calcination			
1	165.9 (2)	1.9016	54.5
2	165.9 (2)	1.9191	54.0
3	165.9 (2)	1.9185	54.0
4	165.9 (2)	1.9299	53.8
5	165.9 (2)	1.9082	54.3
After calcination			
1	155.2 (2)	1.2017	71.2
2	155.2 (2)	1.2462	70.1
3	155.2 (2)	1.2505	70.0
4	155.2 (2)	1.2540	70.0
5	155.2 (2)	1.2222	70.7

*Porosity $= \left(1 - \frac{\rho_{experiment}}{\rho_{XRD}}\right) \times 100\%$, where $\rho_{experiment} = \frac{\pi d^2 hm}{4}$, ρ_{XRD} is calculated during the process of analytical indexing of XRD pattern of the compound.

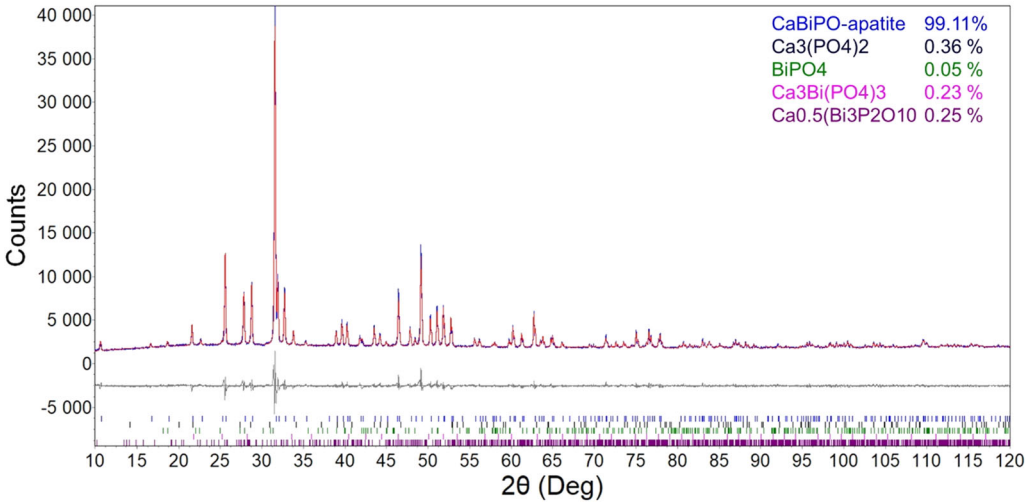

Figure 1. XRD pattern of CaBiPO-apatite. Red line, experimental XRD pattern; blue line, simulated by TOPAS 3.0; grey line, difference line; blue triangles at the bottom, positions of Bragg reflections of all checked compounds.

culture was sterile, mycoplasmas and viruses were not contaminated.

To visualize the cells on the surface of the sample (write the correct designation), a fluorescence microscopy method, implemented on the Cytation 5 multifunction photometer-imager (BioTek, USA), was used.

The intravital staining of the nuclei of cells adhered to the material was carried out using Hoechst 3334 (BD Pharmingen^{TM}) fluorochrome having high specificity for the double-stranded DNA molecule (excitation wavelength of 377 nm, emission wavelength of 447 nm). Staining was performed in accordance with the manufacturer's protocol.

Calcein AM (BD Pharmingen^{TM}) was used to mark living cells and characterize their morphology, having the property due to increased hydrophobicity to easily penetrate into viable cells. Within the esterase cells, amino groups are cleaved from non-fluorescent calcein, which then begins to fluoresce (excitation wavelength of 469 nm, emission wavelength of 525 nm). Calcein staining was performed after staining of Hoechst 3334 cell nuclei according to the manufacturer's protocol.

3. Results and discussion

To be sure that the processes of making ceramics did not affect the functional composition of compound and phase composition of the ceramics, we undertook additional experiments.

Firstly, we grounded a few of ceramic tablets using ball-mill. Then, we compared XRD pattern of obtained powder with data collected from the initial powder sample. In the Bi–Ca–P–O system, many different compounds may form during the synthesis at described conditions [18–22]. We use structure data for main compounds in aforementioned system and made qualitative and quantitative phase analyses using Topas 3.0 software. As it may be found from figure 1, we still did not observe any additional compounds after making ceramics.

IR spectroscopy is one of the most sensitive methods for finding changes in functional composition of the compound. Here, it was used to confirm the absence of pyrophosphate groups $P_2O_7^{2-}$, which may form during condensation of phosphate ions caused by high temperatures. On IR spectrum (figure 2), we detected only stretching asymmetric bonds $\nu_{as}(PO_4)$ (1067, 1036, 999, 979 cm^{-1}), stretching symmetric bonds $\nu_s(PO_4)$ (945 cm^{-1}) and deformation $\delta(PO_4)$ (629, 602, 563 cm^{-1}) modes and no ν(P–O–P) modes, whose presence may confirm the existing of pyrophosphates $P_2O_7^{2-}$ in the final compound even at very small quantitative [23,24].

Owing to the fact that up to 1473 K, there is no phase transitions in the compound (which is confirmed by absolute monotonically changing curve of DTA analysis), the

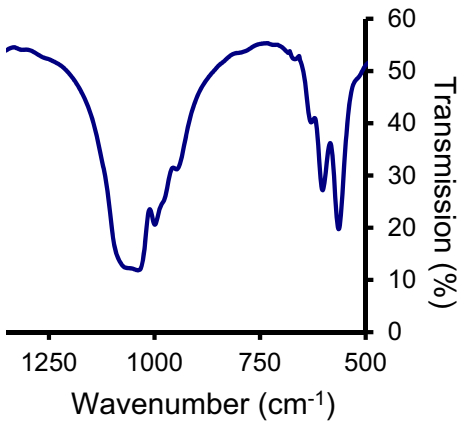

Figure 2. IR spectrum of obtained CaBiPO-apatite.

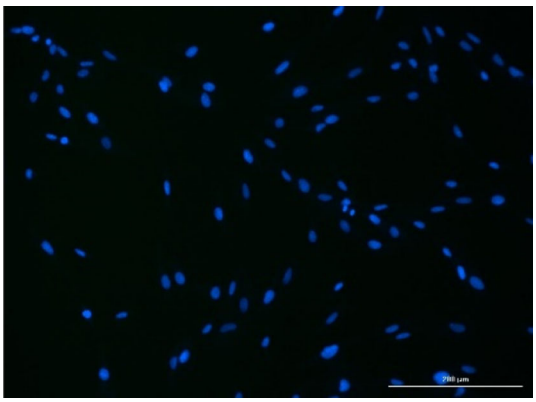

Figure 3. ASM image of nuclei of human mesenchymal stromal cells on the surface and in the volume of the ceramic apatite sample (10×–10× magnification): blue, the cell nuclei (coloured using Hoechst 3334).

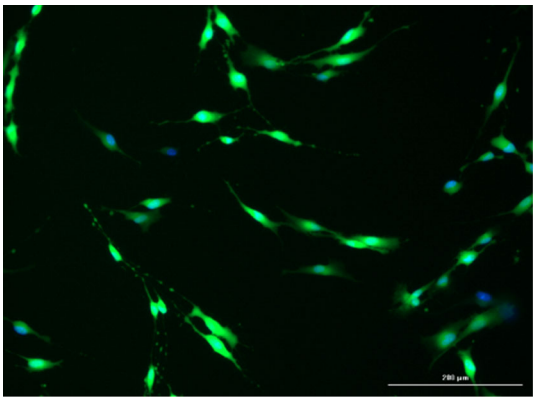

Figure 4. ASM image of human mesenchymal stromal cells on the surface and in the volume of the ceramic apatite sample: blue, the cell nuclei (coloured using Hoechst 3334); green, cytoplasm (coloured using Calcein AM) (10×–10× magnification).

chemical composition of the tablet samples under the aforementioned conditions did not change.

After calcination, the obtained ceramics were sterilized by repeated washing in physiological saline with the addition of antibiotics (penicillin/streptomycin) for 24 h. MSCs are often used to model the behaviour of osteogenic cells on orthopaedic and dental biomaterials. In our study, we evaluated the adhesion properties of the Bi-apatite material using mesenchymal stem cells.

The cells of the third passage in the concentration of 2×10^5 were inoculated onto the test ceramic samples and then, were monitored after 3 days, after the initiation of the interaction. Methods of fluorescence microscopy were used to visualize the cells on the surface of the samples.

Seventy-two hours after sowing the cells, a large number of blue-coloured nuclear cells are fixed on the specimens (figure 3). When using colouration with calcein, a large number of fluorescent cells are determined on the surface of the samples (figure 4). It is known that calcein stains the cytoplasm only in living cells. Staining of the cytoplasm of cells allows simultaneous fixing their morphology on the surface of the samples. Fluorescent cells have a typical spindle shape with pronounced processes, which is typical of fibroblast-like cells. The obtained results testify to the adhesion of MSC on the surface of the test material and their preservation of high viability in the process of cultivation under standard cultivation conditions.

It should be noted that high porosity of samples (table 1, figure 5) allows easier penetration of cells into the apatite ceramics. High porosity of samples in combination with high sorption properties of the material is the most important qualitative characteristic, which implies its effectiveness in clinical application.

The microporosity of the material will allow the circulation of biological fluids, increase the surface area and accelerate the degradation process. Interconnected pores will create capillary nets that will actively promote the penetration of cells and nutrients into the central part of the implant. Macroporosity will ensure the penetration of cells and vessels and the subsequent ingrowth of bone [25].

It is known that for the regeneration of a damaged bone site, MSCs should be recruited into the area of trauma, and then differentiated into osteoblasts. Therefore, the development of new materials that can attract MSC is of great interest for clinical practice [26]. Adhesion of MSC to the test material and maintenance of the cells with a

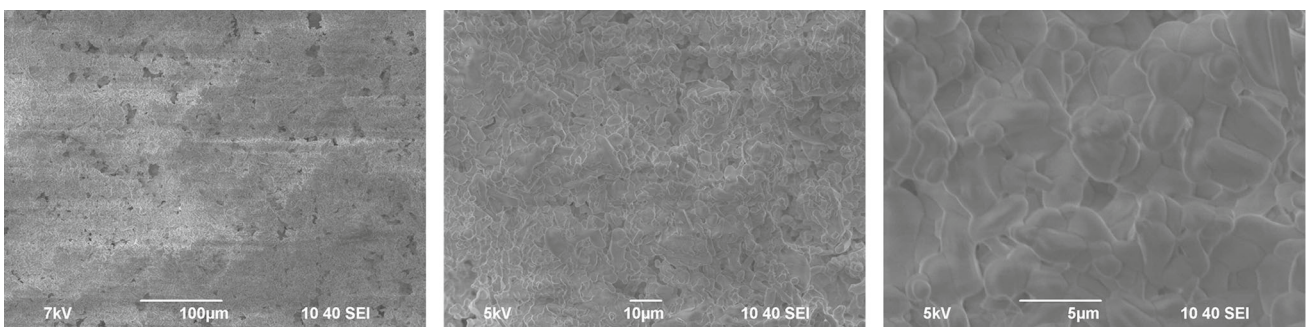

Figure 5. Atomic scanning micro-images of porous structure of CaBiPO ceramic tablets. Branched porosity and a wide pore size distribution are observed.

characteristic morphology when they are cultivated on it. *In vitro* indicates that in the future (*in vivo*) Bi-apatite-based implants will promote the regeneration of damaged bone. The results obtained are consistent with literature data that bismuth-containing materials based on hydroxyapatite can be characterized by lack of cytotoxicity, a high osteogenic potential and a good biomimetic microecology for scaffold mineralization [27]. Based on the data presented, it can be assumed that the material under investigation is promising for clinical use in the treatment of bone defects in the future.

4. Conclusions

Apatite-structure compound $Ca_4Bi(PO_4)_3O$ was synthesized using solid-state reaction and characterized by the complex of physico-chemical methods. Thermal stability of the compound allows to make ceramic materials using simple approach: pressing of the powder with pore-generating compound (for example, ammonium carbonate) with subsequent calcination at high temperatures. Obtained ceramic samples have about 70% porosity, which makes possible penetration of the cells into the sample. Bi-apatite ceramic samples combine antimicrobial properties and biocompatibility with human cells, and hence, they can be a basis of new artificial bone implants. It is obvious that such properties of the material are insufficient to make a final decision on its use. The behaviour of bismuth in the body after implantation is of fundamental importance. There are several approaches to solve this issue. In our opinion, the most technically available and, which is important, relatively inexpensive, is based on thermodynamic modelling of the behaviour of a material taking into account the environment. We applied a similar approach to analyse the stability of the model bone tissue (stoichiometric hydroxyapatite) when radioactive isotopes of strontium 90 enter the body [28]. To carry out such a simulation, data on the thermodynamic functions of the basic substance (in our case—for bismuth-apatite) are required. Based on the results of our previous work, we calculated the standard entropy of formation [16], but to determine the standard enthalpy of formation, a thermochemical scheme is needed, for all other participants in which the standard enthalpies of formation are known. Unfortunately, at the moment, the problem has not been solved in view of the fact that bismuth compounds are either insoluble in suitable solvents or are not characterized thermodynamically. But even without such modelling, it can be argued that the extraction of bismuth by body fluids will most likely be negligible, since compounds of the apatite class (especially calcium phosphates) are characterized by record-low values of the solubility product in neutral and weak acidic media [29], which means that the material can be considered as the basis for new artificial bone implants.

Acknowledgements

We are grateful to the staff of the Laboratory for Ceramics Technology (UNN Research Institute for Physics and Technology), for their help in conducting microscopic studies on ceramics. The work was performed with the financial support of the Russian Foundation of Basic Research (Project No. 16-33-601721 мол_а_дк).

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