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Biocompatibility of biphasic α,β-tricalcium phosphate ceramics *in vitro*

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ABSTRACT

The biocompatibility of biphasic α,β-tricalcium phosphate ceramics, obtained by annealing a compact preform based on β-tricalcium phosphate powder, was studied *in vitro*. It was found that within 10–30 days the adhesion of primary dental pulp stem cells located on the surface of biphasic α,β-tricalcium phosphate ceramics is suppressed. Decrease of the cell number on the surface of biphasic α,β-tricalcium phosphate ceramics, most likely, can be associated with both the pH level (acidic) as a result of hydrolysis of the more soluble phase of α-tricalcium phosphate and with the nature of surface that changes as a result of the formation and growth of hydroxyapatite crystals.

1. Introduction

For many years, materials based on tricalcium phosphate $Ca_3(PO_4)_2$ remain in the focus of researchers in the field of inorganic medical materials science [1]. Tricalcium phosphate has three polymorphs: β-$Ca_3(PO_4)_2$, α-$Ca_3(PO_4)_2$, α′-$Ca_3(PO_4)_2$ [2]. α′- $Ca_3(PO_4)_2$ exists above a temperature of ~1430 °C and completely turns into α-$Ca_3(PO_4)_2$ upon cooling. The conversion of β-$Ca_3(PO_4)_2$ to α-$Ca_3(PO_4)_2$ occurs upon heating at ~1125 °C, while α-$Ca_3(PO_4)_2$ can be preserved upon cooling and exist at room temperature [3]. Ceramics containing β-$Ca_3(PO_4)_2$ are commonly used in medical applications [4]. But α-$Ca_3(PO_4)_2$ is often used as a component of a powder mixture for obtaining a biocompatible calcium phosphate cements [5]. The reason for producing ceramics based on α-$Ca_3(PO_4)_2$ or containing the α-$Ca_3(PO_4)_2$ phase may be the intention to obtain a calcium phosphate material with a higher dissolution rate [6]. In fact, there are a lot of materials under investigation having a deviation of pH from 7 when soaked in water, in body fluids or being implanting [7]. Due to the ability to be hydrolyzed in body fluids and to form orthophosphoric acid α-$Ca_3(PO_4)_2$ as a special phase can be used as a component with ability to compensate alkalinity of other phases of new ceramic composites consisted of additional phases with variable resorbability or strength. For example, Na-rhenanite ($NaCaPO_4$) or K-rhenanite ($KCaPO_4$) when soaked in water or in body fluids generate pH about 8–9 [8,9].

Calcium phosphate materials consisting of β-$Ca_3(PO_4)_2$ and/or α-$Ca_3(PO_4)_2$, for which the Ca/P molar ratio is 1,5, can be obtained using various techniques, primarily related to the preparation of the initial powder or components of the original powder mixture. In order to obtain ceramics with phase composition represented by tricalcium phosphate (β-$Ca_3(PO_4)_2$ and/or α-$Ca_3(PO_4)_2$), calcium phosphate powder or a powder mixture with a Ca/P molar ratio of 1,5 can be used [10]. Tricalcium phosphate cannot be obtained by precipitation from solutions. This calcium phosphate can be obtained either as a result of thermal conversion of Ca-deficient hydroxyapatite [11,12] or as a result of heterogeneous reactions occurring when heated [13–15]. One of the most well-known, reliable and simple techniques for obtaining β-tricalcium phosphate β-$Ca_3(PO_4)_2$ powder for ceramics is solid-phase synthesis [16].

The term «biphasic calcium phosphate ceramics» is well known and usually refers to ceramics consisting of calcium hydroxyapatite and β-tricalcium phosphate [17] or sometimes to ceramics consisting of β-tricalcium phosphate and β-calcium pyrophosphate [18] or even to ceramics consisting of β-calcium pyrophosphate and β-calcium polyphosphate [19]. There are also methods for producing biphasic ceramics, which contain two different (α- and β-) modifications of tricalcium phosphate [20]. Moreover, it is known that biphasic ceramics,

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including α- and β-modifications of tricalcium phosphate, can be obtained from β-$Ca_3(PO_4)_2$ powder by firing the preformed powder at a temperature that is higher than the β-$Ca_3(PO_4)_2$ to α-$Ca_3(PO_4)_2$ phase transition temperature [21]. This study is aimed to investigate the *in vitro* biocompatibility of biphasic ceramics consisting of β-$Ca_3(PO_4)_2$ and α-$Ca_3(PO_4)_2$ modifications of tricalcium phosphate obtained from β-$Ca_3(PO_4)_2$ powder.

2. Materials and methods

2.1. Synthesis of the β-$Ca_3(PO_4)_2$ powder

The initial components of the powder mixture for the synthesis of β-$Ca_3(PO_4)_2$ were calcite $CaCO_3$ (GOST 4530-76) and calcium γ-pyrophosphate γ-$Ca_2P_2O_7$ obtained by thermal conversion of brushite $CaHPO_4{\cdot}2H_2O$, which was synthesized from 1 M solutions of calcium nitrate $Ca(NO_3)_2{\cdot}4H_2O$ (GOST 4142-77) and ammonium hydrogen phosphate $(NH_4)_2HPO_4$ (GOST 3772-74) according to reaction (1).

$$Ca(NO_3)_2 + (NH_4)_2HPO_4 + 2H_2O = CaHPO_4{\cdot}2H_2O + 2NH_4NO_3 \quad (1)$$

Calcium γ-pyrophosphate γ-$Ca_2P_2O_7$ powder was obtained by heat treatment of brushite powder $CaHPO_4{\cdot}2H_2O$ at a temperature of 500 °C for 2 h reaction (2).

$$2CaHPO_4{\cdot}2H_2O = Ca_2P_2O_7 + 5H_2O \quad (2)$$

The preparation of β-tricalcium phosphate β-$Ca_3(PO_4)_2$ was carried out by the solid-phase synthesis method according to the reaction (3).

$$CaCO_3 + \gamma\text{-}Ca_2P_2O_7 = \beta\text{-}Ca_3(PO_4)_2 + CO_2\uparrow \quad (3)$$

The calculated amounts of calcium carbonate $CaCO_3$ and γ-calcium pyrophosphate γ-$Ca_2P_2O_7$, were placed in a ZrO_2-containers. Acetone and ZrO_2 grinding media were added into the ZrO_2-containers. Then the powder mixture was homogenized in a planetary ball mill for 10 min (300 rpm rotation speed). After evaporation of acetone, the dry mixture was sieved through a polyester sieve with a mesh size of ~400 μm. The powder mixture was then fired in a Nabertherm muffle furnace (Germany) at a temperature of 900 °C (heating rate 5 °C/min, holding at a final temperature for 6 h).

2.2. Preparation of biphasic α,β-$Ca_3(PO_4)_2$ ceramics

In order to obtain biphasic α,β-tricalcium phosphate ceramics, samples based on β-$Ca_3(PO_4)_2$ powder were pressed on a Carver Laboratory Press model C (USA) hand press in the form of tablets with a diameter of 8 mm and a height of 5–6 mm. The average weight of sample was about 0.3 g. The forming pressure was set to 100 MPa. Prepared tablets were fired at a temperature of 1200 °C (heating rate - 5 °C/min, holding at the final temperature - 12 h).

2.3. The study of phase composition of powders and ceramics

Phase composition of the prepared powder and fired ceramic samples was examined by means of X-ray diffraction (XRD) (2θ 20–60°, Cu Ka radiation, Rigaku D/MAX 2500 (Rigaku Corporation, Tokyo, Japan) with rotating anode, Japan). The phases were identified using the ICDD PDF2 database [22]. The content of phases in the samples of ceramic material after calcination at 1200 °C for 12 h was determined from quantitative X-ray diffraction analysis according to a procedure based on a standard method. According to this method the proportions of α- and β-modifications of tricalcium phosphate $Ca_3(PO_4)_2$ can be quantified using XRD from integrated intensities of characteristic diffraction peaks.

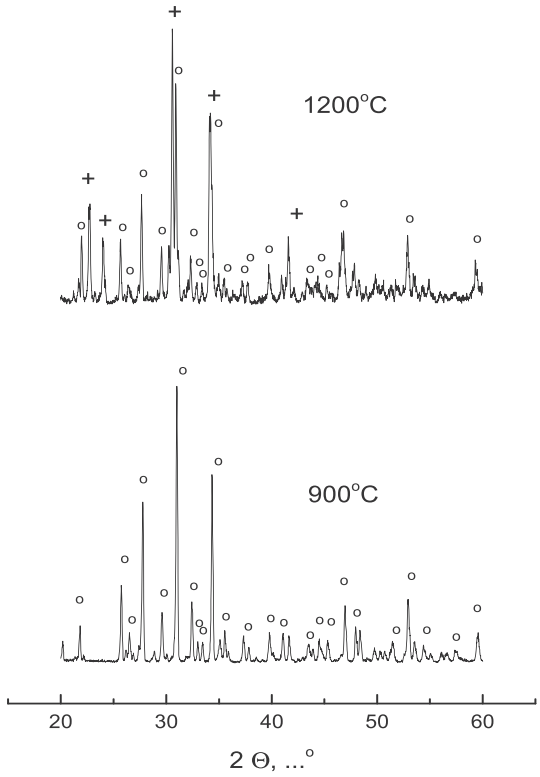

Fig. 1. XRD data for starting tricalcium phosphate powder after firing at 900 °C and for calcium phosphate ceramics after firing at 1200 °C.
+ – α-tricalcium phosphate α-$Ca_3(PO_4)_2$ (PDF card 9–348).
o – β-tricalcium phosphate β-$Ca_3(PO_4)_2$ (PDF card 9–169).

2.4. Cell culture

Primary dental pulp stem cells were used to study the biocompatibility of the biphasic α,β-tricalcium phosphate ceramics. The dental pulp stem cells culture was obtained from freshly extracted third molar teeth (donor age 16 years) with a root at least two thirds formed, which were extracted for orthodontics reasons [23]. The cell cultures were maintained in DMEM/F12 medium supplemented with 10% FBS, 100 units mL^{-1}penicillin and 100 mg mL^{-1} streptomycin under an 80% humidity, 5% CO_2 atmosphere at 37 °C.

2.5. Direct contact method for assessing cytotoxicity

The samples were placed onto 24-well culture plates. The cells were seeded on the surfaces of ceramic samples at 40,000 cell cm^{-2} and cultured in DMEM/F12 (1:1) medium supplemented with 10% FBS, 100 units mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin at 80% humidity in a 5% CO_2 atmosphere at 37 °C. The cytotoxicity of the ceramic samples was estimated by evaluating the cell viability through a double-staining fluorescence assay in a direct contact procedure 1, 4, 10 and 30 days after the beginning of experiments. In this study, the ability of the biphasic α,β-tricalcium phosphate ceramics to support the adhesion of the primary dental pulp stem cells and to stimulate their proliferation was also examined. We used a double-staining assay with SYTO9 (green fluorescent nucleic acid stain), which stains all cells, and propidium iodide (red fluorescent nucleic acid stain), which stains the nuclei of dead cells (L-7007 LIVE/DEAD Bac Light Bacterial Viability Kit, Invitrogen). The cells were visualized using fluorescence microscopy (Axiovert 200, Zeiss, Germany).

2.6. SEM observation of the cell-containing surfaces

The surfaces of biphasic α,β-tricalcium phosphate ceramic

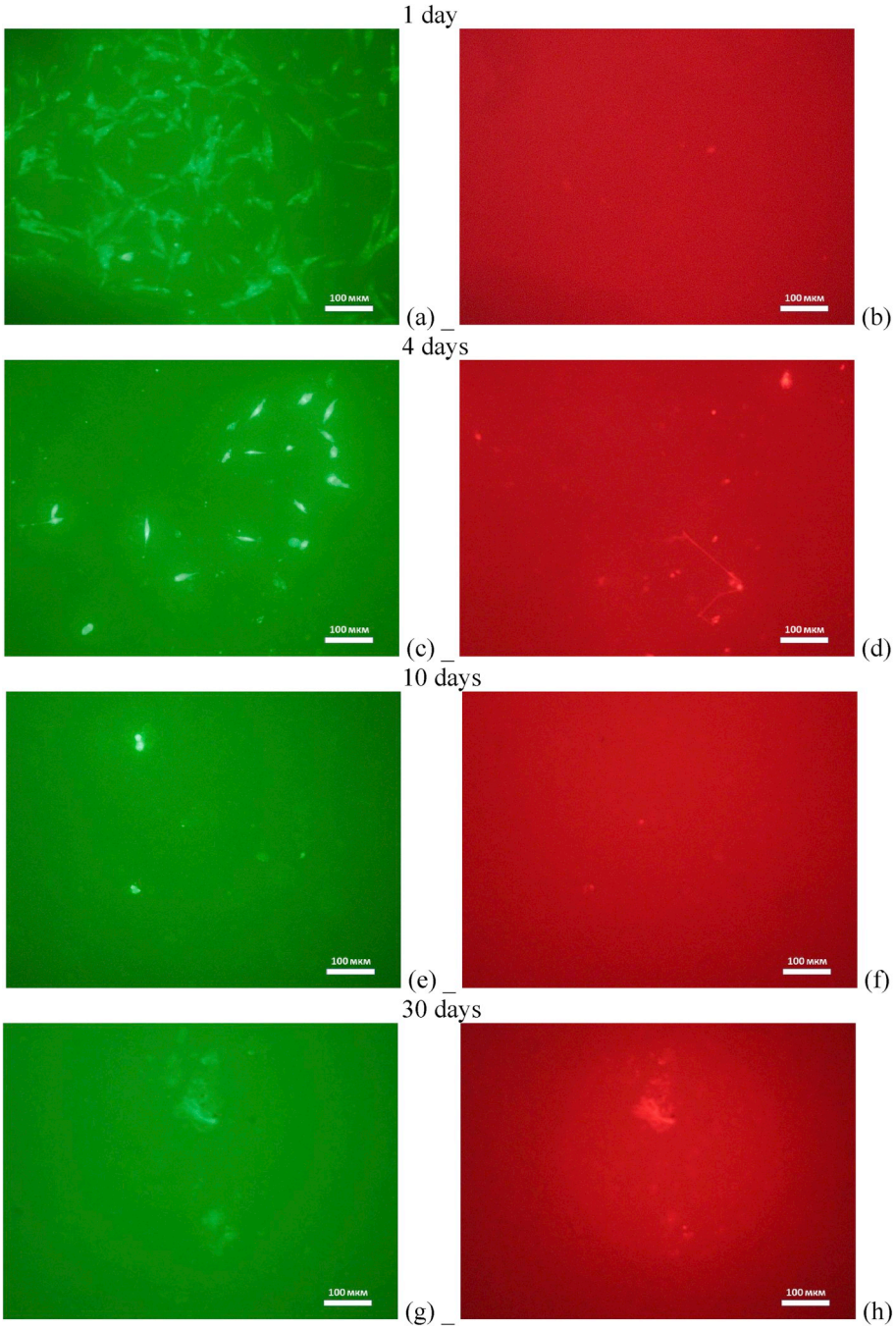

Fig. 2. The appearance of the dental pulp stem cells of the surface of biphasic α,β-tricalcium phosphate ceramics after direct contact procedure for 1 (a, b), 4 (c, d), 10 (e, f), 30 (g, h) days. Fluorescent staining was made with SYTO 9 (a, c, e, g) and propidium iodide (b, d, f, h).

specimens after primary dental pulp stem cells cultivation were studied using a SUPRA 50 VP scanning electron microscope (SEM) (Carl Zeiss, Germany); the imaging was performed in a low vacuum mode at an accelerating voltage of 21 kV (VPSE secondary electron detector) or of 3–21 kV (SE2 detector).

The samples were placed onto 24-well culture plates. The cells were seeded on the surfaces at 40,000 cells cm^{-2}, cultured in DMEM/F12 (1:1) medium supplemented with 10% FBS, 100 units mL^{-1}penicillin, and 100 mg mL^{-1}streptomycin, and cultivated for 1, 4, 10 and 30 days at 80% humidity in a 5% CO^2 atmosphere at 37 °C. To prepare samples of the cell-containing surfaces for SEM analysis, the cells were fixed and dehydrated. Briefly, the samples were washed three times with PBS and fixed with glutaraldehyde (2.5% in PBS, pH 7.4) for 2 h. After fixation, the samples were rinsed with PBS once before being dehydrated using a series of solutions.

2.7. MTT-test

The cytotoxicity of the ceramics was evaluated using MTT test according to ISO 10993-5. The samples were incubated in polypropylene tubes containing DMEM/F12 supplemented with 100 U mL^{-1}penicillin/streptomycin for 3 day at 37 °C under aseptic conditions. In the liquid extracts of materials, the ratio of the mass of the samples (g) to the volume of the culture medium (ml) was 0,1 ÷ 0,2. DMEM/F12 medium was used as a control. The NCTC L929 cells were used at 40,000 cells cm^{-2} for 24 h before adding the liquid extracts of the material. The extracts were transferred onto a layer of cells and incubated. The viability of the cells was evaluated 1 day after the

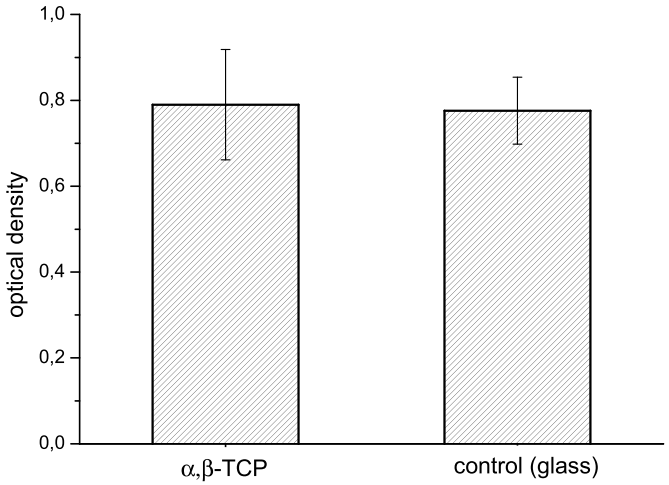

Fig. 3. The MTT viability assay of NCTC L929 cells in the presence of liquid extracts from biphasic α,β-tricalciumphosphate ceramic samples after 24 h cultivation (mean ± SD, n = 10).

beginning of experiments by measuring the reduction of the colorless salt tetrazolium(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) by mitochondrial and cytoplasmic dehydrogenases of living metabolically active cells through the formation of intracellular water-insoluble purple-blue crystals of formazan. The cells were treated with MTT (0.5 mg mL^{-1}) at 37 °C for 3 h in air with 5% CO_2 and 90% humidity. The medium was removed and the formazan was solubilized with 100 μl dimethylsulfoxide (DMSO). The absorption at 540 nm was measured using a microplate spectrophotometer (model 680 BioRad, USA). The value is an average of three separate experiments. The statistically significant difference between the groups was estimated using the Mann–Whitney *U* test. Values of $p < 0.05$ were considered significant.

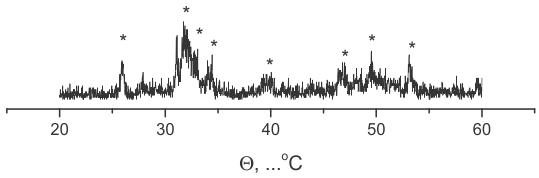

Fig. 5. XRD data of sample surface after cells culturing for 30 days.

3. Results and discussion

The XRD data for β-tricalcium phosphate powder β-$Ca_3(PO_4)_2$ synthesized at 900 °C and a biphasic ceramic obtained based on this powder and including β-$Ca_3(PO_4)_2$ and α-$Ca_3(PO_4)_2$ phases, are presented in Fig. 1. The XRD data of starting powder synthesized at 900 °C from highly homogenized powder mixture of calcium carbonate $CaCO_3$ and γ-pyrophosphate γ-$Ca_2P_2O_7$ confirms that powder consisted of the only phase of β-$Ca_3(PO_4)_2$. According to the XRD data ceramics based of β-$Ca_3(PO_4)_2$ powder after firing at 1200 °C consisted of two phases: β-$Ca_3(PO_4)_2$ and α-$Ca_3(PO_4)_2$. In this work receiving of two phase tricalcium phosphate ceramics realized due to known phase transaction β-$Ca_3(PO_4)_2$ to α-$Ca_3(PO_4)_2$ which take place at 1125 °C. According to quantitative XRD analysis, the content of α-$Ca_3(PO_4)_2$ was about 65 mass %. Density of ceramic samples was determined as 2,2 g/cm^3.

Images of the surface of biphasic α,β-tricalcium phosphate ceramic samples obtained using fluorescence microscopy after culturing cells for 1, 4, 10, and 30 days are presented in Fig. 2. These images reveal that the total number of live cells on the surface of biphasic α,β-tricalcium phosphate ceramics decreases over time from about 36,000 cells/cm^2 after 1 day, then 5 000 cells/cm^2 after 4 days, then 1 000 cells/cm^2 after 10 days and to practically total absence of cells after 30 days. Comparison of «green» (Fig. 2 - a, c, e, g) and «red» (Fig. 2 - b, d, f, h) pictures gives the opportunity for conclusion about a weak adhesion of the cells at the surface during the experiment.

Results of MTT-test are presented in Fig. 3. The MTT assay showed that there was no difference between viability assay of NCTC L929 cells

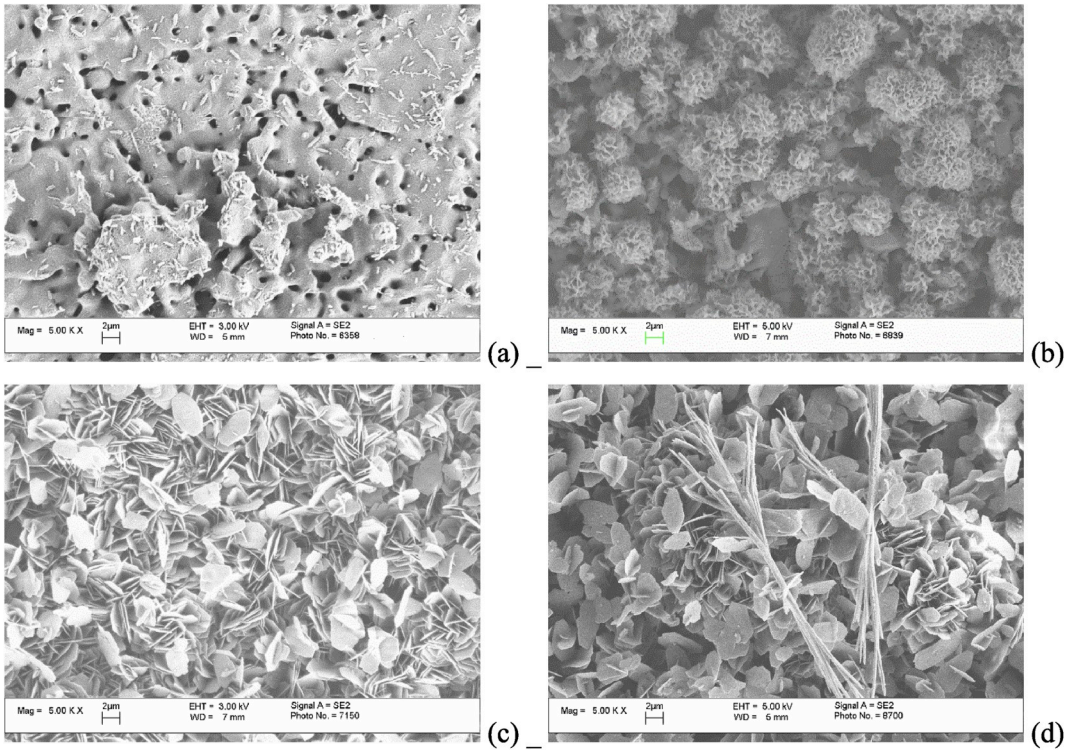

Fig. 4. SEM images of the surface of biphasic α,β-tricalcium phosphate ceramics after culturing cells for 1 (a), 4 (b), 10 (c) и 30 (d) days.

in the presence of liquid extracts from biphasic α,β-tricalcium phosphate ceramic samples after 24 h cultivation sample and control. The decrease in the cell vitality is not statistically significant in comparison with control.

SEM images of the surface of biphasic α,β-tricalcium phosphate ceramic samples after culturing cells for 1, 4, 10, and 30 days are presented in Fig. 4.

Hydroxyapatite crystals grow on the surface of biphasic α,β-tricalcium phosphate ceramics in the form of hexagonal plates, which is more clearly visible for samples after testing for 10 and 30 days. The formation of calcium hydroxyapatite on the surface of biphasic α,β-tricalcium phosphate ceramics can be described by following reactions (4) [24] и (5) [25]:

$$3Ca_3(PO_4)_2 + H_2O \rightarrow Ca_9(OH)(HPO_4)(PO_4)_5 \quad (4)$$

$$10Ca_3(PO_4)_2 + 6H_2O \rightarrow 3Ca_{10}(PO_4)_6(OH)_2 + 2H_3PO_4 \quad (5)$$

Reaction 4, due to which the formation of Ca-deficient hydroxyapatite with Ca/P = 1,5 occurred, was previously considered in the study of the process of obtaining apatite cement as a result of hydrolysis of α-tricalcium phosphate α-$Ca_3(PO_4)_2$. Reaction 5 also indicates the hydrolysis of α-tricalcium phosphate α-$Ca_3(PO_4)_2$, resulting in the formation of both hydroxyapatite and phosphoric acid. The XRD data of the surface presented at Fig. 5 confirm the hydroxyapatite formation.

Over time, as can be seen from Figs. 2 and 4, upon the interaction of the surface of biphasic α,β-tricalcium phosphate ceramics, apatite crystals grow larger, and the surface of the material becomes unsuitable for cell activity. After 30 days of cultivation, in addition to particles with a morphology characteristics of calcium hydroxyapatite, several crystals with a ribbon morphology characteristics of octacalcium phosphate appear on the surface of the material. As reported in the article [26], hydrolysis of α-tricalcium phosphate α-$Ca_3(PO_4)_2$ can also induce the formation of octacalcium phosphate $Ca_8H_2(PO_4)_6 \cdot 5H_2O$.

Earlier studies were conducted comparing bone formation during implantation of porous ceramic materials based on α-tricalcium phosphate α-$Ca_3(PO_4)_2$ and β-tricalcium phosphate β-$Ca_3(PO_4)_2$ [22]. The article emphasized that there is no bone growth in porous materials based on α-tricalcium phosphate α-$Ca_3(PO_4)_2$ due to its rapid dissolution, which (see reaction 4) makes the environment near the surface of the ceramic material more acidic. SEM images of the ceramic samples containing α-$Ca_3(PO_4)_2$ after being in a culturing medium (Fig. 4) suggest that the presence of large, hexagonal plate crystals formed on the surface of biphasic α,β-tricalcium phosphate ceramics is also a factor in preventing the conservation and survival of cells.

4. Conclusions

In vitro studies of biocompatibility of biphasic α,β-tricalcium phosphate ceramics with high (~65%) contents of α-$Ca_3(PO_4)_2$ in the presence of cells indicate that despite the similarity to the chemical composition of the inorganic component of bone tissue, such ceramics should be examined with caution when used in vivo. Among the reasons for the suppression of cell activity in the study of biocompatibility of biphasic α,β-tricalcium phosphate ceramics for 10–30 days *in vitro*, it is worth pointing out both acidification at the environment near the surface of ceramics containing α-tricalcium phosphate and a change in surface morphology as a result of the formation of a plate crystallite layer with sharp edges.

Declaration of competing interest

The authors declare no conflict of interest.

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