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Article

Bioceramics based on calcium pyrophosphate

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Abstract: Ceramic samples based on β-calcium pyrophosphate β-$Ca_2P_2O_7$ were prepared using firing at 900, 1000, and 1100 °C from powders of γ-calcium pyrophosphate γ-$Ca_2P_2O_7$ with preset molar ratios Ca/P=1; 0,975 and 0,95. To prepare powders of γ-calcium pyrophosphate γ-$Ca_2P_2O_7$ with preset molar ratio Ca/P=1; 0,975 and 0,95 powder mixtures based on calcium lactate pentahydrate $Ca(C_3H_5O_3)_2{\cdot}5H_2O$ and, monocalcium phosphate monohydrate $Ca(H_2PO_4)_2{\cdot}H_2O$ were treated in an aqua medium in mechanical activation conditions, dried, disaggregated in acetone, and heat-treated at 600 °C. The phase composition of powder mixtures after treatment if planetary mill in aqua medium included both brushite $CaHPO_4{\cdot}2H_2O$ or monetite $CaHPO_4$, and starting salts. The phase composition of all powder mixtures after disaggregation in acetone in planetary mill included monetite $CaHPO_4$ and starting salts. After heat treatment at 600 °C according to the XRD data phase composition of all powder mixtures was presented by γ-calcium pyrophosphate γ-$Ca_2P_2O_7$. The grain size of ceramics increased both with the growth of firing temperature and with decreasing of molar ratio Ca/P of powder mixtures. Calcium polyphosphate (t_{melt} =960–968 °C) formed from monocalcium phosphate monohydrate $Ca(H_2PO_4)_2{\cdot}H_2O$ acted like a liquid phase sintering additive. It was confirmed by tests in vitro, that prepared ceramic materials with preset molar ratio Ca/P=1; 0,975 and 0,95 and phase composition presented by β-calcium pyrophosphate β-$Ca_2P_2O_7$ according to XRD data were biocompatible and could maintain bone cells proliferation.

Keywords: calcium lactate pentahydrate; monocalcium phosphate monohydrate; mechanical activation; powder; brushite; monetite; calcium pyrophosphate; ceramics; biocompatibility

1. Introduction

Ceramics based on calcium phosphates are widely used for bone defect treatment [1,2]. Resorbable calcium phosphate ceramic materials are necessary for the implementation of bone defect treating methods of regenerative medicine [3]. It is known from the scientific literature that the ability of inorganic materials to resorb is connected with the ability to solve in an aqua medium [4]. And this ability to a great extent depends on the crystal structure of an inorganic substance [5]. Calcium phosphate's ability to solve in an aqua medium can be enhanced with decreasing of Ca/P molar ratio [6]. At the same time pH generated during the dissolution of calcium phosphate has to be close to neutral as a necessary feature of biocompatibility [7]. So, the ceramics based on calcium pyrophosphate $Ca_2P_2O_7$ can be an interesting object of investigation both due to molar ratio Ca/P=1

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(it is less than molar ratio Ca/P=1.67 for insoluble hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ and Ca/P=1,5 for tricalcium phosphate $Ca_3(PO_4)_2$) and due to pH close to neutral (pH ~ 7) during immersion to the water [8].

Different powders and powder mixtures with molar ratio Ca/P=1 can be used as precursors of high-temperature β-modification of calcium pyrophosphate $Ca_2P_2O_7$ as the ceramic phase. Powders of brushite $CaHPO_4{\cdot}2H_2O$ [9], monetite $CaHPO_4$ [10], hydrated amorphous calcium pyrophosphate $Ca_2P_2O_7{\cdot}xH_2O$ [11, 12], calcium pyrophosphate $Ca_2P_2O_7$ in forms of γ or β modifications [13, 14] can be used as starting direct powders precursors for β-calcium pyrophosphate β-$Ca_2P_2O_7$ ceramics preparation.

Solid-state sintering of calcium phosphate ceramics has some difficulties due to the complexity of mass -transfer because of the lower diffusion of the large, multiply charged phosphate or pyrophosphate ions [15]. It is impossible to help sintering of calcium pyrophosphate ceramics with elevating of firing temperature because of β-α phase transition at 1150 °C [16]. Using fine powders, special atmospheres of firing, and using sintering additives can enhance the sintering ability of any ceramic material. Chemical synthesis of calcium phosphate powders for ceramic preparation is used for enhancement of powder sintering activity [17]. CO_2 or H_2O atmosphere can intensify the sintering of hydroxyapatite [18]. Sintering additives with the ability to introduce defects in the crystal structure can help solid-state sintering [19,20]. Quite an ordinary decision to overcome the difficulties in sintering of calcium phosphate ceramics consists in using liquid phase sintering [21]. Liquid phase sintering can be realized when the sintering additive is presented in a quantity of a wide interval from 1% to 40%. Sodium phosphates [22, 23], sodium carbonates [24], sodium/potassium nitrate [25], and calcium polyphosphate [9 Telic, 26, 27] were used as sintering additives for calcium pyrophosphate ceramic preparation. Potassium carbonate [28,29], potassium chloride [30], and calcium chloride [31] used as sintering additives for ceramic based on hydroxyapatite can also be used as sintering additives for calcium pyrophosphate ceramics. Application of low temperature melting salts with biocompatible cations like potassium or sodium has slide disadvantage which consists in the possibility of drift of phase composition of bioceramics to the oxide systems Na_2O-CaO-P_2O_5 or K_2O-CaO-P_2O_5 and the formation of phases of double phosphates due to heterophase reactions. The possibility of these reactions can lead to the diminution of the quantity of sintering additive when processing ceramics, and then formation in ceramics of those phases of double phosphates which in case of notable amount can generate basic pH harmful for a patient organism if implanted. Ceramic materials in the CaO-P_2O_5 system with low content of $Ca(PO_3)_2$ as expected will be more friendly to the living organism if implanted.

As precursors of the calcium polyphosphate phase in ceramics, the different compounds can be used [32]. The following compounds with molar ratio Ca/P=0,5 also can be used as precursors of calcium polyphosphate: amorphous hydrated calcium polyphosphate $Ca(PO_3)_2{\cdot}xH_2O$ [33], $CaH_2(HPO_3)_2$ [34], $CaH_2P_2O_7$ [35], $CaH_2P_2O_7{\cdot}H_2O$ [36], $CaNH_4HP_2O_7$ [37, 38], $Ca(NH_4)_2P_2O_7{\cdot}H_2O$ [39, 40], $CaNH_4HP_2O_7$, $Ca_2NH_4H_3(P_2O_7)_2$ H_2O, $Ca_2NH_4H_3(P_2O_7)_2{\cdot}3H_2O$ [41], $Ca(H_2PO_4)_2$ and $Ca(H_2PO_4)_2{\cdot}H_2O$ [42,43].

Earlier it was shown that the synthesis of fine grain powder of monetite $CaHPO_4$ can be synthesized in conditions of mechanical activation from powder mixture including hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2{\cdot}H_2O$ [10 Sadil]. It also was shown that treatment in a water solution of lactic acid allowed preparing powder of monocalcium phosphate monohydrate $Ca(H_2PO_4)_2{\cdot}H_2O$ with lower dimensions of particles as far lactic acid can act as surface-active substance [44]. It is well known that the smaller particle dimensions of starting components the more homogeneous powder mixture can be prepared. So in the present work powder mixtures for ceramics production were prepared in conditions of mechanical activation from calcium lactate pentahydrate $Ca(C_3H_5O_3)_2{\cdot}5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2{\cdot}H_2O$ when the last was taken in access and used also as a precursor of calcium polyphosphate $Ca(PO_3)_2$ for playing the role of a liquid phase sintering additive.

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The aim of the present work consisted of the preparation of ceramics based on calcium pyrophosphate with the assistance of calcium polyphosphate as a liquid phase sintering additive and investigation of biocompatibility of prepared ceramics in vitro.

2. Materials and Methods

The target phase compositions of ceramics are shown in Table 1. Calcium polyphosphate was introduced as a sintering additive with a low temperature of melting (960–968 °C [16 Hill])

Table 1. Description of target phase composition of ceramics.

sample	Ca/P molar ratio	The phase composition, mol. %		The phase composition, mas. %	
		β-$Ca_2P_2O_7$	β-$Ca(PO_3)_2$	β-$Ca_2P_2O_7$	β-$Ca(PO_3)_2$
Pyro	1	100	0	100	0
Pyro_05Poly	0,975	95	5	96	4
Pyro_10Poly	0,95	90	10	92	8

[1] Table may have a footer.

Powders of calcium lactate pentahydrate $Ca(C_3H_5O_3)_2{\cdot}5H_2O$ (CAS no. 814-80-2, food-grade E327 of FCC, Henan Jindan Lactic Acid Technology Co., Ltd, China) and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2{\cdot}H_2O$ (CAS no. 10031- 30-8, puriss. 99%, Sigma-Aldrich) were used for powder mixtures preparation. Compositions of powder mixtures before treatment in mechanical activation conditions are presented in Table 2.

Table 2. Composition of powder mixtures before treatment in mechanical activation conditions

sample	Ca/P molar ratio	Starting components, mol. %		Starting components, mas. %	
		$Ca(C_3H_5O_3)_2{\cdot}5H_2O$	$Ca(H_2PO_4)_2{\cdot}H_2O$	$Ca(C_3H_5O_3)_2{\cdot}5H_2O$	$Ca(H_2PO_4)_2{\cdot}H_2O$
Pyro	1	50,0	50,0	55,0	45,0
Pyro_05Poly	0,975	48,7	51,3	53,7	46,3
Pyro_10Poly	0,95	47,4	52,6	52,4	47,6

Reaction (1) corresponding to powder mixture “Pyro” was used to calculate quantity of starting components.

$$Ca(C_3H_5O_3)_2{\cdot}5H_2O + Ca(H_2PO_4)_2{\cdot}H_2O \rightarrow 2CaHPO_4{\cdot}2H_2O + 2C_3H_5O_3H + 2H_2O \quad (1)$$

10 g of starting components in ratios presented in **Table 2**, 50 g of grinding media made from zirconia, and 40 ml of distilled water were placed in containers made from zirconia. Containers with starting components were fixed in the planetary mill (Fritch Pulverisette, Germany). Mechanical activation of suspension initially containing distilled water, calcium lactate pentahydrate $Ca(C_3H_5O_3)_2{\cdot}5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2{\cdot}H_2O$ was conducted for 15 min at a rotation speed of 600 rpm. Then, powder mixtures after drying for 72 hours were disaggregated in a planetary mill in acetone medium for 15 min at a rotation speed of 600 rpm. After drying powder mixtures were passed through the sieve with 200 mm mesh. Then powder mixtures were heat-treated at 600 °C for 30 minutes. Powder mixtures prepared this way were used for ceramics preparation. Powder compacts were pressed at 100 MPa in form of disks with a diameter of 12 mm and height of 1 mm in steel mold using the manual press (Carver Laboratory Press model C, Fred S. Carver, Inc., Wabash, USA). Then powder compacts were fired in the air at 900, 1000 и 1100°C with a heating rate of 5 °C/min and 2 hours holding at a specified temperature.

The phase composition of the prepared powder mixtures, powders after heat treatment at 600 °C, and ceramic samples after firing was determined by X-ray powder

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diffraction (XRD) analysis using Rigaku D/Max-2500 diffractometer (Rigaku Corporation, Tokyo, Japan) with a rotating anode (Cu–Ka radiation), angle interval 2Θ: from 2° to 70°, step 2Θ - 0.02°). Phase analysis was performed using the ICDD PDF2 database [45]

Thermal analysis (TA) was performed to determine the total mass loss of the powder mixtures at heating up to 1000 °C in the air using NETZSCH STA 449 F3 Jupiter thermal analyzer (NETZSCH, Selb, Germany). The gas-phase composition was monitored by the quadrupole mass spectrometer QMS 403 Quadro (NETZSCH, Selb, Germany) combined with a thermal analyzer NETZSCH STA 449 F3 Jupiter. The mass spectra (MS) were registered for the following m/Z values: 18 (H_2O); 44 (CO_2); the heating rate was 10 °C/min.

Powders after heat treatment at 600 °C and ceramics after firing were examined by scanning electron microscopy (SEM) on LEO SUPRA 50VP electron microscope (Carl Zeiss, Germany; auto-emission source). This investigation was carried out at an accelerating voltage of 3-20 kV using SE2 detectors. The surface of the ceramic samples was coated with a layer of chromium (up to 10 nm).

Ceramic samples fired at 1100 °C were used for the investigation of cytotoxicity in vitro.

Primary dental pulp stem cells (cell culture) were used to study the biocompatibility of the prepared 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 [46]. The cell cultures were maintained in DMEM/F12 medium supplemented with 10% FBS and 100 units mL^{-1}penicillinand 100 mg mL^{-1} streptomycin under an 80% humidity, 5% CO_2 atmosphere at 37°C.

For assessing cytotoxicity of ceramics direct contact method was used. 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 2 and 7 days after the beginning of experiments. In this study, the ability of the prepared 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).

The cell-containing surfaces of prepared ceramic specimens after primary dental pulp stem cells cultivation were studied using a Tescan Vega II scanning electron microscope (SEM, Tescan Vega II, Czech Republic); the imaging was performed in a low vacuum mode at an accelerating voltage of 20 kV (SE detector). To prepare samples of the cell-containing surfaces of ceramics after 2 days of cells cultivation 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. Samples were coated with a thin layer of gold to prevent surface charging (Q150R ES, Quorum Technologies, East Sussex, UK).

The cytotoxicity of the ceramics was evaluated using the 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 days 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 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

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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.

3. Results and Discussion

According to XRD analysis (**Figure 1**) after homogenization of starting salts in mechanical activation conditions in a planetary mill in aqua medium powder mixture "Pyro" (Ca/P=1) included brushite $CaHPO_4 \cdot 2H_2O$, and monetite $CaHPO_4$ in small quantity and starting components, i.e., calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$. Powder mixtures "Pyro_05Poly" (Ca/P=0,975) and "Pyro_10Poly" (Ca/P=0,95) included monetite $CaHPO_4$ and starting components, i.e., calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$. The presence of starting salts in all powder mixtures under investigation can be explained by the incompleteness of the reactions (1). Moreover, monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$ was intentionally introduced in powder mixtures "Pyro_05Poly" (Ca/P=0,975) and "Pyro_10Poly" (Ca/P=0,95) in excess to provide formation of calcium polyphosphate $Ca(PO_3)_2$ at the firing stage. Intentionally introduced an excess of monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$ provided more acidic pH of water solution formed during treatment of powder mixtures "Pyro_05Poly" (Ca/P=0,975) and "Pyro_10Poly" (Ca/P=0,95) in a planetary mill. More acidic pH of water solution as it was shown before in other investigations [47,48] can explain the preferable formation of monetite $CaHPO_4$ (calcium hydrophosphate anhydrate) in powder mixtures "Pyro_05Poly" (Ca/P=0,975) and "Pyro_10Poly" (Ca/P=0,95) instead of brushite $CaHPO_4 \cdot 2H_2O$ (calcium hydrophosphate dihydrate) as it was for powder mixture "Pyro".

After drying the prepared powder mixtures were aggregated to a great extent and the stage of disaggregation was highly necessary. So, after drying powder mixtures were disaggregated in acetone medium in a planetary mill. According to XRD data (**Figure 2**) after disaggregation in acetone medium in planetary mill phase composition of all powder mixtures included monetite $CaHPO_4$ and starting components, i.e., calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$. One can see that the more content of monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$ in powder mixture the more noticeable its main reflex at normalized graphs. Chemical reaction (2) of brushite dehydration taking place during disaggregation in acetone medium is presented below.

$$CaHPO_4 \cdot 2H_2O \rightarrow CaHPO_4 + 2H_2O \quad (2)$$

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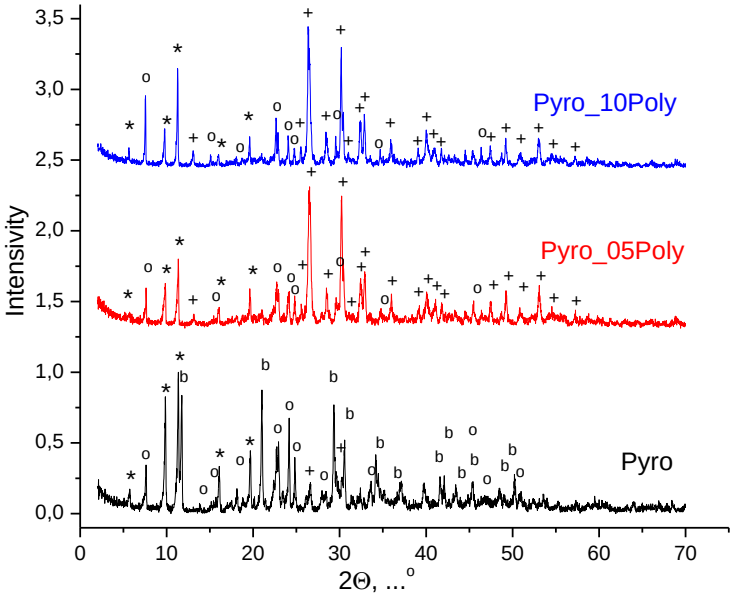

Figure 1. XRD data for powder mixtures prepared in mechanical activation conditions from calcium lactate pentahydrate $Ca(C_3H_5O_3)_2{\cdot}5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2{\cdot}H_2O$: * - $Ca(C_3H_5O_3)_2{\cdot}5H_2O$ (according scientific literature data [49, 50, 51]); o - $Ca(H_2PO_4)_2{\cdot}H_2O$ (PDF card 9-347); + - $CaHPO_4$ (PDF card 9-80); b - $CaHPO_4{\cdot}2H_2O$ (PDF card 9-77).

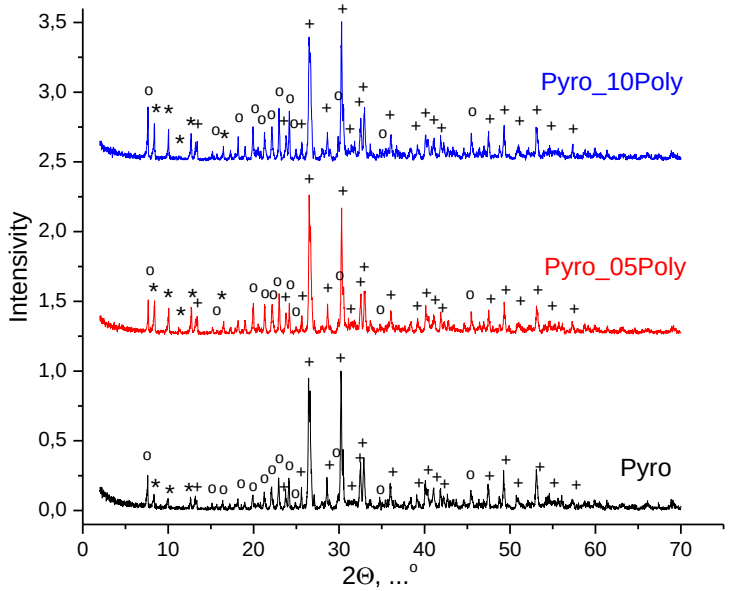

Figure 2. XRD data for powder mixtures prepared in mechanical activation conditions from calcium lactate pentahydrate $Ca(C_3H_5O_3)_2{\cdot}5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2{\cdot}H_2O$ after disaggregation in acetone medium: * - $Ca(C_3H_5O_3)_2{\cdot}5H_2O$ (according scientific literature data [49, 50, 51]); o - $Ca(H_2PO_4)_2{\cdot}H_2O$ (PDF card 9-347); + - $CaHPO_4$ (PDF card 9-80); b - $CaHPO_4{\cdot}2H_2O$ (PDF card 9-77).

TA data of powder mixtures "Pyro" (Ca/P=1) and "Pyro_10Poly" (Ca/P=0,95) prepared in mechanical activation conditions from calcium lactate pentahydrate $Ca(C_3H_5O_3)_2{\cdot}5H_2O$ and disaggregated in acetone medium, and TA of starting components (calcium lactate pentahydrate $Ca(C_3H_5O_3)_2{\cdot}5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2{\cdot}H_2O$) are presented at **Figure 3.** Total mass loss for powder mixtures "Pyro" (Ca/P=1) was 40%. Total mass loss for powder mixtures "Pyro_10Poly" (Ca/P=0,95) was 39%. All processes provided mass loss of the powder mixtures under investigation during heating finished up to 500 °C. As we can assume according data of XRD analysis of powder mixtures after treatment in acetone medium and according reaction (1) composition of powder mixtures included monetite, lactic acid and starting

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components (calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$).

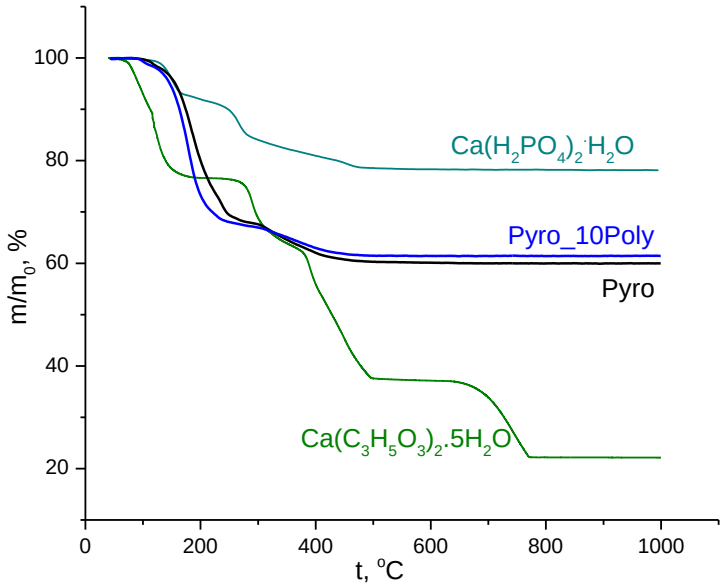

Figure 3. TA of powder mixtures "Pyro" (Ca/P=1) and "Pyro_10Poly" (Ca/P=0,95) prepared in mechanical activation conditions from calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and disaggregated in acetone medium; and TA of starting components (calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$).

So, we can suggest the following reactions which can take place during heating: dehydration of hydrated salts (reactions (3) and (4)), decomposition of lactic acid (reaction (5)), formation of calcium pyrophosphate via condensation (6), synthesis of calcium pyrophosphate due to interaction of monocalcium phosphate with calcium lactate (reaction (7)) or due to interaction of monocalcium phosphate monohydrate with calcium lactate pentahydrate (reaction (8)) and formation of calcium polyphosphate due to condensation (reaction (9)).

$$Ca(H_2PO_4)_2 \cdot H_2O = Ca(H_2PO_4)_2 + H_2O \text{ (~200 [10 Sadil])} \quad (3)$$

$$Ca(C_3H_5O_3)_2 \cdot 5H_2O = Ca(C_3H_5O_3)_2 + 5H_2O \text{ (~200 [55])} \quad (4)$$

$$C_3H_5O_3H + 6O_2 = 3CO_2 + 3H_2O \text{ (melting ~120 [52].)} \quad (5)$$

$$2CaHPO_4 = Ca_2P_2O_7 + H_2O \text{ (~300–475 °C [10 Sadil])} \quad (6)$$

$$Ca(H_2PO_4)_2 + Ca(C_3H_5O_3)_2 + 6O_2 = Ca_2P_2O_7 + 6CO_2 + 7H_2O \text{ (~110-500)} \quad (7)$$

$$Ca(H_2PO_4)_2 \cdot H_2O + Ca(C_3H_5O_3)_2 \cdot 5H_2O + 6O_2 = Ca_2P_2O_7 + 6CO_2 + 13H_2O \text{ (~110-500)} \quad (8)$$

$$Ca(H_2PO_4)_2 = Ca(PO_3)_2 + 2H_2O \text{ (~300-500 [10 Sadil])} \quad (9)$$

The form of curves $m/m_0=f(t)$ at **Figure 3.** of powder mixtures under investigation are very smooth and differ from curves of starting salts. The smoothness of the lines indicates the possibility of overlapping temperature intervals for listed reactions and their simultaneous occurrence. This difference confirms the possibility both of reactions of condensation (reactions (6) and (9)) and the possibility of formation of calcium pyrophosphate from starting components preserved during treatments in mechanical activation conditions. Differentiation of curves $m/m_0=f(t)$ for powder mixtures under investigation allows finding several temperatures with maximum mass loss rate. There are 130 °C, 180 °C (the

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biggest), 240 °C, 320 °C and 400 °C for powder mixture "Pyro" and 100 °C, 180 °C (the biggest), 230 °C and 360 °C for powder mixture "Pyro_10Poly". Mass loss due to CO_2 evolving took place in interval 110-500 °C with a maximum of 190 °C. Mass loss due to H_2O evolving took place in three intervals: 80-130 °C (with the maximum at 105 °C), 130-260 °C (with the maximum at 190 °C), 290-500 °C (with wide maximum 330-400 °C).

XRD data for powder mixtures prepared in mechanical activation conditions from calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$ after heat treatment at 600 °C is presented in Figure 4. The phase composition of prepared powders "Pyro", "Pyro_05Poly", "Pyro_10Poly" after heat treatment at 600 °C was presented by γ-calcium pyrophosphate γ-$Ca_2P_2O_7$.

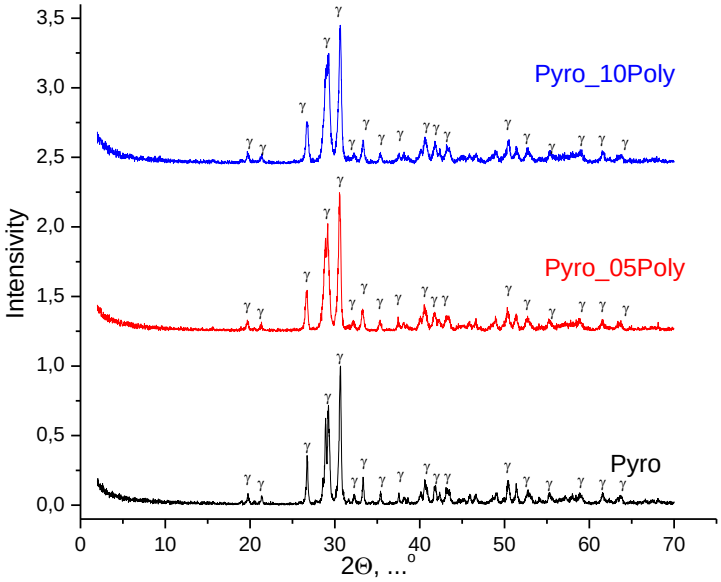

Figure 4. XRD data for powder mixtures prepared in mechanical activation conditions from calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$ after heat treatment at 600 °C: γ - γ-$Ca_2P_2O_7$ (PDF card 17-499.

SEM images of powders "Pyro", "Pyro_05Poly", "Pyro_10Poly" after heat treatment at 600 °C are presented in **Figure 5.** One can see that particles of powders are fine and the dimension of particles is dependent on the preset molar ratio of powders. Particles have plate-like morphology. The lower the molar ratio Ca/P the smaller the dimensions of particles. The dimensions of particles of powder "Pyro" are in interval 0,2-2 μm. The dimensions of particles of powder "Pyro_05Poly" are in interval 0,1-1 μm. The dimensions of particles of powder "Pyro_10Poly" are in interval 0,1-0,5 μm. Mechanical activation conditions and reactions with fast big volumes evolving can be the reason for fine powder formation. It should be noted that small particles of all powders are collected in aggregates. Particle size distribution of powder prepared in mechanical activation conditions from calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$ after heat treatment at 600 °C presented in **Figure 6.** Middle dimension of aggregates of particles for powder "Pyro" estimated as 5 μm, for powder "Pyro_05Poly" - 12,1 μm and for powder "Pyro_10Poly" - 12,5 μm.

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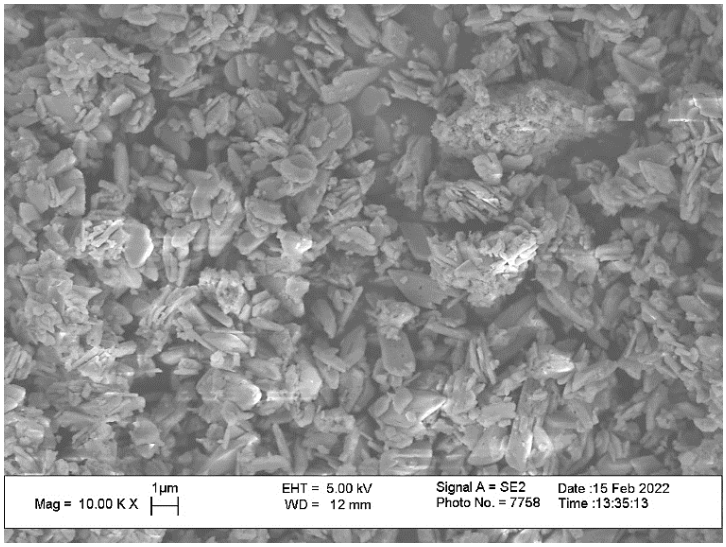
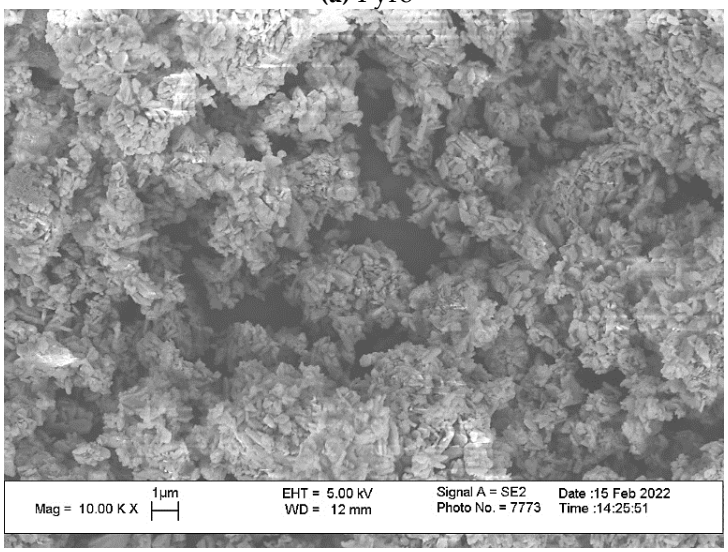
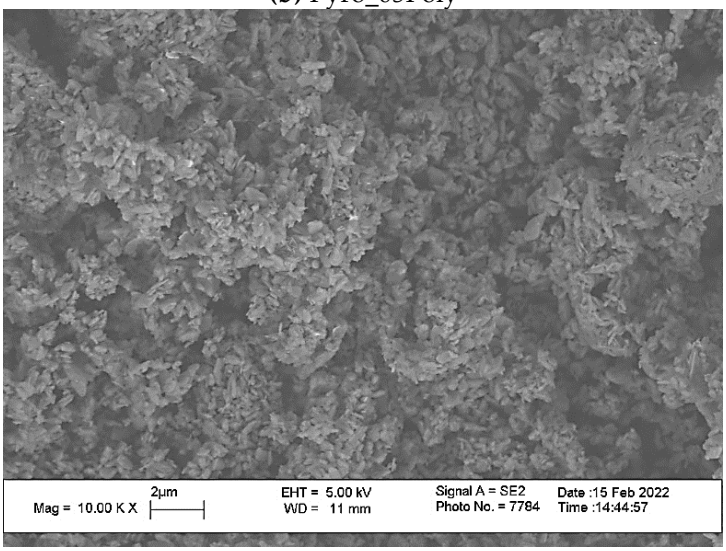


(a) Pyro



(b) Pyro_05Poly



(c) Pyro_10Poly

Figure 5. Micro photos of powders after heat treatment at 600 °C: "Pyro" (**a**); "Pyro_05Poly" (b); "Pyro_10Poly" (c).

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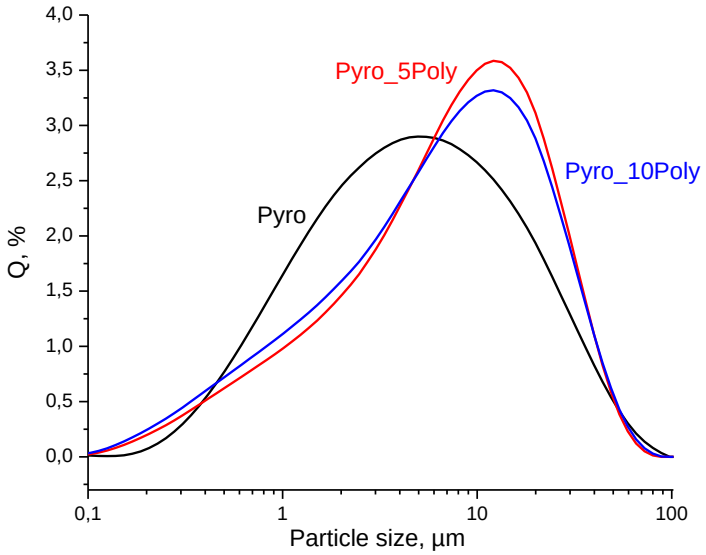

Figure 6. Particle size distribution of powders prepared in mechanical activation conditions from calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$ after heat treatment at 600 °C.

XRD data for ceramic samples based on powders "Pyro", "Pyro_05Poly", "Pyro_10Poly" fired at 900 °C, 1000 °C and 1100 °C are presented in **Figures 7, 8 and 9**. The phase composition of all samples was presented by β-calcium pyrophosphate β-$Ca_2P_2O_7$. So we can conclude that presence of calcium polyphosphate up to 10 mol % introduced via excess of monocalcium phosphate monohydrate cannot be detected using XRD analysis.

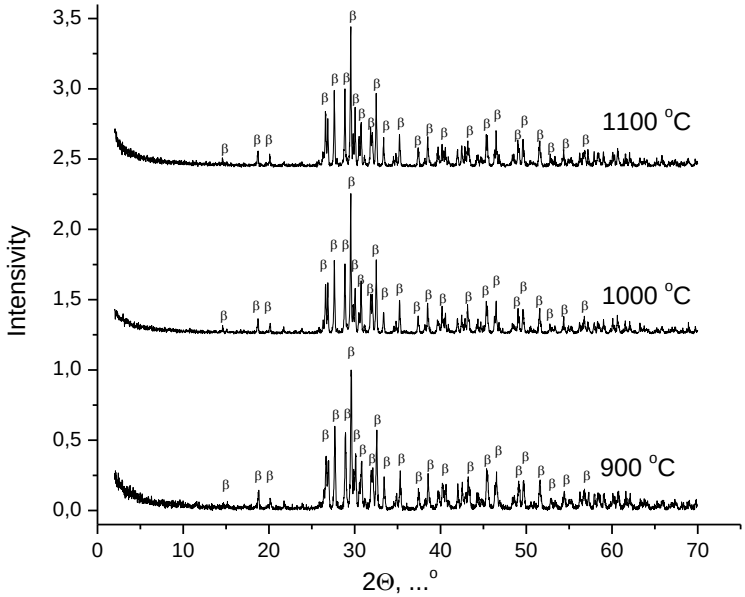

Figure 7. XRD data for ceramic samples "Pyro" fired at 900 °C, 1000 °C and 1100 °C: β – β-$Ca_2P_2O_7$ (PDF card 9-346).

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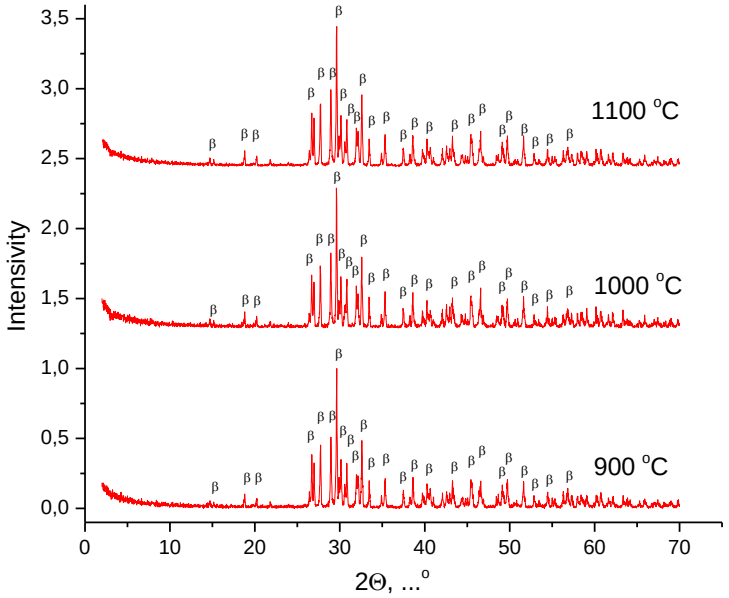

Figure 8. XRD data for ceramic samples "Pyro_05Poly" fired at 900 °C, 1000 °C and 1100 °C: β – β-$Ca_2P_2O_7$ (PDF card 9-346).

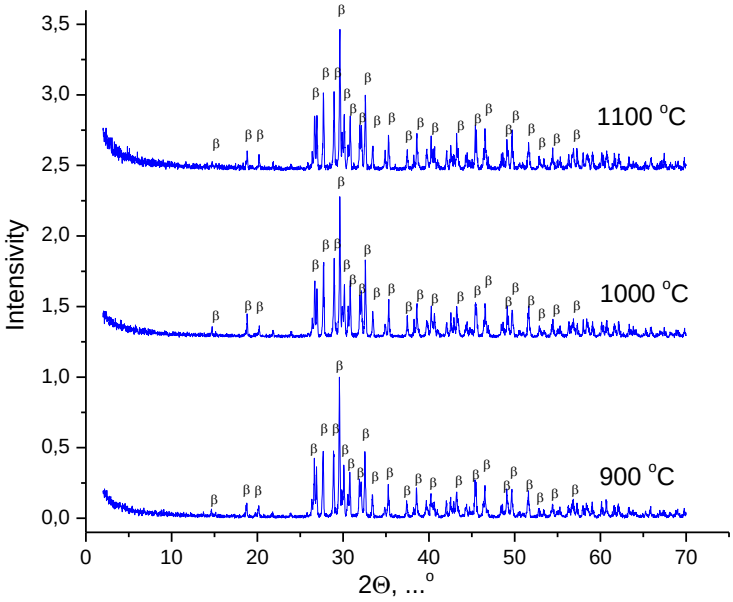

Figure 9. XRD data for ceramic samples "Pyro_10Poly" fired at 900 °C, 1000 °C, and 1100 °C: β – β-$Ca_2P_2O_7$ (PDF card 9-346).

At the same time **Figures 10** ("Pyro"), **11** ("Pyro_05Poly"), and **12** ("Pyro_10Poly") presented SEM micrographs of surface and cross-section of samples allow us to conclude the influence of firing temperature and quantity of sintering additive on the microstructure of ceramics.

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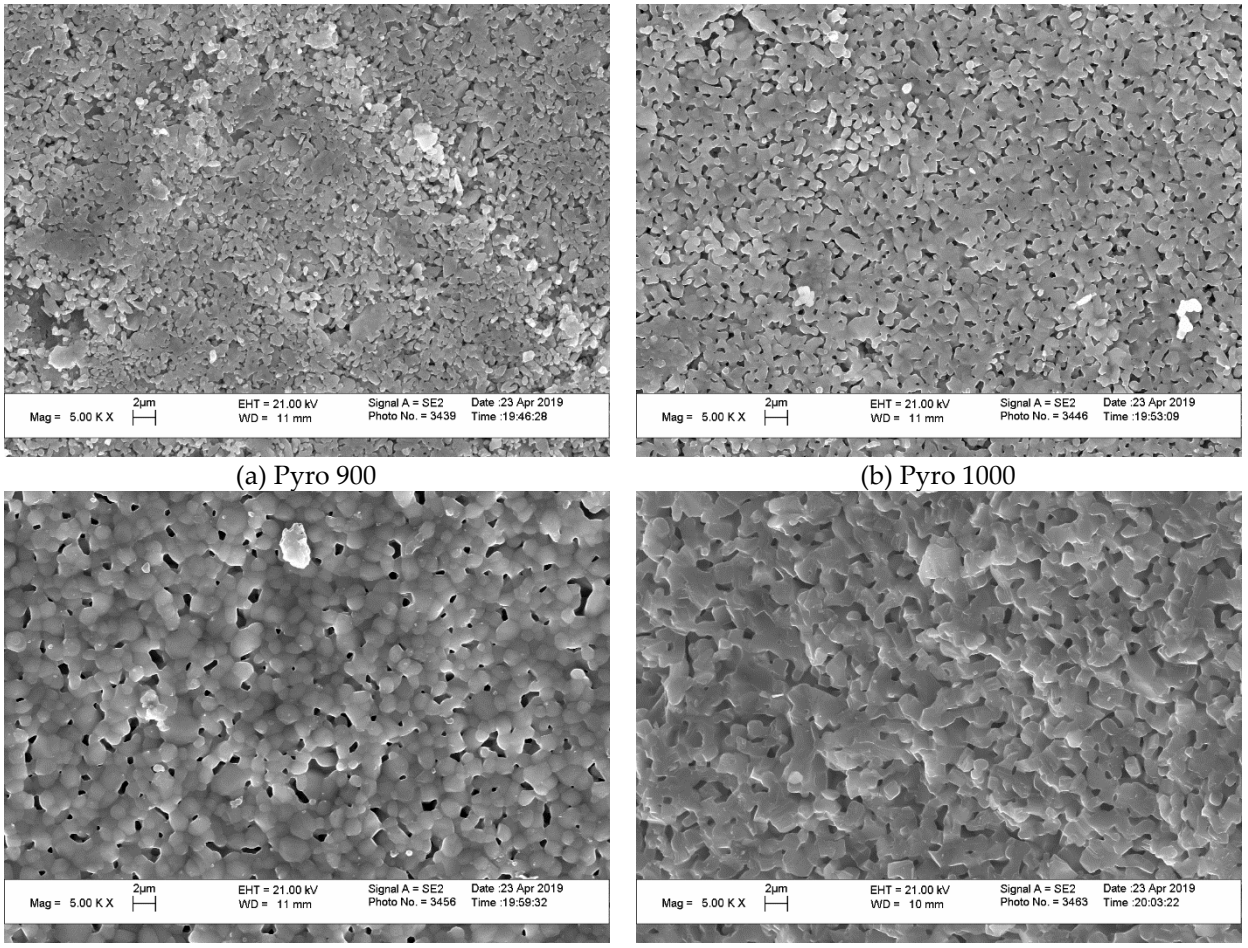

(a) Pyro 900 (b) Pyro 1000

(c) Pyro 1100 (d) Pyro 1100

Figure 10. SEM micrographs of surface (a, b, c) and cross-section (d) of ceramic samples "Pyro" fired at 900 °C (a), 1000 °C (b) and 1100 °C (c, d).

The grain size of ceramics increased both with the growth of firing temperature and with decreasing of molar ratio Ca/P of powder mixtures. The grain size of ceramic samples based on powder "Pyro" (**Figure 10**) increased from 1 µm after firing at 900 °C (**Figure 10, a**) to 2 µm after firing at 1100 °C (**Figure 10, c, d**). It should be noted that impressions from the microstructure of surface and microstructure of cross-section are very much similar.

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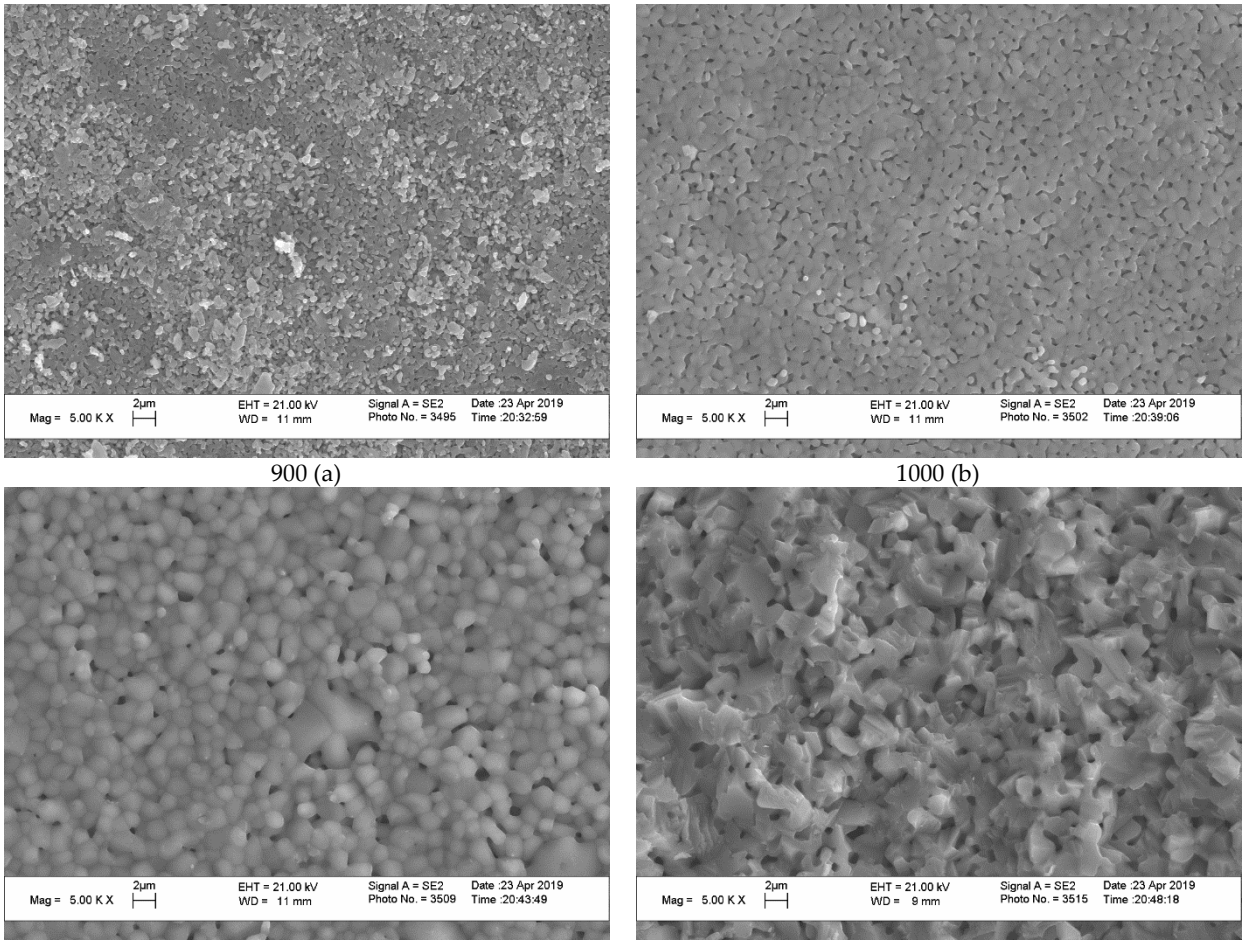

Figure 11. SEM micrographs of surface (a, b, c) and cross section (d) of ceramic samples "Pyro_05Poly" fired at 900 °C (a), 1000 °C (b) and 1100 °C (c, d).

The grain size of ceramic samples based on powder "Pyro_05Poly" (**Figure 11**) increased from ~0,5 - 1 µm after firing at 900 °C (**Figure 11, a**) to ~2 µm after firing at 1100 °C (**Figure 11, c, d**). It should be noted that the sample despite the same development in grain size as it was for ceramics based on powder "Pyro" looks sintered to a greater extent. Microstructure of cross-section of the ceramic sample after firing at 1100 °C (**Figure 11, d**) formed at the presence of liquid phase. Temperatures of firing 1000 and 1100 °C are higher than eutectic temperature (955 °C) in the $CaO-P_2O_5$ system according to literature data [16 Hill]. So, the presence of calcium polyphosphate with the preset quantity of 5 mol. % create the conditions for liquid phase sintering in ceramics based on powder "Pyro_05Poly"

The microstructure of ceramics based on powder "Pyro_10Poly" (**Figure 12**) demonstrates the influence of additive provoking liquid phase sintering. One can see grains with dimensions 1- 4 µm after firing at 900 °C (**Figure 12, a**). Some grains have an elongated form. After firing at 1000 °C (**Figure 12, b**) grains of ceramics based on powder "Pyro_10Poly" have dimensions 2- 4 µm. And after firing at 1100 °C on the surface one can see grains 2-4 µm (**Figure 12, c**). But cross-section (**Figure 12, d**) of ceramics based on powder "Pyro_10Poly" does not allow seeing any grain and determining their dimension.

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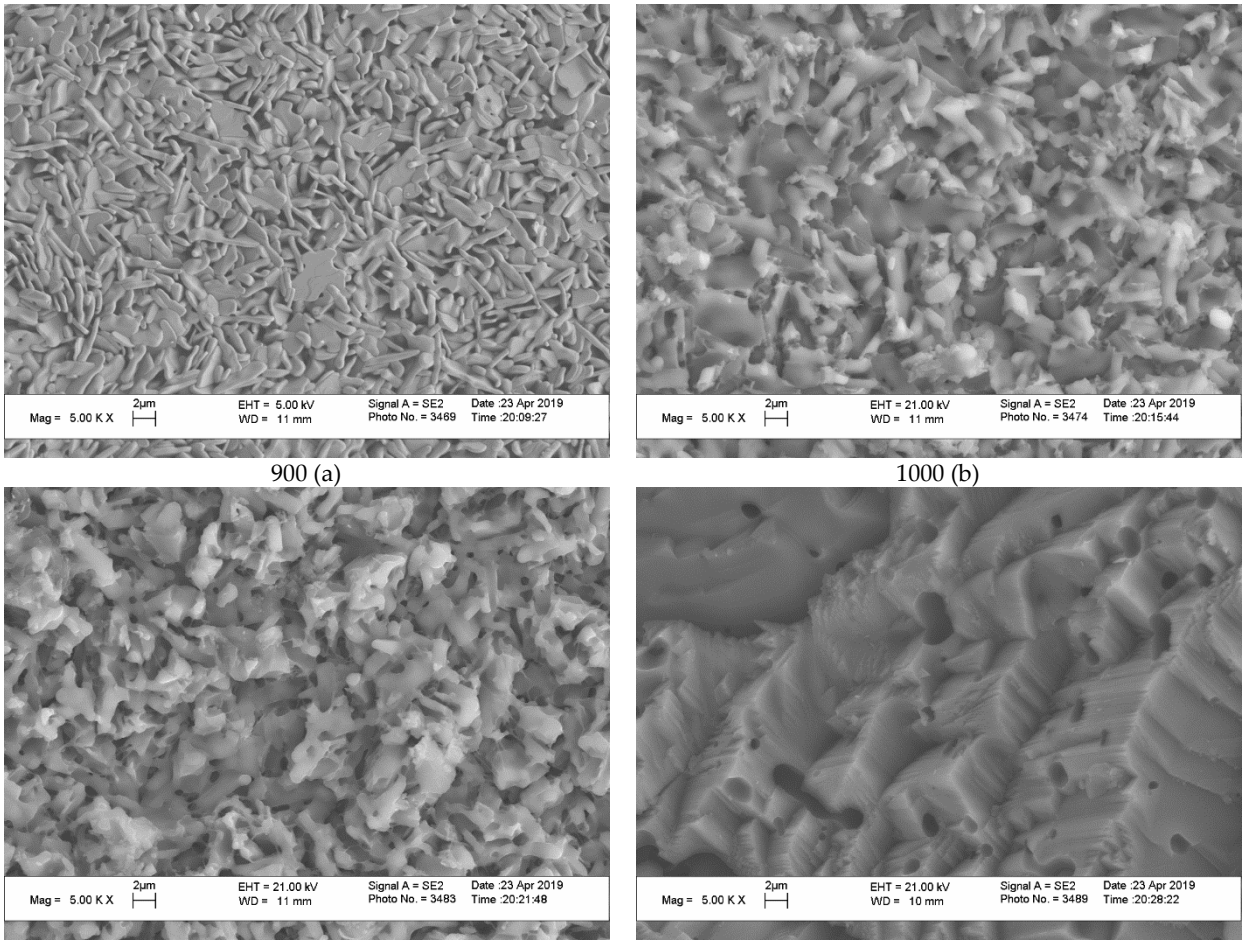

Figure 12. SEM micrographs of surface (a, b, c) and cross section (d) of ceramic samples "Pyro_10Poly" fired at 900 °C (a), 1000 °C (b) and 1100 °C (c, d).

Dependence of apparent density (g/cm^3) and relative diameter (D/D$_0$, %) of ceramic samples from firing temperature are presented in **Figure 13**.

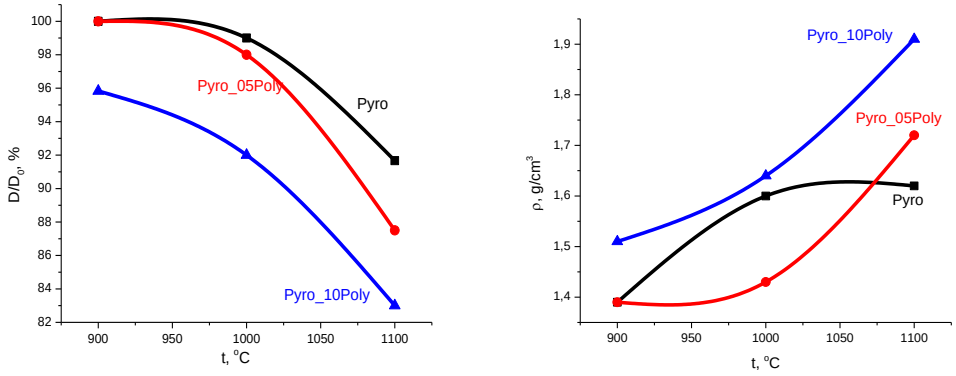

Figure 13. Dependence of relative diameter (a) and an apparent density (b) of ceramic samples from firing temperature.

Linear shrinkage (**Figure 13, a**) increased for all samples with the growth of firing temperature. The maximum liner shrinkage of ceramics based on powder "Pyro" was ~10% after firing at 1100 °C. The maximum liner shrinkage of ceramics based on powder "Pyro_05Poly" was ~13% after firing at 1100 °C. The liner shrinkage ceramics based on powder "Pyro_10Poly" increased from 4% at 900 oC to 17% at 1100 °C.

Density of ceramic samples (**Figure 13, b**) prepared from the powders "Pyro_05Poly" and "Pyro_10Poly" increased with growth of firing temperature from 1,4 g/cm^3 and 1,51,4

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g/cm^3 at 900 ^{o}C to 1,7 g/cm^3 (55%) and 1,9 g/cm^3 (60%) at 1100 ^{o}C respectively. The density of ceramic samples based on powders "Pyro" achieved 1,6 g/cm^3 (50%) after firing at 1000 ^{o}C and after firing at 1100 ^{o}C this value became the same. In comparison with theoretical density (3,12 g/cm^3), we have to admit that as a result, we prepared quite porous ceramic samples from fine powders of γ-calcium pyrophosphate.

The results of the MTT-test are presented in **Figure 14**. The MTT assay showed that there was no big difference between viability assay of NCTC L929 cells in the presence of liquid extracts from ceramic samples under investigation, i.e. "Pyro", "Pyro_05Poly", "Pyro_10Poly" after 48 h cultivation sample and control. The decrease in cell vitality is not statistically significant in comparison with control.

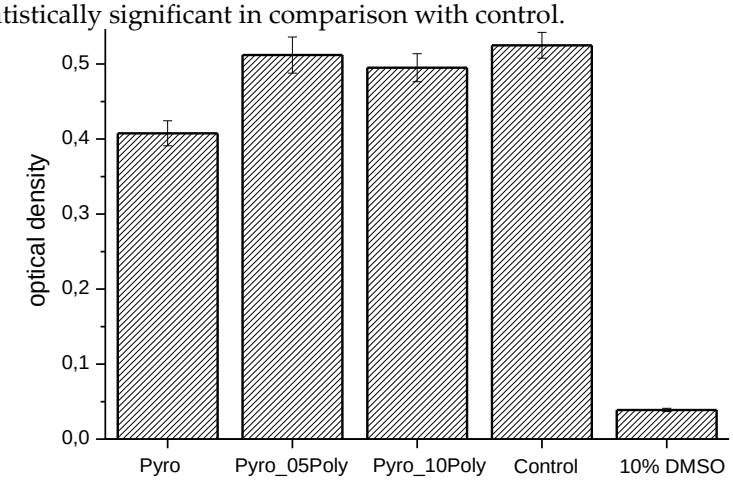

Figure 14. The MTT viability assay of NCTC L929 cells in the presence of liquid extracts from ceramic samples under investigation, i.e. "Pyro", "Pyro_05Poly", "Pyro_10Poly", control and 10%DMSO after 48 h cultivation (mean ±SD, n=10).

The results of determining the viability of cells cultured on the surface of the studied materials on the second (**Figure 15**) and the seventh day (**Figure 16**), confirm that the proliferative activity of cells was observed on the surface of all the samples studied. **Figure 17** presents micrographs cells fixed of the ceramic surface after cultivation for two days.

Normal morphology of DPSC 32 cells is observed on all the studied samples. However, the density of the cell layer on the surface of the studied samples after cultivation for two days was slightly lower than in the control (on the cover glass). This phenomenon was apparently due to the conditions of initial cell adhesion. Nevertheless, the absence of dead cells whose nuclei are stained with propidium iodide indicates the absence of cytotoxic effects of the ceramic materials prepared based on powders "Pyro", "Pyro_05Poly", "Pyro_10Poly".

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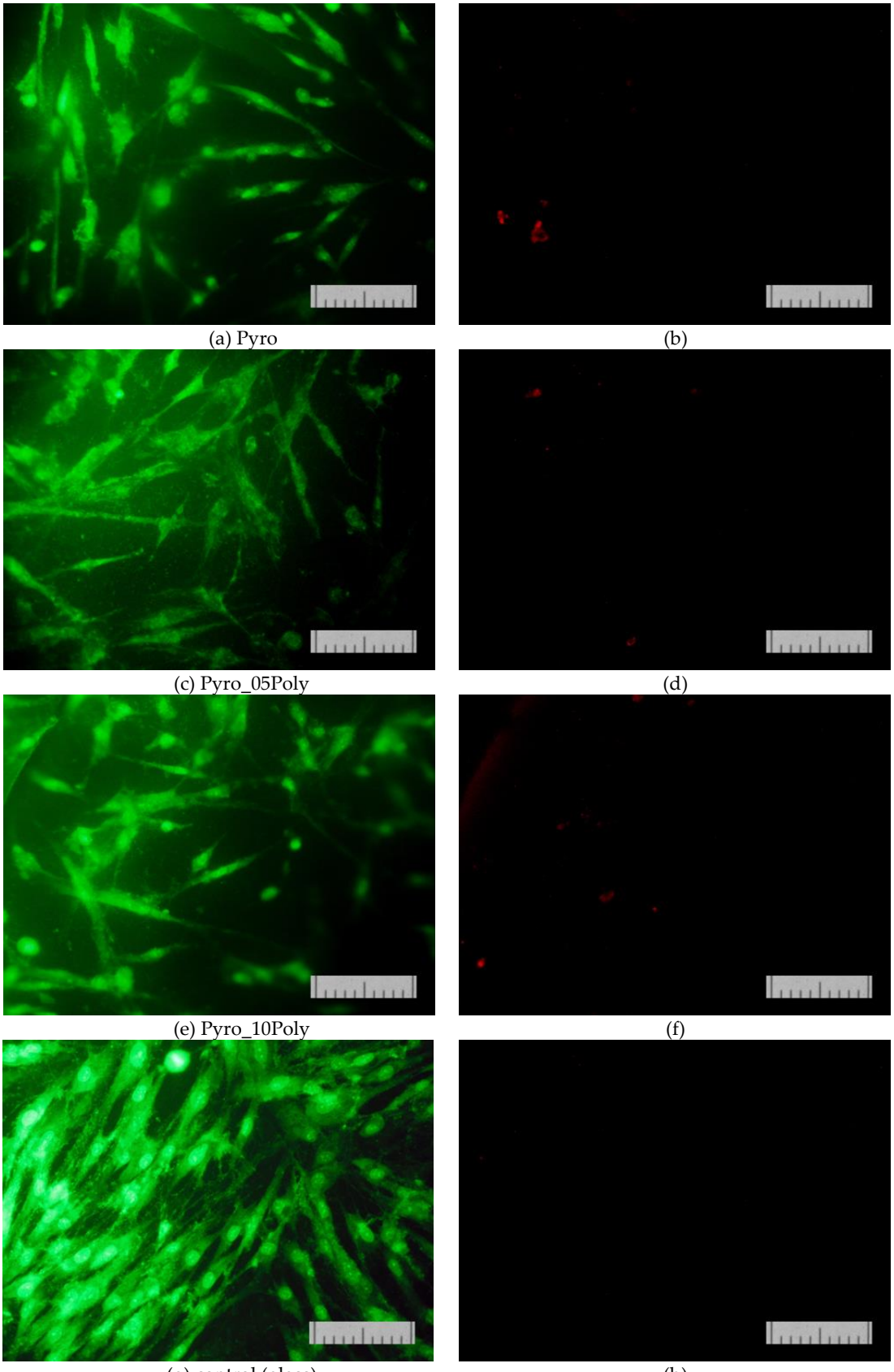

(a) Pyro (b)

(c) Pyro_05Poly (d)

(e) Pyro_10Poly (f)

(g) control (glass) (h)

Figure 15. The appearance of the dental pulp stem cells on the surface of ceramic samples under investigation, i.e. ceramic samples prepared based on powders "Pyro" (a, b), "Pyro_05Poly" (c, d), "Pyro_10Poly" (e, f), and control (g, h) after direct contact procedure for 2 days. Fluorescent staining was made with SYTO 9 (a, c, e, g) and propidium iodide (b, d, f, h). Bar 100 μm.

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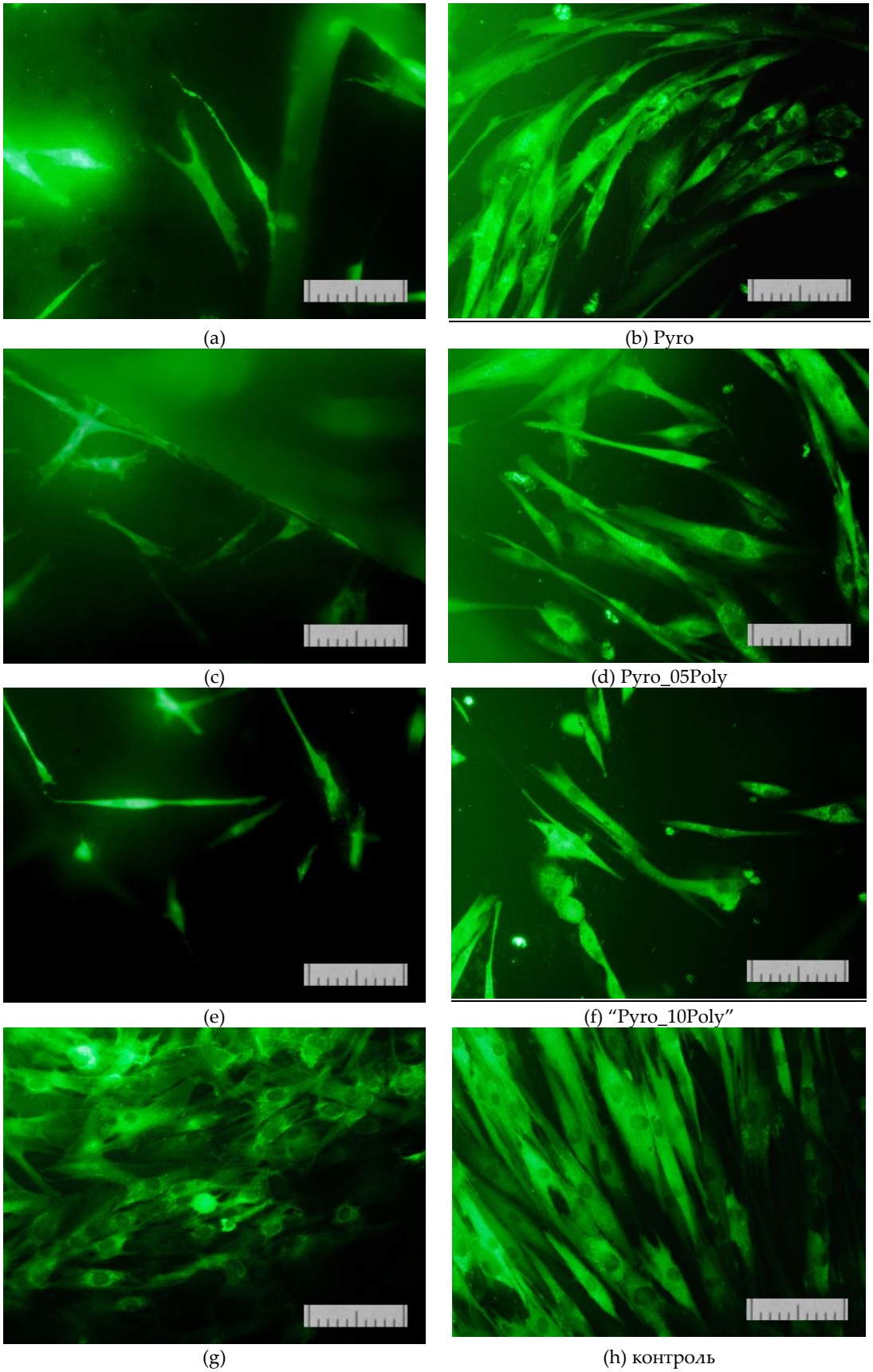

(a) (b) Pyro

(c) (d) Pyro_05Poly

(e) (f) “Pyro_10Poly”

(g) (h) контроль

Figure 16. The appearance of the dental pulp stem cells of the surface of ceramic samples under investigation, i.e. “Pyro” (a, b), “Pyro_05Poly” (c, d), “Pyro_10Poly” (e, f), and control (g, h) after direct contact procedure for 2 (a, c, e, g) and 7 (b, d, f, h) days. Fluorescent staining was made with SYTO 9. Bar - 100 μm.

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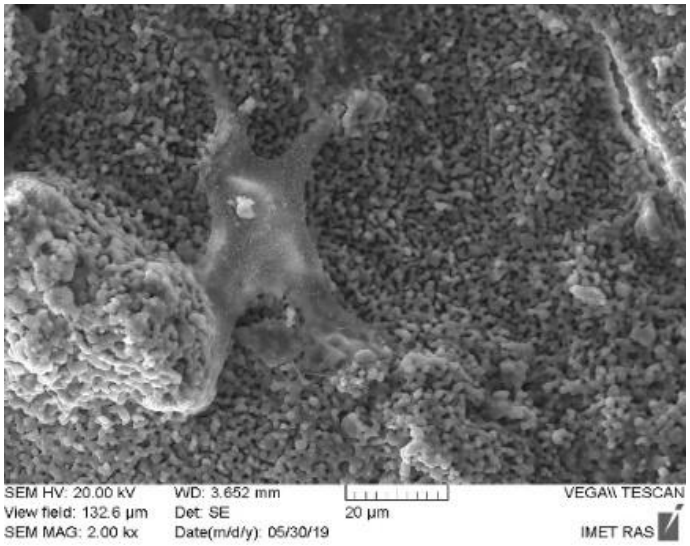
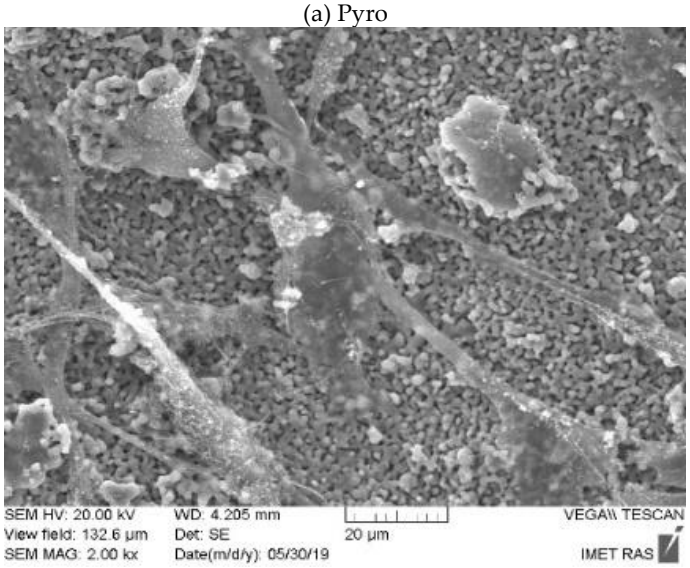
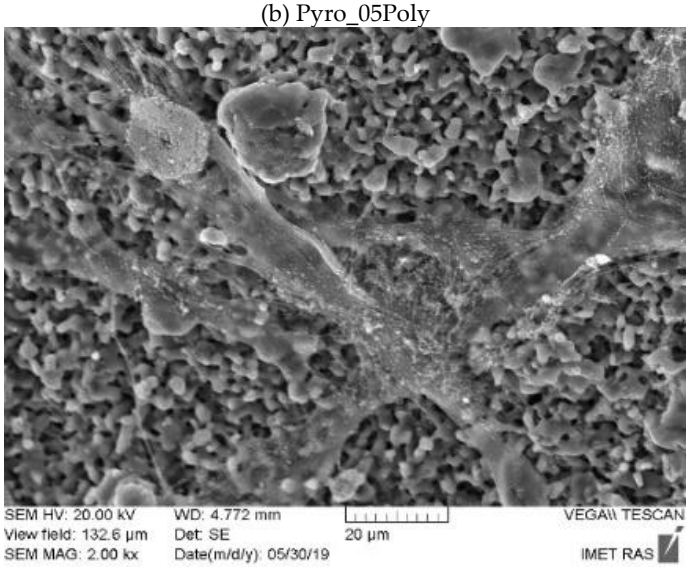


(a) Pyro



(b) Pyro_05Poly



(c) Pyro_10Poly

Figure 17. SEM images cells on the surface of ceramic samples (firing temperature 1100 °C) under investigation, i.e. "Pyro" (a), "Pyro_05Poly" (b), "Pyro_10Poly" (c) after 2 days of cultivation.

Data from in vitro biological experiments confirm the biocompatibility of the obtained ceramic materials and their ability to support cell proliferation.

4. Conclusions

Original method of γ-calcium pyrophosphate γ-$Ca_2P_2O_7$ powder preparation was used. To prepare powders of γ-calcium pyrophosphate γ-$Ca_2P_2O_7$ with preset molar ratio Ca/P=1; 0,975 and 0,95 powder mixtures based on calcium lactate pentahydrate $Ca(C_3H_5O_3)_2 \cdot 5H_2O$ and, monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$ were treated in an aqua medium in mechanical activation conditions, dried, disaggregated in acetone, and heat-treated at 600 °C. Porous ceramic samples with the relative density of 50, 55, and 60% in the CaO-P_2O_5 system were created based on prepared powders after firing at 1100 °C. The grain size of ceramic samples increased both with the growth of firing temperature and with decreasing of molar ratio Ca/P of powder mixtures. Calcium polyphosphate (t_{melt} =960–968 °C) formed from monocalcium phosphate monohydrate $Ca(H_2PO_4)_2 \cdot H_2O$ acted like a liquid phase sintering additive. It was confirmed by tests in vitro, that prepared ceramic materials with preset molar ratio Ca/P=1; 0,975 and 0,95 and phase composition presented by β-calcium pyrophosphate β-$Ca_2P_2O_7$ according to XRD data were biocompatible and could maintain bone cells proliferation.

Author Contributions: Conceptualization, T.S. (Tatiana Safronova); methodology, T.S. (Tatiana Safronova); investigation, A.K. (Andrey Kiselev), I.S., T.S. (Tatiana Shatalova), Y.L., Y.F., O.T., S.T., O.A., A.K. (Alexander Knotko), and T.S. (Tatiana Safronova); writing—original draft preparation, A.K. (Andrey Kiselev) and T.S. (Tatiana Safronova); writing—review and editing, T.S. (Tatiana Safronova); visualization, A.K. (Andrey Kiselev), I.S., T.S. (Tatiana Shatalova), Y.L., Y.F., O.T., S.T., O.A., A.K. (Alexander Knotko), and T.S. (Tatiana Safronova); supervision, T.S. (Tatiana Safronova); project administration, T.S. (Tatiana Safronova); funding acquisition, T.S. (Tatiana Safronova). All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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