Novel approach to obtain composite poly-L-lactide based films blended with starch and calcium phosphates and their bioactive properties

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PAPER

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Abstract

A novel approach for preparation of composite materials based on the poly-L-lactide (PL), starch and calcium phosphates is provided, applying the polymer crazing process in liquid absorption active medium. For composite films preparation, PL and blends of PL and starch have been chosen as polymeric matrixes. Pores formation in the polymer films occurred during the process of uniaxial stretching in the presence of ethanol or water-ethanol mixtures via the solvent-crazing mechanism. The diameter of pores and fibrils in crazed PL was 20–30 nm. Porous polymer matrixes have been loaded with starch, using crazing mechanism, and filled with calcium phosphates (CP) by the countercurrent diffusion method, the content of starch and CP being up to 11.0 and 14.5 wt%, respectively. The obtained composites were investigated by XRD, SEM-EDS and TEM methods. It was found out that the starch filled the porous crazed structure of polymer, while the CP synthesis in the PL pores resulted in the formation of amorphous particles with the diameter of about 20 nm. These CP nanoparticles aggregated into the submicron particles of 100–300 nm in diameter. The bioactivity of the initial and porous poly-L-lactide films, and composites, based on PL with starch and calcium phosphates, was investigated. It was shown that the bioactivity depends on the chemical composition and surface morphology of the prepared materials. After 10 days of cultivation of the pre-osteoblastic MC3T3E1 cells, an intense, 6 times, growth of osteoblast population was achieved for the composite containing calcium phosphates. This result is perceptibly higher than those obtained for other samples (4 times growth) and for the control sample (5 times growth). Based on the results of *in vitro* tests, it can be concluded that the prepared materials are promising for biomedical applications.

1. Introduction

Calcium phosphate (CP) based materials are of great interest due to their similarity to the mineral part of the natural bone tissue [1]. Biological apatite of bone tissue is a non- stoichiometric hydroxyapatite (BHA, $Ca_{10-x-y/2}(HPO_4)_x(CO_3)_y(PO_4)_{6-x-y}(OH)_{2-x}$), the BHA crystal particle size being approximately of 30–50 nm (length), 15–30 nm (width) and 2–10 nm (thickness).

CP based materials are widely used in medicine in the form of ceramics, cements, and coatings on metal implants. Furthermore, they are applied as components of toothpastes, gels and as wound dressings in combustology. However, the CP ceramics application is limited basically due to its poor mechanical properties. The composite materials based on CP and biopolymers enable to combine the CP bioactivity and polymers plasticity, and CP/bioresorbable-polymer composite biomaterials are promising materials for bone tissue reconstruction.

Within the last decade, there is a growing interest towards the use of poly-L-lactide (PL) as a matrix for the nanocomposites manufacturing. Among the PL

advantages, its transparency, strength and biodegradability can be underlined. Furthermore, its production is possible from the renewable sources that makes of the PL one of the most commercially attractive and promising biodegradable materials [2, 3]. Especially, it should be stressed that this polyester is able to compete with traditional petrochemical polymers. However, the combination of hydrophobic PL matrixes with hydrophilic CP component could be a problem due to their thermodynamic incompatibility [4]. This problem can be solved using compatibilizers, e.g. PL-maleic anhydride copolymer [5] or, otherwise, CP particle surface and polymer matrix could be modified with L-lactide, in the presence of Sn compounds [6, 7]. In the literature, an activation of ossification in the presence of CP/PL composites has been clearly demonstrated [8]. Despite the variety of manufacturing methods for producing hydroxyapatite/poly-L-lactide (HA/PL) composites, up to now only hot pressing and forging have been successfully used to produce blocks with mechanical properties similar to those of the natural bone tissue. By dissolving the PL polymer mixed with HA, highly porous HA/PL hybrid biomaterial was obtained [9]. Such composite biomaterial blocks have a compressive strength of up to 140 MPa and an elasticity module of up to 10 GPa. After implantation, host tissue shows good adhesion to the implant surface, as well as better integration due to the higher biocompatibility.

On the other hand, there are two main issues to be faced while using the PL as a biodegradable matrix, i.e. its low resorption rate and comparatively high price. To solve these problems, the blends of bioresorbable polymers or copolymers are employed, such as polyglycolide, polysaccharides [10], etc Following this route, mixtures with starch might be used considering that this latter is available and cheap. In this regard, highly dispersive mixtures of hydrophobic PL with hydrophilic starch are of considerable interest. The main problem in this case (and when PL is combined with CP) is the thermodynamic incompatibility of the components [11]. To enhance the affinity, the standard methods, such as the addition of methylenediphenyldiisocyanate [12] and polyvinyl alcohol [13] compatibilizers are employed. The additional components must have high affinity for both the components and reduce the excess of the interphase energy. Alternatively, PL chemical modification, in order to get copolymers with maleic anhydride [14], acrylic acid [15] and starch [16], could be performed. Thus, it should be stressed that the standard method of the PL-starch or the PL-CP mixtures preparation are based on the application of toxic compatibilizers or necessitate additional synthesis use.

Therefore, the challenge is to obtain starch/CP/PL composites with high mutual dispersity and controllable bioresorption. In this work, the method, based on the fundamental property of glassy (amorphous) and partially crystalline polymers, to get a finely dispersed oriented structure by uniaxial deformation in adsorption active media, is proposed [17–19]. Polymer crazing process gives rise to a system of interpenetrating nanopores with diameter of about 10 nm, whose walls are combined with polymer fibrils. Previously, we reported [20] the hydroxyapatite formation in porous polyethylene matrixes (PE), produced by the crazing mechanism using countercurrent diffusion method. According to the XRD results, carbonated apatite as a single crystalline phase was formed after the HA/PE composite treatment at 600 °C, and the size of apatite nanocrystals was 5–10 nm.

After the analysis of the available literature on PL-polysaccharides and PL-CP blends, the development of a three-component composite, providing all the required properties, seems to be reasonable. Indeed, the PL provides the optimal mechanical features combined with resorbability, polysaccharide phase permits to regulate the decomposition velocity, while the CP particles procure bioactive characteristics. It should be noticed that there are no literature data on such three-component composite systems, which is likely due to the complexity of their practical realization. In [21], the manufacturing of three-component (aliphatic polyesters, chitosan and HA) system is described. It turned out that such kind of composites are rather brittle due to the low adhesion strength between polymer matrixes and inorganic filler. For this reason, the crazing mechanism to fill the PL film with starch and CP particles appears to be perspective and promising. The main purpose of this paper is, therefore, to show the advantages of crazing process for the preparation of nanocomposites based on PL, starch and CP nanoparticles, and to study the peculiarities of structure and bioactive characteristics of the obtained hybrid systems.

2. Materials and methods

2.1. Samples

An industrial non-oriented commercial film of amorphous PL by Cargill DOW, USA (M_w = 200 kDa, PDI of 1.603, glass transition temperature of 63 °C, thickness of 300 μm) was used. The porosity in the polymer structure was achieved via uniaxial stretching in ethanol-water mixture media. Concentration of ethyl alcohol (96%, chemically pure) has been varied from 0% up to 96%. Insertion of carboxymethyl starch in the PL matrix was carried out via stretching of polymer film, in the presence of the ethanol solution (ethyl alcohol content of 30–40 wt%) and of starch (5 wt%) at the rate of 25% per min. Tensile strain of PL was 80%. The CP was synthesized by the reaction between water solutions of 1M $Ca(NO_3)_2$ (chemically pure) and 1M $(NH_4)_2HPO_4$ (chemically pure), in the pore volume of polymer matrixes using the countercurrent diffusion technique [20]; the reaction duration was 3 h.

Table 1. Composition of the investigated samples.

N	Sample	Tensile strain of PL, %	Starch content, wt%	CP content, wt%
1	PL	0	0	0
2	CP/PL	80	0	11
3	Starch/PL	80	6	0
4	Starch/CP/PL	80	14.5	3
5	PL	80	0	0

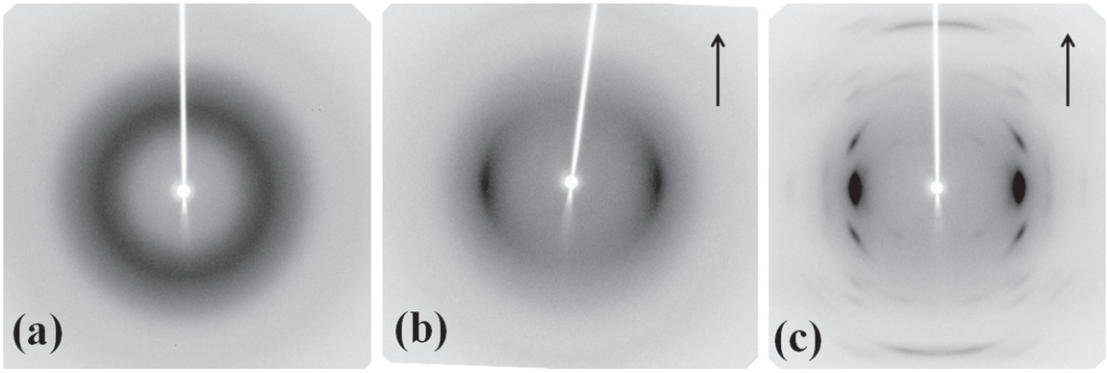

Figure 1. WAXS patterns of PL samples stretched in the liquid adsorption active medium to the different tensile strains: 0 (a), 80 (b), and 400% (c).

2.2. Characterization techniques

Phase composition of the obtained substances was analysed by the conventional x-ray diffraction technique (DRON-3M, Ni-filtered Cu-target, $\lambda = 1.54$ Å). The SEM analysis was carried out with the JEOL JSM-6390LA microscope. The cleavages were prepared applying the method of fragile fracture in liquid nitrogen. For SEM observations, all the samples were coated with 50–70 nm of gold on the sputtering device Giko IB-3 (Japan). Carbon, Calcium and Phosphorus atoms distribution was analysed by the Energy Dispersive x-ray Spectrometry (EDS), by means of the JEOL EX-175JMH detector. The TEM analysis was carried out using the LEO 912 AB OMEGA microscope. Ultrafine cuts were prepared by the ultramicrotome applying the diamond knife method.

Structure of original and stretched PL-films was investigated by the WAXS (Wide-angle x-ray Scattering) technique. X-ray scattering patterns were obtained on the Station 'Protein' (Channel 4.4e of Kurchatov Synchrotron radiation source), equipped with a two-dimensional CCD-detector Rayonix SX165 with a resolution of 2048 × 2048 pixels, goniometer Marresearch dtb, low-temperature device Oxford Cryosystems Cryostream 700 Plus with a temperature range of 80–500 K. The synchrotron radiation wavelength was 0.987 Å. For pore diameter estimation, the SAXS (Small-angle x-ray Scattering) technique was used. It consists of the Nanostar instrument (Bruker AXS), supplied with a two-dimensional coordinate detector, based on the CuKα radiation, at the primary beam point collimation, with the angular resolution of 7′.

3. *In vitro* tests

Pre-osteoblastic MC3T3E1 cells were purchased from the ECAAC, Salisbury, UK. The cells were removed from culture flasks by enzymatic digestion and then re-suspended in culture medium. The cell suspension was adjusted at a density of 5×10^4 cells/ml. All the samples were sterilized in a box under the UV irradiation for 30 min.

The complete osteogenic differentiation culture medium for our experiments was composed of the α-modification minimum essential medium Eagle, 10% FBS with osteogenic supplements L—ascorbic acid 50 μg ml^{-1}, 50 nm Dexametasone, 10 mM β-glycerophosphate and 1% penicillin, streptomycin, fungizone (purchased from Sigma-Aldrich). Sterile samples were embedded into each of the 96-well (untreated) polystyrene plates (pureGrade, Brand), and afterwards 200 μl of the complete culture medium was added. After 1 h of soaking, the medium was aspirated and exchanged for 200 μl of the cell suspension, containing 1×10^4 MC3T3E1 pre-osteoblastic cells. This procedure was performed three times for each sample. As negative control samples, the wells containing cells without samples at a seeding density of 1×10^4 cells per well were considered. They were cultured under the same conditions as samples in the cellGrade culture plate (Brand). The complete culture medium was changed every two days.

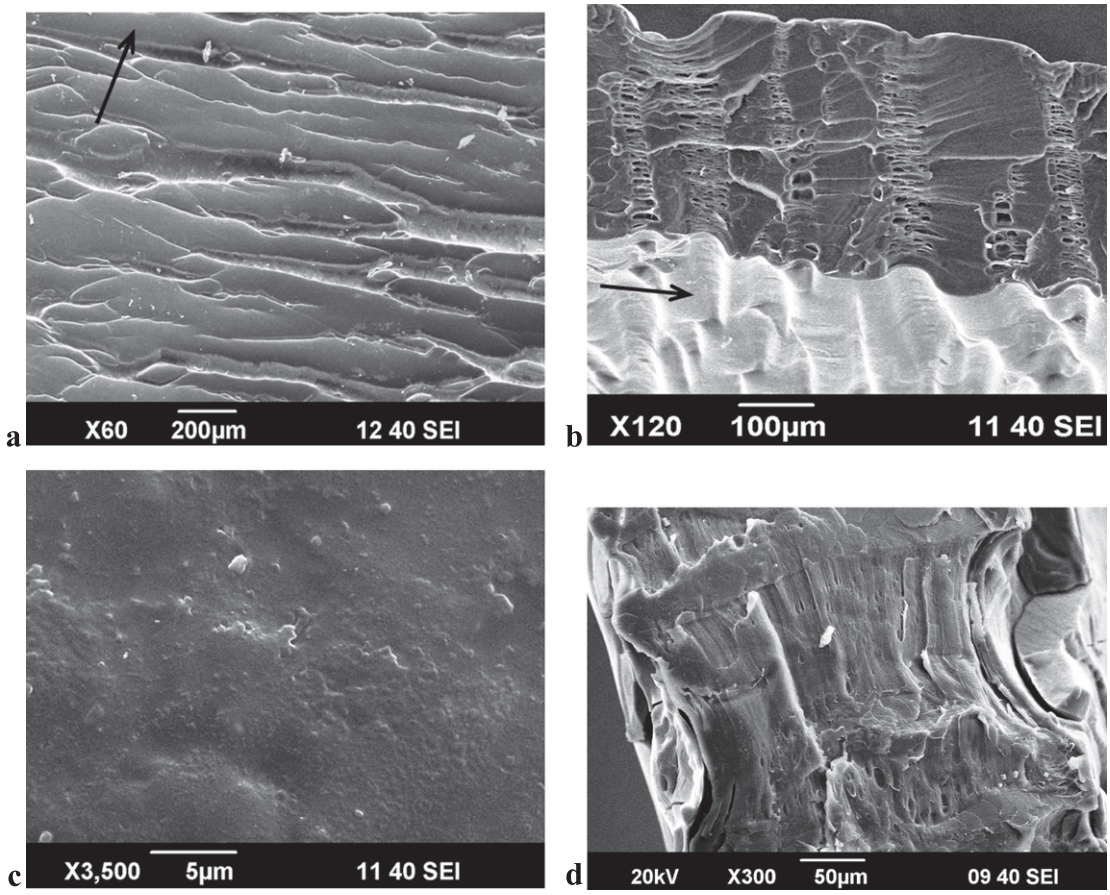

Figure 2. SEM micrographs of (a), (c) the surface and of (b), (d) the surface of a brittle cleavage, of (a), (b) porous PL and of (c), (d) starch/PL composite. The arrow indicates the direction of the polymer stretching during crazing.

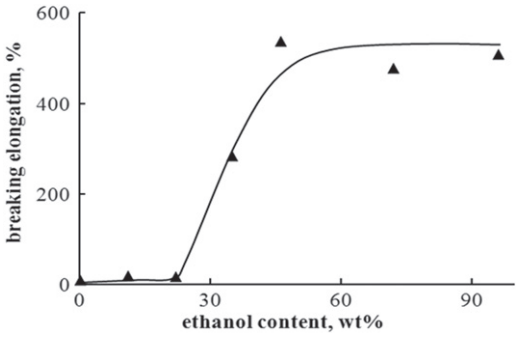

Figure 3. Breaking elongation of PL film during uniaxial stretching in water-ethanol solutions versus ethanol content.

The cytotoxicity of samples was evaluated after 48 h and after 10 days of cultivation by a commercially purchased proliferation test (the Cell titer 96 aqueous solution cell proliferation assay, Promega, USA) according to the manufacturer´s instructions. The absorbance of the final product (formazan) was determined at 490 nm by an UV–vis spectrophotometer (UV-1800, Shimadzu).

The ALP activity of osteoblasts was determined in cell lysates after lysis in solution containing 0.1% Triton X-100, 1 mM $MgCl_2$ and 20 mM Tris. The cell lysates were carefully transferred into the 1.5 ml microcentrifuge polypropylene tubes, frozen at −20 °C and centrifuged at 10.000 RPM for 10 min after thawing. The 100 μL of cell supernatant was added to 100 μl of p-nitrophenyl phosphate in dietanolamine buffer (0.5 mM $MgCl_2$, pH 9.8) and incubated at 37 °C. The reaction was stopped after 60 min by adding 50 μL of 3 M NaOH. The amount of p-nitrophenol, produced during the enzyme catalysis of the p-nitrophenyl phosphate substrate, was determined from the calibration curve of p-nitrophenol at 405 nm using the UV–vis spectrophotometer.

The following film samples were tested:

№1—initial PL film;

№2—PL with CP obtained via countercurrent diffusion 1M $Ca(NO_3)_2$/1M $(NH_4)_2HPO_4$;

№3—PL and starch composite obtained via crazing;

№4—PL, starch, and CP composite obtained via crazing;

№5—porous (38 vol%) PL with neither starch nor CP obtained via crazing.

The compositions of the studied PL-based samples are presented in table 1.

The density, distribution and morphology of the MC3T3E1 cells grown on tested samples were visualized with fluorescent live/dead staining (fluorescein

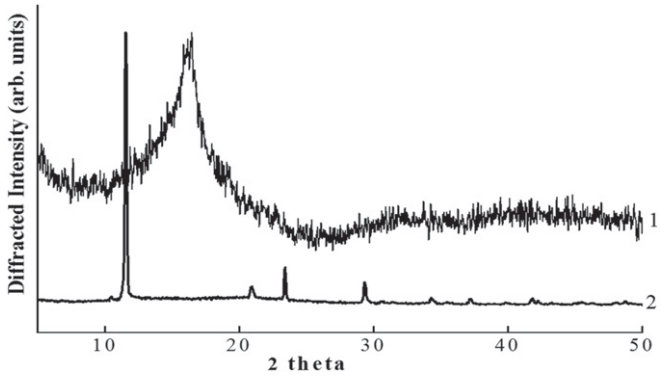

Figure 4. XRD patterns of CP/PL composite (1) and DCPD powder (2).

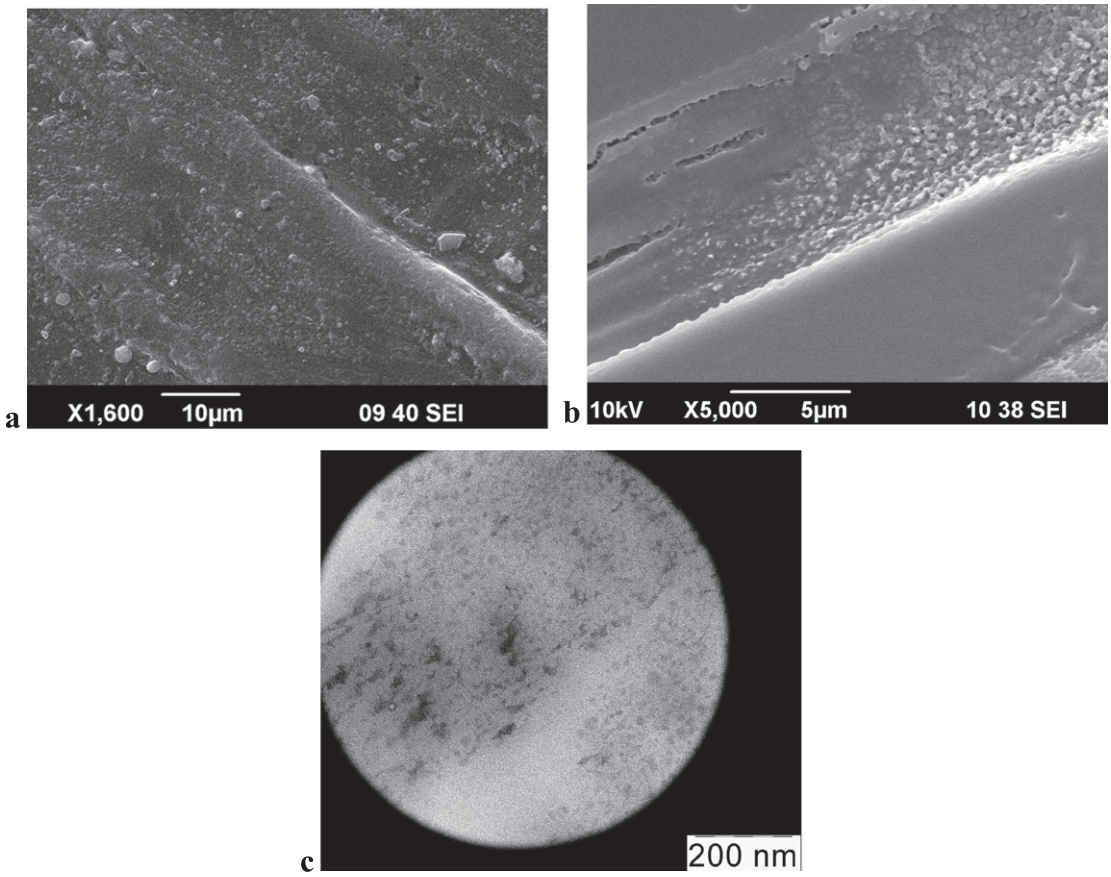

Figure 5. SEM micrographs of (a) the surface and of (b) the surface of a brittle cleavage. TEM image of (c) thin section of CP/PL composite.

diacetate/propidium iodide) by an inverted optical fluorescence microscope (Leica DM IL LED, blue filter).

4. Results and discussion

It is known that poly-L-lactide is quite fragile at the temperatures lower than glass transition point (50–70 °C), and maximum degree of its deformation usually does not exceed 10–15% [22]. Recently, it has been reported that in the presence of liquid adsorption active media, especially in aliphatic alcohols, the PL deformability tends to increase noticeably up to the tensile strain of 500% [23]. In this case, the deformation evolves according to the classic crazing mechanism via the formation of a regular superfine fibrillar-porous system with the pore diameter of about 20–30 nm, estimated by means of the SAXS method. In figure 1, the WAXS patterns of the original amorphous PL film and the films after deformation in the adsorption active medium, leading to different tensile strains, are presented. It is clear that the crazing

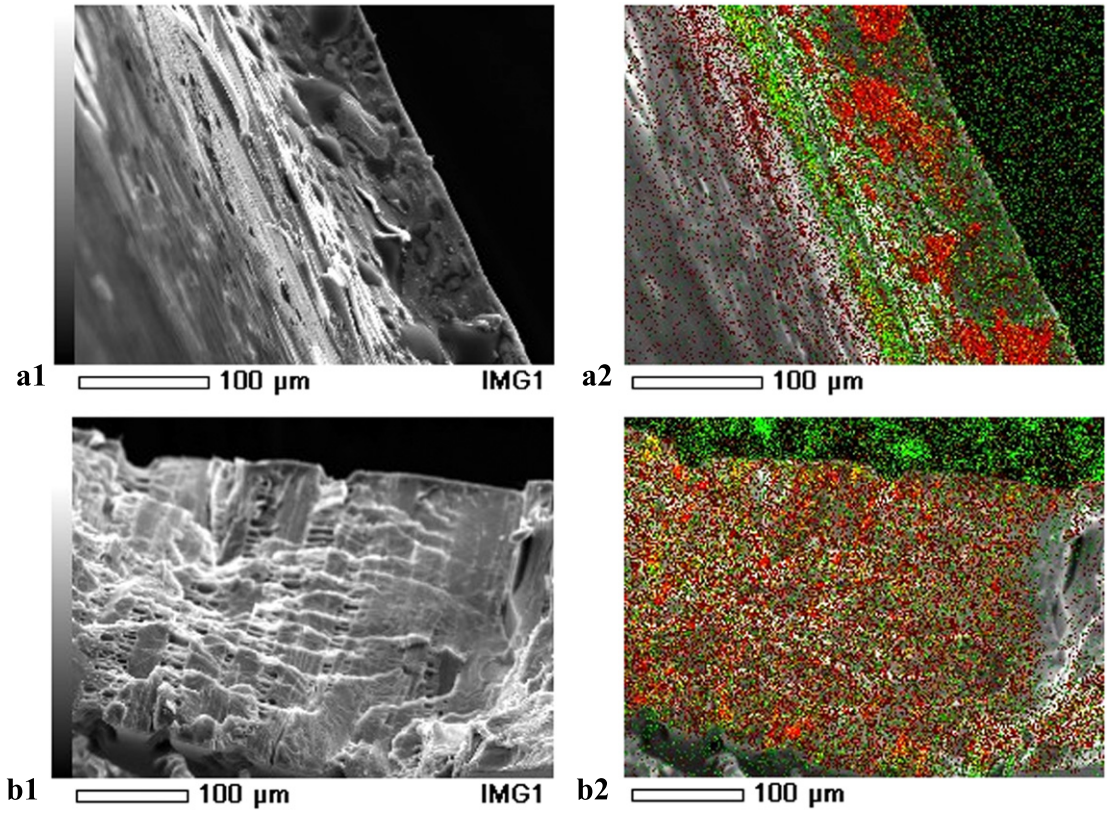

Figure 6. SEM images and elemental distribution of Ca and P on the cleavage surface of (a) CP/PL and (b) starch/CP/PL composites.

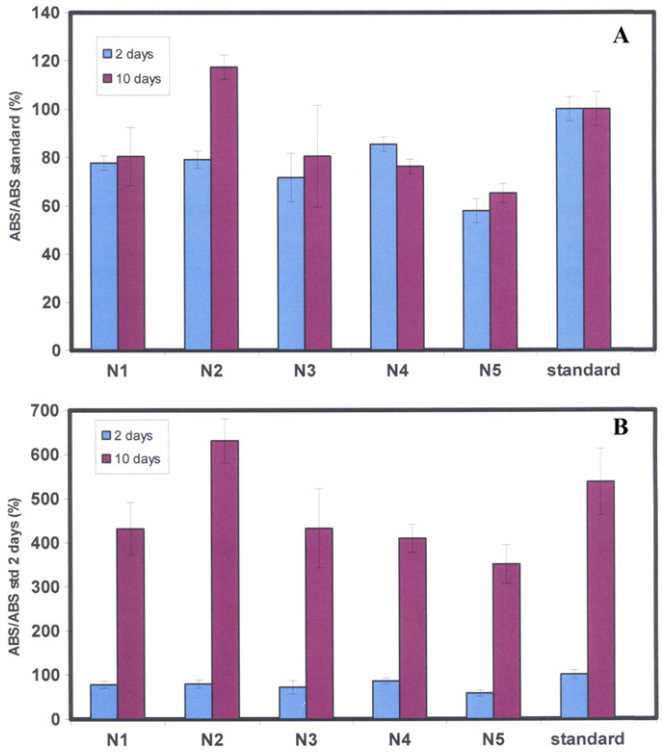

Figure 7. Relative proliferation of osteoblasts on samples №1–5 expressed as the ratio of formazan absorbance (490 nm) in wells with the control sample at corresponding time (A) and with the control sample after 2 days (B) (mean ± SD, n = 3, statistical significant differences with $P < 0.05$) cultured for 2 and 10 days at 37 °C, 95% humidity and 5% CO_2.

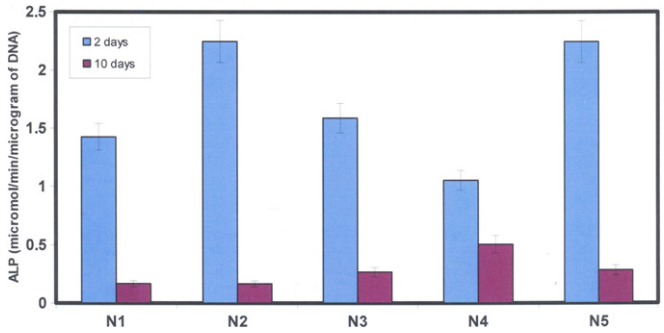

Figure 8. ALP activities of osteoblasts after 2 and 10 days cultivation at 37 °C, 95% humidity and 5% CO_2 .

is accompanied by the processes of fibrils orientation and crystallization, previously observed by the DSC technique [23]. In figures 2(a), (b), SEM micrographs, showing the surface and cleavage of the PL film after 30% strain in ethyl alcohol, are presented. The structure consists of alternating porous regions (crazes), and a block polymer observed on these images corresponds to the classical structure usually formed during the crazing. Crazes on the film surface appear as fissures and grooves, oriented perpendicularly to the direction of the PL film extension during the crazing process. On the crazes fractures, porous regions can be observed. It should be mentioned that during the removal of adsorption active liquid phase, the initial craze nanostructure significantly changes, resulting in the decrease in the volume porosity due to the capillary forces action. Moreover, a thin layer of non-porous polymer (about 1 μm of thickness) is formed on the surface.

In the present work, an application of the solvent-crazing process, in order to load porous PL matrix with the starch, is suggested. The main problem of this process is the low starch solubility in the ethanol media. In order to overcome these difficulties, the uniaxial strain process in the ethanol-water mixtures has been thoroughly studied. In figure 3, the breaking elongation as a function of the ethanol concentration in the media is presented. At the 30–40 wt% concentration of ethanol in the solution, a sharp enlargement of polymer deformability has been detected. This was the reason for using the solutions of slightly higher than 30–40 wt% ethanol concentration, in order to reach both tensile strain higher than 150% and stable starch solubility, not lower than 5 wt%. In figures 2(c), (d), SEM micrographs of the starch/PL composite surface and cleavage are presented. This hybrid material has been previously produced by the strain of the polymer film in the ethanol-water mixture of starch up to 100%. As can be observed, the porous structure, developed via the classic crazing, is filled with the starch, and a considerable part of the starch is located on the surface of the PL sample, forming a layer of about 25 μm of thickness. Therefore, the chemical composition of PL film and PL-starch composite is substantially different, leading to the significant difference in their bioactive properties.

The reaction between calcium nitrate and ammonium hydrophosphate in the crazed PL or starch/PL pore volume has some peculiarities. According to the XRD data, only amorphous CP is produced during the reaction. A peak at $2\theta = 16°$, which can be assigned to the PL crystal phase (see the diffraction pattern (1) in figure 4), has been registered, indicating the intensive polymer crystallization during the crazing process in the ethanol-water solution. An amorphous halo at $2\theta = 28–50°$ may be confidently attributed to the HA amorphous phase. Although during the reaction, occurred in a free volume under the same conditions, fine-crystalline residue is obtained consisting of dicalcium phosphate dihydrate (DCPD) phase, exhibiting the most intensive peaks on the diffraction pattern (2) at 11.5, 21, 23 and 29°, well matched with the DCPD pattern of the JCPDS database (file No.72-0713). The observed differences between the processes, occurring in the crazed polymer matrix and in solution, can be ascribed to the decisive effect of the nanoporous structure.

In figure 5, micrographs of the CP/PL composite surface, cleavage and thin section are presented. The CP nanoparticles with the diameter of about 20 nm and their aggregates with submicron dimension of 100–300 nm are located mostly in the crazes or on the sample surface. This is confirmed also by the elemental distribution map for Ca and P, obtained by the EDS analysis (see figure 6(a)). It should be noticed that in the case of the starch/PL matrix application, the arrangement of the CP particles is more homogeneous (see figure 6(b)).

Thus, the crazing is really quite simple and effective approach for production of the composites with a high level of mutual dispersion of the thermodynamically incompatible components, namely, the hydrophobic matrix of PL and hydrophilic fillers of calcium phosphate and starch. The morphology of composites is determined by the structure of the porous polymer matrix realized via the crazing mechanism. Furthermore, there is a possibility to directionally change the

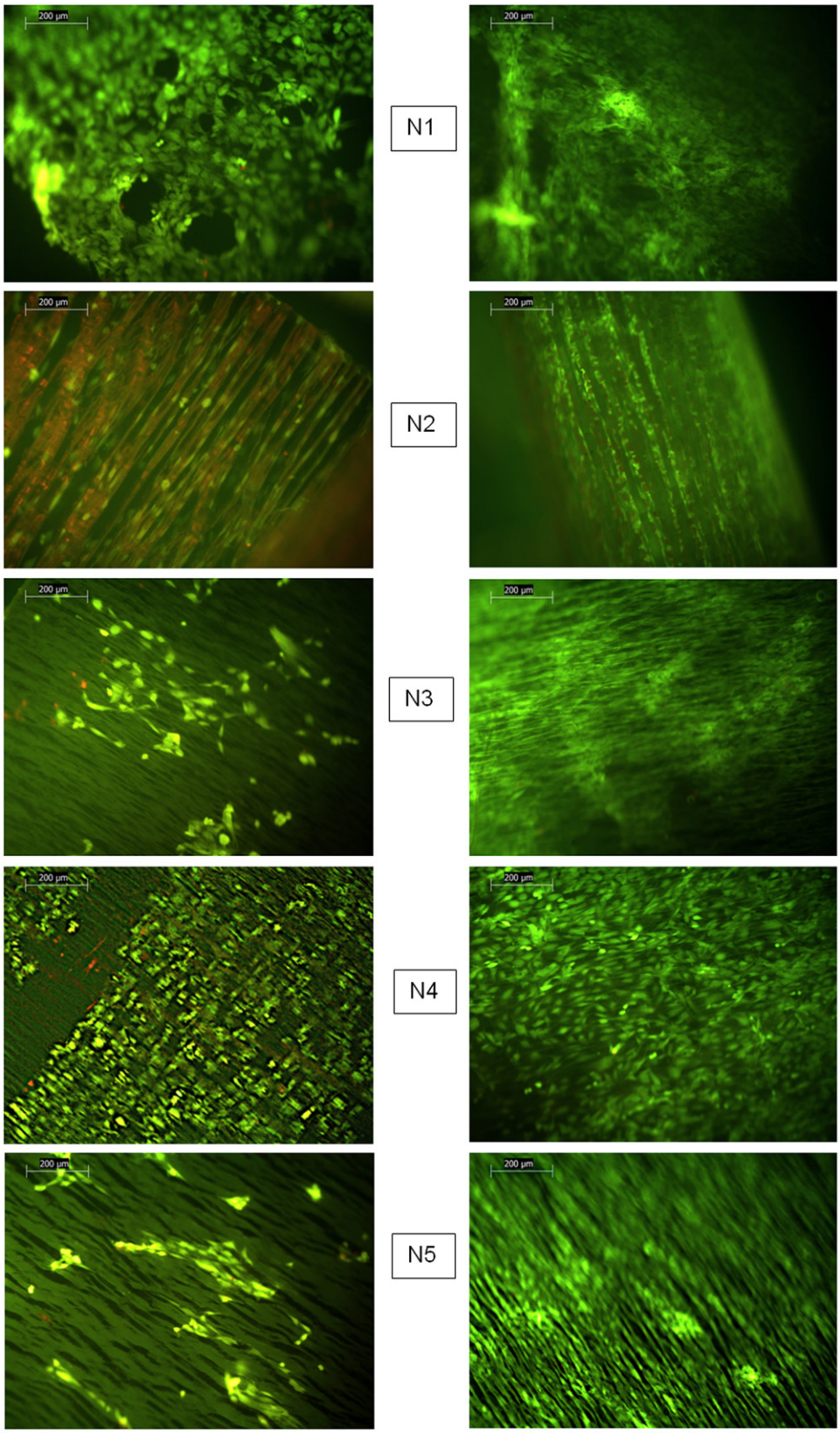

Figure 9. Density, distribution and morphology of the MC3T3E1 cells grown on samples (№1–5) during 2 (left) and 10 (right) days, in medium visualized with live/dead staining (fluorescein diacetat/propidium).

chemical composition and, apparently, the surface properties of the obtained composites.

The bioactivity of the composites based on poly-L-lactide, starch and calcium phosphates has been investigated. In figure 7(A), the relative proliferation of osteoblasts on samples №1–5 is shown. The figure clearly demonstrates that osteoblast proliferation during the first two days of cultivation was approximately

20% lower with respect to the control sample (wells free of samples). Much lower osteoblast population (around 60% with respect to the control sample) was found in the porous sample №5, composed of pure PL. After 10 days of cultivation, an intense, 6 times growth in the osteoblast population, in comparison with other samples (4 times growth) and with the control sample (5 times growth), was observed for the sample №2, containing calcium phosphates (figure 7(B)). The obtained results indicate that the polymer's porous structure, according to the crazing mechanism (for sample №5), leads to the reduction of bioactive properties of the PL film (sample №1). It may be caused by the changes in the chemical composition of the polymer surface because of the possible occurrence of mechanical or hydrolytic degradation of the PL chains during the crazing and adsorption of liquid medium molecules, such as ethanol. Loading such a porous structure with bioactive calcium phosphates (sample №2) results in a significant increase in the cells growth. Therefore, the composition and surface topography of the sample №2 are much more appropriate for proliferation and attachment of osteoblasts. On the other hand, a higher osteoblast ALP activity after 2 days of culture was measured on the film №2, containing calcium phosphates, and on the pure porous film №5. A decreased ALP osteoblast activity (see figure 8) on all the films after 10 days of culture likely can be explained by fact that the ALP activity is a marker for extracellular production during the initial period of osteoblast proliferation. During this period the collagen is the main secretion product, followed by the matrix calcification with osteocalcin marker during the next stage.

In figure 9, the morphology and density of osteoblasts on composite films are shown. As it can be seen, the osteoblasts were randomly oriented on the sample №1 (the initial PL film) irregardless of cultivation time. A high density of osteoblasts verifies their population growth with cultivation time, after 10 days of culture. In the case of other samples, the osteoblasts were oriented along the polymer fibres or pores, clearly visible in the film's texture. The surface morphology shown in figure 9 responds to the SEM micrographs presented in figures 2(a), (c) and in figure 5(a). Previously, similar orienting effect of the fibrillar-porous structure, developed via the crazing mechanism, has been observed during the process of various inorganic and organic substances crystallization [24], including liquid crystals [20, 25], on polymer's surface and in the pore's volume. This experimental fact is likely linked to a high specific surface of nanofibrils, causing improved cells adhesion. After 10 days of culture, higher amount of starch in the film composition (the film №4) caused the change in osteoblasts orientation into a more random, whereas a dense cells layer was found on the samples №2, №3 and №5, characterized by the presence of elongated thin osteoblasts along crazes. Apparently, the obtained images reveal a low cytotoxicity of composite polymer fibres in the films, supporting cells proliferation.

5. Conclusions

In this paper, a novel approach of preparation of composite materials based on the poly-L-lactide, starch and calcium phosphates is proposed. It is based on the polymer crazing process taking place in liquid absorption active medium. Influence of the fibrillar-porous polymer structure, formed upon the crazing, on the CP formation is reported. The appeal of suggested method is based on the fact that high mutual dispersity of hydrophobic PL matrix with hydrophilic fillers, such as starch and CP, can be reached without any preliminary chemical modification or the use of toxic compatibilizers. The performed biological *in vitro* tests with the pre-osteoblastic MC3T3E1 cells showed that the developed materials are promising for medical applications.

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