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Obtaining of Biodegradable Polylactide Films and Fibers Filled Hydroxyapatite for Medical Purposes

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Abstract. Relevance of the work is due to the need for new materials that are used in medicine (orthopedics, surgery, dentistry, and others) as a substitute for natural bone tissue injuries, fractures, etc. The aim of presented work is developing of a method of producing biocompatible materials based on polyesters of hydroxycarboxylic acids and calcium phosphate ceramic (hydroxyapatite, HA) with homogeneous distribution of the inorganic component. Bioactive composites based on poly-L-lactide (PL) and hydroxyapatite with homogeneous distribution were prepared. The results of scanning electron microscopy confirm homogeneous distribution of the inorganic filler in the polymer matrix. The positive effect of ultrasound on the homogeneity of the composites was determined. The rate of hydrolysis of composites was evaluated. The rate of hydrolysis of polylactide as an individual substance is 7 times lower than the rate of hydrolysis of the polylactide as a part of the composite. It was found that materials submarines HA composite and do not cause a negative response in the cells of the immune system, while contributing to anti-inflammatory cytokines released by cells.

INTRODUCTION

Research and development of different medical materials based on phosphate ceramics and biodegradable polyesters suitable for replacement and recovery of bone tissue are perspective areas of investigation on the border of chemistry and medicine. The main advantage of composite biomaterials in contrast with metals and ceramics is the absence of such deficiencies as low biocompatibility and corrosion or fragility of ceramics. Composites provide an opportunity to improve many of the undesirable properties of the individual materials. An important principle in creating biomaterials for implantation is a reproduction of the main characteristics of natural bone tissue, because it is the unique bone structure (chemical composition and morphology) has strong influence on the process of regeneration [1-3]. Hydroxyapatite as the main inorganic component of the bone tissue takes on the cardinal mechanical load in the body, and collagen as the biopolymer is adding the required elasticity and flexibility to the bone [4].

The use of HA ceramics for substitution of bone defects is associated with certain difficulties, since it is very difficult to give a desired shape of the ceramic implants for accurate filling of the defect, providing thus a snug fit of the implant to the bone tissue to form the proper joining *in vivo* [5]. These materials do not cause negative reactions of the immune system [6, 7]. Previous research for obtaining bioactive materials was based on oligomers of lactic acid and HA [8], but they have a low mechanical strength. Development of the methods for improving the strength, biochemical and other important properties of the composites on HA basis consists in three main challenges:

1. obtaining composites from the components which are able to bind HA and to reinforce the composites;
2. searching of the optimal ratio of selected components;

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3. investigation of physical and chemical interactions between the components in order to control the functional properties of the materials.

The aim of the work is to develop the method of preparation of biocompatible materials based on polyesters of hydroxycarboxylic acids and calcium phosphate ceramics with a homogeneous distribution of HA in it. We used poly-L-lactide as polyester, which is widely used in medicine for surgical sutures manufacture, guide pins etc. PL substrates are used as biodegradable porous substrates or matrixes for reconstruction of damaged tissues and organs. PL serves as a cover for drugs delivery systems of prolonged action [9-11].

EXPERIMENTAL PART

PL was obtained by ionic ring-opening polymerization of L-lactide. The molecular weight (M) was determined by means of capillary viscosimetry in chloroform (M = 90000).

Preparation of hydroxyapatite (HA) was carried out by a liquid phase method using microwave radiation at pH ~ 11 [12]:

$$10Ca(NO_3)_2 + 6(NH_4)_2HPO_4 + 8NH_4OH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 6H_2O.$$

Initial solutions were carefully mixed on a magnetic stirrer. The mixture was subjected to microwave exposure, the resulting suspension was kept for three days followed by filtration and drying. Further on a number of composite materials was obtained from PL and HA (powder or suspension). Composites 1 and 2 were obtained by mixing of the HA powder with PL dissolved in chloroform preliminarily. Composites 3-6 were prepared by mixing PL solutions with HA suspension, which was pre-washed with water (3 and 4 composites) or with ethyl alcohol (composites 5 and 6) up to the neutral pH. The mixture of the components to prepare composites 2, 4 and 6 was treated additionally with 40 kHz ultrasound, and then dried in air at room temperature until complete removal of the solvent. In order to prepare composite 7, the PL solution was mixed with HA powder and treated with ultrasound. The resulting mixture was added drop wise to the tenfold excess of cold ethanol (96%) and the precipitated fibrous material was separated by decantation and dried at room temperature.

Analysis of the composites by IR spectroscopy showed that in the spectra of composites 1-7 there are bands that are typical for both HA and for PL. The band shifts or new bands were not detected, indicating at the absence of chemical interactions between the components of the composite material. It is visible on the microphotographs (Fig. 1) obtained by SEM Tescan Vega LMU, that composites 3-6 obtained from the suspension of HA, contain the inorganic component (HA) in the polymer in a heterogeneity manner.

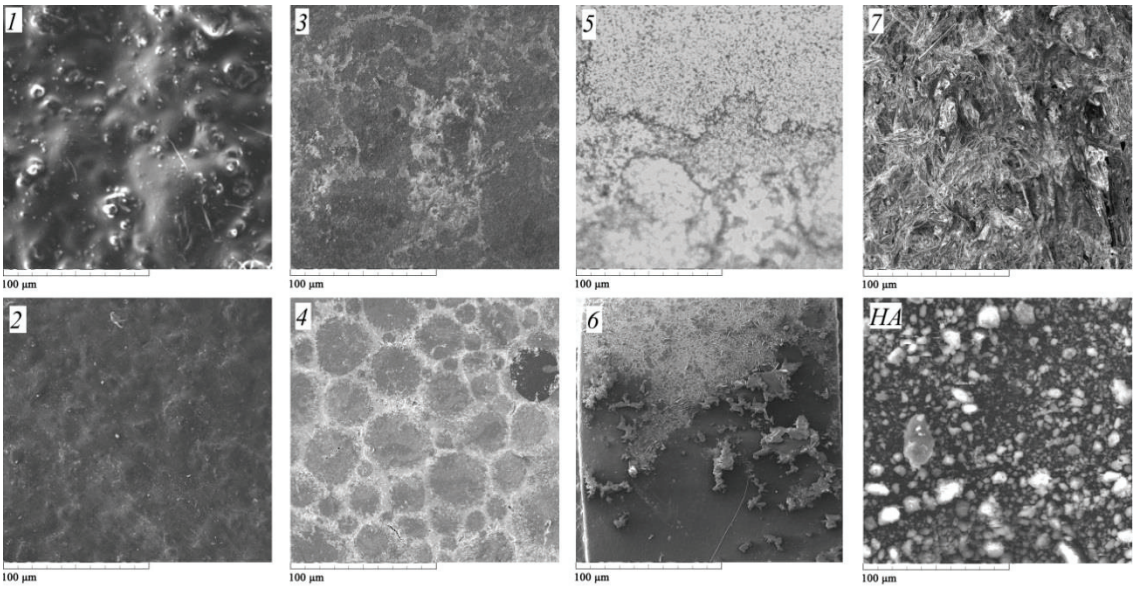

FIGURE 1. The micrographs of composites 1-7 and powder HA

The powder of HA is distributed in composite 2 more homogeneously than that in composite 1, which shows at the positive effect of ultrasound on the HA dispersing. Composite 7 represents a porous material with pore sizes up to 1 micron with HA particles dispersed in the polymer fibers. A porous structure of composite 7 provides the proliferation and differentiation of a living organism cells. This creates the possibility for uniform attachment and growth of bone cells of an organism, and this phenomenon, in its turn, will lead to uniform and gradual growth of the new bone tissue. It is necessary to note that changing the conditions of the composite particles preparation, the agglomeration of HA does not take place because the particles' size of all samples does not change. Thus presence of polymer does not influence the dispersion degree of the inorganic phase in the composites.

The solubility of HA is an important indicator for the bioresorbable composite. Pure HA and composites were placed in a 0.9% solution (NaCl) with constant stirring for 3 days. Further a sample and the amount of calcium in the solution measured by atomic emission spectroscopy. The results are shown in Tab. 1.

TABLE 1. The solubility of HA phase in the composite

Sample	Concentration of $Ca^{2+} \cdot 10^4$, mol/l
HA	9,187
Composite 1	7,194
Composite 2	4,57
Composite 7	5,49

Solubility of the HA phase in the composite is lower because of «blocking» effect of polymer matrix. It is possible that over time, the solubility of HA will increase due to the PL hydrolysis. It should be noted that the solubility of the HA in the composite 1 was greater than other composites. This is associated to the fact that HA powder in the polymer is not homogeneously dispersed. Therefore PL does not cover all HA particles as a result they dissolve faster.

An important requirement to composite materials, aimed at the recovery and replacement of a damaged bone tissue, is the rate of the composite material degradation, which should be similar to the rate of the new tissue growth. The polylactide hydrolysis rate in composite 7 was evaluated by the following procedure: tablets (m = 0.5 g) formed from the composite 7 were kept in a physiological saline (0.9 wt.% NaCl) for 21 days at 37 °C. The amount of lactic acid formed as a result of the PL hydrolysis was determined by HPLC method. It is seen on the kinetic curves (Fig. 2) that during the first 4 days rapid hydrolysis occurs in the incompact areas of the polymer matrix, and then the hydrolysis slows down, because the process shifts on the surfaces of the PL microcrystals.

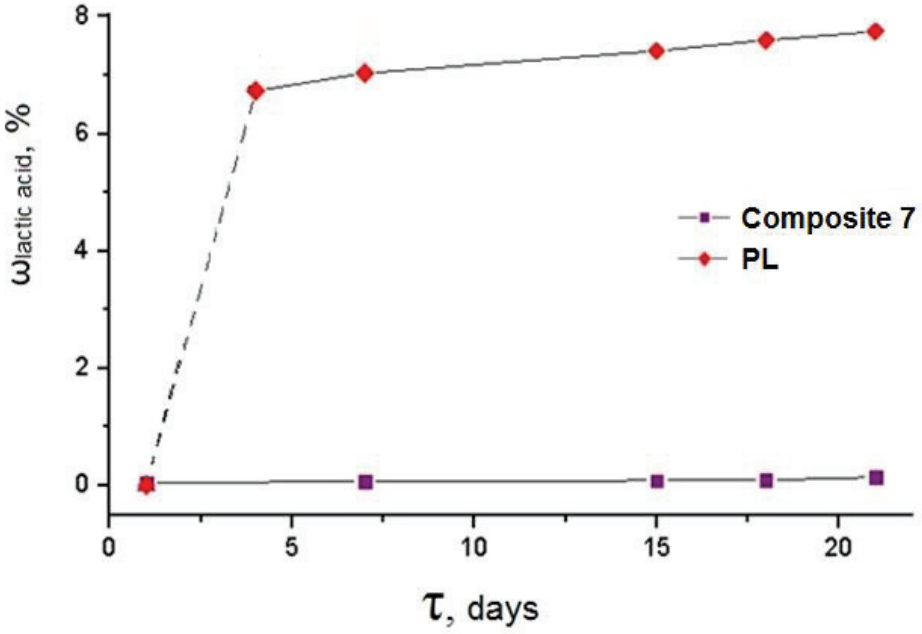

FIGURE 2. Estimate of the rate of hydrolysis of pure PL and as a part of a composite 7

The hydrolysis rate of PL as that of an individual substance is 7 times lower than the rate of PL hydrolysis in the composite. This indicates that introduction of hydroxyapatite particles increases the surface area of the composite and facilitates the water diffusion to the PL macromolecules.

Biocompatibility and presence of inflammatory reactions (pro-inflammatory and anti-inflammatory activity) of PL, HA and composite 2 was studied on cell-mediated immune response of individual donors in vitro, using cells of the human immune system ($CD14^+$ monocytes) [13-15]. Before the biological research all samples were sterilized. The study also included the problem of choice of the proper sterilization method of the polymer composite materials, because the composites based on HA and PL are designed for replacement and restoration of bone tissue. Since any sterilization includes thermal, chemical exposure or ionizing radiation, the method chosen should not influence the properties of the material.

For destruction of the microorganisms, the composite samples were stored in ethyl alcohol (70% aqueous solution) for 30 minutes, and then subjected to UV treatment (30 minutes). To carry out the enzyme-linked immunosorbent assay (ELISA) we used a 96-well plate, and in each well antibodies, specific to the test antigen (antibodies substrate, ATP) at a working concentration of 1:180 in PBS, were overlaid (laminated). The incubation period was 18 hours. After the incubation the washing was carried out 4 times with a solution of 0.05% Tween-20 in PBS.

In order to block the nonspecific binding places, the wells were filled with 1% BSA in PBS, then incubation for 1 hour and one-time washing was carried out. Test samples (AG) were added in the wells followed by 2 hours incubation. Thereafter, the wells were washed 4 times, and 100 µl of a specific antibodies solution (ATD) conjugated to the enzyme label (M) were added, followed by 2 hours of incubation. After 4 washings of the wells the horseradish peroxidase working solution based on streptavidin (Streptavidin-HRP) was added, followed by 20 minutes incubation in the dark place.

To carry out the enzymatic reaction the wells were washed four times, the substrate was added (mixture A staining reagent (H_2O_2) and B (3,3',5,5'-tetramethylbenzidine) in the ratio of 1: 1), followed by 20 min of incubation (the reaction is accompanied by the appearance of a colored product). Use of 3,3',5,5'-tetramethylbenzidine is based on its ability to form an intensely blue compound upon oxidation by hydrogen peroxide in the presence of peroxidase.

ELISA results demonstrated that a small release of pro-inflammatory cytokines such (TNFα) (Fig. 3) is observed in the control sample (without the material) and in the presence of HA, PL and of composite 2 as well.

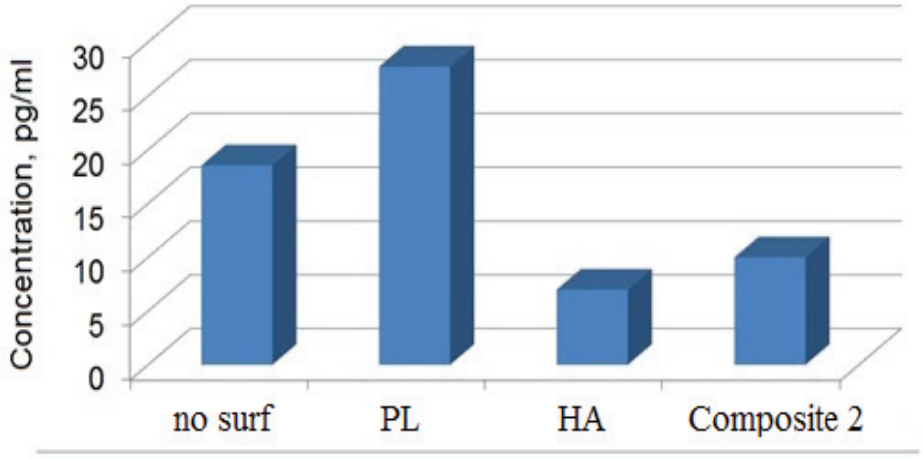

FIGURE 3. Influence PL, HA and composite 2 for allocation of macrophages TNFα in the presence of IFN-γ

The number of cytokines released by macrophages in the presence of HA and composite 2 does not exceed the TNFα concentrations in the control sample, i.e. these materials do not cause an adverse cellular response. The concentration of pro-inflammatory cytokines in the presence of PL is higher than that in the control sample, but it does not exceed the standard value (~ 40 pg / ml). PL contributes to isolation of anti-inflammatory cytokines (CCL18) on the 6-th day of cultivation (Fig. 4), which may indicate the manifestation of the potential anti-inflammatory properties of the polymer material.

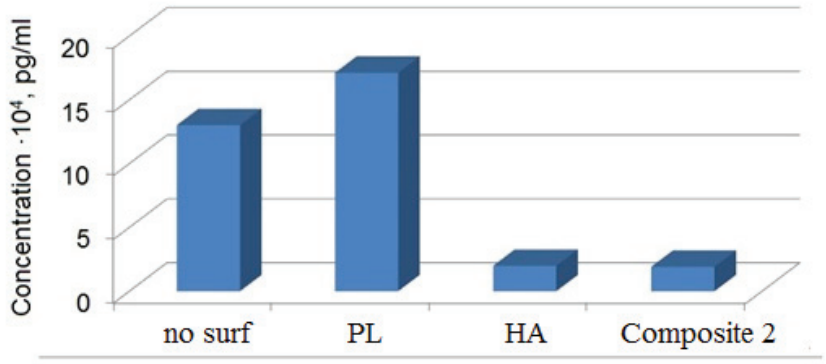

FIGURE 4. Influence PL, HA and composite 2 for allocation of macrophages CCL18 in presence of IL-4

SUMMARY

Thus a novel process for preparing porous bioactive composites based on hydroxyapatite and poly-L-lactide is developed. Electron micrographs of the composite 7 (Fig. 1) show a homogeneous distribution of HA particles in the fibers of poly-L-lactide, what will have a positive effect on the mechanical properties of the material. It is established that ultrasound has a positive effect on dispersion of the inorganic powder in the polymeric matrix. During the pilot experiment on a single human donor it is found that such materials as hydroxyapatite, poly-L-lactide and composite 2 do not cause any negative response of the immune system cells, at the same time they promote releasing of anti-inflammatory cytokines by the cells. These experimental facts allow one to recommend the above materials for further research as biocompatible materials in the bone implants area.

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