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Biodegradable polymer composites with osteogenic potential

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ABSTRACT

The aim was to study the basic physico-mechanical properties of hydroxyapatite (HA) composites (up to 25–50 wt%) with polylactide (PLA-HA) and poly(e-caprolactone) (PCL-HA) prepared by melt compounding, as well as the osteogenic potential of PLA-HA *in vivo*.

Materials and methods. All biodegradable polymer composites were prepared by hot melt compounding and studied by dielectric spectroscopy in frequency domain, optical microscopy, X-ray diffraction analysis and tensile tests. An ability of PLA-5 wt% HA composites prepared by 3D-printing to induce bone tissue growth *in vivo* was detected with the help of ectopic subcutaneous test in inbred mice.

Results. Values of the real part of complex permittivity of PLA-HA and PCL-HA composites are increased by 15–30% compared to those for initial PLA and PCL, while tand loss factor tanδ does not exceed 0.02 for PLA-based composites and 0.2 for PCL-based composites. The crystallinity degree of PLA-HA composites is increased by 3 and 6 times with an increase of HA content from 25 to 50 wt% respectively compared to the indicator for PLA. The crystallinity degree of PCL-HA composites with 25 wt% HA is increased by 2 times compared to the value for PCL. It is due to the fact that HA powder particles play the role of additional nucleation centers. For all this, mechanical strength of composites diminished statistically. Even lowest HA content (5 wt%) in PLA-HA composites prepared by 3D-printing increased the incidence of ectopic osteogenesis by 40%.

Conclusion. Designed biodegradable composites have a practical use potential for bone tissue engineering.

Key words: poly(lactic acid), poly(ε-caprolactone), hydroxyapatite, melt compounding, physicochemical properties, ectopic osteogenesis, *in vivo.*

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Биоразлагаемые полимерные композиции с остеогенным потенциалом

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РЕЗЮМЕ

Цель. Исследование основных физико-механических свойств композитов гидроксиапатита (ГА) (до 25–50%) с полилактидом (ПЛА-ГА) и поли(ε-капролактоном) (ПКЛ-ГА), полученных методом смешения в расплаве, а также остеогенного потенциала ПЛА-ГА *in vivo.*

Материалы и методы. Все биоразлагаемые полимерные композиции изготовлены методом горячего компаундирования в расплаве, исследованы методами диэлектрической спектроскопии в частотном ходе, оптической микроскопии, рентгеноструктурного анализа и испытаний на растяжение. Способность композитов ПЛА-5% ГА, полученных методом 3D-печати, к *in vivo* индукции роста костной ткани изучена при помощи теста подкожного эктопического костеобразования на линейных мышах.

Результаты. Значения действительной составляющей комплексной диэлектрической проницаемости композиций ПЛА-ГА и ПКЛ-ГА увеличиваются на 15–30% по сравнению с исходными ПЛА и ПКЛ, при этом тангенс угла потерь не превышает 0,02 для композиций на основе ПЛА и 0,2 – для композиций на основе ПКЛ. Степень кристалличности для композиций ПЛА-ГА, по сравнению с показателем для ПЛА, увеличивается в 3 и 6 раз при повышении содержания ГА с 25 до 50% соответственно. Для композиции ПКЛ-ГА при 25% ГА степень кристалличности увеличивается в 2 раза по отношению к значению для ПКЛ. Это обусловлено тем, что частицы порошка ГА играют роль дополнительных центров кристаллизации. При этом статистически значимо снижается прочность композитов на разрыв. Композиты ПЛА, полученные методом 3D-печати, даже с низким (5%) содержанием ГА на 40% повышают результаты эктопического остеогенеза.

Заключение. Разработанные биоразлагаемые композиции имеют потенциал практического применения в приложении к биоинженерии костной ткани.

Ключевые слова: полилактид, поли(ε-капролактон), гидроксиапатит, компаундирование в расплаве, физико-механические свойства, эктопический остеогенез, *in vivo.*

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INTRODUCTION

Biodegradable polymers such as poly(lactic acid) (PLA) and poly(ε-caprolactone) (PCL) are used extensively in the field of biomedical applications. In this case, biodegradable polymers are used as polymer scaffolds to assist tissue and cell growth during the bone tissue regeneration, and for drug delivery, when drugs are mixed with polymer matrix and they are gradually released as the polymer is dissolved in the body [1–8]. PLA and PCL can be degraded by microorganisms under environment conditions. In addition, PCL is susceptible to both alkaline and enzymatic hydrolysis [9–10].

Conventional single-phase polymer materials do not always meet the essential requirements of regenerative medicine therefore there is a great need to design multi-phase composites with properties similar to that of natural bone. For instance, poly(lactic acid) has a good biodegradability, however it has a very low elongation at break and brittleness limits its usage. On the other hand, poly(ε-caprolactone) has a higher elongation at break, while its degradability rate is lower than for PLA. Various organic and inorganic fillers are used to improve the properties of biodegradable composites such as mechanical properties, water uptake, degradability rate, and biocompatibility, making these materials suitable for use in the field of biomedicine. Carbon nanotubes (CNT) and carbon fibers (CF), derivatives of graphite, graphene, layered silicates, ferroelectric and piezoelectric ceramics powders, and hydroxyapatite (HA) are often used as fillers with required properties in such multi-phase biodegradable composites [7, 11–18].

For example, in [19–21], the authors showed the effectiveness of "Osteomatrix" biocomposite material, consisting of natural hydroxyapatites, aminoglycans, and collagens, for bone tissue regeneration. Defects in damaged bones were filled with "Osteomatrix" biocomposite material in the form of powder, pellets or blocks, which significantly reduced the formation time of a new bone tissue in patients.

Numerous authors have demonstrated that the addition of HA into PLA or PCL matrices can improve both biocompatibility and mechanical properties of polymer-ceramic composites because hydroxyapatite has the most similar chemical composition to human bone [1, 22–32]. Akindoyo et al. [23] reported that adding HA into PLA allows for better cell attachment and proliferation to the PLA-matrix. Adding the biostrong impact modifier (BSIM) into PLA-HA composites results in increase of impact strength, tensile and flexural properties at 5 wt% BSIM content.

Russias et al. [28] observed that PLA-based composites filled with either a fine-grained powder (average particle size – 5 μm) or larger whiskers (25–30 μm long and 5 μm in diameter) of hydroxyapatite with about 70–85 wt.% ceramic contents can be used to prepare scaffolds with mechanical properties close to those of human cortical bone.

Zhang et al. [31] showed that PLA-HA composites improve interfacial adhesion and the bending strength. Furthermore, these composites can be processed by 3D-printing [30, 31]. Shen et al. [32] reported that PLA-based biocomposites filled with HA and carbon fiber (CF) (PLA-HA-CF) were manufactured of PLA-HA-CF prepreg by hot pressing. PLA-HA-CF prepreg was prepared by mixing components in solution. They have found that biocomposites have excellent mechanical properties, for example, flexural strength, flexural modulus and shear strength reach 430 MPa, 22 GPa, 212 MPa, respectively. Water uptake of PLA-HA-CF biocomposites increased up to 5%, while the rate of mass loss was only 1.6%. After *in vitro* degradation for 3 months, the pH value of phosphate buffer solution decreases by less than 0.1, indicating that the alkaline of HA neutralizes the acid formed following the degradation of PLA by hydrolysis, which prevents the harm of acidity for the patient's body. Park et al. [33], Kim et al. [34] and Jiang et al. [35] demonstrated that the PCL-HA composites can be successfully applied to manufacture biodegradable scaffolds by 3D-printing for bone tissue engineering.

However, the majority of PLA-HA and PCL-HA composites were prepared by mixing in solution, but even for these composites, studies of the main properties are episodic in nature.

The aim of this work was to study the basic physico-mechanical properties of PLA-HA and PCL-HA

composites obtained by melt compounding, as well as *in vivo* osteogenic potential of PLA-HA composites.

MATERIALS AND METHODS

Biodegradable poly(lactic acid) (PLA, Ingeo 4043D, NatureWorks LLC, USA) and poly(ε-caprolactone) (PCL, purchased from Sigma-Aldrich, USA) were used in this work as polymer matrices. Powder of hydroxyapatite (HA) produced by mechanochemical method [36] provided by Sintel RPC LLC (Tomsk, Russia) was used as filler. All materials were used as received without an additional treatment. The filler content (*C*) in polymer composites was changed from 0 to 50 wt%. All composites were prepared by melt compounding in a measuring mixer 50 EHT (Brabender, Germany). Mixing time and processing temperature were 10 min and 190–210 °C for PLA-based composites and 80–100 °C for PCL-based composites respectively. The speed of counter-rotating blades of the mixer was changed from 30 to 90 rpm as shown in Fig. 1. Filler was gradually introduced into the polymer melt up to the required fraction, while mixing until all fillers were evenly distributed in the polymer matrix. After preparation, all composites were granulated with the granulator (Brabender, Germany).

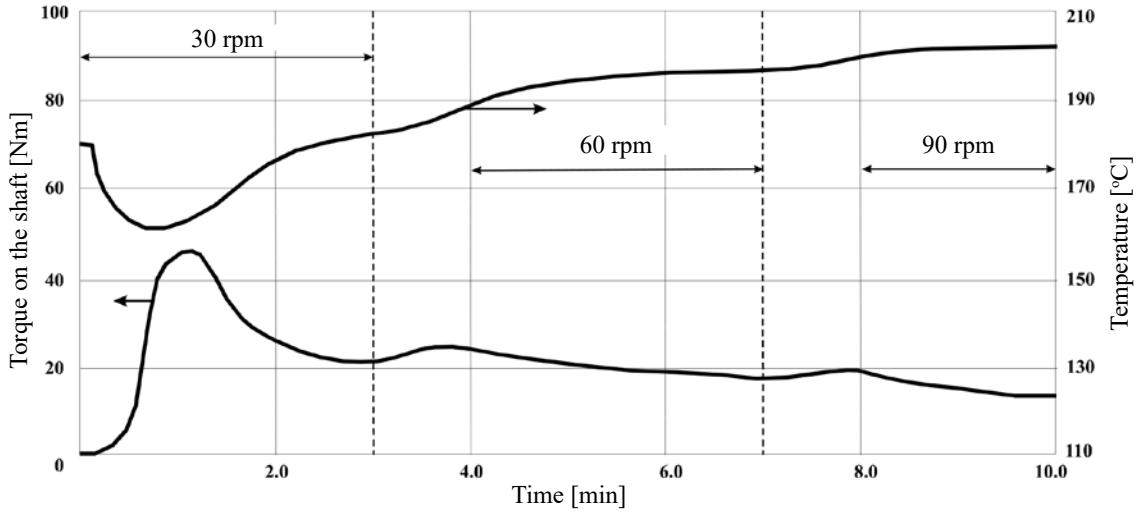


Fig. 1. Temporal diagrams of the melt temperature and torque on the agitator shaft for PLA-based composite

To prepare the samples with dimensions of 65 × 85 × (0.05–1.0) mm by hot pressing, compression molds filled with polymer composite pellets were placed into a vacuum furnace heated to 200 °C (for PLA-based composites) and to 100 °C (for PCL-based composites) for 3 hours. After that, compression molds were pressed in a hydraulic press at 20 MPa for 20 minutes. Then, the compression molds were cooled at a cooling rate of 4°C / min to ambient temperature under pressure in air.

Measurements of the ε′ real part of the $\varepsilon^*(f) = \varepsilon'(f) - \varepsilon''(f)$complex permittivity and the $\tan\delta = \varepsilon''(f)/\varepsilon'(f)$ loss factor, where ε″ is the imaginary part of the complex permittivity; $\delta = 90° - \varphi$ is the loss factor angle; and φ is the angle between voltage and current, were carried out under 3V AC voltage in the frequency range from 1 to 10^5 Hz using a "Solartron Analytical" instrument (Great Britain). From seven to ten measurements per decade in the entire investigated frequency range were carried out for all samples. Not less than three samples for each material were tested.

The morphology of developed composites, shape and dimensions of filler powder particles were studied by an optical microscopy (OM). The thickness of samples for the OM study was 50 μm.

The study of crystalline structure of polymer matrices and composites was carried out by wide angle X-ray diffraction method (XRD) by means of the "Shimadzu XRD-7000" diffractometer (CuK_α radiation λ = 1,54 Å) at an accelerating voltage on an X-ray tube of 40 kV and current of 30 mA, in the angle range of 2θ = 5–90°.

The mechanical properties of the developed polymer composites were studied using an "Instron 3345" universal tensile testing machine (USA). The elastic modulus, elongation at break, and tensile strength for all tested samples were determined from the experimentally obtained stress-strain curves.

To estimate the bioengineering potential of PLA-HA composites, three-dimensional (3D) samples prepared of 95 wt% PLA + 5 wt% HA composite were prepared in the form of disks (11 mm in diameter and 1.2 mm thick) by computer-aided design in Blender software with open source code and subsequent layer-by-layer deposition of filaments (diameter 1.75 mm) using a CreatBot Duo 3D printer (CreatBot 3D Printer, PRC) as described previously [37]. Samples prepared of pure PLA were used as control ones. To improve the bone marrow adhesion to the surface of samples, one of sample surfaces was textured with 0.3–0.5 mm wide grooves.

One of the modern methods to determine the osteogenic properties of materials and products is the ectopic (heterotopic) subcutaneous test, which makes it possible to evaluate the *in vivo* induction of differentiation of mesenchymal stem cells into osteoblasts [38].

The experiments were carried out in compliance with the principles of humane treatment of laboratory animals in accordance with the "Rules for the use of experimental animals" (Appendix to the order of the Ministry of Health of USSR No. 755 dated 12.08.1977). Animals were kept according to RD-APK 3.10.07.02-09. Drinking water was supplied to laboratory animals from the water supply system; water quality was in accordance with SanPiN 2.1.4.1074-01 (Russia). Animals were kept under artificial and natural light in accordance with the requirements of SNiP 23.05-95 (Russia).

BALB/c inbred mice were anesthetized and operated under sterile conditions. The skin was sterilized with 70% ethanol, samples with syngeneic bone marrow from femur were implanted into the lateral subcutis pockets of the venter, and the wound was sutured and treated with 70% ethanol. The procedure was described previously [39]. The bone marrow of an adult organism is the central pool and the source of mesenchymal stem cells (MSCs) [40].

Forty days later, the animals were sacrificed via CO_2 asphyxiation, and the status of the tissues around the implants was assessed. The implants were removed together with the adjacent soft tissues (tissue plates) on the "working" (textured) surface of the samples. Standard histological technique for preparing thin sections of fixed tissue lamellae and their hematoxylin and eosin staining was used [39]. Six implants were tested in both the control and experimental groups. A formation of bone and/or bone tissue with marrow in the tissue lamellae was considered as a positive result.

The ability of implants to induce bone growth was calculated as the percentage of test samples promoting the induction of bone tissue growth (IBT) with or without bone marrow in the tissue plate per the total number of implanted samples according to the formula: IBT (%) = $[N_2/N_1] \times 100\%$, where N_1 is the number of implanted samples; N_2 is the number of samples on which bone tissue growth was detected.

Statistical analyses were conducted using the STATISTICA 13.3 software package for Windows. The normality of the data distribution was defined by the Kolmogorov – Smirnov test. The mean and mean error ($M \pm m$), median and Me (Q_1–Q_3) interquartile range were calculated. To assess the statistical significance of differences, in the event that the distribution of data does not correspond to the normal distribution law, the nonparametric Mann – Whitney test was used. Statistically significant differences were considered at $p < 0.05$.

RESULTS AND DISCUSSION

The results of dielectric spectroscopy in frequency domain are presented in Fig. 2. It is obvious that frequency dependencies of $\varepsilon'(f)$ for polymer composites exhibit typical behavior: the $\varepsilon'(f)$ is monotonically decreased with frequency for both polymer matrices and composites based on them. When filling PLA with HA powder, the permittivity of PLA + 25 wt% HA composite is increased by about 12–15% compared to the initial PLA over the frequency range studied due to the higher permittivity of HA, which is about 4–9 [41]. Increase in the permittivity of PLA + 50 wt% HA composite is 23–32% compared to the initial PLA. Increase in the permittivity of PCL + 25 wt% HA composite is 1–4% compared to the initial PCL. Tan$\delta(f)$ loss factor over the studied frequency range for PLA-based composites does not exceed 0.02, while for PCL-based composites it does not exceed 0.2.

The results of OM are shown in Fig. 3. However, the filler particles form agglomerates sized around 100 μm, which are visible both for initial HA powder (Fig. 3,*a*) and for composite with filler content 25 wt% (Fig. 3,*b*). It can be seen that filler particles are quasi-uniformly distributed in the polymer matrix. Decrease in the average size of filler agglomerates for the composite with 50 wt% HA (Fig. 3,*c*) compared to that for composite PLA + 25 wt% HA is explained by de-agglomeration of filler powder particles during compounding due to an increase in the stiffness of composites.

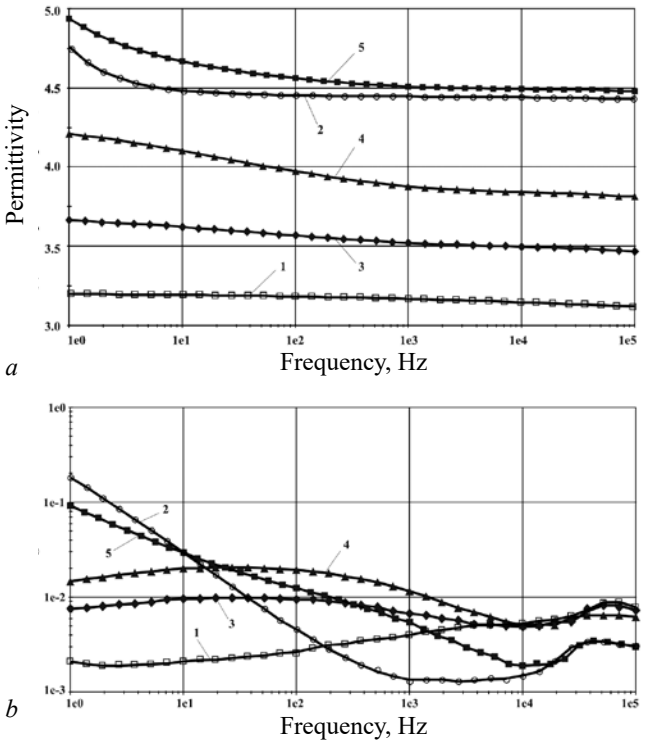


Fig. 2. Frequency dependencies of the real part of complex permittivity (relative units) (*a*) and tanδ (*b*) for: 1 – PLA; 2 – PLA+25% HA; 3 – PLA+50% HA; 4 – PCL; and 5 – PCL+25% HA

X-ray diffraction data (X-ray diffraction analysis, or XRD) for polymer matrices (PLA and PCL) and composites (PLA+HA and PCL+HA) are shown in Fig. 4. These results indicate that for PLA broad diffraction peak around 2Q » 16.2° due to reflections from (200) or (110) crystallographic planes is observed (Fig. 4,*a*). On the other hand, peaks around 26.12°, 29.16°, 32°, 40°, 46.9°, and 49.7° observed for PLA-HA composites are attributed to the characteristic diffractions peaks of HA (Fig. 4,*b* and 4,*c*) due to reflections from (002), (210), (211), (310), (222) and (213) crystallographic planes.

For initial PCL two strong reflections at 2Q » 21.4°, and 23.7°, corresponding to the (110) and (200) crystallographic planes of the PCL crystalline form are observed (Fig. 4,*d*). The characteristic peaks around 26.12°, 29.16°, 32°, 40°, 46.9°, and 49.7° for PCL-HA composites are due to the HA presence (Fig. 4,*e*), as well as for PLA-HA composites.

The degree of crystallinity for the used polymer matrices and composites based on them are listed in Table 1.

It can be seen that the crystallinity degree of PLA-HA composites is increased by 3 and 6 times for PLA + 25 wt% HA and PLA + 50 wt% HA composites, respectively, compared with the initial PLA.

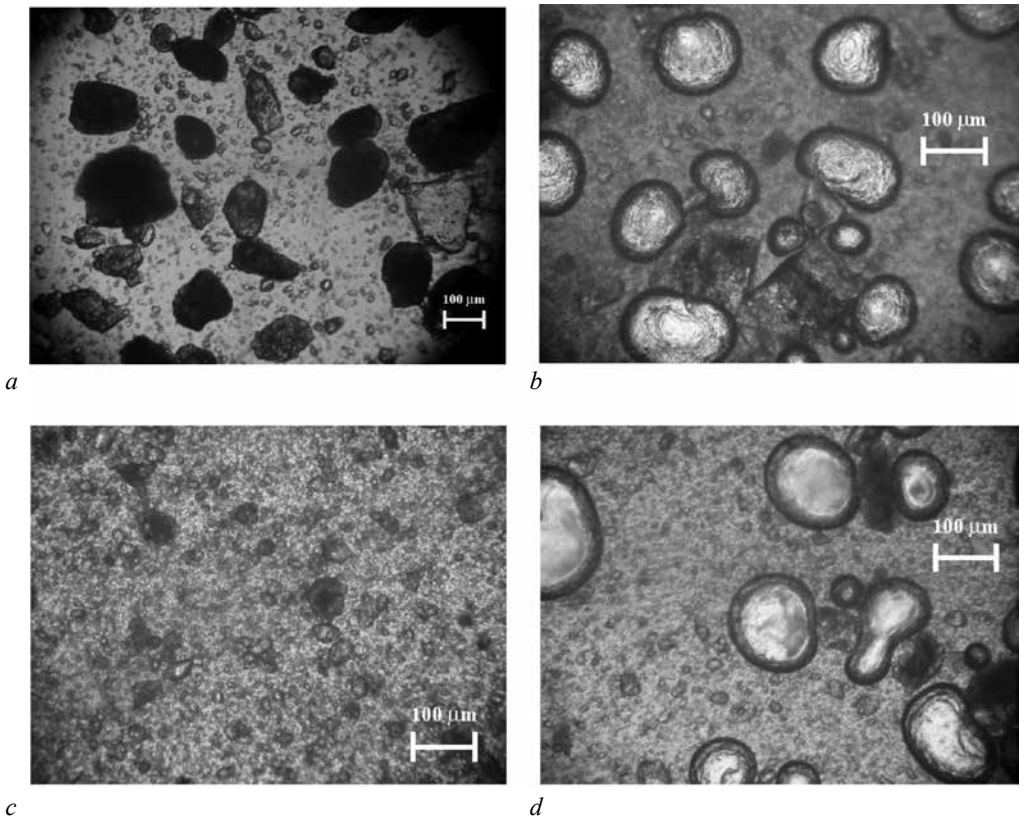


Fig. 3. OM micrographs of the HA filler powder (*a*) and PLA-HA composites: *b* – 25 wt% HA, *c* – 50 wt% HA, and *d* – PCL-HA composite with 25 wt% HA

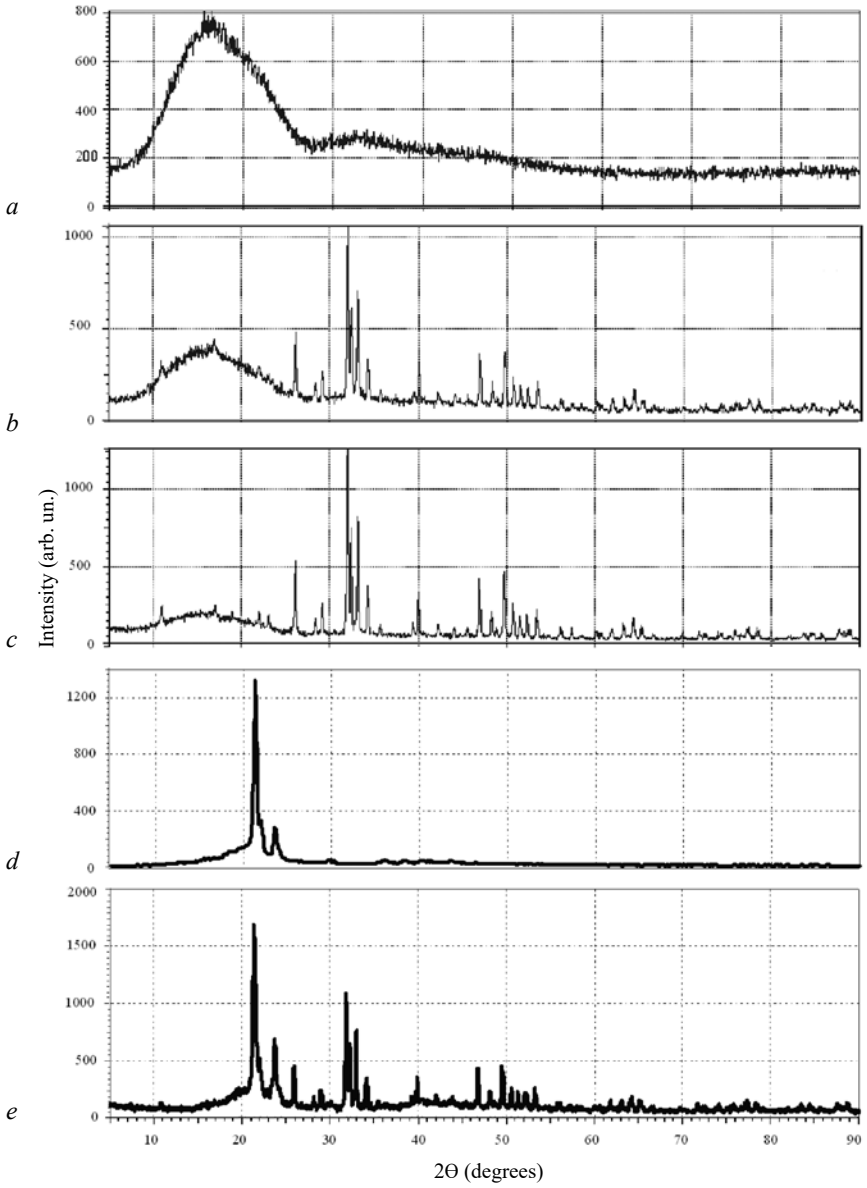


Fig. 4. XRD patterns (*a*) initial PLA; *b* – PLA-HA composite with 25 wt% HA; *c* – PLA-HA composite with 50 wt% HA; *d* – initial PCL; and *e* – PCL-HA composite with 25 wt% HA

Table 1

The degree of crystallinity of polymer matrices and composites, $M \pm m$

Material	PLA	PLA + 25 wt% HA	PLA + 50 wt% HA	PCL	PCL + 25 wt% HA
The degree of crystallinity, %	11.6 ± 0.35	37.9 ± 1.14	71.45 ± 2.14	34.7 ± 1.04	75.53 ± 2.27

The crystallinity degree of PCL + 25 wt% HA composite is increased by 2 times compared to the initial PCL. When polymer matrix is filled with HA powder, the filler particles act as an additional nucleating agent for the polymer matrix resulting in the increase of the crystallinity degree of polymer composites.

Fig. 5 demonstrates the results of tensile tests. It is seen that the addition of HA to the PLA results in a decrease in elongation and tensile strength, and only for the Young's modulus there is a tendency to increase with increasing HA content.

For PCL-based composites the changes in the studied mechanical properties are similar, with the only difference being that the values of the Young's modulus and tensile strength are significantly lower than those obtained for PLA-based composites, while the elongation for PLA-based composites is negligible compared to with PCL-based composites.

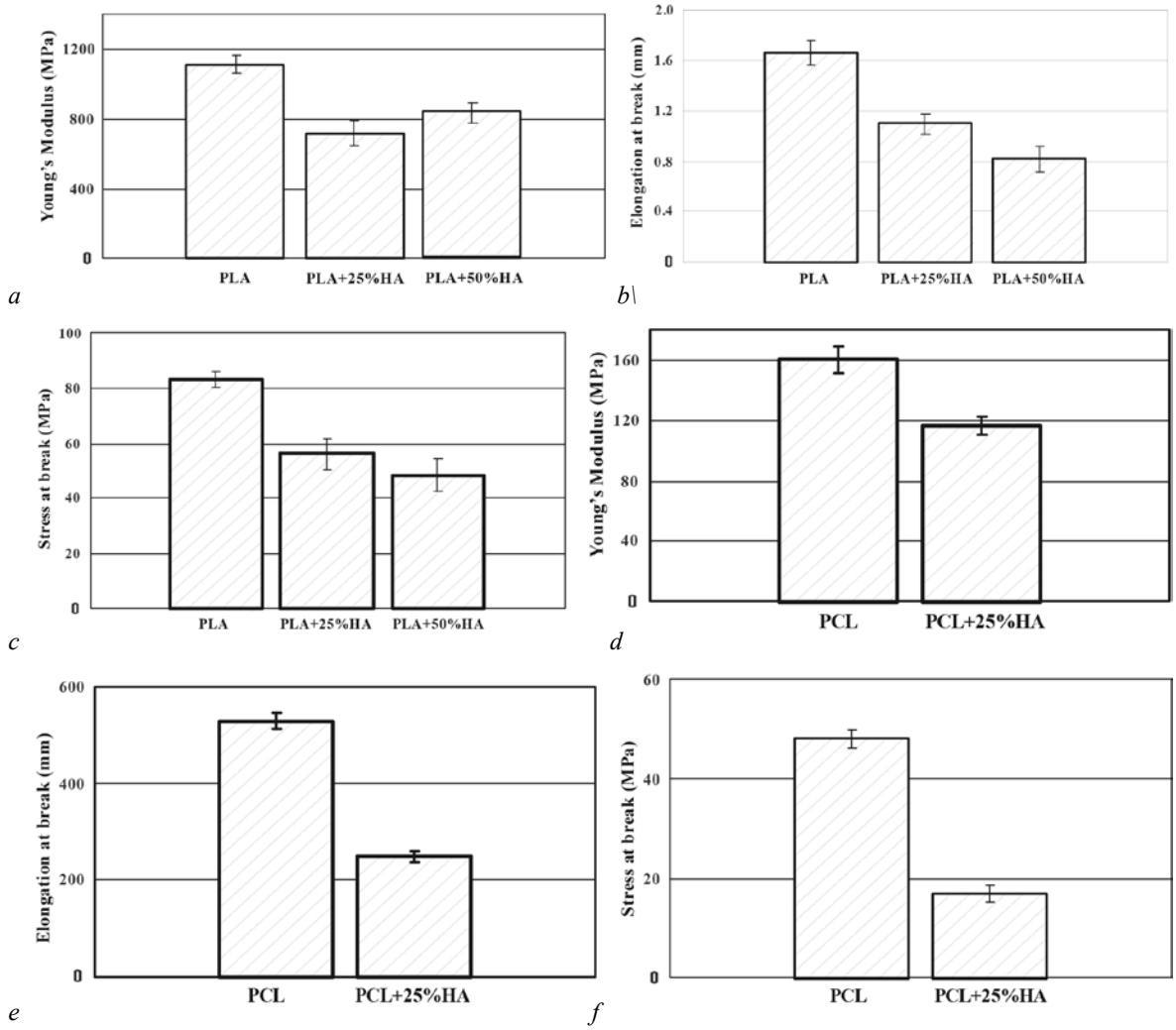


Fig. 5. The results of mechanical tests for PLA-based (*a*, *b*, *c*) and PCL-based (*d*, *e*, *f*) composites: *a*, and *d* – Young's modulus; *b* – and *e* – elongation at break; *c*, and *f* – tensile strength

Currently, biodegradable polymers and their composites are widely used in tissue bioengineering. PLA implants are one of the most popular products in clinical practice. PLA products are resorbable over time and are replaced by bone tissue, according to various authors, not earlier than 7 years after implantation [42]. It can lead to complications in the form of an inflammatory reaction to a foreign body [43]. In this regard, a development and biomedical testing of new technologies for the manufacture and modification of composites of (co)polymers and HA with controlled biodegradation are very relevant. However, at high HA content, the potential enhancement of the osteogenic properties of composites [44] is offset by a real decrease in their physicomechanical properties (Fig. 5).

During subcutaneous implantation, test materials experience a biomechanical load (shock, compression, and lateral displacement) resulting in simulated *in vivo* conditions to bone implantation. While PLA composites with a low HA content (5 wt%) were subcutaneously tested, after 40 days there were no macroscopic signs of implant destruction and inflammatory reaction (in particular, severe hyperemia of the recipient bed, and the presence of exudate). The disks were surrounded by a thin (less than 50 μm) connective tissue capsule, fixing them in the lateral subcutaneous pocket.

PLA and its composites promoted the conduction of grown tissues on the surface of the test samples. It was recorded by more than 3-fold increase in the area of tissue plates (Table 2). Histological evaluation of tissue lamellae showed induction of bone growth (IBT) as follows: 20% in the case of pure PLA samples; 60% in the case of PLA + 5 wt% HA composite scaffolds (Table 2).

Table 2

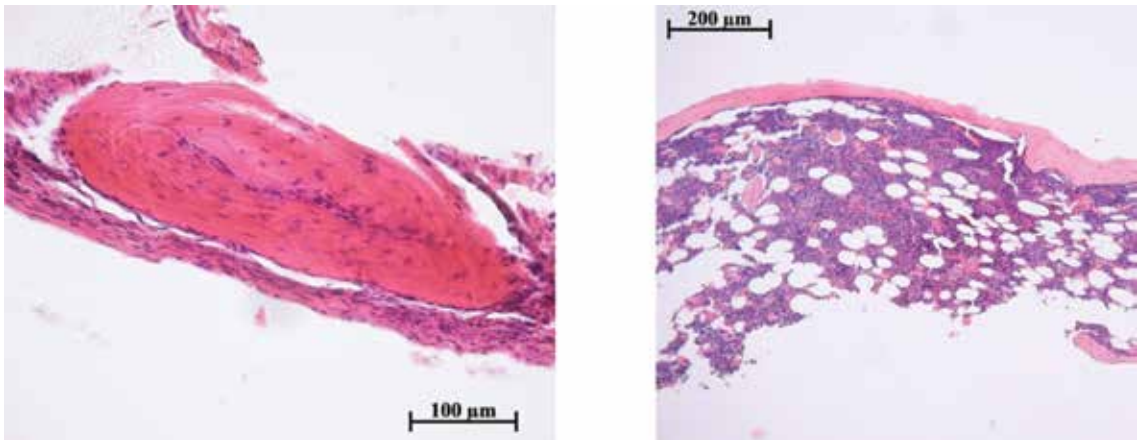
Sample effect on geometric and histological features of tissues grown from bone marrow under subcutaneous ectopic test in BALB/c mice, *Me* (Q_1–Q_3)

Test groups of samples, $n = 6$	Incidence of tissue lamella formation	IBT	Bone marrow (initial level before implantation)	Properties of tissue lamellae (after implantation)	
	%	%	Area, mm²	Area, % of bone marrow area	Histological compound
PLA	100	20	3.54 (3.49–3.72)	306 (217–504)	Bone with marrow (1 case); connective and fat tissues (4 cases)
PLA + 5 wt% HA	100	60	4.53 (3.89–4.87)	382 (327–443)	Membrane reticulated bone (2 cases); bone with marrow (1 case); connective tissue (2 cases)

Note: n – the number of test samples in each group; PLA – polylactide; PLA+5 wt% HA –hydroxyapatite (5 wt%) composite with polylactide.

Membrane reticulated bone, as well as the bone/bone marrow system formed *de novo* due to *in situ* remodeling of bone marrow applied *in vitro* were revealed on histological sections of tissue lamellae (Fig. 6).

Ectopic metaplasia of syngeneic bone marrow proceeds through the activation of a pool of donor MSCs differentiating into the precursors of chondro/osteoblasts. In turn, recipient hematopoietic stem cells repopulate the implantation site [45].



a *b*\

Fig. 6. The positive result examples of histological slices of tissue lamellae grown on PLA and PLA + 5 wt% HA samples after 40-day subcutaneous implantation: *a* – bone tissue, ×200; *b*) bone with marrow, ×100 hematoxylin-eosin staining

CONCLUSION

Biodegradable polymer PLA- and PCL-based composites filled with HA prepared by melt compounding were obtained, which allows introducing up to 25–50 wt% HA into polymer matrices. HA particles and their agglomerates with a diameter of up to 100 µm are uniformly distributed over the matrix volume, increasing the degree of surface polarization and crystallinity of composites. As a consequence of high crystallinity, the mechanical strength of the composites decreases with filling. Developed biodegradable composites can be used for practical application in various areas of regenerative medicine. In the field of bone tissue bioengineering, PLA composites obtained by 3D printing, even with a low (5 wt%) HA content, increase ectopic osteogenesis by 40%. It allows considering MSCs as a cellular target of their biological activity.

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