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MATERIALS FOR ENSURING HUMAN LIFE ACTIVITY AND ENVIRONMENT PROTECTION

Selective Laser Sintering of Bioactive Composite Matrices for Bone Tissue Engineering

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Abstract—A method for surface-selective laser sintering which produces mineral-polymeric materials based on calcium phosphates and aliphatic polyesters was developed. Three-dimensional matrices of the given architectonics for replacement of bone defects and bone tissue engineering were obtained. The microstructure of the experimental samples obtained and their surface morphology and internal structure were investigated by scanning electron microscopy (SEM). The characteristic values of the compressive strength and relative deformation of the mineral–polymer composite samples obtained by surface-selective laser sintering of fine powders consisting of 80 wt % ceramic granules based on tricalcium phosphate and 20 wt % D,L-polylactide PDL04 corresponded to the characteristic indices of the similar parameters for the trabecular bone tissue. As a result of the initial study of the biological properties of mineral–polymer composite scaffolds made by surface-selective laser sintering, it was shown that they had low cytotoxicity and no adverse effects on the proliferative potential of mesenchymal stem cells. The technology of surface-selective laser sintering suggested could be effectively used to create scaffolds for bone tissue engineering.

Keywords: selective laser sintering, scaffold, aliphatic polyesters, tricalcium phosphate, bone tissue engineering, cytotoxicity

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INTRODUCTION

One of the most urgent and promising fields of modern reconstructive and restorative surgery is development and creation of individual grafts and tissue-engineered constructs (TEC) providing optimal conditions for replacement of defects and regeneration of solid tissues [1]. Key elements of these biomedical products are three-dimensional synthetic and, as a rule, bioresorbable matrices with given architectonics [2]. The matrix should have certain chemical composition and surface morphology providing effective invasion of osteogenic cells with further formation of native tissues in the implantation region. It should be also resorbed in vivo with the time correlating with the rate of formation of the new bone tissue [3]. Nowadays, to create such matrices different mineral-polymer composites based on calcium phosphates are used having high osteoconductive potential and regulated biodegradation rate [4], and aliphatic polyesters (polylactides, polylactoglycolides, poly-ε-caprolactones, etc.) widely applied in different biomedical studies and clinical practice are used as polymers [5]. Here, the most promising approach known to date for making individual grafts and scaffolds for TECs with the given architectonics and desired physical, chemical, and biochemical characteristics is rapid prototyping (RP) technology (or additive manufacturing (AM) technology) [6, 7]. They are based on program partitioning of a three-dimensional model of an object into cross sections (layers) and further layerwise manufacturing of this product from the material chosen. An advantage of the RP technology became particularly obvious upon their use in combination with up-to-date techniques of medical diagnostics (computed and nuclear magnetic resonance (NMR) tomography). Thus, on the basis of a three-dimensional model of a tissue fragment of a real patient obtained, for example, on an X-ray tomograph, a personalized graft could be rapidly made from biocompatible material. Upon manufacturing of the product, it could be additionally introduced with certain elements or certain elements could be removed and desired architectonics, including that with gradient porosity, could be created.

One of the most developed and successfully applied RP technologies for manufacturing of biomedical products to date is selective laser sintering (SLS) [8, 9]. SLS is

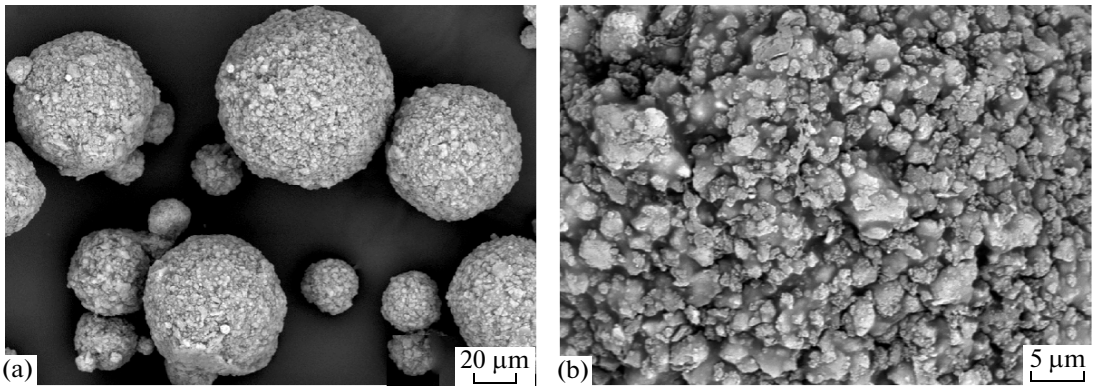

Fig. 1. SEM image of particles of powders of the first type (a) and microstructure of their surface (b).

based on layerwise sintering of fine (50–100 μm) powder materials (metals, ceramics, polymers, and composites on their basis). SLS makes it possible to obtain individual implants and matrices for tissue-engineered constructs using computer-aided design/production (CAD/CAP) systems with minimal time expenditures, high reproducibility, accurate geometry, and given structure. An advantage of the method is the practical absence of restrictions for a mix of powder materials used. At the same time, initially, almost all works in this field were as a rule oriented toward the use of the most economical and cheapest CO_2 continuous wave lasers, their infrared (at a wavelength λ = 10.6 μm) radiation being absorbed by polymer particles. Such a mode of heating and sintering could not be optimal for thermolabile bioresorbable polymers (in particular, polylactides and polylactoglycolides), since they could be extremely overheated, resulting in degradation and formation of crosslinks and toxic components.

Thus, despite a great number of scientific publications and patents on this topic (more than a hundred only in the last five years, mainly published outside Russia), the problem of choosing the methods and parameters of manufacturing osteoconductive materials and osteoconductive scaffolds based on calcium phosphates and aliphatic polyesters has not yet been completely solved.

In [10, 11], surface selective laser sintering (SSLS) was suggested and developed, where, in contrast to conventional selective laser sintering (SLS) based on volume absorption by a polymer powder of radiation of a CO_2 laser, the controlled melting of its particles occurred owing to absorption of laser radiation of the near IR range (λ = 0.97 μm) by a small number (≤0.1 wt %) of carbon or gold nanoparticles uniformly distributed on the polymer surface. SSLS makes it possible to avoid overheating of internal regions of polymer particles owing to delicate melting of only their surface. This method was successfully applied by us for manufacturing of individual scaffolds from thermally unstable bioresorbable polymers (such as polylactides and their copolymers) without noticeable structural and chemical alterations.

This work was aimed at the development of a method of formation of mineral-polymer scaffolds for bone TECs with a gradient distribution of porosity and phase composition from ceramic materials based on calcium phosphates, which provides reliable binding of finely divided mineral particles owing to SSLS of polyester particles located between them, and at the study of structural, morphological, physical, and chemical characteristics of the samples obtained and their cytotoxicity in vitro.

MATERIALS AND METHODS

The initial components for the experiments were represented by two types of finely divided materials (average particle size was from 50 to 200 μm) based on tricalcium phosphate (TCP) and denatured collagen (DC). The first type of materials (Fig. 1) represented microparticles of a spherical shape based on the TCP and DC powder obtained by a technology using immiscible liquids [12]. The second type of materials (Fig. 2) was ceramic granules based on TCP preliminarily sintered at the temperature of 1300°C. To implement surface-selective laser sintering of spherical particles of both types, i.e., their structural integration under laser radiation of moderate (up to 10 W) power, bioresorbable amorphous D,L-polylactide (PLA) particles PURASORB PDL04 (Purac, Netherlands) with intrinsic viscosity of 0.4 dL/g and glass transition temperature of 56°C were used.

There were two variants of using PDL04 particles in our experiments. In the first variant, a thin film of aliphatic polyester (thickness ~2–5 μm) was deposited on TCP microparticles from its solution in dichloromethane with further drying and dispersion in an air stream (Fig. 3). It can be seen that, after deposition of the polymer coating, the shape of the TCP particles was preserved. However, their surface became smoother and the pores closed.

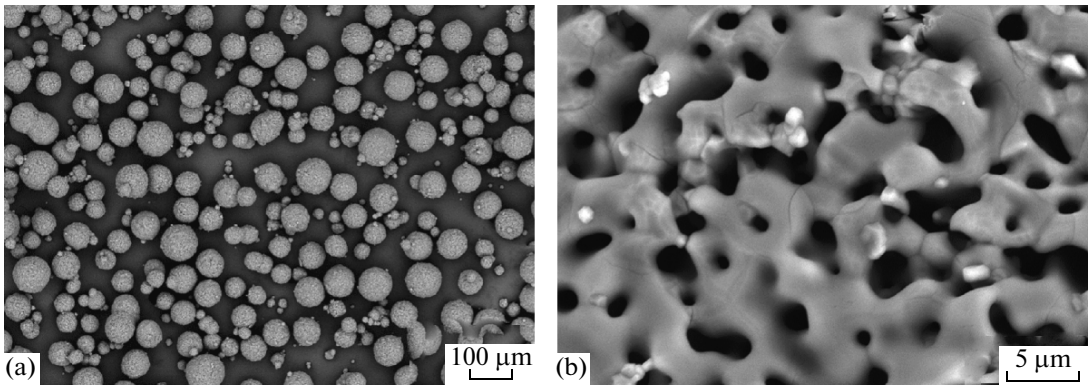

Fig. 2. SEM image of TCP microparticles of the second type.

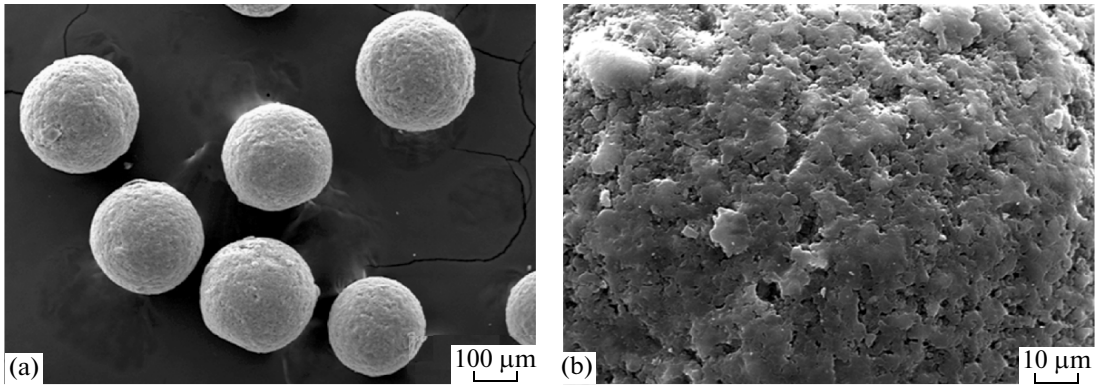

Fig. 3. SEM image of a microparticle of powders of the first type with polymer coating (a) and its surface (b).

In the second variant, TCP particles were mixed with a preliminarily milled finely divided (particle size ~100 μm) powder of amorphous PDL04. For this purpose, initial granules of the polymer (typical size of 2–3 mm) were ground in a rotary mill. The powder milled was sieved through a series of sieves, and the fraction with particle sizes less than 100 μm was chosen. Then, the powders of TCP and polylactide milled were thoroughly mixed at different weight proportions (from 20 : 80 to 80 : 20).

The surface morphology and internal structure of initial TCP powders, polylactide, and samples of three-dimensional scaffolds obtained were studied using scanning electron microscopy (SEM) using a Tescan VEGA II microscope (Tescan, Czech Republic). Layerwise sintering of particles of powder mineral-polymer mixtures not absorbing and weakly absorbing (TCP) laser radiation by SSLS occurred owing to absorption of the radiation by nanoparticles of a sensitizer added to the powder sintered in small amounts. The sensitizer consisted of carbon nanoparticles with a size of ~100 nm in the amount of 0.1 wt %. Heating of the surface of the powder particles in this case occurred upon absorption of the energy of the laser radiation by carbon nanoparticles located on their surface. In this case, the absorption coefficient hardly depended on the wavelength of the laser radiation (carbon was considered as a blackbody) and different most energy efficient and stable optical fiber lasers with near infrared diode pumping (emission wavelength of ~1 μm) could be used.

The experiments on sintering of three-dimensional matrix structures from materials based on TCP of the types described above were performed on an SLS-100 setup for selective laser sintering developed and produced at the Institute on Laser and Information Technologies of the Russian Academy of Sciences [13]. It includes a specially developed single-mode fiber continuous wave diode-pumped laser with an emission wavelength $\lambda = 1.06$ μm and power up to 10 W (NTO, IRE-Polyus, Fryazino), a system of focusing and scanning of laser radiation (making it possible to obtain on the surface of the powder being sintered a focal spot with a diameter of ~80 μm), a working sintering chamber, a system of powder delivery and formation of its layers, a thermostating system, and special program software to control all the elements of the setup. The maximum dimensions of voluminous objects manufactured could be $100 \times 100 \times 50$ mm^3 with reproduction accuracy of about ±100 μm. The

architectonics of the scaffolds formed, their structure, and porosity (shape and size of the cells) were specified by the computer model. The optimum composition of the powder blends, thickness of the layers sintered, and intensity and scanning rate of the laser irradiation were chosen experimentally.

Mechanical tests of the samples were performed using an Instron 5581 universal test machine for uniaxial experiments within a load range up to 5 t with an electric actuator and follower arrangement (Great Britain). For this purpose, the surfaces of butt ends of cylindrical samples were preliminarily cleaned in a special template of textolite to make them parallel. The polishing was performed using diamond abrasive tools. The cylindrical samples were subjected to uniaxial compression (along the cylinder axis) at a loading rate of 100 N/s. The height of the samples was determined in every case by the follower arrangement according to the appearance of the load at the moment of the contact between the cylinder and working surface of the setup. The effect of the rate of conducting the experiment on mechanical characteristics was tested in the experiments, where the loading rate was 5 and 20 N/s. To assess the reproducibility of the values of the strength, from three to six samples were tested. The load curves of the samples converted into stress–strain coordinates were recorded in a digital form using a computer.

Cultures of mesenchymal stem cells (MSC) of the pulp of a baby tooth were taken from the cryobank of the Orekhovich Institute of Biomedical Chemistry. After thawing, the cells were put into culture flasks with a bottom area of 75 cm^2 and cultivated in a growth medium (DMEM/F12 (1 : 1) with addition of 10% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin sulfate, and 2 mM L-glutamine (all the reagents listed were from Gibco, USA)) in a CO_2 incubator under standard conditions (37°C, 5% CO_2, 80% humidity), changing the medium twice a week. At confluence of 80–90%, the cells were taken from the plastic surface by incubation in 2 mL of a mixture of 0.25% tripsin and Versen solutions at a ratio of 1 : 1 (PanEko, Russia) at 37°C for 5–10 min. The cells were suspended and precipitated by centrifugation; the sediment obtained was resuspended in the culture medium by seeding at a ratio of 1 : 3.

To assess biocompatibility of the materials developed, porous scaffolds were produced in the form of disks with a diameter of 12 mm and thickness of 2 mm. Before invasion, the scaffolds were sterilized by immersing them in a 70% C_2H_5OH solution with further preincubation for 24 h in the growth medium. The cells were suspended as described above and then precipitated by centrifugation. However, the sediment was resuspended in the culture medium at concentration of ~250000 cells/mL, and then aliquots of 2 mL were deposited on the scaffolds located in wells of a 24-well plate. To provide efficient cell adhesion, the plate with the scaffolds and cells deposited on it were put into a CO_2 incubator and incubated for 12 h. After that, the TECs obtained were transferred into clean wells of a plate containing 3 mL of the medium and cultivated under standard conditions for 5 days. The number of living cells in the composition of the TEC was assessed by staining the nuclei with a solution of 4,6-diamidino-2-phenylindole (DAPI; Sigma, United States) and using CytoTox 96 Cytotoxicity Assay (Promega, United States). The TECs were taken from the plate and washed in phosphate buffer saline (PBS). A part of the TECs was fixed in a 4% paraformaldehyde solution for 4 min at room temperature and then stained with DAPI at concentration of 1 μg/mL for 10 min. In the end, the samples were embedded in a medium for fluorescence protection (DakoCytomation, United States) and studied using fluorescence microscopy on a ZEISS AxioPlan 2 microscope (Carl Zeiss, Germany). Another part of the TECs was placed into a 24-well plate with the wells containing 300 μL of PBS, and then the cell membranes were lysed by three cycles of freezing down to −70°C with further thawing. Aliquots containing the lysate with a volume of 50 μL were transferred into a 96-well plate, and the study of the lactate dehydrogenase activity was performed using a CytoTox 96 Cytotoxicity Assay kit according to the manufacturer's protocol. The optical density of the content of the wells was measured using an Infinite 200 PRO microplate reader (Tecan, Germany).

RESULTS AND DISCUSSION

To determine the sintering threshold of particles of the materials studied and choose optimal parameters of SSLS, first, a test scanning of their surface was performed by a laser beam, varying the intensity of the laser radiation (within a range from 1×10^3 to 4×10^4 W/cm^2) and scanning rate (within a range from 1 to 20 mm/s).

None of the modes of sintering of the powders based on TCP with a polylactide coating made it possible to obtain stably integrated (sintered) structures. The melted tracks (filaments) scattered into individual agglomerates of the initial particles. To carry out reliable sintering of this type of particles, in our opinion, it is necessary to increase the thickness of the polymer film on their surface by about an order of magnitude, which would be comparable with the size of the TCP core. The technology for production of such composite particles is fairly complex and quite expensive.

A stable mode of SSLS was found in the other (second) variant upon addition of polymer particles in the amount of 30 wt % to the initial ceramic TCP granules. The most critical parameter for obtaining solid homogeneous structures was the scanning rate of the laser beam. An increase in the scanning rate resulted in a decrease in the width of the tracks (improvement of space resolution). In this case, the ratio of the height of the track to the width increased. The track became more compact, the relative melting depth increasing

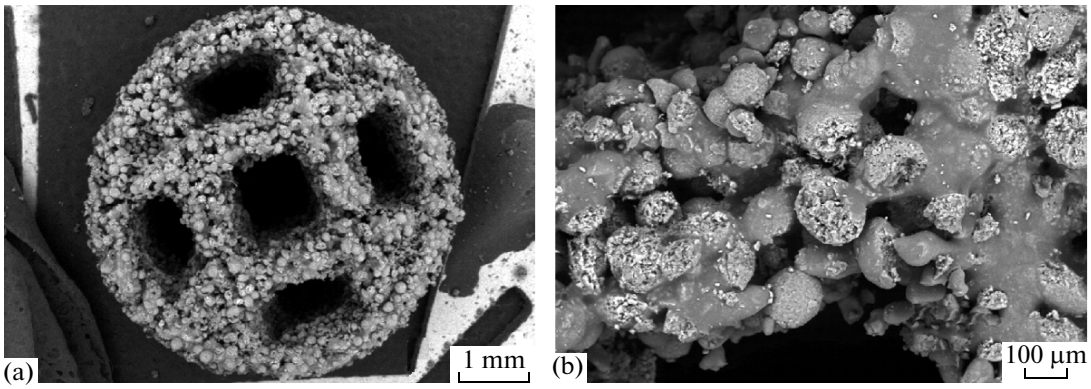

Fig. 4. SEM image of mineral-polymer composite matrices based on TCP and PDL04: (a) top view, (b) surface microstructure.

and integration of the subsequent layer with the previous one improving. Thus, optimal sintering modes for achievement of the best geometrical and mechanical characteristics of the structures were implemented at the greatest scanning rates of the radiation beam. The operating scanning rate chosen by us was in the end 20 mm/s.

The thickness of the powder layer deposited on the working surface was determined by the melting depth of the powder at the given parameters of the sintering. According to the study of the thickness of individual tracks obtained as a result of the scanning of the powder layer with the thickness significantly exceeding the melting depth, the thicknesses providing robust integration of the layers sintered and not impeding at the same time free deposition of the next layer of the powder with particle sizes of about 200 μm were determined.

On the basis of the results of the experiments performed, SSLS of three-dimensional scaffold structures from mixtures of tricalcium phosphates and aliphatic polyesters for their further studies was performed upon meeting the following conditions: (1) the sintered powder consisted of 30–80 wt % of initial TCP and 20–70 wt % of the polymer; (2) the polymer thickness was ≈250 μm; (3) the intensity of the laser radiation at a scanning rate of the surface of the powder being sintered of 20 mm/s was 1.5×10^4 W/cm^2.

These conditions having been satisfied, mineral-polymer materials and matrix structures based on calcium phosphates with a gradient distribution of porosity having a total volume of freely interconnecting pores up to 80% and a characteristic size from several microns to 1.5 mm were obtained using SSLS. At the same time, the size of the pores and their total number could programmatically decrease from the internal regions of the scaffold to its surface. Figure 4 demonstrates the results of SEM analysis of the samples obtained by SSLS.

For comparative studies of mechanical properties of the mineral-polymer matrices obtained by SSLS using the optimal modes (described earlier), three series of cylindrical samples with a diameter of 5 mm and height of 10 mm were manufactured, each of them containing 30 samples consisting of 70 : 30, 75 : 25, and 80 : 20 wt % of ceramic granules based on TCP and D,L-polylactide PDL04, respectively.

Figure 5 shows compressive deformation curves of the samples. The samples of all three series demonstrated fairly high values of compressive strength (up to 4 MPa) at relative deformation up to 9%. The data obtained correspond to characteristic values of the similar parameters for trabecular (spongy) bone tissue, which gives grounds for considering these materials as very promising for creation on their basis of mineral-polymer composite implants and matrices for TECs.

A model cell culture for primary estimation of biological properties of the matrices was represented by MSC of the pulp of a human baby tooth. The scaffolds manufactured according to SSLS were invaded by the cells, and, as a result, experimental TEC samples were made, which were then cultivated for 5 days. Comparison of the number of living cells in the TEC composition at the beginning and at the end of the study was

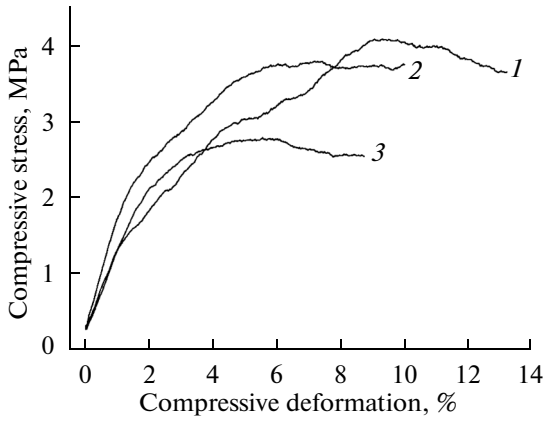

Fig. 5. Strength properties of mineral-polymer composites obtained by SSLS: (*1*) TCP : PL = 70 : 30, (*2*) TCP : PL = 75 : 25, (*3*) TCP : PL = 80 : 20.

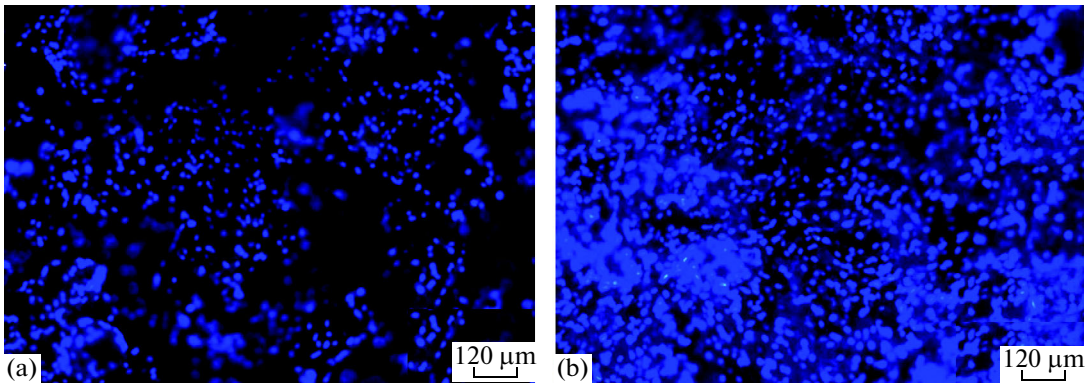

Fig. 6. MSC of the pulp of a human baby tooth cultivated on mineral-polymer matrices obtained by SSLS: (a) 1 day, (b) 5 days. DAPI nuclear staining.

performed in two ways—by direct visual observation and measuring lactate dehydrogenase activity.

Since the scaffolds made according to SSLS were not transparent, to visualize the cells in the composition of the TEC, we stained them with DAPI nuclear fluorescent dye and made photographs of the surface of the scaffolds using a fluorescence microscope. As can be seen from the microphotographs obtained (Fig. 6), the number of the cells significantly increased 5 days after the cultivation.

To prove the results obtained, a study of the activity of lactate dehydrogenase was performed in the cell lysate obtained after three cycles of freezing–thawing of the TEC. Lactate dehydrogenase is a stable cytosolic enzyme, the content of which can be quantitatively measured by colorimetric methods such as a CytoTox 96 Cytotoxicity Assay. The optical density of the solutions obtained as a result of the study was directly proportional to the number of living cells in the TEC samples (Fig. 7). It was shown that the number of cells in the composition of the TEC significantly increased during 5 days of cultivation.

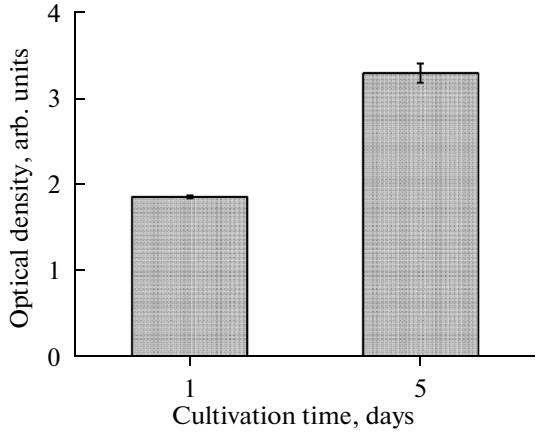

Fig. 7. Change in the number of living cells in the composition of TEC during cultivation.

As already mentioned in the Introduction, upon making synthetic materials for replacement and regeneration of the bone tissue for them to be able to function in a living organism as a natural tissue, it is necessary to make their chemical and phase compositions and structure and morphology of the surface maximally close to the content and structure of the bone tissue. Nowadays, the necessity of the presence of different forms of calcium phosphates (hydroxyapatite (HAP), TCP, etc.) in the composition of tissue engineered constructs is generally accepted. Calcium phosphates as a mineral component of native bone tissue could simultaneously provide the desired characteristics of the TEC framework structure and increase its osteoinductive and osteoconductive potentials. Besides the composition, the internal architectonics of synthetic scaffolds, in particular, their structure and volume distribution of the pores, significantly affects the efficiency of formation and remodeling of the bone tissue [14]. Here, all the components of the scaffold material should be biocompatible with the organism. It should be osteoconductive and ideally osteoinductive (owing to the presence in it of biologically active components enhancing the restorative capacity of the system implanted such as morphogenetic proteins). The scaffold should be biodegradable with time correlating with the kinetics of osteosynthesis and have gradient content and architectonics optimal for stimulation of neovascularization and vital activity of osteogenic cells. The chemical properties of the scaffold materials, its architectonics, and the surface topography should not exclude the possibility of its use as a depot for drugs with controlled release kinetics.

At present, the main bone replacement material in clinical practice is often HAP [3]. It has high biological compatibility owing to its chemical and structural similarity to the mineral phase of natural bone tissue. HAP increases growth, proliferation, and differentiation of osteoblasts [15]. However, the chemical, mechanical, and biological properties of polycrystalline HAP do not satisfy all requirements for modern

biomaterials for engineering of bone tissue. Its wide application is limited at present by a number of factors such as brittleness, complexity in the formation of products of the desired shape, and extremely low rate of bioresorption [16]. Moreover, it was found that application of voluminous (more than several cubic centimeters) blocks of HAP lead to unacceptably high probability of implant rejection in clinical application [14].

It is also of importance to take into account that HAP differently affects the formation and growth of cells of different types. Thus, in [17], the biocompatibility of HAP of different dispersity (from 0.5 to 800 μm) with fibroblasts and myoblasts was studied. It was shown that individual HAP microparticles suppressed the growth of these cells, this process being the most active for particles with the smallest sizes. At the same time, application of nanoscale HAP (nano-HAP) increases the area of its surface, when compared to microscale HAP, and correspondingly increases the rate of its bioresorption. This, in turn, increases its bioactivity and improves osteointegration of the implant with the surrounding tissues [18]. Nanostructured calcium phosphates have specific biological properties: high protein adsorption; increased function of stimulation of osteoclasts and osteoblasts, the cells involved in the remodeling of bone tissue; and decreased function of proliferation of competing cells, in particular, fibroblasts responsible for formation of connective tissue [19]. These properties are believed to be connected with a high energy state and surface morphology of nanocrystalline ceramics. Thus, the study of the effect of nano-HAP on cell growth and formation of tissues, which has begun recently, is extremely urgent and is one of the most actively developed fields of modern tissue engineering.

Thus, it is clear that the use of HAP as a finely divided bioactive filler of different polymer scaffolds makes it possible to avoid almost all insufficiencies of voluminous HAP constructs mentioned above, thus significantly improving osteointegration properties [20].

Among the different forms of calcium phosphates having a controlled biodegradation rate, the greatest attention is paid nowadays to composite biphasic ceramics based on HAP–TCP. An important advantage of these biphasic composite materials is the possibility of precision regulation of the kinetics of their biodegradation by simple measurement of the ratio HAP : TCP having different rates of bioresorption of soluble phases in one material. More rapid dissolution of TCP in the organism promotes mineralization in the implantation region, and a more stable phase of HAP provides the required mechanical characteristics of the scaffold during the time necessary for the formation of the native bone intercellular matrix and a further cycle of remodeling of the bone tissue de novo.

The approach developed in this study makes it possible to satisfy all the requirements on composite ceramic materials with gradient structure mentioned above, which, in turn, could provide creation of high-quality implant systems and biomedical products for effective replacement of defects and directed regeneration of bone tissues.

CONCLUSIONS

The SSLS method developed makes it possible to obtain mineral-polymer materials based on calcium phosphates and aliphatic polyesters and to manufacture on their basis three-dimensional scaffolds with the given architectonics for replacement of bone defects and directed regeneration of bone tissue.

The microstructure of the experimental samples obtained and their surface morphology and internal structure were investigated by scanning electron microscopy (SEM).

It has been shown that effective binding of finely divided particles of calcium phosphates could be implemented owing to softening or melting of the surface of the polymer particles between them. To implement such a mode of SSLS of spherical particles of TCP, continuous wave laser radiation of moderate (up to 10 W) power is sufficient.

The architectonics of the scaffolds formed and their structure and porosity (shape and size of the cells) is specified by the computer model used. In preparing the process of formation of ceramic composite materials and voluminous structures based on calcium phosphates with gradient distribution of porosity, optimal powder mixtures, thicknesses of the layers sintered, and intensity and rate of scanning laser radiation could be varied and chosen experimentally.

The stable mode of SSLS could be achieved upon addition to the initial TCP powders of polylactide particles at a ratio from 20 to 30 wt %. Here, the most critical parameter for obtaining solid homogeneous structures is the scanning rate of the laser beam. The characteristic values of the compressive strength and relative deformation of the mineral-polymer composite samples obtained by SSLS from finely divided powders consisting of 80 wt % ceramic granules based on TCP and 20 wt % D,L-polylactide PDL04 corresponded to the characteristic values of the similar parameters of trabecular bone tissue.

As a result of the primary study of biological properties of mineral-polymer composite scaffolds manufactured by SSLS, it has been shown that they have low cytotoxicity and have no adverse effects on the proliferative potential of MSC upon cultivation in the composition of the TEC. This indicates that the SSLS technology proposed by us could be successfully used to make cellular carriers for tissue engineering.

All this makes it possible to consider the materials obtained as very promising for creating on their basis using SSLS individual mineral-polymer composite implants and scaffolds for bone TECs capable of providing acceleration of reparative processes and effective osteointegration.

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