ISSN 0096-3925, Moscow University Biological Sciences Bulletin, 2017, Vol. 72, No. 4, pp. 190–195. © Allerton Press, Inc., 2017.
Original Russian Text © M.S. Kotliarova, A.Yu. Arkhipova, A.M. Moysenovich, D.A. Kulikov, A.V. Kulikov, A.S. Kon'kov, M.A. Bobrov, I.I. Agapov, M.M. Moisenovich, A.V. Molochkov, A.V. Goncharenko, K.V. Shaitan, 2017, published in Vestnik Moskovskogo Universiteta, Seriya 16: Biologiya, 2017, Vol. 72, No. 4, pp. 222–228.

METHODS

Bioresorbable Scaffolds Based on Fibroin for Bone Tissue Regeneration

M. S. Kotliarova[a, *], A. Yu. Arkhipova[b], A. M. Moysenovich[a], D. A. Kulikov[c], A. V. Kulikov[d], A. S. Kon'kov[a], M. A. Bobrov[c], I. I. Agapov[e], M. M. Moisenovich[c], A. V. Molochkov[c], A. V. Goncharenko[b], and K. V. Shaitan[a]

[a] *Chair of Bioengineering, Department of Biology, Moscow State University, Moscow, 119234 Russia*

[b] *Laboratory of Confocal Microscopy, Department of Biology, Moscow State University, Moscow, 119234 Russia*

[c] *Vladimirsky Moscow Regional Research and Clinical Institute (MONIKI), Moscow, 129110 Russia*

[d] *Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia*

[e] *Shumakov Federal Research Center of Transplantology and Artificial Organs, Ministry of Health of the Russian Federation, Moscow, 113182 Russia*

***** *e-mail: kotlyarova.ms@gmail.com*

Received July 7, 2017; in final form, September 9, 2017

Abstract—Using the tissue-engineered constructs based on scaffolds that imitate the extracellular matrix of living tissues unveils new opportunities in the treatment of various pathologies and injuries associated with tissue and organ damage. Silk fibroin of silkworm *Bombyx mori* is a biocompatible and bioresorbable polymer with high mechanical strength and elasticity that allows creating scaffolds on its basis for regeneration of various tissues, including bone. In the present work, fibroin scaffolds were obtained. They were designed in the form of porous sponges, films, and hybrid scaffolds of a bilayer structure in which the porous sponge three-dimensional structure is limited on one side by a film. The structure of the scaffolds and their biocompatibility were studied: immortalized and primary fibroblasts, as well as the osteoblast-like cells, have been shown to successfully adhere and proliferate on the surface of the studied scaffolds. Numerous osteogenesis foci have been observed in the implant region 4 weeks after the fibroin porous scaffold implantation in the in vivo experiments in a rat femoral bone defect model indicating the osteoconduction of the scaffolds.

Keywords: regeneration, bone substitute, bone defect model, fibroin, scaffold, tissue engineering,

DOI: 10.3103/S0096392517040095

INTRODUCTION

Bone tissue is characterized by a unique capacity to restore its original structure in the course of injury regeneration. However, in case of extensive damage, elderly age, and in some pathological conditions, the normal structure of the bone cannot be restored. In these cases, bone grafts are used to activate the process of bone regeneration.

The ability of bone grafts to stimulate the tissue regeneration is featured by osteogenic, osteoconductive, and osteoinductive potentials [1]. The bone implants must ensure the structural integrity of the bone and be able to allow the osteoconduction, that is, to contribute to the bone integrity restoration by the ingrowth of tissue into the material [2]. The implants having osteogenic potential contain osteoblasts or their progenitors to restore the bone structure [3]. The osteoinductive properties the grafts may have, lead to the migration of the recipient mesenchymal stem cells into the damaged area and their succeeding differentiation into osteoblasts initiates the regeneration process [4].

Autografts and allografts are the most common types of bone implants used in transplantation of bone tissue. The former means tissue from the same body, the latter from a donor, respectively. Allografts are characterized by osteoconductive and, in some cases, osteoinductive properties; autografts also have the osteogenic potential [2]. However, the use of such implants is limited bby a number of complications. For allografts there is a risk of an induced infection and the potential rejection. For autografts the lack of material may be the problem [5]. The ability of bone grafts to stimulate the tissue regeneration is featured by osteogenic, osteoconductive, and osteoinductive potentials.

One solution to this problem is using the tissue-engineered scaffolds. They support the integrity of the tissue or organ and provide a substrate for adhesion, migration, and proliferation of cells involved in the regeneration process [6]. The scaffold material should

be bioresorbable, allowing the scaffold to dissolve in the process of a new tissue being formed. Silk fibroin can be used as a material for these structures. The use of fibroin as a basis for the creation of bone implants is highly promising, since this material is one of the mechanically strongest natural polymers and also has high biocompatibility, stability, and controllable bioresorption [7].

In the present work, we investigated the properties of various fibroin scaffolds: flat scaffolds in the form of a film (FS), porous sponge scaffolds having a complex three-dimensional structure (PSS), and hybrid bilayer scaffolds (HS) having a porous structure formed on a film. In the study, we examine certain aspects of applying these scaffolds for bone tissue regeneration.

MATERIALS AND METHODS

Scaffold Formation

PSS and FS were obtained from the aqueous fibroin according to the method described previously [8].

HS were obtained by forming the porous scaffold on the FS surface.

To introduce the fluorescent label, the scaffolds were incubated in a tetramethylrhodamine-5-isothiocyanate (TRITC, Sigma) solution; the unbound dye was washed away with saline phosphate buffer.

Scanning Electron Microscopy (SEM)

The scaffold structure was studied with a Camscan S2 microscope (Cambridge Instruments, United Kingdom). To prepare the specimens for the examination, they were dehydrated in stepwise increasing concentrations of ethanol and acetone and then dried on a Hitachi critical point dryer HCP-2 device (Hitachi, Ltd., Japan), and a layer of platinum of 20 nm thick was sprayed by means of IB3 Ion Coater device (Eiko Engineering Co., Japan).

Cell Lines and Culture Condition

The isolation of mouse embryonic fibroblasts (MEF) expressing green fluorescent protein (GFP) was performed as previously described [9]. MEF and immortalized mouse line 3T3 fibroblasts were cultured in the Dulbecco modification (DMEM) of Eagle's minimal essential medium from Paneko (Russia) containing 10% of fetal calf serum (FCS) from HyClone (United States). Osteoblast-like cell MG-63 line was cultured in Eagle's minimal essential medium (EMEM) from Lonza (Belgium), to which 1% solution of nonessential amino acids (NEAA) from Lonza (Belgium) and 10% FCS were added. All cell types were cultured at 37°C in 5% CO_2.

Cultivation of Cells on a Scaffold

All scaffolds were sterilized in 70% ethanol. For studies in vitro, the scaffolds were washed with cultivation media without FCS. The round fragments of the scaffolds with a diameter of 15 mm were placed into the wells of 24-well cell culture plates, and 1 mL aliquots of the cells in appropriate cultivation media were put onto them. After being incubated for 5 hours, the scaffolds were transferred to Petri dishes 35 mm a diameter, containing 2 mL of the medium. During culturing cells on the scaffolds, the medium was replaced for fresh every 3 days.

The line 3T3 fibroblasts were cultured on FS and on a porous surface of HS. The initial density of the cells was 2×10^4 cells per scaffold. On the first, third, and seventh days, the MTT test was carried out as previously described [10].

For the MEF cultivation, FS, PSS, and HS labeled with TRITC were used. The cells were added in quantity of 2×10^4 cells per a scaffold. On the first, third, and seventh day of cultivation, the cells were fixed and studied by confocal microscopy.

Osteoblast-like cells (MG-63) were placed onto the TRITC-labeled scaffolds of all types (8×10^4 cells per one sample) and cultivated for 1 day. Then the cells were fixed with 10% formalin in saline phosphate buffer (BioVitrum, Russia), stained with phalloidin-Alexa Fluor™ 488 from Invitrogen (United States) to detect the actin cytoskeleton, and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich, United States) to detect the cell nuclei. The obtained preparations were studied with confocal microscopy.

Confocal Laser Scanning Microscopy (CLSM)

The study was performed on an Eclipse Ti-E microscope with a confocal module A1 (Nikon, Japan). The series of optical slices were obtained by means of CFI Plan Apochromat VC 20 × 0.75 Plan Fluor DIC 40 × 1.30 Oil lenses.

Model of an Artificial Defect of the Femur

The study of bone regeneration in vivo was performed in Wistar rats under anesthesia in sterile conditions. The following mixture was used: ZoletilT 100 anesthetic (Virbac, France) and Rometar muscle relaxant (Bioveta, Czech Republic) in sterile PBS at concentrations of 10 and 20% by volume, respectively. The linear incision 25 mm in length was created after shaving the surgical field and skin treatment with 70% ethanol. The thigh muscle fascia were delaminated lengthways to access the femur. The bone defect in the diaphysis of the bone was formed by a dental drill with a working head diameter of 0.8 mm. The defect size was 1 to 2 mm. The appropriate size fragment of the porous scaffold was implanted into the cavity.

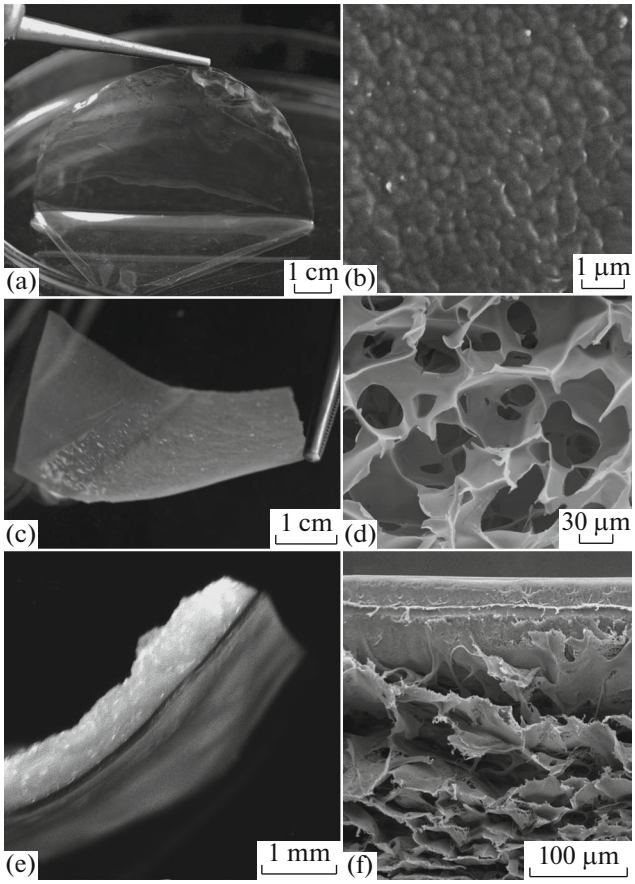

Fig. 1. Silk fibroin based scaffolds: (a, b) FS, (c, d) PSS, and (e, f) HS. On the left—macro-photography views; on the right—surface structure of scaffolds (SEM).

Preparation of Histological Samples

The specimens were fixed in 10% Bouin solution (a saturated aqueous solution of picric acid, formalin, and acetic acid in the ratio 15 : 5 : 1). In addition, decalcification in 25% solution of Trilon B was performed. The bone tissue fragments adjacent to the injury were embedded in paraffin, and slices with a thickness of 5–6 μm were prepared. The slices were rehydrated, stained with hematoxylin and eosin, and covered with a balm. The preparations were examined under an Axiovert 200M LSM510 META microscope (CarlZeiss, Germany) with an AxioCam MRC 5 camera (Carl Zeiss, Germany).

RESULTS

Obtaining Different Types of Scaffolds from Silk Fibroin

To explore the possibility of creating a bioresorbable bone substitute based on silk fibroin, three types of scaffolds were obtained: FS (Figs. 1a, 1b), three-dimensional PSS (Figs. 1c, 1d), and bilayer HS (Figs. 1e, 1f). The surface structure of the obtained scaffolds was characterized by SEM. FP had a microrelief (Fig. 1b). Fibroin PSS had a three-dimensional porous structure with a complex topography (Fig. 1d). A notable peculiarity of HS (Figs. 1d, 1e) is that a porous scaffold on the fibroin film is formed, which leads to the formation of a hybrid bilayer structure having an open porous structure on one side and a flat surface on the other.

Cultivation of Fibroblasts and Osteoblast-Like Cells on Different Types of the Fibroin Based Scaffolds

To study the ability of the silk fibroin scaffolds to support adhesion and proliferation of the cells, the following parameters were investigated: the adhesion and growth of the MEF primary culture, immortalized 3T3 fibroblasts, and the MG-63 human osteosarcoma cells on different types of fibroin scaffolds. Figure 2a shows MEF on the surface of a spongy part of the hybrid scaffold, the structure of which is shown in Fig. 2b. The proliferation of the cells on the surface of scaffolds was assessed by MTT assay (Fig. 2c). The results of the experiments demonstrated the ability of FS, PSS, and HS to support the adhesion and proliferation of all cell types used in the experiments. The 3T3 line fibroblasts continued to proliferate on the HS porous surface up to the seventh day of the experiment, while they stopped to grow after 3 days of cultivation on the FS.

When MG-63 human osteosarcoma cells were cultured on the fibroin scaffolds having a three-dimensional structure (PSS and HS), a significant part of the cells formed contacts with scaffold material and other cells lying in different planes (Figs. 2e–2h), which was not the case for the cells cultured on FS (data not shown).

Regeneration of the Femur Defect

Histological examination of the implantation area 4 weeks after the surgery revealed that there was a slight lymphocytic infiltration and osteogenesis foci inside the scaffold were formed (Figs. 3a, 3b). Osteogenic cells—osteoclasts, osteoblasts, and osteocytes—were identified in the area of the implant.

DISCUSSION

Creating various types of bioresorbable scaffolds for tissue repair is one of the crucial tasks for regenerative medicine development. This paper presents three types of scaffolds from fibroin (Fig. 1), which can be used for producing bone implants.

Bone grafts are typically applied to fill large defects when the use of flat scaffolds for this purpose is limited. They are mainly used as a model to compare with the three-dimensional structures [11]. Moreover, FS can also have its own application area, in particular, for creating the bioresorbable barrier membranes used in the restoration of periodontium. Their functional

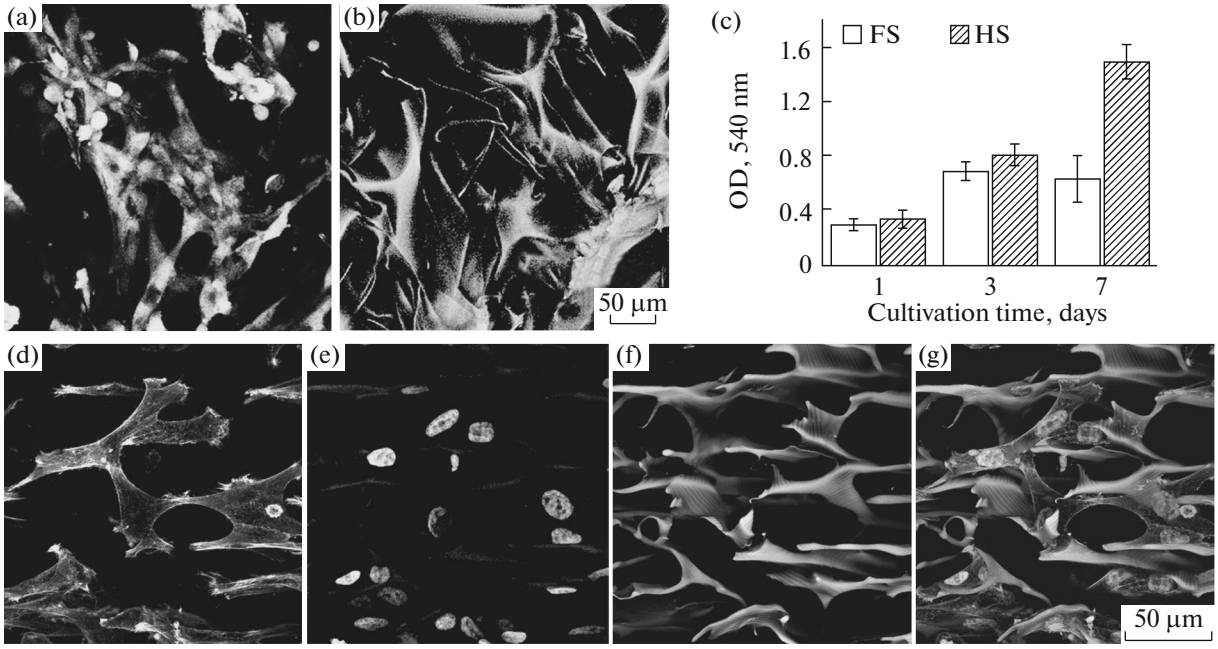

Fig. 2. Adhesion and proliferation of the cells on different type fibroin scaffolds. (a–c) Top row, fibroblasts on the surface of fibroin scaffolds: (a) MEF on the surface of HS (identified by GFP accumulated in the cells, CLSM data), (b) HS spongy surface, stained with TRITC (CLSM data), (c) proliferation of 3T3 fibroblasts on FS and HS (the data of MTT–test). (d–g) Bottom row, MG-63 osteoblastoma cells on the surface of PSS stained by TRITC (CLSM data): (d) MG-63 cytoskeleton revealed with phalloidin–Alexa Fluor™ 488, (e) cell nuclei visualized with DAPI, (f) scaffold material associated with TRITC, (g) merge of the channels e–g.

role is to physically separate the defect from the surrounding soft tissues and to prevent the fast-growing epithelial cells to filling it before the tissue of the periodontal ligament and alveolar bone will grow into this area [12]. Multilayer membranes can be also used for this purpose, which have a smooth surface on one side to ensure the barrier function and a porous surface on the other side for adhesion and proliferation of specific cells of periodontium [13]. Thus, the use of HS having a similar structure to restore periodontal tissues seems to be quite promising. The same type of hybrid design was successfully used to restore the rat skull defect [14].

The porous scaffolds are most frequently used to restore the bone defects [15, 16]. The porous structure of a scaffold ensures the migration and proliferation of the cells involved in bone regeneration and vascularization of the newly formed tissue [17].

Assessing the cell growth on the studied scaffold surfaces have revealed the biocompatibility of the material and the ability to support the adhesion and proliferation of different cell types on their surface (Fig. 2).

Fibroblasts, the resident cells of connective tissue, have a profound impact on the regeneration of various tissues and organs [18]. The ability of the scaffolds to support adhesion and proliferation of this cell type is an important indicator for biocompatibility.

When PSS and HS were used, the cells were detected on the surfaces of the scaffolds; a significant fraction of the cells formed contacts with the scaffold material and other cells in different planes (Figs. 2e–2h). Such cells as fibroblasts and osteoblasts are in a three-dimensional environment in vivo, where they can form contacts with the extracellular matrix and other cells in all planes [19]. Thus, culturing the cells on PSS and HS is close to physiological conditions.

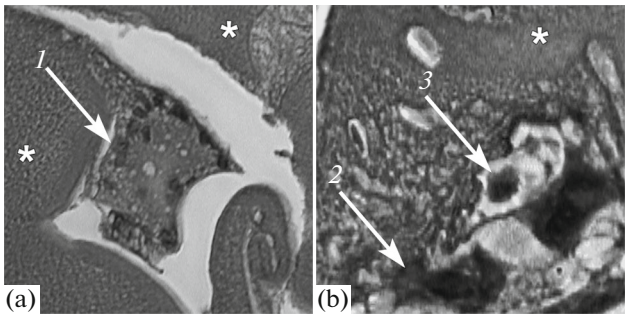

Fig. 3. Fibroin scaffold application for bone tissue regeneration. (a–b) Histological examination of the tissue formed in the rat femur defect filled with the fibroin PSS, stained with hematoxylin-eosin. (a) Osteoclast (*1*) interacting with the scaffold material (*); (b) osteoblasts (*2*) and osteocytes (*3*) inside the scaffold material (*).

The creation of three-dimensional conditions is one of the factors contributing to the differentiation of osteoblasts [20, 21].

Implantation of the porous fibroin scaffold in the bone defect area led to the restoration of the bone integrity to some extent that helped to preserve its function during regeneration. Four weeks after the operation, the cells involved in the bone regeneration were detected in the implantation area (Fig. 3). Osteoblasts normally producing extracellular matrix bone components have been present; osteocytes, which have specific appendages, have been detected in the bone lacunae. Osteoclasts, the polynuclear macrophages of bone tissue resorbing the scaffold material, have been found as well.

The presence of osteospecific cells interacting with the scaffold material indicates its osteoconductive properties, which are characterized by the ability of the surrounding bone tissue to grow into the implant [22].

Therefore, the developed fibroin scaffolds are an effective substrate for adhesion and proliferation of the cells involved in tissue regeneration. PSS and HS are proposed to be the most optimal for the bone substitute development, since they possess a three-dimensional porous structure, which is necessary to restore this type of tissue. It has been shown that implantation of the porous scaffold in the femur defect leads to the ostegenesis foci formed within the implant.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education and Science of the Russian Federation under the Agreement on the provision of subsidies no. 14.607.21.0119 from October 27, 2015 “Creating a Product Prototype Set from Bioartificial Bone Tissue and Osteogenesis Modulators for Regenerative Medicine” (unique identifier RFMEFI60715X0119).

The study was performed using the equipment of the Equipment Center for Collective Use at Moscow State University purchased at the expense of Program of Development of Moscow State University under financial support of the Ministry of Education and Science of the Russian Federation.

REFERENCES

1. Albrektsson, T. and Johansson, C., Osteoinduction, osteoconduction and osseointegration, *Eur. Spine J.,* 2001, vol. 10, suppl. 2, pp. S96–S101.
2. Schroeder, J.E. and Mosheiff, R., Tissue engineering approaches for bone repair: Concepts and evidence, *Injury,* 2011, vol. 42, no. 6, pp. 609–613.
3. Fillingham, Y. and Jacobs, J., Bone grafts and their substitutes, *Bone Joint J.,* 2016, vol. 98, suppl. A, pp. 6–9.
4. Miron, R.J. and Zhang, Y.F., Osteoinduction: A review of old concepts with new standards, *J. Dent. Res.,* 2012, vol. 91, no. 8, pp. 736–744.
5. Laurencin, C., Khan, Y., and El-Amin, S.F., Bone graft substitutes, *Expert Rev. Med. Devices,* 2006, vol. 3, no. 1, pp. 49–57.
6. Wu, S., Liu, X., Yeung, K.W.K., Liu, C., and Yang, X., Biomimetic porous scaffolds for bone tissue engineering, *Mater. Sci. Eng. R. Rep.,* 2014, vol. 80, pp. 1–36.
7. Melke, J., Midha, S., Ghosh, S., Ito, K., and Hofmann, S., Silk fibroin as biomaterial for bone tissue engineering, *Acta Biomater.,* 2016, vol. 31, pp. 1–16.
8. Bagrov, D., Zhuikov, V., Chudinova, Y., Yarisheva, A., Kotlyarova, M., Arkhipova, A., Khaydapova, D., Moisenovich, M., and Shaitan, K., Mechanical properties of films and three-dimensional scaffolds made of fibroin and gelatin, *Biophysics,* 2017, vol. 62, no. 1, pp. 17–23.
9. Orlova, A.A., Kotlyarova, M.S., Lavrenov, V.S., Volkova, S.V., and Arkhipova, A.Y., Relationship between gelatin concentrations in silk fibroin-based composite scaffolds and adhesion and proliferation of mouse embryo fibroblasts, *Bull. Exp. Biol. Med.,* 2014, vol. 158, no. 1, pp. 88–91.
10. Moisenovich, M.M., Kulikov, D.A., Arkhipova, A.Y., Malyuchenko, N.V., Kotlyarova, M.S., Goncharenko, A.V., Kulikov, A.V., Mashkov, A.E., Agapov, I.I., Paleev, F.N., Svistunov, A.A., and Kirpichnikov, M.P., Fundamental bases for the use of silk fibroin-based bioresorbable microvehicles as an example of skin regeneration in therapeutic practice, *Ter. Arkh.,* 2015, vol. 87, no. 12, pp. 66–72.
11. Huang, Y., Ren, J., Ren, T., Gu, S., Tan, Q., Zhang, L., Lv, K., Pan, K., and Jiang, X., Bone marrow stromal cells cultured on poly (lactide-co-glycolide)/nano-hydroxyapatite composites with chemical immobilization of ARG-GLY-ASP peptide and preliminary bone regeneration of mandibular defect thereof, *J. Biomed. Mater. Res. A,* 2010, vol. 95, no. 4, pp. 993–1003.
12. Sheikh, Z., Hamdan, N., Ikeda, Y., Grynpas, M., Ganss, B., and Glogauer, M., Natural graft tissues and synthetic biomaterials for periodontal and alveolar bone reconstructive applications: A review, *Biomater. Res.,* 2017, vol. 21, p. 9.
13. Liao, S., Wang, W., Uo, M., Ohkawa, S., Akasaka, T., Tamura, K., Cui, F., and Watari, F., A three-layered nano-carbonated hydroxyapatite/collagen/PLGA composite membrane for guided tissue regeneration, *Biomaterials,* 2005, vol. 26, no. 36, pp. 7564–7571.
14. Liao, S., Yokoyama, A., Zhu, Y., Watari, F., Ramakrishna, S., and Chan, C.K., In vitro and in vivo behaviors of the three-layered nanocarbonated hydroxyapatite/collagen/PLGA composite, *J. Bioact. Compat. Polym.,* 2010, vol. 25, no. 2, pp. 154–168.
15. Zhang, W., Zhu, C., Ye, D., Xu, L., Zhang, X., Wu, Q., Zhang, X., Kaplan, D.L., and Jiang, X., Porous silk scaffolds for delivery of growth factors and stem cells to enhance bone regeneration, *PLoS One,* 2014, vol. 9, no. 7.
16. Muraev, A.A., Bonartsev, A.P., Gazhva, Yu.V., et al., Development and preclinical studies of orthotopic bone implants based on a hybrid construction from

poly (3-hydroxybutyrate) and sodium alginate, *Sovrem. Technol. Med.,* 2016, vol. 8, no. 4, pp. 42–49.

17. Polo-Corrales, L., Latorre-Esteves, M., and Ramirez-Vick, J.E., Scaffold design for bone regeneration, *J. Nanosci. Nanotechnol.,* 2014, vol. 14, no. 1, pp. 15–56.
18. Costa-Almeida, R., Soares, R., and Granja, P.L., Fibroblasts as maestros orchestrating tissue regeneration, *J. Tissue Eng. Regen. Med.,* 2017. doi 10.1002/term.2405
19. Akhmanova, M., Osidak, E., Domogatsky, S., Rodin, S., and Domogatskaya, A., Physical, spatial, and molecular aspects of extracellular matrix of in vivo niches and artificial scaffolds relevant to stem cells research, *Stem Cells Int.,* 2015, vol. 2015.
20. Tseng, P.C., Young, T.H., Wang, T.M., Peng, H.W., Hou, S.M., and Yen, M.L., Spontaneous osteogenesis of MSCs cultured on 3D microcarriers through alteration of cytoskeletal tension, *Biomaterials,* 2012, vol. 33, no. 2, pp. 556–564.
21. Goncharenko, A.V., Malyuchenko, N.V., Moisenovich, A.M., Kotlyarova, M.S., Arkhipova, A.Y., Kon'kov, A.S., Agapov, I.I., Molochkov, A.V., Moisenovich, M.M., and Kirpichnikov, M.P., Changes in morphology of actin filaments and expression of alkaline phosphatase at 3D cultivation of MG-63 osteoblast-like cells on mineralized fibroin scaffolds, *Dokl. Biochem. Biophys.,* 2016, vol. 470, no. 1, pp. 368–370.
22. Oryan, A., Alidadi, S., Moshiri, A., and Maffulli, N., Bone regenerative medicine: Classic options, novel strategies, and future directions, *J. Orthop. Surg. Res.,* 2014, vol. 9, p. 18.

Translated by E. Puchkov