ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info/jasr

ISSN
0976-9595
Research Article

POROUS TINI-BASED ALLOY SCAFFOLD FOR CELL TISSUE ENGINEERING

[1]Kokorev O.V., [1]Hodorenko V.N., [1*]Chekalkin T.L., [2]Dambaev G.Ts., [1]Gunther V.E.

[1] Tomsk State University, Research Institute of Medical Materials, (Tomsk, Russia)

[2] Siberian State Medical University, Department of Clinical Surgery, (Tomsk, Russia)

*Corresponding author: tc77@rec.tsu.ru

ABSTRACT

Recently, a new fabrication method of scaffolds based on porous TiNi alloy has been developed for tissue engineering. This provides significant advantages over conventional scaffolds, including: versatility, reproducibility and industrial scalability. Structural properties of porous TiNi-based scaffolds have been investigated. The microporous surface of walls was shown to create the condition for cells adhesion and ingrowth, it facilitates the penetration of nutrients from outside and metabolite's outflow during the study. Features of cell growth, which makes different tissue populations and step-by-step cellular interaction with the porous-permeable structure of TiNi-based scaffold were established. Methods used in this study proved the tissue ingrowth within scaffold's pores. Cells implanted to be well protected from the allogenic environment were comprehensively found *in vitro* to fill all the pore space and to create specific tissues.

Keywords: Biomaterials, Tissue Engineering, Porous TiNi-based Scaffold.

1. INTRODUCTION

Three-dimensional porous biomaterials (3D-scaffolds) for bioengineering technology are recently considered to be most suitable ones. Porous, biodegradable and bioresorbable scaffolds are utilized in various tissue engineering applications and are shaped typically via 3-D printing, solvent casting, gas foaming etc. However, significant issues and limitations remain in the existing ones that can be used: cost, reproducibility of the microstructure, properties of the fabricated scaffolds, and scalability of the fabrication processes to realistic manufacturing rates. These remaining major challenges require the development of alternative materials. At the same time, materials creating the matrix have to correspond to certain properties: optimal physicochemical surface providing the specific initial adhesion for cells; large pore space; appropriate pore size distribution and number of pores to stimulate the cell proliferation and to enhance their viability. Mechanical properties of the structure are to be the same as for a real biological tissue to be replaced, exhibiting the hysteretic behavior [1-3]. Of course, the material of a scaffold is supposed to be subjected to the sterilization without changing its properties [4-6].

Currently, up-to-date methods of testing to evaluate the influence of biomaterials on cellular and intracellular processes are rapid, sensitive and informative as follows: adhesion; proliferation; intracellular metabolism; reproduction and cell differentiation. Important role in this is played by the structure of biomaterial: topography, porosity, pore size distribution, resorption etc. [7-10]. An interconnected open pore system providing nutrients and metabolite's outflow is required. Porous materials having intertwined pores are crucially essential for ingrowths of blood vessels.

The increase of degenerative diseases and injuries as well as expensive surgery brings us to the research related to the creation of scaffolds for cell cultures of different bone and cartilage. The actual aims of modern replacement therapy using stem cell technologies are cultivation of pancreatic islet's cells for treatment of diabetes mellitus and hepatocytes for correction of liver failure [11-13].

Recently, tissue engineering uses porous incubators (scaffolds) commonly applied in combination with the cell population adding various differentiation factors. These complexes are further implanted into the organism, reconstructing or restoring the fail organ. Moreover, the implantation of scaffolds showing the ingrowing tissue within the pore structure results in fast tissue regeneration and the acceleration of defect recovering compared with other types of treatment [14-16].

In Research Institute of Medical Materials there were created porous-permeable materials of TNi-based alloy for their use as scaffolds of cell cultures of different organs. Porous and cast TiNi-based alloys meet many strict requirements, which are necessary for the medical application: physical and mechanical properties; biochemical factors; non-toxic; non-carcinogenic; no inflammatory or immune response etc. [17-22].

The aim of this study is to research the application of porous-permeable materials made of TiNi-based alloy as

scaffolds for tissues equivalents making, which are based on bone marrow mesenchymal cells, hepatocytes and pancreatic islet's cells.

2. MATERIALS AND METHODS

2.1. Porous- permeable scaffolds

In this study we used permeable scaffolds (4x4x10 mm) made of porous TiNi-based alloy having mean porosity 70%, which has been produced by the SHS process. Samples were ED cut-off and then autoclaved at 180°C for one hour, cooled and placed into the culture medium.

Surface topography and the structure of pore space were investigated by using metallographic microscope Axiowert 40MAT and the SEM Quanta 200 3D.

The phase composition of the surface was determined by electron microprobe analysis.

2.2. Laboratory animals

We used male mice-hybrids of 10-week-old line F1 CVA/j and Wistar male rats provided by nursery area of experimental animals. All the experiments were carried out with strict adherence to European Convention for the Animals used for experimental and other scientific purposes (Strasburg, 1986) and with the European Communities Council Directive 86/609/EEC.

2.3. Isolation and cultivation of pluripotential mesenchymal stem cells (MSC) in scaffolds

Bone marrow mesenchymal cells of mice-hybrids line F1 CVA/j have been isolated from adult bone marrow following the procedure previously described [10]. Briefly, a bone marrow aspirate was diluted in expansion medium (DMEM supplemented with 10% FBS, 0.1 mM non-essential amino acids and 2.5 mg/ml Fungizone) and plated in tissue culture flasks at density of 2×10^5 cells/cm^2. Non-adherent cells were removed with subsequent media changes and the adherent cells (MSC) were cultured to 80% confluence, trypsinized. The cells were used for differentiation studies and scaffold seeding. Concentration of cells was about 5×10^6 cells/ml in complete medium and seeding onto scaffolds and packaged in 50 ml plastic vials «Corning». Three different culture media were established. Control medium consisted of DMEM with 10% FBS («HyClone», USA), gentamycin 40 mkg/ml, 250mg/l glutamine ("PanEko", RF). Chondrogenic medium consisted of control medium further supplemented with 0.1 mM non-essential amino acids, 5 mg/ml insulin and 5 ng/m hTGF-β («Sigma», USA). Osteogenic medium (OSM) consisted of control medium further supplemented with 50 mg/ml ascorbic acid-2-phosphate, 10 nM dexamethasone, 7 mM β-glycerophosphate and 1 mg/ml rhBMP-2 («Sigma», USA). Scaffolds were kept at 37° C and 100% humidity with 5 % CO_2. Cultivation was for 28 days and medium was changed twice a week. Samples for the SEM analysis were taken in 7, 14, 21 and 28 days. In 28 days, in order to compare the mass of wet and dried samples, they were twice weighed before and after the drying.

2.4. Isolation and cultivation of hepatocytes in scaffolds

In our study of cellular material we used hepatic cells of Wistar male rats. Hepatocytes are isolated by Seglen' modified method [23]. Isolated cells were seeded onto TiNi scaffold having final concentration 15×10^6cells/ml. Scaffolds were kept at 37°C and 100 % humidity with 5 % CO_2 for 24 hours, then under ketamine anesthesia (1mg/10g dose) they have been implanted into abdominal cavity of Wistar male rats. Samples to be investigated were taken every 7 days.

2.5. Isolation and cultivation of pancreatic islet cells in scaffolds

Pancreatic glands of Wistar male rats have been taken under intramuscular anesthesia in a sterile box. Islet cells were isolated by Dexter' modified method [24]. Isolated cells were seeded onto TiNi scaffold having final concentration 15×10^6cells/ml. Scaffolds were kept at 37°C and 100 % humidity with 5 % CO_2 for 24 hours, then under ketamine anesthesia (1mg/10g dose) they have been implanted into abdominal cavity of Wistar male rats. Samples to be investigated were taken every 7 days.

2.6. DNA contents assay

DNA was extracted from scaffolds in the following manner. Scaffolds frozen in 3 ml ddH2O were thawed and sonicated for 30 s to release the ALP and DNA into the solution. The solution was centrifuged for 5 min at 500g to remove scaffold debris. DNA was extracted from 850 ml of the solution and the rest was kept for the ALP activity assay. To the DNA portion was added 95 ml heated 10 ml TE-buffer with 1.5M NaCl and 5% SDS. Then, it was treated with RNAase (20 mg) and Proteinase K (800 mg). The solution was chloroform extracted and the DNA was precipitated with isopropanole, washed with 70% ethanol and dissolved in TE-buffer. The amount and purity of DNA per scaffold was determined by absorbance readings at 260 and 280nm in a UV/VIS spectrophotometer.

2.7. Alkaline phosphatase (AP) activity assay

Alkaline phosphatase activity was evaluated using the p-nitrophenol method in cells cultured in culture medium alone. Cells were washed twice with ice-cold phosphate buffered saline (PBS), and protein extracts were prepared in 10 mM Tris– HCl (pH 7.6) and 0.1% Triton X-100. Total protein extracts were prepared in 0, 7, 14, 21 and 28 days after OS medium induction. Alkaline phosphatase enzyme activity was calculated by measuring the absorbance of p-nitrophenol product formed at 405 nm on a microplate reader (Bio-Rad, Hercules, CA).

2.8. Scanning electron microscopy (SEM)

Scaffolds were washed twice in PBS and fixed with 2.5% glutaraldehyde for a hour. Then each one was flushed triply in PBS for 15 min and fixed for a hour in 1% tetra oxide osmium (SIGMA). Then each one was again flushed triply in PBS and gradually dehydrated by ethanol (30, 50, 70, 90 and 100% strength) for 15 min in each solution. Each sample has been dried before the SEM study of its structure. The microstructure of the inner surface was investigated by the SEM Quanta 200 3D (Japan) having the operating voltage 30 kV.

2.9. Statistical analysis

Results are expressed as mean±standard deviation with n=10. Error bars in figures represent standard deviations. Differences between experimental groups were analyzed according to a paired nonparametric Mann-Whitney's U-test, with p<0,05 considered statistically significant. Pore size effects were verified according to a single factor ANOVA.

3. RESULTS

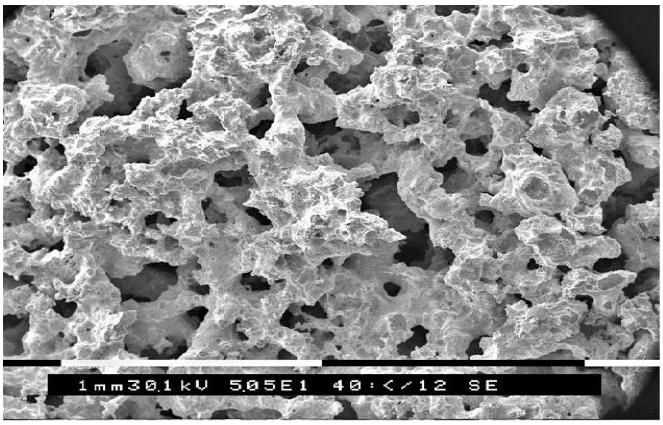
3.1. Study of porous TiNi-based alloy structure

The structure of TiNi-based alloy is the 3D pore space. The porous material has a large specific surface due to the presence of open and interconnected pores (Fig. 1a). Open pores (up to 90%) and the hydrophilic surface allow this material to be wettable and permeable. Pore walls have relief and roughness of nanoporous surface (Fig. 1b). Samples used in this study had the porous (mean porosity 70%) disordered structure with pore size distribution 0.1÷1000 μm.

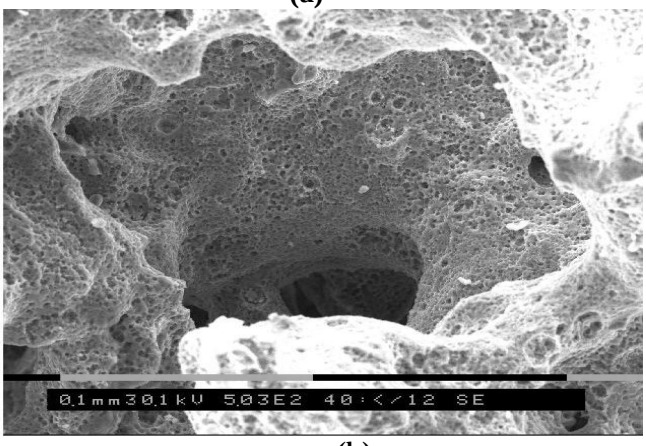
3.2. Physical and biochemical characterization of cultured samples/scaffold controls

Samples were harvested from cultural medium after 28 days and characterized with respect to wet weight (ww), dry weight (% ww), DNA content (% ww) and AP activity (units/ww) (Fig. 2–4). Samples from all groups had comparable wet weights (Fig. 2), whereas osteogenic medium yielded samples with significantly higher dry weight compared to chondrogenic and control media (1.3-fold and 2-fold, respectively, Fig. 2). This fact indicates an appearance of specific matrix of osteogenic cells in scaffold' structure, containing the most Ca than the matrix of chondrogenic cells.

Both DNA content and alkaline phosphatase activity tests also confirm the difference in the culturing of mesenchymal cells having various factors of differentiation. Chondrogenic medium yielded construct with significantly higher DNA content compared to osteogenic and control media (5.5-fold and 1.8-fold, respectively, Fig. 3). However DNA concentrations were low (<0.015% ww) in all groups.



(a)



(b)

Fig. 1. Macrostructure (a) and pore wall microstructure (b) of the porous TiNi-based alloy scaffold

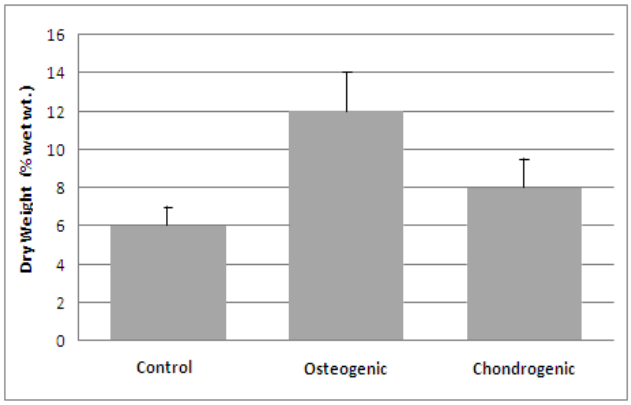


Fig. 2. Dry weight of samples (% wet weight) in 28 days of cultivation

Osteogenic medium yielded samples with significantly higher AP activity (an earlier marker of osteoblastic phenotype) compared to chondrogenic and control media (3.8-fold and 3.2-fold, respectively (Fig. 4). Samples cultered in chondrogenic medium exhibited only minimal AP activity and contained negligible amounts of Ca^{+2}, confirming the specificity of differentiation towards chondrogenic phenotype (Fig. 4). High amounts of AP activity presumably contributed

to the high dry weight of samples in the osteogenic group.

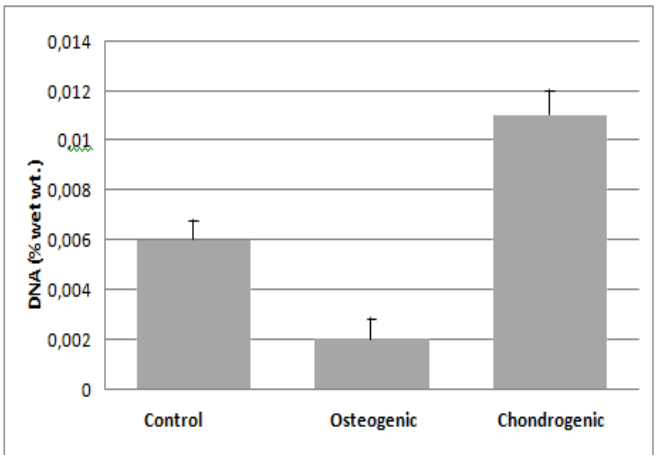


Fig. 3. DNA content in 28 days of cultivation

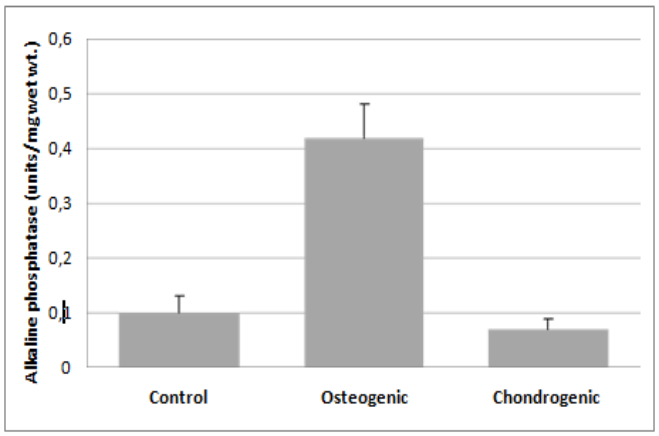


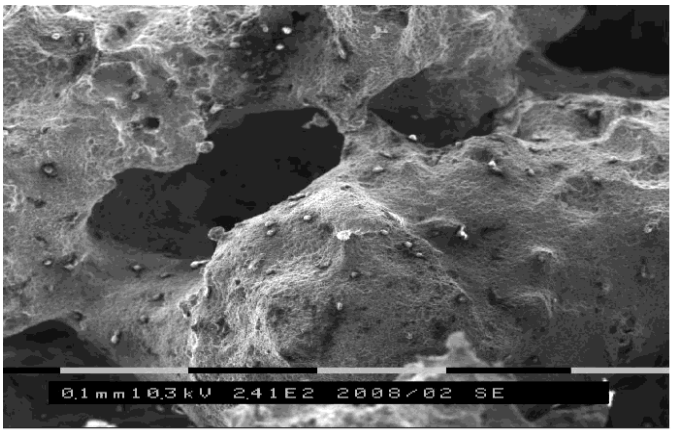
Fig. 4. Alkaline phosphatase activity test in 28 days of cultivation

This confirms the possibility to apply the porous TiNi-based alloy scaffold as an incubator-carrier for the culturing of multipotent mesenchymal stromal cells, as well as the possibility of cell differentiation in the pore space of the scaffold in two directions: chondro- and osteogenic one. The surface structure of the porous alloy was shown by SEM study to have very good adhesive and biocompatible distinctive features, which are necessary for the scaffold when culturing such cells.

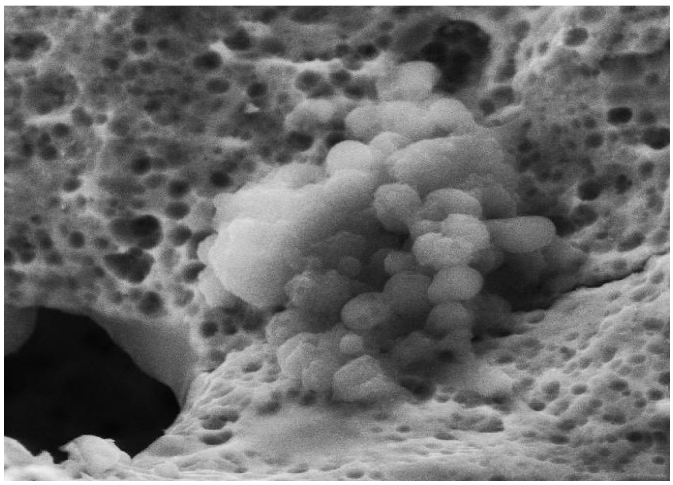
Since mesenchymal bone marrow cells are multipotent population, we can assume that the scaffold is also biocompatible including other cells: adipogenic, neural, muscular derived mesenchymal ones.

3.3. SEM examination

The SEM study of pore space was carried out in 7-day time interval and the following features were noted: at the end of first week there was cell' attaching and their proliferation; most of cells fixed in local cavities, where a lot of small pores having the size less than 3μm were, and then cells grew actively (Fig. 5).



(a)



(b)

Fig. 5. Porous structure of TiNi-based alloy scaffold after seeding by cells culture (a), group of pancreas islet cells in a local cavity (b)

Cell population increase, their growth in pores and the synthesis of extracellular matrix further proceeded. 3D porous structure of the scaffold allows the proliferation to happen intensively by synthesizing the extracellular matrix and forming the spatial incrustations having different shapes and sizes (Fig. 6a).

Beginning from 14th day the tissue gradually lined the inner surface of pores and then the growth vector was from the periphery towards the center, filling all pore space. During the fourth week, most of pores are completely filled by cells and the differentiating matrix (Fig. 6b). This effect was consistently observed since one week both *in vitro* (mesenchymal cells) and *in vivo* (hepatocytes and pancreas islet cells). Samples have been intergrown by tissues, and pores have been filled in 28 days by mesenchymal cells and in 40-60 days by hepatocytes and pancreas islet cells.

Experiments carried out have *in vitro* shown hereby that cells implanted into the porous TiNi-based alloy scaffold attach actively, grow, differentiate and create the specific tissue structure even *in vivo* allogenic condition of a recipient.

In terms of this study it is to be concluded that the internal structure of the permeable scaffold and its pore'surface facilitate to consecutive growth and preservation of neogenic tissue.

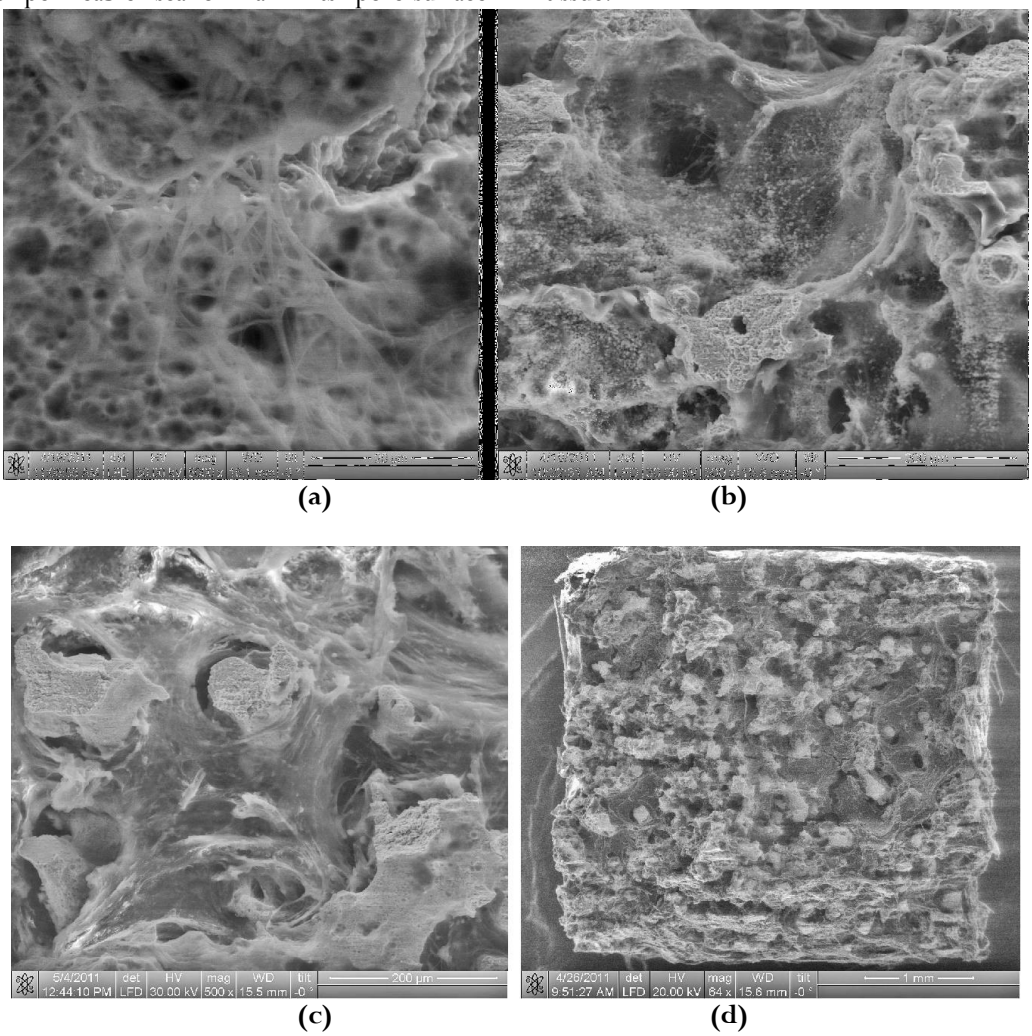


Fig. 6. Cellular evolution: synthesis of extracellular fibers and formation of spatial pseudopodium (a) in 7 days; cellular growth in pores and formation of extracellular matrix (b) in 14 days; phase of active pore filling by cell population (c) in 21 days; view of scaffold entirely filled by tissues (d) in 28 days

4. DISCUSSION

Applying various modes of the SHS one can get the different structure of porous-permeable TiNi-based alloy having specific pore size distribution and, that's very important in cell and tissue engineering, specific topography as well as the surface of pore space, which is required for the culturing of certain cells.

The complex of mentioned features concerning the structure and properties of porous TiNi-based alloy is determined as follows:

- Advanced microporous surface of pore walls (large number of interconnected small pores and roughness of walls themselves), which increases adhesiveness of cells;
- Wetting ability of material and permeability of pore system, which increases a penetrating of water-soluble substances;
- Optimal porosity;
- Phase and chemical composition of the surface showing no inhibitory and toxic effects on cells implanted and growing tissues in pores;
- Hysteresis delay law of the alloy, which exhibits the same bio-mechanical behavior as an alive tissue, what allows to fix a problem of bio-mechanical compatibility [20, 25].

All mentioned above are necessary conditions for initial attachment, growth and reproduction of cells and this set is a driving force for a tissue to be grown. Steady, consistent growth of cells in the scaffold and short time when the porous structure was being filled by a tissue allows to make a conclusion that this scaffold is an unique biocompatible incubator of cell cultures and can be prosperously used to create artificial bio-engineering organs. Directed differentiation of multipotential mesenchymal stromal cells in a cartilage or bone tissue inside the scaffold proves this material

indifference toward the ingrowing tissue. A life cycle of xenogeneic cells *in vivo* implanted without the scaffold is considerably decreased because of immune response. The structure configuration of scaffold' pore space enables to protect cells implanted, to form a high-integrating biosystem of different organs and to extend a life functional cycle of cells implanted, prolonging a curative action.

The technology described is a significant contribution to bioengineering science when studying mechanisms of morphological/functional properties of cells modified in scaffolds, and it can find an application in treatment of locomotor apparatus diseases, metabolic disorders of liver and pancreas.

5. ACKNOWLEDGMENTS

The authors gratefully acknowledge the support for this research provided by the Tomsk State University Competitiveness Improvement Program, Siberian State Medical University (Tomsk), Research Institutes of Pharmacology (Tomsk). Their support is greatly appreciated.

6. REFERENCES

1. Murphy CM, O'Brien FJ. *Cell. Adh. Migr.,* 2010; **4(3)**:377-381.
2. Mitragotri S, Lahann J. *Nature Mater.,* 2009; **8**:15-23.
3. Bettinger CJ, Bruggeman JP, Misra AB. *Biomaterials,* 2009; **30**:3050-3057.
4. Bensch L, Jones D. Biomaterials, artificial organs and tissue engineering. Moscow: Technosphere, 2007.
5. Griffith EG, Naught OG. *Science,* 2002; **295**:1009-114.
6. Sakiyama-Elbert SE, Hubbell JA. *Ann. Rev. Mater. Res.,* 2001; **31**: 183-201.
7. Shinoka TS, Shum-Tim VC, Ma PX *et al. J. Thorac. Cardiovasc. Surg.,* 19981; **15(3)**:536-545.
8. Kirkpatrick CJ, Bittinger F, Wagner M *et al*. *Journal of Engineering in Medicine,* 1998; **212(2)**:75-84.
9. Rikli DA, Regazzoni PA, Perren SM. *Injury,* 2002; **33**:B2-3.
10. Rose FR, Oreffo RO. *Biochem. Biophys. Res. Commun.,* 2002; **292(1)**: 1-7.
11. Vacanti JP, Langer RA. *The Lancet,* 1999; **354**:532-534.
12. Kokorev OV, Hodorenko VN, Anikeev SG, Dambaev GTs, Gunther VE. *Bulletin of new medical technologies,* 2012; **XIX(1)**:32-36.
13. Kokorev OV, Hodorenko VN, Anikeev SG, Dambaev GTs, Gunther VE. *Siberian Medical Journal,* 2012; **1(27)**:130 -136.
14. Cancedda R, Dozin B, Giannoni P. *Matrix. Biol.*, 2003; **22**:81-91.
15. Kuo CK, Li WJ, Mauck RL. *Curr. Opin. Rheumatol.,* 2006; **18**:64-73.
16. Sharma BH, Elisseeff JH. *Ann. Biomed. Eng.,* 2004; **32**:148-59.
17. Wever DJ, Veldhuizen AG et al. *Biomaterials,* 1997; **18**:1115-1120.
18. Prymak O, Bogdanski D et al. *Biomaterials,* 2005; 26: 5801-5807.
19. Ryhanen J, Kallioinen M et al. *J. Biomed Mater Res.,* 1998; **41**:481-488.
20. Gunther VE, Hodorenko VN, Chekalkin TL et al. Medical materials and shape memory implants: In 14 vol. / Shape memory medical materials. Tomsk: MIC, 2011; **1**.
21. Dambaev GTs, Gunther VE, Ziganshin RV et al. Medical materials and shape memory implants: In 14 vol. / Shape memory implants in surgery. Tomsk: MIC, 2012; **11**
22. Choinzonov EL, Gunther VE, Muhamedov MR et al. Medical materials and shape memory implants: In 14 vol. / Shape memory implants in oncology. Tomsk: MIC; **13**
23. Shen L, Hillebrand A, Wang D, Liu M. *J. Vis. Exp.* 2012; **64**: e3917.
24. Stull ND, Breite A, McCarthy R, Tersey SA, Mirmira RG. *J. Vis. Exp.* 2012; **67**:e4137.
25. Gunter VE, Chekalkin TL, Kim JS, Hodorenko VN. *Adv. Mater. Lett.*, 2014; **5(9):**551-556.