MATERIALS AND METHODS OF BONE TISSUE ENGINEERING

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The bone tissue engineering seeks alternative solution to the problem of skeletal injuries. Creation of a tissue-engineered equivalent of a bone tissue using multipotent cells, matrixes carriers of these cells and osteogenic factors is the cornerstone of this method. The process of creation of tissue-engineered bone analog begins with the production of a matrix for cell cultivation. The paper presents the review of the most promising materials and methods used for production of cellular matrices. The modern materials used in creation of cellular matrices replicate the groups of substances composing the natural extracellular bone matrix. Modern technologies of creation of cellular matrices seek to imitate a structure of natural extracellular bone matrix at the micro- and nano-levels. Simulating natural composition and structure is necessary to create optimum conditions for cell activity on a device, as well for creation of favorable physic-mechanical properties of the matrix.
Key Words: bone tissue engineering, cellular matrix, electrospinning, 3D printing, synthetic biodegradable polymers, compounds of natural extracellular bone matrix, signaling molecules.

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According to the World Health Organization [5], there are about 50 million non-fatal injuries each year worldwide resulting in the problems of the musculoskeletal system and causing disabilities. In Russia, about 16 % of bone injuries are treated surgically, i.e. by implantation of metal structures [4] or osteoplastic materials [2] in the body. This approach is accompanied by inconveniences for the patient, it is of long-duration and, what is the most crucial, it does not guarantee a positive outcome. Thus, surgical defect is the cause of pseudarthrosis in 42 % of cases, which, in fact, is a bone defect [1].

Regenerative medicine offers the method of alternative implantation of metal in the body. The main advantage of the proposed approach is the theoretical possibility of complete restoration of anatomical integrity of the bone. The three components constituting the basis of the method are: multipotent cells, osteogenic factors, which are capable of directing cells along the osteogenic pathway, and matrix (cell carrier and substrate).

To date, the compounds mimicking natural bone extracellular matrix are known to be the best for cell cultivation [18]. Thus, scientists have faced with the problem of creating matrices with the surface structured at the micro and nano levels like natural extracellular matrix. In addition, there are requirements for matrices arising from the need to perform the functions of organs they replace, as well as properties that enable their use in implantation in the living body. These requirements include mechanical strength in order to perform, for example, support function of the bone, high porosity for nutrient supply to the cells, bioreactivity for cell interaction, biodegradability for the possibility of construct replacement with natural tissue.

The described requirements for the matrices for cell cultivation can be satisfied by a combination of various materials and methods for their generation.

The purpose of the study is the analysis of materials and methods for creation of cellular matrices in bone tissue engineering for deciding on the application of the most promising ones of them in further experiments.

Methods for creation of cellular matrices in bone tissue engineering

The size of the majority of cells varies from 2 to 120 μm. Recent studies have shown that the best conditions for cell activity are created when the size of the substrate structures is comparable to the size of the cells [15]. Thus, the method of matrix creation for cell cultivation should allow the possibility of structuring of the final product at the micro and nano levels and the possibility of using materials that are the most suitable for the creation of bone tissue analog.

Electrospinning, imprint lithography and 3D printing are among instrumental methods that can accomplish this task.

Electrospinning. Electrospinning device includes a needle through which a polymer solution is supplied and a collector, where the polymer jet released from the needle accumulates. All components of the device (needle, jet, collector) are elements of the same electrical circuit. The essence of the electrospinning process is to overcome the strength of the surface tension of the polymer solution at the end of the needle with the strength of the electric field. As the voltage of the electric field rises, the Taylor cone, a cone-shaped polymer drop, is first formed at the tip of the needle. When the voltage is enough, the polymer jet, the diameter of which depends on many conditions, is streamed from the cone apex towards the collector. While in the air, part of the solvent evaporates, and pure polymer is accumulated in the form of randomly or directionally stacked fibrils on the collector (Fig. 1).

This method is not only technically simple, but it also has a number of advantages. For instance, the possibility of using almost any material in the synthesis has been shown for electrospinning. These materials include synthetic biodegradable polymers: poly(lactide-co-glycolide) [11], polyethylene oxide [36], polycaprolactone [19], polylactide [57]; proteins of the natural extracellular matrix: chitosan [47], collagen type I [54], gelatin [19], elastin [6]; inorganic compounds of the extracellular bone matrix: β-tricalcium phosphate [45], hydroxyapatite [72], and carbon nanotubes [71]. The main issue is still the choice of a universal non-toxic solvent for preparation of composite solutions, which are subsequently to undergo the process of electrospinning.

Using electrospinning, it is possible to structure the matrices at the nano and micro levels [67] and form both parallel and divergent fibrils even within the same structure [26].

Special attention should be given to the work by scientists from South Korea [28] who created a 3D construct using the method of electrospinning (for comparison: 3D-structured films were obtained in the works mentioned above).

Thus, electrospinning can be used for production of 3D composite constructs structured at the micro and nano levels. The disadvantage of this method is that the physical and mechanical properties obtained during electrospinning of constructs do not achieve strength characteristics required from the bone tissue analog. Apparently, this fact is connected with the fibrillar structure of cellular matrices obtained during electrospinning (Fig. 2).

Imprint lithography. Imprint lithography is application of the imprint of an arbitrary form on a film of the desired material. This method allows one to quickly obtain a large number of planar cellular matrices with 3D-structured surface (Fig. 3).

It is possible to obtain a 3D structure by combining a numerous variety of such films. Resolution of the method begins with tens of nanometers. The imprint produced with the stamp can be almost of any shape, which is very advantageous for mimicking microarchitectonics of the bone.

The possibility of using polylactide and polycaprolactone for obtaining cellular matrices by imprint lithography and the possibility of using these matrices for cell culturing have been shown [3, 40].

Due to the complexity of formation of large dimensional structures from structured films as well as the lack of data about the possibility of using composite materials, imprint lithography has not become widespread in the production of the tissue-engineered bone analog.

3D printing. While microarchitectonics of matrices created using electrospinning and imprint lithography can be controlled, their microarchitectonics is limited by the method of creation and, in most cases, is represented by a film.

3D printing is capable of providing personalized matrices using computer imaging techniques, MSCT and MRI, by providing a complete control over microarchitectonics of the final construct. Thus, the possibility of control over the matrix structure at the macro and micro levels appeared with the advent of 3D printing.

Typically, the process of 3D printing involves the following steps: creation of a computer 3D model with precise micro- and macroarchitectonics, transfer of the model to the 3D printing device, and printing.

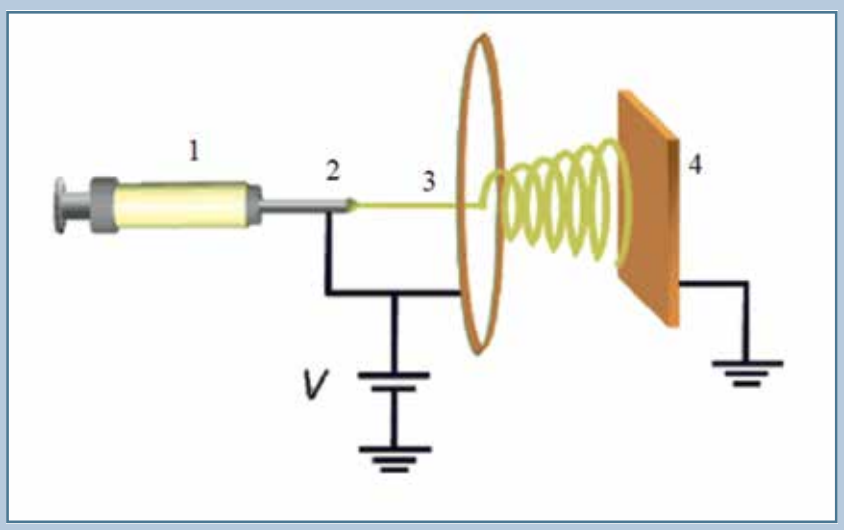

Fig. 1
Schematic representation of the electrospinning process [45]: 1 – syringe with polymer solution; 2 – needle and the Taylor cone formed on the top of it; 3 – jet of polymer solution; 4 – receiving collector

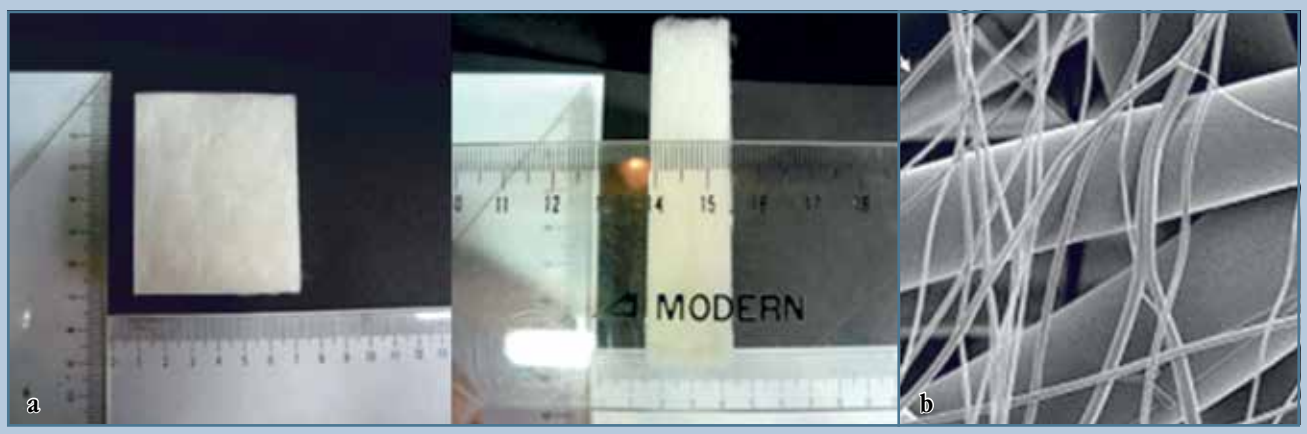

Fig. 2
An example of a matrix produced by electrospinning [28]: **a** – macro view of the device; **b** – microstructure of the device

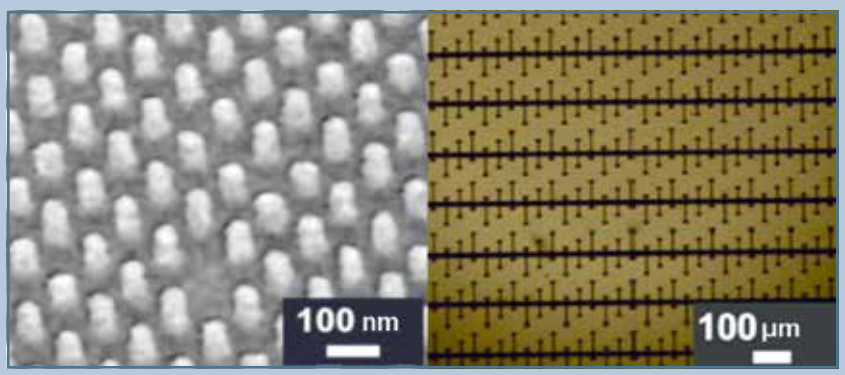

Fig. 3
Examples of matrices generated by imprint lithography: matrices are films with a randomly structured surface [3]

There are several 3D printing technologies, which are different in the methods of construct creation and materials used for production. Some of them will be described in more detail below. Examples of matrices generated using 3D printing methods are shown in Fig. 4.

Agglutination of powder material. The method includes application of an adhesive solution to the powder layer only at sites of projections of the future construct. After application of one layer, a new layer of powder is poured on the top, which is also subjected to gluing only at sites of projection of the future construct. Thus, a glued structure surrounded by unglued powder is created layer by layer.

The resolution of such technology is 300 µm. One of its advantages is the possibility of creating large interconnected pores, which promotes construction infiltration by cells [29]. The process takes place at room temperature, which makes it possible to add such biological agents as proteins to the construct [65].

Synthetic polymers polycaprolactone, polylactide, poly(lactide-co-glycolide) with an organic solvent as the adhesive material [29, 65, 70] as well as gelatin and dextran with water as the adhesive material were used as powder [31, 58].

Hydroxyapatite became widespread as a powder for this method. It is possible to obtain ceramics with porosities of up to 90 % when adding porogen to hydroxyapatite and its gluing with a synthetic polymer. Such structures exhibit pronounced osteoconductive properties [60].

The advantage of this method is the possibility of using a wide range of materials, while the disadvantage is low resolution of printing.

Extrusion technology. Extrusion 3D printers create a model from molten thermoplastic using layer-by-layer method. The main criteria of the materials for this type of printing are the melting temperature and rheology of the molten plastic.

With this technology, it is possible to control sizes of the elements in the layer, distance between the elements and layer thickness. It allows creating constructs with a precise pore size, interpore connections and desired microarchitectonics.

The key advantage of the method is the possibility of creating structures with relatively high porosity without the loss of sufficient mechanical strength. The complexity of the method is the necessity to heat the material to the melting temperature, which makes it impossible to apply a whole range of materials that are unstable upon heating, e.g. proteins.

Polycaprolactone, due to its low melting point (60 °C) and high thermal stability, became the most widely used compound for printing of biocompatible objects by this technology [69]. The

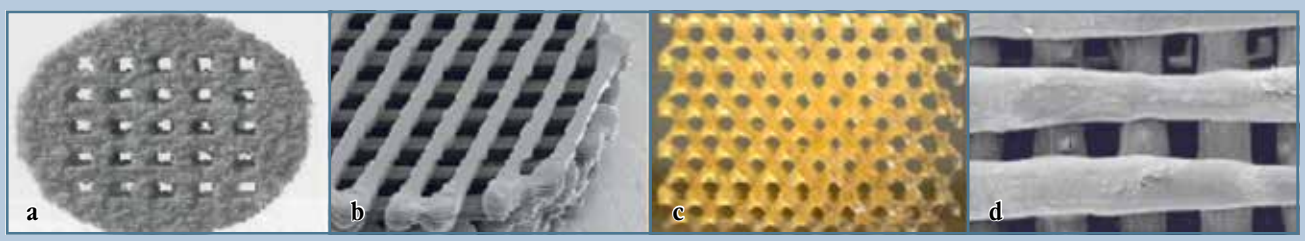

Fig. 4
Matrices formed by 3D printing [23, 36, 61, 65]: **a** – agglutination of the powder material; **b** – extrusion method; **c** – stereolithography; **d** – bioplotting

printing using poly(lactide-co-glycolide) is more difficult since the temperature of 110–140 °C is required for obtaining the desired rheology of the molten polymer [27]. The possibility of adding collagen [68], tricalcium phosphates [61], hydroxyapatite [27], and gelatin [62] to the structure for obtaining composite materials using this method has been shown.

Stereolithography. This method is based on polymerization of photopolymer by ultraviolet. The layer is created when the projector illuminates the dish with photopolymer only at sites of projection of the future construct. Next, the base is immerged and a new layer is illuminated. Finally, the finished object is surrounded by unpolymerized photopolymer solution.

To date, the method has reached high printing resolution (about 1.2 μm), which allows creating objects with rather complex internal microarchitectonics.

The disadvantage of this method is that there is a small amount of biocompatible photopolymers that can be used. The possibility of using poly(propylene fumarate) and diethyl fumarate has been shown for creation of 3D cellular matrices [14, 36]. However, the mechanical properties of the resulting constructs turned out to be insufficient for their use in bone tissue engineering.

Later studies have demonstrated the possibility of using polycaprolactone and polylactide in stereolithography. It is noteworthy that living cells were added to liquid photopolymer for their encapsulation into matrix [17, 46], which can be called bioprinting. Many adjuvants can be added to photopolymer solution, for example bone morphogenetic protein (BMP) [34].

3D plotting (bioplotting). This technology is based on the injection of a solution from a syringe into the liquid collector, the density of which coincides with the density of the solution in the syringe. The collector can also contain polymerizing substances. The process can be performed both at room and elevated temperatures. This method is particularly suitable for obtaining soft matrices from hydrogels.

The first polymers to be used in this technology were natural polymers such as agar and gelatin, while Ca^{2+} was the polymerizing agent [42, 53].

The advantages of this method are the possibility of using a large number of biocompatible materials and low temperature of the process. Among disadvantages are the impossibility of generating sufficiently solid constructs due to the use of hydrogels, and, hence, the impossibility of formation of complex microarchitectonics of the constructs. Resolution of the method is about 400 μm [23].

Bioplotting is similar to this method, with the extension that cell suspensions are also added to the solutions of polymers, for example, in alginate gel. This technology allows achieving a uniform distribution of cells and signaling molecules in the construct, which is especially essential for further tissue formation.

Bioplotting can be used with a poly(lactide-co-glycolide) [39], tricalcium phosphates, chitosan [39], hydroxyapatite [32], collagen [49], polycaprolactone [33]. It should be noted that preservation of the activity of cells that have passed through the process of bioprinting is observed in the mentioned studies regardless of the type of the material used.

Combination of methods. The benefit of combining various methods such as electrospinning and 3D printing in the production of a construct should be noted [52]. This approach is appropriate due to the complex structure of the bone as an organ, which contains not only bone tissue but also the periosteum, endosteum, nerves, blood vessels, and bone marrow. In order to recreate such complex structures, both dimensional constructs obtained by 3D printing techniques and films with a structured surface produced by electrospinning and (or) imprint lithography are likely to be needed.

Each of the presented methods has both advantages and disadvantages. Development of 3D printing technologies is necessary for resolution improvement, complication of construct shapes and increase in the strength of the resulting structures. This way will make it possible to create a sample most effectively imitating the natural extracellular matrix, physical and mechanical properties of which have not yet been achieved.

Materials applied in bone tissue engineering

Bone is a solid organ consisting of bone tissue, bone marrow, endosteum, periosteum, cartilage, nerves and blood vessels. Bone composition and structure

directly depend on its localization, the applied load, age and gender of the individual, as well as diseases that he has suffered. As the composite structure, 60–70 % of the bone consists is the mineral phase, 5–10 % is water, and the remaining part is presented with the organic matrix of collagen and other proteins.

Mineral phase of the bone is composed of calcium phosphate, hydroxyapatite represented in the form of nanocrystals of 25 to 50 nm in size. Up to 90 % of the organic phase of bone tissue is presented with collagen type I in the form of strands with a nanometer thickness.

Such a composite structure provides unique physical and mechanical properties of the bone: strength and elasticity. Characterization of these properties is presented in Table 1.

In the analytical study of 2015 using electronic search, 1458 articles on bone tissue engineering in the period of 2004 to 2013 have been examined. The result has showed that synthetic biodegradable polymers, natural organic compounds, inorganic compounds of the natural bone extracellular matrix, and signaling molecules are most commonly used in the production of cellular matrices.

It should be noted that scientists used combinations of the substances described above in the vast majority of cases, which corresponds to the principle of composite structure of the natural bone tissue and provides favorable bioreactive and physical and mechanical properties of the resulting constructs [51].

However, the optimum composition of cellular matrix for the production of tissue-engineered bone analog is still not identified. The properties and functions of the listed groups of materials will be considered below in order to resolve the issue on the matrix composition that can be possibly used.

Synthetic polymers. Synthetic polymers, in comparison with other materials, provide great flexibility of the synthesis of various constructs and their modification. Bioactivity of these polymers is very low, which allows eliminating the adverse impact on the macro-organism.

The most widely used compounds in the production of tissue-engineered bone analog are D- and L-chiral forms of polylactide [8, 46], poly(lactide-co-glycolide) with different content of copolymers [28], polycaprolactone [17] polypropylene fumarate [14], polyethylene glycol [59], and polyethylene oxide [55].

All of the listed polymers differ in their physical, mechanical, chemical and biological properties. Characteristics of the main properties are summarized in Table 2.

Synthetic polymers are used as the basis for matrices, which may contain adjuvants that significantly alter physical and mechanical properties of the constructs, as well as cell behavior. The gradual biodegradation of such basis should enable the replacement of the construct with natural body tissue.

Natural organic compounds. The purpose of the addition of natural organic compounds to the tissue-engineered matrices is an attempt to most accurately imitate the composition of natural extracellular matrix of the bone. In contrast to synthetic polymers, natural compounds are bioactive substances, which positively affects cell adhesion, proliferation and differentiation on the constructs.

Currently, such compounds as chitosan [50], collagen [16], gelatin [19], elastin, [10], and silk [37] became widely used in bone tissue engineering. Their impact on both composition properties and cell behavior is well studied.

Recent studies have shown the possibility of using minor proteins of the natural bone tissue, such as fibronectin and osteocalcin [64].

Natural compounds are used as adjuvant substances in bone tissue engineering. Their mass fraction in the construct does not usually exceed 10 %. However, even such an amount is sufficient to significantly improve the physical and mechanical properties of the structure itself, as well as to provide more favorable conditions for cell activity compared to pure synthetic polymers.

Calcium phosphates and other inorganic compounds. Calcium phosphates are a family of minerals containing Ca^{2+} and PO_4^{3-} ions. These compounds are contained in large amounts in natural bone extracellular matrix, which provides its rigidity. Calcium phosphates, as a bioactive material, promote the expression of genes responsible for osteogenic differentiation of cells. In addition to this, the possibility of accumulation of various signaling molecules in calcium phosphates has been shown, which provides the material with osteoinductive properties [56].

In bone tissue engineering, calcium phosphates are used as a separate substance forming a matrix with different porosity, as an adjuvant to synthetic polymers for improvement of the mechanical properties of the construct, and as a surface modifier for metal products [21].

Hydroxyapatite [24], including nanohydroxyapatite, β-tricalcium phosphate [10], and calcium carbonate [11] became the most widespread compound in bone tissue engineering. These substances can be used in tissue-engineered constructs in the form of powders, nanopowders, cements, and coatings.

Among other inorganic substances, various metal compounds: MgO, SrO, Au,

Table 1
Physical and mechanical properties of compact and cancellous bone [9, 30]

Properties	Compact bone	Cancellous bone
Tensile strength, MPa	50–150	10–100
Compressive strength, MPa	130–230	2–12
Young's modulus of elasticity, GPa	7,00–30,0	0,02–0,5
Elongation at break, %	1–3	5–7
Shear modulus, GPa	3	3

became widespread in tissue engineering. As natural bone minor compounds of the bone tissue, these materials have a positive effect on cell proliferation and differentiation in constructs, presumably due to the occurrence of electromagnetic stimuli. Various effects on cell cultures depending on metal valence have been shown for gold nanoparticles [12, 38].

The positive effect of non-metals on cell activity has been shown in culture. In order to investigate this, Si [41] and B [22] have been studied. Both substances have shown a positive effect on cell adhesion, proliferation and differentiation. Osteoinductive properties of B turned out to be extremely high.

Signal molecules. Signaling molecules are substances regulating body processes at the cellular level. Therefore, the addition of these materials to biodegradable matrices for bone tissue engineering can direct cells along the osteogenic pathway and enhance their adhesion and proliferation.

BMP family proteins are the most widely used molecules in bone tissue engineering. BMP are responsible for proliferation of chondro- and osteocytes, they increase the production of the extracellular matrix by these cells. BMP support differentiation of stem cells along the osteogenic pathway. BMP 2, 4, 7 cause the production of the extracellular matrix *in vitro*. BMP 1–3 increase collagen type I and osteocalcin production by cells. Studies based on BMP embedment into a biodegradable matrix have showed that addition of this signaling molecule causes formation of the bone tissue inside such construct [7, 20, 34, 35].

Fibroblast growth factors (FGFs) stimulate proliferation of mesenchymal stem cells, osteoblasts and chondroblasts. FGFs promote the formation of various tissues due to their angiogenic potential. FGF-2 is the most studied cytokine in bone tissue engineering [7].

Insulin-like growth factors (IGFs) promote proliferation of chondro- and osteoblasts and stimulate the secretion of natural extracellular matrix by both types of cells. IGFs cause synthesis of collagen and mineralization of the extracellular matrix in the bone tissue [44].

Platelet-derived growth factors (PDGF) enhance proliferation of chondro- and osteoblasts. However, they have a dose-dependent effect. For instance, their effect is resorptive in relation to the bone tissue at certain concentrations. PDGF acts as a mitotic factor on osteoblasts and other cell types [25].

Transforming growth factor-β (TGFs-β) promotes differentiation of mesenchymal stem cells into chondrocytes, has a positive effect on proliferation of chondro- and osteoblasts. Like PDGF, it can cause resorptive action on the bone tissue at certain concentrations, which is due to its role in the regulation of the processes of bone tissue formation and resorption [13].

RGD proteins containing «arginine – glycine – aspartic acid» sequence are cell adhesion proteins and cause attachment of osteoblasts to them and further ostheogenesis. Addition of RGD proteins to the construct significantly improves cell adhesion, proliferation and promotes their differentiation along the osteogenic pathway [63].

Thus, signaling molecules as highly bioreactive natural compounds exert a robust effect on cell activity in matrices. However, their impact on the physical and mechanical properties of the construct is so negligible that they are not taken into account by scientists.

Conclusion

To date, the technologies of bone tissue engineering allow the development of the cellular matrices that are close enough in their structure and composition to the natural extracellular bone matrix, which provides favorable conditions for adhesion, proliferation and differentiation of various cell lines.

Among the methods of creation of cellular matrices in bone tissue engineering, an emphasis should be put on the rapidly evolving 3D printing technology, which provides total control over macro- and microarchitectonics of the final product. This technology demonstrates promising results with use of a variety of materials, as well as in cultivation of various cell lines on the obtained constructs. The level of 3D printing development even to date allows accurate mimicking of the structure of the natural bone extracellular matrix. In this connection, it is advisable to use 3D printing in the production of tissue-engineered bone analog. However, in order to recreate bone as an organ, with its vessels, nerves and periosteum, it is possible that a combination of different techniques, such as electrospinning, imprint lithography and 3D printing, with intro- or post-processing asso-

Table 2
Main characteristics of synthetic polymers [43, 48, 66]

Polymers	Compressive/tensile strength, MPa	Young's modulus, GPa	Elongation, %	Melting temperature, °C	Biodegradation period, months
Poly(L-lactide)	28,0–2300,0	4,8	5–10	175	24–68
Poly(D,L-lactide)	29,0–150,0	1,9	3–10	165–180	12–16
Polyglycolide	350,0–920,0	12,5	15–20	200	6–12
Poly(lactide-co-glycolide) (85/15)	41,4–55,2	2,0	3–10	–	5–6
Poly(lactide-co-glycolide) (50/50)	41,4–55,2	2,0	3–10	–	1–2
Polycaprolactone	23,0	0,4	300–500	57	>24

ciation of the resulting cellular matrices should be used.

Groups of materials used in bone tissue engineering mimic the groups of substances comprising natural bone extracellular matrix. Such approach will provide favorable physical and mechanical properties of the resulting cellular matrices, and create optimal conditions for adhesion, proliferation and differentiation of cellular elements within construct. Biodegradation of the applied materials will enable to provide the implication of the key task of bone tissue engineering: the possibility of replacing natural tissues of the body with constructs, which will result in restoration of the original anatomical integrity of the bone. In the production of tissue-engineered bone analog, it is advisable to use composite materials for cellular matrices that contain biodegradable synthetic polymers, natural organic compounds, inorganic compounds of bone natural extracellular matrix, and signaling molecules.

Due to the large number of established materials and methods that can be possibly applied to date, further production should move towards a thorough working-out of protocols on development of the final product, the content of which will depend on the particular type of the created construct.

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