Archives of Materials Science and Engineering

Volume 91
Issue 2
June 2018
Pages 56-85

International Scientific Journal published monthly by the World Academy of Materials and Manufacturing Engineering

The new generation of the biological-engineering materials for applications in medical and dental implant-scaffolds

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ABSTRACT

Purpose: The publication aims to find the relationship between the proliferation of surface layers of living cells and the deposition of thin atomic layers deposition ALD coatings on the pores internal surfaces of porous skeletons of medical and dental implant-scaffolds manufactured with the selective laser deposition SLS additive technology using titanium and Ti6Al4V alloy.

Design/methodology/approach: The extensive review of the literature presents the state-of-the-art in the field of regenerative medicine and tissue engineering. General ageing of societies, increasing the incidence of oncological diseases and some transport and sports accidents, and also the spread of tooth decay and tooth cavities in many regions of the world has taken place nowadays. Those reasons involve resection of many tissues and organs and the need to replace cavities, among others bones and teeth through implantation, more and more often hybridized with tissue engineering methods.

Findings: The results of investigations of the structure and properties of skeleton micro-porous materials produced from titanium and Ti6Al4V alloy powders by the method of selective laser sintering have been presented. Particularly valuable are the original and previously unpublished results of structural research using high-resolution transmission electron microscope HRTEM. Particular attention has been paid to the issues of surface engineering, in particular, the application of flat TiO_2 and Al_2O_3 coatings applied inside micropores using the atomic layers deposition ALD method and hydroxyapatite applied the dip-coating sol-gel method, including advanced HRTEM research. The most important part of the work concerns the research of nesting and proliferation of live cells of osteoblasts the hFOB 1.19 (Human ATCC - CRL - 11372) culture line on the surface of micropores with surfaces covered with the mentioned layers.

Research limitations/implications: The investigations reported in the paper fully confirmed the idea of the hybrid technology of producing microporous implants and implant-scaffolds to achieve original Authors' biological-engineering materials. The surface engineering issues, including both flat-layered nonorganic coatings and interactions of those coverings with flat layers of living cells, play a crucial role.

Originality/value: Materials commonly used in implantology and the most commonly used materials processing technologies in those applications have been described. Against that background, the original Authors' concept of implant-scaffolds and the application of microporous skeleton materials for this purpose have been presented.

Keywords: Regenerative medicine, Tissue engineering, Materials engineering, Surface engineering, Biological-engineering material, Implant-scaffold, Selective laser sintering, Titanium alloys, Atomic layer deposition, Flat layers of living cells

Reference to this paper should be given in the following way:

L.A. Dobrzański, A.D. Dobrzańska-Danikiewicz, Z.P. Czuba, L.B. Dobrzański, A. Achtelik-Franczak, P. Malara, M. Szindler, L. Kroll, The new generation of the biological-engineering materials for applications in medical and dental implant-scaffolds, Archives of Materials Science and Engineering 91/2 (2018) 56-85.

BIOMEDICAL AND DENTAL MATERIALS AND ENGINEERING

1. Introduction

In engineering practice, the requirements for product ownership often boil down to provide them exclusively or, among others, on the surface of the product. A commonly used method of improving the surface properties is the application of surface coatings, often multilayer ones [1,2]. In the case of surface engineering, the quality of adhesion or connection of the surface layer to the substrate plays an essential role. The connection may be an adhesive one that often exposes the coating or coating to exfoliation or delamination. In the case of a diffusion joint, its quality is better in that respect. In the case of surface layers, the type of connection generally exists. Of course, in the case of a multilayer, but also somewhat gradient coatings, there is a problem of a connection between individual layers, analogously to the one-layer coating and the substrate. In this case, there are the connections between the individual layers of the covering, between the substrate and the most in-depth coating's layer, as well as between the outer coating's layer and the surrounding environment. The interactions between the surface and the environment, often chemically active, physical or biological, are essential. The issues of surface engineering play an essential role in many areas of life, and not only technologies but also others as among others medicine.

Essential and costly problems of modern medicine include the need to replace or supplement organs or tissues, among others in orthopaedics and traumatology as well as maxillo-facial surgery and regenerative dentistry, to prevent biological and social degradation of patients and restore their vital functions [3,4]. The problem also applies to a large extent of removing the effects of tooth decay, classified as extraordinarily onerous and one of the most expensive diseases of civilisation, including due to the local, but also systemic body's complications [5-13]. Given the increasing share of oncological diseases, there is a need for intensive work to continually search for therapeutic solutions to alleviate the fate of thousands of people affected by those diseases. The patients have the analogous situation in cases of various accidents and as a result of ageing and the resulting surgical removals of various organs including limbs and bones and skin defects, including extensive burns. The maxillo-facial parts as well as numerous dental cavities as a result of injuries and extensive caries also require appropriate surgical operations. It is also essential to simultaneously reduce the economic pressure on the sickness, disability and retirement insurance system, in the face of the inability to return patients to work. It is about protection against serious health threats, and not just such as civilisation diseases, pandemics and bioterrorism, but also to support research, especially using modern technologies, to ensure complete disease prevention and safe treatment of patients, paying attention to the links between health and economic prosperity. The citizens' action programme in that regard recognises their right to their health and healthcare and includes promoting health in an ageing society, protecting citizens against health and life threats, and supporting dynamic health systems and new technologies, especially those related to technical support for medicine, including dentistry.

The paper is a part of the mentioned technical support of medicine and dentistry. The paper concerns the continuation of research into the development of Authors' advanced types of implant-scaffolds [14-28]. Implant-scaffolds are a newly developed original category of pioneering innovative technical devices and implantable materials, representing a completely new class of composite biological-engineering materials developed by the Authors of the paper [14-28]. Implant-scaffolds can help and give hope to thousands of people affected by various diseases, often resulting in the partial or full loss of organs and tissues. The hybrid effort in materials engineering, materials processing, tissue engineering and surgical implantation techniques will provide them with the opportunity to supplement those losses and thus enable them to return to normal or at least quasi-normal life and professional activity. The aim of those activities is a vision to alleviate the fate of people affected by severe illness, including oncological ones. In one procedure, along with the removal of diseased tissues, including bones or teeth, reconstruction could be performed by implanting implant-scaffolds from biological-engineering composite materials with autologous cells of the patient, which would immediately begin to connect and proliferate into his/her tissues after surgical treatment.

2. State-of-the-art and the research aim of the paper

The material solutions proposed in the paper, deriving from a synergy of methods of materials engineering and manufacturing technologies, in coincidence with the Authors' assumptions. It depends on the specificity of biological and medical sciences, clinical conditions and tissue engineering, require a multi-aspect state-of-the-art analysis by the literature review and the resulting specific scientific problems resolve in the paper, including its pioneering character and the impact of its outcomes on the evolvement of research in the mentioned scientific disciplines, as well as societal and economic relevance. The general analysis of the problem is impossible, because of lack of references in the literature, except own Authors' works. Therefore, separate aspects are analysed further in this description. The attention is paid to a substrate with an engineering composite material matrix used for scaffolds and newly developed implant-scaffolds and biologically cellular structures active on it.

The research undertaken in the paper concerns regenerative medicine, which is constantly developing and was first mentioned in 1992 [29]. Those issues have been widely presented in the Authors' works [3,4,14-17]. Regenerative medicine is a relatively new branch of medicine that creates numerous new challenges, including in combating the symptoms and effects of diseases, and even their causes [30-34]. The goal of regenerative medicine is treatment by replacing old and diseased cells with young cells, including tissue engineering methods and cell therapy or regeneration of the body through gene therapy. Tissue engineering, which is technical support for regenerative medicine, was introduced in 1985 [35] and is a discipline of technical sciences that uses medical knowledge and material engineering methods. It aims to develop materials that can restore, maintain or improve the functions of individual damaged tissues or organs [36,37] and the production of functional replacements [38,39] for clinical use [40]. The use of cell-based therapy in medicine is a relatively new concept, as the first successful allogeneic transplant of human hematopoietic stem cells was made in 1968 [41]. The therapeutic strategies include direct transplantation of the desired cell type, taken by biopsy or stem cell culture, in both autologous and allogenic systems. The effectiveness of cell-based therapies depends on maintaining their viability after implantation [42,43]. A review of the current situation indicates the diversity of currently available cell-based treatment methods in clinical trials [44], and the global cell-based therapy market includes revenues of over USD 1 billion per year [45]. The interest in explaining the interaction of the surface structure of engineering materials forming the substrate with the cellular structures applied to it is presented among others in the paper.

In pure therapies, stem cells are directly injected into the peripheral circulation or deposited in specific tissues. In many clinical cases, it is necessary to use stem cell carriers for their transport, especially skeletons to three-dimensional grouping them in a specific area of the body, and research such are continuously developed [46-55]. The concept of scaffolds is quite roomy, because it may refer to a modified extracellular matrices' scaffolds, but also rigid microporous materials in which osteoblasts can grow. The microporous mats of polymer nanofibers could be applied into which live cells grow and they can be used as specific patches, for the treatment of, for example, burns or the reconstruction of large patches of skin [21]. Recent publications [4,19,20] also highlighted the desirability and needed to develop those therapeutic methods about the stomatognathic system. A microscopic, porous structure of the scaffolds is required, allowing the diffusion of nutrients and metabolic products through them. The tasks of scaffolds, including bones' ones, should allow both adherence and migration of cells and ensure the necessary conditions for their growth in the natural microenvironment

[52,56] and the growth of new blood vessels [57-60]. Recently, porous metals have been used in orthopaedic surgery to replace damaged bones, and porous scaffolds are geometrically similar. The scaffolds have similar properties to natural hard tissues [61] which consist of a skeleton penetrated by interconnected pores [62] and in most cases serve as supporting devices [63]. Therefore: (1) biocompatibility is required; (2) an appropriate area for cell adhesion, proliferation and differentiation; (3) a network of open pores of porous structure for ingrowing cells and transferring nutrients and metabolic waste; (4) mechanical properties adapted to the requirements of the surrounding tissues to reduce or eliminate excessive stresses and meet anatomical requirements to avoid mechanical damage [64-68]. Bone regeneration in porous implants in vivo involves the recruitment and penetration of cells from surrounding bone tissue and vascularization [69]. Increased porosity, pore size and pore connectivity are vital factors that significantly improve bone growth and the transport of cells and nutrients, undoubtedly improving the quality of biological processes, but can significantly reduce stiffness and strength of scaffolds [63]. Higher porosity can facilitate those processes and allow bone growth [70,71], although there is still much controversy on the subject [63,72]. Pores sizes of artificially created scaffolds should be small, because they provide more space for the growth of bone tissue cells [63], but not so small that the pores will close [52]. However, they should be large enough to prevent blood clots [73], allow for the migration of cells and provide conditions for filling the pores by the reconstructed cells, guaranteeing neovascularisation [74]. The optimal pore size for growing bone cells is estimated to be 100-400 μm [63,72], although, in the case of pores with sizes of 600-900 μm, bone cell ingrowth is higher [63]. Reproduction of bone properties by porous metals requires the digital design of their cells structure and manufacturing using advanced technologies [75]. The considerations described in the paper relate to new implantable devices called implant-scaffolds (Fig. 1). One of the essential parts are scaffolds, and the other inseparably connected are solid cores, and both of those aspects are essential for the success of the concept.

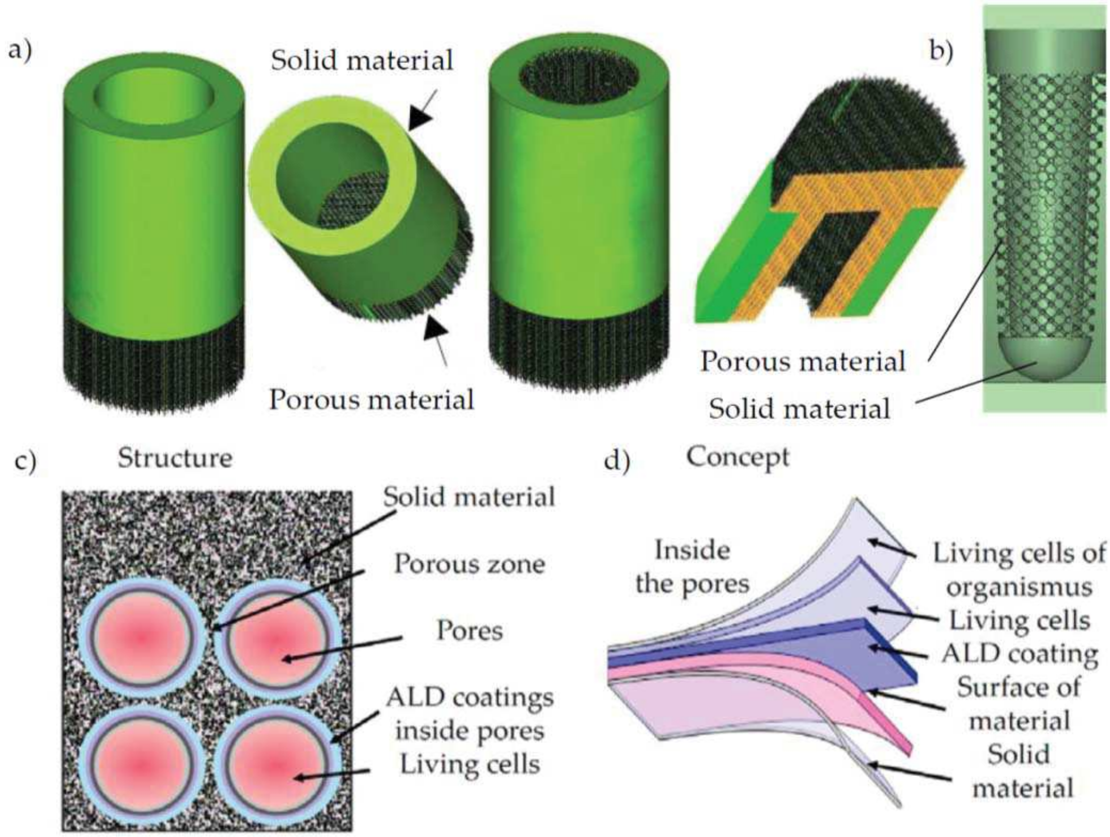

Fig. 1. Scheme of composite biological-engineering materials: a) orthopaedic implant-scaffolds; b) dental implant-scaffold; c) the structure of solid-porous materials; d) the concept of multilayer engineering and biological coatings inside the pores

Although the shape and size of the pores can be regulated by changing the conditions of even traditional manufacturing processes, e.g. casting or powder metallurgy, however, only a randomly organised porous structure can be obtained, and it is generally not fully open or at all [76]. To produce porous scaffolds, as well as other medical implants, including dental implants, additive technologies in combination with previous computer-aided CAD / CAM design/manufacturing are most often used. The additive technologies are highly competitive compared to traditional manufacturing methods, such as casting or cutting [77-82]. The additive technologies have found broad application in the production of various, individualised elements used in medicine. Those elements, among others, could be scaffolds with the required porosity and strength with live cells implanted into the body [84-85], models of dental implants and bridges [86-88], individualised mandibular bone, hip joint, skull fragments [86-96]. The most fabulous opportunities are offered by selective laser sintering (SLS) and its technological variants [78,80,97-106]. Selective laser sintering (SLS) allows providing an open pore structure, e.g. a mesh structure promoting osseointegration while maintaining a variety of external shapes of the entire implant [107]. Selective Laser Sintering (SLS) resembles three-dimensional printing. In earlier own works [16, 19, 20], the state-of-the-art has been presented in the field of materials and technologies for the manufacturing of scaffolds. In those works, the description of the concept of synergistic use of the previous achievements in regenerative medicine and surgery in the field of prosthesis/implantation in the treatment of civilisation diseases and their consequences have been included. The manufacturing engineering and materials engineering in the field design and manufacture of prostheses/implants using and tissue engineering and different engineering materials, including the selection of materials and technologies for the manufacturing of scaffolds also have been presented. It is possible to use various highly specialised technologies, against the results of own previous work in that area [14-28, 108]. The paper presents the results of own research concerning selected engineering materials.

The selection of the right material for the production of porous scaffolds and implant-scaffolds, as well as the concepts of separating the unnecessary fragments of cell-based products after the completing the therapy with them [21], have been analysed in own works [4,19-21]. Regeneration in the natural state forces the removal of the artificial elements [109-111]. Metallic materials are one of the largest groups of engineering materials used for that purpose and include titanium, tantalum, niobium and their alloys, and in dentistry also cobalt-based alloys and precious metal alloys, to which, among others, SLS technologies have been used [112,113]. The corrosion-resistant steels have often been used so far using SLS technology also [76]. One of the main components of austenitic corrosion-resistant steels that can be used for medical purposes is nickel, currently considered one of the most common allergens [114], to which 17% adults [115] and 8% children [116] are sensitised, e.g. in the European Union, approximately 50-60 million people. The element is the cause of many diseases [117,118], including rejection of orthopaedic implants [119], as well as numerous complications related to the implantation of dental implants [120]. Therefore, a directive has been issued in Europe [121] prohibiting the use of nickel and materials with its participation in prosthetic and implants purposes. Co alloys: Co-Cr-Mo, Co-Cr-W and Co-Cr-W-Mo produced using various technologies, including SLS [112,113,122-129], are used, among others for dental restorations [130-134]. The interest in light metals and their alloys is growing. It is pointed out that Mg and its alloys can be used for non-biodegradable scaffolds [135]. Although further studies are needed regarding biomedical applications of Mg in the presence of body fluids [136,137] The use of Ti and its alloys and the SLS technology applied to them is extensive [4,17-20,24-28]. Ti can also be used for non-biodegradable scaffolds [138], including after pore surface treatment [139]. Ti and its alloys do not cause allergic reactions and are resistant to corrosion, show high strength and hardness, as well as thermal conductivity several times smaller than traditional prosthetic materials [140]. The introduction of alloying elements can improve the biocompatibility, especially thrombo-compatibility [141]. The porous Ti6Al4V alloy may be used for non-biodegradable scaffolds [142-146]. In some publications [147-156] information on the toxic effects of V as an alloying element in the alloy have appeared. However, it has been shown by direct comparison under the same conditions that the differences between the effect of Nb and V in Ti alloys are not very significant [154], and even that Ti6Al4V alloy may have better properties than Ti6Al7Nb alloy [154,157-159]. The paper concerns Ti, and Ti6Al4V alloy used to produce implants-scaffolds with the use of additive SLS technology.

Cells grow also affected by texture and surface smoothness [140,160-164]. Technological methods of improving the surface smoothness of materials used in medicine include the addition of surface-active substances, lithography, covering with a layer of self-assembling molecules SAM [161, 167, 168]. Cell adhesion is difficult on less developed surfaces with greater smoothness [169].

The osteoblasts grow much faster on less smooth surfaces [170-172], in contrast to fibroblasts that proliferate more rapidly on more smooth surfaces [173-177]. The topography of the surface of the material affects the ability of cells to adhesion [165,178], while they exhibit the ability to adapt to the specifics of the surface of the material [164,166,179-181]. The wettability of the surface affects the ability of adhesion proteins to attach to the substrate [161]. In general, cells preferentially join, divide and grow on the hydrophilic surfaces of the material, while their ability to adhere to the substrate material decreases on the hydrophobic surfaces [161,179,182-188]. Adhesion and growth of living cells depend on the type and characteristics of the substrate. That is why the idea of producing biological-engineering materials and implants-scaffolds requires searching for the most favourable conditions for the proliferation of living cells inside the pores of the microporous skeleton made of titanium and its alloys and requires improving those conditions. It turns out that they are much more beneficial on the substrate from fully compatible materials, which include TiO_2, Al_2O_3 and hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ [19]. In our works [1,189,190] various technologies of applying surface coatings have been characterized, among others physical and chemical vapour deposition methods PVD, CVD and atomic layer deposition ALD, and in our works [1,191-195], we presented a general methodology for the selection of optimal surface engineering technologies for industrial applications. Among the technologies for applying thin coatings to the internal surfaces of pores, the atomic layer deposition (ALD) technology and the technology of deposition of sol-gel coatings from the liquid phase by immersion are important [1]. Those surface covers and methods for their deposition are used in the paper. The concept of applying thin layers using the ALD method was formulated in the 1970s [187,195] In essence, the ALD method is a variant of the CVD technology, distinguished by the cyclic use of precursors supplied alternately in the form of so-called pulses between which the chamber is rinsed with an inert gas. Ultrathin layers with a thickness of several nanometers are successively applied in several cycles; each cycle increases the thickness of the layer by a precisely defined value of 0.01-0.3 nm. The number of cycles depends on the expected layer thickness. Proper selection of process conditions ensures stable growth of subsequent layers, uniform in each deposition cycle [1]. Deposition of sol-gel coatings from the liquid phase (sol-gel method of nanometric surface layers obtaining) starts with a liquid solution of suitable compounds called precursors and leads to the formation of glass-like materials [1]. The starting material is converted into a sol form in an aqueous medium or a dilute aqueous acid solution. The removal of liquid from the sol leads it into a gel (the sol-gel transition is used to control the shape and size of molecules). The sol-gel coating process is a multi-step process, consisting of hydrolysis of the precursor (obtaining a sol), gelation (condensation), drying and densification. The sols are colloidal systems with a liquid dispersing phase and solid colloidal particles (size 0.1-1 μm). The gel is a two-component system, in which each of components forms a separate continuous phase throughout the entire volume. Calcination (i.e., heating the chemical compound below its melting point) of the gel gives the corresponding oxide. For the application of sol-gel coatings to the surface of processed materials, numerous methods are used, including those used in the present paper and the most frequently used dip-coating method [1]. In the last 20 years, there has been a surge in interest in the use of the sol-gel technique [196-202]. Sol-gel method of deposition of liquid phase coatings can be applied to various types of substrates, not only metals [203-207]. The thickness of the coatings deposited by the sol-gel method from the liquid phase of coatings is usually several hundred nanometers [1]. The sol-gel method allows for uniform coverage of only the surface with low roughness, and the quality of the obtained layer depends to no small extent on the method of preparing the substrates.

Concluding the literature review, it is necessary to say that two separate aspects need to be considered: the selection of engineering materials of which scaffolds are produced, and a porous structure, including the pores size and manufacturing technologies of adequate porous materials. The activities described in the paper envision to create new implantable devices, called implant-scaffolds, in which one of the basic parts are scaffolds, and the other is a permanently linked solid core, and both aspects are of primary importance for the undertaking's success and hence each of them has been analysed based on the state-of-the-art.

The determination of optimum geometric features of scaffolds as a result of computer-aided design in conjunction with the optimisation of technological conditions of the applied additive technology and broad experimental verification in the engineering and biological aspect is of key importance and represents a set of important research issues covered by the paper.

Due to an effect of pH, concentration and type of ions, adsorption of proteins on orthopaedic implants and the biochemical activity of the surrounding cells in the presence of body fluids, the research of titanium and Ti6Al4V alloy was made. Powders are serially manufactured for, SLS technology. The general criteria of materials selection for tissue scaffolds relate to the material type and its structure, osteoconductivity ability, mechanical strength, ease of production and manipulation in clinical applications. The pertinent studies are made in the paper.

The presented biological-engineering materials designed, manufactured and explored in the paper are innovative, and avant-garde and the existing state of the art in this field is negligible. A review of the global and domestic references and the preliminary, limited own investigations' results before preparing this paper indicate that the issue is very interesting. It is justified, therefore, to undertake comprehensive research works in this area which are presented in the paper.

The presented review of the literature indicates that the subject matter concerns a multi-faceted and broad cognitive, biological, medical, material and technological issue. The problem is entirely open and very developmental, and it only addresses its selected aspects. It deals only with surface issues and aims to analyse the relationship between the proliferation of surface layers of living cells and the deposition of thin ALD coatings on the pores internal surfaces of porous skeletons of medical and dental implant-scaffolds, which are the substrates.

3. Production technology, structure and properties of porous skeletons as metallic substrates

Additive technologies as a special method of powder metallurgy are used with very high success for the production of solid materials from many metals and their alloys, as well as other groups of engineering materials and for the production of porous materials. The potential of additive technologies for the production of the microporous materials is much higher than other technologies. Non-waste improves this potential and bringing the whole technology to two main stages of design and manufacturing. The method of selective laser sintering is shown in Figure 2.

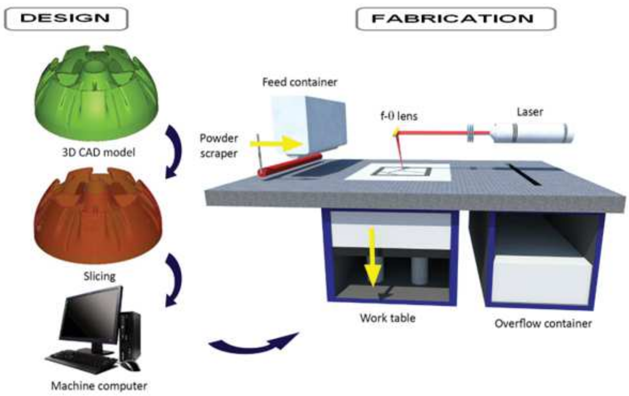

Fig. 2. Scheme of selective laser sintering technology process

The result of designing a given element is a three-dimensional CAD model (computer-aided design) in STL (stereolithography) format, using a layered technique. After giving the model the appropriate size and structure, it is divided into layers of assumed thickness. The number of layers in the virtual model corresponds to the designed number of powder layers, during the actual execution of a given element, which will be sintered to obtain a ready implant or implant-scaffold.

The software also allows to set manufacturing conditions, including layer thickness, laser power, laser beam diameter, scanning speed, a distance between successive laser remelting paths. When designing microporous titanium and titanium alloy skeletons, a set of basic unit cells is used. The base cell, which has been selected for further testing as a result of experimental optimisation, is shown in Figure 3.

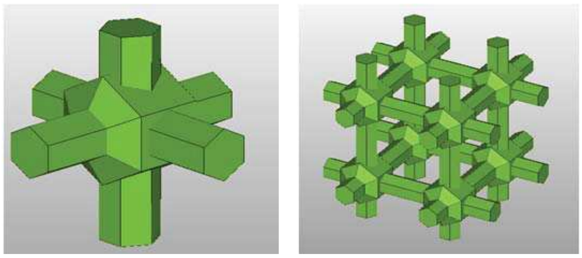

Fig. 3. Hexagonal cross-unit cell

Unit cells are multiplicate for obtaining microporous skeletons with symmetrical spatial network built of nodes and individual network fibres connecting individual skeleton nodes. As a result of multiplication and selection of appropriate values characterising the spatial network, such as height, depth and width, it is possible to define the structure of microporous skeletons with the desired pore size, for example in the range of 250-750 µm. The orientation of unit cells relative to the work platform plays an important role (Fig. 4). It is also possible to design solid elements and solid-porous elements, such as implant-scaffolds.

After designing the model, taking into account all the established production conditions, the designed model is transferred to the machine software, where the selective laser sintering of the powder takes place. Powders of pure titanium and Ti6Al4V titanium alloy are the input material. Both examined materials received appropriate certificates, enabling use for medical and dental purposes, among others in Poland. The chemical composition of powders is given in Table 1, also confirmed by spectral studies using the Energy Dispersive Spectrometry EDS method. The shape of the powder grains is spherical (Fig. 5).

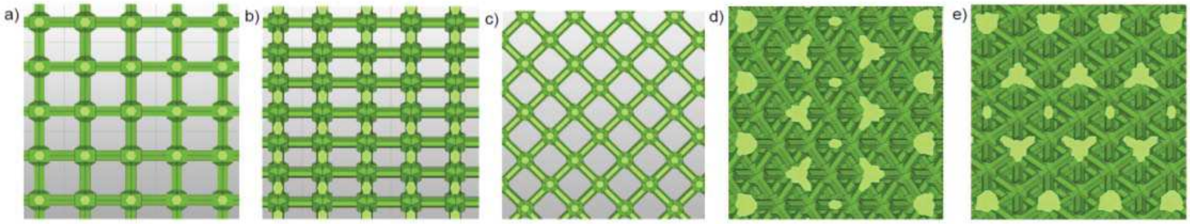

Fig. 4. Image of the structure of computer models showing the arrangement of unit cells in the space of the coordinate system: a) unit cells arranged at an angle of 0° relative to the coordinate system, b) unit cells arranged at an angle of 45° with respect to the axis x of the coordinate system, c) unit cells arranged at 45° to the y-axis of the coordinate system, d) unit cells at an angle of 45° to the x-axis and 45° to the y-axis of the coordinate system, e) unit cells at an angle of 45° to the y-axis and 45° to the x-axis of the coordinate system

Table 1.
Mass concentration of chemical elements in powders subjected to experiments

Powder	Mass concentration of elements, %									
	Al	V	C	Fe	O	N	H	all others together	any other	Ti
Ti	–	–	0.01	0.03	0.14	0.01	0.004	< 0.4	< 0.01	rest
Ti6Al4V	6.35	4.0	0.01	0.2	0.15	0.02	0.003	≤ 0.4	≤ 0.1	

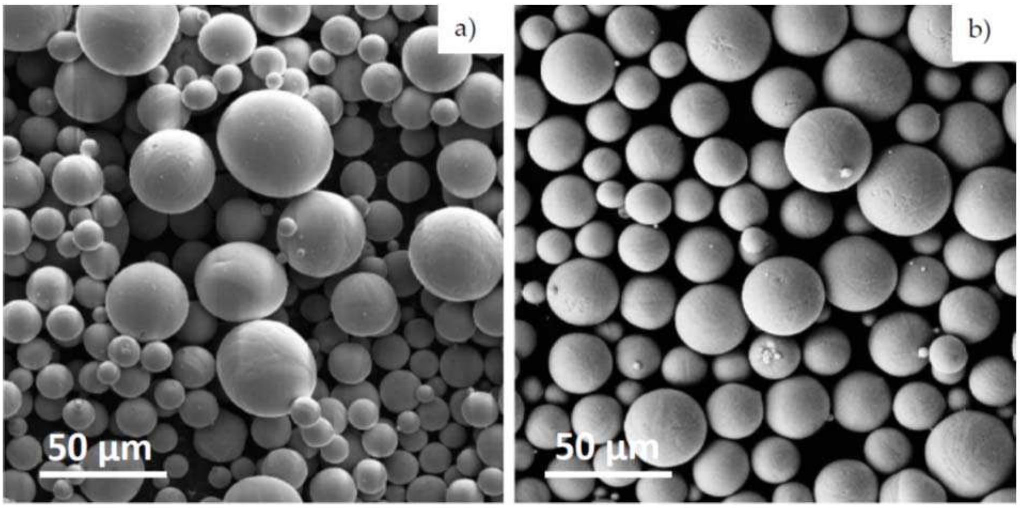

Fig. 5. Microscopic images of powders used for experiments of a) Ti, b) Ti6Al4V alloy (SEM)

For the production of porous micro skeletons using the SLS method, the RENISHAW AM 125 system integrated with the AutoFAB computer software has been used. The technological device uses a fibre YFL laser with active yttrium-doped material and a maximum power of 200 W. The system is equipped with a vacuum chamber in which previously designed elements are manufactured. Porous samples of the shape given in Figure 6 have been made.

The experimental basis for the comparison of the skeleton structure of microporous materials made of pure titanium, but also of the TiAl6V4 alloy produced by selective laser sintering are materials produced using different laser power 200, 175, 150, 125, 100, 75, 65, 60 W and with different diameters laser spots 30, 70, 110, 150, 170 and 200 µm. The selection of these basic manufacturing conditions has a fundamental impact on the surface quality of the microporous material. The essential technological issues include experimental determination of the correlation between the size of micropores calculated by the AutoFab computer program and the actual sizes of these pores, which are possible to ensure as a result of selective laser sintering based on such calculated models (Fig. 7). Using the virtual unit of the element predicted for the production by the method of selective laser sintering, using the basic hexagon cross-unit considered the most advantageous of the considered and assuming its subsequent dimensions 1x1x1 mm, 0.9x0.9x0.9 mm, 0.8x0.8x0.8 mm, 0, 7x0.7x0.7 mm, 0.6x0.6x0.6 mm and 0.5x0.5x0.5 mm, the conditions for the production of microporous skeletons, including the power and diameter of the laser spot, were selected (Fig. 7). The accuracy of the production of microporous elements increases with increasing the size of the base unit cell, which is associated with an increase in the diameter of the laser spot.

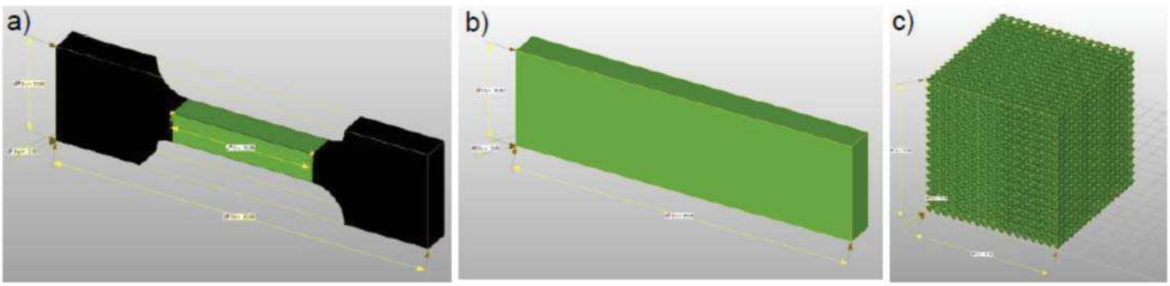

Fig. 6. Image of a computer model of samples for static tests: a) tensile, b) three-point bending, c) compression; b,c) samples for testing of surface layers and their structures; b) samples for biological research

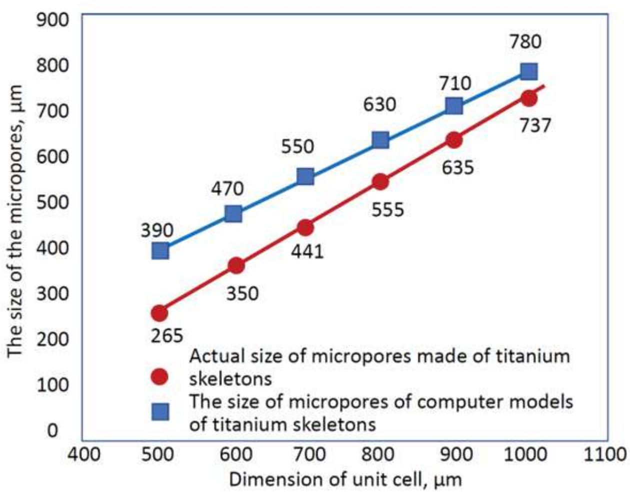

Fig. 7. Diagram of pore size dependencies designed using AutoFab software and titanium micro skeletons produced by selective laser sintering from the unit cell base dimension

In the production of microporous elements with models composed of base unit cells with sizes 500-700 µm, the resulting actual pore size is more than 100 µm smaller than the size designed in the AutoFab computer program, while for micro-cutlets with larger base unit cell sizes 800-1000 µm the precision of the calculations is higher, and the differences between the pores have been experimentally found in the range of 40-75 µm. For even smaller sized cells, the accuracy of calculations is even worse. The series of experimental tests made enables the use of the correction due to the identified deviations from the data derived from the program calculations (Fig. 7). The pore sizes in the produced microporous scaffolds of titanium depend to a large extent on the thickness of the individual fibres of the titanium frame backbone. The difference in the thickness of single fibres of the titanium micro titanium net produced from unit cells with various sizes of 0.5-1 mm is small and amounts to about 30 µm, which corresponds to the average grain size of the powder used for sintering. However, the size of the produced pores and their number change with the increase of the base size of the unit cell in the range of 0.5-1 mm. By using a unit cell of larger sizes, larger pores and smaller numbers are obtained, and vice versa. With an increase in the number of pores with smaller unit sizes, the mass of the skeleton produced increases, which in turn affects the strength properties of titanium micro-brooches, presented in one of the following sub-chapters.

All these factors have an indirect or direct impact on the selection of necessary conditions for the production of microporous titanium skeletons, i.e. laser power and laser spot diameter. The too large diameter of the laser spot 170 and 200 µm prevents the creation of any skeleton, due to the thickness of the fibre, which in the case of a unit cell size 1x1x1 mm is 220 µm. In turn, due to the small diameter of the 30 and 70 µm laser spot, the pores in the manufactured skeleton are practically non-existent, and even though the material produced in such conditions is hardly considered solid, the pores in it are almost invisible. The 200 W laser power enables the creation of a microporous backbone in the case of the laser spot diameter 110 and 150 µm, and the pore sizes that are 301.70 and 416.43 µm respectively (Tab. 2). It was found that during selective laser sintering of titanium micro titaniums composed of unit cells with dimensions 1x1x1 mm, the actual diameter of the laser spot is 150 µm. At the same diameter of the laser spot, reducing the laser power in the range of 200 to 60 W causes an increase in the pore size (Tab. 2) to reach ~ 740 µm at a power of 60 W, which is closest to the computational value of ~ 780 µm.

Important technological factors include the ratio of the laser spot diameter to the distance between individual laser spots, defined as laser path I or II, and the distance between the laser tracks (Fig. 8).

Table 2.
Pore sizes of titanium skeletons obtained at a different diameter of the laser spot and different laser power

<table>
<tr><td rowspan="3">Laser power, W</td><td rowspan="3">200</td><td colspan="6">Diameter of the laser spot, µm</td></tr>
<tr><td>30</td><td>70</td><td>110</td><td>150</td><td>170</td><td>200</td></tr>
<tr><td colspan="2">no visible pores</td><td>301.70</td><td>416.43</td><td>–</td><td>–</td></tr>
<tr><td rowspan="3">Diameter of the laser spot, µm</td><td rowspan="3">150</td><td colspan="8">Laser power, W</td></tr>
<tr><td>60</td><td>65</td><td>75</td><td>100</td><td>125</td><td>150</td><td>175</td><td>200</td></tr>
<tr><td>743.63</td><td>692.63</td><td>667.14</td><td>650.16</td><td>616.15</td><td>548.13</td><td>433.43</td><td>416.43</td></tr>
</table>

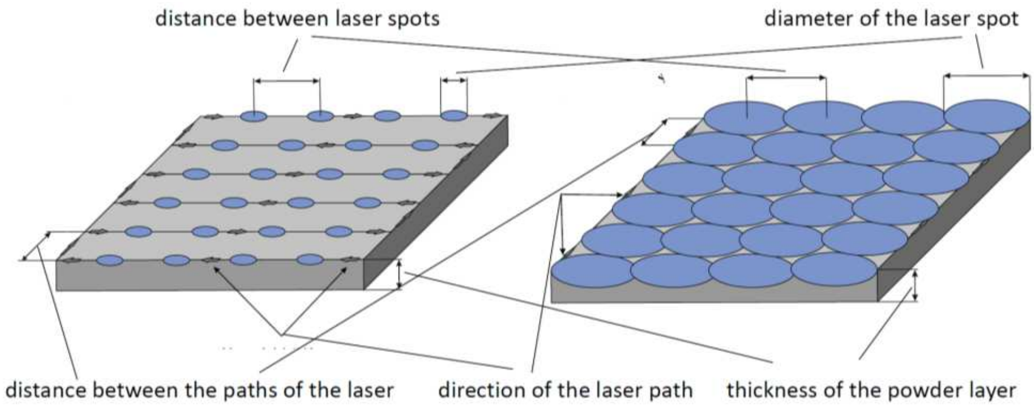

Fig. 8. The characteristics of the laser beam work with the diameter of the spot: a) smaller than the distance between the laser spots, b) greater than the distance between the laser spots

Regardless of the pore size ~ 450, ~ 350 and ~ 250 µm, using the first laser path the diameter of the spot is smaller than the distance between the laser spots and the distance between the laser tracks, whereas if the diameter of the laser is larger than the distance between the laser spots and distances between its laser tracks, defined as II (Fig. 8). In this case, the structure of the microporous titanium is uniform and consistent, with the marked path of the laser to powder and the boundary between the laser tracks, occasional empty spaces occur, and the number of closed pores is significantly smaller than at the laser path I settings. Both porosities, as well as any other structural features of titanium microspheres produced by selective laser sintering, depend in no small extent on the spatial arrangement concerning the axis of the unit cell coordinate system designated as the most advantageous (Fig. 4). The method of arranging the produced sample relative to the plane of the work platform in the production equipment is also of great importance when developing the technology for the production of titanium micro briquettes. In the case of production of elements with successive sintered layers of powder arranged parallel to the work platform, when the laser beam is perpendicular to the manufactured element, there may be no connection between the grains of the next powder deposited with the previous sintering layer, which significantly weakens the connection of individual layers in the produced porous element. A significant improvement of the situation occurs when the element is manufactured by arranging the individual powder layers in parallel with the work platform, with the product being laid at an angle of 45° relative to the work platform. Such a positioning angle on the working platform in the working chamber of the production device also allows an even distribution of the laser energy acting on the powder and a reduction in the number of supports – mainly in the middle part of the volume of the element that is attached to the platform surface. Limiting the number of supports is of great importance because in the process of their removal damage to the porous structure of the manufactured element may occur. This important technological aspect is important due to its mechanical properties.

The process of elements constitution begins with the distribution of the powder layer on the table with an adjustable position relative to the z-axis. The layer acts as a substrate for the resulting object. The laser beam is guided over the surface of the powder by the previously introduced and appropriately configured information regarding successive layers of the cross-sectional spatial image of the object. Then the table with the powder is lowered by the height set by the user, corresponding to the thickness of the layer. Another thin layer of powder is distributed, where the grains sinter, which occurs through the surface melting of the grains of the new metal powder with the already existing fragment of the constituted element. Subsequent layers of the cross-section are sintered together. The cycle is repeated until the constitution of the assumed element is completed, followed by a lowering of the temperature, and the produced element is removed from the powder bed.

On the surface of sintered micro skeletons of the titanium and Ti6Al4V alloy, there are also grains of powder loosely connected to the skeleton, which have not been fully melted with the micro-skeleton, as well as the effects of local melting. The topography of the surface of the porous skeletons is exemplified in Figure 9. To remove those unfavourable surface effects, the porous element after selective laser sintering has been pre-treated in an

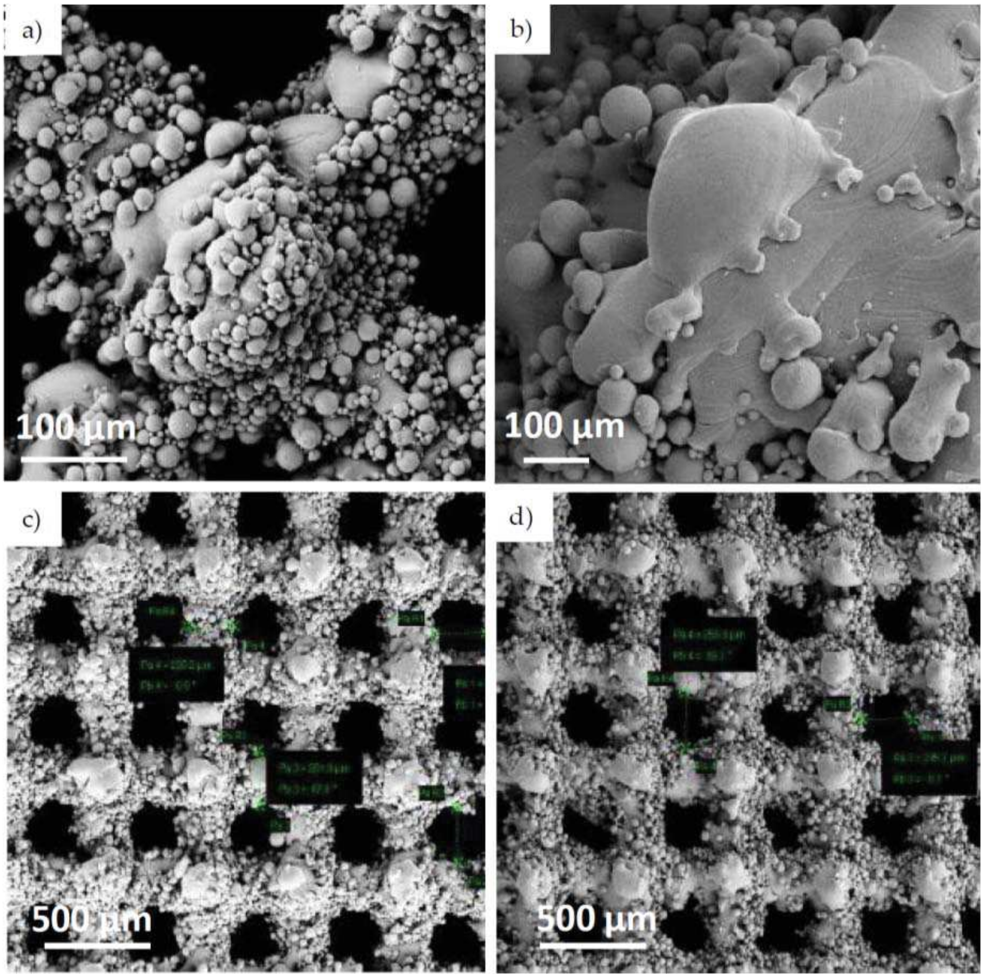

Fig. 9. Topography of porous surface of titanium skeleton with pore size ~ 450 µm and arrangement of unit cell: a) 45° with respect to x-axis, b) 45° with respect to x-axis and 45° with respect to y-axis, c) d) with pore size ~ 250 µm, c) after ultrasound cleaning, d) after etching in a royal water (SEM)

isopropanol solution using an ultrasonic cleaner (Fig. 9). After removing the excess powder from the pore of the titanium skeleton, it has been etched in a solution of royal water with a volume ratio of 3: 1 HCl: HNO_3, for 1 hour using an ultrasonic cleaner (Fig. 9), and alternatively in a 14% aqueous solution of hydrofluoric acid. In the case of TiAl6V4 alloy, etching in an aqueous hydrofluoric acid solution has been more effective.

Selective laser sintering is a complex thermophysical process, the course of which depends on the type of material, laser's and environment's characteristics, and the kind of manufactured elements. The laser power, laser spot diameter, size of the laser spots, distances between laser spots and between successive laser remelting paths as well as the thickness of the powder layer should be adequately selected. It is crucial whether the distance between the laser spots and the distance between the laser remelting paths is higher than the laser spot diameter, or the distance between the laser spots and the distance between the laser remelting paths is equal to or smaller than the laser spot diameter. The essential technological issues also include the experimental determination of the correlation between the size of micropores calculated by the computer software and the actual sizes of those pores, which are possible to ensure as a result of selective laser sintering based on such designed computational models. Experimental tests allow for the correction due to identifying differences between the data derived from the model calculations. The pore sizes in the made microporous scaffolds of titanium or Ti6Al4V alloy depend to no small extent on thickness of individual fibres of a metallic frame backbone. By a wide range of experimental research, technological conditions have been selected taking into account the abovementioned factors. The tests have been carried out on a universal strength machine, in principle, corresponding to static tensile tests, three-point bending and compression, using miniaturised solid and porous samples (Fig. 6). It has been shown that irrespective of the pore size in the examined range, the strength properties of the laser sintered materials tested demonstrate mechanical properties that enable the use of scaffolds and implant-scaffolds. The course of the tensile, compressing and bending curves of microporous titanium or Ti6Al4V alloy scaffolds selectively laser-sintered depending on the power, and various laser path is exemplified in Figure 10.

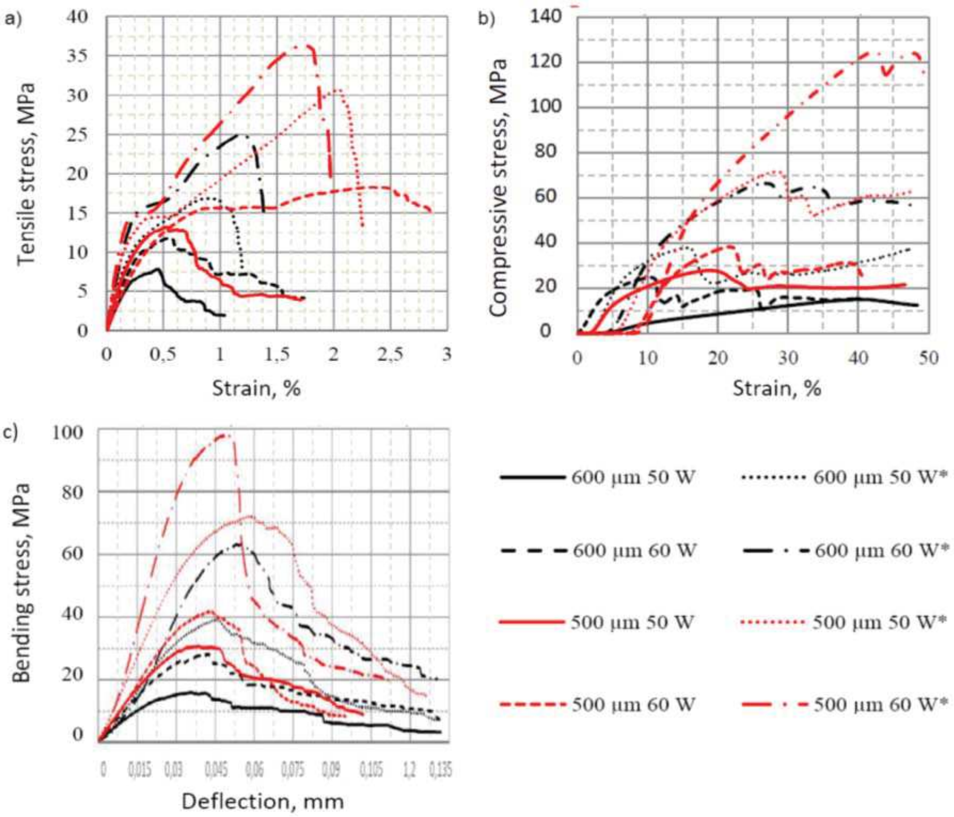

Fig. 10. Strength diagrams a) for tensile b) for compressing c) for bending of selectively laser sintered titanium microporous skeleton with a unit cell size of 500 and 600 μm in unit cell situated with 45° relative to the x-axis produced using different laser power 50 and 60 W and with different laser paths: I* (non-contacting) and II* (partially overlapping)

The skeleton microporous materials produced by selective laser sintering have a porosity depending on the manufacturing conditions, mainly on the laser power and laser spot diameter, and on the distance between the laser spots and the distances between the laser remelting paths (Fig. 8). With the use of 110 W laser power, it is possible to obtain solid titanium with a density of 4.51 g/cm^3 corresponding to the density of solid titanium given in the literature. The smallest porosity of 61-67% corresponds to the most weight laser-sintered skeletons with average pore sizes ~ 250 µm, while the largest porosity of 75-80% corresponds to the lowest weight and average pore size ~ 450 µm. The average reduction in the pore size by 100 µm reduces the porosity from 5-9%. The spatial orientation is of the basic unit cells relative to the coordinate system for the 0°, 45° x, 45° y and 45° xy layouts of slightly ± 1% influences the differences in porosity and mass of the manufactured elements. The spatial arrangement of unit cells of 45° yx causes a 6% difference in the size of porosity compared to other spatial orientation variants with an increase of element weight by up to 20%. It has been found out that the higher the porosity of the metallic micro skeleton, the lower the strength and vice versa. Other detailed information is contained in earlier own publications [1,17-20].

The skeleton microporous materials produced by selective laser sintering from titanium powder and Ti6Al4V alloy are composed exclusively of the lath martensite solid solution grains of the titanium α phase, which has been confirmed by X-ray analysis. The presence of the titanium α phase has also confirmed the results of diffraction studies in the high-resolution transmission electron microscope HRTEM (Fig. 11).

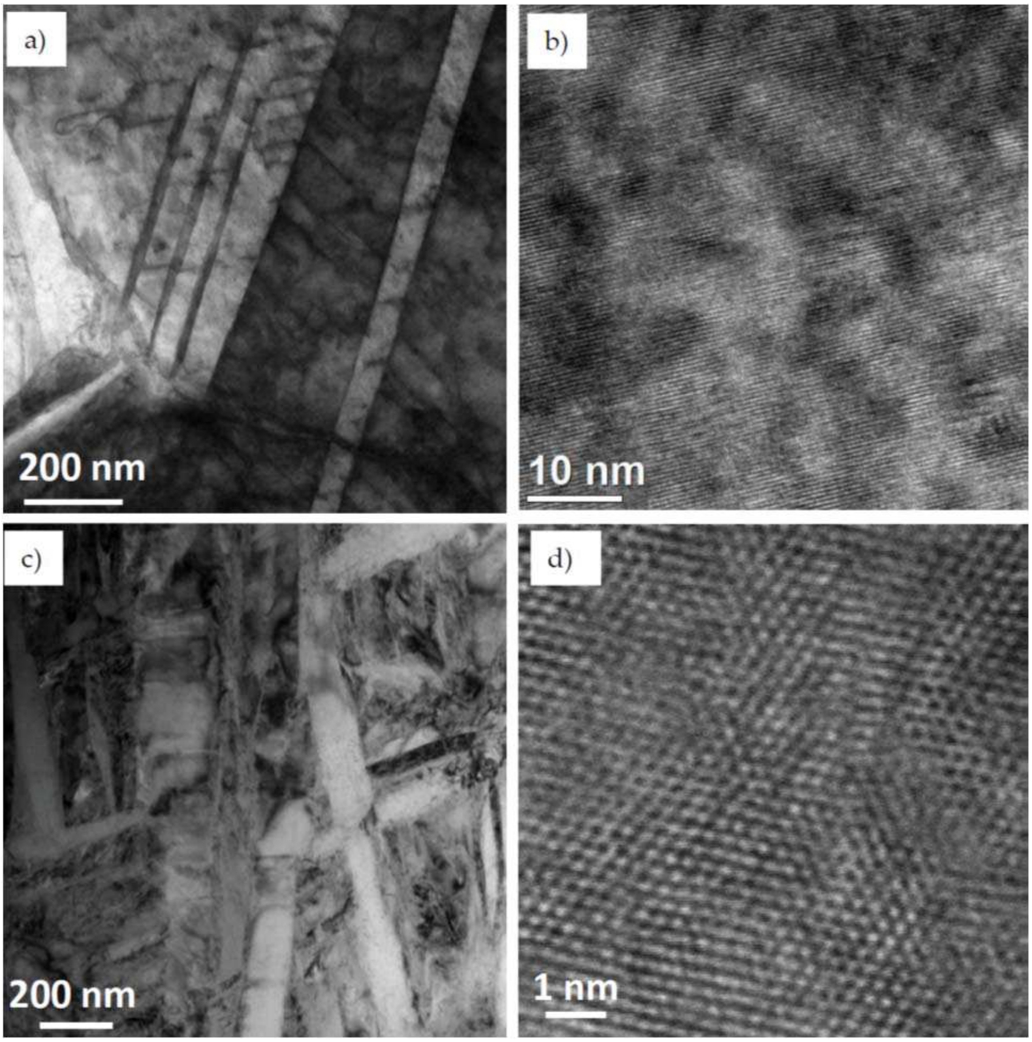

Fig. 11. Crystalline structure of lath martensite: a, b) pristine titanium c, d) Ti6Al4V alloy (HRTEM)

Porous materials from both tested Ti and Ti6Al4V materials produced by selective laser sintering have been the basis for applying appropriate coatings on internal pore surfaces.

4. Coatings technology inside the pores skeletons as metallic substrates

Both pure Ti and its Ti6Al4V alloy, intended to be installed in the human body as implants or implant-scaffolds belong to the currently widely used in medicine and dentistry. The reason for those applications is their low density, favourable strength to yield strength ratio, good corrosive resistance and bio-compatibility. The next improvement of properties of those materials, such as biotolerance and osteoconductivity, is possible due to the use of surface treatment. The permanent application of thin layers to the surface of implants made of metal biomaterials intended for long-term use in the human body is carried out with various methods. In the study, the atomic layer chemical vapour deposition ALCVD method also called atomic layers deposition ALD, as well as the sol-gel dip method, has been used. In the case of applying layers to the surface of porous biomaterials with complicated shapes, it is essential to control precisely the mechanisms of growth. The process controlling allows for the creation of a very thin layer with thickness measured at the nanoscale, but above all, it is crucial to cover geometrically complex surfaces on all sides evenly.

The use of the ALD method allows to achieve such defined final effects, which justifies the choice of the method to cover the microporous surface of the metallic skeletons for medical and dental applications. The internal surface of pores from pristine titanium and Ti6Al4V alloy has been subsequently coated with layers of TiO_2 and Al_2O_3 in the process of deposition of individual atomic layers. An application of the ALD monolayers is preceded by careful preparation of the substrate including degreasing in water with detergent, purification successively in methanol and ethanol using an ultrasonic cleaner and drying in a laboratory drier to evaporate the alcohol. In the role of reference material that allows for the comparison of surface topography and thickness of ALD layers on the treated surface, the BK7 microscope slide and polished monocrystalline silicon have been used.

Monolayers of TiO_2 and Al_2O_3, respectively, have been deposited with the ALD method using the Picosun R-200 system. Layer deposition takes place in a reaction chamber, into which precursors are alternately introduced. The ALD R-200 reactor is equipped with a double chamber system, consisting of a reaction chamber placed inside the vacuum chamber. The advantage of the solution is the ease of changing the reaction chambers without the need to change the vacuum chamber. The precursors may initially be in the solid, liquid or gaseous phase. In the first two cases, it is necessary to ensure the optimum temperature for the formation of a gas phase with adequate vapour pressure. The precursor lines are heated before the gases are transported to the reaction chamber, which improves the quality of the applied layers. The solution also prevents condensation on the walls of the vacuum chamber and its corrosion. The use of double chambers causes the temperature of the reaction chamber to be the same as the precursors, which prevents side reactions inside the reaction chamber. The ALD reactor is adapted to deposit layers under an inert gas atmosphere, which is also a carrier gas. Own preliminary research has determined experimentally technological conditions for deposition of single atomic layers of TiO_2 and Al_2O_3. The carrier gas velocity, time of introducing the precursor, time of reagent introduction and time of chamber rinsing after dispensing of the precursor and reagent belong to those factors (Tab. 3).

The selection of relevant precursors plays an important role in the process of applying atomic monolayers. The highly reactive precursors immediately react with the substrate to form a monolayer and do not allow for further reaction. The implementation of each cycle means the increase of the thickness of the layer by a strictly defined value in the range of 0.01-0.3 nm. The number of cycles depends on the expected thickness of the layer. Table 3 lists the precursors used in this paper.

Table 3.
Precursors used with the ALD method together with appropriate reagents

No	Material	Precursor	Reagent
1	TiO_2	$TiCl_4$, $Ti(OMe)_4$	H_2O
2	Al_2O_3	$AlCl_3$, $AlMe_3$	H_2O, O_3

The most important technological condition for the deposition of individual atomic layers by the ALD method is the temperature that allows controlling the mechanism of growth of the applied layer. The influence of temperature on the layer deposition process can be described thanks to the introduction of the so-called temperature window. Incorrect temperature selection may result in a significant slowdown of the layer's growth and its low stability. The temperature in the chamber must be high enough to prevent condensation of the reagents. If the temperature is too low, the activation energy will not be reached, which may result in incomplete bonds in the monolayer. At too high temperature decomposition of the reagents to volatile products may occur, which in turn slows down the growth of the layer, and the formed monolayer may be unstable, and as a consequence, its evaporation may take place. Variable technological conditions are the deposition temperature within the range of 200-400°C and the number of cycles performed ranging from 500 to 1500 (in the case of TiO_2) or 2000 (in the case of Al_2O_3). Temperature control and the number of cycles allows for the control of the deposition rate and thickness of the deposited layers.

Thin layers of hydroxyapatite have been deposited by the sol-gel method with the dip coating technique. The sol-gel coating process is a multi-step process, consisting of hydrolysis of the precursor (obtaining a sol), gelation (condensation), drying and densification. The thickness of the applied coatings is usually several hundred nanometers. The sol-gel method allows for the uniform coverage of only the surface with low roughness. The quality of the obtained layer depends in no small extent on the method of preparing the substrates. Hence, the coated substrate is degreased, and then rinsed with an ultrasonic cleaner inappropriate compounds (e.g. acetone and methanol) and dried using a laboratory centrifuge. The first step in the production of sol-gel coatings is the preparation of colloidal solutions (sols) as a result of the hydrolysis and condensation of the used precursors. The solution has been prepared using nanopowder hydroxyapatite (HA), polyethene glycol (PEG), glycerol and ethyl alcohol. Thin layers of hydroxyapatite have been made using a PTL-MMB01 device for immersion coatings equipped with holders for fixing samples with a maximum length of 80 mm. The coated substrates are dipped during deposition at a maximum speed of 200 mm/s. The maximum soaking temperature is 200°C.

The deposition of individual TiO_2 and Al_2O_3 atomic layers, as well as hydroxyapatite coatings using the sol-gel method on the surface of pristine Ti and TiAl6V4 titanium alloys, has been confirmed by investigations, including topographical survey results, surface morphology, qualitative chemical composition, and X-ray structural analysis. Also, the porous skeletons with the layers have been subjected to microscopic examination using the Atomic Force Microscope (AFM) of the XE-100 Park System, and the thickness of the deposited layers has been examined using a Sentech ellipsometer, equipped with specialised software. To obtain accurate information on the structure of deposited layers, tests have been carried out using the Renishaw inVia Reflex device, which is an automated Raman Spectrometer (RS), Leica's Research Grade microscope, TITAN 80-300 high-resolution transmission electron microscope FEI. Microscopic examination has been preceded by the preparation of samples for testing in the form of thin foils using the ionic scanning microscope (Focused Ion Beam – FIB). The results of those studies have been extensively discussed in earlier own Authors' publications [1,17-20].

The ALD method allows the coating to be applied very evenly over the entire surface of the workpiece, also if this element has a porous structure, as is the case with skeletons.

An interesting phenomenon observed with the bare eye and in the Discovery V12 Zeiss stereoscopic microscope, enabling the viewing of coloured enlarged images is the colour change of the sample depending on the number of ALD cycles performed, and thus the thickness of the applied TiO_2 layer (Fig. 12). The silver-metallic uncoated element, surface-treated using the ALD method becomes successively brown-gold (500 cycles), dark blue (1000 cycles) and blue with a silvery shade (1500 cycles).

Measurements of the thickness of the layers using a spectroscopic ellipsometer for each sample have been made in 25 places, and statistical calculations have been made, which has allowed making a series of three-dimensional histograms and two-dimensional maps of the thickness distribution of embedded atomic layers (Fig. 13). In the analysed cases regarding the implementation of 500, 1000 and 1500 cycles, the average thickness of the layers deposited by the ALD method is 55.95; 98.90 and 148.73 nm. The difference in thickness of deposited TiO_2 layers in the test area does not exceed 2 nm. The surface of the scaffold contains unevenness measured on a nanometric scale, the number of which increases in direct proportion to the number of layers applied. In particular, the layer deposited during 500 cycles is characterized by a somewhat even granular structure, and only sporadically there are more large concentrations of atoms. In the case of a layer deposited during 1000 cycles, clusters of atoms with a diameter of about one µm occur, spaced every few microns. The largest atomic aggregations that form "islands" with a length up to several microns appear in the case of a layer deposited during 1500 cycles (Fig. 13).

The nanometric thickness of the TiO_2 layers deposited by the ALD method makes those layers possible to be observed in the scanning electron microscope only at very high magnifications of about 150 kx. The surface of the scaffold immediately after production is smooth with distinct longitudinal strips spaced every few dozen / several hundred nanometers, corresponding to the laser's moving direction. The applied TiO_2 atomic layer is visible, at approximately 150 kx magnification, as an "orange peel" (Fig. 14), i.e. a collection of numerous adjacent oval granules, of which a larger diameter distinguishes only a few.

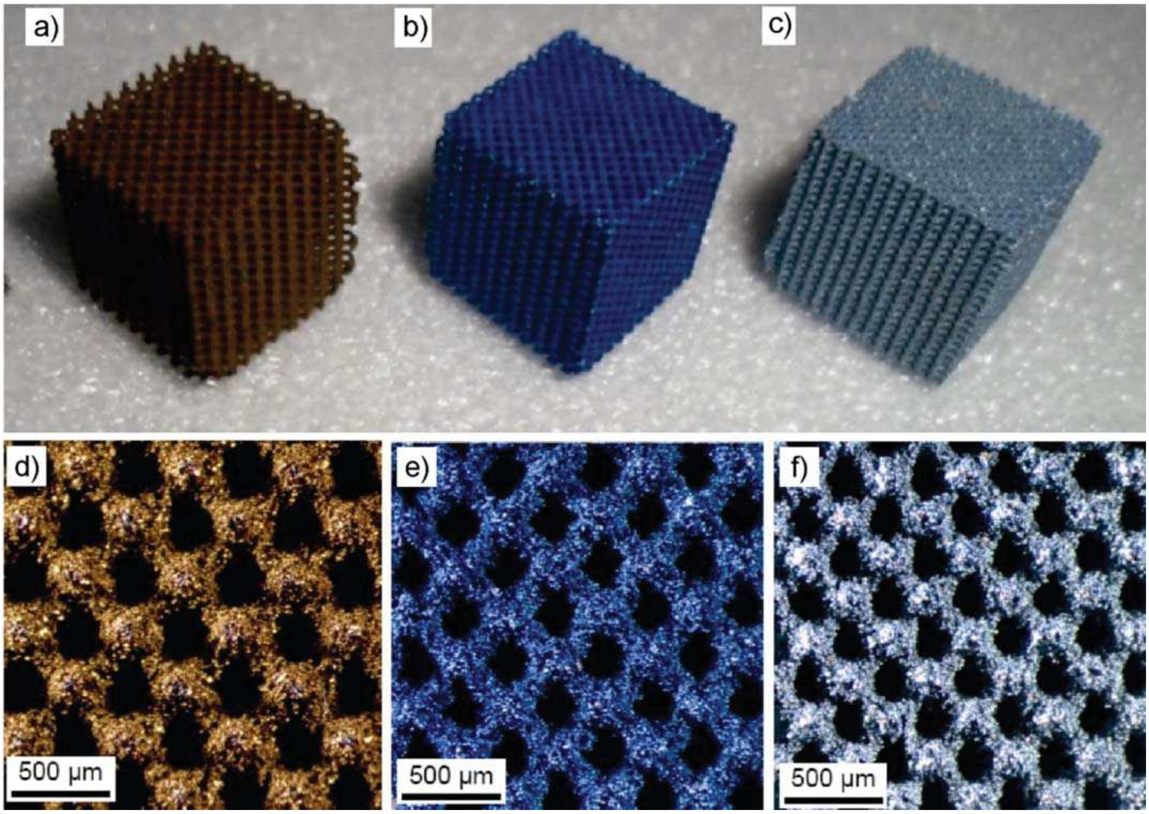

Fig. 12. a-c) Cubic scaffolds viewed with a bare eye, d-f) stereoscopic scaffold images; scaffolds coated with TiO_2 layer after a,d) 500 cycles; b,e) 1000 cycles; c,f) 1500 cycles

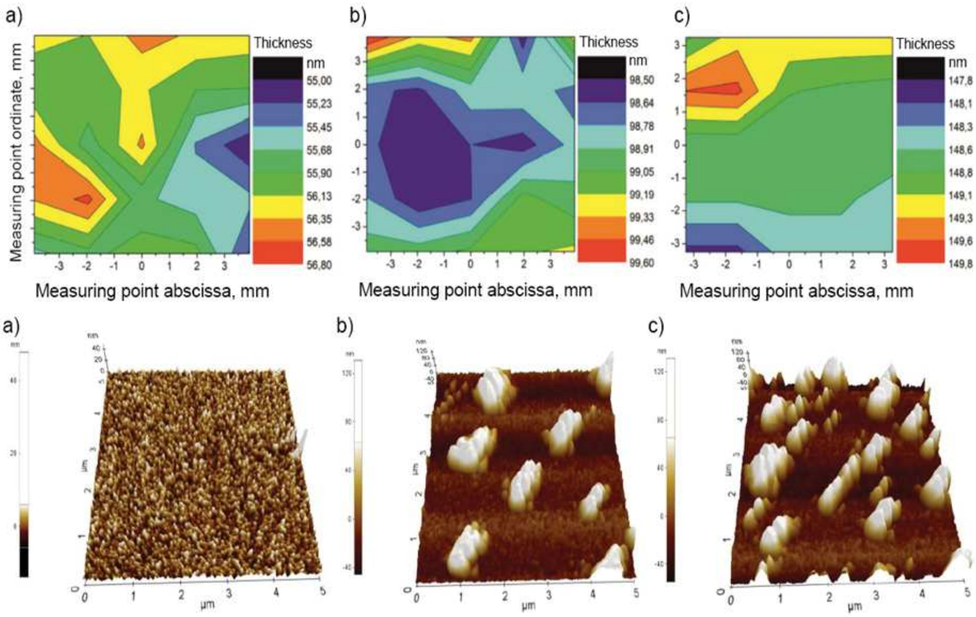

Fig. 13. Thickness deposition maps of the TiO_2 layer deposited by ALD on titanium and AFM image of 3D surface topography a) 500, b) 1000, c) 1500 cycles

Research carried out in a high-resolution transmission electron microscope (Figs. 15 and 16) indicate that the TiO_2 layer deposited to the surface of the scaffolds from the investigated materials has an amorphous structure, in contrast to the crystalline structure of titanium and Ti6Al4V alloy visible in the TEM images. Depending on the number of cycles, the thickness of TiO_2 layer applied by the ALD method varies from several dozens to one hundred and several dozen nanometers. The constant angle of incidence method examined the deposited TiO_2 layers due to its small thickness not exceeding 150 nm, thereby suppressing peaks originating from the substrate. Reflections from three polymorphic forms of titanium dioxide: anatase, rutile and brookite have been identified in the study.

Scaffolds produced by selective laser sintering from titanium powders and Ti6Al4V alloy have also been covered with a thin layer of Al_2O_3 in the deposition process of single ALD atomic layers (Fig. 17). Scaffolds on which Al_2O_3 layers have been deposited during 500 cycles are dark brown, those on which Al_2O_3 layers were deposited during 1000 cycles are navy blue, and those on which Al_2O_3 layers have been deposited during the 1500 cycles are dark blue. Examination of the surface morphology of the produced layers using scanning electron microscope with the highest magnification allows concluding a clear difference between the uncoated scaffolds surface and the surface of the scaffolds with the deposited aluminium oxide layer. The thickness of the layers is changed from about 10 nm in case of 500 cycles, to about 200 nm, when the layers

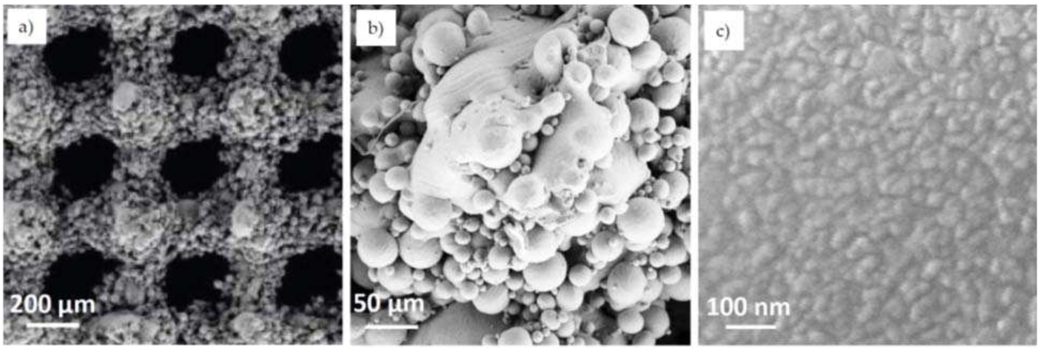

Fig. 14. Structure of scaffold surface; a-c) with TiO_2 layer deposited after 1500 cycles (SEM)

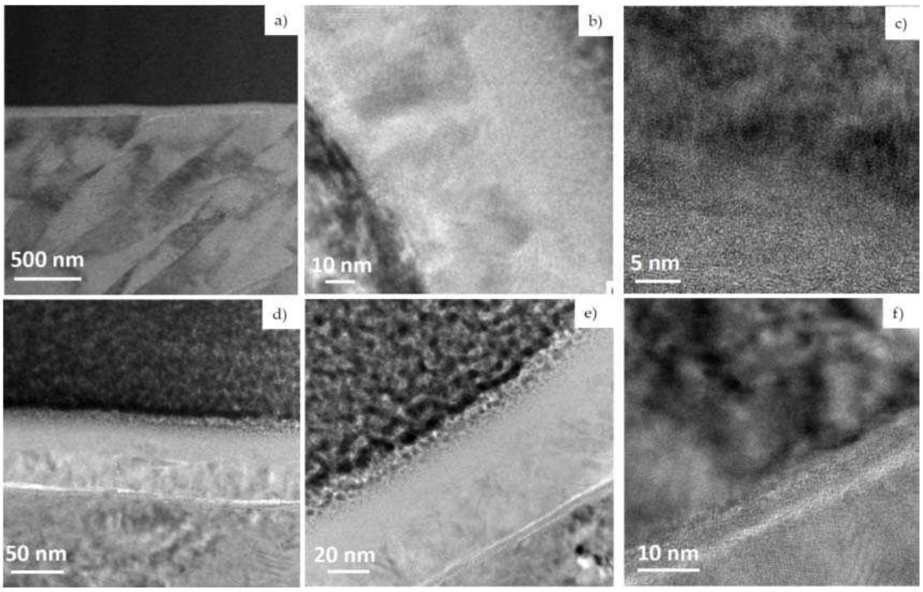

Fig. 15. Structure of TiO_2 layer deposited after 1500 cycles and bonding zone of crystalline a-c) titanium and d-f) Ti6Al4V substrate matrices with visible rows of atoms with an amorphous TiO_2 layers (HRTEM)

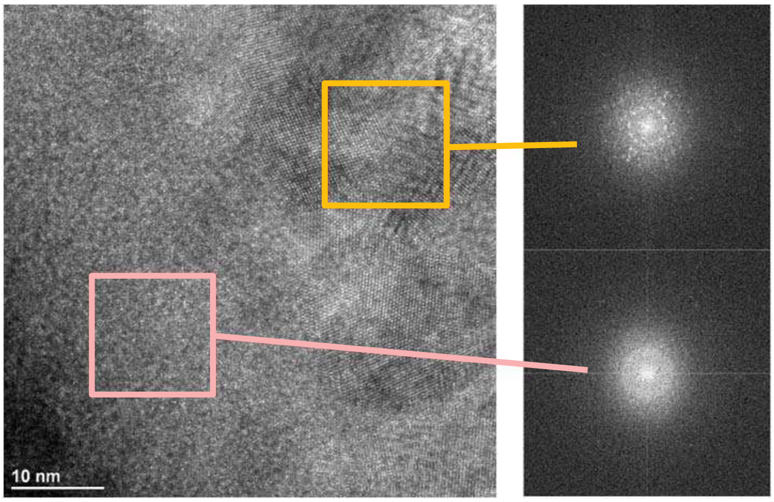

Fig. 16. a) Structure of bonding zone of crystalline titanium substrate matrix with visible rows of atoms with an amorphous TiO_2 layer deposited after 1500 cycles; b, c) fast Fourier Transform FFT confirming b) crystalline structure of Ti substrate, c) amorphous structure of TiO2 layers deposited using ALD method (HRTEM)

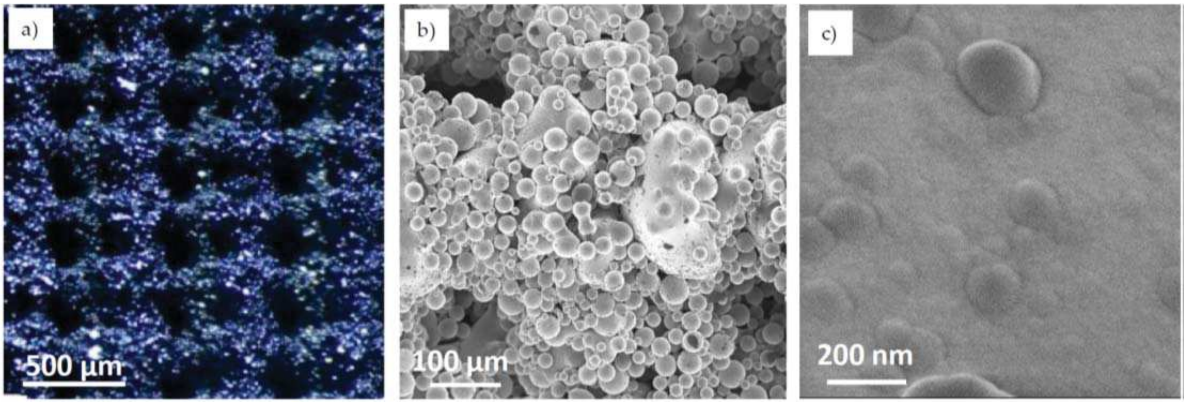

Fig. 17. The surface structure of scaffolds manufactured from) Ti6Al4V alloy deposited with a layer of Al_2O_3 after 1500 cycles; a) stereoscopic scaffold images b,c) SEM

have been deposited during 1500 cycles. X-ray examinations have confirmed the amorphous structure of the aluminium oxide ALD layers. The images of HRTEM clearly show the amorphous structure of the deposited layers as opposed to the crystalline titanium or Ti6Al4V alloy being the substrate.

Thin layers of hydroxyapatite have been deposited by the dip-coating sol-gel method on the porous skeleton produced by selective laser sintering from titanium powders and Ti6Al4V alloy. The structural investigations have confirmed the presence of hydroxyapatite layers (Fig. 18).

5. Results of biological tests of human osteoblast cell cultures on porous substrates of titanium and Ti6Al4V alloy with pores internally coated with nanocoatings

From the assumptions and aims of the study, biological research has been the most important. The responses to several research problems have been expected. It has been necessary to confirm whether it has been possible to produce a skeleton structure from titanium and Ti6Al4V alloy using the method of additive selective laser sintering with expected mechanical properties. It has been necessary to determine whether it is possible to nest and proliferate living cells inside the pores of the so-formed scaffolds and implant-scaffolds. Moreover, perhaps the most important from the goals of the Special Issue has been to investigate whether the use of nanostructural coatings inside cells has been beneficial and whether it has had a beneficial effect on the proliferation of living cells. Finally, it has been crucial to optimise the type of nanostructural layers deposited inside the pores and the conditions of their deposition due to the efficiency of the proliferation of living cells.

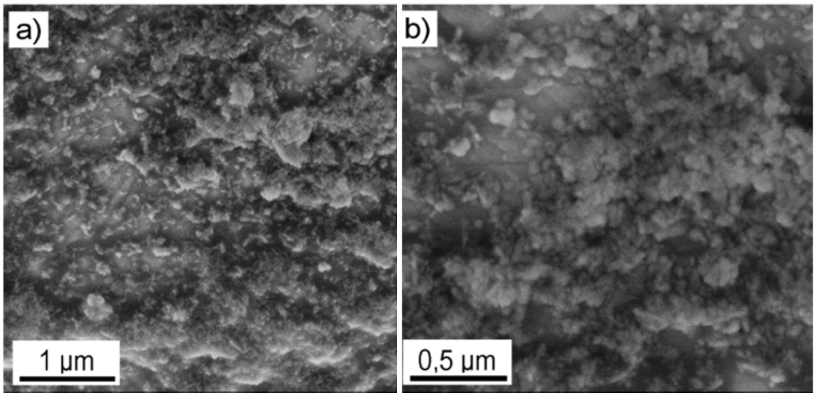

Fig. 18. SEM image of a scaffold made of a) Ti and b) Ti6Al4V; with the sol-gel layer of hydroxyapatite deposited after ten immersions

The biological research has been performed using human osteoblast cells from the hFOB 1.19 (Human ATCC - CRL - 11372) culture line. Osteoblast line cells have come from the collection of the American Type Culture Collection ATCC (USA). ATCC is a nonprofit organisation which collects, stores, and distributes standard reference microorganisms, cell lines and other materials for research and development. At the moment it is the most significant general culture collection in the world.

The culture was carried out in Thermo Scientific Nunc (Denmark) polystyrene bottles with an area of 25 cm^2. A medium consisting of one part of Dulbecco's Modified Eagle's Medium DMEM without phenol red and one part of Hanks F12 medium has been used. Dulbecco's Modified Eagle's Medium DMEM is a variation of Eagle's minimal essential medium (EMEM) which is a cell culture medium that can be used to maintain cells in tissue culture. It contains amino acids, salts (calcium chloride, potassium chloride, magnesium sulfate, sodium chloride, and monosodium phosphate), glucose and vitamins (folic acid, nicotinamide, riboflavin, B12). DMEM is used for most types of cells, including also human ones. DMEM contains four times as much of the vitamins and amino acids present in the original EMEM formula and two to four times as much glucose. Additionally, it contains iron and can contain phenol red. Hanks Balanced Salt Solution (HBSS) is a balanced salt solution to be used in a wide variety of tissue culture applications: to maintain a physiological pH for cells maintained in non-CO_2 conditions, diluting cells before counting, washing cells before dissociating and transporting cells or tissues. Also, the medium has been enriched with L-glutamine (2.5 mM), sodium pyruvate (0.5 mM), gentamicin sulfate (0.3 mg/ml) and fetal bovine serum FBS (10%) and 4- (2-hydroxyethyl) -1-piperazineethanesulfonic acid HEPES (15 mM). HEPES is widely used in cell culture. HEPES is a zwitterionic organic chemical buffering agent. Despite the cellular respiration and changes in carbon dioxide concentration it is better at maintaining physiological pH comparing to bicarbonate buffers also widely used in cell culture. The dissociation constants (pK) of many other buffers do not change much with temperature, but the dissociation of water decreases with falling temperature. HEPES is similar to water because its dissociation decreases with the temperature decreasing. It makes HEPES more effective buffering agent for maintaining enzyme structure and function at low temperatures.

The polystyrene bottles have been incubated at 33.5°C and atmosphere containing 5% of CO_2 and 95% of air. The cells can adhere to the surface of the culture vessel. For the tests, the cells have been separated from the surface using trypsin digestion. After rinsing the resulting cells, the medium has been prepared with working suspensions containing 2.5x105 osteoblasts/ml.

Cell culture with test materials with base dimensions 1x1 cm has been made in a six-well polystyrene standard plate from Thermo Scientific Nunc. The unmodified hydrophobic surface of such a plate provides very good cell growth. The raised rims of the wells protect against contamination of adjacent wells. The excellent transparency of the material allows for the direct microscopic examination. The plates are sterile and no pyrogenic. 3 ml of the cell working suspension was added to one well with a flat bottom plate. Plates with cells and test materials have been incubated for 72 hours. During the culture, photos of the bottoms of the plate with the tested material have been taken in the inverted microscope Olympus IX51 (Japan) with ColorView II Soft Imaging System Cell F (Germany) using a magnification of 100x. The photographs show cells at the edge of the material being examined. Cell growth has been controlled after 24, 48 and 72 hours. The paper contains the final documentation of the tests performed.

After 72 hours of culture, 300 µl of MTT solution (5 mg/ml) has been added to the medium and incubated for 4 hours. The MTT assay is a colourimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. Those enzymes are capable of reducing the tetrazolium dye MTT 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple colour. Then the culture medium has been removed and the purple formazan formed has been dissolved in DMSO (dimethylsulfoxide) added to the well in a volume of 3 ml. The flasks have been shaken for 10 min, absorbance has been taken at 150 µl and the wells (with a flat bottom) of a 96-well microplate made of polypropylene have been placed in the wells. Figure 19 shows photos of the surface of microporous skeletons containing different surface nanolayers in pores or without coatings after 72-hour cells culture and the addition of formazan solutions taken in Dimethylsulfoxide DMSO.

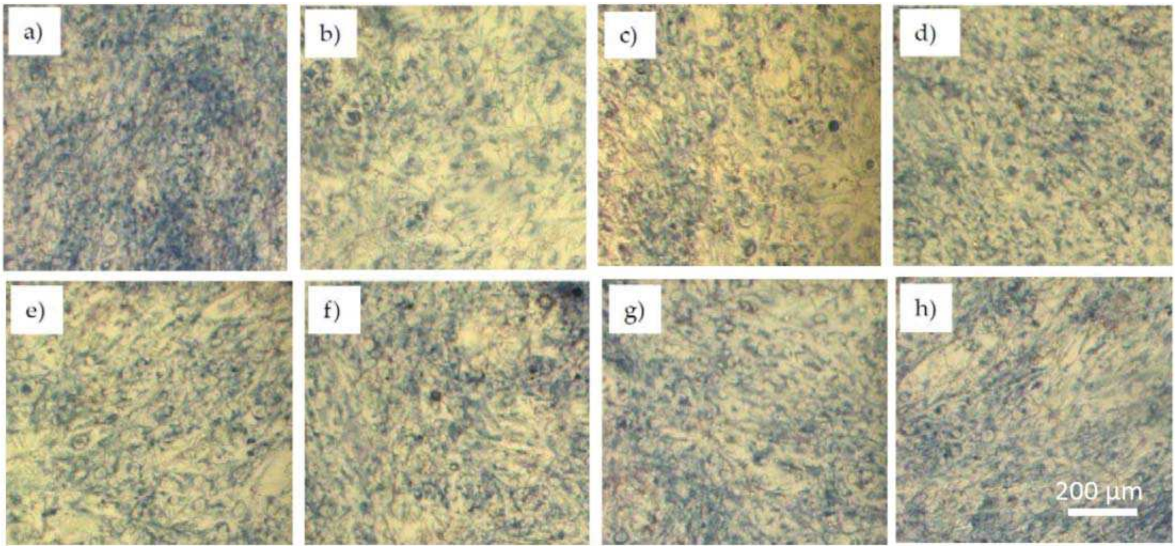

Fig. 19. Photographs of the surface of a) a standard sample; b-h) microporous skeletons of b-e) Ti; f-h) Ti6Al4V; b, f) without surface layers; c, d, g, e, h) containing surface nanolayers c) TiO_2; d, g) Al_2O_3; e, h) hydroxyapatite; inside the pores after a 72-hour culture and the addition of formazan solutions taken in Dimethylsulfoxide DMSO

Absorbance has been measured at a wavelength of 550 nm using the EON High-Performance Microplate Spectrophotometer (BioTek, USA) which combined high performance and high value in microplate-based absorbance readings. Eon's monochromator-based optics have allowed for flexible, filter-free 200-999 nm wavelength selection in 1 nm increments and spectral scanning for 6- to 384-well microplates. Eon has been powered by the Gen5 2.0 Data Analysis Software for reader control and unsurpassed data analysis. From the obtained absorbance results, the value obtained for pure DMSO has been subtracted and the percentage of cell viability has been calculated for cells cultured without the materials tested using the following formula:

$$\% \text{ viability} = \text{absorbance of test samples} * 100\% / \text{absorbance of control samples} \quad (1)$$

Each time measurements have been made on eight occasions in the dimethylsulfoxide DMSO. The results have been analysed statistically, specifying the standard deviation. The measurement results are presented in Table 4.

Table 4.
Viability per cent measurement results as the quotient of absorbance of test samples * 100% and the absorbance of control samples

Skeleton material	Coatings	% viability = absorbance of test samples * 100% / absorbance of control samples % life (average of 8 determinations of formazan solutions taken in Dimethylsulfoxide DMSO)		
% life		85 90 95 100	Result	Standard deviation
Controlling sample			96,4	2,1
Ti	-		88,9	1,8
	TiO_2		92,7	1,5
	Al_2O_3		90,9	3,8
	HA		97,6	3,3
Ti6Al4V	-		97,0	1,5
	Al_2O_3		100,2	3,1
	HA		97,2	3,1

6. Discussion and conclusions

The results of the investigations shown in chapter 5 indicate that both on the substrates of titanium and Ti6Al4V it is possible to grow living cells without any problems. Contrary to the expectations and literature reports described in chapter 2 of the paper, pristine titanium behaves much worse than the Ti6Al4V alloy. Similarly, the scaffolds produced by the method of selective laser sintering from pristine titanium coated inside the pores with TiO_2 and Al_2O_3 layers also are analogously worse. In those cases, the application of those coatings does not play a significant role.

In this respect, the Ti6Al4V alloy has similar properties as the reference material, i.e. neutral laboratory glass. It is worth noting that the obtained results do not confirm the fears pointed out in some of the literature items given in chapter 2 about the harmful role of V as an alloying element. The application of Al_2O_3 coatings to the porous Ti_6Al_4V alloy substrate significantly improves the proliferation of living cells.

The application of hydroxyapatite coatings within the pores of skeletons produced by selective laser sintering requires the commentary. In the case of both analysed substrates the material's ability to nest and proliferate living cells improves. Despite expectations, this impact is not competitive compared to TiO_2 and Al_2O_3 layers, although hydroxyapatite has a much stronger effect on pure titanium substrates. Hydroxyapatite belongs to biodegradable materials, which has been pointed out in chapter 2. It should be remembered that the thickness of the hydroxyapatite layer is relatively small. It should be taken into account that after a relatively short period of a contact with living cells in the body after implantation of such a scaffold, its biodegradation will occur. Contact conditions will, therefore, change so severely, because living cells will come into contact with metal substrate, especially with titanium that is not covered with any coating. The solution, therefore, seems to be non-perspective, giving only short-term benefits that cannot be beneficial in the case of long-term implants. For this reason, it is not interesting to continue the research thread.

The given results need a confirmation in further research to increase the probability of significance of differences between the average obtained results. Those observations, however, indisputably point to the correctness of the assumptions made while developing the original implant-scaffold concept. In the concept, the surface layers applied by the ALD method play an important role inside the pores of microporous skeletons. Owing to the presence of Al_2O_3 and TiO_2 layers deposited by the ALD method, the conditions of nest and proliferation of living cells are significantly improved within the pores.

It is unquestionably advantageous to use the technology of additive selective laser sintering for the production of scaffolds, and especially implant-scaffolds. The porous zones of the implant-scaffolds are hybridized with a solid core in one element. In the porous zones, the live cells can grow. The construction solution is a very useful, unrivalled in many clinical cases including orthopaedics, maxillofacial surgery, as well as dentistry and other areas of regenerative medicine. The solution, combined with specialised methods of computer-aided design CAD and manufacturing CAM, enables the quick and efficient design of individualised implants, especially implant-scaffolds. Particular attention is paid to input materials, due to the almost complete no-waste technology and ecological advantages of technologies due to their purity.

Ensuring the required high dimensional tolerances requires hybridisation of additive laser sintering technology with precise milling on a 5-axis CNC milling machine. The issue is not addressed in the article, although both the concept and the results of the work carried out in the area are presented in our publications [19,108]. The tests of mechanical properties carried out also in the paper confirms the full suitability of even porous and also porous zoned materials to the mentioned applications.

To conclude all the investigations reported in the paper, it should be stated that the Authors obtained the hybrid technology of producing microporous implants and implant-scaffolds to achieve the original Author's biological-engineering materials. The surface engineering issues, including both 2D flat-layered nonorganic coatings and interactions those coverings with flat layers of 2D dimensional living cells, play a crucial role. The issue requires continuing research to obtain conditions for clinical applications, especially after a full cycle of essential in vivo research. The Authors of the paper together with the team of Co-workers continue to research and other work in the area.

Notice

The research presented in the paper was realised in connection with the Project POIR.01.01.01-00-0485/16-00 on "IMSKA-MAT Innovative dental and maxillofacial implants manufactured using the innovative additive technology supported by computer-aided materials design ADD-MAT" realised by the Medical and Dental Engineering Centre for Research, Design and Production ASKLEPIOS in Gliwice, Poland and co-financed by the National Centre for Research and Development in Warsaw, Poland.

Fundusze Europejskie Inteligentny Rozwój ASKLEPIOS Unia Europejska

Additionally the Authors used in the paper some results of the former Project „BIOLASIN Research of the structure and properties of the new obtained biomimetic materials produced by the selective laser sintering methods" realised in the framework of the Competition „Harmonia 4" according to the decision No DEC 2013/08/M/ST8/00818 of the National Science Centre in Cracow, Poland.

Acknowledgements

The Authors would like to thank Ms M. Kraszewska, MA for the correction of English text.

Dedication

We would like to dedicate this paper to the Holy Memory of our beloved Late Father and Grandfather Bolesław Dobrzański Veterinary Doctor on the occasion of the hundredth Anniversary of Birth (30th April 1918 – 30th April 2018). Prof. Leszek A. Dobrzański & Prof. Anna Dobrzańska-Danikiewicz.

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