

Effect of the Surface of Medical Titanium Endoprostheses on the Efficiency of Fibrointegration

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Abstract—Titanium alloys approved for clinical application are used to manufacture various endoprostheses. Engraftment of the implant in bone tissue (osseointegration) is characterized by direct contact and functional connection between the implant and the bone tissue. The process of implant engraftment in soft tissue is characterized by fibrointegration, i.e., interaction between the endoprosthesis material and soft tissue; as a result, connective tissue having a fibrous structure is formed on the endoprosthesis surface. The process of the engraftment of titanium implants greatly depends on the properties of the implant surface; therefore, to improve the efficiency of fibrointegration, various methods for modifying the implant surface are designed to impart them with the necessary biomedical properties. Implants with a polished surface, with surfaces of varying degrees of roughness, as well as those coated with titanium dioxide with the anatase structure are considered. The use of atomic force microscopy, scanning electron microscopy, and profilometry in studies of mesenchymal stem-cell adhesion and in vitro studies of implants with differently treated surface, which were embedded into the soft tissues of experimental animals, made it possible to determine the requirements for the optimal surface treatment of titanium implants used in maxillofacial surgery.

Keywords: titanium endoprosthesis, anatase, atomic-layer deposition, profilometry, atomic force microscopy, scanning electron microscopy, fibrointegration, mesenchymal stem cells, in vitro studies of implants

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INTRODUCTION

Titanium alloys approved for clinical use in Russia [1, 2] are widely applied in traumatology, maxillofacial surgery, and dentistry mainly for the manufacture of various endoprostheses, i.e., devices that are placed inside the human body and replace any damaged organ. The most optimal type of interaction between a metal implant and bone tissue is considered to be osseointegration, which is a whole complex of physiological reactions directly depending on the morphology of the implant surface and its chemical composition [3].

The process of implant engraftment in soft tissue is characterized by fibrointegration, i.e., interaction between the endoprosthesis material and soft tissue, as a result of which a connective tissue with a fibrous structure is formed on the endoprosthesis surface.

Titanium alloys are widely used for the manufacture of medical implants, because they have a combination of such favorable properties as sufficient mechanical strength, sufficient formability, low spe-

cific gravity, excellent corrosion resistance, and biocompatibility [4, 5].

However, the process of engraftment of titanium implants greatly depends on their surface properties [5]; and the biopassive properties of surfaces often impede the healing process [6]. To solve this problem, various methods for modifying the implant surface are being developed to impart them with the necessary biomedical properties [7–11]. For example, it has been noted that the implementation of the synthesis of carbon nanostructures in the form of clusters, fullerenes, and tubes on the surfaces of various materials can manage their biomedical parameters [10]. It has been shown that improving the bioactive properties of a titanium implant can be achieved by applying a layer of titanium dioxide with the anatase structure to the implant surface, which converts the implant from biocompatible materials to the category of bioactive materials [11]. It has been demonstrated that in order to achieve high efficiency of the fibrointegration of titanium endoprostheses used in maxillofacial sur-

gery, treatment of the endoprosthesis surface is necessary, which provides an average roughness of $(4-5) \times 10^2$ nm and a root-mean-square roughness of $(6-7) \times 10^2$ nm [12]. Applying a coating of TiO₂ with the anatase structure and a thickness of 10 nm to this surface by the method of atomic-layer deposition (ALD) increases the well-developed surface and provides an average roughness of $(4-8) \times 10^2$ nm, a root-mean-square roughness of $(0.5-1) \times 10^3$ nm, and a profile height of $(3-6) \times 10^3$ nm. This treatment increases the adhesion between the titanium endoprosthesis and connective tissue and contributes to the efficiency of fibrointegration at the interface of soft tissues and titanium endoprostheses.

In this work, we consider the effect of the surface roughness of titanium endoprostheses on the efficiency of fibrointegration taking into account the biochemical activity of the samples, which is assessed by analyzing the migration of mesenchymal stem cells, their expansion and proliferation, and the structuring of the connecting capsule around the titanium substrate.

EXPERIMENTAL

Preparation of test samples. The object of research in this paper was samples of titanium alloy Ti6Al4V in the form of disks with a diameter of 5 mm and a thickness of 1 mm with various surface treatments: (i) samples with a polished surface; (ii) samples with a polished surface and a bioactive coating of titanium dioxide with the anatase structure; (iii) samples with a rough surface after sandblasting; (iv) samples with a rough surface after sandblasting and coated with titanium dioxide with the anatase structure.

Polishing of the surface of titanium samples was carried out in two stages: first, the samples were machined on a Struers Roto Pol-21 grinding and polishing machine using oxide paste and an etchant, and then after washing they were subjected to chemical etching.

The developed microrelief of the titanium surface with given roughness parameters was formed by sandblasting treatment with corundum Al₂O₃ particles with a particle size of 10–100 μm.

The application of a bioactive titanium-dioxide coating with the anatase structure was carried out at Konmet (Russia) by the ALD method using an ALD reactor of the vertical type, model Sunale-R150 (Picosun Oy). Titanium dioxide was deposited with the application of ethoxytitanium Ti(OC₂H₅)₄ (97%) and water. Because of the low saturation vapor pressure of Ti(OC₂H₅)₄, ethoxytitanium was supplied to the reactor from a heated source at a temperature of

150°C. In this case, the substrate temperature was 250°C [13].

In order to clean mechanical and organic impurities from the surface of the samples, a washing operation consisting of two stages was carried out.

Stage I: washing (preliminary) was carried out only for samples without a TiO₂ coating. In this case, the samples were placed in an ultrasonic bath with a mixture consisting of equal parts of solvents (acetone, dichloroethane, and isopropyl alcohol). The washing time was 5 min.

Stage II: washing was carried out for all types of substrates; double washing for 5 min in isopropyl alcohol using a UZDN-2T ultrasonic installation.

To assess the effect of surface treatment of test samples on the intensity of cell adhesion, their spreading and proliferation on the surface, we used a standard cytotoxicity test [14] that involves the incubation of mesenchymal stem cells directly in contact with the test samples. Test samples intended for these experiments were washed with a detergent (Sigmaclean Glassware Cleaning Concentrate) and distillate, and then dried in air. Next, sterilization treatment in 70% ethanol solution for 1 h and washing with sterile phosphate-buffered saline (PBS) with pH 7.4 were carried out.

To study the interaction between human skin fibroblasts and the prepared samples, mesenchymal stem cells were cultured in 24-well culture plates in a CO₂ incubator at 37°C in a humid atmosphere containing 95% air and 5% CO₂. Cells were seeded on the surface of samples with a density of 35 thousand/cm² and cultured for 72 h. For examination by scanning electron microscopy (SEM), the cells were fixed on the surface of the samples. After 72 h from the time of cell seeding, the samples were washed with a 0.1-M solution of PBS, and then fixed with a 2.5% glutaraldehyde solution in PBS for 2 h. After removing the fixing solution, the samples were washed with PBS, and the material was dehydrated; after the removal of ethanol, the samples were placed in hexamethyldisilazane for 30 min, and then dried in air. Final drying of the samples was carried out by passage through a critical point on a Hitachi CPD-1 apparatus (Critical Point Dryer). After that, they were fixed on object tables and sprayed with a gold-palladium mixture using an Eiko-IB3 installation (Ioncoater) in the following mode: ion current 6 mA, interelectrode voltage 1.5 kV, which allowed a thickness of the sprayed layer of approximately 25 nm to be obtained.

To assess the efficiency of fibrointegration, test samples with different surface treatments (implants) were introduced into the soft tissues of experimental animals for a period of 30 days. As we have shown ear-

lier, the indicated times are sufficient for such experiments [15, 16].

To study the efficiency of fibrointegration and implants using the SEM method, we carried out the following sample preparation: to obtain a conductive surface, after drying, a thin (10–100 nm) gold film was deposited onto allocated and removed implants by cathodic sputtering in argon in a BAL-TECSCD 005 device. Cathodic sputtering is characterized by low thermal effects on samples (since only the surface of the cathode is heated), which prevents the destruction of biological tissues. Then, the samples were frozen to a temperature of -25°C in a Deben Coolstage freezing attachment of a LEO-1430 VP scanning electron microscope. The effective freezing of samples provides the longer preservation of biological tissues under the action of an electron beam.

Methods of investigation. Atomic force microscopy studies. Samples with a relatively small roughness (polished as well as polished and covered with a layer of titanium dioxide TiO_2 with the anatase structure) were examined in an MFP-3D atomic-force microscope (AFM) (Asylum Research, United States). To study the surface topography, we used the semicontact atomic-force mode, which allows the surface relief with to be measured with nanometer horizontal and subnanometer vertical resolution. The following statistical characteristics of the topography (surface morphology) were calculated using the Gwyddion program for AFM image processing in accordance with the standards [18–20]: average roughness, root-mean-square roughness, arithmetic mean deviation of the profile, and mean deviation of all points of the roughness profile from the centerline on the length of the estimate.

Profilometric measurements. The relatively large surface roughness of the samples were measured on an Alpha-Step IQ Surfaceprofiler ASIQ profilometer (KLA-Tencor, United States), which allowed the microroughness to be measured with a resolution of up to 0.1 nm both at short and long (up to 10 mm) scanning distances. The computer control of the device, analysis, and processing of the obtained data provide an opportunity to reduce the effects of nonparallelism and surface curvature of the samples.

The roughness of the sample surface was determined as a set of irregularities in the surface profile. To determine the numerical values of the surface roughness parameters, we used the “centerline system”, which corresponds to the ISO recommendations and is taken into account in the GOST (State Standard) of the Russian Federation [18]. The surface roughness was assessed quantitatively by the following main parameters.

(i) Average roughness (W_a , nm) is the arithmetic mean of the absolute values of the deviation of the profile height within the studied length.

(ii) Root-mean-square roughness (W_q , nm) is the root-mean-square deviation of the profile height within the studied length. This parameter corresponds to the standard deviation of the peak height distribution.

(iii) The maximum peak height (W_p , nm) is the largest value of the peak height; it is measured from the centerline within the studied length.

(iv) The maximum depth of the depressions (W_v , nm) is measured from the centerline within the studied length.

(v) Profile height (W_t , nm) is the sum of the largest peak height and the greatest depth of the depression determined within the studied length.

Scanning electron microscopy studies. To assess cell expansion and proliferation on the surface of the test samples, as well as to evaluate the efficiency of fibrointegration (based on specially prepared samples that were introduced into the soft tissues of experimental animals and were taken out after the animals were removed from the experiments), we used a Cam Scan S-2 scanning electron microscope (Cambridge Scanning). Studies were performed in the mode of secondary electrons with an accelerating voltage of 20 kV. Video processing was carried out using the Microcapture 2.2 software-hardware complex (a system for microscopy and analysis). The study and microimaging of the disk surface were carried out in the system of an Axioplan 2 imaging microscope with a Hitachi HV-C20A analog camera connected to a personal computer, which was equipped with Kontron Elektronik Imaging System KS 400 software, version 2.0 (Carl Zeiss, Germany).

To assess the efficiency of the fibrointegration of implants with different surface treatments, which were implanted into the soft tissues of experimental animals for a period of 30 days, the samples prepared using the above method were examined using a LEO-1430VP scanning electron microscope (Carl Zeiss, Germany) equipped with a Deben Coolstage freezing attachment using a QBSD four-quadrant detector for reflected electrons. The measurements were carried out at an accelerating voltage of 20 kV.

RESULTS AND DISCUSSION

Study of the test samples. As follows from the SEM microimages, there are unidirectional scratches on the surface of the polished test samples (Fig. 1a). After incubation of mesenchymal stem cells, their intensive adhesion, spreading, and proliferation are observed in the region of such scratches (Fig. 1b).

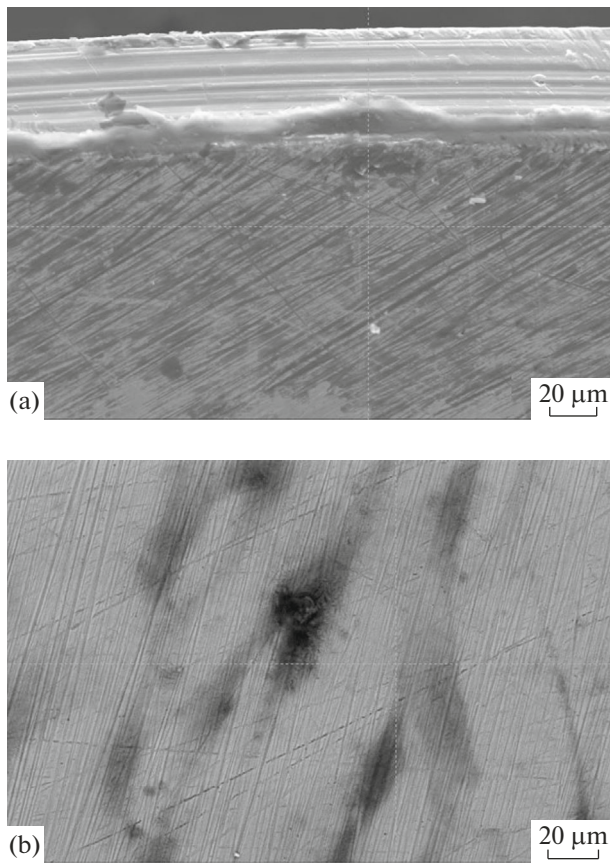


Fig. 1. SEM image of the test sample with a polished surface (a) and visualization of the adhesion and spreading of mesenchymal stem cells after their incubation on a polished surface (b).

The AFM study of the surface of the polished test samples has shown that the average roughness W_a , which was averaged over all such samples and measurement points on these samples, is 44.6 nm, and the average value of the root-mean-square roughness W_q is 55.79 nm. The results of AFM measurements of one of these samples are presented in Fig. 2 [12].

Thus, the application of a bioactive TiO_2 coating with the anatase structure and a thickness of 10 nm on a polished surface slightly reduces the surface roughness.

Figure 3 shows the results of analysis on an Alpha-Step profilometer of the sample surface after polishing and the application of a bioactive coating. Fig. 4 shows the results of analysis of the rough (after sandblasting) surface of the sample after applying the bioactive coating.

Table 1 shows the results of measurements of the surface roughness of the samples with different treatment on an Alpha-Step IQ Surfaceprofiler ASIQ profilometer. Analysis of the results presented in Table 1 allows us to conclude that the application of a TiO_2 coating with an anatase structure and a thickness of 10 nm on test samples with a polished surface slightly

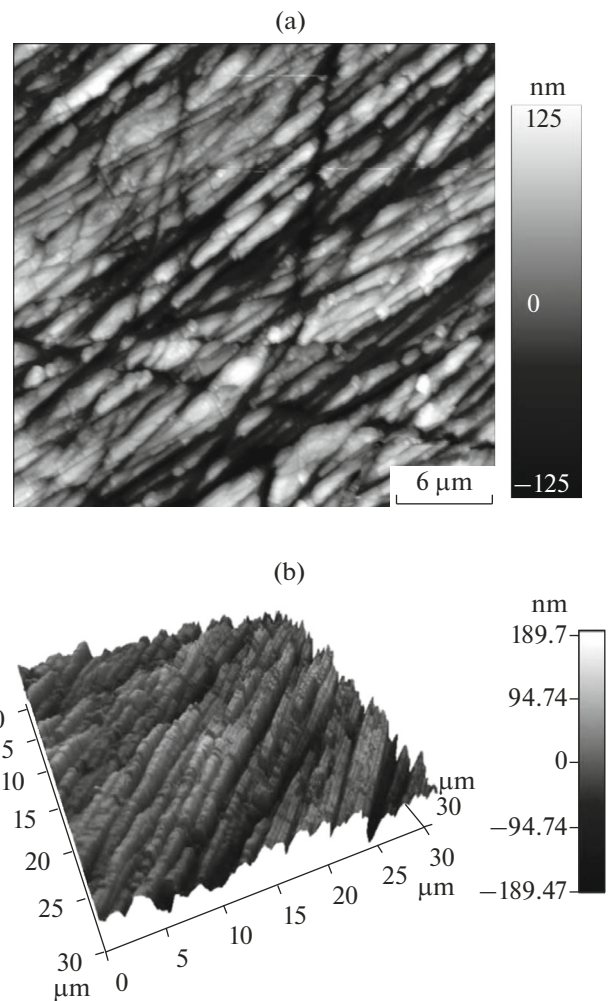


Fig. 2. AFM images of the samples with a polished surface and bioactive coating of titanium dioxide TiO_2 with the anatase structure: (a) 2D topography; (b) 3D topography.

reduces the surface roughness, i.e., the surface is smoothed. This result coincides with the results of studying the same samples by the AFM method.

Another situation occurs on samples with a rough surface. The formation of a rough surface by sandblasting determines the presence of a large number of deep multidirectional scratches and, as a result, the roughness parameters W_a and W_q have values on the order of 10^3 – 10^4 nm. Therefore, the TiO_2 coating with a thickness of 10 nm on such a surface barely affects its roughness. In addition, the sandblasting-induced large spread of the roughness parameters over the surface of the test sample and spread of these parameters from sample to sample do not allow correct evaluation of their averaged values.

Study of the cellular element adhesion. The process of integrating implants into the tissue environment, in particular, into soft tissues, includes as an obligatory

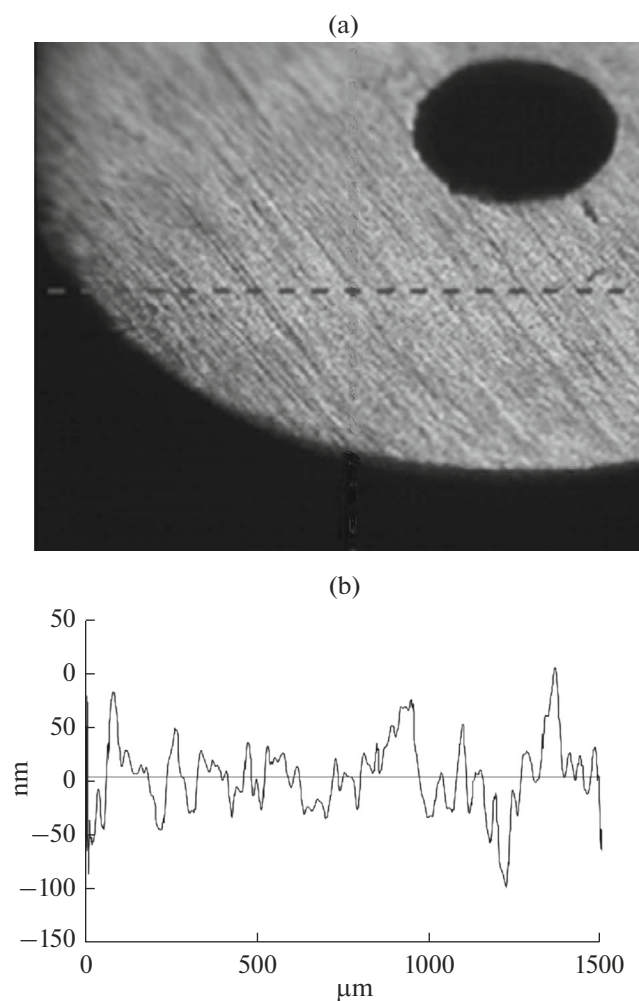


Fig. 3. Results of analysis (Alpha-Step profilometer) of the sample surface after polishing and applying a bioactive coating: (a) scan line; (b) profilogram.

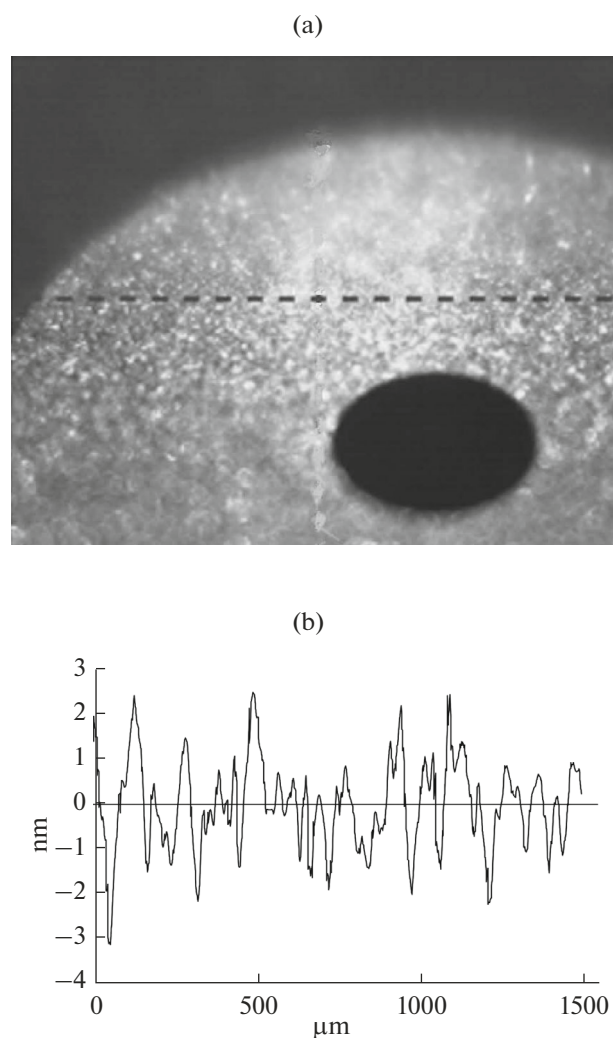


Fig. 4. Results of analysis (Alpha-Step profilometer) of the rough (sandblasted) surface of the sample after applying a bioactive coating: (a) scan line; (b) profilogram.

stage, the process of the adhesion of cellular elements, their surface-spatial spreading, and the proliferative process followed by differentiation. The procedure for studying the state of these cells on the surface of the

test samples is the officially accepted method for assessing the toxicity of the materials under study [14].

To study the intensity of adhesion, spreading, and the proliferation of mesenchymal stem cells on test

Table 1. Results of measurements of the surface roughness of differently treated samples

Characteristics of the sample surface	Scatter of the average roughness values W_a , nm	Averaged values of the average roughness W_a , nm	Scatter of the root-mean-square roughness W_q , nm	Averaged values of the root-mean-square roughness W_q , nm
Polished surface	22.7–37.8	29.35	28.2–49.3	37.24
Polished surface coated with TiO_2	17.8–38	24.35	21.6–48.5	31.68
Rough surface	471–537	–	627–683	–
Rough surface coated with TiO_2	414–823	–	529–1053	–

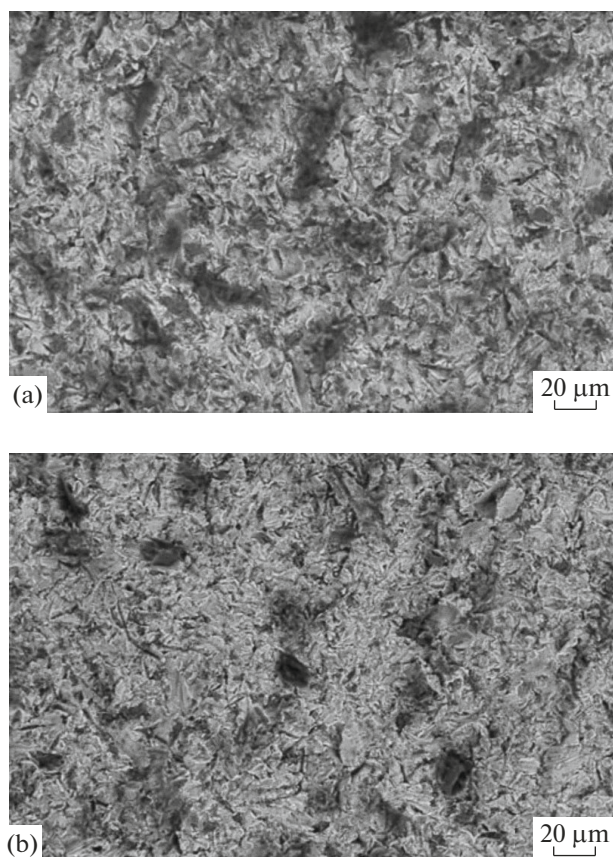


Fig. 5. SEM images of the test samples with a rough surface after the incubation of mesenchymal stem cells: (a) TiO₂-coated sample; (b) sample without a TiO₂ coating.

samples with a rough surface (coated with TiO₂ and without it), these cells were seeded and cultivated for 72 h as described above. SEM images of test samples with a rough surface (coated with TiO₂ and without it) after the incubation of mesenchymal stem cells are shown in Fig. 5.

As follows from Fig. 5 (magnification $\times 300$), a more concentrated arrangement of cellular elements and more active attachment of cells is observed on the TiO₂-coated samples than on samples without a coating. Many of the cells are spread and have an elongated shape. The cellular structure is robust and oblong-volumetric, intracellular structures are noticeable, and attachment zones (locomotor tendency) are observed. A wide area of homogeneous or fine-grained material, which is the product of the vital activity of a cell (extracellular matrix), is determined around some cells.

Using SEM images, we can estimate the morphology of the cell structure. It is noted that the cells are deformed, there are no clear supporting zones, and internal organelles are not detected. There is a low concentration of cells that are not fixed on the surface of the sample (either randomly scattered or collected

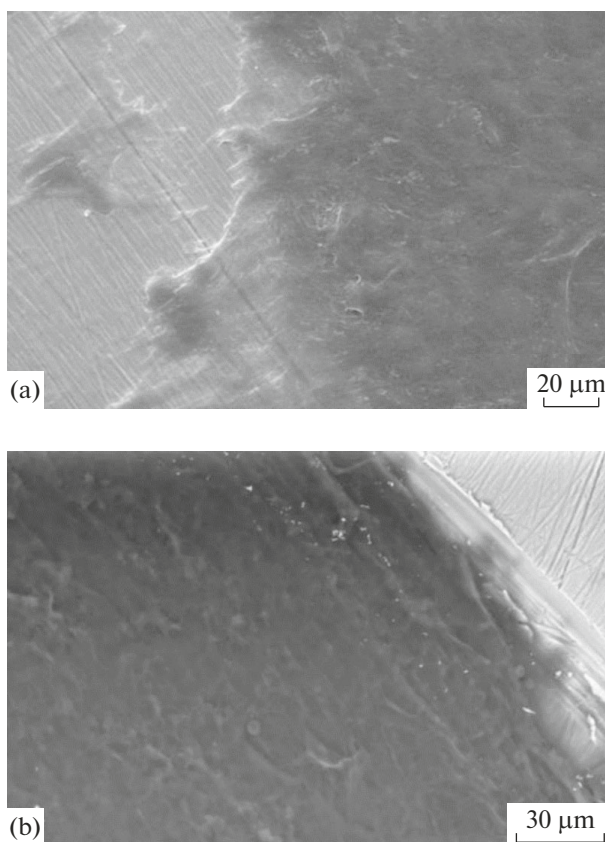


Fig. 6. SEM images of the surface of titanium implants embedded in the soft tissue of the experimental animals for a period of 30 days: (a) implant with a polished surface without a TiO₂ coating; (b) implant with a polished surface coated with TiO₂.

in clusters). Moreover, the tendency to the spreading of such cells is not observed, since the filamentous and lamellar outgrowths characteristic of spreading are lacking.

Thus, the analysis of SEM images of samples with a rough surface (with TiO₂ coating and without it) after the incubation of mesenchymal stem cells has shown that the cell-culture growth, its adhesion, and proliferation tendency are more pronounced on samples coated by TiO₂ with the anatase structure where spreading cells with cytological signs of functional activity prevail.

Study of the fibrointegration of implants. To assess the efficiency of fibrointegration, test samples with different surface treatments (implants) were introduced into the soft tissues of experimental animals and stayed there for 30 days. Based on allocated and removed implants, samples for SEM studies were prepared according to the procedure described above.

Fibrointegration on titanium endoprostheses with a polished surface (coated with TiO₂ of the anatase

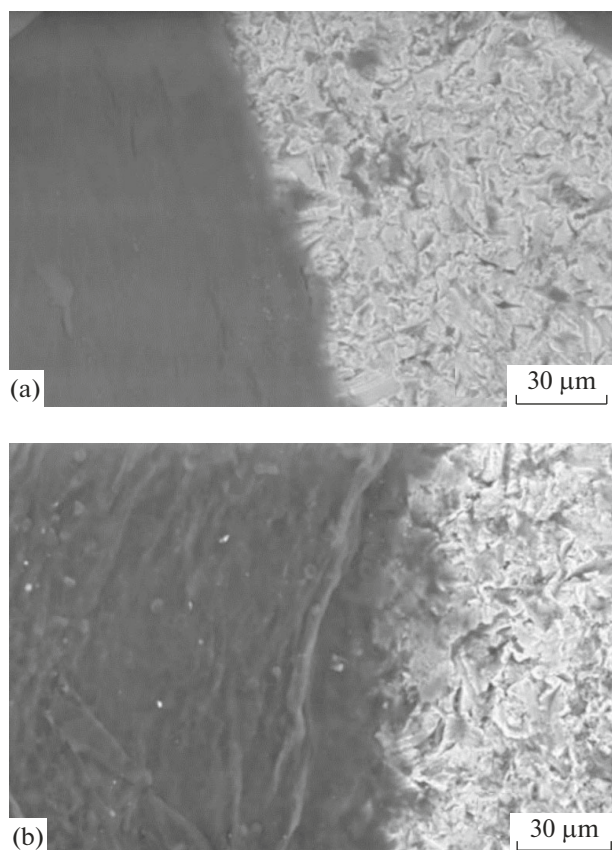


Fig. 7. SEM images of the surface of titanium implants embedded in the soft tissue of experimental animals for a period of 30 days: (a) implant with a sandblasted surface without a TiO₂ coating; (b) implant with a sandblasted surface coated with TiO₂.

structure and without it) can be assessed using the SEM images shown in Fig. 6.

The SEM image of an implant with a polished surface without a TiO₂ coating (Fig. 6a) shows that the connective-tissue capsule, which forms the contact area with the implant, is insignificant. The thinnest fibers form a mat covering the interface of the implant bed; a fibrous interlayer of connective tissue is visible between the disk and soft tissues. The process of formation of the connective-tissue interlayer is negative, since the cell structures on the surface do not form a complete connection between them.

Fibrointegration on titanium endoprostheses with a polished surface (coated with TiO₂ of the anatase structure and without it) was estimated by SEM images shown in Fig. 7. In the case of an implant with a polished surface without a TiO₂ coating (Fig. 7a), there are thin fibers of an interlayer of connective tissue between the disk and soft tissues. The tight fit of the tissue to the disk due to the branched microrelief of the disk surface is associated with increased cell adhe-

sion to each other. The process of the formation of a connective-tissue connection is positive, since the cellular structures form a complete bond between them on the surface. A wide interlayer of loose connective tissue with dense focal lymphomacrophagal infiltrates is determined on top of the implant with a polished surface coated with TiO₂ (Fig. 7b). The periosteal layers of the connective-tissue substance spread almost over the entire surface of the implanted titanium disk. A thick connective-tissue interlayer with a large number of cells of the fibroblastic series with admixtures of lymphomacrophage elements is observed on these samples. The fibrillar skeleton represented by dense bundles of collagen fibers, is well developed in the connective-tissue interlayer.

CONCLUSIONS

Thus, from the obtained data it follows that cell adhesion is better on titanium endoprostheses with a rough surface than on implants with a polished surface. The process of fibrointegration is more intense due to the fact that there is a greater accumulation of cellular structures that are tightly attached and interact with each other. On titanium endoprostheses with a polished surface, the number of cellular structures is less, and strong connections are not formed because of the large distance between them, which prevents the process of fibrointegration.

Analysis of the obtained results shows that coating the surface of titanium endoprostheses with a bioactive titanium-dioxide TiO₂ layer with the anatase structure insignificantly increases the efficiency of fibrointegration; however, the fibrointegration of titanium endoprostheses mainly depends on the roughness of their surface, which determines the concentration of cellular structures, the intensity of their adhesion, and ability to fibrointegration.

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