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The Functionalizing Bioactive Surface of Screw Titanium Implants with Chitosan: Fabrication and Surface Features

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Abstract

The necessity to develop different approaches to increasing dental implant osseointegration should be of high prevalence of dental diseases in current world, the massive expansion of dental implantation technologies, and the advent of technical possibilities to functionalize bioactive surfaces using modern molecular biotechnology.

To improve bioactive surface of dental implants by functionalization, we selected chitosan because it had the properties of biocompatibility and osteoinductive effect, also we had literature data about the possibility of its application as nano-films and nano-coating. Chitosan, applied using epy cathodic sputtering technique, significantly altered nano-surface topography of dental implants. Using atomic force microscopy it is shown that after chitosan applying, the nano-roughness parameter has 5.6-fold increase, and the developed surface area ratio has increased 3.7 times in comparison with surface properties of commercial titanium screw implant without chitosan spraying.

The application of chitosan on bioactive surface of the screw titanium implants was shown to improve the morphological characteristics of osseointegration after implantation into the rat femoral bone. The bone volume fraction in osseointegration zone exceeded at different periods of the experiment the value of the same parameter in the comparison group 1.56-1.64 times. Implants with a surface chitosan-based functional coating provided the additional osteoconductive effect appeared in more intensive and rapid osteogenesis around implants, and the more expressed remodeling and thickening of the surrounding trabecular bone.

Keywords: dental implantation, titanium implants, functional coating, bioactive surface, chitosan, osseointegration, atomic force microscopy

1. Introduction

Biomimetic approach to the principles of maximum biocompatibility and as full compensation for the missing functional properties is crucial for solving the major problems of tissue engineering and regenerative medicine (TERM) (Wang et al., 2014; Hwang et al., 2015; Park

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et al. 2016). With regard to dental implants, this approach can be deciphered as the establishment of artificial or semi-artificial structures that could completely support a function of the tooth for a long time. This paradigm is formulated in the concept of complete osseointegration (Jang et al., 2011; Albertini et al., 2015; Trindade et al., 2015).

From the biomedical standpoint, the osseointegration represents the implantation of the implant, that is, forming a strong bond between its surface and the surrounding bone. The remodeling of this bone is necessary to sustain the loads after the prosthesis. Currently, titanium is the most commonly used material for intraosseous implants, also its alloys with nickel, aluminum, and vanadium used also, alloys with other metals used less frequently (Chang et al., 2010; Mas-Moruno et al., 2015; Ogle, 2015).

Active modification of the implant surface, providing maximum contact area with the adjacent bone and, simultaneously, stimulating the remodeling in this bone, that is, possessing osteoconductive effect, is a fundamental approach to improve osseointegration of the implants. Technically this is achieved through a lot of methods creating three-dimensional porous surface (sandblasting, sintering, deposition, electric arc or plasma spraying, micro-implosion technique, chemical etching, etc.) (Coelho et al., 2009; Chang et al., 2010; Stanford, 2010).

The number of analytic reviews described technique to improve the osseointegration through creation of biomimetic micro-relief of surfaces, and the implant coating by various materials with high osteoinductive properties. The particles of the same alloy, the oxides of titanium, tantalum, hydroxyapatite, or other substances, which were similar to bone mineral matrix, may be useful for improvement of implant surface (Beutner et al., 2010; van Oirschot et al., 2013; Xuereb et al., 2015; Kalita et al., 2016).

A new possibility to produce implants and to control their surface properties at the nanoscale level deserves a special attention and offer great opportunities for fundamental improvement in these properties (Dzenis, 2008; Tomsia et al., 2011; Gao et al., 2015). The idea of this use is in a controlled fixation on the implant surface of molecules with biological effects (adhesive, growth factors, etc.), allowing to achieve the most rapid initialization of osteogenesis on the entire implant surface (Dohan Ehrenfest et al., 2010; Luo et al., 2012; Berglundh, 2013; Correa et al., 2015).

Chitosan proved to be one of the most promising components for this procedure, because it is non-toxic, has complete biocompatibility, bioresorbability, and moderate antibacterial properties.

The use of chitosan as a material for TERM technologies has been documented in an article written by scientific group from Italy led by R. Muzzarelli and published in 'Biomaterials' in May 1988. In subsequent years, these researchers successfully applied chitosan scaffolds for replacement of defects of the Dura mater, the wound surface and the fibrous cartilage, noting the adequate morphological reconstruction of defects without any functional disorders. The authors believe that the start of chitosan application for recovery of lost supporting tissue has opened a new milestone in tissue engineering (Kumar et al., 2004; Muzzarelli, 2011).

The availability of raw materials for chitosan production (exoskeleton of arthropods, fungi etc.) and lightness improvement of its physico-chemical properties with enzymatic treatment are a major reason to consider chitosan to be a very promising basis for the fabrication of modern scaffolds. The strong chondroinductive and osteoinductive effects of three-dimensional porous chitosan were shown experimentally (Di Martino et al., 2005; Abarrategi et al., 2010; Yang, 2011).

Chitosan-based scaffolds have a high ability to induce cellular migration, adhesion, proliferation and induction of necessary chondral or osteogenic phenotype, resulting in intensive remodeling bone and cartilage, it does not activate the resorption of surrounding tissue (Venkatesan, 2010; Correia et al., 2011). The material has adequate wettability and degree of bioresorption, it able to induce bone formation in osteoblast culture (Park et al., 2012). We previously also showed a positive effect of chitosan on the osseointegration of titanium implants (Novochadov et al., 2013).

The goal of this work was to study structural features of nano-sized chitosan-based bioactive coating and opportunities to improve the osseointegration of implants with such procedure.

2. Material and Methods

Chitin as a raw material for chitosan production was extracted from external skeleton of crustaceans (genus Pandalus) by rinsing with tap water, followed by 10 % $NaHCO_3$ solution in the presence of surfactants. The cleaning included being re-deproteinization, washing the intermediate

product, demineralization, final rinse and lyophilization to dry-air condition. The chitosan was obtained by deacetylation of chitin, previously milled to sizes not exceeding 2 mm in diameter. Vacuum conditions promoted the minimum concentration of oxygen in the reaction zone to prevent the oxidative degradation of chitin. The filtered chitosan was a highly hydrated product with a water content of more than 70%. To prevent keratinization this material was dried in a thermostat at 35,0-40,0 °C to a dry-air state (Lyabin, 2012). The obtained chitosan met the Russian standart (Technical Specification 9289-067-00472124-03), it had a mass moisture fraction of 9.4 %, pH of 1% solution in 2 % CH3COOH of 3.85, and the deacetylation degree of 93 %.

As initial products for fabrication of functional coatings we used commercial screw titanium implants for dentistry (MIS BioCom, Israel). Before the chitosan coating all implants were ultrasonically cleaned in MilliQ water and organic solvents. The procedure of deposition was consistently provided to form a finely porous film of chitosan using the freeze-drying technology (1), 1 % suspension in 2 % acetic acid (2), grinding the film with separation of the fraction of microparticles 10-20 microns in diameter (3), ultrasonic dispersion of fragments with a diameter of 1 μm from the surface of micro-particles (4), and cathode coating on the implant surface (5).

Four implants with or without functionalization were used to characterize their surface. Qualitative visualisation of the implant surface morphology was performed using atomic force microscope Solver Pro, equipped with conductive cantilevers coated with diamond DCP-11 (NT–MDT, Zelenograd, Russia). Before each experiment the calibration was performed in the mechanical properties of the tip using NT-MDT software. Images were obtained in Nova software, using semi-contact mode, in which the tip of the cantilever oscillates with a high frequency over the sample and its deformation is captured by the reflected laser beam. Typical digital images contain up to 256 x 256 dpi, 2048 points on curves. Length of curves correspond to a 9.8 microns, the speed of image formation was in the range of 0.7 to 2.1 lines per second. The interaction of the 'tip – sample' was deemed valid, since the maximum height of nano-roughness did not exceed 25 nm.

For quantitative assessment of surface topography, several parameters were investigated at the top and valley regions of the implant threads, on two implants of each type. In accordance with known schemes of analyzing the implants topography (Dohan Ehrenfest, 2011; Shah et al., 2016), we used the following indices of surfase: the maximum peak height (SP, μm), arithmetic mean deviation (SD, nm), ten point height (S10, μm), and developed surface area ratio (SDR, %).

Tissue samples of femur of twenty white skeletally-mature male Wistar rats were used for study in vivo. The Protocol of the experiments conformed to the ethical standards set out in Directive 2010/63/EU on the protection of animals used for scientific purposes. In main group the implants with chitosan-based coating were placed in distal femoral metaphyses of eight skeletally-mature Wistar rats (one implant in each femur) and were followed for four or eight weeks. In comparison group (six rats) the same surgery was using non-coated-implants. Prior to surgery, all animals were anaesthetised by intra-peritoneal injection of Zoletil (20 mg/kg BW; Virbac Sante Animale, France). The animals were fed ad libitum. As a control, we investigated 8 tissue samples of four intact femoral bones from the rats, which have been all the time of the experiment in standard vivarium conditions. The animals were euthanised with an intraperitoneal overdose of Zoletil (200 mg/kg BW; Virbac Sante Animale, France).

After removing the skin, the superficial soft tissue, and careful out-twisting of implants, the remaining tissue specimens of femoral bone, were prefixed by neutral formalin for one week, underwent by decalcification in EDTA solution, dehydrated in a graded series of ethanol, and paraffin embedded. Then we used the staining with toluidine blue for qualitative histology using optical microscopy (Leica DM 4000 (Germany). Quantitative analysis included determining and estimation of following bone structure indicators: the cortical bone thickness (μm), cancellous bone volume fraction (%), trabecular thickness (μm), osseointegration zone thickness (μm), and bone volume fraction in this zone (%).

Immunohistochemical study was to identify CD68 as markers of macrophages/osteoclasts and osteonectin as marker of the osteogenic series using monoclonal antibodies (Novocastra, UK). The numerical density of these cells (103/mm3) was a quantitative parameter for this analysis. For video documentation and quantitative morphological analysis the software Image J (U.S.A) was applied, which allowed determining values of the above parameters in semi-automatic mode.

Quantitative data were processed using with the calculation of the indices adopted to characterize the non-parametric samples in biomedical research. Results were shown as Median

[1st quartile ÷ 3rd quartile]. To prove the validity of differences for multiple groups was applied. P values less than 0.05 were considered significant. The non-parametric Mann-Whitney U and Friedman criterions were used for all statistical analyses between the implant types for atomic force microscopy and quantitative histomorphometry (Statistica 10.0, StatSoft Inc., USA); P values < 0.05 were considered statistically significant. Mean values ± standard deviations are presented.

3. Results

The functionalization of implant surface using chitosan does not affect the properties at the macro level, they were indistinguishable from ordinary commercial samples of these products.

Atomic force microscopy allowed characterizing the nano-relief of the implant surface with or without chitosan coatings. Visualization by NT-MDT software allows us to see that the chitosan application onto implant surface was associated with a considerable alteration of the surface nano-relief. It became nano-rough, while basic micro-relief was almost unchanged (Fig. 1).

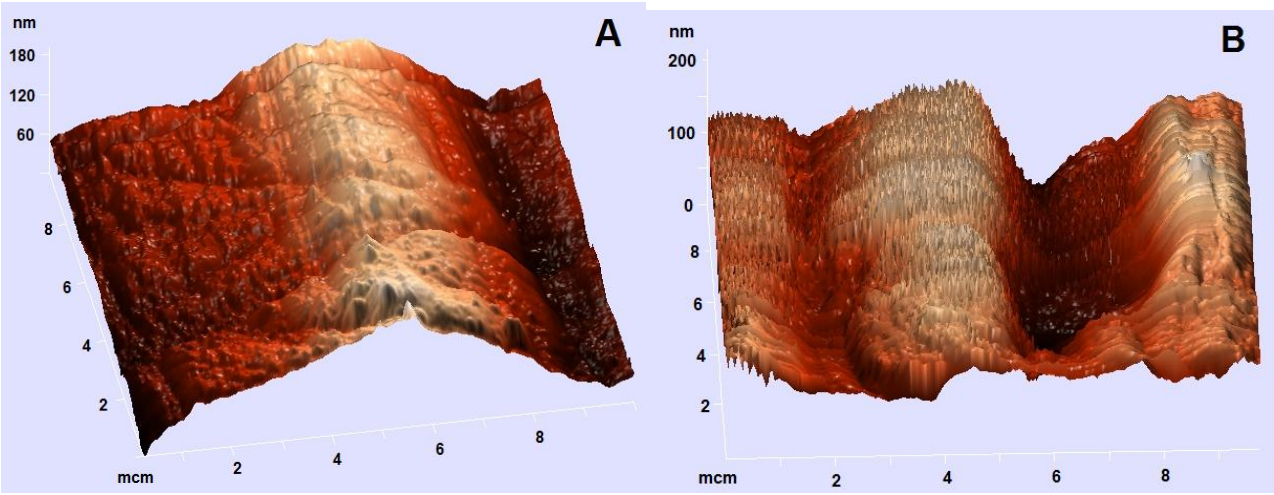

Fig. 1. Functionalization of titanium implants by chitosan-based coating leads to a significant complication of the surface nano-relief, as seen on three-dimensional reconstructions, obtained using atomic force microscopy. A. Non-coated screw titanium implant MIS BioCom (Israel). B. The same implant with chitosan-based coating.

Quantitative characteristics of the surface topography presented in Table 1, confirm and detail these observations. The maximum peak height did not differ between implants with or without surface functionalization; in 75 % of cases its value was in the range from 1.02 to 2.08 μm. Parameter named as ten point height indicating the homogeneity of micro-relief, was also the similar in two variants of implants; it ranged from 1.19 to 2.71 μm. The nano-roughness, estimated from SD parameter, has 5.6-fold increase, and the meaning of SDR has increased 3.7 times in comparison with surface properties of titanium screw implant without chitosan spraying.

Table 1. Surface topography characterization of commercial screw titanium implants for dentistry with or without functional chitosan-based coating (Median [1st quartile ÷ 3rd quartile])

Parameter	Non-coated implants	Chitosan-coated implants
Maximum peak height of the surface, μm	1.44 [1.02 ÷ 1.90]	1.49 [1.14 ÷ 2.08]
Arithmetic mean deviation of the surface, nm	49 [34 ÷ 75]	274 [195 ÷ 340] *
Ten point height of the surface, μm	1.85 [1.19 ÷ 2.62]	1.97 [1.16 ÷ 2.71]
Developed surface area ratio, %	11.34 [6.85 ÷ 15.21]	41.75 [29.71 ÷ 55.84] *

* – Mann-Whitney criterion is less than < 0.05

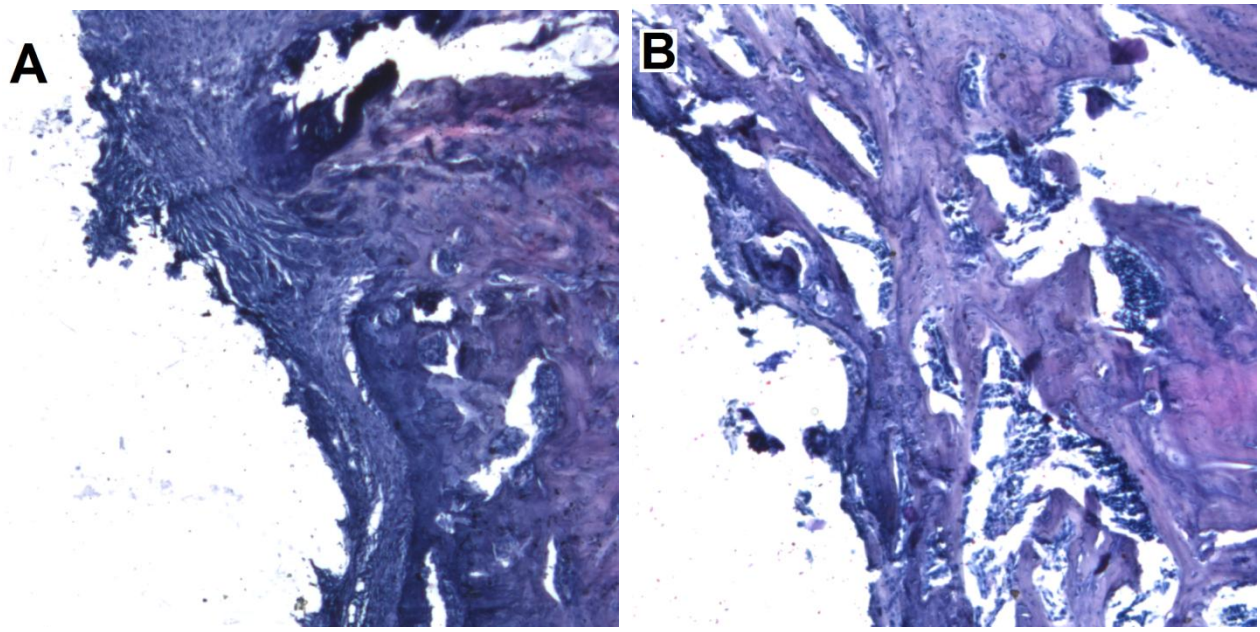

Fig. 2. Histological data at 4 weeks demonstrate the prevalence of osteogenic processes around titanium implants with chitosan-based coating, while osseointegration of implants without coating provided osseointegration through temporary connective tissue layer. A. Osseointegration zone after removing non-coated screw titanium implant MIS BioCom (Israel). B. The same, but implant had chitosan-based coating. Stain with toluidine blue, × 120.

Histological study confirmed the successful osseointegration after setting the titanium implants onto rat femoral bone. Four weeks after installation around the implants without chitosan-based coating we can see a thin layer of connective tissue. It contained a lot of blood vessels and small foci of osteogenic cells. In adjacent bone the simultaneous presence of small osteoresorption areas and chains of osteoblasts at the edges of the osseous beams testified about the active bone remodeling (Fig. 2A). Around implants coated by chitosan, a layer of formed connective tissue contained a variety of osteogenic and chondrogenic foci. The border of tissue after implant removing was irregular in shape, with partial separation of tissue, indicating successful osseointegration. The restoration and remodeling of the surrounding bone was very intense (Fig. 2B).

At 8 weeks, the zone around the implants in the comparison group was presented by a mixed regenerate in the form of connective tissue and fibrous bone with concentric direction of the fibers around the implant. On the border with the surrounding cancellous bone, this regenerate more resembled the structure of the bone. The cancellous bone demonstrated the traits of remodeling. The shape and structure the border with the cavity after implant showed a high degree of osseointegration. In the main group the zone around implants remained appearance of mixed osteogenesis. The fibrous tissue was thin, and the bulk of osseointegration zone look like a dense cover of the newly formed bone surrounding the implant. Adjacent trabecular bone was in a state of intense remodeling, with a clear increase of bone formation.

The data of quantitative analysis are shown in Table 2 and Fig. 3, indicate that the surface coating of the implant with chitosan was accompanied by a significant increase in the thickness of the zone of integration. The bone volume fraction in osseointegration zone exceeded at different periods of the experiment the value of the same parameter in the comparison group 1.56-1.64 times. Changes in the adjacent cancellous bone in group with using chitosan-based coating revealed the activation of osteosynthesis, which is clearly prevailed over osteoresorption. By the 8th week of the experiment, in the main group the cortical bone thickness near the osseointegration zone was 1.21 times higher than in the comparison group; at the same time the cancellous bone volume fraction was exceeded values in the comparison group 1.24 times; and the trabecular thickness was higher than 1.32 times (all differences were significant).

Table 2. Surface topography characterisation of commercial screw titanium implants for dentistry with or without functional chitosan-based coating (Median [1st quartile ÷ 3rd quartile])

Parameter	Control group	Time	Experimental groups	
			Main	Comparison
Osseointegration zone				
Osseointegration zone thickness, μm	-	4 weeks	255 # [198 ÷ 319]	150 [127 ÷ 179]
		8 weeks	412 # [373 ÷ 361]	244 [212 ÷ 270]
Bone volume fraction in osseointegration zone, %	-	4 weeks	44.8 # [41.6 ÷ 47.5]	27.3 [24.1 ÷ 31.0]
		8 weeks	59.9 # [52.8 ÷ 63.1]	38.2 [35.9 ÷ 40.4]
Surrounding bone				
Cortical bone thickness, μm	1035 [945 ÷ 1123]	4 weeks	1195 * [1040 ÷ 1332]	1070 [971 ÷ 1157]
		8 weeks	1384 *# [1210 ÷ 1538]	1136 [1005 ÷ 1275]
Cancellous bone volume fraction, %	41.0 [36.8 ÷ 47.1]	4 weeks	45.0 [41.2 ÷ 50.4]	40.7 [38.3 ÷ 43.4]
		8 weeks	58.6 *# [52.0 ÷ 55.9]	47.2 * [45.4 ÷ 52.5]
Trabecular thickness, μm	238 [214 ÷ 252]	4 weeks	284 [239 ÷ 302]	249 [205 ÷ 289]
		8 weeks	355 * [298 ÷ 410]	268 [220 ÷ 306]

* = p < 0.01 by Friedman test (comparing control group); #= p< 0.05 by Mann-Whitney test (between experimental groups)

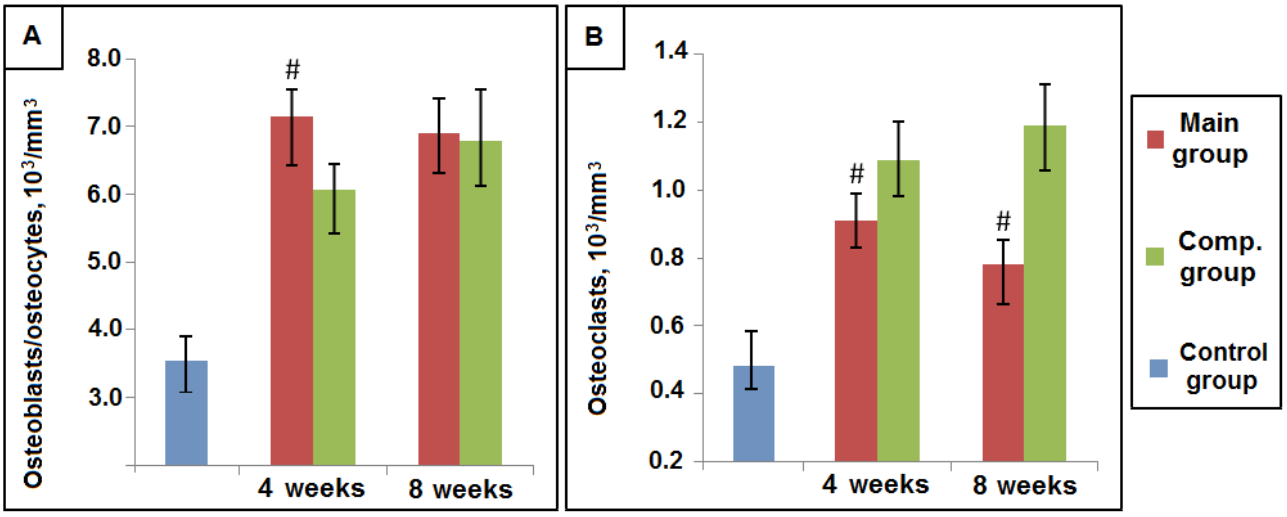

Fig. 3A. The cellular composition of osseointegration zone around of dental implants with chitosan-based coating in rat femur demonstrates more rapid arising the pool of osteogenic cells and more intensive increment of osteoclasts, comparing the dynamics of the same indices in group with non-coated implants. A. Numeral density of osteoblast and osteocytes. B. Numeral density of osteoblast. Data are represented as Me [Q1 ÷ Q3], mark '#' means p<0,01.

Fig. 3B. demonstrates the friendly increase in the numerical density of osteoblasts, osteocytes and osteoclasts, a few more intensive at the 4th week of the experiment in main group. In contrast, the number of osteoclasts in the primary group was significantly lower than in the comparison group, which showed relatively lower capacity of ostertibble in this group.

4. Discussion

According to modern concepts, osseointegration involves a series of events, including the activation of several protein cascades, cell apposition, vascular invasion, de novo bone formation and maturation to achieve the result of primary and secondary stability of the endosseous implants (Sailaja, 2016). This process is successfully accelerated by changing the surface roughness of the implant and the development of a biomimetic interface (Chang et al., 2010; O'brien, 2011). The available preclinical and clinical evaluation of the biomechanical properties showed an ambiguous dependence between the structural parameters of implants, preimplantation tissues, and indicators of functional integration of the implant (Tonetti et al., 2012; Bassi et al., 2013; Morachini et al., 2015). As a result, very little commercial implants contain nanostructures (e.g. Osseospeed, AstraTech, Sweden and Ossean, Intra-Lock, USA), and the prevalence micro-relief coatings (e.g., Bicon, USA) is relatively low (Coehlo, 2009; Dohan Ehrenfest, 2011). The main reason seems to be impossible to fabricate dental implants with enough efficiency in the experiment and clinics, using all suitable nanotechnologies.

We obtained coverage will definitely improve the ability of dental implants to osseointegration. But at the same time, they are still very far from ideal, and need to be studied from the standpoint of uniformity of the obtained properties, repeatability, storage stability, and other important characteristics in the transition to clinical implementation. Most likely, the ideal functional coatings are not manufactured with any one procedure or material, and by combining several approaches, taking into account all (from chemical to macroscopic) levels of interaction between implant and host tissue. The future the problem can be solved based on the principles of functional tissue engineering, which considered the osseointegration as the formation of hybrid biomechanical systems with the construction of computational models based on the principle of achieving the desired end results of the operation (Guliak et al., 2014).

5. Conclusion

Chitosan is a suitable agent for bioactive functionalization of the surface of dental implants. The original functional coating based on chitosan, applied using the technique of cathodic sputtering, alter significantly the surface nano-relief of dental implants. The results of atomic force microscopy confirm that implants with chitosan-based coating had a record of nano- roughness 7.7 times more than non-coated products, and the rate of development of the surface was increased by coating 4.5 times.

The obtained experimental results indicate that after setting the screw titanium implants with bioactive functionalization of their surface with chitosan in the femur of rats, an additional osteoconductive effect is revealed. As a result, more intense and anticipating the timing the formation of bone tissue is in the area of osseointegration, it combines with morphological signs of intensive remodeling and compaction of the surrounding bone. In the end, to 8 weeks after installation, the complete osseointegration is present. This suggests for this method of surface functionalization to be promising for implementation in the dental practice.

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