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MICRO-CT AND HISTOLOGICAL ANALYSIS OF TI_6AL_7NB CUSTOM MADE IMPLANTS WITH HYDROXYAPATITE AND SIO_2-TIO_2 COATINGS IN A RABBIT MODEL

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Abstract

Background and aim. *Bone defect reconstruction in the maxillofacial area comes as a necessity after traumatic, oncological or congenital pathology. Custom made implant manufacturing, such as selective laser melting (SLM), is very helpful when bone reconstruction is needed. In the present study we assessed the osseointegration of custom made implants made of* Ti_6Al_7Nb *with two different coatings:* SiO_2*-*TiO_2 *and hydroxyapatite, by comparing the bone mineral density (BMD) measured on micro-CT and the histological mineralized bone surrounding the implants.*

Methods. *Custom made – cylindrical type – implants were produced by selective laser melting, coated with* SiO_2*-*TiO_2 *and hydroxyapatite and implanted in the rabbit femur. The animals (divided into 3 groups) were sacrificed at 1, 3 and 6 months and the implants were removed together with the surrounding bone. Bone mineral density and histological examination of the bone-implant surface was performed for each group.*

Results. *BMD and histological examination of the samples determined the quantity of mineralized bone at the implant site, showing a good percentage of mineralized bone for the coated implants at 1, 3 and 6 months. The measurements for the implants without coating showed a significant lower quantity of mineralized bone at 3 months compared with the implants with coating, and a good quantity of mineralized bone at 6 months, showing a process of demineralization followed by remineralization in the last month. The measurements of BMD showed similar results with the histological examination.*

Conclusions. *The use of micro-CT and the measurement of BMD are a reliable, minimally invasive and a quick method of osseointegration assessment.*

Keywords: micro-CT, Ti_6Al_7Nb, SLM, osseointegration, implant coating, custom made implant

Background and aim

Technological progress has made it possible for additive manufacturing technologies to be applied successfully in the medical sciences . One of these techniques is Selective Laser Melting (SLM), which consists of producing irregular shape devices by fusing titanium alloy powder, such as Ti_6Al_7Nb. Thus, custom-made implants for bone defect reconstructions can be produced [1]. Although the surgical procedure of implant placement is of great importance, the clinical outcome stands in osseointegration, which represents the ultimate test in morphological and functional rehabilitation of the patient. The study of bone implants osseointegration should observe the device in a 3D setting in which it is present in the living tissues, considering that the histological examination, despite its great value, is just a 2D representation of the present situation. Modern 3D imaging techniques such as micro-CT offer this possibility [2].

Whatever the technology used to obtain the implants, foreign body rejection stands as one of the most difficult obstacles the human body has to overcome. Osseointegration is a process that can be hard to induce, which is why, in this study, hydroxyapatite and SiO2-TiO2 coatings were applied on the implant surface, to enhance the osseointegration process.

Micro-CT analysis has proved itself to be appropriate in the measurement of certain in vivo/in vitro parameters such as mineral bone density and cortical bone thickness [3]. Bone mineral density (BMD) is defined as the volumetric density of calcium hydroxyapatite (CaHA) in a biological tissue in terms of g/cm^3. The combined density of a well-defined volume which contains a mixture of both bone and soft tissue, such as a selected volume of medullar trabecular bone in a femur or tibia, is measured as "**bone mineral density"**, or BMD. This parameter relates to the amount of bone within a mixed bone-soft tissue region.

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Materials and methods

Ti_6Al_7Nb alloy **(**ATI Allvac, Monroe NC, USA) was used to create the sample implants, by selective laser melting technology (Realizer SLM 250 machine, Realizer GmbH, Borchen, Germany). The samples were designed with a cylindrical screw-type shape in order to have a good penetration in the bony structure of the rabbit femur, and perfect primary stability at insertion. The dimensions and properties of the implants were: 10 mm length and 3.3 mm diameter, with a controlled porosity of 24–25%, determined through Archimede's method ISO 2738–99. The implants were divided into three groups: one group uncoated, the second group with HA coating and the third one with SiO_2-TiO_2 coating (Figure 1).

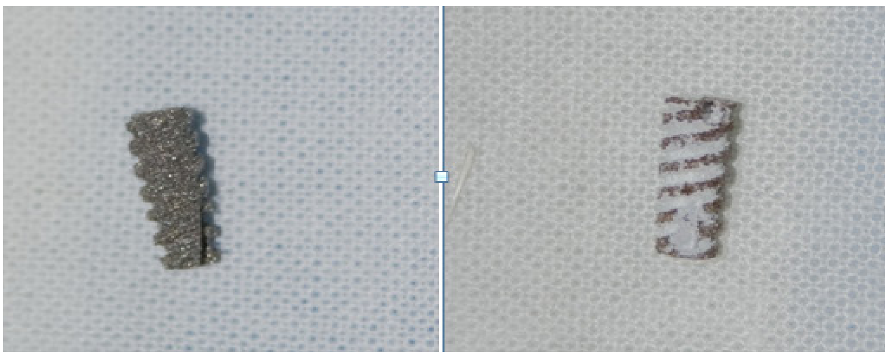


Figure 1. Implants with SiO_2-TiO_2 (left) and HA (right) coating.

The coating procedure was done by immersing the implants into a hydroxyapatite and SiO_2-TiO_2 solution. They were kept in preliminary void for 15 minutes. After that, they were dried in a special oven at 100°C for 30 minutes. The thermal treatment was performed at 600°C for 30 minutes for the implants infiltrated with HA and at 400˚C for 60 minutes for the implants immersed in SiO_{2}-TiO_2 (Rotaru et al.) [4].

Eighteen *New Zealand White Rabbit* (*Oryctolagus cuniculus*) were included in this study, divided into 3 groups of 6 individuals. All rabbits were of the same age (six months) and approximately the same weight, kept in standard conditions of temperature, humidity, day/night cycle and they all had the same access to food and water, *ad libitum*, throughout the experiment. The vivarium conditions were according to the EU Directive 63/2010. The rabbits were anesthetized with a Xylazine/Ketamine cocktail using a dosage of 8 mg Xylazine and 80 mg Ketamine per kg of body weight. The study was approved by the Ethical Committee of the Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania (No. 407/03.12.2014).

The lateral aspect of the femur was shaved and disinfected with iodine solution. A super-inferior incision was performed in order to expose the quadriceps muscle. The femur approach was done through the muscle bodies without tampering with the muscle fibers. A periosteal scraper was used to fully expose the antero-lateral part of the femur. Two cylindrical orifices were created at the proximal area of each femur, using cylindrical 10 mm long burs with ascending dimension: 1 mm - 2 mm – 2.8 mm under continuous cooling with saline solution at 800 rotations/min and a 30 Nm torque. In the left femur at the upper proximal area the Ti_6Al_7Nb-HA implant was placed and in the inferior orifice the Ti_6Al_7Nb-SiO_2-TiO_2 implant was inserted (Figure 2).

In the right femur the control Ti_6Al_7Nb with no coating was placed at the upper area. All the implants were placed with a 30 Nm torque having perfect initial stability. Suture in layers was performed after the implantation procedure. The rabbits were sacrificed at one, three and six months intervals after the implants were placed, by a Potassium Chloride intravenous injection, after inducing general anesthesia. The samples consisting in implant and surrounding bone were immersed in 10% formalin. Micro-CT was performed for each specimen in order to evaluate the position of the implant, the bone apposition between the threads of the implant, and the BMD at the implant site. The measurements were done using the CT-Analyser ***CTAn*** dedicated soft (Figure 3). The samples were scanned with a Bruker Skyscan 1172 MicroCT at 80 kV and 100 mA, using a 1 mm Cu+Al filter, a rotation step of 0.5 and a resolution of 20 µm. Before scanning rehydration by overnight storage in 0.9% physiological saline, was performed. The calibration of the machine was done with 8mm diameter rods (calibration phantoms). The samples were wrapped in paper tissue, loaded into a plastic tube, thenmoistened with saline, same as the BMD phantom rods. The obtained slices were reconstructed using the NRecon software (Bruker, Belgium) and analyzed using CTAn (Bruker, Belgium). The bone surrounding the implant was assessed by selecting it as the region of interest (Figure 3) and measuring the bone mineral density as compared to the scanned phantom rods.

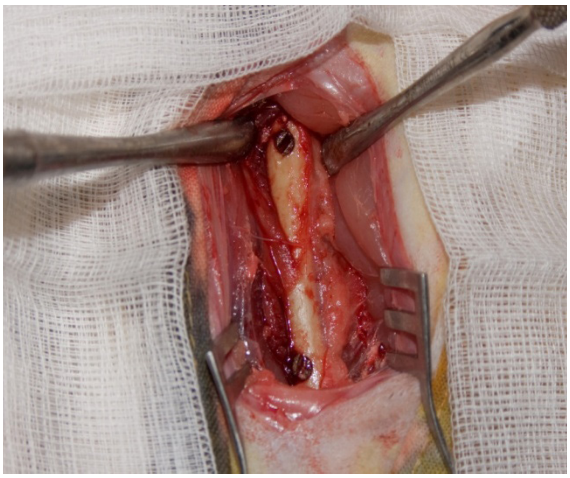


Figure 2. Intra-operative image of implant placement.

Histological examination

After formalin immersion for 2 days the samples were decalcified in an nitric acid solution for 3 days and then prepared for histological examination. The histological slices were colored with Tricrom Masson, which gives two separate colors for mineralized bone and for osteoid, thus an image analysis could be performed.

The histological slices were obtained with a Leica microtome cutting system with 4 micrometer thickness, and examined with a Leica ICC50HD cam microscope.

Image analysis technique

Adobe Photoshop software technique described by Gamal M et al. [5] and Gheban D et al. [6] was used for the image analysis. Panoramic image of the slice was used by merging images done at 50x magnification (CS6 version Photomerge). Cleaning of the artifacts (bone marrow, muscle fiber etc.) was then performed in order to have the best result bone (blue)/osteoid (red) Figure 4.

By pixel quantification for each color, the percentage of mineral bone and osteoid was determined. The measurements by this protocol were performed for each sample.

Statistical analysis was performed using the MedCalc Statistical Software version 15.2.1 (MedCalc Software bvba, Ostend, Belgium). Quantitative variables were analyzed using the Spearman's rho correlation. The level of significance was considered at $p<0.05$.

Results

The rabbits had no post-operative complications and the tissue specimens required for the intended analysis could be processed at state-of-the-art standards. Micro-CT scan performed before the histological analysis of the specimens, showed no implant displacement or osteolysis around the implant threads. No inflammatory reaction or fibrous tissue was noticed for the implant site at 1, 3 and 6 months interval. Table I shows the average BMD for the three implant types, and the average percentage of mineralized bone present at the implant surface, seen at the histology examination.

At one month both analyses done (BMD and histological examination) were showing a high degree of bone mineralization at the implant site, same as the results for 6 months. At 3 months period a demineralization process for the Ti_6Al_7Nb sample was observed, both when calculating the mineralized bone by histology exam and the BMD by the micro-CT exam, while the values for the coated implants are much higher. The comparison for the two examinations is shown in the Figures 5 and 6.

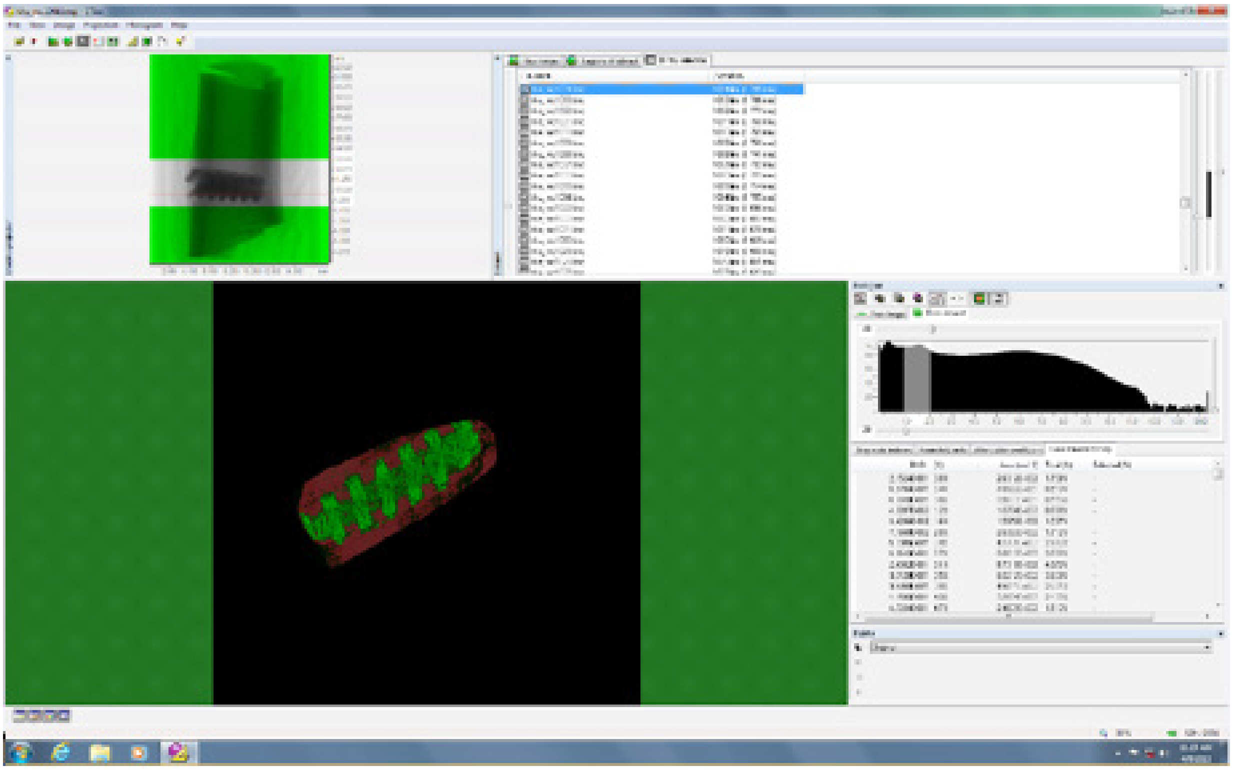
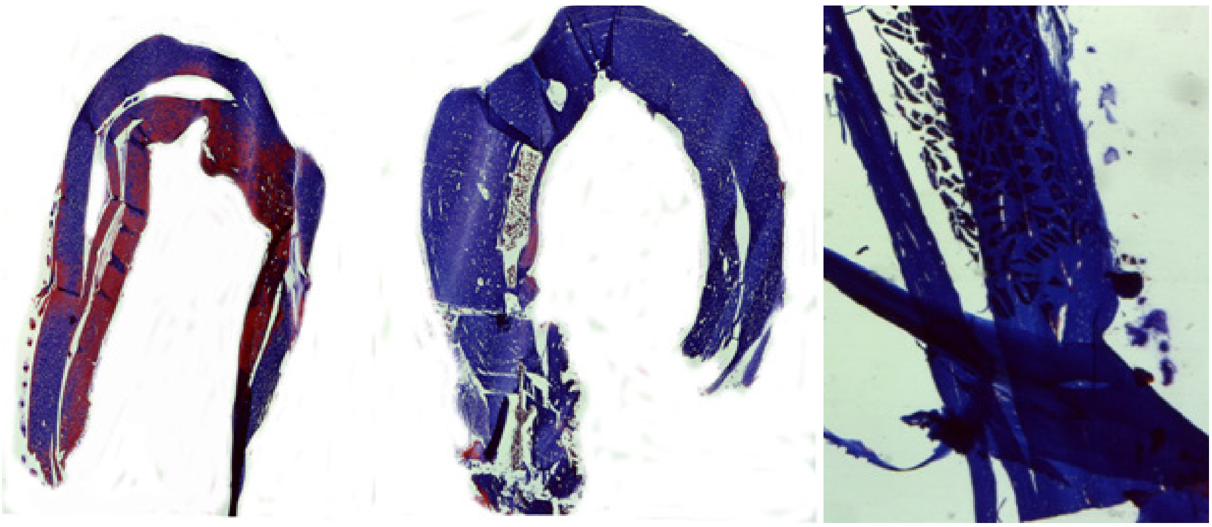


Figure 3. Micro-CT reconstruction and BMD determination for Ti_6Al_7Nb – HA at 1 month.



a **b** **c**

Figure 4. Image at 50x magnification showing mineralized bone (blue) at three months for Ti_6Al_7Nb (a), Ti_6Al_7Nb - SiO_2–TiO_2 (b) and Ti_6Al_7Nb - HA (c).

Table I. Mineralized bone and BMD for the three implant types.

	Mineralized bone 1 month	BMD 1 month	Mineralized bone 3 months	BMD 3 months	Mineralized bone 6 months	BMD 6 months
Ti6Al7Nb	93.83%	2.31	58.33%	1.43	94.67%	2.01
Ti6Al7Nb-SiO2–TiO2	96.83%	2.02	98.50%	2.61	97.00%	2.12
Ti6Al7Nb -HA	99.50%	2.47	99.00%	3.09	97.50%	3.20

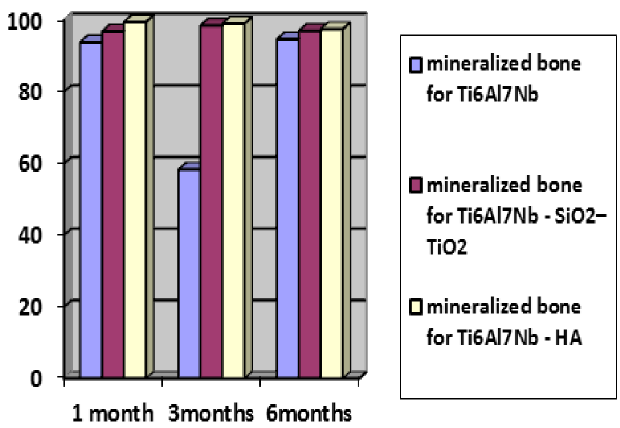


Figure 5. Mineralized bone for the 3 samples.

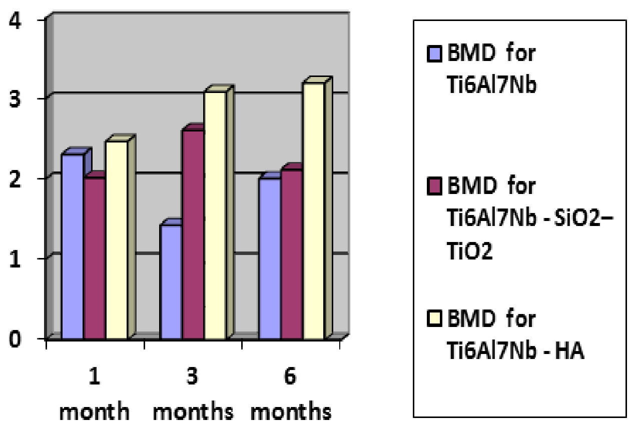


Figure 6. BMD for the 3 samples.

The statistical analysis of the results for the three samples shows that there was a positive correlation between the BMD measurement and the histological results, which is highly statistically significant (r=0.750; p=0.02).

Discussion

One of the best treatment options in bone defect reconstruction is the use of 3D custom made implants [1]. Selective laser melting of titanium alloys is the best option to obtain an irregular shape implant, which can be biocompatible. The bone growth for a specific implant depends on the design of the implant, surface structure and coating material [7]. Osseointegration improvement of this type of alloys, and their clinical assessment , represents a challenging task. Usually the degree of osseointegration is proven by the histology exam that shows the bone cells proliferation, direct bone-implant contact and the mineralization degree at the implant surface [8]. Micro-CT can be another useful method in the osseointegration assessment. Micro-CT images allow us to quantify volumetric bone-implant contact, and bone mineral density (BMD) of a specific region that are of help in the osseointegration evaluation [9,10].

Usually, the titanium alloys and the different coatings used to enhance their osseointegration, were studied for a period of 2, 4, 6 and 12 weeks after implantation implantation, but none studied the behavior of the alloys for more that 18 weeks [11], thus we considerered that a longer period of time (six months), would be more appropriate for integration assessment. It is a known fact that mineralization and demineralization balance require time to be achieved [12]. This can explain why the demineralization process was continuous, for the Ti_6Al_7Nb sample with a peak at about 3 months, followed by remineralization of the site within the following months, reaching a percentage of mineralized bone for 6 months comparable with the one at 1 month (94.67% vs. 93.83%). The remineralization process happened in the absence of any tempering force, the implants having a passive condition inside the femoral bone, without any stress applied directly. The niobium component of the studied alloy has the potential to stabilize the Ti_6Al_7Nb samples in time, due to the niobium oxides (Nb_2O_5) that are formed at the surface of the implants. The mineralization process that occurs after 3 months can happen due to the surface oxide layer, which is less soluble and biocompatible, that forms in time [13].

The micro-CT data, same as the histology examination, showed the same results in terms of BMD evaluation, proving the demineralization process that happens for the Ti_6Al_7Nb samples.

Usually an implant is considered to be osseointegrated when bone develops directly on the implant surface, without any fibrotic tissue grown at the interface [14]. Clinically there is no mobility between the bone and the implant, under regular loading conditions, after an appropriate healing period. In our study we have not seen any fibrotic tissue at the bone-implant interface, also no inflammation was observed. Micro-CT analysis is useful in understanding the circumstances that lead to a poor bone activity response, such as microcracks, plastic deformations or manufacturing deficiencies that can lead to failure [15]; in our study none of the above was seen.

It has been clinically validated that titanium and its alloys present a superior biocompatibility and fewer foreign body reactions in comparison to other conventional materials [16]. The bioinert nature of titanium is its principal quality which consigns itself biocompatibility properties, stating that it does not induce a positive reaction in the tissues, but more likely it conditions the absence of a negative reaction [13], even in the case of demineralization showed for the Ti_6Al_7Nb implant, no inflammation or adverse reaction were seen at the histological analysis.

Implant surface roughness is also responsible for the proportion of osteoblasts proliferation, demonstrating its growth in the case of silicium dioxide sandblasted implants [17], that can explain the higher value of mineralized bone for the $Ti_6Al_7Nb\text{-}SiO_2–TiO_2$ than for the Ti_6Al_7Nb implants. Modification of the surface roughness of implants might hasten osseointegration [18]. Coating the implants with hydroxyapatite enhances their viability in time, and influences positively the cellular and protein adhesion, resulting in improved osseointegration [19].

Similar with our work, Alzubaydi [11] showed in his study that the HA coated Ti-6Al-7Nb screws after 18 weeks of the implantation seem to be well-tolerated by the bone since no adverse tissue reaction was evident. However, there was a faster reaction of bone towards the coated implants compared to the uncoated one. In another study, Cook et al. had observed that histologic evaluations of orthopedic implants revealed mineralization of interface bone directly onto the HA-coated implant surface, with no fibrous tissue layer interposed between the bone and HA visible at the light microscopic level [7,20].

In vitro study done by Brie IC on Ti_6Al_7Nb with HA and SiO2 coatings found that the behavior of osteoblasts depends on the type of implant and culture conditions. Ti-SiO2 scaffolds sustain osteoblast adhesion and promote differentiation with increased collagen and non-collagen protein production. Ti-HA implants have a lower ability to induce cell adhesion and proliferation but an increased capacity to induce **early mineralization**. Both types of infiltrates have their advantages and limitations, which can be exploited depending on local conditions of bone lesions that have to be repaired [21]. The early mineralization process, present at the Ti-HA coating could explain our results of high mineralized bone for the Ti_6Al_7Nb -HA implants.

The use of coated implants is of help whenever local or general condition could temper with the normal osseointegration process.

Conclusions

For a good long-term outcome, the Ti_6Al_7Nb implant need a period of stabilization of about 6 months, any stress applied in this period of time, especially at 3 months could lead to the onset of complications, even implant displacement. Osseointegration outcome of Ti based implants can be improved by SiO_2–TiO_2 and HA coating.

The HA coating represents the best option for the Ti_6Al_7Nb alloy, that beside the osteoinductive and osteoconducting property (Rotaru el al.) [4] can induce early mineralization, that supports implant osseointegration.

The histology examination of titanium based implants needs a long time period (weeks) in order to have a result, meanwhile the micro-CT examination can produce reliable results in a matter of hours, thus the micro-CT examination is easier and faster to be performed.

Further studies that will involve the loading of the implants or force applied to the implant site for the 6 months period of osseointegration should be taken into consideration.

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