Histological and three-dimensional evaluation of osseointegration to nanostructured calcium phosphate-coated implants

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1. Introduction

Recent developments in the surface properties of osseointegrated implants have significantly enhanced both the quality and rate of osseointegration. Current advances enable modifications of implants at the macro, micro, as well as nano levels, and several commercially available implants possess modifications on all these levels [1]. Recent studies have reported that the application of nanostructures to implant surfaces seems to further increase bone apposition [2–4]. Possible explanations for this phenomenon are widely sought, since it has been suggested that it may be due to the cumulative effect of various factors. From a topographical perspective, both in vitro and animal studies have suggested that the enhanced surface area seems to create an optimal basis for bone responses. Lamers et al. [5] reported that osteoblasts sensitively reacted to nanogrooves (width: 50 nm, diameter: 17 nm), resulting in osteogenic gene expression, and suggested that nanogrooves on implant surfaces enhanced the bone response. In another study, Puckett et al. [6] reported reduced bacterial attachment on nano-scale rough surfaces than on other surfaces; furthermore, the same nano-scale surface showed higher affinity to fibronectin, which is essential for the initial osseointegration process [7]. Meirelles et al. [8] conducted an animal study with implants containing nanostructures and polished implants deliberately lacking them, and found that the former had higher bone-to-implant contact than the latter implant surfaces. These reports suggest that cells, particularly osteoblasts, respond to topographical alterations at the nanometer scale.

In addition to topography, the physical and chemical properties of the deposited nano-size materials could enhance the osseointegration cascade. Studies have reported the beneficial...
influences of physical properties such as the wettability of the nanostructured surface [9–11] and this hydrophilicity is one of the factors speculated to be closely involved with plasma proteins and osteogenic cells [12,13].

Nanometer-size calcium phosphate (CaP) coating has drawn considerable attention for its chemical composition, as it mimics the structure and chemical composition of the surrounding bone. Moreover, the possibility of chemical binding of implants to the bone is of great interest, since reports have indicated that implant surfaces incorporated with ions such as Ca, Mg, P and Si exhibit significant plasma protein adsorption and strong bonding to the bone [14–19]. Biomimetic modification is one of the current trends in biomaterial development, where the material is preferably "bioactive" rather than "bioinert" [20–22].

In this study, we focused on rod-shaped nanometer CaP material. This unique topography has been reported to accelerate bone metabolism [23–27]. In our previous study on this nano-CaP coating, we reported enhanced osseointegration to the implant surface and higher bioactivity in comparison to implants whose surfaces lacked CaP coating [3].

Although histological evaluations carried out in several studies have proved that nanometer length scale modification effectively enhanced osseointegration, some other studies did not detect the effects of nano-scale modification; thus, further understanding of these delicate alterations would be worthwhile [28,29]. Therefore, we conducted a three-dimensional (3-D) evaluation using microcomputed tomography (micro-CT) in order to investigate the unique surface modifications at the nano-scale. In this study, we aimed to complement 2-D histomorphometry and histological observation with 3-D imaging and quantification in order to obtain further information of bone formation in nanostructured CaP-coated implants.

2. Materials and methods

2.1. Implant surface preparation and characterization

Thirty-six turned commercially pure titanium implants 8 mm in length and 3.3 mm in diameter were used (Grade 4, Elos Medtech Pinol, Denmark). Half of these implants (test) were coated with nano-sized CaP according to the Promimic HAnano method; detailed information regarding the chemical composition can be found elsewhere [3,30]. In brief, the implants were dipped into a stable nanoparticle suspension containing CaP particles (diameter: 10 nm) followed by heat treatment at 550°C for 5 min in nitrogen atmosphere. A nanoparticle suspension is considered stable when sedimentation or precipitation of the particles is prevented. This was achieved by electrostatic nanoparticles stabilization at pH 9. Furthermore, surfactants were added to maintain the suspension stable through steric stabilization. The uncoated implants (control) were subjected to the same heat treatment as the coated test implants.

2.2. Scanning electron microscopy

The surfaces of the implants were examined by scanning electron microscopy (SEM) with a LEO Ultra 55 FEG instrument (Zeiss, Oberkochen, Germany) at an accelerating voltage of 5 kV. Three implants from each group were investigated.

2.3. Interferometry

The topography of the implants was characterized using an interferometer (MicroXam; ADE Phase Shift Technology, Inc., Tucson, AZ). We randomly selected three implants per group for the analysis. Each implant was measured at nine positions (three top areas, three thread valleys and three flank areas). The parametric calculation was performed after form errors and waviness were removed with a 50 μm × 50 μm Gaussian filter. The following 3-D parameters were selected: Sd (μm) = the arithmetic average height deviation from a mean plane, Sa (μm2) = the density of summits, and Sdr (%) = the developed surface ratio.

2.4. Animals, implantation and sample preparation

Eighteen lap-eared rabbits (mean body weight 3.9 kg) were used in this study. Two implants were inserted in each rabbit: one test and one control implant into the left and right femurs, respectively. This planned animal study was approved by the Malmö/Lund regional animal ethics committee (approval no. M282-09).

Before surgery, the rabbits' hind legs were shaved and disinfected with 70% ethanol and 70% chlorohexidine. The animals were anaesthetized by intramuscular injection of a mixture of 0.15 ml kg⁻¹ medetomidine (1 mg ml⁻¹ Dormitor; Orion Pharma, Sollentuna, Sweden) and 0.35 ml kg⁻¹ ketamine hydrochloride (50 mg ml⁻¹ Ketalar; Pfizer AB, Sollentuna, Sweden). Lidocaine hydrochloride (Xylocaine; AstraZeneca AB, Södertälje, Sweden) was administered as the local anaesthetic at each insertion site at a dose of 1 ml. The implants were inserted using a W&H implant unit (Elcomed, W&H SA310; Burmoos, Austria) at a rotation speed of 20 rpm.

Postoperatively, buprenorphine hydrochloride (0.5 ml Temgesic; Reckitt Benckiser, Slough, UK) was administered as an analgesic for 3 days.

2.5. Micro-computed tomography and 3-D reconstruction

At 2 and 4 weeks postoperatively, the rabbits were killed with an overdose (60 mg ml⁻¹) of sodium pentobarbital (Apoteksbolaget AB, Stockholm, Sweden). Samples were retrieved and placed in 4% formaldehyde for 24 h, after which they were placed in 70% ethanol. The 3-D bone formations surrounding the implants were examined using micro-CT (μCT 40; Scanco Medical, Basserdorf, Germany) at a slice resolution of 36 μm. A total of 500 micro-CT slices for each bone–implant sample were imaged at an X-ray energy level of 55 kVp, and a current of 145 μA. The integration time was 200 ms with a total scanning time of 36.3 min (128 mA).

Data were exported as DICOM files and reconstructed with computer software (MRicro, Atlanta, USA) for further analyses. First, the 3-D images were oriented and cropped to a rectangular shape that fitted the implant and the surrounding bone. The images were then divided into three regions of interest: implant, prosthetic screw-hole and bone.

Thereafter, the images were exported to another software program (3D Slicer v. 3.6, BSD-style; www.slicer.org) to calculate the volume of the different sections. The percentage of 3-D bone surrounding the implant was calculated from the following formula: (bone volume × 100)/[total volume – implant volume – screw-hole volume).

2.6. Ground section preparation and histological analysis

After the micro-CT analysis, all the samples were processed for undecalcified ground sectioning. In brief, after a series of dehydrations and infiltrations in resin, the samples were embedded in light-curing resin (Technovit 7200 VLC; Heraeus Kulzer Wehrheim, Germany). Thereafter, one central undecalcified cut and ground section was prepared from each implant by using Exakt sawing and grinding equipment. The sections were ground to a final
thickness of approximately 40 μm and stained with toluidine blue and pyronin.

Histological evaluations were performed using a light microscope (Eclipse ME600; Nikon, Japan), and the histomorphometrical data were analyzed by image analysis software (Image J v. 1.43u; National Institutes of Health). The bone–implant contact (BIC) percentage along the entire implant was calculated at ×10 objective magnification, and the same amount of bone area defined in the 3-D analysis was calculated.

2.7. Statistical analysis

Statistical analyses were performed using KaleidaGraph software (Synergy Software; Essex Junction, VT, USA) and SPSS (SPSS Inc., Chicago, IL, USA) software. The mean values of surface roughness were compared by one-way ANOVA, followed by a post hoc Tukey–Kramer test with the value of statistical significance set at 0.01. The non-parametric Wilcoxon signed-rank test was used for bilaterally inserted implants with the significance level set at 0.01.

3. Results

3.1. Implant surface characterisation

SEM micrographs of pure titanium (control) and CaP-coated titanium (test) implants are presented in Fig. 1. At high magnification, it is evident that the test implant surface (Fig. 1b) was fully covered with rod-shaped CaP particles approximately 10–15 nm wide and 100–200 nm in length.

Fig. 2a and b present the 3-D optical interferometry images of the control and test surfaces, respectively. The mean Sa value (SD) was 1.02 (0.15) and 0.94 (0.12) for the control and test implants, respectively. The mean Sd (SD) was 134806.23 (6982.94) and 139358.82 (9366.51) for the control and test implants, respectively. The mean Sd percentage was 29.85 (5.88) and 27.44 (8.01) for the control and test implants, respectively. There were no significant differences between the compared parameters.

3.2. Histomorphometry

The postoperative course was uneventful, and there were no clinical signs of infection. All the implants were already immobilized by the time the animals were killed.

The histological sections presented newly formed trabeculae with deeply stained mineralized tissue for both groups after 2 and 4 weeks of healing (Fig. 3a–d).

Both cortical and newly formed bone was in close contact with the implant surface at both time points. The mean BIC (SD) values for the control and test groups at 2 weeks were 24.7% (8.4) and 30.2% (10.4), respectively. There were no significant differences between the two groups at this time point (P = 0.25). At 4 weeks after the surgery, the BIC (SD) of control and test groups was 24.3% (7.2) and 33.44 (7.94), respectively. The BIC was significantly higher in the test implants than in the control implants at 4 weeks (P = 0.0078, Fig. 3e).

The mean bone area (SD) at 2 weeks was 37.21% (3.58) and 32.92% (8.38) for the control and test groups, respectively, and that at 4 weeks was 30.72% (10.14) and 33.21% (11.28), respectively. There were no significant differences in the mean bone area between the test and control between 2 and 4 weeks (control, P = 0.4375 and test, P = 0.5469). In addition, no differences were found in the test and control implants between 2 and 4 weeks (P = 0.153 and P = 0.87, Fig. 3f).

From the histological observation, there seemed to be less trabecular bone and scattered bone formation around the control implants after 4 weeks compared to the test implants; however, this histological observation could not be confirmed by histomorphometry.

3.3. 3-D imaging and 3-D quantification

Fig. 4a–d presents representative 3-D reconstructed images with a focus on the interfacial bone formation on the implants in the control and test groups at 2 and 4 weeks. From the image, significant bone formation can be observed at the implant interface in

Fig. 1. Scanning electron microscopy images (bars: 500 nm) of (a) non-coated (control) and (b) CaP-coated (test) implant surfaces. The test surface is fully covered with rod-shaped structures. A magnified image (bar: 200 nm) shows further details of the coating.

Fig. 2. Interferometry images of (a) the control and (b) the test implant surfaces. Measurement area: 200 μm × 260 μm.

the test group at 4 weeks; these results are in accordance with the BIC measurements.

The mean 3-D bone density (SD) for the control and test groups at 2 weeks was 38.89% (5.59) and 33.88% (10.25), respectively, and 30.38% (4.79) and 30.25% (6.27) at 4 weeks, respectively (Fig. 4e). No significant differences between the test and control groups were observed at both 2 and 4 weeks ($P = 0.085$ and $P = 0.965$). However, there was a significant decrease in the 3-D bone density of the control implants at 4 weeks ($P = 0.007$), while the bone density of the test implants did not significantly change between 2 and 4 weeks ($P = 0.491$).

Additionally, there was no significant difference between the bone area determined by histomorphometry and bone density determined by micro-CT ($P = 0.820$), and there was a significant correlation coefficient value of 0.350 ($P = 0.05$).

4. Discussion

Our results showed that the rod-shaped CaP-coated implants significantly influenced osseointegration. Evaluation of the topography evaluation by interferometry revealed no significant differences, indicating that the topography was only altered at the nanometer scale below the possible detection level (the analysis affords a lateral resolution of 0.3 μm and vertical resolution of 0.05 nm). Since the coating is a monolayer with approximately 10–15 nm developed crystal structures, we characterized its structural characteristics by high-resolution SEM.

The rod-shaped crystal structure of CaP has been reported to enhance the osteogenic activity surrounding it. Ono et al. [27] reported that CaP blocks with rod-shaped structures enhanced the bone metabolism cycle, exhibiting higher levels of osteoclast activity and progressive bone formation. In their study, increased TRAP-positive multinucleated cells existed on the surface of the CaP blocks, and this process was accompanied by cuboidal active osteoblasts with potent alkaline phosphatase (ALP) activity in the newly formed bone. Okuda et al. [25] demonstrated that rod-shaped CaP showed superior bone formation as compared to globular CaP structures. It is speculated that cells or their signalling pathways respond to the rod-shaped structure by upregulating and balancing the osteoclastic and osteoblastic activity for enhanced bone formation.

In the current study, the rod-shaped CaP-coated implant presented significantly higher bone-to-implant contact than the control at 4 weeks. Although no significant differences could be
observed, the mean values were higher for 2 weeks and active bone apposition to the implant surface appears to have continued in the case of the test implant, whereas that of the control implant reached a plateau.

The bone area measurements and the 3-D bone density evaluation did not differ between the non-coated and CaP-coated implant groups. However, the bone density in the control implants significantly reduced over time, although this difference was only significant when evaluated using micro-CT. Although a high rate of bone formation at an earlier time point that reduces over time is a part of the biological process due to periosteal reaction [31,32], and the bone reduction is compensated by mineralized bone formation, i.e. maturation, 2-D histology may not have captured the actual process due to its limited scope of evaluation, since it may depend on where the section was taken from. On the other hand, micro-CT is advantageous in that it enables evaluation of the total circumferential space, while in histomorphometry the evaluation is limited to one or a few 2-D slices. Therefore, the 3-D evaluation is a reliable method and is more accurate than histological analysis, since the evaluation includes all the acquired data. However, one must keep in mind that it is important to understand the biological responses by combining information from 2-D histomorphometry and from 3-D evaluation. Since there exist certain biological events that cannot be observed by micro-CT, e.g. cell alignment, cell shape, localization of proteins or enzyme detection, the biological information that can be obtained by 2-D histology can never be neglected. For example, the enzyme histochemical findings of Ono et al. [27] would never have been possible without histology, and it would have been difficult to interpret the results without this technique. It must be emphasized that the intention of the current study was not to explore for a possible alternative to 2-D histology, but to possibly complement and further clarify biology from a different perspective.

Three-dimensional evaluation with micro-CT has received considerable attention in recent years because it facilitates the observation and quantification of bone formation surrounding implants or bone substitutes in a 3-D plane [33–35]. Although the amount of bone surrounding the implant did not differ across different implant treatments, bone formation at the implant interface was found to be higher (i.e. direct bone contact to the implant) in the test implants at 4 weeks. Along with image reconstruction, it was easy to observe the state of trabecular bone formation to the implant surface, which provides additional information from both qualitative and quantitative perspectives. However, in this study, quantifying the amount of BIC three-dimensionally was considered a challenge due to metallic halation artifacts and the potentially significant effect of these on the evaluation [36]. Although the quantification may still be possible by the application of various filtering techniques [37], it remains to be known whether the actual bone or artifact has been fully differentiated. In order to improve the technique and obtain clearer results, determination of the optimal conditions such as power voltage or the focus size to control the X-ray absorption is necessary at the time of scanning; moreover, optimal software that can efficiently filter out artifacts during the reconstruction is necessary. Hence, in future studies, we intend to focus on improving the novel 3-D evaluation technique to further clarify the effect of various surface modifications on osseointegration.

5. Conclusion

Implants coated with rod-shaped nanostructured CaP showed enhanced osseointegration compared to non-coated implants. Three-dimensional analysis using micro-CT proved to be an effective method to further understand the temporal characteristics of implants in bone.
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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 2–4, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.07.017.

References