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In vivo tensile tests of biomimetic titanium implants pulsed laser coated with nanostructured Calcium Phosphate thin films

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Bioactive titanium coatings made of commercial hydroxyapatite, Mn^{2+}-doped carbonated hydroxyapatite, and octacalcium phosphate were obtained by pulsed laser deposition. The functionalized biomimetic titanium implants coated with nanostructured calcium phosphate layers were successfully tested in vivo. Eight weeks after implantation in the tibia bones of 6-month-old New Zealand White female rabbits the attachment between bone and implants was tested in a tensile (pullout) test. The pullout force was found to more than double in the case of CaP-coated Ti coins, compared with the uncoated ones. Furthermore, compared with the commercial HA-coated implants, the pullout force increased by 25% in implants with Mn^{2+}-doped carbonated hydroxyapatite, and 10% in octacalcium phosphate-coated implants. The results suggest that nanostructured CaP deposition has a significant potential for improving the performance of titanium implants in bone, and that the composition and structure of the calcium phosphate coating have a significant influence on their biological effect.

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1. Introduction

In search of new biomaterials to replace hard tissue, scientists have increasingly turned to biomimetic strategies, on the assumption that a material similar to bone will more easily integrate into the living host tissue. Calcium phosphate coatings of metallic implants are expected to enhance surface bioactivity and stimulate bone apposition [1, 2] as compared to standard Ti alloy implants. We previously reported [3-5] the physicochemical and biological performances of structures consisting of Ti substrates that had been covered by pulsed laser deposition with thin layers of calcium phosphates (CaPs), namely, hydroxyapatite (HA), octacalcium phosphate (OCP), and manganese-doped carbonated hydroxyapatite (Mn-CHA). Octacalcium phosphate, $Ca_8H_2(PO_4)_6*5H_2O$, is a basic calcium phosphate, which hydrolyzes in aqueous solution to the more stable hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ [6]. OCP's structural resemblance to HA and its greater solubility [7, 8] recommend it as a promising alternative to HA for metal coating. HA and Mn-CHA are less soluble than OCP and provide the advantage of a composition more similar to that of bone tissue in inorganic phase. Moreover, the presence of Mn^{2+} in the coating should increase the ligand binding affinity of integrins [9]. Also, manganese is a common structural component, of bones and cartilage.

Pulsed laser deposition (PLD) is one of the most promising methods to deposit calcium phosphate films, as it ensures perfect thickness control and makes it possible to preserve complex stoichiometry, while providing high quality, good performance coatings [10-13]. Our studies showed great versatility in obtaining controlled compositional and morphological characteristics of the coatings, depending on the nature of CaP and PLD experimental conditions. Thus, the OCP PLD coatings were prevalently amorphous with nanocrystalline domain inclusions, had a tree-like, porous morphology, and dissolved faster in simulated body fluid [3,5]. The HA and Mn-CHA coatings were more crystalline, with a granular, more compact morphology, and showed almost no biodegradability in SBF [4,5].

In vitro tests indicated that primary human osteoblasts, when cultured on the surface of pulsed laser deposited CaP thin films for up to 21 days, had a normal morphology, a very high proliferation rate, and a significantly better viability compared with those growing on uncoated Ti. The levels of alkaline phosphatase (ALP) activity, collagen type I (CICP) production, and transforming growth factor beta 1 (TGF-β1) suggested that both types of CaP coatings favored osteoblast differentiation [5, 14].

An *in vivo* biological evaluation of HA, OCP, and Mn-CHA nanostructured coatings, pulsed laser deposited on Ti substrates, is here reported for the first time. The effects of the coatings were studied on bone implants in rabbits. The tensile strength test was chosen over SEM investigations or histology of the bone-implant interface, because it has the advantage of being a functional, more clinically relevant measurement. The model used makes it possible to measure the strength of the bone to implant attachment directly with minimal influence of the effects of friction and mechanical interlocking caused by surface roughness [15]. The performance of the test implants was compared to uncoated titanium implants.

2. Materials and methods

2.1. Implants: Ti samples/coins

Coins of commercial pure titanium, grade 2 (ASTM B 348) were obtained by machining. The 48 implants used were all manufactured from 10 mm cylindrical bars, machined down into disks, 1.95 mm thick and 6.25 mm in diameter. On the reverse of every coin shaped implant, a threaded non-penetrating hole for the connector was drilled in preparation of the tensile test. To remove waviness resulting from machining, the test surfaces of all coins were standardized by finishing with a diamond abrasive polishing paste with 6 μm grain size (according to Struers® Metalog Guide).

As suggested in [16], rough-surfaced implants display a better performance than smooth ones, as the surface geometry distributes the load over a broad surface area and thus provides increased resistance against an overload of the bone structures. Consequently, all the Ti coins were chemically etched. After standard chemical etching procedure, all surfaces were characterized by a mean roughness of 0.7 μm.

2.2. Powders and PLD targets

The HA pellets were made of high purity commercial Osprovit ® HA powder. The Mn-CHA (0.55% Mn^{2+}, 5% CO_3) and OCP powders were synthesized by precipitation methods [17, 18]. The HA and Mn-CHA targets were prepared by pressing at 5 MPa, followed by sintering at 380 ºC for 8 hours. The OCP targets were prepared by pressing at 8 MPa. All targets were dense and compact.

2.3. CaP PLD coatings

Note that PLD consists in the stoichiometric transfer of a material from a solid target into a thin film, as an effect of high intensity pulsed laser irradiation [19]. Each laser pulse expels an infinitesimal amount of ablated material in a plasma plume which evolves toward the collector substrate. The collector is usually parallel to the target and a few centimeters away from it and is generally heated in order to improve coating adherence and/or crystallinity. The deposition process takes place in vacuum or at low pressure in a controlled atmosphere.

The coatings were grown using a PLD facility (Fig. 1) equipped with a KrF* laser source (λ = 248 nm, τ > 7 ns) running at a repetition rate of 2 Hz. After optimization, the laser fluence incident on target surface was set to 2 J/cm^2. To avoid piercing during ablation and improve films' morphology, the targets were both rotated with 0.04 Hz frequency and translated along two orthogonal axes. The target-substrate distance was 4 cm. To deposit each film, 15,000 subsequent laser pulses were applied, resulting in a thickness ≥ 400 nm, as measured by profilometry and electron microscopy in cross-section investigations.

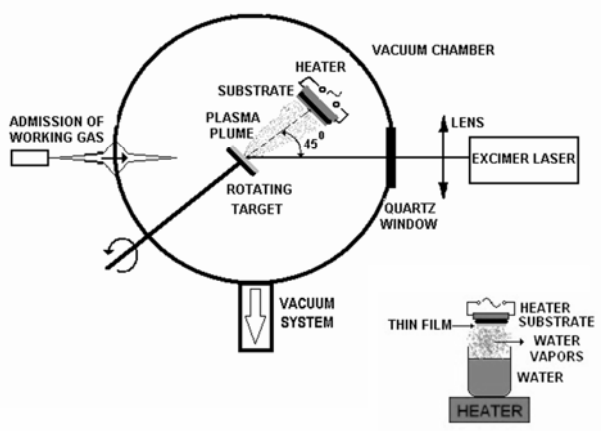

Fig. 1. Pulsed Laser Deposition equipment used in the experiments.

In accordance with the results of physico-chemical characterization, the following optimum deposition regimes were applied throughout the deposition experiments. Thin HA and Mn-CHA films were deposited at a constant temperature of 400°C in 13 Pa O_2 [4], while the OCP coatings were synthesized at 150°C in 50 Pa water vapors [3]. The constant rate of substrate heating/cooling was set at 6°C/min in all cases and monitored with a Eurotherm 2146 instrument. The samples were allowed to cool down in the same atmosphere that had been used for deposition. To improve hydroxylation and crystallinity status, all coatings were then subjected to 6-hour post-deposition heat treatments in water vapor enriched atmosphere at the same temperature as during deposition.

2.4. Roughness investigation

To inspect surface roughness, randomly chosen coins were examined by confocal laser scan microscopy. The average roughness of the surface (R_a) was calculated from the vertical position of each point (Z_i) of the coin topography.

The equipment used in the experiment included:

i) a True Confocal Scanned 4D objective 10x/0.3 (oilless) (Leica) for the optical/scanning part,

ii) the software SCANWare 5.10 (Leica Lasertechnick GmbH) to set the parameters and analyze

the data, and

iii) an (Omnichrome) Ion Laser power supply as laser beam.

To get representative values of the surface morphologies and effectively compare coins from different groups, the coin surfaces were divided into four equal areas. The topography of every quarter was scanned, using the maximal scan size of the confocal laser beam, 1 x 1 mm^2.

Based on the four roughness values obtained by scanning for each coin, the average roughness value (arithmetic mean deviation of the surface) and standard deviation characteristic of every particular surface were calculated.

2.5. Animals

Twelve New Zealand White female rabbits, 6 months old and weighing 3.0 to 3.5 kg each, were used in our studies. The animals were kept in separate cages during the experimental period. According to local regulations, room temperature and humidity were standardized at 19 ± 1 °C and 55 ± 10%, respectively.

The experiments were approved by and registered with the Norwegian Animal Research Authority (NARA). All procedures were conducted in accordance with Norway's National Animal Welfare Act of December 20, 1974, No 73, chapter VI, sections 20-22, and Regulation on Animal Experimentation of January 15, 1996.

2.6. Sedation

The rabbits were sedated by injection with fluanisone-fentanyl (Hypnorm®, Janssen, Belgium) 0.05-0.1 ml/kg s. c. and further anesthetized with midazolam (Dormicum®, Roche, Switzerland) 2 mg/kg bw i.v. The sedation was maintained with diluted Hypnorm® (1 ml Hypnorm and 9 ml sterile water). Lidocaine/adrenaline (Xylocain/Adrenalin®, Astra, Sweden) 1.8-ml s.p. was used for infiltration analgesia at the site of surgery.

2.7. Surgical procedures

Four groups of 12 coin implants - uncoated etched Ti controls and Ti etched and coated with HA, Mn-CHA, or OCP - were tested for tensile/pullout strength. All implants were placed in randomly chosen animals. Since we did not know whether the implants would induce systemic responses, the groups were not intermixed, thus each animal only received implants from one test group.

Prior to surgery the operation sites were depilated, washed with soft soap, and disinfected with colored chlorhexydine gluconate 5 mg/ml (Klorhexidin, Galderma Nordic AB, Sweden). The animals were placed on their back on the operation table, covered with sterile cloths, and the operating sites were disinfected once more with chlorhexydine gluconate 5 mg/ml. An incision was made on the proximal-anterior part of the tibia, penetrating all soft tissue layers. The periosteum was elevated, and four small guide holes were made into the bone marrow with a twist drill (Medicon®, Germany), while a drill guide was used to ensure standardized positioning. The two outermost holes were used for fixing the bone plate, while the two central ones were for stabilizing the custom-made stainless steel bur in leveling the platforms for the implants in the cortical bone. All bone tissue handling was executed under copious irrigation with a physiological saline solution. The implants were then placed on the cortical preparations with only the coated surface being in contact with bone. The outer aspects of the implants were covered with polytetrafluoroethylene (PTFE) caps in order to inhibit bone growth onto the vertical sides of the implant, as well as bone overgrowth [15]. The PTFE-covered implants were then fixed to the bone with a pre-shaped bone plate (Medicon® CMS, Germany), fastened with two titanium screws (Medicon® CMS, Germany), as can be seen in Fig. 2.

Fig. 2. Test implants were placed on the prepared sites on tibia bone in rabbits. To avoid influence of bone contact onto the vertical aspects of the implants during the tensile test, the implants were covered with Teflon® caps that were removed prior to the pull out procedure. The test surface of the implants were placed directly onto the prepared bone surface, and were left unaffected by the Teflon caps and the retention apparatus used to ensure good stability and contact between implant and bone during healing.

Once implant procedures had been completed, the soft tissue layers were repositioned and the wound closed using a polyglycolic acid suture. Following surgery, each animal received an s. c. injection with buprenorphine (Temgesic ®, Reckitt & Colman, England). A second injection of Temgesic was given 3 hrs post-surgery. The animals'

health condition was monitored throughout the study period, and the operation sites were examined daily until wound healing was complete. After 8 weeks, the animals were sacrificed to measure bone-implant attachment by the tensile test. They were euthanized using an i.v. injection of 1.0 ml fentanyl-fluanisone (Hypnorm®, Janssen, Belgium), followed by 1 ml/kg bodyweight pentobarbital (Mebumal ®, Rikshospitalets Apotek, Norway) i.v..

2.8. Tensile test

The pullout (tensile) test was selected to evaluate the strength of the biological-chemical bonding between the bone and the implant surface, as it ensured minimal interference from other implant attachment mechanisms, namely mechanical interlocking and friction. To minimize the different mechanical effects, the flat coin-shaped implants were placed onto a flat bone surface and passively attached to the cortical bone by a titanium band retainer [15].

The 8-week term is considered a proper observation period for tensile tests covering the bone formation and early maturation stages [15, 20, 21]. Immediately after euthanasia, the titanium plates covering the implants were exposed and removed. A hole was made at the centre of the PTFE cap with a small injection needle. Pressurized air was then applied using a syringe to remove the caps and expose the reverse side of the implant without applying any force on the implant itself. The tibia bone was then fixed in a specially designed device that stabilized it during the tensile test procedure. Subsequently, the implants were connected to the load-cell by a threaded pin with a ball-head connected to a 300 mm long wire. This setup made it possible to ensure the load force was perpendicular on the test surface of the implants, thus minimizing the influence of shear forces during tensile testing.

The tensile tests were performed with a Lloyds LRX Materials testing machine fitted with a calibrated load-cell of 100 N. Crosshead speed was set at 1.0 mm/min. Force measuring accuracy was +/- 1% (Certificate of calibration: NAMAS Calibration No. 0019 Issued by Instron Calibration Laboratory no. 1000356). Load was applied until the implants detached from the bone and was recorded on a load vs. time plot.

Statistical analyses were carried out with Student t-test in a pairwise comparison in order to determine statistically significant differences in measured tensile strength between the test groups and controls and estimate the confidence intervals.

3. Results

The CaP coatings deposited by PLD on Ti substrates display significant morphological and structural differences. In fact, the thin films deposited from OCP targets have an amorphous-poor crystalline structure, whereas coatings deposited from apatitic targets are better crystallized. Furthermore, the surface of the OCP coating consists of grains and cauliflower-like aggregates, whereas HA, as well as Mn-CHA thin films display a more granular surface, with the mean dimensions of the grains smaller than those of OCP [14].

Roughness analysis results showed that the test surface of all the implants had the same designed average roughness of (4.8 +/- 0.6) μm**.**

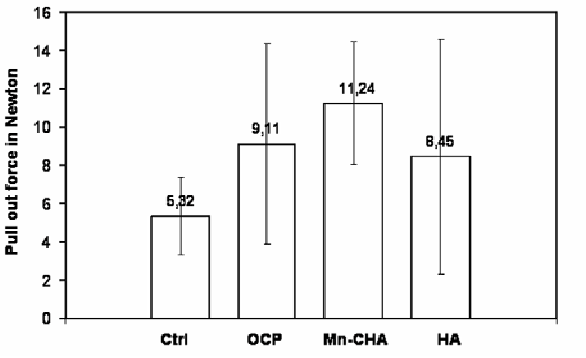

Fig. 3. Diagram of pullout force (tensile strength) for different types of samples. Error bars are ± SD, n = 12 in each group. The values are averaged over data collected for implants of the same type. Asterisks indicate a significant difference between test and control ($p < 0.05$). Ctrl denotes the control group, OCP is the octacalcium phosphate group, Mn-CHA stands for the manganese-doped carbonated hydroxyapatite group, and HA is the hydroxyapatite group.

The results of pullout test measurements for the CaP-coated Ti compared with the uncoated Ti control implants were given in Fig. 3. They clearly demonstrated that all three tested groups (HA, Mn-CHA, and OCP) had a significantly improved bone attachment strength (statistical significance values $p \leq 0.05$), about twice as high as that of control implants (average 5.32 N). Very important too, this strength was 25% higher for Mn-CHA (11.2 N) and 10% higher for OCP (9.11 N), as compared with commercial HA coated implants (8.45 N).

4. Discussion

Having the same roughness**,** all the CaP-coated implants should provide the same degree of mechanical interlocking with bone. According to earlier tensile test studies [21, 22], an average roughness of about 4 μm is considered optimum for the study of direct bone bonding. Consequently, our investigations focused on the relative merits of different material coatings on titanium implant surfaces having a 4 μm average roughness.

It should be emphasized that the tensile test was performed to provide a direct measurement of the attachment between the bone and the implant surface that results from chemical bonding. In animal experiments, increased bone retention of the implant, as measured in our tensile test, is considered a clinically relevant indication of improved stability and capacity of the implants to carry

load without detaching.

This kind of information cannot be acquired through SEM investigations or histology, which provides only limited information on the functional performance of implants. As a matter of fact, a significant correlation between the amount of new bone formed onto the implant surface and the necessary force to detach the implant is rarely established, as the bone will fracture at the weakest region, which could be at the implant-bone interface, in the applied coating or in the adjacent bone.

Since the implant surfaces tested in this study were made relatively smooth to minimize the influence of mechanical interlocking, there was not enough tissue left on the implant surface for histological analysis, microscopy or biochemical analyses. This suggests that the detachment of the implant appears through disruption of the bone-implant interface. Since the test implants all performed better than the controls it is unlikely that detachment occurred through loosening of the surface coating. However, more studies are needed to address how the functionalized implants detach from the bone, and thus to fully understand the molecular and chemical nature of the observed improvement in bone attachment.

5. Conclusions

Tensile strength tests on the CaP PLD-coated titanium implants showed an improved bone-implant attachment after eight weeks of healing. Our studies also suggest that using Mn-CHA or OCP for nanostructured implant coatings significantly increases the osseointegration potential of titanium, as visualized by the tensile test. Moreover, the test results show that while all CaP coatings improved the performance of the titanium implants, the novel coatings reported here (OCP and Mn-CHA) perform significantly better than classical HA, suggesting that these coatings are more bioactive.

The positive outcome of this *in vivo* test demonstrates that nanostructured CaP coatings can be utilized to improve bone attachment to titanium surfaces. Further development of nanostructured biomimetic coating based on OCP and Mn-CHA is encouraged for developing bone implants with improved clinical performance.

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