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# Improvement of hydroxyapatite deposition on titanium dental implant using ArF laser ablation: effect on osteoblast biocompatibility *in vitro*

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## Abstract

To develop a better surface conformation of titanium dental implants, we examined the *in vitro* biocompatibility of a thin natural apatite (NA) film deposited by laser ablation. Thin (2000-Å) hydroxyapatite (HA) and NA films were deposited on titanium discs using an ArF excimer laser operating at a repletion rate of 10 Hz and annealed by heating at 360°C for 1 h. Energy dispersive analysis of the NA film revealed peaks of Na and Mg in addition Ca and P. X-ray diffraction showed that crystalline HA was present in the HA and NA films. Primary mouse osteoblast grew faster and had higher alkaline phosphatase activity when grown in NA films than on HA films or a bare titanium surface. In addition, osteocalsin production by these cells was higher on HA and NA than bare titanium, but there was no significant difference between cells grown on HA and NA. Thus surface modification with NA film may contribute to successful osteoblast function and differentiation at titanium interface.

## Introduction

Because hydroxyapatite (HA) has an inorganic structure similar to bone, it has unique advantages for bone reconstruction; however, sintered HA alone is too weak to be used for bone repair devices. Despite this, the osteoconductivity of HA makes it popular as bioactive coating material for dental implants [1] [2].

HA or apatite coatings are typically obtained by plasma spray, ion sputtering, pulsed-laser deposition (PLD), and methods similar to PLD [3] [4]. PLD methods allow the deposition of very thin films as well as control of surface roughness and ablation of any material, therefore providing strong bonding between the film and substrate. A previous study [4] showed that PLD can be used to produce an ultra-thin film of HA with improved mechanical properties, including increased tensile strength and decreased film thickness. Moreover, *in vivo* [5] and *in vitro* studies [6] have shown that titanium implants coated by PLD with an ultra-thin film of HA have good biocompatibility.

Some applications have employed natural apatite (NA) in place of chemically synthesized HA. NA is not pure HA but contains trace elements including magnesium and sodium [7], and it is expected to be more effective for bone cell seeding and proliferation than chemically synthesized HA. In the current study, to help develop a better surface conformation of titanium dental implants, we examined the *in vitro* biocompatibility of NA films deposited by laser ablation in an osteoblast culture.

## Materials and methods

### Starting material

Raw HA (Japan Chemical Co., Sapporo, Japan) and NA (Fujita Kasei Giken, Tokyo, Japan) powder was pressed at 150 MPa in a cylindrical steel die to form 20-mm diameter, 2-mm thick disks. These HA and NA disks were used as targets for PLD.

### Preparation of the titanium surfaces

Titanium disks (15 mm in diameter and 1 mm thick) were certified according to JIS (Japanese Industrial Standards) Grade II specifications. Discs were prepared by manual parallel polishing using silicium carbide papers (grade 80 to 4000) and automatic mirror polishing using 0.05- to 3-μm $Al_2O_3$ particles. Deposition of HA targets was carried out in water vapor generated by bubbling $O_2$ gas through a water bath at 0.8 m Torr ($1.1 \times 10^{-1}$ Pa). An ArF excimer laser (wavelength = 193 nm; pulse duration = 20 ns) operating at a repetition rate of 10 Hz was used for deposition. The film was deposited at substrate temperature of 300°C and at a rate of 10 nm/min. The film thickness was estimated on the basis of the deposition rate. Film thicknesses of 2000 Å were prepared and annealed by heating at 360°C for 1 h (at heating and cooling rates of 1.5°C/min) in water vapor generated by bubbling $O_2$ gas through a water bath. After annealing, the crystallinity of the coatings was characterized by X-ray diffraction (XRD). The surface conformation of the HA film on the titanium disc was observed by energy dispersive analysis (EDX). Prior to analysis and *in vitro* experiments, test samples were cleaned by a series of 10-min ultrasound treatments in pure acetone, pure alcohol, and distilled water. For cell culture experiments, test samples were sterilized in an autoclave. Four samples were tested for each group.

### Cell culture

Primary mouse osteoblasts (PMOs) were prepared from calvariae of newborn ddY mice by five sequential digestions with 0.1% collagenase and 0.2% dispase. Calvarial osteoblasts were collected

from the second through fifth digestions, and cultured for 3 days in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum.

**Cell proliferation**

PMOs were cultured in 75-$cm^2$ flasks in α-MEM. When monolayers reached confluence, the cells were removed with trypsin. Cells (1.5 x $10^4$/ml) were then seeded on disks placed in the wells of 24-well tissue culture plates containing α-MEM supplemented with 10% fetal bovine serum. The cells were incubated for 24 h at 37°C in an atmosphere containing 5% $CO_2$. Cells were fixed for 10 min in phosphate-buffered saline (PBS) containing 0.5% (w/w) Triton X-100, 4% (w/w) paraformaldehyde, and 5% (w/w) sucrose. Fixed cells were fluorescently stained for 1 h with PBS containing 1/1000 dilution of SYBR Green (Sigma, MO, USA) and $6X10^{-8}$ M rhodamine-phalloidin (Molecular Probes, OR, USA). Cell number and morphology were determined by automated fluorescence microscopy with a DMR 1200 upright microscope (Leica, Cambridge, England) equipped with a computer-controlled translation stage.

**Cell differentiation**

PMOs (4 x $10^4$ cells/ml) were seeded on coated and uncoated titanium disks placed in the wells of 24-well tissue culture plates containing α-MEM supplemented with 10% fetal bovine serum. The cells were incubated for 3 days at 37°C in an atmosphere containing 5% $CO_2$. The medium was removed from the wells, and the cells were cultured in α-MEM containing 10% fetal bovine serum and osteogenic supplements (10 mM β-glycerophosphate, 50 mg/l ascorbic acid, and $10^{-8}$ M dexamethasone) for 7 or 14 days. At each time point, the conditioned medium was removed from the wells to measure osteocalcin (OCN) production.

**Assay of alkaline phosphatase (ALP) activity**

PMOs were washed with PBS and then frozen and thawed three times. The lysates obtained from the cells were homogenized in 10 mM Tris (pH 7.4) and sonicated for 15 min on ice. A fluorimetric assay was performed to ascertain the total amount of DNA. Briefly, immediately prior to analysis, half of the lysate was adjusted to Hoechst 33528 (DOJINDO Laboratories, Kumamoto, Japan). The fluorescence was then quantified on a CytoFluor 4000 fluorescence plate reader (Applied Biosystems, Ca, USA) at an excitation wavelength of 350 nm and an emission wavelength of 455 nm. The remaining half of the lysates was used for measurement of ALP activity. ALP activity was assessed colorimetrically at pH 10.3 using *p*-nitrophenyl phosphate (Sigma, St. Louis, MO, USA) as the substrate. Conversion of the substrate by ALP was quantified at 405 nm using a Maxline ELISA Plate Reader (Molecular Devices**,** CA, USA). The amount of ALP activity was normalized by the amount of total DNA in the cell lysate.

**Assay for OCN production**

The OCN levels in the conditioned media were measured using a Mouse OCN RIA Kit (Biomedical Technologies, MA, USA). This sandwich enzyme immunoassay is specific for mouse OCN and can measure its levels directly in cell culture supernatants. The amount of OCN was normalized by the amount of DNA in the cell lysate.

**Statistical analyses**

One-way analysis of variance was used to determine the statistical significance of the differences of results between different film thicknesses. When a statistically significant difference was found, a Scheffé test was used to examine the significance of the differences between individual groups.

## Results

### Structure of HA films

X-ray diffractograms of the targets and coatings are shown in Figs. 1 and 2. A few peaks in the X-ray diffractograms of both the HA and NA coatings corresponded with HA, and, overall, the X-ray diffractograms for of the HA and NA targets were as expected for these materials. EDX showed peaks of Na and Mg in addition to Ca and P for the NA film (Figs. 3 and 4).

### Effect of the films on the proliferation of PMOs

Fig. 5 shows the proliferation and morphology of PMOs grown on bare titanium or titanium coated with thin films of HA or NA. Cell proliferation was measured by staining the cells with SYBR Green and determining cell numbers by fluorescence microscopy. The extent of cell proliferation was the highest on the NA film.

### Effect of the films on ALP activity

Fig. 6 shows the ALP activity of cells growth on bare titanium or titanium coated with thin films of HA or NA. At both 7 and 14 days, the level of ALP activity on the different substrates decreased in the following order: NA> HA> bare titanium.

### Effect of the films on OCN production

Fig. 7 shows the effect of the films on OCN production as assessed by immunoassay. The production of OCN by cells cultured on HA and NA films for both 7 and 14 days was significantly higher than cells cultured on a bare titanium, but the differences between cells on HA and NA films were not significant.

## Discussion

In the current studies, we used ArF PLD to coat a highly polished titanium substrate with thin films of HA and NA. PMOs grew most rapidly and had the highest level of ALP activity on NA films.
Previous plasma spraying methods do not allow coating of a substrate with NA. PLD is suitable for producing calcium phosphate coatings of different compositions and crystallinities [8], and in the current studies, XRD and EDX results showed that the chemical contents and crystallinity of the films generated by PLD here were similar to those of the raw materials used as a source of apatite.
We found that PMOs proliferate more rapidly on crystallized HA and NA films than on bare titanium. Apatite formed on titanium is known to more effectively adsorb serum proteins than a bare titanium surface. In addition, HA films enhance cell attachment and proliferation [9] due to the adsorption of high levels of fibronectin [10] [11]. In agreement with our current findings, Chou et al. [12] showed that human gingival fibroblasts grow faster on high-crystallinity HA coatings than on uncoated titanium. Therefore, the differences in cell proliferation on HA and NA films may be due to differential absorption of serum proteins. Specifically, a higher adsorption of serum fibronectin may be responsible for the higher rate of osteoblast proliferation on NA films.
PMOs may be a useful for studying the effects of bone replacement biomaterials. These cells are known to synthesize ALP and noncollagenous extracellular bone matrix proteins such as an OCN. Thus, ALP and OCN are useful markers of osteogenesis [13] [14]. Compared to cells grown on HA films, cells grown on NA films for 7 or 14 days produced higher levels of ALP and slightly higher levels of OCN after 14 days. This difference in the level of OCN between cells grown on HA and NA films may become greater with extended culture because the expression of OCN occurs later

during extracellular matrix mineralization.
Our results also suggested that the NA film was the most effective at promoting the differentiation of PMOs. This may be due to the release of ions that mimic magnesium ions. Smith et al. [15] demonstrated that a magnesium deficiency significantly and progressively diminishes bone formation, leading to osteoporosis. In addition, spectroscopic analysis indicates that, in the NA used for the current studies, both $OH^-$ and $PO_4^{3-}$ sites are substituted by carbonate ions [4]**.** Although the levels of carbonate ions in bone mineral are small, they may play a significant role in the biochemistry of hard tissues [16]. Consequently, a number of studies have focused on the production of synthetic carbonate-substituted HA ceramics for bone replacement [16] [17].
Our previous study[18] of materials implanted in canine mandibles showed that implants coated with NA films by PLD are better integrated and more biocompatible than uncoated implants. Taken together the current data suggest that surface chemistry modification of Ti with NA films may prove beneficial in the design of new implant materials. Further studies are needed to determine the operability, stability, and other clinical features of the implants coated with NA films.

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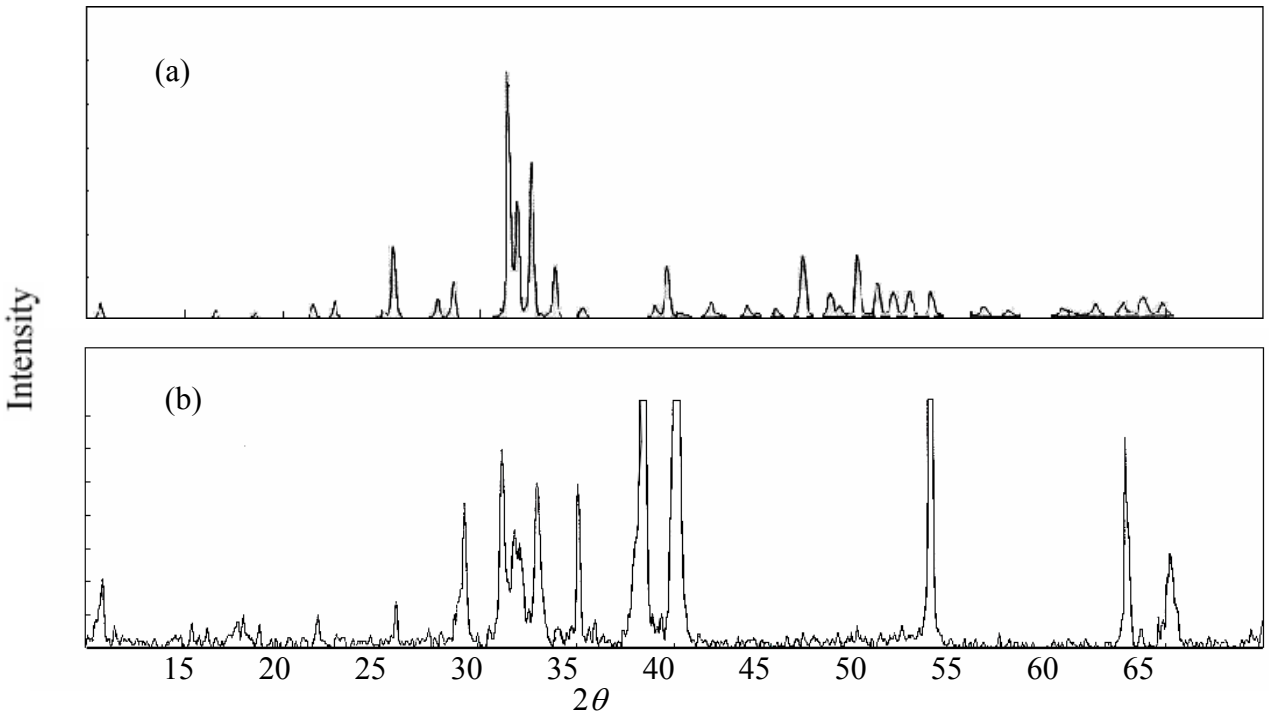


Fig. 1 XRD patterns of an HA film formed on a titanium substrate. (a) HA target. (b) HA film after post-annealing.

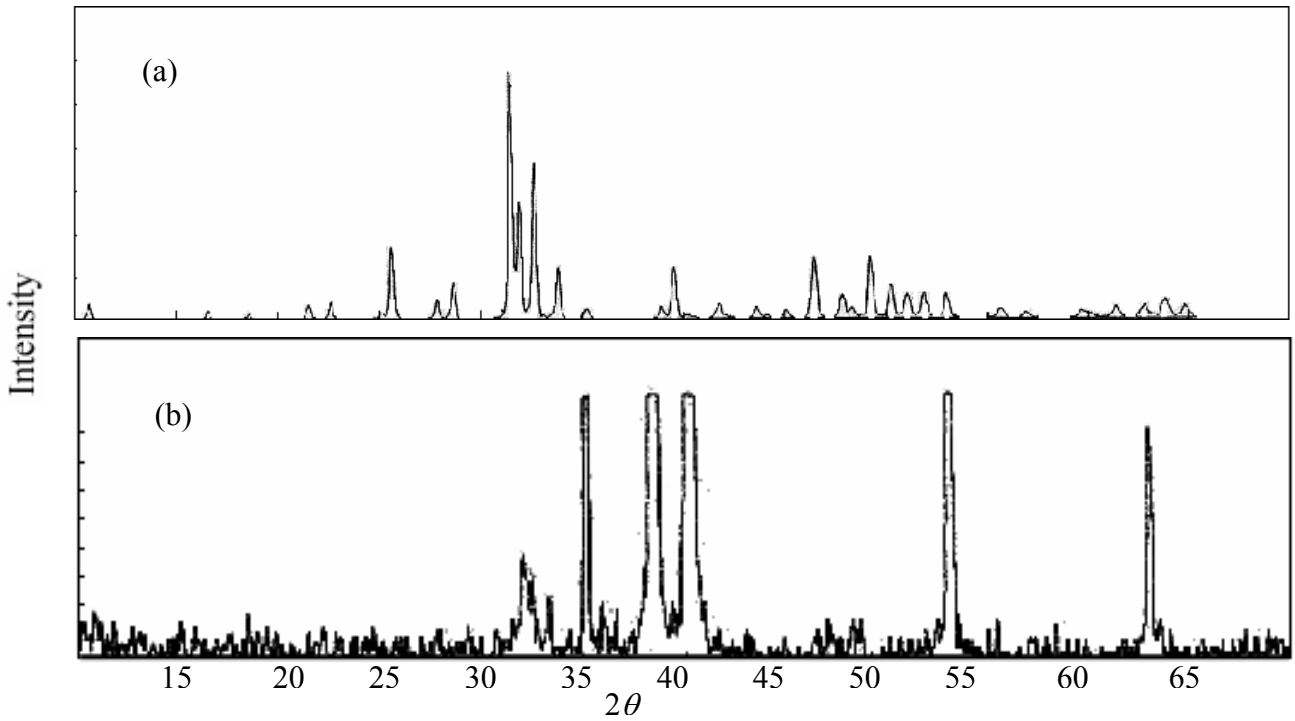


Fig. 2 XRD patterns of an NA film formed on a titanium substrate. (a) NA target. (b) NA film after post-annealing.

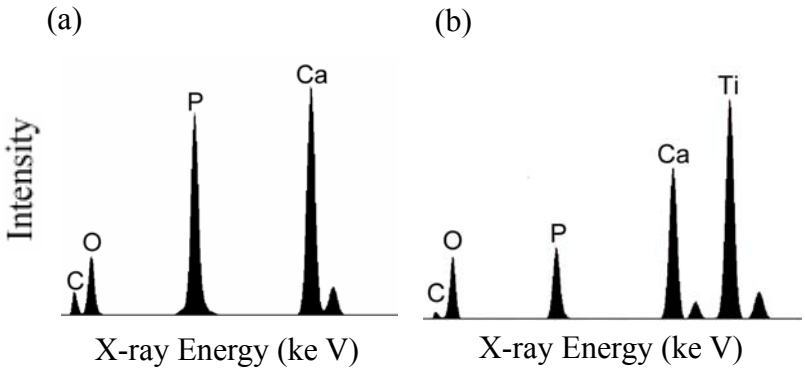


Fig. 3 Composition analysis of the HA film on the titanium substrate by EDX.
(a) HA target. (b) HA film on the titanium substrate.

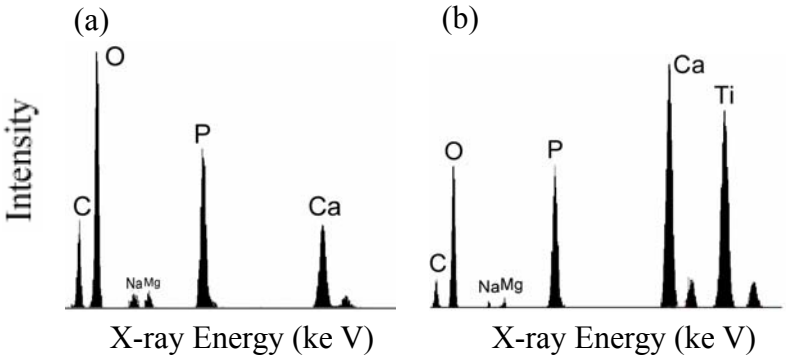


Fig. 4 Composition analysis of the NA film on the titanium substrate by EDX.
(a) NA target. (b) NA film on the Ti substrate.

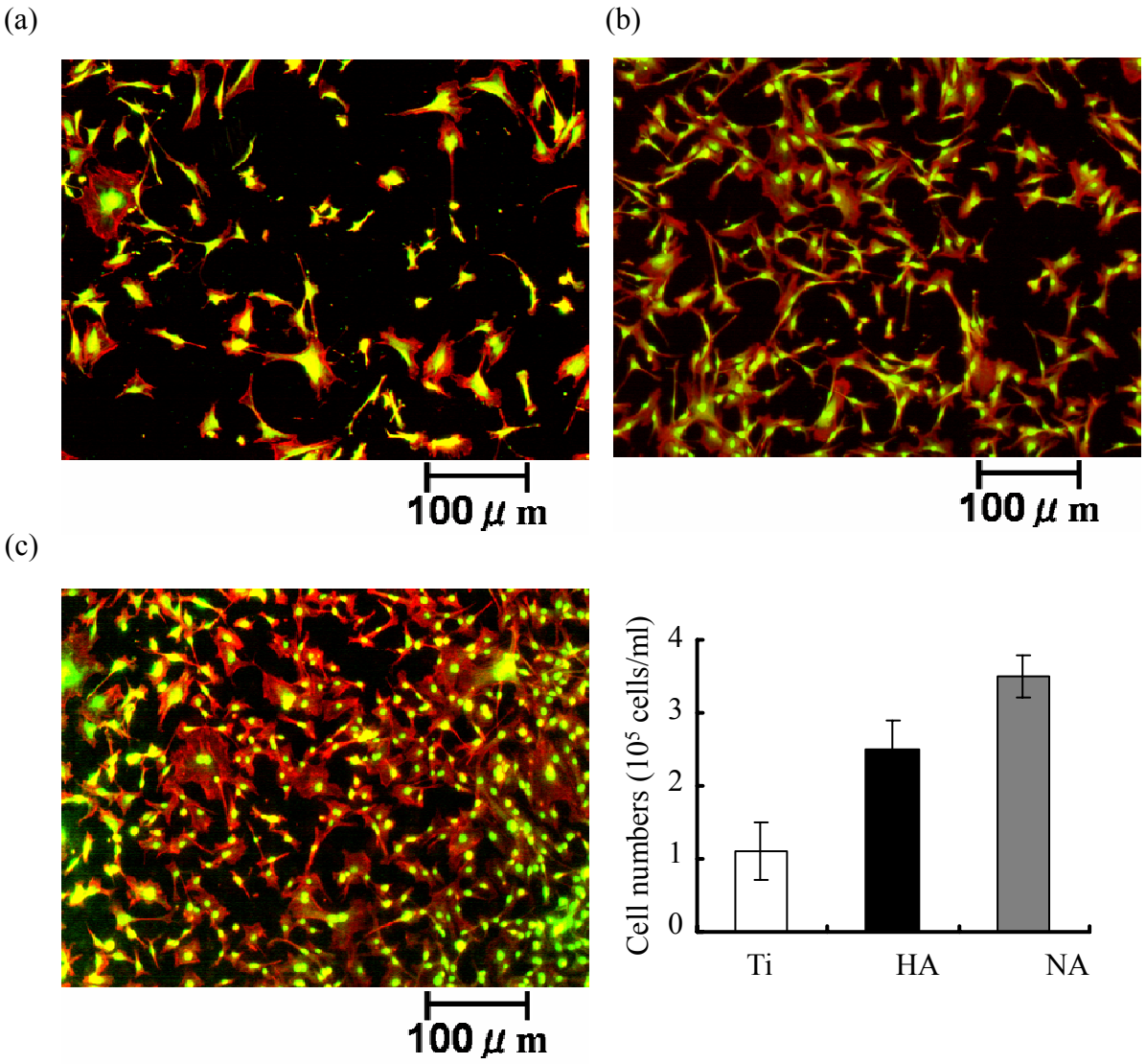


Fig. 5. Morphology (a–c) and proliferation (d) of PMOs. Cells were grown on bare titanium (Ti) or titanium coated with thin films of HA (b) or NA coated (c), stained with SYBR Green and rhodamine-phalloidin, and visualized by fluorescence microscopy. The cell numbers were counted and are shown in (d)

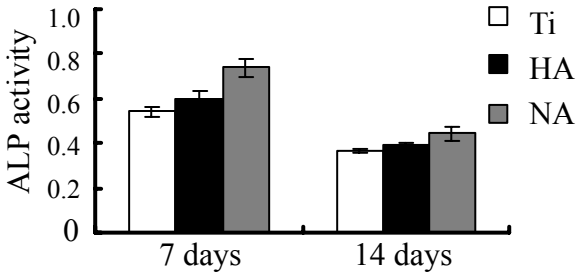


Fig. 6 ALP activity in lysates from PMOs grown for 7 or 14 days on bare titanium (Ti) or on thin films of HA or NA deposited by PLD on titanium surfaces. Bars represent the μmol of ALP per μg of DNA.

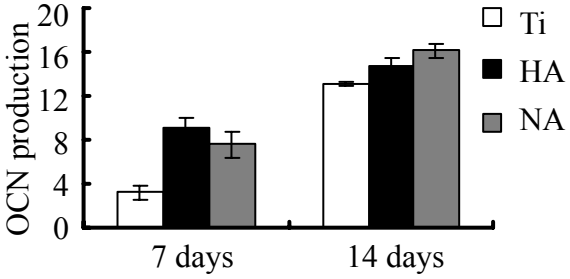


Fig. 7 OCN levels in PMOs grown for 7 or 14 days on bare titanium (Ti) or on thin films of HA and NA deposited by PLD on titanium surfaces. Bars represent the ng of OCN.

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