# Osteoblast cell activity on calcium phosphate layers grown on glass by a laser-liquid-solid interaction

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A calcium phosphate layer was grown on glass by a laser-liquid-solid interaction (LLSI) process in simulated body fluid (SBF). Glass samples with a layer grown by simple soaking in the SBF (i.e. without laser irradiation) were prepared for comparison. Formation of an inhomogeneous calcium phosphate (CaP) layer on both laser-treated and non-treated samples was observed. The results showed that the laser irradiation did not change the layer structure and morphology but yielded the growth of a thicker CaP layer. With increasing load the elasticity and the hardness increased for both laser-treated and non-treated samples. Furthermore, we tested the osteoblast cell activity of the CaP layers grown on the laser-treated and non-treated samples. Toxicity test showed that the viability of the cells on the layer grown by the LLSI process was over 95%. A permanent increase in the cell number was observed for both groups of samples, and it was more stable on the laser-treated surfaces. The latter showed a higher cell number after 7 days of cell culturing. A slower increase, resulting in a lower cell numbers was observed for the samples untreated with laser irradiation.

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## 1. Introduction

CaP phases are present as inorganic components of human bones and teeth. One method for CaP deposition on solid surfaces is their soaking in a solution resembling the human plasma (SBF) [1]. Despite its similarity to mineral formation in nature, the method requires a long time to obtain CaP precipitates. The application of external energy (ultrasound, ultraviolet or microwaves) in the solution-substrate system influences the CaP formation *in vitro* [2-4]. On the other hand, laser sources of energy are used for processing living tissue and the synthesis of biocompatible materials, pulsed laser deposition, biofilms modification, bone defect healing, and the modification of root canal dentine [5,6].

Interactions of materials with cells have been intensely investigated, since materials compatible with cells are important in medical applications [7]. Cells are highly sensitive to topography, roughness, chemistry, surface charge, and hardness [8,9]. Cell-material interactions *in vitro* may be approximated by the process of cell adhesion and spreading, which is a convenient way to determine the biocompatibility of a material. Proteins can also enhance the cell adhesion and thus to modulate the cellular interactions.

This study was divided into two parts: growth of CaP layers by laser irradiation of glass samples in the SBF (the LLSI process) and an *in vitro* cell culture study of the CaP layer with osteoblast cells.

## 2. Experimental

### 2.1. CaP growth by LLSI

The SBF was prepared according to the procedure described in [1]. CaP layers were grown on glass by LLSI in the SBF, as well as by simple soaking in the fluid (i.e. without laser irradiation)for comparison. The applied LLSI method allows simultaneous interaction between the laser beam and the glass immersed in the SBF [1]. The laser beam of a CuBr pulsed laser ($\lambda$ = 578.2 nm, 400 mW) was directed perpendicularly to and focused on the substrate surface. The time of interaction with each glass sample was within 5 min. After the irradiation, all samples were immersed in a 500 ml total volume of irradiated SBF for 6 hours under physiological conditions ($37^0$C, pH 7.4).

### 2.2. *In vitro* test with osteoblast cells

We tested the *in vitro* biocompatibility of the CaP layers grown on the laser-treated and non-treated samples with osteoblast-like cells (MG-6, concentration of $125x10^3$ cells/ml). The cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 0.5% antibiotics, incubated at 37°C and 5% $CO_2$ for 1, 3, 5, and 7 days. Osteoblasts grown in 6-well plates were prepared as controls. Samples from both groups were also covered with the protein fibronectin (FN) for 30 min, to study the initial interactions of the cells cultivated for 2 hours.

### 2.3. Analysis

The topography of the grown CaP layers was observed by coherence probe microscopy (CPM; Leitz Linnik interferometer based system, $\lambda$ = 350-1100 nm, axial and lateral resolution of 10 nm and 0.45 µm, respectively). The layer structure was studied by micro-Raman spectroscopy (Renishaw Rama-scope, $\lambda$ = 488 nm, reflection mode). The elasticity and hardness of the CaP layers was measured by a scanning force microscope (SFM, NanoScan, Russia, loads of 200 to 500 µN).

The cell morphology was observed by SEM (JEOL 5400 LV) after a standard procedure for cell preparation. Cell viability was evaluated by the trypan blue exclusion test. A Kruskal-Wallis nonparametric test was performed for statistical analysis ($p< 0.05$ was considered statistically significant). The overall cell morphology of the osteoblasts was observed using a laser scanning confocal microscope (magnification 20x).

## 3. Results and discussion

### 3.1. CaP layer

An inhomogeneous layer was grown on the glass surfaces from the SBF, either by applying the LLSI (Fig. 1a) or without it (Fig. 1b), as shown by the CPM images.

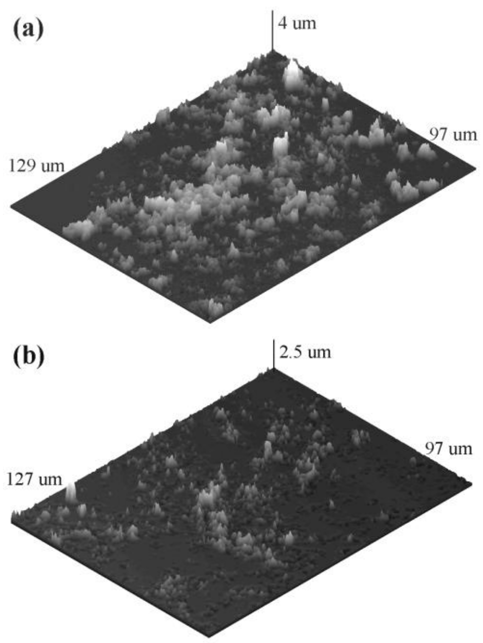



*Fig. 1. Typical CPM images of the grown CaP layers on: (a) laser-treated and (b) non-treated glass samples.*

The average layer thickness and rms roughness on the laser-treated samples were 3.7 and 0.9 µm, respectively, and on the non-treated samples these values were 2.3 and 0.5 µm. Laser-treated surfaces yielded higher CaP roughness.

The Raman spectra of the laser-treated (Fig. 2, spectrum 1) and non-treated samples (spectrum 2) showed the formation of a CaP layer through the P-O vibrational modes of the $PO_4$ group. An increased layer thickness was observed when applying the LLSI.

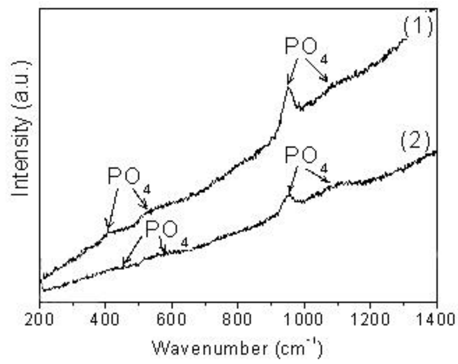



*Fig. 2. Raman spectra of laser-treated (1) and non-treated (2) samples showed the formation of a CaP layer and an increased layer thickness after the LLSI.*

Nanoscan images of the grown CaP on laser-irradiated and non-irradiated surfaces are shown in Fig. 3. SFM showed that upon increasing the load from 200-500 µN, the elasticity of the laser-irradiated samples increased from 34-119 GPa, and for non-irradiated samples it was 37-56 GPa. The hardness of the samples also increased: under the maximum applied load of 500 µN, the measured value was 7.2 for the laser-treated and 3.6 GPa for the non-treated samples.

### 3.2. *In vitro* tests with osteoblast cells

*In vitro* tests revealed good cell adhesion and spreading on both laser-treated and non-treated samples, after 1 and 3 days. As can be seen in Fig. 4a, more cells were observed after 1 day on the laser-treated, in comparison to the non-treated samples (Fig. 4c). The cell numbers on both types of surface increased after 3 days and they formed a confluent monolayer (Figs. 4 b,d). No specific cell orientation was found.

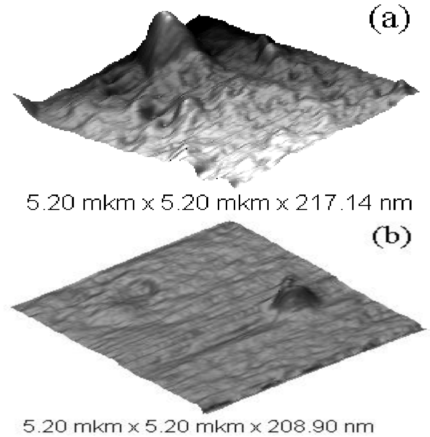



*Fig. 3. Nanoscan images of the laser irradiated (a) and non-irradiated (b) surfaces with grown CaP.*

Proliferation results (Fig. 5) showed a permanent increase of the cell number on the two groups of samples with increasing of incubation time. The increase was more stable on the laser-treated samples (spectrum 1). After 7 days of cell culturing, these samples showed a higher cell number. A slower increase in the cell number, which resulted finally in a lower cell number, was observed for the non-treated samples (spectrum 2).

Toxicity tests showed viability of the cells on the CaP grown on laser-treated samples of over 95%. No difference from this value was observed for the layer obtained without a laser irradiation.

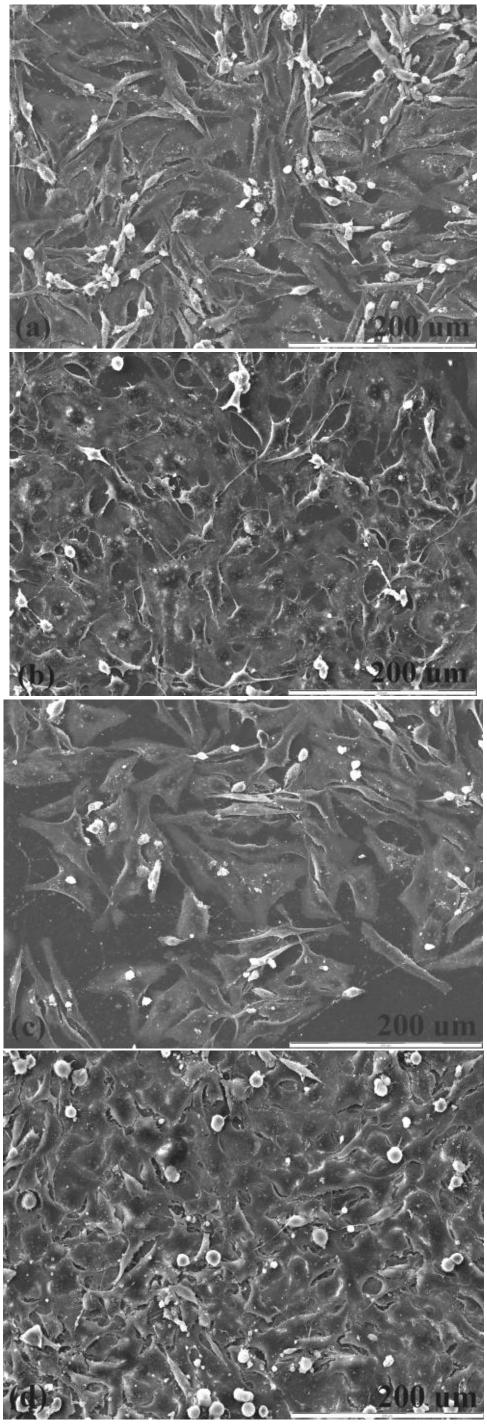



*Fig. 4. SEM images of osteoblast incubated on CaP grown on laser-treated samples for 1 and 3 days (a,b) and on non-treated samples for 1 and 3 days (c,d).*

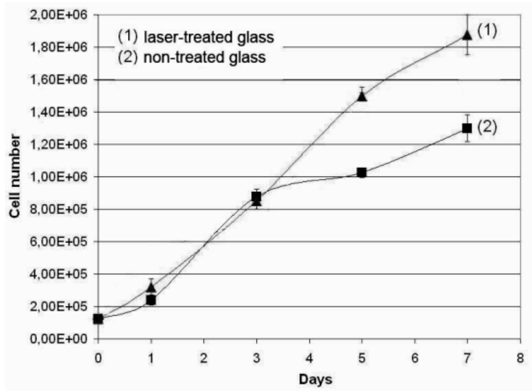
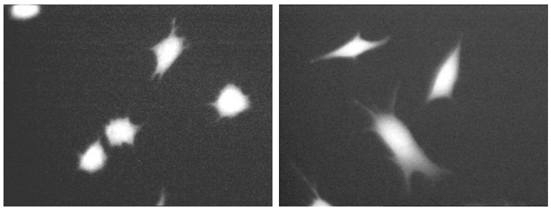



*Fig. 5. The proliferation results show a permanent increase in the cell number on laser-treated (1) and non-treated samples (2) with increasing days of incubation.*

FN coating was found to improve the initial interactions of osteoblast cells with both types of CaP-coated surfaces (laser-treated and non-treated) in terms of adhesion and spreading (Fig. 6). The number of cells increased after FN coating, and they also revealed a flattened morphology. The higher number of cells on the non-treated FN-coated samples (Fig. 6, bottom row) was assigned to the lower surface roughness of the CaP layer beneath.

**Glass + CaP after LLSI:**

Plain FN-coated



**Glass + CaP without LLSI:**

Plain FN-coated

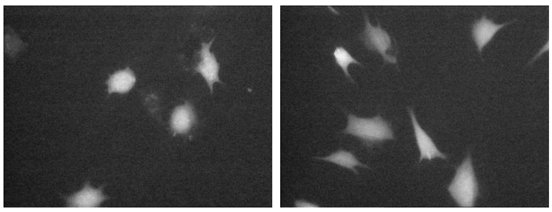


*Fig. 6. Overall cell morphology of osteoblasts adhering on laser-treated (upper figures) and non-treated (down figures) glass samples, plain and after FN coating (magnification 20x).*

## 4. Conclusions

The results show that laser irradiation does not change the structure or morphology of the CaP layer but yields the growth of a thicker layer.

Layers grown by LLSI in SBF or without laser irradiation are not toxic for the osteoblasts, which proliferate rapidly and after 3 days form a confluent monolayer. The initial interaction of the cells is improved by coating the surfaces with the protein FN. The CaP layer roughness influences the number of adhering cells.

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