

# Influence of Hydroxyapatite Crystallinity on the Growth of Keratinocytes

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**Abstract:** Dental implantology is a field, which has made a great progression recently. The main task nowadays is to shorten the healing period and so improve the comfort for the patients. One possibility how to full fill this task is to coat the surface of the implant. Very promising material seems to be hydroxyapatite, which is a natural component of human body and suitable method is the pulsed laser deposition. In our study we tried to evaluate difference between crystalline and amorphous hydroxyapatite coated dental implants from the biological point of view. We found that the cells were able to adhere to all of our studied samples. The worst proliferation of fibroblasts was found on the amorphous coating, whereas the adhesion was fully comparable with other surfaces. The level of keratinocyte differentiation was the same on both of the studied surfaces.

### Introduction

Studies on dental implants are nowadays focused at their coatings which can provide a surface improvement in order to obtain a better healing of the dental implant into the bone, called osseo-integration [1]. Hydroxyapatite (HA) is one of the most attractive materials for human hard tissue implants because it closely resemble to the bone and the teeth. The brittle nature of HA requires to be coated onto a more mechanical resistant material such as titanium or titanium alloy [2]. HA dissolves in body fluids when its crystallinity is low. This means that amorphous HA reveals a poor biocompatibility [3].

The commercially most used method of coating implants is the plasma spraying; however it produces mostly amorphous HA coating [4]. Plasma spraying presents few drawbacks including thermal decomposition of HA from the high temperature plasma and fractures in thick film coatings of more than  $40\ \mu\text{m}$  [5]. On the other hand vacuum deposition techniques such as pulsed laser deposition (PLD) give a finer control over layer thickness, crystallinity and composition [6]. A sputtered HA coating of  $1\ \mu\text{m}$  thickness showed higher bone bonding strength than a plasma spray coating [5]. The research in PLD is directed towards formation of crystalline apatite films at a low substrate temperature and creates high quality coatings with effective bonding to either smooth or rough titanium surfaces [6]. The conversion of the amorphous to crystalline HA depends in case of PLD on both temperature and water vapour pressure [2]. The stability of the HA film determines the success of the coated implants [2].

The influence of the ratio between crystalline and amorphous HA phases in the layer on the biological properties of the coating and on the coating properties to be used in various implants has been frequently discussed and tried to contribute to this theme with our work. The positive role of HA coating, which enhanced osseo-integration, has been proved [7]. Increasing the degree of crystallinity could essentially improve the stability and cell activity.

The aim of study was to find out if there is any difference between amorphous and crystalline hydroxyapatite from the biological point of view.

## Material and Methods

HA films were fabricated by KrF excimer laser. Using this ablation method we obtained three different implant models; model consisting from titanium alloy, titanium alloy with amorphous hydroxyapatite coating and titanium alloy with crystalline hydroxyapatite coating.

In biological testing, human dermal fibroblasts a keratinocytes isolated from epidermis were used. To study the cell adhesion on the surface of implant models we enoculated 30 000 fibroblasts/cm<sup>2</sup> and the cell growth was evaluated after 24 hours. For the test of proliferation fibroblasts were seeded in the density 5 000 cells/cm<sup>2</sup> and they were cultured for 7 days. The fibroblasts were cultured in the D-MEM with 10% FBS (fetal bovine serum) at 37 °C and 5% CO<sub>2</sub>.

Keratinocytes were inoculated in the density of 30 000/cm<sup>2</sup> and were cultured in DMEM + F12 mixture with 10% fetal bovine serum supplemented with EGF, hydrocortisone, cholera toxin and insulin at 37 °C and 5% CO<sub>2</sub>.

The cell growth was evaluated using MTT test [8]. To characterize better the morphology and phenotype of cultured cells the specific proteins (vimentin, fibronectin, pankeratin, keratin 14) were visualized using immunohistochemical detection. The nuclei were visualized using DAPI (4',6-diamidino-2-phenylindol) which is fluorescent stain that binds strongly to DNA. Specimens were analyzed using Nikon fluorescence microscope equipped with CCD camera and computer assisted image analyzer LUCIA.

To detect fibronectin production, fibroblasts were cultivated for a period of 14 days. The cells were seeded in the density of 3 000 cells/cm<sup>2</sup>.

## Results

The cells were able to adhere to all surfaces of our studied samples. The worst proliferation of fibroblasts was found using amorphous HA coating, whereas the

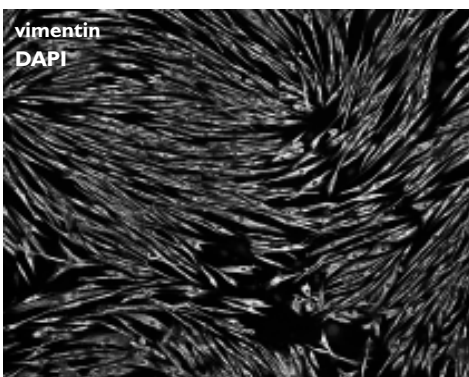


Figure 1 – The growth of fibroblasts on titanium alloy visualized using immunohistochemical detection of vimentin. The cell nuclei are detected with DAPI.

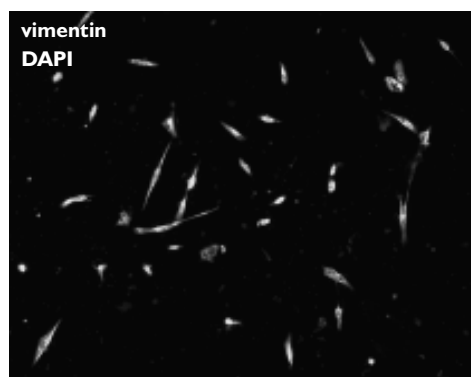


Figure 2 – The growth of fibroblasts on amorphous hydroxyapatite visualized using immunohistochemical detection of vimentin. The cell nuclei are detected with DAPI.

adhesion was well comparable with other surfaces. In the case of titanium alloy and the samples covered by crystalline HA, the cell proliferation was almost the same – see Figures 1, 2 and 3.

Using vimentin as a marker of fibroblast we found out that they create uniform growth with proper cell morphology. On the other hand even after 14 days of cultivation production of fibronectin was not detected, neither extracellular, nor intracellular in all samples (Figure 4).

Keratinocytes grew better using the amorphous HA coating; they were widespread and their proliferation was faster (Figure 5). A little bit worse proliferation was seen on crystalline HA coating (Figure 6). They were visualised using

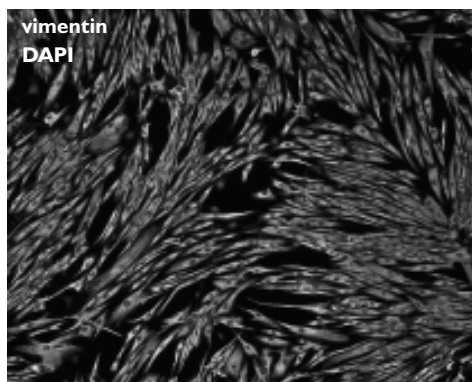


Figure 3 – The growth of fibroblasts on crystalline hydroxyapatite visualized using immunohistochemical detection of vimentin. The cell nuclei are detected with DAPI.

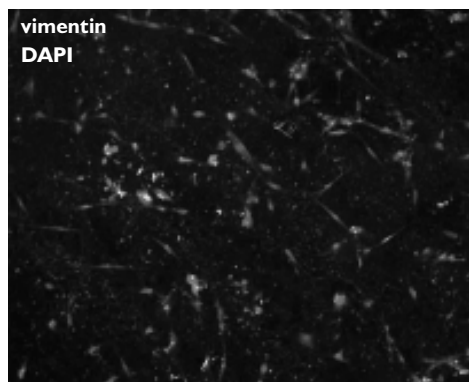


Figure 4 – The long-term cultivation of fibroblast was evaluated by immunohistochemical detection of fibronectin. The cell nuclei are detected with DAPI.

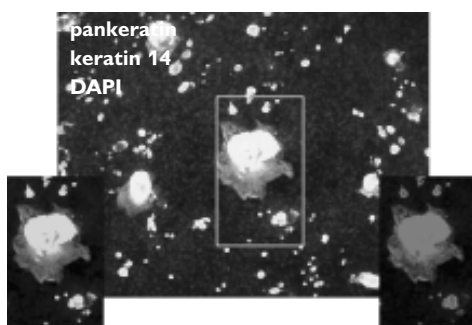


Figure 5 – Immunohistochemical evaluation of the growth of keratinocytes on amorphous hydroxyapatite by detection of pankeratin and keratin 14. The cell nuclei are detected with DAPI.

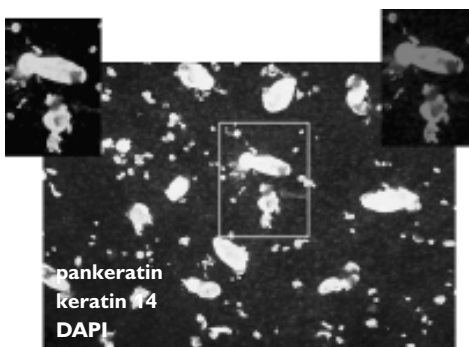


Figure 6 – Immunohistochemical evaluation of the growth of keratinocytes on crystalline hydroxyapatite by detection of pankeratin and keratin 14. The cell nuclei are detected with DAPI.

pankeratin. To determine the differentiation state we used keratin14 which is characteristic for basal keratinocytes at the beginning of differentiation.

The level of keratinocyte differentiation was the same on both HA coated surfaces (Figures 5 and 6).

## Discussion

Development of the ideal HA coating of dental implants started in 1980s. HA is the most stable, least soluble in aqueous media of all calcium phosphates. The natural mineral component of bone (~50% weight and ~70% volume) basically consists of HA [9]. The first coating of the dental implant was used in 1984. Clinical evaluations were encouraging, however the stand-alone HA coating on the titanium alloy needed another research because of the resorption, the crystalline structure, the thickness of the coating and also finding the optimal fabricating method [4]. The widespread method for dental implant coating became plasma spraying with all its advantages and disadvantages. The poor mechanical bond strength between the coating and the substrate was probably one of the reasons why some dentist did not want to rely only on the coated implant formed by plasma spraying. However Trisi et al. [10] analyzed long-term clinical data, which indicated high survival rates for HA coated, implants and compared the studies with an objective measurement lying in bone implant contact (BIC). BIC objectively considered the bone quality and the contact of the lamellar compact bone in contact with the HA coating on the implant surface [10]. Some of the negative reports came out as a poor quality of the coated implants with no reference to the bone quality.

None of technical methods produces automatically HA coatings with a high rate of the crystallinity and the good adhesion. The crystallinity increases with the modification of the experimental parameters in the PLD, which includes also heating of the substrate [11]. Garzia-Sanz et al. also confirmed in his comparative study PLD and plasma spraying that the differences in HA coating morphology were in the direct relation to the experimental parameters [12]. The conversion from amorphous to crystalline HA depends on both the temperature and water vapour pressure [2]. Crystallinity and impurities in HA coating were directly responsible for material melting and disintegration [4]. The influence of the HA bonding to the substrate depended on the ratio of crystalline and amorphous HA, and the crystalline HA is much more resistant [2].

The structure of culture surface plays the key role in the cell adhesion and subsequently in the cell proliferation. For the testing of these new materials two different cell types that are in a direct contact with the tooth were used. Every type of cells is characterized by expression of specific markers that can be used for its detection. In the case of fibroblasts the main marker is vimentin, a member of the intermediate filament family of proteins that plays a significant role in supporting and anchoring the position of the organelles in the cytosol and is also

important when offering flexibility to the cell [13]. Detection of vimentin enabled us to evaluate the fibroblasts morphology on different surfaces in more details than in our previous work [14, 15]. The production of fibronectin and the formation of extracellular matrix are characteristic for fibroblasts *in vivo*. In our conditions we found only weak intracellular expression of fibronectin.

In the case of keratinocytes the intermediate filaments are formed by spectrum of keratines. Their expression characterizes the differentiation status of the cells. Pankeratin is a mixture of antibodies that detect multiple types of keratin in a single test and is used to detect keratinocytes in the culture. On the other hand, keratin 14 is characteristic for the cells of basal layer that are on the very beginning of their differentiation and are still able to proliferate. In our samples the adhesion of keratinocytes was not very high, however, the cells retained their low differentiation status (characterized by expression of keratin 14) for a long time. This finding is rather promising for the further research.

## Conclusion

Crystalline and amorphous hydroxyapatite dental implant coatings may become materials which could shorten the healing period. We did not confirm neither crystalline, nor amorphous layer will be better in *in vitro* conditions. More detailed testing will be therefore needed.

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