Physical and Biological Evaluation of Hydroxylapatite Films Formed on Ti6A14V Substrates by Excimer Laser Ablation

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PHYSICAL AND BIOLOGICAL EVALUATION OF HYDROXYLAPATITE FILMS FORMED ON Ti6Al4V SUBSTRATES BY EXCIMER LASER ABLATION

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Abstract

Thin films of hydroxylapatite, about 500 nm, were formed on Ti6Al4V by excimer laser ablation. Ten different deposition conditions were used such as deposition atmosphere (vacuum, pure water vapor, mixture of Ar and water vapor), substrate temperature, target-substrate distance and energy density. Mechanical and physical analyses were carried out by Rutherford backscattering and particle induced X-ray emission; crystallinity of films was determined by X-ray diffraction, surface topography by scanning electron microscopy and adhesion by a scratch test. Biological evaluation of samples was also performed. The fibroblast proliferation test was used, morphological evaluation of macrophages was carried out, and the activity of lymphocytes cultured in the presence of implants was observed.

Key Words: dental implants, hydroxylapatite, titanium, fibroblasts.

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Introduction

Probably the best way how to develop implants having suitable mechanical, physical and biological properties is to cover metal or metal-alloy prostheses by a thin film of biocompatible material. One of the best known bioceramics is hydroxylapatite (HA) - $Ca_{10}(PO_4)_6(OH)_2$. Many researchers have attempted to find a viable deposition method and optimum deposition conditions for coating of metal prostheses with a layer of HA. What are the requirements on the properties of the HA layer formed on metal prostheses? Supposedly, this has to be high adhesion of the layer to the substrate. 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 is often discussed. Chemical dissolution of the HA film could probably depend on the Ca/P ratio (Singh et al., 1994). For different implants and various bioceramic-tissue attachments, different calcium phosphate phases and rates of dissolution are required (Jelinek et al., 1994). Some authors also think that a low permeability of the layer can prevent the diffusion of ions from the metallic substrate (Bagratashvilli et al., 1995) or that dense layers are to be formed (Knöfler, 1994). Open is also the question of preference of smooth or rough coatings, or the appropriate film thickness (Lin et al., 1992, Klein et al., 1983). Solving the question of the required film thickness is also connected with choosing the desired deposition method, because there are some limitations with regard to the layer thickness and deposition time. Using the plasma-sprayed technique usually creates films with a thickness of 50-200 μm (Transquilli et al., 1994); with thermal spraying the coatings range from 50 to 400 μm (Gross and Berndt, 1994), with electrophoretic deposition the HA layer thickness is usually about 20 μm (Transquilli et al., 1994). Ion sputtering creates a film several hundred nanometers to several micrometers thick. A greater thickness of highly crystalline coatings will result in a brittle material prone to cracking (Transquilli et al., 1994).

The aim of the study was to evaluate the influence

of thin films (nm-range) of hydroxylapatite, which were formed on Ti6Al4V by excimer laser ablation on the morphology and rate of proliferation of cells exposed to this material.

Materials and Methods

Deposition conditions

For deposition a KrF excimer laser (Lambda Physic LPX 200; Goettingen, Germany) was used. The laser beam (pulse duration 30 ns, repetition rate 10 Hz) was focused on the sintered HA target at an angle of 45°. The stainless steel vacuum chamber was pumped to a basic pressure of 10^{-4} mbar by a turbomolecular pump. The films were formed in vacuum, pure water vapor atmosphere and Ar-H_2O vapor mixture. Pure water vapor was supplied to the deposition chamber from a tank containing liquid water at room temperature. The water vapor flowed into the chamber under low pressure.

During the first set of experiments we wanted to choose a suitable deposition atmosphere. Therefore the films were formed on pure Ti substrates in vacuum, in pure water vapor atmosphere (for a wide range of vapor pressures, i.e., from 2×10^{-3} to 2×10^{-1} mbar), and also in the Ar-H_2O vapor mixture (for a fixed Ar/H_2O ratio). The influence of laser energy density on the target (E_T) and substrate temperature (Ts) on the film properties was also considered and studied (Jelinek et al., 1995a, 1995b). In the next (second) set of experiments, the films were formed only in the Ar-H_2O atmosphere. In these experiments the Ar-H_2O flow rate was slightly varied, two laser energy densities (3 J.cm^{-2} and 7 J.cm^{-2}) were tested, and the target-substrate distance (dt-s) vas varied between 3 cm and 9 cm. The argon mass flow rate into the interaction chamber varied from 8 to 17.5 sccm (standard unit per cm^3 per minute), and water vapor flow from 0.7 to 10 sccm. The films were formed on flat titanium-alloy substrates (Ti6Al4V) and Ts varied from 620°C to 780°C. All films and identification of phases were formed under the same deposition conditions three times; one coated specimen was used for physical analysis and the other two for in vitro biological analysis (Table 1).

Mechanical and physical analysis

The content of Ca and P in the films was determined by Rutherford backscattering (RBS) and particle induced X-ray emission (PIXE). The crystallinity of films was determined by X-ray diffraction (XRD): peaks of HA, tetracalcium phosphate (TeCP) - $Ca_4O(PO_4)_2$, tricalcium phosphate (TCP) - $Ca_3(PO_4)_2$ and TiO_2 and CaO). Surface topography was studied by scanning electron microscopy (SEM) using a Tescan TS 5120 (Proxima Tescan, Brno, Czech Republic) at an accelerating voltage of 15 kV. Adhesion was studied by a scratch test - a diamond tip was pressed with a continuously increasing force against the slowly moving tested layer (Table 1) (Jelinek et al., 1994, 1995a, 1995b).

Activity of lymphocytes

Immersion of bioceramic samples into the medium. The specimens were rinsed in distilled water and wrapped in aluminum foil. Then, the samples were sterilized in a hot air sterilizer at 180°C for 30 minutes. Samples were placed in 1 ml multiwell dishes and incubated for seven days with 0.5 ml of RPMI 1640 medium at a pH of 7.3. The eluate obtained in this way was used for the biological tests.

Cells. Mononuclear cells were isolated from 50 ml heparinized (5 U/ml) venous blood from 1 healthy donor. The blood was diluted to twice its volume by RPMI 1640 culture medium and covered with a Ficoll-Paque gradient (density 1.077 g/ml) according to Boyum (1968). Then, the blood was centrifuged at 400 g for 30 minutes, the mononuclear cell interface was carefully collected and washed three times in RPMI 1640 medium adjusted with $NaHCO_3$ (ÚSOL, Prague, Czech Republic) to pH=7.3 for cell culture. The lymphocytes were suspended at a concentration of 2×10^6 cells/ml in RPMI 1640 medium with HEPES buffer (4 mM, Sigma Chemical Co., St. Louis, MO, USA), 5% fetal calf serum (BIOCOM, Brno, Czech Republic) and 0.1 ml of gentamicine (40 mg/ml) (Lek, Ljubljana, Slovenia).

The tests were carried out as follows: We used 3 multiwell dishes (Microwell plate, Nunc, Roskilde, Denmark), i.e., a total of 288 wells. To each well, 100 μl of cell suspension was added. To 12 wells, 50 μl RPMI 1640 medium were added: these wells served as controls. To the remainder of the wells (276 wells) 50 μl of eluate was added in each well (experimental wells). To half of both experimental wells (138 wells) and controls (6 wells) 50 μl phytohemagglutinin (PHA) was added (stimulated cells). To the remaining half, 50 μl RPMI 1640 medium was added (nonstimulated cells). The cells were cultured for 48 hours at 37°C in a humidified atmosphere with 5% CO_2 (Procházková and John, 1986).

Evaluation. Lymphocyte activity was evaluated by a proliferation test based on the activity of stimulated and nonstimulated cells. The ratio of the activity of stimulated over nonstimulated cells was called the stimulation index (SI). The stimulation index of pure titanium was 1.45. The rate of proliferation was determined from the incorporation of radioactively labeled ^{3}H-thymidine. 50 μl medium containing 3.7 MBq of ^{3}H-thymidine was added to each well. All cells

cultured for 4 hours at 37°C in a humidified atmosphere with 5% CO_2. After that, specimens were harvested on glass paper (Whatman GF/A, Maidstone, England), dried and transferred into small glasses with 3 ml scintillation solution prepared from 0.2g POPOP (1,4-bis-2,5-phenyloxazolyl-benzene) (Lachema, Brno, Czech Republic) and 5g of PPO (2,5-diphenyloxazol) (Lachema) dissolved in 1l toluene (Lachema). The radioactivity of the specimens was measured by a β-counter for 1 minute.

Activity of fibroblasts and macrophages

Preparation of test samples: Before the start of the experiments, the specimens were rinsed in distilled water and wrapped in aluminum foil. Then, they were sterilized in a hot air sterilizer at 180°C for 30 minutes.

Cell lines: To determine cell proliferation in contact with an implant and the toxicity of the extract from implants, LEP fibroblasts (SEVAC, Prague, Czech Republic) were used. LEP fibroblasts were isolated from human embryonal lungs, and LEP is a verified, stable, diploid cell line. Cells from passage 24-31 were used after thawing. The influence of the presence of the implant on cell morphology was evaluated on mouse peritoneal macrophages.

The cells were cultured in Eagle's minimal essential medium (MEM; SEVAC) with streptomycin (100 μg/ml), penicillin 200 U/ml (both SEVAC) and 10% fetal calf serum (Veterinary University, Brno, Czech Republic). The cells were cultured for 5-7 days at 37°C, in a 5% CO_2 environment.

Test by direct contact of dental implants with fibroblasts. LEP fibroblasts were cultured in plastic 24-well dishes (Gama, České Budějovice, Czech Republic) on various pads:

a) Directly on plastic pads prepared specially for tissue culture (negative controls), material without cytotoxic response.

b) On plastic pads prepared for bacteriological purposes (positive controls: Petri dishes with a surface for bacteriology)

c) On the tested materials.

Inoculation was carried out with 12000 cells/cm^2 and 1 ml MEM was added to each well. Each material was tested 7 times. After 7 days of cultivation the medium was removed. After cleaning the bottom side (non-tested part) of each sample, the samples were placed in a clean well and the cells were released enzymatically (0.2 % trypsin; Gibco, Paisley, Scotland, UK) from the upper surface of the implant. The number of cells was determined by counting in a Coulter counter (Coulter Electronics, Luton, UK).

Test by indirect contact of implant with cells. For this test, 24-well dishes with macrophages (10^6 cells per
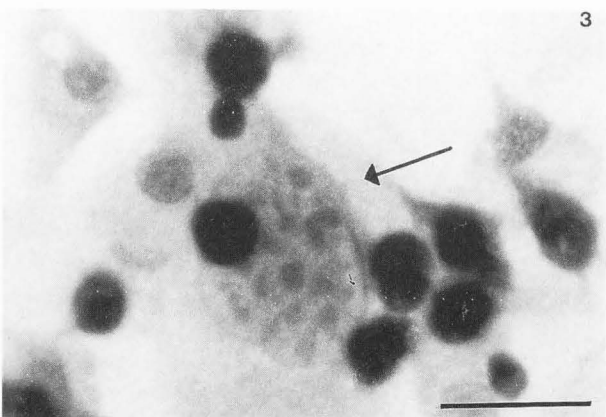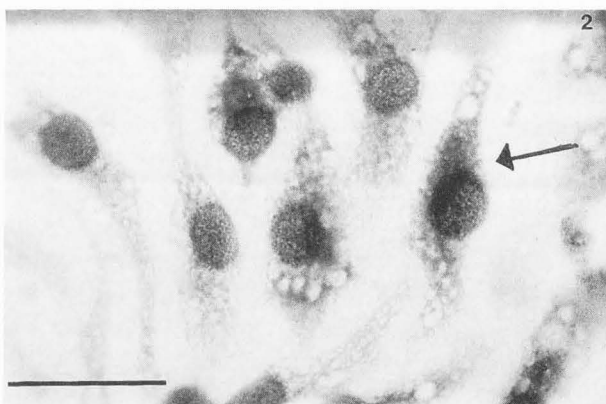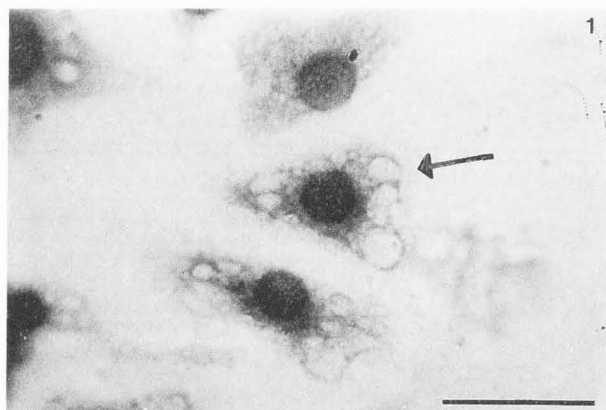
Figure 1. Typical mononuclear macrophage with condensed chromatin, dark nucleus, vacuoles in the cytoplasm (arrow), indicating relatively inactive metabolism, bar = 100 μm.

Figure 2. Elongated, fibroblast-like macrophage (arrow), bar = 100 μm.

Figure 3. Macrophage with blastic shape: mononuclear, but with many nucleoli, almost without vacuoles, the light nucleus (arrow) indicates active metabolism, bar = 100 μm.

1 ml well) were used. The culture was performed on the glass pad with the implant. After 5-days of culture in MEM the implant and medium were removed, a monolayer of cells with the glass pad was taken out, washed in saline solution, fixed with methanol and stained according to the method of Pappenheim (Disbrey *et al.*, 1970). The stained preparations were dried, cleared and mounted in Solacryl (Sanitas, Ričany, Czech Republic). The morphology of 100 cells from each sample was evaluated and the cells were divided into three groups:

a) active macrophages with a typical appearance (condensed chromatin, vacuoles in the cytoplasm, smooth shape, short or few projections) (Fig. 1).

b) fibroblast-like macrophages (light nucleus, elongated or starlike shape with long projections, without or with only few vacuoles in the cytoplasm) (Fig. 2).

c) macrophages with a blastic shape (strikingly light large nucleus with obvious nucleolus, sometimes several nucleoli, light cytoplasm) (Fig. 3).

Test for extracts

LEP fibroblasts were cultured for 48 hours in 96-well dishes (Gama) using a medium prepared from extracts of the tested materials. The enzymatic activity of living cells was determined by the Craig Laughton color reaction (Monner, 1988).

Inoculation was carried out with 30×10^3 cells/well to which 250 μl medium was added. After culture the mitochondria were stained with dimethylthiazol-phenyltetrazolium bromide and after a short incubation at 37°C the staining intensity was measured using an Elisa Reader 2550 (Bio-Rad Laboratories, Richmond, VA). The staining method is based on the fact that mitochondrial enzymes convert dimethylthiazol-phenyltetrazolium bromide to formazone crystals.

Growth of cells in extracts of tested materials prepared under physiological conditions. The samples were extracted in 2 ml MEM at 37°C, and after incubation they were removed. 10% fetal calf serum was added and the extract was used for cell culture. Control fibroblasts were grown under the same conditions, but in MEM only.

Growth of cells in extracts of tested materials prepared under extreme conditions. 11 samples of tested materials were sterilized in deionized water (60 min, 120°C). From the extracts, MEM was prepared with 10% fetal calf serum. As a control freshly prepared medium was used.

Results

Mechanical and physical properties
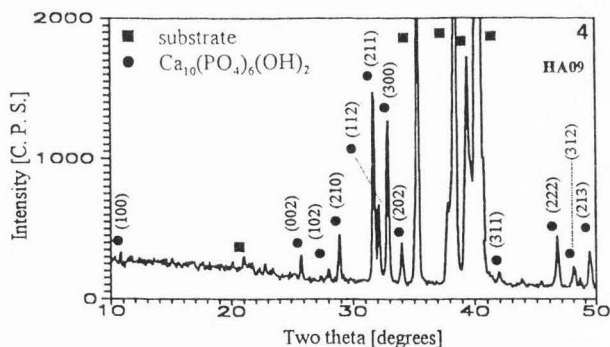
In the first set of experiments (in vacuum, fine scale

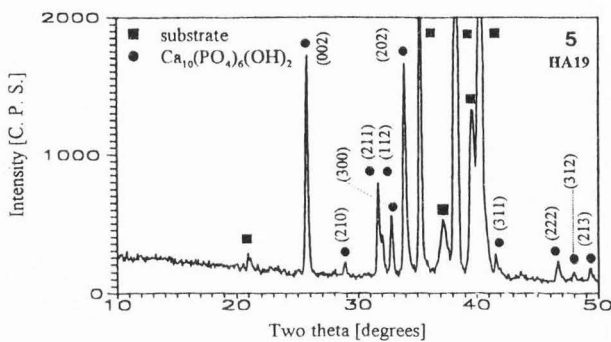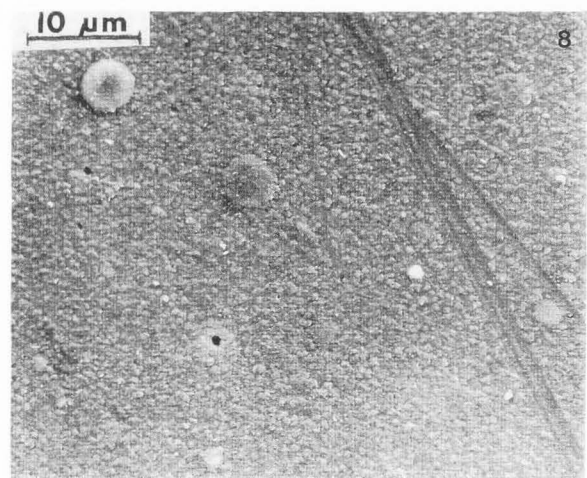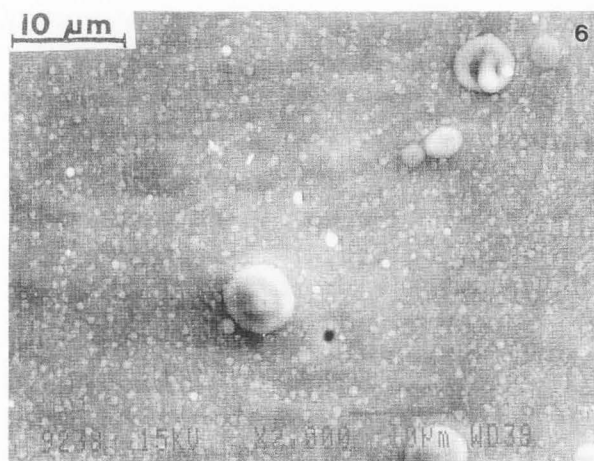Figure 4. The effect of biological treatment of HA/Ti6Al4V on XRD: XRD of sample No. 9.

Figure 5. The influence of biological treatment of HA/Ti6Al4V on XRD: XRD of sample No. 20.

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of water vapor pressures and one value of Ar-H_2O mixture ratio), the best XRD patterns of HA were observed mainly in layers formed in the Ar-H_2O atmosphere. Weak intensity HA peaks were also found for samples formed in pure H_2O vapor atmosphere, but under vacuum conditions non-crystalline HA films were formed. For vacuum deposition and low Ts (200-400°C) an amorphous calcium phosphate phase was usually found (Jelinek *et al.*, 1995a, 1995b). The Ca/P molar ratio was dependent on Ts. This dependence was mainly evident for films deposited under vacuum or in pure water vapor atmosphere. In the second set of experiments (fine scale of Ar-H_2O mixture ratio; Ar flow from 8 to 17.5 sccm and H_2O vapor flow from 0.7 to 10 sccm), the crystalline HA films were formed mainly for Ts in the region of 600°C to 700°C and a water/Ar flow ratio of 0.2-1.2 sccm. The Ca/P ratio slowly increased with Ts and decreased with increasing water vapor content (Jelinek *et al.*, 1995a; Liu *et al.*, 1994). Film adhesion was usually between 30-40 Newtons. Adhesion was less for deposition conditions with higher Ts (760°C) and an atmosphere of low water content (0.7 sccm of water). Some film properties and
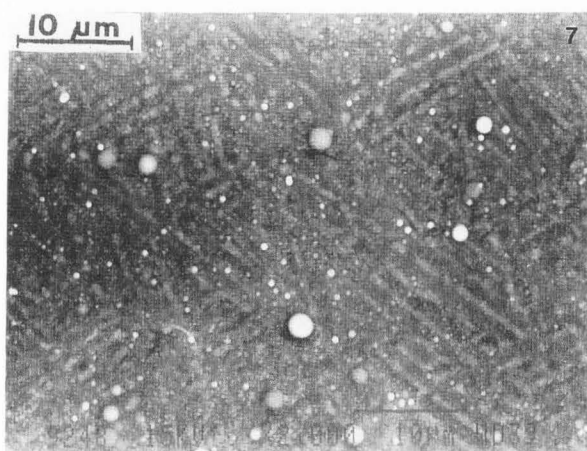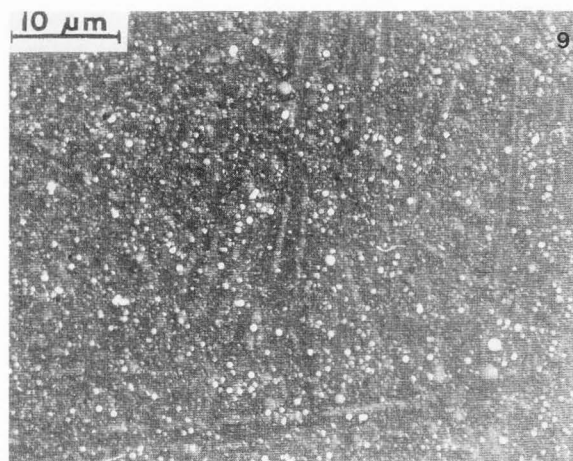
Figure 9. The influence of biological treatment of HA/Ti6Al4V on surface structure: sample No. 20 after biological treatment. Scanning electron micrograph, bar = 10 μm.

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deposition conditions are summarized in Table 1.

Under the best deposition conditions (No. 9, 10, 11, 19, 20, 21) the X-ray spectra contained HA peaks, peaks of $Ca_4O(PO_4)_2$ and phosphate phases (Figs. 4 and 5). Preferential hydroxyapatite film orientations were found to be changed according to the conditions of deposition from (300) to (002) and (112). The optimal Ca/P ratio was 1.68 and 1.78, close to the theoretical value of 1.67. The adhesion was mainly brittle. The structure of the surface was flat with typical bubbles (Figs. 6 and 7). The surface and morphology after biological treatment was without changes (Figs. 8 and 9).

Proliferation test

Measured values of the samples are given in Table 1. The basic value for their classification was the stimulation index (SI) of pure titanium (1.45). Samples with lower SI values had inferior tolerance and would probably be less acceptable to host tissues.

Test by direct contact

The growth of the cells was evaluated by the light microscopy. From the beginning to the end of the culture period, typically elongated fibroblasts were observed close to the samples. In Fig. 10 it can be seen that the number of cells growing on different films is higher than on the negative control (non-toxic material, specially prepared for tissue culture). The implants show better results than the positive control. The pure titanium sample has an optimal surface for the growth of fibroblasts. The tests confirm that this new type of implant materials is biocompatible and has positive effects on cell proliferation. Low stimulation of proliferation was observed with samples 32 and 36 (depo-

Figures 6-8. The effect of biological treatment of HA/Ti6Al4V on surface structure. Scanning electron micrographs, bar = 10 μm.

Figure 6. Sample No. 9 before biological treatment.
Figure 7. Sample No. 20 before biological treatment.
Figure 8. Sample No. 9 after biological treatment.

Table 1. Deposition conditions and some physical properties of HA layers formed on Ti6Al4V substrates by laser ablation.

	Deposition conditions						Physical properties		Biological evaluation			
Sample number	Film thick-ness (nm)	Temper-ature (°C)	Depos. time (min)	Ar flow (sccm)	Water flow (sccm)	Energy density (J.cm^{-2})	Adhesion (N)	Ca/P	SI	Fibro-blasts	Macro-phages	Test of extracts
34,35,36	509	780	10	9.0	10.0	3.0	40	2.05	5.09	-	-	-
4,5,6	349	620	10	17.0	0.7	3.0	35	2.74	3.64			-
30,31,32	441	780	10	17.8	4.0	3.0	35	2.20	3.55	-		
9,10,11	**1090**	**620**	**13**	**9.0**	**10.0**	**3.0**	**brittle**	**1.68**	**3.35**			
1,2,3	331	620	14	17.5	4.4	3.0	brittle	1.91	3.14			-
19,20,21	**675**	**700**	**6**	**9.0**	**10.0**	**3.0**	**brittle**	**1.78**	**2.23**			
12,13,14	823	700	10	17.0	4.0	3.0	brittle	1.86	1.25			
41,42,43	557	700	25	17.0	4.0	7.4	36	2.24	0.80			
38,39,46	678	620	25	17.0	4.0	7.4	brittle	2.00	0.77			-
15,16,17	631	700	8	18.0	0.7	3.0	48	2.60	0.52			
Ti												-

SI Ti = 1.45

Table 2: Evaluation of liquid extracts of implants (culture medium without serum, 37°C, 14 days) and evaluation of liquid extracts of implants (deionized water, 1 hour at 121°C).

Evaluation of liquid extracts of implants (culture medium without serum, 37°C, 14 days)				Evaluation of liquid extracts of implants (deeionized water, 1 hour at 121°C)			
Implant	Absorption 490 nm assay 1	Implant	Absorption 490 nm assay 2	Implant	Absorption 492 nm assay 1	Implant	Absorption 492 nm assay 2
43	0.444	21	0.451	control	0.303	control	0.295
11	0.443	11	0.446	2	0.303	2	0.288
21	0.435	2	0.437	32	0.300	32	0.286
14	0.434	43	0.429	3	0.298	6	0.284
2	0.431	3	0.425	43	0.285	21	0.283
32	0.426	6	0.423	21	0.285	11	0.283
46	0.424	32	0.422	6	0.282	17	0.279
17	0.424	17	0.421	11	0.282	36	0.278
6	0.424	46	0.416	14	0.282	14	0.275
36	0.422	36	0.411	36	0.278	43	0.270
3	0.418	14	0.407	17	0.276	3	0.270
				46	0.276	46	0.267

sition conditions shown in Table 1).

Test by indirect contact

From the observations on changes in cell morphology (Fig. 11) it can be stated that the lowest number of macrophages with blastic shape was found in samples 11, 14, and 46 and on the pure titanium implant. Most macrophages with blastic shape were found in samples 6 and 36. The lowest number of fibroblast-like macrophages was found in sample 43 and most fibroblast-like macrophages were found in the pure titanium sample. Macrophages in the pure titanium sample contained vacuoles; they are probably functional,
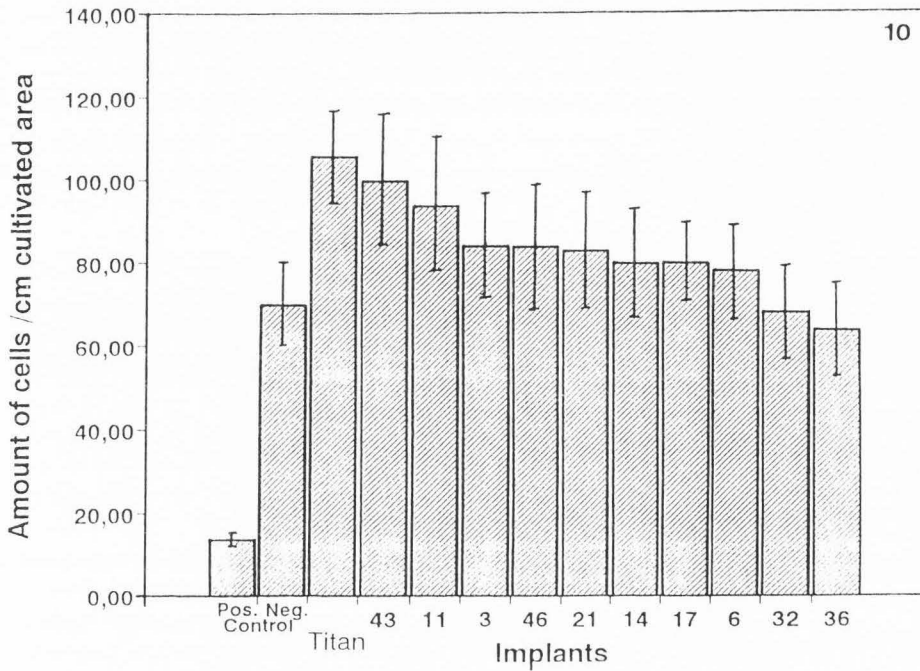
Figure 10. Proliferation of fibroblasts cultured on different supports.
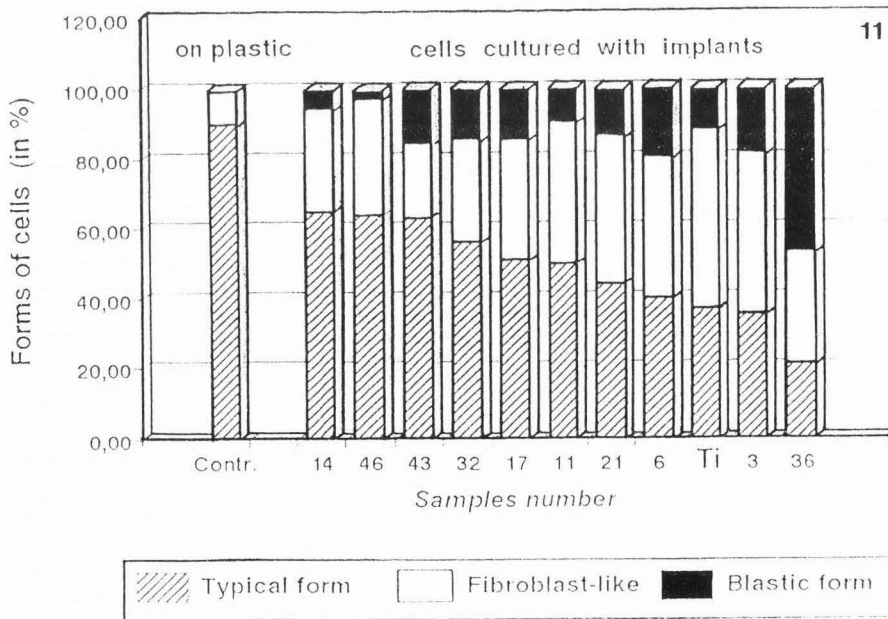
Figure 11. Morphological evaluation of cells cultured in the presence of implants.

but changes in the phenotype can be caused by a different adherence to the substrate.

Test of extract

Low metabolic activity was found in samples 6, 36, and 46 (Table 2).

Discussion

The long-term presence of a dental implant in a host body depends on many factors, but mainly on the properties of the material. Mechanically highly resistant titanium implants do not have optimal chemical properties and implants made only from ceramics are

more fragile and more often mobile than metal implants (Basquill *et al.*, 1994). Probably the best way for developing implants with suitable properties is to cover metal or metal-alloy prostheses with a film of biocompatible material. The commonly used coating material is hydroxyapatite. The protection is based on the close relation between the hydroxyapatite-coat and the bone (Murray *et al.*, 1989; Radin and Ducheyne, 1992).

It is well known that it is the physical properties of bioceramic materials that mainly affect the optimal connection (adhesion) of hydroxyapatite, the titanium implant and the bone (Solnick-Legg and Legg, 1989), and that the strong bond of HA film to the metal post is a very influential property, because the soft tissue could grow into the cleft between the metal and the HA film, so that the implant could be lost (Schliephake *et al.*, 1995) or the metallic part could release ions.

On the other hand, the biological properties of HA are not sufficiently known. Murray *et al.* (1989) state that a high energy surface (hydrophobic) attracts macrophages much stronger than a surface with low energy (hydrophilic). Similarly, rough surfaces have a hydrophobic behavior and smooth surfaces a hydrophilic behavior. Frayssinet *et al.* (1994) discovered that highly crystalline coatings are not soluble, but have an inhibiting effect on cell proliferation. Poorly crystalline coatings dissolve in cell cultures and change the roughness parameters during the process. The ratio of crystalline and amorphous parts is not well known yet, neither is the Ca/P ratio.

Our study tested samples of thin HA films deposited on titanium alloy discs by the laser ablation technique. Quality, thickness and area of deposited thin film were influenced by many factors such as: parameters of the laser (laser wavelength, pulse length, repetition rate), interaction of laser radiation with target (laser power density, spot size, target material properties, environment in the interaction chamber), interaction of plasma plume with the gaseous environment and substrate (gas pressure, target-substrate distance, ratio of ions and neutrals in plasma plume), parameters of the substrate (lattice parameters, thermal conductivity, thermal expansion coefficient, substrate temperature) and regime of film growth (deposition rate, laser repetition rate, film thickness, time deposition regime).

The conditions, and therefore the properties, of each sample were different. Most of the samples had a positive effect on the proliferative activity of the cells, did not cause appreciable changes in macrophage morphology and their extracts did not considerably decrease the metabolic activity of the cells. According to the results of all the tests, optimal deposition conditions were encountered in specimens 9-11 and 19-21. This optimum does not depend on film thickness (1090 nm and 675 nm) or deposition time (13 and 6 min). The optimal temperature was higher (620°C and 700°C). The adhesion was brittle and Ca/P ratio was near the optimum of 1.67 (1.68 and 1.78). Optimal deposition conditions are directly connected with low energy density (both 3.0) and low Ar and water flow (both 9.0 and 10.0).

These results show the influence of deposition conditions on the physical properties of bioceramics; however, the relations of the essential biological properties depending on these physical properties are not yet well known.

The aim of the present study was to evaluate the biological properties of laser deposited HA coatings. From this study it is evident that biocompatible coatings (from the same type of coating material, HA) can have different physical properties (within a certain range) and that suitable biological properties depend on the type of implant used. The Ca/P ratio, adhesion, and surface structure are only some of the most influential factors in developing bioceramic coating with the best physical and biological properties. Therefore it is necessary to balance several factors to achieve an optimal structure of the biocompatible film.

References

Bagratashvilli VN, Antonov EN, Sobol EN, Popov VK, Howdle SM (1995) Microparticle distribution and chemical composition of laser deposited apatite coatings. Appl Phys Lett **66**: 2451-2453.

Basquill PJ, Steflik DE, Brennan WA, Horner J, Van Dyke TE (1994) Evaluation of the effects of diagnostic radiation on titanium dental implant osseointegration in the micropig. J Periodontol **65**: 872-80.

Boyum A (1968) Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest **21**: 77-89.

Disbrey DB, Rack JH, Livingstone E, Livingstone S (1970) Histological Laboratory Methods. Edinburg and London. p 334.

Frayssinet P, Tourenne F, Rouquet N, Conte P, Delga C (1994) Comparative biological performance of HA plasma-sprayed coatings having different crystallinities. J Mater Sci: Mater Med **5**: 11-17.

Gross KA, Berndt CC (1994) In vitro testing of plasma-sprayed hydroxyapatite coatings. J Mater Sci: Mater Med **5**: 219-224.

Jelínek M, Olšan V, Jastrabík L, Studnička V, Hnatowicz V, Kvítek J, Havránek V, Dostálová T, Zergioti Y, Petrakis A, Hontzopoulos E, Fotakis C (1994) Effect of processing parameters on the properties

of a HA films grown by PLD. Thin Solid Films **257**: 125-129.

Jelínek M, Olšan V, Jastrabík L, Dostálová T, Himmlová L, Kadlec J, Pospíchal M, Šimečková M, Fotakis C (1995a) Laser deposited thin films of biocompatible ceramic. In: Proceedings International Conference on Advance of Laser Technologies ALT ´94, Konstanz, Germany. Pustovoy V (ed) Proc SPIE **2498**: 72-78.

Jelínek M, Dostálová T, Fotakis C, Grivas C (1995b) Biocompatible thin films formed on titanium prostheses by laser. Advanced Materials '95. Ul Haq A, Habiby F, Ahmad N, Mazhar AA, Khan AQ (eds). AQ Khan Publisher, Rawalpindi, Pakistan.

Klein CPAT, Driessen AA, de Groot K (1983) Biodegradation behavior of various calcium phosphate materials in bone tissue. J Biomed Mater Res **17**: 769-784.

Knöfler WE (1994). Implants - Results of animal experiments. In: Yearbook for Oral Implantology '94. Myska W, Duelund H, Fischer Brandies (eds). Quintessenz Verlags-GmbH, Berlin, pp. 71-80.

Lin YS, Hlady V, Janatova J (1992) Adsorption of complement proteins on surfaces with a hydrophobicity gradient. Biomaterials **13**: 497-504.

Liu DM, Chou HM, Wu JD (1994) Plasma-sprayed hydroxyapatite coating: effect of different calcium phosphate ceramics. J Mater Sci: Mater Med **5**: 147-153.

Monner DA (1988) An assay for growth of mouse bone marrow cells in microtiter liquid culture using the tetrazolium salt MTT and its application to studies of myelopoiesis. Immunol Lett **19**: 261-268.

Murray DW, Rae T, Rushton N (1989) The influence of the surface energy and roughness of implants on bone resorption. J Bone Joint Surg **71**: 632-637.

Procházková J, John C (1986) Diagnostic Methods for Clinical Immunology (in Czech). Avicenum, Prague. pp 28-31, 194, 248, 258-263.

Radin SR, Ducheyne P (1992) Plasma spraying induced changes of calcium phosphate ceramic characteristics and the effect on in vitro stability. J Mater Med **3**: 33-42.

Singh RJ, Qian F, Nagabushnam V, Damodaran R, Moudgil BM (1994) Excimer laser deposition of hydroxylapatite thin films. Biomaterials **15**: 522-528.

Sisk AL, Steflik DE, Parr GR, Hanes PJ (1992) A light and electron microscopic comparison of osseointegration of six implant types. J Oral Maxillofac Surg **50**: 709-717.

Schliephake H, Neukam FW, Hutmacher D, Wustenfeld H (1995) Experimental transplantation of hydroxylapatite-bone composite grafts. J Oral Maxillofac Surg **53**: 46-52.

Solnick-Legg H, Legg K (1989) Ion beam and plasma technology for improved biocompatible surfaces. MRS Bulletin, Materials Research Society, Pittsburgh, PA. pp. 27-30.

Transquilli PL, Meroli A, Palmacci O, Gabbi C, Cacchioli A, Gonizi G (1994) Evaluation of different preparations of plasma-spray hydroxyapatite coating on titanium alloy and duplex stainless steel in rabbit. J Mater Sci: Mater Med **5**: 354-349.

Discussion with Reviewer

J.D. de Bruijn: The last sentence under the heading "Deposition conditions" (Materials and Methods) mentions that "one coated specimen was used for physical analysis and the other two for biological analysis". This would be an insufficient number of samples to be able to draw any conclusions from this study.

Authors: What we wanted to say is that for all deposition conditions always several samples were made for analysis. For each analysis we used a new specimen to avoid problems of possible modification of the sample after analysis, and we wanted to avoid to use only one sample for all analyses. Since the deposition process is somewhat complicated (deposition in Ar-water vapor atmosphere, exact pressure and gas flow, CO_2 laser heating of the dental implant and temperature control), much attention was paid to the reproducibility of deposition. At first, samples were coated under the same conditions several times on flat substrates and the physical parameters of the created HA layer was studied. On the basis of the experience obtained, we believe that specimens created under the same deposition conditions, exhibited the same physical properties. Because we analyzed all specimens by three different methods of analysis, one physical (XRD, SEM) and two biological, we wrote that all specimens were prepared in triplicate.

J.D. de Bruijn: Please explain the rationale for the chosen biological tests.

Authors: Reproducibility of deposition conditions for creating hydroxyapatite films on implants is a problem for every method of deposition. It was observed that there were small variations in samples prepared under "the same" deposition conditions. Only the same deposition conditions gave rise to the same physical properties. In our previous study (Dostálová *et al.*, 1995) we found differences in activity of lymphocytes due to small variations in deposition conditions. We observed interactions between cells and surface of hydroxyapatite (HA) thin films and between cells and extract from samples. Neither samples nor sample

extracts showed a toxic reaction. Proliferation of fibroblasts depends on the attachment to the material. The shape of macrophages is directly connected with the digestion of particles released from the material. Small differences in deposition condition could alter cell shape and function of macrophages in particular. The quantitative response of tissue to similar deposition conditions could be checked by fibroblast tests.

J.D. de Bruijn: A positive (cytotoxic) control should not be a bacteriologic grade polystyrene since this material is not toxic for cells. The cells are just not able to adhere due to its hydrophobicity.

Authors: We are aware that the material is not toxic to the cells. Therefore, we only compared the samples to each other and to the pure titanium sample.

Additional Reference

Dostálová T, Himmlová L, Jelínek M, Bártová J (1995) Some biological and physical properties of laser deposited hydroxylapatite based films. Cells Mater **5**: 255-260.