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Research Article

Amorphous Calcium Phosphate Layer Prepared Ultrasonically on Titanium

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Abstract An inert titanium surface was treated with the BIO treatment (developed by the company Lasak, Ltd.), i.e. its surface area was increased by mechanical and chemical treatment with blasting and exposure to HCl and NaOH. The treated surface was provided with thin layers of calcium phosphate OCP (Octacalcium Phosphate) or ACP (Amorphous Calcium Phosphate) by means of precalcification (using a supersaturated SCS2 solution). In vitro tests in SBF (Simulated Body Fluid) under dynamic conditions have shown that precalcified layers have the ability to induce development of HAp (Hydroxyapatite) on the surface much faster than BIO surfaces without such layers. The OCP crystals are well adhesive but cytotoxicity tests have shown that a bioactive layer prepared in this manner is slightly toxic. The ACP prepared ultrasonically is incorporated directly into the TiO_2 gel and thus does not increase implant's dimension and moreover ACP is not toxic (confirmed by cytotoxicity tests).

Keywords hydroxyapatite; titanium; amorphous calcium phosphate; SCS2; ultrasonic bath

1 Introduction

In order to ensure the bioactive behavior of an otherwise inert material, titanium (Ti) or its alloy (Ti6Al4V), it is necessary to increase its reactive surface as much as possible (by creating layers of TiO_2 gel). The so-called BIO treatments, that is mechanical treatment (blasting) and chemical treatment (etching in HCl and NaOH), increase the surface area up to 1000 times [6]. The principle of bioactivity of many glass- and ceramics-based materials consists in their ability to release biogenic elements Ca and P (the basic building units of HAp) into their proximity. Hydroxyapatite precipitation starts on the implant surface immediately after a local increase of HAp supersaturation on the surface of the material. Therefore it is desirable to create a resorbable interlayer of calcium phosphate on the surface of an inert implant, which in body fluids dissolves easier than HAp as such. The prepared interlayer shall meet not only high requirements for adhesion to the substrate but also requirements for dimensional stability of the implant. One of the simple and cheap methods to prepare bioactive (resorbable) interlayers is a method of surface precalcification [1,3]. In earlier works published by our laboratory we used mechanically and chemically treated surfaces to prepare OCP layers by precalcification from SCS2 solution. OCP forms little tabular crystals arranged in spherulites and it is a convenient precursor for nucleation and crystallization of HAp [4]. If placed freely into SCS2 solution a layer of OCP or ACP (shorter exposure) spontaneously precipitates on the sample surface within several hours. The objective of the submitted work was to prepare a layer of calcium phosphate on a Ti substrate surface, whose bioactivity was to be comparable with that of OCP layer, but without significantly changing the implant dimensions, and whose preparation was to be reproducible. Therefore we used an ultrasonic bath for the precalcification, as the ultrasonic waves support incorporation of elements (Ca and P) into the previously prepared TiO_2 gel (BIO treatment). The ability of the layer (identified as 1 SCS-UTZ) to induce formation of HAp was tested with an in vitro test in SBF under dynamic conditions (which simulate the conditions in a living organism better) and its behavior was compared with (ACP and OCP) layers prepared under static conditions for periods of 6 or 24 hours in SCS2 (identified as 6 SCS and 24 SCS). The reference sample was a BIO treated Ti (0 SCS). The prepared layers were tested for cytotoxicity. The cytotoxicity in vitro tests are conducted on live cells (mice) and they represent the next level of evaluation for the potential application of tested samples.

2 Materials and methods

Ti in form of disc 9 mm (or 15 mm diameter $\times$ 1 mm height, with BIO surface treatment, made by Lasak) was used. The Ti surface was sandblasted with 250 μm Al_2O_3 particles and

SCS2	Na^+	Ca^{2+}	Cl^-	$H_2PO_4^-$	HCO_3^-
$mmol.dm^{-3}$	4.0	5.0	10.0	2.5	1.5

Table 1: Supersaturated calcifying solution (SCS2) [3].

	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
SBF	142.0	5.0	2.5	1.5	148.0	4.2	1.0	0.5

Table 2: Ion concentrations of corrected SBF ($mmol.dm^{-3}$) [2].

treated with HCl and then with NaOH (4M solution), that is the so-called BIO treatment. The sample was identified as 0 SCS.

Precalcified Ti surface with BIO treatment was exposed for 6 h (sample 6 SCS) or 24 h (sample 24 SCS) to an SCS2 solution at a laboratory temperature. Another sample was prepared by means of exposure to SCS2 solution for one hour in an ultrasonic bath (sample 1 SCS-UTZ). The S/V ratio was in all cases 0.1 cm^{-1}, while the sample area was calculated from geometric dimension of the Ti disc. The composition of SCS2 is shown in Table 1.

2.1 Experimental methods

Simulated body fluid (Table 2) was buffered with TRIS (tris (hydroxyethyl) aminomethane), pH 7.4 at 37 °C under dynamic conditions (with a continual flow of fresh SBF solution).

The testing conditions were as follows:

- temperature: 37 ± 0.5 °C;
- duration: 10 days;
- flow rate of the solution: 48 mL/day;
- testing cell volume: 5.5 ml or 15 ml (cm^3).

The parameters related to the samples are as follows: the surface area of Ti (9 mm or 15 mm in diameter) in testing cell was 9 cm^2 (without the BIO treatment effect).

2.2 Analytical methods

The concentration of Ca was measured at λ = 442 nm by AAS. KCl was added to each sample as a releasing buffer in the concentration of 4000 ppm. Acetylene and N_2O were used as a carrier gas. $(PO_4)^{3-}$ ions concentration was measured with spectrophotometry. The analysis was based on the determination of the concentration of phosphate ions at λ = 830 nm.

Weight increases were monitored both after precalcification and after *in vitro* tests in SBF by weighing on analytical scales Mettler Toledo AG 204.

SEM/EDS: the samples were examined with an electro raster microscope Hitachi S-4700 with energy-dispersive spectroscopy. The acceleration voltage of the primary electrons was 15 kV and the working distance was 11.9 mm.

X-ray microdiffraction: microdiffraction experiments were conducted on PANalytical X'PertPRO equipped with CoKalfa X-ray tube (voltage 40 kV, current 30 mA) and semi-conductor detector X'Celerator. The measurements were performed in the Institute of Inorganic Chemistry of the Czech Academy of Science in Řež u Prahy.

The cytotoxicity test monitored toxic effects of extracts from the solid material on a cell line of mice fibroblasts NIH 3T3 or Balb/c 3T3 in the cell culture under EN ISO 10933 and 7405. The cytotoxicity tests were performed in the Laboratory of Cell Cultures at the Medical Faculty of the Palacký University in Olomouc. The tests were contracted by the company Lasak, s.r.o.

3 Results and discussion

BIO treated samples were precalcified for 6 and 24 hours under static test conditions (without stirring or agitation). An amorphous layer of Ca-P (ACP) developed on the surface of the sample 6 SCS. After 24 hours of precalcification (24 SCS) the X-Ray micro diffraction method detected crystals of OCP on the Ti surface and, as a minority phase, also DCPD (brushite). The thickness of the layer was 20 μm [5]. The appearance of small tabular OCP crystals arranged into spherulites is well visible in Figure 1 showing an SEM image. Precalcification in an ultrasonic bath for one hour produced a layer of amorphous nature or nanocrystalline particles of amorphous calcium phosphate (ACP) (Figure 2), which has been also confirmed by X-ray microdiffraction.

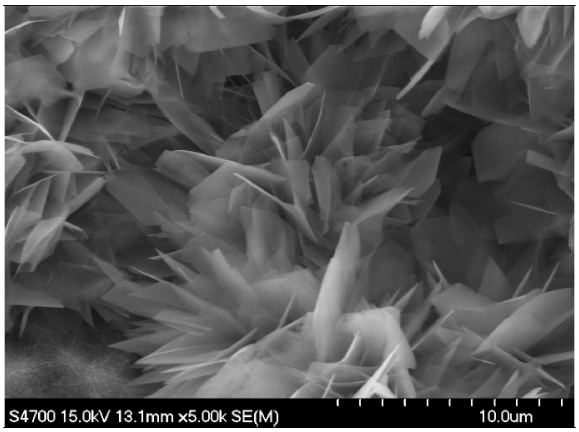

Figure 1: OCP crystals on the surface of the 24 SCS sample after precalcification, measured by SEM.

Tests of reproducibility of precalcification were performed by weighing of the samples before and after the precalcification process. The weight increases were

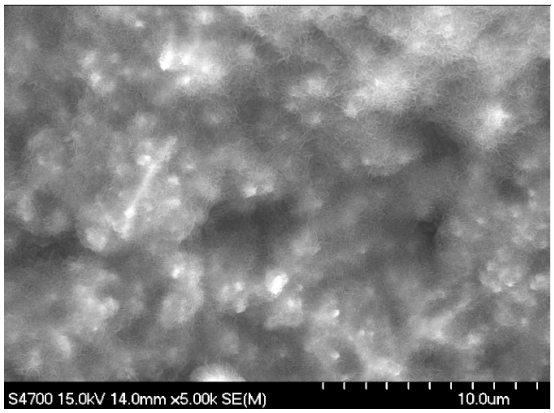

Figure 2: An ACP layer on the surface of the 1 SCS-UTZ sample after precalcification (SEM).

used to calculate the rate of the precalcification process. The results indicate that the precalcification solution SCS2 gets quickly exhausted and the rate of Ca-P formation on the surface decreases with longer precalcification times (Table 3). The table also indicates the total quantity of the Ca-P phase formed by precalcification under the specified conditions.

Sample	Increase Ca-P ($mg.cm^{-2}$)	Rate ($mg.cm^{-2}.hour^{-1}$)
1 SCS-UTZ	0.0312	0.0312
6 SCS	0.1633	0.0272
24 SCS	0.2990	0.0125

Table 3: Quantities of the Ca-P phase formed by precalcification and growth rates of the Ca-P phase on Ti samples.

The prepared layers were tested using in vitro cytotoxicity and dynamic tests (continual flow of fresh SBF solution) and a BIO-treated Ti surface was used as a reference sample (identified as 0 SCS).

The cytotoxicity tests suggested a slight toxicity of the crystalline layer (OCP) developed on the sample 24 SCS. On the contrary, the amorphous layer created by means of an ultrasonic bath (1 SCS-UTZ) was evaluated as non-toxic.

Analyses of Ca and $(PO_4)^{3-}$ in effluents (Figures 3 and 4) have shown that the amorphous layer, spontaneously developed after six hours in SCS2 (6 SCS), behaves differently from the amorphous layer developed ultrasonically (1 SCS-UTZ). The dissolving rate of the amorphous layer on 6 SCS at the beginning of its exposure (increase of concentration of Ca and $(PO_4)^{3-}$ above the original levels in SBF) is higher than the rate of HAp formation. On the contrary, the samples 24 SCS and 1 SCS-UTZ indicate that both surfaces induce HAp formation immediately and at the same rates, although the nature of the two layers is totally different (crystalline OCP versus amorphous layer).

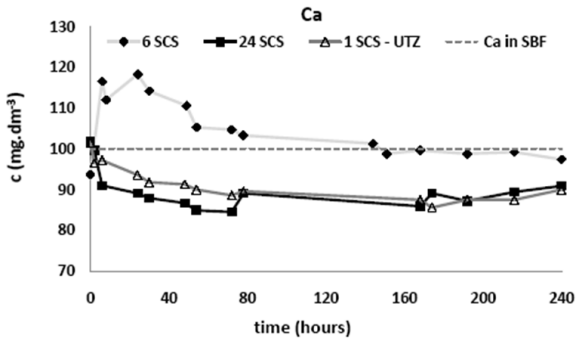

Figure 3: Concentration of Ca in the effluent-dynamic test in SBF.

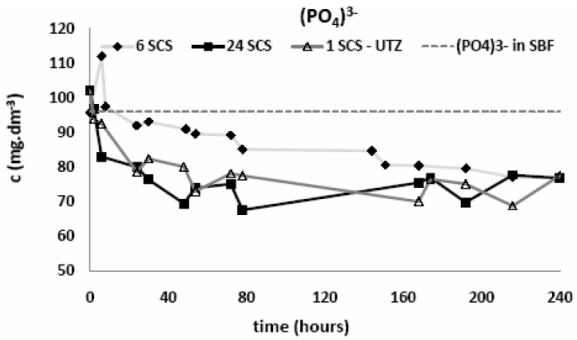

Figure 4: Concentration of $(PO_4)^{3-}$ in the effluent-dynamic test in vitro in SBF.

This fact has been confirmed also by the weight increase of HAp found after the dynamic in vitro test and the subsequently calculated rate of Ca-P phase formation per day. The calculated rate of HAp formation on the Ti sample with a BIO-treated surface (0 SCS) was $0.0134\,mg.cm^{-2}.day^{-1}$. For samples 6 SCS, 24 SCS and 1 SCS-UTZ, the rates of HAp formation were ten times higher (0.1301, 0.1148 and $0.111\,mg.cm^{-2}.day^{-1}$, resp.). A higher increase of HAp on the 6 SCS sample is probably due to a higher quantity of amorphous Ca-P phase developed after precalcification (see Table 3).

4 Conclusions

A layer of amorphous calcium phosphate has been created ultrasonically, with the following characteristics:

(a) the layer was incorporated into the TiO_2 gel layer, it did not alter the sample dimensions;
(b) the ultrasonically prepared amorphous layer (1 SCS-UTZ) was different from the amorphous layer on the sample 6 SCS;
(c) the preparation of bioactive layers on Ti surfaces by precalcification in an ultrasonic bath was simple and reproducible;

(d) during in vitro tests (in SBF) of 1 SCS-UTZ the layer demonstrated the same behavior as the crystalline OCP layer 20 μm thick (24 SCS).

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