Salvador Clavell, R.; Martín De Llano, J.; Carda-Batalla, C.; Gómez Ribelles, JL.; Vallés Lluch, A. (2016). In vitro assessment of the biological response of Ti6Al4V implants coated with hydroxyapatite microdomains. Journal of Biomedical Materials Research Part A. 104(11):2723-2729. doi:10.1002/jbm.a.35817

In vitro assessment of the biological response of Ti6Al4V implants coated with hydroxyapatite microdomains

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ABSTRACT

Dental implantology is still an expanding field of scientific study because of the number of people that receive dental therapies throughout their lives worldwide. Recovery times associated to dental surgery are still long and demand strategies to improve integration of metallic devices with hard tissues. In this work, an *in vitro* ceramic coating is proposed to improve and accelerate osseointegration of titanium surfaces conceived to be used as dental implants or hip or knee prosthesis, shaped either as dishes or screws. Such coating consists of hydroxyapatite microdomains on the implant surfaces obtained *in vitro* by immersion of titanium alloy samples (Ti6Al4V) in a simulated body fluid. This titanium alloy is highly used in implant dentistry and trauma surgery, among other fields. Once the immersion times under physiological conditions yielding to different ceramic topographies on this alloy were set, the acellular coating time of major interest so as to optimize its biological development was determined. For this purpose, dental pulp mesenchymal cells were cultured on titanium coated surfaces with different hydroxyapatite outline, and cell adhesion, proliferation and morphology were followed through histological techniques and scanning electron microscopy. It was found that 4 days of acellular hydroxyapatite coating led to a significant cell adhesion on the titanium alloys at an early stage (6 h). Cells tended though to detach from the surface of the coating over time, but those adhered on domains of intricated topography or hydroxyapatite cauliflowers proliferated on them, leading to isolated large cell clusters.

KEYWORDS: dental implantology; titanium alloy; hydroxyapatite; osseoinduction; osseointegration; simulated body fluid

INTRODUCTION

Dental implantology is still an expanding field of scientific study because of the number of people that receive dental therapies throughout their lives worldwide. Recovery times associated to dental surgery are still long and demand strategies to improve integration of metallic devices with hard tissues. For example, osseointegration processes can conventionally take 3-6 months. Thus, there is room to investigate in this field, to find ways to deal with these implants, which can accelerate osseointegration and be of use in the dental or trauma surgery field.

Dental implants are usually manufactured in either pure titanium or their alloys [1-3], because of their mechanical, chemical and biological properties. Among them, Ti6Al4V alloy can be highlighted because of its biostability in biological tissues.

The fixation of a dental implant in the bone starts with the formation of a ceramic layer on the implant surface; this hydroxyapatite (HAp) mimics native bone in terms of its composition, though not as for its morphology. Hydroxyapatite [$Ca_5(PO_4)_3(OH)$] nucleation, growth and crystallization occurs through chemical interactions between the material surface and ions from the body fluid, such as calcium, sodium, magnesium, carbonate and others [4-8]. Once the HAp layer is formed, mesenchymal stem cells interrogate it, adhere, proliferate and finally differentiate to osteoblasts, which eventually will start bone formation.

Calcium phosphates usually precipitate in two steps: the first step is the formation of a precursor phase, most commonly amorphous calcium phosphate or octacalcium phosphate (OCP, ($Ca_8H_2(PO_4)_6$), which may transform in a second step into calcium-deficient apatite or hydroxyapatite [9-10]. The calcium phosphate system is complicated

as it involves a number of salts of variable stability [11], but HAp is the only thermodynamically stable calcium phosphate that exists in aqueous solutions at pH greater than 4.2 [13].

A good osseointegration refers to the continuity of bone tissue with the implanted material, through only mineralized tissue as interface in place of a fibrous capsule. As regards metallic devices, it has been reported that HAp coating on Ti6Al4V titanium alloys before implantation promotes an *in vivo* osseoinductive behavior that promotes a faster cell proliferation and maturation [13]; along with that, clinical recovery times shorten. Moreover, this inorganic layer on the surface improves the mechanical properties of the material [14-15], and prevents the formation of a deleterious fibrotic capsule around the implant.

The aim of this work was to enhance the biological performance of titanium surfaces conceived to be used as dental implants or hip or knee prosthesis, shaped either as dishes or screws, from the osseointegration and osseoconduction standpoint. To do that, the process invented by Kokubo [see 16-17, among others] to generate controlled hydroxyapatite microdomains on materials of different chemical nature was followed. Published findings corroborate that such previous treatment facilitates cell adhesion and proliferation of mesenchymal stem cells. These procedures are based on hydroxyapatite nucleation and growth by immersion in simulated body fluid (SBF). It is then to be expected that these surface treatments will modify the cellular and surrounding tissue responses when implanting the samples *in vivo* or when used in cell culture approaches, and they will have an effect, mediated by changes of adsorbed proteins, on cell adhesion, proliferation and differentiation. The objectives of this work are, thus, on the one hand, to determine the optimal times of *in vitro* hydroxyapatite coating on Ti6Al4V-based samples leading to stable bone-like surfaces, and, secondly, to assess

cell adhesion and proliferation on these coated samples to determine the role played by HAp topographies on the biological development of these alloys and point out the acellular coating time of most interest to optimize their biological development.

MATERIALS AND METHODS

Samples conditioning

Original samples were manufactured as discs of 25 mm in diameter and 2 mm of thickness as described in [18-19] and kindly provided by M.L. González-Martín (Department of Applied Physics, University of Extremadura, Badajoz, Spain). The composition of these titanium alloys was 6.1 Aluminium, 4.2 Vanadium, 0.01 Carbon, 0.12 Oxygen, 0.006 Nitrogen and the rest was pure Titanium (in wt%). Each disc was cut into 8 triangular parts using a disc saw and sample surfaces were polished with diamond paste of 1 μm particle size. Next, samples where cleaned up with detergent and rubbed with a cloth and thoroughly rinsed with regia water [HNO_3:HCl:H_2O, at 1:3:2 vol.]) to remove residues. Finally, samples were soaked in deionized water and sonicated (Bandelin Sonicator) with short pulses of 0.4 s at 0.2 s intervals for 5 minutes at a power of 50 W.

Hydroxyapatite surface coating

Sterile Simulated Body Fluid (SBF) was prepared following the protocol described in [16-17], but doubling ion concentrations. Briefly, a first solution consisted of 7.995 g of NaCl (99%, Scharlau), 0.225 g of KCl (Scharlau), 0.550 g of $CaCl_2 \cdot 6H_2O$ (Fluka) and 0.305 g of $MgCl_2 \cdot 6H_2O$ (Fluka) in 250 mL of sterile deionized ultrapure water. The second solution consisted of 0.160 g of $Na_2SO_4 \cdot 10H_2O$ (Fluka), 0.355 g of $NaHCO_3$

(Fluka) and 0.230 g of $K_2HPO_4 \cdot 3H_2O$ (Aldrich) in 250 mL of sterile deionized ultrapure water. Both solutions were mixed and its pH adjusted to 7.4 with 0.1M HCl and 0.1M NaOH. This SBF was next filtered in a laminar flow hood using a PES filter of 0.2 μm pore size (Nalgene).

Titanium samples were immersed in 10 mL SBF each in closed glass containers and placed in an incubator at 37ºC for 12 h and 1, 2, 4, 6, 8, 10, 12 and 14 days. At withdrawal, samples were rinsed in 5 mL of Phosphate-Buffered Saline (PBS) for 5 min to remove non-adhered salts. PBS was previously prepared according to the procedure established by Dulbecco [20]. Briefly, 4.00 g NaCl (Scharlau), 0.10 g KCl (Scharlau), 0.72 g Na_2HPO_4 (Panreac) and 0.12 g KH_2PO_4 (Scharlau) were dissolved in 500 mL of sterile deionized ultrapure water, and its pH was adjusted to 7.4 with 0.1 M HCl and 0.1 M NaOH. This solution was filtered as described above.

Characterization of ceramic coatings

Scanning Electron Microscopy (SEM) images of the hydroxyapatite coatings were taken in a Jeol JSM-6300 microscope, with the samples previously sputter-coated under vacuum with gold, 15 kV of acceleration voltage and 15 mm of working distance. Quantification of elements was achieved by Electron Dispersive X-ray Spectroscopy (EDS) in the same device. Coatings were carefully cut and samples were observed transversally to estimate the coating thickness and roughness. Roughness was measured as the average distance between ridges and valleys.

Cell culture and fixation

Human dental pulp stem cells (hDPSC), corresponding to a passage earlier than 6th, obtained from 3rd molar of young patients were seeded on hydroxyapatite coated

samples and on raw ones as control. Cells were cultured following standard procedures. Culture medium was prepared with Minimum Essential Media: (MEM)-alpha medium (Gibco-Life Technologies) containing 10% Fetal Bovine Serum (FBS; Gibco-Life Technologies), 1× penicillin-streptomycin antibiotic solution (Gibco-Life Technologies) and 2 mM glutamine (Gibco-Life Technologies). Resuspended cells were seeded on titanium samples in 24-well plates (1 mL culture medium per well) at a density of 5 x 10^3 cells·mL^{-1}. Cells were incubated at 37ºC and 5% CO_2/95% air atmosphere. Culture medium was changed each 2-3 days. For studying cell adhesion, culture lasted 6 h; to assess cell proliferation, culture time was 7 days. At withdrawal, cellular samples were rinsed in PBS, fixed with glutaraldehyde (TAAB Laboratories Equipment Ltd.) at 2% for 20 min at 4ºC and rinsed in PBS anew.

Cell distribution and morphology

Samples were incubated in the dark for 60 min with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) at a 100 nM concentration in DPBS (Gibco-Life Technologies) for the fluorescence staining of cell nuclei and for 30 min with eosin (Merck) at 0.001% in DPBS, to stain cellular cytoplasm components. Samples were rinsed with DPBS after each staining step. Fluorescence images were next obtained under a Leica DM4000B microscope.

A series of samples were air dried and SEM images of cellular surfaces were obtained under the microscope previously stated, again with the samples sputter-coated under vacuum with gold, 15 kV of acceleration voltage and 15 mm of working distance.

RESULTS AND DISCUSSION

Morphology and composition of hydroxyapatite coatings

By immersing titanium alloys in SBF for different times, a variety of ceramic coatings were obtained (Fig. 1 and 2). A continuous HAp layer consisting in porous cauliflowers packed together was already formed on these materials after only 12 h in SBF (Fig. 1a). The thickness of the HAp layer increased with coating times from approx. 100 μm after 12 h to 140 μm at 1 to 8 days and 180 μm at 12 and 14 days, (Fig. 1b-i and Fig. 2) though it did not grow as monolayer, as also occurs on PET surfaces using different concentrations of SBF and times of coating [21]. Indeed, long coating times (beyond 2 days) allowed hydroxyapatite to grow from secondary nucleating points leading to isolated clusters with abrupt topography together with porous flat surfaces (Fig. 1d-f) and furthermore to very abrupt porous surfaces (Fig. 1g-i). The roughness of the HAp coatings grew to 10-15 μm at day 4, 15-20 μm at day 6 and 20-30 μm at day 8 days, and decreased thereafter back to 10-15 μm.

From this experiment, 4, 8 and 12 days times were selected for cell culture experiments for they yielded different topographies, from isolated small clusters on a flat outline (mean size of 12 μm^2 after 4 days, Fig. 1d, and 25 μm^2 after 8 days, Fig. 1f) to homogeneous truly three-dimensional surfaces at 12 days (Fig. 1i).

As for the evolution of the chemical composition of the surface (see Fig. 3 for selected times as an example) at short times titanium predominates over other elements such as Na, Mg, K, Ca, Cl and P forming salts. Over time, titanium fades and calcium phosphate gains predominance. Indeed, the Ca/P atomic ratio increases with time (Fig.

4) towards the hydroxyapatite physiological ratio (1.67); after 14 days (336 h) the Ca/P ratio attained is 1.21.

Analysis of cell adhesion and distribution on coated titanium-based samples

A 6 h cell culture revealed that cell adhesion on this titanium alloy improved significantly following 4 days of ceramic coating (Fig. 5a,b). However, longer surface treatments led to heterogeneously adhered cell clusters, likely located mainly on calcium phosphate clusters (Fig. 5c,d). Cells tended to detach from flat surfaces, but those deposited on trully three-dimensional aggregations of porous secondary cauliflowers, remained adhered forming large clusters. Cytoplasm morphology is mainly fusiform on titanium control samples (Fig. 6a), as it is also on cellular apatite- and apatite/collagen-coated PLLA flat surfaces [22]. A rounded morphology is though observed onto porous HAp ridges and grooves when such cells are cultured on abrupt topographies (Fig. 6b).

Cell proliferation was greater on control samples (Fig. 7a,b) than on ceramic coated ones, the former yielding a dense cell layer. On the latter, cell proliferated preferably on areas of steep and irregular topography, avoiding continuous flat surfaces of cauliflowers lying alongside each other (Fig. 7c,d).

Cells are fusiform and tend to align unidirectionally on Ti-based samples (Fig. 7b), as has also been found in other studies with Ti6Al4V samples [18-19]. However, when cells are cultured on abrupt topographies consisting of a flat HAp coating with cauliflower clusters onto it, they tend to set down on selected points of this coating and acquire an elongated morphology as described in other works [23-24].

CONCLUSIONS

A variety of HAp coatings can be obtained onto titanium alloys by means of their immersion in simulated body fluid for different times. The resultant topography and thickness of the coating depends on the SBF concentration and incubation time. After 8-12 days porous clusters of HAp cauliflowers sized around 50 micrometers are formed on a continuous flat coating of cauliflowers lying alongside each other.

The composition of the ceramic layer evolves over time, being initially an assembly of salts (containing Na, K, Ca, Mg, Cl, P) and at longer immersion times in SBF being an irregular layer of apatite with a Ca/P ratio approximating physiological hydroxyapatite. Further exposure of scarcely coated samples to culture medium also seems to allow such evolution of the ceramic coating towards physiological hydroxyapatite.

At relatively short incubation times (4 days) the ceramic coating facilitates cell adhesion. Cells are deposited and adhere over the entire coated surface with homogeneous density. At longer culture times, though, the ceramic coating dissolves partially seeking chemical equilibrium between phases and forces the deposition of hydroxyapatite more similar in composition to bone. Throughout this process, some cells detach from the surface and the remaining ones preferably regroup and proliferate onto apatite cauliflowers clusters and in the voids of porous truly three-dimensional structures formed on the flat coating, leading to heterogeneous cell distributions.

ACKNOWLEDGMENTS

This work was supported by CIBER-BBN through R. Salvador i Clavell grant and the BIOSURFACES Intramural Project 2014-15. M.L. González-Martín (Department of Applied Physics, University of Extremadura, Badajoz, Spain) is thanked for kindly providing the Ti6Al4V samples used in this work.

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Figures

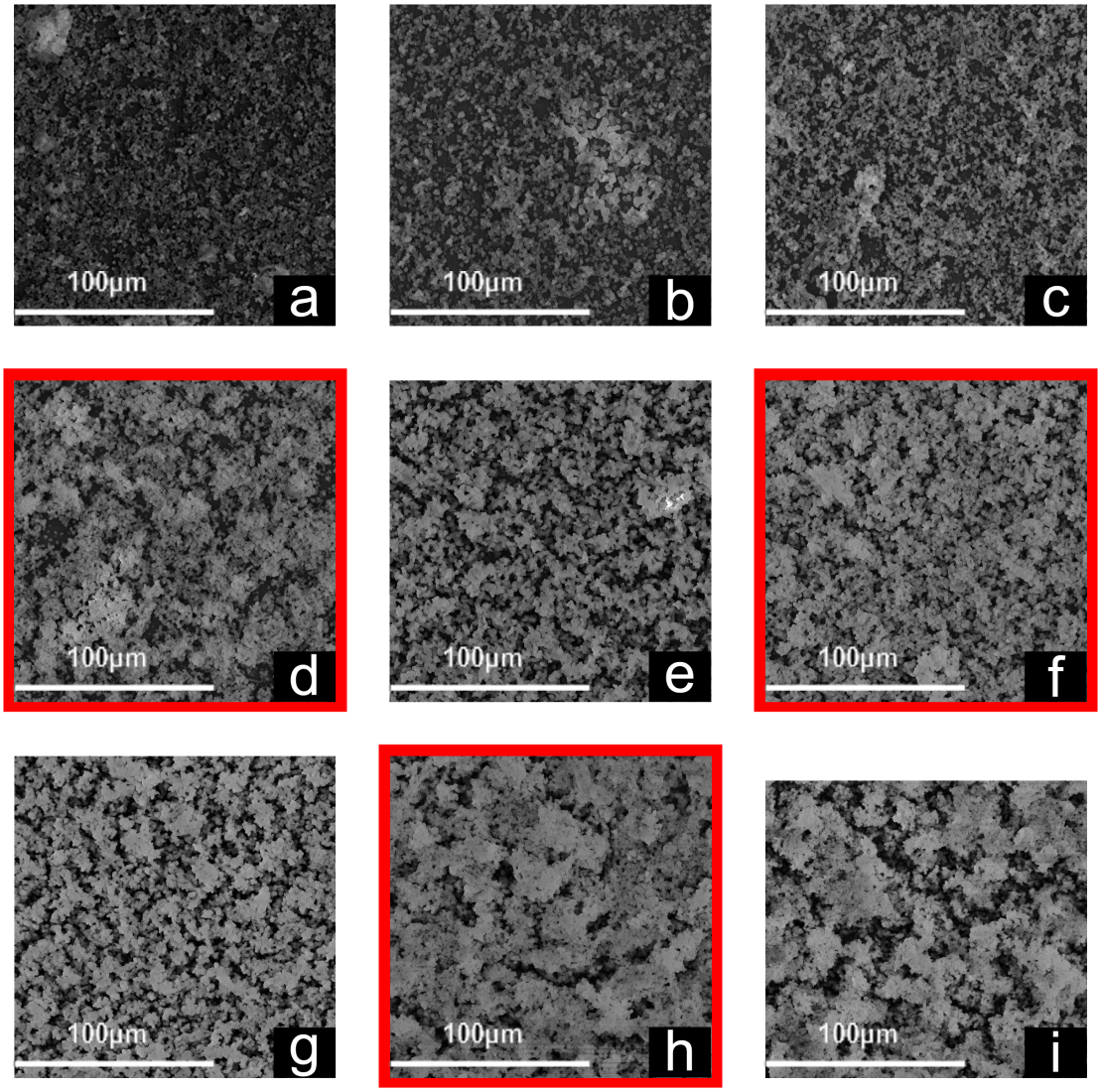

Fig. 1. SEM surface images of HAp coatings on titanium alloys after different times of immersion in SBF: 12 h (a), 1 (b), 2 (c), 4 (d), 6 (e), 8 (f), 10 (g), 12 (h) and 14 days (i). Red frames outline the treatment times chosen for cell culture experiments. Images were taken at 500×.

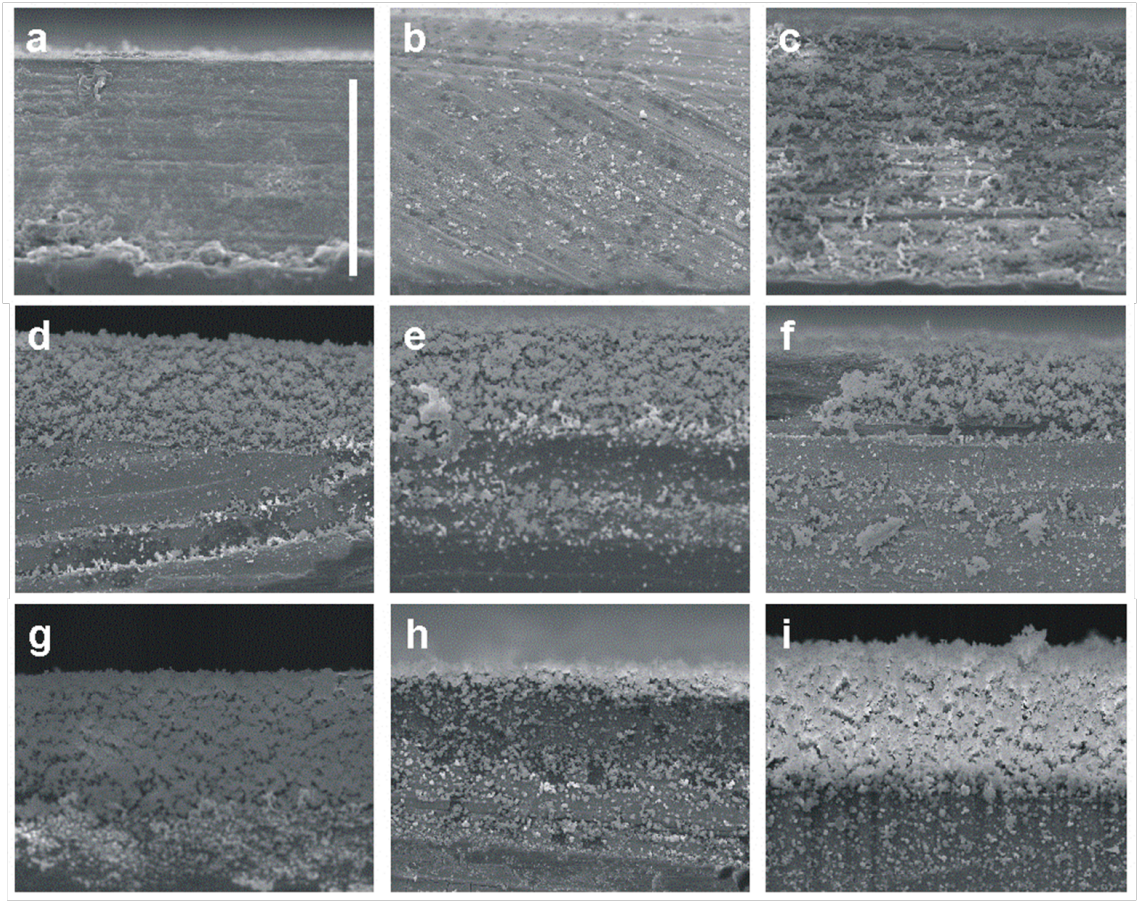

Fig. 2. SEM transversal images of HAp coatings on titanium alloys after different times of immersion in SBF: 12 h (a), 1 (b), 2 (c), 4 (d), 6 (e), 8 (f), 10 (g), 12 (h) and 14 days (i). Scale bar: 100 μm in (a-c) and 200 μm in (d-i).

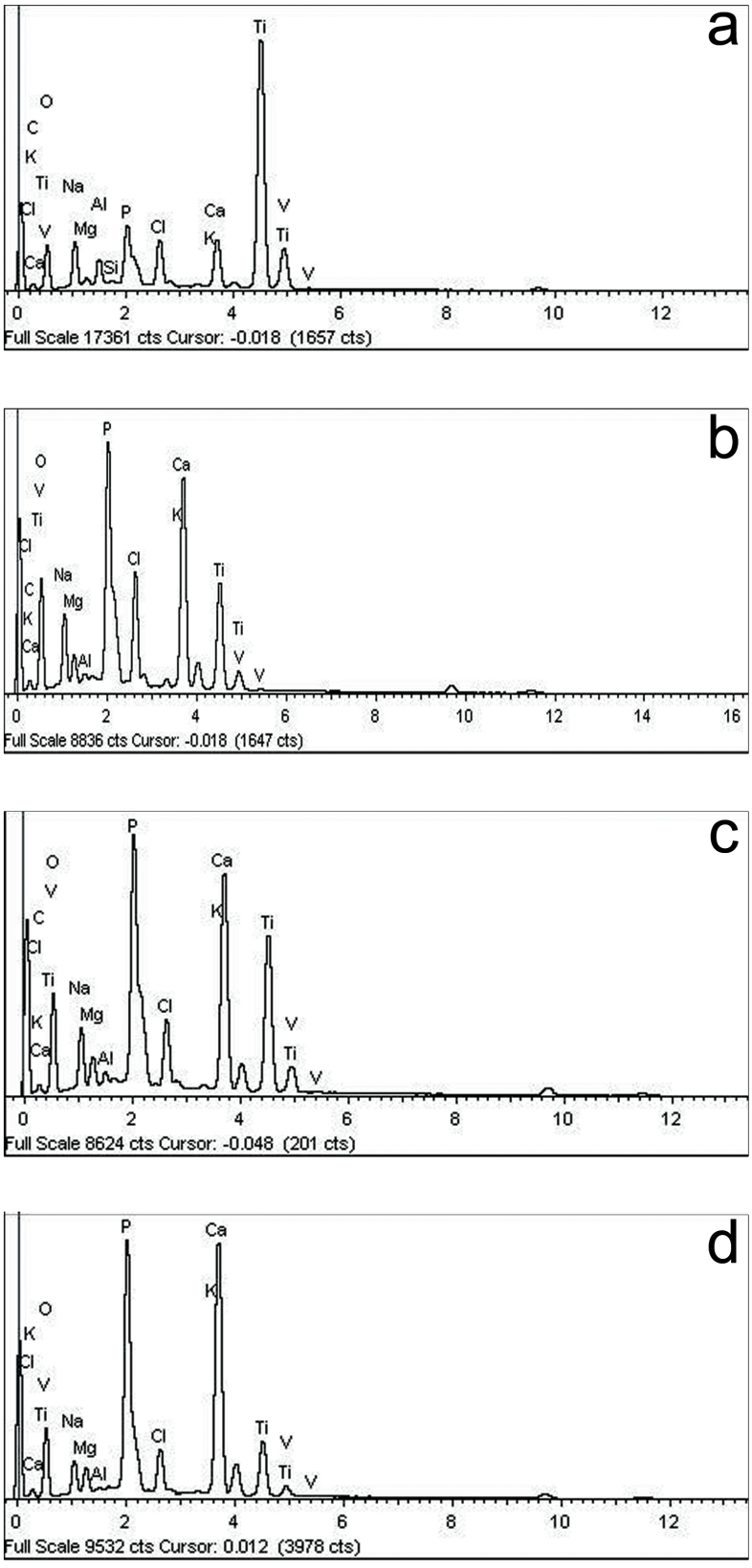

Fig. 3. Evolution of the EDS spectrum with coating times: 12 h (a), 4 (b), 8 (c) and 14 days (d).

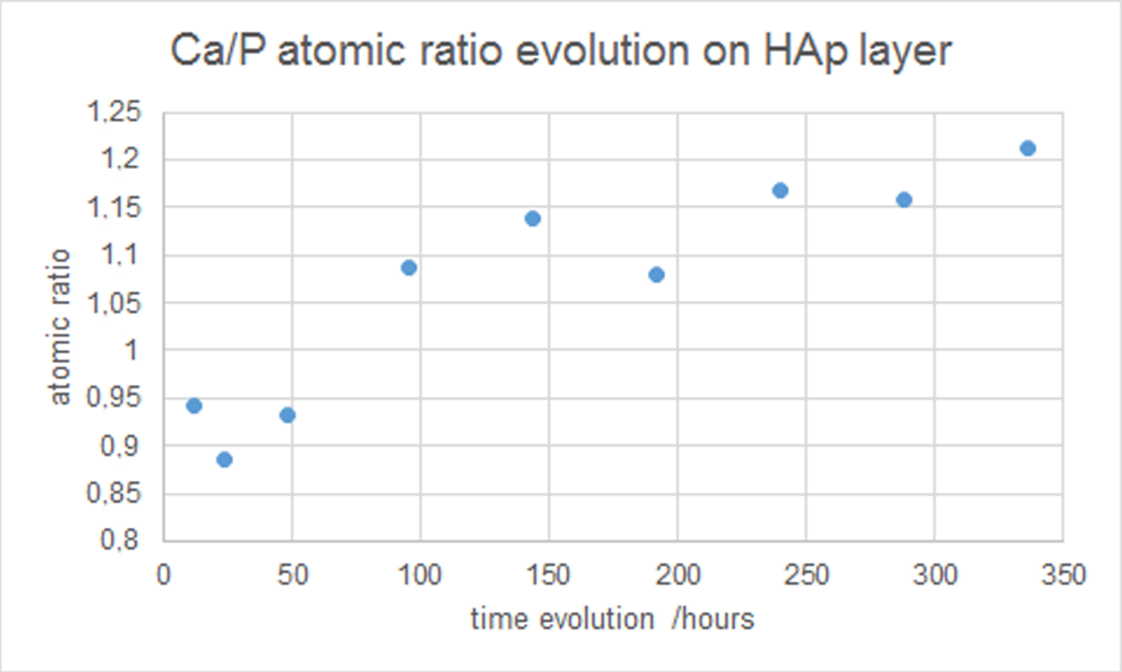

Fig. 4. Ca/P atomic ratio of the ceramic layer on titanium alloys vs. immersion time in SBF.

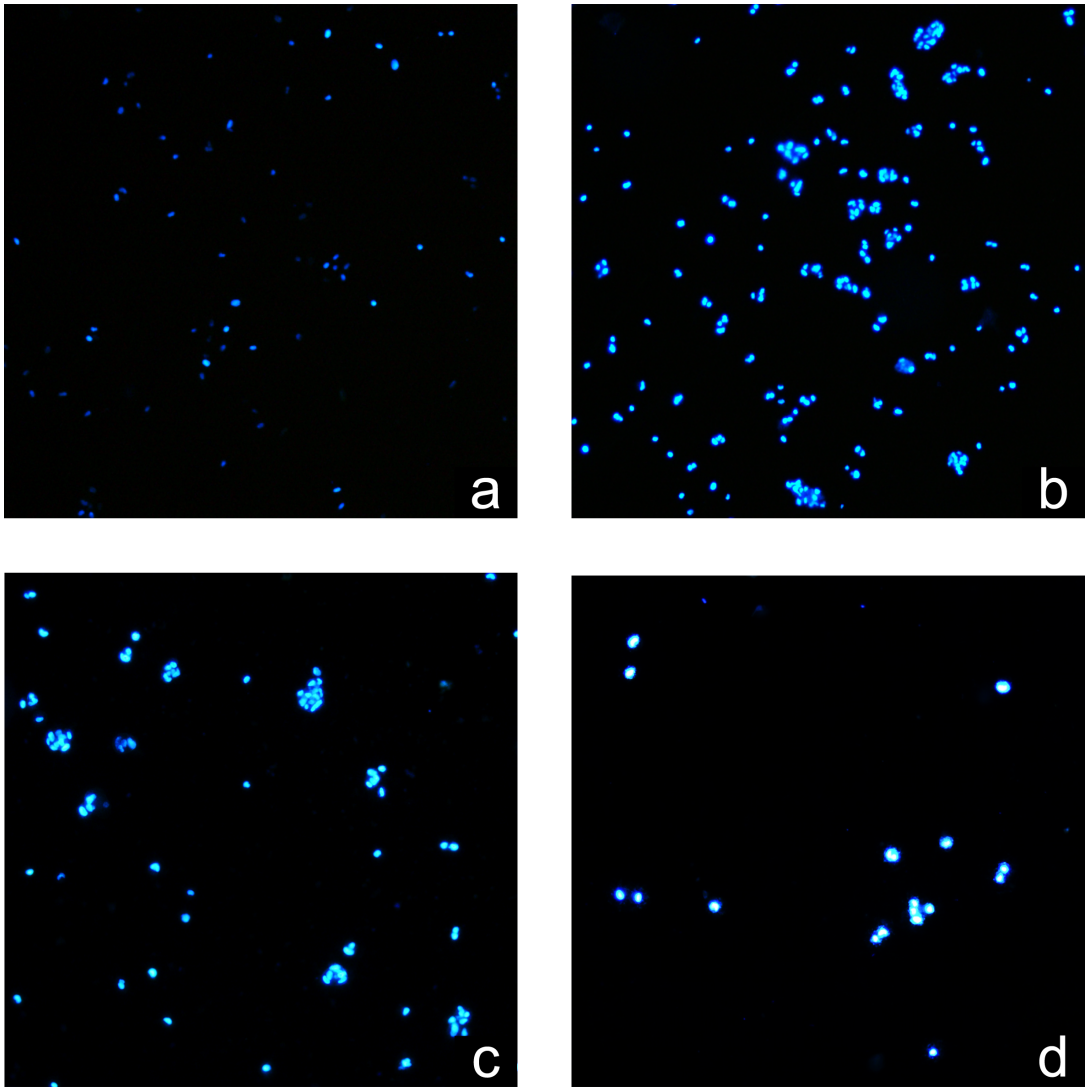

Fig. 5. Fluorescence microscopy images of samples after 6 h cell culture. DNA cell nuclei stained with DAPI (in blue). Raw Ti-based sample as control (a) and after 4 (b), 8 (c) and 12 days (d) in SBF. Images were taken at 10×.

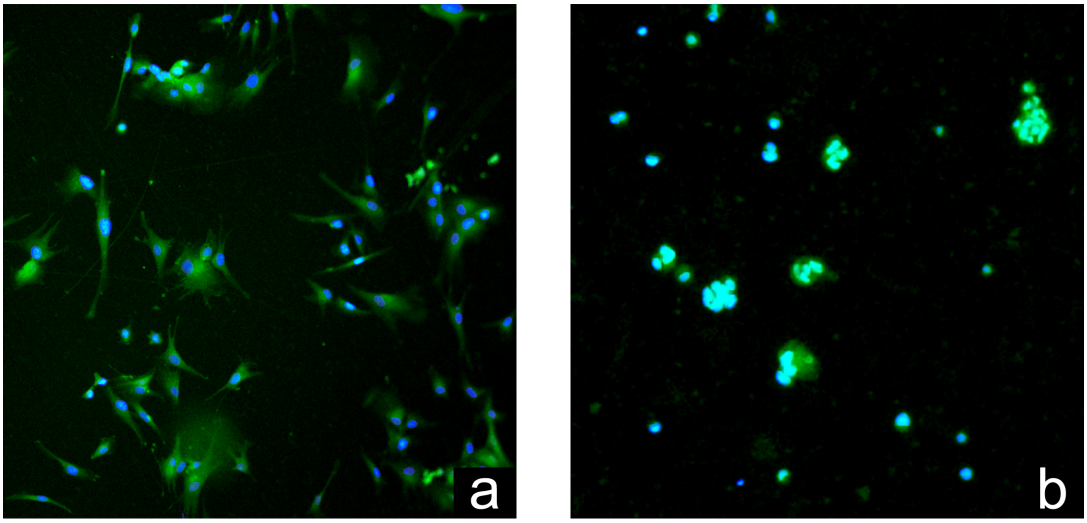

Fig. 6. Fluorescence microscopy images after 6 h culture: raw Ti-based sample as control (a) and after 8 days of ceramic treatment (b). Cell nuclei and cytoplasm are fluorescence stained with DAPI and eosin, respectively (nuclei in blue and cytoplasm in green). Images were taken at 10×.

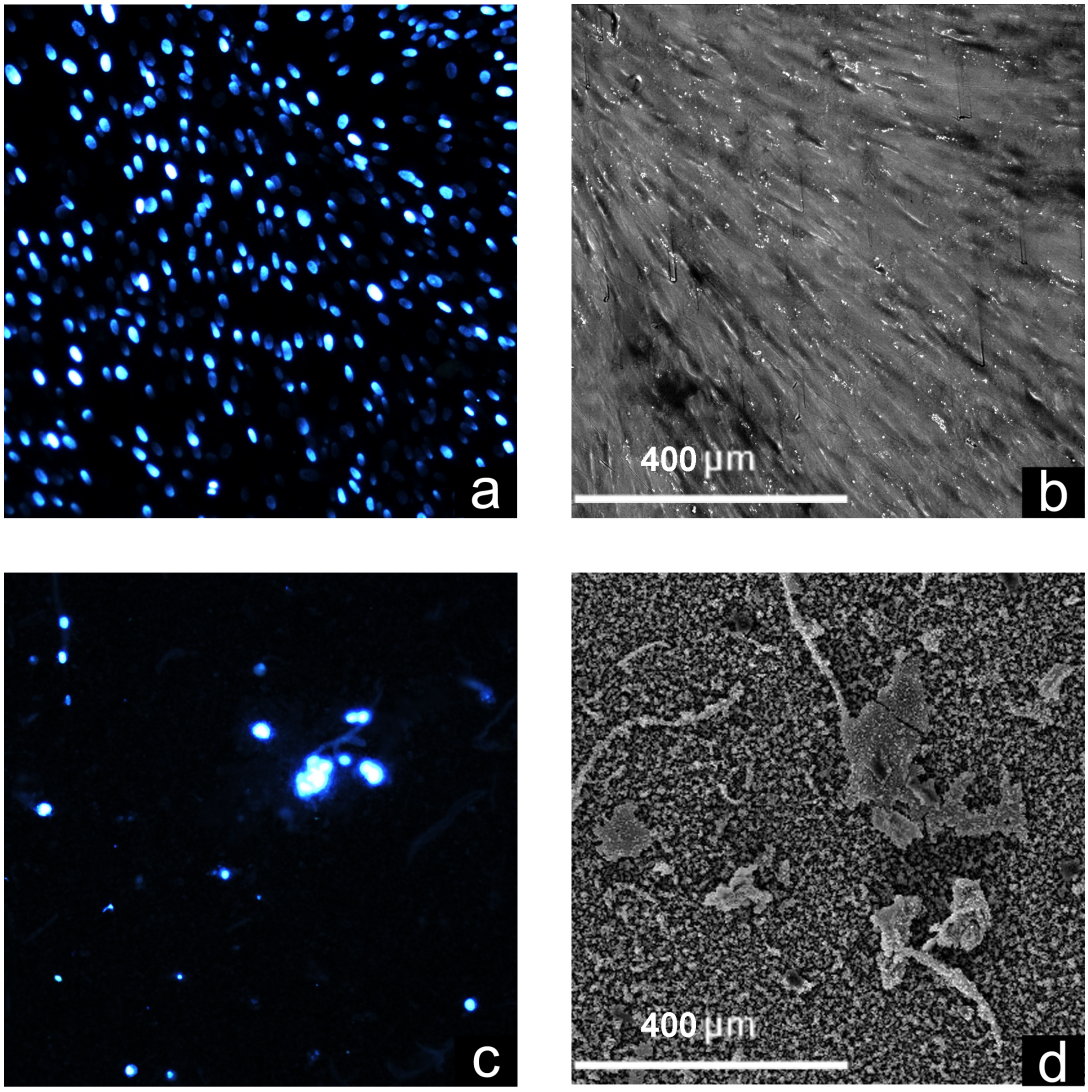

Fig. 7. Cell distribution and morphology after 7 days cell culture. Images were taken under fluorescence microscopy (a and c) and SEM (b and d) after 7 days cell culture, showing the distribution of cell nuclei (fluorescence stained in blue with DAPI) and cell morphology, respectively. Raw titanium samples as control (a, b) and following 8 days HAp-coating (c, d). Images were taken at 10× (a, c) and 150× (b, d).