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Apatite formation and cellular response of a novel bioactive titanium

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Abstract The modification of titanium and titanium alloy surface properties by chemical and electrochemical techniques has opened new possibilities to improve the bioactivity and, in general, the biological performance of the implants once in vivo. One of the main aims is the achievement of a surface oxide layer that stimulates hydroxylapatite mineralization and, also, shows osteoconductive properties once in the host. In the present study, two different bioactive surfaces have been prepared following the method purposed by the group of Kokubo and a new method, BioSpark^{TM}, involving high voltage anodic polarisation and alkali etching both on surface mineralization potential. The aim of the present work was to evaluate and compare the mineralization capability and the early cell response of titanium modified with a new bioactive method and with a well-known and widely tested biomimetic treatment, both compared to non treated titanium. Physical and chemical (energy dispersion spectroscopy, thin film X-ray diffractometry) and morphological (scanning electron microscopy) characterisation of the novel surface features has been performed. Also the effect of the novel surface properties on both hydroxyapatite precipitation and early cellular response has been investigated using in vitro models. The results have shown that both treatments produce an active outer layer on titanium but do not impair cells activity and support osteoblasts processes. BioSpark^{TM} showed high bioactivity and good mineral phase deposition even after early incubation time, these properties were found in Kokubo's surface as previously published. Mineralisation mechanisms of the two materials were different, and while this mechanisms was well characterised and reported for Kokubo's surface, it was still unclear for BioSpark^{TM}. In this paper an explanation was given and catalytic properties of the latter surface was bound to both well known crystal titanium oxide exhibiting anatase lattice and a certain level of calcium and phosphorus doping, which promoted chemical and physical variation in anatase properties. At the same time early osteoblasts response to Kokubo's and BioSpark^{TM}'s surface was characterised and, no significant differences was found.

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Introduction

Progress in recent years in the modification of surfaces by chemical, electrochemical, photochemical and plasma-chemical techniques has opened new possibilities to tailor the surface properties of widely exploited implantology materials such as titanium and titanium alloys. A great deal of current research focuses on the effect of different chemical and electrochemical treatments on bioactivity of such materials [1–4]. The aim of these treatments is to modify titanium in order to achieve surface titanium oxide mimicking hydroxylapatite mineralization and osteoconductive properties.

Calcium phosphate represent the most important and widely exploited class of bio-inorganic materials used to modify titanium surfaces for bone-bonding biomedical applications. In particular, hydroxylapatite (HA) has attracted a lot of attention in view of its brilliant osteoconductive properties. A part from HA other calcium phosphate phases such as α- and β-tricalcium phosphate, tetracalcium phosphate, octacalcium phosphate, fluorapatite and amorphous calcium-phosphate phases has been studied and used as coatings on titanium and its alloys. However, the influence of the physico-chemical properties of calcium phosphate and of its degradation kinetic on new bone rate formation and on the long-term stability of bone-implant interface is still subject of investigation [5–8].

Spontaneous calcium phosphate deposition on titanium surfaces from aqueous electrolyte containing calcium and phosphate ions, such as simulated body fluid solutions, has been observed and is believed to be related to the excellent bonding capability in contact with bone tissue [9, 10]. The formed calcium-phosphate films are generally amorphous or low crystalline, at least in the earlier stages of spontaneous nucleation [11, 12]. Furthermore, after longer simulated body fluid exposure low crystalline HA clusters growth has been reported [13]. Calcium enriched titanium oxide by chemical techniques [14, 15] and thickened gel-like titanium oxide achieved by hydrogen peroxide solutions [16] have been reported to greatly enhance calcium-phosphate nucleation of modified titanium surfaces during soaking in calcium and phosphate containing solutions (SBF). Alkali chemically etched and heat treated titanium [17] has been reported to speed up both calcium phosphate nucleation [18, 19] and bone-bonding with no bone-implant interface fibrous tissue interposition [20, 21]. This treatment has been widely studied in the last decade and has been clinically trailed in Japan with success.

Thus, the aim of this work was to evaluate the mineralization capability and the early cell response of titanium modified with a new bioactive method involving high voltage anodic polarisation and alkali etching [4], and to compare these new surfaces behaviours with a well-known and widely tested biomimetic treatment purposed by the group of Kokubo [17–19] and also with pure titanium as control.

Surfaces properties were investigated by: scanning electron microscopy (SEM) to analyse surface morphology; energy dispersion spectroscopy (EDS), to both assess the procedures influence on surface chemical composition and evaluate the chemical composition of new phases clustered grown on the surfaces after SBF soaking. Also, thin film X-ray diffractometry (TF-XRD) has been performed, in order to achieve higher knowledge of the surface crystalline structure, to assess the presence of crystalline titanium oxides and, the nature of new nucleated phases after SBF soaking on the very titanium surface.

Finally, cells adhesion (SEM) and direct viability (Alamar Blue test) and non contact cyto-toxicity of cells seeded directly on material surfaces and in contact with media previously kept in contact with tested surfaces were assessed in order to evaluate cellular response and correlate it to surface features.

Materials and methods

Samples preparation

All titanium samples used in this work were obtained from a titanium sheet of commercially pure grade 2 titanium (Loterios, Milano), HF/HNO_3 pickled for surface decontamination by Loterios itself. Coupons ($10 \times 10 \times 1$ mm^3) were cut with a mechanical cutter available at IENI (CNR Milan) and rinsed by ultrasonic rinsing (Branson Automatic Cleaner, UK) in acetone (RPE Carlo Erba or Sigma Aldrich, Italy) for 5 min and in distilled water for the same time to degrease and to clean the surface from contaminants. After cutting, cleaned and de-greased titanium were polished with Si–C abrasive paper (meshes 320 and 400). Three different surface finishing were prepared:

- TI: smooth titanium without any further treatment.
- BSP: electrochemical surface treatment by Anodic Spark Deposition and alkali treatment [4, 22] carried out on TI surface.
- KOK: chemical and heat treated surface following the procedure already published by the group of Kokubo [17–19] carried out on TI surface. The procedure published by Prof. Tadashi Kokubo was carefully reproduced step by step for achieving a surface as similar as possible to which was used previously for his numerous studies.

BSP treatment procedure

Titanium surface was prepared in an electrochemical cell by a double step Anodic Spark Deposition technique [23–24], followed by alkali etching. A variable dc power supply (BVR1200-500-1.5 Belotti Variatori s.r.l., Italy) and two voltmeters were used to supply power to the circuit and to monitor the voltage gap between cathode and anode as well as between cathode and points of the electrolytic solution in

between the two electrodes. The solution was kept stirred by magnetic stirring in a double wall glass beaker. Electrolytic solution temperature was monitored and kept at 0 °C (±2 °C) by the flowing of a refrigerating fluid through the external and the inner wall of the beaker. Specimens were connected one by one to the anode. A Teflon sleeve was used to shield the specimens surface and to avoid sparking at the atmosphere-sample-electrolyte solution interface. The cathode was formed by a titanium (c.p. grade 2) cylindrical shaped net, whose surface was about 60 times bigger than the surface of the anodising sample.

BSP protocol involves a two consecutive step ASD process (label ASD1 and ASD2) carried out in different electrolyte at different voltage ranges. ASD1 process is carried out in a 15 mM Ca^{++} and 15 mM Phosphate ions water solution, and ASD2 is carried out in a 100 mM Ca^{++} water solution, and a further hot alkali etching process through which the final BSP surface is achieved [4]. Full details of the BSP surface modification technique and sample preparation techniques have been reported elsewhere [25].

KOK treatment procedure

Smooth titanium after polishing with Si–C abrasive paper (meshes 320 and 400) sample was soaked in 5 ml of 5 M NaOH aqueous solution at 60 °C for 24 h (Ti–Na), gently washed in distilled water and air dried at 40 °C for 24 h. This alkali treatment produces on the very surface of titanium a bioactive sodium titania gel-like layer [26]. After air drying samples were placed in an electric furnace, heated at 600 °C at a rate of 5 °C/min, kept at 600 °C for 1 h and then cooled in the furnace for 24 h (KOK). This heat treatment has been reported to stabilize the gel-like layer covering titanium surface after alkali etching and to produce a dehydrated amorphous sodium titanate mixed with low crystalline titanium oxide thin surface layer.

Physicochemical properties analysis

Samples surface properties were investigated with thin film X-rays diffraction (TF-XRD) (Siemens D500 Kristalloflex) at 40 mA and 40 kV, the assignation of detected peaks to crystalline phases was carried out by using data from the Powder diffraction file, International centre for diffraction data, 1986.

Scanning electron microscopy SEM (Leica Cambridge Instruments, STEREOSCAN 430, uploaded with backscattered electrons detector) at an accelerating voltage of 20 kV for all samples, before SBF soaking and after soaking, furthermore surface analysis was performed before cell seeding and at 10 kV for all cultured samples. Specimen for scanning electron microspcopy were sputter coated (Edwards, Sputter Coater S150B, 1 min at 15–20, 10^{-1} mmHg) with gold before examination. Samples were investigated also by electron dispersion spectroscopy (EDS) (Link eXL analyser, with Si (Li) detector PENTAFET PLUS with ATW window for light elements detection starting from carbon; Software package ZAF-4/FLS for massive sample analysis). A standard cobalt reference was used for calibrating quantitative analyses. Samples prepared for EDS analysis were not sputter coated with gold for avoiding phosphorus and gold peaks overlapping.

Soaking of the specimen in simulated body fluid

Ti, BSP, and KOK samples were all separately placed in a 24 wells tissue culture plate (TCP) and soaked in 6 ml of simulated body fluid (SBF) [27], whose ionic composition resembles plasma ionic concentration (Table 1). TCPs were placed in an incubator at 37 °C with 5% CO_2 and a humidified atmosphere (Forma). The SBF was prepared by dissolving 6.057 g of tris-hydroxyl-methil-amino-methane (0.05 M TRIS, Sigma Aldrich) in 600 ml of distilled water through agitation with a magnetic stirrer in a clean glass beaker. The solution pH was then adjusted to 7.4 by adding 5 M HCl dropwise. The volume of the solution was finally brought up to 1 l with distilled water. The solution buffered at 7.4 pH was divided in two batches of 500 ml in which the calcium component and the remaining ion component were separately dissolved. In the first batch were dissolved 0.386 g of $CaCl_2 \cdot 2H_2O$. The second batch was prepared dissolving the following amount of these reagents, (i) 7.995 g NaCl, (ii) 0.353 g $NaHCO_3$, (iii) 0.224 g KCl, (iv) 0.174 g K_2HPO_4, (v) 0.071 g Na_2SO_4 and (vi) 0.305 g $MgCl_2 \cdot 6H_2O$.

After dissolving all the reagent in an equal amount of the two solutions were mixed together and used to soak samples previously placed in TCP. The ions concentration of the final solution is reported in Table 1. SBF was replaced every 24 h for avoiding ionic depletion produced by new phases nucleation on the

Table 1 Kokubo simulated body fluid ions concentration

Kokubo SBF ions concentration (mM)							
Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	$HC03^-$	HPO_4^{2-}	$S0_4^{2-}$
142	5	1.5	2.5	147.5	4.2	1.0	0.5

bioactive surfaces. Six time-points (1–3–5–8–11–15 days soaking) were carried out for BSP and KOK while TI samples were kept soaked for longer times (1–7–15–21–28 days). Every time-point was characterized by three incubated sample per type of material. After soaking for selected periods, the specimen were removed for the soaking solution, gently washed in distilled water three times by stirring for 30 s and, then dried in air on a clean bench, before further analysis.

In vitro biological response

Sample treatments and controls

The samples were tested after sterilisation by ethanol (70% v/v in distilled water) followed by UV irradiation (254 nm). TCP polystyrene (Corning-Costar) was used as control for cellular elution and cellular metabolic activity studies. All estimations were performed at least in triplicate.

HOS cells

The human osteosarcoma cell line MG63 was obtained from the European Collection of Animal Cell Cultures (ECACC) and cultured as previously described [28].

Cell seeding

After trypsinisation, cell viability was assessed using the Trypan Blue exclusion dye (Sigma-Aldrich). The test materials were placed in a 24 well TCP and seeded with 50 µl of 2×10^5 cells/ml suspension allowed to adhere in the incubator for 1 h, flooded with 1 ml of culture medium and placed back to the incubator. Furthermore, after one more our in cell incubator seeded samples were gently placed one by one in a new 24 well TCP, in order to avoid the risk of compromising metabolic activity test results due to cells adhesion on the old TCP surface. Indeed, the bottom of every wells, where square samples were placed was checked and samples excluded by tests if cells were adhering on the well and not only on the specimen surface.

Cellular morphology

For the SEM analysis**,** the cells were fixed with 1.5% w/w glutaraldehyde (Fluka) buffered in 0.1 M sodium cacodylate (Fluka) (pH 7.2–7.4) at 4 °C after the required incubation period. The cells were dehydrated through a series of ethyl alcohol (BDH) solutions (from 20 up to 100% v/v of ethyl alcohol in distilled water). The final dehydratation was through a series (from 25% up to 100% v/v in ethyl alcohol) of hexamethyldisilazane (Sigma-Aldrich) solutions, followed by air drying. The samples were sputter coated (Edwards, Sputter Coater S150B, 1 min at 15–20, 10^{-1} mmHg) before examination by scanning electron microscopy at an accelerating voltage of 10 keV. Seeded samples were labelled TI6, BSP6 and KOK6 when culture lasted 6 h and TI24, BSP24 and KOK24 when cultured lasted 24 h.

HOS Elution study

Sterile samples were placed into a 24 well tissue culture plate, flooded with 1 ml of culture medium and incubated. At the selected time points, the aqueous extracts were collected in sterile condition and replaced with 1 ml of fresh medium. About 100 µl of 1×10^5 cell/ml cell suspension was seeded into a sterile 96 well culture plate and incubated to almost 90% confluence. The culture medium was then removed and replaced with 100 µl/well of the test extract and the plates incubated for a further 72 h. The extract was then removed and replaced with 100 µl of MTT (Sigma-Aldrich) solution (0.5 mg/ml in culture medium) in each well. The plates were incubated for 4 h at 37 °C, 5% CO_2 in a humidified atmosphere. Following this, the medium was then removed and replaced with 100 µl of DMSO (Merk) to each well and the plate mixed until complete dissolution of the crystals was obtained. Absorbance was measured using a Tecan Genius Plus plate reader (test wavelength: 570 nm; reference wavelength: 630 nm).

Metabolic activity assay

Cells were seeded onto the sample surface as previously described. At each time point (day 1, day 2, day 3 and day 7), the culture medium was replaced with 1 ml of Alamar Blue (Serotec) solution (10% v/v in culture medium) and the plate incubated for 4 h. About 100 µl of Alamar Blue solution was removed from each well, transferred to a 96 well plate and the absorbance measured by a Tecan Genius Plus plate reader (test wavelength: 570 nm; reference wavelength: 630 nm). The samples were rinsed with PBS, 1 ml of culture medium was then added to each well and the plate was returned in the incubator.

Statistical analysis

One-Way ANOVA was carried out for comparing data of more than two populations contemporary and a significance *p*-value of 0.05 was chosen as threshold. One-Way Analysis of Variance (ANOVA) is appropriate when making a single test to find out whether two or more populations have the same mean. Assumptions of this test are that the data follow a normal distribution and have constant variance.

T-Test was exploited when comparing two population and a significance *p*-value of 0.05 was chosen as threshold. With two samples of data, one *X* and one *Y* column, that are independent and that follow a normal distribution with constant variance, a two-population *t*-test can be employed to test whether or not the population means are equal.

Results

Physicochemical properties analysis

TI (Fig. 1a) presents an irregular texture, and grain boundaries are not pronounced. Titanium texture showed smooth peaks alternate to low valleys following the direction of the last abrasive paper polishing.

BSP texture was regular; many round crests and small pores were clearly visible on the surface (Fig. 1b). Moreover, the whole surface was covered by a thin nano-textured roughness overlapped to the micro porous morphology. This netlike surface roughness was not revealed after ASD2 and formed only after alkali treatment [4]. The EDS analysis (Table 2) showed the presence of calcium, phosphorous and oxygen peeks besides the peeks of titanium.

KOK presents the typical morphology achieved by high concentrated sodium hydroxide water solution corrosion on titanium, with many small cracks spread all over the surface. Anyway, KOK surfaces appears quite regular and at least as flat as BSP surface (Fig. 1c). Surface chemical analysis carried out with EDS (Table 2) showed that the surface layer of KOK was still mainly composed by titanium with traces of sodium and oxygen enrichment.

TF-XRD analysis carried out on TI showed that the only crystalline phase detectable was titanium (Fig. 2a), while BSP surface was covered by a thin layer of titanium oxide, mainly in the anatase phase (Fig. 2b). TF-XRD analysis carried out on ASD1 and ASD2 samples showed that the second anodisation step of BSP treatment was the most effective in

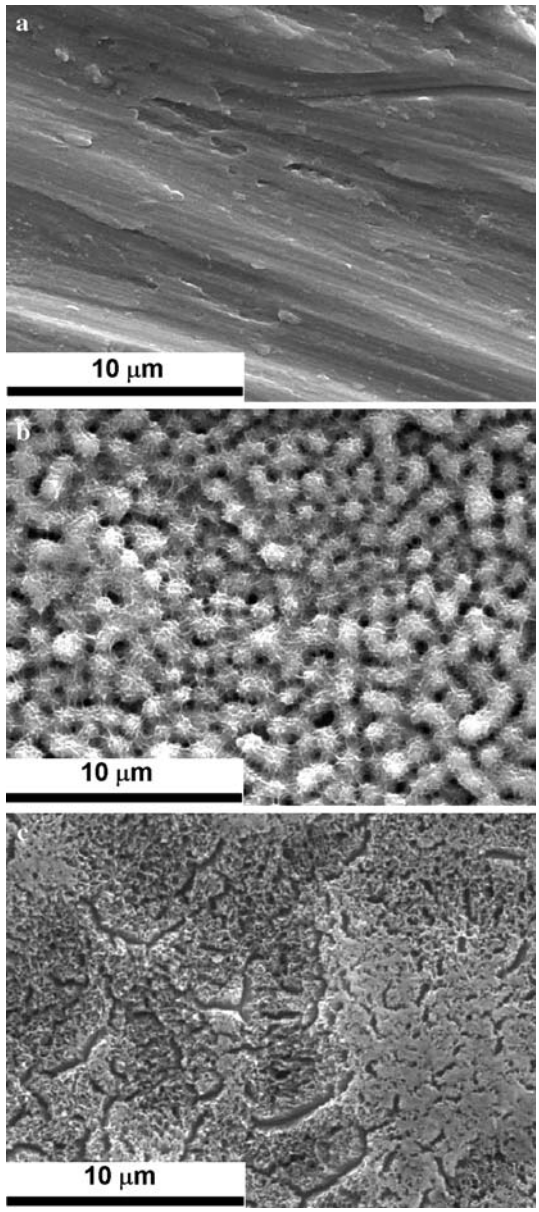

Fig. 1 Scanning electron microscopy and energy dispersive spectroscopy of (**a**) TI, non treated titanium used as substrate; (**b**) BSP treated titanium; (**c**) KOK treated titanium

Table 2 semi-quantitative chemical analysis assessed by energy dispersive spectroscopy (EDS) on BSP, KOK and TI samples before soaking in SBF. Atomic weight percentage of calcium, phosphorus, titanium, oxygen and sodium are reported. Peak calibration for quantitative analysis was carried out with a standard cobalt reference sample

Atomic %	Tl	BSP	KOK
Ca	/	0.956 ± 0.018	/
P	/	0.294 ± 0.028	/
O	1.235 ± 0.047	79.087 ± 0.137	75.092 ± 0.134
Ti	98.568 ± 0.142	19.646 ± 0.131	12.860 ± 0.121
Na	/	/	12.130 ± 0.154

achieving crystalline titanium oxide growth. The comparison of ASD2 spectra and BSP final spectra showed that alkali treatment slightly modified surface crystalline phases, favouring the growth of rutile phase (Fig. 2b).

TF-XRD analysis applied to KOK surface revealed the presence of a crystalline oxide film mainly composed by rutile phase and only a small amount of crystalline sodium titanate was detected (Fig. 2a).

Soaking in simulated body fluid

After soaking in simulated body fluid (SBF), untreated titanium TI and treated BSP and KOK surfaces were investigated by TF-XRD, SEM and EDS. Titanium coupon soaked from 1 to 28 days did not show as expected any massive nucleation of new crystalline phases. By TF-XRD analysis anything but titanium peaks were detected even on samples soaked for 28 days in SBF (data not shown). TI surface after 28 days incubation in SBF (Fig. 3a) exhibited only few new heterogeneous phases. EDS analysis demostrated that this phases were mainly composed by calcium (Fig. 3b) and phosphorous (Fig. (3c), whose distribution was not homogeneous. In particular, EDS maps showed that almost all TI surface even after 28 days soaking in SBF emitted only titanium signal (Fig. 3d).

Both KOK and BSP surfaces showed consistent amount of new formed clusters covered the surfaces treated by BSP and KOK methods during the soaking time in SBF.

A heterogeneous precipitation was found on BSP after 3 days soaking in SBF (Fig. 4a). At this incubation time was still possible to observe the typical porous morphology of BSP underlying the forming mineral phase (Fig. 4a). BSP surface was still visible under the new globular growing layer even after 5 days soaking (Fig. 4b). Only after 15 days the newly nucleated layer completely covered the BSP surface (Fig. 4c).

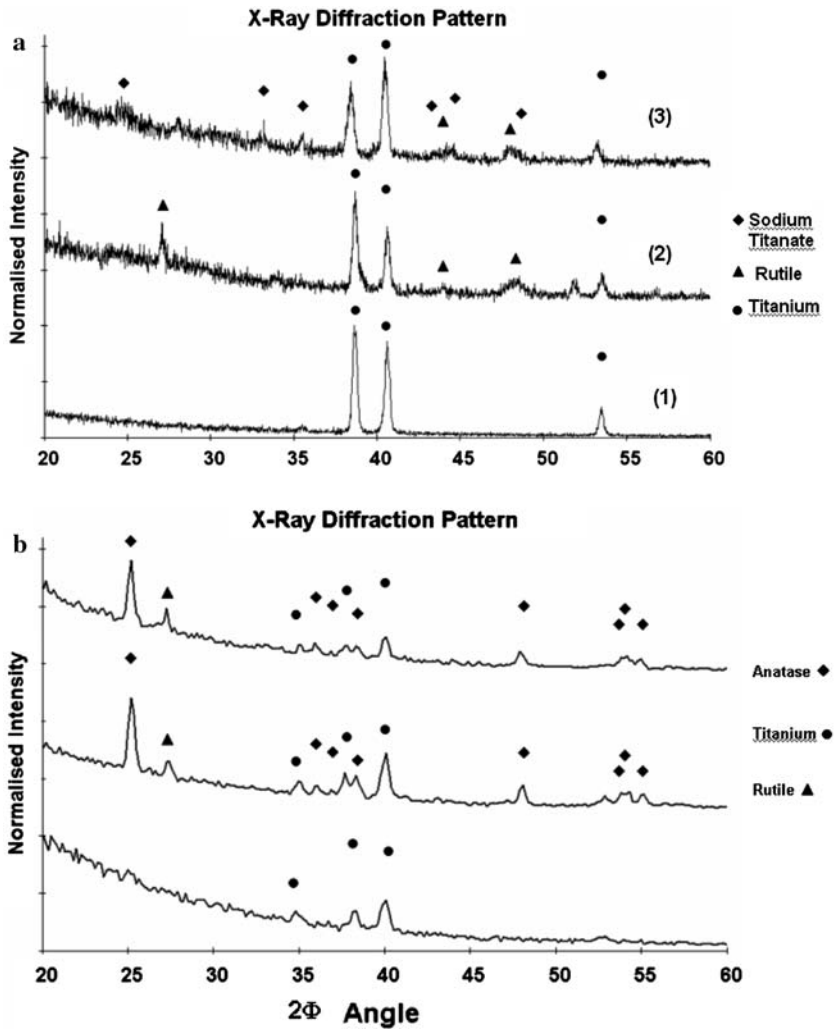

Fig. 2 Thin Film X-ray diffractometry of (**a**–1) TI non treated titanium, (**a**–2) Ti–Na treated titanium and (**a**–3) KOK treated titanium; (**b**–1)ASD1, (**b**–2) ASD2 and (**b**–3) BSP treated titanium

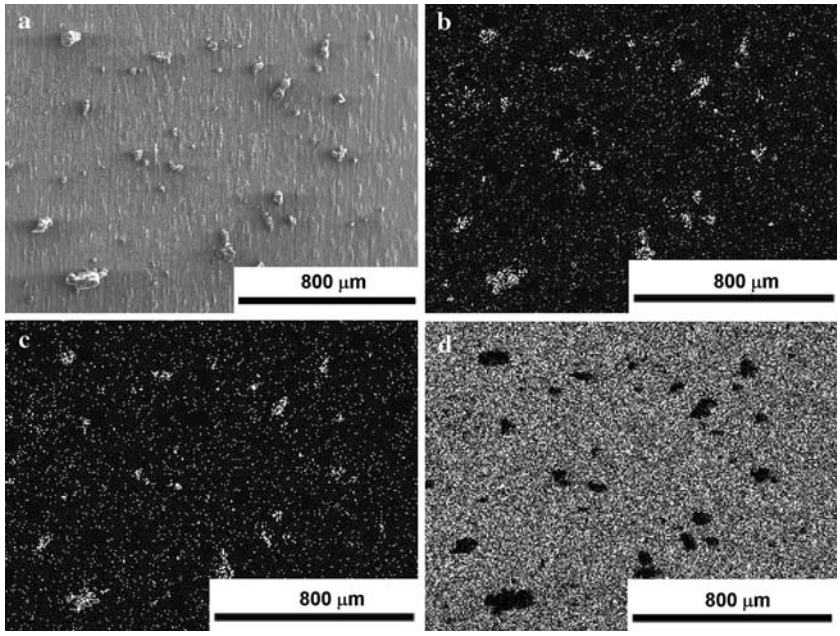

Fig. 3 Scanning electron microscopy and energy dispersive spectroscopy of TI surface after 28 days of soaking in SBF: (**a**) SEM; and (**b**) calcium EDS map; (**c**) phosphorous EDS map; (**d**) titanium EDS map, white spots means effective presence of such element while black field means that no trace of such element was detected

EDS analysis allowed the identification of the mineral phase as mainly composed of calcium and phosphorous, whose amount on BSP surface grew during soaking time (Fig. 4d). This newly formed calcium-phosphate layer thickened until covering the whole surface. This layer was richer in calcium than phosphorus and grew slowly during the first 5 days incubation in SBF, its growing rate exhibited a strong speed up between day 5 and day 8, and thickening rate slowed down from day 8 to day 15 (Fig. 4d). As reported in Table 3 the nucleation of new phases mainly composed of calcium-phosphate involved also sodium and magnesium uptake from SBF as detected by EDS (Table 3).

Scanning electron microscopy was performed also on KOK samples after soaking in SBF for the same time points of BSP. After 3 days soaking, no nucleation was detected (Fig. 5a), and new heterogeneous phases appeared as spots on the surface within 5 days (Fig. 5b). Eventually a thick layer coated the whole surface after 15 days soaking n SBF (Fig. 5c). Energy dispersive spectroscopy was carried out at the six

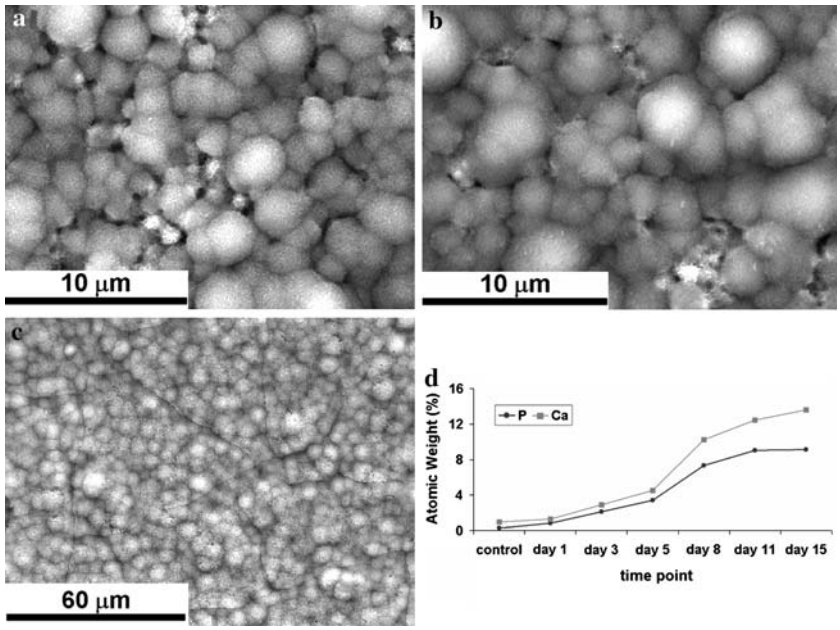

Fig. 4 (**a**–**b**–**c**) Scanning electron microscopy of BSP surface after soaking in SBF: (**a**) SEM after 3 days; (**b**) after 5 days; and (**c**) after 15 days of SBF soaking (**d**) plot of calcium and phosphorus amount on BSP samples soaked in SBF after different time points. Data were collected by EDS analysis after calibration of microanalysis detector through a cobalt standard reference. Calcium and phosphorus amount were reported as atomic weight percentage on the total amount of collected signal of titanium, sodium, magnesium and oxygen

Table 3 semi-quantitative chemical analysis assessed by energy dispersive spectroscopy (EDS) on BSP samples after soaking in SBF for different time points (1, 3, 5, 8, 11, 15 days). Atomic weight percentage of calcium, phosphorus, titanium, oxygen, magnesium and sodium are reported. Peak calibration for quantitative analysis was carried out with a standard cobalt reference sample

BSP	% P	% Ca	% Na	% Mg	% O	% Ti
Day 1	0.84	1.27	–	–	70.08	27.81
Day 3	2.13	2.90	–	–	65.35	26.63
Day 5	3.41	4.53	–	–	66.94	2542
Day 8	7.33	10.23	–	0.63	62.85	18.96
Day 11	9.02	12.47	1.05	0.70	55.28	21.48
Day I8	9.12	13.58	1.57	0.82	58.72	16.19

chosen time points, the newly formed layer exhibited almost the same composition of that one detected on BSP. During the first 5 days soaking KOK surface released sodium (Fig. 5d) into the solution, furthermore this process showed its highest rate between the early 24 h soaking and almost stopped after 5 days in SBF (Fig. 5d). Moreover, while nucleation mechanism started after only three days, even if clustering processes were very slow and not detectable by SEM, only after 5 days a strong speed up of nucleation rate was detected (Fig. 5d) and in few days the surface resulted completely covered by a thick layer of calcium-phosphate (Fig. 5c).

EDS analysis showed that this new phase was mainly composed by calcium and phosphorus as expected, anyway other elements uptake from solution occurred and magnesium as well as for BSP was found as a component of these new phases (Table 4).

When TF-XRD was performed on BSP after incubation in SBF and it was observed that the calcium phosphate phase was low crystalline hydroxyapatite and the first HA peaks were detected only after 3 days incubation in SBF (Fig. 6a). Diffraction analysis confirmed the nature of this new clustered material on KOK as well. Diffraction spectra exhibited low crystalline hydroxylapatite peaks, growing with longer soaking time (first detection of crystal phases at 8 days soaking in SBF) and hiding titanium substrate peaks (Fig. 6b). Thus, SEM, EDS and TF-XRD analysis confirmed the capability of KOK surface to speed up calcium-phosphate uptake form SBF and to favour hydroxyl-apatite nucleation on titanium surface after short time soaking in SBF. These findings agreed with what already published by the group of Kokubo [7, 18, 19].

In vitro biological responses

Cell morphology

At 6 h post-seeding, cells were observed attaching and spreading on the surface of all the tested materials. On TI6 (Fig. 7a) the number of round-shape cells seemed to be lower than on KOK6 and BSP6. On KOK6 (Fig. 7b) cells spreading seems to be reduced if compared to TI6, on which almost all cells are flat and follow clearly the polishing line edges. On the other hand, on BSP6 (Fig. 7c) samples even if round-shaped cells still appear, a high amount of cells showed advanced flattening and cuboidal shape. After 24 h,

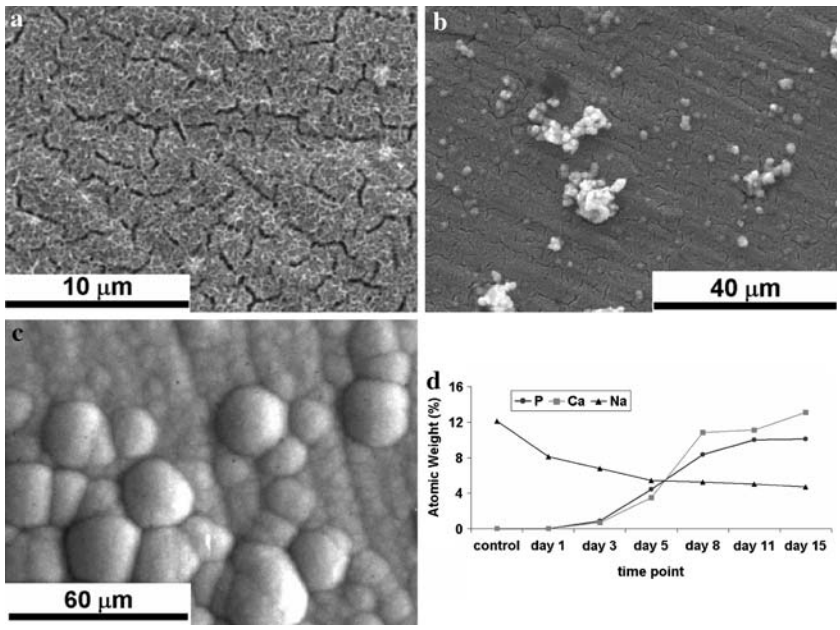

Fig. 5 (**a**–**b**–**c**) Scanning electron microscopy of KOK surface after soaking in SBF: (**a**) SEM after 3 days; (**b**) after 5 days; and (**c**) after 15 days of SBF soaking (**d**) plot of calcium and phosphorus amount on BSP samples soaked in SBF after different time points. Data were collected by EDS analysis after calibration of microanalysis detector through a cobalt standard reference. Calcium, sodium and phosphorus amount were reported as atomic weight percentage on the total amount of collected signal of titanium, magnesium and oxygen

Table 4 semi-quantitative chemical analysis assessed by energy dispersive spectroscopy (EDS) on KOK samples after soaking in SBF for different time points (1, 3, 5, 8, 11, 15 days). Atomic weight percentage of calcium, phosphorus, titanium, oxygen, magnesium and sodium are reported. Peak calibration for quantitative analysis was carried out with a standard cobalt reference sample

KOK	% P	% Ca	% Na	% Mg	% O	% Ti
Day 1	0.00	0.00	8.13	0.00	12.95	78.92
Day 3	0.88	0.70	6.80	0.00	15.95	75.67
Day 5	4.41	3.52	5.46	0.00	16.70	69.91
Day 8	8.33	10.84	5.24	1.93	18.24	55.42
Day 11	10.02	11.10	5.01	2.90	20.36	50.62
Day 15	10.12	13.11	4.71	3.86	22.12	46.08

cells on TI24 (Fig. 7d) exhibited rather the same morphology and distribution of cells on TI6. Furthermore, cells cultured on KOK (Fig. 7e), and BSP24 (Fig. 7f) exhibited a more flattened morphology with a decrease in the round-shaped cell amount. *Elution study:* the bar graph showing elution study results is reported in Fig. 8, different materials are reported separately and results are represented for each time point as mean value and standard deviation for $n = 12$. The only important significant difference ($p = 0.0034$) was detected for cells cultured in medium previously incubated with KOK samples between day 1 and day 2, and cell activity value reached at day 2 and kept at day 3 and 7 a level comparable with activity levels detected on the other materials at every time point ($p > 0.05$).

Metabolic activity assay

The bar graph showing metabolic activity results is reported in Fig. 9, different materials are reported separately and results are represented for each time point as mean value and standard deviation for $n = 12$. At day 1 all three materials showed a metabolic activity comparable to the negative control

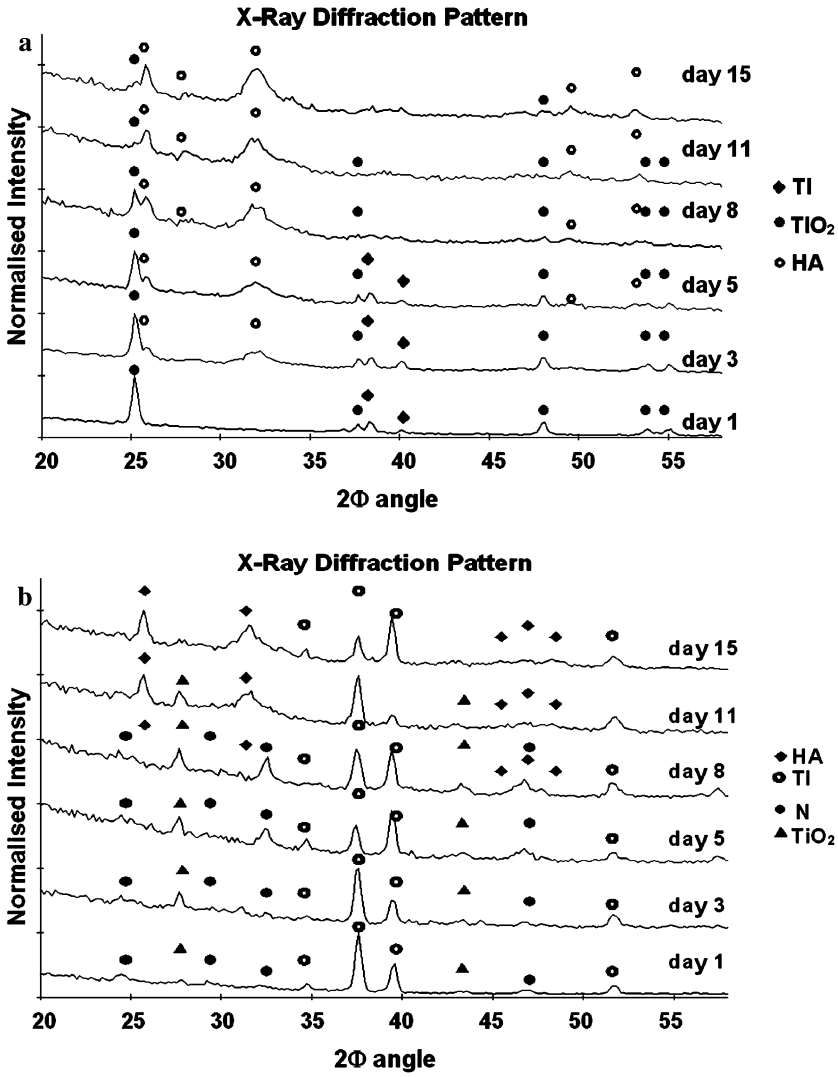

Fig. 6 TF-XRD of (**a**) BSP treated titanium after 1–3–5–8–11–15 days SBF soaking (HA = hydroxylapatite peaks, TI = alpha-titanium peaks, TiO_2 = anatase peaks); (**b**) KOK treated titanium after 1–3–5–8–11–15 days SBF soaking, (HA = hydroxylapatite paeks, TI = titanium peaks, N = sodium titanate $Na_2Ti_5O_{11}$ peaks, TiO_2 = rutile peaks)

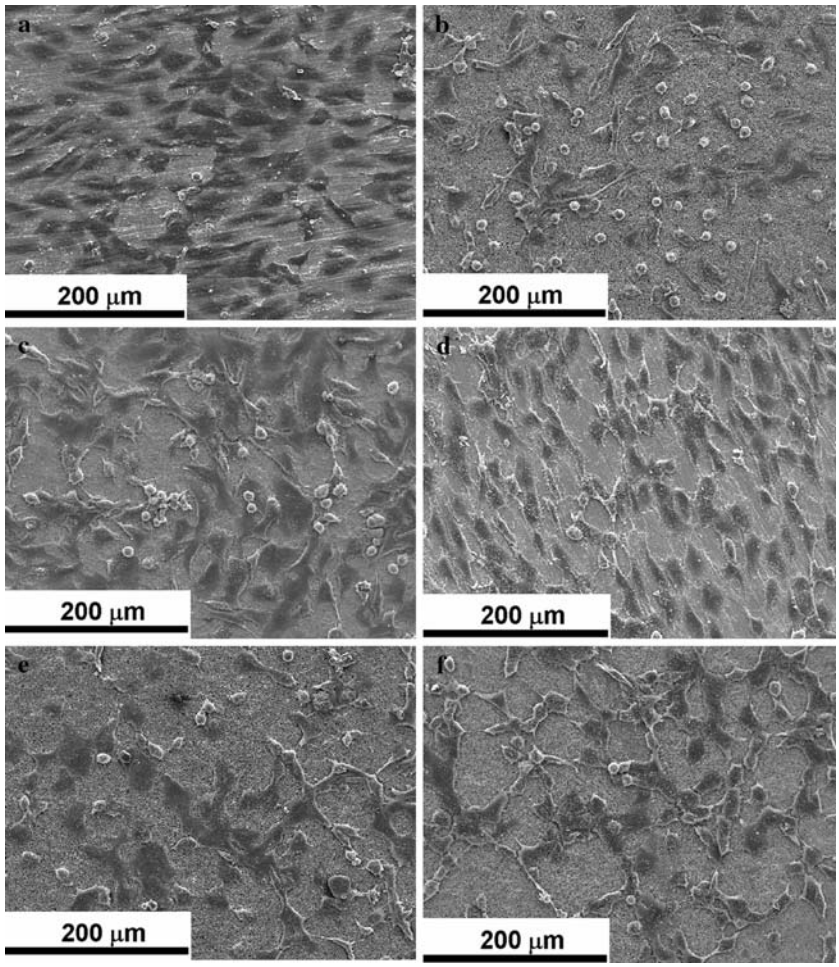

Fig. 7 SEM micrograph of MG63 cells cultured on (**a**) TI untreated titanium for 6 h; (**b**): KOK treated titanium for 6 h; (**c**): BSP treated titanium for 6 h; (**d**) TI untreated titanium for 24 h; (**e**): KOK treated titanium for 24 h; (**f**): BSP treated titanium for 24 h

($p = 0.53247$, ANOVA test) while significant differences were detected at day 2 ($p = 0.00138$, ANOVA test), day 3 ($p = 0.03198$, ANOVA test) and day 7 ($p = 0.01836$, ANOVA test). Cells cultured on BSP, KOK and TCP did not show remarkable differences in cellular activity from day 1 to day 2 ($p > 0.05$ for all three tested substrates), while cells activity reduced abruptly on TI sample from day 1 to day 2 ($p = 0.028$). Furthermore, on every single material significant differences in metabolic activity were detected from day 2 to day 3 and from day 3 to day 7 ($p < 0.05$) and MG63 cells reached the highest activity on day 3 for all the three tested materials and the TCP. At day 2, day 3 and day 7 metabolic activities of osteoblasts on TCP, on BSP and on KOK were comparable ($p < 0.05$), and cells on BSP and TCP showed significantly higher

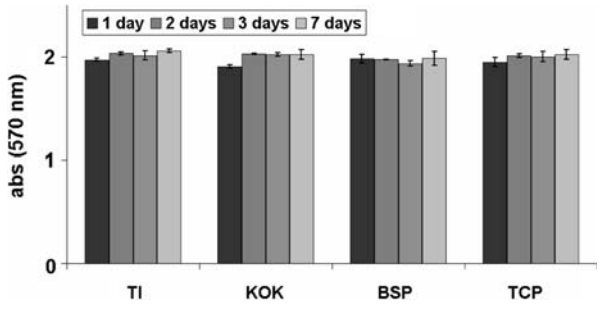

Fig. 8 Cell viability (assessed by elution study) assessed by MTT test of cell culturing in medium kept in contact with samples for different time points (1, 2, 3, 7 days)

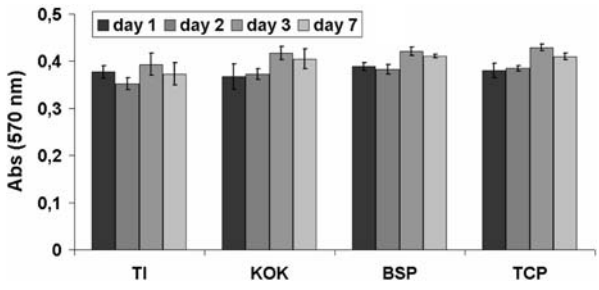

Fig. 9 Cell proliferation (assessed metabolic activity assay) assessed by Alamar Blue test (1, 2, 3, 7 days) of cell culturing on sample surface

activity levels when compared to cells on non-treated titanium TI (at day 2 BSP versus TI $p = 0.00414$, TCP versus TI $p = 6{,}54249E\text{-}4$; at day 3 BSP versus TI $p = 0.00665$, TCP versus TI $p = 0{,}01891$; and at day 7 BSP versus TI $p = 0.0112$, TCP versus TI $p = 0.01511$), while cells activity on KOK did not show significantly higher activity levels than cells cultured on TI ($p > 0.05$). Average activity levels on KOK were slightly lower than on TCP and BSP.

Discussion

The aim of the present study was to evaluate the mineralization capability and the early cell response of titanium modified with a new purposed bioactive method (BSP) [4], and to compare these new surface behaviours with pure titanium used as control and with a well-known and widely tested biomimetic treatment labelled KOK [18, 19]. As expected, pure grade 2 titanium, simply smoothed by silicon carbide abrasive papers, did not show any significant bioactivity and apatite formation after 28 days simulated body fluids incubation. Thin film XRD analysis carried out on TI did not evidence the presence of crystalline titanium oxide phases, even if titanium surface is naturally covered by a thin oxide layer, this layer is too thin to be detected by TF-XRD and still amorphous. Furthermore, titanium shows in vivo a certain kind of bioactivity which is the main reason for the good osteointegration in vivo of titanium and its alloys. Indeed, it was verified from ex vivo implants retrieval that intimate bonding between metal surface and bone tissue is achieved through a thickened oxide layer, which grew as mixed titanium oxide enriched with other elements belonging to body fluids, such as calcium, phosphorus, sodium, magnesium and silicon as well [29]. These new element oxide enrichment and thickness growing might be achieved in vitro only after long incubation time, which is necessary for titanium oxide to grow as crystalline phase and to uptake ions composing SBF and body fluids as well. The anodic spark deposition technique allows to achieve a thickened oxide layer before implantation and, at the same time it produces tailored outer ion doped oxide film by choosing the right electrolyte composition. Furthermore, apatite success as bone bonding material was related to intimate fusion between synthetic ceramic crystal and bone tissue, this mechanism is supported by the chemical composition of apatite, its reactivity in body fluids and its capability to enhance calcium and phosphate uptake from human plasma, thus promoting new apatite nucleation processes, which were reported to be necessary for supporting new bone tissue formation. Therefore, a bioactive material need to exhibit such catalytic properties. Then, the further analysis with in vitro SBF model, carried out on KOK and BSP, supported the evidence that these treated surfaces enhance Ca- and P- uptake from SBF. This surface property has been correlated to higher and swifter osseointegration in vivo by many authors [19–21]. While nucleation mechanisms of KOK surface have been largely discussed [17]. As previously published by Kokubo group a mechanism of release of sodium into solution and SBF pH changing might play an important role in calcium-phosphate nucleation from SBF, and this phenomena of Na^+ release form surface into solution exhibited its highest rate in the first 24 h soaking (Fig. 5d) and slowed down until almost stabilising after day 5. Calcium-phosphate massive nucleation followed sodium dissolution and started after day three, exhibiting a high growing rate between day 5 and day 8. This meant that a nucleation delay onto KOK surface occurred while this nucleation delay did not take place for BSP, whose nucleating capability lacked anyway of explanation. Recently, the group of Kokubo again published a paper [1], in which the role of different crystalline titanium oxide phases was investigated. It was reported that thick crystalline titanium oxide exhibits high bioactivity by enhancing hydroxyl-apatite nucleation on treated surface when soaked in SBF. This bioactive response might be due to the matching of the crystal structures of rutile (101) and of apatite (0004). Furthermore, it was previously reported by other authors that crystal structure matching could be the nuclei for crystal growth [30]. These findings may partially explain the high nucleation capability shown by BSP treated surfaces, which are covered by a thick crystalline titanium oxide layer. Furthermore, it was already published that epitaxial growth effect on anatase might to some extent play a role in the apatite deposition [31]. Moreover, BSP exhibited a Ca- and P-doped crystalline oxide layer and the effect of such doping elements might be both slightly distorting the crystal structure by favouring hydroxyl-apatite structure matching, and enhancing free charges movement, thus improving surface nuclei generation probability. This distortion enhances surface to body fluid exchange activity of the non-stoichiometric n-type semiconductor oxide (TiO_2), whose defects are mainly oxygen vacancies. Indeed, vacancies concentration is influenced by alloying elements and impurity constituents incorporated as a result of interaction with the environment or as a consequence of processing history [29]. It might be concluded that (i) anatase

crystal structure, (ii) crystal lattice distortion due to Ca and P impurity, (iii) P dopant effect in reducing band gap and promoting oxygen vacancies formation, and (iv) last but not least a high Ca/P surface ratio play a synergy in endorsing a swift nucleation process of low crystalline hydroxylapatite. This process reaches almost the same thickness and chemical composition both on KOK and BSP, anyway BSP surface was found to be covered quicker than KOK surface. A little delay in nucleation on KOK was due to the necessity of KOK to loosen Na^+ into solution, so to both provoke a pH change into SBF and free charged groups on the very surface contemporary. On the other hand, calcium-phosphate grow on BSP is strictly bound to BSP surface chemistry and physical properties. Furthermore, nucleation started immediately after SBF soaking and did not need to wait for the simulated body fluid to be modified through an ion release phenomena.

Moreover, Na^+ release into solution might play a slight effect on cultured cells, without involving toxicity, but causing a little slower attachment of HOS cells (Fig. 7b) and a little reduction of metabolic activity (Fig. 8) when HOS were cultured in medium previously kept in contact with KOK surface. Low attachment and a slight metabolic reduction in the early stages of cell contact with KOK seemed not to impair further activity, and from day 2 cell response to KOK surface was as good as on TCP and BSP (Fig. 9). Recapping, the response to osteoblast cells show suitable cellular attachment (Fig. 7a–f) and good proliferation, which is strongly related to metabolic activity (Fig. 9), towards the studied surfaces, thus enhancing the possible exploitation of these treatments for implantable materials, such as titanium and its alloys. Cell adhesion was supported by both bioactive tested materials (KOK and BSP). Osteoblasts colonised BSP and KOK surfaces with cytoplasmatic extensions communicating between cells or anchoring the cells to the sharp peaks of the surface, demonstrating typical viable healthy osteoblasts traits [31]. Furthermore, cells concentration on KOK and BSP was slightly lower than on polished titanium. Anyway, different adhesion rate and cells concentration on polished titanium was predictable, because it is well known that on polished titanium osteoblasts proliferation is increased and differentiation is lowered and, at the same time osteoblasts cells exhibit flat and elongated and well spread shape similar to the morphology seen on tissue culture plate [31]. Also proliferation test further demonstrated that, KOK and BSP exhibited almost equivalent cells response.

Conclusion

In the present study, the tests performed on novel BSP treated surface demonstrated that the treatment produce an active improved outer layer of titanium oxide that do not impair cells activity and support osteoblasts processes, as showed by comparison with KOK and titanium non treated samples. It is possible to conclude that the BSP treated titanium is an intriguing candidate for implant surface design and it clearly showed in vitro performance comparable with well known and characterised KOK treated titanium both for its remarkable catalytic activity and for early cell response, thus hopefully suggesting potential successful performance in vivo too, while avoiding high temperature thermal treatment involved in KOK process and exploiting a very easy-to-use and relatively low cost technology.

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References:

1. BANGCHENG YANG, MASAIKI UCHIDA, HYUN-MIN KIM, XINGDONG ZHANG and TADASHI KOKUBO, *Biomaterials* **25** (2004) 1003
2. MEI WEI, MASAKI UCHIDA, HYUN-MIN KIM, TADASHI KOKUBO and TAKASHI NAKAMURA, *Biomaterials* **23** (2002) 167
3. S. KANEKO, K. TSURU, S. HAYAKAWA, S. TAKEMOTO, C. OHTSUKI, T. OZAKI, H. INOUE and A. OSAKA, *Biomaterials* **22** (2001) 875
4. E. SANDRINI, R. CHIESA, G. RONDELLI, M. SANTIN and A. CIGADA, *J. Appl. Biomater. Biomechan.* **1** (2003) 33
5. R. D. BLOEBAUM and J. A. DUPONT, *J. Arthroplasty* **8** (1993) 195
6. P. BUMA and J. W. GARDENIERS, *J. Arthroplasty* **10** (1995) 389
7. X. S. ZHANG, P. A. REVELL, M. A. EVANS, M. A. TUKE and P. J. GREGSON, *J. Biomed. Mater. Res.* **46** (1999) 279
8. T. W. BAUER, R. C. GEESINK, R. ZIMMERMANN and J. T. MAMAHON, *J. Bone Joint Surg.* **73** (1991) 1439
9. T. HANAWA and M. OTA, *Biomaterials* **12** (1991) 767
10. T. HANAWA, K. ASAMI and A. ASAOKA, *J. Biomed. Mater. Res.* **40** (1998) 530
11. F. BARRERE, P. LAYROLLE, C. A. VAN BLITTERSWIJK and K. DE GROOT, *Bone* **25** (1999) 107S
12. H. M. KIM, Y. KIM, S. J. PARK, C. REY, H. LEE, M. J. GLIMCHER and J. S. KO, *Biomaterials* **21** (2000) 1129
13. O. L. FENG, F. Z. CIU, H. WANG, T. N. KIM and J. O. KIM, *J. Cryst. Growth* **210** (2000) 735
14. H. B. WEN, J. R. DE WIJN and K. DE GROOT, *J. Biomed. Mater. Res.* **41** (1998) 227
15. T. HANAWA, M. KON, H. UKAI, K. MURAKAMI, J. MIYAMOTO and K. ASAOKA, *J. Biomed. Mater. Res.* **34** (1997) 272

16. C. OHTSUKI, H. IDA, S. HAYAKAWA and A. OSAKA, *J. Biomed. Mater. Res.* **35** (1997) 39
17. T. KOKUBO, P. LI, C. OHTSUKI, K. NAKANISHI, N. SOGA and K. DE GROOT, *J. Biomed. Mater. Res.* **28** (1994) 7
18. T. KOKUBO, H. KIM, F. MIYAJI and T. NAKAMURA, *J. Biomed. Mat. Res.* **32** (1996) 409
19. T. KOKUBO, H. KIM, M. OKA, M. NAO, H. KATO, S. NISIGUSHI, T. NAKAMURA, *J. Biomed. Mater. Res.* **54** (2001) 198
20. T. KOKUBO, H. KIM, J. TAMURA, S. NISIGUSHI, T. NAKAMURA, S. FUJIBAYASHI and M. UCHIDA, *J. Biomed. Mater. Res.* **56** (2001) 562
21. FUJIBAYASHI SHUNSUKE, MASASHI NEO, HYUN-MIN KIM, TADASHI KOKUBO and TAKASHI NAKAMURA, *Biomaterials* **25** (2004) 443
22. R. CHIESA, E. SANDRINI, G. RONDELLI, M. SANTIN and A. CIGADA, *J. Appl. Biomater. Biomech.* **1**(2) (2003) 91
23. Kurze, Kryssman, Dittrich, Schneider. Process Charachteristics and Parameters of Anodic Oxidation by Spark Deposition (ANOF). *Crystal Res. Technol.* **19** (1984) 973
24. Kurze, Kryssman, Dittrich, Schneider. Structure and Properties of ANOF Layers. *Crystal Res. Technol.* **19** (1984) 93
25. E. Sandrini, R. Chiesa, G. Rondelli, M. Santin and A. Cigada. Biomimetic treatments for an improved osteointegration of orthopaedic and dental prostheses . Bachelor Thesis Politecnico di Milano 2002 ; T.D.L. 10116 : 0TN900006823
26. H. M. KIM, F. MIYAJI, T. KOKUBO and T. NAKAMURA, *J. Ceramic Soc Jpn* **105** (1997) 111
27. TADASHI KOKUBO, H. KUSHITANI, S. SAKKA, T. KITSUGI and T. YAMAMURO, *J. Biomed. Mater. Res.* **24** (1990) 721
28. C. GIORDANO, E. SANDRINI, B. DEL CURTO, E. SIGNORELLI and L. DI SILVIO, *J. Appl. Biomater. Biomech.* **2** (2004) 35
29. M. TEXTOR, C. SITTIG, V. FRAUCHIGER, S. TOSATTI and D. BRUNETTE, *Titanium Med.* **7** (2001) 171
30. M. C. BRIAM, *Nature* **260** (1976) 727
31. X. X. WANG, S. HAYAKAWA, K. TSURU and A. OSAKA, *J. Biomed. Mater. Res.* **52** (2000) 171
32. J. Y. MARTIN, Z. SCHWARTZ, T. W. HUMMERT, T. M. SCHRAUB, J. SIMPSON, J. LANKFORD, D. D. DEAN and D. L. COCHRAN, *J. Biomed. Mater. Res.* **29** (1995) 389