Controlling porous titanium/soft tissue interactions with an innovative surface chemical treatment: Responses of macrophages and fibroblasts

Controlling Porous Titanium/Soft Tissue Interactions with an innovative surface chemical treatment: Responses of macrophages and fibroblasts

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ABSTRACT:

In order to create a stable interface with the host tissue, porous implants are widely used to ensure the in-growth of the cells and the colonization of the implant. An ideal porous implant should have a 3D architecture that enables fast migration of incoming cells while not inducing a significant pro-inflammatory response by the immune cells. Moreover, in patients where the healing is impeded (patients with co-morbidities and metabolic diseases), porosity by itself is not enough for fast colonization, and the surface properties of the implant should also be controlled. In this study, we present a controlled oxidation-based surface treatment of microbead-based porous titanium implants which not only increases the colonization by connective tissue cells but also decreases the macrophage attachment. The treatment created a nanotextured surface on the implants with an acidic shift of Isoelectric point (from 4.09 to 3.09) without endangering implant's mechanical integrity. The attachment and metabolic activity of activated macrophages were significantly lower on treated surfaces with an increase in the secretion of anti-inflammatory IL-1RA and a decrease in pro-fibrotic CCL-18. Human fibroblasts proliferated faster on the treated surfaces over 14 days with near complete colonization of the whole thickness of the implant with an accompanying an increase in the secretion of TGF-beta. The surface treated samples demonstrated partial filling of the entire pores. We demonstrated that the use of nanoscale surface treatments that can be applied to the whole internal surface of porous titanium implants can significantly alter both the immune response and the colonization of the implants and can be used to fine-tune and personalized implant interfaces according to patient needs.

INTRODUCTION

Titanium is a widely used biomaterial particularly in dental implants and orthopaedic implants [1, 2]. The specific needs of these applications in general has driven the research on the optimization of the surface properties of titanium implants. For orthopaedic implants, the main concern is fast and strong osseointegration; whereas in dental implants the situation is more complicated in which the implant needs to integrate with the jaw but at the same time establish a tight barrier with the gingiva to avoid peri-implantitis.

Traditionally, different surface treatments of titanium and its alloys are used on devices in contact with hard (bone) or soft tissues (gum, skin, muscles, epithelium) based on the consideration that osteoblasts are rough-philic (higher adhesion on rough surfaces) while fibroblasts are rough-phobic (higher adhesion on smooth surfaces) when roughness on microscale is considered.

For the contact with bone tissue, treatments inducing roughness on microscale are widely used and clinically proven to improve osseointegration. Micro-topographies with size ranges on the same magnitude with cellular dimensions promote the formation of focal adhesion points by osteoblasts and bone deposition [3]. In order to obtain a good compromise between osteoblast adhesion/proliferation and implant-bone interlocking, avoiding increasing ions release, pro-inflammatory response and to introduce any element that would weaken the structure of the implant (lower fatigue resistance), the size of roughness should be around 0.2-2 micron [4, 5]. A large number of treatments were tried and developed over years, such as grit blasting, sand blasting, acid etching, anodization and titanium plasma spray (porous coatings) [4]. Nanoscale roughness on the same scale with integrins and protein dimensions can eventually further stimulate cell adhesion and two treatments can be used in combination in order to couple micro and nanoroughness (eg. sand blasting + chemical etching) [6-8].

On the other side, usually very smooth surfaces are used for the contact with soft tissues according to preference of fibroblasts and endothelial cells for surfaces with low roughness; smooth surfaces are also less prone to bacterial colonization as it can occur through soft tissues. Recently, research is moving behind this simplistic approach, but no clinical experimental result is available up to now. For instance, introduction of a specific surface topography acting through contact guidance on fibroblasts orientation has been suggested and it is under further investigation [9].

All these considerations are based on bulk implants where the body of the implant does not have any porosity. Recent advances in additive manufacturing and other techniques enabled the production of titanium structures with high interconnected porosity which can have applications beyond the conventional uses of titanium. In most of the additive manufacturing techniques, titanium powders are utilized with selective laser sintering or electron beam melting or with similar techniques. Another method to obtain porous implants is to use titanium particles of bigger sizes (microbeads of 100 µm up to 500 µm) and to sinter them into stable structures. This way, the problems stated above related to high microscale roughness can be avoided. One application of such porous structures was laryngeal replacement (an artificial larynx system) were porous titanium structures based on size-controlled microbeads were used clinically as a tissue connector with trachea [10, 11]. The long-term follow-up these implants has shown a fibrovascular tissue in-growth to the implants, where the titanium microbeads provide a continuous surface for incoming cell attachment [12]. How the surface treatment of such 3D architectures based on titanium would affect the in-growth of soft tissues has not been elucidated yet.

The rationale used in this research is that, if we move from microscale to nanoscale roughness, the same topography can have positive effects on different type of cells, that means that the same surface treatment can be effective in contact with different tissues. M.J. Dalby et al. demonstrate that different type of cells (endothelial cells, fibroblasts, osteoblasts, leucocytes and platelets) strongly respond to nano-islands (13 nm tall), increasing spreading and proliferation [13]. In a further study, they observed there was an interaction between a surface with nanofeatures 10 nm high and fibroblast filopodia [14]. The upper limit to roughness in order to positively stimulate fibroblasts, could be around 100 nm: in fact, a decrease of cell adhesion and spread of fibroblasts was registered on polymer demixed nanocolumns 160 nm high and 100 nm in diameter [15, 16]. Surface nanotopography can be also effective in polarization of macrophages [17]. Thus, for controlling the behavior of incoming cells (first the immune cells and then fibroblasts) over a porous titanium implant, the nanoscale surface features must be taken into account.

Concerning surface chemistry, it is reported that surfaces with high hydrophilicity (such as in the case of high density of hydroxyl functional groups), are able to induce fast adhesion, spreading and organization of cytoskeleton of fibroblasts, production of collagen fibers and formation of well-vascularized connective tissue [18, 19]. Roughness on nanoscale is also advantageous concerning the risk of infection: in order to avoid bacterial contamination, it would be better not overcome the threshold of 0.2 μm Ra [20].

The treatment used in this work was originally developed and tested for bone contact applications [14, 15]. However, we have previously demonstrated that, in 3D porous implant conditions treatments that are generally reserved for osseointegration (such as anodization and acid etching) can be effective in soft tissue context also both in vitro and in vivo [21]. In this context, the responses of fibroblasts and macrophages are particularly relevant as macrophages are instrumental to the overall reaction to an implant, be it a chronic inflammation or healing and fibroblasts establish the initial granulation tissue to be remodeled and replaced by the local tissue type in the best-case scenario. The treatment used consists in a surface chemical treatment: etching in diluted hydrofluoric acid, followed by controlled oxidation in hydrogen peroxide [22]. The obtained surface shows a specific nanotopography overlapped to roughness on the sub-micron scale; it has been shown that it does not increment, even slightly reduced, biofilm formation [16]. Moreover, the treated surface exposes high density of hydroxyls groups.

In this study, we demonstrated the positive effect of a nanoscale coating on 3D porous titanium surfaces based on titanium microbeads as a potential soft tissue connector for laryngeal replacement. The response of human fibroblasts and human macrophage cell lines were studied for determining the potential of the coating in decreasing inflammatory reaction to titanium implants and increase fibroblast migration and extracellular matrix formation via induction of cytokine/growth factor secretion.

MATERIALS AND METHODS

MATERIALS

Pure medical grade titanium microbeads (500µm diameter) were purchased from Nyco SA (Paris, France). Human monocytic cell line (THP-1, ATCC® TIB-202), human fibroblasts (BJ2, ATCC® CRL-2522) and EMEM media (ATCC®-30-2003) were obtained from ATCC (Manassas, US). RPMI-1640, Dulbecco's phosphate buffered saline, fetal bovine serum (FBS), 0.05% trypsin/0.02% EDTA, Phalloidin (Alexa Fluor 568 phalloidin) and β-mercaptoethanol were purchased from Life Technologies (Carlsbad, USA). Phorbol Myristate Acetate (PMA, P-1585), TritonTM X-100 (T8787) were provided by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Resazurin biochemical assay (Fluorimetric cell viability kit I, PK-CA707-30025-0), DAPI (PK-CA707-40043) were obtained from Promokine (Heidelberg, Germany). For Elisa assays, kits were provided by the following companies: TGF-beta (R&D Systems DY240), CCL-18 (R&D Systems DY394); and IL-1RA (PeproTech 900-K474), TNF-alpha (Peprotech 900-K25).

Titanium Implant preparation

Titanium microbeads are placed in molds and porous 3D structures are obtained under high voltage electrical charge with subsequent sintering steps as described before for obtaining cylindrical structures with 1 mm thickness and 14 mm diameter [23].

Samples Surface treatments

The samples were washed in acetone for 5 minutes and 10 minutes in ultrapure water for two times under sonication in order to remove possible debris due to sintering process and surface contamination from samples handling. The samples were then dried at room temperature. As a final step, the samples were surface treated with a patented chemical treatment which consists in an acid etching followed by controlled oxidation [22, 24, 25].

Characterization of surface topography

Field Emission Scanning Electron Microscopy (FESEM - SUPRATM 40, Zeiss) was used in order to investigate the surface morphology of the samples before and after the chemical treatment and to check the maintenance of the spherical shape of the titanium beads and preservation of the sintering necks.

Chemical characterization

X-Ray Photoelectron Spectroscopy (XPS, PHI 5000 Versa Probe, Physical Electronics, MN, USA) measurements were performed (survey spectra and high-resolution analyses of carbon and oxygen region) on the samples before and after the treatment in order to investigate the presence of hydroxyl groups exposed by the surface and to check the presence of surface contaminants.

Electrokinetic measurements

Before and after the chemical treatment, electrokinetic measurements of z potential were performed on the samples with an electrokinetic analyzer (SurPASS, Anton Paar). A couple of samples was inserted in the adjustable gap cell and the z potential of the surfaces was investigated in function of pH in an electrolyte solution (0.001M KCl). During titration, pH was changed with the addition of 0.05 M NaOH or of 0.05 M HCl solutions by the automatic titration unit of the instrument following the standard procedure [26].

Cell lines

THP-1 cells were cultured in RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin, 0,05mM β-mercaptoethanol and 0,2% fungizone. THP-1 cells were differentiated from monocytes to macrophages using phorbol myristate acetate (PMA) treatment. Briefly, cells were treated with 50ng of PMA dissolved in RPMI medium without β-mercaptoethanol for 24 hours at 37°C, 5% CO_2. Non-adherent cells were then removed with

DPBS and activated THP-1 cells (macrophages) were collected after trypsin treatment, centrifugation and resuspension in RPMI medium.

BJ2 cells were cultured in EMEM medium supplemented with 10% FBS, 1% penicillin/streptomycin and 0,2% fungizone. Then cells were collected after trypsin treatment, centrifugation and resuspension in EMEM medium.

Cell seeding on porous titanium disks

Prior seeding, porous titanium disks were sterilized for 24 hours using dry heat sterilizer and then put in 24 well plate. Then both THP-1 and BJ2 cells were seeded on top of porous titanium disks in microbiological safety cabinets at the same density (2.5 *105cells in 60µL of medium). So 60µL of cell solution was dropped on top of porous titanium disk and then samples were put in incubator (37°C, 5% CO_2) for 15 minutes before adding 1mL of cell culture medium (EMEM for BJ2 and RPMI for THP-1 cells). Cell culture experiments were performed for 14 days with a follow up of metabolic activity and cytokines secretions in supernatant at different time points.

Metabolic Activity

Metabolic activity was assessed at different time points of cell culture: days 1, 3, 7, 10 and 14. To do that, samples were incubated in10 %v/v resazurin solution (prepared in EMEM for BJ2 and RPMI for THP-1) for 2 hours in incubator (37°C, 5% CO_2). This assay is based on the reduction of resazurin to fluorescent resofurin when incubated with viable cells. Fluorescence intensity was monitored using a spectrofluorimeter (SAFAS Xenius XML, Monaco) with the following emission/excitation wavelengths (560nm/590nm).

Cytokines secretions using ELISA kits

Cell culture media were collected at days 7 and 14 and cytokines were quantified by Elisa kit. Absorbance measurements were performed at 450nm and the amount of cytokines was calculated using a standard curve. For THP-1 cells, the following cytokines were quantified: TNF-alpha (pro-inflammatory), CCL-18 and IL-1RA (anti-inflammatory). For BJ2 cells, only TGF-beta was quantified.

DAPI/Phaloidin cell staining

Before staining, cells were first fixed with a 3,7 % v/v paraformaldehyde solution in PBS for 15 minutes washed three times with PBS and then incubated in a Triton X solution (0.1% in PBS) for 5min. After that, samples were incubated with bovine serum albumin solution (1% v/v) in PBS for 20 minutes. For both THP-1 and BJ2 experiments, F-actin filaments from cytoskeleton were labeled with phalloidin. Samples were incubated for 1 hour with phalloidin solution at a dilution of 1/40 in PBS and then two rinsing steps of 5 minutes in PBS were performed. Finally, nuclei were stained by incubating samples in DAPI (1mg/mL) at a dilution of 1/100 in PBS and two rinsing steps in PBS were performed.

Microscopy characterization

Fluorescent images were acquired using confocal microscope (Zeiss LSM 710, Germany) and processed using ImageJ software. Excitations/Excitations wavelengths were 568/600 nm for phalloidin and 358/461 nm for DAPI.

For scanning electron microscopy (SEM), the samples were fixed with 4% glutaraldehyde. The specimens were washed with DPBS, prior to a dehydration protocol using an alcohol series of increasing concentrations (70, 95, and 2 × 100%), with incubation periods of 5 min each.

Subsequently, samples were incubated in 100% ethanol/hexamethyldisilazane (HMDS) (1:1) for 5 min, then only in HMDS for 2 × 5 min and were dried overnight. Samples were made to adhere onto titanium discs using a carbon tape and were coated with gold/palladium in a sputter coater. The samples were sputtered at 7.5 mA for 3 min under argon atmosphere. Analysis with SEM was performed with a Quanta 400 ESEM (FEI Company, Eindhoven, the Netherlands) at an accelerating voltage of 10 kV.

Statistical analysis

The statistical significance of the obtained data was assessed using the t-test, or Mann–Whitney tests ($n \geq 3$). The error bars were representative of standard deviation (SD). Differences at $p \geq 0.05$ were considered statistically insignificant.

RESULTS

Characterization of the porous sintered structure and surface topography

Since the chemical treatment involves an acid etching, as first, it was checked if any damage was introduced by the treatment into the sintered porous structure. In Figure 1, the FESEM images of the samples before and after the treatment at different magnifications are reported.

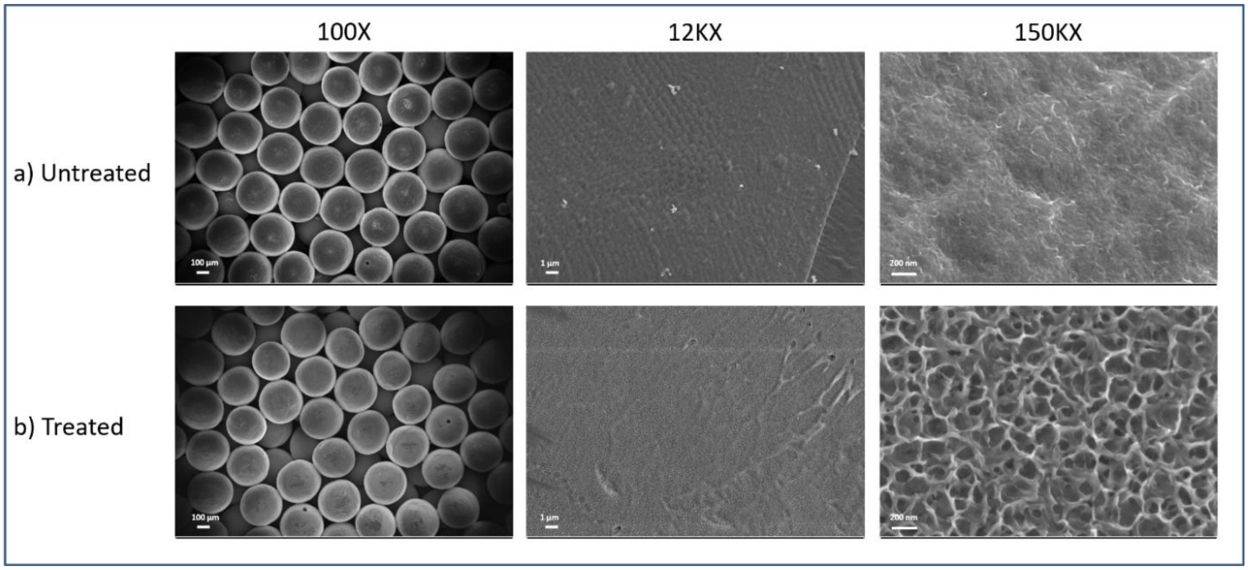

Figure 1: FESEM Images of the samples a) Untreated, b) Treated at 100X, 12000X and 150000X magnifications.

The treatment was applied without compromising the integrity of the sintered structures and also without deforming the shape of the beads (no damages can be evidenced nor in the spheres nor in the sintering necks upon observation of numerous areas). As second, the aim of the treatment is to add a surface oxide layer with topography on the nanoscale onto the surface of the beads. Pictures at high magnification in Figure 1b (treated samples) show that the treatment was able to create on the surface a nanotextured sponge-like oxide structure.

Surface chemical characterization

The effects of the surface treatment in terms of surface chemical composition were investigated by XPS analysis. In Table 1, the atomic surface composition obtained by the XPS survey spectra is reported.

Table 1: XPS atomic composition of the surfaces of the samples

	Sample	
Elements [at%]	**Untreated**	**Treated**
O	42.6	50
C	42.4	32.4
Ti	14.4	16.2
Others	0.7	1.4

Only titanium and oxygen can be detected on the outermost surface layer of both surfaces, despite of a certain amount of carbon due to surface contaminants, as usual on titanium based materials [27, 28]. In Figure 2, the high resolution XPS spectra of the oxygen region are reported.

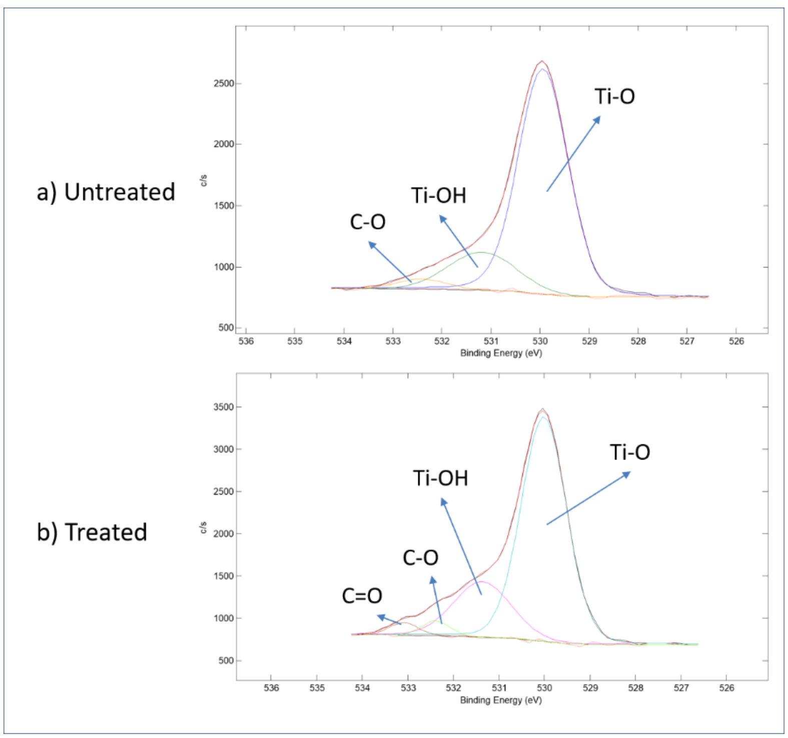

Figure 2: High resolution XPS spectra of the oxygen region of the samples a) Untreated, b) Treated.

The untreated sample showed three peaks (at 529.94, 531.18 and 532.40 eV) which can be correlated with Ti-O, Ti-OH and C-O groups respectively. The treated sample showed four peaks (at 530.01, 531.37, 532.38 and 533.09 eV) which can be attributed to Ti-O, Ti-OH, C-O and C-O groups respectively [22, 28]. The signal attributed to OH groups is higher on the treated sample, consequently it can be deduced, that the treatment induces the formation of a surface oxide layer with high degree of hydroxylation.

Electrokinetic measurements

In Figure 3, the z potential titration curves vs pH of the treated and untreated samples is reported. This characterization gives several information, the focus of the present paper will be the IsoElectric Point (IEP) and presence of functional surface groups with acidic/basic behavior on the surfaces.

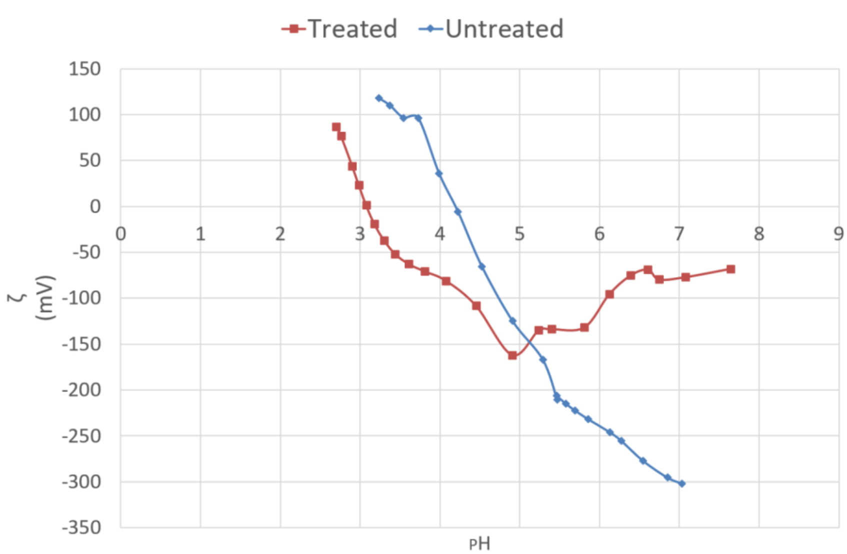

Figure 3: Z potential titration curves vs pH of treated and untreated samples.

The isoelectric point of the untreated sample is 4.19 while after the treatment it is 3.09. This can be associated with acidic functional groups introduced on the surface oxide layer by the treatment. Moreover, a plateau (with onset at pH 5) can be observed on the curve related to treated samples and not on the curve of untreated one. The presence of this plateau can be also correlated with surface functional groups with acidic behavior.

Macrophage response to the treated porous titanium surfaces

Once an implant is put in place, following the clearance of bacterial contamination by neutrophils, monocytes from the blood arrive on the implant surface and differentiate into macrophages. These macrophages, as a function of the implant microenvironment including the implant surface properties, will secrete pro- and/or anti-inflammatory cytokines/chemokines in order to recruit both other immune cells, endothelial cells and fibroblasts. In order to mimic the incoming monocyte reaction, treated and untreated samples were seeded with THP-1 monocytes and the presence of the monocytes on the top and the bottom of the implants were determined by confocal microscopy and SEM (Figure 4 and 5). In confocal images, the number of cells infiltrated through the implant material to the bottom of the implant was significantly higher in the case of non-treated implants on day 1 and by day 7 the number of the attached macrophages on the surface of the treated implants was significantly lower. This behavior was even more obvious in SEM images, where after 7 days the macrophages on the implant surface was scarce for the treated surfaces (Figure 5).

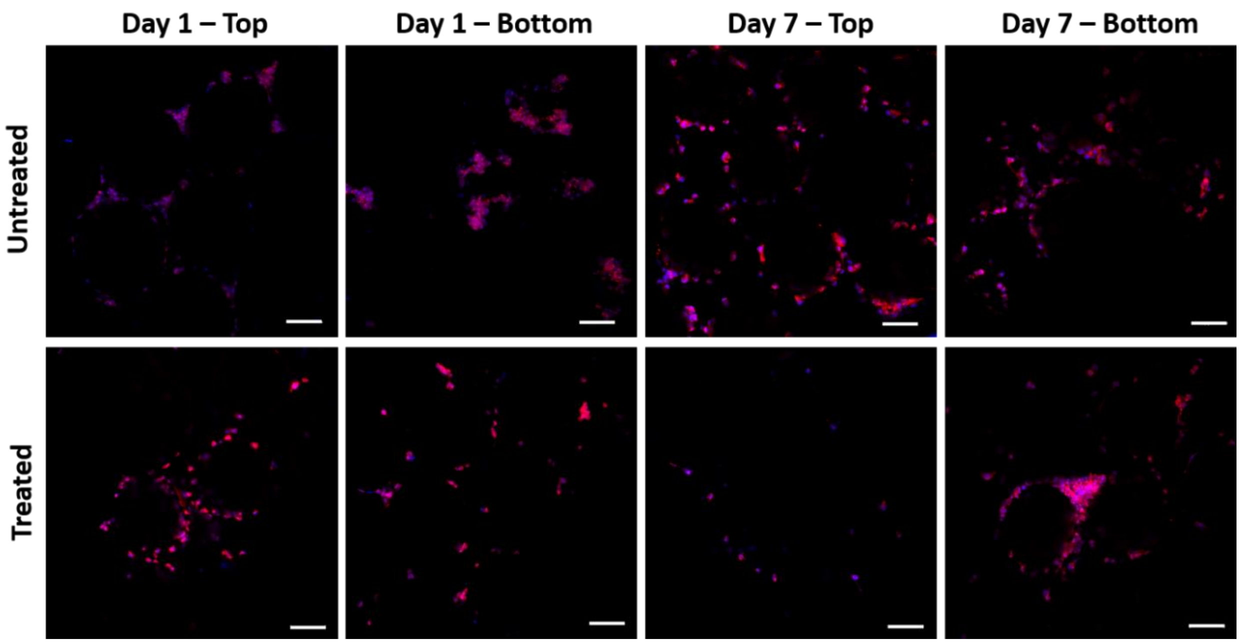

Figure 4. Confocal pictures of DAPI/Phalloidin (F-Actin) staining of activated THP-1 cells in contact with porous titanium with (Treated) and without (Untretated) treatment at different time point of cell culture (scale bar = 100µm).

By day 7, the coverage of the implant surface and also cluster formation was more evident on the untreated samples. Also, the behavior of the adhered macrophages on the surface was significantly different, where on the untreated surfaces after 14 days, well spread macrophages can be observed, on the treated samples the macrophages stayed mostly spherical (Figure S1).

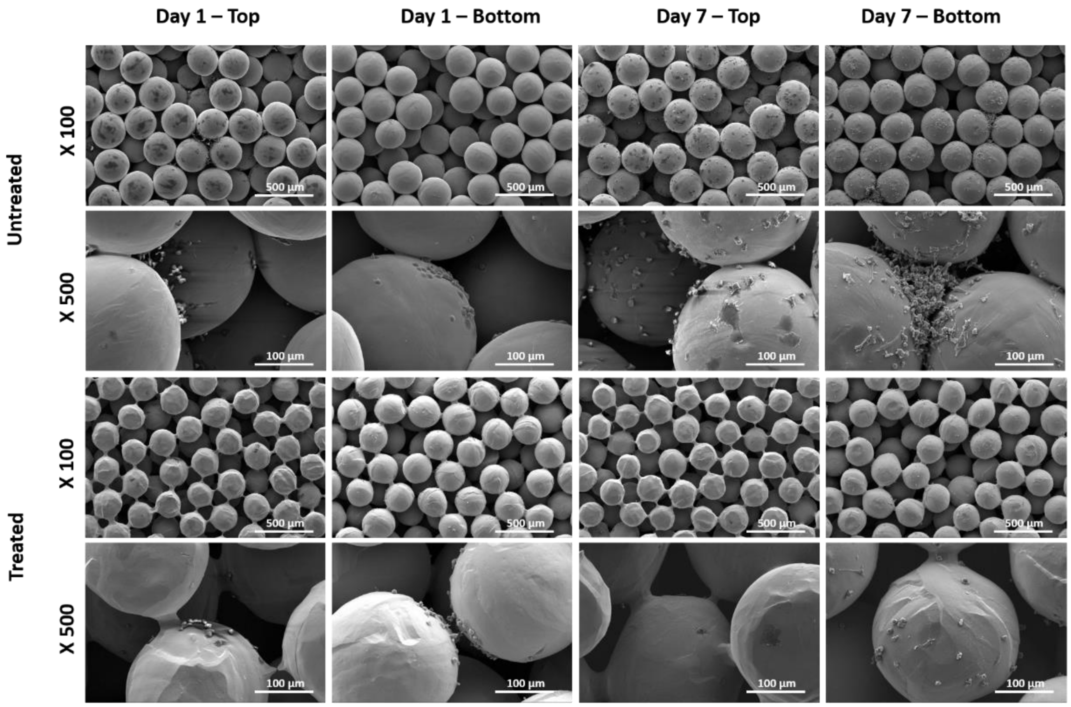

Figure 5. SEM pictures of activated THP-1 cells in contact with porous titanium with (Treated) and without (Untreated) treatment at different time point of cell culture.

The visual results were well-correlated with the biochemical tests where there was significantly more metabolic activity of macrophages on the untreated samples over the course of a 14-day culture period (Figure 6a). Over the panel of 8 cytokines/chemokines, for 3 there was detectable secretion. TNF-α, a potent pro-inflammatory cytokine was secreted at low amounts and at similar levels for both treated and untreated samples. Whereas for IL1-RA, an anti-inflammatory cytokine generally used as a marker for M2 macrophages, the secretion was significantly higher on the treated samples; even though the cell numbers are significantly lower (Figure 6b). CCL-18, a cytokine with pro- and anti-inflammatory properties, but actively involved in fibrous capsule formation (foreign body response) was also secreted less by macrophages on the treated samples; although the overall secretion levels are low.

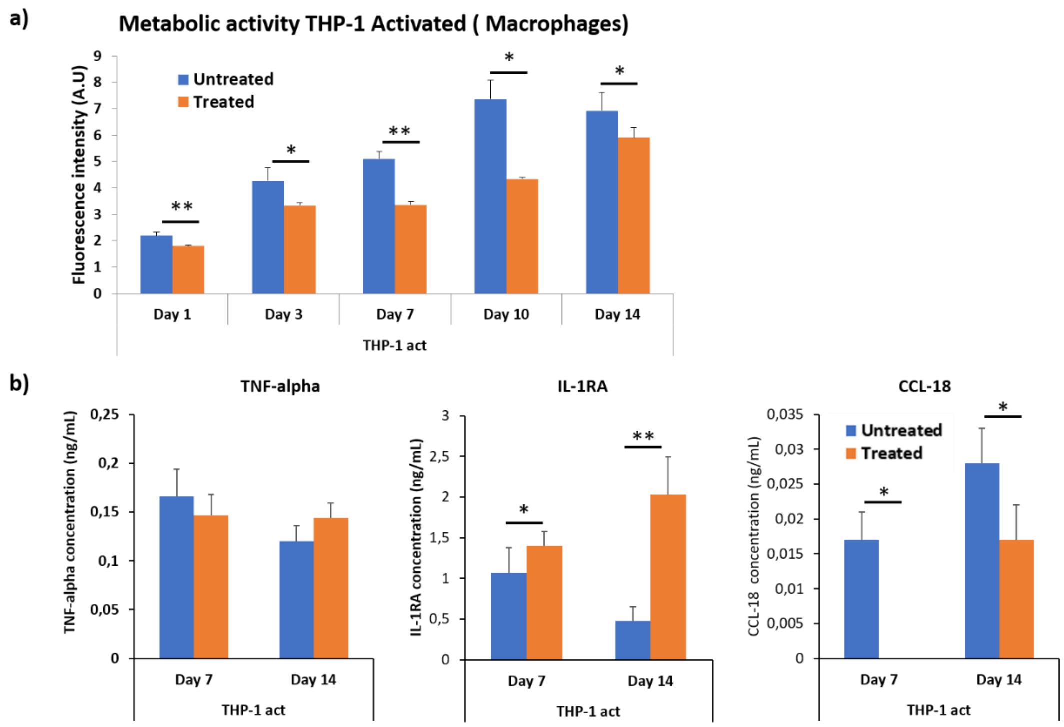

Figure 6. a) Follow-up of metabolic activity of activated THP-1 cells for 14 days (macrophages) in contact with porous titanium with (Treated) and without (Untreated) treatment. b) Quantification of pro-inflammatory (TNF-alpha) and anti-inflammatory (IL-1RA) and pro/anti-inflammatory (fibrotic) (CCL-18) markers secreted by activated THP-1 cells in the supernatant at different time point of cell culture in the different conditions (Treated vs Untreated) (n=3, * $p<0,05$, ** $p<0,001$).

Fibroblast response to the treated porous titanium surfaces

After the initial inflammatory response, following implantation, triggered by the immune system, implants need to be colonized by soft tissue cells such as fibroblast to be fully integrated by the host. To check the effect of the surface treatment on soft tissue ingrowth, fibroblast colonization and growth on titanium microbeads treated and untreated was studied using human fibroblast (BJ2 cells). Human fibroblasts were seeded and the presence and the organization of the cytoskeleton (F-Actin Filaments) of the cells on the top and the bottom of the implant were determined by confocal microscope (Figure 7a). After 14 days of experiment, a significant higher number of cells was observed in the case of the treated samples both at the top and the bottom of the implants. This means that the treated surface promotes both fibroblasts adhesion and migration within the implants. The F-Actin filament staining also showed a denser tissue like structure at the surface of the treated samples with a better spreading of the cells demonstrating that this surface is a better substrate for fibroblast. After 14 days of experiment, samples were also visualized with SEM (Figure 7b) and a significant higher number of fibroblasts were also observed at the surface of the treated sample. On the treated samples, a significant higher collagen secretion was noticed on the treated sample. On the untreated sample, no collagen secretion was observed. This demonstrates that fibroblast on top the treated sample are able to secrete ECM component which is in favor of better implant integration with newly formed tissue.

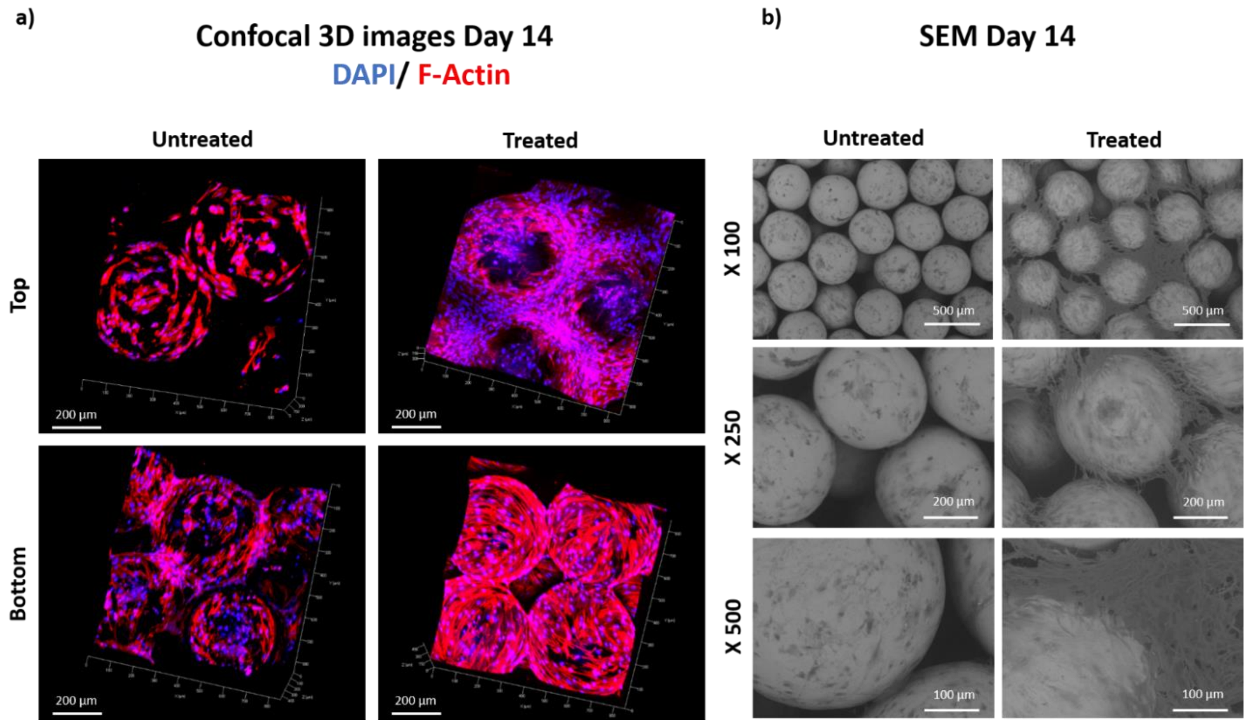

Figure 7. a) Confocal pictures of DAPI/Phalloidin (F-Actin) staining and b) SEM pictures of BJ cells (human fibroblasts) in contact with porous titanium with (Treated) and without (Untreated) treatment after 14 days of cell culture.

These visual results in term of better ingrowth and colonization were confirmed with biochemical tests where a significant higher metabolic activity of fibroblasts was found on the treated sample after 14 days of culture (Figure 8a). TGF-beta secretion in supernatant, a growth factor implicated in would healing which is a promotor of fibroblast proliferation and ECM secretion was also monitored after 7 and 14 days of culture [29-31]. The level of TGF-beta was significantly higher after both 7 and 14 days of culture for the treated samples (Figure 8b) which confirms the SEM results showing a higher collagen secretion in this condition.

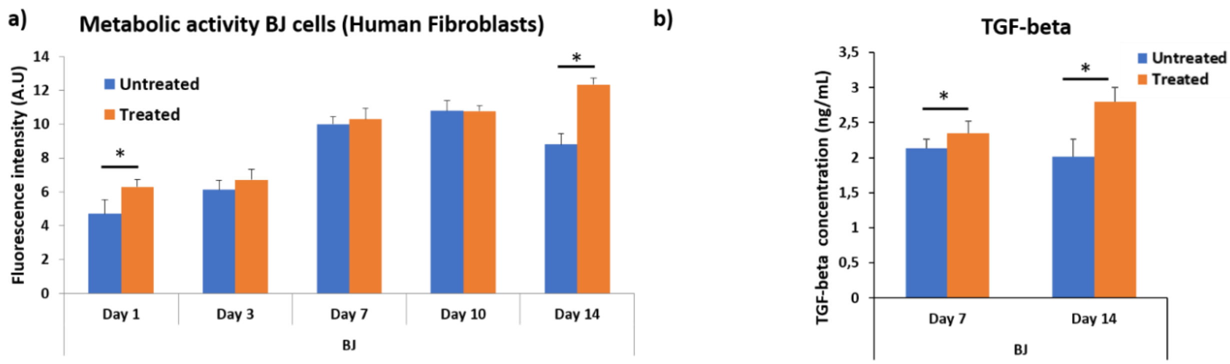

Figure 8. a) Follow-up of metabolic activity of BJ cells for 14 days (human fibroblasts) in contact with porous titanium with (Treated) and without (Untreated) treatment and b) Quantification of TGF-beta secreted by activated THP-1 cells in the supernatant at different time point of cell culture in the different conditions (Treated vs Untreated) (n=3, * $p<0,05$, ** $p<0,001$).

DISCUSSION

The FESEM images reported in Figure 1 evidence that no alteration is introduced by the treatment at the macro and micro scale, in fact shape and dimension of the microbeads and the sintering necks are unaltered as well as surface topography at the micro scale. On the other hand, a well-developed sponge-like nanotexture completely covers the surface of the treated beads at higher magnification. These results are important because they confirm that the treatment allows to preserve the structure of the sintered structures and to create on the surface of the beads a sponge-like oxide structure analogously to what observed in previous works on flat surfaces and on dental screws [22, 25, 32].

XPS survey data show an increase of the oxygen atomic percentage from 42.6% to 50.0% after the chemical treatment which is due to the formation of an oxide layer thicker than the native one. Carbon surface contamination are detected on both the surfaces as usually on titanium based materials [15], [20], [21]. The C=O groups detected on the surface after the treatment can be ascribed to higher reactivity of the treated surface. XPS high resolution spectra of the oxygen region evidence the increase in the signal attributed to hydroxyl groups, in accordance with previous observation of the authors [22, 32].

The isoelectric point of the untreated surface is close to 4, in accordance with values reported in the literature for titanium [33, 34]. Similar values are typical for a surface with no strong acidic/basic surface functional groups (or with a balance between them) [26]. Zeta potential measurements evidence an acidic shift of the isoelectric point after the treatment (from 4.19 to 3.09). This means that acidic functional groups are introduced on the surface oxide layer by the treatment: according to XPS data, they can be connected to hydroxyl groups. The presence of acidic functional groups is confirmed by the appearance of a plateau in the basic region for the treated sample, in fact the onset of a plateau at pH 5 gives the information that they act like a strong acid and they are completely deprotonated at pH values higher than 5. At this pH, the surface reaches an equilibrium with a coverage of negative charges due to the deprotonated acidic groups (O-) and even if the concentration of hydroxyl groups in the solution is further increased (by increasing pH), no more hydroxyl groups are adsorbed on the surface from the solution.

On the other hand, no plateau is observed on the titration curve of the untreated surface and a progressive increasing/lowering of zeta potential can be observed by decreasing/increasing pH in the solution: it can be ascribed to a progressive adsorption of hydronium/hydroxyl groups from the solution with the decreasing/increasing of pH.

Pro-inflammatory Macrophage response to treated surfaces is attenuated

Macrophages adhere to the surface of foreign bodies in their double function as phagocytotic cells (the clearance of the foreign material) and also as the modulators of innate immune response. For degradable materials, they also act as antigen-presenting cells for the adaptive immunity; for structures such as titanium, where the arsenal of macrophages (enzymes, ROS, NO, phagocytosis) is not adequate for their removal, their inability to remove the foreign body leads to a stage described as frustrated phagocytosis which would create a chronic inflammatory environment with continuous secretion of pro-inflammatory cytokines and ROS with significant collateral damage to the surrounding tissues. Thus, for nondegradable implants, attracting a lesser number of macrophages can be considered advantageous. In our system, the surface treatment has resulted in a surface where attachment of macrophages and their migration was significantly less. For secretion, the rough, treated titanium surfaces (such as acid-etched surfaces) have been shown to induce secretion of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 [35] even though they have been demonstrated to be better for osteoblast attachment also. An initial decrease on the adhered macrophages with a pro-inflammatory profile will have downstream regulatory effect on the additional immune cell recruitment, thus facilitating the resolution of inflammation. Recently, it was shown that the induction of anti-inflammatory macrophage phenotype happens in a narrow range of surface roughness [36] and also aided by hydrophilization of the titanium surface [37], which were the effects of the current surface treatment presented.

Soft tissue ingrowth and colonization on treated surfaces is promoted

Soft tissue cells adhesion, colonization and ingrowth at the surface of medical implants is a key factor to promote implant integration and new tissue formation around it. So, implant surface treatment has the ultimate goal of promoting these events in order to ensure the implant integration and consequently prevent implant failure. In our context, soft tissue cells were represented by human fibroblasts as in the prior in vivo and clinical use of microbead-based porous titanium structures we observed a fibroconnective tissue formation between the beads which is both microbead size and surface property dependent. Our data demonstrated that the controlled oxidation of the porous implant surface resulted in better in-growth, colonization and ECM secretion on the treated titanium samples. These results can be explained by the change of surface chemistry after treatment and more specifically by the introduction of functional acidic group (COOH) at the surface of the titanium microbeads together with nanotexturation. In previous studies, it was shown that cells exhibit higher adhesion and spreading in relation to the increase of surface concentration of acidic functional group (COOH) and these findings are in accordance with our data [38, 39]. The positive effect of such surface treatment for soft tissue in-growth stems from the fact that the initial higher adhesion and spreading on the inner architecture of the porous titanium provides an exponential effect on the secretion of ECM molecules and aids the filling of the pore structures much faster than untreated titanium structures.

CONCLUSION

Nanoscale surface treatment of 3D microporous structures for implantable device purposes should ensure an improved cellular in-growth of the functional cell types of the surrounding tissue while limiting the initial immune reaction to the implanted material to ensure that the inflammation resolution is not prolonged. Herein, we presented a controlled oxidation based modification of medical grade porous titanium structures which resulted in limiting of the attachment of macrophages while significantly improving the population of the implant by fibroblasts. Such additional surface treatments of porous structures can aid in facilitation of the integration of the implant internal volume thus improving the functionality of the implants while decreasing the potential complications, such as loosening and bacterial colonization due to limited penetration.

Supporting Information. Figure S1. SEM pictures of activated THP-1 cells in contact with porous titanium with (Treated) and without (Ctrl) treatment after 14 days of cell culture. (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. # These authors contributed equally.

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ABBREVIATIONS

TNF-α, Tumor Necrosis Factor Alpha; CCL-18 CC chemokine receptor 18; IL-1RA Interleukin 1 receptor antagonist; TGF-β Transforming Growth Factor Beta.

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SYNOPSIS

Controlled Oxidation of Porous Microbead based Titanium implants result in more control over immune response and increased in-growth by connective tissue cells.