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Article

Engineering a Hybrid Ti6Al4V-Based System for Responsive and Consistent Osteogenesis

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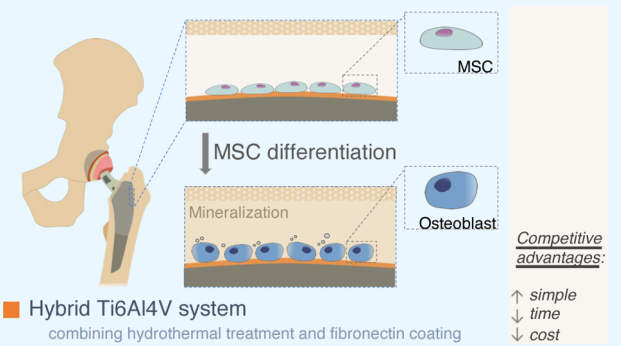
ABSTRACT: As the aging population increases worldwide, the incidence of musculoskeletal diseases and the need for orthopedic implants also arise. One of the most desirable goals in orthopedic reconstructive therapies is de novo bone formation. Yet, reproducible, long-lasting, and cost-effective strategies for implants that strongly induce osteogenesis are still in need. Nanoengineered titanium substrates (and their alloys) are among the most used materials in orthopedic implants. Although having high biocompatibility, titanium alloys hold a low bioactivity profile. The osteogenic capacity and osseointegration of Ti-based implantable systems are limited, as they critically depend on the body−substrate interactions defined by blood proteins adsorbed into implant surfaces that ultimately lead to the recruitment, proliferation, and differentiation of mesenchymal stem cells (MSCs) to comply bone formation and regeneration. In this work, a hybrid Ti6Al4V system combining micro- and nanoscale modifications induced by hydrothermal treatment followed by functionalization with a bioactive compound (fibronectin derived from human plasma) is proposed, aiming for bioactivity improvement. An evaluation of the biological activity and cellular responses in vitro with respect to bone regeneration indicated that the integration of morphological and chemical modifications into Ti6Al4V surfaces induces the osteogenic differentiation of MSCs to improve bone regeneration by an enhancement of mineral matrix formation that accelerates the osseointegration process. Overall, this hybrid system has numerous competitive advantages over more complex treatments, including reproducibility, low production cost, and potential for improved long-term maintenance of the implant.



1. INTRODUCTION

Osteoarthritis (OA) is a heterogeneous disorder that, according to the Osteoarthritis Research Society International, "manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation, and loss of normal joint function) that can culminate in illness".[1] It is estimated to affect more than 240 million persons worldwide and more than 32 million only in the US.[2] Obesity, prior joint injuries, and genetics are the main risk factors for OA, whose dominant symptom is pain. Radiographs of patients with advanced osteoarthritis typically reveal osteophytes and narrowed joint spaces due to cartilage loss. When physical rehabilitation and pharmacological treatments are not sufficient to relieve pain, joint replacement surgery is recommended.[2] Despite advancements in orthopedic implant research and the fact that total joint replacement is considered a clinically relevant and cost-effective treatment for end-stage OA, implant failures still occur. Up to 25% of patients report ongoing pain and disability 1 year following surgery,[3] and even when the results are satisfactory, the success rate tends to decrease a decade after surgery, leading to revision surgeries and implant replacement.[4]

Endosseous implants are made of commercially pure titanium (Ti) or titanium alloys, such as the titanium 6-aluminum 4-vanadium (Ti6Al4V) alloy, whose implantation success and stability are multifactorial-dependent. The patient's bone quality, the selected surgical protocol, and the properties of the implant, such as its surface and geometry determined by the manufacturer, affect the clinical outcome. Considering the implant's environment, its stability is determined by the biomechanical properties of the bone−implant interface.[5] Nowadays, cementless implants are preferred and inserted in the bone cavity using the "press-fit" technique, providing stability just after surgical insertion, i.e., primary stability.

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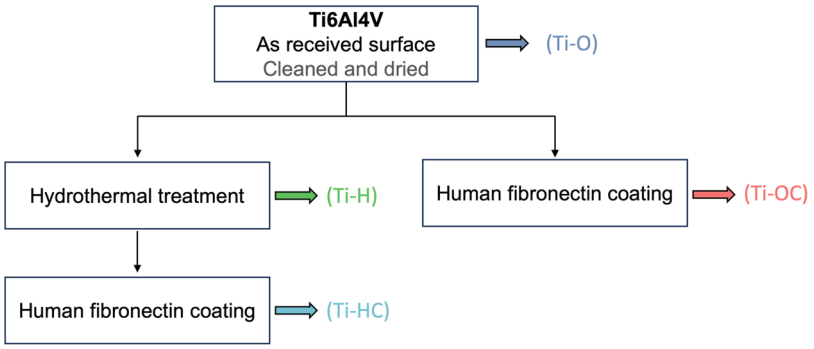
Scheme 1. Detailed Description of the Groups



Afterward, during the healing period, the bone adapts its structure in response to mechanical stress and starts to remodel. The properties of the newly formed bone tissue dictate the secondary stability, which depends on the implant properties, such as its stiffness and surface topology.[5]

Modifications on the surface of biomaterials may affect their physical and chemical properties, which remarkably affect the cell behavior. Therefore, in recent years, efforts have been made to provide an optimal microenvironment able to promote the adhesion and differentiation of mesenchymal stem cells (MSCs) into bone cells and ensure early and long-term biological stability of the implant.[6,7] Biocoatings confer bioactivity to Ti-based implants. Recent developments in antimicrobial, protective, and functional coatings of orthopedic implants were reviewed by Kumar et al.[6] Coatings with biomolecules, such as the extracellular matrix (ECM) proteins, collagen[8−10] and fibronectin,[11−14] enhance osteoinduction. Another alternative is the formation of a titanium dioxide layer whose wettability, roughness, and crystallinity accelerate cellular regeneration.[15,16] This layer may be formed by different surface modification techniques, from complex, such as anodization,[17,18] to simple and economic, such as hydrothermal treatment.[19,20] In a previous study, hydrothermally treated Ti6Al4V samples presented a moderate hydrophilic layer with both anatase and rutile crystalline phases.[19] Besides improving the corrosion resistance of the Ti6Al4V substrate due to thickness increase[21] and the presence of rutile, the most resistant crystallographic form of TiO_2,[22,23] this protective layer has been reported to elicit a similar[24] or even mitigate[25,26] the inflammatory response. In addition, the single-step hydrothermal treatment on Ti-based substrates confers antibacterial properties,[27] promotes osteoblastic lineage,[28] and enhances osteoconductivity in vivo.[29]

Current orthopedic implants lack the necessary biomechanical stimulus at the bone−implant interface, which compromises their long-term success. This study aims to investigate the effectiveness of three treatments in promoting the adhesion and osteogenic differentiation of MSCs, ultimately enhancing the adhesiveness and sustainability of the substrate−cell complex. The treatments considered are hydrothermal treatment to form a titanium dioxide (anatase and rutile) layer, biocoating with fibronectin (an osteogenic matrix protein), and a combination of both treatments. The goal of this study is to improve implant stability during the early healing phase and induce osteogenic differentiation, thus potentially prolonging the lifespan of orthopedic implants.

2. EXPERIMENTAL SECTION

2.1. Preparation of Ti6Al4V Samples. Ti6Al4V alloy (Ti grade 5) plates of dimensions 45 mm × 10 mm × 0.5 mm were acquired from Titanium Products Limited (U.K.) and cut into two-dimensional (2D) substrates of 9 mm × 10 mm × 0.5 mm using a Nd:YV04 laser (XM-30D, XianMing Laser, China).

2D substrates were characterized in terms of roughness and wettability, as previously described.[19] The as-received samples presented an arithmetic average roughness value (R_a) of 10.89 ± 2.01 nm and a water contact angle of 85 ± 3.5°, after autoclaving.[19] Samples were cleaned with ultrasonic rinsing with ethanol, dried in air, and then divided into four groups: control (Ti−O), hydrothermal treatment (Ti−H), human fibronectin coating (Ti−OC), and hydrothermal treatment followed by human fibronectin coating (Ti−HC), as displayed in Scheme 1. Samples subjected to hydrothermal treatment were immersed in a poly(tetrafluoroethylene) (PTFE, Teflon) beaker with 80 mL of distilled water and placed inside a proprietary designed reactor. Treatment was carried out at 180 °C for 180 min, and samples were removed and cooled at room temperature. Subsequently, all samples were sterilized by immersion in ethanol 70% (v/v) for 1 h (each side) followed by irradiation by UVC light for 2 h (each side). Sterile Ti6Al4V samples were stored in 5 times concentrated phosphate-buffered saline (5× PBS) for 24 h. The PBS solution was prepared from PBS tablets (A9201; PanReac AppliChem ITW Reagents), whose composition is 2.7 mM KCl, 140 mM NaCl, and 10 mM phosphate, at a pH of 7.4 (25 °C).

2.2. Cell Culture and the Osteogenic Differentiation of MSCs. Bone marrow-derived human mesenchymal stem cells (MSCs) were purchased from ATCC (PCS-500-012TM; LGC Standard, Spain). Cells were thawed and expanded in clinically compatible xeno-free, animal-serum-free culture media according to the Sartorius AG protocols. MSCs were cultured in a complete MSC medium, composed of NutriStem MSC basal medium (05-200-1A; Sartorius AG, Spain), NutriStem MSC supplement mix (05-201-1U; Sartorius AG, Spain), and PLTGold human platelet lysate (PLTGold27R; Sartorius AG, Spain) in a CO_2 incubator (95% air and 5% CO_2 at 37 °C), and a complete medium change was performed every 3 days. MSCs were passaged using an animal-component-free recombinant trypsin solution (03-078-1; Sartorius AG, Spain). MSCs from the third to seventh passages were used for osteogenic differentiation and the remaining were cryopreserved in MSC freezing solution (05-712-1E; Sartorius AG, Spain).

MSCs were seeded on Ti6Al4V groups with no treatment (Ti−O) and with hydrothermal treatment (Ti−H) and on both groups coated with human plasma-derived fibronectin (05-752-

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Table 1. RT-PCR Primers Used for Osteogenic Gene Expression

gene	forward (5′−3′)	reverse (3′−5′)
OC	GAAGCCCAGCGGTGCA	CACTACCTCGCTGCCCTCC
OPN	CTCAGGCCAGTTGCAGCC	CAAAAGCAAATCACTGCAATTCTC
BMP-2	AACACTGTGCGCAGCTTCC	CTCCGGGTTGTTTTCCCAC
ALP	GACCCTTGACCCCCACAAT	GCTCGTACTGCATGTCCCCT
RUNX2	TGTCATGGCGGGTAACGAT	AAGACGGTTATGGTCAAGGTGAA
GAPDH	TGGAGTCTACTGGCGTCTT	TGTCATATTTCTCGTGGTTCA

1, PRIME-XV human fibronectin; FUJIFILM Irvine Scientific, The Netherlands), corresponding to Ti−OC and Ti−HC, respectively. The plates corresponding to groups Ti−OC and Ti−HC were immersed in human fibronectin solution (5 μg/mL) and incubated overnight at 4 °C. MSCs were seeded on all samples at a cellular density of 3×10^4 cm^{-2}. After 24 h of MSC seeding, the medium was changed to a calcium-free osteogenic differentiation medium (05-440-1; Sartorius AG, Spain) containing dexamethasone (DEX) and ascorbic acid. Cells were incubated for up to 21 days, and the medium was changed every 3 days.

2.3. Evaluation of the Osteogenic Differentiation Capacity of MSCs. *2.3.1. Measurement of Calcium Deposition.* The osteogenic capacity of MSCs in different Ti-treated substrates and at different time points (0−21 days) was determined using Alizarin Red solution (ARS). Briefly, the supernatant was removed, and cells were washed with PBS and incubated with cold EtOH 70% for 30−60 min at room temperature. After washing three times with double-distilled water (DDW), fixed cells were stained with 2% ARS at pH 7.2 (TMS-008-C; Merck Life Science, Germany) and incubated at room temperature for 30−60 min. The excess dye was removed and washed at least four times with DDW. In this step, the calcium secreted from cells was washed out, whereas the nodular structures remained with positive staining for calcium content. DDW was then added to each well to prevent cells from drying and to prepare the plates for visual inspection and image acquisition with a Nikon Eclipse LV 100 ND (Nikon, Japan). Afterward, a semiquantitative assessment of extracellular matrix mineralization was performed by ARS elution. 10% (w/v in DDW) cetylpyridinium chloride (CPC; Merck Life Science, Germany) was added to each well and incubated at room temperature for 1 h to destain cultures. The absorbance was then read at 550 nm using a Biotek Synergy H1Microtiter plate reader spectrometer (Agilent). The mineralization mean was determined by averaging five to six absorbance values, and data were provided as mean ± standard error of the mean. The mineralization kinetics was estimated in intervals of 7 days by calculating the slope (Δabsorbance/Δtime).

2.3.2. Analysis of Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The expression of osteogenic genes in MSCs was examined by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from cultured cells at 0 and 21 days, in triplicate, using the NZY total RNA isolation kit (MB13402; NZYTech, Portugal), according to the manufacturer's instructions. RNA was treated with DNase I with the RNase-free DNase set (79254; Qiagen GMBH) and concentrated with the RNeasy MinElute cleanup kit (74204; Qiagen GMBH). Purified RNA was transcribed into complementary DNA (cDNA) using the NZY first-strand cDNA synthesis kit (MB12501; NZYTech, Portugal), according to the manufacturer's protocol, in a Veriti thermal cycler (Applied Biosystems, Foster City). Real-time PCR was performed using the NZY qPCR green master mix (2×), ROX plus (MB21902; NZYTech, Portugal), according to the manufacturer's instructions. Briefly, the PCR reaction volume included 5 μL of one-step NZY qPCR green master mix (2×), ROX, 0.4 μL of each primer (400 nM), 1 μL of cDNA template, and 3.2 μL of RNA-free water, for a total of 5 μL per sample dispensed in each well.

All of the experiments were performed in a StepOnePlus real-time PCR system (Applied Biosystems) with StepOne Software v2.3. For the reaction conditions, initial activation was performed at 50 °C for 20 min and 95 °C for 10 min, followed by 40 cycles of thermal denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 1 min. The melting curve stage was performed at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and finally 60 °C for 15 s. Melting curve analysis was performed at the end of the program to determine the validity of experimental results. All reactions were performed in triplicate.

Gene specific primers for human osteocalcin (OC), osteopontin (OPN), bone morphogenetic protein-2 (BMP-2), alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed by Eurofins Genomics (Austria), as shown in Table 1.

The expression levels of the tested genes were determined by calculating cycle threshold (C_t) values on StepOne Software version 2.3. Data were normalized based on the mRNA levels of the housekeeping gene GAPDH and by using the $2^{-\Delta\Delta Ct}$ method, as described in ref 30. Data are presented as the mean ± standard error of the mean.

2.3.3. Statistical Analysis. Statistical software GraphPad Prism v.9 (GraphPad Software) was used for all analyses, and the level of $p < 0.05$ was considered statistically significant, considering the normal distribution of data. Differences in mineralization at different time points and treatments were assessed by two-way analysis of variance (ANOVA) with a post hoc Tukey's multiple comparison test and differences in gene expression between time points were assessed by a multiple unpaired t test.

Heatmaps were constructed in RStudio version 2023.6.0.421.[31] For the mineralization data, outliers were detected and removed using the interquartile range (IQR) method within groups, where values exceeding 1.5 times the interquartile range (IQR) below the first quartile (Q1) or above the third quartile (Q3) were deemed as outliers. Then, the standard deviation of each group was used as a measure of the variability in mineralization for each treatment at different time points and plotted as a heatmap. Gene expression at day 21 was divided by that at day 0 for each experimental group and plotted as a heatmap.

3. RESULTS AND DISCUSSION

Total joint replacement is recommended for patients with advanced osteoarthritis. Despite it being considered the most

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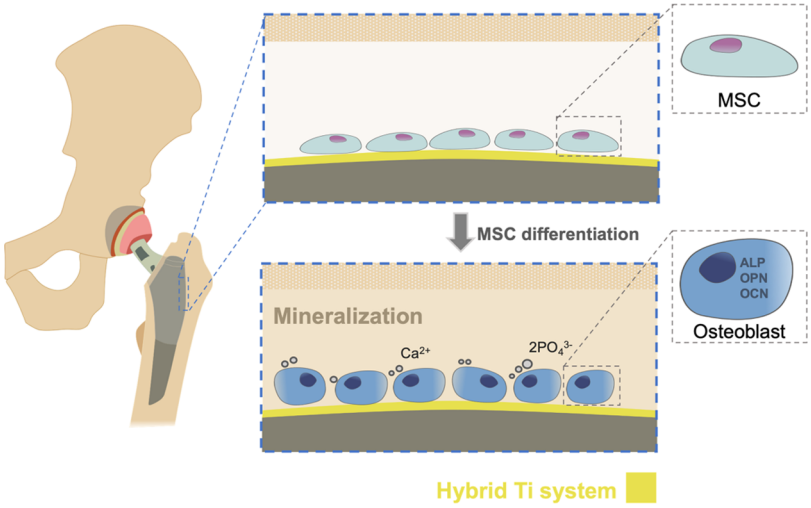

Figure 1. Conventional implants result in bone resorption, compromising their success implantation outcomes. On the other hand, implants with a modified surface present unique features capable of improving the surface properties of the implant, resulting in the activation of cellular response and long-term maintenance. The programming and responsiveness of bone cells are expected to increase the longevity of the implant, which would improve the patient's quality of life and reduce healthcare cost burdens by minimizing the need for revision surgeries.

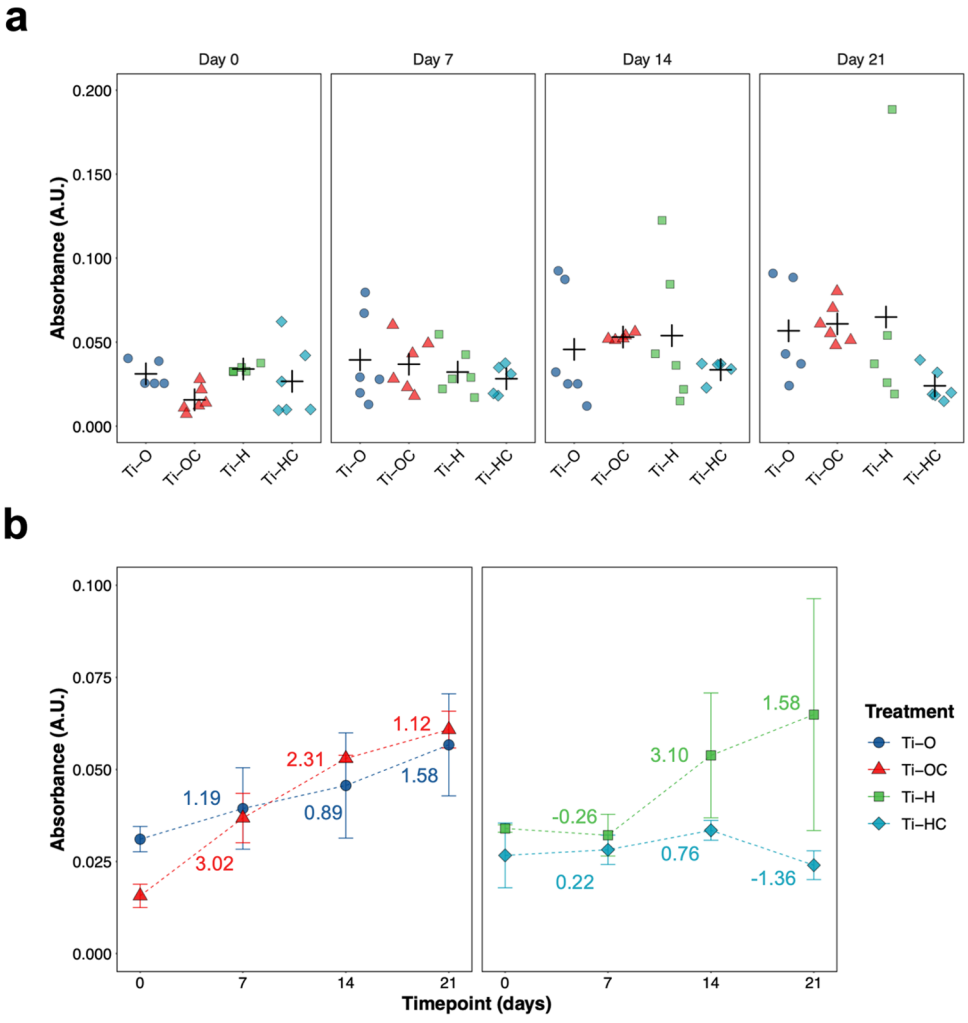

Figure 2. (a) Mineralization values (absorbance read at 550 nm) from 0 to 21 days are shown in a scatter plot, in which the mean of each group is displayed by a cross. (b) Mineralization is displayed for both Ti−O and Ti−OC (left) and both Ti−H and Ti−HC (right) as the mean ± standard error of the mean, and kinetics is displayed on the top of each line (A.U./day, $\times 10^3$).

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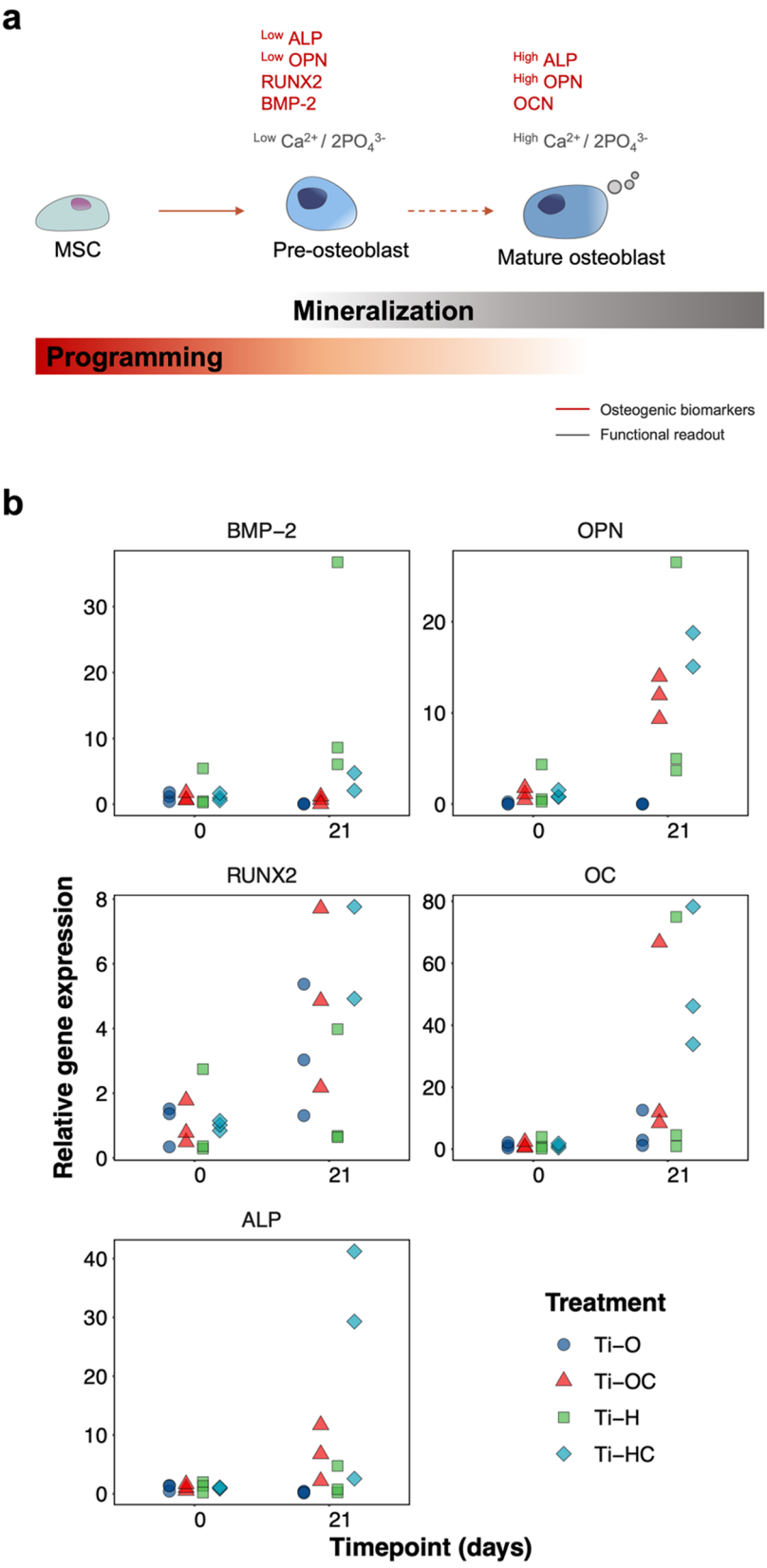

Figure 3. (a) Biogenesis of osteoblasts. MSCs are programmed to commit and differentiate into osteoblasts, which express specific osteoblastic markers (highlighted in red), some of which are responsible for extracellular matrix mineralization, consisting of collagen, calcium, and phosphorus. (b) Expression of osteogenic markers during osteogenic differentiation. Values are reported as the mean ± standard error of the mean.

effective intervention for severe, painful, and disabled osteoarthritis, orthopedic failures are still observed due to an improper biomechanical compliance at the bone−implant interface. Therefore, the surface of the endosseous implant

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must be improved to promote adhesiveness of MSCs and subsequent programming to differentiate into bone cells. In this work, we report the development of a hybrid system aiming to promote bone regeneration and long-term stability of the implant, as illustrated in Figure 1.

3.1. Human Fibronectin-Based Functionalization of Ti6Al4V Substrates Improves the Stability of the Substrate−Cell Complex. Alizarin Red is the water-soluble sodium salt of alizarin sulfonate acid, which chelates with calcium salts in calcium carbonate or calcium phosphate to form an orange−red complex. Therefore, it is used to assess calcium-rich deposits in cultured cells, and matrix mineralization is crucial to guarantee the bone tissue quality. Mineralization increased in all groups compared to the start of the experiment (day 0) (Figure 2), suggesting that MSCs cultured in all Ti6Al4V surfaces successfully differentiated into bone cells. In addition to the extracellular mineralization observed in all groups, the stability of the cell-material complex was evaluated by the dispersion of mineralization values (Figure 2a). Ti6Al4V groups with no fibronectin coating (Ti−O and Ti−H) presented a low dispersion at the beginning of the experiment, but it increased at 21 days of the experiment. On the contrary, Ti6Al4V groups coated with fibronectin (Ti−OC and Ti−HC) had the opposite behavior, presenting a low dispersion of mineralization values at 21 days under osteogenic differentiation. Therefore, fibronectin coating improves the adhesion of cells during the cell culture period, resulting in a higher stability of the cell-material complex over time. These differences in mineralization are not due to differences in cell number, given that no statistically significant differences were observed during incubation (data not shown).

The bone extracellular matrix is composed of organic (collagen and noncollagenous proteins) and inorganic components (hydroxyapatite and other minerals), which act as the template for cellular activities, including attachment, proliferation, and differentiation.[32] The communication between osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells) occurs through membrane-bound ligands, secreted cytokines, growth factors deposited in the bone matrix, and extracellular vesicles. Osteoblasts are known to release a subset of extracellular vesicles, the so-called matrix vesicles, which are involved as initiators of matrix mineralization.[33] The release of these vesicles is first detected during the maturation of osteoblasts and anchors to protein components of the surrounding extracellular matrix, particularly to the collagen matrix. These accumulate calcium and inorganic phosphate, which may be amorphous or may crystallize into hydroxyapatite, eventually disrupting the vesicles' membrane. Their growth continues in the extracellular matrix space to form more stable crystals or propagate on the collagen fibrils, correspondent to matrix mineralization.[34] Besides incorporating mineralization-specific components, such as calcium, these vesicles may function as carriers of osteogenic-related proteins, such as BMPs, and noncollagenous matrix proteins.[35] The osteogenic culture medium used in this study is calcium-free. Therefore, all calcium stained by ARS is a result of the extracellular mineralization that occurred during the osteogenic differentiation of MSCs. Despite the increase in mineralization found in all groups compared to day 0, fibronectin coating was shown to promote the stability and sustainability of mineralization. Fibronectin is a glycoprotein that mediates many cellular interactions with the ECM.[36] It has been reported to facilitate cell adhesion and consequent spreading because it contains the arginine−glycine−aspartic acid (RGD) peptide which binds specifically to the receptors of integrins,[7,13,37] therefore promoting an initial contact of MSCs with the implant's surface. Additionally, fibronectin exhibits both structural and temporal stability when employed as a coating on metallic substrates suitable for biomedical applications.[38,39] These results confirm other studies that the use of human fibronectin coating facilitates the attachment of MSCs to the Ti6Al4V surface, improving the stability of the cell−substrate complex.

3.2. Hydrothermally Treated Ti6Al4V Interface Promotes Osteogenic Programming. During fracture healing, several growth factors, including BMPs as well as platelet-derived growth factors (PDGFs), are secreted and rapidly stimulate MSC recruitment to the site of injury.[40] Afterward, bone formation by intramembranous ossification initiates with the formation of condensations by mesenchymal progenitor cells, which grow and eventually mature, ultimately differentiating into osteoblasts.[41] Osteogenic differentiation is initiated with cell programming, culminating in extracellular mineralization. BMP-2, RUNX2, ALP, OPN, and OC have been identified as key markers involved in osteogenesis.[40,42,43] The biomarker expression profile during osteogenic differentiation and the functional readout as a result of mineralization are illustrated in Figure 3a, and the gene expression of those osteogenic-related genes at 0 and 21 days is displayed in Figure 3b.

During the commitment of the MSCs, each biomarker presents a particular profile and function, as summarized in Table S1. BMPs, including BMP-2, play a crucial role in bone remodeling and homeostasis. In the study conducted by Dragoo et al., human processed lipoaspirate cells treated with either exogenous recombinant BMP-2 (rh-BMP-2) or with an adenovirus containing the cDNA for BMP-2 (Ad-BMP-2) were shown to yield more osteogenic precursors than osteoblasts.[44] Gromolak et al. used ovine BM-MSCs and observed an increase of expression of BMP-2 at 14 days, RUNX2 and osterix at 14 days, and collagen type I, OC, and OPN at 21 days when αMEM was supplemented with both fibroblast growth factor-2 (FGF-2) and BMP-2.[45] Ti6Al4V substrates that underwent hydrothermal treatment (Ti−H and Ti−HC) exhibited an increase of the relative expression of the gene encoding BMP-2, a powerful osteogenic factor responsible for promoting MSC differentiation into osteoblasts, despite not being significant. RUNX2, a specific transcription factor, plays a crucial role in osteogenic differentiation by guiding the differentiation process toward preosteoblasts.[40,46] Its expression showed an overall increase in all groups, with a particularly significant rise in the Ti−HC group ($*p < 0.05$). Additionally, RUNX2 is essential for the expression of noncollagenous proteins such as BSP and OC, further emphasizing its importance in early osteogenic differentiation.[40] ALP facilitates extracellular mineralization, and its overexpression was observed in all groups except the Ti−O group. Besides the overexpression of ALP, mature osteoblasts express OPN and OC. The gene encoding OPN was found to be upregulated in all groups except for Ti−O and the highest overexpression was found for Ti−OC and Ti−HC ($**p < 0.01$). This bone matrix glycoprotein has been reported to regulate collagen organization and mineralization.[43] Finally, osteocalcin is the most abundant noncollagenous protein in the bone ECM[43] and we found that its expression was upregulated in all four groups, particularly in Ti−HC ($*p < 0.05$). OC has affinity for calcium and thus it has an important role during bone matrix mineralization.[43]

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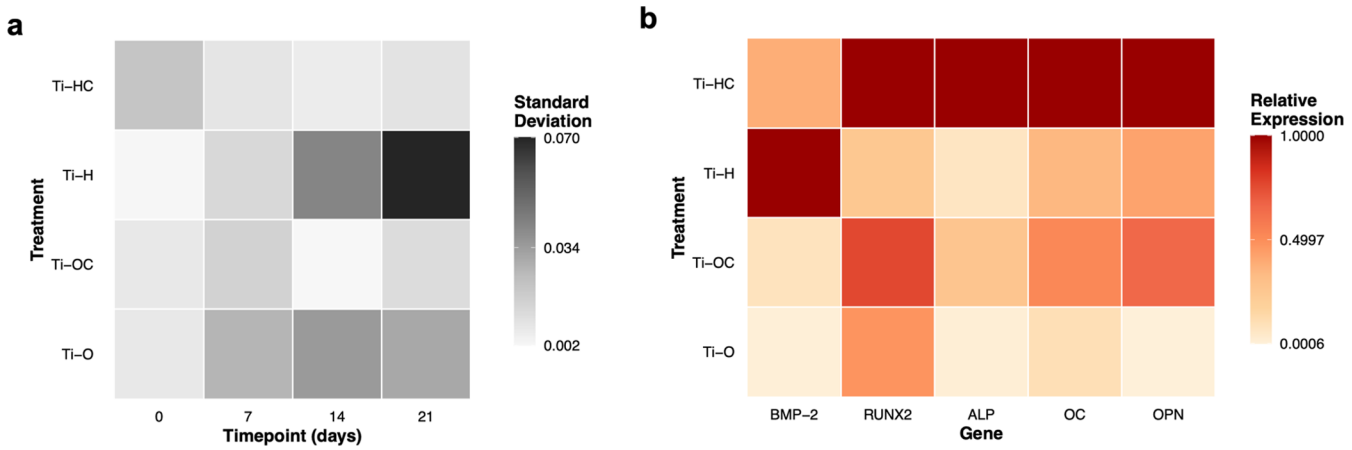

Figure 4. Fibronectin coating reduces variability in mineralization and when combined with hydrothermal treatment, it upregulates the expression of the gene associated with osteogenic differentiation. (a) Variability (standard deviation) in mineralization at different time points of bone marrow-derived human mesenchymal stem cells (MSCs) seeded on Ti6Al4V subjected to different treatments. (b) Relative gene expression levels of hallmarks of osteogenic differentiation in MSCs seeded on Ti6Al4V subjected to different treatments. Gene expression data are presented as fold changes at day 21 relative to the expression level on day 0.

The overexpression of these key osteogenic markers suggests MSC differentiation priming into mature bone cells. The nontreated Ti6Al4V substrate (Ti−O) and the hydrothermal treatment (Ti−H) had no significant effect on gene expression, whereas Ti−OC increased the expression of OPN and Ti−HC increased the expression of OPN, RUNX2, and OC. Therefore, among the four groups, Ti−HC exhibited increasing trends of expression of the transcription factors and osteoblast differentiation markers after 21 days in xeno-free and serum-free osteogenic medium. Furthermore, the analysis of data at 21 days also suggests that fibronectin coating per se allows sustainability of the osteogenic capacity of MSCs.

Surface features of Ti6Al4V biomaterials, such as roughness and wettability, are critical modulators of cell adhesion and activity. Meta-analysis was carried out to assess the effect and contribution of these and others factors in the osteointegration potential both in vitro and in vivo, and it was concluded that an optimized titanium implant surface should have a R_a between 1.5 and 3.0 μm and surface treatment to form an anatase layer.[47,48] We have previously described an extensive surface characterization carried out on Ti6Al4V substrates either with no surface treatment or subjected to hydrothermal treatment, which correspond to Ti−O and Ti−H groups, respectively.[19] Compared to Ti−O, hydrothermal treatment increased the roughness values from 10.89 ± 2.01 to 20.42 ± 1.71 nm, but no major differences were observed regarding wettability. Non-treated and treated substrates were moderately hydrophilic, as the water contact angles were 85 ± 3.5 and 83.5 ± 4.7° for Ti−O and Ti−H, respectively. However, the reported results were obtained for samples autoclaved at 125 °C for 15 min. On the other hand, in the present study, samples were sterilized in alcohol followed by UV irradiation, which has been reported to increase surface wettability without changing the topography and roughness.[49] Besides altering the roughness of Ti6Al4V samples, hydrothermal treatment was shown to effectively create a TiO_2 hydrophilic layer with both anatase and rutile crystalline phases.[19] Lorenzetti et al. investigated the effect of hydrothermally grown TiO_2-anatase coatings on Ti substrates followed by photofunctionalization by UVB irradiation on the biological behavior of human MSCs, and it was observed that they became osteogenically active, presumably due to the 5 h UVB photoinduced hydrophilicity.[50] In addition to the creation of a TiO_2 layer, fibronectin coating on the Ti6Al4V substrate facilitates the binding to osteogenic cell integrin receptors, promoting cell adhesion. In fact, Lv et al. fabricated TiO_2 thin films of anatase and rutile phases by atomic layer deposition and postdeposition annealing on Si substrates and observed a more active conformation of adsorbed fibronectin, which resulted in better osteoblast compatibility in terms of adhesion, proliferation, differentiation, mineralization, and osteogenesis-related gene expression.[16] Rapuano et al. found that fibronectin adsorbed on Ti6A4V did not accelerate osteoblast differentiation but instead increased ECM protein expression during the mineralization stage of osteogenic differentiation.[11]

3.3. Summary of the Results. The foundation for achieving clinical success with implants in bone, whether it is a dental implant or a joint replacement system, lies in the stability of the implant. This stability is influenced by various factors, such as surgical exposure, bone preparation, advancements in manufacturing, surface technology, and geometry. These elements contribute to primary stability and osseointegration during the healing process. Osseointegration refers to the direct connection between the living bone and the implant, which enables load transfer, bone remodeling, and long-term fixation. Stimulating new bone formation on the implant surface can be achieved through material properties, surface topology, porosity, and chemistry. Enhancing the rate, quantity, and quality of osseointegration has been a subject of research for many years. When the optimal implant surface is selected, considerations should include design, manufacturing, cleaning, sterilization, mechanical properties, biocompatibility, implantation, and in vivo response.

The treatment of the Ti6Al4V surface is a critical step in achieving sufficient osseointegration of the implant within the bone. Inadequate healing of the implant can lead to serious complications such as infection, inflammation, aseptic loosening, or stress-shielding effects, which may require additional surgical procedures. In this work, Ti6Al4V surfaces were modified by different strategies aiming to improve their bioactivity and thus promote adhesion and consequent differentiation of MSCs into bone cells. The osteogenic potential of each treatment is summarized in Figure 4.

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Among the many methodologies discussed in the literature, hydrothermal treatment is a simple approach that is able to produce oxide films, in which phase composition and wetting properties have been proved to be beneficial for cell behavior. However, a variety of conditions were found, namely, the choice of aqueous solution (such as sodium tripolyphosphate (STPP) and calcium hydroxide ($Ca(OH)_2$),[51,52] NaOH,[27] calcium phosphate (Ca−P) solution,[46,53] or simply distilled water[29]), temperature (up to 300 °C),[28] and duration (up to 72 h). Here, hydrothermal treatment consisted simply of the immersion of samples in distilled water at 180 °C for 180 min. The use of this method has several competitive advantages over more complex treatments, including ease of use, time reduction, and cost-effectiveness. In addition, the benefits of a surface biocoating with fibronectin were evaluated either per se or combined with hydrothermal treatment. Fibronectin mediates cell adhesion and spreading and is crucial during tissue repair and, in its soluble form, it is a major component of blood plasma.[54] In this study, only xeno- and serum-free reagents were used to closely replicate the human body chemical conditions, and the biocoating was performed using human-derived fibronectin (carrier-free), limiting immunogenicity risks.

MSCs were cultured on Ti6Al4V substrates, which underwent different surface treatments (Ti−O, Ti−H, Ti−OC, and Ti−HC) and their osteogenic differentiation was evaluated based on extracellular mineralization. Compared to the beginning of the experiment, extracellular mineralization increased regardless of the surface treatment. Ti6Al4V surfaces coated with fibronectin (Ti−OC and Ti−HC) exhibited the lowest variability at day 21 (Figure 4a). This finding aligns with previous observations regarding the positive impact of this glycoprotein in promoting the stability of the substrate−cell complex and enhancing the sustainability of osteogenic differentiation. Furthermore, in addition to its effect on mineralization, fibronectin coating also upregulated the expression of key osteogenic markers. The upregulation of pivotal genes associated with osteogenesis, including BMP-2, RUNX2, ALP, OC, and OPN, indicates successful cell programming. Among the four surface treatments evaluated in this study, Ti−HC demonstrated the highest upregulation of the majority of genes (Figure 4b). In summary, our findings indicate that hydrothermal treatment does not impair cell viability and reactivity in Ti6Al4V substrates but rather holds a positive effect. In fact, hydrothermal treatment followed by fibronectin coating offers a combination of minimal variability in extracellular mineralization rates and maximum upregulation of osteogenic genes. Thus, a simple hydrothermal treatment (preparation ease, cost, and duration effectiveness) combined with fibronectin coating represents a simple and cost-effective surface treatment that enhances the adhesiveness of MSCs to Ti6Al4V implant surfaces while also activating their commitment and responsiveness to osteogenic differentiation.

4. CONCLUSIONS

Surface modification of Ti6Al4V implants is a critical step in achieving sufficient osseointegration of the implant within the bone. In the present study, the in vitro response of a titanium alloy implant coated with fibronectin was compared to that of a titanium implant by using an established model. Both noncoated (Ti−O) and coated (Ti−C) groups demonstrated an osteoconductive surface that would potentially promote bone growth. However, hydrothermally treated titanium with fibronectin coating exhibited a more robust bone−implant interface, which is particularly important for long-term implant stability.

It is important to note that this study has limitations regarding the number of time points examined. Future studies with longer observation periods could provide valuable insights into different implant fixation strategies. Nonetheless, the strength of this study lies in its detailed reporting of implant substrate characterization and mechanical properties at the bone−implant interface. This level of detail allows for meaningful comparisons and contrasts, which can be challenging to achieve with clinical implantations. In conclusion, Ti6Al4V subjected to hydrothermal treatment and coated with fibronectin demonstrated a stronger bone−implant interface, which is crucial for long-term implant stability and load transfer.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c07232.

Osteogenic markers and their impact on osteogenesis (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ARS, alizarin red solution; BM, bone marrow; BMP-2, bone morphogenetic protein-2; BSA, bovine serum albumin; CPC, cetylpyridinium chloride; DDW, double-distilled water; DEX, dexamethasone; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSCs, mesenchymal stem cells; OA, osteoarthritis; OC, osteocalcin; OPN, osteopontin; PDGFs, platelet-derived growth factors; PTFE, poly(tetrafluoroethylene); qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction; RGD, arginine−glycine−aspartic acid; RUNX2, runt-related transcription factor 2

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