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Different compact hybrid Langmuir–Blodgett-film coatings modify biomineralization and the ability of osteoblasts to grow

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Received 27 July 2017; revised 28 November 2017; accepted 10 December 2017
Published online 00 Month 2018 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.34069

Abstract: Calcium phosphates (CaPs) are biomaterials widely used in tissue regeneration with outstanding biological performance. Although the tremendous improvements achieved in CaP's materials research over the years, their interaction with physiological environments still need to be fully understood. The aim of this study is to explore a biomimetic Langmuir–Blodgett (LB) membrane to template the growth of hydroxyapatite (HAp) coatings on Ti surfaces and the ability of these coatings in inducing biomineralization by osteoblasts cultured *in vitro*. Changing the phospholipids (i.e., dihexadecyl phosphate (DHP) or octadecylphosphonic acid (OPA)), we also tuned the surface Ca^{2+} concentration. This structural feature gave rise to different LB-hybrid surfaces where the concentration of Ca^{2+} in the OPA/HAp was higher than the concentration of Ca^{2+} in DHP/HAp coating. The higher Ca^{2+} amount on OPA/HAp coatings, allied to the physical–chemical features, lead to different responses on osteoblasts, stimulating or inhibiting the natural biomineralization. The OPA/HAp coating caused a delay in the osteoblast proliferation as indicated by the decrease in the cell viability at the 7th culture day. Improved cell differentiation triggered by the DHP/HAp coating resulted in higher osteoblast biomineralization. The present data underscore that besides both coatings being composed by HAp, the final interfacial composition and physical–chemical properties influence differently the osteoblast behavior. Although the best osteoblast's viability was found to OPA/HAp, our dataset attested that DHP/HAp induced mineralization more effectively than that. This unexpected finding highlight the importance of deeply understanding the biomaterial interface and suggest a promising approach to the design of biofunctional LB-based coatings with tunable properties. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2018.

Key Words: biomineralization, Langmuir–Blodgett, hydroxyapatite, osteoblasts, titanium

How to cite this article: de Faria AN, Cruz MAE, Ruiz GCM, Zancanela DC, Ciancaglini P, Ramos AP 2018. Different compact hybrid Langmuir–Blodgett-film coatings modify biomineralization and the ability of osteoblasts to grow. J Biomed Mater Res Part B 2018:00B:000–000.

INTRODUCTION

The development of implant surfaces that can promote biomineralization upon contact with the host tissue and therefore, correct skeletal and craniofacial fractures is still a challenge. The physical–chemical characteristics of titanium (Ti) makes it the most often used material to correct bone fractures.[1] However, the chemical inertness of Ti may decreases osseointegration.[2] Therefore, several methodologies have been applied to enhance the biocompatibility of Ti surfaces by means of controlling its topography or its surface chemistry. At first glance, better implant-tissue interactions can be accomplished by simply controlling its surface roughness and oxidization by acid-etching, anodization and sandblasting techniques.[3] However, tuning the implant surface chemistry by addition of bioactive compounds seems more promising.[4] Addition of polymers,[5] peptides[6] or biominerals[7] has allowed researchers to design materials with optimized and specific composition, topography and functionality.[8]

Controlling parameters like surface free energy, wettability, roughness, and composition is essential to tailor the interaction between the biomaterials and the host tissue.[9–11] Surface features guide the first contact of an implanted material with the physiological medium. The adsorption of proteins, ions and other biomolecules are important events taking place before the cells begin to adhere.[12] Thus, bone-implant interfaces have been manipulated in order to

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Contract grant sponsor: Conselho Nacional de Desenvolvimento Científico e Tecnológico; contract grant number: 141645/2013-0 (to A.N.F.), 442834/2014-4 (to A.P.R.)
Contract grant sponsor: São Paulo Research Foundation- Fundação de Amparo à Pesquisa do Estado de São Paulo; contract grant number: 2004/08868-0, 2014/11941-3 (to P.C.)

control and stimulate the interaction between the metallic surface and the surrounding environment. Designing materials able to specifically interact with proteins and cells after the implantation is a key point to boosting osseointegration. Osseointegration is the stable anchorage of an implant achieved by direct bone-to-implant contact.[13] The guidance of the body's response to a biomaterial by addition of bioactive compounds to its surface or by tuning the surface properties (i.e., roughness and wettability) have allowed the anchoring and maintenance of long-term implants in favorable conditions to rehabilitate patients and to ensure comfort and bone functionality.[14]

Development of good osseointegration of an implant surface relies on the material's ability to stimulate the osteogenesis and attain a strong bone-implant interface. The initial deposition of mineral during osteogenesis is due to the action of osteoblasts. Osteoblasts produce alkaline phosphatase (ALP), large amounts of collagen type 1α1 (Col1α1), and various other collagen and noncollagen matrix proteins.[15,16] Osteoblasts release matrix vesicles (MVs) containing hydroxyapatite (HAp), which are deposited simultaneously with the collagen fibers, to form the rigid matrix of mineralized bones.[17–19] Studies have shown that biomineralization starts around the 14th day of culture, when the activity of ALP, the release of inorganic phosphate, and the production of collagen approach their peak. The maximum formation of HAp takes place around the 21st day of culture.[20–23] Therefore, the ability of a surface in controlling the biomineralization should be evaluated in order to design biomaterials for improved osseointegration.

Calcium phosphates (CaP), especially HAp, have been widely used as coatings for metallic implants due to its resemblance with the mineral portion of bone.[24] Nevertheless, low homogeneity and lack of knowledge of the ideal concentration of calcium to stimulate proper biomineralization limit most of Ti coating processes.[25,26] Studying the ideal concentration and size of HAp crystals is crucial to stimulate or inhibit mineralization.[27–29] In this sense, the phospholipid-guided growth of HAp crystals is an effective and underexplored strategy to modify the surface of Ti.[30,31] Langmuir–Blodgett (LB) films of negatively[31,32] or positively[33] charged lipid molecules can be transferred to solid supports in the presence of Ca^{2+} ions. The polar heads of lipids interact electrostatically with Ca^{2+}, saturating the ion concentration on the surface and creating an organized array for the crystal nucleation and growth. This biomimetic approach is advantageous over techniques such as radiofrequency magnetron sputtering,[34] sol-gel process,[28] ion-beam-assisted deposition,[35] and plasma-spray[36] for the creation of HAp coatings. The advantages are mostly related to the requirement of a relatively low-cost equipment and use of mild conditions to create highly homogeneous and reproducible coatings. Moreover, the LB-based technique allows the incorporation of proteins in the coating,[32] association with polymers,[37] and mediation of the growth of different biominerals, such as $CaCO_3$ thin films.[38] Additionally, phospholipid-mediated HAp mineralization on Ti surfaces is an approach inspired on the process of bone formation, where HAp crystals first nucleates on the lipid-membrane inside MV's.[18,19] Being so, Ti surfaces modified with LB-films may optimize osseointegration and increase the biocompatibility of Ti because the surface mimics the structure of the cell membrane.[30,37]

In this study, we built LB films of dihexadecylphosphate (DHP) or octadecylphosphonic acid (OPA) on Ti surfaces to mediate the growth of HAp coatings. We choose these lipids due to an interesting structural feature: both lipids bear a phosphate group as polar head but the DHP has a double-carbonic tail. As consequence, the monolayer formed by OPA is more condensed than the DHP one. Therefore, the transference of OPA monolayer formed on subphases containing Ca^{2+} to the Ti surface results in a higher concentration of this ion per area unit (Figure 1). This concentration effects lead to the creation of coatings with different morphology and surface properties.[31,32] In this way, the aim of this study was to investigate the effect of changing surface concentration of Ca^{2+} on the HAp growth and the ability of these coatings in inducing biomineralization by osteoblasts cultured *in vitro*. We want to evidence the versatility of LB-mediated coatings on Ti surfaces to stimulate specific responses on biomaterials surfaces.

MATERIALS AND METHODS

Reactants

DHP, OPA, $CaCl_2$, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), β-glycerophosphate, 2-amino-2-methyl-propan-1-ol, DHP, OPA, Bovine serum albumin (BSA), dexamethasone, tris-hydroxymethylaminomethane (Tris), *p*-nitrophenylphosphate (pNPP), alizarin red, chloramine T, *p*-dimethylaminobenzaldehyde, and soluble collagen were purchased from Sigma-Aldrich Co., St. Louis, MO. Ascorbic acid, α-minimum essential medium (MEM), fetal bovine serum, gentamicin, fungizone, and trypsin were acquired from Gibco-Invitrogen Technologies. Sodium acetate, citric acid, perchloric acid, *n*-propanol, sodium hydroxide, and acetic acid were obtained from Fisher Scientific. The reactants used to prepare SBF (calcium chloride, sodium chloride, potassium chloride, sodium bicarbonate, magnesium chloride, sodium sulfate, sodium phosphate dibasic, and tris-hydroxymethylamine methane) were analytical grade. All the aqueous solutions were prepared with ultrapure dust-free Milli-Q® water. The Ti discs (ϕ 13 mm) were provided by REALUM-Brazil.

Langmuir monolayers and DHP/HAp and OPA/HAp coatings

The surface pressure-surface area (π-A) isotherms of DHP and OPA were analyzed in a 216-cm^2 Langmuir trough (Insight-Brazil) at 25.0 ± 0.5°C by spreading 1.0 mmol L^{-1} DHP or OPA dissolved in chloroform/methanol (3:1, v/v—HPLC grade) on 1.0 mmol L^{-1} $CaCl_2$ aqueous subphase. The interaction between the negatively charged phospholipids and Ca^{2+} at the air/liquid interface was attested by the decreased minimum molecular area in the presence of the Ca^{2+} ions.[31,32]

The Langmuir monolayers of DHP or OPA were transferred to Ti discs (ϕ = 13 mm) previously cleaned with acetone, alcohol, and KH_2PO_4/NaOH buffer solution (pH = 7.5)

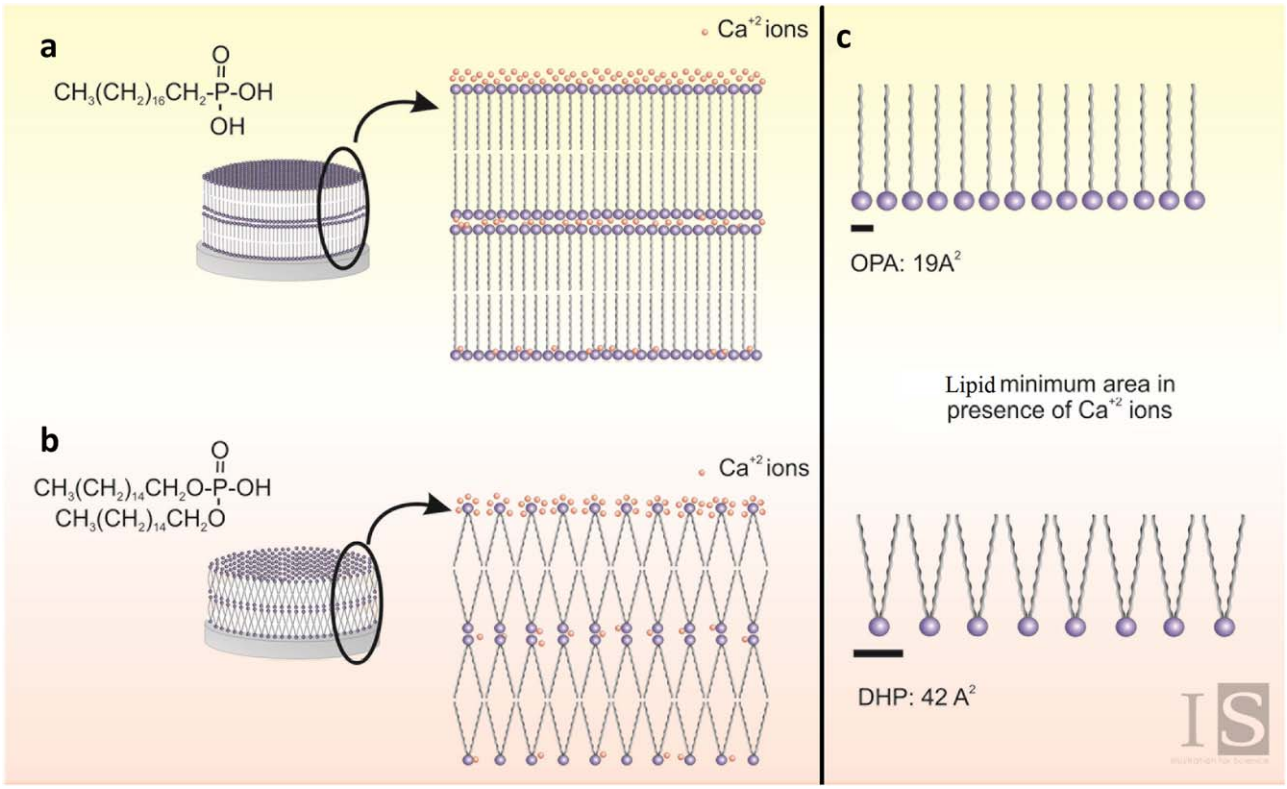

FIGURE 1. Schematic representation: (a) OPA structure, and titanium coated with OPA LB-film; (b) DHP structure, and titanium coated with DHP LB-film; (c) OPA and DHP minimum molecular area in the presence of Ca2+, extracted from Langmuir isotherms.

containing the surfactant Span 20 (4.0×10^{-5} mol L^{-1}) at 65°C, under ultrasound, for 5 min. The DHP and OPA monolayers were rinsed with water before use. Y-type LB films (four layers) containing either DHP or OPA phospholipids were deposited by keeping π constant at 30 mN m^{-1}, at an immersion/withdrawal rate of 0.038 mm s^{-1}.

CaP crystals were grown on the surface of Ti after deposition of the LB-films onto this same surface. To this end, Ti coated with DHP or OPA films was submitted to alternate exposure to $CaCl_2$ solution and 100 mmol L^{-1} phosphate buffer (KH_2PO_4/NaOH, pH 7.5), as described elsewhere.[31,32] HAp growth was then accomplished by exposure to SBF solution, which resulted in the DHP/HAp or OPA/HAp coatings. The SBF solution was prepared as described by Tas et al.[39] The ionic concentration and the pH of the SBF solution were similar to the ionic concentration and the pH found in the human plasma.[40] The morphology of Ti surfaces after the deposition of DHP/HAp and OPA/HAp coatings was investigated by scanning electron microscopy (SEM) by a Shimadzu SS-550 Superscan microscope. Before the analysis, the samples were coated with gold by using cathodic pulverization (Bal-Tec, SCD-050 Sputter Coater).

Cell lineage

The mesenchymal stromal cells of the Wistar rat strain were isolated from the bone marrow, cultured, and differentiated into primary cultures of osteoblasts according to the procedure described by Maniatopoulos et al.,[41] with modifications standardized by de Faria et al.[22] The differentiation process involved β-glycerophosphate, ascorbic acid, and dexamethasone. The Local Ethics Committee of the Ribeirão Preto Medical School, University of São Paulo (protocol number 011/2014–1) approved the isolation procedure.

Cell viability assays

The classic MTT assay described by Mosmann[42] and adapted from de Faria et al.[22] was used to assess cell viability and to evaluate proliferation and survival of osteoblasts on the biomaterials prepared herein. The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide produced the formazan dye, which reflected the presence of living cellular dehydrogenase. After the color reaction finished, the absorbance of each well was measured at 560 and 690 nm on a spectrophotometer (Genesys 2).

To investigate the viability of the cultures of osteoblasts grown on clean Ti and on the DHP/HAp and OPA/HAp coatings, a suspension of 2×10^4 cells in 1 mL of culture medium was added to each well of a 24-well plate. The culture medium was changed each 3 days. The monolayer cultures of osteoblasts were incubated with culture medium for 7, 14, or 21 days. The viability of the osteoblasts was expressed as the percentage of the average of three experiments as compared to the control (CT-polystyrene discs) on the 7th day of culture (100%). The absorbance was measured at 560 and 690 nm on a spectrophotometer Genesys 2

Quantification of proteins

The total concentration of proteins in the cultures of osteoblasts grown on clean Ti and on the DHP/HAp and OPA/HAp coatings was estimated in the presence of 2% (w/v) sodium dodecyl sulfate.[43] BSA was used as standard. The assays were performed on the 7th, 14th, and 21st day of culture for clean Ti, and after the modifications with the DHP/HAp and OPA/HAp coatings. In addition, the concentration of collagen in all the samples was measured according to the methodology of Reddy and Enwemeka.[44] The concentration of proteins was expressed as μg/mL, and the assays were performed in triplicate.

Activity of alkaline phosphatase

ALP is a nonspecific enzyme. Its activity was determined by its action on the substrate PNPP. The *p*-nitrophenylphosphatase activity of ALP was determined in the supernatant of the cell culture after treatment of a homogenized cell suspension with phospholipase C and ultracentrifugation at 100,000*g* according to the procedure described by Camolezi et al.[45] The tests were performed on the 7th, 14th, and 21st day of culture, in triplicate.

Formation of the mineralized matrix

Formation of the mineralized matrix was evaluated on the 14th and 21st day of culture, when the formation of nodules due to the presence of HAp crystals in the matrix was evident.[20] Two techniques were used. In the first technique, the Alizarin Red S methodology described by Gregory et al.[46] was employed. In this technique, the mineralized nodules were fixed with formalin, dehydrated through a graded series of alcohol, and stained with Alizarin Red, which colored the mineralization nodules rich in calcium in red. Next, the contents of the wells were solubilized with acetic acid and neutralized with ammonium hydroxide. The absorbance at 405 nm was read in a spectrophotometer. The mineralization assays were carried out in triplicate. Clean Ti and the DHP/HAp and OPA/HAp coatings without osteoblasts were used as controls because Alizarin Red also stained materials containing Ca^{2+} in their compositions. The second technique consisted in evaluating the Ca/P molar ratio of the nodules by X-ray dispersive energy (EDX) (IXRF system 500 Digital Processing spectrometer). The deviation of the EDX quantitative analysis was close to 10%.

Morphology, attachment, and proliferation of cells

The morphology of living osteoblasts after were evaluated by SEM images recorded with a JSM6610LV–JEOL microscope operating at an accelerating voltage of 20 kV. To this end, the cells were previously treated with glutaraldehyde and osmium, dehydrated with a gradient of CO_2[47] and covered with gold. The samples collected on the 14th and 21st day of culture were analyzed. Confocal microscopy was also applied to investigate cell morphology; a Leica TCS SP5 microscope was employed. The osteoblasts were stained with acridine orange (0.005 mg/mL), a fluorophore that provides a contrast image by binding to the nucleic acids of osteoblasts (RNA in red; DNA in green).[48] To visualize DNA, the sample was excited with the 488-nm line of an Argon laser, and emission was collected between 499 and 541 nm. RNA was visualized by exciting the sample with the 458-nm line of an Argon laser, and emission was collected between 642 and 682 nm.[27,49] The samples were also analyzed on the 14th and 21st day of culture. The morphological analyses were taken in three samples, and the best images were selected.

Statistical analysis

Statistical comparisons were accomplished by two-way variance analysis (ANOVA) followed by Bonferroni's test for all the dataset. *p* Values lower than 0.05 were considered significant.

RESULTS

Surface pressure versus surface area isotherms of OPA and DHP

The interaction of Ca^{2+} ions with the lipid monolayers was investigated by the construction of π-A isotherms. The minimum molecular area of the Langmuir monolayers of DHP, which consisted of two 16-carbon chains linked to a phosphate polar head, and OPA, which comprised a single 18-carbon chain bound to a phosphate [Figure 1(a,b)], decreased in the presence of $CaCl_2$ as compared to the monolayers in the presence of pure water: from 50 to 42 $Å^2$ for DHP molecules, and from 23 to 19 $Å^2$ for OPA molecules (Figure 2). The Ca^{2+} present in the subphase interacted preferentially with the negatively charged phosphate group present in the polar head of both DHP and OPA, diminishing the repulsion between these heads and favoring packaging of the lipids.[31] Increasing Ca^{2+} concentration at the LB film by changing the polar head of the lipid caused the formation of HAp coatings with different topography, as shown in Figure 2. Moreover, this structure feature of the organic matrix allowed the crystallization of coatings where the DHP/HAp has higher roughness and wettability than the OPA/HAp coating.[31,32] Allied to this difference in the surface properties, we will see in the next sections that the coatings exhibit different Ca^{2+} concentration that directly affect the osteoblast behavior.

Cell viability assays

We assayed the viability of osteoblasts to investigate how these cells behaved upon contact with Ti coated with DHP/HAp or OPA/HAp coatings at three distinct phases of osteoblast growth: initial, intermediate, and stationary.[23] Figure 3 shows that, compared to clean Ti, the viability of osteoblasts decreased on the 7th day and increased on the 14th day when the cells were cultured on the OPA/HAp coatings. Growth increased sharply, and the ability of osteoblasts to proliferate was recovered by the 14th day. In turn, the DHP/HAp coating boosted the viability of osteoblasts on the 7th, 14th, and 21st day of culture as compared to clean Ti. The Ti samples coated with unmineralized LB-films (DHP/Ca^{2+} or OPA/Ca^{2+}) decreased the viability of osteoblasts. Ti coated with the unmineralized LB-coatings (DHP/Ca or OPA/Ca) decreased the viability of osteoblasts due to the

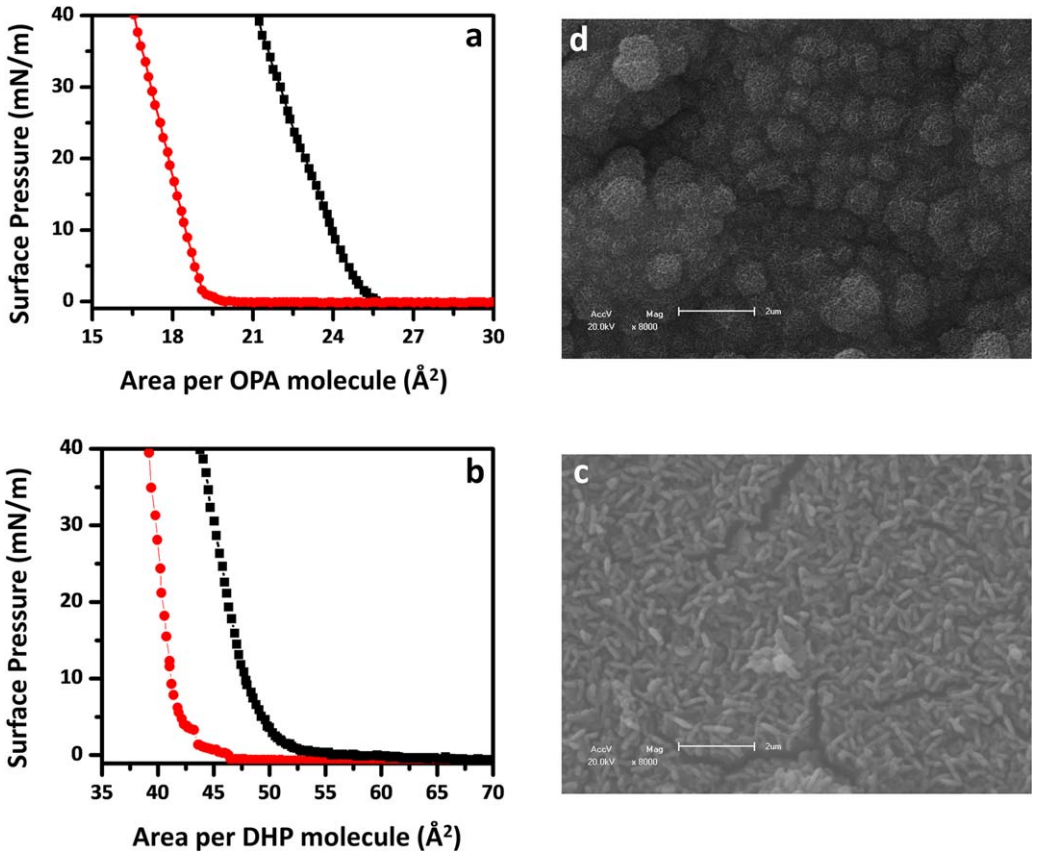

FIGURE 2. Surface pressure–surface area isotherms obtained for A) OPA and B) DHP on pure water subphase (black) and on 1 mmol L^{-1} $CaCl_2$ subphase (red). SEM images of the HAp coatings deposited on Ti surfaces mediated by the OPA (C) and DHP (D) LB films.

lower wettability of the surfaces in the absence of HAp. A hydrophilic environment favors the attachment of osteoblasts to the Ti surface.[50]

Determination of the total content of proteins and of the activity of ALP

The content of proteins is closely linked to the number of viable osteoblasts and their ability to secrete collagen, ALP, and membrane proteins, among others. Figure 4(a) shows that the total content of proteins increased significantly on the 7th, 14th, and 21st day of culture for osteoblasts grown on DHP/HAp coating as compared to the OPA/HAp coating and clean Ti. The concentration of proteins increased between the 7th and the 21st day for all the cultures of osteoblasts.

ALP is one of the main proteins produced by osteoblasts. It is an enzyme labeled as a phenotypic biomarker of biomineralization in cultures of osteoblasts.[17] Figure 4(b) reveals increased activity of ALP on the 14th day of culture for osteoblasts grown on DHP/HAp coating as compared to osteoblasts grown on clean Ti or on OPA/HAp coating. OPA/HAp coating led to decreased activity of ALP on the 21st day of culture as compared to the DHP/HAp coating and clean Ti.

Determination of the concentration of collagen

On the basis of Figure 4(c), the concentration of collagen produced in the culture of osteoblasts grown on Ti modified with the DHP/HAp coating decreased on the 14th day of culture as compared to clean Ti. Interestingly, on the 21st day of culture, the concentration of collagen in the culture of osteoblasts grown on DHP/HAp coating increased significantly as compared to clean Ti or OPA/HAp coating. OPA/

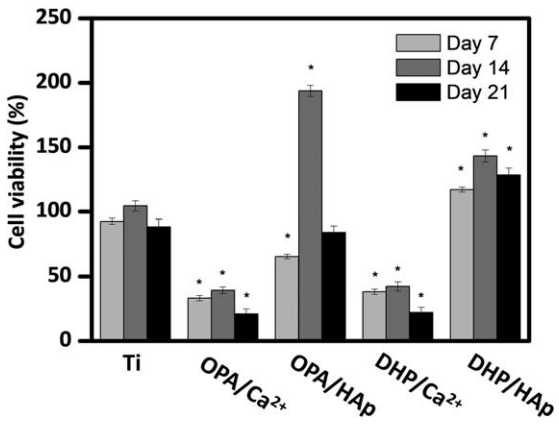

FIGURE 3. Cell viability of osteoblasts grown on clean Ti, Ti surfaces modified with LB films OPA/Ca^{2+} and DHP/Ca^{2+} and modified by the hybrid coatings OPA/HAp and DHP/HAp on days 7, 14, and 21 days. Osteoblasts (2×10^4 cells/well) were cultured in 24-well plates, and the results are expressed as the percentage of the average of three experiments performed in triplicate as compared to the control (100%). *p-value < 0.05 vs control.

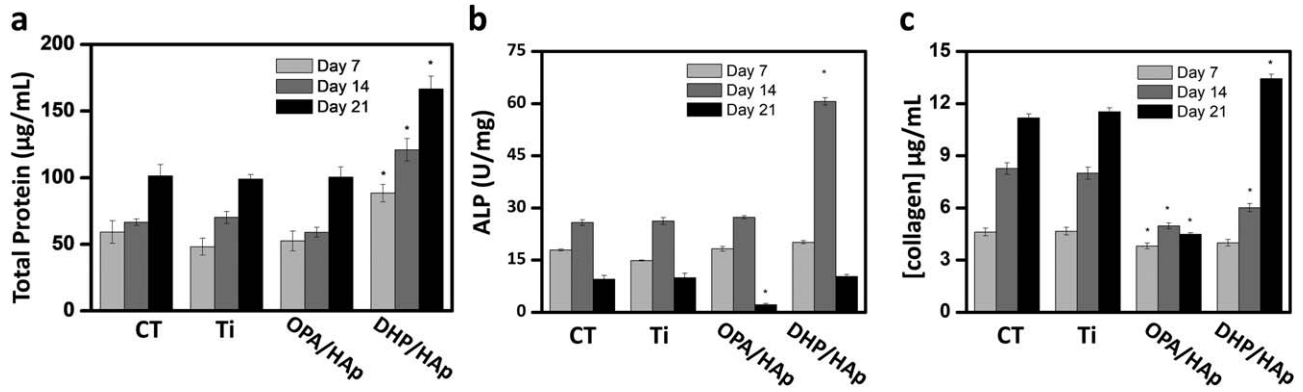

FIGURE 4. a) Determination of total protein in cultures of osteoblasts grown on clean Ti, and Ti modified with the OPA/HAp and DHP/HAp coatings; b) Activity of ALP from osteoblasts grown on clean Ti, and Ti modified with the OPA/HAp and DHP/HAp coatings; c) Concentration of collagen produced by osteoblasts grown on clean Ti, and Ti modified with the OPA/HAp and DHP/HAp coatings. The results are expressed as the average of three experiments performed in triplicate as compared to the control. *p-value < 0.05 vs control. CT refers to the cells cultured on polystyrene discs.

HAp coating afforded decreased concentration of collagen both on the 14th and 21st day of culture as compared to clean Ti.

Formation of mineralized matrix

We quantified the mineralized matrix by the Alizarin Red S method. The Alizarin dye can stain mineralized nodules rich in calcium, and the resulting product absorbs at 405 nm. Formation of the mineralized matrix corresponds to the synthesis of natural HAp by osteoblasts, which starts around the 14th day of culture.[23] Figure 5 evidences that OPA/HAp coating decreased mineralization of the matrix on both the 14th and 21st day of culture as compared to clean Ti. In contrast, DHP/HAp coating increased mineralization of the matrix on the 14th and 21st day of culture.

Knowing that DHP/HAp and OPA/HAp coatings also contained calcium ions, we tested these biomaterials in the absence of osteoblasts (Figure 5, insert). We found that OPA/HAp coating possessed 1.88-fold higher levels of Ca^{2+} than DHP/HAp coating. Therefore, this increase in the Ca^{2+} content for the OPA/HAp coating can be linked to its decreased ability in generate positive responses in the osteoblasts.

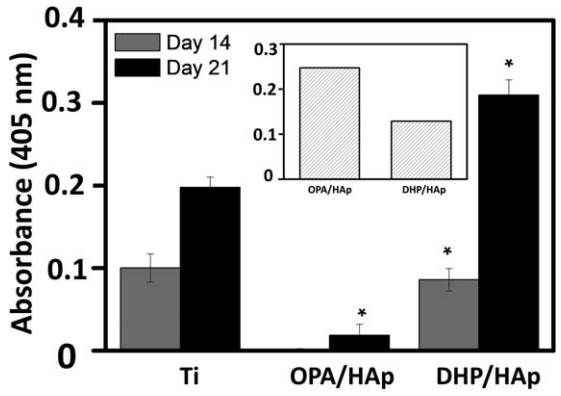

FIGURE 5. Formation of mineralized matrix at 14 and 21 days of culture of osteoblasts grown on clean Ti and modified by the OPA/HAp and DHP/HAp coatings. Insert: Absorbance (at 405 nm) of OPA/HAp and DHP/HAp coatings before the osteoblast culture. The osteoblasts (2×10^4 cells/well) were cultured in 24-well plates, and the absorbances of alizarin red stain are expressed as the average of three experiments performed in triplicate as compared to the control. *p-value < 0.05 vs control.

Formation of mineralized matrix is part of the natural role of osteoblasts. HAp crystals formed within matrix vesicles in osteoblasts are deposited simultaneously with collagen, to afford a rigid matrix of mineralized bone.[17] Biomineralization begins around the 14th day of culture, when ALP starts to deliver inorganic phosphate for the synthesis of HAp crystals; the formation of crystal nodules peaks around the 21st day.[20] Formation of the mineralized matrix by osteoblasts grown on the DHP/HAp coating increased significantly as compared to clean Ti (Figure 5) probably because the physical–chemical features of the coated Ti surface enhanced the proliferation of osteoblasts,[31] the total concentration of proteins concentration, and the activity of ALP. On the other hand, formation of the mineralized matrix by osteoblasts grown on OPA/HAp coating decreased formation of the mineralized matrix, which could be related to lower activity of ALP activity and consequent lack of inorganic phosphate. Our results agreed with the data published by Yang et al.,[51] who verified increased mineralization of osteoblasts grown on HAp coatings after 7 and 14 days of culture. Ozawa and Kasugai[52] detected enhanced formation of mineralized nodules when they used rat bone marrow cells differentiated into osteoblasts grown on a HAp implant material as compared to clean Ti.

We analyzed the mineralized surfaces of the samples collected on the 14th and 21st day of culture by EDX to follow the mineralization of osteoblasts on DHP/HAp and OPA/HAp coatings. The calculated Ca/P molar ratio—1.67 ± 0.17—was close to the value found for natural HAp (1.66).

Cell morphology and proliferation

The SEM images (Figure 6) revealed differences in the shape of osteoblasts in the 14th and 21st day of culture on clean Ti and Ti modified by the DHP/HAp and OAP/HAp

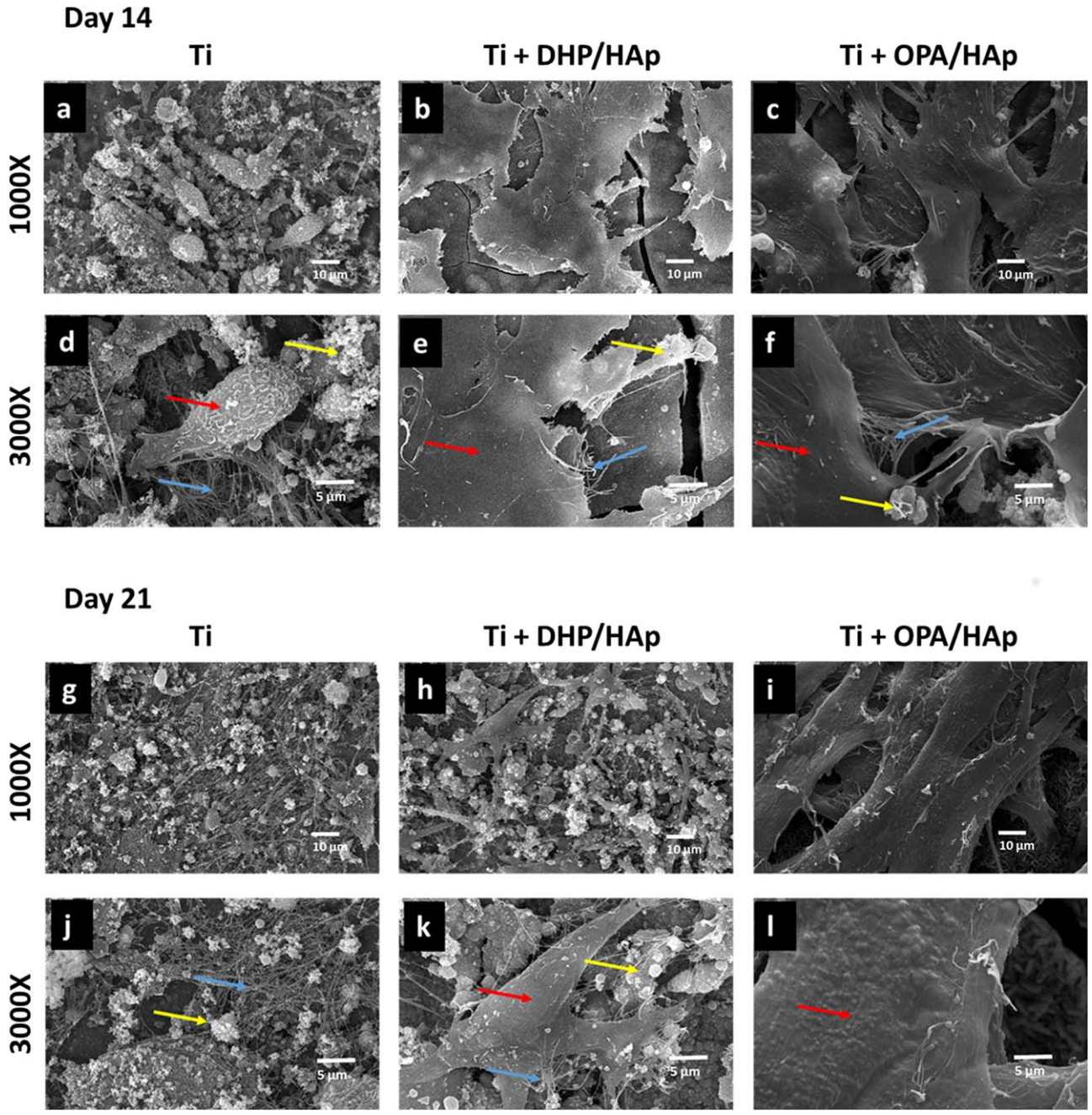

FIGURE 6. SEM images at 1000X and 3000X magnification of osteoblasts grown on clean Ti (a, d, g, j), DHP/HAp (b, e, h, k), and OPA/HAp coatings (c, f, i, l) after 14 (a–f) and 21 days (g–l) of culture. The osteoblasts (2×10^4 cells/well) were cultured in 24-well plates. The red arrows point to osteoblasts, the yellow arrows point to HAp crystals, and the blue arrows point to collagen fibers.

coatings. Figure 6(a,d) show that osteoblasts grown on clean Ti (red arrows) were at an advanced stage of maturation, as seen by the presence of many collagen fibers (blue arrows) and HAp crystals (yellow arrows); however, the cells were smaller than the osteoblasts cultivated on Ti in presence of the hybrid coatings. The osteoblasts were favorably adhered to and spreaded on the DHP/HAp and OPA/HAp coatings [Figure 6(b,c,e,f)] as attested by the longer cytoplasmic extensions (red arrows) as compared to clean Ti. Nonetheless, the osteoblasts were at a pre-maturation stage, as indicated by the onset of formation of collagen fibers at the cell borders (blue arrows).

The SEM images registered on the 21st day of culture [Figure 6(g–l)] confirmed total mineralization of clean Ti [Figure 6(g,j)], as evidenced by the absence of living cells and the presence of HAp crystals (yellow arrows) and collagen (blue arrows). However, on the basis of Figure 6(h,k), DHP/HAp coatings produced a markedly mineralized matrix (yellow arrows) and generated a large amount of collagen (blue arrows) at this stage, with the presence of intact

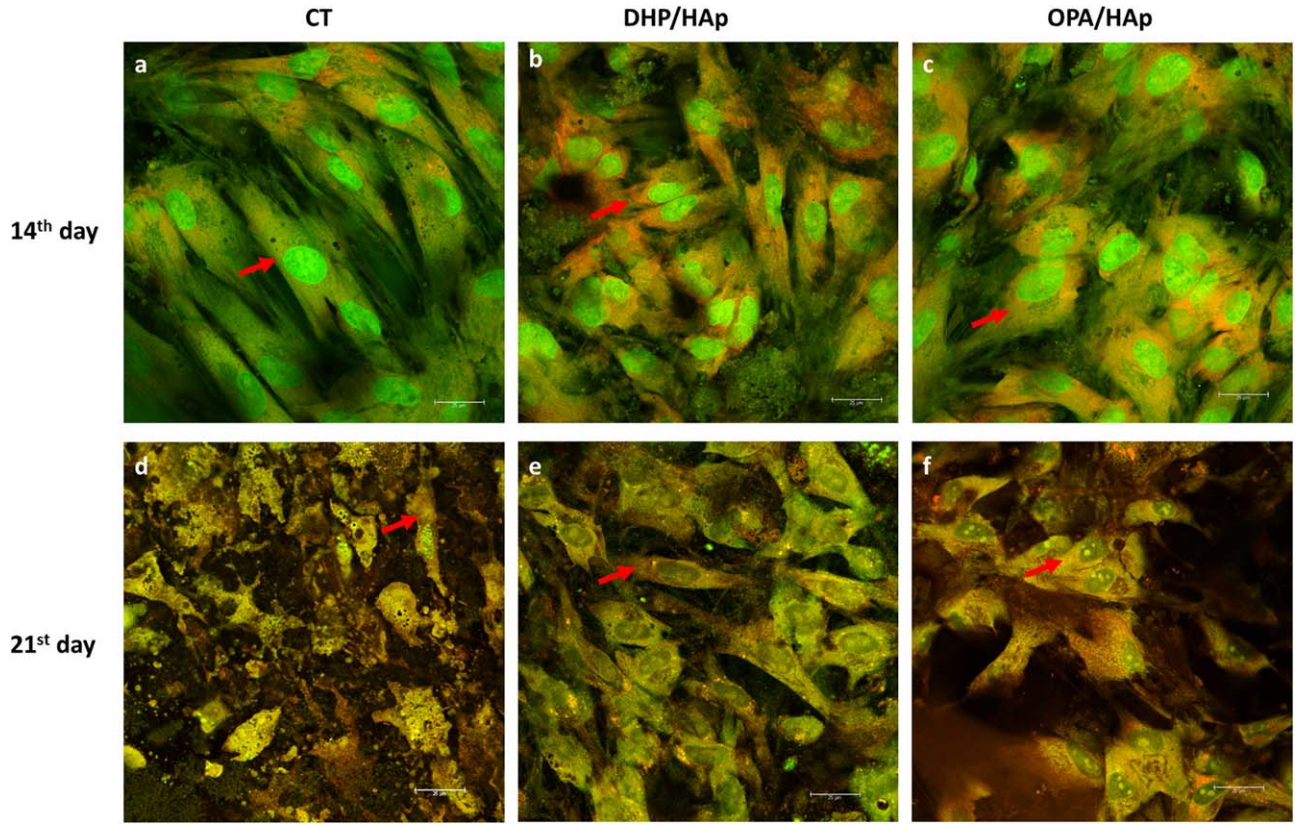

FIGURE 7. Confocal microscopy images at 14 (a–c) and 21 days (d–f) of culture of osteoblasts grown on clean Ti (a, d), and Ti modified by the DHP/HAp coating (b, e), or OPA/HAp coating (c, f). The osteoblasts (2×10^4 cells/well) were cultured in 24-well plates. The red arrows point to osteoblasts.

osteoblasts (red arrows). In contrast, OPA/HAp coating [Figure 6(i,l)] stimulated the growth of osteoblasts along with only a small production of mineralized matrix and collagen.

Confocal microscopy of osteoblasts (red arrows) stained with acridine orange (Figure 7) conducted on the 14th and 21st day of culture for cells grown on clean Ti [Figure 7(a,d), respectively], and Ti modified by the DHP/HAp [Figure 7(b,e), respectively), and OPA/HAp coatings [Figure 7(c,f), respectively] revealed that on the 14th day the osteoblasts attached favorably to both clean and coated Ti, as evidenced by the branches and cytoplasmic extensions emerging from the central body. Cells cultured on clean Ti had more polarized growth direction, whereas osteoblasts grown on the hybrid coatings did not have a crop polarization and appear to occur in larger numbers per field as compared to clean Ti. The several layers of cells (stratified cells) prevented us from estimating the exact number of osteoblasts. Concerning osteoblasts on the 21st day of culture, an advanced stage of senescence and death was evident. Delimitation of the cytoplasm and nucleus were less clear than on the 14th day. Intact cells were present on DHP/HAp and OPA/HAp coatings, whereas the death process was more advanced on clean Ti. Both the SEM and confocal images presented here corroborated the findings of the viability and mineralization assays: compact LB-film coatings stimulate the proliferation of osteoblasts, but just the DHP/HAp coating can increase biomineralization.

DISCUSSION

CaPs have been extensively studied as coatings for metallic surface for bone regeneration applications. However, despite the vast *in vitro* research findings, it is difficult to highlight the most significant properties that lead to a better cellular response.[53] One concern little explored is the optimum Ca^{2+} concentration in CaP coatings that will generate positive cellular responses. Therefore, in this study, we explored the *in vitro* osteoblast response to Ti surfaces modified with HAp coatings where in one the Ca^{2+} amount is twice as the other. Our proposal was to use a biomimetic approach to generate HAp coatings displaying different properties and evaluate the influence of those coatings on the osteoblast biomineralization. We observed that the coating containing high Ca^{2+} amount (OPA/HAp) lead to a decreased osteoblast response and mineralization.

It has been reported that CaP coatings are not always beneficial for the proliferation of osteoblastic cells.[31,54,55] Besides of differences in crystallinity[56] and coating topography,[29] the amount of Ca^{2+} in the coating may have a negative impact during the cell attachment and proliferation. Surfaces with high Ca^{2+}-content by the deposition of $CaCO_3$ thin films have been described to diminish the initial osteoblast proliferation.[38,57] Moreover, implantation of HAp in the body initially dissolves the top surface layer of HAp to deliver Ca^{2+} ions until the dissolution equilibrium is reached.[58–60] In fact, the surface dissolution of CaP to

release Ca^{2+} and phosphate ions in the cell-material interface directly affects the osteoblast behavior,[61] evidencing the importance in studying the Ca^{2+} content on CaP coatings. Nayab et al.[62] reported that Ti surfaces with high levels of Ca^{2+} significantly decreased the adhesion of cells of the MG-63 lineage within the first hours after plating. Nevertheless, the latter authors did not mention the late cell response on the 14th and 21st day observed in the present study. Herein, we assigned the initial decrease in the viability of osteoblasts grown on OPA/HAp coatings on the 7th day and the subsequent increase in the viability of these cells on the 14th day of culture (Figure 3) to the higher concentration of Ca^{2+} on this coating as compared to clean Ti and the DHP/CaP coating. The increase in the total content of proteins and type I collagen for the osteoblasts cultured on DHP/HAp coating agreed with their increased cell viability. The total content of proteins did not increase in the case of osteoblasts grown on OPA/HAp coating. It is well-reported that CaP coatings enhance cellular adhesion, proliferation and differentiation to promote bone regeneration, as evidenced by increase of cell viability, protein synthesis and activity in osteoblasts cultured *in vitro*. Thus, our focus here was to evidence that instead of the well-known beneficial effect of CaP coatings, it may have an optimum Ca^{2+} amount that act coordinately with the surface features of the coating to stimulate a positive cell response, as observed for the DHP/HAp coating. Allied to its optimal Ca^{2+} content, the DHP/HAp coating exhibits higher roughness and wettability than the OPA/HAp, which contributed to the positive response of osteoblasts on those surfaces. It has been reported that osteoblast adhesion and differentiation is improved on rougher HAp coatings.[63]

The influence of the Ca^{2+} content in coatings on the osteoblast behavior is evidenced by the ALP activity. ALP is a nonspecific phosphomonohydrolase that can hydrolyze phosphate monoesters, pyrophosphate, and phosphodiesters in alkaline pH, releasing inorganic phosphate for the synthesis of HAp crystals in the extracellular matrix.[21] The activity of this enzyme is directly related to the rate at which osteoblasts mineralize and can be assigned with a shift toward a more differentiated state.[20,21] Figure 4(b) shows the activity of ALP increased only for osteoblasts cultured on DHP/HAp coatings. Therefore, the abrupt cell proliferation observed for the OPA/HAp coating affect its differentiation as noticed by the decrease ALP activity on the 14th day. At this stage, it is expected a high level of ALP activity and collagen production by the osteoblastic cells, indicating a high stage of differentiation that will culminates in the mineralization of the extracellular matrix. As a result of the low ALP activity in the osteoblasts cultured on the OPA/HAp coatings, these samples resulted in the formation of low mineralized matrix, as observed by the mineralized nodules quantification on Figure 5. The presence of a Ca^{2+}-rich environment might have delayed the onset of osteogenic differentiation. The lower ALP activity observed for the coating with higher Ca^{2+} content (OPA/HAp) agreed with the results described by Hoylaerts et al.,[64] Leone et al.,[65] and Pizauro et al.,[66] who correlated inactivation of ALP to high levels of Ca^{2+}. ALP becomes inactive when Mg^{2+} and Zn^{2+} ions are displaced from its catalytically sites.[64,65] Here, OPA/HAp coating contained 1.88-fold higher level of Ca^{2+} than DHP/HAp coating, which may have favored such displacement and inactivated the enzyme.

SEM images showed that osteoblasts cultured on clean Ti and on coated Ti had different shapes. The increased wettability and surface free energy related to the homogenous coatings increased the spreading and the number of osteoblasts adhered. The SEM images of the Ti control agreed with the images presented by Ramires et al.,[28] who cultivated human MG63 osteoblast-like cells for up to 10 days on clean Ti or on TiO_2 coatings containing different concentrations of HAp, to find osteoblasts with dorsal edges in close contact with each other, connected by filopodia arranged in multilayers. Moreover, the SEM images evidenced the higher differentiation stage for the osteoblasts cultured on DHP/HAp coatings, with the presence of a high number of mineralized nodules.

Therefore, the results shown herein evidence that HAp coatings are far complex, and properties such as Ca^{2+} content and surface features should be taken together in consideration to proper stimulate biomineralization on osteoblast cells. We observed that higher Ca^{2+} content on OPA/HAp stimulated an expressive cell proliferation that culminated in low cell differentiation and activity, as evidenced by the decreased formation of mineralized nodules. Therefore, we claim that DHP/HAp coating provide an auspicious Ca^{2+} environment for the osteoblasts, that allied to its increased surface roughness and wettability[31] promoted the formation of a densely mineralized matrix after 21 days of culture. Moreover, we attested the versatility of using LB films to mediate the growth of CaP coatings with a tunable nanostructured topography. Designing highly controllable surfaces is a new trend in biomaterials research, since osteoblast cells seems to prefer and recognize nanometric HAp crystals.[67] The biomimetic approach used herein allows the control of Ca^{2+} content and topography of HAp coatings, being a promisor technique for the design of biomaterials to proper stimulate biomineralization.

CONCLUSION

The type of phospholipid used to obtain LB-films directly affects the concentration of Ca^{2+} in the final hybrid coating. Phospholipids with reduced minimum molecular area (i.e., OPA) bind to a higher quantity of Ca^{2+}/area in the LB-films. Using this structural feature of the LB films, we created coatings on Ti surfaces where the Ca^{2+} content on the OPA/HAp coating is twice the one observed for the DHP/HAp coating. As consequence, higher activity of ALP, larger concentration of total protein, and enhanced formation of mineralized matrix nodules was observed for the DHP/HAp coating when compared to the OPA/HAp coating. These results reveal that an optimum Ca^{2+} content on CaP coatings influences osteoblast behavior. The present data underscore that besides of both coatings being composed by HAp, the final interfacial composition and physical–chemical properties influence

differently the osteoblast behavior. Although the best osteoblast's viability results were found to OPA/HAp, our dataset attested that DHP/HAp induced mineralization more effectively than that. Ours unexpected findings highlights the importance of deeply understanding the biomaterial interface and suggest a promising approach to design biofunctional LB-based coatings with tunable properties.

ACKNOWLEDGEMENT

We also thank Cynthia Manso for the revision of the English language.

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