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Osteogenic effects of simvastatin-loaded mesoporous titania thin films

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Abstract

The use of statins in the field of bone regeneration is under current investigation due to the existing demand for non-toxic anabolic agents capable of enhancing bone formation in cases of substantial loss. Simvastatin, a coenzyme currently prescribed in clinics to inhibit cholesterol biosynthesis, has been proven to promote osteogenic differentiation by stimulating bone formation and inhibiting osteoclasts activity. We present the loading of simvastatin in mesoporous TiO_2 thin films toward combining the pro-osteogenic properties of this molecule with the demonstrated bioactivity of titania. TiO_2 thin films processing and characterization were carried out, as well as evaluation of MC3T3-E1 pre-osteoblasts viability when directly incubated with different concentrations of simvastatin, followed by the analysis of osteogenic activity promoted by simvastatin upon loading in the thin films. The accessible porosity of 36 % quantified on the 95±5 nm thick mesoporous thin films, together with pore diameters of 5.5 nm, necks between pores of 2.8 nm and interpore distances of 12±2 nm allow the loading of the simvastatin molecule, as confirmed by FTIR spectroscopy. Simvastatin was found to promote MC3T3-E1 pre-osteoblasts viability at concentrations ≤ 0.01 g/L, with a cytotoxicity threshold of 0.05 g/L. We additionally found that film loadings with 0.001 g/L simvastatin promotes statistically higher MC3T3-E1 pre-osteoblast proliferation whereas a higher concentration of 0.01 g/L leads to statistically higher osteogenic activity (ALP synthesis), after 21 days of incubation, as compared to unloaded films. These results demonstrate the potential of simvastatin local administration based on bioactive mesoporous thin films to promote pro-osteogenic properties. By focusing this strategy on the coating of metallic prostheses, the supply of simvastatin to the target tissue can be favored and risks of systemic side effects will be reduced while enhancing the osteointegration of the implants.

Keywords: simvastatin, mesoporous titania, thin films, osteogenic activity, drug delivery.

1. Introduction

The main strategy in clinics to restore functionality in cases of critically damaged bone tissue, such as in osteoporosis (Kanis *et al* 2013) or periodontal diseases (Sayar *et al* 2016), comprises its replacement by metallic alloys. These metallic implants (such as Ti_6Al_4V alloy) present

appropriate mechanical properties and biocompatibility. However, limitations in terms of bioactivity and osteointegration are commonly experienced, which give rise to problems at implant fixation, apart from infection and inflammation cases after the implant surgery (Xia *et al* 2012). Thus, to increase the bioactivity and osteointegration of these implants, cell-biomaterial interactions must be improved. These interactions have been demonstrated to be highly influenced by the stiffness, topography and chemical composition of the implant surface (Bellino *et al* 2013). Bioactive materials favor direct surface formation of apatite *in vivo*, which subsequently enables bone bonding (Karlsson *et al* 2015a). Among the wide variety of bioactive materials, titania is considered as a highly suitable biocompatible material for bone-anchoring implants exhibiting little or no toxicity, both *in vitro* and *in vivo* (Gertler *et al* 2010).

Moreover, these ceramics can be deposited as thin films over metallic alloys to provide the cells with a bioactive surface. Evaporation-induced self-assembly (EISA) is a very versatile chemical methodology (Brinker *et al* 1999) by which mesoporous titania thin films can be easily obtained onto a wide variety of substrates with a tunable mesoscopic topography and controlled wettability, porosity and chemical properties, based on a combination of sol gel chemistry, supramolecular templates and surface modifications (Soler-Illia *et al* 2012, Sanchez *et al* 2008). These mesoporous titania thin films have been proven to promote *in vivo* enhanced initial bone contact and improved distribution of bone tissue. This is due to the highly ordered arrays of monodisperse pores, high specific surface areas and bioactive properties (Meretoja *et al* 2007), which influence and eventually lead to the control of osteoblast adhesion and proliferation. The improvement of the initial attachment of cells onto the implant surfaces promotes enhanced integration of the implant and longer term stability (Bellino *et al* 2013).

Mesoporous materials have additionally shown great potential in drug delivery applications to provide and maintain drug concentrations within the therapeutic window for the desired period of time. The unique features of mesoporous biomaterials, such as high specific surface area, and tunable pore-size (with pore diameters between 2 and 50 nm), -volume and -symmetry (ordered distribution), allow drug release to occur in a highly reproducible and predictable manner (Karlsson *et al* 2015a). This is of great interest since it is believed that the local drug loading of

antibiotics, anti-inflammatory medicines and growth factors, commonly prescribed orally, intravenously, intramuscularly or topically after the implant surgery, contributes to enhancing the efficiency of these drugs, while the classic systemic routes hinder them from reaching the interface of implants and tissues (Xia *et al* 2012). The controlled local delivery of drugs at the implant might bring an efficient therapeutic treatment, since it would administrate the drug directly at the targeted cells for a prolonged time period, and thus reduce the risk of systemic side effects (Karlsson *et al* 2015a).

In the specific case of bone-anchoring implants, it has been demonstrated that their osseointegration capacity can be improved by using an inbuilt drug delivery system, such as modified mesoporous composites and mesoporous bioactive glasses, which can locally administer drugs. For instance, Karlsson *et al* (2015b) proved that the local loading of the osteoporosis bisphosphonate drug alendronate, clinically used by oral administration, promoted more extensive bone formation. Different antibiotics were also loaded at hosting molecules for efficient prevention of infections, as demonstrated by Xia *et al* (2012) by loading cephalothin in titanium dioxide coatings. Anti-inflammatory drugs (ibuprofen) were also tested by McMaster *et al* (2012) in collagen-templated bioactive titanium dioxide porous networks.

Simvastatin, a hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is known to inhibit cholesterol biosynthesis and is currently clinically prescribed for that purpose. Several recent studies have demonstrated that this cholesterol-lowering drug also promotes osteogenic differentiation, stimulating bone formation *in vitro* and *in vivo* (Maeda *et al* 2001, Mundy *et al* 1999, Yin *et al* 2012). The mechanism is thought to be related with the promotion of mitochondrial function (cell proliferation) and stimulation of the expression of the bone morphogenic protein-2 (BMP-2), a growth factor involved in osteoblast activation. In addition, simvastatin seems to participate in osteoclast inhibition by stimulating neovascularization (increasing the secretion of vascular endothelial growth factor) (Kheirallah and Almeshaly 2016).

The topical administration of simvastatin has been proposed to improve the pro-angiogenic and pro-osteogenic properties of bioglass putty in rat calvaria critical-size defects without significant

inflammation, stimulating BMP-2 and VEGF mRNA expression in osteoblasts (Allon *et al* 2012) compared to bioglass without topical simvastatin up to 4 weeks of implantation. The effects of simvastatin on osteoblastic differentiation *in vitro* were evaluated by Maeda *et al* (2001) to demonstrate that simvastatin enhances alkaline phosphatase (ALP) activity and mineralization in a dose- and time-dependent fashion. Mundy *et al* (1999) injected simvastatin (together with lovastatin) subcutaneously in calvaria of mice, resulting in increased bone formation; moreover when simvastatin was orally administered in rats, increased cancellous bone volume was also quantified. The use of statins is of great current interest as there is a clear need for non-toxic anabolic agents that will substantially increase bone formation in people who have already suffered substantial bone loss (Mundy *et al* 1999).

However, the systemic administration of statins has been related to serious side effects that can be avoided by means of local delivery of the molecule. Moreover, clinical studies suggest that orally administered statins may be degraded in the liver, so little of the drug is available to accumulate in bone. If higher doses of systemic applied simvastatin are provided, the risk of liver failure, kidney disease, rhabdomyolysis, myalgia and other side effects increase as well (Kheirallah and Almeshaly 2016). Therefore TiO_2-based biomaterials loaded with simvastatin present good prospect as coatings of metallic implants that can provide a combination of local drug delivery and bioactivity (direct bone-bonding capability) to be used for biomedical applications, such as treatment and regeneration of bone defects.

These small molecules that activate the promoter of the bone morphogenetic protein-2 gene can be readily loaded into the meso-porosity of titania thin films to provide metallic prosthesis with higher osteointegration (due to the bioactivity of the titania film) and with pro-osteogenic properties (due to local simvastatin loading), thereby promoting increased expression of the bone morphogenetic protein-2 (BMP-2) gene in bone cells. The processing and characterization of TiO_2 mesoporous coatings, their loading with different concentrations of simvastatin as well as the biological response in terms of proliferation and osteogenic activity of MC3T3-E1 pre-osteoblasts are the main objectives of the present work.

2. Materials and Methods

2.1 Mesoporous thin films

Glass discs (6 mm in diameter, Thermo Scientific) were sonicated in ethanol for 30 min and then copiously rinsed with Milli-Q water and stored in water until use. Titania mesoporous thin films were fabricated on the glass disc surfaces through the EISA approach as previously reported (Crepaldi *et al* 2003). Briefly, a precursor mixture of $TiCl_4$ (Aldrich), Pluronic F127 ($(HO(CH_2CH_2O)_{106}(CH_2CH(CH_3)O)_{70}(CH_2CH_2O)_{106}OH$), Sigma), EtOH (pure grade, ACS) and Milli-Q water was prepared. $TiCl_4$/EtOH/F127/H_2O mixtures presented a 1:40:0.005:10 molar ratio of the reagents. Then, 125 µL of the freshly prepared mixture was spin-coated (4000 rpm) on top of the glass discs. The deposited films were then placed in 50 % relative humidity chambers (obtained with a NaBr saturated solution in water) for 24 h and subjected to a stabilizing thermal treatment comprising two successive 24 h heating steps at 60 ºC and 120 ºC and a final step at 200 ºC for 2 h. The F127, acting as template, was finally removed by immersing the films in ethanol for 3 days.

2.2 Physicochemical characterization

Transmission electron microscopy (TEM) images were obtained with a JEOL JEM 1010 microscope operating at an accelerating voltage of 100 kV. Samples for TEM were obtained by scratching the films from the substrate and depositing them on carbon- and FORMVAR-coated copper grids. Scanning electron microscopy (SEM) images from the top and the cross section of the films were obtained with a JEOL JSM-6700 FEG scanning electron microscope operating at an accelerating voltage of 10 kV.

The porosity and pore size distribution were assessed by Environmental Ellipsometric Porosimetry with a SE SOPRA GES-5 spectroscopic ellipsometer in microspot configuration. This technique consists of performing spectroscopic ellipsometry measurements while varying water vapor pressure in a controlled humidity chamber. From those measurements, refractive index variation as a function of humidity is obtained and total accessible volume is calculated by using a Lorentz-Lorenz effective medium approximation. The size distribution of pores and

necks was determined from the adsorption and desorption branches of the isotherms, through the Kelvin equation, taking into account the water contact angle of the sample. The data were analyzed with Winelli software (Boissiere *et al* 2005, Soler-Illia *et al* 2012).

Raman spectra were collected using a Renishaw InVia Reflex system. The spectrograph uses high resolution grating (1200 cm^{-1}) with additional band-pass filter optics, a confocal microscope and a 2D-CCD camera. All measurements were made in a confocal microscope in backscattering geometry using a 20X objective with accumulation times of 10 s and a excitation laser line of 532 nm (Nd:YAG).

Fourier transform infrared (FTIR) spectra were recorded in Transmission mode using a Nicolet 5PCFT-IR spectrometer in the 4000−400 cm^{-1} range. Samples were prepared by scratching the film and mixing it with KBr, to form a pellet.

2.3 Determination of cytotoxicity threshold and loading of simvastatin

The cytotoxicity threshold was first determined in order to select the optimal simvastatin concentrations to be tested. Direct cell seeding of the MC3T3-E1 pre-osteoblasts (ECACC, UK) with a wide range of simvastatin ($C_{25}H_{38}O_5$, Sigma S6196, >97 % purity) concentrations in ethanol (5; 1; 0.5; 0.1; 0.05; 0.01; 0.001; 0 g/L) was carried out up to 4 days of incubation. Thus, 20 µL aliquots from each concentration were added to the corresponding well (96 well microplate) and once ethanol was evaporated, 100 µL of cell suspension ($1.7x10^5$ cel/mL) in supplemented MEM-alpha medium (Lonza BE02-002F) were added. After 4 days of incubation, and without medium refreshment to maintain the simvastatin concentrations, cell proliferation was quantified with the Cell Proliferation Kit I (MTT assay, Roche), based on the reduction of the yellow tetrazolium salt MTT (3-(4,5-dimethyltiazolyl-2)-2,5-diphenyl tetrazolium bromide) into insoluble purple formazan crystals by the mitochondrial enzyme succinate dehydrogenase, only present in living cells. To this end, 10 µL of MTT labeling reagent in phosphate buffered saline (PBS) was added to each well for 4 hours (37 ºC, 5 % CO_2). Formazan crystals were subsequently solubilized with 100 µL of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl. The plate was incubated overnight and the resulting colored solution was quantified at 595 and

655 nm using a Bio-Rad Model 550 microplate spectrophotometer. Four replicates per condition were evaluated.

The mesoporous TiO_2 thin films deposited on glass substrates were then sterilized by immersion in 70 % ethanol for 48 h at 2-8 ºC. After that, ethanol was removed and samples dried in a laminar flow chamber. The loading of TiO_2 thin films with simvastatin was performed by addition (discs already placed in 96-well microplates) of 20 µL of simvastatin solution in ethanol and maintained for 48 h at 2-8 ºC. Different simvastatin concentrations, 0.01 g/L, 0.001 g/L and 0 g/L (reference material), were tested. Finally, the samples were dried in the laminar flow chamber before cell seeding. Simvastatin loaded thin films with a concentration of 5 g/L were also characterized by using a Nicolet 5PCFT-IR spectrophotometer in the 4000−400 cm^{-1} range. Samples were prepared by scratching the film and mixing it with KBr, to form a pellet.

2.4 Biological response of simvastatin-loaded mesoporous thin films

A concentration of $1.7x10^5$ cells/mL of MC3T3-E1 pre-osteoblasts in 100 µL of MEM-alpha supplemented with 10 % Fetal Bovine Serum (FBS; Lonza DE14-801E) and 1 % Antibiotic Antimycotic Solution with 10000 units of penicillin, 10 mg of streptomycin and 25 µg of amphotericin B per mL (Sigma A5955) were seeded on the TiO_2 + 0.01, TiO_2 + 0.001 and TiO_2 + 0 discs. Empty wells on the microplates (Tissue Culture Polystyrene, TCPS) were also seeded to be used as the gold standard of the cell culture. Cells were cultured up to 21 days in a humidified atmosphere with 5 % CO_2 at 37 ºC. To induce the differentiation to osteoblasts 2-phospho-L-ascorbic acid trisodium salt (2 mM, Sigma 49752), β-glycerophosphate disodium salt hydrate (10 mM, Sigma G9422) and dexamethasone (10^{-5} M, Sigma D4902, ≥97 %) were added to the supplemented MEM-alpha. Culture medium was renewed every 2–3 days.

Cell morphology was analyzed by SEM (Philips XL30) after 7 and 21 days. After each incubation time, cells were fixed with 2.5 % glutaraldehyde solution in PBS (Lonza BE17-512F) for 2 h at 4 ºC. Samples were then washed three times for 30 min with PBS and dehydrated in graded acetone solutions (30 %, 50 %, 70 %, 80 %, 95 %) for 30 minutes in each solution and in absolute acetone for 1 h. After dehydration, samples were submitted to critical

point in CO_2, at 75 atm and 31.3 ºC, mounted on metal stubs and sputter-coated with gold prior to their analysis using a Philips XL30 scanning electron microscope (CACTI, University of Vigo).

Cell proliferation was quantified after 7 and 21 days with the Cell Proliferation Kit I (MTT assay, Roche), as described above. The plate was then incubated overnight and, after removing the discs, the resulting colored solution was quantified at 595 and 655 nm using a Bio-Rad Model 550 microplate spectrophotometer. Four replicates per material per condition were evaluated and the complete experiment was repeated twice. TiO_2 discs without cell seeding were also incubated as blanks.

Osteogenic activity was evaluated by quantifying the activity of the enzyme alkaline phosphatase (ALP) after 7 and 21 days of incubation. After each incubation time, discs were carefully rinsed twice with PBS and immersed in water. The cell lysates were obtained after the osmotic shock followed by a thermal shock by transferring the lysates from 37 to -80 ºC. ALP activity was measured using a p-nitrophenol assay. Briefly, p-nitrophenyl phosphate, which is colorless, was hydrolyzed by alkaline phosphatase at pH 10.5 and 37 ºC to form free p-nitrophenol, which is yellow. The reaction was stopped by adding NaOH and the absorbance read at 405 nm in a microplate reader (Bio-Tek, USA). The kit (Alkaline Phosphatase Assay Kit, Abcam) provides with ALP standards to calibrate the results and obtain the corresponding concentrations of enzyme (U/mL) from the absorbance data. Both cell proliferation and osteogenic activity protocols were also applied to the TCPS controls, as the gold standard reference. Four replicates were used per material per condition and the complete experiments were repeated twice. Discs without cell seeding were also incubated as blanks.

2.5 Statistical analysis

Data are presented as mean ± mean standard deviation (typical error) in error bars (n=4). Differences between groups were analyzed according to Student´s t-test with $p<0.01$ and $p<0.05$ considered statistically significant (99 % and 95 % of cases, respectively).

3. Results

3.1 Mesoporous thin films characterization and simvastatin loading

Mesoporous titania thin films were fabricated on glass disks by spin coating following the EISA procedure (Crepaldi *et al* 2003). An amphiphilic block-copolymer (Pluronic F127) was employed as pore template and $TiCl_4$ was used as TiO_2 precursor, both dissolved in ethanol. The controlled evaporation of the solvent led to assembly of the Pluronic F127 into micelles and the condensation of the precursor, generating an ordered mesophase. Stiffening of the inorganic network and removal of the template required a thermal treatment and ethanol extraction. Figure 1 shows scanning and transmission electron micrographs of the obtained thin films. Highly ordered pores forming a 3D cubic arrangement compatible with the Im3m space group (body centered cubic order, commonly obtained when using Pluronic F127 as a template (Crepaldi *et al* 2003)) were observed. The interpore distance (from center to center of neighboring pores) for these mesoporous films was of 12±2 nm, as determined from TEM and SEM image analysis. This value is in accordance with previously reported results (Crepaldi *et al* 2003). A film thickness of 95±5 nm was measured from SEM cross section images (inset in figure 1(a)).

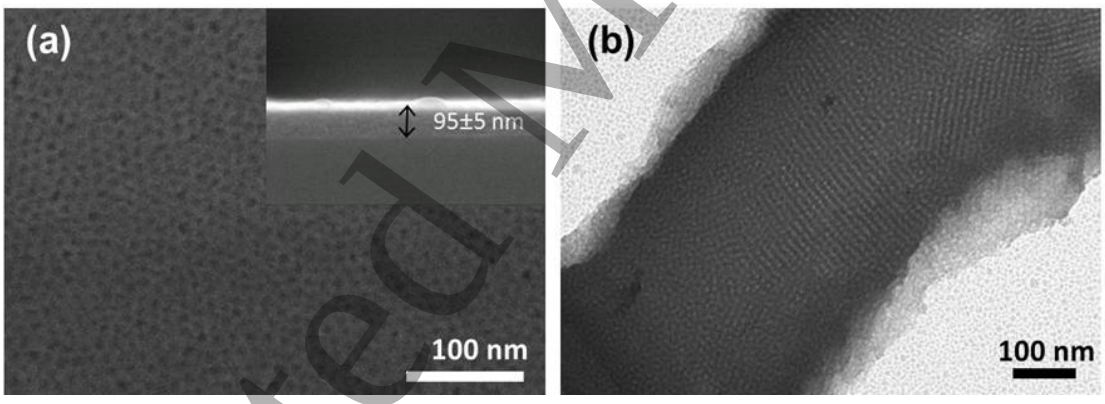

Figure 1. SEM (a) and TEM micrographs (b) of mesoporous titania thin films deposited on a glass disc. In (a) the inset is an image of the cross section of the same mesoporous thin film.

The removal of Pluronic F127 upon ethanol extraction (see experimental part for details) was evaluated by FTIR analysis and confirmed by the absence of the strong IR signals at around 2850-2930 cm^{-1} assigned to the C-H stretching vibration of the template (figure S1(a) in supporting material) (Socrates *et al* 2004). The crystalline structure of TiO_2 was evaluated by Raman spectroscopy, which revealed the characteristic lines of anatase TiO_2 phase at 150 (Eg),

194, 400 (B1g), and 636 (Eg) cm^{-1} (figure S1(b) in supporting material) (Wang *et al* 2013). However, broad of peaks at 400 and 636 cm^{-1} indicates that the titania walls present a low degree of crystalline order, in agreement with previous results (Angelomé PC *et al* 2007).

Finally, the accessible porosity and the pore size distribution of the mesoporous thin films were evaluated by Environmental Ellipsometric Porosimetry (figure 2). The adsorption-desorption isotherms shown in figure 2(a) are characteristic of mesoporous materials and demonstrate that the accessible porosity of the obtained titania mesoporous thin film was around 36 %. The pore size distribution plotted in figure 2(b) corresponds to pores of 5.5 nm (obtained from the adsorption branch) and necks between the pores of 2.8 nm (obtained from desorption branch). The porosity and pore sizes are again in agreement with previous results obtained for calcined mesoporous films (Violi *et al* 2012), which confirms the efficiency of the extraction approach to eliminate the template.

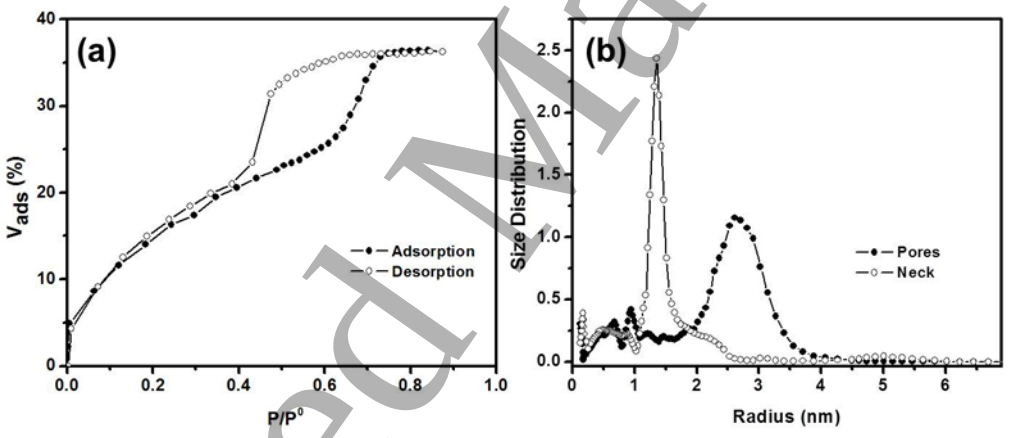

Figure 2. Water adsorption-desorption isotherms for a mesoporous titania thin film (a). Pore size (solid circles) and neck size (empty circle) distributions obtained from adsorption-desorption branches of isotherms through the Kelvin equation (b).

The high porosity of the mesoporous titania thin films together with their bioactive properties motivated us to load the mesostructures with simvastatin. Importantly, simvastatin is a molecule (see figure S2 in supporting information) with dimensions smaller than the mesoporous pore and neck sizes. In addition, the molecule presents ester and hydroxyl groups, which are known to be complexing agents for mesoporous TiO_2 (Angelomé and Soler-Illia 2005).The ability of

simvastatin to diffuse within the mesoporous thin film and attach to the TiO_2 walls was confirmed by FTIR spectroscopy. As shown in figure 3, the FTIR spectrum of a titania thin film loaded with 5 g/L of simvastatin displays the characteristic FTIR signals of this molecule, including symmetric and asymmetric stretching vibration of C–H (2800-3000 cm^{-1} and 1456 cm^{-1}), esteric carbonyl C=O stretch (1695 cm^{-1}) and bending of both C–O–C lactone (1265 cm^{-1}) and ester (1161 cm^{-1}) (Ledeti *et al* 2015), together with the ν_{TiOTi} vibrations of the oxide.

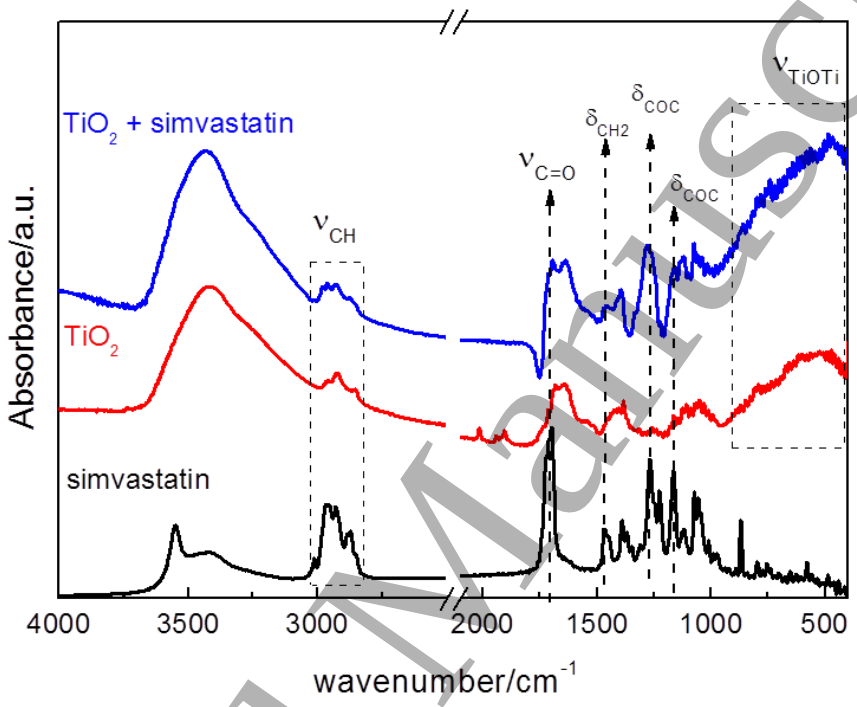

Figure 3. FTIR spectra of simvastatin (black), a mesoporous titania thin film (red) and a mesoporous titania thin film loaded with simvastatin (blue).

3.2 Biological response of simvastatin-loaded mesoporous thin films

Figure 4 presents the direct evaluation of MC3T3-E1 pre-osteoblasts viability with a wide range of simvastatin concentrations from 0 g/L to 5 g/L, in the growth media. The samples with 0 g/L were considered as the positive control for optimal cell viability. After 4 days of incubation, simvastatin concentrations $\leq$ 0.01 g/L promoted cell viability within the same range as the 0 g/L (for 0.01 g/L) or statistically significantly higher ($p < 0.01$) (for 0.001 g/L) than both 0 g/L and 0.01 g/L concentrations. However, higher simvastatin concentrations $\geq$ 0.1 g/L (from 0.1 to 5 g/L) were found to promote low cell viability, with optical density values below 0.4 which is

considered as a cytotoxic level, and significantly lower cell viability ($p < 0.01$) than the positive control (0 g/L) for all cases. The 0.05 g/L samples promoted higher cell viability (> 0.4), but still statistically significantly lower ($p<0.01$) than the positive control. Therefore, taking into account these results, the cytotoxicity threshold for simvastatin with MC3T3-E1 pre-osteoblasts could be established on simvastatin concentrations ≥ 0.05 g/L. Moreover 0.01 g/L and 0.001 g/L were considered as the optimal simvastatin concentrations to be loaded within the mesoporous titania thin films, as they seemed to ensure the same levels of cell viability or higher (in case of 0.001 g/L) than the growth medium (without simvastatin).

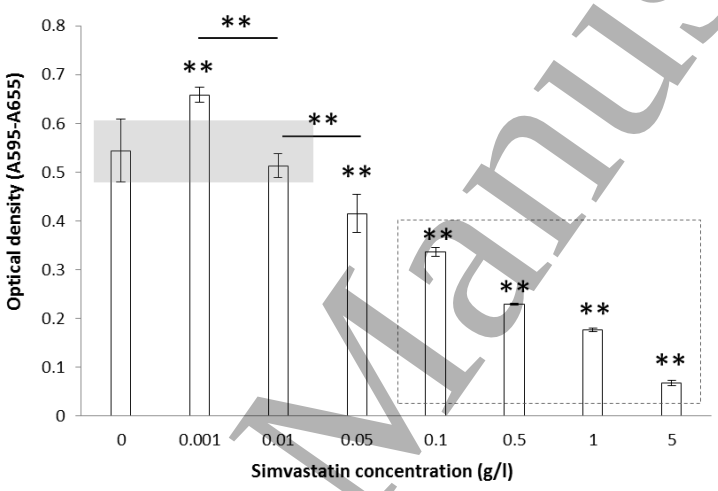

Figure 4. MC3T3-E1 pre-osteoblasts viability after 4 days of incubation with different concentrations (g/L) of simvastatin in supplemented MEM-alpha growth medium. Significant statistical differences are presented as ** for $p<0.01$ (99%) and * for $p<0.05$ (95%).

TiO_2 thin films were then loaded with the two optimal simvastatin concentrations of 0.01 g/L and 0.001 g/L and incubated with MC3T3-E1 pre-osteoblasts to evaluate their proliferation and osteogenic activity. MC3T3-E1 pre-osteoblasts proliferation on the loaded thin film coatings together with the experimental controls (0 g/L and TCPS) is presented in figure 5, as a function of incubation time (7 and 21 days). Cell proliferation on the three tested mesoporous titania thin films was significantly lower ($p<0.01$) than on the TCPS after 7 days of incubation, where the optical density value above 1.0 at this latter validated the healthy stage of the cells. After 21

days of incubation, no statistical differences were found between cell proliferation at 0 g/L mesoporous titania thin films and TCPS, thereby validating the biocompatibility of the films together with their proper sterilization and the optimal evaporation of ethanol during the loading procedure (as these 0 g/L samples were subjected to the same process as the loaded ones). Moreover, cell proliferation increased for all tested conditions, as compared to each value at 7 days. In more detail, cell proliferation at 0.01 g/L was significantly lower ($p<0.01$) than on 0.001 g/L and 0 g/L mesoporous thin films. On the other hand, the 0.001 g/L films promoted significantly higher cell proliferation than the 0.01 g/L ($p<0.01$) and the 0 g/L ($p<0.01$) films. We therefore conclude that, loading of mesoporous titania thin films with 0.001g/L simvastatin significantly promoted the proliferation of MC3T3-E1 pre-osteoblasts after 21 days of incubation, while the cell proliferation rate in case of 0.01 g/L loading was significantly slowed down.

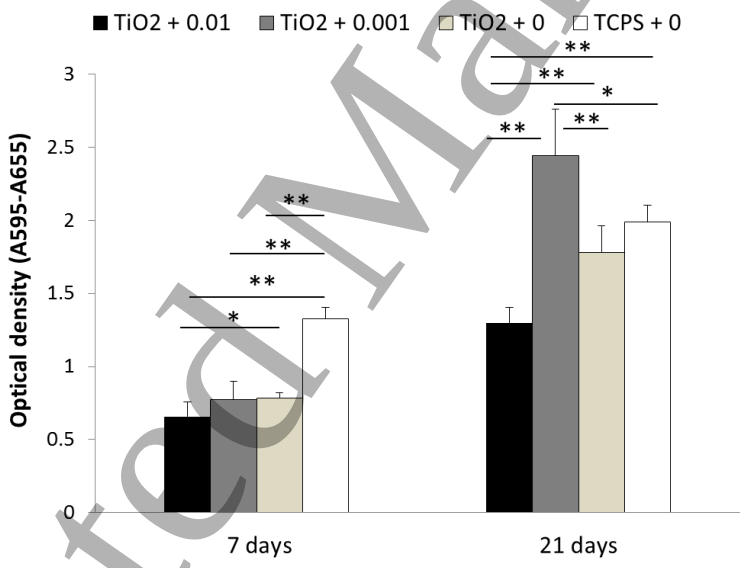

Figure 5. MC3T3-E1 pre-osteoblasts proliferation on mesoporous titania thin films loaded with 0.01 g/L, 0.001 g/L and 0 g/L of simvastatin up to 21 days of incubation. Cell proliferation for tissue culture polystyrene (TCPS) is also presented.

The effect of simvastatin loaded titania thin films on cell morphology after 21 days of incubation was analyzed by SEM. Figure 6 shows SEM micrographs of MC3T3-E1 pre-osteoblasts monolayers on thin films loaded with simvastatin at the 0.01 g/L (a, b), 0.001 g/L (c,

d) and 0 g/L (e, f). It is important to note that a cell monolayer covering the whole film surface was observed in all cases (figure 6a,c,e). Meanwhile, formation of complex filament networks and synthesis of pre-mineralized matrix were evident in 0.01 g/L (figure 6(b)) and 0 g/L (figure 6 (f)) thin films, and less intense in the case of 0.001g/L (figure 6(d)).

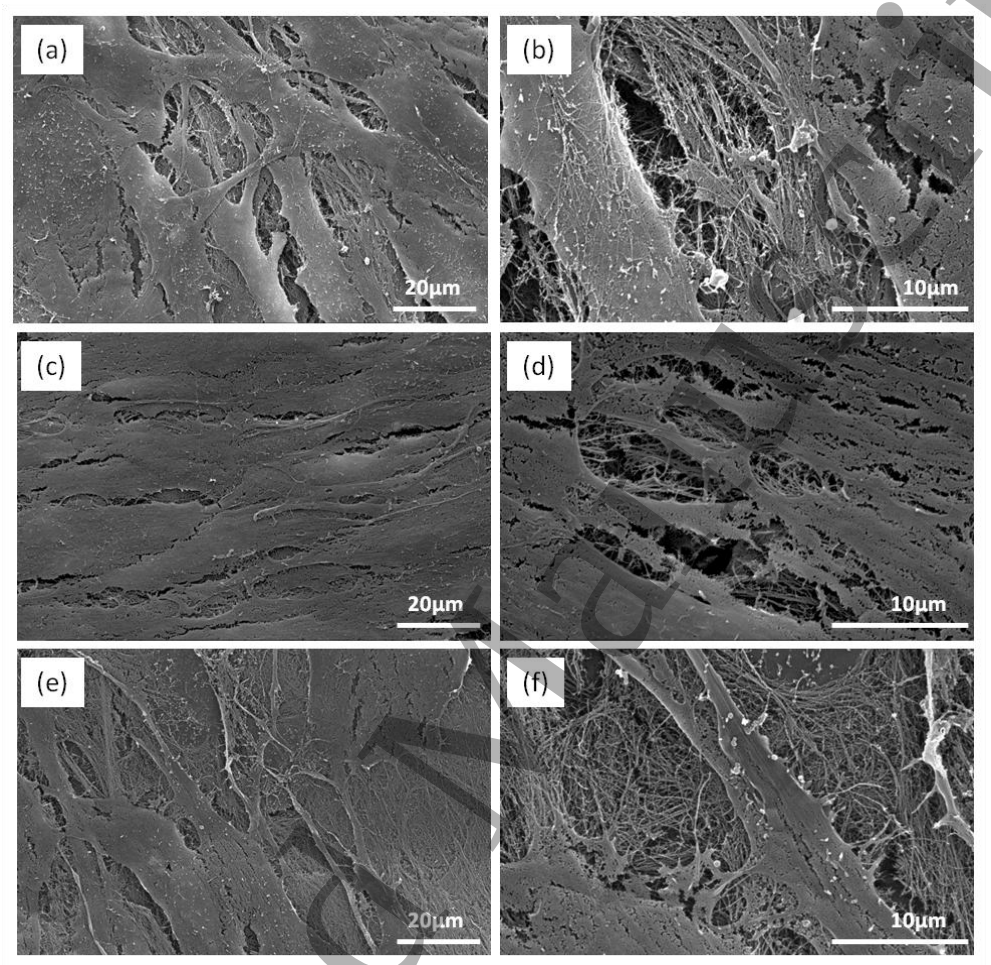

Figure 6. SEM micrographs of MC3T3-E1 pre-osteoblasts morphology on 0.01 g/L (a,b), 0.001 g/L(c,d) and 0 g/L (e,f) simvastatin loaded mesoporous titania thin films after 21 days of incubation.

The osteogenic activity of the MC3T3-E1 pre-osteoblasts was evaluated by quantifying the synthesis of the early marker alkaline phosphatase (ALP enzyme) after 7 and 21 days (figure 7). Thus, the dramatic and expected increase at the ALP synthesis by the cells at 21 days of incubation on the TCPS, compared to the value quantified at 7 days, validated the healthy stage of the cells and, therefore, the experiment. Focusing on the titania thin films, the absence of

ALP synthesis was observed at 7 days of incubation, being significantly lower ($p<0.01$) than in TCPS. A dramatic increase of ALP synthesis was also detected for the three tested titania thin films at 21 days of incubation, in analogy to TCPS, which proved the differentiation of the MC3T3-E1 pre-osteoblast like cells into osteoblasts. However differences were found between them, with significantly higher values for 0.01 g/L of simvastatin than for the two other films with lower concentrations: 0.001 ($p<0.05$) and 0 g/L ($p<0.01$). These two lower concentrations presented also significantly lower ALP synthesis than the TCPS ($p<0.05$ and $p<0.01$, respectively), while the 0.01 promoted the osteogenic activity at the same level than the gold standard.

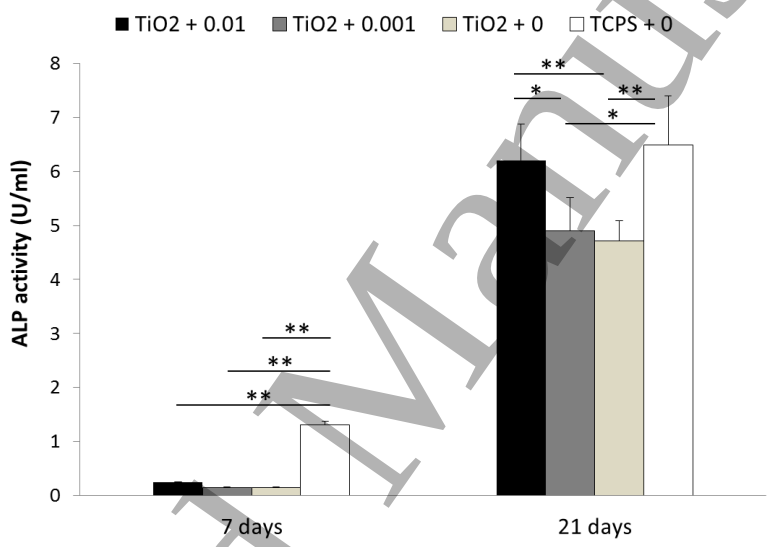

Figure 7. MC3T3-E1 pre-osteoblasts osteogenic activity (ALP synthesis) on mesoporous titania thin films loaded with 0.01 g/L, 0.001 g/L and 0 g/L of simvastatin up to 21 days of incubation. ALP synthesis quantified on the tissue culture polystyrene (TCPS) is also presented.

In figure 8 the SEM analysis of the cell monolayer microstructure at 21 days of incubation on the 0.01g/L thin films is presented. At this time of incubation, cells are found to spread on a flat morphology (figure 8(a)) with abundant lamellipodia to establish cell to cell contacts covering the entire surface and growing directly over cell layer (figure 8(b)), being the thin film under the thick layer of cells. The synthesis of pre-mineralized extracellular matrix can be observed with the formation of a complex network of pre-collagen filaments (figure 8c,d).

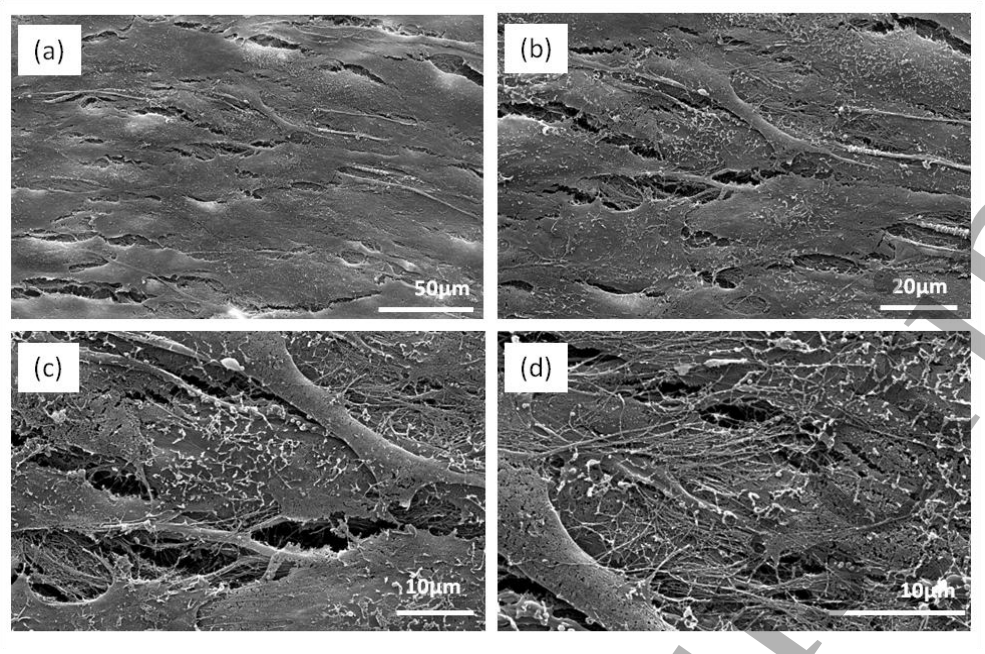

Figure 8. SEM micrographs of MC3T3-E1 pre-osteoblasts morphology on mesoporous titania thin films loaded with 0.01 g/L of simvastatin after 21 days of incubation.

4. Discussion

In the present work, the promotion of osteogenic activity by simvastastin upon loading in mesoporous titania thin films was evaluated on MC3T3-E1 pre-osteoblasts, along with the cytotoxicity threshold.

In detail, simvastatin loading within mesoporous titania thin films was confirmed together with the physicochemical characterization of the films. The abundance of highly ordered pores forming a 3D cubic arrangement was proved on the mesoporous thin films of TiO_2, with an interpore distance of 12±2 nm and a layer thickness of 95±5 nm, as presented in figure 1. The accessible porosity was quantified (figure 2) to be 36 %, with pore diameters of 5.5 nm and necks between the pores of 2.8 nm. These data, together with the simvastatin dimensions (1.2 nm x 1.2 nm) ensure the ability of the molecule to diffuse within the pores and attach to the titania walls, through ester and hydroxyl groups. Simvastatin loading was confirmed by FTIR spectroscopy (figure 3) where $\nu_{C=O}$, ν_{CH} and ν_{COC} vibrations were detected, together with ν_{TiOTi} vibrations assigned to the mesoporous film. An enhanced osteointegration was previously

observed for mesoporous titania with 6 nm of pore diameter (Harmankaya *et al* 2013, Galli *et al* 2014, Karlsson *et al* 2015b).

The ability of mesoporous titania to form chemical bonds with apatite through Ca^{2+} cations (Karlsson *et al* 2014), in combination with its suitability as drug delivery system, render it unique properties when used as implant coatings. The loading of different bone formation promoters in mesoporous titania films (alendronate (Karlsson *et al* 2015b), raloxifene (Harmankaya *et al* 2013) or magnesium (Galli *et al* 2014)) has been reported. In this context, the loading and osteogenic activity promoted by simvastatin are of interest, as it is currently prescribed to inhibit cholesterol biosynthesis and already proven as a promoter of osteogenic differentiation (Maeda *et al* 2001).

When simvastatin was directly added to the cells in growth media (figure 4) promoted MC3T3-E1 pre-osteoblasts viability at concentrations $\leq$ 0.01 g/L, but inhibited viability at higher concentrations $\geq$ 0.1g/L, with a cytotoxicity threshold at 0.05 g/L. This viability trend was maintained upon simvastatin loading into mesoporous titania thin films. Indeed, concentrations of 0.01 g/L and 0.001 g/L of simvastatin promoted the MC3T3-E1 proliferation, being statistically higher for 0.001 g/L (figures 5 and 6), and the osteogenic activity, being statistically higher for 0.01 g/L (figures 7 and 8) after 21 days of incubation. Maeda *et al* (2001) proved higher levels of osteoblast differentiation and mineralization when 10^{-7} M simvastatin was directly added to MC3T3-E1, as compared to 10^{-8} and 10^{-9} M. In our work the highest osteoblast differentiation was achieved for 0.001 g/L ($2.4x10^{-6}$ M) and 0.01 g/L ($2.4x10^{-5}$ M) upon loading in the thin films. Moreover, the cytotoxicity threshold obtained when cells were directly incubated with simvastatin was established as 0.05 g/L ($1.2x10^{-4}$M). These results demonstrate that there was still margin *in vitro* to enrich with higher concentrations of simvastatin for this cell line.

It is important to note that despite the concentrations of statins in the bone marrow have not been well established yet, it is accepted that osteoblasts and osteoclasts may be exposed to very low concentrations (Kheirallah and Almeshaly 2016). In fact, within the strategy of local administration, the coating of a porous and well interconnected scaffold of TiO_2 with alginate

hydrogel containing 10 nM of simvastatin was reported to promote the osteogenic differentiation of human mesenchymal stem cells (Pullisaar *et al* 2014). The presence of alginate and the entire scaffold of titania (8 mm in height and 9 mm in diameter) have surely contributed to these good results for such a low dose of simvastatin, against the thin film titania coatings (95 nm in height and 6mm in diameter) used at the present work.

On the other hand, higher concentrations of simvastatin were also successfully evaluated in tibial defect of rabbit model in terms of osteointegration, bone ingrowth and neovascularization. Thus, Liu *et al* (2016) evaluated porous titanium alloy scaffolds (5 mm diameter and 6 mm thickness) filled with simvastatin/poloxamer 407 hydrogel with final simvastatin concentrations of 0.1 g/L and 0.5 g/L. A significantly increased cortical bone was quantified with the 0.5 g/L simvastatin group (0.5 mg), being nearly identical to that of normal cortical bone at 8 weeks.

Moreover, in a recent review about the simvastatin influence on animal model studies (Sendyk *et al* 2016 the difficulty to equalize the optimal range of doses/concentrations of this drug was discussed. Successful results were found for simvastatin doses which range from 10^{-4} to 10^{-7} M, when directly applied on titanium implants´ surfaces or in calcium phosphate coatings, and from 50 µg to 10 mg on oxidized surface of implants, combined with chitosan as a coating or directly injected in to the femurs. However, topical application has been shown to cause local inflammation in high doses (Sendyk *et al* 2016). There is, therefore, a lack of consensus in the literature in relation to the effective doses of simvastatin, even when being tested alone directly with cells, without any carrier. Moreover, it seems to strongly depend on the drug release patterns from the loading material or administration strategy used.

5. Conclusions

The ability of simvastatin to be loaded in mesoporous titania thin films was demonstrated together with its effectiveness in terms of pro-osteogenic activity. In detail, 36 % of accessible porosity with pore diameters of 5.5 nm interpore distances of 12±2 nm in TiO_2 thin films with thicknesses of 95±5 nm, were proven to favor the diffusion of simvastatin within the pores and its attachment to the titania walls through ester and hydroxyl groups. Simvastatin concentrations $\leq$ 0.01 g/L were proven to promote MC3T3-E1 pre-osteoblasts viability, while concentrations $\geq$

0.1 g/L inhibited it, being the cytotoxicity threshold established as 0.05 g/L. Finally, a concentration of 0.001 g/L of simvastatin upon loaded on the mesoporous titania thin films promoted statistically higher MC3T3-E1 proliferation and a concentration of 0.01 g/L statistically higher osteogenic activity after 21 days in comparison to unloaded films. The *in vitro* effectiveness of simvastatin loading in mesoporous titania thin films was demonstrated, with potential application in the field of bone regeneration, especially when an implant is required. This work opens the possibility of using TiO_2 mesoporous thin films as carriers for local delivery of simvastatin to coat metallic prosthesis and provide them with pro-osteogenic activity and better osteointegration. Deep evaluation on the release pattern of this molecule is required as well as the performance *in vivo* of metallic implants coated with these simvastatin-loaded TiO_2 thin films.

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