

Laminin Coating Promotes Calcium Phosphate Precipitation on Titanium Discs *in vitro*

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ABSTRACT

Objectives: The objective of this study was to investigate the effect of a laminin coating on calcium phosphate precipitation on three potentially bioactive titanium surfaces in simulated body fluid.

Material and Methods: Blasted titanium discs were prepared by alkali and heat treatment (AH), anodic oxidation (AO) or hydroxyapatite coating (HA) and subsequently coated with laminin. A laminin coated blasted surface (B) served as a positive control while a blasted non coated (B-) served as a negative control. Surface morphology was examined by Scanning Electron Microscopy (SEM). The analysis of the precipitated calcium and phosphorous was performed by Energy Dispersive X-ray Spectroscopy (EDX).

Results: The thickness of the laminin coating was estimated at 26 Å by ellipsometry. Interferometry revealed that the coating process did not affect any of the tested topographical parameters on µm level when comparing B to B-. After 2 weeks of incubation in SBF, the alkali-heat treated discs displayed the highest calcium phosphate deposition and the B group showed higher levels of calcium phosphate than the B- group.

Conclusions: Our results suggest that laminin may have the potential to be used as a coating agent in order to enhance the osseointegrative performance of biomaterial surfaces, with the protein molecules possibly functioning as nucleation centres for apatite formation. Nevertheless, *in vivo* studies are required in order to clarify the longevity of the coating and its performance in the complex biological environment.

Keywords: laminin; titanium; biomaterials; calcium phosphates; dental implants; osseointegration.

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INTRODUCTION

Bone anchored titanium implants are widely used in the rehabilitation of edentulism. In order to enhance bone growth around implants, various chemical modifications of titanium surfaces have been proposed. Some techniques are alkali-heat treatment (AH) [1], anodization [2], coatings of calcium phosphates in sol-gels [3] and immobilization of organic bio-molecules on the surface [4]. The simulated body fluid (SBF) model has been extensively utilized for *in vitro* evaluation of various materials and surface modification methods [5-7]. The SBF is defined as a solution with ion concentrations approximately equal to those of human blood plasma [8-10]. The nucleating capacity of a biomaterial can be observed by immersing it in SBF [11]. It has been suggested that the nucleation of calcium/phosphates from SBF, mimics the initial mineralization of bone on the implant surface. The correlation between apatite formation in SBF models and bone bioactivity *in vivo* has been described in a review on the usefulness of SBF in predicting *in vivo* bone bioactivity [12]. However, compared to the SBF model, the *in vivo* process is much more complex and proteins, enzymes and biological factors play a crucial role in this process [13].

Laminin is an organic biomolecule previously utilized with success for improving the attachment of mesenchymal stem cells on TiO₂ nanotubes [14] and for enhancing the epithelial cell attachment on Ti-6Al-4V implants *in vitro* [15,16]. Laminins are heterotrimeric glycoprotein molecules that bind to a protein family known as integrins, especially $\beta 1$ and $\beta 2$ integrins [17]. Integrins are integral membrane glycoproteins which mediate cell-to-cell and cell-to-matrix interactions. Integrins are known to mediate cell adhesion to extracellular matrix and to facilitate the cell communication [18]. One of their key functions is to participate in the assembly of the cytoskeleton and thereby resulting in cell migration, adhesion of epithelial cells and hemidesmosome formation by their cytoplasmic domains [17]. *In vitro* studies indicate that laminin-1 selectively recruits osteoprogenitors through an integrin $\beta 1$ -dependent cell attachment effect [19,20] and possesses the ability to stimulate osteoblasts to produce alkaline phosphatase and deposit mineral [21]. Our hypothesis is that by coating titanium surfaces with laminin-1 enhanced precipitation of CaP in an SBF model may be achieved.

The aim of the present study was to investigate the effects of laminin coating on titanium surfaces modified by three methods claimed to provide bioactivity in terms of Ca/P precipitation, surface morphology and

surface chemistry in simulated body fluid.

MATERIAL AND METHODS

Surface preparations

Ninety discs (diameter = 8 mm, thickness = 1 mm) of titanium grade 4 were included in the study. The samples were blasted with Al₂O₃ powder with an average particle size of 120 μm with a force of 3.5 kg and from a distance of 15 mm and subsequently ultrasonically cleaned in diluted Extran MA01 and absolute ethanol and dried at 60 °C for 24 h. The specimens were then divided into five equally sized groups (n = 18). One group of blasted discs was coated with laminin and served as a positive control (B), while a non laminin-coated group of blasted specimens served as a negative control (B-). The other three groups were treated as follow and were ultimately coated with laminin.

Alkali and heat treatment (AH)

Alkali and heat treatment was performed as described in the literature [10,22,23]. In brief, the discs were soaked in 5 M aqueous NaOH for 24 h at 60 °C, rinsed with distilled water and dried at 40 °C for 24 h. Subsequently, the discs were heated until reaching 600 °C by increasing the temperature by 5 °C/min in an electrical furnace (Bitatherm, Beta Laboratory Furnaces, Israel) and were kept at 600 °C for 2 h. At the end of the process, the discs were left in the furnace until they cooled down to room temperature.

Anodic oxidation (AO)

The samples were prepared in a mixed electrolyte containing calcium ions by the Micro Arc Oxidation (MAO) method in a galvanostatic mode as described in the literature [24]. More specifically, the electrochemical cell was composed of platinum plates as cathodes with a titanium anode at the centre. A computer interfaced with a DC power supply was used to record currents and voltages at milliseconds intervals. The content of ripple was controlled to less than 0.1% [25]. Surface analysis of the oxidized surfaces demonstrated the following properties: a calcium content of 11 atomic percent in the newly formed oxide of 1.2 μm thickness, 24% porosity and mixed anatase and rutile crystal structure [26,27].

Hydroxyapatite coating (HA)

A thin hydroxyapatite layer (< 50 nm) was obtained by dipping the titanium discs (Ti-discs) into a solution containing surfactants, water, organic solvent and crystalline hydroxyapatite particles with a Ca/P ratio of 1.67. The diameter of the hydroxyapatite particles

was approximately 10 nm. After the dipping procedure, the discs were let to dry in open air for 30 min, allowing the organic solvent to evaporate. To remove all dispersing agents, the discs were subjected to heat treatment at 550 °C for 5 min [28].

Laminin coating and quantification

Laminin coating

Laminin (Sigma-Aldrich, L2020, Stockholm, Sweden) was diluted to a concentration of 100 µg/ml in PBS containing 0.15M NaCl, at pH 7.4 at room temperature. The titanium discs belonging to groups B, AH, AO and HA were subsequently incubated for 1 h at room temperature in 48 well plates (Nunclon Surface, Nunc, Roskilde, Denmark) containing 250 µl per well of the laminin solution. The discs were then rinsed with Milli-Q water and blown dry in order to avoid deposition of salts and remove non-adsorbed proteins.

Optically smooth titanium surface preparation

For quantification of adsorbed laminin layer optically smooth titanium surfaces were prepared as described by Linderbäck et al. [29]. Cleaned SiO₂ surfaces were placed in an evaporation chamber with a final pressure below 1×10^{-8} Torr. Approximately 200 nm of titanium was physical vapour deposited (PVD), and thereafter spontaneously oxidized at room conditions. The static water contact angle of optically smooth titanium was $\Theta < 10^\circ$ at room conditions and $> 50^\circ$ after heating to 300 – 500 °C at room conditions for 30 min. The effect of UVO-treatment (Ultra Violet Ozone) was investigated on a set of surfaces illuminated for up to 96 h in a UVO preparation chamber (Jelight Company Inc., Irvine-USA). The wavelengths of the emitted light were 253.7 nm (100%) and 184.9 nm (19%), respectively. The samples were placed within 2 cm from the lamp (Novakemi AB, Handen-Sweden), and the UVO-chamber temperature was below 100 °C. Changes in surface hydrophilicity prior and subsequent to UVO-illumination or annealing was characterized using an OCA 15 plus contact angle microscope (CAM) used in sessile drop mode (Dataphysics Instruments GmbH, Filderstadt, Germany).

Ellipsometry

The amount adsorbed laminin was calculated on the optically smooth titanium surfaces. The surface treated discs were not possible to analyze, since these surfaces did not reflect the laser beam in a measurable manner. Optically smooth titanium surfaces were fixed in the ellipsometric cuvette filled with PBS at room temperature. The ellipsometry angles Δ_0 and Ψ_0 were measured with a Rudolph Research

AutoEL III ellipsometer operating in a wavelength of 632.8 nm at a 70° angle of incidence. Thereafter, the cuvette was emptied and filled with laminin solution and new angles Δ and Ψ calculated. The protein layer thickness was iterated from the ellipsometer angle changes under the assumption that the protein refractive index was $n = 1.465$. The McCrackin algorithm was used for the calculations [30].

SBF immersion

The revised SBF (r-SBF) used in this study was prepared according to the literature [31]. In brief, 5.403 g NaCl (Merck, Darmstadt, Germany), 0.740 g NaHCO₃ (Merck, Darmstadt, Germany), 2.046 g Na₂CO₃ (Merck, Darmstadt, Germany), 0.225 g KCl (Merck, Darmstadt, Germany), 0.230 g K₂HPO₄ · 3H₂O (Merck, Darmstadt, Germany), 0.311 g MgCl₂ · 6H₂O (Merck, Darmstadt, Germany), 11.928 g 2-(4-[2-hydroxyethyl]-1-piperazinyl) ethanesulfonic acid (HEPES) (Reach Organics Inc., Cleveland, Ohio, USA), 0.293 g CaCl₂ (KEBO Lab AB, Spånga, Sweden) and 0.072 g Na₂SO₄ (Merck, Darmstadt, Germany) were dissolved in 1000 ml distilled water. HEPES was dissolved in 100 ml distilled water before being added to the solution and the final pH was adjusted to 7.4 at 37 °C.

The discs were immersed in 25 ml r-SBF in separate sealed polystyrene vials at 37 °C. After immersion for 1 h, 1 day, 3 days, 1 week and 2 weeks, the r-SBF immersion was interrupted and the specimens rinsed with distilled water in order to remove any loosely attached calcium phosphate. Thereafter, the specimens were left to dry at room temperature and ultimately sealed in dry vials. Three samples of each type of surface were not immersed in r-SBF (0 hours).

Topographic characterization

The specimens were topographically characterized after immersion in r-SBF with an interferometer MicroXam (Phase-Shift, Tucson, Arizona, USA) operating in a wave length of $\lambda = 550$ nm.

A Gaussian filter with size $50 \times 50 \mu\text{m}^2$ was applied to separate roughness from form and waviness. Thereafter, the surface roughness was calculated by using the following topographical parameters defined as essential for describing the topography of biomaterial surfaces [32]:

S_a = Arithmetic mean height deviation from a mean plane (μm).

S_{ds} = Density of summits, i.e. the number of summits of a unit sampling area (μm^{-2}).

S_{dr} = Developed interfacial area ratio, i.e. the ratio of the increment of the interfacial area of a surface over

the sampling area (%).

Calculations of group means and standard errors for each surface preparation and time point were performed.

Scanning electron microscopy/energy dispersive X-ray analysis (SEM/EDX)

For the SEM analysis, a LEO Ultra 55 FEG SEM equipped with an Oxford Inca EDX system, operating at 8 and 10 kV was used. The samples were examined without surface sputtering. Micrographs were recorded at different magnifications to investigate both the surface coverage and the morphology of the crystals. EDX analysis at a magnification of 150 times was performed to describe the atomic composition. Two samples from each surface composition. Three titanium discs for each preparation and incubation time were analyzed and a mean value calculated.

Statistical analysis

The normal distribution of the variables was confirmed by Kolmogorov – Smirnov test. Statistical analysis was performed with Statistical Package for the Social Sciences for Windows, version 18 (SPSS®, Chicago, Illinois, USA) using one-way ANOVA (Analysis of Variance). The multiple paired comparisons were performed by Bonferroni Post-Hoc test. The statistical significance level was defined at 0.05.

RESULTS

Topographical analysis

As demonstrated by Table 1, the laminin coating elicited no difference on the examined topographic parameters S_a , S_{ds} and S_{dr} when comparing group B and group B- ($P > 0.05$). Treatment of the titanium discs with alkali and heat resulted in the lowest S_a and the highest density of summits ($P < 0.05$) among the tested surface modifications. As a total, the AH – group possessed the highest developed interfacial area ratio resulting in larger total surface.

Ellipsometry

The thickness of the adsorbed protein was estimated to 26 Å, approximating 180 ng/cm² [30]. The layer approximates two monolayers in thickness.

SEM/EDX

SEM-images were acquired with a × 5000 magnification

Table 1. S_a , S_{ds} and S_{dr} for the five different surface groups. Mean values and standard errors are presented

Surface	S_a (µm)	S_{ds} (µm ²)	S_{dr} (%)
B-	1.36 ± 0.05	153873.2 ± 2585.8	60.99 ± 2.33
B	1.30 ± 0.07	157884.7 ± 6021.2	60.13 ± 5.85
AH	1.24 ± 0.09 ^a	234634.8 ± 8454.7 ^a	84.61 ± 2.32 ^a
AO	1.38 ± 0.05	171155.8 ± 5768.1	73.84 ± 3.14
HA	1.33 ± 0.09	168952.5 ± 3574.2	66.00 ± 6.07

^aSignificant at the level $P < 0.05$ (Bonferroni Post-Hoc test).

B- = uncoated blasted titanium; B = blasted and laminin coated; AH = alkali heat treated and laminin coated; AO = anodic oxidized and laminin coated; HA = hydroxyapatite and laminin coated.

prior to and after 2 weeks of incubation in r-SBF. The images prior to incubation demonstrated no differences in surface morphology when comparing the uncoated (Figure 1a) to the coated control blasted surface (Figure 1b). On the contrary, the alkali and heat treated titanium surfaces (Figure 1c) demonstrated a smooth surface, covered with microscopic spike-like structures and the anodic oxidated ones possessed a porous appearance (Figure 1d). However, no differences were detected in surface morphology when comparing the nano-sized hydroxyapatite coated surface (Figure 1e) to the controls (Figures 1a and 1b). All three surfaces displayed a sharp-edged appearance, which probably depended on the blasting procedure, and included some Al₂O₃ crystals. Nano-sized hydroxyapatite crystals could not be observed at this magnification. In general, no laminin molecules were observed at this magnification probably depending on the fact that the magnification was not high enough to reveal nano-sized features.

After 2 weeks of incubation in SBF, the surfaces B, AH and AO were fully covered with a homogenous calcium phosphate layer (Figures 2b, 2c and 2d). On the contrary, the HA surface (Figure 2e) and the B- surface (Figure 2a) appeared to be only partially covered by crystals.

EDX

Calcium phosphate (CaP)

The total amount of calcium phosphate on surfaces of titanium discs was assessed with EDX by measuring and adding the relative elemental amount of calcium (Ca) and phosphorous (P) present on the surface. AH surfaces showed the highest Ca and P content after 72 h, 1 week and 2 weeks. After 2 weeks no significant differences were detected among the test surfaces AO, HA and the positive control B. At the same time, the negative control B- demonstrated the lowest Ca and P precipitation (Figure 3).

Calcium/Phosphorous ratio (Ca/P ratio)

The proposed bioactive surfaces, i.e. AH, AO and HA treated samples, demonstrated a higher Ca/P ratio than both blasted control samples (B and B-) during the first 24 hours. Noteworthy, calcium and phosphate signals were detected at an earlier time point on bioactive surfaces compared to blasted surfaces, depending on the bioactive modification process. The high early Ca content on the AH surface contributed to a high Ca/P ratio. After 2 weeks of SBF immersion, all the surface groups presented a Ca/P ratio around 1.67, corresponding to hydroxyapatite crystalline formation (Figure 4). Interestingly, the high Ca/P ratio of AH and AO surfaces at early time points decreases with increasing SBF incubation time.

DISCUSSION

Protein coatings have previously been used as a method to stimulate bone formation around implants in different experimental models with promising results [33-36]. Although there is a general agreement that a proper protein coating is promising in terms of enhanced osseointegration, there is still little known regarding the involved mechanisms. The main objective of this study was to study the etiology of the phenomenon in a controlled *in vitro* milieu as the SBF model.

The results demonstrate that all laminin coated surfaces induced a higher final CaP deposition as compared to uncoated blasted titanium after 2 weeks. The fact that all the “bioactively” modified surfaces, i.e. AH, AO, HA, demonstrate a higher CaP formation is in agreement with the findings of Arvidsson et al. [37]. The present study demonstrates that a thin laminin coating on a blasted titanium surface results to enhanced CaP precipitation as compared to a surface without laminin (B-) and

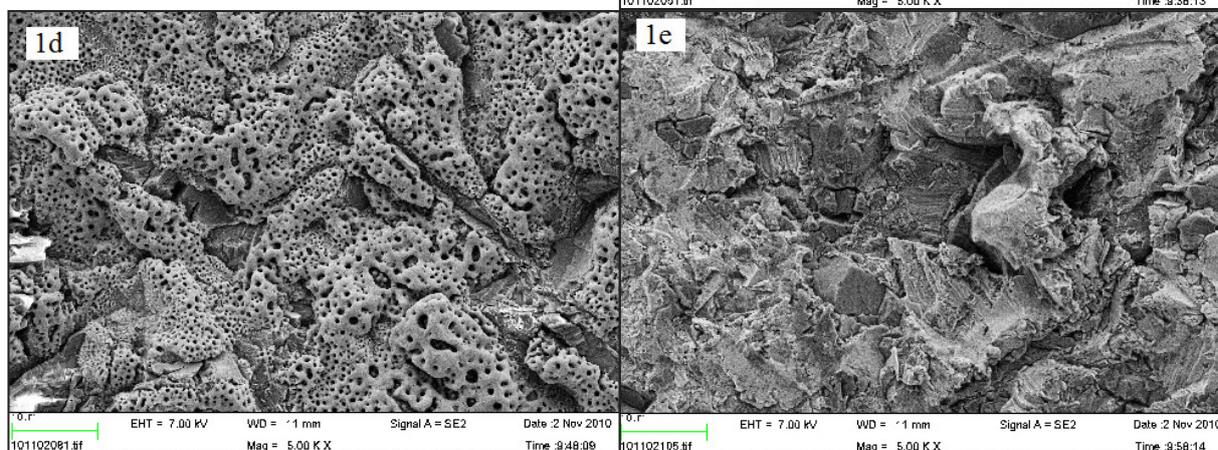


Figure 1. SEM images of titanium discs prior to incubation in SBF (× 5000): (a) B- = uncoated blasted; (b) B = blasted and laminin coated; (c) AH = alkali heat treated and laminin coated; (d) AO = anodic oxidized and laminin coated; (e) HA = hydroxyapatite and laminin coated. The bar presents 10µm.

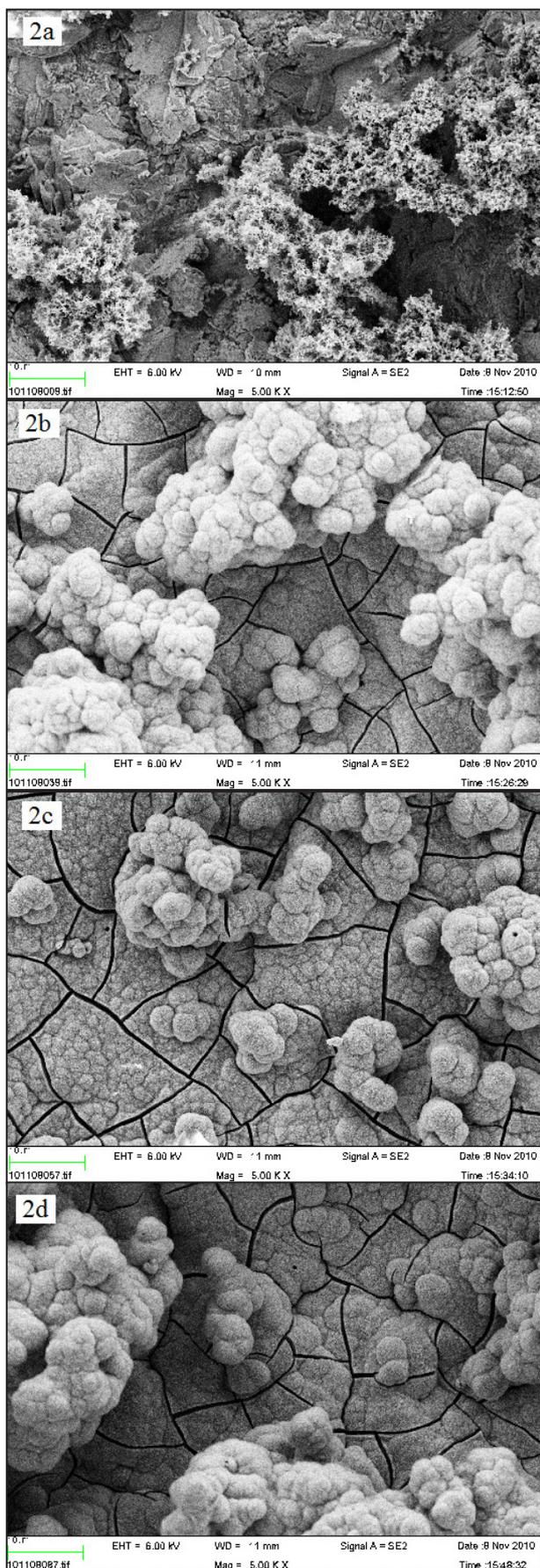


Figure 2. SEM image of a titanium discs after incubation in SBF for 2 weeks ($\times 5000$): (a) B- = uncoated blasted; (b) B = blasted and laminin coated; (c) AH = alkali heat treated and laminin coated; (d) AO = anodic oxidized and laminin coated; (e) HA = hydroxyapatite and laminin coated. The bar presents 10 μm .

to similar CaP precipitation as compared to bioactively modified surfaces. Therefore, we have reasons to believe that a laminin coating may be utilized as a method to bioactively modify a non bioactive surface.

The SEM image demonstrating a laminin coated blasted disc after 2 weeks in SBF (Figure 2b), is similar to the images corresponding the AH (Figure 2c) and AO specimens (Figure 2d). In those images, the underlying titanium is not visible depended on deposition of CaP and large crystals. Interestingly, as examined with EDX, after 2 weeks the Ca/P ratio is approximately 1.67 corresponding to hydroxyapatite, indicating that the formed CaP is hydroxyapatite. A possible explanation for the high performance of the AH surface, in terms of

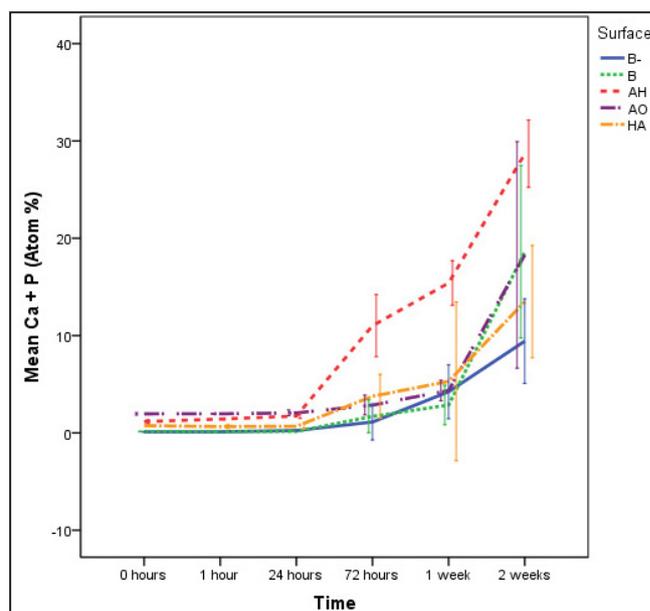
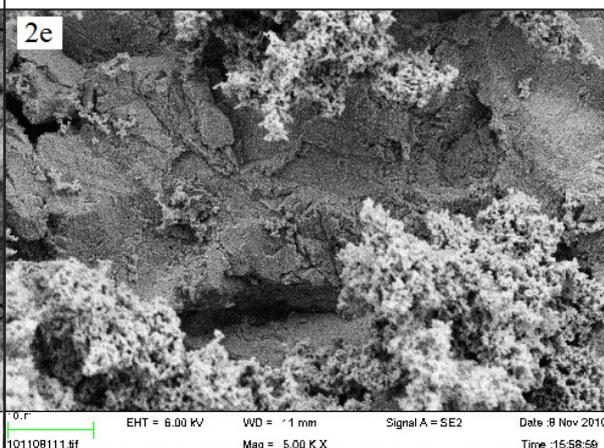


Figure 3. Total amount of precipitated calcium and phosphate on titanium discs calculated by EDX: B- = uncoated blasted; B = blasted and laminin coated; AH = alkali heat treated and laminin coated; AO = anodic oxidized and laminin coated; HA = hydroxyapatite and laminin coated. Mean values and standard errors are presented.



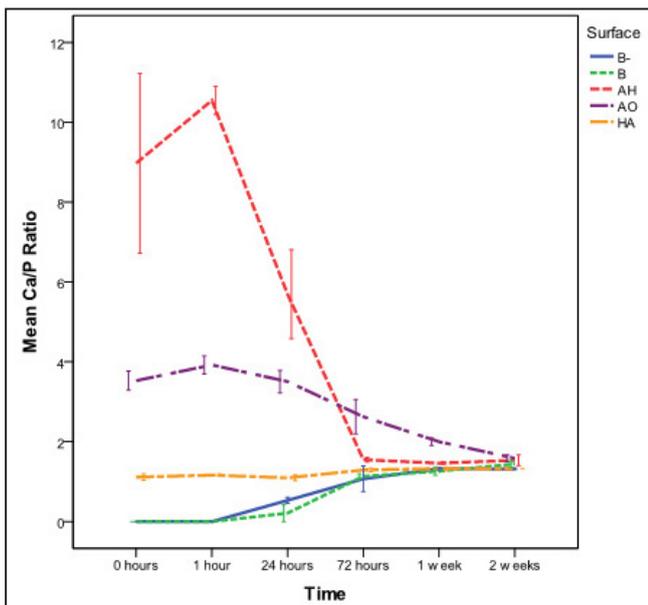


Figure 4. Calcium/phosphorous ratio on titanium discs calculated by EDX: B- = uncoated blasted titanium; B = blasted and laminin coated; AH = alkali heat treated and laminin coated; AO = anodic oxidized and laminin coated; HA = hydroxyapatite and laminin coated. Mean values and standard errors are presented.

CaP precipitation, may be the fact that the developed interfacial area ratio S_{dr} according to Table 1 is the highest among the tested surfaces, thereby providing a larger interface area with the SBF. This observation demonstrates that it is imperative to take into account at least one hybrid factor when characterizing surface topography, since considering solely S_a may lead to false conclusions.

The results of a previous study from our group [38] suggest that a possible mechanism for laminin promoting CaP precipitation *in vitro*, may be its function as a nucleation centre. According to a morphological study examining the same laminin molecule that we utilized in the present study, laminin tends to assume a globular form when used for surface coating [39]. Some of the domains exposed on this protein conformation may act as nucleation centres for calcium ions thereby increasing the local calcium concentration, leading to enhanced nucleation ratio. In a recent study on osteoblasts the effect of elevated extracellular calcium concentration was proposed to stimulate osteoblasts through the receptor activator of NF- κ B ligand [40]. Hence, it may be possible that the enhanced calcium and phosphate precipitation triggers osteoblast differentiation around a laminin coated implant also when applied *in vivo*. There is some indication that enhanced CaP precipitation may be relatively specific for laminin since coating with bovine serum albumin instead reduced CaP precipitation when compared to non-coated

hydroxyapatite surfaces [41]. An additional mechanism suggesting that laminin may be a bone stimulating coating agent is the possible interaction of the arginine-glycine-aspartic acid (RGD) motif with the integrin receptors of the surrounding cells. It has been reported that when RGD-sequences are coated on implants, osseointegration [36,42] seems to be enhanced, and the osteoblast adhesion appears to be upregulated through integrin-mediated mechanisms [43]. Nevertheless, the complexity of the *in vivo* environment makes it imperative to further investigate parameters such as the retention and the clearance of the coating before any further conclusions may be drawn regarding the suitability of laminin as a biomaterial coating agent.

CONCLUSIONS

The results of the present study demonstrate that after 2 weeks of incubation in SBF all the laminin coated titanium surfaces, including the laminin coated blasted controls, induced a higher CaP deposition as compared to uncoated blasted titanium discs. Among the tested surface modifications, alkali and heat treatment seemed to induce a more rapid CaP precipitation. Our study demonstrates that laminin may have the potential to be used as a coating agent in order to enhance the osseointegrative performance of biomaterial surfaces with the protein molecules possibly functioning as nucleation centres for apatite formation. *In vivo* studies are however needed in order to further investigate the possible effect of laminin as a coating agent in the osseointegration of titanium implants.

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