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Alumina-Zirconia composites functionalised with laminin-1 and laminin-5 for dentistry: effect of protein adsorption on cellular response

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Abstract

The present paper describes a study on laminin interaction with the surface of two alumina-zirconia composites with different percentages of ZrO_2, both with submicrometric grain size. As major molecules within the basement membrane (BM), laminins are important protein fragments for epithelial cell adhesion and migration. On the other hand, alumina zirconia composites are very attractive materials for dental applications due to their esthetic and mechanical properties. X-Ray photoelectron Spectroscopy and Atomic Force Microscopy were used to study the adsorption of two types of laminin, Laminin-1 (Ln-1) and Laminin-5 (Ln-5), onto the ceramics surfaces. The *in-vitro* cell response was determined by intracellular phosphorylation of major kinases. Ceramics samples functionalised with laminins showed better cellular activation than untreated specimens; furthermore, cellular activation was found to be greater for the composite with higher percentage in

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zirconia when functionalised with Ln-5, whereas the adsorption of Ln-1 resulted in a greater activation for the alumina-rich oxide.

Keywords : Laminin 5, Laminin 1, Zirconia, Alumina, X ray Photoelectron Spectroscopy, Atomic Force Microscopy, Cell Response.

1. Introduction

Oxidic materials, such as alumina and yttria-stabilised zirconia (Y-TZP) ceramics combine good mechanical and tribological properties with biocompatibility. They are therefore suitable for biomedical applications [1-4]. However, downsides exist: monophase alumina is not used in applications where osseointegration is required, while the low temperature degradation (LTD) of zirconia, the so-called ageing process, is responsible for failures in vivo. These limits were recently overcome with the preparation of composite materials made of alumina and zirconia[1], which have been successfully used for femoral heads. From a mechanical point of view, the technical advantages achieved owed mainly to the limited transition from the tetragonal to monoclinic phase – thus avoiding failures [5] – and to an increase of the material toughness [6-8]. Within the oxide composite, zirconia allows for the formation of apatite-like calcium phosphate deposits, whenever appropriate surface treatments are carried out [9], which is recognized as an index of bioactivity for materials at the interface with bone.

Moreover, the work of Ko *et al.*[10] has proven that these ceramics may show at least similar, if not slightly better, biological responses than the commercial pure titanium usually employed for dental implants. Because of their color, ceramic materials are well-suited for aesthetic oral rehabilitations, e.g. for frontal dental implants. Dental implants are known to interface with three kinds of cells, namely i) osteoblasts/ostecytes (bone), ii) fibroblasts (connective tissue), iii) epithelial cells. The good integration of dental implants depends not only on the bone healing, but also on a proper gingival epithelium attachment [11]. Indeed, when dealing with normal dental anatomy, epithelial cells interact with enamel or cementum via an extracellular matrix called the internal basal lamina (IBL). This matrix cements the epithelium and the tooth together via hemidesmosomes [12]. In implants, such structures could only be found in the apical part of the junctional peri-implant epithelium and they look discontinuous with respect to the dento-gingival interface. This discontinuity is supposedly related to a lack of laminin 5 (Ln-5) in the upper part of the IBL [13] and it has been claimed that the loss of adhesive structures enhances the probability of

bacterial infection on the implant [14]. The basal lamina contains laminins, type IV collagen, nidogens and fibronectin, but the composition of the IBL, located on the tooth surface side, is not well established. However, the presence of laminin-1 (Ln-1) [15] and Ln-5 [13] has been reported. The laminins binding with integrin $\alpha_6\beta_4$ have a major role in the nucleation of the hemidesmosome. Ln-5 binds more integrin $\alpha_6\beta_4$ than Ln-1 [16] and more human epidermal keratinocytes cells on Ti-6Al-4V surfaces coated with Ln-5 than with Ln-1[17] were observed, therefore Ikeda *et al.* suggest that both laminins contribute to the hemidesmosomal organization and cell adhesion[15].

Pre-treatment of oxidic based implants with laminins might be a way to favor the healing of gingival tissues along the surface. For implant materials, surface composition, morphology, roughness and wettability are different factors that can influence protein adsorption, and therefore cellular response. Also the hydroxyls density has be found to affect the laminin adsorption: for instance, by varying the amount of OH groups on surfaces functionalised with different co-polymers, Hernández *et al.*[18] showed that the quantity of Ln-1 adsorbed affects hydrophobic materials more than the hydrophilic ones. Differences in surface roughness are also an important factor influencing protein adsorption[19, 20].

The adsorption of laminins on titanium implants has already been reported. In particular, Werner *et al.* demonstrated how Ln-5 strongly favours the *in vitro* formation of adhesion structure (hemidesomsomes)[21]. A recent study reports that silane-linked Ln-1 on a PDMS surface significantly improves cells adhesion and proliferation[22]. However, no studies regarding the influence of this protein adsorbed onto composite oxidic materials are present in literature, in spite of their suitability as dental implants.

This study is focused on the effect of the adsorption of Ln-1 and Ln-5 onto Zirconia Toughened Alumina (ZTA, 16wt% in zirconia) and Alumina Toughened Zirconia (ATZ, 80wt% of Zirconia). The choice of the two compositions arises from the absence of a phase transition, in the case of ZTA, and the limited phase transition combined with high bioactivity for ATZ [9]. The protein functionalised surfaces were studied via AFM and XPS with the goal of assessing the distribution

and the relative quantities of laminin. The response of epithelial cells towards the oxidic materials after laminin adsorption was then evaluated for cell viability, cell spreading, activation of some major intra-cellular pathways and analysis of the conditioned cell media.

2 Material and Methods

2.1 Sample preparation

High purity powders were used to produce the oxidic disks: Taimei Al_2O_3-16wt % ZrO_2 (ZTA, Taimicron, Taimei, Japan) and ZrO_2-20wt% Al_2O_3, (ATZ, TZ-3Y20AB, Tosoh, Japan), in form of "ready to press" powders, so that no additional mixing was required before pressing. Green samples were obtained by linear pressuring at 80 MPa followed by Cold Isostatic Pressing at 200MPa. The best conditions for the sintering process were: heating at a rate of 50°C/h up to 700°C, followed by a 2 hour dwell; heating at a rate of 100°C/h up to temperature sintering of 1500°C, followed by a 2 hour dwell. Fully dense materials were obtained by this procedure, as reported elsewhere[9]. The resulting samples were 12 mm disks with thickness ranging between 4 and 5 mm. They were mirror polished with diamond suspension in ethanol with decreasing granulometry to the final surface roughness of less than one micron. Hardness, toughness and strength were measured. Further details about the experimental setup are reported elsewhere[9].

2.2 Microstructure analysis and surface characterization

Microstructure was studied by means of a Scanning Electron Microscope Zeiss EVO 50 with Energy Dispersion Spectroscopy analyzer for elemental composition detection. XPS signals were collected using a SPECS (Phoibos MCD 150) X-ray photoelectron spectrometer, with Mg Kα radiation (1253.6 eV) as X-ray source, having a power of 150 W (12 mA, 12.5 kV). The spot size of the analyzed region is 7 mm x 20 mm. The emissions of photoelectrons from the sample were analyzed at a take-off angle of 90° under Ultra High Vacuum conditions. No charge compensation was applied during acquisition. After collection, the binding energies (BEs) were calibrated on the Al 2p signal of Al_2O_3 having a BE of 74 eV. The accuracy of the reported BEs values can be

estimated to be ± 0.1 eV. The XPS peak areas were determined after subtraction of a background. The atomic ratio calculations were performed after normalisation using Scofield factors of element X. All spectrum processing was carried out using the Casa XPS v2.3.13 software (Casa Software Ltd.) package and Origin 7.1 (Origin Laboratory Corp.). The spectral decomposition was performed by using Gaussian-Lorentzian (70%/30%) functions, and the full width at half maximum (FWHM) is fixed for each given peak.

The surface morphology of the ZTA and ATZ samples was characterised using an atomic force microscope (AFM, Park System XE100) in intermittent mode. The scan size was 3×3 μm^2 with a scan rate of 0.4 Hz. The measurements of the root mean square (RMS), average (R_a) and peak-to-valley (R_{pv}) values of the roughness of these samples were evaluated from images taken in five random areas on three samples.

2.3. Laminin adsorption

To ensure a proper surface cleaning before laminin and cellular adsorption, the samples were placed in an oven at 900°C for 24 hours. They were further cleaned in an ultrasonic bath with three common solvents of different polarities, i.e. milliQ water, ethanol and acetone, which were used for 30 min each. Human Laminin 5 (Ln-5) and Natural Mouse Laminin (Ln-1) were purchased from Immundiagnostik (AP1002AG.1, Immunodiagnostik AG, Germany) and Sigma (L2020, Sigma-Aldrich, USA), respectively.

A 0.3 mg/ml sterile stock solution for the two laminins was prepared in phosphate buffer (PBS), from which a concentration of $2.35x10^{-5}$ mol^{-1}, was obtained. The solutions were mixed in a vortex for a few seconds and 212 µL were dispensed onto the planar ceramic samples in a multi-well plate to adsorb laminins at room temperature. After 1 hour, the specimens were rinsed with a milliQ water spray, then immersed for 5s second in milliQ water and finally dried under N_2 flow. The reagents and samples needed for cell culturing were prepared and manipulated under a laminar flow hood to preserve sterility in all phases of manipulation.

2.4 Cell culturing

The biological effects of laminin functionalisation were assessed in-vitro using HeLa epithelial cells, which were purchased from ATCC (ATCC number: CCL-2). Cells were maintained in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum (Gibco Life Technologies, Milan, Italy), 100 U/ml penicillin, 100 µg/ml streptomycin, were passaged at subconfluency to prevent contact inhibition and were kept under a humidified atmosphere of 5% CO_2 in air, at 37°C. Cells were seeded onto the ATZ and ZTA samples (12 mm diameter), which were treated with Ln-1 and Ln-5 or left uncoated, at a concentration of $5x10^5$ cells/well in a 24-well plate (BD, Milan Italy).

2.5 Cell attachment assay

The ATZ and ZTA discs were placed in 24-well culture dishes and HeLa cells seeded into the wells at a density of $5x10^4$ cells/well. The cells were incubated for 4, 12, 24 and 48 hours. After washing in PBS, cells were detached from the discs using 0.05% trypsin-EDTA at each time point and resuspended in 0.4% Trypan Blue. Finally, the cells were counted using an automated cell counter (Countess, Life Technology, Milan Italy) able to estimate also cell viability.

2.6 Cell viability (MTT assay)

Cells were plated at density of $5x10^5$cells/well in 24-well culture dishes and the viability was assessed by colorimetric MTT assay (Chemicon International, Billerica, MA, USA) according to the manufacturer's protocol at 1, 2 and 3 days.

2.7 Analysis of the major phosphorylated proteins present in cell lysates

The intracellular concentration of specific phosphorylated proteins (p-p90, p-ERK1/2, p-JNK, p-p38 MAPK, MEK, c-jun, HSP27) was measured within cell lysates obtained from the HeLa cells cultured overnight and starved for four hours. The Phosphorylated Protein Bio-Plex Assay (Bio-Rad Laboratories, Hercules, CA, USA), which is based on a capture sandwich immunoassay, allowed for the simultaneous dosage of different biomolecules to be done in a single microplate well. Briefly, fluorescent-dyed microspheres (Luminex Corp, Austin, TX, USA) are conjugated with a specific primary antibody and the secondary antibody is biotin-labeled. A strepatividin-biotin reaction is

used to quantify the amount of analyte using the fluorescent system to identify the analyte. All samples were analysed following the manufacturer's protocol. At least three independent repetitions in duplicate were made per experimental condition type. The analyte concentrations were expressed in pg/ml. The data regarding each phosphorylated protein was normalized with respect to the corresponding non-phosphorylated protein. A High Photomultiplier Tube Setting (PMT setting) was prepared and then fitted by Bio-Plex Manager software.

2.8 Analysis of the cytokines, chemokines and growth factors present in cell conditioned media

The concentration of specific cytokines and a vast number of growth factors released by starved cells was measured within four-hour conditioned media [interleukin-1b (IL-1b), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12 (IL-12), interleukin-15 (IL-15), interleukin-17 (IL-17), tumor necrosis factor-α (TNF-α), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-gamma (INF-γ), monocyte chemoattractant protein-1 (MCP-1), CXCL10 chemokine (IP-10), eotaxin, platelet derived growth factor (PDGF), basic-fibroblastic growth factor (bFGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF β), insulin growth factor (IGF-1) hepatocyte growth factor (HGF), nerve growth factor (NGF), stem cell factor (SCF), stromal derived factor 1-a (SDF 1-a)]. The Cytokine Assay Bio-Plex (Bio-Rad Laboratories, Hercules, CA, USA) allowed for the simultaneous dosage of different biomolecules to be performed in a single microplate well. All samples were analysed following the manufacturer's protocol. At least three independent repetitions in duplicate were made per experimental condition type. The analyte concentrations were expressed in pg/ml. A standard curve ranging on average from 0.15 pg/ml to 3700 pg/ml (High Photomultiplier Tube Setting -PMT setting) was prepared and then fitted by Bio-Plex Manager software.

2.9 Expression of FGFb

208 Total RNA samples isolated from HeLa cells after 1 day of incubation onto ceramic samples
209 (RNeasy Mini Kit, Quiagen, Valencia, CA, USA) and 4 hours of starvation were subjected to
210 Taqman-based reverse transcription real time polymerase chain reaction (RT-PCR) for human
211 FGFb using the Universal Probe Library System (Roche Diagnostics Corp., Milano, Italy) as
212 previously described [23]. Primers and probes were designed using the ProbeFinder software
213 (www.roche-applied-science.com).
214 FGFb Fw primer: ttcttcctgcgcatccac
215 FGFb Rev primer: tgcttgaagttgtagcttgatgt
216 Probe #7 18S rRNA (4319413E Applied Biosystems) was used as endogenous control.
217

3. Results

3.1 Microstructure and surface composition

Figure 1 shows the morphologies of the ZTA and ATZ composites. The grains of both materials are sub-micrometric with some differences between them. For ZTA the zirconia grains are one third the size of the alumina grains (0.3 ± 0.1 vs. 0.9 ± 0.3 μm), whereas the composite with higher percentage of zirconia shows similar grains sizes (0.5 ± 0.2 μm) for both oxides. As reported in previous work[9], the lower amount of zirconia led to an increase in alumina particle size.

AFM images of the polished ATZ and ZTA samples are shown in Figure 2. The characteristic microstructure of the alumina and zirconia grains is clearly visible.

The surface roughness clearly decreases, when the samples are polished, from 168 ± 28 nm to 13 ± 3 nm in the case of ZTA and from 107 ± 24 nm to 17 ± 2 nm in the case of ATZ (Table 1).

Before laminin adsorption, aluminium, zircon, yttrium, oxygen and carbon atoms were revealed on the surfaces by XPS spectra. The presence of carbon is due to surface contamination from species adsorbed from the air. Other contaminants are also detectable in very small amounts (Na and Si) on the different survey spectra (*cf.* Figure 3). They are probably related to traces present in the starting powders or collected during the preparation procedure.

The spectra were calibrated by setting the Al2p signal of Al_2O_3 at a binding energy of 74 eV. A complete discussion of such spectra is reported in previous work [9]. They are mentioned here for comparison with spectra collected after the adsorption. In this context, it is helpful to remember that the quantitative fractions of aluminium, zircon, yttrium and oxygen for the two samples show surface chemical compositions for ZTA and ATZ which are very close to that of the bulk, and relative quantities of surface hydroxyls that are comparable on both samples (20 % of the oxygen contribution).

3.3 Laminin adsorption

XPS results

After laminin deposition, a nitrogen peak appears in the XPS survey (*cf.* figure 3), in addition to the aluminium, zircon, yttrium, oxygen and carbon already present on the surface of ATZ and ZTA without the protein. Moreover, after the adsorption, the Al2p, Zr3d, Y3d and the O1s peaks decreased in intensities, indicating the presence of an additional layer on the surface. The high resolution spectra of each element were decomposed in the same way for the ZTA and the ATZ samples. Table 2 summarises the main XPS data. The N1s XPS core level spectrum of adsorbed protein presents one symmetric peak centered at 399.9 ± 0.1 eV, which is assigned to the nitrogen of amine and amide groups of the laminins, as expected for a protein [24] (*cf.* Figure 4). The C1s peaks are fitted with three contributions at 284.6 ± 0.1, 286.2 ± 0.1 and 288.1 ± 0.1 eV. On the untreated ATZ and ZTA samples, these contributions are related to C-C C-H (C_1), C-O (C_2) and O=C-O(C_3), respectively. The high resolution spectra of the C1s regions on the samples functionalised with proteins (*cf.* Figure 4) show the increase of the C_2 and C_3 contributions due to the carbon bonds present in the laminins molecules (C-N C-O C-S and O=C-O O=C-N, respectively) [17]. The O1s peak results from the superposition of the spectra of the laminin overlayer and the alumina-zirconia substrate. The contribution at 529.7 ± 0.1 eV, relative to the zirconia (O_1) and the contribution at 530.8 ± 0.1 eV, relative to alumina (O_2) are reduced in intensity. The contribution at 532.0 ± 0.1 eV corresponds mainly to the oxygen atoms present in the laminin (O_3) and finally the latest contribution at 533.3± 0.1 eV is assigned to the hydrated surface layer (O_4). The characteristic functional groups of the protein, therefore, confirm the presence of adsorbed laminin on the ZTA and ATZ samples.

In order to compare the relative amounts of protein adsorbed on the different surfaces, the atomic ratio between the nitrogen signal intensity (N1s), which is a marker of the protein, and the Al2p and Zr3d signals, which are characteristic of the substrates N1s/(Al2p+Zr3d), was calculated, as it was proposed in previous work [25] (*cf.* Table 3). The ratios obtained for one type of protein are equal regardless of the surface, showing that the relative amounts of alumina and zirconia in the substrate has no impact on the amount of protein adsorbed. Moreover, the ratio for Ln-1 is twice as the one

for Ln-5. Given that Ln-1 has a molecular weight of 810 kD, compared to 430 kD for Ln-5, it may be inferred that the same molar quantity of the different proteins is adsorbed on the surfaces.

AFM results

Figure 5 shows the AFM images obtained with Ln-1 and Ln-5 adsorbed from solutions at $2.35x10^{-5}$ M on the polished surfaces of ATZ and ZTA samples. In the topography images, the Ln-1 molecules are clearly visible on the surfaces in a globular-like morphology (~60 nm), whereas it is impossible to observe the Ln-5 molecules, probably partly because the difference in height relative to the organic layer is not detectable compared to what is obtained on very smooth surfaces. The difference in protein weights (Ln-1 ≅ 810kD and Ln-5 ≅ 430kD) may help in explaining this phenomenon: the heavier protein probably induces a bigger height difference. Even though the height signal did not reveal the Ln-5 adsorption, it was however possible to observe the Ln-5 molecules arranged in a globular-like morphology with a size of ~30-40 nm, by relying on the phase magnitude in intermittent mode[18, 26]. However, the surfaces seem to be less covered by the Ln-5 than by Ln-1.

3.4 Biological effects of laminin adsorption

Cell vitality and spreading on the ATZ and ZTA samples

Based on the MTT assay, ATZ and ZTA specimens were both able to properly sustain cell growth and proliferation without statistically significant differences, within 3 days (data not shown). The functionalisation with laminins did not affect, at least in a statistically significant way, the count of viable cells after 4, 12, 24 and 48 hours since seeding (*cf.* Figure 6), according to the Dunnett's test. Neither was the percentage of dead cells different among the experimental conditions tested (data not shown).

Cellular response to ATZ and ZTA samples functionalised with laminins

Cell reaction to the laminin functionalisation was consistently enhanced when Ln-1 and Ln-5 were adsorbed onto ZTA and ATZ, respectively, according to the activation of major intracellular phosphorylated proteins such as MEK and pERK1/2 (*cf.* Figure 7). Instead, the other key-steps of paramount pathways analyzed were not substantially modulated. Overall ATZ was more active than ZTA, although not always in a statistically significant way. The same pattern was detected, with statistical significance, in the high release of FGFb into the conditioned media within 4 hours (*cf.* Figure 8), but not while investigating the de-novo synthesis of this growth factor by Real Time PCR (*cf.* Figure 9). As for this assay, ATZ proved to be more effective than ZTA in inducing the FGFb transcription independently from the type of laminin adsorbed.

4. Discussion

Scientific evidence on alumina-zirconia composites supports their reputation of suitability for manufacturing orthopedic prosthetics, due to their satisfactory biological response [27] and mechanical properties superior to those of monolithic oxides [2, 28]. Also, since the visual characteristics of these ceramic materials lead to advantageous aesthetic features, which are most appreciated in dentistry, alumina-zirconia composites may be proposed as materials for dental implant fabrication. Thus, ATZ and/or ZTA might become a viable alternative to the monolithic zirconia devices that are already clinically used [29], despite some concerns have recently arisen regarding their possibly low survival rate [30].

The dental implants available on the market have not been functionalised with biomolecules yet, although a vast corpus of scientific literature deals with this theoretically intriguing opportunity [31-34]. More specifically, as major molecules of the basement membrane, laminins are important proteins for the epithelial function, and their grafting is known to favor the adhesion of epithelial cells onto titanium samples [11, 17]. In this context, the current study aimed to assess whether the adsorption of Ln-1 and Ln-5 onto ZTA and ZTA samples could elicit a biological response in a prototypic epithelial system such as the HeLa cell line.

First, disk shaped samples were prepared starting from powders. Then, the ATZ and ZTA samples surfaces were polished so as to obtain samples suitable for proper surface analysis like AFM and XPS. The AFM analyses showed that both surfaces exhibited a comparable surface roughness in the range 11-17 ± 2 nm after polishing. The XPS analyses confirmed that the surface composition of the materials was the same as that of the bulk. Moreover, it appears from the O1s peak decomposition that the OH fraction and water percentage were very similar in both oxides (20% of the O1s peak). All these similarities made it possible to compare the effect of substrate composition (*i.e.* the zirconia percentage) on protein adsorption.

The functionalisation of the surface with Ln-1 and Ln-5 was clearly confirmed by the XPS data, given the N1s peak, which is characteristic of proteins [25, 35], and the concomitant decrease of the substrate peaks' intensity. An estimation of the relative amounts of proteins present on the surfaces was obtained by comparing the ratio N1s/(Al2p+Zr3d) of the different protein/oxide pairs. Taking into account the molecular mass of the two different proteins, it was found that the same molar protein quantity was adsorbed on both composites, regardless of the surface or the protein. This result, adding to the analyses of the AFM images, show that the surfaces are less covered with the Ln-5 than with the Ln-1. Thus the two proteins probably have different adsorption ability on these surfaces. Furthermore, the AFM images show that the two proteins appeared folded into a globular form on both types of surfaces which was already observed for Ln-1 on surfaces having the same molar fraction of -OH groups[18].

As for the cell vitality test, ATZ and ZTA performed similarly in sustaining cell growth, consistently with previous reports in the literature regarding monophase zirconia [36, 37]. Likewise, cell spreading did not differ significantly between the two materials. However, when screening the activation of some important cell pathways elicited in the four experimental conditions chosen in this study (ATZ Ln-1, ATZ Ln-5, ZTA Ln-1, ZTA Ln-5), an interesting response pattern was observed repeatedly: ATZ Ln-5 and ZTA Ln-1 were more efficient in inducing MEK and ERK phosphorylation than the two other combinations. Laminins are known to activate extracellular

346 signal-regulated kinases (ERKs) via integrin interactions, in some settings, though the control
347 mechanisms have not yet been completely elucidated [38]. There is mounting evidence indicating
348 that three laminin globular (LG)[39] domains, LG1–3, in the α chains [40, 41] as well as the
349 glutamic acid residue in the C-terminal tail of the gamma chain [42] are prerequisites for integrin
350 binding by laminins [43]. Among the 24 integrins described in mammals, α3β1, α6β1, α6β4, and
351 α7β1 integrins have been shown to serve as the major laminin receptors in various cell types [44].
352 In HeLa cells, β1 integrin forms heterodimers with α1, α2, α3, α5 and α6 integrin subunits [45].
353 When analyzing the conditioned media after 1 day of cell culture, the same response pattern
354 described for the MEK/ERK activation was discovered for FGFb. Although the FGF secretion
355 pathway remains poorly understood, it has been proposed that FGFb may be transiently
356 phosphorylated by Tec kinase before membrane translocation [46]. Even though active Tec kinases
357 are thought to be primarily localised in plasma membranes, a soluble form of Tec kinase can both
358 bind and phosphorylate FGFb *in vitro* [47]. Therefore, the phosphorylation of FGF2 may serve as a
359 signal for FGF2 transport into the cell periphery. Furthermore, TEC activity has been linked with
360 the Ras-MAPK pathway [48], which may explain why the early FGFb release was correlated to the
361 MEK/ERK activation. This is in accordance with previous studies demonstrating integrin-mediated
362 release of FGF-2 in other experimental settings [49, 50].
363 Instead, the de-novo synthesis of FGFb after 1 day could not be related to the type of laminin, but
364 reflected more generally the ceramic type, being higher for ATZ than ZTA. Other extracellular cues
365 may have possibly guided this phenomenon. Indeed, the zirconia content is known to affect the
366 chemical characteristics of the interface and was shown to be dramatically different when the
367 Kokubo test was performed for both ceramic composites[9].
368 Furthermore, given that the same protein molar quantity was adsorbed onto both ATZ and ZTA,
369 under a globular morphology in both cases, the different effect elicited by Ln-1 and Ln-5 might be
370 due to differences in protein folding, namely the number of putative integrin-binding sites exposed,
371 possibly because of the peculiar surface reactivity of the materials. Dissecting the mechanisms

behind this phenomenon, however, would require an additional series of experiments, as well as a proper *in silico* simulation, the complexity of which goes beyond the scope of the present work.

5. Conclusions

Based on the XPS and AFM data, it can be concluded that both Ln-1 and Ln-5 were adsorbed onto the alumina zirconia composites under the same molar quantity and both on a globular shape. Some of the most important cell kinases were induced within the epithelial cells grown onto the two alumina-zirconia composites by the presence of laminins. In particular, ATZ Ln-5 and ZTA Ln-1 were more efficient in inducing MEK and ERK phosphorylation than the two other combinations and a similar pattern was detected as for FGFb secretion. Although further studies are required to assess precisely how Ln-1 and Ln-5 react on ATZ and ZTA, the authors deem that the present research shed an interesting light on the unprecedented use of Ln-1 and Ln-5 to functionalise alumina-zirconia composites suitable for preparing dental implants in order to favor their biological activity.

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Figure legends

graphical abstract : Surface characterization and cell surface activity of ATZ and ZTA surfaces functionalised with Laminin-1 or Laminin-5

Figure 1: SEM images of the microstrure of (a) ZTA and (b) ATZ composites

Figure 2: Topographic images of ATZ (a) and ZTA (b) and phase images of ATZ (c) and ZTA (d) obtained by AFM

Figure 3: Survey spectra recorded before and after adsorption of Ln-1 and Ln-5 on ATZ and ZTA

Figure 4: N1s, C1s and O1s XPS high-resolution spectra recorded after adsorption of Ln-1 and Ln-5 on ATZ and ZTA

Figure 5: Topographic images of Ln-1 adsorbed on ATZ (a) and ZTA (b) with phase images included as inserts (a,b) phase images of Ln 5 adsorbed on ATZ (c) and ZTA (d) with topographic images included as inserts.

Figure 6: Number of HeLa cells attached to ATZ, ATZ Ln-1, ATZ Ln-5, ZTA, ZTA Ln-1, ZTA Ln-5 measured using an automated cell counter at 4, 12, 24 and 48 hours culture. Control refers to cells cultured on the plastic surface of the 24 well-plate. Error bars represent standard deviation.

Figure 7: Comparison of major intracellular HeLa phosphorylated protein responses on ATZ or ZTA with different surface modifications. Control refers to cells cultured on the plastic surface of the dish. The data results from the normalization of each phosphorylated protein on the correspondent unphosphorylated protein, e.g. phospho-ERK on total ERK, and correspond to $5x10^5$ cells/well. Error bars represent standard deviation. *P <0.05

Figure 8: Comparison of cytokines released into HeLa conditioned media for ATZ or ZTA with different surface modifications. Concentrations are expressed as pg/mL and correspond to $5x10^5$ cells/well. Control refers to cells cultured on the plastic surface of the dish. Error bars represent standard deviation. *P <0.05

Figure 9: Synthesis of FGFb in Hela cells coated on ATZ or ZTA with different surface modifications. Control refers to cells cultured on the plastic surface of the dish. Error bars represent standard deviation.

Table 1: Roughness of the different surfaces obtained by AFM

Table 2: XPS Atomic Percentages of ATZ, ATZ Ln-1, ATZ Ln-5, ZTA, ZTA Ln-1 and ZTA Ln-5

Table 3: Comparison of the ratios %N1s/(%Al2p+%Zr3d) for ATZ-Ln1, ATZ-Ln5, ZTA-Ln1 and ZTA-Ln5.

Table 1

Samples	RMS (nm)	Ra (nm)	Peak-to-valley distance
ATZ non Polished	107±24	87±26	584±142
ZTA non Polished	168±28	133±24	962±224
ATZ Polished	17±2	13±2	112±15
ZTA Polished	13±3	11±2	111±20

600

601 **Table 2:**

Element (%)	ATZ			ZTA		
	untreated	Laminin-1	Laminin-5	untreated	Laminin-1	Laminin-5
Al2p	**9.20±0.92**	**10.40±1.04**	**13.10±1.31**	**27.30±2.73**	**23.60±2.36**	**29.80±2.98**
Zr3d	**13.50±1.35**	**12.20±1.22**	**6.00±0.60**	**1.90±0.19**	**1.30±0.13**	**1.90±0.19**
Y3d	**2.20±0.22**	**1.30±0.13**	**0.50±0.05**	**0.20±0.02**	**0.20±0 .02**	**0.20±0.02**
N1s	**-**	**2.00±0.20**	**0.90±0.09**	**-**	**1.50±0.15**	**0.70±0.07**
O1s	**48.20±4.82**	**42.20±4.22**	**39.50±3.95**	**43.20±4.32**	**35.00±0.35**	**45.20±4.52**
O_1	49.10±4.91	41.10±4.11	41.60±4.16	10.20±1.02	5.10±0.51	6.10±0.61
O_2	27.60±2.76	25.90±2.59	26.20±2.62	71.50±7.15	68.80±6.88	70.90±7.09
O_3	19.50±1.95	30.50±3.05	28.50±2.85	15.90±1.59	23.20±2.32	20.00±2.00
O_4	3.80±0.38	2.50±0.25	3.70±0.37	2.40±0.24	2.90±0.29	3.00±0.30
C1s	**26.90±2.69**	**33.70±3.37**	**40.00±4.00**	**27.40±2.74**	**38.30±3.83**	**22.20±2.22**
C_1	91.20±9.12	82.20±8.22	89.30±8.93	87.00±8.70	85.80±8.58	83.30±8.33
C_2	5.30±0.53	11.50±1.15	7.20±0.72	7.20±0.72	8.10±0.81	10.00±1.00
C_3	3.50±0.35	6.30±6.30	3.50±0.35	5.80±0.58	6.20±0.62	6.70±0.67

602

603 **Table 3:**

Samples	ATZ-Ln1	ATZ-Ln5	ZTA-Ln1	ZTA-Ln5
%N1s/(%Al2p+%Zr3d)	0.09±0.02	0.05±0.01	0.06±0.02	0.020±0.006

604