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Alumina–zirconia composites functionalized 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 functionalized with laminins showed better cellular activation than untreated specimens; furthermore, cellular activation was found to be greater for the composite with higher percentage in zirconia when functionalized with Ln-5, whereas the adsorption of Ln-1 resulted in a greater activation for the alumina-rich oxide.

1. Introduction

Oxidic materials, such as alumina and yttria-stabilized 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

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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 functionalized 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 favors the in vitro formation of adhesion structure (hemidesmsomes) [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, 16 wt% of zirconia) and alumina toughened zirconia (ATZ, 80 wt% 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 functionalized 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 toward 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_3–16 wt%ZrO_2 (ZTA, Taimicron, Taimei, Japan) and ZrO_2–20 wt%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 200 MPa. The best conditions for the sintering process were: heating at a rate of 50 °C/h up to 700 °C, followed by a 2 h dwell; heating at a rate of 100 °C/h up to temperature sintering of 1500 °C, followed by a 2 h 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 1 μm. 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 $K\alpha$ 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 × 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 normalization 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) was fixed for each given peak.

The surface morphology of the ZTA and ATZ samples was characterized using an atomic force microscope (AFM, Park System XE100) in intermittent mode. The scan size was $3 \times 3\ \mu m^2$ 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 h. They were further cleaned in an ultrasonic bath with three common solvents of different polarities, i.e. Milli-Q 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 a phosphate buffer (PBS), from which a concentration of $2.35 \times 10^{-5}\ mol^{-1}$ 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 h, the specimens were rinsed with a Milli-Q water spray, then immersed for 5 s in Milli-Q 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 functionalization 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 5×10^5 cells/well in a 24-well plate (BD, Milan, Italy).