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Nanogrooves and keratin nanofibers on titanium surfaces aimed at driving gingival fibroblasts alignment and proliferation without increasing bacterial adhesion

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ABSTRACT

Periimplantitis and epithelial downgrowth are nowadays the main conditions associated to transmucosal dental implants. Gingival fibroblasts can play an important role in periimplantitis because they are the promoters of the inflammatory process and eventual tissue homeostasis and destruction. Moreover, the related inflammatory state is commonly driven also to counteract bacteria implants colonization.

In the present research, a new technology based on mechanically produced nanogrooves (0.1–0.2 μm) and keratin nanofibers deposited by electrospinning has been proposed in order to obtain titanium surfaces able to drive gingival fibroblasts alignment and proliferation without increasing bacterial adhesion. The prepared surfaces have been characterized for their morphology (FESEM), chemical composition (FTIR, XPS), surface charge (zeta potential) and wettability (contact angle). Afterwards, their performances in terms of cells (human primary gingival fibroblasts) and bacteria (*Staphylococcus aureus*) adhesion were compared to mirror-like polished titanium surfaces.

Results revealed that gingival fibroblasts viability was not negatively affected by the applied surface roughness or by keratin nanofibers. On the opposite, cells adhesion and spread were strongly influenced by surface roughness revealing a significant cell orientation along the produced nanogrooves. However, the keratin influence was clearly predominant with respect to surface topography, thus leading to increased cells proliferation on the surfaces with nanofibers, disregarding the presence of the surfaces grooves. Moreover, nor nanogrooves nor keratin nanofibers increase bacterial biofilm adhesion in comparison with mirror polished surfaces.

Thus, the present research represents a promising innovative strategy and technology for a surface modification finalized to match the main requirements for transmucosal dental implants.

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1. Introduction

Prevention of implant infection and formation of healthy soft tissue around the implant are key issues in several trans-skin orthopedic, and trans-mucosal bone anchored dental implants [1,2]. Periimplantitis and epithelial downgrowth are among the main conditions associated to dental implants failure. Periimplantitis is an infection affecting the tissues around osseointegrated implants with loss of supporting bone associated to clinical signs of inflammation. Recent papers report that they affect >40% of oral implants after 10 years from installation [3–5]. In transmucosal implants, bacterial infection is due to bacterial penetration through the soft tissues in contact with the implant and biofilm formation on the implant surface. The defect in soft tissue sealing and the apically epithelial downgrowth till to the bone level, is responsible of bone resorption and implant mobilization promoted by inflammation of the connective tissue and fibroblasts interplay. In fact, fibroblasts are the promoters of the inflammatory process and eventual tissue homeostasis and destruction [6,7] and they are able to counteract bacteria release of metalloproteinases (MMPs) [8].

In view of this, an ideal surface for prevention or tissue repair after periimplantitis should be able to promote fibroblasts repopulation, thus supporting their protective and regenerative activity, without providing coupling places for biofilm formation. However, despite of certain knowledge on behavior of gingival fibroblasts and bacteria on titanium surfaces, the optimal solution for implant collars and abutments is far from being reached.

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Numerous innovative surfaces have been developed and reported in the scientific literature, in international patents and even in commercial products for the improvement of bone integration of dental implants. On the other hand, few studies can be mentioned with a focus on interaction between the dental implants (collar and abutments) and the adjacent soft tissues: some examples are reported below. A porous titanium layer, obtained by assembly of smooth titanium beads, coated with laminin-5 enriched PLL/PGA, has been proposed in order to improve soft tissue adhesion to the transmucosal part of dental implants [9]. Collagen grafting onto titanium surfaces has also been proposed for the improvement of fibroblasts adhesion on dental implant collar by the formation of a biological sealing [10]. Moreover, biological functionalization with multi-layer polymeric coatings has been reported in patents [11,12] for the enhancement of titanium-soft tissue interaction or for reducing the infection risk. Surface modifications at the same time aimed at improving soft tissue adhesion and at avoiding bacterial colonization, overcoming the above mentioned issues of dental implants, are almost neglected so far.

Fibroblasts are highly sensitive to surface grooves and align along them, this phenomenon is called contact guidance [13,14]. The effect of micro and nanogrooves on fibroblasts alignment and proliferation has been investigated in the literature. Some significant examples are briefly reported below and more deeply analyzed by the authors in [15]. Ti surfaces with different surface finishing (polished, machined, acid-etched, modified by cathodic polarization) have been produced and compared concerning fibroblasts and bacterial adhesion in [16,17]. It has been observed that the surfaces machined and modified by cathodic arc polarization showed micro-grooves able to orient fibroblasts and the ability to increase cell attachment and gene expression for the production of collagen rich ECM [16]. On the other hand, a reduced biofilm formation has been observed on polished titanium [17]. V-shaped 2 μm microgrooves obtained by ultraprecision micromachining have been described for the improvement of soft tissue anchoring and to avoid bacterial penetration from dental implant collar in [18]. Walbomers and co-workers [19] studied the behavior of fibroblasts on smooth and microgrooved (0.5 μm depth, 1–10 μm width) polystyrene substrates. It has been evidenced that at short culture times (30–45 min) cells proliferate more on the smooth one while at longer culture times (4 h) proliferation increases on the microgrooved material and alignment can be observed along the grooves. Microgrooved (15–60 μm width, 5–10 μm depth) dental implants have been investigated in [20]. An increase in fibronectin absorption and surface wettability has been evidenced for 60 μm large and 10 μm deep groves. An effective improvement in proliferation, gene expression and contact guidance has been reported in [21,22] for human gingival fibroblasts cultured on substrates with analogous topography. An oriented connective tissue has been observed also on abutment with micro-channels (8–12 μm width and depth) [23]. The grooves dimensions and their relation to cellular size affect sensitivity of the cells to topography and their behavior. In case of an excessive depth of the grooves (>10 μm), cells tend to spread on the edges without reaching the bottom [24]. Moreover, if the width of the groove is in the same dimensional range of the cells (20–30 μm), the cells spread inside the groove; on the contrary, if the grooves are smaller (2–5 μm) cells cannot penetrate and grow only on the edges and lateral walls [25]. Finally, it has been observed that ridges should be <2 μm large in order to guarantee crosslinks between cells [25]. In the patent databases, micro-grooves with width and depth in the 2–25 μm [26,27,28]and 25–600 μm [29,30] ranges, as well as micro-threads [31,32], roughness 2–10 times higher than traditional implants [33] and micro-holes (1 μm depth and 3 μm wide) [34]have been reported for the improvement of soft tissue integration in dental implant collars.

As far as nanopatterns are concerned, it has been evidenced [35] that the lower limit in order to induce contact guidance on fibroblasts is 35 nm depth and 100 nm width. These values are in accordance with the dimensions of collagen fibrils (few tens of nanometers in diameter).

Also the shape of the surface features significantly affects cellular behavior. In fact, despite good proliferation and alignment of fibroblasts on micro/nanogrooves a significant reduction of their adhesion has been observed on surfaces with holes/pillars (300 nm height) [36], as a confirmation of the rugo-phobic nature of this cell type.

As far as bacteria are concerned, there is a general agreement on the fact that bacterial adhesion increases with surface roughness [37,38]. A roughness of 0.2 μm has been reported as the lower limit below which no increase in bacterial adhesion can be observed [39,40,41] and a roughness of 150 μm is the upper limit over which no variation can be noticed [42].

In conclusion, looking at the literature, the grooves should have a width higher/equal to 100 nm, but lower than 70 μm, a depth higher than 35 nm and a distance lower than 2 μm [15] in order to obtain fibroblasts alignment and their connection. Moreover, the surface roughness should be lower than 0.2 μm in order to limit bacterial contamination [40,41,42]. A specific investigation of fibroblasts and bacteria adhesion on surfaces with this range of grooves is still missing.

In addition to topographical stimuli, also biological ones can be used in order to get an effective tissue healing. Surface functionalization and coatings can be employed for a smart material with active biological properties: according to this strategy, the effect of keratin nanofibers deposition has been tested in the present research work. Keratin is a protein abundant in nature (hair, feathers, nails and horns in mammals, reptiles and birds) able to support fibroblasts cells growth [43]. Keratin extracted from wool has many useful properties, including biocompatibility and biodegradability [44] and it supports the growth and adhesion of fibroblasts [45] and osteoblasts [46]. Moreover, it can be recovered from wastes (e.g. poor quality wools) supporting a green and sustainable use of resources. Because of its low molecular weight (65–11 kDa) and its poor mechanical properties, regenerated keratin is very fragile and difficult to handle. Recently, extracted keratin has been regenerated in films from ionic liquids by the addition of methanol, ethanol, and water as coagulation solvents [47] and keratin has been used for the production of nanofibers by electrospinning [48,49]. The electrospinning process is a low-cost and simple method to produce nanofibers with high surface-to-volume ratio and high porosity; this makes them promising candidates for several applications, such as filter membranes, cell-growth scaffolds, wound dressings and drug-delivery vehicles. In applications such as liquid filtration and biomedical fields, water stability is required and, in a recent work, heating treatments were tested at this purpose [50].

In the present research, an innovative combination of nanogrooves and keratin nanofibers [51] has been obtained on commercially pure titanium substrates in order to investigate new surface technologies for soft tissues contact. The final aim is to promote and drive gingival fibroblasts adhesion/proliferation (through keratin nanofibers) and orientation (through nanogrooves) without increasing bacteria colonization. Oriented nanogrooves with an average surface roughness lower than 0.2 μm have been obtained on commercially pure titanium by a simple and low cost procedure (abrasive papers) and characterized by means of Field Emission Scanning Electron Microcopy (FESEM) and roughness measurements. The deposition of keratin nanofibers has been performed by means of the electrospinning technique and then investigated by means of FESEM observation, Fourier Transformed Infrared Spectroscopy (FTIR) and X-ray Photoelectron Spectroscopy (XPS)

Table 1
Samples names and treatments.

Sample name	Treatment
Ti-pol	Mirror polishing
Ti-rough	Abrasive paper roughening
Ti-pol + ker	Mirror polishing + keratin nanofibers deposition
Ti-rough + ker	Abrasive paper roughening + keratin nanofibers deposition

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