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Aligned keratin submicrometric-fibers for fibroblasts guidance onto nanogrooved titanium surfaces for transmucosal implants

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ABSTRACT

Biomaterials surface modification represents a very attractive tool to modulate host tissue response. Surface modifications of titanium for bone contact applications have been widely investigated, while only few papers can be found regarding modifications aimed at soft tissue contact. However, soft tissue healing represents a crucial step for percutaneous/transmucosal titanium implants success. Fibroblasts, the soft tissues most representative cells, are known to be sensitive to surface topography. Anyhow, fibroblasts adhesion and spread can be more influenced by the presence of attractive macromolecules; so, within a grooved and keratin-coated Ti surface, cells will align following the strongest biochemical guide provided by keratin submicrometric-fibers. In order to obtain the most effective cell stimulation/guidance keratin submicrometric-fibers should be aligned to substrate nanogrooves. Despite the very promising cells guidance provided by keratin submicrometric-fibers, the strong limitation to their synthesis is related to the macromolecules high sensitivity to environmental parameters (temperature/humidity/pH) which can seriously compromise the feasibility and reproducibility of the final submicrometric-fibers layer. Accordingly, this work has the aim to obtain, for the first time, a coating of submicrometric keratin fibers aligned to the nanogrooves of a Ti substrate, in order to validate the attitude of the coated surface to impart additive topographical plus biochemical signals in the same direction for the stimulation of fibroblasts repopulation.

1. Introduction

Biomaterials surface modification is a widely explored tool to modulate host tissue response. Accordingly, surface treatments of titanium for bone contact applications have been widely investigated [1]; conversely, only few papers can be found regarding titanium surface modifications aimed at soft tissue contact. Despite the main application of titanium based materials is in contact with hard tissues, important medical devices, such as transmucosal dental implants, are made of titanium and are intended to both hard and soft tissue contact. This situation requires an accurate surface design able to match the functional requirements of the different areas. In fact, it is well known that fibroblasts (differently from osteoblasts) are rugophobic cells that can be stimulated to repopulate an implantable material surface following a controlled and orientated geometry (grooves) by the "contact guidance" phenomenon [2,3]. This behavior can be of interest for the development of innovative surfaces aimed to favor fibroblast adhesion/alignment with the final aim to improve soft tissue adhesion and healing in percutaneous/transmucosal titanium implants. In a previous work [4], the authors demonstrated that human primary fibroblasts were able to adhere and align onto nanogrooves mechanically obtained onto commercially pure titanium substrates (Ti-cp); however, when randomly oriented keratin submicrometric-fibers were introduced onto these substrates (in order to exploit keratin ability to favour fibroblasts adhesion/proliferation), fibroblasts resulted as more sensitive to the biochemical stimulus of the protein thus losing nanogrooves orientation [4]. From the standpoint of material development, these results were very encouraging as keratin macromolecules are generally very sensitive to the environmental parameters (temperature/ humidity/pH) which may strongly influence fiber formation

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and deposition mechanisms, thus largely limiting the feasibility and reproducibility of the final submicrometric-fibers layer [5,6]. Previous studies performed on highly hydrophobic polymers (e.g. polycaprolactone) demonstrated that in that case only relative humidity was sufficient to interfere with the system capability to impart a preferential fiber orientation by altering the critical collector rotation rate required to reach fiber alignment [7]. Regarding structural proteins (i.e. Keratin), humidity degree may also influence the chemical properties of the macromolecules (i.e, molecular folding), thus compromising fiber integrity during the electrospinning process [8]. Hence, the proposed work is aimed to investigate the possibility to obtain, for the first time, submicrometric keratin fibers aligned to the nanogrooves of a Ti-substrate in order to overcome the main issues related to the randomly oriented ones: in this way, topographical and biochemical stimuli will be in the same direction and their effect on fibroblast adhesion and orientation can be maximized.

2. Materials and methods

Commercially pure titanium foils (Titanium foil 0.025 mm, 99.94%, Alfa Aesar) were used as substrates for keratin fiber deposition. The shape of the substrates was selected according to the requirements of the rotating collector necessary for the deposition of aligned submicrometric-fibers. The morphology and roughness of these commercial titanium foils were compared with the one previously obtained onto titanium disks [4]. Keratin was obtained by a green approach, in fact freeze-dried keratin, extracted from discarded wool by sulfitolysis with sodium metabisulfite, as previously described [4], was dissolved in 1,1,1,3,3,3-hexafluoro-2-pro panol (HFIP, Sigma-Aldrich, Italy) by stirring at room temperature for 24 h until obtaining a clear solution (10% (wt/v). Electrospun fibers were processed by using a commercial electrospinning system (NANON 01, MECC, Japan) equipped with a rotating collector and 18 Ga needle as similarly reported elsewhere [9]. Aligned submicrometric-fibers were fabricated by setting optimal parameters as follows: flow rate 0.5 ml/h, applied voltage 25 kV, spinneret/collector distance 150 mm, collector rotating rate 2000 rpm. Noteworthy, the process was performed at room temperature and controlled humidity degree (35–40%) to assure optimal environmental conditions for keratin fibers formation. Two different times of deposition (15 and 45 min) were considered. Morphology of the fibers was investigated by Field Emission Scanning Electron Microscopy (FESEM; Quanta 200 FEI, the Netherland) under low vacuum conditions. Average diameter of the fibers was measured from selected SEM images (n = 10) using open source image analysis software (ImageJ 1.50i, National Institutes of Health, USA) whereas fiber alignment via FFT analysis (i.e., conversion from spatial to the frequency domain).

The coated samples were subjected to a thermal treatment (2h at 180 °C) in order to stabilize the fibers [4,10]. This process produces at the same time sterile samples suitable for biological characterizations. In this case, morphology and spatial distribution of submicrometric keratin fibers onto titanium substrates was further investigated after sputter coating with a thin Pd-Au layer (ca. 18 nm).

Human primary gingival fibroblasts (HGFs) from normal human gingiva were used for cytocompatibility and cellular alignment evaluation as previously reported [4]. Cells were seeded in a defined number (2×10^4 cells/specimen) directly onto keratin-coated specimens' surface and cultivated for 24 and 48 h. At each time points, cells viability was evaluated by the Alamar blue assay (alamarBlue, ThermoScientific, USA); cells cultivated onto polystyrene were considered as control. 48 h was selected as significant time point for the evaluation of fibroblasts alignment onto keratin coated substrates on the basis of previous studies [4]. Accordingly, after 48 h cultivation, cells morphology was evaluated by fluorescence imaging. After fixation with paraformaldehyde (20 min, room temperature), specimens were stained with phalloidin to visualize cytoskeleton f-actins filaments and then co-stained with DAPI to visualize nuclei (both from Sigma-Aldrich, Italy).

3. Results and discussion

The selected titanium foils displayed a surface topography and mean surface roughness Ra = 0.091 ± 0.008 μm comparable to the mechanically roughened samples showed in a previous work [4] (Ra = 0.104 ± 0.010 μm). So these materials were considered as suitable for a first attempt of submicrometric keratin fibers alignment onto nanogrooved Ti-cp substrates.

Commercially pure titanium is naturally covered by a native oxide layer and hydroxyl groups are exposed [4]. Chemical and/or electrostatic bonding between keratin fibres and these functional groups can be supposed. Moreover, alignment of the fibers to the nanogrooves of the substrate favors the mechanical anchorage increasing the stability of the coating through mechanical bonding.

FESEM images of submicrometric keratin fibers aligned onto titanium substrates are reported in Figs. 1 and 2 as well as the

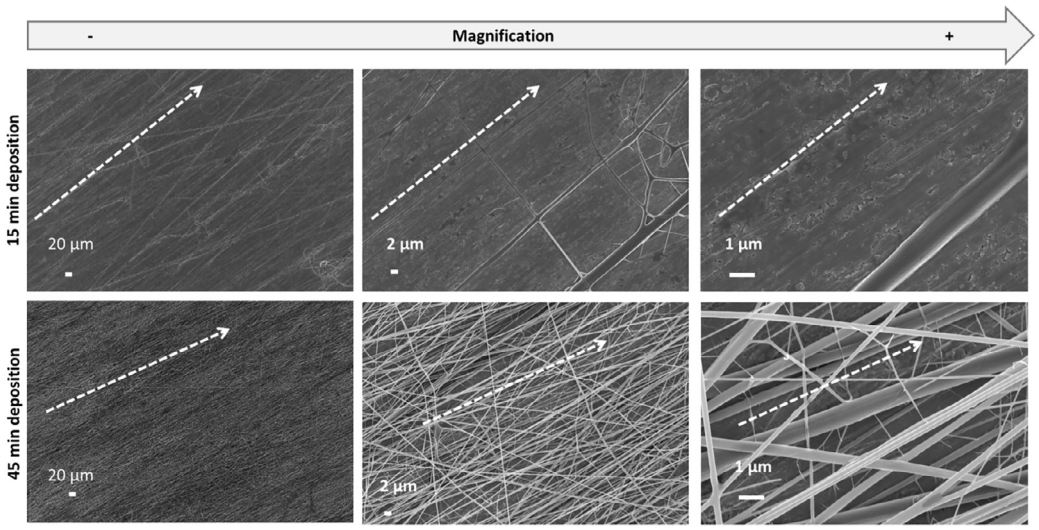

Fig. 1. FESEM images of Ti-cp coated with submicrometric keratin fibers.