Colloids and Surfaces B: Biointerfaces 122 (2014) 601–610

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Collagen-functionalised titanium surfaces for biological sealing of dental implants: Effect of immobilisation process on fibroblasts response

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ARTICLE INFO

Article history:
Received 26 April 2014
Received in revised form 18 June 2014
Accepted 22 July 2014
Available online 31 July 2014

Keywords:
Titanium
Collagen
Fibroblast
Silane
Roughness
Implants

ABSTRACT

The clinical success of a dental implant requires not only an optimum osseointegration, but also the development of a biological sealing; i.e., a soft tissue seal around the transmucosal part of the implant. A promising approach to improve the biological seal of dental implants is the biomimetic modification of titanium surfaces with proteins or peptides that have specific cell-binding moieties. In this work we investigated the process of immobilising collagen on smooth and rough titanium surfaces and its effect on human dermal fibroblast (HDF) cell response. Titanium samples were activated by either oxygen plasma or acid etching to generate a smooth or nanorough surface, respectively. Subsequently, collagen grafting was achieved by either physisorption or covalent bonding through organosilane chemistry. The biofunctionalised titanium samples were then tested for stability and characterised by fluorescent labelling, wettability, OWLS and XPS studies. Biological characterisation was also performed through HDF adhesion, proliferation and gene expression. Covalent-bonded collagen showed higher stability than physisorbed collagen. A significant overexpression of the genes involved in fibroblast activation and extracellular matrix remodelling was observed in the collagen-coated surfaces. This effect was more pronounced on smooth than on rough surfaces. Immobilised collagen on the smooth plasma-treated surfaces favoured both fibroblast adhesion and activation. This study provides essential information for the design of implants with optimal biological sealing, a key aspect to avoid peri-implantitis and ensure long-lasting implant fixation.

1. Introduction

The success of a dental implant requires not only an optimum osseointegration, but also the development of a soft tissue seal around the transmucosal part of the implant. Dental implants are placed in the mouth, a highly septic medium with several types of pathogenic bacteria [1], and a good sealing is vital to prevent bacterial colonisation, which leads to peri-implantitis, one of the main causes of long-term implant failure.

Different strategies have been explored to improve the biological sealing of dental implants, usually based on either topographical or chemical modifications of the implant surface. Surface texture is known to influence epithelial cells and fibroblast attachment, although there is no complete agreement in the literature on the exact effect. Thus, whereas some authors have shown that smooth surfaces are more favourable for epithelial cell proliferation [2–5],

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http://dx.doi.org/10.1016/j.colsurfb.2014.07.038
0927-7765/

other studies suggest that a certain combined micro- and nanotopography can enhance fibrointegration and an optimal healing of soft tissue [6–8].

On the other hand, biomimetic surface modification with proteins or peptides that have specific cell-binding moieties is another promising approach to improve the biological seal. Titanium functionalisation with fibronectin [9,10] or laminin [9,11], or with biologically relevant peptide sequences [12,13], has been shown to promote cell adhesion in different gingival cells (endothelial, keratinocyte and fibroblast).

Collagen contains also bioadhesive motifs, like the glycine–phenylalanine–hydroxyproline–glycine–glutamate–arginine sequence (GFOGER) or the aspartic acid–glycine–glutamate–alanine (DGEA), which are known to be binding ligands for β1 subgroup of integrins, with higher affinity for α2β1 integrin in the case of type I collagen [14–17].

This has been exploited to enhance osteoblast adhesion and differentiation in vitro [18–21], and in vivo osseointegration [19,22] of Ti or Ti alloys. However, little is known about the effect of collagen functionalisation of metallic implants on soft tissues. Some studies reported that physisorbed type I collagen on Ti or Ti6Al4V alloy surfaces had beneficial effects on fibroblast adhesion [23,24]. More recently Kado et al. reported that type I collagen immobilisation on titanium surfaces through the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide improved significantly human periodontal ligament cells (HPDLCs) adhesion and spreading when compared to uncoated titanium surfaces [25].

Collagen immobilisation on metallic surfaces has been performed in most cases by physical adsorption [18,20,21]. However, some studies have highlighted the advantages of covalent immobilisation, [26,27], for improving the stability of the coating. Organosilanes are commonly used to create a link between the surface oxide of titanium and the biomolecules. However, to the best of our knowledge, there are no studies comparing the efficiency and the in vitro response of fibroblasts to physisorbed vs covalently immobilised collagen.

Therefore, in this work the organosilane 3-(chloropropyl)-triethoxisilane was used to generate collagen covalent bonding at the metal surface. Previous studies in our group proved that this organosilane can provide stable covalent bond at the Ti surface of protein-like biopolymers and oligopeptides [28–31]. Before the silanisation step, the surface must be activated by different oxidation techniques, such as oxygen plasma or acid etching, to create reactive hydroxyl-rich oxide layers that are the basis for the bonding of silane molecules [32,33]. However, these techniques, in addition to activating the surface affect other properties, like topography and wettability, which are known to influence the biological performance of the implants, and more specifically their integration with the soft tissues [2–5]. Thus, we also aimed to investigate the physical and chemical properties of the Ti surfaces activated by either oxygen plasma or acid etching, and their correlation with: (i) the amount and stability of immobilised collagen, either by physisorption of by covalent bonding; (ii) the in vitro response of fibroblastic cells, in terms of adhesion, proliferation and activation, by gene expression studies (real time-PCR).

It is well known that fibroblasts play a critical role in the development of the biological seal. After placement of a dental implant, fibroblasts are recruited to the injury site as part of the healing process, and are activated to a transitional stage called myofibroblasts. The fibroblasts activation level can be determined through the detection and quantification of the expression of α-SMA gene (α-smooth muscle actin) [34]. Subsequent to activation, myofibroblasts synthesise extracellular matrix (ECM) through secretion of some proteins like collagen or fibronectin. After initial ECM deposition, fibroblasts are able to remodel it through secretion of some remodelling enzymes like matrix metalloproteinases (MMPs).

Thus, this study aims to provide a better insight into the interplay between topography and biochemical stimuli in the interaction of functionalised titanium surfaces with soft tissues, as a way to identify the adequate strategies to improve biological sealing of dental implants. This was assessed by investigating the effect of surface roughness and either physisorbed or covalently bound collagen, on the in vitro adhesion, proliferation and activation of fibroblasts.

2. Materials and methods

2.1. Titanium surface preparation

Commercially pure Grade 2 Titanium discs (9 mm in diameter, 2–3 mm thick) were cut from Ti bars (Zapp AG, Ratingen, Germany) and polished with 1200 and 4000 grit silicon carbide paper, and subsequently with colloidal silica (0.06 μm). Then the discs were ultrasonicated in a sodium hydroxide–acetone solution to remove residues of colloidal silica. Next, the polished samples were further cleaned by ultrasonication in cyclohexane, isopropanol, ethanol, deionised water (Mili-Q Plus) and acetone (Sigma–Aldrich, Madrid, Spain) and dried with N_2 gas. Subsequently, the polished surfaces were activated by two alternative processes to remove contaminants and form reactive hydroxyl ions on the surface: (i) either O_2 plasma (PDC-002, Harrick Scientific Corporation, USA), 5 min treatment (PL); (ii) or chemical etching for 1 h with 'piranha' (PH), a 1:1 mixture of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2). After etching, the samples were rinsed with distilled water and acetone and dried with N_2 gas.

2.2. Silanisation

After activation the samples were introduced in a N_2-saturated glass vessel and immersed for 1 h at room temperature in a pentane solution containing 0.05 M of N,N-diisopropylethylamine and 0.5 M of 3-chloropropyl-(triethoxy)silane (CPTES). The nomenclature used throughout the text for plasma and piranha-treated surfaces silanised with CPTES was PL-CP and PH-CP, respectively. All chemicals were purchased from Sigma–Aldrich, Madrid, Spain. Afterwards the samples were ultrasonicated successively in isopropanol, ethanol, deionised water (Mili-Q Plus) and acetone, and dried with N_2 gas.

2.3. Collagen immobilisation

Collagen was immobilised on the titanium surface by two different methods: (1) physical adsorption on the plasma or piranha-treated surfaces (the samples were coded as PL-col or PH-col, respectively); and (2) immobilisation through a silanisation process of the previously plasma or piranha-treated surfaces with CPTES (samples coded as PL-CP-col and PH-CP-col, respectively). Type I Collagen was obtained from bovine pericardium as described elsewhere [35]. Collagen was dissolved in a 0.05 M acetic acid solution (1 mg/mL), and subsequently the pH was adjusted to 6 adding 0.01 M sodium hydroxide, which resulted in a final collagen concentration of 150 μg/mL. The Ti discs were immersed in this solution for 16 h. Afterwards, the samples were removed and rinsed twice with a 0.05 M acetic acid solution to remove excess of adsorbed collagen.

2.4. Surface characterisation

2.4.1. Roughness characterisation

Surface roughness of plasma- and piranha-treated surfaces was examined using a white light interferometer microscope (Wyko NT9300 Optical Profiler; Veeco Instruments, New York, NY, USA) in