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Role of wettability and nanoroughness on interactions between osteoblast and modified silicon surfaces

Miguel Padial-Molina [a,b], Pablo Galindo-Moreno [a,b,*], Juan Emilio Fernández-Barbero [c], Francisco O'Valle [d], Ana Belén Jódar-Reyes [e], Juan Luis Ortega-Vinuesa [e], Pedro J. Ramón-Torregrosa [e]

[a] Oral Surgery and Implant Dentistry Department, School of Dentistry, University of Granada, Campus de la Cartuja S/N, 18071 Granada, Spain
[b] Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA
[c] Human Anatomy and Embryology Department, School of Medicine and IBIMER, University of Granada, Avenida de Madrid 11, 18012 Granada, Spain
[d] Pathology Department, School of Medicine and IBIMER, University of Granada, Avenida de Madrid 11, 18012 Granada, Spain
[e] Applied Physics Department, School of Science, University of Granada, Campus de Fuentenueva S/N, 18071 Granada, Spain

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ABSTRACT

Development of new biomaterials is a constant in regenerative medicine. A biomaterial's surface properties, such as wettability, roughness, surface energy, surface charge, chemical functionalities and composition, are determinants of cell adhesion and subsequent tissue behavior. Thus, the main aim of this study was to analyze the correlation between changes in wettability without topographical variation and the response of osteoblast-like cells. For this purpose oxidized silicon surfaces were methylated to different degrees. Additionally, the influence of nanoroughness, and the subsequent effect of hysteresis on cell behavior, was also analyzed. In this case oxidized silicon pieces were etched with caustic solutions to produce different degrees of nanoroughness. Axisymmetric drop-shape analysis and atomic force microscopy confirmed that the proposed surface treatments increased the nanometer roughness and/or the water contact angles. MG-63 osteoblast-like cells were cultured on the altered surfaces to study proliferation, and for ultrastructural analysis and immunocytochemical characterization. Increasing the nanometer surface roughness or water contact angle enhanced osteoblast behavior in terms of cell morphology, proliferation and immunophenotype, the effect provoked by methylation being more significant than that caused by nanoroughness.

1. Introduction

Bone substitutes, as well as biomedical devices for dental and orthopedic applications, must be able to induce an optimal bone response for optimal clinical success. The surface properties of these biomaterials, such as wettability, roughness, surface energy, surface charge, chemical functionalities and composition, as well as the characteristics of the cells, determine the amount and quality of cell adherence to and proliferation and differentiation on the biomaterial and, consequently, tissue growth and clinical success [1–3]. In addition, wettability and surface energy are key parameters in the adhesion and spreading of osteoblastic cells [4] and determine subsequent processes such as cell morphology, proliferation and differentiation [5]. Moreover, the surface characteristics of the biomaterial are considered to be one of the most important parameters in improving the success of such implants [1]. However, the role played by different surface properties has not been clearly established.

Surface microtopography directly influences osteoblast responses in terms of differentiation and proliferation [6], as well as the expression of differentiation markers and the local production of growth factors and cytokines [4]. However, there is incomplete knowledge concerning the potential influence of surface nanoroughness [7]. In addition, changes in surface roughness also affect wettability (hysteresis) [8]. Wettability determines cell behavior due to dynamic biomolecule adsorption onto surfaces [9]. However, to the best of our knowledge, independent influence by both properties, wettability and roughness, has not been previously studied.

Silica derivatives have been introduced as bone substitutes [10], with reported good clinical success rates and promotion of new vital bone around these materials [11], and as biomimetic agents to coat implant surfaces [12]. One of the most important features of silica derivative biomaterial surfaces is the possibility of easily altering their physico-chemical properties by simple chemical treatments [13]. In this sense, it is possible to alter silicon surface wettability without causing topographical changes by surface methylation, as has been previously established by our group [14]. This enables analysis of the influence of the surface–water

* Corresponding author at: Oral Surgery and Implant Dentistry Department, School of Dentistry, University of Granada, Campus de la Cartuja S/N, 18071 Granada, Spain. Tel./fax: +34 958 520658.
E-mail address: pgalindo@ugr.es (P. Galindo-Moreno).

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contact angle independently of the surface roughness, avoiding the associated effect caused by hysteresis.

Thus, the aim of this study was to analyze a model oxidized silicon surface modified by adding methyl groups. This allows to study the correlation between changes in the wettability without topographical variation and the response of osteoblast-like cells in terms of proliferation, morphological modification and immunophenotype. Additionally, the influence of nanoroughness and the subsequent effect on cell behavior were also analyzed.

2. Materials and methods

2.1. Oxidized silicon surfaces

Single-side polished silicon wafers (100 mm diameter, P-type, Bor ⟨1 0 0⟩ orientation, 1–30 Ω cm resistivity, 525 μm thickness, Si-Mat®, Landsberg, Germany) were cut into 1 × 1 cm squares using a special wafer saw. These pieces were cleaned by immersion for 5 min in a boiling basic peroxide solution [H_2O:H_2O_2 (30 vol.%):NH_4OH (25 vol.%), ratio 5:1:1], rinsed in MilliQ water, immersed for 5 min in a boiling acidic peroxide solution [H_2O:H_2O_2 (30 vol.%):HCl (37 vol.%), ratio 6:1:1] and rinsed in deionized water. This process creates a thin, hydrated silicon dioxide layer that is highly hydrophilic [15].

2.2. Surface alteration

Some pieces were rinsed twice in ethanol and once in trichloroethylene (TCE) (Fluka, Schnelldorf, Germany). Then they were incubated at room temperature for 10 min in solutions of dimethyldichlorosilane (DDS) (Fluka) in TCE, added at concentrations of 0.003%, 0.006%, 0.012% and 0.062% in order to produce different degrees of methylation. Finally, these methylated silicon wafers were rinsed in ethanol, TCE, ethanol again and, finally, deionized water. These samples were designated H1, H2, H3 and H4 (for changes in "hydrophobicity"), corresponding to the increasing DDS concentrations above.

Another group of clean oxidized silicon pieces was subjected to caustic etching in aqueous sodium hydroxide solution (0.1 M NaOH) at room temperature for 0, 22, 60 or 165 min. The specimens were then rinsed with water to remove the excess alkali and rinsed in deionized water. These samples were designated R1, R2, R3 and R4 (for changes in "roughness"), corresponding to increasing etching time (0, 22, 60 and 165 min, respectively).

In order to avoid any possible bacterial contamination all surfaces were stored in 1 mg ml^{-1} sodium azide solution, a concentration sufficient to inhibit bacterial growing [16] but not alter the surface properties, as had been checked on another group of samples by characterizing the surface before and after immersion in sodium azide.

2.3. Surface characterization

To characterize the surface wettability three samples per surface alteration were rinsed with deionized water and dried in N_2 gas before measuring the static contact angle (θ) of a sessile water drop (5 μl volume). Six different and independent experiments were performed per sample. The contact angle values were determined by axisymmetric drop-shape analysis (ADSA) [17]. Images for the analyses were taken with a monochrome CCD camera (Sony SSC-M370CE) coupled to a microscope (Apozoom, Leica Microsystems, Wetzlar, Germany), horizontally oriented in a thermostatically controlled room (20 °C). Droplet images were processed with a PC using a frame grabber (DT 3155, Data Translation GmbH, Bietigheim-Bissingen, Germany).

Surface roughness was characterized by quantitative analysis using an atomic force microscope (Nanoscope IV MultiMode®, Digital Instruments, Santa Barbara, CA), operated in tapping mode with a Si_3N_4 V-shaped cantilever (stiffness $k = 63 \pm 8$ N m^{-1}). Three samples per group were used and three different and independent analyses were performed for each sample, analyzing areas of 2.5 × 2.5 μm. The parameters used to characterize the roughness and the texture of the surface were [18]: the arithmetic average roughness (R_a) (average vertical deviation of the absolute surface value from the mean line); the root mean square roughness (R_q or RMS) (deviation of the surface roughness respect to the R_a value); the roughness surface ratio (r) (ratio between the surface area and the projected surface area that quantifies topographical variations with respect to the planar coordinates). Additionally, the fractal dimension (D_f) of the surface was calculated by the box-counting method [19]. It gives information about structural complexity and is related to the degree of compactness of the space-filling structure.

2.4. Cell culture on modified surfaces

Human MG-63 osteoblast-like cells (supplied by the Scientific Instrumentation Center of the University of Granada, Spain) were added at the same density to three silicon wafers per group, face up in 24-well culture plates. 0.1 ml of a 5000 cell ml^{-1} suspension was used in order to cover the entire wafer surface with a thin layer of cells guaranteeing that the cell culture was not saturated in advance. Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1% penicillin/streptomycin was then added up to 2 ml. Culture plates were maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere. Culture medium was exchanged every 48 h until 21 days after plating. The experiment was repeated three times for the proliferation study, ultrastructural analysis and immunophenotypic characterization.

2.5. Proliferation study

Twenty-one days after plating adherent cells were released from the surface by two sequential incubations in a non-enzymatic cell dissociation solution (0.05% trypsin and 0.02% ethylene diamine tetra-acetic acid (EDTA), Sigma, St Louis, MO) for 5 min at 37 °C. The number of adherent cells was determined by counting in a Neubauer chamber. Four counts were made for each cell suspension released from each sample. The mean number of cells was quantified per milliliter.

2.6. Ultrastructural analysis

After culture on the silicon wafers another group of cell cultures were fixed in 4% sodium cacodylate-buffered formaldehyde/glutaraldehyde fixative for 24 h at room temperature after removing the culture medium, post-fixed in 20% osmium tetroxide for 2 h and dehydrated by serial passage through ascending concentrations of acetone (50–100%). Then they were infiltrated with liquid carbon dioxide below the critical drying point and made electrically conductive by mounting them on aluminum slabs with a silver point. Finally, samples were sputter coated with gold/palladium to a thickness of approximately 250 Å. Field emission scanning electron microscopy (FESEM) (LEO 1530 Gemini, Zeiss/LEO, Oberkochen, Germany) at an accelerating voltage of 5 keV intensity was used for ultrastructural analyses in order to evaluate the cell morphology.