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Leading Opinion

Cells preferentially grow on rough substrates☆

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ABSTRACT

Substrate nanotopography affects cell adhesion and proliferation and is fundamental to the rational design of bio-adhesives, to tissue engineering and to the development of assays for in-vitro screening. Cell behavior on rough substrates is still elusive, and the results presented in the open literature remain controversial. Here, the proliferation of cells on electrochemically etched silicon substrates with different roughness and nearly similar surface energy was studied over three days with confocal and atomic force microscopy. The surface profile of the substrates is a self-affine fractal with a roughness R_a growing with the etching time from ~2 to 100 nm and a fractal dimension D ranging between about 2 (nominally flat surface) and 2.6. For four cell types, the number of adhering cells and their proliferation rates exhibited a maximum on *moderately rough* (R_a ~ 10–45 nm) nearly Brownian (D ~2.5) substrates. The observed cell behavior was satisfactorily interpreted within the theory of adhesion to randomly rough solids. These findings demonstrated the importance of nanogeometry in cell stable adhesion and growth, suggesting that moderately rough substrates with large fractal dimension could selectively boost cell proliferation.

1. Introduction

It is becoming clear that nano/micro-topography stimulates behavioral changes in cells and plays a critical role in modifying proliferation and vitality, as well as the strength of adhesion to substrates. Nano/micro-topography has been recognized as fundamental in the design of bio-inspired materials with controlled adhesion [1–4]; in the development of high-throughput micro-fluidic bio-assays for rapid in-vitro screening [5–7]; in tissue engineering and fabrication of implants [8–10]; in eliciting specific cell responses and controlling the fate of undifferentiated stem cells [11,12].

A variety of techniques have been reported for creating substrates with a controlled topography exhibiting short and long range order on different materials. These include lithographic-based methods, as well as electrochemical etching, polymer demixing, electrospinning, and the use of block-copolymers [13]. This has fostered the analysis of cell proliferation, adhesion, migration and differentiation on ordered, structured surfaces over multiple scales. Stemming from the pioneering work of Curtis and his group [14], it has been extensively documented [15] how cells tend to respond to micro features and how such a response is affected by the geometry, material properties, surface functionalization and cell type. For instance, it has been consistently demonstrated that several cell types tend to align, elongate and more avidly adhere over line-grated substrates; whereas reduced adhesion has been observed over pillars and posts. More recently, new insights on the mechanisms regulating the early interaction of cells membranes with nanometer features have been proposed by the group of Bongrand [16] and Spatz [17].

Still, the results presented in the literature for cell adhesion on un-structured randomly rough surfaces, which constitute the majority of natural surfaces, remain controversial, and currently there is no available framework to interpret or even summarize such results. Some studies have documented a decrease in

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proliferation and adhesion with an increase in surface roughness [10], whereas others have shown precisely the opposite [18,19]. A few papers have demonstrated a minor influence of roughness [20], and more interestingly, some studies have observed an 'optimal' roughness for maximum proliferation [9,21].

In this work, four different cell lines from two different species, namely A549 human lung carcinoma, human HeLa, human umbilical vein endothelial cells (HUVECs) and mouse 3T3 fibroblasts, were cultured over electrochemically etched silicon substrates with a surface roughness varying from $R_a \sim 2$ to 100 nm. The rate of proliferation and surface density of cells were monitored through confocal and atomic force microscopy, over three days. The surface roughness of the silicon substrates was analyzed within the realm of fractal theory, and the average roughness R_a, the root mean square roughness R_{rms} and the surface roughness power spectrum were measured.

2. Materials and methods

2.1. Preparation of the rough silicon substrates

(111)-oriented Si wafers were used as substrates. The superficial layer of SiO_2 was removed by immersion in HF:H_2 = 1:5 v/v solution for 30 s. A fresh silicon surface was exposed, and the samples were then wet etched in KOH solution (KOH:H_2O = 1:4 v/v) at different times and at the constant temperature $T = 70\,^{\circ}C$ to obtain surfaces with different roughness. The average surface roughness R_a and the root mean square roughness R_{rms} were readily calculated following the definitions $R_a = \int_l |z(r)|dr/l$ and $R_{rms} = \sqrt{\int_l z(r)^2 dr/l}$, where l is the sampling length and $z(r)$ is the profile of the surface along the r direction, measured using an atomic force microscopy probe operated in tapping mode [22]. R_a and R_{rms} were assessed over multiple regions of the substrates.

2.2. Atomic force microscopy characterization of the rough silicon substrates

Atomic Force Microscopy (diCaliber, Veeco Instruments) was used for deriving the surface roughness profile and for imaging adhering cells. All the measurements were performed in a dry environment at room temperature in tapping mode (oscillating frequency ~270 kHz) over a sampling area of 50 × 50 and 90 × 90 μm^2 for the rough substrates and the cells, respectively. An anisotropic pyramidal tip with a radius of about 15 nm was used as a probe (TESP, NanoWorld Ldt. Co.). The tip was made of Silicon and was mounted onto a rectangular shaped cantilever with a typical spring constant between 20 and 80 N/m. Multiple measurements were made in different scan directions. At least four images in height mode (trace and retrace) were recorded per sample. The images had a resolution of 256 × 256 pixels and were acquired at a scanning rate of about 1 Hz. The images obtained were processed with the diSPMLab software (Veeco).

2.3. Surface contact angle measurement

Surface hydrophilicity of the samples was determined by measuring the water contact angle with one drop (5 μl) of deionized water using an automatic contact angle meter (KSV CAM 101, KSV INSTRUMENTS LTD, Helsinki, Finland) at room temperature. Four measurements were performed on each substrate to evaluate the average contact angle θ, at 5 s. Following the Young–Dupre equation, the energy of adhesion γ per unit area at the silicon/water interface was defined as $\gamma = \gamma_{LG}(1 + \cos\theta)$, where γ_{LG} is the air/water surface tension (~ 72.8 mJ/m^2 at 20 °C).

2.4. Fourier analysis and fractal dimension of the substrate

The profiles of the substrates, obtained by the procedures described above, were processed to obtain the corresponding power spectrum density functions $C(q)$, defined over the surface (x, y) as [23]

$$C_{2D}(q) = \frac{1}{(2\pi)^2}\left(\int \langle z(x)z(o)\rangle e^{-iqx} dx^2\right) \quad (1)$$

where $\boldsymbol{x} = (x, y)$ is the planar coordinate; $z(\boldsymbol{x})$ is the surface profile measured from the average surface plane, defined as $\langle z\rangle = 0$; and q is the wavenumber, related to the characteristic wavelength λ as $q = 2\pi/\lambda$. The symbol $\langle\ldots\rangle$ stands for ensemble averaging over a collection of different surfaces with identical statistical properties. Since the 2D power spectrum density introduced in Eq. (1) is impractical for comparison purposes, a 1D power spectrum density was conveniently extracted using the FACA (Fractal Analysis by Circular Averaging) approach [24]. Considering the polar variables q and ψ ($q = \sqrt{q_x^2 + q_y^2}$ and $\psi = \arctan(q_y/q_x)$) in the plane (x, y) of interest, the power spectrum $C(q)$ is derived as an average taken over every circumference Γ of radius q and origin ($q_x = 0$, $q_y = 0$), that is to say

$$C(q) = \frac{1}{\Gamma}\oint_{\Gamma} C_{2D}\left(q_x, q_y\right)d\gamma = \frac{1}{2\pi}\int_0^{2\pi} C_{2D}(q\cos\psi,\ q\sin\psi)d\psi \quad (2)$$

The resulting function $C(q)$ can be plotted as in Fig. 4 (and Fig. S5).

In the case of self-affine surfaces, for which a rescale in the planar coordinates $x \to bx$ and $y \to by$ is accompanied by a rescaling in the normal direction z ($b\boldsymbol{x}$) $\to bHz(\boldsymbol{x})$, the power spectrum $C(q)$ takes the form [23]

$$C(q) = \frac{H}{2\pi}\left(\frac{h_o}{q_o}\right)^2\left(\frac{q}{q_o}\right)^{-2(H+1)} \quad \text{for } q > q_o(\lambda < \lambda_o) \quad (3)$$

where q_o is the lower cut-off wavenumber corresponding to an upper cut-off wavelength $\lambda_o = 2\pi/q_o$; and h_o is related to the rms roughness amplitude as $h_o = \sqrt{2}R_{rms}$. From Eq. (3), a self-affine fractal surface can be univocally identified by specifying the surface roughness (R_{rms}), the cut-off wavenumber q_o and the coefficient H, known as the Hurst coefficient. In a log–log plot, the power spectrum density of Eq. (3) appears as a line with a slope β for $q > q_o$. The slope β is related to the Hurst parameters as $\beta = 2(H + 1)$. The fractal dimension D of the surface can be derived from β or H as $D = (8 - \beta)/2$ or $D = 3 - H$. The fractal dimension D for a surface ranges from 2 ($H = 1$), representing a perfectly flat surface (Euclidean dimension of a surface), to 3 ($H = 0$), representing an extremely rough surface. For $D = 2.5$ ($H = 0.5$), the so-called Brownian surfaces are identified which have totally random and uncorrelated profiles.

2.5. Cell culture

Four different cell lines were used: a primary human endothelial cell line (HUVEC-C), two human epithelial cancer cell lines (A549 and HeLa cells), and one mouse mesenchymal normal cell line (NIH-3T3). All the cells were obtained from the American Type Culture Collection. The A549 cells were cultured in RPMI (Invitrogen) with 10% FCS (Invitrogen), L-glutamine 200 mM (Invitrogen) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.5 g/ml, Invitrogen). The human HeLa cells were cultured in DMEM supplemented with 10% FBS, penicillin G (100 U/ml, Invitrogen) and streptomycin (100 μg/ml, Invitrogen). The human umbilical vein ECs (HUVECs) were cultured in M199 medium containing 20% newborn calf serum (NCS, GIBCO-BRL), 5% human serum (Gemini Bio-Products, Inc), 50 mg/mL ascorbic acid, 1.6 mmol/L L-glutamine, 5 mg/mL bovine brain extract (Clonetec Corp), 7.5 mg/mL endothelial growth supplement (Sigma), 100 U/mL penicillin, 100 mg/mL streptomycin, and 10 U/mL heparin. HUVECs of the third to fifth passage were used for all experiments. The 3T3 cells were kept in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), penicillin G (100 U/ml, Invitrogen), streptomycin (100 mg/mL, Invitrogen), L-Glutamine 2 mM (Invitrogen), Sodium Pyruvate 1 mM (Invitrogen). All the cells were cultured at 37 °C in a humidified 5% CO2 atmosphere; were detached by trypsinization, collected by centrifugation and resuspended in culture medium. Sterilized rough Si wafer specimens (15‰15 mm approximately) were individually placed into single wells of a 6-well plate (Corning Incorporated) and the nominally flat silicon surface was placed in a 30 mm petri dish (Corning Incorporated) (control experiment). Thereafter, the wafer specimens were washed with phosphate-buffered saline solution (PBS, Invitrogen). The cells were finally seeded in complete cell culture medium and incubated for 24, 36, 48 and 60 h at 37 °C in a humidified 5% CO_2 air atmosphere. After incubation the cell culture medium was removed and the cells were washed twice in PBS and fixed with BD Cytofix (BD Biosciences). 100 μl of Cytofix were put on each sample and were in dark incubated for 30 min at 4 °C. The cells were washed twice with Cytoperm (a permeabilization solution, BD Biosciences). All the cells fixed upon the Si substrates were stained with 100 μl DAPI (4′, 6-Diamidino-2-phenylindole, Sigma–Aldrich) solution for 5 min at 4 °C. Finally, the DAPI solution was removed and each sample was washed with PBS. The total number of cells initially deposited in each well for incubation was $n_{tot} \sim 60000$, 28751, 18,0000 and 20,000 for the mouse 3T3 fibroblasts, human HeLa, human lung carcinoma cells and HUVECs experiments, respectively. The cells were sub-confluent throughout the duration of the experiment.

After 48 h the cells were fixed according to the protocol above and stained with 100 μl of mouse anti clathrin (AbD Serotec) solution and incubated for 30 min. The samples were washed twice with Cytoperm and a secondary antibody (Alexa Fluor 488 chicken anti-mouse from Invitrogen) was added. After 45 min of incubation the cells were washed twice with PBS and incubated with Alexa Fluor 546 phalloidin (Invitrogen) to stain F-Actin (a fibrous actin polymerized in the form of a double helix). Finally the samples were washed twice with PBS.

2.6. Counting the number of adhering cells

An inverted Leica TCS-SP2® laser scanning confocal microscopy system was used to image cells adhering on the substrates. All the measurements were performed using a ArUv laser. The pinhole (~80 μm, or equivalently ~1.5 Airy units) and laser power (80% power) were maintained throughout each experiment. Confocal images