Leading Opinion

Cells preferentially grow on rough substrates

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A B S T R A C T

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 $R_\text{s}$ 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 ($R_\text{s}$ ~ 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
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 Rs ~ 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 Rs, the root mean square roughness 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 SiO2 was removed by immersion in HF:H2O = 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: H2O = 1:4 v/v) at different times and at the constant temperature T = 70°C to obtain surfaces with different roughness. The average surface roughness Rs and the root mean square roughness Rms were readily calculated following the definitions Rs = \int [z(l)]^2 dl and Rms = \sqrt{\int [z(l)]^2 dl}, where l is the sampling length and z(l) is the profile of the surface along the l direction, measured using an atomic force microscopy probe operated in tapping mode [22]. Rs and Rms were assessed on 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 x 50 and 90 x 90 μm2 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 Ltd., 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 x 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 γ - γ_w (1 + cosθ), where γ_w is the air/water surface tension (~ 72.8 mJ/m2 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]

\[ C(q) = \frac{1}{2\pi^2} \left( \int (2\pi x) e^{-4\pi i x} \, dx \right) \]

where x = (x, y) is the planar coordinate; z(x) is the surface profile measured from the average surface plane, defined as z0 ~ 0, and q is the wavenumber, related to the characteristic wavelength λ as q = 2π/λ. The symbol (…) 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 ϕ (q = \[q_1^2 + q_2^2\] and ϕ = \[\arctan(q_2/q_1)\]) in the plane (x,y) of interest, the power spectrum C(q) is derived as an average taken over every circumference l of radius q and origin (q1 = 0, q2 = 0) that is to say

\[ C(q) = \frac{1}{2\pi} \int C_{2D}(q_1, q_2) \, dq_2 \]

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 → bx and y → by is accompanied by a rescaling in the normal direction z (bα) → bαz(bα), the power spectrum C(q) takes the form [23]

\[ C(q) = \frac{H}{q^{2.5}} (q_0^2 < q < q_o) \]

where q0 is the lower cut-off wavenumber corresponding to an upper cut-off wavelength λ0 = 2π/q0; and H is related to the rms roughness amplitude as λ0 = 2R horizon. From Eq. (3), a self-affine fractal surface can be univocally identified by specifying the surface roughness (Rms), the cut-off wavenumber q0 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 > q0. The slope β is related to the Hurst parameters as β = 2(α – 1). The fractal dimension D of the surface can be derived from β or H as D = (8 – β)/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 EECs (HUVEC) 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 mM 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 200 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 10 mm petri dish (Corning Incorporated) (control experiment). Thereafter, the wafer specimens were washed with phosphate-buff ered 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% CO2 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 Ntot = 60000, 28751, 180000 and 20000 for the mouse 3T3 fibroblasts, human HeLa, human lung carcinoma cells and HUVEC 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 A laser scanning confocal microscopy system was used to image cells adhering on the substrates. All the measurements were performed using a Ar/UV laser. The pinhole (~ 80 μm, or equivalently ~ 1.5 Airy units) and laser power (80% power) were maintained throughout each experiment. Confocal images
of blue (DAPI) fluorescence were collected using a 405 nm excitation line and a 10× dry objective, so that cells with a characteristic size of a few microns could be clearly observed. For each substrate a large number of images (≥40) was taken for statistical analysis. Each image was acquired over a region of interest of 882×882 μm² (pixel size = 1.72 μm) and averaged over 4 lines and 10 frames to improve quality and reduce noise. The images were digitized into 512 × 512 pixels and stored on a computer. The fluorescent confocal images of the cells were exported into MatLab® and Mathematica® for deconvolution using in-house developed software. The number of cells adhering to the substrate within the region of interest at different roughness and time steps was measured. The cells were analyzed over time after 24, 36, 48 and 60 h from incubation. The number of adhering cells was normalized with respect to the total number of cells initially deposited in each well for incubation and divided by the well area \( A = 707 \text{ mm}^2 \) and \( n_{	ext{tot}} \) as reported above.

The fluorescence distribution of Actin and Clathrin in mouse 3T3 fibroblast cells was analyzed on unetched and rough (\( R_a = 37.87 \text{ nm}; R_{\text{rms}} = 53.78 \text{ nm} \)) substrates, using an inverted Nikon TE2000-C1 confocal microscope. All the images were acquired using a 60×/1.49 NA. TIRF oil immersion objective. The pinhole (30 μm, or equivalently 1.0 Airy units) was maintained throughout each experiment, thus yielding the maximum confocality for the system. Sample fluorophores were excited using a 488 nm Ar Laser (for clathrin) and a 543 nm He–Ne laser (for actin). Each image was acquired over a region of interest of 68 × 68 μm² and 83 × 83 μm² for the flat and rough substrate, respectively, and averaged over 4 frames to improve quality and reduce noise. The images were digitized into 512 × 512 pixels and conveyed to a computer for storage.

2.7. Statistical analysis

In terms of cell count and surface characterization (\( R_a, R_{\text{rms}}, \theta \)), all data were expressed as mean ± standard deviation, and analyzed statistically by the paired Student’s t test method. Significant difference was determined at \( P \) values smaller than 0.05.

3. Results

(111)-oriented silicon wafers were etched in diluted KOH solutions at 70 °C. The surface roughness of the wafer was observed to increase with the etching time \( \tau \) (Fig. 1a). Six different etching times were used ranging from 2 to 60 min leading to surfaces with an average roughness varying from \( R_a \approx 10 \) to 100 nm. The unetched silicon wafer showed a \( R_a = 2.33 \text{ nm} \). The corresponding values of the root mean square roughness \( R_{\text{rms}} \) were provided in Table 1. Representative images of the etched silicon substrates taken through atomic force microscopy are shown in Fig. 1, for \( R_a = 2.33 \text{ nm} \) (unetched wafer), 37.87 and 101.47 nm. Topographical images for all the substrates are given in the Supplementary data (Fig. S1). From Fig. 1, different geometrical features of the silicon substrates are depicted: the unetched silicon substrate appeared as almost uniformly flat with short peaks in the few nanometer range; at the other extreme, the highly etched silicon substrate (\( R_a \approx 100 \text{ nm} \)) appeared as a set of regular flat ridges a few microns apart; whereas the moderately etched silicon substrate (\( R_a \approx 10–45 \text{ nm} \)) was evenly ‘corrugated’ with individual high peaks. Notice that the individual spikes visible on the unetched silicon substrate in Fig. 1b are experimental noise. The hydrophilicity of the silicon substrates was also characterized by measuring the contact angle \( \theta \). For the unetched wafer, \( \theta \) was about 50° whereas for the rough substrates it varied between 8° (\( R_a = 18.93 \text{ nm} \)) and 18° (\( R_a = 101.47 \text{ nm} \)). Therefore, using the Young–Dupre’ relation, the average surface energy for the rough substrates was \( \gamma = 0.144 \text{ J/m}^2 \), with a variation smaller than 2%. The surface energy for the nominally flat substrate was measured to be \( \approx 0.120 \text{ J/m}^2 \). The etching time \( \tau \), the characteristic surface roughness parameters (\( R_a \) and \( R_{\text{rms}} \)) and the contact angle \( \theta \) are listed in Table 1 for all seven analyzed substrates.

The proliferation rate on the silicon substrates was analyzed by incubating the cells with fragments of silicon wafers (\( \approx 15 \times 15 \text{ mm} \)) placed into the wells of a 6-well plate. At each time point, the silicon substrates were first washed to remove loosely adhering cells; then the cells were fixed and stained with DAPI. Fig. S2, in the Supplementary Data, shows representative images

![Fig. 1](image-url) The variation of the average and root mean square surface roughness (\( R_a, R_{\text{rms}} \)) as a function of the etching time \( \tau \) (a) and the surface profiles detected through atomic force microscopy for three sample substrates, namely \( R_a = 2.33 \text{ nm} \) (b); \( R_a = 18.93 \text{ nm} \) (c) and \( R_a = 101.47 \text{ nm} \) (d).
Table 1
Surface properties of the silicon substrates generated through electrochemical etching. The numbers in bold represent the values for moderately rough silicon substrates (Rq ≈ 10–45 nm).

<table>
<thead>
<tr>
<th>t [min]</th>
<th>Rq [nm]</th>
<th>Rms [nm]</th>
<th>θ</th>
<th>β</th>
<th>D</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.15</td>
<td>3.24</td>
<td>50.29</td>
<td>-1.56</td>
<td>3.09</td>
<td>2.45</td>
</tr>
<tr>
<td>2</td>
<td>0.21</td>
<td>5.20</td>
<td>9.11</td>
<td>-0.49</td>
<td>2.91</td>
<td>2.54</td>
</tr>
<tr>
<td>3</td>
<td>0.26</td>
<td>10.45</td>
<td>7.90</td>
<td>0.77</td>
<td>3.02</td>
<td>2.49</td>
</tr>
<tr>
<td>4</td>
<td>0.30</td>
<td>15.81</td>
<td>15.81</td>
<td>0.88</td>
<td>3.51</td>
<td>2.24</td>
</tr>
<tr>
<td>5</td>
<td>0.37</td>
<td>24.02</td>
<td>12.20</td>
<td>0.68</td>
<td>2.96</td>
<td>2.52</td>
</tr>
<tr>
<td>6</td>
<td>0.45</td>
<td>37.87</td>
<td>9.11</td>
<td>0.49</td>
<td>2.91</td>
<td>2.54</td>
</tr>
<tr>
<td>7</td>
<td>0.51</td>
<td>53.78</td>
<td>5.12</td>
<td>0.48</td>
<td>2.91</td>
<td>2.54</td>
</tr>
</tbody>
</table>

Fig. 2. Relative density of stably adhering cells nd as a function of the silicon substrate average roughness Rq at different time points, for mouse 3T3 fibroblasts (a); human HeLa cells (b); human A549 lung carcinoma (c) and HUVEC-C (d).

of 3T3 fibroblasts growing on a silicon substrate (Rq = 37.87 nm) taken at four time points, namely 24 h, 36 h, 48 h and 60 h after seeding. The cells adhering over the region of interest with fixed area (882 × 882 μm²) were counted by analyzing the confocal fluorescent microscopy images with an ad-hoc imaging analysis software. For each silicon substrate, more than 40 ROIs were monitored to provide a meaningful sample size for the statistical analysis. The cell morphology was also imaged through atomic force microscopy (Fig. S3). For all substrates and each cell line, a well spread cytoskeleton and the formation of long lamellipodia protruding out of the cell membrane were observed, both signs indicating healthy, growing cells.

For a quantitative analysis of proliferation, the number of cells adhering to the substrate was normalized by the total number of cells originally incubated and divided by the surface area of the well. The resulting number is the relative density of cells nd which was measured as a function of time, substrate roughness and cell type. The parameter nd was plotted as a function of the silicon surface roughness and at different times points in Fig. 2, for the four different cell lines considered. As expected, the number density nd increased with time. More interestingly, Fig. 2 showed the existence of a preferential substrate roughness for which the number density of adhering cells and the corresponding proliferation rate were maximized. For the 3T3 fibroblasts (Fig. 2a), the optimal roughness Rq was observed to range between about 10 and 45 nm. A similar behavior was observed for the HeLa tumor cells (Fig. 2b). Differently, for the A549 human lung carcinoma (Fig. 2c) and the HUVEC-C, a more clear absolute maximum was depicted for Rq = 37.8 nm and Rq = 18.9 nm, respectively, at 60 h. Introducing the ratio G between the absolute maximum for nd and the corresponding value over the unetched silicon substrate, at 60 h, the 3T3 fibroblasts were observed to proliferate 3-times faster (G ≈ 3) on moderately rough substrates; whereas for the HeLa, HUVEC-C and the A549 lung carcinoma the ratio G was much larger being equal to about 5, 26 and 50, respectively. In Fig. 3, the surface densities of the cells on the optimal (blue bar), nominally flat (green bar; Rq = 2.33 nm) and highly rough (pink bar; Rq = 101.47 nm) substrates were compared. These experimental evidence support the notion that cell proliferation can be enhanced over moderately rough substrate already after 48 h.

Electrochemical etching generates roughness over multiple scales with characteristic features at the micron, sub-micron and nano scale. The roughness parameters Rq and Rrms cannot capture the multiscalar essence of the surface profile [25]. Therefore, to gain a deeper insight into the mechanisms favoring cell stable adhesion and proliferation, the surface profiles were analyzed within the theory of fractals [23]. The surface roughness power spectrum density C(q) was determined using atomic force microscopy [24], as described in the Materials and Methods. The relation C(q) was shown in Fig. 4 for three representative substrates, namely Rq = 2.33, 37.87 and 101.47 nm. In the Supplementary data (Fig. S5), images of the power spectrum C(q) for all the analyzed substrates were presented. Interestingly, Fig. 4 and Fig. S5 showed that the
The power spectrum $C(q)$ can be quite accurately represented through a straight line with a slope $\beta$ in a log–log diagram, for a sufficiently large $q$ (\(q_0 = 0.5-2 \mu m^{-1}\)). This demonstrated that the silicon substrates were self-affine fractals and could be univocally described by the two independent variables $R_{rms}$ and $D$. By a least square fitting of the experimental results (Fig. 4 and Fig. S5), the slope $\beta$ was estimated and from this, the fractal dimension $D$ and the Hurst exponent $H$ were readily derived following the definitions $D = (8 - \beta)/2$ and $H = (\beta - 2)/2$, respectively. In Table 1, the values for $\beta$, $D$ and $H$ are listed for all the analyzed silicon substrates. The fractal dimension $D$ and contact angle $\theta$ are plotted in Fig. 4d. The parameter $D$ was measured to be about 2 for the unetched and highly etched wafer and was about 2.5 (Brownian surface) for moderately rough substrates ($R_a = 10-45$ nm). On the other hand, the contact angle $\theta$ was about $50^\circ$ for the unetched wafer and about $14^\circ$ for all other substrates.

As shown in Figs. 2 and 4d, the larger number of adhering cells and the highest proliferation rates were associated with the moderately rough silicon substrates ($R_a \sim 10-45$ nm) and the fractal dimensions $D = 2.502 \pm 0.038$ ($H = 0.497 \pm 0.039$). These evidences support the notion that cells proliferate more on moderately rough Brownian substrates ($D = 2.5$) as compared to nominally flat or extremely rough substrates.

4. Discussion

The increased proliferation rate observed on moderately rough Brownian substrates could be attributed to several, possibly concurring, mechanisms among which (i) the increased effective surface energy $\gamma_{eff}$ typically associated with moderately rough substrates [26,27] and (ii) the non-uniform surface adsorption and preferential conformation of proteins over non planar substrates [28–31]. The effective surface energy $\gamma_{eff}$ of a cell adhering to a substrate is defined as the total work per unit area required for full detachment. In general, this work would depend on the surface roughness, and it would be $\gamma_0$ for perfectly planar surfaces.

![Fig. 3. The maximum relative density of stably adhering cells $n_d$ compared to the corresponding values for $R_a = 2.33$ nm (unetched silicon substrates) and $R_a = 101.47$ nm at 60 h (The star symbol means statistically significant difference with $P < 0.01$). The ratios between the $n_d$ max and on unetched substrates are 2.7; 5; 30 and 30 respectively for the Fibroblasts, HeLa, Lung Carcinoma and Huvecs. Similarly the ratios between the $n_d$ max and on $R_a = 101.47$ nm substrates are 2; 1.5; 3 and 2.2.](image)

![Fig. 4. Power spectrum analysis of three representative silicon substrates, namely $R_a = 2.33$ nm (a); $R_a = 37.87$ nm (b) and $R_a = 101.47$ nm (c). The variations of the surface fractal dimension $D$ and contact angle $\theta$ over the seven silicon substrates (d).](image)
Cell adhesion is mediated by the formation of discrete ligand–receptor bonds (specific interactions) and short ranged interfacial forces as van der Waals, double layer electrostatic and steric (non-specific interactions), which together contribute to the surface energy of specific and non-specific adhesion $g_{\text{adh}}$ [26]. This grows with the density of the ligands adsorbing on the silicon substrate, the density of the receptors expressed on the cell membrane and the affinity of the ligand–receptor bonds.

Under the simplistic assumption that cells can be represented as thin elastic layers of modulus $E$ sitting over a rigid wavy surface with a fixed wavenumber $q$ and an amplitude $h$, the ratio $\gamma_{\text{eff}}/\gamma_0$ has been shown [26] to be larger than unity for $h$ smaller than $h_c$. In other words, cell stable adhesion and, consequently, proliferation would be energetically favorable on rough surfaces as long as $h < h_c$, which would then identify the subset of moderately rough substrates for a given $q$. Also, $\gamma_{\text{eff}}/\gamma_0$ and $h_c$ have been predicted to grow with the normalized parameter $q g_{\text{adh}}/E$ [26].

As schematically shown in Fig. 5a, cells settling down in the quiescent fluid of the culture well would make first contact with the peaks of the silicon substrate. These would be candidate spots for the formation of focal complexes (FXs), the first step in the adhesion process. The subsequent enlargement of the adhesive spots would be driven by a reduction in free energy of the system regulated by the interplay between the surface energy $g_{\text{adh}}$, which drives for adhesion, and the energy required for stretching the cell membrane and recruiting new adhesive molecules [26], which opposes adhesion. For $h < h_c$, the free energy reduction associated with the increase in the size of the adhesion spot would be larger, in modulus, than the free energy increase associated with the stretching of the cell membrane and the recruitment of new cell receptors at the adhesion site (i.e. $\gamma_{\text{eff}}/\gamma_0 > 1$). Conversely, for $h > h_c$ the stretching and ‘recruitment’ energies would outgrow the contribution of $g_{\text{adh}}$, thus impairing stable cell adhesion and proliferation (i.e. $\gamma_{\text{eff}}/\gamma_0 < 1$). Evidently as $q g_{\text{adh}}/E$ increases, adhesion would be facilitated in that the free energy reduction is proportional to $g_{\text{adh}}$ and the energy for cell stretching reduces with $E$ reducing.

Similar conclusions have been drawn in the more general context of adhesion on randomly rough substrates [27], where $\gamma_{\text{eff}}/\gamma_0$ and $h_c$ have been shown to depend also on the fractal dimension $D$, in addition to the surface roughness ($h$ for a single wave substrate) and the material parameter $q g_{\text{adh}}/E$. In particular, $\gamma_{\text{eff}}/\gamma_0$ and $h_c$ have been predicted to grow with $D$ in that for a given surface roughness (fixed $R_a$ or $R_{\text{rms}}$), the surface area available for adhesion would grow with the fractal dimension $D$ [27]. Therefore, the theory of adhesion of elastic solids on randomly rough substrates could explain the observed preferential stable adhesion and proliferation of cells on moderately rough surfaces. Also, the same theoretical reasoning would suggest that for a given roughness interval (moderately rough substrate), the effective energy $\gamma_{\text{eff}}$ would steadily grow with the fractal dimension $D$. However, in this study, it was not possible to etch silicon substrates beyond $D = 2.6$, possibly because of the extremely high surface reactivity of such substrates.

![Fig. 5. Pictorial sketch of a cell membrane adhering on a rough substrate. Initially, the cell membrane makes point contact with the higher peaks on the substrate (a). As time passes, the cell membrane stretches and wraps around the peaks expanding the initial adhesion spot, almost mimicking an endocytic process (b). The wrapping of the cell membrane induces tensile intracytoskeletal stresses that could favor the recruitment of new adhesive molecules (c).](image-url)
In the discussion above, it has been tacitly assumed that cells act as passive systems insensitive to external mechanical stimuli. Indeed, it is well known that the maturation of FXs into more stable focal adhesions (FAs) is regulated by mechanical intracytoskeletal stresses arising at the original adhesion spot, which are believed to trigger the recruitment of additional ligand molecules [32,33]. Cell adhesion on nanoposts has been associated with localized membrane stretching and wrapping around the geometrical features, in a process similar to endocytosis [34], which is associated with local mechanical stresses and cytoskeletal reorganization. This could be responsible for the recruitment of new molecules at the adhesion site (peaks on the substrate), which would eventually increase locally $\gamma_{adh}$ and therefore facilitate adhesion. Cell staining with clathrin, a marker associated with endocytic vesicles, was demonstrated to reproduce the substrate nanotopography. Fig. 6 shows clusters of clathrin molecules (green spots) smaller and more uniformly distributed over the whole cell membrane for the unetched wafer (uniformly distributed small peaks) compared to substrates with higher roughness (large individual peaks).

Finally, a few studies have also tried to address the role played by protein adsorption and conformation on cell adhesion. The surface energy of the rough substrates was demonstrated to be nearly constant ($\sim 0.144 \text{ J/m}^2$) before cell culturing. However, even if the silicon substrates were not functionalized, proteins such as fibronectin, vitronectin and fibrinogen would tend to deposit on the silicon substrate from the cell culture medium supporting the formation of specific molecular bonds [29]. In this context, a few authors have observed an increased adsorption and a better orientation of the proteins on rougher or nanostructured substrates [28–31]. The larger adsorbed amount as well as the proper orientation and conformation of the proteins would lead to an increase in $\gamma_{adh}$, thus favoring adhesion on rougher and nanostructured substrates as compared to nominally flat surfaces.

Preferential stable adhesion and proliferation of cells on moderately rough surfaces has been observed also in the case of non silicon substrates and for cell lines different from those considered in this analysis. On nanotextured substrates, made up of demixed poly(lactic acid) and polystyrene, human fetal osteoblastic cells have shown optimal adhesion and spreading for $R_a$ ranging between 5 and 15 nm [35]. With a similar technique but using different polymers (polybromostyrene and polystyrene), fibroblasts have been documented to grow and adhere more avidly on the same small range of $R_a$ [21]. For bacteria, as the staphylococcus epidermidis growing on PMMA, the optimal surface roughness $R_a$ was found to range from 40 to 100 nm [36]. For silicon
etched substrates, again, neurons have been shown to adhere more strongly for $R_a \sim 25$ nm [9]. Macrophages have been traditionally considered as rugophobic cells adhering more easily on planar rather than rough surfaces [37]. However, recent studies seem to support again the notion that moderate roughness (from a few nanometers to a few tens of nanometers) could help macrophages to adhere even on cytotoxic surfaces made by zinc oxides [38].

5. Conclusions

We show that stable cell adhesion and proliferation can be maximized on moderately rough substrates. Although this was observed for four different cell lines, belonging to two different species, growing over seven electrochemically etched silicon substrates with different surface roughness for three days, additional studies are needed to assess the generality of the results. The present study emphasized also the importance of (i) fully characterizing the substrate topography, by measuring the power spectrum density rather than just the average or root mean square roughness; and (ii) developing effective micro/nano fabrication protocols for generating substrates where roughness and fractal dimension could be controlled independently.

The use of silicon in biomedical applications is becoming more and more important with successful examples in the development of brain–machine interfaces [39], retinal implants [40] and in the design of microfluidic chips for high-throughput screening [41]. This study would suggest that designing silicon substrates with moderate roughness could enhance the proliferation rate of specific cells and, thus boost the performances of implantable devices.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2010.06.016.

References


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