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Orthogonal nanometer-micrometer roughness gradients probe morphological influences on cell behavior

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ABSTRACT

Surface gradients facilitate rapid, high-throughput, systematic investigations in cell biology, materials science, and other fields. An important surface parameter is the surface roughness on both the micrometer and nanometer scales in the lateral direction. Two approaches have been combined to create two-dimensional roughness gradients by adding a nanoparticle density gradient onto a gradient of micro-featured roughness. All fabricated gradients were extensively characterized by SEM, AFM and optical profilometry to ensure their quality and to determine the roughness parameter R_a along the gradient. Additionally, a Fourier-transform approach was applied that allows a wavelength-dependent analysis of the surface topography. Since cell-culture assays require replicate experiments, a replica technique was used to create copies of the master gradient. Creating a negative replica in an elastomeric material served as a mold for a subsequent ceramic-casting process. A positive replica was then formed from epoxy resin, which was subsequently coated with titanium and used for cell studies. Finally, these gradients were used in cell-culture assays to determine cellular response to surface roughness. The results clearly demonstrate the influence of surface roughness on the production by osteoblasts of markers for osteogenesis. It was shown that high roughness in the micrometer range, combined with an intermediate nanofeature density (30–40 features/μm^2), leads to the highest degree of osteopontin production after 14 days.

1. Introduction

The investigation of the influence of a surface parameter (e.g. chemistry or morphology) on a phenomenon such as cell adhesion or tribological properties traditionally involves experiments on a series of individual, homogeneous samples, each with a different value of the parameter of interest. This method, however, can introduce an additional source of error due to culture-to-culture variation and requires repeated experiments to ensure reliable results. By creating samples with one gradually changing parameter, many of these problems, including the labor and time of processing multiple samples, can be overcome. When testing such a gradient surface, the entire range of the parameter is covered on a single sample and homogeneous conditions during the experiment are ensured. Thus, gradient surfaces allow thorough, systematic studies of the effects of changing a surface parameter, while time and resources can be saved.

Many methods for the fabrication of surface-chemical gradients have been developed [1–5]. Generally, they are based on two principles: either the outermost layer of a surface is gradually modified by a high-energy beam or by chemical etching, or a surface coating (e.g. self-assembled monolayer, polymer brush) is attached to the surface in a gradual manner.

In materials science, another equally important surface parameter is the surface roughness. It plays a significant role in tribology and adhesion [6,7] but also affects biological response to a surface, e.g. cell adhesion, morphology, proliferation and differentiation [8]. Although the influence of the roughness is indisputable in these fields, only few studies have specifically concerned the effect of surface topography. One difficulty is that surface roughness is not readily characterized by a single parameter — the choice of parameters depends mainly on the type of surface and the type of problem to be studied, but also on the characterization technique.

The influence of surface topography on cell behavior has been known from the very beginning of cell culture. In 1912 Harrison [9] studied cells that were seeded on a mesh of spider silk. He noticed that they assumed a bipolar form on a single fiber and tri- or quadripolar form on a fiber crossing. He also observed that the cells

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move along the fibers. This phenomenon was later termed "contact guidance" by Weiss [10] in 1945. However, for many years, studies on this topic were limited by the paucity of methods to fabricate micro and nanoscale features.

With the development of modern implants, control of surface roughness became increasingly important as it became evident that topography alters cell behavior on the implant and plays a crucial role in the success of the implant in a biological environment [11]. For dental implants — one of many examples of so-called bone-anchored implants — a major requirement is rapid attachment and ingrowth in the jawbone, to ensure rapid and stable osseointegration. With the need for better-performing implants (along with the development of better characterization techniques for surface morphology) more studies were carried out in this field, resulting in a good general understanding [12–17] as to how cells respond to surface roughness. However, most studies were of an empirical nature, in which a specific feature type was compared to a smooth surface. The general consensus is that osteoblasts function more effectively on rough surfaces [8], and macrophages select rough surfaces, while other cells such as fibroblasts actively select smooth surfaces [18].

Roughness gradients are a very promising tool to investigate the effect of surface topography on a biological system. Apart from reducing the number of samples drastically, they provide far more insight into the phenomena than can be obtained from a few individual samples. Roughness is influenced by the feature size, spacing, but also shape. Therefore creating one roughness gradient cannot cover all types of surface roughness. However, combining two roughness gradients to form a two-dimensional, orthogonal gradient allows a much wider range of surface roughness to be covered. For example, a micro-featured roughness gradient combined with a nano-featured gradient will yield countless combinations of nano- and micrometer-scale roughness values.

A technique is introduced that allows the fabrication of two-dimensional roughness gradients combining micro-roughness and nano-roughness gradients, as indicated in Fig. 1.

2. Materials and methods

2.1. Micro-featured gradient

A micro-featured roughness gradient master was prepared, as described previously [19]. In brief, pure aluminum substrates were sandblasted and then fully immersed into a chemical polishing solution, prior to their being slowly withdrawn in a controlled manner, yielding a stochastic roughness gradient with micrometer-scale lateral feature size and with R_a values ranging from 1 to 6 μm.

2.2. Ceramic micro-featured-gradient replica

First, a negative mold of the aluminum master was prepared in polyvinylsiloxane (PROVIL novo light, Heraeus-Kulzer, Switzerland) [19]. Then, a high-solids-loading alumina slurry (57 vol% solids loading) was prepared by slowly adding alumina powder (200 nm grain size, C517475, Ceralox, USA) under constant stirring to a 0.05 M solution of NH_4Cl (Fluka, Switzerland) in deionized water [20]. The pH of the suspension was kept between 4 and 5 by adding 2 M hydrochloric acid (Merck, Germany).

Once all the components were mixed together, the suspension was ball-milled with alumina balls for 18 h to break up agglomerates and to homogenize the slurry. After separating the slurry from the milling balls, a few drops of 1-octanol (Sigma–Aldrich, Switzerland) were added to reduce the surface tension of the suspension, which was then degassed under constant stirring in a mild vacuum. The slurry was cast into the polyvinylsiloxane negative via a 20 ml syringe, to ensure a well-controlled dosing. To ensure a slow, but homogenous drying of the alumina replicas, the molds were covered with a flat piece of polyvinylsiloxane. After at least 48 h of drying the green bodies were carefully removed from the molds and sintered in a high-temperature oven (HT08/17, Nabertherm, Switzerland). The samples were slowly heated to 400 °C at 1 °C/min and then to 1650 °C at 5 °C/min where they were sintered for 4 h.

After sintering, the alumina replicas were coated with 200 nm of SiO_2 by PECVD (Plasma Enhanced Chemical Vapor Deposition, FIRST Laboratory, ETH Zürich). This layer not only adjusted the surface chemistry for subsequent particle adsorption, but it also smoothened asperities at the grain boundaries of the alumina ceramic. Since the PECVD process generates a slightly rough surface, the samples were annealed for 8 h at 1100 °C prior to use.

The surface of the substrates was sterilized and cleaned for 2 min in an oxygen plasma (Harrick, PDC-32G, USA) at a pressure of roughly $2 \cdot 10^{-3}$ mbar on the "high" RF setting. To render the surface positively charged, the substrates were coated with a monolayer of poly(ethylene imine) (PEI, $M_W = 750{,}000$, 50 wt% in water, Sigma Aldrich, Switzerland) by immersing them for 30 min in a 1 mg/ml solution.

2.3. Nanoparticle adsorption

Nanoparticles were adsorbed as follows [21]: A silica particle suspension (particle diameter, 40 nm) with a concentration of 0.005 wt % was prepared by diluting a homogenized stock solution with deionized water. Then, the suspension was degassed for 5 min under a light vacuum to prevent gas bubbles from forming during the adsorption process. Prior to gradient fabrication, the substrates were pre-wetted with ultra-pure water and immersed into the suspension to the starting point of the gradient, after which a controlled immersion program was initiated. During the entire adsorption process, the suspension was kept slightly agitated with a magnetic stirrer (100 rpm) and an ultrasonic horn (UP 200s, Hielscher GmbH, Germany) at the lowest settings (Cycle = 0.2, Amplitude = 20%).

The computer-controlled immersion program was set to $x(t) = a \cdot t^2$, with $a = -3.09 \cdot 10^{-6}$, x being the distance and t the time of immersion, respectively. After 30 min of adsorption, the substrates were immediately removed from the

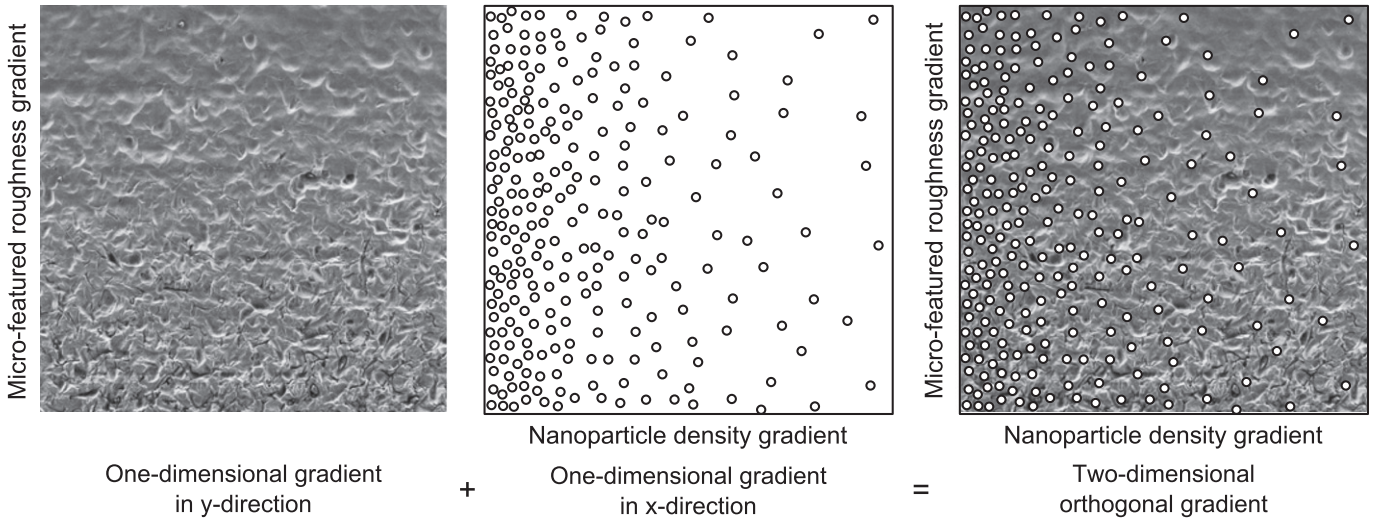

Fig. 1. A sketch showing the formation of a two-dimensional, orthogonal gradient: A micro-featured roughness gradient on one axis is combined with a nanoparticle density gradient on the other axis to form a 2D gradient that combines roughness on the μm and nm scales.