Biomaterials 55 (2015) 96–109

Contents lists available at ScienceDirect

Biomaterials

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

Stem cell behavior on tailored porous oxide surface coatings

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ARTICLE INFO

Article history:
Received 12 December 2014
Received in revised form
16 March 2015
Accepted 20 March 2015
Available online 13 April 2015

Keywords:
Nanotopography
GLAD
Mesenchymal stem cells
Porosity
Titanium dioxide
Silicon dioxide

ABSTRACT

Nanoscale surface topographies are known to have a profound influence on cell behavior, including cell guidance, migration, morphology, proliferation, and differentiation. In this study, we have observed the behavior of human mesenchymal stem cells cultured on a range of tailored porous SiO_2 and TiO_2 nanostructured surface coatings fabricated via glancing angle electron-beam deposition. By controlling the physical vapor deposition angle during fabrication, we could control systematically the deposited coating porosity, along with associated topographic features. Immunocytochemistry and image analysis quantitatively revealed the number of adherent cells, as well as their basic cellular morphology, on these surfaces. Signaling pathway studies showed that even with subtle changes in nanoscale surface structures, the behavior of mesenchymal stem cells was strongly influenced by the precise surface structures of these porous coatings.

1. Introduction

The use of substrates fabricated with micro- and nano-topographic features like roughness, porosity, grooves, and pillars for the culture of mammalian cells [1–4] has the advantage of creating surface environments that allow for systematic studies of cell adhesion, growth, and differentiation. In using such tailored and well-characterized surface environments, in conjunction with detailed observation of cellular behavior, a growing picture of the signaling pathways involved in the cell/surface interaction is beginning to emerge. This is essential to understand the profound roles that physicochemical interactions play between biological systems and their surrounding surface environment. Prominent examples include the use of engineered surfaces to influence cellular behavior and potentially forgo the need for harmful chemicals in the body [1,5,6], and in bone implants to induce osteogenesis without the use of growth factors [1] or to accelerate bone apposition and bone bonding strength [7].

Adult stem cells, such as mesenchymal stem cells (MSCs), are defined as a multipotent population of slow growing, self-renewing cells with low rates of metabolism within a defined tissue niche. It has been shown that the first cells to colonize the surface after implantation are MSCs. Peripheral MSCs are attracted into the peri-implant region via chemo-attractant molecules and are able to migrate through the blood clot to colonize the implant surface [5,8]. MSCs, present in blood, bone marrow and other tissue at low levels, have been shown to differentiate into various cell types including osteoblasts [9], chondroblasts [10], myoblasts [11] and adipocytes [12]. *In vitro*, MSC differentiation requires the use of differentiation factors such as dexamethasone for osteogenic, insulin for adipogenic and hydrocortisone for smooth muscle differentiation. The use of these factors is one of the main obstacles for MSC clinical application. Recent reports have shown that mechanical factors such as stresses [13,14], adhesion area [15,16], substrate elasticity [11] and micro- and nanoscale topography [1,17] can also induce differentiation of stem cells. While the nature of cell adhesion and the degree of cytoskeletal tension are widely accepted as affecting stem cell behavior, the precise role of nanotopography on the adhesion, morphology and differentiation of cells has not been established. In order to control stem cell differentiation using only

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http://dx.doi.org/10.1016/j.biomaterials.2015.03.033
0142-9612/

topography, the elucidation of the mechanisms by which a cell perceives these topographical cues and then relays the information into the nucleus to initiate a cellular response is critical. This knowledge could then be applied to the design and fabrication of topographic surfaces that enable tailored control of specific behaviors such as differentiation or self-renewal of cells.

Studies of the influence of nanoscale environments on cell behavior have proven to be challenging. Relevant cell-surface interactions at nanometer and micrometer size regimes and interdependencies among physical parameters at the nanoscale can often lead to complex changes in substrate properties. For example, it has been shown that a change in the complexity of surface topography such as mixing micro- and nanotopography by superposition, may induce a change in wettability and/or surface roughness [18]. To understand the effects of surface topography on cell behavior, characterizing fundamental surface properties, e.g., topography, wettability, chemistry, ζ potential and roughness, remains a critical concern. To that end, numerous studies on cell-surface interactions have been performed at both the nano- and microscales [5,19–21].

Although there have been many studies of cells on surfaces with features in the 10–100 nm regime, there have been relatively few studies of nanostructured surfaces with features less than 10 nm in size. So far, it is known that important extracellular matrix protein adsorption and activation properties are strongly influenced by topographic features in the 10–100 nm scale [4,22]. For example, fibronectin-surface interactions have been investigated [23], yet minimal efforts have been devoted to understanding the influence of topographical parameters at this scale range and also below 10 nm [1,16,17,24–26]. While such studies have demonstrated that there are important cell-surface interactions that occur below 100 nm, a concise picture of these processes has not yet emerged.

Porous coatings fabricated utilizing glancing angle deposition (GLAD) techniques [27,28] exhibit several favorable characteristics that make them both a potentially important class of biomaterial and a valuable investigative tool for understanding the specific nuanced roles that biomaterial surfaces play in influencing cell behavior, including adhesion, cell morphology, mineralization, and gene expression [25]. Specifically, surface coatings fabricated utilizing GLAD techniques: i) have demonstrated reliable and tailorable control of biologically relevant physical properties at the micro- and nanoscale, such as porosity or mass density, surface roughness, and hydrophobicity [27,29,30]; ii) can be fabricated from a variety of material systems, including metals, dielectrics, semiconductors and their composites; iii) can be fabricated on polymer surfaces, which can then be subsequently bent, molded or otherwise reshaped while remaining well-adhered to their substrate [31]; iv) can be fabricated in large and uniform quantities, on the order of tens of cm^2; and v) can be integrated with a variety of other coating fabrication techniques. These characteristics of GLAD coatings allow for a wealth of novel surface structures, which make them a near ideal material system for a great diversity of cell-surface studies.

In this work we describe the behavior of human mesenchymal stem cells (hMSCs) cultured on coatings fabricated using GLAD across a systematic range of porous SiO_2 surfaces with topographic features in the 1–100 nm size regime. Similar TiO_2 surface coatings were also investigated for comparison. Cellular behavior on the surface coatings was studied by immunostaining and compared to the tailorable physical parameters of porosity, feature sizes, and surface roughness. This technique allows a clear characterization of the cell morphology that is shown to be dependent on a coating's porosity and associated surface structures at the nanoscale. Then, the cell proliferation, adhesion and differentiation were characterized for four selected surfaces, and gene and protein expression were also investigated allowing a clear characterization of cell behavior. We have shown that cell behavior is dependent on the angle of deposition and the resulting surface structures at the nanoscale and that cellular behavior can be strongly modulated by subtle changes in nanotopography, highlighting the importance of nanotopographic surface features below 10 nm.

2. Materials and methods

2.1. Coating fabrication on silica glass

SiO_2 and TiO_2 (99.999% purity source) porous coatings were fabricated by electron-beam evaporation utilizing the GLAD technique on standard laboratory glass cover slides (15 mm round, Propper, select quality, 0.14 mm thickness, 2 cm^2 square surface area source). Substrates were cleaned prior to deposition using isopropanol (IPA) for 10 min and then washed twice (5 min each) with deionized water rinse. Samples were fabricated in batch numbers of 40 glass cover slides. Pressure during depositions was at/or below 1.5×10^{-6} Torr, and occurred at approximately room temperature. Coating thickness and deposition rate were monitored using a quartz crystal sensor. The deposition rate was manually maintained at a constant rate of 0.2 nm/s and 0.08 nm/s for SiO_2 and TiO_2 coatings, respectively. Calibrated coating thickness was targeted to be 200 nm for SiO_2 samples and 100 nm for TiO_2 samples. Deposition angle was manually controlled using a fixed-variable angle substrate mount. Prior to cell culture, the surfaces were sterilized in 70% ethanol for 15 min and then rinsed in phosphate buffered saline (PBS).

2.2. Surface characterization

AFM measurements were carried out on an Asylum Research MFP-3D AFM (Oxford Instruments). The AFM-cantilevers used were Nanosensors Super-SharpSilicon – SPM-Sensor, Material n^+ – silicon, resistivity: 0.01–0.02 Ω cm. Thickness: 7.0 ± 1 μm/Length: 225 ± 10 μm/Width 38 ± 7.5 μm, Resonance Frequency: 146–236 kHz, Force Constant: 21–98 N/m, Tip height: 10–15 μm/Tip radius: ~2–10 nm. To remove adsorbed water on the nanostructured surfaces prior to AFM imaging, samples were baked in a standard laboratory oven at 175 °C for at least 2 h. Samples were imaged over 1 μm^2 areas in intermittent contact mode at a scan rate of 1 Hz with 512 DPI resolution at three separate locations. The height of the individual nanorough surface features was determined by analyzing AFM images with the grain analysis tool in the open source software Gwyddion. Feature height is defined as the vertical distance between the highest point of a surface feature and the lowest point of the valley separating one surface feature from surrounding ones. Feature radius was calculated from the circular area equivalent of the projected boundary area as described above for feature height. The root-mean square (RMS) roughness values were defined as in Dolatshahi-Pirouz et al. [30]. Owing to finite resolution of the AFM tip apex and surface convolution effects, it is likely that nanotopographic feature height values are underestimated and feature radius values are overestimated. Furthermore, for porous coatings exhibiting nanocolumnar nature, because nanocolumns are tilted in the direction of the vapor flux deposition angle, the AFM tip cannot penetrate the mesoporous voids where the nanocolumns overlap.

Images of nanoporous coating surfaces on silicon substrates were obtained utilizing a Carl Zeiss Supra SEM. Coatings were prepared prior to imaging by a diamond scribe cleave parallel to the deposited vapor flux and an ultra-thin (ca. 10 nm) platinum sputter coat to reduce charging issues of the non-conductive samples. An accelerating voltage of 5 kV with a working distance of 5 mm, and an aperture of 10 μm was used during imaging.

Coating thickness and refractive index were measured optically using a J.A. Woollam variable-angle ellipsometer. Thickness non-uniformity within each batch of samples was measured to vary by ±25%, for coatings located closest to the vapor source as compared to samples located farthest from the vapor source (6 cm). The porosity of a coating is defined as the ratio between the volume of air in the film and the total volume of the film. The porosity is then estimated from the effective refractive index based on the linear volume approximation between source material and air [32]. Porosity values are normalized to that of a coating deposited at normal incidence. It is known that the GLAD surface coatings experience an increase in porosity and surface feature size as a function of film thickness [30,33]. However, in the thickness regime used in the present study, no apparent change in cellular behavior was observed as a result of non-uniform coating thicknesses. Indirect measurements of porosity were obtained for SiO_2 and TiO_2 surface coatings by ellipsometry inferred through optical measurements of the coating's effective refractive index. Such optical measurements are only an approximation of a coating's bulk porosity and do not describe potentially important pore size distribution information. It is noted here that only surface coatings at certain deposition angles were fully characterized, since it was clear from our earlier work [32] on the optical behavior of these coatings that their properties varied little at the lowest deposition angles.