Acta Biomaterialia 9 (2013) 6158–6168

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

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

Complex protein nanopatterns over large areas via colloidal lithography ☆

Stine H. Kristensen [a], Gitte A. Pedersen [b], Ryosuke Ogaki [a], Vladimir Bochenkov [a,c], Lene N. Nejsum [a,b], Duncan S. Sutherland [a,*]

[a] iNANO Interdisciplinary Nanoscience Center, Aarhus University, Ny Munkegade 120, DK-8000 Aarhus C, Denmark
[b] Department of Molecular Biology and Genetics, Aarhus University, C.F. Møllers Allé 3, DK-8000 Aarhus C, Denmark
[c] Department of Chemistry, Lomonosov Moscow State University, Leninskie Gory 1/3, 119991 Moscow, Russia

ARTICLE INFO

Article history:
Available online 16 January 2013

Keywords:
Sparse colloidal lithography
Protein nanopattern
Vitronectin
E-cadherin
Cell adhesion

ABSTRACT

The patterning of biomolecules at the nanoscale provides a powerful method to investigate cellular adhesion processes. A novel method for patterning is presented that is based on colloidal monolayer templating combined with multiple and angled deposition steps. Patterns of gold and SiO_2 layers are used to generate complex protein nanopatterns over large areas. Simple circular patches or more complex ring structures are produced in addition to hierarchical patterns of smaller patches. The gold regions are modified through alkanethiol chemistry, which enables the preparation of extracellular matrix proteins (vitronectin) or cellular ligands (the extracellular domain of E-cadherin) in the nanopatterns, whereas the selective poly(L-lysine)–poly(ethylene glycol) functionalization of the SiO_2 matrix renders it protein repellent. Cell studies, as a proof of principle, demonstrate the potential for using sets of systematically varied samples with simpler or more complex patterns for studies of cellular adhesive behavior and reveal that the local distribution of proteins within a simple patch critically influences cell adhesion.

1. Introduction

Interactions between cells and their surroundings (neighboring cells, extracellular matrix components or biomaterials) both in vivo and in vitro are mediated at the molecular and macroscopic levels. The specific interactions of cells with the environment provide vital communication through cellular signaling pathways [1]. Biochemically nanopatterned surfaces can provide insight into different intercellular signaling pathways and the ability to spatially control the immobilization of macromolecules; consequently, these surfaces provide a platform for investigating single molecular events [2–4]. Therefore, during the last few decades, several strategies to produce and study nanometer-scale surfaces that contain features with dimensions of 10–1000 nm have been developed utilizing both lithographic "top-down" approaches and self-assembling "bottom-up" approaches [5]. These features have a similar size range to the biochemical and structural features found in the in vivo microenvironment that surrounds the cells and in the cells themselves. Therefore novel strategies to study cellular systems have been and are continuously being developed using these materials [6–9]. By combining soft lithography techniques and efficient approaches to minimize non-specific protein binding, microscale patterns have widely been used as model systems and have had considerable impact in understanding cellular behavior [8,10]. While micropatterns of biomolecules are of considerable interest, the size and length scales at which proteins and other macromolecules are arranged are often at the nanometer scale [11].

A number of lithographic approaches can be used to prepare submicron and nanometer-scale protein patterns. By using direct write and replication techniques, it is possible to obtain sub-100 nm resolution, and patterning at the single molecule level has been demonstrated [12–14].

A significant limitation for the replication approaches is associated with the fabrication of large area nanoscale master patterns and their durability. A set of alternative lithographic routes that employ self-assembled building blocks have been developed for the patterning of biomolecules, which allow for considerably faster direct fabrication methods. Block copolymers have been widely used as building blocks and provide features with sizes that range from a few to several nanometers [7,15,16]. Through the use of these self-assembly approaches, patterned surfaces can be achieved over large areas without the use of complex equipment. However, significant limitations of the self-assembling approaches are associated with minimal control over the pattern, the limited number of patterns available and the relatively low level of achievable ordering.

☆ Presented at the E-MRS Biomaterials Symposium, organized by Prof. Kurosch Rezwan, Dr. Laura Treccani, Prof. Giovanni Marletta and Prof. Miguel Manso Silván.

* Corresponding author. Tel.: +45 23385789; fax: +45 87154041.
E-mail address: duncan@inano.au.dk (D.S. Sutherland).

1742-7061/$ - see front matter © 2013 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.actbio.2013.01.011

Our focus has been on using colloidal monolayer masks as a self-assembly route for creating complex nanostructured features [17]. This approach has the capability for producing patterns on the length scale of 100–1000 nm over large areas (up to 100s of cm^2) through self-assembly. Colloidal particles can be arranged into close-packed arrays through self-assembly, and they can be used as templates for advanced materials [18,19]. Alternatively, dispersed arrays can be formed using electrostatic self-assembly to obtain short-range ordered arrays that do not have long-range order [17]. Each particle can be used to create a single structure that can be produced over large areas. A number of different structures have been previously demonstrated (e.g. holes, disks, rings and pits) [20–23]. Control of the pattern transfer step, for example, through tilted deposition and annealing, can yield a wider range of structural features [22].

Patterned substrates produced through colloidal lithography have been used in biological studies, e.g. to model biomaterial surfaces or nanotopographies [24,25]. Cells interacting with a surface placed in a biological environment interact with the material that is pre-coated with proteins adsorbed from the environment, which critically determines the cellular response. The composition and functional state of the protein coating will be influenced by the architecture of the surface, e.g. the topography and chemistry of the material [26,27]. By using coatings to minimize non-specific protein interactions, materials that allow the study of cellular interactions with their surroundings can be prepared [26]. Nanoscale engineering has been used to immobilize cell-binding ligands into nanoscale patterns [6,7,28–32].

In this study, we present a novel way to fabricate complex protein nanopatterns over large areas using a relatively simple and scalable approach that combines colloidal monolayer masks with multiple steps and angled deposition, and subsequent biochemical functionalization. This simple but powerful approach provides new routes for investigating cellular processes at the molecular scale. In proof-of-principle experiments, we present nanoscale patterns of vitronectin and E-cadherin:Fc, and explore the role of the local ligand distribution on the development of cell adhesion complexes.

2. Materials and methods

2.1. Sparse colloidal lithography

Pre-cut oxidized silicon wafers were sputter-coated with 4 nm of Ti and 30 nm of Au. A triple-layer precursor film was adsorbed to make the surface positively charged: (i) 2% poly(diallyldimethylammonium chloride) (MW 200,000–350,000, Sigma–Aldrich), 30 s, rinse in water, dry; (ii) 2% poly(sodium 4-styrenesulfonate) (MW 70,000, Sigma–Aldrich), 30 s, rinse in water, dry; and (iii) 5% poly(aluminum chloride) (KemiraMiljø), 30 s, rinse in water, dry. Negatively charged polystyrene particles (110, 200, 300, 800, 1000 and 3000; Invitrogen) were adsorbed onto the charged substrates from solution through electrostatic interactions. Particle concentrations between 0.1% and 1% were used. The adsorption time was varied between 2 min and 12 h to allow the adsorption to reach saturation in all experiments. Excess particles were rinsed off under running water, and the samples were transferred to pressure chambers with degassed Milli-Q purified (MQ) water and heated to 120–130 °C for 1 h.

The samples were dried under a stream of nitrogen gas and subsequently coated with 11 nm of silica or gold by evaporation, according to Table 1. A titanium adhesion layer was added between each layer, also by evaporation. All samples were rotated at 6 rpm while the layers were being deposited. Subsequently, the particles were removed by stripping with tape and ultrasonication in ethanol and MQ water. Domains of smaller gold patches were produced by the adsorption of smaller particles (ø = 0.1 μm) on the larger nanopatterns (0.8 μm), then a second silica layer was deposited by evaporation.

Table 1
Evaporation parameters.

Structure	Initial coating	Substance	Angle (deg.)
Gold patches, ø = 0.1–3.0 μm	Au	SiO_2	0
Gold patches, ø = 0.3 μm, gold ring	Au	SiO_2	45
		Au	15
		SiO_2	0
Silica patches, ø = 0.3, gold ring	SiO_2	Au	45
		SiO_2	0

The samples were characterized using scanning electron microscopy (SEM; Magellan™ XHR scanning electron microscope, FEI) to determine the size of the hole and the center-to-center distance. The diameter distribution of each patch size was measured using the ImageJ software program [33].

The characteristic spacing of the holes on each type of sample was determined from the analysis of a minimum of four SEM micrographs from each type of sample. The micrographs were used to identify the center of each hole, which was then used to calculate the hole radial distribution functions for each type of sample; the average peak position marks the characteristic spacing, whereas the average full-width at half-maximum of the peak determines the error bars.

2.2. Biofunctionalization

Buffers were created using MQ water that was adjusted to pH 7.4 by adding hydrochloric acid or sodium hydroxide, and were filtered through 0.2 μm pore filters before use. The buffers used included 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma–Aldrich) and 10 mM HEPES with 154 mM NaCl and 7.2 mM KCl (Ringers, Sigma–Aldrich). The prepared samples (6 × 6 mm) were initially incubated with poly(L-lysine)–poly(ethylene glycol) (PLL-g-PEG; Surface Solution, 0.25 mg ml^{-1} sterile filtered in HEPES buffer without salt, incubated for 30 min), rinsed with buffer and then added to a buffer with salt. The samples were incubated with either vitronectin (VN; R&D systems, 10 μg ml^{-1}, 5 h at room temperature (RT)), or a series of three proteins to correctly orient the extracellular portion of E-cadherin, E-cad:Fc. Neutravidin was first deposited (Fisher Scientific, 0.5 mg ml^{-1}, incubated for 2 h at RT), followed by incubation with protein A (Fisher Scientific, 20 μg ml^{-1}, 1 h at RT) and subsequently with E-cad:Fc (20 μg ml^{-1}, 2 h at RT). E-cad:Fc was produced by recombinant expression in HEK293 cells and purified from cell culture medium using a Protein A Sephadex column (GE Healthcare). The E-cad:Fc had the extracellular domain of E-cadherin fused at the C-terminus to the Fc domain of human IgG1 [34]. Each adsorption step was followed by rinsing with buffer. A blocking step with 2% bovine serum albumin (BSA; Sigma–Aldrich) was performed after the first protein adsorption. The samples were transferred to 24-well low-adherent tissue culture plates and rinsed with Ringer's solution.

2.3. Surface chemical imaging by ToF-SIMS

A time-of-flight secondary ion mass spectrometer (ToF-SIMS V, IONTOF GmbH, Muenster, Germany) was used in the "high current bunched" (HCB) and "burst alignment" (BA) modes. The SIMS data were acquired using 15 keV Bi_1^+ ions rastered in a 256 × 256 format over a 100 μm × 100 μm and a 30 μm × 30 μm area in the HCB and BA modes, respectively. The ion current was measured in the