Hydrogels with precisely nano-functionalized micro-topography for cell guidance

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Abstract

In vivo, cells encounter different physical and chemical signals in the extracellular matrix (ECM) which regulate their behavior. Examples of these signals are micro- and nanometer-sized features, the rigidity, and the chemical composition of the ECM. The study of cell responses to such cues is important to understand complex cell functions, some diseases, and is basis for the development of new biomaterials for applications in medical implants or regenerative medicine. Therefore, the development of new methods for surface modifications with controlled physical and chemical features is crucial. In this work, we report a new combination of micelle nanolithography (BCML) and soft micro-lithography, for the production of polyethylene glycol (PEG) hydrogels, with a micro-grooved surface and decoration with hexagonally precisely arranged gold nanoparticles (AU-NPs). The Au-NPs are used for binding adhesive ligands in a well-defined density. First tests were performed by culturing human fibroblasts on the gels. Adhesion and alignment of the cells along the parallel grooves of the surface were investigated. The substrates could provide a new platform for studying cell contact guidance by micro-structures, and may enable a more precise control of cell behavior by nanometrically controlled surface functionalization.

1 Introduction

Features of the extracellular environment co-regulate complex cell behavior and functions *in vivo* and *in vitro*. The signals can be of chemical nature, for example given by the chemical composition of the extracellular matrix (ECM), or physical, for example by the ECM stiffness and topography on nanometer and micrometer size scale. Cells are able to sense these signals, transduce them into intracellular biochemical signals, finally leading to biological cell responses [1]. Although many molecular details of these biological processes are not in detail understood, there is a long history on using micro-sized textures of surfaces to control adhesion and behavior of cells in contact with them [2-4]. Several techniques of surface modifications have been conceived. Very often, structures with sizes in the micrometer range have been used to study the behavior of different cell types such as fibroblasts or osteoblasts [2,5,6]. One well-examined example of cell response to surface topographies on micro-manufactured substrates is the so-called contact guidance. There, cells respond to topography by adapting their cell morphology or migration with respect to elongated surface features such as parallel grooves. This phenomenon was first reported by R. Harrison in 1914 [3]. Next to this phenomenon, there are other *in vitro* cell responses. Surface topographies can alter the gene expression profiles of cells and could be utilized as a signaling modality for directing differentiation. One impressive example is the reprograming of somatic cells into pluripotent stem cells [7]. Cultivation of somatic cells on a surface with parallel microgrooves could partially substitute the effects of epigenetic modifiers and improved reprogramming efficiency.

While many previous studies have used micron-sized surface features produced by conventional technologies such as photolithography, improved methods for fabricating nanostructured surfaces have enabled novel studies in the past decade. Several reports have impressively demonstrated that even surface features with heights in the range of a few nanometers or small variation in adhesive ligand distance of a few nanometer can drastically influence the behavior of cells growing on such surfaces *in vitro* [8-11].

Since the size of the topographical structures influencing cells can be in the range of nanometer and micrometer, the combination of fabrication techniques promises the production of substrates that offer topographic cues with both appropriate length scales. One technique for producing polymer substrates with specific µm-sized surface topography is based on the well-established method of soft lithography. Invented in the mid-1990s it found many applications in biomedical research [12]. In a first step, a master wafer is produced by conventional optical lithography (Image 1). The desired lateral structure of the surface topography is transferred to a silicon wafer covered with photo resist by an optical illumination process and appropriate developing steps. The resulting surface topography is transferred to a polymer substrate, usually by casting a polymer solution of poly(dimethyl)siloxane (PDMS) on the master wafer and peeling it off the wafer after curing. The surface of the polymer substrate is now structured with the desired topography. Although such methods pro-

vide valuable surface modifications down to the submicron-range, more precise control of adhesive ligand immobilization on a surface down to a few nanometers is required to control the geometric exposure of adhesive proteins epitopes on a surface. To achieve such spatial resolution, block copolymer micelle nanolithography (BCML) has been proved to allow for the nanoscale positioning of ECM ligand molecules (Figure 1). This methods immobilizes 4 to 15 nm-sized nanoparticles arranged in a quasi-hexagonal pattern, with a adjustable inter-particle distance between 20–200 nm [1]. Nanoparticles are then used for binding of adhesive ligands (Figure 1). Besides its unique ability to precisely position single molecules at nanometer length scale, it is suitable for functionalizing large areas and can be used with hydrogel polymers such as Poly(ethylenglycol) (PEG).

Objective of this study was to optimize surface properties of micro-structured polymer biomaterials that might be finally used in basic cell biology studies, regenerative medicine or medical implants [13]. Combining soft lithography and BCML, we developed PEG-surfaces with a micrometer-sized topography, with nanometrical control of adhesive cell ligands distribution. Using these substrates, we investigated in a model study the contact guidance behavior of human fibroblasts.

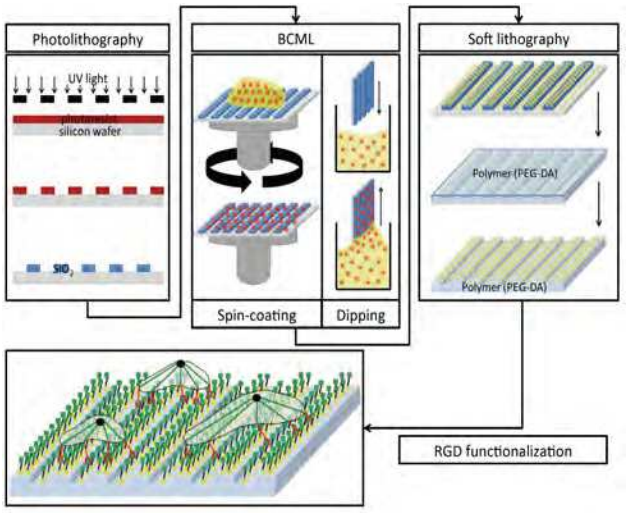

Figure 1. Scheme representing the combination of the two methods for micro- and nanofabrication of hierarchically structured hydrogels.

2 Materials and Methods

Photolithography Photolithography was performed as previously described [13], briefly silicon wafers were covered with positive photoresist (ma-P1210 photoresist Micro Resist Technology, Germany) by spin-coating (Laurell Technologies Corp., USA). After curing the resist was exposed with UV light through a micropatterned photomask (ML & C, Jena, Germany) using a mask aligner MJB4 (Süss Microtech, Germany). The line pattern with widths of 2, 3, 4, 5, 8 and 10 µm are arranged on the mask in 10x10mm squares. After developing processes, SiO_2 was deposited onto the micro-structured wafer by physical vapor deposition yielding in the desired micro-topography.

Block copolymer micelle nano lithography (BCML) The basic principle of this method is described in [14] and illustrated in Figure 1. A 5mg/ml O-xylene (Merck Schuchardt OHG, Germany) concentrated micelle solution of the block copolymer PS (1056)-b-P2VP(495) (Polymer Source Inc. Dorval, Canada) was created, 35mg of gold (III) chloride acid ($HAuCl_4 \cdot 3H_2O$) (ABCR GmbH & Co.KG, Germany) were added, and stirred for 24h. Before use, the micelle solution was purified by using a 0.2-micron membrane filter (Roth). To nanopattern micro-structured wafers with the block copolymer solution, two methods were tested: dipping and spin coating. In the first method, wafers were dipped at a defined speed in the block copolymer solution. In the second method, wafers were spin coated (Npp/A2/AR2, Laurell Technologies Corp., USA), for one minute, with 20 µl of the polymer solution. Once monolayer of micelles were loaded onto wafers, these were treated for 45 minutes in a reactive ion etcher (Microwave plasma system PS 210 PVATePla America, Inc., USA) with W10 gas (10% Argon and 90% Hydrogen) at a power of 200 W and a pressure of 0.1 mbar.

Soft lithography By means of soft lithography, nanopatterned microstructures were transferred on PEG-DA hydrogels (PEG-DA 700, Sigma-Aldrich, USA). First, wafers containing both micro- and nanopattern were incubated 1h with a linker solution (N-bis (acryloyl)cystamine) (Alfa Aeasar, Germany) and protected from light, in order to covalently bind gold nanoparticles to PEG. After, 1 mL presynthesized PEG-DA 700 was mixed with 1 mL of dd H_2O. The mixture was stirred under a nitrogen blanket until it was transparent. Then 65 µl of the initiator, (4 - (2-hydroxyethoxy) phenyl-(2-propyl) ketone) (Sigma-Aldrich, USA) was added and stirred for 10 min under a nitrogen blanket protected from the light. For hydrogel casting, functionalized wafers were covered with a quartz glass ¾, with the polymer mixture in between. Polymerization was achieved under a UV lamp for 10 minutes at λ = 365nm. Finally, slides with PEG were immersed in dd-H_2O and stored in a fridge for 48 hours. Wafers and PEG-gels were characterized by scanning electron microscopy (SEM)(Zeiss Ultra).

RGD functionalization and preparation of the PEG-DA hydrogels Nano-micro-structured hydrogels were sterilized with 70% ethanol for 15min. After that, they were rinsed 3 times with PBS, 10min each. Then, hydrogels were incubated for 90 min at room temperature, with a 25 µM RGD solution (RGD-peptide sequence: cRGD T3, Peptide Specialty Laboratorie GmbH, Germany), followed by 3 rinses of PBS, 10min each. Subsequently, hydrogels were washed for 30 min in PBS. Before seeding cells, substrates were rinsed for 2 hours in the appropriate cell medium.

Cell culture Human fibroblasts cells from bone were cultured in D-MEM cell media (Gibco) supplemented with 10% fetal bovine serum and 1% antibiotic. The culture medium was changed every second day. Cells were seeded onto hydrogels at a cell density of 50/mm^2. After 24h, micrographs from cells on substrates were taken with an Observer Z1 inverted light microscope (Carl Zeiss, Germany)

Data analysis Microscope pictures were analyzed with ImageJ. The contours of at least 30 isolated cells per condition, were marked manually. The mean cell orientation was quantified through the non-polar order parameter S = <cos (2φ)>. For S=-1 cells are oriented perpendicular to the microstructures direction, for S=0 cells are randomly oriented, and for S=1 cells are totally parallel to the microstructures [15].

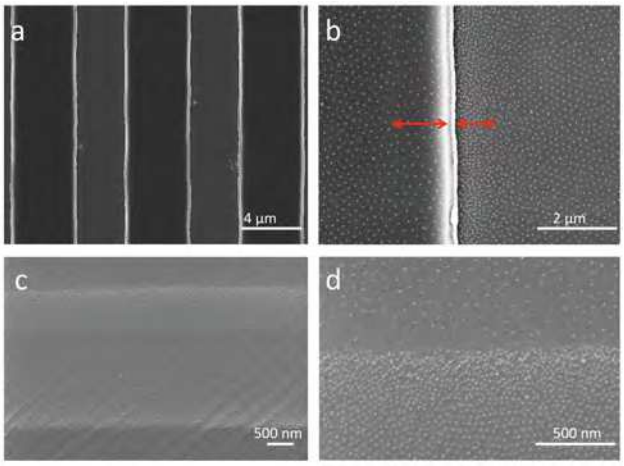

Figure 2. (a) SEM images of micro-structured silicon wafer with a groove width of 4 microns. (b) Micro-structured silicon wafers with gold nanoparticles, the arrows indicate the influence of the capillary effect. (c, d) Gold nanoparticle on PEG-DA hydrogels at two different magnifications (SEM images).

3 Results

Nanopattern on microstructures In order to obtain a homogeneously and precise distribution of gold nanoparticles on the micro-structured silicon wafer two different methods were used. In one method, micro-structured wafers were dipped into the micelle solution at various speeds. Orientation of micro-channels was parallel to the dipping direction. In the other method, the micro-structured wafers were modified by spin coating the micelle solution at different rpm. To determine the best conditions for achieving a precise hexagonal immobilization of the gold nanoparticles on the wafers, the resulting pattern were quantitatively evaluated by analyzing SEM images such as in Figure 2 and calculating the mean distance between particles and standard error. In all conditions and with all microstructure groove sizes, a more homogeneous Nano pattern, indicated by smaller standard errors, was found on spin-coated samples than on samples prepared by the dipping process.

To optimize the spin coating process and adapting the method to different microstructure sizes, further experiments were performed with variation in spin-coating speed and other parameters. SEM micrographs of wafers with 200 and 350 nm deep microgrooves (2 and 5 µm width) were quantitatively analyzed by measuring the nanoparticles hexagonality and the width of regions with decreased or increased particle density along the edges of the grooves (Figure 2b). Locally varied capillary forces provoked this formation of areas with different nanoparticle densities in comparison with central regions of grooves and ridges. Further optimization of the protocol led to reduced widths of these inhomogeneous regions. At a spin-costing speed of 9000 rpm, the width of these areas was between 340-380 nm (for 200 nm groove height, and 2 µm groove width), and between 515 and 535 nm on wafers with 5 µm wide grooves. On wafers with 350 nm deep grooves, the width of the areas with altered particle density was typically increased by ~100-150 nm. The conditions, at which the Au-nanoparticles are arranged with highest hexagonality was also obtained with a spinning velocity of 9000 rpm on wafers with 200 nm deep microgrooves and 2 and 5 µm groove width. In all cases, hexagonality was always highest in the central part of the microstructure and reduced along the edges of the grooves. Average nanoparticles distance was ~95±16 nm for both wafers with 200 nm deep grooves (2 and 5 µm width).

Nanopatterned microstructure transfer to PEG hydrogels and cell test Gold nanoparticles on micro-structured silicon wafers were successfully transferred to PEG-DA 700 hydrogels (Figure 2). Both, the microstructure and the pattern with nanoparticles were preserved. The order of the nanoparticle arrangement was not drastically changed and is similarly ordered as on the master wafer. To promote integrin-mediated cell adhesion to the non-adhesive PEG-surface, covalent functionalization of the particles with RGD ligands was carried out. Human fibroblasts were used to test the new micro-nanostructured surfaces.

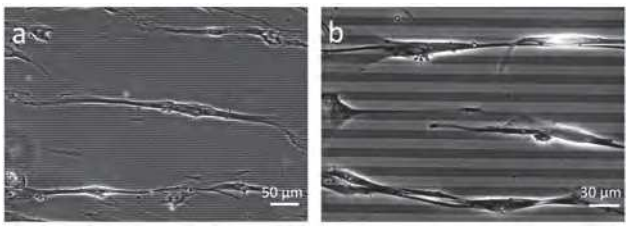

Figure 3. Phase contrast microscopy images of human fibroblasts on a PEG-DA hydrogel with 200 nm deep microgrooves and a groove width and separation of 2 µm (a) and 10 µm (b). The Au-nanoparticles are not visible at this resolution.

For these test experiments, PEG-gels with 200 nm deep microgrooves and separation width of 2x2 µm and 10x10 µm were used. As shown in Figure 3, fibroblast cells could adhere very well to the otherwise non-adhesive gels. They adapt their morphology and align parallel to the micro-grooves (contact guidance). Cells were better aligned on 2 µm wide grooves ($S=0.94\pm0.02$) than on 10 µm wide grooves ($S=0.83\pm0.06$). The determined order parameters are significantly higher than the ones found for cells growing micro-structured PDMS substrates with physisorbed fibronectin ($S\approx0.45$ on 10 µm wide grooves, and $S\approx0.85$ on 2 µm grooves, [15]). We suggest that the small areas with high particel density along the groove edges, as discussed above, provide an additional elongated chemical signal for the cells leading to an improved alignment.

4 Conclusions

In this work, we present a combination of methods for micro-structuring of PEG-hydrogels surfaces and decorating the surface with ordered arrays of gold nanoparticles serving as anchor for cell adhesion ligands. Both methods haven been used, the combination provides a technique to obtain surfaces with structures on two length scales - the molecular length scale of nanometers, and micrometer-sized structures in the order of cell sizes. First tests with human fibroblasts indicate an increased contact guidance of the cells than on conventional micro-grooved surfaces. This new hierarchically structured surfaces, could provide a new platform to study cell responses to a combination of different surface chemistries, topographies, and stiffness, to eventually control cell behavior.

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