Patterning Function and Shape for Application from Microelectronics to Biotechnology

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Introduction

Lithography and other patterning processes are powerful tools catalyzing many developments in science and engineering. The controlled formation of nanometer scale structures in 2 and 3 dimensions is therefore of increasing importance in many applications ranging from biotechnology to nanotechnology. This presentation will discuss new approaches for the construction of small-scale (a few tens of nm) structures using both 1- and 2-photon processes. Several approaches to fine feature lithography including the use of molecular glasses will be described. Such small scale structures can be used in a variety of biological applications including study of cell function and will be described.

As shown in Figure 1, the remarkable progress in nanofabrication made possible by the microelectronics industry has led to the ability to create small structures overlapping many length scales of biological systems.

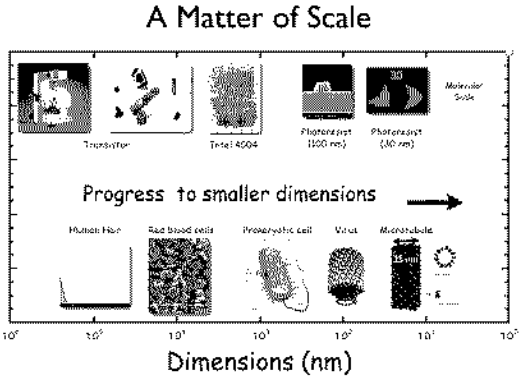

Figure 1. Progress in nanofabrication compared to length scales of biological systems.

Fabrication techniques can be divided in two major categories - "top-down" and "bottom-up" approaches. The former includes well-known electron beam or photolithographic techniques [1] currently used in microelectronics, and the latter focuses on chemical design and includes the use of self-assembling materials for producing surfaces ordered at the nanoscale level. The bottom-up approach allows for a strict control on the physicochemical properties of a surface and represents a versatile method for the production of a variety of surfaces. It also shows promise for sub-100 nm feature fabrication [2].

Typically patternable materials are rigid, glass forming organic materials to enable maximum resolution of resulting features. In the growing field of nanobiotechnology, soft materials such as hydrogels, have found because of their biocompatible nature applications in drug-delivery [3], tissue engineering [4] and specific cell/protein interactions [5] [3]. Elastomers have proven to be useful materials for fabricating microfluidic channels [6]. The patterning of such soft materials in two-dimensions has already been achieved by conventional 2D lithography [7,8]. However, to achieve the full potential of these materials, patterning in three-dimensions is necessary as one can add a higher level of functionality.

This presentation will discuss fabrication strategies for making small-scale structures with controlled surface functionality with the goal of interactions with biomolecules and living cells.

Experimental

Materials. 2-Hydroxy ethyl methacrylate (HEMA) and poly(ethylene) glycol diacrylate (PEGDA) were purified by passage through a MEHQ inhibitor removal column. 7-benzothiazol-2-yl-9,9-diethylfluoren-2-yl)diphenylamine, designated as AF-240 [9] was supplied by Air Force Research Laboratory. All other chemicals were obtained commercially and used as received.

Ligand Synthesis. DNP-NH-$(CH_2)_5$-CO-O-$(CH_2CH_2$-O$)_4$-CO-$(CH_2)_{16}$-S$]_2$,(DNPcap-PEG$_4$C$_{16}$ disulfide) was synthesized according to a literature procedure in 65% yield [10].

Electron Beam Lithography. A bilayer electron beam resist process [10] was used to define the molecular tethering sites. First, a 4% solution of 495 K MW poly(methyl methacrylate) (PMMA) in anisole was spun at 4000 rpm for 60 sec, and baked in air at 170 °C for 15 min. A 2% solution of 950 K MW PMMA in methyl isobutyl ketone (MIBK) was then spun at 2000 rpm and baked at 170 °C for 15 min. The resist was patterned using electron beam lithography (100 keV Leica VB6) and developed in MIBK: 2-propanol 1:3 for 1 minute. After reactive plasma etching for 2 min on the patterned PMMA, gold (50 nm, 1 Å/s) with a titanium (10 nm, 0.2 Å/s) adhesion layer was deposited using electron beam evaporation and subsequently PMMA/gold was removed by lift off in a solution of methylene chloride leaving gold posts (50 nm in height) on silicon.

Monolayer Preparation and Mast Cell Studies. SAMs were prepared by immersing cleaned substrates immediately into a solution of DNP-cap-PEG4C16 disulfide in anhydrous tetrahydrofuran (THF) as previously described [10]. Cell stimulation was carried out using RBL-2H3 cells that were sensitized with fluorescently labeled monoclonal anti-DNP IgE (1 µg ml-1) for 4 h at 37 °C. The cell suspension (5 x 106 cells ml-1) was added on top of the ligand patterned substrate as described previously [10] and characterized using confocal microscopy.

Polymer for two-photon lithography. Equimolar amounts of HEMA and PEGDA (Mn 575) were mixed. To this mixture, 1 weight % of 2,2-dimethoxy-2-phenyl acetophenone (DMPAP) was added as a free radical initiator. Ethylene glycol dimethacrylate (0.003 mole %) was added as a crosslinking agent. The TPA chromophore, AF-240 was added at a concentration of 0.4 weight %. Toluene was used as the solvent.

Two-Photon Lithography. An Olympus IX70 confocal laser scanning microscope with Lambda Physik titanium: sapphire laser operating mode-locked (~80 MHz, ~100 fs) at the near-IR wavelength of 780 nm was used for fabrication. The focus of the beam was controlled by a motorized stage and mirrors. The femtosecond pulse was scanned through a 40x ~1.15 NA water objective along the surface of the liquid resin resting on a coverslip (no. 1). Exposures were performed voxel by voxel with each voxel separated by 0.533 µm in the x, y-plane and 0.3 to 0.6 µm in the z-plane. Exposure times were 100 ms per voxel. The laser power was measured with a Spectra Physics 407A power meter and the percentage of the total power reaching the surface of the resin was controlled through COMOS software. After fabrication, the structures were developed with ethyl acetate.

***Pheochromocytoma* (PC12) Cell Culture.** PC12 cells (passage 9) were re-seeded in media DMEM (DMEM with 5% FBS and 10% HS) for 3 days prior to experiments. Cells were plated at a density of 50,000 cells/coverslip. Nerve growth factor (mNGF 2.5S, Roche Diagnostics, MN) was loaded into the fabricated hydrogels for 24 hours. It was then released in the cell culture. Cells were fixed and stained after 3 days.

Results and discussion

Ligand Synthesis. The ligands synthesized, contained a long alkyl chain, $(CH_2)_n$ (n=16) with a thiol or disulfide terminal group to bind to the gold surface and an oligo ethylene glycol (OEG) spacer to reduce the non-specific adsorption which is terminated by a DNP-cap to bind to anti-DNP antibodies (Figure 2). The terminal DNP group can also bind to anti-DNP IgE in solution and bound to specific receptors (IgE-FcεRI) on mast cells [11].

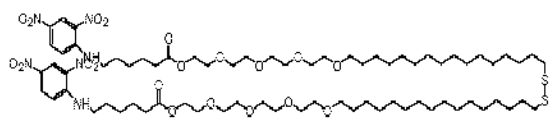

Figure 2. Chemical structure of the ligand

Electron Beam Lithography. Electron beam lithography was used for fabrication of arrays with a 1µm feature size. After patterning

the PMMA was then removed from the unexposed regions and titanium followed by gold was evaporated onto the substrate. Using a lift-off method, PMMA/gold was removed leaving gold posts on the silicon. Figure 3 shows SEM images of gold posts (height of 50 nm) on silicon with feature size of 1μm.

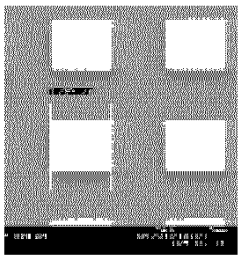

Figure 3. SEM image of gold post with feature size of 1μm

Mast Cell Studies. Mast cells are activated by ligand-mediated clustering of IgE-FcεRI receptors. Stimulated cellular responses were examined by incubating the patterned substrates with a suspension of RBL-2H3 cells that had been sensitized with AlexaFluor-488 labeled anti-DNP IgE. The cells settled on the silicon chip and became adherent within 2-3 min at room temperature (RT). The fluorescently labeled IgE receptor complexes that were originally dispersed over the cell surface became clustered after binding to the DNP-cap-PEG_4C_{16} self-assembled monolayer (SAM) modified features (Figure 4). Two control samples confirmed the specific binding and resulting colocalization [10]. Consequent cellular activation was confirmed by stimulated tyrosine phosphorylation in the region of the clustered receptors [10].

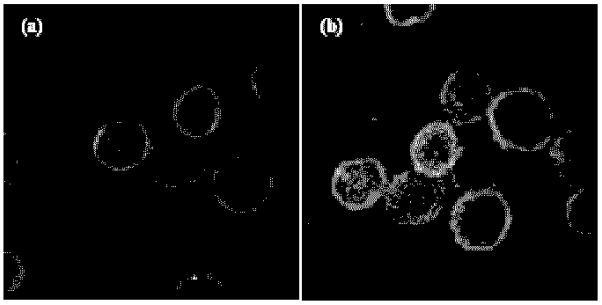

Figure 4. Confocal images of RBL stimulated by DNP-ligand SAM. a) View of the cell at the interface interacting with the ligand patterned substrate. Fluorescently labeled IgE concentrates over the patterned features. b) Overlay of the reflectance (red) and fluorescence (green).

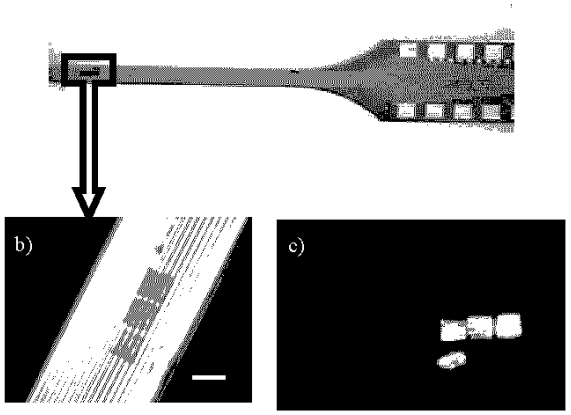

Figure 5. Microfabricated structures on a neural prosthetic device. a) Optical image of the device with patterned structures. b) Magnified view of the structures c) Fluorescence image of the structures. Scale bars = 60 μm (Figure 5a), and 25 μm (Figure 5b)

Two-Photon Lithography. Figure 5a,b shows optical images of 3 cubic structures (25μmx25μmx2μm) formed on a neural prosthetic device (University of Michigan, USA). These hydrogel structures were then loaded with a fluorescently labeled Dextran (molecular weight 10kDa.) (Figure 5c): Dextran serves as a model for the nerve growth factor (NGF) whose molecular weight is around 26 kDa.

Swelling Studies. A reciprocal relationship between cross-link density and swelling of gels was observed. As the laser power decreased, a gel of lower cross-link density resulted. This was verified from fluorescent images of 3D microstructures (Figure 6). Approximately 15 % swelling was observed after addition of water when waffle shaped objects were produced using laser intensity of 3mW (compare 6a and 6b).

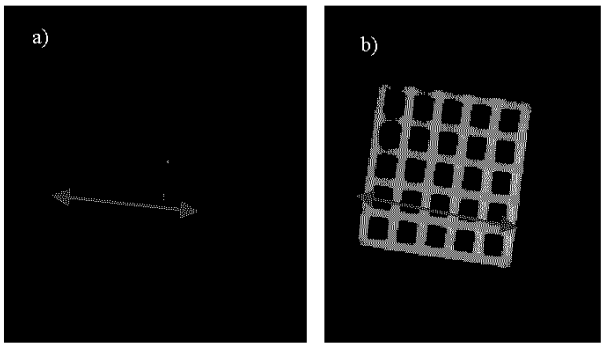

Figure 6. Fluorescence image of a microfabricated waffle a) Before addition of water; b) After addition of water.

Conclusions

Using high-resolution lithography, precisely patterned gold regions were formed on silicon. To these sites DNP-ligand modified alkanethiols were tethered and tested for molecular recognition with anti-DNP antibodies as well as for visualizing stimulated cellular response. Using two-photon lithography, hydrogels were successfully microfabricated on a neural prosthetic device. Different types of hydrogel microstructures were fabricated and tested for their swelling properties. On releasing growth factors from these hydrogels PC12 cells extended their processes towards the device. These *in vitro* results demonstrate that neuronal-like cells are attracted to the device and indicate that *in vivo* testing is justified. Both these research areas demonstrate the importance of patterning and formation of the appropriate chemistry and topography of the material systems to elicit the most favorable response from biological systems.

Acknowledgments

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