Supporting Information

Nanolithographic control of the spatial organization of cellular adhesion receptors at the single-molecule level

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Content:

Fabrication of Nanodot Arrays

NIL molds, either from diamond-like carbon (DLC)[1-3] or hydrogen silsesquioxane (HSQ) on silicon, were patterned by electron beam lithography. DLC based molds were made of Si substrates with 100 nm DLC films deposited by PECVD. The substrates (10 mm x 20 mm) were patterned by e-beam lithography using an FEI Sirion scanning electron microscope equipped with a Nabity NPGS pattern generator. A 20 nm film of HSQ (Fox-12, Dow-Corning) was used as a negative tone e-beam resist, which was developed after the exposure in TMAH solution (LDD 26W) for 2 min., following by DI water rinse and nitrogen gun drying. The pattern transfer to DLC was done by O_2 RIE (Oxford PlasmaLab 80, RF power of 150 W, 30 mTorr) to a depth of 30 nm, followed by stripping of the HSQ mask in a buffered HF solution. Finally, an anti-adhesion fluorocarbon plasma treatment process was applied to the DLC template (Oxford PlasmaLab 80, C_4F_8, 88 mTorr, 100W, 30s), to ensure clean separation of the mold from the imprinted resist.

For the HSQ based molds (Fig. S1), Si substrates (10 mm x 20 mm) were patterned by HSQ in the same manner as described above. The patterning was followed by annealing at 550^o C in a nitrogen atmosphere for 1 hr., and application of a commercial mold release agent (Nanonex NXT-130).

Thermal nanoimprint was done either on Si substrates or glass cover slips covered with a 60 nm film of PMMA (35K, Microresist Technology GmbH) using a Nanonex BX-200 nanoimprinter. Typical imprint parameters used were: an imprint temperature of 180^{o}C, a pressure of 500 psi and an imprint time of 5 min.

For the hard mask, 12 nm of Ti was deposited in a Semicore SC2000 e-beam evaporator, at an evaporation rate of 0.2 Angstrom/sec. During this evaporation the substrates were tilted 30 degrees to the vertical direction. After Ti mask evaporation,

the residual PMMA layer was removed by etching in an oxygen plasma asher (Technics 800, 200mTorr, 50 W, 30s). 3 nm of AuPd with a 1 nm Ti adhesion layer were deposited by e-beam evaporation after the etching, followed by liftoff and thermal annealing at temperature of 450^{o}C for 1 hr to provide the fabricated nanodots with a uniform spherical shape along with a reduced diameter.

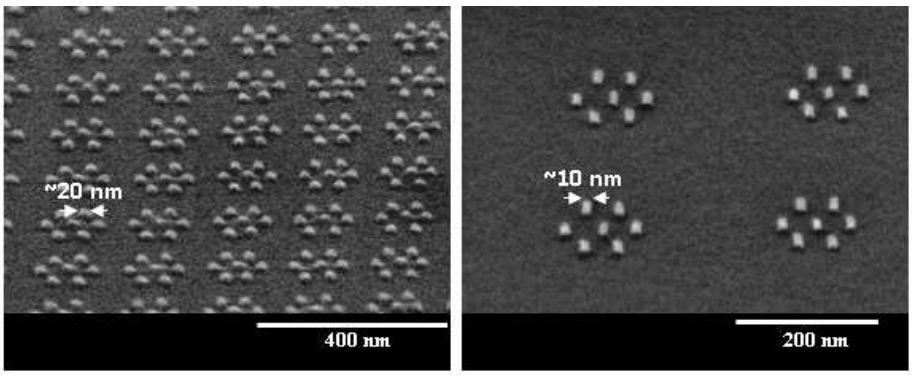

Figure S1. SEM of HSQ mold, patterned with heptagonal clusters (45° tilt).

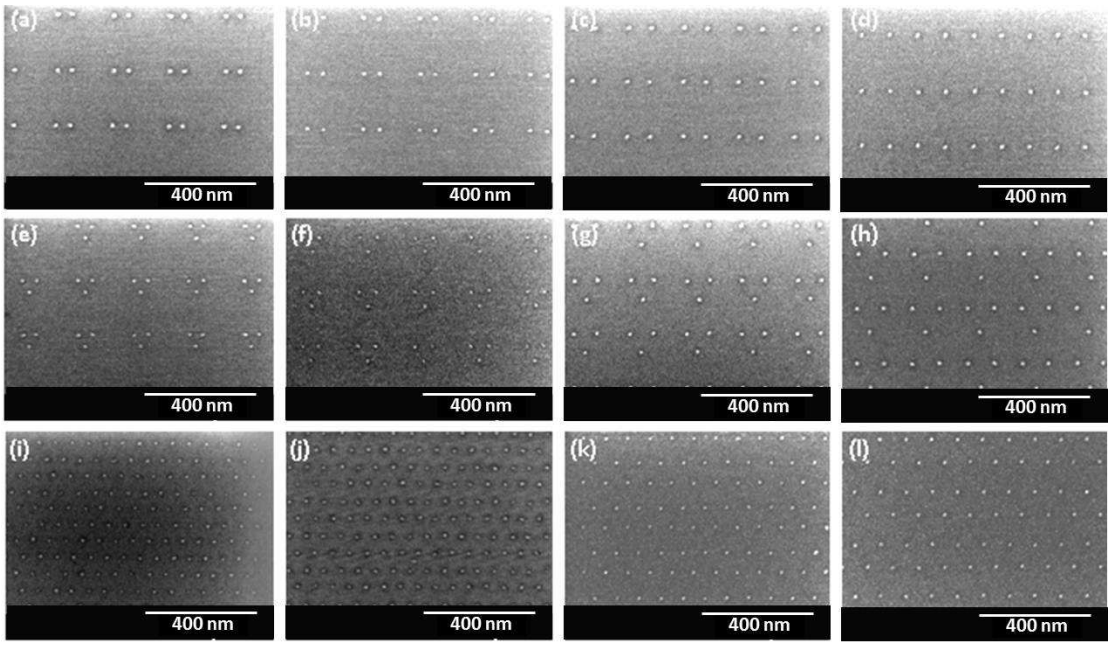

Figure S2. SEM of nanodot arrays of dimers ((a)-(d)), trimers ((e)-(h)), and extended hexagons ((i)-(l)), with interdot spacings of 50 nm, 60 nm, 80 nm, and 100 nm respectively

Biofunctionalization Process

The nanodot biofunctionalization process is schematically shown in Fig S3. Glass substrates, patterned with arrays of AuPd nanodots were immersed in a 1h 30 minutes aged piranha solution ($^1/_3$ H_2O_2, $^2/_3$ H_2SO_4) for two minutes, followed by DI water rinsing, ethanol rinsing and nitrogen gun drying. The piranha treatment was followed by an additional cleaning in UV-ozone for 10 min.

Immediately after the cleaning, the substrates were immersed in a 1mM mixed thiol solution of HS-C_{11}-$(EG)_3$-OH and HS-C11-$(EG)_3$-Biotin (3:1) in anhydrous ethanol overnight, rinsed with ethanol and dried with a nitrogen gun.

The unpatterned glass surface was passivated by a self-assembled monolayer of PEG in order to prevent non-specific binding of proteins to surface. The PEG solution was prepared by dissolving ~2mg of $(PEG)_n$-Si-$(OEt)_3$ (Mw=600Da, Nektar) in 25 mL of anhydrous toluene, and adding 20 μL of glacial acetic acid as a catalyst. After the thiolation step the samples were rinsed in ethanol, blown dry (Ar), then immersed into the PEG solution and kept there for at least 24 hrs. The immersion was followed by immediate rinsing with ethanol, DI water and drying.

Streptavidin solution was prepared by adding streptavidin (10μg/mL), labeled with AlexaFluor 488 fluorescent dye (Invitrogen), to the phosphate buffer saline (PBS, Gibco™ Dulbecco's Phosphate Buffer Saline 1X) solution, and adding chicken egg albumin (1mg/ml) (Sigma). Samples were immersed in the solution right after the PEGylation step, previous rinsing, for one and a half hours, followed by PBS rinsing. Finally, the streptavidinated samples were rinsed in PBS and then immersed into a ~10μM solution of peptide of interest for one hour, following by rinsing with DI water and PBS. In all the cell assays, biotinylated RGDfK with -$(PEG)_2$ linker (Peptides International) was used as the final peptide. For DNA imaging we first

hybridized in a ThermoScientific BupH™Phosphate Buffered Saline (pH=7.2) (TPBS) solution the following oligonucleotides (IDT): one 20-mer with a biotin functional group at the 5' position (5'- /52-Bio/GTC ACT TCA GCT GAG ACG CA -3') and the complementary strand with a Cy3 fluorophore at the 5'-end (5'- /5Cy3/TGC GTC TCA GCT GAA GTG AC -3'); the samples were then immersed in a 1μm TPBS solution of the hybridized oligonulceotides (i.e the Cy3-labeled dsDNA) for 2 hours, and then rinsed in TPBS.

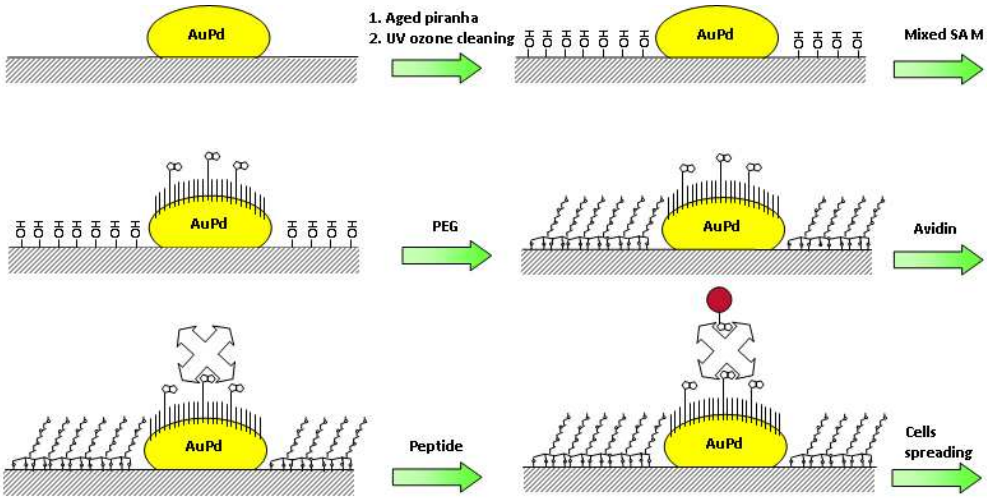

Figure S3. Biofunctionalization of AuPd nanodot arrays

Cell Assays and Imaging

NIH 3T3 mouse fibroblast cells were plated on the biofunctionalized arrays, with the cell concentration high enough to plate ~ 20 - 40 cells per 200 μm x 200 μm array. Both fluorescence imaging of streptavidin and DIC imaging of cells were performed with an Olympus IX-81 microscope. Dynamic behavior of cell spreading on chips on multiple arrays was captured by simultaneous imaging of the arrays using

an automatic stage with programmed positioning, usually taking 1 frame per 4 minutes for each array.

For SEM imaging, the cells were first fixed with 2% glutaraldehyde in PBS for one hour, washed in PBS, and soaked in 50% ethanol/water, 70%, 80%, 90% and 100% ethanol, 7 minutes in each solution. Critical point drying was used to dry the samples, and then 3 nm of AuPd was deposited by e-beam evaporation (Semicore SC2000) to prevent surface charging during the SEM imaging. SEM imaging was done using an Hitachi 4700 microscope, at an accelerating voltage of 10kV.

In order to verify that cell spreading is caused only by the integrin-binding molecules anchored to the nanodots – and not by any other external factors (such as the surface topography in the patterned areas, or the presence of streptavidin there), a control experiment was done, in which the last step of adding an integrin-binding peptide was omitted from the pattern functionalization. Figure S5 shows the cells plated on such an array of hexagonally arranged dots with 50 nm spacing. The cell behavior was very similar to that of cells on PEG-passivated glass surface, and this behavior was mostly characterized by high motility, especially in the first half hour, and complete lack of spreading.

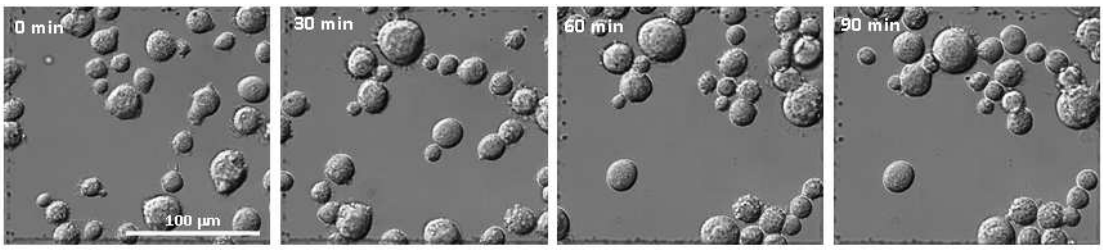

Figure S4. Control experiment - cells spreading on nanodots array pattern (hexagonal arrangement, 50 nm spacing), functionalized up to the avidin step, with no integrin-binding group.

Figure S5 shows cell spreading efficiency curves for dimers, trimers, extended hexagonal arrays and planar AuPd with inter-dot spacings of 80 nm and 60 nm, demonstrating the effect of spacing on cell spreading. This is also reflected in the total area of spread cells (Fig. S6). The cell area was determined using the public domain image processing software ImageJ.

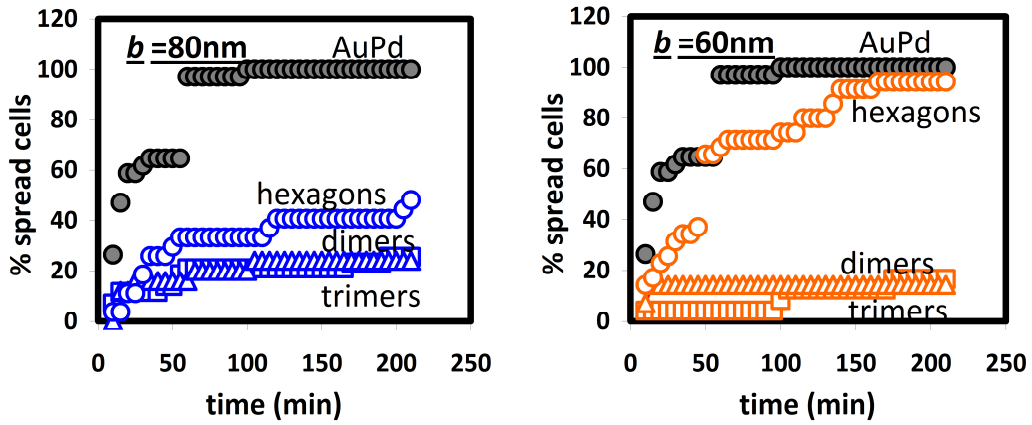

Figure S5. Cell spreading curve for different arrays with b = 80 nm, and b = 60 nm.

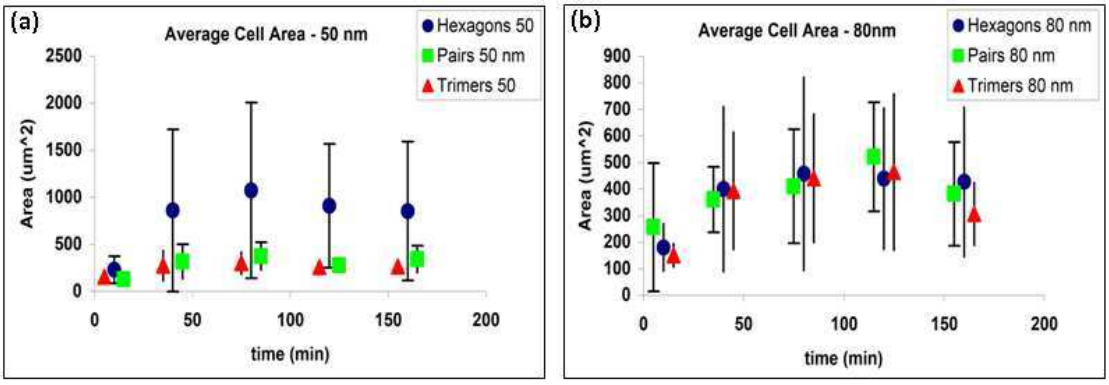

Figure S6. Average area of spread cells on arrays with inter-dot spacing of (a) 50 nm and (b) 80 nm.

1 Schvartzman, M., Mathur, A., Hone, J., Jahnes, C. & Wind, S. J. Plasma fluorination of carbon-based materials for imprint and molding lithographic applications *Appl. Phys. Lett.* **93**, 153105 (2008).

2 Schvartzman, M. *et al.* Fluorinated diamondlike carbon templates for high resolution nanoimprint lithography. *Journal of Vacuum Science and Technology B: Microelectronics and Nanometer Structures* **26**, 2394-2398 (2008).

3 Schvartzman, M. & Wind, S. J. Plasma fluorination of diamond-like carbon surfaces: mechanism and application to nanoimprint lithography. *Nanotechnology* **20**, 145306-145312 (2009).