# Soft Lithographic Patterning of Hyaluronic Acid on Hydrophilic Substrates using Molding and Printing

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## 1. Introduction

Hyaluronic acid (HA, also called hyaluronan) is a linear anionic polysaccharide composed of disaccharide repeat units exhibiting one carboxyl group. HA is an attractive building block for novel biocompatible and biodegradable polymers that have applications in drug delivery, tissue engineering, and viscosupplementation. Interestingly, HA not only provides excellent resisting properties toward adhesion of many proteins and cells, but it can also interact with receptors such as CD44 and p32, allowing for a number of cell recognition processes. Therefore, HA may act as attractive or repulsive spacers between cells depending on the presence of HA receptors (CD44 or p32). Thus, once immobilized, HA patterned surfaces could be potentially useful in detecting or repelling specific biological species.

In this study, we describe two methods of patterning HA on various substrates including glass, silicon dioxides, poly (hydroxyethyl methacrylate) [poly (HEMA)], polystyrene cell culture dishes, and biodegradable polylactic glycolic acid (PLGA), without the use of chemical modification or with slight modification with NaOH or oxygen plasma treatment.1 Such versatile use of soft lithographic methods would be potentially useful for many biomedical applications

## 2. Experimental section

### Microcontact printing (μCP)

PDMS stamps were plasma cleaned for 5 min (model PDC-001, Harrick Scientific Inc.) to ensure proper cleaning and to increase wettability. After cleaning, a 5 mg/mL solution of HA (Genzyme Inc., $M_n$ = 2.1 MDa.) in deionized water was applied to the surface using a cotton swap. HA was coated three times to ensure complete coverage with HA and subsequently placed onto glass slides that were plasma cleaned for 1 min. The stamp was left for 20 min and then peeled off. The printed slides were allowed to sit for 12 h before further usage.

### Molding

A few drops of the solution were placed on a glass slide and a thin film of HA was coated by spin coating (Model CB 15, Headaway Research, Inc.) at 1000 rpm for 10 s. To make conformal contact, PDMS stamps were carefully placed onto the surface and the samples were stored overnight at room temperature to allow for evaporation of the solvent. The film thickness after solvent evaporation is about 606 nm as determined by ellipsometry (Gaertner L116A, Gaertner Scientific Corp.) and AFM.

### Protein adsorption

FITC-labeled BSA and IgG and FN (Sigma) were dissolved in phosphate buffered saline (PBS) solution (pH = 7.4; 10 mM sodium phosphate buffer, 2.7 mM KCl, and 137 mM NaCl) at a concentration of 50 μg/mL, 50 μg/mL, and 20 μg/mL, respectively. To measure FN patterns, the surfaces were stained with anti-FN antibody (Sigma) for an additional 45 min, followed by 1 h incubation with the FITC-labeled anti-rabbit secondary antibody. Protein patterns were realized by evenly distributing a few drops of the protein solution onto the patterned HA surfaces, storing at room temperature for 30 min, rinsing subsequently with PBS solution and water and then blowing dry in a stream of nitrogen. The surface was then imaged using an inverted microscope (Axiovert 200, Zeiss).

### Cell cultures and adhesion

NIH-3T3 murine embryonic fibroblasts (American Type Culture Collection) were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco Invitrogen Corp.) supplemented with 10 % fetal bovine serum (Gibco Invitrogen Corp.) at 37 °C and 5% $CO_2$ environment. For cell attachment experiments, the patterned surfaces of HA were treated with 500 g/mL of FN in PBS for 15 min and then the cells were trypsinized and washed and directly seeded on the patterned surfaces at a cell density of ~$10^4$ cells/$cm^2$. The cell patterns were examined under phase-contrast microscope after removing non-adhered cells by rinsing with PBS. A schematic diagram is shown in Fig. 1

## 3. Results

To test the effectiveness of the HA patterned surfaces for protein patterning, HA modified surfaces were exposed to fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA), goat anti-rabbit immunoglobulin G (FITC-IgG) and fibronectin (FN). Experiments demonstrated that the adhesion of BSA (0.46%), IgG

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(7.81%), and FN (6.22%) was significantly reduced on HA coated glass substrates in comparison to bare glass controls (100%) ($p<0.001$). Figure 2 shows protein patterns on various substrates such as (A-C) glass, (D) silicon dioxides, (E) poly(HEMA), (F) PLGA, and (G-I) polystyrene culture dishes when HA was used as a resistant layer. Despite the surface modification by NaOH, the pattern generation on biodegradable PLGA surface was not satisfactory compared to other substrates due to a significant swelling by water at the time of contact. For the other substrates, the pattern generation was successful in that the images reveal sharp contrast between binding and non-binding areas for a number of "sticky" proteins, suggesting excellent resisting properties of the HA surfaces and direct applicability of the current approach to various biomaterials with μCP and molding methods.

Cells were also patterned on various substrates using HA as the resisting layer. Cell seeding experiments indicated that <1% of the cells adhered onto HA coated glass surfaces, which is significantly lower than FN treated controls or glass substrates (>90%). To pattern cells, HA patterned surfaces were treated with FN for 15 minutes and subsequently seeded with cells. Figure 3 shows typical images of HA patterned surfaces that were seeded with NIH-3T3 murine embryonic fibroblasts and incubated for 6 h. As expected, cells deposit only on the exposed substrate regions, ranging from aggregated cell arrays (150 μm holes in Fig. 3A) to single cell arrays (15 μm holes in Fig. 3B) depending on the pattern size. Poly(HEMA) also provides neat arrays of 3T3 cells whereas PS culture dishes need to be treated with NaOH or oxygen plasma to ensure successful patterning. In addition to fibroblasts, murine hepatocyte cells and embryonic stem cells were also patterned on HA coated surfaces, which indicate the feasibility of this approach for various cell types (data not shown).

## 4. Summary

In summary, we have demonstrated soft lithographic application of HA (or polysaccharides in general) by means of μCP and molding and constructed well-defined patterns of proteins and cells on various substrates including glass, silicon dioxides, poly(HEMA), polystyrene culture dishes, and biodegradable PLGA. These results suggest that HA could be used as a general platform on hydrophilic substrates.

## 5. References


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(2) A. Khademhosseini, K. Y. Suh, J. M. Yang, G. Eng, J. Yeh, S. Levenberg, and R. Langer, *Biomaterials 25*, 3583 (2004).


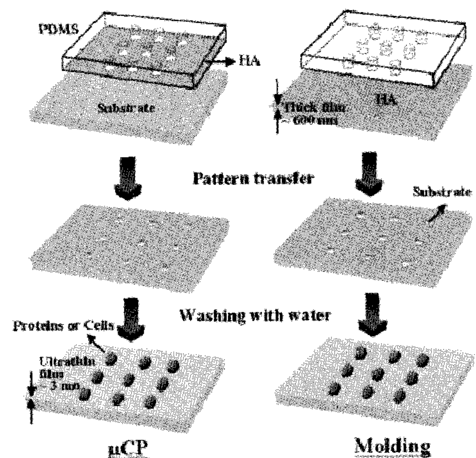


**Figure 1.** Schematic illustration of the two soft lithographic methods used in the experiment: μCP (left) and molding (right). μCP utilizes direct transfer from a stamp to a substrate whereas molding deals with pattern formation from a uniform polymer film into the features of the stamp.

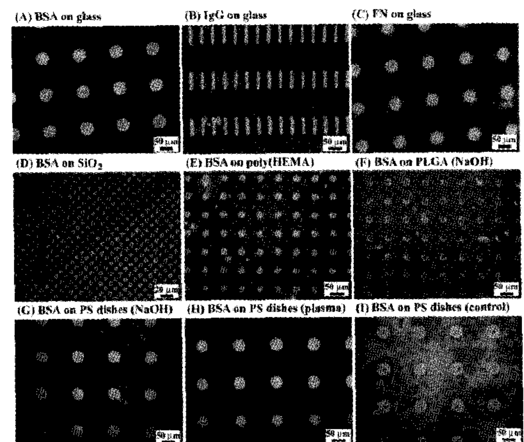


Figure 2. Fluorescent images for protein patterns on various substrates.

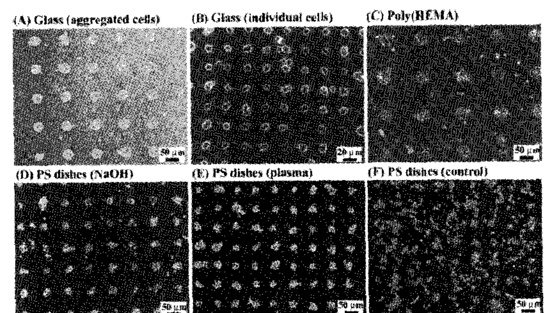


Figure 3. Optical images for cell arrays on various substrates. Using FN as an adhesion layer, NIH-3T3 cells were seeded on glass with 150 μm holes (A) and 15 μm holes (B), leading to aggregated and individual cell arrays depending on the feature size. In addition, poly(HEMA) and PS culture dishes were also tested with suitable modifications if