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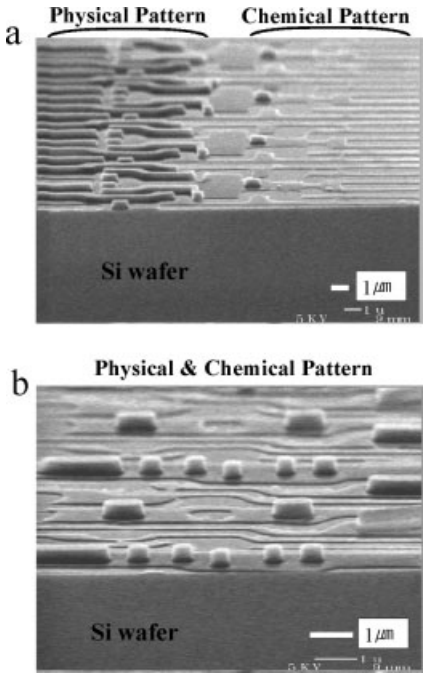

Figure 4. SEM images of physical and chemical polymer (P4VP) micro- and nanostructures on a Si wafer in polymer spin transfer printing: a,b) Micro- and nanostructures with complex physical and chemical nanostructures on a Si wafer.

Finally, because polymer spin-transfer printing can be used to make nanostructures with general polymer systems, as opposed to a very specific type of polymer, it is easily applicable to different surface, thermal, mechanical, and chemical properties. Some issues such as the reduction of adhesion between the stamp and the transferred polymer layer must be perfected to achieve perfect transfer fidelity each time. However, this new unconventional lithographic technique—polymer transfer printing—introduces a range of interesting applications in electronic, optical, and biomaterials device areas.

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Soft Lithographic Patterning of Hyaluronic Acid on Hydrophilic Substrates Using Molding and Printing**

By *Kahp Y. Suh*,* *Ali Khademhosseini*, *Jen Ming Yang*, *George Eng*, and *Robert Langer**

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 for applications in drug delivery, tissue engineering, and viscosupplementation.[1–4] Interestingly, HA not only has excellent resistant properties toward adhesion of many proteins and cells,[5–7] but it can also interact with receptors such as CD44 and p32, allowing for a number of cell recognition processes.[8,9] 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 communication, we describe two methods of patterning HA on various substrates including glass, silicon dioxides, poly(hydroxyethyl methacrylate) (poly(HEMA)), polystyrene (PS) cell culture dishes, and biodegradable polylactic glycolic acid (PLGA), without the use of chemical modification or with slight modification using NaOH or oxygen plasma treatment. Such versatile use of soft lithographic methods would be potentially useful for many biomedical applications. The motivation for this study arises from two features. First, to our knowledge there have been no previous reports dealing with soft lithographic uses of polysaccharides (including HA),

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since they are highly hydrophilic (i.e., not compatible with a stamp prior to oxygen plasma treatment) and soluble in water (i.e., coatings would be washed away in the subsequent steps). Second, soft lithographic patterning of hydrophilic biopolymers is intriguing since it is characterized by a large contact angle (> 90°) on the hydrophobic stamp and high repulsion at the water/stamp interface. As polysaccharide coatings are emerging as new elements for constructing low-fouling, biocompatible, and bioactive surfaces,[10,11] an approach to the patterning of polysaccharides could be potentially useful for bioanalytical and biomedical applications. Moreover, we have employed HA as a new ink/medium in protein and cell patterning, providing new potential for the use of HA in many other applications.

Here, we report that HA is compatible with microcontact printing (μCP) and molding approaches (Fig. 1). To pattern HA using μCP, poly(dimethylsiloxane) (PDMS) stamps were treated with oxygen plasma to improve adhesion of HA to the stamp.[12] In addition, the transfer of the HA layer to the substrate surface was dependent on the wetness of the stamp at the time of contact, such that pattern transfer was successful only when the stamp was wet (i.e., prior to complete evaporation of solvent). The high contrast in the height and phase atomic force microscopy (AFM) images (Figs. 2A,B, 10 μm box stamp) indicate that the pattern transfer by μCP was successful with good edge definition. The height of the printed HA layer was typically ~90 nm for the conditions used in the experiment, which is much higher than the few nanometers typically obtained for self-assembled monolayers (SAMs).[13–15] This may be attributed to the sluggish movement of highly viscous, entangled HA chains during the contact process. It is observed that washing with water leads to the formation of an ultrathin HA pattern (~3 nm) regardless

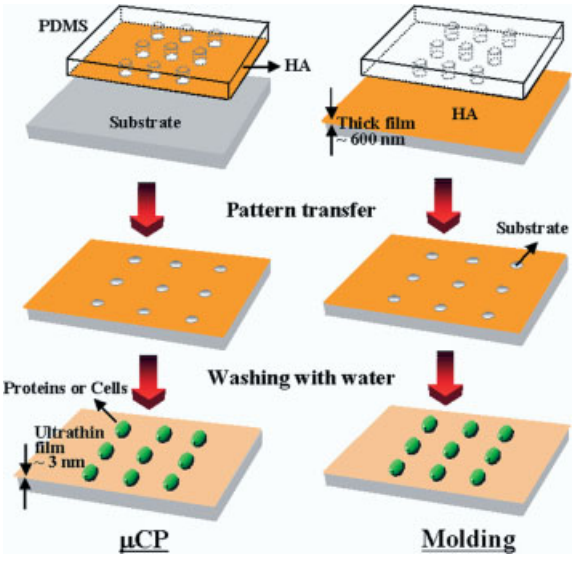

Figure 1. Schematic illustration of the two soft lithographic methods used in the experiment: μCP (left) and molding (right). μCP utilizes direct transfer from a stamp to a substrate whereas molding deals with pattern formation from a uniform polymer film into the features of the stamp.

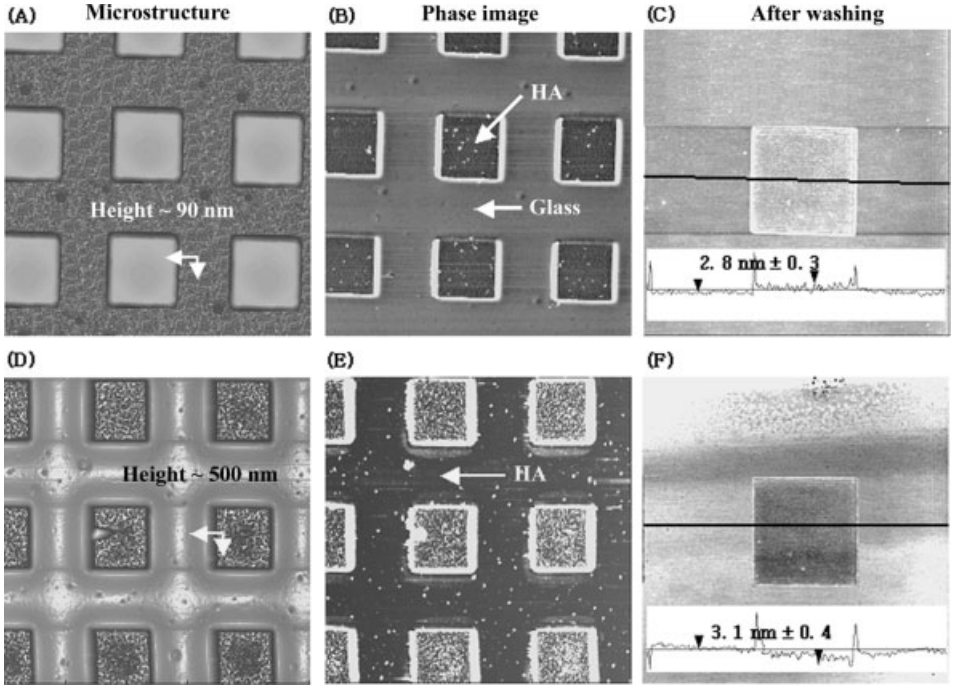

Figure 2. AFM height and phase images for printed (A–C) and molded (D–F) HA patterns onto glass substrates before (the first two columns) and after washing (the third column). Note that the washing step ultimately leads to a chemisorbed HA layer of ~3 nm. The scan size is 50 × 50 μm^2 for A, B, D, E and 30 × 30 μm^2 for C and F.

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of the heights of the initial microstructures for the conditions used in the experiment (Fig. 2C). It has been shown that HA can be stabilized on the surface through hydrogen bonding between the hydrophilic moieties in HA, such as carboxylic acid (–COOH) or hydroxyl (–OH) groups, with silanol (–SiOH), carboxylic acid, or hydroxyl groups on the hydrophilic substrates (submitted elsewhere).

An alternative to μCP is a molding process, in which a polymer layer is molded into the features of the stamp.[16,17] Interestingly, the pattern transfer by the molding method was not affected by the use of plasma treatment of the stamp as well as the substrate, thus providing a more convenient route to patterning. As shown in the experimental procedure (Fig. 1), HA was spin-coated onto the desired substrates and a PDMS stamp was immediately brought into conformal contact with the substrate. Since HA is a highly hydrophilic polymer, the wetting environment for the molding is very different from those of previous studies.[16,17] Hydrophobic polymers such as polystyrene partially wet the hydrophobic PDMS wall (acute contact angle), thus rising into the void space ("capillary rise"). Alternatively, hydrophilic polymers (such as HA) under the void space recede downwards until the substrate surface becomes exposed, which is readily understood in that the contact angle with the PDMS stamp in Young's equation is larger than 90° ("capillary depression").[18] In general, capillary rise or depression was determined by the competition between the hydrodynamic repelling force at the contact region and capillary force within the void space. For example, the HA filled up the void space despite an obtuse contact angle of water on PDMS stamp (~105°)[19] when the hydrodynamic force was dominant over the capillary force. In this case, the HA solution under the contact region was squeezed outside the protruding features of the stamp until the substrate surface became completely exposed. When the capillary force was dominant, however, the HA receded towards the bottom. If we define r as a ratio of the size under a void space (L_1) to that under a contact region (L_2), i.e., $r = L_1/L_2$, then positive stamps correspond to $r > 1$ (features sticking out) and negative stamps to $0 < r < 1$ (features indented). As expected, capillary depression was observed for negative stamps since the capillary force is inversely proportional to the length scale of the void space, as given by the Laplace equation.[18] Interestingly, the hydrodynamic force was dominant over the capillary force for $r = 1$ (Figs. 2D–F, 10 μm positive box stamp). The height and phase images (Figs. 2D,E) indicate well-defined HA microstructures after the molding process. According to the cross-sectional analysis, the polymer completely filled up the void space. In parallel, a large amount of polymer was squeezed outside the stamp during the molding process, resulting in a reduction of the initial pattern height. For example, Figure 2D shows that the average thickness of the HA pattern was reduced from ~600 nm to ~230 nm as calculated from the cross-sectional profile of the AFM image. It was observed that the substrate surface was easily exposed without fine control over film thickness and surface conditions, which is a unique advantage of the molding process involving hydrophilic polymers.[20] However, in some cases patterns were only formed at edge regions, possibly due to repulsion between water and PDMS and poor wetting of the stamp. To ensure good pattern fidelity, this problem needs to be addressed. Although the aspect ratio of the original HA structures could be controlled using different concentrations and coating speeds, washing with water ultimately leads to a chemisorbed layer, similar to μCP results (Fig. 2F).

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 (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 3 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 (see the experimental protocol). Glass, silicon dioxides, and poly(HEMA) surfaces are intrinsically hydrophilic in terms of hydroxyl groups on the surface, such that direct immobilization of HA and patterning can be achieved without any further treatment of the substrates. On the other hand, a slight modification by NaOH or oxygen plasma treatment is necessary for polystyrene culture dishes and PLGA to generate surface hydroxyl or carboxyl groups (see the experimental protocol). It was reported that the hydroxide anion acts as a catalyst and cleaves the polymer chain more effectively than water, leading to surface hydrolysis.[21] 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. Thus HA provides flexibility for use in various soft lithographic methods. Furthermore, a wide range of feature sizes (ranging from 5 μm to 500 μm) is easily attainable without additional modifications. One example of such fine patterning is shown in Figure 3D, over a large area with high pattern fidelity, in which a 5 μm box pattern was used.

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 min and subsequently seeded with cells. Figure 4 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. 4A) to single cell

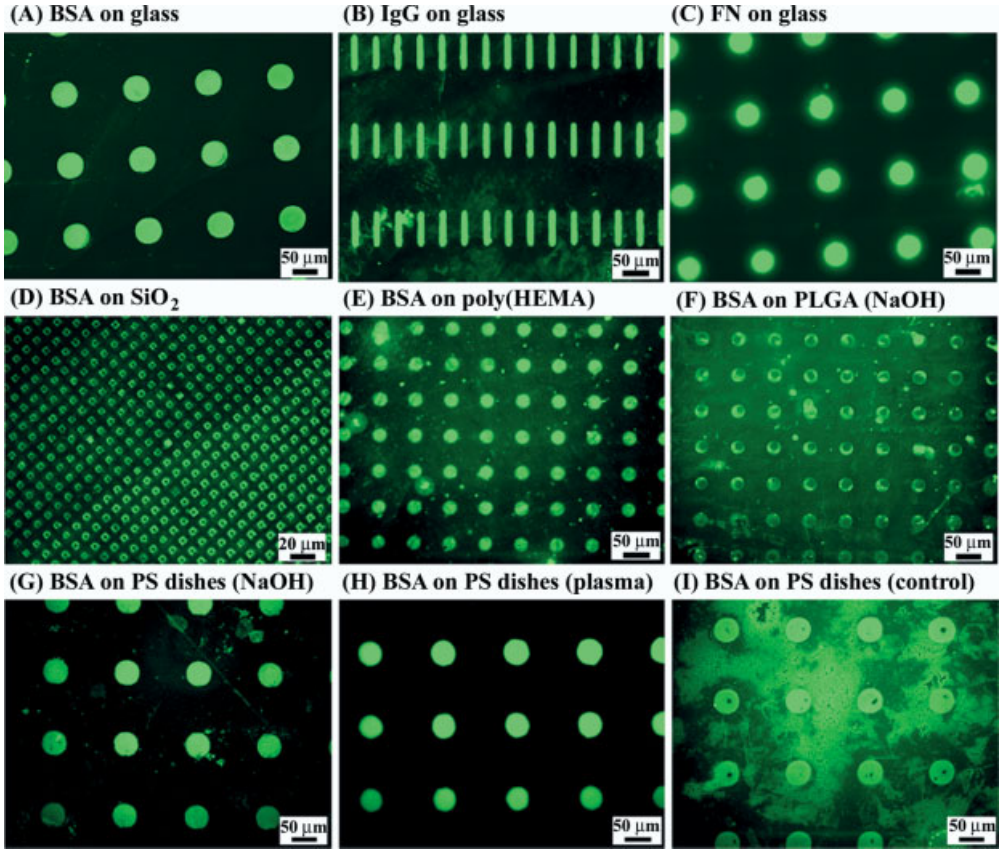

Figure 3. Fluorescent images for protein patterns on various substrates. A number of proteins including BSA (A), IgG (B), and FN (C) were tested on glass substrate to examine the effectiveness of the HA surfaces. D) An example of fine pattern on SiO_2 substrate for a 5 μm box stamp. The use of other biomaterials substrates was also tested on poly(HEMA) (E), PLGA with NaOH treatment (F), and PS culture dishes with different modifications (G–I).

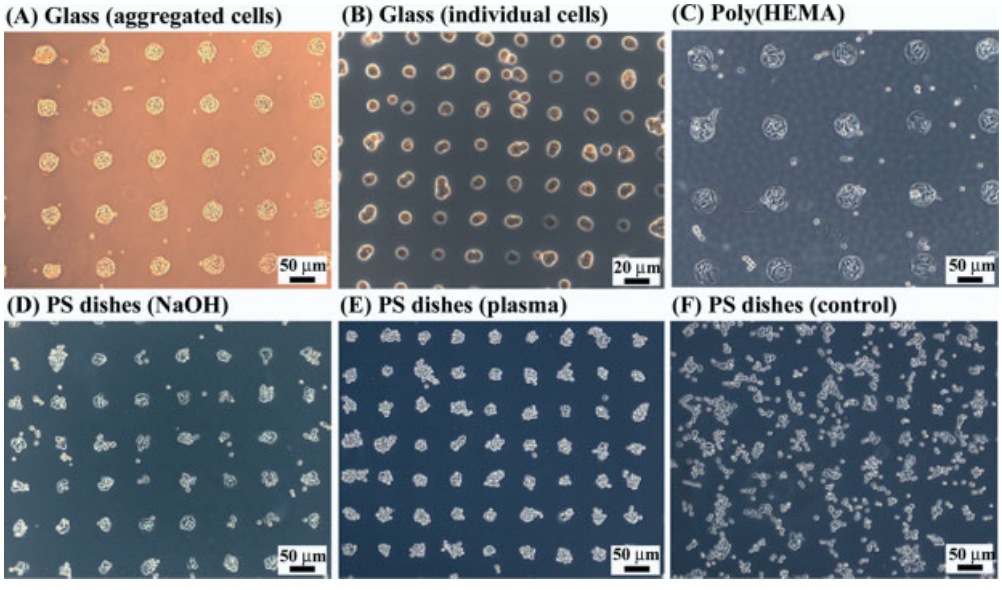

Figure 4. Optical images for cell arrays on various substrates. Using FN as an adhesion layer, NIH-3T3 cells were seeded on glass with 150 μm holes (A) and 15 μm holes (B), leading to aggregated and individual cell arrays depending on the feature size. In addition, poly(HEMA) and PS culture dishes were also tested with suitable modifications if necessary.

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arrays (15 μm holes in Fig. 4B) 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).

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 for cell and protein patterning or even on hydrophobic substrates provided that the surface is made hydrophilic through simple modification of NaOH or oxygen plasma treatment. As hydrophilic nature is ubiquitous to most biopolymers, the current approach could be beneficial in fabricating patterns of other hydrophilic polymers as well.

Experimental

Fabrication of PDMS Stamps: PDMS stamps were fabricated by casting PDMS (Sylgard 184 elastomer, Essex Chemical) against silicon masters prepared by photolithography (1:10 ratio of the curing agent). Then the pre-polymer was mixed well and incubated at 70 °C for 1 h. After curing, PDMS stamps were detached from the master and cut prior to use.

Atomic Force Microscopy (AFM): Atomic force micrographs were taken in tapping mode on a Digital Instruments Dimension 3100 (Veeco Instruments Inc.) in air. The scan rate was 0.5 Hz and 256 lines were scanned per sample. Tapping mode tips, NSC15–300 kHz, were obtained from MikroMasch (Portland). Data were processed using Nanoscope III 4.31r6 software (Veeco Instruments Inc.).

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^{-1} solution of HA (Genzyme Inc., $M_n = 2.1$ MDa.) in deionized water was applied to the surface using a cotton swab. HA was coated three times to ensure complete coverage 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 use.

Molding: A few drops of the solution were placed on a glass slide and a thin film of HA was applied 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^{-1}, 50 μg mL^{-1}, and 20 μg mL^{-1}, 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 μg mL^{-1} 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. The cell patterns were examined under phase-contrast microscope after removing non-adhered cells by rinsing with PBS.

Surface Modification by NaOH: Poly(DL-lactide-*co*-glycolide) (85:15) (PLGA, $M_w = 50\,000$–$75\,000$) and poly(HEMA) were purchased from Aldrich. A few drops of a PLGA solution in toluene (8 wt.-%) and a poly(HEMA) solution in ethanol (6 wt.-%) were evenly distributed on glass slides respectively and then spin coated at 1000 rpm for 2 min. The samples were stored in a vacuum oven at 70 °C overnight to remove the residual solvent. To treat with NaOH, PLGA films and PS cell culture dishes were immersed in 1 N NaOH for 30 s to generate surface hydroxyl or carboxyl groups [21].

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