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Original Research

Aqueous micro-contact printing of cell-adhesive biomolecules for patterning neuronal cell cultures

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Abstract Micro-contact printing (μCP) technique has been widely used for generating micro-scale patterns of biomolecules for patterning live cells. The contact-printing process is carried out in air, while most of the biomolecules including proteins and antibodies should be handled in a solution to preserve their bioactivity. Here we attempted to print biomolecules under aqueous conditions by modifying certain steps that are known to be critical for the bioactivity. The proposed contact-printing process is as follows: After inking the stamp with biomolecule in a solution, the stamp was rinsed in ultra-sonication bath to remove excessive inked biomolecules on the stamp and the following contact-printing process ('stamping') was carried out in a buffered solution. By this way, inked biomolecules were consistently handled under a well-defined aqueous condition. Results showed that high-resolution micropatterns of biomolecules can be printed under the aqueous condition (aqueous micro-contact printing, aq-μCP) and it was readily applicable for patterning neuronal cell cultures. Using the modified process, we were able to print widely separated patterns (2 μm-wide lines with 400 μm spacing), which was not achievable with conventional μCP. Extracellular matrix proteins (laminin and fibronectin) were readily printed in a few micrometer scale patterns and their biological activities were confirmed by immunoassays and neuronal cell cultures. We also demonstrated that pH sensitive surface biofunctionalization scheme can be implemented with the proposed aq-μCP for patterning neuronal cell cultures. The aq-μCP improves the existing surface patterning strategy by extending printable patterns and proteins for neuronal cell chip design.

Keywords: Surface micropatterning, Micro-contact printing, Neuronal cell culture, Soft-lithography, Protein printing

Introduction

Micro-contact printing (μCP) has been widely used for generating nano- or micro-scale patterns of molecules on various surfaces. Since Kumar *et al*. initially suggested this technique to make patterns of self-assembled monolayers (SAMs) such as alkanethiols on the gold surface[1], this has been applied to make patterns of various molecules such as organic molecules[1,2], metals[3], and polymers[4]. This simple patterning technique has been further highlighted due to its ability to print micropatterns of various biomolecules on surfaces for biochips. In addition, the ease of replication molding by soft-lithography made it suitable for the fabrication of biological assays in biology laboratory environments. Various biological substances have been printed: poly-lysine[5], proteins[6-8], antibodies[8], enzymes[9], DNA[10], and living organisms[11]. The versatility of the technique has not only brought an experimental model for studying fundamental mechanisms of biological system[12,13], but also led to develop DNA microarrays[14] or cell chips[15]. In neurobiological applications, surface-bound chemical cues were printed on various substrates to locate somata (cell bodies) and guide neurite outgrowth; surface gradients of axon guidance molecules were printed to study the growth cone behavior[6]. Soma and axon specific regions were printed using a double stamping method[16]. Micropatterns of poly-D-lysine were printed

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on microelectrode array surfaces through covalent linking chemistry to construct long-lasting neuronal networks for the study of neural information processing of cultured neurons[5,17].

There are several steps to print biomolecules such as peptides, proteins or antibodies using μCP. First, a bare hydrophobic polydimethoxysiloxane (PDMS) stamp surface is converted into a hydrophilic surface by air/O_2 plasma[18] or surfactant coating[17], which facilitate the inking of biomolecules on the stamp. After loading the ink on the stamp, the inked stamp is rinsed in a fresh solution to dissolve excessive inks and the stamp surface is blow-dried with compressed air or inert gas (e.g. nitrogen) to remove the moisture. Next, the stamp is brought in contact with the substrate surface in air and a pressure is applied to transfer the inked biomolecules from the stamp surface to the substrate. Bernard *et al.* pointed out that the blow-drying step for removing extra ink molecules is critical to protein-contact printing[7]; they showed that several proteins partially lost their activity after being printed[6]. Oliva *et al.*, suggested 'indirect' patterning scheme to avoid the drying step that can denature the target protein[19]; 'linker' molecules were first patterned by means of μCP and subsequently the target molecules were selectively bound to patterned linkers under aqueous condition. In this way, they were able to preserve the desired target bioactivity while using the simple μCP technique. Considering the fact that other protein patterning techniques such as microfluidic patterning[20], micro-stenciling[21], or photolithographic patterning[22] delivers proteins in aqueous states to prevent protein denaturation, in-air printing process of the conventional μCP is not desirable and may be problematic for certain biomolecules. So far, contact-printing of biomolecules under the aqueous condition has not been attempted, as biomolecules are likely to diffuse in the water-based solvents.

Here we report the printing of biomolecules under aqueous conditions using μCP. The main idea is to process inking, rinsing, and contact-printing in a buffer solution so that the whole printing process would be carried out under a defined aqueous condition. Results showed that various types of biomolecules can be successfully patterned in a solution without compromising the printing resolution. Micropatterns of 2 μm-wide lines were readily achievable with the proposed technique and the aqueous stamping also improved the 'roof-collapse' problem that frequently occurred in the conventional 'dry' μCP condition. Extreme geometrical patterns which was not very successful with conventional printing procedure was tested and synthetic biopolymer and extracellular matrix proteins that were frequently used in fabricating neuron chips were successfully printed and validated by culturing E18 rat hippocampal neurons. In addition, we demonstrated that pH controlled surface biofunctionalization chemistry can be used. To the best of our knowledge, this is the first report on the successful demonstration of printing biomolecules under aqueous conditions for cell chip design.

Results and Discussion

Optimization of printing process

Figure 1 shows the procedure of conventional dry-μCP (Figure 1a) and modified aq-μCP (Figure 1b). The main difference is that the inking and stamping steps were performed under aqueous condition so that biomolecules were not exposed to drying conditions. Two representative fluorescent images show 30 μm-wide FITC-PLL patterns on a substrate (glass coverslip). Printed patterns were clearly discerned by both visual inspection and the measured intensity profiles (Figure 1c). The mean intensity profile shows that there was a significant difference in the FITC-PLL intensity in on-pattern and off-pattern areas. When the size of the printed patterns was measured from the intensity profile, it matched well with the original dimension of pattern (30 μm line and spacing). Figure 1c also shows the change of intensity profile by increasing the stamp cleaning time under ultra-sonication bath. Up to 1 min, the cleaning process did not alter the intensity significantly, while long cleaning resulted in substantial decrease of the mean intensity. Figure 1d shows the relation between the amount of FITC-PLL and the stamp sonication time after inking. As we cleaned the inked stamp longer under the sonication bath, less amount of FITC-PLL was printed on the surface. The measurement indicated that there was a linear relationship between the sonication time and the amount of printed molecules. Figure 1e shows the relationship between the amount of transferred ink molecules and the stamping pressure. More ink molecules were transferred to the substrate as the stamping pressure increased. Furthermore, contact-printing time of 1 min was sufficient to transfer FITC-PLL to the surface and their fluorescent intensity was higher than those obtained from dry-μCP. It was also possible to print patterns without the sonication step (indicated as 0 min in Figure 1c, 1d, and 1e), but it can potentially degrade the quality of printed patterns due to the diffusion.

Contact-printing of fine features with wide spacing

Two different patterns including lines and nodes with a wide spacing were used to test the performance of aq-μCP under extreme pattern geometries. One pattern

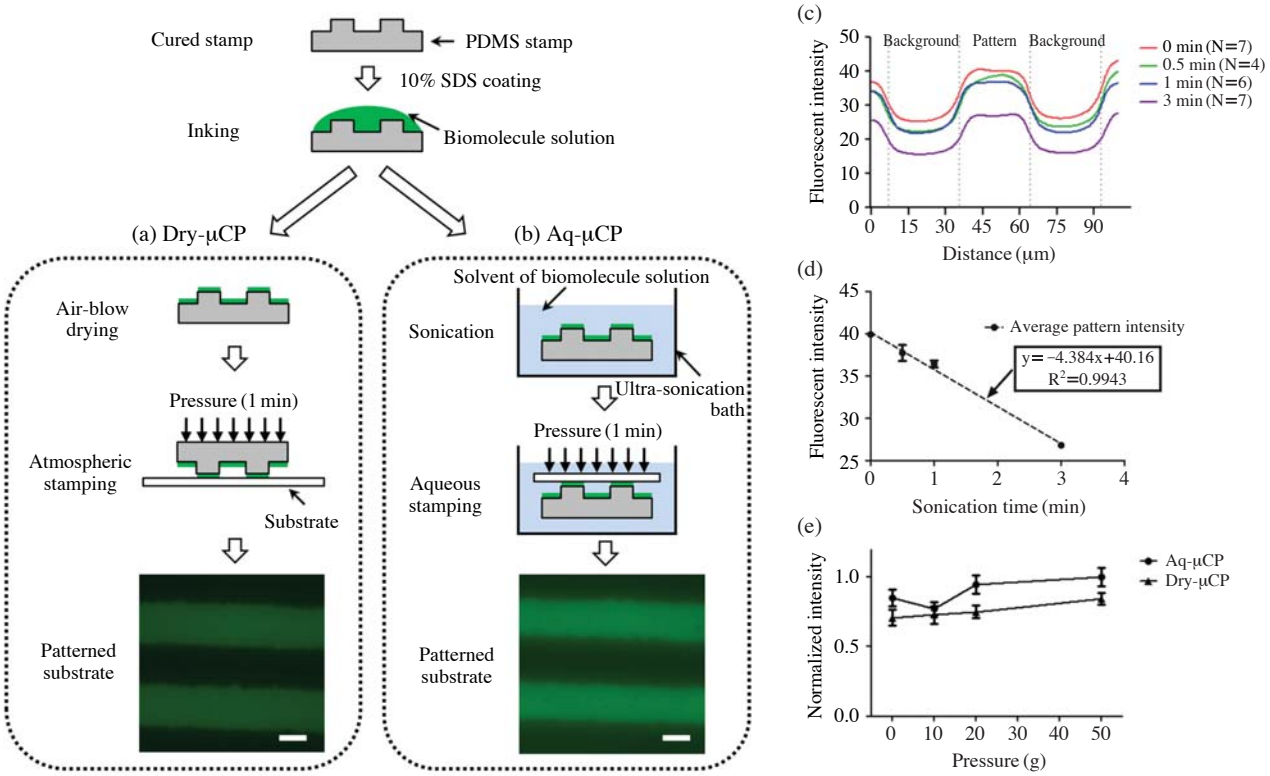

Figure 1. The procedure of dry-μCP and aq-μCP. After the modification of the stamp with SDS solution, the solution of biomolecules was loaded on a stamp for 30 minutes (inking). (a) In dry-μCP, excessive biomolecules were removed by gently dipping the stamp in DI water and blowing with airstream (air-blow drying). Then the pattern was printed on the substrate by applying a pressure (20 g) for 1 min (atmospheric stamping). (b) In case of aq-μCP, excessive biomolecules were removed using ultra-sonication bath (sonication). Then the substrate was stamped in an aqueous condition (aqueous stamping). The shown pattern was printed with 20 g of pressure after 1 min sonication process. Scale bars: 20 μm. (c) Fluorescence profile of the patterned substrate, (d) intensity of printed FITC-PLL patterns versus sonication time, (e) normalized FITC-PLL intensity versus stamping pressure. (mean ± S.D.)

was composed of arrays of lines (width: 2 μm; length: 400 μm) spaced by 50 μm and each line arrays were spaced by 200 and 500 μm in vertical and horizontal directions, respectively (Figure 2a and 2b). The other pattern was complex characters composed of 15 μm-diameter nodes and 3 μm-wide lines. Each character occupied an area of 100-by-200 μm and a set of characters was 400 μm apart (Figure 2c and 2d). As the test patterns had a wide spacing (or gap) compared to the printing area (lines), it was difficult to obtain the desired FITC-PLL patterns (lines and nodes) by dry-μCP. Rather, the large background (off-pattern) regions were also printed due to the roof-collapsing (shown as large green background area in Figure 2a and 2c). In contrast, when the same patterns were printed using aq-μCP, we were able to obtain a high precision and high fidelity FITC-PLL patterns over the large area (inset area: 2.6 × 2 mm) without background printing. The smallest feature size (2 μm) was readily obtained with high precision. This indicated that aq-μCP was capable of eliminating roof-collapse with the same elastic PDMS stamp.

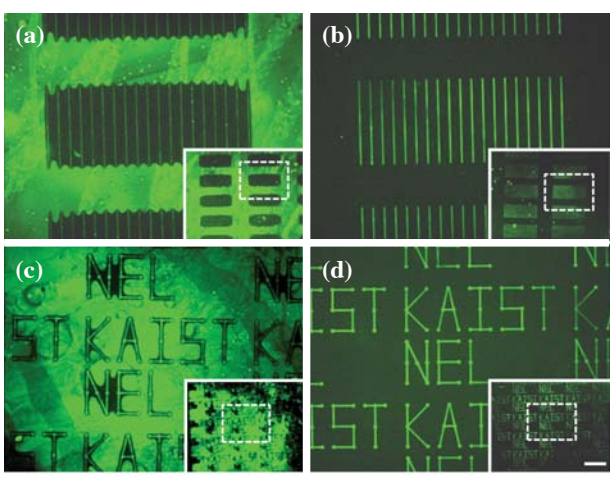

Figure 2. Comparison of dry- vs. aq-μCP of wide-spacing fine patterns of FITC-PLL. (a, b) Thin line array patterns printed by dry-μCP (a) and aq-μCP (b). (c, d) KAIST/NEL patterns printed by dry-μCP (c) and aq-μCP (d). All images are the magnified part of inset images. Each pattern was printed with 20 g of pressure after the sonication for 1 min. Scale bar is 50 μm.

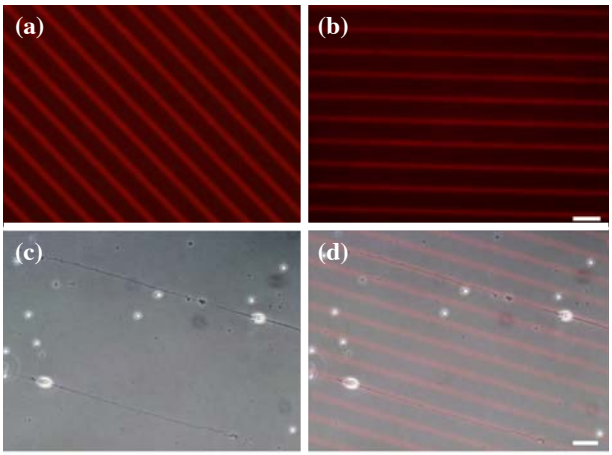

Figure 3. Immunolabeled (a) laminin patterns (line width: 5 μm; spacing: 25 μm) and (b) fibronectin patterns (line width: 3 μm; spacing: 30 μm) printed on glass coverslips. (c) Phase-contrast image of cultured hippocampal neurons on laminin patterns and (d) overlaid with laminin patterns (on-pattern area: red).

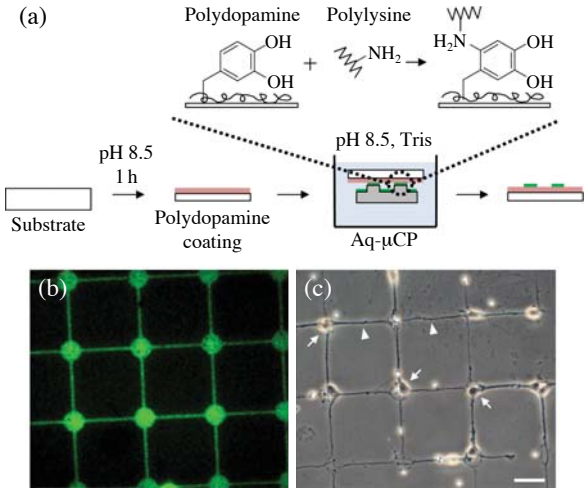

Figure 4. (a) The schematic procedure of covalently linking FITC-PLL patterns to polyDAs. (b) FITC-PLL patterning on polyDA-coated glass coverslips at pH 8.5. (c) Hippocampal neurons cultured for 3 DIV (arrows: cell bodies; arrowheads: neurites). The pattern was printed with 20 g of pressure after the sonication for 1 min. Scale bar is 50 μm.

The result was consistent with the submerged contact printing proposed by Bessueille *et al*. for patterning alkanethiol self-assembled monolayers on gold substrates[24]. Our results confirmed that the same mechanism can be utilized for printing proteins without roof-collapsing, which is important for the design of sparse protein patterns for cell chip designs.

Patterning extracellular matrix proteins

Aq-μCP was capable of patterning active proteins on the glass substrate (Figure 3). We printed laminin patterns composed of 5 μm-wide lines with 25 μm-spacing (Figure 3a), and fibronectin patterns composed of 3 μm-wide lines with 30 μm-spacing, respectively (Figure 3b). The activity of the printed proteins was confirmed by immunoassays and the fluorescent labeling of the antibody indicated that surface proteins were intact after the printing. The fluorescent micrographs also showed the uniformity of the printed proteins. Next, we confirmed the activity of the proteins (laminin) by culturing E18 rat hippocampal neurons on the patterned substrate (Figure 3c and 3d). Neurons only attached on laminin printed areas and a directional neurite outgrowth was observed (Figure 3c). The overlay of fluorescent micrograph and phase-contrast micrograph confirmed that the neurite outgrowth was guided by the underlying laminin patterns. As laminin is known to stimulate integrin receptor on the cell membrane and induces neurite growth, the guided neurite outgrowth validated the biological activity of the printed laminin. These results imply that other biomolecules such as DNA, proteins, and antibodies could be printed in micro-meter scales in a similar manner while maintaining their biological activities.

Surface micro-patterning under pH controlled condition

Next, we showed that aq-μCP could be applied for immobilizing biomolecules on the surface through covalent linking scheme. We chose polydopamine (polyDA) based surface biofunctionalization as polyDA film is known to form a covalent bond with amine groups in proteins and it occurs favorably at pH 8.5[25]. A glass coverslip was coated with polyDA film, and FITC-PLL was printed in Tris buffer (Figure 4a). To ensure the chemical reaction, the stamping time was elongated to 1 hour. Figure 4b shows the printed FITC-PLL patterns on the polyDA-coated substrate. The transfer of inked FITC-PLL to the substrate only occurred at the area that was physically contacting with the substrate. The FITC-PLL on the off-pattern area was not transferred to the substrate despite the elongated printing time. When we repeated the printing process following the conventional dry-μCP, we were not able to obtain any visible FITC-PLL patterns on polyDA film (data not shown). Figure 4c shows hippocampal neurons growing on the printed patterns, which verified the high quality of the printed FITC-PLL patterns. The present demonstration implies that aq-μCP could be applied to fabricate biomolecule patterns that are chemically linked to the chip surface using the vast

amount of linking scheme available in the literature[26].

Conclusions

Previously, Besseuille *et al.*[24] proposed 'submerged microcontact printing' for the purpose of printing alkanethiol patterns on the gold substrate inside water. Submerging a PDMS stamp coated with organic ink molecules (thiols) into inorganic solution (water) prevented the diffusion of ink molecules and also resolved roof-collapsing owing to the incompressibility of water. In our work, the diffusion of biomolecules into the solvent did not seem to occur during the printing process. Even a prolonged 1-hour stamping process did not result in any background printing due to the diffusion process. We reason that biomolecules are tightly bound to the stamp surface coated with SDS and they are only transferred to the substrate in case of physical contact. The ultra-sonication process after the inking has removed unbound or weakly bound biomolecules from the stamp surface.

Here we showed that biomolecules can be contact-printed under aqueous conditions, which overcame some technical and fundamental limitations of conventional μCP. Eliminating roof-collapsing made it easy to print sparse patterns, and versatile surface biofuncationlization schemes can be utilized for immobilizing active proteins on a chip surface. Inked biomolecules that made physical contacts with the surface were selectively transferred to the surface, while background biomolecules did not show any evidence of diffused adsorption onto the substrate. This simple modification of conventional methods without additional resources improved the existing strategy and also would be very useful for generating biological patterns or chemical patterns which require the certain condition to generate patterns or maintain their ability.

Materials and Methods

Aqueous micro-contact printing procedure

PDMS stamps were fabricated by soft-lithography. A silicon master was made of SU-8 2002 (Microchem, MA) and it was treated with trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane (448931, Aldrich, MO) for 45 min in a vacuum. Then a mixture of Sylgard 184 and curing agent was poured and cured for more than 2 hours in the oven (60°C). Cured PDMS stamps were gently released from the mold and cut into 1-by-1 cm of stamps.

Stamps were sequentially washed with acetone, isopropyl alcohol (IPA), and deionized (DI) water for 5 min each in an ultra-sonication bath. After blow-drying the stamp with an air stream, stamps were coated in 10% sodium dodecyl sulfate (SDS, L3771, Sigma, MO) solution for 15 min (5 min at ultra-sonication bath, 10 min at rest), which is reported to enhance inking and stamping of polylysine[16]. After extra molecules of SDS were removed by blowing with an air-gun, biomolecules were loaded on the stamp (inking in Figure 1). We tested various biomolecules such as FITC labeled poly-L-lysine (FITC-PLL, 0.1 mg/mL in DI water, P3069, Sigma, MO), laminin (0.1 mg/mL in PBS, L2020, Sigma, MO), and fibronectin (0.1 mg/mL in PBS, F2518, Sigma, MO) in this study. After 30 min of inking, stamps were gently rinsed once in DI water and then immersed in a solvent of biomolecule solution. Next, the stamp was sonicated for 1 min (sonication in Figure 1b). After the sonication, a substrate was placed on top of the stamp and a uniform pressure was applied with a weight for 1 min (aqueous stamping step in Figure 1b). In case of dry-μCP, the SDS treated stamp was rinsed in DI water once and blow-dried using a compress air. Then, the stamp was placed on the substrate and stamping was processed in an atmospheric condition (atmospheric stamping in Figure 1a). Note that the position of the stamp and substrate (stamp-up and substrate-bottom in Figure 1a) was reversed during aq-μCP (stamp-bottom and substrate-up in Figure 1b) for experimental convenience.

Glass coverslips were used as printing chip substrates. Chips were cleaned with acetone, IPA, and DI water for 5 min in an ultra-sonication bath and blow-dried using a compressed air stream. To coat with polydopamine (polyDA); the chip was immersed in a solution of dopamine (2 mg/mL in 10 mM Tris-HCl, pH 8.5; H8502, Sigma, MO) for at least 3 hours[23]. Then aq-μCP was performed in this solvent (10 mM Tris-HCl, pH 8.5) and stamping time was 1 hour to induce chemical reactions.

Cell culture

Hippocampal neurons from E18, Sprague-Dawley rat (S. D. rat, Koatech, Korea) were cultured on the patterned substrate. Dissected from the brain, hippocampi were dipped into Hank's balanced salt solution (HBSS, LB003-03, WelGENE) and dissociated with a pipette. After centrifuging for 2 min at 1,000 rpm, supernatant were removed and plating medium (Neurobasal medium (21103, Gibco, CA) supplemented with B27 (17504-044, Gibco, CA), 2 mM GlutaMAX (35050, Gibco, CA), 12.5 μM L-glutamate (G8415, Sigma, MO) and penicillin-streptomycin (15140, Gibco, CA)) were added. Cell suspensions were sieved with cell strainer

(352340, BD Falcon, NJ), then plated on the patterned substrates. All procedures were done according to approved animal use protocols of the KAIST Institutional Animal Care and Use Committee (IACUC).

Immunoassay

In order to confirm the activity of proteins after being patterned, we labeled patterned proteins with fluorescence markers that are bound to the antibody of each protein. After aq-μCP, we immediately immersed patterned substrates into PBS for preventing drying. Primary antibodies of each protein (anti-laminin produced in rabbit, L9393, Sigma, MO; anti-fibronectin produced in rabbit, F3648, Sigma, MO) were added to PBS (1 : 500). After 1 hour, samples were washed with PBS about 2-3 times and secondary antibody (Alexa Flour® anti-rabbit, A11012, Invitrogen) was added to each sample (1 : 100). Samples were washed with PBS after 1 hour then fluorescence was measured by a microscope (Olympus IX71).

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