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Nanopatterning of Proteins Using Composite Nanomold and Self-Assembled Polyelectrolyte Multilayers

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Abstract: This paper describes the simple nanopatterning of proteins on polyelectrolyte surfaces using microcontact printing with a nanopatternable, hydrophilic composite nanomold. The composite nanomold was easily fabricated by blending two UV-curable materials composed of Norland Optical Adhesives (NOA) 63 and poly(ethylene glycol) dimethacrylate (PEG-DMA). NOA 63 provided stable nanostructure formation and PEG-DMA induced high wettability of proteins in the nanomold. Using the composite mold and functionalized surface with polyelectrolytes, the fluorescent, isothiocyanate-tagged, bovine serum albumin (FITC-BSA) was successfully patterned with 8 nm height and 500 nm width. To confirm the feasibility of the protein assay on a nanoscale, a glycoprotein-lectin assay was successfully demonstrated as a model system. As expected, the lectins correctly recognized the nano-patterned glycoproteins such as chicken ovalbumin. The simple preparation of composite nanomold and functionalized surface with a universal platform can be applied to various biomolecules such as DNA, proteins, carbohydrates, and other biomolecules on a nanoscale.

Keywords: nanomold, polyelectrolyte multilayer, protein patterning.

Introduction

Precise patterning of biomolecules on desired surface is essential technique in wide range of applications such as biosensor, biochip, lab on a chip, proteomics, and genomics.[1,2] Especially, high-resolution printing of biomolecules with high fidelity is an important step in the nanoscale manufacturing of emerging applications in microfluidics, micro electro mechanical system (MEMS), and biological sensors.[3-5] Current research focuses on printing processes instead of standard photolithography to decrease the costs of nanofabrication and to increase the range of patterning applications.[6] Among several methods, soft lithographic techniques are currently most useful for the fabrication of micro or nanopatterns.[7-11] Especially, contact printing is well known for a simple, fast, efficient, and inexpensive method.[7,8] CP process means contact printing method. First, the stamps are created from a master, which is typically fabricated from wafers patterned by lithography. The stamps are first dipped in the "ink" and then the stamp is brought into contact with the surface to transfer the ink. Poly(dimethylsiloxane) (PDMS) has shown good performance of micropattern because unique properties of PDMS are useful for the formation of high-quality patterns. First, PDMS can provide conformal contact with substrate because of elastomeric property. Second, PDMS is homogeneous, isotropic, and optically transparent down to about 300 nm, which construct optical devices for adaptive optics.[12,13] Third, PDMS is a durable polymer. The same stamp can be used up to about 100 times.[14] Fourth, the surface properties of PDMS can be modified with self-assembled monolayers (SAMs).[15] However, PDMS have detrimental effects in nanometer-scale printing process owing to its mechanical property.[16] First, gravity and adhesion force cause stress on the elastomeric features and cause them to collapse and generate defects in the pattern. High aspect ratio of the features induce the PDMS structures fall down because of their own weight or collapse due to the adhesion forces during the printing process. Second, when the aspect ratios are too low, the structures are not able to withstand the adhesion force between the stamp and the substrate; these interactions result in sagging. Third, the fabrication process of nano-scale structures without distortion is more difficult with a flexible elastomer.

To solve these problems, several methods have been proposed for a harder mold than soft PDMS. Recently, urethane-related acrylate prepolymer is attempted as a new

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mold material.[17] A nanopatterned surface was successfully fabricated and contact printing of hydrophobic molecules was successfully performed with this material.[17,18] However it is difficult to make patterning of biomolecules because biomolecules are hydrophilic, which can induce the wetting problem. Therefore, the fabrication of micro or nanomold with reliable wettability of biomolecules and higher mechanical rigidity is important for the construction of successful nanopatterning. In addition, the substrate transferred biomolecues have to be modified to provide easy and rapid interaction between biomolecules and surface. Covalent binding, physical adsorption, and specific affinity interaction are general approaches to immobilize biomolecules. Although covalent binding is mechanically stable method, it needs for several tedious chemical steps and chemical binding between surface and biomolecules could induce loss of their activity due to the denaturation of three-dimensional conformation and steric hindrance of active sites.[19] Biomolecules can also attach onto surface through a specific affinity interaction. For example, some proteins are fused with affinity tag for the attachment to the surface via their affinity force. Although immobilized biomolecules have high possibility of maintaining in their native conformation and the partner or analytes have easier access to the active sites of biomolecules, the modification of biomolecules with fusion-tag and modification of substrate with appropriate their ligand requires several times and labors.[20] The most simple and straightforward method might be an adsorption through non-covalent interaction containing hydrophobic interaction, electrostatic interaction, and entrapment.[21,22] However, the simple adsorption of biomolecules results in non-uniform orientation, unstable bonding, and releasing problem.

Therefore, there are great needs for novel surface modification technique which provide stable immobilization. This study demonstrates simple approach to modified surface with self-assembly of polyelectrolyte multilayer as a substrate for the stable formation of nanopatterns of proteins. Polyelectrolyte multilayers have an attractive approach for the fabrication of functionalized surface through simple alternative adsorption of oppositely charged polyelectrolyte from aqueous solution.[23] It is a simple and repeatable method but produces uniform and ultra-thin film at a time. Polyelectrolyte multilayer becomes also promising biomaterials in the wide applications because it offers easy control of thickness and it is able to adapt biomaterials without losing their functionality. In addition, nanopatterned proteins are easily immobilized in semi-wet microenvironment by the combination of electrostatic interaction, entrapment of porous structure, and hydrophobic interaction because of the amphiphilic property of polyelectrolytes.

In this study, a novel combining approach of nanopatterning is performed with hydrophilic composite nanomold and functionalized surface by self assembly of polyelectrolyte multilayers. This composite nanomold is obtained by blending Norland Optical Adhesives (NOA 63) and poly(ethylene glycol) dimethacrylate (PEG-DMA). NOA 63 provides stable nanostructure of mold and PEG-DMA induces high wettablity of proteins. Using this composite hydrophilic nanomold, nanopatterning of protein and protein assay were examined via contact printing method.

Experimental

Fabrication of Stamp Having Nanopatterns. The schematic diagram for the fabrication of a nanomold is illustrated in Figure 1. First, PDMS replica was fabricated from the master having nanometer line patterns. The first PDMS replica was a negative feature of the master. Then, the mixture of NOA 63 prepolymer and PEG-DMA precursor at a ratio of 6:4 (w/w) was poured on the PDMS replica and polymerized under UV light (λ=365 nm) for 30 min.[9] Finally, the fabricated nanomold was peeled from the PDMS mold, which resulted in a nanomold with an identical topography with the original master. The analysis of topographies of the master, PDMS, and NOA63/PEG-DMA nanomold was performed with atomic force microscopy (PSIA, XE-150, Korea).

Coating of Polyelectrolyte Multilayer. The glass wafers (1 cm×2 cm) were used for the deposition of the polyelectrolyte multilayers. Polyelectrolyte coating on the glass wafer were easily achieved by simple dipping method. The cleaned glass wafers were first dipped into the solution of 20 mM poly(allylamine hydrochloride) (PAH, *Mw* 70,000) dissolved in water for 30 min and then rinsed 3 times washings with

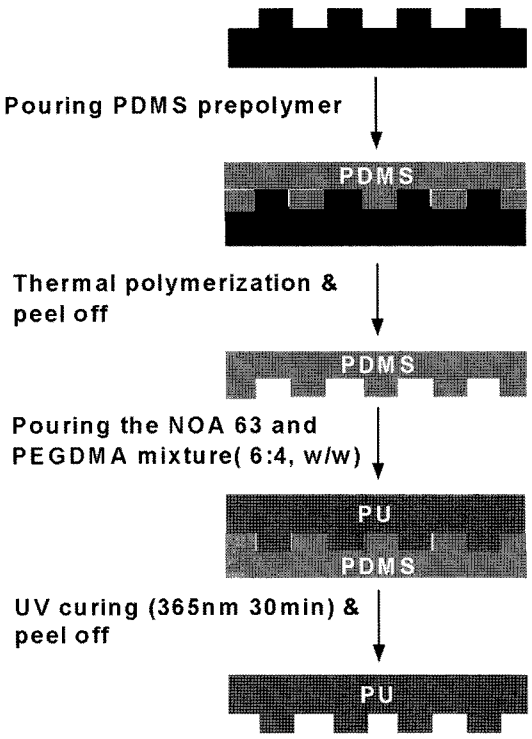

Figure 1. The schematic procedure for fabrication of the nanomold.

distilled water for 90 s. After the first layer deposition, the PAH-coated glass wafers were immersed in the solution of 60 mM poly(4-ammonium styrene sulfonate) (PSS, *Mw* 200,000) dissolved in water for 30 min followed by the same rinse procedure. Again these wafers were dipped in the poly(allylamine hydrochloride) (PAH) solution once again and were rinsed with distilled water for 90 s. Finally, the wafers were dried under nitrogen gas. We deposited 3-layer polyelectrolyte layers were assembled with a positively charged PAH on the outermost layer.

Nanopatterning of Proteins. For the fabrication of nanopatterning of protein, FITC-BSA (1 μg/mL) was dropped on the nanomold for 30 min. After drying step, it was brought into conformal contact with a polyelectrolyte deposited glass wafer for 30 min and then detached. Nanopatterns were analyzed using atomic force microscopy (XE-150, PSIA, Korea) and analyzed with XEP software. The topographic images were obtained in tapping mode (scan size: 10 μm × 10 μm, scan rate: 0.3 Hz). In the case of protein-protein interaction assay, image was obtain with other AFM (Dimension 3100, Veeco, USA) and analyzed with Nanoscope software.

In the case of interaction assay, concanavalin A (Con A) and ovalbumin were investigated for the feasibility of quantitative assay. First, ovalbumin (1 mg/mL) was transferred with contact printing method. Then, 0.1% solution of BSA dissolved in 50 mM tris buffer (pH 7.5) was dropped on the patterned surface for 30 min to block the unpatterned regions. After the washing with 50 mM tris buffer (pH 7.5), 50 μg/mL tetramethylrhodamin conjugated concanavalin A (Rho-ConA) in 50 mM tris buffer (pH 7.0) was dropped on the patterned surface for 30 min. To remove unreacted ConA, the substrate was washed with 50 mM tris buffer (pH 7.0) for three times.

For the study about the relationship between ovalbumin and concanavalin A, we performed interaction assay only about one concentration of ovalbumin (1 mg/mL) using various concentration of concanavalin A from 5 to 100 μg/mL. Fluorescent microscopy images were obtained using an inverted fluorescence microscope (Nikon, TE2000-U, Japan) and image analysis was performed using image analysis software (Media Cybernetics, Image-Pro Plus, USA).

Statistics. The data were expressed as means and standard deviations (SD). Comparisons of means between groups were performed by using the Student's unpaired *t*-test, and *p* values of less than 0.05 were considered significant.

Results and Discussion

The selection of suitable materials capable of preparing nanostructures and retaining high wettability of biomolecules and applicable fabricating methods are prerequisite for the success of nanopatterning of proteins. Here, we pre-

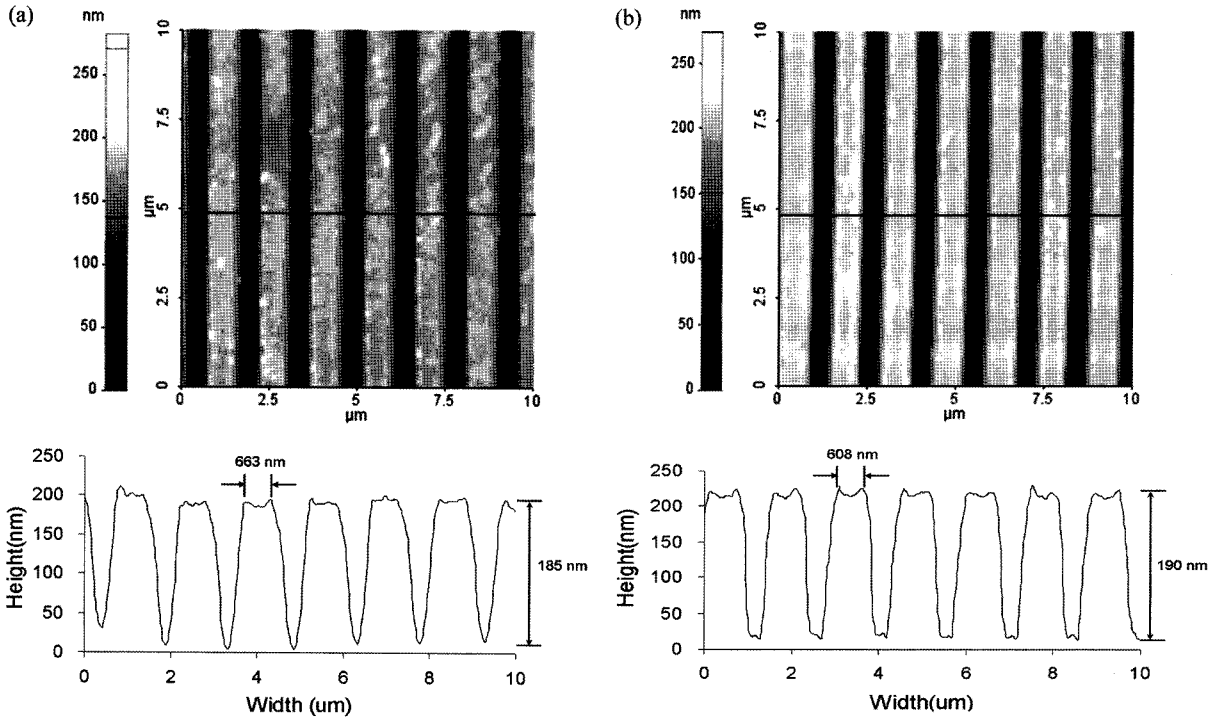

Figure 2. Cross-sections AFM images of the master, PDMS and nanomold. Images in the left-hand sides are planar views, and those in the right-hand sides are cross-sectional views of the line. (a) Master, the length of master line pattern was 663 nm and the height of pattern was 185 nm. (b) Nanomold replicated from the PDMS replica. Nanomold has the 608 nm width and 190 nm pattern. These images indicate that line patterns are successfully replicated from master to nanomold. The scan size was 10 μm × 10 μm.

sented the fabrication method of composite nanomold using soft-lithographic techniques (Figure 1). The patterned nanostructures of the master were exactly replicated in the PDMS mold with width reduction less than 5%. Then, the nanomold including opposite pattern to PDMS were correctly fabricated, which is identical to original nanostructure of master. Figure 2 shows atomic force microscopy (AFM) images of the master, PDMS replica, and composite nanomold (NOA63/PEG-DMA). Although PDMS replica has correct nanostructures replicated from master, the inappropriate mechanical property is limited to application of nanopatterning process owing to deformation, buckling, or collapsing of relief features. Thus, composite nanomold is promising candidate for nanopatterning of biomolecules. In this process, the width of finally fabricated nanomold (608 nm) was smaller than that of original master (663 nm). The narrow patterns were resulted from shrinkage of PDMS or NOA63/PEG-DMA during polymerization. In addition, the capillary filling of prepolymer in the mold wall was faster than at the center because the adhesion force between prepolymer and mold wall is stronger than the cohesive force of prepolymers, which give rise to meniscus. After reaching the bottom of mold, the viscous prepolymer reflowed to fill the entire space of the void. Thus, the obtained height was measured to be 189 nm (PDMS) and 190 nm (NOA63/PEG-DMA), which was well correlated with the height of the original master (185 nm).

The effectiveness of the nanomold and polyelectrolyte multilayer deposited surface for protein patterning is investigated. The sequential deposition of oppositely charged polyelectrolytes by layer-by-layer method is well known for an efficient surface modification. In this study, we demonstrated a simple procedure to modified glass by self-assembly of the polyelectrolyte multilayers as a platform surface for protein patterning with the combination of strong electrostatic interactions, hydrophobic interactions, and entrapment in the porous structure.[24,25] To select optimum thickness of polyelectrolyte multilayers for efficient nanopatterning of proteins, we had investigated the effect of thickness of multilayer for the immobilization efficiency of proteins onto the polyelectrolytes coated surface. However, we had found that there was no strong increase of the efficiency of protein patterning at above 3 layers and the surface roughness were increased with the increase of number of layer (data not shown). This result of surface roughness is well matched with previous report.[26]

Firstly, a positively charged surface at the outmost layer (3 layer) was constructed for the immobilization of FITC-BSA because the net charge of BSA in phosphate buffer (pH 7.5) is negative considering its isoelectric point (pI = 4.7). Figure 3 showed that line patterns of FITC-BSA were successfully transferred onto the PEL coated surface with high regularity and selectivity. Previous studies have found that the transfer of proteins from the stamp to the surface is

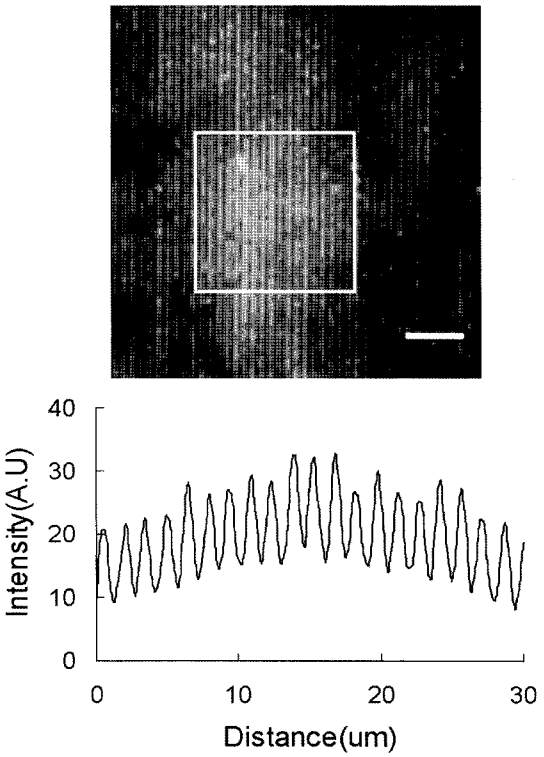

Figure 3. Fluorescence image of FITC-BSA (1 μg/mL) nanopatterns and rectangular profile of the patterns. Scale bar in the image indicates 10 μm.

determined by the reversible work of adhesion of water to the substrate.[27] Our experiment demonstrated that the increase of wettability with water soluble PEL induced the easy printing of protein from the stamp to a surface. Thus, the result of protein pattern confirms that the composite nanomold has advantages not only in the high wettability of polar biomolecules but also in the high mechanical stability in the process of nanopatterning of proteins.

Next, we examined the topographic evolution of the FITC-BSA patterns using AFM (tapping mode in air). The topographical features are well defined on the polyelectrolyte multilayers deposited the glass substrate. The background of the surface between patterns is not perfectly flat because of inherent property of flexible polyelectrolyte multilayers. Considering of the thickness (4 nm) of three layer depositions in this experiment, the average thickness of polyelectrolyte monolayer was calculated about 1.33 nm. The thickness of polyelectrolyte monolayer is well corresponded to the previous reported values of 1~2 nm.[28] And the height of protein patterns was about 8 nm higher than the assembled polyelectrolyte multilayers, which indirectly confirmed the height of FITC-BSA. Although it is well known that the height of BSA monolayer is 4 nm, the experimental result indicates the thickness of patterned BSA are double layers. The top morphology of the transferred protein patterns also indicated little rough and irregular because of the imperfect

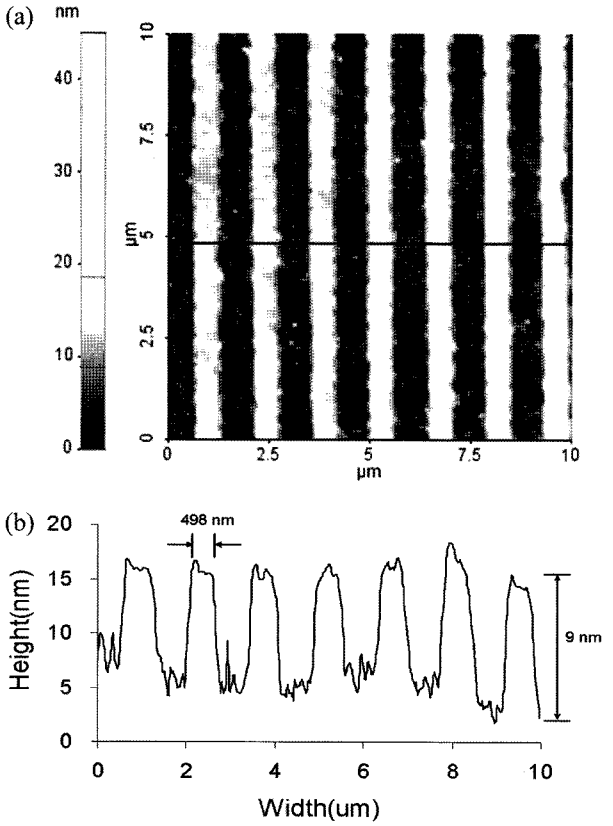

Figure 4. AFM images of the FITC-BSA line patterns. (a) A planar views of FITC-BSA line pattern. (b) A cross-sectional views of the line in (a). The scan size was 10 μm × 10 μm.

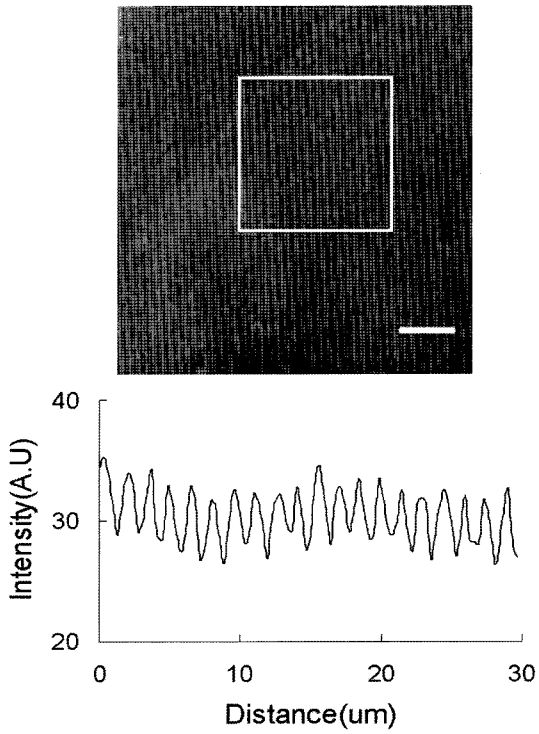

Figure 5. Interaction assay using conjugated concanavalin A (50 μg/mL) and ovalbumin (1 mg/mL). Scale bar indicates 10 μm.

flatness of nanomold feature, diffusion of the FITC-BSA, and inhomogeneous surface coverage of polyelectrolyte on the substrate (Figure 4). Compare to nanomold in Figure 2, the width of transferred protein patterns (500 nm) on PEL surface was narrower than that of the nanomold (The width of nanomold is 600 nm). The difference might be attributed from of the rapid evaporation of protein solution on nanomold. It is well known that cake pattern (center bright while periphery dark) can be formed when printing is conducted when the water begin to evaporate, but not fully dried.[29] For biomacromolecule solutions, it is difficult to achieve thin layer of solution on micro or nanomold because solvent, which is generally water, is not simple to be dried to retain a homogeneous thin layer of liquid on mold. Thus, the small shrinkage of pattern size, especially nanoscale patterns with biomacromolecules, is inevitable phenomena when printing process is used.

It is crucial for protein pattern to have binding ability of target molecules on the chip surface. To confirm protein interaction assay on the surface, ovalbumin-concanavalin A as a model was selected because carbohydrate-protein interactions that involve specific protein-linked oligosaccharides and their complementary binding proteins are known to directly be participated in several important biological processes including the targeting of proteins, mechanism of inflammation, and adhesion of microbes to host cells.[30] Firstly, unlabeled ovalbumin solution was used for protein patterning, and then tetramethylrhodamin conjugated concanavalin A (Rho-ConA) solution were incubated with patterned ovalbumin surface to analyze binding of concanavalin A. Figure 5 shows the fluorescent image of line patterns and indicated concanavalin A could easily recognize the nanopatterned ovalbumins on the surface.

To understand the whole binding processes at nanoscale better, AFM study is used for the analysis of patterned proteins and interacted protein layers (Figure 6). Figure 6(a) is a 10 μm×10 μm AFM image of a 600 nm wide nanomold stamped ovalbumin lines. The initial height of patterned ovalbumin lines was measured about 10 nm, which indicated that the patterned ovalumins are bilayer structure because the height of monolayer of ovalbumin is known for 5 nm.[31] Although mechanism of protein patterning on surfaces is a very complex process, our system can be understood by a number of interactions including electrostatic interaction between positively charged PEL surface and negative ovalbumin, lateral and vertical interactions between adsorbed proteins and free proteins, hydrogen bonding among PEL, proteins, and water, and van der Waals interaction. Ovalbumin not only has a higher affinity for the PEL coated surface than the composite nanomold because of strong electrostatic interaction force but also has lateral and vertical interactions between adsorbed proteins onto PEL

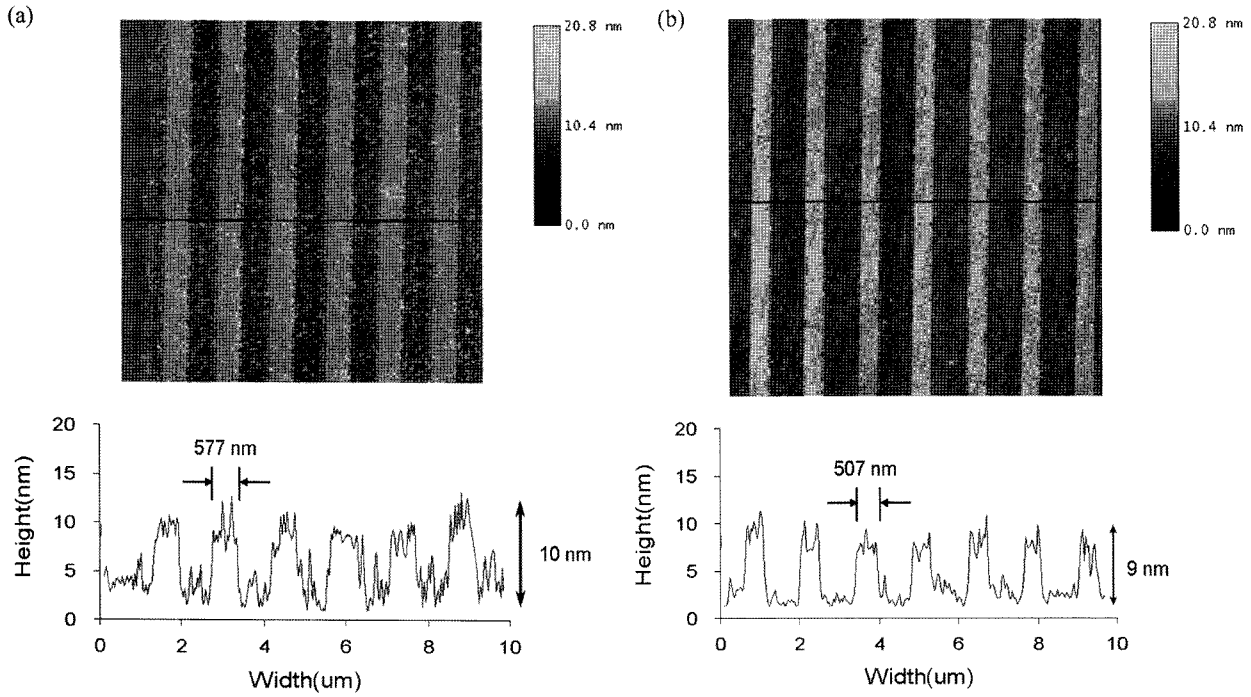

Figure 6. AFM images of the ovalbumin line pattern and interacted concanavalin A. (a) A planar views and cross-section of ovalbumin line pattern. (b) A planar view and cross-section of concanavalin A. These images show that concanavalin A successfully interacts with glycoprotein, ovalbumin. The scan area was 10 μm × 10 μm.

surface and free proteins. Thus, the patterned ovalbumins formed bilayer on the surface. Next, unpatterned regions (free PEL surface) have to be treated with BSA to reduce nonspecific binding because the false signal might be attributed from free PEL surface region. After blocking with BSA, the topology of surface showed flat surface on the contrary of expectation because the height of ovalbumin is theoretically higher (1 nm) than that of BSA, which suggest that the upper ovalbumin could be easily separated from ovalbumin monolayer during the washing step because the washing solution containing surfactant might result in segration of vertically stacked ovalbumins from bilayer of ovalbumin. After incubating the patterned ovalbumin bilayer in concanavalin A solution, the step height of concanavalin A was typically increased to around 9 nm, indicating that the concanavalin A was in a more vertical orientation with respect to previously patterned ovalbumin and bound concanavalin A to ovalbumin is monolayer because the height is consistent with previous study of X-ray crystallography measurement of concanavalin A's dimension (63.1, 87.0, and 89.2 Å).[32]

Finally, we attempted to analyze the relationship of the fluorescence intensity according to concentration of concanavalin A. Figure 7 shows the magnitude of the fluorescence intensity obtained by the analysis of patterned lines per one substrate from three separated batch experiments. The fluorescence signal intensity linearly increases with the increase in the concentration of the ConA up to 50 μg/mL. The limit of detection of this assay was 5 μg/mL, corresponding to 116.3 nmol/L of ovalbumin in considering the start point of linearity. These results indirectly display the sensitivity and effectiveness of this method for quantitative analysis of interesting targets.

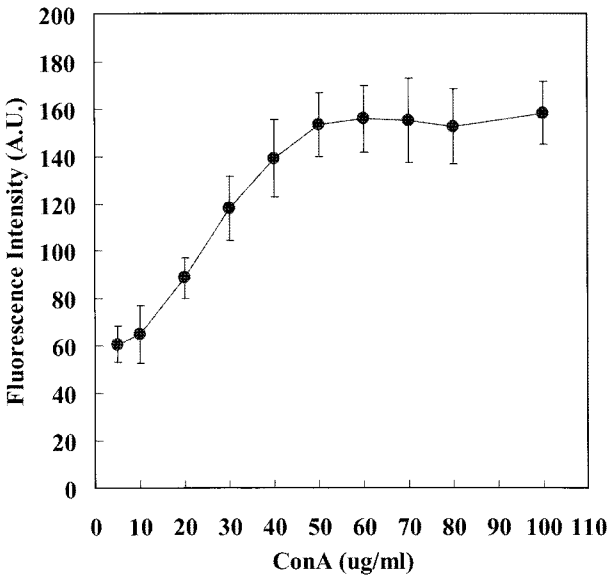

Figure 7. The quantitative analysis with concanavalin A-ovalbumin system; firstly, ovalbumin (1 mg/mL) was patterned onto surface and then free polyelectrolytes was blocked by bovine serum albumin. The patterned protein activity was measured using from 5 to 100 μg/mL concanavalin A solution.

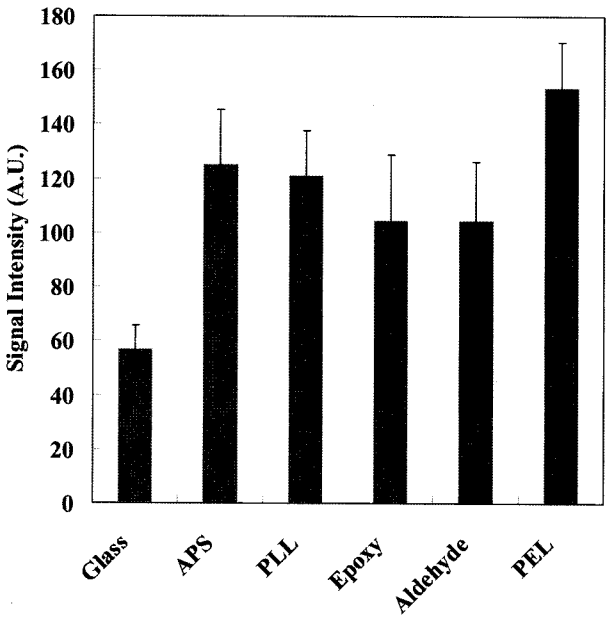

Figure 8. Comparison of the signal intensities of nanopatterns produced on the glass surface modified with various materials; bare glass after cleaning treatment, amine groups, lysine groups, epoxy groups, aldehyde groups, polyelectrolytes. Signal intensity of patterns using PEL modified glass was higher than surface modified with other various materials.

To compare between polyelectrolyte modified surface and other modified surface, glass surface modified with various materials was prepared. Contact printing onto modified glass surfaces was performed with the same concentration of ovalbumin and concanavalin A. Signal intensity was analyzed from the fluorescence images. As shown the Figure 8, Signal intensity of patterns using PEL modified glass was higher than surface modified with other various materials. Although charge interaction could be occurred, poly-L-lysine modified glass surface or amine modified surface was less intense than polyelectrolyte modified surface (poly-L-lysine: 21.09%, amine: 19.57%). The reason for this could be the high-density adsorption of polyelectrolyte. More proteins could be transferred onto the surface because of a lot of charge of polyelectrolyte multilayer which can react with the protein. Surface modification with covalent bonding like as epoxy or aldehyde groups was lower than poly-L-lysine or amine modified surface (epoxy: 16.50%, aldehyde: 13.84%, the intensity of poly-L-lysine modified surface and amine modified surface was the same) and polyelectrolyte modified surface (epoxy: 32.00%, aldehyde: 21.09%) due to problem of protein structure stability. Thus, we can confirm that surface modification by polyelectrolytes could be contributed to the increase of signal intensity.

In summary, this method has several attractive points: (1) By using simple surface modification (alternative dipping in charged polyelectrolyte solution), functionalized surface is easily obtained from aqueous solution. (2) Although this study only performs glass surface, this method can be applied to various substrates including silicon, gold, and polymer. (3) This method can be selectively used to pattern biomolecules or cells without spotting or dispensing machine and have flexibility in pattern shape and size depending on stamp. (4) This method is applicable to quantitative analysis for target molecules by either changing the size of the patterns or the concentration of patterning proteins. Technique can be applied to other systems requiring distinct protein surfaces such as protein biosensors, protein chip, cell chip and lab on a chip. (5) Surface modification by polyelectrolytes could be contributed to the signal intensity inducing. (6) The simple, reliable fabrication method is desirable to mass production.

Conclusions

In this study, we demonstrated a simple and efficient method for nanopatterning of proteins using simple contact printing, functionalized surface with self-assembled polyelectrolytes, and with nanopatternable hydrophilic composite nanomold. The hydrophilic composite nanomold provides stable formation of nanostructure and solves dewetting problem of protein solution in inking step because it consist of two UV-curable materials including rigid Norland Optical Adhesives (NOA) 63 and hydrophilic poly(ethylene glycol) dimethacrylate (PEG-DMA).

Our proposed approach, therefore, can remove a limitation of conventional printing methods for the fabrication of nanopatterns of biomolecules. In addition, the simple approach did not require hazardous pholithography steps, use of toxic solvent, and expensive elegant machine for the formation of protein nanopatterns and will be a valuable platform technology for the study of protein based interactions including protein-protein, protein-small molecules, and protein-cell. In the future, we can apply these techniques to estimate the influence of engineered nanopatterns of proteins on physiological responses of cells.

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