Selective Nanopatterning of Protein via Ion-Induced Focusing and its Application to Metal-Enhanced Fluorescence

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The surface immobilization of protein as a form of micro/nanoarray is very important for fundamental biological studies including proteomics and cell research.\cite{1–3} Minimizing the spot size not only leads to the minimized use of protein, but also maximizes the efficiency of reaction.\cite{4,5} Selective immobilization onto the designated sites is particularly important as well as maintaining high spatial resolution down to the nanoscale to prevent unwanted nonspecific protein interactions.\cite{6–7} Most previous attempts to form protein nanorays were done based on dip-pen nanolithography, which has been demonstrated as able to ensure nanoscale resolution with high selectivity.\cite{8–13} However, dip-pen lithography is inherently a serial process, even though a parallel approach was recently reported.\cite{12} Previously developed parallel methods for patterning proteins include microcontact printing,\cite{14,15} ink jet printing,\cite{16,17} dielectrophoretic deposition,\cite{18} photolithography,\cite{19} and electrospray deposition.\cite{20–24} However, they have difficulty in providing nanoscale resolution over large areas. Recently, an interesting study of microcontact printing was reported to produce submicrometer-scale virus arrays.\cite{25}

Here, we report the parallel generation of protein nanorays with 50–130 nm features ensuring high selectivity, as well as microarrays, by utilizing the ion-induced focusing concept.\cite{26,27} Moreover, we demonstrate that protein nanoparticles can be precisely guided and selectively deposited onto the deep bottom surface within microchannels that may be used as a platform for novel microfluidic devices for fundamental biological studies such as the guided growth of cells.\cite{28} The protein activity after deposition is confirmed to be preserved. To demonstrate the viability of the present approach, a new design of metal-enhanced fluorescence (MEF) substrate was prepared by sequentially patterning silver nanoparticles and then fluorescence-tagged protein nanoparticles precisely on the top of silver nanoparticle pattern, without nonspecific adsorption.

To form micro/nano protein arrays in a parallel way, we first generate charged protein aerosols and the same-polarity ions as the particles via the electrospraying of protein solution, which is then injected into an electrostatic precipitator chamber under a given electric field where a SiO$_2$ prepatterned silicone substrate is located. Figure 1 illustrates the experimental set-up for producing micro/nano protein arrays utilizing both electrospraying of the protein solution and the concept of an ion-induced electrostatic lens. In injecting conductive protein suspension into the needle and putting it under a high voltage, a Taylor cone jet is formed where the equilibrium between the electric-field-induced force and the surface tension is established. Through the cone jet, charged droplets are generated and sprayed.\cite{29} The droplet includes protein particles that were originally dispersed in the solvent and eventually evaporates, generating charged protein nanoparticles and ions having mostly the same polarity. Even though positive electrospray could generate negative droplets, their fraction is an order of magnitude less compared to positive droplets.\cite{30} Since negative potential is applied to the substrate, only positive droplets are attracted to the substrate. While ions are first deposited on both the conducting (Si) and nonconducting (SiO$_2$) surfaces due to higher mobility of ions before the arrival of charged protein nanoparticles at the substrate, the ions deposited on the conducting Si surface are immediately neutralized, leaving ion charges on the SiO$_2$ surface, which generates ion-induced nanoscopic electrostatic lenses. Through these lenses, the charged protein particles are guided to be convergently deposited only within the centre region on the open Si surface. In this way, the feature size can be significantly reduced and nanoscale protein arrays with selectivity can be realized within submicrometer patterns that can be readily fabricated by conventional photolithography. Previously, the particle focusing phenomenon was observed after a sufficient amount of charged particles were deposited on a Tellon mask\cite{31} or a photosensitive prepatterned substrate.\cite{32,33} This approach could guide charged
protein nanoparticles into the specific desired locus to avoid unwanted nonspecific adsorption.

We show that human IgG can be patterned using this method (see Figure 2) where protein particles are convergently deposited only in the centre regions (feature sizes approximately 500 nm in width and 400 nm in height) within 2 μm circular and line patterns of SiO₂. This implies that the electrostatic lens developed by ion deposition reduces the feature size by approximately 4 times. It also confirms that the selective immobilization of protein onto the desired locus is possible. To verify the protein activity after deposition, the protein arrays were reacted with dilute Alexa Fluor 488-tagged anti-human IgG solution (diluted with phosphate buffered saline (PBS), where the remnant anti-human IgG was later washed away using PBS and Tween-20 (PBST) solution). Figure 2 shows the fluorescence image of Alexa 488 through confocal laser scanning microscope (CLSM), which confirms the reaction of anti-human IgG and the deposited human IgG (a detailed procedure for fluorescent immunostaining is described in the Supporting Information (SI)).

The focused deposition of protein has also been done within submicrometer-scale windows formed by 200 and 500 nm square photoresist (poly(methyl methacrylate), PMMA) patterns, as shown in Figure 3a,b. Due to the focusing effect mentioned, the feature size of protein patterns has been reduced to approximately 50 nm within 200 nm rectangular patterns and 130 nm within 500 nm patterns, which demonstrates that protein nanoarrays can be successfully made in a parallel fashion. A similar result was also obtained using protein G (SI, Figure S1). We also attempted to guide protein nanoparticles into the specific desired locus to avoid unwanted nonspecific adsorption.
into a deep trench microchannel structure having various aspect ratios (height/width). Figure 3c,d shows focused collagen patterning on the bottom surfaces within two microchannels of different aspect ratios. Figure 3c shows a cross-sectional view of deposition within a 1 μm-deep and 2 μm-wide trench. Figure 3d is that of a 4 μm-deep trench. In all cases, protein particles are deposited only in the centre region on the bottom surface. It is interesting to see the microtip structure of the proteins that have grown due to this focusing effect (see Figure 2c,d). Simulations of protein particle trajectories and deposition agree with experimental results. This may provide a possibility of engineering 3D micro/nanostructures consisting of protein nanoparticles.[34] Other cases with different aspect ratios can be found in the SI, Figure S2.

We simulated the deposition process of charged protein nanoparticles by calculating Lagrangian particle trajectories.[35] This has been done by solving the following Langevin equation for charged particle motion including the fluid drag force \( F_D \), the Brownian force \( F_B \), due to the random bombardment of surrounding gas molecules, the Coulomb force \( F_C \) and a van der Waals force \( F_{vdW} \).

\[
\frac{d \mathbf{v}}{dt} = F_D + F_B + F_C + F_{vdW}
\]

In the simulation, we used a representative size (26 nm) and a charge (108 elementary charges) of collagen protein particles which were determined from size and charge distribution measurements[36,37] using a condensation nuclei counter (CNC, TSI 3022A) and transmission electron microscopy. The surface charge density on the SiO2 was assumed to be saturated and calculated to be about \( 5.4 \times 10^{-5} \) C m\(^{-2} \). After solving the Poisson equation to obtain the electric fields using commercial finite element code (COMSOL 3.2), the trajectories of particles were obtained by solving the above Langevin equation (details for the simulation of collagen particle deposition are described in the SI). SI, Figure S2e shows the trajectories of collagen particles. The particles initially move downward nearly straight lines and are deflected near SiO2 pattern surface following the converging field lines and finally deposit in the centre region. SI, Figure S2f,g show the deposition profile (top and cross-sectional view, respectively) confirming the feature size reduction. SI, Figure S2g shows calculated deposited structure that looks like a Gaussian function shape, which is in agreement with experimental results.

To show the viability of our approach, we prepared a new design of MEF substrate. Conventional MEF substrates are prepared by dipping the metal-patterned substrate into a protein solution, then washing away proteins adsorbed on the surface except the metal-pattern portions. This may require complicated surface modification steps.[38,39] A complete removal of protein on the areas except the metal patterns may pose a problem. Our approach could guide different nanoparticles onto the same desired locus with nanoscale resolution. We first deposited silver nanoparticles generated by a spark discharge method[40] into a triangular SiO2 pattern (see SI Figure S3) via our approach. Then, on top of the silver nanoparticle patterns, cysteine and human IgG-FITC (fluorescein isothiocyanate) generated by electrospraying are selectively deposited using the same principle. In this way, we eliminated the problem of unwanted nonspecific adsorption of proteins. For comparison, we also prepared a sample having patterns of only human IgG-FITC without silver nanoparticle patterns. Figure 4 shows that fluorescent signals of the present design (human IgG-FITC on the top of silver nanoparticle) have been significantly improved compared to the no-silver case. Since reactants are precisely delivered to the desired locus, efficient use of each reactant is possible and background noise can be minimized.

In summary, we report the selective patterning of protein nanoparticles to designated loci on the substrate with nanoscale resolution utilizing both electrospraying of a protein solution and the ion-induced focusing principle. We have demonstrated the nanoarrays of proteins with a 50 nm feature size within 200 nm SiO2 square patterns and a 130 nm feature size within 500 nm square patterns. We also successfully guided proteins into the deep bottom surface of high-aspect-ratio microchannels. The immobilization and the activity of protein were examined. Protein particle trajectory calculations support our experimental data. We fabricated MEF substrate having selective deposition of protein on the top of silver nanoparticle patterns and showed much enhanced fluorescence signals compared to the case with protein only. The simplicity, parallel nature and nanoscale resolution for selective patterns onto the desired location can be advantageous for biological and chemical analysis using protein micro/nanoarrays, cellular engineering, and related research.[28]
Preparation of Prepattern: SiO$_2$ micropatterning on p-type Si wafer was done by chemical vapor deposition (CVD) using Tetraethyl orthosilicate (TEOS). Photoresist AZ1512 was spin-coated and the pattern was transferred using typical photolithography. For the preparation of submicrometer patterns, PMMA solution is spin-coated to 130 nm-thick layer and pattern was formed using e-beam lithography. The detailed procedure is described in the SI.

Electrospray Deposition of IgG and Protein G: All chemicals were purchased from Sigma except anti-IgG. Proteins are filtered using syringe filter (purchased from Corning) to separate agglomerated particles prior to use. IgG (from human serum, Sigma) was diluted with distilled water to 1 mg mL$^{-1}$. Flow rate of syringe pump when supplying antibody into the electrospray needle was 20 μL h$^{-1}$. The voltage applied to the needle was +5 kV and −300 V was applied to the substrate. Between the needle and the substrate, the grounded copper plate with a hole of 5 mm is located. The carrier gas of generated protein particle was carbon dioxide (CO$_2$) and its flow rate was 1 lpm. The deposition time was 3 min. Protein G (G4689, Sigma) was diluted to 1 μg mL$^{-1}$ with distilled water and patterned to ZEP 520A (e-beam resist, Zeon Corp.) nanopattern with the same condition as done for IgG nanopatterning.

Collagen Electrospray Deposition: Type-III collagen (from human placenta, Sigma) was diluted to 0.2 mg mL$^{-1}$. Flow rate of syringe pump to supply collagen into the electrospray needle was 20 μL h$^{-1}$. About +4 kV was applied voltage to the needle and −600 V was applied to the substrate. The carrier gas was CO$_2$ and its flow rate was 1 lpm as well. The deposition time was 30 min.

Metal-Enhanced Fluorescence of Silver Nanoparticle and IgG-FITC: N$_2$ ions are injected to substrate for 20 min using a corona charger. N$_2$ gas was fed to the charger at 4 lpm. Silver nanoparticles are generated by spark discharge method. N$_2$ carrier gas was supplied to the chamber with the flowrate of 4 lpm and the deposition time was 5 min. The thickness of SiO$_2$ layer was 1 μm. 10$^{-3}$ M cystein (Aldrich, 168149) solution was electrosprayed for 15 min and the substrate was incubated overnight. Human IgG-FITC (Sigma, F9636) solution (0.2 mg mL$^{-1}$) was electrosprayed for 10 min and the substrate was incubated at 0 °C for 4 h. Fluorescence images were taken using a confocal microscope (Carl Zeiss LSM510).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.
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