The Large Scale Fabrication of Protein Nanoarrays Based On Nanosphere Lithography

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ABSTRACT

Using 300 nm nanosphere monolayer mask on COOH terminated silane surface as mask, we fabricate a chemical pattern which has polyethylene glycol (PEG) terminated surface patterned with carboxylic terminated holes. Lysozyme selectively absorbs on the COOH terminated holes forming a hexagonal packed lysozyme monolayer array. The patterned protein islands cover the whole 1x1 cm² wafer and have a ring shape, with ring diameter of 118nm and ring width of 50nm. This new method offers a fast and economical way to fabricate protein arrays over large areas with feature sizes comparable to scanning probe based techniques.

Keywords: protein array, nanosphere lithography, protein pattern, silane; AFM

Protein microarray has been widely used in biotech research. Advancing the protein arrays into nanoscale is an important step towards next generation biological and medical applications. [1] Since biological processes occur on the nanoscale, miniaturization of protein arrays provides not only a higher array density, but also provides a novel approach for monitoring biological process, such as single molecule detection, for tissue engineering, and for the fabrication of nano-mechanical devices such as nano-propeller, nano-linear motor. [2,3] Compared with uniform protein films or protein microarrays, nanoarrays of proteins provide a much greater exposed edge area. Since the protein molecules in these edge areas are highly exposed, i.e., not blocked by other proteins, they have higher bioactivity, which is an important factor in improving the signal quality of biosensors. Also, the nanometer sized discrete protein islands offer a nice scaffold for incorporating other nano-gadgets in fabricating complex nano-biofunctional device.

In protein patterning, the use of traditional patterning methods such as high temperature, organic solutions, strong acid/base is quite limited. These factors make the resolution of protein patterning usually much lower than the traditional patterning. Currently, there are two approaches in fabricating protein patterns on the surface: The scanning probe microscope (SPM) based high-resolution techniques and the photolithography/soft lithography based high throughput techniques. Dip-pen nanolithography (DPN), [2-5] nanografting [5-8] and conducting tip AFM writing [9,10] are emerging scanning probe based lithographies capable of creating protein patterns with ~100 nm resolution over small surface regions. High throughput patterning techniques, such as photolithography [11], micro-contact printing, [12,13] and laser lithography [14] are used to pattern protein over large area, with a feature resolution around 1-2 micron.

Here, we demonstrate a nanosphere based method to direct the assembly of proteins into an array of 120 nm diameter islands over large area. The self-assembly of a monolayer of polystyrene nanospheres on a solid surface provides a simple and effective means of creating a hexagonally patterned lithographic mask for further surface processing. The geometry of the spheres restricts deposition processes and chemical modification directly beneath the nanospheres. Nanosphere lithographic patterns have been employed to create metal hexagonally packed nano-clusters. In the present study, we combine nanosphere lithography and silane chemistry [15-17] to chemically pattern macroscopic surface regions with ~100nm scale features over cm² sample sizes. We show that the protein lysozyme selectively adsorbs on the carboxylic acid terminated 120nm islands, each containing about 640 protein molecules, and not on the polyethylene glycol (PEG) interstitial regions.

We have chosen to use lysozyme because it is readily available and its structure and properties are well known. With an isoelectric point of 11, lysozyme is positively charged, it will selectively absorb on the negatively charged carboxylic acid terminated regions in a pH 7 buffer solution. We use polyethylene glycol silane (PEG-silane) to passivate the unpatterned surface to resist the protein non-specific adsorption in these regions. [10,18]

Protein pattern fabrication process is illustrated in Figure 1. Briefly, a monolayer of 300 nm diameter nanosphere mask was formed on pre-fabricated COOH terminated silane surface. The PEG-silane grows between the nanospheres and forms a second layer that resists protein adsorption. After removing the mask, the surface is a chemically patterned surface with hexagonally packed carboxylic terminated holes. Figure 2 shows the AFM friction image of this surface. The protein is deposited onto the surface next. In pH=7 HEPES buffer, lysozyme has a positive charge, which drives it to selectively absorb on the negatively charged carboxylic terminated holes, and not absorb on the PEG-silane surface.

The AFM image in Figure 3 shows the existence of hexagonally packed lysozyme islands with a 300nm spacing. In the friction image 3b, the protein islands also appear as dark pits (high friction) that look similar to the template before protein adsorption. This is not surprising since the lysozyme is a water soluble protein and also has a hydrophilic surface. However, in the topography image 3a, the surface evolves from the surface with negative pits

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Two control experiments were also carried out. In the first, a buffer solution without lysozyme was applied onto the chemically patterned surface. AFM scans showed no topography change from the PEG-silane/UTS$_{ox}$ template. In the second control experiment, after lysozyme adsorption the patterned surface was treated with trypsin. The trypsin is known to specifically cut the peptide bond after Arginine and Lysine, both of which are abundant in lysozyme. Hence the lysozyme would be cleaved into many small pieces. (Figure 1g) After this treatment, the AFM image (Figure 4) shows that the protrusion pattern shown in Figure 3 has vanished. These controls prove that the observed hexagonal packed array after the lysozyme solution deposition is indeed a lysozyme array.

The AFM image in Figure 3 also reveals additional features of the protein array. Each lysozyme island is actually a protein ring since no protein adsorbs in the central region. Here the friction image (Figure 3b) shows that in the island center the friction is low, which indicates a hydrophobic region. We believe that this central region may result from residual polystyrene which is not removed with the polystyrene nanosphere during the mask lift-off. Such remnant is common in nanosphere lithography and has been reported elsewhere. [19]

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**Figure 2** Friction image (8x8 µm) of a UTS$_{ox}$ and PEG-silane patterned surface. Darker contrast corresponds to higher friction force. The hexagonal order is clearly observed. The hydrophilic UTS$_{ox}$ terminus has higher friction when imaged by a hydrophilic tip and appears as dark holes in the image.

**Figure 3** Lysozyme pattern on the surface in (a) topographical and (b) friction modes. The island-island distance is 300nm. The island is ring shaped with an outer ring diameter of 118±6 nm. The ring width is about 50nm. The inset in (a) shows the Fourier transform of the image in (a). In (b) the darker color corresponds to higher friction force. The higher friction of the protein islands shows the protein surface is hydrophilic. The inset shows the height profile corresponding to the blue line in (a).

and the width of the ring is about 50nm. The overall size of the protein islands (118nm) is comparable to the pattern written by the SPM techniques.[2-10] The line profile across the rings (Fig. 3b inset) shows the height of the island is 1.0-1.5 nm higher than the surrounding PEG-silane. Since the PEG-silane film has a height of 1.5-1.7 nm, which is based on the X-ray reflectivity data, [20] we infer that the height of the protein immobilized inside the UTS$_{ox}$ hole is about 3nm. This height is comparable to the dimension of lysozyme (5x3x3 nm). We can not exclude weakly adsorbed protein layer(s) floating on the first adsorbed protein layer. These layers might be removed by the AFM tip during scanning, thus invisible to the AFM. Although we cannot
rule out this possibility, from AFM derived thickness, we believe that the stable absorbed protein is a monolayer. From the measured height of 3nm, we assuming that the lysozyme is oriented with the long axis parallel to the surface. Then each protein will cover an area $3 \times 5 = 15\text{nm}^2$. Thus, each protein island incorporates about 640 lysozyme molecules, of which 16% are at the edge of the islands.

![Image](image.png)

**Figure 4** The surface after treated with trypsin. The hexagonal packed islands disappear, leaving a featureless surface. $7 \times 7\text{micron}$, $Z$ scale: $13.8\text{Å}$.

In summary, by incorporating nanosphere lithography and surface silane chemistry, we have developed a method to fabricate a hexagonal array of nanoscale protein rings over $\text{cm}^2$ regions with the feature resolution close to the SPM based protein patterning techniques. This method could be readily adopted to potentially smaller spacing and offers an efficient and economic alternative for protein patterning.

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**REFERENCES**