Direct Confinement of Individual Viruses within Polyethylene Glycol (PEG) Nanowells

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ABSTRACT

Individual M13 viruses were spatially confined within wells fabricated from nanomolding of a PEG-based random copolymer. The viruses were selectively adhered to the region pretreated with an antibody against the virus, resulting in individual virus arrays. The polymer surface was found to be highly resistant to the attachment of the virus ($\sim 0.02 \ \mu m^{-2}$), approximately 2 orders of magnitude lower than that on a bare silicon surface. The physical height of the template provided an additional barrier to the attachment of the virus due to entropic penalty in bending of a semi-flexible M13 virus. The effects of pattern size and barrier height were investigated, revealing that a certain critical height is needed to ensure successful confinement within the template for a given pattern size.

Recently, biological nanostructures have attracted much attention because of their unique molecular recognition, leading to self-assembly and templating of atomic and molecular structures.^{1–5} Biologically inspired materials offer many advantages over conventional nanoprocessing methods such as top-down approaches (e.g., photolithography) and chemically oriented synthetic techniques. In particular, genetically engineered M13 bacteriophages were proposed by Belcher and co-workers as promising building blocks for constructing well-ordered nanostructures.^{6,7} It was shown that these engineered viruses could recognize specific semiconductor surfaces through the method of selection by combinatorial phage display.⁶ More recently, a highly ordered two-dimensional monolayer structure of viruses has been achieved via alternating electrostatic assembly.⁸

Virus microarrays may provide a potentially powerful biosensing platform. One such example is the fabrication of viral arrays using scanning probe nanolithography (SPN) in combination with chemical templates for protein-to-surface interactions.⁹ The strategy used in the assembly is to introduce a chemoselective linker on the virus surface to enable the attachment onto a patterned template created by SPN. However, SPN patterning is expensive and difficult to adapt for large-scale patterns. Another example is a hybridization-based approach using a genetic modification of the virus coat protein, hybridization with nucleic acid, and subsequent attachment to electropatterned chitosan-coated silicon chips.¹⁰ Hybridization-based methods are highly specific and reliable but often require cumbersome multistep chemistries.

The motivation of this study thus arises from two aspects. First, the use of soft lithographic methods with regard to direct patterning of biological species has been limited to micrometer-length scale.¹¹ With growing interest in nanoscale entities such as proteins, viruses, and DNA,^{1,12} a soft lithographic technique for patterning submicrometer or nanometer features would be of great benefit. For example, the technique could be an alternative to SPN for fabricating nanoscale biomolecular arrays or biosensors. Second, spatially patterned M13 viruses can be used as components for

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Figure 1. Schematic illustration of the experimental procedure. Prior to virus seeding, the patterned surface was treated with the P3 antibody to promote adhesion of the virus.

miniaturized electronic devices or biosensors.¹³ To address these challenges, we have demonstrated the feasibility of patterning arrays of single M13 viruses using a simple soft lithographic approach.

Construction of PEG Copolymer Nanowells and Patterned Arrays of P3 Antibody Specifically Interacting with M13 Virus. To fabricate submicron PEG patterns, we used a simple nanomolding method (Figure 1).^{14,15} In this technique, a patterned elastomeric mold such as poly-(dimethylsiloxane) (PDMS) is placed on a spin-coated polymer film and then the temperature is raised above the polymer's glass transition temperature (temperature-assisted capillarity).¹⁴ Alternatively, a patterned PDMS mold can be placed directly on a wet, spin-coated polymer before solvent evaporation (solvent-assisted capillarity).¹⁵ Because the PEG copolymer does not soften with temperature, the latter scheme was used throughout the experiment. In solventassisted capillarity, the feature height can be controlled relatively easily depending on the geometry of PDMS stamp and initial film thickness. Moreover, the resulting structures are intrinsically dense and robust with fewer defects. A feature of this technique is that the substrate surface can be completely exposed, which is a prerequisite to enabling successful patterning of biological species. Recently, we found that the surface can be exposed easily with PEG-based polymers by means of precise control over capillarity and dewetting.¹⁶ In comparison to hydrophobic polymers such as polystyrene and polymethyl methacrylate, the PEG polymers were neatly patterned upon conformal contact with a patterned PDMS stamp, resulting in a negative replica without dewetting.



Height image



Figure 2. AFM images of 700 nm PEG nanowells in (a) height and (b) deflection modes. Scan area was $15 \times 15 \,\mu\text{m}^2$. The inset shows the corresponding fluorescent image treated with the P3 antibody and a FITC-labeled secondary antibody.

Figure 2a and b represents atomic force microscopy (AFM) images of PEG templates in height (a) and deflection (b) modes, respectively. For the PEG copolymer, a PEG-based random copolymer, poly(3-trimethoxysilyl)propyl methacrylate-r-poly(ethylene glycol) methyl ether) [poly(TMSMAr-PEGMA)] was used. This polymer can generate features varying in height from a few nanometers to a few hundred nanometers.¹⁷ Furthermore, the PEG copolymer has an anchoring group and thus can bind covalently with silicon surfaces, allowing for water stability for at least two weeks.¹⁷ In the figure, 700 nm nanowells were fabricated on silicon wafer using PDMS stamps having protrusions with 1 μ m height (positive stamp). The initial film thickness was \sim 570 nm after evaporation of the solvent. During the molding process, the PEG copolymer was repelled from the contact area until the substrate surface became exposed, as shown clearly in the deflection image. Also, a considerable amount



Figure 3. (a) SEM image showing the M13 viruses that were cast from a solution onto a silicon wafer. The width of the virus was increased from ~ 6 nm to ~ 10 nm because of the gold coating (inset). (b-c) SEM images showing the transition of virus morphology on the P3 and P9 antibody treated silicon wafer, respectively.

of liquid was squeezed outside of the stamped area such that the final thickness was reduced to ~ 280 nm.

The incubation of an antibody onto the PEG template resulted in a selective adsorption of the antibody onto the exposed substrate because the PEG copolymer surface is highly resistant to protein adhesion.^{18,19} The immobilized antibody acts as an adhesion site for subsequent virus attachment. Two antibodies specifically interacting with binding domains of the virus were tested in this study (P3 and P9 antibody). The major coat protein of M13 virus is the product of phage gene 8 (g8p) and there are 2700–3000 copies of this protein per virus particle, together with approximately 5 copies per each of four minor capsid proteins, g3p, g6p, g7p, and g9p, which are located at the ends of the filamentous particle. In our experiments, the P9 antibody specifically interacts with g3p at one end while the P3 antibody recognizes g8p, the coat proteins on M13 virus.

It was found that the presence of the P3 antibody increased the adhesion of M13 virus significantly compared to the P9 antibody. As shown in the inset of Figure 2b, a P3 antibody that has been treated with a FITC-labeled secondary antibody can be selectively adsorbed within the exposed substrate of the nanowells.

Patterned Arrays of Individual M13 Virus. Using the P3 antibody as an anchoring agent, the M13 viruses were subsequently seeded onto the pattern. It turned out that the polymer surface was highly resistant to the attachment of the virus so that the average population was less than ~0.02 μ m⁻² on a bare PEG surface. This density is approximately 2 orders of magnitude lower than that on a bare silicon wafer. Figure 3a shows a scanning electron microscopy (SEM) image of the attached viruses on a bare silicon surface. According to previous work, the genetically engineered M13

virus was monodisperse in size and shape, having a filamentous shape (~880 nm in length and 6.6 nm in diameter).^{6,7} An enlarged view of a single virus in the inset indicates that the diameter was increased slightly to ~10 nm because of the gold coating. When cast from a solution onto a silicon surface (Figure 3a), the viruses had various sizes, ranging from 400 nm to 3 μ m. Genetically, the length of a single virus particle should be 880 nm, suggesting that some particles were broken or self-assembled to form aggregates. Also, the virus is known to be fairly stiff or semi-flexible²⁰ such that it has a rodlike shape. For aggregates, the viruses showed a curved shape or a loop (not shown).

When the silicon surface was treated with the P3 or P9 antibody, the number density was increased significantly by approximately 1 order of magnitude for the P3 antibody but not changed for the P9 antibody. Representative images of a single virus adhered to the silicon surface were shown in Figure 3b and c. Because the P3 antibody interacts with g8p along the long edges of the virus, the virus was adhered horizontally (b), whereas the virus on the P9 antibody-treated surface was adhered vertically at one end (c). This can explain the reduced ability of the P9 antibody to bind M13 because there are fewer accessible binding sites being present on an end, whereas there are abundant binding sites on the side surface.

After constructing a PEG template followed by treatment with the P3 antibody, the sample was incubated in a virus solution, washed with deionized water, and dried for SEM analysis. A typical SEM image of an individual virus array within 1 μ m circular wells is shown in Figure 4 along with four insets indicating a magnified view at different locations. Although not shown, the other wells were also occupied with an individual virus (coverage was ~70% on the whole



Figure 4. SEM image of an individual virus array on six wells. Four insets show the captured single virus at different locations. The arrows indicate the location of the virus.

surface). Because the well diameter was larger than the average length of the virus (~880 nm), a rodlike virus less than ~1 μ m in size was seen only within the wells. Viruses larger than 1 μ m were generally restricted to adhere to the surface.

Interestingly, a critical physical height existed between confinement and nonconfinement for a given pattern size. Examples are shown in Figure 5a–d where various heights of PEG templates were tested to examine the effects of barrier height. For line patterns (Figure 5a and b), the viruses were neatly confined with 800 nm lines for the higher barrier (260 nm) while some of the viruses (not all) exceeded the boundary of the well for the lower barrier (51 nm). Similarly, the virus must bend itself or form a loop to be entrapped within a circular well smaller than the length of the virus. Such examples are shown in Figure 5c and d using (c) 1 μ m and (d) 500 nm circular wells. As seen from the figure, the virus was even confined within the 500 nm well with a bending, which is quite intriguing considering the filamentous nature of M13 virus and an accompanying entropic penalty.²⁰

To model the patterning behavior of viruses within nanowells, we summarized the effects of pattern size and barrier height in Figure 5e. We defined two regions in the scheme, that is, Region I and Region II. When the pattern size is larger than or comparable to the length of the virus, it is expected that the virus could be confined easily without bending even with a relatively low height (Region I). However, as the pattern size gets smaller than the length of the virus, the virus must bend itself or form a loop to minimize the energy cost in contact with the polymer surface. This confinement, in turn, generates an entropic penalty from the bending so that there would be a competition between these two contributions (Region II). As expected, the critical barrier height that is required for the successful confinement gradually increased in region I. By contrast, a sharp increase in slope was observed in region II, suggesting that the barrier should be sufficiently high to suppress the entropic penalty for the size less than 800 nm.

In summary, we have demonstrated that individual M13 viruses can be confined using PEG copolymer nanowells aided by repulsive interactions of the virus with PEG surfaces and enhanced physical height. Distinct confinement and nonconfinement regions were observed depending on the pattern size and physical height, suggesting that a high physical barrier is needed to compensate for an entropic penalty generated from the bending of the virus. Recently, a similar direct patterning method was developed using a different template material and patterning technique,²¹ suggesting that the current approach would find uses in virus patterning and its applications.

Experimental Section. *Materials.* A poly(ethylene glycol) (PEG)-based random copolymer, poly(3-trimethoxysilyl)-propyl methacrylate-*r*-poly(ethylene glycol) methyl ether) [poly(TMSMA-*r*-PEGMA)] was used in the experiments. Details on properties and characterizations of this polymer were published elsewhere.¹⁸

Escherichia coli (*E. coli*) and M13 Culture. The M13 Phase display containing the M13 phase and *E. coli* was obtained from New England Biolabs (Beverly, MA). The *E. coli* was grown in Erlenmeyer flasks that were filled with 20 mL LB medium (10 g/L Bacto-Tryptone, 5 g yeast extract, 5 g NaCl) and shaken continuously at 37 °C. To maintain the cultures, we diluted the cells 1:100 in fresh LB medium every 24 h.



Figure 5. (a–d) SEM images of the confined individual virus within various PEG templates with different heights: (a) 800 nm lines with 260 nm height (confined), (b) 800 nm lines with 51 nm height (nonconfined), (c) 1 μ m circles with 82 nm height (confined), and (d) 500 nm circles with 283 nm height (confined). For line features shown in a and b, the virus particles assembled to form long filamentous aggregates. For the 1 μ m circles shown in c, the well size is similar to the length of a single virus particle (880 nm), resulting in the deposition of individual viruses without bending within the wells. For the 500 nm circles shown in d, however, the well size is smaller than that of a single virus, resulting in a bending of individual viruses to fit into the well size. (e) A scheme for the effects of well size and barrier height on the confinement of individual viruses: symbols represent confinement (\bigcirc), nonconfinement (\times), and intermediate case (\triangle) where confinement and nonconfinement were observed concurrently. Note that the slope increases sharply as the well size decreases below \sim 800 nm.

To prepare the M13 phage, the *E. coli* was grown in an overnight culture of 1:100 in 20 mL LB medium and incubated overnight. Subsequently, a solution containing $\sim 1.5 \times 10^{11}$ virions was added to the mixture and shaken vigorously for 4.5 h at 37 °C. To isolate the M13 from the *E. coli*, the cultures were then centrifuged at 3000 rpm for 10 min. The supernatant was then transferred to a new vial and centrifuged again. The top 80% of the supernatant was then moved to a fresh tube and stored at 4 °C until use.

Fabrication of PDMS Stamps. PDMS stamps were fabricated by casting PDMS (Sylgard 184 elastomer, Essex Chemical) against silicon masters prepared by photolitho-

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graphy (1:10 ratio of the curing agent). Then the pre-polymer was well mixed and incubated at 75 °C for 1 h. After curing, PDMS stamps were detached from the master and cut prior to use.

Nanomolding. A few drops of a 1-5 wt % solution of poly(TMSMA-*r*-PEGMA) solution in ethanol were placed on a silicon wafer, and a thin film of the copolymer was obtained by spin coating (Model CB 15, Headaway Research, Inc.) at 1000 rpm for 10 s. To make conformal contact, we carefully placed PDMS stamps onto the surface and the samples were left undisturbed for 20 min at room temperature to allow for evaporation of the solvent. The film thickness after solvent evaporation ranges from 32 to 370 nm as

determined by ellipsometry (Gaertner L116A, Gaertner Scientific Corp.) and AFM.

Patterning M13 and Proteins on the Surface. To pattern the antibody on the surface, the P3 (Sigma-Aldrich Co., MO) and P9 (New England Biolabs, MA) antibodies were diluted at 1:100 in PBS. Here is the description of the P9 antibody: Anti-M12 pIII Monoclonal antibody (mouse isotype IgG2a) is derived from BALB/c mice immunized with the C-terminal half of M13 coat protein III (residues 259-406). Monoclonal anti-M13 pIII recognizes nonreduced and denatured reduced forms of wild type pIII or pIII fusions in immunoblotting and ELISA. A few drops of the antibody solution were then spread evenly on the patterned surface for 1 h. The resulting patterns were either stained with secondary antibodies to determine protein distribution or treated with a solution of M13. To pattern the M13 virus on the surfaces, the patterns were placed within a dish containing the M13 containing solution for 4 h, prior to analysis.

Atomic Force Microscopy (AFM). AFM images were taken in tapping mode on a NanoScope III Dimension (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.). Some of the images shown were flattened but not further manipulated.

Scanning Electron Microscopy (SEM). Virus images were taken using a high-resolution SEM (JEOL 6320FV, MIT) at an acceleration voltage of 3 eV. Samples were coated with Au layer to \sim 5 nm prior to analysis to prevent charging.

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References

- Seeman, N. C.; Belcher, A. M. Proc. Natl. Acad. Sci. U.S.A. 2002, 99, 6451–6455.
- (2) Hyun, J.; Ahn, S. J.; Lee, W. K.; Chilkoti, A.; Zauscher, S. Nano Lett. 2002, 2, 1203–1207.
- (3) Liu, M. Z.; Amro, N. A.; Chow, C. S.; Liu, G. Y. Nano Lett. 2002, 2. 863–867.
- (4) Niemeyer, C. M. Angew. Chem., Int. Ed. 2001, 40, 4128-4158.
- (5) Demers, L. M.; Ginger, D. S.; Park, S. J.; Li, Z.; Chung, S. W.; Mirkin, C. A. Science 2002, 296, 1836–1838.
- (6) Whaley, S. R.; English, D. S.; Hu, E. L.; Barbara, P. F.; Belcher, A. M. *Nature* 2000, 405, 665–668.
- (7) Lee, S. W.; Mao, C. B.; Flynn, C. E.; Belcher, A. M. Science 2002, 296, 892–895.
- (8) Yoo, P. J.; Nam, K. T.; Qi, J. F.; Lee, S. K.; Park, J.; Belcher, A. M.; Hammond, P. T. *Nat. Mater.* 2006, *5*, 234–240.
- (9) Cheung, C. L.; Camarero, J. A.; Woods, B. W.; Lin, T. W.; Johnson, J. E.; De Yoreo, J. J. *J. Am. Chem. Soc.* **2003**, *125*, 6848–6849.
- (10) Yi, H. M.; Nisar, S.; Lee, S. Y.; Powers, M. A.; Bentley, W. E.; Payne, G. F.; Ghodssi, R.; Rubloff, G. W.; Harris, M. T.; Culver, J. N. Nano Lett. 2005, 5, 1931–1936.
- (11) Whitesides, G. M.; Ostuni, E.; Takayama, S.; Jiang, X.; Ingber, D. E. Annu. Rev. Biomed. Eng. 2001, *3*, 335–373.
- (12) Lee, K. B.; Park, S. J.; Mirkin, C. A.; Smith, J. C.; Mrksich, M. Science 2002, 295, 1702–1705.
- (13) Mao, C. B.; Qi, J. F.; Belcher, A. M. Adv. Funct. Mater. 2003, 13, 648-656.
- (14) Suh, K. Y.; Kim, Y. S.; Lee, H. H. Adv. Mater. 2001, 13, 1386–1389.
- (15) Kim, Y. S.; Suh, K. Y.; Lee, H. H. Appl. Phys. Lett. 2001, 79, 2285–2287.
- (16) Suh, K. Y.; Langer, R. Appl. Phys. Lett. 2003, 83, 1668-1670.
- (17) Khademhosseini, A.; Jon, S.; Suh, K. Y.; Tran, T. N. T.; Eng, G.; Yeh, J.; Seong, J.; Langer, R. *Adv. Mater.* **2003**, *15*, 1995–2000.
 (18) Jon, S. Y.; Seong, J. H.; Khademhosseini, A.; Tran, T. N. T.; Laibinis,
- P. E.; Langer, R. *Langmuir* 2003, *19*, 9989–9993.
 Suh, K. Y.; Khademhosseini, A.; Yoo, P. J.; Langer, R. *Biomed.*
- (19) Sun, K. 1., Knadenniossenn, A., 100, P. J., Langer, K. Biomea. Microdevices 2004, 6, 223–229.
- (20) Dogic, Z.; Fraden, S. Phys. Rev. Lett. 1997, 78, 2417-2420.
- (21) Yoo, P. J.; Nam, K. T.; Kim, Y. S.; Belcher, A. M.; Hammond, P. T., to be submitted for publication.

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