Assembly and Functionalization of Phage onto Substrates Patterned by Dip-Pen Nanolithography

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Submitted to the Department of Materials Science and Engineering
in Partial Fulfillment of the Requirements for the Degree of
Bachelor of Science in Materials Science and Engineering

at the

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by

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Submitted to the Department of Materials Science and Engineering on May 22, 2006 in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science in Materials Science and Engineering at the Massachusetts Institute of Technology.

ABSTRACT

Advances in nanochemistry will drive the development of technologies at the scale of 1 – 100 nm. Principles of biology are used for the self-assembly of structures and devices at this scale. The M13 bacteriophage, a virus employed in phage-display libraries, serves as a scaffold for nanoscale structures. Phage are functionalized with inorganic materials, and controlled placement of phage at the nanoscale may lead to useful devices. Substrates patterned with dip-pen nanolithography (DPN) serve as templates for the deposition of phage. On gold substrates, 16-mercaptohexadecanoic acid (MHA) is deposited to form patterned lines. After surface passivation and activation chemistry, phage are deposited and adhere to the patterned substrate. Images from atomic force microscopy support that phage are covalently coupled to MHA lines and that cobalt precipitates on patterned phage.

Thesis Supervisor: Professor Angela Belcher
Title: Professor of Biological Engineering and Materials Science and Engineering

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1 INTRODUCTION

1.1 Motivation

Advances in nanochemistry will drive the development of technologies at the scale of 1 – 100 nm.[1] Two approaches to produce devices at this dimension are the top-down and bottom-up approach. The former, consisting of techniques that begin with a macro-assemblage and scale down through processing, is reaching its lower limit in dimension.[2] In contrast, the bottom-up approach involves an ever-broadening array of technologies to produce smaller structures.[3] Biological molecules are key actors in building from the bottom.[4] In nature, biological systems have a great degree of control over the assemblage of inorganic materials.[5] Principles of biology may be used to develop processes for the self-assembly of structures and devices at the nanoscale. Developments in this area will result in lower cost, improved efficiency, and advancements perhaps otherwise impossible.

1.2 Nanoengineering with Biology

There are likely as many examples of biological organisms as there are interactions of organic with inorganic materials.[4] In the biomineralization of abalone shells, a layer of calcite crystals first grows on a sheet of proteins, and the interaction of differently expressed proteins directs phase changes.[6] Important for hearing in higher vertebrates is the biomineralization of calcium carbonate to form otoconia in the inner ear.[7] Expression levels of homologous genes in zebrafish are responsible for controlling the shape of otoliths, or calcium biominerals.[7] Magnetotactic bacteria deposit iron oxide to create permanent, single-domain crystals

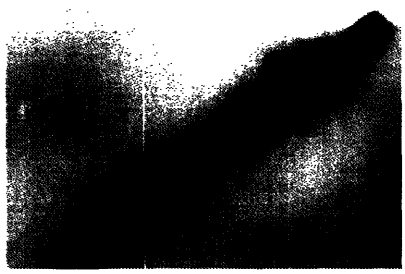

Figure 1. TEM image of magnetotactic bacteria. The length of each magnetic particle is approximately 100 nm.

(Figure 1).[4] Proteins mediate the shape, size, and crystallographic orientation of inorganic materials.

In this research, the biological modes of interaction and recognition of materials is mimicked synthetically to create structures at the nanoscale. In particular, viruses are versatile entities employed in novel ways to interact with inorganic materials and create new structures and devices.[8] This thesis summarizes attempts to adhere phage to patterned substrates and subsequently functionalize them. Organic-inorganic interactions are utilized with a burgeoning printing technique, dip-pen nanolithography, as a step towards creating novel structures and devices.

1.3 Nanoengineering with Bacteriophage

The use of phage as a vehicle of interaction was a revolution in the development of biomimetics. As discussed, biological organisms found means of controlling the assemblage of nanoscale components. Unfortunately, they did not develop interactions with useful electronic and optical materials.[9] Enter the M13KE bacteriophage, a virus employed in phage-display libraries traditionally used to find interactions useful in pharmaceutical applications.[10] This cylindrical phage is 6.5 nm wide by 860 nm long.[8] It consists of four minor coat proteins, including the pIII and pIX, and 2700 copies of a major coat protein, pVIII (Figure 2).[7] A combinatorial library consisting of billions of random peptides fused to the pIII phage is screened against targets of interest to identify peptides with affinity for

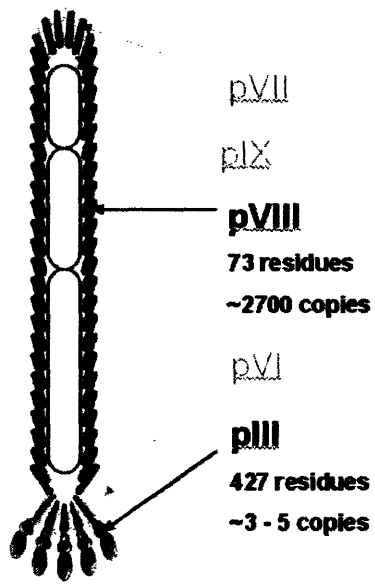

Figure 2. M13 bacteriophage with labeled proteins The pIII and pVIII are key proteins for building structures with phage

inorganic materials.[9] By genetically engineering the phage to express these proteins, the phage catalyzes reactions to nucleate inorganic materials and form useful structures and devices.[11]

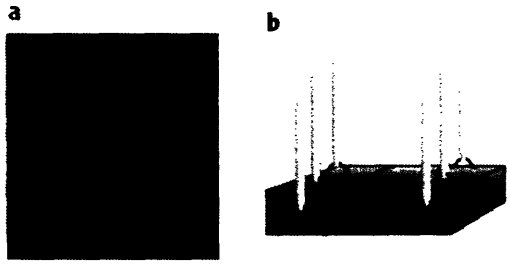

Figure 3. Evolved affinity of phage to substrates (a) Flourescently labeled phage binding preferentially to GaAs over SiO_2. **(b)** Schematic representation of preferential

This bottom-up approach to building nanostructures is used to select phage with particular affinity for materials, bind nanocrystals, form nanowires, and assemble electronic devices. Combinatorial phage-display libraries are used to determine proteins with high specificity for semiconductor surfaces.[12] The affinity depends on crystallographic orientation and composition of the materials used. In one set of experiments, labeled phage specifically bound GaAs and not SiO_2 (Figure 3).[12] Examples of other materials specifically recognized by peptides include GaN, ZnS, CdS, Fe_3O_4, and $CaCO_3$.

Engineered phage are capable of nucleating materials on the pIII and pVIII proteins; for example, phage are engineered to bind nanocrystals.[13] An initial step is screening a library of random peptides against a surface of interest through combinatorial phage-display. Screening against ZnS, a dominant binding motif was found after five rounds of selection. A peptide insert with this motif is expressed in the pIII protein of the

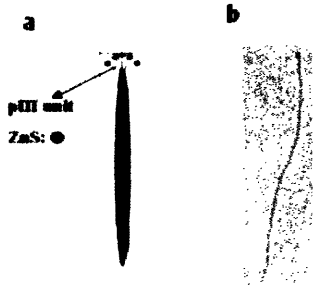

Figure 4. Binding of nanocrystals to phage (a) Schematic depiction of phage binding ZnS at the pIII protein. **(b)** TEM image of individual phage and ZnS nanocrystal

engineered phage, and the phage binds nanocrystals after exposure to ZnS precursor solutions (Figure 4).

Techniques to bind 0D materials are extended to produce 1D structures. After screening with a library of phage, peptides with affinity for substrates of interest are determined; through genetic modification, these peptides are fused to the N terminus of the pVIII protein.[14] After incubating the phage with metal salt precursors at low temperature, nanocrystals nucleate with preferred crystallographic orientation. In the synthesis of ZnS and CdS nanowires, the process of annealing produces single-crystals (Figure 5).[14] Through genetic modification, the properties of phage may be tuned, and they can serve as universal templates for the control of crystalline semiconducting, metallic, oxide, and magnetic materials.

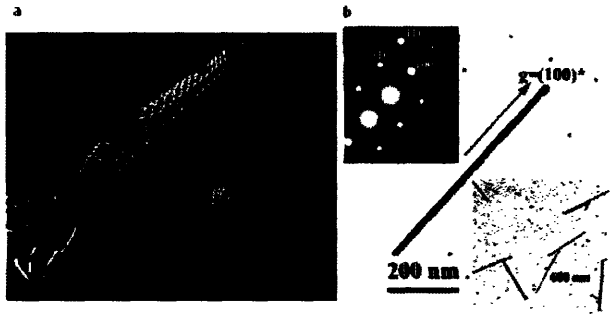

Figure 5. Phage as templates for 1D structures (a) Schematic depiction of inorganic materials nucleating on phage and subsequent annealing. **(b)** TEM image of nanowires produced from mineralization on phage

1.4 Patterning Substrates with Dip-Pen Nanolithography (DPN)

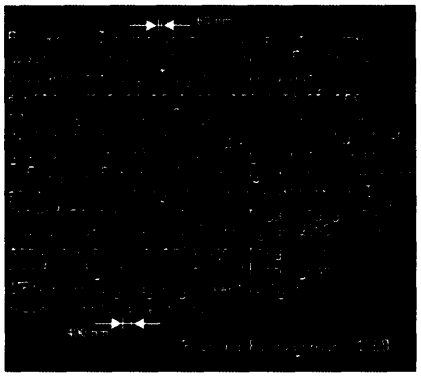

Figure 6. Patterned gold substrate using DPN.

Direct-write dip-pen nanolithography (DPN) is a technique used to pattern molecules at nanoscale dimensions (Figure 6).[15] An atomic force microscope (AFM) tip is soaked in a solution containing a molecule capable of deposition on a substrate of interest, and the tip is allowed to dry. When in close proximity to the substrate, a water meniscus forms between the tip and substrate, and this

serves as a conduit for flow of the binding molecule. As the tip traverses the substrate, molecules are deposited through capillary transport (Figure 7).[16] Lines, circles, and polygons of nanometer dimension are easily written through DPN.[15]

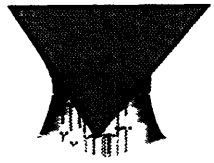

Figure 7. Schematic of molecules depositing from atomic force microscope tip to substrate. A water meniscus forms between tip and surface, serving as conduit for the flow of molecules.

As opposed to microcontact printing, DPN is a serial technique, but micromachining technology is currently being developed to create high-density parallel DPN probes.[17] Dip-pen nanolithography is a promising technique for precisely patterning and functionalizing substrates.[18]

1.5 Research Goal and Prior Art

The goal of the work is to pattern substrates using DPN, adhere phage through surface chemistry, and functionalize the immobilized phage to create useful structures or devices. Previous research demonstrates tobacco mosaic virus (TMV) binding to substrates patterned with DPN (Figure 8).[19] These virus particles consists of 2130 coat protein molecules that form 300 nm long cyclinders with an 18 nm diameter.[20] The virus has been used as a template for the controlled deposition of inorganic materials, including silver, platinum, and gold. Reaction precursors are believed to bind to amino acids such as glutamic and aspartic acid, arginine, and lysine in the virus coat proteins.[20] The

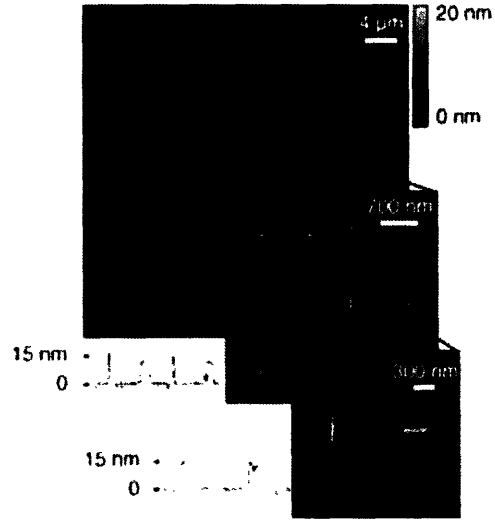

Figure 8. Topographic arrays and height profiles of TMV nanoarrays

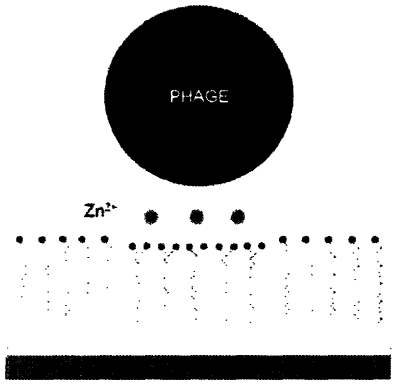

Figure 9. Phage interact with Zn^{+2} ions chelated to patterned MHA

deposition of materials is influenced by modification of the surface chemistry of the virus. [20]

In the controlled deposition of TMV, features 350 x 110 nm^2 are patterned on gold with 16-mercaptohexadecanoic acid (MHA), and the substrate is backfilled with a self-assembled monolayer of 11-thioundecyl-penta(ethylene glycol).[19] Electrostatic interaction between phage and lines is possible through chelating Zn^{2+} with carboxyl groups of MHA (Figure 9).[19] Individual virus particles assemble on the features through interaction with cations.[19] Many viruses have metal-binding sites in their coat, so this appears to be a broad technique for the patterning of phage on substrates.[19]

1.6 Patterning M13 Phage using Dip-Pen Nanolithography

The M13 bacteriophage is a promising candidate for creating devices through the patterning of phage by DPN. The M13 phage may interact with patterned substrates in a similar fashion to TMV. Metals and semiconductors can coat the phage, and direct placement onto substrates could lead to patternable devices. The M13 bacteriophage may function as a template for batteries, circuits, and sensors.

2 EXPERIMENTAL METHODS

2.1 Substrate Preparation

A flat surface is required for patterning with DPN and obtaining clear images of phage. Electron-beam evaporation with gold on silicon generates smooth substrates.

Dip-pen nanolithography is a technique used to pattern substrates at the nanoscale, and the patterned molecules are activated for subsequent coupling with phage.

2.1.1 Electron-Beam Evaporation

Electron beam evaporation is a technique used to deposit metals and some non-metals onto a variety of substrates. A substrate of interest is positioned above the material to be deposited. The electron beam directed at the deposition material evaporates the material, and it is deposited on the surface of the sample. A slow deposition rate is used to deposit layers with small grain sizes; typical deposition rates are 0.5 angstroms per second.

Smooth gold substrates are used in DPN and the patterning of phage. The underlying substrate is silicon, and to promote the adhesion of gold, a layer of titanium or chromium is first deposited. With titanium adhesion layers, thicknesses are typically 50 - 100 nm for gold and 5 – 10 nm for titanium.[21,22] When employing chromium, the thickness of gold and chromium is 25 – 30 nm and 5 nm, respectively.

2.1.2 Dip-Pen Nanolithography

Dip-pen nanolithography is the technique used to pattern substrates prior to phage exposure. In DPN, an AFM contact mode tip is soaked in a solution of acetonitrile and MHA, and patterns are created on gold substrates in AFM contact mode. A saturated solution of 6 mM MHA in acetonitrile is sonicated for five minutes. Tips are soaked in solution for approximately five seconds, and after blotting on tissue paper they are allowed to dry in atmosphere. To

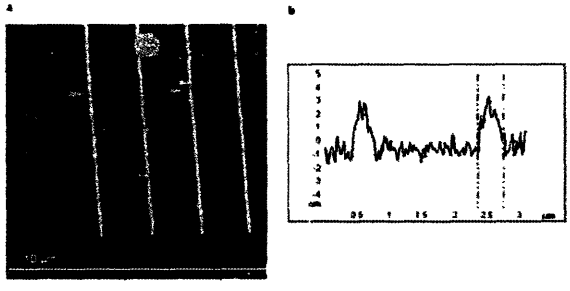

Figure 10. Patterned MHA lines on gold substrate (a) MHA lines created by DPN on gold substrate. **(b)** Distance-height profile of patterned lines.

generate lines, the slow-scan axis of the AFM is disabled, and lines several hundred nanometers in width are deposited by scanning the surface for roughly twenty seconds (Figure 10).

2.1.3 Surface Passivation and Activation

The DPN patterned gold substrates with MHA are passivated by backfilling with molecules capable of generating a SAM on gold. Soaking the substrate for at least two hours in a 6 mM solution of ethanol and octadecane thiol (ODT) or a 5 mM solution of methoxy poly(ethylene) glycol thiol (PEG-SH) in ethanol generates a single layer of covalently bound molecules on the surface of gold. The monolayer of ODT or PEG-SH is resistant to the attachment of phage.

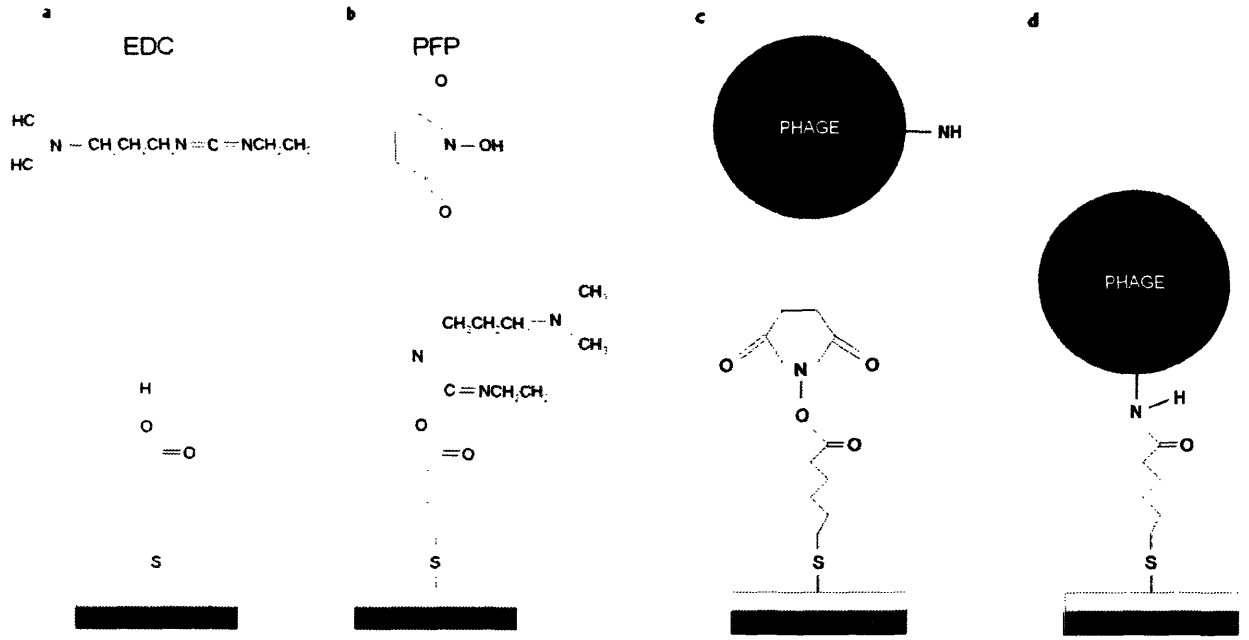

Figure 11. Covalent coupling chemistry of phage to carboxylate (a) The carboxyl group of MHA is activated with EDC, which **(b)** subsequently reacts with PFP. **(c)** The NH_2 group of the amine reacts with the activated carboxyl group, and **(d)** the phage attaches covalently to the substrate.

After backfilling with these molecules, the patterned lines are activated with ions or other chemistry for electrostatic or covalent coupling with phage. Zinc ions are

believed to interact favorably with the phage coat proteins. To chelate zinc ions with carboxyl groups of MHA, substrates patterned with MHA are soaked in an aqueous solution of zinc chloride. In order to covalently bind phage to MHA, the carboxyl groups of MHA are activated with 0.1 mM EDC and 0.2 mM PFP in ethanol. An amine on the coat protein of the phage is believed to covalently bond to the activated carboxyl group (Figure 11).

2.2 Phage Exposure and Functionalization

After substrate fabrication, patterning, passivation, and activation, the sample is exposed to a solution of phage. A wash removes non-selectively bound phage, and atomic force microscopy is used to image the surface.

2.2.1 Phage Exposure

The intent of exposing a patterned substrate to a solution of phage is to selectively adhere phage to lines of MHA. Phage of interest are amplified in cultures of *E. Coli*, and the final step in amplification is resuspending the phage in tris-buffered saline (TBS). Substrates are placed in a microcentrifuge tube and filled with several hundred microliters of phage solution. The tube is gently rocked for at least several hours, routinely overnight.

2.2.2 Surface Wash

The substrate is washed after phage exposure; the purpose is to remove phage non-selectively bound to the substrate. In the first step, substrate is removed from the tube and placed in a small plastic box sanitized by washing with 10% bleach solution. One milliliter of purified water is pipetted in and out of a box a total of three times. The sample dries in atmosphere prior to imaging.

2.2.3 Cobalt (II) Chloride Exposure

The E4 phage is a modified M13 phage engineered to bind inorganic materials, including cobalt. Patterned substrates with phage are exposed to a 2 mM aqueous solution of cobalt chloride for fifteen minutes. Cobalt ions are reduced by adding a 5 mM aqueous solution of sodium borohydride. After fifteen seconds, the sample is removed, washed with purified water, and allowed to dry.

2.3 Imaging

Atomic force microscopy is used to image surfaces after exposure to phage. In contact mode AFM, a tip in contact with the sample scans across the surface. Deflections in the tip correspond to local changes in height; corresponding changes in position of a laser reflected off a tip and onto a photodetector are used to determine the topology of the surface (Figure 12). In tapping mode AFM, a tip vibrating at resonance frequency scans across a sample. Interactions with the surface affect the amplitude and phase of the oscillations, and these changes are analyzed to yield information regarding the topology and modulus of the sample. Operation of the tip in contact mode may disrupt oriented phage; to image phage, the AFM is operated in tapping mode.

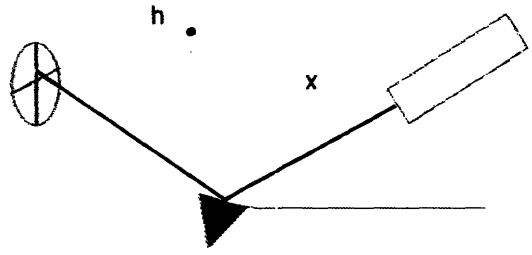

Figure 12. Schematic of the operation of an AFM. A tip scans across the surface and deflections are measured by corresponding changes in position of a laser reflected off a tip

3 RESULTS AND ANALYSIS

Experiments involved patterning gold substrates with MHA, passivating the gold surface with a SAM, adhering and coupling phage to the MHA, and functionalizing the phage by exposure to cobalt (II) chloride.

3.1 MHA Lines, M13KE Phage Exposure

The first set of experiments involved patterning MHA lines on gold substrates and exposing the sample to phage. Lines several hundred nanometers in width were deposited on gold. A two dimensional oval of MHA on the surface results from scanning a soaked tip in the shape of a rectangle. The fact that the width of lines is dependent on scan times and ovals are produced by rectangular deposition evinces that MHA diffuses on the surface. The height of the line is larger than an MHA molecule, and this is believed to result from hydration of water to the carboxyl groups, contaminant interacting with carboxyl groups, or MHA multilayer formation.

Images of the substrate after phage exposure support the hypothesis that phage bind preferentially to lines of MHA (Figure 13). Long, filamentous shapes oriented on the lines are believed to correspond to bundles of phage. The orientation of phage amidst swirls of phage binding non-selectively to gold indicates a favorable interaction with MHA. Contrast in phase images indicates a material binding to MHA of different modulus.

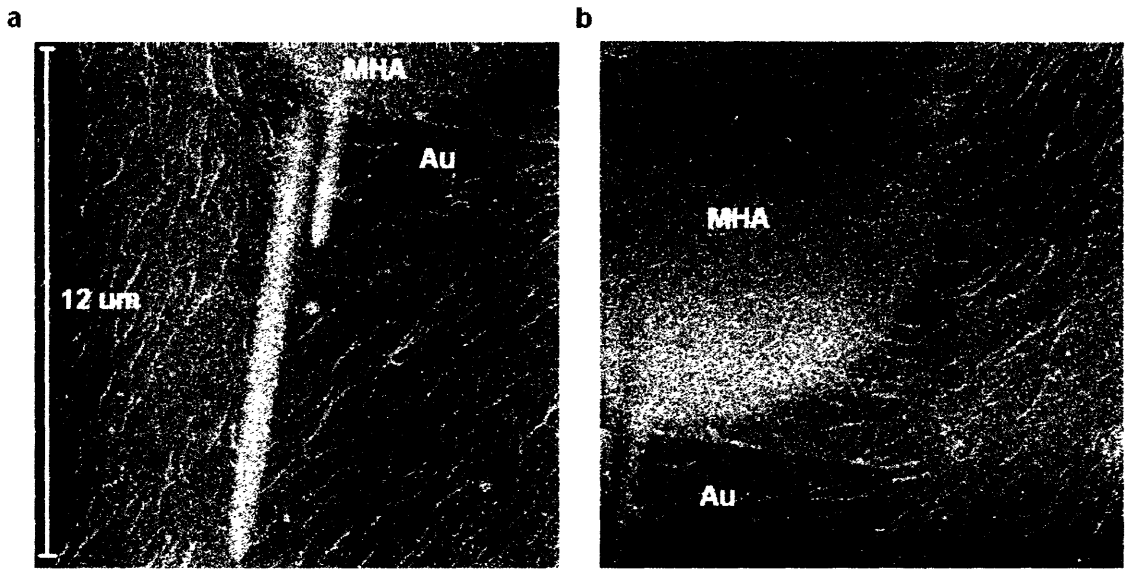

Figure 13. AFM height images of MHA patterned gold substrate exposed to phage. (a) The orientation of phage along the patterned line contrasts the direction of non-specifically bound phage. **(b)** The hypothesis that phage interact favorably with MHA is demonstrated by the change of orientation of phage towards MHA. Both images are 12 μm in width.

3.2 MHA Lines, PEG-SH Backfill, M13KE Phage Exposure

The surface of gold needs to be passivated to avoid non-selective attachment of phage to gold. Biological species do not favor binding to hydrophobic surfaces, and ODT and PEG-SH are two molecules that form hydrophobic SAMs on gold. To generate a SAM, a patterned substrate is soaked in a 5 mM solution of PEG-SH for several hours. After exposure to phage, the surface of the substrates are viewed with AFM. Images indicate that phage preferentially adhere to the patterned MHA lines and do not attach to the backfilled monolayer (Figure 14).

Atomic-force microscopy images and height-distance profiles suggest individual phage adhere to portions of the MHA line and that bundles of phage fully bind to the lines. The height associated with phage partially attached to lines is approximately 5 nm, which corresponds to the diameter of phage. Height-distance profiles of phage fully

attached to MHA demonstrate a height two to three times as large. The bridging of phage between lines separated by small distances support that phage interacts favorably with MHA or neighboring phage.

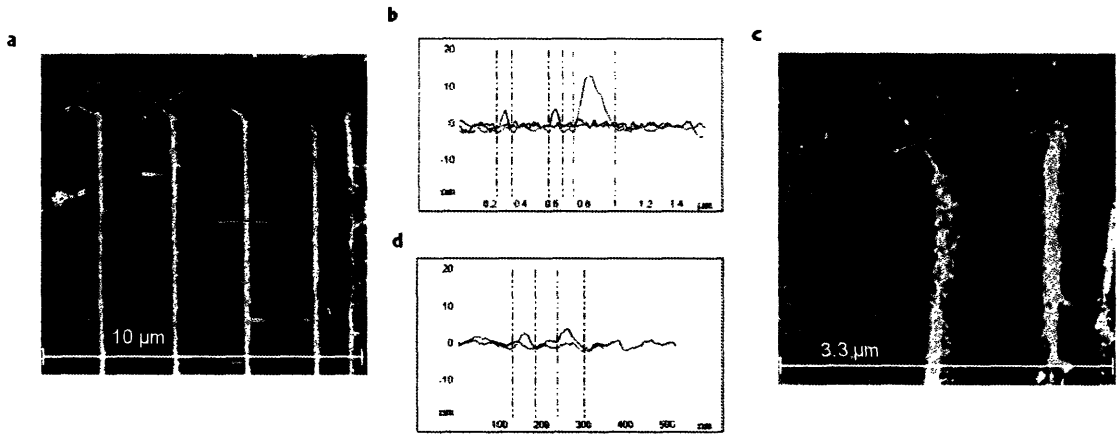

Figure 14. Phage binding to MHA lines with PEG-SH backfill (a) Phage bind specifically to the patterned lines of MHA; they bridge short distances between lines but do not bind to the SAM backfill. **(b)** Each peak in the height-distance profile corresponds to lines in image (a) from left to right. The magnitude of the third peak supports multiple phage binding to the MHA line. **(c)** Individual phage appear to bind to portions of the MHA line. **(d)** The heights of peaks in the height-distance profile support that individual phage bind to portions of the MHA lines.

3.3 MHA Lines, PEG Backfill, Covalent Chemistry, M13KE Phage Exposure

To increase the robustness of patterned phage, MHA is activated to covalently bind phage. The surface is backfilled with a SAM, and EDC and PFP are employed to activate the carboxyl group of MHA. Figure 15 demonstrates phage bound to lines of MHA. The height-distance profiles evince that phage adhere in bundles on the lines, and the topology of the phage is smooth.

Attempts to elute the phage are used to test whether they are covalently bound to the lines. The sample is soaked in a solution of glycine-HCl. Protons in the low pH solution are believed to bind proteins on the phage coat and disrupt interactions between MHA and the protein coat. Images of the AFM after the acidic soak demonstrate the disruption of the bundles of phage. Both the height and phase images of the surfaces

after the acid wash support that individual phage remain covalently bound to the substrate after the acidic wash. Individual phage binding is particularly evident in the images of phage partially bound to the MHA lines. Comparison of the height-distance profile is further support of individual phage adhering to the lines (Figure 16). Before the wash, the height associated with the bundle of phage is approximately 12 nm. Multiple peaks of roughly 5 nm remain after the acidic soak.

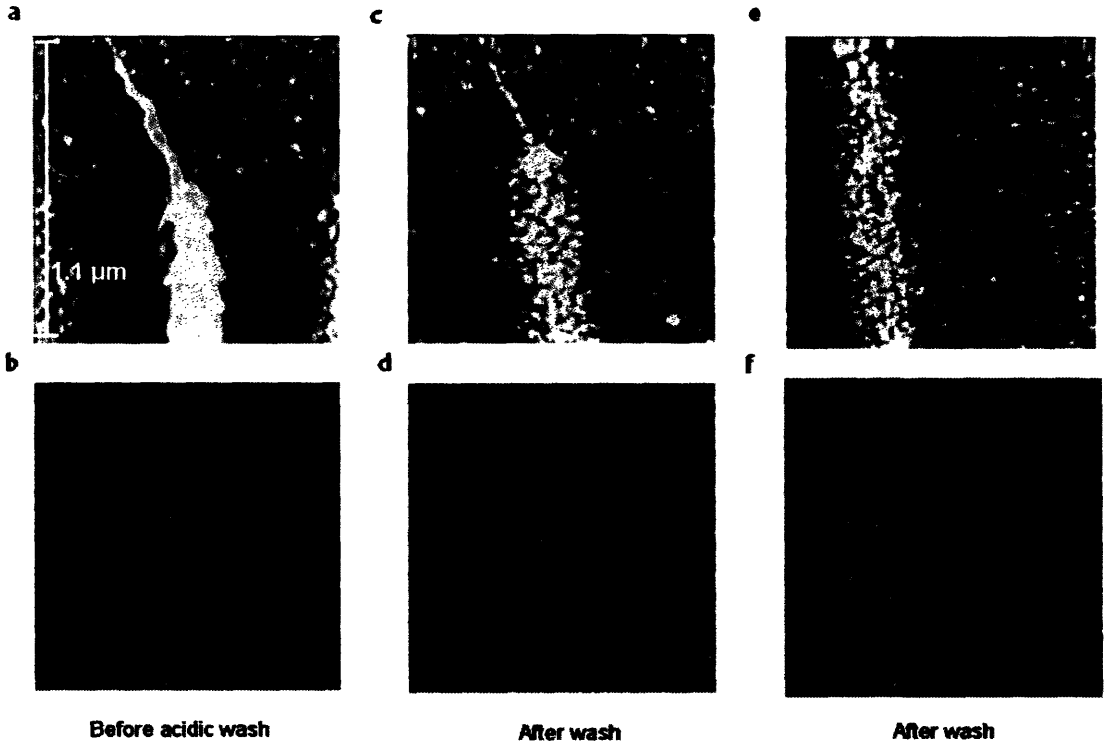

Figure 15. Height and phase images of phage binding to MHA lines before and after elution (a) The height image and height-distance profile of the surface prior to acidic wash supports bundles of phage adhering to the lines. **(b), (c)** After the wash, individual phage appear to bind the lines. All images are 1.1 µm in width.

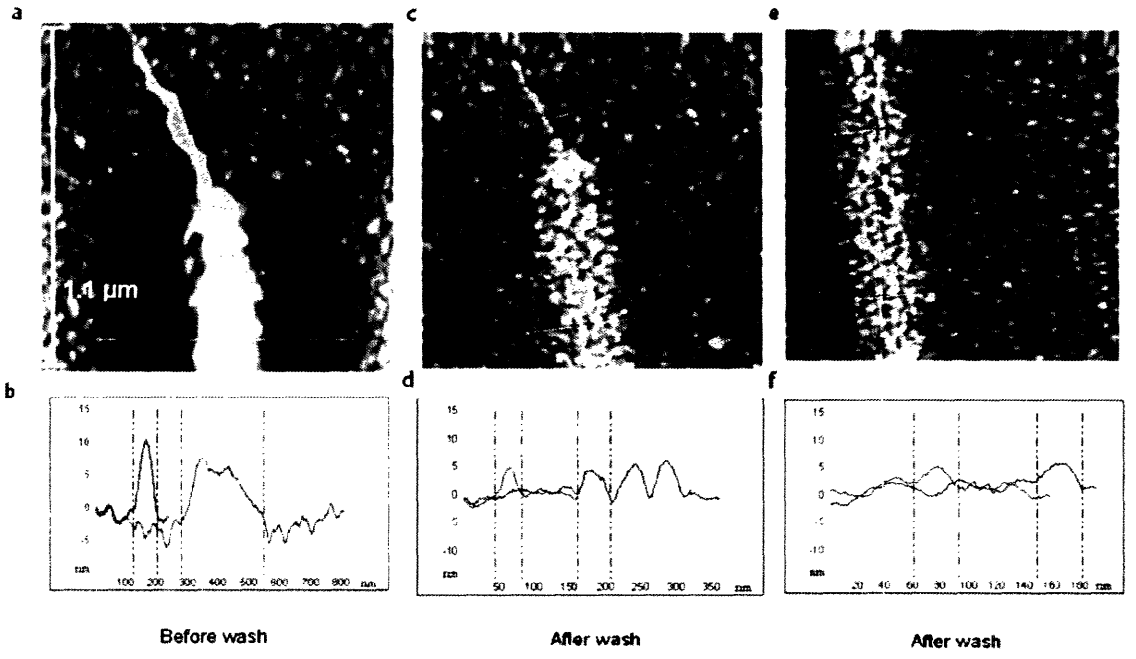

Figure 16. Height images and height-distance profiles of phage binding to MHA lines before and after elution (a), (b) Height and height-distance profiles of phage adhered to patterned substrates before acidic wash. Bundles of phage appear covalently bound. **(c), (d), (e),** and **(f)** After the soak, the height and height-distance images show a change in topology. Individual strands of phage appear to adhere to the surface, and multiple peaks of approximately 5 nm remain after the wash. All images are 1.1 µm in width.

3.4 MHA Lines, PEG Backfill, E4 Phage Exposure, Cobalt Exposure

The ability to selectively pattern and adhere phage was demonstrated; to create devices, the phage must be functionalized. The E4 phage is engineered to precipitate materials on its coat, and AFM height and phase images support that cobalt mineralizes on the phage. Lines of MHA were patterned on the substrate, and the surface was backfilled with PEG-SH. After determining that phage adhered to the lines, the sample was exposed to a 2 mM solution of cobalt (II) chloride. The sample was reduced, washed with water, and imaged.

The AFM images support cobalt mineralizing on the coat of phage. The phage adhered to the lines, and after soaking in solution of cobalt (II) chloride, the phage are

partially washed off the lines. It appears that nanocrystals mineralize on the phage, and the contrast in the phase images support that an inorganic material adheres to the phage.

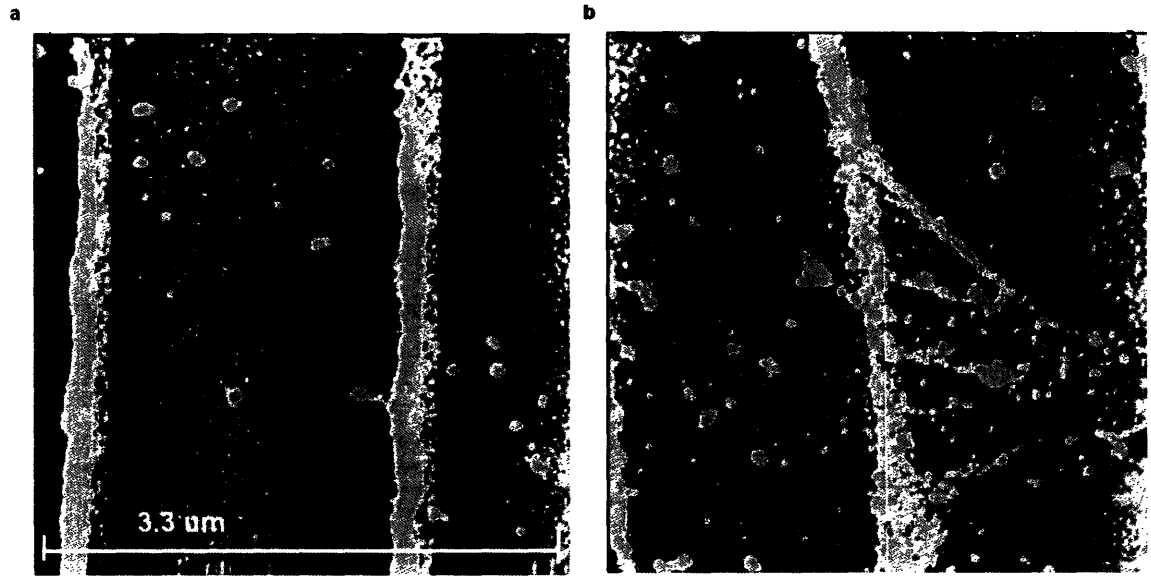

Figure 17. Height images of phage adhered to MHA lines before and after cobalt (II) chloride exposure (a) Lines of MHA with E4 phage adhered. **(b)** After exposure to cobalt (II) chloride and reduction with sodium borohydride, phage are displaced from the line, and nanocrystals appear to mineralize on the phage coat. Both images are 3.3 μm in width.

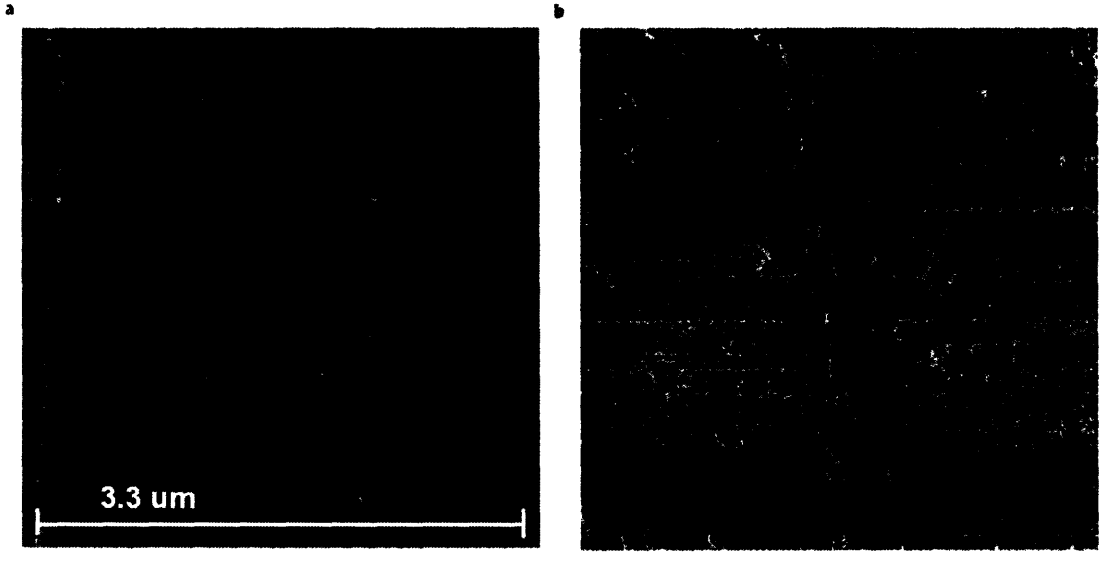

Figure 18. Phase images of phage adhered to MHA lines before and after cobalt (II) chloride exposure (a) Height image before wash. Bundles of phage appear to attach to the MHA lines. **(b)** The soak in solution of cobalt (II) chloride, reduction, and subsequent wash results in release of phage from the line. Spherical nanocrystals appear to mineralize on the coat of the phage. Both images are 3.3 μm in width.

4 CONCLUSIONS AND DISCUSSION

Evidence supports that phage bind to MHA lines, may be covalently attached, and may mineralize material on its coat. It appears that a SAM of PEG-SH functions well as a passivating backfill. A plausible mechanism of phage preferentially binding to lines of MHA involves the hydrophilic character of MHA. During phage exposure, the solution appears to evaporate last over regions patterned with MHA; phage may collect on lines due to this process.

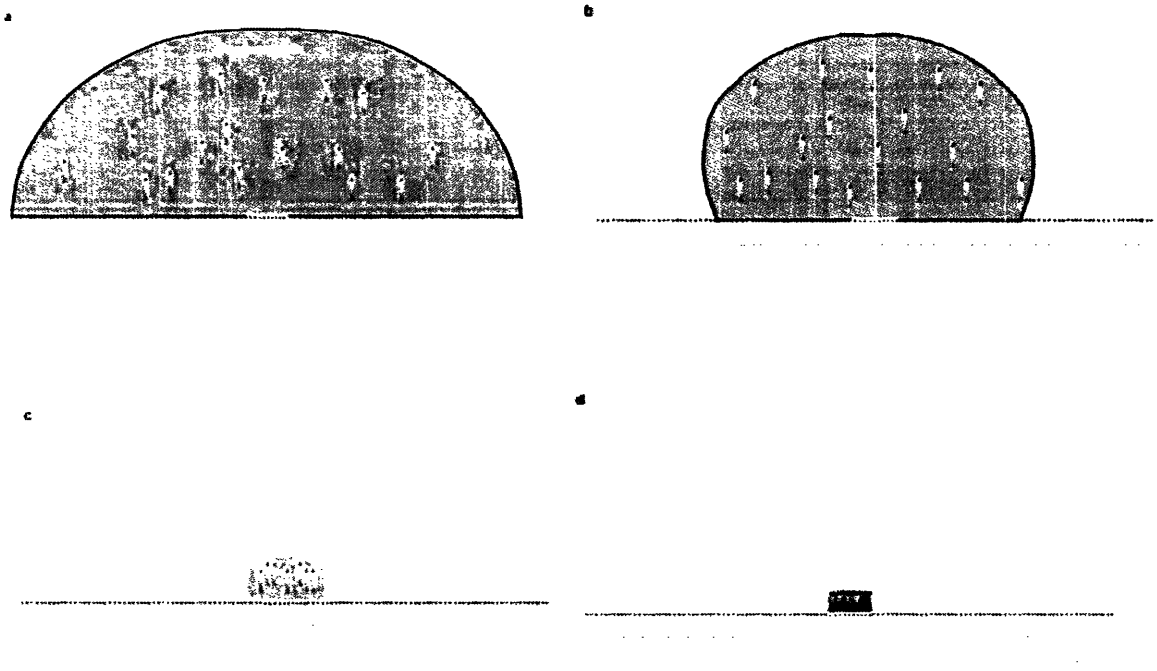

Figure 19. Mechanism of phage binding to patterned substrates. (a) A drop of phage solution is exposed to a patterned and passivated substrate. **(b)** and **(c)** The solution evaporates last over regions of MHA. **(d)** Phage orient on the patterned line after solution evaporates

5 FUTURE RESEARCH

There are many opportunities for future investigation. First, antibodies with conjugated nanoparticles could be employed to detect phage; this would serve to confirm the visual data. Previous experiments support that phage bind after exposing the substrate to zinc; it may be worthwhile to measure the relative strength of adhesion of phage to lines of MHA activated with positive chelating ions. Further experiments with covalent coupling chemistry could ensure that phage is indeed covalently bound to MHA. Elemental analysis of the surface may validate that cobalt is mineralized on the coat of phage. Combining coupling chemistry with mineralization would be a next step in developing devices.

Building devices on gold substrates is not ideal, for it may prove difficult to measure conductivity of nanowires that are nanometers from a gold substrate. Patterning the phage on a gold substrate and subsequently transferring to an insulating material may be necessary. Patterning on non-conducting substrates is an alternative. Single-crystal nanowires may form from annealing mineralized phage, and techniques to check the conductivity of crystallized material on phage would be useful A device will likely consist of heterogeneous materials; it will be necessary to develop techniques to pattern multiple types of phage and mineralize several different materials. Carbon nanotubes adhere to MHA patterned substrates, and they may be incorporated in devices with phage.[23]

A hypothetical device constructed from dip pen nanolithography is demonstrated in Figure 20. On a non-conducting substrate, molecules are patterned between four metallic contacts. The sample is exposed to phage, and a p-type semiconductor material

coats the phage. Lines are deposited from the phage to the contacts, and oxide, metallic, and n+ wires are created by patterning and functionalizing phage. This device would function as a MOSFET

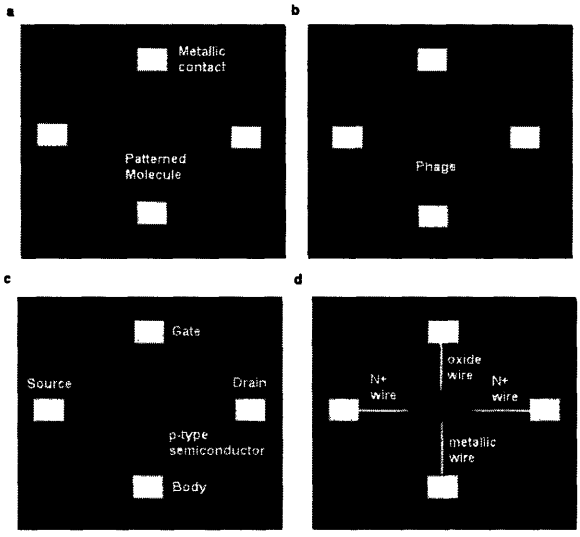

Figure 20. Hypothetical MOSFET device constructed from DPN and functionalized phage. (a) On a non-conducting substrate, patterned molecules are deposited between metallic contacts. **(b)** Phage engineered to bind a p-type semiconductor are deposited **(c)** Lines are patterned between the phage and contacts. **(d)** Oxide, metallic, and n+ wires are constructed.

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