Patterned Protein Microarrays for Bacterial Detection

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Last edit: Oct. 24, 2002

Abstract

Patterned microarrays of antibodies were fabricated and tested for their ability to bind targeted bacteria. These arrays were used in a series of bacterial immunoassays to detect *E. coli 0157:H7* and *Renibacterium Salmoninarum* (RS). Microarrays were fabricated using microcontact printing (μCP) and characterized using scanning probe microscopy (SPM). The high-resolution SPM imaging showed that targeted bacteria had a higher binding selectivity to complementary antibody patterns than to unfunctionalized regions of the substrate. Additional studies indicated a significant reduction in binding of bacteria when the microarrays were exposed to non-specific bacteria. These studies demonstrate how protein microarrays could be developed into useful platforms for sensing microorganisms.

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1. Introduction

Recent events have triggered a strong interest in sensitive detection of viruses and bacteria. Various strategies have been proposed to detect the presence of bacterial contaminants. The traditional technique, in which specimens are collected, cultured and then counted, presents problems because of the long time scales required. To solve this problem, indirect techniques have been proposed in which the presence of bacteria is rapidly inferred from changes in a transducer output signal. These indirect techniques include electrochemical methods [1-4], and quartz microbalance detection. [4-7]. While these techniques offer promise of a reagentless detection scheme, they in turn often suffer from false readings caused by variable flow rates, changes in pH, or fluctuations in temperature. The work reported here investigates a new methodology for detecting bacteria that uses immobilized antibodies to capture them on a patterned substrate and direct observation in order to overcome these difficult issues. A benefit of patterning antibodies in a microarray is the possibility of rapid detection through pattern recognition algorithms.

Protein microarrays of antibodies are used in this work to perform immunoassays with two different bacterial species. Patterning antibodies into a microarray was accomplished by the use of microcontact printing (μCP). The μCP method, introduced and developed by Whitesides *et al.* [8,9], has been used to pattern a variety of different proteins [10-16]. Moreover the μCP method, also known as a "soft lithography technique", has been successfully applied to pattern cells. Substrates patterned with alkanethiols allowed for the adsorption of a protein, which then bind cells to the substrate

from an aqueous solution [17]. Microcontact printed substrates were also used in cell biology studies to capture cells at specific sites and then grow them as they adhered to the surface [18, 19].

Microcontact printing techniques, described in our previous work on protein immunoassays, [20] proved to be an easy and inexpensive method for the patterning of proteins onto substrates without loss of biological activity. Reports are now beginning to appear in the literature on the use of patterned antibodies for detection of bacteria. F. Morhard *et al.* and P. M. St. John *et al.* used micro-patterned antibodies on gold [21] and silicon [22] to create patterns of bacteria. A diffraction-based method was used for detecting the presence of bacteria in both studies. Neither study focused on the cross-reaction between their antibody microarrays and non-specific bacteria. These studies would also have benefited from high-resolution lateral microscopy analysis of the substrates after performing the bacterial immunoassay.

The objective of the work described here was to assess the utility of high resolution scanning probe microscopy (SPM) to detect bacteria captured from solution onto an antibody microarray. The advantage of this approach is that it allows direct interrogation of the physical features of captured bacteria. Topographical features of bacteria can be very unique and aid in confirming their presence. The high lateral resolution of current SPM technology can theoretically lower the limits of detection, since the presence of a single bacterium can be determined from an SPM image. Specificity of the patterned antibody system for specific bacteria was examined by exposing the microarrays to a broad selection of non-immunologically targeted bacteria.

2. Experimental

2.1 Stamp fabrication

Poly(dimethylsiloxane) (PDMS) stamps are fabricated by casting and curing Sylgard 184 (Dow Corning, Midland, MI, USA), an elastomeric polymer, against photoresist micropatterned silicone masters. The master relief pattern used to make a PDMS stamp was a negative relief of the stamp mold and was manufactured at the Stanford University Nanofabrication Facility. These masters were made by first spin coating a positive photoresist, SPR 220-7 from Shipley (Marlborough, MA, USA) on a silicon wafer. Irradiation with UV light through a chromium mask was used to render any exposed photoresist soluble, which was then removed by washing with the developer LDD26W from Shipley (Marlborough, MA, USA). The patterns used for our experiments consisted of a 10 μm x 10 μm square pattern with a 5 μm separation between squares on all sides. The PDMS stamps were cured for 2 days at room temperature or 12 hours at 60^{o}C and sonicated in an ethanol – water (1:2) solution before oxidation in a commercially available plasma cleaner.

2.2 Chemicals and Reagents

Immunoassay reagents included affinity-purified antibody to *Escherichia* coli 0157 H:7 (*E. coli*), affinity purified antibody to *Renibacterium Salmoninarum* (RS), as well as positive controls of *E. coli 0157 H:7* bacteria and RS bacteria. Control samples were obtained from Kirkegaard & Perry Laboratories, (Gaithersburg, MD, USA). The positive control bacteria used in our work were heat killed. Although the bacteria are dead, studies conducted in solution still showed specific antibody binding. Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). Tween 20, Tween 80,

chlorodimethylooctadecylsilane and anhydrous toluene were received from Aldrich (Milwaukee, WI, USA) and used without further processing.

2.3. Substrate preparation

Functionalized substrates used for this study were glass slides (Gold Seal, Hungary) coated with silane. Protein antibodies to the targeted bacteria were immobilized on these substrates by physical adsorption to the silane layer. The glass slides were chosen because they are inexpensive, transparent and a good material on which to deposit the proteins.

Preparation of the glass substrates is described in detail elsewhere [20]. The substrates were cleaned with sulfochromic acid and silanized in a solution of chlorodimethyloctadecylsilane (0.02 M) in an anhydrous toluene solution [23].

The RMS surface roughness of the glass substrates used in this study was found to be ~ 1.3 nm using an SPM. The roughness of the substrate plays a critical role in the rapid determination of relevant topographical changes when imaging with SPM after exposure to bacteria.

2.4 Microcontact printing (μCP)

The PDMS elastomer is a common choice for microcontact printing (μCP). Hydrophobic PDMS surfaces can be made hydrophilic by an appropriate treatment. This is accomplished by placing the stamp in a plasma cleaner (Harrick Scientific, Ossining, NY, USA) prior to use. Thus treated PDMS is an acceptable material for the directed transfer of proteins from solution to a micro-pattern on a solid substrate. The process of

loading a protein onto a PDMS stamp will be referred to as "inking" and the protein loaded onto the stamp or substrate as the "inking protein".

The surface of the PDMS stamp was exposed to solutions of inking antibodies for various lengths of time. The concentrations of inking antibodies to bacteria were in the range of 100-200 μg/ml ($6.6x10^{-7}$ – $1.3x10^{-6}$ M). All antibodies solutions were made in phosphate buffer saline (PBS) at pH=7.4. After 10 to 30 min, excess solution was removed and the stamp was dried under a stream of nitrogen gas. After inking, the stamp was brought into contact with the glass substrate and a small force was applied to make a better contact between both surfaces. The stamp was removed after approximately 1 minute and the glass slide washed with PBS followed by a deionized water wash.

Imaging the patterned array of antibodies with the SPM optimized this entire process. Both topographical and phase-contrast imaging were employed to determine if the desired micron-size pattern of antibodies had been faithfully transferred to the silanized substrate.

2.5 Performing the Bacterial Assay

The substrates with printed microarrays of antibodies were incubated in a solution containing complementary bacteria. To prevent non-specific adsorption to regions between the antibody arrays, BSA and Tween 20 or Tween 80 were used as backfilling agents. The concentration of BSA was 5-10 mg/ml (10^{-5} - 10^{-4} M); the typical concentration of Tween was 0.1-0.5%.

The substrates with patterned antibody were incubated with an analyzed solution containing complimentary bacteria for 30 –40 minutes and washed with phosphate buffer

and water before microscopic and SPM studies. Concentration of the original stock solution of heat-killed *E. coli* and RS was approximately $7.0x10^9$ cells/ml and $3.0x10^9$ cells/ml respectively. Before incubation with the antibody array, the bacteria were diluted 1:100 or 1:1000 with phosphate buffer (0.01M) pH=7.4. A schematic representation of this particular immunosystem is given in Figure 1. The reproducibility of the performed bacterial assay was approximately 70%.

To determine the magnitude of cross-reaction between the two antibody-bacteria systems used for this study, antibody microarrays of *E. coli* were exposed to RS bacteria and vise versa (results discussed below).

2.6 Detection

2.6.1. Microscopy

An optical microscope was used to verify the performance of the immunoassay before utilizing more precise scanning probe techniques. A Nikon TE-300 optical microscope was used, at magnifications 50x or 100x to investigate the emergence of detectable patterns on the substrates after exposure to bacteria. An attractive feature of this simple approach is that patterns of adsorbed bacteria can be seen directly.

2.6.2 Scanning Probe Techniques

Precise verification of the quality of printing and the efficiency of the bacterial-antibody binding were obtained using a commercially available scanning probe microscopy (SPM) system [24]. The SPM system was modified to allow mounting on an inverted optical microscope, which greatly aided in the positioning of the SPM tip over

the antibody arrays. The substrates were imaged in non-contact modes using a cantilever with a force constant of ~ 2 N/m [25].

3. Results and Discussion

Figures 2a and 3a show false-colored non-contact SPM topographic images of the immobilized antibodies to both the *E. coli* and RS bacteria before performing a bacterial assay. These images were used to confirm the quality of μCP antibody transfer. μCP fabricated arrays of both antibodies were observed to have a submicron sharpness.

The SPM imaging shows the height of the *E. coli* antibody is 6 ± 3 nm (higher morphology results in a lighter color). Similarly the height of RS antibody is also found to be 5 ± 2 nm. Both images show defects in the transferred antibody monolayers. The μCP antibody to *E. coli* consistently appeared to have micrometer-sized defects located in the center of the printed region. Other investigators have also reported this tendency for higher quality printing around the edges of micron-size patterns [14]. Observed defects on the microarrays of RS antibody were on average much smaller (~100 nm) than defects present in *E. coli* antibody arrays. The area of the defects observed on both types of antibody arrays was relatively small compared to the total area coated by the antibody monolayer.

The images also show the absence of large foreign objects between the printed regions of antibodies. This control over the preparation of the patterned arrays is critical for a conclusive immunological identification and is only possible by optimizing each step in the array fabrication process. As an example, foreign objects present on the RS antibody array, before incubation, had an average height of ~10 nm and are much

smaller than the measured height of the RS bacteria which typically are greater than 150 nm. We conclude that their presence will not adversely affect the determination of bacterial binding.

Figure 2b is an SPM image of an antibody microarray to *E. coli* after incubation in a solution of *E. coli* bacteria at a concentration of $7x10^7$ cell/ml. These images show the presence of large objects, with an average height of 130 ± 40 nm. Most importantly, these objects are observed to have a tendency to selectively bind to the printed antibody regions.

The large objects attached to the printed antibody regions in Figure 2b had a large distribution of topographic heights. This is to be expected due to the fact that heating killed the E. coli bacteria. This caused lysing of cells, which results in fragmentation of the bacterial cells into many small particles of random sizes. Figure 6a is a larger scan of the *E. coli* antibody microarray after incubation. From the SPM images, it is estimated that ~ 4% of the antibody area is coated by the *E. coli* bacteria at this concentration during an exposure time of 40 minutes. This low coverage is consistent with published studies of electrochemical detection of *E. coli* [1,2].

Figure 3b shows a noncontact SPM image of an antibody microarray to RS after exposure to a solution of RS bacteria at a concentration of $3x10^6$ cell/ml. Unlike the *E. coli*, the RS bacteria had a more definite shape and size, making their identification easier. The average height of the RS bacteria was found to be 180 ± 50 nm. Figure 3c is a phase image made simultaneously with Figure 3b. The phase image is useful for verifying the presence of the patterned antibody region, since it is often difficult to

observe the antibody pattern in the presence of the bacteria due to the extreme difference in their heights.

Figure 4 is a similar non-contact SPM image of patterned antibody to RS after incubation with a solution of RS bacteria at a concentration of $3x10^7$ cell/ml. Depending on bacteria concentration, different amounts of bacteria are bound to the printed antibody surface (see Figure 3b and Figure 4a). Bacteria bound to the RS antibody in Figure 4 were aggregated at this concentration. A reference grid was added in Figure 4a to help show that the aggregate RS cells form a pattern on the microarray. The centers of the antibody patterns are 15 microns apart as expected from the known geometry of the stamped array. Addition of Tween to the buffer solution prevents non-specific adsorption to the glass substrate and also helps to disperse the cells. At this concentration of bacteria and for an exposure time of 40 minutes, it was estimated that 30% of the microarray surface was coated by the RS bacteria.

Because of the checkerboard pattern of the antibody arrays, it is possible to use optical microscopy to detect the presence of the bacteria. While a positive identification of bacteria on any one square of the pattern might require careful optical microscopy examination, the appearance of a periodic pattern of squares can be quickly detected using ordinary optical microscopy. Digitized images clearly show that at these concentrations, the bacteria confined to antibody regions produce well-defined optical patterns that can be easily detected.

Cross-reaction studies were also conducted to determine the degree to which antibody to RS bacteria will interact with *E. coli* bacteria. Figure 5a shows a printed microarray of *E. coli* antibody after incubation with a solution of *E. coli* (concentration $7x10^7$ cell/ml)

for 40 minutes. There is clear evidence for the selective binding of the *E. coli* to its antibody. Similarly, a printed microarray of RS antibody was also exposed to a solution of *E. coli* at a concentration of $7x10^7$ cell/ml for 40 minutes. A comparison between image 5a and 5b shows a dramatic reduction in the number of bound objects when the RS antibody array was exposed to *E. coli 0157:H7*. Similar experiments also showed that RS bacteria do not exhibit a selective binding to the *E. coli* antibody (data not shown). These initial experiments support the claim that only specific bacteria will bind with complementary antibody. The results also indicate that the bacteria remain immunologically active even after death.

4. Conclusion

We describe a simple technique for fabrication of antibody microarrays capable of detecting bacteria. The microarrays were fabricated by inexpensive μCP methods. The fabrication of the arrays was optimized by careful characterization by scanning probe microscope techniques. SPM studies of exposed antibody arrays indicated a high specificity of bacteria binding to their complementary antibody. The results of the cross-reaction studies show that the bacteria exhibit a low binding selectivity to non-complementary antibodies. In addition to the above observations, the incubation times used in this study did not produce any detectable degradation of the patterned antibody arrays. We are currently in the process of quantifying the strength of the antibody-bacterium bond and these results will be reported at a later date.

This work demonstrates how microarrays coupled with the high-resolution scanning probe capabilities can be a sensitive tool for bacterial detection. Our next step towards the

development of a practical bacterial-immunosensor requires the fabrication of multiple antibody microarrays for the simultaneous detection of a wide variety of bacteria.

Acknowledgements

The authors gratefully acknowledge support from U.S. Navy grant No. N00164-00-C-0047, Ben Slentz for the use of his photoresist master. In addition we also thank Dr. Mary Tang of the Stanford University Nanofabrication Facility for the fabrication of photoresist micropatterned silicone masters. The authors would also like to acknowledge the encouragement and many helpful discussions with Prof. Y.E. Kim, the Director of the Center for Sensing Science and Technology at Purdue University, during all phases of this work.

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24. PicoSPM manufacture by Molecular Imaging™, Phonex, AZ 85044, www.molec.com

25. ThermoMicroscopes™ Non-Contact Ultralevers (ULNC-AUHW)

Figure Captions

1) Schematic of immunosystem

2) Noncontact topological SPM images of antibody microarray to *E. coli* a) before incubation (scan size: 9 x 9 μm) and b) after incubation with a solution *E. coli* bacteria (scan size 14 x 14 μm). The images clearly show a high selectivity of the bacteria to the antibody.

3) Noncontact topological SPM images of antibody microarray to RS a) before incubation (scan size 25 x 25 μm) and b) after incubation with a solution RS bacteria (scan size 21.5 x 21.5 μm). Similarly these images show that the RS bacteria only adhered to the patterned antibody. c) An accompanying phase map to b). The phase image is useful for determine the presence of the antibody which has a low contrast in image b) do to the large difference in the heights of the bacteria and antibody.

4) a) Large-area (50 x 30 μm) SPM image of an antibody microarray to RS exposed to the RS bacteria. The overlying grid is used to show the organization of the bacteria aggregates on the array. The antibody pattern is not visible in the image due to the large height of the bacteria. b) Zoom image of one element of the antibody microarray with bound RS bacteria (scan size 16 x 16 μm). c) Further zoom in of aggregated RS bacteria (scan size 3.1 x 3.1 μm).

5) a) SPM image of microarray of *E. coli* antibody after incubation with *E. coli* bacteria (scan size 35 x 25 μm). b) SPM image of a microarray of RS antibody after incubation with *E. coli* bacteria (scan size 40 x 28 μm). Comparison of the

images shows a dramatic change in the binding affinity of the *E. coli* bacteria to the RS antibody.

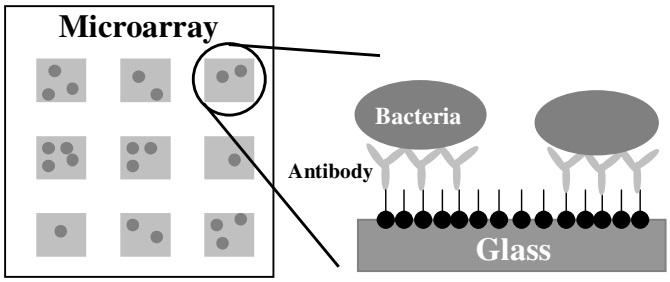
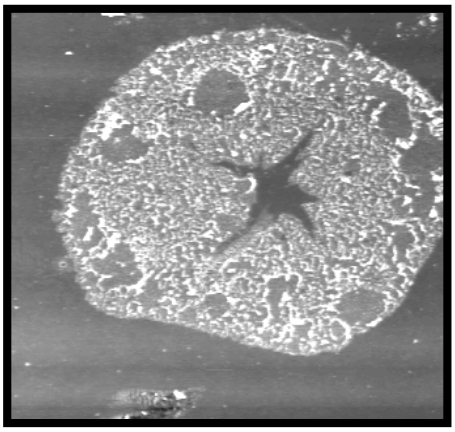


Figure 1

a)



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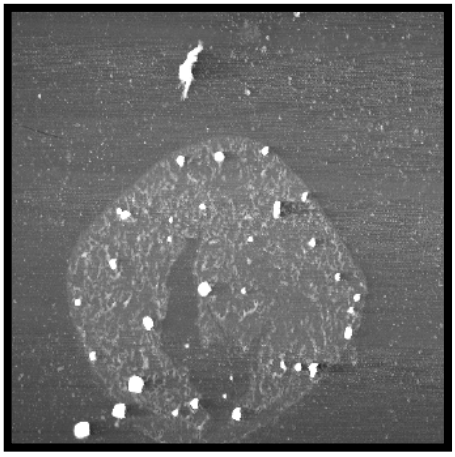
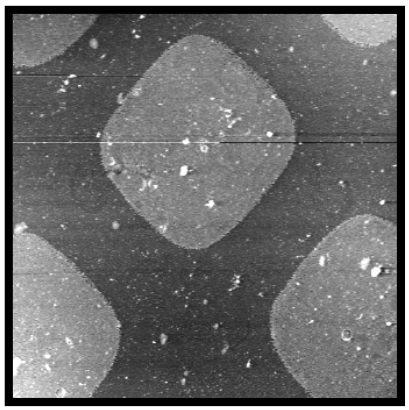


Figure 2

a)



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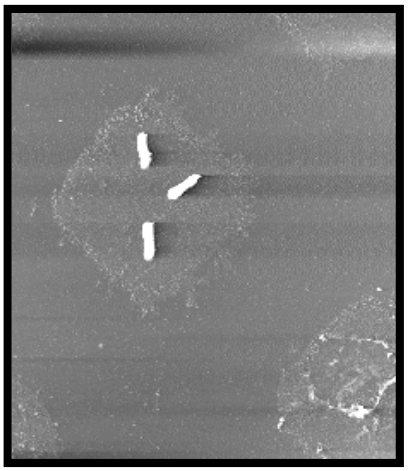


c)

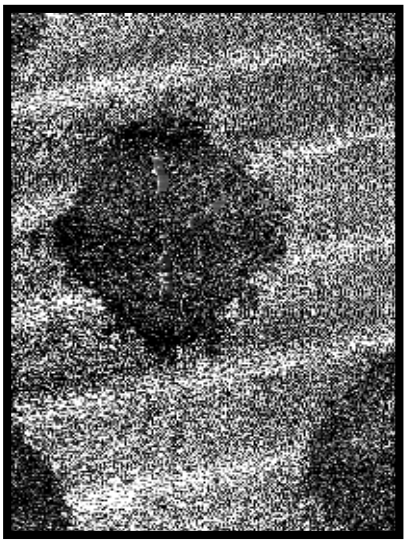


Figure 3

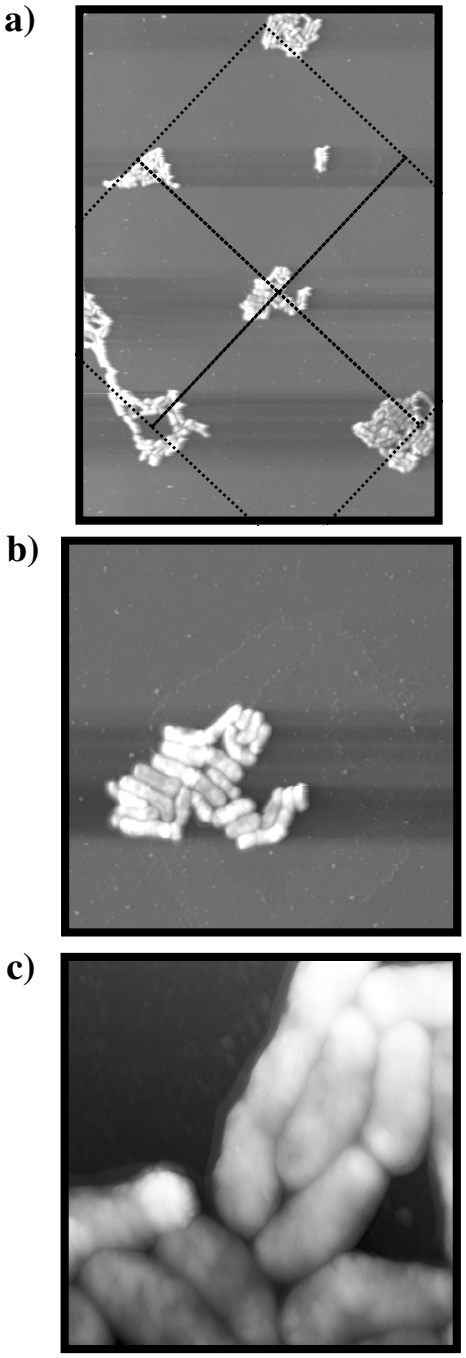
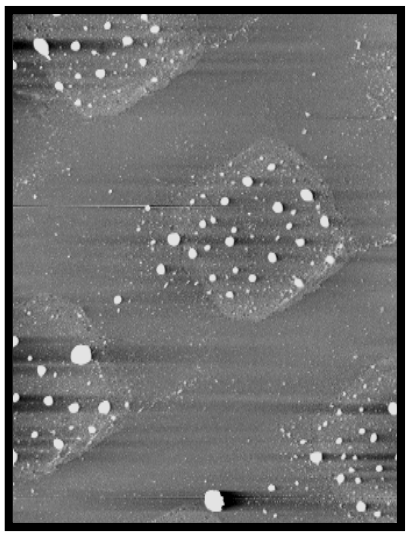


Figure 4

a)



b)

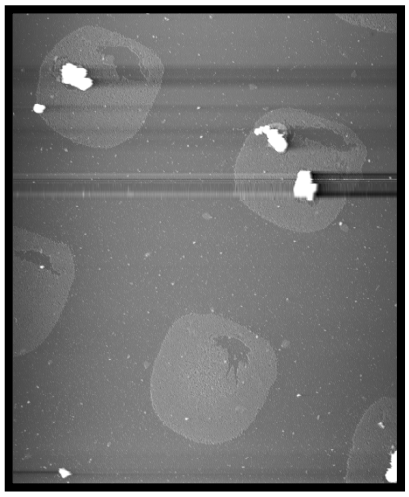


Figure 5