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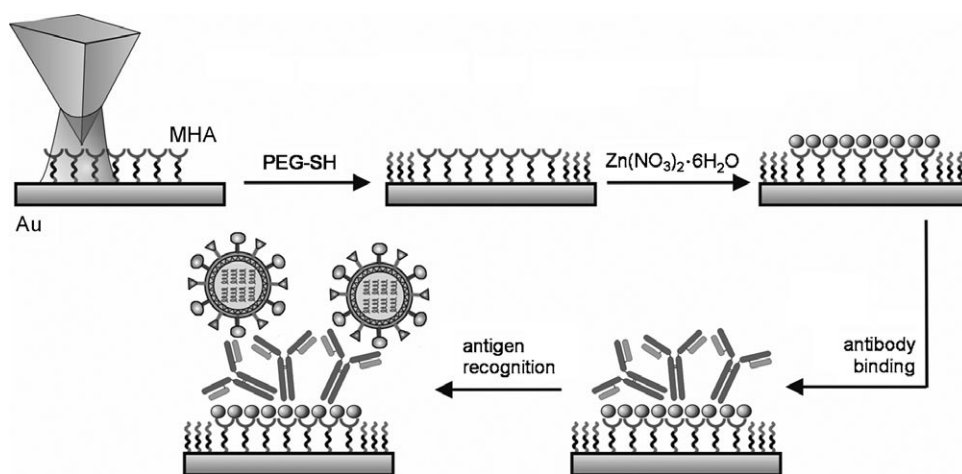
Functional Antibody Arrays through Metal Ion-Affinity Templates

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In the post-genomic era, surface-based proteomics tools in high-throughput formats are becoming crucial for analyzing protein expression, protein–protein interactions, signal-transduction pathways, and the processes underlying cellular functions.^[1] Protein micro- and nanoarrays hold great promise in areas of health-related research,^[2] drug discovery,^[3] and diagnostics in which well-defined features and their spacing are important for studying surface–cellular interactions^[4] and detecting biomacromolecules.^[5] Thus far, a variety of techniques have been developed for immobilizing proteins, specifically antibodies, on surfaces. These techniques have relied primarily on antibody-binding proteins (proteins A, G, A/G, and L),^[6,7] genetic-engineering technologies to produce unnatural binding tags for directed surface attachment,^[8] electrostatically driven adsorption,^[9,10] covalent linking,^[5,11] or a combination thereof.^[12] Although these approaches have been widely used, they have drawbacks ranging from cost and complexity to inactivation of the antibody structures due to denaturation, which leads to poor antigen binding.^[13] Here, we present a novel method that utilizes metal ions to immobilize unmodified antibodies in a way that preserves their biorecognition properties in the context of micro- and nanoarrays. This approach is related to ones used for immobilizing antibodies with metal ions (i.e. Zn^{II}, Cu^{II}, Ni^{II}, Co^{II}) on three-dimensional chromatographic supports.^[14–17]

Researchers have utilized direct-write techniques, such as robotic spotting^[13] and dip-pen nanolithography (DPN),^[18] in combination with metal ions as linking groups to generate oligo-

nucleotide^[19] and virus particle^[20] micro-/nanoarrays. Metal ions as surface linking groups have the advantages of being readily accessible, robust, economical, and stable over long periods of time. Moreover, they are not susceptible to denaturation, as are many of the proteins used for antibody immobilization (i.e. proteins A, G, A/G, and L, etc.). In this report, we demonstrate how the Zn^{II} ion can be used as a versatile linker to immobilize antibodies on surfaces in an active state. This includes the assembly of a large class of unmodified poly- and monoclonal antibodies (goat IgG, rabbit IgG, mouse IgG1, chicken IgY, and mouse IgM) in the context of micro- and nanoscale features generated by microcontact printing (μ -CP)^[21] and DPN, respectively. The activity and utility of metal ion-immobilized antibody arrays are demonstrated with protein and viral antigens. Moreover, we show that one can use nanoarrays as a “litmus-like test” for evaluating the activity of surface-immobilized antibodies. The reduced spot area in a nanoarray demands efficient and relatively uniform immobilization of antibody structures in active states to exhibit uniform activity from spot to spot within the array. With larger features, such as those found in microarrays, inefficient immobilization (i.e. smaller percentage of active antibodies) still leads to apparent uniform activity from feature to feature within the array. Finally, we also demonstrate that we can use this approach to make nanoarrays of antibody structures, such as IgY and IgM, that others have claimed cannot be immobilized in active forms using the protein A/G approach.^[22]



Scheme 1. Schematic illustration of the steps associated with the generation of functional antibody nanoarrays.

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In a typical experiment, 16-mercaptohexadecanoic acid (MHA, Aldrich) features were patterned on a gold thin-film substrate (30 nm Au and 10 nm Ti on a silicon wafer, Silicon Sense, Inc., (Nashua, NH) by DPN or μ -CP (Scheme 1). The regions surrounding these features were then passivated with 11-mercaptoundecylpenta(ethylene glycol) (PEG-SH)^[23] by immersing the substrate in the alkanethiol solution (5 mM in ethanol) for 30 min followed by copious rinsing with ethanol. The passivation layer minimizes nonspecific binding of the proteins or virus particles to the unpatterned areas. The carboxylic acid

groups of MHA were then coordinated to Zn^{II} ions by exposing the substrate to an ethanolic solution of $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (5 mM, 99%, Fluka) for 6 h, followed by rinsing with ethanol (200 proof, HPLC grade), and the substrate was dried under a stream of N_2 to remove any residual metal ions from the surface.^[20,24] The functionalized substrates were finally exposed to a solution of the desired antibody (100 $\mu\text{g mL}^{-1}$) in phosphate-buffered saline (PBS, 10 mM with NaCl (0.15 M), pH 7) for 2 h at room temperature in an air-tight humidity chamber. Excess antibodies were removed by washing the substrates with PBS buffer. The antibody arrays were characterized by tapping-mode AFM (TM-AFM, taken with a Nanoman AFM system, Veeco Instruments) and polarization modulation-infrared reflection-absorption spectroscopy (PM-IRRAS; see the Supporting Information). Furthermore, the presence of Zn^{II} ions before and after the immobilization of antibodies was confirmed by using time-of-flight secondary ion mass spectrometry (TOF-SIMS; see the Supporting Information). The immunoreactivity of immobilized antibodies was confirmed by an increase in AFM height profiles and fluorescence microscopy (Zeiss Axiovert 100 A, an optical microscope equipped with a Zeiss MRC5 CCD camera, Thornwood, NY) upon treatment with the appropriate antigens (proteins/virus particles) and fluorophore-labeled antibodies.

To investigate the immobilization of antibodies onto metal ion-modified arrays, we first studied a polyclonal goat IgG (anti-Udorn)^[25] that specifically recognizes proteins of the influenza virus (A/Udorn/307/72; H3N2), including the major surface antigen, hemagglutinin (HA). Micro- and nanoarrays of anti-Udorn immobilized through Zn^{II} ions were generated as described above (Figure 1 A and Supporting Information). The

TM-AFM images of anti-Udorn nanoarrays confirm adsorption of the antibody and are consistent with the formation of a monolayer of antibodies (a uniform height of 6.5 ± 1.0 nm is observed for each feature; the typical dimensions of an antibody are $8.5 \times 14.5 \times 4.0$ nm).^[26,27] Note that these images were collected under ambient conditions under which contraction of the antibody is expected.^[9]

To evaluate the biorecognition properties of the immobilized anti-Udorn, the arrays were incubated for 2 h at 37°C with a solution of purified influenza virus (A/Udorn/72).^[28,29] Excess virus particles were removed by washing the substrates with PBS, Tween-20 solution (0.05%), and NANOpure water. TM-AFM imaging showed a height increase of 23 ± 2 nm; this is consistent with virus particle assembly on the anti-Udorn features of the nanoarray (Figure 1 B and Supporting Information). In addition, the virus attachment was also screened by fluorescence microscopy. For these studies, an array of anti-Udorn with captured influenza virus particles was treated with anti-HA (mouse IgG1, 12 A5, Covance Inc., Berkeley, CA) for 1 h at 37°C to form a sandwich complex, followed by the addition of a fluorophore-labeled secondary goat anti-mouse IgG (Alexa Fluor 488, Covance Inc., Berkeley, CA) for the same amount of time. Excess antibodies were removed by washing, as previously described. When the arrays were treated with virus particles, green fluorescence was observed on the features (Figure 2 A). However, when the array was not treated with the virus solution, only background signal was observed (Figure 2 B). These substrates were also measured by TM-AFM, and a height increase of approximately 15.1 ± 2.3 nm was observed upon formation of the sandwich complex; this is consistent with both antibodies binding onto the captured virus particles (Figure 2 C).

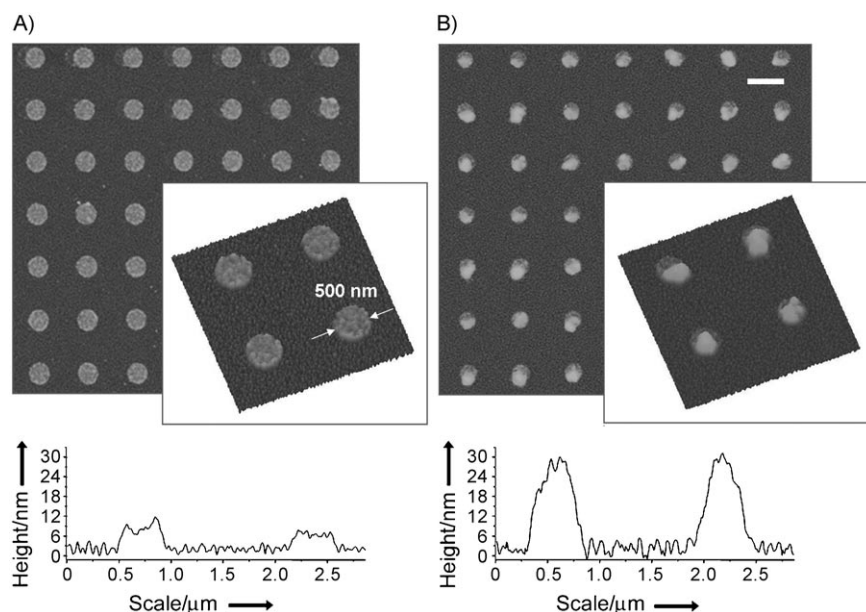


Figure 1. A) TM-AFM topography image and height profiles of anti-Udorn (polyclonal goat IgG) immobilized on 500 nm MHA dot arrays pretreated with $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$. B) TM-AFM topography image and height profile of the anti-Udorn nanoarray after the addition and binding of the influenza virus. All AFM images were taken at the same location (with zoomed-in 3D topographical insets) at a scan rate of 0.5 Hz (silicon cantilever, spring constant = 40 N m^{-1}). Scale bar = 1 μm for the large arrays.

The generality of the metal ion-mediated immobilization scheme was studied with a series of different antibody subclasses. Polyclonal chicken IgY (anti-HA, YPYDVPDYA [influenza HA-epitope], US Biological, Swampscott, MA, in PBS 0.1% Tween 20), polyclonal rabbit IgG (anti-SV5),^[25] monoclonal mouse IgM (anti-estrogen receptor β (ER- β), Sigma-Aldrich), and monoclonal mouse IgG1 (anti-p24, Fitzgerald Industries Inc., Concord, MA) antibodies were assembled on nanoscale metal ion-based affinity templates and exhibited height increases of 5.8 ± 1.3 nm, 8.0 ± 1.2 nm, 2.8 ± 0.5 nm,^[30] and 7.8 ± 1.1 nm, respectively. The biological recognition of the antibodies was probed by incubating the arrays in solutions of their corresponding antigens for 2 h at 37°C . A

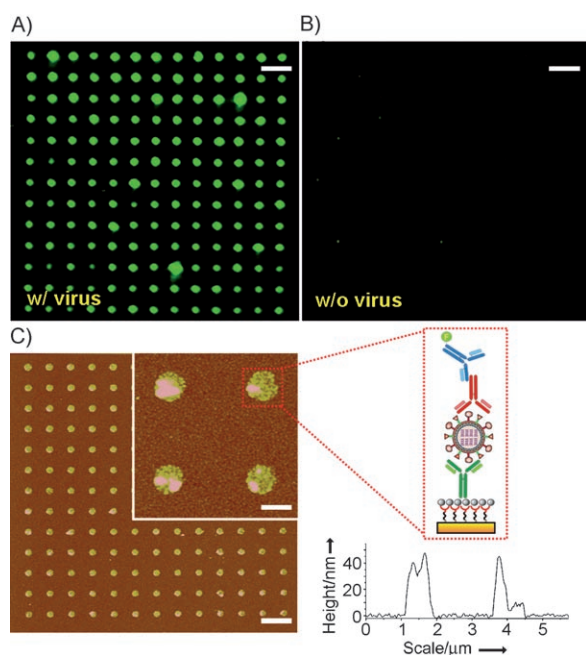


Figure 2. Fluorescence images showing the successful binding of Alexa Fluor 488-labeled goat anti-mouse (secondary antibody) to array features A) treated with and B) without influenza virus particles sandwiched with anti-HA (mouse IgG) on anti-Udorn (goat IgG) nanoarrays immobilized with Zn^{II} -MHA affinity templates. C) The TM-AFM image confirms the binding of the secondary antibody to the sandwiched virus particles. The scale bars on the fluorescence and AFM images are $3\ \mu\text{m}$ for the array and $1\ \mu\text{m}$ for the insert. AFM images were taken at a scan rate of $0.5\ \text{Hz}$.

comparison of the AFM images of all antibody arrays before and after incubation with their corresponding antigen solutions shows antigen binding. Indeed, the height increase of $29 \pm 1\ \text{nm}$ confirms that anti-HA (chicken IgY) recognizes influenza virus particles (Figure 3A and B),^[29] whereas a height increase of $25 \pm 2\ \text{nm}$ shows that the immobilized anti-SV5 binds to SV5 particles (Figure 3C and D).^[29] Similarly, anti-ER- β nanoarray features recognize estrogen receptor β ($10\ \mu\text{g mL}^{-1}$, Sigma-Aldrich) as confirmed by a height increase of $5.6 \pm 1.5\ \text{nm}$ (Figure 3E and F). Fluorescence microscopy also confirmed the presence of fluorescein-labeled HIV-1 p24 ($50\ \mu\text{g mL}^{-1}$; Fitzgerald Industries) on anti-p24 nanoarrays^[31] immobilized through metal ion-affinity templates (Figure 3G and H).

The specificity of our metal ion-immobilized methodology was also tested by exposing an anti-SV5 nanoarray to a solution of tobacco mosaic virus (TMV), a rod-shaped virus that is approximately $300\ \text{nm}$ in length and $18\ \text{nm}$ in diameter. A comparison of the TM-AFM images of the arrays before and after treatment with a solution of TMV ($100\ \mu\text{g mL}^{-1}$, American Type Culture Collections, MA) under the same conditions as before shows no evidence of the virus binding to the immobilized antibodies or any of the exposed Zn^{II} ions on the patterned area, despite the fact that TMV shows an affinity for Zn^{II} (Figure 4A).^[20] Moreover, when a 1:1 mixture of the SV5 and TMV virus particles was incubated with anti-SV5 nanoarrays, only SV5 particle recognition took place on the entire array, as

confirmed by TM-AFM images (Figure 4B). This supports our hypothesis that the Zn^{II} -antibody features are homogeneously covered with captured antigens and indicates that many of the immobilized antibodies are in an active state that results in immunoreactivity.

The ability to sort and detect multiple antigens on the same substrate with negligible cross-reactivity was also confirmed by using this metal-ion approach (Figure 4C and D). Following the aforementioned method, we created two separate microarray patterns of metal ion-based affinity templates using $\mu\text{-CP}$ on the same substrate. One side of the array was then immersed in a solution of anti-cholera toxin (mouse IgG1 ($100\ \mu\text{g mL}^{-1}$), Biodesign International, Saco, ME), whereas the other side was immersed in a solution of anti-trypsin inhibitor from soybean (rabbit IgG ($100\ \mu\text{g mL}^{-1}$), Biodesign International) for 2 h at room temperature. Excess antibody was removed by rinsing the substrates with PBS. The substrate was incubated with a 1% BSA solution in PBS for 30 min to block any unreacted sites on the array. The entire substrate was then challenged with a 1:1 solution of the corresponding fluorophore-labeled antigens (Alexa Fluor 594-labeled cholera toxin subunit B and Alexa Fluor 488-labeled trypsin inhibitor from soybean, Molecular Probes, Eugene, OR ($100\ \mu\text{g mL}^{-1}$ in PBS)) for 1 h at 37°C . After the substrate had been rinsed with PBS, fluorescence microscopy confirmed the specific attachment of labeled antigens to their respective antibody array with no observable cross-reactivity, thus demonstrating that Zn^{II} -immobilized antibodies retain their activity and antigen-recognition capabilities.

An informative comparison is the activity of antibodies immobilized on MHA features with and without the metal ions. Interestingly, on the micron-to-macroscopic length scales, both strategies, in certain cases, will result in immunoreactive features. However, reducing the size of the features to nanoscopic dimensions shows the importance and increased efficiency of the metal-mediated route. For example, when nanoarrays of anti-SV5 were fabricated by DPN (feature size = $300\ \text{nm}$) with and without the Zn^{II} coordination-complex layer and studied in the context of SV5 virus recognition and binding, the arrays without Zn^{II} showed binding to less than 1% (two virus particles imaged per four hundred nanoscopic features ($n=3$)) of the features while those prepared with Zn^{II} showed binding to over 95% of the features (see the Supporting Information). These experiments were also repeated with anti-Udorn on MHA to capture influenza virus particles, and similar results were obtained (data not shown). On the other hand, a similar experiment was also performed with protein A/G to immobilize anti-Udorn so as to capture influenza virus particles, and comparable results were obtained to those prepared by the Zn^{II} -mediated approach (see the Supporting Information). Therefore, nanoarrays allow one to readily observe the efficiency of different types of immobilization methods, and suggest that, for the majority of antibodies studied, the activities of the immobilized antibodies are comparable to the protein A/G route. Interestingly, the Zn^{II} ion approach allows one to immobilize IgY and IgM in their active states when, according to other researchers,^[22] these antibodies cannot be immobilized by protein A/G.

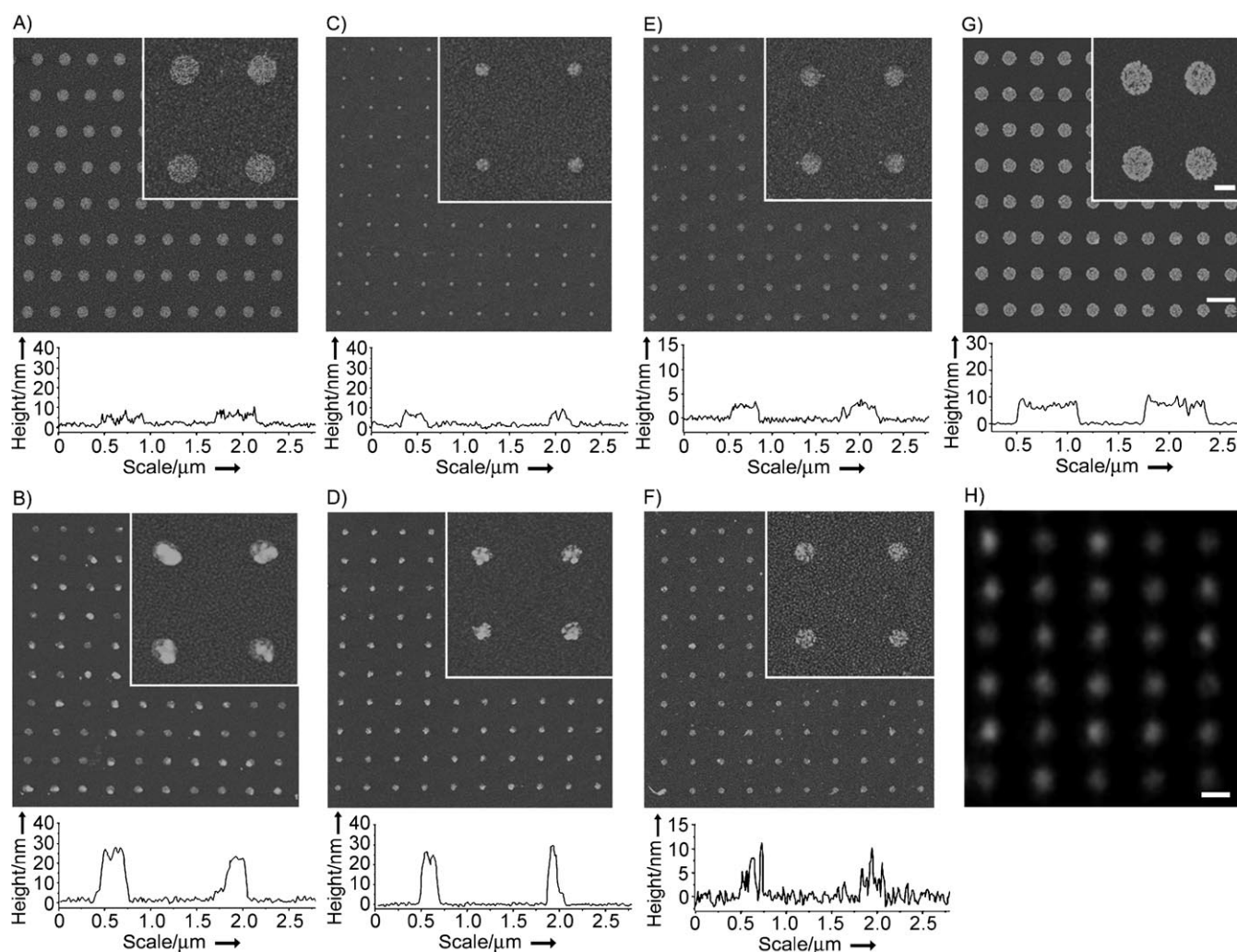


Figure 3. AFM tapping-mode images and height profiles of antibody nanoarrays immobilized with Zn^{II} affinity templates before and after treatment with complementary virus or protein antigens. Anti-HA (chicken IgY) nanoarray A) before and B) after treatment with influenza virus. Anti-SV5 (rabbit IgG) nanoarray C) before and D) after the addition of paramyxovirus SV5. Anti-ER- β (mouse IgM) nanoarray E) before and F) after addition of a recombinant long form of ER- β . G) Anti-p24 (mouse IgG1) nanoarray TM-AFM image before and H) fluorescent image after addition of recombinant p24 (HIV-1)-FITC. The scale bars on the AFM images are 1.5 μm for the large arrays, 450 nm for the insets, and 700 nm for the fluorescence images. AFM images were taken at a scan rate of 0.5 Hz.

In conclusion, we have demonstrated a straightforward and versatile methodology for immobilizing antibodies in the context of micro- and nanoarrays by utilizing Zn^{II} metal ions. While the nature of the chemistry behind how the antibodies are immobilized by the metal ions has not yet been elucidated, and is the focus of current studies, this method allows one to immobilize many of the antibodies that can be immobilized in an active form with proteins A, G, A/G, or L.^[6] It even allows one to immobilize antibodies (e.g. chicken IgY and mouse IgM) that generally have a low affinity for proteins A, G, A/G, or L.^[22] The enormous price differential between the commonly used protein A/G and $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ makes this new methodology a highly practical consideration for antibody immobilization in the context of ELISAs, protein chips for proteomic analysis, particle probe development in protein diagnostics, and protein arrays.

Experimental Section

DPN-patterning experiments were carried out with an AFM (CP, Veeco/Thermomicroscopes, Sunnyvale, CA) equipped with a 100 μm scanner, closed-loop scan control, and commercial lithography software (DPNWriteTM, DPN System-1, Nanoink Inc., Chicago, IL). Gold-coated commercial AFM cantilevers (sharpened, Si_3N_4 , type A, Nanoink Inc.) with a spring constant of 0.05 Nm^{-1} were used for patterning and subsequent imaging. All DPN patterning experiments were carried out under ambient conditions ($\sim 30\%$ relative humidity, 24°C). Tips were soaked in a solution of MHA in acetonitrile (10 mM) and then blown dry with N_2 . MHA features were generated on a gold thin-film substrate by traversing the tip over the surface in the form of the desired pattern.

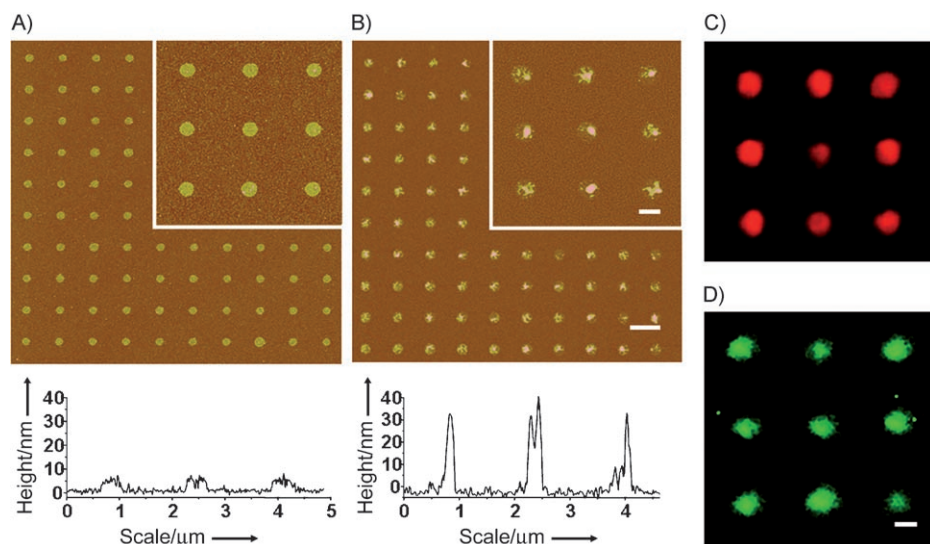


Figure 4. A) TM-AFM image of the anti-SV5 on a Zn^{II}-MHA nanoarray after the addition of the TMV (note the lack of TMV on the features). B) TM-AFM image of the anti-SV5 on a Zn^{II}-MHA nanoarray after the addition of SV5/TMV (1:1). The insets of all AFM images show enlarged topographic images of 3 × 3 dot arrays. C) Fluorescence image of Alexa Fluor 594-labeled cholera toxin subunit B, and D) Alexa Fluor 488-labeled trypsin inhibitor from soybean (1:1) captured on their respective antibody microarrays, imaged on the same substrate with negligible cross-reactivity. Scale bars of AFM images are 1.5 μm for the large arrays, 500 nm for the insets, and 2.5 μm for the fluorescence images. AFM images were taken at a scan rate of 0.5 Hz.

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