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Immobilization of Histidine-Tagged Proteins on Nickel by Electrochemical Dip Pen Nanolithography

Gunjan Agarwal,[†,‡] Rajesh R. Naik,[†,‡] and Morley O. Stone*,[†]

Contribution from the Materials and Manufacturing Directorate, Air Force Research Laboratory, Wright-Patterson Air Force Base, Ohio 45433, and Technical Management Concepts, Inc., Beavercreek, Ohio 45434

Received December 20, 2002; E-mail: morley.stone@wpafb.af.mil

Abstract: Dip-pen nanolithography (DPN) is becoming a popular technique to "write" molecules on a surface by using the tip of an atomic force microscope (AFM) coated with the desired molecular "ink". In this work, we demonstrate that poly-histidine-tagged peptides and proteins, and free-base porphyrins coated on AFM probes, can be chelated to ionized regions on a metallic nickel surface by applying an electric potential to the AFM tip in the DPN process. DPN has been accomplished in the Tapping Mode of AFM, which creates many possible applications of positioning and subsequently imaging biomolecules, especially on soft surfaces.

Introduction

The immobilization of biomolecules on substrates is a potentially important prerequisite for the fabrication of biosensors and/or nanotechnology devices. Various strategies for linking biomolecules to solids involve covalent interactions such as thiols to gold,[1−2] acrylamides to silanized surfaces,[3] and amines to aldehyde-treated surfaces.[4−5] These interactions generally require tailoring the biomolecules to include a specific functional moiety. Immobilization via naturally occurring electrostatic attractions, though popular for imaging purposes,[6−7] is unsuitable for most device applications where the samples need to undergo multiple solution/buffer treatments that can disrupt or weaken the electrostatic binding. A relatively strong natural bond is the nickel−histidine (Ni−His) bond commonly used in immobilized metal-affinity chromatography (IMAC). IMAC is a well-established technique for protein purification based on the observation that proteins with exposed histidine and cysteine side chains have a high affinity for certain metal ions[8] like Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}, and Mn^{2+}. Protocols for immobilization utilizing the Ni−His bond have been based on the commonly used chelator for nickel, nitrilotriacetic acid (NTA).[8−12] However, the surface roughness and nonuniformity of Ni−NTA coatings on glass slides is not appropriate for nanoscale deposition and patterning of biomolecules.[13] Nickel-treated mica has been used as a smoother alternative[14] but is unsuitable for solvent treatment after immobilization, as the nickel ions adsorbed on the mica surface wash off.

Here, we demonstrate the nanoscale patterning of histidine-tagged (His-tagged) peptides and proteins to metallic nickel surfaces using electrochemistry at the probe (tip) of an atomic force microscope (AFM). The AFM has been previously utilized to write on surfaces by a technique now commonly known as Dip-Pen Nanolithography (DPN).[15−16] Our process resembles the DPN technique conducted in the presence of electric fields, henceforth termed as electrochemical DPN (E-DPN).[17−18] In addition, all DPN/imaging experiments have been carried out in the Tapping Mode of AFM, a mode more conducive to biological imaging.[13] The AFM tip is coated with peptide/proteins and scanned at slow speeds, with a negative bias applied to the tip and a nickel-coated substrate held at ground potential. As in the DPN process, a small water meniscus is formed at the end of the AFM probe due to capillary condensation. The potential applied between the probe and substrate results in the ionization of the nickel surface. The six histidine (hex-His)

* Corresponding author.
† Wright-Patterson Air Force Base.
‡ Technical Management Concepts, Inc.

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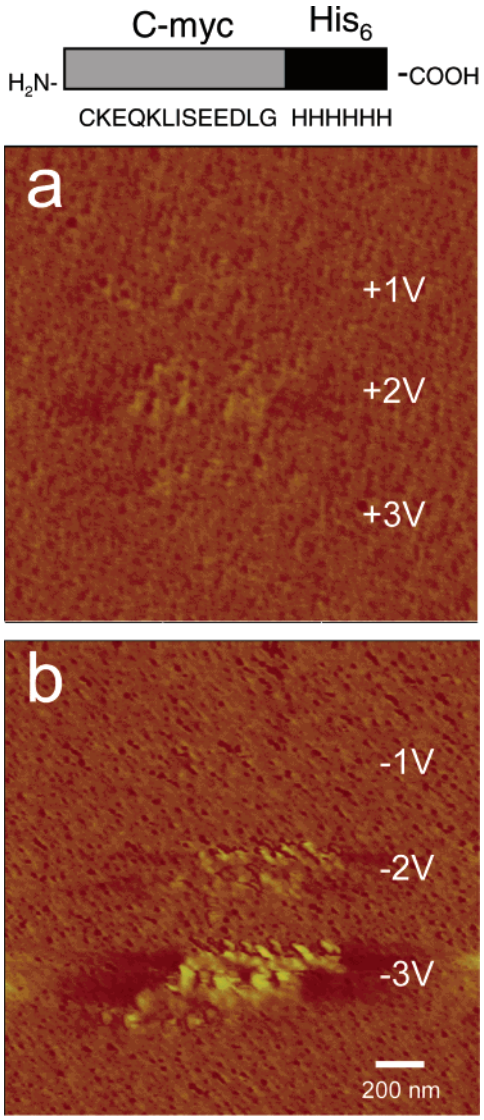

Figure 1. AFM height images of E-DPN with the peptide MH2 (shown above) on metallic nickel surface at various potentials (indicated). Negligible deposition is observed between −1 and +3 V. Potential range for optimal deposition was between −2 and −3 V.

tagged peptide/protein or a free-base porphyrin coated on the AFM tip dissolves in the water meniscus and attaches to the ionized nickel surface. The process presented here offers finer patterning capabilities than that obtained by using electrochemistry in nanopipets for biomolecular deposition.[19]

Experimental Section

Materials. The peptide MH2 was chemically synthesized by New England Peptide, Inc. (Fitchburg, MA). The histidine-tagged proteins, TlpA,[20] its truncated mutant TlpA-8 and the green fluorescent protein (GFP) fused with R5 peptide[21] (henceforth called GFP−R5) were expressed in bacteria and purified using Ni−NTA affinity resin.[22] The purified proteins were dialyzed against distilled water. Protein purity was >90%, as determined by SDS-poylacrylamide gel electrophoresis.

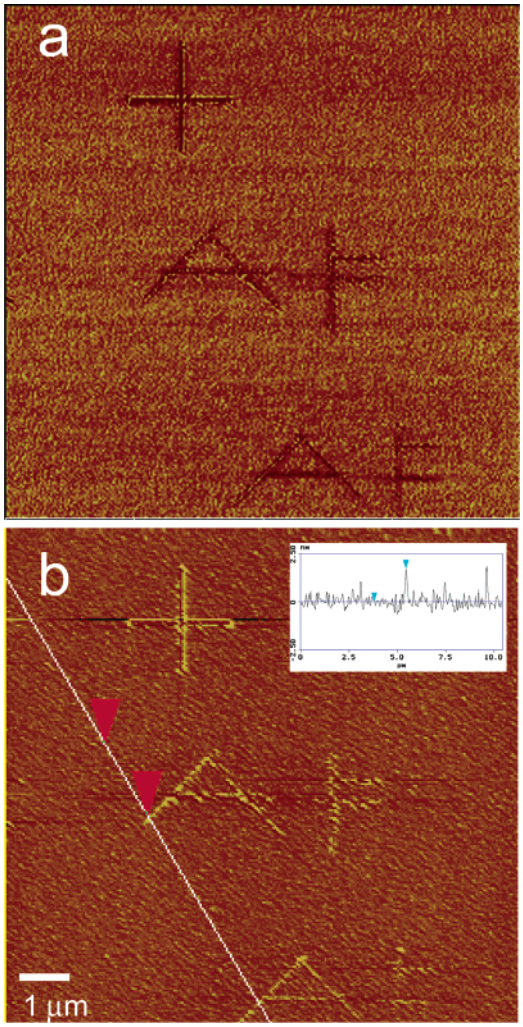

Figure 2. Patterns written with MH2 peptide using E-DPN on a metallic Ni surface. Both (a) phase and (b) height images show clear contrast in the patterned area. Height of the patterns estimated by a line across it was ∼1.0−1.7 nm (inset, b). The three patterns were written consecutively from top to bottom. The patterns written later are weaker in contrast as compared to earlier ones due to depletion of ink from the probe.

The free-base of tetra(*N*-methylpyridinium)porphyrin chloride salt, synthesized via Rothmund condensation by use of a modified Adler procedure was used.[23−24] Muscovite mica, grade 1 was from S & J Trading Inc., NJ. Nickel coating (50−70 nm) on freshly cleaved mica was performed using a thermal evaporator (Edwards). Nanoscope IIIa Multimode Scanning Probe Microscope (Digital Instruments, Santa Barbara, CA) was used for all AFM/DPN experiments. NSC15 probes for Tapping Mode AFM, were purchased from MikroMasch (Estonia).

Sample Preparation. All solutions were made in doubly distilled deionized water at a concentration of ∼20 μg/μL (MH2), 1.0 μg/μL (TlpA), 1.3 μg/μL (TlpA-8), 1.5 μg/μL (GFP−R5) and 25−35 μg/μL (porphyrin). AFM probes were dipped into the desired solution for 15−30 s and air-dried. Freshly cleaved mica coated with metallic nickel was used as the substrate.

DPN/AFM Experiments. All experiments were carried out using Tapping Mode in ambient air. Silicon cantilevers with a nominal spring constant of 40 N/m were used at a frequency of 312 to 320 Hz. Scanner type used was J; scanning rate was 2−3 Hz for imaging and height and phase images were recorded with 256 lines per scan direction. Electric potentials ranging from −10 to +10 V could be applied to the

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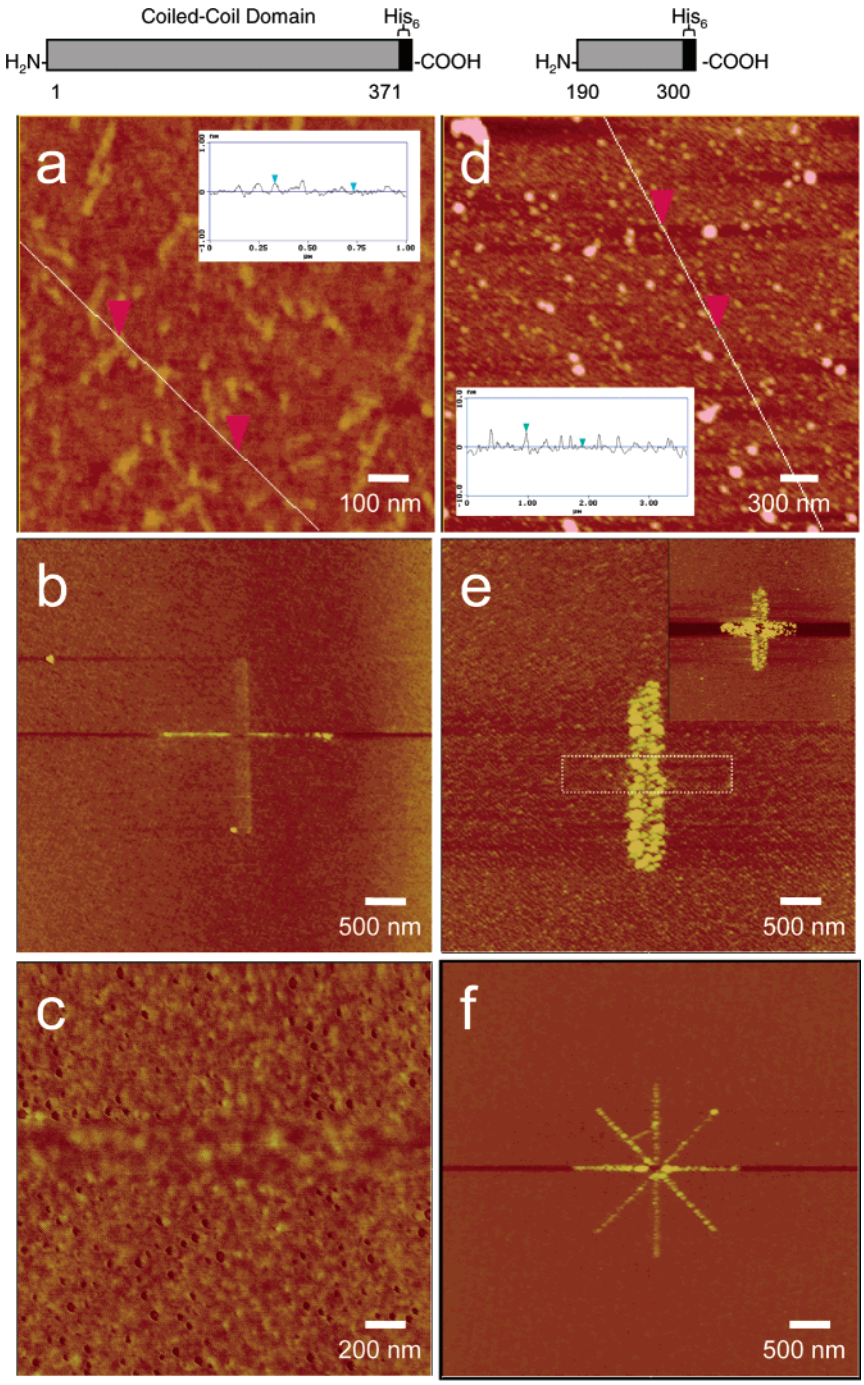

Figure 3. Patterning of TlpA and its truncated mutant, TlpA-8 on nickel via E-DPN. (a) AFM height image of TlpA on mica shows filaments <1 nm in height (inset) and 100−300 nm long. (b) Patterning with TlpA on nickel. (c) A magnified image of the patterned region shows structures similar to those in panel a, but the contrast in the image is diminished due to granularity of the metallic nickel surface. (d) AFM height image of TlpA-8 on mica shows globules 2−5 nm in height (inset). In patterned regions on nickel, an identical morphology of the protein globules is seen. In panel e, the missing horizontal line of "+" was written at zero potential and the vertical line at −2 V. Inset e, an identical "+" written with −2 V along both directions. (f) A thin array of globules of TlpA-8 deposited on nickel.

probe and the substrate was always held at ground potential. As demonstrated earlier,[13] a high drive-amplitude (∼5 to10 times that used for imaging) and slow scan speeds (∼0.5 Hz) enabled DPN in Tapping Mode. Patterns could be written using appropriate scan sizes, scan angles and aspect ratios.[13]

Results

Deposition of Peptide. In DPN experiments, when probes coated with the His-tagged peptide, MH2, were used at zero potential on a nickel surface, no deposition was observed (see Supporting Information, Figure S1). Using a high force (drive amplitude) simply resulted in indentation of the surface as observed by the dark regions in the height images. Similarly, with native, uncoated probes, there was no evidence of deposition (in height images) on nickel surfaces upon the application of potentials. The phase images exhibited a contrast in the regions where potential was applied, suggesting that the nickel surface was being ionized (see Supporting Information, Figure S2). These observations motivated us toward the development

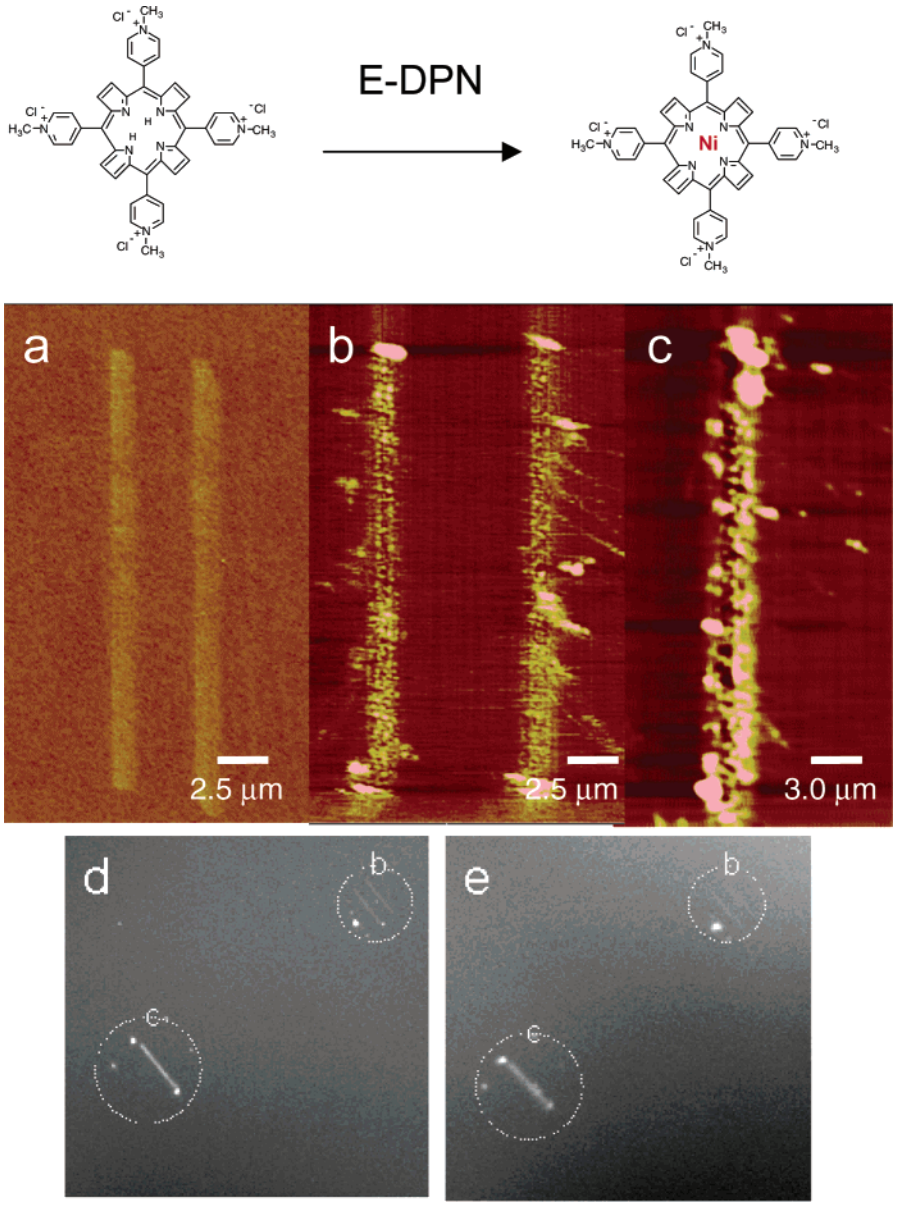

Figure 4. AFM images of porphyrin patterns deposited using E-DPN for a period of (a) 1, (b) 4 (left) and 6 (right), and (c) 30 min. (d) Fluorescence microscopy images of the E-DPN patterns. The pattern in panel a could not be detected, while patterns in panels b and c show an increase in fluorescence intensity as deposition times are increased. (e) Patterns imaged using fluorescence microscopy after washing.

of an E-DPN technique, where the nickel surface could be ionized for binding of His-tagged proteins and/or peptides.

E-DPN of MH2 on a nickel surface was carried out at various probe potentials using a peptide-coated probe (Figure 1). Little or no deposition was observed in the range −1 to +3 V. A potential less than −2 V resulted in the rapid, uncontrollable deposition of the peptide; therefore, −2 to −3 V was determined as the optimum potential for deposition. Figure 2 shows patterns of MH2 written via E-DPN. The phase and height images show clear contrast in the patterned regions. The vertical height of the patterns ranged from 1.0 to 1.7 nm.

E-DPN of Proteins. To explore if Ni−His binding achieved using electrochemical DPN is applicable to larger peptides and proteins, a thermally active protein TlpA[20] and its truncated mutant, TlpA-8, both with a carboxy terminus His tag were used. TlpA, an autoregulatory repressor in the bacterium *Salmonella typhimurium*, is a 43 kDa, alpha-helical coiled-coil protein that makes use of a rapid structural change in its coiled-coil motif to sense temperature changes.[20] Immobilization and patterning of this protein on a solid substrate could be of potential use in a thermal sensor. TlpA self-assembles to form filaments <1 nm in height and 100−200 nm in length,[20] as verified by AFM images (Figure 3a and inset). E-DPN of full length TlpA, with an attached His tag was accomplished (Figure 3b,c). The patterned regions exhibited a height consistent with the height of TlpA filaments. We have identified a smaller (∼12 kDa) region of TlpA, amino acid residues 190−300 (herein called TlpA-8), which appears to possess the same thermal sensing properties as full length TlpA.[25] AFM images of TlpA-8 on mica show globular particles 2−5 nm in height and few larger aggregates, ∼10 nm in height (Figure 3d). The self-assembly of TlpA-8 into globular entities as opposed to the filamentous structure of TlpA is possibly due to the shorter coiled-coil domain. E-DPN of TlpA-8 showed similar characteristic

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globular features when patterned on the nickel surface. The deposition is sensitive to the potential applied to the tip, consistent with the results obtained using the peptide MH2. When DPN is performed at zero bias, no deposition is observed (missing horizontal line in Figure 3e). For Ni−His binding to occur, a potential of ∼ −2 V is required (Figure 3e, inset). Thinner (20 nm) lines show an array of the deposited globular protein (Figure 3f). The presence of a His tag was confirmed as a requisite in these E-DPN experiments, as peptides and proteins without a His tag did not show similar deposition on nickel (see Supporting Information, Figure S3).

Fluorescence of E-DPN Patterns. As a verification of the deposition process, a histidine-tagged GFP−R5 fusion protein was used. GFP is a widely used fluorescent marker with a strong excitation peak at 398 nm and emission at 509 nm. GFP−R5 was deposited using the E-DPN process on nickel and fluorescence of the patterned regions could be detected using fluorescence light microscopy before and after washing the patterned sample with water (see Supporting Information, Figure S4). To confirm the fluorescence in the E-DPN patterns, a smaller molecule which is a strong chromophore like a free base, water-soluble porphyrin was used in the E-DPN experiments (Figure 4). Porphyrins are an important class of molecules that regulate many biological processes such as the transport and activation of oxygen.[26] Free-base porphyrins are strong fluorophores and can be functionalized with metal ions (such as Ni^{2+}) and yet preserve their fluorescent properties.[27] E-DPN was used to functionalize and immobilize the free base porphyrin (see Figure 4, top) to the nickel surface. To achieve detectable fluorescence intensity, it was necessary to rewrite the patterns several (>20) times in order to obtain a greater molecular density. E-DPN patterns created using porphyrin could be seen under a fluorescence microscope using filters with an excitation wavelength of ∼440 nm and an emission of ∼650 nm. The fluorescence in the patterns remained intact even when the substrates were imaged after washing with water, confirming the stable immobilization of the molecules onto the metallic nickel surface.

Discussion

Our results demonstrate that E-DPN in the Tapping Mode can be used to immobilize biomolecules via novel linker chemistry. Attachment of His-tagged peptides/proteins to a metallic nickel surface requires ionization of the nickel surface. Optimum deposition was found to occur ca. −2 to −3 V. These observations are consistent with the standard potential (1.593 V) of NiO_2 being reduced to Ni^{2+}, which is inevitable as a layer of NiO_2 is always present on the nickel surface when exposed to ambient air and applying a potential in the E-DPN process results in hydrolysis of the water meniscus formed at the AFM tip.[28] Our proposed reaction scheme for the electrochemistry occurring at the interface of the nickel substrate and the AFM tip is[29]

$$NiO_2 + 4H^+ + 2e^- \rightarrow Ni^{2+} + 2H_2O$$

Diffusion experiments in E-DPN, where the probe was held at a particular spot and MH2 was allowed to diffuse from the tip, showed no deposition of peptide even with the application of various potentials (data not shown). We found that it was necessary for the biased tip to scan and ionize the area in order for the deposition of peptide to occur via histidine binding to exposed nickel ions.

We preferred to use native, un-functionalized silicon AFM probes as other molecules present on the probes could possibly interfere with the electrochemistry occurring between the proteins and nickel surface. We found that using a gold-coating on tips interfered with the protein deposition as gold from the tips could be deposited onto the substrate (data not shown). Additionally, the molecules used in this work could adhere to the native silicon tips and functionalization of the tips was not necessary.

The vertical height of the patterned peptide, MH2, is consistent with the length of the peptide and suggests that the peptide is attached via histidine residues at one end and is standing upright on the nickel surface. Hence, by appropriately positioning the His tag, one can possibly control the orientation of the biomolecule immobilized on the nickel surface, as opposed to a horizontal/random orientation when the deposition is mediated through electrostatics.

The protein morphology in the patterned regions was identical to morphology in the native, unpatterned regions. Furthermore, the GFP−R5 and porphyrin patterns could be identified using fluorescence microscopy before and after washing the patterned substrates. These results suggest that the E-DPN process does not adversely affect the native properties of the deposited molecules but enables stable immobilization of the molecules.

Conclusions

We have demonstrated the precise, patterned and stable immobilization of peptides, proteins and small synthetic molecules on a metallic nickel substrate. Our results demonstrate that electrochemistry at the nanoscale can be used to achieve immobilization via metal chelation (eg. Ni−His) and/or metallochemistry. The Ni−His bond can provide strong linker chemistry based on the hex-His repeat incorporated in recombinant biomolecules. Moreover, orientation of the molecules can be controlled by suitably positioning the His tag at the end of the molecule. Additionally, E-DPN carried out in the Tapping Mode is advantageous for patterning on soft surfaces—an important aspect to patterning polymers and/or biological macromolecules. The technique described herein enables a novel method for the controlled patterning of proteins on surfaces for the purposes of biosensors, biophysics and nanotechnology.

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Supporting Information Available: Experiments illustrating necessity of an electric potential and a His tag on the molecule for Ni−His binding are available as Figures S1, S2, and S3. E-DPN experiments using GFP-R5 are illustrated in Figure S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supplemental Information

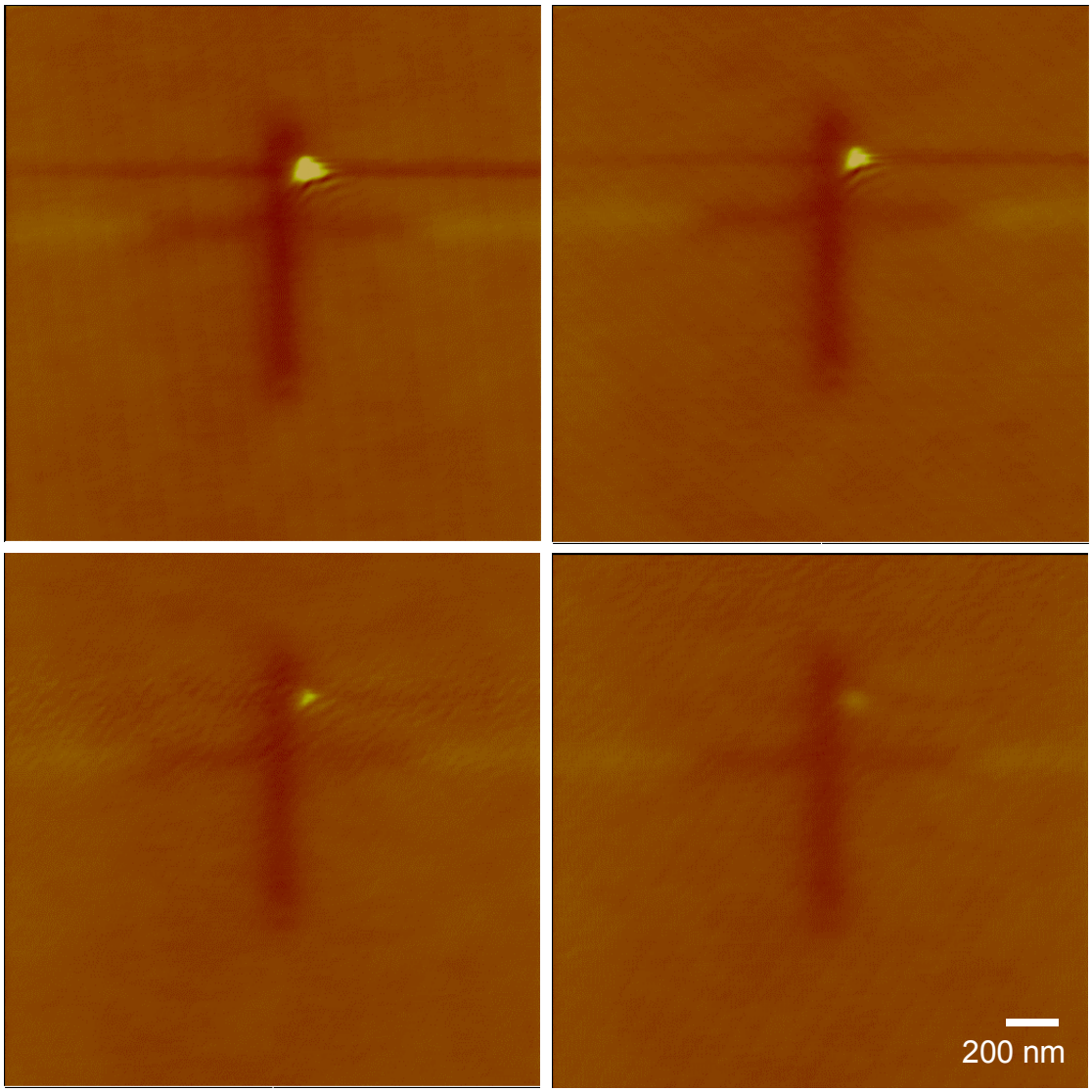


Figure S1 AFM height images of E-DPN conducted on metallic Ni surface with Si probes coated with His-tag peptide at zero potential. No deposition occurs as observed in height images. Large force (drive amplitude) used in these experiments scrapes (indents) the surface. The white globular particle is most likely a water droplet, which evaporates upon repeated scanning.

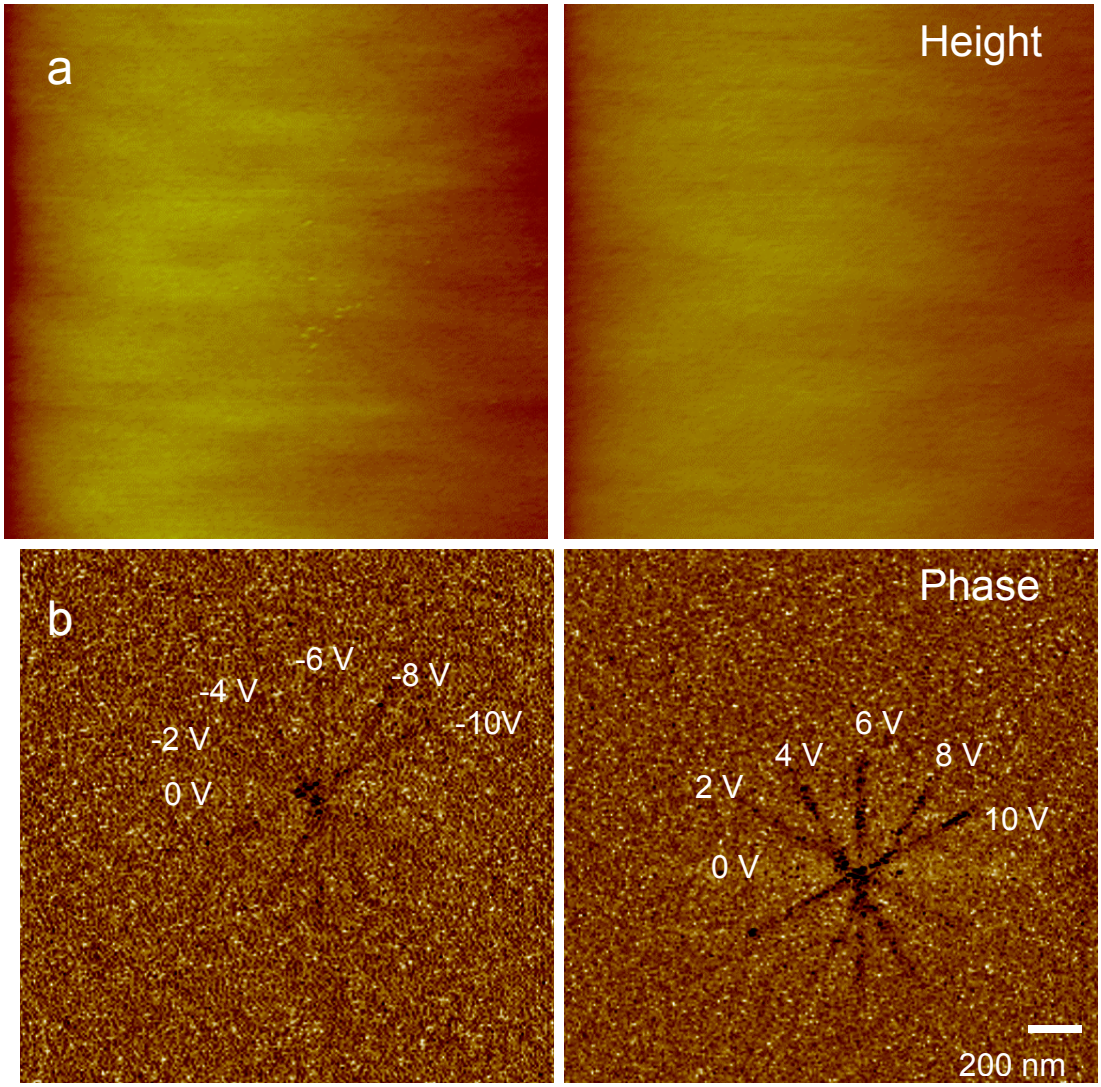


Figure S2. AFM images of E-DPN conducted with native, uncoated Si probes on metallic nickel surface. No deposition was observed as seen by lack of topographical features in (a) height images. Phase images (b) of the corresponding regions exhibit a contrast in the patterned regions, indicating ionization of the nickel surface.

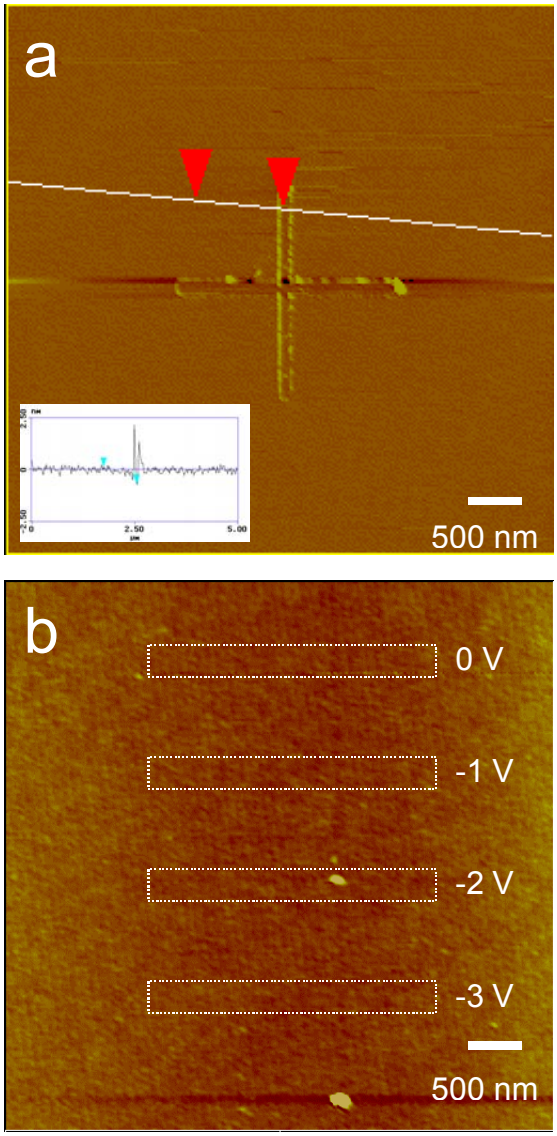
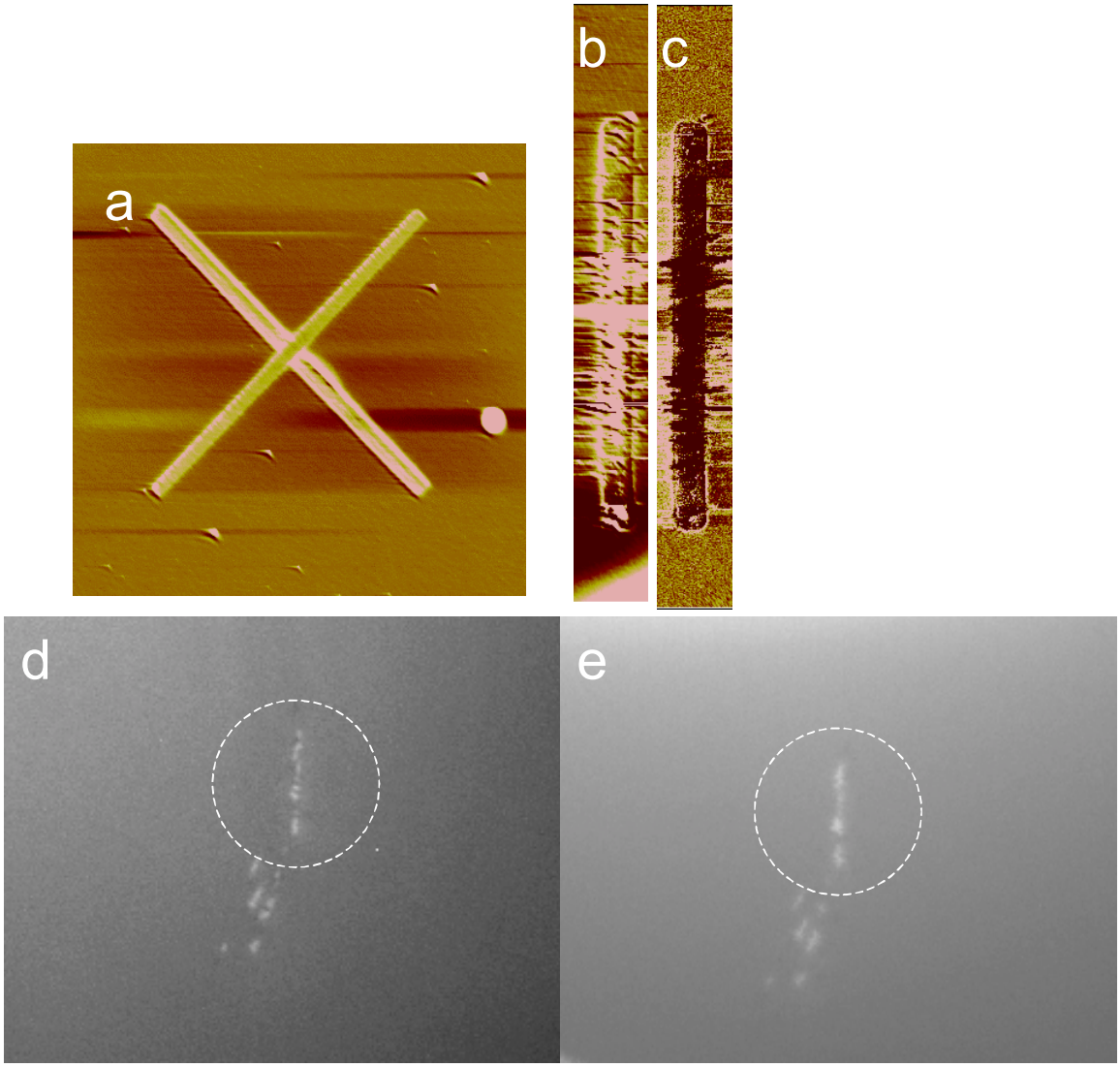


Figure S3. (a) E-DPN with a peptide without a His-tag. No deposition occurs. Using a large force results in surface scratching leading to scraping of the peptides onto the scratch edges when the AFM tip is lifted off the surface. Height profile shows a dip (indent) in the surface with deposition only on the edges. (b) E-DPN of collagen type 1 without His-tag. No deposition of the protein occurs even as the potentials are varied.



S4: E-DPN of histidine tagged GFP-R5 on nickel. (a) AFM height image of an "x" with each axis of length 25 μm written for 5 minutes. (b) AFM height and (c) phase image of a vertical 25 μm line written for 10 minutes. Pattern in (a) was not detectable using fluorescence light microscopy. Fluorescence light microscopy images of the pattern in (b) and (c) using λ_{ex} ~400 nm (d) before and (e) after washing.