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Monitoring the Activity of Tyrosinase on a Tyramine/Dopamine-Functionalized Surface by Force Microscopy

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ABSTRACT

Tyrosinase activity is monitored by π-donor−acceptor force interactions between a bipyridinium-modified AFM tip and the biocatalytic reaction product generated on a tyramine- (or dopamine-) modified surface. Upon oxidation of the surface to dopaquinone as a result of tyrosinase activity, force interactions are switched "OFF". After reduction of the resulting surface with ascorbic acid, forces are quantitatively reestablished as a result of the formation of the dopamine-functionalized surfaces. The method provides a general approach to design biosensors using force interactions as the readout signal.

Chemical force microscopy (CFM) is an analytical tool that detects[1−5] bond-rupture events using a modified atomic force microscope (AFM) tip and quantifies intermolecular forces with single-molecule sensitivity. Primarily, this technique has been used to study force interactions in biological systems such as the separation of complementary DNA strands,[6−8] antigen−antibody complexes,[9] protein−ligand binding,[10−17] or carbohydrate−protein interactions.[18] Force measurements on biomolecules have shown[18] that mechanical stresses alter the structure of proteins and the activities of biomolecules. Thus, force must be taken into account as another important physical parameter that controls biological functions. Force microscopy has been extended to the study of noncovalent interactions such as hydrogen bonding,[19−21] hydrophilic−hydrophobic,[22−24] ionic,[25−28] and recently, donor−acceptor interactions.[29−30] The understanding of supramolecular force interactions has significant consequence in developing sensors,[31−33] "smart" surfaces of controlled wettabilities,[34] and the design of optimized molecular machines.[35]

Studying proteins by way of single-molecule CFM has led to important insights into biomolecular binding and activity. Typically, a surface is functionalized with the enzyme of interest at low concentration, and the rupture forces measured upon separation of the interfaces have been used to assess the substrate binding strengths, mechanism, and the effects of mechanical stress on enzyme activity. Several studies have analyzed biotin−avidin complexes by CFM.[10−12] By functionalizing an AFM tip with avidin and an agarose bead with either biotin or iminobiotin, researchers[10] were able to quantitatively determine differences in rupture forces required to retract the tip from the different beads, and these differences were attributed to differential binding strengths between the avidin and the two substrates. Also, CFM provided new insights[16] into the molecular mechanism of heptaprenyl diphosphate synthase. In this study, subunit I of the enzyme was attached to the surface and subunit II to the tip. Force interactions between the functionalized surface and tip were only detected in the presence of Mg^{2+}, thereby demonstrating that Mg^{2+} was necessary for enzymatic activity, a hypothesis which had been previously unconfirmed. A further study elaborated[17] the role of mechanical stress on the activity of angiostatin. Within the extracellular matrix, where angiostatin is active, forces of 5.5 nN μm^{-1} are generated and these can unfold proteins. By anchoring one end of the angiostatin peptide chain onto a surface and the opposite end to an AFM tip, it was revealed that a partially unfolded structure is the active form of the enzyme, thereby demonstrating a mechanical trigger for biocatalytic activity. The present study differs from previous reports in two aspects: (1) in this study, we detect interactions that are the result of the activity of an enzyme rather than carrying out direct measurements on the biomolecule, thereby using force microscopy as a biosensor, and (2) we measure forces that result from many weak interactions, which, in turn, lead to an amplified signal for detection that is stronger than would originate from a single-molecule measurement.

One of the goals of nanobiotechnology[36] is to use biomolecules to create emergent properties on the nanometer scale. Tyrosinase catalyzes[37] the oxidation of tyramine to dopaquinone (Scheme 1), thus transforming a π-donor

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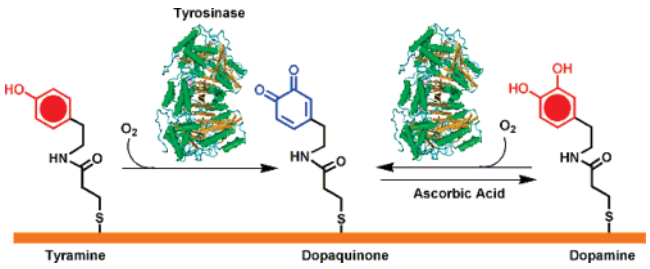
Scheme 1. Tyrosinase-Promoted Conversion of the π-Electron Donor, Tyramine-Modified Surface, to the π-Electron Acceptor, Dopaquinone[a]



[a] Addition of ascorbic acid regenerates a π-electron donor, dopamine. The molecule can be cycled from donor to acceptor by successive oxidation/reduction cycles.

tyramine-modified surface to a π-acceptor dopaquinone-functionalized interface. In the present study, we use a bipyridinium π-acceptor-functionalized tip to probe the force interactions between the tip and the modified surface that is subjected to the biocatalytic process. The tyrosinase-induced oxidation of the tyramine-modified surface results in the turning "OFF" of attractive interactions between the π-donor-functionalized surface and a π-electron acceptor-functionalized AFM tip, thus enabling the detection of the tyrosinase activity. The presence or absence of such forces, which is the direct result of tyrosinase activity, are readily detected by CFM because the ensemble of many weak interactions between complementary groups on the tip and the surface results in significant forces. It should be noted that the detection of tyrosinase has important analytical implications because elevated levels of tyrosinase appear in melanoma cancer cells, and it is considered a biomarker for these cells.[38] Previous studies have reported on the development of electrochemical[39] or optical[40−41] biosensors for the detection of tyrosinase. The present report introduces a new method to detect enzyme activities at the nanoscale.

Functionalization of Surfaces. *N*,*N*′-Dialkyl-4,4′-bipyridinium-coated cantilevers were prepared by cleaning gold-coated AFM cantilevers (Mikromasch) with a 10 min exposure to ozone, followed by washing with pure EtOH and H_2O. The cantilevers were immersed in a 1 mM solution of 2,2′-dithio-bis(ethaneamine) (cystamine) in H_2O by following[42] a literature procedure. After washing with EtOH and H_2O, the cantilevers were immersed in a 1 mM solution of *N*-methyl-*N*′-undecanoic acid-4,4′-bipyridiunium[43] and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (3 mM) in 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES) buffer (10 mM, pH = 7.5) for 2 h. The cantilevers were washed with H_2O, pure EtOH, and then dried prior to use. Tyramine- and dopamine-coated Au surfaces were prepared[40] by cleaning gold-coated glass surfaces (Evaporated Coatings, Willow Grove, PA) with Piranha solution (3:1 H_2SO_4:H_2O_2 (30% aq)) for 3 min and washed thoroughly with H_2O. The clean Au surfaces were reacted with an 3 mM DMSO (dimethyl sulfoxide) solution of 3,3′-dithio-bis(propionic acid)-*N*-hydroxysuccinimide ester for 2 h.[44] After rinsing thoroughly with H_2O, the cystamine-modified surfaces were reacted with a solution of either dopamine or tyramine (1 mM) for 1 h. The surfaces were rinsed thoroughly with H_2O and EtOH prior to use. Enzymatic oxidation of surfaces was carried out by immersing the π-donor-functionalized surfaces in a stock solution of tyrosinase (0.5 mg mL^{-1}, 200 units of activity) that was prepared in phosphate buffer (pH = 6.5, 10 mM) and was used for no longer than 1 week. For each oxidation, 20 μL of stock solution were diluted into 0.5 mL of phosphate buffer (5 units activity). The donor-functionalized Au surfaces were removed from the liquid cell, immersed in the dilute enzyme solution for 40 min, washed thoroughly with water, pure EtOH, and then dried before forces were measured. The ascorbic acid reduction of surfaces was carried out by immersing the quinone-functionalized Au surface in a solution of ascorbic acid (20 mM) in phosphate buffer (10 mM, pH = 6.5) for 30 min. The surface was washed thoroughly with H_2O, pure EtOH, and then dried before forces were measured.

Acquisition of Force Curves. Force measurements were carried out at room temperature using a Multimode scanning probe microscope with a Nanoscope 3A controller and a Pico Force module (Digital Instruments/Veeco Probes/Santa Barbara, CA). The spring constants of the cantilevers were

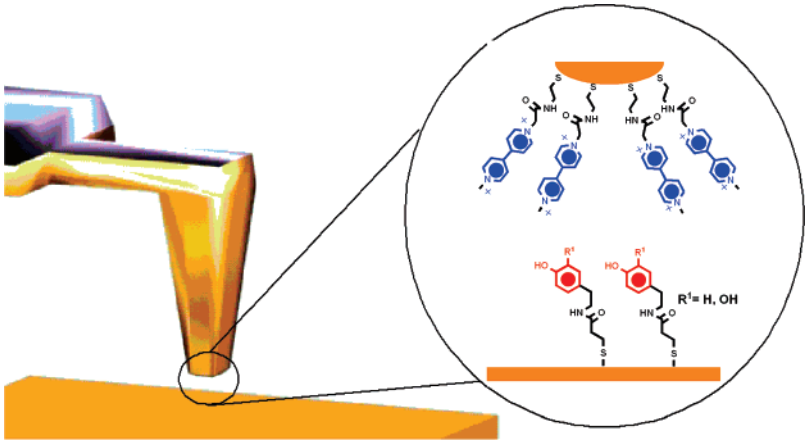


Figure 1. Schematic presentation of the attractive π-donor−acceptor interactions between a bipyridinium-modified tip and a tyramine/dopamine-functionalized surface measured using force microscopy.

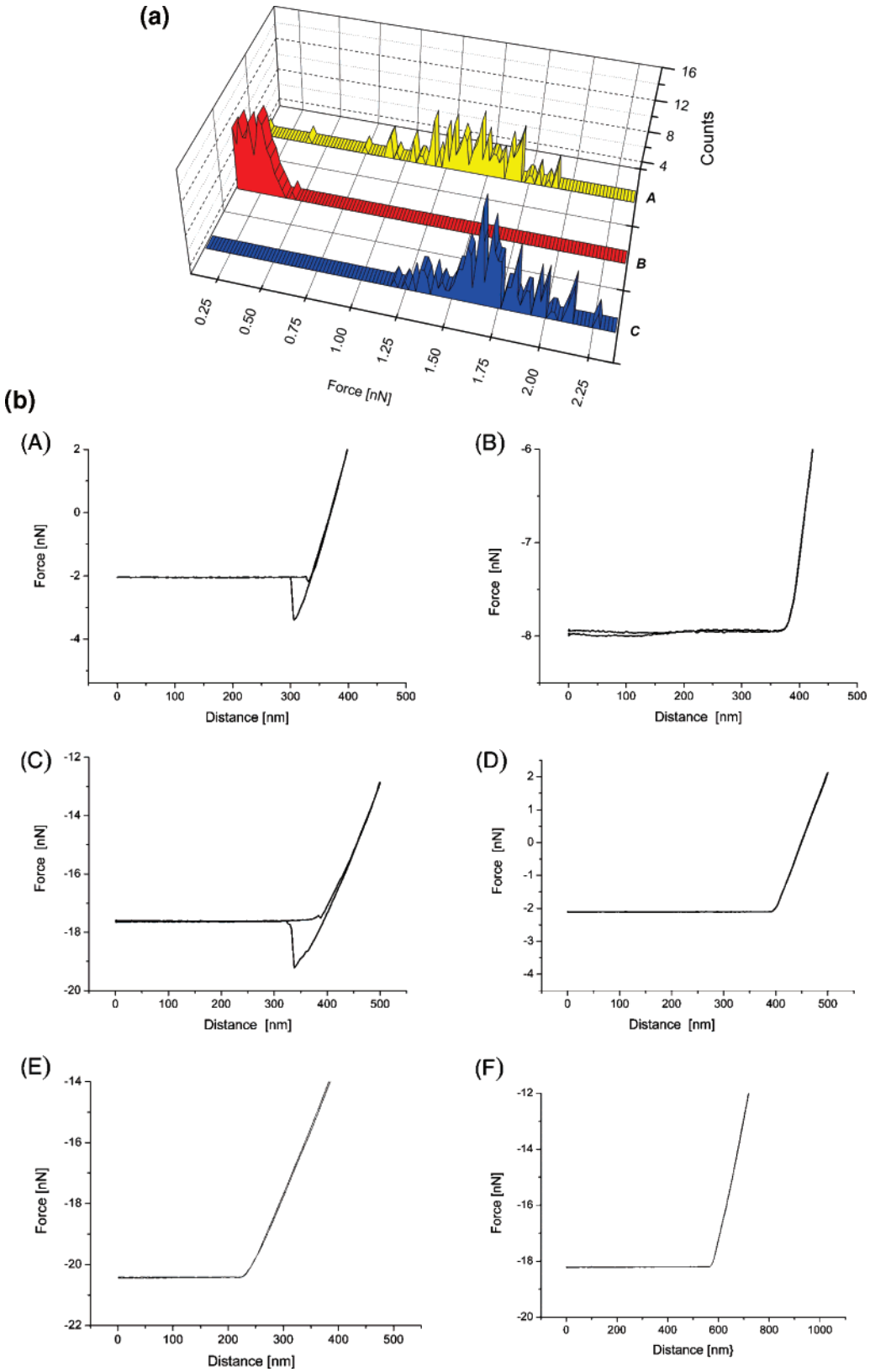


Figure 2. (a) Histograms of forces measured after 300 pulling experiments between the tyramine-functionalized surface and bipyridinium-functionalized tip before reaction with tyrosinase (A), after reaction with tyrosinase (B), and following the addition of ascorbic acid (C). (b) Representative force curves of a viologen tip with a tyramine surface (A), a viologen tip with a dopaquinone surface (B), a viologen tip with a dopamine surface (C), a bare gold surface with a bare gold tip (D), a viologen tip with a bare gold surface (E), and a bare gold tip with a tyramine surface (F).

determined in air using the thermal noise method[45] to give an average spring constant of 0.06 $N \cdot m^{-1}$. All experiments were conducted in phosphate buffer ($K_2H_2PO_4$ 10 mM, pH = 6.5) in a fluid cell. Prior to measurement, all samples were

equilibrated in the fluid cell for 30 min. To measure the force interactions, the probe tip was lowered to the surface and immediately retracted at a rate of 0.5 μm·s^{-1}, and data points were analyzed with their associated spring constants. Histograms were prepared using ORIGINPRO software (OriginLab, Northampton, MA). Different bin sizes did not affect the conclusions drawn from the data. Peaks were fitted[27] using a Lorentzian function that best fit our data with a Levenberg−Marquardt fitting algorithm. Each histogram was the result of at least 300 separate force measurements.

Effects of Biocatalytic Transformations on Functionalized Surfaces. Tyrosinase converts the π-electron donor tyramine to the π-electron acceptor dopaquinone (Scheme 1), and thus we saw the opportunity to detect the enzymatic activity by measuring the forces between the substrate-modified surface and an AFM tip functionalized with π-electron-accepting *N*,*N*′-dialkyl-4,4′-bipyridinium (viologen) units (Figure 1). Initially, attractive interactions exist between the π-donor, tyramine, on the surface and the acceptor, viologen, on the tip. Upon oxidation of tyramine to dopaquinone, the attractive interactions between the tip and the functionalized surface are switched "off" because of the lack of associative interactions between the acceptor−acceptor couple. Reduction of the dopaquinone with ascorbic acid generates[46] dopamine, a molecule that exhibits strong π-donor properties. This results in the reestablishment of attractive force interactions with the π-acceptor-modified tip that can be turned "ON" and "OFF" repeatedly through successive reduction/oxidation of the modified surface by ascorbic acid and tyrosinase, respectively.

The π-electron donating ability of both dopamine and tyramine have been previously established[47] with the bipyridinium-containing cyclophane host cyclobis(paraquat-*p*-phenylene)$^{4+}$. While dopamine is a slightly stronger donor, and therefore forms a stronger complex with the host, both are relatively weak guests. As a result, we expect that the bond rupture force for a single donor−acceptor interaction between a viologen unit on the tip and either donor on the surface will be below the sensitivity of the force microscopy technique, as well as other more sensitive techniques such as optical tweezers. Accordingly, we have prepared monolayers on both the tip[24] and the surface[30] to access the combined force of many simultaneous bond-rupture events during each measurement that results in the amplification of the detection signal, i.e., the rupture force. The activity of tyrosinase was detected by measuring changes in the force upon retracting the viologen-modified tip from the surface. The histograms for the rupture forces, as well as representative force curves, between a tyramine-functionalized surface before and after being subjected to the enzymatic oxidation followed by the chemical reduction with ascorbic acid are shown in Figure 2. The average force measured upon retraction of the AFM tip from the tyramine surface was 1.53 ± 0.14 nN. Following this measurement, the surface was immersed in a dilute solution of tyrosinase (5 units of activity), and the pulling experiments were repeated. The negligible forces (average force = 0.34 ± 0.09 nN) are consistent with the hypothesis that tyrosinase oxidizes the

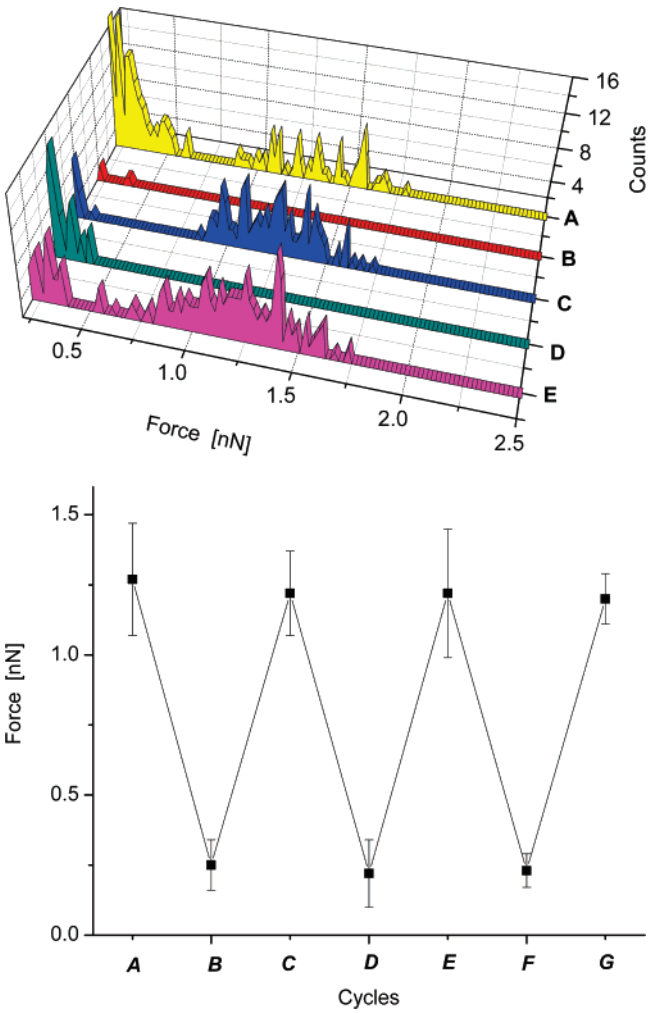


Figure 3. (a) Histograms showing the forces measured between the viologen-modified tip and a dopamine-modified surface after 300 pulling experiments. Forces were initially measured with the dopamine-functionalized surface (A). The surface was repeatedly subjected to tyrosinase oxidation (B,D) followed by ascorbic acid reduction (C,E). (b) Switchable forces between the bipyridinium-modified tip and the dopamine-functionalized surface upon enzymatic-oxidation (B,D,F)/chemical-reduction (C,E,G) cycles.

tyramine surface to the π-electron donor dopaquinone, thereby shutting "OFF" attraction between the tip and the surface. Following exposure of the surface to a 20 mM solution of ascorbic acid, we were able to observe again the attractive forces between the tip and the surface, however, at a higher magnitude, 1.80 ± 0.25 nN, than was initially measured with the tyramine surface. The higher values of force are consistent with our expectations for the system because ascorbic acid reduces dopaquinone to dopamine, which is a stronger π-electron donor[47] than the tyramine that was initially present on the surface. Thus, the ensemble of forces created between a dopamine-functionalized surface and the viologen-modified tip will have a stronger interaction than with the tyramine surface because of the amplification of many slightly stronger interactions involved with the dopamine surface.

To demonstrate that the disappearance of forces between the tip and the surface was the result of enzymatic oxidation of the surface-bound tyramine ligand to dopaquinone, a

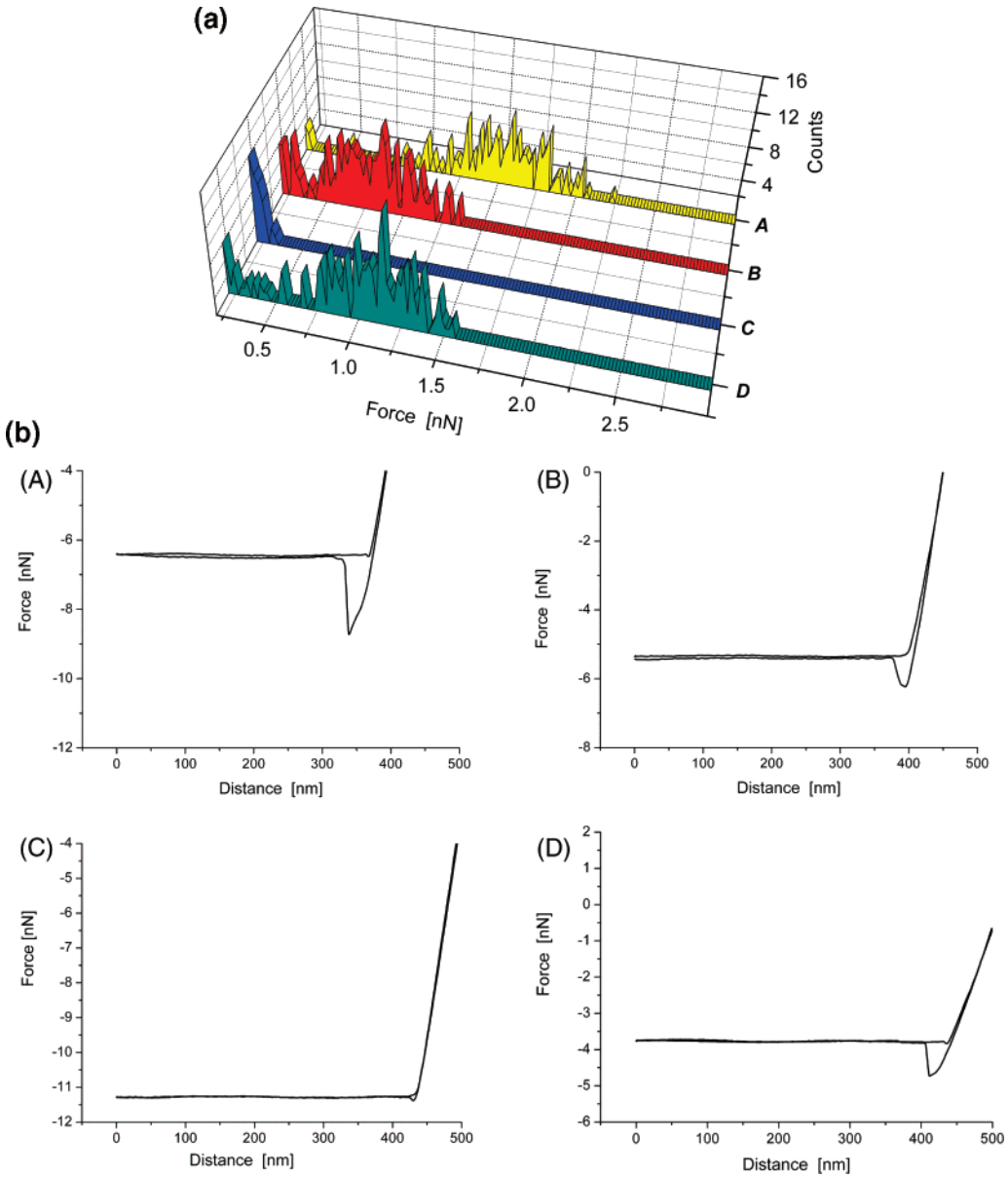


Figure 4. (a) Titration of the force interactions between the bipyridinium-modified tip and the dopamine-functionalized surface by the external *para*-hydroquinone (HQ), π-donor. The histograms show the forces that are measured between the viologen-modified tip and the dopamine-functionalized surface (A), in the presence of a 20 mM solution of HQ (B), in the presence of a 50 mM solution of HQ (C), and after the HQ has been washed away (D). (b) Representative force curves of a viologen tip with a tyramine surface (A), in the presence of 20 mM hydroquinone (B), in the presence of 50 mM hydroquinone (C), and after removing hydroquinone from the experimental cell (D).

dopamine-terminated surface was subjected to several cycles of enzymatic oxidation followed by chemical reduction with ascorbic acid, and the forces between tip and surface were measured at each step. Figure 3 shows the histograms for three successive oxidation/reduction cycles carried out on the same dopamine-functionalized surface. Initially, an average force of 1.27 $\pm$ 0.40 nN was determined between the viologen-modified tip and the surface, consistent with the presence of donor−acceptor interactions between dopamine and the viologen-functionalized tip.[48] Upon exposure to the dilute enzyme solution, the attractive force interactions disappeared, consistent with the formation of dopaquinone. However, following reduction of the surface with ascorbic acid, the initial force was quantitatively reestablished, in agreement with the regeneration of the dopamine-modified surface. For three successive cycles, the force could be reestablished without noticeable degradation of the surfaces; however, we did not explore further cycling of our system to eliminate the possibility of gradual degradation of the tip/surface coatings from the force measurements. These observations demonstrate that the disappearance of forces, and ascorbic acid-induced reappearance, is the result of the cyclic tyrosinase-induced oxidation of dopamine and the subsequent reduction of the resulting dopaquinone by ascorbic acid.

Finally, we proceeded to establish that donor−acceptor interactions were indeed the cause of the attractive forces measured between the tyramine-functionalized surface and the viologen-modifed tip. Toward this end, a solution of *para*-hydroquinone (HQ) as a π-electron donor that com-

petes[49] with the surface-bound π-electron donors for binding sites on the viologen-containing tips was added to the experimental cell (Figure 4). Forces initially measured between a dopamine-terminated surface and the viologen tip, in the absence of HQ, resulted in a force of 1.53 ± 0.25 nN.[50] By replacing the pure buffer solution with a 20 mM solution of HQ, we observed a significantly lower measured force of 0.88 ± 0.35 nN. We interpret the lower force as the result of fewer rupture events occurring between the tip and the surface upon retraction of the tip because the HQ occupies some of the available binding sites on the modified tip. In the presence of a 50 mM HQ solution, no significant forces were detected (average force $= 0.27 \pm 0.53$ nN). Upon flushing out the HQ from the liquid cell, we observe a strong force of 1.09 ± 0.31 nN, which is a lower force than 1.53 ± 0.25 nN that was initially measured, and we attribute it to incomplete rinsing of the HQ from the modified tip. This titration experiment demonstrates that donor−acceptor interactions are the cause of the forces measured in this study. A series of control experiments were carried out to eliminate the possibility that the measured forces were caused by other effects besides donor−acceptor interactions. These included the measurement of forces with a bare tip and a bare surface, a bare tip and a tyramine-functionalized surface, as well as a viologen-functionalized tip and a bare surface. The pull-off curves for these studies are shown in Figure 2b and are notable for the absence of any significant forces, which is representative of the more than 300 individual force curves measured in each control study.

In conclusion, we report on a novel method for monitoring the biocatalytic activity of tyrosinase using force microscopy. We have modified Au-coated AFM cantilevers and Au surfaces with complementary π-electron acceptors and donors, respectively, and the attractive interactions between these donor−acceptor pairs create an emergent effect that results in forces that could be measured using CFM. The activity of tyrosinase oxidizes tyramine or dopamine on a surface to the π-electron acceptor, dopaquinone. This transformation results in the elimination of forces between the π-electron acceptors on the tip and the surface-bound molecules. However, forces were regenerated by reducing the dopaquinone to dopamine with ascorbic acid, and the forces could be cycled "ON" and "OFF" by successive enzymatic oxidation/chemical reduction cycles. Titration with HQ and a series of control experiments established that forces measured were the result of donor−acceptor interactions. As far as we are aware, there is no precedent to the application of force microscopy as a means to detect the biocatalytic activity without having to directly modify the AFM tip with the biomolecule of interest. We believe that monitoring biocatalytic transformations by force microscopy is a technique with broader potential for the sensitive detection of biological species and events such as hydrolytic biotransformations or redox-catalyzed reactivities involving different cofactors.

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(48) Identical viologen[44] and tyramine[40] monolayers on Au have been investigated in our group. We have reported on the orientation (30° pitch), surface density (80 $Å^2$ $molecule^{-1}$), and monolayer height of the viologen surface[44] as well as the surface density (55 $Å^2$ $molecule^{-1}$) of a tyramine surface,[31] both prepared identically to the surfaces used in the present study. Because the surface density of the viologens (80 $Å^2$ $molecule^{-1}$) is lower than the tyramine surface as a result of charge repulsion between adjacent bipyridiniums, forces were calculated on a per-viologen basis. We used the established model[15] to estimate the contact area between the tip our 30 nm diameter tips. Although the contact area between the surfaces is dependent of the height of the monolayer on the tip, we have neglected this parameter in our calculatons because it is small (6 Å) compared to the radius of the tip (15 nm) to estimate at a contact area of 700 nm^2. Thus, for a viologen-coated tip, we arrive at forces of 1.7 pN $molecule^{-1}$ and 2.1 pN $molecule^{-1}$ for tyramine and dopamine surfaces, respectively. While this value is small compared to that obtained for other supramolecular systems,[19−30] the low value is in agreement with our expectations for the substantially weaker interactions that we are measuring.
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(50) In different experiments, we observe small differences in the magnitudes of force between identical tip−surface pairs because the measured force is sensitive to slight differences in tip radius.

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