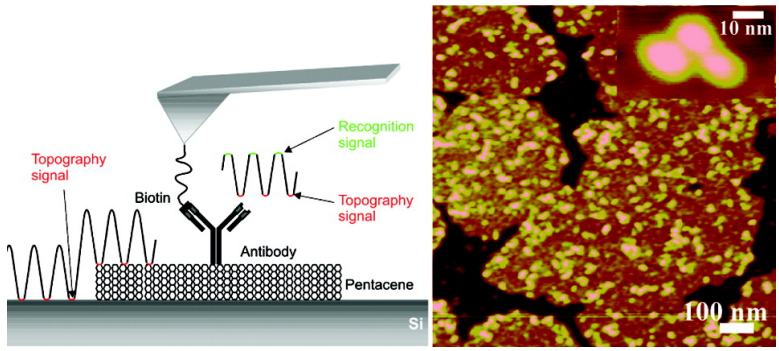
NANO LETTERS

Letter

Imaging and Detection of Single Molecule Recognition Events on Organic Semiconductor Surfaces

J. Preiner, N. S. Losilla, A. Ebner, P. Annibale, F. Biscarini, R. Garcia, and P. Hinterdorfer

Nano Lett., **2009**, 9 (2), 571-575• DOI: 10.1021/nl802721g • Publication Date (Web): 30 December 2008



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Nano Letters is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

NANO LETTERS

2009
Vol. 9, No. 2
571-575

Imaging and Detection of Single Molecule Recognition Events on Organic Semiconductor Surfaces

J. Preiner,[†,||] N. S. Losilla,[‡,||] A. Ebner,[†] P. Annibale,[§] F. Biscarini,[§] R. Garcia,[*,‡] and P. Hinterdorfer[†]

Christian Doppler Laboratory for Nanoscopic Methods in Biophysics, Institutte for Biophysics, Johannes Kepler University of Linz, A-4040 Linz, Austria, Instituto de Microelectrónica de Madrid, CSIC, Isaac Newton 8, 28760 Tres Cantos, Madrid, Spain, CNR-Istituto per lo Studio dei Materiali Nanostrutturati (ISMN), Via P. Gobetti 101, I-40129 Bologna, Italy

Received September 8, 2008; Revised Manuscript Received December 2, 2008

ABSTRACT

The combination of organic thin film transistors and biological molecules could open new approaches for the detection and measurement of properties of biological entities. To generate specific addressable binding sites on such substrates, it is necessary to determine how single biological molecules, capable of serving as such binding sites behave upon attachment to semiconductor surfaces. Here, we use a combination of high-resolution atomic force microscopy topographical imaging and single molecule force spectroscopy (TREC), to study the functionality of antibiotin antibodies upon adsorption on pentacene islands, using biotin-functionalized, magnetically coated AFM tips. The antibodies could be stably adsorbed on the pentacene, preserving their functionality of recognizing biotin over the whole observation time of more than one hour. We have resolved individual antigen binding sites on single antibodies for the first time. This highlights the resolution capacity of the technique.

Reliable and low-cost methods to detect tiny amounts of biological molecules are critical for the development of ultrasensitive and/or field-portable diagnostic devices. Different approaches are being pursued,[1−11] among those, sensor devices based on micro and nanoscale field effect transistors.[8−11] The versatility of organic synthetic techniques makes organic thin film transistors promising candidates for making a variety of electronic applications,[12−17] and in particular ultrasensitive biological sensors. The implicit assumption in electrical sensors based on transistors is that the biological functionality of single biomolecules remains intact upon adsorption on the semiconductor surface. Here, we image and detect single antibody−antigen interactions between a single immunoglobulin (IgG)-type antibody deposited on top of a pentacene monolayer and a biotin-functionalized probe. The biotin serves as an antigen in this case. The recorded single molecule recognition events on pentacene are comparable to those performed on canonical surfaces such as mica. This demonstrates the suitability of pentacene as a semiconductor material to fabricate nanoscale devices for single molecule detection. The experimental approach, which is based on a dynamic force microscopy method that combines topography and recognition imaging, is easily adaptable to either test or confirm the detection readings by the proposed ultrasensitive diagnostic devices.

Thin film transistors are being applied to detect biological or chemical species.[9−12] It was shown that the binding of a charged molecule on a receptor placed on top of an active part of the transistor alters the conductance of the device, which results in a change in the gate voltage. The sensitivity of those devices has been remarkably increased by using nanowire-based transistors.[9,10] One attractive property of organic semiconductor devices is the incorporation of functionality by design[12] which could make the selective positioning of biomolecules on the nanoscale transistor easier.

A central issue in electric signal transduction associated with molecular recognition processes is the biological functionality of the biomolecule upon adsorption on the semiconductor surface. This is also valid for sensors based on micro and nanomechanical transducers[1−3] or optical properties.[4−7] One basic requirement is that the biomolecular functionality must not be altered by either the deposition protocol or by unspecific semiconductor-biomolecule interac-

* To whom correspondence should be addressed. E-mail: rgarcia@imm.cnm.csic.es.
[†] Johannes Kepler University of Linz.
[‡] CSIC, Isaac Newton 8.
[§] CNR-Istituto per lo Studio dei Materiali Nanostrutturati (ISMN).
[||] These authors have contributed equally to the realization of the work.

10.1021/nl802721g CCC: $40.75
Published on Web 12/30/2008

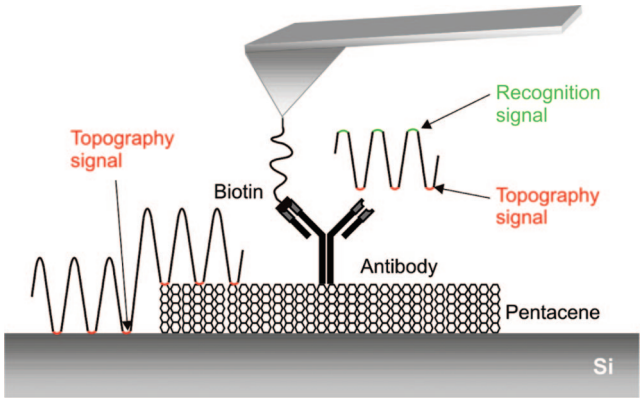


Figure 1. Scheme of the simultaneous topography and recognition AFM imaging of antibiotin antibodies adsorbed on pentacene islands. A magnetically driven cantilever oscillates across the surface. The oscillation signal is split into two parts of which the lower part is used to generate the topography (topography signal, red) and the upper part is used for the recognition image (recognition signal, green).

tions. Here, we imaged and detected single molecule recognition interactions on top of organic semiconductor surfaces. By using a force microscopy method providing simultaneously recorded topography and molecular recognition images of biological samples in buffer solutions,[18,19] we measured specific antigen−antibody interactions on top of pentacene monolayers. The recognition signals were comparable to the recognition events recorded in canonical flat substrates for force microscopy. Furthermore, we recorded the biological activity of single antibodies deposited on a pentacene monolayer island for about 1 h without observing any degradation.

Results and Discussion. To assess the morphological and functional integrity of a biomolecule deposited on a flat surface, a technique that provides both topographic and interaction information is required. Atomic force microscopy methods have demonstrated molecular resolution images of several protein assemblies.[20−24] Intermolecular interactions have been measured with great accuracy with a force spectroscopy based on AFM methodologies.[25−29] Simultaneous imaging of topography and molecular recognition processes in liquids has been achieved by a dynamic atomic force microscopy method that takes advantage of the low quality factors of microcantilevers in liquids ($Q \sim 1$). As a consequence, the cantilever's oscillation is no longer symmetric with respect to its resting position. This enables the cantilever oscillation to split into lower and upper parts. When a ligand is tethered to the microcantilever tip via a flexible PEG (polyethylene glycol) linker,[30] the upper part of oscillation carries information on specific binding events, whereas the lower part, conveniently processed, is used to record the topography (see Figure 1). Another advantage of the linkage via PEG is that unspecific tip−surface interactions that could mask the recognition process are avoided. Pentacene monolayers were deposited by organic molecular beam epitaxy on top of Si(100) surfaces (see Figure 1). The growth conditions were adjusted to achieve a submonolayer coverage. In this way, a good contrast between the Si substrate and the pentacene surfaces was achieved. Then, a

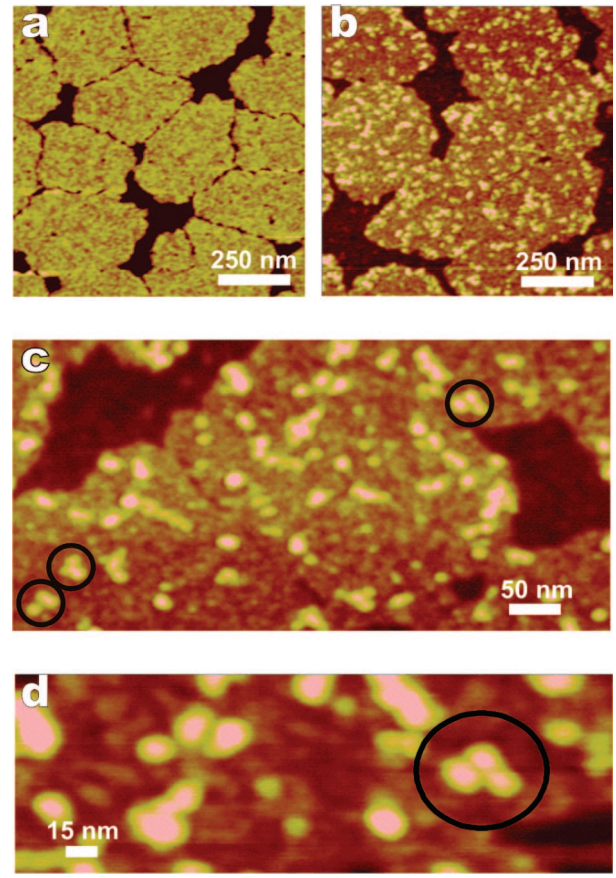


Figure 2. Amplitude modulation AFM images of pentacene islands before and after antibody deposition. (a) The pentacene islands (∼1.5 nm in height) show a relatively rounded shape. The silicon appears dark in background between islands. (b) After antibody deposition, the pentacene surface is covered by many small particles. Many of them show the characteristic Y-shape of IgG antibodies. (c) High-magnification image of a pentacene layer containing physisorbed antibodies. The antibodies showing a Y-shape morphology are marked. (d) Zoom of a region of panel c showing an antibody molecule on pentacene with its expected size and shape.

20 μL drop containing antibiotin antibodies was deposited. Figure 2 shows an amplitude modulation AFM image obtained with a silicon tip of several antibodies on top of pentacene islands. As in previous AFM studies of IgG antibodies deposited on mica, many morphologies were observed.[23−31] In particular, several molecules that exhibit the characteristic Y-shape morphology of IgG antibodies (Figure 2b,c), each subunit with a size of ∼6 nm in consistency with X-ray characterization were found. Detailed analysis of the observed morphologies on pentacene do not reveal any difference with those observed on mica and on purple membranes.[23]

Then we proceeded to image the specific binding events between the antibiotin antibodies and a biotin molecule attached via a distensible PEG linker to a magnetically coated silicon nitride tip. A time series of this experiment is shown in Figure 3.

The topographical images, Figure 3a,c,e,g reveal several antibodies on top of the pentacene islands. The recognition images (Figure 3b,d,f,h) contour spots that are associated

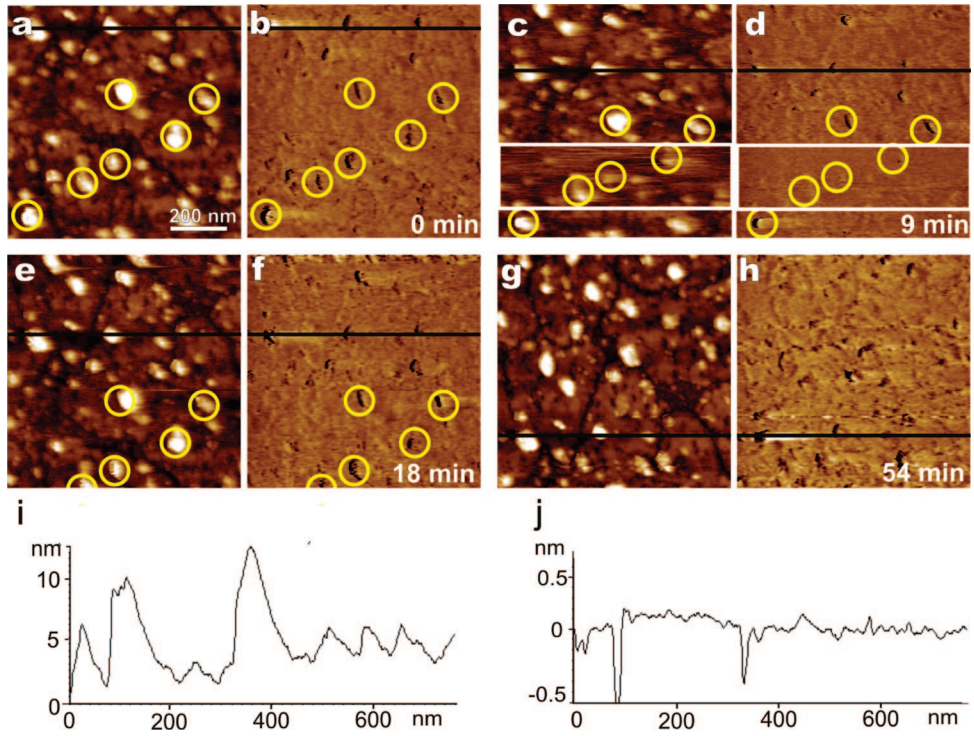


Figure 3. Time series of topography (a,c,e,g) and recognition image (b,d,f,h) of antibiotin antibodies adsorbed on pentacene islands obtained using a biotin-functionalized tip. Within the framed area in c and d, the setpoint-amplitude was increased to a value bigger than the PEG-linker length, which resulted in a disappearance of the recognition spots (black circles between the white lines; compare to panels b and f). Note that the antibodies before and after the setpoint-amplitude increase are recognized by the biotin-functionalized tip (white circles). (i,j) Cross-section indicated by the lines in panels a and b. (Color scale range: topography images, 0−13 nm; recognition images, −0.5−0.7 nm).

with the antibodies. The cross-section along the lines in Figure 3a,b shows that the recognition event is associated with a decrease in the upper part of the oscillation (Figure 3i,j). The recognition images, Figure 3b,d,f,h, show a constant recognition pattern throughout the whole observation time of 54 min. Because of thermal drift (∼11 nm/min) the cross-sections (black lines) are steadily shifted in each image. In the marked region of Figure 3c,d, we have performed an additional experiment that underlines the genuine character of the recognition process. The set-point amplitude was increased to a value where the actual peak to peak amplitude exceeds the PEG-linker length. This prevents or considerably restricts the binding, so the most of the binding events disappear in the recognition image (yellow circles between the white lines; compare to Figure 3b,f). The biotin on the tip was no longer able to bind continuously to an antibody on the surface since the increased peak to peak amplitude leads to a rupture of the biotin from the antibody in each oscillation cycle. Thus, the dwell time close to the antibodies on the surface is reduced, which in turn decreases the binding probability. Importantly, before and after the set-point amplitude increase (i.e., outside the framed area, and also Figure 3b,f), the antibodies were recognized by the biotin-functionalized tip. We note that not all the features observed in the topography image should give a recognition event. On one hand, some of the topographic features may not correspond to antibodies (see below), and on the other hand the antibodies may lie on the pentacene in a position that hinders the recognition process. Taking this into account, the ratio of accessible binding sites to the total number of sites can be estimated to be about 60%.

To unambiguously confirm our adscription of the spots observed in the AFM recognition image to specific antibody-biotin interactions we have performed two types of control experiments, one unspecific and the other specific. The unspecific control experiment consisted in performing topography and recognition imaging on a pentacene surface covered with inorganic nanoscale particles (Figure 4a,b). The AFM topography image reveals objects with sizes comparable to those of IgG molecules; however, the recognition image does not record any specific antibody-biotin binding event. The specific check consisted in blocking the biotin-functionalized tip with streptavidin, a molecule that has four binding sites to specifically bind biotin. The cantilever used for recording the previous images was immersed into a PBS buffer with a high streptavidin concentration (10 mg/ml) for 20 min. After this treatment, the biotin molecule on the tip was blocked by a streptavidin molecule. Figure 4 shows the simultaneously recorded topography (Figure 4e) and recognition image (Figure 4f) obtained with the blocked tip on the same sample as in Figure 3. The recognition spots disappeared (flat cross-section, Figure 4h), since the streptavidin-blocked biotin was no longer able to bind to the antibodies on the pentacene. In contrast, the topography still shows the same features (the resolution was slightly affected, which might be caused by unspecific attachment of some streptavidin molecules to the tip).

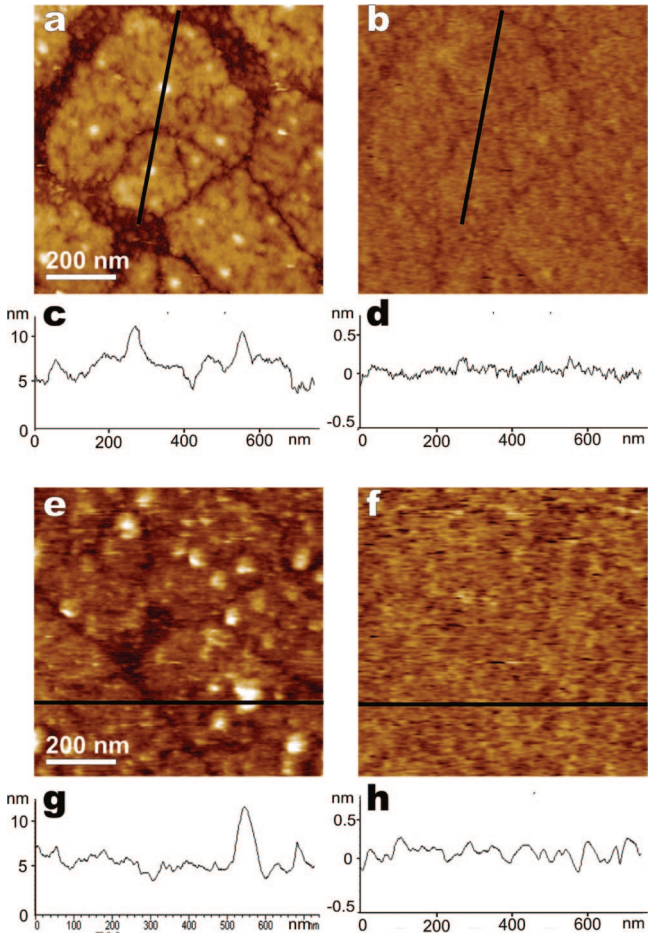


Figure 4. AFM topography and recognition images in control experiments. (a) Pentacene islands imaged with a functionalized tip in liquid (antibody-free PBS). The small white dots are some unidentified inorganic nanoparticles. (b) The recognition image shows no specific interaction events. (c,d) Corresponding topography and recognition sections along the green line in panels a and b. (e) Topography and (f) recognition image on the same sample as in Figure 3 but taken with a tip where the biotin was blocked by adding free streptavidin. The topography shows no relevant differences with respect to previous topographic images. In contrast, all binding events have disappeared in the recognition image. (g,h) Respective topographic and recognition cross section along the green line in panels e and f. (Color scale range: topography images, 0−13 nm; recognition images, −0.5−0.7 nm.)

IgG antibodies, like the antibiotin antibodies, have two flexibly linked Fab (fragment antigen binding) fragments, each carrying a specific binding site for biotin at the end. Antibodies are very flexible molecules and can therefore adopt many different conformations when adsorbed to a surface. Depending on the actual conformation (i.e. the angle between the Fabs) on the surface, these binding sites can be accessible for the biotin. A closer look on the observed recognition spots (Figure 5, bottom) and the corresponding topography images (Figure 5, top) reveals (although blurred due to the finite PEG linker length) various different spot shapes.

Consequently, these different shapes can be attributed to the conformations of the antibodies on the surface leading to different distances between the two binding sites on each antibody. In some cases (Figure 5, first column), the binding

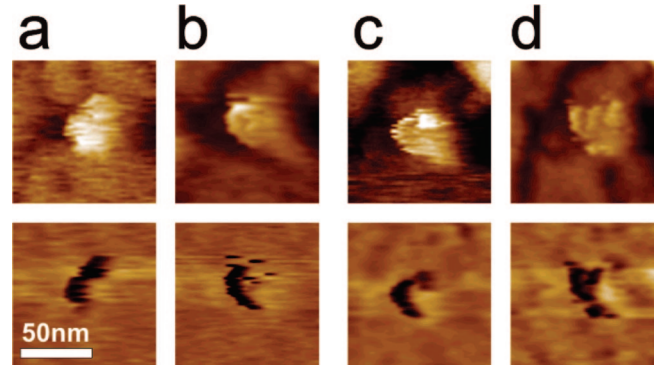


Figure 5. Topography (top) and recognition images (bottom) of antibiotin antibodies adsorbed on pentacene islands. Different shapes of recognition spots were observed, which can be attributed to different orientations of the Fab fragments of the adsorbed antibodies. (Color scale range: topography images, 0−13 nm; recognition images, −0.5−0.7 nm)

sites were efficiently far away from each other and appeared spatially separated, proving that both antigen binding fragments preserved their functionality upon adsorption to pentacene.

Conclusions. Simultaneous topography and recognition imaging has proven to be a powerful tool for the detection and localization of single molecule interactions. Here we used this technique to unambiguously confirm that single antibodies remained fully functional after deposition on the organic semiconductor pentacene. The observed recognition patterns remained stable for at least 1 h, indicating a strong immobilization of the adsorbed antibodies. Additionally, spatially separated binding sites on single antibodies were observed, demonstrating the resolution capacity of the TREC method. The specificity of recognition events was shown in an unspecific and a specific control experiment. These findings suggest the use of antibodies as specific addressable binding sites on the surface of organic thin film transistors for a broad range of antigens. Since antibodies specifically recognize antigens, thin film devices based on pentacene decorated with antibodies appear to be promising for the detection of antigens as immunological diagnostic tools.

Materials and Methods.

Atomic Force Microscopy. Amplitude modulation AFM imaging in liquid was performed by magnetic excitation using a Pico Plus AFM (Agilent, Tempe, AZ, U.S.A.) with magnetically coated cantilevers (MAC levers, 0.1 Nm^{-1} nominal spring constant). The excitation frequency of the cantilever was tuned about 10% below resonance frequency in liquids (7 kHz). Far from the surface, the oscillation amplitude (free amplitude) was 10 nm (peak-to-peak value). The feedback amplitude (set-point amplitude) was about 85% of the free amplitude. The topography and recognition data where recorded using a commercially available electronic unit (PicoTREC, Agilent).[18,19] In both cases, the oscillation amplitude was split into lower and upper parts. All images (512 × 512 pixel) were taken at a scanrate of 1 line/second, resulting in a total scanrate of 512 s per image frame.

Amplitude modulation AFM images in air were taken with a Multimode AFM with Nanoscope III controller (Digital

Instruments, U.S.A.) operated in the noncontact mode. Sharp silicon cantilevers with force constant of about 10 N/m were used for imaging (SSS-SEIHR-W, Nanosensors, Germany).

AFM Tip Chemistry. Magnetically coated AFM tips (Agilent, Tempe, AZ, U.S.A.) were functionalized with ethanolamine and further derivatized with biotin-poly(ethylene glycol (PEG)-amino-reactive *N*-hydroxysuccinimide (NHS).

Antibodies. Pentacene samples were incubated with monoclonal IgG antibiotin antibody (Ab 38C2, Sigma) solution (1 mg/ml in phosphate buffered saline (PBS) buffer solution) for 30 s and subsequently rinsed with PBS.

Pentacene Growth. Pentacene (Sigma Aldrich Oekanal, Analytical Standard) was grown by high vacuum sublimation on a silicon (100) surface native silicon oxide surface. The surface is covered by a native silicon oxide. Base pressure in the evaporation chamber was 8×10^{-7} mbar. The evaporation rate of 10 Å/min and substrate temperature (298 K) were chosen to obtain layered structures with a thickness of 1.5 nm which corresponds to the molecule van der Waals length.

Acknowledgment. This work was financially supported by the European Commission (FORCETOOL, NMP4-CT-2004-013684 and BIODOT, NMP4-CT-2006-03265) and the Ministerio de Educación y Ciencia (MAT2006-03833).

References

(1) Fritz, J.; Baller, M. K.; Lang, H. P.; Rothuizen, H.; Vettiger, P.; Meyer, E.; Güntherodt, H. J.; Gerber, Ch.; Gimzewski, J. K. *Science* **2000**, *288*, 316–318.
(2) Wu, G.; Datar, R. H.; Hansen, K. M.; Thundat, T.; Cote, R. J.; Majumdar, A. *Nat. Biotechnol.* **2001**, *19*, 856–860.
(3) Tamayo, J. *Nat. Nanotechnol.* **2007**, *2*, 342–343.
(4) Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* **2003**, *301*, 1884–1886.
(5) Sia, S. K.; Linder, V.; Parviz, B. A.; Siegel, A.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **2004**, *43*, 498–502.
(6) Cooper, M. A. *Anal. Bioanal. Chem.* **2003**, *377*, 834–842.
(7) Sikes, H. D.; Hansen, R. R.; Johnson, L. M.; Jenison, R.; Birks, J. W.; Rowlen, K. L.; Bowman, C. N. *Nat. Mater.* **2008**, *7*, 52–56.
(8) Hafeman, D. G.; Parce, J. W.; McConnell, H. M. *Science* **1988**, *240*, 1182–1185.
(9) Cui, Y.; Wei, Q.; Park, H.; Lieber, C. M. *Science* **2001**, *293*, 1289.
(10) Patolsky, F.; Timko, B. P.; Zheng, G. F.; Lieber, C. M. *MRS Bulletin* **2007**, *32*, 142–149.
(11) Stern, E.; Klemic, J. F.; Routenberg, D. A.; Wyrembak, P. N.; Turner-Evans, D. B.; Hamilton, A. D.; LaVan, D. A.; Fahmy, T. M.; Reed, M. A. *Nature* **2007**, *445*, 519–522.
(12) Reese, C.; Bao, Z. *Mater Today* **2007**, *10*, 20–27.
(13) Jurchescu, O. D.; Baas, J.; Palstra, T. T. M. *Appl. Phys. Lett.* **2004**, *84*, 3061–3063.
(14) Facchetti, A.; Mushrush, M.; Yoon, M. H.; Hutchison, G. R.; Ratner, M. A.; Marks, T. J. *J. Am. Chem. Soc.* **2004**, *126*, 13480–13874.
(15) Gelinck, G. H.; Huitema, H. E. A.; Van Veenendaal, E.; Cantatore, E.; Schrijnemakers, L.; Van der Putten, J. B. P. H.; Geuns, T. C. T.; Beenhakkers, M.; Giesbers, J. B.; Huisman, B. H.; Meijer, E. J.; Benito, E. M.; Touwslager, F. J.; Marsman, A. W.; Van Rens, B. J. E.; De Leeuw, D. M. *Nat. Mater.* **2004**, *3*, 106–110.
(16) Roberson, L. B.; Kowalik, J.; Tolbert, L. M.; Kloc, C.; Zeis, R.; Chi, X.; Fleming, R.; Wilkins, C. *J. Am. Chem. Soc.* **2005**, *127*, 3069–3075.
(17) Herwig, P. T.; Mullen, K. A. *Adv. Mater.* **1999**, *11*, 480–483.
(18) Stroh, C.; Wang, H.; Bash, R.; Ashcroft, B.; Nelson, J.; Gruber, H.; Lohr, D.; Lindsay, S. M.; Hinterdorfer, P. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12503.
(19) Ebner, A.; Kienberger, F.; Kada, G.; Stroh, C. M.; Geretschlager, M.; Kamruzzahan, A. S. M.; Wildling, L.; Johnson, W. T.; Ashcroft, B.; Nelson, J.; Lindsay, S. M.; Gruber, H. J.; Hinterdorfer, P. *ChemPhysChem* **2005**, *6*, 897–900.
(20) Engel, A.; Muller, D. J. *Nat. Struct. Biol.* **2000**, *7*, 715–718.
(21) Scheuring, S.; Seguin, J.; Marco, S.; Levy, D.; Robert, B.; Rigaud, J-L. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1690–1693.
(22) Hörber, J. K. H.; Miles, M. J. *Science* **2003**, *302*, 1002–1005.
(23) Kienberger, F.; Muller, H.; Pastushenko, V.; Hinterdorfer, P. *EMBO Rep.* **2004**, *5*, 579–583.
(24) Muller, D. J.; Engel, A. *Nat. Protocol.* **2007**, *2*, 2191–2197.
(25) Lee, G. U.; Chrisey, A. C.; Colton, R. J. *Langmuir* **1994**, *10*, 354–357.
(26) Florin, E. L.; Moy, V. T.; Gaub, H. E. *Science* **1994**, *264*, 415.
(27) Hinterdorfer, P.; Baumgartner, W.; Gruber, H. J.; Schilcher, K.; Schindler, H. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 3477.
(28) Hinterdorfer, P.; Dufrene, Y. F. *Nat. Methods* **2006**, *3*, 347–355.
(29) Ebner, A.; Hinterdorfer, P.; Gruber, H. J. *Ultramicroscopy* **2007**, *107*, 922–927.
(30) San Paulo, A.; Garcia, R. *Biophys. J.* **2000**, *78*, 1599–1605.
(31) Patil, S.; Martinez, N. F.; Lozano, J. R.; Garcia, R. *J. Mol. Recognit.* **2007**, *20*, 516–523.

NL802721G