DNA Hybridization Detection

Label-Free Detection of DNA Hybridization at the Nanoscale: A Highly Sensitive and Selective Approach Using Atomic-Force Microscopy**

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The hybridization of oligonucleotides in solution to surface-immobilized DNA is of crucial importance to microarray-based genetic diagnostic devices.[1–3] Most microarray-based approaches rely on the labeling of samples with a fluorophore or a radioactive tag. This allows for very sensitive detection but can be time-consuming and expensive. Alternative label-free methods, using a quartz crystal microbalance,[4] surface plasmon resonance (SPR),[5–9] and cantilever arrays[10] have been shown to be capable of detecting DNA quantitatively, however, their relatively large detection areas have limited their detection sensitivity. To date, the highest sensitivity for the detection of label-free DNA probes by these methods is in the femto to attomol range.[8,10] Attomol-scale sensitivity was achieved by modifying the probe strand with gold colloids.[11]

Atomic-force microscopy (AFM) is a powerful tool in nanotechnology not only because of its ability to detect surface topography at sub-nanometer precision, but also because it may be used for nanoscale construction and manipulation. AFM has been used to measure DNA surface coverage, layer thickness,[12,13] and to construct and read DNA and protein micro- or nanoarrays.[14–19] The recent development of AFM-based nanolithography techniques, nanoshaving and nanografting,[20] make it possible to produce surface features as small as $2 \times 4\ nm^2$, and follow their evolution in situ.[21] A wide range of surface features have been constructed from alkyl thiols,[20,22] DNA,[23,24] protein,[25] and active enzymes.[26] We have recently used nanoshaving to produce and manipulate DNA nanofeatures through hybridization and chemical treatment.[24] Herein, we show this method can be used to detect label-free DNA hybridization quantitatively and follow the hybridization dynamics at the nanoscale. We also show the successful construction of multisequenced DNA nanofeatures on a single surface through sequential nanoshaving and self-assembly, which can selectively detect unlabeled target DNA in the presence of 100-fold excess of nonmatching DNA. This approach can detect less than 10^{-21} mol of label-free probe DNA, which makes it one of the most sensitive methods for the detection of DNA hybridization with label-free DNA.

The experimental approach involved the following steps: 1) A self-assembled monolayer (SAM) resist of a 12-mer thiolated double-stranded (ds) DNA was prepared on a template-stripped gold (TSG) surface.[24] TSG was selected as the substrate because its extreme flatness made it possible to detect small changes in topography by AFM. A resist coating of DNA was selected in favor of conventional alkyl thiols because self-assembled monolayers of DNA may be removed by a soft cantilever, thereby improving sensitivity, and because of its biocompatibility.[24] 2) Nanoshaving[20] was used to remove the dsDNA resist at preselected nanoareas to create reactive surface domains. 3) The nanoholes were filled with a 20-mer thiolated single-stranded DNA (ssDNA) by self-assembly to obtain ssDNA (target) nanofeatures. 4) Finally the surface was incubated with a solution of a 24-mer nonthiolated ssDNA whose sequence is complementary to that of the ssDNA nanofeatures. The topographic changes of the DNA nanofeatures were monitored as a function of time. Hybridization of the probe strands in solution with complementary target strands tethered on the Au surface within the nanofeature led to the formation of rigid dsDNA that "stood up" and aligned more perpendicular from the surface. This occurs since extra space is needed to hold the extra volume of the newly introduced probe DNA. The resulting increase in the height of the DNA nanofeatures was detected in situ and measured by AFM. Hybridization was highly sequence specific, no detectable nonspecific adsorption of probe DNA onto the dsDNA resist background was observed (Figure 1).

Table 1 lists the DNA sequences used in this study. A DNA SAM resist was prepared by incubation of a freshly prepared TSG surface with a 2 μM solution of HS-dsDNA-0 in tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5) for 48–72 h.[24] An AFM topographic image showed the formation of a complete SAM. Nanoholes created within the dsDNA resist SAM by nanoshaving had a depth of 2.8 ± 0.3 nm (Figure 1a). After incubation of the surface with HS-DNA-1 (1 μM) for 60 minutes, the holes were filled and the average height of the protrusions was approximately 0.5 nm above the background (Figure 1b). When the surface was incubated with a solution of the complementary probe DNA (DNA-1C) at concentrations between 10 nM to 1 μM, height increases as a result of hybridization were observed on the

Table 1: The DNA sequences and their nomenclatures.

Name		Sequence
HS-dsDNA-0	$HS\text{-}C_6H_{12}$-	5′-GATCCTCATCGA-3′
		3′-CTAGGAGTAGCT-5′
HS-dsDNA-1	HSC_6H_{12}-	5′-CTCTAGACATATGGGTACCG-3′
		3′-GAGATCTGTATACCCATGGCTTAA-5′
HS-DNA-1	HSC_6H_{12}-	5′-CTCTAGACATATGGGTACCG-3′
DNA-1C		3′-GAGATCTGTATACCCATGGCTTAA-5′
HS-DNA-2	HSC_6H_{12}-	5′-CGTGCACCCATGGCTCGAGA-3′
DNA-2C		3′-GCACGTGGGTACCGAGCTCTTCGA-5′
DNA-3		3′-GGAATTCTCTAGAGACGTCCCTAG-5′

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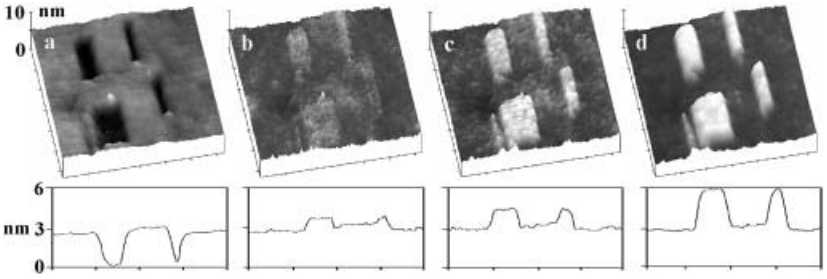

Figure 1. Representative AFM topographic images showing the hybridization process of HS-DNA-1 nanofeatures. All images were in the same *x*-, *y*- ($1.25\times1.25\ \mu m^2$), and *z*-scales. a) A series of nanoholes (20×300, 50×400, 100×400, and $200\times400\ nm^2$) created within the HS-dsDNA-0 SAM by nanoshaving. b) After incubation with 1 μM HS-DNA-1 for 60 mins, the holes were filled and protruded by about 0.5 nm above the background. Incubation of the surface with the complementary DNA-1C (1 μM) for c) 15 and d) 140 mins. The height of the features increased to 1.5, and 3.0 nm above the background, respectively. Longer incubation times resulted in no further height increase of the nanofeatures. The corresponding averaged line-scan profiles across the top two DNA features in the AFM images are shown underneath each image. All line scans are in the same z-scale. The standard deviation on the measured DNA feature height is about ±0.2 nm.

DNA nanofeatures in all cases. Representative images are shown in Figure 1 c–h. The changes in feature height versus incubation time at a variety of DNA-1C concentrations are shown in Figure 2 a.

It is clear that the rate of increase in height and the maximum height increase are strongly dependent on the DNA-1C concentration. For example,, saturation was not reached until over 400 minutes at 10 nM, with a maximum height increase of only 1.1 nm, while saturation was observed within 100 minutes using a more concentrated DNA-1C solution (1 μM), and the height increase was 2.5 nm. It took longer to reach saturation in these experiments than has been reported in SPR or cantilever array studies, most probably because a flow system was used in those experiments.[7–10] Quantitative analysis of these data is possible if it is assumed that the height increase is linearly proportional to hybridization efficiency. Normalized hybridization efficiencies were obtained by division of the increase in the saturated height by the difference in the thickness between SAMs of HS-DNA-1 (0% hybridization) and HS-dsDNA-1 (100% hybridization, which is 7.0 ± 0.8 nm thick).[24] The parameters of a Langmuir adsorption model were fitted to the data to derive a thermodynamic surface–solution equilibrium dissociation constant K_d. If it is assumed that the target–probe bindings are independent of the surface coverage (hybridization efficiency) the Langmuir isotherm can be described as hybridization efficiency $= a\,c/(K_d + c)$,[10] where c is the concentration of the target DNA-1C, and a is the maximum hybridization efficiency under the above conditions (not all surface-tethered ssDNA strands are available for hybridization).[7–10] The plot of the reciprical hybridization efficiency versus the reciprical concentration (Figure 1 b) gives a linear fit ($R = 0.992$), which yields $K_d = 19$ nM and $a = 0.69$. The K_d value for the 20-mer is in good agreement with values obtained from SPR studies and cantilever arrays, (for example, $K_d = 17$ nM for a 25-mer, 33 nM for a 18-mer,[7] and 41 nM for a 12-mer[10]). The maximum hybridization efficiency of 69% for the HS-DNA-1 nanofeatures observed in this study is higher than those reported from SPR studies.[6–8] Perhaps the much smaller feature size and longer incubation time permit greater accessibility to the complementary strand.

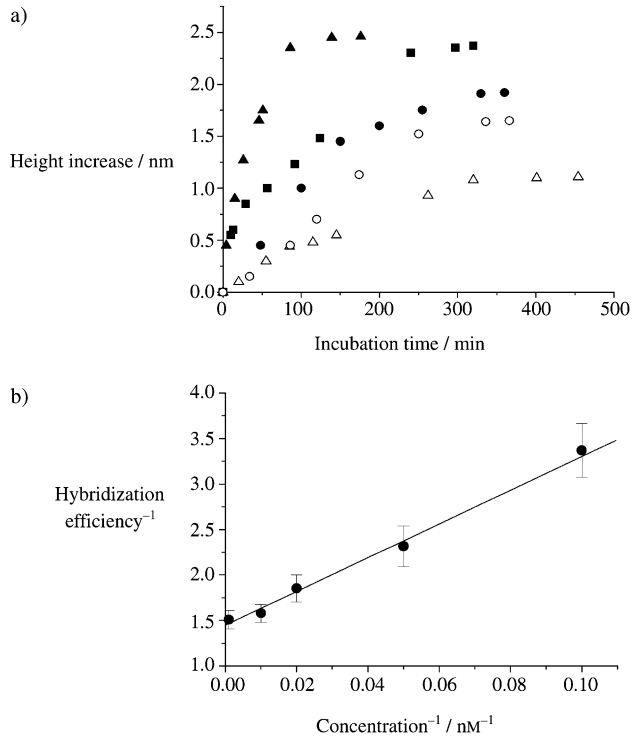

Figure 2. a) Plot of the height changes for the HS-DNA-1 nanofeatures versus the incubation time of the DNA-1C at 1 μM (▲), 100 nM (■), 50 nM (●), 20 nM (○), and 10 nM (△). b) Plot of the hybridization efficiency^{-1} versus concentration^{-1} of the probe DNA.

Experiments have been conducted on smaller-sized HS-DNA-1 features, typically $20\times300\ nm^2$ (Figure 1) which have an area more than six orders of magnitude smaller than those used in SPR hybridization experiments (typically $500\times500\ \mu m^2$)[5] or cantilever arrays ($100\times500\ \mu m^2$).[10] In a $20\times300\ nm^2$ feature there are only about 1500 HS-DNA-1 molecules.[27] A hybridization efficiency of 20%, which corresponds to about 300 hybridized molecules (5×10^{-22} mol), produces significant, readily detectable topographic changes (Figure 1). This is the most sensitive method for detection of label-free DNA through hybridization so far reported.[8,10]

It is important that a hybridization experiment exhibits selectivity. To demonstrate sequence specificity, we employed nanoshaving[20] to produce multisequenced DNA features on a single surface. Nanoshaving was used first to produce two nanoholes (2.5-nm deep) within the DNA resist layer (Figure 3 a). After incubation with the HS-DNA-1 (1 μM) for 1 h, the holes were filled and the features protruded by

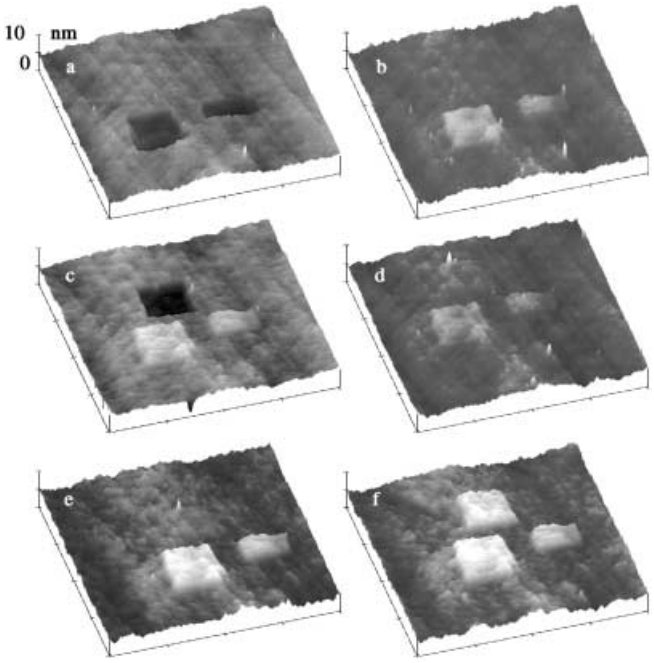

Figure 3. AFM topographic images showing the process for the construction of multisequenced ssDNA nanofeatures on a single surface and selective DNA sequence detection. All images are in the same *x*-, *y*- ($2\times2\ \mu m^2$) and *z*-scales. a) Two nanoholes (400×400 and $400\times200\ nm^2$) were made within HS-dsDNA-0 SAM resist. b) After incubation with HS-DNA-1 (1 μM) for 1 h. c) A new hole was constructed. d) Incubation with HS-DNA-2 (1 μM) for 1 h. No detectable changes were observed upon exposure of the surface to a noncomplementary DNA-3 (10 μM) for 1 h. e) After incubation of the surface with 91 nM DNA-1C in the presence of 9.1 μM DNA-3 for 4 h. f) After introducing 0.5 μM DNA-2C into the incubation solution and incubating for 80 minutes.

about 1 nm above the DNA resist background (Figure 3b). A new hole was produced at a different location of the surface with a depth of 3.0 nm (Figure 3c), and filled by further incubation with HS-DNA-2 (1 μM) for 1 h. The new feature protruded by about 0.4 nm above the DNA background (Figure 3d). No topographic changes were observed on exposure of this surface to a solution of the noncomplementary DNA-3 (10 μM) for 1 h. However, when the surface was exposed to a solution containing the complementary DNA-1C (91 nM) in the presence of 100-fold excess of DNA-3 (9.1 μM) for 4 h, a height increase of about 1.2 nm was observed for the HS-DNA-1 features, with no observable topographic changes from the HS-DNA-2 feature (Figure 3e). Following the introduction of 0.5 μM DNA-2C (complementary to HS-DNA-2), the height of the ssDNA-2 feature was found to have increased by about 2 nm after incubation for 80 minutes (Figure 3f). This observation confirmed that the integrity of the HS-DNA-2 feature had been maintained, and no detectable exchange of thiolated DNA had occurred during the sequential nanoshaving and self-assembly processes. These results demonstrate that the multisequenced DNA nanofeatures have a high selectivity towards probe sequences, which can specifically detect probe sequences even in the presence of a 100-fold excess of noncomplementary strands.

In summary, this work demonstrates the use of AFM as a tool for systematic and quantitative studies of the DNA hybridization dynamics by monitoring the topographic changes of the DNA nanofeatures produced by nanoshaving and self-assembly. This method is extremely sensitive. We have demonstrated that it is possible to image approximately 300 molecules of unlabeled DNA and detect hybridization from 10 nM solutions in situ. We have also demonstrated the use of sequential nanoshaving and self-assembly to produce multisequenced DNA nanofeatures on the same surface, which can selectively detect probe DNA sequences in the presence of 100-fold excess of noncomplementary DNA.

Experimental Section

All single-stranded (ss) DNAs were purchased from MWG Biotech AG (Ebersberg, Germany) and their sequences are listed in Table 1. MilliQ water (resistance $>18\ M\Omega\,cm$) was used to make the Tris buffer (10 mM Tris·HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.5). TSG surfaces were prepared following the literature procedure.[24] Once prepared, the TSG surfaces were immediately used to minimize contamination. Prehybridized dsDNA was prepared following the literature procedure,[24] and diluted to a working concentration with the Tris buffer.

All AFM experiments were carried out on a Digital Instrument (Veeco, CA, USA) Dimension 3100 AFM with a Nanoscope IV controller and a fluid cell under Tris buffer at $24\pm1\,°C$ in contact mode.[24] Standard oxide-sharpened Si_3N_4 probes (DI, spring constant: $0.12\ N\,m^{-1}$) were used in all image collections and nanoshaving experiments. Images were collected at 2 Hz, and analyzed using the Nanoscope image analyzing software with first-order flattening. Before conducting the nanoshaving experiment, the dsDNA SAM resist surface was first imaged under minimum loading force (0.2–0.5 nN) to select a suitable small flat area that avoided domain boundaries. The chosen area was repeatedly scanned at 4–5 Hz under high loading forces (30–50 nN) to scratch away the DNA resist till the gold interatomic steps were clearly visible.[24] The AFM tip was then fully retracted from the surface, zoomed out to a bigger scan area, then re-engaged with minimum force to take topographic images. After each manipulation, the remaining unreacted solution was removed from the fluid cell, and the surface was thoroughly washed with Tris buffer, before a new DNA solution (ca. 150 μL) was introduced and incubated.

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[1] C. M. Niemeyer, D. Blohm, *Angew. Chem.* **1999**, *111*, 3039; *Angew. Chem. Int. Ed.* **1999**, *38*, 2865.
[2] S. V. Tillib, A. Mirzabekov, *Curr. Opin. Biotechnol.* **2001**, *12*, 53.
[3] E. Southern, K. Mir, M. Shchepinov, *Nat. Genet.* **1999**, *21*, 5.
[4] F. Caruso, E. Rodda, D. F. Furlong, K. Niikura, Y. Okahata, *Anal. Chem.* **1997**, *69*, 2043.
[5] B. P. Nelson, T. E. Grimsrud, M. R. Liles, R. M. Goodman, R. M. Corn, *Anal. Chem.* **2001**, *73*, 1.
[6] A. W. Peterson, R. J. Heaton, R. M. Georgiadis, *Nucleic Acids Res.* **2001**, *29*, 5163.
[7] A. W. Peterson, L. K. Wolf, R. M. Georgiadis, *J. Am. Chem. Soc.* **2002**, *124*, 14601.

[8] F. Y. Song, F. M. Zhou, J. Wang, N. J. Tao, J. Q. Lin, R. L. Vellanoweth, Y. Morquecho, J. Wheeler-Laidman, *Nucleic Acids Res.* **2002**, *30*, e72.
[9] R. Georgiadis, K. P. Peterlinz, A. W. Peterson, *J. Am. Chem. Soc.* **2000**, *122*, 3166.
[10] R. McKendry, J. Y. Zhang, Y. Arntz, T. Strunz, M. Hegner, H. P. Lang, M. K. Baller, U. Certa, E. Meyer, H. J. Guntherodt, C. Gerber, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 9783.
[11] L. He, M. D. Musick, S. R. Nicewarner, F. G. Salinas, S. J. Benkovic, M. J. Natan, C. D. Keating, *J. Am. Chem. Soc.* **2000**, *122*, 9071.
[12] S. O. Kelley, J. K. Barton, N. M. Jackson, L. D. McPherson, A. B. Potter, E. M. Spain, M. J. Allen, M. G. Hill, *Langmuir* **1998**, *14*, 6781.
[13] M. Sam, E. M. Boon, J. K. Barton, M. G. Hill, E. M. Spain, *Langmuir* **2001**, *17*, 5727.
[14] R. D. Piner, J. Zhu, F. Xu, S. H. Hong, C. A. Mirkin, *Science* **1999**, *283*, 661.
[15] J. C. O'Brien, J. T. Stickney, M. D. Porter, *J. Am. Chem. Soc.* **2000**, *122*, 5004.
[16] J. R. Kenseth, J. A. Harnisch, V. W. Jones, M. D. Porter, *Langmuir* **2001**, *17*, 4105.
[17] K. B. Lee, S. J. Park, C. A. Mirkin, J. C. Smith, M. Mrksich, *Science* **2002**, *295*, 1702.
[18] L. M. Demers, D. S. Ginger, S. J. Park, Z. Li, S. W. Chung, C. A. Mirkin, *Science* **2002**, *296*, 1836.
[19] S. J. Park, T. A. Taton, C. A. Mirkin, *Science* **2002**, *295*, 1503.
[20] G. Y. Liu, S. Xu, Y. L. Qian, *Acc. Chem. Res.* **2000**, *33*, 457.
[21] G. Y. Liu, N. A. Amro, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5165.
[22] X. Z. Wang, D. J. Zhou, T. Rayment, C. Abell, *Chem. Commun.* **2003**, 474.
[23] M. Z. Liu, N. A. Amro, C. S. Chow, G. Y. Liu, *Nano Lett.* **2002**, *2*, 863.
[24] D. J. Zhou, K. Sinniah, C. Abell, T. Rayment, *Langmuir* **2002**, *18*, 8278.
[25] K. Wadu-Mesthrige, N. A. Amro, J. C. Garno, S. Xu, G. Y. Liu, *Biophys. J.* **2001**, *80*, 1891.
[26] C. H. Jang, B. D. Stevens, P. R. Carlier, M. A. Calter, W. A. Ducker, *J. Am. Chem. Soc.* **2002**, *124*, 12114.
[27] A HS-DNA-1 SAM prepared on a TSG surface under identical condition (1 μM in Tris, 1 h) has a dry DNA thickness of 1.6 ± 0.4 nm from the ellipsometry measurement, which corresponds to $2.6 \pm 0.7 \times 10^{13}$ DNA cm^{-2} assuming a DNA density of 1.7 g cm^{-3} (see Y. K. Cho, S. Kim, G. Lim, S. Granick, *Langmuir* **2001**, *17*, 7732). The value is in very good agreement with XPS and radiolabeling data (see D. Y. Petrovykh, H. Kimura-Suda, L. J. Whitman, M. J. Tarlov, *J. Am. Chem. Soc.* **2003**, *125*, 5219; A. B. Steel, R. L. Levicky, T. M. Herne, M. J. Tarlov, *Biophys. J.* **2000**, *79*, 975).

