Probe Microscopy, 2002 Vol. 2 (3–4), pp. 227–234

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Aminomodified Probes for Atomic Force Microscopy

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(Received 14 March 2000; Revised 6 August 2000; In final form 1 September 2000)

A procedure for direct functionalization of silicon nitride atomic force microscope (AFM) probes with 3-aminopropyltriethoxy silane (APTES) was developed. The probes were modified in vapors of APTES at ambient conditions (AP-tips). The functionalization of tips was monitored by fluorescent microscopy and the force measurements with AFM. AP-tips were derivatized with a non-fluorescent quinoline analog (FQ) which reacts with primary amines to form a strongly fluorescent isoindole. AP-tip fluorescence was significantly higher than control probes. Amino modified tips manifest a strong adhesion to freshly cleaved mica and this behavior is qualitatively similar to the interaction of unmodified silicone nitride probes with AP-mica. The adhesion of AP-tips to mica or AP-mica to unmodified tips decreases as ionic strength increases indicating a dominant electrostatic component of the adhesion effect. This interpretation is supported by the experiments at different pH and the reaction of AP-tips with glutaraldehyde. The adhesion effect is very small at basic pH due to the deprotonation of amino groups. The reaction of immobilized amines by the treatment of the tips with glutaraldehyde decreases the tip–surface interaction dramatically. Like AP-mica, AP-trips are stable during storage and thus can be used for additional derivation steps.

Keywords: Atomic force microscopy (AFM); Probes; Functionalization; Adhesive forces; Aminosilanes

INTRODUCTION

Despite their recent invention, atomic force microscopes (AFM) are widely available commercially. AFM and its special modifications (tapping mode and non-contact operation in solution) have been successfully used for topographic studies of a large number of biological objects including DNA, RNA, proteins, cell membranes and even whole cells (reviewed in Bottomley, 1998). Specifically, AFM was very successfully applied to studies of nucleic acids (Bustamante *et al.*, 1992; 1994; Hansma and Hoh, 1994; Lyubchenko *et al.*, 1995). Part of this success is due to development of reliable sample preparation procedures (Vesenka *et al.*, 1992; Yang *et al.*, 1992; Lyubchenko *et al.*, 1992; 1995; 1996). In addition to topographic studies, AFM is becoming a powerful tool for investigating intermolecular interactions (Chemical Force Microscopy, CFM, e.g. Moy *et al.*, 1994; Hinterdorfer *et al.*, 1996; Vezenov *et al.*, 1997). This field of AFM application is of great importance and the availability of simple and reliable methods for preparation of surfaces and AFM probes with desirable characteristics facilitates a routine use of this type of AFM for numerous pure and applied biological and pharmacological studies.

We earlier developed a procedure for modification of mica with 3-aminopropyltriethoxy silane (APTES) under very mild conditions (AP-mica) (Lyubchenko *et al.*, 1992; 1995; 1996; Lyubchenko and Lindsay, 1998). This enables routine visualization of DNA with AFM, achieving resolution as good as that of traditional electron microscopy (EM) (reviewed in Lyubchenko *et al.*, 1995). Remarkably, we have achieved resolution for DNA in solution exceeding that of EM for dried DNA samples (Lyubchenko and Shlyakhtenko, 1997). We have also found the conditions for a long-term storage of AP-mica in active form preventing contamination of the surface (Lyubchenko *et al.*, 1995; Lyubchenko and Lindsay, 1998). These attractive features of AP-mica together with its high stability, activity in a board range of ionic strength, pH and temperatures motivated us to study the possibility of preparation of AFM probes with similar characteristics. Silicon nitride ceramic can be modified with silanes

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(Tsukruk and Bliznyuk, 1998), so it is reasonable to propose that silicone nitride AFM probes will react with APTES to provide a functionalized surface (Lyubchenko and Lindsay, 1998). We have shown recently that AP-mica is terminated with an active amino group capable of coupling with amine reagents (Shlyakhtenko *et al.*, 1999). Specifically, we showed that DNA can be covalently attached to AP-mica using an amine-coupling reagent. This is a very important feature of AP-surfaces that opens prospects for preparation of functionalized AFM probes with similar immobilization characteristics. In the current report we test the possibility of functionalization silicon nitride tips with APTES (AP-tips). We describe the procedure for preparation of AP-tips characterized by fluorescence microscopy and force measurements. The latter appeared to be a comparatively simple, convenient and sensitive tool for testing AP-tips.

MATERIALS AND METHODS

Chemicals and Materials

3-Aminopropyltriethoxy silane purchased from Aldrich (Milwaukee, WI, USA) or United Chemical Technologies, Inc. (Bristol, PA, USA) was vacuum distilled, aliquoted and stored under argon.

Modification of Silicone Nitride Probes

Silicon nitride tips (Park Scientific Instruments, Sunnyvale, CA) were washed with ethanol for 2 min, thoroughly rinsed with ultrapure water (resistivity ~ 17 MΩ/cm, ModuPure Plus, Continental Water System, San Antonio, TX), blown dry with argon and finally cleaned by irradiation with a short-wave high intensity UV lamp for 45 min (Shlyakhtenko *et al.*, 1999). The distance between lamp and cantilever surface was 2.5 cm. Cleaned Si_3N_4 tips were mounted inside the top of a glass 2L desiccator for the modification. Vapor deposition was performed as previously described (Lyubchenko and Lindsay, 1998). Briefly, 30 μl of APTES in a plastic cap was placed at the bottom of the desiccator which was then filled with argon. The probes were incubated under these conditions for the desired time period, usually 15 min, 1 or 2 h. Tips functionalized in this fashion tips were stored in a desiccator under argon atmosphere. Mica sheets were modified simultaneously with the tips for their use in parallel experiments for monitoring the functionalization process.

Primary Amine Labeling

Primary amines on APTES-treated probes (AP-tips) were detected by labeling with ATTO-TAG FQ [3-(2-furoyl) quinoline-2-carboxaldehyde] (Amine Derivatization Kit, A-2334, Molecular Probes, Eugene, OR, USA) and examination with epifluorescence confocal microscopy. AP-tips were taken from storage under argon and anchored in small petri dishes (60 mm, Fischer Scientific) with a strip of double-sided tape. Individual cantilever substrates were immersed in 425 μl of borate buffer, pH 9.5, to which 50 μl of ATTO-TAG FQ (10 mM stock) and 25 μl KCN (200 mM stock) were added. The derivatization reaction was carried out for 3.5 h at room temperature followed by three washes with borate buffer. The FQ-treated tips were kept borate buffer and immediately examined with epifluorescence microscopy. Control Si_3N_4 probes received identical treatment in parallel with the AP-tips.

Epifluorescence Microscopy

The highly-fluorescent products of FQ-derivatized primary amines are maximally excited at 480 nm with an emission maximum at 590 nm. Owing to the relatively small surface area of the AP-tips, fluorescent counts were detected with a confocal microscope (Noran Odyssey, Middleton, WI, USA). To minimize photobleaching, probe tips were located and evaluated for integrity with reflectance mode microscopy. The confocal microscope was then switched to the fluorescence channel collection mode and fluorescence images were acquired with the following parameters: 488 nm excitation, 515 nm emission filter, 100 μm slit, 55% laser power, 2600 brightness, 128 contrast, 3 × digital zoom, 40 × objective (Plan-Neoflur, NA 0.7, Zeiss). Images were summed over 256 frames to enhance the signal-to-noise ratio. These parameters were held constant across all image acquisitions, both for AP-tips and controls. All AP-tips and two of five controls were evaluated in one session.

Image Analysis

Images were first corrected for aspect-ratio differences between the video acquisition system and computer-based digital presentation (Debabelizer 3, Equilibrium Software, Sausalito, CA, USA). The corrected images were then quantitatively analyzed with IP Lab Spectrum (v3.1a, Signal Analysis, Vienna, VA, USA). Fluorescent counts were detected within a fixed region of interest (40 × 40 pixels), corresponding to the base of the pyramidal tips, appropriately positioned on each image. The 1-D raw fluorescent count data was imported into Excel98 (Microsoft, Redmond, WA) and arranged for further analysis. Histograms and box plots were generated in Origin 6.0 (Microcal Software). Fluorescence images presented in the figures were enhanced in Photoshop (v5.5, Adobe Software, Mountain View, CA, USA). Minitab (v12, Minitab) was used for ANOVA and homogeneity of variance tests. All statistical analyses and plots were done with the raw data.

Atomic Force Microscopy

A NanoScope III MultiMode System (Digital Instruments, Santa Barbara, CA) with a D-scanner and commercial

fluid cell was used in all experiments. Imaging was performed in air with TappingMode operation. Force distance curves were recorded at 1–3 Hz frequency and 200–400 nm amplitude at the conventional force calibration plot mode for randomly selected points. Adhesion forces were obtained by averaging over 10–30 force distance plots. Number of data points collected in one approach-retract cycle varied from 256 to 512. V-shaped silicon nitride cantilevers with a nominal spring constant 100 pN/nm were used for force measurements, unless otherwise stated. The cantilever spring constants were estimated using the resonant frequency method (Cleveland *et al.*, 1993). This parameter for different tips varied between 44 and 80 pN/nm (Table I). The same tip was used for measurements at different ionic conditions.

The fluid cell was cleaned by soaking in chromic/sulfuric acid solution at room temperature for 5 min followed by a thorough rinsing with deionized water. The buffer solution was changed by using 1 ml tuberculin syringes with plastic tips attached to the fluid cell. The glass fluid cell was used without an O-ring.

The buffers used were 2 mM Tris–HCl (pH 7.6), 0.2 mM EDTA (0.2 TE buffer), 10 mM Tris–HCl pH 7.6, 1 mM EDTA (1 TE buffer), 100 mM Tris–HCl pH 7.6, 10–mM EDTA (10 × TE buffer), 10 mM Tris −7 mM NaOH, pH 11.2, phosphate buffer pH 7–9 with ionic strength $I = 10$ mM and 10 mM NaOH, pH 12. pH values were monitored by a pH meter Model 8000 (VWR Scientific Products, USA). To achieve reproducible force values for force–distance curves at different ionic conditions one tip was used throughout one set of force titration experiments. After injection of ~20 ml of TE buffer into the fluid cell, force plots were obtained. Then the glass cell was gently washed with ~200 μl of a new buffer solution and force curves were measured for these ionic conditions, and so on.

RESULTS AND DISCUSSION

The Tip–substrate Interaction

Calibration of the Probes

The cantilever resonance frequency shift method (Cleveland *et al.*, 1993) was used for estimation of the spring constant for each tip used. Difference in spring constants of cantilevers taken from a restricted region of a wafer is considerably smaller than manufacturer's specification provides—from 60 to 150 pN/nm.

TABLE I Adhesion forces (nN) for aminomodified mica (AP-mica) and unmodified silicone nitride cantilevers in different solutions

Cantilever	TE-buffer	10 × TE	pH 11.2
1 ($k = 0.5$ N/m)	1.96 ± 0.40	0.84 ± 0.09	0.78 ± 0.15
2 ($k = 0.1$ N/m)	4.62 ± 0.47	2.28 ± 0.28	1.08 ± 0.36
3 ($k = 0.05$ N/m)	1.75 ± 0.19	1.24 ± 0.19	0.55 ± 0.13

Force Measurements

Contact AFM in liquid was used to measure the tip–surface interaction. Tip adhesion was measured in force distance cycles as the force necessary to break the tip away from mica surface. A typical force–distance curve for an unmodified tip on freshly cleaved mica is shown in Fig. 1A. There is no adhesion effect, which is the expected result for surfaces that are negatively charged at neutral pH (Butt, 1991). Modification of the surface changes the interaction dramatically. A strong adhesion effect for the AP-mica/unmodified silicone nitride tip pair in a comparatively low ionic strength solution is shown in Fig. 1B. The adhesion is considerably less if the force–distance curves are obtained at higher ionic strength (Fig. 1C). Note that only the solution was exchanged—the same substrate and the tip were used in the experiments. These data indicate that electrostatic interaction between the AP-surface and unmodified tip is responsible for the adhesion effect. The adhesion is substantially less if alkaline solution is substituted for the neutral TE buffer (Fig. 1D). AP-mica, similar to other immobilized amines, deprotonates at pH 9 and higher (Bezanilla *et al.*, 1995) and silicone nitride remains negatively charged in this region (Tsukruk and Bliznyuk, 1998; Müller *et al.*, 1999), so these data are consistent with the proposal of an electrostatic component dominating the tip–AP-mica interaction. The results for pull-off forces for three different pairs tip/AP-mica are shown in Table I. There is a substantial variability in adhesion forces that very likely reflects differences in tip geometry. However, the most important results of these observations is that despite the difference between the tips the effect of ionic strength is the same.

The results for the interaction of amino functionalized Si_3N_4 tips and freshly cleaved mica are shown in Fig. 2. The data are shown for three cantilevers with different spring constants. Strong adhesion is observed in low salt, neutral pH solution (TE buffer) for all the cantilevers (plots A, D, and G). At elevated ionic strength (10 × TE) the adhesion effect is considerably lower for cantilevers 1 and 2 (plots B and E, respectively) and a soft cantilever 4 showed no adhesion (plot H). The data obtained for a basic buffer (pH 11.2) are shown in plots C, F, and I for cantilevers 1,2, and 4, respectively. The adhesion effect for all cantilevers drops dramatically at these conditions. The pull-off forces calculated from these plots are shown in

TABLE II Adhesion forces (nN) for aminomodified cantilevers AP1, AP2, and AP15 (spring constants are given in parenthesis) and unmodified mica at different ionic conditions and concentration of glutaraldehyde (GA)

Cantilever type (spring constant)	TE-buffer	10 × TE	pH 11.2
1 ($k = 0.5$ N/m)	3.25 ± 0.42	1.58 ± 0.46	0.80 ± 0.25
2 ($k = 0.1$ N/m)	1.67 ± 0.23	0.91 ± 0.19	0.08 ± 0.02
4 ($k = 0.01$ N/m)	4.60 ± 0.65	0.19 ± 0.15	0.44 ± 0.13

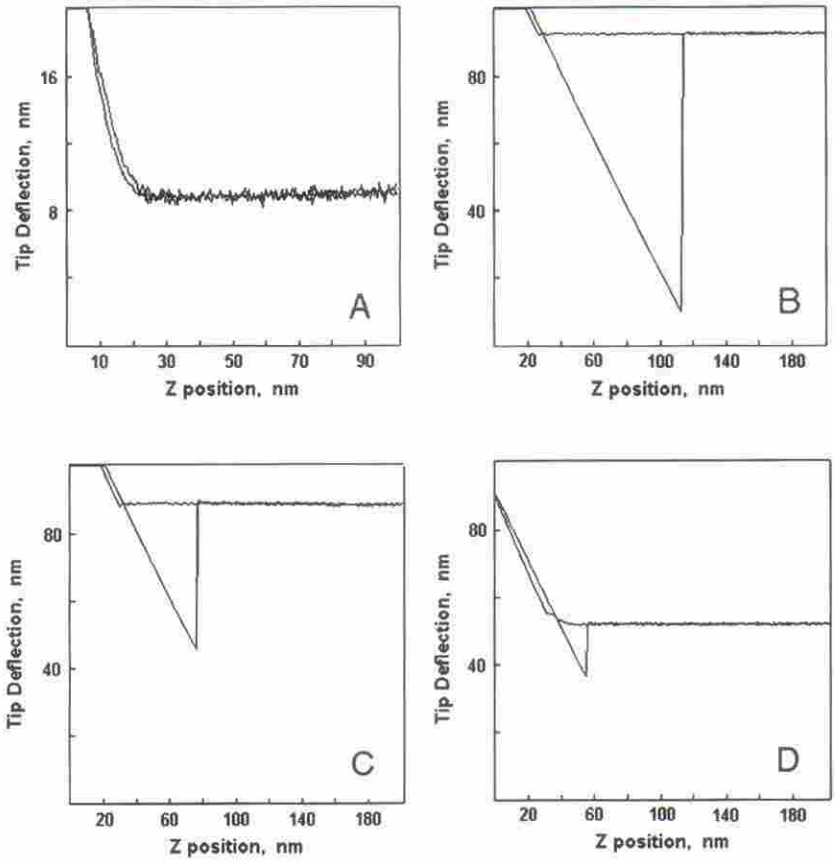

FIGURE 1 The force–distance curves for different probe–substrate combinations. The data were taken in aqueous solutions. (A) Silicon nitride tip-freshly cleaved mica, TE buffer; (B–D) AP-mica-cantilever 2 ($k = 0.1$ N/m); B: TE buffer, C: 10 × TE buffer, D: pH 11.2; (F–H) unmodified tips.

Table II. There is a remarkable coincidence of these data with the results for the interaction of AP-mica with silicon nitride tips (Table I), suggesting that the characteristics of APTES-functionalized tips and AP-mica are similar.

A key feature of AP-mica is its capacity for binding DNA (Lyubchenko *et al.*, 1992; 1995; 1996; Lyubchenko and Lindsay, 1998; Lyubchenko and Shlyakhtenko, 1997). To demonstrate that AP-tips bind DNA in a similar manner, AP-tips were immersed into DNA solutions, dried and then the adsorption of DNA to this surface was monitored by taking force–distance curves (Fig. 3). Plot A indicates that there is pure repulsion between both surfaces. Because DNA is a negatively charged polymer, its binding leads to the conversion of a positively charged surface AP-tip into a negative one. However, the force–distance curves change over repeating approach-retract cycles and one curve taken after dozens of such cycles is shown in Fig. 3B. A clear adhesion peak is formed indicating that DNA is partially displaced from the apex of the tip allowing exposed regions of the AP-tip to interact with mica. This observation is qualitatively consistent with the AFM imaging of DNA on AP-mica in solution demonstrating a high mobility of DNA on AP-mica. The profile of this curve is quite broad, indicative of a complex tip morphology.

We recently showed that amino groups of AP-mica surface are active and are capable of reaction with amine-reactive compounds (Shlyakhtenko *et al.*, 1999). In particular, we conjugated a trifluorester of trioxalen to amines of AP-mica that allowed us to covalently attach DNA molecules to surface. To test the reactivity of AP-tips we studied their reaction with glutaraldehyde, a common reagent for modification of free amines. Glutaraldehyde should decrease the surface charge density of AP-tips due to the formation of the Schiff bases with amines (Knapp, 1979), so the measurements of the AP-tip/mica interaction is a straightforward means for monitoring the reactivity of amines of AP-tips. The force measurements were performed with the same tip in different solutions. The data in Fig. 4 show that adhesion forces drop dramatically upon adding glutaraldehyde providing strong support for the proposed chemistry of AP-tips.

One of the typical applications of surfaces functionalized by amines is the immobilization of biological molecules through the coupling reaction with amino-reagents like succinimydyl esters, trifluoresters, etc. (e.g. Shlyakhtenko *et al.*, 1999). The reactivity of AP-tips was tested by FQ [3-(2-furoyl)quinoline-2-carboxaldehyde]. FQ is a non-fluorescent quinoline which reacts with primary amino groups in the presence of KCN forming a strongly fluorescent isoindole. Both aminated tips and untreated control probes were derivatized with FQ [3-(2-furoyl)quinoline-2-carboxaldehyde]. The results of fluorescent microscopy studies for unmodified and AP-probes are shown in Fig. 5. To minimize photobleaching,

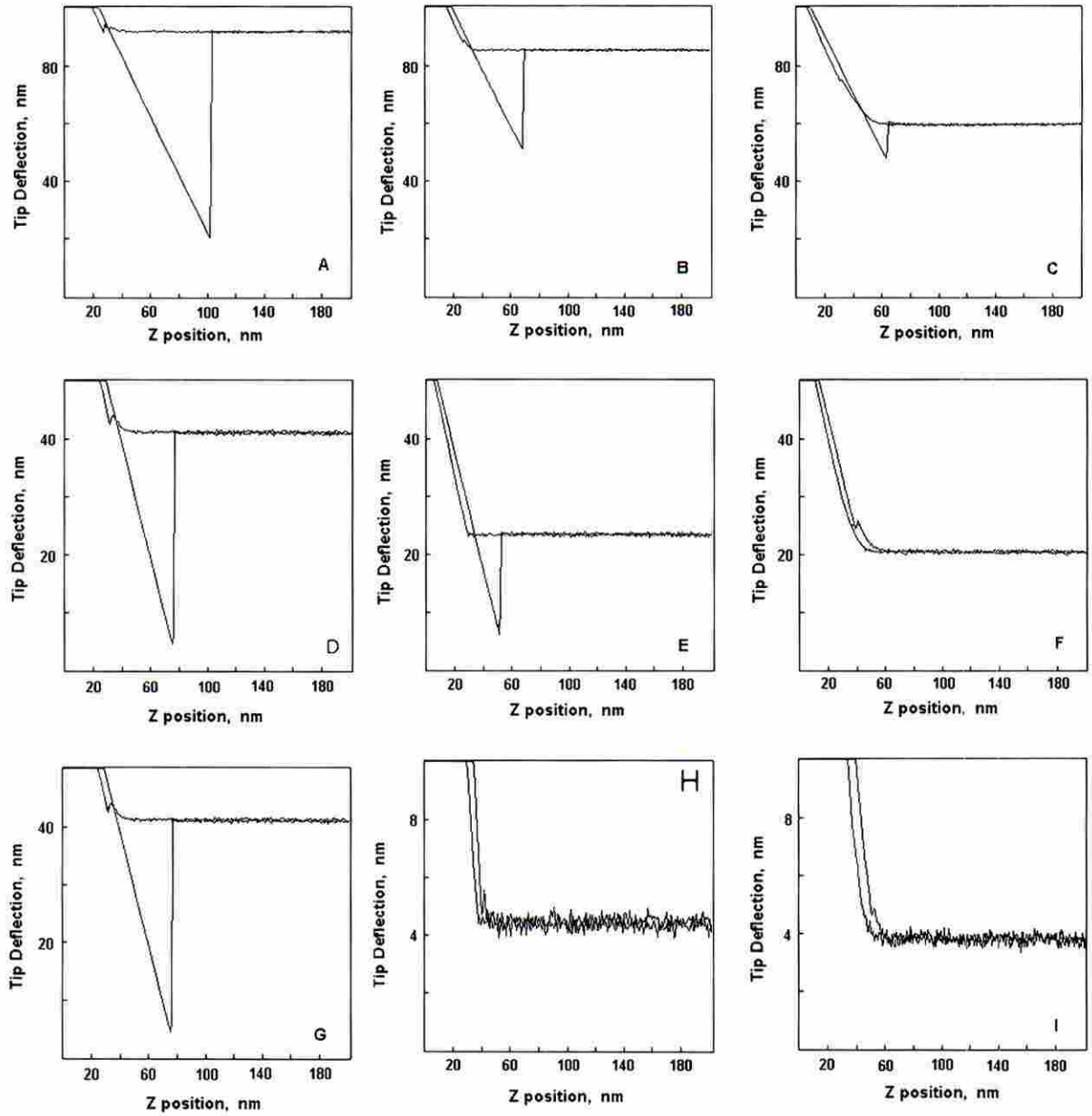

FIGURE 2 The force–distance curves for the interaction of AP-tips (different spring constants) with a freshly cleaved mica at different conditions. The plots (A–C) are for data cantilever 1 ($k = 0.5$ N/m); (D–F) are for the cantilever 2 (0.1 N/m) and (G–I) are for cantilever 4 (0.01 N/m). The data for TE buffer; (A,D,G); 10 × TE buffer (B,E,H) and pH 11.2 (C,F,I).

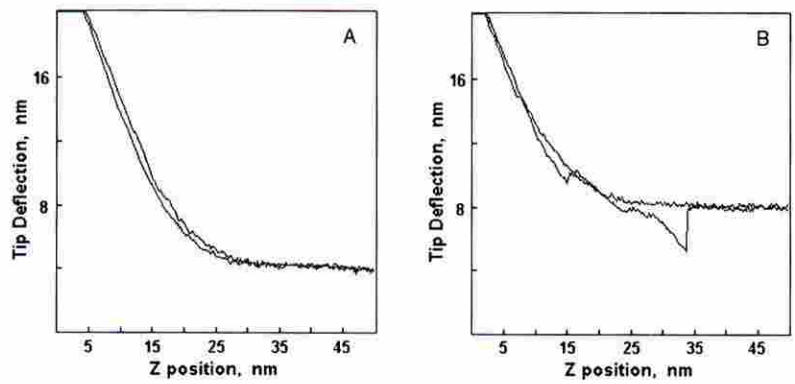

FIGURE 3 The effect of DNA binding on the interaction of AP-tip ($k = 0.1$ N/m) with freshly cleaved mica in TE buffer. Plots A and B were taken within a few second interval.

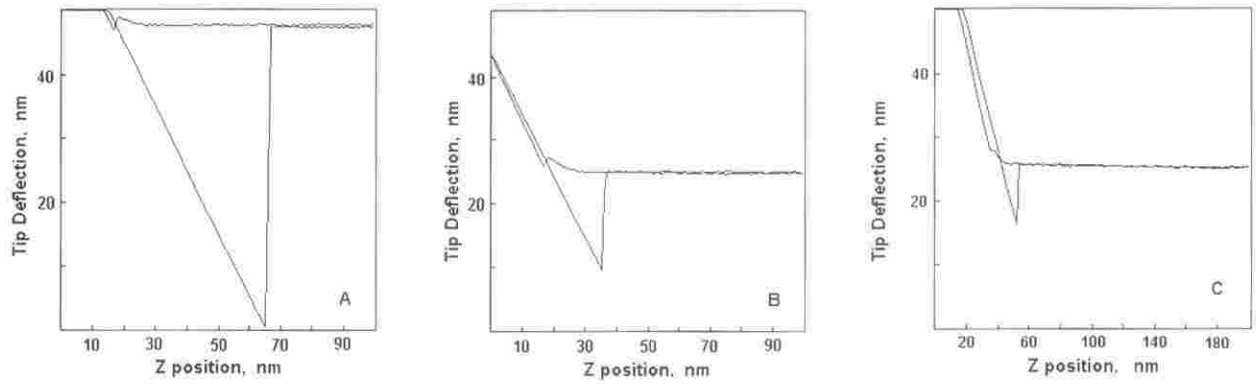

FIGURE 4 The effect of glutaraldehyde on the interaction of AP-tip ($k = 0.1$ N/m) with freshly cleaved mica. (A) No glutaraldehyde, phosphate buffer (pH 7.0); (B) 0.3 mM of glutaraldehyde and (C) 3 mM of glutaraldehyde.

probe tips were located and evaluated for integrity with reflectance mode microscopy. The confocal microscope was then switched to fluorescence channel collection mode and fluorescence images were acquired. Panels A and C are control probes; panels B and D are AP-tips. In general, the fluorescence of AP-tips was higher than control samples. However, quantitative analysis was necessary to buttress this qualitative evaluation. Fluorescence of both types of the sample was quantitated with confocal microscopy and statistical analyses were performed on the raw pixel count data (grayscale levels 0–255). The histograms are presented in Fig. 6. Assuming a 10% significance level, the balanced ANOVA results indicate that the difference in average grayscale value between the control and experimental groups is statistically significant ($p = 0.091$). The average grayscale value is 21.530 for the control group and 28.493 for the AP-tip group. However, homogeneity of variance tests (Levene's test) show there is significant tip-to-tip variability within both control and AP-tips groups. The ANOVA results show that within-group variability is statistically significant at $p = 0.000$, implying that the grayscale average varies substantially between tips within group. The strong fluorescence in the aminated probes relative to control indicates that the AP-tips are capable of conjugating with succinimydyl esters and corroborates the other evidence presented here that they are effectively derivatized with primary amine groups.

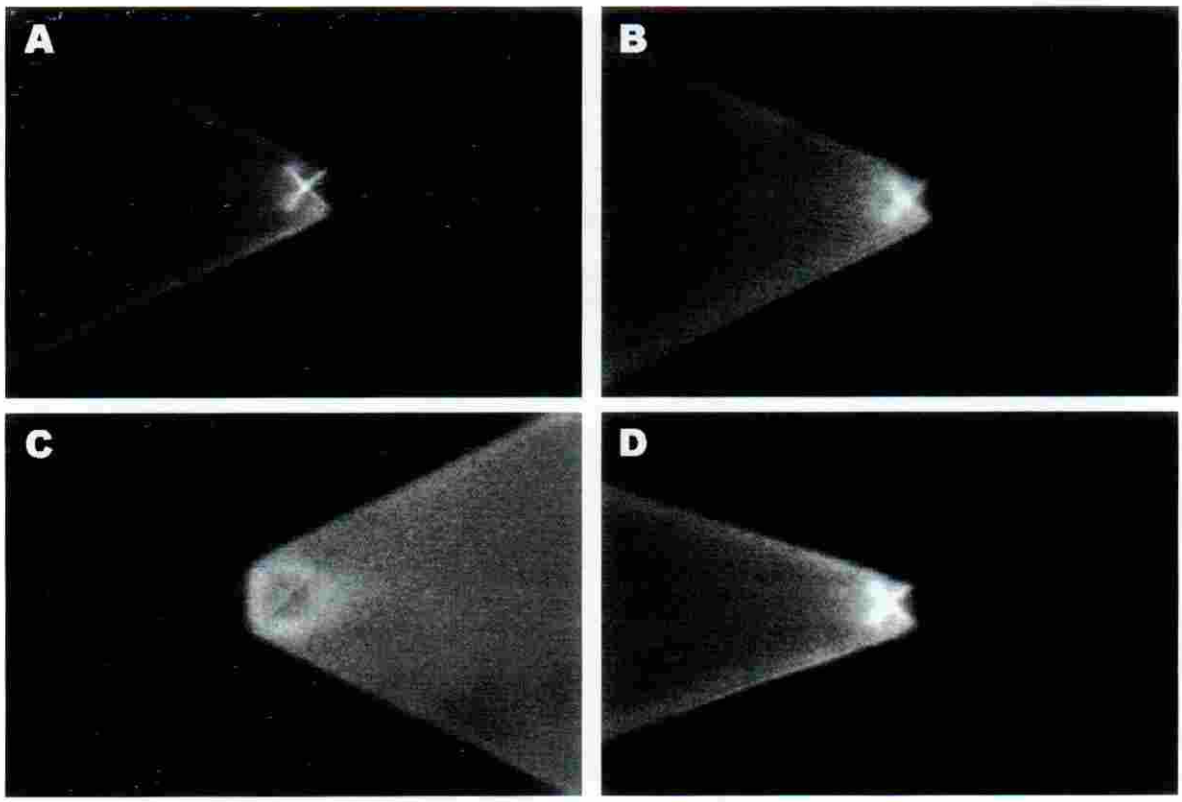

FIGURE 5 Fluorescence micrographs of control probes and AP-tips. Amino groups were derivatized with ATTO-TAG FQ and examined with confocal microscopy. Panels A and C are control probes; panels B and D are AP-tips. Image levels were enhanced for display purposes only.

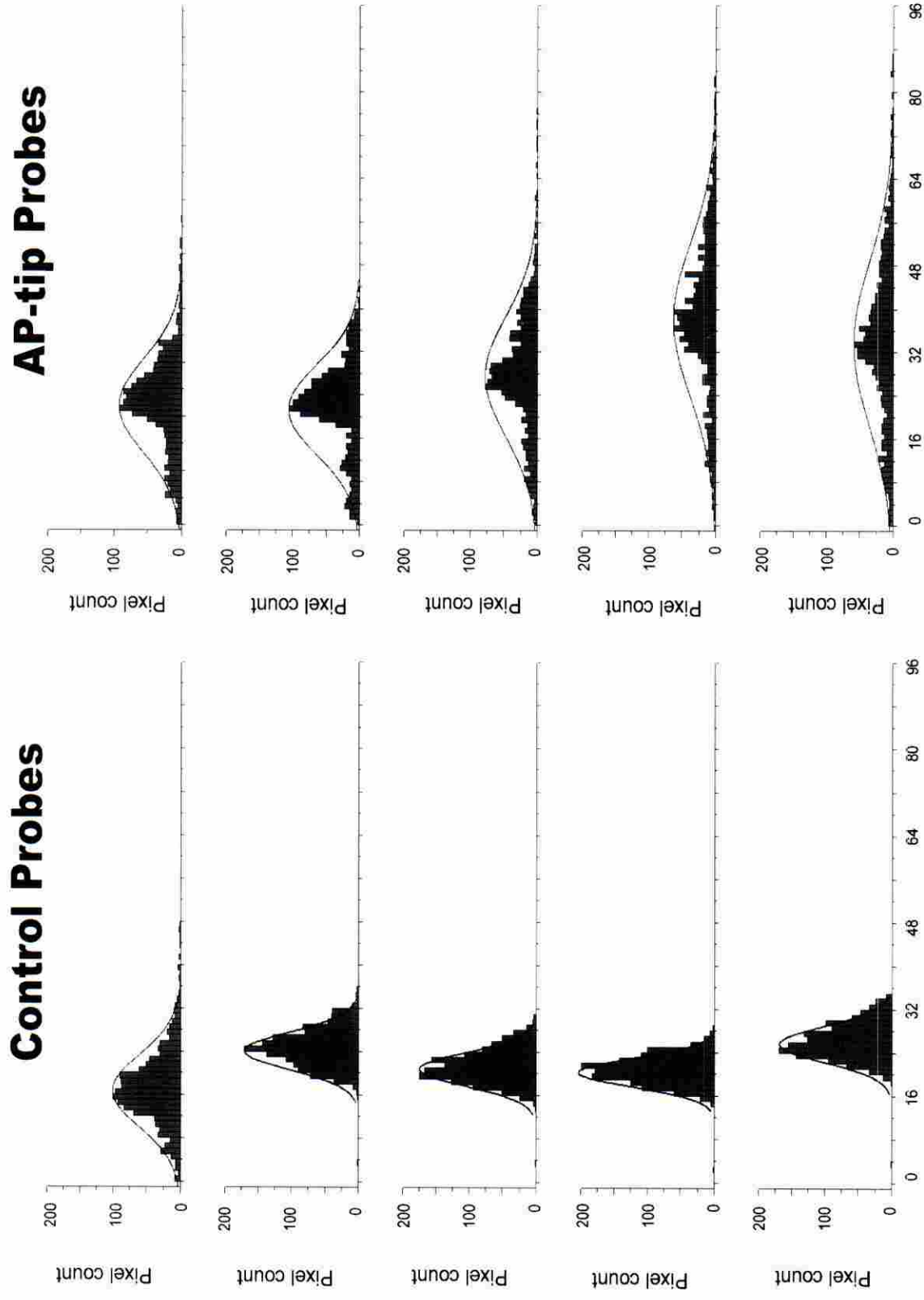

FIGURE 6 Histograms of fluorescence data from primary amino group labeling. Left column is control probes, right column is APTES-treated probes (AP-tips). Control probes exhibit nearly normal distribution of grayscale values over a narrow range ($x = 21.517 \pm 5.161$), whereas AP-tip values do not follow a normal distribution and are spread over a greater range ($x = 28.480 \pm 13.413$). ANOVA results indicate the average grayscale value is significantly different between control and AP-tip groups ($p = 0.091$).

CONCLUSIONS

The results presented here demonstrate that amino functionalized tips can be prepared by the modification of Si_3N_4 probes under very mild conditions. We also demonstrate a very simple way of testing amino modified AFM probes based on the analysis of force–distance curves acquired by a conventional AFM instrument. In addition, we demonstrate that amino groups of AP-tips are reactive, and they can be conjugated with biological molecules using a well-known chemistry for immobilization of amines. This establishes the prospects for the use of this type of amino-functionalized tip for numerous CFM applications.

Acknowledgements

This work was supported partially by the grants GM 54991 and GM 62235 (NIH) and LST.CNS.976242 (NATO). The authors thank I. Yaminsky for useful comments and suggestions.

References

Bezanilla, M., Manne, S., Laney, D.E., Lyubchenko, Y.L. and Hansma, H.G. (1995) "Adsorption of DNA to mica, silylated mica, and minerals: characterization by atomic force microscopy", *Langmuir* **11**, 655–659.

Bottomley, L. (1998) "Scanning probe microscopy", *Anal. Chem.* **70**, 425R–475R.

Bustamante, C., Vesenka, J., Tang, C.L., Rees, W., Gutfold, M. and Keller, R. (1992) "Circular DNA molecules imaged in air by scanning force microscopy", *Biochemistry* **31**, 22–26.

Bustamante, C., Erie, D.A. and Keller, D. (1994) "Biochemical and structural applications of scanning force microscopy", *Curr. Opin. Struct. Biol.* **3**, 750–760.

Butt, H. (1991) "Measuring electrostatic, van der Waals, and hydration forces in electrolyte solutions with an atomic force microscope", *Biophys. J.* **60**, 1438–1444.

Cleveland, J.P., Manne, S., Bocek, D. and Hansma, P.K. (1993) "A non-destructive method for determining the spring constant of cantilevers for scanning force microscopy", *Rev. Sci. Instr.* **64**, 403–405.

Hansma, H.G. and Hoh, J. (1994) "Biomolecular imaging with the atomic force microscopy", *Ann. Rev. Biophys. Biochem. Struct.* **23**, 115–139.

Hinterdorfer, P., Baumgartner, W., Gruber, H., Schilcher, K. and Schindler, H. (1996) "Detection and localization of individual antibody—antigen recognition events by atomic force microscopy", *Proc. Natl Acad. Sci. USA* **93**, 3477–3481.

Knapp, D.R. (1979) Handbook of Analytical Derivatization Reactions (Wiley, New York).

Lyubchenko, Y.L. and Lindsay, S.M. (1998) "DNA, RNA and nucleoprotein complexes immobilized on AP-mica and imaged with AFM", In: Colton, R.J., ed, Procedures in Scanning Probe Microscopy (Wiley, Toronto), pp 493–496.

Lyubchenko, Y.L. and Shlyakhtenko, L.S. (1997) "Visualization of supercoiled DNA with atomic force microscopy *in situ*", *Proc. Natl Acad. Sci. USA* **94**, 496–501.

Lyubchenko, Y.L., Gall, A.A., Shlyakhtenko, L.S., Harrington, R.E., Oden, P.I., Jacobs, B.L. and Lindsay, S.M. (1992) "Atomic force microscopy imaging of double stranded DNA and RNA", *J. Biomol. Struct. Dyn.* **9**, 589–606.

Lyubchenko, Y.L., Jacobs, B.L., Lindsay, S.M. and Stasiak, A. (1995) "Atomic force microscopy of protein–DNA complexes. Review article", *Scanning Microsc.* **9**, 705–727.

Lyubchenko, Y.L., Blankenship, R.E., Lindsay, S.M., Simpson, L. and Shlyakhtenko, L.S. (1996) "AFM studies of nucleic acids, nucleoproteins and cellular complexes: the use of functionalized substrates", *Scanning Microsc.* **10**(Suppl.), 97–109.

Moy, V., Florin, E.-L. and Gaub, H. (1994) "Intermolecular forces and energies between ligands and receptors", *Science* **266**, 257–259.

Müller, D., Fotiadis, D., Scheuring, S., Muller, S. and Engel, A. (1999) "Electrostatically balanced subnanometer imaging of biological specimens by atomic force microscope", *Biophys. J.* **76**, 1101–1111.

Shlyakhtenko, L.S., Gall, A.A., Weimer, J.J., Hawn, D.D. and Lyubchenko, Y.L. (1999) "Atomic force microscopy imaging of DNA covalently immobilized on a functionalized mica substrate", *Biophys. J.* **77**, 568–576.

Tsukruk, V. and Bliznyuk, V. (1998) "Ashesive and friction forces between chemically modified silicon and silicon nitride surfaces", *Langmuir* **14**, 446–455.

Vesenka, J., Guthold, M., Tang, C.L., Keller, D. and Bustamante, C. (1992) "Substrate preparation for reliable imaging of DNA molecules with the scanning force microscopy", *Ultramicroscopy* **42–44**, 1243–1249.

Vezenov, D., Noy, A., Rozsnyai, L. and Lieber, C. (1997) "Force titrations and ionization state sensitive imaging of functional groups in aqueous solutions by chemical force microscopy", *J. Am. Chem. Soc.* **119**, 2006–2015.

Yang, J., Takeyasu, K. and Shao, Z. (1992) "Atomic force microscopy of DNA molecules", *FEBS Lett.* **301**, 173–176.