

# Simple, Clickable Protocol for Atomic Force Microscopy Tip Modification and Its Application for Trace Ricin Detection by Recognition Imaging

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A simple two-step protocol for modification of atomic force microscopy (AFM) tip and substrate by using a “click reaction” has been developed. The modified tip and substrate would be applied to detect trace amounts of ricin by using atomic force microscopy. A key feature of the approach is the use of a PEG (polyethylene glycol) derivative functionalized with one thiol and one azide ending group. One end of the PEG was attached to the gold-coated AFM tip by a strong Au–thiol bond. The azide group hanging at the other end of the immobilized PEG was used for the attachment of an antiricin antibody modified with an alkyne group using a “click reaction”. The latter reaction is highly efficient, compatible with the presence of many functional groups and could proceed under mild reaction conditions. In a separate step, ricin was immobilized on the gold substrate surface that was modified by active esters. For this process, a novel bifunctional reagent was employed containing an active ester and a thioctic acid moiety. By these modification processes, AFM recognition imaging was used to detect the toxin molecules and the results show fg/mL detection sensitivity, surpassing the existing detection techniques. With measurement of the unbinding force between the antiricin antibody and ricin, which was statistically determined to be  $64.89 \pm 1.67$  pN, the single molecular specificity of this sensing technique is realized.

## 1. Introduction

Atomic force microscopy (AFM), which was first described by Binnig et al. in 1986,<sup>1</sup> has found wide application in biological areas.<sup>2</sup> In particular, AFM recognition imaging is gaining increasing attention.<sup>3–5</sup> The recognition ability of AFM combines chemical force microscope and magnetic alternative current (MAC) imaging mode AFM, thereby producing both images and force spectroscopic curves. It offers a rapid and sensitive tool for molecular identification and interaction studies.<sup>6</sup> Due to its high sensitivity and fast imaging process, it could be a high-resolution alternative for detection of deadly biological warfare agents (BWA), such as ricin toxin.

Ricin toxin, which is a byproduct of castor oil production, was discovered in the seeds of the castor bean plant, *Ricinus communis*. It is a heterogeneous proteinaceous toxin, which consists of many different proteins.<sup>7,8</sup> The average lethal dose of ricin in humans is approximately 0.2 mg, which makes it 6000 times more toxic

than cyanide and 12000 times more lethal than rattlesnake venom by weight. There is no antidote for ricin toxin once introduced above the lethal dosage.<sup>9</sup> In addition, ricin toxin can be formulated in a variety of physical forms including powder, mist, and pellet or can be dissolved in water or weak acids. Due to its ready availability, high toxicity, and stability, ricin has the potential to be used in bioterrorism attacks. For this reason, a rapid, sensitive, and quantitative detection method for trace amounts of the toxin is needed.<sup>10,11</sup>

The current detection approach for ricin is based on traditional immunological methods, such as radioimmunoassay,<sup>12</sup> enzyme-linked immunosorbent assay (ELISA),<sup>13</sup> and enhanced colorimetric and chemi-luminescence ELISA.<sup>14</sup> However, lengthy assay time and limited throughput make these methods impractical for rapid responses. Numerous alternative techniques have been developed, and examples include functionalized gold nanoparticle,<sup>15,16</sup> capillary electrophoresis,<sup>17</sup> hybrid combination of quadrupole with time-of-flight,<sup>18</sup> and hydrogel-based protein

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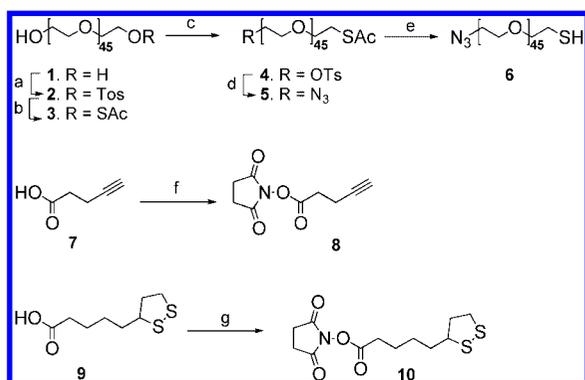
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**Scheme 1. Synthesis of Bifunctionalized PEG Linker 6 and Succinimidyl Active Esters 8 and 10<sup>a</sup>**

<sup>a</sup> Reagents and conditions: (a) TsCl, Py, 0 °C; (b) KSAc, CH<sub>3</sub>OH, reflux; (c) TsCl, Py, 0 °C; (d) NaN<sub>3</sub>, DMF, 60 °C; (e) NaOCH<sub>3</sub>, CH<sub>3</sub>OH, rt; (f) EDC, NHS, DCM, rt; (g) DCC, NHS, dioxane, rt.

microchips.<sup>19</sup> Although the detection time is greatly decreased, these methods still do not meet all the requirements for sensitive, rapid, and inexpensive detection of toxins.

AFM recognition imaging, which combines the unique imaging and spectroscopy capabilities, could serve as a better alternative technique for detection work. Because this AFM technique is based on the molecular interaction of the probe molecule hanging on the tip with the target molecules immobilized on the substrate, tip modification is a critical step<sup>20,21</sup> for successful detections. Several protocols have been applied to chemically functionalize AFM tips and generally entail three steps, namely, modification of a tip surface, grafting of a cross-linker, and conjugation of a biomolecule.<sup>6,22–24</sup> There are three main classes of methods for carrying out the first step: (i) silanization of the tip surface;<sup>6</sup> (ii) hydrosilylation and polymerization on the tip surface;<sup>25</sup> and (iii) formation of SAM on a metal-coated tip surface.<sup>9</sup> The latter approach is the most popular and convenient one.<sup>21</sup> However, these techniques generally are complicated and time-consuming.

We report here a simplified method to modify AFM tip based on the powerful “click chemistry”,<sup>26,27</sup> which facilitated practical and sensitive detection of toxins. A key feature of the approach involves the use of a polyethylene glycol derivative (compound **6**, Scheme 1) modified with a thiol and azide end group. The thiol moiety of the PEG derivative enables attachment to a gold-coated tip without the need for initial tip modification. The azide of the immobilized PEG can then be employed in a Cu<sup>I</sup>-catalyzed 1,3-dipolar cycloaddition with an alkyne moiety of an appropriate biomolecule. This cycloaddition reaction, which has been coined “click chemistry”, is attractive because it can be performed at room temperature to provide stereospecifically stable triazoles in high yield and is compatible with a variety of solvents and functional groups.<sup>28</sup> We have applied the technique for immobilization of an antiricin antibody (antiricin Ab), which made

it possible to qualitatively and quantitatively detect ricin by AFM.<sup>11</sup> In addition, the AFM spectroscopy information and the interaction force between these two biomolecules were also measured and analyzed to ensure the detection specificity.

## 2. Experimental Section

**2.1. Materials.** The materials used were fluorescein-labeled *Ricinus communis* Agglutinin II (RCA II, RCA60, ricin) (Vector Laboratories Inc.), affinity purified goat antiricin communis agglutinin I and II (1 mg/mL, Vector Laboratories AS-2084), regenerated cellulose dialysis membrane (MWCO = 3500) (Membrane Filtration Products, Inc.), 1-(5-[1,2]dithiolan-3-yl-pentanoyl)-pyrrolidine-2,5-dione (LA-NHS, as described), pentynoic acid 2,5-dioxo-pyrrolidin-1-yl ester (Alkyne-NHS, as described), HS-PEG<sub>(2000)</sub>-N<sub>3</sub> (as described), PNS buffer (10 mM phosphate, pH = 7.8, 0.15 M NaCl, 0.08% Sodium Azide), and CS-10 silicon AFM probe (Nanoscience Instruments).

All chemicals used for synthesis were purchased from Aldrich or Fluka. Dichloromethane was distilled from CaH<sub>2</sub> and stored over 4 Å molecular sieves. Pyridine was distilled from P<sub>2</sub>O<sub>5</sub> and stored over 4 Å molecular sieves. Prior use THF was distilled from sodium. Other chemicals were used without further purification.

All reactions were performed in anhydrous conditions under an atmosphere of Ar. Reactions were monitored using thin layer chromatography (TLC) on Kieselgel 60 F254 (Merck). Detection was performed by examination under UV light (254 nm) or by charring with 5% sulfuric acid in methanol. Flash chromatography was performed on silica gel (Merck, 70–230 mesh). Iatrobeds (60 μm) were purchased from Bioscan. <sup>1</sup>H NMR (1D, 2D) and <sup>13</sup>C NMR were recorded on a Varian Merc 300 spectrometer as well as Varian 500 and 600 MHz spectrometers equipped with Sun workstations. Negative ion matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) measurements were recorded on a VOYAGER-DE Applied Biosystems using dihydrobenzoic acid as a matrix. High-resolution mass spectra were obtained using a VOYAGER-DE Applied Biosystems in the positive mode by using 2,5-dihydroxylbenzoic acid in THF as matrix.

**2.2. Synthesis of N<sub>3</sub>(CH<sub>2</sub>CH<sub>2</sub>O)<sub>44</sub>CH<sub>2</sub>CH<sub>2</sub>SAc (**5**).** TsCl (190 mg, 1.0 mmol) was added to a solution of an uncapped PEG diol (2.0 g, average molecular weight 2000 Da, 1.0 mmol) in pyridine (15 mL). The resulting mixture was stirred at 0 °C for 12 h. The precipitate was removed by filtration and the solvent of the filtrate evaporated. The residue and potassium thioacetate (0.26 g, 2.0 mmol) in anhydrous DMF (5 mL) was stirred at 80 °C for 4 h. DCM (25 mL) was added and the resulting solution was washed with water (5 mL). The organic layer was dried (MgSO<sub>4</sub>), and the solvents were removed in vacuo. The residue was dissolved in pyridine (15 mL) and TsCl (190 mg, 1.0 mmol) was added. The resulting mixture was stirred at 0 °C for 12 h. Next, the mixture was filtered and the residue was concentrated under reduced pressure. The residue and sodium azide (130 mg, 2.0 mmol) in anhydrous DMF (5 mL) were stirred at 80 °C for 8 h. CH<sub>2</sub>Cl<sub>2</sub> (50 mL) was added and the resulting solution was washed with water (5 mL). The organic layer was dried (MgSO<sub>4</sub>) and the solvents were removed under reduced pressure. The crude product was purified by silica gel chromatography (DCM: CH<sub>3</sub>OH 10:1) to afford **5** (43% over four steps). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ: 3.87 (m, 2H), 3.83–3.60 (m, 80H), 3.41 (m, 2H), 2.07 (s, 3H).

**2.3. Synthesis of N<sub>3</sub>(CH<sub>2</sub>CH<sub>2</sub>O)<sub>44</sub>CH<sub>2</sub>CH<sub>2</sub>SH (**6**).** NaOCH<sub>3</sub> in CH<sub>3</sub>OH (1 M) was added to a solution of **5** (0.4 mmol) in CH<sub>3</sub>OH to adjust the pH value to 10, under an atmosphere of Ar. The resulting reaction mixture was stirred at room temperature for 5 h. The solvent was evaporated and the residue was purified by silica gel chromatography (CH<sub>2</sub>Cl<sub>2</sub>:CH<sub>3</sub>OH, 8:1) to afford **6** (78%). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ: 3.87 (m, 2H), 3.83–3.60 (m, 80H), 3.31 (m, 2H).

**2.4. Synthesis of Pentynoic Acid 2,5-Sioxo-pyrrolidin-1-yl Ester (Alkyne-NHS) (**8**).** 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (0.42 g, 2.2 mmol) was added to a stirred solution of 4-pentynoic acid (0.2 g, 2 mmol) and NHS (0.25

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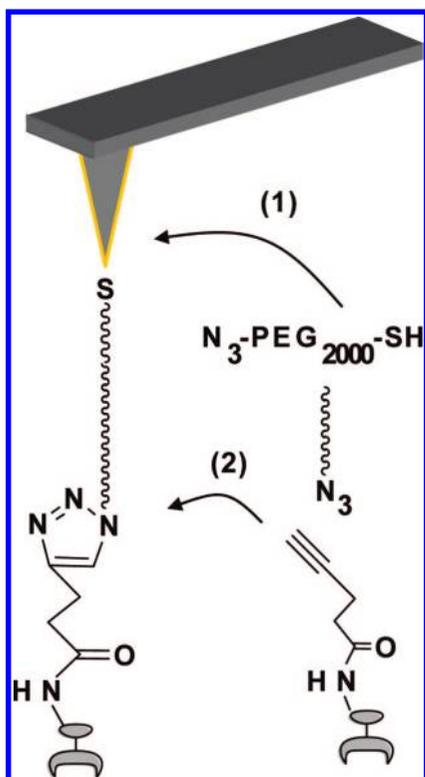
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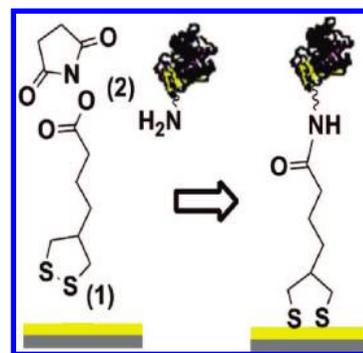


**Figure 1.** Schematic representation of AFM tip modification. (1) The thiol moiety of PEG<sub>2000</sub> formed a SAM on the gold-coated tip; (2) the antiricin Ab with an alkyne group attached to the tip through click chemistry.

g, 2.2 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL).<sup>29</sup> The resulting mixture was stirred at room temperature overnight and was then filtered through a pad of Celite. The filtrate was concentrated in vacuo and the resulting residue was purified by silica gel chromatography (hexane:ethyl acetate = 2:1) to afford **8** (85%). <sup>1</sup>H NMR (500 Hz, CDCl<sub>3</sub>) δ: 2.88–2.83 (m, 6 H), 2.60 (td, *J*<sub>1</sub> = 2.4 Hz, *J*<sub>2</sub> = 7.8 Hz, 2 H), 2.04 (t, *J* = 2.4 Hz, 1 H).

**2.5. Synthesis of 1-(5-[1,2]Dithiolan-3-yl-pentanoyl)-pyrrolidine-2,5-dione (LA-NHS) (10).** *N*-Hydroxysuccinimide (0.310 g, 2.7 mmol) and DCC (0.56 g, 2.7 mmol) was added to a stirred solution of thioctic acid (0.5 g, 2.6 mmol) in dioxane (10 mL) cooled on an ice bath.<sup>30</sup> The resulting mixture was stirred at room temperature for 48 h and then filtered through a pad of Celite. The filtrate was concentrated in vacuo and the obtained residue was redissolved in 2-propanol (5 mL) and left overnight at 4 °C, to give a white precipitate which was filtered off and dried in vacuo to give **10** (57%). <sup>1</sup>H NMR (DMSO) δ: 2.78 (t, 4H), 2.42–2.51 (m, 3H), 2.29 (t, 2H), 1.84 (m, 2H), 1.37–1.62 (m, 4H), 1.25 (m, 2H). <sup>13</sup>C NMR (DMSO) δ: 176.0, 166.4, 53.7, 42.9, 37.3, 36.8, 32.6, 25.9, 24.1, 21.3.

**2.6. Alkyne Modification of Antiricin Ab.** Commercial antiricin Ab in a PNS buffer (200 μL, 1 mg/mL) was dialyzed using a regenerated dialysis membrane (MWCO: 3500 Da, two Quick Seps) in purified water (18.2 MΩ) at 0 °C for 3–5 h. Salt removal was deemed important to avoid possible interference with the conjugation reaction. The resulting antiricin Ab (200 μL) was mixed with active ester **8** (200 μL, approximately 1 mg/mL) in dimethyl sulfoxide/CH<sub>2</sub>Cl<sub>2</sub> (1:1). The mixture was stirred at 0 °C for 1 h. Next, the solution was washed with ethyl acetate (3 × 400 μL) and the aqueous solution was placed in Quick Seps and dialyzed against purified water at 0 °C for 3–5 h.



**Figure 2.** Schematic representation of ricin immobilization on the gold surface. (1) Active ester **10** attached to the gold-coated mica surface by the thioctic acid moiety; (2) ricin binds to active ester **10** by forming an amide bond with amine groups of the protein.

**2.7. Functionalization of AFM Tip.** Tips were coated with 20–30 nm of gold using an E-beam evaporator and then immersed in HS-PEG<sub>(2000)</sub>-N<sub>3</sub> (**6**) in chloroform (5 mg/mL) at 4 °C for 10–12 h to form a compact SAM.<sup>31</sup> The PEG modified tips were washed with dimethyl sulfoxide followed by purified water three times. The tips were placed into alkyne-modified antibody solution (200 μL, 0.1 mg/mL) and then sodium ascorbate (3 μL, 1 M) and copper(II) sulfate (1 μL, 0.3 M) in water were added to the solution.<sup>32</sup> The click reaction was allowed to perform for 10–12 h in the dark at 0 °C. Finally, the tips were washed with PNS buffer three times and stored in PNS buffer at 4 °C. See Figure 1.

**2.8. Ricin Immobilized on the Functionalized Gold Substrate.** A fresh thermal gold-coated mica substrate was annealed by a hydrogen flame for 2 min, and then active ester **10** in dichloromethane (200 μL, 5 mg/mL) was immediately added to the surface. The substrate was kept at 4 °C for 3 h.<sup>33</sup> Following a cleaning by dimethyl sulfoxide and purified water, 200 μL (24 fg/mL) of ricin solution were put on the surface. The mixture was maintained at 4 °C for 30 min. See Figure 2.

**2.9. AFM Experimental Setup.** An Agilent 5500 AFM system equipped with an inverted light microscope (ILM) system (Agilent, Chandler, AZ) was used. An Agilent multipurpose AFM scanner was used for scanning an area of 10 μm<sup>2</sup>. Silicon cantilever tips with a nominal spring constant of about 0.1 N/m were used throughout the experiments. All the images were taken in water using recognition imaging<sup>6</sup> based on Agilent magnetic AC (MAC) mode AFM with a magnetically coated lever. The detailed parameters for the image are as follows: drive is approximately 46%, resonance gain is 2, resonance frequency is 12.95 KHz, resonance amplitude is 1.68 v, and scan rate is 1.22 line/s.

### 3. Results and Discussion

**3.1. Tip and Substrate Modification.** A novel approach for AFM tip modification has been developed employing bifunctional PEG derivative **6**. The thiol moiety of **6** allows convenient attachment to a Au-modified tip because of the high bond energy of the Au–S bond which ranges from 20 to 35 kcal/mol (85–145 kJ/mol) and its ability to form compact monolayers.<sup>33</sup> The azide moiety of the resulting SAM can then be employed in click reactions with biomolecules modified with alkynes.

The bifunctional PEG derivative **6** was prepared by a convenient four-step procedure in an overall yield of 43%. Thus, commercially available PEG diol **1** (2000 Da) was desymmetrized by reaction with 1 equiv of *p*-toluenesulfonyl chloride in pyridine to give a monotosylate **2**, which could easily be separated from starting material and disubstituted product using silica gel column

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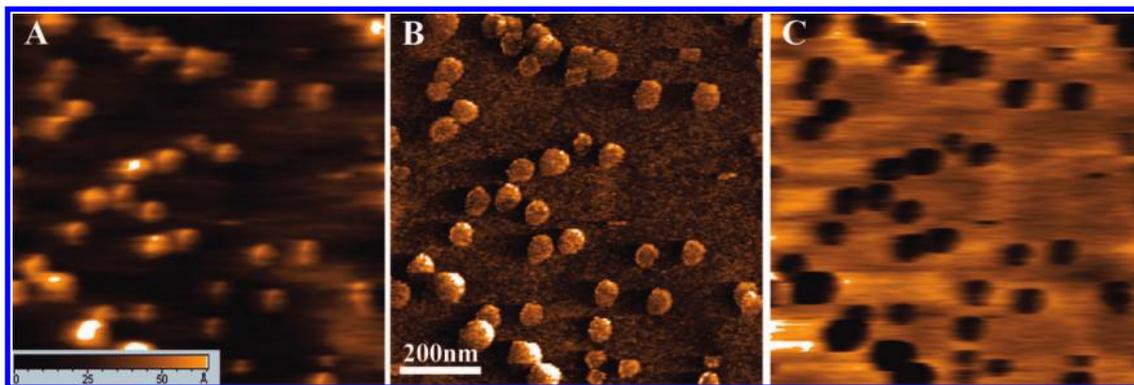
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**Figure 3.** (A) Topographical image for 24 fg/mL; (B) corresponding amplitude image; (C) corresponding recognition image.

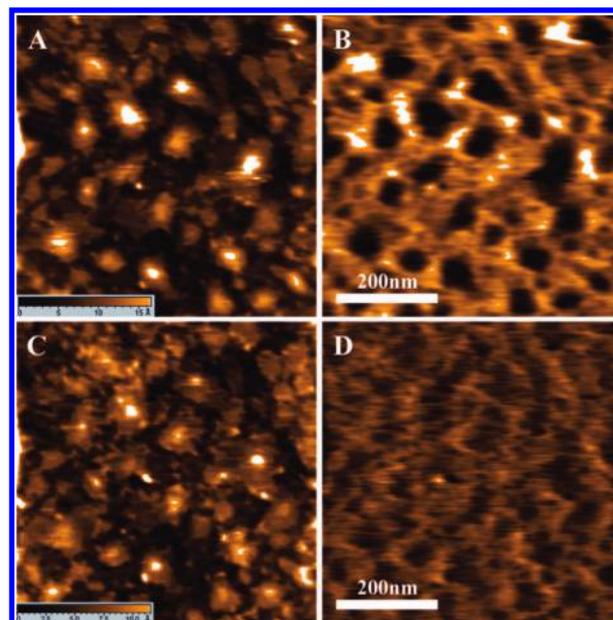
chromatography (Scheme 1). The resulting monotosylate **2** was converted into thioacetate **3** by nucleophilic displacement with the potassium salt of thioacetic acid in DMF. The alcohol of **3** was tosylated using standard conditions to give **4**, which was subjected to a displacement reaction with sodium azide in DMF to yield compound **5**. Prior to SAM formation, the thioacetyl ester of **5** was saponified using sodium methoxide to give target compound **6**.

An antiricin Ab was modified by alkyne groups by aminolysis with NHS ester of 4-pentynoic acid (**8**). The NHS ester could easily be prepared by the esterification of the carboxylic acid with hydroxysuccinimide using EDC (Scheme 1). Alkyne-modified antiricin Ab was then attached to the SAM using a Cu(I)-catalyzed reaction between the azide of the tip and the alkyne of the toxin.

For ricin immobilization to gold-coated mica substrate surface, novel compound **10** was employed which contains a thioctic acid moiety for immobilization to the gold surface and an activated ester for capture of proteins. The thioctic was deemed attractive because it forms more stable complexes with gold with two Au–S bonds compared to monothiol ligands.<sup>33</sup> Compound **10** was easily prepared in good yield by the esterification of the carboxylic acid of **9** with NHS using DCC (Scheme 1).

**3.2. Ricin Detection.** During scanning, the antiricin antibody functionalized tip interacts more strongly with ricin immobilized to gold-modified mica than with other parts of the surface. Therefore, in the recognition image, the ricin molecules are visualized as black dots. The scanned topography image was simultaneously recorded to provide further proof of detection. By using this method, one could not only detect the presence of the ricin but also estimate the quantity of the ricin. Thus, 200  $\mu\text{L}$  of a ricin solution (24 fg/mL) was kept on the modified gold surface for 30 min; then the chip was placed into the AFM system for scanning. Clear topography, amplitude, and recognition images were obtained (Figure 3A–C, respectively). As can be seen in Figure 3, each ricin molecule has a spherical shape (dimensions 20–30 nm). By comparing the topographical and recognition images, we could easily identify which feature belonged to a ricin molecule: each bright spot in the topographic image, if representing a ricin molecule, will result in a dark spot in the recognition image at the same location. Based on the obtained results, it was concluded that the detection method based on AFM could easily be achieved in the femto- or sub-femto-level. Compared with other detection methods, this method is much easier and faster and offers a significant advantage in both sensitivity and resolution.

To confirm the detection results, a control experiment was carried out adopting a flow-cell technique.<sup>6</sup> When the AFM system was stable, 200  $\mu\text{L}$  of ricin protein (24 pg/mL) was injected into

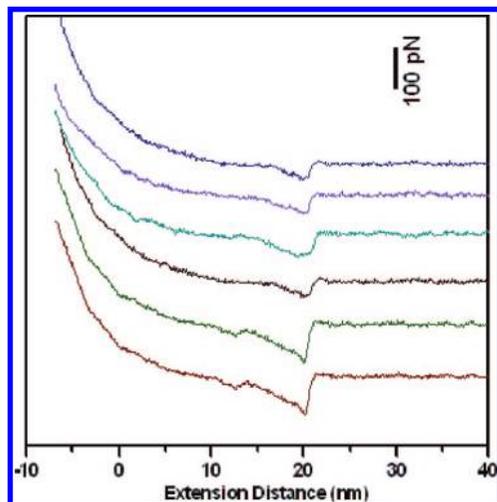


**Figure 4.** Topographic image before (A) and after (C) the blocking of antibodies on the AFM tip and the corresponding recognition image before (B) and after (D) the blocking.

the liquid cell using an inlet syringe. Meanwhile, 200  $\mu\text{L}$  of water in the liquid cell was drawn out by the outlet syringe to prevent the liquid in the cell from overflowing. The real time changes in the recognition image were monitored at the same location. It was observed that features were still clear in the topography image (Figure 4A,C). However, the corresponding black dots fully disappeared in the recognition image (Figure 4B,D). This observation can be explained by the binding of the injected ricin molecules to the antiricin Ab on the tip and blocking of the interaction between the antiricin Ab and the ricin on the surface. These results strongly support the belief that the detection results (Figure 3) were reliable and indicate that AFM recognition imaging is a powerful technique for providing insight into molecular binding events.

**3.3. Binding Force Study.** In addition to images, the AFM recognition imaging technique can also provide force measurements of pico-Newton accuracy.<sup>34</sup> Therefore, during the detection of ricin, the unbinding force was also measured through recording the force–distance curve ( $F$ – $D$  curve). To measure the unbinding force as accurately as possible, the spring constant of the AFM cantilever was calibrated through the resonance frequency changes

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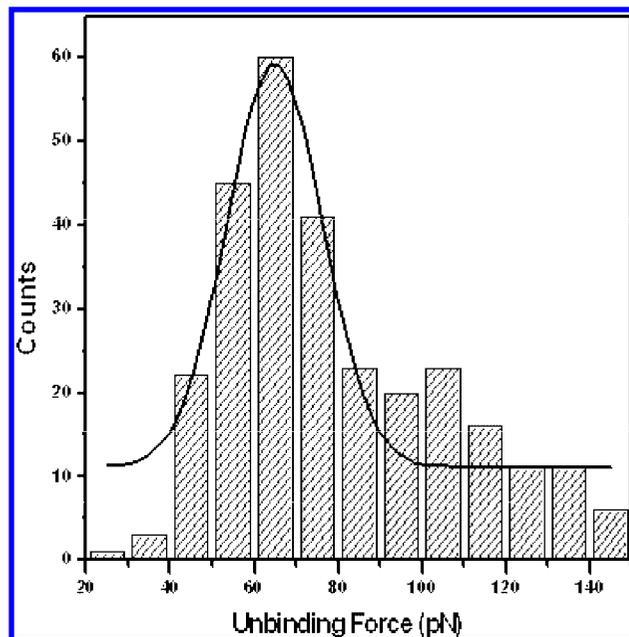


**Figure 5.** Typical unbinding force versus distance curves.

which were induced by small mass.<sup>35</sup> The force measurement was accomplished under the MAC mode to ensure that the tip was directly positioned above the ricin molecules. The typical unbinding force–distance curves are shown in Figure 5. The measured curves agreed with a one-barrier bond dissociation model.<sup>36,37</sup> Figure 5 shows that the typical  $F$ – $D$  curves have some small variations, which are probably due to microscopic complexity. To determine accurately the unbinding force, the force histogram was built from 3000  $F$ – $D$  curves and Gaussian fitting was applied to identify the peak values (Figure 6), which gave an expected unbinding force of  $64.89 \pm 1.67$  pN.<sup>38</sup>

#### 4. Conclusions

A new and efficient protocol for tip functionalization for AFM has been developed using a PEG derivative containing a thiol and azide end group. This bifunctional linker could be employed for SAM formation on a gold-modified tip and the azides of the resulting SAM could be exploited for the immobilization of an antiricin Ab modified by alkynes using a click reaction. The high efficiency, mild reaction condition, and simple operation of the click reaction greatly simplified the tip modification process. Furthermore, ricin could be immobilized on a gold-coated mica substrate by employing another novel bifunctional compound **10** composed of an *N*-hydroxysuccinimidyl ester and a thioctic acid moiety. The thioctic acid moiety of this reagent binds strongly with a gold-coated mica substrate and the active esters of the resulting surface can then be reacted with amines of the protein. With use of the recognition approach, qualitative and quantitative



**Figure 6.** Unbinding force distribution and its Gaussian fitted curve (black solid line).

information could be easily obtained from the images attained by the AFM system. Compared with other detection methods, this approach has significantly higher sensitivity (sub-femto-gram level), faster responses, and easier operation protocol. The detection results were further confirmed by blocking experiment. Therefore, we expected this AFM-based technique to be an attractive method for single-molecule toxin detection as well as for studies of molecular recognition events.

The approach also allowed the measurement of the unbinding force of the Ab–ricin interaction. The obtained  $F$ – $D$  curves were consistent with the well-established one-barrier bond dissociation model.<sup>36,37</sup> The average unbinding force obtained from the histogram was  $64.89 \pm 1.67$  pN, which is in good agreement with values for antibody and antigen unbinding events. Therefore, a detailed understanding of antigen–antibody interactions has also been provided.

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