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

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Received October 22, 2008. Revised Manuscript Received December 6, 2008

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 thiolic 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 ± 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 1986,¹ has found wide application in biological areas.² In particular, AFM recognition imaging is gaining increasing attention.³–⁵ 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.² 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, Ricinis communis. It is a heterogeneous proteinaceous toxin, which consists of many different proteins.⁷,⁸ 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.⁹ In addition, ricin toxic 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.¹⁰,¹¹

The current detection approach for ricin is based on traditional immunological methods, such as radioimmunoassay,¹² enzyme-linked immunosorbent assay (ELISA),¹³ and enhanced colorimetric and chemi-luminescence ELISA.¹⁴ 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,¹⁵,¹⁶ capillary electrophoresis,¹⁷ hybrid combination of quadrupole with time-of-flight,¹⁸ and hydrogel-based protein...
it possible to qualitatively and quantitatively detect ricin by AFM.\textsuperscript{11} 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 \textit{Ricinus communis} Agglutinin II (RCA II, RCA60, ricin) (Vector Laboratories Inc.), affinity purified goat antiricinus communis agglutinin I and II (1 mg/mL, Vector Laboratories AS-2084), regenerated cellulose dialysis membrane (MWCO = 3500) (Membrane Filtration Products, Inc.), 1,3-dipolar cycloaddition with an alkyne moiety of an appropriate 1,3-dipolar cyclization with an alkyne moiety of an appropriate

2.2. Synthesis of Pentynoic Acid 2,5-Sioxo-pyrrolidin-1-yl ester (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 MALDI-TOF mass spectrometer (Finnigan MAT, San Jose, CA) in the positive mode by using 2,5-dihydroxybenzoic acid in THF as matrix.

2.3. Synthesis of N\textsubscript{3}(CH\textsubscript{2}CH\textsubscript{2}O\textsubscript{4})\textsubscript{4}CH\textsubscript{2}CH\textsubscript{2}SCAc (5). TsCl (190 mg, 1.0 mmol) was added to a solution of 4-pentynoic acid (0.2 g, 2 mmol) and NHS (0.25 mmol, 33% over four steps). 1H NMR (CDCl\textsubscript{3}, 300 MHz) δ 3.87 (m, 2H), 3.83 (m, 2H), 3.60 (m, 80H), 3.31 (m, 2H).

2.3. Synthesis of N\textsubscript{3}(CH\textsubscript{2}CH\textsubscript{2}O\textsubscript{4})\textsubscript{4}CH\textsubscript{2}CH\textsubscript{2}SH (6). NaOCH\textsubscript{3} in CH\textsubscript{3}OH (1 M) was added to a solution of 5 (0.4 mmol) in CH\textsubscript{3}OH to adjust the pH value to 10, under an atmosphere of Ar. The resulting mixture was stirred at 80 °C for 4 h. The precipitate was removed by filtration and the solvent of the filtrate evaporated. The residue and potassium thiocacetate (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\textsubscript{4}), 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\textsubscript{3}Cl\textsubscript{2} (50 mL) was added and the resulting solution was washed with water (5 mL). The organic layer was dried (MgSO\textsubscript{4}), and the solvents were removed under reduced pressure. The crude product was purified by silica gel chromatography (DCM: CH\textsubscript{3}OH 10:1) to afford 6 (35% over four steps). 1H NMR (CDCl\textsubscript{3}, 300 MHz) δ 3.67 (m, 2H), 3.53–3.60 (m, 20H), 3.41 (m, 2H), 2.07 (s, 3H).

2.4. Synthesis of Pentaerythritol 1,2-ethyl 3-(dimethylamino)propyl) 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...
thiol moiety of PEG2000 formed a SAM on the gold-coated tip; (2) the acetate residue was purified by silica gel chromatography (hexane:ethyl acetate 8:2) to afford 8 (85%). 1H NMR (500 Hz, CDCl3) δ: 2.88–2.83 (m, 6 H), 2.60 (td, J1 = 2.4 Hz, J2 = 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)-pyrrolodine-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 for 48 h and 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 30:70) to afford 8 (85%). 1H NMR (CDCl3) 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). 13C 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. Alkene 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/CH2Cl2 (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.

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-PEG2000-N3 (6) in chloroform (5 mg/mL) at 4 °C for 10–12 h to form a compact SAM.11 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.32 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.31 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 × 10 µm. Imaging6 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.33 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 monosulfate 2, which could easily be separated from starting material and disubstituted product using silica gel column

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-pentyneoic 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. 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 µL 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. When the AFM system was stable, 200 µL of ricin protein (24 pg/mL) was injected into the liquid cell using an inlet syringe. Meanwhile, 200 µL 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. 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.

which were induced by small mass.\(^{35}\) 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.\(^{36,37}\) 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.\(^{38}\)

### 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 thiotic acid moiety. The thiotic 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 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-femtogram 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.\(^{36,37}\) 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.

**Acknowledgment.** We thank Dr. Anna Jagielska for helpful discussions. Bingqian Xu acknowledges the University of Georgia UGARF faculty research fund and the National Science Foundation (ECCS-0823849) for financial support. Geert-Jan Boons acknowledges support by the Research Resource Center for Biomedical Complex Carbohydrates (P41-RR-5351).

**Note Added after ASAP Publication.** This article was released ASAP on January 7, 2009. The supporting information was removed, and the article was reposted on February 6, 2009. LA803523B

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