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Application of Catalyst-free Click Reactions in Attaching Affinity Molecules to Tips of Atomic Force Microscopy for Detection of Protein Biomarkers

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Abstract

Atomic Force Microscopy (AFM) has been extensively used in studies of biological interactions. Particularly, AFM based force spectroscopy and recognition imaging can sense biomolecules on a single molecule level, having great potential to become a tool for molecular diagnostics in clinics. These techniques, however, require affinity molecules to be attached to AFM tips in order to specifically detect their targets. The attachment chemistry currently used on silicon tips involves multiple steps of reactions and moisture sensitive chemicals, such as (3-aminopropyl)triethoxysilane (APTES) and N-hydroxysuccinimide (NHS) ester, making the process difficult to operate in aqueous solutions. In the present study, we have developed a user-friendly protocol to functionalize the AFM tips with affinity molecules. A key feature of it is that all reactions are carried out in aqueous solutions. In summary, we first synthesized a molecular anchor composed of cyclooctyne and silatrane for introduction of a chemically reactive function to AFM tips and a bi-functional polyethylene glycol linker that harnesses two orthogonal click reactions, copper free alkyne-azide cycloaddition and thiol-vinylsulfone Michael addition, for attaching affinity molecules to AFM tips. The attachment chemistry was then validated by attaching anti-thrombin DNA aptamers and cyclo-RGD peptides to silicon nitride (SiN) tips respectively, and measuring forces of unbinding these affinity molecules from their protein cognates human α -thrombin and human $\alpha_5\beta_1$ -integrin immobilized on mica surfaces. In turn, we used the same attachment chemistry to functionalize silicon tips with the same affinity molecules for AFM based recognition imaging, showing that the disease-relevant biomarkers such as α -thrombin and $\alpha_5\beta_1$ -integrin can be detected with high sensitivity and specificity by the single molecule technique. These studies demonstrate the feasibility of our attachment chemistry for the use in functionalization of AFM tips with affinity molecules.

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Supporting Information

Detailed descriptions of synthesis and characterization of organic compounds, reactions of PEG linkers with DNA aptamer and cyclo-peptide, chemical reactions on silicon substrates can be found in the supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

The human proteome consists of millions of proteins, many of which occur in minute concentrations below limits of detection (LOD) of current technologies such as ELISA, mass spectrometry and protein microarrays.^{1, 2} Therefore, there is a long felt need of a molecular tool capable of directly detecting those disease relevant protein biomarkers present in low abundance without any additional manipulation such as post-assay signal amplification. AFM has been envisioned as a mean of nanodiagnostics due to its single molecule sensitivity.³ It has been demonstrated that in combination with irreversible binding, AFM can reach a concentration sensitivity limit of 10^{-17} M.⁴ While AFM has been exploited in the analysis of DNA, proteins and cells, its chemical sensibility has grown tremendously as well. As illustrated in Figure 1, AFM is capable of “seeing and counting” target molecules when its tip is equipped with an affinity molecule. The interactions between antibody and antigen, ligand and receptor, DNA probe and target etc. can be determined and characterized at a single molecule level by AFM force measurements, termed as Molecular Recognition Force Spectroscopy (MRFS).^{5–11} Also, AFM has been enabled to scan individual proteins immobilized on a surface with an affinity molecule tethered to its tip, known as Recognition Imaging (RI).^{12–16} It is conceivable to employ both MRFS and RI for identification and detection of protein biomarkers in a clinic setting. This requires that these techniques are robust, supported with well-designed chemistry and bioassays. Recent advances in automated AFM-based force spectroscopy should facilitate the instrument operation.¹⁷ One of our efforts has been directed towards developing simple attachment chemistry that works in aqueous solutions without any of organic solvents involved so that it can easily be adapted in biological laboratories and clinics.

A molecular linker is often employed to attach affinity molecules to AFM tips, which provides an advantage in distinguishing between specific and nonspecific interactions.¹⁸ The heterobifunctional poly[ethylene glycol] (PEG) has become a commonly used linker.^{19, 20} In general, the attachment is a three-step process that begins with functionalizing an AFM tip with a chemically reactive group, tethers the PEG linker to the AFM tip, and then reacts with an affinity reagent. (3-Aminopropyl)triethoxysilane (APTES) is a choice reagent for amination of silicon tips,^{21, 22} but it is notoriously problematic for forming uniform monolayers, especially when the reaction is carried out in a liquid phase.^{23, 24} APTES should be freshly redistilled before use in order to achieve reproducible results. Chemical vapor deposition of APTES has been developed to improve the outcome,²¹ but the process is tedious, requiring a thorough purge of the deposition chamber with argon to remove trace of moisture. Without developing an automated apparatus, it is difficult to be scaled up. The reaction of amine with NHS (N-Hydroxysuccinimide) ester has been one of the most commonly used methods for tethering carboxylated PEG linkers to AFM tips.^{25–30} The NHS ester is sensitive to moisture, and prone to rapid hydrolysis with increase in pH (a half-life time of 4–5 hours at pH 7 and one hour at pH 8).^{31, 32} On the other hand, the amine exists in an aminium form at the neutral pH, requiring a basic condition to be deprotonated for its nucleophilic activity. These caveats make it difficult to handle the NHS ester reaction in aqueous solutions and one has to fine tune pH, reaction time in order to achieve optimal outcomes.

Here, we report on a new scheme of attaching affinity molecules to AFM tips based on click chemistry (Figure 2). Chen *et al* have employed a copper-catalyzed alkyne-azide reaction to attach antibodies to a gold coated AFM tip through an azido-PEG-thiol linker.³³ To take it further, we implement two orthogonal catalyst-free click reactions for the attachment of affinity molecules to silicon tips. First, we have synthesized a molecular anchor composed of cyclooctyne and silatrane for the introduction of an alkyne function to the silicon tip. In aqueous solution, the silatrane moiety reacts with silanol on silicon surfaces to form a

monolayer. It has been known that silatrane is less reactive than alkoxysilanes and extremely resistant to polymerization at a neutral pH.³⁴ Gruber and Lyubchenko *et al* have employed 1-(3-aminopropyl)silatrane (APS) as a substitute of APTES in functionalization of AFM tips and mica surfaces.^{35–38} Thus, we expected that the new anchoring molecule would form a uniform cyclooctyne monolayer on the silicon tips. The ring strained cyclooctyne promotes the alkyne-azide reaction without the copper catalyst.³⁹ In addition, we have synthesized a new class of molecular linkers, azido-PEG-vinyl sulfone with defined lengths, for connection of affinity molecules to AFM tips. In the present study, we have focused on attaching thiolated oligonucleotide aptamers and affinity peptides to AFM tips because they are rapidly growing areas in molecular diagnostics.⁴⁰ The reaction of vinyl sulfone with thiol in aqueous solution comprises another category of click chemistry in bioconjugation,⁴¹ being used for the labeling of proteins⁴² and proteomes.⁴³ We adapt it as a first click reaction to connect thiolated affinity molecules to the PEG linker as illustrated in Figure 2. The second click (azide to alkyne) finishes the process of the attachment. These two click reactions are orthogonal so that there are no cross talks between each other.

Results and Discussion

Synthesis

The molecular anchor (**3**) was synthesized simply by reacting APS (**1**)³⁶ with 2-(cyclooct-2-yn-1-yloxy)acetic acid (**2**)³⁹ in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Scheme 1-a). The desired product was separated as a white solid by silica gel chromatography with a yield of 60% (see Supporting Information for details). The molecular linker for RI (**6a**, Scheme 1-b) was synthesized starting from hexaethylene glycol. First, azido-(CH₂CH₂O)₆-Ts (**4**, Ts = tosyl) was synthesized in a multi-gram scale following a method reported in literature.^{44, 45} The azido-(CH₂CH₂O)₁₂-H (**5**) was prepared in a 71% yield by reacting **4** with sodium hexaethylene glycoxide (3 times excess) that was generated *in situ* by treating hexaethylene glycol with sodium hydride. In presence of potassium *t*-butoxide, **5** reacted with divinyl sulfone to furnish the desired product **6a** in a fairly good yield (64%). In the same manner, the linker azido-(CH₂CH₂O)₃₆-vinyl sulfone (**6b**) was synthesized by reacting azido-dPEG[®]₃₆-alcohol with divinyl sulfone in a yield close to that of **6a** (Scheme 1-c). These two products were characterized with FTIR, NMR, and mass spectroscopy (see supporting information). Although it has been reported that vinyl sulfones react with azides in presence of CuSO₄ and sodium ascorbate,⁴⁶ we found by NMR monitoring that **6a** and **6b** were stable both in its pure form and in chloroform at room temperature at least for two days. They have been stored at –78°C already for one year and no degradation has been observed. Maleimide is another widely used reactive group that functions similarly to vinyl sulfone in bioconjugation,⁴⁷ but it may not be amenable to coexisting with azide because a [3 + 2] cycloaddition could spontaneously take place between these two functions in some circumstances.⁴⁸ In addition, maleimide can undergo the thiol exchanges and ring hydrolysis (above pH 8),^{49, 50} which would complicate outcomes of the conjugating reaction. It is also a reason why we chose vinyl sulfone as a Michael addition receptor of thiols in our attachment chemistry.⁴²

Click 1: tethering molecular linkers to affinity molecules

Two affinity molecules, thrombin-binding DNA aptamer (TBA)⁵¹ and cyclic RGDfC peptide containing a RGD motif that binds to integrin receptors such as $\alpha_5\beta_1$,⁵² were chosen to study the attachment chemistry. First, the disulfide at the 3'-end of the DNA aptamer from custom synthesis was reduced to thiol using tris(2-carboxyethyl)phosphine (TCEP), which then reacted with linker **6a** and **6b** at pH 8.0 in phosphate buffered aqueous solutions, respectively. Through the Michael addition of thiol to vinyl sulfone (Scheme 2-a), the DNA

aptamer was converted to azido-PEGylated products **D-1a** with a 95% yield and **D-1b** with a 89% yield, based on HPLC analysis (see Supporting Information). The disulfide DNA was used as a negative control and it did not react with **6a** and **6b**, implying that the vinyl sulfone is specific to thiol under the current conditions. Also, we observed that the reaction between the vinyl sulfone and the thiolated DNA at pH 7–7.5 was very slow and did not complete even after one day. The thiol reaction is driven by the thiolate that is a much stronger nucleophile than its conjugate acid thiol. Since the alkylthiol is fairly acidic with pK_a of about 10 to 11, the increase of pH surely increases existence of the thiolate anion, resulting in an increased reaction rate. This is consistent with what has been reported in literature.⁵³ Under the similar conditions, the thiolated RGDfC was converted to products **P-1a** and **P-1b** quantitatively (Scheme 2-b). We did not observe any side products by MALDI mass spectrometer and reverse phase HPLC analysis. In addition, no apparent time differences between reacting with **6a** and **6b** were observed (see Supporting Information for details). In sum, all these reactions were completed within three hours when starting with DNA or peptides in a range of millimolar concentrations.

It should be noted that the vinyl sulfone also reacts with alkyl amines under basic conditions.⁵⁴ In our case, the amine functionalized aptamer and the cyclic RGDfK reacted with both **6a** and **6b** in phosphate buffered solutions at pH 8.8, but the reactions were very slow and not completed even after ten hours. This shows that the vinyl sulfone can specifically react with thiol in the presence of amino function with well-tuned pH.

Click 2: Attaching affinity molecules to AFM tips

At present, there is no effective way to directly monitor chemical reactions and characterize their products on AFM tips. To have insights into our new attachment chemistry, we first carried out a pilot study on planar thermally oxidized silicon substrates, presumably the surface of which has a chemical reactivity similar to that of silicon AFM tips. We found that compound **3** formed a monolayer with its physical properties close to those of the monolayer generated by reacting cyclooctynyloxy-acetic acid **2** with the APTES functionalized silicon substrate. As illustrated in Scheme 3, when APTES was deposited on a silicon oxide surface by chemical vapor deposition (route i-a), it changed the contact angle of water on the surface from 0° to ~46° (Table 1), a value that is consistent to data reported in literature.⁵⁵ The measured thickness of the organic layer was about 7.3 Å, slightly larger than the calculated distance from nitrogen to oxygen of APTES (see S-2 in Scheme 3), indicating formation of a monolayer (See Supporting Information for details about contact angle and thickness measurement). Treating the APTES monolayer with compound **2** in the presence of EDC increased the contact angle to ~76° and thickness to ~15.9 Å, close to the expected value (see S-3 in Scheme 3). When the same silicon substrates were treated directly with an aqueous solution of compound **3** (route ii in Scheme 3), the measured contact angle and thickness were ~78° and 15.3 Å, respectively, well matching with those data just mentioned above. This indicates that compound **3** may form a monolayer with a structure as suggested in S-3. In turn, we treated the cyclooctyne surface with a solution of fluorescent TBA containing an azide at its 3'-end and it became highly fluorescent after one hour incubation, whereas the same surface treated with the fluorescent aptamer containing disulfide at the 3'-end (negative control) had negligible fluorescence (see Figure S1 in Supporting Information). Note that we had confirmed that the azide functionalized TBA and cyclo-peptides reacted with the cyclooctyne effectively in the liquid phase (monitored by MALDI mass) before applying them to silicon substrates or tips.

Based on the study above-mentioned, we have developed a two-step protocol for the attachment. As illustrated in Figure 3, a bare AFM tip is first functionalized with the molecular anchor **3** in aqueous solution, followed by reacting with the azide functionalized

affinity molecules under physiological conditions (see Experimental Section). It is worth noting that all the reactions were carried out in aqueous solutions without using any of organic solvents. The reaction between cyclooctyne and azide may yield two regioisomeric triazoles,⁵⁶ but it will be challenging for us to confirm their existence on the AFM tip with currently available analytical tools. However, we have not observed any apparent regioisomeric effects on the following AFM measurements. The attachment chemistry has worked well on two different AFM tip materials: SiN tipped probes (from Olympus and Bruker) and silicon probes (from NanoWorld). Before the chemical functionalization, these tips were cleaned sequentially with UV-ozone and oxygen plasma to increase the silanol density on the silicon surface for the silanization reaction.

Force Measurement

The attachment chemistry was validated by measuring forces of affinity molecules tethered to SiN tips unbinding from their protein cognates. The protein samples were immobilized on APS-modified mica substrates using glutaraldehyde as a crosslinker according to a procedure reported in literature.⁵⁷ Initially, we collected about 1000 force-distance curves from each of measurement experiments with either **D-1b** against thrombin or **P-1b** against integrin $\alpha_5\beta_1$. The blue solid lines in Panel A and D of Figure 4 show typical retracting force-distance curves we used for data analysis, which accounts for more than one forth of the collections. The selection was based on an assumption that a rupture directly related to unbinding of an affinity molecule from its protein cognate is likely to take place around the distance corresponding to the stretched length of a PEG linker (~ 13.5 nm in our case). A distance histogram was created from each data set (Panel B and E of Figure 4). They show that the unbinding events were mainly distributed in the regions of 2–7 nm and 13–16 nm. Ratios of the rupture events between these two regions were 1 : 1.2 for the TBA tip against the thrombin and 1 : 1.3 for the RGD tip against the integrin respectively. After finishing each of the initial measurements, a thrombin or integrin solution was injected to the flow cell accordingly, and then another set of force curves were collected to determine the specificity of unbinding.⁵⁸ We expected disappearance of the specific unbinding ruptures from the force-distance curves because the interactions of the affinity molecule tethered to the tip with its cognates on the substrate were blocked by protein from the solution. In fact, we obtained force-distance curves appearing like those red dotted lines in Panel A and D of Figure 4. Overall, the ruptures around the longer distances were reduced to a great extent and those around the shorter distances remained (Panel C and F of Figure 4) in comparison with those prior to blocking. The rupture ratios between these two regions were changed to 12.3 : 1 for the TBA tip against the thrombin modified surface and 12 : 1 for the RGD tip against the integrin modified surface. To best interpret these results shown in Panel B and E of Figure 4, we assigned the rupture events occurring at the distance around ~ 13.5 nm as specific unbinding of the affinity molecule from its protein cognate and those events in the range of 2 to 7 nm are a consequence of multi-molecular interactions and unraveling that do not involve stretching of the PEG linker between the surface and the tip.

In total, there were 26.5 % of force-distance curves containing the specific ruptures of TBA unbinding from thrombin in the initial collection. They were plotted as a force histogram and fitted into a Gaussian function, yielding a curve with the peak at ~ 80 piconewton (pN) (Panel A of Figure 5). Similarly, 29.4 % of the initial force-distance curves showed the specific unbinding ruptures for the RGD-integrin interactions, which results in a Gaussian curve with the peak at ~ 48 pN (Panel B of Figure 5). These unbinding force data were comparable with those reported in literature.^{59, 60}

We further examined the non-specific interactions between functionalized AFM tips with both bare and bovine serum albumin (BSA) immobilized mica substrates. The results are

given in Table 2. In summary, the functionalized AFM tips generally formed featureless force-distance curves on these surfaces. Only 6.1% of collected curves show unbinding ruptures from the nonspecific TBA-BSA interaction (median force 14.4 pN) and 5.6% from the nonspecific RGD-BSA interaction (median force 15.3 pN) around the expected distance, respectively. The functionalized tips interacted with the bare mica surfaces with even lower statistics and smaller unbinding forces. All of non-specific unbinding forces we measured were significantly smaller than those specific ones. These data demonstrate that our attachment chemistry has effectively tethered affinity molecules to AFM tips as well as maintained their specificity.

Recognition Imaging (RI)

The AFM based recognition imaging has great potential to be an effective tool for clinical diagnostics. It is important for us to confirm that our chemistry works with the RI technique. Unexpectedly, the recognition imaging of clinically relevant proteins thrombin and integrin has not been reported. It has been demonstrated that a PEG linker with 12 ethyleneoxy ($\text{CH}_2\text{CH}_2\text{O}$) units can effectively produce quality recognition images.¹³ Linker **6a** was tailored for RI. Its conjugate **D-1a** or **P-1a** was attached to Ni-coated MacMode tips (from Nanoworld) following the same protocol above mentioned. The protein samples (thrombin or $\alpha_5\beta_1$ integrin) were deposited on mica using the same glutaraldehyde chemistry. However, the optimal protein concentration (50 pg/ μL in 1X PBS buffer, pH 7.4) for the RI was 20 times lower than that for the force measurements, which was pre-determined by imaging the surface with bare AFM tips in the air mode, ensuring that the protein molecules were well distributed in a predefined area. For one measurement, only 2–3 μL of protein sample is needed in the current setup. Thus, a few femtomoles of proteins can readily be detected by the AFM based recognition imaging. Figure 6 shows the images obtained from our RI experiments. In general, RI simultaneously produces both topographic and recognition images. Each bright round spot in the topographic image presumably represents a protein molecule (thrombin in Panel A and integrin in panel D of Figure 6). This can be verified by examining the recognition images (Panel B and E of Figure 6) where the dark spots represent recognition of those bright ones within the corresponding locations in the topographic image as expected protein molecules. We obtained about 77% recognition of thrombin and 84% recognition of integrin by comparison between their topography and recognition images. The recognition was further confirmed by the same blocking experiments as in the force measurements. After injecting a protein (thrombin or integrin) solution to the flow cell, most of the dark spots disappeared from the recognition images (Panel C and F of Figure 6). These experiments demonstrate that our attachment chemistry works effectively for RI as well.

Conclusions

We have developed a new scheme to attach affinity molecules to AFM tips for force spectroscopy and recognition imaging, based on two orthogonal click chemistries: catalyst free azide-alkyne cycloaddition and thiol-vinyl sulfone Michael addition. All the reactions can be carried out in aqueous solutions without the use of organic solvents. We synthesized two new reagents for this implementation. The first one is an APS derivative of cyclooctyne for introduction of a chemically reactive group to AFM tips. The silatrane chemistry allows for the formation of a uniform monolayer in aqueous solution, which is particularly useful when the chemical is not volatile and the vapor deposition would not work. The operation is more convenient compared to the vapor deposition technique and the resulting surface is highly reproducible. The second one is a class of heterobifunctional linkers with a form of “azido-PEG-vinyl sulfone”. Our data show that it works for both AFM based force measurement and recognition imaging. The attachment process is easy to follow since there

are no special requirements for the chemical reactions. With an increasing number of affinity oligonucleotides and peptides, more and more proteins will be detected with these synthetic materials. Incorporating thiol to peptides and oligonucleotides has become a routine process in custom synthesis. Hence, our attachment method should be applicable to a broad range of affinity molecules.

Experimental Section

General procedure for attaching affinity molecules to AFM tips

AFM tips (a batch of four or five) were first immersed in ethanol for five minutes, dried with ultrapure argon, and then treated with ultraviolet-ozone in a Boekel UV cleaner for 15 minutes and oxygen plasma (medium power) in a Harrick Plasma Cleaner for 2 minutes. These tips were immediately placed in a petri dish, to which an aqueous solution of compound **3** (50 mM) was added. After one hour, the tips were taken out, rinsed with water thrice, and dried gently with nitrogen.

In a humid surrounding, the cyclooctyne functionalized tips were placed in a petri dish and a solution of **D-1a** (50 μ M, 20 μ l) in 1X PBS buffer (pH 7.4) was added to cover all the tips, incubated at room temperature for one hour, and then the tips were rinsed thrice with the same buffer and used immediately. Other conjugates including **D-1b**, **P-1a**, and **P-1b** were also attached to the AFM tips under the exactly same conditions.

Immobilization of proteins on mica substrates

Freshly cleaved mica sheets ($1.5 \times 2.0 \text{ cm}^2$) were immersed in an aqueous solution of APS (50 mM). After one hour, the mica sheets were taken out and rinsed thoroughly with water thrice. In a humid surrounding, an aqueous solution of glutaraldehyde (1 mM, 200 μ L) was added on the APS mica sheet. After 10 minutes, the mica substrates were rinsed with water thrice, and then a solution of protein in a 1X PBS buffer (3 μ L) was placed on it, incubated for one hour, and rinsed with the 1X PBS buffer thrice. In general, protein concentrations were made around 10 ng/ μ L for force measurements and 0.05 ng/ μ L for recognition imaging. It should be noted that integrin we used was a lyophilized product from a solution containing: 0.26 mg/ml $\alpha_5\beta_1$, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM MgCl_2 , 0.2% Triton X-100, which was reconstituted by dissolving it in 1X PBS buffer.

AFM experimental setup

An Agilent AFM 5500 (with inverted light microscope) system was used for the AFM experiments. Both force measurement and recognition imaging were carried out in 1X PBS buffer (pH 7.4). For force measurements, Veeco probes (Bruker, SiN tips) were used, having a force constant 0.05 N/m and a gold back coating, and the loading rate was 25000 pN/s.

For Recognition Imaging, magnetic cantilevers were used in AC (MAC) mode operation. Tips from NanoWorld were made of silicon and had a length of 125 μ m, width 35 μ m and thickness 800 nm with force constant value of 0.14 N/m. Backsides of these tips were coated with 1 nm Ti/40 nm Ni. They have a remarkably low spread in force constant (a few percentage) and give stable MacMode operation in even quite reactive solution. Also, Olympus tips (silicon nitride, a force constant 0.08 N/m) were functionalized and used for few recognition experiments. Each of images was taken by scanning a $1 \times 1 \mu\text{m}^2$ area.

For a blocking experiment, a protein solution (50 μ L, 0.01 ng/ μ L in 1X PBS buffer, pH 7.4) was added to the flow cell, and the surface was imaged again. In general, a 15–20 minute waiting time is needed to effectively block the tip. The blocking for force measurements proceeded in the same way.

Data analysis

All the topography images, recognition images and force spectra were recorded using Agilent PicoView software. The force-distance curves were analyzed in PicoView, and the corresponding unbinding forces were plotted in the form of histograms and fitted into the Gaussian function using MathWorks-MATLAB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Illustration of an AFM tip with an affinity molecule tethered at its apex to specifically recognize its protein cognates immobilized on a substrate (A). Using contact mode, a force of the affinity molecule unbinding from its cognate can be determined by retracting the tip along the Z direction (B). By tapping the functionalized tip on the surface along the X to Y direction, topographic and recognition images can be generated (C, D). In general, the affinity molecule can be a ligand, an antibody, a nucleic acid aptamer, and so on

Figure 2.
Illustration of a new chemical approach for attaching affinity molecules to an AFM tip

Figure 3.

Process of functionalizing AFM tips with affinity molecules: (a) coupling cyclooctyne to an AFM tip through silanization; (b) attaching affinity molecules to an AFM tip through alkyne-azide click reaction (only one regioisomeric product is drawn).

Figure 4.

(A) Blue solid line: a force-distance curve of a TBA functionalized tip retracting from a thrombin immobilized mica surface; red dotted line: a force-distance curve from repeating the measurement after blocking the TBA tip with thrombin; (B) A distance histogram of the ruptures taking place with the TBA functionalized tip retracting from the surface; (C) A distance histogram of the ruptures taking place after blocking the TBA tip with thrombin; (D) Blue solid line: a force-distance curve of a RGDfC functionalized tip retracting from a $\alpha_5\beta_1$ integrin immobilized mica surface; red dotted line: a force-distance curve from repeating the measurement after blocking the RGDfC tip with integrin; (E) A distance histogram of the ruptures taking place with the RGD functionalized tip retracting from the surface; (F) A distance histogram of the ruptures taking place after blocking the RGD tip with integrin.

Figure 5.

(A) A force histogram of TBA unbinding from thrombin immobilized on the mica surface;
(B) A force histogram of RGD unbinding from integrin immobilized on the mica surface.

Figure 6.

(A) Topographic image of thrombin proteins on mica; (B) Corresponding recognition image of A; (C) a recognition image from using a thrombin solution to block the TBA tip; (D) Topographic of integrin proteins on mica; (E) Corresponding recognition imaging of D; (F) a recognition image from using a integrin solution to block the RGD tip; (the circles in the images indicate those protein molecules that were recognized whereas the square indicates the protein that was not recognized)

Scheme 1.
Schematic diagram of the chemical syntheses

Scheme 2.

Schematic description of tethering the molecular linkers to affinity molecules through the Michael addition of thiol to vinyl sulfone

Scheme 3.

Functionalization of the silicon oxide surface: (i-a) chemical vapor deposition of APTES; (i-b) coupling of compound **2** to the APTES surface in DCM; (ii) reacting with compound **3** in an aqueous solution. The numbers in red are estimated molecular lengths from ChemDraw 3D modeling.

Table 1

Physical properties of surfaces derivatized with chemical functions

	Contact Angle (°)	Thickness (Å)
S-2 (Route i-a)	45.8±0.9	7.3±0.3
S-3 (Route i-b)	75.8±0.8	15.9±0.5
S-3 (Route ii)	77.9±1.2	15.3±0.3

Table 2

Statistical data of functionalized AFM tips interacting with varied surfaces based on force-distance curves

On the tip	On the substrate	Unbinding events (%) [*]	Unbinding force (pN)
TBA	thrombin	26.5	80.2±34.5
TBA (blocked)	thrombin	7.0	15.6±12.9
TBA	BSA	6.1	14.4±9.4
TBA	bare mica	3.0	6.9±3.8
RGD	integrin	29.4	48.0±27.8
RGD (blocked)	integrin	6.7	11.1±10.7
RGD	BSA	5.6	15.3±14.4
RGD	bare mica	2.5	6.7±4.0

^{*} The percentage of ruptures taking place around the specific unbinding distance over total collected force curves.