Attaching Single Biomolecules Selectively to the Apex of AFM Tips for Measuring Specific Interactions

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ABSTRACT We present a general approach for preparing well-defined AFM tips for probing single target molecules. We demonstrated that carboxylic acid groups could be generated by electrochemical oxidation selectively at the apex of an AFM tip that is coated with a monolayer of oligo(ethylene glycol) derivatives for resisting nonspecific interactions. These carboxylic acid groups were used as handles to tether only one ligand molecule, such as biotin, to the tip apex for measurement of specific interactions with biomolecules.

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Atomic force microscopy (AFM) has become a powerful tool for studying biological samples at nanoscale (1). Tethering probe molecules to the AFM tip allows for measurement and mapping of specific interactions between the probe molecules and the target molecules on the sample (2). To obtain reliable results and to improve the contrast and spatial resolution, it is highly desirable to prepare nanometer-sized probe tips that resist nonspecific interactions and are tethered with a defined number of the probe molecules at the tip apex. To this end, we recently developed a new method for modifying silicon AFM tips with an alkyl monolayer terminated with oligo(ethylene glycol) (OEG) groups (3). We demonstrated that the modification greatly reduced the nonspecific interactions with proteins while maintaining a small tip size for high-resolution imaging. In this communication, we show that carboxylic acid groups can be selectively generated at the apex of these OEG-modified tips, which can serve as handles for tethering other functional moieties to the tip apex for measuring specific interactions.

Our method stems from a nanolithography technique referred to as conductive AFM (c-AFM) in which the substrate surface under a biased AFM tip in air can be electrochemically oxidized (4). We used this method to fabricate nanometric templates on OEG-terminated monolayers on silicon surfaces, followed by attachment of proteins to the templates to form protein nanoarrays (5). Our recent study showed that c-AFM can generate nanoarrays presenting COOH groups with a feature size down to 10 nm on OEG-terminated monolayers. Herein, we show that this electrochemical oxidation can also take place on the OEG-modified AFM tips, generating a few COOH groups at the tip apex (Fig. 1).

OEG-terminated monolayers were grown on silicon AFM tips using surface hydrosilylation (3). Briefly, soft silicon cantilevers (NSC12, MikroMasch, Moscow) with a force constant of ~0.05 N/m were mounted on a Teflon holder. After cleaning with Piranha solution, the oxide layer on the silicon surface was removed with a buffered HF solution to generate a hydrogen-terminated silicon surface. Reacting this surface with methoxy tri(ethylene glycol) undecene led to tip A (Fig. 1) with an OEG-terminated monolayer attached to the tip surface via Si–C bonds (3). The cantilever was then mounted to an AFM (Nanoscope IIIa, Digital Instruments, Santa Barbara, CA) equipped with a voltage-pulse generator connecting the tip and a flat Au(111) electrode. A droplet of mesitylene was placed on the gold surface, and the tip was then immersed in the droplet and engaged to the surface in contact mode. Mesitylene was used to reduce the water meniscus between the tip and gold surface. A voltage pulse of 0.5 V with duration of 5 s was applied to the gold electrode while the tip was grounded (Fig. 1).

The presence of COOH groups at the tip apex was probed by force titration curves (6) that plot the adhesion forces between the tip and a hydroxy-terminated self-assembled monolayer (SAM) in 0.01 M PBS buffer as a function of the solution pH. Curve *a* in Fig. 1 is characteristic for a tip A, showing that the adhesion forces were unaffected by the pH. However, upon the above electrooxidation process, the resulting tip B exhibited a drop of adhesion force at pH ~ 4.0 as shown by curve *b* in Fig. 1. These results strongly indicate the presence of COOH groups on the tip apex B. These carboxy groups are mostly in the protonated form (COOH) at pH $<$ 5.0, which interacts with the SAM surface more strongly via hydrogen bonding than the deprotonated form (COO^-) at high pH (6). The difference of the adhesion forces (ΔF_a) at pH $<$ 3 and pH $>$ 7 in the force-titration curves provides a rough estimation of the number of COOH groups on the tip apex. Lieber and co-workers suggested that ~100 pN of ΔF_a corresponded to one COOH group at the end of a single-walled carbon nanotube tip (6). The OEG-coated tips modified by the present method had ΔF_a in the range of 200–400 pN, substantially lower than the reported values

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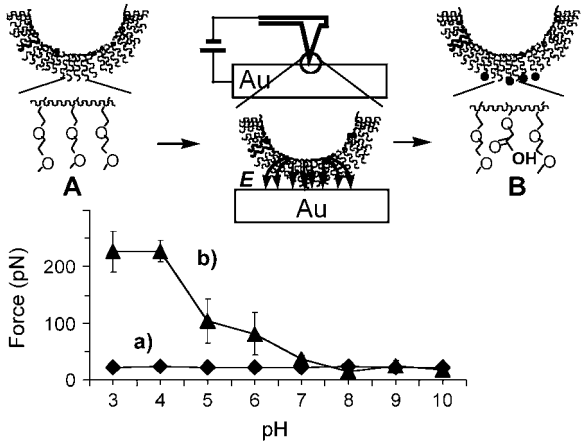
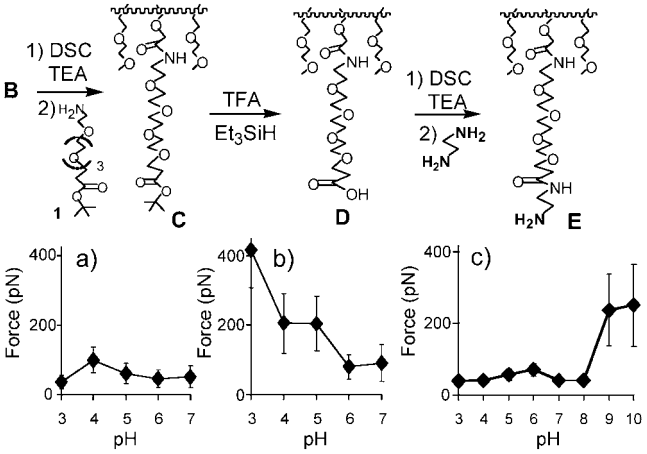


FIGURE 1 (*Top*) Illustration of the electrooxidation selectively at the apex of OEG-coated tip. (*Bottom*) Plots of adhesion force versus pH obtained with an OEG-coated tip interacting in 0.01 M PBS buffer with an OH-terminated SAM prepared from 11-mercaptoundecanol on gold substrate before (*a*, tip *A*) and after (*b*, tip *B*) subjected to electrochemical oxidation. Each data point corresponds to the mean of 300–400 consecutive measurements at more than five locations and the error bars represent mean ± 1 SD.

(~500 pN) for the oxidized carbon nanotube tips (6), indicating that only a few COOH groups were present. These groups were most likely located only at the tip apex due to the confinement of the electric field (Fig. 1). Upon derivatization, ligands can be introduced only to the apex of the AFM tip. In comparison, other methods for tip modification generally led to a random distribution of ligands on the whole cantilever tip (2). The mechanical properties of such cantilevers could be significantly affected by the binding of proteins to the large number of ligands on the cantilever surfaces. Furthermore, the above local oxidation process did not change the tip size (~11 nm) as shown by blind reconstruction of the tip shape.

The oxidized tips can be easily derivatized via formation of amide bonds. We attached amino-dPEG$_4$ *t*-butyl ester (1) to the oxidized tips B to afford the tips C (Scheme 1). The force titration curve *a* in Scheme 1 obtained with a tip C interacting with the above OH-terminated SAM shows that the adhesion forces were nearly independent to pH, consistent with the absence of COOH groups on the tip apex. The *t*-Bu protecting group in the tip C was then removed to generate the tip D. The presence of COOH group in the tip D is clearly indicated by the force titration curve *b* in Scheme 1. The COOH groups could be converted to amino groups as in the tip E that can serve as handles for attaching carboxylic acid derivatives to the tip. The force titration curve *c* in Scheme 1 obtained with the tip E showed that the adhesion forces sharply increased from ~30 pN at pH 8 to ~250 pN at pH 9, consistent with the presence of amino groups at the tip apex (6). In addition, the adhesion forces remained about the same within pH 3–7, indicating that the COOH groups had been consumed.



SCHEME 1 Derivatization of the oxidized tips B and characterization with force titration curves.

The OEG-coated tips with COOH groups at the tip apex (tip B) can be derivatized to attach biomolecules for measurement of specific interactions with target molecules, as demonstrated here with the biotinated tips. As illustrated in Fig. 2, the mono-biotinated tips F were prepared by activating the carboxylic acids in the tips B with differential scanning calorimetry followed by treatment with the amine 2. The unbound forces between the tips F and an avidin film (7) were measured in 0.01 M PBS buffer at pH 7.4. The histogram of a tip F is presented in Fig. 2 *a*. The mean adhesion force of this tip with avidin was 51 ± 18 pN. Previously reported forces for unbinding one biotin-avidin pair were ~45–50 pN (7), measured under conditions similar to ours (force constant of the cantilever ~0.05 N/m, loading rate ~1 nN/s, retraction velocity ~200 nm/s, loading force ~100 pN). Upon addition of a biotin solution (20 μg/ml) into the system to block the binding sites of avidin, the adhesion forces significantly decreased (Fig. 2 *b*), strongly indicating that the measured forces were originated from the specific biotin-avidin interactions. On average, each

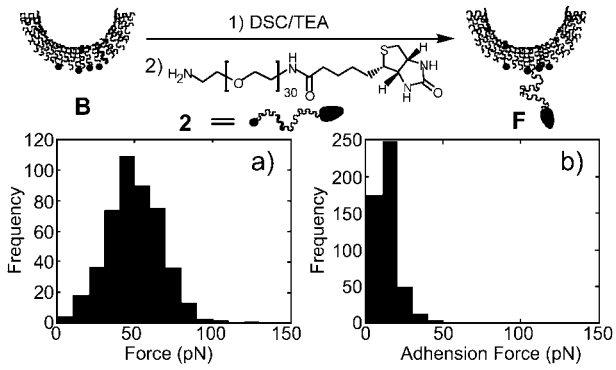


FIGURE 2 Histograms of adhesion forces measured from consecutive force curves obtained with a tip F interacting with an avidin film on five different locations in 0.01 M PBS buffer at pH 7.4 before (*a*) and after (*b*) adding a biotin solution.

immobilized avidin molecule should have two free binding sites for biotin. Therefore, if multiple biotin groups are present on the tip apex with a diameter of ~11 nm, they may form multiple bindings with one or several avidin molecules. With such tips, some of the force-extension curves should show the sequential or simultaneous breakage of multiple bonds during the retraction of the AFM tip. The absence of such events in the force curves leading to Fig. 2 *a* indicates that this particular tip had only one biotin group at the tip apex.

In conclusion, we have developed a general method for attaching one biomolecule selectively to the apex of an OEG-coated AFM tip. Such well-defined single-molecule tips are ideal for measuring and probing specific biomoleculear interactions.

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