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Functionalization of Cantilever Tips with Nucleotides by the Phosphoramidite Method

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In atomic force microscopy (AFM) a sharp cantilever tip is used to scan surfaces at the atomic level. One further application is force spectroscopy, in which force–distance curves between binding partners located on the cantilever and substrate surface are determined. This requires specifically immobilized molecules. Herein we describe the covalent binding of single adenosine and thymidine nucleotides on an amino-PEGylated cantilever tip by the phosphoramidite method. Force–distance curves between these cantilever tips and gold surfaces were recorded. The rupture forces of the coordination bond between the primary amine of adenosine and the undercoordinated gold atoms were determined to be 145 pN, which is in agreement with previously published data. The force–distance curves of thymidine-functionalized tips did not show rupture events, because this nucleotide does not possess a primary amine function. Nucleotide-functionalized tips could aid in the understanding of binding mechanisms of nucleotide binding molecules such as polymerases immobilized on surfaces or membranes.

Atomic force microscopy is a type of high-resolution scanning probe microscopy, in which a cantilever with a sharp tip is used to scan the probe surface.[1] This method is also applicable to living cells,[2] and even membrane proteins can be monitored with sub-nanometer resolution.[3] A major application of AFM methodology is force spectroscopy, in which force–distance curves from single biomolecules or binding partners between the cantilever tip and surface are measured. Binding forces between single ligand receptor pairs,[4] complementary DNA strands,[5] and the adhesion of DNA strands to charged surfaces have been measured. Furthermore, the unfolding patterns of single proteins, the force activation of single enzymes,[6] and even the strengths of single covalent bonds can be determined.[7] All these methods require specifically immobilized target structures.

It is well known that thiol-functionalized polymers interact covalently with gold surfaces via a sulfur–gold bond. The force required to rupture this bond was recently determined to be 1.4 nN[7] at an applied force-loading rate of 10 nN s^{-1}. In a related pathway, amines can form covalent bonds with gold atoms via the free electron pair of the nitrogen atom.[8] To determine the binding forces of amine functions of short DNA molecules or single nucleotides to gold surfaces via the amine–gold bond, it is necessary to provide specifically functionalized cantilever tips—that is, the attachment mechanism and location are known.

Herein we describe the functionalization of such cantilever tips with nucleotides by phosphoramidite methodology (Scheme 1).[9] First, silicon nitride cantilever tips were functionalized with epoxy moieties by direct incubation with 3-glycidoxypropyl trimethoxysilane (**1**). In the following step an α-hydroxy-ω-aminopolyethylene glycol (HO-PEG-NH_2, 10000 Da) was introduced to the surface by reaction of the amino functionality with the epoxy group of the silane at pH 8.5 in a buffered aqueous solution (**2**). After removal of unreacted PEG by washing the tip with ddH_2O, the cantilever tips were incubated with either adenosine- or thymidine-phosphoramidite solutions in acetonitrile with tetrazole as the activating agent, resulting in a phosphite triester nucleotide functionalized PEG (**3**). The PEG-functionalized tip acts as a solid support, which enables a high success rate of educts and the ability to repeatedly rinse the surface and perform the reaction multiple times. After the reaction, the phosphite triester was oxidized to a phosphate triester by 0.1 M iodine in THF in the presence of piperidine.

The DMT protecting group of the 5′-hydroxy functionality of the deoxyribose can be cleaved with 2% trichloroacetic acid (TCA) in dichloromethane. Because this group shows an absorption maximum at λ 498 nm with an extinction coefficient of 78000 $M^{-1}cm^{-1}$,[10] the liberated group can be used to determine the amount of bound nucleotide. By relating the total reactive surface area of the cantilever to the surface area of the tip, the total number of molecules that are able to interact with a surface can be calculated with the Beer–Lambert law. Assuming an accessible cantilever tip radius of 10 nm, 309 ± 7 adenosines or 219 ± 54 thymidine residues can theoretically come into contact with the surface. Finally, the protecting group of the primary amine of adenosine was removed by 20% ammonia solution (**4**). The functionalized tips were used to investigate the interaction forces of single nucleotides with surfaces.

For a detailed validation of quality and yield of the phosphate ester formation, the PEG was reacted with adenosine- or thymidine-functionalized phosphoramidites in solution. After oxidization with iodine, the reaction mixtures were purified by HPLC and analyzed by MALDI-TOF mass spectrometry (Supporting Information). The yield for adenosine PEG was 71% and for thymidine PEG, 45%. MALDI-TOF MS analysis of the collected HPLC fractions showed an increase in the average

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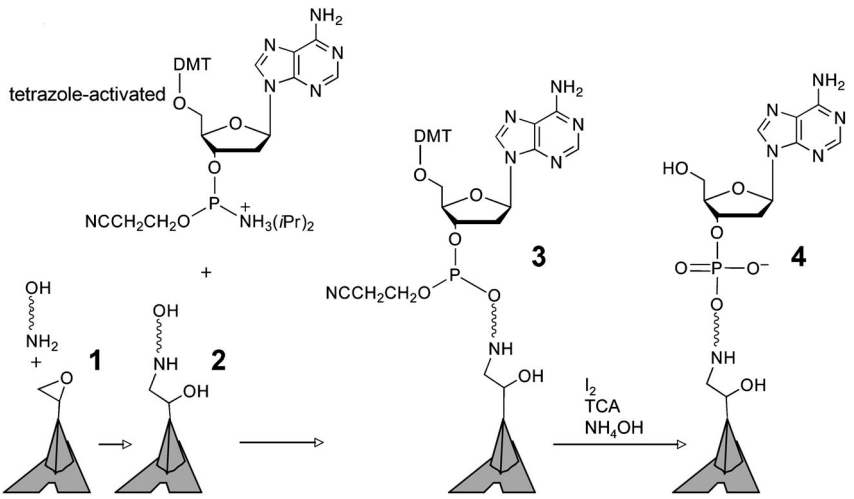

Scheme 1. Functionalization of cantilever tips with nucleotides by the phosphoramidite method. First the silicon oxide tip is functionalized with an epoxysilane. The epoxy function reacts in a second step with the amine group of a bifunctional polyethylene glycol (OH-PEG-NH_2). Reaction of the hydroxy group with a phosphoramidite–nucleotide leads to a PEG–phosphite triester, which is then oxidized to a PEG–phosphate triester. Removal of the protecting groups results in a nucleotide-functionalized tip.

mass of 652 Da in the case of adenosine coupling and 619 Da in the case of thymidine coupling relative to unfunctionalized PEG. These masses correspond to nucleotides containing the DMT protecting groups (Figure S2, Supporting Information). Thus it was shown that the formation of the phosphate ester works in general. Because the tip acts as a solid support, the yield could be enhanced with an increased concentration of educts and repeated reaction steps. To determine the applicability of the nucleotide-functionalized cantilever tips for investigating interactions of nucleotides or short DNA strands with DNA binding enzymes, surfaces, or other structures, we chose a gold-coated surface as the target. As described previously, primary amines can form a coordination bond with undercoordinated gold atoms.[8,11] The force to disrupt such a single coordination bond was recently determined by single-molecule force spectroscopy to be in the range of 150–170 pN.[12] Previous studies describe typical energies for the Au–N bond of 32–42 kJ mol^{-1} and a bond length of nearly 0.3 nm, which corresponds to a barrier force of ~185 pN.[13] Thus the coordinative Au–N bond is clearly weaker than the covalent Au–S bond.

Providing clearly detectable forces, the amine–gold bond is a good model to probe binding forces of nucleotide-functionalized tips with target structures. Both the adenosine- and thymidine-functionalized cantilevers were brought into contact with the gold surface for 0.5 s in water containing 100 μM $MgCl_2$ and then removed with a pulling speed of 1500 nm s^{-1} in the AFM (Figure 1). A pH value of 8.5 was chosen to enhance the number of deprotonated amines with free electron pairs to react with the gold surface.

To provide a chemically reactive gold surface without passivating oxide layers, cyclic voltammograms were performed prior to the force spectroscopy experiments. In the case of adenosine, force–distance curves could be recorded, which showed an average rupture force of 145 pN (Figure 1C) and average contour length of 40 nm (Figure 1D). This length is typical for a 10-kDa PEG, and the force–distance curve was fit with a two-state freely jointed chain (FJC) model.[14] In the case of thymidine, no specific force–distance curves could be detected. Because thymine does not possess a primary amino function with a free electron pair, it is unable to covalently interact with the gold surface to form a coordinate nitrogen–gold bond.

Taken together, we provide a simple and inexpensive method for the introduction of single nucleotides to cantilever tips. This study demonstrated

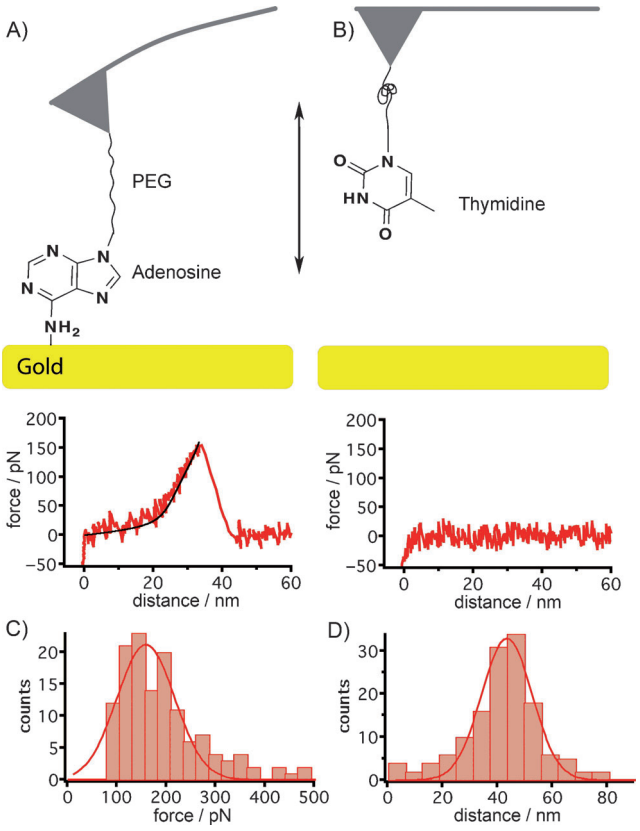

Figure 1. Probing the interaction between nucleotides and gold surfaces by AFM. Adenosine (A) or thymidine (B) nucleotides were attached to a 10-kDa PEG spacer molecule, which itself was covalently bound to the AFM tip via an epoxysilane. Typical examples of force–extension curves, with adenosine and thymidine as the nucleotides, were recorded. No measurable interaction could be observed for the thymidine–PEG, whereas for adenosine, stretching of the PEG spacer (two-state FJC fit, black curve) and rupture of the N–Au bond in the range of 145 pN was repeatedly observed. The corresponding histograms of 140 force–extension curves delivers an average rupture force of 145 ± 83 pN (C) and an average rupture length of 40 ± 13 nm (D), which is typical for a 10-kDa PEG spacer.

the ability to recognize different binding behaviors of single nucleotides to gold surfaces. The presented tips could help to investigate binding forces of nucleotide binding molecules and structures, as previously shown for a receptor–ligand system.[15] In recent years, nuclease-resistant nucleic acids consisting of unnatural nucleotides as therapeutic agents have gained increasing attention.[16] Cantilever tips functionalized with single nucleotide analogues may help to investigate the binding behavior of polymerases with altered substrate specificity.[17] Thus, this technique may facilitate the optimization of specific polymerases to generate nuclease-resistant nucleic acids and enable their use as therapeutic agents. Subsequently, the synthesis of longer stable nucleic acids such as aptamers on the cantilever tip might enable specific investigations of aptamer drug–target interactions for late-stage candidate molecules in drug development.

Experimental Section

All chemicals and organic solvents were purchased from Roth, unless mentioned otherwise in the text. Silicon nitrite cantilever tips (MLCT-AUHW, Veeco, Germany) were cleaned by a UV cleaner (UVOH 150 Lab, FHR) for 20 min and then directly incubated in glycidoxypropyl trimethoxysilane (Sigma–Aldrich) for 1 h. In the following step tips were rinsed three times in toluene and water and then incubated for 30 min at 80 °C. Then a solution of NH_2-PEG-OH (10 mM; RAPP Polymere GmbH, Tübingen, Germany) in borate buffer (pH 8.5) was added and incubated for 1 h and rinsed three times with water afterward.

Nucleotide phosphoramidites were dissolved to 150 mM in tetrazole/CH_3CN activation reagent as provided by the supplier and added to the cantilever tips 2×15 min. After rinsing with CH_3CN, *i*PrOH and THF, the phosphite triester was oxidized to a phosphate triester with 50 mM iodine in THF/H_2O/pyridine (7:2:1 *v/v/v*) for 20 min. Tips were washed with THF, *i*PrOH and CH_2Cl_2. The DMT protecting group was removed with 2% TCA in CH_2Cl_2 for 5 min, and the supernatant was used to determine the coupling efficiency photometrical at λ 498 nm with an extinction coefficient of 78000 $M^{-1}cm^{-1}$. Finally, the DMT protecting group of the 5′-OH group was removed by incubation with 20% NH_3 in 25% EtOH at 60 °C for 2 h.

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