

Linking of Sensor Molecules with Amino Groups to Amino-Functionalized AFM Tips

Linda Wildling,[†] Barbara Unterauer,[‡] Rong Zhu,[†] Anne Rupprecht,[⊥] Thomas Haselgrübler,[§] Christian Rankl,^{||} Andreas Ebner,^{†,‡} Doris Vater,[§] Philipp Pollheimer,[†] Elena E. Pohl,[⊥] Peter Hinterdorfer,^{†,‡} and Hermann J. Gruber^{*,†}

[†]Institute of Biophysics and [‡]Christian Doppler Laboratory for Nanoscopic Methods in Biophysics, J. Kepler University, Altenberger Str. 69, A-4040 Linz, Austria

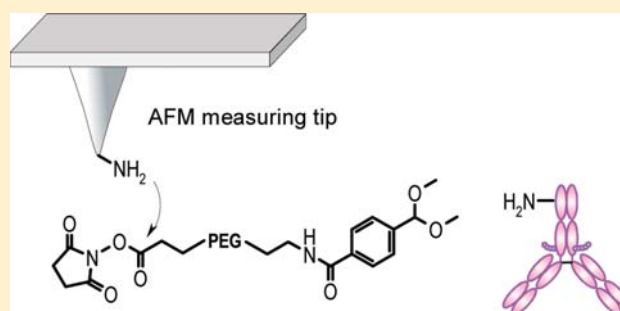
[§]Center for Advanced Bioanalysis, Scharitzer Str. 6-8, A-4020 Linz, Austria

^{||}Agilent Technologies Austria, Aubrunnerweg 11, A-4040 Linz, Austria

[⊥]Department of Biomedical Sciences, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria

S Supporting Information

ABSTRACT: The measuring tip of an atomic force microscope (AFM) can be upgraded to a specific biosensor by attaching one or a few biomolecules to the apex of the tip. The biofunctionalized tip is then used to map cognate target molecules on a sample surface or to study biophysical parameters of interaction with the target molecules. The functionality of tip-bound sensor molecules is greatly enhanced if they are linked via a thin, flexible polymer chain. In a typical scheme of tip functionalization, reactive groups are first generated on the tip surface, a bifunctional cross-linker is then attached with one of its two reactive ends, and finally the probe molecule of interest is coupled to the free end of the cross-linker. Unfortunately, the most popular functional group generated on the tip surface is the amino group, while at the same time, the only useful coupling functions of many biomolecules (such as antibodies) are also NH₂ groups. In the past, various tricks or detours were applied to minimize the undesired bivalent reaction of bifunctional linkers with adjacent NH₂ groups on the tip surface. In the present study, an uncompromising solution to this problem was found with the help of a new cross-linker (“acetal-PEG-NHS”) which possesses one activated carboxyl group and one acetal-protected benzaldehyde function. The activated carboxyl ensures rapid unilateral attachment to the amino-functionalized tip, and only then is the terminal acetal group converted into the amino-reactive benzaldehyde function by mild treatment (1% citric acid, 1–10 min) which does not harm the AFM tip. As an exception, AFM tips with magnetic coating become demagnetized in 1% citric acid. This problem was solved by deprotecting the acetal group before coupling the PEG linker to the AFM tip. Bivalent binding of the corresponding linker (“aldehyde-PEG-NHS”) to adjacent NH₂ groups on the tip was largely suppressed by high linker concentrations. In this way, magnetic AFM tips could be functionalized with an ethylene diamine derivative of ATP which showed specific interaction with mitochondrial uncoupling protein 1 (UCP1) that had been purified and reconstituted in a mica-supported planar lipid bilayer.



INTRODUCTION

Atomic force microscopy (AFM) can operate in aqueous solution under physiological conditions¹ and reveal fine details not resolved by electron microscopy.² AFM is thus well-suited for the structural analysis of biomolecules and their assemblies.^{1,3} In addition, an AFM tip can be functionalized with one or several probe molecules (e.g., antibodies) whereupon it can be used as a specific biosensor by which cognate target molecules (e.g., antigens) are detected when the tip is moved over the sample surface.^{4–6} Binding is detected as a rupture event which is sensed by a vertically oscillated cantilever⁷ preferably under simultaneous monitoring of sample topography.^{8,9} Alternatively, the tip can be vertically

oscillated at a fixed X–Y position above a target molecule, in which case repeated binding–unbinding events are recorded as force–distance profiles. When repeating force–distance cycles at different force loading rates,¹⁰ detailed biophysical parameters of the non-covalent bond can be calculated from the force data.^{4,5,11–14}

Linear polymers, such as carboxymethylamylose,^{15–18} poly(*N*-succinimidyl acrylate),¹⁹ or poly(ethylene glycol) chains (PEG),^{6,12,14,20–31} have regularly been used as flexible tethers

Received: February 23, 2011

Revised: April 13, 2011

Published: May 04, 2011

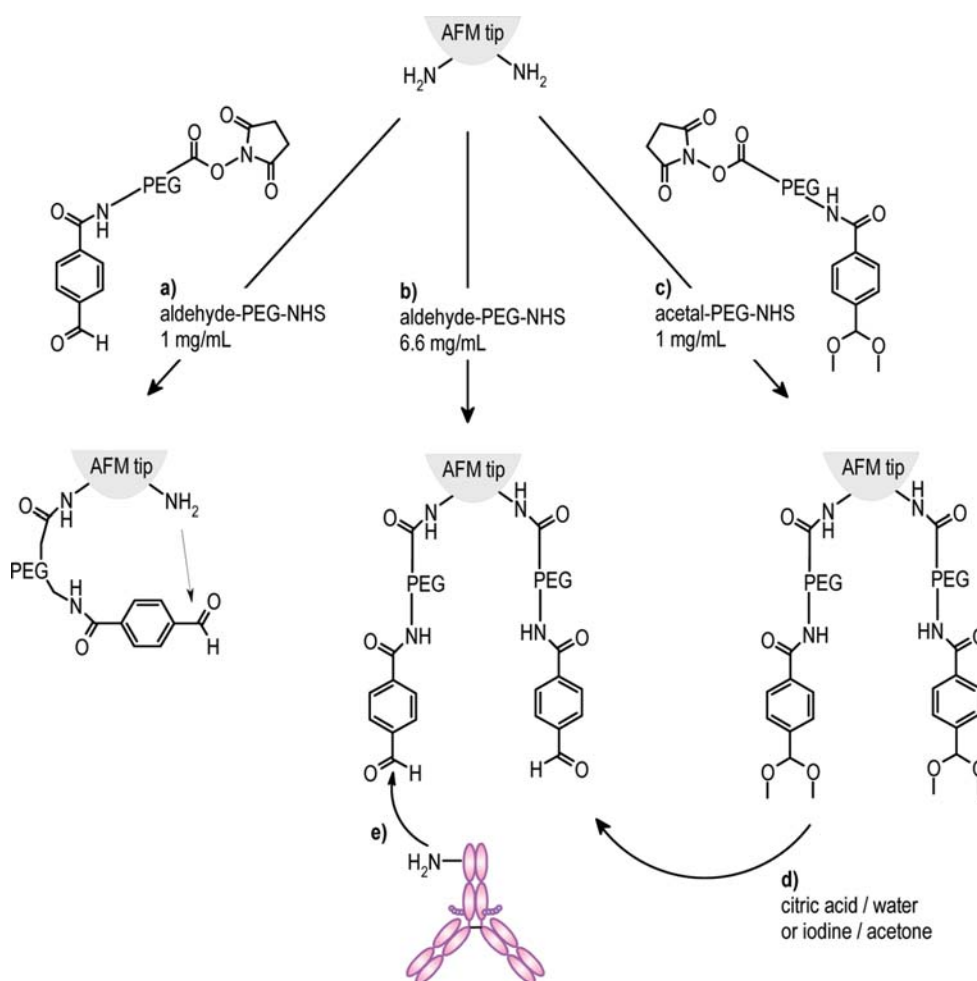


Figure 1. AFM tip functionalization with aldehyde-PEG-NHS as compared to acetal-PEG-NHS. Loop formation between adjacent amino groups can occur with aldehyde-PEG-NHS (a); it is largely suppressed at high concentrations of aldehyde-PEG-NHS (b). No loop formation occurs with acetal-PEG-NHS (c), since the acetal is converted into aldehyde only after derivatization of all NH_2 groups on the AFM tip (d). Lysine residues of proteins can be coupled to terminal benzaldehyde functions (e).

between the tip and the probe molecule, resulting in much higher probability for binding between the probe molecule on the tip and the target molecules on the sample surface. Tethering of probe molecules via linear polymers is usually performed in three stages. First, reactive sites are generated on the tip surface. Second, a linear polymer (“cross-linker”) is attached with one reactive end while reserving the other end for the probe molecule. Third, the probe molecule is coupled to the free end of the polymer chain. The most straightforward scheme comprises (i) amino-functionalization of the tip, (ii) amide bond formation with a heterobifunctional cross-linker that has one amino- and one thiol-reactive end group, and (iii) attachment of a thiol-carrying probe molecule to the free end of the cross-linker.^{6,8,9,25,28,32}

Unfortunately, antibodies and many other proteins possess no free thiols (cysteines) but lots of reactive amino functions (lysines, e.g., 80–90 per antibody).³³ At the same time, amino-functionalization is the predominant method of tip surface activation. This poses the problem of connecting tip- NH_2 with NH_2 -protein with a bifunctional cross-linker, while preventing cross-linker loops between adjacent NH_2 groups on the tip surface. The nontrivial task has been solved by several strategies, each having its own advantages and drawbacks.

The simplest approach is to use a heterobifunctional cross-linker, as described above, and to pre-derivatize the protein with a reagent that introduces free thiol residues^{20,24,25} or pentynoyl groups (for coupling to an azide on the linker via click chemistry).^{22,34} These methods work well, yet ~ 0.2 mg of precious antibody is required per batch, and gel filtration is needed for rigorous removal of reagents. Consequently, the derivatized antibody is rather dilute which prevents refreezing of unused portions.

For minimization of antibody consumption, several methods have been developed in which the lysine residues of antibodies (or other proteins) are directly coupled to tip-bound cross-linker, without pretreatment of the protein. (i) Gold-coated tips were covered with a SAM containing exposed thiol groups to which a heterobifunctional linker (maleimide-PEG-NHS) was bound with its maleimide function.^{12,14} In this way, the amino-reactive NHS ester group was spared for subsequent coupling of protein via lysine. (ii) Gold-coated tips were amino-functionalized with cystamine, and carboxymethylamylose was bound via some of its many NHS ester functions, leaving the other NHS ester groups for coupling of protein.^{17,18} In both methods, the large radius of the gold-coated tips reduces their usefulness for high-resolution imaging. (iii) Silicon nitride tips were amino-silanized and

NHS-activated carboxymethylamylose was applied in the analogous way.^{15,16} (iv) Silicon nitride tips were silanized with a mixture of aminosilane and a large excess of an inert silane, aiming at such a low surface density of NH₂ functions that homobifunctional NHS-PEG-NHS could react with one NHS ester function only, whereupon protein could react with the second NHS ester group.³⁰

All NHS ester methods (i–iv) have in common that coupling of protein is fast and efficient, provided that the concentration of protein is high enough and the optimal pH value is chosen to outrun premature hydrolysis of the NHS ester functions.^{35,36} In this respect, aldehyde functions are a promising alternative because they cannot hydrolyze. Under comparable conditions, coupling of amino groups to aldehydes is much slower than to NHS esters.^{36,37} In a recent study, we took advantage of this kinetic difference by devising a new heterobifunctional cross-linker (aldehyde-PEG-NHS)³⁸ for AFM tip functionalization (Figure 1a,b). When applying the same concentration as with other cross-linkers (1 mg/mL), the tendency for loop formation between adjacent NH₂ groups was very pronounced (Figure 1a). At high concentration (6.6 mg/mL), however, the majority of NH₂ functions on the tip was rapidly consumed by reaction with NHS esters (Figure 1b); thus, most benzaldehyde functions were spared for coupling of protein.³⁸ This method was successfully used in a number of AFM studies.^{21,23,38–49}

In the present study, the benzaldehyde function was kept in a protected form until all NH₂ groups on the tip surface had formed amide bonds with a new linker (acetal-PEG-NHS, Figure 1c). Subsequently, the acetal functions were converted into reactive aldehyde moieties. Dilute citric acid was found to cleave all acetal functions without harmful effects on the tip surface and on the gold mirror on the top side of the cantilever. This strategy affords effective tip functionalization at low cross-linker concentration, which adds to the advantage that synthesis of acetal-PEG-NHS is much simpler than of aldehyde-PEG-NHS.

EXPERIMENTAL PROCEDURES

Materials. Analytical-grade solvents and reagents were used as long as they were commercially available. Muscovite mica sheets were supplied by Christiane Gröpl (Austria). Si₃N₄ measuring tips were purchased from Veeco (Santa Barbara, CA) and Si₃N₄ chips from Wacker-Chemitronic GmbH (Germany). APTES was obtained from Aldrich. NaCNBH₃ was purchased from Fluka. Avidin, D-biotin, EAP, and ethanolamine hydrochloride were obtained from Sigma-Aldrich. EDA-ATP was obtained from BioLog (Bremen, Germany). NH₂-PEG-COOH was synthesized as described.⁵⁰ Acetal-PEG-NHS and aldehyde-PEG-NHS were synthesized as outlined in Figure 3 (see Supporting Information for details). Biotin-IgG was prepared by derivatization of goat IgG with biotin-cap-NHS as described.²⁵ Buffer A contained 100 mM NaCl, 50 mM NaH₂PO₄, and 1 mM EDTA (pH 7.5 adjusted with NaOH). Buffer B contained 300 mM NaH₂PO₄ and 1 mM EDTA (pH 7.5 adjusted with NaOH). PBS contained 150 mM NaCl and 5 mM NaH₂PO₄ (pH 7.4 adjusted with NaOH). Mitochondrial uncoupling protein 1 (UCP1) was purified and reconstituted into mica-supported bilayers as described in the Supporting Information.

Functionalization of AFM Tips with Acetal-PEG-NHS and Antibodies. AFM Tips (Si₃N₄) were amino-functionalized either with a solution of ethanolamine hydrochloride in DMSO (“room temperature method”)⁵¹ or with APTES from gas phase,^{51,52} as specified. Attachment of acetal-PEG-NHS was

performed by 2 h incubation of the tips in 0.5 mL of chloroform containing 1 mg/mL acetal-PEG-NHS and 0.5% (v/v) of TEA. Subsequently, the tips were rinsed in chloroform (3×). Two alternate methods were used for conversion of the acetal functions into aldehyde groups: (i) immersion in acetone containing 0.1% iodine for 5 min and rinsing in pure acetone (3 × 5 min), (ii) immersion in water containing 1% citric acid for 10 min and rinsing with water (3 × 5 min). For the coupling of the antibody, tips were transferred into a mixture of 50 μL biotin-IgG solution (0.2 mg/mL in buffer A) with 2 μL 1 M NaCNBH₃ stock solution (to reach a final concentration of 40 mM NaCNBH₃) for one hour. Subsequently, 5 μL of ethanolamine hydrochloride stock solution (1 M, preadjusted to pH 9.6 with 20% NaOH) was mixed into the biotin-IgG solution and incubation was continued for 10 more minutes. Finally, the tips were washed in PBS (3 × 5 min) and stored in PBS at 4 °C until use (up to 3 days). A detailed illustration of tip functionalization is found in the Supporting Information (Figure S5).

Monitoring of Single Unbinding Events between Tip-Bound Biotin-IgG and Mica-Bound Avidin by AFM. Avidin (1 mg/mL in PBS) was diluted with 1 mM NaCl to a final concentration of 0.5 μg/mL and adsorbed to freshly cleaved mica. After 15 min, the substrate was rinsed with 1 mM NaCl (10×) and subsequently with PBS (10×). Force measurements with biotin-IgG-functionalized AFM tips were performed in a fluid cell of a commercial AFM (PicoSPM, Agilent, Tempe, AZ). Force–distance cycles were recorded at a 1 Hz vertical scan rate and 100–300 nm z-amplitude. The measurements were performed at a constant trace and retrace velocity. All measurements were done in PBS. For specific blocking of avidin–biotin interaction, the biotin residues on tip-bound IgG were masked by addition of free streptavidin (0.5 mg/mL final concentration) to the bath solution. The spring constants of the levers were determined by thermal noise method.^{53,54}

Functionalization of Silicon Nitride Chips with Amino Groups, Acetal-PEG-NHS, and Biotin-IgG, with Quantitative Assay for Chip-Bound Biotin-IgG. Silicon nitride chips were pre-cut, cleaned with chloroform (3×), dried with nitrogen, immersed in a piranha solution (70/30, H₂SO₄/H₂O₂, caution: piranha is extremely corrosive and can explode) for 30 min, extensively rinsed in water, dried with nitrogen, and heated to 160 °C for 5 min. The amino-functionalization was performed with the “room temperature method” as described before.⁵¹ After washing in DMSO (3×) and ethanol (3×), the chips were dried in nitrogen and derivatized with acetal-PEG-NHS as described above for AFM tips. Cleavage of the acetal functions was always performed with citric acid (1%, in water, 1 or 15 min) and washing with water (3 × 5 min) or with iodine (0.1% in acetone, 5 min) and washing with acetone (3 × 5 min), as specified. Coupling of biotin-IgG was performed as described above for AFM tips, except that the concentration of biotin-IgG was varied as specified in the tables. Unused aldehyde functions were blocked with ethanolamine or with glycine, as specified. The surface density of biotin-IgG bound to the silicon nitride chips was determined by specific binding of ExtrAvidin-Peroxidase (EAP) as described before.⁵¹ A detailed illustration of chip functionalization and estimation of its efficiency is found in the Supporting Information (Figure S6).

RESULTS AND DISCUSSION

Synthesis of Acetal-PEG-NHS and Aldehyde-PEG-NHS.

The synthesis route for the new cross-linker acetal-PEG-NHS followed the typical scheme for long heterobifunctional

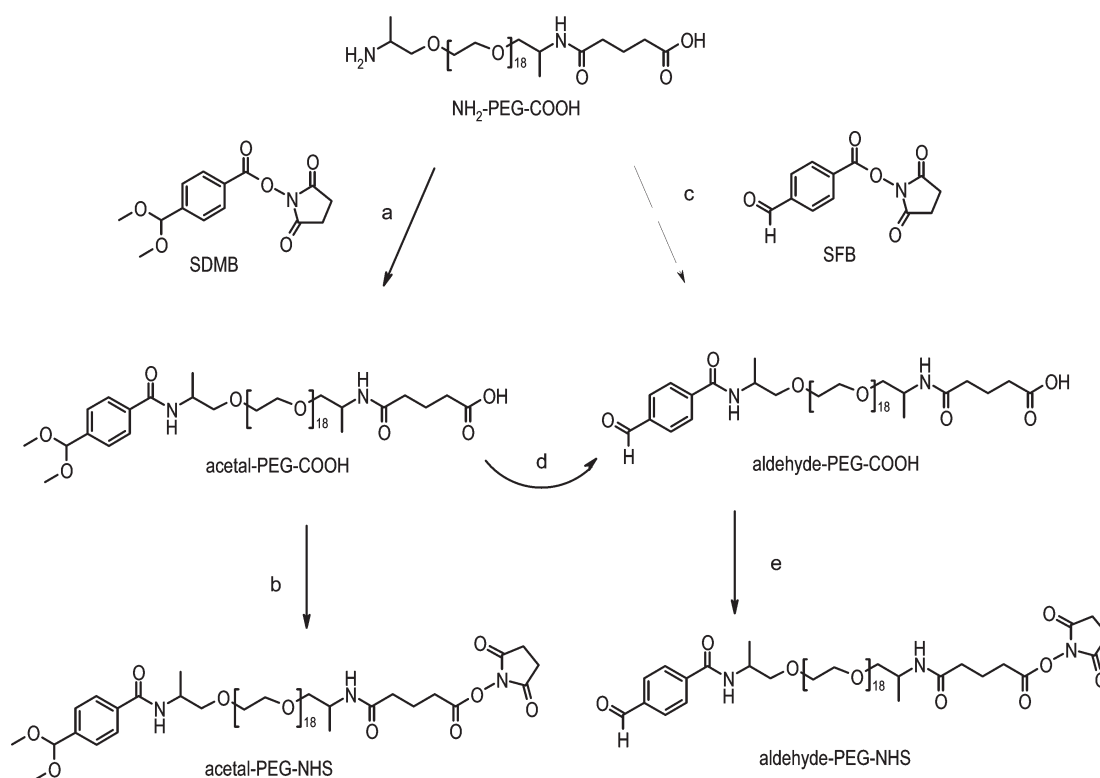


Figure 2. Synthesis of acetal-PEG-NHS and aldehyde-PEG-NHS. (a) $\text{NH}_2\text{-PEG-COOH}$ was reacted with SDMB to give acetal-PEG-COOH. (b) The terminal carboxyl function was activated by conversion into an NHS ester (acetal-PEG-NHS). (c) Previously published method of preparing aldehyde-PEG-COOH by use of SFB.³⁸ (d) New synthesis of aldehyde-PEG-COOH by cleavage of the acetal function. (e) Conversion of the terminal COOH group into an aminoreactive NHS ester (aldehyde-PEG-NHS). Experimental details of the syntheses are found in the Supporting Information.

cross-linkers^{25,38,50,51} in the sense that a short cross-linker (SDMB) was attached to the *N*-terminus of $\text{NH}_2\text{-PEG-COOH}$ in the first step (Figure 2a). To our knowledge, the short cross-linker SDMB has been synthesized for the first time (see Figure S1 in the Supporting Information). After removal of all small molecules (excess of SDMB, free NHS, TEA), the carboxyl group of acetal-PEG-COOH was converted into an NHS ester, resulting in the heterobifunctional cross-linker acetal-PEG-NHS (Figure 2b).

As can be seen from Figure 2, the new module acetal-PEG-COOH served a second purpose also: cleavage of the acetal by iodine/acetone (0.1%, 10 min) or citric acid/water (1%, 10 min) afforded pure aldehyde-PEG-COOH (Figure 2d) which was easily converted into the previously published linker acetal-PEG-NHS (Figure 2e). In contrast to the new synthetic route (Figure 2a,d), the old method of reacting $\text{NH}_2\text{-PEG-COOH}$ with SFB (Figure 2c)³⁸ had given a significant fraction of Schiff base adducts besides the desired amide product; thus, a cumbersome extraction scheme was required for purification of aldehyde-PEG-COOH from undesired PEG derivatives.³⁸ The new synthetic route for preparation of the old linker aldehyde-PEG-NHS (Figure 2, steps a, d, and e) is important because the new linker acetal-PEG-NHS can replace the old one in most but not all AFM applications (see Figure 5).

AFM Tip Functionalization, Using Iodine/Acetone for Cleavage of Tip-Linked Acetal Functions. As can be seen from Figure 1, the essential improvement of the new tip functionalization method rests on the use of a masked aldehyde (i.e., an acetal) which remains unreactive while amide bonds are formed between the

linker and the amino groups on the tip surface. In the next step, the acetal function on the free end of the tip-bound linker was converted into an amino-reactive benzaldehyde function (step d). Hereby, it was critical to select the mildest available method in order to prevent damage to the tip surface and to the light-reflecting metal film on the top side of the cantilever. In a recent study, it had been found that dimethylacetals of aromatic aldehydes are cleaved within 5 min by 0.6% iodine in anhydrous acetone under neutral conditions.⁵⁵ In the present study, we further lowered the iodine concentration to 0.1% and found it fully sufficient to completely deprotect acetal-PEG-COOH within 5–10 min in bulk solution (Figure 2, step d). Consequently, acetone with 0.1% iodine was also applied to tip-linked acetal functions (Figure 1, step d).

After deprotection, the functionality of “tip-PEG-acetal” was examined by a established validation method.^{25,38} Biotin-IgG was coupled to the tip-bound benzaldehyde functions (Figure 1, step e), using NaCNBH_3 for concomitant fixation of the initially formed Schiff base bonds (see Figure S5). The tip was then mounted in the AFM and the ligand function of the biotin residues was tested by repeated approach to, and retraction from, the test sample which consisted of a dense monolayer of avidin, bound to mica by electrostatic adsorption (Figure 3A).⁵¹ A typical example of the resulting force curves is shown in Figure 3B. The *X*-axis corresponds to the vertical movement of the piezo which carries the cantilever, while the *Y*-axis mirrors the deflection of the cantilever (converted into force values by multiplying the deflection amplitude with the force constant of the particular cantilever). During the approach (gray line), the cantilever is in the resting position up to the point of contact.

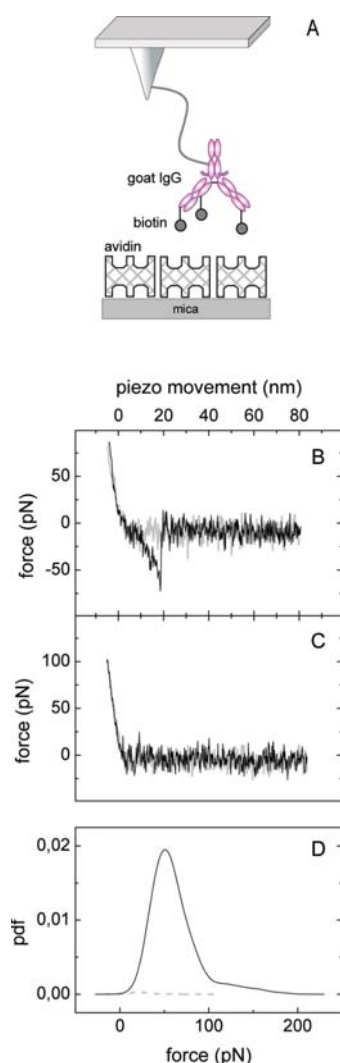


Figure 3. Force spectroscopy of single avidin–biotin interactions. (A) The AFM tip was functionalized with biotin–IgG, as outlined in Figure 1cd (or in greater detail in Figure S5, Supporting Information), using ethanolamine hydrochloride for tip amino-functionalization and iodine/acetone for cleavage of the acetal functions. In parallel, avidin was irreversibly adsorbed onto freshly cleaved mica. (B) Example of a force–distance cycle showing specific avidin–biotin unbinding. The piezo movement in the approach-and-retraction cycles was ± 100 nm in 1 s, the force constant of the cantilever was 18 pN/nm, and the loading rate at unbinding was calculated as 1180 pN/s. Unbinding occurred at a rupture force of 51 pN and a tip–mica distance of 22 nm. (C) Example of a force–distance cycle performed in the presence of free streptavidin (0.5 mg/mL final concentration in PBS). (D) Probability density function (pdf) of the unbinding forces observed in 199 out of 948 force–distance cycles (solid line, 21% binding probability). In the presence of free streptavidin, only 3 out of 198 force–distance cycles showed apparent unbinding events (dashed gray trace, 1.5% binding probability).

Further approach causes upward bending of the cantilever. Retraction causes relaxation of cantilever bending, which does not stop at zero force but proceeds to negative values at further retraction. The reason is binding of a tip-tethered biotin residue to mica-bound avidin which causes progressive stretching of the PEG chain. At the critical force of 51 pN, the avidin–biotin bond is suddenly ruptured and the cantilever jumps to its resting position. The probability density function (pdf)⁵⁶ of 199

unbinding events (Figure 3D, solid line) shows the expected average avidin–biotin rupture force, as previously observed at the same force loading rate.^{25,38,51}

The specificity of avidin–biotin interaction was demonstrated by blocking the biotin residues on the tip with free streptavidin from solution. As a consequence, most force–distance cycles showed only upward bending of the cantilever (Figure 3C) and only 1.5% of the force distance cycles showed downward deflection, in contrast to 21% before the addition of free streptavidin. This difference in binding probability is reflected by the much lower area of the unbinding force distribution function in the presence of streptavidin (dashed gray line in Figure 3D), as compared to the condition with no streptavidin (solid line).

For independent validation of the new tip functionalization method (Figure 1c,d,e), the procedure was applied to chips (5 mm \times 5 mm) consisting of the same material as the AFM tips (Si_3N_4) and the area density of immobilized biotin–IgG was estimated from binding of EAP which is a conjugate between avidin and horse radish peroxidase.^{25,38,51} Specific binding (Table 1, column 5) was calculated by subtracting nonspecific binding in the presence of free D-biotin (column 4) from total binding in the absence of free D-biotin (column 3). The results show that specific binding of EAP to immobilized biotin–IgG is far more pronounced than nonspecific adsorption. The actual numbers in columns 3–5 are similar as previously obtained with aldehyde-PEG-NHS,³⁸ except that aldehyde-PEG-NHS must be used at high concentrations (Figure 1b,e) in order to suppress loop formation on the surface (Figure 1a), whereas the new linker acetal-PEG-NHS can be used at the low concentration of 1 mg/mL (Table 1, column 1). From these findings, we conclude that all available amino groups on the tip surface fully react with the NHS ester function of acetal-PEG-NHS (1 mg/mL in chloroform with 0.5% TEA, 60 min), thereby eliminating the danger of loop formation on the tip surface when/after the acetal is converted into a benzaldehyde function in the next step (Figure 1d).

Table 1 also shows that there is no need to use antibody concentrations higher than 0.15 mg/mL (column 2). The same phenomenon has been observed when other PEG linkers were used for antibody linking to AFM tips.^{25,38} It was conclusively explained by weak preadsorption of protein to the tip surface, with the effect that one protein molecule adsorbed within reach of a 6-nm-long PEG linker has an effective local concentration of 3 mM.²⁵ As a consequence, very low protein concentrations suffice to equip all PEG linkers on the AFM tip with a protein molecule. In practice, we have seen efficient tip functionalization with ≥ 0.03 mg/mL antibody concentration (data not shown).

AFM Tip Functionalization, using Dilute Citric Acid for Cleavage of Tip-Linked Acetal Functions. The above-described method of tip functionalization has successfully been applied in several AFM laboratories when suddenly the users complained that the gold mirror on the top side of the cantilever got destroyed during tip functionalization. Detailed analysis showed that this destruction occurred during acetal cleavage with iodine/acetone (Figure 1d) and that it only happened with cantilevers purchased after the producer had changed the production method. As a consequence, several publications could only be finished by switching back to tip functionalization with aldehyde-PEG-NHS (Figure 1b,e).^{39,41,49}

In search for an alternative to iodine/acetone a study was found in which the diethylacetal of a PEG-linked benzaldehyde had completely been hydrolyzed within 5 h at pH 5, which had been adjusted with HCl.⁵⁷ This condition seemed promising in

Table 1. Tethering of Biotinylated IgG to Silicon Nitride Chips via Acetal-PEG-NHS (Iodine Method)^a

[PEG linker] (mg/mL)	[biotin-IgG] (mg/mL)	total sites (μm^{-2}) ^b	nonspecific sites (μm^{-2}) ^b	specific sites (μm^{-2}) ^b
1	2	805 ± 124	27 ± 4	778 ± 143
10	2	742 ± 81	27 ± 4	715 ± 94
1	0.15	777 ± 136	151 ± 51	626 ± 153

^a Silicon nitride chips were derivatized according to the scheme shown in Figure S6. Ethanolamine hydrochloride was used for amino-functionalization of silicon nitride (step a in Figure S6). Iodine (0.1%, in acetone, 5 min) was applied for cleavage of the acetal function (step c). ^b The number of biotin-IgG molecules per μm^2 (specific sites) was estimated from the difference EAP binding, measured in the absence (total sites, step f) and in the presence of free D-biotin (nonspecific sites, step e).

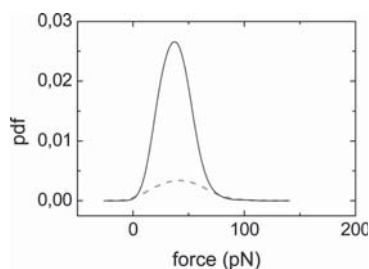


Figure 4. Force measurements of single avidin–biotin interactions. Tip functionalization and force spectroscopy were performed as in Figure 3, except that citric acid (1%, in water, 15 min) was used in place of iodine/acetone for conversion of acetal into aldehyde functions. The piezo movement in the approach-and-retraction cycles was ± 150 nm in 1 s, the force constant of the cantilever was 37 pN/nm, and the loading rate at unbinding was calculated as 720 pN/s. The graph shows the probability density function (pdf) of the unbinding forces observed in 321 out of 985 force–distance cycles (solid line, 32.6% binding probability). In the presence of free streptavidin, only 41 out of 938 force–distance cycles showed apparent unbinding events (dashed gray line, 4.4% binding probability).

terms of mildness, but shorter reaction times and a more reliable way to adjust pH were required for practical purposes. For a first test, we treated acetal-PEG-COOH with 1% citric acid (pH 2.2, Figure S4) for 10 min and found that at least 99.6% (or more) of the acetal functions had been converted into benzaldehyde functions (see Supporting Information). Consequently, the AFM test experiment from Figure 3 was repeated, except that conversion of “tip-PEG-acetal” into “tip-PEG-aldehyde” was performed with 1% citric acid instead of iodine/acetone. The results are summarized in Figure 4. The unbinding force distribution shows the expected average unbinding force of ~ 50 pN, as well as high binding probability (32.6%, solid line) before and low binding probability (4.4%, dashed line) after specific block of avidin–biotin interaction with excess of streptavidin.

The modified tip functionalization protocol with 1% citric acid in place of iodine/acetone was again validated by imitation of tip functionalization on silicon nitride chips (Table 2). Columns 1 and 5 confirm the well-known trend that higher densities of amino groups are achieved with ethanolamine/DMSO as compared to gas-phase silanization with APTES.^{51,52} In addition, the ethanolamine/DMSO method is less critical and easier to perform by newcomers. Nevertheless, the APTES method is to be preferred for high-quality AFM measurements.⁵⁸ When varying the incubation time with 1% citric acid (column 2), it was found that even the short time interval of 1 min was sufficient to deprotect 60–90% of the benzaldehyde functions on the chip (see column 5). From these data, it appears that 3–5 min of treatment with 1% citric acid is the best compromise between efficient acetal cleavage and minimal chemical stress on AFM tips.

Table 3 addresses the question regarding whether it is necessary to block potentially unused benzaldehyde functions on the AFM tip. Now, all chips were amino-functionalized with APTES in the gas phase, followed by reaction with acetal-PEG-NHS, cleavage of tip-bound acetal functions with 1% citric acid, and coupling of biotin-IgG to the resulting benzaldehyde functions on the chip. In case unused benzaldehyde functions are present on the chip surface, this should lead to covalent coupling of the ExtrAvidin-Peroxidase (EAP), which we use for “counting” of the biotin-IgG molecules on the chip surface. Such covalent coupling would contribute to nonspecific binding (column 4 in Table 3), in contrast to the specific interaction of EAP with biotin-IgG (column 5) which is blocked by excess of free D-biotin. Previously, it was shown that the terminal benzaldehyde is efficiently blocked by free ethanolamine in presence of NaCNBH_3 .³⁸ Now, free ethanolamine was applied after coupling of biotin-IgG (lines 3 and 4) and compared to controls in which no attempt was made to block unused benzaldehyde functions (lines 1 and 2). However, no significant difference was found with respect to the extent of nonspecific binding. The same result was also obtained when glycine was used in place of ethanolamine (lines 5 and 6).

Lines 1, 3, and 5 in Table 3 differ from all chip functionalization assays in this and previous studies^{25,38,51,52} by omission of Tween 20 in the incubation step with ExtrAvidin-Peroxidase (EAP). Absence of detergent was expected to enhance adsorption, and thereby also to accelerate covalent coupling of EAP to surface-bound benzaldehyde residues,²⁵ in case not all of them had reacted with biotin-IgG in the preceding step. The data in Table 3, however, do not show a significant difference in the extent of nonspecific binding of EAP to the chip surface, whether Tween 20 was present or not (columns 2 and 4). It is, therefore, concluded that nonspecific binding was merely caused by physical adsorption of ExtrAvidin-Peroxidase and not by covalent coupling to unused benzaldehyde functions. With respect to AFM tip functionalization, this means that all (or almost all) benzaldehyde groups on the tip surface appear to react with a protein molecule under the standardized reaction conditions and that blocking of putatively unused benzaldehyde groups is merely optional. In case blocking is desired, glycine is to be preferred over ethanolamine, because the lower pK_a of glycine allows for a more neutral pH during the blocking step (see legend to Table 3). An alternate method for blocking of potentially unused aldehyde functions at pH ~ 7.7 is described in the Supporting Information (part 4).

Rapid Imaging of Mitochondrial Uncoupling Protein with ATP-Functionalized AFM Tips. As describe above, citric acid (1%) allows for conversion of “tip-PEG-acetal” into “tip-PEG-aldehyde” without adverse effects on standard AFM cantilevers. Unfortunately, this is not true for MAC levers, the magnetic

Table 2. Tethering of Biotinylated IgG to Silicon Nitride Chips via Acetal-PEG-NHS (citric acid method).^a

amino-functionalization	1% citric acid (min)	total sites (μm^{-2}) ^b	nonspecific sites (μm^{-2}) ^b	specific sites (μm^{-2}) ^b
ethanolamine	1	1389 ± 146	159 ± 49	1230 ± 169
	15	1486 ± 151	159 ± 49	1327 ± 185
APTES	1	645 ± 141	118 ± 27	527 ± 163
	15	1009 ± 95	118 ± 27	891 ± 117

^a Silicon nitride chips were derivatized according to the scheme shown in Figure S6. The concentration of acetal-PEG-NHS was 1 mg/mL (step b in Figure S6). Citric acid (1%, in water, treatment time either 1 or 15 min) was applied for cleavage of the acetal function (step c). The concentration of biotin-IgG was 0.22 mg/mL (step d). ^b The number of biotin-IgG molecules per μm^2 (specific sites) was estimated from the difference in EAP binding, measured in the absence (total sites, step f) and in the presence of free D-biotin (nonspecific sites, step e).

Table 3. Comparison of Different Methods for Blocking of Unused Aldehyde Functions (citric acid method).^a

blocking method	Tween 20 ^d	total sites (μm^{-2}) ^e	nonspecific sites (μm^{-2}) ^e	specific sites (μm^{-2}) ^e
none	-	933 ± 133	142 ± 39	791 ± 92
	+	898 ± 344	96 ± 17	802 ± 344
ethanolamine ^b	-	775 ± 24	146 ± 28	629 ± 37
	+	715 ± 107	138 ± 26	577 ± 110
glycine ^c	-	1053 ± 345	81 ± 12	972 ± 345
	+	865 ± 146	90 ± 32	775 ± 149

^a Silicon nitride chips were derivatized according to the scheme shown in Figure S6. APTES was used for amino-functionalization of the silicon nitride chips (step a in Figure S6). The concentration of acetal-PEG-NHS was 1 mg/mL (step b). Citric acid (1%, in water, 15 min) was used for cleavage of the acetal function (step c). The concentration of biotin-IgG was 0.15 mg/mL (step d). ^b Ethanolamine hydrochloride (0.05 volumes of a 1 M stock solution, adjusted to pH 8.3 with NaOH) was added to the biotin-IgG solution on the AFM tips after 1 h incubation and allowed to react for 10 min (step f in Figure S5). ^d The minus or plus signs indicate absence or presence of Tween 20 (0.1%) during incubation with EAP. In any case, Tween 20 was used in the subsequent washing steps. ^e The number of biotin-IgG molecules per μm^2 (specific sites) was estimated from the difference in EAP binding, measured in the absence (total sites, step f in Figure S6) and in the presence of free D-biotin (nonspecific sites, step e in Figure S6).

coating of which has been observed damaged to variable degrees by treatment with 1% citric acid (data not shown). MAC levers are indispensable for simultaneous “topography and recognition imaging” (TREC). In the so-called TREC mode, a biofunctionalized tip is magnetically oscillated near its resonance frequency while rapidly scanning the sample surface. Hereby, topographic features dampen the downward amplitudes of tip oscillation, while recognition of a target molecule on the sample surface by a tip-bound sensor molecule causes stretching of the PEG linker between the tip and the sensor molecule, which dampens the upward amplitudes of tip oscillation. Consequently, the envelope of the lower amplitudes yields the topographic image (Figure 5B), while the envelope of the upper amplitudes yields a map of recognition sites (Figure 5C). A more detailed explanation of the TREC imaging method can be found at the end of the Supporting Information.

Given the poor resistance of the magnetic coating of MAC levers to 1% citric acid, the new tip functionalization protocol with acetal-PEG-NHS cannot be applied to tips used for recognition imaging, and the older method with aldehyde-PEG-NHS must be used instead.³⁸ Nevertheless, the present study provides for a major improvement of the older method, albeit in an indirect way. Figure 2 depicts a new route for the synthesis of the old linker aldehyde-PEG-NHS (steps a, d, and e). Previously, the old linker was prepared by reaction of NH_2 -PEG-COOH with succinimidyl formylbenzoate (SFB, step c). Unfortunately, SFB has two amino-reactive ends, thus several side products are formed in the reaction with NH_2 -PEG-COOH which are difficult to remove and cause low yields of aldehyde-PEG-COOH. No such problem is encountered when reacting the new module SDMB with NH_2 -PEG-COOH (step a) because it possesses only

one amino-reactive group. Consequently, the yield of acetal-PEG-COOH is high and the intermediate is obtained in good yield. Cleavage of the acetal function (step d) and activation of the terminal carboxyl (step e) are also simple, thus the old linker aldehyde-PEG-NHS is obtained in high yield from the valuable starting material NH_2 -PEG-COOH.

For the “proof of principle”, the binding of ATP to the mitochondrial uncoupling protein, UCP1, was studied.⁵⁹ UCP1 is an anion transporter, which dissipates the mitochondrial inner membrane proton gradient and can be inhibited by di- or triphosphate nucleotides. Although some plausible models were proposed (for review, see Krauss et al.⁶⁰), the exact mechanism of UCP1 inhibition remains unknown. In the absence of the protein crystallographic structure, the studies using tip-tethered ATP molecules are a powerful tool to elucidate different parameters of ATP-UCP interaction, such as location of the nucleotide binding site inside the membrane and a binding force of the nucleotide-protein interaction. The latter is a subject of our subsequent study.

The newly synthesized “old linker” acetal-PEG-NHS was used for flexible attachment of an ATP derivative to a MAC lever, as depicted in Figure 5A (for details, see Supporting Information). The functionalized tip was scanned over a mica-supported phospholipid bilayer which contained purified UCP1. The individual UCP1 molecules were nicely visible as round structures of defined size in the topographic image (Figure 5B). The specific recognition of the ATP-binding sites is mirrored in the recognition image (Figure 5C) of the TREC mode. For the competitive blocking of ATP-binding sites in the control experiment, 4.8 mM of free ATP was added to the buffer solution before scanning. Figure 5D shows that the protein spots in the

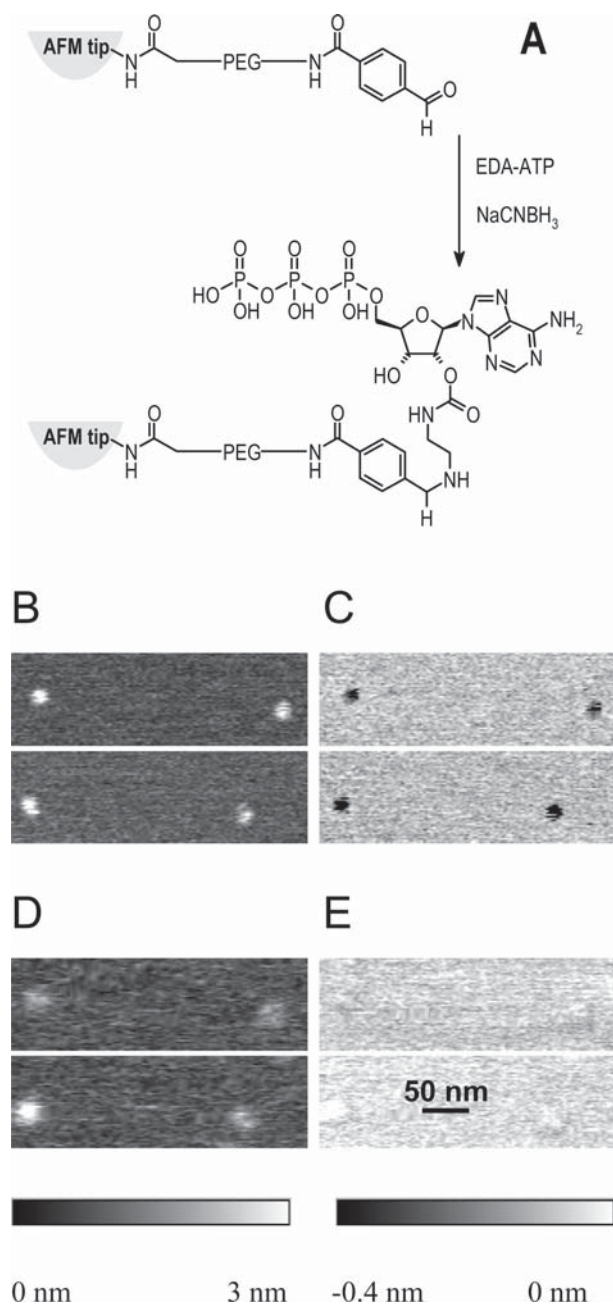


Figure 5. Topographic and recognition imaging of uncoupling protein 1 (UCP1) in planar supported bilayer with an ATP-functionalized AFM tip. (A) Reaction of tip-PEG-aldehyde with an ethylene diamine derivative of ATP (EDA-ATP) with concomitant reduction of the C=N bond by NaCNBH₃. Topographic images (B) and simultaneously acquired recognition images (C) of two different bilayer patches (top and bottom strip) in the absence of free ATP. Topographic images (D) and simultaneously acquired recognition images (E) of the same sample areas as in (B) and (C) after the addition of free ATP (final concentration 4.8 mM).

topographic image persisted, whereas only very dim shadows with the opposite amplitude were observed in the recognition image (Figure 5E), revealing the tiny amplitude of the spillover from the topographic image into the recognition image. The complete loss of the recognition after addition of free ATP (Figure 5E) clearly confirms that all recognition spots in

Figure 5C were caused by specific binding of tip-linked ATP to UCP1 molecules in the planar lipid membrane.

CONCLUSIONS

The present study offers an uncompromised and simple solution to the nontrivial problem of linking the NH₂ groups of biomolecules to the NH₂ groups of amino-functionalized AFM tips. In contrast to previous methods, a bifunctional linker with a single amino-reactive end is used which is reacted with all available NH₂ groups on the tip surface. Subsequently, the free-hanging end of the PEG linker is converted into an amino-reactive aldehyde function which provides for coupling of sensor molecules via their NH₂ functions. In this way, the undesired cross-linking of adjacent NH₂ functions on the tip surface is perfectly avoided. The method is particularly suited for proteins, which are usually abundant in lysine residues. Other obvious applications are synthetic oligonucleotides with NH₂ groups on their 5' or 3' end, as well as small biomolecules or drugs with endogenous or artificially introduced amino functions.

The only weak point of the new tip functionalization protocol is the adverse effect of the acetal-to-aldehyde conversion step on the magnetic coating of cantilevers which are needed for fast imaging of recognition sites on the sample surface. Such cantilevers must be functionalized with the old linker aldehyde-PEG-NHS. Nevertheless, this older method has also profited from the present study because a simpler and more efficient synthesis route to aldehyde-PEG-NHS was found. The experiments with reconstituted UCP1 demonstrate a great application potential of recognition force microscopy for the functional studies of biomolecules which is not easily gained from other techniques.

ASSOCIATED CONTENT

S Supporting Information. Syntheses of 4-(dimethoxymethyl)-benzoic acid, *N*-succinimidyl 4-(dimethoxymethyl)-benzoate, aldehyde-PEG-COOH, aldehyde-PEG-NHS, acetal-PEG-COOH, acetal-PEG-NHS. Kinetics of acetal cleavage at different pH values in aqueous solution. Detailed illustration of AFM tip functionalization via acetal-PEG-NHS. Illustration of silicon nitride chip functionalization and characterization with ExtrAvidin-Peroxidase. Method of AFM tip functionalization with EDA-ATP. Purification of UCP1 and reconstitution into mica-supported bilayer. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*To whom correspondence should be addressed at the Institute of Biophysics in Linz. Tel.: +43 (732) 2468-9271. Fax: +43 (732) 2468-9280, e-mail: hermann.gruber@jku.at.

ACKNOWLEDGMENT

We are indebted to Prof. Norbert Müller for use of NMR spectrometer facilities. This work was supported by the Austrian Science Foundation (FWF project SFB-F35, to P. H.), by the Austrian Research Promotion Agency (FFG, Austrian Nanoscience Initiative VO104-08-BI, project 819703 NSI-NABIOS, to H. J. G.), by the Christian Doppler Society, by EC grant SMW (grant number 213717, to P. H.), and by Deutsche Forschungsgemeinschaft (Po-524/3-1 and Po 524/5-1 to E. P.).

■ ABBREVIATIONS

AFM, atomic force microscope (or microscopy); acetal-PEG-COOH, see Figure 3; acetal-PEG-NHS, see Figure 2; acetal-PEG₂₇-NHS, see Figure 2; aldehyde-PEG-COOH, see Figure 2; aldehyde-PEG-NHS, see Figure 2; aldehyde-PEG₂₇-NHS, see Figure 2; ATP, adenosine-5'-O-triphosphate; biotin-cap-NHS, N-succinimidyl 6-(N-biotinoyl)-aminohexanoate; DMSO, dimethyl-sulfoxide; EAP, ExtrAvidin-Peroxidase; EDA-ATP, 2'-/3'-O-(2-aminoethylcarbamoyl)-adenosine-5'-O-triphosphate; EDTA, ethylene diamine N,N,N',N'-tetraacetic acid; NH₂-PEG-NH₂, O,O'-bis-(2-aminopropyl)-poly(ethylene glycol) 800; NH₂-PEG-COOH, N-glutaryl derivative of NH₂-PEG-NH₂, see Figure 3; NHS, N-hydroxysuccinimide; pdf, probability density function; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); SDMB, N-succinimidyl 4-(dimethoxymethyl)-benzoate; SFB, N-succinimidyl 4-formylbenzoate; TEA, N,N,N-triethylamine; TREC mode, topography-and-recognition mode

■ REFERENCES

- (1) Hörber, J. K., and Miles, M. J. (2003) Scanning probe evolution in biology. *Science* 302, 1002–1005.
- (2) Scheuring, S., Müller, D. J., Ringler, P., Heymann, J. B., and Engel, A. (1999) Imaging streptavidin 2D crystals on biotinylated lipid monolayers at high resolution with the atomic force microscope. *J. Microsc.* 193, 28–35.
- (3) Parot, P., Dufrene, Y. F., Hinterdorfer, P., Le Grimmelc, C., Navajas, D., Pellequer, J. L., and Scheuring, S. (2007) Past, present and future of atomic force microscopy in life sciences and medicine. *J. Mol. Recognit.* 20, 418–431.
- (4) Hinterdorfer, P., and Dufrene, Y. F. (2006) Detection and localization of single molecular recognition events using atomic force microscopy. *Nat. Methods* 3, 347–355.
- (5) Kienberger, F., Ebner, A., Gruber, H. J., and Hinterdorfer, P. (2006) Molecular recognition imaging and force spectroscopy of single biomolecules. *Acc. Chem. Res.* 39, 29–36.
- (6) Raab, A., Han, W., Badt, D., Smith-Gill, S. J., Lindsay, S. M., Schindler, H., and Hinterdorfer, P. (1999) Antibody recognition imaging by force microscopy. *Nat. Biotechnol.* 17, 902–905.
- (7) Radmacher, M., Cleveland, J. P., Fritz, M., Hansma, H. G., and Hansma, P. K. (1994) Mapping interactions forces with the atomic force microscope. *Biophys. J.* 66, 2159–2165.
- (8) Stroh, C. M., Wang, H., Bash, R., Ashcroft, B., Nelson, J., Gruber, H., Lohr, D., Lindsay, S. M., and Hinterdorfer, P. (2004) Single-molecule recognition imaging microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12503–12507.
- (9) Stroh, C. M., Ebner, A., Geretschläger, M., Freudenthaler, G., Kienberger, F., Kamruzzahan, A. S. M., Smith-Gill, S. J., Gruber, H. J., and Hinterdorfer, P. (2004) Simultaneous topography and recognition imaging using force microscopy. *Biophys. J.* 87, 1981–1990.
- (10) Evans, E., and Ritchie, K. (1997) Dynamic strength of molecular adhesion bonds. *Biophys. J.* 72, 1541–1555.
- (11) Baumgartner, W., Hinterdorfer, P., Ness, W., Raab, A., Vestweber, D., Schindler, H., and Drenckhahn, D. (2000) Cadherin interaction probed by atomic force microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4005–4010.
- (12) Hukkanen, E., Wieland, J., Gewirth, A., Leckband, D., and Braatz, R. D. (2005) Multiple-bond kinetics from single-molecule pulling experiments: Evidence for multiple NCAM bonds. *Biophys. J.* 89, 3434–3445.
- (13) Nevo, R., Stroh, C., Kienberger, F., Kaftan, D., Brumfeld, V., Elbaum, M., Reich, Z., and Hinterdorfer, P. (2003) A molecular switch between alternative conformational states in the complex of Ran and importin β 1. *Nat. Struct. Biol.* 10, 553–557.
- (14) Wieland, J. A., Gewirth, A. A., and Leckband, D. E. (2005) Single molecule adhesion measurements reveal two homophilic neural cell adhesion molecule bonds with mechanically distinct properties. *J. Biol. Chem.* 280, 41037–41046.
- (15) Cross, B., Ronzon, F., Roux, B., and Rieu, J. P. (2005) Measurement of the anchorage force between GPI-anchored alkaline phosphatase and supported membranes by AFM force spectroscopy. *Langmuir* 21, 5149–5153.
- (16) Desmeules, P., Grandbois, M., Bondarenko, V. A., Yamazaki, A., and Salesse, C. (2002) Measurement of membrane binding between recoverin, a calcium-myristoyl switch protein, and lipid bilayers by AFM-based force spectroscopy. *Biophys. J.* 82, 3343–3350.
- (17) Touhami, A., Hoffmann, B., Vasella, A., Denis, F. A., and Dufrene, Y. F. (2003) Probing specific lectin-carbohydrate interactions using atomic force microscopy imaging and force measurements. *Langmuir* 19, 1745–1751.
- (18) Touhami, A., Jericho, M. H., and Beveridge, T. J. (2007) Molecular recognition forces between immunoglobulin G and a surface protein adhesion on living *staphylococcus aureus*. *Langmuir* 23, 2755–2760.
- (19) Cecchet, F., Lussis, L., Jérôme, C., Gabriel, S., Silva-Goncalves, E., Jérôme, R., and Duwez, A.-S. (2008) A generic chemical platform for molecular recognition and stimuli-responsive probes based on scanning probe microscopy. *Small* 4, 1101–1104.
- (20) Avci, R., Schweitzer, M., Boyd, R. D., Wittmeyer, J., Steele, A., Toporski, J., Beech, W., Arce, F. T., Spangler, B., Cole, K. M., and McKay, D. S. (2004) Comparison of antibody-antigen interactions on collagen measured by conventional immunological techniques and atomic force microscopy. *Langmuir* 20, 11053–11063.
- (21) Bonanni, B., Kamruzzahan, A. S. M., Bizzarri, A. R., Rankl, C., Gruber, H. J., Hinterdorfer, P., and Cannistraro, S. (2005) Single molecule recognition between cytochrome C 551 and gold-immobilized azurin by force spectroscopy. *Biophys. J.* 89, 2783–2791.
- (22) Chen, G., Ning, X., Park, B., Boons, G.-J., and Xu, B. (2009) Simple, clickable protocol for atomic force microscopy tip modification and its application for trace ricin detection by recognition imaging. *Langmuir* 25, 2860–2864.
- (23) Francius, G., Alsteens, D., Dupres, V., Lebeer, S., De Keersmaecker, S., Vanderleyden, J., Gruber, H. J., and Dufrene, Y. F. (2009) Stretching polysaccharides on live cells using single molecule force spectroscopy. *Nat. Protoc.* 4, 939–946.
- (24) Hinterdorfer, P., Baumgartner, W., Gruber, H. J., Schilcher, K., and Schindler, H. (1996) Detection and localization of individual antibody-antigen recognition events by atomic force microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3477–3481.
- (25) Kamruzzahan, A. S. M., Ebner, A., Wildling, L., Kienberger, F., Riener, C. K., Hahn, C. D., Pollheimer, P. D., Winklehner, P., Hölzl, M., Lackner, B., Schörkl, D. M., Hinterdorfer, P., and Gruber, H. J. (2006) Antibody linking to atomic force microscope tips via disulfide bond formation. *Bioconjugate Chem.* 17, 1473–1481.
- (26) Kienberger, F., Pastushenko, V. P., Kada, G., Gruber, H. J., Riener, C., Schindler, H., and Hinterdorfer, P. (2000) Static and dynamical properties of single poly(ethylene glycol) molecules investigated by force spectroscopy. *Single Mol.* 1, 123–128.
- (27) Langry, K. C., Ratto, T. V., Rudd, R. E., and McElfresh, M. W. (2005) The AFM measured force required to rupture the dithiolate linkage of thioctic acid to gold is less than the rupture force of a simple gold-alkyl thiolate bond. *Langmuir* 21, 12064–12067.
- (28) Lin, L., Wang, H., Liu, Y., Yan, H., and Lindsay, S. (2006) Recognition imaging with a DNA aptamer. *Biophys. J.* 90, 4236–4238.
- (29) Lower, B. H., Yongsunthon, R., Shi, L., Wildling, L., Gruber, H. J., Wigginton, N. S., Reardon, C. L., Pinchuk, G. E., Droubay, T. C., Boily, J.-F., and Lower, S. K. (2009) Antibody-recognition force microscopy shows that outer membrane cytochromes OmcA and MtrC are expressed on the exterior surface of *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* 75, 2931–2935.
- (30) Ratto, T. V., Langry, K. C., Rudd, R. E., Balhorn, R. L., Allen, M. J., and McElfresh, M. W. (2004) Force spectroscopy of the double-tethered concanavalin-A mannose bond. *Biophys. J.* 86, 2430–2437.

- (31) Ros, R., Schwesinger, F., Anselmetti, D., Kubon, M., Schafer, R., Pluckthun, A., and Tiefenauer, L. (1998) Antigen binding forces of individually addressed single-chain Fv antibody molecules. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7402–7405.
- (32) Puntheeranurak, T., Wimmer, B., Castaneda, F., Gruber, H. J., Hinterdorfer, P., and Kinne, R. K. H. (2007) Substrate specificity of sugar transport by rabbit SGLT1: Single-molecule atomic force microscopy versus transport studies. *Biochemistry* 46, 2797–2804.
- (33) Dorner, M. M., Bassett, E. W., Beiser, S. M., Kabat, E. A., and Tanenbaum, S. W. (1967) Studies on human antibodies: V. Amino acid composition of antidextrans of the same and of different specificities from several individuals. *J. Exp. Med.* 125, 823–831.
- (34) Van Dongen, S. F. M., Teeuwen, R. L. M., Nallani, M., van Berkel, S. S., Cornelissen, J. J. L. M., Nolte, R. J. M., and van Hest, J. C. M. (2009) Single-step azide introduction in proteins via an aqueous diazo transfer. *Bioconjugate Chem.* 20, 20–23.
- (35) Johnsson, B., Lofas, S., and Lindquist, G. (1991) Immobilization of proteins to a carboxymethyl-dextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal. Biochem.* 198, 268–277.
- (36) Lahiri, J., Isaacs, L., Tien, J., and Whitesides, G. M. (1999) A strategy for the generation of surfaces presenting ligands for studies of binding based on an active ester as a common reactive intermediate: A surface plasmon resonance study. *Anal. Chem.* 71, 777–790.
- (37) Hölzl, M., Tinazli, A., Leitner, C., Hahn, C. D., Lackner, B., Tampe, R., and Gruber, H. J. (2007) Protein-resistant self-assembled monolayers on gold with latent aldehyde functions. *Langmuir* 23, 5571–5577.
- (38) Ebner, A., Wildling, L., Kamruzzahan, A. S. M., Rankl, C., Wruss, J., Hahn, C. D., Hölzl, M., Kienberger, F., Blaas, D., Hinterdorfer, P., and Gruber, H. J. (2007) A new, simple method for linking of antibodies to atomic force microscopy tips. *Bioconjugate Chem.* 18, 1176–1184.
- (39) Alsteens, D., Dupres, V., McEvoy, K., Wildling, L., Gruber, H. J., and Dufrene, Y. F. (2008) Structure, cell wall elasticity and polysaccharide properties of living yeast cells, as probed by AFM. *Nanotechnology* 18, 384005.
- (40) Chtcheglova, L. A., Waschke, J., Wildling, L., Drenckhahn, D., and Hinterdorfer, P. (2007) Nano-scale dynamic recognition imaging on vascular endothelial cells. *Biophys. J.* 93, L11–13.
- (41) Francius, G., Lebeer, S., Alsteens, D., Wildling, L., Gruber, H. J., Hols, P., De Keersmaecker, S., Vanderleyden, J., and Dufrene, Y. F. (2008) Detection, localization and conformational analysis of single polysaccharide molecules on live bacteria. *ACS Nano* 2, 1921–1929.
- (42) Kloss, F. R., Gassner, R., Preiner, J., Ebner, A., Larsson, K., Hachl, O., Tuli, T., Rasse, M., Moser, D., Laimer, K., Nickel, E. A., Laschober, G., Brunauer, R., Klima, G., Hinterdorfer, P., Steinmüller-Nethl, D., and Lepperdinger, G. (2008) The role of oxygen termination of nanocrystalline diamond on immobilisation of BMP-2 and subsequent bone formation. *Biomaterials* 29, 2433–2442.
- (43) Rankl, C., Kienberger, F., Wildling, L., Wruss, J., Gruber, H. J., Blaas, D., and Hinterdorfer, P. (2008) Multiple receptors involved in human rhinovirus attachment to live cells. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17778–17783.
- (44) Tang, J., Badelt-Lichtblau, H., Ebner, A., Preiner, J., Kraxberger, B., Gruber, H. J., Sleytr, U. B., Ilk, N., and Hinterdorfer, P. (2008) Fabrication of highly ordered gold nanoparticle arrays templated by crystalline lattices of bacterial S-layer protein. *ChemPhysChem* 9, 2317–2320.
- (45) Tang, J., Ebner, A., Ilk, N., Lichtblau, H., Huber, C., Zhu, R., Pum, D., Leitner, M., Pastushenko, V., Gruber, H. J., Sleytr, U. B., and Hinterdorfer, P. (2008) High-affinity tags fused to S-layer proteins probed by atomic force microscopy. *Langmuir* 24, 1324–1329.
- (46) Tang, J., Ebner, A., Kraxberger, B., Badelt-Lichtblau, H., Gruber, H. J., Sleytr, U. B., Ilk, N., and Hinterdorfer, P. (2001) Mapping short affinity tags on bacterial S-layer with an antibody. *ChemPhysChem* 11, 2323–2326.
- (47) Zhu, R., Ebner, A., Kastner, M., Preiner, J., Howorka, S., and Hinterdorfer, P. (2009) Topography and recognition imaging of protein-patterned surfaces generated by AFM nanolithography. *ChemPhysChem* 10, 1478–1481.
- (48) Zhu, R., Howorka, S., Proll, J., Kienberger, F., Preiner, J., Hesse, J., Ebner, A., Pastushenko, V. P., Gruber, H. J., and Hinterdorfer, P. (2010) Nanomechanical recognition measurements of individual DNA molecules reveal epigenetic methylation patterns. *Nat. Nanotechnol.* 5, 788–791.
- (49) Tang, J., Krajcikova, D., Zhu, R., Ebner, A., Cutting, S., Gruber, H. J., Barak, I., and Hinterdorfer, P. (2007) Atomic force microscopy imaging and single molecule recognition force spectroscopy of coat proteins on the surface of *Bacillus subtilis* spore. *J. Mol. Recognit.* 20, 483–489.
- (50) Riener, C. K., Kienberger, F., Hahn, C. D., Buchinger, G. M., Egwin, I. O. C., Haselgrübler, T., Ebner, A., Romanin, C., Klampfl, C., Lackner, B., Prinz, H., Blaas, D., Hinterdorfer, P., and Gruber, H. J. (2003) Heterobifunctional crosslinkers for tethering single ligand molecules to scanning probes. *Anal. Chim. Acta* 497, 101–114.
- (51) Riener, C. K., Stroh, C. M., Ebner, A., Klampfl, C., Gall, A. A., Romanin, C., Lyubchenko, Y. L., Hinterdorfer, P., and Gruber, H. J. (2003) Simple test system for single molecule recognition force microscopy. *Anal. Chim. Acta* 479, 59–75.
- (52) Ebner, A., Hinterdorfer, P., and Gruber, H. J. (2007) Comparison of different aminofunctionalization strategies for attachment of single antibodies to AFM cantilevers. *Ultramicroscopy* 107, 922–927.
- (53) Butt, H.-J., and Jaschke, M. (1995) Calculation of thermal noise in atomic force microscopy. *Nanotechnology* 6, 1–7.
- (54) Hutter, J. L., and Bechhoefer, J. (1993) Calibration of atomic-force microscope tips. *Rev. Sci. Instrum.* 64, 1868–1873.
- (55) Sun, J., Dong, Y., Cao, L., Wang, X., Wang, S., and Hu, Y. (2004) Highly efficient chemoselective deprotection of *O*,*O*-acetals and *O*,*O*-ketals catalyzed by molecular iodine in acetone. *J. Org. Chem.* 69, 8932–8934.
- (56) Baumgartner, W., Hinterdorfer, P., and Schindler, H. (2000) Data analysis of interaction forces measured with the atomic force microscope. *Ultramicroscopy* 82, 85–95.
- (57) Akiyama, Y., Nagasaki, Y., and Kataoka, K. (2004) Synthesis of heterotelechelic poly(ethylene glycol) derivatives having α -benzaldehyde and ω -pyridyl disulfide groups by ring opening polymerization of ethylene oxide using 4-(diethoxymethyl)benzyl alkoxide as a novel initiator. *Bioconjugate Chem.* 15, 424–427.
- (58) Ebner, A., Wildling, L., Zhu, R., Rankl, C., Haselgrübler, T., Hinterdorfer, P., and Gruber, H. J. (2008) Functionalization of probe tips and supports for single-molecule recognition force microscopy. In: *STM and AFM studies on (bio)molecular systems: Unraveling the nano-world* (Samori, B., Ed.) pp 29–76, Vol XIV, Springer, Berlin.
- (59) Urbankova, E., Voltchenko, A., Pohl, P., Jezek, P., and Pohl, E. E. (2003) Transport kinetics of uncoupling proteins. Analysis of UCP1 reconstituted in planar lipid bilayers. *J. Biol. Chem.* 278, 32497–32500.
- (60) Krauss, S., Zhang, C. Y., and Lowell, B. B. (2005) The mitochondrial uncoupling-protein homologues. *Nat. Rev. Mol. Cell Biol.* 6, 248–261.