Supporting Information to:

**Linking of Sensor Molecules with Amino Groups to Aminofunctionalized 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*

*hermann.gruber@jku.at

*Institute of Biophysics, J. Kepler University, Altenberger Str. 69, A-4040 Linz, Austria;

**Abbreviations Used in the Supporting Information Only.** DCM, dichloromethane; DMB, 4-(dimethoxymethyl)-benzoic acid; DMF, \(N,N\)-dimethylformamide; PEG\textsubscript{18} diamine, see Figure S3; RT, room temperature; SLS, sodium lauryl sulfate; TLC, thin layer chromatography; TSTU, \(N,N,N',N'\)-tetramethyl-(\(O\)-succinimidyl)-uronium tetrafluoroborate.

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PART 1

SYNTHESES OF CROSSLINKERS

Materials listed in the Supporting Information only. 4-Formylbenzoic acid and NHS were obtained from Aldrich. Ninhydrine was purchased from Merck. DCC and TSTU were obtained from Fluka. PEG\textsubscript{15} diamine was purchased from Polypure (Norway).

Thin layer chromatography. Merck plastic sheets (silica gel 60) with fluorescent indicator were used for TLC. Eluents I, II, and III contained 70 parts of chloroform, 30 parts of methanol, and 4 parts of concentrated ammonia, or water, or acetic acid, respectively. Eluent IV contained 8 parts of chloroform and 2 parts of methanol. Acetal-protected and unprotected benzaldehyde residues were selectively detected as dark spots on plates with fluorescent indicator when illuminating at 254 nm. Amino groups were specifically stained with ninhydrine (0.1% ninhydrine and 2% acetic acid in 1-butanol) and heating to 120°C. All other components were visualized in iodine vapor.

Figure S1. Synthesis of DMB (a, methanol/NH\textsubscript{4}Cl) and SDMB (b, DCC/NHS).

Synthesis of 4-(Dimethoxymethyl)-benzoic Acid (DMB). The product was synthesized as described [Roberts et al., 2003], with slight modifications. 4-Formylbenzoic acid (2 g, 13.3 mmol) was dissolved in dry methanol (40 mL, pre-dried with 3 Å molecular sieves) and NH\textsubscript{4}Cl (4 g, 74.8 mmol) was added. The reaction mixture was heated under reflux for 20 h. For maximization of the degree of acetalization, 3Å molecular sieves (~5 g) were added and the reaction mixture heated under reflux for an additional 2 h. The solid material was removed by filtration and the mixture was taken to dryness. The residue was dissolved in chloroform and the solution was filtered again. The solvent was removed and the product was recrystallized from n-hexane/chloroform (v/v, 3/1), filtered while still hot, and allowed to crystallize overnight (10 °C), yielding 1.83 g (9.33 mmol) product. According to TLC, the recrystallized product (R\textsubscript{f}\textsuperscript{IV} = 0.4) appeared to contain a small fraction educt (R\textsubscript{f}\textsuperscript{IV} = 0.2). However, no aldehyde signal was seen in the NMR spectrum. This finding suggests that the product was partially deprotected on the TLC plate. \textsuperscript{1}H-NMR (200 MHz, CDCl\textsubscript{3}) \(\delta\) (ppm): 3.36 (6H, s, CH\textsubscript{3}-O-) 5.48 (1H, s, -CH\textsubscript{-}(O-CH\textsubscript{3})\textsubscript{2}) 7.26 (s, trace of CHCl\textsubscript{3} in CDCl\textsubscript{3}) 7.58 (2H, d, J = 8.4 Hz, H3 and H5 of aromatic ring, next to the acetal function) 8.14 (2H, d, J = 8.4 Hz, H2 and H6 of the aromatic ring, next to the COOH group).
Synthesis of N-Succinimidyl 4-(dimethoxymethyl)-benzoate (SDMB). DMB (1.33 g, 6.8 mmol) and hydroxysuccinimide (806 mg, 7 mmol) were dissolved in THF (25 mL, dried over basic Al₂O₃) and the solution was cooled using an ice bath. A solution of DCC (1.40 g, 6.8 mmol) in THF (10 mL) was added drop wise under argon while stirring. After 2 hours the ice-bath was removed and the reaction mixture was allowed to react overnight at RT. The supernatant was filtered and taken to dryness. The oily residue was dissolved in DCM, filtered, taken to dryness, and recrystallized from 2-propanol to yield 1.34 g (4.6 mmol) product which was pure by TLC (R_f = 0.83), except for a trace of free acid (R_f = 0.4). ¹H-NMR (200 MHz, CDCl₃) δ (ppm): 2.90 (4H, s, NHS) 3.32 (6H, s, C₆H₃-O-) 5.46 (1H, s, -CH=(O-CH₃)₂) 7.26 (s, trace of C₆HCl₃ in CDCl₃) 7.60 (2H, d, J = 8.4 Hz, H3 and H5 of aromatic ring, next to the acetal function) 8.13 (2H, d, J = 8.4 Hz, H2 and H6 of the aromatic ring, next to the COOH group).

Figure S2. Synthesis of acetal-PEG-NHS and aldehyde-PEG-NHS. a: SDMB/chloroform/TEA, b: DMF/pyridine/TSTU, c: citric acid/water, d: DMF/pyridine/TSTU. According to the manufacturer of NH₂-PEG-NH₂, the molecular mass of the PEG chain was 800 g/mol. Mass spectrometry revealed the chain length distribution was n = 18 ± 4 [51].
Synthesis of \( \alpha-[2-[4-(\text{Dimethoxymethyl})-\text{benzoyl}]-\text{aminopropyl}]\)-\( \omega \)-[2-[4-\text{carboxybutanoylamido}]-\text{propyl}]-\text{poly}(\text{oxyethylene})-800 \) (acetal-PEG-COOH). NH2-PEG-COOH (503.10 mg, 471.38 \( \mu \)mol) was dissolved in chloroform (6.5 mL) and SDMB (247.80 mg, 845.36 \( \mu \)mol, dissolved in a minimal volume of chloroform) was added, followed by addition of TEA (232 \( \mu \)l, 1.68 mmol). The progress of the reaction was monitored by TLC in eluent II. fractions were combined, taken to dryness and dried under vacuum over night, flow 0.4 mL/min). Fractions of 3.2 mL were collected and analyzed by TLC in Eluent II.

NMR (200 MHz, CDCl\( _3 \)) \( \delta \) (ppm): 1.04-1.36 (10 H, m, NH\( _2 \)-CH\( _2 \)-(CH\( _3 \))-CH\( _2 \)-O and NH\( _2 \)-CH\( <(\text{CH}_2\text{-CH}_3\text{-CH}_2\text{-O of PEG}_{800}) \) 1.92 (2 H, quin, J = 6.8 Hz, CO-CH\( _2 \)-CH\( _2 \)-CH\( _2 \)-CO, position \( \beta \)) in glu) 2.28 (2 H, t, J = 7 Hz, NH-CO-CH\( _2 \)-CH\( _2 \)-CH\( _2 \)-COOH, position \( \gamma \)) in glu) 2.38 (2 H, t, J = 6.7 Hz, NH-CO-CH\( _2 \)-CH\( _2 \)-CH\( _2 \)-COOH, position \( \alpha \)) in glu) 3.29 (6 H, s, \( \text{CH}_3 \)-O)-CH\( _2 \)-CO) 3.39-3.51 (6 H, m, N-CO-OH) 3.63 (72 H, s, O-COOH) 3.80 (3 H, s, CO-OH) 4.05 (3 H, s, NH) 7.26 (s, trace of C(\text{\text{H}}\text{O})_2\text{C}) 7.50 (2 H, d, J = 8.2 Hz, H3 and H5 of aromatic ring) 7.90 (2 H, d, J = 8.2 Hz, H2 and H6 of aromatic ring).

Synthesis of \( \alpha-[2-[4-(\text{Dimethoxymethyl})-\text{benzoyl}]-\text{aminopropyl}]\)-\( \omega \)-[2-[4-(\text{N-succinimidyl}oxycarbonyl}]-\text{butanoylamido}]-\text{propyl}]-\text{poly}(\text{oxyethylene})-800 \) (acetal-PEG-NHS). Acetal-PEG-COOH (570.3 mg, 468 \( \mu \)mol) was dissolved in DMF (6 mL) and pyridine (3 mL) and TSTU (216 mg, 717 \( \mu \)mol) was added. After 2 h of stirring at RT, the mixture was taken to dryness. The residue was dissolved in a mixture of chloroform (50 ml) and methanol (10 mL), stirred for 20 min, the solid material was removed by filtration, and additional chloroform (40 mL) was added. The solution was swirled with cold buffer A (100 mM NaCl, 50 mM NaH\( _2 \)PO\( _4 \), 1 mM EDTA, pH 7.5 adjusted with NaOH) for 2 min (2 \( \times \) 100 mL). It was washed with water (3 \( \times \) 40 mL), dried with as little Na\( _2 \)SO\( _4 \) as was required to clarify the solution, and filtered through paper. The organic solvents were removed and the residue dried at 1-10 Pa overnight yielding 510.9 mg (393.6 \( \mu \)mol) of product which was pure by TLC (iodine: \( R_t^I = 0.66, R_t^{II} = 0.68, R_t^{III} = 0.65 \)). \( 1^H \) NMR (200 MHz, CDCl\( _3 \)) \( \delta \) (ppm): 1.04-1.36 (10 H, m, NH\( _2 \)-CH\( _2 \)-(CH\( _3 \))-CH\( _2 \)-O and NH\( _2 \)-CH\( <(\text{CH}_2\text{-CH}_3\text{-CH}_2\text{-O of PEG}_{800}) \) 2.08 (2 H, quin, J = 6.8 Hz, CO-CH\( _2 \)-CH\( _2 \)-CH\( _2 \)-CO, position \( \beta \)) in glutaryl residue) 2.28 (2 H, t, J = 6.6 Hz, NH-CO-CH\( _2 \)-CH\( _2 \)-CH\( _2 \)-COOH, position \( \gamma \)) in glutaryl residue) 2.68 (2 H, t, J = 6.8 Hz, NH-CO-CH\( _2 \)-CH\( _2 \)-CH\( _2 \)-COOH, position \( \alpha \)) in glutaryl residue) 2.85 (4 H, s, CO-CH\( _2 \)-CH\( _2 \)-CO of succinimidylyl group) 3.32 (6 H, s, \( \text{CH}_3 \)-OCH\( _2 \)-CH\( _2 \)-COH) 3.49-3.51 (6 H, m, N-CO-OH) 3.80 (3 H, s, CO-OH) 4.05 (3 H, s, NH) 7.26 (s, trace of C(\text{\text{H}}\text{O})_2\text{C}) 7.50 (2°H, d, J = 8.2 Hz, H3 and H5 of aromatic ring) 7.90 (2 H, d, J = 8.2 Hz, H2 and H6 of aromatic ring).
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Synthesis of $\alpha$-[2-[4-Formylbenzoyl]-aminopropyl]-ω-[2-[4-carboxybutanoylamido]-propyl]-poly(oxyethylene)-800 (aldehyde-PEG-COOH). Acetal-PEG-COOH (736.4 mg, 591 µmol) was dissolved in acetone (30 mL) under argon, iodine (30 mg) was added and the solution was allowed to stir for 10 min. For removal of iodine the acetone fraction was mixed with water (50 mL) and washed with toluene (3 x 50 mL). The aqueous layer was taken to dryness and the residue dried at 1-10 Pa overnight yielding 679.4 mg (580 µmol) of product which was pure by TLC (iodine: $R_f^I$ = 0.59). $^1$H NMR (200 MHz, CDCl$_3$) δ (ppm): 1.04-1.26 (10 H, m, NH$_2$-CH<(C$_3$H$_3$)-CH$_2$-O and NH$_2$-CH<(C$_2$H$_2$)-CH$_2$-O of PEG$_{800}$) 2.08 (2 H, t, J = 6.6 Hz, CO-CH$_2$-CH$_2$-CO, position β’ in glutaryl residue) 2.26 (2 H, t, J = 6.8 Hz, NH-CO-CH$_2$-CH$_2$-CO-NH, position γ’ in glutaryl residue) 2.68 (2 H, t, J = 7.0 Hz, NH-CO-CH$_2$-CH$_2$-CO-NH, position α’ in glutaryl residue) 2.85 (4 H, s, CO-CH$_2$-CH$_2$-CO of succinimidyl) 3.33-3.58 (6 H, m, N-CH<(CH$_3$)-CH$_2$-O and N-CH<(CH$_2$-CH$_3$)-CH$_2$-O of PEG$_{800}$) 3.66 (72 H, s, O-C$_2$H$_5$-C$_6$H$_5$-O, 18 ethylene glycol units) 3.95-4.15 (2 H, broad s, CO-NH-CH<(C)-CH$_2$-O of PEG$_{800}$) 7.26 (s, trace of CHCl$_3$ in CDCl$_3$) 7.9-8.3 (4 H, m, aromatic ring) 10.09 (1 H, s, aldehyde). As a more convenient alternative to iodine/aceton, the acetal function was hydrolyzed with 1% citric acid (in water, 10 min, room temperature) in a parallel batch, whereupon the product was extracted into chloroform. The chloroform solution was washed with brine, filtered, and taken to dryness, yielding pure aldehyde-PEG-COOH, as well.

Synthesis of $\alpha$-[2-[4-Formylbenzoyl]-aminopropyl]-ω-[2-[4-(N-succinimidylcarbonyl)-butanoylamido]-propyl]-poly(oxyethylene)-800 (aldehyde-PEG-NHS). The synthesis of aldehyde-PEG-NHS followed the same protocol as the synthesis of acetal-PEG-NHS (see above) yielding a viscous product which was pure by TLC (iodine: $R_f^I$ = 0.05, 0.68, $R_f^{II}$ = 0.70, $R_f^{III}$ = 0.66). $^1$H NMR (200 MHz, CDCl$_3$) δ (ppm): 1.04-1.26 (10 H, m, NH$_2$-CH<(CH$_3$)-CH$_2$-O and NH$_2$-CH<(CH$_2$-CH$_3$)-CH$_2$-O of PEG$_{800}$) 2.08 (2 H, t, J = 6.6 Hz, CO-CH$_2$-CH$_2$-CO-NH, position α’ in glutaryl residue) 2.68 (2 H, t, J = 7.0 Hz, NH-CO-CH$_2$-CH$_2$-CO-NH, position γ’ in glutaryl residue) 2.85 (4 H, s, CO-CH$_2$-CH$_2$-CO of succinimidyl) 3.33-3.58 (6 H, m, N-CH<(CH$_3$)-CH$_2$-O and N-CH<(CH$_2$-CH$_3$)-CH$_2$-O of PEG$_{800}$) 3.66 (72 H, s, O-CH$_2$-CH$_2$-O, 18 ethylene glycol units) 3.95-4.15 (2 H, broad s, CO-NH-CH<(C)-CH$_2$-O of PEG$_{800}$) 7.26 (s, trace of CHCl$_3$ in CDCl$_3$) 7.9-8.3 (4 H, m, aromatic ring) 10.09 (1 H, s, aldehyde).

Synthesis of $a$,$\omega$-bis-[2-[4-(Dimethoxymethyl)-benzoyl]-aminoethyl]-octadeca(oxyethylene) (acetal-PEG$_{18}$-acetal). PEG$_{18}$ diamine (466 mg, 0.52 mmol) was dissolved in 6 mL chloroform and stirred under argon atmosphere. SDMB (245 mg, 0.84 mmol) and TEA (230 µL, 1.65 mmol) were added, and stirring was continued at room temperature under argon atmosphere. After 2 h the mixture was taken to dryness at the
rotavap, the residue was dissolved in methanol (5 mL) and subjected to chromatography on Sephadex LH-20 in methanol (column dimensions 1.5 cm × 95 cm, flow 0.4 mL/min, fraction size 3.2 mL). Elution of the product was detected by TLC in eluent II. The fractions containing pure product were combined and taken to dryness, yielding 570 mg (0.45 mmol). 

$^1$H-NMR (200 MHz, CDCl$_3$) δ (ppm): 3.30 (12 H, s, CH$_3$-O-) 3.61 (80 H, broad s, PEG chain, including the two terminal O-CH$_2$-CH$_2$-NH- groups) 5.40 (2 H, s, -CH=$\text{O-}$CH$_3$)$_2$ 6.98 (2 H, broad s, NH) 7.48 (2 H, dd, J$_1$ = 8.2 Hz, J$_2$ = 1.4 Hz, H3 and H5 of both aromatic residues) 7.26 (s, trace of CHCl$_3$ in CDCl$_3$) 7.80 (2 H, dd, J$_1$ = 8.2 Hz, J$_2$ = 1.4 Hz, H2 and H6 of both aromatic residues).

**Figure S3.** Synthesis of "acetal-PEG$_{18}$-acetal" and kinetics of acetal cleavage at different pH values. (A) PEG$_{18}$ diamine was derivatized with SDMB on both amino functions (see above). (B) The time course of acetal cleavage at a given pH value was measured by dissolving 100 mg of acetal-PEG$_{18}$-acetal in 10 mL aqueous buffer (prepared by adjusting 5% citric acid to the specified pH with NaOH) and removing 1 mL aliquots at defined time intervals. The PEG components of the aliquot were extracted into chloroform. The chloroform solution was dried with Na$_2$SO$_4$, filtered, and taken to dryness and redissolved in CDCl$_3$. The change in the acetal content was calculated from the decrease of the methoxy signal (3.30 ppm) relative to the PEG signal (3.61 ppm) in the $^1$H-NMR spectrum. The time courses were fit to monoexponential functions (lines in panel B) and the resulting half times are shown as a function of pH (panel C).
PART 2

KINETICS OF ACETAL CLEAVAGE BY ACID IN WATER

Figure S3 describes a series of experiments in which the pH dependence of acetal cleavage was studied under controlled conditions. The logic model compound for such a study would have been "acetal-PEG-COOH" (see Figure S2). Unfortunately, large amounts of this valuable compound would have been destroyed in the five time series at different pH values (Figure S3B), therefore "acetal-PEG\textsubscript{18}-acetal" was prepared as a cheaper substitute (Figure S3A). Five different citric acid buffers were prepared by 5\% citric acid (pH 1.9, see Figure S4) to pH 2, 3, 4, 5, or 6 with concentrated NaOH. For each time series, acetal-PEG-acetal was dissolved in the corresponding buffer to start the acetal cleavage process at the defined pH value. Aliquots were removed at different time intervals and the cleavage process was terminated by instantaneous partitioning of all PEG components into chloroform and the extent of acetal cleavage was calculated from the $^1\text{H}$-NMR spectrum (see legend to Figure S3). The time series data were fit to monoexponential decay functions (shown in a linearized form in Figure S3B) and the corresponding half times were plotted as a function of pH (Figure S3C).

It is interesting to compare the results obtained with acetal-PEG-acetal (Figure S3) with the kinetics of acetal cleavage observed for free acetal-PEG-COOH (Figure S2) in 1\% citric acid. According to Figure S4, an aqueous solution of citric acid (1\%) has a pH value of 2.2. When synthesizing aldehyde-PEG-COOH from acetal-PEG-COOH (Figure S2), we regularly performed acetal cleavage by 10 min treatment in 1\% citric acid. The $^1\text{H}$-NMR spectrum of the resulting product was completely free from any trace of the methoxy signal (3.30 ppm, in spite of the fact that this 6-proton singlet is particularly sharp and intense. Consequently, we estimate that treatment with 1\% citric acid cleave at least 99.6\% of the acetal functions within 10 min. In a monoexponential time course, 99.6\% turnover is reached after 8 half times. Consequently, a maximal half time of $10/8 = 1.25$ min is calculated for the 10 min treatment of acetal-PEG-COOH. This estimate agrees reasonably well with the half time of 1.6 min interpolated in Figure S3C for cleavage of acetal-PEG\textsubscript{18}-acetal in 5\% citric acid (adjusted to pH 2.2 with NaOH). The minor discrepancy may relate to slight differences in the buffer systems and in the molecular structures.
Figure S4. Dependence of pH on the concentration of unbuffered citric acid or acetic acid in pure water.
**PART 3**

**ILLUSTRATIONS OF TIP / CHIP FUNCTIONALIZATION WITH ACETAL-PEG-NHS**

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**Figure S5.** Scheme for tethering of proteins to AFM tips, using of acetal-PEG-NHS as heterobifunctional crosslinker. a) Aminofunctionalization of silicon nitride tip with ethanolamine hydrochloride in DMSO or with APTES in the gas phase. b) Amide bond formation in chloroform with TEA (0.5%). c) Deprotection with 0.1% iodine in acetone (5 min, iodine method) or with 1% citric acid in water (1-15 min, citric acid method). d) Schiff base formation with the lysine residue of a protein (e.g. 0.2 mg/mL antibody) in PBS (pH 7.4). e) Schiff base reduction with NaCNBH$_3$ (20 mM). In practice, step d and e are performed simultaneously since NaCNBH$_3$ is applied together with the antibody (1 h at RT). f) Blocking of unused benzaldehyde functions on the AFM tip by addition of 0.05 volumes of ethanolamine hydrochloride solution (1 M, pH 9.6 pre-adjusted with NaOH) or 0.05 volumes of glycine solution (1 M, pH 8.0 pre-adjusted with NaOH) and incubation for 5 min.
Figure S6. Schematic representation of chip functionalization and characterization. (a) Silicon nitride chips (A) were aminofunctionalized, either with ethanolamine hydrochloride in DMSO or with APTES in the gas phase (B). (b) Aminofunctionalized silicon nitride chips were reacted with acetal-PEG-NHS, resulting in amide-bond linked PEG chains with an acetal function (i.e., a protected aldehyde) on their free end (C). (c) Conversion of the acetal function into an aldehyde function (D) by use of iodine/acetone (0.1%, 5 min) or citric acid/water (1%, 15 min or 1 min, as specified), as stated for particular data. (d) Coupling of protein (biotin-IgG) by Schiff base formation with a lysine residue and concomitant fixation of the C=N double bond by reduction with NaCNBH₃ gives a stable C-N single bond (E). Continued on the next page.
Figure S6 (continued from the previous page). (e) Control experiment for non-specific adsorption of ExtrAvidin®-peroxidase (EAP) to chips carrying biotin-IgG covalently bound via PEG linkers. The biotin-binding sites of EAP were pre-blocked by a large excess of free d-biotin, thus EAP cannot bind to immobilized biotin-IgG (F) and only nonspecific adsorption of EAP to the chip can occur. The amount of adsorbed EAP is estimated from the rate of color production, as explained below. (f) In the absence of free d-biotin, EAP is bound to the chip both by nonspecific adsorption (not shown) and by specific interaction (stage G). After removal of unbound EAP by washing, a chromogenic substrate is added and the amount of bound enzyme is calculated from the rate of color production [38, 51].
PART 4

TIP FUNCTIONALIZATION WITH ALDEHYDE-PEG-NHS AND EDA-ATP

Magnetically coated cantilevers (MAC levers, Agilent) were functionalized with the ethylene diamine-derivative of ATP (EDA-ATP, 2’/-3’-O-(2-aminoethylcarbamoyl)-adenosine-5’-O-triphosphate, BioLog) by a well established three-step procedure: (i) aminofunctionalization, (ii) attachment of the heterobifunctional polyethylene glycol linker (aldehyde-PEG-NHS, synthesized by the new synthesis route Figure S2acd), and (iii) reaction of "tip-PEG-aldehyde" with the amino group of EDA-ATP, using NaCNBH₃ for reduction (fixation) of the initially formed Schiff base conjugate (compare steps d and e in Figure S5).

Commercial MAC® levers were cleaned with chloroform and aminofunctionalized with APTES in the gas phase as described previously [51]. For attachment of aldehyde-PEG-NHS, the cantilevers were put in the solution of the crosslinker in chloroform at a concentration of 6.6 mg/ml. TEA at final concentration of 0.5% was added to deprotonate the amino functions on the tip and ensure amide bond formation. After about 1.5 hours of reaction, the cantilevers were washed in chloroform (3 ×) and dried in air.

Fifty µL aliquots of the commercial EDA-ATP solution (10 mM, in water, from BioLog) were pipetted into Eppendorf reaction vials, and frozen in liquid nitrogen. The vials were placed in a pre-cooled round-bottomed flask which was attached to a cold trap and evacuated at 1-10 Pa, in order to evaporate the water content in the frozen state. For coupling to “tip-PEG-aldehyde”, one dried portion of EDA-ATP was redissolved in 9 µL of buffer B (1 mM EDTA and 300 mM NaH₂PO₄, pH adjusted to 7.5 with NaOH) and 1 µL of a 0.2 M solution of NaCNBH₃ (freshly prepared by dissolving 32 mg NaCNBH₃ in a mixture of 50 µL 100 mM NaOH plus 450 µL water, and diluting with 2 mL buffer B) was added. After mixing, this solution was transferred onto a sheet of clean Parafilm in a polystyrene Petri dish. Several cantilevers were immersed in the 10 µL droplet with their tips and incubated for 1 h. Subsequent deactivation of residual aldehyde functions on the tip was performed by addition of 0.5 µl 1 M ethanolamine hydrochloride solution (pre-adjusted to pH 9.6 with NaOH and stored in small aliquots at -25°C) and incubation for 10 min. The tips were washed 3 times and stored in buffer B treated with argon at 4°C.

The addition of 0.5 µL (0.05 vol) 1 M ethanolamine (pH 9.6) to 10 µL buffer B caused little change in pH. This was demonstrated by mixing 5 mL buffer B with 0.25 mL (0.05 vol) 1 M ethanolamine (pH 9.6) whereby the final pH rose from 7.5 to 7.7. The small extent of the pH change is due to the high phosphate concentration (300 mM) of buffer B. In contrast, regular buffer A does not have sufficient buffering capacity, as shown by mixing of 5 mL buffer A (50 mM phosphate only!) with 0.25 mL (0.05 vol) 1 M ethanolamine (pH 9.6) which gave a final pH of 9.2.
PART 5

RECONSTITUTION OF UCP1 IN PLANAR BILAYER
AND RECOGNITION IMAGING WITH ATP-FUNCTIONALIZED TIPS

The coding region of murine Uncoupling protein 1 (UCP1) was amplified by PCR from a mouse EST clone and cloned into the expression vector pET-24a (Novagen, Germany), as described in [Rupprecht et al., 2010]. For protein expression E. coli strain Rosetta (DE3) (Novagen, Germany) was transformed. Inclusion body purification was performed according to the procedures, described previously [Smorodchenko et al., 2009; Rupprecht et al., 2010]. In brief, cells were disrupted by French press and centrifuged to eliminate cell debris. The inclusion bodies were obtained after supernatant centrifugation at 14000 g for 10 min. 2.5 mg protein from inclusion bodies per 100 mg lipid was used for the subsequent UCP1 purification. The inclusion bodies were washed in TRIS/glycerol buffer (100 mM Tris base, 5 mM EDTA, 10% glycerol, pH 7.5) with additional 2 % Triton X-100 first and then with 0.1 % sodium lauryl sulfate (SLS). For solubilization, the inclusion bodies were incubated at room temperature in TRIS/glycerol buffer with 2 % SLS and 1 mM DTT. After centrifugation the supernatant was incubated overnight with the mixture of 100 mg E. coli polar lipid (Avanti Polar Lipids, USA), 400 mg Triton X-114, 100 mg octyl-polyoxyethylene, 1 mM DTT and 1 mM ATP, then concentrated with Amicon Ultra-15 filters (Millipore, Germany) and dialyzed for two hours against TRIS/glycerol buffer supplemented with 1 mg/mL BSA and 1 mM DTT. Two further dialysis cycles were performed without DTT for a minimum of 12 h. Three final dialyses were completed with the assay buffer (see below).

Aggregated proteins were eliminated by centrifugation of the dialysate at 14000 g for 10 min. To eliminate incorrectly folded proteins, the supernatant was run over a column containing 1 g hydroxyapatite (Bio-Rad, Germany) [Lin & Klingenberg, 1980; Lin & Klingenberg, 1982]. For removal of non-ionic detergent, the sample was incubated with Bio-Beads SM-2 (Bio-Rad, Germany) [Rigaud et al., 1995]. The protein content of the obtained proteoliposomes was measured by Micro BCA Protein Assay (Perbio Science, Germany).

The AFM sample plate (Agilent Technologies, USA) with the coil for magnetic field application was covered with a small piece of aluminum foil, the centre of which was marked with a small dot to indicate the cantilever position for the charge-coupled device (CCD) camera. Freshly cleaved mica was placed on the aluminum film and mounted with a flow-through fluid cell. Ten µl stock solution of UCP1 proteoliposomes (with a lipid concentration of 4-5 mg/mL and a protein/lipid ratio of 0.02-0.03) was diluted with 240 µL of assay buffer (50 mM Na₂SO₄, 20 mM MES, 20 mM TRIS, 0.6 mM EDTA, pH 7.2). After short vortexing, the solution was injected into the fluid cell on the mica surface. After incubation for 8 min, the mica surface was thoroughly washed with buffer. Finally, 600 µL buffer was left in the fluid cell for measurements.
Mac-mode AFM imaging was performed using a PicoPlus 5500 AFM (Agilent). Recognition images [8, 9] were simultaneously recorded using the ATP-functionalized cantilever, which was driven by an alternating magnetic field at its resonant frequency. Images were recorded at an amplitude set-point of 2.3-8.4 nm, and with a ratio of 88-97 % to the amplitude observed before the cantilever touched the sample surface. The scanning speed for imaging was 2-4 µm/s. Experiments on the blocking of the recognition were conducted by injecting free ATP solution into the measurement solution at a final concentration of 4.8 mM. Both topography and recognition images were displayed after leveling by mean plane subtraction and scanning line correction (Gwyddion 2.9).

PART 6

EXPLANATION OF SIMULTANEOUS TOPOGRAPHY AND RECOGNITION IMAGING WITH MAGNETICALLY COATED AFM CANTILEVERS.

Topography and recognition imaging (TREC) is based on tapping mode AFM. A magnetic coil mounted either above or below the magnetically coated cantilever generates a sinusoidal oscillation of the cantilever when using a driving frequency which is adjusted to ~0.5 kHz below the resonance frequency of the cantilever. This allows for gentle and noninvasive scanning of the surface which is a prerequisite when using ligand-functionalized cantilevers for imaging purposes. In contrast to the conventional magnetical tapping mode (MAC®-Mode, Agilent), recognition imaging not only yields sample topography but also a simultaneously acquired map of recognition sites. For this purpose, the lower and the upper parts of the cantilever oscillation trace are separated by an electronic circuit which is termed the TREC box [8, 9] (see Figure S7A). The lower parts are only affected by the sample topography and used for the piezo feedback loop, resulting in the topographical image. In the case of a complex formation of a tip-bound ligand with a surface-embedded receptor, changes of the upper parts of the amplitude become observable. An important prerequisite is the stable tethering of the ligand to the tip with a distensible polymer linker. The chain length is typically chosen to be 3-9 nm and the working amplitude of cantilever oscillation is kept smaller than the sum of linker chain length plus the height of the receptor-ligand complex, thereby avoiding a forced rupture of the receptor-ligand complex while scanning above the receptor site [Preiner et al., 2009] A typical example is shown in Figure S7. [Ebner et al., 2005]. Individual avidin molecules were adsorbed to mica and the surface was scanned with a tip carrying a biotin residue via a 6 nm long PEG linker. The height of the avidin molecules resulted in a damping of the lower parts of the amplitude used for driving the piezo feedback loop (Figure S7A, blue trace). In parallel, the formation of an avidin-biotin complex while scanning over avidin hinders the upwards movement in the oscillation and results in a ~1nm lowered upper peak amplitude when using a peak-to-peak amplitude that is smaller than the length of the PEG chain (~6 nm) plus the height of avidin (~4 nm). Changes in the upper part
of the amplitude can directly be translated in recognition signals. As a result TREC allows for mapping of recognition sites simultaneously with gentle imaging of the sample surface, both at high resolution and a fast lateral scan rate.

Figure S7. TREC imaging (Ebner et al. 2005). (A) Principle: The cantilever oscillation is split into lower and upper parts, resulting in simultaneously acquired topography and recognition images. (B) Avidin was electrostatically adsorbed to mica and imaged with a biotin-tethered tip. A good correlation between topography (left image, bright spots) and recognition (right image, dark spots) was found (exemplified by the three solid circles). Topographical spots without recognition indicate structures lacking specific interaction (dashed circle). The scan size was 500 nm × 500 nm. Reproduced with permission from [Ebner et al., 2005].
PART 7

LITERATURE CITED in the Supporting Information only

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