Supporting Information to

Antibody Linking to AFM Tips via Disulfide Bond Formation

A. S. M. Kamruzzahan, Andreas Ebner, Linda Wildling, Ferry Kienberger, Christian K. Riener, Christoph D. Hahn, Philipp D. Pollheimer, Peter Winklehner, Martin Hölzl, Bernd Lackner[§], Daniela M. Schörkl, Peter Hinterdorfer, and Hermann J. Gruber*

Institute of Biophysics and [§]Institute of Organic Chemistry, J. Kepler University, Altenberger Str. 69, A-4040 Linz, Austria;

*To whom correspondence should be addressed at the Institute of Biophysics in Linz. Phone.: +43 (732) 2468-9271. Fax: +43 (732) 2468-9270, e-mail: hermann.gruber@jku.at

[1]**Abbreviations:**

AFM, atomic force microscope (or microscopy); APTES, 3-aminopropyltriethoxysilane; biotin-cap-COOH, 6-(biotinoylamino)-hexanoic acid; biotin-cap-NHS, *N*-succinimidyl ester of biotin-cap-COOH; biotin-IgG, goat IgG derivatized with biotin-cap-NHS; biotin-IgG-SATP, goat IgG derivatized with both biotin-cap-NHS and SATP (see Figure 10S in this Supporting Information); biotin-NHS, succinimidyl ester of *d*-biotin; biotin-PEG-NHS, analogous to PDP-PEG-NHS in Figure 2, with biotin in place of the PDP group; BSA, bovine serum albumin; DACA, *p*-dimethylaminocinnamaldehyde; DCC, *N,N′*-dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropyl-*N*-ethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; 2,2'-DTDP, 2,2'-dithiodipyridine; 4,4'-DTDP, 4,4'-dithiodipyridine; DTT, 1,4-dithiothreitol; EAP, ExtrAvidin®-peroxidase; EDTA, ethylene diamine-*N,N,N′,N′*-tetraacetic acid; IgG, immunoglobulin G; NH_2-PEG-NH_2, *O,O′-bis*(2-aminopropyl)-poly(ethylene glycol) 800; NH_2-PEG-COOH, mono-*N*-glutaryl derivative of NH_2-PEG-NH_2; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; NTA, nitrilotriacetate; OPD, *o*-phenylenediamine·2HCl; PBS, phosphate-buffered saline; PBT, PBS with 0.5% Tween®-20; PDP-, 3-(2-pyridyldithio)-propionyl group; PDP-OH, 3-(2-pyridyldithio)-propionic acid; PDP-PEG-BSA, BSA derivatized with ~1 PDP-PEG- per BSA; PDP-PEG-COOH and PDP-PEG-NHS, see Figure 2; PEG, poly(ethylene glycol); Pyr-S-S-PEG-NHS, see Figure 2; RT, room temperature; SATP, *N*-succinimidyl 3-(acetylthio)-propionate; SPDP, *O*-succinimidyl 3-(2-pyridyl)-dithiopropionate; SMRFM, single molecule recognition force microscope (or: microscopy); SMRFS, single molecule recognition force spectroscopy; TEA, *N,N,N*-triethylamine; THF, tetrahydrofuran; TLC, thin layer chromatography; Tris, *tris*-(hydroxymethyl)-aminomethane; 2-TP, 2-thiopyridone; 4-TP, 4-thiopyridone; TSTU, *N,N,N′,N′*-tetramethyl-*O*-(*N*-succinimidyl)-uronium tetrafluoroborate.

EXPERIMENTAL PROCEDURES IN THE SUPPORTING INFORMATION

Materials. Analytical grade materials were used as long as they were commercially available. The starting materials were of highest available purity. For synthesis of NH_2-PEG-COOH[1], *O,O′-bis*(2-aminopropyl)-poly(ethylene glycol) 800 was obtained from Fluka. Muscovite mica sheets were supplied by Christine Gröpl Elektronen-mikroskopie (Tulln, Austria). Si_3N_4 measuring tips were bought from Veeco Instruments (Dourdan, France) and Si_3N_4 chips from Wacker-Chemitronic GmbH (Germany). Pyr-S-S-PEG-NHS was purchased from Polypure (Oslo, Norway).

Buffers and aqueous reagents. Buffer A contained 100 mM NaCl, 50 mM NaH_2PO_4, and 1 mM EDTA (pH 7.5 adjusted with NaOH).

Buffer B contained 600 mM NaH_2PO_4 (pH adjusted to 6.0 with NaOH). Buffer C contained 100 mM NaH_2PO_4 (pH adjusted to 6.0 with NaOH). Buffer D contained 100 mM NaCl and 35 mM boric acid (pH 8.6 adjusted with NaOH). Buffer E contained 50 mM citric acid (pH adjusted to 5.5 with NaOH). Buffer F contained 0.1 M phosphoric acid (pH adjusted to 2.0 with NaOH). Buffer G contained 100 mM NaCl and 20 mM sodium acetate (pH adjusted to 4.5 with HCl). PBS contained 150 mM NaCl and 5 mM NaH_2PO_4 (pH 7.4 adjusted with NaOH). PBT was prepared by dissolving Tween®-20 at a final concentration of 0.5% in PBS and re-adjusting the pH to 7.5.

Hydroxylamine reagent (500 mM hydroxylamine·HCl, 25 mM EDTA, pH 7.5) was prepared by dissolving 1.74 g hydroxylamine hydrochloride, 0.355 g anhydrous Na_2HPO_4, and 0.466 g $EDTANa_2{\cdot}2H_2O$ in 40 mL of water, adjusting the pH to 7.5 with NaOH and addition of water to obtain a final volume of 50 mL. Aliquots of the hydroxylamine reagent were stored at -25°C.

A 100 mM *d*-biotin stock solution (24.4 mg/mL, for blocking of EAP) was prepared by suspending *d*-biotin in buffer A and re-adjusting the pH to 7.5 with NaOH with stirring, whereupon *d*-biotin was dissolved. Aliquots were stored frozen at –25°C. A 10 mg/mL *d*-biotin stock solution (for blocking of avidin-biotin recognition in the AFM) was prepared by diluting 100 µL of 100 mM *d*-biotin stock solution with 144 µL of buffer A.

Unblocked EAP reagent was prepared by diluting 50 µL of commercial EAP solution with 950 µL of PBT. Blocked EAP reagent was prepared in the same manner but including 10 µL of 100 mM *d*-biotin in addition.

Solid OPD was stored in small aliquots together with blue gel at –70°C (*Caution:* OPD is highly carcinogenic). OPD reagent was always freshly prepared by dissolving solid OPD in buffer E at a final concentration of 0.8 mg/mL.

Thin layer chromatography. Merck plastic sheets (silica gel 60) with fluorescent indicator were used. Eluents I, II, and III contained 70 parts of chloroform, 30 parts of methanol, and 4 parts of conc. ammonia, or water, or acetic acid, respectively. Amino groups were specifically stained with ninhydrin reagent (0.1% ninhydrin and 2% acetic acid in butanol) and heating to 120°C, biotin residues were specifically stained with DACA reagent (equal volumes of 2% sulfuric acid in ethanol and 0.2% DACA in ethanol were mixed immediately before use) and heating to 120°C [McCormick and Roth, 1970], all other components were visualized in iodine vapor. Eluent IV and V contained chloroform and methanol at a ratio of 80/20 and 90/10, respectively (v/v).

Synthesis of 2,2'-DTDP. 2-Thiopyridone (22.4 g, 200 mmol) was dissolved in water (600 mL) and cooled to ≤15°C but not below 10°C at which temperature precipitation was observed. The ice bath was removed and a mixture of 155 mL water and 155 mL 30% H_2O_2 was gradually added with stirring and stirring was continued for 30 min. At different times, a 200 µL aliquot of the reaction mixture was diluted with 20 mL of buffer C and insoluble product was removed by filtration through cotton. The UV-vis spectrum was recorded between 300 and 400 nm. The peak at 343 nm reflected unreacted 2-thiopyridone (ε_{343} = 8080 M^{-1} cm^{-1}) (*31*). When 2-thiopyridone was no longer detectable, the solution was cooled to ~3°C, the precipitated product was collected by filtration and washed with 50 mL of ice-cold water. Drying at 1-10 Pa overnight gave 19.2 g of 2,2'-DTDP (87 mmol, 87% of theory) which was pure by TLC (R_f^{I} = 0.87, R_f^{II} = 0.80, R_f^{III} = 0.86, R_f^{V} = 0.69) which was distinctively different from the educt (R_f^{I} = 0.71, R_f^{II} = 0.71, R_f^{III} = 0.76, R_f^{V} = 0.51).

^{1}H-NMR (200 MHz, $CDCl_3$) δ (ppm): 7.08-7.15 (2H, m, *H5* and *H5′*) 7.60-7.64 (4H, m, *H3*, *H3′*, *H4*, and *H4′*) 8.45-8.48 (2H, d, *J* = 5.0 Hz, *H6* and *H6′*).

Synthesis of PDP-OH. In a 500 mL 3-necked flask, 2,2'-DTDP (15 g, 68 mmol) was dissolved in 100 mL of ethanol. Subsequently, 20 mL of 37% HCl and 100 mL of water were slowly added with stirring. The flask and the attached dropping funnel were flushed with argon. Ethanol (20 mL) and 3-mercaptopropionic acid (4.74 mL, 55 mmol) were mixed in the dropping funnel under argon and this solution was slowly added with vigorous stirring. After 20 and 40 min, a 10 µL aliquot was withdrawn and mixed with 20 mL of buffer C and filtered from insoluble material. The reaction was found to be complete after 20 min already, as judged from the absorbance at 343 nm

(0.9 cm^{-1}) which was the same at both times and close to the expected value for complete turnover (0.85 cm^{-1}) calculated from the known molar absorptivity of 2-thiopyridone (ε_{343} = 8080 M^{-1} cm^{-1}) (*31*). The volume was reduced by a factor of ~4 on the rotavap at 20°C water bath temperature. The solution was transferred into a glass beaker and stirred in a hood. Chloroform (50 mL) was added and the pH was raised to ~7.5 (examined by different kinds of pH paper) by gradual addition of 15% Na_2CO_3.

For re-oxidation of 2-thiopyridone into 2,2'-DTDP, 8.7 mL of 30% H_2O_2 was added dropwise with stirring. The completeness of the reaction was examined by withdrawing aliquots (3, 30, and 300 µL) from the aqueous layer, mixing with 3 mL of buffer B each, and measuring the UV-vis absorption spectrum. Absence of the 2-thiopyridone peak at 343 nm indicated completeness of re-oxidation.

The mixture was transferred into a separatory funnel, the organic layer was collected and the aqueous layer was extracted with chloroform (5 × 20 mL). The combined chloroform phases were taken to dryness, yielding pure 2,2'-DTDP which was re-used in subsequent batches. The aqueous phase was transferred into a new beaker and the pH was lowered to 2.5-3.0 at which point part of the product PDP-OH precipitated and was extracted into chloroform (5 × 50 mL). The chloroform phase was treated with 5 g of sodium sulfate to obtain a clear solution and filtered. The filtrate was taken to dryness, yielding crude PDP-OH which contained 3,3'-dithiodipropionic acid as byproduct.

Crude PDP-OH was purified by anion exchange chromatography on QAE Sephadex® A-25 (5 × 20 cm bed volume, chloride form). PDP-OH (1.94 g, 9 mmol, partially purified by extraction at pH 8 and pH 3, see above) was suspended in water (30 mL), and the pH was adjusted to 7.5 with 15% Na_2CO_3 whereupon all solid was dissolved. Water was added to obtain a final volume of 200 mL and the solution was loaded on the column at 4 mL/min. After washing with water (600 mL), 250 mM NaCl (600 mL), and 500 mM NaCl (600 mL), the product was eluted with 1 M NaCl while collecting 10 mL fractions. Product was found in fractions #15-45, as detected by TLC in eluent IV. These fractions were pooled and concentrated to 50 mL by rotary evaporation. The pH was adjusted to ~2.5 with 1 M phosphoric acid and the solution was extracted with chloroform (5 × 50 mL). The organic phase was treated with the minimum of sodium sulfate to get a clear solution and filtered. The filtrate was taken to dryness and dried at 1-10 Pa for 2 h. The product (1.13 g) was pure by TLC in eluent IV.

As an alternative, crude PDP-OH (0.68 g) was purified by flash chromatography on Silica 60 (50 g, 30 mm column diameter, flow ~30 mL/min), using dichloromethane / methanol (95 / 5) as eluent (1.5 L for column packing and elution). 60 mL fractions were collected and analyzed by TLC in eluent II. The product (0.59 g) contained traces of 2,2'-DTDP and 3-mercaptopropionic acid which were only visible on an overloaded TLC.

^{1}H-NMR (200 MHz, $CDCl_3$) δ (ppm): 2.75 (2H, t, *J* = 6.7 Hz, C*H*$_2$-COOH) 3.05 (2H, t, J = 6.7 Hz, S-S-C*H*$_2$) 7.15-7.22 (1H, m, *H5* in pyridine) 7.55–7.75 (2H, m, *H3* and *H4* in pyridine) 8.47 (1H, d, 4.8 Hz, *H6* in pyridine).

Synthesis of SPDP. PDP-OH (1 g, 4.7 mmol) and NHS (0.802 g, 7.0 mmol) was added to dry THF (15 mL) and stirred at RT until NHS was dissolved. The solution was cooled to 0°C and DCC (1.44 g, 7.0 mmol) was added. Precipitation of dicyclohexylurea was observed. The mixture was stirred for 1 h at 0°C and overnight at RT. The precipitate was removed by filtration and the filtrate taken to dryness. The solid residue was dissolved in 90% ethanol at 40°C, cooled in an ice bath, kept at 4°C for 3 h and then at –20°C for 3 days. The white crystals were collected by filtration, washed with diisopropyl ether, and dried at 1-10 Pa overnight. The product (1.15 g, 3.7 mmol) was pure by TLC (R_f^{II} = 0.83, R_f^{III} = 0.87).

^{1}H-NMR (200 MHz, $CDCl_3$) δ (ppm): 2.84 (4H, s, *N*-hydroxysuccinimide residue) 3.0-3.3 (4H, m, S-S-C*H*$_2$-C*H*$_2$-COOH) 7.12 (1H, dd, J_1 = 8.5 Hz, J_2 = 4.8 Hz, *H5* in pyridine) 7.60–7.75 (2H, m, *H3* and *H4* in pyridine) 8.51 (1H, d, J = 4.7 Hz, *H6* in pyridine).

Synthesis of PDP-PEG-COOH. NH_2-PEG-COOH (416 mg, 400 µmol) was dissolved in chloroform (4.5 mL) and SPDP (184 mg, 590 µmol) was added under argon atmosphere. The reaction was started by addition of TEA (180 µL, 1290 µmol) and monitored by TLC in

eluents I and II. After 2 h, all ninhydrin-positive educt had disappeared and the reaction mixture was taken to dryness. The solid residue was treated with 200 mL of water (pH pre-adjusted to 2 with dilute phosphoric acid) and insoluble material was removed by centrifugation in Falcon tubes (10 min, 4000 RPM, 1800 g_{max}). The supernatant was filtered (0.45 µm) and the pH of the filtrate was raised to 7.7 with triethanolamine base. After washing with ethyl acetate (3 × 120 mL), the aqueous layer was stirred under argon atmosphere for 3 h at RT to hydrolyze residual unreacted SPDP. The aqueous solution was extracted with chloroform (5 × 100 mL) and the combined chloroform layer was washed with buffer F (3 × 120 mL), dried with sodium sulfate, filtered, and taken to dryness. The residue was dried at 1-10 Pa overnight, yielding 302 mg of PDP-PEG-COOH which was pure by TLC (R_f^{II} = 0.54).

^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 1.04-1.26 (10H, m, NH_2-CH<(C*H*$_3$)-CH_2-O and NH_2-CH<(C*H*$_2$-C*H*$_3$)-CH_2-O of the PEG termini which also contain 2-aminobutyl groups besides 2-aminopropyl) (*16*) 1.96 (2H, quin, *J* = 7.2 Hz, CO-CH_2-C*H*$_2$-CH_2-CO, β' position in glu) 2.23-2.48 (4H, m (2t), NH-CO-C*H*$_2$-CH_2-C*H*$_2$-CO-NH, position α' und γ' in glu) 2.56 (2H, t, *J* = 6.4 Hz, NH-CO-C*H*$_2$-CH_2-S-S) 3.06 (2H, t, *J* = 6.5 Hz, NH-CO-CH_2-C*H*$_2$-S-S) 3.33-3.58 (6H, m, N-C*H*<(CH_3)-C*H*$_2$-O in 2-aminopropyl termini of PEG chain, N-C*H*<(CH_2-CH_3)-C*H*$_2$-O in 2-aminobutyltermini of PEG chain) 3.66 (72H, s, O-C*H*$_2$-C*H*$_2$-O) 3.95-4.15 (2H, broad s, CO-N*H*-CH<(C)-CH_2-O, aminotermini of PEG chain) 7.12 (1H, m (dd), position *H5* in pyridine) 7.65 (2H, m (dt), position *H3* and *H4* of pyridine) 8.45 (1H, m (dd), position *H6* of pyridine).

Synthesis of PDP-PEG-NHS. PDP-PEG-COOH (179 mg, 145 µmol) was dissolved in DMF (1 mL) and pyridine (1 mL) was added while stirring under argon atmosphere. TSTU (67 mg, 222 µmol) was separately dissolved in DMF (1 mL) and added dropwise. After 2 h, the pyridine content of the mixture was removed by rotary evaporation without heating, the remaining DMF solution (together with a stir bar) was frozen in liquid nitrogen and attached to a nitrogen-cooled cold trap at 1-10 Pa. Stirring was resumed upon thawing whereupon DMF was removed. The residue was dissolved in a mixture of chloroform (50 mL) and methanol (10 mL) and insoluble material was removed by filtration. Chloroform (30 mL) was added and the solution was washed with buffer A (2 × 50 mL) with the minimal time per cycle (~2 min). The organic layer was washed with water (3 × 20 mL), dried with sodium sulfate, filtered, and taken to dryness. Drying at 1-10 Pa gave 146 mg of PDP-PEG-NHS which was pure by TLC (R_f^{II} = 0.67).

^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 1.04-1.26 (10H, m, NH_2-CH<(C*H*$_3$)-CH_2-O and NH_2-CH<(C*H*$_2$-C*H*$_3$)-CH_2-O, termini of PEG chain) (*16*) 1.96 (2H, quin, *J* = 7.2 Hz, CO-CH_2-C*H*$_2$-CH_2-CO, β' position in glu) 2.23-2.48 (4H, m (2t), NH-CO-C*H*$_2$-CH_2-C*H*$_2$-CO-NH, position α' und γ' in glu) 2.56 (2H, t, *J* = 6.4 Hz, NH-CO-C*H*$_2$-CH_2-S-S) 2.85 (4H, s, CO-C*H*$_2$-C*H*$_2$-CO in NHS residue) 3.06 (2H, t, *J* = 6.5 Hz, NH-CO-CH_2-C*H*$_2$-S-S) 3.33-3.58 (6H, m, N-C*H*<(CH_3)-C*H*$_2$-O, N-C*H*<(CH_2-CH_3)-C*H*$_2$-O, termini of PEG chain) (*16*) 3.66 (72H, s, O-C*H*$_2$-C*H*$_2$-O) 3.95-4.15 (2H, broad s, CO-N*H*-CH<(C)-CH_2-O, termini of PEG chain) (*16*) 7.12 (1H, m (dd), position *H5* in pyridine) 7.65 (2H, m (dt), position *H3* and *H4* in pyridine) 8.45 (1H, m (dd), position *H6* in pyridine).

Synthesis of Biotin-cap-COOH. Biotin-NHS was prepared as described [Wilchek and Bayer, 1990]. The product which still contained non-activated *d*-biotin was triturated with chloroform and the suspension was filtered. The solid remainder was treated in the same way several times. The combined filtrates were taken to dryness and the solid residue was found to be pure biotin-NHS. 6-Aminohexanoic acid (1.3 g, 10 mmol) was dissolved in 10 mL of water, followed by addition of 2 mL acetonitrile and 2 mL DMSO. The mixture was stirred under argon atmosphere throughout the following procedure. DIEA (10 µL) was added which raised the appeared pH from ~7 to ~8 according to pH paper. Biotin-NHS (341 mg, 1 mmol) was dissolved in 2 mL of DMSO, and 0.75 mL of this solution was added dropwise, causing a decrease of the apparent pH to ~7. Dropwise addition of DIEA (120 µL) brought the apparent pH back up to ~8. In two more cycles, 0.75 mL of the biotin NHS ester and 120 µL of DIEA each were added as described above. After another 5 min of stirring, a last portion of DIEA (120 µL) was added to raise the apparent to pH 8 again. After 30 min, the mixture was slowly acidified to an apparent pH of ~2 with 1 M HCl. Precipitated product was collected by filtration and washed with 5 mL of 0.1 M HCl. The

solid was re-dissolved in a minimum of 1 M Na_2CO_3 and again acidified to pH~2 with 1 M HCl. The precipitate was again collected and washed as before. Drying at 1-10 Pa overnight gave 166 mg of Biotin-cap-COOH which was pure by TLC (R_f^I = 0.08, R_f^{II} = 0.33, R_f^{III} = 0.54). Although these R_f values were not too different from free *d*-biotin (R_f^I = 0.10, R_f^{II} = 0.33, R_f^{III} = 0.62), the product was clearly distinguished on TLC plates when both compounds were spotted next to each other. 6-Aminohexanoic acid did not migrate in these eluents while biotin-NHS was more much mobile (R_f^I = 0.57, R_f^{II} = 0.69, R_f^{III} = 0.71), especially in eluent I.

^{1}H-NMR (200 MHz, D_2O/NaOD) δ (ppm): 1.2-1.9 (12H, m, -C*H*$_2$-C*H*$_2$-C*H*$_2$-CH$_2$-CO in biotin and in aminohexanoic acid) 2.1-2.35 (4H, m, -C*H*$_2$-CO in biotin and in aminohexanoic acid) 2.7-2.85 (1H, d, $^2J_{gem}$ = 12.9 Hz, position 6$_I$ (trans to 6a) in biotin) 2.9-3.1 (1H, dd, $^3J_{vic}$ = 4.8, $^2J_{gem}$ = 12.9 Hz, position 6$_I$ (cis to 6a) in biotin) 3.1-3.25 (2H, m, -CO-NH-C*H*$_2$-) 3.25-3.4 (1H, m, position 4 in biotin) 4.4 (1H, m, position 3a in biotin) 4.6 (1H, m, position 6a in biotin)

Synthesis of Biotin-cap-NHS. Conversion of biotin-cap-COOH into biotin-cap-NHS was performed as described [Wilchek and Bayer, 1990].

Preparation of Biotin-IgG-SATP and Biotin-IgG. Biotin-IgG-SATP was prepared in two steps, closely following the preparation of fluorescein-IgG-SATP (*35*). Goat IgG (Sigma I-5256, 5 mg, 33 nmol) was dissolved in buffer D (0.5 mL) and 5 µL of 66 mM Biotin-cap-NHS in DMSO (30 mg/mL, 330 nmol) was slowly added while vortexing.. The mixture was incubated at RT with occasional vortexing. After 30 min, the pH was lowered to 7.5 by addition of 4.2 µL 1 M NaH_2PO_4, 5 µL of 66 mM SATP in DMSO (16.2 mg/mL, 330 nmol) was slowly added while vortexing, and the mixture was incubated at RT for another 30 min with occasional vortexing. Derivatized goat IgG was separated from the reagents by gel filtration in buffer A on a PD-10 column (Amersham). The column was pre-washed with buffer A (25 mL), the outlet was closed, the reaction mixture (0.5 mL) was loaded, and the outlet was opened to allow for elution of 0.5 mL by gravity flow. Two 1 mL portions of buffer A were applied to the column, and after the flow had ceased, one portion of buffer A (1.3 mL) was applied and the eluent (the protein fraction) was collected. One 100 µL aliquot of the protein fraction was diluted to 1 mL with buffer A and the absorption spectrum was measured from 200–400 nm. The absorbance at 280 nm (0.303 cm^{-1}) was divided by the molar absorptivity of goat IgG (ε_{280} = 210000 M^{-1} cm^{-1}) (*32*) to yield the IgG concentration (14.4 µM, 2.2 mg/mL). The rest of the protein fraction was split into 40 µL aliquots which were frozen in liquid nitrogen and stored at –25°C.

For measurement of protein-bound 3-(acetylthio)-propionyl residues on biotin-IgG-SATP, 38 µL of the protein fraction was mixed with 260 µL buffer A in a styrene tube and the tube was capped with a rubber septum. The gas phase in the tube was perfused with argon for 2 min through two hypodermic needles with occasional vortexing. Hydroxylamine reagent (500 mM, 15 µL) was injected through the septum with a Hamilton syringe and the tube was incubated for 60 min at RT to unmask the protected mercaptopropionyl residues on IgG (see Figure 15S below). The septum was removed and 300 µL of argon-purged buffer A was added. Ellman reagent (10 mM, 10 µL) was added from an Eppendorf Multipette while vortexing. After 15 min incubation at room temperature, the solution was transferred to a microcuvette and A_{412} was measured against water (0.185 ± 0.006 cm^{-1}, from triplicates). For measurement of reagent blank absorption (A_{412b} = 0. 105 ± 0.003 cm^{-1}) 38 µL of buffer A was used in place of 38 µL protein sample. Consequently, the sulfhydryl concentration in the cuvette was calculated as (0.185 cm^{-1} – 0.105 cm^{-1}) / 14150 M^{-1} cm^{-1} = 5.6 × 10^{-6} M (*32*), corresponding to 93 µM 3-(acetylthio)-propionyl residues in the undiluted protein fraction from the PD-10 column (14.4 µM IgG). This means that out of the 10 SATP offered per IgG molecule, 6.5 3-(acetylthio)-propionyl residues were actually bound, in good agreement with previous findings with fluorescein-IgG-SATP (*35*).

The biotin content of biotin-IgG-SATP was not explicitly measured but a similar number of biotin residues (>5 per IgG) is assumed since (i) the biotin-cap-NHS concentration was the same as that of SATP, (ii) the reaction mechanism is the same, and (iii) under these conditions of pH, protein concentration and NHS-ester concentration, the fraction of NHS-ester that

covalently reacts with protein is typically around 50% [Gruber et al., 2000] (*35*).

Biotin-IgG was prepared by the analogous procedure, applying gel filtration immediately after reaction of IgG with biotin-cap-NHS. Aliquots (30 µL) with 24.9 µM (3.74 mg/mL) protein concentration were frozen in liquid nitrogen and stored at -25°C.

Functionalization of AFM tips with PEG linkers and antibodies. For aminofunctionalization of AFM tips, ethanolamine hydrochloride (3.3 g), DMSO (6 mL), and molecular sieves (4 Å) were heated to 100°C in a flask until all salt was dissolved. The mixture was allowed to cool to RT and, after the appearance of gas bubbles had ceased, the AFM tips were immersed and incubated overnight. The cantilevers were washed in DMSO (3 ×) and ethanol (3 ×) and dried in a stream of nitrogen gas. The tips were stored in a desiccator for up to 4 days.

The standard protocol for covalent coupling of Pyr-S-S-PEG-NHS (commercial crosslinker, Figure 2) or PDP-PEG-NHS (Figure 2) to AFM tips (Figure 1) was the following: One aliquot of linker (1 mg in the standard version) was dissolved in 1 mL of chloroform containing 0.5% TEA. For comparison, 10 mg portions of crosslinker were used in some series (see Table 2). The solution was transferred into tiny "beaker" which had been produced by cutting off the top part of a 4 mL screw cap glass vial and using the bottom part. This beaker was placed into a small glass chamber with a tightly fitting lid (Kimax weighing bottle, Aldrich). The AFM cantilevers were immersed for 2 h at RT, washed with chloroform (3 ×), dried with a stream of nitrogen and immediately used for functionalization with IgG (see below).

For tip functionalization with antibody, 40 µL of biotin-IgG-SATP (2.2 mg/mL) was mixed with 380 µL of buffer A and 80 µL of 500 mM hydroxylamine reagent. The mixture was transferred to a small polystyrene petri dish that was in a slightly tilted but stable position to let the solution accumulate in one place. Tips pre-functionalized with Pyr-S-S-PEG-NHS or PDP-PEG-NHS (see Figures 1 and 2) were immersed for 1 h. The tips were washed in buffer A (2 ×) and PBS (2 ×) and stored in PBS at 4°C until used (up to 3 days).

Preparation of dense avidin monolayers on mica and imaging with AFM. Avidin (1 mg/mL) was dissolved in PBS and aliquots were stored frozen at –25°C. Immediately before use, an aliquot was thawed and 10 µL thereof was diluted with 90 µL of water and placed on freshly cleaved mica which had been mounted in an AFM fluid cell. After 15 min incubation time the mica was washed extensively with a 9/1-mixture of water and PBS. A bare silicon nitride tip was used to image a 1.5 × 1.5 μm^2 area of the avidin monolayer at a pressing force of 200 pN, showing the typical 1-2 nm roughness of an avidin monolayer (not shown). After zooming in to an area of 100 × 100 nm, the pressing force was increased to 10 nN in order to scratch away all adsorbed avidin molecules from this area. Subsequently, the original settings were restored and the avidin layer with the rectangular hole was imaged (Figure 3).

Monitoring of single unbinding events between tip-bound biotin-IgG and mica-bound avidin by force spectroscopy. All force measurements were done on a commercial AFM (Pico-SPM, Molecular Imaging, Phoenix, AZ) using a Nanoscope IIIA™ controller (Digital Instruments, Santa Barbara, CA). The mica sheet with the adsorbed avidin molecules was placed in a commercial fluid cell. The cantilever with the PEG-tethered biotin residues was mounted on the piezo tube and force-distance cycles (conventional force calibration mode) were recorded at a 1 Hz vertical scan rate and 300 nm z-amplitude. The measurements were done in PBS at a constant trace and retrace velocity of 100 nm/s. The spring constants of the levers were determined by the thermal noise method (*23, 24*).

Functionalization of silicon nitride chips with amines, PEG linkers and biotin-IgG-SATP, and quantitative assay for chip-bound biotin-IgG-SATP. The chips were precut (5 × 5 mm^2), weighed, washed in chloroform (3 × 10 min), dried in a stream of nitrogen and immersed in piranha for 30 min. *Caution*: piranha is explosive and quickly destroys skin, tissue, gloves, and other organic materials. Piranha was prepared by cautiously adding small portions of 98% sulfuric acid (7 volume parts) to 30% H_2O_2 (3 volume parts) while swirling the flask in cold water (*warning*: the reverse order may cause explosion!). After extensive rinsing with water, the chips were heated to 160°C on a hot plate and dried with a stream of nitrogen while holding the

chip with tweezers. The chips were immediately immersed in the same kind of ethanolamine hydrochloride/DMSO solution as described above for the AFM tips (with two layers of 4 Å molecular sieves beads underneath the chips) and incubated at RT for 16 h. After washing in DMSO (3 ×) and ethanol (3 ×), the chips were dried with nitrogen and derivatized with Pyr-S-S-PEG-NHS or PDP-PEG-NHS exactly as described above for AFM tips.

The aminofunctionalized chips were washed in chloroform (3 ×), dried with nitrogen, and placed in a plastic petri dish. Biotin-IgG-SATP (2.2 mg/mL) or biotin-IgG (3.2 mg/mL) was mixed with buffer A and 500 µL hydroxylamine reagent to adjust final concentrations of 0.5 mg/mL for IgG and 50 mM for hydroxylamine and 50 µL of this solution was pipetted onto the surface of each chip. The petri dishes were covered with a lid and incubated for 60 min. The chips were removed and washed in buffer A (3 × 10 min) and PBS (3 × 10 min).

For quantification of chip-bound biotin-IgG-SATP or biotin-IgG, most of the buffer was removed from the chips and they were placed on teflon plates (pre-washed with ethanol and dried well) which were lying in disposable petri dishes. 50 µL of unblocked (or blocked) EAP reagent was placed in each chip and incubated in the closed chamber for 60 min. The chips were removed and washed in PBT (3 × 10 min) and PBS (3 × 10 min). A UV-vis spectrometer cuvette was filled with 3 mL of OPD reagent and 30 µL of 3% H_2O_2 was added with stirring.

The derivatized chip was immersed into the stirred assay mixture for exactly one minute after which time the chip was transferred into buffer B and the absorbance (A_{490}) of the cuvette was measured. Subsequently, the cuvette was again stirred and the chip was re-immersed for another time interval. This process was repeated until the plot of A_{490} versus time showed a significant rise. The enzyme densities were estimated by comparing the observed $\Delta A_{490}/\Delta t$ with the $\Delta A_{490}/\Delta t$ values of calibration experiments in which known amounts of ExtrAvidin®-peroxidase (5 µL of a 200-fold dilution of the purchased stock solution) were dissolved in the same volume of assay mixture. According to the supplier, the protein concentration in the stock solution was 2.0-2.5 mg/mL and the molar ratio of avidin/peroxidase 0.5 - 0.9. For estimation of the number of marker enzymes per μm^2 (see Table 1) it was assumed that the protein concentration in the stock solution was 2 mg/mL, that on average 1.5 peroxidase molecules were bound per avidin tetramer, and that the average molecular weight was 120 kDa.

Kinetics of thiol coupling to pyridyldithio groups. PDP-BSA was prepared by dissolving BSA (402 mg, 6 µmol) in buffer A (6 mL) and reacting with SPDP (9.4 mg, 30 µmol, pre-dissolved in 300 µL DMSO) for 1 h at RT. The pH was lowered to 5.5 with 10% acetic acid and further to 4.5 with 1 M HCl. Turbidity was removed by centrifugation in an Eppendorf centrifuge at 14000 RPM. An aliquot (0.5 mL) was withdrawn and gel filtered in buffer A on a PD-10 column, as described above for biotin-IgG-SATP. The protein concentration (284 µM) was calculated from A_{280} (ε_{280} = 44300 M^{-1} cm^{-1}, [Elgersmaa et al., 1990]) and the 2-pyridyldithio group content (628 µM) was measured as described (*35*).

The rest of the above reaction mixture (5.5 mL) was treated with DTT (0.7 mL of a 250 mM solution in buffer G) for 15 min during which time the progress of the reaction was repeatedly examined by withdrawing a 50 µL aliquot, mixing with 1 mL buffer G, and measuring A_{343}. Under these conditions of pH and DTT concentration, only PDP groups but no cystines in proteins are being cleaved (*34*). Buffer E (1 mL) was added and the sample was gel filtered (Sephadex® G25F, 1.5 cm × 48 cm, 1 mL/min) in argon-bubbled buffer E while collecting 5 mL fractions under argon in septum-covered polystyrene tubes. The BSA peak was detected by mixing 50 µL aliquots with buffer G (1250 µL) and measuring A_{280}. Fractions #4-10 were combined and found to contain 179 µM BSA (according to A_{280}, ε_{280} = 44300 M^{-1} cm^{-1}, [Elgersmaa et al., 1990]) and 367 µM of sulfhydryl groups (as measured by reaction with 4,4'-DTDP) (*32*).

PDP-PEG-BSA was prepared by dissolving BSA (100 mg) in buffer A (3 mL) and reacting with PDP-PEG-NHS (4 mg, 3 µmol, pre-dissolved in 200 µL DMSO) for 75 min at RT. The product was isolated by gel filtration in buffer A on Sephadex G100 (1.5 x 48 cm, 0.5 mL/min [Kaiser et al., 1997]). The protein and PDP group content in the fractions (4 mL) was measured as described above for PDP-BSA. Fractions #7-11 were

combined and found to contain 93 µM of BSA, as well as 93 µM of intact PDP groups.

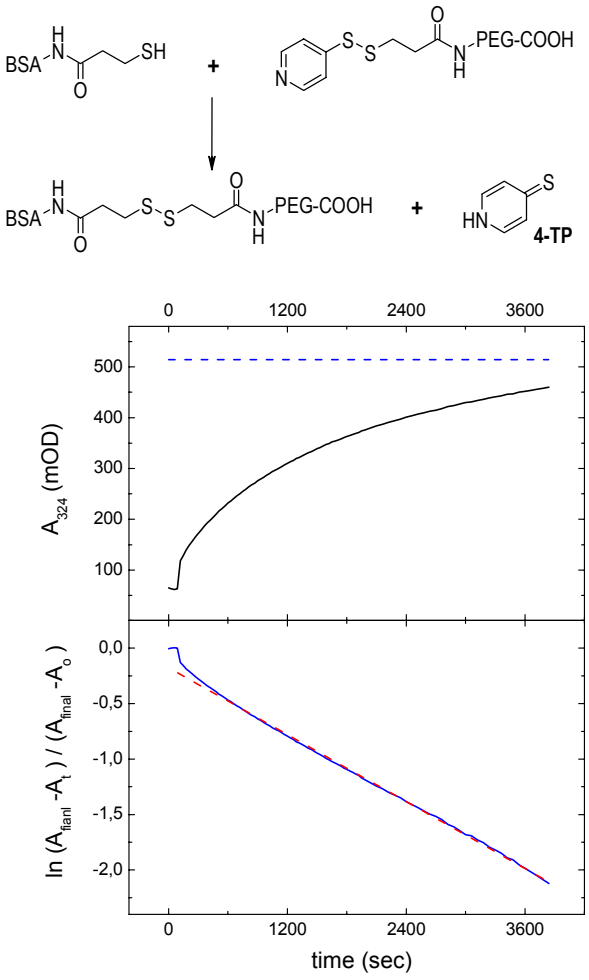

Figure 8S. Time course of disulfide bond formation when 4-pyridyldithiopropionyl-NH-PEG-COOH (80 µM, in buffer A) was stirred and mercaptopropionyl-BSA was added at a final concentration of 20 µM. The release of 4-TP was monitored by light absorption at 324 nm. (ε_{324} = 21400 M^{-1} cm^{-1}) (*32*). The dashed horizontal line in panel A indicates the expected absorbance after completion of the reaction (A_{final}).

In a quartz cuvette, a 1 mL sample of buffer A with 80 µM of a pyridyldithio component (see Table 2) was shortly bubbled with argon and A_{343} was monitored over time in a UV-vis spectrophotometer. The observed time course was re-plotted semilogarithmically according to

$$\ln [(A_{343,t} - A_{343,final}) / (A_{343,t=0} - A_{343,final})] = -k_1 t \quad (1)$$

and the kinetic constant k_1 was obtained from the linear portion of the graph (data not shown). This k_1 which was divided by the approximately constant concentration of the excess component (80 µM) to obtain an estimate for the bimolecular kinetic constant k_2 according to:

$$-d[thiol] / dt = k_2 [pyr\text{-}S\text{-}S\text{-}R] [thiol] \quad (2)$$

$$k_1 = k_2 [pyr\text{-}S\text{-}S\text{-}R] \sim k_2 \times 80\ \mu M \quad (3)$$

$$-d [thiol] / d t = k_1 [thiol] \quad (4)$$

$$\ln ([thiol]_t / [thiol]_{t=0}) = -k_1 t \quad (5)$$

with equation (5) being equivalent to equation (1) above. (As an example, the time course of reaction of 4-pyridyldithiopropionyl-NH-PEG-COOH (80 µM) with mercaptopropionyl-BSA (20 µM) is shown in panel A of Figure 8S, and the semilogarithmic plot is shown in panel B.)

The bimolecular kinetic constant k_2 was multiplied with 1 mM to calculate the apparent kinetic constant $k_1^{1\ mM}$ for the reaction a minute concentration of pyr-S-S-R with 1 mM of free thiol component which is in excess (compare equation (3)). The expected half time $t_{1/2}^{1\ mM}$ (see Table 2) was calculated as

$$t_{1/2}^{1\ mM} = \ln 2 / k_1^{1\ mM} \quad (6)$$

and the analogous procedure was used to calculate the predicted half time $t_{1/2}^{1\ \mu M}$ (see Table 2) for the reaction of <<1 µM pyr-S-S-R with 1 µM free thiol component which is in excess.

A modified protocol was used to measure the reaction of pyr-S-S-ethanol and pyr-S-S-ethylamine since the former is not commercially available and the latter is rather unstable in pure form (unpublished observation). A 1 mL sample of buffer A with 60 µM of 2,2'-DTDP was bubbled with argon and A_{343} was monitored over time when

suddenly 2-mercaptoethanol or 2-mercaptoethylamine hydrochloride were added from a 1 mM stock solution (in 1 mM acetic acid, freshly prepared by dilution of 500 mM stock solutions in 500 mM acetic acid) at a final concentration of 120 µM with stirring for 3 s. Within 6 s, all free sulfhydryls were converted into pyr-S-S-groups, as was obvious from the sudden increase in A_{343}. The rise in A_{343} was much slower, reflecting the reaction of pyr-S-S-groups with 2-mercaptoethanol or 2-mercaptoethylamine. The first part of slow time course was fitted by a monoexponential function yielding the apparent k_1 at 60 µM pyr-S-S-component. From this, k_2 and the half times $t_{1/2}^{1\ mM}$ as well as $t_{1/2}^{1\ \mu M}$ (Table 2) were calculated as described above.

SUPPORTING RESULTS

Modified synthesis of biotin-cap-NHS. Biotin-NHS was synthesized in DMF by the DCC method, as described [Wilchek and Bayer, 1990], whereas the subsequent reaction with 6-aminocaproic acid described in the same publication could not be reproduced because Biotin-NHS always precipitated in the dropping funnel, in spite of preheating of the DMF solution. Probably the described method works on a very hot summer day or if the dropping funnel is externally heated. As an alternative, (i) biotin-NHS was dissolved in DMSO, and (ii) the large excess of aminocaproic acid was dissolved in water/DMSO/acetonitrile (see Figure 9S). The inclusion of organic co-solvents in the aminocaproic acid solution served two purposes: (i) It prevented precipitation of biotin-NHS when the latter was added from the dropping funnel. (ii) It lowered the dielectric constant, thereby facilitating deprotonation of the amino group of aminocaproic acid by the relatively weak base DIEA and disfavoring the formation of hydroxyl anions which would cause hydrolysis of biotin-NHS into free *d*-biotin.

By the above method it was possible to completely convert all biotin-NHS into biotin-cap-COOH, without any hydrolysis of biotin-NHS into the free carboxylic acid (= *d*-biotin). The latter was essential because biotin-cap-COOH and free *d*-biotin differ just enough to resolve them on analytical TLC, while preparative separation by flash chromatography seems tricky.

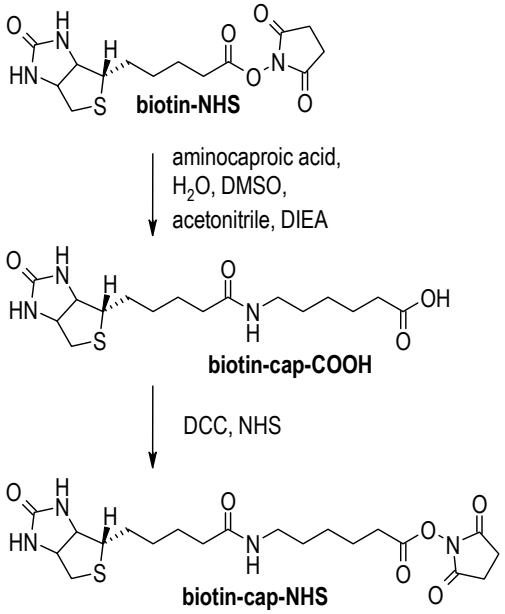

Figure 9S. Synthesis of Biotin-cap-NHS. Aminocaproic acid was dissolved in water / DMSO / acetonitrile, a DMSO solution of biotin-NHS was added and the apparent pH (according to pH indicator paper) was repeatedly re-adjusted to 8 by additions of DIEA. Biotin-cap-COOH was precipitated by acidification, re-dissolved at neutral pH and again precipitated with acid. Subsequent activation of biotin-cap-COOH with DCC / NHS was done as described [Wilchek and Bayer, 1990].

The method of adding a DMSO solution of a non-polar NHS ester to a DMSO/acetonitrile/water solution of a small primary amine that carries ≥1 negatively charged carboxylates has since been used in the derivatization of $N_\omega N_\alpha$-*bis*-carboxymethyl-lysine (often termed lysine-NTA, manuscript in preparation). It may generally be of help for reaction a non-polar NHS ester with highly polar primary amines (e.g. oligonucleotides with primary amino groups), thereby reconciling opposing solvent needs of different educts.

Modification of Goat IgG with biotin-cap-NHS and SATP. In a preceding study (*35*), IgG was modified both with the NHS ester of carboxyfluorescein and with SATP, yielding fluorescent protein which was then coupled to 2-pyridyl-S-S-PEG linkers on liposomes (*35*), as well as to AFM tips and silicon nitride chips (*9*). The fluorescent label was coupled to IgG by the procedure recommended by the supplier, i.e. at pH

8.6. For attachment of SATP, however, a lower pH (7.5) was preferred because a significant fraction of the thioester function gets hydrolyzed at pH 8.6 (unpublished observations). In order to circumvent dialysis, the necessary pH drop from 8.6 to 7.5 was accomplished by adding a pre-determined volume of a 1 M NaH_2PO_4 stock solution to the labeling mixture. The same method has now been used for preparation of biotin-IgG-SATP (see Figure 10S), i.e. IgG was first reacted with biotin-cap-NHS at pH 8.6, and after lowering the pH to 7.5, the same protein was further derivatized with SATP. In spite of the lower pH, protein derivatization with SATP was quite efficient since 6.5 out of 10 offered SATP molecules were covalently bound to IgG.

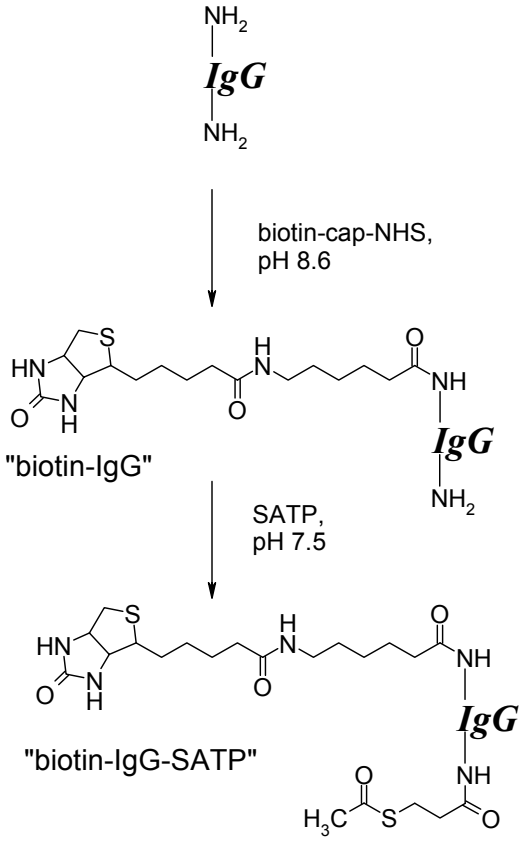

Figure 10S. Preparation of biotin-IgG and biotin-IgG-SATP by modification of goat IgG with biotin-cap-NHS (at pH 8.6), followed by a defined drop in pH and further modification with SATP (*35*) in order to introduce either protected thiol groups (in biotin-IgG-SATP). The pH 7.5 for reaction with SATP was chosen because of the base sensitivity of the thioeter function in SATP, as well as in biotin-IgG-SATP.

Synthesis of SPDP. Since large quantities of expensive SPDP are needed for a constant supply of PDP-PEG-NHS, the synthesis of SPDP was reinvestigated. Carlsson et al. (*34*) were the first synthesize SPDP. In the first step, PDP-OH was formed by the simple reaction of 2,2'-DTDP with mercaptopropionic acid (similar as in Figure 12S) in ethanol/acetic acid. Since 2,2'-DTDP is also expensive and must be used in large excess, we tried to prepare it from the cheap educt 2-TP (the tautomeric form of 2-mercaptopyridine) by oxidation with H_2O_2 in pure water which has not been used before to prepare 2,2'-DTDP. Indeed, the product was immediately precipitated at neutral pH and could be isolated by simple filtration and washing with water (see Figure 11S). The well defined oxidation of 2-TP into 2,2'-DTDP with H_2O_2 was not only used for synthesis of 2,2'-DTDP, it proved particularly helpful in the synthesis of PDP-OH also, as described below.

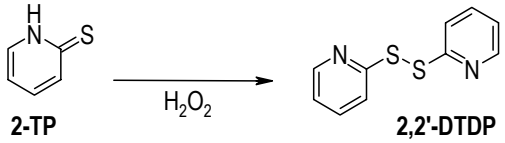

Figure 11S. Synthesis of 2,2'-DTDP from 2-TP by oxidation with H_2O_2 in pure water. The product is insoluble at neutral pH and precipitates. Further oxidation of 2,2'-DTDP had to be prevented by cooling to 10-15°C and short reaction time (30 min).

The synthesis of PDP-OH is depicted in Figure 12S. The main problem of this reaction is that, in spite of using a large excess of 2,2'-DTDP, a large fraction of PDP-OH undergoes further reaction with $HS\text{-}CH_2\text{-}CH_2\text{-}COOH$ and gives $HOOC\text{-}CH_2\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}CH_2\text{-}COOH$ which is difficult to separate from PDP-OH, especially by the method reported by Carlsson et al. (*34*) (chromatography on Alox). In this study, the extent of formation of the symmetric side product was reduced by slow addition of 3-mercaptopropionic acid to a strongly acidic solution of 2,2'-DTSP. Acidic conditions are known to slow all thiol-disulfide exchange processes (*33*). After completion of the reaction, all released 2-TP was re-oxidized into 2,2'-DTDP of which a large excess was present anyway (see Figure 12S). Conversion of 2-TP into 2,2'-DTDP was very advantageous

because, in the subsequent extraction at neutral pH, the latter quantitatively partitioned into chloroform (in contrast to 2-TP which has similar solubility in water and chloroform). At this stage, the aqueous solution contained the product (PDP-OH) and the symmetric byproduct (HOOC-CH_2-CH_2-S-S-CH_2-CH_2-COOH). Switching to pH ~2.5 allowed for preferential back-extraction PDP-OH into chloroform. Unfortunately, some symmetric byproduct was also collected in the chloroform phase, thus chromatography was required for isolation of pure PDP-OH. Ion exchange on QAE Sephadex A-25 was found to afford >99% purity but this method is expensive and time-consuming. Fortunately, flash chromatography on silica 60 in dichloromethane/methanol (95/5) also gave ~99% pure PDP-OH, speed and simplicity being the trade-off for partial loss of product by irreversible adsorption to silica.

It should be noted that the two-step extraction (first at neutral pH and then at pH 2-3) was taken from the SPDP synthesis of Shval'e and Ofitserov [1985]. These authors used rigorously dry conditions to react 2-TP with dangerous chlorine gas to prepare pyridine-2-sulfenyl chloride in their first step. The latter was then reacted with HS-CH_2-CH_2-COOH to obtain PDP-OH. When reproducing this procedure, we found that the high reactivity pyridine-2-sulfenyl chloride did not suffice to instantaneously convert all HS-CH_2-CH_2-COOH into PDP-OH (in spite of rigorously dry working conditions), as was obvious from appearance of the undesired HOOC-CH_2-CH_2-S-S-CH_2-CH_2-COOH in the reaction mixture. In conclusion, the use of chlorine gas and rigorously dry conditions did not lead to suppression of side product formation. This is the reason why we prefer to use the simpler and less hazardous reaction scheme of Carlsson et al. (*34*), together with the improvements shown in Figure 12S. However, the multi-step extraction scheme of Shval'e and Ofitserov [1985] was found very useful for removing 2,2'-DTDP and a large extent of HOOC-CH_2-CH_2-S-S-CH_2-CH_2-COOH.

The conversion of PDP-OH into SPDP (Figure 13S) was done as described by Shval'e and Ofitserov [1985], except that 90% and not 70% ethanol was used for re-crystallization to minimize hydrolysis. Pure white crystals of SPDP were obtained which were stable for a long time when stored at –25°C.

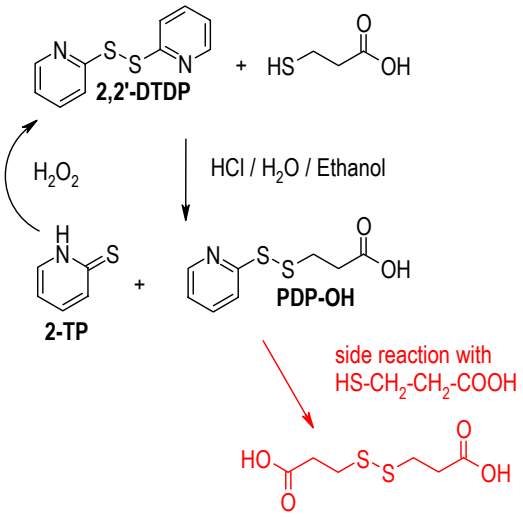

Figure 12S. Synthesis of PDP-OH. Excess of 2,2'-DTDP and acidic reaction helped to minimize the undesired formation of symmetric 3,3'-dithiodipropionic acid. The byproduct 2-thiopyridone was re-oxidized to 2,2'-DTDP which was selectively extracted into chloroform at pH 8. PDP-OH was further purified, either by anion exchange chromatography or by silica chromatography, in order to remove symmetric 3,3'-dithiodipropionic acid.

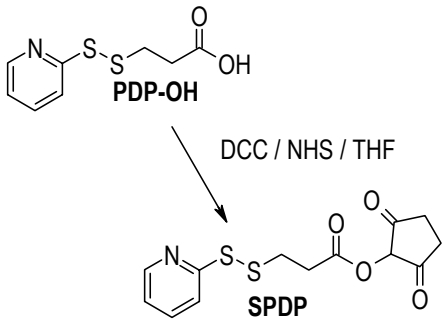

Figure 13S. Synthesis of SPDP from PDP-OH. After activation of PDP-OH with DCC/NHS, the final product was re-crystallized from 90% ethanol.

Synthesis of PDP-PEG-COOH. NH_2-PEG-COOH was synthesized and reacted with excess of SPDP, as described before (*22*). In this previous study, we have successfully used gel filtration in aqueous buffer to separate the product (PDP-PEG-COOH) from excess of SPDP and unreacted PDP-OH. However, this method was found to yield variable results; sometimes the small molecules were well separated but more often they were found to co-migrate with PDP-PEG-COOH, as if solubilized by the large PEG molecules. Surprisingly, the same phenomenon was also observed when gel filtration was done in methanol on Sephadex LH-20. For this reason, we developed a multi-step extraction scheme (see Materials and Methods) which finally gave pure PDP-PEG-COOH in a reproducible way.

Synthesis of PDP-PEG-NHS. PDP-PEG-COOH was converted into PDP-PEG-NHS by the TSTU method [Bannwarth et al., 1991] which has the advantage that small quantities of carboxylic acid can be reacted with several-fold excess of the activator (TSTU), whereas only stoichiometric amounts of DCC and NHS should be applied in the conventional method. Two significant modifications were introduced in the TSTU method: (i) Pyridine was used as base instead of triethylamine because this mild base can be used at an invariant high concentration (33-50%), irrespective of the carboxylic acid concentration, and (ii) the NHS ester was isolated in pure form by dissolving in chloroform, washing with neutral buffer, drying with sodium sulfate and evaporating the chloroform phase. The ease of perfect byproduct removal by this method nicely contrasts with the difficulty to remove all dicyclohexylurea after the DCC method. The modified TSTU method has since been used to yield perfectly pure NHS esters with a large variety of carboxylic acids (including PDP-OH, unpublished results), only in case of biotin it failed because biotin-NHS did not sufficiently partition into the chloroform phase to be protected from hydrolysis while washing with neutral aqueous buffer solution.

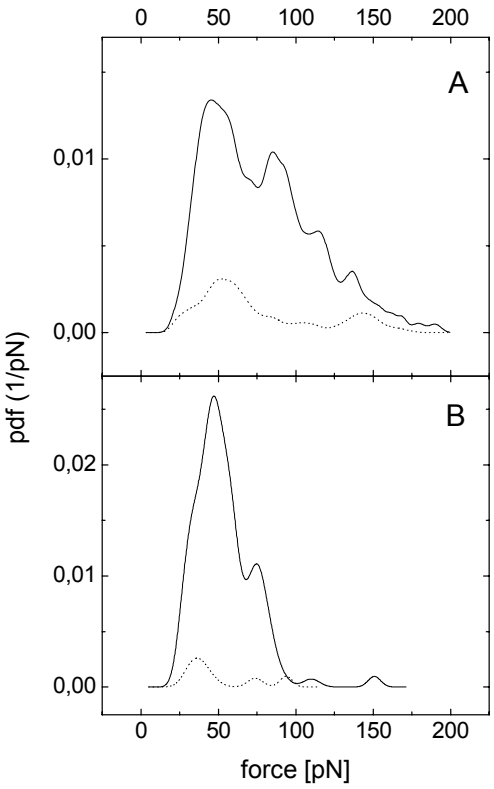

Figure 14S. Unbinding force distribution obtained with two different tips (panel A for the first tip and panel B for the second tip). Both tips had been reacted with the commercial linker Pyr-S-S-PEG-NHS (10 mg/mL in chloroform containing 0.5% triethylamine) after aminofunctionalization, followed by coupling of biotin-IgG-SATP in presence of hydroxyl amine, as usual. With each tip, 999 approach-and-retraction cycles (unbinding cycles) were performed, first in absence of *d*-biotin (solid lines) and then in presence of *d*-biotin (0.1 mg/mL, 0.4 mM, dotted lines). In absence of *d*-biotin, the binding probabilities were 22% and 10% in panels A and B, respectively (solid lines). In presence of *d*-biotin, the binding probabilities were reduced to 3.7% and 0.7% in panels A and B, respectively (dotted lines). The areas underneath the unbinding force distribution curves are set proportional to the binding probability in the 999 force-distance cycles performed for this data set.

AFM Results with Commercial Pyr-S-S-PEG-NHS in place of PDP-PEG-NHS. Figure 14S shows unbinding force distributions obtained with two different AFM tips which had been functionalized by the scheme shown in Figure 1, using commercial Pys-S-S-PEG-NHS in place of PDP-PEG-NHS. With each tip, 999 approach-and-retraction cycles were performed, first in absence of *d*-biotin (solid lines) and then in presence of *d*-biotin (0.1 mg/mL, 0.4 mM, dotted lines). The tip in panel A showed a more multi-modal force distribution, indicating that a heavily biotinylated antibody had been linked to the tip. The tip in panel B showed a mostly monomodal unbinding force distribution which is attributed to a tip-linked IgG molecule which either carries a single biotin residue only. Alternatively, the IgG molecule may carry several biotin residues which, however, cannot simultaneously bind to mica-bound avidin, due to an unfavorable spatial arrangement of the biotins on IgG. Both tips showed specific loss of their recognition function in the presence of *d*-biotin, as graphically indicated by reduction of area underneath the force distribution curve. From these experiments it is concluded that the commercial Pyr-S-S-PEG-NHS can well be used in place of PDP-PEG-NHS. Only in the TREC mode (simultaneous measurement of "Topography and RECognition") (*19-21*) it is less suited, due to its greater effective length (S. M. Lindsay, personal communication).

Schematic illustration of chip functionalization. Figure 15S depicts the sequence of chemical reactions and of biospecific binding steps, as they occur during functionalization and characterization of silicon nitride chips. The first three stages (panels A-C) are the same as in the coupling reaction of aminofunctionalized AFM tips with PDP-PEG-NHS and biotin-IgG-SATP (see Figure 1).

LITERATURE CITED IN THE SUPPLEMENTS ONLY

Bannwarth, W., and Knorr, R. (1991) Formation of carboxamides with *N,N,N′,N′*-tetramethyl (succinimido) uronium tetrafluoroborate in aqueous/organic solvent systems. *Tetrahedron Lett. 32*, 1157-1160.

Elgersma, A. V., Zsom, R. L., Norde, W., and Lyklema, J., (1990) The adsorption of bovine serum albumin on positively and negatively charged polystyrene latices. *J. Coll. Int. Sci. 138*, 145-156.

Gruber, H. J., Hahn, C. D., Kada, G., Riener, C, Harms, G. S., Ahrer, W., Dax, T. G., and Knaus, H.-G. (2000) Anomalous fluorescence enhancement of Cy3 and Cy3.5 versus anomalous fluorescence loss of Cy5 and Cy7 upon covalent linking to proteins and noncovalent binding to avidin. *Bioconjugate Chem. 11*, 696-704.

Kaiser, K., Marek, M., Haselgrübler, T., Schindler, H., and Gruber, H. J. (1997) Basic studies on heterobifunctional biotin-PEG conjugates with a 3-(4-pyridyldithio)propionyl marker on the second terminus. *Bioconjugate Chem. 8*, 545-551.

McCormick, D. B., and Roth, J. A (1970) Colorimetric determination of biotin and analogs. *Methods Enzymol. 18*, 383-385.

Shval'e, A. F., Ofitserov, V. I., and Samurov, V. V. (1985) New synthesis of *N*-hydroxysuccinimide ester of 3-(2-pyridyldithio)-propionic acid. *Zh. Obshch. Khim. 55*, 2152-2152.

Wilchek, M., and Bayer, E. A. (1990) Biotin-containing reagents. *Methods Enzymol. 184*, 123-138.

Figure 15S on next page ➔

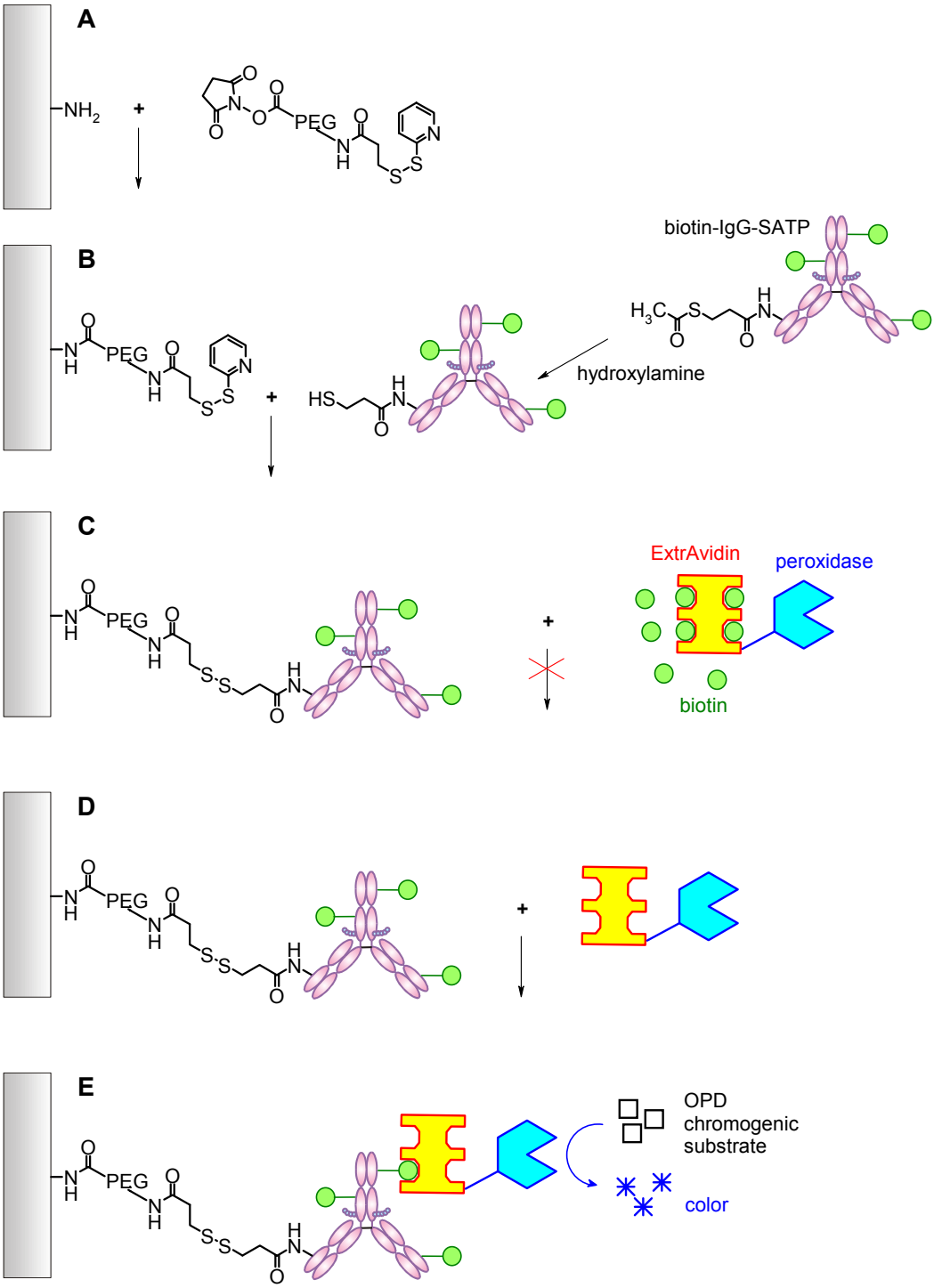

Figure 15S. Schematic representation of chip functionalization and characterization. (A) Aminofunctionalized silicon nitride chips were reacted with PDP-PEG-NHS (or Pyr-S-S-PEG-NHS, see Figure 2), resulting in amide-bond linked PEG chains with 2-pyridyl-S-S groups on their free end. (B) Biotin-IgG-SATP is deprotected with hydroxylamine to obtain biotin-IgG with free thiol functions. The thiol reacts with the 2-pyridyl-S-S group on chip-bound PEG, resulting in a stable disulfide linkage. (C) Control for non-specific adsorption of ExtrAvidin®-peroxidase to the pre-functionalized chip by inclusion of excess of *d*-biotin which blocks the biotin-binding sites in ExtrAvidin®-peroxidase. (D) Binding of unblocked ExtrAvidin®-peroxidase to the functionalized chip, by specific binding to the biotin residues of biotin-IgG and potentially also by non-specific adsorption. (E) Quantification of chip-bound ExtrAvidin®-peroxidase via the enzymatic activity of the peroxidase molecules on the chip.