General

Unless otherwise stated, all solvents used were reagent grade. Dichloromethane was dried over P₂O₅ and distilled prior to use. DMF was stored over 3 Å molecular sieves. Water was deionized prior to use. HBTU (Iris Biotech), DIPEA (Aldrich), N-hydroxysuccinimide (Iris Biotech or GE Healthcare), tert-butyl phenylcarbonate (Aldrich), ethylenediamine (Aldrich), trifluoroacetic acid (Aldrich), EDC (GE Healthcare), 4-mercapto phenylacetic acid (Aldrich), tris(carboxyethyl)phosphine hydrochloride (Aldrich), methoxyamine hydrochloride (Aldrich), ribonuclease S (grade XII-S, Sigma), Ellman’s reagent (Merck), S-trityl 3-mercaptopropionic acid (Sigma), trisopropylsilane (Aldrich), p-cresol (Aldrich) were all used as supplied. All t-Boc-protected amino acids and MBHA resins (0.92 mmol g⁻¹) were obtained from NovaBiochem. ¹H and ¹³C NMR spectra were acquired on a Varian 400-MR spectrometer at 400 MHz and 100 MHz, respectively, in deuterated solvents containing 0.5% v/v TMS; chemical shifts are listed in ppm relative to TMS as an internal standard. ESI-MS spectra were recorded on an Applied Biosystems Single Quadrupole Electrospray Ionization Mass Spectrometer API-150EX in positive mode. Reversed phase HPLC was performed on a Varian Prostar 320 HPLC system by using a VYDAC protein & peptide C18 column. A gradient of acetonitrile in water, both containing 0.1% TFA was used to elute the peptides. Detection was performed by an UV-detector (λ = 214 nm). All SPR experiments were performed on a Biacore T100 instrument (GE Healthcare) using CMS type chips. HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.5% v/v surfactant P20, pH 7.4) was obtained from GE Healthcare as a 10x concentrated solution and diluted appropriately before filtering through 0.2 μm nylon ZAPCAP-CR filters (Whatman).

Deprotection Kinetics of Thiazolidine 4 in Aqueous Buffer. Thiazolidine deravitive 4 was prepared as a 25 mM solution in 50 mM acetate buffer (pH 4.0) containing 250 mM methoxyamine hydrochloride. The deprotection of the thiazolidine ring at either 20 °C or 37 °C was monitored by LC-MS over 10 h. Figure S1 shows the conversion of 4 to the free N-terminal cysteine with time at the two different temperatures. At 37 °C, a conversion of more than 90% was observed after 2 h.

Figure S1. Deprotection kinetics of the thiazolidine ring in 4.
**NCL Chip Preparation.** All modifications described were performed on CM5 chips (GE Healthcare). The running buffer was HBS-EP at pH 7.4 at a flow rate of 10 μL min⁻¹ unless otherwise stated. Thiazolidine derivative 4 was immobilized in flow channels 2 and 4 at 25 °C using the following three step procedure: (1) 7 min injection of an equimolar solution of EDC and NHS (1.0 M); (2) 7 min injection of 4 (250 mM) in 50 mM borate buffer at pH 8.5; (3) 7 min injection of 1.0 M ethanolamine HCl at pH 8.5. Using this procedure, we were able to reproducibly immobilize approximately 150 RU of 4 onto the surface. For the reference surfaces, ethanolamine HCl was immobilized in flow channels 1 and 3 using a similar approach. The temperature of the chip was raised to 37 °C before 2 x 70 min injections of a 250 mM methoxyamine HCl in 50 mM acetate buffer at pH 4.0 over flow channels 1 – 4 at a flow rate of 5 μL min⁻¹. After cooling the chip back to 25 °C, regeneration of the surface was initiated using 10 x 30 s pulses of 50 mM NaOH in 0.5 M NaCl over all channels at a flow rate of 10 μL min⁻¹. Chips prepared according to the above procedure were used directly in ligation experiments, or were stored 4 °C in HBS-EP buffer at pH 7.4 containing 2 mM TCEP until needed. Typical sensorgrams at each stage of the chip preparation are given in Figure S2.

**Solid Phase Peptide Synthesis.** All peptides were prepared by manual solid phase peptide synthesis on a 0.25 mmol scale using the in situ neutralization/HBTU activation procedure for t-Boc chemistry. Each synthetic cycle consisted of 3-4 minutes activation of the t-Boc-amino acid (1.1 mmol) by HBTU (1.0 mmol; 0.5 M in DMF) in the presence of N,N-disopropylethylamine (DIPEA) (3 mmol). All activated amino acids were coupled for 10 minutes, except for Arg, Ser and Asn which required a coupling time of 20 minutes. Unbound amino acids were removed by a DMF flow wash (2 x 20 seconds of rinsing). The tBoc group was removed by TFA (2 x 1 minute) followed by a second DMF flow wash. Additional flow washes with dichloromethane were performed directly before and after the TFA deprotection step of Asn residues. Peptide were synthesized on a TAMPAL resin to yield C-terminal MPAL.
thioesters. The TAMPAL resin was prepared as follows. t-Boc-Leu was coupled to the 4-methylbenzhydrylamine (MBHA) resin, following the same synthetic cycle as mentioned above. Next, 1.1 mmol of S-tritylmercaptopropionic acid was activated with 1.0 mmol of HBTU in the presence of 3 mmol DIPEA and coupled for 30 minutes to Leu-MBHA resin. The protecting trityl group was removed by the addition of TFA containing 2.5% triisopropylsilane and 2.5% H₂O. The thioester bond was formed after coupling of the next coupling of a C-terminal amino acid to the resin. After formation of the MPAL thioester on the resin, the remaining amino acids were coupled in the typical manner. After synthesis, the peptide was flow-washed with dichloromethane (DCM) and 50% MeOH in DCM to remove all DMF and dried \textit{in vacuo}. HF treatment over 1 hour at 0 °C with 4% p-cresol was used to cleave the peptide from the resin and to remove the protecting groups from the side chains (except for the Dnp groups on the histidine side-chains). After cleavage, the peptide was precipitated with ice-cold diethyl ether, dissolved in acetonitrile and lyophilized.

\textbf{Streptavidin-Binding HPQ Peptide MPAL Thioester – SLLAH(Dnp)PQGGG-MPAL.} After solid phase synthesis, the peptide was purified by RP-HPLC using a gradient of 35 – 55% acetonitrile in water (containing 0.1% TFA) over 90 min. The pure peptide was obtained in 62% overall yield. ESI-MS: found mass: 1301.5 Da; calculated mass: 1301.6 Da.

\textbf{S-Peptide MPAL Thioester - KETAAKFERQH(Dnp)MDSSTSAAGG-MPAL.} After solid phase synthesis, the peptide was purified by RP-HPLC using a gradient of 15 – 35% acetonitrile in water (containing 0.1% TFA) over 90 min. The pure peptide was obtained in 54% overall yield. ESI-MS: found mass: 2646.3 Da; calculated mass: 2646.7 Da.

\textbf{Figure S3.} Structure of the SLLAH(Dnp)PQGGG-MPAL thioester.

\textbf{Figure S4.} Structure of the S-peptide MPAL thioester.

\textbf{Purification of S-Protein.} Preparative RP-HPLC was used to isolate S-protein from RNase S. RNase S (20 mg, 1.46 \textmu mol) was dissolved in deionized water and eluted using a linear gradient of 20 – 40% acetonitrile in water (containing 0.1% TFA) over 20 min. The S-protein was obtained in 71% yield (12.0 mg, 1.04 \textmu mol). Mass spectrometry revealed that the protease subtilisin does not hydrolyze the amide bond between residues 20 and 21 with complete selectivity; the bond between residues 21 and 22 is also cleaved, although to a much lesser extent.

ESI-MS: found mass: 11534.6 Da (S₁₂₋₁₂₄, predominant species), 11447.8 Da(S₁₂₋₁₂₄); calculated mass: 11534.0 Da (S₁₂₋₁₂₄), 11446.9 Da(S₁₂₋₁₂₄).

\textbf{Binding Reproducibility of Streptavidin to HPQ-Modified Biosensor Surface.} As described in the text, we performed multiple injections of a 2 \textmu M solution of SA in HBS-EP across flow channels 1 and 2, where flow channel 2 represents the biosensor surface modified with 750 RU of the HPQ-containing SA-binding peptide. The sensorgrams are shown in Figure S5. The sensor response relating to binding reproducibility was determined to be within 1 – 2%.
Concentration-Dependant Binding of Streptavidin to HPQ Surface. SA was dissolved in HBS-EP and diluted into the proper concentrations for kinetic binding experiments (32 µM to 10 nM). A kinetic experiment was set up using the Biacore T100 method builder. A flow rate of 70 µl min\(^{-1}\) with an association phase of 300 s and a dissociation phase of 600 s were employed. Regeneration at the end of each run was performed with a 30 s injection of 50 mM NaOH in 0.5 M NaCl. Kinetic data (Figure S6) could not be fit to conventional binding models due to the nature of the multivalent interaction between SA and the HPQ peptide.

Sequential HPQ Immobilization on the Same Biosensor Surface. The HPQ peptide (0.5 mM) was preincubated with 4-mercaptophenylacetic acid (50 mM) and tris(carboxyethyl)phosphine (10 mM) in HBS-EP at pH 7.4 (30 min, RT). At a flowrate of 5 uL min\(^{-1}\), the ligation mixture was injected over flow channel 1 and 2 for 60 sec. Subsequent regeneration using 50 mM NaOH in 0.5 M NaCl yielded a surface with 105 RU peptide immobilized. Streptavidin (2 µM) was injected for 600 s, followed by regeneration of the surface with 50 mM NaOH in 0.5 M NaCl. This cycle was repeated 3 times, yielding the immobilization levels and binding response as indicated in Table S1.
Table S1. Sensor responses for SA-binding in the reference and sample channels after sequential ligations of SLLAHPQGGG-MPAL to the sensor chip.

<table>
<thead>
<tr>
<th>Immobilized Peptide (RU)</th>
<th>Reference Channel Binding (RU)</th>
<th>Sample Channel Binding (RU)</th>
<th>Ref. Subtracted Binding (RU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>105</td>
<td>39</td>
<td>75</td>
<td>36</td>
</tr>
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<td>193</td>
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<td>289</td>
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<td>498</td>
<td>456</td>
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</table>

Native Chemical Ligation of GFP-MESNA. GFP-MESNA was obtained as described previously. The protein thioester (0.1 mM) was preincubated with 4-mercaptophenylacetic acid (50 mM) and tris(carboxyethyl)phosphine (10 mM) in HBS-EP at pH 7.4 (30 min, RT). At a flow rate of 5 μL min⁻¹, the ligation mixture was injected over flow channels 1 and 2 for 60 min. Subsequent regeneration using 50 mM NaOH in 0.5 M NaCl yielded the surface with 450 RU protein immobilized (Figure S7). Binding experiments with α-GFP (JL-8, Clontech) were performed at a 500-fold dilution (approx. 14 nM) in HBS-EP at a flow rate of 5 μL min⁻¹.

Figure S7. Ligation of GFP-MESNA to the biosensor chip.

S-Protein Binding. S-Protein was dissolved in HBS-EP and diluted into the proper concentrations for kinetic binding experiments (1 μM to 10 nM). A kinetic experiment was set up using the Biacore T100 method builder. A flow rate of 70 μL min⁻¹ with an association phase of 300 s, a dissociation phase of 300 s, and a single regeneration step of 10 mM glycine at pH 1.5 were employed. Kinetic data were fitted to a standard 1:1 binding model using BIAevaluation software.
References

