Site-Specific Immobilization on Surface Plasmon Resonance Chips via Strain-Promoted Cycloaddition

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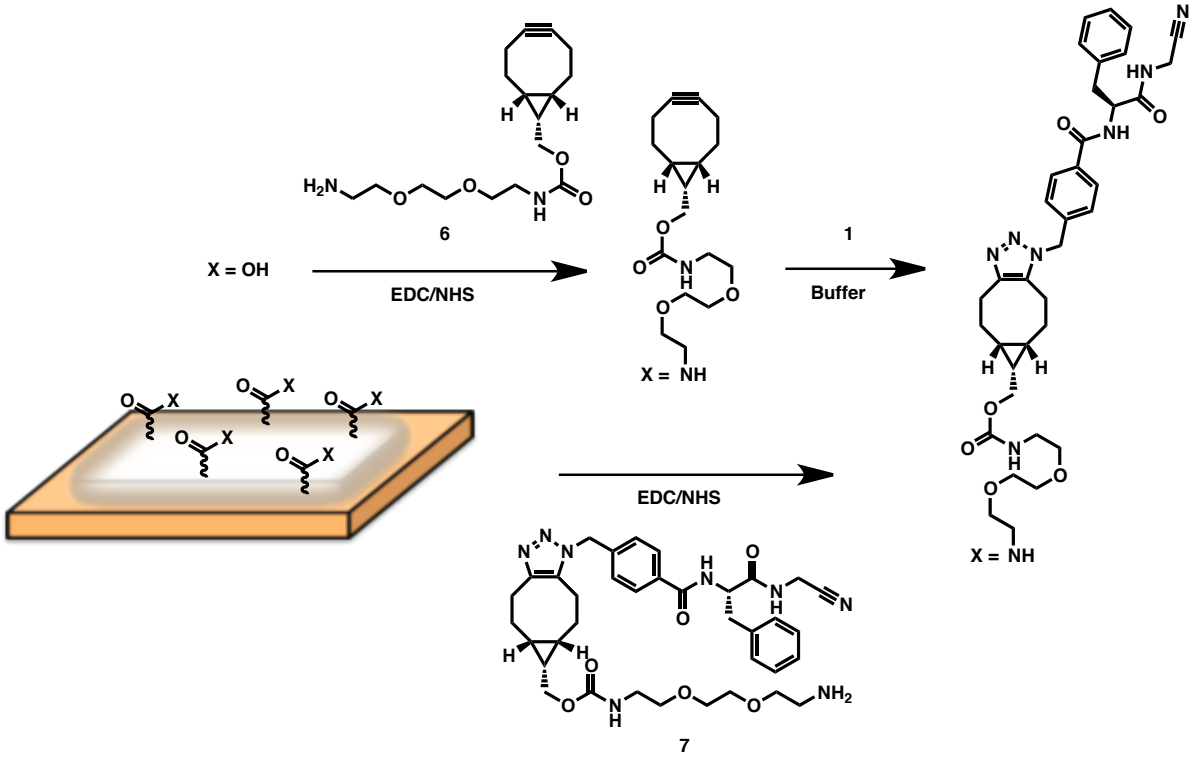
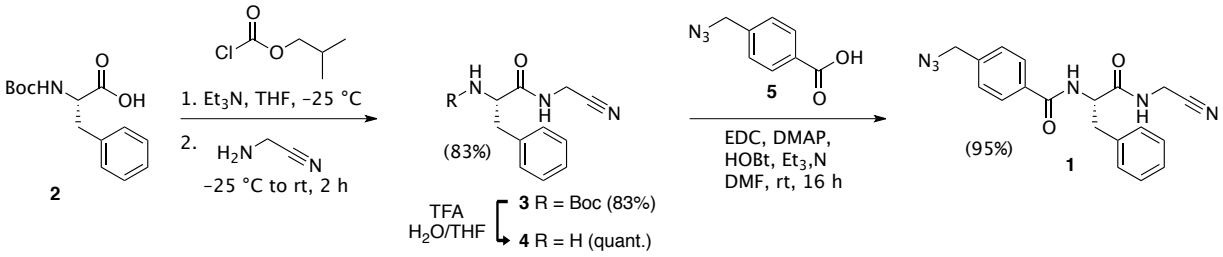
Supporting Information

Overview

1. Papain model system



Scheme S1 Schematic overview of preparation SPR surface, starting from a Biacore CM-5 chip.



Scheme S2 Synthesis of nitrile **1**

1.1 Synthesis papain ligands

General

Reactions were followed and R_F values were obtained using thin layer chromatography (TLC) on silica gel-coated plates (Merck 60 F254) with the indicated solvent mixture. Detection was performed with UV-light, and/or by charring at ~150 °C after dipping into a solution of either ninhydrin or Cl-TDM (4,4'-tetramethyldiamino-diphenylmethane). Melting points were analyzed with a Büchi melting point B-545. IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer, or a Bruker Tensor 27 FTIR spectrometer. NMR spectra were recorded on a Bruker DMX 300 (300 MHz), and a Varian 400 (400 MHz) spectrometer in $CDCl_3$ solutions (unless otherwise reported). Chemical shifts are given in ppm with respect to tetramethylsilane (TMS) as internal standard. Coupling constants are reported as *J*-values in Hz. Peak assignments in ^{13}C spectra are based on 2D-GHSQC and GHMBC spectra. Column or flash chromatography was carried out using ACROS silica gel (0.035–0.070 mm, and ca 6 nm pore diameter). Optical rotations were determined with a Perkin Elmer 241 polarimeter. High-resolution mass spectra were recorded on a JEOL AccuTOF (ESI), or a MAT900 (EI, CI, and ESI).

Boc-Phe-NHCH$_2$CN (3)

Under nitrogen atmosphere, Boc-Phe-OH (1.33 g; 5.0 mmol) was dissolved in dry THF (15 mL) and cooled to -25 °C. Subsequently, freshly distilled Et_3N (695 µL; 5.0 mmol) and isobutyl chloroformate (652 µL; 5.0 mmol) were sequentially added under vigorous stirring. After 30 minutes, a solution of aminoacetonitrile sulfate (850 mg; 5.5 mmol) in H_2O (0.5 mL), precooled on ice, and Et_3N (764

µL, 5.5 mmol) were added. The resulting mixture was allowed to warm to room temperature. After 2 h, THF was removed under reduced pressure and the residual aqueous mixture was diluted with a small volume of H_2O, adjusted to pH 1 (10% $KHSO_4$) and extracted with ethyl acetate (3 x 20 mL). The combined organic layers were washed with H_2O (10 mL), sat. $NaHCO_3$ (2 x 10 mL) and brine (10 mL), dried over Na_2SO_4 and evaporated to dryness. The solid residue was recrystallized from cyclohexane/ethyl acetate to obtain the expected product (1.26 g; 83%) as white crystals. R_F = 0.45 (2.5% MeOH/CH_2Cl_2); m.p. = 136-138 °C; $[\alpha]^{20}_D$ = -0.60. ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.32 (s, 9H, $C(CH_3)_3$), 3.00-3.04 (m, 2H, CH_2Ph), 4.03-4.05 (d, *J* = 5.6 Hz, 2H, CH_2CN), 4.33-4.35 (d, *J* = 7.2 Hz, 1H, C<u>H</u>CH_2Ph), 5.07-5.09 (d, *J* = 7.2 Hz, 1H, NH), 6.82 (bs, 1H, NH), 7.13-7.29 (m, 5H, Ph). ^{13}C NMR (CDCl3, 75 Hz) δ = 27.16, 27.27, 28.22, 38.13, 55.29, 80.82, 115.51, 127.17, 128.82, 129.20, 136.01, 155.64, 155.73, 171.84. HRMS (ESI) *m/z* calc. for $C_{16}H_{22}N_3O_3$ $[M+H]^+$: 304.1675; found 304.1661.

H-Phe-NHCH$_2$CN (4)

To a cold solution (0 °C) of Boc-Phe-NHCH$_2$CN (600 mg, 1.98 mmol) in H_2O/THF (1:1 v/v, 0.1 M) was added TFA (8 portions of 10 eq). The mixture was stirred for 2 h at room temperature, after which the solvent was removed under reduced pressure and the reaction mixture was lyophilized to obtain the expected product (594 mg, quant.) as white crystals. m.p. = 146-155 °C; ^{1}H NMR (MeOD, 400 MHz) δ = 3.07-3.13 (dd, *J* = 7.2, 13.8 Hz, 1H, CH_2Ph), 3.16-3.21 (dd, *J* = 7.2, 13.8 Hz, 1H, CH_2Ph), 4.08-4.12 (t, *J* = 7.2 Hz, 1H, C<u>H</u>CH_2Ph), 4.14-4.16 (d, *J* = 7.6, 2H, CH_2CN), 7.25-7.37 (m, 5H, Ph). ^{13}C NMR (DMSO, 75 MHz) δ = 27.09, 36.82, 53.39, 116.90, 127.27, 128.60, 129.49, 134.60, 158.32, 158.74, 168.81. HRMS (ESI) *m/z* calc. for $C_{11}H_{14}N_3O_1$ $[M+H]^+$: 204.1138; found 204.1137.

4-(Azidomethyl)benzoic acid (5)

At 0 °C, 4-(bromomethyl)benzoic acid (1.05 g; 4.88 mmol) was dissolved in water/DMSO (1:1 v/v; 1M) and sodium azide (1.27 g; 19.5 mmol) was added. The reaction mixture was allowed to stir for 24 h at room temperature, after which extra water was added and the pH was set to 3 with a 1M HCl solution. The resulting mixture was extracted with EtOAc (3 x 10 mL), the combined organic layers were washed with brine (15 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The product was formed in quantitative yield as a slightly yellow oil.

R_F = 0.55 (1:1 EtOAc/Heptane + 1% AcOH); ^{1}H NMR (DMSO, 400 MHz) δ = 4.56 (s, 2H, CH_2N_3), 7.47-7.49 (d, *J* = 8.0 Hz, 2H), 7.95-7.97 (d, *J* = 8.5 Hz, 2H). ^{13}C NMR (DMSO, 75 MHz) δ = 53.07, 128.32, 129.68, 130.42, 140.60, 166.97. FTIR = 2539, 2105, 1678, 1290.

4-(Azidomethyl)benzoyl-Phe-NHCH$_2$CN (1)

To a solution of 4-(azidomethyl)benzoic acid (183 mg; 1.03 mmol) and H-Phe-NHCH$_2$CN (295 mg; 0.98 mmol) in EtOAc (0.1 M) was added DMAP (240 mg; 1.96 mmol), Et_3N (274 µL; 1.96 mmol), HOBt (150 mg; 0.98 mmol) and EDC (198 mg; 1.03 mmol). The reaction mixture was allowed to stir for 16 h, after which the mixture was washed with $KHSO_4$ (2 x 5 mL), $NaHCO_3$ (2 x 5 mL) and brine (5 mL), dried over Na_2SO_4 and concentrated in vacuo. Column chromatography (5% MeOH/CH_2Cl_2) afforded the expected product (230 mg; 95 %) as white crystals. R_F = 0.39 (5% MeOH/CH_2Cl_2); m.p. = 90-93 °C; $[\alpha]^{20}_D$ = -38.3. ^{1}H NMR (acetonitrile, 400 MHz) δ = 3.09 (dd, *J* = 13.9, 9.4 Hz, 1H, CH_2Ph), 3.35 (dd, *J* = 14.0, 5.2 Hz, 1H, CH_2Ph), 4.10 (dd, *J* = 5.9, 4.0 Hz, 2H, CH_2CN), 4.49 (s, 2H, CH_2N_3), 4.82 (m, 1H, $C_\alpha H$), 7.26 (m, 1H), 7.33 (m, 5H, CH_2Ph), 7.49 – 7.43 (m, 2H), 7.81 – 7.76 (m, 1H) ppm. ^{13}C NMR (acetonitrile, 75 MHz) δ = 28.22, 37.88, 54.62, 55.83, 118.31, 127.66, 128.67, 129.25, 129.41, 130.25, 131.01, 134.55, 138.51, 140.69, 167.54, 172.65 ppm. HRMS (ESI) *m/z* calc. for $C_{19}H_{19}N_6O_2$ $[M+H]^+$: 363.1579; found 363.1570. FTIR = 3273, 3057, 2932, 2552, 2223, 2105, 1683, 1634, 1533, 1294, 1240 cm^{-1}.

Nitrile 7

To a solution of BCN-POE$_3$-NH$_2$ (4.48 mg; 14.0 µmol) in MeOH (1.0 mL) was added ligand **1** (5.0 mg; 14.0 µmol) and the mixture was allowed to stir for 15 min, after which full conversion was seen. The product was concentrated *in vacuo* and purified using column chromatography (5% MeOH/CH_2Cl_2 + 1% Et_3N) affording the expected product in 70% yield as colorless oil. R_F = 0.34 (5% MeOH/CH_2Cl_2 + 1% Et_3N). HRMS (ESI) *m/z* calc. for $C_{36}H_{47}N_8O_6$ $[M+H]^+$: 687.3619; found 687.3619.

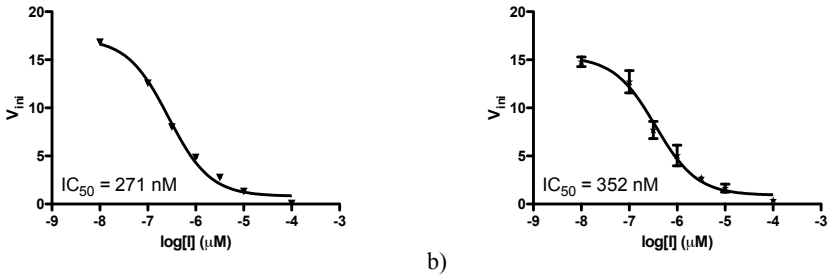
Amine 8

To a solution of BCN-POE$_3$-NH$_2$ (4.48 mg; 14.0 µmol) in MeOH (1.0 mL) was added azidoethanol (5.0 mg; 14.0 µmol) and the mixture was allowed to stir for 15 min, after which full conversion was seen. The product was concentrated *in vacuo,* affording the expected product in 95% yield as colorless oil. HRMS (ESI) *m/z* calc. for $C_{19}H_{34}N_5O_5$ $[M+H]^+$: 412.2557; found 412.2560.

1.2 Enzymatic measurements

1.2.1 UV measurements ligand 1 and 7

Enzyme activities were calculated from kinetic measurements performed by spectrometric detection as described by Löser *et al.*[1] For ligand **1** and **7** an IC_{50} value of 271 nM, respectively 352 nM was found (Figure S1).



a) b)

Figure S1 IC_{50} values found for a) ligand **1** and b) ligand **7.**

1.2.2 Isothermal Titration Calorimetry

In order to validate the values found with the SPR experiments, isothermal titration calorimetry (ITC) was applied as a label-free method to measure the binding affinity of a ligand with an analyte. Thus, after mixing of solutions of 10 µM papain and 50 µM ligand **7**, ITC measurements and fitting of data, a K_D between ligand **7** and papain was established as 235±45 nM (Figure S2), which is in the same range as values obtained by the SPR experiments.

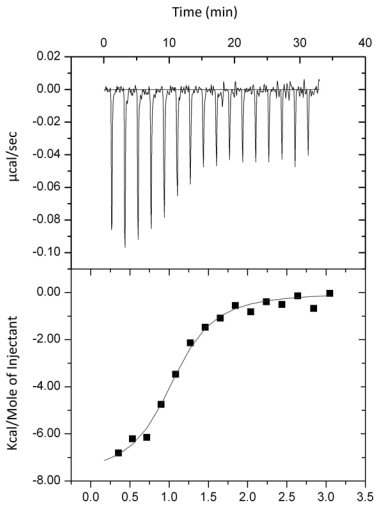


Figure S2 Isothermal titration calorimetry of papain with ligand **7**.

Experimental - Heats of interaction were measured at 25 °C with an ITC_{200} automated system (GE Healthcare – Microcal). The protein and ligands were dissolved in a degassed 0.1 M phosphate buffer (pH 6.5) containing 2.5 mM EDTA and 15 mM DTT with a final concentration of 0.5% DMSO. The cell was filled with 200 µL of a 10 µM papain solution and a 50 µM solution of ligand **7** was injected in 16 steps of 2.5 µL. Experiments were performed in triplicate. Heats of dilution were assayed from endpoint signals after saturation of binding. Blank experiments involving the titration of buffer against papain were carried out and used for subtraction of the background heat change. The corrected data were fitted with a single site-binding model with floating values for stoichiometry, binding constant (K_A), and enthalpy change (ΔH), using the Microcal – Origin software delivered with the ITC_{200} system.

1.2.3 Competition experiment

Competition experiments were performed in order to observe the influence of the cyclooctyne-derived triazole moiety on the affinity between the ligand and papain. To this end, the ligand containing the azide moiety (**1**) was compared to ligand **7** S, the triazole structure obtained by SPAAC reaction of **1** with BCN-containing amine. After 10 minutes incubation of papain with a concentration range of either ligand **1** or ligand **7**, the mixture was injected and the SPR signals were measured (Figure S3a). As expected, more inhibitor led to reduced binding of papain to the surface. More importantly, the competition experiments showed that the influence of the BCN moiety is minimal (Table S1), which is consistent with results from the UV assay (Figure S1).

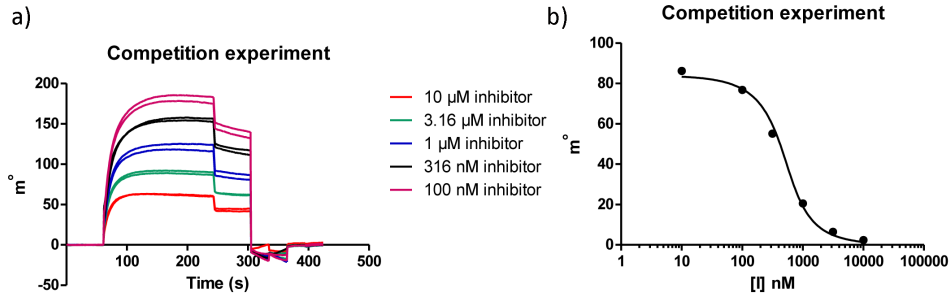


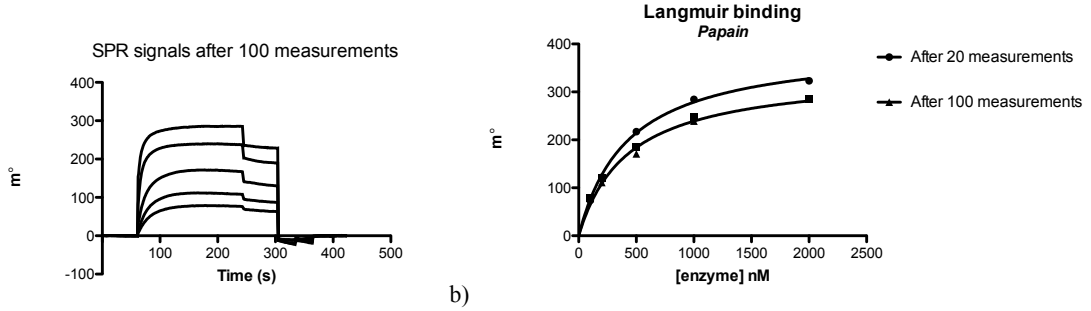
Figure S3 a) SPR signals for the competition experiments (performed in duplo) and b) SPR competition experiments in the presence of 500 nM papain and various concentrations of competing ligand **1** or **7**. Data are fitted with a competition model yielding the affinity in solution (K_S) as described by de Mol et al.[2]

Table S1 Competition experiment to obtain K_S in the presence of 500 nM papain and different ligand concentrations.

Compound	SPAAC protocol	Amine protocol
1	500 ± 100 nM	1200 ± 300 nM
	500 ± 50 nM	1200 ± 150 nM
7	600 ± 100 nM	1700 ± 400 nM
	700 ± 100 nM	1600 ± 400 nM

1.2.4 Experiment to determine stability of functionalization

The papain Langmuir binding experiment, as described above, was performed on a freshly modified SPR surface and after 100 measurements. In both cases good Langmuir binding graph were obtained resulting in a consistent K_D value.



a) b)

Figure S4 a) SPR signals after 100 measurements on the CM5 chip modified via SPAAC. Papain concentrations from 100 to 2000 nM were added to the surface; b) Langmuir binding on the same chip after 20 and 100 measurements. Although the B_{max} is lower, the K_D is for both measurements 350 ± 50 nM.

2. Anl-GFP model system

2.1 Synthesis of Anl-GFP and reactivity test

To introduce an azide into GFP, an azidonorleucineresidue (Anl) was incorporated into GFP by employing residue-specific non-canonical amino acid incorporation to produce Anl-GFP.[3] Here, Anl was used as a methionine (Met) analogue and to reduce the number of azides in the protein, an engineered GFP gene with only one methionine codon (start codon) was used. The GFP was also equipped with an N-terminal histidine and a C-terminal FLAG-tag for purification and detection (Figure S5). Because Anl is not a substrate for wild-type methionyl-tRNA synthetase (MetRS), a mutant MetRS (L13N, Y260L, H301L; NLL-MetRS), which is able to efficiently activate Anl, was used for the expression of the engineered GFP in methionine auxotrophic *E. coli*.[4]

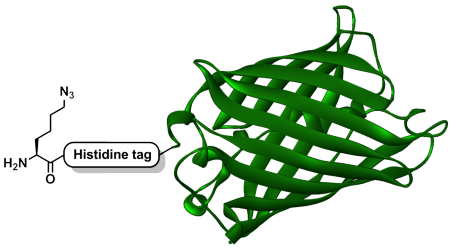


Figure S5 Graphical representation of Anl-GFP

The expression was carried out as described by Tirrell *et al.*[4] After expression of Anl-GFP and Met-GFP (control protein) the proteins were purified via affinity chromatography with a Ni^{2+}NTA column. The purity was verified by SDS-PAGE (Figure S6a) and the exact mass was determined by mass spectrometry (Figure S6b). The deconvoluted masses corresponded to the theoretical masses (Anl-GFP = 29018.4 Da and Met-GFP = 28995.4 Da). The absorbance spectrum of Anl-GFP was measured (Figure S6c) and a yield of 10-25 mg/L of culture was determined for Anl-GFP using the molar extinction coefficient.

To demonstrate the reactivity of the azide in Anl-GFP, a conjugation reaction with BCN-PEG_3-NH-lissamine rhodamine B conjugate (BCN-LisRhoB (**9**), Figure S8) was performed. The product was then analyzed by SDS-PAGE (Figure S7a) and size exclusion chromatography (SEC) on a Superdex 75 3.2/30 column (Figure S7b). In the fluorescence analysis of the SDS-PAGE a fluorescent band corresponding to the labeled product was observed for Anl-GFP, while no reaction was observed for the control protein. The same result can be found in the SEC analysis. GFP eluted from the column at a volume of 1.2 mL and only in the case of Anl-GFP coelution of **9** was observed, showing that **9** is attached to Anl-GFP.

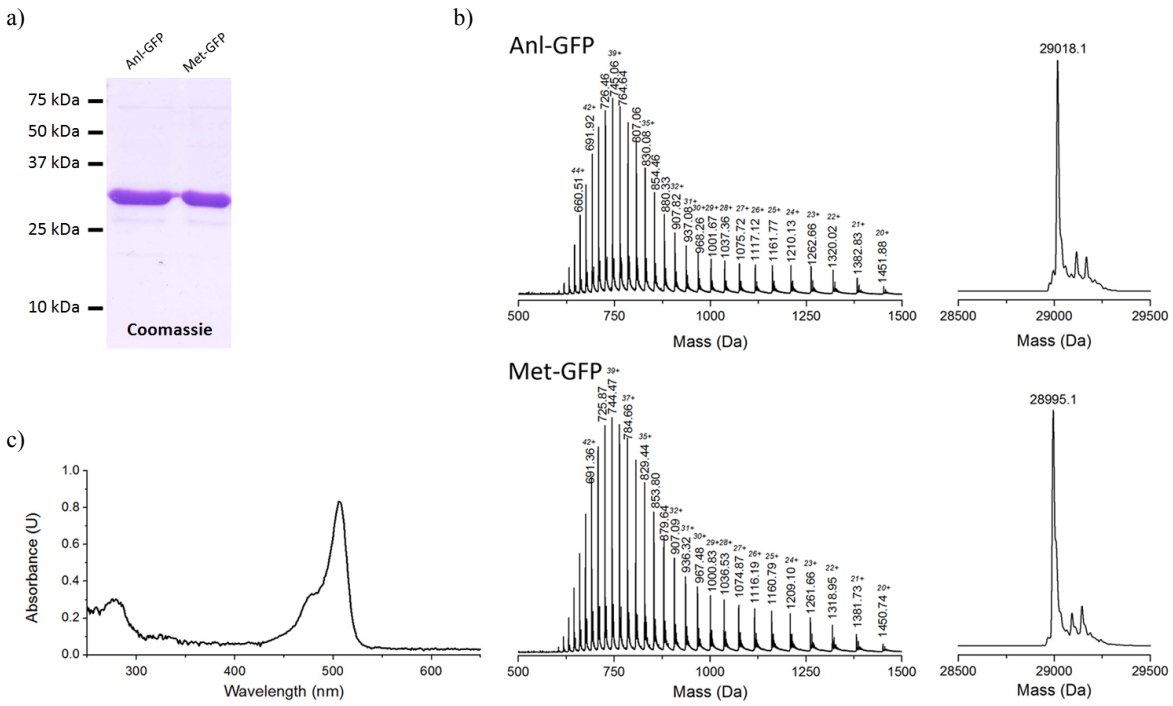


Figure S6 Coomassie-stained SDS-PAGE (a) and mass spectra(b) multiply charged ion series (left) and deconvoluted total mass spectrum (right) of purified Anl-GFP and Met-GFP. The absorbance spectrum of Anl-GFP (c)

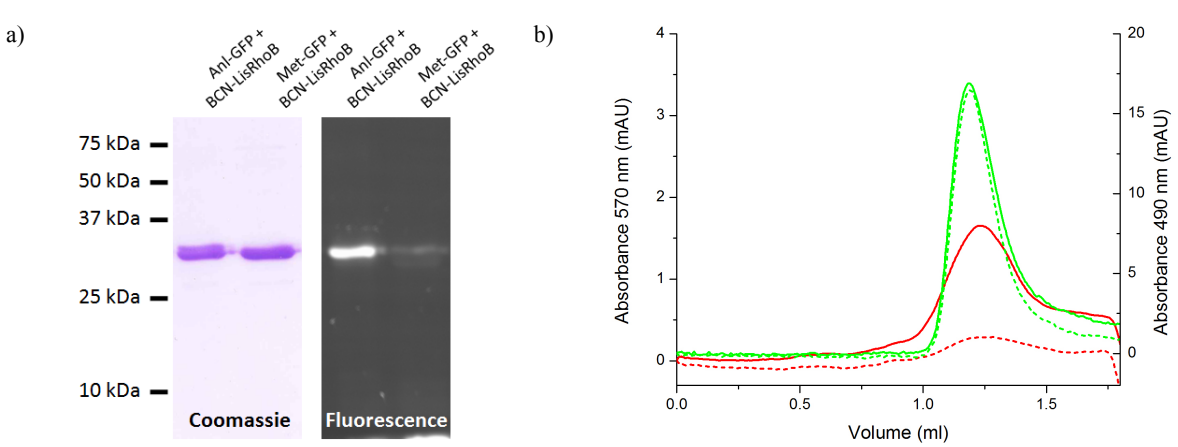
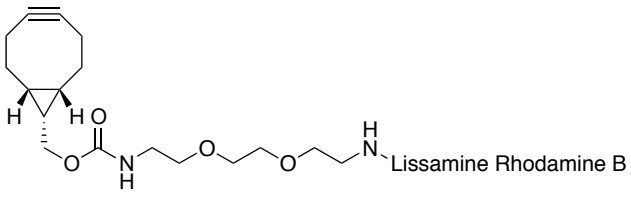


Figure S7 Analysis of reaction by Coomassie-stained and fluorescent SDS-PAGE (a) and by SEC (b) of purified Anl-GFP (solid lines) and Met-GFP (dashed lines) with BCN-LisRhoB. The green lines correspond to the GFP absorbance at 490 nm and the red lines correspond to the lissamine rhodamine absorbance at 570 nm.



9

Figure S8 BCN-PEG_3-NH-lissamine rhodamine B conjugate **9**.

2.2 SPR Langmuir binding of Anl-GFP with monoclonal Anti-polyHistidine

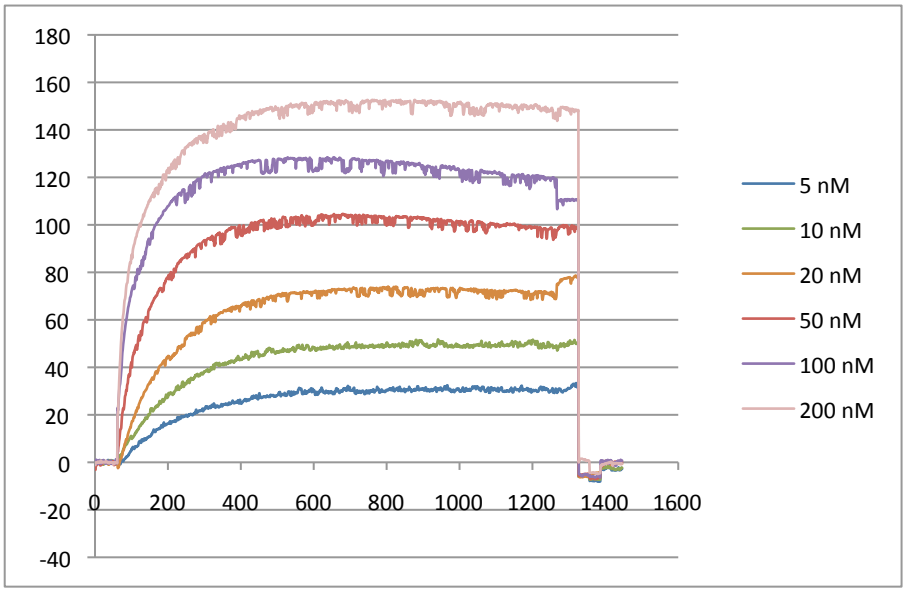


Figure S9 SPR data of Anti-His binding to immobilized Anl-GFP. Different anti-His concentrations were added to the surface.

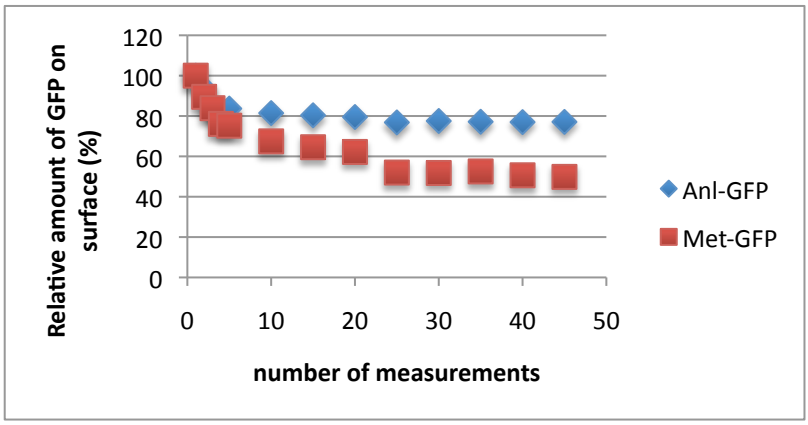


Figure S10 Stability check of SPR surfaces for both immobilization strategies. For the Met-GFP more material is lost after 50 measurements, then with Anl-GFP.

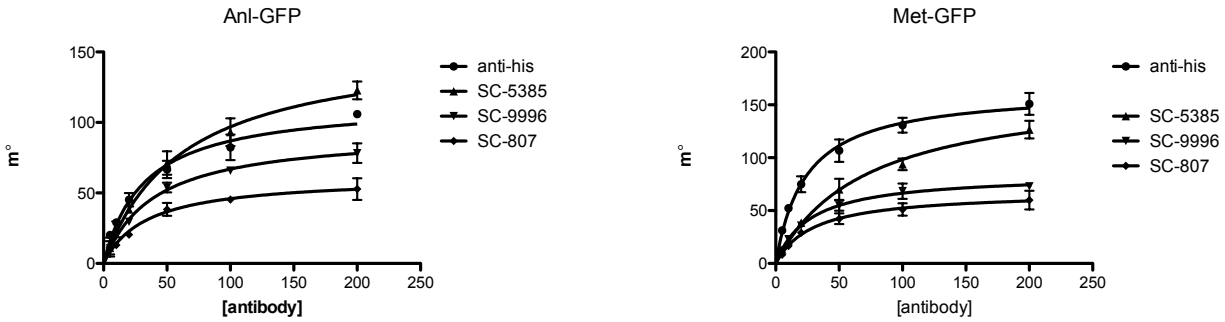


Figure S11 Langmuir binding curves for Anl-GFP and Met-GFP for the four different antibodies

Table S2: K_D values obtained of different antibodies for Met-GFP.

Antibody	K_D *(nM)*
Anti-His	23.8 ± 2.5
Anti-GFP (1)	71.0 ± 8.7
Anti-GFP (2)	27.0 ± 2.5
Anti-FLAG	29.8 ± 4.7

3. References

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