Supporting Information

Proximity-Enabled Sulfur Fluoride Exchange Reaction in Protein Context

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Reagents and molecular biology

Primers were synthesized and purified by Integrated DNA Technologies (IDT), and plasmids were sequenced by GENEWIZ. All molecular biology reagents were obtained from either New England Biolabs or Vazyme. His-HRP antibody was obtained from ProteinTech Group. Wild-type Spike RBD (SRD-C52H3) were purchased from ACROBiosystems. Mutant SARS-CoV-2 (COVID-19) S proteins (RBD) were purchased from ACROBiosystems: 1. E484K (#SRD-C52H3); 2. F490L (#SRD-C52Hf); 3. N501Y (#SRD-C52Hn); 4. N439K (#SRD-C52Hg); 5. K417N, E484K, N501Y (#SPD-C52Hp).

pBAD-MBP-Z(24TAG), pBAD-Afb4A-7X (X = Lys, His, Tyr), pBAD-7D12(109TAG) and pBAD-SR4(57TAG) were used as previously described.[1] pEvol-FSYRS, pEvol-mFSYRS and pEvol-SFYRS were used as previously described.[1a]

Incorporation of FSY into MBP-Z(24TAG), 7D12(109TAG), and SR4(57TAG)

pBAD plasmid encoding MBP-Z(24TAG), 7D12(109TAG), or SR4(57TAG) was co-transformed with pEVOL-FSYRS into *E. coli* DH10β cells. Cells were plated on LB agar plate supplemented with 50 μg/mL ampicillin and 34 μg/mL chloramphenicol. After 12 h, a single colony was picked and inoculated into 1 mL 2xYT (5 g/L NaCl, 16 g/L Tryptone, 10 g/L Yeast extract) supplied with 50 μg/mL ampicillin and 34 μg/mL chloramphenicol. The cells were left grown at 37 °C, 220 rpm for overnight. Cells were then diluted 20 times in fresh 2xYT supplemented with relevant antibiotics and cultured at 37 °C. When cells reached to an OD_{600} of 0.8, 0.2% *L*-arabinose and 1 mM FSY was added. The cells were then induced at either 30 °C for 6 h or 18 °C for overnight. Cells were then collected by centrifugation for protein purification.

Incorporation of mFSY or SFY into ecGST107X(103TAG) for *in vivo* cross-linking

pBAD plasmid encoding ecGST107X(103TAG) was co-transformed with pEVOL-mFSYRS or pEvol-SFYRS into DH10β cells. Cells were plated on LB agar plate supplemented with 50 μg/mL ampicillin and 34 μg/mL chloramphenicol. After 12 h, a single colony was picked and inoculated into 1 mL 2xYT (5 g/L NaCl, 16 g/L Tryptone, 10 g/L Yeast extract) supplied with 50 μg/mL ampicillin and 34 μg/mL chloramphenicol. The cells were left grown at 37 °C, 220 rpm for overnight. Cells were then diluted 20 times in fresh 2xYT supplemented with relevant antibiotics

and at cultured at 37 °C. When cells reached to an OD_{600} of 0.8, 0.2% *L*-arabinose was added. 1 mM mFSY or 1 mM SFY was also added. The cells were then induced at 30 °C. At the indicated time points, 100 µL *E. coli* cells were extracted, centrifuged and resuspended in 50 µL Laemmli loading buffer. The loading buffer was then boiled at 95 °C for 10 min. The covalent dimerization of ecGST was analyzed on denatured Western blot using anti-6His antibody.

His-tag protein purification

Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, 1% v/v Tween 20, 10%v/v glycerol, DNase 0.02 mg/mL, and protease inhibitors). The cell suspension was lysed by sonication (60% output, 3 sec off, 3 sec on) in an ice-water bath, followed by centrifugation (20,000 g, 15 min, 4 °C). The supernatant was collected and incubated with pre-equilibrated Protino®Ni-NTA Agarose resin at 4 °C for 1 h with constant mechanical rotation, after which the slurry was loaded onto a Poly-Prep® Chromatography Column. The resin was then washed with 20 mL wash buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole) for 3 times, and eluted with 500 µL elution buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 300 mM imidazole). The eluates were buffer exchanged into 200 µL of PBS (pH 7.4) using Amicon Ultra columns and stored at -80 °C.

Band intensity quantification and k_{obs} calculation

The linear plot of natural logarithm (ln) of the protein intensity versus time (h) gives k_{obs}. k_{obs} was calculated based on the time-dependent intensity decrease of the protein band. The band intensities were quantified with Bio-rad imaging software. The band intensities at 0 h was set as 100%. The band intensities at other time points were quantified as the percentage (%) of the band intensity at time 0. The linear plot of natural logarithm (ln) of the band intensity percentage versus time (h) gives k_{obs}.

Dissociation constant measurement between Spike RBD and SR4 nanobody

To facilitate detection, a biotin tag was installed on the SR4 nanobody using genetic code expansion and click chemistry (**Scheme S1**). Briefly, unnatural amino acid 4-azido-L-phenylalanine (AzF) was incorporated to the 5th position of SR4 nanobody using the orthogonal AzF-specific tRNA/AzFRS to prepare mutant nanobody SR4(5AzF). To conjugate biotin via the

azido group through copper-free click chemistry, 1 mg/mL SR4(5AzF) was react with 0.5 mM DBCO-biotin (Sigma-Aldrich, # 760706) in PBS (pH 7.4) at room temperature for 3 h. Excess DBCO-biotin was removed by 10 kDa cut-off spin column. The biotin labeled SR4 was concentrated to 1.5 mg/mL and store at -80 °C.

Binding constants between Spike RBD and SR4 nanobody was measured by biolayer interferometry (BLI) using Octet Red384 systems (ForteBio). Biotinylated SR4 was firstly loaded to streptavidin (SA) sensor (ForteBio #18-5019) by incubating the SA sensor in 200 nM biotinylated SR4 in Kinetic Buffer [0.005 % (v/v) Tween 20 and 0.1 % BSA in PBS, pH = 7.4] at 25 °C. The sensor was equilibrated (baseline step) in Kinetic Buffer for 120 s, after which the sensor was incubated with Spike RBD (association step) for 180 s. The concentrations for Spike RBD were 0, 250, 500, 1000, and 2000 nM. The sensor was then moved into Kinetic Buffer (dissociation step) for 300 s. Data was fitted for a 1:1 stoichiometry and K_d, k_{on}, and k_{off} were calculated using the built in software.

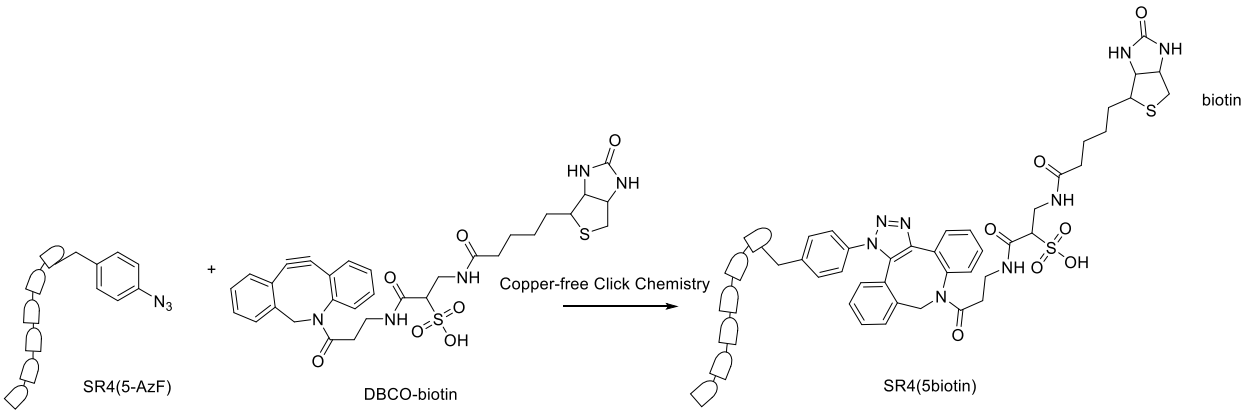
Dissociation constant measurement between wild-type MPB-Z and Afb-7K, Afb-7H, or Afb-7Y.

Biotinylated MBP-Z was prepared using the same method as for the preparation of biotinylated SR4 nanobody, which was described in detail above. The biotinylated MBP-Z was firstly loaded to streptavidin (SA) sensor (ForteBio #18-5019) by incubating the SA sensor in 200 nM biotinylated MBP-Z in Kinetic Buffer [0.005 % (v/v) Tween 20 and 0.1 % BSA in PBS, pH = 7.4] at 25 °C. The sensor was equilibrated (baseline step) in Kinetic Buffer for 120 s, after which the sensor was incubated with Afb (association step) for 300 s. The concentrations for Afb were 0, 25, 50, 100, and 200 μM. The sensor was then moved into Kinetic Buffer (dissociation step) for 600 s. Data was fitted for a 1:1 stoichiometry and K_d, k_{on}, and k_{off} were calculated using the built in software.

Cross-linking of 7D12(109FSY) with endogenous EGFR on cell surface

A431 cells were seeded into 12-well plate at a density of 2.5×10^6 cells per well and cultured overnight. The cells were treated with 3.2 μM, 0.8 μM, 0.4 μM, 0.2 μM or 0.1 μM 7D12(109FSY) for 2, 4, 6, 8 or 10 h. Cells were then collected by centrifugation at 500 g for 3 min and lysed by

RIPA buffer. The cell lysates were separated on SDS-PAGE and subjected to Western-blot detection with 1:10000 anti-Hisx6 antibody. GAPDH was used as loading control.



Scheme S1. Preparation of biotinylated SR4 using genetic code expansion and click chemistry.

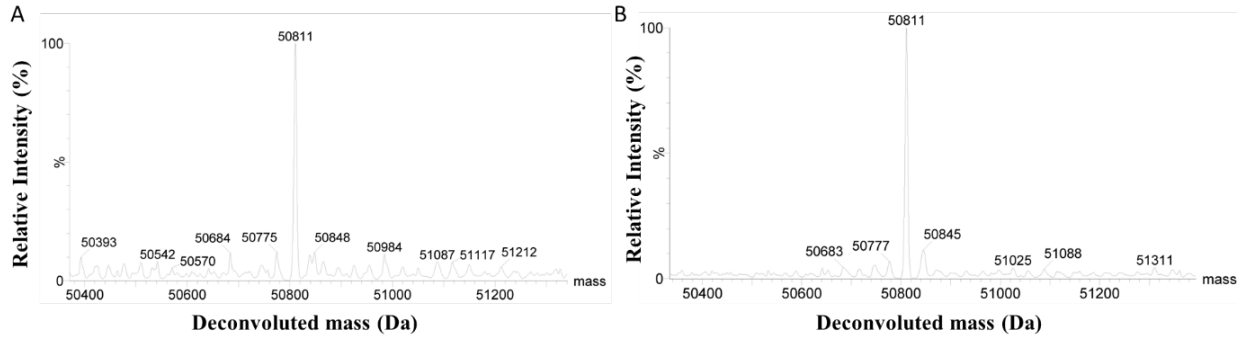


Figure S1. ESI-TOF MS spectrum of intact MBP-Z(24FSY) before (A) and after 24 h incubation in PBS (pH = 7.4) at 37 ℃ (B). 1 mg/mL MBP-Z(24FSY) was incubated in PBS (pH = 7.4) at 37 ℃. At the time point of 0 h and 24 h, the protein sample was extracted and analyzed by high resolution electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS). For sample at t = 0 h, the major peak observed at 50811 Da corresponds to intact MBP-Z containing FSY at site 24 (**A**). After 24 h incubation in PBS (pH = 7.4) at 37 ℃, mass spectrum showed that the peak at 50811 Da remained as the major peak and no peaks corresponding to FSY hydrolysis (minus 2 Da) or intramolecular cross-linking (minus 20 Da) were detected (**B**).

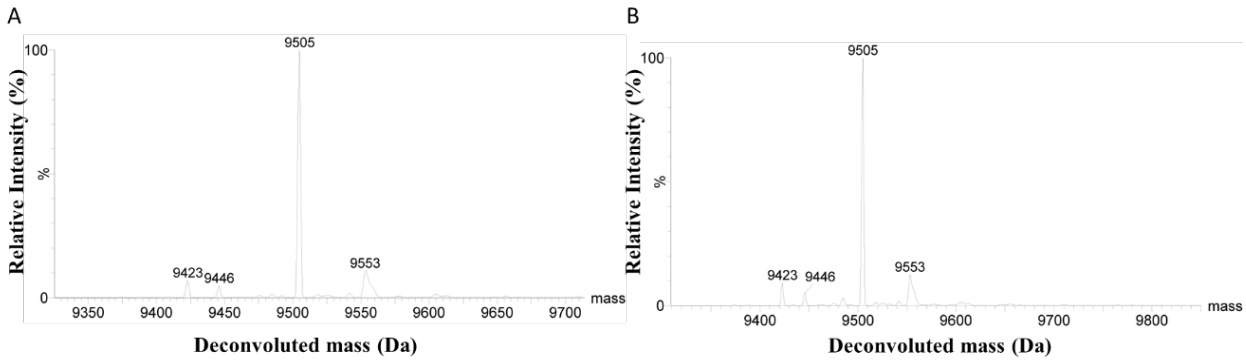


Figure S2. ESI-TOF MS spectrum of intact Ub(6FSY) before (A) and after 24 h incubation in PBS (pH = 7.4) at 37 °C (B). 1 mg/mL Ub(6(FSY) was incubated in PBS (pH = 7.4) at 37 °C. At the time point of 0 h and 24 h, the protein sample was extracted and analyzed by ESI-TOF MS. The major peak observed at 9505 Da corresponds to intact ubiquitin containing FSY at site 6 (**A**). After 24 h incubation in PBS (pH = 7.4) at 37 °C, the peak at 9505 Da remained as the major peak and no peaks corresponding to FSY hydrolysis (minus 2 Da) or intramolecular cross-linking (minus 20 Da) were observed (**B**).

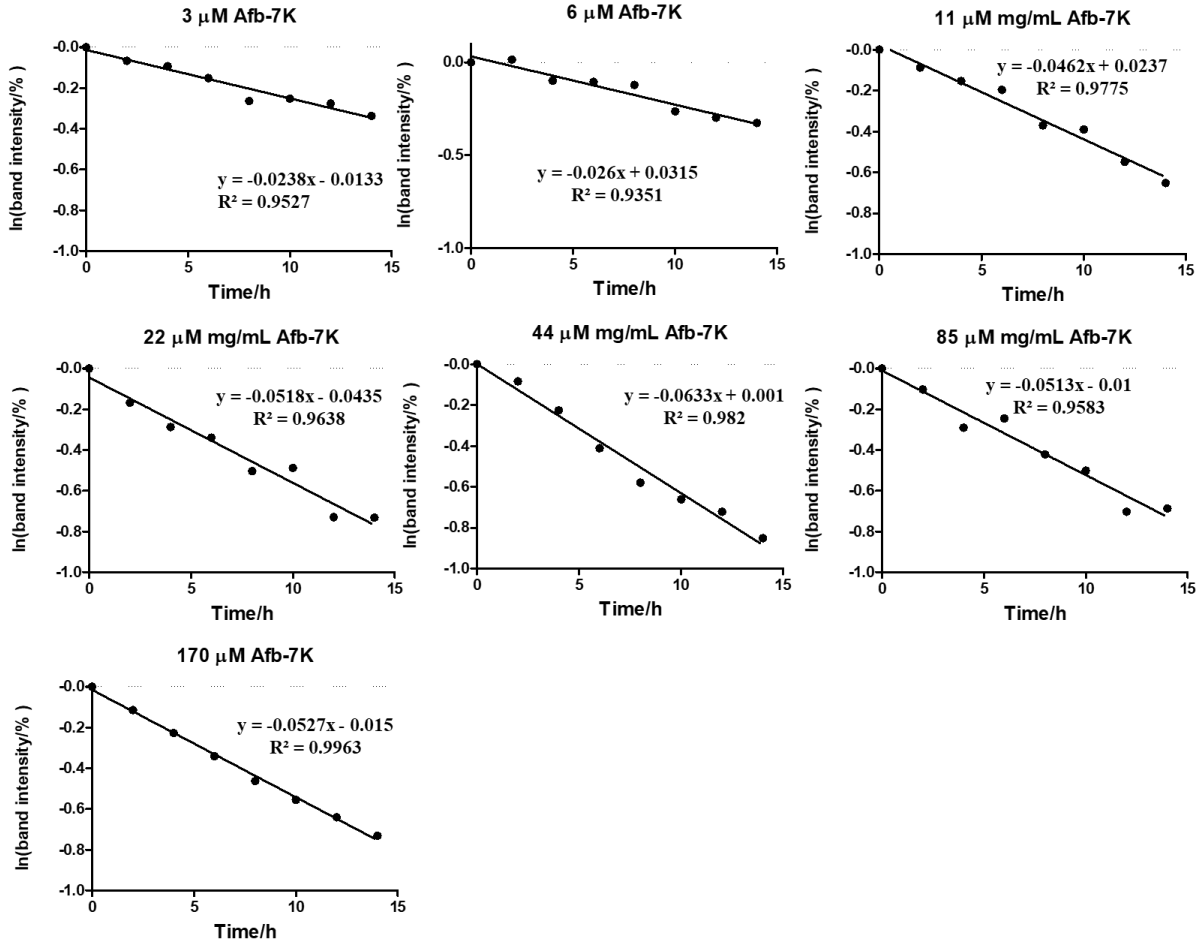


Figure S3. Kinetics of MBP-Z24FSY (6 μM) cross-linking with various concentrations of Afb-7K *in vitro*. k_{obs} was calculated based on the time-dependent intensity decrease of the MBP-Z24FSY protein band. The band intensities in the SDS-page were quantified with Bio-rad imaging software. The linear plot of natural logarithm (ln) of the MBP-Z24FSY band intensity (%) versus time (h) gives k_{obs}. The experiments were independently repeated three times, and the data for one time are shown here.

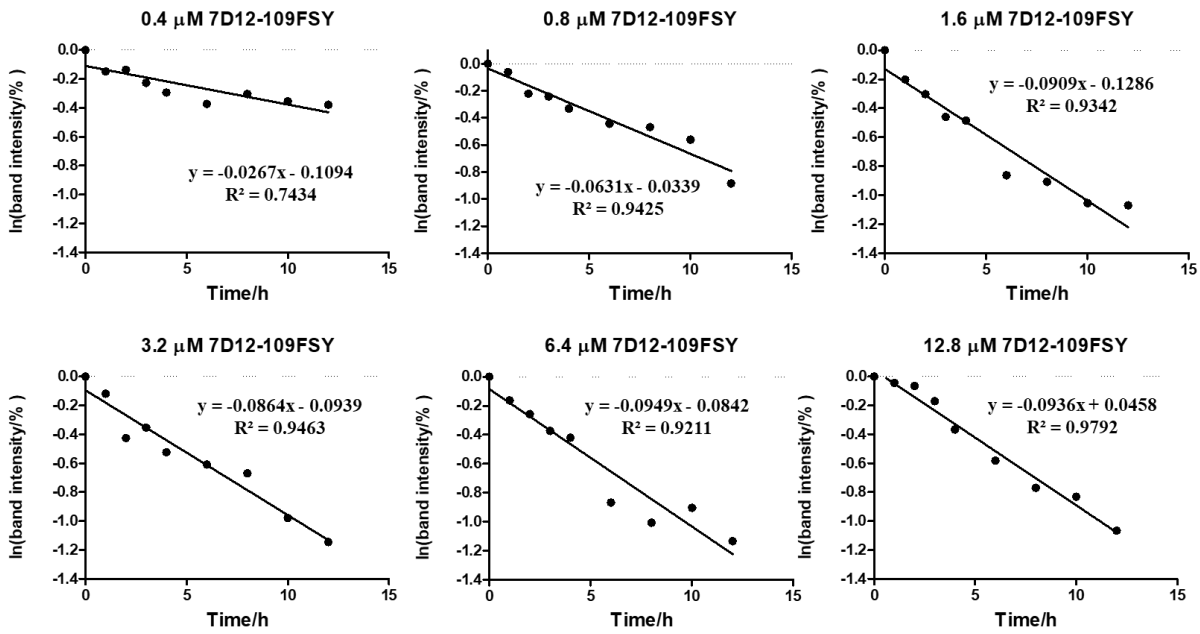


Figure S4. Kinetics of EGFR (0.2 μM) cross-linking with various concentrations of 7D12-109FSY *in vitro*. k_{obs} was calculated based on the time-dependent intensity decrease of the EGFR protein band. The band intensities in the SDS-page were quantified with Bio-rad imaging software. The linear plot of natural logarithm (ln) of the EGFR band intensity (%) versus time (h) gives k_{obs}. The experiments were independently repeated three times, and the data for one time are shown here.

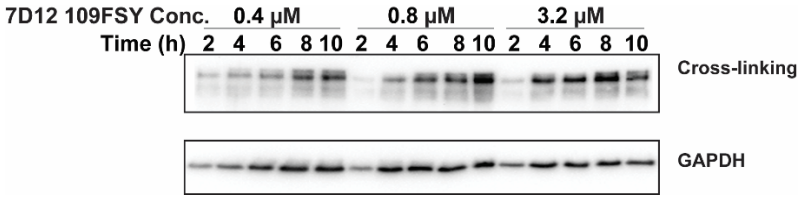


Figure S5. Cross-linking between 7D12(109FSY) and EGFR on cell surface. Cells were incubated with varying concentration of 7D12(109FSY) for the indicated time, after which cell lysate was analyzed with denatured Western blot and visualized using anti-Hisx6 antibody. GAPDH was used as loading control.

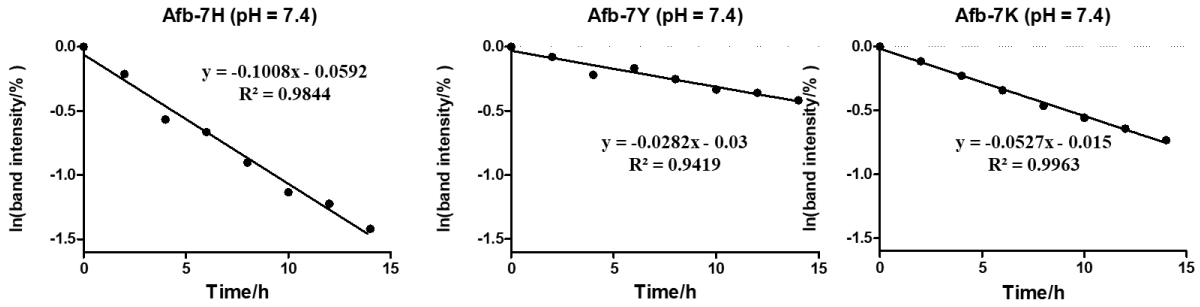
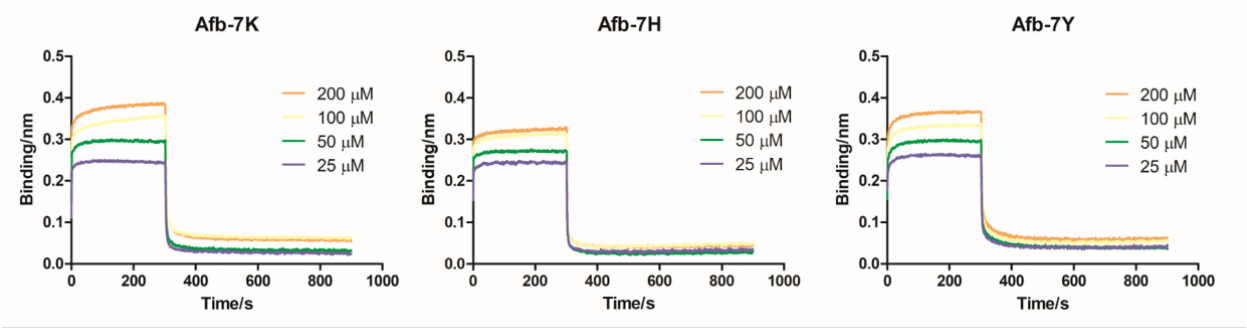


Figure S6. Kinetics of MBP-Z24FSY (6 μM) cross-linking with 192 μM Afb-7K, Afb-7H, or Afb-7Y at pH 7.4 *in vitro*. k_{obs} was calculated based on the time-dependent intensity decrease of the MBP-Z24FSY protein band. The band intensities in the SDS-page were quantified with Bio-rad imaging software. The linear plot of natural logarithm (ln) of the MBP-Z24FSY band intensity (%) versus time (h) gives k_{obs}. The experiments were independently repeated three times, and the data for one time are shown here.



Affibody	K_d (μM)	k_{on} (1/Ms)	K_{off} (1/s)
7K	4.7	1.95×10^4	9.10×10^{-2}
7H	6.9	2.47×10^4	17.05×10^{-2}
7Y	3.7	1.50×10^4	5.79×10^{-2}

Figure S7. Biolayer interferometry assay of the dissociation constant K_d between wild-type MPB-Z and Afb-7K, Afb-7H, or Afb-7Y.

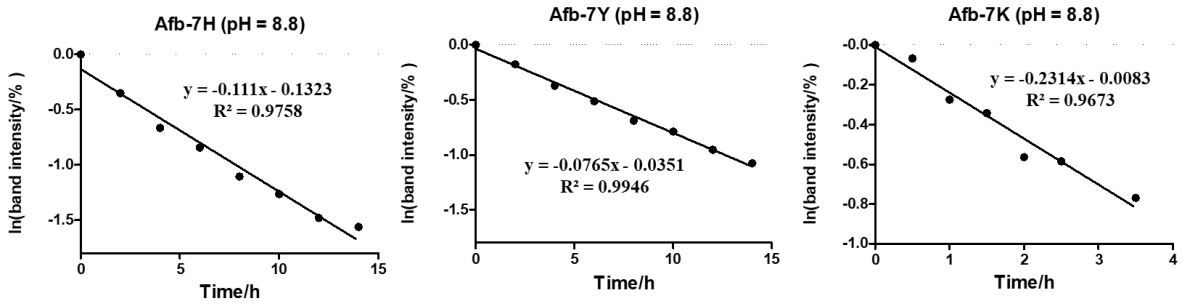


Figure S8. Kinetics of MBP-Z24FSY (6 μM) cross-linking with 192 μM Afb-7K, Afb-7H, or Afb-7Y at pH 8.8 *in vitro*. k_{obs} was calculated based on the time-dependent intensity decrease of the MBP-Z24FSY protein band. The band intensities in the SDS-page were quantified with Bio-rad imaging software. The linear plot of natural logarithm (ln) of the MBP-Z24FSY band intensity (%) versus time (h) gives k_{obs}. The experiments were independently repeated three times, and the data for one time are shown here.

Supporting References

[1] a) N. Wang, B. Yang, C. Fu, H. Zhu, F. Zheng, T. Kobayashi, J. Liu, S. Li, C. Ma, P. G. Wang, Q. Wang, L. Wang, *J. Am. Chem. Soc.* **2018**, *140*, 4995-4999; b) J. Liu, L. Cao, P. C. Klauser, R. Cheng, V. Y. Berdan, W. Sun, N. Wang, F. Ghelichkhani, B. Yu, S. Rozovsky, L. Wang, *J. Am. Chem. Soc.* **2021**, *143*, 10341-10351; c) B. Yang, S. Tang, C. Ma, S.-T. Li, G.-C. Shao, B. Dang, W. F. DeGrado, M.-Q. Dong, P. G. Wang, S. Ding, L. Wang, *Nat. Commun.* **2017**, *8*, 2240.