Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting information

Freezing-Assisted Synthesis of Covalent C-C Linked Bivalent and Bispecific Nanobody

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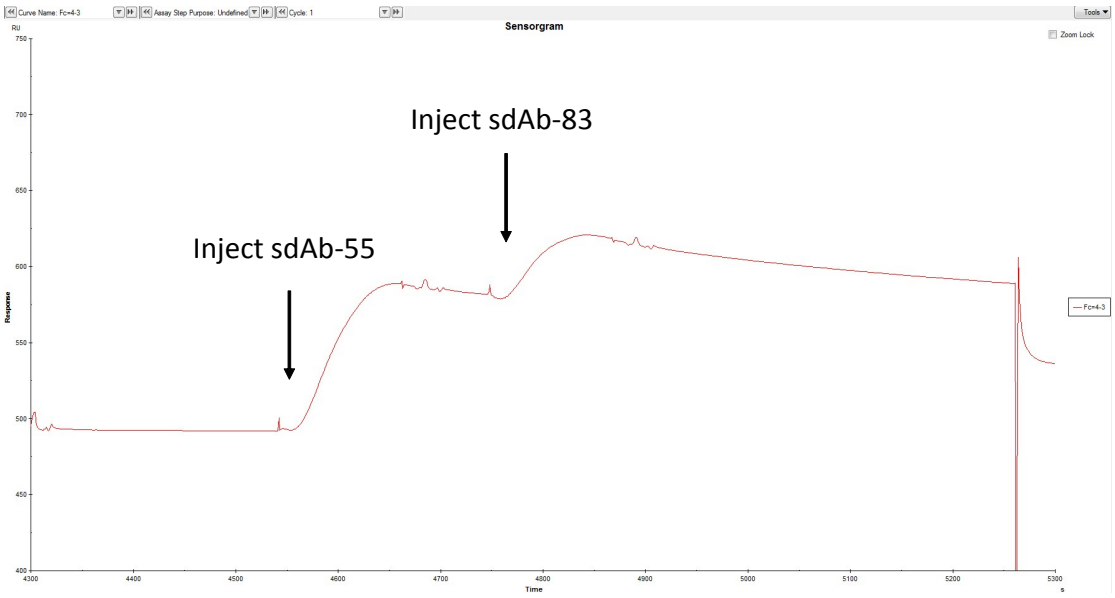
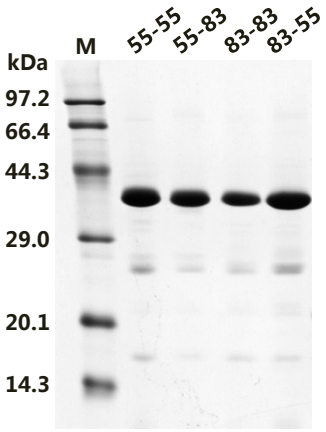


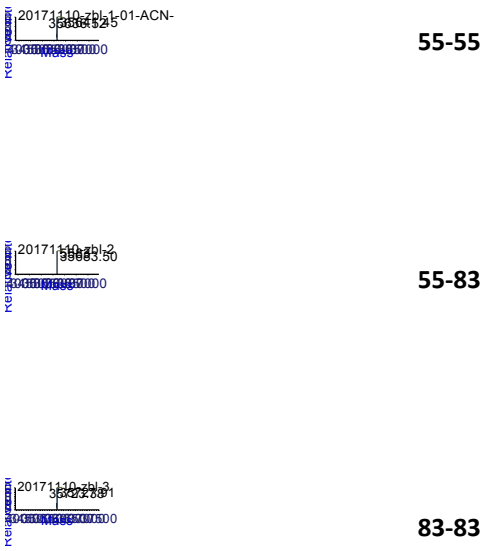
Figure S1. Epitope mapping. The nanobody sdAb-55 was added at saturating concentration to the cuvette containing immobilized hβ2M and allowed the two proteins to bind top each other. Thereafter, the second nanobody, sdAb-83, was added, and the association of the sdAb-83 was recorded.

(a)

(b)



(d)



(c)

sdAb	Yields (mg/L cell culture)
55-55	66
55-83	80
83-83	67
83-55	77

(e)

C-N bivalent/specific sdAb	Calculated Mass (Da)	Measured Mass (Da)
55-55	35640.12	35641.45
55-83	35683.12	35683.50
83-83	35726.12	35727.91
83-55	35683.12	35686.10

Figure S2. (a) The composition of C-N linked bivalent sdAb 55-55 and 83-83, bispecific sdAb 55-83 and 83-55**.** SDS-PAGE analysis (b) and the expression and purification yields (c) of the C-N linked sdAbs. Deconvolution of mass spectra (d) and the calculated molecular weight (e) of the C-N linked sdAbs.

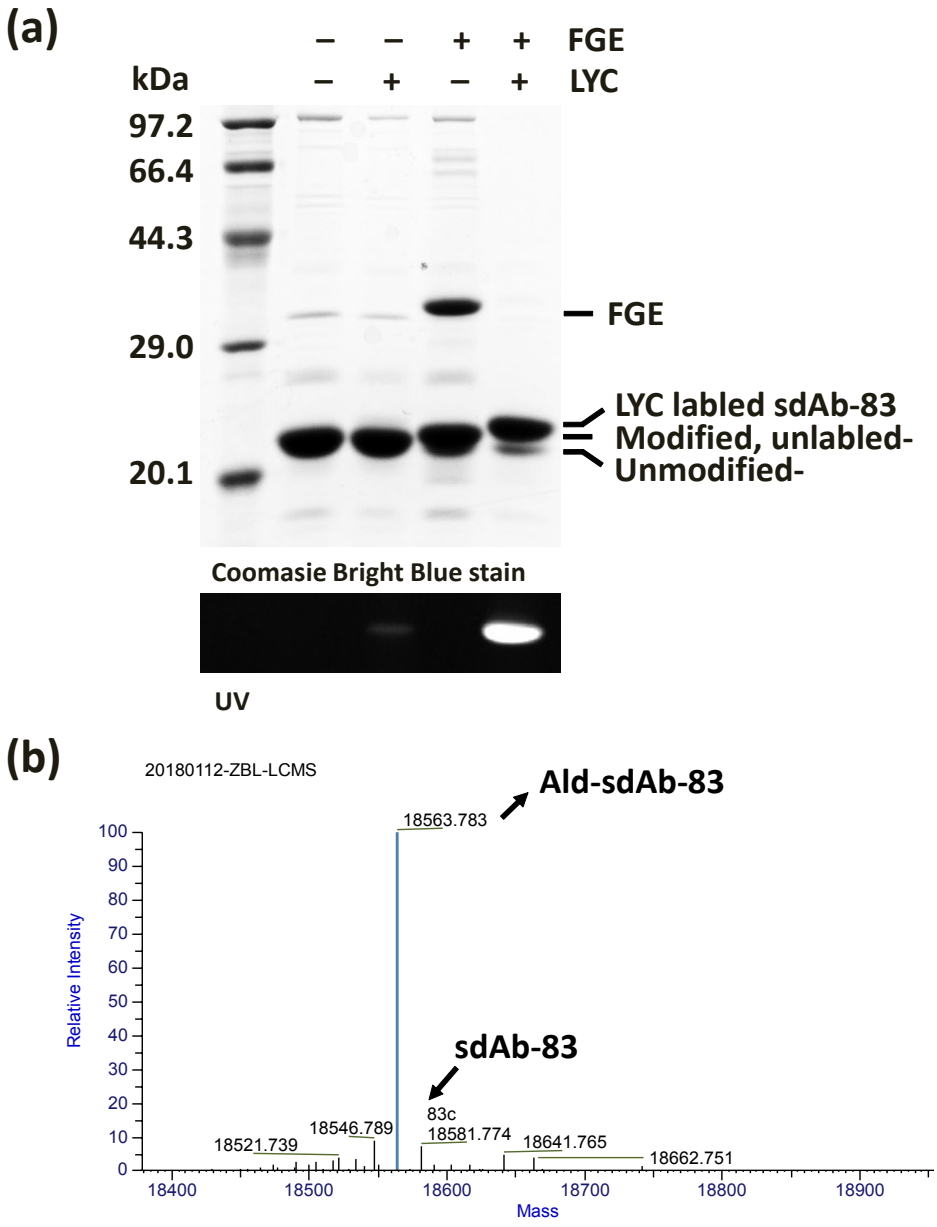


Figure S3. Introduction of site-specific aldehyde group into the C terminus of sdAb-83. (a) SDS-PAGE analysis of native sdAb-83, aldehyde modified sdAb-83 and the lucifer yellow-CH (LYC) labeled sdAb-83. Upper panel: Coomassie bright blue staining; bottom panel: image of the gel taken under UV light. (b) Deconvolution of the mass spectrum of the native and aldehyde-modified sdAb-83. Before modification, theoretical mass: 18581.82 Da/measured value: 18581.78 Da (-Met, two disulfide bonds). After modification, theoretical mass: 18563.82 Da/measured: 18563.78 Da (-Met, two disulfide bonds).

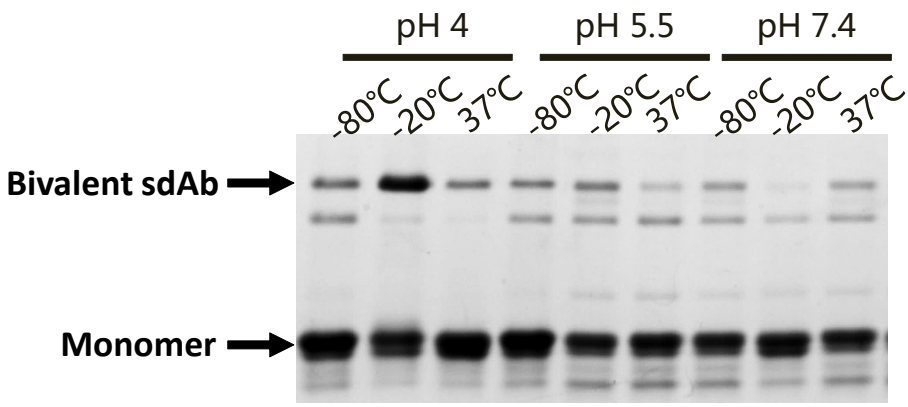
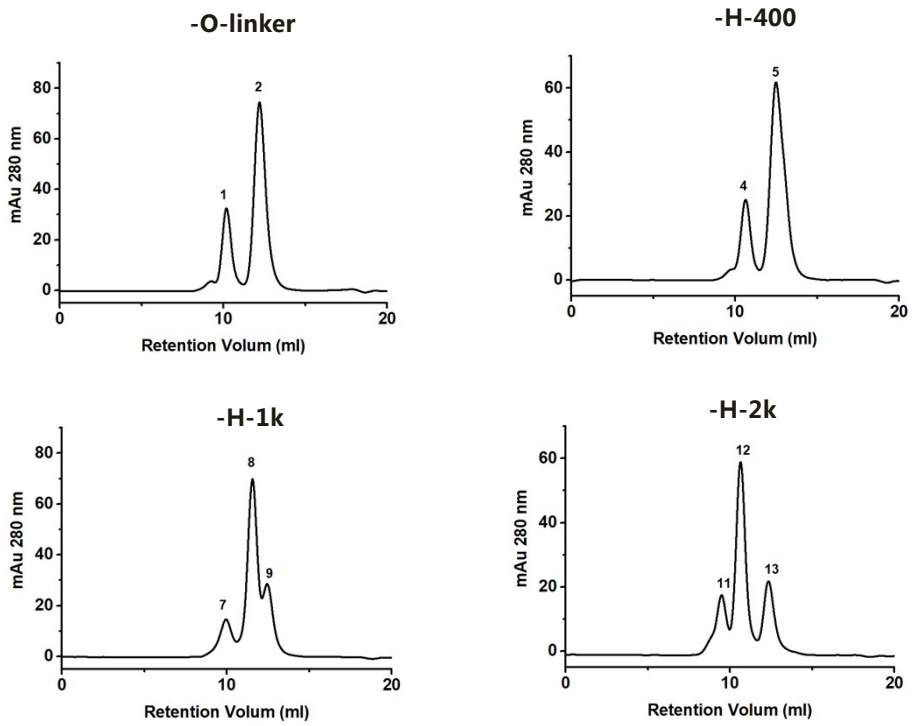


Figure S4. SDS-PAGE analysis of the bivalent sdAb produced under different temperatures and pHs.



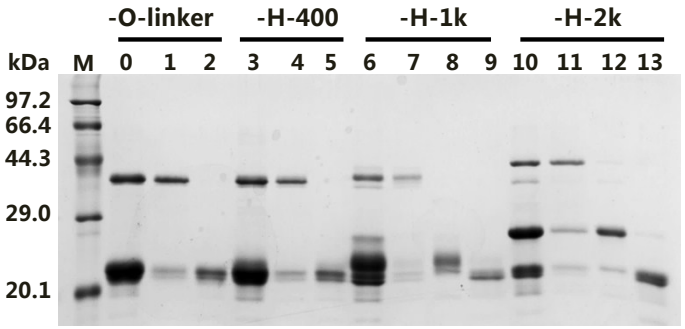


Figure S5. SEC purification of the linker-ligated sdAb-83. Excess linkers were added to the sdAb-83, and the linker ligated sdAb were then purified by SEC (peak NO. 2, 5, 8, 12) and used for the synthesis of bispecific sdAbs. The various samples in the CBB gel were also retained from SEC.

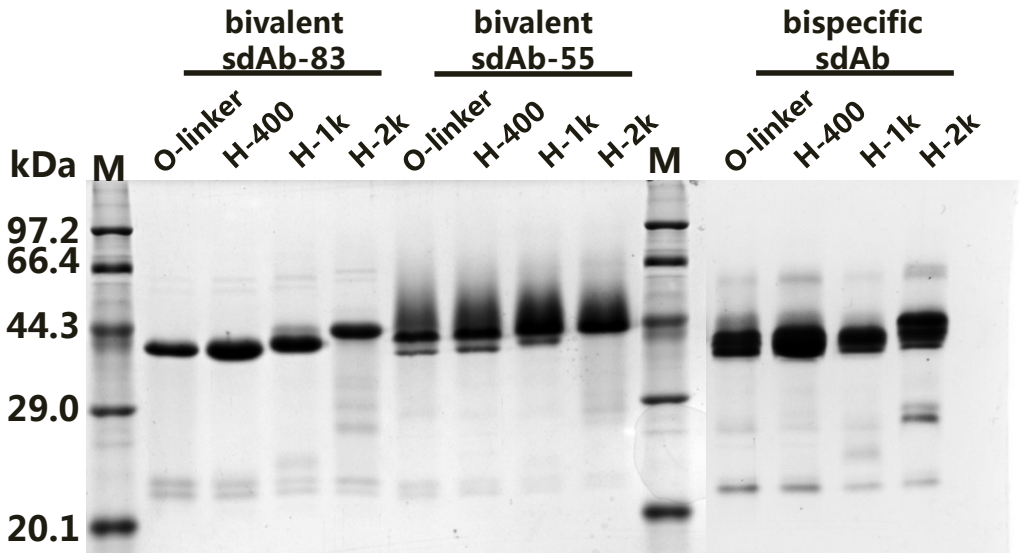
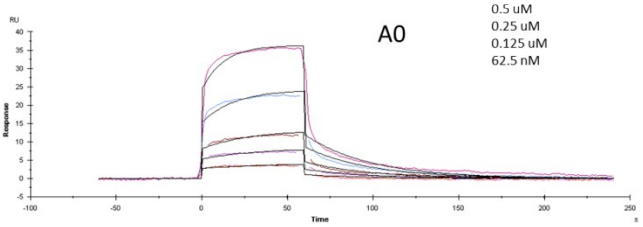
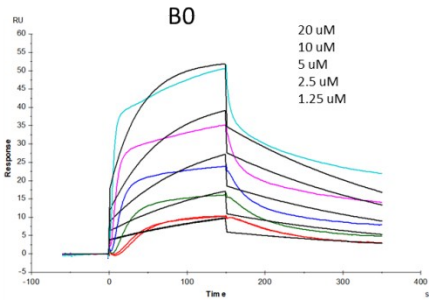


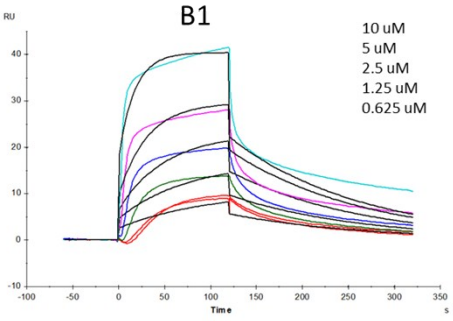
Figure S6. SDS-PAGE analysis of the C-C linked bivalent and bispecific sdAbs purified by SEC.

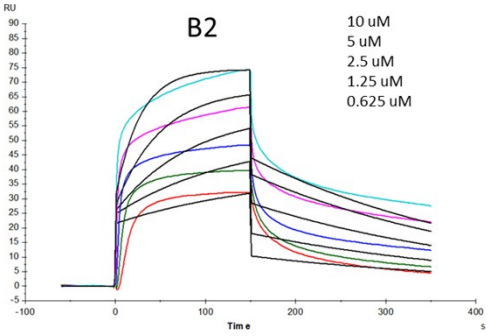
Table S1. Kinetic rate constants, k_a and k_d, and equilibrium dissociation constant K_D of O-bivalent- (linked by oxime bond), H-bivalent- (linked by hydrazine bond) and their single-domain antibody fragments (Mono-) as determined with Biacore™ biosensor

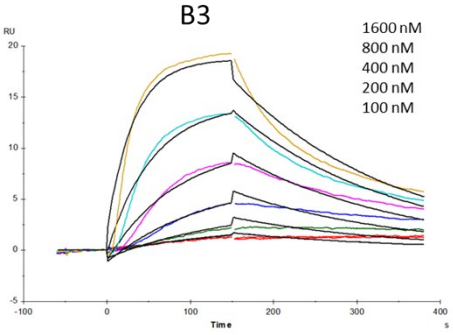
NO.	SdAb Name	MW (Da)	k_a (10^4/Ms)	k_d (10^{-3}/s)	K_D (10^{-8}M)
Mono					
A0	Mono-55	18588	0.633	16.63	262
B0	Mono-83	18593	0.1016	3.637	358
Bivalent					
A1	C-C 55-Olinker	37401	3.23	0.682	2.11
A2	C-C 55 -H400-55	37689	1.99	0.763	3.84
A3	C-C 55-H1k-55	38289	4.42	17.55	39.7
A4	C-C 55-H2k-55	39289	3.00	6.1	20.7
A5	C-N 55-$(G_4S)_3$-55	35683	0.469	3.662	78
B1	C-C 83-Olinker-83	37408	0.4818	6.992	145
B2	C-C 83-H400-83	37696	0.2406	3.21	133
B3	C-C 83-H1k-83	38296	1.61	5.112	31.8
B4	C-C 83-H2k-83	39296	9.3	9.907	10.7
B5	C-N 83-$(G_4S)_3$-83	35640	0.236	1.504	63.74
Bispecific					
C1	C-C 83-Olinker-55	37403	13.0	2.455	1.89
C2	C-C 83-H400-55	37691	20.8	6.213	2.99
C3	C-C 83-H1k-55	38291	16.2	5.024	3.10
C4	C-C 83-H2k-55	39291	21.7	6.088	2.81
C5	C-N 55-$(G_4S)_3$-83	35683	0.236	2.29	86.9
C6	C-N 83-$(G_4S)_3$-55	35683	0.138	1.483	107.4

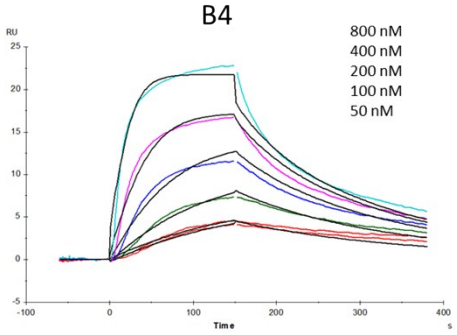

A0
1 uM
0.5 uM
0.25 uM
0.125 uM
62.5 nM
RU
Response
Time
s

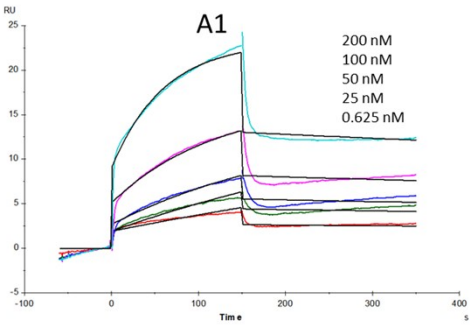

B0
20 uM
10 uM
5 uM
2.5 uM
1.25 uM
RU
Response
Time
s

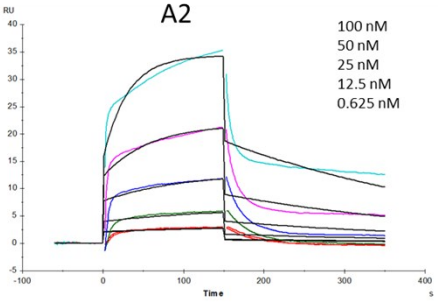

B1
10 uM
5 uM
2.5 uM
1.25 uM
0.625 uM
RU
Time
s

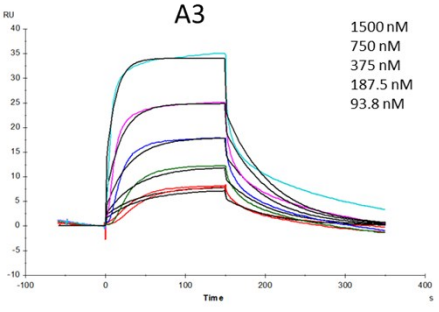

B2
10 uM
5 uM
2.5 uM
1.25 uM
0.625 uM
RU
Time
s

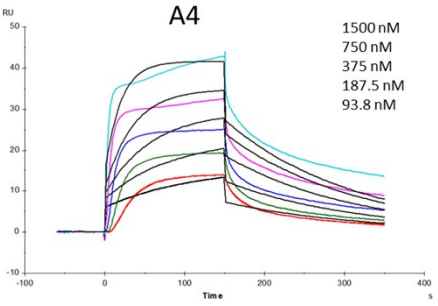

B3
1600 nM
800 nM
400 nM
200 nM
100 nM
RU
Time
s


B4
800 nM
400 nM
200 nM
100 nM
50 nM
RU
Time
s


A1
200 nM
100 nM
50 nM
25 nM
0.625 nM
RU
Time
s


A2
100 nM
50 nM
25 nM
12.5 nM
0.625 nM
RU
Time
s


A3
1500 nM
750 nM
375 nM
187.5 nM
93.8 nM
RU
Time
s


A4
1500 nM
750 nM
375 nM
187.5 nM
93.8 nM
RU
Time
s

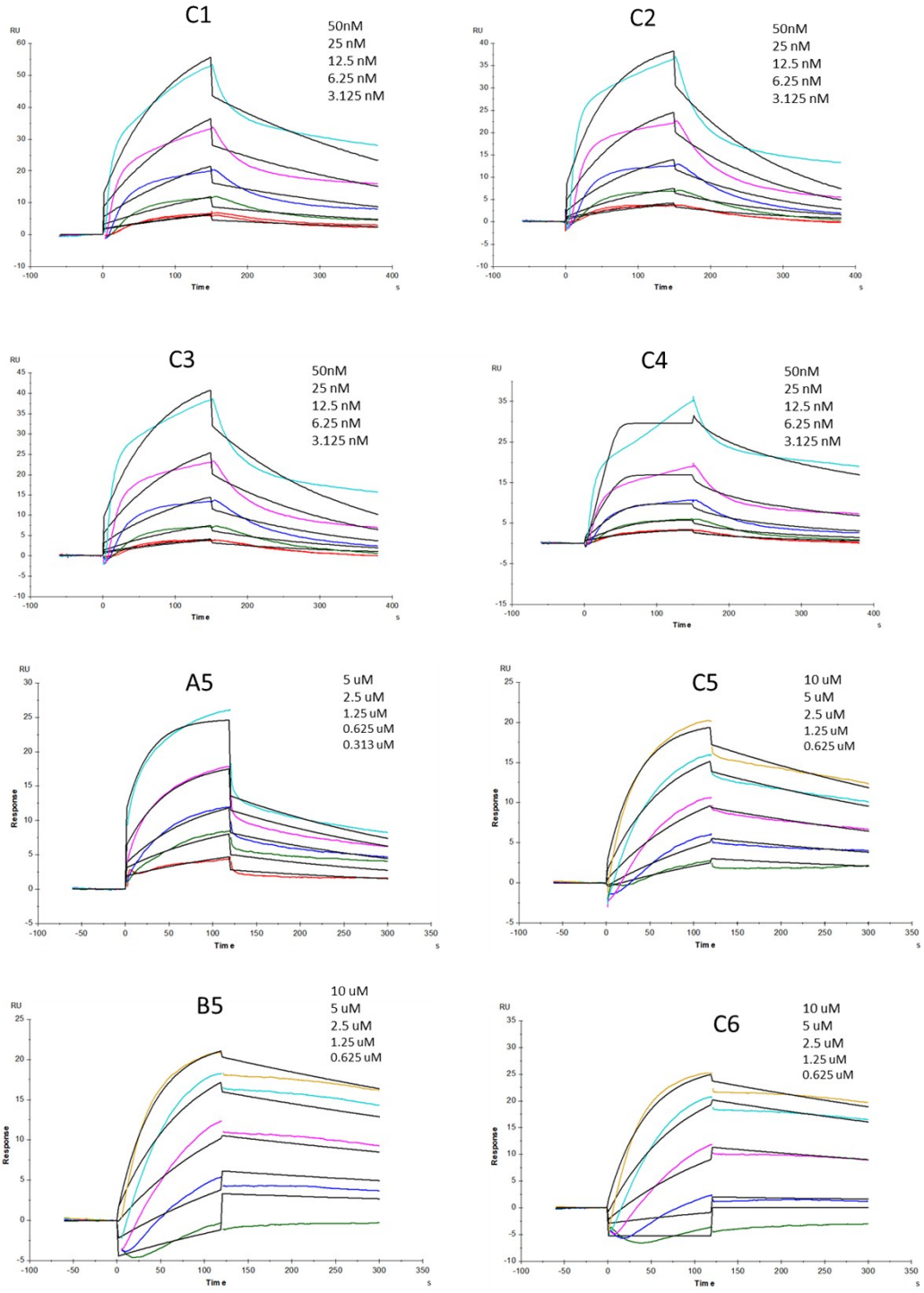


Figure S7. SPR sensorgrams of A0-A5, B0-B5 and C1-C5 showing the binding of bivalent and bispecific sdAbs to immobilized human β2-microglubolin.

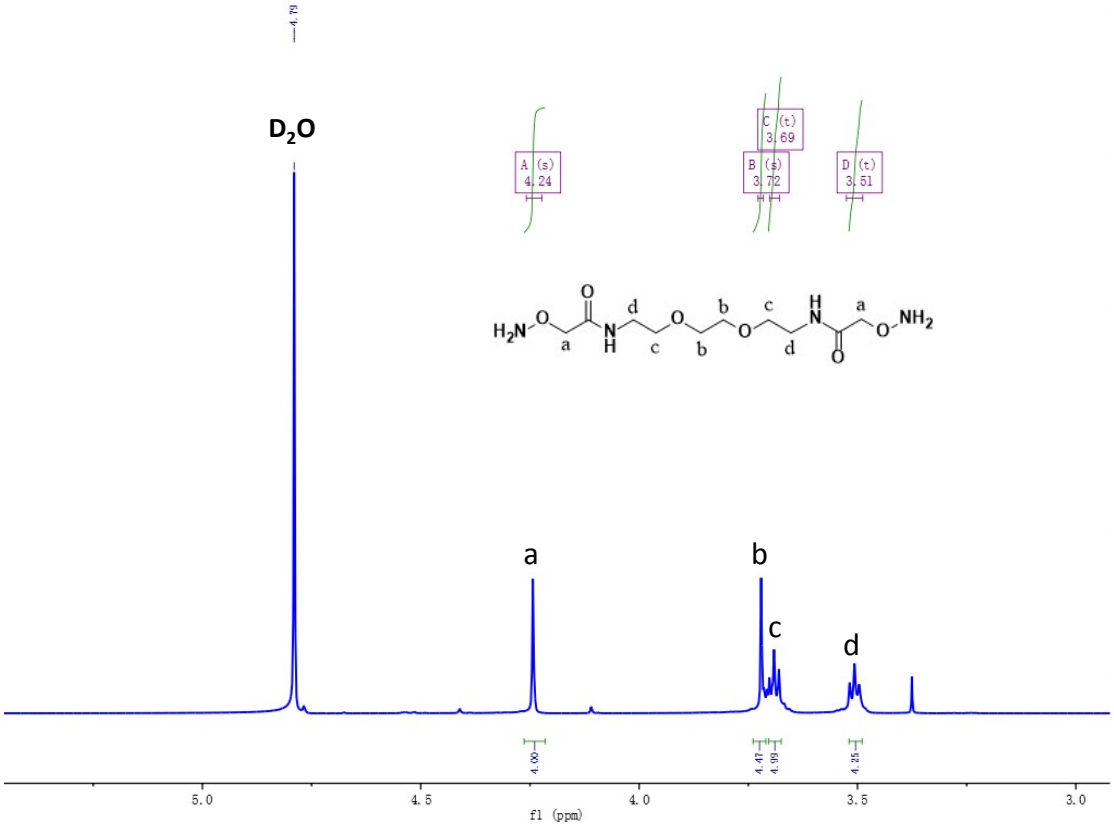


Figure S8. ^{1}H NMR spectra of bis-aminooxy linker.

Experimental Section

General

All reagents and solvents were obtained commercially from J&K Chemical (Beijing, China) and used as received unless otherwise stated. All biochemical reagents were purchased from Solarbio Inc. (Beijing, China) unless otherwise stated. Hydrazide-Poly (Ethylene Glycol) –Hydrazide (400 Da, 1000 Da, 2000 Da, HZ-PEG-HZ) were purchased from Laysan Bio (Arab, Alabama). Lucifer yellow dye (Lucifer yellow CH, Lithium Salt) which contains a carbohydrazide (CH) group was purchased from Thermo Fisher Scientific (lot NO. L453). NMR spectra were recorded on a Bruker Avance III 500 spectrometer. Chemical shifts are reported in parts per million (ppm) and coupling constants (J) are reported in hertz (Hz).

Cloning, expression and purification of formylglycine-generating enzyme

The gene encoding formylglycine-generating enzyme (FGE) was synthesized by Xuguan Biotechnology (Shanghai, China). The FGE gene was inserted into pET28a vector at the NcoI and XhoI restriction sites, and the resulting construct was transformed into *E.coli* SHuffle T7 Express (New England Biolabs). The transformed cells were cultured in Terrific Broth media supplemented with 50 mM of copper sulfate and 100 μg/mL of Kanamycin at 37^{o}C. When the culture reached an OD600 of 3.0-4.0, the growth temperature was decreased to 18^{o}C and IPTG was added to a final concentration of 0.5 mM to induce the expression of FGE. The cells were harvested 20 h post induction by centrifugation at 8000 × g for 5 min and then resuspended in 5 ml of lysis buffer (50 mM Tris, 500 mM NaCl, 10% glycerol, 20 mM imidazole, 1 mM dithiothreitol, 1 mM methionine, pH 7.5) per gram of wet cells and lysed by passage through a French press (3 cycles, 700 bar). The cell lysate was clarified by centrifugation and applied to a 5-ml HisTrap HP column (GE Healthcare). The column was washed with lysis buffer containing 60 mM imidazole, and FGE was

eluted with lysis buffer containing 100 to 300 mM imidazole. The fractions eluted with 100-300 mM imidazole were pooled and exchanged into the storage buffer (25 mM TEAM pH 8.0, 1 mM DTT, 0.1 mM EDTA, 150 mM NaCl, 10% glycerol) by repeated dilution during ultracentrifugation (Amicon Ultra-15 centrifugal filter, 10,000 MWCO) and concentrated to 6 mg/ml. The FGE stock was stored at -80^{o}C. This expression system could achieve 100 mg/L of FGE.

Cloning, expression and purification of single-domain antibody (sdAb) and C-N linked bivalent/bispecific sdAbs

The gene for sdAb-55 and sdAb-83 anti-human β2-microglobulin (hβ2M) were selected by phage-display in our lab and cloned into pET23a, and the resulting constructs were transformed into *E. coli* SHuffle T7 Express. The bivalent C-terminus to N-terminus linked sdAbs were designed as bisdAb-55: 55-$(G_4S)_3$-55-G4S-8xHis; bisdAb-83: 83-$(G_4S)_3$-83-G4S-8xHis, and the bispecific sdAb 55-83: 55-$(G_4S)_3$-83-G4S-8xHis; bispecific sdAb 83-55: 83-$(G_4S)_3$-55-G4S-8xHis. Expression and purification of the C-N linked sdAbs were as described above. The eluted fractions containing the majority of the target protein were pooled and the buffer was exchanged to the sdAb storage buffer (20 mM Potassium phosphate pH 7.5, 150 mM NaCl, 5% sucrose) and concentrated to about 15 mg/ml. The concentrated sample was stored at -80°C until further use. The C-N linked sdAbs were also successfully expressed in the cytoplasm of E. coli with yields of about 50 mg per liter of cell culture. The four C-N linked sdAbs were purified with a TALON@ colum with purity of >90%.

Site-specific aldehyde modification of sdAb

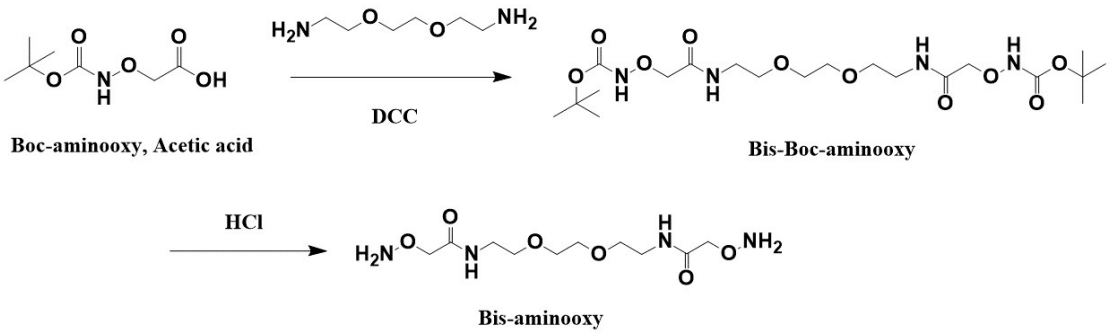
Using the prokaryotic FGE from Mycobacterium tuberculosis, sdAb with C-terminal Ald-tag (amino acid sequence: LCTPSR) was attached with an aldehyde group in a site-specific manner. The sdAb was mixed with FGE in molar ratio of 1:10 in reaction buffer (50 mM TEAM, 150 mM NaCl, 2 mM 2-Mercaptoethanol, pH 9.0) and

incubated at 18°C. After 6 hours of incubation, the buffer in the sample was exchanged into labeling buffer (0.1 M acetic sodium, 150 mM NaCl, pH 4.0) and the generated aldehyde was labeled by addition of 0.5 mM lucifer yellow-CH (LYC) followed by incubation at 37°C for 6 h. The efficiency of the FGE reaction was determined by 15% SDS-PAGE, and the Coomassie bright blue-stained and fluorescent images of the gel were obtained with a ChemiDoc XRS+ system (Bio-Rad). Subsequently, the proteins in the gel were quantitated using the Image Lab™ Software (Version 5.2.1).

LC-MS analysis of intact proteins

The precise efficiency of cysteine-to-formylglycine conversion was determined by HPLC-ESI-MS by comparing the mass spectral abundance of native sdAb with that of aldehyde-modified sdAb, assuming the two components have the same ionization efficiencies. HPLC-ESI-MS measurement was obtained using an Accela LC that was equipped with a XBridgeTM C4 column (4.6 mm × 250 mm, Waters) and connected in-line with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The instrumentation is located in the F502/ State Key Laboratory of Fine Chemicals at the Dalian University of Technology. Raw mass spectra were viewed using Thermo Xcalibur (version 2.2 SP1.48) and mass spectral deconvolution was performed using BioPharma Finder 2.0 Software (Thermo Fisher Scientific).

Synthesis of Acetamide, N,N'-[1,2-ethanediylbis(oxy-2,1-ethanediyl)]bis[2-(aminooxy)- (9CI) (bis-aminooxy linker)



Boc-aminooxy, Acetic acid

Bis-Boc-aminooxy

Bis-aminooxy

2-(2-(2-aminoethoxy)ethoxy) ethanamine (0.37 g, 2.5 mmol) was dispensed into an ice-bath cooled suspension of (Boc-aminooxy) acetic acid (1 g, 5.2 mmol) and dicyclohexylcarbodiimide (DCC, 1.13 g, 5.4 mmol) in anhydrous DCM (20 mL). The mixture was stirred at room temperature for 6 h and the insoluble matters were separated by filtration. The resulting mixture was washed successively with saturated aqueous $NaHCO_3$ (2 × 30 mL), H2O (2 × 30 mL) and brine (3 × 30 mL), dried over anhydrous Na_2SO_4, and then filtered and concentrated in vacuo. The crude product (1.01 g, 82%) was used directly for the next reaction without purification. To a solution of the above diBoc linker (1.01 g, 2.05 mmol) in DCM (15 mL) at 0^{o}C was added 15 mL trifluoroacetic acid. The resulting mixture was stirred at room temperature for 3 h. It was then concentrated and 5 mL DCM was added to the residue followed by the addition of 2 mL 4N HCl in dioxane. Ether (400 mL) was added to precipitate the dihydroxylamine as a white solid, giving a yield of 0.48 g or 80%. ^{1}H NMR (500 MHz, D_2O) δ 4.24 (s, 4H), 3.72 (s, 4H), 3.69 (t, J = 3.7 Hz, 4H), 3.51 (t, J = 5.4 Hz, 4H).

Preparation of bivalent or bispecific sdAb under freezing

The general procedure for the preparation of bivalent or bispecific sdAb under freezing was as follows: The bis-aminooxy linker or HZ-PEG-HZ linker (400 Da, 1000 Da, 2000 Da) (0.5 eq, in labeling buffer) was added to sdAb-55 and sdAb-83 (2 mg/mL). Then sodium cyanoborohydride (10 eq) were added to reduce the formed hydrazone or oxime bond. The mixture was first rapidly frozen at -30^{o}C and then

annealing at -10°C for about 24 h. The reaction was then quenched by adding 1 M NaOH to adjust the value of the pH from 4.0 to about 7.5 to assess the yield of the bivalent sdAb. Next, the bivalent sdAb was purified on a Superdex 75 Increase 10/300 GL (GE Healthcare) column and its affinity kinetics was then measured. In addition, we provided a two-step strategy for the synthesis of bispecific sdAb. First, we modified sdAb-83 with the bi-functional linker by adding excessive linkers (50-fold molar equivalents), the excess linkers were removed by SEC. The protein concentrations were adjusted to 2 mg/mL. The linker-ligated sdAb-83 and sdAb-55 were mixed at 2:1 molar ratio (about 6 mg in total), froze the mixture at -10°C for over 24 h, and then thawed it and quenched it with 1 M NaOH, and purified the bispecific sdAb by SEC.

Biacore Assay

The kinetic binding parameters k_a and k_d were determined with a Biacore T200 (GE Healthcare) instrument. hβ2M was coupled to the CM5 sensor via primary amine groups with a binding value of 700 Ru. After the flow cell had been equilibrated with HEPES EP+ buffer, different concentrations of the bivalent or bispecific protein were directly injected into the flow cell and allowed to bind and dissociate for 120 seconds, and 180 seconds, respectively. The kinetic parameters of the sdAb monomer, C-C linked and C-N linked bivalent and bispecific sdAbs were determined by surface plasmon resonance using a Biacore T200 instruments (GE Healthcare). hβ2M was covalently coupled to a CM5 chip via amine coupling. Subsequently, different sdAb concentrations (between 10 μM and 2 nM) were injected into the chip that was immobilized with hβ2M. The kinetic rate constants k_a and k_b and the equilibrium dissociation constant K_D were determined with the Biacore T200 evaluation software ver 3.1 (GE Healthcare). The kinetic rate values of every sdAb were plotted on a two-dimensional diagram so that data points located on the same diagonal line would have identical K_D values, i.e., a rate plane with isoaffinity diagonals (RaPID) plot.

Amino acid sequences of expressed proteins:

sdAb-55:
AQVQLVESGGGLVQAGGSLRLSCAASGSTLDSYYIGWFRQAPGKEREGVSCISSSGNSIRYVDSV
KDRFTISRDNGKNTAYLHINSLKPEDTAVYYCAASRRGRIPGLPCSLVRERYAYWGQGTQVTVSS
EPKTPKPQPQPQPQPQPDPTTEGGGGSHHHHHHGGGGSLCTPSR

sdAb-83:
AQVQLVESGGGLVQPGGSLRLSCAASGFSLDYYAIGWFRQAPGKEREGVSCISSSDDSTYYAGS
VKGRFKISRDNAKSTVYLQMNSLKPEDSAVYYCAALHSGNYYYTPSYICDAEMDYWGKGTLVTV
SSEPKTPKPQPQPQPQPQPNPTTEEFHHHHHHGGGGSLCTPSR

mtFGE:
ALTELVDLPGGSFRMGSTRFYPEEAPIHTVTVRAFAVERHPVTNAQFAEFVSATGYVTVAEQPL
DPGLYPGVDAADLCPGAMVFCPTAGPVDLRDWRQWWDWVPGACWRHPFGRDSDIADRA
GHPVVQVAYPDAVAYARWAGRRLPTEAEWEYAARGGTTATYAWGDQEKPGGMLMANTW
QGRFPYRNDGALGWVGTSPVGRFPANGFGLLDMIGNVWEWTTTEFYPHHRIDPPSTACCAP
VKLATAADPTISQTLKGGSHLCAPEYCHRYRPAARSPQSQDTATTHIGFRCVADPVSG