Supplementary Material (ESI) for Chemical Communications

Construction of Di-scFv through a Trivalent Alkyne-Azide 1,3-Dipolar Cycloaddition

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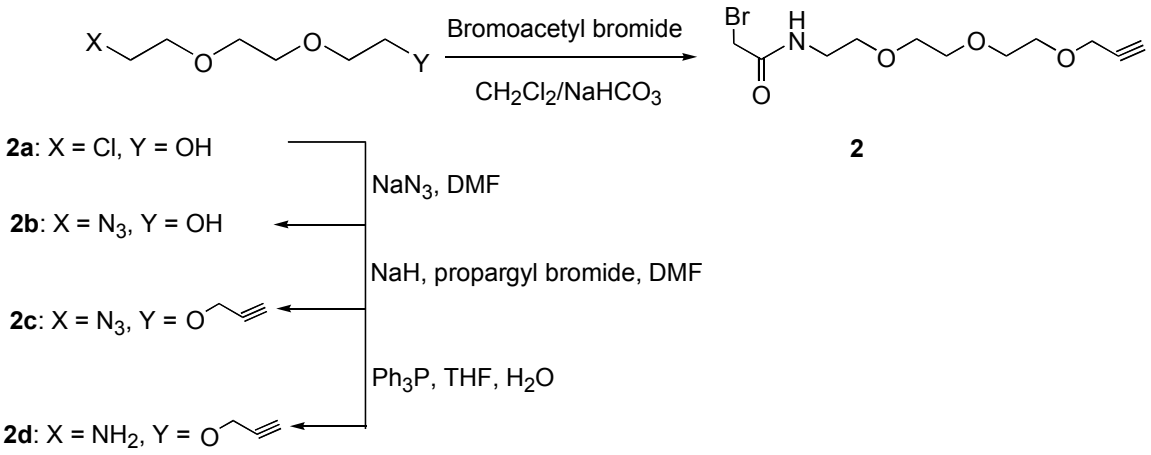
Supporting Information

All materials were obtained from commercial sources and used without additional purification. Solvents used in the syntheses were purchased in capped DriSolv™ bottles and used directly without further purification and stored under argon. All glassware utilized was flame-dried before use. Glass-backed TLC plates (Silica Gel 60 with a 254 nm fluorescent indicator) were used without further manipulation and stored over desiccant. Developed TLC plates were visualized under a short-wave UV lamp, stained with an I_2 mixture, and /or by heating plates that were dipped in ammonium molybdate/cerium (IV) sulfate solution. Flash column chromatography (FCC) was performed using flash silica gel (32-63 μm) and employed a solvent polarity correlated with TLC mobility. NMR experiments were conducted on either a Varian 400 MHz or 600 MHz instrument using $CDCl_3$ (99.9% D) as a solvent. Chemical shifts are relative to the deuterated solvent peak and are in parts per million (ppm). Low resolution mass spectra were acquired using a ThermoQuest Surveyor™ LC/MS or Qtrap LC/MS instruments (Applied Biosystem Inc., Foster City, CA). FT-IR spectra were acquired from a Galaxy FTIR 3000 instrument at 25 ^oC.

Tris(2-carboxyethyl)phosphine (TCEP) and monobromobimane (MBB) were purchased from Molecular Probes Inc (Eugene, OR). Triazole ligand **6** was synthesized according to the literature.[1] Protein scFv (**1**) was produced and purified as reported previously.[2] SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was purchased from Pierce Biotechnology Inc. (Rockford, IL) and operated according to the manufacturer's directions. The

fluorescence intensities were measured by a Cary Eclipse Fluorescence spectrophotometer (Varian Inc., Palo Alto, CA). Protein bands were detected by Coomassie Blue (CB) staining and band intensities were measured by a S1 Densitometer (Molecular Dynamics Inc., Sunnyvale, CA). The band intensities were converted to digital data from densitometry performed on the scanned gel images and ligation efficiency was calculated according to the literature.[3]

The Synthetic Scheme of linker **2** is shown below:



2-[2-(2-azidoethoxy)ethoxy]ethanol (**2b**): In a 250 mL round bottom flask, 2-[2-(2-chloroethoxy)ethoxy]ethanol (**2a**, 3.0 g, 18 mmol), NaN_3 (3.5 g, 54 mmol) and TBAI (662 mg, 1.8 mmol) were added to 15 mL DMF. The temperature was slowly raised to 65 ^oC and the reaction mixture was stirred under argon for 26 h. Solvent was evaporated and H_2O (15 mL) was added. The solution was extracted with 3×40 mL EtOAc. The organic layers were combined and dried with Na_2SO_4. Solvent was evaporated and the residue was applied to FCC (CH_2Cl_2:MeOH = 90:10; R_f = 0.40) to afford **2b** as a slightly yellow oil (2.77 g, 88%). ^1H NMR (400 MHz, $CDCl_3$): δ 3.29 (t, J = 5.2 Hz, 2H), 3.50 (m, 2H), 3.57-

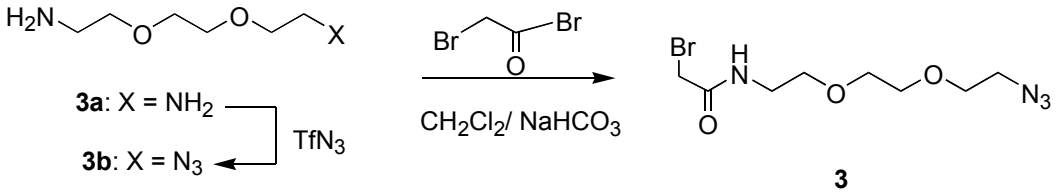
3.64 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 50.6, 61.6, 70.0, 70.3, 70.6, 72.6. ESIMS calcd for $C_6H_{13}N_3NaO_3$ $[M+Na]^+$ 198.09, found: 198.12.

3-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)prop-1-yne (**2c**): In a 50 mL flask, **2b** (350 mg, 2 mmol) was dissolved in 10 mL DMF. NaH (58 mg, 2.4 mmol) and propargyl bromide (476 mg, 4.0 mmol) were added. The reaction mixture was stirred at rt overnight. Solvent was evaporated and the residue was applied to FCC (hexane:EtOAc = 7:3; R_f = 0.41) to afford **2c** as slightly yellow oil (361 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.43 (t, J = 1.2 Hz, 1H), 3.38 (t, J = 5.2 Hz, 2H), 3.66-3.69 (m, 10H), 4.20 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 50.9, 58.6, 69.3, 70.3, 70.7, 70.87, 70.89, 74.7, 79.8. ESIMS calcd for $C_9H_{15}N_3NaO_3$ $[M+Na]^+$ 236.22, found: 236.28. FTIR (NaCl plate, CH_2Cl_2): 1118 (C-O str), 2107 (N≡N and C≡C str), 2905 (C-H str).

3-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)prop-1-yne (**2d**): In a 50 mL round bottom flask, **2c** (150 mg, 0.7 mmol) and Ph_3P (275 mg, 1.05 mmol) were dissolved in 10 mL THF. The reaction mixture was stirred at rt for 3 h. H_2O (1 mL) was added and the reaction was stirred at rt for 36 h. Solvent was evaporated and the residue was applied to FCC (CH_2Cl_2:MeOH:TEA = 80:20:1; R_f = 0.35) to afford **2d** as a slightly yellow oil (131 mg, quant.) ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.46 (br, 2H), 2.35 (t, J = 1.6 Hz, 1H), 2.73 (t, J = 5.2 Hz, 2H), 3.38 (t, J = 5.2 Hz, 2H) 3.50-3.59 (m, 10H), 4.08 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 41.7, 58.3, 69.0, 70.2, 70.4, 70.5, 73.4, 74.6, 79.6. ESIMS calcd for $C_9H_{18}NO_3$ $[M+H]^+$ 188.13, found: 188.16. FTIR (NaCl plate, CH_2Cl_2): 1118 (C-O str), 2111 (C≡C str), 2869 (C-H str), 3249 (N-H str).

Mono-alkyne linker 2: To a solution of **2d** (50 mg, 0.27 mmol) in CH_2Cl_2 (3 mL) was added sat. $NaHCO_3$ (3 mL). The reaction mixture was cooled to 0 ^oC. Under stirring, bromoacetyl bromide (80 mg, 0.40 mmol) was added dropwise. The pH was adjusted in the range of 8-9 by adding sat. $NaHCO_3$. The reaction was stirred at 0 ^oC for 15 min.; CH_2Cl_2 (5 mL) was added and the reaction mixture was extracted 3×5 mL CH_2Cl_2. The organic layers were combined, washed with brine and dried with Na_2SO_4. The solvent was evaporated and the residue was applied to FCC (CH_2Cl_2:MeOH = 95:5; R_f = 0.34) to afford **2** as a clear oil (65 mg, 78%). ^1H NMR (400 MHz, $CDCl_3$): δ 2.42 (t, J = 2.4 Hz, 1H), 3.48 (dd, J = 5.2, 5.2 Hz, 2H), 3.57 (t, J = 5.2 Hz, 2H), 3.62 (m, 4H), 3.66-3.70 (m, 4H), 3.85 (s, 2H), 4.18 (d, J = 2.4 Hz, 2H), 6.91 (bs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 29.4, 40.1, 58.6, 69.3, 69.6, 70.5, 70.6, 70.8, 74.9, 79.7, 165.9. ESIMS calcd for $C_{11}H_{18}BrNNaO_4$ $[M+Na]^+$ 330.03, 332.03, found: 330.33, 332.46.

The synthetic scheme of linker **3** is shown below:

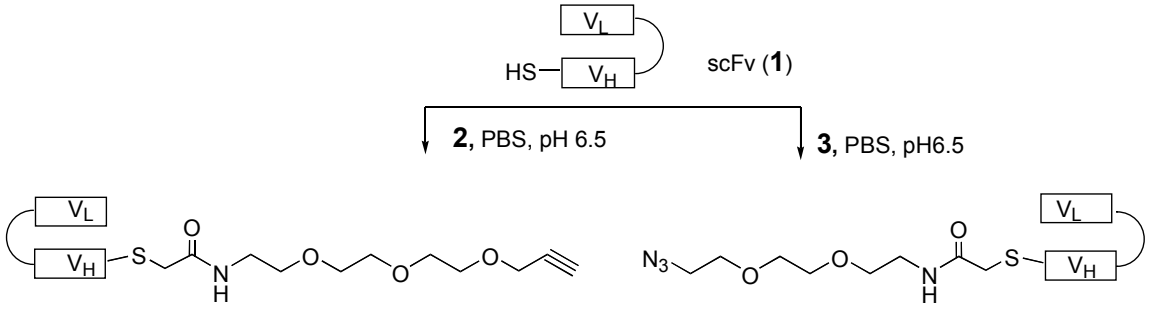


2-(2-(2-azidoethoxy)ethoxy)ethanamine (**3b**): To a solution of NaN_3 (3.45 g, 53 mmol) in H_2O (12 mL) was added CH_2C1_2 (18 mL). The resulting biphasic mixture was cooled in an ice bath. Tf_2O (3.0 g, 10.6 mmol) was added dropwise over a time period of 60 min. The reaction mixture was stirred at 0 ^oC for 4 h. The organic phase was separated and the aqueous phase was extracted once with CH_2C1_2 (20 mL). The organics were washed with saturated Na_2CO_3 solution to give a crude TfN_3 solution, which was used without further

purification. **3a** (3.14 g, 21.2 mmol) was dissolved in MeOH (10 mL) and was treated with potassium carbonate (1.7 g, 12.3 mmol, in 3.0 mL H_2O) and $CuSO_4$ pentahydrate (14 mg, 0.056 mmol, in 1.0 mL H_2O). The TfN_3 solution was added dropwise over a time period of 18 h. The reaction was allowed to stir at rt for 24 h and solvent was evaporated. The residue was applied to FCC (CH_2Cl_2:MeOH:TEA = 80:20:1; R_f = 0.40) to afford **3b** as a slightly yellow oil (1.85 g, 50%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.86 (s, 2H), 2.88 (t, *J* = 5.4 Hz, 2H), 3.39 (t, *J* = 5.4 Hz, 2H), 3.52 (t, *J* = 5.4 Hz, 2H), 3.63-3.68 (m, 6H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 41.9, 50.9, 70.3, 70.5, 70.9, 73.4. ESIMS calcd for $C_6H_{15}N_4O_2$ $[M+H]^+$ 175.21, found: 175.25. FTIR (NaCl plate, CH_2Cl_2): 1118 (C-O str), 2110 (N≡N str), 2918 (C-H str), 3300 (N-H str).

***N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-2-bromoacetamide** (**3**): To a solution of **3b** (32 mg 0.18 mmol) in CH_2Cl_2 (3 mL) was added sat. $NaHCO_3$ (3 mL) in 0 ^{o}C. Bromoacetyl bromide (55 mg, 0.27 mmol) was added dropwise. Sat. $NaHCO_3$ was added to maintain the pH at approx. 8-9. The reaction was stirred at 0 ^{o}C for 15 min. CH_2Cl_2 (5 mL) was added and the reaction mixture was extracted 3×5 mL with CH_2Cl_2. The organic layers were combined, washed with brine, and dried with Na_2SO_4. Solvent was evaporated and the residue was applied to FCC (CH_2Cl_2:MeOH = 95:5; R_f = 0.38) to afford **3** as a slightly brown oil (39 mg, 0.13 mmol, 78%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.35 (t, *J* = 4.8 Hz, 2H), 3.49 (dd, *J* = 10.4, 5.2 Hz, 2H), 3.55 (t, *J* = 4.8 Hz, 2H), 3.60-3.66 (m, 6H), 3.83 (s, 2H), 6.86 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 29.3, 40.0, 50.8, 69.5, 70.3, 70.5, 70.7, 165.8. ESIMS calcd for $C_8H_{15}BrN_4NaO_3$ $[M+Na]^+$ 317.02, 319.02, found: 317.38, 319.29. FTIR (NaCl plate, CH_2Cl_2): 1120 (C-O str), 1653 (C=O str), 2110 (N≡N str), 2954 (C-H str), 3307 (N-H str).

Synthesis of ScFv (**1**) containing linkers **4** and **5:**

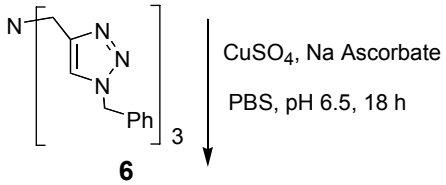


4 **5**

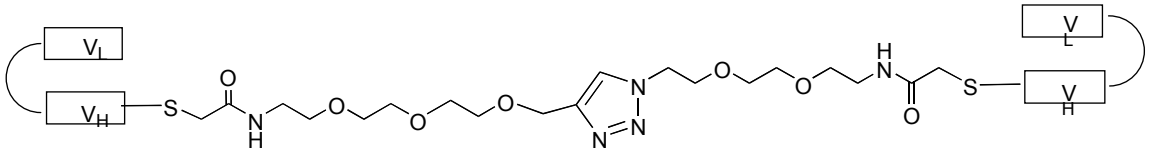
In two separated microtubes, protein scFv (2 nmole in 100 μL PBS) was pre-treated with TECP (4 nmole) at pH 6.5 for 4 h. Linkers **2** and **3** (50 nmole of each) were added separately. The reaction mixtures were incubated overnight at rt. Un-conjugated linkers and TECP were removed by 10 kDa cutoff membrane dialysis. Un-reacted cysteine was evaluated by reaction with monobromobimane (MBB) accord to the literature.[4] The amount of free –SH were evaluated by comparing the relative fluorescence intensities of the reaction mixture+MBB and scFv+MBB. The conjugation efficiencies of scFv with linker **2** and linker **3** were 82% and 78%, respectively.

The synthesis of di-scFv 7 is shown below:

4 + 5



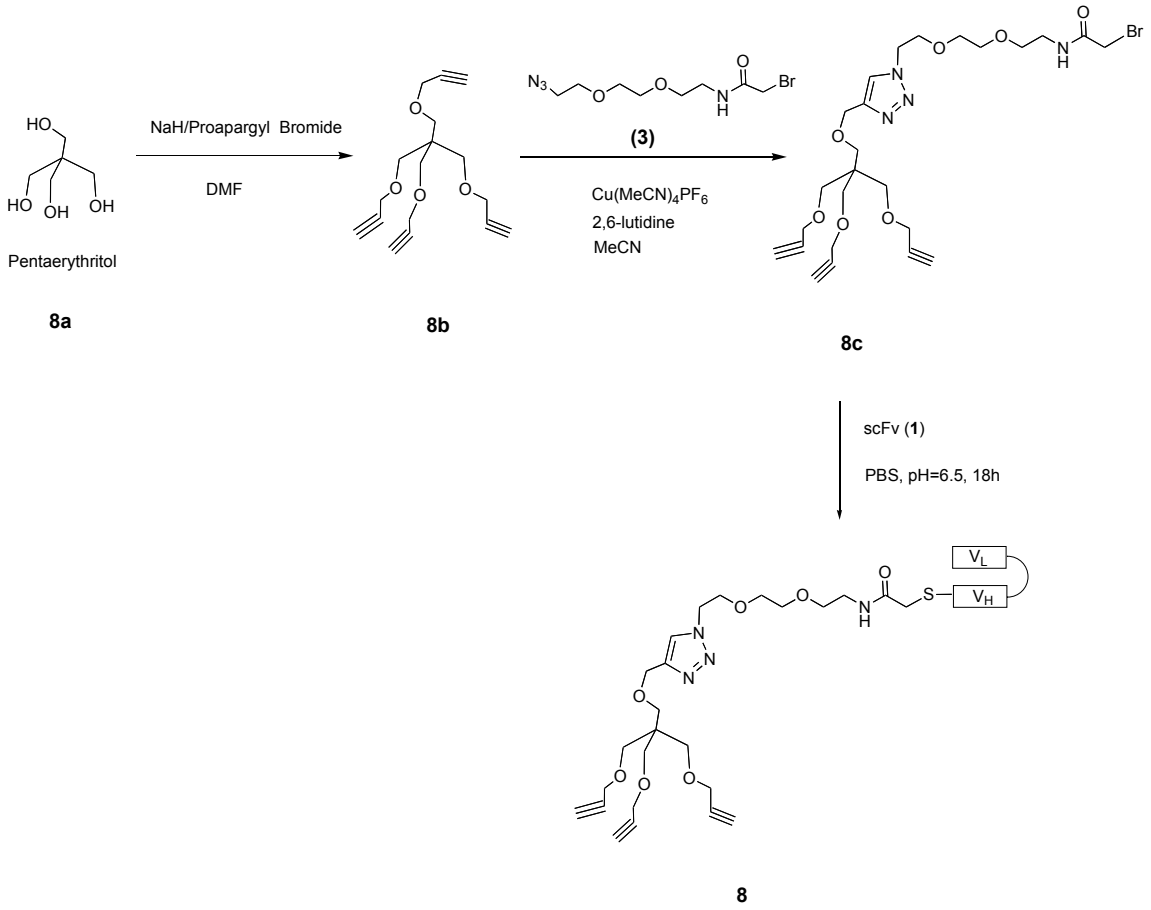
6



di-scFv (7)

The scFv conjugated linker **4** (2 nmol) was mixed with linker **5** (10.0 nmol) in PBS buffer (300 μL total volume) at pH 6.5. Ligand **6** (0.1 nmol), $CuSO_4 \cdot 5H_2O$ (0.1 nmol) and sodium ascorbate (0.2 nmol) were added sequentially. The reaction mixture was incubated at rt for 18 h. The reaction mixture was purified by 10 kDa cut-off dialysis and concentrated by centriprep. PAGE digitally scanned by densitometry indicated a 33% yield. The concentrate was further purified by HPLC (1mL/min, SEC 3000, pH 7 PBS buffer).

The synthesis of 8 is shown below:



Tetra-alkyne **8b**: Pentaerythritol (**8a**, 272 mg, 2.0 mmol) was added in DMF (20 mL) and the temperature was raised to 65 ^{o}C. NaH (288 mg, 12.0 mmol) and propargyl bromide

(1.4 g, 12 mmol) were added. The reaction was stirred at 65 ^{o}C overnight. Solvent was removed and the residue was applied to silica gel column chromatography (CH_2Cl_2:Hexane = 8:2; R_f = 0.60) to afford **8b** as a slightly yellow oil (434 mg, 1.5 mmol, 75%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.39 (s, 4H), 3.45 (s, 8H), 4.05 (s, 8H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 44.9, 58.8, 69.1, 74.5, 80.1. ESIMS calcd for $C_{17}H_{20}NaO_4$ $[M+Na]^+$ 311.33, found: 311.22.

Tri-alkyne linker **8c**: Linker **3** (30 mg, 0.11 mmol) and tetra-alkyne **8b** (62 mg, 0.22 mmol) were dissolved in acetonitrile (3 mL), 2,6-lutidine (35 μL) and $Cu(MeCN)_4PF_6$ (8 mg, 0.022 mmol) were added. The reaction was stirred at rt overnight. Solvent was evaporated and the resulting residue was applied to silica gel column chromatography (CH_2Cl_2:MeOH = 10:1; R_f = 0.38) to afford **8c** as a slightly yellow oil (45 mg, 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.43 (m, 3H), 3.47-3.60 (m, 18H), 3.91 (m, 4H), 4.11 (m, 6H), 4.55 (t, *J* = 5.4 Hz, 2H), 4.64 (s, 2H), 6.93 (bs, 1H), 7.70 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.5, 40.2 45.2, 50.5, 58.9, 65.5, 69.2, 69.5, 69.6, 69.7, 70.6, 70.7, 74.5, 80.3, 123.7, 145.8, 165.9. ESIMS calcd for $C_{25}H_{35}BrN_4NaO_7[M+Na]^+$ 605.16, 607.16, found: 605.46, 607.44.

ScFv conjugated linker **8** was synthesized by mixing linker **8c** and scFv (**1**) in the same manner as described for **4** and **5**.

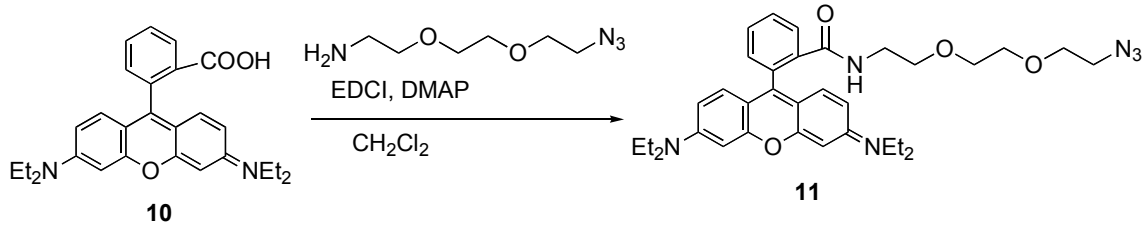
Synthesis of di-scFv (9): Linker **8** (2 nmol) was mixed with linker **5** (2 nmol, 4 nmol and 6 nmol) in PBS buffer (300 μL total volume) at pH 6.5. Ligand **6** (0.1 nmol), $CuSO_4 \cdot 5H_2O$ (0.1 nmol) and sodium ascorbate (0.2 nmol) were added sequentially. The reaction mixture

was incubated at rt for 18 h. The ligated di-scFv (**9**) was purified by 10 kDa cut-off dialysis and concentrated by centriprep. The product (100 μg) was applied to SDS-PAGE followed by CB staining (Figure 3A), which indicated the presence of the desired band for di-scFv (52 kD) as well as a small band at approx 40-45 kD. This band was also present in lane 0 and therefore was attributed to an impurity. Lane 0 also contained a faint 52 kD band, which was attirbuted to non-covalent dimerization. To confirm that the product was formed by 1,3-dipolar cycloaddition and not disulfide formation, the reaction mixtures were treated with tri(carboxyethyl)phosphine (TCEP, 5 eq.) for 4 h and then subjected to SDS-PAGE (Figure 3B). Under these conditions, lanes 1-3 were not reduced. The band intensities of lanes 0-3 in Figure 3A were digitized from densitometry performed on the scanned gel images. The band intensities were converted to digital data and the reaction efficiency was calculated as described previously[3] to give the yields of 1,3-diploar cycloaddition of lanes 1-3 as 58%, 74% and 66%, respectively.

Estimation of conjugation efficiency of Br-PEG linkers to scFv-c: In two separated microtubes, protein scFv (2 nmoles, in 100 μL PBS) was pre-treated with TECP (4 nmoles, 2×) at pH 6.5 for 4 hr. Linkers **2** and **3** (50 nmoles of each) were added separately. The reaction mixtures were incubated overnight at rt. Unconjugated linkers and TECP were removed by 10 kDa cutoff membrane dialysis. Unreacted cysteine was evaluated by reaction with monobromobimane (MBB). The conjugation efficiency was determined by comparing the relative fluorescence intensities of scFv+linkers+MBB with scFv+MBB, assuming that MBB reacted with scFv in a quantitative manner. The conjugation efficiencies with linker **2** and linker **3** were 82% and 78% respectively.

Purification and characterization of di-scFv: The click reaction product (**9**) was dialyzed with 10kDa molecular weight cut-off membrane. After membrane purification, it was further purified by Beckman coulter system Gold 128 Chromatography. Superdex-12 HR column (Amersham biosciences, CA) with PBS (pH 7) as elution solvents was performed at 0.5 mL per minute. UV absorbance was monitored simultaneously at 234, and 280 nm. Pure di-scFv fractions were pooled and concentrated for further characterization by 4-12% SDS PAGE (Figure 4, left).

The synthesis scheme for Rhodamine –azide 11 is shown below:



Rhodamine B (**10**, 409 mg, 0.85 mmol) and the azido amine (100 mg, 0.57 mmol) were dissolved in 10 mL CH_2Cl_2, under stirring, DIPEA (0.3 mL), EDCI (326 mg, 1.17 mmol) and DMAP (cat.) were added. The reaction mixtures were stirring at rt overnight. Solvent was evaporated and the residue was applied to FCC to afford **11** as bright pink oil (345 mg, 94%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.15 (t, J = 6.8 Hz, 2 H), 3.17 (t, J = 7.2 Hz, 12 H), 3.26-3.37 (m, 14H), 3.47 (dd, J = 5.6, 3.6 Hz, 2H), 3.56 (dd, J = 5.6, 5.2 Hz, 2H), 6.23 (d, J = 2.4 Hz, 1H), 6.25 (d, J = 2.4 Hz, 1H), 6.35 (d, J = 2.8 Hz, 2H), 6.40 (d, J = 8.8 Hz, 2H), 7.03 (m, 1H), 7.39 (m, 2H), 7.86 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.69, 39.34, 44.44, 50.71, 64.89, 67.96, 70.04, 70.08, 70.51, 97.82, 105.59, 108.12, 122.78, 123.86, 128.04, 128.93, 131.06, 132.46, 148.81, 153.33, 153.85, 168.30. ESIMS calcd for

$C_{34}H_{42}N_6NaO_4$ $[M+Na–HCl]^+$ 621.73, found: 621.72. FTIR (NaCl plate, CH_2Cl_2): 1098 (C-O str), 1660 (C=O str), 2113 (N≡N str), 2909 (C-H str), 3310 (N-H str).

ELISA. ELISA was performed on MUC-1 peptides, cell lysates and live cells. Ninety-six well plates were coated with the MUC-1 peptide (1 μg/100 μl/well) or DU145 cell lysates containing the cell membranes (100 μl per well). The latter was prepared as follows: Cells in log-phase were harvested and homogenized using a homogenizer. The concentration of cell lysates was adjusted to 1mg protein per ml. For ELISA on live cells, cells in log-phase were harvested, re-suspended in fresh medium and added into flat 96-well plate ($1x10^6$cells per well). Medium was changed to BSA-PBS solution (PBS buffer containing 1.5% BSA). All reagents and washing were performed by spinning the cells at the bottom and by maintaining cell integrity. ELISA assays were performed triplicate, following the approach described previously [2]. In ELISA studies the scFv and di-scFv binding effects were compared on synthetic MUC-1 peptide and DU145 cell lysates. The di-scFv showed increased binding effects of 2-5 folds than scFv (Figure 4 right).

Immunohistochemistry (IHC). Formalin-fixed, paraffin-embedded human prostate cancer tissues were sectioned and slides were deparaffinized. Cultured cells were smeared onto slides, air dried and fixed in 10% formalin. Endogenous peroxidase in both fixed tissues and cells was quenched by submerging slides in 0.3% H_2O_2 in methanol for 15 min. For fixed tissues, antigen retrieval was performed using a microwave approach in sodium citrate buffer (10 mM, pH 6.0). After rinsing in PBS, all slides to be stained were blocked for 30 min with 10% goat serum in PBS. ScFv-c and scFv-PTP-scFv proteins were separately added to corresponding slides followed by incubation for 3 hours at 37 ^{0}C. After rinsing, HRP conjugated anti-E Tag antibody or HRP-conjugated anti-mouse IgG was

added and incubated for one hour at room temperature followed by rinsing in PBS. The peroxidase reaction was developed with 3,3'-diaminobenzidine (DAB) reagent (Vector Laboratories, Burlingame, CA). After rinsing, sections were counterstained with Hematoxylin. Before photography, images of the slides were reviewed with a pathologist, Dr. Linlang Gun, photographed and electronically saved. Immunohistochemical staining of DU145 and MCF-7 cells (x650) and formalin-fixed, paraffin-embedded prostate cancer tissue (x400), showed tumor staining of the cells and cancer tissues with di-scFv. The brown membrane staining can be easily detected on the cells with scFv-c and di-scFv proteins. The membranes of these tumor cells were strongly stained with the di-scFv. In all IHC experiments, Jurkat cells were used as negative control (Figure 5).

References:

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