Supporting Information

Chemistry Letters

Dendritic Molecular Glues with Reductively Cleavable Guanidinium Ion Pendants: Highly Efficient Intracellular siRNA Delivery via Direct Translocation

Kou Okuro,*[1] Harei Nemoto,[1] Rina Mogaki,[1] and Takuzo Aida*[1,2]

[1]*Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*

[2]*Riken Center for Emergent Matter Science, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*

Received June 16, 2018; CL-180551; E-mail: okuro@macro.t.u-tokyo.ac.jp (K. Okuro), aida@macro.t.u-tokyo.ac.jp (T. Aida)

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Kou Okuro,*[1] Harei Nemoto,[1] Rina Mogaki,[1] and Takuzo Aida*[1,2]

[1]*Department of Chemistry and Biotechnology, School of Engineering*

The University of Tokyo,

7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

[2]*Riken Center for Emergent Matter Science*

2-1 Hirosawa, Wako, Saitama 351-0198, Japan

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1. General

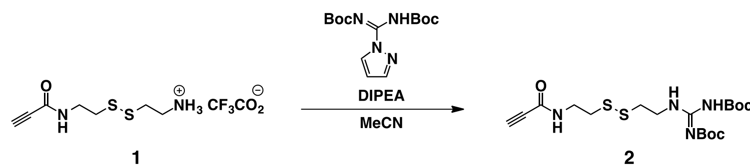
^{1}H and ^{13}C NMR spectra were recorded on a JEOL type GSX-270 or GSX-500 spectrometer or Bruker type AV-500 spectrometer equipped with a TCI cryo probe, where chemical shifts for ^{1}H NMR spectroscopy were determined with respect to non-deuterated solvent residues; $CHCl_3$ (δ 7.26), $CHD_2(CD_3)SO$ (δ 2.50) and HDO (δ 4.79), and those for ^{13}C NMR spectroscopy were determined with respect to $CDCl_3$ (δ 77.2). Matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF-MS) spectrometry was performed using α-cyano-4-hydroxy cinnamic acid (CCA) or sinapic acid (SA) as a matrix on an Applied Biosystems BioSpectrometry Workstation™ model Voyager-DE™ STR spectrometer. Recycling preparative gel permeation chromatography (GPC) was performed on a Japan Analytical Industry models LC908-C60 or LC-918 high performance liquid chromatography using a column set consisting of JAIGEL 1H, 2H, 2.5H, 1H-40, and 2H-40. Normal-phase column chromatography was performed using Wakogel silica C-300HG (particle size 40–60 μm) or Merck alumina 90 standardized. Confocal laser scanning microscopy was performed on a Carl-Zeiss model LSM 510 confocal laser-scanning microscope. Image analysis of agarose gels was performed on a FUJIFILM model LAS-3000 luminescent image analyzer. Electronic absorption spectra were recorded on a Beckman Coulter type FP-6500 spectrometer. Luminescence spectra were recorded on a Promega model GloMax® 96 microplate luminometer. Dynamic light scattering (DLS) and zeta potential measurements were performed using a Malvern model Zetasizer Nano ZS particle size analyzer equipped with a 532 nm frequency doubled diode-pumped solid-state (DPSS) laser light source.

Unless otherwise noted, reagents and solvents were used as received from commercial sources without further purification. Luciferase siRNA ($siRNA^{luc}$; sense: 5’-CUU ACG CUG AGU ACU UCG AdTdT-3’; antisense: 5’-UCG AAG UAC UCA GCG UAA GdTdT-3’), Cy3-labeled luciferase siRNA ($^{Cy3}siRNA$; sense: 5’-[Cy3]CUU ACG CUG AGU ACU UCG AdTdT-3’; antisense: 5’-UCG AAG UAC UCA GCG UAA GdTdT-3’), and mismatch siRNA (mis-siRNA; sense: 5’-GCA GCA CGA CUU CAA GdTdT-3’; antisense: 5’-CUU GAA GAA GUC GUG CUG CdTdT-3’) were purchased from Hokkaido System Science. Human epithelial carcinoma HeLa cells, human hepatocellular carcinoma Huh-7 cells, and human lung adenocarcinoma A549 cells were purchased from ATCC. Huh-7 cells and A549 cells stably expressing the luciferase gene (Huh-7-luc and A549-luc) were obtained by transfecting the cells with luciferase plasmid DNA according to the previously reported methods.[S1] Luciferase-expressing mutants of HeLa cells and mouse melanoma B16F10 cells

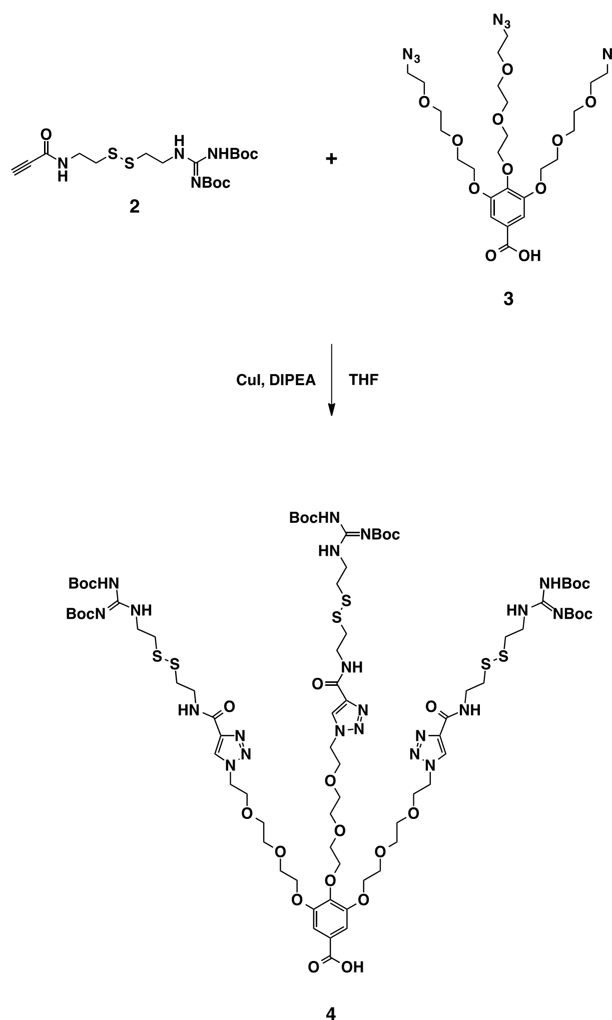
(HeLa-luc and B16F10-luc) were purchased from Caliper Life Sciences. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), Dulbecco's phosphate buffer saline (D-PBS), and Lipofectamine 2000 (LF2000) were purchased from Invitrogen. Cell Counting Kit-8 was purchased from Dojindo. Glutathione (reduced form), free acid (GSH) was purchased from Nacalai Tesque. DL-Buthionine-(*S*,*R*)-sulfoximine (BSO) was purchased from Sigma-Aldrich. Passive Lysis Buffer and Luciferase Assay System were purchased from Promega.

2. Synthesis

2-1. Synthesis of Glue^{SS}-BP

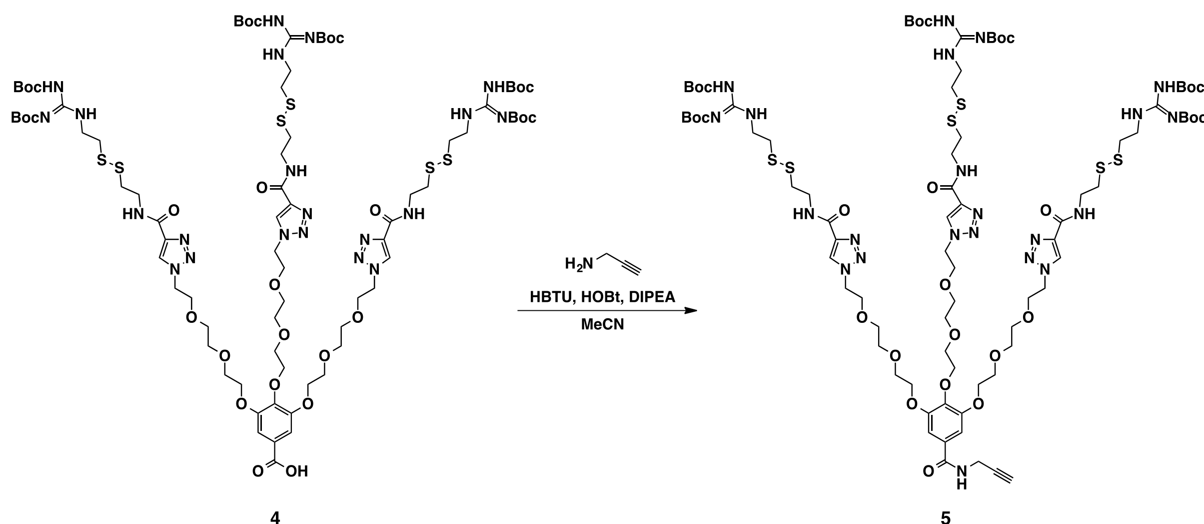


Compound 2. To a MeCN (20 mL) solution of **1**[S2] (149 mg, 0.494 mmol) was successively added *N*,*N*'-bis(*t*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine (169 mg, 0.543 mmol) and diisopropylethylamine (DIPEA, 173 μL, 1.00 mmol), and the mixture was stirred overnight at room temperature. Then, the reaction mixture was evaporated to dryness under reduced pressure, and the residue was dissolved in AcOEt (20 mL) and washed with brine (20 mL $\times$ 3). An organic extract separated was dried over Na_2SO_4 and filtered off from an insoluble fraction. The filtrate was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with AcOEt/hexane (1/2) as an eluent to allow isolation of **2** as white solid (155 mg, 70%). ^{1}H NMR ($CDCl_3$; ppm): δ 1.45–1.51 (m, 18H; $C(CH_3)_3$), 2.78 (s, 1H; CCH), 2.84–2.95 (m, 4H; SCH_2), 3.59 (m, 2H; CH_2NHCN), 3.76 (m, 2H; CH_2NHCO), 7.20 (br, 1H; CH_2NHCO), 8.64 (br, 1H; CH_2NHCN), 11.51 (s, 1H; $NHCO_2$). ^{13}C NMR ($CDCl_3$; ppm): δ 28.0, 28.2, 36.4, 38.5, 38.9, 39.4, 73.3, 76.5, 79.4, 83.3, 152.2, 152.9, 156.0, 163.0.

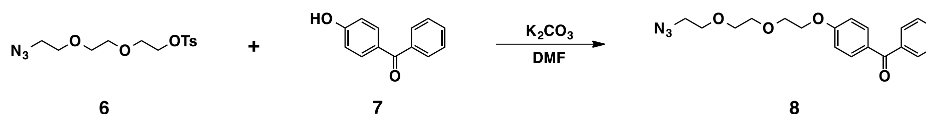


Compound 4. To a THF (20 mL) solution of a mixture of **2** (648 mg, 1.45 mmol) and **3**[S3] (282 mg, 0.440 mmol) was successively added diisopropylethylamine (DIPEA, 505 μL, 2.92 mmol) and copper(I) iodide (276 mg, 1.45 mmol), and the mixture was stirred for 3.5 h at room temperature. Then, the reaction mixture was evaporated to dryness under reduced pressure, and the residue was dissolved in $CHCl_3$ (20 mL) and washed with saturated aqueous NH_4Cl (20 mL) followed by brine (20 mL). An organic extract separated was dried over Na_2SO_4 and filtered off from an insoluble fraction. The filtrate was evaporated to dryness under reduced pressure, and the residue was subjected to recycling preparative GPC with $CHCl_3$ as an eluent to allow isolation of **4** as white solid (688 mg, 79%). ^{1}H NMR ($CDCl_3$; ppm): δ 1.49–1.50 (m, 54H; $C(CH_3)_3$), 2.89 (m, 12H; SCH_2), 3.58–3.64 (m, 12H; SCH_2CH_2), 3.76–3.82 (m, 18H; OCH_2), 3.84–3.90 (m, 6H; triazole-CH_2), 7.41 (m, 2H; ArH), 7.56–7.65 (m, 3H; triazole-H), 8.26–8.34 (m, 3H; triazole-CONH), 8.66 (s, 3H; CH_2NHCN), 11.49 (br, 3H; $NHCO_2$). ^{13}C NMR ($CDCl_3$; ppm): δ 28.1, 28.3, 37.5, 37.9, 38.2, 39.5, 50.6, 69.1, 69.8, 70.4, 70.7, 72.4, 79.2, 83.2, 126.5, 142.9, 152.2, 153.0, 156.0, 160.1, 160.3, 163.2. MALDI-

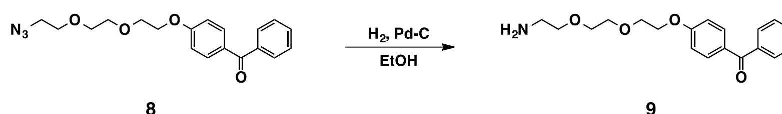
TOF-MS: *m/z* found: 1380.49 ([M – 6Boc + H^+] calcd: 1380.47).



Compound 5. To a MeCN (25 mL) solution of a mixture of **4** (688 mg, 0.347 mmol), propargylamine (191 mg, 3.47 mmol), and diisopropylethylamine (DIPEA, 605 μL, 3.50 mmol) was successively added *o*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU, 132 mg, 0.347 mmol) and 1-hydroxybenzotriazole (HOBt, 53 mg, 0.347 mmol), and the mixture was stirred for 8 h at room temperature. Then, the reaction mixture was evaporated to dryness under reduced pressure, and the residue was dissolved in $CHCl_3$ (20 mL) and washed successively with saturated aqueous NH_4Cl (20 mL), brine (20 mL), saturated aqueous $NaHCO_3$ (20 mL), and brine (20 mL). An organic extract separated was dried over Na_2SO_4 and filtered off from an insoluble fraction. The filtrate was evaporated to dryness under reduced pressure, and the residue was chromatographed on alumina with AcOEt/MeOH (95/5) as an eluent. The obtained fraction was subjected to recycling preparative GPC with $CHCl_3$ as an eluent to allow isolation of **5** as pale yellow solid (623 mg, 89%). ^{1}H NMR ($CDCl_3$; ppm): δ 1.49–1.50 (m, 54H; $C(CH_3)_3$), 2.86–2.93 (m, 12H; SCH_2), 3.59–3.65 (m, 13H; SCH_2CH_2, CCH), 3.73–3.82 (m, 18H; OCH_2), 3.84–3.90 (m, 6H; triazole-CH_2CH_2), 4.16–4.23 (m, 8H; $ArOCH_2$, $ArCONHCH_2$), 4.57–4.59 (m, 6H; triazole-CH_2), 7.55–7.63 (m, 3H; triazole-H), 7.74–7.78 (br, 1H; ArCONH), 8.24–8.25 (m, 3H; triazole-CONH), 8.61–8.64 (m, 3H; CH_2NHCN), 11.48 (br, 3H; $NHCO_2$). ^{13}C NMR ($CDCl_3$; ppm): δ 29.7, 37.6, 38.1, 38.3, 39.6, 50.6, 69.1, 69.8, 70.5, 70.6, 70.7, 71.2, 72.4, 79.2, 80.2, 83.2, 107.7, 126.4, 126.6, 129.1, 142.9, 152.3, 153.0, 156.0, 160.2, 160.3, 163.3, 166.4. MALDI-TOF-MS: *m/z* found: 1417.26 ([M – 6Boc + H^+] calcd: 1417.50).

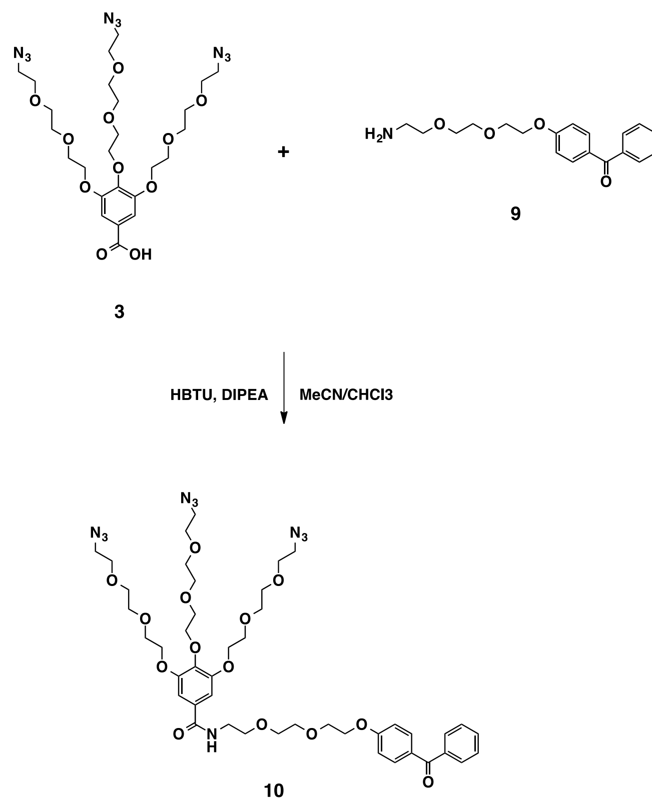


Compound 8. To a DMF (15 mL) solution of a mixture of **6**[S4] (1.66 g, 5.04 mmol) and **7** (1.00 g, 5.04 mmol) was added K_2CO_3 (2.09 g, 15.1 mmol), and the mixture was stirred for 18 h at 80 °C. An insoluble fraction was filtered off, and then the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in AcOEt (50 mL) and washed with water (50 mL) followed by brine (50 mL). An organic extract separated was dried over Na_2SO_4 and filtered off from an insoluble fraction. The filtrate was evaporated to dryness under reduced pressure, and the residue was chromatographed on alumina followed by silica gel using $CHCl_3$ as an eluent to allow isolation of **8** as yellowish clear oil (1.60 g, 89%). ^{1}H NMR ($CDCl_3$; ppm): δ 3.38 (t, J = 5.0 Hz, 2H; CH_2N_3), 3.63–3.80 (m, 6H; OCH_2), 3.90 (t, J = 4.7 Hz, 2H; $ArOCH_2CH_2$), 4.21 (t, J = 4.7 Hz, 2H; $ArOCH_2$), 6.98 (d, J = 8.6 Hz, 2H; 3,5-ArH), 7.41–7.61 (m, 3H; 3',4',5'-ArH), 7.70–7.88 (m, 4H; 2,5,2',5'-ArH). ^{13}C NMR ($CDCl_3$; ppm): δ 50.6, 67.5, 69.5, 70.0, 70.6, 70.8, 113.9, 127.9, 129.5, 130.0, 131.6, 132.2, 138.0, 162.2, 195.1. MALDI-TOF-MS: m/z found: 394.29 ([M + K$^+$] calcd: 394.12), 378.32 ([M + Na$^+$] calcd: 378.14), 356.35 ([M + H$^+$] calcd: 356.16).



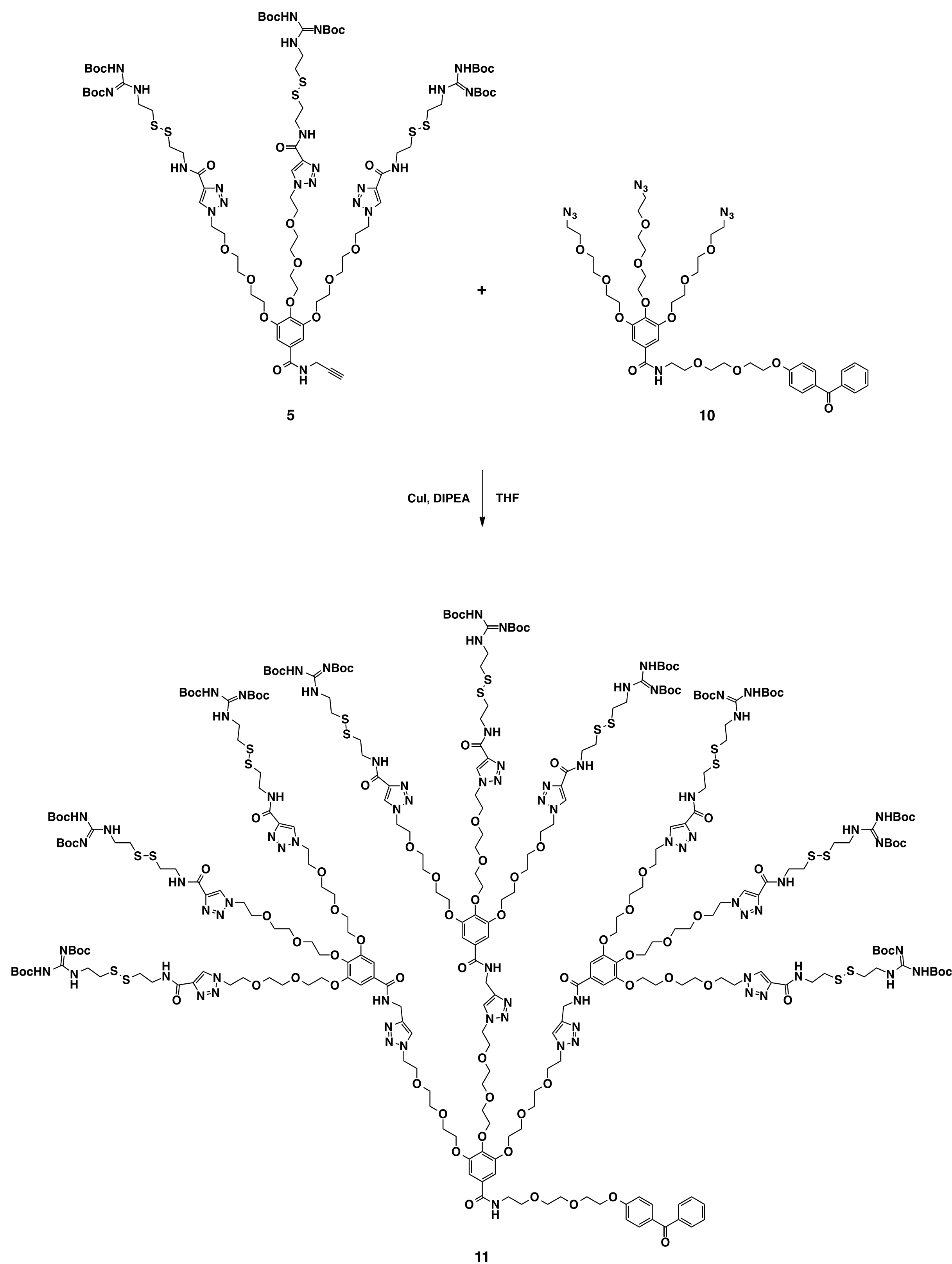
Compound 9. To an EtOH (10 mL) solution of **8** (1.45 g, 4.08 mmol) was added 10% palladium on carbon (100 mg), and the suspension was bubbled with H_2 overnight at room temperature. Then, the reaction mixture was filtered off with celite from an insoluble catalyst residue, and the filtrate was evaporated to dryness under reduced pressure, affording **9** as yellow solid (1.24 g, 93%). ^{1}H NMR ($CDCl_3$; ppm): δ 3.15 (t, J = 5.1 Hz, 2H; CH_2N_3), 3.55–3.97 (m, 8H; OCH_2), 4.22 (t, J = 4.7 Hz, 2H; $ArOCH_2$), 5.39 (br, 2H; NH_2), 7.00 (d, J = 9.2 Hz, 2H; 3,5-ArH), 7.41–7.61 (m, 3H; 3',4',5'-ArH), 7.70–7.88 (m, 4H; 2,5,2',5'-ArH). ^{13}C NMR ($CDCl_3$; ppm): δ 39.8, 67.5, 69.4, 70.0, 70.6, 70.8, 114.1, 128.1, 129.6, 130.3, 131.9,

132.4, 138.0, 162.0, 195.5. MALDI-TOF-MS: *m/z* found: 370.46 ([M + K^+] calcd: 368.13), 352.41 ([M + Na^+] calcd: 352.15), 330.43 ([M + H^+] calcd: 330.17).



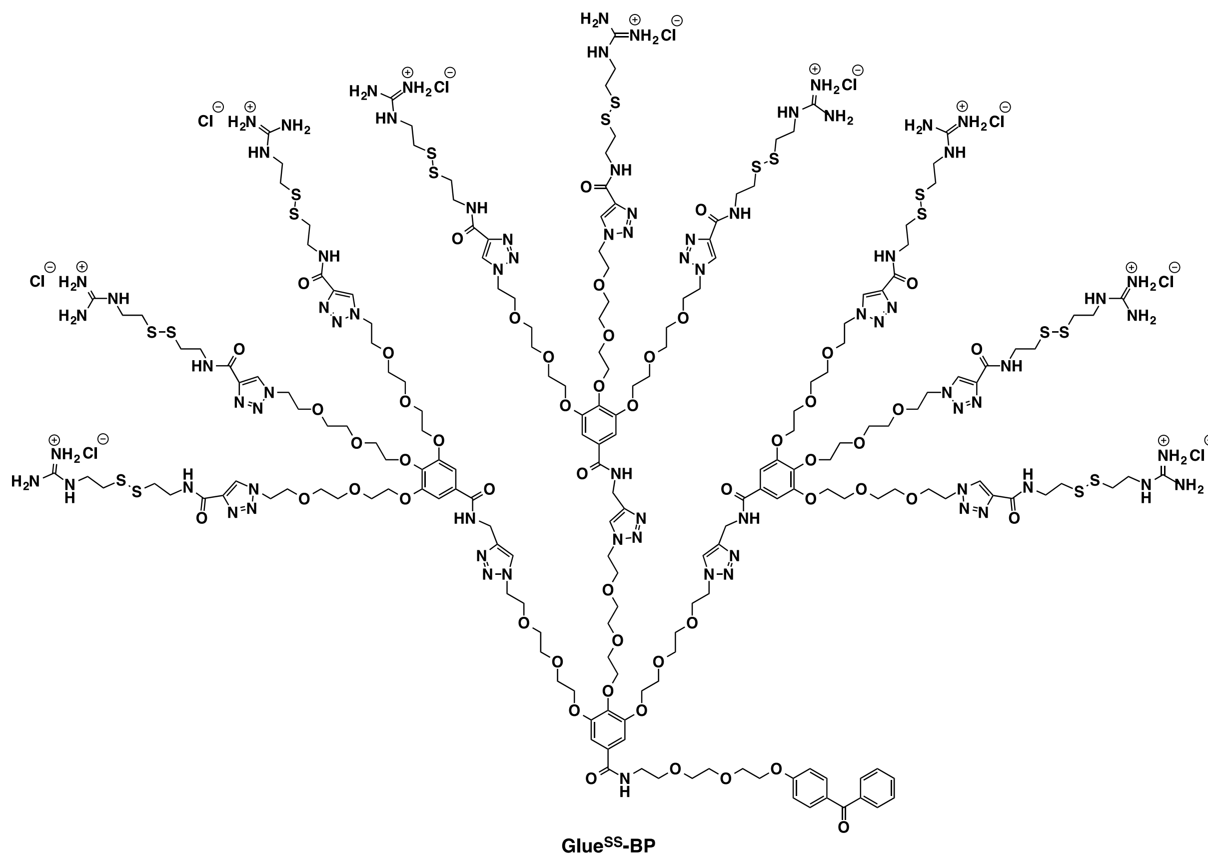
Compound 10. To a MeCN/$CHCl_3$ (10 mL, v/v = 1/1) solution of a mixture of **3** (500 mg, 0.779 mmol), **9** (254 mg, 0.771 mmol), and diisopropylethylamine (DIPEA, 200 μL, 1.16 mmol) was added *o*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HBTU, 192 mg, 0.818 mmol), and the mixture was stirred for 3 h at room temperature. Then, the reaction mixture was evaporated to dryness under reduced pressure, and the residue was dissolved in AcOEt (20 mL) and washed successively with aqueous $NaHSO_4$ (2 M, 20 mL), brine (20 mL), saturated aqueous $NaHCO_3$ (20 mL), and brine (20 mL). An organic extract separated was dried over Na_2SO_4 and filtered off from an insoluble fraction. The filtrate was evaporated to dryness under reduced pressure, and the residue was chromatographed on alumina with AcOEt as an eluent and then on silica gel with AcOEt/hexane (4/1 to 1/0) as an eluent. The obtained fraction was subjected to recycling preparative GPC with $CHCl_3$ as an eluent to allow isolation of **10** as pale yellowish oil (373 mg, 51%). ^{1}H NMR ($CDCl_3$; ppm): δ 3.36 (m, 6H; CH_2N_3), 3.52–3.94 (m, 34H; OCH_2, CONHCH_2), 4.18 (m, 8H; ArOCH_2), 6.73 (br, 1H; CON*H*CH_2), 6.93 (d, *J* = 8.6 Hz, 2H; 3,5-benzophenone-H), 7.08 (s, 2H; ArH), 7.40–

7.60 (m, 3H; 3',4',5'-benzophenone-H), 7.67–7.83 (m, 4H; 2,5,2',5'-benzophenone-H). ^{13}C NMR ($CDCl_3$; ppm): δ 39.8, 50.6, 67.5, 69.0, 69.7, 69.8, 69.9, 70.1, 70.4, 70.6, 70.7, 72.3, 106.9, 113.9, 128.0, 129.6, 130.1, 131.7, 132.3, 137.9, 141.2, 152.2, 162.0, 166.7, 195.5. MALDI-TOF-MS: *m/z* found: 991.81 ([M + K^+] calcd: 991.39), 975.84 ([M + Na^+] calcd: 975.42).



Compound 11. To a THF (1 mL) solution of a mixture of **5** (140 mg, 69.3 μmol) and **10** (20.0 mg, 21.0 μmol) was successively added diisopropylethylamine (DIPEA, 24 μL, 0.139 mmol) and copper(I) iodide (13.2 mg, 69.3 μmol), and the mixture was stirred overnight at

room temperature. Then, the reaction mixture was diluted with $CHCl_3$ (20 mL) and washed with saturated aqueous NH_4Cl (20 mL) followed by brine (20 mL). An organic extract separated was dried over Na_2SO_4 and filtered off from an insoluble fraction. The filtrate was evaporated to dryness under reduced pressure, and the residue was subjected to recycling preparative GPC with $CHCl_3$ as an eluent to allow isolation of **11** as pale yellow solid (50 mg, 34%). ^{1}H NMR ($CDCl_3$; ppm): δ 1.42–1.49 (m, 162H; $C(CH_3)_3$), 2.83–2.90 (m, 36H; SCH_2), 3.54–3.84 (m, 142H; OCH_2), 4.06–4.13 (m, 27H; $ArOCH_2$), 4.42 (br, 6H; $OCH_2C\mathit{H}_2$-triazole), 4.52–4.54 (br, 18H; triazole-CH_2), 4.59–4.60 (m, 6H; triazole-$C\mathit{H}_2NH$), 6.87–6.89 (m, 3H; triazole-H), 7.11–7.15 (s, 9H; triazole-H), 7.42–7.45 (m, 2H; 3,5-benzophenone-H), 7.52–7.54 (m, 2H, 3,5-benzophenone-H), 7.59–7.61 (m, 3H, 3',4',5'-benzophenone-H), 7.68–7.75 (m, 12H; ArH, 2,6,2',6'-benzophenone-H), 8.04 (br, 1H; $ArCON\mathit{H}CH_2CH_2O$), 8.58–8.60 (m, 9H; $CH_2N\mathit{H}CN$), 11.45 (s, 9H; $NHCO_2$). ^{13}C NMR ($CDCl_3$; ppm): δ 28.0, 28.2, 37.0, 37.5, 37.9, 39.1, 50.1, 50.5, 67.5, 68.7, 69.0, 69.1, 69.2, 69.4, 69.6, 70.3, 70.5, 72.3, 79.3, 83.2, 106.8, 114.0, 123.6, 126.5, 126.6, 128.2, 129.7, 131.9, 132.4, 138.1, 140.9, 142.9, 152.2, 153.1, 156.1, 160.4, 162.2, 163.4, 166.8, 167.0.



Glue^{SS}-BP

Glue^{SS}-BP. A 1,4-dioxane (5 mL) solution of HCl (4 M) was added to **11** (50.1 mg, 7.19 μmol), and the mixture was stirred overnight at room temperature. The reaction mixture was evaporated to dryness under reduced pressure, affording **Glue^{SS}-BP** as yellow solid (27.4 mg, 69%). ^{1}H NMR (DMSO-d_6; ppm): δ 2.80–3.01 (m, 36H; SCH_2), 3.22–3.88 (m, 144H; CH_2NHCN, OCH_2, CH_2CH_2NHCO), 3.94–4.19 (m, 26H; $ArOCH_2$), 4.40–4.63 (m, 30H; OCH_2CH_2-triazole, triazole-CH_2NH), 7.01–7.98 (m, 20H; ArH, triazole-H, benzophenone-H), 8.53 (s, 9H; triazole-H), 8.68 (s, 9H; triazole-CONH), 9.03 (br, 3H; triazole-CH_2NH). MALDI-TOF-MS: m/z found: 5207.00 ([M – 9HCl + H^+] calcd: 5202.91).

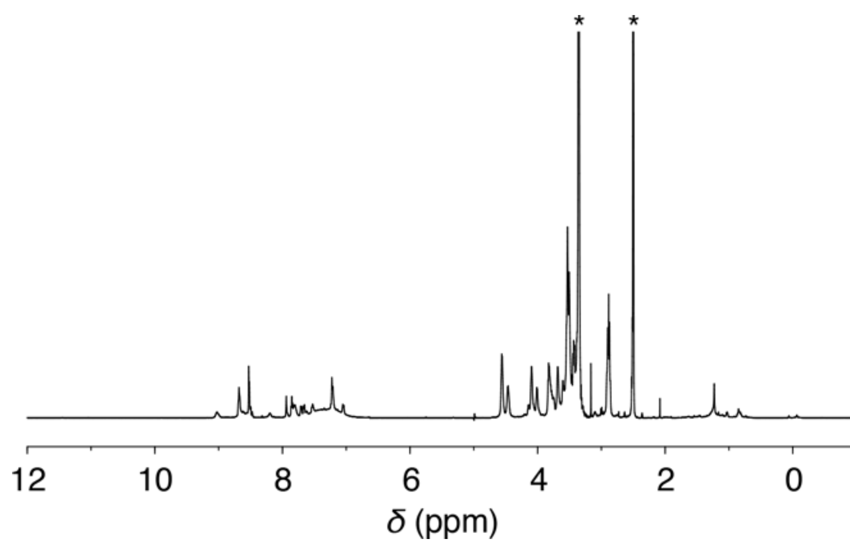
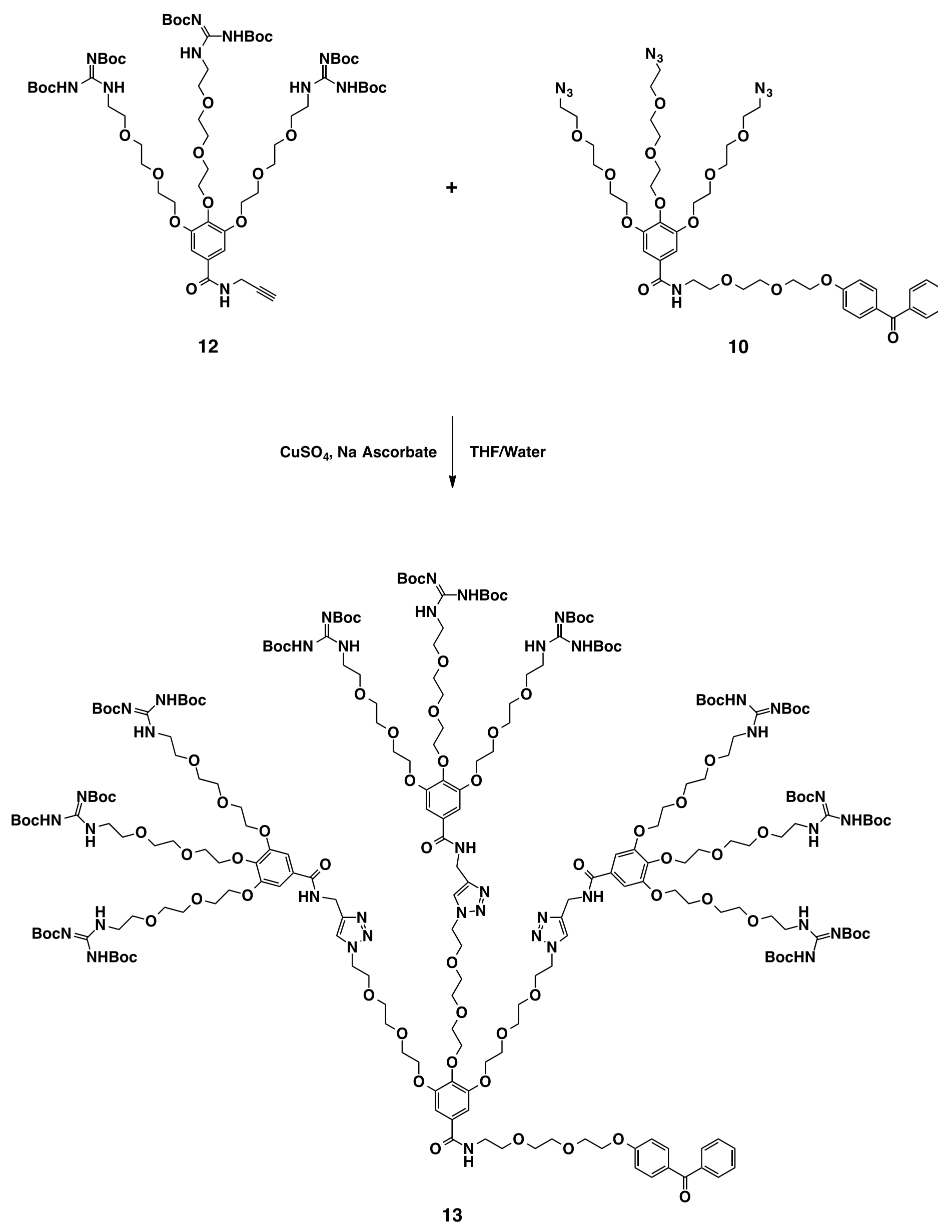


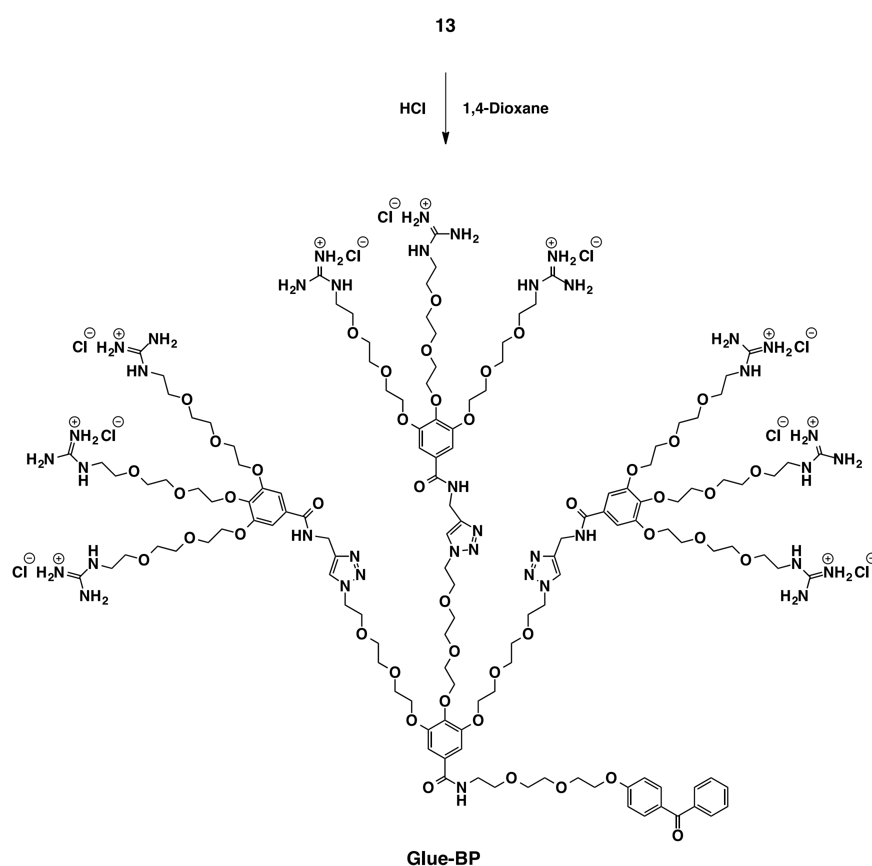
Figure S1. ^{1}H NMR spectrum of Glue^{SS}-BP in DMSO-d_6 at 25 °C. The signals marked with asterisks at δ 2.50 and 3.35 ppm are due to DMSO and water, respectively.

2-2. Synthesis of Glue-BP



Compound 13. A THF/water (10 mL, v/v = 1/1) solution of a mixture of **12**[S5] (555 mg, 0.418 mmol), **10** (119 mg, 0.125 mmol), copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 11 mg, 40 μmol), and sodium ascorbate (166 mg, 0.836 mmol) was stirred for 12 h at room temperature. Then, the reaction mixture was diluted with AcOEt and washed with brine (20 mL). An organic extract separated was dried over Na_2SO_4 and filtered off with celite from an insoluble fraction. The filtrate was evaporated to dryness under reduced pressure, and the residue was subjected to recycling preparative GPC with $CHCl_3$ as an eluent to allow isolation of **13** as pale brown solid (420 mg, 68%). ^{1}H NMR ($CDCl_3$; ppm): δ 1.39 (m, 162H;

$C(CH_3)_3$), 3.33–3.85 (m, 122H; OCH_2, CH_2NHCN), 3.93–4.18 (m, 26H; $ArOCH_2$), 4.38 (br, 6H; OCH_2CH_2-triazole), 4.53 (br, 6H; triazole-CH_2NH), 6.82 (m, 3H; triazole-H), 7.08 (br, 6H; ArCON*H*, 3,5-benzophenone-H), 7.29 (br, 10H; ArH, 3,5-benzophenone-H), 7.33–7.53 (m, 3H; 3',4',5'-benzophenone-H), 7.55–7.86 (m, 4H; 2,5,2',5'-benzophenone-H), 8.52 (br, 9H; CH_2N*H*CN), 11.40 (br, 9H; $NHCO_2$). ^{13}C NMR ($CDCl_3$; ppm): δ 27.9, 28.1, 35.2, 40.4, 49.9, 68.8, 69.0, 69.2, 69.5, 69.9, 70.2, 70.4, 70.5, 72.1, 78.8, 82.6, 106.9, 113.8, 123.2, 127.8, 128.8, 129.3, 129.5, 129.9, 131.5, 132.0, 137.8, 141.1, 152.1, 152.6, 155.8, 161.9, 163.0, 166.4, 166.6, 194.8.



Glue-BP. A 1,4-dioxane (5 mL) solution of HCl (4 M) was added to **13** (405 mg, 82 μmol), and the mixture was stirred for 6 h at room temperature. The reaction mixture was evaporated to dryness under reduced pressure, affording **Glue-BP** as pale brown solid (282 mg, quant.). ^{1}H NMR (D_2O; ppm): δ 3.20–3.34 (m, 18H; CH_2NHCN), 3.37–3.95 (m, 106H; OCH_2, CH_2CH_2NHCO), 3.96–4.18 (m, 26H; $ArOCH_2$), 4.49 (br, 12H; OCH_2CH_2-triazole, triazole-CH_2NH), 6.65 (m, 3H; triazole-H), 6.94–7.15 (m, 10H; ArH, 3,5-benzophenone-H), 7.19–7.56 (m, 7H; 3',4',5'-benzophenone-H, 2,5,2',5'-benzophenone-H). ^{13}C NMR (D_2O; ppm): δ

29.0, 40.5, 42.0, 51.4, 67.2, 69.0, 69.5, 69.6, 69.7, 70.1, 70.3, 70.4, 70.7, 72.7, 106.6, 106.8, 114.7, 129.6, 130.2, 130.3, 133.2, 133.4, 137.4, 140.2, 152.4, 152.6, 157.8, 162.9, 166.7, 167.5, 168.8, 198.0. MALDI-TOF-MS: *m*/*z* found: 3132.72 ([M − 9HCl + H^+] calcd: 3132.64).

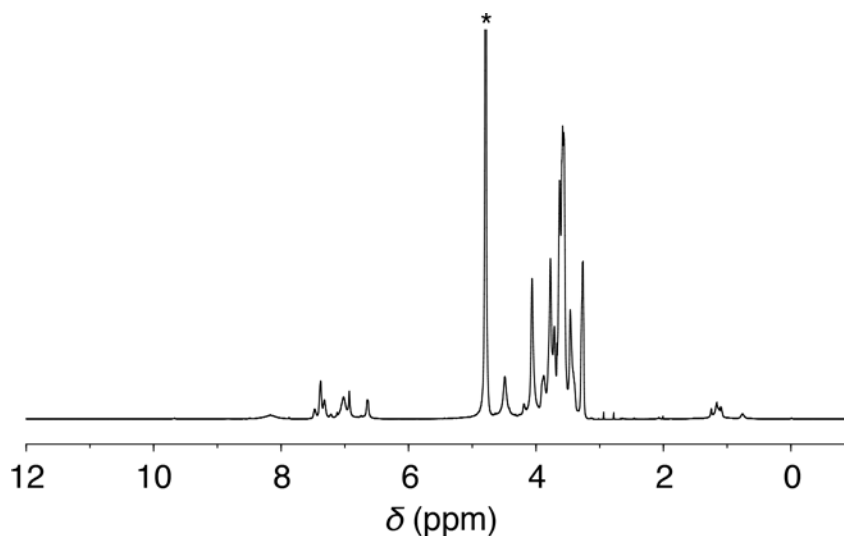


Figure S2. ^{1}H NMR spectrum of Glue-BP in D_2O at 24 °C. The signals marked with an asterisk at δ 4.79 ppm is due to water.

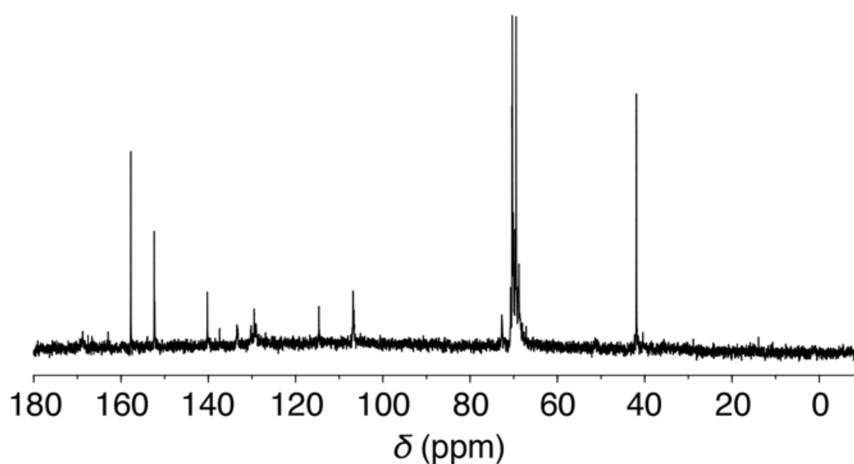


Figure S3. ^{13}C NMR spectrum of Glue-BP in D_2O at 24 °C.

3. Agarose Gel Electrophoresis

A HEPES buffer (10 mM, pH 7.4) solution of a mixture of siRNA (1.5 μM), Glue^{SS}-BP (0–420 μM), and glutathione (GSH, 10 mM) was incubated for 2 h at 37 °C and subjected to agarose gel electrophoresis. Likewise, reference samples before incubation with GSH were obtained under conditions otherwise identical to the above procedures. The gel was stained with ethidium bromide and visualized using a FUJIFILM model LAS-3000 luminescent image analyzer (λ_{ext} = 460 nm). siRNA and Glue^{SS}-BP were mixed at $[Gu^+]/[PO_4^-]$ = 0–60.

4. Dynamic Light Scattering (DLS) and Zeta Potential Measurements

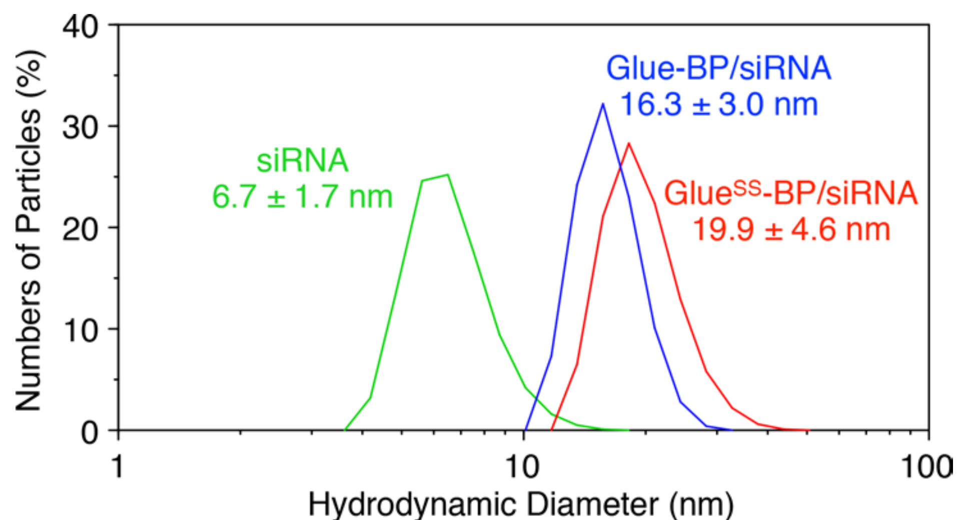


Figure S4. DLS histograms of siRNA (100 nM) in the absence (green) and presence of Glue-BP (5 μM, blue) and Glue^{SS}-BP (5 μM, red) in HEPES buffer (10 mM, pH 7.4) at 25 °C.

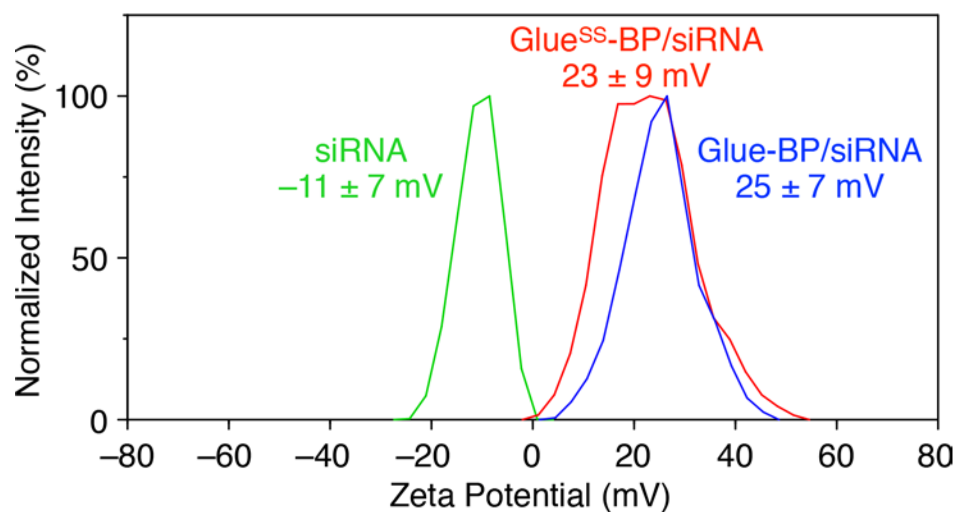


Figure S5. Zeta potential profiles of siRNA (100 nM) in the absence (green) and presence of Glue-BP (5 μM, blue) and Glue^{SS}-BP (5 μM, red) in HEPES buffer (10 mM, pH 7.4) at 25 °C.

5. Confocal Laser Scanning Microscopy

5-1. Structure of Cy3-labeled siRNA (^{Cy3}siRNA)

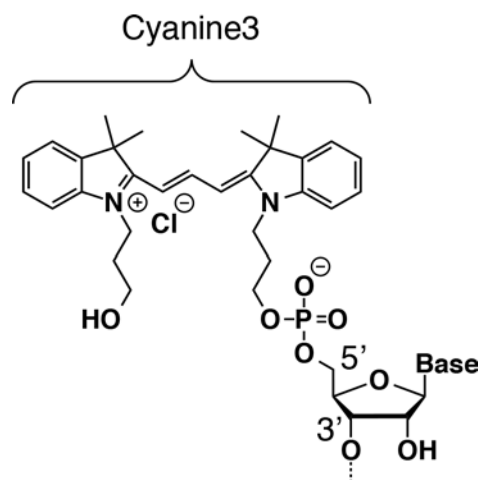


Figure S6. Terminal structure of the Cy3 (Cyanine3)-labeled siRNA sense strand.

5-2. Cellular Uptake of ^{Cy3}siRNA

Human epithelial carcinoma HeLa cells (5.0×10^3 cells/dish; 35-mm glass bottom culture dish, culture area = 3.14 cm^2/dish) were incubated at 37 °C under 5% CO_2 for 24 h in Dulbecco's modified Eagle's medium (DMEM, 1 mL) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin). The cell sample was rinsed with Dulbecco's phosphate buffer saline (D-PBS, 1 mL × 3) and treated with DMEM (500 μL) containing ^{Cy3}siRNA (100 nM) and Glue^{SS}-BP (5 μM). After incubation at 37 °C under 5% CO_2 for 1 h, the cell sample was rinsed with D-PBS (1 mL), treated with DMEM (10% FBS, 1 mL), and then subjected to confocal laser scanning microscopy (λ_{ext} = 435 nm). An analogous cell sample incubated at 4 °C was likewise prepared under conditions otherwise identical to the above procedures. A reference cell sample treated with DMEM (500 μL) containing ^{Cy3}siRNA (100 nM) and Glue-BP (5 μM) was likewise prepared under conditions otherwise identical to the above procedures. Lipofectamine 2000 (LF2000) was used according to the procedure provided by the supplier (Invitrogen).

Likewise, HeLa cells were incubated at 37 °C under 5% CO_2 for 24 h in DMEM (1 mL) containing 10% FBS and buthionine sulfoximine (BSO, 100 μM). The cell sample was rinsed with D-PBS (1 mL × 3) and treated with DMEM (500 μL) containing BSO (100 μM), ^{Cy3}siRNA (100 nM), and Glue^{SS}-BP (5 μM). After incubation at 37 °C under 5% CO_2 for 1 h,

the cell sample was rinsed with D-PBS (1 mL × 3), treated with DMEM (10% FBS, 1 mL), and then subjected to confocal laser scanning microscopy (λ_{ext} = 435 nm).

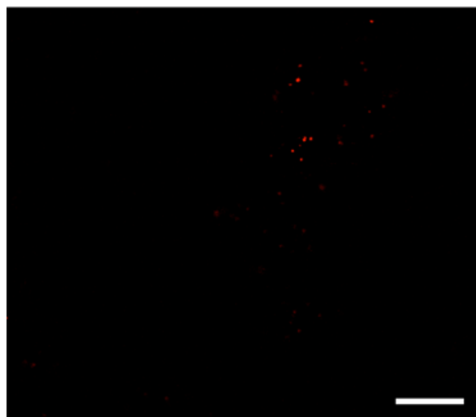


Figure S7. A confocal laser scanning microscopy (CLSM, λ_{ext} = 435 nm) image of HeLa cells after 1-h incubation at 4 °C in DMEM containing ^{Cy3}siRNA (100 nM) and LF2000. Scale bar = 20 μm.

6. Gene Knockdown Assay

6-1. Gene Knockdown in HeLa Cells

HeLa-luc cells (1.5 × 10^4 cells/well; 96-well culture plate, culture area = 0.33 cm^2/well) stably expressing the luciferase gene were incubated at 37 °C under 5% CO_2 for 24 h in DMEM (100 μL) containing 10% FBS and 1% antibiotics (penicillin-streptomycin). The cell sample was rinsed with D-PBS (100 μL × 3) and treated with DMEM (100 μL) containing siRNA (100 nM) and $Glue^{SS}$-BP (10 μM). After incubation at 37 °C under 5% CO_2 for 4 h, the cell sample was rinsed with D-PBS (100 μL) and treated with DMEM (10% FBS, 100 μL). After incubation at 37 °C under 5% CO_2 for 24 h, the cell sample was rinsed with D-PBS (100 μL × 2) and supplied with Passive Lysis Buffer (100 μL). The obtained suspension (20 μL) was added to the Luciferase Assay Reagent (100 μL), and the resultant mixture was subjected to luminescence spectroscopy. A reference cell sample treated with DMEM (100 μL) containing siRNA (100 nM) and Glue-BP (10 μM) or Lipofectamine 2000 (LF2000) was likewise prepared under conditions otherwise identical to the above procedures. An analogous cell sample supplied with DMEM (10% FBS, 100 μL) containing siRNA (100 nM) and $Glue^{SS}$-BP (10 μM) or LF2000 was likewise prepared under conditions otherwise identical to the above procedures.

6-2. Gene Knockdown in Various Cell Lines

B16F10-luc, Huh-7-luc, and A549-luc cells (B16F10-luc: 5.0 × 10^3 cells/well; Huh-7-luc and A549-luc: 1.5 × 10^4 cells/well) stably expressing the luciferase gene were incubated at 37 °C under 5% CO_2 for 24 h in DMEM (100 μL) containing 10% FBS and 1% antibiotics (penicillin-streptomycin). The cell sample was rinsed with D-PBS (100 μL × 3) and treated with DMEM (100 μL) containing siRNA (100 nM) and $Glue^{SS}$-BP (10 μM). Luciferase activities were likewise evaluated under conditions otherwise identical to the procedure 5-1.

7. Cell Viability Assay

HeLa cells (5.0×10^3 cells/well; 96-well culture plate, culture area = 0.33 cm^2/well) were incubated at 37 °C under 5% CO_2 for 24 h in DMEM (100 μL) containing 10% FBS and 1% antibiotics (penicillin-streptomycin). The cell sample was rinsed with D-PBS (100 μL × 3) and treated with DMEM (100 μL) containing $Glue^{SS}$-BP (0.05–10 μM) or Glue-BP (0.05–10 μM). After incubation at 37 °C under 5% CO_2 for 4 h, Cell Counting Kit-8 reagent (10 μL) was added to the cells followed by incubation of the resultant mixture at 37 °C under 5% CO_2 for 30 min. Cell samples thus prepared were subjected to absorption spectroscopy (λ = 450 nm).

8. References

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