Supplementary Material (ESI) for Molecular BioSystems

Supporting Information

Effective Delivery of IgG-antibodies into Infected Cells via Dendritic Molecular Transporter Conjugate IgGMT

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General Methods. All reagents were purchased either from AAPER Alcohol and Chemical Co. (Shelbyville, KY), Acros Organics, Alfa Aesar, Chem-Impex Int. (Wood Dale, IL), EMD, Fisher, Invitrogen (Carlsbad, CA), J.T. Bakker, or Sigma-Aldrich, and used without further purification unless otherwise noted. Synagis (palivizumab) monoclonal antibody (IgG1κ) was obtained from MedImmune, Inc. (Gaithersburg, MD). Alexa Fluor® 568 Protein Labeling Kit (A10238) was purchased from Molecular Probes (Eugene, OR). Uncoated, 14 mm diameter Microwell, No. 1.5 MatTek dishes were purchased from MatTek Corp. (Ashland, MA). Analytical TLC was performed on commercial Merck plates coated with silica gel 60 F_{254} and spots located by UV light (254 and 366nm) or via iodine or potassium permanganate staining. Silica gel for flash chromatography was Sorbent Technologies 60 Å (40-63μm, technical grade). Spectra/Por® Biotech Regenerated Cellulose Dialysis Membranes (3,500 MWCO) obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Nuclear magnetic resonance was performed on a Bruker DPX-300, a Bruker AV-400, Bruker DRX-500, or Bruker AV-II spectrometer. Chemical shifts are reported in ppm and referenced to the corresponding residual nuclei in deuterated solvents. Nitro triacid, Behera's amine, and G1 were synthesized according to the previously published procedures.[1]

Amine G1, 1 (Hydrogenation of G1). A solution of G1 (8.33 g, 5.67 mmol) in ethanol (500 mL) in a Parr hydrogenation bottle with Raney-Nickel (8.11 g, 138.18 mmol) was shaken at 65 psi for 6 days at room temperature. The reaction was then filtered through Celite, rinsing with additional ethanol, and the removal of the solvent under reduced

pressure gave amine G1 as a white solid (6.71 g, 82.3 %). ^{1}H NMR (500 MHz, MeOD) δ 7.39 (2H, s, NH_2), 6.92 (3H, s, NH,), 2.19 (24H, m, CH_2), 1.94 (18H, m, CH_2), 1.61 (6H, m, CH_2), 1.45 (81H, s, CH_3); ^{13}C NMR (400 MHz, MeOD) δ 175.7, 174.4, 81.7, 58.7, 36.0, 32.0, 30.7, 30.5, 28.5.

SSG1, (PDPOH attachment to amine G1) 3-(2-pyridinyldithio)propanoic acid (PDPOH) (91.46 mg, 4.25 mmol) in anhydrous tetrahydrofuran (THF) (100 mL) was stirred under argon at room temperature with 1-hydrobenzotriazole (HOBt) (68.90 mg, 5.10 mmol) and DCC (1.05 g, 5.10 mmol). After 1 h, amine G1, **1**, (7.34 g, 5.10 mmol) was added to the solution and the reaction proceeded for 48 h, after which, it was filtered and concentrated under reduced pressure. The crude material was purified via flash column chromatography eluting with 10:1 hexanes:ethyl acetate increasing to 100% ethyl acetate to give white SS-G1 (4.67 g, 67.1 %). ^{1}H NMR (400 MHz, MeOD) δ 8.42 (1H, d, J=4.6Hz, ArH), 7.83 (1H, td, J=4.9Hz, J=8.2Hz, ArH), 7.61 (1H, s, ArH), 7.33 (4H, s, NH), 7.24 (1H, ddd,J=1.5Hz, J=4.9Hz, J=6.7Hz, ArH), 3.08 (2H, t, J=7.0Hz, CH_2), 2.64 (2H, t, J=6.9Hz, CH), 2.91-1.92 (48H, m, CH_2), 1.44 (81H, m, CH_3); ^{13}C NMR (400 MHz, MeOD) δ 175.4, 174.4, 172.9, 161.2, 150.5, 139.2, 122.5, 121.3, 81.7, 59.2, 58.8, 36.8, 35.6, 32.3, 32.1, 30.7, 30.5, 28.5.

SSG1OH, 2 (Deprotection of SSG1). SSG1 (4.67 g, 2.85 mmol) was dissolved in formic acid (100 mL) and the reaction stirred at room temperature overnight. Upon completion, the formic acid was removed azeotropically with toluene under reduced pressure to yield the product SSG1OH (3.29 g, 100.0 %). ^{1}H NMR (400 MHz, MeOD) δ 8.41 (1H, d, J=4.4Hz, ArH), 8.07 (9H, s, COOH), 7.84 (1H, m, ArH,), 7.53 (1H, td, J=7.4Hz, J=25.5Hz, ArH), 7.33 (4H, m, NH), 7.22 (1H, m, ArH), 3.08 (2H, t, J=7.0Hz,

CH_2), 2.64 (2H, t, J=7.0Hz, CH_2), 2.32-1.89 (48H, m, CH_2); ^{13}C NMR (400 MHz, MeOD) δ 177.1, 175.6, 175.5, 164.5, 150.4, 139.4, 122.5, 121.5, 59.2, 58.7, 36.9, 35.6, 32.0, 31.9, 30.5, 29.3.

SSGGLL, 3 (N-Boc-1,6-diaminohexane attachment to SSG1 and deprotection of pheripheral Boc groups) SSG1OH, **2** (3.29 g, 2.91 mmol) in anhydrous THF (100 mL) was stirred under argon at room temperature with HOBt (4.23 g, 31.25 mmol) and DCC (6.45 g, 31.25 mmol). After one hour, N-boc-1,6-diaminohexane (6.76 g, 31.25 mmol) was added to the solution and the reaction proceeded for 48 h at room temperature. Upon completion, the reaction solution was filtered to remove the DCC salt and the filtrate concentrated and purified via flash column chromatography eluting with 1% methanol in dichloromethane and gradually increasing to 10% methanol in dichloromethane to yield a white solid (4.42 g, 52.0 %). ^{1}H NMR (400 MHz) δ 8.43 (1H, m, ArH), 7.98 (1H, d, J=4.8Hz, ArH), 7.81 (18H, m, NH,), 7.52 (1H, d, J=17.8Hz, ArH), 7.23 (1H, dd, J=4.6Hz, J=8.0Hz, ArH), 6.55 (4H, s, NH), 3.15 (18H, t, J=6.6Hz, CH_2), 3.08 (2H s, CH_2), 3.02 (18H, t, J=6.9Hz, CH_2), 2.67 (2H, s, CH_2), 2.17-1.98 (48H, m, CH_2), 1.46 (117H, m, CH_2, CH_3), 1.33 (36H, s, CH_2); ^{13}C NMR (400 MHz, MeOD) δ 175.4, 174.8, 172.8, 161.0, 158.3, 150.5, 139.1, 122.4, 121.1, 79.6, 59.4, 59.0, 41.2, 40.4, 36.9, 35.6, 31.9, 31.8, 31.7, 31.3, 30.9, 30.3, 28.9, 27.7, 27.5. The resulting solid was dissolved in 1,4-dioxane (20 mL), the solution was cooled to 0 ˚C, and 4 M HCl in 1,4-dioxane (20 mL) was added and the reaction stirred for 24 h at room temperature. Removal of the solvent under pressure gave a white solid, SSGGLL(3.55 g, 100.0%). ^{1}H NMR (400 MHz) δ 9.15 (9H, m, NH), 8.89 (1H, s, ArH), 8.58 (1H, m, ArH), 8.42 (1H, d, J=5.9Hz, ArH), 8.11 (18H, s, NH_2), 7.92 (5H, m, NH, ArH), 3.35 (2H, d, J=1.8Hz, CH_2), 3.27

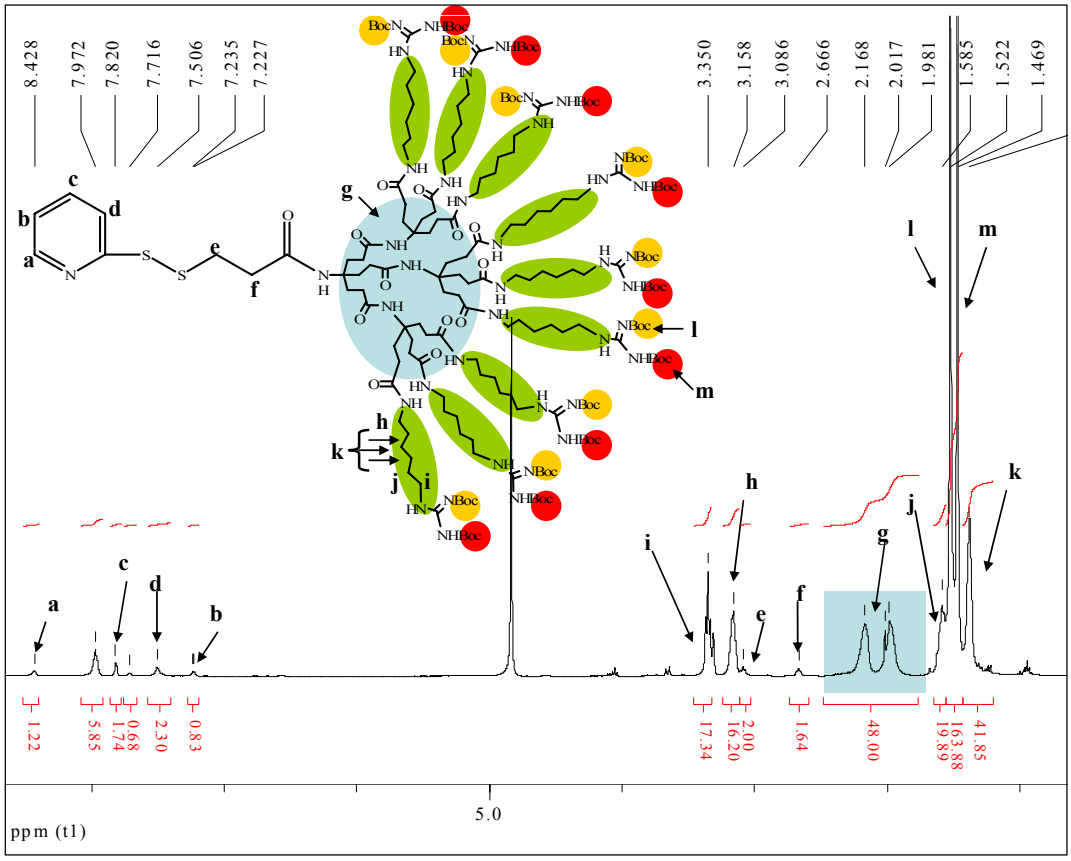
(18H, s, CH_2), 2.96 (18H, t, J=6.7Hz, CH_2), 2.83 (2H, s, CH_2), 2.41-2.05 (48H, m, CH_2), 1.60 (36H, s, CH_2), 1.42 (36H, s, CH_2); ^{13}C NMR (400 MHz, MeOD) δ 176.8, 176.1, 175.3, 157.7, 147.4, 144.2, 126.9, 125.5, 59.7, 59.3, 41.2, 40.7, 32.0, 32.0, 31.8, 30.6, 29.6, 28.3, 27.3, 27.0.

Attachment of Goodman's reagent to SSG1LL. To a cooled solution of SSG1LL HCl salt (3.55 g, 1.51 mol) in methanol (50 mL) was added triethylamine (TEA) (3.41 mL, 24.56 mmol) followed by the addition of N,N'-diboc-N''-trifylguanidine (6.94 g, 17.74 mmol) and the reaction was stirred for 24 h at room temperature. After removal of the solvent under reduced pressure, the crude product was purified via flash column chromatography eluting with 1% methanol in dichloromethane and gradually increasing to 10% methanol in dichloromethane to yield a white solid (838.2 mg, 13.13 %). ^{1}H NMR (400 MHz, MeOD) δ 8.44 (1H, m, ArH), 7.97 (27H, s, NH), 7.80 (1H, d, J=13.0Hz, ArH), 7.49 (5H, d, J=14.9Hz, NH, ArH), 7.23 (1H, dd, J=4.8Hz, J=8.0Hz, ArH), 3.35 (18H, t, J=7.0Hz, CH_2), 3.16 (18H, d, J=4.6Hz, CH_2), 3.08 (2H, dd, J=9.1Hz, J=15.5Hz, CH_2), 2.67 (2H, d, J=5.5Hz, CH_2), 2.17-1.98 (48H, m, J=74.5Hz, CH_2), 1.59 (18H, t, J=10.9Hz, CH_2), 1.50 (162H, d, J=21.2Hz, CH_3), 1.38 (54H, s, CH_2); ^{13}C NMR (400 MHz, MeOD) δ 175.6, 175.5, 172.9, 164.5, 161.1, 157.5, 154.2, 150.7, 139.2, 122.5, 121.2, 84.4, 80.3, 59.5, 59.2, 41.8, 40.7, 40.5, 32.0, 31.9, 31.7, 31.4, 30.4, 30.1, 28.7, 28.4, 27.7, 27.7.

MT,4 (Deprotection of Molecular Transporter). The guanidinylated dendrimer (288.3 mg, 68.71 μmol) was dissolved in 1,4-dioxane (20 mL), the solution was cooled to 0 ˚C, and 4 M HCl in 1,4-dioxane (20 mL) was added and the reaction stirred for 24 h at room temperature. Removal of the solvent under pressure gave a white solid (164.5 mg,

100.0%). ^{1}H NMR (600 MHz, MeOD) δ 8.65 (1H, d, J=4.3Hz, ArH), 8.37 (19H, q, J=8.5Hz, ArH, NH_2), 8.08 (1H, d, J=8.5Hz, ArH), 7.74 (1H, t, J=6.8Hz, ArH), 7.47 (18H, br s, NH), 7.31 (9H, br s, NH), 6.79 (4H, br s, NH), 3.28 (2H, s, CH_2), 3.20 (36H, d, J=5.9Hz, CH_2), 2.6 (2H, s, CH_2), 2.38-1.90 (48H, m, CH_2), 1.58 (36H, d, J=39.0Hz, CH_2), 1.40 (36H, s, CH_2); ^{13}C NMR (600 MHz, MeOD) δ 176.0, 176.0, 159.2, 158.6, 158.6, 146.2, 146.1, 143.0, 127.8, 127.7, 123.8, 123.6, 59.6, 59.2, 42.4, 40.5, 32.1, 31.8, 31.3, 30.1, 29.8, 27.5, 27.3.

1H NMR Molecular Transporter MT, 4:



Preparation of IgG Antibody. Twenty microliters of a 100 mg/mL Synagis solution (2.0 mg, 13.51 nmol, 20 μL of 100 mg/mL solution) was diluted with 980 μL of Dulbecco's phosphate buffered saline (DPBS) (calcium chloride and magnesium chloride free) and was dialyzed against DPBS (pH = 7.4) using regenerated cellulose dialysis membrane (MWCO = 3,500) at 4 °C for 2 days to remove histidine and glycine from the antibody liquid solution.

Alexa-IgG, Attachment of Alexa Flour® 568 to Antibody. An Alexa Fluor® 568 Protein Labeling Kit was used to attach Alexa Fluor® 568 to the antibody. To the 2.0 mg/mL antibody solution (2.0 mg, 13.51 nmol, 1.0 mL) in DPBS was added 1.0 M sodium bicarbonate solution (100.0 μL). The solution (pH = 8.5) was divided in half and each portion the antibody solution (550.0 μL) was transferred to a vial of reactive Alexa Fluor® 568 dye. The vials were capped and inverted a few times to fully dissolve the dye and the solutions combined into one vial and allowed to stir at room temperature for 3 h. The reaction was then dialyzed against DPBS using regenerated cellulose dialysis membrane (MWCO = 3,500) at 4 °C for 6 days to remove any unreacted dye.

IgGMT, 5 (Attachment of Molecular Transporter to labeled Antibody). A solution of molecular transporter in PBS (pH = 7.0) (186.02 μg, 81.08 nmol, 17.22 μL) was added to the labeled antibody solution (pH = 7.4) (2.0 mg, 13.51 nmol, 2.0 mL) in DPBS and stirred at room temperature for 4 h and at 4 °C overnight. The reaction was then dialyzed using regenerated cellulose dialysis membrane (MWCO = 3,500) at 4 °C, first against DPBS, then gradienting to sterile water (pH = 5.5) and back to DPBS for 6 days to remove any unreacted molecular transporter.

Cell Studies:

Mammalian cell lines and viruses: Human Epithelial cells line 2 (HEp-2) (ATCC CCL-23) were grown in modified eagle medium supplemented with 10% heat inactivated fetal bovine serum, L-glutamine, penicillin streptomycin sulfate antibiotic-antimycotic mixture and gentamicin. Cells were maintained at 37 ºC with 5% CO_2 in a 95% humidity incubator. The recombinant RSV expressing GFP was used for all infections and has been described previously.[2, 3]

Uptake Study. Fluorescent bioconjugate uptake by mammalian cells was assessed using HEp-2 cells grown in uncoated, 14 mm diameter Microwell, No. 1.5 MatTek dishes in Opti-MEM and incubated at 37 ºC and 5% CO_2 in a 95% humidity incubator. Imaging was performed on a Zeiss LSM 510 META confocal microscope through the use of the VUMC Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, HD15052, DK59637 and EY08126). The cells were treated with 100 μL of the fluorescent bioconjugate (IgG+MT+Alexa Fluor® 568) (100 μg/mL) for the series of incubation times, washed twice with PBS, and fed with Opti-MEM media supplemented with 2% FBS for visualization by confocal microscopy.

Neutralization Study.

Infection and microscopic analysis of RSV-infected cells. HEp-2 cells were split in Mattek dishes and simultaneously infected with rRSV-GFP (diluted 1:50). After 24 h of infection, the supernatants of the cells were removed and the cells washed twice with PBS and then treated with 100 μL of the fluorescent bioconjugate solution (2 mg/2.918 mL) for 30 min. A control dish was only treated with PBS for 30 min. After treatment,

the cells were washed twice with PBS and fed with Opti-MEM media supplemented with 2% FBS for visualization by confocal microscopy daily until 96 hours post infection. Wells were harvested by flash freezing 100µl of the supernatants from each well before daily imaging for determination of virus titers. Plaque titration assays were done on HEp-2 cells as previously described.[4]

Virus Titrations:

	24 hr of infection (before treatment)	48 hr of infection (treatment 24 hr)	72 hr of infection (treatment 48 hr)
Well #1 pfu/mL (untreated) 1:3 cell split infection 10^3	$7x10^3$	$7.8x10^4$	$1.8x10^5$
Well #1 pfu/mL (treated) 1:3 cell split infection 10^3	$1\ x10^3$	$1x10^1$	$1x10^2$
Well #1 pfu/mL (untreated) 1:3 cell split infection 10^4	$1.4x10^4$	$7x10^3$	$3.6x10^4$
Well #1 pfu/mL (treated) 1:3 cell split infection 10^4	$8x10^4$	$2.4x10^5$	$4x10^3$

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3. L. K. Hallak, D. Spillmann, P. L. Collins and M. E. Peeples, *J Virol*, 2000, **74**, 10508-10513.
4. H. V. Coates, D. W. Alling and R. M. Chanock, *Am J Epidemiol*, 1966, **83**, 299-313.