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Aggregation-Mediated Macromolecular Uptake by a Molecular Transporter

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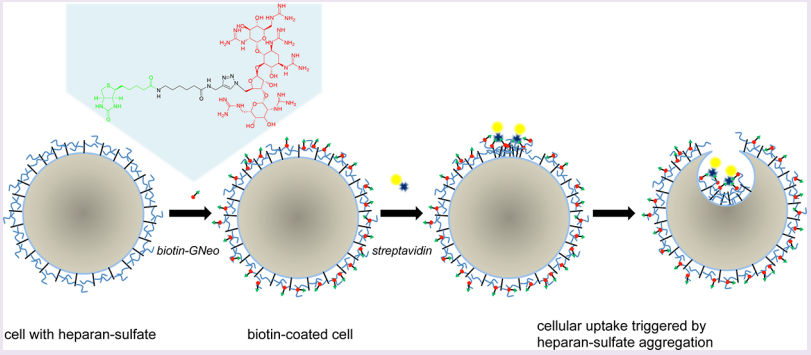
Supporting Information



ABSTRACT: Endocytosis is a key process in cellular delivery of macromolecules by molecular transporters, although the mechanism of internalization remains unclear. Here, we probe the cellular uptake of streptavidin using biotinylated guanidinoneomycin (biotinGNeo), a low molecular weight guanidinium-rich molecular transporter. Two distinct modes were explored: (i) incubation of cells with a preformed tetravalent streptavidin-(biotinGNeo)$_4$ conjugate and (ii) preincubation of cells with the biotinGNeo before exposure to streptavidin. A significant enhancement in uptake was observed after preincubation with biotinGNeo. FRET studies showed that the enhanced uptake was accompanied by extensive aggregation of streptavidin on the cell surface. Because guanidinylated neomycin was previously found to exclusively bind to heparan sulfate, our observations suggest that heparan sulfate proteoglycan aggregation is a pivotal step for endocytic entry into cells by guanidinoglycosides. These observations put forward a practical and general pathway for the cellular delivery of diverse macromolecules.

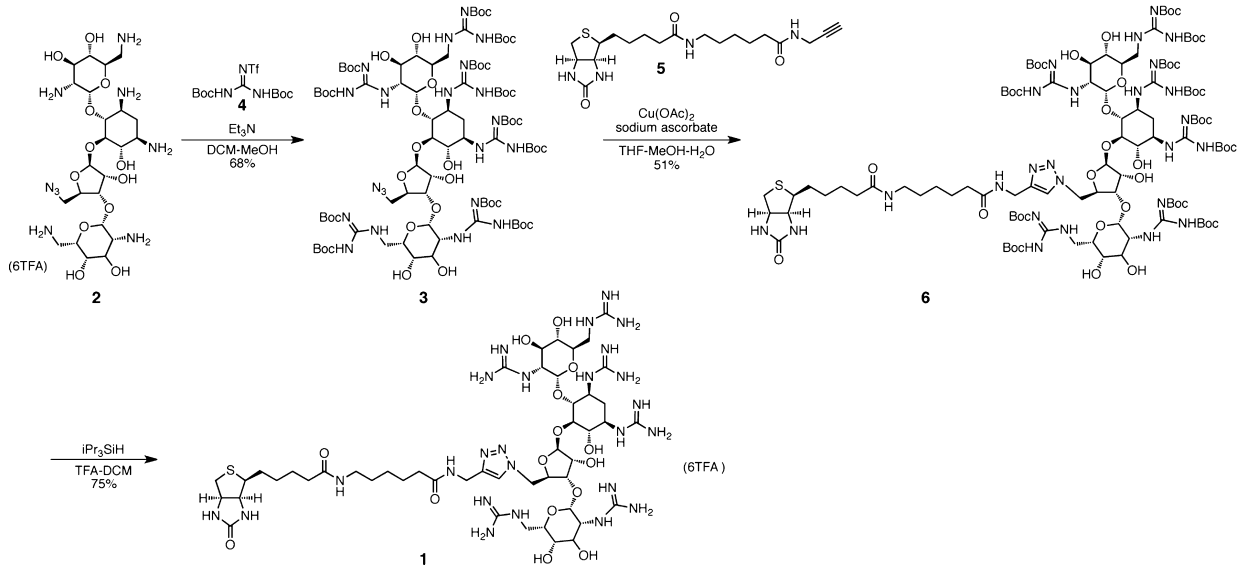
Cell-penetrating peptides (CPP) and related guanidinium-rich transporters have been explored as cellular delivery agents of biologically relevant macromolecules, such as peptides, proteins, and nucleic acids for both research and potential pharmaceutical uses.[1−4] The mechanistic understanding of the cellular uptake and internalization of these transporter molecules remains, however, incomplete. Transport mediated by specific receptors would appear to be inconsistent with the structural diversity of the guanidinium-based transporters described to date. Endocytosis-based mechanisms have been both supported and questioned.[5] Several models have also been proposed for the primary transporter−membrane interactions, including electrostatic interactions of the positively charged peptides with membrane phospholipids and with negatively charged cell surface proteoglycans.[6]

Virtually every type of animal cell produces proteoglycans, which consist of one or more glycosaminoglycan chains covalently linked to a core protein.[7,8] The proteoglycans can be classified according to location (intracellular granules, plasma membrane, or secreted), glycosaminoglycan composition (heparan sulfate, chondroitin sulfate/dermatan sulfate, or keratan sulfate), or by core protein (e.g., the membrane spanning syndecans, the GPI-anchored glypicans, CD44, and several others). Heparan sulfate proteoglycans (HSPGs) are enriched on the cell surface and bind to numerous ligands, which can then be internalized as the proteoglycans enter the cells via a nonclathrin mediated pathway enroute to lysosomes.[9]

We recently showed that guanidinoglycosides, a family of synthetic carriers made by replacing all ammonium groups on aminoglycoside antibiotics with guanidinium groups, can transport bioactive, high molecular weight cargo into diverse cells.[10−13] Unlike other guanidinium-rich transporters, the cellular uptake of guanidinylated aminoglycosides occurs at nanomolar concentrations and is exclusively dependent on HSPGs. The valency of the carriers has been demonstrated to be of importance, suggesting complex interactions with the inherently multivalent cell surface HSPGs.[12] In this contribution, we interrogated the role of HSPG aggregation on the cell

Received: September 29, 2012
Accepted: April 16, 2013
Published: April 26, 2013

Scheme 1. Synthesis of BiotinGNeo 1



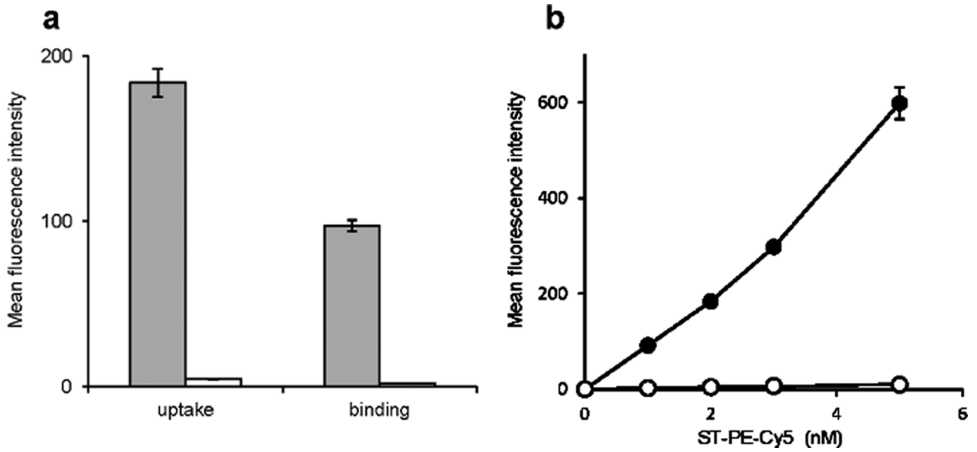


Figure 1. Cellular binding and uptake for conjugate of ST-PECy5 and compound **1**. (a) Binding and uptake activity was studied with either wild-type cells (gray) or pgsA-745 cells (white). For the binding study, cells were incubated with conjugate (2 nM ST-PECy5 and 10 nM compound **1**) for 0.5 h at 4 °C. For the uptake study, incubation was done for 1 h at 37 °C. (b) Dose−response of cellular uptake for conjugate of ST-PECy5 and compound **1** with wild-type cells (black) or pgsA-745 cells (white). The molar ratio of compound **1** to streptavidin was 5:1.

surface as a determinant in the delivery of high molecular weight cargos into the cell. To shed light on this complex phenomenon, we used a fluorescently tagged streptavidin as a model cargo and evaluated its cellular uptake either by preforming a multivalent carrier−cargo complex, or by allowing the complex to be formed on the cell surface. Dramatically different outcomes are seen, and their implications on the endocytic mechanism and utility of these carriers are discussed.

To interrogate carrier−cell surface HSPG interactions, a biotinylated guanidinoneomycin-based carrier was designed and synthesized (**1**, Scheme 1). A relatively short linker (amino-caproic acid) was selected to restrict the distance between the HSPG-binding module (guanidinoneomycin) and the bound streptavidin cargo, to minimize flexibility of the linker and to facilitate effective monitoring of HSPG aggregation. Its preparation followed established procedures (Scheme 1), where guanidinylation of compound **2**[14] by reagent **4**[15] gave Boc-protected guanidinoneomycin **3** in moderate yield. Subsequent 1,3-dipolar cycloaddition with the biotinylated linker **5** afforded the protected BiotinGNeo **6**. Final deprotection with trifluoroacetic acid yielded the desired biotinGNeo **1** in good yield.

To first test if compound **1** serves as a HSPG-dependent molecular transporter, fluorescent streptavidin-phycoerythrin-Cy5 (ST-PECy5) was used as a model payload, and cellular uptake was measured by flow cytometry as previously reported.[11] Two different cells were used for the study: wild-type Chinese hamster ovary (CHO) cells and CHO-derived mutant cells (pgsA-745). The latter produce <2% of the wild-type level of heparan sulfate chains on the cell surface.[16] The tetravalent conjugate ST-(biotinGNeo)$_4$ was obtained by mixing ST-PECy5 and compound **1** at a 1:5 ratio for 20 min at room temperature, followed by dilution with cell culture medium to a final ST-PECy5 concentration of 2 nM. Cells were incubated with the conjugate for 1 h at 37 °C, washed and harvested with trypsin/EDTA, and analyzed by flow cytometry. Under these conditions, all cell-surface-bound material is cleaved, and the fluorescence signal represents internalization of the conjugates.[13] As shown in Figure 1a, pgsA mutant cells exhibited far less uptake of ST-(biotinGNeo)$_4$ compared to

dx.doi.org/10.1021/cb400172h | *ACS Chem. Biol.* 2013, 8, 1383−1388

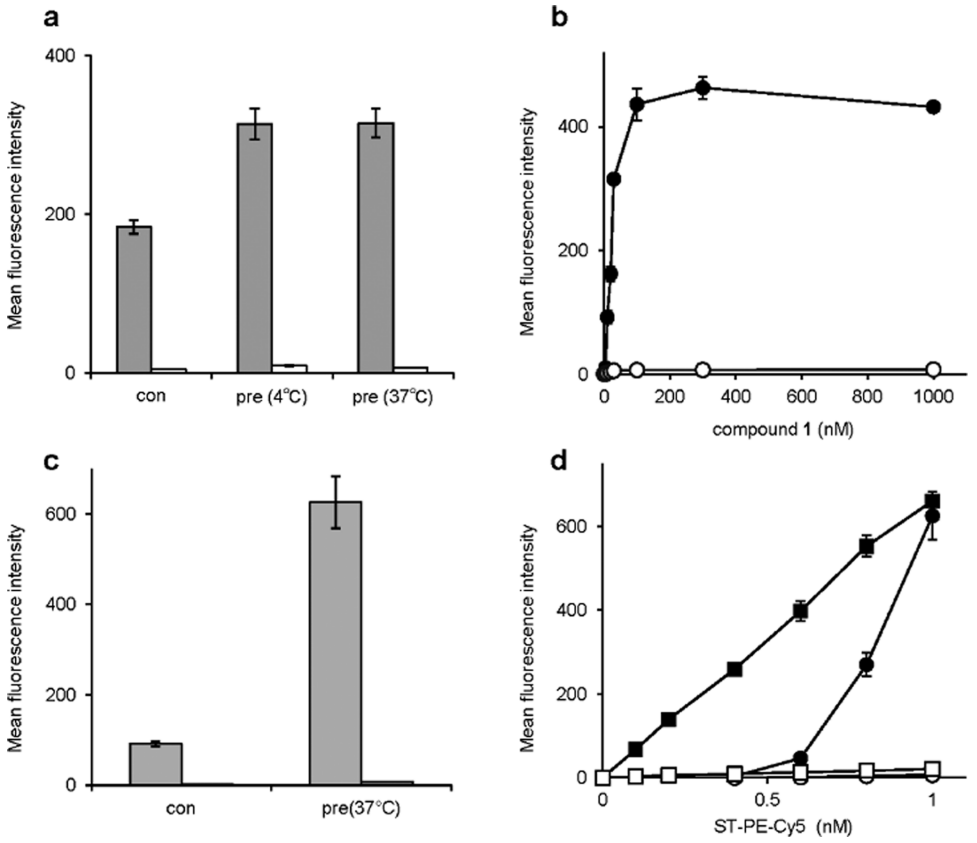


Figure 2. Cellular uptake for preformed conjugates and after precoating cells with compound **1**. (a) Conjugates were made of 2 nM ST-PECy5 and 10 nM compound **1** (con). Preincubation was done by incubating cells with 10 nM compound **1** either at 4 °C (pre-4) or 37 °C (pre-37). (b) Cells were preincubated for 1 h at 37 °C with various concentrations of compound **1**. ST-PECy5 (1 nM) was added and uptake was measured. (c) Comparison of uptake at 37 °C for 1 nM ST-PECy5 under conjugate conditions (10 nM compound **1**; con) and after preincubation conditions (100 nM compound **1**; pre-37). (d) Dose−response curve of ST-PECy5 under preincubation conditions with 100 nM compound **1** at 37 °C. Dose of ST-PECy5 was varied in the absence (circle) or presence (square) of 20 nM streptavidin. Cellular uptake is shown for wild-type cells (gray in a and c, black in b and d) and pgsA-745 cells (white).

wild-type CHO cells, demonstrating that cellular uptake was HSPG-dependent. The uptake linearly increased with increasing concentration of the conjugate (Figure 1b). To probe only cell surface binding, cells were incubated with the fluorescent conjugate at 4 °C, where no uptake occurs.[11] After harvesting the cells with EDTA, significantly more binding was observed with wild-type cells compared to pgsA-745 cells (Figure 1a). Thus, compound **1** was found to bind exclusively HSPG on the cell surface and enter cells in a HSPG-dependent manner as we have previously reported for other guanidinoglycoside-based transporters.[11−13]

We next examined if streptavidin can be taken up by cells that were precoated with carrier **1**. The cells were first tested for their capacity to bind compound **1**. CHO cells were preincubated with 10 nM of **1** for 1 h at 4 °C, at which no internalization occurs. The preincubated cells were washed with fresh medium and treated with 2 nM ST-PECy5 at 37 °C. Cellular uptake of ST-PECy5 was observed with wild-type cells (Figure 2a), indicating that compound **1** bound cells and remained at the cell surface as expected. However, pgsA-745 cells exhibited far less uptake of ST-PECy5 under these conditions than wild-type cells, showing that bioitnGNeo **1** precoated cells in a HPSG-dependent manner. The preincubation was also tested at 37 °C and uptake was found to be independent of the preincubation temperature. Reducing the preincubation time from 1 to 0.5 h did not significantly impair uptake, suggesting binding of **1** on the cell surface was complete within this time range (Supplementary Figure 1). Interestingly, uptake was found to be higher than that with preformed ST-$(biotinGNeo)_4$ conjugate at the same streptavidin and compound concentrations (2 and 10 nM, respectively). We rationalized that coating the cell-surface with **1** facilitates the interaction between streptavidin and HSPG and triggers endocytosis. To find the critical concentration, where the carrier completely coats the cells, uptake of ST-PECy5 was tested after treating the cells with different concentrations of **1** (Figure 2b). Cellular uptake saturated at ∼100 nM of **1**, suggesting that the cells were fully coated at this concentration. The uptake observed with 1 nM ST-PECy5 after preincubation with 100 nM biotinGNeo was ∼6-fold higher than that seen with the same concentration of the preformed ST-$(biotinGNeo)_4$ conjugate (Figure 2c), illustrating the highly efficient uptake induced by preincubation with **1**. Importantly, the uptake remained exclusively dependent on HSPG because the selectivity between wild-type and pgsA-745 cells was retained even at the highest concentration tested (1 μM). The impact of preincubation was retained even after incubation of the precoated cells in fresh medium for 1 h at 37 °C (Supplementary Figure 2). The improved uptake under preincubation conditions was not likely due simply to slow kinetics of binding of **1** to HSPG because uptake was not increased by incubating the preformed conjugate with cells

dx.doi.org/10.1021/cb400172h | *ACS Chem. Biol.* 2013, 8, 1383−1388

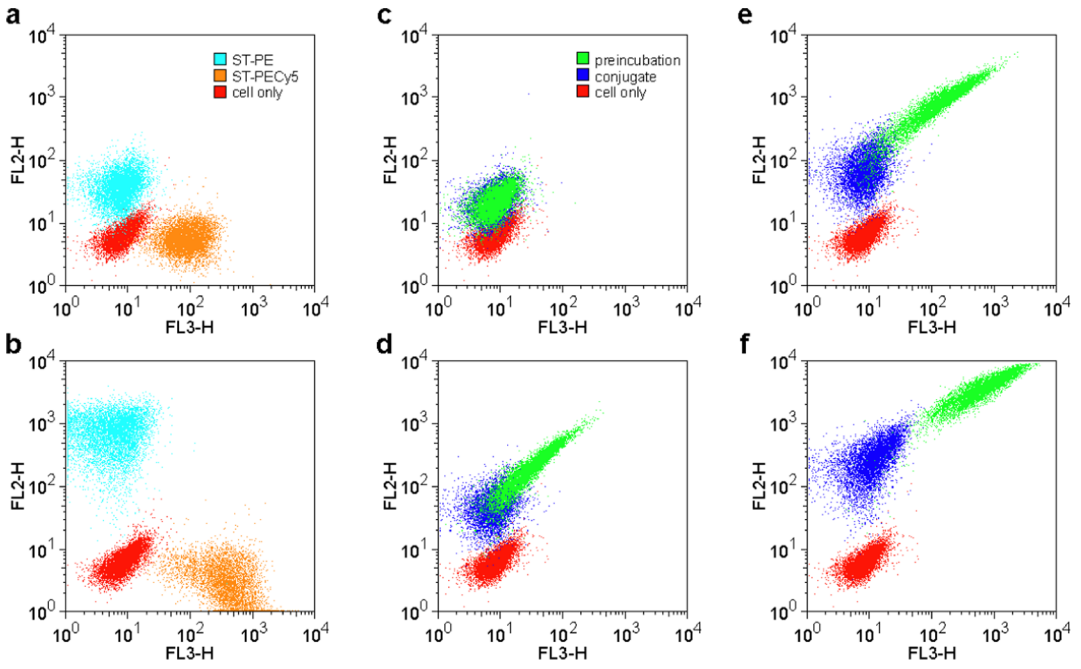


Figure 3. Intermolecular FRET using ST-PE and Cy5-PE. Wild-type cells were incubated at 4 °C for 30 min with preformed conjugate of compound **1** and ST-PE/ST-Cy5 mixture. Compound **1**/ST-PE/ST-Cy5 was set as 10:1:1. Under preincubation conditions, wild-type cells were preincubated with 100 nM compound **1** at 37 °C for 1 h, washed with fresh medium, and then treated with ST-PE/ST-Cy5 mixture (1:1) at 4 °C for 30 min. Cells were detached by Versene (EDTA) and analyzed by flow cytometry. *Y*-axis (FL2-H) and *X*-axis (FL3-H) represent fluorescent intensity of PE (λ_{em} = 560 nm) and PE/Cy5 FRET complex (λ_{em} = 670 nm), respectively. As a control experiment, compound **1** with either ST-PE or ST-PECy5 was tested as preformed conjugates (a) and after precoating the cells with compound **1** (b). ST-PE (ST-Cy5) concentration was varied from 0.5 nM (c), 0.75 nM (d), 1 nM (e), to 2 nM (f).

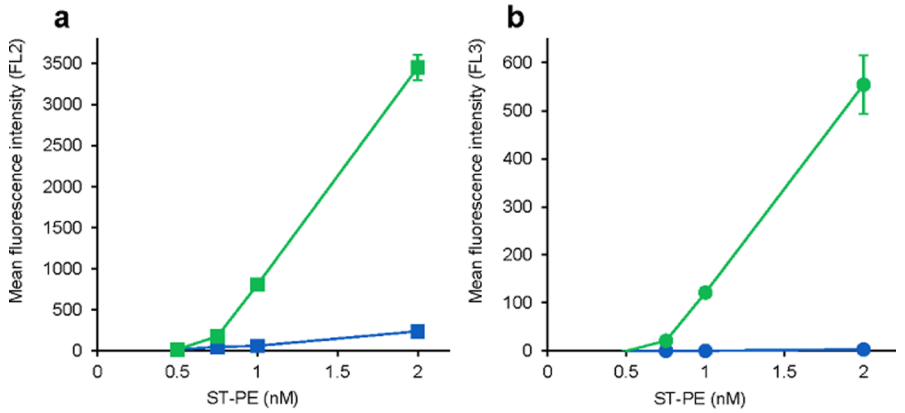


Figure 4. Dose−response of mean fluorescent intensity for the intermolecular FRET study (Figure3). Geometrical mean of (a) FL2 (emission signal from PE, left graph) and (b) FL3 (FRET emission signal from PE/Cy5, right graph) under conjugate conditions (cyan) and preincubation conditions (green) are shown.

under binding conditions (4 °C, 1 h) prior to the uptake experiment (Supplementary Figure 3).

To determine the lowest amount of streptavidin needed to trigger cell entry, cellular uptake was probed with subnanomolar concentrations of ST-PECy5 after preincunation of cells with 100 nM **1**. Interestingly, uptake had characteristics of a switch-like mechanism, with negligible uptake at concentrations below 0.6 nM (Figure 2d). Adding 20 nM nonfluorescent streptavidin induced a linear response at subnanomolar concentration of ST-PECy5, suggesting that a minimum number of bound streptavidin was required to induce internalization. Uptake under these conditions remained dependent on HSPGs, as shown by negligible uptake in pgsA-745 cells.

The observations outlined above suggested that the extent of clustering of streptavidin on the cell surface could impact cellular uptake. To test this hypothesis, FRET studies using two different fluorescent streptavidins, streptavidin−phycoerythrin (ST-PE; λ_{max} = 496 nm and λ_{em} = 575 nm) and streptavidin−Cy5 (ST-Cy5; λ_{max} = 649 nm and λ_{em} = 666 nm) as a mixture, instead of ST-PECy5, were performed. With this combination, the extent of aggregation can be monitored by the FRET signal intensity between ST-PE and ST-Cy5. Thus, cell binding experiments were done using 1:1 mixture of ST-PE and ST-Cy5 as fluorescent probes either under the preincubation conditions or with preformed tetravalent conjugate to compare the FRET signal intensity between the two distinct modes. As shown in Figure 3, significant FRET signal, shown as FL3-H,

dx.doi.org/10.1021/cb400172h | *ACS Chem. Biol.* 2013, 8, 1383−1388

was observed under preincubation condition starting at 0.75 nM ST-PE (or ST-Cy5), and the signal intensity increased as the concentration of streptavidin increased (Figure 4), indicating that FRET signal intensity was correlated with the degree of cellular uptake. However, exposure to the preformed conjugate mixtures of ST-PE(biotinGNeo)$_4$ and ST-Cy5-(bioitnGNeo)$_4$ gave negligible FRET signals even at 2 nM ST-PE (or ST-Cy5). The results show that significant clustering of streptavidin is observed under the preincubation conditions, whereas less aggregation occurred with the preformed conjugates. Knowing that compound **1** exclusively binds HSPG, the strong FRET signal observed under preincubation conditions is attributed to HSPG aggregation on the cell surface. Since the FRET signal intensity is also correlated with the degree of cellular uptake, our observations suggest that HSPG aggregation is a pivotal step for endocytic entry into cells by guanidinogycoside-based transporters.

The clustering of HSPG in endocytosis has been previously suggested, including in studies with the TAT peptide.[17−21] Unlike Tat and other CPPs, guanidinoglycosides penetrate cells at nanomolar concentrations and with specificity for heparan sulfate.[11] We speculate that HSPG aggregation, caused by binding streptavidin to HSPG bound compound **1**, may contribute to membrane curvature cooperatively, thereby triggering endocytosis. Such a cooperative mechanism of membrane deformation has been recently suggested by simulating endocytosis of ligand coated nanoparticles.[22] The existence of a threshold concentration of streptavidin for uptake as shown in Figure 2d is also consistent with a cooperative mechanism. The present observations clearly demonstrated that HSPG aggregation is a key step for cellular uptake by guanidinoglycoside-based carriers. As all cells express HSPGs, which, due to their size, provide a high density of binding sites for ligands, such cell surface molecules offer a privileged high capacity uptake pathway for delivery of bioactive cargo.[7] The preincubation system shown here can be used for further mechanistic and kinetic analysis of endocytic entry. Additionally, the present protocol provides a unique, very effective and, perhaps, general approach for pretargeting cells for streptavidin-modified pharmaceutically relevant payloads, considering that the compound shows no toxicity even after prolonged exposure to cells (Supplementary Figure 4). In this approach, cells would be preincubated with molecules such as **1** and then exposed to the payload of interest as it is conjugated to streptavidin or encapsulated in streptavidin-coated vesicles/nanoparticles.

METHODS

Synthesis of Compound 1. To a solution of compound **2** (200 mg, 0.151 mmol) in methanol (0.2 mL) was added triethylamine (0.421 mL, 3.02 mmol), dichloromethane (2 mL), and *N*,*N*′-di-*tert*-butoxycarbonyl-*N*″-trifluoromethanesulfonylguanidine (**4**, 710 mg, 1.81 mmol) at ambient temperature under an argon atmosphere. The mixture was stirred for 60 h. The mixture was partitioned between dichloromethane and 10% aqueous citric acid. The organic layer was washed with brine, dried over anhydrous $MgSO_4$ and filtered, and the filtrate was evaporated. Silicagel column chromatography (dichloromethane−methanol, a linear gradient of methanol from 0 to 1.8%) afforded compound **3** as a colorless amorphous solid (215 mg, 68%).

^{1}H NMR (400 MHz, CD_3OD): 1.38−1.75 (109H, m), 2.25 (1H, m), 3.21−3.57 (8H, m), 3.67−3.99 (8H, m), 4.124.32 (5H, m), 4.58 (1H, m), 5.06 (1H, m), 5.11 (1H, d, J = 2.4 Hz), 5.97 (1H, d, J = 4.0 Hz). MS (ESI) m/z: exact mass calcd. for $C_{89}H_{153}N_{21}O_{36}$. 2092.08; found, 1046.92 $[M + 2H]^{2+}$, 1057.97 $[M + H + Na]^{2+}$, 1069.05 $[M + 2Na]^+$

To a solution of compound **3** (51.7 mg, 0.025 mmol) and compound **5** (14.6 mg, 0.037 mmol) in tetrahydrofuran/methanol/water (5:4:1, 1 mL) was added sodium ascorbate (6.4 mg, 0.032 mmol) and $Cu(OAc)_2$ (5.8 mg, 0.032 mmol) at ambient temperature. The mixture was sonicated for 30 min (3 cycles of each 10 min with 5 min intervals; no rise in temperature noted). The mixture was then partitioned between dichloromethane/methanol and satd. aqueous ammonium chloride. The organic layer was dried over anhydrous $MgSO_4$ and filtered, and the filtrate was evaporated. Silicagel column chromatography (dichloromethane/methanol, a linear gradient of methanol from 0 to 20%) afforded compound **6** as a faint greenish amorphous solid (31.5 mg, 51%).

^{1}H NMR (400 MHz, CD_3OD): 1.40−1.57 (m, 121H), 2.18−2.28 (5H, m), 2.71 (1H, d, J = 12.4 Hz), 2.93 (1H, dd, J = 5.0 and 12.6 Hz), 3.26−4.65 (m, 29 H), 5.01 (1H, m), 5.06 (1H, d, J = 3.6 Hz), 5.64 (1H, d, J = 3.6 Hz), 7.85 (1H, s). MS (ESI) m/z: exact mass $C_{108}H_{183}N_{25}O_{39}S$, 2486.28; found, 1244.13 $[M + 2H]^{2+}$, 1255.14 $[M + H + Na]^{2+}$, 1266.01 $[M + 2Na]^+$

To a solution of compound **6** (31.5 mg, 0.013 mmol) in dichloromethane (0.1 mL) was added triisopropylsilane (0.039 mL, 0.19 mmol) followed by trifluoroacetic acid (0.5 mL) at ambient temperature. The mixture was stirred for 13 h. The volatiles were evaporated off. The residue was azeotroped with toluene (three times), dissolved in water, and washed with dichloromethane. The aqueous solution was lyophilized to give a colorless amorphous solid (24.2 mg). HPLC (C18, acetonitrile/water containing 0.1% trifluoroacetic acid, a linear gradient of acetonitrile from 0 to 40% over 16 min) afforded compound **1** (R_t = 12.42 min) as a colorless amorphous solid (18.7 mg, 75%).

^{1}H NMR (500 MHz, D_2O): 1.26−1.72 (13H m), 2.19−2.23 (3H, m), 2.28 (2H, t, J = 7.5 Hz), 2.74 (1H, d, J = 13.2 Hz), 2.96 (1H, dd, J = 4.6, 13.2 Hz), 3.09−3.18 (2H, m), 3.30−3.36 (2H, m), 3.46−3.71 (12H, m), 3.72 (1H, m), 3.80 (1H, m), 4.13−4.18 (2H, m), 4.29−4.31 (2H, m), 4.38−4.48 (4H, m), 4.57−4.73(3H, m), 5.02 (1H, m), 5.07 (1H, m), 5.69 (1H, d, J = 4.6 Hz), 7.84 (1H, s). MS (ESI) m/z: exact mass calcd. for $C_{48}H_{87}N_{25}O_{15}S$, 1285.65; found 644.05 $[M + 2H]^{2+}$, 700.93 $[M + TFA + 2H]^{2+}$, 1286.65 $[M + H]^+$. High-resolution mass found 643.8337 $[M + 2H]^{2+}$; calcd 643.8340

Cell Culture. Wild-type Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61). Mutant pgsA745 was described previously.[16,23] All cells were grown under an atmosphere of 5% CO_2 in air and 100% relative humidity in Dulbecco's Modified Eagle's Medium (DMEM, low glucose, Life Technologies) supplemented with 10% (v/v) fetal bovine serum, 100 g/mL of streptomycin sulfate, and 100 units/mL of penicillin G.

Preparation of Conjugates and Cellular Binding/Uptake Studies. In a typical experiment, 7.5 μM biotinGNeo was incubated with 1.5 μM ST-PECy5 (BD Biosciences) for 20 min at ambient temperature and then diluted with ice-chilled DMEM cell culture medium to give conjugate solutions (final ST-PECy5 of 2 nM).

Wild-type and mutant CHO-pgs cells were seeded onto a 24-well tissue culture plate (100 000 cells/well) and grown for 24 h to achieve 80% confluence. For binding studies, cells were treated with 300 μL of the conjugate solutions after removal of the medium and incubated for 0.5 h at 4 °C. Cells were washed with 300 μL of ice-chilled phosphate buffered saline (PBS) twice, detached with 100 μL of Versene (EDTA, Life Technologies), diluted with PBS containing 5% BSA, and analyzed by flow cytometry. For uptake experiment, incubation was done for 1 h at 37 °C under an atmosphere of 5% CO_2. The workup procedure was the same as the binding study, except 50 μL of trypsin/EDTA (37 °C, 3 min) was used to release the cells and remove any cell surface bound streptavidin conjugates.

Preincubation of the Cells and Cellular Binding/Uptake/FRET Studies. In a typical experiment, 100 nM of biotinGNeo was incubated with CHO cells for 1 h at 37 °C under an atmosphere of 5% CO_2. The cells were then washed with ice-chilled DMEM cell culture medium twice, and treated with 1 nM ST-PECy5 (for the binding/uptake study) or 1:1 mixture of ST-PE and ST-Cy5 (for the FRET study) in DMEM cell culture medium. After incubating for 1 h at 37 °C (for the uptake study) or for 0.5 h at 4 °C (for the binding/FRET

study) under an atmosphere of 5% CO_2, cells were washed with PBS twice, detached with trypsin/EDTA (for the uptake study) or Versene (for the biding/FRET study), followed by the workup procedure as described above, and analyzed by flow cytometry.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis of linker **5** and its analytical data; additional uptake data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the National Institutes of Health for support (GM077471 to Y.T. and J.D.E.). We acknowledge N. Freeman at Department of Chemistry and Biochemistry, University of San Diego for technical help with HPLC purification. We also acknowledge University of California, San Diego, Chemistry and Biochemistry Mass Spectrometry Facility for help with mass spectrometry analysis.

■ REFERENCES

(1) Chugh, A., Eudes, F., and Shim, Y. S. (2010) Cell-penetrating peptides: Nanocarrier for macromolecule delivery in living cells. *IUBMB Life 62*, 183−193.

(2) Grdisa, M. (2011) The delivery of biologically active (therapeutic) peptides and proteins into cells. *Curr. Med. Chem. 18*, 1373−1379.

(3) Shim, M. S., and Kwon, Y. J. (2010) Efficient and targeted delivery of siRNA *in vivo*. *FEBS J. 277*, 4814−4827.

(4) Wender, P. A., Galliher, W. C., Goun, E. A., Jones, L. R., and Pillow, T. H. (2008) The design of guanidinium-rich transporters and their internalization mechanisms. *Adv. Drug Delivery Rev. 60*, 452−472.

(5) Schmidt, N., Mishra, A., Lai, G. H., and Wong, G. C. L. (2010) Arginine-rich cell-penetrating peptides. *FEBS Lett. 584*, 1806−1813.

(6) Walrant, A., Bechara, C., Alves, I. D., and Sagan, S. (2012) Molecular partners for interaction and cell internalization of cell-penetrating peptides: how identical are they? *Nanomedicine 7*, 133−143.

(7) Bishop, J. R., Schuksz, M., and Esko, J. D. (2007) Heparan sulfate proteoglycans fine-tune mammalian physiology. *Nature 446*, 1030−1037.

(8) Esko, J. D., and Lindahl, U. (2001) Molecular diversity of heparan sulfate. *J. Clin. Invest. 108*, 169−173.

(9) Belting, M. (2003) Heparan sulfate proteoglycan as a plasma membrane carrier. *Trends Biochem. Sci. 28*, 145−151.

(10) Luedtke, N. W., Carmichael, P., and Tor, Y. (2003) Cellular uptake of aminoglycosides, guanidinoglycosides, and poly-arginine. *J. Am. Chem. Soc. 125*, 12374−12375.

(11) Elson-Schwab, L., Garner, O. B., Schuksz, M., Crawford, B. E., Esko, J. D., and Tor, Y. (2007) Guanidinylated neomycin delivers large, bioactive cargo into cells through a heparan sulfate-dependent pathway. *J. Biol. Chem. 282*, 13585−13591.

(12) Dix, A. V., Fischer, L., Sarrazin, S., Redgate, C. P. H., Esko, J. D., and Tor, Y. (2010) Cooperative, heparan sulfate-dependent cellular uptake of dimeric guanidinoglycosides. *ChemBioChem 11*, 2302−2310.

(13) Sarrazin, S., Wilson, B., Sly, W. S., Tor, Y., and Esko, J. D. (2010) Guanidinylated neomycin mediates heparan sulfate-dependent transport of active enzymes to lysosomes. *Mol Ther. 18*, 1268−1274.

(14) Childs-Disney, J. L., Wu, M., Puschechnikov, A., Aminova, O., and Disney, M. D. (2007) A small molecule microarray platform to select RNA internal loop−ligand interactions. *ACS Chem. Biol. 2*, 745−754.

(15) Baker, T. J., Luedtke, N. W., Tor, Y., and Goodman, M. (2000) Synthesis and anti-HIV activity of guanidinoglycosides. *J. Org. Chem. 65*, 9054−9058.

(16) Esko, J. D., Stewart, T. E., and Taylor, W. H. (1985) Animal cell mutants defective in glycosaminoglycan biosynthesis. *Proc. Natl. Acad. Sci. U.S.A. 82*, 3197−3201.

(17) Dehio, C., Freissler, E., Lanz, C., Gómez-Duarte, O. G., David, G., and Meyer, T. F. (1998) Ligation of cell surface heparan sulfate proteoglycans by antibody-coated beads stimulates phagocytic uptake into epithelial cells: a model for cellular invasion by *Neisseria gonorrhoeae*. *Exp. Cell Res. 242*, 528−539.

(18) Fuki, I. V., Meyer, M. E., and Williams, K. J. (2000) Transmembrane and cytoplasmic domains of syndecan mediate a multi-step endocytic pathway involving detergent-insoluble membrane rafts. *Biochem. J. 351*, 607−612.

(19) Ziegler, A., and Seelig, J. (2004) Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters. *Biophys. J. 86*, 254−263.

(20) Ziegler, A., Nervi, P., Dürrenberger, M., and Seelig, J. (2005) The cationic cell-penetrating peptide CPP^{TAT} derived from the HIV-1 protein TAT is rapidly transported into living fibroblasts: optical, biophysical, and metabolic evidence. *Biochemistry 44*, 138−148.

(21) Ziegler, A., and Seelig, J. (2011) Contributions of glycosaminoglycan binding and clustering to the biological uptake of the nonamphipathic cell-penetrating peptide WR9. *Biochemistry 50*, 4650−4664.

(22) Yue, T., and Zhang, X. (2012) Cooperative effect in receptor-mediated endocytosis of multiple nanoparticles. *ACS Nano 6*, 3196−3205.

(23) Bai, X. M., Wei, G., Sinha, A., and Esko, J. D. (1999) Chinese hamster ovary cell mutants defective in glycosaminoglycan assembly and glucuronosyltransferase I. *J. Biol. Chem. 274*, 13017−13024.