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Aggregation-mediated macromolecular uptake by a molecular transporter

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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

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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>.

enriched on the cell surface and bind to numerous ligands, which can then be internalized as the proteoglycans enter the cells via a non-clathrin 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 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 performing 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 (aminocaproic 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 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 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 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 pre-coated 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 temperature no internalization occurs. The preincubated cells were washed with fresh medium, and treated with 2 nM of ST-PECy5 at 37°C. Cellular uptake of ST-PECy5 was observed with wild-type cells, indicating

that compound **1** bound cells and remained at the cell surface as expected. On the other hand, pgsA-745 cells exhibited far less uptake of ST-PECy5 under these conditions than wild-type cells, showing that bioitnGNeo **1** pre-coated 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 (Figure 2a). Reducing the preincubation time from 1 to 0.5 h did not significantly impair uptake, suggesting binding of **1** on the cell surface is 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 of 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), consistent with exclusive binding of **1** to HSPGs on the cell surface. The impact of preincubation was retained even after incubation of the pre-coated cells in fresh medium for 1 h at 37°C (Supplementary Figure 3). 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 under binding conditions (4°C, 1 h) prior to the uptake experiment (Supplementary Figure 2).

To determine the lowest amount of streptavidin needed to trigger cell entry, cellular uptake was probed with subnanomolar concentrations of ST-PECy5 after preincubation of cells with 100 nM of **1**. Interestingly, uptake had characteristics of a switch-like mechanism, with negligible uptake at concentrations below 0.6 nM (Figure 2d). Adding 20 nM of non-fluorescent 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; $\lambda_{\text{max}}=496$ nm, $\lambda_{\text{em}}=575$ nm) and streptavidin-Cy5 (ST-Cy5; $\lambda_{\text{max}}=649$ nm, $\lambda_{\text{em}}=666$ 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, was observed under preincubation condition starting at 0.75 nM of 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. On the other hand, exposure to the preformed conjugate mixtures of ST-PE(biotinGNeo)₄ and ST-Cy5(bioitnGNeo)₄ gave negligible FRET signals even at 2 nM of 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 condition 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 guanidinoglycoside-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 exclusive specificity for heparan sulfate.¹¹ We speculate 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 from the computer simulation study 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 MgSO₄, 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%).

¹H-NMR (400MHz, CD₃OD): 1.38–1.75 (109H, m), 2.25 (1H, m), 3.21–3.57 (8H, m), 3.67–3.99 (8H, m), 4.12–4.32 (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 C₈₉H₁₅₃N₂₁O₃₆: 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 MgSO₄, 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%).

$^1\text{H-NMR}$ (400MHz, 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 $\text{C}_{108}\text{H}_{183}\text{N}_{25}\text{O}_{39}\text{S}$: 2486.28, found 1244.13 $[\text{M} + 2\text{H}]^{2+}$, 1255.14 $[\text{M} + \text{H} + \text{Na}]^{2+}$, 1266.01 $[\text{M} + 2\text{Na}]^{+}$

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%).

$^1\text{H-NMR}$ (500MHz, 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 $\text{C}_{48}\text{H}_{87}\text{N}_{25}\text{O}_{15}\text{S}$: 1285.65, found 644.05 $[\text{M} + 2\text{H}]^{2+}$, 700.93 $[\text{M} + \text{TFA} + 2\text{H}]^{2+}$, 1286.65 $[\text{M} + \text{H}]^{+}$, High resolution mass found 643.8337 $[\text{M} + 2\text{H}]^{2+}$, calcd. 643.8340

Cell culture—Wild type Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61). Mutants 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 of biotinGNeo was incubated with 1.5 μM of 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 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 . Work-up procedure was the same as 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 of 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 under an atmosphere of 5% CO_2 , cells were washed with

PBS twice, detached with trypsin/EDTA, followed by the work-up procedure as described above, and analyzed by flow cytometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 activity 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.

Figure 2.

Cellular uptake for preformed conjugates and or after pre-coating cells with compound **1**. (a) Conjugates were made of 2 nM of ST-PECy5 and 10 nM of compound **1** (con). Preincubation was done by incubating cells with 10 nM of 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 by 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/black) and pgsA-745 cells (white).

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 of 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) represents fluorescent intensity of PE ($\lambda_{em} = 560$ nm) and PE/Cy5 FRET complex ($\lambda_{em} = 670$ nm), respectively. As a control experiment, compound **1** with either ST-PE or ST-PECy5 was tested as preformed conjugates (a) and after pre-coating 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 (Figure 3). Geometrical mean of (a) FL2 (emission signal from PE, left graph) and (b) FL3 (FRET emission signal from PE/Cy5, right graf) under conjugate conditions (cyan) and preincubation conditions (green) are shown.

Scheme 1.
Synthesis of BiotinGNeo 1