UNIVERSITY OF CALIFORNIA, SAN DIEGO

Probing the Cellular Delivery Capabilities of Modified Aminoglycosides

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by

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ABSTRACT OF THE THESIS

Probing the Cellular Delivery Capabilities of Modified Aminoglycosides

by

Kaivin Hadidi

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Professor Yitzhak Tor, Chair

Advancement and investigation of cellular delivery vehicles using guanidinoglycosides for the transport of biologically relevant cargos are reported. Insertion of the PEG-modified phospholipid DSPE-PEG-OMe 2000 and a lipophilic guanidinoneomycin derivative on the surface of liposomes diminishes uptake of the

nanoassemblies and is dependent on the amount of DSPE-PEG-OMe inserted on the liposome surface. To illustrate this, liposomes with guanidinoneomycin on the bilayer surface (i.e., GNeosomes) were treated with varying amounts of the PEG-modified phospholipid and subsequently analyzed to study the effects on liposomal size, zeta potential, and cellular uptake. By systematically protecting GNeosomes through inserting varying amounts of PEG chains into liposomal bilayers, methodical control over cellular uptake is obtained.

Additionally, an analysis of modified amino- and guanidino-glycosides derived from kanamycin, tobramycin, and neomycin in native and mutant CHO cells is examined using confocal microscopy and flow cytometry, illustrating the significance of multivalency for mammalian cell internalization of carriers that specifically interact with cell surface heparan sulfate proteoglycans.

CHAPTER 1

1. Introduction

The fight to circumvent bodily defense mechanisms remains a daunting challenge for modern medicinal chemistry and drug design.[1] Following successful trials *in vitro*, many compounds synthesized for therapeutic purposes have difficulty reaching their intended targets *in vivo* due to protective barriers.[2] Pharmaceutical companies notably devote gargantuan financial sums towards developing novel compounds intended to alleviate issues introduced by various medical conditions, yet also bypass these defenses.[3] Notably, the human body encompasses a plethora of methods that exist solely for the destruction and purging of foreign agents to preserve overall health, regardless of benefit or harm.[4] In addition, the epithelium restricts the entrance of foreign pathogens, toxins, and in the context of drug delivery, remedial therapeutics.[5] Therefore, few approaches remain towards introducing a beneficial remedy even before the consideration of defenses within the body.

Possible entrances, which can potentially be utilized for drug administration, include the eyes, ears, naval cavities, mouth, lungs, and anal cavities. They each possess unique functions and interactions with the body's environment. While these entrances deter foreign agents through unique methods, primary responsibility for protection against environmental threats lies with the skin. Within the context of drug delivery, the mouth, lungs, and direct injections through the skin are preferred routes for drug administration.[6–8] Oral ingestion through the mouth sees high popularity due to low invasiveness.[6] Unfortunately, the breakdown and processing of consumed nutrients is the

primary function of the digestive system. If administered orally, any potential therapeutic must withstand destructive enzymes produced through salivary glands in addition to the stomach's harsh acidic environment.[9] Upon passing the stomach, compounds enter the bloodstream through membranes within the gut. Lipophilic membranes restrict cellular translocation of compounds with high solubility in water, favoring compounds with hydrophobic properties. Despite these issues, oral ingestion remains attractive. The general population favors pill consumption more than invasive methods, although at times the latter is needed due to factors beyond our control.

Delivery through the nose is also highly preferred, especially when targeting the sinuses or the respiratory system, as access towards the mucosal surfaces are desirable for delivery of drugs and vaccines.[10] Treatment of diseases in the nose also benefit from the application of potential medicines directly to the afflicted area. If appropriate, this method may also aid in circumventing solubility and bioavailability issues through bypassing the gastrointestinal tract and metabolism in the liver. However, some attention should be given towards the fact that the primary purpose of nasal airways is protection of the lungs from harmful exposures, not as a method for delivering therapeutics.[7] While often the attractive features of nasal ingestion are highlighted in various publications, imposition of the functional features on nasal delivery are commonly disregarded, and have historically been difficult to address.[7] Such functions include filtration and conditioning of incoming air, in addition to the regulation of fluid retention and gas exchange.[11] Additionally, nasal delivery is an attractive route towards targeting the brain through quickly passing the blood brain barrier.[12]

Introducing a drug is often done through injection, which places the drug directly into the bloodstream to reach its target via the circulatory system. This bypasses the epithelia, the primary frontier that an organism possesses to defend itself from the environment, and is mostly attributed with the skin.[8] However, this method, although powerful in its ability to quickly deliver remedial agents, includes drawbacks. Various enzymes in the blood destroy specific functional groups upon binding, such as esterases and proteases, which threaten the stability of various compounds.[13] Additionally, while hydrophobic characteristics augment translocation across nonpolar membranes, poor water solubility restricts incorporation into the bloodstream.[14] Yet given that administration through other means is difficult, injection presents a simple method towards bypassing mentioned complications.

Once within the bloodstream, the endothelium and the cell membrane are primary frontiers for expelling foreign agents. The responsibility for the forefront of this defense is the former, which consist of tightly packed sheets of cells that line the interior of the cardiovascular system.[5] Its structure as a physical barrier aids the numerous functions it possesses, such as filtration and cardiovascular regulation.[15] Therapeutics may cross endothelium through two primary routes, the transcellular and paracellular pathways. Transcellular pathways are employed by lipophilic drugs and molecules that take advantage of the numerous mechanisms cells utilize for the passage of desired materials, such as pumps, channels, and carriers.[16] However, hydrophilic drugs, unable to benefit from this pathway, move through the paracellular pathway, which is tightly regulated by tight junctions.[17] Analysis of tight junctions has afforded the design of procedures able to reversibly open them, with potential for drug delivery across the endothelium.[18] Common

procedures include gene silencing and applying small molecules/peptides with the ability to target junction proteins.[19] Once bypassed, the cell membrane remains. Comprised primarily of amphiphilic phospholipids, the cell membrane is a highly complex network of multiple families of biomolecules expressed on both sides of the membrane. While investigation of cellular functions began near half a century ago, great strides have been made in understanding the cell membrane's regulation of internalization.[20] Various endocytosis pathways are responsible for internalizing diverse biological molecules, and can be read about in this 2009 review by Doherty and McMahon.[21]

Often when formulating new drugs to combat a certain disease, candidates frequently emerge with excellent potency for its target yet are eliminated as potential therapeutics for *in vivo* applications due to poor solubility and cellular translocation.[14] To combat such challenges, scientists have developed a class of agents termed molecular transporters responsible for ferrying biologically active cargo inside cells.[22] These molecular transporters vary greatly in type and effectiveness. Transporters in the form of small molecules are generally very common, yet formulations have also emerged from biomacromolecules (most commonly peptides/proteins),[23] as well as nanoparticle therapies such as liposomes and micelles.[24,25]

Molecular transporters are frequently studied due to their relative abundance and ease of derivatization compared to larger biomolecules, yet a primary source for differentiation among this subclass is through interactions with cargo.[26] Direct conjugation, complexation, and encapsulation are examples of the various chemical interactions small molecule transporters employ to deliver therapeutics. It is important to note that derivatizing wild-type cargo is unfavorable, especially therapeutic enzymes, as

modifications often lead to changes in activity. However, this is often a necessary compromise to reach the target of interest. Direct conjugation perhaps is the harshest approach due to covalent linkages between transporter and cargo, yet has been shown to be effective, as Dubikovskaya and coworkers exemplified in their Taxol derivative.[27] The anticancer molecule Taxol was linked to octaarginine through a disulfide linker and overcame Taxol resistance in Taxol-resistant ovarian carcinoma cells. Its performance improved compared to Taxol alone.[27] Yet most advantageous interactions are complexation through electrostatic interactions and encapsulation, as cargo can be left unmodified. Gelhe and coworkers utilized an organocatalytic ring-opening cyclization to produce amphipathic block co-oligomers that non-covalently complex with and deliver siRNA *in vitro* and report 90% knockdown of target protein.[28] Additionally, digestion of the carbonate scaffold in cells produces non-toxic components, which presents an attractive angle for a range of beneficial purposes.[29]

Surprisingly, the most effective small molecule molecular transporters consist of scaffolds that express positively charged guanidinium groups.[22] It has been known for nearly half a century that polybasic proteins enhance the cellular uptake of biomolecules,[30] yet incredible progress has been made in the past two decades to advance these tools.[3] This intellectual explosion began in 1988 where two groups reported the discovery that the arginine-rich region of the HIV Tat peptide was responsible for the translocation of viral DNA into the nucleus.[31,32] Despite the attractive qualities of the guanidinium group, numerous other naturally occurring peptides do exist that display similar cellular uptake properties, but efforts have centralized around the guanidinium group.[33,34] Further experiments indicated that conjugation of the RNA binding domain of

the TAT peptide to proteins enhanced cellular uptake of said proteins, and studies with $(Arg)_9$ and D-Tat gave insight into the effects of stereochemistry.[23] This discovery fueled an explosion of new molecular transporters inspired by the Tat peptide, termed the guanidinium-based molecular transporters.[35] Attaching guanidinium groups to various scaffolds is still investigated nearly twenty years later with Wender and coworkers functionalizing oligo-phosphoesters with guanidinium groups.[36] However, the scaffold with the highest uptake efficiency is derived from a class of antibiotics known as the aminoglycosides.[35]

Aminoglycoside antibiotics were among the first antibiotics to be discovered and used clinically with Waksman's discovery of the first aminoglycoside Streptomycin.[37] Kanamycin, Neomycin, and Gentamycin are naturally occurring aminoglycosides whose discovery followed suit, and various semi-synthetic derivatives have also been introduced such as amikacin and dibekacin.[38,39] While the popularity of these compounds decreased over the past half century, alternate applications for these molecules have emerged, prominently as scaffolds for guanidinium-based molecular transporters. Conversion of the amino groups on the parent aminoglycosides into guanidinium groups yields the guanidinoglycosides, a family initially synthesized to study RNA small- molecule binding.[40] Compared to their parent compounds, the guanidinoglycosides showed increased uptake efficiency. Of significance is the derivative created by the guanidinylation of neomycin, termed guanidinoneomycin (GNeo), which has been shown to enter cells in sub μM concentrations. The versatility of GNeo's cellular delivery capabilities has been studied through delivery of both small and large biomolecules.[35] Initially, delivery of a fluorescently tagged 60 kDa Streptavidin protein was reported

using a biotin functionalized GNeo derivative (**Figure 1.1**). The corresponding streptavidin-GNeo tetramer exhibited potent uptake efficacy using sub μM concentrations.[41,42] Upon discovering cell surface heparan sulfate was responsible for conjugate uptake, monomeric and dimeric guanidinoglycosides were synthesized to study the effects of multivalency and proteoglycan sulfation patterns on cellular uptake, indicating multivalency lowered necessary binding fidelity towards specific sulfation patterns for the initiation of endocytosis.[43]

While certainly impressive, the GNeo-biotin conjugation suffers from significant drawbacks. Principally, its uptake capabilities are limited to streptavidin and streptavidin-conjugated proteins.[42] To circumvent this, amphiphilic GNeo derivatives were synthesized to probe the use of liposomal technologies for cellular delivery.[24,25] Liposomal formulations are among the most well-investigated nanocarriers for targeted drug delivery, and have improved therapies for a range of biomedical applications by stabilizing therapeutics, overcoming uptake issues, and improving biodistribution to target sites *in vivo*.[44] They are phospholipid vesicles consisting of a bilayer(s) that enclose discrete aqueous spaces and possess a unique ability to entrap both hydrophobic and hydrophilic compounds. This allows for the encapsulation and subsequent protection of a diverse range of drugs, including macromolecules. When combined with the capacity for self-assembly and modifiable biological characteristics, liposomes remain powerful tools for drug delivery *in vivo*. Additionally, they are considered pharmacologically inactive and express minimal toxicity due to being composed of natural phospholipids. These properties have not gone unnoticed, and various liposomal formulations are currently used in the clinic, despite slow translation.[45] DOXIL, a liposomal formulation of

doxorubicin, is widely used as a method of slowly introducing doses of the anticancer drug over longer periods of time.[45,46] The bilayer of the liposomes of DOXIL are surrounded with oligomers of polyethylene glycol, which prevent enzymes present in the blood from destroying the liposomes and by association doxorubicin purely through steric interactions.[45–47] By delaying degradation, the nonpolar doxorubicin slowly escapes the vesicles and provides small doses of the harsh anticancer drug, alleviating harsh side effects.

Post-inserting an amphiphilic GNeo derivative into pre-formed liposomes functionalizes the outer face of the bilayer with molecular transporters, allowing for the delivery of entire vesicles into cells. When inserted into liposomal membranes, a derivative with a stearic acid handle termed stearyl-GNeo exhibited the highest uptake and assembly stability. This construct, termed GNeosomes, has delivered small molecules and enzymes necessary for enzyme replacement therapy with similar efficacy compared to the GNeo-Streptavidin conjugate.[24,25]

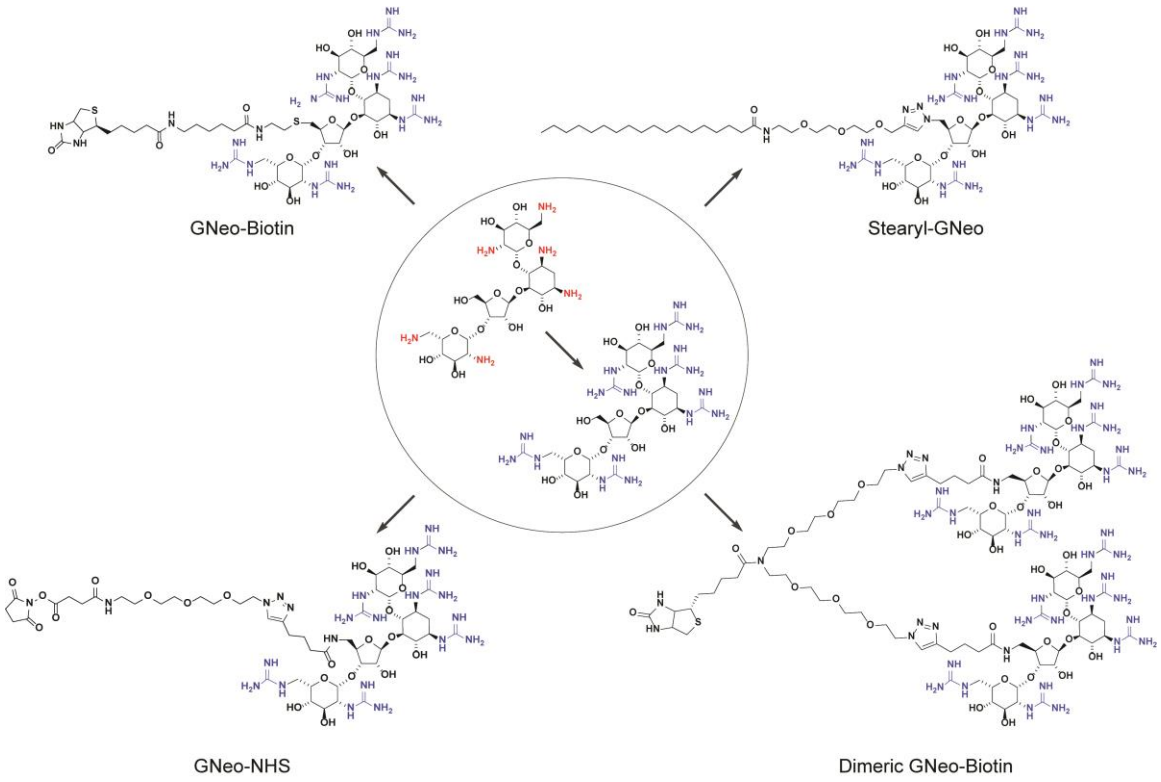

Figure 1.1. Previously studied derivatizations of guanidinylated neomycin, including biotinylated guanidinoneomycin (both monomeric and dimeric), GNeo-NHS, and stearyl-GNeo.

The work presented here centers on fine-tuning the uptake properties of GNeosomes by functionalizing the liposomal membrane with DSPE-PEG-OMe 2000 to regulate uptake, increase nanoparticle stability, and protect encapsulated therapeutics. It is well established that conjugation of poly-ethylene glycol (PEG) oligomers with biological cargo increases biodistribution, bioavailability and overall stability of the therapeutic *in vivo* through repellence with steric hindrance.[48] This method has been employed in liposomal formulations as well, termed "stealth" liposomes, which exhibit

extended bioavailability once PEG-modified phospholipids are introduced to liposomal membranes.[49]

2. Results

2.1 Analysis of the effects of PEG on the liposomal bilayer

To study the effect of increasing amounts of PEG-modified phospholipid on the surface of the bilayer, liposomes were incubated with various mol percentages of DSPE-PEG-OMe 2000 to determine the relationship between size and zeta potential of the PEGylated liposomes. Upon incubating with 0, with 0, 0.5, 1, 2 and 5 mol % DSPE-PEG-OMe 2000, a sharp decay in zeta potential was detected which correlated with an increase in the diameter of the liposomes (**Figure 2.1**). Interestingly, only a slight change in zeta potential was observed upon adding 10 mol % DSPE-PEG-OMe vs. adding 5 mol % DSPE-PEG-OMe. Increasing concentration of modified phospholipid decreases surface charge density and overall particle size increases.

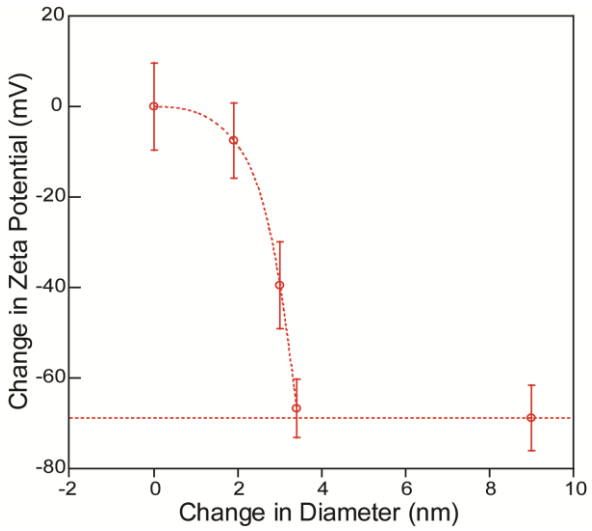

Figure 2.1. Plot of the change in zeta potential (mV) of PEGylated liposomes vs difference in average diameter (nm). All values are normalized using blank liposomes. Each point represents a unique concentration of DSPE-PEG-2000 (left to right: 0, 0.5, 1, 2 and 5 mol % in solution)

2.2 Cellular Uptake of Modified GNeosomes

To study the effects of DSPE-PEG-OMe 2000 on GNeosomes, plain liposomes encapsulating 400 µM Cy5 were initially post-inserted with stearyl-GNeo to express GNeo on liposomal membranes. This formulation differs from established procedures in that a 60 mg/mL lipid solution was used instead of the reported 15 mg/mL solution, altering encapsulation efficiency. Upon forming desired vesicles, the liposomal solution was split into 5 and treated with varying amounts of a concentrated solution of DSPE-PEG-OMe until the desired concentrations were obtained (0, 1, 1.5, 2 and 4 mol %). Particle size and surface charge were measured using dynamic light scattering, indicating a similar decay pattern. Subsequently, internalization of the PEGylated GNeosomes was studied by treating wild type Chinese hamster ovarian (CHOK1) cells with a solution of

the vesicles at 0.5 mg/ml. As the concentration of PEG-modified phospholipid on the surface of the liposomes increases, a decrease in overall uptake is observed (**Figure 2.1**)

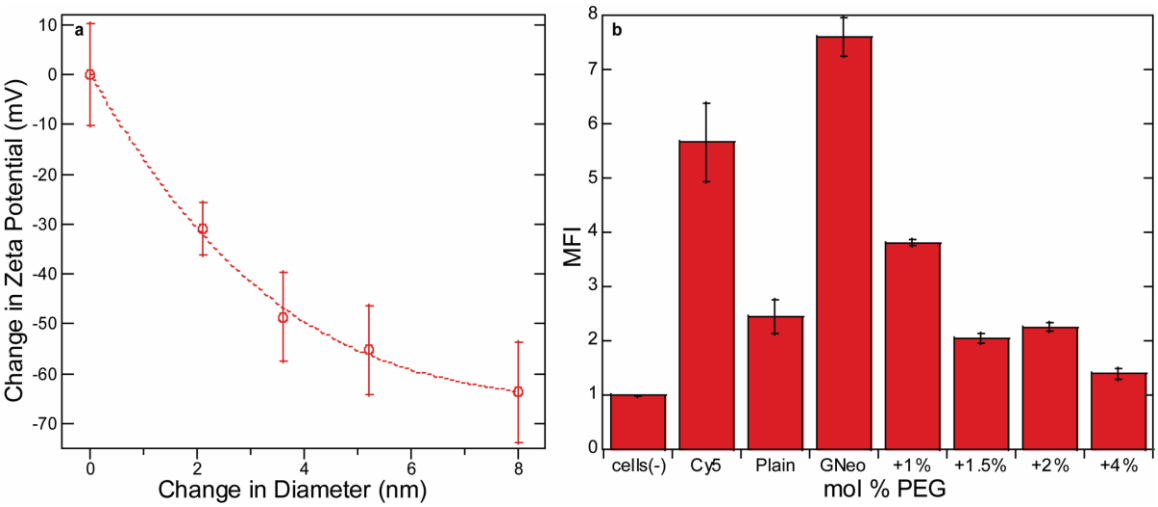

Figure 2.2. (a) Plot of the change in zeta potential (mV) of PEGylated GNeosomes vs. difference in average diameter (nm). All values are normalized using unmodified GNeosomes. Each point represents a unique concentration of DSPE-PEG-2000 (left to right: 0, 0.5, 1, 2 and 5 mol % in solution). (b) Cellular uptake of PEGylated GNeosomes formulated from a 60 mg/ml lipid solution with 400 µM encapsulated Cy5, including blank, 5 µM Cy5, blank liposomal and GNeosomal negative controls.

To resolve signal/noise issues observed in the cellular uptake of PEGylated GNeosomes encapsulating 400 µM of Cy5, GNeosomes were initially formulated by encapsulating 800 µM of Cy5 in 1 mL of a 60mg/mL solution of liposomal lipids. The encapsulation efficiency of concentrated GNeosomes matched published values through measuring the fluorescence intensity of lysed liposomes. Subsequently, the PEGylated GNeosomes were prepared again and incubated with CHOK1 cells for 1, 3 and 24 hr(s) to examine the effects of longer incubation times on cellular uptake (**Figure 2.3 a–c**). Blank GNeosomes and GNeosomes with 1% DSPE-PEG-OMe used exhibited higher

cellular uptake than blank liposomes at 1 and 3 hr incubation times. At similar times, PEGylated GNeosomes with a higher surface density of oligomer exhibited lower levels of cellular uptake comparable to controls. During the first three hours of incubation, the trend of decreasing uptake with higher DSPE-PEG-OMe concentration is retained. However, all MFI values fell to baseline levels at 24 hr, and MFI values of all samples decrease over time. The relationship between average MFI and particle size corresponds to a linear trend, compared to the exponential curve seen between average MFI and change in surface charge density (**Figure 2.3 d–e**).

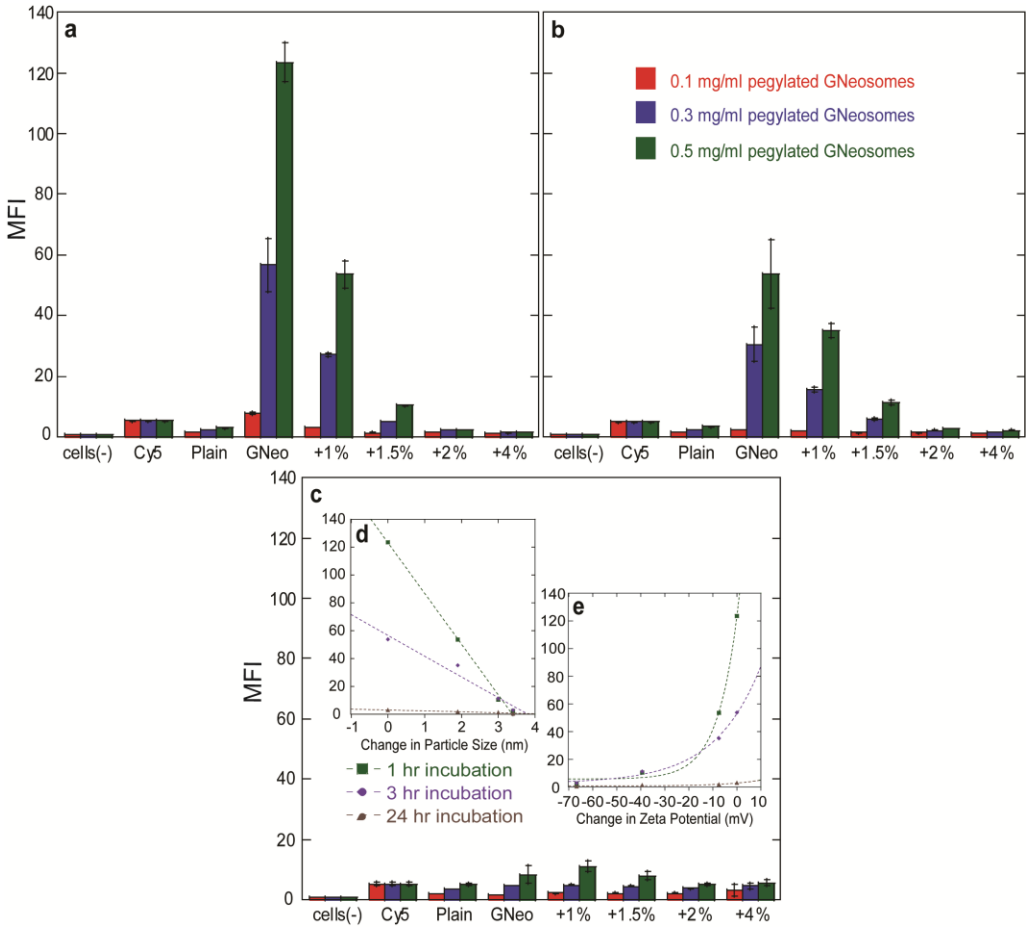

Figure 2.3. Cellular uptake of PEGylated GNeosomes after (a) 1 hr, (b) 3 hr, and (c) 24 hr incubation. (d) Plot of cellular uptake vs. change in particle size and (e) vs. change in surface charge density. All measurements were taken in triplicate.

3. Discussion

Based on these results, a sensitive quantification method for determining the concentration of surface DSPE-PEG-OMe on liposomal bilayers is needed. However, very few quantification methods are reported in literature. A few mentioned in this 2010 review[50] include a phospholipase C assay using the Stewart method, a method involving extraction with picric acid,[51] and HPLC[52]. Unfortunately, none of these methods are practical for this system. The phospholipase C and picric acid extraction methods are impractical due to detection of other liposomal lipids, while separation and resolution of phospholipid peaks using HPLC remains a daunting challenge. It is important to note that liposomal lipids are in huge excess compared to GNeo and the PEG-modified phospholipid, and low concentrations of either are likely undetectable by HPLC due to poor UV absorbance.

Of peculiar notice is the difference between plots of particle size and zeta potential between PEGylated liposomes and GNeosomes (**Figure 2.1–2.2a**). In the absence of surface GNeo, DSPE-PEG-OMe changes surface charge density drastically, approaching the surface charge limit within adding a few nm to particle size. Amongst GNeosomes, addition of DSPE-PEG-OMe to the surface gradually alters surface charge, approaching the surface charge limit within adding 8 nm to the diameter. A potential explanation for this phenomenon lies in the surface packing of the oligomer. Positive charges provided by cationic lipids on the surface of plain liposomes may interact with the oxygen atoms within the PEG, wrapping the chain tightly around the vesicle. Adding GNeo to the bilayer presents irregularity to surface topology, hindering this interaction through increased difficulties in stacking on a rugged surface. Therefore, the same

amount of oligomer added to GNeosomes may additionally increase particle size compared to plain liposomes due to a larger possibility of chains extending out into solution.

Although increasing surface expression of DSPE-PEG-OMe leads to lower cellular uptake, overall internalization decreases across all samples during extended periods of incubation. While decreasing cellular uptake of unmodified GNeosomes over time is logical due to substantial internalization initially, the reduction in uptake over time of PEGylated GNeosomes remains baffling, considering the introduction of steric interactions through surface PEG oligomers. Through steric inhibition of GNeo-cell surface heparan sulfate interactions, cellular uptake should ideally remain constant over time, especially for PEGylated GNeosomes with higher PEG surface density. We speculate that excess ingestion of the nanoassemblies may inhibit endocytosis and hinder the internalization of subsequent vesicles. It is important to note that uptake of modified GNeosomes includes PEG, which may be particularly difficult for cellular digestion.

Considering GNeo is the primary source for surface charge and responsible for cellular uptake through heparan sulfate mediated endocytosis, an exponential relationship is logical. This same relationship is not observed with particle size, which exhibits a linear decrease in uptake with increasing liposome diameter. The PEG chains of pegylated GNeosomes block interactions between GNeo and Heparan Sulfate, slowing rates of endocytosis. Yet strangely, over time the internalization rate fluctuates rather than staying constant. This indicates the absence of infamous “stealth” properties of large PEG oligomers. While uptake appears constant for PEGylated GNeosomes with larger PEG surface density, internalization is diminished to insignificant levels.

4. Conclusion

GNeosomal bilayers post-inserted with DSPE-PEG-OMe 2000 demonstrate systematically reduced uptake efficacy compared to unmodified GNeosomes. Additionally, increasing incubation times lead to drastic declines in overall internalization of the vesicles independent of DSPE-PEG-OMe concentration. These findings establish a solid foothold for tuning cellular uptake while maintaining assembly stability.

5. Future Directions

While the results are promising, a crucial factor that remains unstudied is the effect of chain length on stability and delivery of the nanoparticles. An ideal balance between these two factors is of crucial importance when considering delivery *in vivo*, in which large varieties of cells are present with unique rates of endocytosis, as well as toxicity effects potentially introduced through the ingestion of PEG.

6. Experimental

6.1 Materials

Reagents were purchased from Sigma-Aldrich and were used without further purification unless otherwise specified. All experiments were carried out in Corning 24-well plates. PBS, FBS and F-12 nutrient mixture (HAM) were purchased from Life Technologies (San Diego, CA). Trypsin-EDTA was purchased from VWR. Particle size, surface charge, and polydispersity measurements were measured by dynamic light

scattering on a Zestier Nano ZS (model ZEN3600 from Malvern Instruments). FACS experiments were carried out on a BD FACS Calibur instrument.

6.2 Cell Culture

Cells were grown under a 5% CO_2 atmosphere in air with 100% relative humidity at 37 °C. Wild-type Chinese hamster ovarian cells were obtained from the American Type Culture Collection (CCL-61) and were grown in F-12 medium with 10% v/v fetal bovine serum, penicillin, and streptomycin.

6.3 Synthetic Procedures

Stearyl-GNeo was synthesized according to previously published procedures.

6.3.1. Fabrication of PEGylated liposomes and GNeosomes

GNeosomes were formulated based on established procedures. Briefly, a solution of lipids in chloroform (73:11:16 DOPC: DOPE: Cholesterol) was dried to produce a lipid layer on the bottom of a round bottom flask. The layer was then rehydrated with milliq (or any solution containing the potential cargo of interest) and subjected to 6 freeze-thaw cycles to form unilamellar and multilamellar liposomes. The solution was then extruded through a filter with 100 nm pores to unify the diameter and lamellarity, and subjected to size-exclusion chromatography to remove any excess cargo. Liposomes were quantified using the Stewart method. 2.5 mL of 4 mg/mL liposomes was incubated for 1 hr at rt with 250 µL of a 2.7 mol % Stearyl-GNeo solution. Excess GNeo was removed with size-exclusion chromatography and the liposomes were quantified using

the Stewart method. The PEGylated liposomes and GNeosomes were then formulated by incubating 500 ml of 2 mg/mL regular liposomes/ GNeosomes with 500 µL of 0, 1, 2, 4, and 10 mol % DSPE-PEG-OMe 2000, purifying with size exclusion and quantifying concentration with the Stewart method. 100 µL of PEG GNeosomes was then diluted to 1 mL to measure particle size, zeta potential, and polydispersity using dynamic light scattering.

6.4 Stewart Method

The concentration of liposomal lipids in solution were measured based on established procedures.[53] Briefly, 50 µL of the liposomal solution was added to 1.5 mL chloroform in a falcon tube and vortexed vigorously for 10 s. Subsequently, 1.5 mL of ammonium ferrothiocynate (0.1 M) was added and vortexed for 15 seconds. The biphasic system was centrifuged at high speed for 2 min. The optical density of the organic phase was measured at 480 nm using chloroform as a blank.

6.5 Flow Cytometry Protocol

To measure the cellular uptake of PEGylated liposomes, all nanoassemblies were tested against wild type Chinese hamster ovarian cells (CHOK1). Cells were seeded onto a 24-well plate and grown to 80% confluency overnight. Subsequently, cells were washed with Dulbecco's phosphate-buffered saline (PBS). PEGylated liposomes were then diluted to desired concentrations in F-12 growth medium containing 10% Fetal Bovine Serum (FBS) and cells were treated with 300 µL of the corresponding solutions for 1 hour at 37 °C in a 5% CO_2 atmosphere. Cells were then washed twice with 300 µL PBS

and treated with 60 µL Trypsin-EDTA for 10 minutes, followed by dilution a dilution with 0.1% BSA in PBS, and analyzed by FACS. This was repeated for PEGylated GNeosomes containing 800 µM Cy5, and incubation times were changed to 1, 3, and 24 hours accordingly.

7. Appendix

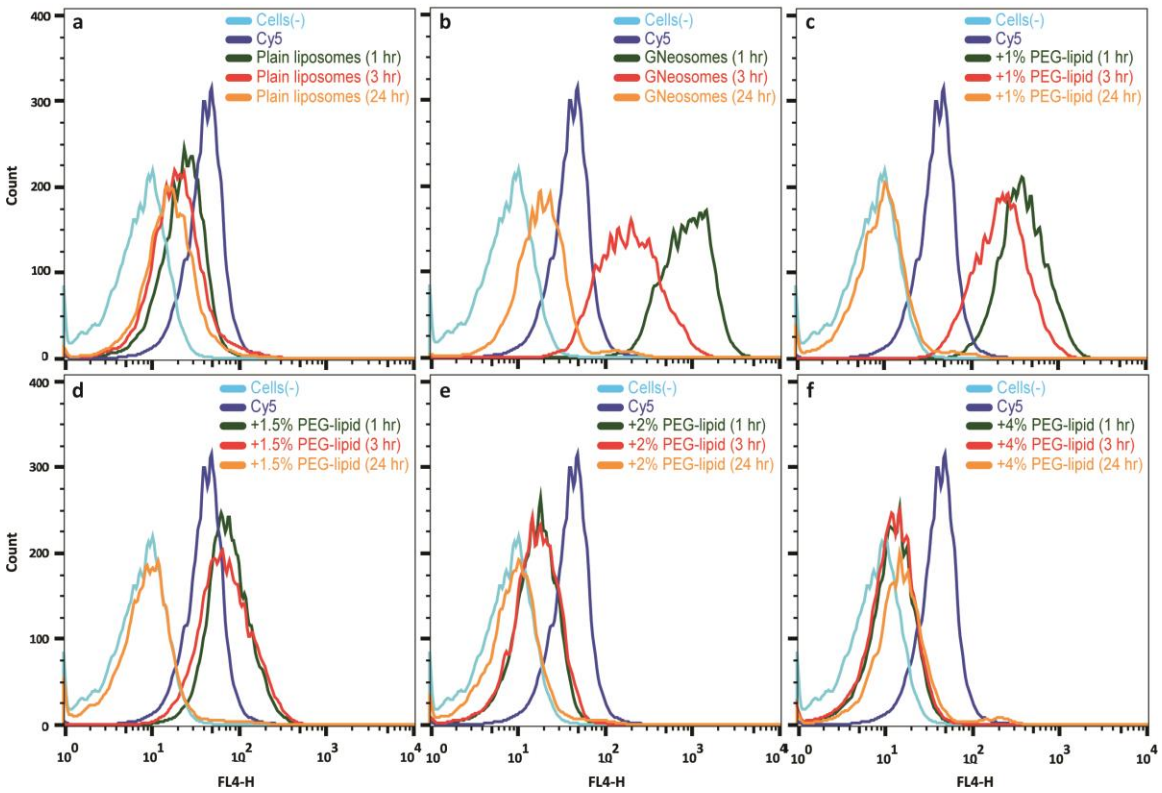

Figure S3. FACS histograms of PEGylated GNeosomes after 1 hr, 3 hr, and 24 hr incubation. (a) Plain liposomes, (b) Plain GNeosomes, (c) GNeosomes +1% DSPE-PEG-OMe, (d) GNeosomes +1.5% DSPE-PEG-OMe, (e) GNeosomes +2% DSPE-PEG-OMe, (f) GNeosomes +4% DSPE-PEG-OMe.

CHAPTER 2

1. Introduction

Aminoglycoside antibiotics were among the first antibiotics discovered and used clinically.[54] Streptomycin, isolated from *Streptomyces griseus* was the first aminoglycoside discovered,[37] and quickly employed clinically to treat tuberculosis. Since then, various naturally occurring aminoglycosides such as kanamycin, neomycin (fradiomycin) and gentamycin have been discovered, and various semi-synthetic analogues have been introduced such as amikacin and dibekacin.[38,39] Aminoglycosides, while still clinically relevant, are not commonly employed due to their adverse effects and emergence of antibiotic resistance impeding their efficacy.[55,56] Nevertheless, while the prevalence of aminoglycoside antibiotics in the clinic may have decreased, the past half century of research has given way to alternate applications for this class of molecules.[57,58] One such application is as a scaffold for guanidinium-based molecular transporters, where they can serve as delivery vehicles for various biological entities into cells.[35,59]

Over the last two decades, our group has developed and investigated various derivatives of guanidinium-rich cellular delivery agents based on aminoglycosidic scaffolds, commonly referred to as guanidinoglycosides (**Figure 1**).[10–12] This subcategory of molecular transporters is synthesized by converting all amino groups on the aminoglycoside scaffolds into guanidinium groups.[60] Whereas the majority of cellular delivery agents and guanidinium-based molecular transporters are often used at micromolar concentrations, guanidinoglycosides have been shown to deliver large bioactive macromolecules into cells at nanomolar concentrations when both covalently or

non-covalently bound to a cargo of interest.[24,25,41–43,61] The presence of heparan sulfate proteoglycans on the cell surface has been crucial to the observed cellular uptake.[12,42] Of significance is that multivalent systems have dominated these studies, with less information available for monomeric arrangements. Since minimizing the number of molecular transporters used per cargo is desirable, we have sought to compare and contrast the uptake features of mono- and multi-valent amino- and guanidino-glycosides. In this communication, we report on cellular uptake studies with such highly charged carriers in their monovalent low MW (fluorescently tagged) form and multivalent arrangement (when bound to a high MW fluorescently labeled streptavidin via a biotin-linker) in both wild type (CHOK1) and heparan sulfate deficient (pgsA-745) cells.[62] These results illustrate intriguing carrier/uptake relationships that may impact the design of future transporters.

2. Results

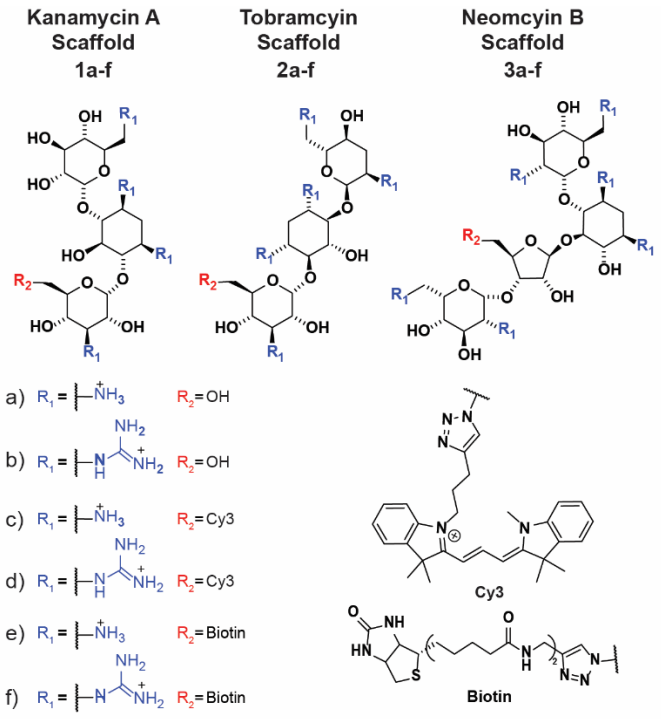

Figure 2.1. Structures of unmodified (**1-3, a-b**), Cy3-conjugated (**1-3, c-d**) and biotinylated (**1-3, e-f**) amino- and guanidino-glycosides.

To investigate the effect of multivalency on cellular uptake, biotinylated and Cy3-conjugated amino- and guanidino-glycoside derivatives (**Figure 1**) were prepared according to previously reported synthetic procedures (full details to be published elsewhere). All compounds were tested in cell culture with wild type Chinese hamster ovarian (CHOK1) and with mutant Chinese hamster ovarian cells (pgsA-745) devoid of cell surface heparan sulfate. The internalization was quantified using flow cytometry (FACS).

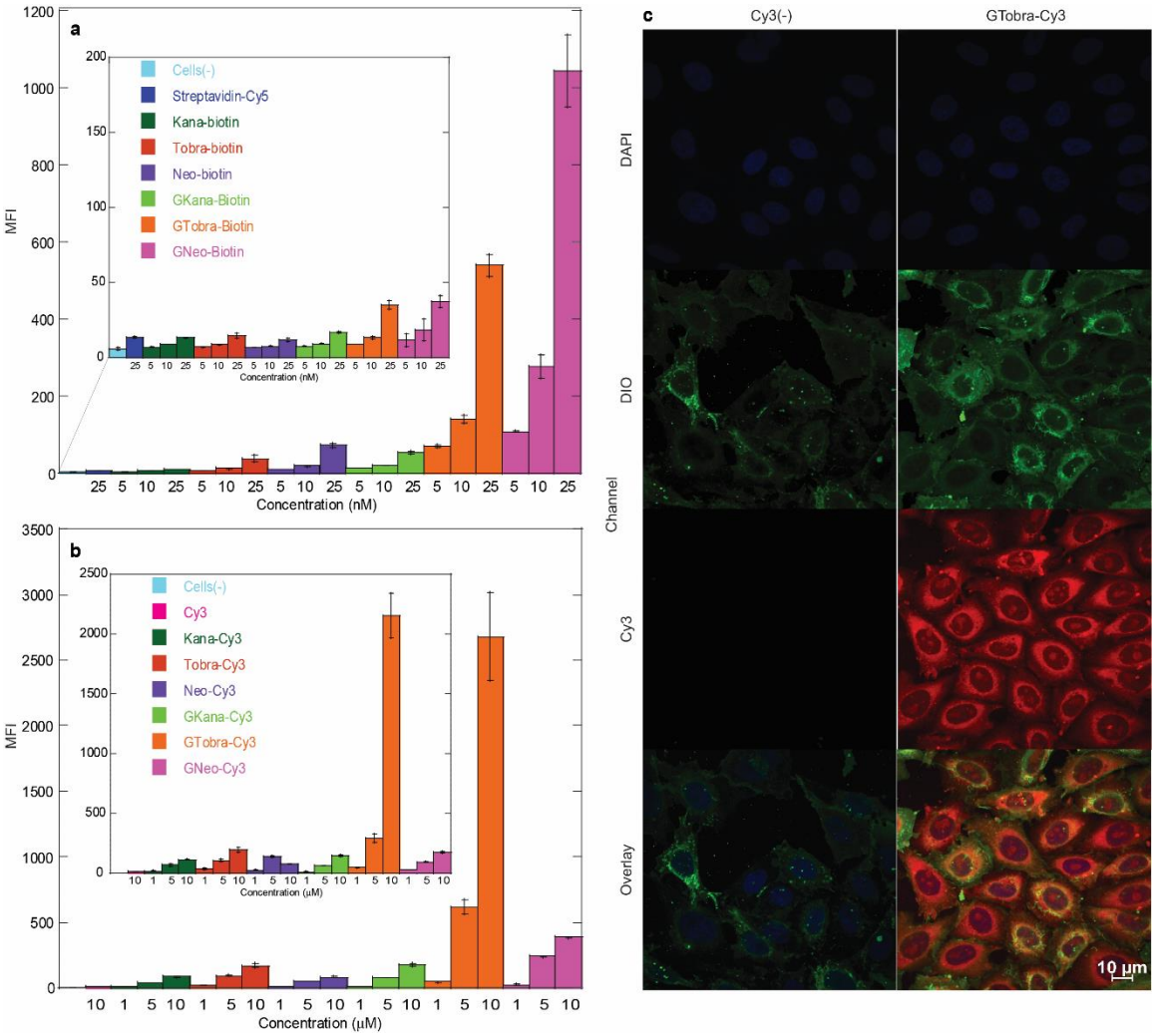

Figure 2.2. Mean fluorescence intensity of amino- and guanidino-glycosides bound to Cy5-labelled streptavidin in (a) wild type Chinese hamster ovarian (CHOK1) cells and mutant pgsA-745 cells (inset). (b) Mean fluorescence intensity of Cy3-labelled amino- and guanidino-glycosides in CHOK1 cells and pgsA-745 cells (inset). All experiments were performed in triplicate. (c) Confocal microscopy images of CHOK1 cells treated with Cy3 (left) and guanidinotobramycin-Cy3 (right).

Amongst the amino- and guanidino-glycoside streptavidin conjugates **1e**–**3e** and **1f**–**3f**, respectively, the guanidinylated neomycin carrier (**3f**) conjugate was shown to enter cells with higher efficacy than the other guanidino- and amino- conjugates in CHO cells (**Figure 2a**). The internalization of the guanidinoglycoside conjugates was

significantly higher compared to their aminoglycoside counterparts when bound to streptavidin-Cy5, demonstrating the impact on the guanidinium group upon cellular delivery of macromolecules. This is consistent with previously published work performed solely on the GNeo-streptavidin conjugate.[12,61] The GNeo-streptavidin (**3f**) conjugate entered cells twice as well as the guanidinotobramycin (**2f**) conjugate, highlighting the benefit of a higher number of guanidinium moieties on the transporter. Comparatively, the guanidinokanamycin (**1f**) conjugate showed minimal cellular uptake. The same behavior, albeit significantly reduced, was observed in mutant pgsA-745 cells, which do not express cell surface heparan sulfate.

3. Discussion

The monomeric amino- and guanidino-glycosides showed lower levels of entry into mammalian cells compared to their multivalent counterparts (**Figure 2b**, note different scales). While the GNeo-biotin conjugate depicted high fluorescence intensities, the only monomeric derivative that approached this fluorescence intensity was guanidinotobramycin-Cy3 (**2d**) albeit at 10 μM, nearly three orders of magnitude higher than the concentration used in multivalent system (25 nM). It is important to note, however, that the quantum yield of Cy3 is significantly lower than that of Cy5, and that such a significant difference in concentration could potentially originate from this disparity. Amongst the fluorescently tagged monomeric amino- and guanidino-glycosides, the guanidinoglycosides entered cells better than their aminoglycoside parents, similar to the multivalent system, indicating that the guanidinium group is important for these monomeric transporters as well. Cells treated with compound **2d**,

guanidinotobramycin-Cy3, exhibited nearly six-fold higher fluorescence intensity compared to guanidinoneomycin-Cy3 (**3d**) and guanidinokanamycin-Cy3 (**1d**), which is intriguing as it suggests lack of correlation between the number of charges on the molecular transporter and cellular uptake. This might appear to be contradictory to the obvious trend in the multivalent system, where increasing the number of positive charges augmented cellular uptake, hinting at distinct cell entry pathways. Indeed, the same fluorescence intensities are observed when pgsA-745 cells are treated with the monomeric derivatives, indicating that uptake of the guanidino-Cy3 derivatives (**1d**, **2d**, **3d**) is not exclusively dependent on cell surface heparan sulfate, as is well established for the multivalent derivatives. Other monomeric derivatives containing a different fluorescent probe were analyzed previously,[63] yet those compounds exhibited different uptake behavior than the Cy3 derivatives, which demonstrates that the uptake of fluorescently tagged monomeric guanidinoglycosides is dependent on the label (i.e., cargo) of interest as well as the molecular transporter. This is not entirely unthinkable, as under such circumstances the fluorescent tag is almost as large as the carrier itself.

The results discussed above provide insight into the reduced efficacy of the monomeric carriers, yet fail to explain why compound **2d** enters cells more efficiently than its other monomeric counterparts, including compound **3d**. We speculate that compound **2d**, having a total of 6 positive charges, may possess an effective combination of functional groups and positive charges in the optimal orientation to take advantage of multiple cell entry pathways (e.g., endocytosis, micropinocytosis). Preliminary confocal microscopy experiments in CHO cells, fixed after treatments with the fluorescently

tagged carriers, indeed show broad cytoplasmic distribution, clearly distinct from previous observations (**Figure 2c**).[42]

4. Conclusion

In summary, we have demonstrated the significance of multivalency in cellular uptake of aminoglycoside-based guanidinium-rich cellular delivery agents that exclusively utilize heparan sulfate as a cell entry pathway. Furthermore, when selectivity drops and this internalization route into mammalian cells is no longer exclusively exploited, other factors appear to become significant, including the number of positive charges present as well as the cargo of interest. These observations may have universal significance as structural features and fluorescent labels may be playing a role in the cellular delivery of other cell penetrating agents. This chapter has been submitted for publication. Hadidi, K.; Wexselblatt, E.; Esko, J.D.; and Tor, Y. Cellular Uptake of Modified Aminoglycosides. The thesis author was the primary investigator and author of the material.

5. Future Directions

Further experiments are required to cement alternative localization and cellular distribution mechanisms for guanidinylated tobramycin derivatives. Although micropinocytosis was previously shown to be the major endocytosis pathway mediated by heparan sulfate, the internalization mechanisms involved in the cellular uptake of much smaller derivatives remains unclear. A full study examining various charges of the

cargo with each unique carrier and their relationship towards various cellular internalization pathways remains a potentially attractive route to pursue.

6. Experimental

6.1 Synthetic Procedures

Biotinylated and Cy3-conjugated amino- and guanidino-glycoside derivatives (**Figure 1**) were prepared based on previously reported synthetic procedures.[61]

6.2 Cell Culture

Cells were grown under a 5% CO_2 atmosphere in air with 100% relative humidity at 37 °C. Wild-type Chinese hamster ovarian cells were obtained from the American Type Culture Collection (CCL-61). CHOK1 cells were grown in F-12 medium with 10% v/v fetal bovine serum, penicillin, and streptomycin.

6.3 Flow Cytometry Protocol

Experimentally, wild-type CHOK1 and mutant pgsA-745 cells were seeded onto a 24-well plate at a density of 100,000 cells per well and grown to 80% confluency overnight. Biotinylated amino- and guanidino-glycosides were then bound to a Cy5-labelled streptavidin to form tetravalent biotin-streptavidin conjugates by incubating the compounds with streptavidin-Cy5 (5:1) in a MilliQ:PBS (1:1) solution protected from light for 20 minutes. Both the streptavidin conjugates and the Cy3-guanidinoglycosides were diluted to the desired concentrations in F-12 growth medium containing 10% FBS. The cells were washed with PBS and incubated with 300 μL of the fluorescent carrier

solutions for 1 h at 37 °C in a 5% CO_2 atmosphere. The cells were then washed twice with 300 μL of PBS and detached with 60 μL of trypsin-EDTA for 10 minutes, followed by a dilution with 0.1% BSA in PBS, and analyzed by FACS (**Figure 2**).

7. Appendix

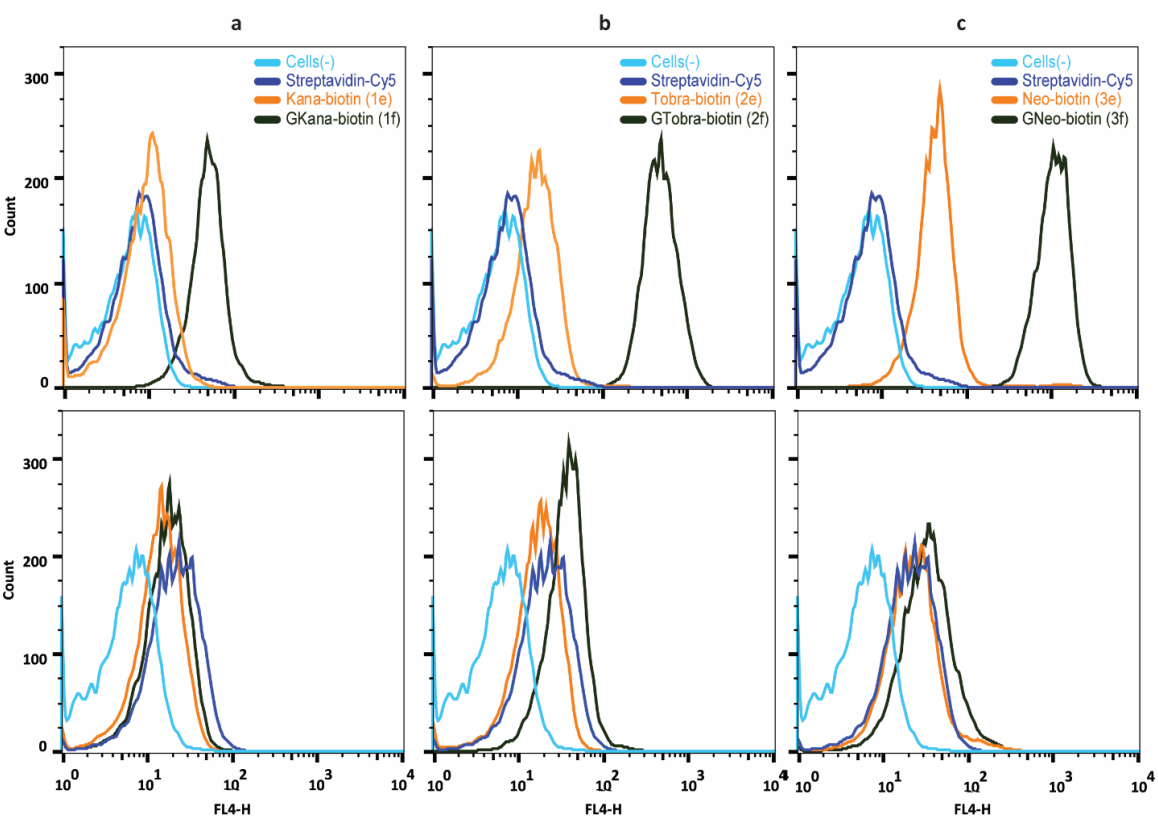

Figure S3. FACS histograms of amino- and guanidino-glycosides bound to Cy5 labelled streptavidin, 25nM. (a) Cellular uptake of kanamycin-biotin (compound **1e**) and guanidinokanamycin-biotin (compound **1f**) in CHOK1 (top) and pgsA745 cells (bottom). (b) Cellular uptake of tobramycin-biotin (compound **2e**) and guanidinotobramycin-biotin (compound **2f**) in CHOK1 (top) and pgsA745 cells (bottom). (c) Cellular uptake of neomycin-biotin (compound **3e**) and guanidinoneomycin-biotin (compound **3f**) in CHOK1 (top) and pgsA745 cells (bottom)

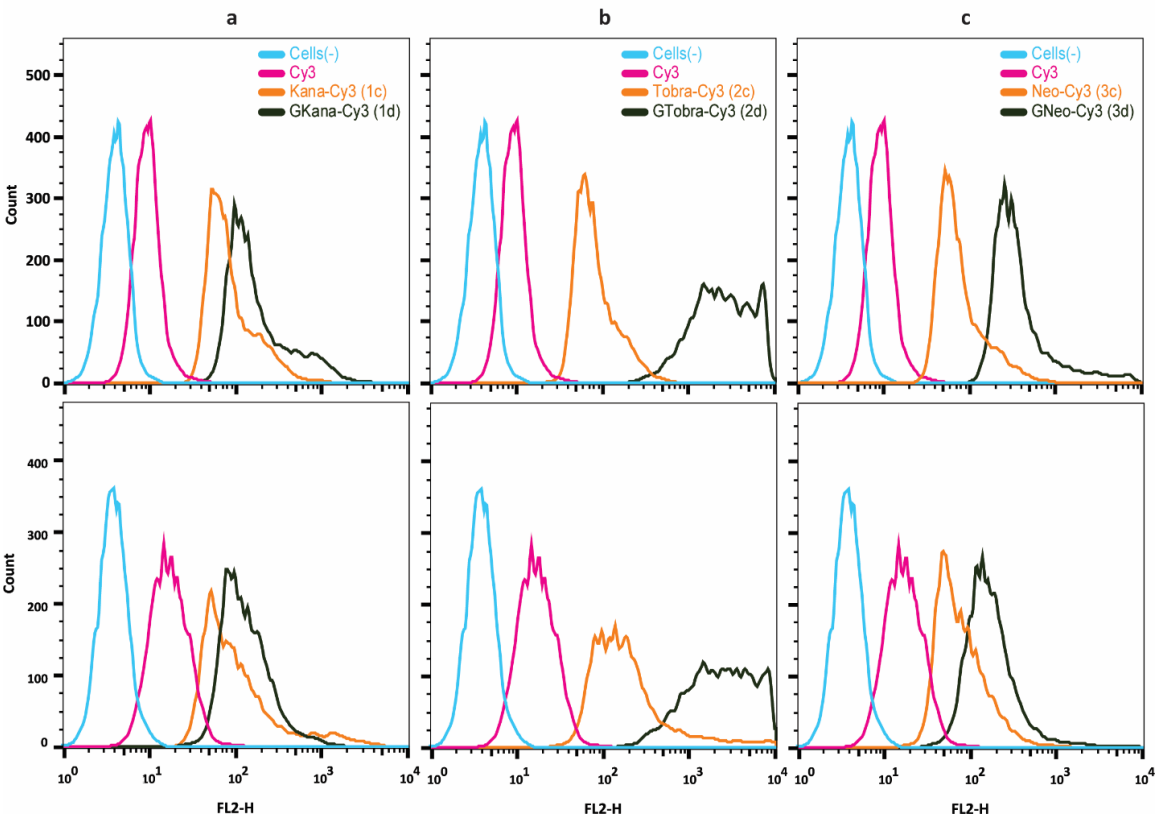

Figure S4. FACS histograms of Cy3 conjugated amino- and guanidino-glycosides, 10µM. (a) Cellular uptake of kanamycin-Cy3 (compound **1c**) and guanidinokanamycin-Cy3 (compound **1d**) in CHOK1 (top) and pgsA745 cells (bottom). (b)Cellular uptake of tobramycin-Cy3 (compound **2c**) and guanidinotobramycin-Cy3 (compound **2d**) in CHOK1 (top) and pgsA745 cells (bottom). (c) Cellular uptake of neomycin-Cy3 (compound **3c**) and guanidinoneomycin-Cy3 (compound **3d**) in CHOK1 (top) and pgsA745 cells (bottom).

REFERENCES

1. Chertok, B., Webber, M. J., Succi, M. D. & Langer, R. Drug Delivery Interfaces in the 21st Century: From Science Fiction Ideas to Viable Technologies. *Mol. Pharm.* **10,** 3531–3543 (2013).

2. Heitz, F., Morris, M. C. & Divita, G. Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br. J. Pharmacol.* **157,** 195–206 (2009).

3. Milletti, F. Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov. Today* **17,** 850–860 (2012).

4. Masereeuw, R. & Russel, F. G. M. Mechanisms and clinical implications of renal drug excretion*. *Drug Metab. Rev.* **33,** 299–351 (2001).

5. González-Mariscal, L., Nava, P. & Hernández, S. Critical Role of Tight Junctions in Drug Delivery across Epithelial and Endothelial Cell Layers. *J. Membr. Biol.* **207,** 55–68 (2005).

6. Goldberg, M. & Gomez-Orellana, I. Challenges for the oral delivery of macromolecules. *Nat. Rev. Drug Discov.* **2,** 289–295 (2003).

7. Djupesland, P. G. Nasal drug delivery devices: characteristics and performance in a clinical perspective—a review. *Drug Deliv. Transl. Res.* **3,** 42–62 (2013).

8. Wong, J. *et al.* Suspensions for intravenous (IV) injection: A review of development, preclinical and clinical aspects. *Adv. Drug Deliv. Rev.* **60,** 939–954 (2008).

9. Woodley, J. F. Enzymatic barriers for GI peptide and protein delivery. *Crit. Rev. Ther. Drug Carrier Syst.* **11,** 61–95 (1994).

10. Kublik, H. & Vidgren, M. T. Nasal delivery systems and their effect on deposition and absorption. *Adv. Drug Deliv. Rev.* **29,** 157–177 (1998).

11. Mygind, N. & Dahl, R. Anatomy, physiology and function of the nasal cavities in health and disease. *Adv. Drug Deliv. Rev.* **29,** 3–12 (1998).

12. Tiwari, G. *et al.* Drug delivery systems: An updated review. *Int. J. Pharm. Investig.* **2,** 2–11 (2012).

13. Williams, F. M. Clinical Significance of Esterases in Man. *Clin. Pharmacokinet.* **10,** 392–403 (1985).

14. Savjani, K. T., Gajjar, A. K. & Savjani, J. K. Drug Solubility: Importance and Enhancement Techniques. *ISRN Pharm.* **2012,** (2012).

15. Salama, N. N., Eddington, N. D. & Fasano, A. Tight junction modulation and its relationship to drug delivery. *Adv. Drug Deliv. Rev.* **58,** 15–28 (2006).

16. Laksitorini, M., Prasasty, V. D., Kiptoo, P. K. & Siahaan, T. J. Pathways and Progress in Improving Drug Delivery through the Intestinal Mucosa and Blood-Brain Barriers. *Ther. Deliv.* **5,** 1143–1163 (2014).

17. Cereijido, M., Shoshani, L. & Contreras, R. G. Molecular Physiology and Pathophysiology of Tight Junctions I. Biogenesis of tight junctions and epithelial polarity. *Am. J. Physiol. - Gastrointest. Liver Physiol.* **279,** G477–G482 (2000).

18. Gonzalez-Mariscal, L., Chávez de Ramírez, B. & Cereijido, M. Tight junction formation in cultured epithelial cells (MDCK). *J. Membr. Biol.* **86,** 113–125 (1985).

19. Underwood, J. L. *et al.* Glucocorticoids regulate transendothelial fluid flow resistance and formation of intercellular junctions. *Am. J. Physiol. - Cell Physiol.* **277,** C330–C342 (1999).

20. Korn, E. D. Structure and Function of the Plasma Membrane. *J. Gen. Physiol.* **52,** 257–278 (1968).

21. Doherty, G. J. & McMahon, H. T. Mechanisms of Endocytosis. *Annu. Rev. Biochem.* **78,** 857–902 (2009).

22. Goun, E. A., Pillow, T. H., Jones, L. R., Rothbard, J. B. & Wender, P. A. Molecular Transporters: Synthesis of Oligoguanidinium Transporters and Their Application to Drug Delivery and Real-Time Imaging. *ChemBioChem* **7,** 1497–1515 (2006).

23. Futaki, S. *et al.* Arginine-rich Peptides AN ABUNDANT SOURCE OF MEMBRANE-PERMEABLE PEPTIDES HAVING POTENTIAL AS CARRIERS FOR INTRACELLULAR PROTEIN DELIVERY. *J. Biol. Chem.* **276,** 5836–5840 (2001).

24. Wexselblatt, E., Esko, J. D. & Tor, Y. GNeosomes: Highly Lysosomotropic Nanoassemblies for Lysosomal Delivery. *ACS Nano* **9,** 3961–3968 (2015).

25. M. Hamill, K., Wexselblatt, E., Tong, W., D. Esko, J. & Tor, Y. Delivery of an active lysosomal enzyme using GNeosomes. *J. Mater. Chem. B* **4,** 5794–5797 (2016).

26. Stanzl, E. G., Trantow, B. M., Vargas, J. R. & Wender, P. A. Fifteen Years of Cell-Penetrating, Guanidinium-Rich Molecular Transporters: Basic Science, Research Tools, and Clinical Applications. *Acc. Chem. Res.* **46,** 2944–2954 (2013).

27. Dubikovskaya, E. A., Thorne, S. H., Pillow, T. H., Contag, C. H. & Wender, P. A. Overcoming multidrug resistance of small-molecule therapeutics through conjugation with releasable octaarginine transporters. *Proc. Natl. Acad. Sci.* **105,** 12128–12133 (2008).

28. Geihe, E. I. *et al.* Designed guanidinium-rich amphipathic oligocarbonate molecular transporters complex, deliver and release siRNA in cells. *Proc. Natl. Acad. Sci.* **109,** 13171–13176 (2012).

29. Tezgel, A. Ö. *et al.* Novel Protein Transduction Domain Mimics as Nonviral Delivery Vectors for siRNA Targeting NOTCH1 in Primary Human T cells. *Mol. Ther.* **21,** 201–209 (2013).

30. Ryser, H. J.-P. Uptake of Protein by Mammalian Cells: An Underdeveloped Area: The penetration of foreign proteins into mammalian cells can be measured and their functions explored. *Science* **159,** 390–396 (1968).

31. Green, M. & Loewenstein, P. M. Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* **55,** 1179–1188 (1988).

32. Frankel, A. D. & Pabo, C. O. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **55,** 1189–1193 (1988).

33. Matile, S. & Fyles, T. Transport Across Membranes. *Acc. Chem. Res.* **46,** 2741–2742 (2013).

34. Derossi, D., Chassaing, G. & Prochiantz, A. Trojan peptides: the penetratin system for intracellular delivery. *Trends Cell Biol.* **8,** 84–87 (1998).

35. Wexselblatt, E., Esko, J. D. & Tor, Y. On Guanidinium and Cellular Uptake. *J. Org. Chem.* **79,** 6766–6774 (2014).

36. McKinlay, C. J., Waymouth, R. M. & Wender, P. A. Cell-Penetrating, Guanidinium-Rich Oligophosphoesters: Effective and Versatile Molecular Transporters for Drug and Probe Delivery. *J. Am. Chem. Soc.* **138,** 3510–3517 (2016).

37. Schatz, A., Bugie, E. & Waksman, S. A. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram negative bacteria. *Proc. Soc. Exp. Biol. Med.* **55,** 66–69 (1944).

38. Kumazawa, J. & Yagisawa, M. The history of antibiotics: The Japanese story. *J. Infect. Chemother.* **8,** 125–133 (2002).

39. Becker, B. & Cooper, M. A. Aminoglycoside Antibiotics in the 21st Century. *ACS Chem. Biol.* **8,** 105–115 (2013).

40. Luedtke, N. W., Baker, T. J., Goodman, M. & Tor, Y. Guanidinoglycosides: A Novel Family of RNA Ligands. *J. Am. Chem. Soc.* **122,** 12035–12036 (2000).

41. Elson-Schwab, L. *et al.* Guanidinylated Neomycin Delivers Large, Bioactive Cargo into Cells through a Heparan Sulfate-dependent Pathway. *J. Biol. Chem.* **282,** 13585–13591 (2007).

42. Sarrazin, S., Wilson, B., Sly, W. S., Tor, Y. & Esko, J. D. Guanidinylated neomycin mediates heparan sulfate-dependent transport of active enzymes to lysosomes. *Mol. Ther. J. Am. Soc. Gene Ther.* **18,** 1268–1274 (2010).

43. Dix, A. V. *et al.* Cooperative, Heparan Sulfate-Dependent Cellular Uptake of Dimeric Guanidinoglycosides. *ChemBioChem* **11,** 2302–2310 (2010).

44. Sercombe, L. *et al.* Advances and Challenges of Liposome Assisted Drug Delivery. *Front. Pharmacol.* **6,** (2015).

45. Immordino, M. L., Dosio, F. & Cattel, L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *Int. J. Nanomedicine* **1,** 297–315 (2006).

46. Torchilin, V. P. Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug Discov.* **4,** 145–160 (2005).

47. Gabizon, A., Tzemach, D., Mak, L., Bronstein, M. & Horowitz, A. T. Dose Dependency of Pharmacokinetics and Therapeutic Efficacy of Pegylated Liposomal Doxorubicin (DOXIL) in Murine Models. *J. Drug Target.* **10,** 539–548 (2002).

48. Poly(ethylene glycol) in Drug Delivery: Pros and Cons as Well as Potential Alternatives - Knop - 2010 - Angewandte Chemie 10.1002/anie.200902672

49. Uster, P. S. Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time. *FEBS Lett.* **386,** 243–246 (1996).

50. Cheng, T.-L., Chuang, K.-H., Chen, B.-M. & Roffler, S. R. Analytical Measurement of PEGylated Molecules. *Bioconjug. Chem.* **23,** 881–899 (2012).

51. Shimada, K. *et al.* Determination of incorporated amounts of poly(ethylene glycol)-derivatized lipids in liposomes for the physicochemical characterization of stealth liposomes. *Int. J. Pharm.* **203,** 255–263 (2000).

52. Zhong, Z., Ji, Q. & Zhang, J. A. Analysis of cationic liposomes by reversed-phase HPLC with evaporative light-scattering detection. *J. Pharm. Biomed. Anal.* **51,** 947–951 (2010).

53. Stewart, J. C. M. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal. Biochem.* **104,** 10–14 (1980).

54. Fair, R. J. & Tor, Y. Antibiotics and Bacterial Resistance in the 21st Century. *Perspect. Med. Chem.* **6,** 25–64 (2014).

55. McCoy, L. S., Xie, Y. & Tor, Y. Antibiotics that target protein synthesis. *Wiley Interdiscip. Rev. RNA* **2,** 209–232 (2011).

56. Houghton, J. L., Green, K. D., Chen, W. & Garneau-Tsodikova, S. The Future of Aminoglycosides: The End or Renaissance? *ChemBioChem* **11,** 880–902 (2010).

57. Hainrichson, M., Nudelman, I. & Baasov, T. Designer aminoglycosides: the race to develop improved antibiotics and compounds for the treatment of human genetic diseases. *Org. Biomol. Chem.* **6,** 227–239 (2008).

58. Malik, V., Rodino-Klapac, L. R., Viollet, L. & Mendell, J. R. Aminoglycoside-induced mutation suppression (stop codon readthrough) as a therapeutic strategy for Duchenne muscular dystrophy. *Ther. Adv. Neurol. Disord.* **3,** 379–389 (2010).

59. Wender, P. A., Cooley, C. B. & Geihe, E. I. Beyond Cell Penetrating Peptides: Designed Molecular Transporters. *Drug Discov. Today Technol.* **9,** e49–e55 (2012).

60. Baker, T. J., Luedtke, N. W., Tor, Y. & Goodman, M. Synthesis and Anti-HIV Activity of Guanidinoglycosides. *J. Org. Chem.* **65,** 9054–9058 (2000).

61. Inoue, M., Tong, W., Esko, J. D. & Tor, Y. Aggregation-mediated macromolecular uptake by a molecular transporter. *ACS Chem. Biol.* **8,** 1383–1388 (2013).

62. Esko, J. D., Stewart, T. E. & Taylor, W. H. Animal cell mutants defective in glycosaminoglycan biosynthesis. *Proc. Natl. Acad. Sci.* **82,** 3197–3201 (1985).

63. Luedtke, N. W., Carmichael, P. & Tor, Y. Cellular Uptake of Aminoglycosides, Guanidinoglycosides, and Poly-arginine. *J. Am. Chem. Soc.* **125,** 12374–12375 (2003).