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Structure−Activity Examination of Poly(glycoamidoguanidine)s: Glycopolycations Containing Guanidine Units for Nucleic Acid Delivery

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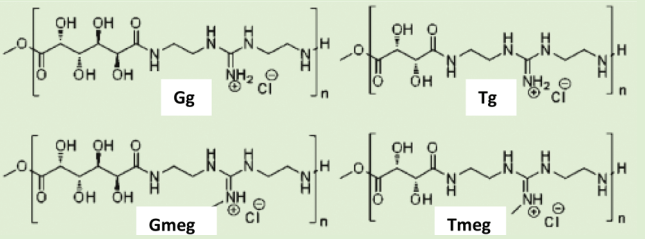
Supporting Information

ABSTRACT: In this study we synthesized a new series of polymers known as poly(glycoamidoguanidine)s (PGAGs). These new polymer structures were synthesized by copolymerizing a carbohydrate monomer (diester; galatarate or tartarate) with a diamine incorporating guanidine or methylguanidine as a charge center to create a polyamide backbone. These materials were strategically designed and compared to our previously studied DNA delivery vehicles, poly(glycoamidoamine)s (PGAAs), which contain secondary amines as the charge groups along the polymer backbone to examine the effect of charge center type on the cellular delivery efficiency of plasmid DNA (pDNA). The guanidine moieties within the PGAGs facilitate electrostatic binding with the negatively charged phosphate backbone of plasmid DNA (pDNA). Stable polymer−pDNA complexes (polyplexes) with sizes in the range of 60−200 nm are formed at polymer/pDNA charge ratios (*N*/*P*) of 5 and above. When the PGAGs are complexed with Cy5-labeled pDNA (Cy5-pDNA) at *N*/*P* ratios of 10 and 25, between 80 and 95% of HeLa cells were positive for Cy5 fluorescence, indicating effective cellular internalization of the polyplexes. The toxicity of both PGAA and PGAG polyplexes was studied via MTT assays, and over 95% cell survival was observed at *N*/*P* ratios of 5, 10, 15, 20, 25, and 30 in HeLa cells. Transgene expression was examined via luciferase assays at various *N*/*P* ratios in the absence and presence of serum. In the absence of serum, the PGAG polyplexes revealed similar transgene expression when compared to polyplexes formed with their analogous PGAA structures. In the presence of serum, one analog (Gg) consisting of galactarate copolymerized with the guanidine monomer yielded gene expression similar to the positive control, Glycofect Transfection Reagent. This new series of guanidine-containing oligomers are promising as a new design strategy to incorporate an alternative charge center type within the backbone of glycopolymer-based nucleic acid delivery vehicles.



INTRODUCTION

The cellular delivery of nucleic acids has been the focus of increasing attention due to the incredible promise of this new therapeutic modality.[1] Many polycation macromolecules are under investigation as non-viral polynucleotide delivery vehicles as they have the ability to bind and compact nucleic acids into nanoparticle complexes termed polyplexes.[2−4] In addition, many studies have shown that polymeric vehicles can facilitate cellular uptake and transport of polynucleotides into various regions of the cell, such as the cytoplasm and nucleus.[5,6] Non-viral delivery vehicles are advantageous as they are easy to synthesize, can carry a high therapeutic payload, and can be easily tailored to display stabilizing and targeting groups to increase circulation time and tissue specific delivery.[6−10]

Our group has focused on the design and development of a novel class of polymers that we have termed poly(glycoamidoamine)s (PGAAs).[3,7,8,11−14] These structures were strategically designed and synthesized to incorporate both oligoethyleneamine and carbohydrate moieties in the repeat units.[3,8,10,15] Numerous analogous structures have been created and examined;[11,12,15,16] several comprehensive studies using a variety of mammalian cell lines have shown that PGAAs exhibit similar high transfection efficiency when compared to the positive control that contains repeated ethyleneamines, polyethyleneamine (PEI), but with significantly reduced toxicity, similar to chitosan, which consists of repeated carbohydrates. Moreover, earlier studies have shown that the carbohydrate groups in our PGAA structures provide a biologically benign attribute, contribute to polyplex stability via hydrogen-bonding to the nucleic acids,[14] and when copolymerized with the amine structures, help to facilitate rapid polymer degradation through hydrolysis of the amide bond.[17,18] Among the library of PGAAs we have created and studied, the compounds with four secondary amines in the oligoethyleneamine unit exhibited the best biological delivery efficiency results. The interaction of the polyplexes with glycosaminoglycans (GAGs) on the cell surface has been shown to be essential for the internalization of these polyplexes.[19,20] We have also performed extensive mechanistic studies to show that, while PGAA polyplexes are internalized via

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clathrin and caveolae-mediated endocytosis, caveolae appear to be the major route leading to nuclear delivery of pDNA for subsequent expression.[21] Although PGAAs have revealed interesting biological behavior, it is essential to have a thorough understanding of the complex factors that dictate the cellular delivery of nucleic acids.[11] To this end, a systematic investigation of structure−bioactivity relationships is critical for the design of more efficient delivery vehicles for therapeutic compounds.

One of the critical biological barriers in non-viral polynucleotide delivery involves the transit of the polyplexes from endosomes to their destination. While the mechanisms of polymer-mediated nucleic acid delivery are still under intense investigation, endosomal release of polyplexes into the cytoplasm is thought to be essential to facilitate high delivery efficiency, particularly in therapeutic modalities that function in the cytoplasm, such as siRNA. A number of theories have been proposed for this process, including pore formation,[22,23] the fusion and flip-flop[24] mechanism, and the proton sponge hypothesis,[25] which is associated with the endosomal release of the polyplexes due to their high buffering capacity. This theory states that, during the acidification event of the early endosomes, the protonation of amines in the polymer backbone provide a buffering capacity, which causes the subsequent influx of H^+ ions to further reduce the pH of these vesicles (and, in turn, it is thought that an influx of Cl^- ions also takes place as a charge neutralization event). As a result of this osmotic imbalance across the endosomal membrane, water rushes in and swells the endosomes, which eventually rupture and release their cargo into the cytoplasm. Although this theory has been widely studied, the various mechanisms involved in this process are debated within this field as much evidence both for and against this theory has been published.[12,26−30]

In the current study, we synthesized a novel group of macromolecules that we have termed poly(glycoamidoguanine)s (PGAGs), which contain guanidine cationic groups copolymerized with a *meso*-galactarate or L-tartarate moiety. These structures were created as a series of oligomers analogous to original PGAAs structures;[3,7,8,11−14,17,18,21] however, we have replaced the "buffering" oligoethyleneamine groups with cationic charges that do not have a buffering capacity. These oligomers were developed to study and compare the structure−property relationships, in particular, the biological effect of changes in amine (PGAAs) versus guanidine (PGAGs) charge type present on the backbone. As discussed earlier, subtle changes in the chemical structure of a delivery vehicle can affect its complexation with DNA and, thus, the biological activity of the complex. Although some polymer vehicles have been reported that contain guanidine functionalities, the cationic groups are typically grafted as a pendant on the polymer backbone (i.e., poly-L-arginine, PLA),[31] or grafted to the ends of the macromolecule vehicle.[32,33] While those polymers have shown to facilitate the cellular delivery of polynucleotides, they also have been shown to vary in their toxicity profile (typically between 60 and 80% cell survival at low *N*/*P* ratios) in vitro.[32,33]

The results of the current study revealed that the low molecular weight PGAG structures created from galatarate or tartarate are able to bind with pDNA and form stable complexes (polyplexes) in the range of 60 to 200 nm. Biological assays with HeLa cells revealed that the polyplexes formed with the PGAGs displayed higher cellular uptake over polyplexes formed with their analogous PGAAs created by copolymerizing galactarate or tartarate with diethylenetriamine. Despite the lack of buffering capacity of the guanidine group, our studies have revealed that the galactaramide and tartaramide-based PGAG polyplexes transfect HeLa cells and yield higher gene expression than their PGAA analogs at low molecular weights. In addition, this study has found that the PGAG polyplexes exhibit nontoxic behavior at a variety of polymer-to-DNA ratios (*N*/*P* ratios), and it could be related to the presence of carbohydrates and the low molecular weight of these materials.

EXPERIMENTAL SECTION

General. Unless specified otherwise, almost all chemicals used in the monomer synthesis were purchased from Sigma Aldrich Chemical Co. and were used without any further purification. Di-*tert*-butyl dicarbonate was purchased from Alfa Aesar Chemical Co. Monomers dimethyl-*meso*-galactarate and dimethyl-L-tartarate and polymers G1 and T1 were synthesized as previously described.[3,7] Glycofect Transfection Reagent was obtained as a gift from Techulon, Inc. (Blacksburg, VA). Poly-L-Arginine (PLA; M_w = 5000−15000 Da) was purchased from Sigma Aldrich Chemical Co. NMR spectra were collected on an Inova MR-400 MHz spectrometer and mass spectra were obtained on an IonSpec HiResESI mass spectrometer. Cell culture media and supplements were purchased from Gibco/Invitrogen (Carlsbad, CA). pCMV-lacZ was labeled with a Cy5 nucleic acid labeling kit (Mirus, Madison, WI) and purified by a QIAquick PCR purification kit (QIAGEN, Valencia, CA). HeLa cells were purchased from ATCC (Rockville, MD). The luciferase assays were completed with a Promega Luciferase Assay Kit (Madison, WI). The toxicity assays were performed with a Bio-Rad DC Protein Assay Kit (Hercules, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen (Carlsbad, CA).

I. Monomer Synthesis. *1,3-Bis(2-((tert-butoxycarbonyl)amino)-ethyl)thiourea (**2**).* Dry methylene chloride (200 mL) was added to a three-neck flask and was brought to −78 °C. Thiophosgene (1.665 mL, 21.84 mmol) was added carefully to this flask under nitrogen. It should be noted that thiophosgene is a highly toxic liquid and this procedure should be completed strictly in a hood with proper safety precautions. A mixture of mono-*N*-Boc-ethylenediamine (**1**; 7.00 g, 43.68 mmol) and diisopropylethylamine (DIPEA; 5.60 g, 43.68 mmol) in 200 mL of dry methylene chloride was added slowly and carefully to the dark orange thiophosgene in methylene chloride over a period of 2 h. This reaction was stirred for 2 h at −78 °C and then warmed to room temperature and stirred for an additional 2 h. After, the reaction mixture was refluxed for another 2 h and then washed with ultrahigh purity water. The organic layer was dried over Na_2SO_4 and the methylene chloride was evaporated in vacuo, yielding a sticky yellow solid. Ethyl acetate (100 mL) was added to this residue, which was then sonicated for 30 min, and the white precipitate was filtered and isolated, washed again with cold ethyl acetate, and dried to yield 5.06 g (13.98 mmol, 64%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 1.44 (s, 18H, $C(CH_3)_3$), 3.33 (m, 4H, CH_2NHCO), 3.54 (m, 4H, CH_2NHCS), 5.14 (br, 2H, NHCO), 6.70−7.06 (br, 2H, NHCS). ^{13}C NMR ($CDCl_3$): δ 28.26, 39.73, 44.78, 79.88, 157.18, 181.99. ESI-MS $[C_{15}H_{31}N_4O_4S]^+$ (m/z): observed, 363.2065; calcd, 363.2061.

*1,3-Bis(2-((tert-butoxycarbonyl)amino)ethyl)-2-methylisothiouronium Iodide (**3**).* Compound **2** (3.00 g, 8.28 mmol) was dissolved in 100 mL of acetonitrile in a round-bottom flask and then a 10-fold molar excess of methyl iodide (11.76 g, 82.80 mmol) was added. This reaction mixture was then stirred at 40 °C for 8 h. Acetonitrile and excess methyl iodide were evaporated in vacuo, and the resulting pure product was dried and characterized to yield 3.84 g (7.62 mmol, 92%) of the final product. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 1.42 (s, 18H, $C(CH_3)_3$), 2.75 (s, 3H, CH_3S), 3.43−3.56 (m, 6H, CH_2CH_2NHCO), 3.54 (m, 2H, $CH_2{}^+NH{=}CS$), 5.58, 5.72 (2s, 2H, NHCO), 8.74, 8.98 (2*s*, 2H, $^+NH{=}CS(CH_3)NH$). ^{13}C NMR ($CDCl_3$): δ 15.27, 28.33, 38.25, 39.30, 45.94, 79.74, 80.59, 156.65, 158.28, 168.28. ESI-MS $[C_{16}H_{34}N_5O_4 - I]^+$ (m/z): observed, 377.2216; calcd, 377.2222.

*1,3-Bis(2-(tert-butoxycarbonylamino)ethyl)-2-methylguanidinium Iodide (**4**).* Compound **3** (1.00 g, 1.98 mmol) was dissolved in 100 mL of chloroform and was stirred at 50 °C. To this mixture, 2 mL of 4.0 M methylamine in tetrahydrofuran (THF) was added every 1 h for the first 4 h After 20 h, the reaction mixture was filtered to remove the precipitated impurity, and then the low boiling compounds were evaporated under reduced pressure to leave a very viscous colorless oil. This oil was dried under vacuum and then weighed to recover 0.93 g (1.90 mmol, 96%) of compound **4**. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 1.43 (s, 18H, $C(CH_3)_3$), 2.99 (s, 3H, $CH_3{}^+NH{=}C$), 3.39 (q, 4H, CH_2NHCO), 3.51 (m, 4H, CH_2NHCS), 5.78 (t, 2H, NHCO), 7.30 (s, 2H, NHCS), 7.32 (s, 1H, $^+NH{=}CS$). ^{13}C NMR ($CDCl_3$): δ 28.32, 28.67, 39.24, 43.27, 80.31, 155.00, 157.95. ESI-MS $[C_{16}H_{34}N_5O_4 - I]^+$ (m/z): observed, 360.2615; calcd, 360.2610.

*1,3-Bis(2-(tert-butoxycarbonylamino)ethyl)-2-guanidinium Iodide (**5**).* Compound **3** (1.00 g, 1.98 mmol) was dissolved in 100 mL of chloroform and this reaction mixture was brought to 40 °C. To this mixture, 2 mL of 7.0 N ammonia in methanol was added every hour for the first 4 h. After 20 h, the reaction mixture was filtered to remove the precipitated impurity, and then the solvent was evaporated in vacuo, yielding a viscous colorless oil. This oil was then dried under vacuum to obtain 0.89 g (1.88 mmol, 95%) of compound **5**. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): 3.33 (m, 4H, CH_2NHCO), 3.40 (m, 4H, CH_2NHCS), 5.44 (s, 2H, NHCO), 7.02 (s, 2H, NHCS), 7.68 (s, 2H, $^+NH_2{=}CS$). ^{13}C NMR ($CDCl_3$): δ 28.36, 39.37, 41.92, 80.21, 156.38, 157.19. ESI-MS $[C_{15}H_{32}N_5O_4 - I]^+$ (m/z): observed, 346.1934; calcd, 346.2449.

*1,3-Bis(2-aminoethyl)-2-methylguanidine Trihydrochloride (**6**).* Compound **4** (0.50 g, 1.03 mmol) was dissolved in 10 mL of trifluoroacetic acid (TFA) and stirred at room temperature. After 2 h, the TFA was evaporated under reduced pressure, which resulted in a dark brown oil. This oil was dissolved in 20 mL of ethanol, to which 2 mL of concentrated hydrochloride was added. A white precipitate resulted that was subsequently filtered and washed with cold ethanol. This product was then dried and characterized. Yield (0.16 g, 0.61 mmol, 59%). ^{1}H NMR (400 MHz, D_2O): δ 2.82 (s, 3H, CH_3N), 3.19 (CH_2NHC), 3.53 (CH_2NH_2). ^{13}C NMR ($CDCl_3$): δ 27.81, 38.07, 38.66, 155.52. ESI-MS $[C_6H_{18}N_5 - Cl]^+$ (m/z): observed, 160.1554; calcd, 160.1562. Purity from NMR: >99%.

*1,3-Bis(2-aminoethyl)-2-guanidine Trihydrochloride (**7**).* Compound **5** (0.50 g 1.06 mmol) was dissolved in 10 mL of trifluoroacetic acid (TFA) and stirred at room temperature. After 2 h, the TFA was evaporated under reduced pressure, which resulted in a dark brown oil. This oil was dissolved in 20 mL of ethanol, to which 2 mL of concentrated hydrochloride was added. A white precipitate resulted that was then isolated via filtration and washed with cold ethanol. The product was then dried and characterized, yield 0.17 g (0.67 mmol, 63%). ^{1}H NMR (400 MHz, D_2O): δ 3.19 (CH_2NHC), 3.53 (CH_2NH_2). ^{13}C NMR ($CDCl_3$): δ 38.06, 38.74, 156.26. ESI-MS $[C_5H_{16}N_5 - Cl]^+$ (m/z): observed, 146.1402; calcd, 146.1406. Purity from NMR: >95%.

II. Polymer Synthesis. *Poly(L-tartaramidodiethyleneamine) (**T1**).*[7] Diethylenetriamine (0.10 g, 0.97 mmol) was weighed out in a round-bottom flask and 0.96 mL (2.0 M) of methanol was added to dissolve the diethylenetriamine. Dimethyl-L-tartarate (0.17 g, 0.97 mmol) was added to the round-bottom flask. This mixture was then stirred for 24 h at room temperature, after which time 5 mL of water was added to this reaction mixture. This reaction mixture was then dialyzed in 1000 Da molecular weight cut off (MWCO) membrane against ultrapure water for 24 h to purify the polymer material from the residual low molecular weight compounds. This reaction mixture was then lyophilized until dry. The resulting polymer was then characterized via GPC (Table 1). Yield 0.087 g (41%, M_w = 1.1 kDa, n = 4).

*Poly(galactaramidodiethyleneamine) (**G1**).*[3] Diethylenetriamine (0.3 g, 2.91 mmol) was dissolved in 58.1 mL (0.1 M) of methanol and dimethyl *meso*-galactarate (0.693 g, 2.91 mmol) was added to this reaction mixture. This reaction mixture was stirred for 24 h. Methanol was evaporated under reduced pressure water to afford a white solid. This solid was dissolved in 10 mL of ultrapure and the mixture was transferred in a 1000 Da MWCO membrane water and was exhaustively dialyzed against ultrapure water for 24 h to remove the relatively smaller MWCO compounds. This mixture was then flash frozen in a scintillation vial and lyophilized to afford a white fluffy solid. The resulting polymer was then characterized via GPC. Yield 0.32 g (38%, M_w = 1.3 kDa, n = 5).

Table 1. Weight Average Molecular Weight (M_w), Polydispersity Index (M_w/M_n), and Degree of Polymerization (n) for the Guanidine and Amine Containing Oligomers

polymer	M_w (kDa)	M_w/M_n	n
Gg	1.3	1.2	4
Gmeg	1.3	1.3	4
Tg	1.3	1.2	5
Tmeg	1.4	1.2	5
G1	1.3	1.3	5
T1	1.1	1.3	4

*Poly(galactaramidodiethylenemethylguanidine) (**Gmeg**).* Compound **6** (0.15 g, 0.56 mmol) was dissolved in 2 mL of methanol. To this, 400 μL of triethylamine (TEA) was added in 5 × 0.556 mmol aliquots while stirring over a period of 15 min to obtain a homogeneous mixture. Next, dimethyl *meso*-galactarate (0.13 g, 0.56 mmol) was added, and the reaction was stirred at room temperature for 48 h. The reaction mixture was then concentrated under reduced pressure. Ultrapure water (5 mL) was added to the reaction mixture and it was then pipetted into a 1000 Da MWCO membrane and exhaustively dialyzed for 24 h against ultrapure water to remove lower molecular weight impurities. This mixture was then flash frozen in a scintillation vial and was lyophilized to afford a sticky white solid. The resulting polymer was then characterized via GPC. Yield 0.073 g (36%, M_w = 1.3 kDa, n = 4).

*Poly(L-tartaramidodiethylenemethylguanidine) (**Tmeg**).* Compound **6** (0.15 g, 0.56 mmol) was dissolved in 1 mL of methanol in a round-bottom flask. Triethylamine (400 μL) was added to this mixture in 5 × 0.56 mmol aliquots and stirred for 15 min to obtain a homogeneous mixture. Next, dimethyl L-tartarate (99.1 mg, 0.56 mmol) was added and stirred for 120 h at room temperature. The methanol was then removed under reduced pressure to afford a white solid and the product was dissolved in 5 mL of ultrapure water, transferred to a 1000 Da MWCO membrane, and was exhaustively dialyzed against ultrapure water for 24 h. This mixture was then flash frozen in a scintillation vial and lyophilized to afford a sticky yellowish solid. The resulting polymer was then characterized via GPC. Yield 0.043 g (23%, M_w = 1.4 kDa, n = 5).

*Poly(galactaramidodiethyleneguanidine) (**Gg**).* Compound **7** (0.10 g, 0.39 mmol) was added to a round-bottom flask and dissolved in 2 mL of methanol. To this mixture, 275 μL of triethylamine (TEA) was added in 5 × 0.393 mmol aliquots while stirring over the period of 15 min to obtain homogeneous reaction mixture. Next, dimethyl *meso*-galactarate (0.094 g, 0.39 mmol) was added and the reaction was stirred for 48 h at room temperature. The reaction mixture was concentrated under reduced pressure. Ultrapure water (5 mL) was then added to this reaction mixture and the solution was transferred in a 1000 Da MWCO membrane and was exhaustively dialyzed against ultrapure water for 24 h to remove low molecular weight impurities. This mixture was then flash frozen in a scintillation vial, after which the remaining water was lyophilized to afford a sticky white solid. The resulting polymer was then characterized via GPC. Yield 0.043 g (31%, M_w = 1.3 kDa, n = 4).

*Poly(L-tartaramidodiethyleneguanidine) (**Tg**).* Compound **7** (0.15 g, 0.59 mmol) was dissolved in 1 mL of methanol in a 5 mL round-bottom flask. Triethylamine (425 μL) was added in 5 × 0.59 mmol aliquots and

stirred for 15 min to obtain a homogeneous mixture. Next, dimethyl L-tartarate (105.5 mg, 0.59 mmol) was added and the reaction mixture was stirred for 120 h. Next, the methanol was removed under reduced pressure to afford an off-white solid. To purify, the solid was dissolved in 5 mL of ultrapure water and transferred into a 1000 Da MWCO membrane and exhaustively dialyzed against ultrapure water for 24 h. This mixture was then flash frozen in a scintillation vial and lyophilized to afford a sticky yellowish solid. The resulting polymer was then characterized via GPC. Yield 0.063 g (36%, M_w = 1.3 kDa, n = 5).

III. Polymer Characterization. Molecular weight and polydispersity were characterized using a Viscoteck GPCmax with a GMPWXL column coupled to a triple detector (static light scattering, viscometry, and refractive index). A solution of 0.5 M sodium acetate in 80:20 water to acetonitrile was used as the mobile phase. The pH of the mobile phase was adjusted to pH 7 by adding acetic acid. Sample preparation was done by dissolving 2 mg of polymer sample in 1 mL of mobile phase, and injecting 100 μL of this solution onto the column at a flow rate of 0.6 mL/minute.

IV. Polyplex Characterization. *Gel Electrophoresis Shift Assays.* The binding between **Gg**, **Gmeg**, **Tg**, and **Tmeg** polymers and pDNA was tested via gel electrophoresis under 60 V for 60 min. Agarose gel (0.6% w/v) was created using 1× TAE buffer (IBI Scientific, IA), and ethidium bromide (0.6 μg/mL) was added to the contained gel (Invitrogen, CA). Plasmid DNA (pCMV-*lacZ*) was diluted to create a 0.1 mg/mL stock solution with nuclease-free water (Gibco). Stock solution of every polymer and control were made with nuclease free water at N/P 50 (where N = number of guanidine groups (for PGAGs) or secondary amines (for PGAAs) on polymer, P = number of phosphate groups on DNA) based on 0.1 mg/mL pDNA solution). Polymer stock solutions were further diluted to the desired N/P ratios using ultrapure water. Polymer/pDNA complexes (polyplexes) were then formulated at various N/P ratios between 0 (pDNA only) to 30. To create the polyplexes at different N/P ratios, 10 μL of each polymer solution (diluted from the stock) was added to 10 μL of pDNA solution. The polyplex solutions were then incubated for 60 min before 2 μL loading buffer (Blue Juice) was added. A 15 μL aliquot of each polyplex solution was loaded onto the gel for electrophoresis testing. The pDNA migration was visualized by exciting ethidium bromide at 322 nm UV light. The gels were visualized and photographed with a FOTO/FX gel digital imaging system (FOTODYNE, WI).

Dynamic Light Scattering. The hydrodynamic diameter of the polyplexes were measured in triplicate at 25 °C (633 nm and detection angle of 173°) on a Zetasizer (Nano ZS) dynamic light scattering instrument (Malvern Instruments, Malvern, U.K.). Polyplexes were formed at N/P ratios between 2 and 30 by mixing each aqueous polymer solution (150 μL in H_2O) with pCMVβ (plasmid factory, Germany; 150 μL, 0.02 μg/μL in H_2O) and allowing the solution to sit for 40 min. The average size of the polyplexes and standard of deviation are reported for each polyplex solution in Figure 2 and Figures S3 and S4.

Transmission Electron Microscopy. Polymer−pDNA complexes were prepared at N/P = 20, as described above for the dynamic light scattering studies. Samples (5 μL of the polyplex solution in water) were applied in duplicate to 400 mesh carbon-coated grids (EMS, Fort Washington, PA) and incubated for 60 s. Excess liquid was removed by blotting with a kimwipe. Samples were negatively stained with uranyl acetate (2%, w/v) for 90 s and again blotted with a kimwipe. TEM images were recorded with a JEOL JEM-1230 transmission electron microscope operated at 60 kV and the images have been presented in the Supporting Information (Figure S5).

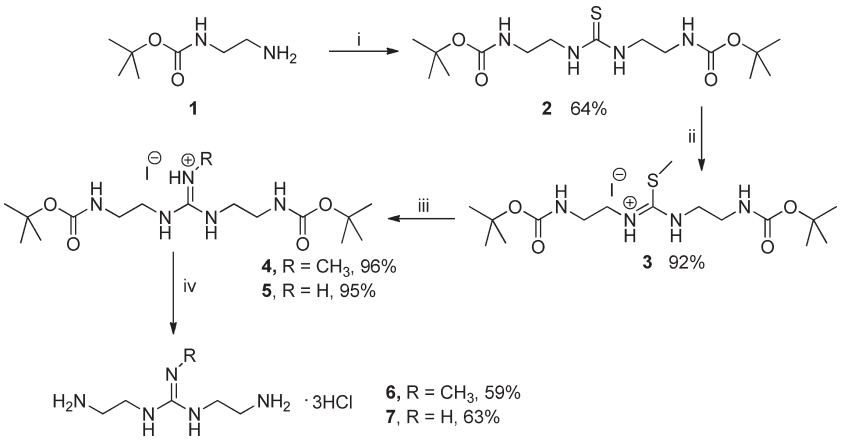
V. Cell Culture Experiments. *General.* HeLa (human cervix adenocarcinoma) cells were purchased from ATCC. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 50 units/mL of penicillin, and 50 μg/mL of streptomycin in 5% CO_2 at 37 °C. Plasmid DNA including pCMV-*lacZ* and luciferase DNA (pCMV-*luc*) were purchased from PlasmidFactory (Germany). All experiments used an identical passage of cells and the same batch of reagent; all samples were prepared with Gibco nuclease-free water and all materials were autoclaved or treated under UV light for 15 min to ensure aseptic conditions and reproducible results.

Luciferase and Protein Assays in Serum-free Culture Medium. HeLa cells were cultured in a 24-well plate with a density of 5×10^4 cells/well and were incubated for 24 h prior to transfection. To form the polyplexes, 150 μL of an aqueous solution of each PGAG (**Gg**, **Gmeg**, **Tg**, and **Tmeg**) or positive control polymer (**G1**, **T1**, poly-L-arginine (PLA), and Glycofect Transfection Reagent) were added into 150 μL pCMV-*luc* DNA (0.02 mg/mL) at N/P ratios of 5, 10, 15, 20, 25, and 30 at room temperature. The solutions were allowed to incubate for 60 min to allow polyplex formation, after which 600 μL of serum-free medium (Opti-MEM) was added to each Eppendorf containing the polyplex solutions and mixed gently with a pipet. The HeLa cells were washed with 0.5 mL PBS, after which 300 μL of polyplex solution (containing 1 μg pDNA and the designated N/P ratios of polymer) was added to the cells in each well. Untranfected cells and cells transfected with pCMV-luc pDNA only were used as the negative controls. Experiments were completed in triplicate. Four hours after initial transfection, 800 μL of DMEM solution containing 10% FBS was added to each well. A total of 24 h after initial transfection, the medium in each well was replaced with 1 mL of fresh DMEM containing 10% FBS. A total of 48 h after initial transfection, cells were washed with PBS and lysed with 100 μL of 1× cell culture lysis buffer (Promega). A Bio-Rad DC protein assay kit was used to determine the amount of protein in the cell lysates. For luciferase activity, the cell lysates were analyzed by adding luciferase substrate and measuring the relative light units. Luminescence was measured over 10 s with a luminometer (GENios Pro, TECAN US, Research Triangle Park, NC).

Luciferase and Protein Assays in Culture Medium Containing Serum. The luciferase and protein assays in the presence of DMEM containing 10% serum carried out via an identical protocol to the serum-free study with the exception that 600 μL of DMEM containing 10% FBS was added directly to the polyplex solutions and the transfections were completed solely in the presence of this media. Also, only two representative N/P ratios (10 and 25) were used for transfection studies with the PGAGs and positive control polymers in the medium containing serum.

MTT Assay. HeLa cells were cultured in a 24-well plate with a density of 5×10^4 cells/well and incubated for 24 h prior to transfection. Polyplexes were formed by pipetting 150 μL of the PGAG or positive control polymers (**Gg**, **Gmeg**, **Tg**, **Tmeg**, **G1**, **T1**, PLA, or Glycofect Transfection Reagent) into 150 μL of pCMV-*lacZ* DNA (0.02 mg/mL) and pipetting up and down to mix the solutions. The polyplexes were formed via this method at N/P ratios of 5, 10, 15, 20, 25, and 30 at room temperature. The polyplex solutions were allowed to sit for 60 min prior to addition of 600 μL of serum-free medium (Opti-MEM). HeLa cells were washed with 0.5 mL of PBS, after which 300 μL of each polyplex solution (containing 1 μg DNA and different N/P ratios of each polymer) was added to the cells in each well. Untransfected cells and cells transfected with pCMV-luc pDNA only were used as negative controls. Each experiment was completed in triplicate. A total of 4 h after initial transfection, 800 μL 10% FBS DMEM solution was added to each well. A total of 24 h after initial transfection, the cells in each well were washed with 0.5 mL of PBS. Then 0.5 mL of a 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 10% FBS DMEM solution was added to each well. After incubation at 37 °C for 1 h, the cells in each well were washed with 0.5 mL of PBS and lysed with 600 μL of DMSO. Cell lysates were shaken for 20 min in darkness. A 200 μL aliquot of cell lysate was loaded to a 96-well plate for colorimetric measurement. Samples were measured with a spectrophotometer (GENios Pro, TECAN US, Research Triangle Park, NC) with a wavelength of 570 nm. Cell viability profiles were characterized by

Scheme 1. Synthesis of the Guanidine Containing Monomers[a]



[a] Reagents and conditions: (i) Thiophosgene, DIPEA, −78 °C reflux, $CHCl_3$; (ii) methyl iodide, 40 °C, acetonitrile; (iii) 4.0 M methylamine and THF for **4** and 7.0 N ammonia in methanol for **5**; (iv) TFA and wash with conc. HCl in EtOH.

absorbance results. Cell viability of transfected cells was normalized to that of untransfected cells.

Flow Cytometry. HeLa cells were cultured in a 6-well plate with a density of 2.5×10^5 cells/well and were incubated for 24 h prior to transfection. Polyplexes were formed by adding 250 μL of each polymer and control (**Gg**, **Gmeg**, **Tg**, **Tmeg**, **G1**, **T1**, PLA, and Glycofect Transfection Reagent) into 250 μL of Cy5-labeled pCMV-*lacZ* DNA (excited at 633 nm) (0.02 mg/mL). Polyplexes were formed at *N*/*P* ratios of 10 and 25 at room temperature and allowed to sit for 60 min at room temperature. After this incubation period, 1 mL of serum-free medium (Opti-MEM) was added. HeLa cells were washed with 1 mL of PBS and 1.5 mL of polyplex solution (containing 5 μg DNA and each polymer to examine the effect of *N*/*P* ratios of 10 and 25) was added to cells in each well. Untransfected cells and cells transfected with pDNA only were used as negative controls for the cellular uptake study. Cells treated with PLA and Glycofect Transfection Reagent were used as the positive controls and all of the experiments were completed in duplicate. A total of 2 h after initial transfection, cells were rinsed with PBS several times to remove any polyplexes bound to the cell surface. After, 1 mL of 10% FBS DMEM was then added to allow further endocytosis for 30 min. A total of 4.5 h after initial transfection, cells were washed with PBS, trypsinized, and pelleted, the supernants were removed, and the cells were resuspended in PBS for flow cytometry analysis. Cells positive for Cy5 (and the average Cy5 intensity) were analyzed with a FACSCanto II (Becton Dickenson, San Jose, CA). Approximately 20000 events were recorded.

RESULTS AND DISCUSSION

Herein, we have synthesized and studied a new polymer series we have termed poly(glycoamidoguanidine)s (PGAGs) that have been created as analogues to our previous PGAAs. To examine the biological effects of the charge center, we have replaced the amines in our original PGAA structures with guanidine and methylguanidine groups. The guanidine group has a high pK_a value of around 12.5 (dependent on the substituent) and is consistently protonated at physiological pH, as opposed to the amine groups in the PGAAs; in the case of **T1**, only about 50% of the secondary amines are protonated at physiological pH.[14] Because subtle changes in the chemical structure[34,35] of the delivery vehicle can largely impact the resulting biological properties of a polymer, the different charge types in the PGAAs and PGAGs can heavily impact the biological properties, such as polyplex formation,[14,16] polyplexes internalization[19] into cells, intracellular trafficking, and possible release from the endosome. Also, according to the proton sponge hypothesis, the presence of PGAGs in a polyplex should not provide buffering capacity in the endosomes, thereby supposedly hindering endosomal release, and our study seeks to explore how these structural changes alter the delivery profiles.

Monomer and Polymer Synthesis. Poly(glycoamidoguanidine)s (PGAGs) were designed based on a similar aminolysis chemistry used previously for synthesizing the PGAAs. However, because the diamine with a guanidine moiety in the architecture was not commercially available, we used similar synthetic methods related to those developed by Sambrook et al.[36] As shown in Scheme 1, mono-*N*-Boc-ethylenediamine (**1**) synthesized according to an earlier reported procedure[37] was reacted carefully with thiophosgene at −78 °C in the presence of diisopropylethylamine (DIPEA). The reaction mixture was warmed to room temperature and then refluxed. After purification, the thiourea compound (**2**) was obtained and was subsequently reacted with methyliodide, yielding the methyl thiourea species (**3**). The methyl thiourea was then treated with either ammonia or methylamine, which afforded the desired methyl guanidine (**4**) and guanidine (**5**) compounds, respectively. Both compounds (**4** and **5**) were then treated with trifluoroacetic acid (TFA) to remove the Boc groups and liberate the amines. The final methyl guanidine-containing (**6**) and guanidine (**7**) monomers were obtained by dissolving the materials in ethanol and precipitating out the structures by treating each respective solution with concentrated HCl.

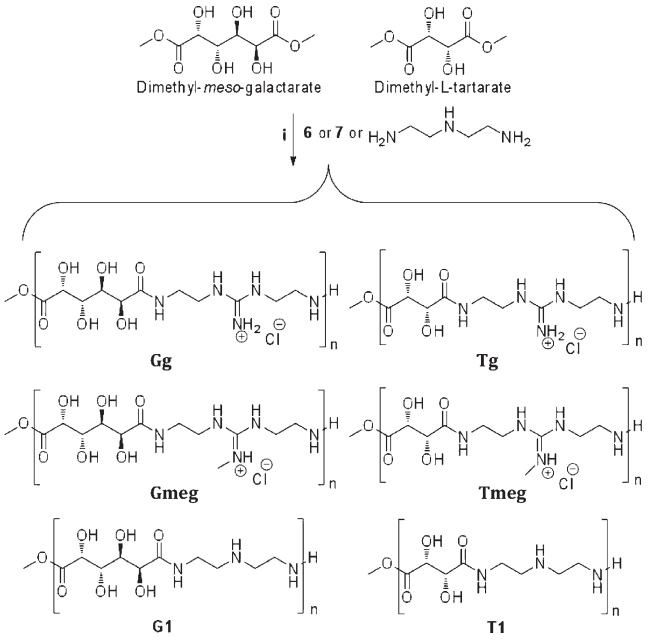
The PGAG oligomers were synthesized by reacting the guanidine (**g**, **7**) or methylguanidine (**meg**, **6**) moieties with dimethyl *meso*-galactarate (**G**) or dimethyl-L-tartarate (**T**) to yield four structures (Scheme 2). To synthesize the galactarate polymers, monomers **6** or **7** were each dissolved in methanol separately in the presence of TEA and then reacted with dimethyl *meso*-galactarate for 48 h.

The tartarate series was synthesized by reacting monomers **6** and **7** with dimethyl-L-tartarate under similar conditions for 120 h. After polymerization of each mixture, the reaction mixtures were each dialyzed with a 1000 MWCO membrane against ultrapure water for 24 h to purify the polymers from unreacted monomer and each product was characterized via GPC. The PGAAs with one secondary amine created with galactarate or tartarate monomers (**G1** and **T1**) were also synthesized to obtain similar short oligos to more accurately compare the effects of the

charge center on the biological properties and to ensure that the differences observed in the biological activity are related to the charge center alterations. Table 1 reveals the characterization data for each of the polymers created for this study.

Polyplex Characterization. *Gel Electrophoresis Shift Assay.* The ability of each PGAG polymer to bind with the pDNA was examined using a gel electrophoresis shift assay to observe the inhibition of pDNA migration with increasing the polymer concentration (N/P ratio). Polyplexes were prepared at a variety of N/P ratios from zero (pDNA only) to 30 and loaded onto the gel. As shown in Figure 1, at N/P zero (pDNA only) pDNA migrates toward the positive electrode and all four of the guanidine polymers were shown to bind with pDNA and inhibit gel migration at low N/P ratios of 2.5 or lower. Gel electrophoresis shift assays were conducted with the guanidine monomers (compound **6** and **7**) and binding of the monomers only to pDNA was not observed (see SI). For this reason, further transfection experiments were not performed using the monomes only.

Scheme 2. Polymerization of the Diamine Monomers, Such As 6, 7, or Diethylenetriamine with Dimethyl-*meso*-galactarate or Dimethyl-L-tartarate[a]



[a] Reagents: (i) TEA, MeOH.

The analogous PGAA polymers, **T1** and **G1**, showed pDNA migration inhibition at N/P of 10 or higher. Thus, at similar degrees of polymerization, the guanidine-containing oligomers (PGAGs) exhibited a higher affinity for pDNA binding compared to the amine-containing polymers (PGAAs). The galactarate-based, guanidine-containing polymers (**Gg** N/P = 1.5, **Gmeg** N/P = 2) were shown to bind pDNA at a lower N/P ratio as compared to the tartarate-based polymers (**Tg** N/P = 2,

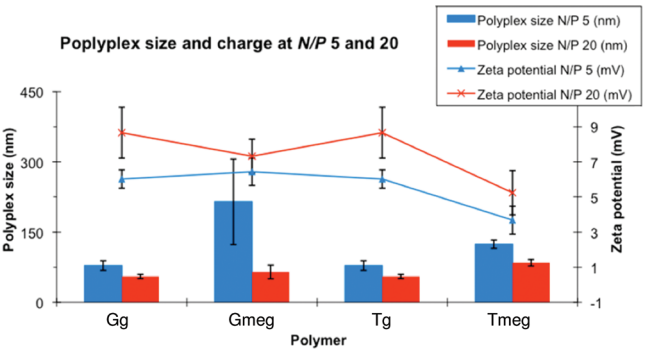


Figure 2. PGAG polyplex size and zeta potential. PGAG polymers were dissolved in water and complexed with pDNA at N/P = 5 and 20. Polyplex data for N/P ratios from 0 (pDNA only) up to 30 (increasing the polymer ratio) is available in the Supporting Information.

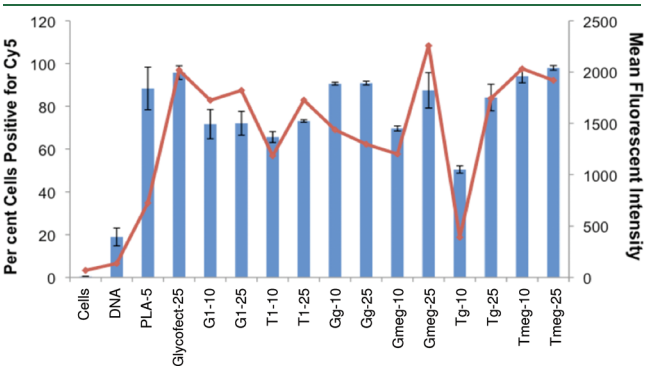


Figure 3. HeLa cell uptake of Cy5-pDNA delivered with the PGAG and PGAA polymers complexed with pDNA at N/P ratios of 10 and 25. Cells only and DNA only are used as negative controls and Glycofect Transfection Reagent (N/P 25) and poly-L-arginine (PLA) were used as the positive controls. The bars represent the percentage of cells positive for Cy5 fluorescence; the line represents the mean fluorescence intensity of the cells.

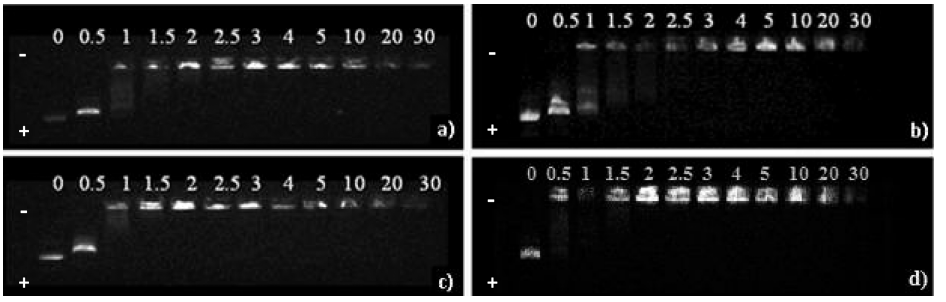


Figure 1. Gel electrophoresis shift assay for the PGAGs. Each polymer was complexed with pDNA in different N/P ratios between 0 (pDNA only, migrates with the electrophoretic field) and 30. These data show that (a) **Gmeg** binds with pDNA at N/P = 2, (b) **Tmeg** binds with pDNA at N/P = 2.5, (c) **Gg** binds with pDNA at N/P = 1.5, and (d) **Tg** binds pDNA at N/P = 2.

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Tmeg $N/P = 2.5$). This supports our previous data showing that the galactarate moiety appears to play a role at enhancing the polymer binding affinity to pDNA. In addition, the PGAGs with a methyl guanidine group (**Tmeg** and **Gmeg**) bound pDNA at a higher N/P ratio when compared to the PGAGs with a guanidine group, indicating that the methyl group may slightly weaken the binding affinity due to a lower electrostatic interaction. Interestingly, the zeta potential data supports this hypothesis (see Supporting Information).

Polyplex Size. Polyplex size was characterized via dynamic light scattering (DLS) at various N/P ratios between 2 and 20. Figure 3 shows the data for polyplexes formed with each of the new PGAGs at $N/P = 5$ and 20 (data for all N/P ratios can be found in the Supporting Information). The average size of the polyplexes at a low N/P ratio of 2 was approximately 600 nm likely due to the incomplete plasmid compaction (and negative zeta potential, vide infra). However, at an N/P ratio of 5, the polyplex size significantly decreased to between 78 and 124 and remained consistent at this size, with the exception of polyplexes formed with **Gmeg**, which revealed larger sizes of around 200 nm. The polyplex size of **Gmeg** at N/P 20 was also determined using TEM (see Figure S5 in the Supporting Information), and it was found that these polyplexes might be forming dimers, which could be the reason for the observed DLS measurements. Interestingly, **G1** and **T1** polyplexes revealed large particle sizes of between 400 and 700 nm even at higher N/P ratios, revealing that these materials may not fully compact pDNA into polyplexes effectively (see Supporting Information). However, the positive controls, PLA and Glycofect, were both very effective at compacting pDNA into polyplexes (Supporting Information). The disparity in polyplex size when comparing the small PGAG polyplexes to the complexes formed with the PGAAs is likely due to differences in the electrostatic interaction of these short oligomers with pDNA (PGAGs are more cationic and appear to promote a higher binding affinity at lower N/P ratio).

Zeta Potential. As shown in Figure 2 (and the Supporting Information), the zeta potential of the PGAG polyplexes in an aqueous solution was dependent on the N/P ratio of formulation. At a low N/P ratio of 2, the polyplex zeta potential values were all neutral or slightly positive (0 to +2 mV), with the exception of **Gmeg** complexes (−7 mV). At polyplex formulations greater than 2, the polyplexes all resulted in a moderately positive surface charge on the polyplexes (4−12). Polyplexes formed with **Tmeg** yielded the lowest zeta potential values, which were near neutral (+5 mV), even at $N/P = 20$; this result supports the gel shift assays that had the highest N/P ratio of complexation. Interestingly, with the controls, **T1** exhibited lower zeta potential values at N/P ratios of both 10 and 25 (Supporting Information).

Cell Culture Experiments. *Cellular Uptake and Transgene Expression Studies.* To examine the cellular uptake of the PGAG-based polyplexes (**Gg**, **Gmeg**, **Tg**, **Tmeg**) and compare the results to the controls cells, pDNA, poly-L-lysine (PLA, $N/P = 5$), Glycofect Transfection Reagent ($N/P = 25$), **G1**, and **T1** ($N/P = 10$ and 25), a flow cytometry assay was carried out to ascertain both the number of cells positive for Cy5-labeled pDNA and the average intensity of Cy5 florescence in the cells. PGAG polyplexes were formed at $N/P = 10$ and 25, and HeLa cells were incubated with each polyplex solution and control for 2 h, after which, the cells were washed multiple times with PBS, trypsinized, pelleted, washed with PBS, and pelleted. This procedure was repeated several times to remove surface bound polyplexes for accurate flow cytometry characterization. Results

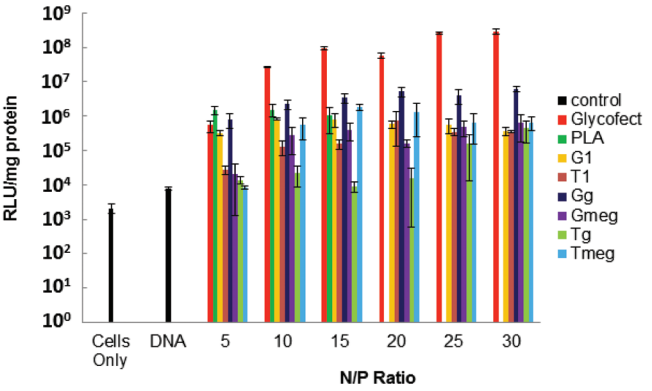

Figure 4. Luciferase gene expression observed with polyplexes formed with the PGAGs, PGAAs, and the positive controls, Glycofect Transfection Reagent, and poly-L-arginine Opti-MEM (reduced serum culture medium). The polyplexes are formed at different N/P ratios from 5 to 30. The gene expression values are shown as relative light units (RLU/mg of protein). The data is reported as the mean and standard of deviation of three replicates and the transfection data has been normalized to the controls.

are shown in Figure 3 for the percentage of cells positive for Cy5 fluorescence, as well as for mean fluorescence intensity. Specifically, only 20% of the cells were positive for Cy5 fluorescence with pDNA only. All of the polymers (PGAGs, PGAAs, and positive controls) facilitated an increase in the uptake of pDNA in HeLa cells over the negative controls. It is also interesting to note that the PGAGs promoted similar cellular uptake of pDNA as the positive controls, Glycofect, a glycopolymer that consists of repeated galatarate residues along with four ethyleneamines and poly-L-arginine (PLA), a polypeptide consisting of repeated pendant guanidine charge centers, and also revealed higher cellular uptake than the other PGAA analogues (**G1** and **T1**). Polyplexes formed with **G1** and **T1** were internalized by about 70% of cells, while the PGAGs promoted higher cellular internalization, where, on average, 90% of the cells were positive for Cy5-pDNA (with the exception of **Tg** polyplexes at $N/P = 10$). While the uptake of the **Tg** polyplexes at $N/P = 10$ is low, we currently do not understand this result. When comparing the mean Cy5 intensity per cells, surprisingly, the **Tg** ($N/P = 10$) and the control PLA polyplex samples revealed the lowest intensity values, indicating that these formulations do not facilitate effective nucleic acid transfer into cells. On the high end, both of the methylated guanidine analogs, **Gmeg** ($N/P = 25$) and **Tmeg** (N/P 10 and 25) yielded high Cy5 internalization, similar or higher than Glycofect. This result was very interesting and prompted our comparison of the gene expression profile for these systems.

Luciferase Assays in Serum and Serum-Free Media. Luciferase reporter gene expression assays were carried out in order to characterize how efficiently the guanidine-containing oligomers can deliver pDNA to HeLa cells and promote transgene expression. Similar to the flow cytometry studies, two positive controls were used, Glycofect Transfection Reagent and poly-L-arginine. The luciferase reporter gene delivery experiments were carried out both in the absence and presence of serum (Figures 4 and 5, respectively) for each polyplex type and control and the data revealed some interesting trends. In the absence of serum (Opti-MEM), it was found that transgene expression with the PGAG polyplexes, increased with an increase in the N/P ratios from 5 to 30. Overall, of the new polymers examined, **Gg** was found to yield

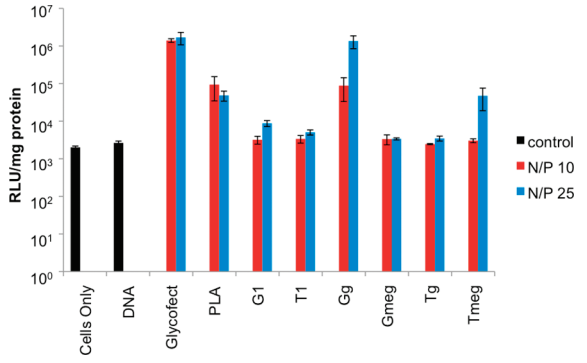

Figure 5. Luciferase gene expression observed with polyplexes formed with the PGAGs, PGAAs, and the positive controls Glycofect Transfection Reagent and poly-L-arginine in DMEM containing 10% serum. The polyplexes are formed at *N*/*P* ratio 10 and 25. The gene expression values are shown as relative light units per milligram of protein (RLU/mg). The data is reported as the mean and standard of deviation of three replicates.

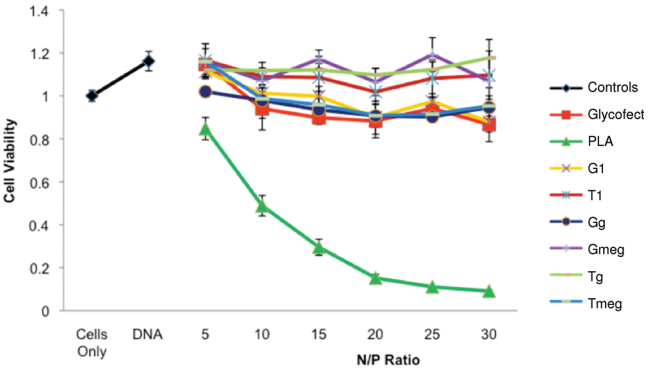

Figure 6. HeLa cell viability following a 24 h exposure to polyplexes formed with pDNA and each PGAG, PGAAs, Glycofect Transfection Reagent, and poly-L-arginine at different *N*/*P* ratios from 5 to 30 in reduced serum culture medium. The fraction cell survival is normalized to the untransfected cells and pDNA only was also used as an experimental control.

the highest transgene expression within this series. Also, at lower *N*/*P* ratios, the galactarate polymers **Gg** and **G1** revealed the highest gene expression (similar to PLA), which correlates to our findings that the galactarate units in the polymer appear to enhance interactions with pDNA and promote stable polyplex formation.[14] As the *N*/*P* ratio is increased, transgene expression with the tartarate polymers (in particular, **Tmeg**) increases. Overall, Glycofect revealed the highest gene expression in serum-free media. PLA revealed a similar gene expression profile to the PGAGs and PGAAs (**G1** and **T1**), however, above an *N*/*P* ratio of 15, total cell death was observed and expression values were not able to be recorded.

Figure 5 shows the transgene expression data for the PGAG and control polyplexes formed at *N*/*P* ratios of 10 and 25 in the presence of serum. At both *N*/*P* ratios, compounds **G1**, **T1**, **Gmeg**, **Tg**, and **Tmeg** (*N*/*P* 10 only) revealed minimal luciferase expression (similar to the negative controls). However, polyplexes formed with **Gg**, particularly at *N*/*P* = 25, revealed high gene expression similar to Glycofect and higher than PLA. This result is intriguing because the guanidine oligomers are completely protonated at physiological pH and therefore do not have a buffering capacity, however, **Gg** revealed gene expression values similar to Glycofect, which has secondary amines in the polymer backbone. These results do not agree with the proton sponge hypothesis and suggest that the galactarate residues may play a role in the transfection. Interestingly, when comparing the positive controls, PLA revealed lower gene expression than Glycofect Transfection Reagent with Hela cells, and this result is likely related to its high toxicity (polyplexes formulated at *N*/*P* ratios higher than 15 caused complete cell death in the experiment, which is why the gene expression data is not available in Figure 4 for these *N*/*P* ratios). Overall, polyplexes formed with **Gg** appeared to be the most effective pDNA delivery vehicles of the PGAG oligomer series. This agent promoted binding at the lowest *N*/*P* ratio (1.5), the smallest polyplex sizes, and the highest number of cells positive for Cy5-pDNA of all the PGAG structures created and studies here. It is also interesting to note that **Gg** had a higher gene expression level than **Gmeg**, while a reverse trend was observed for the tartarate-based, guanidine-containing polymers; interestingly**Tmeg** promoted luciferase expression at *N*/*P* = 25, where **Tg** polyplexes were complexly inactive. These data indicate that both the charge center and the carbohydrate type play a large role in the delivery of pDNA to the cellular nucleus for transgene expression.

MTT Assay. The cell viability was characterized via an MTT assay using HeLa cells. As shown in Figure 6, all of the PGAG and PGAA polyplexes exhibited low toxicity, as more than 90% of the cells were viable at *N*/*P* ratios ranging from 5 to 30 over the 48 h experimental time course. The positive control, Glycofect Transfection Reagent, showed similar low toxicity. PLA, however, revealed a very high cytotoxic response. Even though it displayed 80% cell viability at an *N*/*P* ratio of 5, cell viability drastically decreased to about 16% as the *N*/*P* ratio increased to 20 and above. The large difference in cytotoxic response when comparing the PGAGs to PLA is likely 2-fold: (i) the low molecular weight of the PGAG oligomers likely plays a major role in their low cytotoxic profile and (ii) the presence of a carbohydrate group could also be a factor in promoting a biocompatible response.

CONCLUSIONS

Herein, a new series of carbohydrate-containing nucleic acid carriers [poly(glycoamidoguanidine)s or PGAGs] have been synthesized by copolymerizing either dimethylgalatarate or dimethyl-L-tartarate with monomers containing either a guanidine or methyl-guanidine charge center. These polymers have been created as analogs to the extensively studied previous systems that contain oligoethyleneamine charge centers (PGAAs) to compare the effect of charge center type on the biological properties of these glycopolymers. The PGAG vehicles bound pDNA at lower *N*/*P* ratios when compared to their PGAA analogs (**T1** and **G1**) and readily formed polyplexes with pDNA at sizes approximately around 100 nm, as characterized from gel electrophoresis shift assay and DLS studies, respectively. Interestingly, the PGAG oligos were not cytotoxic and all analogs revealed high cellular uptake and transgene expression in HeLa cells as compared to their amine-containing analogs (**G1** and **T1**). It is interesting to note that others have reported that guanidine groups within the backbone of polymer vehicles can possibly assist in the cellular entry of nanoparticles.[38,39] This study also revealed that while the PGAGs do not provide any buffering capacity in the endosomal pH range, two of the derivatives, **Gg** and **Tmeg**, promoted higher transgene expression values than their amine-containing analogs

T1 and **G1**. While we still do not fully understand these intriguing results, we have found that a number of our polymer systems do not strictly obey the proton sponge hypothesis, as analogous PGAA polymers (with a higher buffering capacity in the cellular pH range) do not necessarily lead to higher transgene expression.[12] To this end, extensive studies in our lab are currently focused on understanding how the polymer structure affects the routes of polyplex intracellular trafficking and results of these studies will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information. Gel electrophoresis shift assays for polymers **T1**, **G1**, and the guanidine monomers (compounds **6** and **7**), dynamic light scattering and zeta potential data for all polyplexes, transmission electron micrograph of **Gmeg** polyplexes, ^{1}H and ^{13}C NMR and MS data for all monomers. This material is available free of charge via the Internet at http://pubs.acs.org.

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