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Poly(glycoamidoamine) Vehicles Promote pDNA Uptake through Multiple Routes and Efficient Gene Expression via Caveolae-Mediated Endocytosis

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Abstract: The use of synthetic polymers for the delivery of nucleic acids holds considerable promise for understanding and treating disease at the molecular level. This work aims to decipher the cellular internalization mechanisms for a series of synthetic glycopolymer DNA delivery vehicles we have termed poly(glycoamidoamine)s (PGAAs). To this end, we have performed cellular delivery experiments in the presence of pharmacological endocytosis inhibitors. Confocal microscopy analysis showed colocalization of labeled pDNA in polyplexes with immunolabeled endocytic molecules to identify the cellular internalization pathways in HeLa cells. Direct membrane penetration was also investigated through various methods, including cellular energy depletion and leakage of a cytosolic enzyme from the cell. The data suggests that the cellular internalization of PGAA polyplexes occurs through a multifaceted internalization mechanism primarily involving caveolae, yet clathrin-coated vesicles and macropinosomes were also involved to a lesser degree. The primary mechanism that leads to efficient nuclear delivery and transgene expression appears to be caveolae/raft-mediated endocytosis. The cellular internalization pathways for PGAAs were not identical to those for polyethylenimine, illustrating that differences in the chemical structure of materials directly impacts the cellular internalization mechanisms.

Keywords: Nonviral DNA delivery; caveolae; clathrin; flow cytometry; transfection; membrane disruption

Introduction

The intracellular delivery of nucleic acids is unlocking the mysteries of biological processes and facilitating the development of new treatments for a myriad of inherited and acquired diseases. While nucleic acids have exceptional affinity and specificity for their intracellular targets, many complex factors dictate the accuracy, reproducibility, and relevance of using these biomacromolecules to regulate gene expression for biological research and therapeutic development. In particular, delivery systems are needed to compact nucleic acids into nanostructures, protect them from enzymatic damage, facilitate their cellular entry, and provide the possibility of targeting their delivery to specific tissue types and sites within the cell.[1]

The delivery vehicle plays a central yet elusive role in dictating the efficacy, safety, cellular transport mechanisms, and kinetics of gene regulation in a spatial and temporal manner. While viral-based delivery methods are the most commonly studied vectors, there are many shortcomings with

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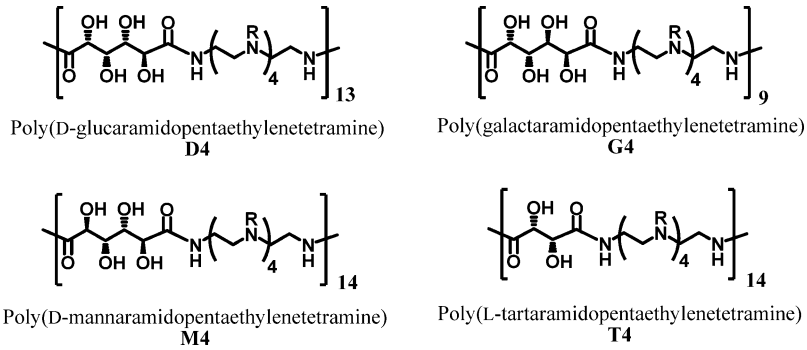

Figure 1. Structures of the poly(glycoamidoamine) (PGAA) nucleic acid delivery polymers.[12−15] As shown, the polymers differ by the stereochemistry of hydroxyl groups in the carbohydrate portion of the repeat unit. Each polymer was created by the copolymerization of a carbohydrate-based monomer and pentaethylenehexamine (the R groups = H or point of polymer branching).

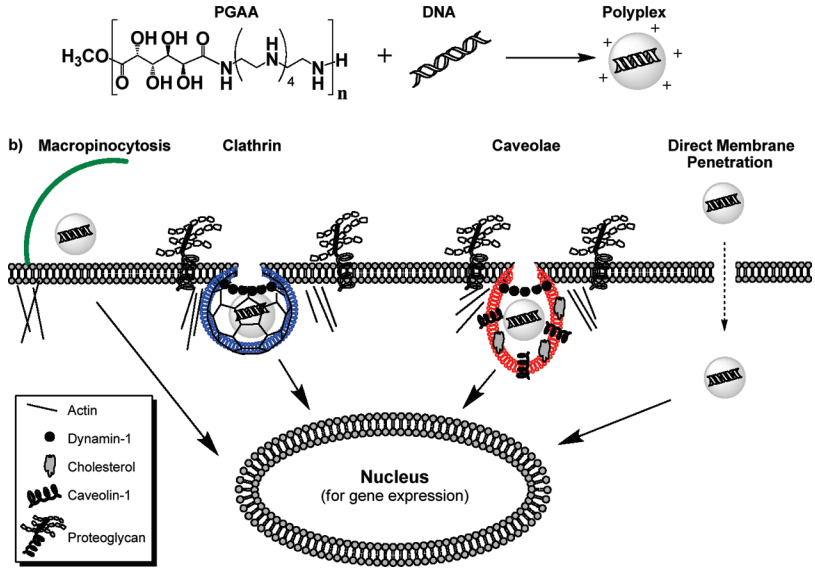

Figure 2. Summary of cellular internalization pathways of polyplexes formed with PGAA polymers and DNA. (a) Polyplexes form spontaneously by mixing DNA and polymer, forming cationic spherical "polyplexes." (b) The polyplex can be internalized by multiple pathways that can lead to nuclear trafficking, including macropinocytosis (far left), clathrin-mediated endocytosis (middle left), caveolae (middle right), and nonendocytic direct membrane penetration (far right). These pathways are explored in this study to investigate the mechanism of cellular internalization of PGAA polyplexes.

these systems such as toxicity, immunogenicity, and difficulties in biological engineering.[2,3] These issues have inspired the creative design of synthetic materials that promote cellular entry of nucleic acids. Several subclasses of nonviral delivery vehicles are being developed, including cationic polymers,[4,5] liposomes,[6] and peptides.[7,8] These synthetic vehicles have the potential to avoid immunogenic and toxic side effects.[9,10] They offer a facile means to incorporate functionality for increasing specificity of cellular entry and transport with the potential to target specific cell types *in vivo*.[11]

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Indeed, many diverse synthetic polymeric delivery vehicles are being developed to encapsulate nucleic acids into nanoparticle complexes, termed polyplexes. However, polyethylenimine (PEI), a material originally used in ion exchange and epoxy resins,[16] still remains among the most heavily studied materials for nucleic acid delivery. The high *in vitro* transfection efficiency of PEI is routinely used as a benchmark for new vehicle development;[4] yet, its clinical relevance is limited by high cytotoxicity.[12,17] For this reason, we have rationally designed a family of cationic glycopolymer vehicles termed poly(glycoamidoamine)s (PGAAs, Figure 1) by copolymerizing a PEI-like polyamine with a variety of monosaccharide monomers (dimethyl-*meso*-galactarate, dimethyl-D-glucarate, D-mannaro-1,4:6,3-dilactone, and dimethyl-L-tartarate).[12,15,18] These polymers were originally developed to reduce the toxicity of PEI-based vehicles and to gain an understanding of the structure−bioactivity relationships. Through many studies on these structures, we have found that both hydroxyl stereochemistry and amine number play a role in delivery efficacy. We have shown that the structures with four ethyleneamines generally have the best delivery and biocompatibility profiles.[12,13,15,18]

While the development of safe and effective delivery materials is important to this area, the elucidation of the intracellular mechanisms of polyplex transport as a function of polymer structure is paramount to the advancement of nucleic acid research tools and medicines. Indeed, several recent reviews have highlighted the fundamental importance of understanding these biological mechanisms of delivery.[10,19,20] We are particularly interested in how copolymerizing carbohydrates with PEI-like oligoethyleneamine groups affects cellular internalization routes, as we have shown that subtle changes in polymer structure can greatly influence bioactivity. These studies are essential for the future design of new vehicles that can be tuned to deliver nucleic acids through specific cellular trafficking pathways, as different nucleic acid types require distinctive final destinations inside the cell. For example, plasmid DNA must be trafficked to the nucleus for transcription but siRNA requires delivery to the cytoplasm to inhibit translation. Using a multidisciplinary approach, we can begin to understand how the polymer chemistry affects cellular processing of polyplexes, with the end goal of rationally-designed vehicles to deliver DNA into cells in an unambiguous manner.

Herein, we elucidate the cellular entry mechanisms of PGAA nucleic acid delivery vehicles with human cervical adenocarcinoma (HeLa) cells. This was achieved by comparing the PGAA results to those of linear PEI (Jet-PEI) in order to observe differences in cellular uptake of polyamine vehicles with those that contain carbohydrates in the backbone. The results presented herein suggest that, while multiple pathways of endocytosis are involved in the cellular uptake of PGAA polyplexes, one pathway is dominant in promoting transgene expression. Caveolae/raft-mediated and clathrin-mediated endocytosis appear to be the primary pathways of PGAA polyplex uptake by HeLa cells, and macropinocytosis is involved to a lesser degree (Figure 2). Caveolae/raft-mediated uptake appears to be the major uptake route leading to nuclear delivery and efficient transgene expression with the PGAA delivery vehicles. We also show that nonendocytic uptake via direct membrane penetration cannot be ruled out as a slight contributor to total polyplex uptake, but this effect is less for PGAA polyplexes than for those made with Jet-PEI. Jet-PEI appears to be less dependent on these mechanisms, suggesting alternate mechanisms contribute to PEI uptake. These fundamental studies are crucial for understanding the structure−bioactivity relationships for synthetic materials and will aid researchers to rationally design novel materials for biological use and targeted delivery systems.

Experimental Section

Reagents. All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. All four poly(glycoamidoamine)s [poly(galactaramidopentaethylenetetramine) (G4); poly(D-glucaramidopentaethylenetet-

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ramine) (D4), poly(D-mannaramidopentaethylenetetramine) (M4), and poly(L-tartaramidopentaethylenetetramine) (T4)] were synthesized and purified as previously described.[12,13,15] Plasmid DNA, both pCMVβ and gWizLuc (containing the luciferase reporter gene), were purchased from Aldevron (Fargo, ND) and Plasmid Factory (Bielefeld, Germany), respectively. pCMVβ was labeled with Cy5 using a Label *IT* kit (Mirus, Madison, WI) at 1/10 the labeling ratio suggested by the manufacturer, and purified using QIAquick PCR purification kit (Qiagen, Valencia, CA). FITC-pDNA was purchased from Mirus. Jet-PEI solution was purchased from Avanti Polar Lipids (Birmingham, AL). Tetramethylrhodamine-labeled dextran (TAMRA-Dextran, 25 kDa), propidium iodide (PI) and dimethyl sulfoxide were from Molecular Probes (Eugene, OR). Unless noted otherwise, all dilutions, including N/P dilutions of the PGAAs and pDNA, were done in DNase/RNase-free H_2O (Gibco, Carlsbad, CA).

Polyplex Preparation. For all experiments herein, PGAA polyplexes were formed at an N/P ratio of 20, as we have previously shown that this N/P ratio leads to high transgene expression and minimal toxicity in HeLa and other mammalian cell types.[12,13] Jet-PEI polyplexes were formed at N/P 5. For cellular uptake experiments, polyplexes were formed with Cy5-pCMVβ, and for transfection experiments, polyplexes were prepared using the gWizLuc luciferase reporter plasmid. The concentration of DNA used to form to polyplexes was 0.02 mg/mL, and an equal volume of polymer solution at the appropriate N/P ratio was added to the DNA solution and incubated at room temperature for at least 30 min. N/P ratios were calculated using only the secondary amines (and not the amide nitrogens). Uncomplexed DNA control (DNA only) was prepared using nuclease-free water in place of the polymer solution.

Cell Culture. All cell culture products, unless otherwise noted, were purchased from Gibco/Invitrogen (Carlsbad, CA). HeLa (human adenocervical carcinoma) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA), and were subcultured once per week in Advanced DMEM, supplemented with 2% fetal bovine serum, 1% L-glutamine, 100 units/mg penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin. Transfection medium was Dulbecco's modified Eagle's medium containing GlutaMAX, supplemented with 10% fetal bovine serum, 100 units/mg penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin. Cells were determined to be free of mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, ME).

Endocytic Pathway Inhibition. HeLa cells were seeded at 1.5×10^5 cells/well in transfection media in 6-well tissue culture plates (Corning; Corning, NY) and incubated for 24 h at 37 °C in a humid 5% CO_2 atmosphere. Polyplexes were formed as described previously. Medium was removed from the wells, and the wells were rinsed with PBS. Opti-MEM (2 mL) was added to each well, containing the following concentrations of the endocytic pathway inhibitors: chlorpromazine, 10 μg/mL; filipin III, 1 μg/mL; dimethylamiloride, 100 μM; phorbol 12-myristate 13-acetate, 2 μM; cytochalasin D, 2 μg/mL; Dynasore, 80 μM. Cells were incubated with the drugs for the following amounts of time: filipin III, 1 h; chlorpromazine, 30 min; Dynasore, 30 min; phorbol myristate acetate, 15 min; cytochalasin D, 15 min; dimethylamiloride, 5 min. The drug concentrations and incubation times used in this study were based upon previously published studies[5,21−23] and drug toxicity studies (not shown). Polyplex solution (300 μL) was added directly to each well containing 2 mL of Opti-MEM and the drugs at the above concentrations. Negative controls were cells only or uncomplexed DNA (DNA only). Cells were transfected for 2 h at 37 °C. After 2 h, 3 mL of transfection medium was added to each well and the cells were incubated an additional 30 min. Cells were detached $2^1/_2$ h post-transfection with 500 μL of Trypsin-EDTA and quenched with transfection medium (1 mL), and the contents of each well were collected into Falcon Tubes (BD Biosciences, San Jose, CA). Cells were centrifuged at 4 °C and 1250 rpm for 10 min. The supernatants were removed, and the cell pellets were rinsed with PBS and centrifuged again at identical conditions. The cell pellets were suspended in 2% FBS in PBS. Cellular uptake of Cy5-pCVMβ was measured on a FACS Canto II flow cytometer (BD Biosciences). Propidium iodide (PI, 5 μg/mL) was added to each tube and mixed 2−5 min prior to analysis. Appropriate gating was done against the cells-only control to ensure that autofluorescence and dead cells (PI-positive) were excluded from subsequent analysis. Cy5 was excited using a 633 nm helium−neon laser and detected with a 660/20 nm bandpass filter, and propidium iodide was excited with a 488 nm solid-state laser and fluorescence emission was detected by a 670 nm long-pass filter. 10,000−20,000 gated events were collected for each sample, and data analysis was completed using FACSDiva software (BD Biosciences) and FCS Express (De Novo Software, Los Angeles, CA). Presented data is an average of at least two replications.

Transgene Expression Experiments. For the non-endocytic transfection experiments, HeLa cells were seeded at 5×10^4 cells/well in 24-well tissue culture plates in transfection media and incubated for 24 h at 37 °C and 5% CO_2. The control cells were kept at 37 °C, and the inhibited cells were preincubated at 4 °C prior to transfection. The control cells were rinsed with 1−2 mL of room temperature PBS and the inhibited cells with ice-cold PBS. Polyplexes (300 μL) were diluted with 600 μL of Opti-MEM, and 300 μL of resulting polyplex solution in Opti-MEM was added to each well. Negative controls were cells only and uncom-

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plexed DNA. Control cells were allowed to transfect at 37 °C and inhibited cells at 4 °C for 2 h, at which point the medium was removed and the cells were rinsed twice with 1 mg/mL heparin in PBS and refreshed with 1 mL of transfection medium. Medium was replaced 24 h post-transfection, and the cells were lysed and assayed for luciferase gene expression 48 h post-transfection, using a Luciferase Assay Kit (Promega, Madison, WI) at 750 nm, with luminescence measured in duplicate in 10 s intervals using a plate reader (GENios Pro, TECAN US, Research Triangle Park, NC). Cell viability was measured using the DC Protein assay (Bio-Rad, Hercules, CA) and normalized against a cells-only negative control using a standard curve of bovine serum albumin.

To investigate the effect of endocytic pathway inhibition on transgene expression, cells were incubated in 500 μL of Opti-MEM containing inhibitors at identical concentrations and incubation times as for the uptake experiments. After incubation, inhibitors in Opti-MEM were removed, cells were rinsed with PBS to remove the drugs, and transfection was completed as described above, using Opti-MEM containing the drug inhibitors.

Liposome Disruption. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA, monosodium salt, T_c = 67 °C) were obtained as powders from Avanti Polar Lipids (Birmingham, AL). Lipids were dissolved in chloroform/methanol/distilled water at approximately 10 mg lipid/mL solvent. A dry lipid film was obtained by rotary evaporation of the solvent in a round-bottomed flask and dried under vacuum overnight. The final lipid composition was approximately 95% DPPC/5% DPPA. Lipid film was stored at −20 °C until further use.

A 45−50 mM solution of 5(6)-carboxyfluorescein (CF) was prepared in PBS. This solution was added to the dry lipid film and agitated for 1 h in 75 °C water bath, and then allowed to rest at the same temperature for 10 min. Large unilamellar vesicles (LUV) were prepared by subjecting vesicle suspension to 4−5 freeze−thaw cycles prior to extrusion 15× through 1 μm polycarbonate membranes using a miniextruder (Avanti). Nonencapsulated CF was removed by passing vesicles through Sephadex G-25 microspin columns (GE Healthcare, Piscataway, NJ). Vesicle size and negative surface charge were verified by dynamic light scattering and ζ-potential with a Zetasizer NanoSizer ZS (Malvern; Worchestershire, U.K.). Encapsulation of fluorophore was verified by addition of 1 mL of 1% triton X-100 to 1 mL of diluted vesicles and observation of increased fluorescence intensity. Vesicles were stored at 4 °C. To assess leakage, vesicle suspension was added to a fluorescence cuvette or black 96-well microplate and diluted with PBS. Fluorescence of diluted vesicles was measured, and polyplex solution was titrated in aliquots and measurements were taken over time. To assess fluorescence of completely ruptured vesicles, 10% Triton X-100 was added. Measurements were taken using a Cary Eclipse spectrophotometer (Varian, Palo Alto, CA), with λ_{ex} = 492 nm and λ_{em} = 500−600 nm, λ_{max} = 525 nm, an excitation slit width of 1.5 nm, and emission slit width of 10 nm, or with the plate reader. Measurements were normalized to controls of untreated vesicles in PBS.

Adenylate Kinase Leakage Assay. HeLa cells were plated at 5 × 10^4 cells/well in transfection media in 24-well culture plates and allowed to adhere to the plate for 24 h at 37 °C in a humid atmosphere containing 5% CO_2. Opti-MEM (600 μL) was added to 300 μL of polyplex solution, and 300 μL was transfected into each well. After four hours, 800 μL of transfection medium was added to each well. Adenylate kinase activity was assessed up to 48 h post-transfection using the Toxi-Light Bioassay kit (Lonza) and measured with the plate reader. For samples taken prior to 4 h post-transfection, 5 μL of media was used for the measurement, with all subsequent measurements using 20 μL to compensate for dilution. Results are the mean of triplicate measurements, and data was normalized to DNA only at each time point.

Cell Imaging by Confocal Microscopy. All primary antibodies were obtained from AbCam (Cambridge, MA). HeLa cells were plated at 1.2 × 10^4 cells/well in transfection media in 12-well culture plates containing sterile 15 mm No. 1 coverslips. Cells were incubated for 48 h at 37 °C in a humid 5% CO_2 atmosphere to allow adherence to the coverslip. Polyplexes were prepared in the dark. After removing media and rinsing coverslips with PBS, 1 mL of Opti-MEM was added to each well, and each well was transfected with 100 μL of polyplex solution, Opti-MEM (cells only), or uncomplexed DNA. Controls of primary antibodies only (no secondary antibody) and secondary antibody only (no primary antibodies) ensured that nonspecific fluorescence was not occurring (not shown). Polyplexes were allowed to transfect in HeLa cells for 1 h at 37 °C/5% CO_2. After transfection, coverslips were rinsed 3× with PBS. For antibody samples, cells were fixed in 1 mL of 4% PFA in PBS for 10 min at 4 °C. For dextran samples, 300 μL of TAMRA-dextran (Molecular Probes, 10 mg/mL in PBS) was added to each well and incubated for 15 min, then fixed. Cells were permeabilized with 0.5 mL of 0.25% Triton X-100 in PBS, and blocked with 1 mL of 1% BSA in PBS (blocking buffer). Primary antibodies were diluted in blocking buffer, and labeling concentrations of primary antibodies were as follows: anti-clathrin (rabbit polyclonal to clathrin heavy chain), 1 μg/mL; anti-caveolin-1 (rabbit polyclonal to caveolin-1), 2 μg/mL. For primary antibody labeling, blocking buffer was removed from each well, and 500 μL of the appropriate antibody was added to each well, and incubated at room temperature for 1 h. The excess primary antibody was removed, cells were rinsed 3× with PBS, and coverslips placed onto 100 μL of secondary antibody (5 μg/mL, Alexa Fluor 555 goat, anti-rabbit monoclonal Ab), and incubated 1 h at room temperature in a humid environment. Coverslips were counterstained with DAPI (Molecular Probes) or Draq-5 (Alexis Biochemicals, San Diego, CA) for nuclear staining, mounted using Prolong antifade mounting media (Molecular Probes), and dried overnight. Cell imaging was done using a 63× oil immersion objective (NA = 1.4) on a LSM510 confocal system fitted onto an Axioplan 2 upright microscope

or 40× oil immersion objective (NA = 1.3) on an Axiovert 100 inverted microscope (Zeiss, San Diego, CA). Images were acquired using laser excitation and filter sets appropriate for the dyes of interest (DAPI, λ_{ex} = 364 nm, λ_{em} = BP 385−470 nm; FITC, λ_{ex} = 488 nm, λ_{em} = BP 505−550 nm; AF-555, λ_{ex} = 543 nm, λ_{em} = BP 560−615 nm; Draq5, λ_{ex} = 633 nm, λ_{em} = LP 650). The confocal pinhole was adjusted to image a vertical optical slice of 1.0 μm (40× objective) or 0.8 μm (63× objective). Minimal modifications to image brightness, contrast and noise reduction were completed using NIH Image J software,[24] and all modifications were applied to the entire image.

Statistical Analysis. Statistical significance of the data was assessed by Student's *t* test, using JMP 7.0 software.

Results and Discussion

Endocytosis Inhibition on PGAA Uptake. The cellular uptake mechanisms of polyplexes formed with pDNA and each of our glycopolymer delivery vehicles were investigated using cellular transfection experiments in the presence of pharmacological inhibitors to block various cellular uptake pathways. Polyplexes were formed using Cy5-labeled pDNA, and HeLa cells were transfected in the presence of endocytic inhibitors. Chlorpromazine, filipin III, or dimethylamiloride (DMA) were used to inhibit clathrin, caveolae and macropinocytosis, respectively.[10,20] Similarly, phorbol myristate acetate (PMA) was used to stimulate macropinocytosis. Cytochalasin D (depolymerizes actin) and Dynasore (inhibits dynamin) were also used in these experiments to understand the role of these proteins on polyplex endocytosis. In these experiments, a decrease in cellular uptake or gene expression in the presence of an inhibitor indicates that a specific mechanism is involved in polyplex uptake. The mean intracellular fluorescence intensity of labeled pDNA was measured by flow cytometry to characterize the effect on cellular internalization. Each experiment was compared to the negative controls of untreated cells (HeLa cells only) and uncomplexed DNA (DNA only), as well as the positive controls of cells transfected with PGAA polyplexes without the drug inhibitors. The results of the polyplex uptake experiments in HeLa cells cultured in the presence of the inhibitors are shown in Figure 3 and are presented as the percent decrease in cellular uptake as normalized to transfected cells not exposed to the drug inhibitors. It should be noted that one of the potential issues with pharmacological endocytic pathway inhibition is the possibility of side effects occurring in the cell as a result of the treatment.[25] For example, inhibiting one cellular pathway sometimes leads to upregulation of another, as biological systems will adapt

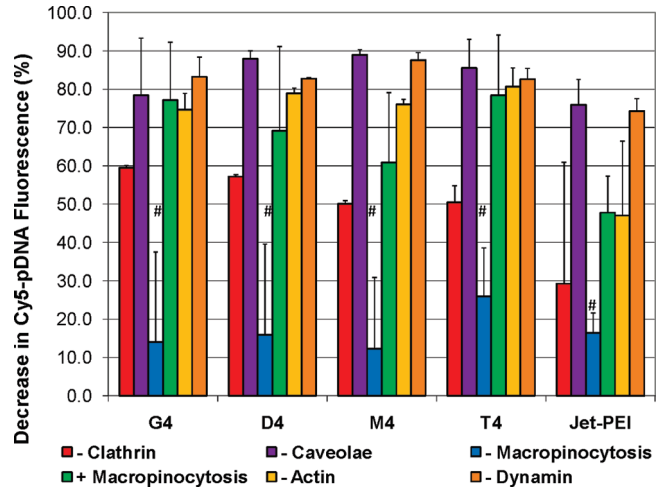

Figure 3. Percentage decrease in the cellular uptake of polyplexes upon pharmacological inhibition of the endocytic pathways. HeLa cells were incubated with the inhibitors prior to polyplex exposure, and the decrease in polyplex uptake was determined after $2^1/_2$ h via flow cytometry. Chlorpromazine, filipin III, and dimethylamiloride (DMA) were used to inhibit clathrin, caveolae and macropinocytosis, respectively. Phorbol myristate acetate (PMA) were used to stimulate macropinocytosis. Cytochalasin D and Dynasore were used to depolymerize actin and inhibit dynamin, respectively. The percentage decrease in Cy5-pDNA fluorescence is determined relative to cells transfected with each polyplex type without the inhibitors. All values are statistically different from DNA only ($p < 0.05$), unless marked with # (not significant).

to readily ensure the needs of the cell are satisfied. However, the signaling pathways that lead to upregulation of other internalization mechanisms are poorly understood.[26] With these phenomena in mind, these studies were carried out using the most specific known inhibitors for a given pathway with the least known potential side effects.[20,27]

As shown in Figure 3, upon inhibition of caveolae using filipin III, cellular uptake was nearly abolished for all the PGAA polyplexes (formulated with G4, D4, M4, and T4), giving 78−89% inhibition of polyplex uptake into HeLa cells. Polyplexes formed with Jet-PEI were also inhibited to a large degree with filipin III. These data suggest that caveolae-mediated endocytosis is highly involved in uptake of PGAA and PEI complexes. Likewise, chlorpromazine was used as a specific inhibitor of clathrin-mediated endocytosis. Inhibiting clathrin also had a pronounced effect on PGAA polyplex uptake, reducing the internalization of polyplexes by between 50% and 60%, suggesting that clathrin, like caveolae, also contributes significantly to total PGAA

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polyplex uptake, but perhaps to a lesser extent. On the other hand, Jet-PEI polyplex uptake was affected to a much lower degree by clathrin inhibition, as inhibition decreased uptake by less than 30%. This suggests that caveolae-mediated endocytosis is the dominant mechanism leading to PGAA and Jet-PEI polyplex uptake, but clathrin is more involved in PGAA polyplex uptake than for Jet-PEI polyplexes with HeLa cells. The apparent favorability of the caveolar pathway for the PGAA polymeric vectors could be advantageous for therapeutic administration, as caveolae are known to be an efficient mechanism of transcytosis of cargo,[28,29] which could be beneficial for vascular and deep tissue penetration of nucleic acid drugs. For example, it has been shown that HIV particles can bind to certain receptors present in caveolar microdomains and be directly shuttled (transcytosed) across epithelial cells.[29]

Formation of macropinosomes in cultured cells can be specifically inhibited by adding dimethylamiloride (DMA) into the culture media.[10,20,30] Inhibiting macropinocytosis with DMA caused a small, statistically-insignificant decrease in cellular uptake of PGAA polyplexes. T4 polyplex internalization was inhibited to the largest degree (26%), and the remainder of the PGAA polyplexes formed with G4, D4, and M4 were inhibited by 16% or less. The results suggest that macropinocytosis is a minor internalization pathway for PGAA polyplexes compared to clathrin and caveolae. To confirm these results, the transfections were done in the presence of phorbol 12-myristate 13-acetate (PMA), which has been shown to stimulate macropinocytosis.[5,20,31] Thus, cells treated with PMA were expected to internalize more polyplexes through macropinocytosis. Instead, treatment with PMA caused a substantial decrease in polyplex uptake. For all four PGAAs, the decrease in uptake was greater than 60%, with polyplexes formed with G4 and T4 approaching 80% uptake DMA inhibition (Figure 3). Similarly, inhibition of macropinocytosis causes decreased PEI polyplex uptake, as inhibition led to 16% inhibition, and PMA stimulation gave nearly 50% inhibition, which is less than observed for PGAA-based polyplexes. It is well-known that one of the primary fates of macropinosomes is recycling to the cell surface for exocytosis,[32,33] so stimulating macropinocytosis could increase the rate of polyplex recycling back to the cell membrane. In addition, stimulating this minor pathway could also decrease the rate of other primary cellular internalization routes because polyplexes may be quickly internalized, decreasing interactions with other endocytic machinery. This hypothesis is supported by the previous experiments that reveal caveolae and clathrin uptake routes are more efficacious pathways.

The three pathways of endocytosis described thus far all involve actin for the import of vesicles into the cells. The actin cytoskeleton has been previously demonstrated to be involved in endocytosis; ligands bound to receptors interact with actin filaments, which presumably play a role in vesicle budding.[34,35] Clathrin and caveolae have been shown to require actin for vesicle budding and detachment from the plasma membrane for release into the cytosol,[5,36] and macropinosomes are formed through the collapse of actin extensions onto the cell surface.[37] The importance of an intact actin cytoskeleton was studied using cytochalasin D, which caps the ends of f-actin and leads to depolymerization of actin filaments.[5,20,38] Actin disruption caused a decrease of 75−81% in the uptake of all the PGAA polyplexes (Figure 3), suggesting that an intact actin cytoskeleton is imperative for efficient polyplex uptake. Jet-PEI uptake was decreased by a lesser degree, with 47% uptake inhibition. This outcome suggests that Jet-PEI uptake is less contingent on an actin-dependent pathway.

Dynamin is an enzyme involved in many pathways of endocytosis, including clathrin and caveolae,[22,26,36] and possibly macropinocytosis,[39] as it is responsible for pinching off budded vesicles from the plasma membrane, freeing them into the cytoplasm.[22,40] Inhibiting dynamin activity should halt endocytic processes that require its action for vesicle scission. To test this hypothesis, a similar experiment was conducted using Dynasore, a reversible, noncompetitive inhibitor of dynamin function that has been recently discovered.[22] Polyplex uptake was decreased dramatically (between 83% and 88%) upon dynamin inhibition (Figure 3), strongly suggesting that PGAA uptake is dynamin-dependent. Jet-PEI also appears to rely heavily on dynamin-dependent endocytosis, albeit slightly less than PGAAs, since uptake decreased by 74%. This result suggests that PEI polyplexes

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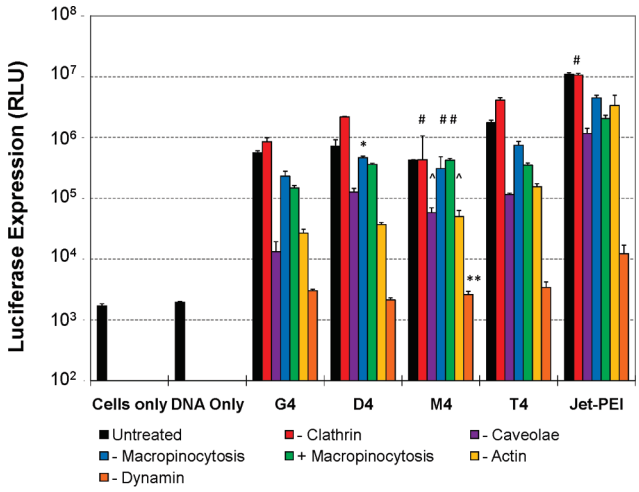

Figure 4. Effect of endocytic pathway inhibition on pDNA trafficking to the nucleus as measured by luciferase gene expression (shown as relative light units, RLU). The endocytic pathways were inhibited in an identical manner as the experiments in Figure 2, and the effect to transgene expression was assessed after inhibition of clathrin (chlorpromazine), caveolae (filipin III), macropinocytosis (DMA), actin (cytochalasin D), and dynamin (Dynasore), as well as after stimulation of macropinocytosis (PMA). Luciferase gene expression was assessed 48 h post-transfection. Inhibition experiments are compared against controls of gene expression in the absence of pharmacological inhibitors, which are represented as the first (black) bars in each series. Unless denoted otherwise, all values are significant compared to uninhibited control ($p < 0.0001$) * $p < 0.001$; ** $p < 0.005$; ^ $p < 0.1$; # not significant.

can possibly be internalized by dynamin-independent routes more efficiently that PGAA polyplexes.

To further pinpoint the specific endocytic route of PGAAs, polyplexes were transfected into cells in which multiple pathways of endocytosis were inhibited by adding two drug inhibitors to the experiment. Results of these experiments are presented in Figure S1 in the Supporting Information. Inhibiting clathrin and actin using chlorpromazine and cytochalasin D, respectively, caused a decrease in uptake of 68%, 74%, 72%, and 80% for G4, D4, M4, and T4 polyplexes, respectively. These extents of uptake reduction are lower than what was observed upon inhibition of actin values are lower than inhibiting actin alone but higher than inhibiting clathrin only, suggesting that polyplexes enter the cell through other actin-dependent pathways. When clathrin and macropinocytosis were inhibited using chlorpromazine and DMA, respectively, uptake was inhibited by 80%, 66%, 82%, and 75% for G4, D4, M4, and T4 polyplexes, respectively. The inhibition of both pathways significantly decreased uptake, and the percent inhibition nearly equaled the sum of inhibiting clathrin and macropinocytosis alone. This was unexpected, as caveolae were also shown to significantly contribute to uptake. These results support that clathrin-mediated endocytosis is a significant pathway in PGAA polyplex uptake. Inhibiting clathrin-mediated endocytosis with chlorpromazine while stimulating macropinocytosis with PMA showed a similar degree of inhibition as inhibiting both clathrin and actin; polyplex uptake was inhibited by 71%, 73%, 70%, and 76% for G4, D4, M4 and T4 polyplexes, respectively. For G4 and T4 polyplexes, these inhibition levels were similar to stimulating macropinocytosis alone and higher than inhibiting clathrin alone. However, D4 and M4 polyplex uptake decreased to an intermediate degree when compared to treatments with chlorpromazine (inhibiting clathrin) or PMA (stimulating macropinocytosis) alone. To our surprise, inhibiting macropinocytosis and disrupting actin resulted in a net increase (less inhibition) of polyplex uptake compared with internalization of polyplexes in cytochalasin D-treated cells. Since macropinocytosis is reliant on actin for formation of macropinosomes, it was expected that the results would be similar to the actin-inhibited cellular uptake. This increase in uptake could be due to upregulation of other pathways.[27] These poorly characterized actin-independent pathways may play a small role in total polyplex uptake normally, but interfering with the cytoskeleton and Na^+/H^+ pumps could stimulate the cell to upregulate other pathways to ensure that vital endocytic function is not lost. It should be noted that the likelihood of drug-induced side effects to internalization are amplified in these experiments, and further experiments are needed to discern these possible alternative pathways.

Endocytosis Inhibition on Gene Expression. Transfection efficiency experiments were carried out using endocytic pathway inhibition to determine how uptake influences nuclear trafficking. This data allows us to determine the most critical pathways for successful shuttling of the pDNA to the cell nucleus with the studied vehicles and if they differ from the pathways most prominent for uptake. The results, shown in Figure 4, support the results of the uptake experiments with an interesting finding: polyplexes formed with each of the four PGAAs show similar trends in transfection in the presence of these drug inhibitors, suggesting similar mechanisms lead to efficient polyplex trafficking to the nucleus. Inhibition of caveolae-mediated endocytosis with filipin III resulted in decreased luciferase reporter gene expression for all of the PGAA polyplexes. In the presence of filipin III, luciferase expression was lower with G4 and T4 polyplexes than with D4 and M4 polyplexes, suggesting that G4 and T4 polyplexes are shuttled to the nucleus through caveolar uptake to a greater extent than D4 and M4, possibly substantiating the high transfection efficacy commonly observed with T4 and G4.[12,13,18] Depolymerizing actin with cytochalasin D had a similar effect by decreasing luciferase expression dramatically for all PGAAs. Inhibiting dynamin activity with Dynasore nearly completely abolished transfection for all PGAA polyplex types. These data support our previous data suggesting that actin and dynamin-dependent pathways are primary methods of polyplex uptake and trafficking within the cell. Inhibiting and stimulating macropinocytosis had similar effects on transfection and cellular uptake, since PMA-induced stimulation of macropinocytosis caused a decrease in reporter gene expression.

These results support our previous data suggesting a limited role of macropinocytosis in the cellular internalization of polyplexes (Figure 3). Surprisingly, treatment with chlorpromazine to inhibit the clathrin-mediated pathway resulted in an enhancement of transfection with respect to untreated cells. This unexpected result indicates that efficient trafficking of PGAA polyplexes to the nucleus occurs through a clathrin-independent mechanism.

Transfection experiments with Jet-PEI resulted in differences when compared to the PGAAs. Clathrin inhibition showed little change in transfection as compared to untreated cells, again supporting our observations that polyplexes internalized via the clathrin mechanism may not be efficiently transported to the nucleus. Others have indicated previously that PEI transfection occurs primarily through caveolae, as filipin III (blocks caveolae) and genistein (a tyrosine kinase inhibitor also shown to inhibit caveolae)[20] lead to reduced transfection, but little effect on transfection was seen with chlorpromazine and K^+ depletion (both inhibitors of clathrin-mediated endocytosis) in HeLa cells.[41,42] Filipin III caused a marked decrease in PEI-mediated transfection, continuing to suggest that caveolae play a role in polyplex uptake and shuttling to the nucleus. Dynasore caused a substantial decrease in transfection, but unlike the PGAA polyplexes, significant gene expression was still observed with the Jet-PEI polyplexes. Similarly, depolymerization of actin resulted in only a slight decrease in transgene expression, supporting our suggestion that an accessory uptake mechanism is involved in cellular internalization with Jet-PEI polyplexes, such as passive membrane disruption, that does not require actin and dynamin for entry. Drugs affecting macropinocytosis had similar effects on the Jet-PEI and PGAA polyplexes, suggesting that macropinocytosis is a minor pathway leading to gene expression for these delivery vehicles.

Cellular Imaging of Endocytosis. Confocal microscopy was used to support the data above by visualizing the endocytic pathways involved in polyplex uptake. Proteins involved in the specific mechanisms of endocytosis, clathrin and caveolin-1, were labeled using immunostaining and imaged after transfection. Additionally, TAMRA-labeled dextran was used as a marker for macropinocytosis. HeLa cells were exposed to polyplexes formed with FITC-labeled pDNA, fixed after 1 h, and analyzed via confocal microscopy for colocalization between pDNA and the endocytic markers. In these studies, polyplexes formed with polymers G4 and T4 were chosen for these imaging experiments due to our previously published observations that these vehicles generally have the most promising transfection properties among the PGAAs.[12,13] Polyplexes formed with Jet-PEI were also imaged for comparison. Figure 5a shows an example of the component images and the overlaid images of cells transfected with FITC-labeled pDNA (within polyplexes containing G4, T4, or Jet-PEI) and immunolabeled with the relevant fluorescent endocytic marker. Remaining component images are included in the Supporting Information for clarity (Figure S2 in the Supporting Information). Arrows in the figure point to regions of colocalization (pseudocolored white), as highlighted in Image J software. As shown in Figure 5b, G4 polyplexes colocalized with clathrin and caveolin-1 in several small regions near the cell membrane. This was expected since fixation was done quickly (1 h) after transfection to capture the endocytic events. These data corroborate the role of clathrin and caveolin-based mechanisms for G4 polyplex entry. Cells labeled with the TAMRA-dextran (for macropinocytosis) showed sparse colocalization with polyplexes at the cell periphery.

As shown in Figure 5b, cells transfected with T4 polyplexes show a few areas around the cell periphery of colocalization of caveolin-1 and a large aggregate of DNA. This phenomenon is similar to that seen with G4 colocalization with clathrin. As we have previously published,[12,13] PGAA polyplexes are known to aggregate to some degree in buffer, so it is possible that aggregated polyplexes may be partially located on the cell surface, and, depending on the internalization route, the whole or part of the aggregate (a few or single polyplexes) may be internalized over time. Indeed, glycosaminoglycans are known to cluster receptors for internalization, which may serve to bring in polyplexes as aggregates.[20] Others have demonstrated that latex beads as large as 500 nm may be slowly internalized by a caveolar mechanism.[43] However, not all polyplexes congregate in aggregates, as noted from the smaller colocalized regions near the cell periphery (Figure 5). The T4 polyplexes colocalized with clathrin (Figure 5a), supporting previous findings that uptake occurs in a clathrin-dependent manner. Dextran colocalization experiments (Figure 5b) revealed T4 polyplexes in macropinosomes near the cell surface, as well as in small vesicles localized near the nuclear membrane. These results confirm that uptake of G4 and T4 polyplexes occurs through multiple pathways. It is to be noted, however, that many FITC-labeled polyplexes do not colocalize with any of the labeled endocytic molecules, suggesting that other uptake mechanisms may play a role in intracellular transport.

Jet-PEI polyplexes also show some colocalization with caveolin-1 and clathrin (Figure 5). Colocalization with caveolin-1 was observed after 1 h, which supports internalization of Jet-PEI polyplexes by caveolae-mediated endocytosis. Colocalization of Jet-PEI with clathrin and caveolin-1 appears to be in regions near the cell surface, possibly indicating Jet-PEI polyplexes in the process of being internalized. Colocalization of Jet-PEI polyplexes and dextran was not observed. This corroborates the inhibition data and further suggests that macropinocytosis does not account for significant uptake of Jet-PEI polyplexes.

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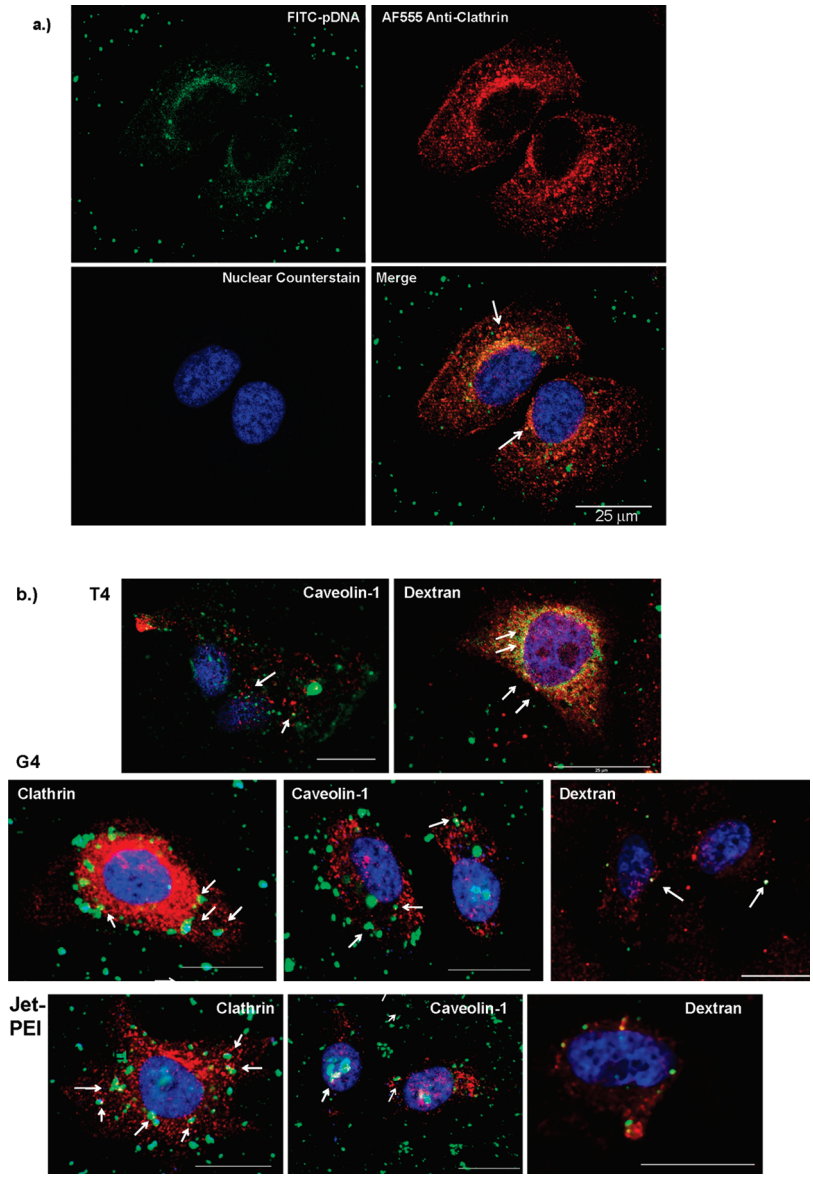

Figure 5. Confocal micrographs of HeLa cells transfected with each polyplex type and stained for the various routes of endocytosis. Polyplexes were formed with FITC-labeled pDNA and each polymer vehicle (PGAA or Jet-PEI). Cells were fixed and immunostained one hour post-transfection, and colocalization between FITC-pDNA and the labeled markers of endocytic pathways were imaged. (a) Component images of cells transfected with T4 polyplexes (containing FITC-pDNA shown in green), regions labeled with primary antibodies against clathrin heavy chain and fluorescently labeled with Alexa Fluor 555 secondary antibodies (red), and nuclear counterstain (blue). The merged image is the overlay of the three previous images. The component images for the colocalization experiments with T4, G4, and Jet-PEI polyplexes (from which this figure was derived) are included in Figure S2 in the Supporting Information. (b) Merged images of transfection experiments using polyplexes formed with T4, G4, or Jet-PEI and FITC-pDNA (green), labeled clathrin heavy chain, caveolin-1 (labeled with Alexa Fluor 555-conjugated secondary antibodies), or TAMRA-dextran. Arrows indicate calculated colocalization (white) between polyplexes (green) and the endocytic marker (red). Scale bars = 25 μm.

Despite the inhibition data suggesting a prominent role for caveolae, it is interesting that the polyplexes do not colocalize with caveolin-1 after a one hour transfection to the degree that we expected. It is reasonable to speculate that the polyplexes may be associating with lipid rafts, which are functionally similar to caveolae but do not contain caveolin-1.[36] These domains are also filipin III-sensitive,[44] as the drug binds to membrane cholesterol, and are dependent on actin and dynamin for internalization. These endocytic vesicles are internalized and traffic through the cell in a similar fashion,[36] and may contain the majority of internalized polyplex. Polyplexes may also be internalized by

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caveolae at a different rate than other endocytic pathways, such that the timing of the experiment may be very important in capturing these events, and fixation at 1 h may be suboptimal for visualizing these events. Studies are currently ongoing to investigate these possibilities.

It is noteworthy that none of the pharmacological treatments alone completely inhibited polyplex internalization. Furthermore, many polyplexes did not colocalize with markers for the clathrin, caveolae, or macropinocytic pathways, so we cannot rule out that additional internalization mechanisms independent of these pathways may be involved in polyplex uptake. Indeed, several recent studies have begun to discover clathrin- and caveolae-independent pathways within the cell, and these mechanisms may be involved in PGAA polyplex uptake.[23,45,46] For example, a clathrin- and caveolae-independent mechanism has been reported that involves the cell surface marker protein flotillin-1, which buds into the cell and accumulates in vesicles that are discrete from clathrin- and caveolae-containing vesicles,[46] and traffic to late endosomes.[23] These mechanisms are likely to be significant to polyplex internalization and warrant future investigation.

Direct Membrane Penetration by the Polyplexes. Since we were unable to link all internalized polyplexes to known mechanisms using available markers and inhibitors, we were interested in whether these materials have the ability to directly penetrate the plasma membrane to gain access to the cytoplasm. This phenomenon has been suggested previously; Hong and co-workers have shown by AFM that polycations such as PAMAM dendrimers (G5) and branched PEI can induce hole formation in supported lipid bilayers, possibly by lipid removal from the membrane.[47,48] The following experiments were focused on determining the role nonendocytic uptake plays in total polyplex uptake. We have approached this question in three ways: (i) monitoring cellular uptake at 4 °C, where active uptake mechanisms do not occur,[49] (ii) monitoring plasma membrane puncture via leakage of a small cytosolic enzyme, adenylate kinase (AK), and (iii) modeling the interaction of polyplexes with lipid bilayer vesicles to observe vesicle leakage.

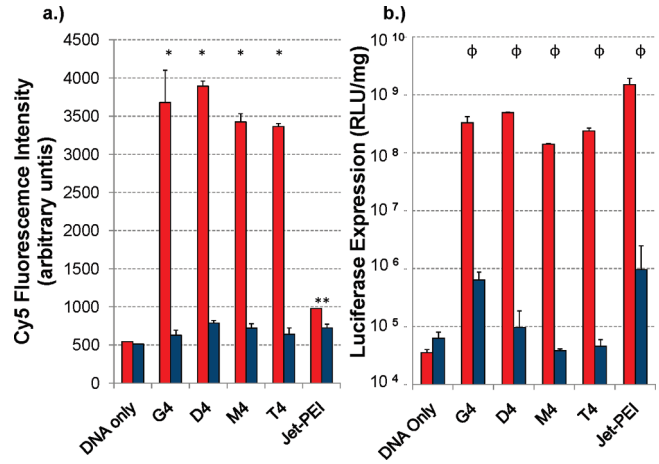

Figure 6. Effect of inhibiting active endocytosis on (a) cellular uptake, as determined via flow cytometry analysis of cells positive for Cy5-labeled pDNA, and (b) transfection efficiency of pDNA containing the luciferase reporter gene. Polyplexes were transfected at 4 °C (blue bars) and compared to polyplexes transfected at 37 °C (red bars), to observe energy-independent nonendocytic uptake, and the ability of the internalized polyplexes to traffic to the cell nucleus for transgene expression. * $p < 0.0001$; ** $p < 0.05$; φ $p < 0.001$.

The role of direct membrane passage was investigated with experiments designed to observe nonendocytic internalization and transgene expression in HeLa cells. Cells were transfected with PGAA or Jet-PEI polyplexes at 4 °C; at this low temperature, active transport processes, such as endocytosis, cannot occur.[49] Cells were exposed to polyplexes for 2 h, then removed and washed with 1 mg/mL heparin, a polyanion which dissociates surface-bound polyplexes,[25] to remove bound polyplexes from the cell surface. These cells were then assessed for cellular uptake. A complementary experiment at 4 °C was completed to measure transgene expression. In this case, after polyplexes were removed with the heparin solution, the cells were provided with fresh media at 37 °C, and polyplexes were allowed to traffic for an additional 46 h before measuring luciferase expression. The results of these experiments are shown in Figure 6. Cellular uptake (Figure 6a) analysis showed a small degree of direct membrane penetration for the PGAAs and Jet-PEI polyplexes at 4 °C, with Jet-PEI and D4 showing slightly higher cell internalization than G4, M4, or T4. These results cannot rule out that that a small degree of nonendocytic uptake may occur for these polyplexes. Total cellular uptake (at 37 °C) of Jet-PEI polyplexes is less than that of PGAA polyplexes (Figure 6b), a phenomenon that is consistently observed between these two vehicle types.[13,14] The luciferase expression experiments, however, show that the vehicle has a dramatic effect on cellular processing of polyplexes internalized by nonendocytic means. Polyplexes formed with G4 show considerably higher transgene expression at 4 °C than the other PGAAs in HeLa cells, possibly indicating that G4 encourages

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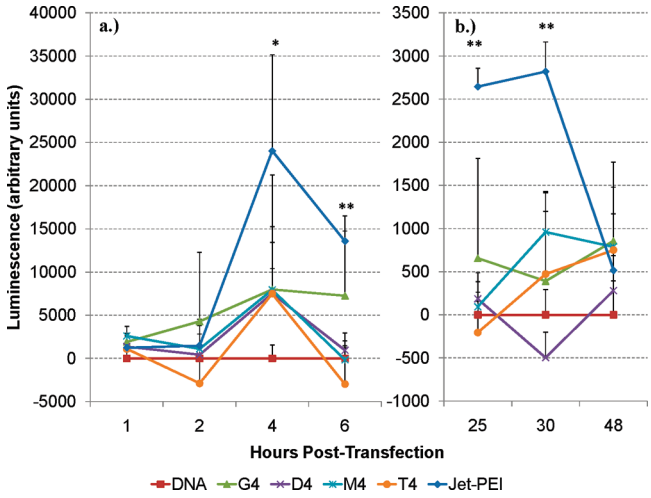

Figure 7. Penetration of the cell plasma membrane by polyplexes as determined by adenylate kinase (AK, a cytosolic enzyme) leakage from cultured HeLa cells. (a) 0−6 h post-transfection; (b) 25−48 h post-transfection. AK outside the cell generates ATP which is required for luciferase activity. Luminescence values (from luciferase activity) are normalized against a negative control of cells treated with DNA only at each measured time point. Error bars represent the standard deviation of the mean of triplicate measurements. Jet-PEI shows statistically higher AK leakage; * $p < 0.05$, ** $p < 0.01$. G4 and Jet-PEI are not statistically different at $t = 6$ h.

trafficking to the cell nucleus or more efficiently dissociates from the plasmid in a more efficient manner. Other PGAA polyplexes did not show nonendocytic transfection in these experiments, but Jet-PEI polyplexes, internalized by a nonactive endocytic pathway, exhibit significant transgene expression. The low temperature data also suggests a nonendocytic component to cellular uptake, which may help to account for the higher observed transfection of Jet-PEI with respect to the PGAAs *in vitro*. PEI may be able to passively enter the cell and traffic to the nucleus in a manner independent of an active vesicular transport mechanism. These findings may also help explain the lower degree of drug inhibition observed with PEI polyplexes (Figures 2, 3, and S1 in the Supporting Information); a non active transport pathway could play a role in polyplex internalization and toxicity.

To assess plasma membrane puncture using AK leakage, HeLa cells were transfected with polyplexes and AK leakage into the culture media was monitored over time to determine if and when membrane leakage is occurring. Figure 7 represents AK leakage over time, which is normalized to DNA only control. AK leakage was observed between 2 and 4 h post-transfection. The highest AK leakage induction among PGAAs was observed for G4, consistent with earlier results (Figure 6). AK was released from HeLa cells faster with Jet-PEI than with the PGAAs, suggesting a higher level of membrane puncture for Jet-PEI than the PGAAs. PGAA polyplexes caused statistically insignificant levels of AK leakage compared to uncomplexed DNA, suggesting a lower degree of membrane disruption compared to PEI. Indeed, the leakage was nearly 5-fold higher for Jet-PEI than for the PGAAs, indicating a higher membrane-disrupting effect. This suggests nonendocytic internalization of Jet-PEI polyplexes or cytotoxicity due to compromise of the membrane during cell death. These results are in agreement with other results that have shown that Jet-PEI can potentially damage membranes by expanding defects in model membranes.[47] These results demonstrate that PGAA polyplexes do not cause significant membrane disruption with respect to PEI.

Further insight into the ability of PGAA polyplexes to disrupt cell membranes was gained by investigating whether these materials can directly penetrate model phospholipid bilayer membranes. To study this, anionic phospholipid bilayer vesicles were formed that encapsulated a fluorophore, 5(6)-carboxyfluorescein, that was quenched inside the crowded environment of the vesicle and extruded to form 1 μm vesicles. The presence of vesicles of desired size and negative charge were verified by dynamic light scattering and ζ-potential (Figure S4 in the Supporting Information), which showed an average size around 1 μm and a slightly anionic surface charge of −4 mV. Diluted vesicles were titrated with polyplex solutions up to a concentration of 0.005 mg/mL DNA in polyplexes. Immediate titration of polyplexes into PBS-diluted vesicles did not lead to vesicle rupture for any of the PGAAs or Jet-PEI, since fluorescence profiles of vesicles titrated with polyplexes were identical to negative controls (titration with water) (Figure S5 in the Supporting Information). To determine the ability of PGAA polyplexes to puncture vesicles in a time-dependent manner, polyplexes at various concentrations were incubated at 37 °C with vesicles in PBS. The results of these experiments are shown in Figure S6 in the Supporting Information. The dilution control consists of vesicles incubated with a volume of water equal to the volume of added polyplex. The dilution control shows a decrease in fluorescence intensity over the time of measurement. This decrease is likely due to photobleaching of unencapsulated fluorophore in the buffer that remained after purification. For vesicles incubated with G4, D4, and M4 polyplexes, vesicle rupture (evidenced by increasing fluorescence) was evident after about 21 h and continued to increase until about 30 h, especially at biologically-relevant concentrations (DNA concentration in transfection experiments is equal to 0.0033 mg/mL). Incubating vesicles with T4 polyplexes resulted in model vesicle penetration after about 18 h and increasing up through 24 h. At 30 h, fluorescence decreases, which is likely attributed to quenching of unencapsulated fluorophore and a slowed vesicle rupture rate. Interestingly, the fluorescence increase with T4 polyplexes was twice that of G4 and four times that of D4 and M4 polyplexes. This suggests that polymer T4 may interact more closely with the membrane lipids, increasing its ability to induce charge-mediated lipid disruption. However, the extended time period required for lipid disruption suggests that most particles are not internalized by a membrane-disrupting mechanism, as endocytosis would occur on a much faster time scale, leaving decreased

concentrations of extracellular polyplex to disrupt the membrane. Interestingly, Jet-PEI showed minimal vesicle puncture in this experiment. It should be noted that PEI polyplexes at N/P 5 have a slightly lower zeta potential ($\sim -12-14$ mV) than PGAA polyplexes, which may help to explain this observation. However, previous observations suggest that PEI can disrupt the plasma membrane.[47] In our experiments, the phospholipid vesicle models account only for direct electrostatic interactions of the polyplex with the lipid bilayer. Indeed, anionic glycosaminoglycans and other cell surface components have been shown to play a role in polyplex association with the plasma membrane,[7,49] and may serve to protect against direct interaction with the membrane. Taken together, these experiments suggest that the majority of polyplexes are internalized through active uptake routes, but direct membrane interactions and passive endocytic routes may contribute to total polyplex uptake or represent a potential interaction leading to toxicity.

Conclusions

Synthetic drug and gene delivery systems are now ubiquitous in novel targeted therapeutic development because of their promise to improve treatment specificity and efficacy and decrease immune response and other side effects. For this reason, the discovery of the endocytosis and intracellular trafficking mechanisms taken by synthetic nucleic acid delivery vehicles is important for the design and optimization of materials that can efficiently enter the cell and deliver nucleic acids to specific intracellular sites. These studies are essential as the number of synthetic materials in human clinical trials is rapidly increasing.

The data presented herein suggests that a complex, multifaceted internalization pathway is taken by the PGAA polyplexes. The dominant mechanism of PGAA polyplex uptake appears be through active transport via an actin- and dynamin-dependent mechanism. Of the better-characterized pathways, clathrin, caveolae, and macropinocytosis all appear to contribute to total polyplex uptake. Macropinocytosis appears to be the least involved of the three pathways, with clathrin- and caveolae-mediated endocytosis playing significant roles in polyplex internalization.

An important discovery in this work is that, while a multitude of pathways contribute to uptake, only certain pathways may lead to significant gene expression. An actin- and dynamin-dependent mechanism, likely caveolae-mediated or lipid raft endocytosis, appears to be the predominant uptake route leading to nuclear trafficking and transgene expression. An unexpected and interesting result was that, while clathrin does contribute to the uptake route, it appears not to be the mechanism of shuttling PGAA polyplexes to the nucleus as transgene expression actually *increased* with clathrin inhibition. This could mean that polyplexes taken up by this route are either shuttled to lysosomes for degradation, released into the cytosol, exocytosed, or lack a viable means of active transport into the nucleus once escaped from intracellular transport vesicles.

Indeed, targeting specific pathways may be advantageous for efficient cellular entry, and certain mechanisms may enable better targeting of the nucleic acid cargo to specific intracellular locations. As a result, future generations of polymers could be designed to utilize different pathways depending on the intended therapeutic target. Since we cannot account for all polyplex internalization using inhibition of components of known pathways, we speculate that there are likely alternate mechanisms at work, including possibly a clathrin- and caveolae-independent mechanism, which likely involves actin and dynamin for vesicular internalization. Nonendocytic uptake routes appear to be minor, but cannot be ruled out as a potential mechanism leading to internalization. Further studies to identify the molecules on the cell surface and along the endocytic pathways with which PGAA polyplexes interact will expand our understanding of how these materials maneuver through the cell. Many of these additional studies are currently in progress.

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Supporting Information Available: Uptake experiments with multiple inhibitors, component microscopy images, and liposome disruption studies, including size and charge characterization of model phospholipid vesicles and initial experimental details on polyplex-induced phospholipid vesicle rupture. This material is available free of charge via the Internet at http://pubs.acs.org.

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