

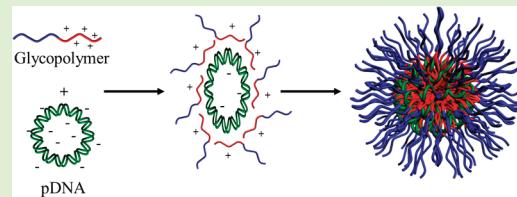
Diblock Glycopolymers Promote Colloidal Stability of Polyplexes and Effective pDNA and siRNA Delivery under Physiological Salt and Serum Conditions

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 Supporting Information

ABSTRACT: A series of glycopolymers composed of 2-deoxy-2-methacrylamido glucopyranose (MAG) and the primary amine-containing *N*-(2-aminoethyl) methacrylamide (AEMA) were synthesized via aqueous reversible addition–fragmentation chain transfer (RAFT) polymerization. The colloidal stability of the polyplexes formed with three diblock glycopolymers and pDNA was assessed using dynamic light scattering, and the polyplexes were found to be stable against aggregation in the presence of salt and serum over the 4 h time period studied. Delivery experiments were performed *in vitro* to examine the cellular uptake, transfection efficiency, and cytotoxicity of the glycopolymer/pDNA polyplexes in cultured HeLa cells and the diblock copolymer with the shortest AEMA block was found to be the most effective. Additionally, the ability of the diblock glycopolymers to deliver siRNA to U-87 (glioblastoma) cells was screened, and the diblock copolymer with the longest AEMA block was found to have gene knockdown efficacy similar to Lipofectamine 2000.



INTRODUCTION

Interpolyelectrolyte nanoparticle complexes or “polyplexes” consisting of cationic polymers complexed to various polynucleotides (i.e., plasmid DNA (pDNA), oligodeoxynucleotides, small interfering RNA (siRNA), and microRNA) have proven to be useful chemical biology tools for understanding genetic and epigenetic mechanisms associated with human health and disease.¹ Moreover, the recent clinical success of a polyplex formulation consisting of poly(ethylene glycol) (PEG)-ylated β -cyclodextrin-based polycations complexed with siRNA has been an exciting breakthrough in the development of targeted nanomedicines for human cancer treatment.² This significant advance has proven that novel polymers and self-assembled complexes are safe and effective in humans. Therefore, the development of novel polymeric delivery vehicles that can consistently complex macromolecular drugs into nanoparticles, protect their payload from enzymatic degradation in the blood, remain colloidally stable, and avoid rapid clearance by the reticuloendothelial system (RES) is warranted for the future success of more specific and selective nanomedicines in the clinic.

In an effort to prevent colloidal aggregation, opsonization, and RES clearance of various nanomedicines such as polyplexes, delivery vehicles often incorporate a neutral hydrophilic component such as PEG to provide steric stabilization.^{3–5} Although PEG is widely utilized in delivery applications, it is not without drawbacks. For example, recent work has shown that PEG can promote nonspecific interactions with blood components, complement activation, and promote antibody production, which signals and accelerates nanomedicine clearance from the blood and negates the use of the delivery system in multiple treatments.⁵

To address these drawbacks, other nonionic, hydrophilic polymers have been proposed as PEG alternatives including poly(amino acid)s, poly(glycerol), poly(2-oxazoline)s, and vinyl polymers.⁵ A need still exists for new systems that can provide colloidal stabilization.

Synthetic glycopolymers have received increasing interest because of their ability to imitate, inhibit, or promote specific biological interactions such as targeting functionality in the delivery vehicle.^{6,7} However, prior to the 1980’s, polymer chemists were limited in their ability to adequately design and customize delivery vehicles with complex functionality such as carbohydrate groups.⁸ With the emergence of controlled/“living” radical polymerization (CLRP) techniques such as nitroxide-mediated polymerization (NMP),⁹ atom transfer radical polymerization (ATRP),¹⁰ and reversible addition–fragmentation chain transfer polymerization (RAFT),¹¹ researchers are now capable of synthesizing well-defined (co)polymers and block copolymers with advanced architectures containing reactive functional groups, without the need for the stringent reaction conditions required for anionic polymerizations. Of the CLRP methods, RAFT polymerization is of particular significance for the development of drug and nucleic acid delivery vehicles because of the ability to polymerize a wide range of functional monomers (including glycomonomers^{12–19}) in a variety of solvents without the need for a transition metal catalyst that could potentially present toxicity.^{8,20}

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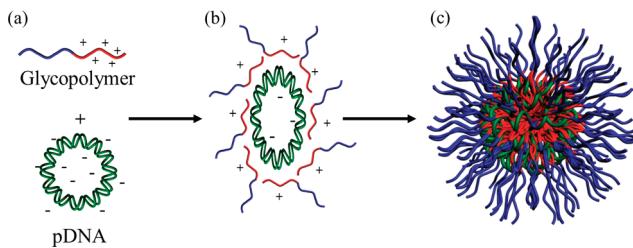


Figure 1. Schematic of polyplex formation with the diblock glycopolymer and pDNA. (a) Cationic AEMA blocks electrostatically associate with the anionic pDNA. (b) Polymer–pDNA binding and compaction. (c) Core–shell polyplex formation with the poly(glucopyranose) block displayed on the surface. Polyplexes can also be formed in a similar manner with siRNA.

Herein, we report the RAFT synthesis and biological efficacy of a new diblock glycopolymer nucleic acid delivery vehicle motif that readily assembles with pDNA and siRNA. A family of diblock glycopolymers was created that incorporated 2-deoxy-2-methacrylamido glucopyranose (MAG) to promote colloidal stability and *N*-(2-aminoethyl) methacrylamide (AEMA) to provide complexation of nucleic acids and polyplex formation. This architecture was conceived to form uniform core–shell polyplexes with the P(MAG) block displayed on the nanoparticle surface to prevent colloidal aggregation in the presence of salt and serum (Figure 1). To examine the effects of the amine block length on colloidal stability and delivery efficiency, three diblock glycopolymers were synthesized and examined for their ability to form polyplexes with and deliver pDNA and siRNA into cultured cells.

EXPERIMENTAL SECTION

Materials. All chemicals were purchased from Aldrich (Milwaukee, WI) at the highest available purity and were used as received unless otherwise noted. AEMA was purchased from Polysciences (Warrington, PA) and was recrystallized twice from ethanol prior to use. MAG²¹ and the chain transfer agent (CTA) 4-cyano-4-(propylsulfanylthiocarbonyl) sulfanylpentanoic acid (CPP)²² were synthesized as previously reported. 4,4'-Azobis(4-cyanopentanoic acid) (V-501) was recrystallized twice from methanol prior to use. Cell culture media and supplements were purchased from Gibco (Carlsbad, CA). Human cervix adenocarcinoma (HeLa) cells were purchased from ATCC (Rockville, MD). Luciferase-expressing U-87 cells (glioblastoma cells) were purchased from Caliper LifeSciences (Mountain View, CA). JetPEI (PolyPlus Transfections, Illkirch, France), Lipofectamine 2000 (Invitrogen, Carlsbad, California), and Glycofect (Techulon, Blacksburg, VA) were used according to the manufacturer's protocol.

Glycopolymer Synthesis and Characterization. A solution of CPP (15.0 mg, 0.0540 mmol), MAG (2.00 g, 80.1 mmol), and V-501 (1.5 mg, 0.0054 mmol) in 16 mL of 4:1 acetate buffer (pH 5.2)/ethanol was added to a 25 mL round bottom flask equipped with a magnetic stir bar. The solution was then sparged with nitrogen for 30 min, and the flask was placed in a preheated oil bath at 70 °C. The reaction was terminated after 3 h by quenching the reaction tube in liquid nitrogen, followed by exposure to air. After purification by dialysis against water (pH 4 to 5) and lyophilization, the PMAG macroCTA was chain-extended with AEMA to yield three diblock copolymers following a similar procedure. In general, AEMA (0.265 g, 1.61 mmol), PMAG₄₆ (0.200 g), and V-501 (0.902 mg, 0.00320 mmol) were dissolved in 2.5 mL of acetate buffer (pH 5.2) and added to a 5 mL round bottom flask equipped with a magnetic stir bar. After sparging with nitrogen for

30 min, the reaction was allowed to proceed at 70 °C for 2.5 h. The reaction mixture was then quenched by cooling the reaction vessel in liquid nitrogen and exposure to air. The product was purified by dialysis against DI water (pH 4 to 5), followed by lyophilization.

Size exclusion chromatography (SEC) was used to determine the number-average molecular weight (M_n) and polydispersity indices (PDIs) for the PMAG macroCTA and the block copolymers of MAG and AEMA using an aqueous eluent of 1.0 wt % acetic acid/0.1 M Na₂SO₄. A flow rate of 0.3 mL/min, Eprogen (Downers Grove, IL) columns [CATSEC1000 (7 μm, 50 × 4.6), CATSEC100 (5 μm, 250 × 4.6), CATSEC300 (5 μm, 250 × 4.6), and CATSEC1000 (7 μm, 250 × 4.6)], a Wyatt HELEOS II light scattering detector ($\lambda = 662$ nm), and an Optilab rEX refractometer ($\lambda = 658$ nm) were used. Astra V (version 5.3.4.18, Wyatt Technologies, Santa Barbara, CA) was utilized for the determination of M_n , PDI, and dn/dc of the (co)polymers.

¹H NMR measurements were performed with a temperature-controlled Varian 400-MR (Palo Alto, CA) spectrometer operating at a frequency of 399.7 MHz. Samples were prepared in D₂O (HOD internal standard), and spectra were recorded for each copolymer at a temperature of 70 °C. Block copolymer compositions were determined by comparing resonances of the PMAG block with those associated with the PAEMA block.

Polyplex Formation and Characterization. The pDNA binding ability of P1, P2, and P3 was examined with gel electrophoretic shift assays. pCMV-luc (Plasmid Factory, Bielefeld, Germany) (1 μg) was mixed with an equal volume of polymer at N/P ratios (the ratio of primary amine (N) on the diblock copolymer to the phosphates (P) on the DNA backbone) between 0 and 10. After an incubation time of 45 min, a 10 μL aliquot was run in a 0.6% agarose gel containing 6 μg of ethidium bromide/100 mL TAE buffer (40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid (EDTA)).

Polyplex sizes were measured by dynamic light scattering (DLS) at 633 nm on a Malvern Instruments Zetasizer Nano ZS (Westborough, MA). pCMV-luc (3 μg) was incubated with an equal volume of P1, P2, and P3 (150 μL in H₂O) at N/P values of 2, 5, and 10 for 1 h to form the polyplexes. Each sample was then diluted to 900 μL with H₂O, Opti-MEM, or supplemented Dulbecco's modified Eagle's medium (DMEM) (10% fetal bovine serum (FBS)) to determine the salt and serum stability of the polyplexes. The particle sizes were then measured at 25 °C at time intervals of 0, 20, 40, 60, and 240 min after dilution using a detection angle of 173°. Measurements were performed in triplicate. The ζ potential measurements were also performed using the same instrument with a detection angle of 17°. The polyplexes were formed in water using the same procedure described above. After 1 h of incubation time, each sample was then diluted to 0.9 mL with nuclease-free water, and the ζ potential was measured in triplicate.

Transmission electron microscopy (TEM) was performed on polyplexes prepared as described above for DLS at an N/P ratio of 5. After 1 h, 5 μL of sample was applied in duplicate to a 400-mesh carbon coated copper grid for 60 s, after which time the excess liquid was removed by blotting the grids with filter paper. The samples were then negatively stained with a 2% uranyl acetate solution for 90 s before blotting. Images were recorded using a Philips EM420 transmission electron microscope (FEI, Hillsboro, OR) operated at 60 kV.

Cellular Uptake, Transfection, and Toxicity Studies. HeLa (human cervix adenocarcinoma) cells were cultured according to established protocol (ATCC, Rockville, MD). Cells were seeded on six-well plates at 250 000 cells/well and allowed to incubate in supplemented DMEM at 37 °C and 5% CO₂ for 24 h. Cyanine (Cy5)-labeled pDNA was prepared with a Label-IT Cy5 DNA labeling kit (Mirus, Madison, WI) according to the manufacturers protocol. The polyplexes were prepared by combining 250 μL of Cy5-labeled pDNA (5 μg) with 250 μL of P1, P2, or P3 at N/Ps of 2, 5, 10 or with 250 μL of JetPEI (N/P = 5) or Glycofect (N/P = 20). The cells were then transfected with

0.5 mL of the polyplex solutions in 1.0 mL of either Opti-MEM (serum-free media) or DMEM (10% FBS). The cells were incubated with each solution for 4 h to allow internalization of the polyplexes. After 4 h, supplemented DMEM was added, and the cells were incubated for an additional 20 h. The cell growth media was replaced with supplemented DMEM 24 h after transfection. 48 h after initial transfection, the cells were incubated in CellScrub for 15 minutes at RT, trypsinized, pelleted, and resuspended in phosphate-buffered saline (PBS). A FACSCanto II (Becton Dickinson, San Jose, CA) equipped with a helium-neon laser to excite Cy5 (633 nm) was used. A total of 10 000 events were collected in triplicate for each sample. The positive fluorescence level was established by visual inspection of the histogram of negative control cells such that <1% appeared in the positive region.

To determine the transgene expression and cytotoxicity of the polyplexes, HeLa cells were cultured in a manner similar to that described above in 24-well plates (50 000 cells/well) and incubated for 24 h prior to polyplex exposure. Polyplexes were prepared immediately before transfection by combining solutions of each polymer (150 μ L) with an equal volume of pDNA (3 μ g) at N/P values of 2, 5, and 10. As positive controls, polyplexes formed with JetPEI (N/P = 5) and Glycofect (N/P = 20) were prepared. The polyplex mixtures were incubated for 1 h and diluted to 900 μ L with either Opti-MEM or DMEM. The cells were transfected with 300 μ L of polyplex solution or with naked pDNA in triplicate. After 4 h, 800 μ L of DMEM was added to each well. The media was replaced 24 h after the transfection with 1 mL of DMEM. After 48 h, the cells were washed with 500 μ L of PBS and treated with lysis buffer (Promega, Madison, WI) for 15 min at room temperature. Aliquots (5 μ L) of cell lysates were examined on 96-well plates with a luminometer (GENios Pro, TECAN US, Research Triangle Park, NC) for luciferase activity. The amount of protein in the cell lysates was measured according a standard curve of bovine serum albumin by following the protocol provided by Bio-Rad (Hercules, CA) DC protein assay kit. Cell viabilities after treatment with polyplexes of P1, P2, P3, and the positive controls were measured by the amount of protein in the cell lysates obtained 48 h after the transfection as well as by an MTT assay. For the MTT assay, cells were plated at a density of 50 000 cells/well in a 24-well plate. The cells were incubated for 24 h prior to transfection. Polyplexes were formed using the same procedure as that used for size and ζ potential measurements with 600 μ L of either OptiMEM or DMEM used to dilute the polyplex solutions. Cells were transfected in triplicate with 1 μ g of plasmid DNA added to each well. After 4 h, 800 μ L of DMEM was added to each well. The media was replaced 24 h after the transfection with 1 mL of DMEM. After 48 h, cells were washed with 0.5 mL of PBS per well, and 1 mL of DMEM containing 10% FBS and 0.5 mg/mL of 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) (Molecular Probes, Eugene Oregon) was added to the cells. Cells were incubated with the MTT-containing media for 1 h at 37 °C, the media was aspirated off, cells were washed with PBS as described above, and 600 μ L of DMSO was added to each well. Cells were incubated on a shaker for 15 min to ensure even distribution of the purple formazan. Subsequently, 200 μ L was pipetted out of each well and into a clear 96-well plate and analyzed for absorbance at 570 nm using a Tecan GENios Pro plate reader (TECAN US).

Confocal Microscopy. To synthesize the fluorescein isothiocyanate (FITC)-labeled P3, 14.19 mg (48 equiv) of P(MAG₄₆-b-AEMA₄₈)·48HCl were dissolved in 1000 μ L of 0.1 M NaHCO₃, which was previously deoxygenated by bubbling N₂ through the solution for 30 min. The polymer solution was cooled in an ice bath, and 20.0 μ L of a DMSO solution containing 0.20 mg (1 equiv) of FITC were added with a pipet. The reaction mixture was allowed to warm slowly to RT. After 12 h of stirring, the reaction mixture was placed in a dialysis bag (MWCO 3500 Da) and dialyzed for ~8 h against the following aqueous solutions: 0.1 M NaCl (4 L \times 2), 0.07 M NaCl (4 L), 0.03 M NaCl (4 L), H₂O (4 L \times 3).

The contents of the dialysis bag were then transferred to a vial and lyophilized, yielding a bright-yellow powder.

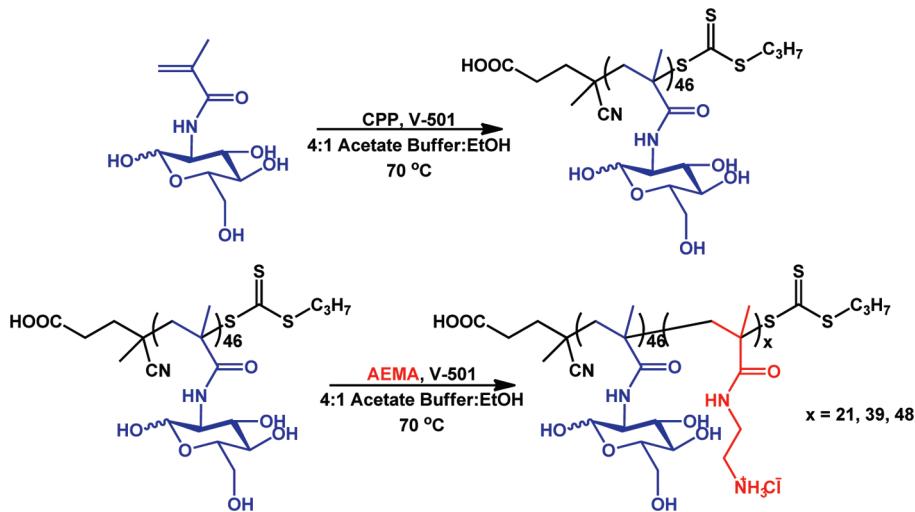
HeLa cells were seeded onto PLL-coated coverslips in a 12-well plate at 15 000 cells/well and incubated in DMEM containing 10% FBS at 37 °C, 5% CO₂ for 48 h to reach confluence. After incubation, polyplexes were formed by adding 50 μ L of FITC-labeled P3 to 50 μ L of a 0.02 mg/mL Cy5-labeled pDNA solution (1 μ g) to give 100 μ L of polyplex solution at an N/P ratio of 5. The DMEM was aspirated off the cells, cells were washed with 1 mL of PBS/well, and 1 mL of Opti-MEM was added to each well. The 100 μ L of polyplex solution was added to each well, giving a total of 1 μ g of pDNA/well. Four hours after transfection, 1 mL of supplemented DMEM was added to each well. Cells were fixed in 4% paraformaldehyde 4, 24, 48 h after transfection, stained with 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) according to manufacturer's protocol and mounted onto microscopy slides using ProLong Gold Antifade Reagent (Molecular Probes) according to manufacturer's protocol. The slides were allowed to dry overnight in the dark before being sealed with clear nail polish and imaged on a Zeiss LSM 510 META confocal microscope (Carl Zeiss MicroImaging, LLC, Thornwood, NY). Images were taken using a slice thickness of 0.8 μ m. Mander's coefficients were used to determine colocalization between P3 and plasmid DNA as previously described^{23,24} and were obtained using the software NIH ImageJ.²⁵

siRNA Gene Knockdown in Glioblastoma Cells. Luciferase expressing cells (U-87, glioblastoma cells), which were purchased from Caliper LifeSciences that have been engineered to incorporate a pGL4 luciferase reporter vector into the genome, were seeded at 50 000 cells/well in 24-well plates. In general, 6.6 μ L of a 10 μ M stock solution of either antiluciferase siRNA (8.8 ng) or scrambled siRNA (siCON) (9.8 ng) (IDT, Coralville, IA) and an appropriate amount of glycopolymers stock solution (1 μ g/ μ L) were diluted to 33 μ L with RNase free water to give an N/P ratio of 20. The polyplexes were then formed by the addition of the glycopolymers solution (33 μ L) to the siRNA solution (33 μ L), followed by incubation for 45 min at room temperature. The polyplex solutions were subsequently diluted with prewarmed Opti-MEM to yield final volume of 660 μ L with an siRNA concentration of 100 nM. Cells were washed with PBS before 200 μ L/well of polyplex solution was added. The lipoplex formation follows the standard protocol for Lipofectamine 2000. The cells were incubated for 4 h before fully supplemented DMEM (1 mL/well) was added. Cell lysates were analyzed 48 h after the transfection for luciferase activity and protein content according to the procedure detailed above. The protein level and the luciferase activity of untransfected cells were utilized for normalizing the data and calculating the extent of knockdown.

Statistical Analysis. All data are presented as mean \pm standard deviation. Measurements were performed in triplicate unless otherwise noted. For statistical analysis of the data, means were compared using a Student's *t* test with *p* < 0.05 being considered as statistically significant.

■ RESULTS AND DISCUSSION

Synthesis and Characterization of P(MAG-*b*-AEMA) Diblock Glycopolymers. RAFT provides a facile technique for preparing well-defined diblock copolymers of preselected compositions to test the effect of cationic block length on nucleic acid complexation, compaction, and delivery. As mentioned, this synthetic technique offers a means to tailor materials for biological use, offers structures with low dispersity, and is ideal for polymerizing highly functionalized monomers that are commonly used in the design of nucleic acid and drug delivery. The diblock copolymers of MAG and AEMA were synthesized according to Scheme 1. A trithiocarbonate, CPP, was used to mediate the aqueous RAFT polymerization of MAG in the presence of the free radical initiator V-501 to yield PMAG₄₆.

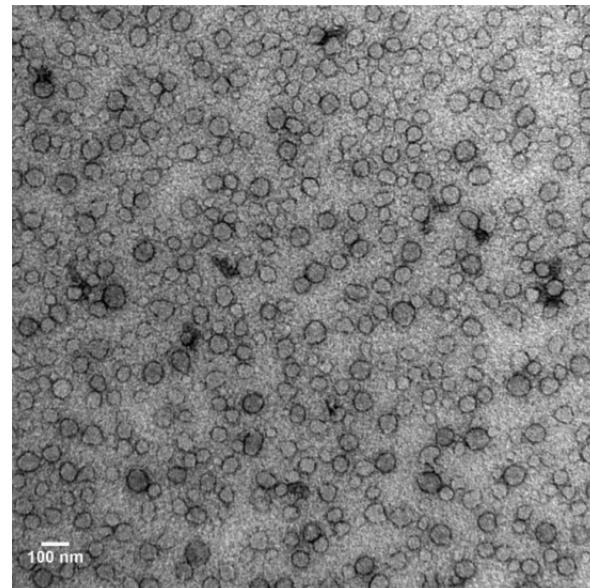
Scheme 1. Synthesis of P(MAG₄₆-*b*-AEMA_x) Nucleic Acid Delivery Vehicles via RAFT Polymerization**Table 1.** Molecular Weight Data for the Series of P(MAG₄₆-*b*-AEMA_x)

polymer	<i>M_n</i> ^a	PDI ^a	MAG DP ^b	AEMA DP ^b
macroCTA	11 700	1.24	46	
P1	14 400	1.15	46	21
P2	16 700	1.12	46	39
P3	17 800	1.12	46	48

^a As determined by aqueous size exclusion chromatography using a flow rate of 0.3 mL/min of 1.0 wt % acetic acid/0.1 M Na₂SO₄, Erogen CATSEC100, CATSEC300, and CATSEC1000 columns, a Wyatt HELEOS II light scattering detector ($\lambda = 662$ nm), and an Optilab rEX refractometer ($\lambda = 658$ nm). ^b As determined by ¹H NMR spectroscopy (1.5 mg/mL in D₂O at 65 °C).

The PMAG₄₆ macroCTA was subsequently chain-extended with AEMA to produce three diblock copolymers with varying AEMA block lengths. All polymerizations were performed in an aqueous acetate buffer to minimize hydrolysis and maintain the trithiocarbonate chain ends.^{26,27} SEC chromatograms of PMAG₄₆ and the three block copolymers, P1, P2, and P3, were unimodal with low PDIs (<1.25) indicating near-quantitative blocking efficiency and controlled polymerization (Supporting Information, Figure S1). ¹H NMR studies of the glycopolymers (Supporting Information, Figure S2) revealed compositions in agreement with those calculated from SEC molecular weight measurements. The molecular weight and composition data of the diblock copolymer series are summarized in Table 1.

The ability of the three diblock glycopolymers to bind pDNA was examined using gel electrophoresis shift assays at N/P ratios between 0 and 10. All three diblock glycopolymers completely bind pDNA below an N/P ratio of 3 (Supporting Information, Figure S3). DLS and ζ potential measurements were performed on polyplexes formed at N/P ratios of 2, 5, and 10 in nuclease-free water (Supporting Information, Figure S4). DLS revealed sizes between 55 and 115 nm, which is within the size range to allow uptake into cells through the endocytic pathway.^{28,29} Zeta potential measurements of the polyplexes in nuclease-free water varied between 15 and 40 mV, and the values increased

**Figure 2.** TEM micrograph of polyplexes formed between P1 and pDNA at an N/P ratio of 5 in water.

with an increase in AEMA block length and N/P ratio. TEM was performed on the polyplexes formed at an N/P ratio of 5. The micrograph for polyplexes formed with P1 and pDNA in H₂O (Figure 2) revealed monodisperse polyplexes with sizes in agreement with DLS measurements.

It is envisioned that the block copolymer architecture of P1, P2, and P3 should lead to an interpolyelectrolyte core consisting of complexed nucleic acid and the AEMA block with a shell of MAG chains. The corona of MAG (Figure 1) was designed to maintain water solubility as well as provide steric stabilization against aggregation in the presence of salts and anionically charged serum proteins. In support of our core–shell structural hypothesis, Ziebarth and Wang recently reported a molecular dynamics simulation of DNA condensation by block copolymers composed of a neutral and a cationic block and concluded that

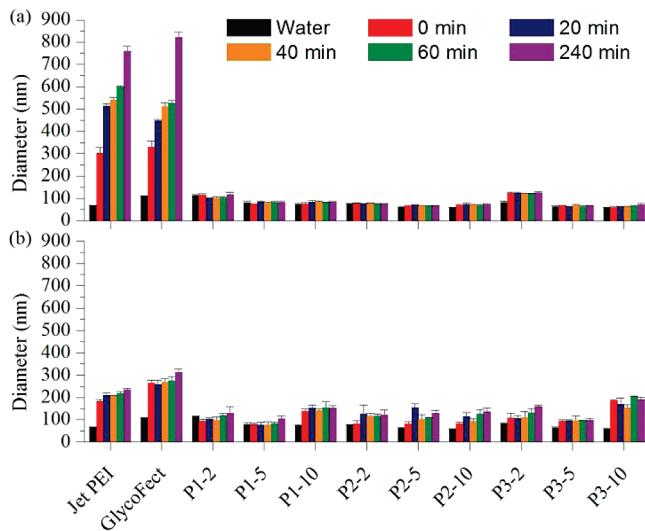


Figure 3. Hydrodynamic diameter of the polyplexes formed between pDNA and P1, P2, and P3 at N/P ratios of 2, 5, and 10 at 0, 20, 40, 60, and 240 min after dilution with (a) Opti-MEM and (b) DMEM (containing 10% FBS). Error bars represent the standard of deviation of analyzed data from three replicates.

the subsequent complexes have a similar core–shell structure.³⁰ To assess the colloidal stability of the polyplexes formed with this series of diblock glycopolymers, the sizes of the polyplexes formed at N/P ratios of 2, 5, and 10 were examined via DLS at 0, 20, 40, 60, and 240 min after dilution with two different types of cell culture media: either Opti-MEM (contains physiological salt and other small molecule nutrients) or DMEM (supplemented with 10% fetal bovine serum). Upon the addition of Opti-MEM, polyplexes formed with two commercially available transfection agents, Jet PEI (N/P = 5) and Glycoflect (N/P = 20), exhibited substantial aggregation, as evidenced by a dramatic increase in hydrodynamic diameter from <100 nm to >750 nm over a time period of 4 h. On the contrary, the hydrodynamic diameter of the sugar-coated polyplexes formed with the diblock glycopolymers was almost completely uniform and did not significantly increase compared with the polyplexes in water (Figure 3a). In a similar study by Wang and coworkers, the colloidal stability of a related series of block copolymers composed of PEG and poly(N-(2-aminoethyl) methacrylate) (PAEM) in the presence of salt was studied.³¹ Interestingly, the block lengths of the PEG (DP = 45) and the PAEM (DPs = 19, 39, and 75) are similar to the block lengths utilized in our current study, which allows for a direct comparison of the incorporation of PEG vs PMAG as a stabilizing block due to the similarity of the DPs and cationic block chemistry. In the presence of 150 mM NaCl, the polyplexes formed with the shortest two polymers, PEG-*b*-PAEM₁₉ and PEG-*b*-PAEM₃₉, doubled in size over a time period of 4 h, whereas PEG-*b*-PAEM₇₅ only slightly increased in size. Our results, coupled with those of Wang and coworkers, illustrate one advantage of using a glycopolymer instead of PEG as a stabilizing block in this core–shell glyco-polyplex motif. After dilution with DMEM (Figure 3b), the glycopolymer-derived polyplexes slightly increased in size, likely because of the interaction of the anionically charged serum proteins with the excess surface charge at the N/P ratios examined. In general, the increase in the size of the polyplexes corresponds to the increase in ζ potential at higher N/P ratios. However, the polyplexes do still remain more stable

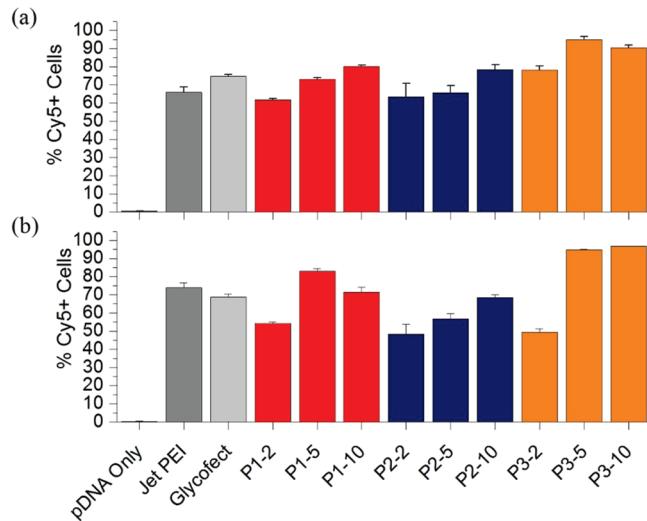


Figure 4. Percentage of Cy5 positive HeLa cells transfected with polyplexes formed at N/P ratios of 2, 5, and 10 with Cy5-labeled pDNA and P1, P2, and P3 in (a) Opti-MEM and (b) DMEM. Error bars represent the standard of deviation of analyzed data from three replicates. All measurements except pDNA only were found to be statistically significant ($p < 0.05$) compared with cells only.

than the controls in 10% serum and are still in a size range appropriate for cellular endocytosis.^{28,29} These results demonstrate that the presence of the MAG block aids in the colloidal stability of the polyplexes in salt and serum conditions by providing a hydrophilic steric barrier to prevent aggregation. Ahmed and Narain recently reported the RAFT synthesis of block and statistical copolymers of 3-gluconamidopropyl methacrylamide (GAPMA, an open-ring carbohydrate form) and either *N*-(3-aminopropyl)methacrylamide (APMA) or AEMA.¹⁹ Interestingly, the polyplexes formed between their copolymers and pDNA were found to aggregate over time in serum-containing media.

The utility of the diblock glycopolymers as pDNA delivery vehicles was examined with cultured HeLa (human cervix adenocarcinoma) cells in Opti-MEM and DMEM. The cellular uptake profile of polyplexes formed with each polymer type complexes with Cy5-labeled pDNA was first analyzed by flow cytometry to determine the number of cells positive for Cy5 fluorescence (Figure 4) and the average fluorescence intensity per cell (Supporting Information, Figures S5). In both Opti-MEM and DMEM, the percentage of Cy5 positive cells generally increased with increasing N/P values and with longer AEMA block lengths. At N/P values of 5 and 10, P1, P2, and P3 showed similar or better internalization compared with the two positive control polymers (JetPEI (N/P = 5) and Glycoflect (N/P = 20)). This trend could be related to the concurrent increase in polyplex ζ potential noticed with the increasing block length (Supporting Information, Figure S4) and is commonly observed in polyplex systems.³¹ Also, polymer P3 was revealed to transfect the highest number of cells as compared with the analogs with shorter AEMA blocks in both the presence and the absence of serum. Interestingly, in the presence of DMEM (contains 10% serum), polymer P1 yielded the highest average Cy5 intensity per cell, indicating that more nucleic acid, on average, was getting into the cells that were successfully transfected under these conditions (Supporting Information, Figures S5).

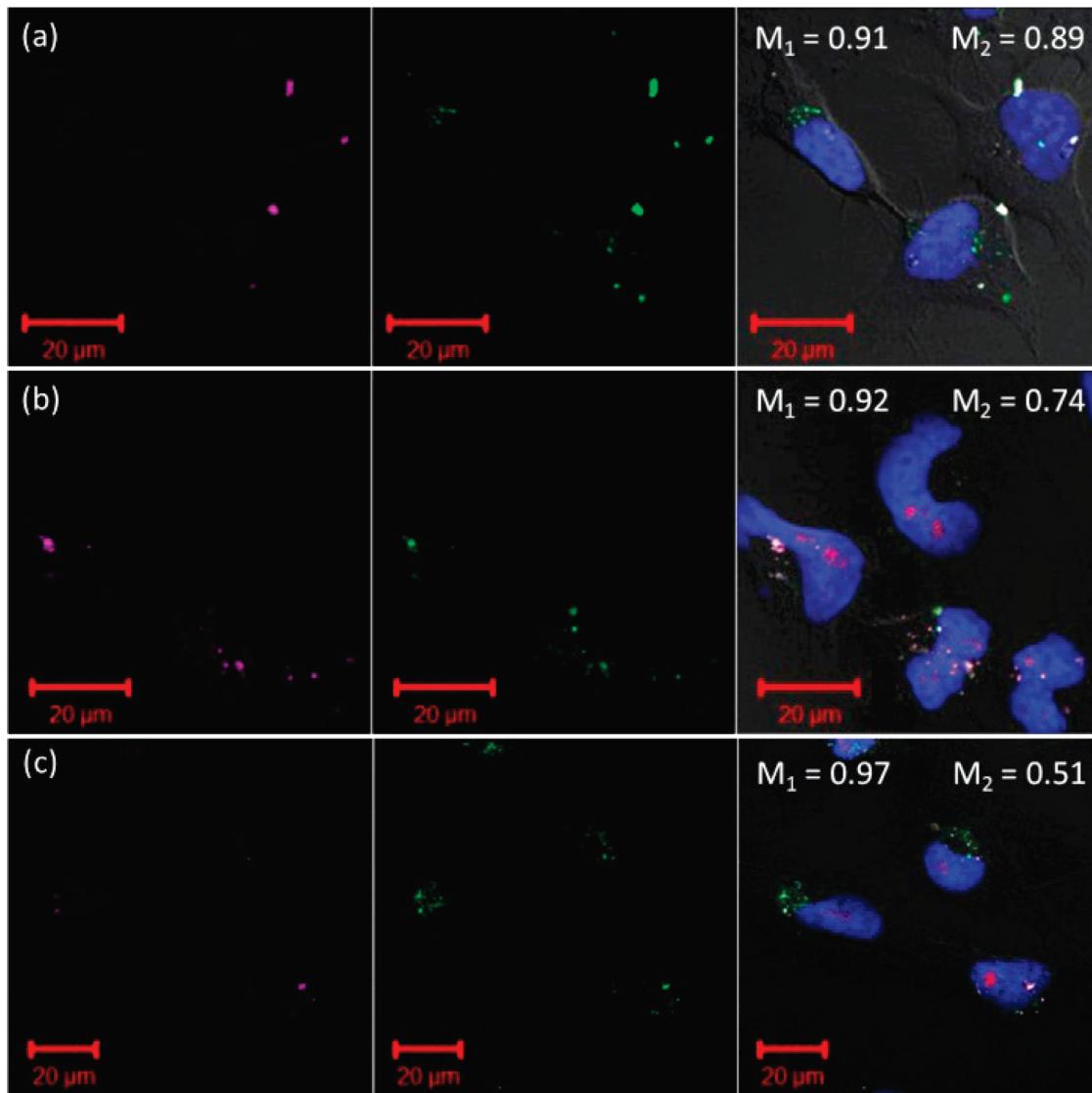


Figure 5. Confocal micrographs of HeLa cells transfected with polyplexes (white) formed between FITC-labeled P3 (shown in green) and Cy5-labeled pDNA (shown in purple). Nuclei (shown in blue) were stained with DAPI. Z-stacked images are available in movie format in the Supporting Information (for 4, 24, and 48 h time points).

Confocal microscopy was also performed to visualize cellular internalization of the polyplexes as well as examine the degree of colocalization of FITC-labeled P3 and Cy5-labeled pDNA (Figure 5). Confocal images were recorded 4, 24, and 48 h after transfection, and movies of the compiled z-stacks for each time point shown in Figure 5 can be found in the Supporting Information. The Mander's coefficients were calculated to determine two parameters: (i) M_1 represents the extent of the Cy5-labeled pDNA overlap with the FITC-labeled P3 and (ii) M_2 represents the extent of overlap of FITC-labeled P3 with Cy5-labeled pDNA.^{23,24} It is interesting to note that although M_1 is >0.9 for the three time points (indicating full colocalization of all pDNA with polymer); however, M_2 decreases with the time course of the experiment from 0.89 at 4 h to 0.51 at 48 h. This observation suggests that the pDNA remains closely associated with the P3 delivery vehicle as polyplexes, but excess polymer appears to dissociate from the polyplexes throughout the time course of the experiment.

The transfection efficiency of the glycopolymer/pDNA polyplexes was determined in cultured HeLa cells using luciferase as a reporter gene (Figure 6). In Opti-MEM, there was no significant increase in luciferase gene expression in cells transfected with polyplexes formulated with the glycopolymers at N/P ratios of 2 and 5. At an N/P ratio of 10, however, there was a two orders of magnitude difference between cells only and cells transfected with P1. Interestingly, pDNA expression decreased with longer AEMA block lengths. These results may be attributed to two possible factors. First, the DNA-binding block is composed of primary amines that are fully protonated at physiological pH and lack buffering capacity and therefore cannot promote endosomal swelling and lysis during the acidification event according to the "proton sponge" hypothesis.^{32,33} However, further studies with siRNA (vide infra) reveal that this may not be the case because siRNA does reach its cytoplasmic destination in a very effective manner. Second, and more likely, the primary amines in the AEMA block may bind too strongly to release the plasmid for

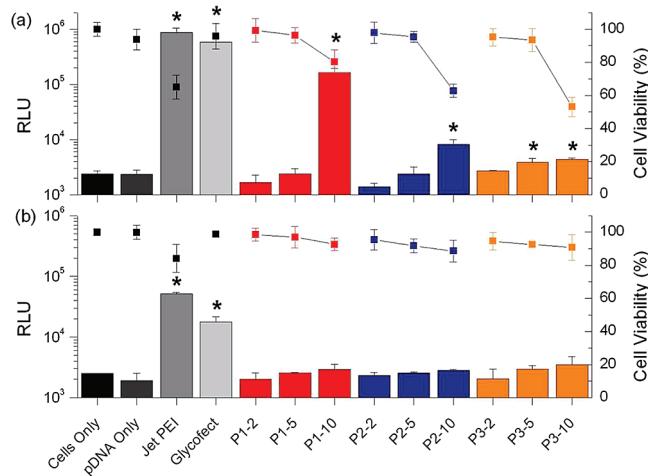


Figure 6. Luciferase gene expression and cell viability observed in HeLa cells transfected with polyplexes formed at N/P ratios of 2, 5, and 10 with pDNA and P1, P2, and P3 in (a) Opti-MEM and (b) DMEM. Error bars represent the standard of deviation of analyzed data from three replicates. Measurements found to be statistically significant ($p < 0.05$) compared with cells only marked with an asterisk.

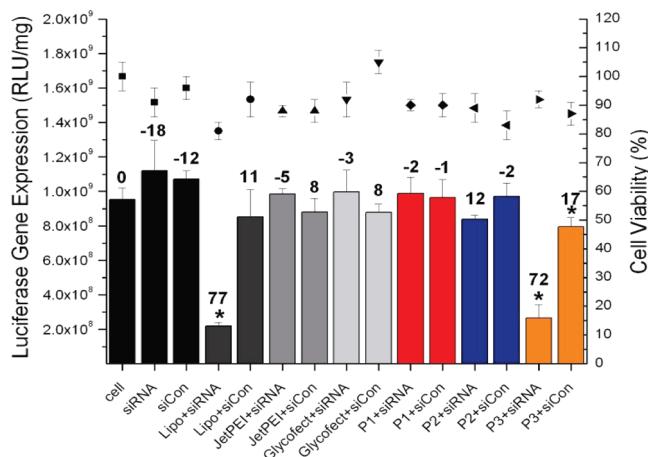


Figure 7. Luciferase gene expression (bars, left y axis) and cell viability (points, right y axis) in U-87 cells that stably express luciferase. The cells treated with polyplexes formed at an N/P ratio of 20 with an antiluciferase siRNA (100 nM) and glycopolymers P1, P2, and P3 in Opti-MEM. The percentage gene knockdown compared with the cells only control are given as numbers above the corresponding bars. Measurements found to be statistically significant ($p < 0.05$) compared with cells only marked with an asterisk. Error bars represent the standard of deviation of analyzed data from three replicates.

transcription. It has been shown previously that longer primary amine blocks bind DNA more strongly than shorter blocks.³¹ This is also supported by the Mander's coefficients for pDNA colocalized with P3 (M_1) remaining >0.9 for the three time points examined by confocal microscopy.

HeLa cell viability was determined 48 h after transfection with the glycopolymer/pDNA polyplexes via an MTT assay (Figure 6). In Opti-MEM, the three glycopolymers maintained $>90\%$ cell viability at N/P values of 2 and 5, comparable to cells treated with pDNA only and with polyplexes formed with Glycofect (N/P = 20). At an N/P of 10, however, there was a marked increase in

toxicity and cell viability appeared to decrease as the AEMA block lengths increased. In the presence of DMEM containing 10% serum, all three glycopolymers maintained cell viability $>90\%$.

The ability of the diblock glycopolymers to deliver siRNA to the cellular cytoplasm was evaluated by conducting luciferase gene knockdown experiments in U-87 (human glioblastoma) cells that stably express luciferase. Polyplexes were formed by mixing the three glycopolymers with an antiluciferase siRNA as well as a scrambled control sequence (siCon) to examine siRNA-mediated gene knockdown and also off target cytotoxic effects (via siCON experiment) of the polymer vehicle. The experiments utilized a final siRNA concentration of 100 nM and a polymer concentration to give an N/P ratio of 20. It should be noted here that the siRNA concentration used in this experiment is lower than the pDNA concentration used. The dose and N/P ratios were optimized in a number of preliminary experiments to determine the proper siRNA and polymer dose to promote gene knockdown while minimizing toxicity and off target effects. As positive controls, the antiluciferase siRNA was complexed with Lipofectamine 2000, JetPEI (N/P = 20), and Glycofect (N/P = 50) using the manufacturers' suggested protocol. After 48 h, the luciferase levels of the treated cells were determined using both protein and luciferase assay kits (Figure 7). Cells treated with polyplexes formed with the glycopolymers P1 and P2 and the antiluciferase siRNA did not show significant gene knockdown compared with the negative controls. Remarkably, glycopolymer P3 demonstrated luciferase gene knockdown on par with Lipofectamine 2000 (72% for P3 and 77% for Lipofectamine 2000). The polyplexes composed of P3 + siCon also demonstrated statistically significant gene knockdown (17%), potentially due to the positive charge on P3 after releasing siCon leading to increased off target effects. As denoted in Figure 7 (right y axis), the cell viability was very high at these polyplex dose ratios, and thus the toxic effects were minimal. These results demonstrate how small differences in the polymer structure can have a large impact on delivery and efficacy of both pDNA (to the nucleus for gene expression) and siRNA (to the cytoplasm for gene knockdown).

CONCLUSIONS

In summary, we have described the synthesis of a small family of diblock glycopolymers for the delivery of nucleic acids. The glycopolymer/pDNA polyplexes were found to be colloidally stable in the presence of salt and serum over a period of 4 h, and this poly(glucopyranose) motif appears to be a viable alternative to PEG for avoiding aggregation. Polyplexes were formed with pDNA, and transfection results in HeLa cells indicated a dependence of the cellular uptake, transfection efficiency, and toxicity on the AEMA block length, with shorter blocks leading to higher expression profiles and lower toxicity. Interestingly, an opposite trend was found when examining polyplex formulations with siRNA and their ability to promote luciferase gene knockdown in U-87 cells; only P3 containing the longer AEMA block revealed effective siRNA delivery and significant gene knockdown.

These results lead us to believe that the shorter AEMA block length is sufficient to complex pDNA, promote cellular internalization, and more importantly, able to effectively unpackage its large polynucleotide payload for subsequent expression. However, it appears that a longer AEMA block length (P3) is needed to promote higher binding affinity and stable polyplex formation with the sensitive siRNA to discourage nuclease degradation while still retaining the ability to unpackage its smaller

oligonucleotide payload for gene knockdown. Current studies are focused on examining the effects of varying both the poly-(glucopyranose) and the AEMA block lengths as well as incorporation of other charge centers on the biological properties.

■ ASSOCIATED CONTENT

S Supporting Information. Additional SEC chromatograms, ¹H NMR spectra, gel electrophoresis shift assay, DLS, and flow cytometry results are provided in the electronic Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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