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Mannosylated poly(beta-amino esters) for targeted antigen presenting cell immune modulation

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Abstract

Given the rise of antibiotic resistance and other difficult-to-treat diseases, genetic vaccination is a promising preventative approach that can be tailored and scaled according to the vector chosen for gene delivery. However, most vectors currently utilized rely on ubiquitous delivery mechanisms that ineffectively target important immune effectors such as antigen presenting cells (APCs). As such, APC targeting allows the option for tuning the direction (humoral vs cell-mediated) and strength of the resulting immune responses. In this work, we present the development and assessment of a library of mannosylated poly(beta-amino esters) (PBAEs) that represent a new class of easily synthesized APC-targeting cationic polymers. Polymeric characterization and assessment methodologies were designed to provide a more realistic physiochemical profile prior to *in vivo* evaluation. Gene delivery assessment *in vitro* showed significant improvement upon PBAE mannosylation and suggested that mannose-mediated uptake and processing influence the magnitude of gene delivery. Furthermore, mannosylated PBAEs demonstrated a strong, efficient, and safe *in vivo* humoral immune response without use of adjuvants when compared to genetic and protein control antigens. In summary, the gene delivery effectiveness provided by

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Author Contributions

CHJ conceptualized and designed the overall study, conducted all biological experiments, analyzed all data, and co-wrote the manuscript. AR conducted polymer experiments and analyzed resulting data. MC conducted polymer experiments. RR assisted with animal experimentation and handling. GZ helped collect and analyze polymeric data. APH planned and analyzed animal studies. BAP analyzed all data and co-wrote the manuscript.

Notes

The authors declare no competing financial interests.

Supporting Information. Monomer information. Compiled polymer characterization data. Confirmatory NMR spectra. Additional cytotoxicity and TGDP comparisons. Raw degradation data of D4A4 and D4A4-Man. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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mannosylated PBAE vectors offers specificity and potency in directing APC activation and subsequent immune responses.

Keywords

gene therapy; nonviral vector; cationic polymers; vaccines; antigen presenting cells

INTRODUCTION

Genetic vaccination is a promising immunization strategy capable of provoking potent protective responses without the need to handle or administer pathogenic strains that provide the target antigens (Ags). Upon administration of plasmid DNA (pDNA) encoding a desired Ag, a process of cellular internalization, expression, and processing occurs, which biases subsequent immune responses towards cellular immunity. However, DNA vaccines have also been documented to induce potent humoral responses[1]. Additionally, through the use of molecular adjuvants and intracellular processing sequences, immune responses can be further directed towards a specific arm of the adaptive immune response[2].

A key mediator of adaptive immunity is the initial Ag uptake and processing by antigen presenting cells (APCs; macrophages and dendritic cells). These cells are sentinels of the innate immune system and are involved in the clearance of cellular debris, pathogens, and others immunogens. Furthermore, APC-targeted delivery of genetic antigens can improve the magnitude and coverage of elicited protective responses. Analogously, by targeting only immune-effector cells, anergy-related responses derived from systemic delivery and expression will be reduced[3]. A prominent method to target APCs is through *ex vivo* loading and re-inoculation[4]. Though effective, this approach cannot be readily applied to vaccines or other therapeutic treatments that require rapid administration or utilization under resource-limited circumstances (e.g., emerging markets). Other approaches being developed for APC targeting include use of dedicated promoters and targeting ligands[5, 6].

Cell-specific promoters have demonstrated the ability to limit off-target expression[5] while providing a tool-set to readily tailor transcription to any desired immune-effector cell type. However, expression-exclusionary approaches to APC-targeting fail to prevent off-target uptake which necessitates larger initial delivery doses. Targeting can be attained with more ease and control using cell-specific endocytic receptors. Cell receptor expression patterns are generally cell- and location-specific,[7, 8] resulting in spatial up- and down-regulation of particular receptors across the body.

Macrophages represent a viable therapeutic target that can be used in the treatment and prevention of cancer and tumor progression,[9–11] auto-immune diseases (such as arthritis), [12, 13] and vaccines[13, 14]. Like dendritic cells, macrophages have demonstrated a comparable ability to process and present Ags *in vivo*[15]. Other reports have shown that macrophages can effectively cross-present Ag[16] or be polarized to either Th1 (cell-mediated)[17] or Th2 (humoral) responses[18]. Targeting of APCs can be attained through the activation of the mannose receptor (MR; CD206)[19, 20]. MR is a member of the calcium-dependent lectin family, found primarily on the surface of macrophages and

dendritic cells (and to a lesser extent on the surface of dermal fibroblasts[21] and keratinocytes[22]), and selectively recognizes polysaccharides (mannose, fucose, and *N*-acetylglucosamine) that are abundant on microbes[23]. Previous studies have indicated that expression of MR on APCs plays an important role in Ag processing and presentation against microbial pathogens[24, 25]. Additionally, MR-mediated endocytosis allows for rapid and successive accumulation of mannosylated Ag for eventual MHC presentation through constitutive recycling[26].

In the past, polymer conjugate approaches have been used to target anatomical or cellular sites, including the MR[27]. However, complications associated with complex biomaterial synthetic schemes, associated polymer cytotoxicity, incomplete ligand conjugation, and the costs associated with attaching biological targeting molecules (such as antibodies) must be addressed for eventual application. Cationic polymers are commonly used as nucleic acid complexation agents for the purpose of gene delivery[28–33]. In particular, the poly(beta-amino ester) (PBAE) family of polymers has garnered attention for simple synthetic schemes, ease of functionalization, and safe degradation profiles[28–30]. As such, a PBAE was utilized as a basis for mannose conjugation and targeted macrophage gene delivery.

In the present study, a mannosylated-PBAE was synthesized to guide gene delivery capabilities to macrophage APCs. In so doing, significantly improved *in vitro* gene delivery metrics were observed when compared to non-mannosylated and commercially-vetted control vectors. These results prompted *in vivo* assessment with similarly significant improvements in immune responses. Specifically, we report improved standardized antibody titers, without the need for adjuvant addition, that outperform DNA and protein antigen controls. In addition, unequivocal MR contributions to antibody titers were observed. Lastly, data from the *in vitro* gene delivery studies suggest a unique processing mechanism of MR-mediated uptake that more readily permits the processing of polyplexes towards transgene expression.

MATERIALS AND METHODS

Measurements

All ^1H -NMR spectra were measured at 500 MHz in d-DMSO using a Varian INOVA-500 spectrometer maintained at 25°C with tetramethylsilane (TMS) as an internal reference standard. Gel permeation chromatography (GPC) data were acquired from a Viscotek GPC system equipped with a VE-3580 refractive index (RI) detector, a VE 1122 pump, and two mixed-bed organic columns (PAS-103M and PAS-105M). Dimethylformamide (DMF; HPLC) containing 0.01 M LiBr was used as a mobile phase with a flow rate of 0.5 mL/min at 55°C. The GPC instrument was calibrated through narrowly-dispersed linear polystyrene standards purchased from Varian. Average hydrodynamic diameters (D_h) and zeta potential of polyplexes were obtained using dynamic light scattering (DLS) on a Zetasizer nano-ZS90 (Malvern, Inc.) at 25°C. All experiments were conducted using a 4 mW 633 nm HeNe laser as the light source at a fixed measuring angle of 90° to the incident laser beam. The correlation decay functions were analyzed by cumulants method coupled with Mie theory to obtain volume distribution. Microplate experiments were analyzed using a Synergy 4

MultiMode Microplate Reader (BioTek Instruments, Inc.). FACS analysis was conducted using a Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Materials

Monomers were purchased from Sigma-Aldrich (St. Louis, MO) and TCI (Portland, OR). Acetone (HPLC), DMF (HPLC), and DMSO (99.7%) were purchased from Fisher Scientific (Pittsburgh, PA). Allyl alcohol (99%), D-(+)-mannose (cell culture grade) and 4-toluenesulfonyl chloride (p-TsCl) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) was purchased from Life Technologies (Grand Island, NY).

Synthesis of Allyl- α -D-mannopyranoside (ADM)

ADM was synthesized by dissolving 3 g of D-(+)-mannose and 18 mg p-TsCl in allyl alcohol (20 mL) at 90°C under reflux for 24 h.

Synthesis of Mannosylated Poly(beta-amino esters)

A library of mannosylated-PBAEs (PBAE-Man) was synthesized using the following three-step procedure (Scheme 1). First, acrylate-terminated PBAEs were synthesized without solvent at a 1:1.2 amine/diacrylate molar ratio (using 1,000 mg amine) for 24 h at 90°C with continuous stirring at 1,000 rpm. Second, acrylate-terminated polymers were reacted with excess ethylenediamine to amine-cap the terminal ends. Specifically, acrylate-terminated polymers were dissolved in DMSO at 167 mg/mL and 500 μ L reacted with 500 μ L of 0.5 M ethylenediamine (in DMSO) at 22°C for 24 h. Amine-capped polymers were purified by dialysis followed by evaporation under vacuum. Dialysis procedures were conducted against acetone using molecular porous membrane tubing (Spectra/Por Dialysis Membrane, Spectrum Laboratories Inc.) with an approximate molecular weight cut off at 3,500 Da. Third, amine-capped PBAEs were then reacted with ADM at a 1:2 molar ratio in DMSO at 90°C for 24 h and then purified via dialysis. Structure and purity of polymers were confirmed using ^1H NMR spectroscopy (Figure S6).

Degradation Studies

Desiccated PBAE samples were resuspended to a concentration of 1 mg/mL. RPMI-1640 (pH 7.4) or 25 mM sodium acetate buffer (pH 5.15), with and without 10% FBS, were used as the suspension media. Solutions were sealed in Eppendorf tubes and incubated at 37°C with mild shaking. At predefined time intervals, aliquots of 1 mL of polymer solution were withdrawn and fully dried under vacuum. The resulting polymer was dissolved in DMF for GPC analysis.

Cell Lines and Reporter Plasmids

A RAW264.7 (murine macrophage) cell line was provided by Dr. Terry Connell (Department of Microbiology and Immunology, University at Buffalo, SUNY). The cell line was maintained in medium prepared as follows: 50 mL of FBS (heat inactivated), 5 mL of 100 mM MEM sodium pyruvate, 5 mL of 1 M HEPES buffer, 5 mL of penicillin/streptomycin solution, and 1.25 g of D-(+)-glucose added to 500 mL RPMI-1640 and filter sterilized. The HeLa (human epithelial) cell line was provided by Dr. Stelios Andreadis

(Department of Chemical and Biological Engineering, University at Buffalo, SUNY) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% (v/v) FBS (GIBCO) and 1% (v/v) Antibiotic-Antimycotic (A/A; Gibco). All cell lines were housed in T75 flasks and cultured at 37°C/5% CO₂.

To determine *in vitro* transfection efficacy, we employed two reporter genes encoding luciferase and EGFP with each driven by a cytomegalovirus promoter within plasmids pCMV-Luc (Elim Biopharmaceuticals) and pCMV-EGFP (Addgene plasmid 11153[34]). Alternatively, *in vivo* transfection was evaluated using pCI-neo-cOVA (Addgene plasmid 25097[35]). Plasmids were transformed into and isolated from an *E. coli* cloning host (GeneHogs, Invitrogen) using a PureYield™ Plasmid Midiprep System (Promega) prior to being used in the experiments outlined below.

Gel-Shift Assay

pDNA (500 ng/well) was mixed by gentle vortexing with each polymer at various ratios in 25 mM NaOAc (pH 5.15). After 15 min incubation at 22°C, mixtures were loaded into a 0.8% agarose gel containing SYBR® Safe DNA Gel Stain (Invitrogen) and electrophoresed at 80 V for 30 min. Gels were visualized under UV after electrophoresis to determine the minimal polymer concentration required.

Preparation of Polymer:pDNA Polyplexes

Polyplexes were prepared by electrostatic interaction between respective PBAEs and pDNA by mixing various weight ratios of polymer to 500 ng of pDNA. First, working dilutions of PBAEs were prepared in 25 mM NaOAc (pH 5.15) to a final concentration of 4 mg/mL. Depending on the desired weight ratio, differing amounts of PBAE stock solution were mixed with pDNA in 25 mM NaOAc (pH 5.15), gently vortexed, and incubated 30 min. Taking a 200:1 polyplex ratio as an example, 125 µL of PBAE stock solution was mixed with 2,500 ng pDNA and 25 mM NaOAc (pH 5.15), as needed, to provide a final volume of 150 µL, which was then distributed as 500 ng pDNA/well.

Polyplex Characterization

Using polyplexes prepared as described above, zeta potential and effective diameter were measured by in either PBS (pH 7.4) or 25 mM NaOAc (pH 5.15). All data points result from three independently formulated polyplex samples measured five times.

Hemolytic activity was assessed using a previous protocol[33]. Briefly, a 5% RBC/PBS solution was prepared by washing sheep blood (HemoStat Laboratories) with PBS until the supernatant became clear. Next, 100 µL of purified 5% RBC solution was incubated with 900 µL of PBAE (at various concentrations) in PBS either at pH 7.4 or 5.15 for 1 h at 37 °C. Water was used as a positive control to construct a % blood lysis standard curve by altering the amount of blood added to each respective sample. For example, for 50% and 100% lysis, 50 and 100 µL of purified 5% RBC solution was mixed with the balance water. PBS was used to generate negative controls. Samples were centrifuged and hemolysis quantified by measuring supernatant at 541 nm and comparing to the % blood lysis standard curve.

***In Vitro* Transfection Studies**

For gene delivery experiments, RAW264.7 and HeLa cell lines were seeded at densities of 3×10^4 and 2×10^4 cells/well, respectively, in two different types of 96-well plates (four plates in total) in 100 μ L medium/well and allowed 24 h for attachment. A tissue culture-treated, flat-bottom, sterile, white, polystyrene 96-well plate was used for luciferase assays; whereas, bicinchoninic acid (BCA) (and also 3-(4, 5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) and EGFP flow cytometry) assays were conducted in tissue culture-treated, sterile, polystyrene 96-well plates. Each plate was carried out as a duplicate of the other.

Following a 24 h incubation, the medium was removed and replaced with 100 μ L growth medium plus 30 μ L of PBAE:pDNA polyplexes, prepared as described above (total volume of 130 μ L), and incubated for an additional 4 h. Polyplex-containing media was then removed using a 12-channel aspirating wand and replaced with 100 μ L fresh, antibiotic-free RPMI-1640 medium preheated to 36°C. Similarly, FuGENE 6 (Promega, Madison, WI), FuGENE HD (Promega), Xfect (Clontech Laboratories, Inc., Mountain View, CA), and JET-PEI (Polyplus-transfection SA, Illkirch, France) were included as positive controls and used according to each manufacturer's instructions. Cells were allowed to incubate for an additional 24 or 48 h prior to gene delivery assessment. After a second 24 h incubation (48 h after initial seeding), plates were analyzed for luciferase expression using the Bright Glo assay (Promega) and protein content using the Micro BCA Protein Assay Kit (Pierce) according to each manufacturer's instructions. Gene delivery was calculated by normalizing luciferase expression by protein content for each well/plate. Results derive from six replicates and two independent experiments.

To measure the transfection efficiency of cells seeded and transfected as described above, flow cytometry was performed. Two days after transfection, cells were washed with ice-cold PBS and detached from the well surface using cell scrapers (RAW264.7) or 0.25% Trypsin/1mM EDTA (HeLa). For HeLa cells, trypsin was inactivated using an equal volume of DMEM supplemented with 10% FBS. Next, HeLa cells were centrifuged (250 g, 5 min) and resuspended in PBS. The fraction of EGFP-expressing cells was quantified using FACS. Results derive from twelve replicates and four independent experiments. Additionally, images of EGFP-expressing cells were captured using a Zeiss Axio Observer (Zeiss, Thornwood, NY) inverted microscope and photographed with an ORCA-ER CCD camera (Hamamatsu, Japan).

Gene Delivery Inhibition Studies

To determine if transfection was affected by the presence of free mannose and increasing levels of serum, polyplexes were prepared at a 200:1 PBAE to pDNA weight ratio. Vectors were then transfected as before using RAW264.7 cells with the following alterations. First, three concentrations of free mannose were added either 30 minutes prior to transfection or concomitantly with polyplexes. Separately, media was replaced with increasing levels of FBS (20, 30, and 50%) 30 min prior to transfection. After a 4-hr incubation, the altered media was replaced with regular growth media.

MTT Assay

Cytotoxicity resulting from polyplexes was determined by the MTT colorimetric assay. RAW264.7 cells were seeded and transfected as described above. Following a 24 h incubation after vector addition, cells were assayed with MTT solution (5 mg/mL), added at 10% v/v, for 3 h at 37°C/5% CO₂. Medium plus MTT solution was then aspirated and replaced by DMSO to dissolve the formazan reaction products. Following agitated incubation for 1 h, the formazan solution was analyzed using a microplate reader at 570 nm with 630 nm serving as the reference wavelength.

Mouse Model Immunization Studies

Female BALB/cByJ mice aged 8 weeks were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed and utilized in accordance with institutional guidelines. Chicken ovalbumin and complete and incomplete Freund's adjuvant (CFA & IFA, respectively) were purchased from Sigma. For immunizations, polyplexes were prepared at a 200:1 weight ratio and diluted in PBS to 10 µg pDNA per 100 µL. Antigens were emulsified with the adjuvants in PBS to yield 1 mg/mL (OVA protein) and 100 µg/mL (OVA pDNA) solutions. Mice (6 animals per group) were immunized at days 0 and 14. Polyplexes were injected (100 µL) subcutaneously and intraperitoneally, while controls (plasmid and protein) were only injected (100 µL) subcutaneously. Initial immunization of controls included CFA, which was replaced with IFA during booster immunizations. At days 14 and 21 post immunization, retro-orbital blood samples were collected and clarified by centrifugation to collect serum. Serum anti-OVA antibody titers were quantified using the Cayman Anti-Ovalbumin IgG₁ (mouse) EIA Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Statistical Evaluation

Unless otherwise indicated, data presented were generated from three independent experiments. Error bars represent standard deviation values. All statistical significance comparisons between groups were performed using a one-way ANOVA with Dunnett (to compare within groups) or Bonferroni (to compare across groups) post-tests.

RESULTS AND DISCUSSION

Polymer Synthesis and Characterization

Synthesis was completed using a modified end-capping scheme with monomers defined in Scheme 1 and Table S1[30, 36]. Resulting polymers were named according to the diacrylate and amine monomers used during synthesis. Similarly, base polymers were end-capped with mannose using a three-part synthetic strategy (Scheme 1A). Each respective mannosylated and non-mannosylated PBAE structure and purity was confirmed using ¹H NMR (Figure S6). GPC and DLS were used to measure the size, polydispersities, and zeta potential of the polymers (Table S2).

Polyplex Formation and Characterization

To form polyplexes, polymer:pDNA weight ratios were evaluated by using an agarose gel retardation assay to determine the minimal amount of each respective PBAE to completely complex pDNA (Table S2). A 200:1 polymer to pDNA weight ratio was selected for all *in vitro* high throughput screening experiments unless otherwise indicated. Mannosylation of PBAEs resulted in an increased amount of polymer required to fully complex pDNA.

To investigate the biophysical properties associated with 200:1 polyplexes, DLS was used to measure the zeta potential and effective diameter in both PBS and 25 mM NaOAc (Figure S1A and B). Polyplexes were generally negatively charged in PBS and positively charged in acidic buffer. Similarly, buffer-specific trends were observed for polyplex particle diameters. Neutral pH resulted in larger particles ranging in size from 200 to 500 nm. However, in acidic pH, polyplexes were generally smaller presumably because of functional group ionization providing stronger pDNA electrostatic interaction. Mannosylation appeared to have no clear impact on the trends observed.

For eventual applicability, a polymer must possess a safe cytotoxicity and hemolysis profile. Thus, PBAEs were examined for their cytotoxicity against RAW264.7 cells using a MTT assay (Figure S2). PBAEs of various doses were incubated with cells for 4 or 24 h. At 4 h (Figure S2A), non-mannosylated PBAEs were non-toxic at the smallest doses but demonstrated increased toxicity with increasing polymer dose. However, several mannosylated PBAEs demonstrated minimal or only slightly reduced toxicities at doses of 0.5 and 1.0 mg/mL (which correspond to 100:1 and 200:1 polyplexes), but cytotoxicity increased henceforth with polymer dose. At 24 h, all cytotoxicity profiles were exacerbated, but mannosylated PBAEs produced low cytotoxicity with viabilities near or above 80% at the lowest polymer dose. One polymer, D4A4-Man, was only slightly toxic after 24 h incubation with 95 and 87% viability at 0.5 and 1.0 mg/mL polymer levels.

To further evaluate biocompatibility, hemolysis assays were performed to assess interaction between PBAE polyplexes and red blood cells (RBCs). It is proposed that cationic polymers can interact with the cell membranes of RBCs to form nanosized pores, resulting in an influx of small solutes and osmotic lysis[37, 38]. The assay was conducted in neutral and acidic conditions to approximate circulation and lysosomal environments (Figure S1C). In neutral pH, low hemolysis (<20%) was observed with non-mannosylated polymers; whereas, mannosylation resulted in negligible hemolysis (<~5%). Reduction in hemolysis suggests a shielding role for mannose, similar to that expected of molecules such as poly(ethylene glycol)[32, 33]. At an acidic pH, hemolysis was slightly depressed in most cases when mannosylated polyplexes were compared to their non-mannosylated counterparts. However, the small relative changes under these conditions suggest that endosomal release mechanisms would remain similar or unchanged.

In Vitro Transfection Studies

To test the transfection capabilities of mannosylated PBAEs with cells predominantly expressing CD206, a RAW264.7 (murine macrophage) line was utilized (Figure 1A). Transfection efficacy of polyplexes was compared with commercial controls that were

previously identified as effective gene delivery agents for RAW264.7 (which are noted for their difficulty of transfection[39]). Luciferase expression and % enhanced green fluorescent protein (EGFP) positive cells were positively correlated with increases in polymer to pDNA weight ratios for all polymers. However, all mannosylated polymers, with the exception of D4A3-Man and D6A5-Man, mediated significantly higher levels of transfection than their respective non-mannosylated variants at 100:1 and 200:1 weight ratios. Of these polymers, only D4A4-Man mediated transfection levels that exceeded all commercial controls. To visually demonstrate the transfection potential of D4A4-Man, fluorescent images were taken after a typical transfection using a 200:1 weight ratio and compared to respective controls (Figure 2). JET-PEI was selected for comparison as it is a mannosylated cationic polymer. D4A4-Man significantly outperformed both the commercial control and the non-mannosylated variant.

To further assess gene delivery properties of polyplexes, the non-phagocytic and physiologically distinct HeLa cell line was utilized[40]. This line also contains the MR but internally processes cargo differently[41]. Transfection (Figure 1B) resulted in a generally higher percentage of EGFP positive cells and improved luciferase expression for all polyplexes and controls. Unlike before, mannosylated polyplexes did not mediate improved transfection and most were unable to outperform positive controls. In previous studies, we observed that conjugation of moieties that do not help bind pDNA lead to reduce polyplex transfection at respective weight ratios[33]. With this cell line, this trend is also observed with mannosylated polyplexes, and only D4A4, D4A5, and D5A5 mediated comparable % EGFP positive values to that of the best performing positive control. D4A4 and D4A5 also provided statistically higher luciferase expression compared to all positive controls. From these results, D4A4 and D4A4-Man were selected as candidates for *in vivo* studies; these two samples were also selected to directly compare the PBAE backbone common to each.

Previously, we developed an improved quantitative metric for the comparison of transfection termed Total Gene Delivery Performance (TGDP). TGDP is the product of APC viability, population-based delivery efficiency (% EGFP positive cells), and gene delivery magnitude (luciferase expression). Analysis using TGDP provides a better representation of vector translation capabilities as many vectors have been unsuccessfully tested in clinical settings due to an insufficient value in one or more of the constituent variables (e.g., high gene delivery but high cytotoxicity). Thus, we applied this approach to our polymeric library (Figure 3A) using transfection data for RAW264.7 cells. Despite promising data for several polymers, only D4A4-Man outperforms commercial controls. In a separate analysis, TGDP was evaluated across polymers with respect to molecular weight, polyplex particle diameter, and polymeric zeta potential (in 25 mM NaOAc [pH 5.15]). Though no clear trends emerged, the highest TGDP values were associated with lower molecular weight and higher zeta potential values. This is an interesting observation as high molecular weight polymers are typically associated with optimal transfection (though also linked to cytotoxicity)[32]. In addition, larger polyplex particle diameter was weakly correlated with TGDP improvements for mannosylated PBAEs. Analogously, we prepared additional comparative schemes to further assess viability, zeta potential, and particle size dependencies as they relate to each other (Figure S3). From this set of comparisons, lower molecular weights resulted in slight positive correlations with cell viability improvements for mannosylated PBAEs. Cell

viability was negatively correlated to polyplex zeta potential for non-mannosylated polymers, supporting previous studies in which increased charge density is attributed to systemic toxicities (such as hemolysis) and cytotoxicities[32].

To further assess the role of mannosylation in improved transfection, a series of CD206 competition studies were designed using exogenous mannose (Figure 4A–C). Mannose was provided either prior to or during polyplex addition. Uninhibited % EGFP positive transfection levels were used as a standardizing element (i.e., 100% corresponds to uninhibited transfection). Pre-treated mannose addition resulted in dose dependent (statistically significant) depression of transfection for mannosylated polyplexes. However, non-mannosylated polyplex-mediated transfection increased with mannose concentration, resulting in several statistically significant improvements as in the case of D6A5. Using these observations, mannose was then added simultaneously with polyplexes (Figure 4B). Unlike before, mannose addition statistically facilitated transfection for mannosylated polyplexes which then decreased with further increased mannose doses. At the highest mannose dose, all mannosylated polyplex transfection values were comparable to uninhibited transfection. In this assay, non-mannosylated polyplexes again mediated statistically significant dose-dependent increases of transfection. Interestingly, vectors ineffective in mediating transfection (e.g., D4A3) demonstrated the most substantial improvements. To better illustrate improvements of non-mannosylated polyplexes, transfection values were re-standardized by their respective uninhibited mannosylated polyplex variants. Accordingly, at higher doses (500 and 1000 μ M) of mannose addition, non-mannosylated polyplexes mediated transfection at values comparable to their mannosylated variants (Figure 4C). These findings suggest that mannose alters the uptake and/or processing of polyplexes towards a mechanism that readily permits transfection.

In our previous studies of delivering pDNA via pegylated polyplexes,[33] we concluded that macrophages' enhanced ability to take up polyplexes through various mechanisms resulted in a higher gene delivery magnitude (i.e., luciferase expression) but did not assist population based transfection values (i.e., % EGFP positive cells). The results presented in this manuscript indicate that specific uptake mechanisms are ultimately tied to improved gene delivery. Mannosylated-mediated processing is commonly associated with endocytic trafficking towards recycling mechanisms (to refresh surface CD206)[42] and through proposed additional endocytic vesicles (early to late endosome)[43]. Whereas, other studies have indicated that general phagocytosis does not contribute significantly to biomolecule release, representing less than 5% of processed cargo[44]. Thus, mannosylation of PBAEs is clearly triggering uptake and processing mechanisms beneficial to eventual gene delivery. At the same time, studies have indicated that gene delivery can be significantly improved by designing synthetic vectors that are capable of avoiding degradative compartments of the cell[45–48] or by escaping endocytic recycling mechanisms[49]. For example, lipid nanoparticles have been associated with a “gentler” endosomal escape mechanism[50]; whereas, cationic polyplexes' pore formation and subsequent burst provide a “rougher” means of compartment escape and cargo release. The combination of biased mannosylated-mediated uptake and rougher release mechanics of cationic polymers may help explain our results and suggests additional combinatorial studies in which variations of targeting

moieties, conjugation methods, and backbone vectors are utilized to further assess, optimize, and tune APC gene delivery, antigen processing, and subsequent immune responses[51].

For PBAE-based polyplexes to be relevant for *in vivo* applications, gene delivery must remain effective in the presence of heightened levels of extracellular protein. Generally, serum proteins bind positively charged complexes through electrostatic interactions, resulting in destabilization, aggregation, and clearance[32]. As such, transfection of RAW264.7 cells was conducted in various percentages of fetal bovine serum (FBS) (Figure 4D). Similar to our previous observations,[33] significant decreases in % EGFP positive cells occur with increasing serum percentage. Interestingly, the rate of transfection efficiency declination was significantly reduced when using mannosylated vectors. This result suggests that mannosylation also serves as a beneficial shielding agent that can simultaneously improve targeting specificity as well as *in vivo* residence time.

Degradation Studies

Upon selection of the top performing polymers, it was necessary to assess the degradation profiles prior to initiation of *in vivo* studies. Cationic polymer degradation is a key determinant in gene cargo unpackaging and vector-associated cytotoxicity[32, 33]. Premature degradation can compromise polyplex stability and may result in undesired cargo release. To more accurately mimic physiologically-relevant settings, we designed a study to determine degradation profiles using a nutrient rich salt solution (RPMI) with and without FBS (Table 1 and Figure S3). The half-life of D4A4 was 2 h with and 13 h without serum addition. D4A4-Man, however, degraded slower for every condition tested.

Furthermore, after APC uptake, polyplexes will be processed through a series of endocytic vesicles that gradually acidify through fusion with other vesicles towards the formation of a lysosome. An acidic environment is thought to be required to release pDNA cargo through the proton sponge effect[52]. As this environment is marked by lower pH and high protein content, we utilized an acidic buffer (25 mM NaOAc pH 5.15) with and without FBS. In these media, both polymers degraded at the same rate as RPMI plus serum, indicating a degree of independence of pH and serum addition. As before, mannosylation resulted in increased stability, presumably by some form of steric inhibition to degradation.

Immunization Studies

To test the capabilities of the top performing PBAEs in an *in vivo* setting, we utilized an ovalbumin (OVA) mouse immunization model (Figure 5A and S5A). At predefined time-points, serum was collected and the antibody response against OVA antigen was quantified. Additionally, at day 14, a secondary immunization was utilized to boost the immune response.

No toxicity, in terms of adverse side effects or death, was observed regardless of whether mice were immunized subcutaneously (S.Q.) or intraperitoneally (I.P.) and total IgG₁ titers elicited from both sets of polyplexes were significantly higher than the OVA pDNA positive control regardless of administration route at day 14. At day 21 (7 days following secondary administration), only D4A4-Man polyplexes elicited significantly higher antibody titers compared to the pDNA control. At this time point, the S.Q. route of administration elicited

higher antibody titers than I.P. injection. D4A4-Man polyplexes produced significantly higher antibody titers than non-mannosylated variants regardless of administration route or time point. No polyplex elicited higher titers than immunization using OVA protein with adjuvant. However, this control represents an upper limit to humoral immune responses and is seldom matched by synthetic gene delivery carriers unless the OVA protein control is administered without adjuvant addition[53].

However, the efficacy of D4A4-Man polyplexes is unequivocally higher when the antibody response is standardized by the amount of antigen (Figure 5B and S5B). The data presented is consistent with the unstandardized results of Figure 5A with the exception of D4A4-Man polyplexes. The mannosylated polyplex demonstrates statistically higher standardized antibody titers than the OVA protein plus adjuvant control at both time points and when using either administration route. Notably, the results emphasize the potential of the mannosylated polyplexes for genetic vaccination with lower antigen doses and without the need for adjuvant addition.

APC-targeted therapeutics represent a next generation vaccination strategy for modulating immune responses towards a desired outcome[54]. In the current study, the mannosylated PBAEs enable APC targeting which initiates uptake and intracellular processing of the vector and genetic antigen. Though the PBAE is not specifically designed to aid immune recognition or response, the polymeric nature of the vector triggers endosomal/lysosomal destabilization as the compartment acidifies, the polymer backbone degrades, and genetic cargo is translocated. Previously, APC-targeted vaccines have been used in the context of generating cell-mediated responses; however, only recently has it been demonstrated that APC immune modulation can elicit effective humoral responses[55], similar to this study. In particular, studies have suggested that targeting of resident tissue macrophages will trigger preferred antibody responses[56]. Presumably, this occurs due to the natural ability of macrophages to express and/or retain Ags for up to 72 h after exposure[57]. Ag are retained due to possible binding of CD169 or MAC1 (macrophage receptor 1) followed by cumulative presentation to respective T and B cells[58, 59]. In the context of particulate-based vaccines, these studies indicate an essential role of macrophages (together with DCs) in the initiation of humoral responses by 1) acting as sentinels at the lymph-tissue interface, 2) concentrating Ag in immune tissue (i.e., lymph nodes), and 3) presenting sustained Ag signals. As such, selection and understanding of particular APC subsets as target cells can further direct the magnitude and type of response elicited from polymer-based genetic vaccination.

CONCLUSION

The library of mannosylated PBAEs developed in this study highlights the ability of APC targeting to elicit statistically significant levels of gene transfection both *in vitro* and *in vivo*. This new class of vector demonstrates the combined impact of vector type and functionalization as evident by significant improvements in transfection assays when compared to commercial controls. Results were further extended by completing similarly successful *in vivo* immunization experiments. In summary, the new vector and approach

offer a broader set of metrics and methodologies to extensively evaluate gene delivery in the context of APC targeting and immune response modulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. RAW264.7 (A) and HeLa (B) cells transfected with polyplexes prepared at three polymer to pDNA weight ratios. *Statistical significance (95% confidence) relative to commercial vector controls. #Statistical significance (95% confidence) for improved performance relative to respective non-mannosylated polymers.

Figure 2. Fluorescent microscopy of top-performing polymers. Images depict EGFP-positive RAW264.7 cells resulting from transfection using naked pDNA (A), JET-PEI (B), D4A4 (C), and D4A4-Man (D).

Figure 3. Total gene delivery performance (TGDP) analysis. TGDP is the product of transfection efficiency (percent EGFP positive cells) and magnitude (luciferase) and cell viability after transfection. Comparisons are made with respect to transfection vector (A), PBAE molecular weight (B), polyplex particle diameter (C), and zeta potential (D). *Statistical significance (95% confidence) relative to commercial vector controls.

Figure 4.

Gene delivery inhibition studies. Transfection of RAW 264.7 was conducted using 200:1 polymer to pDNA polyplexes in media with mannose added either before (A) or concomitantly with polyplexes (B and C). Polyplexes were also transfected in media containing increasing concentrations of FBS (D). Data was standardized relative to polyplex transfection in mannose-free medium with 10% FBS (A, B, D). Panel C is the same data presented in B, but standardization is relative to mannosylated polymers. For example, D3mA4 data used in panel B was standardized by transfection values of D3mA4-Man to yield data in panel C. *Statistical significance (95% confidence) for improved performance relative to standardizing transfection (A, B, C) or between respective mannosylated and non-mannosylated samples (D). #Statistical significance (95% confidence) for decreased performance relative to standardizing transfection.

Figure 5.

Mouse model immunization. Total (A) and normalized (B) antibody responses are compared between genetic (pDNA) and protein OVA antigens delivered with adjuvant and top-performing PBAE polyplexes without adjuvant. Statistical significance (95% confidence) compared to pDNA (*; panel A and B; respective time points) and protein (@; panel B; respective time points) OVA controls. #Statistical significance (95% confidence) compared to non-mannosylated polymer (respective time point and administration route). ‡Statistical significance (95% confidence) of S.Q. administration route compared to I.P. route.

Scheme 1.

Polymer synthesis (A) and associated monomers (B). Diacrylate (D) and amine (A) monomers were copolymerized to create acrylate-terminated base polymers (i) which were then amine-capped using excess ethylenediamine (E, ii) and reacted with allyl- α -D-mannopyranoside (ADM) to create mannosylated polymers (iii). Respective polymers are named by their monomers and mannose content. For example, reacting diacrylate monomer D4 with amine monomer A4 results in the synthesis of polymer 'D4A4.' 'D4A4-Man' represents the mannosylated variant.

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Table 1

Polymer degradation study

Polymer	Half-life in Water (pH 7.4)	Half-life in RPMI (pH 7.4)	Half-life in RPMI (pH 7.4) w/Serum	Half-life in NaOAc (pH 5.15)	Half-life in NaOAc (pH 5.15) w/Serum
D4A4	8 h	13 h	2 h	2 h	2 h
D4A4-Man	17 h	19 h	8 h	8 h	8 h