SYNTHETIC POLYMERS WITH WELL-DEFINED STRUCTURES FOR
DNA VACCINE DELIVERY AND CANCER THERAPY

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Weihang Ji

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Abstract

Non-viral gene delivery continues to suffer from the lack of safe and efficient polymeric carriers because the underlying mechanism of polymer mediated gene transfer and expression is still not fully understood. Current undefined and polydisperse synthetic polymer systems are not good models for investigating structure-function relationship of polymeric gene carriers. Furthermore, in the context of DNA vaccine delivery, there is a lack of understanding of the interaction between polymer/DNA complexes (polyplexes) and antigen presenting cells (APCs). In this work, we aimed to reveal the structure-function relationship of polymers for DNA vaccine delivery by employing polymers with well-defined chemical structure and narrow molecular weight distribution synthesized by living radical polymerization. We also explored the cytotoxicity of certain cationic amphiphilic polymers for anticancer application. First, we focused on understanding the effect of polymer molecular weight of poly(2-aminoethyl methacrylate) (PAEM) on DNA vaccine delivery. Second, the relationship between hydrophobicity of polymers and their performance in gene delivery to dendritic cells was investigated by using well-defined cationic poly(aminoalkyl methacrylate) (PAAM) polymers with varied length of side groups. Lastly, we evaluated the possibility to use a synthetic membrane lytic poly(6-amino-1-hexyl methacrylate) (PAHM) polymer which kills cancer cells effectively as an anticancer agent. We also described a strategy to reversibly mask the cytotoxicity of PAHM by forming acid sensitive PAHM-maleate conjugates for future in vivo applications.
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Chapter 1: Introduction

1.1 Non-viral vectors for gene delivery

Gene therapy is defined as the treatment of human disease by transferring genetic materials into specific cells of patients to direct the synthesis of specific proteins [1]. Gene therapy has been evaluated in the treatment of genetic diseases such as haemophilia [2], muscular dystrophy [3], or cystic fibrosis [4] through replacement of errant genes within affected cells. Gene therapies are also being developed for cardiovascular [5], neurological [6], infectious diseases [7], wound healing [8], and cancer [9-11] by delivering genes to augment naturally occurring proteins, to alter the expression of existing genes, or to produce cytotoxic proteins or prodrug-activating enzymes-for example, to kill tumour cells or inhibit proliferation of endothelial cells to prevent restenosis [12].

Because of its great potential, gene therapy has been heavily investigated during the past two decades. Up to January 2013, 1902 gene-therapy clinical trials have been initiated or approved worldwide since 1989 [13], and 69 of them have entered Phase III stage. But the low number of successes is disappointing compared with the large number of clinical trials.

Although the genetics community has identified many genes that can be potentially used for therapeutic purposes, the efforts of the DNA delivery community have been hindered by a number of as yet unresolved challenges. Various extracellular and intracellular barriers limit transferring enough genetic information into targeted cells
to take effect, such as DNA uptake by cells, endosomal escape and transport from the cytoplasm to the nucleus, etc [12].

Viruses address many of the above barriers. Thus most of gene therapy in clinical trials has used recombinant viruses as gene delivery vehicles [14]. However, virus vectors are still not safe. Although recombinant viral vectors are rendered non-replicative, and therefore non-pathogenic, there still exists the possibility that the virus will revert to a wild-type virion or co-purify with replication-competent virions. Also, viral vectors could induce cancer via insertional mutagenesis-random transgenic insertion into the host chromosome disrupting the normal expression of a critical gene that ordinarily regulates cell growth and division [15-16]. Furthermore, viruses are inherently immunogenic, leading to difficulty with repeat administrations and possibility of dangerous immune reactions. Other challenges with viral gene delivery include limitations in target-cell specificity and cost of manufacturing viral-based gene therapies.

The safety issues of viral vectors have motivated researchers to develop safer, less pathogenic and immunogenic gene delivery alternatives, including lipids [17], inorganic materials [18], peptides [19], and polymers [12, 20-24]. In addition to the potential safety benefits, such non-viral systems offer greater structural and chemical versatility for manipulating physicochemical properties, vector stability upon storage and reconstitution, and a larger gene capacity compared to their viral counterparts [21]. Although lipids have been reported for gene delivery since 1987, lipid based gene delivery has crucial limitations, including difficulties in reproducibly fabricating liposomes and DNA-liposome complexes, toxicity to some cell types in vitro and in vivo, and colloidal stability, especially upon systemic administration. Peptide-based gene delivery is also
limited by the high expense of fabricating and potential immunogenic responses. Because of flexibilities of polymer chemistry, polymers can provide multiple functions required for efficient gene delivery while maintaining biocompatibility, facile manufacturing, and robust and stable formulation. As a result, cationic polymers have great potential for human gene therapy.

1.2 Cationic polymers as gene delivery carriers

Cationic polymers form complexes with DNA via electrostatic interaction between cationic groups in the polymers and anionic groups in DNA. These polymers possess amino groups, which can be completely or partially protonated at physiological pH, at a sufficient nitrogen to phosphate (N: P) charge ratio, the polymers can condense DNA to sizes compatible with cellular uptake while providing steric protection from nuclease degradation [25]. The representative examples of cationic polymer based gene delivery including poly(L-lysine) (PLL), poly(amidoamine) (PAMAM), poly(N-ethyl-4-vinylpyridinium bromide) (PVP), poly(dimethylaminoethyl methacrylate) (PDMAEMA), chitosan, linear and branched poly(ethylenimine) (PEI), poly(β-amino ester), poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) etc. (Figure 1.1).

In the history of non-viral gene delivery, many early gene delivery studies used commercially available polymers, such as PLL [26] and PEI [27]. Because these materials were not designed for gene delivery, their efficacy as gene delivery agents was somewhat serendipitous. Although these off-the-shelf polymers are not perfect for gene delivery, they have nonetheless been widely studied and form the basis for much of the non-viral gene delivery literature.
Figure 1.1 Cationic polymers used as gene delivery carriers.
1.3 Extracellular and subcellular barriers for polymer mediated gene delivery

After putting lots of effort into the off-the-shelf polymers, researchers have known more about how cationic polymers work to transfer genes. Although some of detailed mechanisms of gene delivery are still not clear, past research has shown the major barriers for polymer mediated gene delivery (Figure 1.2) [12, 20, 28].

![Figure 1.2](image)

Figure 1.2 Schematic illustrations showing the three major trafficking barriers of non-viral gene delivery: (1) The passage of DNA across the plasma membrane; (2) Protection and release of the DNA molecules; and (3) The passage of DNA across the nuclear membrane. The process can be divided into (A) DNA complex formation and cell binding, (B) cellular uptake and endocytosis, (C) escaping of the complex from endosome with limited stability, (D) cytosolic transit and nuclear entry, and (E) gene expression. Figure copied from [28]. Figure reprinted with permission from Elsevier: [Biomaterials] (v. 33, p. 3025), copyright (2012).

In general, polymeric vectors need to overcome three major trafficking barriers when delivering the gene cargos: (1) the passage of DNA across the plasma membrane, (2) protection and release of the DNA molecules, and (3) the passage of DNA across the nuclear membrane (Fig. 1.2). Of all the three barriers, the biggest hurdle for polymeric
gene delivery systems is the passage of DNA across the nuclear membrane into the nucleus for expression, which has been identified as a rate-limiting step for gene transfection [29]. In addition, for in vivo applications, those polymeric carriers must overcome organism level challenges including stability within blood circulation, prolonged circulation time, access to target tissue/cells, and minimal toxicity and immunogenicity [20].

Consequently, development of highly efficient and safe gene carriers is the most challenging work in the field of non-viral gene therapy [30]. In the past two decades, more sophisticated polymers aiming at overcoming both the extracellular and intracellular barriers have been developed. The boom in the non-viral gene delivery field helped researchers establish certain polymer design criteria for gene delivery [12, 20]. However, considerable work is still needed towards developing synthetic polymeric delivery systems with efficiencies approaching those of viruses. Also there is still significant gap in our general understanding of how these synthetic polymers work to deliver DNA to the nucleus of cells.

1.4 DNA vaccine and dendritic cell maturation

One important application of gene delivery is in DNA vaccines. DNA vaccination is a simple, scalable method for inducing immune responses without the many limitations of conventional vaccines based on attenuated pathogens and subunit vaccines [31]. In recent years, DNA vaccines have shown considerable promise in defending against a wide range of bacterial and viral pathogens [32] and have been evaluated as immunotherapeutics for cancer [33], where the proper mobilization of both arms of adaptive immunity is desired.
The major component of a DNA vaccine is a plasmid DNA that encodes for protein antigens of interest. Antigen presenting cells (APCs) transfected with the DNA vaccine express the encoded antigen endogenously, process and present the antigen through the Major Histocompatibility Complex (MHC) molecules, resulting in the generation of antigen-specific humoral and cellular immune responses in vivo [34]. Dendritic cells (DCs) are the most important APCs and play a central role in regulating innate and adaptive immunity [35]. Similar to macrophages, DCs are involved in acquiring antigens at the peripheral tissues and presenting antigens to naïve B and T lymphocytes in the spleen and draining lymph nodes. More importantly, DCs serve as a “master switch” of the immune system—between generating potent immunity to reject foreign antigen and inducing tolerance to self-antigen, the decision largely depends on the “context” (such as the maturation state of DCs), within which the antigen is presented [36]. Therefore, it is logical to focus on the ability to modulate the behavior of DCs when it comes to certain practical applications and clinical outcomes—for example, immune activation is required for effective vaccination and immunotherapy, whereas immune tolerance is desired for improving biocompatibility of biomaterial implants [37-38].

The success of DNA vaccination depends not only on the effective delivery of plasmid DNA encoding antigen to target APCs, but also on the activation of the APCs [34, 39]. Some polymeric biomaterials themselves can induce DC maturation under mechanisms which are still not clear [40]. However, adjuvants may still be useful to help improve DC maturation and activate immune response because the interaction between polymers and DCs is not fully understood.

1.5 Combinatorial approach for polymeric carrier design

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Polymer design for gene delivery suffers from the complex biological environment in which the carriers are employed. Combinatorial chemistry can drastically shorten development cycles by producing a large set of system descriptors (chemical structures, physical properties and biological characteristics associated with these structures), which can be used rationally to design vector systems. The concept of high-throughput combinatorial chemistry was previously introduced to the gene therapy field by the Langer lab, synthesizing a library of 140 poly(β-amino ester)s through one step conjugate addition of primary or secondary diamines to diacrylate monomers [41]. To improve the throughput of this approach, the same group further built a larger library containing 2350 poly(β-amino ester) structures [42]. From their screen the authors identified 46 new structures with transfection efficiencies exceeding optimized “gold standard” PEI. These reports outlined a clear strategy that could be used to screen polymer structures efficiently as a function of structure, DNA concentration, DNA/polymer ratio, cell lineage, cell density and so on.

Recently, David et al [43] developed three combinatorial libraries of polymeric vectors to investigate the functional roles of molecular weight (MW), cations, pH-sensitive moieties, and hydrophobic derivitization in polymer mediated gene delivery. In this work, side chains of poly(N-methacryloxy succinimide) were substituted by four cationic and pH-sensitive moieties (imidazole, primary, secondary, and tertiary amino) and three hydrophobic residues (C4 butyl, C6 hexyl, and C8 octyl) in single and serially incremented, binary combinations. After screening all the polymers in the libraries, the highest levels of transfection, comparable to branched PEI (25 kDa), were achieved by 30 kDa and 50 kDa formulations containing primary amino and imidazole groups.
Those two examples indicate clearly that the combinatorial approach is a simple, fast, and robust route to screen potential polymeric gene carriers. However, this approach is not sophisticated enough to reveal detailed underlying structure-functional relationship of polymers for gene delivery. Firstly, there are very limited types of monomers that can be polymerized in the combinatorial approach. Secondly, most of the polymers prepared in this way don’t have their structure or molecular weights well defined because of the polymer chemistry they employed. Lacking of controlled polymerization may make conclusions about performance of different polymers rough and inaccurate. Therefore, simple polymers with precisely controlled chemical structure and narrow molecular weight distribution qualify better as model systems to investigate structure-function relationships in non-viral gene or DNA vaccine delivery.

1.6 Atom transfer radical polymerization (ATRP) in gene delivery

Since 1995, the controlled living radical polymerization technique has been growing fast, which provides a powerful tool to synthesize polymers with well-defined compositions, architectures, and functionalities. In atom transfer radical polymerization (ATRP), polymerization is controlled by establishing a rapid dynamic equilibration between a minute amount of growing free radicals and a large majority of dormant alkyl halides [44]. A general mechanism for ATRP is shown in scheme 1.1.

\[
R-X + M_i^{n-Y} / \text{Ligand} \xrightleftharpoons[k_{\text{act}}]{k_{\text{deact}}} R^* + X-M_i^{n-1-Y} / \text{Ligand}
\]

Scheme 1.1 Transition-metal-catalyzed ATRP. Figure copied from [44]. Figure reprinted with permission from American Chemical Society: [Chemical Reviews] (v. 101, p. 2921), copyright (2001).
The radicals are generated through a reversible redox process catalyzed by a transition metal complex (Mt\textsuperscript{n}-Y/Ligand, where Y may be another ligand or the counterion) which undergoes a one electron oxidation with concomitant abstraction of a halogen atom, X, from a dormant species, R-X. This process occurs with a rate constant of activation, \( k_{act} \), and deactivation \( k_{deact} \). Polymer chains grow with the rate constant of propagation \( k_p \). Termination reactions (\( k_t \)) also occur in ATRP, mainly through radical coupling and disproportionation; however no more than 5% of the polymer chains undergo termination. This process generates oxidized metal complexes, X-Mt\textsuperscript{n+1}, as persistent radicals to reduce the stationary concentration of growing radicals and thereby minimize the contribution of termination. A successful ATRP will have not only a small contribution of terminated chains, but also a uniform growth of all the chains, which is accomplished through fast initiation and rapid reversible deactivation [44].

Using ATRP, polymers with defined molecular weight and narrow distribution can be synthesized in a controlled way. ATRP has wide selection range of monomers, which is good for developing diverse chemical structure and functionality. Through designing or transforming the initiating or ending sites of polymer chains in ATRP, end group functionality can be obtained. ATRP can also be used to synthesize materials with different topology, such as linear, block, graft, star, hyper-branched and dendritic polymers. In addition, most of the ATRP components are commercially available and the polymerization can be conducted under mild conditions.

Because of the benefits of ATRP, different cationic polymers have been developed for gene delivery by this technique [24, 45-46], most of which are polymers based on (meth)acrylate or (meth)acrylamide monomers, like 3-(N,N-dimethylamino)-
propyl methacrylamide (DMAPMA), 2-(N, N-dimethylamino) ethyl methacrylate (DMAEMA) and 2-(N,N-diethylamino) ethyl methacrylate (DEAEMA).

Overall, ATRP provides us a powerful tool to prepare model polymers with defined chemical structure and narrowly dispersed molecular weight for structure-function relationship study for gene or DNA vaccine delivery. Despite its capability of polymerizing a wide range of monomers, the polymerization of methacrylates presenting pendant amino groups remains difficult. This is because amines can provoke displacement of the ligand from the metal complex [47], and in the case of primary amines, react with the halogen end group of the initiator or of the growing polymer chain [48]. To solve this problem, proper protecting chemistry should be applied.

1.7 Hydrophobicity of cationic macromolecules relates to their cytotoxicity

Although lots of polymers with versatile structures have been claimed as potential gene carriers, toxicity of these polymers is still a big concern for non-viral gene delivery, especially for in vivo gene therapy [49]. Polymers are only used to transfect cells below the cytotoxic dose and much effort has been made to decrease or eliminate toxicity of polymeric carrier for gene delivery [49]. The commonly used gene carrier PEI has been found toxic to cells and induce cell death via necrosis or apoptosis based on their molecular weight and structure [50-51]. Our group found that higher N/P ratio of PEI/DNA polyplexes during tranfection resulted in higher cytotoxicity, lower transfection but higher antigen presentation, stronger DC maturation, and greater immune response in PEI-mediated DNA vaccine delivery via bystander cells [52]. Our results indicated that the balance between transfection and polyplex induced toxicity is important for
stimulating immune responses. Also it raises the possibility of harnessing cytotoxicity of polymer carriers for the benefit of DNA vaccine therapy.

The progress in the field of antimicrobial polymers informs us that charge type, molecular weight, chemical structure, and hydrophobicity of cationic polymers relate directly with their cytotoxicity to mammalian cells [53-54]. Among these structural parameters, hydrophobicity is particularly interesting because hydrophobic portions of certain polymers may potentially act as damage-associated molecular patterns to initiate repair, remodeling, and immunity [55], which is crucial for DNA vaccine therapy. Adjusting hydrophobicity of polycations affects the membrane binding of polymers through partitioning of hydrophobic portion into lipid layers, which in turn impacts the membrane integrity of cells [56]. Therefore, the hydrophilic/hydrophobic balance is important to control the cytotoxicity of cationic polymers, which is also true for many amphipathic membrane-disruptive anticancer peptides [57-58].

Instead of simply dumping cationic polymers with high cytotoxicity for general gene delivery or bacteria killing, we may explore their other biomedical applications, such as possible DNA vaccine adjuvants to activate APCs or a new type of anticancer agents.

1.8 Thesis overview

The non-viral gene delivery community continues to suffer from lacking safe and highly efficient polymer carriers mostly because the underlying mechanism of polymer mediated transfection is still not fully understood. Current undefined and molecular weight broadly dispersed polymer systems are not good enough to make accurate conclusions regarding the structure-function relationship of polymeric carriers. As for DNA vaccine delivery, not much
work has been done to reveal the interaction between polyplexes and APCs. In this work, we aimed to reveal the structure-function relationship of polymers for DNA vaccine delivery by employing polymers prepared by ATRP with well-defined chemical structure and narrow molecular weight distribution. We also explored the cytotoxicity of certain cationic polymers for anticancer application.

Chapter 2 focused on understanding the effect of polymer molecular weight on transfection efficiency. Cationic poly(2-aminoethyl methacrylate) (PAEM) polymers with precisely controlled chain length were synthesized and characterized for delivering plasmid DNA to antigen-presenting dendritic cells. Through a comprehensive study we revealed correlations between polymer chain length and colloidal properties of the polyplexes, transfection efficiency, and the capacity of inducing DC maturation and CD8+ T cell activation by a model DNA vaccine. In chapter 3, the relationship between hydrophobicity of polymers and their performance in gene delivery to dendritic cells was investigated. We developed a series of poly(aminoalkyl methacrylate) (PAAM) homopolymers with different alkyl size (ethyl, propyl, butyl, and hexyl) and hydrophobicity by ATRP and applied them for gene delivery to DCs. We studied comprehensively the colloidal properties of the PAAM/plasmid polyplexes (including stability, particles size and charge), uptake and subcellular trafficking in DCs, and biological properties in vitro (including hemolysis, cytotoxicity, transfection efficiency and DC maturation), aiming to reveal the impact of hydrophobicity of polymers on DNA delivery to DCs. In chapter 4, we evaluated the possibility to use a toxic cationic polymer poly(6-amino-1-hexyl methacrylate) (PAHM) as an antitumor agent. We found that the structurally simple, synthetic membrane lytic polymer PAHM was highly efficient in
killing cancer cells in vitro through cell membrane disruptions. In addition, a strategy to
reversibly mask the cytotoxicity of PAHM could be done by forming acid sensitive
PAHM-maleate conjugates. Chapter 5 gives out future directions to improve the outcome
of polymers for their specific applications.
Chapter 2: Poly(2-aminoethyl methacrylate) with Well-defined Chain Length for DNA Vaccine Delivery to Dendritic Cells

2.1 Introduction

In recent years DNA vaccines have shown considerable promise for numerous medical interventions—from prophylactic vaccine strategies that target viral, bacterial or parasitic infections to potential therapeutics for treating infectious diseases, cancer, and autoimmune disorders [59]. DNA vaccination has advantages over conventional vaccines because of its simplicity, flexibility and safety. A plasmid DNA that encodes for a protein antigen of interest is the basic component of a DNA vaccine formulation. Ideally, antigen-presenting cells (APCs) transfected with the DNA vaccine should express the encoded antigen endogenously, process and present the antigenic peptide fragments through the Major Histocompatibility Complex (MHC) molecules, resulting in the generation of antigen-specific immune responses. The most important APCs, the dendritic cells (DCs) [36], are widely considered the ideal target cells of DNA vaccines. Sustained antigen presentation from DCs combined with DC maturation is expected to generate robust adaptive cellular immunity, which is particularly necessary for combating cancer and viral infection [60].

A large number of cationic polymers with diverse structures and properties have been developed for gene delivery [21, 24, 61], some of which have been evaluated for DNA vaccine delivery [34]. Despite much effort in the past, designing polymers as DNA vaccine carriers has been complicated due to vast possible combinations of physicochemical variables and by the complex biological/immunological environment in
which the carriers are used. For many years the molecular weight, or chain length, of polymers has been shown to have significant impact on DNA delivery. The relationship between polymer molecular weight and transfection efficiency has been studied in many polymers such as branched polyethylenimine (PEI) [62], poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) [63], trehalose oligoethylenamine click polymers [64], and polyphosphoramidate (PPA) [65], to name a few. In general, increasing molecular weight will increase gene expression. However, some discrepancies exist [62, 66-67] and a fundamental understanding of the influence of linear polycation chain length on the gene transfer process, especially in the context of DNA vaccine delivery to immune cells, remains elusive.

Controlled polymerization techniques such as atom transfer radical polymerization (ATRP) [44-45] make it possible to prepare cationic polymers with defined chain length in a facile way. Recently, we have synthesized polyethylene glycol-block-poly(2-aminoethyl methacrylate) (PEG-b-PAEM) diblock copolymers with narrow molecular weight distribution by ATRP and have shown that the length of the cationic block exerted significant influence on how the polymer carrier interacted with plasmid DNA and how the polyplexes interacted with DCs [68]. Although the PEG-b-PAEM diblock copolymers are useful model systems, they had low transgene expression efficiency in DCs, a significant drawback that limits their practical usage in DNA vaccination.

In this study we focused on poly(2-aminoethyl methacrylate) (PAEM) homopolymers with defined chain length and narrow molecular weight distribution. We investigated comprehensively the colloidal properties of the PAEM/plasmid polyplexes
(including stability, particles size, and charge) and biological/immunological properties in vitro (including cytotoxicity, transfection efficiency, DC maturation, and activation of native CD8\(^+\) T cells to produce interferon-\(\gamma\)), aiming to further elucidate the impact of polymer chain length on DNA delivery to DCs and to evaluate the practical value of PAEM as DNA vaccine carrier and immunostimulatory adjuvant.

2.2 Materials and methods

2.2.1 Materials

Toluene (Sigma-Aldrich, St. Louis, MO) was dried by refluxing over sodium and distilled. \(N\)-(\(\text{tert}\)-butoxycarbonyl)aminoethyl methacrylate (\(t\)BAM) was synthesized as described [69]. Ethyl \(\alpha\)-bromoisobutyrate, copper (I) chloride (CuCl), and 2,2′-dipyridyl (bPy) were purchased from Sigma-Aldrich. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Dulbecco’s phosphate buffered saline (DPBS), Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from Invitrogen (Carlsbad, CA). Murine DC 2.4 cells (ATCC, Manassas, VA) were incubated in DMEM containing 10% FBS, 10 mM HEPES, 100 U/mL penicillin/streptomycin at 37°C and a humidified atmosphere containing 5% CO\(_2\). Three different DNA plasmids were used in various experiments: (1) a green fluorescent protein plasmid (pEGFP-N1), used for evaluating polyplex properties and gene transfection; (2) a firefly luciferase plasmid (pCMV-Luc), used as a negative control in transfection experiments; (3) a chicken ovalbumin plasmid (pCMV-OVA), used in DC maturation and CD8\(^+\) T cell activation experiments. All three plasmids contained a CMV promoter. Other chemicals and solvents were purchased from Sigma-Aldrich and used without further purification.
2.2.2 Synthesis of poly(2-aminoethyl methacrylate) (PAEM)

The ATRP of tBAM followed a procedure modified from Tang et al [68]. A glass two-neck flask was charged with tBAM, CuCl, bPy, and the system was degassed three times. Dried degassed toluene and ethyl α-bromoisobutyrate as initiator were added, and the mixture was heated at 80°C for 8 h. The reaction was terminated by exposing the system to air. The reaction solution was then diluted by dichloromethane and passed through a basic aluminum oxide column to remove the copper complex. The product was precipitated in hexane twice and dried in vacuum at room temperature for 2 days. Three different monomer-to-initiator feed ratios (50:1, 100:1, 200:1) were used to obtain PtBAM homopolymers with varying chain length. To remove the Boc groups, 0.8 g of PtBAM was dissolved in 5 mL of trifluoroacetic acid (TFA) and stirred for 2 h at room temperature. TFA was then removed by evaporation, and the oil residue was rinsed three times with diethyl ether. The resultant precipitate was collected by filtration, washed twice by diethyl ether, and dried overnight in vacuum. The polymers were then washed with NaOH water solution at pH 9.0, and dialyzed (MWCO 3,500) against distilled deionized water for 3 days. The final PAEM polymers were obtained by lyophilization.

2.2.3 Characterization of polymers

The $^1$H and $^{13}$C NMR spectra of the polymers were acquired on a Varian Unity spectrometer (300 MHz) using CDCl$_3$ (for PtBAM) and D$_2$O (for PAEM) as solvents. Chemical shifts were recorded in ppm and referenced against tetramethylsilane (TMS) and D$_2$O, respectively. Gel Permeation Chromatography (GPC) experiments to analyze PtBAM were performed at 35°C in CHCl$_3$ with a flow rate of 1 mL/min using a Hewlett-Packard 1100 series liquid chromatography equipped with three PL gel 5-µm mixed
columns (Jordi Gel columns of 500, 103, and 104 Å pore sizes) and a Hewlett-Packard 1047A refractive index detector. The GPC instrument was calibrated with polystyrene standards (Polymer Laboratories, Amherst, MA). All sample solutions were filtered through a 0.22-μm filter before analysis.

2.2.4 Gel retardation assay

Polyplexes of N:P ratios ranging from 1/8 to 16 were prepared by adding 25 μL of polymer solution in 20 mM HEPES (pH 7.4) to 25 μL of DNA plasmid solution (0.2 μg/μL in 20 mM HEPES, pH 7.4) vortexed for 10 s, incubated for 30 min at room temperature, and analyzed by electrophoresis on a 1.0% agarose gel containing 0.5 μg/mL ethidium bromide.

2.2.5 Heparin competition assay

To determine the strength of DNA binding by polymers with varying chain length, polyplexes at N:P ratio of 8 were incubated with increasing concentrations of heparin (0.1 to 0.9 IU per μg of DNA) for 20 min at room temperature and analyzed by agarose gel electrophoresis.

2.2.6 Ethidium bromide (EB) exclusion

Polymer solutions were added to premixed EB and plasmid solution with varying N:P ratios from 1/8 to 32 and incubated for 30 min. The intensity of EB fluorescence was recorded by a Bio-Tek Synergy HT plate reader with excitation wavelength of 530/25 nm and emission wavelength of 590/35 nm. DNA/EB solution without any polymer was used as control.

2.2.7 Dynamic light scattering (DLS) and zeta potential measurement
The average hydrodynamic diameter and polydispersity index of polyplexes in HEPES buffer (20 mM) at 25°C were determined using a ZetaPlus Particle Analyzer (Brookhaven Instruments Corporation, Holtsville, NY; 27 mW laser; 658 nm incident beam, 90° scattering angle). Polyplexes with N:P ratios ranging from 1/4 to 32 were prepared as described above and diluted 20 times to a final volume of 2 mL in HEPES buffer before measurement. Simultaneously the zeta-potential of the polyplexes was determined using the ZetaPal module of the Particle Analyzer.

2.2.8 Transmission electron microscopy (TEM)

The morphology and size of polyplexes at N:P ratio of 8 were observed using a JEOL 1200 EXII transmission electron microscope. Samples for TEM were prepared by placing a drop of the polyplex solution onto carbon-coated EM grids followed by negative staining using phosphotungstic acid (1.0%, pH 4~5).

2.2.9 Cytotoxicity assay

The cytotoxicity of free polymers was evaluated by a MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [70]. DC 2.4 cells were seeded into 96-well plates at 6000 cells/well and cultured with polymers of various concentrations for 24 h in DC 2.4 media (DMEM low glucose, 10% FBS, 10 mM HEPES, 100 U/mL penicillin/streptomycin) at 5% CO₂ and 37°C. MTT in PBS (5 mg/mL, 20 µL) was added to each well reaching a final concentration of 0.5 mg/mL. After 4 h, unreacted MTT was removed by aspiration. The formazan crystals were dissolved in 150 µL of DMSO and the absorbance was measured at 570 nm using a Bio-Tek Synergy HT plate reader. Cell viability was calculated by \([\text{Absorbance of cells exposed to polymers}] / [\text{Absorbance of cells cultured without polymers}]\) in percentage.
2.2.10 Gene transfection in vitro

DC 2.4 cells were seeded into 12-well plates at 100,000 cells/well and cultured overnight. For transfection under serum-free condition, the cell media was removed and cells washed by PBS twice followed by adding DC 2.4 media without serum. Transfection with polyplexes of the GFP plasmid lasted for 4 h at 5% CO$_2$ and 37°C. The media was then discarded, cells washed by PBS twice, and cultured in serum-containing media for another 20 h before GFP level was recorded. For transfection in media containing 10% serum, DC 2.4 cells were incubated with polyplexes for 24 h at 5% CO$_2$ and 37°C. The cells were harvested by treating with trypsin-EDTA, dispersed in FACS buffer (PBS containing 1% bovine serum albumin), and analyzed using a BD LSR II flow cytometer. DC 2.4 cells were also transfected by polyplexes of the luciferase plasmid under the same conditions to exclude the autofluorescence of cells. Percentage of GFP$^+$ cells was determined using Flowjo. The GFP positive gate was drawn based on the luciferase control where false positive frequency was restricted to below 0.2%.

2.2.11 DC maturation

DC 2.4 cells were transfected with polyplexes at N:P ratios of 8 and 16 as described above. 24 hours later, the cells were stained with PE-labeled anti-mouse-CD40 antibody (Biolegend, San Diego, CA) and analyzed by flow cytometry. Untreated cells were of the immature phenotype (iDC, CD40$^{\text{low}}$). Positive mature DCs were generated by stimulating with 2 μg/mL of lipopolysaccharide (LPS, from *E. coli* strain 026:B6, Sigma). All cell culture media, buffers, polymer stock solutions, and plasmid stock solution were tested free of endotoxin contamination using a LAL endotoxin detection kit following manufacturer's instruction.
2.2.12 CD8⁺ T cell activation

Naïve mouse CD8⁺ T cells from the lymph nodes of OT-1/PL TCR transgenic mice were isolated as described previously [71]. To determine T cell activation, DC 2.4 cells were transfected using polyplexes containing an OVA plasmid with or without serum for 24 h as described above. Lymph node cells from OT-1/PL TCR transgenic mouse were added to the DCs so that approximately 3 × 10⁵ CD8⁺ T cells were present in each well. After co-culturing for 3 days, the supernatant of the cell media was analyzed for IFN-γ by ELISA using a Ready-Set-Go IFN-γ “Femto-HS” kit (e-Bioscience, San Diego, CA) following manufacturer’s procedure. As a positive control, iDCs without transfection were stimulated with SIINFEKL peptide (New England Peptide, LLC., Gardner, MA) at 1 µM for 1 h and with LPS at 2 µg/mL overnight before T cells were added.

2.2.13 Statistical analysis

Statistical analysis was carried out using a two-sample Student’s t-test with unequal variance. Values of $p<0.05$ were deemed to be different with statistical significance.

2.3 Results and discussion

2.3.1 Synthesis and characterization of PAEM homopolymers

We have chosen a simple cationic polymer, PAEM, as a model carrier for DNA vaccine delivery. The repeating unit of PAEM contains a primary amine in the side chain (Figure 2.1A), which is capable of condensing efficiently any anionic cargos including plasmid DNA. PAEM has been used to prepare copolymers to study properties of polyion complex micelles with heparin [72] or oligodeoxynucleotide [73], to deliver genes to
COS cells in vitro [74-75], or to serve as antimicrobial agents [69]. Recently, we have reported on a series of well-defined diblock copolymers of PEG and PAEM that can condense and deliver plasmid DNA to DC 2.4 cells and have shown that the length of the PAEM block had much influence on the properties of the polyplexes, gene transfection efficiency, and DC maturation [68]. However, due to the presence of the PEG block, the diblock copolymers had rather low transfection efficiency, which poses a potential limitation to practical application of the polymers for DNA vaccination. To this end, here we have synthesized PAEM homopolymers with well-defined chain length and conducted a comprehensive investigation on the physicochemical properties of the polyplexes, polyplex/DC interaction including efficiency of transfection, phenotypic maturation of DCs, and CD8+ T cell stimulation in vitro.

![Chemical structure of PAEM polymer](image1)

**Figure 2.1** Physical properties of PAEM. (A) Chemical structure of the PAEM polymer. (B) GPC traces of PtBAM, the precursor of PAEM with Boc-protected side chains, using CHCl$_3$ as mobile phase and a flow rate of 1 mL/min. Average molecular weight was determined based on polystyrene standards.
PAEM was synthesized using ATRP modified slightly from a previously described process [68]. In the first step, the ATRP of Boc-protected aminoethyl methacrylate monomer (tBAM) was initiated by ethyl α-bromo-isobutyrate with Cu(I)-dipyridyl complex as catalyst. The molar ratio of the monomer and initiator in the feed was varied to achieve PrtBAM with different chain length. Three PrtBAM polymers were synthesized with monomer conversion of over 99% (determined by proton NMR, data not shown) and yield of over 70%. GPC traces showed single peaks (Figure 2.1B) and the number average molecular weight (Mn) of the three polymers was calculated to be 0.98×10⁴, 1.66×10⁴, and 3.37×10⁴ with the polydispersity index (PDI) of 1.19, 1.20, and 1.16, respectively. The narrow molecular weight distribution of the polymers indicated that the ATRP reaction was successful. Proton NMR analysis (not shown) confirms the chemical structure of all three PrtBAM polymers. The average degree of polymerization (DP) of the PrtBAM was calculated to be 45, 75, and 150 based on GPC analysis (Figure 2.1B), and the three polymers were thus named PrtBAM₄₅, PrtBAM₇₅, PrtBAM₁₅₀, respectively. In the second step, the Boc protecting group was removed by TFA treatment. Proton NMR analysis (not shown) confirmed complete disappearance of the methyl proton signal of the Boc group at 1.46 ppm, proving that all the Boc groups have been removed. The final product was obtained by removing the TFA salt in a mild basic solution to give PAEM with free amines. During the TFA removal the pH was strictly controlled to be below 9 so as to prevent aminolysis of the methacrylate ester group [76]. Overall, this two-step synthesis possesses excellent control over the polymerization comparable to the recently reported single-step synthesis of PAEM [76].
2.3.2 The capacity of DNA binding and condensation is dependent on polymer chain length

![Diagram showing gel retardation assay, ethidium bromide (EB) exclusion, and destabilization of polyplexes with increasing concentration of heparin.]

Figure 2.2 Capacity of DNA binding and condensation by the PAEM polymers with different chain length. (A) Gel retardation assay of polyplexes prepared at various N:P ratios. Arrows point to the threshold N:P ratio where retardation of DNA migration through the gel occurred. (B) Ethidium bromide (EB) exclusion due to polyplex formation. Fluorescence intensity of EB mixed with plasmid without addition of polymer was taken as 100%. (C) Destabilization of polyplexes with increasing concentration of heparin. Arrows point to the threshold concentration of heparin beyond which unpackaging of DNA occurred.

The gel retardation assay qualitatively revealed the difference in DNA binding ability among the three PAEM polymers. PAEM$_{75}$ and PAEM$_{150}$ were able to completely prevent the migration of plasmid DNA at the N:P ratio of 1 and beyond. However, at this neutral charge ratio, PAEM$_{45}$ could only partially retard the DNA migration and reach a complete retardation at N:P ratio of 2 (Figure 2.2A). EB exclusion experiment gave a more quantitative assessment of DNA condensation capacity of PAEM. As the chain length of the polymers increased from DP of 45 to 150, the ability to condense plasmid DNA increased accordingly, judged by the lowering N:P ratios at which half of the EB is excluded.
was displaced from intercalating with DNA, resulting in reduced fluorescence intensity (Figure 2.2B).

The stability of polyplexes at N:P ratio of 8 was studied in the presence of increasing amount of heparin, a polyanion that can compete with DNA for the binding to the polycation, followed by agarose gel electrophoresis. As shown in Figure 2.2C, the threshold concentration of heparin at which polyplex disruption occurred was 0.4, 0.5, and 0.7 IU/µg of DNA for polyplexes of PAEM_{45}, PAEM_{75} and PAEM_{150}, respectively, suggesting that polyplexes formed with longer PAEM chains were more stable than shorter one. This conclusion generally agrees with findings of other polydisperse cationic polymer carriers, such as PDMAEM [63]. Moreover, we have previously observed a similar trend in the PEG-b-PAEM/DNA complexes [68]. Interestingly, polyplexes formed with PEG-b-PAEM were more resistant against heparin destabilization than polyplexes formed with the PAEM homopolymer of the same chain length. For example, with a DP of 75 the threshold heparin concentration was 0.8 IU/µg of DNA for the diblock and 0.5 IU/µg of DNA for the homopolymer. This difference is likely due to the steric hindrance to heparin posed by the PEG chains.

2.3.3 Average particle size of the polyplexes and cytotoxicity are independent of polymer chain length

The size of nanoparticles is highly important for gene delivery in general and potentially for DC targeting and internalization [77]. DLS revealed that regardless of PAEM chain length, the average particle size of the polyplexes in aqueous buffer spanned a range of 100 to 200 nm with much dependence on the N:P ratio (Figure 2.3A). The size distribution of each type of polyplex was also quite narrow with PDI ranging from 0.05 to
0.2. The particle size peaked at around 170 nm at the neutral charge ratio. Decreasing or increasing the N:P ratio, the particle size decreased gradually. There’s no distinct difference in particle size among the three PAEM polymers with different chain length. TEM images supported the results of the DLS, showing typical spherical nanoparticles in a representative image of PAEM$_{75}$ polyplex at N:P ratio of 8:1 (Figure 2.3B). At N:P ratio of 8:1, polyplexes of PAEM of different chain length also shared similar zeta potential values at around 35 mV.

![Figure 2.3](image)

**Figure 2.3** Average particle size of the polyplexes and cytotoxicity. (A) Average particle size of polyplexes in aqueous buffer (20 mM HEPES, pH 7.4) as a function of N:P ratio determined by DLS. (B) A typical TEM image of polyplexes (N:P ratio of 8) showing discrete, condensed nanoparticles of $\sim$100 nm in size. Scale bar: 100 nm. (C) Cytotoxicity of PAEM polymers to DC 2.4 cells measured by the MTT assay. Viability of cells cultured without polymer was taken as 100%.

That the hydrodynamic size of polyplexes was independent of the molecular weight of polymers has been observed in other cationic polymer systems, such as poly(2-
methyl-acrylic acid 2-[(2-(dimethylamino)-ethyl)-methyl-amino]-ethyl ester) (pDAMA) at N:P ratio >5 [78] and trehalose click polymers at low N:P ratios [64]. This phenomena was also consistent with our previous report on polyplexes formed by PEG-b-PAEM and plasmid DNA, except at N:P ratio of 1, at which particle size decreased with increasing length of the PAEM block [68].

Cytotoxicity of the PAEM polymers was evaluated using a murine dendritic cell line, DC 2.4. All the PAEM polymers regardless of chain length were not toxic to DC 2.4 cells at concentrations as high as 10 µg/mL (Figure 2.3C). Cell viability, determined by the MTT assay, dropped to around 70% at polymer concentration of 20 µg/mL and to around 40% at 40 µg/mL. Overall, the cytotoxicity of PAEM polymers in DCs was not sensitive to changes of polymer chain length, similar to what was observed with the PDMAEMA system [63].

2.3.4 Transfection of DCs in vitro with and without serum

PAEM-mediated transfection of DC 2.4 cells with a GFP plasmid was conducted in cell culture media in the absence or presence of 10% serum, and the expression level of GFP was determined by flow cytometry (Figure 2.4). To exclude any possible interference from cell autofluorescence, DCs transfected under identical conditions but using a luciferase plasmid served as negative controls to set the GFP$^+$ gate. Under serum-free conditions, transfection efficiency of DC 2.4 cells measured by the percentage of GFP$^+$ cells ranged from around 10% to 35% (Figure 2.4A) and was dependent on both polymer chain length and N:P ratio (Figure 2.4B). Overall, longer polymer chain length enabled higher transfection with the exception of the longest PAEM$_{150}$ at high N:P ratios (16 and 24).
Figure 2.4 Transfection efficiency of DC 2.4 cells by polyplexes determined by flow cytometry. (A) Representative dot plots of transfected cells. The GFP⁺ gate was set based on cells transfected with a luciferase plasmid. (B, C) Quantification of the percentage of GFP⁺ cells transfected in the absence (B) or presence (C) of 10% serum. *p < 0.05.

For transfection in serum-containing media, the overall transfection level was much lower than that in serum-free media at all N:P ratios tested, but longer chain length...
at high N:P ratios (16 and 24) did achieve transfection as high as 10% (Figure 2.4A, C). Similar decrease in transfection efficiency due to the inference of serum has been reported for many cationic polymers with different molecular structures [79-81], and the reason is often attributed to destabilization of polyplexes by serum proteins. We found that longest PAEM$_{150}$ was able to form the most stable polyplexes among the three polymers tested and that it indeed achieved the highest transgene expression level despite the presence of serum.

Compared to PEG-b-PAEM block copolymers we reported earlier [68], the PAEM homopolymers showed much higher transfection efficiency of DCs cultured in serum-free medium, presumably due to the fact that positively charged PAEM homopolymer/DNA polyplexes could be taken up by cells more efficiently. Another potential factor may be that although PEGylation is known to enhance colloidal stability of the polyplexes, too much stability might impede polyplex dissociation inside cells, hence resulting in lower gene transfection.

2.3.5 *DC maturation in vitro with and without serum*

As a connection between the innate and adaptive immune responses, the maturation state of DCs is important to successful DNA vaccination. Several cationic polymers including polylysine derivatives [82], PEI [83], chitosan [84], and poly(β-amino esters) [85] have been reported to enhance immune response, but the potential of inducing DC maturation by such cationic polymers has not been established. Here we examined the maturation state of murine DC 2.4 cells after treatment with PAEM/DNA polyplexes in vitro.
Figure 2.5 Maturation of DC 2.4 cells by polyplexes marked by upregulation of CD40. (A) A representative set of flow cytometry data of cells transfected by polyplexes or LPS or naked DNA only, in the presence or absence of serum. The shaded area is untreated DCs. Numbers represent the values of mean fluorescence intensity. (B) Quantification of DC maturation. CD40 level was normalized against untreated DCs. *p < 0.05.

As positive and negative controls, DCs treated with LPS up-regulated the expression of a maturation marker CD40, while naked DNA plasmid did not have any effect (Figure 2.5). Under serum-free conditions, exposure to the polyplexes at N:P ratios of 8 and 16 induced a strong up-regulation of CD40 at levels similar or higher than LPS,
but the correlation between CD40 level and chain length of PAEM was not pronounced (Figure 2.5). The presence of 10% serum in the cell culture media attenuated the amount of DC maturation at N:P ratio of 8 but not 16. At both N:P ratios, however, the correlation between polymer chain length and DC maturation was apparent-polyplexes with longer PAEM chain length caused higher up-regulation of CD40. These findings suggest that as carriers for DNA plasmid, the PAEM polymers may be potentially good adjuvants because of their ability of stimulating DC maturation, a prerequisite for generating adaptive immune responses.

Furthermore, we have previously reported that PEG-b-PAEM block copolymers induced the maturation of murine bone marrow derived DCs in a chain length-dependent manner [68]. It would be interesting to compare DC stimulatory capacity of the homopolymers with the PEG block copolymers side-by-side. Regardless, it is speculated that such DC maturation stimulated by cationic polymers is at least partially due to the stress and cytotoxicity caused by the polymers [71].

2.3.6 Polyplex transfected DCs activated antigen-specific CD8+ T cells in vitro

To assess the potential practical utility of the PAEM polymers for DNA vaccine delivery, we evaluated the ability of PAEM delivering a model antigen to activate naïve CD8+ T cells. 24 hours after transfecting DC 2.4 cells in the absence or presence of serum, naïve OVA-specific CD8+ T cells harvested from mice were incubated with the DCs for 3 days. OVA-specific T cell activation was determined by quantifying the level of IFN-γ production. We found that under serum-free condition, polyplex-transfected DCs stimulated IFN-γ production by T cells at levels on par with the positive control-DCs stimulated with LPS and the CD8+ T cell epitope from OVA, SIINFEKL peptide
The amount of IFN-$\gamma$ production scaled with the level of gene transfection efficiency of DCs (Figure 2.4B), that is, at N:P ratio of 8, longer PAEM produced more IFN-$\gamma$, whereas at N:P ratio of 16, PAEM with the intermediate chain length (DP = 75) resulted in the highest IFN-$\gamma$ production (Figure 2.6). For DC transfection in the presence of serum, the IFN-$\gamma$ level, once again, correlated with the transfection efficiency under the same condition (Figure 2.4C), dropping to close to background level at N:P ratio of 8 and rebounding to approach the serum-free level at N:P ratio of 16 (Figure 2.6). Taken together, the correlation between polymer chain length, transfection efficiency, and T cell activation was apparent. It is also a strong indication that the PAEM polymers may be potentially effective delivery vehicles of DNA vaccine for inducing antigen-specific T cell immunity. Further evaluation of the effectiveness of PAEM in DNA vaccination in vitro and in vivo is warranted.

Dave Panus in our lab prepared polyplexes using fluorescently labeled PAEM polymers and plasmid DNA and studied correlations between polymer chain length and uptake in dendritic cells and subcellular trafficking [86]. The results show that longer PAEM chains enhance cellular uptake of polyplexes, while shorter PAEM chains facilitate intracellular dissociation of polyplexes. And endolysosomal localization is not clearly dependent on polymer chain length. Longer PAEM chains promote nuclear localization of polyplexes but do not favor the release of free plasmid. In particular, at N:P ratio of 8, the chain length dependence of transfection appeared consistent with the amount of free (transcriptionally active) plasmid found in the cell nucleus.
**Figure 2.6** CD8⁺ T cell activation. DCs were transfected with polyplexes containing a model antigen (OVA) encoding plasmid in the absence or presence of serum. Untreated DCs and DCs stimulated with SIINFEKL peptide plus LPS were used as controls. IFN-γ production by CD8⁺ OVA-specific T cells was quantified by ELISA.

PAEM is a structurally simple member of the poly(amoioalkyl methacrylate) family. It has a very flexible polymethacrylate backbone and high positive charge density at physiological pH, owing to the primary amine side-chains, which favors the formation of compact, nano-sized polyplexes. It would be interesting to see how further variations of the polymer structure might affect gene transfer and interaction with DCs, such as using longer alkyl chains between the charge center and the polymer backbone [43, 87]. Also, a direct comparison between PAEM and a structurally similar yet more extensively studied gene carrier, PDMAEMA, which has tertiary amine side chains [88], may help
understand the influence of the type of positive charge center on the formation and subcellular trafficking of polyplexes in DCs.

2.4 Conclusion

Cationic PAEM polymers with precisely controlled chain length were synthesized and characterized for delivering plasmid DNA to antigen-presenting dendritic cells. Through a comprehensive study we revealed correlations between polymer chain length and colloidal properties of the polyplexes, transfection efficiency, and the capacity of inducing DC maturation and CD8$^+$ T cell activation by a model DNA vaccine. Thus, the structurally simple and defined PAEM polymer is not only a useful model material for understanding structure-function relationship in non-viral gene delivery, but may also be a practically effective, immunostimulatory DNA vaccine carrier.
Chapter 3: Hydrophobicity Matters: Influence of Side Chain Length on Gene Delivery to Dendritic Cells Mediated by Poly(aminoalkyl Methacrylate) Homopolymers

3.1 Introduction

In recent years, a large number of cationic polymers with diverse structures and properties have been developed as non-viral gene delivery carriers [12, 24]. Despite much progress in the past two decades, to successfully design safe and efficient polymers as gene carriers suffers from complicated possible combinations of physicochemical variables and the complex biological environment in which the carriers are applied. Comprehensive structure-function relationship study of polymeric gene carriers is always helpful to develop better carriers through adjusting the structural variables of polymers. Hydrophobicity of polymers is one of the factors that have been shown to exert significant impact on DNA delivery. Lot of work has been done to directly modify the hydrophobicity of existing polymeric gene carriers such as polyethylenimine (PEI), chitosan, and PLL, by introducing a lipid chain or hydrophobic polymer segments [89-90]. Although optimized gene carrier can be dug out by this route, it makes the relationship between polymer hydrophobicity and transfection efficiency even more complicated because this strategy simultaneously changes other structural parameters of polycations, such as chemical composition, charge type, charge density, and chain regularity [43].

Several examples have been reported to better elucidate the hydrophobicity-performance relationship of polymeric carriers by minimizing the interference of other
structural variables caused during aquaphobicity adjustment [87, 91-94]. In these examples, hydrophobicity was altered by increasing the side chain length of polymers while keeping polymer backbone unchanged. Unfortunately, most of the mentioned polymers above may not be good candidates for the structure-function interaction study either because their molecular weights were not defined based on their polymer chemistry. Lacking of controlled polymerization may result in inaccurate conclusions regarding structure-performance relationships of different polymers. Therefore, polymers with precisely controlled chemical structure and narrow molecular weight distribution are needed as model polymers to reveal structure-function relationships.

We are interested in transfecting dendritic cells (DCs) in that DCs are the most important antigen-presenting cells (APCs), which are widely considered the ideal target cells of DNA vaccines [36]. Compared with general gene delivery, a plasmid DNA that encodes for a protein antigen of interest and sustained antigen presentation from DCs combined with DC maturation are expected for DNA vaccine to generate robust adaptive cellular immunity [60], which is particularly necessary for combating cancer and viral infection. However, not much is known about how polymer’s hydrophobic/hydrophilic characteristic would affect the phenotypic maturation state of DCs [95]. In this case, structure and molecular weight defined polymers are very helpful to reveal this relationship.

Atom transfer radical polymerization (ATRP) [44, 47], one of the widely used controlled radical polymerization techniques has been shown to be a powerful and convenient means to prepare cationic polymers with defined structure and molecular weight in a facile way. Recently we have synthesized a series of poly(2-aminooethyl
methacrylate) (PAEM) homopolymers with narrow molecular weight distribution by ATRP and have shown that chain length exerted significant influence on how the polymer carriers interacted with plasmid DNA and how the polyplexes interacted with DCs [86]. This simple poly(amoineethyl methacrylate) can be structurally extended as a model system to explore more about its interaction with DCs in gene delivery.

In currently work, we developed a series of poly(amoinealkyl methacrylate) (PAAM) homopolymers with different alkyl size (ethyl, propyl, butyl, and hexyl) and hydrophobicity by ATRP and applied them for gene delivery to DCs. We investigated comprehensively the colloidal properties of the PAAM/plasmid polyplexes (including stability, particles size and charge), uptake and subcellular trafficking in DCs, and biological properties in vitro (including hemolysis, cytotoxicity, transfection efficiency and DC maturation), aiming to reveal the impact of hydrophobicity of polymers on DNA delivery to DCs.

3.2 Materials and methods

3.2.1 Materials and general methods

Toluene (Sigma-Aldrich, St. Louis, MO) was dried by refluxing over sodium and distilled. Dichloromethane bought from Sigma was dried by refluxing over calcium hydride and distilled. 3-(Boc-amino)-1-propanol, 4-(Boc-amino)-1-butanol and triethylamine were purchased from sigma and used without further purification. Methacryloyl chloride (Sigma) was freshly distilled under vacuum before use. N-(tert-butoxycarbonyl)aminoethyl methacrylate (tBocAEM) [69] and N-(tert-butoxycarbonyl)-6-amino-1-hexyl methacrylate (tBocAHM) [75] were synthesized as described elsewhere. Ethyl α-bromoisobutyrate, copper (I) chloride (CuCl) and 2,2′-dipyridyl (bPy) were also
purchased from Sigma-Aldrich. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Dulbecco’s phosphate buffered saline (DPBS), Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from Invitrogen (Carlsbad, CA). Murine DC 2.4 cells (ATCC, Manassas, VA) were incubated in DMEM containing 10% FBS, 10 mM HEPES, 100 U/mL penicillin/streptomycin at 37°C and a humidified atmosphere containing 5% CO₂. Two different DNA plasmids were used in various experiments: (1) a green fluorescent protein plasmid (pEGFP-N1), used for evaluating polyplex properties and gene transfection; (2) a firefly luciferase plasmid (pCMV-Luc), used in cellular uptake and subcellular trafficking studies and as a negative control in transfection experiments. Both plasmids contained a CMV promoter. Other chemicals and solvents were purchased from Sigma-Aldrich and used without further purification.

The ¹H NMR spectra were acquired on a Varian Unity spectrometer (300 MHz) and chemical shifts were recorded in ppm. High-resolution mass spectra in electrospray (ESI) experiments were obtained on a Bruker BioTOF II mass spectrometer. Gel Permeation Chromatography (GPC) experiments to analyze Boc protected polymers were performed at 35°C in CHCl₃ with a flow rate of 1 mL/min using a Hewlett-Packard 1100 series liquid chromatography equipped with three PL gel 5-μm mixed columns (Jordi Gel columns of 500, 103, and 104 Å pore sizes) and a Hewlett-Packard 1047A refractive index detector. The GPC instrument was calibrated with polystyrene standards (Polymer Laboratories, Amherst, MA). All sample solutions were filtered through a 0.22-μm filter before analysis.
3.2.2 Synthesis of N-(tert-butoxycarbonyl)-3-amino-1-propyl methacrylate (tBocAPM) and N-(tert-butoxycarbonyl)-4-amino-1-butyl methacrylate (tBocABM) monomers

To a solution of 3-(Boc-amino)-1-propanol (10 g, 57.1 mmol) and TEA (12.0 mL, 85.6 mmol) in 80 mL of anhydrous DCM at 0°C was added dropwise a solution of methacryloyl chloride (6.2 mL, 62.8 mmol) in 20 mL of DCM. The reaction mixture was then warmed to r.t. and reacted for additional 24 h under N2 flow. After removing white precipitate, the filtrate was washed with water (3 × 150 mL), saturated NaHCO3, and brine, and then dried overnight over MgSO4. The resultant solution was concentrated and purified through a silica gel column with hexane/ethyl acetate (5/1, v/v) to afford tBocAPM as a colorless oil. Yield: 60.2%. 1H-NMR (300 MHz, CDCl3): δ (ppm), 1.43(s, 9H, −C(CH3)3), 1.86 (m, 2H, −CH2CH2OOC−), 1.94 (s, 3H, −CH3), 3.22 (m, 2H, −CH2NHBoc), 4.21 (t, 2H, −CH2OOC−), 4.75 (br.s, 1H, −NHBoc ), 5.56/6.10 (s, 2H, =CH2). ESI-MS Calcd for (C12H21NO4), 243.15; found m/z, 266.14 (M + Na+).

tBocABM was synthesized similarly using 4-(Boc-amino)-1-butanol as a starting material. Yield: 63.1%. 1H-NMR (300 MHz, CDCl3): δ (ppm), 1.42(s, 9H, −C(CH3)3), 1.55(m, 2H, BocNHCH2CH2− ), 1.69 (m, 2H, −CH2CH2OOC−), 1.92 (s, 3H, −CH3), 3.14 (m, 2H, −CH2NHBoc), 4.14 (t, 2H, −CH2OOC−), 4.60 (br.s, 1H, −NHBoc ), 5.53/6.07 (s, 2H, =CH2). ESI-MS Calcd for (C13H23NO4), 257.16; found m/z, 280.16 (M + Na+).

3.2.3 Synthesis of poly(amoioalkyl methacrylate)

The ATRP of tBocAEM, tBocAPM, tBocABM, and tBocAHM followed a procedure described by Ji et al [86]. The monomer/initiator feed ratios were fixed at 100:1 to afford poly(amoioalkyl methacrylate) (PAAM) homopolymers with close
backbone length. For example, to synthesize PtBocAPM with the target degree of polymerization (DP), 1.0 g of tBocAPM, 8.0 mg of ethyl α-bromoisobutyrate, 4.0 mg of CuCl, 12.8 mg of bPy and 3 mL of dried toluene were added to a two-neck glass flask and degassed via three freeze-thaw cycles. The mixture was then stirred at 80°C for 6 h. The reaction was terminated by exposing the system to air. The reaction solution was diluted by dichloromethane and passed through a basic aluminum oxide column to eliminate the copper complex. The resulting solution was concentrated under vacuum and precipitated in hexane twice and dried in vacuum at room temperature for 2 days. The Boc group was removed by dissolving 0.6 g of PtBocAPM in 4 mL of trifluoroacetic acid (TFA) and stirred for 2 h at room temperature. TFA was then removed by evaporation, and the residue was rinsed three times with diethyl ether. The resultant precipitate was collected by filtration, washed twice by diethyl ether, and dried overnight in vacuum. The polymer was then neutralized with 0.5 M NaOH water solution to reach pH 8.0, and dialyzed (MWCO 3,500) against distilled deionized water for 3 days. The final PAPM was obtained by lyophilization. PAEM, PABM, and PAHM polymers were synthesized similarly.

3.2.4 Gel retardation assay

Polyplexes of N:P ratios ranging from 1/8 to 16 were prepared by adding 25 μL of polymer solutions in 20 mM HEPES (pH 7.4) to 25 μL of DNA plasmid solutions (0.2 μg/μL in 20 mM HEPES, pH 7.4), vortexed for 10 s, incubated for 30 min at room temperature, and analyzed by electrophoresis on a 1.0% agarose gel containing 0.5 μg/mL ethidium bromide.

3.2.5 Ethidium bromide (EB) exclusion
Polymer solutions were added to pre-mixed EB and plasmid solutions with varying N:P ratios from 1/8 to 32 and incubated for 30 min. The intensity of EB fluorescence was recorded by a Bio-Tek Synergy HT plate reader with excitation wavelength of 530/25 nm and emission wavelength of 590/35 nm. DNA/EB solution without any polymer was used as a control.

3.2.6 Heparin competition assay

To determine the stability of complexes formed by polymers with varying side chain length, polyplexes at N:P ratio of 8 were incubated with increasing concentrations of heparin (0.1 to 0.9 IU per μg of DNA) for 20 min at room temperature and analyzed by agarose gel electrophoresis.

3.2.7 Dynamic light scattering (DLS) and zeta potential measurement

The average hydrodynamic diameter and polydispersity index of polyplexes in HEPES buffer (20 mM) at 25°C were determined using a ZetaPlus Particle Analyzer (Brookhaven Instruments Corporation, Holtsville, NY; 27 mW laser; 658 nm incident beam, 90° scattering angle). Polyplexes of each polymer with N:P ratios ranging from 1/4 to 32 were prepared as described above and diluted 20 times to a final volume of 2 mL in HEPES buffer before measurement. Simultaneously the zeta-potential of the polyplexes was determined using the ZetaPal module of the Particle Analyzer.

3.2.8 Transmission electron microscopy (TEM)

The morphology and size of polyplexes at N:P ratio of 8 were observed using a JEOL 1200 EXII transmission electron microscope. Samples for TEM were prepared by
placing a drop of the polyplex solution onto carbon-coated EM grids followed by negative staining using 1% uranyl acetate.

3.2.9 Circular dichroism (CD) spectroscopy

CD spectra of polyplex solutions at N:P 8 were recorded on a Jasco J-815 spectra polarimeter (Jasco Inc., Easton, MD) in a 0.1 cm path length cuvette under nitrogen at 25°C. The spectra were recorded between 220 and 320 nm at a DNA concentration of 0.1 mg/mL in 20 mM HEPES (pH 7.4).

3.2.10 Hemolysis

0.5 mL of blood was collected from the ear artery of a female adult New Zealand White Rabbit (approximately 2 kg, obtained from Bakkom Rabbitry, Red Wing, MN) and centrifuged at 2000 rpm for 10 min at 4°C. The plasma was removed, and erythrocytes were resuspended in 2 mL of ice cold PBS. The cells were again centrifuged at 2000 rpm for 10 min at 4°C. This procedure was repeated more than twice to ensure the removal of any released hemoglobin. After the supernatant was removed, the cells were resuspended in PBS solution and diluted to obtain a cell suspension with Abs = 0.6 at 650 nm. Polymers were also diluted in PBS to reach different concentrations. 0.1 mL of the polymers were added to 0.1 mL of RBC suspension in a 96-well plate and incubated for 1 h at 37°C with mild shaking. Complete hemolysis was attained using a 2% v/v Triton-X yielding the 100% control value. PBS was used as the negative control. After incubation, the 96-well plate was centrifuged at 2000 rpm for 10 min, and 100 µL of the supernatants were transferred to another 96-well plate. The release of hemoglobin was determined by UV at 414 nm. Each sample was tested in triplicates. Degree of hemolysis is defined as % lysis = 100*(A_{polymers} - A_{blank})/(A_{triton} - A_{blank})
3.2.11 Cytotoxicity of free polymers

Cytotoxicity of free polymers was evaluated by a MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. DC 2.4 cells were seeded into 96-well plates at 6000 cells/well and cultured overnight. Then DCs were added polymers of various concentrations and cultured for 24 h at 5% CO₂ and 37°C. MTT in PBS (5 mg/mL, 20 µL) was added to each well reaching a final concentration of 0.5 mg/mL. After 4 h, unreacted MTT was removed by aspiration. The formazan crystals were dissolved in 150 µL of DMSO and the absorbance was measured at 570 nm using a Bio-Tek Synergy HT plate reader. Cell viability was calculated by \([\text{Absorbance of cells exposed to polymers}] / [\text{Absorbance of cells cultured without polymers}]\) in percentage.

3.2.12 Gene transfection in vitro

DC 2.4 cells were seeded into 12-well plates at 100,000 cells/well and cultured overnight. Before adding polyplexes, the cell media was removed and cells washed by PBS twice followed by adding serum-free DC 2.4 media. Transfection with polyplexes of GFP plasmid lasted for 4 h at 5% CO₂ and 37°C. The media was then discarded, cells washed by PBS twice, and cultured in serum-containing media for another 20 h. Afterwards, the cells were harvested by treating with trypsin-EDTA, dispersed in FACS buffer (PBS containing 1% bovine serum albumin), and analyzed using a BD LSR II flow cytometer. DC 2.4 cells were also transfected by polyplexes of luciferase plasmid under the same condition to exclude autofluorescence of cells. Percentage of GFP⁺ cells was determined using Flowjo. The GFP positive gate was drawn based on the luciferase control where false positive frequency was restricted to below 0.2%. Cytotoxicity of polyplexes was also tested right after the transfection by the MTT assay.
3.2.13 Fluorescent labeling of polymers and plasmid

For subcellular trafficking studies, both poly(aminoalkyl methacrylate)s and DNA plasmid were covalently labeled with fluorophores. PAAM polymers were labeled with Oregon Green 488 carboxylic acid succinimidyl ester (Invitrogen) using a method previously described [86] followed by purification via dialysis (MWCO 3,500). The luciferase DNA plasmid was labeled with a Cy5 fluorophore using a MIRUS LabelIT Kit (Mirus, Madison, WI) according to manufacturer’s protocol. After the labeling reaction, the DNA plasmid was purified from excess dye by means of ethanol precipitation according to the manufacturer’s protocol. Both purified labeled polymers and DNA plasmid were stored at -20°C until use.

3.2.14 Cellular uptake and subcellular trafficking

DC 2.4 cells were seeded in 4-well LabTek II glass chamber slides (Fisher Scientific, Pittsburgh, PA) at a density of 100,000 cells per well in 1 mL of media and cultured overnight. To visualize cellular uptake and polyplex intracellular dissociation, polyplexes were formed using fluorescently labeled polymers and luciferase plasmid. Prior to transfection, DC 2.4 media was removed and the cells were washed twice with warm PBS and supplied with serum-free DC 2.4 cell media. Polyplexes were added to cells and incubated for 4 h at 37°C, 95% humidity, and 5% CO₂. The serum-free media was then removed and the cells were washed twice with warm PBS and replaced with complete DC 2.4 media and incubated for additional 20 h. Prior to confocal microscopy imaging, Hoechst 33342 (Invitrogen) was added to each chamber at a final concentration of 30 µM to stain the cell nuclei, and the samples were washed and fixed using BD cytofix fixation buffer (BD Bioscience, San Jose, CA) according to the manufacturer’s
protocol. The cells were then mounted with SlowFade Gold© anti-fade reagent (Invitrogen), covered with a coverslip, and sealed with clear nail polish. Cell images were captured following the same settings described by Ji et al [86] with an Olympus FV-1000 confocal microscope.

To determine endolysosomal colocalization, DC 2.4 cells were transfected with polyplexes formed with non-labeled polymers and Cy5-labeled luciferase plasmid. Polyplexes were added to DC 2.4 cells and incubated for 4 h in serum-free media. The media was then removed and the cells were washed twice with warm PBS and replaced with complete DC 2.4 media and incubated for additional 8h. After the designated incubation time, the cells were washed twice with PBS and fixed with BD cytofix according to the manufacture’s protocol. Next, the cells were permeabilized using 0.5% Saponin (Sigma) solution (in PBS) at room temperature for 30 minutes. The cells were then washed twice with PBS. Non-specific binding was prohibited by incubating the cells with CD16/32 antibody for 5 minutes on ice. Afterwards, FITC labeled anti-LAMP-1 antibody (eBioscience) and Hoechst 33342 were added to the cells and incubated on ice for 30 minutes. The cells were then washed twice with PBS, mounted and imaged as described above.

The confocal fluorescence microscopy images of cellular uptake and subcellular trafficking were analyzed using ImageJ software and image quantification was carried out using a method developed by Akita et al [96]. Cellular uptake of Cy5-labeled plasmid, the percentage of polyplex intracellular dissociation, and the percentage of endolysosomal colocalization were measured and quantified similarly to what Ji et al did
A total of 30 cells from 3 different ROI were analyzed for each experimental group based on the coefficient of variance [96].

### 3.2.15 DC maturation

DC 2.4 cells were transfected with polyplexes at N:P ratio of 8 as described earlier. 24 hours later, the cells were stained with PE-labeled anti-mouse-CD40 antibody (Biolegend, San Diego, CA) and analyzed by flow cytometry. Untreated cells were of the immature phenotype (iDC, CD40<sub>low</sub>). Positive mature DCs were generated by stimulating with 2 μg/mL of lipopolysaccharide (LPS, from E. coli strain 026:B6, Sigma). All cell culture media, buffers, polymer stock solutions, and plasmid stock solution were tested free of endotoxin contamination using a LAL endotoxin detection kit following manufacturer's instruction.

### 3.2.16 Statistical analysis

Statistical analysis was carried out using a two-sample Student’s t-test with unequal variance. Values of \( p<0.05 \) were deemed to be different with statistical significance.

### 3.3 Results and discussion

#### 3.3.1 Synthesis and characterization of poly(aminealkyl methacrylate)

In the past two decades, numerous cationic polymers with versatile structures and physicochemical properties have been developed as gene delivery vehicles. Revealing how the structures of polymers determine their biological performance is crucial to help manufacture clinically safe and efficient gene carriers. Hydrophobicity is one of the fundamental properties of a cationic polymer known to exert profound influence on its
gene delivery capacity. The strategy that directly adds hydrophobic moiety to polymers severely changes other structural parameters of polycations, such as chemical composition, charge density, and chain regularity, thus in turn makes it difficult to clearly study the impact of hydrophobicity on gene delivery. Several polymer systems that minimized the interference of other structural variables caused during hydrophobicity adjustment didn’t have their molecular weights defined based on the polymer chemistry they employed. Lacking of controlled polymerization may make conclusions regarding performance of different polymers not precise and safe. Therefore, polymers with precisely controlled chemical structure and narrow molecular weight distribution are always welcomed as model polymers to reveal structure-function relationships. In addition, numerous pieces of evidence have shown that hydrophobicity of polymers directly affect the immune response [97]. Thus, to investigate the influence of hydrophobicity on the activation state of antigen-presenting cells in context of DNA vaccine delivery is another task needs to be completed.

Previously, we have displayed the potency of chain length well-defined PAEM homopolymers as DNA vaccine carriers [86]. We have also showed that PAEM worked well as a model system to investigate the chain length dependence on the physicochemical properties of the polyplexes, polyplex/DC interaction including subcellular trafficking, efficiency of transfection, phenotypic maturation of DCs, and CD8+ T cell stimulation in vitro. In current study, we synthesized a series of poly(amoalkyl methacrylate) homopolymers bearing same backbone but varied side chain length to explore their structure-function relationship as carriers to deliver DNA to dendritic cells.
PAAMs were synthesized using ATRP slightly modified from a previously described process [86] as shown by Figure 3.1. First, Boc-protected aminoalkyl methacrylate monomers were synthesized with good purity and yield. In the polymerization step, ATRP of monomers were initiated by ethyl α-bromoisobutyrate with Cu(I)-dipyridyl complex as catalyst. The molar feed ratio of monomers to initiator was kept at 100:1 to achieve PtBocAAM with close main chain length (Table 3.1). Four PtBocAAM polymers were synthesized with monomer conversion of over 98% (determined by proton NMR, data not shown) and yield of over 70%. Unimodal and symmetric peaks of GPC traces (Figure 3.2) along with narrow molecular weight distribution (Table 3.1) of the polymers indicated that the ATRP reactions were successful. The average degrees of polymerization (DP) of the PtBocAAM were also quite close based on GPC analysis (Table 3.1).

\[\text{Figure 3.1} \text{ Synthetic route for PAAM polymers with different side chain length. i: CuCl, dipyridyl, ethyl } \alpha\text{-bromoisobutyrate; ii:CF}_3\text{COOH.}\]

\[n=2, \text{PAEM} \]
\[3, \text{PAPM} \]
\[4, \text{PABM} \]
\[6, \text{PAHM} \]
Table 3.1 Molecular weight and MW distribution of poly(tBoc-aminoalkyl methacrylate)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Average Mn ($10^3$)</th>
<th>PDI</th>
<th>Average DPa</th>
<th>Average DPb</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtBocAEM</td>
<td>16.0</td>
<td>1.20</td>
<td>75</td>
<td>99</td>
</tr>
<tr>
<td>PtBocAPM</td>
<td>16.1</td>
<td>1.21</td>
<td>68</td>
<td>98</td>
</tr>
<tr>
<td>PtBocABM</td>
<td>18.8</td>
<td>1.22</td>
<td>73</td>
<td>98</td>
</tr>
<tr>
<td>PtBocAHM</td>
<td>19.5</td>
<td>1.26</td>
<td>70</td>
<td>99</td>
</tr>
</tbody>
</table>

a: Determined by GPC in chloroform; b: determined via conversion by $^1$H NMR

Figure 3.2 GPC traces of PtBocAAM, the precursors of PAAMs with Boc-protected side chains. Mobile phase: CHCl$_3$ at a flow rate of 1 mL/min. Average molecular weight was determined based on polystyrene standards.

In the second step, the Boc protecting group was removed by TFA treatment. Proton NMR analysis (not shown) confirmed complete disappearance of the methyl proton signal of the Boc group at 1.46 ppm, indicating that all the Boc groups have been removed. The final product was obtained by neutralizing the TFA salt in a mild basic
solution to give PAAMs with free amines. During the TFA removal, the pH was strictly controlled below 8 so that to prevent aminolysis of the methacrylate ester group [76]. It is reported that more methylene groups between the ester bond and amines will hinder the aminolysis side reactions [98]. We didn’t find any structural change during our base treatment procedures. And we did find that PAAMs with longer side chain length had lower solubility because of higher hydrophobicity. Overall, this two-step synthesis offered excellent control over the polymerization.

3.3.2 DNA binding and stability of polyplexes

The gel retardation assay was performed to measure DNA binding ability of the four PAAM polymers. PAEM, PAPM, and PABM were able to completely prevent the migration of plasmid DNA at N:P ratio of 1 and beyond. However, at this neutral charge ratio, PAHM could only partially retard the DNA migration and reach a complete retardation at N:P ratio of 2 (Figure 3.3 A).

EB exclusion experiment gave a more quantitative assessment of DNA condensation capacity of PAAMs. As the side chain length increased from 2 to 6, the ability of PAAMs to condense plasmid DNA didn’t show a clear trend, judged by the irregular N:P ratios at which half of the EB was displaced from intercalating with DNA, resulting in reduced fluorescence intensity (Figure 3.3B).

In our previous PAEM work, we showed that N:P 8 worked best to transfect DC 2.4 cells in vitro. In this study, we also focused on N:P 8 to compare other polymers with PAEM. The stability of polyplexes at N:P ratio of 8 was studied in the presence of increasing amount of heparin, a polyanion that can compete with DNA for binding to the polycation, followed by agarose gel electrophoresis. As shown in Figure 3.3C , the
threshold concentrations of heparin at which polyplexes disruption occurred were 0.5, 0.4, 0.4, and 0.5 IU/µg of DNA for polyplexes of PAEM, PAPM, PABM, and PAHM respectively, suggesting that altering side chain length didn’t affect the stability dramatically. In the aspect of DNA binding strength and polyplex stability, side chain length alternation didn’t exert distinctive effect compared with the backbone length change [86].

![Figure 3.3](image)

**Figure 3.3** Capacity of DNA binding and condensation by PAAM polymers with different side chain length. (A) Gel retardation assay of polyplexes prepared at various N:P ratios. Arrows point to the threshold N:P ratio where retardation of DNA migration through the gel occurred. (B) Ethidium bromide (EB) exclusion due to polyplex formation. Fluorescence intensity of EB mixed with plasmid without addition of polymer was taken as 100%. (C) Destabilization of polyplexes with increasing concentration of heparin. Arrows point to the threshold concentration of heparin beyond which unpackaging of DNA occurred.
3.3.3 Colloidal properties of polyplexes

Figure 3.4 Colloidal properties of PAAM polyplexes. (A) Average particle size of polyplexes in aqueous buffer (20 mM HEPES, pH 7.4) as a function of N:P ratio determined by DLS. (B) Zeta potential of PAAM/plasmid polyplexes at N:P ratio of 8, showing surface charge of polyplexes independent of polymer side chain length. (C) Representative TEM images of polyplexes (N:P ratio of 8) showing discrete, condensed round nanoparticles of ~100 nm in size. Scale bar: 100 nm. (D) Average particle size of polyplexes measured from TEM images. Mean ± SE, 30 nanoparticles for each sample were counted, **p < 0.01.

The size of nanoparticles is an important factor for gene delivery in general and potentially for DC targeting and internalization [20, 77]. According to DLS results, all of polyplexes in aqueous buffer averagely sized in a range of 50 to 180 nm with much
dependence on the N:P ratio (Figure 3.4A) We noticed that the size distribution of each type of polyplex was also quite narrow with PDI ranging from 0.05 to 0.2. The particle size peaked at around 170 nm at the neutral charge ratio. Decreasing or increasing the N:P ratio, the particle size decreased gradually. Generally, polyplexes formed by PAEM resulted in the largest average diameter, followed by PAPM and PABM, and PAHM the smallest. One reason may be that more hydrophobic polymers collapse more tightly with DNA in aqueous buffer. And at N:P ratio of 8:1, four types of polyplexes shared similar zeta potential values at around 40 mV (Figure 3.4B).

TEM images supported the results of the DLS, showing typical spherical nanoparticles in representative images of all four types of polyplexes at N:P ratio of 8:1 (Figure 3.4C). The size of PAAM nanoparticles measured by TEM was generally smaller than that measured by DLS (Figure 3.4D), mainly due to dehydration of nanoparticles during the EM process.

3.3.4 Conformation change of pDNA in polyplexes

The interaction of polycations with plasmid DNA can also be studied by using circular dichroism to monitor the DNA conformational changes. Figure 3.5 shows the binding interaction of PAAM polymers with GFP-DNA plasmid. The naked GFP DNA control shows a characteristic B-form spectrum with a negative peak at 244 nm and a positive peak at 274 nm [99]. With the addition of PAAMs to the GFP plasmid the positive peak experienced a red shift as well as decrease in magnitude suggesting that the GFP plasmid underwent a conformational change from B-form structure into a C-form secondary structure [100]. The negative peak also underwent a change in magnitude while also undergoing a red shift affecting the helicity of the GFP plasmid [101]. Among
these four polyplexes, PAEM induced the greatest red shift in both the positive and negative peaks as well as decrease in magnitude, follow by PAPM and PABM but at a similar level, and PAHM resulted in smallest alternation. The PAAM polymers themselves showed no positive reading for a secondary structure.

![Figure 3.5](image.png)

**Figure 3.5** CD spectra of PAAM/DNA complexes at N:P 8 in aqueous buffer (20 mM HEPES).

It’s interesting to note that at N-P 8, all PAAM polymers replaced similar amount of chelated EB from EB-DNA mixtures but shown different capacity to induce DNA conformational change. One explanation may be that similar percentage of EB can survive and keep the chelated format to the extent of DNA conformation change induced by PAAMs. It’s also possible that EB exclusion assay is not as sensitive as CD measurement to look at the DNA higher structure change caused by polycations in our system. The distinct capacity difference of DNA conformation change by PAAMs seems to be subtle in the dye exclusion experiment.
3.3.5 Hemolysis and cytotoxicity of PAAMs

Figure 3.6 Hemolysis and cytotoxicity of PAAM polymers. (A) Hemolysis of PAAM polymers against red blood cells measured by relative hemoglobin release. (B) Cytotoxicity of PAAM polymers to DC 2.4 cells measured by the MTT assay. Viability of cells cultured without polymer was taken as 100%, *p < 0.05.

Hemolysis assay of PAAMs was conducted via direct contact of red blood cells by polymer solutions. As shown in Figure 3.6A, when methylene groups between ester bond and amino group in the side chain are less than 6, no erythrocyte is lysed even at 80 µg/mL. However, PAHM showed strong hemolytic profile by disrupting 40% of the red
blood cells at 5 µg/mL, while increasing concentration of PAHM also resulted in more hemoglobin release. This hemolysis profile indicates that PAHM may possess a different mechanism from other polycations to interact with lipid membranes.

Cytotoxicity of the PAAMs was tested using a murine dendritic cell line, DC 2.4. Again, PAHM showed higher cytotoxicity than the other three polymers in our system, although the difference was not as distinct as hemolysis assay (Figure 3.6B). Till 10 µg/mL, PAEM, PAPM, and PABM were almost nontoxic to DC 2.4 cells (Figure 3.6B). However at the same concentration, cell viability of PAHM treatment was around 80%. More percentage of cells was killed when polymer concentrations were raised. When polymer concentration increased from 10 µg/mL to 40 µg/mL, there was a clear trend in terms of cytotoxicity that PAHM > PAPM ≈ PABM > PAEM.

The hemolysis and cytotoxicity profiles together imply that polycations with longer side chain length in our system have stronger interaction with cell membranes. Other groups have also reported that cytotoxicity would increase with longer side chain length, such as poly(amidoamine)s with pendant primary amines [91, 94]. However, that cytotoxicity decreased with longer side chain length has also been reported in poly(β-aminoester)s with pendant primary amines [93]. Poly(N-(12-aminododecyl)acrylamide), the member with the longest side chain length even had the best biocompatibility among all poly(aminoalkyl acrylamide)s [87]. This contradiction may come from the different backbone structures of cationic polymers. Further work needs to be done to study how PAAMs interact with cells at toxic concentration range. Although PAAMs possess varied hydrophobicity, we didn’t found any secondary structure for anyone in aqueous buffers by circular dichroism. These four cationic polymers may form different complicated
structures with cell membrane components to result in cell death after they attach to negative cell surface through electrostatic forces. And this possible mechanism has also been applied to explain how several anticancer peptides take effect [102].

3.3.6 Transfection of DCs

PAAMs mediated transfection of DC 2.4 cells with a GFP plasmid at N-P 8 was conducted in serum-free cell culture media, and the expression level of GFP was determined by flow cytometry (Figure 3.7A). To exclude any possible interference from cell autofluorescence, DCs transfected under identical conditions but using a luciferase plasmid served as negative controls to set the GFP⁺ gate. Transfection efficiency of DC 2.4 cells measured by the percentage of GFP⁺ cells reached around 25% by PAEM, dropped to 12% by PAPM and 8% by PABM respectively (Figure 3.7B). Mean fluorescence intensity also followed this trend, which meant cells transfected by polymers with shorter pendant chains were brighter than those with longer ones (Figure 3.7C).

To our surprise, PAHM polyplexes killed most cells under above transfection condition so that we couldn’t collect enough cells to run flow cytometry. However, at the same PAHM concentration, most cells survived from free PAHM challenge. PAPM polyplexes also displayed slight cytotoxicity to DC 2.4 cells. But only 60% of cells remained alive after treatment with PABM polyplexes as shown in Figure 3.7D. The toxicity discrepancy between free polymers and their corresponding polyplexes may arise from different conditions where they contact the cells. In the free polymer MTT assay, polymers may bind to negative proteins in the serum and shield partial positive charge to deactivate their interaction with cells, while during transfection, PAAMs got more chance to attach to cells in serum-free cell culture media. The cytotoxicity of PAPM and PABM
can be regarded as a reason for lower level of gene transfection because the gene expression step may be restricted in dying cells.

![Figure 3.7](image)

**Figure 3.7** Transfection efficiency of DC 2.4 cells by polyplexes determined by flow cytometry. (A) Representative dot plots of transfected cells. The GFP<sup>+</sup> gate was set based on cells transfected with a luciferase plasmid. (B) Quantification of the percentage of GFP<sup>+</sup> cells transfected in the absence of serum. (C) Mean fluorescence intensity of GFP<sup>+</sup> cells transfected in the absence of serum. (D) Cytotoxicity of polyplexes after transfection in serum-free media measured by MTT assay. Mean ± SD, *p < 0.05, **p < 0.01.
Researchers got similar conclusions for poly(β-aminoester)s bearing pendant primary amines, the one with the shortest side group made the highest gene transfection efficiency [93], mainly because the side group affected degradation of polymers. However, for poly(amoalkyl acrylamide)s, the shortest member who had good cell viability didn’t gain the best transfection efficiency. Poly(N-(8-aminooctyl)acrylamide) with a fairly medium side chain length reached the highest gene transfer efficiency [87], the reason may be that this member achieved a proper hydrophobic/hydrophilic balance for gene transfection. Also, the poly(amidoamine) with middle-sized pendant group gained the highest gene expression [94].

Considering the high cytotoxicity of PHAM, we didn’t include this polymer in the following biological characterizations.

3.3.7 Subcellular trafficking of polyplexes

In order to know what happened when polyplexes enter cells, we fluorescently labeled PAAMs and plasmid DNA and monitored the cellular uptake and subcellular trafficking of polyplexes using confocal laser microscopy. All transfection experiments for intracellular trafficking study by PAAM/DNA polyplexes except PAHM were done at N-P ratio 8.

Cy5-labeled luciferase plasmid was used for transfection to visualize cellular uptake in DC 2.4 cells after 12 h and 24 h by confocal fluorescence microscopy (Figure 3.8A). The level of Cy5-plasmid fluorescence inside cells was then quantified based on the confocal images using a method reported by Akita et al [96] (Figure 3.8B). Representative confocal images at 24 h showed clearly that PABM resulted in less quantities of Cy5-plasmid inside cells than PAEM and PAPM did. From 12 h to 24 h the
amount of plasmid taken up by the cells continued to rise and there was clearly a dependence on side chain length. The quantity of cellular uptake of plasmid complexed to PAEM was significantly ($p < 0.05$) higher than that to PABM at both 12 h and 24 h. The uptake of plasmid complexed to PAPM arrived at lower levels than that of PAEM by 12 h and 24 h, yet remained significantly higher than the PABM polyplexes at 24 h (Figure 3.8B).

![Confocal fluorescence images and quantification graph](image)

**Figure 3.8** Cellular uptake of plasmid. (A) Representative confocal fluorescence microscopy images of DC 2.4 cells transfected by polyplexes (N:P ratio of 8) at 24 h. Red: Cy5-labeled plasmid. Blue: Hoechst 33342 staining cell nuclei. Shown are fluorescence overlaid with white light images. (B) Quantification of cellular uptake. Mean ± SE, 30 cells for each sample were counted, *$p < 0.05$.

For a successful transgene expression, DNA plasmids must be released from polyplexes at a certain time point after being taken up by the cells. Here confocal
fluorescence microscopy images were captured to assess the effect of polymer hydrophobicity on polyplex dissociation after internalization (Figure 3.9A), which was then quantified by measuring the spatial colocalization of fluorescence signals from the Cy5-labeled DNA plasmid and Oregon Green 488-labeled PAAMs at 24 h (Figure 3.9B).

Figure 3.9 Intracellular dissociation of polyplexes. (A) Representative confocal fluorescence microscopy images of DC 2.4 cells transfected by polyplexes (N:P ratio of 8) at 24 h. Red: Cy5-labeled plasmid. Green: Oregon Green-labeled polymer. Blue: Hoechst 33342 staining cell nuclei. (B) Quantification of intracellular polyplex dissociation. Mean ± SE, 30 cells for each sample were counted, *p < 0.05.

Among all three polymers, PABM with aminobutyl side group had the highest fraction (~50%) of intact polyplexes, significantly different from the other two. Again,
the difference in dissociation between PAEM and PAPM polyplexes was not statistically significant. These findings demonstrated the possibility of utilizing hydrophobicity of polymers to modulate the polyplex dissociation, and in turn, to control transgene expression.

Figure 3.10 Endolysosomal localization of plasmid. (A) Representative confocal fluorescence microscopy images of DC 2.4 cells transfected by polyplexes (N:P ratio of 8) at 12 h. Red: Cy5-labeled plasmid. Green: FITC labeled LAMP-1 antibody. Blue: Hoechst 33342 staining cell nuclei. (B) Quantification of plasmid localized in the endolysosome. Mean ± SE, 30 cells for each sample were counted, *p < 0.05.
The colocalization of Cy5-labeled plasmid DNA and FITC labeled LAMP-1 antibody stained late endosome and lysosome was also observed by confocal fluorescence microscopy (Figure 3.10A) and quantified by calculating the amount of overlap between the fluorescent signals of Cy5 and FITC (Figure 3.10B). Unlike cellular uptake and polyplex dissociation, there was no overall side chain length dependence for endolysosomal colocalization of the polyplexes. All three polymers showed similar level of endolysosomal entrapment at 12 h (around 15 to 20%, no statistical significant difference).

3.3.8 DC maturation by polyplexes

The phenotype maturation state of DCs is important to successful DNA vaccination considering the unique role of DCs to link the innate and adaptive immune responses. Previously, we showed that PAEM with different molecular weight could strongly up-regulate the expression of a maturation marker CD40. Here we examined the maturation state of murine DC 2.4 cells after treatment with PAAM/DNA polyplexes in vitro to see how the hydrophobic state would play a role. LPS was used as a positive control to treat DCs and up-regulate the expression of CD40, while naked DNA plasmid as a negative control did not have any effect (Figure 3.11A). Under serum-free transfection condition, exposure to polyplexes at N:P 8 induced a strong up-regulation of CD40 at levels similar or higher than LPS did. And there’s a clear correlation between CD40 level and pendant chain length of PAAMs (Figure 3.11B): shorter pendant chain stimulated higher CD40 level.
Figure 3.11 Maturation of DC 2.4 cells by polyplexes marked by up-regulation of CD40. (A) A representative set of flow cytometry data of cells transfected by polyplexes or LPS or naked DNA only. The shaded area is untreated DCs. Numbers represent the values of mean fluorescence intensity. (B) Quantification of DC maturation. CD40 level was normalized against untreated DCs.

These findings suggest that as carriers for DNA plasmid, the PAAM polymers may be potentially good adjuvants because of their ability of stimulating DC maturation, a prerequisite for generating adaptive immune responses. Cellular uptake and cytotoxicity of PAAM polyplexes may relate to this trend of CD40 augmentation. It’s reported that proper cytotoxicity of polymers or proteins may be necessary to induce DC maturation [95]. In our system, PAPM and PABM did show modest cytotoxicity and induced higher level of CD40 than LPS. However, PAEM was nontoxic to DCs in the process of transfection and could still induce strong DC maturation. AAM monomers can also be
combined and fabricated to copolymers to precisely control their hydrophobicity and cytotoxicity to get the optimized transgene expression and DC phenotype maturation.

**3.3.9 Discussion**

When adding one more methylene group in the side chain, this structure subtle alternation has been amplified in cytotoxicity and subcellular trafficking steps and resulted in reduced transfection efficiency. At N:P 8, PAEM polyplexes were nontoxic, PAPM polyplexes began to show slight cytotoxicity, while PABM polyplexes killed around 40% of DC 2.4 cells and PAHM polyplexes killed all cells. At that N:P ratio, PAAM polyplexes shared similar DNA binding strength, polyplex stability against heparin, and surface charge, although PAEM polyplexes were larger than the other two in size. However, the uptake of DNA by PAEM polyplexes was higher than that by PAPM, and followed by PABM. In addition, more polymer/DNA dissociation happened in PAEM and PAPM polyplexes than in PABM polyplexes inside cells. Because of different cytotoxicity and subcellular trafficking process caused by structural alternation, transfection efficiency of DC 2.4 cells measured by the percentage of GFP$^+$ cells reached around 25% by PAEM, dropped to 12% by PAPM and 8% by PABM respectively. The elucidation of the internalization mechanisms and intracellular trafficking of the PAAM/DNA polyplexes will require our further studies. And we will focus on the influence of charged membrane components or cellular proteins on the stability and transport of PAAM polyplexes.

Interestingly, we also found that increasing hydrophobicity caused more toxicity and lower gene transfection level in our preliminary study of a poly(2-aminoethyl methacrylate)-co-poly(butyl methacrylate) copolymer system, as shown in Appendix I.
The PAEM-co-PBM random copolymer system was synthesized by ATRP to afford polymers with similar total repeating units but different PBM fractions. With more fraction of PBM incorporated into the copolymer system, gene transfection reduced gradually. For example, at N:P 8, 10% and 30% of PBM in the copolymer reduced transfection to 18% and 4% respectively, while PAEM polyplexes could transfet 25% of DC 2.4 cells. When 50% of PBM was incorporated, the polymer showed strong cytotoxicity. Although PAEM-co-PBM is an interesting polymer system to investigate the influence of hydrophobicity of polycations on cytotoxicity, gene expression level, and DC cell activation level, this copolymer system is not as good as the PAAM system because the chain regularity and charge density are changed simultaneously when the hydrophobicity is altered.

In chapter 2 and chapter 3, we discussed the influence of chain length and hydrophobicity of polycations on their gene transfection performance respectively. And we believe that the structure-function relationship study will enlighten us to design more efficient and reliable polymeric DNA vaccine carriers. Another widely reported polymer structure variation is the charge type polycations bearing. In a preliminary study, we found that tertiary and quaternary amines decreased gene transfection level dramatically while distance from polymer backbone and charge center was kept unchanged, as shown in Appendix II. At N:P 8, poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) still had low level of transfection on DC 2.4 cells, however, poly([2-(methacryloyloxy)ethyl]trimethylammonium chloride) didn’t transfet DCs at all. We will continue to reveal how the charge type affects the properties of polyplexes and subcellular trafficking.
3.4 Conclusion

Cationic PAAM polymers with varied pendant chain length but precisely controlled main chain length were synthesized by ATRP and characterized for delivering plasmid DNA to antigen-presenting dendritic cells. Through a comprehensive investigation we revealed how the increase of side chain length would affect the colloidal properties of the polyplexes, DNA conformation in polyplexes, cytotoxicity and transfection efficiency, uptake in dendritic cells and subcellular trafficking, and the capacity of inducing DC maturation. Increasing the side chain length resulted in higher cytotoxicity and lower cellular uptake of polyplexes, which in turn decreased the transfection efficiency. Thus, the structurally simple and defined PAAM polymers are useful model materials for understanding structure-function relationship in non-viral gene delivery.
Chapter 4: Cytotoxic Synthetic Chemical Polymers as a New Class of Potential Anticancer Agents

4.1 Introduction

Conventional chemotherapeutic drugs against cancer may suffer from multiple drawbacks including toxic side-effects, low bioavailability in vivo, resistance in cancer cells, and efficacy limited to small fractions of patient population [103]. New anticancer agents with high specificity, stability, and being able to overcome multi-drug resistance are always the targets researchers try to develop. In the last two decades, over 150 peptides have been reported to be able to kill cancer cells [104], and some of them also show good selectivity to malignant cells over healthy cells [105]. Most of the anticancer peptides kill cancer cells effectively through membrane disruption, regardless of drug resistance, such as melittin [106] and cecropins[107]. Although anticancer peptides seem to be promising, they may suffer from poor stability in vivo, limited solubility in water, and lack of specificity, which restricted their further usage clinically [57].

Synthetic water soluble polymers have been widely used to incorporate anticancer drugs in the format of micelles [108], polymer-drug conjugates [109], or polymersomes[110] to delivery drugs for cancer therapy. And many synthetic polymers could also be adopted as genetic material carriers to regulate tumor cell proliferation [111]. The prevailing guideline stimulates that the ideal polymer carrier itself must be safe, i.e. chemically and biologically inert and non-toxic. A research field that comes up lots of cytotoxic synthetic polymers is the development of antimicrobial polymers. In the past decade, a vast number of antimicrobial polymers [54] have been designed and
synthesized. During the development process for each type of antimicrobial polymers, large amount of structurally similar polymers were synthesized, however, the majority of the candidates were eliminated because of unacceptable toxicity to mammalian cells. In all cases, those polymers that happen to be cytotoxic are discarded and never considered anymore.

Here we propose to exploit the cytotoxicity of many synthetic polymers for direct killing of cancer cells. We further hypothesize that such cytotoxic polymers would have similar cell killing mechanism and efficiency as anticancer peptides and toxins but are superior in several aspects such as stability in vivo, water solubility, purity, and synthesis and modification with ease and precision. Here we present the proof-of-principle of this approach.

4.2 Materials and methods

4.2.1 Materials

Toluene (Sigma-Aldrich, St. Louis, MO) was dried by refluxing over sodium and distilled. \( N\)-(tert-butoxycarbonyl) aminohexyl methacrylate (tBocAHM) was synthesized as described by Zhu et al [75]. Ethyl \( \alpha \)-bromoisobutyrate, copper (I) chloride (CuCl), 2,2′-dipyridyl (bPy), and doxorubicin hydrochloride were purchased from Sigma-Aldrich. Maleic anhydride, dimethyl maleic anhydride, and citraconic anhydride were also ordered from Sigma-Aldrich. Cis-aconitic maleic anhydride was bought from Alfa Aesar (Ward Hill, MA). 2,4,6-trinitrobenzenesulfonic acid (TNBSA) was a product of Thermo Fisher Scientific Inc. (Rockford, IL). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), ethidium homodimer-1, Dulbecco’s phosphate buffered saline (DPBS), Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), trypsin and
HEPES buffer solution were purchased from Invitrogen (Carlsbad, CA). Other chemicals and solvents were purchased from Sigma-Aldrich and used without further purification.

4.2.2 Synthesis of poly(6-amino-1-hexyl methacrylate) (PAHM)

The atom transfer radical polymerization (ATRP) of tBocAHM followed the method reported by Ji et al [86]. Three different monomer/initiator feed ratios (20:1, 40:1, 100:1) were used to obtain poly(6-amino-1-hexyl methacrylate) (PAHM) homopolymers with varying molecular weights. For example, to synthesize PAHM with the largest degree of polymerization (DP), 1.0 g of tBocAHM, 6.8 mg of ethyl α-bromoisobutyrate, 3.5 mg of CuCl, 10.9 mg of bPy and 3 mL of dried toluene were added to a two-neck glass flask and degassed via three freeze-thaw cycles. The mixture was then heated at 80°C, stirred for 6 h. The reaction was terminated by exposing the system to air. The diluted reaction solution by dichloromethane was then passed through a basic aluminum oxide column to remove the copper complex. The resulting solution was concentrated under vacuum and precipitated in hexane twice and dried in vacuum at room temperature for 2 days. The Boc group was removed by dissolving 0.6 g of PtBocAHM in 5 mL of trifluoroacetic acid (TFA) and stirred for 2 h at room temperature. TFA was then removed by evaporation, and the residue was rinsed three times with diethyl ether. The resultant precipitate was collected by filtration, washed twice by diethyl ether, and dried overnight in vacuum. The polymers were then neutralized with 0.5 M NaOH water solution to reach pH 8.0, and dialyzed (MWCO 3,500) against distilled deionized water for 3 days. The final PAHM polymers were obtained by lyophilization. PtBocAHM with targeting DP of 20 was recovered by removing most of solvents after eliminating the copper complexes. TFA was added directly to the above product, stirred for 2 h, and
removed under vacuum. The residue was dissolved in water, neutralized with NaOH, dialyzed (MWCO 1,000), and lyophilized to harvest PAHM with the smallest DP. Poly(2-aminoethyl methacrylate) (PAEM) with DP100 was also synthesized by ATRP according to procedures by Ji et al [86].

4.2.3 Characterization of PAHM

The $^1$H NMR spectra of the polymers were acquired on a Varian Unity spectrometer (300 MHz) using CDCl$_3$ (for PtBocAHM) and D$_2$O (for PAHM) as solvents. Chemical shifts were recorded in ppm and referenced against tetramethylsilane (TMS) and D$_2$O, respectively. Gel Permeation Chromatography (GPC) analysis of PtBocAHM was performed at 35°C in CHCl$_3$ with a flow rate of 1 mL/min using a Hewlett-Packard 1100 series liquid chromatography equipped with three PL gel 5-µm mixed columns (Jordi Gel columns of 500, 103, and 104 Å pore sizes) and a Hewlett-Packard 1047A refractive index detector. The GPC instrument was calibrated with polystyrene standards (Polymer Laboratories, Amherst, MA). All sample solutions were filtered through a 0.22-µm filter before analysis.

4.2.4 Cytotoxicity in 2D monolayer cell culture

A mouse breast cancer cell line EMT-6 (ATCC, Manassas, VA), two human breast cancer cell lines MDA-MB-231 (ATCC) and MCF-7 (ATCC), and one human glioma cancer cell line T98G (ATCC) were used in standard MTT assays [112] to test the cytotoxicity of PAHM polymers with different molecular weight and concentration after 24 and 48 h of treatment. Dox-HCl was also tested for comparison. All of the four cancer cell lines were incubated in corresponding cell culture media, EMT-6 media (DMEM high glucose, 10% FBS, 100 U/mL penicillin/streptomycin), T98G and MDA-
MB-231 media (DMEM low glucose, 10% FBS, 100 U/mL penicillin/streptomycin),
MCF-7 media (DMEM low glucose, 10% FBS, 10 µg/mL insulin, 100 U/mL penicillin/streptomycin), at 37 °C, and a humidified atmosphere containing 5% CO2.

The MTT assay of PAHM polymers and Dox-HCl on EMT-6 cells exemplified the general experimental procedures. EMT-6 cells were seeded into 96-well plates at 5000 cells/well and cultured for 20 h in EMT-6 media at 5% CO2 and 37°C. Afterwards, polymers of various molecular weight and concentrations were added to the cells and cultured for 24 h or 48 h. MTT in PBS (5 mg/mL, 20 µL) was then added to each well, reaching a final concentration of 0.5 mg/mL. After 4 h, unreacted MTT was removed by aspiration. The formazan crystals were dissolved in 150 µL of DMSO, and the absorbance was measured at 570 nm using a Bio-Tek Synergy HT plate reader. Cell viability was calculated by [absorbance of cells exposed to polymers]/ [absorbance of cells cultured without polymers] in percentage. Cell images were captured before MTT was added using an Olympus IX70 upright microscope under polarized light.

4.2.5 Mechanism of cytotoxicity of PAHM

EMT-6 cells were seeded into 96-well plates at 5000 cells/well and cultured for 20 h in EMT-6 media at 5% CO2 and 37°C. The cells were washed by PBS and supplemented with 180 µL of fresh media for each well. Ethidium homodimer-1 solution in 20 µL of PBS was then added to above cells to reach a concentration of 4 µM and incubated for 10 min according to the manufacturer’s instructions. PAHM polymers with three different chain lengths were added to cells pre-stained with ethidium homodimer-1 or cells without staining to reach 40 µg/mL and incubated for 10 min. Then, images of
cells were captured by an Olympus IX70 inverted fluorescence microscope. Cells stained with ethidium homodimer-1 without PAHM treatment were used as controls.

4.2.6 Hemolysis of PAHM

An eight-week old male C57BL/6 mouse was sacrificed by CO₂ asphyxiation and blood was collected from the heart and centrifuged at 1500 rpm for 5 min at 4°C. The plasma was removed, and the erythrocytes were resuspended in 2 ml ice cold PBS. The cells were again centrifuged at 1500 rpm for 5 min at 4°C. This procedure was repeated more than twice to ensure the removal of any released hemoglobin. After the supernatant was removed, the cells were resuspended in PBS solution and diluted to obtain a cell suspension with Abs = 0.6 at 650 nm. Cationic polymers PAHM and PAEM were also diluted in PBS to reach different concentrations. 0.1 mL of the polymer solutions were added to 0.1 mL of the RBC suspension in a 96-well plate and incubated for 1 h at 37°C with mild shaking. Complete hemolysis was attained using a 2% v/v Triton-X yielding the 100% control value. PBS was used as negative control. After incubation, the 96-well plates were centrifuged at 1500 rpm for 5 min, and 100 µL of the supernatants were transferred to another 96-well plate. The release of hemoglobin was determined by UV at 414 nm. Each sample was measured in triplicates. Degree of hemolysis is defined as % lysis = 100*(A_{polymer} - A_{blank}) / (A_{triton} - A_{blank}).

4.2.7 Synthesis of PAHM-maleamate conjugates

The synthesis of PAHM-maleamate conjugates followed the methods described in a literature [113]. To a solution containing 250 µg PAHM₁₈, 2.8 mg HEPES, and 0.5 mg NaOH in 120 µL water was added 0.45 mg of maleic, dimethylmaleic, or citraconic anhydride in 20 µL ethanol respectively with rapid vortexing. For cis-aconitic anhydride,
there was a substantial amount of hydrolyzed anhydride present in the sample; therefore, 1.26 mg of anhydride, 5.6 mg of HEPES, and 1 mg of NaOH were used. All of the mixed solutions were incubated at room temperature for 1 h.

To determine the amine content of above samples, samples were diluted properly and added to 0.25 mL of 0.01% (w/v) 2,4,6-trinitrobenzenesulfonic acid (TNBSA) solutions in 100 mM borate buffer with pH 8.5. Fifteen minutes later the absorbance of the solution was measured at 420 nm. The amount of amine was calculated by comparing the absorbance between samples and a PAHM control, whose concentration equaled the total concentration of free PAHM and acylated PAHM in the mixture solution. Each sample was measured in triplicates. Degree of acylation is defined as %acylation = 1 - 100*(A_{sample} - A_{blank}) / (A_{PAHM} - A_{blank}).

4.2.8 Hydrolysis kinetics of PAHM-maleamate conjugates

Maleamylated PAHMs were synthesized freshly as described in last section. The acylated PAHM were then added buffer solutions with 3 different pH values respectively: borate buffer (pH 7.4, 400 mM), citric buffer (pH 6.0, 133 mM), and acetic buffer (pH 5.0, 400 mM), and incubated at room temperature with mild shaking. At various time points, 25 µL of the mixed solutions were added to 475 µL of 100mM borate buffer with pH 8.5, then 0.25 mL of 0.01% (w/v) TNBSA solutions in 100 mM borate buffer with pH 8.5 were added to above solutions and mixed well. Fifteen minutes later the absorbance of the solution was measured at 420 nm. A PAHM control bearing total concentration of free PAHM and acylated PAHM in the mixture solution was also tested. The amount of amine was calculated by comparing the absorbance between samples and the PAHM
control. Each sample was measured in triplicates. Degree of amide bonds hydrolysis is defined as \( \% \) hydrolysis = \( 100 \times \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{PAHM}} - A_{\text{blank}})} \).

4.2.9 Cytotoxicity of hydrolysis product of PAHM-maleamate conjugates

PAHM-maleamate conjugates were synthesized freshly based on the same protocol in last two sections. The acylated PAHM were then added acetic buffer (pH 5.0, 400 mM) and incubated at room temperature with mild shaking for 3 days. Afterwards, the mixed solutions were neutralized by the borate buffer with pH 8.5. Then the solutions were further diluted by PBS and added to either EMT-6 cells or red blood cells. Cell viability and hemolysis of hydrolysis product were obtained following procedures described in the cytotoxicity and hemolysis section. PAHM alone with equivalent concentration of the acylated PAHM and free PAHM in mixture solutions were used as controls. All the buffer solutions used in this part were sterilized by passing 0.22-µm filters.

4.2.10 Statistical analysis

Statistical analysis was carried out using a two-sample Student’s \( t \)-test with unequal variance. Values of \( p<0.05 \) were deemed to be different with statistical significance.

4.3 Results and discussion

4.3.1 Synthesis and characterization of PAHM polymers

Most anti-cancer host defense peptides are cationic amphiphilic polymers which can bind to cancer cell membranes and cause cell death by destroying the membrane integrity [58]. Synthetic polymers built with chemical structures containing certain
positive charges and appropriate hydrophobic components can also have strong interaction with cell membranes [114]. The structure of PAHM (Fig. 4.1A) was chosen for sufficiently dense cationic primary amines to bind to cell surface and for appropriate hydrophobicity to disrupt cell membrane. PAHM was used as a copolymer with PDMAEMA to bind and deliver plasmid DNA to cells within its nontoxic concentration [75]. While biocompatibility is important for a polymer to be a good candidate for gene delivery application, cytotoxicity of a polymer may be good to kill cancer cells. Here we synthesized PAHM homopolymers and exploited their capability to kill cancer cells both in vitro and in vivo to show the possibility that synthetic polymers can be a new class of anticancer agent.

PAHM homopolymers were synthesized via a controlled radical polymerization technique-ATRP, which offers a convenient and effective means to generate polymers with well-defined chain length, composition, and molecular architecture [115]. In the first step, ethyl α-bromoisobutyrate was used to initiate the ATRP of the Boc-protected 6-amino-1-hexyl methacrylate (tBocAHM) to give PtBocAHM. The molar ratio of tBocAHM monomer and the initiator was varied from 20, 40 to 100 to achieve three PtBocAHM with monomer conversions of 95%, 98% and 99% respectively (determined by proton NMR, data not shown) and yield of over 70%. The average degree of polymerization (DP) of the PtBocAHM was calculated to be 18, 38, and 100 based on monomer conversion, and the three polymers were thus named PtBocAHM18, PtBocAHM38, and PtBocAHM100, respectively. GPC traces showed single peaks (Figure 4.1B), and the number-average molecular weight (Mn) of the three polymers was calculated to be $0.82 \times 10^4$, $1.20 \times 10^4$, and $2.08 \times 10^4$ with the polydispersity index
(PDI) of 1.13, 1.18, and 1.26, respectively. Monomodal and symmetric GPC peaks along with narrow molecular weight distribution indicated that well-defined PtBocAHM by ATRP were obtained successfully. In the second step, the Boc-protecting group was completely removed from PtBocAHM by acid treatment, yielding the final products of PAHM. The ease of synthesis and availability of a large number of structurally diverse monomers would enable further optimization of cytotoxicity and specificity.

![Chemical structure of PAHM](image1)

![GPC traces of PtBocAHM](image2)

**Figure 4.1** Physical properties of PAHM. (A) Chemical structure of the PAHM polymer. (B) GPC traces of PtBocAHM, the precursor of PAHM with Boc-protected side chains, using CHCl₃ as mobile phase and a flow rate of 1 mL/min. Average molecular weight was determined based on polystyrene standards.

### 4.3.2 PAHM kills 2D monolayer cells efficiently

To determine the cytotoxicity of PAHM, we treated murine EMT-6, three human cancer cell lines MDA-MB-231, MCF-7, and T98G for 24 and 48 hours with varying concentrations and measured cell viability by MTT assay (Fig. 4.2). A widely used chemo drug doxorubicin was also used as a control to compare the potency of PAHM to kill cancer cells. For the three PAHM polymers with varying chain lengths, there’s no
significant difference among their capacities to kill these 4 cancer cell lines in most cases. PAHM maintained its cytotoxicity even with 18 repeating units, whose chain length was in the similar range with those of many anticancer peptides [58].

The potency of PAHM to kill EMT-6 and MDA-MB-231 in 2D monolayer culture was similar to Dox. After 24 h treatment by PAHM and Dox, the IC$_{50}$ of the PAHM and Dox were close around 10-15 µg/mL on EMT-6 and MDA-MB-231, as shown in figure 4.2A & 4.2B. 48h later, the IC$_{50}$ of Dox decreased to 5 µg/mL while IC$_{50}$ of PAHM didn’t change much. Longer treatment time induced smaller IC$_{50}$ of Dox could be attributed to more cellular accumulation of Dox to cause more apoptosis. PAHM showed superior potency to Dox in killing multidrug resistant cells MCF-7 and T98G [116-117] as shown in figure 4.2C & 4.2D. After 24h treatment by PAHM and Dox, IC$_{50}$ of PAHM was close, around 10-15 µg/mL in MCF-7 and T98G cells, while IC$_{50}$ of Dox was 30 µg/mL and far above 40 µg/mL respectively. Increasing treatment time to 2 days didn’t alter the IC$_{50}$ values of PAHM polymers, but did decrease the gap of IC$_{50}$ values between PAHM and Dox. These 2D monolayer cytotoxicity data imply that PAHM kills cancer cells via a mechanism different from Dox, which may be a benefit to overcome the multi-drug resistance of cancer cells.
Figure 4.2 Cytotoxicity of PAHM polymers and Dox to cancer cells for 24 h and 48 h measured by the MTT assay. Viability of cells cultured without polymer was taken as 100%. Each concentration was tested triplicate, mean ± SD. (A) EMT-6; (B) MDA-MB-231; (C) T98G; (D) MCF-7.
4.3.3 PAHM kills cells through membrane lysis

**Figure 4.3** Images showing PAHM polymers killing cancer cells by disrupting membrane integrity within short time. (A) White light image of EMT-6 cells treated by PAHM$_{100}$ at 40 µg/mL after 10 min; (B) White light image of EMT-6 cells without treatment as control; (C) Fluorescent image of ethidium homodimer-1 pre-stained EMT-6 cells incubated with PAHM$_{100}$ at 40 µg/mL for 10 min; (D) Fluorescent image of ethidium homodimer-1 pre-stained EMT-6 cells incubated with 20 mM HEPES buffer for 10 min.

PAHM’s cationic and amphipathic chemical structure, along with non selective cytotoxicity between regular and multi-drug resistant cells remind us that PAHM and most anti-cancer host defense peptides may share similar mechanism to kill cancer cells through cell membrane disruption. We thus select a fluorescent dye ethidium homodimer-1 (EthD-1) to evaluate plasma membrane integrity after PAHM treatment. EthD-1 can undergo a 40-fold enhancement of fluorescence upon binding to nucleic acids when it
enters cells with damaged membranes, while EthD-1 is excluded by the intact plasma membrane of live cells [118]. Thereby there’s a bright red fluorescence coming out in dead cells after EthD-1 staining, but not live cells.

We observed rapid EMT-6 cell death due to membrane permeation (Fig. 4.3C) even after only 10min exposure to PAHM, no matter how large the polymer was. And few red fluorescence signals were found in the control group, which justified the validity of this assay. The bright field images of EMT-6 cells were also recorded 10min after PAHM treatment. EMT-6 showed round, swollen morphology (Fig. 4.3A), which was distinguished from control cells. The above dye permeation experiment suggested strong membrane lytic activity of PAHM as the mechanism of cell killing. Cancer cells may not be able to develop resistance to this rapid cell killing by PAHM.

![Graph](image_url)

**Figure 4.4** Hemolysis of PAHM and PAEM polymers on red blood cells measured by hemoglobin release. Each concentration was tested triplicate, mean ± SD.
Hemolysis experiment also confirmed the capability of PAHM to lyse cell membranes. Longer PAHM has slightly higher hemolytic ability than shorter ones. At 80 µg/mL, all PAHM polymers induced around 90% red blood cell lysis (Fig. 4.4).

Charge type, number and hydrophobicity governed the interaction between polymers with cell membranes. PAEM, holding the same primary amine with PAHM, but having only two methylene groups between ester bond and charge center didn’t cause any blood cell lysis. Probably, PAEM isn’t hydrophobic enough to insert into lipid membranes after they attach to membrane surface through electrostatic attraction. We didn’t find PAHM to form any secondary structure in neutral buffer solution. It is reported that some anti cancer peptides are not necessary to self assemble to a secondary structure in solution [57]. They may form a secondary structure when they bind to the cell membrane to induce cell membrane disruption. PAHM may also assemble to a particular structure after they attach to lipid membranes to cause cell membranes lose their integrity.

4.3.4 Hydrolysis kinetics of PAHM-maleamate conjugates

Although PAHM kills cancer cells efficiently, it can’t distinguish healthy cells from cancer cells. In addition, PAHM lyse red blood cells, which also inhibits the in vivo application of PAHM. Intelligent polymer delivery systems responding to particular tumor microenvironments have been widely used to enhance anticancer drug targeting and decrease nonspecific toxicity [119-120]. Covalent conjugation of anticancer drugs to a polymer backbone via a pH-sensitive linkage has been explored as an option to liberate chemodrug such as doxorubicin in local tumor environment. Common pH responsive linkages include anhydrides, cis-aconityl, hydrazones, orthoesters, and acetals [121].
Scheme 4.1 Maleic anhydride and poly(6-amino-1-hexyl methacrylate)-maleamate conjugates. Maleic anhydride and maleamate derivatives: maleic $R_1$ and $R_2=H$; dimethylmaleic $R_1$ and $R_2=CH_3$; citraconic $R_1=H$ and $R_2=CH_3$, cis-aconitic $R_1=H$ and $R_2=CH_3COOH$.

Here we applied a chemical strategy to reversibly mask the cytotoxicity of PAHM in respond to environmental pH change. Maleic, dimethylmaleic, citraconic, and cis-aconitic anhydride were used to acylate PAHM at mild basic condition (Scheme 4.1). By TNBSA assay there was no detectable amount of amine upon acylation under these conditions no matter which anhydride was used.

All PAHM-maleamate conjugates showed different hydrolysis kinetics under varied pH except PAHM-maleic amide conjugate as shown in Figure 4.5. We didn’t detect any hydrolysis of amide bond of maleic anhydride modified PAHM at pH 7.4, 6.0, and 5.0. At pH 7.4, citraconylated PAHM and cis-aconitylated PAHM kept intact till 3 days, while around 30% of dimethylmaleamylated PAHM hydrolyzed within 1 day. When pH decreased to 6.0, 30% of citraconylated PAHM and 20% of cis-aconitylated PAHM hydrolyzed after 3 days incubation. Dimethylmaleamylated PAHM hydrolyzed very fast compared with the other two conjugates, 30% of amines were recovered within 5 min, 75% within 30 min, and 90% within 2 h. At pH 5.0, all three kinds of amide bonds hydrolyzed much faster. 60% of PAHM-citraconic and 36% of PAHM-cis-aconitic
conjugates hydrolyzed after 3 days incubation. PAHM-dimethylmaleic conjugate still had the fastest hydrolysis speed compared with the other two conjugates, 25% of amines were recovered within 1 min, 65% within 5 min, and 85% within 30 min, following a plateau.

**Figure 4.5** Hydrolysis profile of PAHM$_{18}$-maleamate conjugates in neutral and acidic buffers. PAHM$_{18}$-maleamate conjugates were incubated in buffers and certain amount of samples were taken at different time points to run TNBS assay to determine the amount of free primary amines, which were free PAHM hydrolyzed from the conjugates. PAHM$_{18}$ homopolymer in each buffer was regarded as 100% amide bond hydrolysis. Each time point was tested triplicate, mean ± SD. (A) pH 7.4; (B) pH 6.0; (C) pH 5.0.

Overall, more acidic, faster hydrolysis happened for the PAHM-maleamate conjugates except the maleic one. Maleic anhydride, which has no substitution at the unsaturated carbon-carbon bond forms the most stable maleamic acid. Citraconic and cis-aconitic-derived maleamic acids have one substitution and are more pH-labile. Maleamic
acids derived from disubstituted dimethylmaleic anhydride are the most pH-labile. Similar hydrolysis profiles under varied pH have also been observed on aclyated PLL and melittin [113].

4.3.5 Hydrolysis product of PAHM-maleamate conjugates

![Graph showing hemoglobin release vs concentration](image)

**Figure 4.6** Hemolysis of PAHM₁₈-maleamate conjugates and their hydrolysis product. All three PAHM₁₈-maleamate conjugates were made freshly and added into pH 5.0 buffers to incubate for 3 days to allow hydrolysis. Afterwards, freshly made conjugates and neutralized hydrolysis products were added to RBC cells and incubated with mild shaking for 1 h under 37°C. Hemoglobin release was then measured at 414 nm. The concentrations were calculated to be equivalent with PAHM₁₈ homopolymer. Each concentration was tested triplicate. Mean ± SD, *p < 0.05, **p < 0.01.

We incubated PAHM-maleamate conjugates in acetic buffer with pH 5.0 and added both the conjugates and their hydrolysis products to erythrocytes or EMT-6 cells to see whether cytotoxicity of PAHM could be reversibly covered and recovered. Upon acylation, none of the conjugates caused hemoglobin release. And only hydrolysis
product of dimethylmaleamylated PAHM lysed more than 60% of the red blood cells as shown in Figure 4.6. This could be explained that around 85% of free PAHM had been recovered in dimethylmaleamylated PAHM after acid treatment.

![Graph showing cell viability and concentration](image)

**Figure 4.7** Cytotoxicity of PAHM-18-maleamate conjugates and their hydrolysis product on EMT-6 cells. All three PAHM-18-maleamate conjugates were made freshly and added into pH 5.0 buffers to incubate for 3 days to allow hydrolysis. Afterwards, freshly made conjugates and neutralized hydrolysis products were added to EMT-6 cells and cultured for 24 h. Cell viability was measured by a standard MTT assay. The concentrations were calculated to be equivalent with PAHM-18 homopolymer. Each concentration was tested triplicate. Mean ± SD, *p < 0.05, **p < 0.01.

When the conjugates and their hydrolysis products were added to EMT-6 cells, the situation was quite different from what happened on erythrocytes (Fig. 4.7). Dimethylmaleamylated PAHM and its hydrolysis product both caused substantial percentage of cell death after 24 h incubation with EMT-6. PAHM-maleic and PAHM-cis-aconitic conjugates and their hydrolysis products didn’t alter the cell viability much.
PAHM-maleic didn’t hydrolyze at all and 36% of free PAHM recovered in PAHM-cis-aconitic conjugate wasn’t enough to interact with cell membrane to induce cell death. PAHM-citraconic conjugate showed slightly toxicity to lower the cell viability by around 10%. After hydrolysis, citraconylated PAHM killed 60% and 90% cells at 40 µg/mL and 80 µg/mL equivalent PAHM concentrations respectively.

It’s still not clear why the same hydrolysis product of PAHM-citraconic conjugate didn’t lyse erythrocytes but killed cancer cells. One reason may be that cancer cell membranes are more negatively charged than healthy cells [122]. Although 60% of recovered free amines could not induce red blood cell membrane lysis, it was good enough to bind to the cell membrane and disrupt membrane integrity of EMT-6 cells. Considering the instability of PAHM-dimethylmaleic conjugate at physiological pH and non-pH response of PAHM-maleic conjugate, PAHM-citraconic and PAHM-cis-aconitic conjugates have the potential to be investigated in vivo to kill tumor cells utilizing the mild acidic tumor microenvironment.

Overall, PAHM showed strong potency to kill tumor cells in 2D monolayer cell culture through cell membrane disruption. In addition, the cytotoxicity of PAHM could be reversibly masked by forming PAHM-maleate conjugates. Our results provide a proof-of-concept example that synthetic chemical polymers can be used as a new class of anticancer agents. And there’s still a lot of room to improve its therapeutic efficacy. The primary amines on the pendant chain provide good reaction sites to be modified by ease chemistry techniques to make PAHM a safer prodrug. The cationic charge and toxicity can be temporarily shielded at normal physiology conditions and recovered at tumor tissues utilizing specific physiological characteristics, such as acid pH we adopted here.
[113], hypoxia [123], and tumor associated matrix metalloproteinases [124]. In addition, because of facile ATRP implement and wide monomer tolerance [44], the charge and hydrophobicity of synthetic polymers could be precisely controlled in different copolymer combinations to give polymers with optimized cytotoxicity and specificity. Finally, this water soluble polymer can be formulated with other adjuvants to produce nano- or micro- particles to be delivered systemically.

4.4 Conclusion

A structurally simple, synthetic membrane lytic polymer PAHM was highly effective in killing cancer cells in vitro through cell membrane disruptions. In addition, a strategy to reversibly mask the cytotoxicity of PAHM could be done by forming acid sensitive PAHM-maleate conjugates. These results suggest the possibility of a new class of anticancer agents with remarkable potential for superior efficacy, given their unique, tunable chemistry for optimization and formulation.
Chapter 5: Future Directions

5.1 Improving in vivo gene delivery of PAEM

In chapter 2, we showed that the chain length of PAEM played a key role in its biological performance for DNA vaccine delivery in vitro. Although PAEM exhibited good potency to be a DNA vaccine carrier, it’s also noted that the transfection efficiency of PAEM dropped dramatically when 10% serum was applied in the transfection cell culture media. The reason is still not clear yet, it’s highly possible that cationic polyplexes form large aggregates with the serum components or other polyplexes to weaken the overall uptake by cells. It becomes even worse for further in vivo applications where the whole blood contains more serum and the aggregates can be cleared quickly by the reticuloendothelial system [125-126].

The incorporation of poly (ethylene glycol) (PEG) into vector designs is a common strategy to increase stability of polyplexes and reduce the interactions between individual polyplexes as well as with serum components resulting in prolonged circulation time [127]. This benefit comes from the PEG hydrophilic shell induced steric effects that lead to decreased particle-particle and particle-protein interactions. Although PEGylation can help stabilize positive polyplexes and prolong blood circulation time, this effect is at the cost of decreasing the interaction between positive polyplexes and negatively charged cell membrane, which facilitate cellular uptake [128]. In addition, PEGylation may also limit polyplexes escaping from endosomes [129]. As a result, many PEG coated polyplexes suffer from reduced association with cells, diminished cellular uptake and/or endosomal escape, and inefficient cell transfection [68, 130].
It would be ideal if the polyplexes can be shielded by PEG during circulation in the bloodstream, but de-shielded upon cell entry. Since the pH of endosomes drop to ~5 from ~7 of extracellular environment, it’s a good way to utilize this pH difference to remove the shielding components of polyplexes and unpack the genes from the polyplexes. Recently, several papers about pH-triggered de-shielding approach have been reported. Walker et al. developed an acetal-based pH-triggered de-shielding method in which the shielded positively charged surface of polyplex was re-exposed in the acidic environment of the endosome, enhancing endosomal membrane disruption and subsequent transfection efficiency [131]. Li et al synthesized an ortho ester based acid-labile block copolymer of PDMAEM and PEG as an intelligent PEG de-shielding gene carrier [132]. In vitro transfection efficiency of the acid-labile copolymer greatly increased after 6 h incubation at pH 5.0, approaching the same level of PDMAEM.

Our group is pretty interested in poly (ortho ester)s (POE) because the ortho ester bonds in the polymer are relatively stable at physiological pH but hydrolyze rapidly at around pH 5 [133]. Since POEs show a predominantly surface-confined erosion process, small quantities of acid hydrolysis products of POEs are able to diffuse away, potentially minimizing DNA or protein degradation when they are used for DNA or protein delivery. Since 1970s, POEs have been under investigation as biodegradable materials for controlled delivery of (protein) drugs. Wang et al also reported biodegradable POE microparticles as DNA vaccine carrier to generate immune response and suppress tumor growth [134]. Recently, our group developed new synthetic pathways to synthesize new families of ortho ester-containing polymers for drug or gene delivery [135-137].
For in vivo applications in future, the hydrolysable ortho ester group will be used to conjugate PEG onto PAEM to prolong the circulation time in blood stream and help remove the PEG shielding effect from polyplexes after they enter endosomes. In my design, PEG10k with ortho ester-linked end group will be converted to an acid cleavable active ester and grafted to a short PAEM chain. This acid-labile PEG grafted PAEM will be labeled as PEG-a-g-PAEM. In addition, the graft density can be adjusted by controlling the feed ratio. The stable PEG-g-PAEM polymer will be used as a control to compare the impact of de-shielding on in vivo gene transfer. The physicochemical properties as well as in vivo performance of PEG-a-g-PAEM polyplexes will be thoroughly evaluated.

5.2 Improving gene transfection of PAEM utilizing membrane disruption ability of PAHM

In chapter 3, we developed a series of poly (aminoalkyl methacrylate) (PAAM) homopolymers with different alkyl size (ethyl, propyl, butyl, and hexyl) and hydrophobicity by ATRP and applied them for gene delivery to DCs. And I investigated comprehensively the colloidal properties of the PAAM/plasmid polyplexes (including stability, particles size and charge), uptake and subcellular trafficking in DCs, and biological properties in vitro (including hemolysis, cytotoxicity, transfection efficiency and DC maturation), aiming to reveal the impact of hydrophobicity of polymers on DNA delivery to DCs. As the side group became longer, higher cytotoxicity and lower cellular uptake of PAAM polyplexes resulted in lower transfection efficiency. PAHM showed the highest cytotoxicity and killed most of cells during transfection.
We also showed that PAHM killed cells by directly disrupting cell membranes in chapter 4. This strong interaction with cell membranes seems not good at all for gene delivery since it results high cytotoxicity. However, on the other hand, we may utilize this feature to disrupt certain membranes of cellular organelles to help polyplex escape and improve gene transfer.

Endosome escape is a key step for successful polymer mediated gene transfer by avoiding lysosomal degradation of loaded pDNA. The simplest strategy to improve the transfection efficiency of PAEM polyplexes is to provide them with endosome-escaping functions. Several previous studies have reported about the integration of endosome-escaping elements into other polyplexes, including membrane-fusion peptides [138-139] and buffering units with low pKa, such as histidine [140]. Recently, Kataoka et al developed a charge-conversional polymer and used it to form a ternary polyplex system which showed excellent endosome escape capacity [141-142]. Firstly, they treated the primary amino groups of ethanediamine units in poly (N-(N’-(2-aminoethyl)-2-aminoethyl)aspartamide) (PAsp(DET)) with cis-aconitic anhydride to convert the charge from positive to negative. Then this negative PAsp(DET)-maleamate conjugate could be easily integrated to form the ternary PLL polyplex through electrostatic interaction. More importantly, this resultant charge conversional polymer returned to PAsp(DET) in endosomes (pH~5) via the hydrolysis of cis-aconitic amide bonds. Eventually, because of electrostatic repulsion, PAsp(DET) was released from the PLL polyplex to disintegrate the endosomal membrane and facilitate transport of the polyplex into cytoplasm [143].

In my current system, PAHM may play the same role as PAsp(DET) did to disrupt the endosomal membranes and help polyplexes escape, which can be used to
further improve the transfection efficiency of PAEM. However, integration of PAHM moiety into the cationic PAEM polyplex with simple coating is difficult because of electrostatic repulsion between the two. Therefore, PAHM can also be converted by citraconic or cis-aconitic anhydride to negative PAHM-maleamate conjugates and then incorporated into the PAEM polyplexes. When these ternary complexes enter acidic endosomes, the charge conversional acylated PAHM will be degraded to recover PAHM, which can be released from the PAEM polyplex by electrostatic repulsion. The free PAHM is expected to destabilize endosomal membranes to facilitate transport of the polyplex into cytoplasm.

In addition, there will be other benefits of this ternary polyplex system. First of all, the acute cytotoxicity of PAHM is avoided by converting PAHM to a negatively charged new polymer. Secondly, the anionic nature of acylated PAHM at physiological pH is expected to stabilize the ternary polyplexes in the biological milieu due to the reduced interaction with anionic serum proteins.

Overall, it’s promising to combine the membrane disrupting ability of PAHM and good transfection capacity of PAEM together to make a ternary polyplex system to further augment gene delivery by improving endosome escape.

5.3 Improving tumor therapeutic effect of PAHM by forming biodegradable nanoparticles

In chapter 4, we showed that a structurally simple, synthetic membrane lytic polymer PAHM was highly effective in killing cancer cells in vitro through cell membrane disruptions. In addition, a strategy to reversibly mask the cytotoxicity of PAHM could be done by forming acid sensitive PAHM-maleate conjugates. These results
suggest the possibility of a new class of anticancer agents with remarkable potential for superior efficacy, given their unique, tunable chemistry for optimization and formulation.

To further validate the potential in vivo anticancer effect of PAHM, another group member David Panus investigated PAHM using both in vitro 3D multicellular tumor spheroids and in vivo tumor models on mice. And the results were also inspiring. PAHM could efficiently reduced the growth of human T98G tumor spheroids at 40 μg/mL or higher, whereas Dox was ineffective even at 400 μg/mL. In the updated in vivo tumor challenging experiment, intratumorally injected PAHM was able to retard the progression of subcutaneously inoculated EMT-6 tumors on mice compared with PBS groups. The body weight of polymer-injected mice was not different than PBS injected mice, suggesting the lack of acute systemic toxicity of PAHM at 3.6 and 9.1 mg/kg doses. Histology study of organ harvested after PAHM treatment didn’t show any difference with PBS injected mice, which also confirmed that intratumoral topical treatment didn’t cause acute damage to organs.

Together, PAHM showed strong potency to kill tumor cells in 2D monolayer cell culture, 3D multicellular tumor spheroids, and in vivo tumor model through cell membrane disruptions. Our results provide a proof-of-concept example that synthetic chemical polymers can be used as a new class of anticancer agents.

In our initial work, we applied PAHM on tumor models by intratumoral topical injection. Although intratumoral topical treatment are begun to be considered recently [144-145], we can expand the scope of application by delivering PAHM systematically with appropriate carriers, the strategy employed to administrate many cytotoxic anticancer peptides, such as melittin [106] and (KLAKLAK)2 peptide [146-147]. We
have demonstrated that the cytotoxicity of PAHM could be reversibly masked by forming acid sensitive PAHM-maleate conjugates, where the positively charged primary amines of PAHM were transformed to negative carboxylic groups by citraconic or cis-aconitic anhydrides. These PAHM-maleate conjugates can not only be PAHM prodrugs, but also serve as PAHM delivery carriers. Preliminary results showed that nano-sized polyelectrolyte complexes formed when we mixed free PAHM and acylated PAHM together in buffers at physiological pH. When injected intravenously, we can expect these anionic complexes will keep stable during circulation in blood stream because of the repulsive interaction with anionic serum proteins. At the same time, there’ll be no side toxicity because all PAHM injected is chemically or physically shielded. It’s expected that these nanoparticles will accumulate in tumor tissue through the EPR effect [148-149] where acylated PAHM will be hydrolyzed back to PAHM due to weak acidic environment. Therefore, both newly formed PAHM and original packaged PAHM will take effect to kill cancer cells and shrink the tumor size. The dosage and charge ratio should be optimized to reach the maximum antitumor effect.
References


Appendix I: Preliminary Results of Poly(2-aminoethyl methacrylate)-co-poly(butyl methacrylate) (PAEM-co-PBM) Copolymers for DNA Delivery to Dendritic Cells

**Figure 1** Chemical structure for poly(2-aminoethyl methacrylate)-co-poly(butyl methacrylate) (PAEM-co-PBM) copolymers with different composition. x=100, 89, 69, or 48.

**Figure 2** GPC traces of PtBocAEM-co-PBM, the precursors of PAEM-co-PBM with Boc-protected side chains, using CHCl₃ as mobile phase and a flow rate of 1 mL/min. Average molecular weight was determined based on polystyrene standards.
Table 1 Molecular weight and MW distribution of PtBocAEM-co-PBM

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Figure 3 Transfection efficiency of DC 2.4 cells by polyplexes at N:P 8 determined by flow cytometry. (A) Representative dot plots of transfected cells. The GFP$^+$ gate was set based on cells transfected with a luciferase plasmid. (B) Quantification of the percentage of GFP$^+$ cells transfected in the absence of serum. Mean ± SD, *p < 0.05.
Appendix II: Preliminary Study of the Influence of Charge Type of Polycations on Their Gene Delivery Performance

Figure 1 Chemical structure of cationic polymers with different charge types.

Figure 2 Preliminary serum-free transfection efficiency of DC 2.4 cells by polyplexes at N:P 8 determined by flow cytometry. Representative dot plots of transfected cells are shown. The GFP$^+$ gate was set based on cells transfected with a luciferase plasmid.