Published in final edited form as:

J Biomed Mater Res A. 2011 February ; 96(2): 456–465. doi:10.1002/jbm.a.32994.

Enzyme-synthesized Poly(amine-co-esters) as Non-viral Vectors for Gene Delivery

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Abstract

A family of biodegradable poly(amine-co-esters) was synthesized in one step via enzymatic copolymerization of diesters with amino-substituted diols. Diesters of length C₄–C₁₂ (i.e., from succinate to dodecanedioate) were successfully copolymerized with diethanolamines with either an alkyl (methyl, ethyl, *n*-butyl, *t*-butyl) or an aryl (phenyl) substituent on the nitrogen. Upon protonation at slightly acidic conditions, these poly(amine-co-esters) readily turned to cationic polyelectrolytes, which were capable of condensing with polyanionic DNA to form nanometer-sized polyplexes. *In vitro* screening with pLucDNA revealed that two of the copolymers, poly(*N*-methyldiethyleneamine sebacate) (PMSC) and poly(*N*-ethyldiethyleneamine sebacate) (PESC), possessed comparable or higher transfection efficiencies compared to Lipofectamine 2000. PMSC/pLucDNA and PESC/pLucDNA nanoparticles had desirable particle sizes (40–70 nm) for cellular uptake and were capable of functioning as proton sponges to facilitate endosomal escape after cellular uptake. These polyplex nanoparticles exhibited extremely low cytotoxicity. Furthermore, *in vivo* gene transfection experiments revealed that PMSC is a substantially more effective gene carrier than PEI in delivering pLucDNA to cells in tumors in mice. All these properties suggest that poly(amine-co-esters) are promising non-viral vectors for safe and efficient DNA delivery in gene therapy.

Keywords

poly(amine-co-esters); enzyme catalyst; non-viral vector; gene transfection; cytotoxicity

INTRODUCTION

Gene therapy represents a novel form of medical treatment that is expected to have a major impact on human health in 21st century since a large number of human diseases are caused by genetic disorders¹. Because of its broad potential, gene therapy has been intensively investigated during the past several decades^{2,3}. The success of gene therapy is largely dependent on the development of a vector or vehicle that can selectively and efficiently deliver a gene to target cells with minimal toxicity⁴. Although viral vectors display rather good transfection properties, both *in vitro* and *in vivo*, there are a number of problems associated with the use of these vectors, which include the induction of an immune response

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against the viral proteins, possible recombination with wild-type viruses, limitations on the size of inserted DNA, and difficult pharmaceutical grade production on a large scale⁵. For these reasons, recent studies have focused on non-viral carriers in gene therapy to overcome the inherent disadvantages of viral vectors⁶.

In general, non-viral vectors are materials that electrostatically bind DNA or RNA, condense the genetic material into particles--typically several hundred nanometers in diameter-- that protect the genes and facilitate cellular entry⁷. Current non-viral approaches primarily employ polyplex delivery systems, lipoplex delivery systems, solid polymer nanoparticle systems, and naked DNA injection protocols. In most cases, direct DNA injection is not an effective method for gene delivery since the unprotected DNA material can be easily degraded by endogenous nucleases. On the other hand, lipoplex-based delivery has several crucial disadvantages including difficulty in reproducibly fabricating liposomes and DNA-liposome complexes, significant toxicity, and colloidal instability⁸⁻¹⁰. In contrast, polyplex delivery approach provides opportunities for improved treatment safety, greater formulation flexibility, and more facile manufacturing of polymeric carriers and stable polymer/DNA complexes^{11,12}. In formulating polyplexes, various types of cationic polymeric materials containing amine functional groups have been used to condense DNA^{13,14}, such as poly(ethyleneimine) (PEI)¹⁵, poly(L-lysine)¹⁶, chitosan¹⁷, poly(dimethylaminoethyl methacrylate)¹⁴, poly(trimethylaminoethyl methacrylate)¹⁴, poly(4-hydroxy-L-proline ester) (PHP)¹⁸, poly[α -(4-aminobutyl)-L-glycolic acid] (PAGA)¹⁹, modified polyamidoamine (PAMAM) dendrimers²⁰, and poly(β -amino esters) (PBAE)²¹. Among these materials, biodegradable polyesters bearing tertiary amino substituents are significantly less toxic than PEI or polylysine and mediate the transfer and expression of genes to cells at levels that approach or exceed those using PEI²². Thus, design of biodegradable polycations appears to be a reasonable approach to the development of safe and effective non-viral gene vectors. However, it has been a challenge to synthesize such amino-bearing polyesters, because metal catalysts required for conventional polyester synthesis are often sensitive to and deactivated by amino groups, which means the amino substrates in the monomers must be protected prior to polymerization, necessitating additional post-polymerization deprotection steps^{23,24}. For example, synthesis of PHP and PAGA with only low molecular weight require multiple preparation steps, involving protection and deprotection of the amino substituents^{18,19}. More recently, poly(*N*-methyldiethyleneamine sebacate) (PMSC) was synthesized by polycondensation reaction between sebacoyl chloride and *N*-methyldiethanolamine²⁵. Triethylamine in large excess was used in order to remove the hydrochloric acid byproduct of the reaction. The synthesized PMSC served as an intermediate for preparation of a cholesterol-derivatized, amphiphilic copolymer for gene delivery. Because of side reactions associated with this synthesis method, only low molecular weight polyesters containing amino groups were obtained^{18,19,25}. But polycations with high molecular weight are essential for efficient gene delivery²⁶⁻²⁸. Thus, new selective methods for synthesizing high molecular weight, amino-bearing polyesters are highly desirable.

Enzyme-catalyzed organic reactions have great potential, since they can produce metal-free polyesters with well-defined structures and high molecular weight under mild reaction conditions²⁹⁻³¹. Various polyesters have been successfully synthesized via enzymatic polymerization reactions, including transesterification reactions of diesters with diols^{32,33}, ring-opening polymerizations of lactones^{34,35}, combined ring-opening and condensation copolymerizations of lactones with diesters and diols³⁶⁻³⁹, and syntheses of aliphatic polycarbonates^{29,40,41} and poly(carbonate-*co*-esters)^{42,43}. On the basis of a clear understanding of the mechanisms of transesterification reaction of diesters with diols, we synthesized a series of high molecular weight (M_w up to 44000) poly(amine-*co*-esters) via copolymerization of diesters with amino-substituted diols by employing *Candida antarctica*

lipase B (CALB) as the catalyst⁴⁴. In this report, we show that these poly(amine-*co*-esters) can condense DNA via electrostatic interaction to form nano-sized polyelectrolyte complexes with positive surface charge. Further, we evaluated these copolymers as nonviral vectors for both gene transfection *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Diethyl succinate, diethyl adipate, diethyl suberate, diethyl sebacate, diethyl dodecanedioate, *N*-methyldiethanolamine, *N*-ethyldiethanolamine, *N*-*n*-butyldiethanolamine, *N*-*tert*-butyldiethanolamine, *N*-phenyldiethanolamine, diphenyl ether, and poly(ethyleneimine) (PEI: branched, 25 kDa) were purchased from Aldrich Chemical Co. and were used as received. Immobilized *Candida antarctica* lipase B (CALB) supported on acrylic resin or Novozym 435, chloroform, dichloromethane, hexane, dimethyl sulfoxide (DMSO), and chloroform-*d* were also obtained from Aldrich Chemical Co.

HEK293 cells, U87MG cells, 9L cells, and LLC cells were obtained from American Type Culture Collection (Manassas, VA) and grown at 37 °C under 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Plasmid DNA (pGL4.13) encoding the firefly luciferase (pLucDNA) and Luciferase Assay Buffer were obtained from Promega Co. (Madison, WI). GFP reporter gene pSicoR-GFP (pGFP) was obtained from Addgene⁴⁵.

Synthesis and characterization of poly(amine-*co*-esters)

Poly(amine-*co*-esters) via copolymerization of diesters with amino-substituted diols using *Candida antarctica* Lipase B (CALB) as catalyst were synthesized as described previously⁴⁴. Briefly, equal moles of diester and amino-substituted diol monomers were mixed in diphenyl ether solution with Novozym 435 (10% wt/wt), the reaction was carried out using a parallel synthesizer under the vacuum. The copolymerization reactions were carried out in two stages: during the first stage reaction, the reaction mixtures were stirred at 80 °C under 1 atmosphere pressure of nitrogen for 24 h. Then, for polymerization, the pressure was reduced to 1.6 mmHg and the reactions were continued for an additional 72 h. At the end of the reactions, the formed poly(amine-*co*-esters) was purified and washed by hexane. Subsequently, the poly(amine-*co*-esters) were dissolved in dichloromethane followed by filtration to remove the catalyst particles. The resultant filtrates were concentrated under vacuum and then dried at 40 °C under high vacuum overnight to yield the purified poly(amine-*co*-esters).

The molecular weights (M_n and M_w , respectively) of polymers were measured by gel permeation chromatography (GPC) using a Waters HPLC system equipped with a model 1515 isocratic pump, a 717 plus autosampler, and a 2414 refractive index (RI) detector with Waters Styragel columns HT6E and HT2 in series. The chemical structural were analyzed by ¹H and ¹³C NMR using a Bruker AVANCE 500 spectrometer. The chemical shifts reported were referenced to internal tetramethylsilane (0.00 ppm) or to the solvent resonance at the appropriate frequency.

Preparation of polymer/DNA polyplex

Poly(amine-*co*-esters) were dissolved in DMSO and the resultant polymer solutions were diluted by adding 25 mM sodium acetate buffer (pH = 5.2). Poly(amine-*co*-ester)/DNA complexes with weight ratio ranging from 20:1 to 100:1 were prepared. Typically, 50 µl of a diluted poly(amine-*co*-ester) solution was added to the same volume of a DNA solution at a desired weight ratio and the resultant mixture was vortexed on a medium setting for 5

seconds. The poly(amine-*co*-ester)/DNA polyplexes were incubated at room temperature for 15 min before they were used for DNA transfection experiments with living cells. Control experiments employing Lipofectamine 2000 (Invitrogen Corp.) were performed using the optimal procedures provided by the manufacturer.

Measurement of particle size, zeta potential and morphology of polyplex

Poly(amine-*co*-ester)/pLucDNA polyplexes with a weight ratio of 100:1 were prepared by mixing the diluted poly(amine-*co*-ester) solution and the DNA solution in 25 mM NaAc buffer at pH 5.2. After incubating for 15 min at room temperature, the poly(amine-*co*-ester)/pLucDNA polyplexes samples were diluted in either Hepes buffer (10 mM, pH = 7.2) or NaAc buffer (25 mM, pH = 5.2), and then their particle size and zeta potential were measured immediately by ZetaPals dynamic light scattering (Brookhaven Instruments Corp). The morphology of the polyplexes was analyzed using a XL30 ESEM scanning electron microscope (FEI Company). The poly(amine-*co*-ester)/DNA polyplex nanoparticles were placed on a round cover glass mounted on an aluminum stub using carbon adhesive tape. After drying at room temperature, the stub was sputter-coated with a mixture of gold and palladium (60:40) under low pressure of argon using a Dynavac Mini Coater.

In vitro cell transfection

The *in vitro* gene transfection of poly(amine-*co*-ester)/DNA complexes was performed using HEK293, 9L, and U87MG cells. Cells of each type were seeded in 24-well plates at density of 75,000 cells/well with each well containing 500 μ l of DMEM. After 24 h, the growth medium was replaced, and a selected poly(amine-*co*-ester)/DNA complex solution containing either 1 μ g luciferase reporter gene or 1 μ g GFP reporter gene was added to each well. In case of the luciferase gene transfection, the culture medium was removed after 2 days and the cells in each well were washed with 0.5 ml of cold PBS. Reporter lysis buffer (200 μ l) was then added to each well to lyse the cells. The cell suspension was frozen at -80° C for an hour and then thawed, followed by centrifugation at 12,000 rpm for 5 min. Subsequently, 20 μ l of the supernatant was mixed with 100 μ l Luciferase Assay Buffer. The relative light unit (RLU) of the resultant mixture was measured using a luminometer and normalized to the total protein content measured by the BCA microprotein assay (Pierce, USA). For GFP gene transfection, the cells were transfected in a similar manner, but harvested by a different protocol. After 48 h of incubation in DMEM, the transfected cells in each well were washed twice with 500 μ l of PBS. Subsequently, 100 μ l of trypsin was added to each well, followed by incubation at 37 $^{\circ}$ C for 3 min. The cells were then washed with PBS, suspended in 300 μ l FACs buffer, and finally analyzed by BD FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA).

In vitro cytotoxicity study

The cytotoxicity of PMSC and PESC as representative poly(amine-*co*-esters) was studied against HEK293 cells. The cells were grown in 96-well plates at an initial seeding density of 1.5×10^4 cells/well with each well containing 100 μ l of DMEM. The cells were allowed to grow overnight. Thereafter, the growth medium was removed and replaced with fresh DMEM, followed by addition of 20 μ l of either PMSC/pLucDNA or PESC/pLucDNA complex at different concentrations to each well. For control experiments, instead of the polyplex solutions, an equivalent volume of sodium acetate buffer was used as the negative control and an equivalent volume of PEI/pLucDNA complex (prepared according to optimal conditions defined previously, as in reference ¹) was employed as the positive control. After 24 h incubation, the medium with the polyplexes was replaced with fresh DMEM, and the cells were incubated for an additional 24 h. Subsequently, the cells were assayed for metabolic activity using a MTS cell proliferation assay kit (Promega, WI). Twenty μ l of the MTS reagent was added to each well and the microplates were incubated at 37 $^{\circ}$ C in

darkness for 2 h. Thereafter, the plates were placed on a rotational shaker for 10–15 minutes and were allowed to cool to room temperature. MTS absorbance, which is related to the number of metabolically active cells, was measured using a microplate reader (Molecular Devices). To evaluate the poly(amine-co-ester) cytotoxicity toward different types of cells, instead of HEK293 cells, 9L and U87MG cells were also employed and were treated with PMSC/pLucDNA polyplex at various concentrations following experimental procedures analogous to those described above.

***In vivo* gene transfection**

All animal care and studies were approved by Yale's Institutional Animal Care and Use Committee (IACUC). The *in vivo* gene transfection efficiency of polymer/pLucDNA complexes were evaluated in mice bearing subcutaneous tumors of Lewis lung carcinoma (LLC). LLC cells (1×10^6 cells, 0.1 ml) were transplanted into C57BL/6 male mice (6 weeks old, Charles River Laboratories) subcutaneously. When a convenient tumor size (about 100–200 mm³) was obtained, the polymer/pLucDNA nanoparticles formulated using the aforementioned procedures were directly injected into tumors. At 48 h post-injection, the tumors were harvested for luciferase analysis. Three microliters of ice cold Reporter Lysis Buffer per 1 mg of tumor were added, and the tumors were immediately homogenized. After one freeze-thaw cycle, the samples were centrifuged for 10 min at 4 °C and the luciferase assay was performed using similar procedures as described in the cell transfection section.

Statistical analysis

Statistical tests were performed with a two-sided Student's T-test. A *P*-value of 0.05 or less was considered to be statistically significant.

RESULTS AND DISCUSSION

Synthesis and characterization of poly(amine-co-esters)

The copolymers were enzymatically synthesized using various diesters and amino-substituted diols as comonomers and Novozym 435 as the catalyst. The copolymerization reactions were performed in two stages: oligomerization under 1 atmosphere pressure of nitrogen followed by polymerization under high vacuum. The first stage reaction allows conversion of the monomers to nonvolatile oligomers, thus minimizing monomer loss via evaporation. The use of high vacuum during the second stage reaction efficiently removes the byproduct formed by equilibrium polycondensation reactions to accelerate polymer chain growth. Scheme 1 illustrates the chemical structural of poly(amine-co-esters) via copolymerization of different diesters with amino-substituted diols. NMR analysis showed that during the copolymerization reactions, byproduct ethanol was formed and condensed in the dry ice trap between the reactors and vacuum pump (see reference ⁴⁴).

Nine copolymers were synthesized and subsequently purified from five diethyl diesters (succinate, adipate, suberate, sebacate, and dodecanedioate) and five amino diols (*N*-methyldiethanolamine, *N*-ethyldiethanolamine, *N*-*n*-butyldiethanolamine, *N*-*t*-butyldiethanolamine, and *N*-phenyldiethanolamine). The molecular structures of the polymers were analyzed by both ¹H and ¹³C NMR spectroscopy: this analysis confirmed the chemical structures shown in Scheme 1.⁴⁴ Table 1 shows the molecular weight (*M*_w), and polydispersity (*M*_w/*M*_n) of the purified poly(amine-co-esters), along with their corresponding co-monomers employed and abbreviations.

Characterization of poly(amine-co-ester)/DNA nanoparticles

The first step of non-viral polymeric gene delivery is formation of gene-carrying complexes of appropriate size. To achieve efficient transfection, it is crucial that DNA is condensed into particles that protect the DNA from nuclease degradation and promote its cellular uptake^{22,46,47}. Most cells can efficiently internalize near neutral or slightly charged particles with a size smaller than 200 nm in diameter^{48,49}.

To investigate the relationship between the structure of polymer carriers and the biophysical properties of their corresponding DNA polyplex nanoparticles, we analyzed the particle size and surface charge of several representative poly(amine-co-ester)/pLucDNA complexes (Table 1). As shown in the table, the diameters of the polyplex particles are carrier-dependent, ranging from 41 to >1000 nm. It appears that the particles with the long chain (C₁₀–C₁₂) diester copolymers, such as PMSC/DNA and PESC/DNA polyplexes, tend to form in small sizes (< 110 nm). On the other hand, the presence of relatively short diester chain segments ($\leq$ C₈) in the copolymers substantially increases the size (e.g., > 600 nm) of their complexes with DNA. The zeta potential values of the poly(amine-co-ester)/DNA complexes were measured in both 25 mM NaAc buffer with pH = 5.2 and 10 mM Hepes buffer with pH = 7.2 (Table 1). Results indicate that the tertiary amino groups in the polymer chains were protonated at the lower pH. Protonation of the poly(amine-co-esters) is essential in order to convert the copolymers to polycations capable of forming complexes with negatively charged DNA. In addition, the ability of the polymer carriers to absorb protons indicates that after cellular uptake, the DNA complexes with these polymers should be capable of escaping endosomal/lysosomal disruption via the “proton sponge effect”^{2,50}, which may explain the excellent transfection efficiencies observed for several poly(amine-co-esters) (which is to be discussed in the following section). Upon condensation of the copolymers with DNA, the resultant polyplex particles tend to possess minimal surface charge. Zeta potential values of the poly(amine-co-ester)/DNA complexes changed from slightly positive in NaAc buffer medium to negative in Hepes buffer presumably due to partial deprotonation of the polycations with increasing medium pH (Table 1). We believe that this zeta potential change is important for successful gene transfection. If the poly(amine-co-ester)/DNA complexes are taken up by cells through an endocytic pathway, the protonation of the poly(amine-co-ester) will effectively buffer the acidic environment of the endosome, facilitating endosomal escape and unpacking DNA, with the net effect of a high transfection efficiency. The morphology of the copolymer/DNA complexes was examined using a scanning electron microscope (SEM). The SEM image of free-standing PMSC/pLucDNA nanoparticles revealed a near spherical shape (Figure 1), which was typical of our results. The average size of the nanoparticles shown in the SEM micrograph was comparable to that measured by dynamic light scattering.

In vitro cell transfection

Initial screening tests of *in vitro* gene transfection efficiency on the poly(amine-co-ester)/DNA polyplex nanoparticles were conducted using human embryonic kidney (HEK293) cells and the luciferase reporter gene. The polymer/pLucDNA complexes were prepared in NaAc buffer then incubated with the cells for 48 h. Luciferase gene transfection efficiency depends strongly on the poly(amine-co-ester) structure and the polymer/DNA weight ratio (Figure 2). Luciferase expression levels for the polyplex samples increased as the polymer/DNA weight ratio increased from 20 to 60–100. Among all polyplex particles, the PMSC/pLucDNA and PESC/pLucDNA complexes exhibited outstanding transfection efficiency; an optimal polymer/DNA ratio (i.e., 100) yielded luciferase expression comparable to that obtained with optimized Lipofectamine 2000 (LF2K). More specifically, the luciferase expression levels obtained with the PMSC/pLucDNA and PESC/pLucDNA polyplexes were 6×10^{10} and 1.2×10^{10} RLU/mg protein, respectively, as compared to 1.3×10^{10} RLU/mg

protein for LF2K/pLucDNA reference sample. We note that, even at 100:1 polymer:DNA weight ratio, the active agent DNA comprises 1% of the weight of the delivered complex: results obtained with other poly(amine-*co*-esters) of similar molecule structures use similar, or even higher (up to 150:1), polymer/DNA weight ratios^{21,51}.

Among the factors that may affect gene transfection efficiency, the small size (40–70 nm) of the PMSC/pLucDNA and PESC/pLucDNA complexes is likely an important element in enhancing cellular uptake, thus contributing to their efficient performance; the optimal transfection efficiency was lower for the DNA complexes with copolymers (e.g., PMSN, PMAP, PMSR, and PMDO) that resulted in larger sized particles (100 to >1000 nm) (Figure 2). It is known that large particle size (e.g., >500 nm) results in low cellular uptake of similar particles⁴⁷. Furthermore, solubility tests revealed that poly(amine-*co*-esters) containing sebacate units (e.g., PMSC, PESC, PBⁿSC, PBⁱSC, and PPSC), had lower solubility in aqueous medium when the substituent on nitrogen is changed from methyl or ethyl to more hydrophobic *n*-butyl, *t*-butyl, and phenyl groups. Thus, the unusually low transfection efficiencies observed for complexes with PBⁿSC, PBⁱSC, and PPSC are likely attributable to their low aqueous solubility. It is interesting to note that previously reported PMSC, which was chemically synthesized via polycondensation reaction between sebacoyl chloride and *N*-methyldiethanolamine, showed quite low transfection efficiency in delivering the luciferase gene to several types of living cells including HEK293 cells⁵². This is likely ascribable to the substantially lower molecular weight of the chemically synthesized copolymer²⁵ when compared to the enzymatically synthesized PMSC reported herein (Table 1). It is known that polymer molecular weight has a dramatic effect on gene transfection efficiency for PEI^{26,27} and other polyamine materials²⁸, with higher molecular weight leading to increased transfection efficiency.

To further investigate the performance of the poly(amine-*co*-esters) as carriers for different types of genes in different types of cells, PMSC/pGFP polyplex particles, along with LF2K/pGFP complex, were used to transfect glioblastoma U87MG cells and 9L cells (Figure 3). The LF2K/pGFP complex at an optimal dose exhibited medium transfection efficiency, yielding 40–45% GFP-positive cells. The pGFP polyplex of PMSC (the most effective carrier for pLucDNA delivery as discussed above) showed transfection efficiency for both 9L and U87MG cells that increased as the PMSC/DNA weight ratio increased from 40 to 100. At the optimal PMSC/pGFP DNA weight ratio of 100, the values of GFP positive 9L and U87MG cells were 56% and 57%, respectively, which are significantly higher than the 43% and 45% efficiency observed in cells transfected by LF2K/pGFP complex (Figure 3). The observed weight ratio dependent transfection efficiency of PMSC/pGFP complex toward 9L and U87MG cells is consistent with that of PMSC/pLucDNA nanoparticles toward HEK293 cells (Figure 2).

Images of GFP-positive 9L and U87MG cells 48 h after transfection were obtained (Figure 4). For both cell lines, the cells treated with the PMSC/pGFP complex showed a larger percentage of transfected cells exhibiting higher intensity of GFP fluorescence as compared to LF2K/pGFP particles. Furthermore, a large number of LF2K/pGFP transfected U87MG cells appeared in a nonregular, altered, cellular configuration, indicating possibly significant cytotoxicity of the LF2K/pGFP particles toward these cells. In contrast, the U87MG cells transfected by PMSC/pGFP complex showed normal, healthy, epithelial-like morphology.

***In vitro* cytotoxicity study**

Cationic polymers must be low in cytotoxicity in order to be suitable as non-viral gene vectors. A number of polycations have been shown to elicit considerable cell toxicity, which would likely limit their utility as carriers for gene delivery^{51,53,54}. Examples include high molecular weight polymers with a high density of primary and/or secondary amines. To

determine the cytotoxicity of the poly(amine-*co*-esters), HEK293 cells were treated with PMSC/pLucDNA (100:1, wt/wt), PESC/pLucDNA (100:1, wt/wt), and a reference sample (PEI/pLucDNA) at various concentrations for 24 h and cell viability was measured (Figure 5A). Although all three polymers exhibit low toxicity toward HEK293 cells at low concentrations (≤ 30 $\mu\text{g/ml}$), the cytotoxicity of PMSC and PESC is much lower than that of PEI at higher concentrations (≥ 50 $\mu\text{g/ml}$). For example, even at the highest concentration (500 $\mu\text{g/ml}$), which corresponds to an approximate 300:1 polymer/DNA weight ratio, the nanoparticles of PMSC and PESC are not cytotoxic; the cell survival rates exceed 80% (Figure 5A). In contrast, the PEI particles display strong cytotoxicity ($\sim 40\%$ cell survival rate) against HEK293 cells at concentrations as low as 50 $\mu\text{g/ml}$; cell killing was complete at concentrations above 100 $\mu\text{g/ml}$. To further investigate the cytotoxicity of the poly(amine-*co*-esters) toward different cell lines, 9L and U87MG cells were incubated with the PMSC/pLucDNA nanoparticles at various polymer concentrations for 24 h (Figure 5, B and C). Again, the PMSC/pLucDNA complexes exhibit remarkably low cytotoxicity toward both 9L and U87MG cells; the cell viability trends are comparable to that observed for HEK293 cells. For comparison, the PEI/pLucDNA polyplex shows even higher toxicity on 9L and U87MG cells than on HEK293 cells (Figure 5). Since cell transfection studies demonstrated that PMSC and PESC can achieve high transfection efficiency, as great as lipofectamine 2000, at a polymer/DNA weight ratio of 100:1, which is significantly lower than the concentration we tested for their cytotoxicity, these poly(amine-*co*-esters) have great potential for effective and safe gene transfection.

***In vivo* gene transfection**

To determine whether these poly(amine-*co*-esters) can serve as *in vivo* gene delivery vectors, the top-performing polymer, PMSC, was selected for testing. Mice received a single injection of PMSC/pLucDNA polyplex containing 10 μg pLucDNA; the PMSC/pLucDNA (100:1 wt/wt) nanoparticles were directly injected into LLC flank tumors in the C57BL/6 mice. Since PEI is a better *in vivo* gene carrier than LF2K^{55,56}, PEI/pLucDNA (10:1 N/P ratio) particles were also tested as a positive control. Luciferase expression in the tumor tissue for animals treated with either PMSC/pLucDNA or PEI/pLucDNA were measured at 48 h after the intratumoral injections (Figure 6). PMSC is a substantially more effective gene carrier than PEI in delivering pLucDNA to the tumor. As the result, the observed *in vivo* luciferase expression for PMSC/pLucDNA polyplex is approximately 1.5 times higher than produced by PEI/pLucDNA particles (Figure 6). Under similar conditions, use of naked pLucDNA only produced a weak luciferase expression background at 48 h post-injection.

CONCLUSIONS

We synthesized a series of biodegradable poly(amine-*co*-esters) with diverse structures via enzymatic copolymerization of diesters with amino-substituted diols. Upon protonation at slightly acidic conditions, these poly(amine-*co*-esters) readily turned to cationic polyelectrolytes, which were capable of condensing with polyanionic DNA to form polyplex nanoparticles. *In vitro* cell transfection screening of these polymers identified two poly(amine-*co*-esters), PMSC and PESC, which possess pLucDNA transfection efficiency comparable to or even higher than that of LF2K. Studies on the physical properties and morphology of PMSC/pLucDNA and PESC/pLucDNA nanoparticles revealed that both polyplexes had desirable particle sizes (40–70 nm on average) for cellular uptake and were able to absorb protons upon pH change from 7.2 to ~ 5 in the medium. Thus, like other polyamines (e.g., PEI), the poly(amine-*co*-esters) should act as proton sponges to facilitate endosomal escape of their DNA complexes after cellular uptake. As typical examples of poly(amine-*co*-ester)/DNA complexes, PMSC/pLucDNA and PESC/pLucDNA polyplexes exhibited extremely low cytotoxicity. Furthermore, gene transfection experiments performed

using mouse tumor models showed that PMSC is a substantially more effective gene carrier than PEI in delivering pLucDNA to the tumor cells *in vivo*. All these properties make the poly(amine-*co*-esters) to be promising non-viral vectors for safe and efficient DNA delivery in gene therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by a grant from the National Institutes of Health (EB-000487) with partial financial assistance from the China Scholarship Council (CSC).

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Figure 1. SEM image of PMSC/pLucDNA polyplex nanoparticles. The polyplex nanoparticles were formed at polymer/DNA weight ratio of 100. Scale bar is 500 nm.

Figure 2.

Luciferase expression level in HEK293 cells transfected with pLucDNA polyplex of PMSN (A), PMAP (B), PMSR (C), PMSC (D), PMDO (E), PES (F), PB³SC (G), PB⁴SC (H), or PPSC (I). LF2K was used at the optimal dose recommended by the supplier. The standard deviation is shown by error bars (n = 3).

Figure 3. Percentage of GFP-positive cells observed 48-hours after transfection of 9L and U87MG cells with PMSC/pGFP or LF2K/pGFP nanoparticles. The standard deviation is shown by error bars (n = 3).

Figure 4. Fluorescence images of 9L cells (A) and U87MG cells (B) with GFP expression after transfection with a same dose of pGFP carried by LF2K or PMSC (PMSC/DNA weight ratio = 100:1).

Figure 5. Cell viability vs. polymer concentration for pLucDNA polyplex of PEI (Δ), PMSC (■), or PESC (○) against different cell lines: (A) HEK293; (B) 9L; and (C) U87MG. The standard deviation is shown by error bars (n = 3).

Figure 6.

In vivo luciferase expressions in the tumor tissue after intratumoral injection of the PEI/pLucDNA and PMSC/pLucDNA nanoparticles into subcutaneous LLC tumors in mice. The standard deviation is shown by error bars (n = 4). ** $P < 0.01$

Table 1

Molecular Weight and Polydispersity of Poly(amine-co-esters) and Characterization of Poly(amine-co-ester)/pLucDNANanoparticles

Substrates		Isolated Polymer			Properties of polyplex ^c		
Diethyl Diester	Diol	Name ^a	M_w^b	M_w/M_n^b	Mean Particle Diameters (nm)	Zeta Potential (mV)	
Succinate	<i>N</i> -Methyl/diethanolamine	PMSN	29500	2.3	620	In NaAc	In Hepes
Adipate	<i>N</i> -Methyl/diethanolamine	PMAP	29600	2.3	>1000	8.7	-5.7
Suberate	<i>N</i> -Methyl/diethanolamine	PMSR	30300	2.4	>1000	13.3	-20.6
Sebacate	<i>N</i> -Methyl/diethanolamine	PMSC	31800	2.3	69	20.7	-23.4
Dodecanedioate	<i>N</i> -Methyl/diethanolamine	PMDO	41200	2.4	107	35.4	-31.4
Sebacate	<i>N</i> -Ethyl/diethanolamine	PESC	29900	2.3	41	22.0	-26.2
Sebacate	<i>N</i> - <i>n</i> -Butyl/diethanolamine	PB ⁿ SC	36000	2.2	880	26.7	-23.9
Sebacate	<i>N</i> - <i>r</i> -Butyl/diethanolamine	PB ^r SC	44500	2.0	>1000	10.4	-16.9
Sebacate	<i>N</i> -Phenyl/diethanolamine	PPSC	44200	2.2	724	14.6	-15.3
						11.3	-9.6

^a Abbreviations name of the polymers.

^b Molecular weight and polydispersity are cited from our previous work ⁴⁴.

^c The polyplex nanoparticles were formed at polymer/DNA weight ratio of 100.