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Synthesis of Poly(β-amino ester)s with Thiol-Reactive Side Chains for DNA Delivery

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Abstract: The safe and efficient delivery of DNA remains the major barrier to the clinical application of non-viral gene therapy. Here, we present novel, biodegradable polymers for gene delivery that are capable of simple graft modification and demonstrate the ability to respond to intracellular conditions. We synthesized poly(β-amino ester)s using a new amine monomer, 2-(pyridyldithio)-ethylamine (PDA). These cationic, degradable polymers contain pyridyldithio functionalities in the side chains that react with high specificity toward thiol ligands. This reactivity is demonstrated using both mercaptoethylamine (MEA) and the thiol peptide RGDC, a ligand that binds with high affinity to certain integrin receptors. These two polymer derivatives displayed strong DNA binding as determined using electrophoresis and dye exclusion assays. In addition, the MEA-based polymer and plasmid DNA were shown to self-assemble into cationic complexes with effective diameters as low as 100 nm. Furthermore, this DNA binding ability was substantially reduced in response to intracellular glutathione concentrations, which may aid in DNA unpackaging inside the cell. These complexes also displayed low cellular toxicity and were able to mediate transfection at levels comparable to PEI in human hepatocellular carcinoma cells. These results suggest that PDA-based poly(β-amino ester)s may serve as a modular platform for polymer-mediated gene delivery.

Introduction

Cationic polymers have emerged as promising materials for the intracellular delivery of DNA.[1] Some polycations have demonstrated the ability to electrostatically bind DNA and condense it into nanoparticles sufficient for cellular uptake.[2] However, no material is capable of overcoming all obstacles to efficient gene delivery including selective cellular uptake, endosomal escape, cytoplasmic trafficking, and nuclear import.[3] Each of these barriers has been addressed in design of DNA delivery systems, usually individually and rarely in combination.

Non-viral gene delivery systems usually consist of cationic polymers such as poly-L-lysine (PLL), polyethyleneimine (PEI), poly(amido amine)s (PAMAMs), or chitosan.[4,5] Using these materials as a base, various functional groups have been grafted to the polymers to enhance their delivery properties. For example, cell specific delivery has been demonstrated by conjugating ligands such as growth factors,[6,7] RGD sequences,[8,9] transferrin,[10] and antibodies[11,12] to the polymer chains. Viral mechanisms to promote endosomal escape have also been incorporated into these polycationic materials using peptide transduction domains, such as the HIV TAT sequence and influenza virus hemagglutinin subunit HA-2.[13,14] Also, nuclear localization sequences derived from intracellular proteins have been grafted to the vector to direct DNA to the nucleus.[15,16]

The current non-viral delivery systems suffer from several limitations. The most important is low transfection efficiencies relative to viral vectors.[17] In addition, many are nondegradable and do not provide a mechanism for elimination and DNA release.[18] The high cationic charge densities present in the most common polymers, such as PLL and PEI, result in substantial cytotoxicity.[19,20] In the case of PLL and other protein-based systems, immune responses can be problematic, especially for

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(1) Merdan, T.; Kopecek, J.; Kissel, T. *Adv. Drug Deliv. Rev*. **2002**, *54*, 715−758.
(2) De Smedt, S. C.; Demeester, J.; Hennink, W. E. *Pharm. Res.* **2000**, *17*, 113−126.
(3) Wiethoff, C. M.; Middaugh, C. R. *J. Pharm. Sci.* **2003**, *92*, 203−217.
(4) Thomas, M.; Klibanov, A. M. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 27−34.
(5) Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. *Nat. Rev. Drug Discov.* **2005**, *4*, 581−593.
(6) Blessing, T.; Kursa, M.; Holzhauser, R.; Kircheis, R.; Wagner, E. *Bioconjug. Chem*. **2001**, *12*, 529−537.
(7) Sosnowski, B. A.; Gonzalez, A. M.; Chandler, L. A.; Buechler, Y. J.; Pierce, G. F.; Baird, A. *J. Biol. Chem.* **1996**, *271*, 33647−33653.
(8) Erbacher, P.; Remy, J. S.; Behr, J. P. *Gene Ther*. **1999**, *6*, 138−145.
(9) Hart, S. L.; Harbottle, R. P.; Cooper, R.; Miller, A.; Williamson, R.; Coutelle, C. *Gene Ther*. **1995**, *2*, 552−554.
(10) Kircheis, R.; Kichler, A.; Wallner, G.; Kursa, M.; Ogris, M.; Felzmann, T.; Buchberger, M.; Wagner, E. *Gene Ther*. **1997**, *4*, 409−418.
(11) Suh, W.; Chung, J. K.; Park, S. H.; Kim, S. W. *J. Controlled Release* **2001**, *72*, 171−178.
(12) O'Neill, M. M.; Kennedy, C. A.; Barton, R. W.; Tatake, R. J. *Gene Ther*. **2001**, *8*, 362−368.
(13) Beerens, A. M.; Al Hadithy, A. F.; Rots, M. G.; Haisma, H. J. *Curr. Gene Ther*. **2003**, *3*, 486−494.
(14) Plank, C.; Zauner, W.; Wagner, E. *Adv. Drug Deliv. Rev*. **1998**, *34*, 21−35.
(15) Chan, C. K.; Jans, D. A. *Immunol. Cell Biol.* **2002**, *80*, 119−130.
(16) Cartier, R.; Reszka, R. *Gene Ther*. **2002**, *9*, 157−167.
(17) Varga, C. M.; Tedford, N. C.; Thomas, M.; Klibanov, A. M.; Griffith, L. G.; Lauffenburger, D. A. *Gene Ther.* **2005**, *12*, 1023−1032.
(18) Schaffer, D. V.; Fidelman, N. A.; Dan, N.; Lauffenburger, D. A. *Biotechnol. Bioeng.* **2000**, *67*, 598−606.

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repeat injections.[21] Finally, material modifications are often time-consuming, involve difficult and/or multistep purifications, and may employ harsh conditions at high temperatures and pH's.

Recently, there has been an interest to develop degradable cationic polymers that can mediate gene delivery.[22] One promising class of such materials are the poly(β-amino ester)s. These polymers can be readily synthesized by the conjugate addition of primary amines or bis-secondary amines to diacrylate compounds.[23] Due to the large number of commercially available monomers, libraries of structurally diverse poly(β-amino ester)s have been synthesized and tested for gene delivery.[24−26] Structure−function studies have shown that the combination of hydrophobic diacrylates and amino-alcohols resulted in polymeric vectors with DNA delivery efficiencies that are superior to PEI and Lipofectamine, two commercially available gene delivery reagents.[27]

In our effort to develop degradable polycations for gene delivery, we present here the synthesis of new poly(β-amino ester)s using the primary amine monomer, 2-(pyridyldithio)-ethylamine (PDA). The resulting poly(β-amino ester)s contain pyridyldithio groups in the side chains that display fast and selective reactivity with thiols, without altering the charge density of the polymer backbone. Such reactivity can be useful for conjugating cell targeting peptides, viral transduction domains, and other synthetic peptides into a single polymer chain to improve its gene transduction efficiency. An added benefit of this reaction mechanism is that a molecule of 2-mercaptopyridine (2-MP) is released for each thiol molecule reacted. This compound has a strong absorbance at 343 nm which enables the side chain conversion to be followed colorimetrically.[28] In addition to reactive side groups, the polymer end chains consist of reactive acrylates that can be used to couple amine-containing compounds.

As one potential application, we examined the attachment of mercaptoethylamine (MEA) and the RGDC peptide to PDA poly(β-amino ester)s. Both polymers were shown to condense DNA and, for the MEA derivatives, form cationic DNA complexes with effective diameters around 100 nm. The MEA based polymer was also shown to be responsive to intracellular glutathione concentrations in which a significant decrease in DNA binding was observed. To test the generality of the approach, three different PDA-based poly(β-amino ester)s were synthesized, along with their MEA derivatives, and tested for transfection on human hepatocellular carcinoma cells. In all cases, the transfection efficiency was greatly improved by MEA side chain substitution and rival that observed for optimized PEI-based formulations.

Experimental Section

Materials. Polyethylenimine (water free, Mw ≈ 25 kDa, Mn ≈ 10 kDa), 2,2′-dipyridyl disulfide (98%), mercaptoethylamine (98%), mercaptoethylamine hydrochloride (∼95%), 2-mercaptopyridine (99%) (2-MP) and reduced L-glutathione (99%) were purchased from Sigma-Aldrich (St. Louis, MO). A 25 mM sodium acetate buffer solution pH 5.2 (NaAc buffer) was prepared by diluting a 3 M stock (Sigma-Aldrich). Arginine-glycine-aspartate-cysteine (RGDC) peptide was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). 1,4-Butanediol diacrylate (99+%) (C) was from Alfa Aesar (Ward Hill, MA); Neopentyl glycol diacrylate (>85%) (F) was from Scientific Polymer Products, Inc. (Ontario, NY); 1,6-hexanediol ethoxylate diacrylate (U) was from Sigma-Aldrich. All chemicals were used as received without any further purification. PicoGreen was purchased from Molecular Probes (Eugene, OR). pCMV−LUC plasmid DNA stock solution (1 mg/mL in water) was obtained from Elim Biopharmaceuticals (Hayward, CA). The MTT Cell Proliferation Assay, Bright Glo Luciferase Assay Kits, and recombinant Luciferase protein were purchased from Promega Corporation (Madison, WI). FOCUS hepatocellular carcinoma (HCC) cells were a kind gift from Professor Dane Wittrup and grown at 37 °C, 5% CO_2 in Minimum Essential Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 units/mL of penicillin/streptomycin, and 1 mM sodium pyruvate. All cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA). White and black polystyrene tissue culture treated 96-well plates and half area polystyrene 96-well plates were obtained from Corning Costar. Clear polystyrene tissue culture treated 96-well plates were obtained from Becton Dickinson (Bedford, MA).

Methods. All ^{1}H NMR was conducted on a Varian Unity spectrometer (300 MHz). Gel permeation chromatography (GPC) was performed as described previously[29] to determine weight and number average molecular weights of the polymers relative to polystyrene standards.

Synthesis of 2-(Pyridyldithio)-ethylamine Hydrochloride (PDA*HCl). The synthesis of PDA*HCl was performed following a method similar to that reported by Ebright et al.[30] Briefly, 2-mercaptoethylamine hydrochloride (2.288 g, 20 mmol) was dissolved in 17.5 mL of methanol and added dropwise to a stirred solution of 2,2′-dipyridyl disulfide (8.815 g, 40 mmol) dissolved in 41.6 mL of methanol containing 1.6 mL of glacial acetic acid. The reaction was kept under an argon atmosphere to minimize free thiol oxidation. After 48 h, the mixture was concentrated under reduced pressure to give approximately 10−15 mL of yellow oil. The product was precipitated by the addition of 100 mL cold ether and purified by redissolving in 20 mL methanol and precipitating with 100 mL cold ether six times to give a white powder. ^{1}H NMR (D_2O): δ (ppm) 3.1 (t, J = 6.26 Hz, 2H), 3.3 (t, J = 6.26 Hz, 2H), 7.3 (m, 1H), 7.7 (m, 1H), 7.8 (m, 1H), 8.4 (m, 1H).

Synthesis of 2-(Pyridyldithio)-ethylamine (PDA). Sodium hydroxide (384.7 mg, 9.6 mmol) was dissolved in 1 mL of water and quickly added to 1.95 g of 2-(pyridyldithio)-ethylamine hydrochloride (8.8 mmol) dissolved in 2 mL of water. The solution was briefly vortexed and allowed to stand for 15 min. The PDA free base phase separated and was isolated by draining from the tube bottom. ^{1}H NMR ($CDCl_3$): see Figure S1.

Synthesis of PDA Poly(β-amino ester)s. Polymers were synthesized by adding 187 mg (1 mmol) of freshly isolated PDA to 1 mmol of either C, F, or U diacrylate in a Teflon-lined screw cap vial with a magnetic stir bar. The mixture was stirred at 60 °C for 2 days after which time the polymer was stored at −20 °C until used for each experiment. Letter designations for the diacrylates correspond to those reported by Anderson et al.[25] Weight-average molecular weights and

(19) Brazeau, G. A.; Attia, S.; Poxon, S.; Hughes, J. A. *Pharm. Res.* **1998**, *15*, 680−684.
(20) Moghimi, S. M.; Symonds, P.; Murray, J. C.; Hunter, A. C.; Debska, G.; Szewczyk, A. *Mol. Ther.* **2005**, *11*, 990−995.
(21) Hudecz, F.; Gaal, D.; Kurucz, I.; Lanyi, A.; Kovacs, A. L.; Mezo, G.; Rajnavolgyi, E.; Szekerke, M. *J. Controlled Release* **1992**, *19*, 231−243.
(22) Wagner, E. *Pharm. Res.* **2004**, *21*, 8−14.
(23) Lynn, D. M.; Langer, R. *J. Am. Chem. Soc.* **2000**, *122*, 10761−10768.
(24) Lynn, D. M.; Anderson, D. G.; Putnam, D.; Langer, R. *J. Am. Chem. Soc.* **2001**, *123*, 8155−8156.
(25) Anderson, D. G.; Lynn, D. M.; Langer, R. *Angew. Chem., Int. Ed.* **2003**, *42*, 3153−3158.
(26) Anderson, D. G.; Peng, W.; Akinc, A.; Hossain, N.; Kohn, A.; Padera, R.; Langer, R.; Sawicki, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16028−16033.
(27) Anderson, D. G.; Akinc, A.; Hossain, N.; Langer, R. *Mol. Ther.* **2005**, *11*, 426−434.
(28) Grassetti, D. R.; Murray, J. F., Jr. *Arch. Biochem. Biophys.* **1967**, *119*, 41−49.
(29) Akinc, A.; Anderson, D. G.; Lynn, D. M.; Langer, R. *Bioconjug. Chem.* **2003**, *14*, 979−988.
(30) Ebright, Y. W.; Chen, Y.; Pendergrast, P. S.; Ebright, R. H. *Biochemistry* **1992**, *31*, 10664−10670.

polydispersities (given in parenthesis) were determined to be 4000 (1.35) for C−PDA, 4400 (1.40) for F−PDA, and 4200 (1.38) for U−PDA. PDA polymer stock solutions at 100 mg/mL were made by dissolving 20 mg of polymer in 200 μL of DMSO and used for all subsequent experiments unless otherwise noted.

Synthesis of PDA Poly(β-amino Ester) Derivatives. A C−PDA−MEA polymer solution at 100 mg/mL was prepared by dissolving 20 mg of C−PDA in 200 μL of DMSO containing 20 mg/mL MEA. C−PDA−RGDC polymer solution at 100 mg/mL was prepared by dissolving 20 mg of C−PDA in 200 μL of DMSO containing 23.4 mg RGDC. The C−PDA−MEA solution was always prepared 30 min before usage while the C−PDA−RGDC solution was made 1 day prior to allow for a 24 h reaction period before each experiment. These reaction times were necessary to ensure complete side chain conversion. These solutions were used in all subsequent experiments unless otherwise noted.

Characterization of Polymer Side Chain Reactivity. Concentrated solutions of the thiol reagents MEA and RGDC were each prepared at 20 mg/mL in DMSO. A series of ten 1:2 dilutions in DMSO were performed to generate MEA and RGDC dilution sets. Both thiol dilution sets were plated at 150 μL/well in a 96-well plate. The C−PDA polymer was dissolved in DMSO to a concentration of 1 mg/mL and plated at 100 μL/well. Using a 12-channel pipettor, an equivalent volume of thiol solution (MEA or RGDC) was added to the C−PDA polymer solution and mixed briefly by pipetting. The mixture absorbance was measured at several time intervals using a Molecular Devices SPECTRAmax PLUS384 absorbance plate reader at a wavelength of 343 nm.

Agarose Gel Electrophoresis of Polymer/DNA Complexes. Polymer solutions at 100 mg/mL in DMSO were diluted accordingly into NaAc buffer and 25 μL was added to 25 μL of DNA (60 μg/mL in NaAc buffer) and mixed vigorously. The solutions were left undisturbed for 5 min after which time 10 μL of loading buffer was added to each sample. The loading buffer consisted of 10% Ficoll 400 in 25 mM HEPES buffer. Polymer−DNA solutions were then diluted 4-fold into TAE buffer and 20 μL of these solutions were added to the wells of a 1% agarose gel containing ethidium bromide. Gel electrophoresis of the complexes was performed in TAE buffer at 60 V for 3 h. Imaging of the gel was performed using a Kodak Electrophoresis Documentation and Analysis System 120.

Polymer−DNA Binding Assay with PicoGreen. All of the following manipulations were carried out in black 96-well plates. Polymer solutions at 100 mg/mL in DMSO were diluted according into NaAc buffer and 50 μL/well of each was added to 50 μL/well of DNA (12 μg/mL in NaAc buffer). The solutions were mixed vigorously and allowed to sit undisturbed for 5 min to allow for polymer−DNA complexation. After this time, 100 μL of PicoGreen solution was added to each well. PicoGreen working solution was prepared by diluting 60 μL of the purchased stock into 12 mL of NaAc buffer. After 5 min, the plate fluorescence was measured on a Perkin-Elmer Victor 3 plate reader using a FITC filter set (excitation 485 nm, emission 535 nm). The relative fluorescence (RF) was calculated using the following relationship:

$$RF = (F_{sample} - F_{blank})/(F_{DNA} - F_{blank})$$

Experiments were also conducted in the presence of L-glutathione in essentially the same manner as described above. However, 100 μL/well of the PicoGreen−polymer−DNA solution was added to 100 μL/well of glutathione solution (0, 0.2, or 20 mM) in phosphate buffered saline (PBS; 10 mM phosphates, 137 mM NaCl, pH 7.4) prior to measurement of the plate fluorescence.

Polymer/DNA Complex Size and Surface Charge Measurements. A C−PDA−MEA polymer solution at 100 mg/mL in DMSO was prepared as described above and diluted accordingly in NaAc buffer immediately before use. To prepare polymer−DNA complexes, 100 μL of diluted polymer was added to 100 μL of DNA (60 μg/mL in NaAc) and pipetted vigorously. Complexation was allowed to proceed undisturbed for 5 min after which time 150 μL of the sample was diluted into 1.8 mL of PBS. Polymer−DNA complex size and surface charge were measured on a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, NY; 15 mW laser; 676 nm incident beam, 90 deg scattering angle). Effective particle diameters were calculated from the autocorrelation function using the MAS option of the BIC particle sizing software assuming a log-normal distribution. The solution viscosity and refractive index were assumed equal to pure water at 25 °C. Zeta potentials were calculated from the measured electrophoretic mobilities of the complexes using the BIC PALS zeta potential analysis software and the Smoluchowsky model for aqueous dispersions.

Measurements of Polymer Cytotoxicity. HCC cells were plated at 15 000 cells/well in clear 96-well plates and grown overnight. A 29 mg/mL solution of 2-MP in DMSO was prepared. Polymer and 2-MP solutions in DMSO were diluted accordingly into NaAc buffer and 25 μL of each was mixed vigorously with 25 μL of DNA (60 μg/mL in NaAc buffer) in a half-area 96-well plate. The solutions were left undisturbed for 5 min after which time 30 μL of each was added to 200 μL of Opti-MEM in a separate, clear 96-well plate. The media over the cells was then removed with a 12-channel aspirator wand and followed by the addition of 150 μL/well of polymer−DNA complex solution in Opti-MEM. Complexes were incubated with the cells for 1 h after which time they were removed and replaced by 105 μL/well of fresh cell culture media. Metabolic activity of the cells was measured 24 h later using the MTT Cell Proliferation Assay Kit following the supplied instructions.

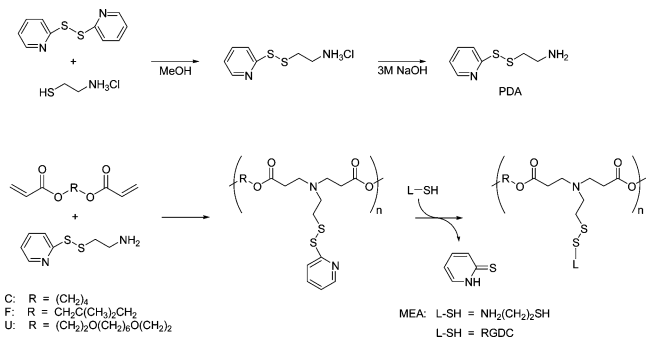
Transfection of Hepatocellular Carcinoma cells. HCC cells were plated at 5000 cells/well in opaque 96-well plates and allowed to adhere overnight. C−PDA, F−PDA, and U−PDA polymer solutions in DMSO were prepared as described above. A 100 μL sample of each was diluted into 900 μL of NaAc buffer to produce a 10 mg/mL aqueous solution. A 10 mg/mL aqueous, C−PDA−MEA polymer solution was prepared by diluting 100 μL of 100 mg/mL C−PDA into 900 μL of NaAc buffer containing 10 mg/mL MEA. Aqueous solutions of F−PDA−MEA and U−PDA−MEA were prepared in a similar manner using the base polymers F−PDA and U−PDA, respectively. The 10 mg/mL aqueous polymer solutions were diluted accordingly into NaAc buffer, and 25 μL of each was mixed vigorously with 25 μL of DNA (60 μg/mL in NaAc buffer) in a half-area 96-well plate. The solutions were left undisturbed for 5 min after which time 30 μL of each was added to 200 μL of Opti-MEM in a separate clear 96-well plate. The media over the cells was then removed with a 12-channel aspirator wand and followed by the addition of 150 μL/well of polymer−DNA complex solution in Opti-MEM. Complexes were incubated over the cells for 1 h after which time they were aspirated off and replaced by 105 μL/well of fresh cell culture media. Cells were allowed to grow for 3 days at 37 °C, 5% CO_2 and were then analyzed for luciferase protein expression.

Luciferase expression was analyzed using Bright-Glo assays kits. Briefly, 100 μL/well of Bright-Glo solution was added to the cell plates. The plates were gently agitated to promote mixing for 2 min. Luminescence was then measured on a Mithras Luminometer using a 1% neutral density filter and a 1 s per well counting time. A standard curve was generated by performing an identical assay with dilutions of recombinant Luciferase protein.

Results

Monomer Synthesis. The synthesis of the amine monomer, 2-(pyridyldithio)-ethylamine hydrochloride, proceeds via the reaction between mercaptoethylamine hydrochloride and 2,2′-dipyridyl disulfide as shown in Scheme 1. Conversion to the free base form was accomplished by dissolving the hydrochlo-

Scheme 1



ride salt in a sodium hydroxide solution and isolating the phase separated PDA liquid. Although the product has residual water as a contaminant, this isolation procedure was necessary due to the instability of the free base form. Attempts to remove the water by distillation or the use of drying agents caused rapid degradation of the free base. Furthermore, alternative preparations involving extraction into organic solvents, drying, and evaporation of the solvent led to substantial breakdown of PDA, as noted previously.[31]

The degradation of PDA occurs through an irreversible disproportionation reaction, similar to other structurally related unsymmetrical disulfides.[32,33] Through this pathway, the unsymmetrical PDA compound is converted into its symmetrical counterparts, namely 2,2′-dipyridyl disulfide and cystamine, as verified by ^{1}H NMR and TLC analysis. The latter is a diamine disulfide that, if present during the polymerization, can lead to substantial crosslinking. From the ^{1}H NMR spectrum of the PDA product, there is evidence of some disproportionation and the formation of symmetrical disulfide molecules (Figure S1). Peak integration indicates that there is an approximate 3.5:1 molar ratio of PDA to cystamine. Much lower ratios (i.e., higher cystamine contamination) were observed following the alternative isolation procedures mentioned above. The phase separation method followed here minimized PDA breakdown through its brief exposure to base and the use of equimolar neutralization conditions. The PDA product was always used immediately upon isolation before any additional transformation could occur.

Polymer Synthesis. The synthesis of three PDA-based poly(β-amino ester)s was carried out by direct mixing of PDA with diacrylates (Scheme 1). Equimolar monomer concentrations were used, assuming pure PDA, and the reaction temperature was maintained at 60 °C to promote polymerization and minimize PDA degradation. Higher temperatures and excess molar equivalents of PDA over diacrylate resulted in substantial crosslinking due to the generation of cystamine from PDA disproportionation. The optimal reaction conditions lead to weight average molecular weights (relative to polystyrene) of approximately 4 kDa. The molecular weights were limited due to an imbalance in monomer ratios from water contamination in the PDA.

Despite the inherent instability of PDA, the ^{1}H NMR spectra of the PDA polymers revealed several peaks that are consistent with intact, thiol-reactive poly(β-amino ester)s (Figures S2−S4). First, multiplet peaks were observed around 2.4 and 2.7 ppm and correspond to hydrogens on the newly formed methylene bonds between the tertiary amine and the esters. In addition to GPC measurements, this peak assignment provides evidence of polymerization through the anticipated reaction between the PDA amine and diacrylates. Second, protons in the pyridyldithio side chain showed characteristic peaks in the 7−8.5 ppm range. No peaks were observed below 7 ppm that would indicate the presence of 2-MP from disulfide hydrolysis or partial disproportionation. Therefore, the polymer is intact and should display thiol-specific reactivity in the side chains. Finally, due to the imbalance in monomer ratios, an excess of diacrylate over PDA resulted in several peaks around 6 ppm. Similar peaks have been seen in other poly(β-amino ester)s generated using excess diacrylate and correspond to acrylate functionalities at the ends of the polymer chains.[29] This structural feature allows for photopolymerization or the conjugation of nucleophilic molecules to the polymer terminus while thiol-containing ligands can be linked specifically to the side chains.

Further analysis of the polymer ^{1}H NMR spectra reveals branched structures. Partial disproportionation of PDA apparently occurs during its isolation and polymerization, leading to the formation of the cystamine crosslinking agent. Peak integration and comparison of pyridyl and ethyl side chain protons indicates that 70% of the polymer consists of PDA side chains, with the balance composed of cystamine crosslinks. Treatment of C−PDA with MEA leads to a 35% reduction in the molecular weight, indicating that these crosslinks may be reducible by thiolated molecules.

Side-Chain Reactivity. The reactivity of PDA polymer side chains to the thiol compounds MEA and RGDC was investigated as a function of concentration and time. Reaction with MEA resulted in a degradable cationic poly(β-amino ester) with pendent primary amines linked to the main chain through disulfide bonds (Scheme 1). The RGDC compound was used to demonstrate the attachment of a peptide useful for targeting endothelial cells and other integrin expressing cell types.[34] In both cases, the reaction was carried out in DMSO instead of aqueous buffer solutions to prevent polymer hydrolysis. As shown in Scheme 1, each thiol molecule reacts with a pyridyldithio side chain of the polymer to release a corresponding 2-MP molecule. This one-to-one correlation, and the strong 2-MP absorbance at 343 nm, permitted the progress of the reaction to be conveniently followed colorimetrically. These results were also confirmed by ^{1}H NMR for each PDA polymer (Figures S5−S7). Reaction with excess MEA caused the disappearance of the three characteristic pyridyldithio side chain protons between 7 and 8.5 ppm and the formation of 2-MP (6.8−7.6 ppm).

The reaction kinetics of MEA and RGDC with the C−PDA polymer are shown in Figure 1. The absorbance of 2-MP was measured and converted into the percent of pyridyldithio side-chains reacted using a 2-MP calibration curve. At low concentrations, both molecules demonstrate a linear increase in conversion with thiol concentration. Nonlinearity becomes apparent when the side chain conversion passes 60%, at which point the thiol concentration is approximately equal to the pyridyldithio concentration. In both cases, an approximate 2-fold molar excess of thiol over pyridyldithio groups is necessary to

(31) Sigurdsson, S. T.; Seeger, B.; Kutzke, U.; Eckstein, F. *J. Org. Chem.* **1996**, *61*, 3883−3884.
(32) Field, L.; Parsons, T. F.; Pearson, D. E. *J. Org. Chem.* **1966**, *31*, 3550−3555.
(33) Bellas, M.; Tuleen, D. L.; Field, L. *J. Org. Chem.* **1967**, *32*, 2591−2595.
(34) Ruoslahti, E. *Annu. Rev. Cell Dev. Biol.* **1996**, *12*, 697−715.

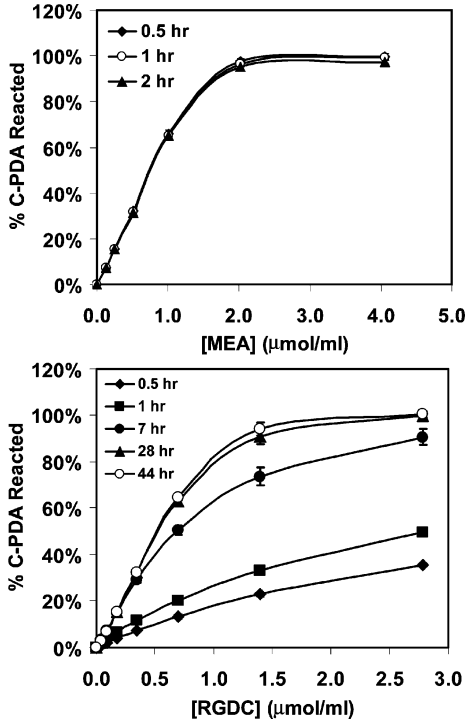


Figure 1. Side chain reactivity of the C−PDA polymer with (a) MEA and (b) RGDC in DMSO as a function of concentration. In both cases, a 1 mg/mL polymer solution was mixed with an equal volume of either of an MEA or RGDC solution. Absorbance measurements were taken at the indicated time intervals and converted into the percentage of side chains reacted using a 2-MP calibration curve. Data are expressed as mean values (±SD, $n = 3$).

attain complete conversion, regardless of the reaction time. Although the concentration dependence is very similar, the MEA and RGDC compounds show very different rates of reaction. MEA reached equilibrium at every concentration tested at the first 30 min time point. In fact, the reaction appeared instantaneous based on the immediate yellow color change of the solution upon mixing, but time points earlier than 30 min were not taken to verify this. Conversely, RGDC demonstrated much slower kinetics, taking over 28 h to approach completion at each concentration tested. Full side chain conversion could be obtained under 30 min but it required an almost 30-fold molar excess of RGDC over pyridyldithio side groups (data not shown). Similar reaction kinetics and ligand concentration dependencies were seen with the F−PDA and U−PDA polymers, indicating that these effects are general for PDA poly(β-amino ester)s (Figure S8).

Polymer−DNA Binding. An important requirement for an effective gene delivery system is the ability to interact with plasmid DNA. Polycationic materials that have high molecular weights and charge densities can bind negatively charged DNA through electrostatic interactions.[2] For the C−PDA based polymers, qualitative assessments of these interactions were made using an agarose gel electrophoresis shift assay. In this experiment, the migration of C−PDA, C−PDA−MEA, and C−PDA−RGDC polymer DNA complexes through the gel was compared to free DNA. The results are shown in Figure 2 for a range of polymer−DNA weight ratios. For this and all other experiments described below, the C−PDA−MEA and C−PDA−RGDC polymers contain 100% MEA and RGDC side chains, respectively. As shown in Figure 2, the free DNA migrated through the gel and resolved into two distinct bands, which

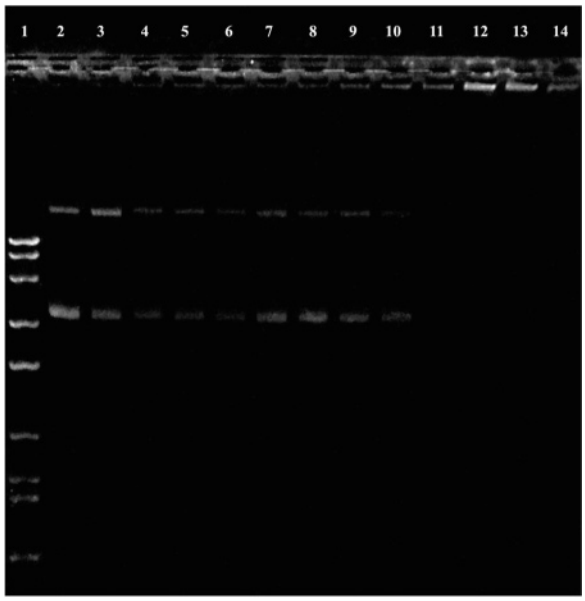


Figure 2. Agarose gel electrophoresis of polymer−DNA complexes. The C−PDA−MEA and C−PDA−RGDC polymers contain 100% MEA and RGDC side chains, respectively. Lane assignments are as follows: (1) DNA ladder; (2) pCMV−Luc DNA; (3)−(6) C−PDA−RGDC/DNA complexes at polymer−DNA ratios of (3) 20:1, (4) 40:1, (5) 60:1, and (6) 100:1; (7)−(10) C−PDA/DNA complexes at polymer−DNA ratios of (7) 20:1, (8) 40:1, (9) 60:1, and (10) 100:1; (11)−(14) C−PDA−MEA/DNA complexes at polymer:DNA ratios of (11) 20:1, (12) 40:1, (13) 60:1, and (14) 100:1.

correspond to the supercoiled and nicked-circular forms of the plasmid. Complexation of the C−PDA polymers with DNA retarded its movement to varying degrees depending on the polymer type and side chain substituent. For both the base C−PDA polymer and C−PDA−RGDC, partial inhibition of DNA migration is seen at all polymer−DNA ratios. As the polymer−DNA ratio is increased, more localization of DNA can be seen adjacent to the loading wells with a corresponding decrease at the levels of unbound DNA. This trend indicates that increasing amounts of polymer are necessary to more effectively bind DNA, but full complexation cannot be realized even at high polymer−DNA ratios. On the contrary, the C−PDA−MEA polymer displays very effective DNA binding at all ratios. For each condition there appear sharp, distinct bands close to the loading wells with no apparent free DNA (Figure 2). The enhanced ability of this C−PDA derivative to bind DNA is the result of its primary amine side chains that significantly increase the cationic charge density of the polymer.

A more quantitative assessment of polymer−DNA interactions was made by performing a dye exclusion assay. In this experiment, the PicoGreen dye was incubated with unbound DNA and polymer−DNA complexes. The dye's fluorescence is significantly enhanced only when it intercalates between the bases of DNA. High fluorescence is typically produced with free plasmid, but significant reductions can occur when a cationic polymer binds the DNA and shields it from dye penetration.[35] Such fluorescence reductions relative to free DNA are shown in Figure 3 for each C−PDA derivative as a function of the polymer−DNA ratio. For the base C−PDA polymer, a gradual reduction in the relative fluorescence (RF) is seen as the polymer−DNA ratio is increased. The extent of polymer−DNA complexation appears to be low, even at high polymer−

(35) Welz, C.; Fahr, A. *Appl. Spectrosc. Rev.* **2001**, *36*, 333−397.

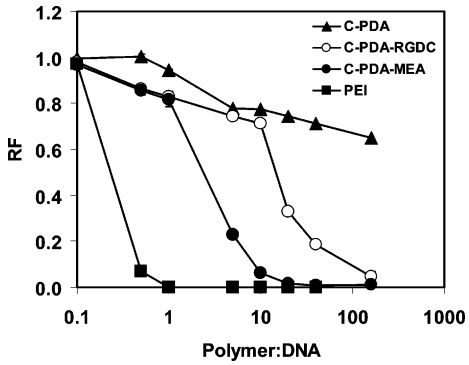


Figure 3. Polymer−DNA binding with PicoGreen. The C−PDA−MEA and C−PDA−RGDC polymers contain 100% MEA and RGDC side chains, respectively. Polymer−DNA complexes in NaAc buffer were mixed with an equal volume of PicoGreen in NaAc buffer and the fluorescence was measured. Fluorescence intensities of polymer−DNA complexes are expressed relative to free DNA and as a function of the polymer−DNA weight ratio. Data are expressed as mean values (±SD, $n = 4$).

DNA ratios. The weak interactions are most likely the result of the low molecular weight and hydrophobicity of the polymer, which greatly limit its solubility and propensity to bind DNA. The C−PDA−RGDC polymer also displayed limited DNA binding up to a ratio of 10:1, after which a significant decrease in the RF occurred. At ratios only exceeding 100:1, near complete inhibition of dye penetration is seen. The highly cationic C−PDA−MEA polymer demonstrated a greater capacity to interact with and shield plasmid DNA. Dye intercalation could be significantly reduced at polymer:DNA ratios as low as 5:1. At ratios greater than 10:1 almost complete complexation of the plasmid occurs. Therefore, the C−PDA polymer and its derivatives display a general increase in DNA binding and association as the polymer−DNA ratio is increased, consistent with the observed electrophoretic mobilities of the complexes. Furthermore, increased hydrophilicity and charge density appear to mediate stronger DNA binding at lower polymer−DNA ratios, as expected. These results are compared to PEI in which the high solubility, molecular weight, and charge density enable this polymer to complex DNA at much lower ratios.

The PicoGreen exclusion assay was also performed in the presence of L-glutathione for C−PDA−MEA polymer DNA complexes. Glutathione is a thiolated tripeptide present at high concentrations in the cytosol (up to 10 mM)[36,37] and at much lower amounts in the extracellular spaces (~4.5 μM in plasma).[38] It is capable of mediating disulfide bond cleavage through disulfide exchange reactions.[39,40] Therefore, it was speculated that this peptide could induce partial C−PDA−MEA breakdown by cleaving the disulfide bonds and separating the primary amine side chains from the polymer backbone. The consequence of this reaction was expected to be a reduction in the polymer charge density and a corresponding decrease in the electrostatic association with DNA. The results of this assay are shown in Figure 4 for C−PDA−MEA polymer DNA complexes at a 40:1 ratio incubated with 0, 0.1, and 10 mM

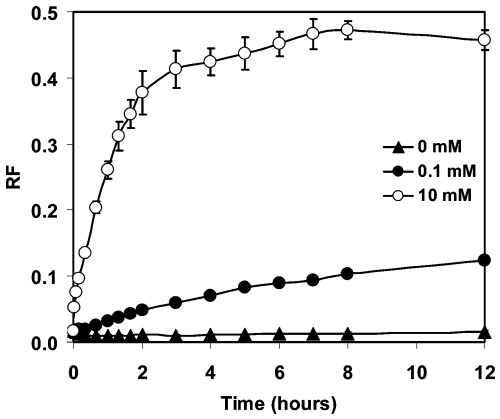


Figure 4. C−PDA−MEA (100% MEA side chains) DNA binding with PicoGreen in the presence of L-gluathione. Polymer−DNA complexes in NaAc buffer were mixed with an equal volume of PicoGreen in NaAc buffer and subsequently mixed with a solution of glutathione in PBS buffer. Fluorescence intensities of polymer−DNA complexes are expressed relative to free DNA as a function of time and glutathione concentration. Data are expressed as mean values (±SD, $n = 4$).

Table 1. Size and Surface Charge Measurement of Polymer−DNA Complexes

polymer	P–D[a]	diameter (nm)	zeta potential (mV)
C−PDA−MEA[b]	100	183.3 ± 29.1	18.87 ± 3.90
	60	133.0 ± 16.0	30.21 ± 4.04
	40	100.8 ± 6.2	10.61 ± 2.14
	20	104.2 ± 2.9	28.31 ± 0.96
	10	255.4 ± 15.2	22.23 ± 0.96
PEI	10	74.4 ± 20.4	10.42 ± 0.89

[a] Polymer−DNA ratio (wt:wt). [b] 100% MEA Side Chains.

glutathione. At the highest concentration, a very rapid RF increase occurs during the first 2 h while a more gradual rise proceeds for the next 2 to 8 h. The overall change in RF is from approximately 0 to almost 0.5. Complete DNA unbinding is not realized because the polymer still contains tertiary amines in the backbone that can remain associated with the DNA. A much smaller RF effect is seen at the lower glutathione concentration tested. In this case, a much slower rise in RF occurs over the 12 h incubation period up to a maximum of 0.1. Therefore, strong DNA binding is still observed at 0.1 mM glutathione, which is over 20 times higher than the blood plasma levels. This result is potentially important for subsequent *in vivo* administration and indicates that substantial decreases in polymer−DNA binding can be expected to occur in the cytosol and not in transit to the target cells.

Polymer−DNA Complex Size and Charge. The size and surface charge of C−PDA−MEA polymer DNA complexes were measured using dynamic and phase analysis light scattering (Table 1). Effective particle diameters of approximately 100 nm were formed at 20:1 and 40:1 polymer−DNA ratios. Higher and lower amounts of polymer tended to give larger nanoparticles. In all cases, the complexes had positive surface charges in the range of 10−30 mV without a clear correlation to the polymer−DNA ratio. The small size and cationic charge of the C−PDA−MEA polymer DNA complexes suggests that they are sufficient for cellular endocytosis.[2,41] For comparison, PEI−polymer DNA particles had effective diameters around 74 nm with a surface charge of approximately 10 mV.

(36) Schafer, F. Q.; Buettner, G. R. *Free Radic Biol. Med.* **2001**, *30*, 1191−1212.
(37) Meister, A.; Anderson, M. E. *Annu. Rev. Biochem.* **1983**, *52*, 711−760.
(38) Chawla, R. K.; Lewis, F. W.; Kutner, M. H.; Bate, D. M.; Roy, R. G. B.; Rudman, D. *Gastroenterology* **1984**, *87*, 770−776.
(39) Bulmus, V.; Woodward, M.; Lin, L.; Murthy, N.; Stayton, P.; Hoffman, A. *J Controlled Release* **2003**, *93*, 105−120.
(40) Saito, G.; Swanson, J. A.; Lee, K. D. *Adv. Drug Deliv. Rev.* **2003**, *55*, 199−215.
(41) Prabha, S.; Zhou, W. Z.; Panyam, J.; Labhasetwar, V. *Int. J. Pharm.* **2002**, *244*, 105−115.

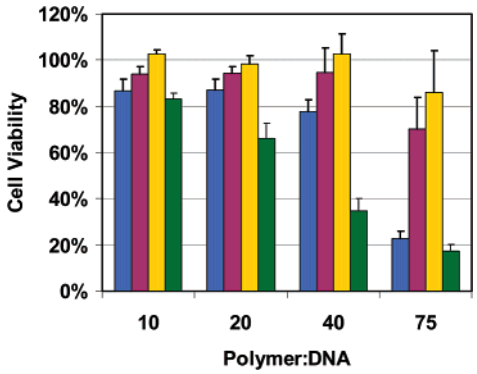


Figure 5. Cytotoxicity of polymer−DNA complexes for C−PDA (blue), C−PDA−MEA (red), 2-MP (yellow), and PEI (green). HCC cells were exposed to polymer−DNA complexes for 1 h and analyzed for metabolic activity 24 h later using the MTT Cell Proliferation Assay. Toxicity of 2-MP solutions were also measured at concentrations in which this molecule is present in polymer−DNA samples at the given weight ratio. The C−PDA−MEA polymer contains 100% MEA side chains. The percent cell viability is shown as a function of the polymer−DNA weight ratio for each sample. Data are expressed as mean values (±SE, $n = 4$).

Polymer Cytotoxicity. Cell compatability studies were performed by incubating C−PDA based polymer DNA complexes on the cells and assaying for metabolic activity 1 day later. The results for C−PDA and C−PDA−MEA are shown in Figure 5 along with PEI for comparison. The C−PDA polymer showed minimal toxicity up to a 40:1 polymer−DNA ratio but induced substantial cell death at very high ratios. In contrast, the more cationic C−PDA−MEA polymer caused little toxicity over the entire range, with a maximum of approximately 30% cell death at 75:1 polymer−DNA ratio. The more toxic effects of C−PDA might be attributed to the hydrophobicity of the polymer that could perturb or lyse the lipophilic cell membranes. In addition to the polymers, the toxicity profile of 2-MP was also measured. Concentrations of 2-MP were set to those at which this molecule is present in polymer−DNA samples at the given weight ratios. As shown, this small molecule has excellent biocompatibility even at very high concentrations. Because of the low 2-MP toxicity, it may be possible to perform thiol ligand conjugations to the C−PDA polymer without necessarily purifying the polymer product from the 2-MP released. Compared to the C−PDA polymers, PEI resulted in higher toxicity levels at almost all polymer:DNA ratios. Cell viability decreased in a linear fashion as the ratio was increased.

HCC Transfections. The ability of PDA based polymers to deliver DNA into HCC cells was determined by performing a Luciferase reporter gene transfection. MEA derivatives were formed for each polymer and tested along with the unmodified PDA base polymers. The results are shown in Figure 6 for each polymer at four different polymer:DNA ratios. The transfection levels using DNA complexes prepared with the base polymers C−PDA, F−PDA, and U−PDA were all very low and not higher than the untreated control. However, protein expression was significantly enhanced when using all of the MEA derivatives. In the case of F−PDA−MEA, an order-of-magnitude increase in Luciferase expression was seen at the higher polymer−DNA ratios over F−PDA. The U−PDA−MEA and C−PDA−MEA polymers displayed an even greater effect with expression levels over 300 times that of naked DNA. These polymers, when used in complexes with DNA, show transfection

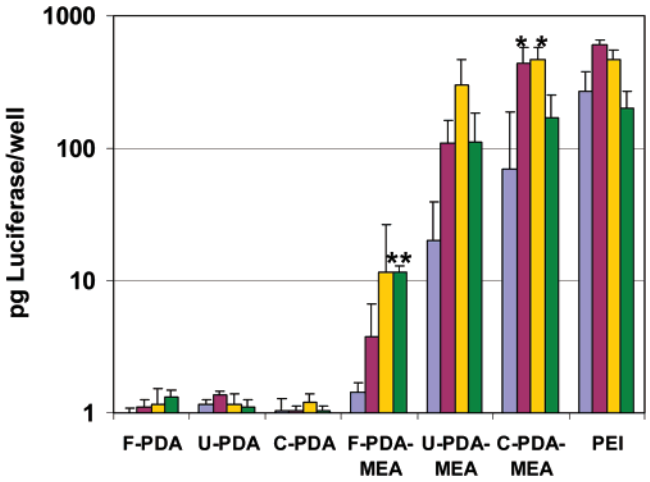


Figure 6. Transfection of HCC cells. PDA polymers and PDA−MEA polymers (100% MEA side chains) were complexed with pCMV−Luc DNA at 20:1 (blue), 30:1 (red), 40:1 (yellow) and 60:1 (green) polymer−DNA weight ratios and incubated with HCC cells for 1 h. Luciferase expression levels were measured 3 days later. The relative light units measured were converted to mass of expressed protein using a Luciferase calibration curve. Data are expressed as mean values as a function of both the polymer and polymer−DNA weight ratio (±SD $n = 3$). Statistical significance of MEA polymers over the base PDA polymers was determined by a t-test (two-tailed, unequal variance) with $p < 0.05$ (*) and $p < 0.01$ (**).

levels equal to that obtained with optimized PEI, one of the best commercially available polymer transfection reagents. All of these results suggest that these are a new effective class of poly(β-amino ester)s. In addition to the pyridyldithio functionalities of the base polymers, the amine side chains in the MEA polymer derivatives can potentially be used to conjugate ligands for cell targeting, but with the added benefit of efficient transfection.

Discussion

For gene delivery to be clinically viable, a safe and efficient delivery system needs to be developed. The shortcomings of viral vectors have elicited the exploration of non-viral alternatives such as cationic lipids and polymers. In order for these materials to approach viral efficiency, they must overcome the cellular barriers to gene delivery. These include DNA condensation for endocytosis, cellular association and uptake, endosomal escape, cytoplasmic transport, nuclear import, and DNA unpackaging. *In vivo* administration presents additional challenges that include serum stability, avoidance of the reticulo−endothelial system, resistance to nucleases, and specific delivery to a target cell population.[42]

To address these challenges, we developed a new class of poly(β-amino ester)s that have thiol-reactive side chains. Similar to other poly(β-amino ester)s, these PDA-based polymers are degradable by hydrolysis of the backbone ester bonds and have tertiary amines to facilitate DNA binding.[23] Furthermore, the side chains consist of pyridyldithio functionalities that are highly reactive with thiol groups through a disulfide exchange reaction.[43] The 2-MP generated following thiol conjugation is a good leaving group due to its resonance stabilization and does not re-react with the side chains.[43] In addition, the synthesis conditions employed here resulted in diacrylate-capped end

(42) Nishikawa, M.; Huang, L. *Human Gene Ther.* **2001**, *12*, 861−870.
(43) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, 1996.

chains that can permit another level of functionalization. In theory, amine compounds and other nucleophiles can be selectively coupled to the polymer ends while thiol molecules can be grafted to the side chains.

The PDA reactive side group provides a platform for further development of polymer functionality, which potentially includes the attachment of (1) thiolated targeting ligands such as peptides and proteins to provide tissue and cell specific delivery without altering the charge density of the backbone, (2) inert polymers (e.g., PEG) to improve serum stability, and (3) peptide transduction domains, nuclear localization sequences and other functionalities to overcome cellular transfection barriers. The pyridyldithio groups allow these potential modifications to be performed over a wide pH range and with very fast colorimetric quantification of the conversion due to 2-MP release.[28] The PDA poly(β-amino ester)s can therefore serve as a cationic, degradable platform to incorporate many levels of gene delivery functionality through the side chains and end groups. Ultimately, this level of convenience and design flexibility may prove valuable in constructing a delivery system that is capable of overcoming obstacles to efficient gene transfection.

As one application of PDA polymer modification, we examined the reaction of the pyridyldithio side groups with the thiol compound MEA. The reaction proceeded almost instantaneously and resulted in the formation of poly(β-amino ester)s containing primary amines in the side chains that are linked to the backbone through disulfide bonds. Furthermore, NMR analysis shows the disappearance and reaction of polymer acrylates with MEA, demonstrating the potential to couple nucleophilic molecules at the polymer terminus (Figures S5−S7). These polymers displayed strong DNA binding ability over a range of polymer:DNA ratios as determined by electrophoresis and dye exclusion assays. Particle sizing and surface charge measurements indicated that the polymer−DNA complexes were cationic with effective diameters as low as 100 nm. These features suggest that the particles are sufficient for association with the negatively charged cell membrane and subsequent endocytosis. Although the polymer/DNA complexes are cationic, cytotoxicity was minimal, even at high polymer−DNA ratios.

The MEA-based poly(β-amino ester)s developed here are among only a few degradable polymers synthesized with amine side chains. Lim et al.[44,45] have demonstrated the synthesis of poly(4-hydroxy-L-proline ester) (PHP ester) and poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) and the utility of these polymers for gene delivery. Both polymers are degradable via ester hydrolysis and contain either secondary (for PHP ester) or primary (for PAGA) amines in the side chains. Transfection levels using these polymers were elevated over poly-L-lysine but required the presence of chloroquine to enable endosomal escape. Presumably, our MEA-based polymers circumvent that requirement since they are capable of delivering DNA without the need for exogenous endosomal buffering agents. This could be a result of the tertiary amines in the backbone of the polymer that may act through a proton sponge mechanism to facilitate endosomal release, similar to PEI.[46]

Another potential advantage of the MEA based poly(β-amino ester)s over other degradable polycations synthesized thus far is its response to glutathione. Using a dye-exclusion assay, we have shown that polymer−DNA binding is significantly reduced in the presence of glutathione. Intracellular glutathione concentrations are sufficient to break the polymer disulfides and decrease the cationic charge density, thereby weakening the strength of DNA binding. Furthermore, lower concentrations of glutathione that are found outside the cell were shown to have a minimal effect. These results indicate that the MEA based polymer delivery system should be relatively stable in the extracellular space, but responsive to intracellular conditions in which partial unpackaging is triggered. A similar response has also been seen by Pichon et al.[47] with a polylysine derivative that contained disulfide linked primary amine side chains. Theoretically, complete dissociation of the amine side chains could be accomplished, resulting in a neutral polymer with little DNA binding capacity. For the C−PDA−MEA polymer, we obtained an almost 50% restoration of dye fluorescence, indicating an increase in the amount of unbound DNA. The fluorescence was not restored to the levels of fully unbound DNA since our polymer also has tertiary amines in the backbone that still enable some DNA binding. This interaction is purely electrostatic in nature since the ^{1}H NMR of the C−PDA−MEA polymer does not show any acrylate end groups that could react with the DNA. Although the unpackaging effect is limited, the tertiary amines confer the potential advantage of endosomal escape, as mentioned above.

The ability of the PDA-based polymers to deliver DNA was tested on a human hepatocellular carcinoma cell line. We show that gene delivery could be markedly enhanced by changing the side chain functionality. In particular, MEA substitution resulted in elevated transfection levels for all three polymers tested. These results extend previous structure−function studies to include poly(β-amino ester)s with primary amine side chains as effective DNA delivery polycations.[25] For the case of C−PDA−MEA and U−PDA−MEA, the transfection levels were almost as high as that observed with an optimal PEI formulation. Although the mechanism behind this increase in transfection efficiency is unclear, both solubility and DNA binding significantly improved with MEA substitution.

The reaction of RGDC to the C−PDA polymer side chains was used to demonstrate the potential to attach thiolated ligands for the development of a targeted delivery system. The RGD peptide sequence has been shown to bind with high affinity to the $\alpha_v\beta_3$ integrin receptor that is overexpressed on the surface of angiogenic endothelial cells.[34,48] Therefore, incorporation of integrin-binding ligands in the polymer side chains may promote specific targeting of the polymer−DNA complexes to the tumor vasculature, as observed for other delivery systems.[49] We show here that RGDC can be grafted to all of the polymer side chains in a 1-day reaction using a small excess of peptide. The reaction can be conveniently carried out in DMSO due to the high solubility of the peptide in this organic solvent. Elevated temperatures and the use of catalysts are not necessary because of the high reactivity of the pyridyldithio group toward thiol

(44) Lim, Y. B.; Choi, Y. H.; Park, J. S. *J. Am. Chem. Soc.* **1999**, *121*, 5633−5639.
(45) Lim, Y. B.; Han, S. O.; Kong, H. U.; Lee, Y.; Park, J. S.; Jeong, B.; Kim, S. W. *Pharm. Res.* **2000**, *17*, 811−816.
(46) Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. *J. Gene Med.* **2005**, *7*, 657−663.
(47) Pichon, C.; LeCam, E.; Guerin, B.; Coulaud, D.; Delain, E.; Midoux, P. *Bioconjug. Chem.* **2002**, *13*, 76−82.
(48) Brooks, P. C.; Clark, R. A. F.; Cheresh, D. A. *Science* **1994**, *264*, 569−571.
(49) Hood, J. D.; Bednarski, M.; Frausto, R.; Guccione, S.; Reisfeld, R. A.; Xiang, R.; Cheresh, D. A. *Science* **2002**, *296*, 2404−2407.

ligands. This may prove a valuable mechanism for the conjugation of sugar, protein, and antibody ligands that are heat-sensitive or unstable in certain aqueous buffers. With RGDC side chains, the polymer displayed the ability to bind and condense plasmid DNA, especially at high polymer−DNA ratios. We are currently testing the targeting effects of this polymer on endothelial cells, and it is envisioned that this delivery system could permit localization and transfection in the tumor vasculature for cancer therapies.

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Supporting Information Available: ^{1}H NMR spectra of the PDA monomer, C−PDA polymer, and C−PDA−MEA polymer. Side chain reactions of the F−PDA and U−PDA polymers with MEA and RGDC. This material is available free of charge via the Internet at http://pubs.acs.org.

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