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Parallel Synthesis and Screening of Polymers for Nonviral Gene Delivery

Sutapa Barua,[†] Amit Joshi,[‡] Akhilesh Banerjee,[‡] Dana Matthews,[†] Susan T. Sharfstein,[‡] Steven M. Cramer,[‡] Ravi S. Kane,*[,‡] and Kaushal Rege*[,†]

Department of Chemical Engineering, Arizona State University, Tempe, Arizona 85287-6006, and Department of Chemical and Biological Engineering and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180

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Abstract: We describe the parallel synthesis and in vitro evaluation of a cationic polymer library for the discovery of nonviral gene delivery vectors. The library was synthesized based on the ring-opening polymerization reaction between epoxide groups of diglycidyl ethers and the amines of (poly)amines. Parallel screening of soluble library constituents led to the identification of lead polymers with high DNA-binding efficacies. Transfection efficacies of lead polymers were evaluated using PC3-PSMA human prostate cancer cells and murine osteoblasts in the absence and presence of serum. In vitro experiments resulted in the identification of a candidate polymer that demonstrated significantly higher transfection efficacies and lower cytotoxicities than poly(ethyleneimine) (pEI), the current standard for polymeric transfection agents. In addition, polymers that demonstrated moderately higher and comparable transfection efficacies with respect to pEI were also identified. Our results demonstrate that high-throughput synthesis and screening of polymers is a powerful approach for the identification of novel nonviral gene delivery agents.

Keywords: Nonviral gene delivery; transfection; DNA-binding; parallel synthesis; cationic polymers; parallel screening; diglycidyl ethers; polyamines; ethidium bromide

Introduction

The premise of gene therapy is based on correcting disease at the genetic level by compensating for abnormal genes using exogenous DNA. However, the low efficacy of passive DNA transport through mammalian cell membranes necessitates the use of appropriate delivery vehicles. Viruses possess efficient machinery for delivering DNA into mammalian cells and are the most efficient gene delivery vectors known.[1−3] However, issues pertaining to safety, immunogenicity, repeated dosage, viral degradation, and production scale-up motivate the investigation of nonviral approaches to gene delivery/therapy.[4−6]

* To whom all correspondence is to be addressed. K.R.: mailing address, Arizona State University, Chemical Engineering, ECG 202, Tempe, AZ 85287-6006; e-mail, Kaushal.Rege@asu.edu; tel, 480-727-8616; fax, 480-965-0037. R.S.K.: e-mail, kaner@rpi.edu.

[†] Arizona State University.

[‡] Rensselaer Polytechnic Institute.

(1) Kafri, T.; Praag, H.; van Gage, F. H.; Verma, I. M. Lentiviral vectors: regulated gene expression. *Mol. Ther.* **2000**, *1* (6), 516–21.

(2) Maheshri, N.; Koerber, J. T.; Kaspar, B. K.; Schaffer, D. V. Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. *Nat. Biotechnol.* **2006**, *24* (2), 198–204.

(3) Medina-Kauwe, L. K. Endocytosis of adenovirus and adenovirus capsid proteins. *Adv. Drug Delivery Rev.* **2003**, *55* (11), 1485–96.

(4) Garnett, M. C. Gene-delivery systems using cationic polymers. *Crit. Rev. Ther. Drug Carrier Syst.* **1999**, *16* (2), 147–207.

(5) Glover, D. J.; Lipps, H. J.; Jans, D. A. Towards safe, non-viral therapeutic gene expression in humans. *Nat. Rev. Genet.* **2005**, *6* (4), 299–310.

(6) Yi, Y.; Hahm, S. H.; Lee, K. H. Retroviral gene therapy: safety issues and possible solutions. *Curr. Gene Ther.* **2005**, *5* (1), 25–35.

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A wide variety of nonviral agents are currently being explored as potential gene delivery vectors. Cationic molecules, including polyamines,[7] lipids,[8] and polymers[9] condense DNA to nanoscale complexes in aqueous solutions by neutralizing the negative charges on the molecule. These complexes often possess an excess positive charge and interact with anionic mammalian cell membranes leading to endocytotic uptake. An effective gene delivery vector is then able to escape the lysosomal degradation pathway by means of early escape from endosomes, ultimately leading to nuclear entry of the DNA and transcription of the target protein.[10]

Cationic polymers have attracted significant attention for delivering exogenous DNA to cells[5,11−18] and for enhancing the efficacy of virus-mediated gene transfer.[19−21] Traditionally, poly(L-lysine), poly(ethylene imine),[22−24] chitosan,[25,26] dendrimers including polyamidoamine or PAMAM dendrimers,[27,28] and poly(vinyl pyrrolidone)[29] have been employed as polymeric gene delivery agents. However, most traditionally used cationic polymers possess high cytotoxicities. To overcome this challenge, recent efforts have focused on the development of biocompatible polymers including those based on cyclodextrin and carbohydrate comonomers.[30−35] Recent work also includes the development of degradable polymers[36−42] and genetically engineered

(7) Osland, A.; Kleppe, K. Polyamine induced aggregation of DNA. *Nucleic Acids Res.* **1977**, *4* (3), 685–95.
(8) Wasungu, L.; Hoekstra, D. Cationic lipids, lipoplexes and intracellular delivery of genes. *J. Controlled Release* **2006**, *116* (2), 255–64.
(9) Lungwitz, U.; Breunig, M.; Blunk, T.; Gopferich, A. Polyethylenimine-based non-viral gene delivery systems. *Eur. J. Pharm. Biopharm.* **2005**, *60* (2), 247–66.
(10) Kamiya, H.; Tsuchiya, H.; Yamazaki, J.; Harashima, H. Intracellular trafficking and transgene expression of viral and nonviral gene vectors. *Adv. Drug Delivery Rev.* **2001**, *52* (3), 153–64.
(11) Davis, M. E. Non-viral gene delivery systems. *Curr. Opin. Biotechnol.* **2002**, *13* (2), 128–31.
(12) Dincer, S.; Turk, M.; Piskin, E. Intelligent polymers as nonviral vectors. *Gene Ther.* **2005**, *12* (Suppl. 1), S139–45.
(13) Ma, H.; Diamond, S. L. Nonviral gene therapy and its delivery systems. *Curr. Pharm. Biotechnol.* **2001**, *2* (1), 1–17.
(14) Neu, M.; Fischer, D.; Kissel, T. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. *J. Gene Med.* **2005**, *7* (8), 992–1009.
(15) Putnam, D. Polymers for gene delivery across length scales. *Nat. Mater.* **2006**, *5* (6), 439–51.
(16) Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. Design and development of polymers for gene delivery. *Nat. Rev. Drug Discovery* **2005**, *4* (7), 581–93.
(17) Roth, C. M.; Sundaram, S. Engineering synthetic vectors for improved DNA delivery: insights from intracellular pathways. *Annu. Rev. Biomed. Eng.* **2004**, *6*, p. 397–426.
(18) Schaffer, D. V.; Lauffenburger, D. A. Targeted synthetic gene delivery vectors. *Curr. Opin. Mol. Ther.* **2000**, *2* (2), 155–61.
(19) Davis, H. E.; Morgan, J. R.; Yarmush, M. L. Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. *Biophys. Chem.* **2002**, *97* (2−3), 159–72.
(20) Davis, H. E.; Rosinski, M.; Morgan, J. R.; Yarmush, M. L. Charged polymers modulate retrovirus transduction via membrane charge neutralization and virus aggregation. *Biophys. J.* **2004**, *86* (2), 1234–42.
(21) Landazuri, N.; Le Doux, J. M. Complexation of retroviruses with charged polymers enhances gene transfer by increasing the rate that viruses are delivered to cells. *J. Gene Med.* **2004**, *6* (12), 1304–19.
(22) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92* (16), 7297–301.
(23) Zanta, M. A.; Boussif, O.; Adib, A.; Behr, J. P. In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. *Bioconjugate Chem.* **1997**, *8* (6), 839–44.
(24) Coll, J. L.; Chollet, P.; Brambilla, E.; Desplanques, D.; Behr, J. P.; Favrot, M. In vivo delivery to tumors of DNA complexed with linear polyethylenimine. *Hum. Gene Ther.* **1999**, *10* (10), 1659–66.
(25) Borchard, G. Chitosans for gene delivery. *Adv. Drug Delivery Rev.* **2001**, *52* (2), 145–50.
(26) Roy, K.; Mao, H. Q.; Huang, S. K.; Leong, K. W. Oral gene delivery with chitosan--DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat. Med.* **1999**, *5* (4), 387–91.
(27) Bielinska, A.; Kukowska-Latallo, J. F.; Johnson, J.; Tomalia, D. A.; Baker, J. R.; Jr. Regulation of in vitro gene expression using antisense oligonucleotides or antisense expression plasmids transfected using starburst PAMAM dendrimers. *Nucleic Acids Res.* **1996**, *24* (11), 2176–82.
(28) Lee, C. C.; MacKay, J. A.; Frechet, J. M.; Szoka, F. C. Designing dendrimers for biological applications. *Nat. Biotechnol.* **2005**, *23* (12), 1517–26.
(29) Mendiratta, S. K.; Quezada, A.; Matar, M.; Wang, J.; Hebel, H. L.; Long, S.; Nordstrom, J. L.; Pericle, F. Intratumoral delivery of IL-12 gene by polyvinyl polymeric vector system to murine renal and colon carcinoma results in potent antitumor immunity. *Gene Ther.* **1999**, *6* (5), 833–9.
(30) Hwang, S. J.; Bellocq, N. C.; Davis, M. E. Effects of structure of beta-cyclodextrin-containing polymers on gene delivery. *Bioconjugate Chem.* **2001**, *12* (2), 280–90.
(31) Pun, S. H.; Davis, M. E. Development of a nonviral gene delivery vehicle for systemic application. *Bioconjugate Chem.* **2002**, *13* (3), 630–9.
(32) Reineke, T. M.; Davis, M. E. Structural effects of carbohydrate-containing polycations on gene delivery. 2. Charge center type. *Bioconjugate Chem.* **2003**, *14* (1), 255–61.
(33) Reineke, T. M.; Davis, M. E. Structural effects of carbohydrate-containing polycations on gene delivery. 1. Carbohydrate size and its distance from charge centers. *Bioconjugate Chem.* **2003**, *14* (1), 247–54.
(34) Liu, Y.; Reineke, T. M. Poly(glycoamidoamine)s for gene delivery. structural effects on cellular internalization, buffering capacity, and gene expression. *Bioconjugate Chem.* **2007**, *18* (1), 19–30.
(35) Liu, Y.; Wenning, L.; Lynch, M.; Reineke, T. M. New poly(d-glucaramidoamine)s induce DNA nanoparticle formation and efficient gene delivery into mammalian cells. *J. Am. Chem. Soc.* **2004**, *126* (24), 7422–3.
(36) Akinc, A.; Lynn, D. M.; Anderson, D. G.; Langer, R. Parallel synthesis and biophysical characterization of a degradable polymer library for gene delivery. *J. Am. Chem. Soc.* **2003**, *125* (18), 5316–23.

protein-based polymers[43] for nucleic acid delivery. Although in vitro results with the above systems are encouraging, nonviral gene delivery systems do not approach the transduction efficacies of viral vectors in vivo.[5] This inherent low efficacy of nonviral vectors is a significant limitation in the development of safer alternatives to viral vectors.

Novel strategies, including both rational and semirational discovery approaches, can lead to the accelerated identification of highly efficient and safe vectors for nonviral gene delivery. Combinatorial/parallel synthesis has gained immense popularity as a means to rapidly synthesize large numbers of small-molecule compounds as potential drug candidates.[44,45] The approach has recently been extended to the synthesis of diverse oligomer and polymer libraries[46−50] for a variety of applications including gene transfer.[36,42,51−53] In the current work, we employed parallel synthesis and screening techniques for the rapid identification of novel DNA-binding cationic polymers for gene delivery. A library of eighty polymers was synthesized in parallel; the ring-opening polymerization of diglycidyl ethers by amines[54] was employed in the generation of the library. Primary screening involved the parallel evaluation of the DNA-binding efficacies of the library constituents using the ethidium bromide displacement assay.[55−58] In vitro transfection, using representative polymers that demonstrated high DNA-binding efficacies, resulted in the identification of a candidate polymer that showed significantly higher transfection activities and lower cytotoxicities than poly(ethylene imine), both in the presence and absence of serum.

Experimental Section

Materials. Eight diglycidyl ethers, 1,4-butanediol diglycidyl ether (1,4B), 1,4-cyclohexanedimethanol diglycidyl ether (1,4C), 4-vinylcyclohexene diepoxide (4VCD), ethyleneglycol diglycidyl ether (EDGE), glycerol diglycidyl ether (GDE), neopentylglycol diglycidyl ether (NPDGE), poly-

(37) Cohen, H.; Levy, R. J.; Gao, J.; Fishbein, I.; Kousaev, V.; Sosnowski, S.; Slomkowski, S.; Golomb, G. Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. *Gene Ther.* **2000**, *7* (22), 1896–905.

(38) Lewis, K. J.; Irwin, W. J.; Akhtar, S. Development of a sustained-release biodegradable polymer delivery system for site-specific delivery of oligonucleotides: characterization of P(LA-GA) copolymer microspheres in vitro. *J. Drug Targeting* **1998**, *5* (4), 291–302.

(39) Little, S. R.; Lynn, D. M.; Ge, Q.; Anderson, D. G.; Puram, S. V.; Chen, J.; Eisen, H. N.; Langer, R. Poly-beta amino ester-containing microparticles enhance the activity of nonviral genetic vaccines. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (26), 9534–9.

(40) Maheshwari, A.; Mahato, R. I.; McGregor, J.; Han, S.; Samlowski, W. E.; Park, J. S.; Kim, S. W. Soluble biodegradable polymer-based cytokine gene delivery for cancer treatment. *Mol. Ther.* **2000**, *2* (2), 121–30.

(41) Petersen, H.; Merdan, T.; Kunath, K.; Fischer, D.; Kissel, T. Poly(ethylenimine-co-L-lactamide-co-succinamide): a biodegradable polyethylenimine derivative with an advantageous pH-dependent hydrolytic degradation for gene delivery. *Bioconjugate Chem.* **2002**, *13* (4), 812–21.

(42) Lynn, D. M.; Langer, R. Degradable Poly(beta-amino esters): Synthesis, Characterization, and Self-Assembly with Plasmid DNA. *J. Am. Chem. Soc.* **2000**, *122* (44), 10761–10768.

(43) Megeed, Z.; Haider, M.; Li, D.; O′Malley, B. W., Jr.; Cappello, J.; Ghandehari, H. In vitro and in vivo evaluation of recombinant silk-elastinlike hydrogels for cancer gene therapy. *J. Controlled Release* **2004**, *94* (2−3), 433–45.

(44) Gallop, M. A.; Barrett, R. W.; Dower, W. J.; Fodor, S. P.; Gordon, E. M. Applications of combinatorial technologies to drug discovery. 1. Background and peptide combinatorial libraries. *J. Med. Chem.* **1994**, *37* (9), 1233–51.

(45) Gordon, E. M.; Barrett, R. W.; Dower, W. J.; Fodor, S. P.; Gallop, M. A. Applications of combinatorial technologies to drug discovery. 2. Combinatorial organic synthesis, library screening strategies, and future directions. *J. Med. Chem.* **1994**, *37* (10), 1385–401.

(46) Brocchini, S.; James, K.; Tangpasuthadol, V.; Kohn, J. Structure-property correlations in a combinatorial library of degradable biomaterials. *J. Biomed. Mater. Res.* **1998**, *42* (1), 66–75.

(47) Freeman, A. W.; Chrisstoffels, L. A.; Frechet, J. M. A simple method for controlling dendritic architecture and diversity: A parallel monomer combination approach. *J. Org. Chem.* **2000**, *65* (22), 7612–7.

(48) Kim, D. Y.; Dordick, J. S. Combinatorial array-based enzymatic polyester synthesis. *Biotechnol. Bioeng.* **2001**, *76* (3), 200–6.

(49) Lavastre, O.; Illitchev, I.; Jegou, G.; Dixneuf, P. H. Discovery of new fluorescent materials from fast synthesis and screening of conjugated polymers. *J. Am. Chem. Soc.* **2002**, *124* (19), 5278–9.

(50) Vogel, B. M.; Cabral, J. T.; Eidelman, N.; Narasimhan, B.; Mallapragada, S. K. Parallel synthesis and high throughput dissolution testing of biodegradable polyanhydride copolymers. *J. Comb. Chem.* **2005**, *7* (6), 921–8.

(51) Lynn, D. M.; Anderson, D. G.; Putnam, D.; Langer, R. Accelerated discovery of synthetic transfection vectors: Parallel synthesis and screening of degradable polymer library. *J. Am. Chem. Soc.* **2001**, *123* (33), 8155–8156.

(52) Green, J. J.; Langer, R.; Anderson, D. G. A Combinatorial Polymer Library Approach Yields Insight into Nonviral Gene Delivery. *Acc. Chem. Res.* **2008**, *41*, 749–759.

(53) Anderson, D. G.; Akinc, A.; Hossain, N.; Langer, R. Structure/property studies of polymeric gene delivery using a library of poly(beta-amino esters). *Mol. Ther.* **2005**, *11* (3), 426–34.

(54) Raman, V. I.; Palmese, G. R. Nanoporous thermosetting polymers. *Langmuir* **2005**, *21* (4), 1539–46.

(55) Boger, D. L.; Fink, B. E.; Brunette, S. R.; Tse, W. C.; Hedrick, M. P. A simple, high-resolution method for establishing DNA binding affinity and sequence selectivity. *J. Am. Chem. Soc.* **2001**, *123* (25), 5878–91.

(56) Geall, A. J.; Blagbrough, I. S. Rapid and sensitive ethidium bromide fluorescence quenching assay of polyamine conjugate-DNA interactions for the analysis of lipoplex formation in gene therapy. *J. Pharm. Biomed. Anal.* **2000**, *22* (5), 849–59.

(57) Rege, K.; Hu, S.; Moore, J. A.; Dordick, J. S.; Cramer, S. M. Chemoenzymatic synthesis and high-throughput screening of an aminoglycoside-polyamine library: identification of high-affinity displacers and DNA-binding ligands. *J. Am. Chem. Soc.* **2004**, *126* (39), 12306–15.

(58) Rege, K.; Ladiwala, A.; Hu, S.; Breneman, C. M.; Dordick, J. S.; Cramer, S. M. Investigation of DNA-binding properties of an aminoglycoside-polyamine library using quantitative structure-activity relationship (QSAR) models. *J. Chem. Inf. Model.* **2005**, *45* (6), 1854–63.

(ethyleneglycol) diglycidyl ether (PEGDE), and poly(propyleneglycol) diglycidyl ether (PPGDE) were purchased from Sigma-Aldrich and were used without any further purification. Ten amines, 1-(2-aminoethyl) piperidine, 1,4-bis(3-aminopropyl) piperazine (1,4Bis), 3,3′-diamino-*N*-methyl dipropylamine (3,3′), 4,7,10-trioxa-1,13-tridecanediamine, aniline, butylamine, diethylenetriamine (DT), ethylenediamine (ED), *N*-(2-aminoethyl)-1,3-propanediamine (N-2amino), and pentaethylenehexamine, were also purchased from Sigma-Aldrich and used as received. Calf-thymus DNA, ethidium bromide, 25 kDa poly(ethylene imine) (M_n = 10 kDa, M_w = 25 kDa; henceforth called pEI-25), and 750 kDa poly(ethylene imine) (M_n = 60 kDa, M_w = 750 kDa; henceforth called pEI-750) were purchased from Sigma-Aldrich. The pGL3 control vector and the Bright Glo kit were purchased from Promega.

Parallel Polymer Synthesis. Eight diglycidyl ethers (2.3 mmol) were reacted with equimolar amounts of amines; neat as-purchased solutions were employed for both reactants. In the case of pentaethylenehexamine, the low solubility of the resulting polymers at a 1:1 ratio of diglycidyl ether to amine necessitated the use of a 10:1 diglycidyl ether:amine molar ratio in subsequent experiments. The polymerization was carried out in 20 mL glass scintillation vials for 16 h. After 16 h, the resulting polymer was diluted to a concentration of 2 mg/mL in 20 mM Tris buffer, pH 7.4. The solution pH was adjusted to 7.4 using 30% hydrochloric acid in deionized (DI) water to compensate for the alkalinity of the polymers. Only those polymers that were soluble at a concentration of 2 mg/mL at pH 7.4 were evaluated for their DNA-binding efficacies.

Parallel Screening of DNA-Binding Activity. The ethidium bromide displacement assay[55−58] was employed to evaluate the DNA-binding affinity of the cationic polymer library in parallel. Briefly, 1.5 mL of 6 μg/mL double-stranded, calf-thymus DNA was equilibrated with 15 μL of 0.5 mg/mL ethidium bromide (all solutions were prepared in 20 mM Tris buffer, pH 8.0). After equilibration, 25 μL of 2 mg/mL polymer was added to the DNA−ethidium bromide mixture and equilibrated for 20 min. 150 μL of the polymer−DNA solution was transferred into a 96-well microtiter plate, and the fluorescence (excitation at 260 nm, emission at 595 nm) was measured using a plate reader (Perkin-Elmer Lambda 6.0). The decrease in fluorescence intensity (percent fluorescence decreased compared to control) was used to rank DNA-binding efficacies of individual polymers.

Polymer-Mediated Transfections. The pGL3 control vector (Promega Corp., Madison, WI), which encodes for the modified firefly luciferase protein under the control of an SV40 promoter, was used in transfection experiments. *Escherichia coli* (XL1 Blue) cells containing the pGL3 plasmid DNA were cultured overnight (16 h, 37 °C, 150 rpm) in 15 mL tubes (Fisher) in 5 mL of Terrific Broth (MP Biomedicals, LLC) containing 1 mg/mL ampicillin (Research Products International, Corp.). The cultures were then centrifuged at 5400*g* and 4 °C for 10 min. Plasmid DNA was purified according to the QIAprep Miniprep Kit (Qiagen) protocol; DNA concentration and purity were determined based on absorbance at 260 and 280 nm determined using a NanoDrop Spectrophotometer (ND-1000; NanoDrop Technologies).

The PC3-PSMA human prostate cancer cell line[59] was a generous gift from Dr. Michel Sadelain of the Memorial Sloan Cancer Center, New York, NY. The cells were cultured in a 5% CO_2 incubator at 37 °C using RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (10,000 units/mL penicillin G and 10,000 μg/mL streptomycin). MC3T3 murine osteoblasts were cultured in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) containing 4.5 g/L glucose and L-glutamine, supplemented with 10% fetal bovine serum (Invitrogen, CA) and 1% penicillin/streptomycin (Invitrogen, CA). PC3-PSMA and MC3T3 cells were seeded in 24-well plates at a density of 50,000 cells/well and allowed to attach overnight. Polymer:pGL3 control plasmid at weight ratios of 25:1 (polymer concentration 10 ng/μL and 200 ng pGL3 plasmid in each well) were incubated for 30 min at room temperature, and the resulting polyplexes were added to cells for 6 h either in the absence or presence of serum (10% FBS), at the end of which fresh serum-containing medium was added to the cells. Following further incubation for 48 h, cells were lysed using the Bright Glo kit (Promega) and analyzed for luciferase protein expression (in relative luminescence units or RLU) using a plate reader (Bio-Tek Synergy 2). The protein content in each well was determined using the BCA assay, and the luminescence value (RLU) was normalized by the protein content. Transfection efficacies of different polymers from the library were compared with the normalized value (RLU/mg protein) obtained for pEI-25.

Polyplex and Polymer Cytotoxicity. PC3-PSMA cells were seeded in a 24-well plate at a density of 50,000 cells/well and incubated overnight at 37 °C. Different weight ratios of polymer−DNA polyplexes (10:1, 25:1, and 50:1 polymer:pGL3 plasmid) and different concentrations of polymers (4−20 ng/μL) were added in the absence of serum, and the cells were incubated for 6 h to determine polyplex- and polymer-induced cytotoxicity, respectively. Following incubation, cells were treated with 100 μL of 4 μM ethidium homodimer-1 (EthD-1; Invitrogen) for 15 min and imaged immediately using Zeiss AxioObserver D1 inverted microscope (10×X/0.3 numerical aperture (NA) objective; Carl Zeiss MicroImaging Inc., Germany). Fluorescence using excitation at 550 nm and emission at 670 nm were used for the microscopy; dead/dying cells with compromised nuclei stained positive (red) for EthD-1.

Quantitative analysis of polymer/polyplex induced cell death was carried out as follows. The number of dead cells

(59) Gong, M. C.; Latouche, J. B.; Krause, A.; Heston, W. D.; Bander, N. H.; Sadelain, M. Cancer patient T cells genetically targeted to prostate-specific membrane antigen specifically lyse prostate cancer cells and release cytokines in response to prostate-specific membrane antigen. *Neoplasia* **1999**, *1* (2), 123–7.

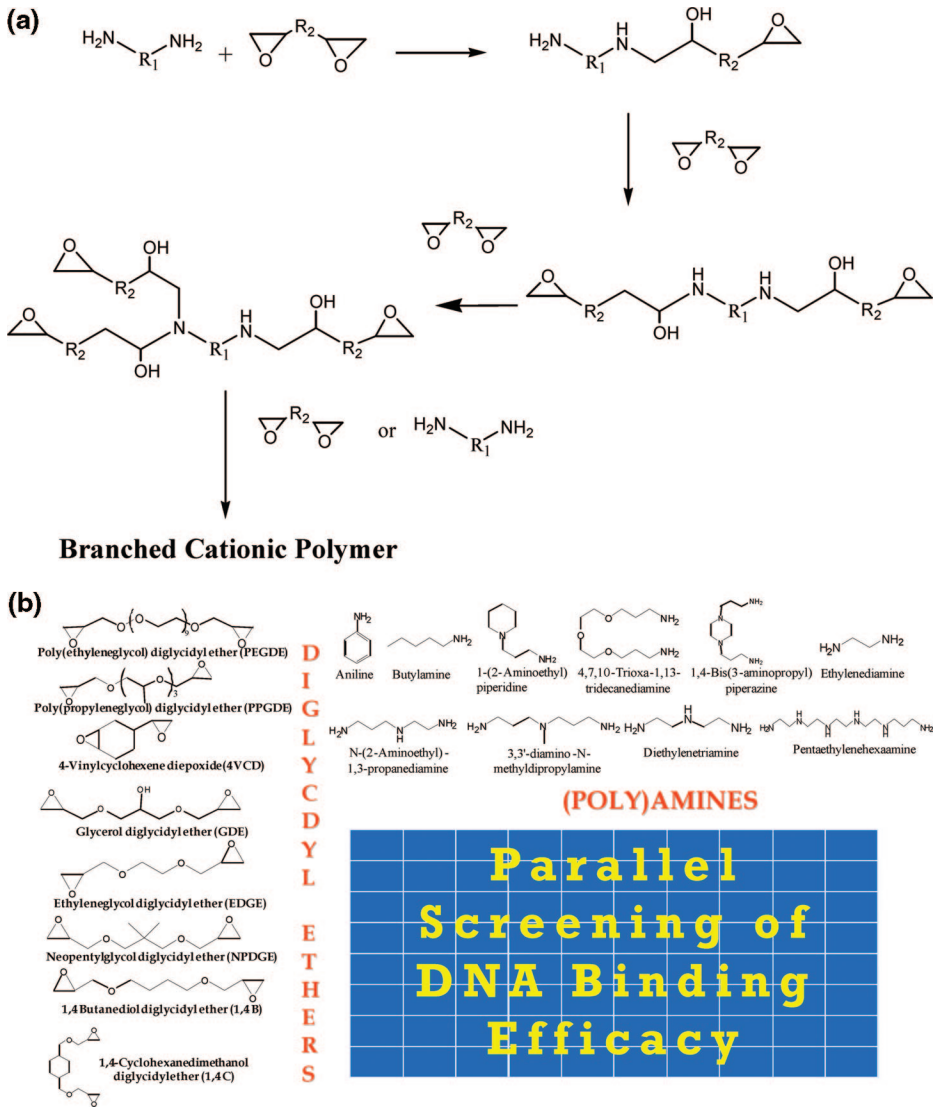

Figure 1. a. Schematic of the reaction employed for the generation of the cationic polymer library based on the ring opening of diglycidyl ethers by amines. b. Combinatorial matrix of the cationic polymer library composed of diglycidyl ethers and (poly)amines.

in each case was counted manually for three individual fields of fluorescence microscopy images by means of the Cell Counter plugin in ImageJ software (Rasband, W. S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997−2005). The number of dead cells in both dead and live controls was determined for at least two fields of view, and their average values were calculated. The number of red fluorescent cells in the case of each polymer or the corresponding polyplex was determined, and the percentage of dead cells was calculated by normalizing the number of dead cells in the sample to the number of dead cells in the dead control.

Polymer Characterization. (a) Polymerization Kinetics. The disappearance of reactive (primary and secondary) amines with time was used to monitor the kinetics of the diglycidyl ether−polyamine reaction (Figure 1a); the ninhydrin assay[60] was used to determine the concentration of reactive amines at each time point. The ninhydrin assay results in a yellow-orange color in the case of secondary amines and a dark blue/purple color in the case of primary amines. Briefly, approximately 2 mg of the polymers were weighed into 1.5 mL microcentrifuge tubes (Fisher) at different time points (0−24 h) during the polymerization reaction. Ninhydrin reagent (Sigma; 100 μL) and DI water (200 μL) were added to the polymers in the centrifuge tubes, following which the tubes were placed in a boiling water bath for 10 min and cooled to room temperature (22 °C). The mixture was diluted by adding 500 μL of 95% ethanol. The mixtures were further diluted 10- and 100-fold using DI water in order to obtain absorbance values within the calibration range (using glycine standards) employed. Ab-

(60) Friedman, M. Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. *J. Agric. Food Chem.* **2004**, *52* (3), 385–406.

sorbance was measured at 570 nm in triplicate for each sample using a microplate reader (BioTek Synergy 2). The amine concentration was monitored every 4 h, and the concentration of amines in the reaction mixture at a given time point was normalized with the concentration of amines at the start of the reaction ($t = 0$) in order to obtain percentage amine values.

(b) Determination of Polymer Molecular Weight. Polymer molecular weight was determined using gel permeation chromatography (GPC) using a ViscoGEL column (MBLMW, Mixed Bed, dimensions: 7.8 mm × 30 cm) using 5% (v/v) acetic acid in water as the eluent (flow rate 1 mL/min).[61] The M_n and M_w values were estimated as an average of two experimental runs using a light scattering Viscotek 270 Trisec Dual Detector; OmniSEC software, $\lambda = 670$ nm.

(c) Fourier Transform Infrared (FT-IR) Spectroscopy. FT-IR spectra were obtained at two different polymerization time points ($t = 0$ and $t = 16$ h) in order to ascertain the formation of the polymers. Polymer samples were loaded on a germanium attenuated total reflectance (GATR) crystal such that they covered the center area of the crystal. The sample chamber was equilibrated to approximately 4 mbar pressure in order to minimize interference from atmospheric moisture and CO_2. The absorption spectrum was measured between 650 and 4,000 cm^{-1} using a Bruker IFS 66 v/S FT-IR spectrometer and the background spectrum was subtracted from all sample spectra.

Statistical Analyses. Data are reported as mean ± one standard deviation of independent replicate experiments. Statistical significance was determined for a given polymer using unpaired Student's *t* test; *p*-values <0.05, with respect to pEI-25, are considered statistically significant.

Results and Discussion

The identification of efficient nonviral gene delivery agents is a critical limitation in the development of safer alternatives to viral-based delivery of DNA. While combinatorial chemistry and high-throughput screening approaches have been widely established in the discovery of small molecule drugs, their utility in the discovery of nonviral gene delivery agents is somewhat underexplored. We have employed this approach for the discovery of polymeric transfection agents. A library of eighty cationic polymers was synthesized in parallel using the ring-opening polymerization between diglycidyl ethers and amines.[54] Primary screening involved the evaluation of the DNA-binding efficacies of each of the individual library components in parallel. Transfection experiments with high DNA-binding polymer leads resulted in the identification of one polymer that possessed significantly higher transfection efficacies than polyethyleneimine (pEI) in addition to the identification of other polymer that possessed similar efficacies compared to pEI.

Parallel Synthesis and Evaluation of the DNA-Binding Activity of the Cationic Polymer Library. We chose the ring opening of the epoxide groups on diglycidyl ethers by polyamines as a suitable platform reaction for the rapid generation of a cationic polymer in a parallel fashion. Figure 1a shows the reaction scheme employed for the generation of the library of eighty cationic polymers. We have previously employed a synergistic combination of chemoenzymatic synthesis and parallel screening for elucidating structure−property relationships involved in polyamine−DNA binding.[57,58] However, while small molecular weight (<2 kDa) polyamines are known to bind DNA, their poor transfection efficacies necessitate the use of cationic polymers or cationic lipids for gene delivery. We hypothesized that the use of diglycidyl ethers to "cross-link" polyamine molecules can result in diglycidyl ether-polyamine based polymers in which an individual polymer molecule will possess multiple copies of the DNA binding polyamine molecule ultimately resulting in high cellular transfection activities. Diglycidyl ethers were chosen as comonomers since epoxide groups readily react with amines and hence can act as linkers between multiple polyamine molecules leading to rapid polymer synthesis. This property of diglycidyl ethers has been exploited for the generation of cross-linked polymers and proteins for a variety of biomedical applications.[62−65] In addition, the use of a diverse set of diglycidyl ether monomers results in the exploration of a wider chemical space for the resulting polymers. Although most diglycidyl ethers contained glycol moieties, they differed in the number of methylene ($-CH_2-$) units between the oxygen atoms as well as side chain functionalities. A relatively wider range of chemical diversity was employed in case of amines employed for generating the cationic polymer library. Not only did the amines vary in the nitrogen atom content (from one to six), they also varied in their chemical composition; aromatic (e.g., aniline), cyclic (e.g., 1-(2-aminoethyl)piperidine), and linear polyamines (e.g., diethylenetriamine and pentaethylenehexamine) were employed in the study. As with the diglycidyl ethers, it was hypothesized that exploring a wider chemical space with amine monomers would accelerate the discovery of effective polymeric gene delivery agents. Figure 1b shows the combinatorial matrix of the polymer library.

Polymerization reactions were carried out for 16 h at 25 °C in order to maintain the parallel nature of the synthesis

(61) Joshi, A.; Saraph, A.; Poon, V.; Mogridge, J.; Kane, R. S. Synthesis of potent inhibitors of anthrax toxin based on poly-L-glutamic acid. *Bioconjugate Chem.* **2006**, *17* (5), 1265–9.

(62) Khanam, N.; Mikoryak, C.; Draper, R. K.; Balkus, K. J., Jr. Electrospun linear polyethyleneimine scaffolds for cell growth. *Acta Biomater.* **2007**, *3* (6), 1050–9.

(63) Swami, A.; Kurupati, R. K.; Pathak, A.; Singh, Y.; Kumar, P.; Gupta, K. C. A unique and highly efficient non-viral DNA/siRNA delivery system based on PEI-bisepoxide nanoparticles. *Biochem. Biophys. Res. Commun.* **2007**, *362* (4), 835–41.

(64) Yu, X. X.; Wan, C. X.; Chen, H. Q. Preparation and endothelialization of decellularised vascular scaffold for tissue-engineered blood vessel. *J. Mater. Sci. Mater. Med.* **2008**, *19* (1), 319–26.

(65) Segura, T.; Chung, P. H.; Shea, L. D. DNA delivery from hyaluronic acid-collagen hydrogels via a substrate-mediated approach. *Biomaterials* **2005**, *26* (13), 1575–84.

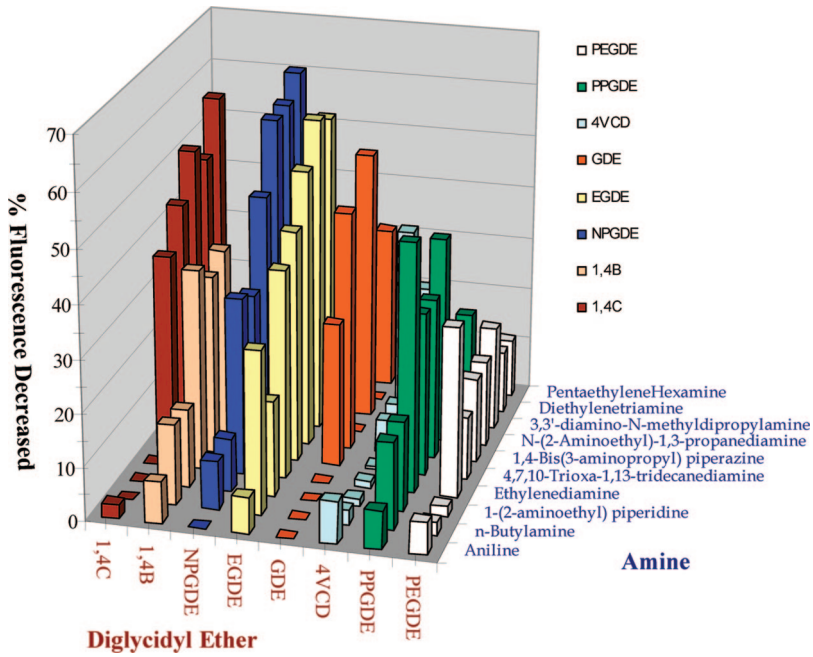

Figure 2. DNA-binding activity of the diglycidyl ether based cationic polymer library determined using the ethidium bromide displacement assay. The percent fluorescence decreased upon polymer binding to calf thymus DNA intercalated with ethidium bromide was used to rank polymer efficacy.

approach. It is acknowledged that different polymerization reactions in the library synthesis can proceed at different rates, resulting in polymers with different molecular weights. However, we chose to carry out polymerizations for 16 h for the following reasons: (1) longer polymerization times (>24 h) resulted in the generation of insoluble polymers in case of a number of higher homologue polyamines, presumably due to extensive cross-linking (not shown), and (2) variability in reaction time was minimized in order to maintain the parallel nature of the polymer synthesis. As a result, all polymerizations with the exception of those based on glycerol diglycidyl ether based polymerizations were carried out for 16 h; reactions with glycerol diglycidyl ether were carried out for 4.5 h since reaction times greater than 5 h resulted in insoluble polymers for all cases.

Sixteen out of the eighty polymers synthesized were not soluble at concentrations of 2 mg/ml; in general, the use of higher amine homologues (polyamines) led to lower aqueous solubilities of the resulting polymers. This lower solubility can be attributed to extensive cross-linking that can take place due to the presence of multiple amines. In particular, pentaethylenehexamine-based polymers were insoluble at a concentration of 2 mg/ml in buffer when equimolar amounts of the amine and diglycidyl ethers were employed. We therefore synthesized polymers with different stoichiometries of pentaethylenehexamine:diglycidyl ether (10:1, 8:1, 6:1, 4:1, 2:1, and 1:1) and tested their solubilities; a ratio of 10:1 was chosen for subsequent experiments based on the solubility of the resulting polymers. Sixty-four soluble polymers were employed in the primary screening which involved an evaluation of their respective DNA-binding efficacies using the ethidium bromide displacement assay.[55] It is important to mention that the polymers were not characterized at this stage in order to maintain the high-throughput/parallel nature of the current approach; instead, the focus was on the rapid identification of lead candidates that demonstrate high DNA-binding efficacies.

Figure 2 shows the DNA-binding efficacy of the cationic polymer library determined using the ethidium bromide displacement assay. As expected, polymers based on monoamines (e.g., aniline and butylamine) demonstrated low values of percent fluorescent decreased (i.e., low DNA-binding efficacies) while those derived from higher homologue polyamines, such as 1,4-bis(3-aminopropyl)piperazine, 3,3′-diamino-*N*-methyl dipropylamine, diethylenetriamine, and *N*-(2-aminoethyl)-1,3-propanediamine demonstrated higher efficacies. While it is possible that the low DNA binding efficacies of some polymers (e.g., polymers based on monoamines) are a result of the lower degree of polymerization, we did not verify this further at this stage and discontinued any further analyses with these polymers. Seven representative polymer leads, with different DNA binding efficacies (percent fluorescence decreased values ranging from 30% to 60%), were chosen for the transfection of PC3-PSMA cells as described below.

In Vitro Transfections. Figure 3a shows the transfection of PC3-PSMA cells with the pGL3 plasmid using a set of lead polymers selected from the DNA-binding screen. Representative polymer leads that possessed moderate (30% fluorescence decreased) to high (>60% fluorescence decreased) DNA binding efficacies were employed in the transfection experiments. It is important to point out that calf-thymus DNA was used only as generic double-stranded DNA in the primary DNA binding screen for identifying lead polymers. However, the lack of a constitutive promoter region implies that this DNA cannot be employed as a reporter for transfection. Consequently, transfections were

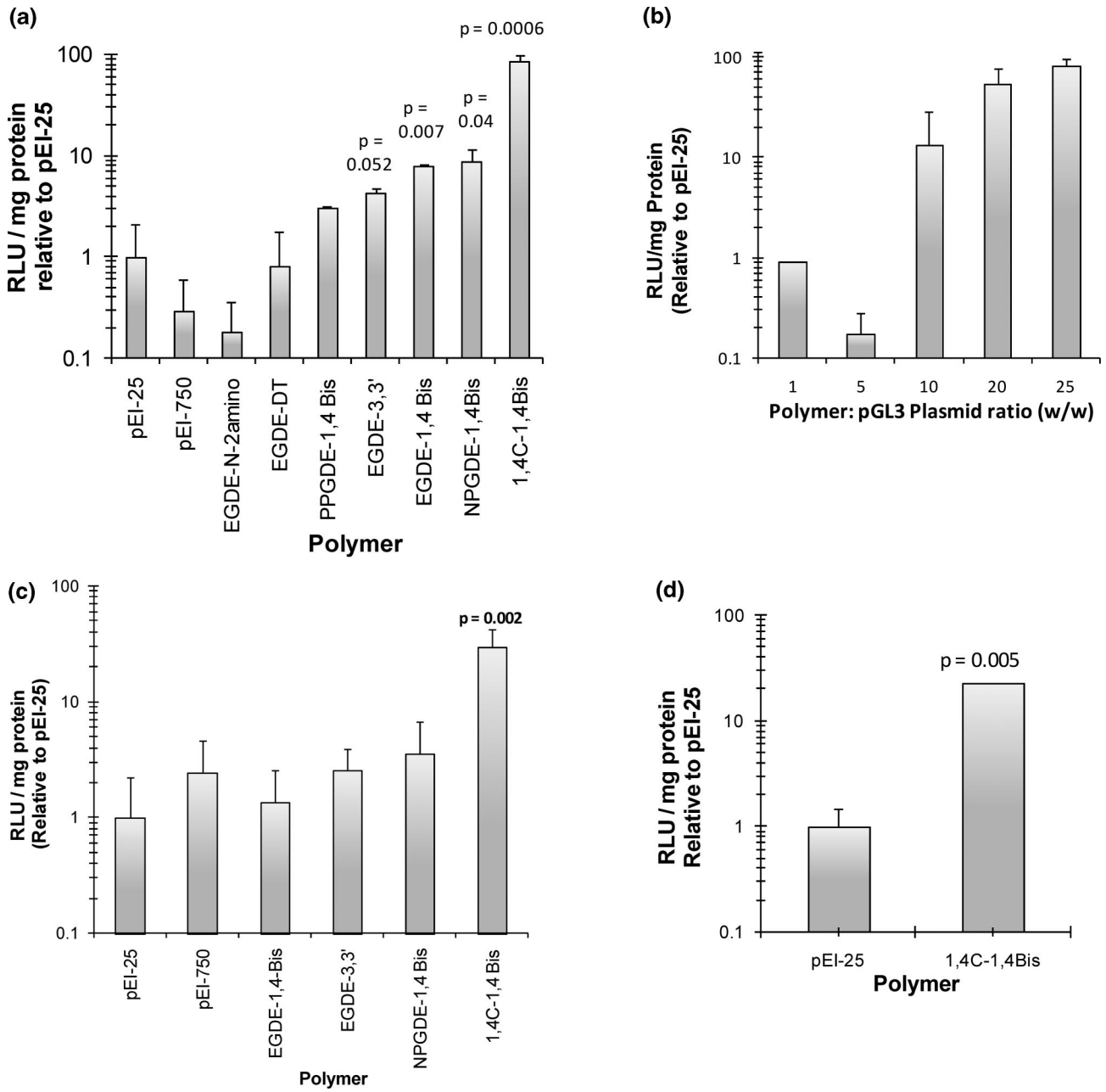

Figure 3. Transfection of PC3-PSMA human prostate cancer cells using polymer leads selected from the DNA binding screen. Transfection efficacy of a given polymer was determined from the luciferase expression (relative luminescence units or RLU) normalized by the total protein amount (mg). Polymer transfection efficacies are reported as relative to that of pEI-25. Relative efficacy data are plotted on a logarithmic scale (*y*-axis), and statistical significance using *p*-values was determined by comparing data for a given polymer with pEI-25. a. Polymer-mediated transfection of PC3-PSMA cells in the absence of serum using a polymer: pGL3 plasmid ratio of 25:1 (w/w). b. Transfection of PC3-PSMA cells in the absence of serum using different polymer:plasmid ratios of the 1,4C-1,4Bis polymer. c. Polymer-mediated transfection of PC3-PSMA cells in the presence of 10% fetal bovine serum using a polymer:pGL3 plasmid ratio of 25:1. d. Polymer-mediated transfection of murine osteoblasts using 1,4C-1,4Bis and pEI-25 polymers in the presence of 10% fetal bovine serum using a polymer:pGL3 plasmid ratio of 25:1.

carried out with the pGL3 control vector which codes for luciferase protein. A polymer to plasmid ratio of 25:1 was employed in order to evaluate the transfection efficacies of the selected polymers. The use of nitrogen:phosphorus (N:P) ratio is common in comparing cationic lipid and cationic polymer meditated gene delivery. However, we used a w/w ratio, which has been previously employed for evaluating polymeric transfection agents.[36,51]

In order to evaluate polymer-mediated transfection efficacy, luminescence (relative luminescence units or RLU) due to the expression of the luciferase protein was normalized

to the protein content in each well, and the normalized values were compared to those determined for pEI-25. A number of polymers demonstrated statistically significant higher transfection efficacies than pEI-25 in the absence of serum. In particular, the 1,4C-1,4Bis polymer, based on the monomers, 1,4-cyclohexanedimethanol diglycidyl ether (1,4C) and 1,4-bis(3-aminopropyl) piperazine (1,4Bis), demonstrated greatly (>80-fold) higher transfection efficacy than pEI-25 in the absence of serum. Other polymers demonstrated moderately higher (4−8-fold) or comparable transfection efficacies compared to that of pEI-25 in the absence of serum. For example, neopentyl glycol diglycidyl ether-1,4-bis(3-aminopropyl) piperazine (NPGDE-1,4Bis) and ethylene glycol diglycidyl ether-1,4-bis(3-aminopropyl) piperazine (EGDE-1,4Bis) polymers demonstrated approximately 8-fold higher efficacies than pEI-25. Clearly, polymers generated using 1,4-bis(3-aminopropyl) piperazine (1,4Bis) as the amine monomer resulted in candidates with higher gene transfection efficacies than pEI-25.

The transfection efficacy of the 1,4C-1,4Bis polymer was further evaluated as a function of polymer dose (i.e., polymer:pGL3 plasmid) in the absence of serum (Figure 3b). While the polymer demonstrated comparable transfection efficacies to that of pEI-25 at low polymer:plasmid ratios (1:1 and 5:1), transfection efficacies with higher polymer ratios (10:1, 20:1 and 25:1) were significantly higher than those for pEI-25. It is important to note that, in addition to the normalized ratios presented in Figure 3b, the actual protein expression values (i.e., RLU/mg) were the highest observed in the experiments (not shown) when the 1,4C-1,4Bis polymer was used, indicating that the polymer resulted in greater protein expression than pEI-25 under all conditions evaluated.

Next, a subset of polymers that demonstrated appreciable transfection efficacies in the absence of serum was employed to transfect PC3-PSMA cells in the presence of serum-containing media. In the presence of serum, the transfection efficacy dropped in all polymers evaluated. Nevertheless, the 1,4C-1,4Bis polymer demonstrated considerably higher efficacies (approximately 30-fold) compared to pEI-25 in the presence of serum (Figure 3c). However, the transfection efficacies of other polymers investigated were not statistically different compared to that of pEI-25. We did not evaluate these polymers at higher polymer:plasmid ratios in order to keep this ratio consistent with experiments carried out in the absence of serum. Nevertheless, the significantly higher efficacy of the 1,4C-1,4Bis polymer makes it an attractive candidate for further evaluation in vivo.

Transfections were also carried out in serum-containing media with murine osteoblasts in order to compare the efficacy of the 1,4C-1,4Bis polymer to that of pEI-25 in cells unrelated to human prostate cancer cells. Lower transfection efficacies were observed in the MC3T3 murine osteoblast cell line for both polymers (not shown) as compared to PC3-PSMA cells, reflecting the challenges of transfecting these cells with nonviral transfection agents.[66] However, as with the PC3-PSMA cells, the 1,4C-1,4Bis polymer was more effective (approximately 23-fold) than pEI-25 in transfecting these cells (Figure 3d), indicating that the polymer might be useful in transfecting different cell types.

Taken together, these results indicate that transfections with polymer leads selected from the DNA binding screen resulted in the identification of one polymer, 1,4C-1,4Bis, that possesses significantly higher transfection efficacies than the current standard pEI-25 (Figures 3a−c). In addition, a number of polymers demonstrated moderately higher or comparable transfection efficacies with respect to pEI-25 (Figures 3a and 3b). However, it is important to note that while DNA binding efficacy is indeed necessary for polymer-mediated gene delivery, DNA binding activity of polymers did not correlate with their transfection efficacies (Figure S1 in the Supporting Information).

Polymer and Polyplex Cytotoxicity. While cationic polymers are attractive agents for nonviral gene delivery, their toxicity limits their use both in vitro and in vivo. We compared the cytotoxicity of the 1,4C-1,4Bis polymer with that of pEI-25 with PC3-PSMA cells. Ethidium homodimer is a red-fluorescent dye that stains DNA in cells with compromised nuclei. It is important to note that the MTT assay consistently underreported PC3-PSMA cell death when compared to what was seen using visual microscopic observation and was therefore not employed for evaluating polymer cytotoxicity with these cells.

Figure 4 shows representative fluorescence microscopy images of polymer and polyplex induced cytotoxicity. As seen in Figures 4a and 4b, the 1,4C-1,4Bis polymer was less toxic to PC3-PSMA cells than pEI-25 at all concentrations (4−20 ng/μL) investigated. Note that a polymer concentration of 10 ng/μL (polymer:pGL3 plasmid 25:1) was used in the transfection experiments in Figure 3a. The higher efficacy of the 1,4C-1,4Bis polymer can be explained, in part, due to its lower cytotoxicity compared to pEI-25 under these conditions. While polymer-mediated cytotoxicity is indeed a limiting factor, we reasoned that a large number of amine groups, that play a role in polymer toxicity, are involved in complexation with plasmid DNA. As a result, we evaluated the cytotoxicity of the polymer:plasmid polyplexes in addition to the cytotoxicity due to the polymer alone. As seen in Figures 4c and 4d, pEI-25-based polyplexes demonstrate significantly higher cytotoxicities toward PC3-PSMA cells compared to polyplexes based on the 1,4C-1,4Bis polymer.

In order to determine the relative toxicities of the two polymers and their polyplexes, we quantified polymer and polyplex toxicity based on at least three different fluorescence microscopy images as described in the experimental section. As seen in Figure 4e, pEI-25 is significantly more cytotoxic when compared to the 1,4C-1,4Bis polymer at concentrations

(66) Kim, S.-W.; Ogawa, T.; Tabata, Y.; Nishimura, I. Efficacy and cytotoxicity of cationic-agent-mediated nonviral gene transfer into osteoblasts. *J. Biomed. Mater. Res. Part A* **2004**, *71A* (2), 308–315.

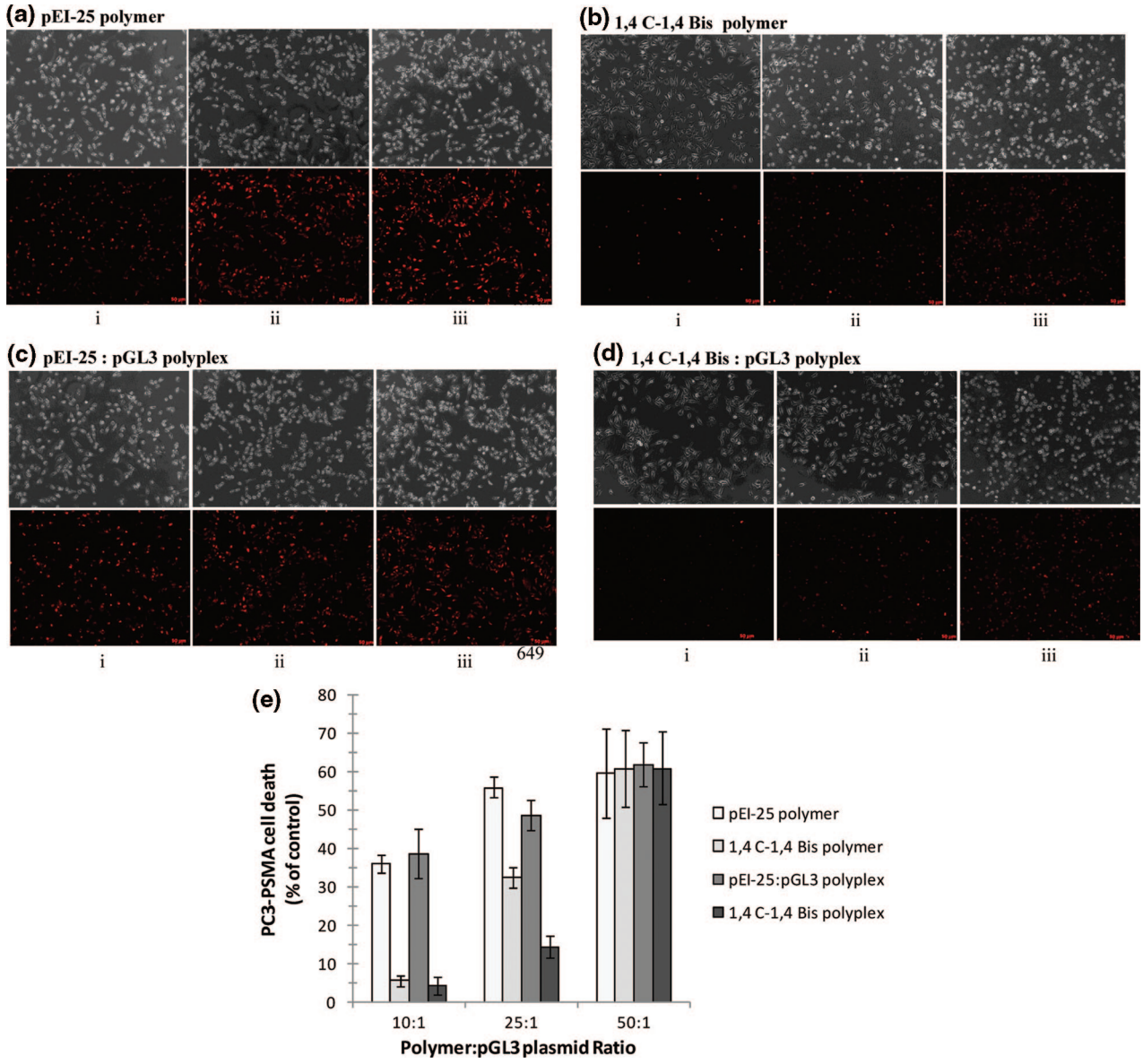

Figure 4. Phase contrast and fluorescence microscopy images that show the cytotoxicity of (a) pEI-25 polymer, (b) 1,4C-1,4Bis polymer, (c) pEI-25: pGL3 polyplex, and (d) 1,4C-1,4Bis:pGL3 polyplex toward PC3-PSMA cells in serum-free media. Red-fluorescent ethidium homodimer, that stains DNA in compromised nuclei, was used for determining cytotoxicity. In panels a,b: i, 4 ng/μL; ii, 10 ng/μL; and iii, 20 ng/μL polymer. In figures c,d: i, 10:1 polymer:plasmid (or 4 ng/μL polymer); ii, 25:1 polymer:plasmid (or 10 ng/μL polymer); iii, 50:1 polymer:plasmid (or 20 ng/μL polymer). The same amount of pGL3 plasmid (200 ng) was used in all polyplex-mediated cytotoxicity experiments. Representative images of at least three independent experiments and at least three different fields of view per independent experiment are shown in the figure. (e). Percentage of dead PC3-PSMA cells following polymer/polyplex treatment; details are described in the Experimental Section.

of 4 ng/μL and 10 ng/μL (equivalent polymer:pGL3 plasmid ratios of 10:1 and 25:1). However, both polymers demonstratred similar cytotoxicities at 20 ng/μL (equivalent polymer:pGL3 plasmid ratios of 50:1). Interestingly, 1,4C-1,4Bis polymer based polyplexes (polymer:pGL3 plasmid ratios of 10:1 and 25:1) demonstrated lower cytotoxicities when compared to the polymer alone. In contrast, pEI-25 based polyplexes showed comparable cytotoxicty compared to the polymer alone at all polymer:pGL3 plasmid ratios investigated. It is possible that lower concentrations of pEI-25 are required for complexation with DNA and the "excess" polymer is responsible for polymer cytotoxicity to cells. The low cytotoxicities of the 1,4C-1,4Bis polymer and its polyplex with pGL3 plasmid are in part responsible for the higher transfection efficacies of this polymer. Taken together, these results indicate that not only does the 1,4C-1,4Bis polymer demonstrate significantly higher efficacies for transfecting PC3-PSMA cells, but it also demonstrates lower cytotoxicities making it an attractive polymeric transfection agent.

Lead Polymer Characterization. Characterization experiments were carried out with the lead polymer (1,4C-1,4Bis)

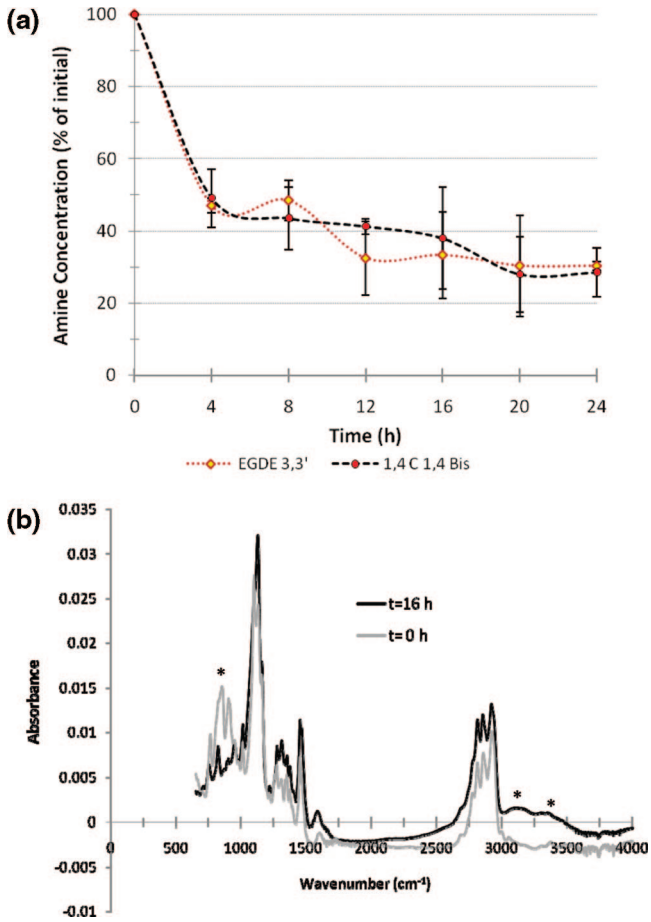

Figure 5. Polymer characterization. a. Polymerization kinetics of EGDE-3,3′ and 1,4C-1,4Bis polymer formation as reported by the disappearance of amines as a function of time. Formation of dark blue/purple color indicated the presence of primary amines. Primary amine concentration was determined at predetermined time points using the ninhydrin reagent and compared to the initial primary amine content. Polymerization kinetics of two representative polymer leads 1,4C-1,4Bis and EGDE-3,3′ used in transfection experiments are shown in the figure. b. Fourier transform infrared (FT-IR) spectroscopy of 1,4C-1,4Bis polymerization at time $t = 0$ h (monomer mixture) and $t = 16$ h following initiation of the polymer reaction. Polymer formation at 16 h can be seen from the disappearance of the epoxide peak in the 860−950 cm^{-1} region and from the emergence of the hydroxyl peak in the 3000−3500 cm^{-1} region.

that demonstrated successful cellular transfection. The polymer EGDE-3,3′ was also used in some cases in order to demonstrate the results for a different polymer used in transfections.

In order to ascertain that 16 h was indeed sufficient time for polymerization, samples of the reaction mixture were evaluated for the disappearance of amines as a function of time (Figure 5a) using a ninhydrin assay with glycine standards. The amine concentration remained largely invariant after 12 h of reaction time as determined using absorbance spectroscopy based on the formation of dark blue/purple color following reaction with primary amines. These results indicate that a reaction time of 16 h was appropriate for the generation of polymers that demonstrated moderate to high DNA binding and cellular transfection efficacies. In addition, the presence of residual primary amines (approximately 30% of the initial primary amine concentration, as reported by the dark blue color of the ninhdyrin reaction), indicated the formation of branched polymers with multiple terminal primary amines and not linear polymers. This is consistent with what can be expected from the employed reaction chemistry (Figure 1a).

Gel permeation chromatography was employed to determine molecular weight values of the 1,4C-1,4Bis polymer which were as follows: $M_n = 3.9$ kDa and $M_w = 23.5$ kDa indicating a polydispersity (PD) of 5.96. These values indicate that the polymer molecular weights are comparable to those of pEI-25 used in the study: $M_n = 10$ kDa, $M_w = 25$ kDa, PD = 2.5 (Sigma). Formation of the 1,4C-1,4Bis polymer was further verified using Fourier transform infrared (FT-IR) spectroscopy by following the appearance and disappearance of certain bands as a function of reaction time. We have carried out similar analyses for other polymers used in the transfection experiments, but only results obtained with the 1,4C-1,4Bis polymer that demonstrated high transfection efficacies are described in this section. The epoxide peak ranging from 858 to 918 cm^{-1} can be seen in the monomer mixture at time $t = 0$ h (Figure 5b) due to stretching and contraction of C−O bonds in the epoxide moiety.[67] However, this peak is significantly reduced after 16 h of reaction time, indicating a reduction in the epoxide content upon formation of the cationic polymer.[67] Characteristic spectral bands of primary amines were seen at 1100−1128 and 3358−3382 cm^{-1} due to the presence of C−N and N−H bonds, respectively. The broad bands from 3382−3402 cm^{-1} upon 16 h of reaction time can be attributed to hydroxyl (−OH) groups generated upon reaction of the epoxy rings with primary and secondary amines.[68] Taken together, FT-IR analysis further confirmed that the diglycidyl ether-polyamine reaction resulted in the formation of the 1,4C-1,4Bis polymer that demonstrated high transfection efficacies in vitro.

Conclusions

Parallel polymer synthesis techniques were employed for the rapid generation of a cationic polymer library based on addition polymerization between diglycidyl ethers and amines. Polymers were screened in parallel for their DNA-binding affinities using an ethidium bromide displacement assay. Parallel screening led to the identification of a number of polymer leads that demonstrated significant DNA-binding

(67) Loos, M. R.; Coelho, L. A. F.; Pezzin, S. H.; Amico, S. C. The effect of acetone addition on the properties of epoxy. *Polim.: Cienc. Tecnol.* **2008**, *18* (1), 76–80.
(68) Gosselin, M. A.; Guo, W.; Lee, R. J. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. *Bioconjugate Chem.* **2001**, *12* (6), 989–94.

properties. In vitro transfection experiments indicated that the 1,4C-1,4Bis polymer based on the monomers, 1,4-cyclohexanedimethanol diglycidyl ether (1,4C) and 1,4-bis(3-aminopropyl) piperazine (1,4Bis), resulted in significantly higher transfection efficacies, in both human prostate cancer cells and murine osteoblasts, compared to pEI-25, a current standard for polymer-mediated gene delivery. The 1,4C-1,4Bis polymer and resulting polyplexes were lower in cytotoxicity than pEI-25. We note that previous reports have employed parallel synthesis methods for the accelerated identification of polymeric transfection agents.[36,53,69−71] However, a direct comparison of our polymers with those used in previous studies is difficult due to differences in the cells or cell lines used, transfection protocols, and/or the plasmids that have been employed. We also note that, in addition to the polymer described above, our approach resulted in the identification of a number of other polymers that demonstrated moderately higher or comparable efficacies to pEI-25. Future work will involve the evaluation of the efficacy and biocompatibility of these polymers in vivo.

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Supporting Information Available: A figure comparing the DNA binding efficacy and transfection efficacy of lead polymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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(69) Akinc, A.; Anderson, D. G.; Lynn, D. M.; Langer, R. Synthesis of poly(beta-amino ester)s optimized for highly effective gene delivery. *Bioconjugate Chem.* **2003**, *14* (5), 979–88.

(70) Anderson, D. G.; Peng, W.; Akinc, A.; Hossain, N.; Kohn, A.; Padera, R.; Langer, R.; Sawicki, J. A. A polymer library approach to suicide gene therapy for cancer. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (45), 16028–33.

(71) Thomas, M.; Lu, J. J.; Zhang, C.; Chen, J.; Klibanov, A. M. Identification of novel superior polycationic vectors for gene delivery by high-throughput synthesis and screening of a combinatorial library. *Pharm. Res.* **2007**, *24* (8), 1564–71.