Degradable Poly(β-amino esters): Synthesis, Characterization, and Self-Assembly with Plasmid DNA

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Abstract: Poly(β-aminoesters) **1**−**3** were synthesized *via* the addition of N,N′-dimethylethylenediamine, piperazine, and 4,4′-trimethylenedipiperidine to 1,4-butanediol diacrylate. Polymerization proceeded exclusively *via* the conjugate addition of the secondary amines to the bis(acrylate ester). Polymers were isolated in up to 86% yields with molecular weights ranging up to 31 200 relative to polystyrene standards. The polymers degraded hydrolytically in acidic and alkaline media to yield 1,4-butanediol and β-amino acids **4a**−**6a** and the degradation kinetics were investigated at pH 5.1 and 7.4. In general, the polymers degraded more rapidly at pH 7.4 than at pH 5.1. In initial screening assays, both the polymers and their degradation products were determined to be noncytotoxic relative to poly(ethylene imine), a polymer conventionally employed as a synthetic transfection vector. Polymers **1**−**3** interacted electrostatically with polyanionic plasmid DNA in water and buffer at physiological pH, as determined by agarose gel electrophoresis, quasi-elastic dynamic light scattering (QELS), and ζ-potential measurements. All three polymers condensed DNA into soluble DNA/polymer particles on the order of 50−200 nm. Particles formed from polymers **1** and **2** aggregated extensively, while particles formed from polymer **3** exhibited positive ζ-potentials (e.g., +10 to +15 mV) and did not aggregate for up to 18 h. The nanometer-sized dimensions and reduced cytotoxicities of these DNA/polymer complexes suggest that these types of polymers may be useful as degradable polymeric gene transfer vectors.

Introduction

The treatment of human diseases through the application of nucleotide-based drugs such as DNA has the potential to revolutionize the medical field.[1] Thus far, the use of modified viruses as gene transfer vectors has generally represented the most clinically successful approach to gene therapy. While viral vectors are currently the most efficient gene transfer agents, concerns surrounding the overall safety of viral vectors, which include the potential for unsolicited immune responses, have resulted in parallel efforts to develop nonviral alternatives.[2] Current alternatives to viral vectors include polymeric delivery systems,[3,4] liposomal formulations,[5] and "naked" DNA injection protocols.[6] While these strategies have yet to achieve the clinical effectiveness of viral vectors, the potential safety, processing, and economic benefits offered by these methods[1a] have ignited interest in the continued development of nonviral approaches to gene therapy.[7−9]

Cationic polymers have been widely used as transfection vectors due to the facility with which they condense and protect negatively charged strands of DNA. Amine-containing polymers such as poly(lysine),[3,4] poly(ethylene imine) (PEI),[7] and poly(amidoamine) dendrimers[9] are positively charged at physiological pH, form ion pairs with DNA, and mediate transfection in a variety of cell lines. Despite their common use, however, cationic polymers such as poly(lysine) and PEI can be significantly cytotoxic.[3,5c,10] As a result, the choice of cationic polymer for a gene transfer application generally requires a tradeoff between transfection efficiency and short- or long-term cytotoxicity. Additionally, the long-term biocompatibility of these polymers remains an important issue for use in therapeutic applications *in vivo*, since several of these polymers are not readily biodegradable.[11] For use *in vivo*, new cationic polymers should be designed incorporating hydrolyzable moieties such that the polymers readily degrade into nontoxic byproducts.

To develop safe alternatives to existing polymeric vectors and other functionalized biomaterials, we[8a,12] and others[8b−c,13] have pursued the synthesis of degradable polyesters bearing

(1) (a) Anderson, W. F. *Nature* **1996**, *392 Suppl*. 25−30. (b) Friedman, T. *Nature Med*. *2* **1996**, 144−147. (c) Crystal, R. G. *Science* **1995**, *270*, 404−410. (d) Mulligan, R. C. *Science* **1993**, *260*, 926−932.

(2) For leading references, see: (a) Luo, D.; Saltzman, W. M. *Nat. Biotechnol*. **2000**, *18*, 33−37. (b) Behr, J.-P. *Acc. Chem. Res.* **1993**, *26*, 274−278.

(3) Zauner, W.; Ogris, M.; Wagner, E. *Adv. Drug Del. Rev.* **1998**, *30*, 97−113.

(4) Kabanov, A. V.; Kabanov, V. A. *Bioconjugate Chem.* **1995**, *6*, 7−20.

(5) (a) Miller, A. D. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 1768−1785. (b) Hope, M. J.; Mui, S.; Ansell, S.; Ahkong, Q. F. *Molec. Membr. Technol.* **1998**, *15*, 1−14. (c) Deshmukh, H. M.; Huang, L. *New J. Chem.* **1997**, *21*, 113−124.

(6) Sanford, J. C. *Trends Biotechnol*. **1988**, *6*, 288−302.

(7) Boussif, O.; Lezoualc'H., F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297−7301.

(8) (a) Putnam, D.; Langer, R. *Macromolecules* **1999**, *32*, 3658−3662. (b)Lim, Y.; Choi, Y. H.; Park, J.-S. *J. Am. Chem. Soc.* **1999**, *121*, 5633−5639. (c) Lim, Y.-B.; Kim, C.-H.; Kim, K.; Kim, S. W.; Park, J.-S. *J. Am. Chem. Soc.* **2000**, *122*, 6524−6525. (d) Gonzalez, H.; Hwang, S. J.; Davis, M. E. *Bioconjugate Chem.* **1999**, *10*, 1068−1074.

(9) (a) Kukowska-Latallo, J. F.; Bielinska, A. U.; Johnson, J.; Spindler, R.; Tomalia, D. A.; Baker, J. R., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4897−4902. (b) Tang, M. X.; Redemann, C. T.; Szoka, F. C., Jr. *Bioconjugate Chem.* **1996**, *7*, 703−714. (c) Haensler, J.; Szoka, F. C., Jr. *Bioconjugate Chem.* **1993**, *4*, 372−379.

(10) (a) Choksakulnimitr, S.; Masuda, S.; Tokuda, H.; Takakura, Y.; Hashida, M. J. *Controlled Release* **1995**, *34*, 233−241. (b) Brazeau, G. A.; Attia, S.; Poxon, S.; Hughes, J. A. *Pharm. Res.* **1998**, *15*, 680−684.

(11) (a) Uhrich, K. *Trends Polym. Sci.* **1997**, *5*, 388−393. (b) Roberts, J. C.; Bhalgat, M. K.; Zera, R. T. *J. Biomed. Mater. Res.* **1996**, *30*, 53−65.

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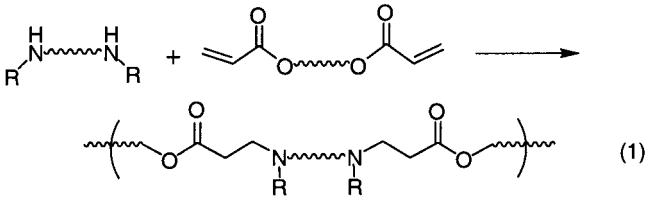
cationic side chains. Examples of such polymers include poly(L-lactide-*co*-L-lysine),[12a] poly(serine ester),[13] poly(4-hydroxy-L-proline ester),[8a,b] and, more recently, poly[α-(4-aminobutyl)-L-glycolic acid].[8c] Poly(4-hydroxy-L-proline ester) and poly[α-(4-aminobutyl)-L-glycolic acid] were recently demonstrated to condense plasmid DNA through electrostatic interactions and mediate gene transfer.[8a−c] Generally, these new polymers are less-toxic than poly(lysine) and PEI and they degrade into nontoxic metabolites.[8] It is clear from these investigations that the rational design of amine-containing polyesters can be a productive route to the development of safe, effective transfection vectors. Unfortunately, however, present syntheses of these polymers require either the independent preparation of specialized monomers[12a,8c] or the use of stoichiometric amounts of expensive coupling reagents.[8a] Additionally, the amine functionalities in the monomers must be protected prior to polymerization,[8,12] necessitating additional postpolymerization deprotection steps before the polymers can be used as transfection agents.

We sought to develop a complementary strategy for the synthesis of amine-containing polyesters that would address several of these synthetic drawbacks. While degradability, reduced cytotoxicity, and an ability to complex DNA remained fundamental design criteria, we also desired an approach that would yield a class of polymers that were structurally different from previously studied polymeric vectors so that structure/activity relationships could be further explored. To date, most cationic polymers synthesized and investigated for transfection efficacy have contained pendant amines in the polymer side chains.[3,4,14] Herein, we report a strategy for the preparation of poly(β-aminoesters) containing tertiary amines in their backbones based on the conjugate addition of bis(secondary amine) monomers to diacrylate esters (eq 1). These polymers interact electrostatically with plasmid DNA at physiological pH and assemble it into nanometer-scale polymer/DNA complexes. The polymers themselves are noncytotoxic and they degrade into nontoxic small molecule byproducts.



(1)

Results and Discussion

Polymer Synthesis and Characterization. The synthesis of linear poly(amido amines) containing tertiary amines in their backbones was reported by Ferruti *et al*. in 1970 by the addition of bifunctional amines to bisacrylamides.[15] Linear poly(amido amines) were initially investigated as heparin and ion complexing biomaterials.[15c−f] Dendritic poly(amido amines) (PAMAMs) have seen increasing use in gene transfer applications due to their ability to complex DNA,[9] and a recent report describes the application of a family of linear poly(amido amines) to cell transfection and cytotoxicity studies.[16] In contrast, analogous poly(ester amines) formed from the Michael-type addition of bifunctional amines to diacrylate esters have received less attention.[15,17]

The poly(ester amine) approach presents a particularly attractive basis for the development of new polymeric transfection vectors for several reasons: (1) the polymers contain the requisite amines and readily degradable linkages, (2) multiple analogues could potentially be synthesized directly from commercially available starting materials, and (3) if the resulting polymers were useful as DNA condensing agents, future generations of polymer could easily be engineered to possess amine pK_a values spanning the range of physiologically relevant pH. This last point was particularly intriguing, because the buffering capacity of polyamines has recently been implicated as a factor influencing the escape of DNA from cell endosomes following endocytosis.[7,9c,18] While complexation of DNA with cationic polymers is required to compact and protect DNA during early events in the transfection process, DNA and polymer must ultimately decomplex to allow efficient transcription.[2a] In view of this requirement, degradable polycations could play an important role in "vector unpackaging" events in the nucleus.[2a,19] Finally, we hypothesized that polymers of this general structure, and the β-amino acid derivatives into which they would presumably degrade, would be significantly less toxic than poly(lysine) and PEI. As outlined above, degradable polycations[8a−c] and linear polymers containing relatively hindered amines located close to the polymer backbone[8d] are less toxic than poly(lysine) and PEI.

We initially investigated the synthesis of polymers **1**−**3** *via* the addition of the bis(secondary amines) N,N′-dimethylethylenediamine, piperazine, and 4,4′-trimethylenedipiperidine to 1,4-butanediol diacrylate.[20] The polymerization of these monomers proceeded in THF and CH_2Cl_2 at 50 °C to yield the corresponding polymers in up to 86% yields (Table 1). Polymers were purified through repeated precipitation into diethyl ether or hexane. Polymer **1** was isolated as a clear viscous liquid; polymers **2** and **3** were obtained as white solids after drying under high vacuum. Alternatively, polymers **1**−**3** could be isolated as solid hydrochloride salts upon addition of diethyl ether/HCl to a solution of polymer in THF or CH_2Cl_2. All three polymers were soluble in organic solvents such as THF, CH_2Cl_2, $CHCl_3$, and MeOH and were also soluble in water at

(12) (a) Barrera, D. A.; Zylstra, E.; Lansbury, P. T., Jr.; Langer, R. *J. Am. Chem. Soc.* **1993**, *115*, 11010−11011. (b) Kwon, H. Y.; Langer, R. *Macromolecules* **1989**, *22*, 3250−3255.

(13) (a) Trollsås, M.; Lee, V. Y.; Mecerreyes, D.; Löwenhielm, P.; Möller, M.; Miller, R. D.; Hedrick, J. L. *Macromolecules* **2000**, *33*, 4619−4627. (b) Zhou, Q.; Kohn, J. *Macromolecules* **1990**, *23*, 3399−3406.

(14) van de Wetering, P.; Moret, E. E.; Schuurmans-Nieuwenbroek, N. M. E.; van Steenbergen, M. J.; Hennink, W. E. *Bioconjugate Chem.* **1999**, *10*, 589−597.

(15) (a) Danusso, F.; Ferruti, P. *Polymer* **1970**, *11*, 88−113. (b) Ferruti, P.; Marchisio, M. A.; Barbucci, R. *Polymer* **1985**, *26*, 1336. (c) Ferruti, P.; Barbucci, R. *Adv. Polym. Sci.* **1984**, *58*, 55−92. (d) Ferruti, P.; Ranucci, E.; Sartore, L.; Bignotti, F.; Marchisio, M. A.; Bianciardi, P.; Veronese, F. M. *Biomaterials* **1994**, *15*, 1235−1241. (e) Ferruti, P.; Ranucci, E.; Trotta, F.; Gianasi, E.; Evagorou, E. G.; Wasil, M.; Wilson, G.; Duncan, R. *Macromol. Chem. Phys.* **1999**, *200*, 1644−1654. (f) Ferruti, P.; Ranucci, E.; Sartore, L.; Bignotti, F.; Marchisio, M. A.; Bianciardi, P.; Veronese, F. M. *Biomaterials* **1994**, *15*, 1235−1241.

(16) Hill, I. R. C.; Garnett, M. C.; Bignotti, F.; Davis, S. S. *Biochim. Biophys. Acta* **1999**, *1427*, 161−174.

(17) (a) Kargina, O. V.; Mishustina, L. A.; Kiselev, V. Y.; Kabanov, V. A. *Vysokomol. Soedin. Ser. A* **1986**, *28*, 1139−1144. (b) Rao, W,; Smith, D. J. *J. Bioact. Compat. Polym.* **1999**, *14*, 54−63.

(18) (a) Behr, J.-P. *Chimia* **1997**, *51*, 34−36. (b) Demeneix, B. A.; Behr, J. P. In *Artificial Self-Assembling Systems for Gene Delivery*; Felgner, P. L., Heller, M. J.; Lehn, P.; Behr, J. P.; Szoka, F. C., Jr., Eds.; American Chemical Society: Washington, D. C., 1996; pp 146−151. (c) Kabanov, A. V.; Felgner, P. L.; Seymour, L. W. In *Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial*; John Wiley and Sons: New York, 1998.

(19) (a) Schaffer, D. V.; Fidelman, N. A.; Dan, N.; Lauffenburger, D. A. *Biotechnol. Bioeng.* **2000**, *67*, 598−606. (b) Kabanov, A. V. *Pharm. Sci. Technol. Today* **1999**, *2*, 365−372.

(20) The preparation of polymers **1** and **2** has been briefly described; however, the synthetic details and structural characterization were not reported (see refs 15a and 17a). A general investigation into the properties of these polymers does not appear in the literature to the best of our knowledge.

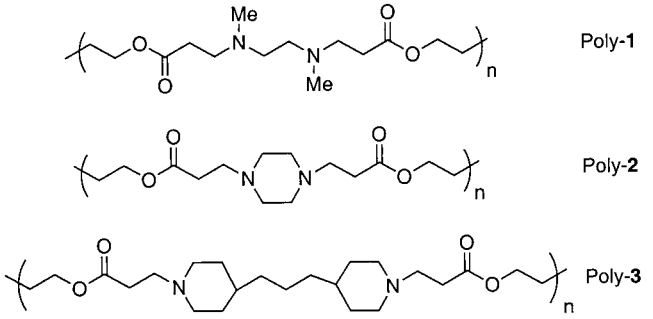
Table 1. Representative Molecular Weight Data for Polymers **1**−**3**

polymer	solvent	M_n[c]	PDI	yield, %
1[a]	THF			[d]
1[a]	CH_2Cl_2			82%[21]
2[a]	THF	10 000	1.77	64%
2[a]	CH_2Cl_2	17 500	2.15	75%
3[a]	THF	24 400	1.55	58%
3[a]	CH_2Cl_2	30 800	2.02	70%
1[b]	THF	5800	2.83	55%
2[b]	CH_2Cl_2	16 500	2.37	80%[e]
3[b]	CH_2Cl_2	31 200	2.55	86%[e]

[a] Conditions: [diamine] = [1,4-butanediol diacrylate] = 0.38 M, 50 °C, 48 h. [b] Conditions: [diamine] = [1,4-butanediol diacrylate] = 1.08 M, 50 °C, 48 h. [c] GPC analysis was performed in THF/0.1 M piperidine and molecular weights are reported versus polystyrene standards. [d] No polymer was isolated under these conditions. [e] The reaction solution became very viscous and eventually solidified under these conditions.

reduced pH. Polymer **1** and the hydrochloride salts of polymers **1**−**3** were freely soluble in water.

The molecular weights of polymers **1**−**3** and their corresponding hydrochloride salts were determined by both organic and aqueous phase gel permeation chromatography (GPC). Polymer molecular weights (M_n) ranged from up to 5800 for polymer **1** to up to 32 000 for polymer **3**, relative to polystyrene standards. Molecular weight distributions for these polymers were monomodal with polydispersity indices (PDIs) ranging



from 1.55 to 2.55. Representative molecular weight data are presented in Table 1. While the synthesis of linear poly(amido amines) is generally performed using alcohols or water as solvents,[15] we employed anhydrous THF and CH_2Cl_2 to minimize hydrolysis reactions during synthesis. The yields and molecular weights of polymers synthesized employing CH_2Cl_2 as solvent were generally higher than those of polymers synthesized in THF (Table 1).[21]

The structures of polymers **1**−**3** were confirmed by ^1H and ^{13}C NMR spectroscopy. These data indicate that the polymers were formed through the conjugate addition of the secondary amines to the acrylate moieties of 1,4-butanediol diacrylate and not through the formation of amide linkages under our reaction conditions. Additionally, the newly formed tertiary amines in the polymer backbones do not participate in subsequent addition reactions with diacrylate monomer, which would lead to branching or polymer cross-linking. This fortunate result appears to be unique to polymers of this type produced from bis-(secondary amine) monomers. In our hands, the synthesis of analogous polymers employing difunctional *primary* amines as monomers (such as 1,4-diaminobutane) generally leads to polymer branching and the formation of insoluble cross-linked polymer networks if conditions are not explicitly controlled.[22]

In view of the juxtaposition of amines and esters within the backbones of polymers **1**−**3**, we were initially concerned that hydrolysis might occur too rapidly for the polymers to be of practical use. For example, poly(4-hydroxy-L-proline ester) and poly[α-(4-aminobutyl)-L-glycolic acid] degrade quite rapidly near neutral pH, having half-lives of roughly 2 h[8b] and 30 min,[8c] respectively.[23] Analysis of polymers **1** and **2** by aqueous GPC using 1% acetic acid/water as eluent, however, revealed that degradation was sufficiently slow in acidic media. For example, the GPC traces of polymers **1** and **2** sampled under these conditions over a period of 4−5 h revealed no changes in molecular weights or polydispersities.[24] We were also concerned that significant backbone hydrolysis might occur during the isolation of the hydrochloride salts of polymers **1**−**3**. To prevent hydrolysis during the protonation and isolation of these polymers, anhydrous solvents were employed and reactions were performed under an argon atmosphere. Analysis of the polymers before and after protonation revealed no observable hydrolysis. For example, the GPC trace of a sample of polymer **3** after precipitation from CH_2Cl_2 with 1.0 M diethyl ether/HCl (M_n = 15 300; PDI = 1.90) was virtually identical to the molecular weight of the polymer prior to protonation (M_n = 15 700; PDI = 1.92) and no lower molecular weight species were evident.[25] Solid samples of polymers **1**−**3** could be stored for several months without detectable decreases in molecular weight.

Polymers **1**−**3** were specifically designed to degrade by hydrolysis of the ester bonds in the polymer backbones. However, an additional concern surrounding the overall stability and biocompatibility of these polymers is the potential for unwanted degradation to occur through retro-Michael reaction under physiological conditions. Because these polymers were synthesized via the Michael-type reaction of a secondary amine to an acrylate ester, it is possible that the polymers could undergo retro-Michael reaction to regenerate free acrylate groups, particularly under acidic conditions. Acrylate esters are potential DNA-alkylating agents and are therefore suspected carcinogens.[26] Because these polymers are expected to encounter the reduced pH environment within the endosomal vesicles of cells (pH = 5.0−5.5) during transfection, we addressed the potential for the degradation of these polymers to occur through a retro-Michael pathway.

Under extreme acidic (pH < 3) or basic (pH > 12) conditions, polymers **1**−**3** degraded rapidly and exclusively to 1,4-butanediol and the anticipated bis(β-amino acid) byproducts **4a**−**6a** as determined by ^1H NMR spectroscopy. We found no spectroscopic evidence for retro-Michael addition under these

(21) We were unable to synthesize polymer **1** in CH_2Cl_2. The color of the reaction solution progressed from colorless to an intense pink color almost immediately after the introduction of a solution of *N*,*N*′-dimethylethylenediamine in CH_2Cl_2 to a solution of 1,4-butanediol diacrylate in CH_2Cl_2 (see Experimental Section). The color progressed to light orange over the course of the reaction, and an orange polymer was isolated after precipitation in hexane. The isolated polymer was insoluble in CH_2Cl_2, THF, and water at reduced pH and was not structurally characterized. This problem was not encountered for the analogous reaction in THF.

(22) A recent report describes the synthesis of polyesters containing secondary amines in their backbones *via* the addition of difunctional primary amines to 1,4-butanediol diacrylate. The authors report the characterization of soluble, linear polymer. In our hands, however, we observe considerable cross-linking and the subsequent formation of insoluble polymer networks. For synthetic details and a description of the application of these polymers to the delivery of NO, see ref 17b.

(23) Such rapid degradation times did not preclude the application of these polymers to gene delivery (see refs 8b and 8c). However, extremely rapid degradation rates generally introduce additional concerns surrounding the manipulation, storage, and application of degradable polymers.

(24) Polymer **3** could not be analyzed by aqueous GPC.

(25) Comparative GPC data were collected employing THF/0.1 M piperidine as eluent (see Experimental Section). The HCl salts of the polymers were insoluble in THF, but were soluble in THF/0.1 M piperidine concomitant with the production of a fine white precipitate which was filtered prior to injection.

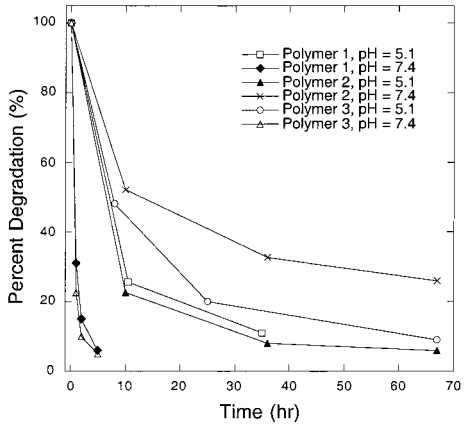


Figure 1. Degradation of polymers **1**−**3** at 37 °C at pH 5.1 and 7.4. Degradation is expressed as percent degradation over time based on GPC data.

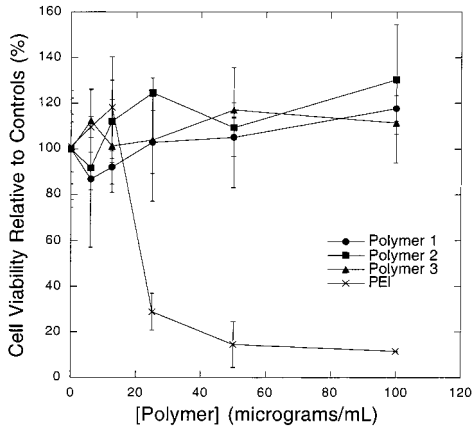
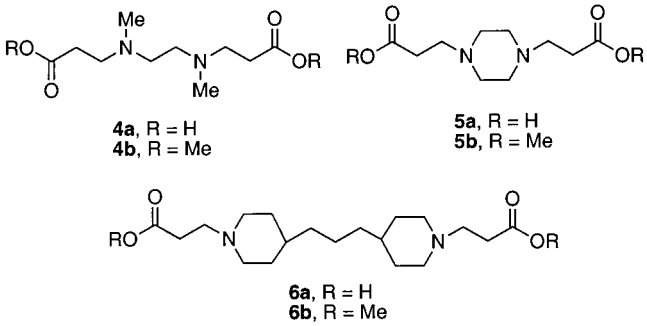


Figure 2. Cytotoxicity profiles of polymers **1**−**3** and PEI. Viability of NIH 3T3 cells is expressed as a function of polymer concentration. The molecular weights of polymers **1**, **2**, and **3** were 5800, 11 300, and 22 500, respectively. The molecular weight of the PEI employed was 25 000.

conditions. It is worth noting that bis(β-amino acid) degradation products **4a**−**6a** would be less likely to undergo a retro-Michael reaction, as acrylic acids are generally less activated Michael addition partners.[27] We did not observe further degradation of compounds **4a**−**6a** under these conditions.



The kinetics of polymer degradation were investigated under the range of conditions likely to be encountered by these polymers during transfection. Degradation was monitored at 37 °C at buffered pH values of 5.1 and 7.4 in order to approximate the pH of the environments within endosomal vesicles and the cytoplasm, respectively. The hydrochloride salts of polymers **1**−**3** were added to the appropriate buffer, incubated at 37 °C, and aliquots were removed at appropriate times. Aliquots were frozen immediately and lyophilized and polymer was extracted into THF/0.1M piperidine for analysis by GPC. Figure 1 shows the degradation profiles of polymers **1**−**3** as a function of time. In general, the polymers degraded more slowly at pH 5.1 than at pH 7.4. Polymers **1**−**3** displayed similar degradation profiles at pH 5.1, each polymer having a half-life of approximately 7−8 h. In contrast, polymers **1** and **3** were completely degraded in less than 5 h at pH 7.4. These results are consistent with the pH−degradation profiles of other amine-containing polyesters, such as poly(4-hydroxy-L-proline ester), in which pendant amine functionalities are hypothesized to act as intramolecular nucleophilic catalysts and contribute to more rapid degradation at higher pH.[8b,c] While we cannot rule out the possibility of intramolecular assistance, it is less likely for polymers **1**−**3** because the tertiary amines in these polymers should be less nucleophilic. The degradation of polymer **2** occurred more slowly at pH 7.4 than at pH 5.1 (Figure 1). This anomalous behavior is most likely due to the incomplete solubility of polymer **2** at pH 7.4 and the resulting heterogeneous nature of the degradation mileu.[28]

Cytotoxicity Assays. Poly(lysine) and PEI have been widely studied as DNA condensing agents and transfection vectors[2−4,7,18] and are the standards to which new polymeric vectors are often compared.[8] Unfortunately, as outlined above, these polymers are also associated with significant levels of cytotoxicity and high levels of gene expression are usually realized only at a substantial cost to cell viability. To determine the toxicity profile of polymers **1**−**3**, we conducted a MTT/thiazolyl blue dye reduction assay using the NIH 3T3 cell line and the hydrochloride salts of polymers **1**−**3** as initial indicators. The 3T3 cell line is commonly employed as a first level screening population for new transfection vectors, and the MTT assay is generally used as an initial indicator of cytotoxicity, as it determines the influences of added substances on cell growth and metabolism (Figure 2).[29]

Cells were incubated with polymer **1** (M_n = 5800), polymer **2** (M_n = 11 300), and polymer **3** (M_n = 22 500) as described in the Experimental Section. As shown in Figure 2, cells incubated with these polymers remained 100% viable relative to controls at concentrations of polymer up to 100 μg/mL. These results compare impressively to data obtained for cell populations treated with PEI ($M_n \approx$ 25 000), included as a positive control for our assay as well as to facilitate comparison to this well-known transfection agent. Fewer than 30% of cells treated with PEI remained viable at a polymer concentration of 25 μg/mL, and cell viability was as low as 10% at higher concentrations of PEI under otherwise identical conditions. We performed an analogous MTT assay using independently synthesized bis(β-amino acid)s **4a**−**6a** to screen the cytotoxicity of the hydrolytic degradation products of these polymers.[30] Compounds **4a**−**6a** and 1,4-butanediol did not perturb cell growth or metabolism

(26) For recent examples, see: (a) Schweikl, H.; Schmalz, G. *Mutat. Res.* **1999**, *438*, 71−78. (b) Yang, J.; Duerksen-Hughes, P. *Carcinogenesis* **1998**, *19*, 1117−1125.

(27) Perlmutter, P., in *Conjugate Addition Reactions in Organic Synthesis* Pergamon Press: New York, 1992.

(28) Polymers **2** and **3** are not completely soluble in water at pH 7.4. While polymer **3** dissolved relatively rapidly during the degradation experiment, solid particles of polymer **2** were visible for several days. We continue to investigate the degradation profiles of solid samples formed from polymers **2** and **3**.

(29) Hansen, M. B.; Neilson, S. E.; Berg, K. J. *Immunol. Methods* **1989**, *119*, 203-210.

(30) Bis(β-amino acid)s **4a**−**6a** should either be biologically inert or possess mild or acute toxicities which are lower than traditional polycationic transfection vectors. In either case, the degradation of these materials should facilitate rapid metabolic clearance.

in this initial screening assay (data not shown).[31] A more direct structure/function-based comparison between polymers **1**−**3** and PEI cannot be made due to differences in polymer structure and molecular weight, both of which contribute to polycation toxicity. Nonetheless, the excellent cytotoxicity profiles of polymers **1**−**3** alone suggested that they were interesting candidates for further study as DNA condensing agents.

Self-Assembly of Polymers 1−3 with Plasmid DNA. The tendency of cationic polyamines to interact electrostatically with the polyanionic backbone of DNA in aqueous solution is well-known. Provided that the polymers are sufficiently protonated at physiological pH, and that the amines are sterically accessible, such interactions can result in a self-assembly process in which the positively and negatively charged polymers form well-defined conjugates.[18c] The majority of polyamines investigated as DNA-complexing agents and transfection vectors have incorporated amines at the terminal ends of short, conformationally flexible side chains [e.g., poly(lysine) and methacrylate/methacrylamide polymers],[3,4,14] or accessible amines on the surfaces of spherical or globular polyamines (e.g., PEI and PAMAM dendrimers).[7,9] Because polymers **1**−**3** contain tertiary amines, and those tertiary amines are located in a sterically crowded environment (flanked on two sides by the polymer backbones), we were initially concerned that the protonated amines might not be sufficiently able to interact intimately with DNA.

The ability of polymers **1**−**3** to complex plasmid DNA was demonstrated through an agarose gel shift assay. Agarose gel electrophoresis separates macromolecules on the basis of both charge and size. Therefore, the immobilization of DNA on an agarose gel in the presence of increasing concentrations of a polycation has been widely used as an assay to determine the point at which complete DNA charge neutralization is achieved.[8] As mentioned above, the hydrochloride salts of polymers **1**−**3** are soluble in water. However, polymers **2** and **3** are not completely soluble at pH 7.2 over the full range of desired polymer concentrations. Therefore, DNA/polymer complexes were prepared in MES buffer (25 mM, pH = 6.0). DNA/polymer complexes were prepared by adding an aqueous solution of DNA to vortexing solutions of polymer in MES at desired DNA/polymer concentrations (see Experimental Section). The resulting DNA/polymer complexes remained soluble upon dilution in the electrophoresis running buffer (20 mM HEPES, pH = 7.2) and data were obtained at physiological pH. As a representative example, Figure 3 depicts the migration of plasmid DNA (pCMV−Luc) on an agarose gel in the presence of increasing concentrations of polymer **1**.

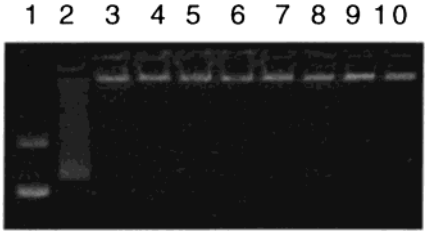


Figure 3. Agarose gel electrophoresis retardation of pCMV-Luc DNA by polymer **1**. Lane numbers correspond to different DNA/polymer weight ratios as follows: (1) 1:0 (DNA only), (2) 1:0.5, (3) 1:1, (4) 1:2, (5) 1:3, (6) 1:4, (7) 1:5. (8) 1:6, (9) 1:7, (10) 1:8.

As shown in Figure 3, retardation of DNA migration begins at DNA/**1** ratios as low as 1:0.5 (w/w) and migration is completely retarded at DNA/polymer ratios above 1:1.0 (w/w).[32] Polymers **2** and **3** completely inhibit the migration of plasmid DNA at DNA/polymer ratios (w/w) above 1:10 and 1:1.5, respectively (data not shown). These results vary markedly from gel retardation experiments conducted using model "monomers". Since the true monomers and the degradation products of polymers **1**−**3** do not adequately represent the repeat units of the polymers, we used bis(methyl ester)s **4b**−**6b** to examine the extent to which the polyvalency and cooperative binding of *poly*cations **1**−**3** is necessary to achieve DNA immobilization. "Monomers" **4b**−**6b** did not inhibit the migration of DNA at DNA/"monomer" ratios (w/w) of up to 1:30 (data not shown).

The reasons for the less-efficient complexation employing polymer **2** in the above gel electrophoresis assays most likely results from differences in the pK_a values of the amines in these polymers. The direct measurement of the pK_a values of polymers **1**−**3** is complicated by their degradability and solubility profiles. However, we predict the range of pK_a values of the amines in polymers **1** and **2** to extend from approximately 4.5 and 8.0 for polymer **1** and to 3.0 and 7.0 for polymer **2**, based on comparisons to structurally related poly(β-amino amides).[33] As a result, polymer **2** should be protonated to a lesser extent than polymer **1** at physiological or near-neutral pH and would therefore be a less effective DNA condensing agent. The range of pK_a values for polymer **3** should be higher than the range for polymers **1** and **2** due to the increased distance between the nitrogen atoms. Accordingly, polymer **3** forms complexes with DNA at substantially reduced concentrations relative to polymer **2**.

Agarose gel retardation assays are useful in determining the extent to which polycations interact with DNA. To be useful transfection agents, however, polycations must also be able to self-assemble plasmid DNA into polymer/DNA complexes small enough to enter a cell through endocytosis. For most cell types, this size requirement is on the order of 200 nm or less,[3] although larger particles can also be accommodated.[18b,c] The ability of polymers **1**−**3** to compact plasmid DNA into nanometer-sized structures was determined by quasi-elastic laser light scattering (QELS), and the relative surface charges of the resulting complexes were quantified through ζ-potential measurements. DNA/polymer particles used for particle sizing and ζ-potential measurements were formed as described above for agarose gel electrophoresis assays and diluted in 20 mM HEPES buffer (pH = 7.0) for analysis, as described in the Experimental Section.

Polymer **1** formed complexes with diameters ranging from 90 to 150 nm at DNA/polymer ratios above 1:2 (w/w), and polymer **2** condensed DNA into particles on the order of 60−125 nm at DNA/polymer ratios above 1:10. These results are consistent with the data obtained from agarose gel electrophoresis experiments above. However, the particles in these experiments aggregated over a period of hours to yield larger complexes with diameters in the range of 1−2 μm. The tendency of these particles to aggregate can be rationalized by the low

(31) It should be noted that the MTT assay is only a preliminary indicator of biocompatibility, and additional work must be done to more firmly establish the safety of these polymers.

(32) We report here DNA/polymer weight ratios rather than DNA/polymer charge ratios. Although both conventions are used in the literature, we find weight ratios to be more practical and universal, since the overall charge on a polyamine is subject to environmental variations in pH and temperature. While DNA/polymer charge ratios are descriptive for polymers such as poly(lysine), they are less meaningful for polymers such as PEI and **1**−**3**, which incorporate less basic amines.

(33) The pK_a values of structurally related poly(β-amino amides) containing piperazine and dimethylethylenediamine units in their backbones have been reported. (a) Barbucci, R.; Ferruti, P.; Micheloni, M.; Delfini, M.; Segre, A. L.; Conti, F. *Polymer* **1980**, *21*, 81−85. (b) Barbucci, R.; Ferruti, P.; Improta, C.; Delfini, M.; Segre, A. L.; Conti, F. *Polymer* **1978**, *19*, 1329−1334. (c) Barbucci, R.; Casolaro, M.; Ferruti, P.; Barone, V.; Lelj, F.; Oliva, L. *Macromolecules* **1981**, *14*, 1203−1209.

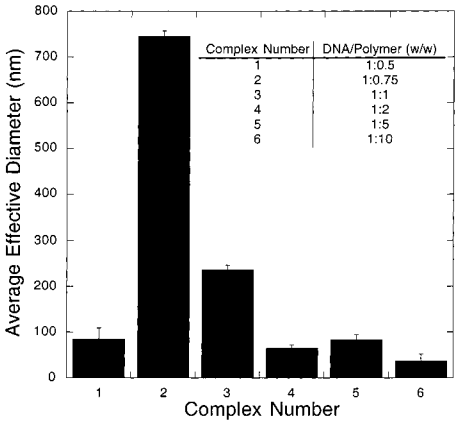


Figure 4. Average effective diameters of DNA/polymer complexes formed from pCMV-Luc plasmid and polymer **3** (M_n = 31 000) as a function of polymer concentration.

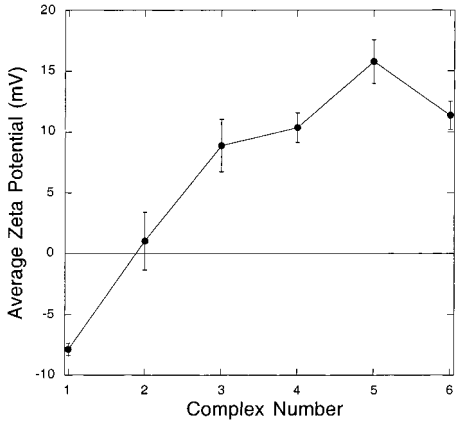


Figure 5. Average ζ-potentials of DNA/polymer complexes formed from pCMV-Luc plasmid and polymer **3** (M_n = 31 000) as a function of polymer concentration. The numbers for each complex correspond to the complex numbers in Figure 4.

ζ-potentials of the DNA/polymer particles observed under these conditions. For example, complexes formed from polymer **1** at DNA/polymer ratios above 1:10 had average ζ-potentials of +4.51 (±0.50) mV. The ζ-potentials of complexes formed from polymer **2** at DNA/polymer ratios above 1:20 were lower, reaching a limiting value of +1.04 (±0.57) mV. These differences correlate with the estimated pK_a values for these polymers, as the surfaces of particles formed from polymer **1** would be expected to be slightly more protonated than particles formed from polymer **2** at pH = 7.0.

Polymer **3** formed complexes with diameters in the range of 50−150 nm at DNA/polymer ratios above 1:2. As a representative example, Figure 4 shows the average effective diameters of particles formed with polymer **3** as a function of polymer concentration. The diameters of the particles varied within the above range from experiment to experiment under otherwise identical conditions, possibly due to subtle differences during the stirring or addition of DNA solutions during complex formation.[34] The ζ-potentials for complexes formed from polymer **3** were on the order of +10 to +15 mV at DNA/polymer ratios above 1:1, and the complexes did not aggregate extensively over an 18-h period (pH = 7, 25 °C). The positive ζ-potentials of these complexes may be significant beyond the context of particle stability, as net positive charges on particle surfaces may play a role in triggering endocytosis.[4,8c,18]

Particles formed from polymer **3** were also relatively stable at 37 °C. For example, a sample of DNA/**3** (DNA/**3** = 1:5, average diameter = 83 nm) was incubated at 37 °C for 4 h. After 4 h, a bimodal distribution was observed consisting of a fraction averaging 78 nm (>98% by number, 70 vol %) and a fraction of larger aggregates with average diameters of approximately 2.6 μm. These results suggest that the degradation of complexes formed from polymer **3** occurred more slowly than the degradation of polymer in solution or that partial degradation did not significantly affect the stability of the particles. The apparently increased stability of DNA/polymer complexes formed from degradable polycations relative to the degradation of the polymers in solution has also been observed for DNA/polymer complexes formed from poly(4-hydroxy-L-proline ester).[8b]

(34) The order of addition of polymer and DNA solutions had considerable impact on the nature of the resulting DNA/polymer complexes. To form small particles, for example, it was necessary to add the DNA solution to a vortexing solution of polymer. For cases in which polymer solutions were added to DNA, only large micrometer-sized aggregates were observed. Thus, it is possible that subtle differences in stirring or rate of addition could be responsible for variation in particle sizes.

The particle size and ζ-potential data in Figures 4 and 5 are consistent with models of DNA condensation observed with other polycations.[4,8] DNA is compacted into small negatively charged particles at very low polymer concentrations and particle sizes increase with increasing polymer concentration.[35] Complexes reach a maximum diameter as charge neutrality is achieved and aggregation occurs. Particle sizes decrease sharply at DNA/polymer concentrations above charge neutrality up to ratios at which additional polymer does not contribute to a reduction in particle diameter. This model is confirmed by ζ-potential measurements made on complexes formed from these polymers. As shown in Figure 5, the ζ-potentials of polymer/DNA particles formed from polymer **3** were negative at low polymer concentrations and charge neutrality was achieved near DNA/polymer ratios of 1:0.75, resulting in extensive aggregation. The ζ-potentials of the particles approached a limiting value ranging from +10 to +15 mV at DNA/polymer ratios above 1:2.

The average diameters of the complexes described above fall within the general size requirements for cellular endocytosis. We have initiated transfection experiments employing the NIH 3T3 cell line and the luciferase reporter gene (pCMV-Luc). Thus far, polymers **1** and **2** have shown no transfection activity in initial screening assays. By contrast, polymer **3** has demonstrated transfection efficiencies exceeding those of PEI under certain conditions.[36] These data suggest that polymers of this general

(35) Accurate light scattering data could not be obtained for DNA alone or for DNA/polymer associated species at DNA/polymer ratios lower than 1:0.5, since flexible, uncondensed DNA does not scatter light as extensively as compacted DNA (ref 18C).

(36) Transfection experiments were performed according to the following general protocol: Cells were grown in 6-well plates at an initial seeding density of 100 000 cells/well in 2 mL of growth medium. Cells were grown for 24 h after which the growth medium was removed and replaced with 2 mL of serum-free medium. DNA/polymer complexes were formed as described in the Experimental Section (2 μg of DNA, DNA/**3** = 1: 2 (w/w), 100 μL in MES (pH = 6.0)] and added to each well. DNA/PEI complexes were formed at a weight ratio of 1: 0.75, a ratio generally found in our laboratory to be optimal for PEI transfections. Transfections were carried out in serum-free medium for 4 h, after which medium was replaced with growth medium for 20 additional hours. Relative transfection efficiencies were determined by using luciferase (Promega) and cell protein assay (Pierce) kits. Results are expressed as relative light units (RLU) per milligram of total cell protein: for complexes of polymer **3**, 1.07 (±0.43) × 10^6 RLU/mg; for PEI complexes, 8.07 (±0.16) × 10^5 RLU/mg). No luciferase expression was detected for control experiments employing naked DNA or performed in the absence of DNA. These transfection data are the results of initial screening experiments, and a full report on the structure/activity relationships of polymers **1**−**3** under optimized conditions will be reported in due course.

structure hold promise as synthetic vectors for gene delivery and are interesting candidates for further study. The relative efficacy of polymer **3** relative to PEI is interesting, as our initial screening experiments were performed in the absence of chloroquine and polymer **3** does not currently incorporate an obvious means of facilitating endosomal escape. It should be noted, however, that the pK_a values of the amines in these polymers can be "tuned" to fall more directly within the range of physiologically relevant pH using this modular synthetic approach. Therefore, it will be possible to further engineer the "proton sponge" character[18] of these polymers, and thus enhance their transfection efficacies, directly through the incorporation of or copolymerization with different diamine monomers.

Summary

A general strategy for the preparation of new degradable polymeric DNA transfection vectors is reported. Poly(β-amino esters) **1**−**3** were synthesized *via* the conjugate addition of *N*,*N*′-dimethylethylenediamine, piperazine, and 4,4′-trimethylenedipiperidine to 1,4-butanediol diacrylate. The amines in the bis(secondary amine) monomers actively participate in bond-forming processes during polymerization, obviating the need for amine protection/deprotection processes which characterize the synthesis of other poly(amino esters). Accordingly, this approach should enable the generation of a variety of structurally diverse polyesters containing tertiary amines in their backbones in a single step from commercially available staring materials. Polymers **1**−**3** degraded hydrolytically in acidic and alkaline media to yield 1,4-butanediol and β-amino acids **4a**−**6a** and the degradation kinetics were investigated at pH 5.1 and 7.4. The polymers degraded more rapidly at pH 7.4 than at pH 5.1, consistent with the pH/degradation profiles reported for other poly(amino esters). An initial screening assay designed to determine the effects of polymers **1**−**3** on cell growth and metabolism suggested that these polymers and their hydrolytic degradation products were noncytotoxic relative to PEI, a nondegradable cationic polymer conventionally employed as a transfection vector.

Polymers **1**−**3** interacted electrostatically with plasmid DNA at physiological pH, initiating self-assembly processes that resulted in nanometer-scale DNA/polymer complexes. Agarose gel electrophoresis, quasi-elastic dynamic light scattering (QELS), and ζ-potential measurements were used to determine the extent of the interactions between the oppositely charged polyelectrolytes. All three polymers were found to condense DNA into soluble DNA/polymer particles on the order of 50−200 nm. Particles formed from polymers **1** and **2** aggregated extensively, while particles formed from polymer **3** exhibited positive ζ-potentials (e.g., +10 to +15 mV) and did not aggregate for up to 18 h. The nanometer-sized dimensions and reduced cytotoxicities of these DNA/polymer complexes suggest that polymers **1**−**3** may be useful as degradable polymeric gene transfection vectors. We are currently evaluating and optimizing the transfection activities of DNA/polymer complexes formed from these polymers and structurally related derivatives. A thorough understanding of structure/activity relationships existing for this class of polymer will expedite the design of safer polymer-based alternatives to viral transfection vectors for gene therapy.

Experimental Section

General Considerations. All manipulations involving live cells or sterile materials were performed in a laminar flow using standard sterile technique. ^{1}H NMR (300.100 MHz) and ^{13}C NMR (75.467 MHz) spectra were recorded on a Varian mercury spectrometer. All chemical shift values are given in parts per million and are referenced with respect to residual proton or carbon signal from solvent. Organic phase gel permeation chromatography (GPC) was performed using a Hewlett-Packard 1100 series isocratic pump, a Rheodyne model 7125 injector with a 100-μL injection loop, and two PL-Gel mixed-D columns in series (5 μm, 300 × 7.5 mm, Polymer Laboratories, Amherst, MA). THF/0.1M piperidine was used as the eluent at a flow rate of 1.0 mL/min. Data were collected using an Optilab DSP interferometric refractometer (Wyatt Technology, Santa Barbara, CA) and processed using the TriSEC GPC software package (Viscotek Corporation, Houston, TX). The molecular weights and polydispersities of the polymers are reported relative to monodisperse polystyrene standards. Aqueous phase GPC was performed by American Polymer Standards (Mentor, OH) using Ultrahydrogel L and 120A columns in series (Waters Corporation, Milford, MA). Water (1% acetic acid, 0.3 M NaCl) was used as the eluent at a flow rate of 1.0 mL/min. Data were collected using a Knauer differential refractometer and processed using an IBM/PC GPC-PRO 3.13 software package (Viscotek Corporation, Houston, TX). The molecular weights and polydispersities of the polymers are reported relative to poly(2-vinylpyridine) standards. For cytotoxicity assays, absorbance was measured using a Dynatech Laboratories MR5000 microplate reader at 560 nm.

Materials. *N*,*N*′-Dimethylethylenediamine, piperazine, and 4,4′-trimethylenedipiperidine were purchased from Aldrich Chemical Co. (Milwaukee, WI). 1,4-Butanediol diacrylate was purchased from Alfa Aesar Organics (Ward Hill, MA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO). Plasmid DNA (pCMV-Luc) was produced in *E. coli* (DH5α, a kind gift from Zycos, Inc., Cambridge, MA), isolated with a Qiagen kit, and purified by ethanol precipitation. NIH 3T3 cells were purchased from American Type Culture Collection (Manassas, VA) and grown at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium, 90%; fetal bovine serum, 10%; penicillin, 100 units/mL; streptomycin, 100 μg/mL. All other materials and solvents were used as received without further purification.

General Polymerization Procedure. In a typical experiment, 1,4-butanediol diacrylate (0.750 g, 0.714 mL, 3.78 mmol) and diamine (3.78 mmol) were weighed into two separate vials and dissolved in THF (5 mL). The solution containing the diamine was added to the diacrylate solution *via* pipet. A Teflon-coated stirbar was added, the vial was sealed with a Teflon-lined screw-cap, and the reaction was heated at 50 °C. After 48 h, the reaction was cooled to room temperature and dripped slowly into vigorously stirring diethyl ether or hexanes. Polymer was collected and dried under vacuum prior to analysis.

Synthesis of Polymer 1. Polymer **1** was prepared according to the general procedure outlined above. ^{1}H NMR δ ($CDCl_3$, 300 MHz) 4.11 (br t, 4H), 2.75 (br t, J = 7.05 Hz, 4 H), 2.53 (br s, 4H), 2.50 (br t, (obsc), J = 7.20 Hz, 4H), 2.28 (br s, 6 H), 1.71, (br m, 4 H). ^{13}C NMR δ ($CDCl_3$, 75.47 MHz) 172.55, 64.14, 55.31, 53.39, 42.47, 32.54, 25.53.

Synthesis of Polymer 2. Polymer **2** was prepared according to the general procedure outlined above. ^{1}H NMR δ ($CDCl_3$, 300 MHz) 4.11 (br t, 4 H), 2.74 (br t, J = 7.35, 4 H), 2.56 (br m, 12 H), 1.71 (br t, 4 H). ^{13}C NMR δ ($CDCl_3$, 75.47 MHz) 172.24, 64.19, 53.55, 52.75, 32.27, 25.52.

Synthesis of Polymer 3. Polymer **3** was prepared according to the general procedure outlined above. ^{1}H NMR δ ($CDCl_3$, 300 MHz) 4.11 (br t, 4 H), 3.00 (br m, 4 H), 2.79 (br m, 4 H), 2.65 (br m, 4 H), 2.11 (br m, 4 H), 1.70 (br m, 8 H), 1.25 (br m, 12 H). ^{13}C NMR δ ($CDCl_3$, 75.47 MHz) 172.37, 64.13, 53.89 (br), 36.74, 35.58, 32.11 (br), 25.45, 24.05.

Polymer Degradation Studies. The hydrochloride salts of polymers **1**−**3** were dissolved in acetate buffer (1 M, pH = 5.1) or HEPES buffer (1 M, pH = 7.4) at a concentration of 5 mg/mL (the use of millimolar concentrations of buffer resulted in substantial reduction of pH during degradation due to the production of acidic degradation products). Samples were incubated at 37 °C on a mechanical rotator and aliquots (1 mL) were removed at appropriate time intervals. Aliquots were frozen immediately in liquid nitrogen and lyophilized. Polymer was extracted from dried buffer salts using THF/0.1 M piperidine (1 mL) and samples were analyzed directly by GPC.

Formation of DNA/Polymer Complexes and Agarose Gel Retardation Assays. DNA/polymer complexes were formed by adding 50 μL of a plasmid DNA solution (pCMV Luc, 2 μg/50 μL in water) to a gently vortexing solution of the hydrochloride salt of polymers **1**−**3** (50 μL in 25 mM MES, pH = 6.0, concentrations adjusted to yield desired DNA/polymer weight ratios). The samples were incubated at room temperature for 30 min, after which 20 μL was run on a 1% agarose gel (HEPES, 20 mM, pH = 7.2, 65 V, 30 min). Samples were loaded on the gel with a loading buffer consisting of 10% Ficoll 400 (Amersham Pharmacia Biotech, Uppsala, Sweden) in HEPES (25 mM, pH = 7.2). Bromphenol blue was not included as a visual indicator in the loading buffer, since this charged dye appeared to interfere with the complexation of polymer and DNA. DNA bands were visualized by ethidium bromide staining.

Quasi-elastic Laser Light Scattering (QELS) and Measurement of ζ-Potentials. QELS experiments and ζ-potential measurements were made using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, NY, 15 mW laser, incident beam = 676 nm). DNA/polymer complexes were formed as described above for agarose gel retardation assays. Samples were diluted with 900 μL of HEPES (20 mM, pH = 7.0), added to a gently vortexing sample of DNA/polymer complex (total volume = 1 mL, pH = 7.0). Average particle sizes and ζ-potentials were determined at 25 °C. Correlation functions were collected at a scattering angle of 90°, and particle sizes were calculated using the MAS option of BIC's particle sizing software (version 2.30), using the viscosity and refractive index of pure water at 25 °C. Particle sizes are expressed as effective diameters assuming a log-normal distribution. Average electrophoretic mobilities were measured at 25 °C using BIC PALS zeta potential analysis software and zeta potentials were calculated using the Smoluchowsky model for aqueous suspensions. Three measurements were made on each sample and results are reported as average diameters and zeta potentials.

Cytotoxicity Assays. Immortalized NIH 3T3 cells were grown in 96-well plates at an initial seeding density of 10 000 cells/well in 200 μL growth medium (90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, penicillin 100 units/mL, streptomycin 100 μg/mL). Cells were grown for 24 h, after which the growth medium was removed and replaced with 180 μL of serum-free medium. Appropriate amounts of polymer were added in 20 μL aliquots. Samples were incubated at 37 °C for 5 h, and the metabolic activity of each well was determined by using a MTT/thiazolyl blue assay: to each well was added 25 μL of a 5 mg/mL solution of MTT stock solution in sterile PBS buffer. The samples were incubated at 37 °C for 2 h, and 100 μL of extraction buffer (20% w/v SDS in DMF/water (1:1), pH = 4.7) was added to each well. Samples were incubated at 37 °C for 24 h. Optical absorbance was measured at 560 nm with a microplate reader and expressed as a percent relative to control cells.

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