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Parallel Synthesis and Biophysical Characterization of a Degradable Polymer Library for Gene Delivery

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Abstract: We recently reported the parallel synthesis of 140 degradable poly(β-amino esters) via the conjugate addition of 20 primary or secondary amine monomers to seven different diacrylate monomers. To explore possible structure/function relationships and further characterize this class of materials, we investigated the ability of each DNA-complexing polymer to overcome important cellular barriers to gene transfer. The majority of vectors were found to be uptake-limited, but complexes formed from polymers **B14** and **G5** displayed high levels of internalization relative to "naked" DNA (18× and 32×, respectively). Effective diameter and zeta potential measurements indicated that, in general, small particle size and positive surface charge led to higher internalization rates. Of the 10 DNA/polymer complexes with the highest uptake levels, all had effective diameters less than 250 nm and nine had positive zeta potentials. Lysosomal trafficking was investigated by measuring the pH environment of delivered DNA. Complexes prepared with polymers **G5**, **G10**, **A13**, **B13**, **A14**, and **B14** were found to have near neutral pH measurements, suggesting that they were able to successfully avoid trafficking to acidic lysosomes. This work highlights the value of parallel synthesis and screening approaches for the discovery of new polymers for gene delivery and the elucidation of structure/function relationships for this important class of materials.

Introduction

The full promise of gene therapy has yet to be realized, due in large part to shortcomings associated with the safety and efficacy of gene delivery systems. The overwhelming majority of current gene therapy protocols employ viral delivery systems. However, significant concerns regarding the safety of viral vectors have been raised.[1,2] Nonviral delivery systems offer a potentially safer alternative, but are less effective because conventional formulations based on cationic lipids and synthetic polymers lack the functional sophistication necessary to overcome one or more of the intracellular barriers to efficient gene delivery. Advanced functional materials continue to evolve through the incorporation of new design elements into polymers, but progress is often made in small steps through the linear, iterative modification of existing materials.[3−8]

Cationic polymers have been investigated broadly in the context of gene delivery, primarily due to the facility with which they form conjugates with negatively charged strands of DNA, condensing it into nanometer-scale structures small enough to enter cells via endocytosis.[9] Although many polycations are effective at overcoming these early entry-based barriers to gene delivery, commonly used polymers such as polylysine and poly(ethylene imine) (PEI) are also associated with high degrees of cytotoxicity.[10,11] We and others have investigated the synthesis of biodegradable polycations for use as gene delivery agents, both from the standpoint of reducing the general toxicities of conventional materials and as a potential means through which to control or trigger the release of transfected DNA inside the cell. Recent examples of biodegradable polycations introduced in the context of gene delivery include poly(4-hydroxy-L-proline ester),[12,13] poly[α-(4-aminobutyl)-L-glycolic acid] (a polyester analogue of polylysine),[14] poly(2-aminoethyl propylene phosphate),[15,16] and degradable hyperbranched analogues of poly(amido amine) dendrimers.[3] As a class of materials, these biodegradable polycations are significantly less toxic than

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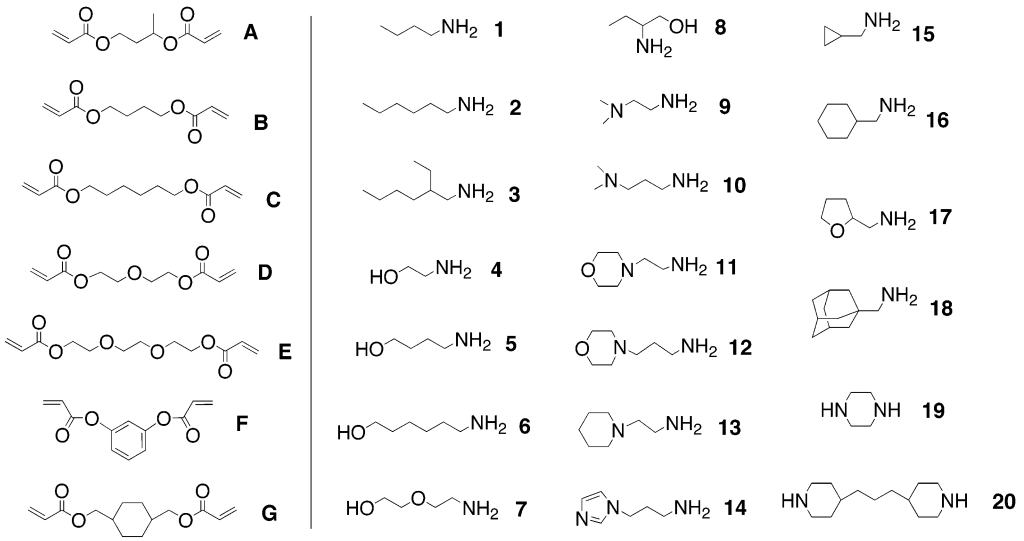
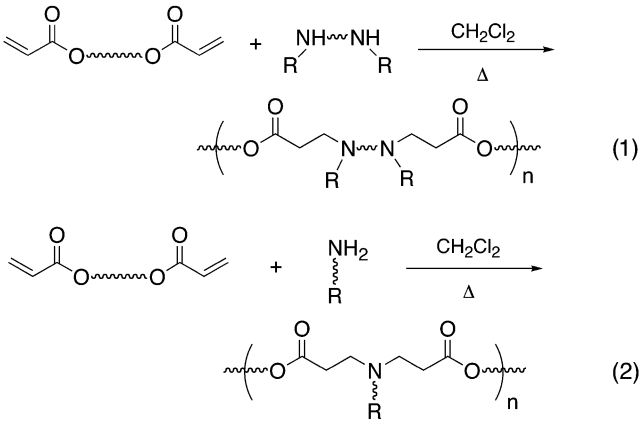


Figure 1. Diacrylate (**A**−**G**) and amine (**1**−**20**) monomers used in the synthesis of the poly(*β*-amino ester) library.

polylysine and PEI and mediate the transfer and expression of genes to cells at levels that approach[12−16] or exceed[3] those using PEI,[17−19] a polymer generally considered to be the current standard and the example to which new polymers are often compared. These results suggest that the design of biodegradable polycations represents a reasonable approach to the development of safe and effective polymeric vectors.

We previously described the synthesis of poly(*β*-amino ester)s, a class of biodegradable polymers having both tertiary amines and esters in their backbones, synthesized via the conjugate addition of amine-based monomers to diacrylate compounds (eq 1).[20] In a more recent paper, we reported the



extension of this work to the parallel synthesis of a 140-member library of structurally unique biodegradable materials and the identification of new gene delivery vectors through rapid cell-based screening assays.[21] This approach led to the discovery of two polymers, **B14** and **G5**, that mediated gene expression at levels 4−8 times higher than that of poly(ethylene imine) (PEI). In addition to providing a means for discovering new vectors for gene delivery, access to a library of structurally related polymers provides new opportunities to investigate structure/function relationships for a family of materials. Here, we analyze the resulting polymer/DNA complexes with respect to their size and surface charge, their ability to mediate cellular uptake, their ability to avoid trafficking to acidic lysosomes, and ultimately, their ability to mediate gene expression. Through this work we have identified several chemical features correlating with improved gene transfer properties.

Results and Discussion

Polymer Synthesis and Initial Screening. Polymers were synthesized as previously described.[21] Diacrylate monomers **A**−**G** and amine monomers **1**−**20** were selected for the synthesis of the polymer library (Figure 1) on the basis of representative degree of hydrophobicity and hydrophilicity and either structural or functional character. Aliphatic amines such as 1-aminohexane and functionalized amines such as 1-amino-6-hexanol reacted rapidly with butanediol diacrylate to produce linear polymer. Aromatic amines such as benzylamine were more sluggish, presumably due to their decreased nucleophilicity, and were not used in subsequent experiments. Likewise, the use of dimethacrylate monomers did not lead to the production of polymer under any of the reaction conditions used. Model reactions employing bis(acrylamide) monomers yielded the expected poly(*β*-amino amide)s, but these reactions required a different set of solvents (e.g., water) and reaction conditions and, therefore, were not considered further in this study. Although, in principle, this set of monomers could be expanded to produce libraries of polymers numbering into the thousands, the size of this library (7 diacrylates × 20 amines = 140 polymers) was limited to facilitate the manual manipulation and characterization of monomers and polymers in proof-of-concept experiments.

Methylene chloride was chosen as a general solvent for these reactions on the basis of the insolubility of certain monomers in THF and our earlier observation that the use of CH_2Cl_2 typically yields higher molecular weight polymer than reactions performed in THF. Model reactions also indicted that reaction

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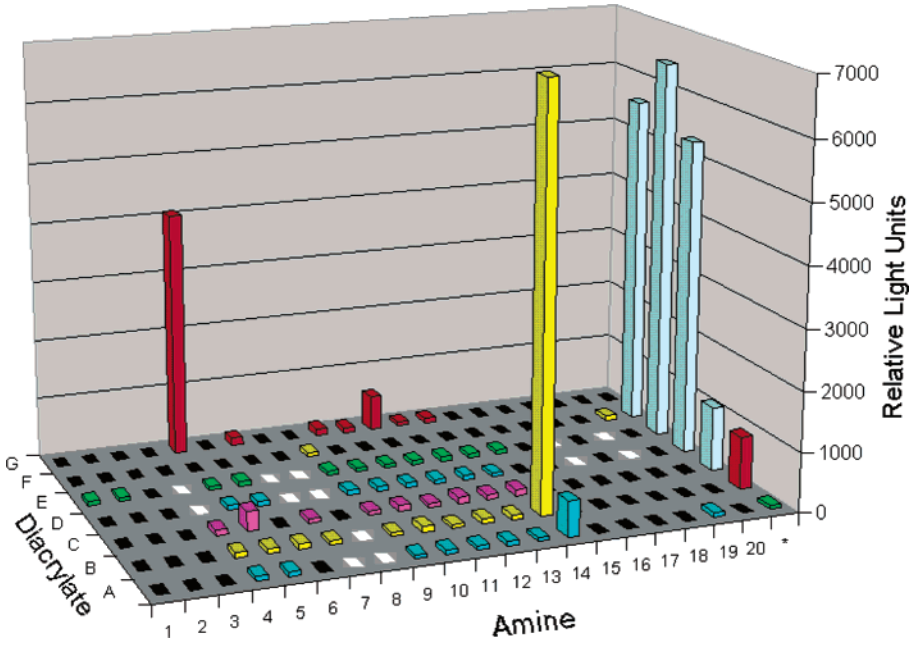

Figure 2. Transfection data as a function of structure for an assay employing pCMV-Luc (600 ng/well, DNA/polymer = 1:20). Light units are arbitrary and not normalized to total cell protein; experiments were performed in triplicate (error bars not shown). Black squares represent water-insoluble polymers; white squares represent water-soluble polymers that did not complex DNA. The right column (marked "*") displays values for the following control experiments: no polymer (green), PEI (red) (DNA/polymer = 1:1), and Lipofectamine 2000 (light blue) at 0.1, 0.2, 0.4, and 0.6 μL quantities.

times of 48 h at 45 °C were generally sufficient to yield polymers of reasonable molecular weight. However, we selected 5 days as a general reaction period for these parallel experiments to accommodate different (and unknown) reaction rates based on significant differences in monomer structures. Removal of solvent under high vacuum after 5 days yielded 600−800 mg of each product as a solid, a gummy material, or a viscous liquid. Seventy-seven samples (representing 55% of the polymer library) were analyzed by gel permeation chromatography, indicating molecular weights ranging from 1000 to 50 000, relative to polystyrene standards.[21]

It was not clear at the outset whether all 140 members of the screening library would be water-soluble or sufficiently able to form electrostatic complexes with plasmid DNA. We conducted a series of solubility and DNA-complexation assays to further characterize these polymers and identify the subset that would be useful in subsequent assays. Seventy polymers were sufficiently water-soluble at a concentration of 2 mg/mL (25 mM acetate buffer, pH = 5.0) to be screened for DNA-binding capacity using an agarose gel electrophoresis retardation assay. Fifty-six of the 70 water-soluble polymers were found to interact with DNA sufficiently at a ratio of 1:20 (w/w, DNA/polymer)[22] to retard the migration of DNA through an agarose gel; those polymers unable to interact sufficiently with DNA did not retard migration in this assay and were discarded from further consideration. The remaining 56 DNA-complexing polymers were screened for their abilities to transfect cells (Figure 2) and formed the basis for our investigations of polymer structure/function relationships.

Biophysical Characterization and Evaluation of Intracellular Barriers to Transfection. In addition to the identification of new materials for use in gene delivery systems, access to a library of novel structures affords the opportunity to test and evaluate vector design criteria. For efficient gene transfer and expression to occur, a vector must be capable of overcoming or avoiding several important intracellular barriers to transfection.[9,23] Two important requirements for efficient delivery are the ability to mediate cell entry (e.g., via endocytosis) and the ability to avoid intracellular trafficking to lysosomes, the main degradative compartment of the cell. Although the observation of gene expression in transfected cells is an important tool for evaluating new materials, it is merely an end-point result and provides no specific mechanistic information about the ability of a given material to overcome individual, intermediate barriers to transfection. For example, the transfection experiments above (Figure 2) clearly demonstrate that polymer/DNA complexes formed from polymers **B14** and **G5** are able to cross the cell membrane. However, the absence of observed expression using other polymers is not sufficient to conclude that these complexes are uptake-limited; that is, these polymers may mediate cellular uptake, but could ultimately be limited by downstream barriers such as lysosomal compartmentalization and degradation, the inability to localize to the nucleus, or the inability to release DNA once inside the nucleus.[4,9,24]

While it is generally straightforward to measure the cellular uptake of polymer/DNA complexes, it is more difficult to quantify the extent to which these complexes avoid trafficking to lysosomes. To address these and related mechanistic questions, we developed a fluorescence-based flow cytometry assay that can be used to quantify levels of cellular uptake of polymer/DNA complexes and the average pH environment of delivered

(22) We report here DNA/polymer weight ratios rather than DNA/polymer charge ratios or nitrogen/phosphate ratios. Although all three conventions are used in the literature, we find weight ratios to be more practical and definitive, 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 polylysine, they are less meaningful for the 140 different polymers described here which incorporate less basic amines.

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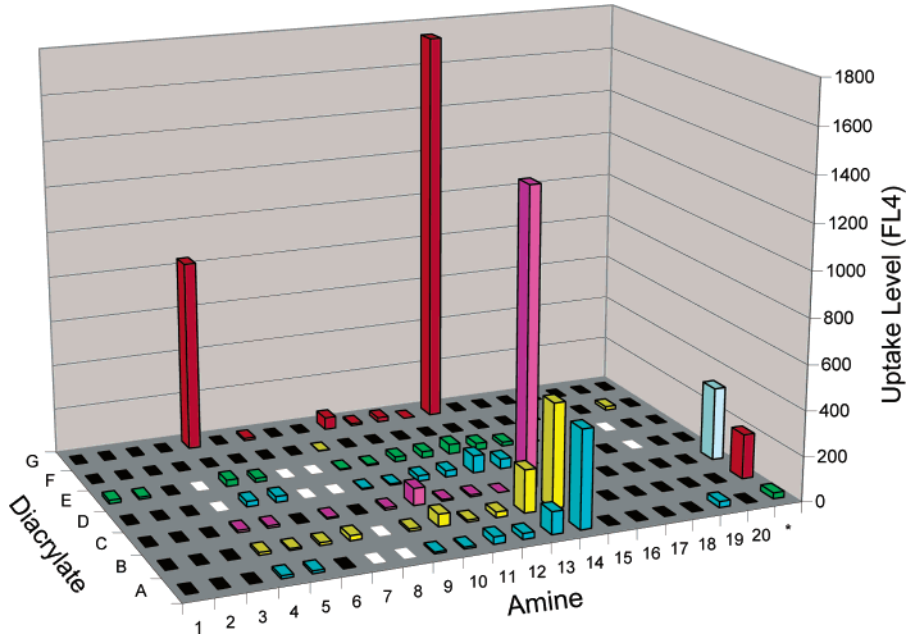

Figure 3. Cellular uptake data as a function of structure for an assay employing Cy5-labeled plasmid DNA (DNA/polymer = 1:20). Relative uptake level determined by median Cy5 fluorescence of cells (measured in FL4 channel of the flow cytometer); experiments were performed in duplicate (sample discrepancies were within 10%). Black squares represent water-insoluble polymers; white squares represent water-soluble polymers that did not complex DNA. The right column (marked "*") displays values for the following control experiments: no polymer (green), PLL (red) (DNA/polymer = 1:1), and PEI (light blue) (DNA/polymer = 1:0.75).

DNA.[25] By directly evaluating these processes, the impact of these barriers on gene transfer can be assessed individually. Such detailed information should yield a more complete biophysical understanding of these polymers in a cellular environment and could lead to the identification of new structural elements useful in the design of improved polymeric vectors. The following sections describe the application of these analytical techniques to the biophysical characterization of the 56 DNA-complexing members of the polymer library.

Analysis of Cellular Uptake. The ability of different polymers to mediate the cellular uptake of DNA was measured by adapting a fluorescence-based flow cytometry protocol used to measure the pH environment of vector-delivered DNA.[25] Polymer/DNA complexes were prepared using plasmid DNA covalently labeled with two fluorescent labels, fluorescein (pH sensitive) and Cy5 (pH insensitive). To permit the direct correlation of cellular uptake data with the gene expression data outlined above, complexes were formed at the same DNA/polymer ratios used in previous transfection experiments (e.g., 1:20 w/w). Double-labeling was used to permit simultaneous measurement of particle uptake and pH environment (as described below); however, only the signal corresponding to Cy5 emission was used for cellular uptake experiments. Labeled complexes were incubated with NIH 3T3 cells for 30 min at 37 °C to allow for uptake (see Experimental Section for details), and the relative intensity of Cy5 fluorescence due to particle uptake was quantified by flow cytometry. The results of these uptake experiments are shown in Figure 3.

The results in Figure 3 indicate that the majority of polymer/DNA particles prepared using the polymer library were uptake-limited, as only a small fraction of the polymer library was able to mediate high levels of cellular uptake under these conditions. As anticipated, polymers **B14** and **G5**, previously identified as "hits" in the transfection assay, had cellular uptake levels significantly higher (18× and 32×, respectively) than that of "naked" DNA. The relative cellular uptake of particles formed from polymer **A14** was equivalent to those measured using polymer **B14**, but levels of gene expression were consistently much lower in transfection experiments. This result suggests that particles formed from **A14** are not uptake-limited, but may be limited by downstream barriers to efficient transfection. Complexes prepared using polymers **C14** and **G14** demonstrated very high levels of uptake, 50× and 67× higher than "naked" DNA, respectively. The discrepancies between these high uptake levels and correspondingly low levels of gene expression could be related to downstream barriers to transfection, but are most likely linked to the apparent toxicity of these two polymers at the concentrations used in this assay.[21] Conversely, it is possible that the extremely high levels of uptake for particles prepared with these polymers could be responsible for the observed cytotoxicities, resulting in lower gene expression (as discussed below).

Effective Diameter and Zeta Potential Measurements. Particle size and surface charge both influence the internalization of polymer/DNA complexes by nonspecific endocytosis. Most cell types efficiently internalize particles less than 200 nm in size,[4,11] and a positive surface charge may lead to a greater interaction with the negatively charged cell membrane, triggering endocytosis.[26] We analyzed the size and surface charge of DNA/polymer complexes prepared using members of the polymer library to investigate the relationships between polymer structure and these important physical factors. Particle size was determined by measuring the effective diameter of complexes using dynamic light scattering, and surface charge was quantified by zeta potential analysis. For these experiments, complexes were formed under the same conditions used in uptake experiments

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Table 1. Effective Diameter (nm) of DNA/Polymer Complexes[a]

	diacrylates					
amine	A	B	C	D	E	G
1					1029	
2					175	
3						
4	350	561	150			
5	302	301	328	352	363	238
6		193			269	
7		271	205			276
8						
9	2471	1004	1583	1418	2033	
10	209	217	195	318	259	300
11	268	317	269	1308	351	185
12	237	298	278	510	425	271
13	176	130	134	168	164	159
14	159	223	111	224	255	114

[a] Complexes were formed at DNA/polymer ratios of 1:20, with DNA added dropwise to the polymer while gently vortexing the mixture. Table entries left blank represent water-insoluble polymers or polymers unable to complex DNA. Measurements were made in 10 mM HEPES buffer (pH = 7.2).

Table 2. Zeta Potential (mV) of Polymer/DNA Complexes[a]

	diacrylates					
amine	A	B	C	D	E	G
1					−9.9	
2					−26.7	
3						
4	−17.0	−17.4	−41.6			
5	−11.5	−19.3	−8.5	−14.0	−12.5	−3.5
6		−15.9			−17.2	
7		−12.9	−20.6			−11.2
8						
9	−6.0	−8.1	−4.4	−5.7	−7.9	
10	6.9	6.0	6.6	2.2	0.1	2.9
11	−10.3	−17.8	−11.6	−12.5	−8.3	−11.5
12	−17.2	−11.8	−12.7	−10.7	−8.3	−8.2
13	19.0	23.6	19.5	15.6	16.5	15.8
14	5.0	1.8	24.8	−2.9	−13.3	22.8

[a] Complexes were formed at DNA/polymer ratios of 1:20, with DNA added dropwise to the polymer while gently vortexing the mixture. Table entries left blank represent water-insoluble polymers or polymers unable to complex DNA. Measurements were made in 10 mM HEPES buffer (pH = 7.2).

with the exception that unlabeled plasmid DNA was used. As we determined that the presence of fluorescent label did not alter the effective diameters or zeta potentials of resulting complexes (data not shown), unlabeled plasmids were used to conserve labeled DNA.

The results of the particle size measurements and zeta potential analyses of the DNA-complexing members of the polymer library are shown in Tables 1 and 2. The vectors with the highest uptake levels, namely, **A**−**C14**, **G14**, **A13**, **B13**, and **G5**, all had measured effective diameters below 250 nm. Conversely, of the complexes with effective diameters greater than 1000 nm (**A**−**E9**, **E1**, and **D11**), none were internalized by cells at appreciable levels. The majority (∼70%) of the complexes in the library had negative zeta potentials at DNA/polymer ratios of 1:20 (w/w) (as measured in 10 mM HEPES buffer, pH = 7.2), and this could explain the poor levels of uptake and expression for these polymers. Complexes formed from the family of polymers synthesized from amine monomers **10**, **13**, and **14** had positive zeta potentials at pH 7.2 and correspond to the group of polymers mediating the highest levels of uptake (Figure 3). In these experiments, a DNA/polymer weight ratio of 1:20 corresponds to a polymer nitrogen/DNA phosphate (N/P) ratio of roughly 20:1 for polymers containing only one amine per repeat unit (taken as an average for the entire library). The N/P ratio for polymers containing two amines per repeat unit (e.g., polymers containing amine monomers **10** and **13**) is roughly 40:1 and helps explain the positive zeta potentials observed using these polymers. In general, the results above suggest that forming polymer/DNA complexes and screening at N/P ratios greater than 20:1 could increase the zeta potentials of these complexes, facilitate internalization, and lead to higher transfection efficiencies. The results of this current study address only those conditions used in initial transfection screening assays.

When the particle sizing and zeta potential measurements are taken together, a number of relationships emerge. For example, of the 10 polymer/DNA complexes with the highest levels of cellular uptake, all had measured effective diameters below 250 nm and all but one had positive zeta potentials (the notable exception being **G5**, with a zeta potential of −3.5 mV). Furthermore, the complexes combining the smallest effective diameters with the largest positive zeta potentials were **C14** and **G14**, the two vectors resulting in the highest levels of cellular uptake. The unusually high level of uptake exhibited by polymer **G5** is difficult to rationalize based solely on particle size and zeta potential measurements, and we continue to investigate the mechanistic aspects of transfection using this polymer.

Analysis of pH Environment. Because DNA is rapidly degraded by lysosomal enzymes, an efficient gene delivery system must be able to avoid the lysosomal trafficking pathway. Design elements generally incorporated into synthetic polymers to overcome this barrier include the incorporation of fusogenic peptides[4,27,28] and the use of pH-buffering "proton sponge" polymers[29] that are thought to promote the osmotic swelling and physical rupture of endosomes and the subsequent release of the vector into the cytoplasm. We,[25] and recently another group,[30] have employed the general flow cytometry-based approach outlined above to investigate the ability of different polymers to overcome the lysosomal barrier to efficient gene transfer. The details of this protocol have been previously described and are similar to the protocol used above for analysis of cellular uptake.[25] Briefly, the formation of polymer/DNA particles using plasmid DNA labeled with both fluorescein (pH sensitive) and Cy5 (pH insensitive) allows the local pH environment of transfected DNA to be determined as a linear function of pH (in the range of pH from 4 to 8), simply by measuring the emission ratios of these two fluorophores. The fluorescence emission ratios of NIH 3T3 cells incubated with labeled polymer/DNA complexes were measured using a flow cytometer and were used to calculate the average pH environment of the delivered DNA. The results of these experiments are displayed in Figure 4.

In this assay, particles unable to avoid lysosomes typically yield average pH measurements less than 5.0, while vectors able to escape the lysosomal pathway yield higher average pH measurements. For example, the local pH environment of DNA

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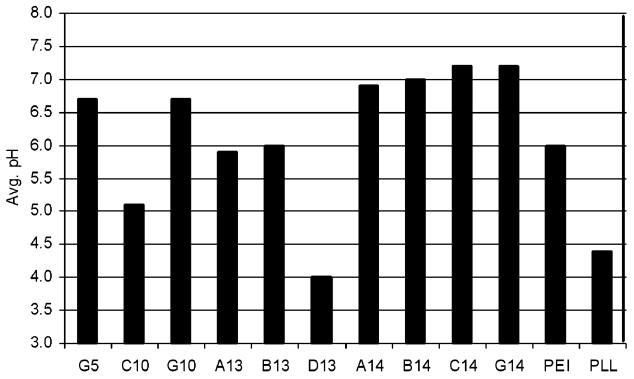

Figure 4. Measured average pH environment of DNA delivered via polymer/DNA complexes formed from members of polymer library (DNA/polymer = 1:20) and the controls PEI (DNA/polymer = 1:0.75) and PLL (DNA/polymer = 1:1). Measurements were performed in duplicate (sample discrepancies were within 0.2 pH units).

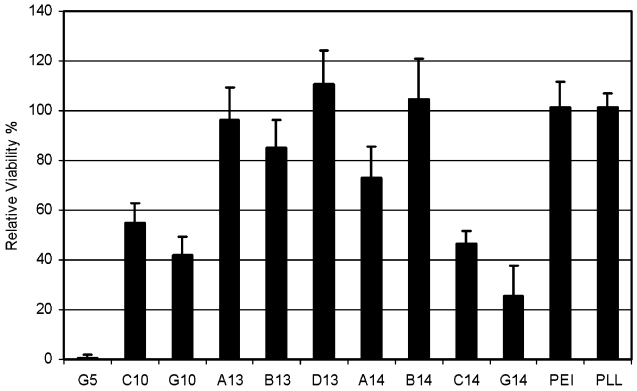

Figure 5. Relative viability of cells treated with DNA/polymer complexes formed from members of polymer library (DNA/polymer = 1:20), PEI (DNA/polymer = 1:0.75), and PLL (DNA/polymer = 1:1). Viabilities were determined relative to untreated control cells. Measurements were performed in triplicate (error bars represent standard deviation).

delivered using PEI, the prototypical "proton sponge" polymer, was measured to be 6.0, while that of DNA delivered using polylysine (PLL), a polymer with no known means for escaping lysosomes, was measured to be 4.4. While not a direct measure of compartmental location, the average pH experienced by delivered DNA is a reasonable indicator of the extent to which vectors have avoided or escaped acidic lysosomes (typical pH = 4.0−5.0).

Measurements of pH environment were made for the 10 complexes with the highest levels of cellular uptake. Of those complexes, those with the highest levels of transfection efficiency (**G5**, **B14**, **A14**, and **G10**) all had measured average pH values above 6.5, suggesting that they were able to successfully avoid trafficking to acidic lysosomes (Figure 4). The results surpassed that of even PEI, used in this experiment as a positive control (measured pH value after internalization = 6.0). We[10,31] and others[32] have previously incorporated imidazoles into polymers as pH-buffering moieties to aid in the escape of vectors from the lysosomal trafficking pathway. The high measured pH values for vectors containing monomer **14** support the hypothesis that the imidazole group ($pK_a \approx 6.2$) acts as a "proton sponge" in these polymers. It should be noted that the pH values measured for **C14** and **G14** (pH = 7.2 for both) could also be due to membrane permeabilization and subsequent pH equilibration with surrounding media resulting from the associated cytotoxicities of these materials. The near-neutral pH environment experienced by DNA complexed to polymer **G5** is surprising in this context, as this material does not incorporate an obvious means of facilitating endosomal escape. Measured pH values for DNA delivered via complexes **C10** (pH = 5.1) and **D13** (pH = 4.0) were consistent with lysosomal pH, suggesting that these vectors were unable to avoid trafficking to lysosomes. Polylysine, a polymer with no known means for escaping the lysosomal trafficking pathway, was used as a negative control in the experiment and resulted in a pH measurement of 4.4.

Analysis of Cytotoxicity. We previously assessed the degradability and cytotoxicity of three different poly(β-amino esters).[20] In general, these polymers are noncytotoxic, and cells remained 100% viable relative to control cells even at concentrations as high as 100 μg/mL. In contrast, for cells treated with PEI, less than 30% were viable at a polymer concentration of 25 μg/mL and less than 10% were viable at a polymer concentration of 100 μg/mL. During the evaluation of transfection and cellular uptake, however, we observed that the toxicity of several members of the polymer library seemed to increase upon the formation of complexes with DNA. We therefore evaluated the cytotoxicity profiles of the polymer/DNA complexes exhibiting the highest levels of cellular uptake using a standard MTT/thiazolyl blue dye reduction assay. Cells were incubated with complexes for 1 h, after which time complexes were removed and fresh growth media was added. The MTT assay was performed 24 h later, as described in the Experimental Section.

The results shown in Figure 5 suggest that complexes prepared with polymers synthesized using monomers **C** and **G** (corresponding to some of the more hydrophobic polymers in the library) are the most cytotoxic. The most notable example is **G5**, which appears to kill nearly all cells under the conditions employed. By contrast, complexes prepared with polymers synthesized using monomers **A**, **B**, and **D** appear to be far less toxic. The control polymers PEI and PLL were not toxic when complexed with DNA under the conditions used in this experiment. Although both polymers **G5** and **B14** resulted in high levels of transfection, **B14** appears to be the more promising polymer for gene delivery, based on their greatly differing levels of toxicity.

Summary

We have described the parallel synthesis and biophysical characterization of a 140-member degradable poly(β-amino ester) screening library. In vitro transfection screening of this library led to the identification of two polymers, **B14** and **G5**, with transfection levels 4−8 times higher than that of PEI and levels rivaling those of a leading commercially available lipid-based reagent. In addition to providing a platform for the discovery of novel gene transfer vectors, a library of structurally diverse polymers affords the opportunity to correlate changes in polymer structure with differences in polymer function. The relatively small size of the current proof-of-concept library, combined with the diverse range of polymer molecular weights produced, currently prevents the assignment of definitive structure/function relationships. However, we have identified

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several structural features that correlate with improved physical and gene transfer properties. From the standpoint of DNA complexation, for example, we have found that polymers containing structural elements in which an oxygen atom is two carbons removed from an amine (e.g., polymers containing amine monomers **4**, **7**, or **8**) are generally unable to form intimate electrostatic complexes with DNA. Characterization of the effective diameters and zeta potentials of polymer/DNA complexes suggests that, in general, small particle sizes and positive surface charges lead to higher levels of cellular uptake in vitro and that polymers with multiple amines per repeat unit tend to yield complexes with smaller particle sizes and larger zeta potential. Evaluation of the pH environment of delivered DNA suggested that polymers incorporating an imidazole group or two amines in close proximity could successfully avoid low pH lysosomes. Finally, measurement of the cytotoxicity of a subset of readily internalized polymer/DNA complexes implies that polymers synthesized using monomers **C** and **G** may be more toxic relative to their less hydrophobic counterparts **A**, **B**, and **D**.

The further elucidation of structure/function relationships will likely require the production of larger, more diverse libraries as well as greater control over polymer molecular weight. We recently synthesized a larger library consisting of several thousand unique structures, and initial transfection screening of this library has led to the identification of additional relationships, including the observation that the efficacy of polymer **G5** in this study appears to extend to several other polymers having different alcohol-terminated side chains.[33] More work will be required to understand the role of specific functional elements with respect to the gene transfer properties of these and other cationic polymers. We believe that this parallel approach to the synthesis, discovery, and evaluation of libraries of cationic polymers and related materials will lead to an accelerated and improved understanding of the factors that currently limit nonviral gene delivery.

Experimental Section

Materials. Polymers were synthesized as previously described.[21] Hyperbranched PEI (M_n = 25 000) and PLL (M_n 34 000) were purchased from Sigma-Aldrich (St. Louis, MO). Plasmid DNA containing the firefly luciferase reporter gene (pCMV-Luc) was purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA) and used without further purification. COS-7 cells used in transfection assays and NIH 3T3 cells used in uptake and pH measurement experiments 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. Luciferase detection kits used in high-throughput transfection assays were purchased from Tropix (Bedford, MA). All other materials and solvents were used as received without additional purification.

General Protocol for Cell Transfection Assays. Transfection assays were performed in triplicate in the following general manner. COS-7 cells were grown in 96-well plates at an initial seeding density of 15 000 cells/well in 200 μL of phenol red-free 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 in an incubator, after which the growth medium was removed and replaced with 200 μL of OPTI-MEM medium (Invitrogen Corp., Carlsbad, CA) supplemented with HEPES (total concentration = 25 mM). Polymer/DNA complexes prepared from the 56 water-soluble/DNA-complexing polymers were prepared at a ratio of 1:20 (w/w) using a commercially available plasmid containing the firefly luciferase reporter gene (pCMV-Luc) in the following manner.

Polymers were dissolved in 25 mM acetate buffer (pH = 5.0) to yield a final concentration of 2 mg/mL and were arrayed into a 96-well cell culture plate. DNA/polymer complexes were formed at a ratio of 1:20 (w/w) by transferring 40 μL of each polymer solution from the stock plate to a new plate using a multichannel pipettor. Each polymer was further diluted with 60 μL of acetate buffer (25 mM, pH = 5.0, total volume = 100 μL), and the plate was shaken for 30 s on a mechanical shaker. An aqueous solution of plasmid DNA (100 μL of a 0.04 μg/μL solution) was added to each well in the plate, and the solutions were vigorously mixed using a multichannel pipettor and a mechanical shaker. DNA/polymer complexes were incubated at room temperature for 30 min, after which 30 μL of each sample was added to the cells using a multichannel pipettor (600 ng DNA/well).

Controls employing poly(ethylene imine) (PEI) and polylysine (PLL), prepared at DNA/polymer ratios of 1:1 (w/w), were prepared in a manner similar to that above and included with DNA and no-DNA controls. Controls employing Lipofectamine 2000 (Invitrogen Corp.) were performed at several concentrations (0.1, 0.2, 0.4, and 0.6 μL), as described in the technical manual for this product (http://lifetechnologies.com). Cells were incubated for 4 h, after which the serum-free growth medium was removed and replaced with 100 μL of phenol red-free growth medium. Cells were incubated for an additional period of time (typically varied between 36 and 60 h), and luciferase expression was determined using a commercially available assay kit (Tropix, Inc., Bedford, MA). Luminescence was quantified in white, solid-bottom polypropylene 96-well plates using a 96-well bioluminescence plate reader. Luminescence was expressed in relative light units and was not normalized to total cell protein in this assay.

Cellular Uptake Experiments. NIH 3T3 cells were seeded on six-well plates at a concentration of 4×10^5 cells/well and grown for 24 h, after which the growth medium in each well was removed and replaced with 0.5 mL of OPTI-MEM. DNA/polymer complexes employing fluorescein-Cy5 double-labeled plasmid[25] were prepared at a DNA/polymer ratio of 1:20 (w/w) in the following manner. Polymers were dissolved in 25 mM acetate buffer (pH = 5.0) to yield a concentration of 2 mg/mL. A 50 μL sample of each polymer solution was pipetted into polypropylene microcentrifuge tubes. While gently vortexing, 50 μL of an aqueous solution of double-labeled DNA (100 μg/mL in HEPES buffer, pH = 7.2) was added dropwise to each microcentrifuge tubes containing polymer solution (100 μL total volume). Controls employing PEI and PLL were prepared in a similar fashion except that they were dissolved in acetate buffer at concentrations of 75 and 100 μg/mL, respectively (resulting in DNA/polymer ratios of 1:0.75 (w/w) and 1:1 (w/w)). All complexes were incubated at room temperature for 30 min, after which they were added to the wells at a concentration of 5 μg of DNA/well and placed in a 37 °C incubator for 30 min to allow for uptake. The loading medium was then removed and washed twice with PBS. Fresh growth medium was then added (2 mL/well) and the plates were returned to the 37 °C incubator to allow for uptake of any remaining surface-bound complexes. After 2 h, cells from each of the wells were harvested using trypsin and pelleted in separate eppendorf vials. We found that with the NIH 3T3 cell line this procedure was sufficient to eliminate surface-bound complexes, but a more rigorous washing or stripping step may be required for other cell lines. Cells were washed twice and finally resuspended in PBS containing 2% fetal bovine serum. Each cell sample was analyzed by flow cytometry to determine the level of Cy5 fluorescence.

Particle Sizing and Zeta Potential Measurements. Particle sizing experiments and zeta potential measurements were made using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, NY, 15 mW laser, incident beam = 676 nm).

(33) Anderson, D. G. Personal communication, 2003.

DNA/polymer complexes were prepared as described above, and each sample was diluted in 1.4 mL of HEPES buffer (10 mM, pH = 7.2). 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 (ver. 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.

Measurement of pH Environment of Delivered DNA. NIH 3T3 cells were seeded on T75 flasks at a concentration of 3×10^6 cells per flask and grown for 24 h at 37 °C. The growth medium was then replaced with 3 mL of OPTI-MEM per flask. DNA/polymer complexes were prepared as described above except that the procedure was scaled up by a factor of 10 (0.5 mL of DNA solution added to 0.5 mL of polymer solution, total volume = 1 mL). The sample (50 μg of DNA) was then added to the flask, and the flask was returned to the incubator. After 30 min, the loading medium was removed, and 12 mL of fresh growth medium was added to the flask. Two hours after addition of the fresh culture medium, the cells were harvested using trypsin and pelleted in six separate microcentrifuge tubes. Two of the aliquots were used to obtain the data in duplicate, and the other four were used to generate a pH calibration curve. Cells in two of the vials were each resuspended in PBS containing 2% fetal bovine serum, while cells in the other four vials were resuspended in each of four intracellular pH clamping buffers (pH = 4.5, 5.8, 6.6, and 7.4). These buffers were prepared by mixing 50 mM HEPES (pH = 7.4) and 50 mM MES (pH = 5.0) buffers (each also containing 50 mM NaCl, 30 mM ammonium acetate, and 40 mM sodium azide). The cells were washed by pelleting and resuspending them once again in the appropriate buffers. One T75 flask yielded one data point (one library member) in duplicate. The fluorescein/Cy5 fluorescence ratio was measured for each of the six samples using a flow cytometer. The four intracellular pH clamped samples were used to generate a pH calibration curve (linear), from which the fluorescein/Cy5 fluorescence ratios of the other two duplicate samples were converted to average pH values.

Flow Cytometry. Flow cytometry was performed with a FACSCalibur (Becton Dickinson) equipped with an argon ion laser to excite fluorescein (488 nm excitation) and a red diode laser to excite Cy5 (635 nm excitation). The emission of fluorescein was filtered using a 530 nm band-pass filter, and the emission of Cy5 was filtered using a 650 long pass filter. The cells were appropriately gated by forward and side scatter, and 30 000 events per sample were collected. The median FL1 (fluorescein) and FL4 (Cy5) values were used to determine the FL1/FL4 ratio for each sample.

Measurement of Cytotoxicity. Cytotoxicity assays were performed in triplicate in the following general manner. COS-7 cells were grown in 96-well plates at an initial seeding density of 15 000 cells/well in 200 μL of phenol red-free 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 in an incubator, after which the growth medium was removed and replaced with OPTI-MEM medium containing DNA/polymer complexes. Complexes were prepared as described above. After 1 h incubation at 37 °C the OPTI-MEM containing the complexes was removed and 100 μL of fresh growth medium was added to each well. After an additional 24 h incubation period, cells were assayed for metabolic activity using the MTT cell proliferation assay kit (ATCC, Manassas, VA). A 10 μL portion of MTT reagent was added to each well. After 2 h incubation at 37 °C, 100 μL of detergent reagent was added to each well. The plate was then left in the dark at room temperature for 4 h. Optical absorbance was measured at 570 nm using a microplate reader and converted to percent relative to control (untreated) cells.

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