Gene Delivery Proteins

Semi-Automated Synthesis and Screening of a Large Library of Degradable Cationic Polymers for Gene Delivery**

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The combinatorial, automated high-throughput synthesis and evaluation of small molecules has revolutionized modern drug discovery. Here we describe the first high-throughput, semi-automated methodology for the synthesis and screening

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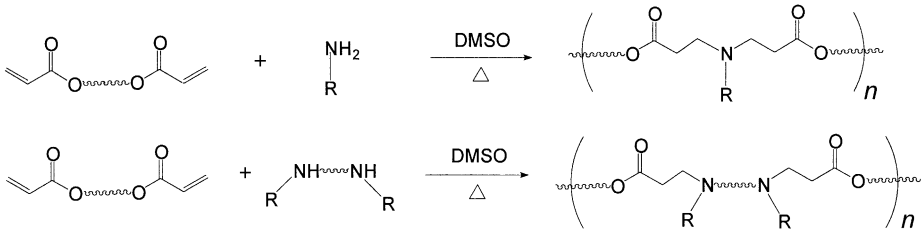
of a large library of polymers for gene delivery. A key feature of these methods is that synthesis, storage, and cell-based testing are all performed without removing solvent, thereby allowing high- throughput manipulations using simple, easily automated fluid-handling systems. By using these methods, we synthesized a library of 2350 structurally unique, degradable, cationic polymers. High-throughput, cell-based screening has identified 46 new polymers that transfect with a higher efficiency than conventional nonviral delivery systems such as poly(ethyleneimine).

The major barrier to the success of gene therapy in the clinic remains the lack of safe and efficient DNA-delivery methods. Currently, the majority of clinical trials use modified viruses as delivery vehicles, which, while effective at transferring DNA to cells, suffer from potentially serious toxicity and production problems.[1,2] In contrast, nonviral systems offer a number of potential advantages, including ease of production, stability, low immunogenicity and toxicity, and reduced vector size limitations.[3] Despite these advantages, however, existing nonviral delivery systems are far less efficient than viral vectors.[4] One promising group of nonviral delivery compounds are cationic polymers, which spontaneously bind and condense DNA.[5–12] We have been particularly interested in poly(β-amino ester)s as delivery agents which have been shown to generally possess low cytotoxicity and are easily synthesized by the conjugate addition of a primary amine or bis(secondary amine) to a diacrylate (Scheme 1).[11,12]

The traditional development of new biomedical polymers has been an iterative process: polymers typically are designed one at a time and then individually tested for their properties. More recently, attention has focused on the development of parallel, combinatorial approaches that facilitate the generation of structurally diverse libraries of polymeric biomaterials.[12–15] However, the methods used in all previous studies are still relatively low-throughput, and therefore were not able to generate large amounts of structure–function information.



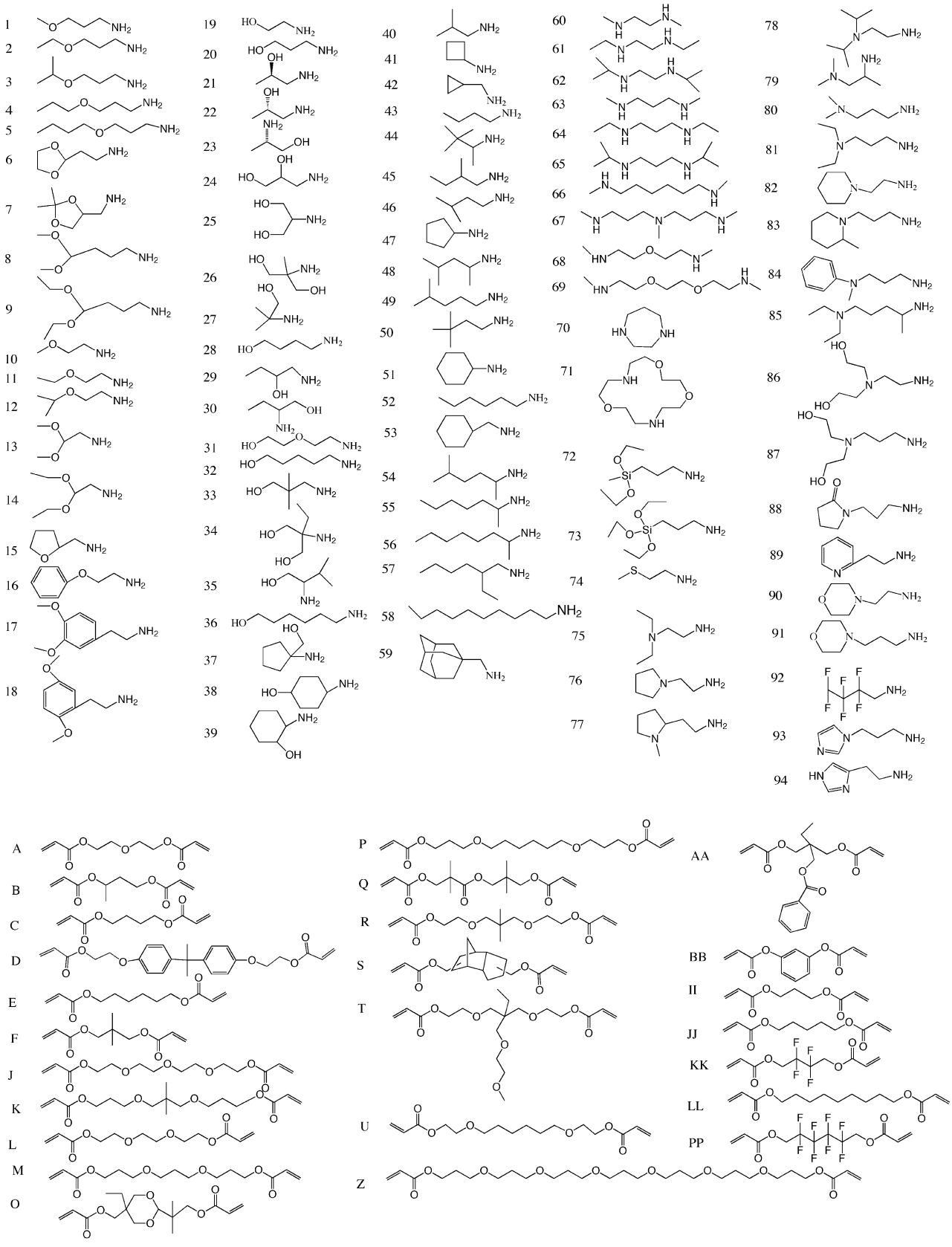
Scheme 1. Synthesis of poly(β-amino ester)s. Poly(β-amino ester)s were synthesized by the conjugate addition of primary or bis(secondary amines) to diacrylates.

Among the primary factors limiting the throughput and automation of poly(β-amino ester) synthesis and testing was the viscosity of the monomer and polymer solutions, and the difficulty with manipulating the solid polymer products. While automation of liquid handling is straightforward using conventional robotics, the manipulation of solids and viscous liquids on a small scale is not. Therefore, we sought to develop a general procedure with which polymers could be synthesized and screened in cell-based assays without leaving the solution phase. Since this would require the presence of residual solvent in the cell assays, we chose to use a relatively nontoxic solvent: dimethyl sulfoxide (DMSO). DMSO is a widely used solvent in the culturing of cells, and is commonly used when storing frozen stocks of cells. It is miscible with water and is generally well-tolerated.

The first step in preparing for high-throughput synthesis was to identify conditions that would allow for the production of polymer and yet possess a manageable viscosity. Small-scale, pilot experiments showed that polymerizations could be performed effectively at 1.6 M in DMSO at 56 °C for 5 days. Based on this, we prepared a general strategy for synthesis and testing. All monomers (Scheme 2) were diluted to 1.6 M in DMSO, and then using both a fluid-handling robot and a 12-channel micropipetter, we added 150 μL of each amine and diacrylate monomer into a polypropylene, deep-well plate, and sealed it with aluminum foil. These were placed on an orbital shaker and incubated at 56 °C for 5 days. To compensate for the increased viscosity of the polymeric solutions, we added 1 mL of DMSO to each well of the plate and then stored them frozen at 4 °C until ready for use. These synthetic methods greatly increased our throughput, and allowed us to set up all 2350 reactions in a single day. Furthermore, the production and storage of polymers in a 96-well format allowed for an easy transition to an automated 96-well format, cell-based testing of the efficiency of polymer transfection.

Once synthesized, all polymers were tested for their ability to deliver the luciferase expressing plasmid, pCMV-luc, into the monkey kidney fibroblast cell-line COS-7. The large size of the polymer library necessitated the development of a high-throughput method for the cell-based screening of transfection efficiency. Since polymers were stored in 96-well plates, we performed all polymer dilution, DNA complexation, and cell transfections in parallel by directly transferring polymers from plate to plate using a liquid-handling robot. All polymers were synthesized using the same concentration of amine monomer, thus comparison between polymers at a fixed ratio of the amine-containing monomer to DNA phosphate (N:P ratio) was straightforward. While the amine monomers contain either one, two, or three amines per monomer, initial broad-based screens for transfection efficiency were greatly simplified by maintaining a constant monomer concentration in all the wells of a single plate, and therefore a constant volume of polymer solution per reaction (see the Experimental Section).

We selected N:P ratios of 10:1, 20:1, and 40:1 for our initial screens based on our previous experience with these types of polymers. We screened all 2350 polymers at these three ratios with our high-throughput system. Transfection values at the best conditions for each polymer were tabulated into a histogram (Figure 1). These results were compared to three controls: naked DNA (no transfection agent), poly-



Scheme 2. Amino (numbers) and diacrylate (letters) monomers.

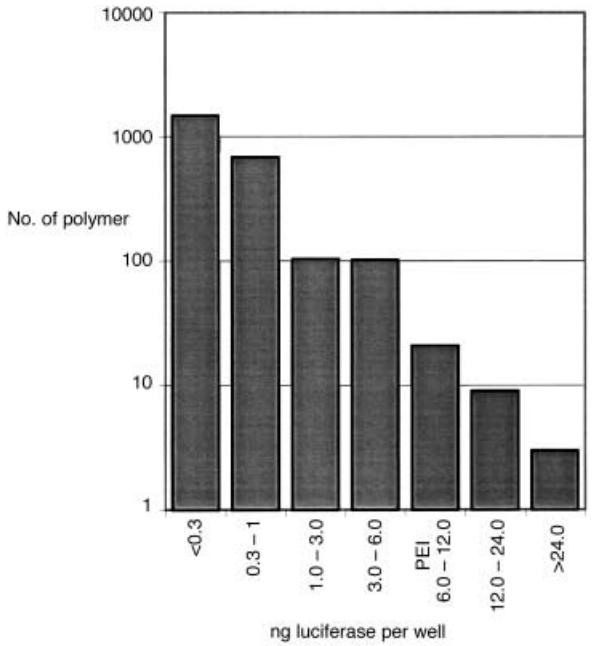


Figure 1. Histogram of polymer transfection efficiencies. In the first screen all 2350 polymers were tested for their ability to deliver pCMV-luc DNA at N:P ratios of 40:1, 20:1, and 10:1 to COS-7 cells. PEI transfection efficiency is shown for reference. COS-7 cells readily take up naked DNA, and in our conditions produce 0.15 ± 0.05 ng of luciferase per well. Under optimized conditions, 1 h transfections with PEI produce 6.0 ± 0.2 ng per well, and the best lipid reagent we have used, lipofectamine 2000, produces 13.5 ± 1.9 ng per well.

(ethyleneimine) (PEI), and lipofectamine 2000. The low, residual levels of DMSO present in the transfection solutions did not affect the transfection efficiency of either naked DNA or PEI. Thirty three of the 2350 polymers were found to be as good or better than PEI in this unoptimized system.

Since cationic polymer transfections tend to be sensitive to the polymer:DNA ratio,[16] we decided to individually optimize the transfection conditions with the best polymers from our preliminary screen. The average transfection efficiencies in the optimized conditions for the best 50 polymers are shown in Figure 2, along with control data. In this experiment, 46 polymers were identified that transfect as good or better than PEI. All 93 of these polymers were also tested for their ability to bind DNA using agarose gel electrophoresis (Figure 2). Interestingly, while almost all of the polymers do bind DNA as expected, two polymers that transfect at high levels do not: M17 and KK89 (Figure 2).

To further examine the transfection properties of these polymers, ten of the best polymers were tested for their ability to deliver the green fluorescent protein plasmid, pCMV-eGFP. Unlike pCMV-luc, pCMV-eGFP provides information concerning what percentage of cells is transfected. High levels of transfection were observed for all ten polymers, and two of the best are shown in Figure 3.

The "hits" identified in the above assays (Figure 2) reveal a surprisingly diverse and unexpected collection of polymers. Particularly surprising is the large collection of polymers containing hydrophobic monomer D. In fact, the diacrylate

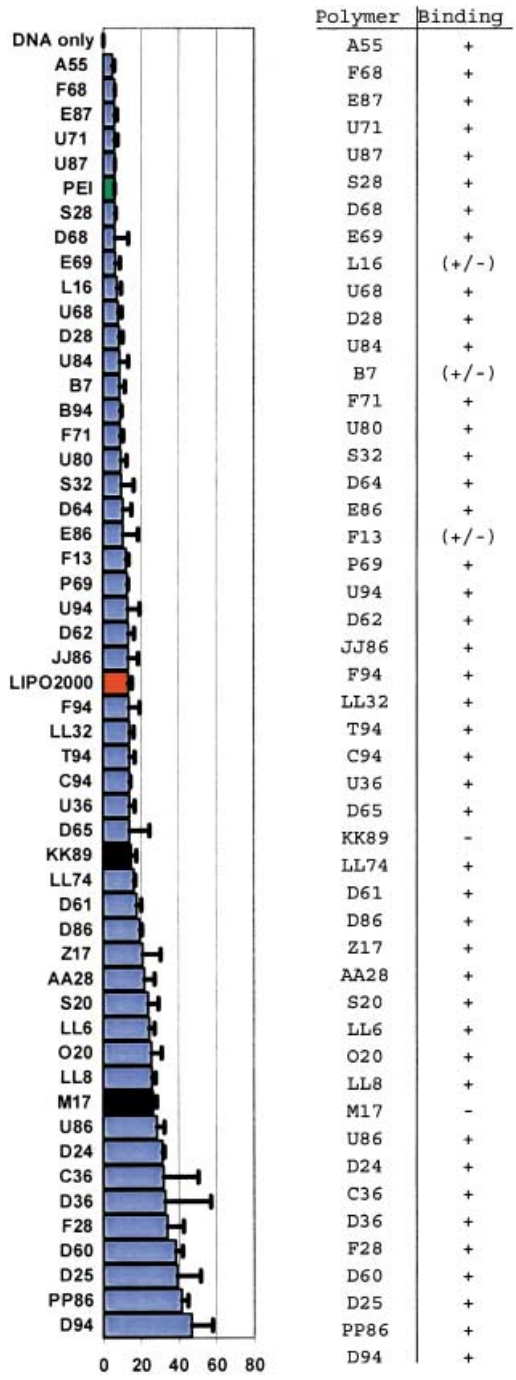


Figure 2. Left) Optimized transfection efficiency of the top 50 polymers relative to PEI and lipofectamine 2000. Polymers were tested as described in the Experimental Section. In the first broad screen N:P ratios of 40:1, 20:1, and 10:1 were tested. The top 93 were rescreened in triplicate at the following N:P ratios: (the optimal N:P ratio from the first screen) $\times 0.5$, 0.75, 1.0, 1.25, 1.5, and 1.75. Control reactions are labeled in red, and polymers that did not bind DNA in a gel electrophoresis assay are shown in black. Right) DNA binding of polymers as determined by agarose gel electrophoresis. The data was tabulated in the following manner: 1) fully shifted DNA is represented by (+), 2) partially shifted DNA is represented by (+/−), 3) unbound DNA is represented by (−).

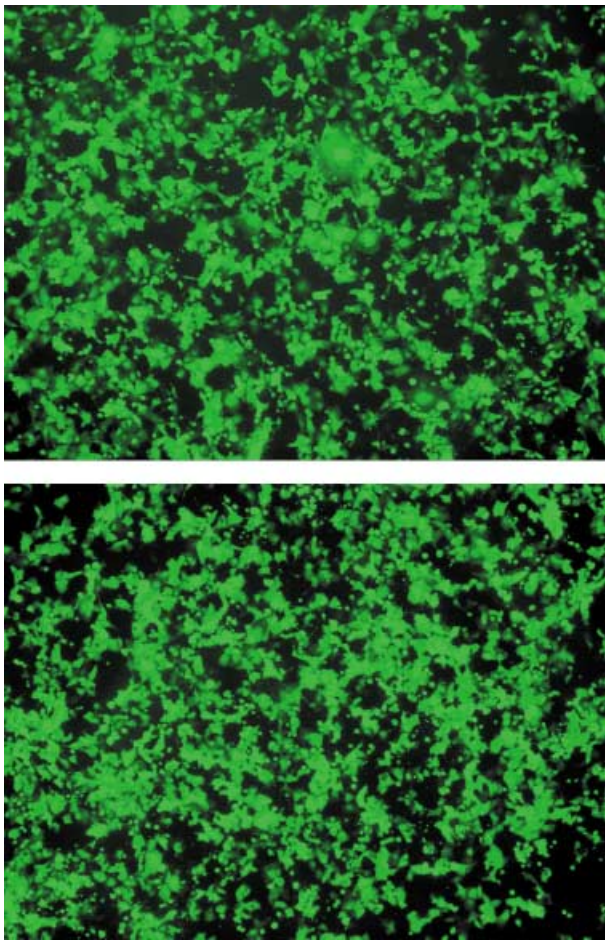


Figure 3. Transfection of COS-7 cells using enhanced green fluorescent protein plasmid. Cells were transfected at an N:P ratio of (optimal N:P from the broad screen) × 1.25 with 600 ng of DNA. Regions of the well showing high transfection are shown for the following polymers: top: C36, bottom: D94.

monomers used to make the 50 best-performing polymers are almost always hydrophobic. Further analysis reveals two more common features of the effective polymers: 1) 12 of the 26 polymers that are better than the best conventional reagent, lipofectamine 2000, have mono- or dialcohol side groups, and 2) linear, bis(secondary amines) are also over represented in the hit structures. Also surprising was the identification of two polymers that transfect at high levels, but do not appear to bind DNA (KK89 and M17). Both are also insoluble at pH 5 and pH 7, and we speculate that their ability to facilitate DNA uptake may be a result of permeabilization of the cellular membrane. While the mechanisms behind the function of these polymers remains unclear at this point, we are developing second-generation DNA delivery polymers using these data as design criteria.

Also important for the function of gene-delivery polymers is length.[17,18] Using these results as a guideline, we are in the process of creating a range of polymer lengths for the most effective DNA-delivery polymers by carefully varying the relative concentration of the monomer. Eventually, in vivo experiments will be critical in determining the safety, efficacy, and ultimate utility of these polymers for gene therapy.

We have described the combinatorial synthesis and testing of 2350 structurally unique poly (β-amino ester)s for their ability to transfect the mammalian cell line, COS-7. We were able to set-up all 2350 synthetic reactions in a single day by using semi-automated methods, and then test the transfection potential of these polymers at a rate of about 1000 per day. The methods we have developed here are not limited to gene-delivery polymers, but can be more broadly applied towards the identification of materials for nucleic acid, protein, and drug delivery, as well as in other areas of medicine.

Experimental Section

Polymer synthesis: Monomers were purchased from Aldrich (Milwaukee, WI), TCI (Portland, OR), Pfaltz & Bauer (Waterbury, CT), Matrix Scientific (Columbia, SC), Acros-Fisher (Pittsburg, PA), Scientific Polymer (Ontario, NY), Polysciences (Warrington, PA), and Dajac monomer-polymer (Feasterville, PA). The monomers were dissolved in DMSO (Aldrich) to a final concentration of 1.6 M. All possible pairwise combinations of amine and diacrylate monomers were added in aliquots (150 μL) to each well of the 96-well polypropylene masterblock deep-well plates (2 ml, Griener America, Longwood, FL). The plates were sealed with aluminum foil, and incubated at 56 °C while rotating them on an orbital shaker. After 5 days, DMSO (1 ml) was added to each well, and the plates were resealed and stored frozen at 4 °C until ready to be used.

Transfection experiments: 14,000 cos-7 cells (ATCC, Manassas, VA) were seeded into each well of a solid white or clear 96-well plate (Corning-Costar, Kennebunk, ME) and allowed to attach overnight in growth medium composed of phenol red free Dulbecco's modified eagle medium (DMEM, 500 mL), heat-inactivated fetal bovine serum (FBS, 50 mL), penicillin/streptomycin (5 mL, Invitrogen, Carlsbad, CA). Each well of a master block 96-well plate was filled with 25 mM sodium acetate at pH 5 (1 ml). The polymer/DMSO solution (40, 20, or 10 μL) was then added. The diluted polymer (25 μL) was added to 60 $\mu g\,mL^{-1}$ pCMV-luc DNA (25 μL; Promega, Madison, WI) or pEGFP-N1 (Invitrogen) in a half-volume 96-well plate. These were incubated for 10 min, and then the polymer-DNA solution (30 μL) was added to optimem (200 μL) containing sodium bicarbonate (Invitrogen) in 96-well polystyrene plates. The medium was removed from the cells using a 12-channel wand (V&P Scientific, San Diego, CA) after which the optimem-polymer-DNA solution (150 μL) was immediately added. The complexes were incubated with the cells for 1 h and then removed using the 12-channel wand and replaced with growth medium (105 μL). Cells were allowed to grow for three days at 37 °C in 5 % CO_2 and then analyzed for luminescence (pCMV-luc) or fluorescence (pEGFP-N1). Control experiments were performed as described above, but using poly(ethyleneimine) $M_r = 25\,000$ (Aldrich) instead of the synthesized polymer, and at polymer:DNA weight ratios of 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, and 2:1. Lipofectamine 2000 (Invitrogen) transfections were performed as described by the vendor, except that complexes were removed after 1 h.

Luminescence was analyzed using bright-glo assay kits (Promega). Briefly, Bright-glo solution (100 μL) was added to each well of the microtiter plate containing media and cells. Luminescence was measured using a Mithras Luminometer (Berthold, Oak Ridge, TN). In some cases a neutral density fliter (Chroma, Brattleboro, VT) was used to prevent saturation of the luminometer. A standard curve for luciferase was generated by titration of luciferase enzyme (Promega) into growth media in white microtiter plates. eGFP expression was examined using a Zeiss Axiovert 200 inverted microscope.

Agarose gel electrophoresis DNA-binding assays were done at an N:P ratio of 40:1, as previously described.[12] All liquid handling was performed using an EDR384S/96S robot (Labcyte, Union City, CA)

or a 12-channel micropipetter (Thermo Labsystems, Vantaa, Finland), in a laminar flow hood.

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