Accelerated Discovery of Synthetic Transfection Vectors: Parallel Synthesis and Screening of a Degradable Polymer Library

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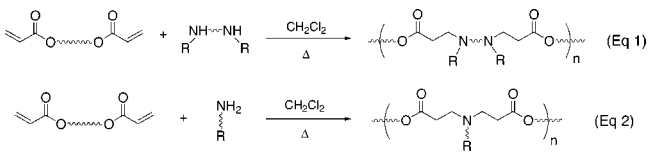
The safe and efficient delivery of therapeutic DNA to cells represents a fundamental obstacle to the clinical success of gene therapy.[1] The challenges facing synthetic delivery vectors are particularly clear, as both cationic polymers and liposomes are less effective at mediating gene transfer than viral vectors. The incorporation of new design criteria has led to recent advances toward functional delivery systems.[2,3] However, the paradigm for the development of polymeric gene delivery vectors remains the incorporation of these design elements into materials as part of an iterative, linear process—an effective, albeit slow, approach to the discovery of new vectors. Herein, we report a parallel approach suitable for the synthesis of large libraries of degradable cationic polymers and oligomers and the discovery of new synthetic vector families through rapid cell-based screening assays.[4]

Poly(β-amino ester)s are hydrolytically degradable, condense plasmid DNA at physiological pH, and are readily synthesized via the conjugate addition of primary or secondary amines to diacrylates (eqs 1 and 2).[5] An initial screen of model polymers identified these materials as potential gene carriers and demonstrated that structural variations could have a significant impact on DNA binding and transfection efficacies.[5] We reasoned that this approach provided an attractive framework for the elaboration of large libraries of structurally unique polymers for several reasons: (1) diamine and diacrylate monomers are inexpensive, commercially available starting materials, (2) polymerization can be accomplished directly in a single synthetic step, and (3) purification steps are generally unnecessary as no byproducts are generated during polymerization.



The paucity of commercially available bis(secondary amines) limits the degree of structural diversity that can be achieved using the above synthetic approach. However, the pool of useful, commercially available monomers is significantly expanded when primary amines are considered as potential library building blocks. Because the conjugate addition of amines to acrylate groups is generally tolerant of functionalities such as alcohols, ethers, and tertiary amines,[6] we believed that the incorporation of functionalized primary amine monomers into our synthetic strategy would serve to broaden structural diversity. Diacrylate monomers **A**−**G** and amine monomers **1**−**20** were selected for the synthesis of an initial screening library (Figure 1).

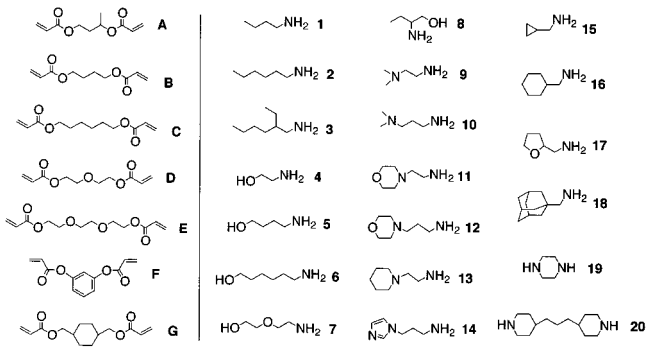


Figure 1. Diacrylate (**A**−**G**) and amine (**1**−**20**) monomers chosen for the synthesis of an initial screening library.

The size of the library constructed from this set of monomers (7 diacrylates × 20 amines = 140 structurally unique polymers) was chosen to be large enough to incorporate sufficient diversity, yet small enough to be practical without the need for automation in our initial studies. It was unclear at the outset whether a polymer such as **G16** (formed from hydrophobic and sterically bulky monomers **G** and **16**) would be water-soluble at physiological pH or be able to condense DNA sufficiently. However, monomers of this type were deliberately incorporated to fully explore diversity space, and in anticipation that this library may ultimately be useful as a screening population for the discovery of materials for applications other than gene delivery.[4,7]

Polymerization reactions were conducted simultaneously as an array of individually labeled vials. Reactions were performed in methylene chloride at 45 °C for 5 days, and polymers were isolated by removal of solvent to yield 600−800 mg of each material. Reactions performed on this scale provided amounts of each material sufficient for routine analysis by GPC and all subsequent DNA-binding, toxicity, and transfection assays. A survey of 55% of the library by GPC indicated molecular weights ranging from 2000 to 50000 (relative to polystyrene standards). As high molecular weights are not required for DNA-complexation and transfection (as shown below),[8] this library provided a collection of polymers and oligomers suitable for subsequent screening assays.

Of the 140 members of the screening library, 70 samples were sufficiently water-soluble (2 mg/mL, 25 mM acetate buffer, pH = 5.0) to be included in an electrophoretic DNA-binding assay (Figure 2). To perform this assay as rapidly and efficiently as possible, samples were mixed with plasmid DNA at ratios of 1:5 and 1:20 (DNA/polymer, w/w) in 96-well plates and loaded into an agarose gel slab capable of assaying up to 500 samples using a multichannel pipettor. All 70 water-soluble polymer samples were assayed simultaneously at two different DNA/polymer ratios in less than 30 min. As shown in Figure 2, 56 of the 70 water-

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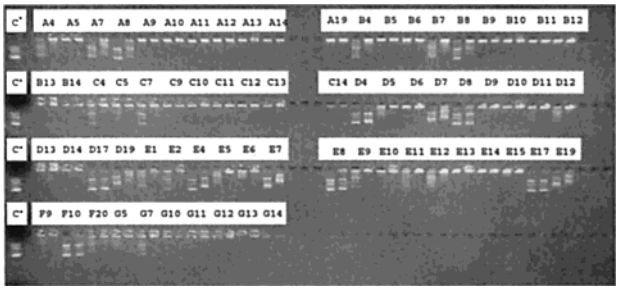

Figure 2. Gel electrophoresis assay used to identify DNA-complexing polymers. Lane annotations correspond to the 70 water-soluble members of the screening library. For each polymer, assays were performed at DNA/polymer ratios of 1:5 (left well) and 1:20 (right well). Lanes marked C* contain DNA alone (no polymer) and were used as a control.

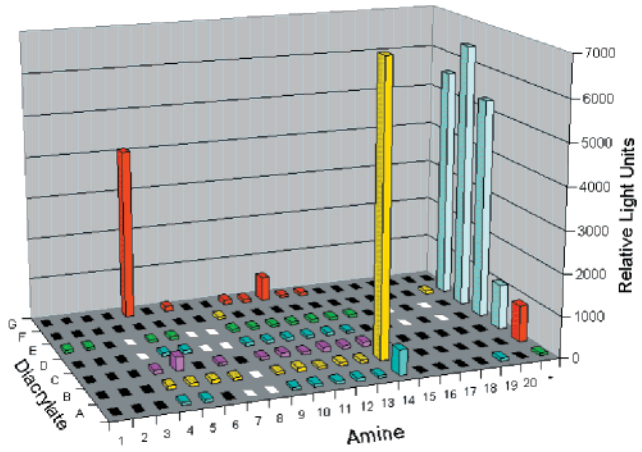

Figure 3. Transfection data as a function of structure for an assay employing pCMV-Luc (600 ng/well, DNA/polymer = 1:20). Light units are arbitrary and not normalized to total cell protein; experiments were performed in triplicate (error bars not shown). Black squares represent water-insoluble polymers, white squares represent water-soluble polymers that did not complex DNA in Figure 2. The right column (marked *) displays values for the following control experiments: no polymer (green), PEI (red), and Lipofectamine 2000 (light blue).

soluble polymer samples interacted sufficiently with DNA to retard migration through the gel matrix (e.g., **A4** or **A5**), employing the 1:20 DNA/polymer ratio as an exclusionary criterion. Fourteen polymers were discarded from further consideration (e.g., **A7** and **A8**), as these polymers did not complex DNA sufficiently.

The DNA-complexing materials identified in the above assay were further investigated in transfection assays employing plasmid DNA and the COS-7 cell line. All assays were performed simultaneously with the firefly luciferase reporter gene (pCMV−Luc), and levels of expressed protein were determined using a commercially available assay kit and a 96-well luminescence plate reader. Figure 3 displays data generated from an assay employing pCMV-Luc (600 ng/well) at DNA/poly ratios of 1:20 (w/w). The majority of the polymers screened did not mediate transfection above a level typical of "naked" DNA (no polymer) controls under these conditions. However, several wells expressed higher levels of protein and the corresponding polymers were identified as "hits" in this assay. Polymers **B14** (MW = 3180) and **G5** (MW = 9170), for example, yielded transfection levels 4−8 times higher than control experiments employing poly(ethylene imine) (PEI), a polymer conventionally employed as a synthetic transfection vector,[9] and transfection levels within or exceeding the range of expressed protein using Lipofectamine 2000,[9b] a leading commercially available lipid-based transfection vector system. Polymers **A14**, **C5**, **G7**, **G10**, and **G12** were also identified as positive "hits" in the above experiment, but levels of gene expression were lower than those for **B14** and **G5**.

Structural differences among synthetic polymers typically preclude a general set of optimal transfection conditions. For example, polymers **C5**, **C14**, and **G14** were toxic at the higher concentrations employed above,[10] but mediated transfection at lower DNA and polymer concentrations (data not shown). The assay system described above can easily be modified to evaluate polymers as a function of DNA concentration, DNA/polymer ratio, cell seeding densities, or incubation times. Additional investigation will be required to more fully evaluate the potential of this screening library, and experiments to this end are currently underway.

The assays above were performed in the absence of chloroquine, a weak base commonly added to enhance in vitro transfection,[3,8] and the results therefore reflect the intrinsic abilities of those materials to mediate transfection. The polymers containing monomer **14** are structurally similar to other histidine containing "proton sponge" polymers,[3] and could enhance transfection by buffering acidic intracellular compartments and mediating endosomal escape.[3,9] The efficacy of polymers containing monomer **5** is surprising in this context, as these materials do not incorporate an obvious means of facilitating endosomal escape. While the efficacy of these latter polymers is not yet understood, their discovery helps validate our parallel approach and highlights the value of incorporating structural diversity, as these polymers may not have been discovered on an ad hoc basis. Polymers incorporating hydrophilic diacrylates **D** and **E** have not produced "hits" under any conditions thus far, providing a possible basis for the development of more focused libraries useful for the elucidation of structure/activity relationships.

We have generated a library of 140 degradable polymers and oligomers useful for the discovery of new DNA-complexing materials and gene delivery vectors. Several of these materials are capable of condensing DNA into structures small enough to be internalized by cells and releasing the DNA in a transcriptionally active form. The total time currently required for library design, synthesis, and initial screening assays is approximately 2 weeks. However, the incorporation of robotics and additional monomers could significantly accelerate the pace at which new DNA-complexing materials and competent transfection vectors are identified. We continue to evaluate the potential of this approach through the elucidation of structure/function relationships and the development of second-generation screening libraries.

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Supporting Information Available: Synthetic details and experimental protocols (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Determined by the absence of cells in wells containing these polymers as observed upon visual inspection. These polymers were less toxic and mediated transfection at lower concentrations.