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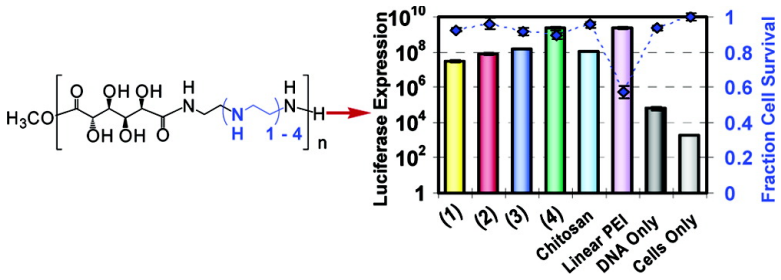

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New Poly(D-glucaramidoamine)s Induce DNA Nanoparticle Formation and Efficient Gene Delivery into Mammalian Cells

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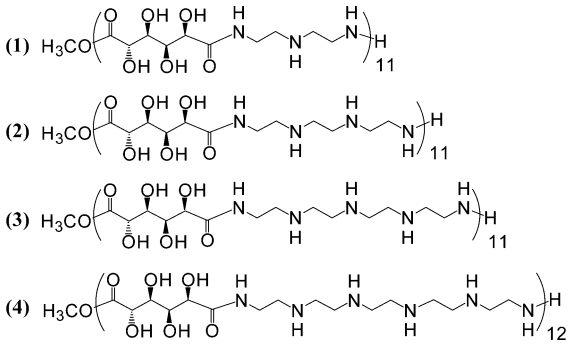
Nucleic acids show great promise as new therapeutics to treat both acquired and inherited diseases. One of the greatest challenges with the successful application of nucleic acid drugs is the development of an efficacious delivery method.[1] Delivery systems are needed to compact genetic material into nanostructures that can be uptaken by cells, protect nucleic acids from enzymatic damage during cellular transport, and provide the possibility of targeting the delivery to specific cell types.[2] Viral vectors are still the most effective and commonly used method of DNA transport even though many problems with this delivery method have been revealed.[2,3]

Polymer-mediated gene delivery has recently emerged as a viable alternative to viral-based transfection systems since polymers may not induce immune and inflammatory responses, have a lower cost of synthesis, and have a large nucleic acid loading capacity.[1,2] Several studies have shown that polycations bind DNA electrostatically and form polyplexes (polymer + DNA complexes) that are endocytosed by many cell types and deliver DNA with varying degrees of delivery efficiency and toxicity.[4,5] Although synthetic delivery systems show great promise, difficulties with polymer toxicity and low delivery efficiency have hampered clinical application of these vectors.[1,2] For example, polyethylenimine (PEI), a polymer of ethylenediamine, exhibits efficient gene delivery but is also very cytotoxic.[6] Conversely, chitosan, a polymer of glucosamine, is completely nontoxic yet reveals low delivery efficiency in many cell lines.[7] Progress toward rationally designed synthetic delivery systems has also been stalled by a lack of understanding the fundamental polymer structure−biological property relationships that exist for synthetic delivery vehicles.[4,5]

Here, we present our efforts toward designing novel polymeric gene delivery vehicles that may circumvent these drawbacks. We designed several systems to lower the toxicity of the conventional polymeric vectors by incorporating a carbohydrate comonomer within a PEI-like backbone. In addition, we have systematically increased the number of secondary amines between the carbohydrates to elucidate how the number of basic groups within a polymer repeat unit facilitates efficient nucleic acid binding, condensation, and intracellular gene delivery. To this end, we selected a series of comonomers that has allowed us to design in these chemical characteristics to yield both a nontoxic and highly efficient delivery vehicle.

Esterified D-glucaric acid comonomer was synthesized according to the method of Kiely et al. and recrystallized from a mixture of methanol and triethylamine.[8] The amine-containing comonomers, diethylenetriamine, triethylenetetramine, and tetraethylenepentamine (Aldrich), were used in the polymer synthesis without further purification. The pentaethylenehexamine comonomer (Acros) was purified via fractional distillation, dissolved in methanol, and precipitated by addition of concentrated HCl before polymerization.[9]

The desired polyamides (**1**−**4**) were prepared by polycondensation of the esterified D-glucaric acid comonomer with each of the four amine comonomers above at room temperature in methanol.[8] The polymerization concentrations and times were all optimized to yield a series of poly(D-glucaramidoamine)s with similar degrees of polymerization so the chemical structure−biological properties could be accurately elucidated without concerns dealing with differences in degrees of polymerization.[10] After polymerization, each polymer was dissolved in ultrapure water, dialyzed to purity in a 1000 MWCO membrane, and lyophilized to dryness. NMR studies as well as the α value in the Mark−Houwink−Sakurada equation obtained via viscometry (for **1**−**4**, $\alpha = 0.6-0.7$) suggest that these polymers are mostly linear.[11] This indicates that polymerization occurs predominantly through the primary amines with a low degree of branching off the secondary amines.[12]



Results of gel electrophoresis shift assays (Figure 1a) completed as previously reported[5b] with plasmid DNA (pDNA) at N/P ratios (the ratio of polymer nitrogens (N) that can be protonated/phosphates (P) on the DNA backbone)[13] between 0 and 50 reveal that polymers **1**−**4** bind pDNA at N/P ratios of 5, 3, 2, and 2, respectively. Also, dynamic light scattering and TEM experiments (Figure 1b) indicate that **1**−**4** all compact DNA into nanoparticles in the approximate size range to be uptaken into cells through the endocytotic pathway.[2b,14]

Next, the cell viability (Figure 2) and gene delivery efficiency (Figure 3) of the poly(D-glucaramidoamine)s were examined with a BHK-21 cell line (baby hamster kidney, American Type Culture Collection). As positive controls for the delivery experiment, we compared the toxicity and delivery efficiency of **1**−**4** to those of linear PEI (JetPEI, Avanti Polar Lipids) and low-molecular weight chitosan (50−190 kDa, Aldrich) that are well studied in the literature as gene delivery agents.[6,7] Untransfected cells and "naked" pDNA were used as the negative controls in this experiment. The polyplexes were formed with gWizLuc pDNA (Aldevron) containing the firefly luciferase reporter gene under the control of the CMV promoter in DNAse, RNAse free water. The BHK-21 cells were cultured as previously reported.[4e,5b] Transfections were completed

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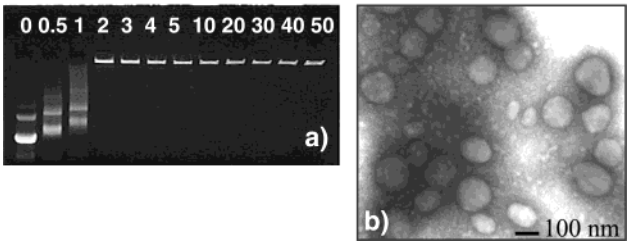

Figure 1. (a) Gel electrophoresis shift assay of **4** bound to pDNA at the indicated N/P ratios. (b) TEM of polyplexes formed with pDNA and **4** at N/P ratio of 30.

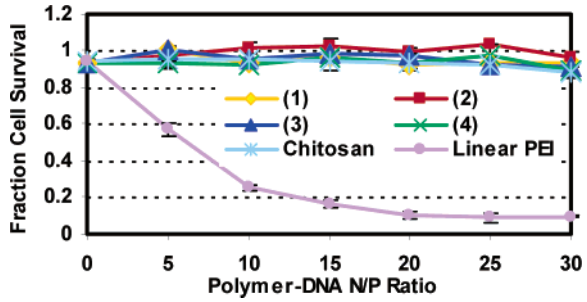

Figure 2. Viability of BHK-21 cells after exposure to polyplexes formed with pDNA and each polymer at N/P ratios between 0 and 30.

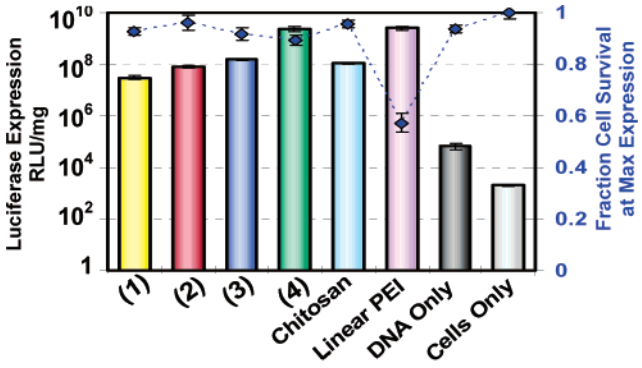

Figure 3. Maximum gene expression (bars) and cell viability at maximum gene expression (line) with BHK-21 cells for polyplexes formed with each vector and pDNA containing the firefly luciferase reporter gene. The N/P ratios that exhibit the maximum gene expression for polymers **1**−**4**, chitosan, and linear PEI are: 20, 30, 30, 30, 5, and 5, respectively.

with polyplexes formed with each polymer bound to 1 μg of pDNA at N/P ratios of 0 (pDNA only), 5, 10, 15, 20, 25, and 30 in triplicate.[15] The cells were lysed 47 h after initial transfection and assayed for luciferase expression (with a Promega luciferase assay system) and cell viability (with a Bio-Rad DC Protein assay kit).[4e,5b]

As shown (Figures 2 and 3), polymers **1**−**4** exhibit the nontoxic properties of chitosan even at an N/P ratio of 30 (Figure 2), and **4** displays high gene delivery efficiency (equivalent to linear PEI, shown in Figure 3). The polymer analogues **1**−**3** exhibit low delivery efficiency where **2** and **3** display about the same relative gene expression as chitosan. In addition, these data suggest that as the number of amine units increases in the repeat unit (**1**−**4**), the gene expression increases. This may be due in part to the "proton sponge" hypothesis proposed by Behr et al.[16] Polymers with primary, secondary, or tertiary amines have been found to exhibit enhanced gene delivery efficiency (due to buffering of the endosomal environment during the acidification process, causing endoosmolytic release of the polyplexes) over polycations that cannot be protonated (i.e. containing quaternary ammoniums).[5c]

In summary, the delivery of therapeutic nucleic acids may yield revolutionary advances to modern medicine, providing that a safe and effective delivery method is discovered. Here, we have synthesized a series of new poly(D-glucaramidoamine)s that exhibit high gene delivery efficiency without toxicity with BHK-21 cells. Currently, we are investigating the delivery profiles in several mammalian cell lines. Moreover, we are synthesizing systems containing differing carbohydrate comonomers to study the effects of the hydroxyl stereochemistry on cellular uptake.

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Supporting Information Available: Experimental details of the synthesis and characterization of **1**−**4** and details of the electrophoresis, cell culture, viability, and reporter gene assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Weight averaged molecular weights and M_w/M_n values were measured with a Viscotek GPCmax Instrument using a Triple Detection System (7 and 90 deg. static light scattering, viscometry and refractive index). Analytical data for **1**−**4** is given as M_w and M_w/M_n respectively: **1**, 3.0 kDa, 2.0; **2**, 3.4 kDa, 1.4; **3**, 3.9 kDa, 1.4; **4**, 4.9 kDa, 1.6.

(11) In general, α values between 0.5 and 0.8 in the Mark−Houwink−Sakurada equation ($\eta = kM^{\alpha}$) indicate randomly coiled linear polymers.

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(13) The amide nitrogens are not counted in this ratio since they will not display a positive charge within the physiological pH range (7.4−5.0).

(14) Polyplex sizes were determined with a Zetapals dynamic light scattering instrument (Brookhaven) at $\lambda = 662$ nm. Polyplexes were formed by combining each polymer (**1**−**4**) at an N/P ratio of 30 with 3μg of pDNA in 300 μL of DNAse free water. After 1 h, each solution was diluted with DNase free water to 0.7 mL. The results are reported as an average of 10 measurements: 256 nm (**1**), 441 nm (**2**), 140 nm (**3**), and 175 nm (**4**). TEM experimental details are given in the Supporting Information.

(15) Cells were plated in 24 well plates at a density of 50, 000 cells per well and transfected with polyplexes formed with polymers **1**−**4**, chitosan, and linear PEI 24 h after incubation at 37 °C and 5% CO_2. Media was replaced after 4 and 24 h following initial transfection (see Supporting Information).

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