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# The Effects of Structure on Gene Delivery with Linear $\beta$- and $\gamma$-Cyclodextrin-Containing Polycations

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**Abstract**

Cyclodextrin (CD)-containing polycations are prepared by copolymerization of $3^A3^B$-dideoxy-$3^A$,$3^B$-diamino-$\beta$- and $\gamma$-CDs with dimethyl suberimidate·2HCl to yield polyamidine products. Both alkyl- and alkoxy-diamines are used to vary the spacing between the CD and the amidine charge centers. It is found that the transfection efficiency and toxicity of such polycations is dramatically affected by the structure of the spacer separating the CD ring from the charge centers and less so by the type of CD used.

## Introduction

Gene therapy holds tremendous promise for the treatment of ailments that are of genetic origin. Selected cationic lipids and polycations are currently under investigation as nonviral gene delivery vectors. The complexes produced through self-assembly of cationic lipids or polycations with DNA obviate the nucleic acid size limitations and immunological concerns of viral vectors, and large-scale synthesis of such complexes will likely be more cost effective than with viral vectors. Unfortunately, nonviral systems continue to fall short of viral-vector-mediated delivery efficiencies and most demonstrate unacceptable levels of toxicity for use as systemic delivery vectors in humans. In response to these shortcomings, chemical synthesis and rational design have increasingly been applied to the development of new nonviral gene delivery vectors. Recent reviews provide some information on observed structure-function relationships among nonviral vectors [1, 2].

A family of linear, $\beta$-cyclodextrin-containing polycations ($\beta$-CDPs) suitable for use as gene delivery vectors has been described [3]. The $\beta$-CDP materials were prepared by polymerization of $6^A$-$6^D$-dideoxy-$6^A$,$6^D$-diamino-$\beta$-CDs with comonomers such as dimethyl suberimidate·2HCl (DMS) to give polyamidines. Initial structure-function studies with the $\beta$-CDPs showed the transfection efficiency and toxicity of these polycations to be a function of the intercharge spacing in the polycationic backbone [4]. Given this information, we expanded the scope of structure-function relationships for linear CD-containing polycations by synthesizing a larger and more diverse number of polycations. In this study, a new series of linear CD-containing polycations based on $3^A$,$3^B$-dideoxy-$3^A$,$3^B$-diamino-$\beta$- and $\gamma$-CDs are explored. Additionally, alkyl- and alkoxy-diamines are used to vary the spacing between the CD and the amidine charge centers. This is also the first report of a linear, $\gamma$-CD-containing polycation.

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## Experimental

### *Chemicals*

$\beta$- and $\gamma$-cyclodextrins were purchased from Wacker Biochem Corp. (Adrian, MI) and dried in vacuo at 120 °C overnight before use. Chlorosulfonic acid (Alfa Aesar; Ward Hill, MA) was distilled before use. Dimethyl suberimidate·2HCl (DMS) was purchased from Pierce Endogen (Rockford, IL) and used without further purification. All other reagents were obtained from commercial suppliers and were used as received. Ion-exchange chromatography was run on a Toyopearl SP-650M (Toso-Haas; Montgomeryville, PA) column ($NH_4^+$ form) and products were eluted with aqueous ammonium bicarbonate up to 0.4 M. Thin-layer chromatography was performed on Silica Gel 60 F 254 plates (EM Separations Technology; Gibbstown, NJ) and compounds were eluted with 5:3:3:1 *n*-PrOH:AcOEt:$H_2O$:NH3(aq) and visualized by reaction with ninhydrin. Mass spectra were obtained on a Hewlett Packard 1100 Series LC/MSD operated in electrospray ionization mode. NMR spectra were recorded on a Bruker AMX500 spectrometer as dilute solutions of either $D_2O$ or DMSO-$d_6$. Dialysis was carried out using a 3500 molecular weight cutoff regenerated cellulose dialysis cassette (Pierce Endogen). Plasmid pGL3-CV (Promega; Madison, WI) was amplified with the DH5$\alpha$ strain of E. coli (Gibco BRL; Gaithersburg, MD) and purified using the Ultramobius 1000 kit (Novagen; Madison, WI). This plasmid encodes the firefly luciferase gene under control of the SV40 promoter.

*Synthesis*

*$2^A,2^B$-disulfonated-$\beta$-cyclodextrin*, **3a** and *$2^A,2^B$-disulfonated-$\gamma$-cyclodextrin*, **3b** were synthesized according to literature methods [5, 6]. NMR and mass spectra data were in agreement with published values.

*Synthesis of $3^A,3^B$-di(aminoalkylamino)- and $3^A,3^B$-di(aminoalkoxyamino)-cyclodextrins (**4a–d** and **5a–d**)*
These syntheses were carried out as exemplified by the following procedure.

*Synthesis of* **5c**
Hexamethylenediamine (5.89 g, 50.7 mmol) was dissolved in 35 mL degassed water. **3b** (1.50 g, 0.88 mmol) was added at once and stirred at 37 °C under nitrogen for 19 hours. The reaction was further carried out at 70 °C for 3 hours then concentrated under reduced pressure. Cyclodextrins were precipitated with 11:1 acetone:methanol and collected by filtration. Ion-exchange chromatography yielded the pure product (855 mg, 54% yield). ESI-MS $[M + H]^+ = 1494$.

*Synthesis of polycations (**6a-d** and **7a-d**)*
These syntheses were carried out as exemplified by the following procedure.

*Synthesis of* **7c**
**5c** (100 mg, 54.7 $\mu$mol) and DMS (15.5 mg, 56.7 $\mu$mol) were taken up in 108 $\mu$L 0.5M $Naa_2CO_3$ and stirred for 13 hours. Acidification followed by exhaustive dialysis yielded 58.4 mg of a white powder (56% yield).

*Light scattering and molecular weight determination*

The specific refractive index (RI) increment, d$n$/d$c$, of each polycation was determined by fitting a linear curve to plots of RI versus concentration (five data points per polycation). Polycations were then analyzed on a Hitachi D6000 HPLC system equipped with a ERC-7512 RI detector and a Precision Detectors PD2020/DLS light scattering detector using a PL aquagel-OH column (Polymer Laboratories, Amherst, MA). The eluent was 0.8 M ammonium acetate with 0.05% sodium azide, adjusted to pH 2.8 with phosphoric acid and flowing at 0.7 mL/min. RI values were measured on a Carl Zeiss refractometer (Max Erb Instrument Co., Burbank, CA) in the same eluent as used for HPLC analysis.

*Polyplex formation and characterization*

Polyplexes were formulated by adding polycation solutions in $dH_2O$ to an equal volume of DNA in $dH_2O$ (0.05 mg/mL final DNA concentration). Desired charge ratios were achieved by using appropriate concentrations of polycation solution. Retardation of polyplexes was investigated by gel electrophoresis in a 0.8% agarose gel (30 $\mu$g ethidium bromide/50 mL TAE buffer). Particle size and $\zeta$ potential of polyplexes were analyzed using a ZetaPALS instrument (Brookhaven Instruments; Holtsville, NY).

*Cell culture and transfections*

BHK-21 cells were maintained at 37 °C in 5% CO2 atmosphere in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 $\mu$g/mL amphotericin B (Gibco BRL). For transfections, cells were seeded at 50,000 cells/well in 24-well plates. Trypan blue exclusion was used to verify cell viability above 95%. At one day, cells were transfected in serum-free medium with 1 $\mu$g pGL3-CV plasmid pre-assembled with CD-polycations at various charge ratios. After four hours, polyplex solutions were removed from the cells and replaced with 1 mL regular growth medium. For measurement of luciferase activity and toxicity, cells were lysed two days after transfection with 1X Cell Culture Lysis Reagent (Promega). The Luciferase Assay System (Promega) was used to measure luciferase activity of cell lysates on a Monolight 2010 luminometer (Becton Dickinson Biosciences; San Jose, CA). Total protein content of cell lysates was assessed with the DC Protein Assay (Bio-Rad; Hercules, CA), a derivative of the Lowry assay.

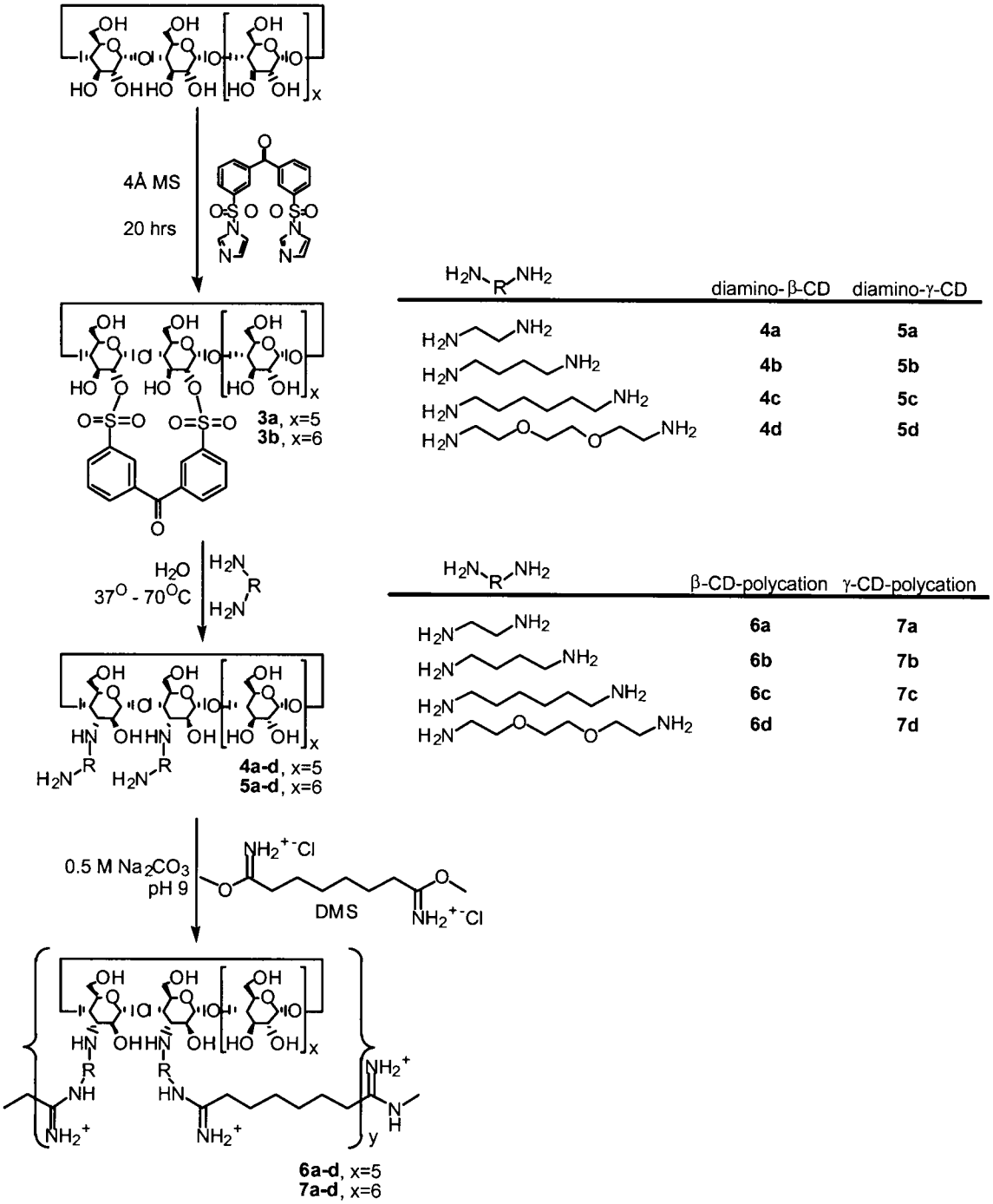
## Results

*Polycation synthesis and characterization*

Cyclodextrin-polycations were synthesized using $\beta$- and $\gamma$-CDs functionalized with alkyl- and alkoxy-diamines (Scheme 1). The cyclodextrin-comonomer clearly influences the polymerization with DMS, as shown by the data given in Table 1. The polymerization yield increased with the distance between the cyclodextrin ring and the primary amine in the CD-monomer. Furthermore, the polymerization yield is quite similar for $\beta$- and $\gamma$-cyclodextrin-monomers with identical spacers. An increase in $M_w$ is observed between cyclodextrin monomers having 4 or fewer methylene units between the cyclodextrin and the amidine and cyclodextrin monomers with longer spacers. This increase in polycation $M_w$ is from an average degree of polymerization (DOP) of 5 or 6 to a DOP of 7 or 8. An increase in polydispersity accompanies the increase in $M_w$.

In vitro *transfection efficiency*

*In vitro* transfection efficiency was assessed at a charge ratio of 10 $\pm$. Lysates of transfected cells were examined for luciferase activity by measure of relative light units (RLU) that were normalized by total protein content (Figure 1). An order of magnitude increase in transfection efficiency is observed as the length of the methylene spacer between the secondary amine and the charge center is increased from two to four, then again as the number of methylenes is increased from four to six. The diaminoalkoxy-CD analogues, **6d** and **7d**, demonstrated an intermediate level of transfection efficiency similar to **6b** and **7b**. No difference in transfection efficiency is observed between otherwise similar $\beta$- and $\gamma$-CD polycations. The luciferase activity of untreated cells, cells treated with polycation alone and cells treated with




*Scheme 1.* Synthesis of cyclodextrin-polycations.

*Table 1.* Effect of cyclodextrin comonomer on the polymerization

| Polycation | Polymerization yield (%) | d*n*/d*c* (mL/g | $M_w$ (kDa) | $M_w/M_n$ | Average degree of polymerization |
|---|---|---|---|---|---|
| **6a** | 32 | 0.1029 | 10.0 | 1.1 | 6 |
| **6b** | 44 | 0.1406 | 8.1 | 1.3 | 5 |
| **6c** | 61 | 0.1515 | 13.9 | 1.7 | 8 |
| **6d** | 74 | 0.1322 | 13.0 | 1.4 | 7 |
| **7a** | 32 | 0.1085 | 9.3 | 1.1 | 5 |
| **7b** | 47 | 0.1386 | 9.6 | 1.4 | 5 |
| **7c** | 56 | 0.1237 | 14.7 | 1.6 | 8 |
| **7d** | 58 | 0.1279 | 13.3 | 1.3 | 7 |

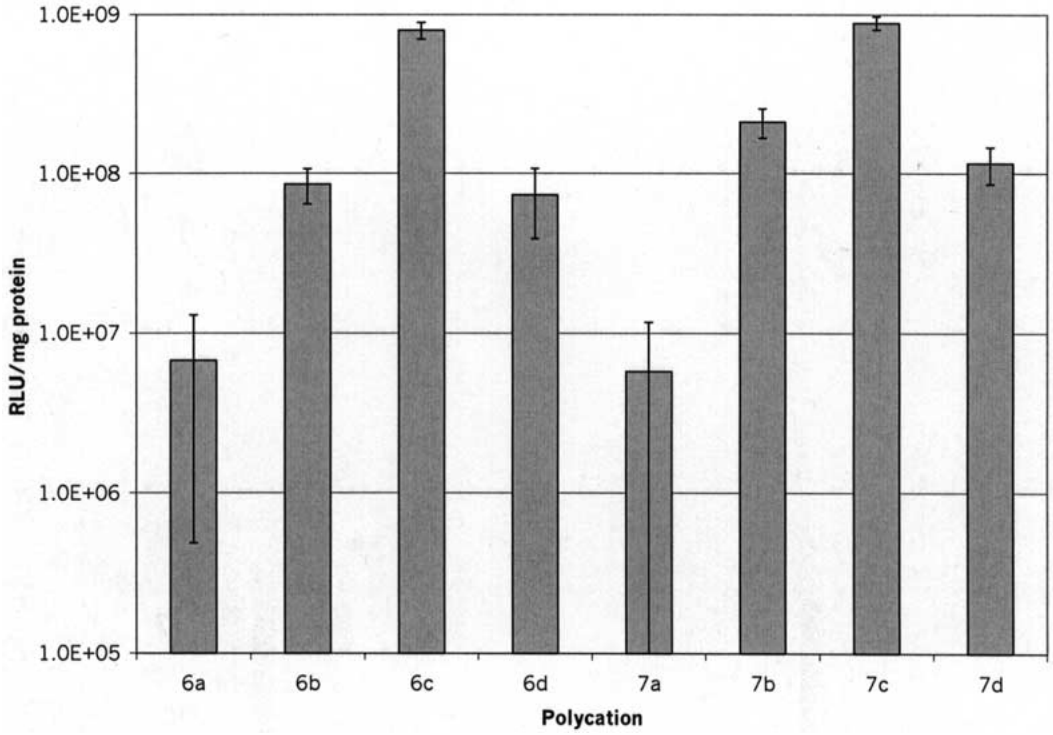



*Figure 1.* Transfection efficiency of polyplexes at a charge ratio of 10 ±.

DNA alone gave approximately $5 \times 10^4$ RLU/mg protein, which is significantly lower than the value obtained from cells treated with polyplexes.

In vitro *toxicity*

The total protein content of cell lysates was used as a measure of polycation toxicity. The fractional cell survival of transfected cells was assessed at a charge ratio of 10 ± by comparison to untransfected cells. Polycations **6c** and **7c** were considerably more toxic than the other polycations. Furthermore, polycation **6c** was more toxic than **7c**. Polyplex toxicity was found to be quite similar to toxicity of the same concentration of polycation without DNA as shown in Figure 2.

## Discussion

The objective of this study is to elucidate the structure-function relationships for gene delivery with a family of linear $\beta$- and $\gamma$-cyclodextrin-containing polycations by investigating their ability to transfect cells *in vitro* with plasmid DNA. A series of cyclodextrin polycations was synthesized with various spacer-linkages between the cyclodextrin ring and the amidine charge centers. The type of cyclodextrin was also varied, such that all polycationic spacers have been prepared with both $\beta$- and $\gamma$-cyclodextrins. This approach allows direct evaluation of the effect of cyclodextrin-type on transfection efficiency and toxicity.

Polymerization yield increased with the distance between the cyclodextrin ring and the reactive primary amine of the cyclodextrin monomers with DMS. Similar yields were found for polymerization of the otherwise identical $\beta$- and $\gamma$-CD monomers. The steric bulkiness of the cyclodextrin could account for the reduced reactivity of the primary amines that are closer to the cyclodextrin cup. Thus, increasing the distance between the reactive center and the bulky cyclodextrin leads to a corresponding increase in the polymerization yield. Polycation molecular weights are found to increase by about 50% as the distance between the cyclodextrin and the reactive amine increases from 4 to 6 methylene units; an associated increase in polydispersity is also observed. The average degrees of polymerization of 5–8 correspond to an average of 10–16 cationic charges per polycation. If the differences in molecular weight are assumed to not significantly affect polycation performance, the variation in observed performance may then be attributed to differences in polycation structure.

Previous work demonstrated that the transfection efficiency and toxicity achieved with CD-containing polycations is affected by the presence of CD moieties and by the alkyl chain length between charge centers [4]. Here, it is demonstrated that the transfection efficiency and toxicity of such polycations is also affected by the structure of the spacer separating the CD ring from the charge centers and the type of CD used.

Transfection efficiencies of the polyplexes were found to increase as the length of the methylene spacer in the polycation increased, with the alkoxy derivatives **6d** and **7d** providing intermediate levels of transfection. Polycations **6c** and **7c**, which contain six-methylene spacers between the secondary amine and the amidine charge center, exhibit cell viability below that of the other polycations while providing the highest transfection efficiencies. Moreover, the $\gamma$-CD-

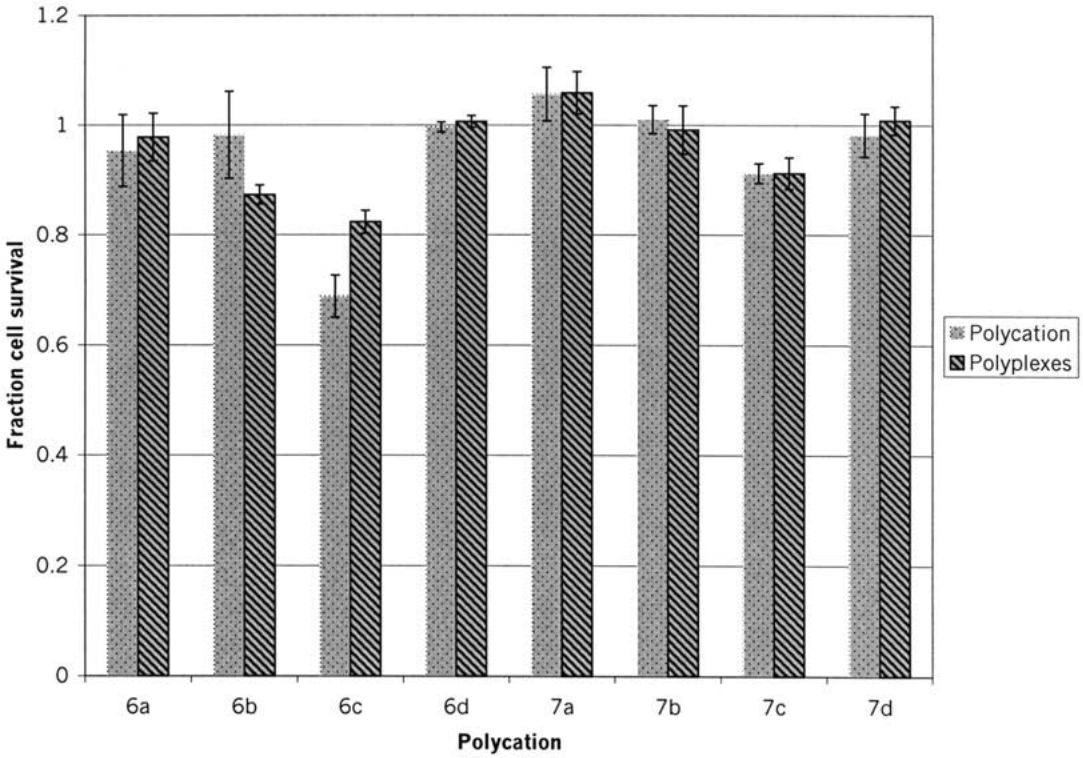



*Figure 2.* Toxicity of polyplexes (diagonally slashed bars) at a charge ratio of 10 ± and of polycation alone at the same polycation concentration in the polyplexes.

derivative, **7c**, is less toxic than the $\beta$-CD-derivative, **6c**. These results suggest that the toxicity-mediating influence of the CD may be a result of steric bulk, shielding intracellular entities from the otherwise toxic amidine charge centers. Toxicity of the polyplexes is largely independent of the presence of DNA and highly dependent on the concentration of polycation (Figure 2), suggesting that free polycation in solution should be minimized by formulating polyplexes at lower charge ratios.

From this initial study, it is clear that polycationic structure has a dramatic effect on performance. More detailed studies are underway and will be published later. Lower charge ratios will be investigated to attempt to show differences in transfection efficiency between the different polycations while likely reducing cell death due to lower concentration of polycation.

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