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Substituted β-Cyclodextrins Interact with PAMAM Dendrimer–DNA Complexes and Modify Transfection Efficiency

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The efficiency of PAMAM dendrimer-mediated DNA transfer can be improved by the addition of substituted β-cyclodextrins (β-CDs) as formulation excipients. *In vitro* CAT expression increased approximately 200-fold when dendrimer/DNA/β-CD formulations were applied on the surface of collagen membranes. The inclusion of β-CD into the formulations resulted in particles that were smaller and more evenly distributed on the surface of the solid support. The average size of the complex formed at 50 μg/ml and at charge ratio of 1 decreased from 156 nm to 5.8 nm and 21.2 nm in 0.025–0.1% w/vol β-CDs. Sulfonated β-CDs bind to dendrimer and in the increased concentration may displace DNA in the dendrimer/DNA complex. High concentrations of amphoteric β-CD do not dissociate dendrimer/DNA complexes; however, they may decrease their ability to transfect cells. At the optimized formulations the surface-modified β-CDs may enhance solid support-based transfection *in vitro,* through modification of dendrimer/DNA complex composition and improved surface distribution.

Polyamidoamine (PAMAM) dendrimers are a class of nanoscopic polymers that in recent years have emerged as suitable carriers for *in vivo* applications in therapeutics as well as DNA delivery. All PAMAM dendrimers have a molecular architecture characterized by regular dendritic branching with radial symmetry. The spheric topological structure of these polymers is achieved by the ordered assembly of polymer subunits in concentric dendritic tiers around an initiator core, which also leads to these polymers having a generational size distribution that is uniform (1). The physicochemical properties of dendrimers include high surface charge density and water solubility, enabling electrostatic interactions with nucleic acids. In turn these consistent and predictable electrostatic interactions have allowed the dendrimer–DNA complexes to be used for the efficient transfection of cells both *in vitro* and *in vivo* (2–6). In addition we have reported that dendrimer–DNA complexes can be used as functional coatings on biodegradable solid supports to facilitate transfection of wounded skin (7).

Cyclodextrins are cyclic carbohydrates that have been used as pharmaceutical excipients for more than two decades (8, 9). These biocompatible α-D(+)-glucose oligomers have been studied extensively and utilized for enhancement of drug stability, solubility and bioavailability (10). β-CDs are generally nontoxic and nonimmunogenic. The majority of pharmaceutical applications exploit the ability of β-CDs to form inclusion complexes with variety of drugs, although these compounds have also been used as excipients (11). Previous studies have also used β-CDs to augment transfections; however, no studies have focused on defining potential interactions between β-CD and polymer-based transfection systems (12). We now report preliminary characterization of the physical interactions that occur between β-CD and dendrimer–DNA complexes. In addition, our data indicate that the addition of β-CDs to dendrimer–DNA complexes can modify dendrimer–DNA complex composition and improve distribution of complexes within aqueous solutions. These monodisperse formulations can be used as functional coatings on biodegradable membranes. The immobilized dendrimer–DNA complexes facilitate transfection of cells *in vitro.*

MATERIALS AND METHODS

Dendrimers. The production and characterization of Starburst PAMAM dendrimers has been previously described in detail (1). Generation 5 of EDA core based dendrimers (G5 EDA) with a MW 28,826 Da and 128 primary surface amine groups were employed in these studies.

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β-Cyclodextrins. The amphoteric (A), sulfonated (S) as well as unsubstituted β-cyclodextrins (β-CDs) were purchased from Supelco. Aqueous solutions of β-CDs were prepared according to the manufacturer's description using double distilled deionized water.

Collagen membranes. Bilayer collagen membranes were made by alkaline initiated polymerization of a Type I bovine collagen (Cell Prime, Collagen Biomaterials, Fremont, CA) solution using phosphate-buffered saline, pH 7.2 (Life Technologies, Grand Island, NY) as a diluent (7).

Preparation of plasmid dendrimer–DNA complexes. Dendrimer–DNA complexes were formed by incubating the two aqueous solutions of components together in 100–200 μL of water for a minimum of 10 min at room temperature. Charge ratios of dendrimer to DNA were based on the calculation of the electrostatic charge present on each component, the number of terminal NH_2 groups on the dendrimer, vs the number of phosphate groups in the DNA as previously described (3, 13).

Dynamic light scattering (DLS) analysis. Size and distribution of dendrimer/DNA complexes in water with and without β-CDs were analyzed using a NICOMP 370 System.

DNA gel retardation assay. 0.5 μg of linearized plasmid DNA (0.05 μg/μl), in the presence of increasing concentrations of amphoteric or sulfonated β-CDs, was complexed with G5 EDA dendrimer at charge ratios 1 or 10 (+/−). After incubation for 10 min at room temperature, samples were electrophoresed through the 0.8% agarose/TAE gels, stained with 0.03 μg/ml ethidium bromide, and photographed under ultraviolet light.

Coating of collagen membranes with dendrimer–DNA complexes. Dendrimer–DNA complexes with or without β-CD were prepared in 100 μl of water and incubated for 10 min at room temperature (RT). The complexes were then overlaid on the surface of the membranes. Coated membranes were air-dried in a laminar flow hood for 1–2 h at RT prior to use. Complexes were formed with DNA at a concentration 0.5 mg/ml (50 μg) and 0.325 mg/ml (32.5 μg) of G5 EDA dendrimer alone (dendrimer–DNA charge ratio of 1) or in the presence 0.01 and 0.1% (w/v) of amphoteric (A) or sulfonated (S) β-CD. During the formation of complexes the DNA was labeled with ethidium bromide (EtBr, 5 nM) added directly to the DNA solution to allow for visualization of the dendrimer–DNA complexes.

Plasmid DNA. The reporter plasmid pCF1CAT has been described in detail previously (14, 15). Plasmid DNA was isolated from bacterial transformants by double cesium chloride gradient and endotoxin levels in each batch of plasmid was determined by limulus assay by the Vector Core Laboratory at the University of Michigan. All batches of plasmid DNA used for experiments had endotoxin levels less than or equal to 3.3 EU/mg.

In vitro transfections. COS-1 cells were maintained in D-MEM medium (Gibco BRL) with 10% FCS (Hyclone), 1% penicillin-streptomycin and 2 mM L-glutamine and incubated at 37°C in 5% CO_2. For *in vitro* transfection experiments cells were seeded at the density $2–3 \times 10^4/cm^2$ on the surface of the membranes coated with dendrimer–DNA complexes described above. Cells were harvested 36–48 h after and the amount of CAT was determined in 10 μl of whole cell lysates by ELISA (Boehringer Manheim GmbH).

Molecular dynamic (MD) simulation. A model building and MD simulations were performed using Onyx workstation (Silicon Graphics, Inc., Mountain View, CA) and the Insight II software package (Molecular Simulations Inc., San Diego, CA). As a model system, a generation 3 PAMAM dendrimer (G3) was first built and simulated with the consistent valence force field (CVFF) for 50 ps at 295°K, after minimization and annealing processes. The primary amines of a PAMAM dendrimer were fully protonated to simulate dendrimer at pH 7. The 12 bp double stranded DNA was modeled to obtain a dendrimer to DNA charge ratio of 1 and simulated with dendrimer for 50 ps. A distance-dependent dielectric constant was used to shield electrical charges. A number of β-cyclodextrin and S β-cyclodextrin (three sulphonate groups per CD) was added to the model for 100 ps simulation. The β-CD concentrations of 0.01 and 0.1% w/v correspond to a stoichiometry of 1.2 and 11.6 β-CD for one G3 EDA/DNA complex, respectively. The Coulomb energy between G3 EDA and DNA was determined as a function of the number of β-CD molecules.

TABLE I

Particle Size Analysis of Dendrimer–DNA Complexes

Formulation	Mean size (nm)
G5EDA/DNA complex	159.5 (SD 34.0)
+0.025% A β-CD	21.2 (SD 3.0)
+0.05% A β-CD	184.2 (SD 39.2)
+0.1% A β-CD	218.5 (SD 46.4)
+0.05% S β-CD	116.7 (SD 24.0)
+0.1% S β-CD	5.8 (SD 0.5)
+0.5% S β-CD	188.9 (SD 36.6)

Note. The number-weighted NICOMP distribution analysis of solid particles present in each formulations was determined using a cycle time of 2 min. Results are presented as means ± standard deviation (S.D.).

Statistical analysis. Statistical analysis was performed using Systat 5.2 software for Macintosh. Errors were calculated as standard deviations and differences between samples were analyzed by ANOVA.

RESULTS AND DISCUSSION

Characterization of Dendrimer–DNA Complexes in the Presence of β-CD

The effect of β-CD on dendrimer–DNA complex size was analyzed by dynamic light scattering (DLS) and the results are presented in Table I. The G5 EDA/DNA complexes formed in water at DNA concentrations of 50 μg/ml at a charge ratio of 1 resulted in a heterodispersed population with the mean particle sizes of 156.0 nm (SD 34.0 nm). The addition of 0.1% and 0.025% of either sulfonated (S) or amphoteric (A) β-CD to the dendrimer–DNA complexes resulted in a monodispersed population with the calculated mean particle size of 5.8 nm (SD 0.5 nm) or 21.2 nm (SD 3.0 nm) respectively. The small size of the particles especially in the 0.1% of S β-CD [comparable to the size of G5 EDA dendrimer (5.6 nm), β-CDs (~8 nm), or compacted DNA alone] suggests possibility of the dissociation of dendrimer/DNA complex. At the lower concentrations of A β-CDs (0.025%) uniform small particles were observed by DLS, suggesting that dendrimer/DNA complexes are stable and possibly include molecules of β-CD electrostatically bound to the dendrimer–DNA complexes. We suggest that in these particular conditions the tripartite interactions are in electrostatic equilibrium. Further increases in β-CD concentrations led to the generation of larger particles possibly representing mixed complexes containing all three components, any combination of β-CD–DNA (in case of

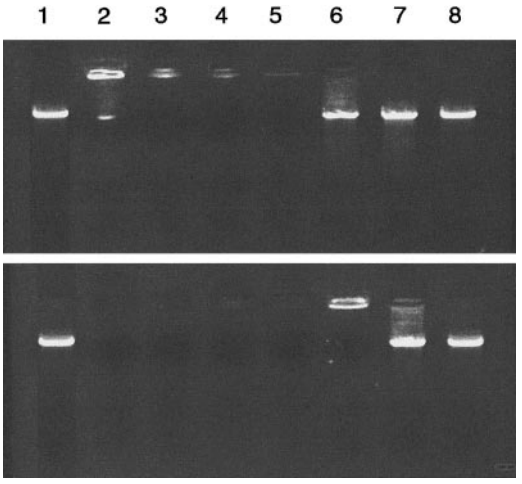

FIG. 1. Electrophoretic behavior of dendrimer/DNA complexes—Effect of modified β-CD on the electrostatic stability of the complex. Upper panel, E5/DNA complex at charge ratio 1; lower panel, G5 EDA/DNA complex at charge ratio 10. Lane 1, DNA alone; lane 2, dendrimer/DNA complex. Lanes 3, 4, and 5: complex in the presence of 0.05, 0.1, and 1% of A β-CD. Lanes 6, 7, and 8: complex in the presence of 0.05, 0.1, and 1% of S β-CD. DNA is visualized with EtBr.

A β-CD) or both A and S β-CD binding to dendrimer (Table I). It is important to underline that those tripartite interactions differ from those most studied with host–guest systems (8). In the typical β-CD-mediated host–guest complexing, the guest molecules inhabit the cavities of the cyclodextrin host, most often in a 1:1 stoichiometric ratio (10, 16). Because free dendrimers or dendrimer/DNA complexes are larger than the diameter of the β-CD cavity, dendrimer–DNA–β-CD interactions are mainly electrostatic and involve charged groups on the surface of the respective molecules. The electrophoretic mobility of DNA is retarded upon binding with G5 EDA dendrimers (13). The presence of 0.05%, 0.1 and 1% of A β-CD does not change mobility of DNA indicating stable complexes (Fig. 1, lanes 2, 3, and 4 in upper and lower panel). However, the same concentrations of S β-CD displace DNA from dendrimer complex at charge ratio 1, whereas dendrimer/DNA complexes at charge ratio 10 are fully dissociated with 1% of S β-CD (Fig. 1, lanes 6, 7, and 8 in upper and lower panel). DNA displacement from the complex by negatively charged S β-CD confirms that tripartite intramolecular interactions are primarily electrostatic. The electrophoretic effects of A and S β-CDs also indicate different modes of molecular interactions of S (with negative net charge) and A (with neutral net charge) β-CDs with dendrimer/DNA complexes. With increasing concentrations, S β-CD competes for binding to dendrimer and may displace DNA from the dendrimer/DNA complexes. With increasing concentrations, A β-CD can interact with either moiety of dendrimer/DNA complex through positive (+) and/or negative (−) local charge. The resulting changes in colloidal characteristics of dendrimer/DNA complexes may affect transfection.

Modified β-Cyclodextrins Improve Surface Distribution of Dendrimer/DNA Complexes

In our previous studies we have documented the feasibility of using dendrimer/DNA complexes as a functional coating for transfection of wounded skin (3). Utilization of solid support membranes—such as *ex vivo* polymerized collagen—as a platform for dendrimer–DNA delivery would likely be optimized by immobilizing monodisperse complexes on the surface. Coating of the membrane with dendrimer/DNA complexes involves electrostatic forces that develop between complexes and a surface of the collagen membrane. Because our current method of membrane coating uses simple dehydration, drying and/or absorption the solvent (water) results in a progressive increase in the concentration of complexes that may lead to aggregation and uneven surface distribution of dendrimer/DNA complexes. This in consequence may limit DNA accessibility for *in situ* transfection. Figure 2 illustrates effect of both A and S β-CD on the distribution of dendrimer/DNA complexes on the surface of collagen membranes containing phosphatidyl glycerol (1% PG). All preparations of plasmid DNA complex with G5 EDA PAMAM dendrimer were formed in 100 μl of H_2O and applied to the surface of the membranes and air-dried approximately 2 h in the laminar flow tissue culture hood (Material and Methods). DNA at a concentration 0.5 mg/ml (50 μg) was complexed with 0.325 mg/ml (32.5 μg) of G5 EDA alone (dendrimer/DNA charge ratio of 1) or with 0.01 and 0.1% (w/v) of A or S β-CD. Membranes coated with complexes alone display uneven distribution of complexes with a majority of DNA in heterogeneous aggregates (Fig. 2, lane 1). The presence of either type of β-CD results in the increasingly disperse and evenly distributed DNA. At 0.01% (w/v) β-CD aggregates of complexes are visibly smaller and more disperse. At 0.1% w/v β-CD the dendrimer/DNA complex appears to be as evenly distributed as naked DNA in the control preparations (Fig. 2, lanes 2, 3, and 4).

Membrane-Based Dendrimer-Mediated Transfection in Vitro in the Presence of Modified β-CD

The effect of β-CD-mediated dendrimer/DNA complex distribution on *in situ* transfection was assessed using COS-1 cells directly seeded onto surface of the membranes. The collagen/1% PG membranes were coated with E5 EDA/DNA complexes (at charge ratio 0.1) containing 1 and 5 μg pCF1-CAT plasmid and 0.1% to 0.5% of (A) or (S) β-CD. The transgene expression in cells transfected with 1 μg and 5 μg of pCF1 CAT complexes yielded 5 and 122 pg/ml of CAT protein, respectively, which suggests that threshold amount DNA is necessary for efficient transfection. The addition of either 0.1% A or

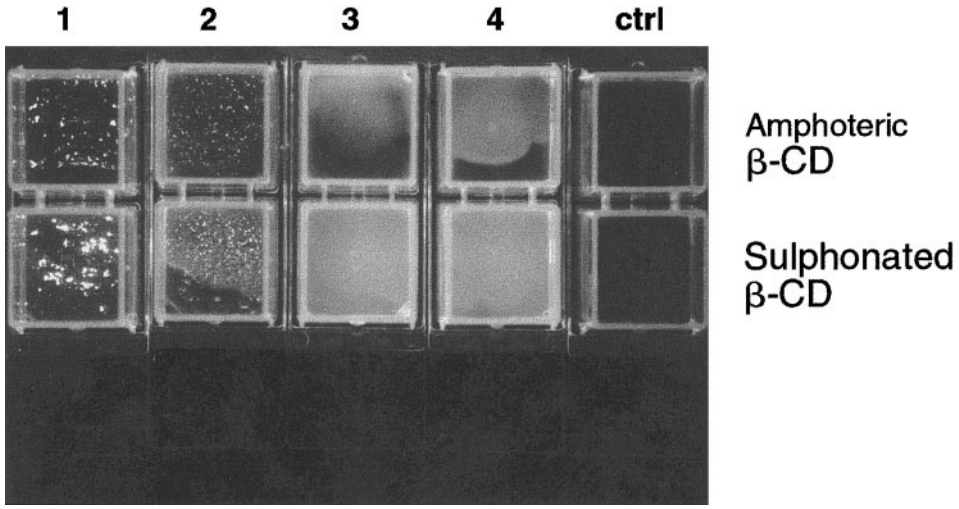

FIG. 2. The effect of modified β-CD on the membrane surface distribution of dendrimer/DNA complexes. The conditions of complex formation: 1, G5 EDA–DNA complex at a charge ratio 1; 2 and 3, complex with addition of 0.01 and 0.1% of β-cyclodextrin; 4, DNA alone; control, membrane not coated with DNA. (Top) Complexes with the addition of amphoteric (A) β-CD (% w/vol); (bottom) complexes with addition of sulfonated (S) β-CD (% w/vol).

0.1% S β-CD to the membrane preparations vastly improved transfection efficiency. The transfection of 1 μg of pCF1CAT DNA in the dendrimer complex in this conditions resulted in 1.21 and 0.867 ng/ml of CAT protein, respectively (Fig. 3). Since neither naked DNA nor any of DNA/β-CD preparations resulted in the detectable CAT expression (data not shown), the 240- and 170-fold increases in the efficiency of transfection can be at least partially attributed to the improved surface distribution of dendrimer/DNA complexes. A further increase of β-CD content leads to a decrease in the expression of transgene. Although β-CD—especially the soluble derivatives—are commonly utilized in variety of pharmaceutical and biochemical applications (Uekama *et al.*, 1998), their interactions with DNA, and particularly electrostatic complexation have not been extensively studied. Recently, Gonzales *et al.* (17) described the synthesis of a family of β-CDs polymers capable of binding DNA and mediating transfection. Our data using both amphoteric or sulphonated β-CD confirms their observations that

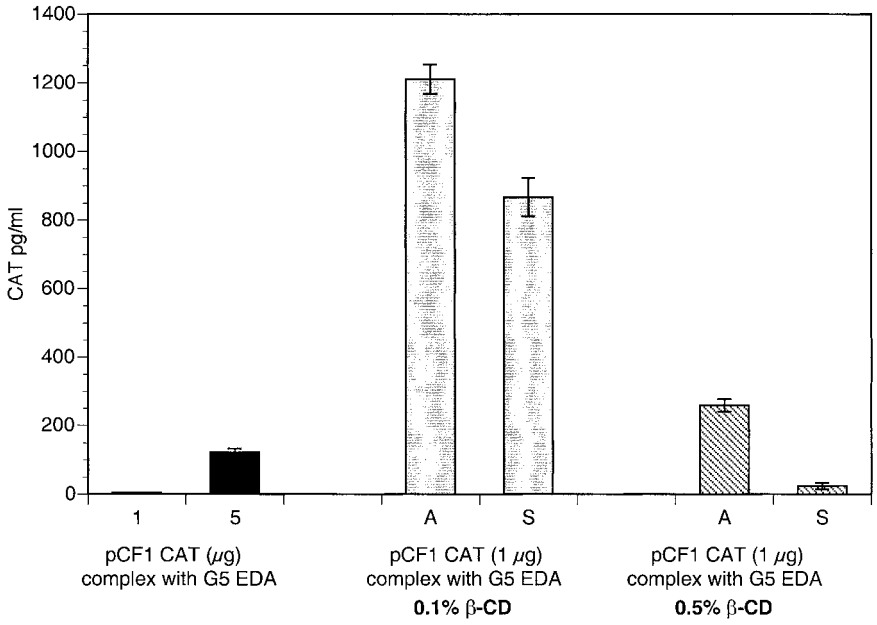

FIG. 3. The effect of β-CD on the efficiency of membrane based dendrimer–DNA complex transfection of COS-1 cells. Data are presented as the mean ± SD of three repeats. Toxicity was not observed in any of transfection condition.

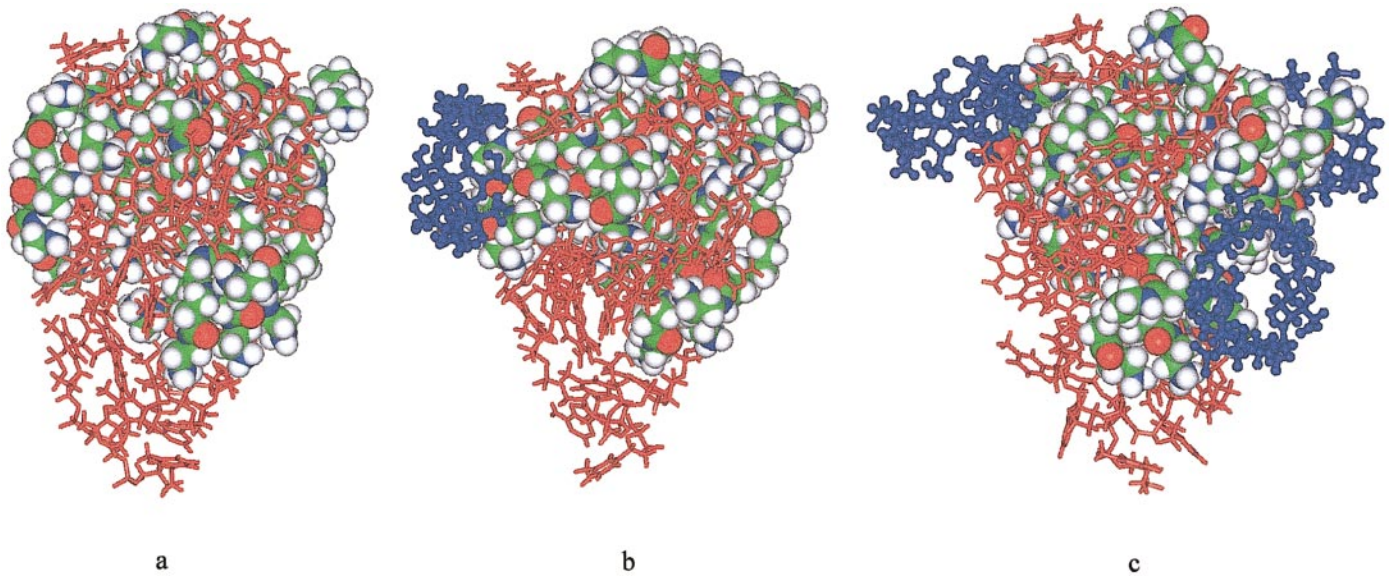

FIG. 4. Molecular dynamics simulation of the dendrimer/DNA complex with β-CD. The final configuration of (a) G3 EDA PAMAM dendrimer and double-stranded 12 bp DNA after 50 ps simulation; (b) one β-CD attached to the G3/DNA complex after 100 ps simulation of 11 β-CDs added to the model; (c) three S β-CD attached to the G3/DNA complex after 100 ps simulation with 11 S β-CDs added to the model. Dendrimers are represented as multicolor spheres (white, green, red, and blue) present within the center of the multimolecular complex. DNA molecules are represented as a scaffold of interconnected rods (red) and cyclodextrin molecules are represented as groups of aggregated knobs (blue).

β-CD monomers alone do not form complexes with DNA that facilitate efficient gene transfer to cells.

Molecular Dynamic Simulation

For the molecular dynamic (MD) simulations of potential tripartite interactions between dendrimer, DNA and cyclodextins; the G3 EDA dendrimer and 12 bp DNA molecules were utilized. The monomeric unit of G3 EDA/DNA complex after 50 ps simulation is shown in Fig. 4a. One to eleven molecules of β-CD were added to this model surrounding the complex and then simulated for 100 ps. Figures 4b and 4c represent final structures of the tripartite complexes simulated with eleven β-CD and (S) β-CD, respectively. The noncharged β-CD does not contribute to the complex when a small number of β-CD was added to the simulation. Among 11 β-CD, only one particle is attached at the random position on the G3 EDA/DNA complex (Fig. 4b). This effect was confirmed by simulation with a different initial configurations (result not shown here). The S β-CD specifically binds to the dendrimer component of the complex. The direct contact of S β-CD with E3 EDA/DNA complex increased from 1 to 3 molecules when 4 to 11 S β-CD were added to the model (Fig. 4c). Interestingly, the MD modeling performed at the variety of conditions revealed that neither A nor S β-CD accommodates any part (branch) of G3 EDA dendrimer or DNA chain into their intramolecular cavities.

The electrostatic interaction between G3 EDA and DNA was calculated in relation to the number of β-CD molecules. After the intermolecular energies within several ranges (1 to 26 Å) of the G3 EDA and DNA interface were calculated using the software docking module, the Coulomb energies within 8 Å range of the dendrimer/DNA interfaces were presented as a function of the β-CD number (Fig. 5). Nonmodified or amphoteric β-CD had little effect on Coulomb energy between dendrimer and DNA, and only a slight reduction of energy was observed with β-CDs within the simulation conditions. However, S β-CD significantly reduced Coulomb interaction between G3 EDA dendrimer and

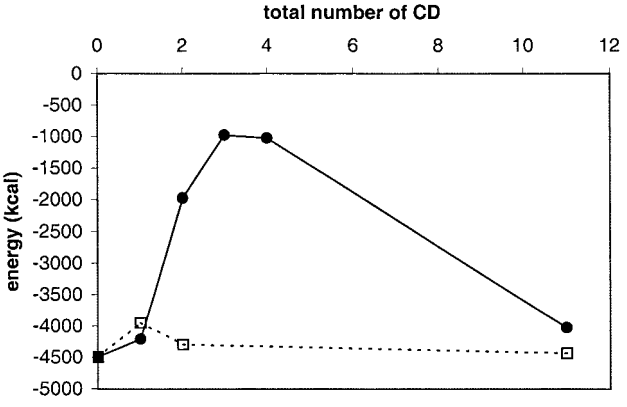

FIG. 5. Calculated energy of dendrimer–DNA interaction in the presence of β-CD. Coulombian energy between G3 dendrimer and 12-bp double-stranded DNA within 8 Å range of the G3/DNA interface as a function of the total number of S β-CD (●), and β-CD (□) in the simulations.

DNA as the total number of S β-CD in the model increased, except in the case of 11 S β-CDs. When 11 S β-CDs were utilized in the model, 3 of the molecules interacted with the G3/DNA complex with some of the S β-CDs located very close to the G3/DNA interface (Fig. 4c). The S β-CDs within 8 Å range of the G3/DNA interface seemed to contribute an additional Coulomb energy for the tripartite complex. This leads to stronger intermolecular interaction then with fewer S β-CDs in the simulation, which is reflected in the dramatic increase reaching the initial level of energy (−4000 vs −4500 kcal). The incorporation of S β-CD to the G3/DNA interface with a larger number of S β-CD molecules might disrupt the dendrimer/DNA interface and result in the dissociation of the complex. This result of molecular simulation is consistent with data in Fig. 1.

In this report we provide initial characterization of interactions between dendrimer/DNA complexes and β-cyclodextrins. Depending on the chemical modifications and concentrations, β-CDs affect the physicochemical properties of dendrimer/DNA complexes. The improvement of *in situ* transfection indicates the potential for development of novel multipolymer formulations including PAMAM dendrimers, β-CDs and plasmid DNA for solid support gene delivery.

REFERENCES

1. Tomalia, D. A., Naylor, A. N., and Goddard, W. A., III (1990) *Agnew. Chem.* **29,** 138–175.
2. Kukowska-Latallo, J. F., Bielinska, A. U., Johnson, J., Spindler, R., Tomalia, D. A., and Baker, J. R., Jr. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4897–4902.
3. Bielinska, A. U., Chen, C., Johnson, J., and Baker, J. R., Jr. (1999) *Bioconj. Chem.* **10,** 843–850.
4. Hudde, T., Rayner, S. A., Comer, R. M., Weber, M., Isaacs, J. D., Waldmann, H., Larkin, D. F., and George, A. J. (1999) *Gene Ther.* **6,** 939–943.
5. Kukowska-Latallo, J. F., Raczka, E., Quintana, A., Chen, C., Rymaszewski, M., and Baker, J. R., Jr. (2000) *Hum. Gene Ther.* **11,** 1385–1395.
6. Eichman, J. D., Bielinska, A. B., Kukowska-latallo, J. F., and Baker, J. R., Jr. (2000) *PSTT* **3,** 232–245.
7. Bielinska, A. U., Yen, A., Wu, H. L., Zahos, K. P., Sun, R., Weiner, N. D., Baker, J. R., Jr., and Roessler, B. J. (2000) *Biomaterials* **21,** 877–887.
8. Uekama, K., Hirayama, F., and Irie, T. (1998) *Chem. Rev.* **98,** 2045–2076.
9. Bibby, D. C., Davies, N. M., and Tucker, I. G. (2000) *Int. J. Pharm.* **197,** 1–11.
10. Connors, K. A. (1997) *Chem. Rev.* **97,** 1325–1357.
11. Irie, T., and Uekama, K. (1997) *J. Pharm. Sci.* **86,** 147–162.
12. Freeman, D. J., and Niven, R. W. (1996) *Pharm. Res.* **13,** 202–209.
13. Bielinska, A. U., Kukowska-Latallo, J. F., and Baker, J. R., Jr. (1997) *Biochim. Biophys. Acta* **1353,** 180–190.
14. Yew, N. S., Wysokenski, D. M., Wang, K. X., Ziegler, R. J., Marshall, J., McNeilly, D., Osburn, W., and Cheng, S. H. (1997) *Hum. Gene Ther.* **8,** 575–584.
15. Raczka, E., Kukowska-Latallo, J. F., Rymaszewski, M., Chen, C., and Baker, J. R., Jr. (1998) *Gene Ther.* **5,** 1333–1339.
16. Leiterman, R. V., Mulski, M. J., and Connors, K. A. (1995) *J. Pharm. Sci.* **84,** 1272–1275.
17. Gonzales, H., Hwang, S. J., and Davies, M. E. (1999) *Bioconj. Chem.* **10,** 1068–1074.