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Sunflower-Shaped Cyclodextrin-Conjugated Poly(ε-Lysine) Polyplex as a Controlled Intracellular Trafficking Device

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Gene therapy is being developed to ameliorate acquired and inherited diseases in a straightforward manner by adding, correcting, or replacing genes.[1,2] Nonviral polymeric gene carriers allow the delivery of therapeutic genes that can be tailored to increase both cellular uptake and transfection efficacy.[3,4] Recently, cyclodextrin (CD) containing polymers have been used as nonviral vectors due to their low cytotoxicity and their ability to modify the polyplex by inclusion complexation. Uekama et al. suggested the potential of CD-dendrimer conjugates as gene-transfer vectors showing much higher transfection efficiency than dendrimers.[5] Davis et al. have studied the use of CD-containing poly(ethylenimine)s (PEIs) for polyplex forma-

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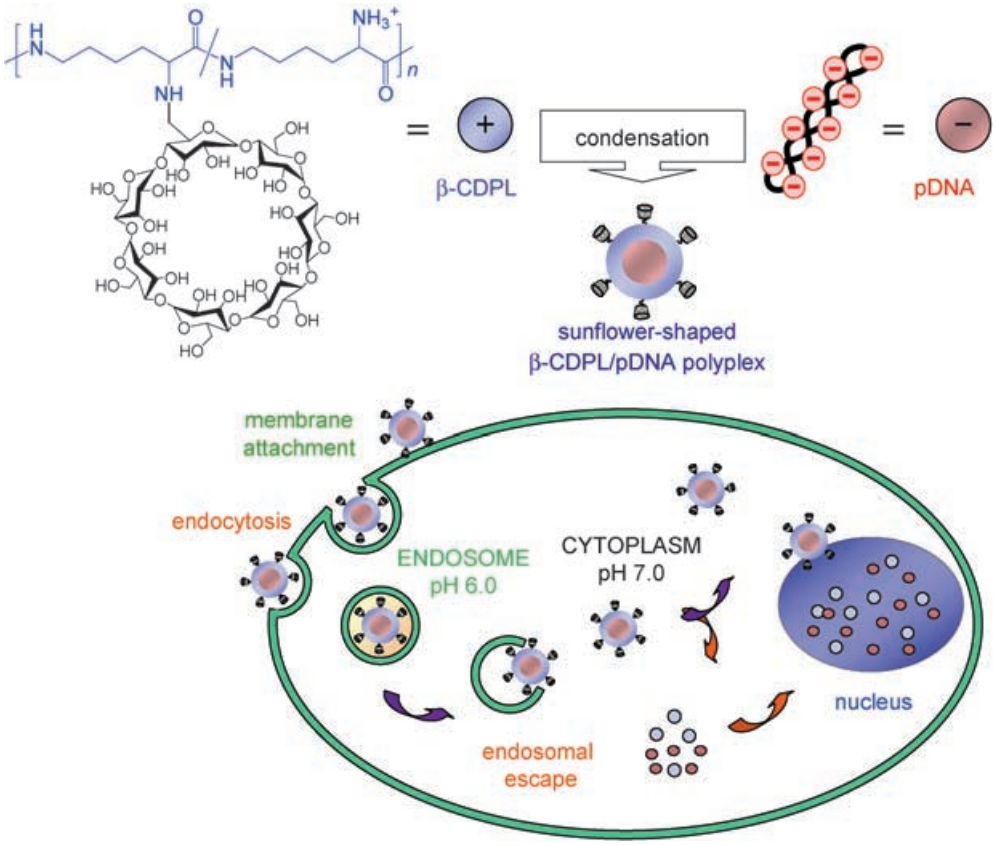
tion with plasmid DNA (pDNA) through their electrostatic interactions.[6] Here we suggest a new strategy for effective gene trafficking using a sunflower-shaped β-CD-conjugated poly(ε-lysine) (β-CDPL) polyplex. The β-CDs facing the outside of the polyplex promote the removal of cholesterol from the cell membrane, this introduces local membrane disturbances and assists the transfer of pDNA into cells, either by endocytosis or by endosomal release into the cytoplasm.[7] Furthermore, we propose that the secondary amines of β-CDPL employ a proton-sponge effect to produce significantly enhanced transfection.

In this study, we sought to test the hypothesis that efficient gene delivery could be mediated by β-CDPL/pDNA complexes that have been designed to promote the efficient escape of pDNA from early endosomes in the endocytosis pathway (Scheme 1).[8,9] We anticipated that this might be induced by the β-CD side chains on the polycations, since the stripping of cholesterol and phospholipids from endosomal membranes would exert membrane-disrupting effects on the endosome.[10] In endosomes in which the pH drops from 7.4 to 6.0, the β-CDPL/pDNA complex forms more condensed particles because the β-CDPL shows pH-dependent complexation with negatively charged guests.[8] Furthermore, the buffering effect of the secondary amines in the β-CDPL synergistically induces membrane destabilization through osmotic swelling arising from the proton-sponge mechanism.[11] This approach could significantly enhance the nuclear delivery of transfection activity of β-CDPL polyplexes relative to linear PEI (LPEI).

We assumed that, during polyplex formation, the pDNA would be condensed by the cationic β-CDPLs such that the β-CD side chains would face the out from the polyplex surface like a sunflower, where they would be free to interact by cholesterol binding and efflux. In order to verify this hypothesis, a hydrophobic fluorescence probe was bound to the surface of the rhodamine-labeled pDNA/β-CDPL polyplex, and the complexation geometry was observed by confocal-laser scanning microscopy (CLSM) and fluorescence spectroscopy (see Figure S4 in the Supporting Information). TNS (6-*p*-toluidino-2-naphthalenesulfonate) forms a stable inclusion complex with β-CD in a manner similar to cholesterol.[12] CLSM images show that the periphery of the polyplex produces blue TNS fluorescence that is localized near a red fluorescence arising from the rhodamine-labeled pDNA. In addition, the fluorescence intensity of the TNS–β-CDPL–pDNA complex significantly increased due to the inclusion complexation between the hydrophobic part of TNS and the outwards-facing β-CD cavities. Considering the chemical rationale, the CLSM data strongly support our initial hypothesis that the majority of the β-CD cavities are located on the outer surface of the polyplex (sunflower shape).

The β-CDPL polyplexes were characterized by dynamic light scattering (DLS) and zeta (ζ) potential measurements at different pHs to reveal the effect of environmental pH on polyplex size and surface charge, factors that will affect their fate during intracellular trafficking. As shown in Figure 1 A, PL polyplexes do not show any pH-dependent complexation in the range of pH 6.0–7.4 because the pK_a of the primary amines in PL is



Scheme 1.

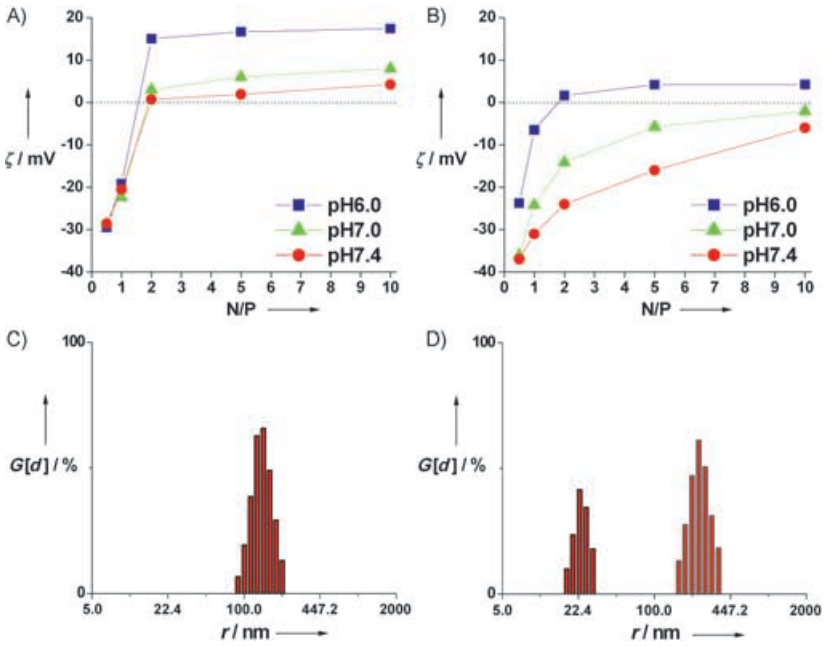


Figure 1. Zeta potentials of the polyplexes transfected with A) PL and B) β-CDPL, and DLS data from β-CDPL polyplexes (N/P = 10) at C) pH 6.0 and D) 7.4. The solutions containing the polyplexes were prepared in a 10 mM phosphate buffer after 20 min incubation at room temperature.

about 9.0. The ζ potentials increased upto N/P = 2, at which point the values show similar tendency; this means that the polyplex surface at N/P = 2 is almost occupied by β-CDPL. Above N/P = 2, the difference results from the degree of protonation of the primary amines of PL. On the other hand, the ζ potentials of the β-CDPL polyplexes were negative at all N/P ratios in pH 7.4 medium (Figure 1B); this indicates that all available primary amines were charge balanced by the phosphate groups of DNA. The ζ potentials at pH 6.0 were positive above N/P ratios of 2; this suggests that the secondary amines are protonated ($pK_a = 6.35$, see Figure S3 in the Supporting Information), while at pH 7.0, the β-CDPL polyplexes were negatively charged due to deprotonation of the β-CDPL secondary amines. The hydrodynamic diameter of the β-CDPL polyplexes at N/P 10 was also measured by DLS. At pH 6.0 the diameter was very small (ca. 150 nm, Figure 1C); this suggests a tight condensation between the protonated amines and pDNA, whereas two diameters of about 25 and 284 nm were observed at pH 7.4 (Figure 1D). The implication of these data is that the tightly packed β-CDPL polyplexes in acidic endosomes (pH 6.0) would be weakened once they are released into the cytoplasm, since the ζ potential of the β-CDPL polyplex was negative at pH 7.0 (Figure 1B). The large particles represent loosely condensed β-CDPL polyplexes, while the small ones match the decondensed β-CDPL aggregates.[13] These pH-dependent properties could significantly affect the intracellular trafficking of the β-CDPL polyplexes within cells that have internalized them by endocytosis.

To address the impact of β-CDPL as a programmed packaging device for efficient gene therapy,[14] the time-dependent changes in cellular uptake and intracellular trafficking of the β-CDPL polyplexes were observed by CLSM imaging of NIH-3T3 cells.[15] The microscopy data show that β-CDPL polyplexes are efficiently internalized even at short incubation times (Figure 2B–D). After a 0.5 h of transfection, significant cellular uptake was observed as numerous discrete yellow patches in the endosome/lysosome: the Lysosensor and the rhodamine-labeled pDNA yield yellow spots due to colocalization of the green and red fluorescence. On the other hand, PL polyplexes (Figure 2A) were localized at the cell surface after 1.5 h, with limited internalization occurring after 3.0 h. The efficient cellular uptake of the β-CDPL polyplexes was also quantitatively confirmed by fluorescence-based flow cytometry (Figure 2F). When pDNA was condensed with β-CDPL, the cellular association was enhanced by nearly one order of magnitude relative to free pDNA. Considering the previous reports showing that β-CDs possess hemolytic and membrane-disrupting activity toward erythrocytes through incorporation of cholesterol and phospholipids,[7,10] we propose a synergistic action of the surface-exposed β-CDs as endosomal membrane-destabilizing agents and the complexation behavior of the cationic β-CDPL, which together lead to increased uptake of pDNA within the cells.

After internalization by endocytosis, the polyplexes have to escape the endosome and enter the cytosol before lysosomal degradation. As shown in Figure 2, the large and numerous red fluorescent spots observed in the β-CDPL polyplexes after a 1.5 h of incubation indicate that the pDNA has extensively escaped from the endosome/lysosome. The multifunctional character of β-CDPL could account for the highly efficient endosomal escape properties; membrane disruption by cholesterol extraction, and endosomal release through the proton sponge effect (see Figures S1–S3 in the Supporting Information).

In the 3.0 h CLSM images, efficient perinuclear accumulation was observed for the β-CDPL/pDNA complexes. Specifically at N/P = 5 and 10, colocalization of pDNA is clearly observed as pink spots within the nuclei. Recently, we reported that LPEI is potent in transfecting cells in the G0/G1 phase when the nuclear membrane is intact; this suggests that the secondary amines of LPEI can function as a nuclear localization signal (NLS).[16] β-CDPLs possess similar chemical characteristic to LPEI, such as polyvalent secondary amines, which might make them function like a NLS. The less effective nuclear colocalization of β-CDPL polyplex at N/P = 2 can be explained as a weak membrane-destabilizing activity due to the reduced number of β-CD moieties on the particle surface. An alternative explana-

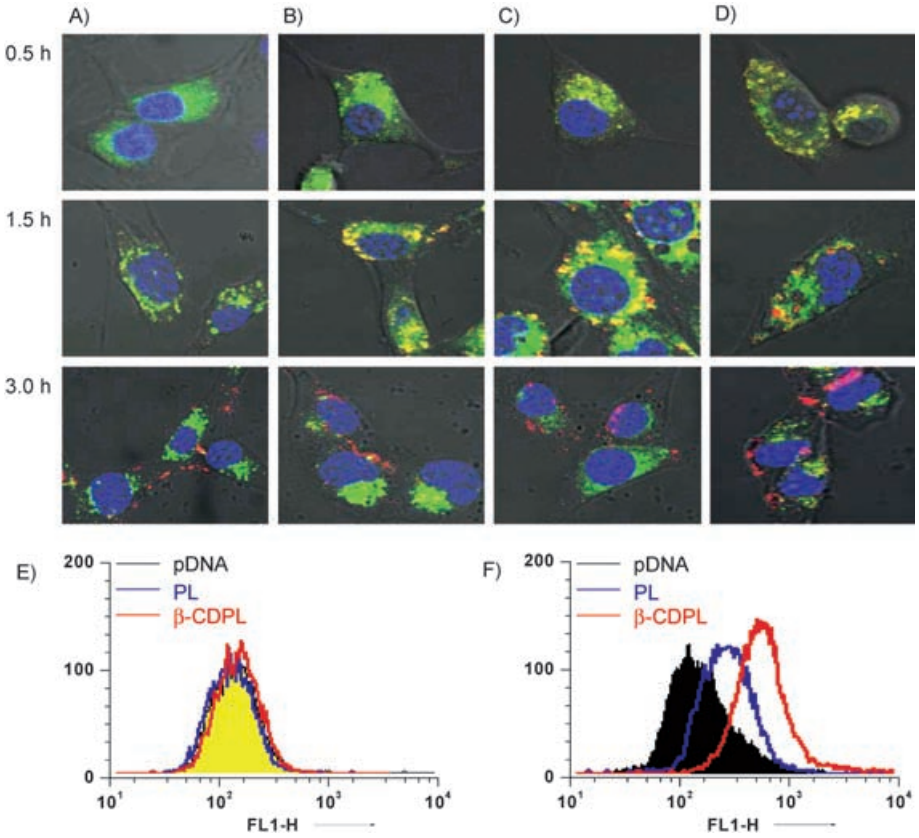


Figure 2. CLSM images for the pDNA polyplexes after 0.5, 1.5, or 3.0 h of transfection; A) PL with N/P = 5, B) β-CDPL with N/P = 2, C) β-CDPL with N/P = 5, and D) β-CDPL with N/P = 10. A total of 2.0 μg pDNA was transfected into 1×10^5 NIH-3T3 cells per dish. Endosome/lysosome (green) was stained with Lysosensor, and the blue fluorescence shows the Hoechst 33258-stained nuclei. Efficient polyplex-mediated uptake of pDNA into NIH-3T3 cells was obtained at E) 4 and F) 37 °C. The influence of preincubation with serum on the cellular association of the rhodamine-labeled pDNA was measured with a flow cytometer 1.5 h after transfection (N/P = 5).

tion for this effect is the inhibition of nuclear approach by the electrostatic interaction between the negative ζ potential of the β-CDPL polyplexes and the negatively charged nuclear membrane lipids.

The transfection activities of β-CDPL polyplexes were compared with those of PL and LPEI at different N/P ratios. As shown in Figure 3A, PL induced very low luciferase activity regardless of the N/P ratios; however, the β-CDPL polyplexes at both N/P = 5 and 10 showed a transfection activity that was four orders of magnitude higher than that of PL and 10 times higher than that of LPEI. This suggests that efficient nuclear transfer might be responsible for the high transfection activities observed for the β-CDPL polyplexes. We attribute this enhancement to the synergistic action of the proton-sponge mechanism, which arises from the protonation of the secondary amines, and the membrane destabilization properties of β-CD. In addition, the cytotoxicity of the polyplexes was tested by MTT assay in terms of the N/P ratio. As shown in Figure 3B, the cell viability of the PL homopolymer gradually decreases with the N/P ratio and shows a sharp decrease to under 50% at N/P = 50. However, for β-CD substitutions onto the PL homopolymer, β-CDPL shows an interesting and remarkable decrease in cellular toxicity due to the low toxicity of the CDs as well as the reduced charge density.[5,6] Thus, the introduction of β-CDs into the polycation backbone led to significant enhancements of the cellular uptake, endosomal escape, nuclear transfer, and cell viability of the β-CDPL polyplexes in spite of the lower number of cationic groups than in the PL homopolymer.

In conclusion, the CLSM and fluorescence spectroscopy evidence support our initial hypothesis that β-CDs face outwards from the polyplex like a sunflower and that this directly affects cell attachment and endosomal release through a membrane-disrupting mechanism. Furthermore, the newly developed secondary amines induced a buffering proton-sponge effect by β-CD conjugation, which might synergistically facilitate the rapid endosomal release. The high cellular uptakes and the low toxicity of this β-CDPL make it a promising molecular template with great potential as an intracellular trafficking device.

Experimental Section

Synthesis of β-CDPL. β-CDPL was made by a coupling reaction between 6-deoxymonoaldehyde-activated CD (ald-CD) and PL (M_w = 4000, DS = 33.2%) as a programmed packaging device for an efficient gene delivery.[14] The primary amino groups in the PL (α-position, pK_a = 9.0) were used for the complexation with pDNA, while proton-sponge effects result from the increased secondary amino groups (pK_a = 6.35) through CD conjugating to the primary amines of the PL.[17] The detailed synthetic method for β-CDPL can be found in the Supporting Information.

Cell culture, transfection, and luciferase assay. NIH-3T3 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum at 37 °C under 5% CO_2 in air. For luciferase assays, NIH-3T3 cells in 24-well plates were seeded at 4×10^4 cells per well and transfected with pDNA (0.4 μg per well) suspended in DMEM (0.25 mL) without serum, then incubated for 3 h at 37 °C. The cells were then washed with PBS, and DMEM (1 mL with 10% serum) was added to the cells, followed by a further incubation for 21 h. The cells were extracted with a reporter lysis buffer (Promega, Madison, WI) and assayed by using a luminometer (Luminescencer-PSN, ATTO, Japan). The amount of protein was determined by using a BCA protein assay kit (PIERCE, Rockford, IL). Each construct within an experiment was transfected in triplicate, and the summarized data are the result of at least three separate transfection experiments.

CLSM and flow cytometry. For the CLSM experiments, rhodamine-labeled NIH-3T3 cells were treated with different N/P ratios for dif-

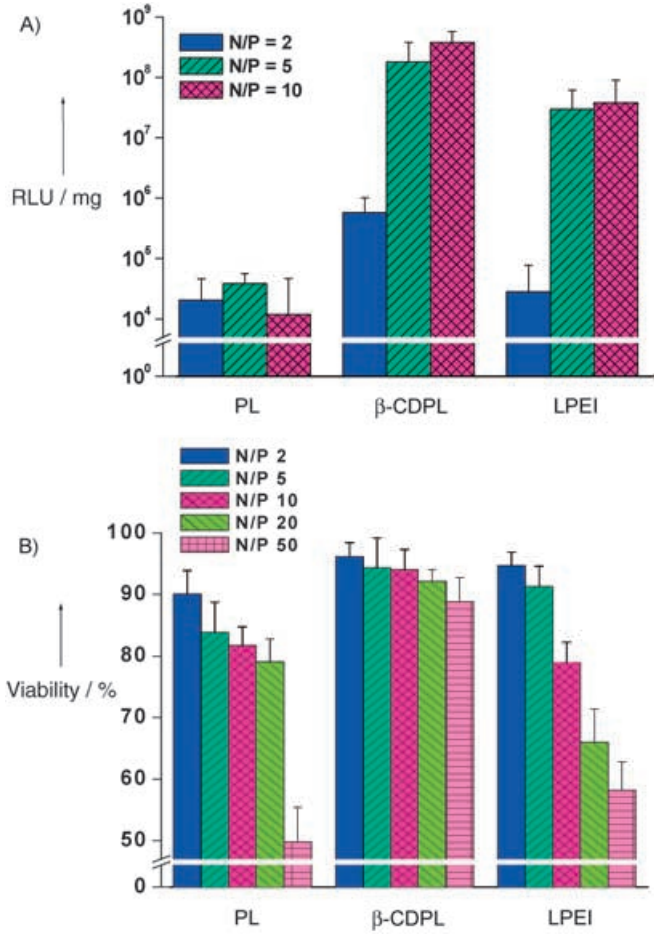


Figure 3. A) Transfection efficiency for pDNA polyplexes in NIH-3T3 cells and B) subsequent viability of these cells. The polyplexes were transfected for 24 h with PL, β-CDPL, and LPEI with varying N/P ratios ($n = 5$). The transfection results were expressed as light units integrated over 10 s per mg of cell protein by using the BCA assay. All transfection experiments were carried out in duplicate and were repeated at least three times to confirm their reproducibility.

ferent incubation times (0.5, 1.5, and 3 h). Endosome/lysosome and nuclei of NIH-3T3 cells were stained with Lysosensor DND-189 (1 μM; Molecular Probes, OR) and Hoechst 33258 (30 μM; Wako Chemical, Osaka, Japan), respectively. Fluorescence and bright-field images were captured by using a Zeiss Axiovert 200 inverted fluorescence microscope equipped with a confocal microscope Zeiss LSM 510 (Carl Zeiss Co. Ltd., Jena, Germany). For flow cytometry, the rhodamine-labeled pDNA was mixed with polycations and added to the NIH-3T3 cells seeded at 5×10^5 cells per dish for transfection in DMEM (10 mM) at pH 7.4. After 1.5 h incubation at 4 and 37 °C, the fluorescence of the cells was quantified by flow cytometry (FACScan, Becton Dickinson).

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[13] To prepare a stable polyplex, β-CDPL was dissolved in PBS at pH 6.0 and mixed with pDNA solution; this mixture was then diluted tenfold in PBS at pH 7.4. We confirmed that the polyplex was stable under these condition, in spite of the deprotonation of some of the β-CDPL secondary amines (Figure 1d). Since the DLS data for the β-CDPL polyplexes were obtained under cation-rich conditions (N/P 10) there is a surplus of cations that are not involved in polyplex formation; this results in the split peaks; see H. S. Choi, T. Ooya, K. M. Huh, N. Yui, *Biomacromolecules* **2005**, *6*, 1200.

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