Cationic Star Copolymers Based on β-Cyclodextrins for Efficient Gene Delivery to Mouse Embryonic Stem Cell Colonies**

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Experimental Section

Materials. The cell culture medium used was Glasgow Minimum Essential Medium (GMEM) (Sigma-Aldrich) supplemented with 2mM L-glutamine, 1mM non essential amino acids (Lonza), 0.5 mM β-mercaptoethanol (GIBCO), 10% (vol/vol) foetal bovine serum (FBS) and 1000 units/ml of LIF (Chemicon). All media contained 1mM sodium pyruvate (GIBCO) and 1X penicillin-streptomycin (Wako). β-Cyclodextrin (98%) was purchased from Sigma–Aldrich Chemical Co., Milwaukee, WI. and used after recrystallization from water and drying at 100 °C under vacuum. (2-Dimethylamino)ethyl methacrylate (DMAEMA, >98%) and 2-hydroxyethyl methacrylate (HEMA, 97%) were obtained from Sigma–Aldrich Chemical Co., Milwaukee, WI. They were used after removal of the inhibitors in a ready-to-use disposable inhibitor-removal column (Sigma–Aldrich). Copper(I) bromide (Fluka, 98%) was washed with glacial acetic acid in order to remove any soluble oxidized species, filtered, washed with ethanol, and dried. 1,1,4,7,10,10-Hexamethyl triethylenetetramine (HMTETA, 99%), 2-Bromo-isobutyric bromide (99%) and anhydrous N,N-dimethylacetamide (99%) were obtained from Sigma–Aldrich Chemical Co., Milwaukee, WI. All reagents were used as received without further purification unless otherwise stated.

Synthesis of β-CD-Based Macroinitiator (4Br-β-CD). The β-CD-based macromonomer was synthesized following a similar method to previous reports. β-CD (5.11 g, 4.5 mmol, vacuum dried at 100 °C overnight before use) was dissolved in 30 mL of anhydrous N,N-dimethylacetamide with stirring and then was cooled to 0 °C. Subsequently, a solution of 2-bromo-isobutyric bromide (4.14 g, 18.0 mmol) in anhydrous N,N-dimethylacetamide (10 mL) was added dropwise to the β-CD solution for a period of 1 h at 0 °C under N_2 atmosphere. After the addition, the reaction temperature was maintained at 0 °C for another 2 h and then allowed to rise slowly to r.t. (room temperature), after which the reaction was allowed to continue for 24 h. The final reaction mixture was precipitated with 900 mL of diethyl ether. The resulting white powder was collected by centrifugation, washed with acetone (2×30 mL). In the purification process, the crude product was suspended in 100 mL of DI water, and the

mixture was stirred at r.t. overnight. The purified product was collected by centrifugation, washed with acetone (2×30 mL), dried under vacuum. Yield = 67.8%. ^{1}H NMR (400 MHz, DMSO-d6, δ): 1.88 (m, 24H, -OCO-C(CH3)2Br), 3.00-6.00 (m, 66H, -OH and -CH- of β-CD)

Synthesis of β-CD-Core Star Polymer, β-CD-P(DMAEMA-co-HEMA)$_4$ via ATRP Method. As an example, the synthesis of H3 is presented. Macroinitiator 4Br-β-CD (0.10 mmol) and DMAEMA and HEMA (2.0 mmol each) were added into a dry flask. The flask was sealed with a septum and subsequently purged with dry nitrogen for several minutes. Then, HMTETA (0.48 mmol) in 3.5 mL of thoroughly degassed isopropanol was added with a degassed syringe. The mixture was stirred at room temperature and purged with dry nitrogen for 10 min. Last, CuBr (0.4 mmol) was added and the mixture was purged with dry nitrogen for another 10 min at room temperature. The mixture was then heated at 45 °C in an oil bath. After 4h, the experiment was stopped by opening the flask and exposing the catalyst to air. The final green mixture was diluted with THF and passed through a short Al_2O_3 column to remove copper catalyst. The resulting eluate solution was concentrated to ca. 10 mL, and precipitated with 500 mL diethyl ether. The white product was collected by centrifugation, washed with diethyl ether, and dried under vacuum. Yield = 20.7 % after purification.

Characterization of Polymer/pDNA Nanocomplexes. Plasmid pRL-CMV encoding Renilla luciferase was purchased from Promega Co. (Cergy Pontoise, France). The plasmid DNA (pDNA) was amplified and further purified. 0.5 mg/mL pDNA in tris-EDTA (TE) buffer and polymer solutions with a nitrogen concentration of 10 mM in highly purified water were prepared before every use. Star-shaped polymers to DNA ratios were expressed as molar ratios of nitrogen (N) in the copolymers to phosphate (P) in pDNA (N/P ratios). An average mass weight of 325 per phosphate group of DNA was assumed. All polymer/pDNA nanocomplexes at different N/P ratios were formed by mixing certain volume of polymer solution and pDNA solution homogeneously and further incubated for 30 min at ambient temperature. The zeta potentials and particle sizes of the nanocomplexes were determined by a Zetasizer Nano ZS (Malvern). The atomic force microscopy (AFM) system was applied to observe the morphology of nanocomplexes and AFM images were obtained on a Nanoscope Multimode III AFM instrument (Vecco, USA) in tapping mode.

DNA binding experiments. Agarose gel electrophoresis was utilized to determine the ability of the copolymers to bind the pDNA into NPs. Gel electrophoresis was implemented in a Sub-Cell system (Bio-Rad Laboratories) and DNA bands were visualized and photographed by a UV transilluminator and BioDco-It imaging system (UVP Inc.).

Cell Viability. The cell viability of polymer/pDNA nanocomplexes at N/P ratios of 20 but at different concentrations in feeder free mouse embryonic stem cell (EB3) was determined by MTT assay. The absorbance was measured using a microplate reader (Spectra Plus, Tecan, Zurich, Switzerland) at a wavelength of 570 nm. The computational method of cell viability (%), relative to that of the control cells cultured in a medium without polymers, followed the formula $[A]_{test}/[A]_{control} \times 100\%$, where $[A]_{test}$ and $[A]_{control}$ are the absorbance values of the wells (with the nanocomplexes) and control wells (only with pDNA), respectively. The cell viability of each sample was obtained from the measurement of six wells in parallel.

***In Vitro* Transfection Assay.** Plasmid pRL-CMV and enhanced green fluorescent protein (EGFP) pDNA (BD Biosciences, San Jose, CA) as reporter genes were used in transfection studies in vitro. The cells were cultured for 24 h before adding different N/P ratios polymer/pDNA nanocomplexes. After incubator for 4 h under standard incubator conditions, the medium was replaced with fresh culture medium and cells were incubated for another 20 h, resulting in a total transfection time of 24 h. At the end of transfection, the cultured cells were washed with PBS and lysed with lysis reagent. Luciferase gene expression was quantified using a luminometer (Berthold Lumat LB 9507) and a commercial kit (Promega Co.). Bicinchoninic acid assay (Biorad Lab) was carried out to analyze the protein concentration in the cells. Results were expressed as relative light units (RLUs) per milligram of cell protein lysate (RLU/mg protein). EGFP pDNA, as another reporter gene, was also used for gene delivery assay. The procedure was similar to those mentioned above. Leica DMIL Fluorescence Microscope and flow cytometry (FCM, Beckman Coulter) were applied to observe and measure the percentage of the transfected cells.

ES cell culture. This embryonic stem cell line was derived from the 129/Ola mouse strain. In these cells, one of the Oct3/4 gene loci is disrupted by Ires-BSD knockout vectors. Cells are cultured in

medium consisting of GMEM supplemented with FBS (10%), NEAA (0.1mM), sodium pyruvate (1mM), LIF (1000U/ml), and 2-mercaptoethanol (0.1mM). Feeder free mouse embryonic stem cell (EB3) was used in all the cell culture studies. The ES cells were cultured on gelatin-coated plates in supplemented GMEM. The ES cells were cultured on the coated coverslips at a cell seeding density of 5 x 10^4 cells / coverslip and incubated at 37 ^oC in 5% CO_2, in humidified air. For maintenance of the ES cells, cells were passaged every 3 days using 0.25% trypsin-1.0 mM EDTA.

Figures

Fig S1. Electrophoretic mobility of pDNA in the nanocomplexes of the cationic polymers, H1-H3 compared with the control PEI.

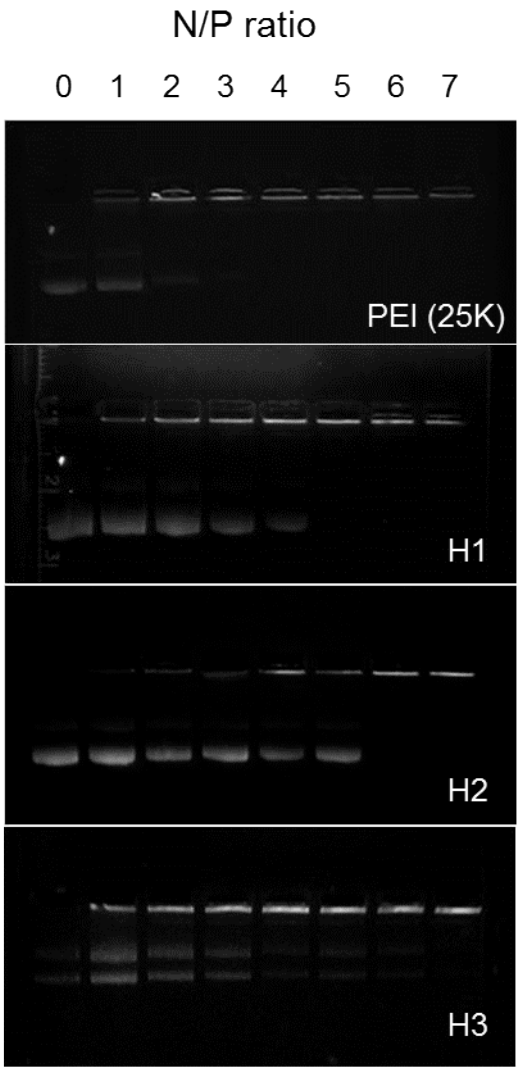

Fig S2. In vitro gene transfection efficiency of the cationic polymer/pDNA nanocomplexes at different N/P ratios in comparison with that of PEI (25KDa) (at the optimal N/P ratio of 10) in HEK293 cell lines

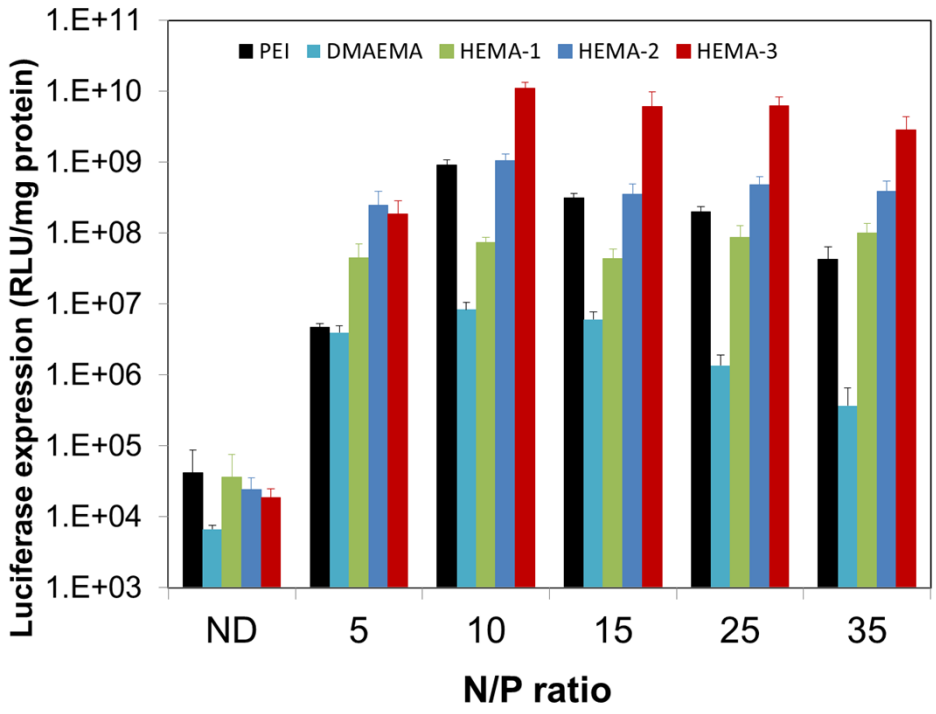

Fig S3. Cell viability of polymer/pDNA nanocomplexes at N/P ratio of 20 in feeder free mouse embryonic stem cells (EB3).