Supplementary Material (ESI) for Chemical Communications

Two Novel Nonviral Gene Delivery Vectors: Low Molecular Weight Polyethylenimine Cross-linked by (2-hydroxypropyl)-β-cyclodextrin and (2-hydroxypropyl)-γ-cyclodextrin

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Materials, Methods and Results

Materials

Polyethylenimine (PEI, MW 600 Da), Polyethylenimine (PEI, MW 25K Da), (2-hydroxypropyl)-β-cyclodextrin, (2-hydroxypropyl)-γ-cyclodextrin, (3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich. 1,1 –carbonyldiimidazole (CDI) was purchased from Merck Corporation. Plasmid DNA pGL3 and Luciferase Kit Assay were purchased from Promega Corporation (Madison, WI, USA). Endofree plasmid mega kit was purchased from Qiagen (Hilden, Gemany). SKOV-3 cell line was purchased from American Type Culture Collection (Rockville, MD).

Methods and Results

Synthesis of 2-hy-β-CD-PEI600 and 2-hy-γ-CD-PEI600

1. Synthesis of 2-hy-β-CD-PEI600 (Fig.1)

(2-hydroxypropyl)-β-cyclodextrin 0.51g (0.37 mmol) dissolved in 6 ml DMSO, added 1,1 –carbonyldiimidazole 0.49 g (3 mmol) (dissolved in 6ml DMSO) and 0.1 ml Et3N. The mixture was stirred in dark and at room temperature for 1.5h. Reaction system was protected by nitrogen. The hydroxyl group on the outside of 2-hy-γ-CD (Compound 1) were activated by CDI[1]. Product is 2-hy-γ-CD-CDI (Compound 2). The mixture was precipitated in cold ethylether, filtered, dissolved in 6 ml DMSO, stored at 4 ℃. Then 1.8 g (3 mmol) PEI600Da dissolved in 6 ml DMSO, the described 2-hy-β-CD-CDI in 6ml DMSO and 0.2 ml Et3N was added dropwise for over 2 h, with additional stirring for over 5 h. So the hydroxy group on the outside of 2-hy-β-CD was cross-linked with amino groups of PEI (Compound 3). The mixture was dialyzed with dialysis tube (MW 2,000) in running-water for two days and the aqueous solutions were lyophilized for three days. A white water-soluble solid was named 2-hy-β-CD-PEI600.

2. Synthesis of 2-hy-γ-CD-PEI600 (Fig.1)

(2-hydroxypropyl)-γ-cyclodextrin 0.58 g (0.37 mmol), 1,1 –carbonyldiimidazole 0.54 g (3.3 mmol), 2 g (3.3 mmol) PEI600 Da were used as described.

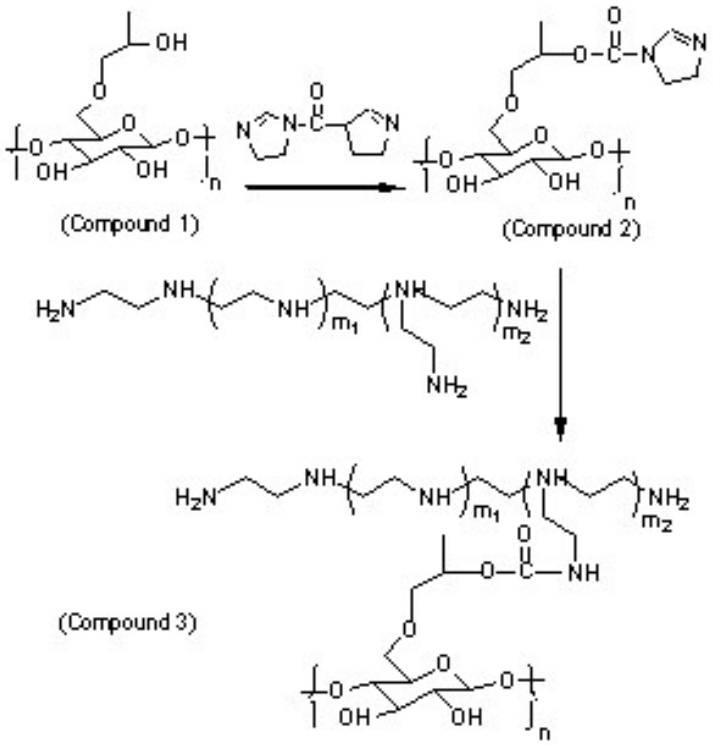


Fig. 1 Synthesis of the Compound 3 （n=7, Compound 3 is 2-hy-β-CD-PEI600 or n=8, Compound 3 is 2-hy-γ-CD-PEI600)..

Cell culture and plasmid preparation

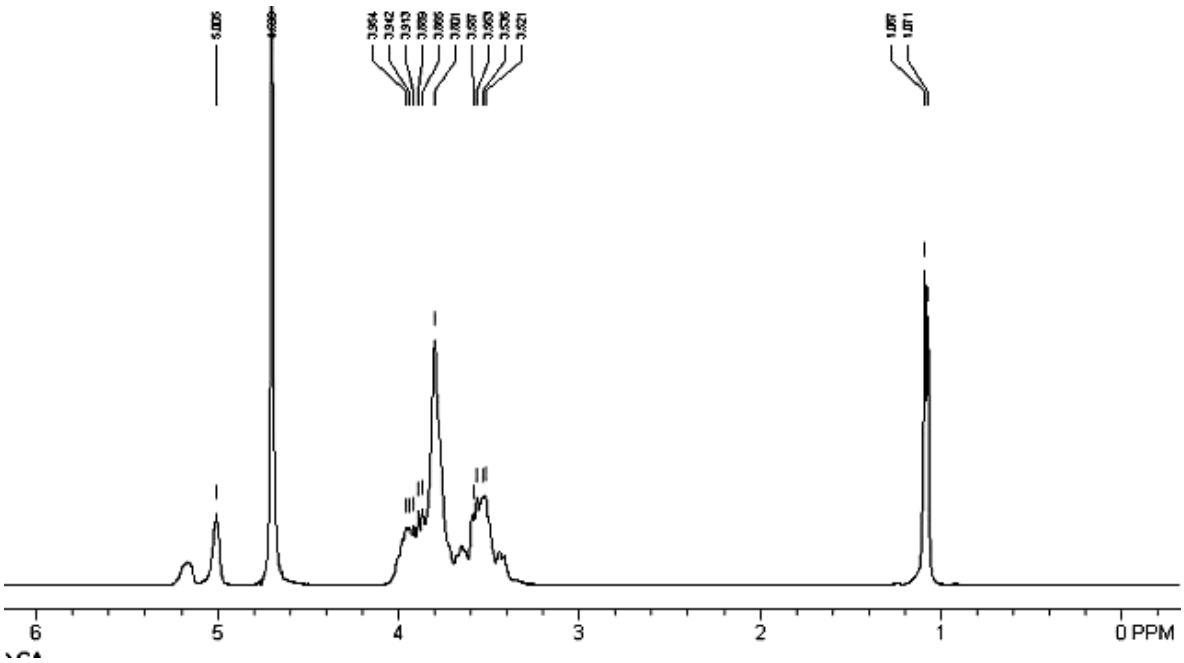
SKOV-3 cells were purchased from the ATCC and cultured in DMEM containing 10% fetal bovine serum (GIBCO Invitrogen Corp. Greenland NY, USA) and 100 units/ml penicillin in a 5% CO_2 humidified atmosphere at 37℃. The plasmids were amplified in E. *coli* and purified according the supplier's protocol (Qiagen, Hilden, Germany).

Measurement

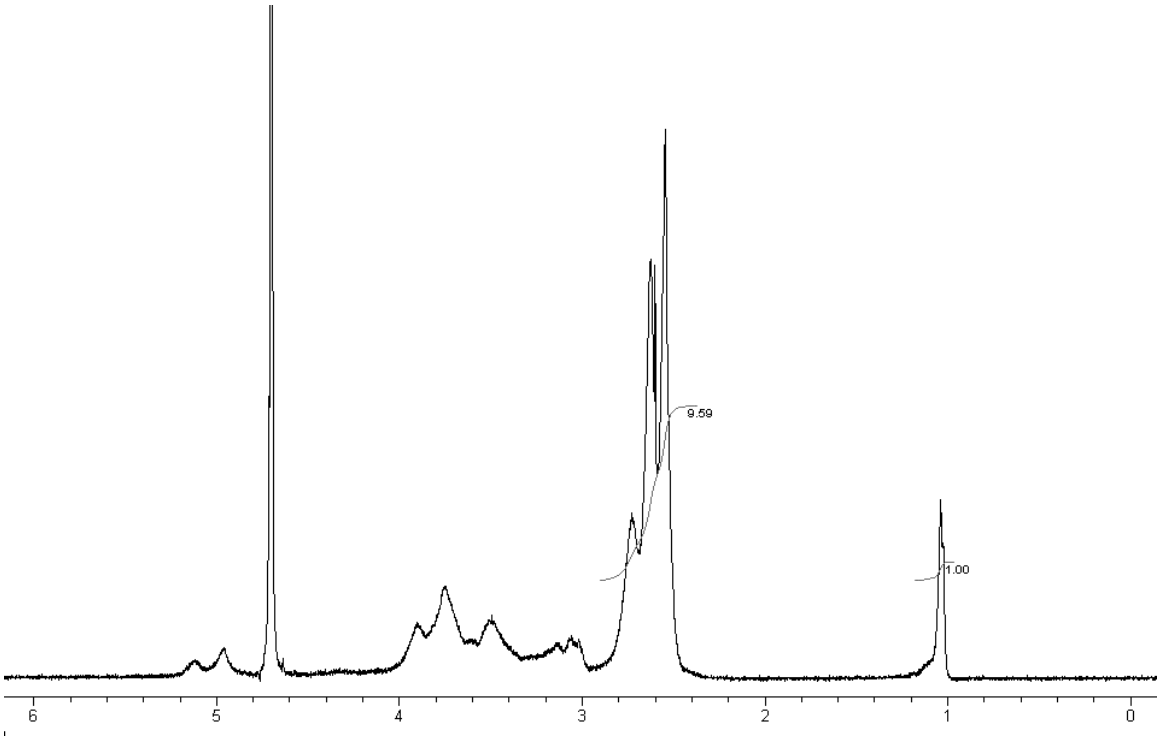
^{1}H NMR

The structure of 2-hy-β-CD-PEI600 and 2-hy-γ-CD-PEI600 were ascertained by ^{1}H NMR. The ratio of cyclodextrin and polyethylenimine of polymer sample was determined from ^{1}H NMR spectra using intergral values obtained for the CH_3 of hydroxypropyl of 2-hy-β-CD (or 2-hy-γ-CD) and CH_2CH_2NH- protons of PEI600Da. It was carried out with 10 mg 2-hy-β-CD-PEI600 and 2-hy-γ-CD-PEI600 sample put into 0.7 ml deuterium oxide (D_2O) in Varian 400MHz spectrometer with 32 scans at room temperature.

The ratio of cyclodextrin and PEI in new polymers was calculated based on the proton integral values of ^{1}H NMR δ1.038 ppm (CH_3 of hydroxypropyl), 2.4-3.0 ppm (CH_2 of PEI)[2]. It was found to be 1:3.3 for CD and PEI600 Da (molar to molar) (Fig.2, Fig.3).

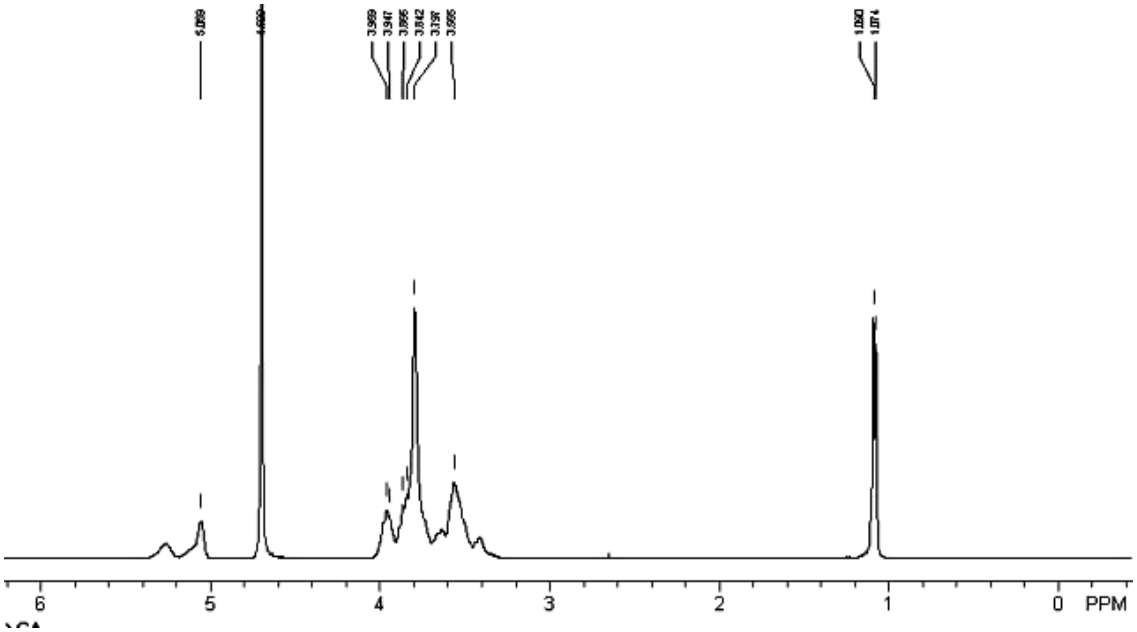


a

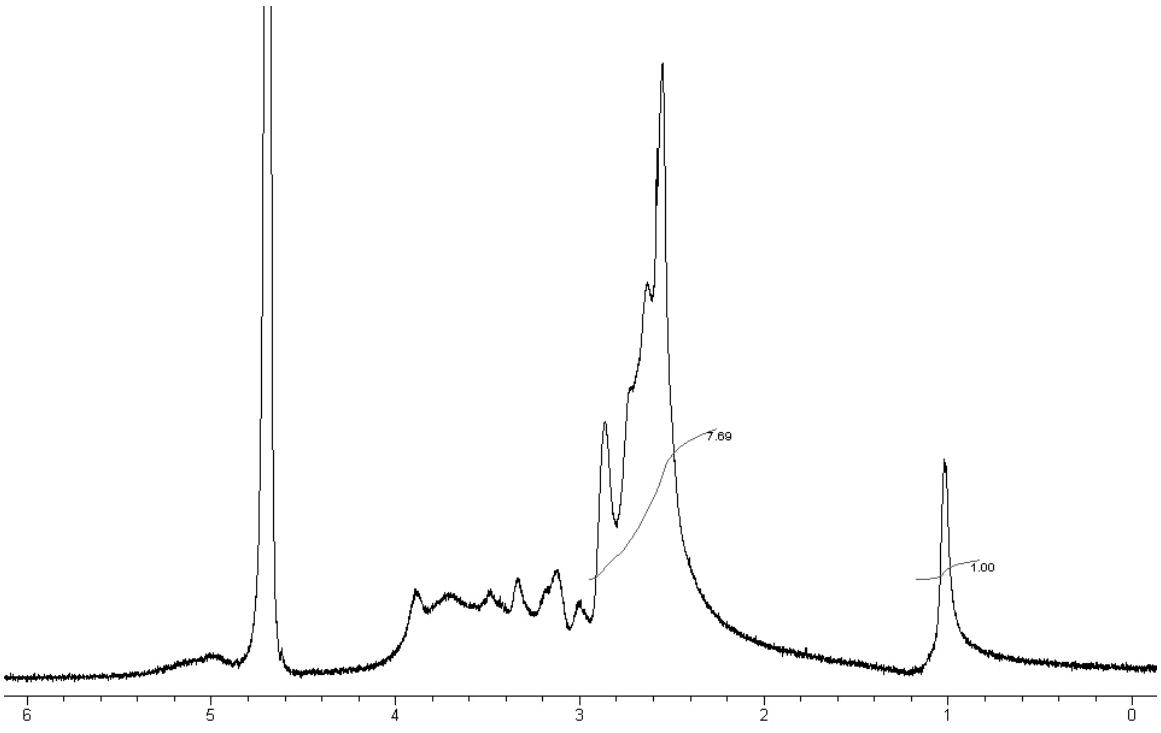


b

Fig. 2 ^{1}H NMR spectrum of **a**) 2-hy-β-CD and **b**) 2-hy-β-CD-PEI600.



a



b

Fig. 3 ^{1}H NMR spectrum of **a**) 2-hy-γ-CD and **b**) 2-hy-γ-CD-PEI600.

X-rays (XRD) Measurements

X-ray diffraction measurements were carried out using D/Max-2550 X-Ray Diffractometer, using Ni-filtered Cu Kα radiation (40 Kv, 300 mA). Powder samples were mounted on a sample holder and scanned in 0.02° steps from 3° to 50° (in 2θ) with 0.5 s per step.

There were two obviously peaks in the 2-hy-β-CD profile. It appeared at 2Θ= 12.7° and 19.1° (Fig 4.(1)b) . When forming 2-hy-β-CD-PEI600 polymer, only one peak existed in the profile. It was at 2Θ= 20°(Fig 4.(1)a). For 2-hy-γ-CD, there were two peaks at 2Θ= 9.5° and 17.2°(Fig 4.(2)b). But in 2-hy-γ-CD-PEI600 polymer, one peak was at 2Θ= 21°(Fig 4.(2)a). We think that in the synthesis polymer 2-hy-β-CD-PEI600 or 2-hy-γ-CD-PEI600 the extent of amorphous phase was increased.

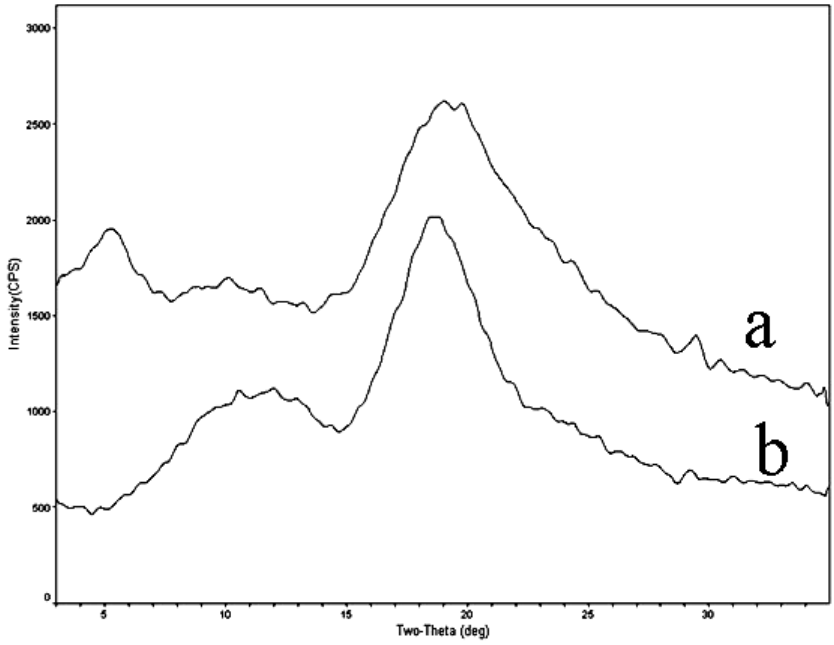


Fig. 4. (1) X-Ray of **a**) 2-hy-β-CD-PEI600, **b**) 2-hy-β-CD

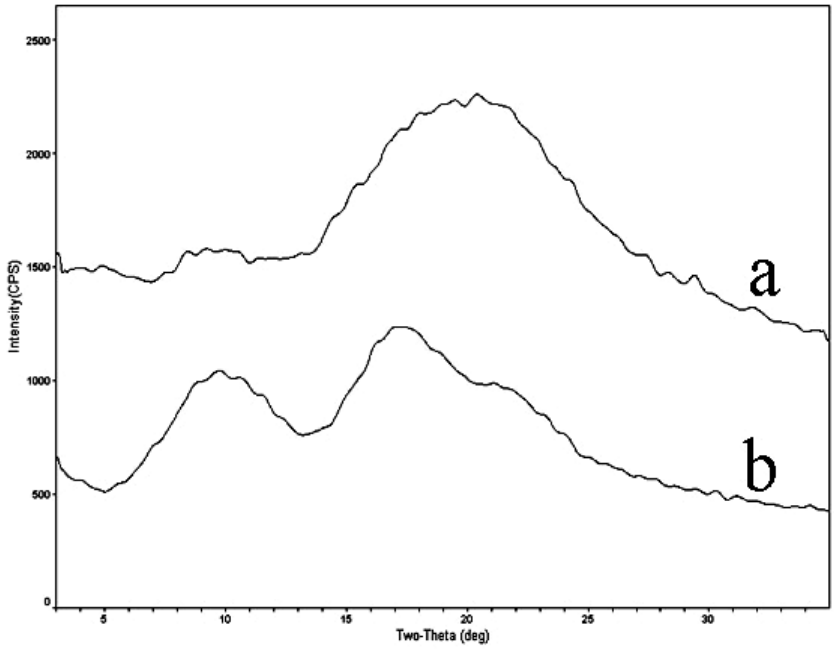


Fig. 4. (2) X-Ray of **a**) 2-hy-γ-CD-PEI600, **b**) 2-hy-γ-CD

Thermogravimetric Analysis (TGA)

TGA was made using a TA Instrument SDT 2960. Samples were heated at 10 °C/min from room temperature to 700 ℃ in a dynamic nitrogen atmosphere (flow rate = 70 ml/min).

The polymer was characterized thermoanalytically. The results were presented for homoPEI600, 2-hy-CD and 2-hy-CD-PEI600 in Fig.5. Fig.5(1)a) was 2-hy-β-CD, the temperature was at 340 °C. Figure 5(1) b was the homoPEI600, it degraded beginning at 250 ℃. Figure 5(1) c was 2-hy-β-CD-PEI600, three mainly steps could be observed in the thermogravimetric profiles of it comparing with PEI600 Da and 2-hy-β-CD. We think the first step near 100°C was due to solvent which residues of the sample. At temperature 250-350°C, the first step, it was PEI600 and close at 400 °C, it coursed by 2-hy-β-CD.

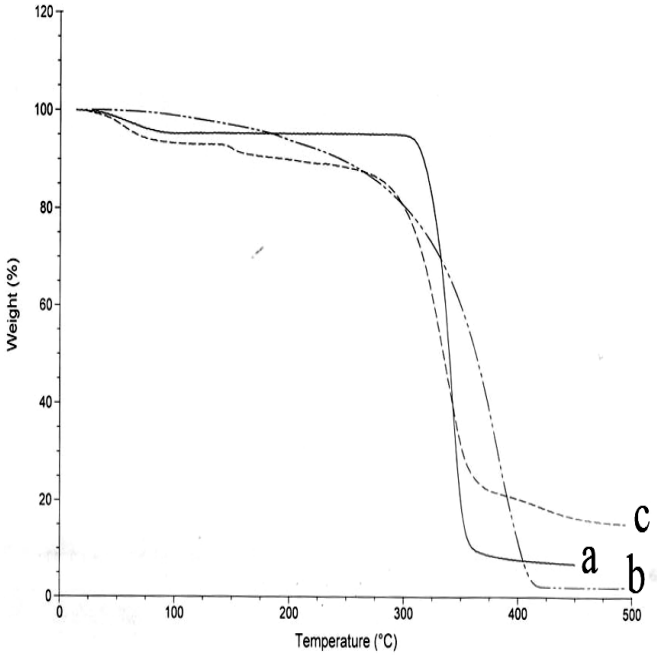


Fig. 5. (1) TGA curves of pure 2-hy-β-CD (**a**), PEI600 Da (**b**), 2-hy-β-CD-PEI600(**c**)

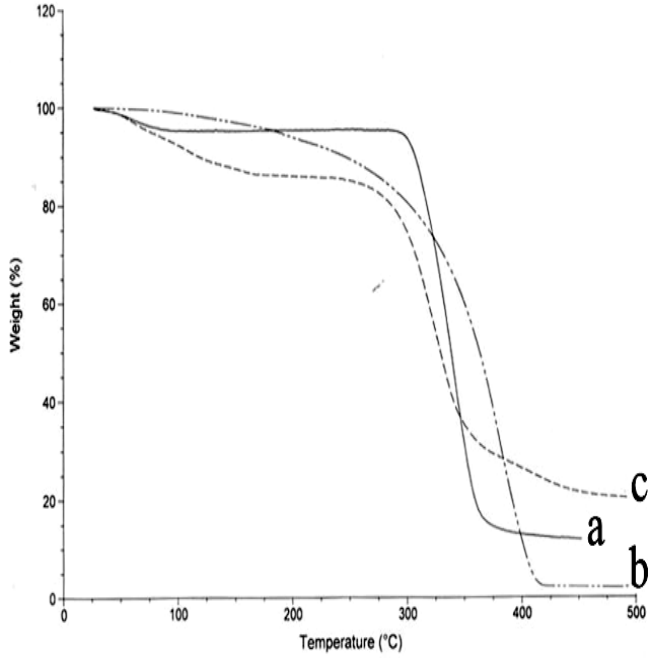


Fig. 5 (2) TGA curves of pure 2-hy-γ-CD (**a**), PEI600 Da (**b**), (c) 2-hy-γ-CD-PEI600(**c**).

Fourier Transformed Infrared Spectroscopy (FT-IR

FT-IR spectra were conducted on a FT-IR spectrometer (Spectrum 2000, PERKIN ELMER), 16 scans were signal-averaged with a resolution of 2 cm^{-1}at room temperature. Samples were prepared by dispersing the samples in KBr and compressing the mixtures to form disks.

Fig. 6 (a) and (b) shows the FT-IR spectra of 2-hy-β-CyD-PEI600 and 2-hy-β-CyD. It provided for the formation of them. For 2-hy-β-CyD a widely peak of OH group was at 3395.7 cm^{-1}. O-C stretch vibration characteristic peaks of 2-hy-β-CyD were appeared at 1030.1, 1082.6 and1156.6 cm^{-1}. For 2-hy-β-CyD-PEI600, besides a widely peak over 3400 cm^{-1} (OH-), the stretch vibration characteristic peaks of proton assigned to methyl and methylene were appeared at 2932 cm^{-1} and 2849 cm^{-1} when PEI600Da conjugate to 2-hy-β-CyD. Due to H-bonding between N-H of PEI600 and the C=O of 2-hy-β-CyD, the ester signal had shifted to 1707.9 cm^{-1}. The same explanation was for 2-hy-γ-CyD and 2-hy-γ-CyD-PEI600(Fig.6(c) and (d)).

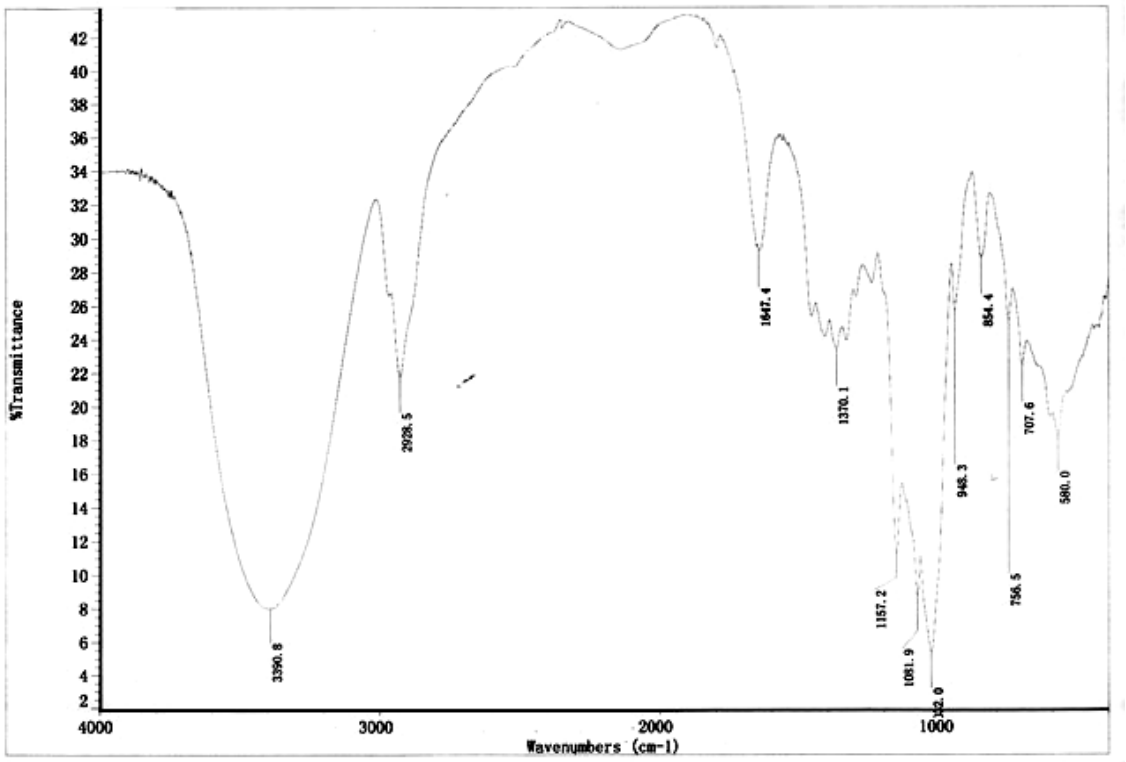


Fig. 6 a) **FT-IR spectra of pure** 2-hy-β-CD.

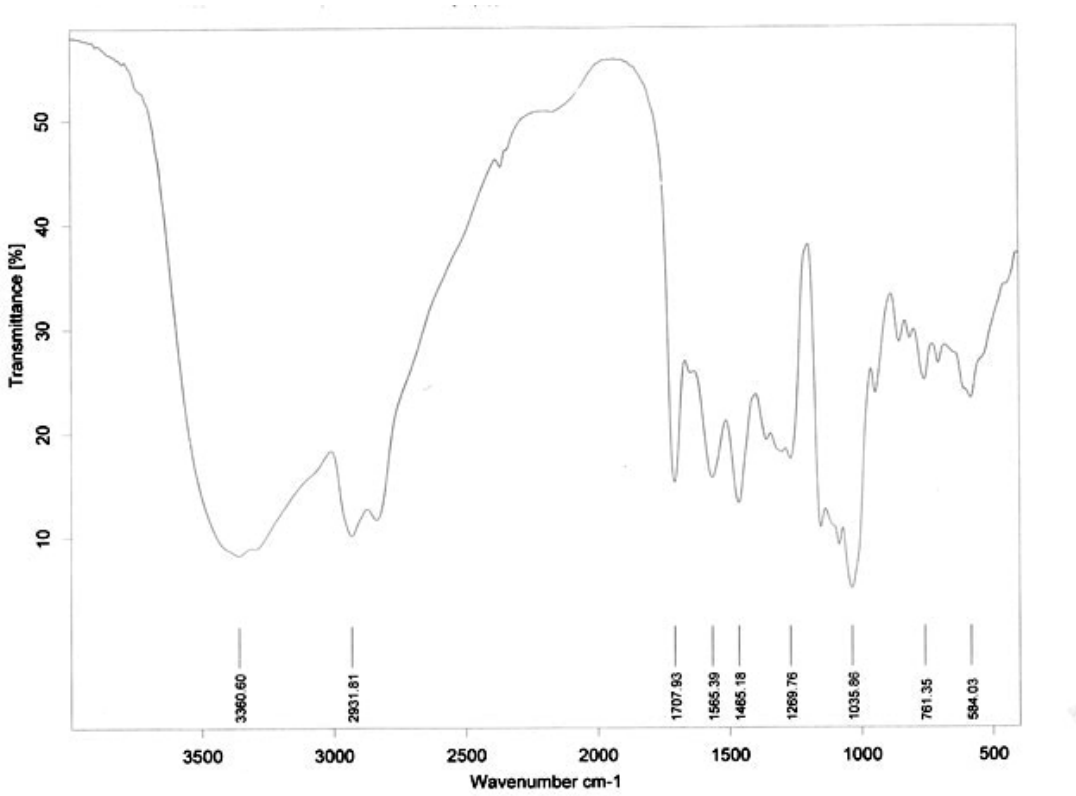


Fig. 6 b) **FT-IR** spectra of pure 2-hy-β-CD-PEI600.

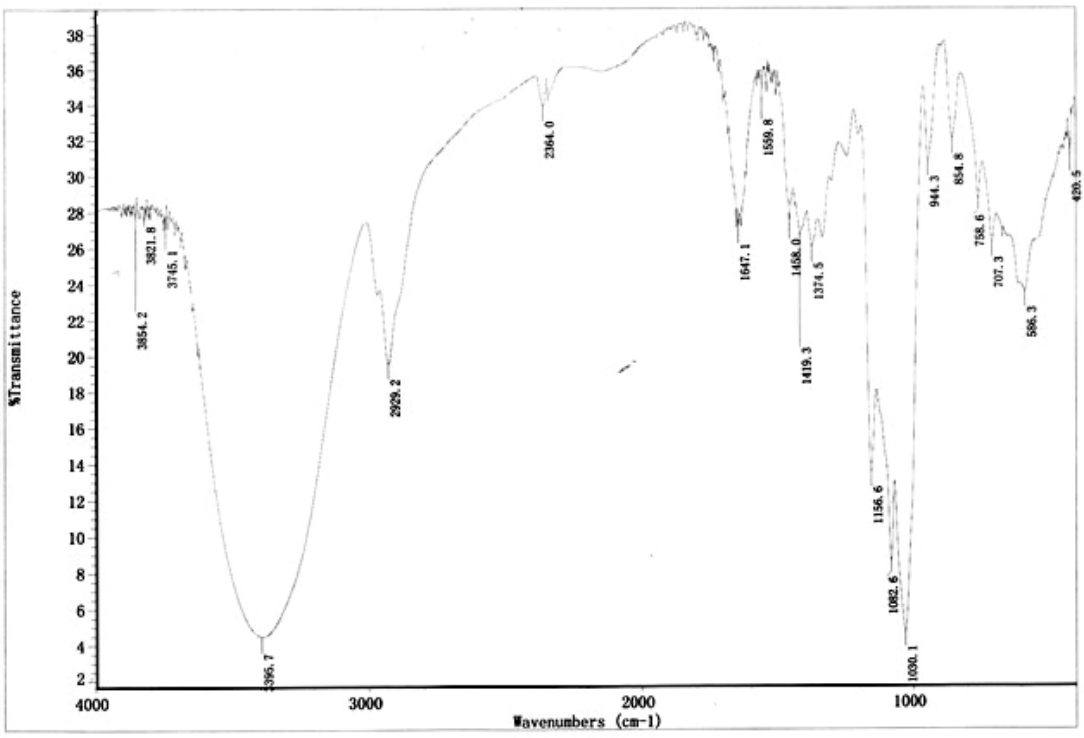


Fig. 6. c) **FT-IR** spectra of pure 2-hy-γ-CD.

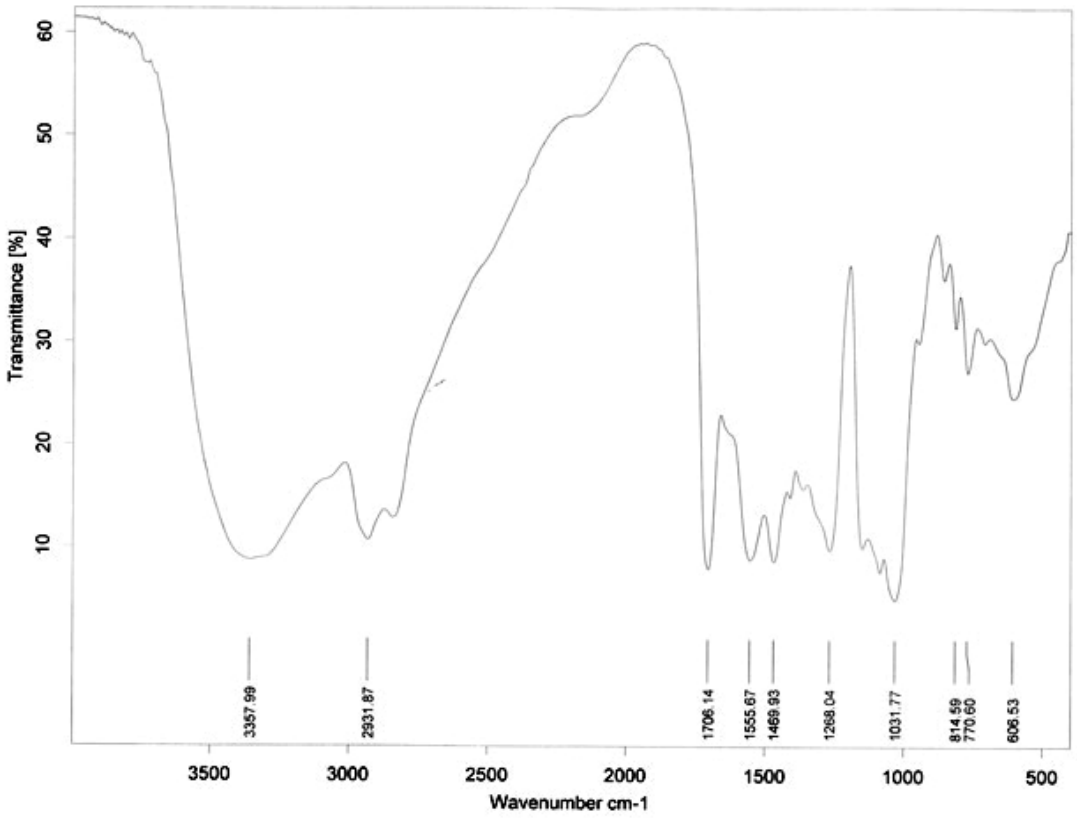
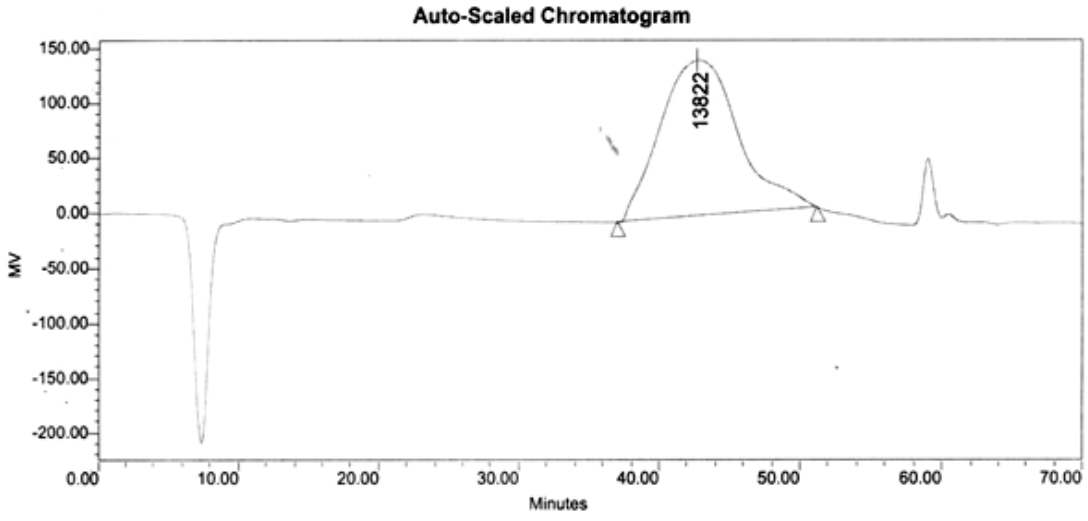


Fig. 6 d) **FT-IR spectra of pure** 2-hy-γ-CD-PEI600.

Gel Permeation Chromatography (GPC)

Analysis The number and weight average weights were determined by GPC using Waters 600 E pump and Waters 2410 Refractive Index Detector (33 °C). The columns were Phenomenex Polysep Guard S/n 70978G, Polysep GFC-P S/n 70977 and Polysep GFC-P S/n 70976 (33 °C). The elution was distill-water, and flow rate was 0.7 ml/ min. Molecular weight analyses were made against five poly (ethylene glycol) standards of number average molecular weights 7100, 10600, 12600, 23600 and 56000.



GPC Results

	SampleName	Mn	Mw	MP	Mz	Mz+1	Polydispersity
1	Tang3	9982	14675	13822	19362	23365	1.470222

Fig.7 GPC of 2-hy-β-CD-PEI600

From the GPC figure, it showed that the Mw of 2-hy-β-CD-PEI600 was closed to 14,500. That means there were more than 4 units for PEI600 Da and 2-hy-β-CD to link together one by one.

MALDI-TOF Mass Spectra

The MALDI-TOF spectra were done on a Voyager Biospectrometry Workstation (Perseptive Biosystens. Inc.) in the linear mode. A N_2 laser radiating at 337nm wavelength with 2 ns pulses was used. The ions generated by the laser pulses were accelerated to22 Kv energy. For analysis, 2-β-hy-CD–PEI600 solution in H_2O at a concentration of 0.5 mg/ml were mixed in a 1:1 (v/v, polymer solution: matrix solution) ratio with a matrix solution of 2, 5-dihydroxybenzoic acid at a concentration of 10 mg/ml on the sample plate and dried in the air).

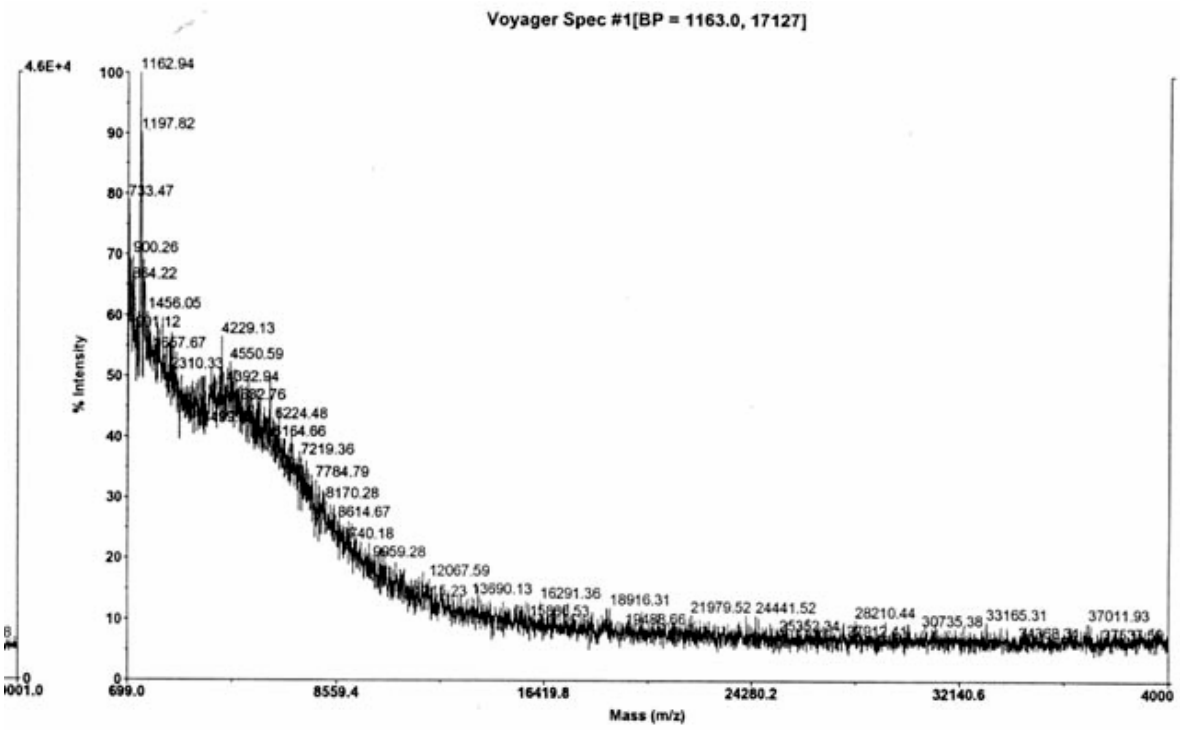


Fig.8 Mass spectrophotometry of 2-hy-β-CD-PEI600

MALDI-TOF mass spectrophotometric analysis of 2-hy-β-CD-PEI600 was showed in Figure 8. Many intensity peaks appeared from 12067 to 2310 (m/z), and the majority peaks were at round 4550 (m/z). We thought that it was difficult in measuring the molecular weight of 2-hy-β-CD-PEI600 exactly using MALDI-TOF mass spectrophotometry at various concentrations and matrices[3].

The formation of DNA/polymer complexes and gel retardation analysis

Plasmid DNA (pGL3) was diluted to a chosen concentration (usually 0.5 μg/μl) using 150 mmol/l NaCl under vortexing. Then an appropriate amount of polymer was added slowly into DNA solutions. The amount of polymer added was calculated based on a designed weight ratio of polymer/DNA. After the solution was incubated at room temperature for 30 min with gently vortexing, the formed polymer/DNA complexes were mixed with a loading buffer and loaded onto 1% agarose gel containing ethidium bromide. Gel electrophoresis was run at room temperature in TAE buffer at 80 V for 60 min. DNA bands was visualized by a UV (254 nm) illuminator. As showed in Fig.9 a) and b), the migration of DNA was completely retarded when the weight ratio of the two new polymers/DNA was at 2:1. In Fig.9 c) and d), the migration of DNA was completely retarded when the N/P ratio of polymer/DNA was at 4:1 (N/P ratio, the number of nitrogen residues of PEI per DNA phosphate).

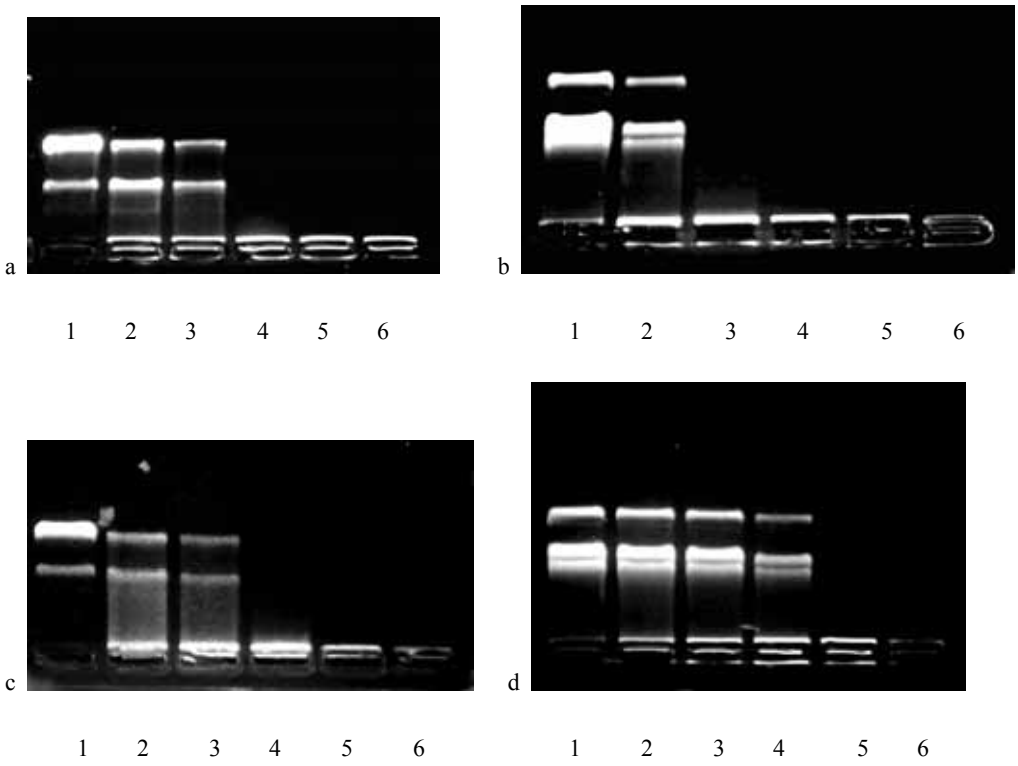


Fig. 9 Agarose gel electrophoresis retardation of pGL3 plasmid DNA by **a**) 2-hy-β-CD-PEI600 and **b**) 2-hy-γ-CD-PEI600. Lane numbers correspond to different polymer / DNA weight ratios: (1) 0:1 (DNA only), (2) 0.5:1, (3) 1:1, (4) 2:1, (5) 3:1, (6) 5:1;

c)PEI25 KD and **d**) PEI600 Da. Lane numbers correspond to different polymer / DNA N/P ratios: (1) 0:1 (DNA only), (2) 1:1, (3) 2:1, (4) 3:1, (5) 4:1, (6) 5:1

Cytotoxicity assay of polymers

SKOV-3 cells were used to investigate the cytotoxicity of co-polymers. For cell viability assay, polymer solutions were prepared in serum supplemented tissue culture medium. PH and osmolarity of the preparations were routinely measured and adjusted to PH 7.4 and 280-320 mos/kg. The cells (1×10^5 cells /well) were seeded into 96-well plate (Costar, Corning Corp. New York). After overnight incubation the culture medium was replaced with 100μl serial dilutions of the co-polymers and the cells were incubated for another 4 h. 20 μl sterilized MTT (5 mg/ml) stock solution in PBS was added to each well reaching a final concentration of 0.5 mg MTT/ml. After 4h, un-reacted dye was removed by aspiration. The formazan crystals were dissolved in 100 μl/well DMSO and measured spectrophotometrically in an ELISA reader (Model 680, Bio-Rad) at a length of 570 nm. The relative cell growth (%) related to controls containing cell culture medium without co-polymer was calculated by test/control×100. The polymers showed significantly lower cytotoxicity than that of PEI25K Da and PEI600 Da showed no cytotoxicity (Fig.10).

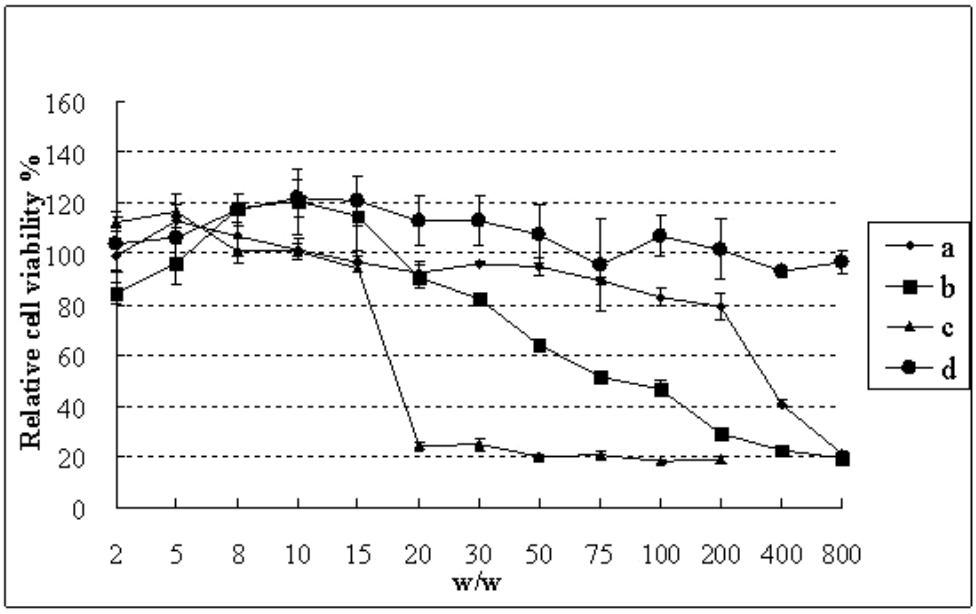


Fig. 10 Comparison of the cytotoxities induced by the **a**) 2-hy-β-CD-PEI600, **b**) 2-hy-γ-CD-PEI600, **c**) PEI 25K Da and **d**) PEI 600 Da in SKOV-3 cells measured by the **MTT assay**.

Particle size of co-polymer/DNA complexes

Polymer/DNA complexes were prepared at a DNA concentration of 25 μg/ml in 150 mmol/L NaCl. A volume of 2 ml was used for the measurement. Size of different weight ratios of polymer/DNA was measured with 90Plus/BI-MAS (Brookhaven Instruments Corporation) at room temperature. Scattering light was detected at 90° angle, Each sample was run for 200 sec and analyzed in the Unimodal Analysis mode. It showed that, the size of polymer/DNA complexes was less than 300 nm (Fig.11).

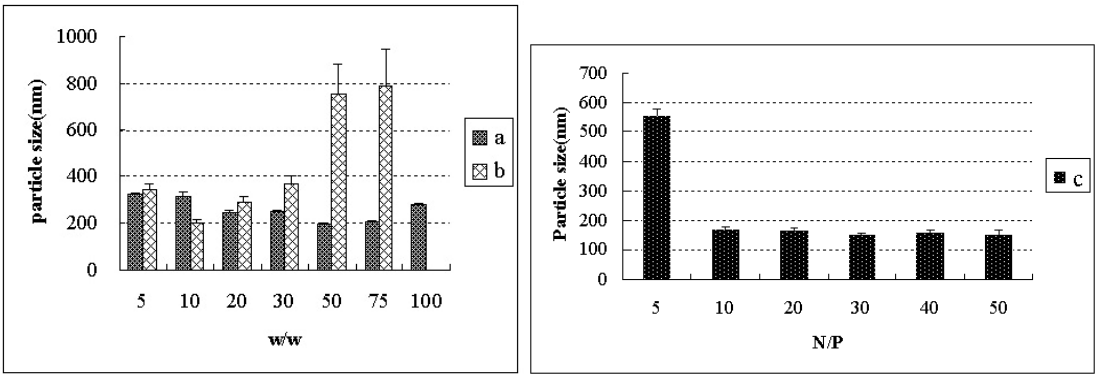


Fig.11 Particle size of **a**) 2-hy-β-CD-PEI600/DNA complex, **b**) 2-hy-γ-CD-PEI600/DNA complex and **c**) PEI 25K Da/DNA complex (N/P=10).

Assay of transfection efficiency

For in vitro transfection studies, SKOV-3 cells were seeded 24 h prior to transfection into 24-well plate at a density of 5×10^4 per well in 0.75 ml of DMEM containing antibiotics. The plasmid DNA (pGL3) in the amount of 1 μg per well was used for transfection. At the time of transfection, the

medium in each well was replaced with 0.5 ml of serum free medium. PEI600 Da/DNA, PEI25 KDa/DNA or polymer/DNA complexes were incubated with the cells for 4 h. Then the medium was replaced with 0.75 ml of fresh DMEM and cells were further incubated for 24h. After the incubation, cells were permeabilized with 100 μl of cell lysis buffer (Promega Corporation). The luciferase activity in cell extracts was measured using a luciferase assay kit (Promega Corporation) on a single-well luminomiter (Berthold lumat LB9507, Germany) for 10 sec. The relative light units (RLU) were normalized against protein concentration in the cell extracts, which was measured using a BCA protein assay kit (Pierce, Rockford, USA). The results showed the optimal ratio of transfection efficiency in vitro. The optimal N/P ratio of transfection efficiency of PEI25K Da/DNA complex was 10, the optimal w/w ratio of 2-hy-β-CD-PEI600/DNA complex was 75, 100 and that of 2-hy-γ-CD-PEI600/DNA complex was 10 (showed in Fig.4). PEI600 Da showed low transfection efficiency(data not shown here). Relative Light Unit (RLU) of two new polymer/DNA complexes was 1.5 -1.7 fold higher than that of PEI25K Da/DNA complexes and over 20-fold higher than that of PEI600 Da/DNA complexes.

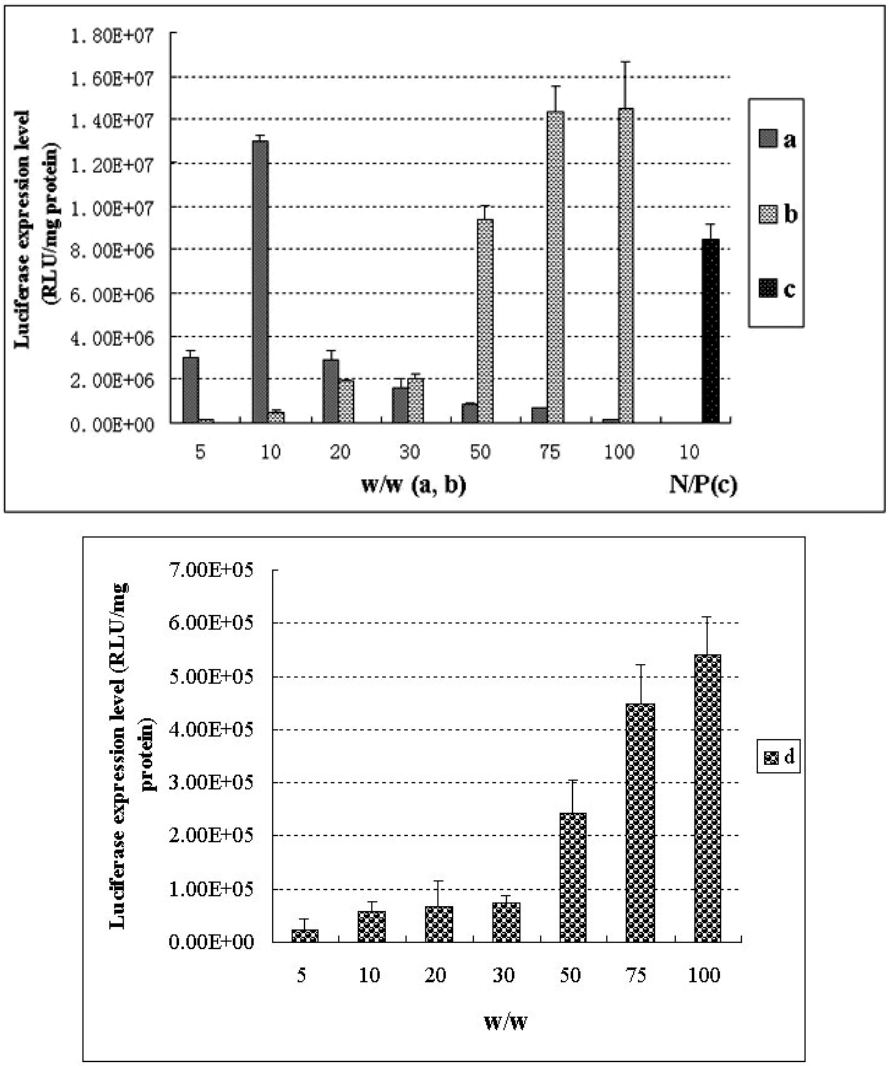


Fig.12 Transfection efficiency of **a**) 2-hy-β-CD-PEI600, **b**) 2-hy-γ-CD-PEI600, **c)** PEI 25K Da (N/P=10), and **d**) PEI600 Da.

Notes and references

1. Greg T. Hermanson. *Bioconjugate Techniques*. 153-155, Academic Press. 1995
2. G.P. Tang, J.M. Zeng, S.J. Gao, Y.X. Ma, L. Shi, Y. Li, H.-P. Too, S. Wang. *Biomaterials* 2003, **24**, 2351–2362.
3. H. Sang-oh, Mahato R. I. and W. K. Sung. *Bioconjugate Chemistry*. 2001, **12**, 337-345.