Electronic Supplementary Information

Supramolecular cyclodextrin nanocarriers for chemo- and gene therapy towards the effective treatment of drug resistant cancers

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Experimental

1. Materials

β-Cyclodextrin, polyethylenimide with typical Mn: 600 (GPC) (OEI-14), branched PEI (MW 25K), 1,1′-Carbonyldiimidazole (CDI), N,N'-dicyclohexylcarbodiimide (DCC), 4-dimehylaminopyridine (DMAP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, and streptomycin were obtained from Sigma-Aldrich. Paclitaxel was obtained from Yunnan Hande Bio-Tech Co., Ltd, China. Qiagen kit and Luciferase kit were purchased from Qiagen and Promega respectively. β-NC-OEI and FA-SS-COOH were synthesized according to our previous reports.[1, 2] Nur77 gene utilized was a Nur77 mutant lacking its DNA binding domain (Nur77/ΔDBD) as previously reported.[3] Bruker AV-400 NMR spectrometers were applied to determine NMR spectra of samples at ambient temperature and HOD as solvent as previously reported.[4] Mass spectrometry was conducted in a thermo scientific Q-Exactive LC-MS/MS (Thermo Fisher, USA) with heated electrospray ionization source (HESI) in the positive ionization mode. The operating conditions of the ESI source: positive ion mode; spray voltage 3 kV, capillary temperature 230 °C, heater temperature 35 °C, sheath gas flow rate 5, S-lens RF level 50. HR-ESI was equipped with ion source, analyzing ions in scan range of full mass 500–6000. Samples were prepared in distilled water, the 1mM samples were diluted 10 times by methanol/ distilled water (1:1) and introduced via direct infusion at a maximum injection time of 50 ms, with syringe pump setting of 5 μl/min.

2. Synthesis of β-NC-OEI-SS-FA and preparation of inclusion complex.

Synthesis of star-shaped cationic β-NC-OEI with β-CD core and multiple OEI arms has been showed in our report.[1] Brifely, β-CD (0.454 g, 0.4 mmol, minimum 98% purity) was dried at 120 °C in vacuum overnight. 40 mL dry DMSO was injected under nitrogen

when the flask was cooled. After all β-CD was dissolved, the DMSO solution of β-CD was added dropwise during a period of 6 h under nitrogen to 40mL of anhydrous DMSO solution in which 1,1′-carbonyldiimidazole (CDI) (5.84 g, 36 mmol) was dissolved, and the mixture was stirred overnight under nitrogen at room temperature. Then, the mixture of 400 mL tetrahydrofuran (THF) and 1700 mL ethyl ether (Et_2O) was used to precipitate the product. The precipitate was centrifuged and washed with THF three times. Then, the resulting sticky solid was dissolved in 40 mL DMSO and this solution was slowly added dropwise during a period of 3 h into 25.92 g (43.2 mmol) of OEI-14 which was dissolved in 40mL of DMSO with stirring at room temperature, followed by stirring the mixture overnight. 900mL dichloromethane (DCM) was poured in the reaction mixture to precipitate the product. The precipitate was centrifuged and washed with DCM three times, and the resulting crude product was purified by size exclusion chromatography (SEC) on a Sephadex G-25 column using DI water as eluent. Finally, 0.580 g sticky product was obtained. Yield, 31%. ^{1}H NMR (400 MHz, D_2O, 22 °C): δ 5.0 (s, broad, 7H, H(1) of CD), 3.2–4.6 (m, broad, 42H, H(3), H(6), H(5), H(2) and H(4) of CD), 3.0–3.2 (m, broad, 13H, methylene of –$CONHCH_2$–), 2.3-2.8 (m, 363H, ethylene of OEI-14). Anal. Calcd. for $C_{218}H_{511}N_{88}O_{35}\cdot 15H_2O$, C: 50.39, H: 10.49, N: 23.72. Elemental Analysis: C: 49.76, H: 9.85, N: 24.59.

Synthesis of FA-SS-COOH has been reported.[2] Firstly, coupling reaction between FA and cystamine dihydrochloride was carried out by DCC and DMAP to introduce disulfide bond. Secondly, the amino-terminated FA was allowed to react with succinic anhydride to give FA-SS-COOH. The feeding ratio of DCC and cystamine was less than 1:1 to make sure that only the γ-carboxyl group of FA was conjugated with disulfide bond because over-modification of α-carboxyl group of FA may result in loss of binding ability to FR. In details, to a suspension of FA (265 mg, 0.6 mmol) and cystamine dihydrochloride (180 mg, 0.8 mmol)

in a mixture of DMSO (4 mL) and pyridine (4 mL) was added DCC (144 mg, 0.7 mmol) and DMAP (cat.). The mixture was stirred at room temperature for 18 h under dark, then poured into acetone (80 mL). The yellow precipitate was collected and washed with acetone (40 mL) twice, and dried under vacuum to yield 330 mg of yellow solid (yield, 96%). To a solution of this product (330 mg, 0.6 mmol) in pyridine (5 mL) was added succinic anhydride (100 mg, 1.0 mmol), and then the mixture was stirred at room temperature for 18 h under dark. The reaction mixture was then poured into acetone (80 mL). The yellow precipitate was collected, washed with acetone (40 mL) twice, and dried under vacuum to yield 348 mg of yellow solid (yield, 90%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.62 (s, 1H, -CH- of pyrazine), 8.01 (brs, 2H, -NH-), 7.63 (d, 2H, *J* = 8.4 Hz, -CH of benzyl ring), 6.90 (brs, 2H, -NH-), 6.62 (d, 2H, *J* = 8.4 Hz, -CH- of phenyl ring), 4.47 (s, 2H, -CH_2-), 4.32 (m, 1H, -CH-), 2.72 (t, 4H, *J* = 6.8 Hz, -CH_2S-), 2.20−2.45 (m, 4H, -CH_2CO- of succinic anhydride), 1.80−2.20 (m, 4H, -CH_2 of FA).

DCC (4.3 mg, 20.8 μmol) and DMAP (cat.) was added to a solution of β-NC-OEI (108 mg, 20.9 μmol) and FA-SS-COOH (9.2 mg, 20.8 μmol) in DMSO/pyridine (1 mL/1 mL). The mixture was stirred at room temperature for 18 h under dark conditions. Purification by dialysis (MWCO, 2000) against water was then conducted under dark conditions for 5 d, before the resulting mixture was freeze-dried to yield 87 mg of β-NC-OEI-SS-FA as a yellow solid (74.2%). ^{1}H NMR (400 MHz, D_2O): δ 8.57 (s, 1H, -CH- of pyrazine on FA), 7.64 (d, 2H, -CH- of phenyl ring on FA), 6.73 (d, 2H, -CH- of phenyl ring on FA), 4.99 (brs, 7H, H-1 of β-CD), 4.53 (2H, -CH_2NHPh- of FA), 4.26 (1H, -CHCOOH- of FA), 3.00–4.50 (m, 55H, H-2–6 of β-CD, -CONHCH_2- of OEI), 2.10–2.92 (m, 372H, -CH_2CH_2NH- of OEI). Quasi-molecular ion peak at *m/z* 5591.3 ($[M+2H]^{2+}$) by HR-ESI-MS.

The general synthesis procedure of inclusion complexes of β-NC-OEI-SS-FA with PTX is as follows. A solution of PTX (8.5 mg, 10 μmol) in ethanol (1 mL) was added to a

solution of β-NC-OEI-SS-FA (56 mg, 10 μmol) in water (1 mL). The suspension was then stirred for five days in the dark until a clear solution was formed. The mixture was concentrated under vacuum to a volume of c.a. 1 mL, centrifuged to remove unreacted PTX, and then freeze dried to yield the product. The β-CD-OEI/PTX inclusion complex was a colorless solid (60 mg, yield 93%). ^{1}H NMR: (400 MHz, D_2O): d 7.18-8.10 (m, 15 H, ArH of PTX), 4.85-5.65 (m, 11 H, H-1 of CD, H of PTX), 2.95-4.50 (m, 58 H, H-2 - H-6 of β-CD, -$CONHCH_2$- of OEI, H of PTX), 2.00-2.95 (m, 384 H, -CH_2CH_2NH- of OEI, H of PTX).

3. Gel retardation experiments

Samples were examined for their ability to bind pRL-CMV-Nur77 plasmid DNA through gel electrophoresis experiments. All the sample stock solutions were prepared at a nitrogen concentration of 1 mM in distilled water and the pH was adjusted to 7.4. Solutions were sterile filtered (0.2 μm) and stored at 4 °C. pRL-CMV-Nur77 (0.2 mg in 2mL of TAE buffer) was mixed with an equal volume of polymer solution at nitrogen/phosphate (N/P) ratios between 0 and 35. Each mixture was vortexed and incubated for approximately 30 min at room temperature and then analyzed on 1% agarose gel containing 0.5 mg/mL ethidium bromide (EtBr). Gel electrophoresis was carried out in TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) at 120 V for 40 min in a Sub-Cell system (Bio-Rad Laboratories, CA). DNA bands were visualized and photographed by a UV transilluminator and Bio-Rad-It imaging system.

4. Stability evaluation of co-delivery system β-NC@Nano-polyplex

The stability of nano-polyplex β-NC@Nano-polyplex was evaluated by particle size measurements by using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA, USA) with a laser light wavelength of 633 nm at a 173° scattering angle.[5, 6] In details, β-

NC@Nano-polyplex with co-delivery of PTX and Nur77 gene at its optimal N/P ratio of 15 was prepared by adding 200 ng Nur77 gene into β-NC-OEI-SS-FA, which was similar to the formulation utilized for cellular or animal study. The nano-polyplex solution was dispersed in 1 mL RPMI 1640 cell culture medium supplemented with fetal bovine serum (FBS), before its particle size measurements at time points of 0, 6, 12, 24 h.

Furthermore, in order to test the DNA protection efficiency of nano-polyplex β-NC@Nano-polyplex, gel retardation experiments were conducted for the supernatants of above β-NC@Nano-polyplex at time points of 0, 6, 12, 24 h. The disassembly or release of free DNA into supernatants was analyzed on 1% agarose gel containing 0.5 mg/mL ethidium bromide (EtBr). Gel electrophoresis was carried out in TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) at 120 V for 40 min in a Sub-Cell system (Bio-Rad Laboratories, CA). DNA bands were visualized and photographed by a UV transilluminator and Bio-Rad imaging system.

5. Cell culture

The cell culture medium was made of FA-free RPMI 1640 medium, supplemented 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cell culture incubator with 5% CO_2 and 37 °C was applied.

6. In vitro gene transfection procedures

Transfection studies were performed in HeLa using the plasmid Renilla as the reporter gene. In brief, 48-well plates were seeded with cells at a density of 5×10^4/well 24 h before transfection in 200 μL of FA-free 1640 RPMI medium with 5% CO_2 at 37 °C. After 24 h incubation, the sample/DNA complexes at various N/P ratios were prepared by adding the PEI-25K, β-NC-OEI-SS-FA/PTX into 200 ng DNA aqueous solutions with volume of 50 μL

dropwise, followed by swinging and incubation for 30 min at room temperature before transfection. At the time of transfection, the medium in each well was replaced with reduced-serum medium or normal medium. The complexes were added into the transfection medium and incubated with cells for 4 h under standard incubator conditions in serum and serum free medium respectively. After 4 h, the medium was replaced with 200 μl of fresh medium supplemented with 10% FBS, and the cells were further incubated for an additional 20 h under the same conditions, resulting in a total transfection time of 24 h. Cells were washed with PBS twice and lysed in 50 μL of cell culture lysis reagent (Promega). Luciferase activities were measured using single-Luciferase Assay System Kit (Thermo).

7. Bcl-2 stably expressing cell lines

For plasmid construction and transfection, the coding sequence of Bcl-2 was amplified by PCR and inserted into the expression PCDH-vector. The Bcl-2 expression vector and empty PCDH-vector in lentivirus were transfected into HepG2 or HeLa cells using PEI-25K reagent (Sigma) following the manufacturer's instructions. After using puromycin selection (1 μg/ml), the resistant cells were pooled and the expression of Bcl-2 mRNA levels and protein levels were further confirmed. And the cell lines successfully stably expressing Bcl-2 were named as HepG2/Bcl-2 or HeLa/Bcl-2 cells.

For reverse transcription polymerase chain reaction (RT-PCR) of mRNA, after infection of the lentivirus (PCDH-Bcl-2, PCDH-vector) into HeLa and HepG2 24h, total RNA was extracted from the HeLa and HepG2 cells (4×10^6) by utilizing Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed into cDNA using a First-Strand cDNA synthesis kit (Takara). Gene expression was performed by gel retardation assay.

For Western blot analysis of protein expression, after infection of the lentivirus (PCDH-Bcl-2 or PCDH-vector) into HeLa and HepG2 for 24h, the cells were subsequently washed with PBS three times and their proteins were extracted by using RIPA lysis buffer (approximately 10 min). Protein lysates were separated by SDS-PAGE and then transferred onto PVDF membranes according to the standard steps. The membranes were blocked with 5% BSA for 1.5 h and then incubated with the primary antibody against Bcl-2 (1:1000, Santa Cruz) at 4 °C overnight.

8. Cytotoxicity studies

For cytotoxicity experiments, HepG2 liver cancer cells, HeLa cervical cancer cells, HepG2/Bcl-2 or HeLa/Bcl-2 cells with high expression of Bcl-2 were incubated in a 96-well plate with a density of 1×10^5 /mL overnight at 37 °C. Before the test, the cell culture medium was replaced with sample solutions with PTX concentrations of 5 μM to 20 μM for 24 h, before the further addition of 10 μl of MTT and 4 h incubation at 37 °C. The precipitated crystals were carefully dissolved in 120 μL DMSO and a microplate reader was used to measure the solution absorbance at 495 nm to determine cell viability.

9. *In vivo* tumor growth

Female 8 week old BALB/c nude mice were fed in the Xiamen University Laboratory Animals Centre and supplied with sterilized air, water, and food. All animal treatments or procedures were approved according to the Animal Care Guidelines of Xiamen University. For developing the drug resistance tumor model, subcutaneous injections of 2×10^6 HeLa/Bcl2 cells suspended in 0.2 ml of PBS was conducted on the backs of the nude mice. When tumor size reached ~60 mm^3 (experimental day 0), the samples with codelivery of PTX and Nur77 gene were injected through the tail vein. BALB/c nude mice bearing HeLa/Bcl-2 tumors were randomly assigned to five groups with four mice in each group. The injection doses were

formulated by mixing 25 μL DNA solution containing 200 ng Nur77 gene mixed with 25 μL β-NC-OEI-SS-FA/PTX (4 μM), with further incubation for 30 min at room temperature. And the resultant β-NC@Nano-polyplex was further mixed with 0.735 mg β-NC-OEI-SS-FA/PTX in 50 μL PBS before injection. Injections were conducted twice a week and the treatment continued for 14 days. At each time point, vernier calipers were applied to measure the size of solid tumors. Calculation of the volume of the tumor was performed by using the following formula: Tumor volume = [length of tumour × (width of tumor)^2]/2. At specific time points on day 14, the sacrificing of the mice was carried out and following that, the tumor weights were recorded.

10. Histological analysis

On day 14, the subcutaneous tumors of sacrificed mice were removed, before fixing in 4% paraformaldehyde and subsequent dehydration with sucrose solution (mass concentration 15%, 30%, respectively) for 24 h. The tumors were also sliced into frozen sections with 2 μm thickness and hematoxylin-eosin (HE) staining was performed.

11. Statistical analysis

Cell viability, tumor volume and tumor weight in experiments were calculated by expressing the mean ± standard deviation. All data and figures in this paper were analyzed and plotted by using Origin 8.

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2. F. Zhao, H. Yin and J. Li, *Biomaterials*, 2014, **35**, 1050-1062.
3. B. Lin, S. K. Kolluri, F. Lin, W. Liu, Y. H. Han, X. Cao, M. I. Dawson, J. C. Reed and X. K. Zhang, *Cell*, 2004, **116**, 527-540.
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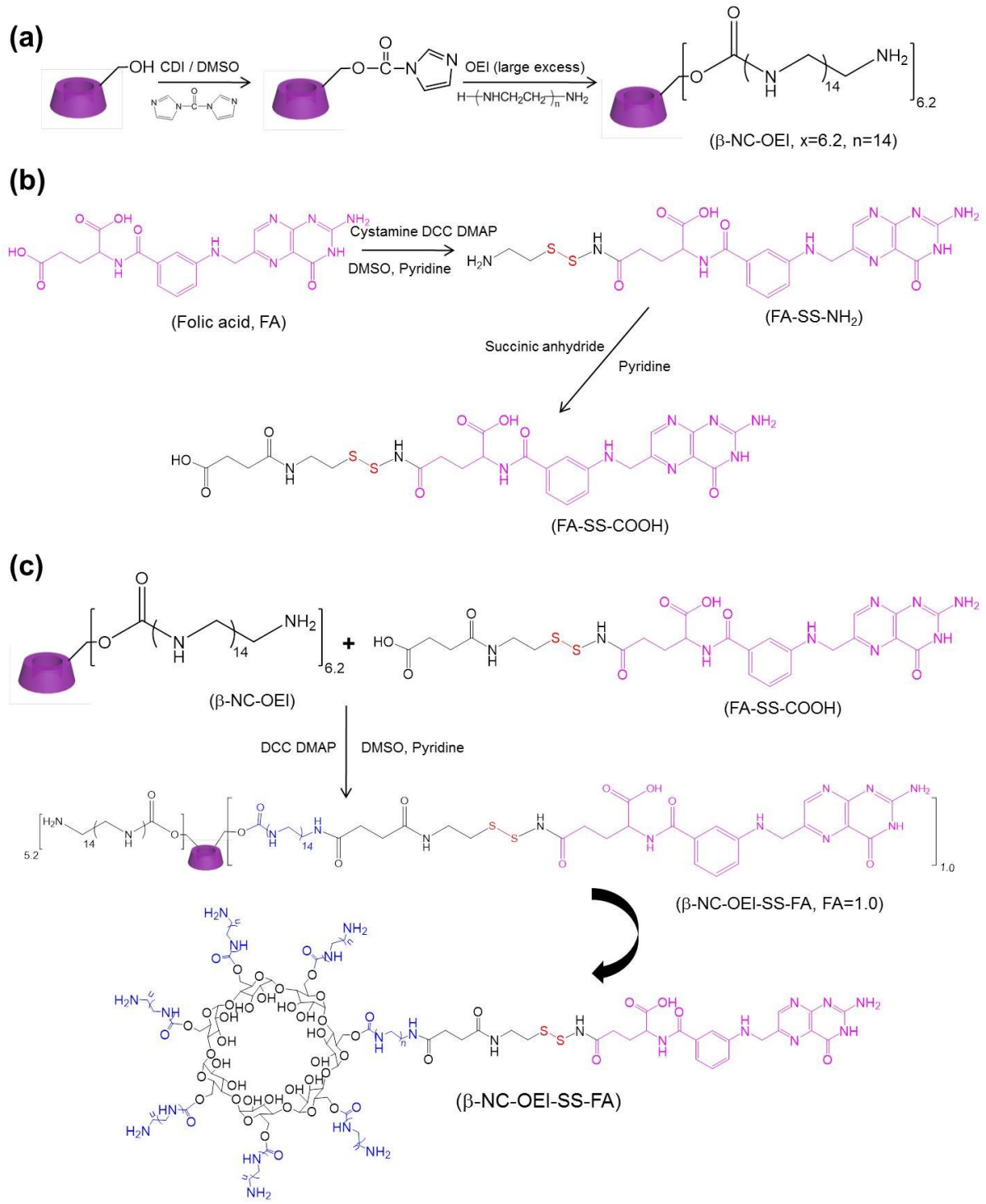
5. S. Swaminathan, L. Pastero, L. Serpe, F. Trotta, P. Vavia, D. Aquilano, M. Trotta, G. Zara and R. Cavalli, *Eur. J. Pharm. Biopharm.*, 2010, **74**, 193-201.

6. X. Zhao, F. Li, Y. Li, H. Wang, H. Ren, J. Chen, G. Nie and J. Hao, *Biomaterials*, 2015, **46**, 13-25.



Scheme S1. Synthesis of cyclodextrin-based nanosponge β-NC-OEI-SS-FA (c), by taking the advantage of β-NC-OEI (a) and FA with disulfide linker FA-SS-COOH (b).

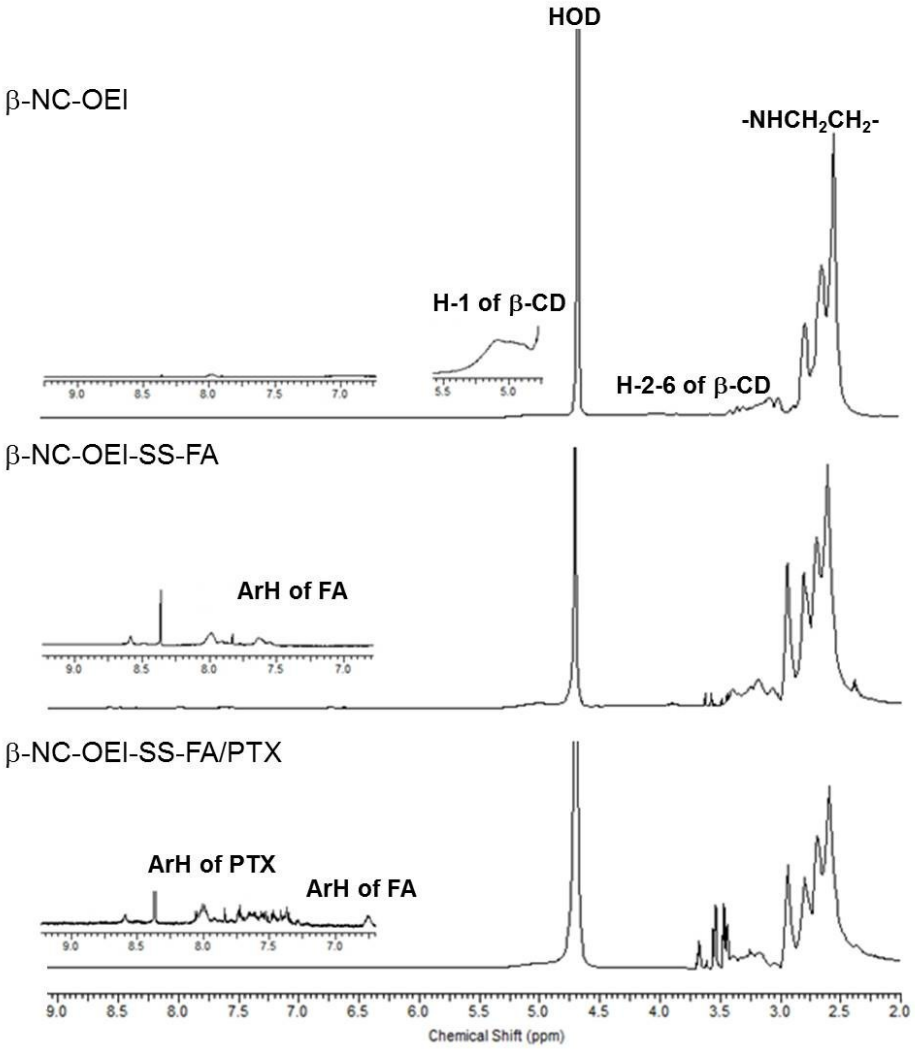


Figure S1. ^{1}H-NMR result of β-NC-OEI, β-NC-OEI-SS-FA, and β-NC-OEI-SS-FA/PTX, by using the solvent of D_2O.

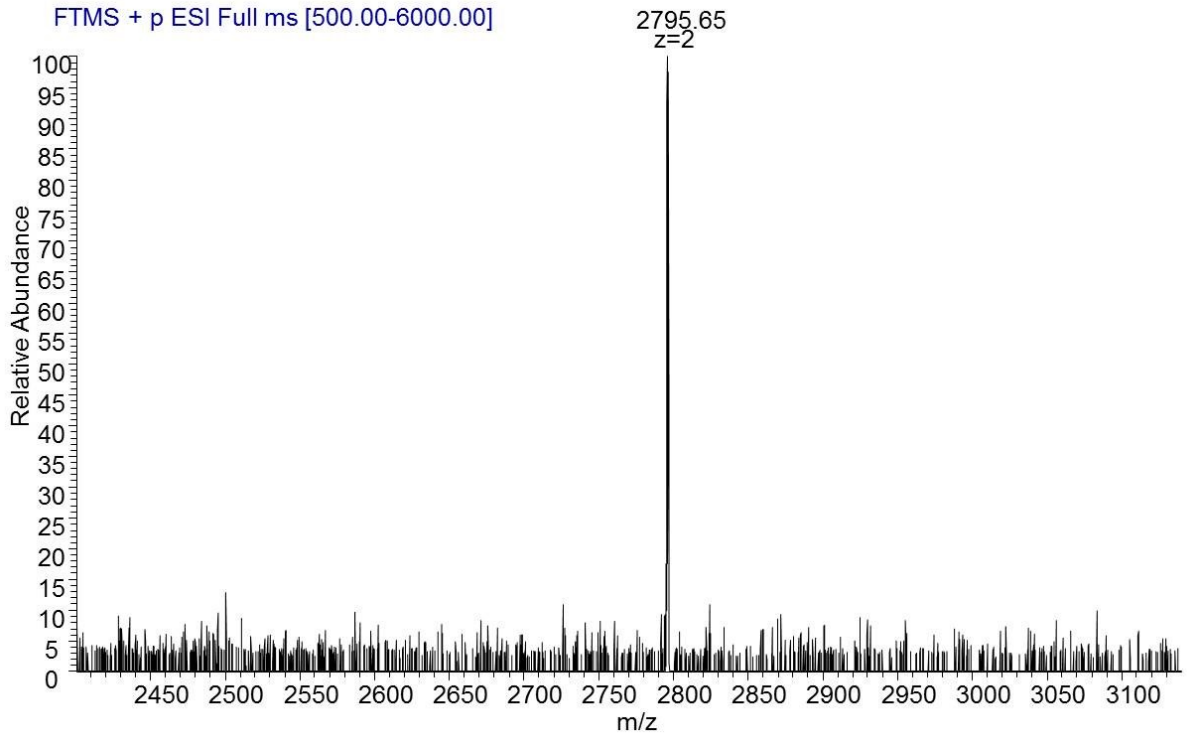


Figure S2. HR-ESI-MR analysis of β-NC-OEI-SS-FA.

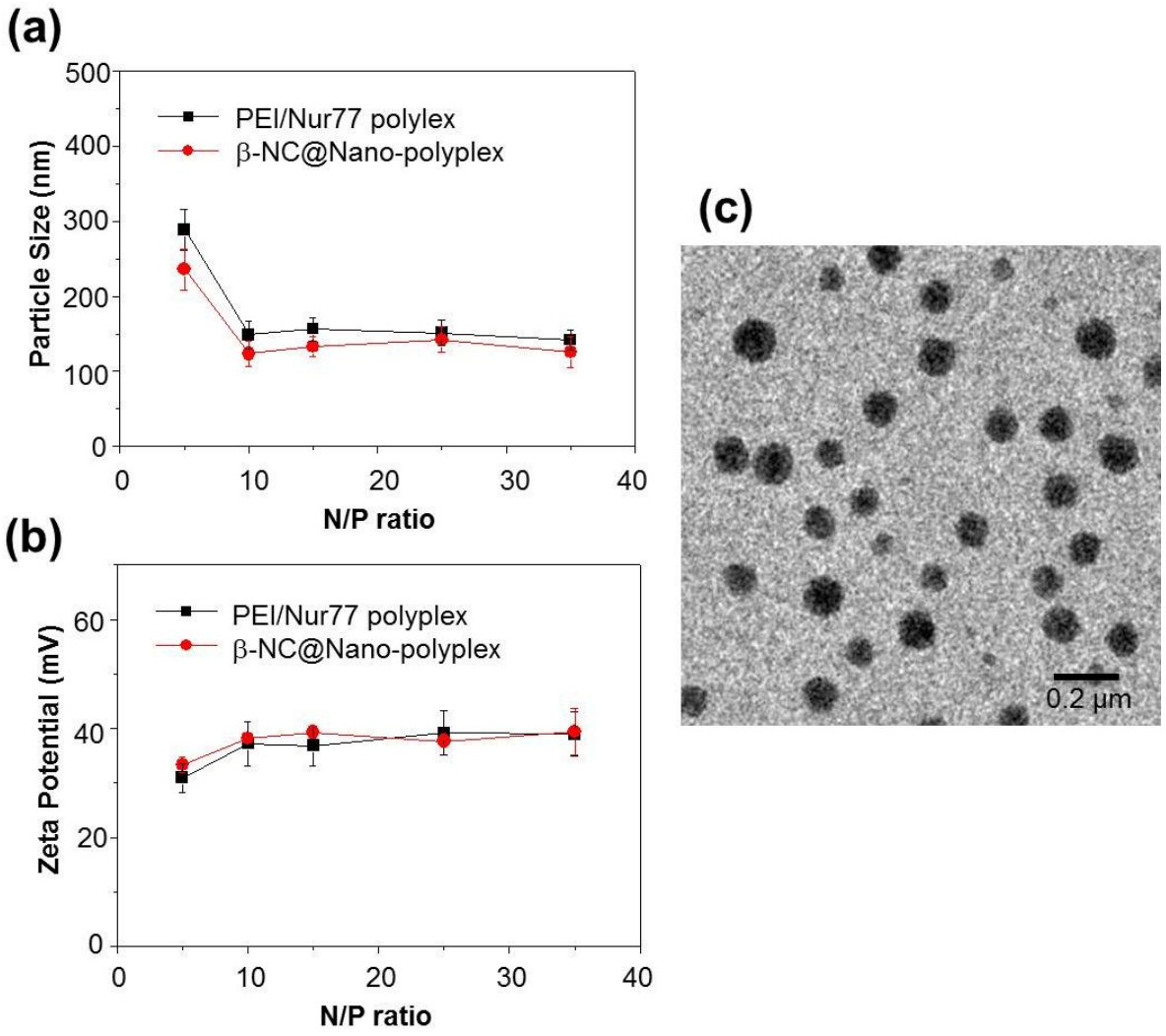


Figure S3. Particle size (a) and zeta-potential (b) measurements by using Malvern Nanosizer of PEI/Nur77 plasmid DNA polyplex or β-NC@Nano-polyplex, at various N/P ratios. (c) Transmission electron microscopy (TEM) image of β-NC@Nano-polyplex at N/P ratio of 15.

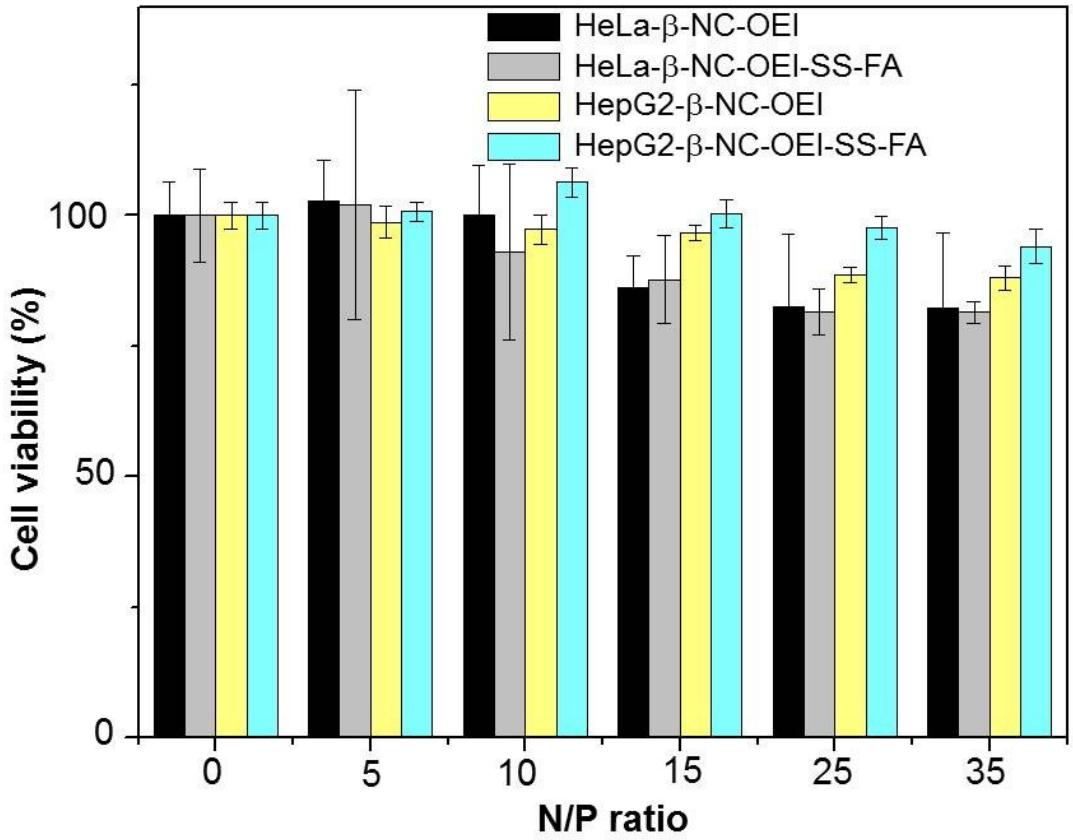


Figure S4. Toxicity of cyclodextrin-based nanocarriers against HeLa cervical cancer cells or HepG2 liver cancer cells.

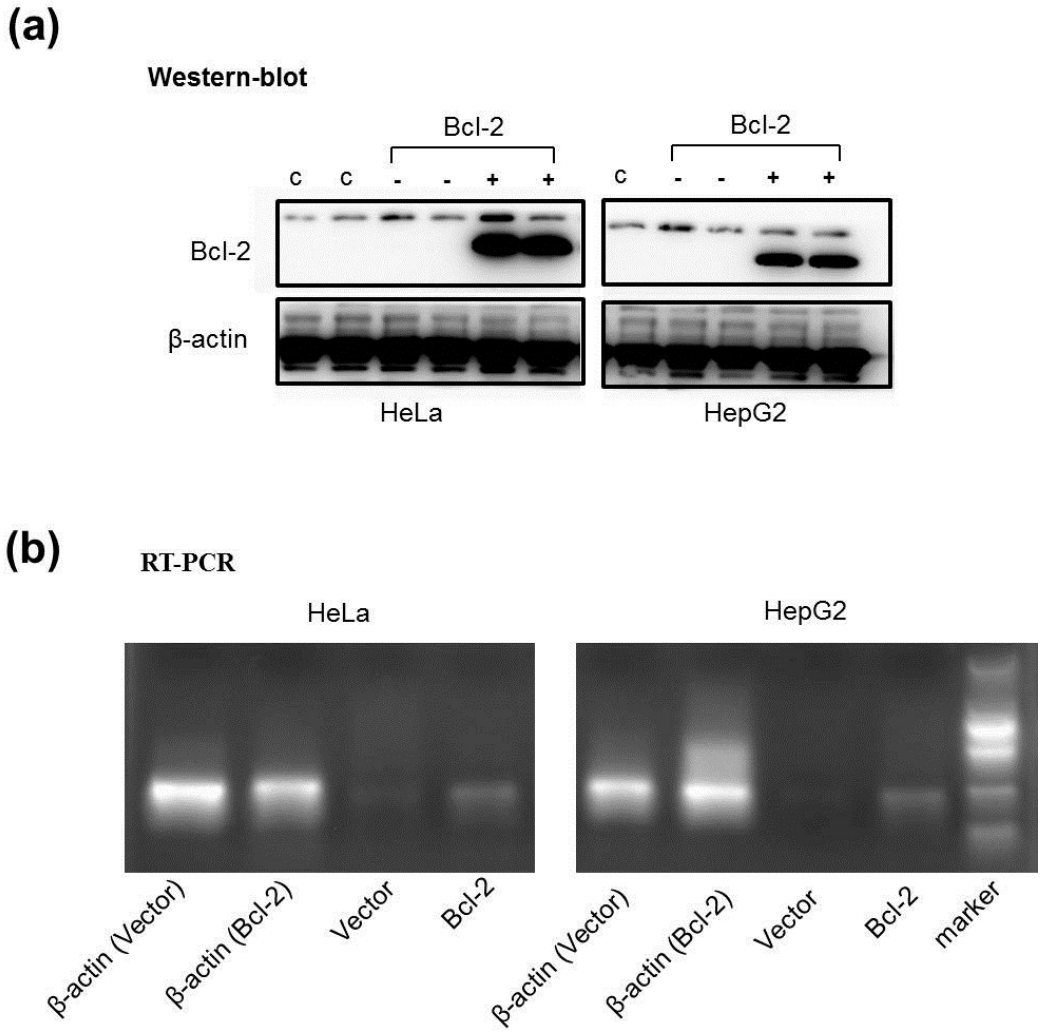


Figure S5. Protein expression (a) and mRNA level (b) analysis of Bcl-2 in normal or lentiviral mediated Bcl-2 stably expressing HeLa or HepG2 cancer cells, by using western-blot or reverse-transcript polymerase chain reaction (RT-PCR).

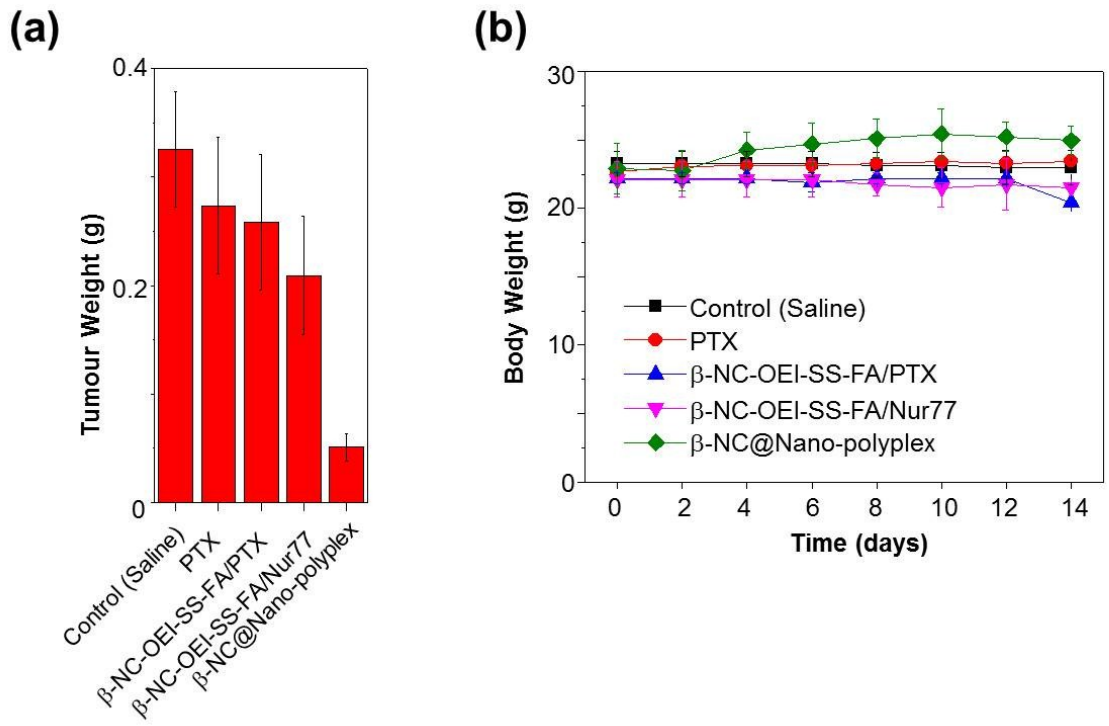


Figure S6. Tumor weights (a) and animal weights (b) of HepG2/Bcl-2-bearing mice with treatments of saline, PTX, β-NC-OEI-SS-FA/PTX, β-NC-OEI-SS-FA/Nur77, or β-NC@Nano-polyplex, as a function of time (days). Data were given as mean (n = 4) with the SD error bars.

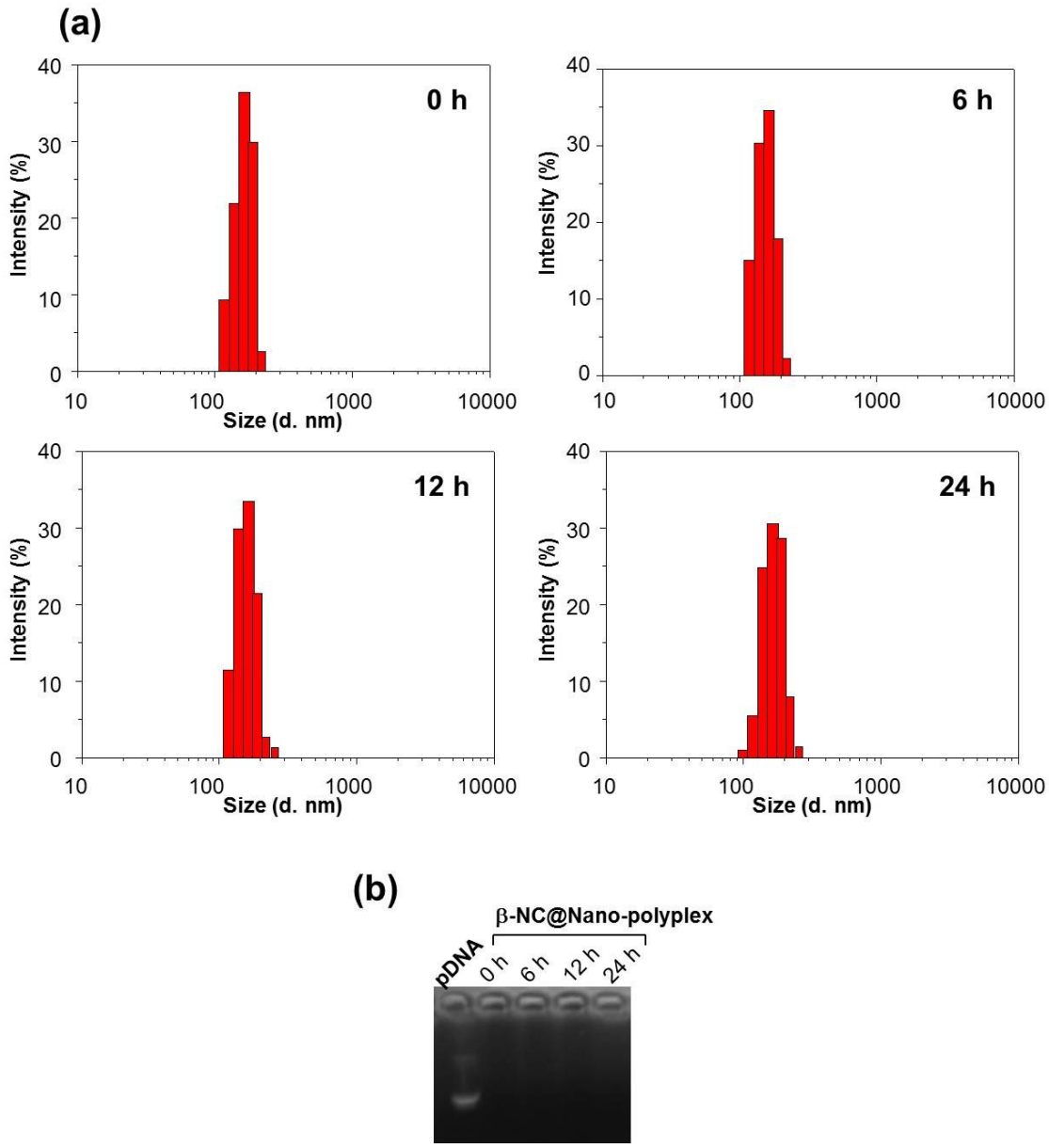


Figure S7. Stability evaluation of nano-polyplex β-NC@Nano-polyplex at optimal N/P ratio of 15. (a) β-NC@Nano-polyplex stability in serum was tested by examing the size changes by dynamic light scattering within 24 h. (b) DNA stability in β-NC@Nano-polyplex over time examined by electrophoretic mobility, in comparison with plasmid DNA only.