Supplementary Materials

Copolymer of poly(ethylene glycol) and poly(L-lysine) grafting polyethylenimine through reducible disulfide linkage for siRNA delivery

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Synthesis of lPEI-NH_2

Linear polyethylenimine (PEI) was synthesized as follows. [see: Cao N, Cheng D, Zou S, Ai H, Gao J, Shuai X. The synergistic effect of hierarchical assemblies of siRNA and chemotherapeutic drugs co-delivered into hepatic cancer cells. Biomaterials 2011;32:2222-32] MPTS (0.93 g, 5 mmol), EtOx (7.9 g, 80 mmol), and CH_3CN (10 mL) were charged into a reaction vial equipped with a stirring bar and a reflux condenser. The vial was closed, placed in an oil bath thermostated at 82 °C, and then stirred for 3 days. The mixture was cooled at 0 °C whereas dry ammonia (NH_3) was slowly blown into the vial for 1 h to terminate the reaction. After polymerization, the solvent was removed under reduced pressure and the residue was dissolved in $CHCl_3$ and then precipitated in excessive diethyl ether. Poly(2-ethyl-2-oxazoline) (MeO-PEtOx-NH_2) was isolated by filtration and dried in vacuum. Then, MeO-PEtOx-NH_2 (7.9 g, 5 mmol) was dissolved in 20 mL of aqueous HCl (10 wt%) and refluxed for 24 h under a nitrogen atmosphere at 100 °C. The pH of the reaction mixture was adjusted to 12 with 1 M NaOH, which led to precipitation of the product. The product was isolated by centrifugation, washed twice with deionized (DI) water and then subsequently freeze-dried to obtain lPEI-NH_2 (M_n = 0.6 kDa, calculated from the ^1H NMR spectrum).

Synthesis of lPEI-COOH

DTDPA (2.1 g, 10 mmol) and NHS (1.15 g, 10 mmol) were dissolved in 20 mL of chloroform/DMSO (1:1) and placed in a flask equipped with a magnetic stirring bar. After the flask was cooled in an ice-water bath, DCC (2.05 g, 10 mmol) was added. The flask was then sealed off under argon. The reaction mixture was stirred at 0 °C for 1 h. Approximately 1.2 g of lPEI-NH_2 (2 mmol) was dissolved in 20 mL

chloroform/DMSO (1:1), treated with TEA (1.2 eq.) at room temperature for 30 min, and then slowly added. The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 24 h. The DCU precipitated was removed by filtration. The filtrate was then added into excessive cold ethanol. The precipitation was collected by centrifugation and washed with 2-propanol. The product lPEI-COOH was vacuum-dried at room temperature to obtain a light yellow powdery product (M_n = 0.8 kDa, calculated from the ^{1}H NMR spectrum).

Synthesis of *m*PEG-*b*-PLL (PPL)

A pre-determined amount of *m*PEG-*b*-PLL was synthesized according to literature [see: Dai J, Zou S, Pei Y, Cheng D, Ai H, Shuai X. Polyethylenimine-grafted copolymer of poly(L-lysine) and poly(ethylene glycol) for gene delivery. Biomaterials 2011;32:1694-1705]. CBZ-L-Lysine NCA was synthesized according to a previously described method [see: Zhang X, Li J, Li W, Zhang A. Synthesis and characterization of thermo- and pH-responsive double-hydrophilic diblock copolypeptides. Biomacromolecules 2007;8:3557-67]. Then, *m*PEG-b-PCBZLLys was synthesized by ring-opening polymerization of CBZLLys-NCA using *m*PEG-NH_2 as a macroinitiator. In brief, 1.0 g (0.5 mmol) of *m*PEG-NH_2 was vacuum-dried at 70 °C for 4 h in a 100 mL flask, and then 20 mL of anhydrous DMF was added to dissolve PEG. After 6.76 g (22 mmol) of CBZ-L-lysine NCA was dissolved in 30 mL of DMF and added to the above solution, the reaction was allowed to proceed for 72 h at 35 °C. The mixture was dialyzed against DI water for 5 days to remove organic solvents and subsequently freeze-dried to achieve *m*PEG-*b*-PCBZLLys (denoted as PPCL) (M_n = 13.0 kDa, calculated from the ^{1}H NMR spectrum, *m*PEG$_{45}$-*b*-PCBZLLys$_{42}$).

Approximately 2.6 g of the copolymer *m*PEG-*b*-PCBZLLys (0.2 mmol) was dissolved

in 30 mL trifluoroacetic acid. After stirring at room temperature, HBr/acetic acid (33%, 5 mL) was added. After stirring at room temperature for an additional 3 h, the copolymer was precipitated by adding an excess amount of diethyl ether. The copolymer was washed with ethyl ether at least four times. After evaporation of solvents in vacuum, the residue was vacuum-dried at room temperature. The polymer was dissolved in DI water, dialyzed against DI water for 5 days, and then freeze-dried to achieve *m*PEG-*b*-PLL (M_n = 7.4 kDa, calculated from the ^{1}H NMR spectrum).

Synthesis of Her2-PEG-*b*-PLL-*g*-(ss-lPEI) (Her2-PLI)

The Her2-encoded copolymer Her2-PEG-*b*-PLL-*g*-(ss-lPEI) (Her2-PLI) was synthesized according to our previous report [see: Chen G, Chen W, Wu Z, Yuan R, Li H, Gao J, et al. MRI-visible polymeric vector bearing CD3 single chain antibody for gene delivery to T cells for immunosuppression. Biomaterials 2009; 30: 1962-1970]. Herceptin bearing free sulphydryl groups was prepared for conjugation to delivery vectors as follows: 1.4 mg Herceptin (10 nmol, antibody molecular weight: 139 kDa) was dissolved in a mixture of 100 μL phosphate-buffered saline (PBS) (pH 7.4) and 25 μL EDTA aqueous solution (0.5 M). 2-Mercapto ethylamine (150 mg) and 25 μL of 0.5 M EDTA aqueous solution were added into PBS (2.5 mL) and then mixed with the antibody solution. After incubation for 90 min at 37 °C, the obtained Herceptin solution was purified by ultrafiltration in an Amicon cell (regenerated cellulose membrane, MWCO = 5 kDa) along with three PBS washes (pH 7.4, each 500 μL containing 10 μL of 0.5 M EDTA solution). Up to 40 μg MAL-PEG-COOH (synthesized according to our previous report) dissolved in 40 μL PBS containing 10 μL of 0.5 M EDTA solution was added to the Herceptin solution, which was incubated overnight at 4 °C. The resultant solution containing Herceptin-functionalized PEG

(Her2-PEG-COOH) was washed three times with PBS (pH 7.4) with the aid of ultrafiltration using an Amicon cell (MWCO = 5 kDa). EDC (5 μg) and NHS (5 μg) were added to the purified Her2-PEG-COOH solution, followed by the addition of PLI (10 mg) solution in PBS (pH 7.4). The solution was incubated overnight at 4 °C. The resultant solution containing Herceptin-functionalized PLI was washed three times with PBS (pH 7.4) with the aid of ultrafiltration using an Amicon cell (MWCO = 5 kDa). Finally, the Herceptin-functionalized copolymer Her2-PLI was obtained.

Particle size and zeta potential measurements

The hydrodynamic sizes and zeta potentials of polyplexes formed between PLI and siRNA were determined *via* dynamic light scattering (DLS). Measurements were performed at 25 °C by using a 90 Plus/BI-MAS equipment (Brookhaven Instruments Corporation, USA). For the ζ–potential measurements, a standard electrophoresis mini-cell from Brookhaven was used. The data for particle size and ζ-potential were collected on an auto-correlator with a detection angle of scattered light at 90° and 15°, respectively. For each sample, the data from five measurements were averaged to obtain the mean particle size and ζ-potential.

Real-time PCR assay for mRNA level of XIAP gene

Total RNA was extracted from the cells by using the RNeasy Micro Kit (Qiagen Inc., USA). First-strand cDNA was synthesized using a PrimeScript® RT reagent Kit (Takara Biotechnology, Japan). The mRNA expression of XIAP was quantified *via* real-time PCR by using a StepOne Plus real-time PCR System (ABI, USA). The real-time PCR reaction was performed in a 20 μL mixture containing primers, Fast Start Universal Probe master reagent (Roche Applied Science, Mannheim, Germany),

and 2 μL cDNA sample. The XIAP and β-actin mRNA targeting forward primer, reverse primer, and probe sequences are shown in Table S3. The mRNA level of the β-actin gene was measured in each sample as an internal normalization standard. The PCR program was carried out in triplicate. The thermal cycling conditions were as follows: 50 °C/2 min, 95 °C/10 min, and 40 cycles of 95 °C /15 s and 57 °C /60 s.

Western blot

Total protein was extracted using a cytoBuster^{TM} protein extraction reagent (Merk, Darmstadt, Germany), and the protein content was determined using a bicinchoninic acid protein assay kit (invitrogen, Carlsbad, CA). About 10 μg of protein was separated on 12% SDS-PAGE (Bio-rad, Hercules, CA) and then transferred to a nitrocellulose membrane. The membranes were then incubated with rabbit anti-human XIAP monoclonal antibody (1:500 dilution in PBS/Tween; Cell Signaling Technology, Danvers, USA) or rabbit anti-human cleaved caspase-3 polyclonal antibody (1:500 dilution in PBS/Tween; Cell Signaling Technology, Danvers, USA). β-actin was used as an internal standard to normalize protein expression, and the protein-containing membranes were simultaneously incubated with β-actin (C4) monoclonal antibodies (1:2000 dilution in PBS/Tween; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Protein-antibody complexes were detected *via* chemoluminescence (ECL Plus, Amersham Biosciences, USA).

TUNEL assay

The cells were seeded at a density of 1×10^5 per well in six-well plates with a glass bottom and were incubated overnight at 37 °C. The complexes prepared as described in the previous section were then added to the cells. After 48 h of cell incubation,

TUNEL assay was performed using a FragEL^{TM} DNA fragment detection kit (colorimetric-TdT enzyme method) according to the manufacturer's protocol (EMD chemicals Inc., Darmstadt, Germany). In brief, after 100 μL of proteinase K (20 μg/mL) in Tris buffer (pH 8.0) was added into the culture medium in each well, the cells were incubated for 20 min at room temperature and then washed with TBS. After 3% H_2O_2 aqueous solution was added to inactivate the endogenous peroxidase at room temperature, the cells were washed with PBS, treated with terminal deoxynucleotidyl transferase (TdT Enzyme) at 37 °C for 90 min, and then washed again with PBS. The exposed 3'-OH ends of the breakage DNA fragment in the apoptotic cells were labeled with biotin-labeled deoxynucleotides. The cells were incubated at room temperature for 30 min with a streptavidin-horseradish peroxidase conjugate. Diaminobenzidine reacted with the labeled sample to generate an insoluble brown DAB signal, whereas a shaded blue-green to greenish tan signifies the presence of nonapoptotic cells.

Cell viability assay

The cytotoxicity after XIAP siRNA transfection was evaluated *via* MTT assay. All experiments were conducted in triplicate. After the culture medium in each well was replaced by the same volume of serum-free RPMI-1640, the cells were incubated for 48 h in the presence of polyplexes with a siRNA final concentration of 100 nM. The method of cytotoxicity detections was executed similar to that in the cell viability assay section.

Histology and immunohistochemistry

The tumors were collected and fixed for 24 h in 10% PBS buffered formalin. After

deparaffinization, tissue sections (5 μm) were stained with hematoxylin/eosin (H&E). At least five paraffin sections from each animal were used for H&E staining. Immunohistochemistry study was then performed. The tumor sections were deparaffinized with xylene and alcohol, washed with PBS, and incubated in 10 mM citrate buffer (pH 7.4) at 90 °C for 15 min. The sections were then treated with 0.3% hydrogen peroxide in methanol at 4 °C for 30 min to inactivate the endogenous peroxidases. After being blocked with 10% normal horse serum, 2% bovine serum albumin, and 0.5% Triton X-100 for 1 h at room temperature, the tissues were incubated in the presence of rabbit polyclonal primary antibodies for XIAP, cleaved caspase-3 (1:500 dilution in PBS/Tween; Cell Signaling Technology, Danvers, USA) for 1 h at 37 °C, washed with PBS, and then further incubated for 1 h with horseradish-conjugated donkey anti-rabbit IgG secondary antibodies (DAKO Corporation, Carpinteria, CA). Finally, the immuno-reactivity on the tissue sections was visualized using the peroxidase substrate diaminobenzidine.

Moreover, tumor apoptosis in paraffin sections was detected *via* TUNEL assay, which was carried out as previous described. For the molecular biology assays, tumor tissues were homogenized after being frozen in liquid nitrogen. Total RNA and protein were then extracted and analyzed as previous described.

In vivo fluorescence imaging

PLI (or Her2-PLI) was labeled with the near infrared fluorescent agent Alexa Fluor® 750 (Life Technologies) NHS-ester as follows: Alexa Fluor® 750 NHS-ester 100 μg (dissolved in 100 μL DMSO) and PLI 5 mg (dissolved in 1 mL DMSO) were mixed, and the reaction was allowed to proceed in the dark overnight at room temperature. The obtained solution was diluted to 10 mL with PBS and concentrated by

ultrafiltration in an Amicon cell (regenerated cellulose membrane, MWCO = 5 kDa). The resultant solution was washed five times with PBS (pH 7.4) by ultrafiltration. Finally, the Alexa Fluor® 750-labeled copolymer PLI (or Her2-PLI) was obtained.

Mice bearing Skov-3 tumors were subjected to in vivo fluorescence imaging. 100 μL Alexa Fluor® 750-labeled PLI/SCR polyplexes or Her2-PLI/SCR polyplexes with the same fluorescence intensity were injected into the tail vein of the tumor-bearing mice (n = 3) respectively. The fluorescence images were captured at different time points (8 h, 12 h, 24 h, and 36 h) on a small animal in vivo fluorescence imaging system (Carestream, USA). Before imaging, the mice were anesthetized by intraperitoneal injection of 150 μL 4% chloralic hydras. The fluorescence intensity in the *in vivo* imaging tests was quantified using the Carestream MI software.

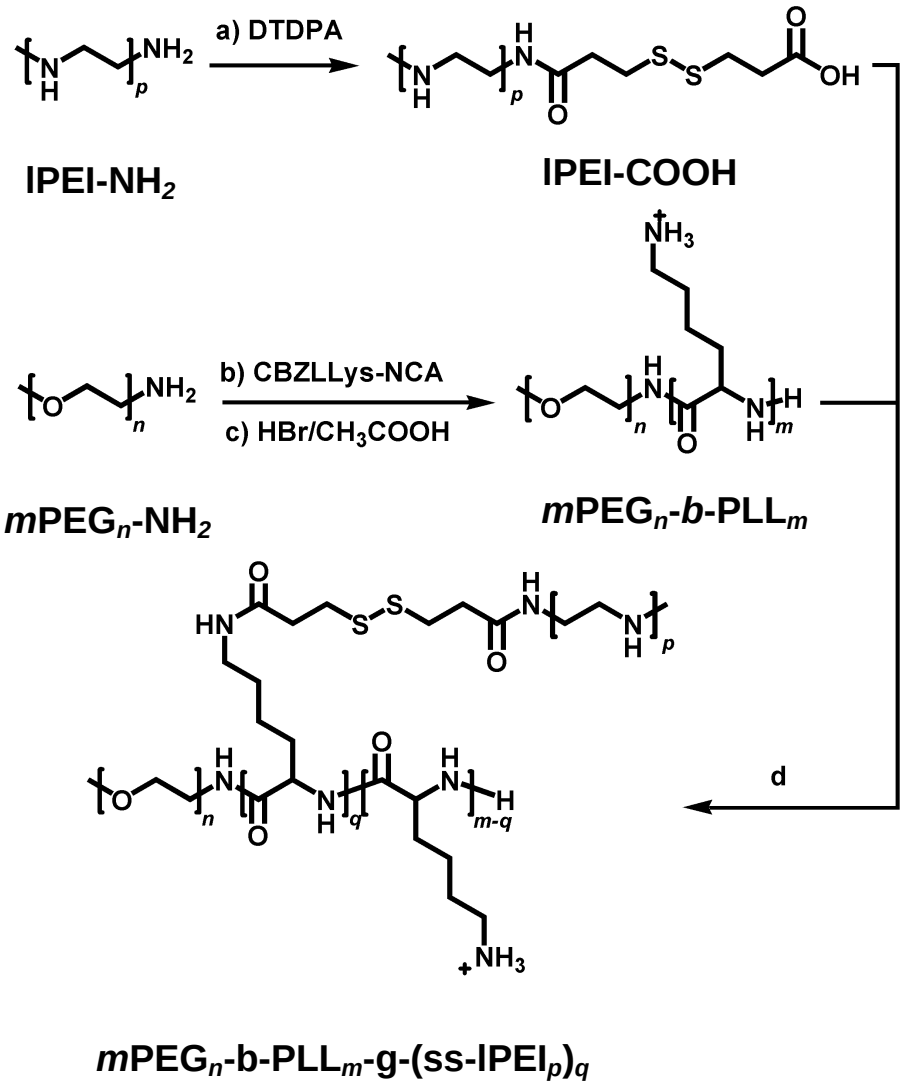
Cytotoxicity of the copolymer

As shown in Fig. S7, compared to the result which was obtained previously, the use of disulfide instead of urethane linkage to graft lPEI did not alter the phenomenon that lPEI grafting obviously decreased the cytotoxicity of the copolymer. Moreover, complexation of siRNA further reduced the cytotoxicity of copolymer.

XIAP gene expression in tumor tissues detected by real-time PCR and western blotting

As shown in Fig. S8a, the mice receiving PLI/XIAP-siRNA exhibited a lower level of XIAP mRNA in the excised tumor tissue than the mice receiving PBS or Her2-PLI/SCR. Moreover, Her2-PLI/XIAP-siRNA demonstrated even better performance than PLI/XIAP-siRNA in decreasing XIAP mRNA level in tumor tissue.

Western blot analysis of tumor tissue at protein level yielded consistent results (Fig. S8b). That is, mice receiving Her2-PLI/XIAP-siRNA had the lowest level of XIAP protein in tumor tissues. Furthermore, the strongest protein bands for activated caspase-3 were observed in tumor tissue from the mice receiving Her2-PLI/ XIAP-siRNA.



Scheme S1. Synthetic route of PLI. Reagents and conditions: (a) DTDPA, DCC, NHS, chloroform, DMSO, 0 °C, 24 h (84%); (b) *m*PEG-NH_2, CBZLL-NCA, DMF, 35 °C, 72 h (95%); (c) TFA, HBr/acetic acid, room temperature, 1 h (85%); (d) DCC, NHS, DMSO, 18 °C, 24 h (94%).

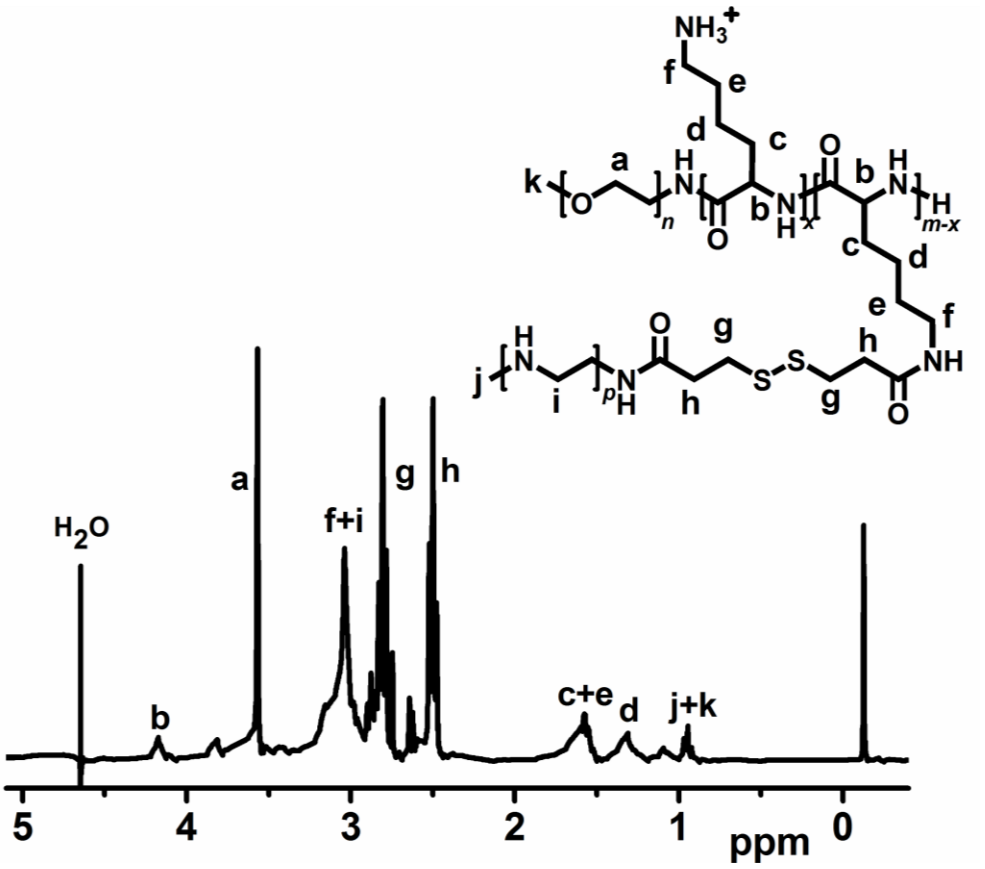


Fig. S1. ^{1}H NMR spectrum of PLI in D_2O recorded on a Varian Unity 300 MHz spectrometer.

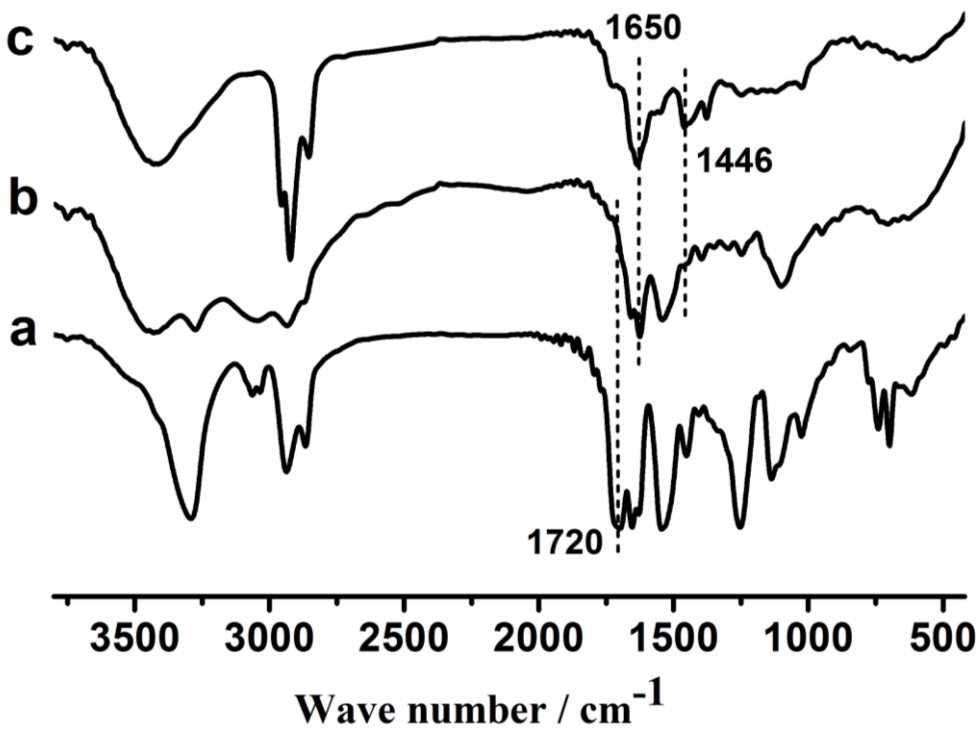


Fig. S2. FTIR spectra of a) PPCL, b) PPL, c) PLI.

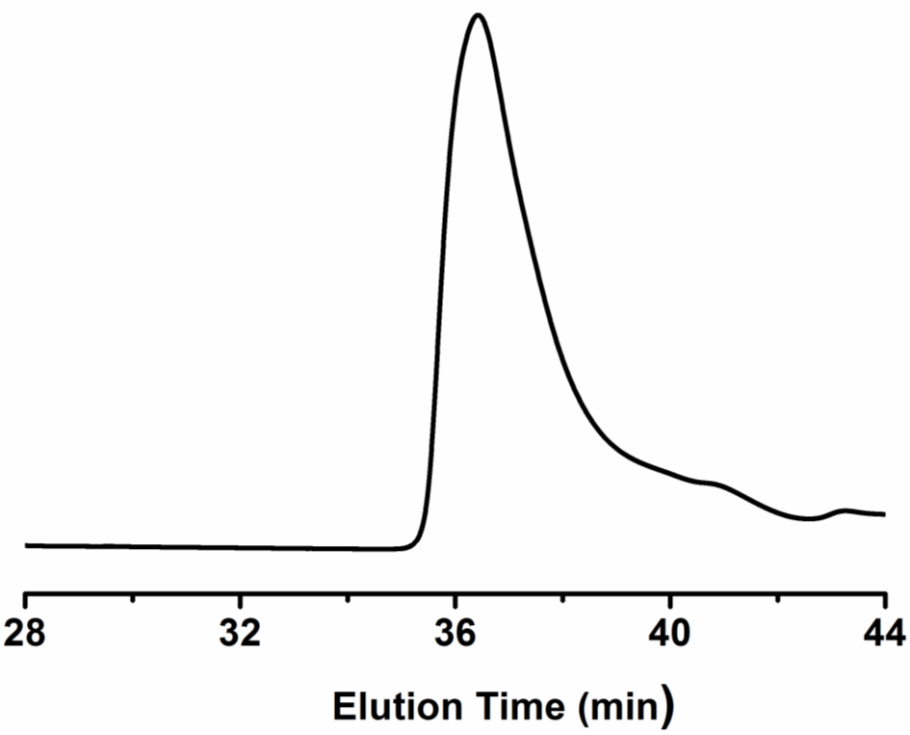


Fig. S3. Gel permeation chromatography of PLI at a flow rate of 0.6 mL/min acetic acid (0.3 mol/L)/sodium acetate (0.2 mol/L)

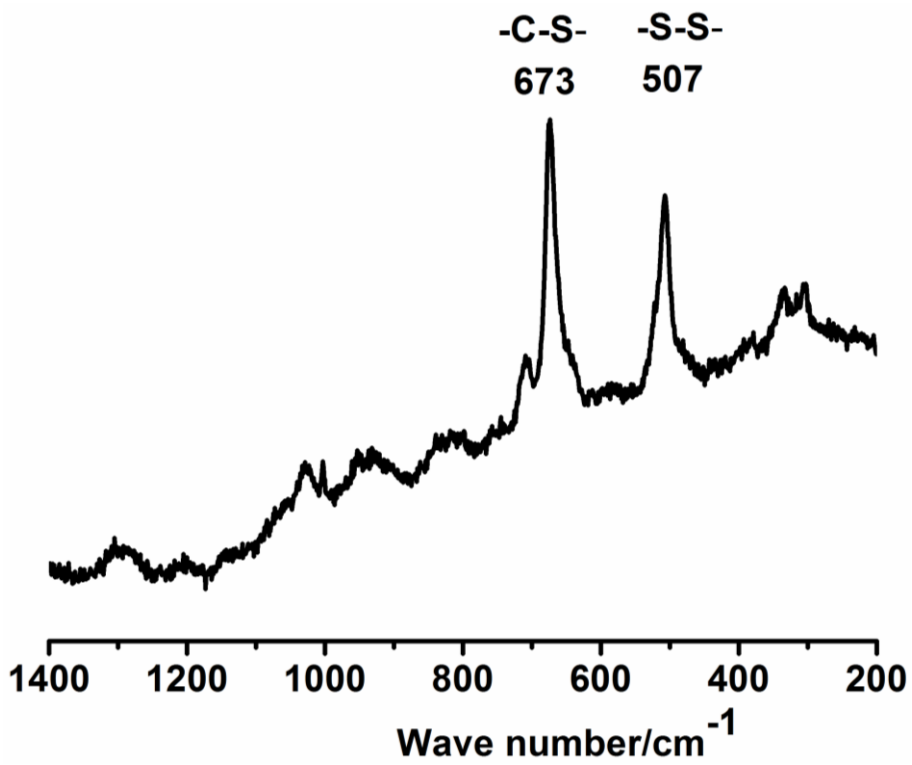


Fig. S4. Raman spectrum of PLI clearly showing the existence of disulfide bonds.

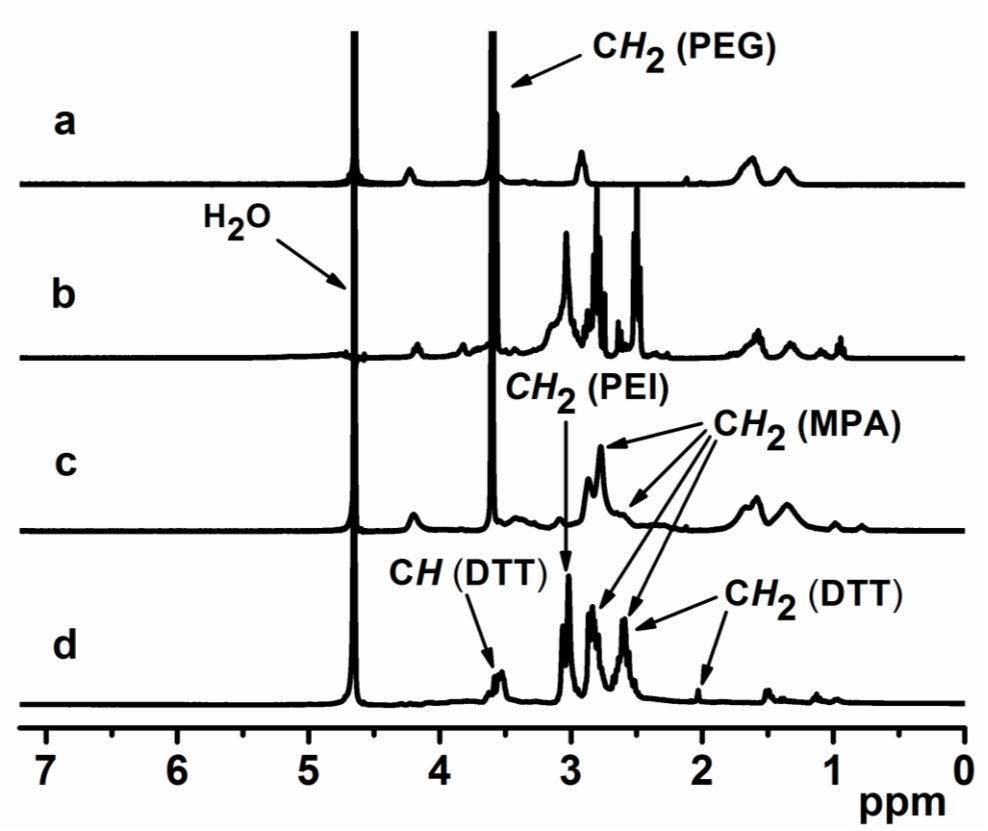


Fig. S5. ^{1}H NMR spectra of a) PPL; b) PLI; c) The upper solution of PLI with adding DTT after ultrafiltration; d) The lower solution of PLI with adding DTT after ultrafiltration. Spectra were recorded on a Varian Unity 300 MHz spectrometer in DMSO-d_6 at 25 °C (polymer concentration: 10 mg/mL). MPA is the abbreviation of 3-Mercaptopropionic acid.

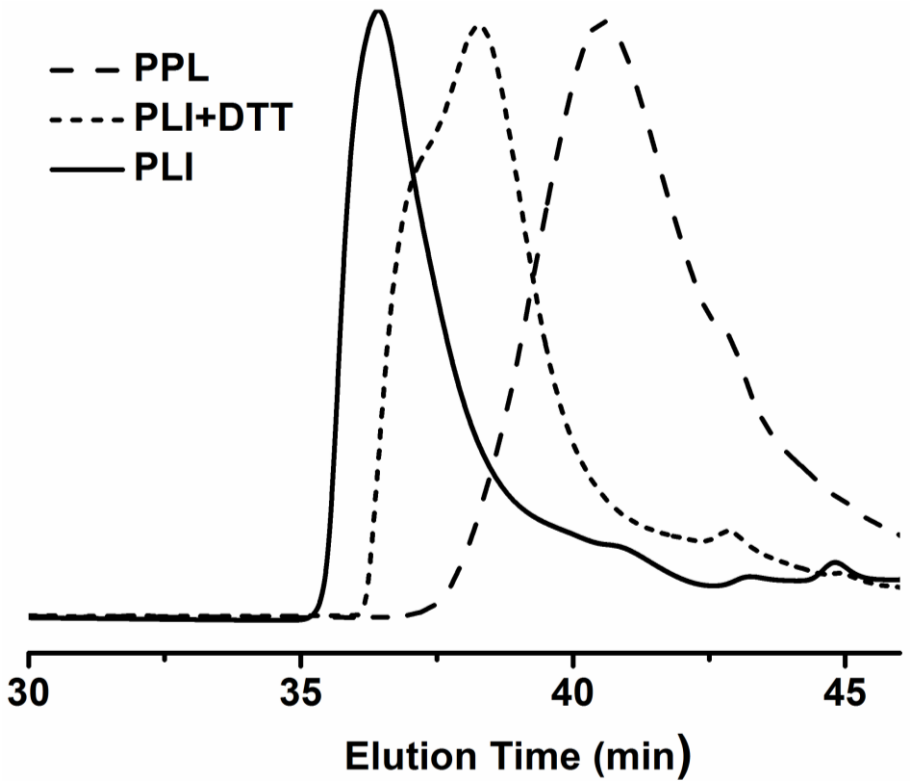


Fig. S6. Gel permeation chromatography of PPL, PLI and PLI with adding DTT in aqueous solution of acetic acid (0.3 mol/L)/sodium acetate (0.2 mol/L) (pH 4.5) at a flow rate of 0.6 mL/min.

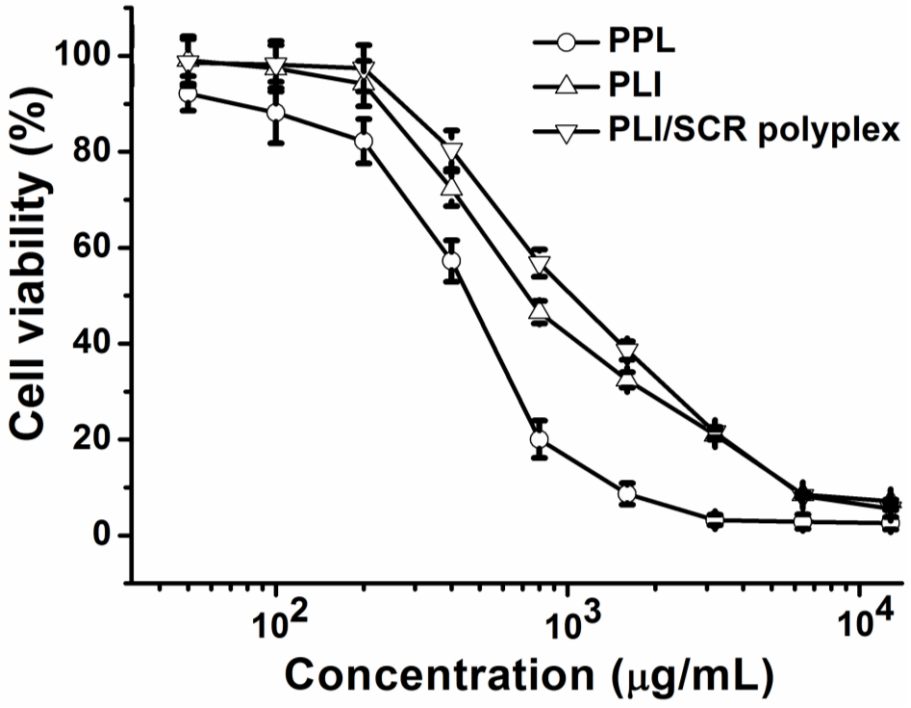


Fig. S7. *In vitro* cytotoxicity in Skov-3 cells determined by MTT assay (mean ± SE, n = 3). Incubation time: 48 h.

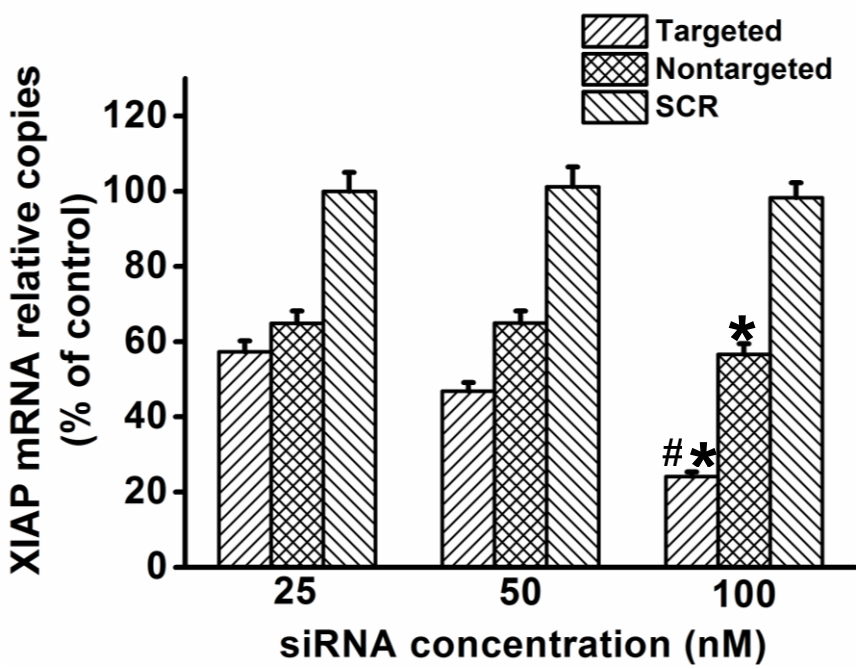


Fig. S8. Suppression on the XIAP mRNA levels quantified by real-time PCR analysis after incubation at different siRNA doses (n = 3). Incubation time: 72 h. Dose: 100 nM siRNA per well. *P < 0.05 vs SCR; #P < 0.05 vs nontargeted.

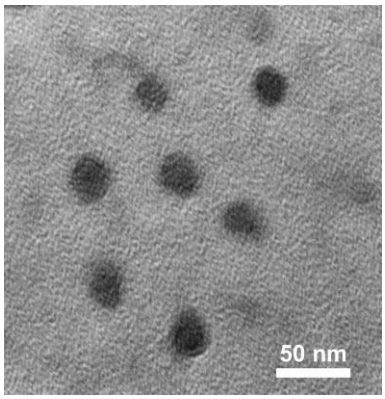


Fig. S9. Transmission electron microscopy (TEM) images of the polyplex (N/P = 20). The samples were stained with uranyl acetate.

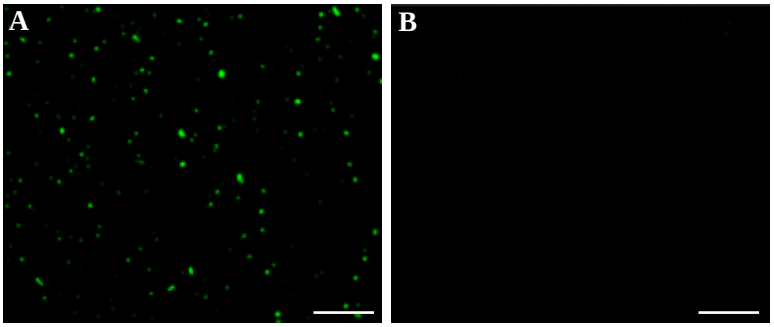


Fig.S10. FITC-labeled secondary antibody for Herceptin binds with the targeted polyplex to show green fluorescent dots under inverted fluorescence microscope (A). In contrast, green fluorescent dots were not observed in the solution of Herceptin-free polyplex (B). Scale bar represent 10 μm.

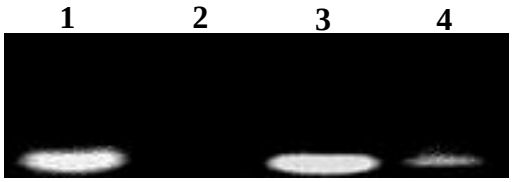


Fig.S11. RNase protection assay. Ribonuclease (Promega, Madison, WI, USA) was used to determine the ability of polyplexes in protecting siRNA from enzymatic degradation. 0.25 μg of naked siRNA or siRNA complexed with polyplexes was incubated at 37°C in 1.5 mL tubes which contain 0.1 unit of ribonuclease dissolved in the reaction buffer consisting of 10 mM Tris-HCl (pH 7.5), 5 mM EDTAand 0.2 M sodium acetate. After 60 min incubation, 10 mg of heparin (Sigma, St. Louis,MO, USA) was added into each solution at room temperature for 60 min. The samples were loaded onto 1% agarose gel with EB (0.1 mg/mL), and then ran with TAE buffer at 170 V for 10 min to examine the structural integrity of siRNA. 0.25 μg of siRNA without enzymatic digestion was also ran the same electrophoresis as a control. Electrophoresis of naked siRNA without ribonuclease digestion (1), naked siRNA with ribonuclease digestion (2), siRNA complexed at N/P 20 with ribonuclease digestion (3), and siRNA complexed at N/P 5 with ribonuclease digestion (4).

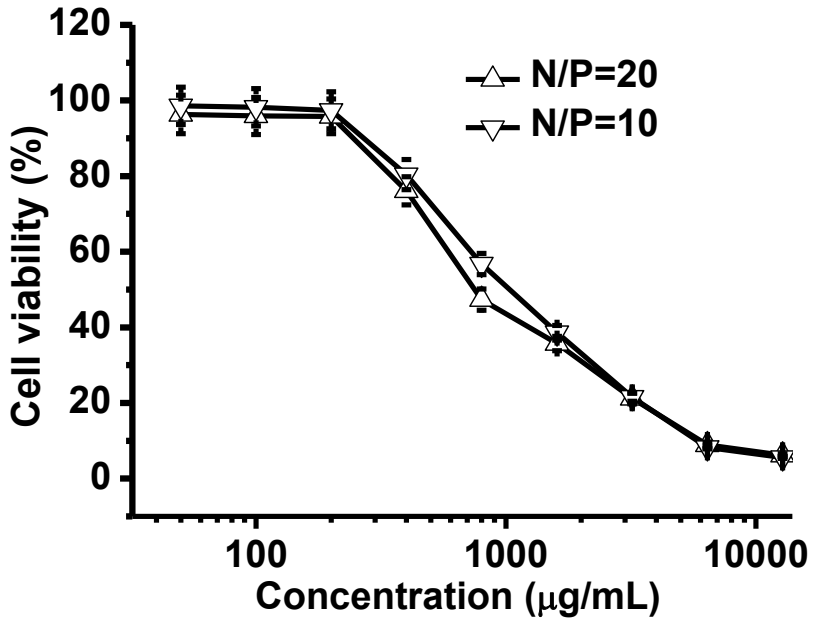


Fig.S12. *In vitro* cytotoxicity of polyplexes complexed with scramble siRNA (SCR) at N/P = 10 or N/P = 20 in Skov-3 cells determined by MTT assay (mean ± SE, n = 3). Incubation time: 48 h.

Table S1. Molecular characteristics of the Copolymers.

Sample	Composition of copolymer [a]	M_n [a] (Da)	M_n [b] (Da)	M_w/M_n [b]
PPCL	PEG_{45}-*b*-$PCBZLL_{42}$	12458	11463	1.18
PPL	PEG_{45}-*b*-PLL_{42}	7418	5372	1.21
PLI	PEG_{45}-*b*-PLL_{42}-g-$(ss\text{-}lPEI_{15})_{10}$	13818	11139	1.08
PLI+GSH	PEG_{45}-*b*-PLL_{42}-g-$(MPA)_{10}$	9338	7587	1.15

[a] As calculated from ^{1}H NMR spectra. [b] As estimated by GPC, PCL using DMF (containing 1 g/L LiBr) as mobile phase and other copolymers using acetic acid (0.3 mol/L)/sodium acetate (0.2 mol/L) (pH 4.5) as mobile phase.

Table S2. The Sequence of XIAP siRNA and SCR

	sense（5’-3’）	anti-sense（5’-3’）
XIAP-1	GCAGGUUGUAGAUAUAUCAdTdT	UGAUAUAUCUACAACCUGCdTdT
XIAP-2	GGUCAGUACAAAGUUGAAAdTdT	UUUCAACUUUGUACUGACCdTdT
SCR	UUCUCCGAACGUGUCACGUdTdT	ACGUGACACGUUCGGAGAAdTdT

Table S3. The primer and probe Sequence of real-time PCR

	Forward primer（5’-3’）	Reverse primer（5’-3’）	Probe
XIAP	AGGAAAGTATCCCCAAATTGCA	AGACTGCGTGGCACTATTTTCA	ATTTATCAACGGCTTTTATC
β-actin	GTACGCCAACACAGTGCTGTCT	TGCATCCTGTCGGCAATG	CACCACCATGTACCCTG