Supporting Information

Improved Cell Transfection of siRNA by pH-Responsive Nanomicelles Self-assembled with mPEG-b-PHis-b-PEI Copolymers

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Methods and Results

S1. Synthesis and characterization of ECE copolymers

meo-PEG_{2000}-PCL_{2500}-COOH (1 g, 0.22 mmol), NHS (50 mg, 0.44 mmol) and DCC (91 mg, 0.44 mmol) were dissolved in 10 mL DMSO and stirred at room temperature for one day under nitrogen atmosphere. Then branched polyethyleneimine (LMw-$PEI_{1.2K}$) (1.848 g, 1.54 mmol) was suspended in 4 mL DMSO and injected into the mixture solution. Then the mixture was stirred at room temperature for one day. After that, the mixture was dialyzed against DMSO for three days and against water for two days (MWCO:3500). The final product was collected after lyophilization. The yield of the product was 0.927 g (73.2%).

Figure S1 showed the H-NMR spectrum of ECE copolymer: ^{1}H-NMR (400 MHz, DMSO-d_6) δ3.98 (-CO-CH_2-CH_2-CH_2-CH_2-CH_2-O-), 3.51 (-CH_2-CH_2-O-), 3.25 (meo-), 2.39-2.72 (CH, CH_2 from PEI), 2.28 (-CO-CH_2-CH_2-CH_2-CH_2-CH_2-O-), 1.58 (-CO-CH_2-CH_2-CH_2-CH_2-CH_2-O-), 1.29 (-CO-CH_2-CH_2-CH_2-CH_2-CH_2-O-).

In Figure S2A, Fourier transform infrared spectra (FTIR) of EHE copolymers showed that the characteristic absorption peaks appeared at 3350 cm^{-1} (-NH_2 of PEI), 1710 $cm^{-1}$and 1450 cm^{-1} (-imidazole of PHis), and 1100 cm^{-1} (-CH_2-O-CH_2 of PEG). Meanwhile, fourier transform infrared spectra (FTIR) of EHE copolymers (Figure S2B) showed that the characteristic absorption peaks appeared at 3350 cm^{-1} (-NH_2 of PEI), 1660 cm^{-1} (-CO-NH- of PCL), and 1100 cm^{-1} (-CH_2-O-CH_2 of PEG).

Gel permeation chromatography (GPC) of EHE and ECE polymers were shown in Figure S3. The results showed there was a similar GPC spectra between EHE and

ECE polymers, indicating that a similar molecule weight in both polymers.

S2. Gel retardation assay

To investigate the loading efficiency and stability of siRNA in EHE/siRNA and ECE/siRNA nanoplexes at different N/P ratios, EHE/siRNA and ECE/siRNA nanoplexes were prepared at different N/P ratios, ranging from 1 to 20 (siRNA concentration was 1 μM). To investigate the stability of loaded siRNA, the nanoplexes was mixed with 3% SDS solution and the released siRNA was analyzed by Gel Retardation assay. Briefly, the samples were mixed with loading buffer and electrophoresed on a 1% agarose gel containing 0.1 ‰ GelRed (a special luminant dye for siRNA staining). Electrophoresis was performed at 80 mV for 3 min, subsequently 100 mV for 12 min, and the final gels were photographed under UV-illumination. Free siRNA was used as control.

The results of Gel Retardation assay were shown in Figure S4 and Figure S5. As shown in Figure S4, the results showed that the EHE micelles could load siRNA effectively above N/P of 4, while ECE micelles could load siRNA effectively above N/P of 8. The effective siRNA encapsulation of PHis-based micelles could be attributed to its electrostatic interaction with negatively-charged RNA. After destroyed by 3% SDS solution, the clear stripe of released siRNA from EHE/siRNA or ECE/siRNA nanoplexes could be seen at each setting N/P ratio (Figure S5), indicating the loaded siRNA were stable in nanoplexes.

S3. Cytotoxicity assay

MTT assay was to measure the cytotoxicity of siRNA-loaded NPs. Briefly, HeLa cells were seeded in 96-well plates at a density of 8000 cells per well. After 24h proliferation, cells were treated with various NPs at different concentration of siRNA for 4 h. After that, the medium was replaced by RPMI-1640 medium supplemented with 10% FPS, followed by another 48h incubation. Then, culture medium was removed and cells were incubated with MTT solution (0.5 mg/mL) for 4 h in the dark. The MTT solution was then removed and resulting formazan crystals were dissolved in 200 μL DMSO per well. Absorbance values were read on a microplate reader at the wavelength of 490 nm.

The cytotoxicities of siRNA-loaded NPs determined in HeLa cells were shown in Figure S6. The results showed that the cell viability treated by EHE/siRNA at each setting concentration was more than 90%, which was significantly higher than that by ECE/siRNA and $PEI_{1.2K}$, indicating the EHE/siRNA nanoplexes was a safe siRNA vector.

S4. Hemolytic activity of siRNA-loaded nanoplexes

To evaluate the safety of siRNA-loaded nanoplexes in blood circulation, the hemolytic effects on erythrocytes were determined. Erythrocytes (RBC) were isolated from SD Rat blood by centrifugation at 1500 g for 5 min at 4 ℃ and washed with physiological saline solution (0.9% sodium choloride, w/v). The cell pellet was suspended into a 2% erythrocyte suspension with pre-chilled different PBS buffer

solution (0.1M, pH=7.4, 6.8, or 5.5). Different siRNA-loaded NP solution were mixed with 0.2% erythrocyte suspension (pH=7.4) and the final concentration of siRNA was 10, 20, 50, 100, 200, 250, or 500nM. Then the mixed solution was incubated at 37 ℃ for 2h. 5% Glu was used as a negative control and Triton X-100 (2%, v/v) was used as a positive control. After centrifugation, the supernatant from each sample was transferred to a new 96 well plate and the absorbance was measured at 540 nm using a microplate reader. Relative Hemolysis Rate=$([Abs]_{sample}-[Abs]_{buffer})/([Abs]_{triton}-[Abs]_{buffer})*100\%$.

As shown in Figure S7, it was revealed that there was no significant hemolysis effects on erythrocytes found in EHE/siRNA group at any concentration (siRNA<500nM), indicating it would be a safe siRNA vector for intravenous injection.

S5. Serum aggregation effects of siRNA nanoplexes

To evaluate the stability of siRNA-loaded NPs in serum, the changes of serum turbidity was examined. Briefly, 100μL 5% glucose (as Control), EHE/siRNA nanoplexes, PEI_{1200}/siRNA nanoplexes and PEI_{25000}/siRNA nanoplexes were individually mixed with 100μL FBS in 96-well plates (Corning Inc., Corning, NY), followed by incubation at 37 °C. Then, serum aggregations (turbidity of the mixtures) were determined by measuring the absorbance at 630 nm using a microplate reader (Bio-Rad Laboratories, Inc. Shanghai, China) at different time points.

As shown in Figure S8, compared to significant aggregation of serum proteins

found in $PEI_{2.5K}$/siRNA or $PEI_{1.2K}$/siRNA, little aggregation of serum proteins was observed in EHE/siRNA group, indicading the EHE/siRNA nanoplexes would be stable in blood circulation.

Figure Captions

Figure S1. ^{1}H-NMR (400 MHz) spectra of ECE in DMSO-d6.

Figure S2. Fourier transform infrared (FTIR) spectroscopy of copolymers. (A)EHE. (B) ECE.

Figure S3. GPC spectra of the copolymers: (A) EHE. (B) ECE.

Figure S4. siRNA encapsulation efficacy of micelles at different N/P. (A) EHE. (B) ECE.

Figure S5. Integrity of loaded siRNA at different N/P. (A) EHE/siRNA. (B) ECE/siRNA.

Figure S6. Cell viability of Hela cells after incubated with EHE/siRNA, ECE/siRNA, $PEI_{1.2K}$/siRNA and PEI_{25K}/siRNA for 4 h. The data were shown as mean ±SD (n=3).

Figure S7. Haemolytic activity of siRNA-loaded NPs at different concentrations. The data were shown as mean ±SD (n=3).

Figure S8. Aggregation of serum proteins induced by siRNA-loaded NPs. The data were shown as mean ±SD (n=3).

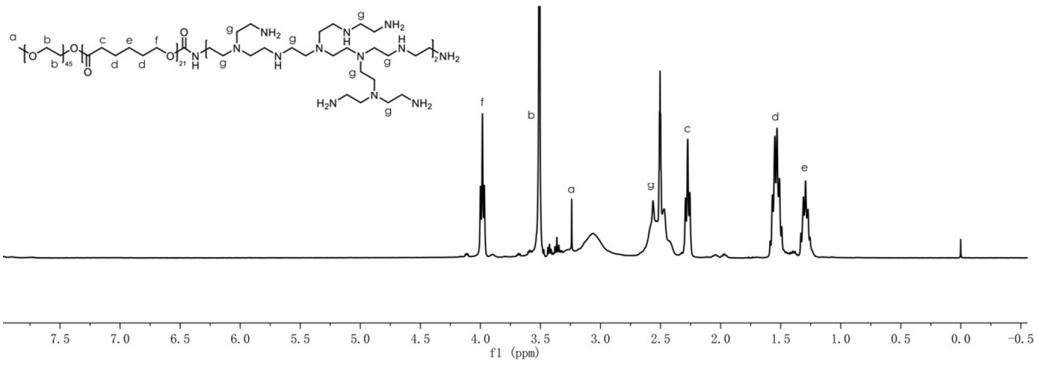


Figure S1. ^{1}H-NMR (400 MHz) spectra of ECE in DMSO-d6.

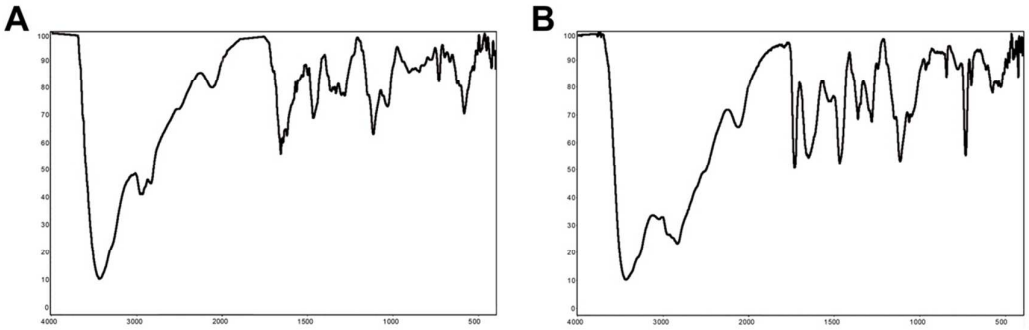
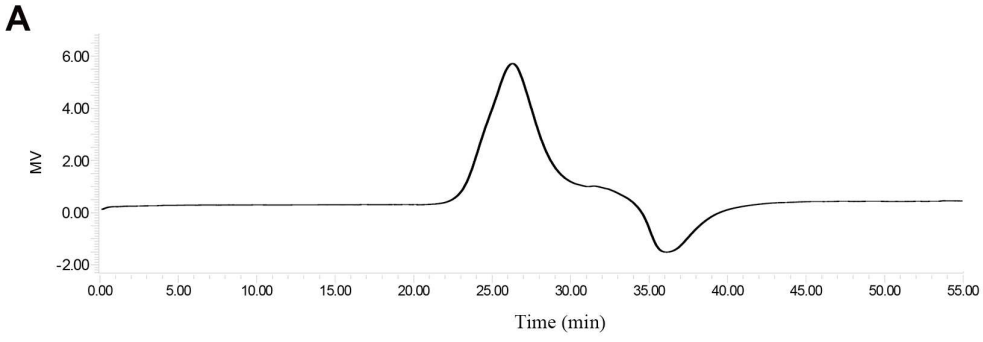
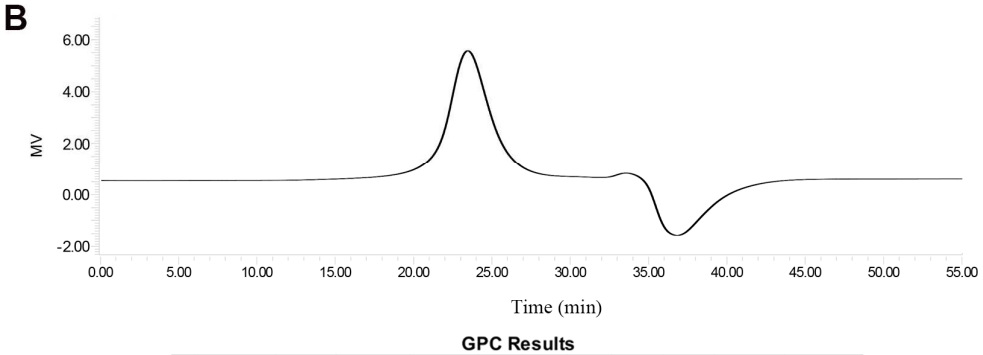


Figure S2. Fourier transform infrared spectra (FTIR) of copolymers. (A) EHE. (B) ECE.



GPC Results

	Dist Name	Elution Volume (ml)	Retention Time (min)	Adjusted RT (min)	Mn	Mw	MP	Mz	Mz+1	Mz/Mw	Mz+1/Mw
1	EHE	24.319	24.319	24.319	3879	5578	5639	7962	8006	1.427393	1.435281



GPC Results

	Dist Name	Elution Volume (ml)	Retention Time (min)	Adjusted RT (min)	Mn	Mw	MP	Mz	Mz+1	Mz/Mw	Mz+1/Mw
1	ECE	23.140	23.140	23.140	5030	6484	6582	8448	8588	1.302899	1.324491

Figure S3. GPC spectra of the copolymers: (A) EHE. (B) ECE.

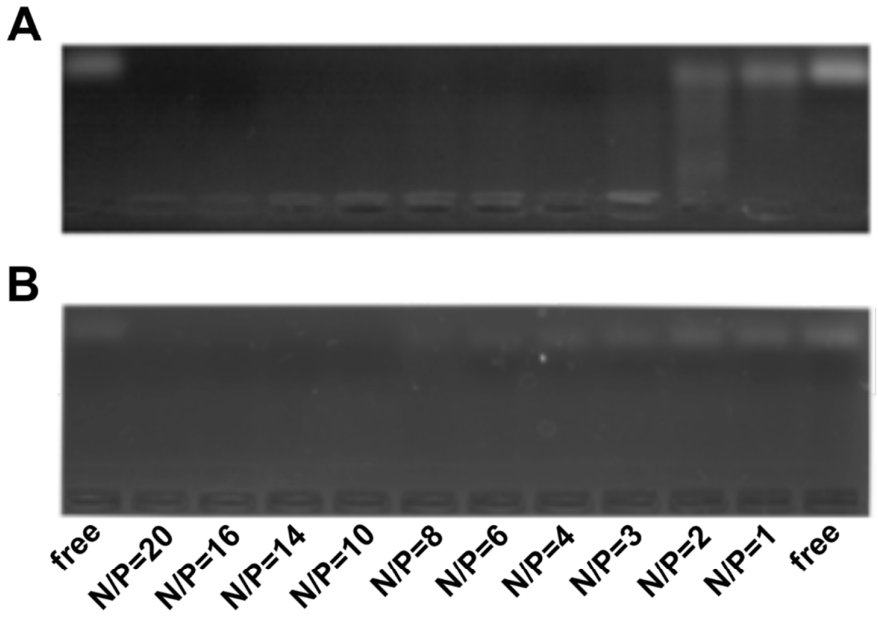


Figure S4. Gel retardation assay for siRNA loading efficacy of various nanoplexes at different N/P ratios. (A) EHE. (B) ECE.

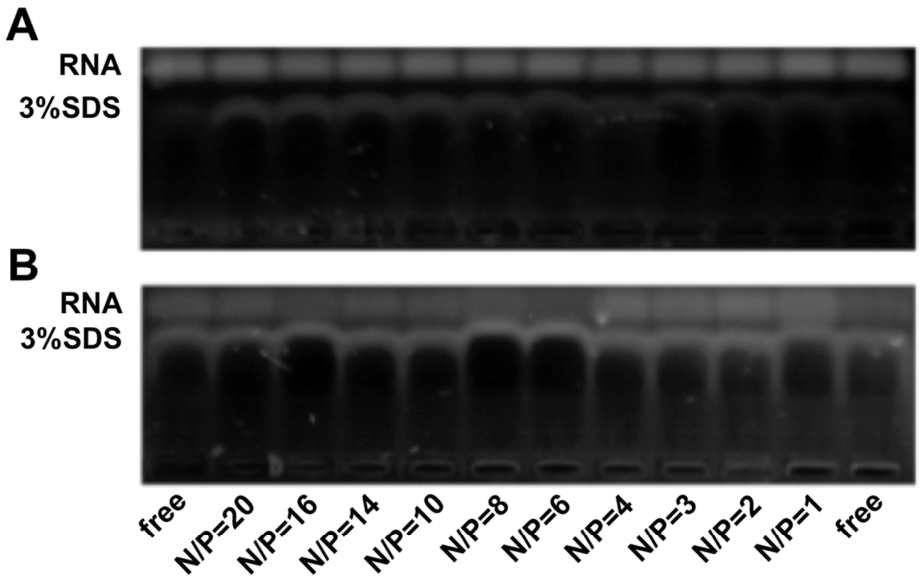


Figure S5. Integrity of loaded siRNA in different nanoplexes at different N/P. (A) EHE/siRNA. (B) ECE/siRNA.

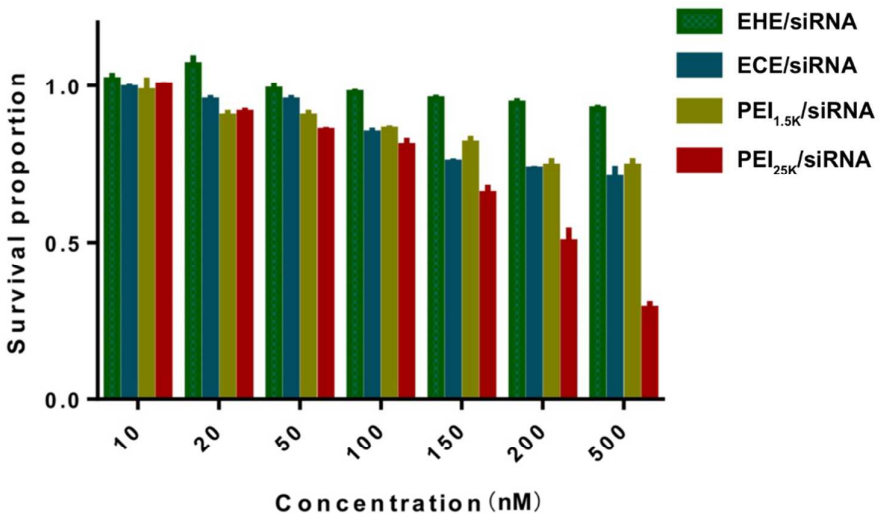


Figure S6. Cell viability of Hela cells after incubated with EHE/siNC, ECE/siNC, PEI_{1200}/siNC and PEI_{25000}/siNC for 4 h. The data were shown as mean ±SD (n=3).

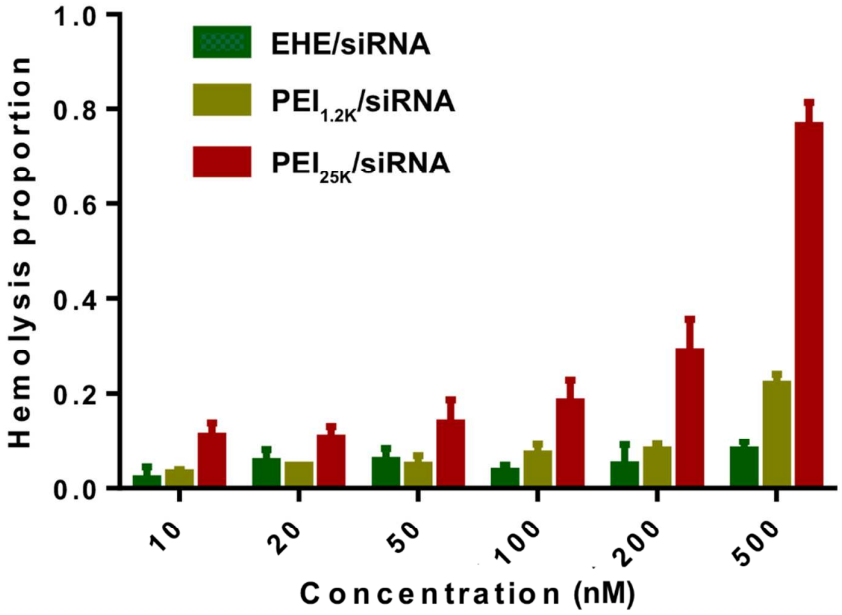


Figure S7. Haemolytic activity of siRNA-loaded NPs at different concentrations. The data were shown as mean ±SD (n=3).

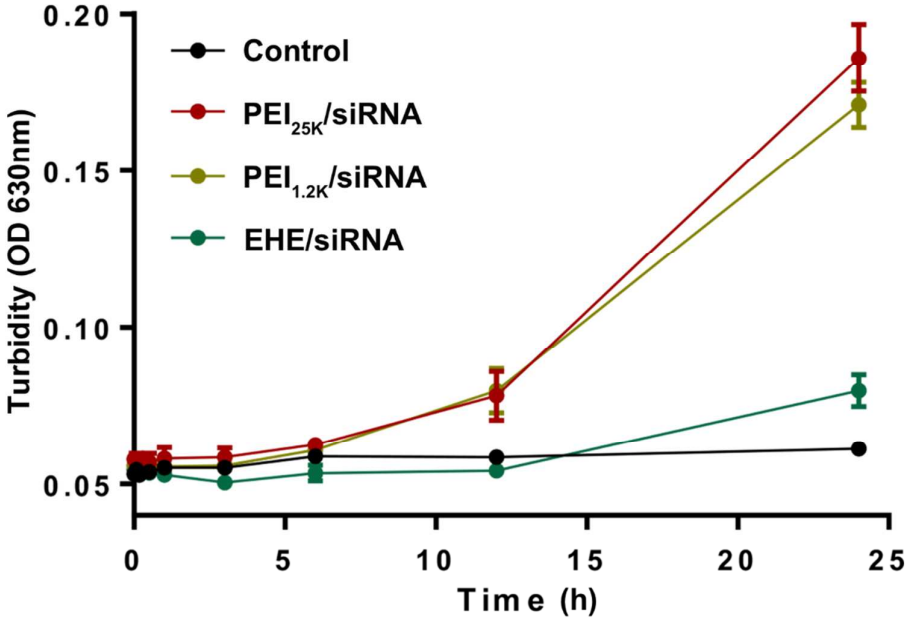


Figure S8. Aggregation of serum proteins induced by siRNA-loaded NPs. The data were shown as mean ±SD (n=3).