

## Electronic Supplementary Information

### The effects of a multifunctional oligomer and its incorporation strategies on the gene delivery efficiency of poly(L-lysine)

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## 1. Chemical synthesis and characterization

### 1.1. Materials and Reagents

*L*-Histidine methyl ester (*L*-His-OMe), poly(L-lysine) (PLL, Mw=150-300 kDa), agarose, trypsin-EDTA, Dulbecco's modified Eagle medium (DMEM), heparin, deoxyribonuclease I (DNase), ethidium bromide (EB), fetal bovine serum (FBS), bovine serum albumin (BSA, Mw=69 kDa, pI=4.8), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumerma bromide (MTT) and phosphate buffer solution (PBS, 0.01M, pH=7.2~7.4) were obtained from Beijing Dingguo Biotech. Co. Ltd. (Tianjin, China). Benzotriazole (Bt), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl, 98.5%)

and N-hydroxysuccinimide (NHS, analytical grade) were purchased from Aladdin (Shanghai, China). 4,4'-Azobis(4-cyanopentanoic acid) (ACVA, Fluka, 98%) was recrystallized from methanol. Anhydrous methanol, dichloromethane (DCM), thionyl chloride (SOCl<sub>2</sub>) and N,N-dimethylformamide (DMF) obtained from The Sixth Chemical Reagent Company (Tianjin, China) were all distilled before use. (4-Cyanopentanoic acid) dithiobenzoate (CPADB) was prepared according to literature.<sup>1</sup> Branched polyethylenimine (PEI, Mw=25 kDa) was purchased from Sigma (St. Louis, MO). Plasmid pEGFP-N1 (4.7 kbp, Clontech, Palo Alto, CA, USA) encoding enhanced green fluorescent protein (EGFP) was driven by immediate early promoter of CMV. The plasmid DNA (pDNA) was propagated in DH5 $\alpha$  strain of E.coli and purified by use of Endfree plasmid kit (Tiangen, China). The purity and concentration were confirmed by spectrophotometry (A260/A280). The lactose monomer MAEL was synthesized according to our previous work.<sup>2</sup> Other agents used were all of the highest purity available.

## 1.2. Characterization Methods

Chemical structure was confirmed by <sup>1</sup>H NMR spectra recorded on a Varian UNITY-plus 400 spectrometer operated at 400 MHz. The molecular weight (Mw) and polydispersity index (PDI) were determined with size exclusion chromatography (SEC) equipped with Waters Shodex KW-804 columns. The gel shift assays were conducted on 1.5% agarose gel in Tris–boric acid–EDTA buffer (pH=8.0) at 100 V for 45 min. The complex sizes and zeta potentials were determined with a Zetasizer Nano Series Nano-ZS (Malvern) instrument equipped with a 4.0 mW He-Ne laser at a scattering angle of 90° producing a wavelength of 633 nm.

## 1.3. Preparation of *L*-MA-His-OMe

Methacryloyl benzotriazole (MA-Bt) was first prepared according to literatures.<sup>3,4</sup> Then, *L*-histidine methyl ester (5.52 mM) was dissolved in deionized water and the pH value was adjusted to 7~8 with saturated sodium bicarbonate solution. 1,4-dioxane containing MA-Bt (5.52 mM) was added slowly to the solution and the reaction was

conducted at room temperature. Completion of the reaction was monitored by thin layer chromatography (TLC). When the reaction was finished, the 1,4-dioxane was evaporated under vacuum. The residues were diluted with deionized water and extracted with ethyl acetate to remove the 1H-benzotriazole and unreacted MA-Bt. Collected water phase was neutralized to pH=6-7 using 10% HCl solution. The water was removed *via* rotary-evaporator. After drying in vacuum, the obtained crude product was dissolved in absolute ethanol and then filtered to remove the remaining *L*-histidine methyl ester. Then the ethanol was removed *via* rotary-evaporator to give *L*-MA-His-OMe. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O, δ): 8.60 (s, 1H; CH), 7.42 (s, 1H; CH), 5.65 (s, 1H; CH<sub>2</sub>), 5.45 (s, 1H; CH<sub>2</sub>), 4.45 (t, 1H; CH), 3.68 (s, 3H; CH<sub>3</sub>), 3.0-3.2 (m, 1H; CH), 1.95 (s, 3H; CH<sub>3</sub>).

#### 1.4. Preparation of P(His-co-DMAEL)

P(His-co-DMAEL) was prepared by copolymerizing *L*-MA-His-OMe with MAEL. *L*-MA-His-OMe (4.0 mM), MAEL (0.66 mM), CPADB (0.08 mM) and ACVA (0.027 mM) were dissolved in DMF (10 mL). After degassing by three freeze-pump-thaw cycles, the polymerization was conducted in a sealing glass tube at 70 °C for 48 h. When the polymerization was finished, the reaction was quenched with ice water. The polymer was dialyzed with deionized water for 2 days (cutoff Mw=3.5 kDa) to remove the remaining *L*-MA-His-OMe and then the product was lyophilized. Afterward, the glycopolymer in the polymer was deprotected. The lyophilized polymer was dissolved in anhydrous methanol, next sodium methoxide (30%, mass concentration) was added and the mixture was stirred for 1 h at room temperature. Then the solution was dialyzed with deionized water for 2 days (cutoff Mw=3.5 kDa) and lyophilized to give P(His-co-DMAEL).

#### 1.5. Preparation of PLL-g-P(His-co-DMAEL)

For preparing PLL-g-P(His-co-DMAEL), P(His-co-DMAEL) was dissolved in distilled water, EDC.HCl and NHS (1 : 1, molar ratio) were added and the mixture was stirred for 1 h, then PLL was added. The mixture was further stirred for 12 h. Afterward, the mixture was dialyzed with deionized water for 2 days (cutoff

Mw=12-14 kDa) and lyophilized.

### **1.6. Preparation of FITC-labeled PLL and PLL-g-P(His-co-DMAEL)**

Briefly, PLL or PLL-g-P(His-co-DMAEL) was dissolved in deionized water. Then the required FITC, which was dissolved in DMSO, was added and the mixture was further stirred for 12 h at room temperature in dark (the molar ratio of FITC to the amino group in PLL was 1:100). Afterward, the mixture was dialyzed with deionized water for 2 days (cutoff Mw=3.5 kDa) and lyophilized in dark to give FITC-labeled PLL or PLL-g-P(His-co-DMAEL).

### **1.7. Preparation of polycations/pDNA Complexes**

For the preparation of PLL/pDNA/P(His-co-DMAEL) ternary complexes, calculated pDNA and P(His-co-DMAEL) were first mixed and left for 15 min at room temperature. Then according to N/P ratio, required PLL was dropped into the mixture. Then mixture was vortexed for 10 s and left for another 15 min. For the preparation of PLL-g-P(His-co-DMAEL)/pDNA complexes, pDNA and PLL-g-P(His-co-DMAEL) were directly mixed and left for 30 min at room temperature.

### **1.8. Gel Shift Assays<sup>5</sup>**

In general procedure, N/P ratios of 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 were employed. The weight of pDNA employed for each sample preparation was 120 ng. Complexes were prepared as mentioned above. Then the samples were transferred to agarose gel well and electrophoresis was conducted. For investigating complex stability in heparin, NaCl, DNase and serum, complexes were prepared as above and N/P ratios of 4, 8, 12, 16 and 20 were employed. For investigating the complex stability in heparin, heparin (135 USP units/ $\mu$ g pDNA) and NaCl (0.5 M final concentration) were added to the complexes and the complexes were incubated at 4 °C for 30 min. For investigating the complex stability in NaCl, NaCl (2 M final concentration) was added to the complexes and the complexes were incubated at 4 °C for 30 min. For investigating the complex stability in DNase, DNase (2 USP units per

1  $\mu\text{g}$  pDNA) and  $\text{MgCl}_2$  (2.5 mM final concentration) were added to the complexes and the complexes were incubated at 37 °C for 10 min. Then the enzyme reaction was stopped by EDTA (15 mM final concentration). For investigating the complex stability in serum, an equivalent volume of DMEM containing 10% FBS was added to the complexes and the complexes were incubated at 37 °C for 24 h. As controls, the naked pDNA (120 ng) was treated in the same way.

### 1.9. Complex Sizes and Zeta Potentials

Complex sizes and zeta potentials (N/P=8 or 16) were measured using dynamic light scattering (DLS). 3  $\mu\text{g}$  of pDNA was employed for each sample preparation. Samples were prepared in high purity water as mentioned above. For investigating the complex stability in protein solutions, the complexes were prepared in PBS at N/P ratio of 16 and the complex sizes were measured. Then BSA solution was added to the complexes (0.5% final concentration) and vortexed for 30 s. 24 h later, the complex sizes were measured again.

## 2. Biological studies

### 2.1. Cell Culture

The human cervical cancer cell line (HeLa), human hepatoma cell line (HepG2) and mouse embryonic fibroblast cell line (NIH3T3) were purchased from ATCC (Teddington, UK) and maintained in DMEM containing 10% FBS without any antibiotics at 37 °C, 5%  $\text{CO}_2$  atmosphere.

### 2.2. Transfection *in Vitro*

For transfection *in vitro*, HeLa, HepG2 and NIH3T3 cells were seeded in 24-well plates at a density of  $4 \times 10^4$  cells per well in 500  $\mu\text{L}$  of complete DMEM media and transfected the next day at 70~80% confluence. Prior to transfection, the media were removed and the cells were washed twice with 500  $\mu\text{L}$  of PBS. Then the cells were

replenished with 500  $\mu\text{L}$  of complete media containing the polycations/pDNA complexes (N/P=8 or 16) at a concentration of 1  $\mu\text{g}$  pDNA/well. The cells were further incubated for 48 h. According to our previous work,<sup>6</sup> the cells transfected with PEI/pDNA complexes at the N/P ratio of 10 were used as the positive controls, and the cells without any treatment were used as the negative controls. The GFP positive cells were observed under an inverted fluorescence microscope and images were recorded with a CCD camera (Nikon ECLIPSE TE2000-U, Japan). The gene transfection efficiency was determined with a flow cytometer (BD FACS Calibur, USA) equipped with an argon laser exciting at 488 nm.

### 2.3. Complex Cellular Uptake and Trafficking

FITC-labeled PLL and P(His-co-DMAEL)-g-PLL were used to prepare the complexes. The transfection was conducted in serum conditions at N/P ratio of 16 as described above. For determining the complex cellular uptake efficiency, at 4 h post transfection, the culture media were aspirated and the cells were washed six times with 500  $\mu\text{L}$  of PBS. Then the cells were collected and analyzed with a flow cytometer (BD FACS Calibur, USA) equipped with an argon laser exciting at 488 nm. To visualize the distribution of the complexes, at 2, 4 and 6 h post transfection, the culture media were aspirated. The cells were washed six times with 500  $\mu\text{L}$  of PBS and fixed in 4% paraformal and then the fixed cells were observed with an inverted fluorescence microscope and images were recorded with a CCD camera (Nikon ECLIPSE TE2000-U, Japan).

### 2.4. Cytotoxicity assay

Complexes with pDNA concentration of 0.5  $\mu\text{g}$ /well were prepared at N/P ratio of 16, PEI/pDNA complexes at N/P ratio of 10 were used as comparison. HeLa and NIH3T3 cells were seeded in 96-well plates at an initial density of 8,000 cells/well in 100  $\mu\text{L}$  of complete media. After 24 h growth, the original media were replaced with 100  $\mu\text{L}$  of fresh media containing the complexes. 48 h later, the media were replaced with 100  $\mu\text{L}$  of MTT solution (1 mg/mL final concentration, diluted with complete media). The

cells were further incubated for 4 h. Afterward, the MTT solution was replaced with 150  $\mu$ L of DMSO and the plates were slightly shaken for 10 min to ensure the complete dissolution of the formed formazan. Absorbance of the dissolved formazan was measured using a microplate reader (Labsystem, Multiskan, Ascent, Model 354 Finland) at the wavelength of 570 nm.

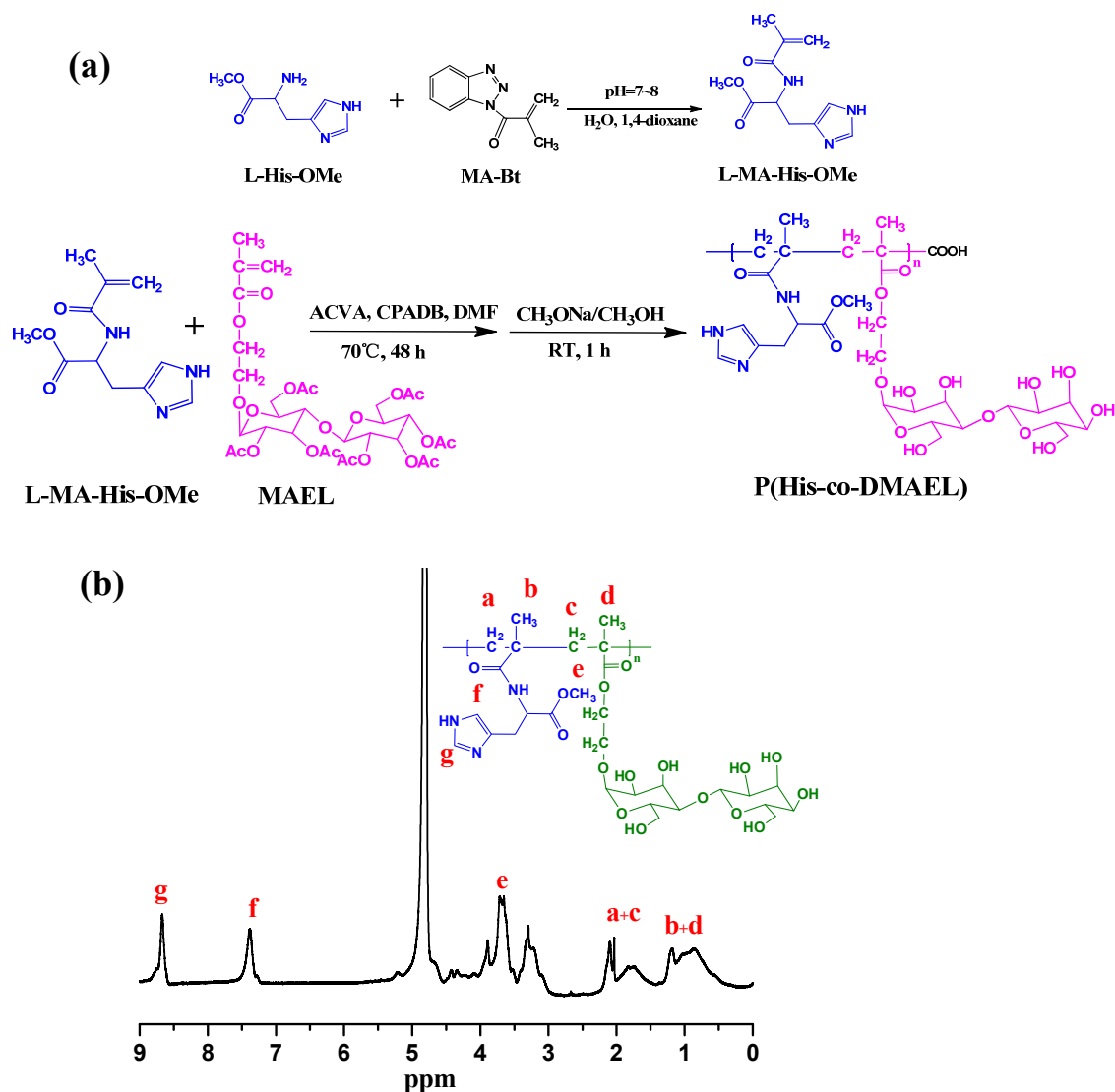
## 2.5. Statistics

Data were expressed as mean $\pm$ standard deviation. Statistical comparisons between the control and treated groups were performed using the Student's t-test. Mean values and standard deviations were calculated for each sample examined from at least three independent experiments. The levels of statistical significance were set at  $P < 0.05$  (\*), 0.01 (\*\*) or  $P < 0.001$  (\*\*\*).

## Reference

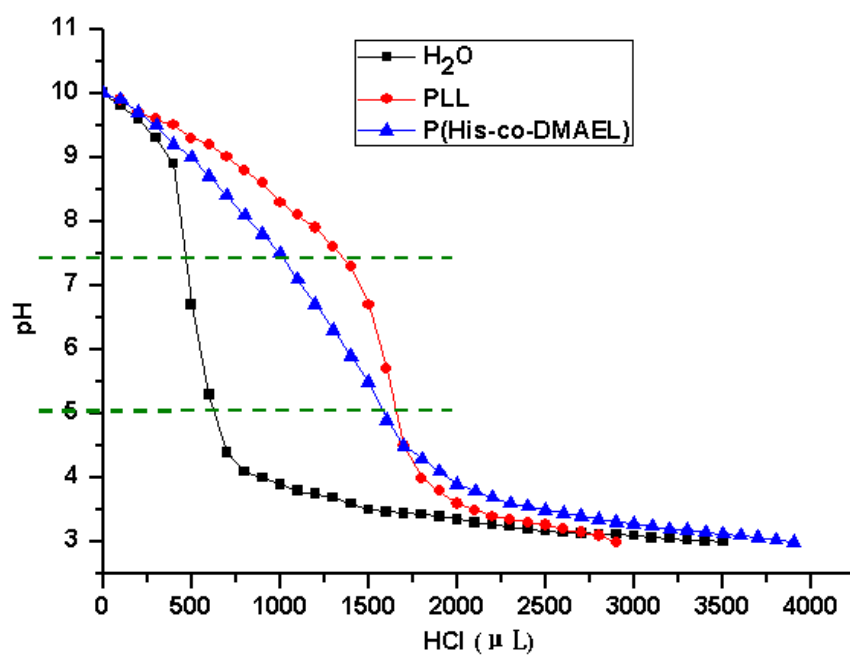
- 1 Y. Mitsukami, M. S. Donovan, A. B. Lowe, C. L. McCormick, *Macromolecules*, 2001, **34**, 2248.
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- 3 A. R. Katritzky, Y. Zhang, S. K. Singh, *Synthesis*, 2003, **6**, 2795.
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## 4. Figures

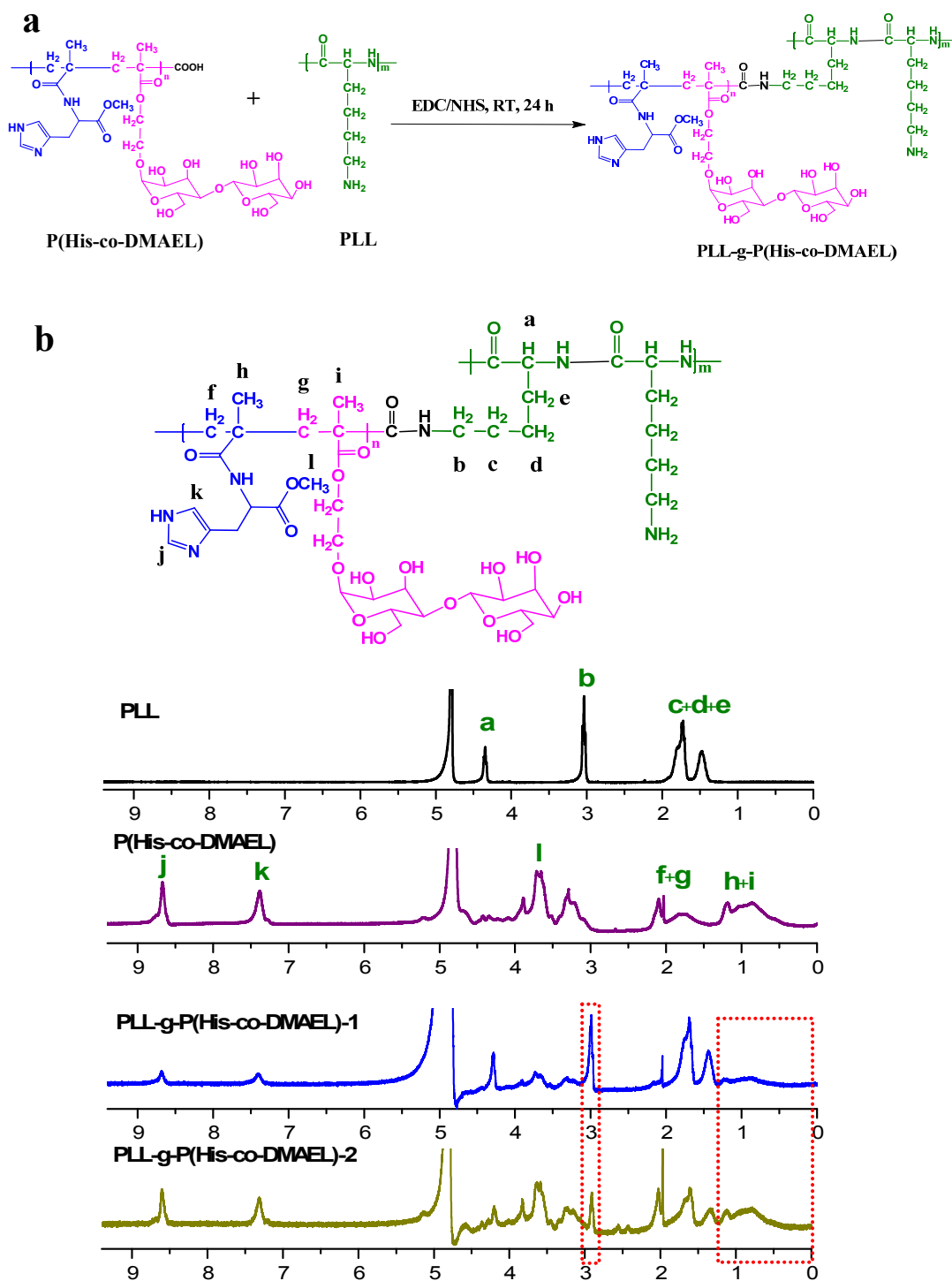


**Fig. S1** (a) Synthetic approach to the oligomer P(His-co-DMAEL). (b)  $^1\text{H}$  NMR spectrum of P(His-co-DMAEL). The imidazole groups can be identified at 7.4 and 8.7 ppm. The methoxyl groups can be identified at 3.7 ppm. The pendant methyl groups were located in 0.5~1.4 ppm region. The methylene groups on the backbone were located in 1.4~2.2 ppm region. Other groups were overlapped in 3.0~5.2 ppm region. By comparing the integral area of the pendant methyl groups with that of the imidazole groups, the molar ratio of MAEL units to L-MA-His-OMe units in P(His-co-DMAEL) was calculated to be 1:10.

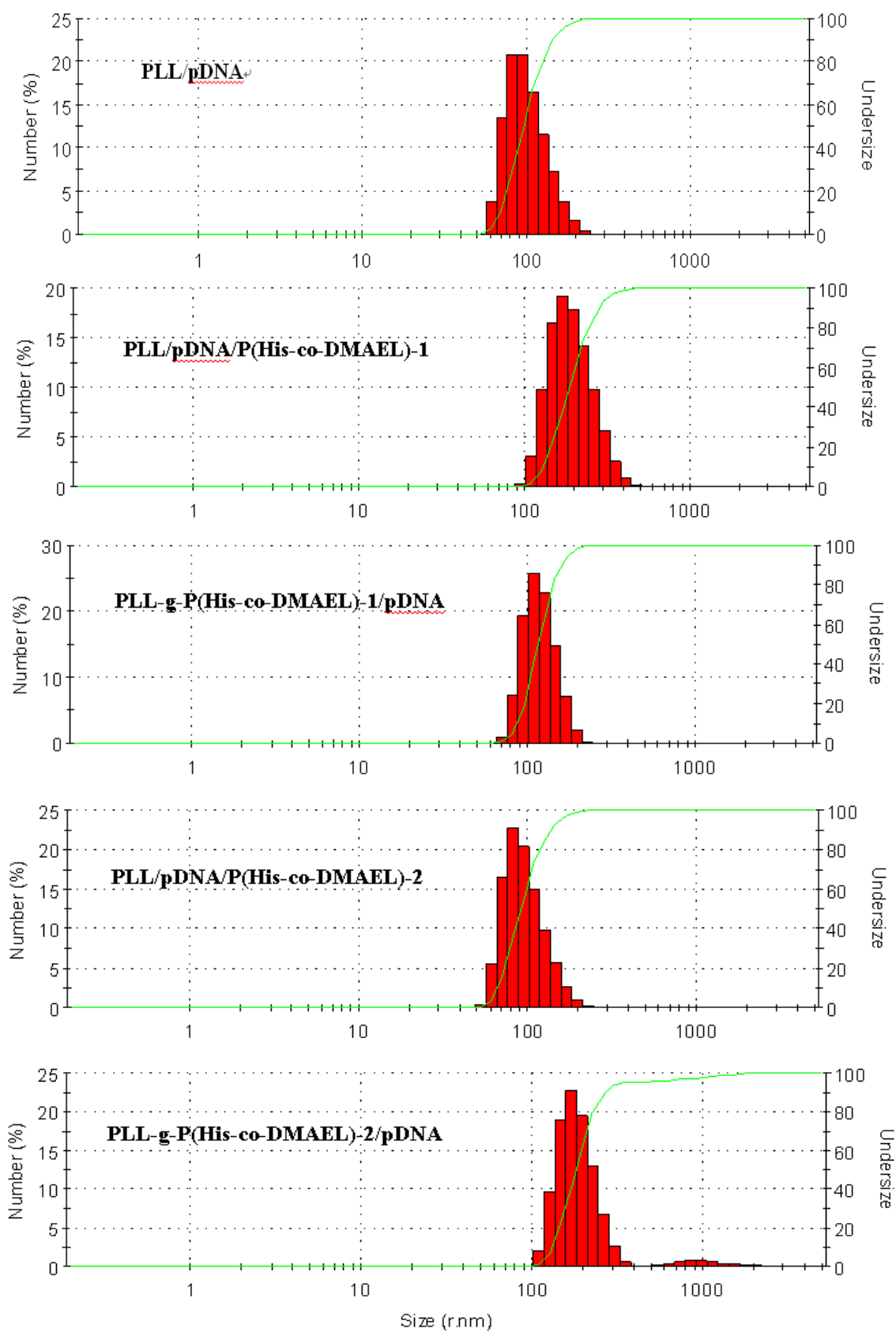




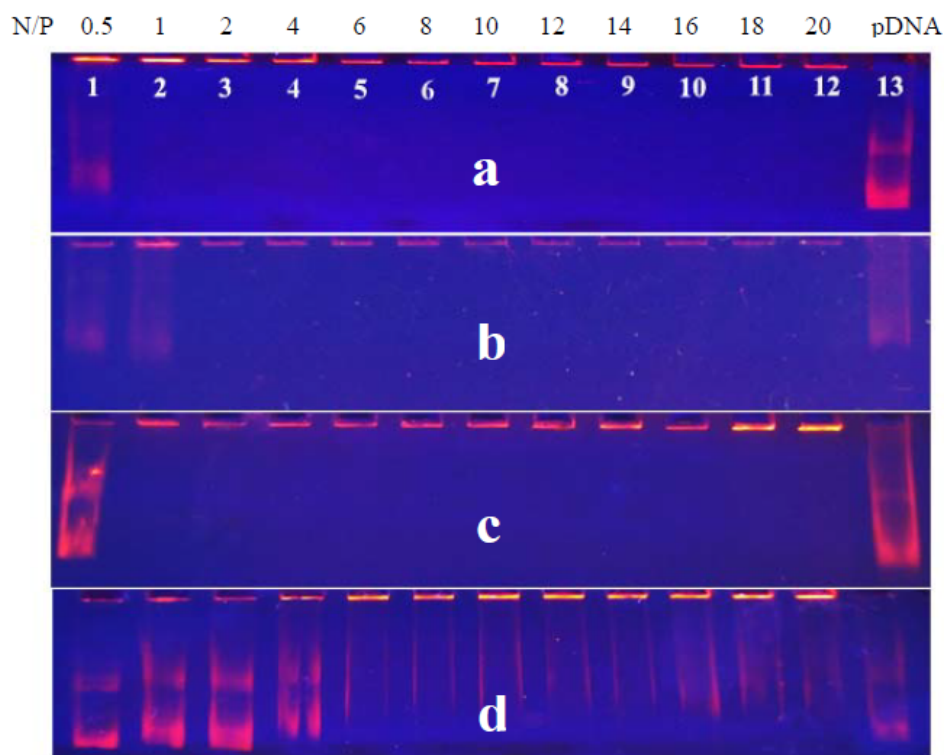
**Fig. S2** Acid-base titration curves of P(His-co-DMAEL), PLL and H<sub>2</sub>O.



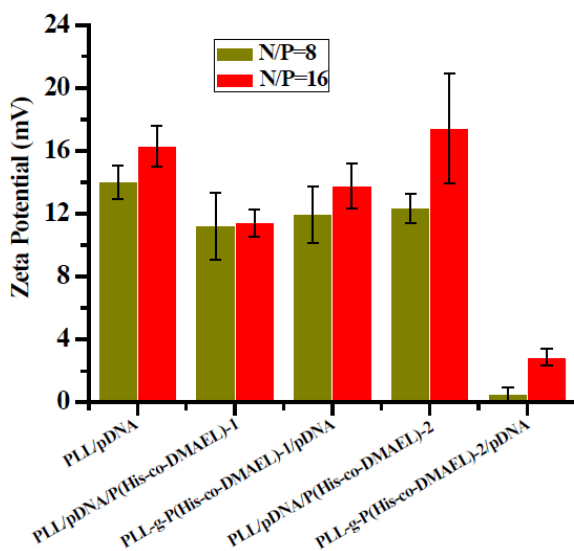
**Fig. S3 (a)** Preparation of PLL-g-P(His-co-DMAEL). **(b)**  $^1\text{H}$  NMR spectrum of PLL, P(His-co-DMAEL), PLL-g-P(His-co-DMAEL)-1 and PLL-g-P(His-co-DMAEL)-2. By comparing the integral area of the group "b" in PLL with that of the group "h+i" in P(His-co-DMAEL), the degree of substitution (DS) was calculated to be 1.35% for PLL-g-P(His-co-DMAEL)-1 and 7% for PLL-g-P(His-co-DMAEL)-2.



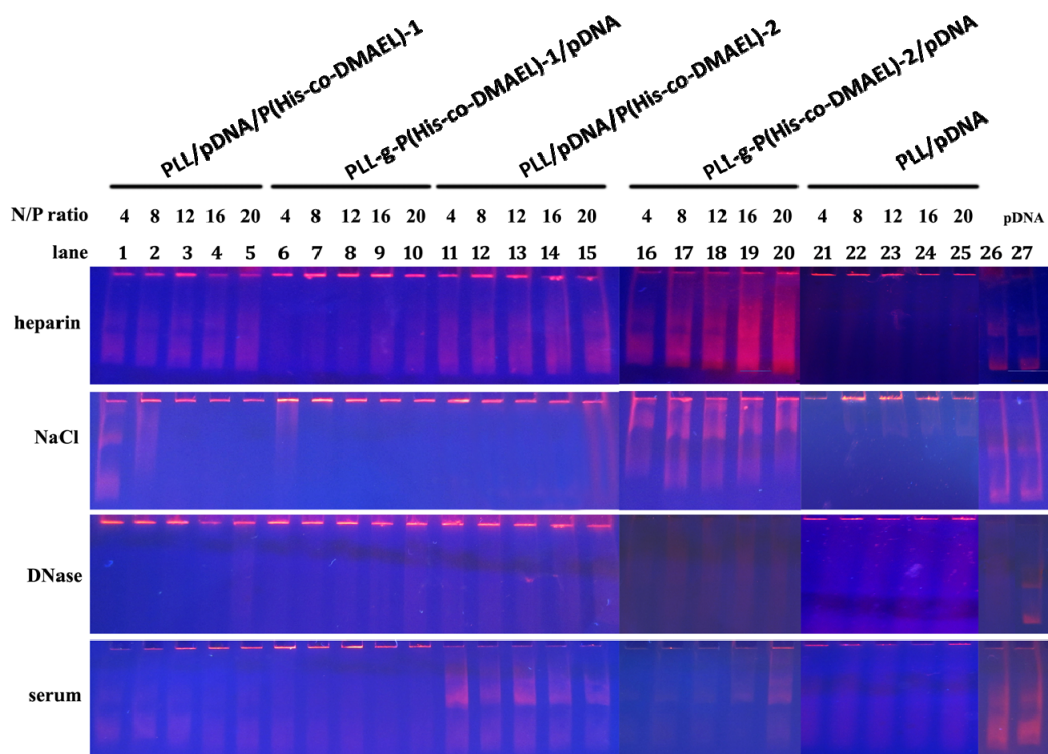
**Fig. S4** Size distributions of complexes by number.



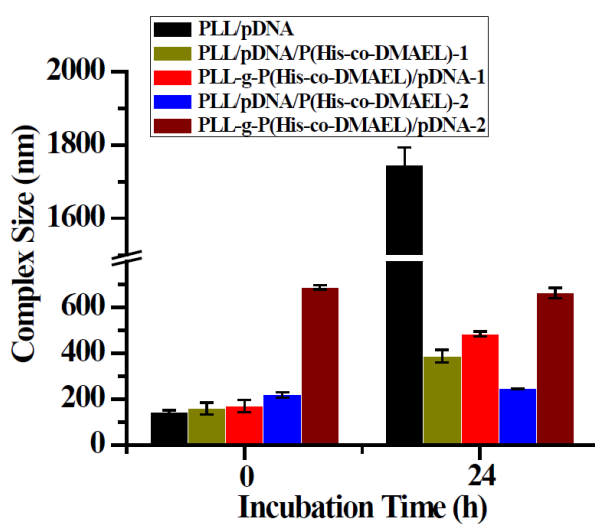
**Fig. S5** Gel shift assays of PLL/pDNA/P(His-co-DMAEL)-1 (a), PLL-g-P(His-co-DMAEL)-1/pDNA (b), PLL/pDNA/P(His-co-DMAEL)-2 (c) and PLL-g-P(His-co-DMAEL)-2/pDNA (d).



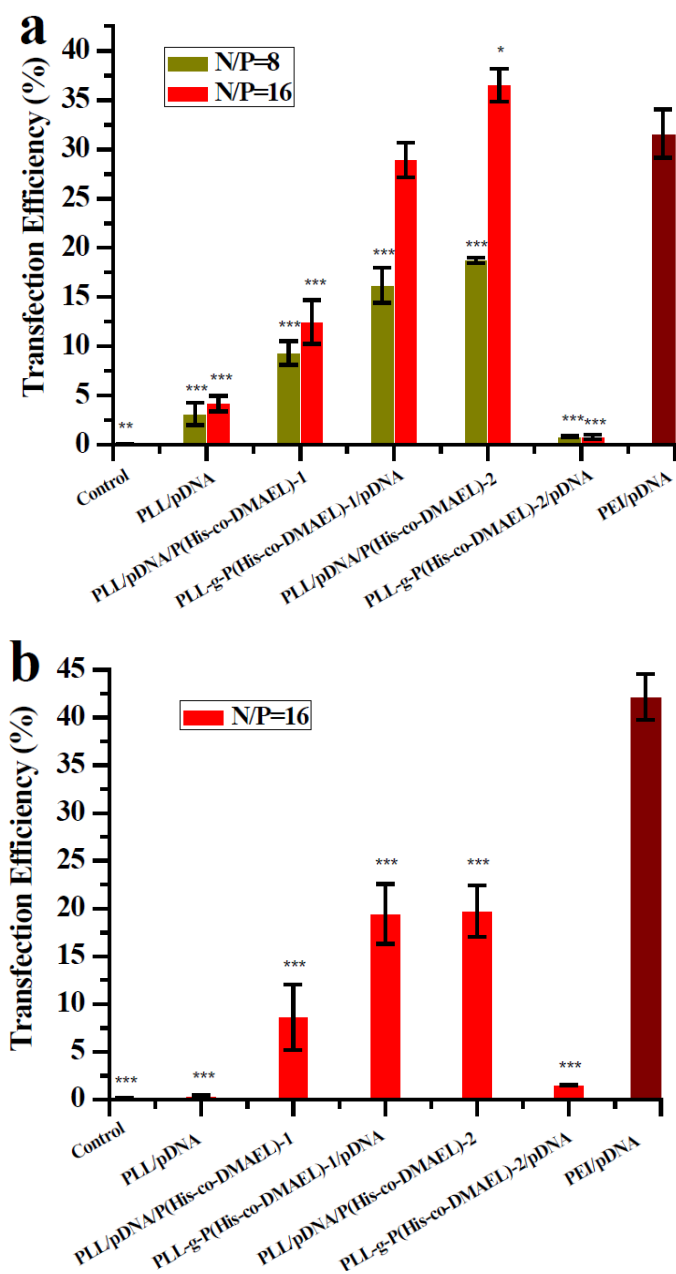
**Fig. S6** Zeta potentials of complexes. Without PLL addition, the zeta potentials of PLL/pDNA/P(His-co-DMAEL)-1 and PLL/pDNA/P(His-co-DMAEL)-2 were  $-32.15 \pm 0.78$  mV and  $-33.23 \pm 1.93$  mV, respectively.



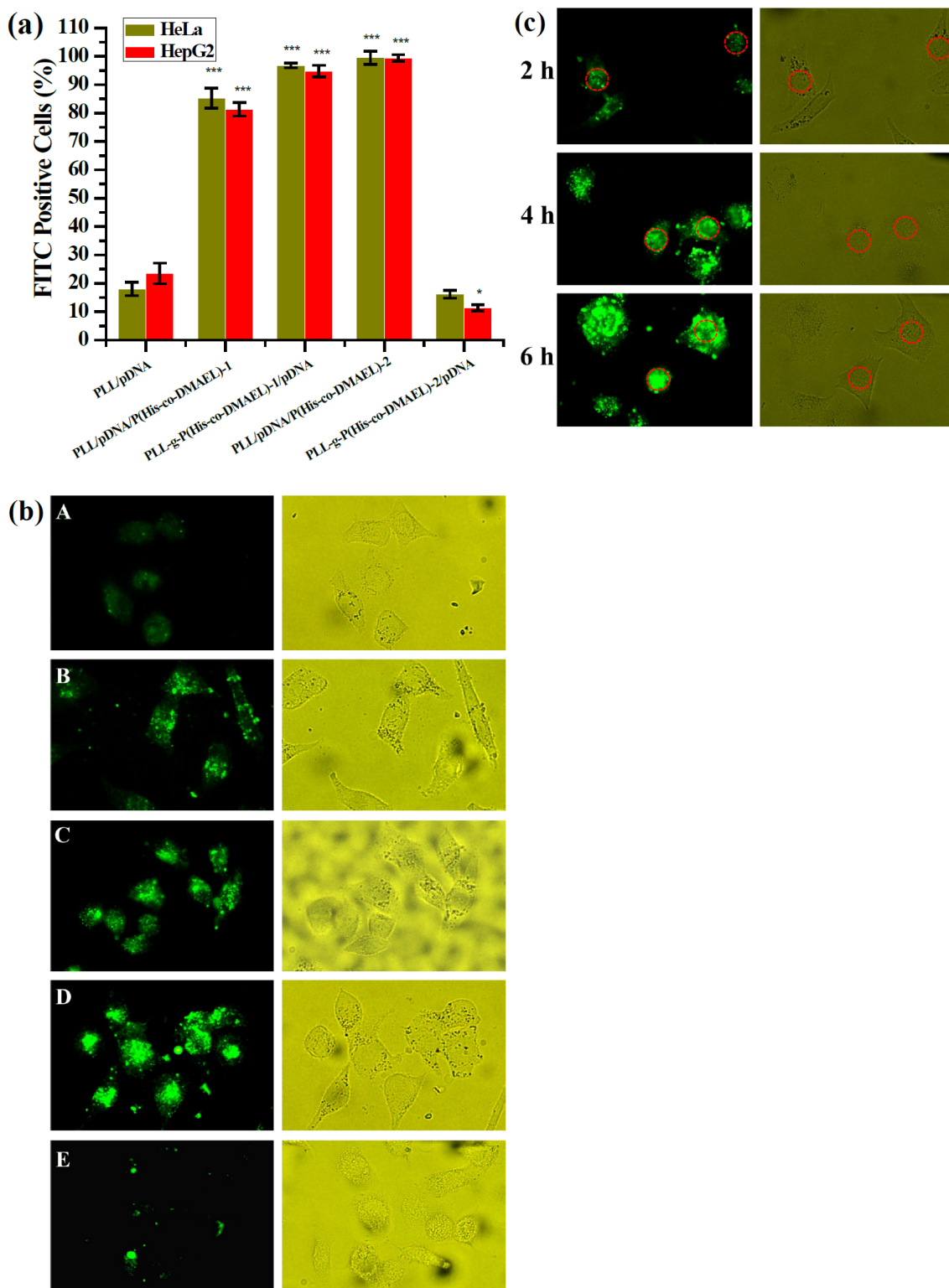
**Fig. S7** Complex stability in heparin, NaCl, DNase and serum at N/P ratios of 4, 8, 12, 16 and 20. Lane 26 was naked pDNA treated in the same way as the complexes. Lane 27 was naked pDNA without any treatment.



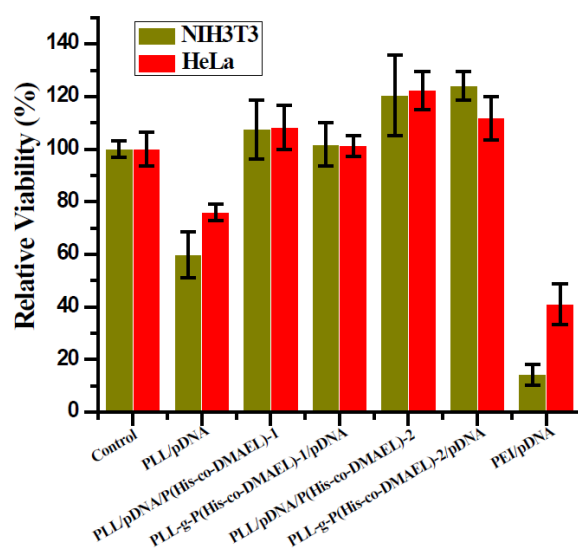
**Fig. S8** Complex sizes in 0.5% BSA solution.



**Fig. S9** Gene transfection efficiency of complexes with HepG2 (a) and NIH3T3 (b) cells in serum conditions.



**Fig. S10** (a) Cellular uptake efficiency of complexes. (b) Distribution of PLL/pDNA (A), PLL/DNA/P(His-co-DMAEL)-1 (B), PLL-g-P(His-co-DMAEL)-1/pDNA (C), PLL/DNA/P(His-co-DMAEL)-2 (D) and PLL-g-P(His-co-DMAEL)-2/pDNA (E) at 4 h post transfection. (c) Distribution of PLL/DNA/P(His-co-DMAEL)-2 complexes at 2, 4 and 6 h post transfection. The magnification was 400 $\times$ .



**Fig. S11** The relative cell viability at 48 h post transfection.