Supporting Information

Histone-inspired biomimetic polymeric gene vehicles with excellent biocompatibility and enhanced transfection efficacy

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Experimental

1.1 Materials

Cystamine dihydrochloride was purchased from J&K (Beijing, China). Histamine dihydrochloride was purchased from Chemlin Chemical Industry Co. Ltd (Nanjing, China). Agmatine dihydrochloride and nigericin was purchased from J&K (Beijing, China). PEI (Mw=25 kDa) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbeco's Modified Eagle Medium (DMEM) and 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) were purchased from KeyGEN Biotech (Nanjing, China). YOYO-1 and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, USA). Trypsin–EDTA solution (0.25 %) was obtained from Gibco (Burlington, Canada). Antibodies against p53 and β-actin were purchased from Cell Signaling Technology (USA). All other chemicals and reagents were obtained from commercial sources and used without further purification.

1.2 Plasmids

Therapeutic plasmid pCMV-Neo-Bam-p53wt (p53, 8.4 kb) and reporter plasmid pEGFP-C3-hYAP1 (pEGFP-C3, 4.7 kb) used in this study were amplified in competent DH-5α Escherichia coli grown in LB medium containing 50

μg/mL kanamycin. Plasmids were collected and purified by a E.Z.N.A.® Fastfilter Endo-free Plasmid Maxi kit (Omega, USA). The quantity and quality of the purified plasmids were assessed by measuring their optical density at 260 nm and 280 nm.[1] DNA (plasmid) used in the following experiments in this study were all obtained from this approach except as otherwise noted.

2. Synthesis of N,N'-cystamine-bis-acrylamide (CBA)

Cystamine dihydrochloride (5.630 g, 25 mmol) was dissolved in 25 mL water. Add a three-necked, 250 mL flask equipped with a stirrer, thermometer, and two dripping funnels. An acryloyl chloride (4.526 g, 50 mmol) solution in dichloro-methane (5 mL) and NaOH solution (4.0 g, 0.1 mol) were added simultaneously under stirring for 60 min at 0 °C. After that, the reaction was performed for 6 h at room temperature. Then the reaction mixture was extracted with dichloromethane and dried over anhydrous Na_2SO_4. The product was obtained by crystallization from ethyl acetate. Pure CBA was vacuum-dried and analyzed by nuclear magnetic resonance (300 MHz, DMSO-d_6).

3. Synthesis of PCA_mH_n

A series of PCA_mH_n were synthesized (m and n are relative molar ratios between agmatine dihydrochloride (Agm·2HCl) and histamine dihydrochloride (His•2HCl) in synthesized polymers, which were determined by ^1H NMR analysis) by Michael addition according the following standard procedure. N,N'-cystamine-bis-acrylamide was dissolved in methanol with agmatine dihydrochloride and histamine hydrochloride. The mixture was stirred for 24 h at 90 °C under a N_2 atmosphere in the dark after adding triethyamine. Then, the product was titrated with 0.1 M HCl until pH reached 4 to remove hydrochloride completely and dialyzed against ultrapure water with dialysis membrane (MWCO=3000) for 48 h, followed by lyophilization to leave PCA_mH_n. The molar ratios between agmatine dihydrochloride (Agm•2HCl) and histamine dihydrochloride (His•2HCl) were varied as follows: n (CBA): n (Agm•2HCl): n (His•2HCl)=1:1:0 (PCA); 1:0.75:0.25 (PCA_3H_1); 1:0.5:0.5 (PCAH); 1:0.25:0.75 (PCA_1H_3); 1:0:1 (PCH).

4. Characterization of PCA_mH_n

4.1. Molecular weight measurements

The molecular weights of PCA_mH_n were measured by gel permeation chromatography (GPC, LC-20 AB, Shimadzu, Japan). The polymers were dissolved at a concentration of 5 mg/mL. The assay was run on an Ultrahydrogel 250 column with 0.05 mol/L Sodium sulfate as an eluent at 1.0 mL/min of flow rate.

4.2. Buffering capacity measurements

The buffering capacity of PCA_mH_n was determined by acid base titrations. Each 2 mg of polymer was dissolved in 10 mL of 150 mmol/L aqueous NaCl solution and the solution was set to pH 7.4 using 1 M NaOH. The solutions were titrated to pH 4 with 0.1 M HCl. pH changes of the solutions were measured by a pH meter (OHAUS, USA). Titration of NaCl and PEI 25K were also performed in the same manner as controls.

4.3. Agarose gel electrophoresis

Agarose gel electrophoresis experiment was performed to determine condensation ability of PCA_mH_n. After 30

min of incubation with DNA at room temperature, the PCA_mH_n/DNA complexes were electrophoresed at 100 V (Mupid-2plus, Japan). In order to examine the DNA condensation ability under reducing condition, the identically prepared polyplexes were additionally incubated for 1 h in the presence of 10 mM DTT at 37 °C before being electrophoresed.

4.4. Protection and release assay

Protection and release of DNA in complexes were measured using a gel electrophoresis method. [2] Briefly, DNase I in DNase I/Mg^{2+} digestion buffer was added to 6μL of complexes solution (weight ratio, PCA_mH_n/DNA=30) or to 0.4 μg naked plasmid DNA, and the mixture were incubated at 37 °C with shaking at 120 rpm for 30 min. Thereafter all samples were treated with 4μL EDTA (250 mM) for 20 min at 65 °C for DNase I inactivation. Afterwards, the samples were mixed with 5 % heparin sodium at a final volume of 17 μL, and the mixture was incubated at room temperature for 2 h, followed by electrophoresis in a 1 % agarose gel containing GEL RED™ in TAE running buffer at 50 V for 30 min.

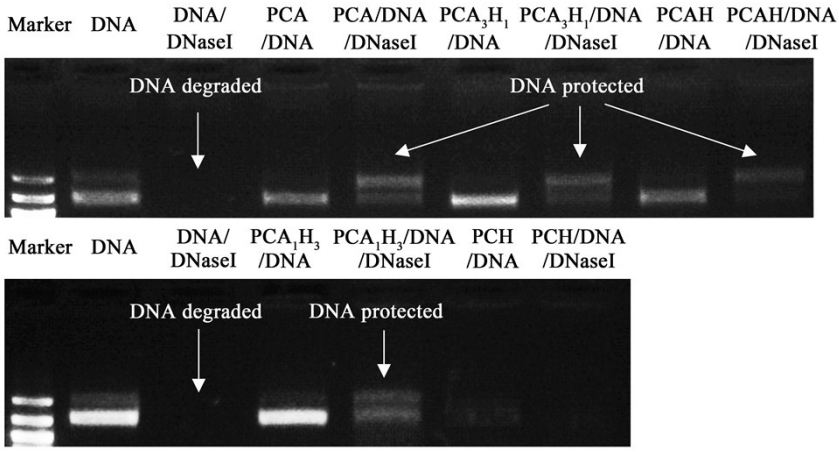

Fig. S1 Protection and release assay of DNA. (DNA was released by adding 5 % heparin sodium to the PCA_mH_n/DNA complexes at weight ratio=30)

4.5. Stability of the PCA_mH_n/DNA complexes in serum

Condensation ability of PCA_mH_n in 50 % FBS was also measured using a gel electrophoresis method. Briefly, equal volume of FBS was added to 9 μL of complexes solution (weight ratio, PCA_mH_n/DNA=30) or to 0.4 μg naked plasmid DNA and the mixture was incubated at room temperature for 2 h and 4 h, followed by electrophoresis in a 1 % agarose gel containing GEL RED™ in TAE running buffer at 50 V for 30 min.

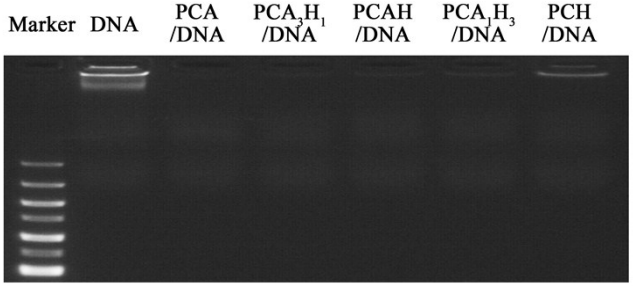

Fig. S2 Agarose gel electrophoresis results of stability of the PCA_mH_n/DNA complexes formed at weight ratio of 30 after incubation with 50 % FBS for 4 h.

4.6. Average particle size and zeta-potential measurements

The particle size and zeta potential of PCA_mH_n/DNA complexes at different weight ratios were determined by ZetaPlus particle size and zeta potential analyzer (Brookhaven Instruments, USA). 1 mL of polyplex solutions (100 μg DNA) were prepared at various weight ratios ranging from 1 to 20. After 30 min incubation, polyplex solutions were determined by ZetaPlus analyzer. Measured average sizes and zeta potential values were presented as the average values of 3 runs.

4.7. Observation of transmission electron microscopy

PCAH samples at weight ratio of 5 for TEM (H-7650; Hitochi; Japan) were placed on a copper grid. After drying under infrared light, the sample was imaged using Tecnai G2 Spirit BioTWIN microscope (FEI Company) operating at 80 kV.

5. *In vitro* hemolysis assay

The hemolysis assay was measured according to previous exported method.[3] Freshly collected rat blood was provided by the Animal Laboratory Center following an animal protocol approved by the Institutional Animal Ethical Committee of China Pharmaceutical University. Fresh diluted rat blood was added to 20 mL normal saline to dissolve different amounts of polymer (0.01, 0.05, 0.1, 1 mg) at 37 °C for 4 h. Then normal saline and Triton X-100 dissolved in normal saline served as the negative control and positive control, respectively. Then, all sample solutions were centrifuged at 3000 rpm for 5 min and 200 μL of the supernatant was seeded in 96-well plate and analyzed for released hemoglobin at 540 nm using microplate reader (ThermoFisher Scientific Multiskan Go, USA). Percentage hemolysis was determined by the following formula:

$$\text{Relative rate of hemolysis (\%)} = \left[\frac{(A_s - A_n)}{A_p - A_n}\right] \times 100\ \%$$

Where A_s, A_n and A_p represent the absorbance of samples, negative and positive controls, respectively. All experiments were conducted in triplicate.

6. Cell culture

HepG2 (Human hepatoma cells) and HUVEC (Human umbilical vein endothelial cells) were purchased from the Chinese Academy of Sciences Shanghai Institute of Cell Bank (Shanghai, China) and cultured in DMEM supplemented with 10 % FBS, 80 units/mL of penicillin, and 80 μg/mL of streptomycin. Cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere.

7. Cytotoxicity

Cytotoxicity of the polymers was measured by MTT assay in HepG2 and HUVEC cells. Cells were seeded in 96-well cell culture plates at a density of 1×10^4 cells/well. When achieving 80 % of confluency after 24 h, the cells were exposed to 100 μL of polymer solutions with concentrations from 10 μg/mL to 200 μg/mL in medium with medium containing 10 % FBS and further incubated for 24 h. Then, the cells were treated with MTT solution for 4

h at 37 °C in the dark. After removing each medium carefully, dissolve the formazan crystal formed by proliferating cell with 150 μL DMSO. The absorbance at 570 nm of the solution in each well was recorded using a Microplate Reader (Thermo, USA). Cells without treatment were used as control. All experiments were conducted in quadruplicate.

Cell lines	Polymer	IC 50[a] (μg/mL)	Polymer	IC 50[a] (μg/mL)
HepG2	PEI	7.128	PCAH	>200
	PCA	104.452	PCA_1H_3	>200
	PCA_3H_1	133.484	PCH	>200
HUVEC	PEI	9.581	PCAH	80.547
	PCA	38.534	PCA_1H_3	114.692
	PCA_3H_1	53.963	PCH	152.939

Fig. S3 IC 50 values of different PCA_mH_n against HepG2 and HUVEC cells. [a] IC 50 values are calculated based on PCA_mH_n concentration (μg/mL).

8. Transfection experiments *in vitro*

Green fluorescent protein (GFP) gene expression was evaluated. HepG2 and HUVEC cells were seeded in 24-well cell culture plates at a density of 1×10^5 cells/well in culture medium. When the cells achieved 80 % of confluency after 24 h, the media were exchanged with DMEM (10 % FBS) for the assay in serum condition, respectively. Then, the cells were treated with polymer/GFP (1.5 μg) complexes at various weight ratios and further incubated for 4 h. PEI 25K (weight ratio=1) was used as a control. After exchange with medium containing 10 % FBS, the cells were further incubated for 24 h. Then, GFP expression was confirmed by microscopic observations. The cellular uptake of fluorescence-labeled polyplexes was examined by using the Flow Cytometer (FACS, BD AccuriC6, USA) at a minimum of 1×10^4 cells gated per sample. All experiments were performed in triplicate.

9. Cellular uptake mechanism study

HepG2 cells were seeded at a density of 1.5×10^5 cells/well in a 24-well plate in DMEM (10 % FBS) and incubated at 37 °C overnight. When cells were grown to reach 70~80 % confluence, the media were exchanged with serum-free DMEM for further 4 h. Then, the cells were pre-treated with different inhibitors for 1 h, including 0.5 mM amiloride hydrochloride, 5 mM Methyl-beta-cyclodextrin hydrate (MβCD), 0.03 mM chlorpromazine hydrochloride. To further verify that this uptake was mediated by endocytosis pathway, the samples were also preincubated in 4 °C for 1 h. The cells were then treated with different kinds of polymer/DNA solutions at a relatively suitable weight ratio for 3 h for transfection with YOYO-1 iodide labeled DNA (1 molecule dye/50 nucleotide base pairs). The weight ratios between different polymer/DNA solutions were varied as follows: PCA, PCA_3H_1, PCAH: 10; PCA_1H_3: 20; PCH: 40. Subsequently, the supernatant was removed and the cells were washing three times with 500 μL PBS. After trypsinization, the cells were suspended in 500 μL serum-free medium and harvested into eppendorf tubes. The cellular uptake of fluorescence-labeled polyplexes was examined by using the Flow Cytometer at a minimum of 1×10^4 cells gated per sample.

10. Uptake of PCA_mH_n in the presence of nigericin

HepG2 cells were seeded at a density of 1.0×10^5 cells/well in a 24-well plate in DMEM (10 % FBS) and

incubated at 37 °C overnight. When cells were grown to reach 70~80 % confluence, the cells were pre-treated with endosomal acidification inhibitors nigericin (10 μM) for 0.5 h. Then the cells were treated with different kinds of polymer/GFP solutions in serum-free DMEM at the weight ratio of 30 for 4 h. After exchange with medium containing 10 % FBS, the cells were further incubated for 24 h. Subsequently, GFP expression was confirmed by the Flow Cytometer at a minimum of 1×10^4 cells gated per sample. All experiments were performed in triplicate.

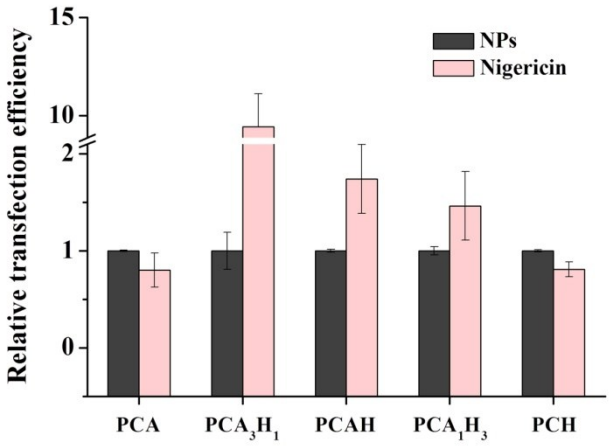

Fig. S4 Uptake of PCA_mH_n in the presence of nigericin (10 μM)

11. Confocal laser scanning microscopy

Intracellular location of PCA_mH_n/DNA was investigated using confocal laser scanning microscopy (CLSM, FV1000, Olympus). HepG2 cells were seeded in thin glass-bottomed 35 mm petridishes at 5×10^4 cells per dish, and incubated for 12 h at 37 °C under 5 % CO_2 atmosphere to reattach. After that, the media were removed and the cells were washed three times with PBS, followed by incubation at 37 °C with LysoTracker Red (100 nM, for 60 min, Molecular Probes, Oregon, USA).Then, the freshly prepared PCA_mH_n /YOYO-1-DNA (2 μg) complexes (weight ratio=20) (100 μg DNA could be fluorescently labelled with 1 μL 1mM YOYO-1 iodide) were added to the dishes for further incubation at 37 °C for 1 h and 10 h relatively. Afterwards, cells were stained with Hoechst 33342 (100 μM in PBS). Before visualized under a confocal laser scanning microscope, cells were rinsed with cold PBS for three times and fixed with 4 % paraformaldehyde for 30 min at 37 °C.

12. Cell proliferation assay

HepG2 cells were seeded at a density of 1×10^4 cells/well in a 96-well plate in DMEM (10 % FBS) and incubated at 37 °C overnight. The PCAH/p53 (0.1 μg) nanocomplex (weight ratio=20) was then added to each well. PEI 25K/p53 (weight ratio=1) nanocomplex was used as a control. After 48 h incubation, 20 μL MTT (5 mg/mL in PBS) was added and the plates were incubated for additional 4 h. Next, the MTT solution was removed and 150 μL dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals. Then absorbance at 570 nm was measured using a Microplate Reader (Thermo, USA). Cells without treatment were used as control. The cell viability (%) was calculated as $A_{sample}/A_{control}$, where A_{sample} and $A_{control}$ are the absorbance values of the treated and untreated cells, respectively.

13. Western blotting analysis

HepG2 cells were seeded in 6-well plates at a density of 1.5×10^5 cells per well and incubated for 48 h before p53

transfection, which was conducted using PCAH/p53 nanocomplex (weight ratio=20) with a plasmid mass of 2 μg. After post-transfection, HepG2 cells were washed with ice-cold PBS twice and proteins of cells were collected by Total Extraction Sample Kit (KeyGEN BioTECH, Nanjing, China). The supernatants were collected by centrifugation at 12,000 rpm for 10 min, and the concentrations of protein were measured using by a standard BCA protein assay kit (KeyGEN BioTECH, Nanjing, China). Afterwards, 30 μg proteins of all samples were subjected to SDS-PAGE analysis and transferred to the PVDF membranes (Merck Millipore, USA) by electroblotting. The membranes were blocked for 2 h with 5 % non-fat milk (prepared with TBST solution), incubated with the corresponding primary antibodies at 4 °C overnight and then probed with HRP antibodies for 2 h at room temperature. Finally, specific proteins were detected by CCD image system (Tanon 4200, China). The protein expression level was normalized against β-actin.

Notes and references

1 C. Wang, X. Bao, X. Ding, Y. Ding, S. Abbad, Y. Wang, M. Li, Y. Su, W. Wang and J. Zhou, *Polym. Chem.*, 2015, **6**, 780.

2 H. L. Jiang, J. L. Zhang, S. Che, T. Jin, P. F. Cui, Y. J. He, J. Y. Lyu, J. Gong, L. Xing and J. B. Qiao, *RSC Adv.*, 2016.

3 R. L. Xie, Y. J. Jang, L. Xing, B. F. Zhang, F. Z. Wang, P. F. Cui, M. H. Cho and H. L. Jiang, *Int. J. Pharm.*, 2015, **478**, 19.