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A protein-polymer hybrid gene carrier based on thermophilic histone and polyethylenimine

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Experimental section

1. Materials

The plasmid pEGFP-N3 encoding green fluorescent protein was kindly provided by Prof. Xuesi Chen (Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, China), and the plasmid pGL-3 was purchased from Promega (Madison, WI, USA). The plasmids were amplified in *E. coli* DH5α and purified using the Axygen Plasmid Maxi kit (Jiangsu, China). The recombinant *E. coli* harboring thermophilic histone gene GK2215 from *G. kastophilus* HTA426 was constructed and stored in our laboratory. Branched PEI25K was purchased from Aldrich and used as received. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amersco (Solon, USA). Lipofectamine^{2000} was purchased from Invitrogen, and used according to the manufacturer's recommendation. N-hydroxysuccinimide (NHS) and 1-[3-(dimethylamino)propyl]-3-ethyl carbodiimine (EDC) were obtained from Aladdin (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, USA). Yeast extract and tryptone were purchased from Oxoid Ltd. (Basingstoke, UK). The cell lysis buffer and luciferin substrate were purchased from Promega (Madison, WI, USA). The BCA protein assay kit was purchased from Dingguo Co. (Beijing, China). All other chemicals were of the highest reagent grade commercially available and used as received.

2. Purification of thermophilic histone

The recombinant *E. coli* harboring thermophilic histone gene GK2215 was cultured and inducted according to our previous report.[26] To purify the thermophilic histone, the cells were mixed with 50 mM phosphate buffer (pH 8.0) at a ratio of 1:8 (w/v) and subjected to the ultrasonic cell disintegration for 15 min. The suspension was sequentially incubated at 75 °C for 30 min, and the crude extract was centrifuged at 8000 r/min for 15 min. The supernatant was collected, desalted by PD-10 column (GE Healthcare), and further purified through ÄKTA Purifier 10 equipped with a HiTrap heparin-Sepharose column (GE Healthcare) and eluted with a linear gradient of 1-2 M NaCl. Finally, the purified thermophilic histone was dialyzed against 50 mM phosphate buffer (pH 8.0) for 24 h (molecular weight cut-off: 3500 Da).

3. Synthesis and characterization of HEP

The hybrid gene carriers were prepared through EDC/NHS-mediated couple reaction of thermophilic histone and PEI25K. Briefly, 1.2 mg EDC and 1.8 mg NHS were added into thermophilic histone solution (1 mg/mL), and the reaction was conducted at room temperature for 15 min. The PEI25K solution was then added into the reaction system with predetermined molar ratios of thermophilic histone/PEI25K. After stirring at 4 ^{o}C for 2 h, the samples were dialyzed against distilled water for 24 h (molecular weight cut-off: 3500 Da). The products were obtained by lyophilization and named as HEP (**1:6**, **1:3**, **1:1**, **3:1**, **6:1** and **10:1**), according to the molar ratio of thermophilic histone to PEI25K. The HEP structure was characterized by FT-IR spectra, which were performed

in the wavenumbers of 500-4000 cm^{-1} on a Nicolet 5700 Instrument (Thermo Electron Co., USA) using the standard KBr disk method.

4. Gel retardation assay

The HEP/pDNA complexes were prepared by gently mixing the carrier and plasmid pEGFP-N3 together at different mass ratios, and then incubated at room temperature for 30 min. The binding ability with pDNA was assessed by 1% agarose gel electrophoresis in Tris-acetate-EDTA buffer solution (120 V, 20 min). For the plasmid degradation assay, HEP/pDNA nanocomplexes were mixed with 10% or 50% FBS, and incubated at 37 ^{o}C for 1.5 h. The HEP/pDNA nanocomplexes were then assessed by 1% agarose gel electrophoresis as above.

5. Hemolytic activity assay

The hemolytic activity of HEP was performed using whole blood from Beagle dogs to estimate its blood compatibility. The erythrocytes were isolated by centrifugation at 1500 r/min for 10 min, washed with 50 mM phosphate buffer (PBS, pH=7.2) three times and re-suspended in the same buffer to a final concentration of 5×10^{9} cells/mL. 100 μL of the suspension was then incubated with 900 μL of carrier solution with different concentrations at 37 °C for 1 h. The erythrocytes treated with PBS and 1% Triton X-100 solution were used as negative and positive control, respectively. After incubation, the cell suspension was centrifuged at 1500 r/min for 10 min, and the

supernatant was used to measure the absorbance of released hemoglobin using a GM-3000 microplate reader (Shandong, China) at 578 nm. The percent of hemolysis was calculated by the following equation:

Hemolysis (%)=(A-A_0)/(A_{100}-A_0)×100%

where A, A_0 and A_{100} were the absorbance values of sample, negative (PBS) and positive control (1% Triton X-100 solution), respectively.

6. *In vitro* cytotoxicity assay

The cytotoxicity of HEPs was measured by MTT assay. HeLa cells were maintained in DMEM supplemented with 10% FBS, and 100 mg/mL of penicillin and streptomycin under 5% CO_2 and 37 ^oC in a humidified atmosphere. The cells were seeded at a density of 8.0×10^3 cells/well onto 96-well plate, and pre-incubated in fresh FBS-free DMEM for 24 h, and further incubated with different concentrations of HEPs. After 24 h, 20 μL MTT solution (5 mg/mL in PBS) was added to each well, and the plate was incubated for an additional 4 h. The MTT solution was then removed from each well and 150 μL DMSO was added to dissolve the formazan crystals. The plate was incubated for an additional 10 min, and the absorbance at 492 nm was recorded using a GF-M3000 microplate reader (Shandong, China). The cell viability (%) was calculated as $A_{sample}/A_{control}$, where A_{sample} and $A_{control}$ represented the absorbance values of the treated and untreated cells, respectively.

7. *In vitro* transfection assay

HeLa cells were seeded in 6-well plate at a density of 1.5×10^5 cells/well, and cultured for 24 h prior to the transfection experiment. After removing the medium from each well, the cells were washed with 1 mL PBS and followed by incubating with carrier/pDNA complexes (2 μg pEGFP-N3 plasmid) for 4 h in DMEM, in the absence or presence of 10% FBS. The transfection medium was replaced with 2 mL DMEM containing 10% FBS, and the cells were incubated for another 40 h. The transfected cells were washed with PBS twice and analyzed through an Olympus IX71 fluorescence microscopy (Osaka, Japan).

For the quantitative transfection, HeLa cells were seeded in 6-well plate at a density of 2.5×10^6 cells/well, and cultured in DMEM containing 10% FBS for 24 h. The medium was removed, and the cells were washed twice with PBS and then incubated with the carrier/pGL-3 complexes at different mass ratios (2 μg plasmid) in FBS-free DMEM for 4 h. After removing the medium harboring the complexes, the cells were incubated in DMEM containing 10% FBS for 40 h, washed twice with PBS, and treated with cell lysis buffer (50.0 μL per well). The suspension (10.0 μL) was mixed with 50.0 μL luciferin substrate, and the emission light was measured by a luminometer (Promega). The relative luciferase units were normalized with the protein concentration determined using a BCA protein assay kit.

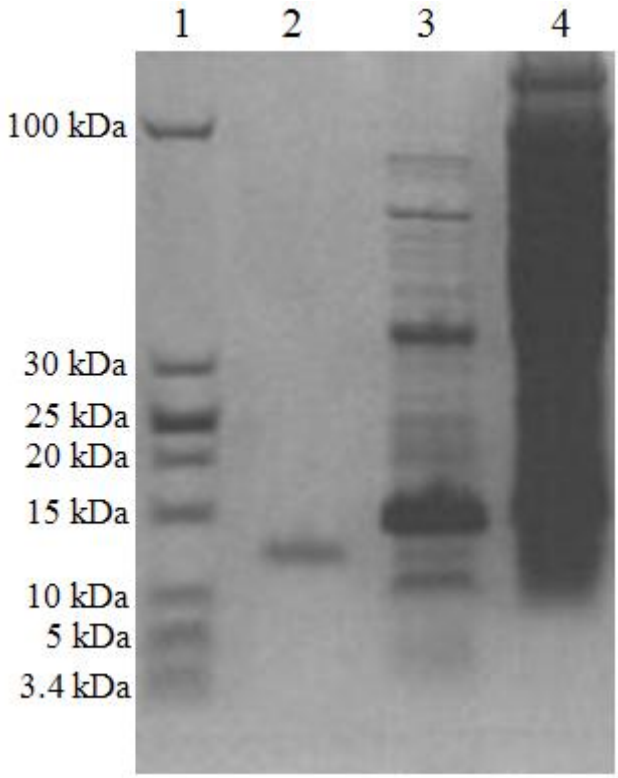

Fig. S1. Tricine-SDS-PAGE analysis for the purification of thermophilic histone. Lane 1: low molecular weight protein markers; lane 2: the purified thermophilic histone after Hitrap heparin-Sepharose affinity column chromatography; lane 3: the sample obtained through thermal treatment at 75 ^{o}C for 30 min and centrifugation; lane 4: the supernatant after ultrasonication.

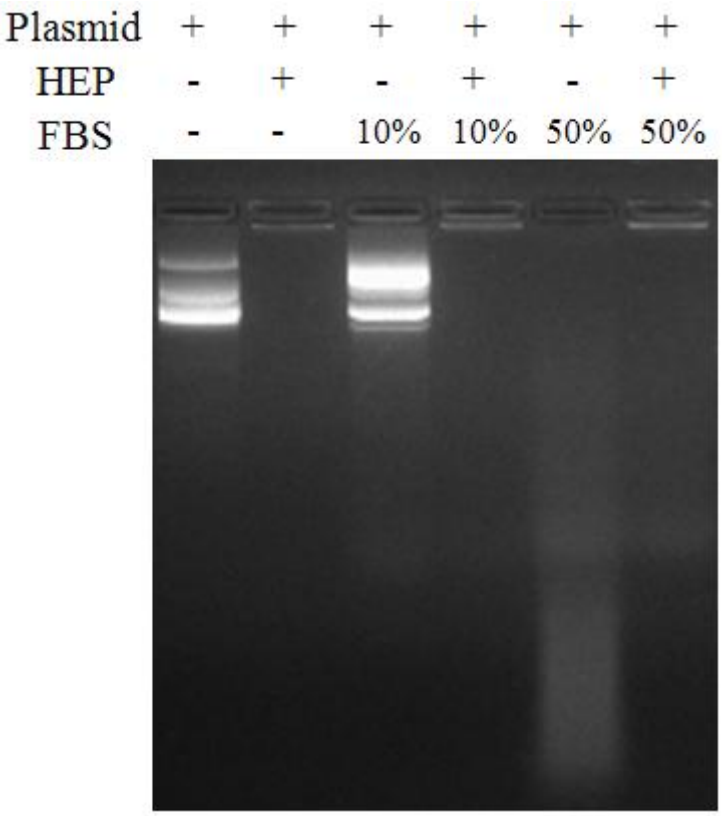

Fig. S2. Gel retardation assay for HEP **1:1**/pDNA nanocomplex (mass ratio of 2:1) in the absence or presence of serum.

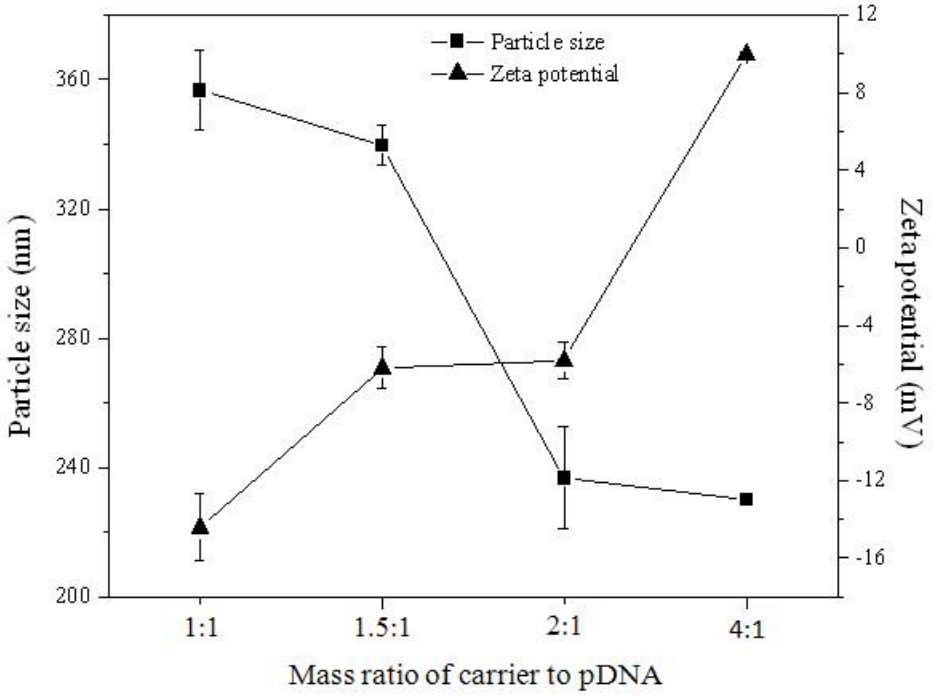

Fig. S3. Particle size and zeta potential of HEP **1:1**/pDNA nanocomplexes at different mass ratios. The data were expressed as mean value ± SD of three experiments.