

Electronic Supplementary Information for

A controlled release system for simultaneous promotion of gene transfection and antitumor effect

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Materials

Trifluoroacetic acid (TFA) and thionyl chloride were purchased from Sinopharm Chemical Reagent Co., Ltd. Di-*tert*-butyl dicarbonate [(BOC)₂O], 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) and *N*-hydroxysuccinamide (NHS) were purchased from Aladin. Rhodamine B isothiocyanate (RB-ITC), 4-{4-[Bis(2-chloroethyl)amino]phenyl}butanoic acid (chlorambucil, CHB), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and polyethylenimine (PEI, 25K) were purchased from Sigma-Aldrich. Triethylamine (TEA), dichloromethane (DCM) and *N,N*-dimethylformamide (DMF) were dried over calcium hydride (CaH₂) and distilled under reduced pressure. Dialysis bag (MWCO=3.5 and 1.0 kDa) was purchased from Shanghai greenbird development Co. Ltd, China. Other reagents were used as received.

Preparation of 4-{4-[bis(2-chloroethyl)amino]phenyl}butanoyl chloride

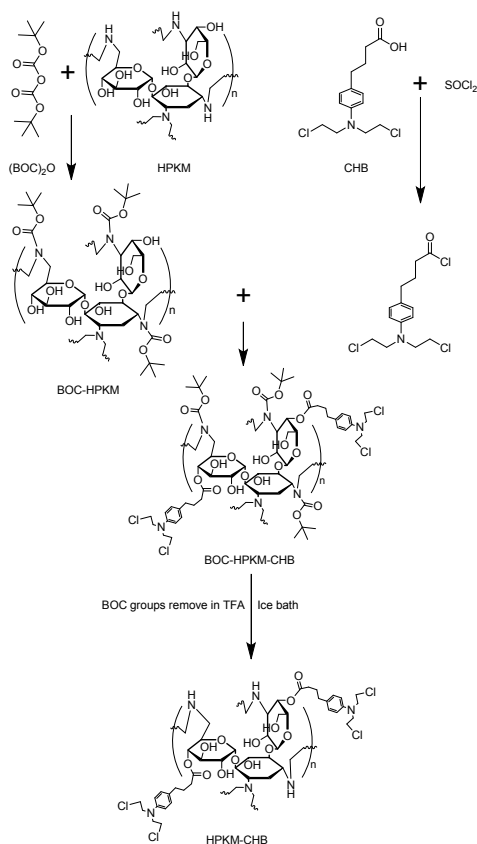
0.3 g CHB was dissolved in 100 mL DCM, and then 3 mL thionyl chloride was added through a syringe. After the solution was refluxed at 60 °C for 16 h, the rotary evaporation was carried out to remove the solvent under reduced pressure. The crude product of 4-{4-[bis(2-chloroethyl)amino]phenyl}butanoyl chloride was prepared for next reaction.

Preparation of hyperbranched poly(kanamycin-MBA) (HPKM)

HPKM was synthesized via Michael-addition polymerization, and the synthetic route was consistent with our previous work.¹ In detail, 11.6 g kanamycin sulfate and 4.7 g MBA were added into a flask and then 400 mL distilled water was carefully introduced. The reaction was conducted with stirring at 60 °C for 80 h. rotary evaporation was used to get concentrated crude products. The polymer was purified by exhaustive dialysis with a dialysis bag (MWC=3.5 kDa). The solvent was evaporated and the product was dried in vacuum oven overnight.

***Tert*-butoxycarbonyl (BOC) protection of HPKM**

The amine groups of HPKM was protected by Di-*t*-butyl dicarbonate. In detail, 2.268 g HPKM was dissolved in 100 mL saturated NaHCO₃. 2.766 g Di-*t*-butyl dicarbonate was dissolved in dioxane and then slowly dropped into HPKM solution through constant pressure funnel for 30 min. After reaction for 24 h at 30 °C, the solution was extracted with ether for 3 times. Then the aqueous phase was transferred into a dialysis bag (MWCO=3.5 kDa). The dialysis against distilled water was carried out for 48 h, and then the product was lyophilized. 2.4 g white product of HPKM protected by BOC (BOC-HPKM) was obtained.



Scheme S1 Synthesis of HPKM-CHB.

Preparation of HPKM-chlorambucil (HPKM-CHB)

4-{4-[Bis(2-chloroethyl)amino]phenyl}butanoyl chloride was dissolved in anhydrous DMF. Then 1 mL TEA and 1.2 g HPKM-BOC were added sequentially. After stirred at 30 °C for 24 h, the crude product of BOC-HPKM-CHB was obtained. The solution was concentrated by rotary evaporation under reduced pressure to remove the solvent. Then the crude product of BOC-HPKM-CHB was dissolved in ice-cold DCM/TFA solution (DCM: TFA=2:1) and stirred at ice bath for 2 h. The solution was precipitated by ether, and the product was dissolved in DMSO. Dialysis against ultra-pure water was carried out for 48 h. Subsequently, the solution was filtered and lyophilized. Finally, 0.65 g bright-yellow product (HPKM-CHB) was obtained and the yields was 61%. ¹H NMR (400 MHz, D₂O, 298 K) δ: 4.4 (NHCH₂NH); 2.8-3.3 (O=CCH₂CH₂); 2.2-2.6 (O=CCH₂CH₂); 1.0-2.2 (CHCH₂CH). ¹H NMR (400 MHz, dimethyl sulfoxide-*d*₆, 298 K) δ: 8.2-9.0 (CH₂CCHCHC); 6.8-7.4(CH₂CCHCHC); 4.4 (NHCH₂NH); 2.5 (O=CCH₂); 2.2 (ClCH₂CH₂); 1.75 (O=CCH₂CH₂).

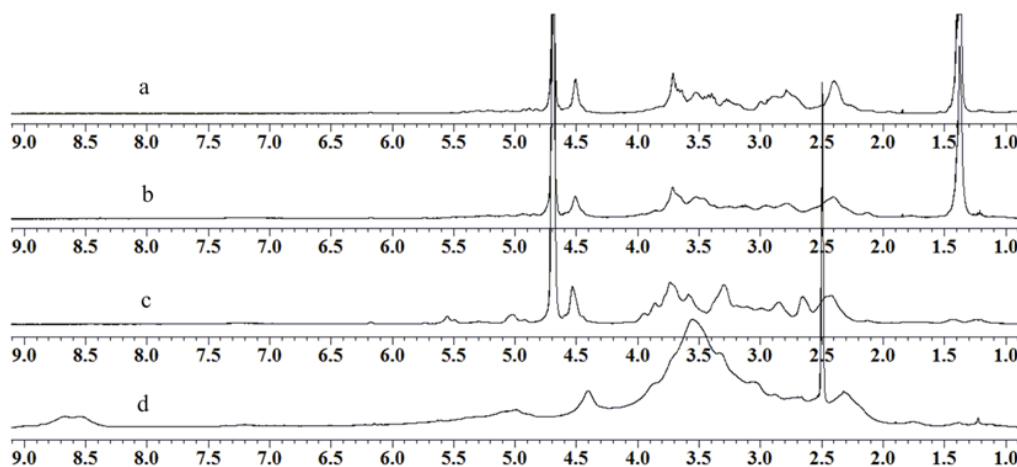


Fig. S1 ¹H NMR spectra of (a) BOC-HPKM in D₂O; (b) BOC-HPKM-CHB in D₂O; (c) HPKM-CHB in D₂O; (d) HPKM-CHB in *d*₆-DMSO.

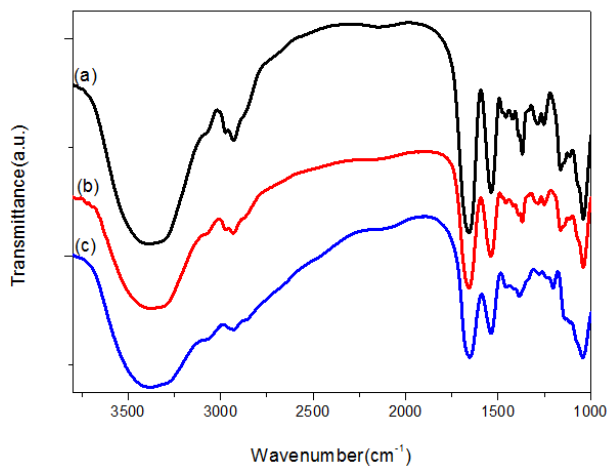


Fig. S2 FTIR spectra of (a) BOC-HPKM, (b) BOC-HPKM-CHB, and (c) HPKM-CHB.

IR (cm^{-1}): 3381 ($\nu_{\text{as OH}}$, $\nu_{\text{as NH}_2}$), 3070 ($\nu_{\text{as benzene}}$), 1656 ($\nu_{\text{as carbonyl}}$), 1542 ($\nu_{\text{as C-N}}$, $\nu_{\text{as benzene}}$, δ_{NH_2} , δ_{NH_2}), 1136 ($\nu_{\text{as C-N}}$), 1038 ($\nu_{\text{as C-O-C}}$).

Table S1 Results of HPKM modified with CHB

Sample	$M_n(\times 10^3)$	$M_w(\times 10^3)$	PDI	CHB content ^a	Nitrogen content
HPKM-CHB	5.5	12	2.2	12.9%	6.28%

^a quantified by NMR

Characterization of HPKM-CHB

^1H NMR analyses were recorded on Bruker Avance III 400 spectrometer with D_2O and *dimethyl sulfoxide- d_6* ($\text{DMSO-}d_6$) as solvents at 298 K. FTIR spectra was performed by KBr sample holder method on Bruker Equinox-55 FTIR spectrometer in the range of 4000-500 cm^{-1} . Because the charge of HPKM-CHB maybe interact with the column, so the HPKM-CHB of amino capped with BOC groups was used for accurate data. TSK guadcolumn AW-H (R0064) GPC system was used to determined the molecular weight and polydispersity index (PDI). This instrument equipped

TSKgel SuperAWM-H*2 column (R0091+R0093 Column lot) and a refractive index (RI) detector. DMF (contained 10 mM LiBr) was used to dissolve product and the flow rate was 0.6 mL/min at 40 °C. PMMA calibration was used as a standard to determine profile of conjugate polymer.

Biophysical properties of HPKM-CHB/pDNA

The pGL3-control vector was used as a model plasmid DNA (pDNA) of gene expression. The pDNA was purified by Endofree Plasmid Purification Kit (QIAGEN). Both pDNA and HPKM-CHB were dissolved in neutral phosphate buffer solution (PBS) at predetermined molar ratios of HPKM-CHB nitrogen to pDNA phosphorus (N/P ratios). The HPKM-CHB/pDNA complex solutions of N/P ratios with 0, 2, 5, 8, 10, 12, 15, and 20 were mixed with 6×loading buffer, which were added into the slots of a 1% agarose gel (containing ethidium bromide) in sequence. The electrophoresis was carried out at 100 V for about 60 min. The results were analyzed with Bio-RAD UV transilluminator.

HPKM-CHB and pDNA were dissolved in neutral PBS to obtain the aqueous solution of polyplexes with N/P=20. After incubation at room temperature for 30 min, a droplet was transferred to carbon-coated of copper grids and dried in the air overnight at 25 °C. The morphology of HPKM-CHB/pDNA polyplexes was examined with transmission electron microscopy (TEM, JEOL 2010 instrument).

Cell cultures

As model cells, COS-7 (an African green monkey kidney cell line) and MCF-7 cells (an adenocarcinoma cell line of human breast) were cultured at 37 °C in a 5% CO₂ atmosphere. Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) was

used. Both streptomycin and penicillin were dissolved with a final concentration of 100 units/mL to kill microorganisms.

Preparation of HPKM-RB

2.1 g tert-butoxycarbonyl (BOC) protection of kanamycin-based hyperbranched glycoconjugated polymer (BOC-HPKM), 2.1 g Rhodamine B (RB), 1.0 g 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), 0.6 g *N*-hydroxysuccinamide (NHS) and 0.54 g 4-dimethylaminopyridine (DMAP) were dissolved in anhydrous dimethyl sulfoxide (DMSO) and stirred at 30 °C for 50 h, then dialysis (MWCO=1.0 kDa) against to water were conducted for 7 days, followed by lyophilization to get the BOC-HPKM-RB conjugate. Subsequently, BOC groups were removed in dichloromethane (DCM) and trifluoroacetic acid (TFA) mixed solution at ice bath condition to get HPKM-RB. Then the precipitation was transferred into a new dialysis (MWCO=1.0 kDa), dialysis were conducted for 7 days. Product was lyophilized. BOC-HPKM-RB and HPKM-RB were characterized by ¹H NMR. As shown in Fig. S3, tert-butyl proton signal in 1.4 ppm disappeared and broad proton signal of methyl unit in RB appear in 1.0-1.5 ppm, these results demonstrated that protective groups have been removed thoroughly and RB have been conjugated to HPKM *via* ester bond. RB content in HPKM-RB was determine by fluorescence spectrometer with the help of RB standard curve (Fig. S4).

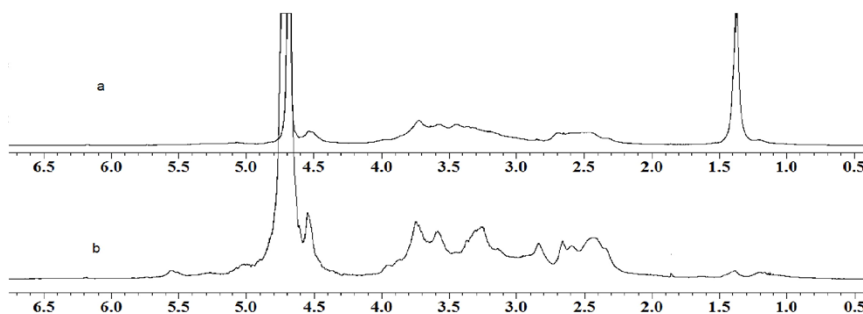


Fig. S3 ¹H NMR spectra of (a) BOC-HPKM-RB and (b) HPKM-RB in D₂O.

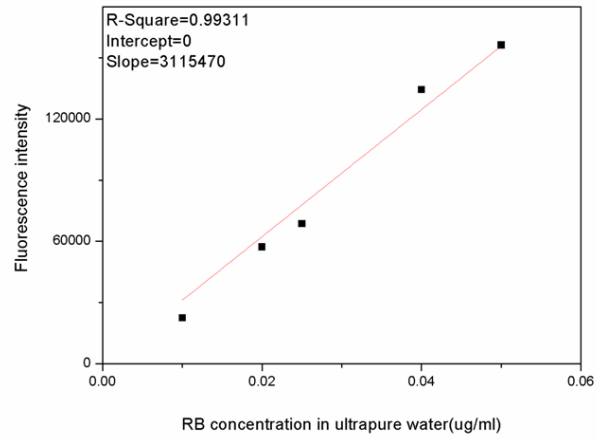


Fig. S4 Standard curve of RB in ultrapure water.

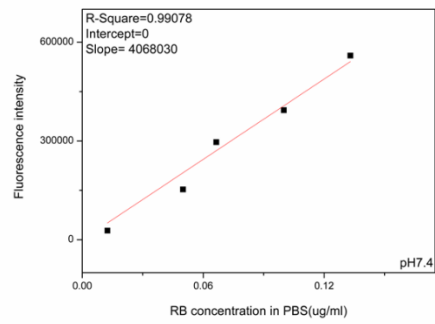


Fig. S5 Standard curve of RB in PBS (pH7.4).

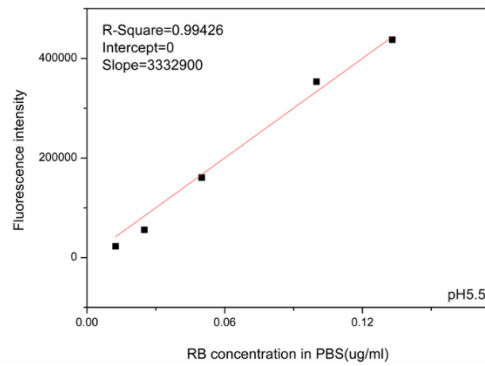


Fig. S6 Standard curve of RB in PBS (pH5.5).

In vitro release study

CHB is a hydrophobic drug and the release behavior is hard to be determined at physiological conditions. At the same time, the release of CHB from HPKM-CHB is the result of ester bond hydrolysis, so the conjugate of HPKM grafted by RB through ester linkage (HPKM-RB) was prepared and used to imitate the CHB release *in vitro*.

A new dialysis bag filled with 5 mL HPKM-RB was immersed neutral PBS (pH7.4) or acetate PBS (pH5.5) in a shaking water bath at physiological conditions (37 °C). At predetermined intervals, 5 mL of the external buffer was sucked out and supplemented with an equal volume of fresh PBS. Withdrawn RB content in external buffer was evaluated by fluorescence spectrofluorometer (excitation at 550 nm) and the quantity of released RB was calculated.

Mark of HPKM-CHB and cell internalization

RB-ITC was used in HPKM-CHB labeling. Briefly, RB-ITC and HPKM-CHB were dissolved in ultrapure water separately, then the two solutions were mixed at the quality ratio of RB-ITC and HPKM-CHB in 1:50 and stirred for 24 h at room temperature. The crude product was transferred into a new dialysis bag (MWCO=3.5 kDa). Dialysis against distilled water was carried out for 48 h and then the product was used for cell internalization examination.

The behavior of cellular uptake and intracellular tracking was observed by CLSM and flow cytometry. For the CLSM photographing, MCF-7 cells were sown in 6-well plates containing coverslips at 1.5×10^5 cells/well and incubated for 24 h, followed by adding RB labeled HPKM-CHB at a final concentration of 2.5 mg/mL. The cells were cultured at 37 °C for 30 min. Subsequently, extracellular dye was washed with neutral PBS and 4% paraformaldehyde was used to fix cells at 25 °C for 30 min. Thereafter, the cells were washed and Hoechst 33342 was filled

with a final concentration of 10 µg/mL, and the rinsed coverslips were mounted and observed with a Leica CLSM.

For flow cytometry, MCF-7 cells were seeded in 6-well plates at 5×10^5 cells/well and incubated for 24 h. Then the RB labeled HPKM-CHB dissolved in the DMEM of predetermined wells at a final concentration of 2.5 mg/mL and the cells were cultured at 37 °C for 30 min. Thereafter, the media was removed and cells were rinsed 3 times with PBS. Then trypsin treated MCF-7 cells were collected. Data for about 10000 total events were observed with flow cytometer (BD) and about 8500-10000 gated events were analyzed by CELLQuest software.

***In vitro* antitumor effects**

Inhibition proliferation of free CHB (The medium contains 0.5% v/v DMSO for the dispersion of CHB) and conjugated CHB was estimated by MTT assay. MCF-7 cells were seeded into 96-well plates at a density of 8000 cells/well. After 24 h incubation, HPKM-CHB solutions with DMEM were prepared and appropriate solution was replaced with 250 medium containing serial HPKM-CHB solution at different concentrations (10, 20, 40, 80, 160 and 320 µg/mL). Cells were incubated for another 96 h. 25 µL 5 mg/mL MTT was added into each well. After cells being lysed for 4 h, solution in every well was removed carefully. DMSO was added with 200 µL per well to dissolve blue formazan crystals. The absorbance was recorded by BioTek at a wavelength of 562 nm. The cell viability was obtained by the formula as follows:

$$\text{Cell viability} = \frac{[\text{OD}_{562}]_{\text{sample}}}{[\text{OD}_{562}]_{\text{control}}} \times 100\%$$

Assessment of gene transfection

HPKM-CHB was dissolved in neutral PBS and sterilized through a Millipore filter. Polyplexes were prepared by mixing HPKM-CHB and pDNA with a serial N/P ratios of 5, 10, 20, 40, and 80. PEI was regarded as the gold standard for gene transfection reagent and it was set as controls at the N/P ratio of 15. 0.2 μg pDNA was used in every well. COS-7 cells were seeded at a density of 10000 cells/well. After cultivating for 24 h, the medium was replaced with 50 μL medium containing 10% FBS. Then, 50 μL polyplex solution was added. Cells were cultured in incubator at 37 $^{\circ}\text{C}$ with CO_2 atmosphere for 4 h, and the previous medium were replaced by fresh with 10% serum. After another 2 days culture, the cell lysis kit was used to lyse transfected cells. The polyplex performance was assessed by Promega luciferase assay kit. Protein concentration was determined according to the protocol of BCA protein assay kit. Relative light units (RLU) were obtained from the expression intensity of reporter gene and corresponding protein concentrations.

Reference

- 1 M. Chen, J. Wu, L. Zhou, C. Jin, C.Tu, B. Zhu, F. Wu, Q. Zhu, X. Zhu and D. Yan. *Polym. Chem.*, 2011, **2**, 2674.