Supporting Information for

Cathepsin B-Sensitive and Biocompatible Dendritic polyHPMA-Gemcitabine Prodrug-Based Nanoscale System Markedly Enhances the Antitumor Activity

Yan Dai,[a,c,‡] Xuelei Ma,[b,‡] Yanhong Zhang,[a] Kai Chen,[a] James Z Tang,[d] Qiyong Gong,[a] Kui Luo[a,*]

[a]*Huaxi MR Research Center (HMRRC), Department of Radiology,* [b]*Department of Department of Oncology, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China*

[c]*Department of Pharmacy of the Affiliated Hospital of Southwest Medical University, Southwest Medical University, Luzhou, Sichuan Province, 646000, China.*

[d]*School of Pharmacy, University of Wolverhampton, Wolverhampton WV1 1LY, United Kingdom*

*Corresponding author

Prof. Luo is to be contacted at Tel./fax: +86 28 85410653. E–mail addresse: luokui@scu.edu.cn

‡Yan Dai and Xuelei Ma contributed equally to this work.

Materials and Methods:

The information about monomers HPMA,[1] MA-GFLG-GEM,[2] MA-GFLGK-MA,[3] MA-N_3,[3] MA-GFLG-CTA were prepared as previous reported.[3] The agents including Akyne-Cy5.5, 2,2'-[azobis(1-methylethylidene)]bis[4,5-dihydro-1H-imidazole] dihydrochloride (VA044) were purchased from Sigma-Adrich (Shanghai, China). ^{1}H NMR data was recorded via a 400 MHz Bruker Advanced Spectrometer under room temperature. Particle size and zeta potential were measured in distilled water by a Zetasizer Nano ZS (Malvern Instruments, UK). The ex vivo fluorescent images were obtained using a Maestro In-Vivo Imaging System (Cri, USA). The cancer cell was 4T1 cell line (murine breast cancer cell). The animals are female BALB/c mice obtained from Chengdu DaShuo Biological Technology Co., Ltd. China.

MW and PDI

The MW and polydispersity (PDI) of the copolymer were tested via size-exclusion chromatography (SEC) on a Superose 6 HR10/30 column and on an ÄKTA/FPLC system (GE Healthcare). Sodium acetate buffer/methanol (7:3, pH 6.5) was used as mobile phase with a corresponding flow rate of 0.4 mL/min. The copolymers were purified by SEC via a Superose 6 HR10/30 column, while the mobile phase was sodium acetate buffer/methanol (7: 3, pH 6.2), and the flow rate was 2.5 mL/min, and the temperature was 4 ^{o}C. The products were fractionated/purified by size exclusion chromatography using Superose 6 HR10/30 (MW range for hydrophilic neutral polymers 15-300 kDa/14 mL separation volume) column on an ÄKTA FPLC system (GE Healthcare) column with sodium acetate buffer containing 30% methanol (pH = 6.5) as the mobile phase.

Click reaction

The azido functionalized dendritic polymer (Dendritic polymer 600 mg), alkyl Cy5.5 (Akyne-Cy5.5, 24 mg) and sodium ascorbate (10 mol%) were in a vial, and the vial was protected with nitrogen. The cooled solution of DMSO/water (1:1, 20 mL) was added. The solution was protected with nitrogen. The copper sulfate in 2 mL of di-water (5 mol%) was added. The solution was bubbled with nitrogen for 30 min, and the vial was closed. The solution was stirred for 4 h, and the solution was dialysized in the aqueous solution water with ethylenediaminetetra-acetic acid disodium salt (0.5 mM) for 10 h and di-water for 12 h, then freeze dried, resulting in a blue powder. The products were purified via size exclusion chromatography using Superose 6 HR10/30 column on an ÄKTA FPLC system. The fractions were collected and dialysized in the aqueous solution water, freeze dried, resulting in a blue powder 450 mg.

Size and Zeta Potential

Zeta potential and size of the conjugate was assayed through DLS. The dendritic prodrug (Dendritic polyHPMA-GEM) was diluted with deionized water (2 mg/mL) and sonicated for 30 s before measurement. The dendritic prodrug was incubated in PBS (pH = 7.4) and PBS with 10%FBS (pH = 7.4) at 37 °C for 1h, 4h, 12h, 24h, 36h and 48h, respectively. The size and zeta potential were measured via DLS. Particle size and zeta potential of the samples were measured using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Each measurement was measured in triplicate, and the results were processed with DTS software version 3.32.

Biodegradability of the conjugate

Biodegradation study of the dendritic prodrug (Dendritic polyHPMA-GEM) was carried out in McIlvaine's buffer (pH 5.4) with 2.8 μM cathepsin B. The concentration of the dendritic prodrug (Dendritic polyHPMA-GEM) was 3 mg/mL. The mixture was incubated for 0, 2, 4, 8, 12 h at 37 °C. Thereafter, tested samples were measured by SEC.

Cell culture and animals

4T1 cell line (murine breast cancer cell) derived from the BALB/c spontaneous mammary carcinoma was obtained from Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China). The cell line was cultured in RPMI 1640 supplemented with 10% (v/v) FBS (HyClone), 100 U/mL of penicillin, and 100 mg/mL of streptomycin. The cells were cultured at 37 °C in a humidified 5% CO_2 incubator. Six- to eight-week-old female BALB/c mice with body weights in the range of 18.0 to 22.0 g were chosen as hosts for the tumour model. The animals were obtained from Chengdu DaShuo Biological Technology Co., Ltd., which were housed in standardized disinfected conditions. All mice were allowed to acclimatize for a week before the start of any experiments. All experiments were performed using protocols approved by the animal experiments ethical committee of Sichuan University and China.

Statistical Analyses

Student's t-test was used to statistically compare the experimental groups to ascertain statistical significance. All data were obtained as mean ± SD, while p values < 0.05 were considered to be statistically significant.

Results

Table S1. Characterization of the prepared dendritic polymers.

Copolymer	MW (kDa)	PDI	Gly%	Phe%	Leu%	Lys%	GEM%	Cy5.5%
Dendritic polymer	154	2.84	5.89	6.56	5.08	1.90	5.8	-
Dendritic polyHPMA-GEM	168	2.24	5.91	6.48	5.11	1.89	5.6	0.35

Table S2. The results of the degraded segments after incubation of the dendritic prodrug (Dendritic polyHPMA-GEM) in PBS (pH = 7.4) or in McIlvaine's buffer with cathepsin B (2.8 μM, pH = 5.4) at 37 °C.

conditions	0 h	2h	4h	8h
PBS (pH 7.4)	168 kDa, PDI 2.24	168 kDa, PDI 2.26	166 kDa, PDI 2.31	165 kDa, PDI 2.34
McIlvaine's buffer (pH , 5.4)	168 kDa, PDI 2.24	78 kDa, PDI 1.85	48 kDa, PDI 1.35	29 kDa, PDI 1.21

PBS: phosphate buffered saline

Table S3. Blood samples tests from the normal mice injected intravenously with saline, free drug GEM and dendritic prodrug (Dendritic polyHPMA-GEM).

Biomarker	Saline	GEM	Dendritic prodrug
ALT (U/L)	22.5±1.3	26±8.7	24.3±5.0
AST (U/L)	85.5±2.9	96.3±5.4	92.3±6.2
TP (g/L)	49.7±2.2	53.6±1.4	46.7±1.4
ALB (g/L)	36.6±0.6	33.5±1.9	36.3±1.3
GLOB (g/L)	13.1±2.0	14.3±0.8	13.2±1.2
UREA (mmol/L)	7.0±1.4	5.6±0.6	9.1±1.4
CREA (μmol/L)	9.5±1.3	8.25±1.3	9.3±1.0
BUN (mmol/L)	19.5±4.2	15.8±1.7	22.5±4.0
LDH (U/L)	463.0±15.9	492.7±22.9	468.6±15.3

CK-MB (U/L)	224.0±24.2	259.5±54.7	230.8±45.9

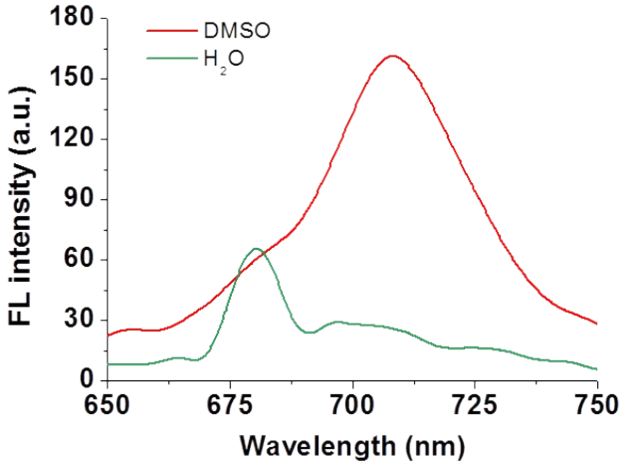

Fig. S1. The fluorescence spectrum of the dendritic prodrug was tested in water and DMSO.

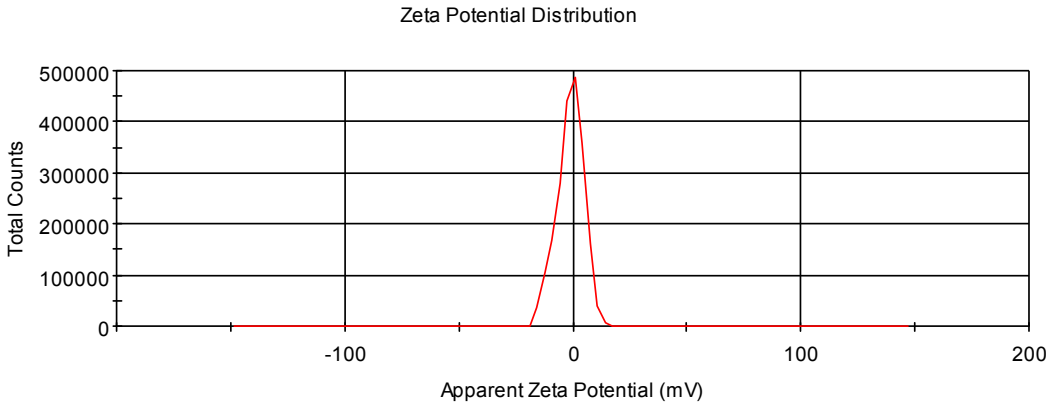

Fig. S2. Zeta potential of dendritic prodrug based nanoscale system (–1.44 mV).

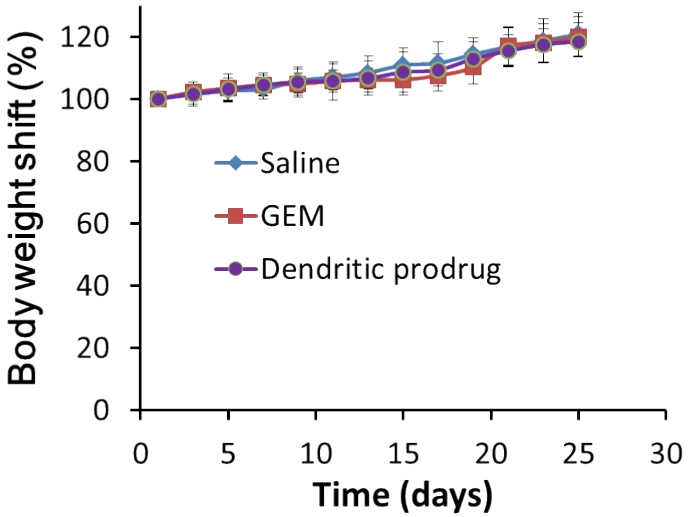

Fig. S3. The body weight shift of the normal mice injected intravenously with saline, free drug GEM and dendritic prodrug (Dendritic polyHPMA-GEM).

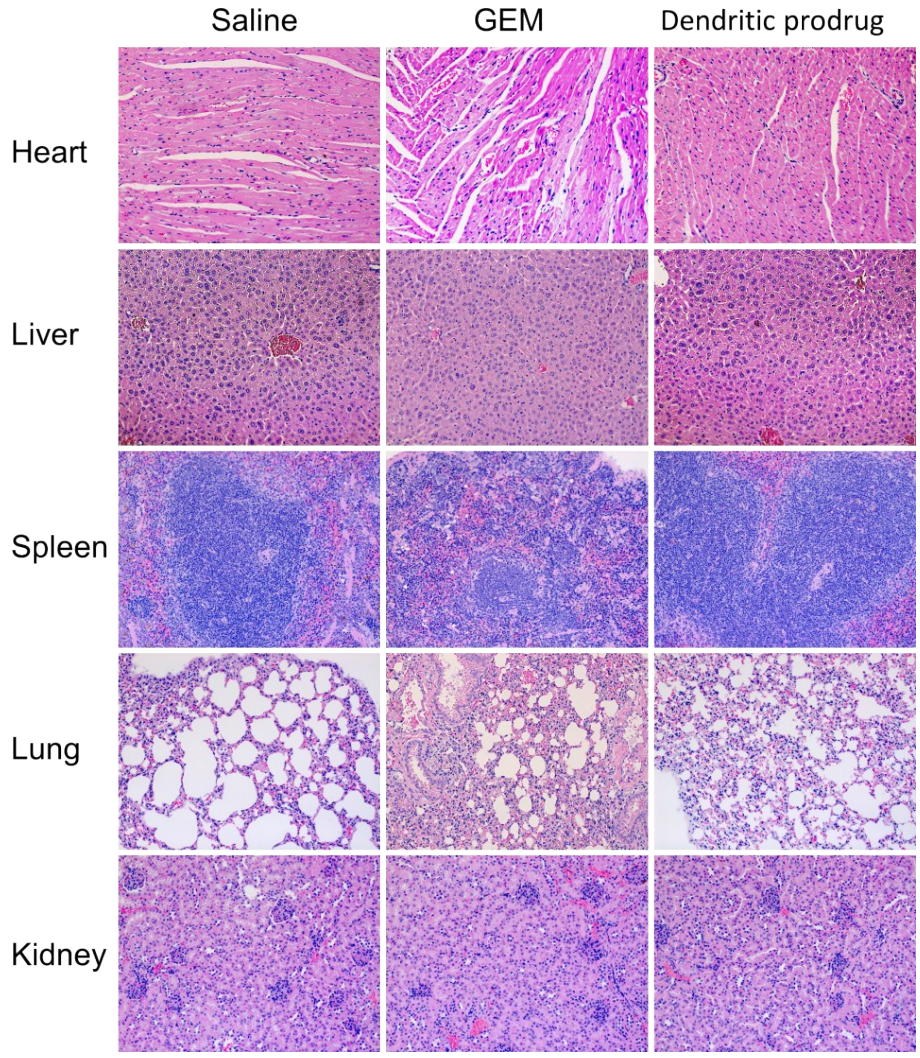

Fig. S4. For the in vivo biosafety, H&E staining of the major organs (i.e., heart, liver, spleen, lung, and kidney) separated from normal mice injected intravenously with saline, free drug GEM and dendritic prodrug (Dendritic polyHPMA-GEM) (×200).

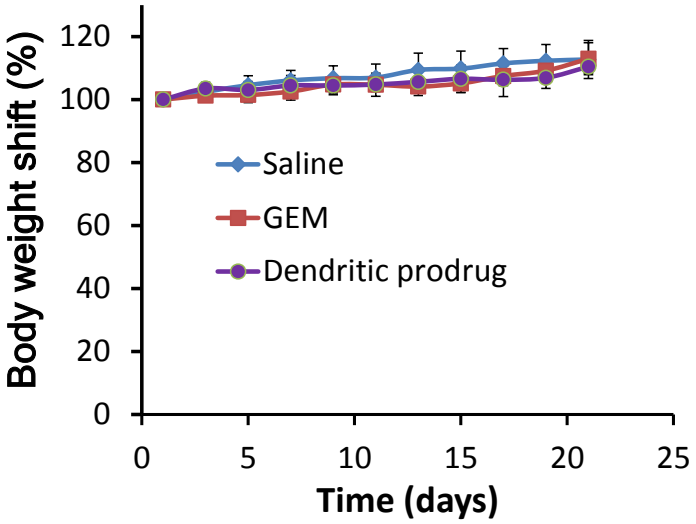

Fig. S5. The body weight shift of the tumor bearing mice, which are injected intravenously with saline, free drug GEM and dendritic prodrug (Dendritic polyHPMA-GEM).

References

1. J. Kopeček, H. B. *Eur. Polym. J.* **1973**, 9, 7-14.

2. Yang, J.; Luo, K.; Pan, H.; Kopeckova, P.; Kopecek, J. *React. Funct. Polym*. **2011**, 71, 294-302.

3. Sun, L.; Li, X.; Wei, X.; Luo, Q.; Guan, P.; Wu, M.; Zhu, H.; Luo, K.; Gong, Q. *ACS Appl. Mater. Interfaces*, 2016, **8**, 10499-10512.