Electronic Supplementary Material (ESI) for New Journal of Chemistry.

Glutathione Responsive Poly(HPMA) Conjugate Nanoparticle for 6-MP Efficient Delivery

Weibing Xu[a]*, Guichen Li[b], Haitao Long[a], Guorui Fu[a], Lumei Pu [a]*

[a] *College of Science, Gansu Agricultural University, Lanzhou 730000, China.*

[b] *Gansu Provincial Key Laboratory of Aridland Crop Science, Gansu Agricultural University, Lanzhou 730070, Gansu, China;*

**Corresponding author, Tel.: +86 15095309279; Email: xuwb@gsau.edu.cn;*

Experimental

Materials

2,2`-Azobis(2-methylpropionitrile (AIBN, Aldrich) was recrystallized from ethanol and dried at room temperature under vacuum. Methacryloyl chloride (>98%), Thiazolyl blue tetrazolium bromide (MTT, 98%), dimethyl sulfoxide (DMSO, 99.8%), dichloromethane, acetone and isopropanolamine were all purchased from Aldrich. Silica gel 60 (0.0632-0.2 mm) was obtained from Merck (Darmstadt, Germany). NMR solvents ($CDCl_3$ and DMSO), 6-mercaptopurine, sodium methylate and propiolic acid were purchased from Fisher. Regenerated cellulose dialysis membrane (Spectra/Por 6, molecular weight cutoff 3000 Da) was purchased from Fisher.

Synthesis of Poly(HPMA)-PTA

HPMA and *cis*-3-(9H-purin-6-ylthio)-acrylic acid (PTA) was synthesized according to the literatures.[1,2] Poly(HPMA)- PTA was prepared as following: PTA (20 mg, 0.093 mmol) and Poly(HPMA) (0.14 g, 10mmol) was dissolved in DMSO (2 ml). The solution was degassed with nitrogen gas in an ice bath for 10 min. DCC (46 mg, 0.23 mmol) and DMAP (3 mg, 0.02mmol) were added and vigorously stirred. The mixture was stirred for 120 h at -5 ^{o}C (ethanol/dry ice mixture). The mixture was precipitated into acetone to give a white powder (0.12 g, yield: 66%). The compound was dissolved in 20 ml of deionized water and dialyzed against water for 48 h in a dialysis bag. The final product was obtained by lyophilizing the dialysis solution.

Characterization

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury plus-400 instrument. The chemical shifts are reported in ppm relative to the solvent residual peak. A Bruker IFS 66v/s IR spectrometer (Bruker, Karlsruhe, Germany) was used for the Fourier transformed infrared (FTIR) analysis in the range of 400–4000 cm^{-1} with the resolution of 4 cm^{-1}. The content of 6MP in the Poly (HPMA)-mustard was determined by UV-visible spectrophotometer (TU-1901, HITACHI). Hydrodynamic diameters were measured at 25 °C using a Malvern Zetasizer NanoZS Instrument equipped with a 4 mW He-Ne solid-state laser operating at 633 nm (The samples were dissolved in phosphate buffered saline (PBS 10mM). Backscattered

light was detected at 173° and the mean particle diameter was calculated from the quadratic fitting of the correlation function over thirty runs each of ten seconds duration. Zeta potentials were calculated from electrophoretic mobilities using the same Malvern Instruments Zetasizer NanoZS instrument equipped with an autotitrator (MPT-2 multipurpose titrator, Malvern Instruments). The molecular weights and distributions were obtained by Gel Permeation Chromatography (Waters Alliance GPCV 2000 chromatograph). The morphologies of the materials were examined using a transmission electron microscope (TEM) (FEITECNAI G2TF20, USA).

6-MP Release Study

10 mg of Poly(HPMA)-PTA was dissolved in 20 mL of phosphate buffer pH 7.4 (10 mM). The solution then was loaded into dialysis bag (MWCO 3000). The dialysis bag was immersed into corresponding buffer medium (200 mL) containing concentration of GSH (0.0 mM and 10 mM) to study the GSH dependent release kinetics. At desired time intervals of 0.5, 1, 2, 4, 6, 8, 12 or 24 h, 5 mL of released medium was taken out and an equal amount of the same concentration of GSH solution was added to the dialysis medium simultaneously. The concentration of 6MP was determined by HPLC (Thermo) with UV detection at 320 nm using a mixture of acetonitrile and water (v/v = 5/95) as the mobile phase. Colum: Sepax GP-C18 (150 mm × 4.6 mm, 5 μm), flow rate: 1.0 mL/min; injection volume: 20 μL, retention time: 5.3 min. The data are presented as mean ± SD (n = 3).The accumulative release rate was calculated as follows:

$$\text{Accumulative release } \% = \frac{M_t}{M \times X} \times 100\%$$

Where M_t is the amount of 6-MP at time point t, M is the total weight of the conjugate, X is the 6-MP content in the corresponding conjugate (%).

Hemolysis assay

The fresh blood of 5.0 mL was drawn from rabbit and placed into saline containing centrifuge tube immediately. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Gansu Agricultural University and approved by the Animal Ethics Committee of Gansu Agricultural

University. The tube was centrifuged at 1500 rpm for 15 min and the supernatant was discarded. The obtained precipitate was repeatedly washed and centrifuged until the supernatant was substantially colorless. The precipitated red blood cells were mixed with physiological saline to prepare a 2% (v/v) cell suspension. Hemolysis tests were performed based on previously published procedures.[3] A series of solutions (0.5 mL) of different mass concentrations were placed in the centrifuge tube to which an equal volume of 2% (v/v) red blood cell suspension was added. Another 2% (v/v) red blood cell suspension (0.5 mL) was added to an equal volume of distilled water and physiological saline as a positive control and a negative control, respectively. The all tubes were thoroughly mixed and incubated for 1.0 h in a 37 °C incubator. Then, the all tubes were centrifuged at 2000 rpm for 15 min and the supernatant (100 uL) was pipetted into a 96-well plate. The OD value of each well at a wavelength of 540 nm on a 96-well plate was measured using a microplate reader. The hemolysis rate is calculated using the following formula:

$$Hemolysis\ rate\ \% = \frac{OD_{sample} - OD_{negative}}{OD_{positive} - OD_{negative}} \times 100\%$$

Each concentration was set to three parallel groups.

MTT Assay

The MTT assay was introduced to study the cytotoxicity of the conjugates. The HepG2 cell cultures were carried out in 96-well plates (cell density: 5×10^3). After 24 h, the Poly(HPMA) conjugate, PTA and 6-MP (equivalent mustard dose, 5, 10, 20 and 40 μg mL^{-1}) were added, respectively. The additional culture lasted for 48 h at 37°C. Before being incubated with MTT (5 mg/mL^{-1}, 20 μL per well), the medium was removed and cells were washed twice with ice-cold PBS. Then the solution was removed before adding DMSO (150 μL per well) into the wells to dissolve the formazane of MTT. The cell viability was calculated *via* ELISA plate reader (Thermo Fisher Scientific, MA, USA) with the ultraviolet absorbance at 570 nm. The optical

density was used to calculate cell viability.

Scratch assay

We evaluated the effect of Poly(HPMA)-PTA on the migratory activity of HepG2 cells compared to its free 6MP counterparts using scratch assay. We plated HepG2 cells in 24-well plates at a seeding density of 50,000 cells per well and allowed the cells to grow under normal culture conditions to 95% confluence. The cell monolayer was scratched with a sterile 200 µl pipette tip to create a "wound" across the center of each well. Each well was washed with PBS to remove the detached cells and the wound was imaged (t = 0 h) at 10X magnification using a Nikon Eclipse Ti inverted microscope equipped with a camera to capture phase contrast images of the wound. Subsequently, the cell monolayer was treated with free 6MP and Poly(HPMA)-PTA nanoparticles following the treatment conditions described in section MTT Assay. After 48 h, we imaged the wound area using the same microscope set-up.

Cellular Uptake

The cellular uptake of Poly(HPMA)-PTA by HepG2 cells was investigated by the live cell imaging system (Cell'R, Olympus). Cells were cultured in the same medium with MTT assay. The culture medium was removed after 24 and 48h incubation. Cells were washed with pH 7.4 PBS buffer solution and stained with Hoechst 33342 (10 mg L^{-1}). During observation, the cells were incubated with RPMI-1640 culture medium and samples at 37 ^{o}C under a 5% CO_2 atmosphere. The images were captured with the excitation wavelength of 340 nm (blue) per 30 min.

Flow cytometric analysis

Cell death was analyzed by fluorescence-activated cell sorting (FACS) using Annexin V and propidium iodide (PI) staining assay. HepG2 cells were seeded in a 6-well late at a density of 2×10^5 cells per well and incubated in RPMI-1640 incubation medium (3 mL) and allowed to settle for 24 h. The medium was replaced with the fresh one containing free 6MP and Poly(HPMA)-PTA, respectively. After incubation for 48 h, the cells were washed twice with cold PBS, trypsinized and centrifuged (4000 g, 3 min). The supernatant was discarded and the cells were resuspended in binding buffer (500 μL), stained with Annexin V and incubated in the dark for 15 min. The cells were treated with PI and analyzed by flow cytometry.

1．S. Gunnarsdottir and A. A. Elfarra, *J. Pharmacol. Exp. Ther.*, 1999, **290**, 950-957.

2．N. Francini, L. Purdie, C. Alexander, G. Mantovani, S. G. Spain, *Macromolecules*, 2015, **48,** 2857-2863.

3. N. Sahiner, S. Sagbas, N. Aktas, *Polym. Degrad. Stab.*, 2016, **129**, 96-105.

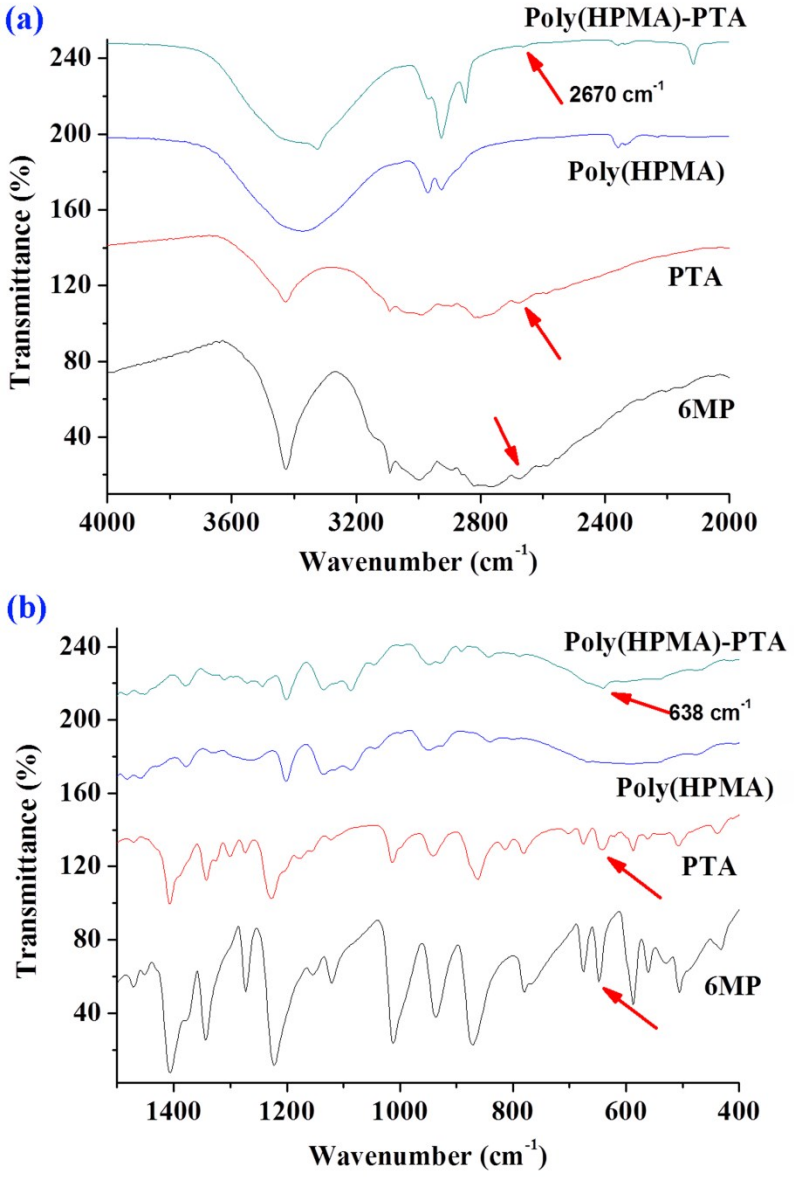

Fig. S1 The IR spectra of 6-MP, PTA, Poly(HPMA) and Poly(HPMA)-PTA.

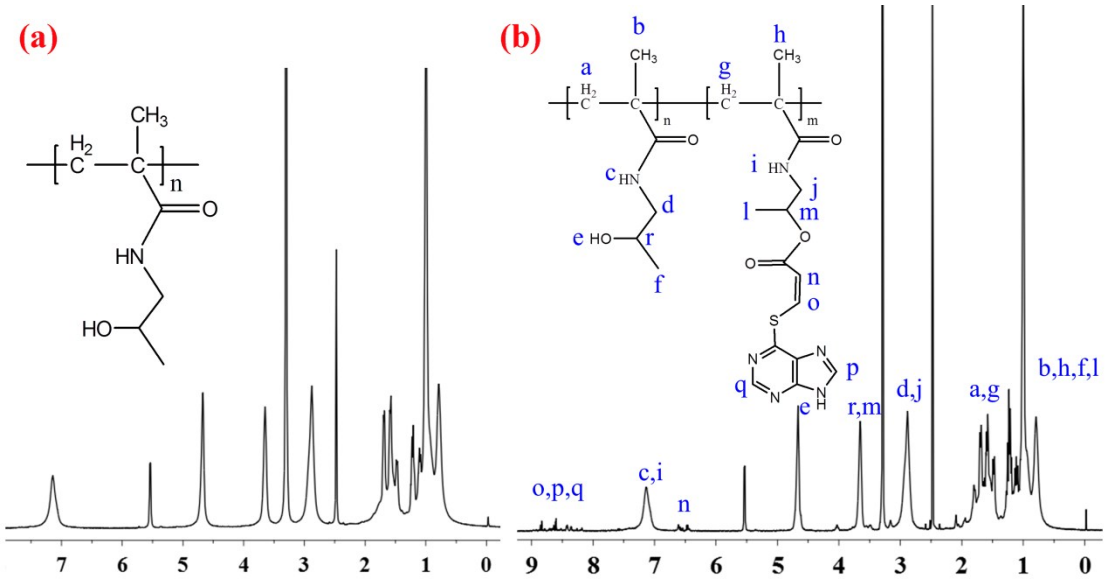

Fig. S2 The NMR spectra of poly(HPMA) (a) and Poly(HPMA)-PTA (b).

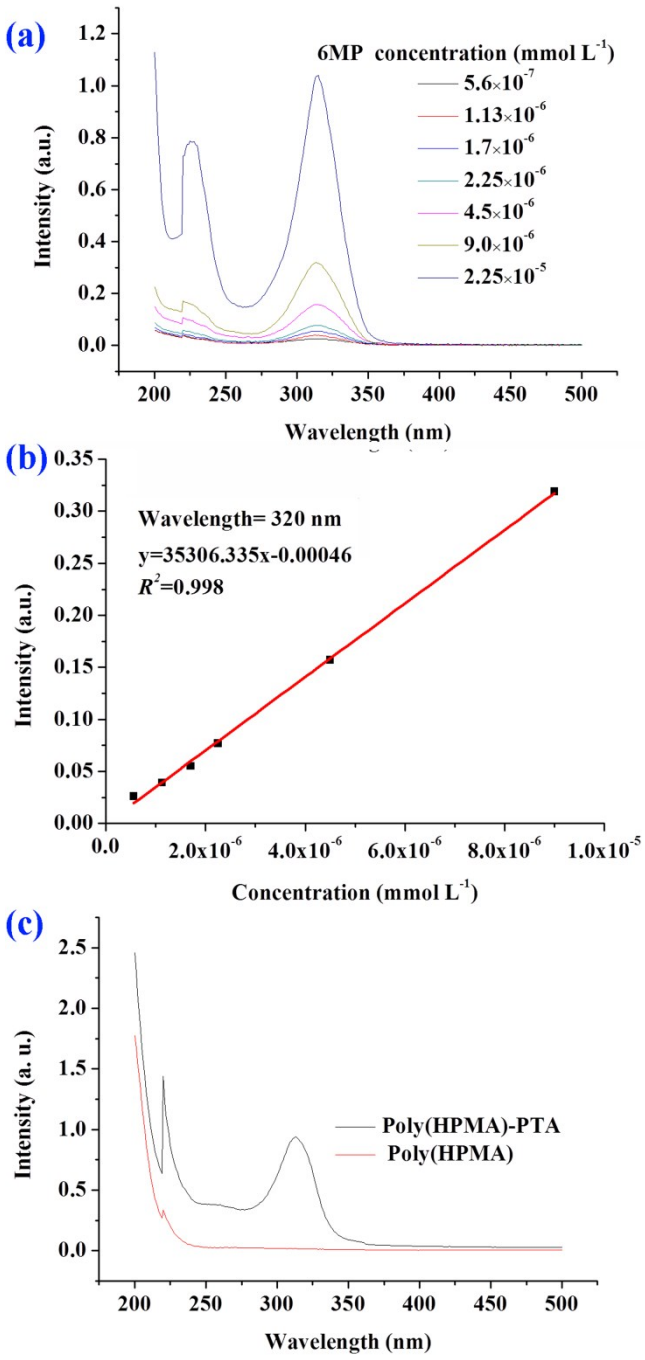

Fig. S3 The UV spectra of PTA (a), the calibration curve (b) and UV spectra of Poly(HPMA) and Poly(HPMA)-PTA conjugate (c).

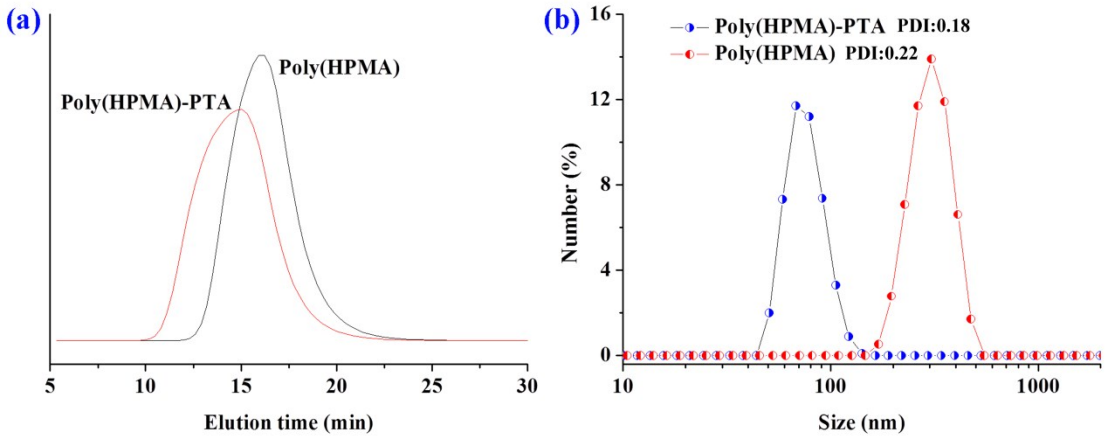

Fig. S4 The GPC trace (a) and dynamic light scattering size distributions (b) of the Poly(HPMA) and Poly(HPMA)-PTA conjugates.

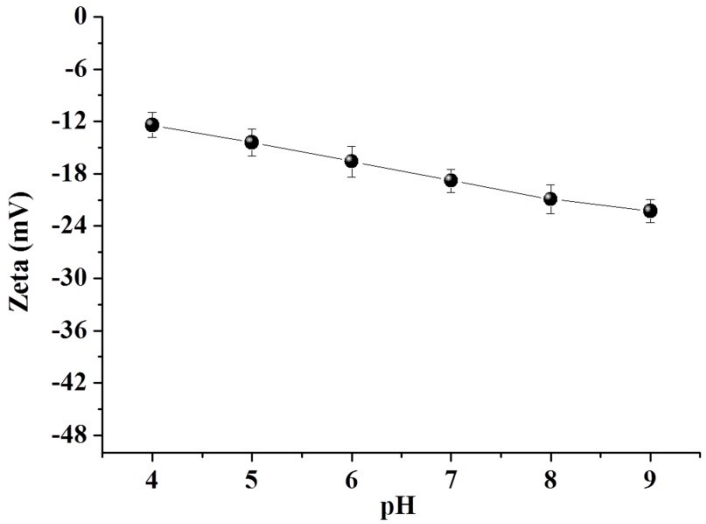

Fig. S5 The zeta potential of the Poly(HPMA)-PTA conjugate.

Table S1. Properties of poly(HPMA) and poly(HPMA)-PTA conjugate.

Samples	PTA (mol %)[a]	M_n (g mol^{-1})[b]	PDI (M_w/M_n)[b]	D_h (nm)[c]
PTA	-	219.06	-	-
Poly(HPMA)	-	1.56×10^4	1.42	317 nm
Poly(HPMA)-PTA	8.8 %	1.67×10^4	1.56	89 nm

[a] Estimated by ^{1}H NMR.

[b] Determined by GPC.

[c] The hydrodynamic diameter (D_h) and polydispersity by DLS analysis.

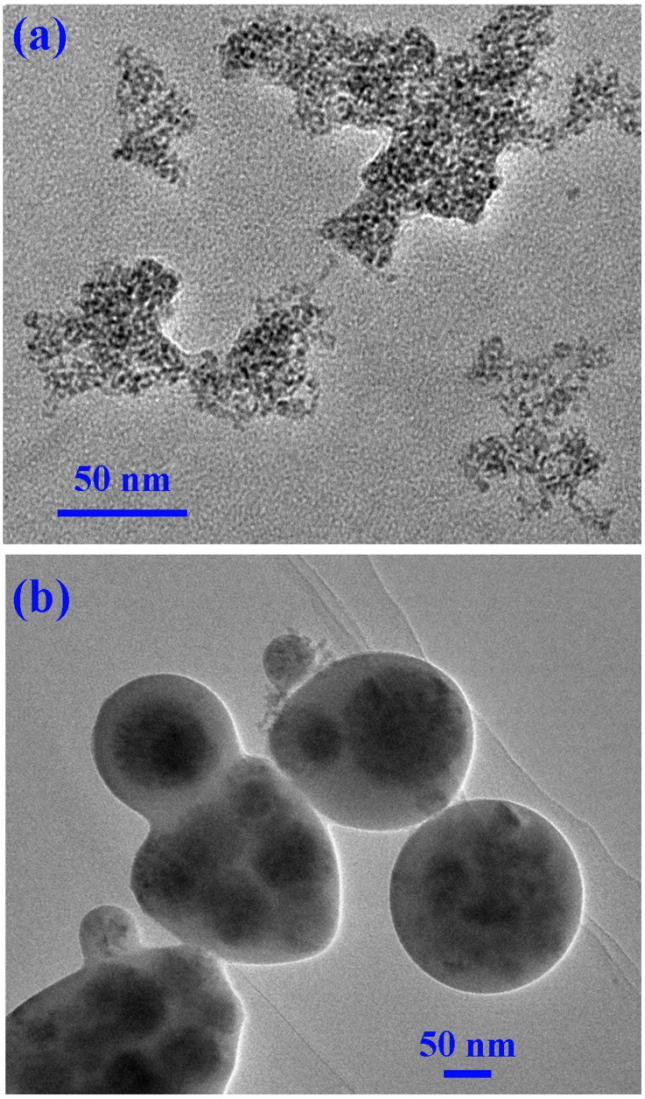

Fig. S6 the TEM of Poly(HPMA) (a) and Poly(HPMA)-PTA (b)

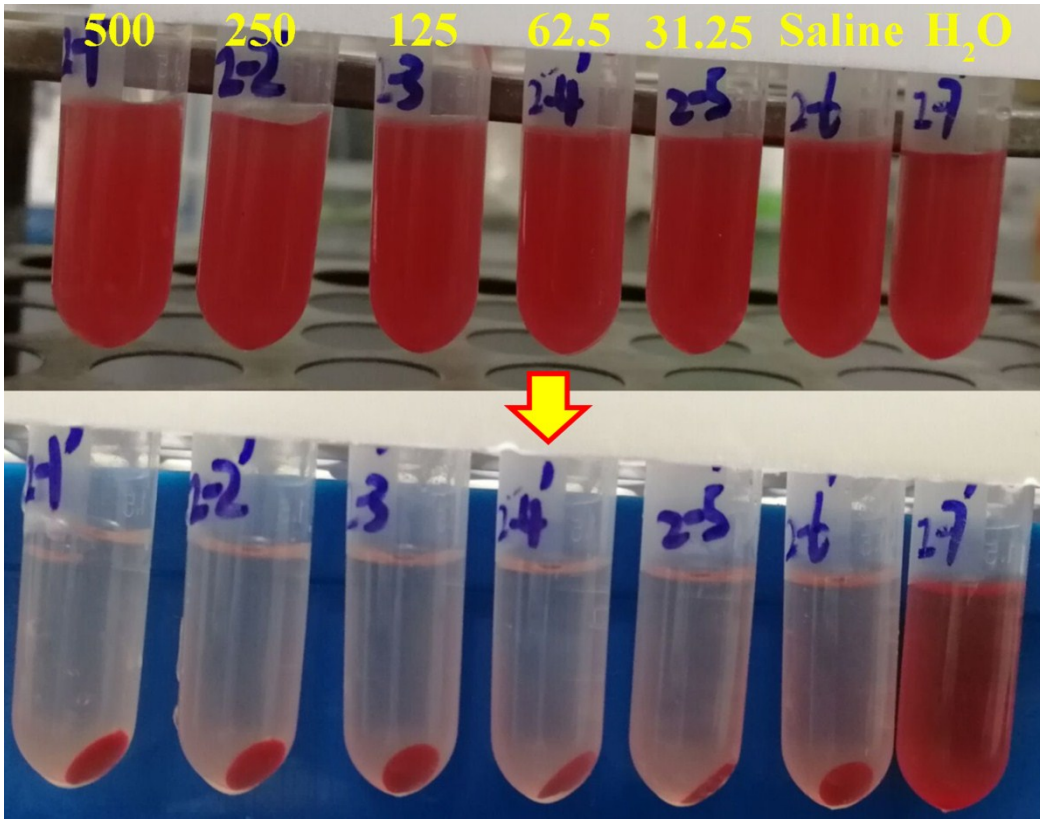

Fig. S7 Erythrocyte hemolysis resulted in the presence of Poly(HPMA)-PTA conjugate.

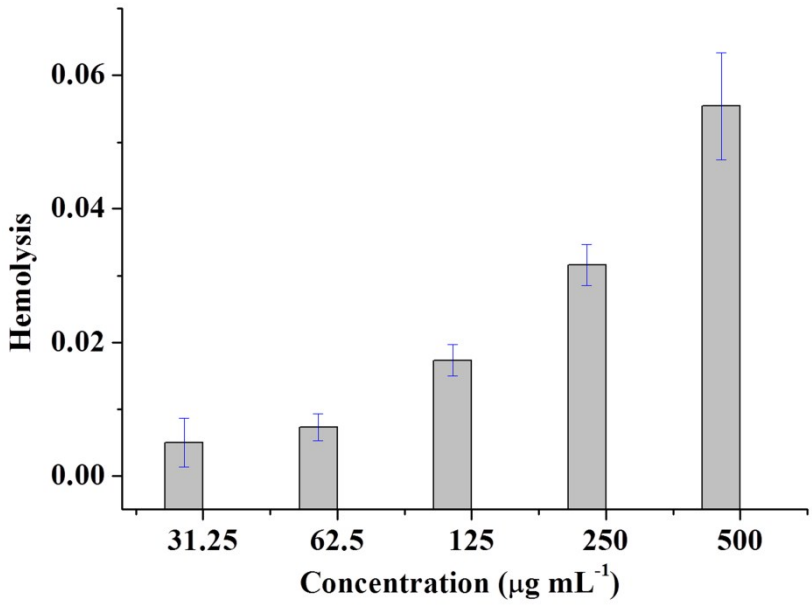

Fig. S8 The hemolysis rate of Poly(HPMA)-PTA conjugate.

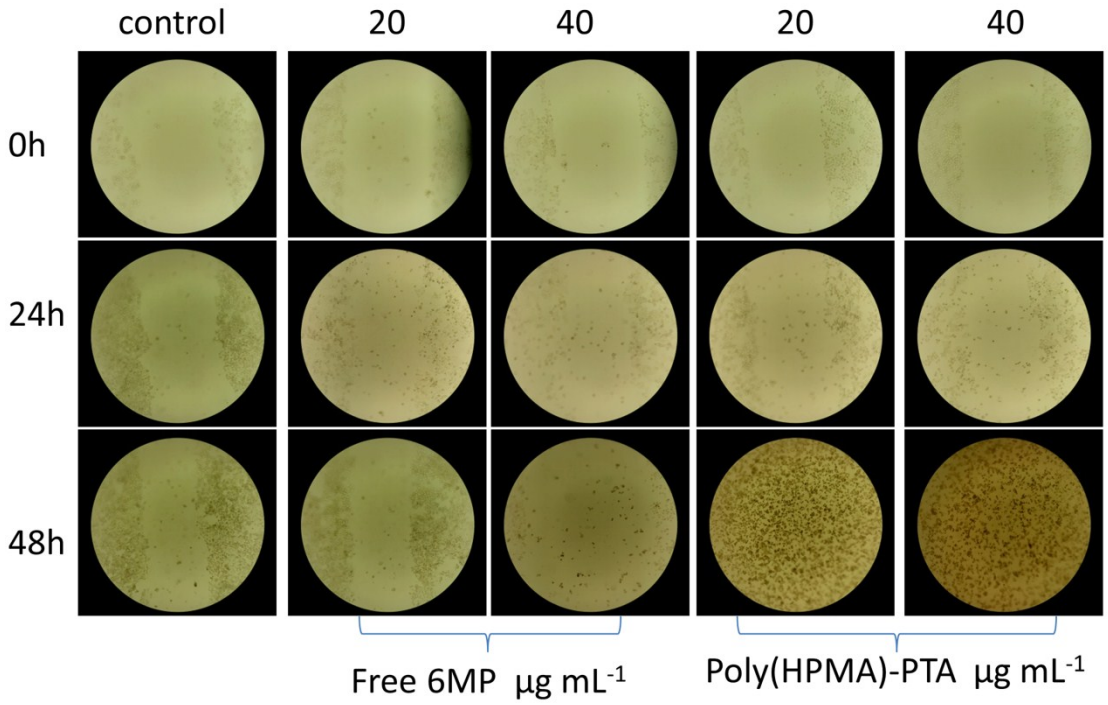

Fig. S9 Inhibition of HepG2 cell migration.

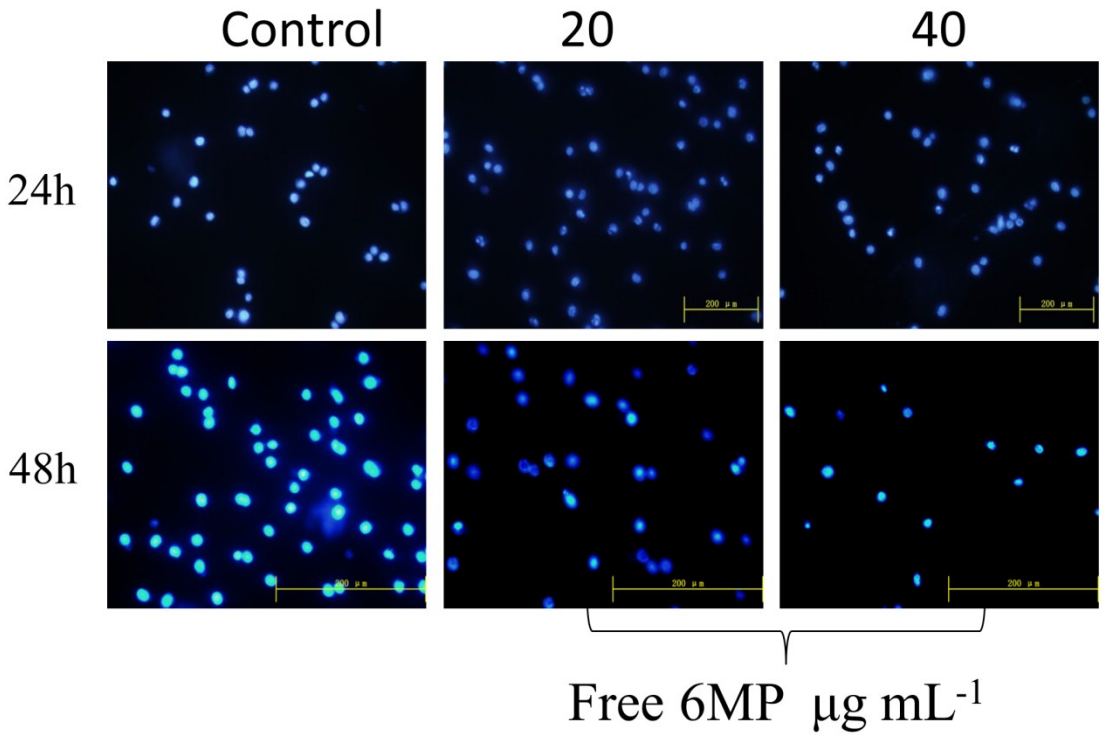

Fig. S10 Fluorescence images of HepG2 cells incubated with free 6MP for different times.

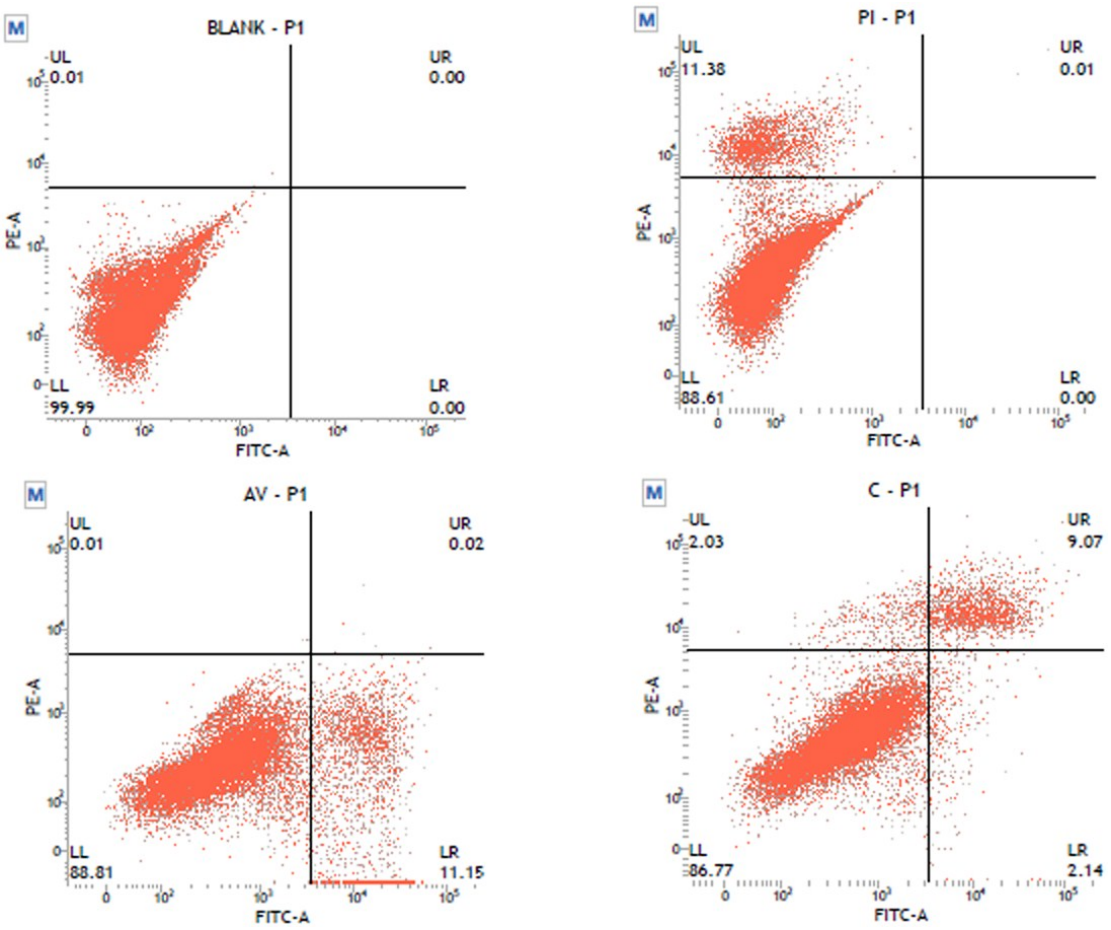

Fig. S11 Flow cytometric analysis of HepG-2 cells in control group.