Supplementary Information

Conjugated polyelectrolytes with galactose-containing side chains for targeted hepatoma cell Imaging

Mingli Zhang,[ab,‡] Wu Pan,[ab,‡] Wei-Tao Dou,[c] Hai-Hao Han,[c] Xiao-Peng He,*[c] Chunyan Tan,*[ab] and Yuyang Jiang*[d]

[a] Department of Chemistry, Tsinghua University, Beijing 100084, P. R. China.

[b] The State Key Laboratory Breeding Base-Shenzhen Key Laboratory of Chemical Biology, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, P. R. China. Email: tancy@sz.tsinghua.edu.cn

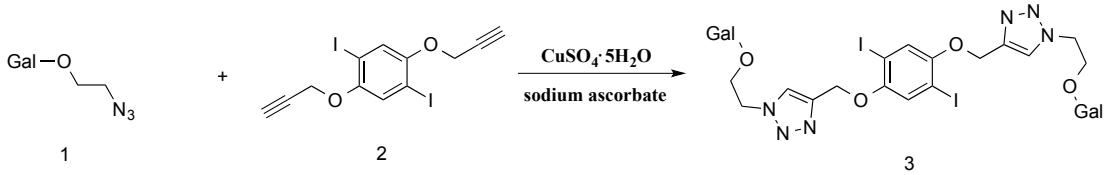
[c] Key Laboratory for Advanced Materials & Institute of Fine Chemicals, School of Chemistry and Molecular Engineering, East China University of Science and Technology, Shanghai 200237, P. R. China. E-mail: xphe@ecust.edu.cn

[d] School of Pharmaceutical Sciences,, Tsinghua University, Beijing 100084, P. R. China. Email: jiangyy@sz.tsinghua.edu.cn

Experimental

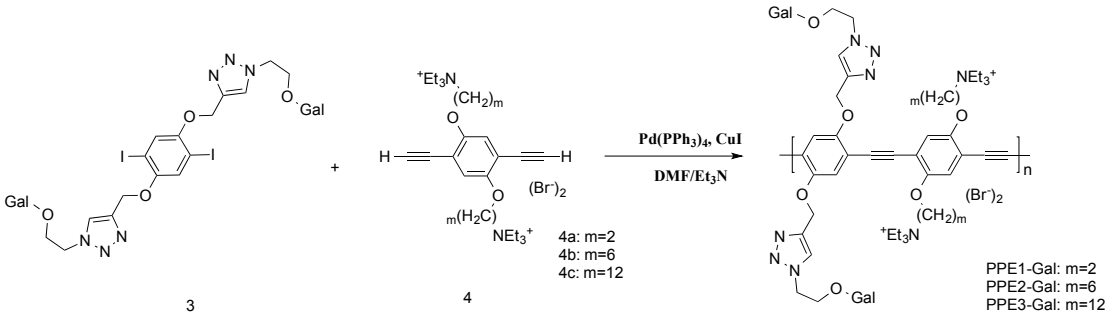
Materials. All purchased chemicals and reagents are of analytical grade. Solvents were purified by standard procedures. Proteins were purchased from Sigma-Aldrich. The water used in all of the experiments was prepared on a Milli-Q water purification system and displayed a resistivity of ≥18.2 MΩ cm^{-1}.

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer. Absorption spectra were recorded on a Cary 100 UV-Vis spectrometer. Fluorescence spectra were recorded on a SPEX Fluorolog 3-TCSPC spectrometer with 1 cm path length cuvettes. Fluorescence images were taken by confocal microscopy (Olympus FV1000-IX81 and Perkinelmer).



Synthesis of compound 3. Compound 1 and compound 2 was synthesized according to literature reported procedures.[1-2] To a solution of 1 (0.206 g, 1.005 mmol) and 2 (0.200 g, 0.4566 mmol) in 20 ml of acetone and water (10:1) solvent mixture, sodium ascorbate (0.181g, 0.9132 mmol) was added, followed by $CuSO_4 \cdot 5H_2O$ (0.114 g, 0.4566 mmol) at room temperature, and the mixture was stirred overnight. At the end of the reaction, the reaction mixture was evaporated using rotary evaporator under reduced pressure. 10 mL of water was added and the mixture was filtrated. The solid was recrystallized to obtain 3 as white solid (0.232g, 60%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.29 (s, 2H), 7.60 (s, 2H), 5.149 (s, 4H), 4.97 (d, 2H, *J*=4.0 Hz), 4.86 (s, 2H), 4.77 (d, 2H, *J*=6.8 Hz), 4.61 (s, 4H), 4.60 (s, 2H),

4.42 (d, 2H, *J*=4.0 Hz), 4.18 (d, 2H, *J*=6.8 Hz), 4.09 (dd, 2H, *J*=5.6 Hz), 3.90 (dd, 2H, *J*=5.6 Hz), 3.62 (s, 2H), 3.51 (s, 4H), 3.30 (s, 4H). ^{13}C NMR (100 MHz, d_6-DMSO) δ 152.2, 142.1, 125.3, 123.2, 103.5, 86.8, 75.3, 73.2, 70.2, 68.0, 67.1, 63.3, 60.4, 49.7.



Polymer synthesis. The polymers were synthesized via the Sonogashira protocol. In this work, three polymers with galactose-containing side chains and different cationic side groups were obtained. Compound 4a-c were synthesized according to literature reported procedures.[3] General polymerization procedures are summarized as below.

PPE1-Gal: A solution of monomers 3 (93.6 mg, 0.1 mmol) and 4a (57.4 mg, 0.1 mmol) in 50 mL of dry 3:1 (v/v) DMF/Et_3N in a three-necked flask fitted with a condenser was degassed with argon for 20 min, and then 5.8 mg of $Pd(PPh_3)_4$ (5 μmol) and 1.0 mg of CuI (5 μmol) were added under argon. The reaction mixture was stirred at 40 °C for 12h. Then the reaction mixture was filtered, the filtrate was dropped into methanol, and the brown solid was collected by centrifugal separation. The solid was dissolved in DMF and purified by dialysis against deionized water using a regenerated cellulose membrane (2 kD molecular weight cut-off) to get a red-brown solution. $\bar{M}_n$=4.49 kD, $\bar{M}_w$=6.17 kD, PDI=1.38.

PPE2-Gal: PPE2-Gal was synthesized according to the same procedure used for PPE1-Gal except that 4b (68.7 mg, 0.1 mmol) was used. $\bar{M}_n$=4.00 kD, $\bar{M}_w$=5.21 kD, PDI=1.30.

PPE3-Gal: PPE3-Gal was synthesized according to the same procedure used for PPE1-Gal except that 4c (85.5 mg, 0.1 mmol) was used. $\bar{M}_n$=4.37 kD, $\bar{M}_w$=6.10 kD, PDI=1.40.

Molecular weight determination. All of the mass spectra were acquired using a 4700 Proteomics Analyzer MALDI-TOF/TOF-MS (Applied Biosystems, Framingham, MA). Samples using 2,5- dihydroxybenzoic acid (DHB) as a matrix and silver trifluoroacetate (AgTFA) as an additive were prepared by dissolving the polymer in water at a concentration of 5 mg/mL. A 5 μL aliquot of this solution was added to a 5 μL aliquot of a 10 mg/mL matrix solution containing 1 μL of AgTFA in water (0.1 M) as the cationization agent. A 1 μL aliquot of the sample solution was hand- spotted on a stainless steel target plate and allowed to dry by air. Samples were analyzed with the operator manually searching for the sample "sweet spot" for data collection. The samples were measured in negative linear ion mode. All of the spectra were taken with signal averaging of 300 laser shots. All of the mass spectrometry (MS) data were further processed using Data Explorer 4.5 (Applied Biosystems).

Cell culture. HepG2, sh-ASGPr and HeLa cells were maintained in a Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Gland Island, NY, USA) and A549 cells were cultured in

F12 supplemented with 10% FBS in a humidified atmosphere of 5% CO2 and 95% air at 37 ºC, and split when the cells reached 90% confluency.

Cell viability assay. Cells were plated overnight on 96-well plates at 8000 cells per well in growth medium. After seeding, cells were treated with the polymers at different concentrations for 15 min. Then, cells were gently washed with PBS once. After exposure for 48 h, 10 μL per well of MTS/PMS (20:1, Promega Corp) solution was added to each well containing 100 μL of serum-free HG-DMEM, followed by a gentle shake. After incubation at 37 °C under 5% CO2 for 2 h, 80 μL of the mixture was transferred to another 96-well plate. The absorbance of the mixture solutions was measured at 490 nm with 650 nm as a reference, using an M5 microplate reader (Molecular Device, USA). The optical density of the result in MTS assay was directly proportional to the number of viable cells.

Cell imaging. HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and 100 000 cells were then seeded on 35 mm culture plates, which were incubated at 37 °C for 24 h. Three polymers (PPE1-Gal, PPE2-Gal, PPE3-Gal) were added to 1 mL of medium containing HepG2 cells in a 20 mm plate (the concentration of polymer was 5 μM). After incubation at 37 °C for 0.5 h, the medium was removed, and the cells were washed six times using 1× PBS. Fluorescence images (450−550 nm) were obtained by excitation with a multiline Hg laser (88.0% 405 nm) and analyzed using ImageJ software.

Cells (HepG2: 25000 per well; sh-ASGPr: 25000 per well; HeLa: 12000 per well; A549: 15000 per well) were seeded on a black 96-well microplate with optically clear

bottom (Greiner bio-one, Germany) overnight. The cells were incubated with PPE1-Gal, PPE2-Gal and PPE3-Gal (5 μM) for 15 min. For the competition assay, the HepG2 cells were preincubated with free D-galactose for 2 h, followed by incubation with **PPE2-Gal** for 15 min. Then, cells were washed with PBS (phosphate buffered saline) three times. The fluorescence images were recorded using an Operetta high content imaging system with an excitation wavelength of 410-430 nm and an emission wavelength of 460-540 nm, and quantified and plotted by columbus analysis system (Perkinelmer, US).

Celluar uptake of polymers by flow cytometry. Cells (HepG2 and HeLa cells) were seeded into a 6-well plate (~200,000/well) overnight. The cells were incubated with **PPE1-Gal**, **PPE2-Gal** and **PPE3-Gal** (5 μM) for 15 min. After washing with 1×PBS buffer three times, cells were digested by trypsin, and then the sample was immediately analyzed on the Becton Dickinson FACS Canto II flow cytometery.

Establishment of the ASGPr-knockdown HepG2 cell line (sh-ASGPr). Plasmids encoding ASGP-R1 specific shRNA was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Lentiviral particles were generated according to the manufacturer's instructions. Briefly, 293T cells were seeded in a six-well tissue culture plate and were grown to 80-90% confluency in antibiotic-free normal growth medium supplemented with FBS. shRNA plasmid (3 μg) was cotransfected with 1.8 μg pCAG-VSVG and 2.7 μg PAX2 into 293T cells using 15 μL lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 6 h, the medium was

changed to fresh DMEM with 10% FBS. After 72 h, the lentivirus-containing supernatant were collected, filtered, and then employed for analysis.

Real-time quantitative PCR. Total RNA was isolated from cells and tissues using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Complementary DNA generated using a PrimeScript® RT reagent kit (TaKaRa, Dalian, China) was analyzed by quantitative PCR using SYBR® Premix Ex TaqTM. Real-time PCR was performed using a 7300 Real-Time PCR system (Applied Biosystems, CA, USA). GAPDH was detected as the housekeeping gene. Primers for qPCR were as follows:

GAPDH forward, 5'-ATCACTGCCACCCAGAAGAC-3'

and reverse, 5'-ATGAGGTCCACCACCCTGTT-3'

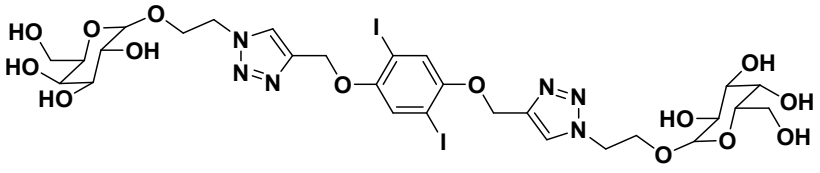
ASGPR1 forward, 5'-CTGGACAATGAGGAGAGTGAC-3'

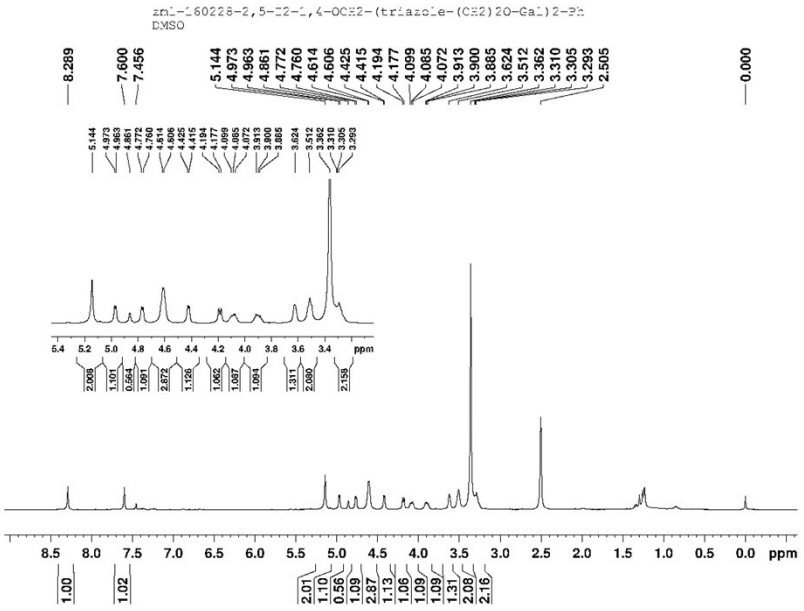
and reverse, 5'-TTGAAGCCCGTCTCGTAGTC-3'

Gal-contents determination. A freshly-prepared anthrone solution was prepared by adding 2 grams of anthrone per milliliter of concentrated H_2SO_4. Take 0, 0.2, 0.4 mL of 100 μg/mL standard D-galactose solution into a tube, replenish water to 2 ml, and then add 5 ml of anthrone solution in each tube and mix them. The UV-vis spectra of the resulting solutions were recorded on a Varian Cary 500 spectrophotometer. The absorbance of the solution at 620 nm was measured and the data were plotted against the concentration of D-galactose. The result was used as the calibration curve for the calculation of the D-galactose density on polymers. The D-galactose density experiments for the three polymers were carried out by polymer (1

mM) in Milli-Q water, and the solutions were treated with anthrone/H2SO4 following the same protocol described above. The density of three polymers' D-galactose was then determined using the calibration curve.

Results





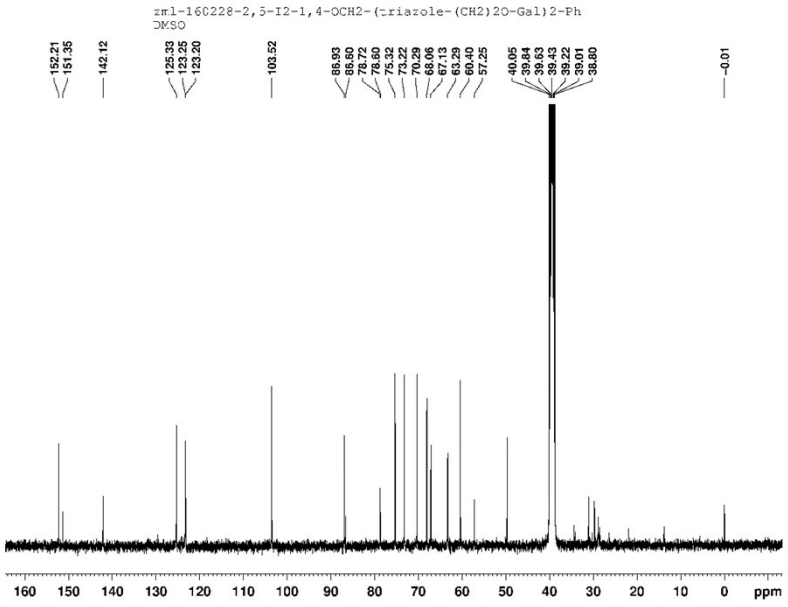


Figure S1. NMR spectra of compound 3.

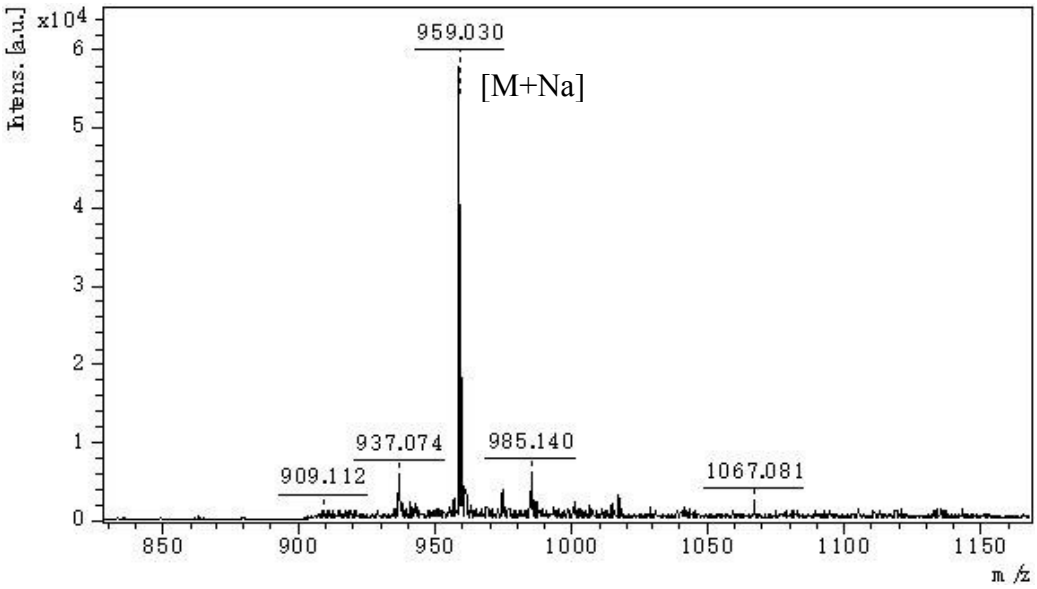


Figure S2. Mass spectra of compound **3**.

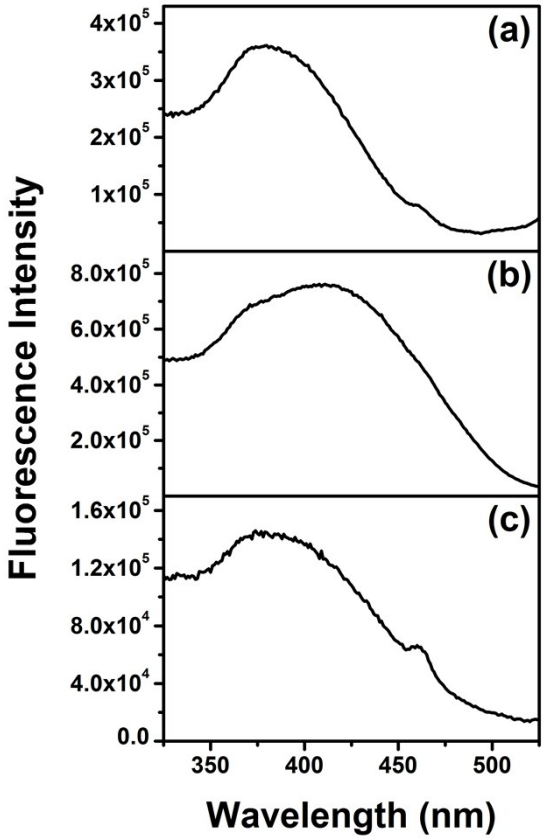


Figure S3. Fluorescence excitation spectra of (a) **PPE1-Gal**, (b) **PPE2-Gal**, and (c) **PPE3-Gal** in water. The excitation wavelength of **PPE1-Gal**, **PPE2-Gal** and **PPE3-Gal** were all from 325 to 525 nm. The concentrations of **PPE1-Gal**, **PPE2-Gal** and

PPE3-Gal were 11.8, 12.2, and 12.3 μM, respectively. Emission was set at 550 nm.

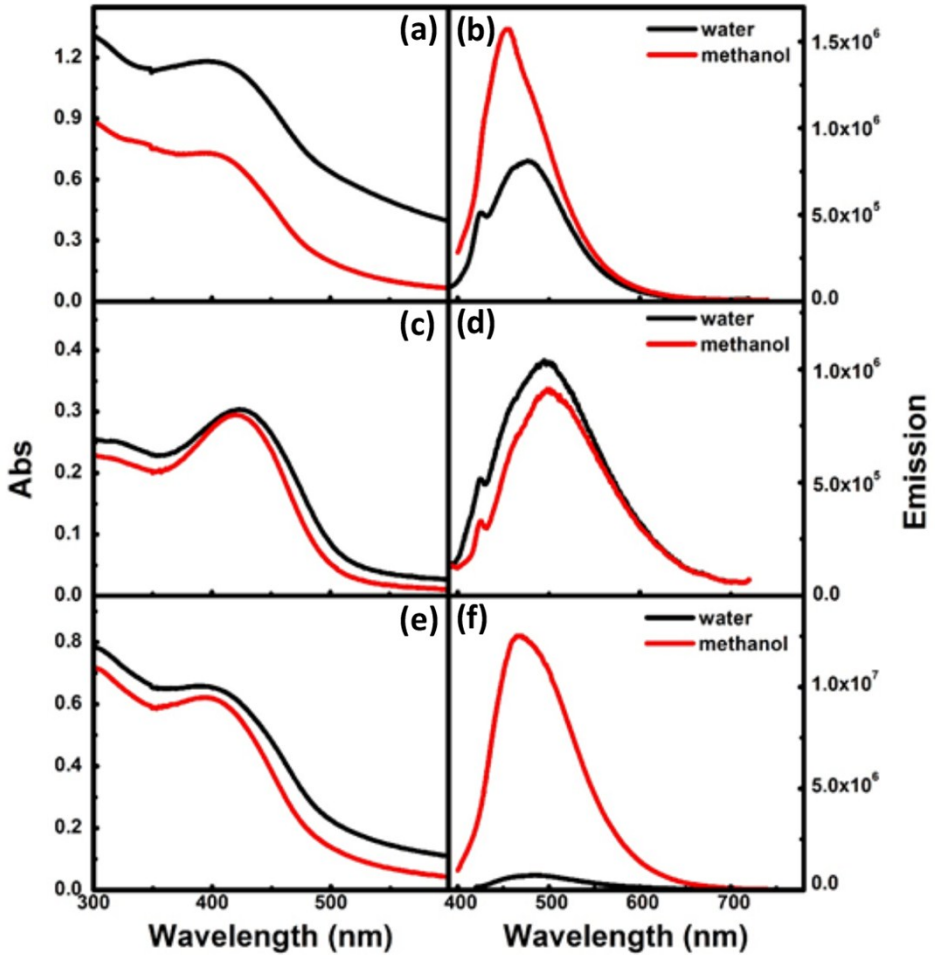


图 1

Figure S4. UV-Vis absorption (a, c and, e) and fluorescence emission spectra (b, d, and f) of **PPE1-Gal**, **PPE2-Gal**, and **PPE3-Gal**, respectively. **PPE1-Gal**, **PPE2-Gal**, and **PPE3-Gal** were excited at 370, 370, and 380 nm, and their concentrations were 1.18, 6.07, and 39.0 μM, respectively.

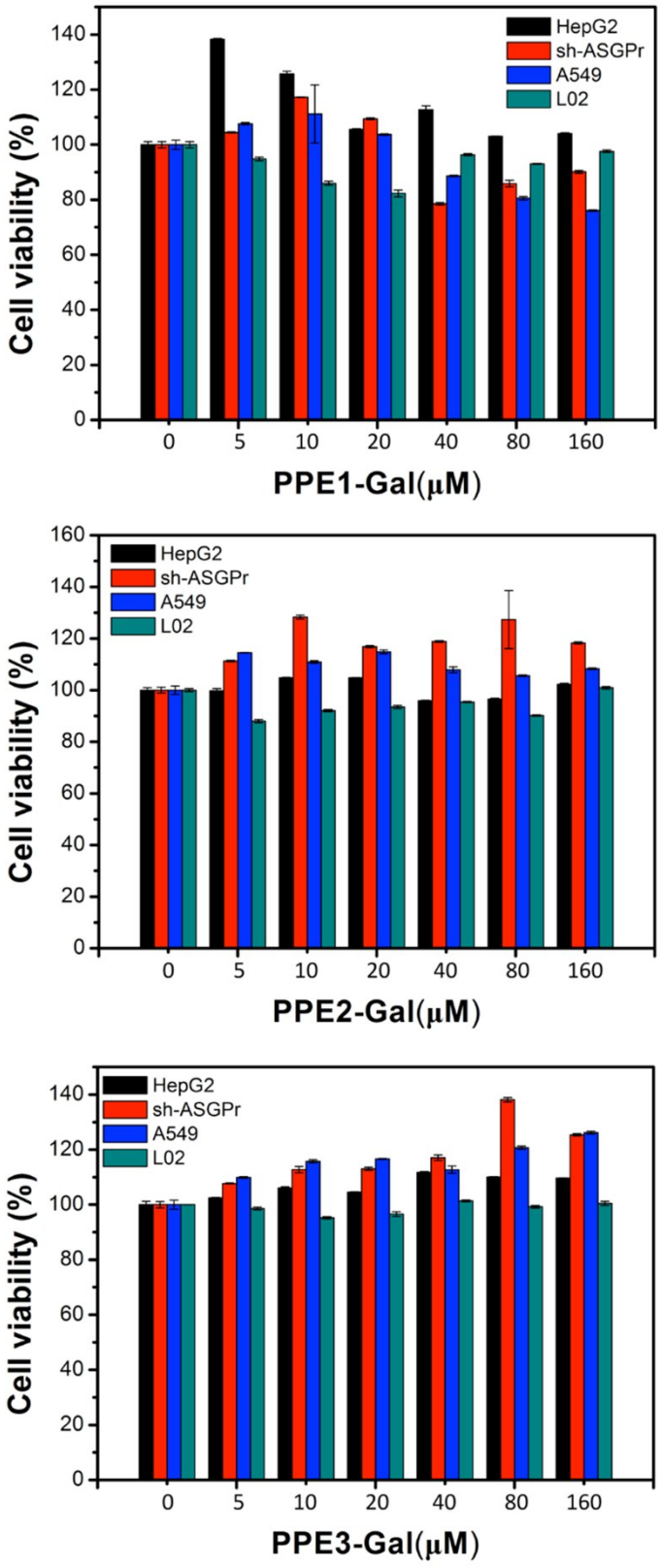


Figure S5. Cell viability of HeLa cells treated with **PPE1-Gal**, **PPE2-Gal**, and **PPE3-Gal** at the concentration of 100 μM for 24h.

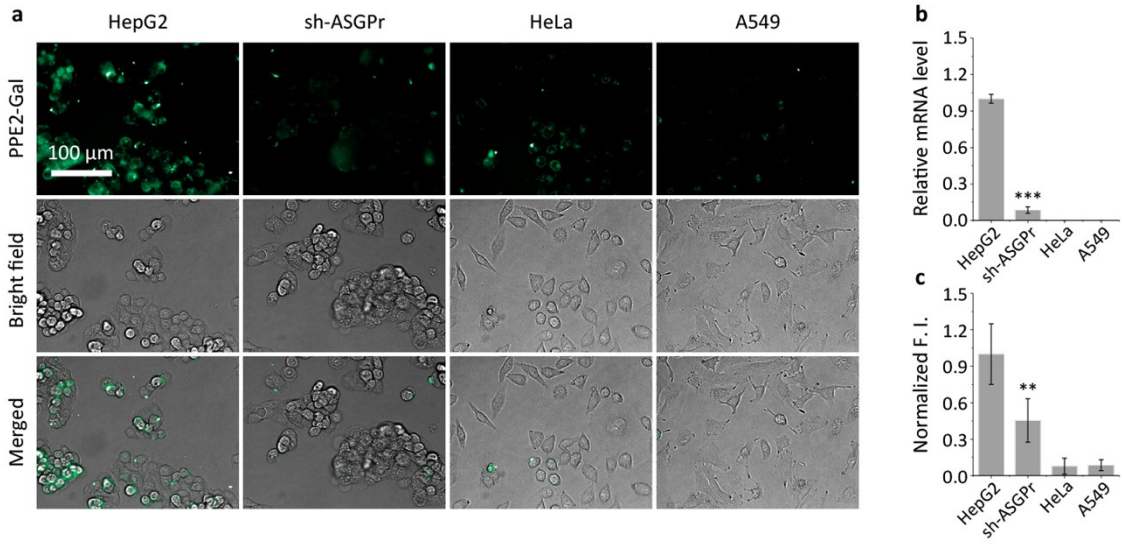
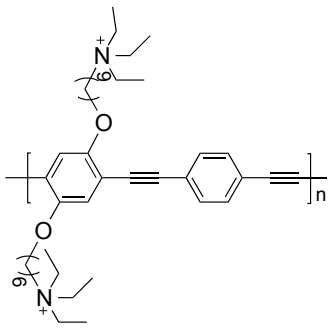


Figure S6. (a) Fluorescence imaging of HepG2 (human hepatoma), sh-ASGPr (HepG2 cells with a reduced ASGPr expression level), HeLa (human cervical) and A549 (human lung) cells treated with **PPE2-Gal** (5 μM). (b) Relative mRNA expression level of the cells determined by real-time quantitative polymerase chain reaction (***P<0.001 with respect to HepG2). (c) Quantification of HepG2 (human hepatoma), sh-ASGPr (HepG2 cells with a reduced ASGPr expression level), HeLa (human cervical) and A549 (human lung) cells treated with **PPE2-Gal** (5 μM) (**P<0.05 with respect to HepG2).



PPE-N2

Fig. S7. Chemical structure of **PPE-N2**.[4]

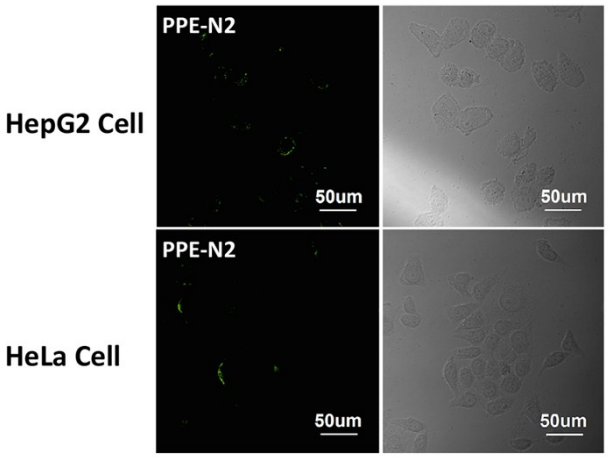
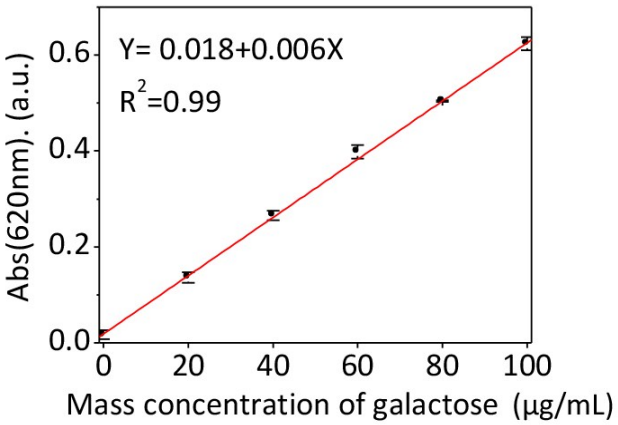


Figure S8. Confocal imaging of **PPE-N2** (5 μM) in HepG2 cells and HeLa cells. Scale bar: 50 μm



	Abs_{620} (a.u.)	**μg/mL**
PPE1-Gal	0.1225	17.42
PPE2-Gal	0.1226	17.43
PPE3-Gal	0.1228	17.47

Figure S9. Calibration curve obtained by treating various concentrations of D-galactose with anthrone/sulfuric acid and measuring the absorption at 620 nm. The density of three polymers' D-galactose was then determined using the calibration curve.

References

1. K.-B. Li, N. Li, Y. Zang, G.-R. Chen, J. Li, T. D. James, X.-P. He and H. Tian, *Chem. Sci.*, 2016, **7**, 6325.
2. B. C. Englert, M. D. Smith, K. I. Hardcastle and U. H. F. Bunz, *Macromolecules*, 2004, **37**, 8212.
3. Z. Chen, P. Wu, R. Cong, N. Xu, Y. Tan, C. Tan and Y. Jiang, *ACS Appl. Mater. Interfaces*, 2016, **8**, 3567.
4. J. Wu, C. Tan, Z. Chen, Y. Z. Chen, Y. Tan and Y. Jiang, *Analyst*, 2016, **141**, 3242.