Supporting information

of

The glyco-regioisomerism effect on lectin-binding and cell-uptake pathway of glycopolymer-containing nanoparticles

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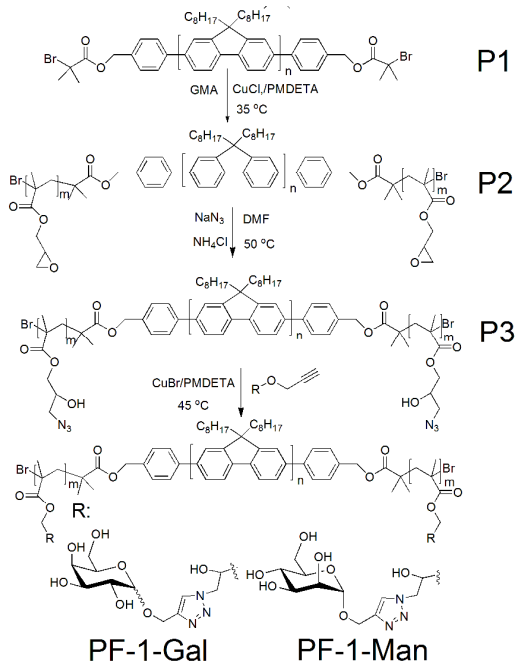
Materials. Mannose and galactose were purchased from Shanghai Bangcheng chemical Co., Ltd. 2,7-Dibromofluorene (97%), 1-bromohexane (98%) and 4-bromobenzyl alcohol (99%) were purchased from Shanghai Darui fine chemical Co., Ltd. Methacryloyl anhydride (98%), 2-bromoisobutyryl bromide (BiBB) (98%), 1,5-cycooctadiene (COD) (99%+), bis(1,5-cyclooctadiene)nickel (0) ($Ni(COD)_2$), 1,1,4,7,7-Pentamethyldiethylene triamine (PMDETA) (97%), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) (97%) and anisole were purchased from J&K Scientific Ltd. CuCl (99.99+%), CuBr (99.99+%) and glycidyl methacrylate (GMA, 98%) were purchased from Sigma-Aldrich. Anisole was distilled from calcium hydride (CaH_2) and stored under argon. GMA was passed through a column of alumina to remove inhibitor and dried over CaH_2. It was then distilled under reduced pressure and stored under argon. THF and toluene were purified by distillation from sodium in the presence of benzophenone. Other organic solvents were used without any further purifications. HEPES buffer (20 mM, pH 7.4) was prepared by NaCl (50 mM), $CaCl_2$ (5 mM) and $MnCl_2$ (5 mM). All reactions were performed under nitrogen atmosphere. 1-(2'-propargyl)-α-D-mannose, 1-(2'-propargyl) D-galactose, 6-*O*-Methacryloyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose, and polyfluorene (PF) macroinitiator (**P1**) were synthesized according to the previous literatures[1]. ASGPR was purchased from Sino Biological Inc. in the form of recombinant human ASGPR1 with a His tag at the N-terminus. ASGPR1 is the only subunit in ASGPR, which binds galactoside. SYTO® 61 red fluorescent nucleic acid stain, CellLight® Early Endosomes-RFP BacMam 2.0, CellLight® Late Endosomes-RFP BacMam 2.0 were purchased from Life Technologies Corporation. Lyso-Tracker Red was purchased from Beyotime Institute of Biotechnology.

Characterization. ^{1}H NMR spectra were recorded with a JEOL ECA-400 spectrometer. Gel permeation chromatography (GPC) analysis was carried out with an Agilent HP1100 columns analysis system with THF as eluents at the flow rate of 1 mL/min and polystyrene as the calibration standard. Photo luminescent spectra were measured on an FLS 920 spectrofluorophotometer from Edinburgh Instruments. Dynamic light scattering (DLS) studies were conducted using ALV/5000E laser light scattering spectrometers at scattering angle of 90°. CONTIN analysis was used for the extraction of $\langle R_h \rangle$ data.

Transmission electron microscopy (TEM) experiments were performed on a Philips CM 120 electron microscope at an accelerating voltage of 80 kV. A drop of a nanoparticle solution (~1 μL) was placed on a carbon-coated copper grid. The grid was left to dry at room temperature for several hours and was further dried under vacuum overnight. Confocal laser-scanning microscopy (CLSM) images were recorded on a Zeiss LSM 710 (Carl Zeiss, Germany) microscope with imaging software (ZEN 2009 Light Edition).



Scheme S1. Synthetic route of **PF-1-Gal** and **PF-1-Man** glycopolymers.

Synthesis of PF-Macroinitiator (P1)

P1 was prepared according to the literature[1d]. ^{1}H NMR ($CDCl_3$, 400MHz, δ): 7.82-7.67 (br, 36H, fluorene aromatic protons), 7.59-7.48 (2d, 8H, phenyl groups), 5.28 (s, 4H, O-CH_2-), 2.11 (br, 24H, -CH_2C$_7$H$_{15}$), 1.98 (s, 12H, -C(CH_3)$_2$Br), 1.25 -0.81 (m, 180H, -CH$_2$C$_7$$H_{15}$). Based on the ^{1}H NMR result, Mn of PF is calculated as 2400 g/mol with DP 6 (degree of polymerization). M_n and polydispersity (PDI) of **P1** are 3600 g/mol and 1.4, respectively, according to the results from GPC.

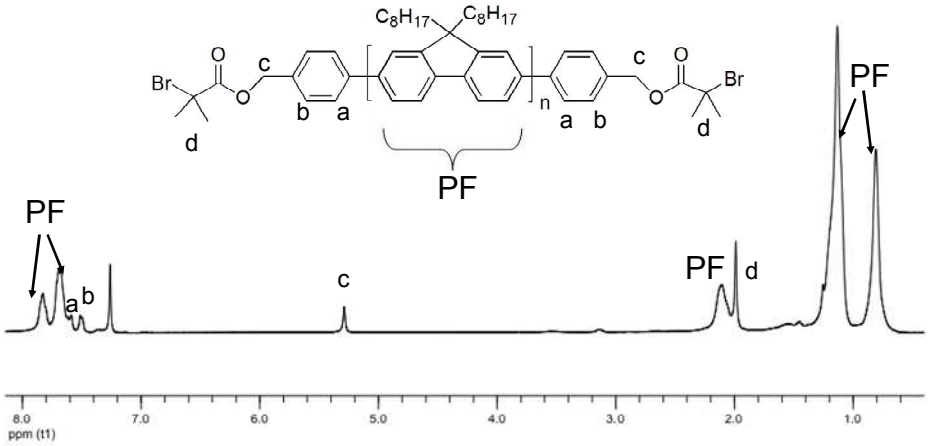


Figure S1. ^{1}H NMR of **P1** in $CDCl_3$.

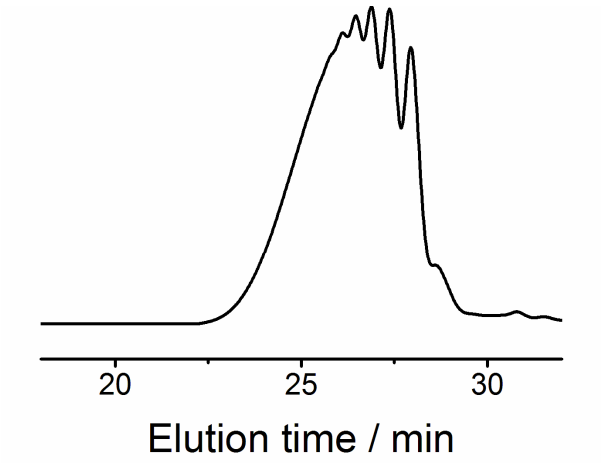


Figure S2. GPC trace of the macro-initiator **P1**.

Synthesis of triblock copolymer PGMA-PF-PGMA (P2)

P2 was synthesized by ATRP according to the previous procedure[2]. Typically, a Schlenk tube was charged with 120 mg (0.04 mmol) **P1**, 6 mg (0.04 mmol) CuBr, 3 mL (21 mmol) degassed GMA monomer and 6 mL anisole before the tube was sealed with a rubber septum. After three freeze-pump-thaw cycles, the glass reactor was immersed in an oil bath at 35 °C. After 5 min, 8 μL (0.04 mmol) PMDETA was quickly injected into the tube to carry out polymerization. After 3 h, the polymerization was quenched by liquid nitrogen. The sample was first passed through a column of neutral alumina to remove the catalyst. Then the eluent was concentrated and precipitated in diethyl ether (1.20 g, 38%).

^{1}H NMR ($CDCl_3$, 400 MHz, δ): 8.1-7.64 (br, fluorene aromatic protons), 7.6-7.4 (br, phenyl groups), 5.6 (s, phenyl- CH_2-O-), 4.30, 3.83 (d, -OCO-CH_2-), 3.23 (s, -CH-CH_2-O-), 2.84, 2.64 (d, -CH-CH_2-O-), 2.2-1.8 (br, -C$H_2C_7H_{15}$ and -CH_2-C(CH_3)), 1.7-0.9 (br, -$CH_2C_7H_{15}$ and -CH_2C(CH_3)). Based on ^{1}H NMR result, M_n of PGMA is calculated as 1.35×10^4 g/mol. M_n and PDI of **P2** are 1.92×10^4 g/mol and 1.4, respectively, according to the results from GPC.

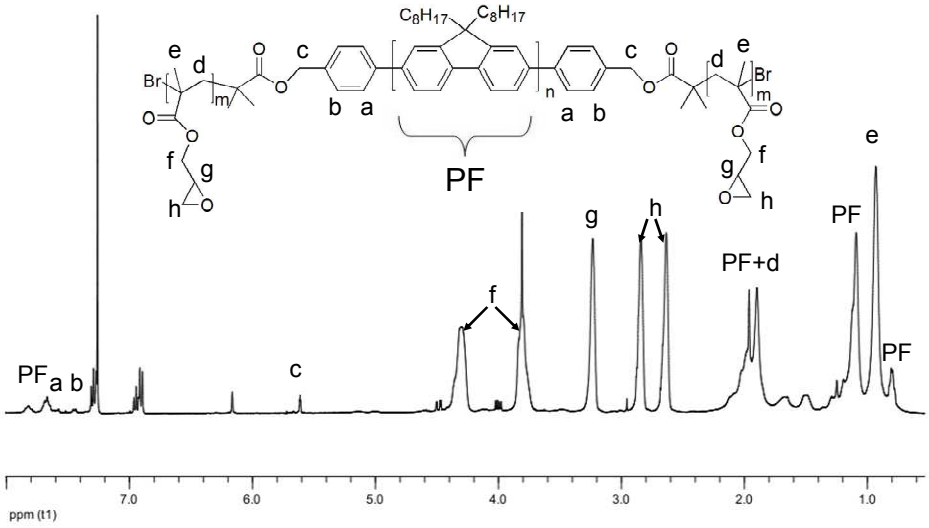


Figure S3. ^{1}H NMR of **P2** in $CDCl_3$.

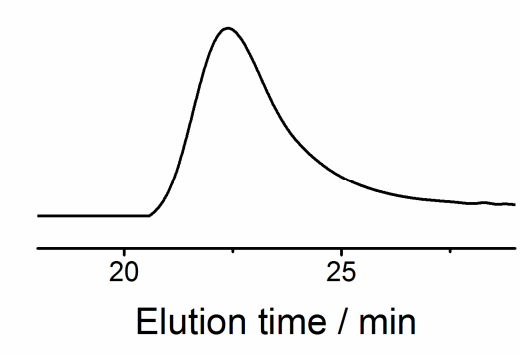


Figure S4. GPC trace of **P2**.

Synthesis of (PGMA-(-N_3)-PF-(PGMA-(-N_3)) triblock copolymer (P3)

Ring-opening of PGMA segment in **P2** with sodium azide and ammonium chloride was performed according to the previous procedure[2]. Typically, 0.77 g PGMA (5.5 mmol of

epoxide moieties) was dissolved in 100 mL *N*,*N*-dimethylformamide (DMF), 1.14 g (17.5 mmol) sodium azide and 0.95 g (17.5 mmol) ammonium chloride were added to the solution, and the mixture was stirred at 50 °C for 20 h. After removal of most DMF by rotavap, the residue was precipitated in water, filtrated, washed with water. The final product was obtained after drying under vacuum (0.8 g, yield: 80%). ^{1}H NMR (DMF-d_7, 400 MHz, δ): 7.9-7.2 (br, fluorene aromatic protons and phenyl groups), 5.7 (s, -OCO-CH_2-CH(*OH*)-CH_2-N_3), 4.09 (d, -OCO-CH_2-C*H*(OH)-CH_2-N_3), 3.46 (s, -OCO-CH_2-CH(OH)-CH_2-N_3), 2.3-1.7 (br, -C$H_2C_7H_{15}$ and -CH_2-C(CH_3)), 1.7 -0.6 (br, -$CH_2C_7H_{15}$ and -CH_2C(CH_3)).

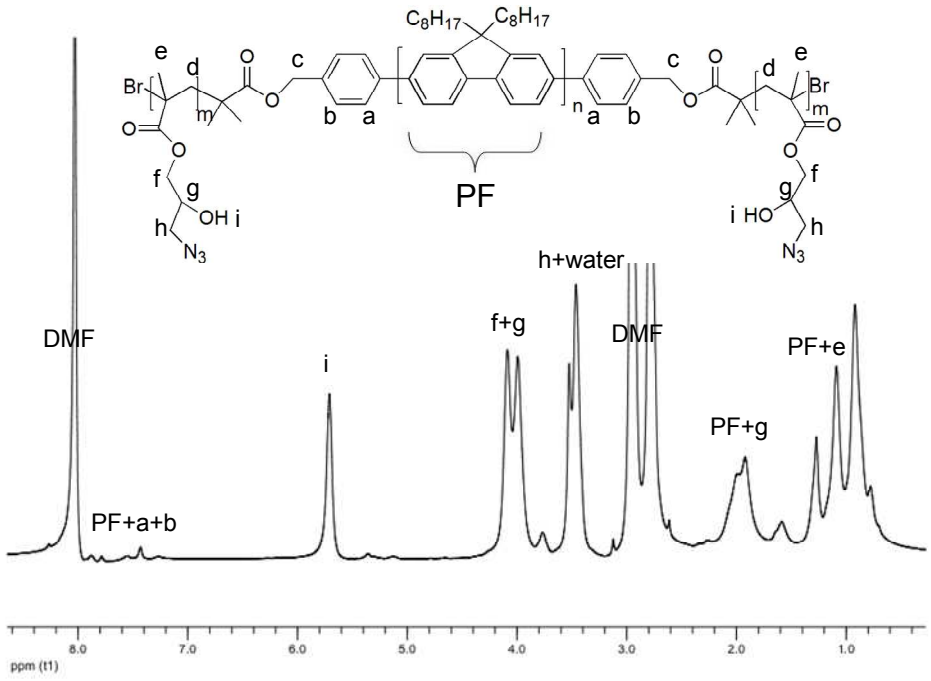
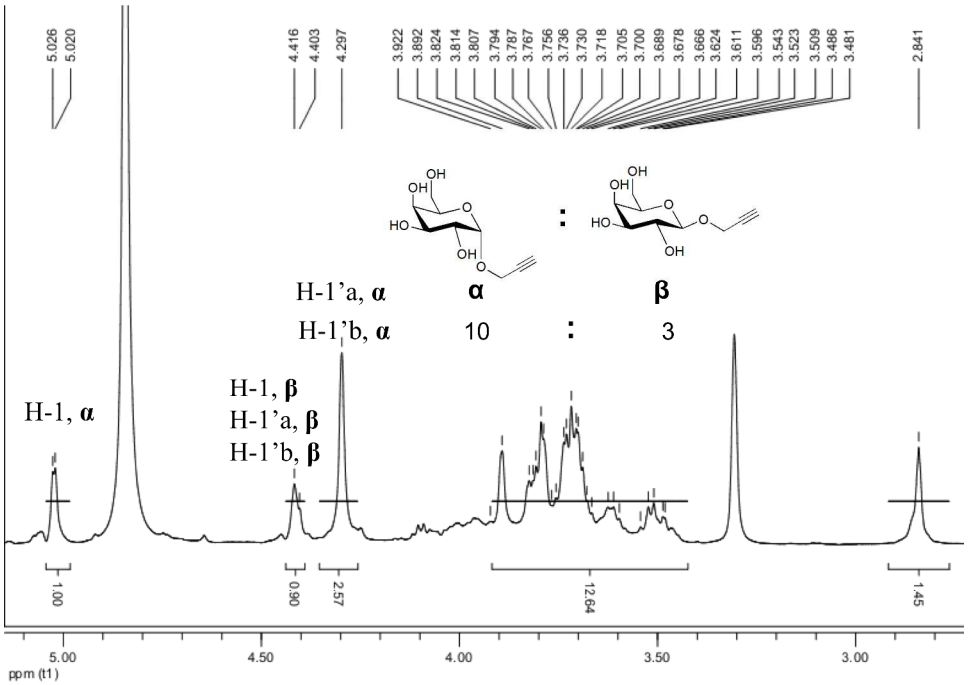
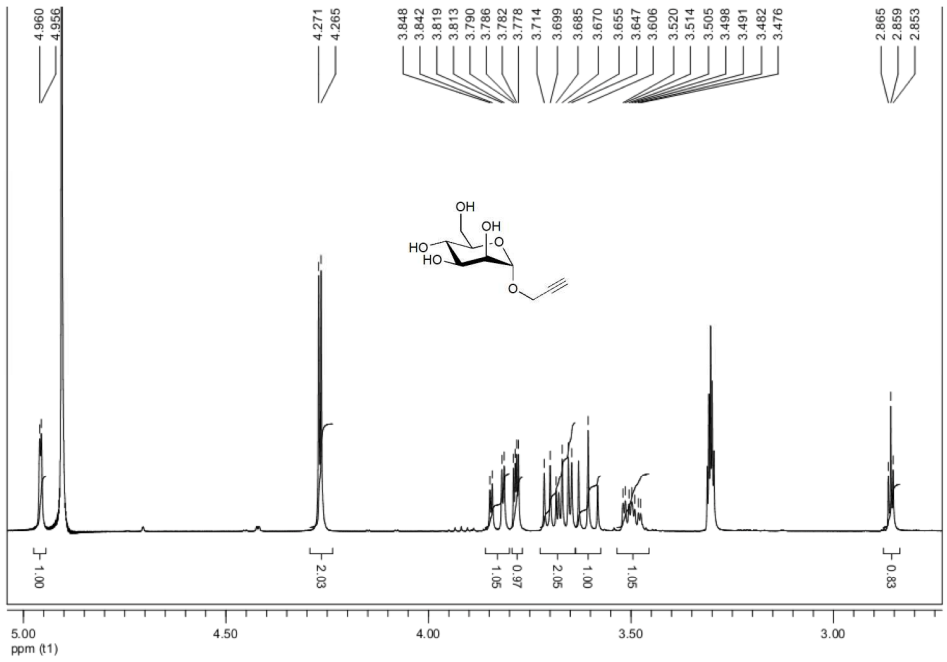


Figure S5. ^{1}H NMR of **P3** in DMF-d_7.



(a)



(b)

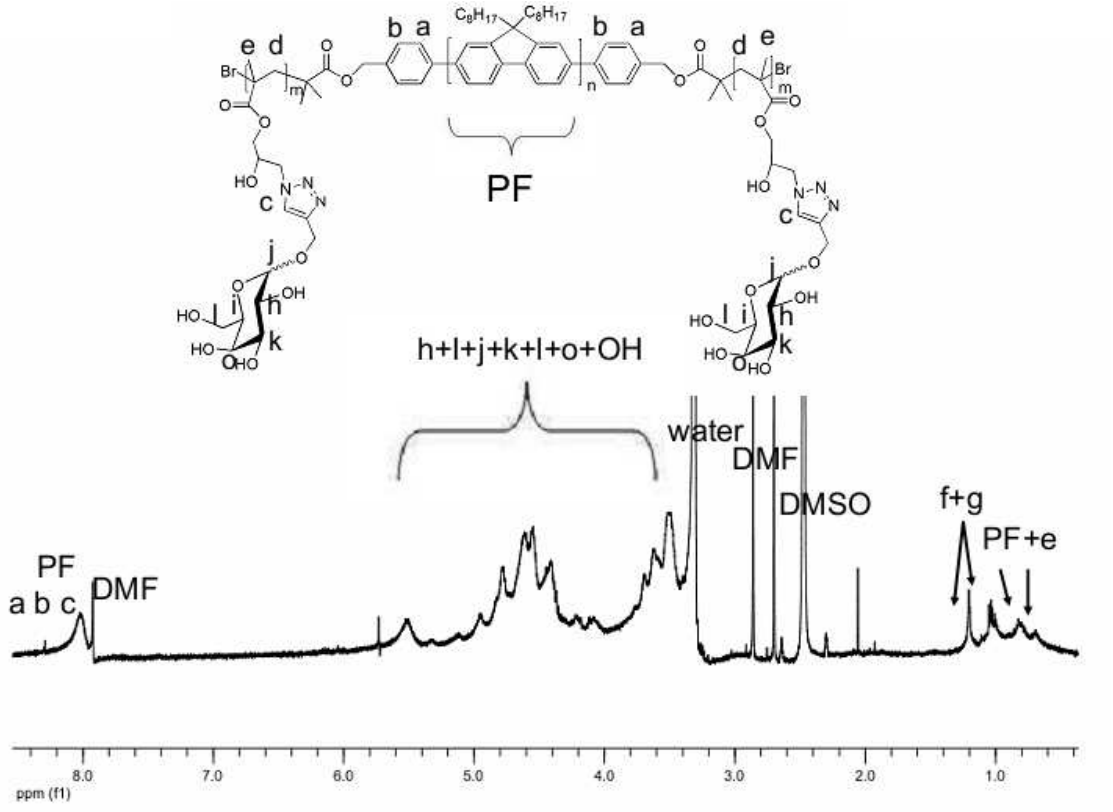
Figure S6. ^{1}H NMR of (a) 1-(2’-propargyl) D-galactopyranoside and (b) 1-(2’-propargyl)-α-D-mannopyranoside in $CDCl_3$.

Synthesis of triblock copolymer PF-1-Gal

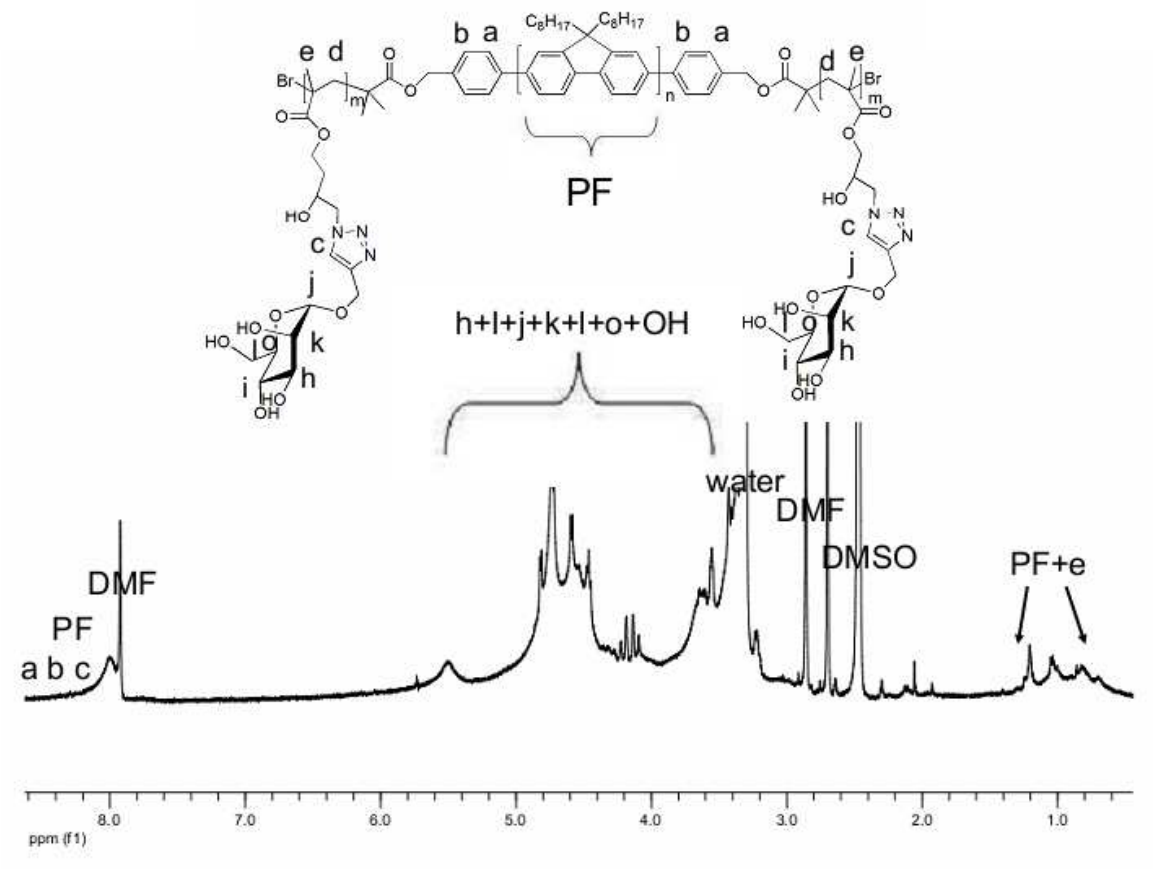
A Schlenk tube was charged with 200 mg 1-(2'-propargyl)-D-galactose, 100 mg (5.5 mmol of N_3-) triblock copolymer **P3**, 18 mg (0.13 mmol) CuBr and 15 mL DMF, before the tube was sealed with a rubber septum. After three freeze-pump-thaw cycles, the glass reactor was immersed in an oil bath at 45 ^{o}C. After 10 min, 28 μL (0.13 mmol) PMDETA was quickly injected into the tube to carry out polymerization. After 24 h, the polymerization was quenched by liquid nitrogen, and the mixture was dialyzed against water in a dialysis bag (MWCO 7000 Da) for 2 d. The final product was obtained after freeze-drying (0.12 g, yield: 63%). ^{1}H NMR spectrum of **PF-1-Gal** is in Figure S7a.

Synthesis of PF-1-Man triblock copolymer

The synthesis of **PF-1-Man** triblock copolymer is similar to **PF-1-Gal**. ^{1}H NMR spectrum of **PF-1-Man** is presented in Figure S7b.

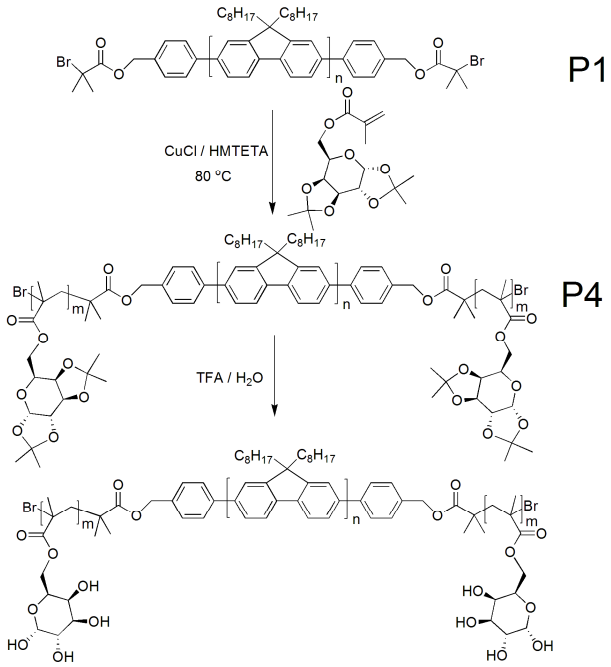


(a)



(b)

Figure S7. ^{1}H NMR of (a) **PF-1-Gal** and (b) **PF-1-Man** in DMSO-d_6.



Scheme S2. Synthetic route of triblock copolymer **PF-6-Gal**.

6-O-Methacryloyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (MalpGa)

To 350 mL dry acetone, concentrated sulfuric acid (10 mL) was added at 0 ^{o}C, followed by 10.0 g D-galctose. The reaction mixture was then stirred at room temperature for 16 h until the glucose was solubilized. The solvent was then neutralized with saturated aqueous NaOH solution, and the aqueous layer was extracted with chloroform, washed with water (150 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to give 1,2:3,4-di-O-isopropylidene-D-galactopyranose as yellow oil. To a solution of 1,2:3,4-di-O-isopropylidene-D-galactopyranose (30.6 g, 115.2 mmol) in 150 mL of anhydrous pyridine, 32 mL of methacrylic anhydride (202 mmol) was added dropwise at room temperature. The mixture was heated at 65 ^{o}C for 4.5 h and another 1 h after the addition of water (100 mL). The mixture was cooled to room temperature overnight and then extracted with petroleum ether for three times. The combined extracts

were washed twice with 150 mL aqueous solution of sodium hydroxide (5%) and three times with 100 mL of water and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the oil residue was purified by column chromatography (EtOAc : petroleum ether (1:8) as eluent) to afford the final product as a crystalline solid (34 g, 90%).[1]H NMR ($CDCl_3$, 400MHz, δ): 6.12 (s, 1H), 5.56 (m, 1H), 5.52 (d, 1H), 4.62 (dd, 1H), 4.36-4.24 (m, 4H), 4.06(m, 1H), 1.93(s, 3H), 1.49(s, 3H), 1.44 (s, 3H), 1.33 (s, 3H),1.32 (s, 3H).

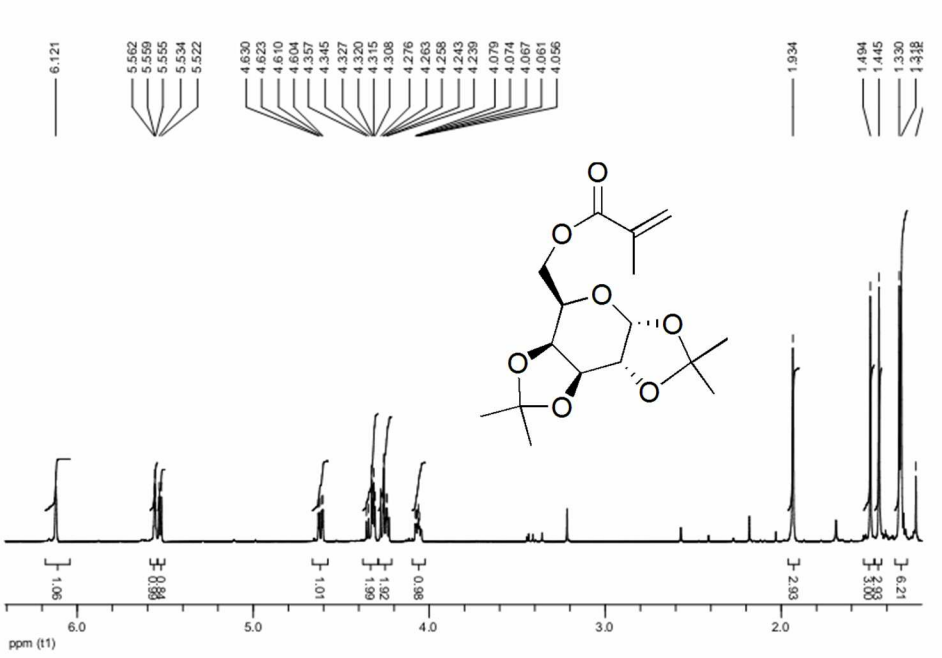


Figure S8. [1]H NMR of 6-*O*-Methacryloyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose in $CDCl_3$.

Synthesis of triblock copolymer P4

P4 was synthesized by ATRP according to the previous procedure[1c]. Typically, a Schlenk tube was charged with 80 mg (0.027 mmol) **P1**, 4 mg (0.04 mmol) CuCl, 500 mg (1.54 mmol) monomer 6-*O*-Methacryloyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose and 6 mL anisole before the tube was sealed with a rubber septum. After three freeze-pump-thaw cycles, the reactor was immersed in an oil bath at 80 ^oC. After 5 min, 12 mL

(0.04 mmol) HMTETA was quickly injected into the tube to carry out polymerization. After 24 h, the polymerization was stopped by transferring the flask into liquid nitrogen. The samples were passed through a column of neutral alumina to remove the catalyst. The eluent was concentrated and precipitated in n-hexane. The precipitate was collected and dried under vacuum (0.4 g, yield: 69%). ^{1}H NMR spectrum of **P4** is in Figure S9, which gave out Mn as 1.66×10^4 g/mol and DP as 42. The Mn and PDI of **P4** are 1.89×10^4 and 1.22 respectively, according the results from GPC. (Figure S10).

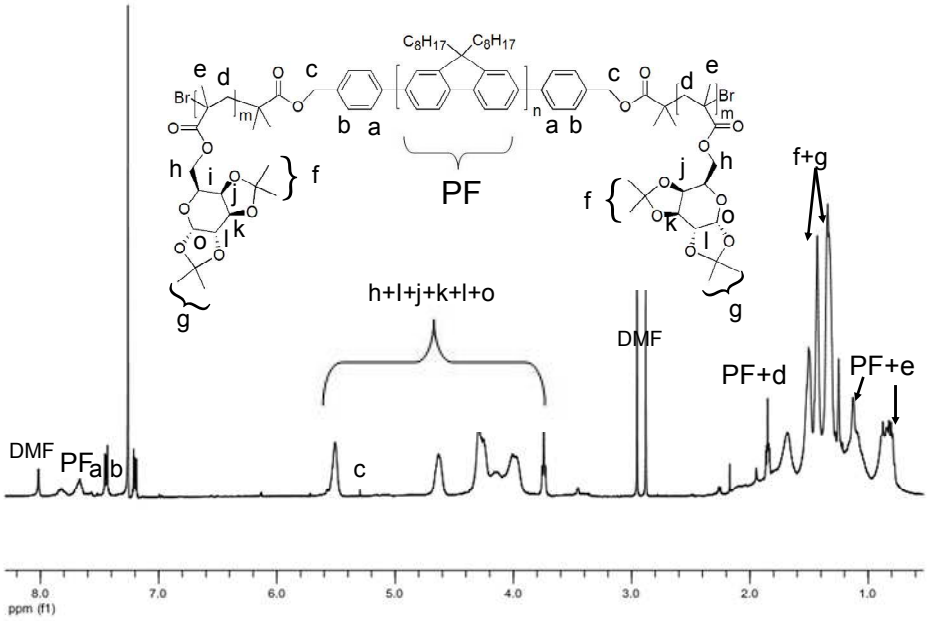


Figure S9. ^{1}H NMR of **P4** in $CDCl_3$.

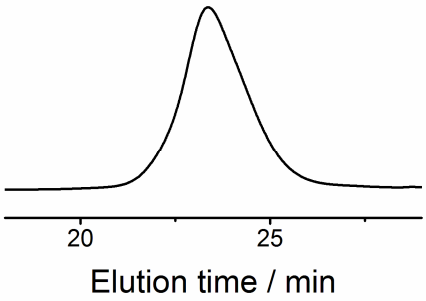


Figure S10. GPC trace of **P4**.

Synthesis of PF-6-Gal triblock copolymer

To deprotect the sugar moieties, 200 mg **P4** was dissolved in 6 mL mixture of trifluoroacetic acid and water (*v*:*v* = 5:1) and stirred for 1 h. After the reaction, the solution was neutralized by addition of sodium bisulfate and subsequently dialyzed against water in a dialysis bag (MWCO 7000 Da) for 2 d before freeze-drying. A white powder was obtained (0.18 g, yield: 90%). ^{1}H NMR spectrum of **PF-6-Gal** is shown in Figure S11.

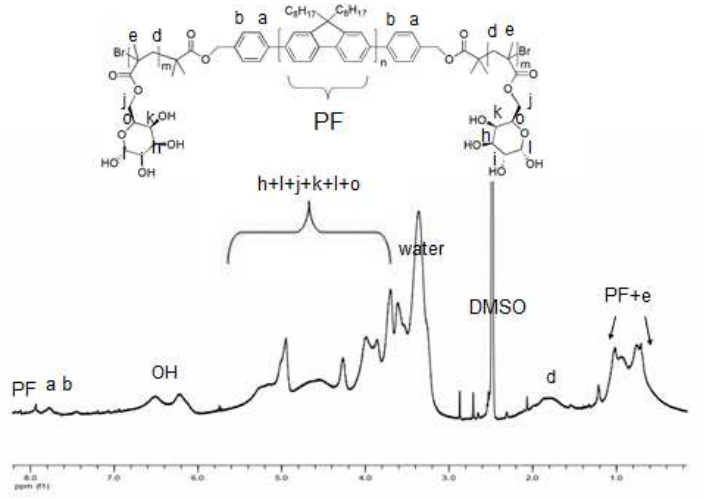


Figure S11. ^{1}H NMR of **PF-6-Gal** in DMSO-d_6.

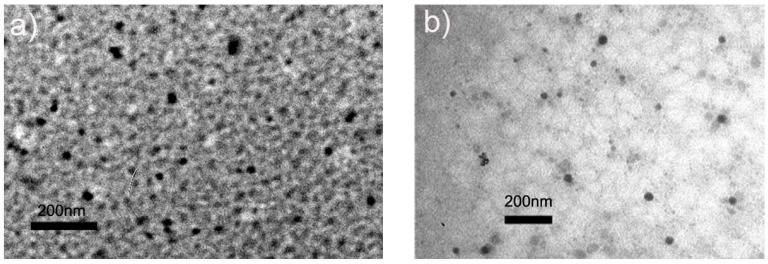


Figure S12. TEM images of (a) **NP-6-Gal** and (b) **NP-1-Man** (scale bar: 200 nm).

Typical procedure for the self-assembly of nanoparticles. Typically, the **PF-1-Gal** triblock copolymer (10 mg) was dissolved in DMF (10 mL). Then the solution was dialyzed in a dialysis bag (MWCO 3500 Da) against HEPES buffer to remove DMF.

After 48 h, a bluish opalescence solution was obtained indicating formation of assembled nanoparticles. At last, HEPES buffer was added into the nanoparticle solution to adjust the volume to 20 mL (concentration 0.5 mg/mL).

Binding measurement by Quartz Crystal Microbalance with Dissipation (QCM-D).
QCM-D measures the changes of resonance frequency (Δf) of a sensor crystal upon interaction of matter with its surface. A decrease in Δf indicates a mass increase. In our experiments, QCM-D measurements were performed with a Q-Sense E4 system (Biolin Scientific AB). His-tag capturing QCM-D sensors with a fundamental resonance frequency $f_1 \approx 4.95$ MHz (QSX340; Biolin Scientific AB, Va□stra Fro□lunda, Sweden) were stored at −80 °C prior to use. The system was operated in flow mode with a flow rate of 20 μL/min using a tubing pump (ISMATEC, IDEX Corporation, Switzerland). The working temperature was 23 °C. Δf was measured as the third frequency. The ASGPR protein was dissolved in HEPES buffer (25 μg/mL). The concentration of **NP-1-Gal** and **NP-6-Gal** are 0.5 mg/mL.

Cell Culture
The hepatocellular carcinoma cell line (Hep G2) cells and human cervical carcinoma (HeLa) cells were cultured in T-75 flasks with Dulbecco's Modified Eagle's Medium (DMEM, Gibco), 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 0.25 μg/mL Fungizone (BioSOURCE). Cells were incubated at 37 °C in ambient air with 5% CO_2 atmosphere and subculture every 3 days.

In vitro Cytotoxicity
The *in vitro* cytotoxicity of polymeric micelles was evaluated by MTT assay with Hep G2 cells. The cells were seeded in a 96-well plates containing 100 μL DMEM supplemented with 10% FBS at an initial density of 1×10^4 cells per well. The cells were incubated at 37 ^oC under an atmosphere of 95% relative humidity and 5% CO_2 for 24 h. Then the cell culture media was replaced with fresh medium containing **NP-1-Gal** and **NP-6-Gal** at different concentrations. After 24 h, 20 μL of 5 mg mL^{-1} MTT solution in

PBS was added to each well and the plates were further incubated for another 4 h. Then the supernatant was removed by centrifugation for 10 min, and 200 μL DMSO was added to each well. The optical absorbance was measured in a Multiskan MK3 microplate reader (Thermo Scientific) at 492 nm. Cells without nanoparticle treatment were used as control.

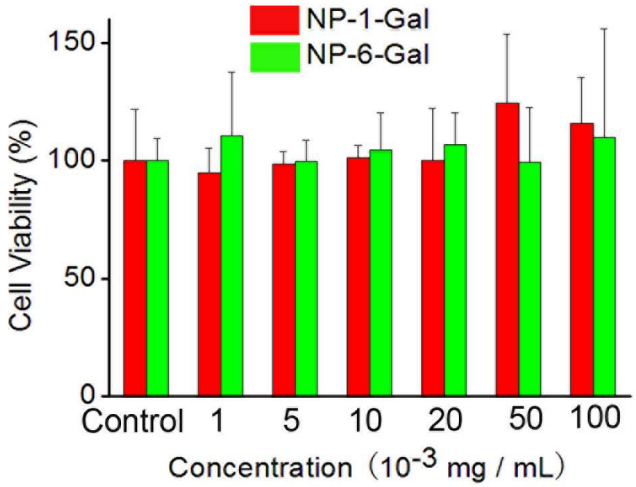


Figure S13. Cell viability of Hep G2 cells after incubation with **NP-1-Gal** and **NP-6-Gal** at different concentrations for 24 h. Standard derivation is obtained from three repeated experiments.

Cell Uptake experiment of NP-1-Gal, **NP-6-Gal** and **NP-1-Man**

Hep G2 and HeLa cells were seeded on a coverslip in a 24-well plate at a density of *ca.* 10^4 cells per well and cultured in DMEM supplemented with 10% FBS separately. After 24 h, the cells were incubated with 50 μg (100 μL × 0.5 mg/mL) of **NP-1-Gal**, **NP-6-Gal** and **NP-1-Man** at 37 °C for 4 h. For competition experiments, Hep G2 cells were pre-incubated with 20 mM lactose for 1 h at 37 °C. To stain the cell nucleus, cells were stained with 2.5 μL SYTO® 61 red fluorescent nucleic acid for 15 min. The cells were washed with PBS buffer (2 mL) three times, to remove unbound particles and excess tracker. Then the cover slip seeded with cells was mounted upside down on a glass slide. Confocal microscopic images were acquired using a Carl Zeiss LSM 710 microscope with a 63× water objective with excitation wavelength at 405 nm for nanoparticles and 561 nm for SYTO® 61 red fluorescent nucleic acid. The experiment has been repeated for three times.

Co-localization of NP-1-Gal and NP-6-Gal with lysosomes

Hep G2 cells were seeded on a coverslip in a 24-well plate at a density of *ca.* 10^4 cells per well and cultured in DMEM supplemented with 10% FBS. After 24 h, the cells were incubated with 50 μg (100 μL × 0.5 mg/mL) of **NP-1-Gal** and **NP-6-Gal** at 37 °C for 4 h. For staining the lysosomes of the cells, cells were stained with Lyso-Tracker Red 2.5 μL for 15 min before observation. The cells were washed with PBS buffer (2 mL) three times, which removed free particles and free tracker. The cover slip seeded with cells was mounted upside down on a glass slide. Confocal microscopic images were acquired using a Carl Zeiss LSM 710 microscope with a 63× water objective with excitation wavelength at 405 nm for nanoparticles or 561 nm for Lyso-Tracker Red. The experiment has been repeated for three times.

Co-localization of NP-1-Gal and NP-6-Gal with early endosomes and late endosomes

Hep G2 cells were seeded on a coverslip in a 24-well plate at a density of *ca.* 10^4 cells per well and cultured in DMEM supplemented with 10% FBS. For staining the early endosomes or late endosomes of the cells, Hep G2 cells were pre-incubated with CellLight® Early Endosomes-RFP BacMam 2.0 or CellLight® Late Endosomes-RFP BacMam 2.0 80 μL for 16 h at 37 °C. Then the cells were incubated with 50 μg (100 μL × 0.5 mg/mL) **NP-1-Gal** or **NP-6-Gal** at 37 °C for 4 h. The cells were washed with PBS buffer (2 mL) for three times, which removed free particles. The cover slip seeded with cells was mounted upside down on a glass slide. Confocal microscopic images were acquired using a Carl Zeiss LSM 710 microscope with a 63× water objective with excitation wavelength at 405 nm for nanoparticles or 561 nm for CellLight® Early Endosomes-RFP BacMam 2.0 and CellLight® Late Endosomes-RFP BacMam 2.0. The experiment has been repeated for three times.

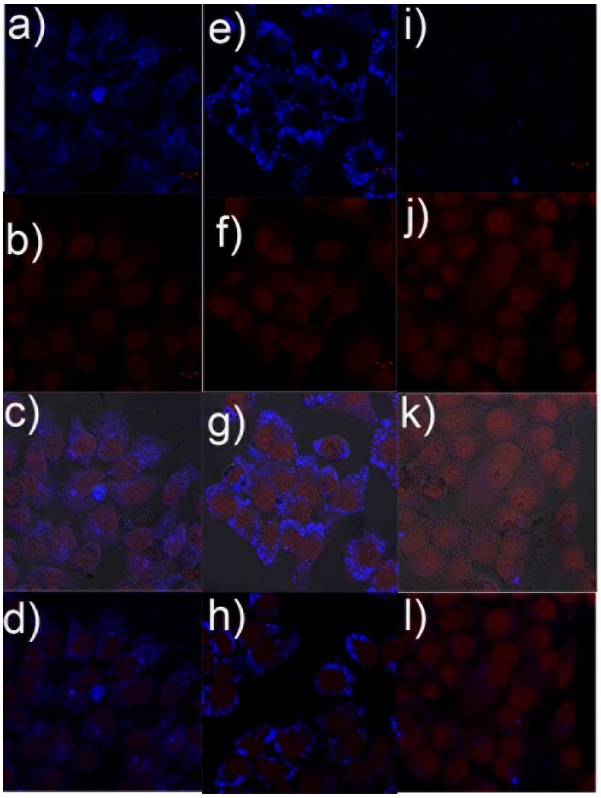


Figure S14. Confocal fluorescence microscopy images of internalization of nanoparticles by Hep G2 cells. The cells were treated with 50 μg of **NP-1-Gal** (a–d), **NP-6-Gal** (e–h) and **NP-1-Man** (i–l) at 37 °C for 4 h incubation. a, e, i) Fluorescence images with blue color from nanoparticles. b, f, j) Cell nuclei stained with SYTO® 61 red fluorescent nucleic acid. c, g, k) Merge of the above images in the same column. d, h, l) Merge of blue and red channel. Scale bar: 10 μm.

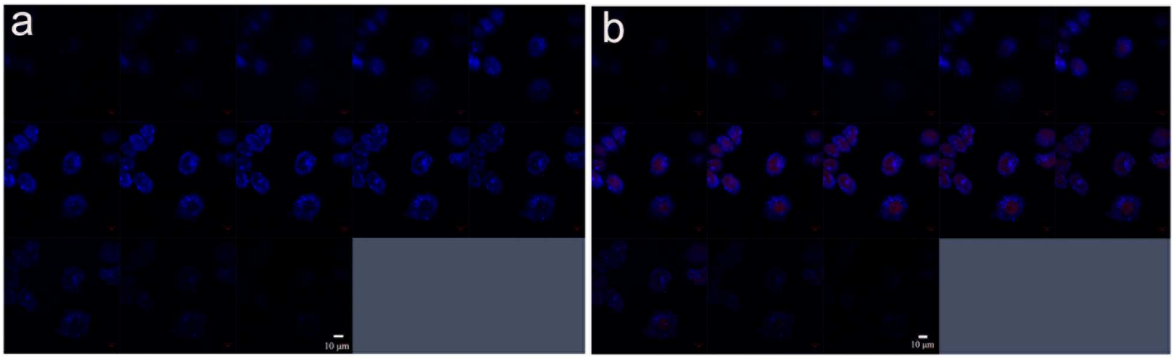


Figure S15. Confocal fluorescence microscopy images of internalization of **NP-1-Gal** by Hep G2 cells scanned via z-axis. The cells were treated with 50 μg of **NP-1-Gal** at 37 °C

for 4 h incubation. a) Fluorescence images with blue color from nanoparticles. b) Merge of blue and red channel. (blue: nanoparticle; red: cell nuclei stained with SYTO® 61 red). Scale bar: 10 μm.

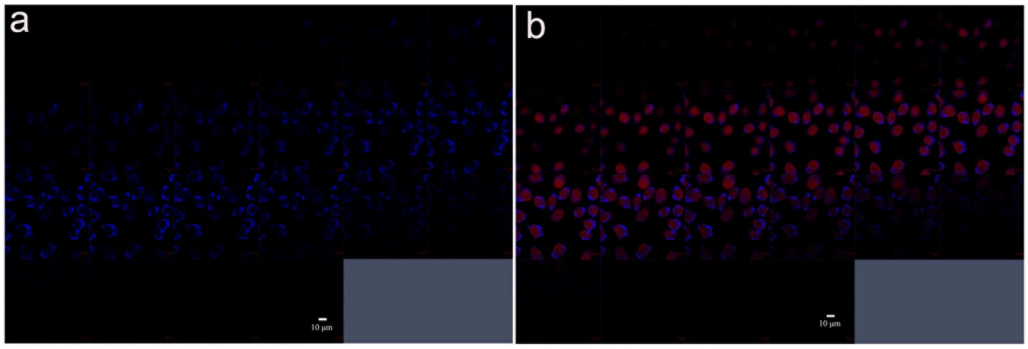


Figure S16. Confocal fluorescence microscopy images of internalization of **PF-6-Gal** by Hep G2 cells scanned via z-axis. The cells were treated with 50 μg of **NP-6-Gal** at 37 °C for 4 h incubation. a) Fluorescence images with blue color from nanoparticles. b) Merge of blue and red channel. (blue: nanoparticle; red: cell nuclei stained with SYTO® 61 red). Scale bar: 10 μm.

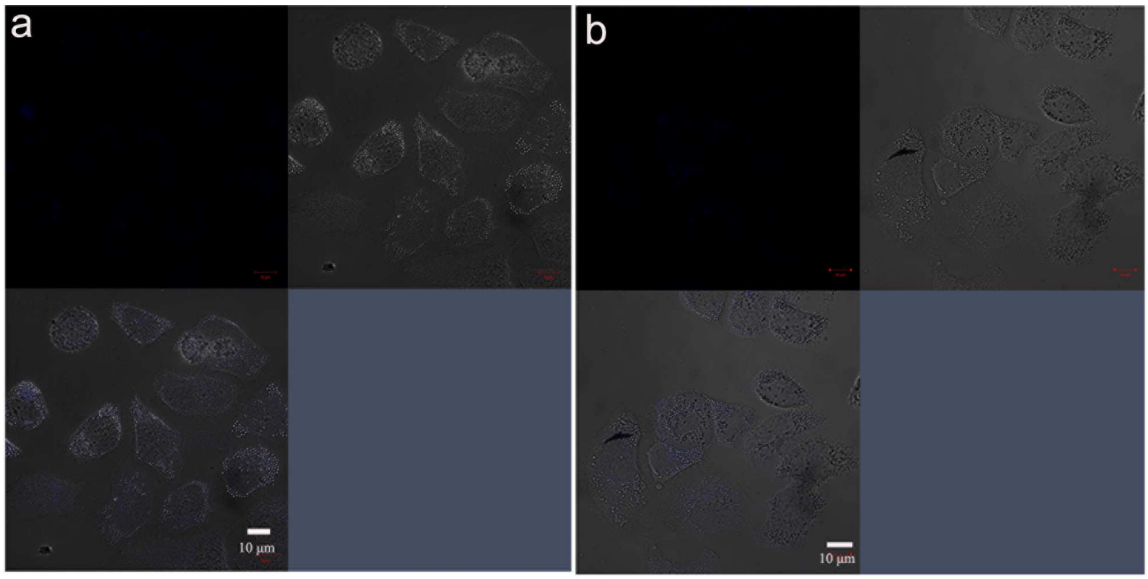


Figure S17. Confocal fluorescence microscopy images of internalization of nanoparticles by Hep G2 cells. The cells were treated with 50 μg of **NP-1-Gal** (a), **NP-6-Gal** (b) and at 4 °C for 4 h incubation. Scale bar: 10 μm.

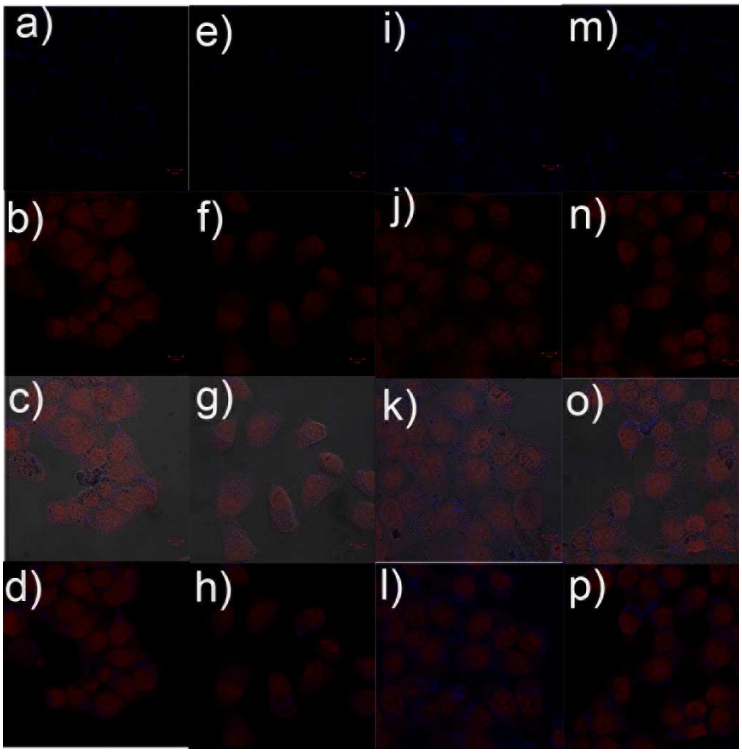


Figure S18. Confocal fluorescence microscopy images of internalization by Hela cells with 50 μg of **NP-1-Gal** (a–d), **NP-6-Gal** (e–h) and Hep G2 cells with pre-incubated 20 mM lactose for 1 h at 37 °C and then incubated with 50 μg of **NP-1-Gal** (i–l), **NP-6-Gal** (m–p) at 37 °C for 4 h incubation. a, e, i, m) Fluorescence images with blue color from nanoparticles. b, f, j, n) Cell nuclei stained with SYTO® 61 red fluorescent nucleic acid stain. c, g, k, o) merge of the above images in the same column. d, h, l, p) Merge of blue and red channel. Scale bar: 10 μm.

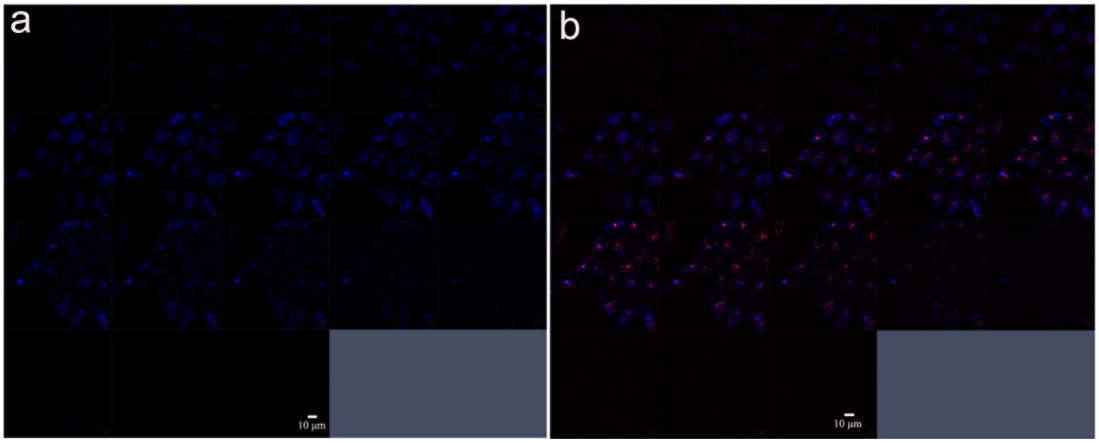


Figure S19. Confocal fluorescence microscopy images of internalization of **PF-1-Gal** by Hep G2 cells in the presence of LysoTracker scanned via z-axis. The cells were treated with 50 µg of **NP-1-Gal** at 37 °C for 4 h incubation. a) Fluorescence images with blue color from nanoparticles. b) Merge of blue and red channel. (blue: nanoparticle; red: lysosome stained with LysoTracker). Scale bar: 10 µm.

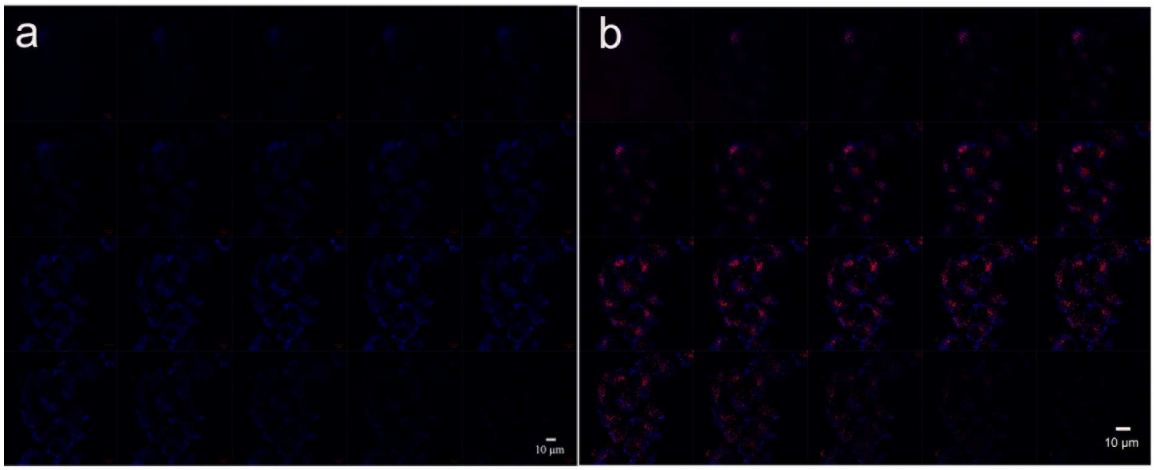


Figure S20. Confocal fluorescence microscopy images of internalization of **PF-6-Gal** by Hep G2 cells in the presence of LysoTracker scanned via z-axis. The cells were treated with 50 µg of **NP-6-Gal** at 37 °C for 4 h incubation. a) Fluorescence images with blue color from nanoparticles. b) Merge of blue and red channel (blue: nanoparticle; red: lysosome stained with LysoTracker). Scale bar: 10 µm.

References:

1 (a) Zhang, Q.; Slavin, S.; Jones, M. G.; Haddleton, A.J.; Haddleton, D.M. *Polym. Chem.* **2012**, *3*, 1016− 1023
(b) Roy, B.; Mukhopadhyay, B. *Tetrahedron Letters* **2007**, *48*, 3783–3787.
(c) Aissou, k.; Pfaff, A.; Giacomelli, C.; Travelet, C.; Mü ller, A. H.; Borsali, R. *Macromol. Rapid Commun.* **2011**, *32*, 912–916.
(d) Lu, S.; Fan, Q. L.; Chua, S. J.; Huang, W. *Macromolecules* **2003**, *36*, 304 -310.
2 Lian, X.M.; Wu, D.X.; Song, X.H.; Zhao, H.Y. *Macromolecules* **2010**, *43*, 7434 -7445.