Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information

Synthesis and biological properties of water-soluble polyphenylthiophene brushes with poly(ethylene glycol)/polyzwitterion side chains

Yajun Zhang, Zhengkui Zhang, Changren Liu, Weizhi Chen, Cheng Li, Wei Wu*, Xiqun Jiang*

Department of Polymer Science & Engineering, College of Chemistry & Chemical Engineering, Nanjing University, Nanjing 210023, People's Republic of China

Methods and Experiments

1. Materials and animals

N,*N*′-dicyclohexylcarbodiimide (DCC), dichloromethane (DCM), tetrahydrofuran (THF), *N*,*N*-Dimethylformamide (DMF), sodium azide and copper(II) sulfate pentahydrate were purchased from Sinopharm Chemical Reagent Co. Ltd, China. Boc-NH-PEG_9-CH_2CH_2-NH_2 and propyne-PEG_3-NH-PEG_{36}-COOH were purchased from Biomatrik Inc., China. *N*-hydroxysuccinimide (NHS), *N,N*-diisopropylethylamine (DIPEA), $Pd(PPh_3)_4$, dimethylaminoethyl methacrylate（DMAEMA）, *tert*-butyl bromoacetate, *N*,*N*,*N*′,*N*′,*N*″-pentamethyldiethylenetriamine (PMDETA) and 2-bromoisobutyryl bromide were purchased from Sigma-Aldrich. $FeCl_3$, phenylmethyl 3-(4-bromophenyl)propanoate, 3-thiophene boronic acid, ascorbic acid and trifluoroacetic acid (TFA) were purchased from Alfa Aesar. DMAEMA were distilled before use, and the first fractions were discarded to remove low molecular mass inhibitor. Human neuroblastoma SH-SY5Y cells, human pulmonary cancer A549 cells and human cervical cancer Hela cells were purchased from Shanghai Institute of Cell Biology (Shanghai, China).

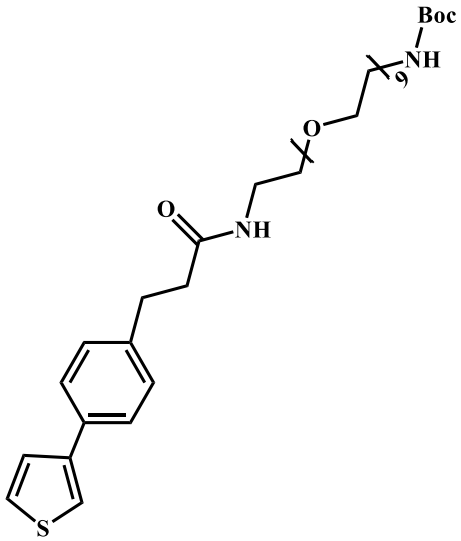
All animal experiments were implemented according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Drum Tower Hospital (Nanjing, China).

2. Instrumentation

Nuclear magnetic resonance (NMR) spectroscopy was recorded on a Bruker DQX-400 spectrometer with tetramethylsilane as internal standard. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses were performed on an autoflex II TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using α-Cyano-4-hydroxycinnamicacid (HCCA) as the matrix. The average molecular weight (Mn) was calculated according to the following equations: $\mathrm{Mn} = \sum(N_i M_i)/\sum N_i$, where N_i and M_i represent the number and mass for the polymer containing i monomers, respectively[1]. The molecular weight and polydispersity index (PDI) of polymers were measured by Gel permeation chromatography (GPC), a PL-GPC 50 integrated GPC system equipped with a PL aquagel-OH 30 8 μm 300 × 7.5 mm column and an internal refractive index (RI) detector. DMF

was used as eluent at 35 ℃ at 1.0 mL/min. UV-vis absorption spectra were recorded on a Shimadzu UV-2401 spectrophotometer. Steady-state emission spectra were measured on a Horiba Jobin Yvon FluoroMax-4 NIR spectrofluorometer at room temperature. Atomic force microscopy (AFM) measurements were taken on a Digital Instruments Dimension 5000 AFM with a Nanoscope IIIa controller (DI/Veeco, Santa Barbara, CA) operated in the tapping mode using silicon probes (Mikromasch USA, resonance frequencies in the range of 175-350 kHz, free amplitude: 20-25 nm) at RT. One drop of a dilute solution of molecular brush (2×10^{-4} mg/mL) in water was placed onto freshly cleaved mica surfaces and dried at ambient temperature. Laser scanning confocal images were recorded on LSM-710 (Zeiss Inc., Germany).

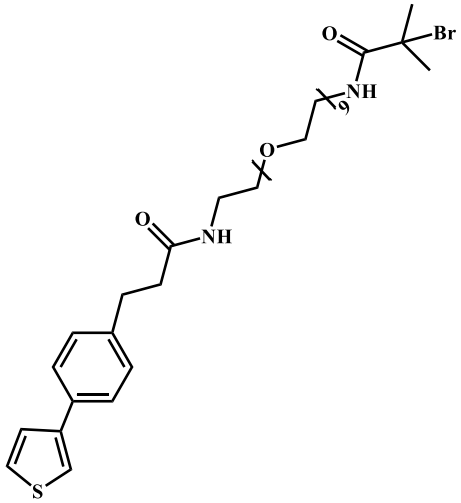
3. Synthesis of TPPA-PEG$_9$-Boc



DCC (0.738 g, 3.58 mmol), NHS (0.412 g, 3.58 mmol) and 3-[4-(3-thienyl)phenyl]propanoic acid (0.624 g, 2.68 mmol, synthesized following published procedures[2]) were dissolved in anhydrous THF (40 mL) and stirred at room temperature for 1 h. To the resulting solution, a solution of BocNH-PEG$_9$-$CH_2CH_2NH_2$ (1 g, 1.79 mmol) in anhydrous THF (20 mL) was added dropwise over 1 h. The resulting mixture was stirred overnight. After the removal of the precipitate by filtration, the filtrate was concentrated under reduced pressure and the product was isolated by column chromatography with an eluent of DCM/methanol (97/3, v/v), giving TPPA-PEG$_9$-Boc as a pale yellow oil (852 mg, 61% yield).^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm): 7.52 (d, 2H; Th), 7.41(d, 1H; Th), 7.36(d, 2H; Ph)7.22 (d, 2H; Ph),6.22 (s, 1H; CONH), 5.04 ppm (s, 1H; BocNH),3.25-3.68(m,40H, -CH_2-in

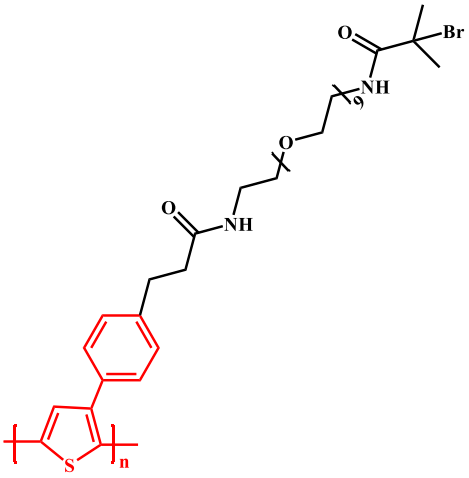
PEG chain), 2.98 (t, 2H; Ph-CH_2), 2.50(t, 2H; $NCOCH_2$), 1.43(s, 9H; Boc). ^{13}C NMR: δ(ppm):172.07, 155.97, 142.05, 140.02, 133.71, 128.83, 126.42, 126.17, 119.81, 79.04, 70.51, 70.16, 69.12, 40.34, 39.21, 38.13, 31.31, 28.42.

4. Synthesis of TPPA-PEG$_9$-Br



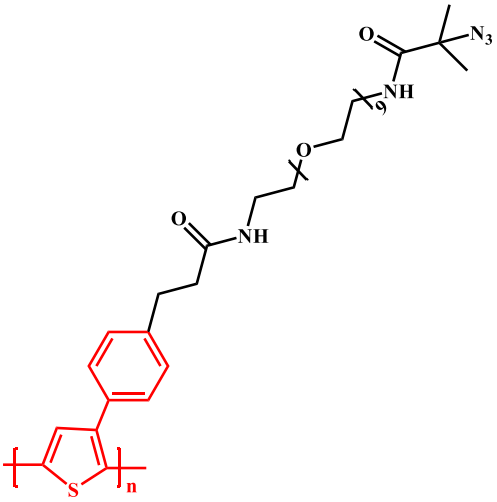
TPPA-PEG$_9$-Boc (1 g, 1.2 mmol) was dissolved in a mixture of TFA/DCM (10 mL, 1/1, v/v). The resulting solution was stirred at room temperature for 24 h. After removal of the solvents under reduced pressure, anhydrous DCM (20 mL) was added. To the ice-cooled resulting solution was added 2-bromoisobutyryl bromide (0.643 g, 2.8 mmol) and DIPEA (0.362 g, 2.8 mmol) dropwise within 1 h. The resulting mixture was stirred in ice-water bath for 2 h and then warmed slowly to room temperature and stirred for 24 h. Thereafter, the resulting solution was washed twice with 1 M HCl, once with saturated $NaHCO_3$ solution, once with 1 M NaCl solution and finally with water, and dried over Na_2SO_4. After removal of the solvent under reduced pressure, the yellow-brownish residue was chromatographed over a silica column DCM/methanol (97/3, v/v), giving TPPA-PEG$_9$-Br as a pale yellow oil (710 mg, 67% yield). ^1H NMR (400 MHz, $CDCl_3$): δ(ppm): 7.52 (d, 2H; Th), 7.41(d, 1H;Th), 7.37(d, 2H; Ph)，7.23 (d, 2H; Ph),7.12 (s, 1H; $Br(CH_3)_2CCONH$), 6.27 ppm (s, 1H; CONH),3.25-3.68(m,40H, -CH_2-in PEG chain), 2.99 (t, 2H; Ph-CH_2), 2.51(t, 2H; $NCOCH_2$), 1.95(s, 6H; $Br(CH_3)_2C$). ^{13}C NMR: δ (ppm):172.59, 172.20, 142.23, 140.09, 133.98, 129.05, 126.63, 126.45, 126.39, 119.84, 70.75, 70.61, 70.37,70.20, 69.67, 62.72, 40.35, 39.56, 38.27, 33.67, 31.61.

5. Synthesis of PTPPA-PEG$_9$-Br



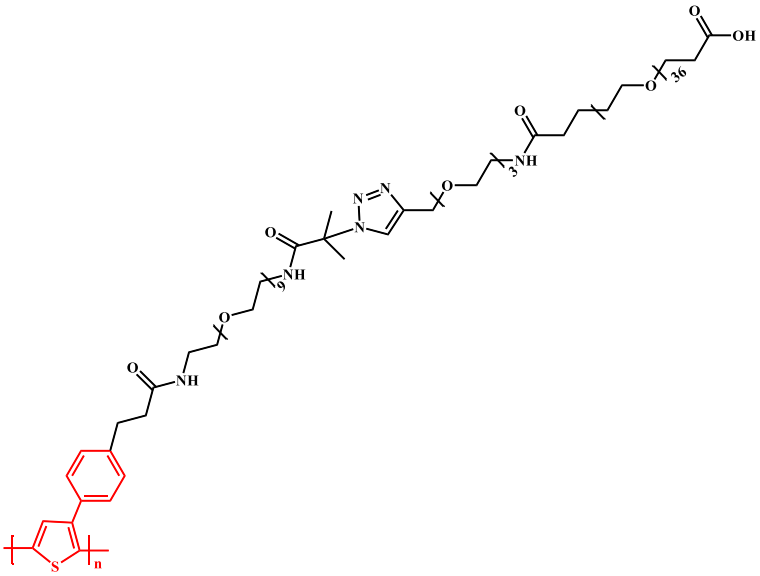
$FeCl_3$ (0.649 g 0.4 mmol) was dispersed in 30 mL of anhydrous $CHCl_3$ under argon. To the resulting mixture, was added dropwise a solution of TPPA-PEG$_9$-Br (0.863 g, 0.1 mmol) in 20 mL of anhydrous $CHCl_3$. The resulting mixture was stirred at room temperature for 48 h. Thereafter, the produced precipitate was collected and dissolved in methanol. The crude product was purified by a Sephadex LH-20 column with methanol as eluant to remove the unreacted $FeCl_3$ and monomer. After removal of the solvent under reduced pressure, the product PTPPA-PEG$_9$-Br was provided as dark red oil (259 mg, 30% yield). ^{1}H NMR (400 MHz, *d6*-DMSO): δ(ppm): 8.03 (s, 1H; CONH), 7.85(s, 1H, Th)，7.23 (s, 4H; Ph),6.93 (s, 1H; Br(CH_3)$_2$CCONH), 3.25-3.68(m,40H, -CH_2-in PEG chain), 2.79 (s, 2H; Ph-CH_2), 2.38 (s, 2H; NCOCH_2), 1.83(s, 6H; Br(CH_3)$_2$C).

6. Synthesis of PTPPA-PEG$_9$-N$_3$



PTPPA-PEG$_9$-Br (100 mg, containing 0.12 mmol Br units) and sodium azide (0.156 g, 2.2 mmol) were dispersed in DMF (10 mL) and stirred at 60 °C for 48 h. After removal of the precipitate by filtration, the filtrate was diluted with 3 times volume of deionized water and dialyzed against 2 L of deionized water in a 14 kDa MWCO membrane for 24 h. After lyophilization, the product PTPPA-PEG$_9$-N$_3$ was provided as red oil (70 mg, 73% yield). ^{1}H NMR (400 MHz, *d6*-DMSO): δ(ppm): 7.90 (s, 2H; CONH and Th)， 7.25 (s, 4H; Ph),6.94 (s, 1H; Br(CH$_3$)$_2$CCONH), 3.25-3.68(m,40H, -CH2- in PEG chain), 2.81 (s, 2H; Ph-CH$_2$), 2.37 (s, 2H; NCOCH$_2$), 1.36(s, 6H; N$_3$(CH$_3$)$_2$C).

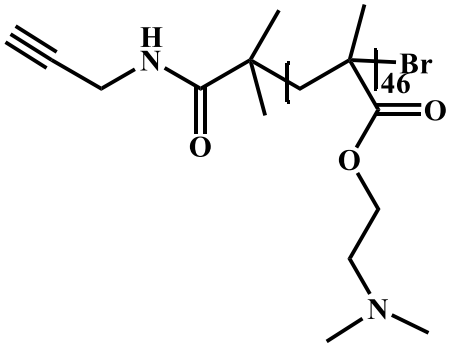
7. Conjugation of Propyne-PEG$_3$-NH-PEG-COOH to PTPPA-PEG$_9$-N$_3$.



Propyne-PEG$_3$-NH-PEG-COOH (300 mg, 0.15 mmol), PTPPA-PEG$_9$-N$_3$ (100 mg, containing 0.12 mmol azido group) and PMDETA (20 μL, 0.096 mmol) were dissolved in DMF (8 mL). The mixture was degassed by three freeze-pump-thaw cycles. CuSO$_4$•5H$_2$O (6 mg, 0.024 mmol) and ascorbic acid (84.5 mg, 0.48 mmol) were added to the frozen mixture. Before the flask was sealed, it was evacuated and backfilled with argon for three times. The resulting mixture was stirred at 40 °C under dark for 48 h. Thereafter, the solution was diluted with 3 times volume of deionized water and dialyzed against 2 L of deionized water in a 14 kDa MWCO membrane for 24 h. The unreacted Propyne-PEG$_3$-NH-PEG-COOH was further removed by centrifugal ultrafiltration on a Millipore Amicon Ultra centrifugal filter unit (10 kDa MWCO). The solution was lyophilized to get the product PPTPEG brush (6) as a dark red

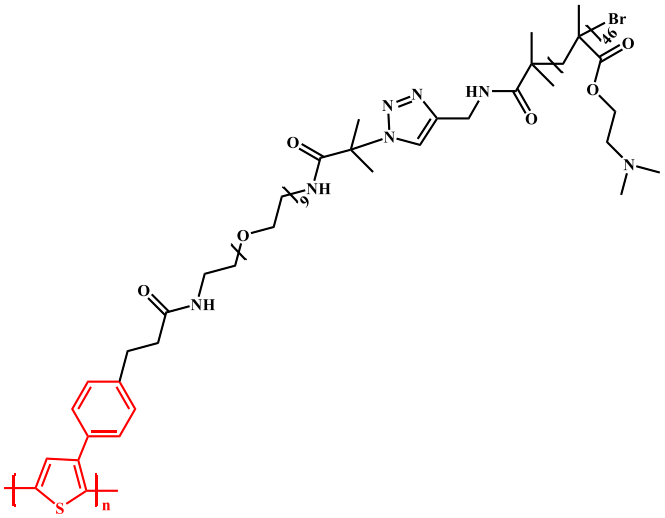
solid (200 mg, 59% yield).

8. Synthesis of PDMAEMA



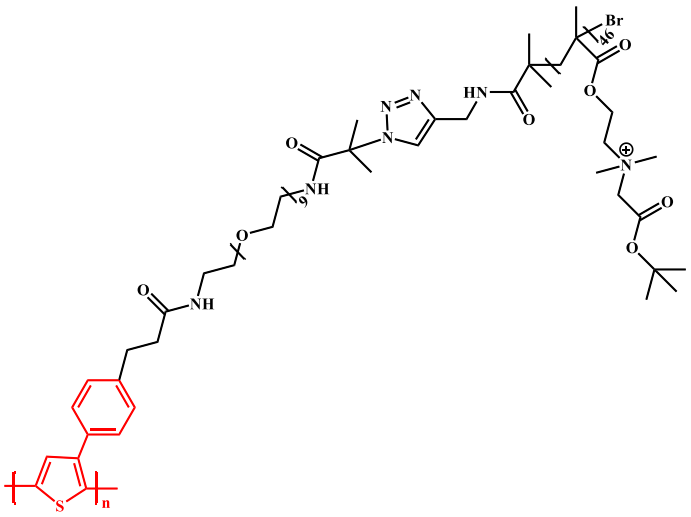
Polymerization of DMAEMA was performed using Propargyl 2-Bromo-2-methylpropionamide (BMP) as an initiator. Briefly, BMP (100 mg, 0.49 mmol, synthesized following published procedures[3]), DMAEMA (8.3 ml, 49 mmol), THF (8.3 mL), Cu (0) wire (l = 10 cm, d = 1 mm) and ligand PMDETA (200 μL, 0.96 mmol) were placed in a 25 mL Schlenk flask. Then the flask was degassed by freeze-pump-thaw for three cycles in liquid N_2. Thereafter, the flask was sealed under vacuum and stirred at 30 ºC for predetermined time. After diluted with THF, the resulting solution was filtered through a column of activated neutral Al_2O_3 to remove copper salts. The crude product was purified by precipitating from DCM to n-hexane three times and dried under vacuum to get the product PDMAEMA as a white solid (4.2 g, Mn = 7230 g mol^{-1} and n = 46, which was calculated on the basis of monomer conversion measured by ^{1}H NMR spectra[4]). ^{1}H NMR (400 MHz, $CDCl_3$): δ(ppm): 4.05 (2H, –O**CH_2**CH_2N–), 2.57 (2H, –OCH_2**CH_2**N–), 2.38–2.20 (6H, –$N(CH_3)_2$), 2.10–1.63 (2H, –CCH_2C–), 1.10–0.80 (3H, –CCH_3).

9. Conjugation of PDMAEMA to PTPPA-PEG$_9$-N$_3$.



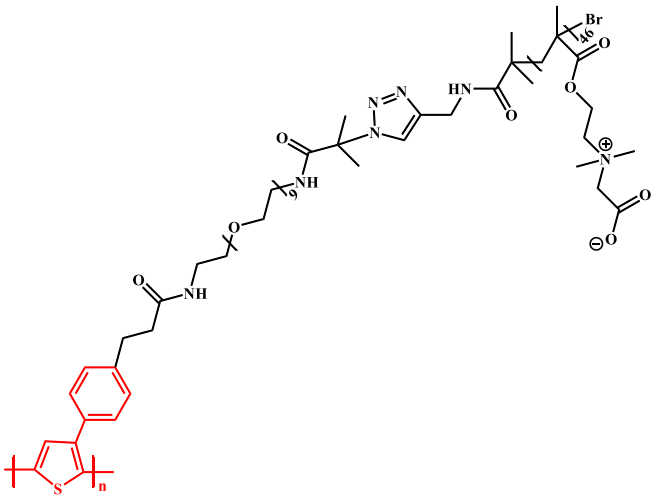
PDMAEMA (500 mg, 0.065 mmol), PTPPA-PEG$_9$-N$_3$ (50 mg, containing 0.06 mmol azido group) and PMDETA (20 μL, 0.096 mmol) were dissolved in DMF (5 mL). The mixture was degassed by three freeze-pump-thaw cycles. $CuSO_4$•$5H_2O$ (10 mg, 0.04 mmol) and ascorbic acid (345 mg, 1.96 mmol) were added to the frozen mixture. Before the flask was sealed, it was evacuated and backfilled with argon for three times. The resulting mixture was stirred at 40 °C under dark for 48 h. Thereafter, the solution was diluted with 3 times volume of deionized water and dialyzed against 2 L of deionized water in a 14 kDa MWCO membrane for 48 h. The unreacted PDMAEMA was removed by centrifugal ultrafiltration on a Millipore Amicon Ultra centrifugal filter unit (100 kDa MWCO). Then, the solution was lyophilized to get the product PPTPDMAEMA brush as a dark red solid (352 mg, 69% yield).

10. Synthesis of PPTPCBtBu brush



PPTPDMAEMA brush (500 mg, containing 2.84 mmol tertiary amino group) and tert-butyl bromoacetate (800 mg, 4.1 mmol) were dissolved in DMF (10 mL) and stirred at 50 °C for 24 h under argon. Thereafter, the crude product was purified by precipitating from DMF to ethyl ether three times and dried under vacuum, providing the product PPTPCBtBu brush as a dark red solid (950 mg, 90% yield).

11. Synthesis of PPTPCB brush



PPTPCBtBu brush (200 mg, containing 0.53 mmol tert-butyl ester group) was dissolved in a mixture of TFA/DCM (6 mL, 1/1, v/v). The resulting solution was stirred for 5 h at room temperature and then precipitated into ethyl ether. To ensure the water solubility of the product, the precipitate was re-dissolved in deionized water and lyophilized to get the product PPTPCB brush as a dark red solid (140 mg, 83% yield).

12. *In vitro* cytotoxicity of molecular brushes

The *in vitro* cytotoxicity of PPTPEG and PPTPCB brushes against the human SH-SY5Y neuroblastoma was tested by MTT [3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyltetrazolium bromide] assay. The cells were seeded in a 96-well plate at a density of 5000 cells per well and incubated with 200 μL of culture medium containing a series of doses of the samples at 37 ℃ for 24 h. After the incubation, the culture medium in each well was removed and the cells were washed three times with PBS. 20 μL of MTT solution (5 mg/mL) was added to each well and cultured for another 4 h. The supernatant was discarded and then 100 μL of DMSO was added to each well. The values of the plate were observed on a microplate reader at 570 nm (Safire, Tecan). The results were expressed as the viable percentage of cells after various treatments

relative to the control cells without any treatment. Cell viability was calculated by following formula:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance test cells}}{\text{Absorbance reference cells}} \times 100\%$$

13. Cellular uptake of molecular brushes

The human SH-SY5Y neuroblastoma cells were used to study the cellular uptake of molecular brushes. The cells were seeded into a 6-well plate at a density of 2.5×10^5 cells per well and incubated for 24 h followed by coincubating with PPTPEG and PPTPCB brushes for 4 h at 37 ℃, respectively. The emission intensities of the feeding solutions of PPTPEG and PPTPCB brushes at their respective $\lambda_{max,em}$ were ensured to be the same to compare the cellular uptake of PPTPEG and PPTPCB brushes. Thereafter, the cells were washed three times with PBS at 4 and 37 ℃ respectively to remove any free molecular brushes. The cell nuclei were stained with Hoechst 33258 in PBS. The cellular uptake images were recorded with a confocal laser scanning microscope (CLSM; LSM 710, Zeiss, Germany). For quantitative studies, the cells were harvested for flow cytometric analysis (Accuri C6, BD Biosciences, USA).

To quantitatively analyze the cellular uptake efficiency of the different molecular brushes, 200 μL of cell lysis solution (150 mM NaCl, 1% Trition X100, 0.1 SDS, 50 mM Tris pH 8.0) was added into 6-well plate at a density of 2.5×10^5 cells per well to disrupt the cell structure after removal of the free brushes. Horiba Jobin Yvon FluoroMax-4 NIR spectrofluorometer with Ex 470 nm/Em 590 nm was used to determine cellular uptake efficiency. The cell uptake efficiency was calculated by following formula:.

$$\text{uptake efficiency (\%)} = \frac{I_{\text{sample}} - I_{\text{negative}}}{I_{\text{positive}}} \times 100\%$$

The I_{sample}, I_{positive} and I_{negative} are the fluorescent intensity of the sample, positive control (brushes in cell lysis solution) and negative control (SH-SY5Y cells without treatment), respectively.

14. Endocytic pathway of molecular brushes

To study the endocytic pathway of the molecular brushes, the cells were preincubated in

serum-free DMEM medium with MβCD (5 mM, 1 h), chlorpromazine (10 mg/mL, 1 h) or cytochalasin B (10 μg/mL, 1 h), respectively. Then the molecular brush sample (PPTPEG 10 mg/mL, PPTPCB 50 mg/mL) in PBS (200 μL, 0.01 M, pH=7.4) was added and the cells were further incubated for 4 h at 37 ºC followed by washing three times with PBS. Hoechst 33258 was employed to dye the nucleus zone of the cells. Then the cells were observed with CLSM. For quantitative studies, the cells were harvested for flow cytometric analysis (Accuri C6, BD Biosciences, USA).

15. Intracellular distribution of molecular brushes

SH-SY5Y cells were first incubated with 100 nM Lyso-Tracker (blue) for 1 h at 37 ºC in a humidified atmosphere of 5% CO_2, and washed with culture medium. The solutions of PPTPEG (10 mg/mL) and PPTPCB (50 mg/mL) brushes in PBS (200 μL, 0.01 M, pH=7.4) were subsequently added into the cell culture medium, respectively. After incubation for 4 h at 37 ºC, the cells were washed three times with PBS. Then the cells were observed with CLSM. Lyso-Tracker (blue) excitation was achieved with a 405 nm laser.

16. Uptake and penetration of molecular brushes in multicellular spheroids (MCs)

The SH-SY5Y MCs were prepared as described in our previous work.[5] SH-SY5Y MCs with diameters between 200-300 μm were harvested after approximately 14 days of growth. For the experiments, about 20 spheroids were handpicked with a Pasteur pipette and transferred to a 5 mL eppendorf tube. The molecular brush sample (PPTPEG 10 mg/mL, PPTPCB 50 mg/mL) in PBS (200 μL, 0.01 M, pH=7.4) was then added to the spheroids suspension and co-cultured at 37 ºC for 24 h. The medium was then removed and spheroids were washed with PBS (pH 7.4) before observation. Individual spheroids were imaged by CLSM every 15 μm section from the top to the center. A program ZEN 2008 was used to calculate the mean fluorescence intensity of each multicellular spheroid.

17. Biodistributions of molecular brushes

To build the subcutaneous hepatic H22 tumor model, 5-6 × 10^6 H22 tumor cells were injected subcutaneously in the right axilla of ICR mice (6–8 weeks, 22–26 g). The molecular

brush samples were dissolved in saline at a concentration of 100 mg/mL and injected into the tumor-bearing mice *via* tail vein, respectively. At different time intervals, the mice were sacrificed with three mice for one time point. Blood samples were collected via eye puncture and centrifuged at 14,000 rpm for 20 min to obtain plasma. All the plasma were intensely homogenated in methanol. After two days of extraction and subsequent centrifugation, the molecular brush concentrations in the supernatant were measured by fluorescence technique with an excitation wavelength of 480 nm and emission wavelength of 590 nm according to pre-established calibration curves. The calibration curves were established by adding respectively a set amount of molecular brush to the blood obtained from untreated mice, followed by the identical processing as described above.The tissues including hearts, livers, spleens, kidneys, lungs and tumors were excised. Then the tissues were imaged together with a molecular brush solution in saline (10 mg/ml) as an internal standard by a Maestro™ EX fluorescence imaging system (Cambridge Research& Instrumentation, CRi, USA). The biodistributions of the molecular brushes were expressed as the mean fluorescence intensities of different tissues normalized to that of the internal standard. The mean fluorescence intensities of tissues and internal standard were calculated by a program Nuance 3.0.0.

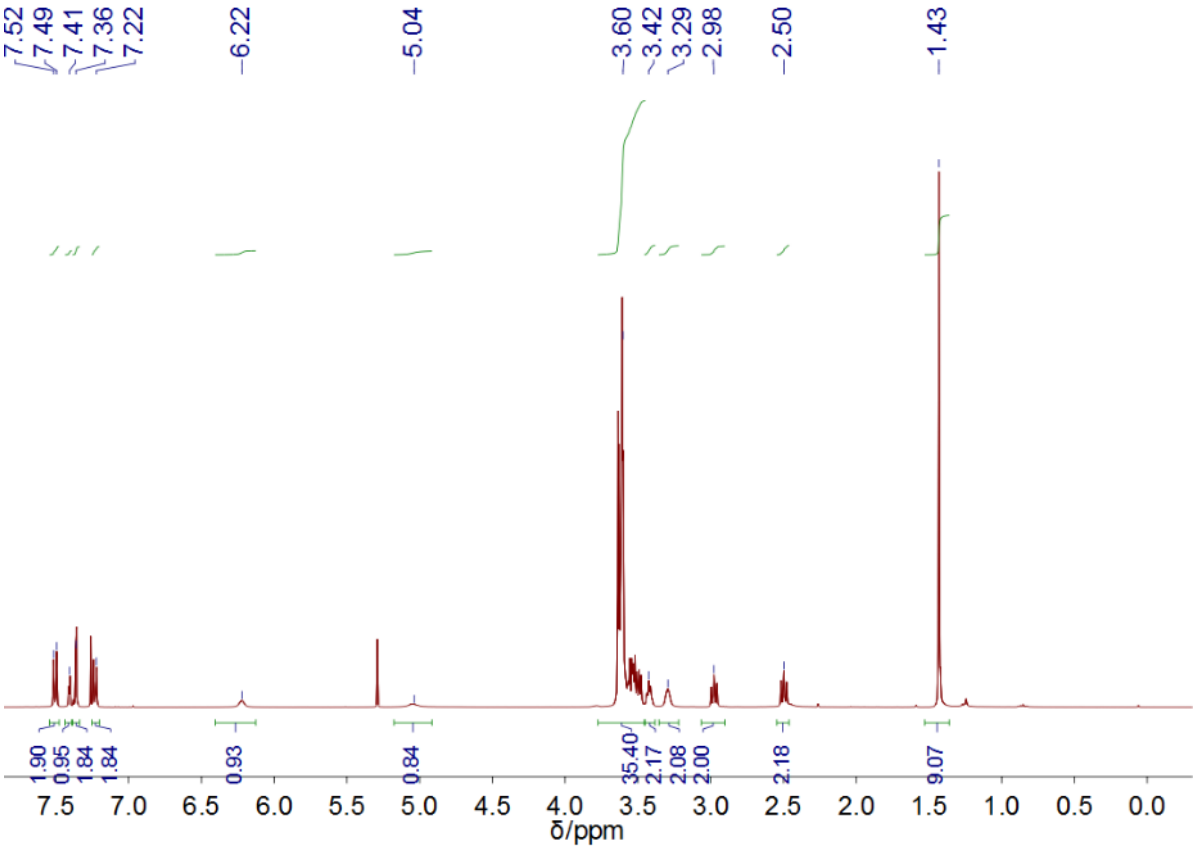


Fig. S1 ^{1}H NMR spectrum of TPPA-PEG$_9$-Boc in CDCl$_3$.

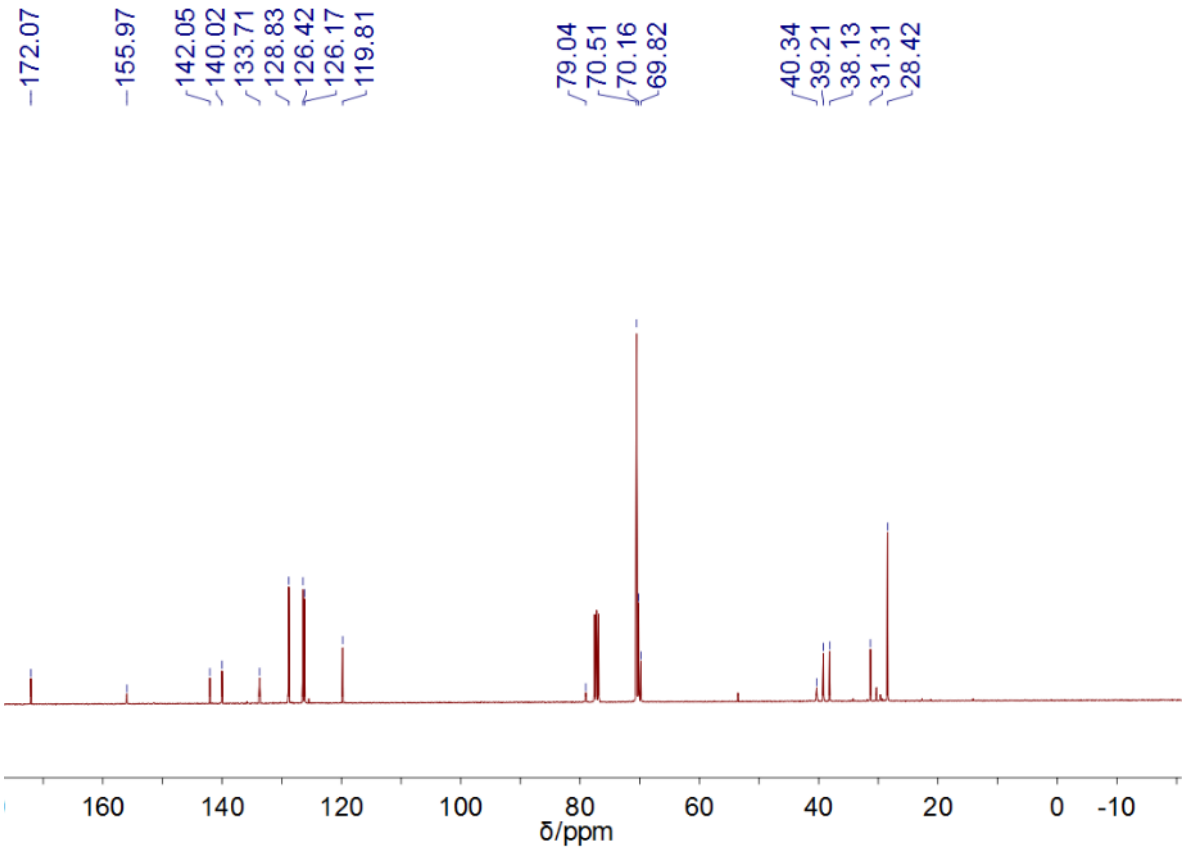


Fig. S2 ^{13}C NMR spectrum of TPPA-PEG$_9$-Boc in $CDCl_3$.

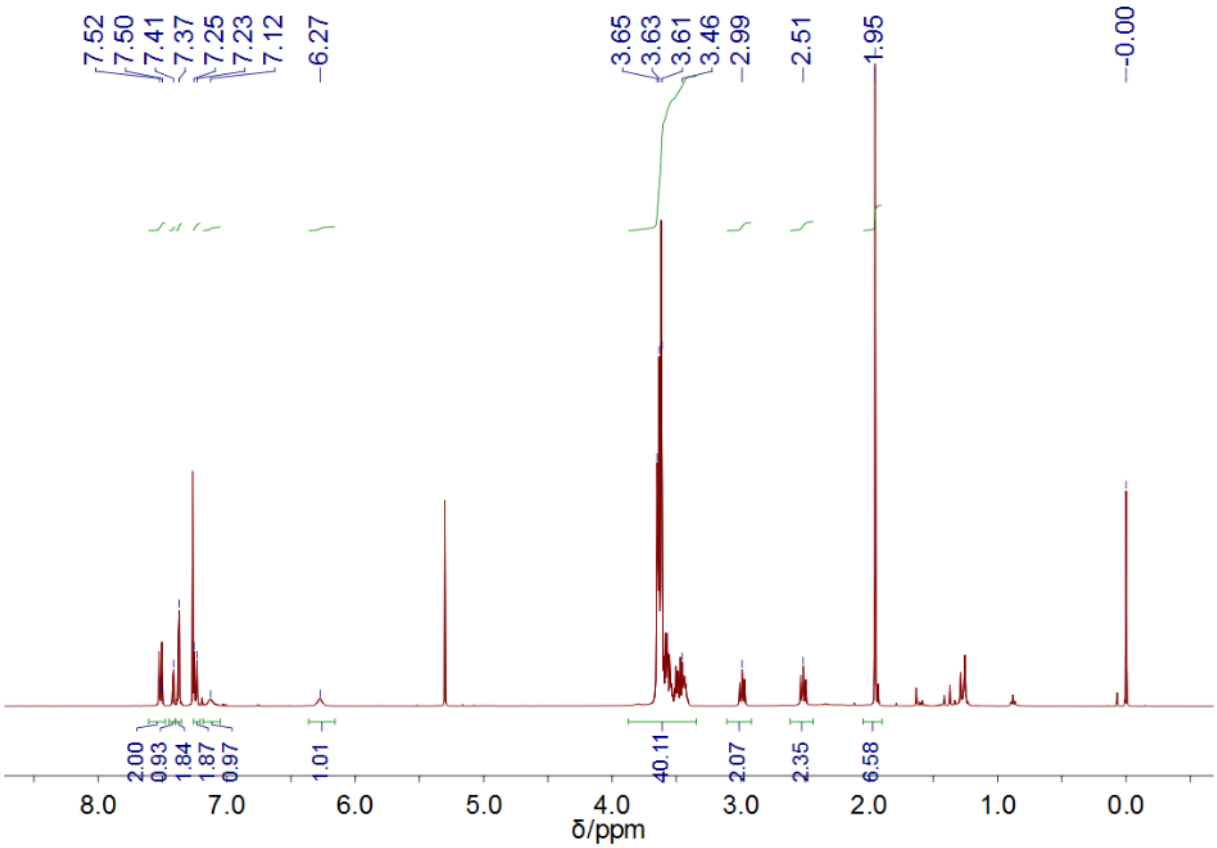


Fig. S3 ^{1}H NMR spectrum of TPPA-PEG$_9$-Br in $CDCl_3$.

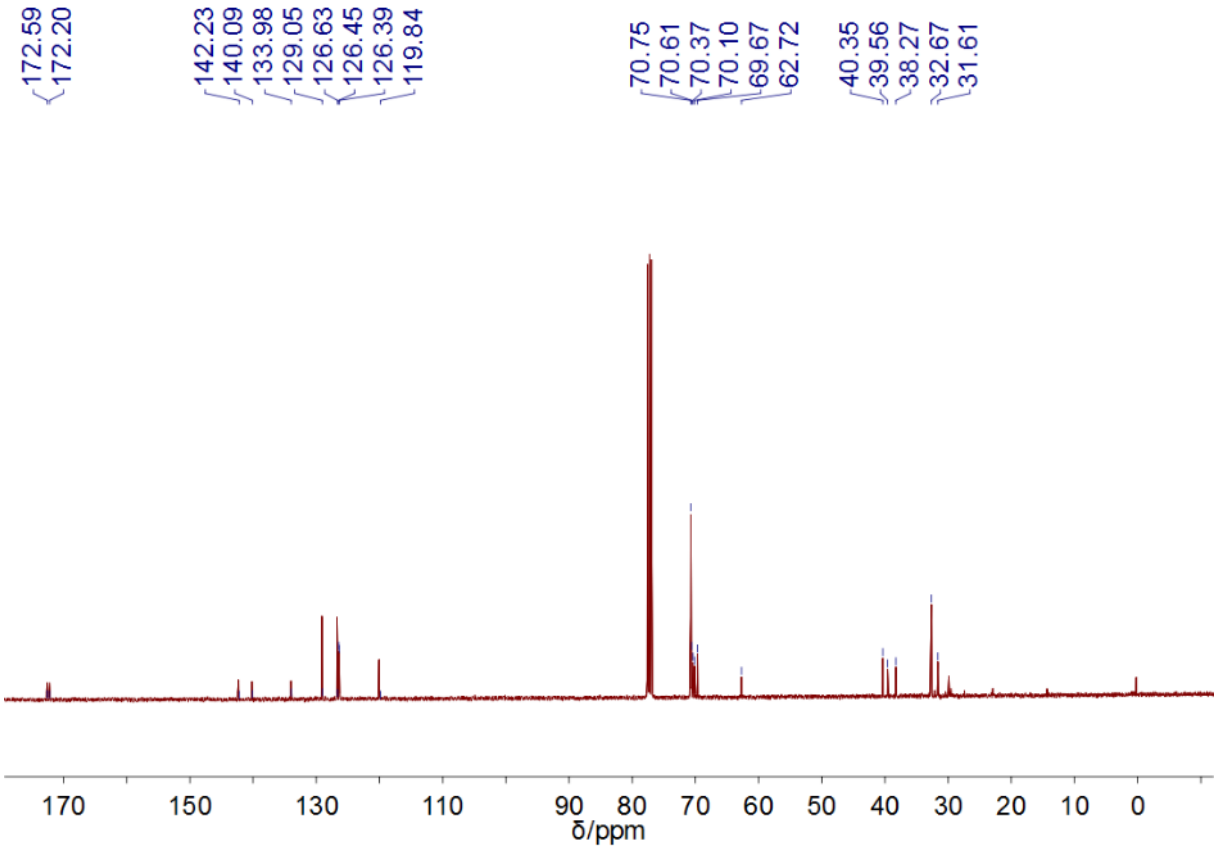


Fig. S4 ^{13}C NMR spectrum of TPPA-PEG_9-Br in $CDCl_3$.

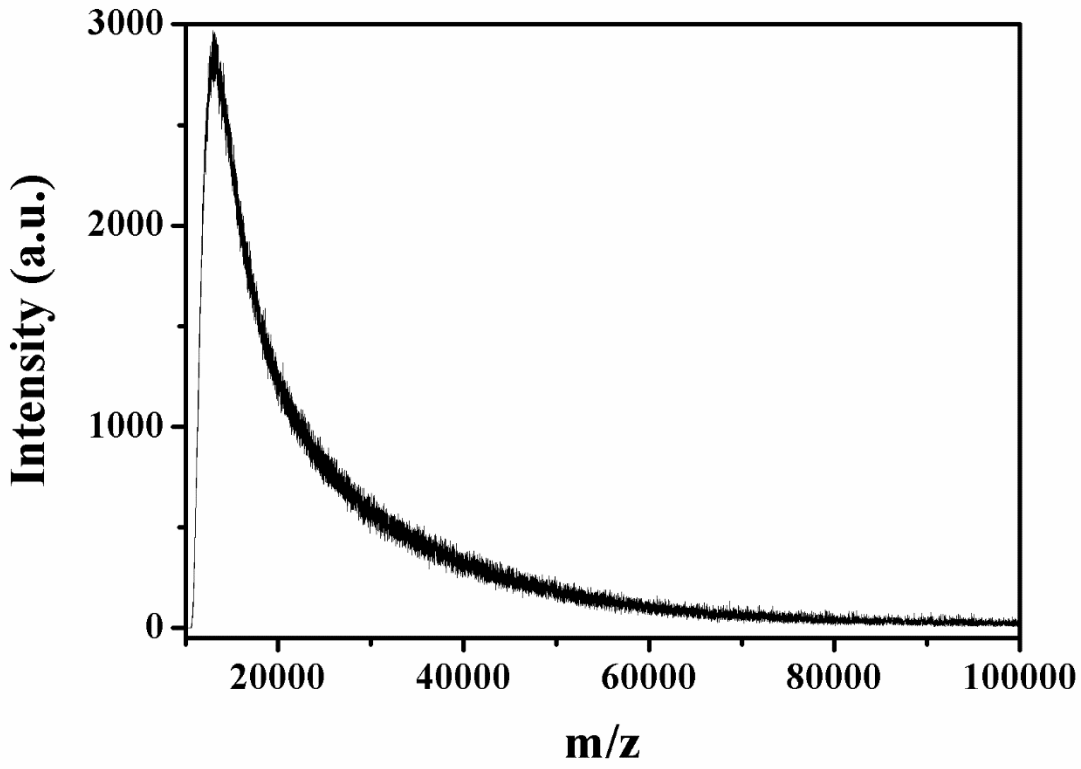


Fig. S5 MALDI-TOF MS of PTPPA-PEG$_9$-N$_3$.

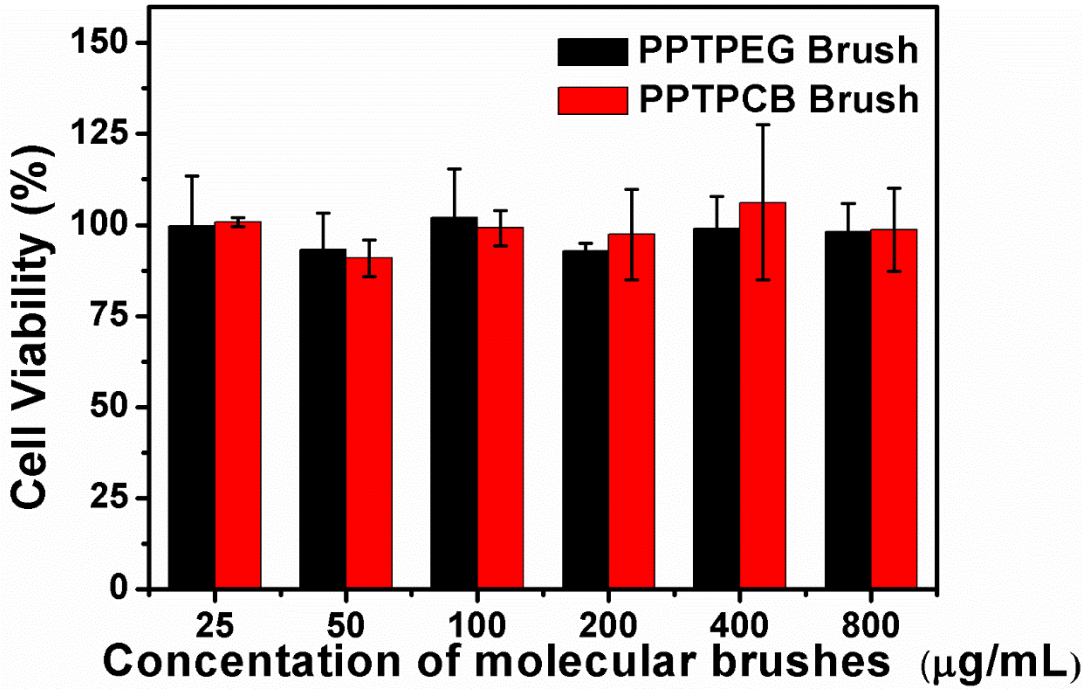


Fig. S6 *In vitro* cytotoxicities of PPTPEG and PPTPCB brushes against SH-SY5Y cells. Data are presented as mean values ± S.D. (n = 3).

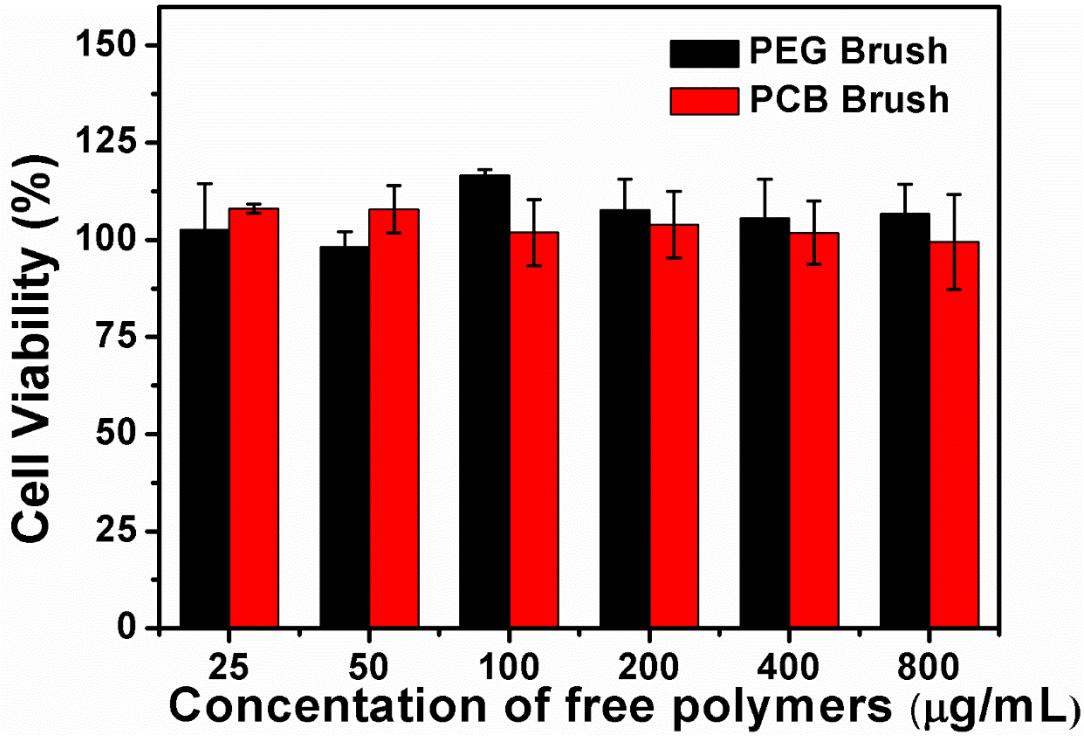


Fig. S7 *In vitro* cytotoxicities of PPTPEG and PPTPCB brushes against A549 cells. Data are presented as mean values ± S.D. (n = 3).

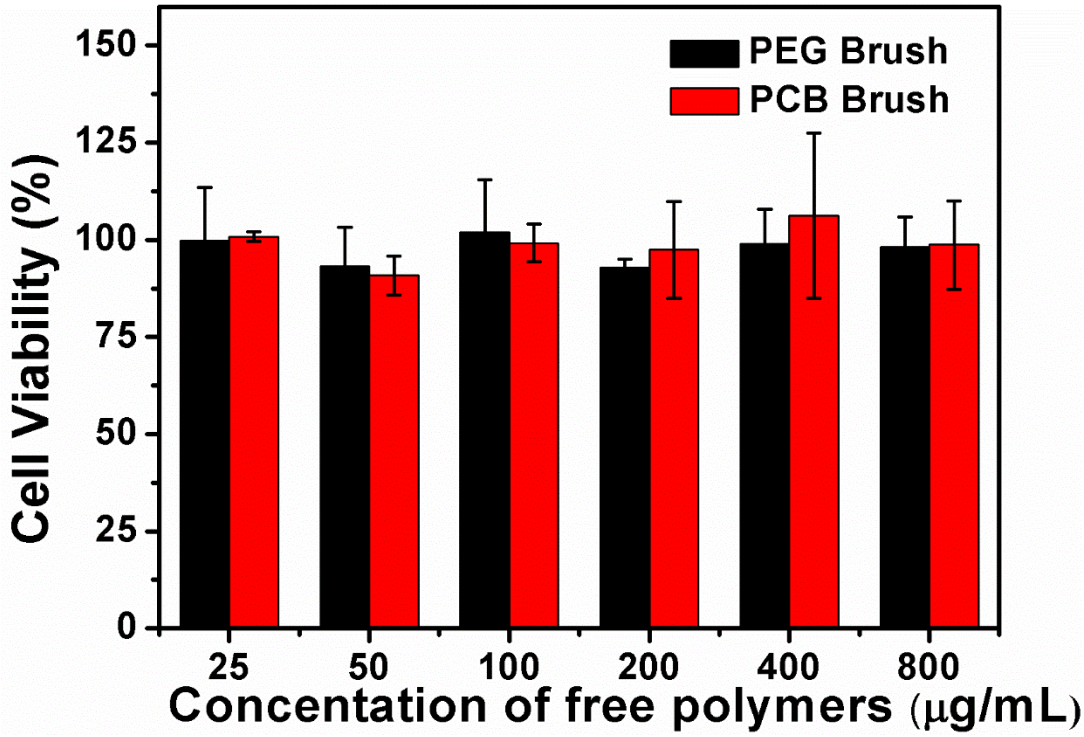


Fig. S8 *In vitro* cytotoxicities of PPTPEG and PPTPCB brushes against A549 cells. Data are presented as mean values ± S.D. (n = 3).

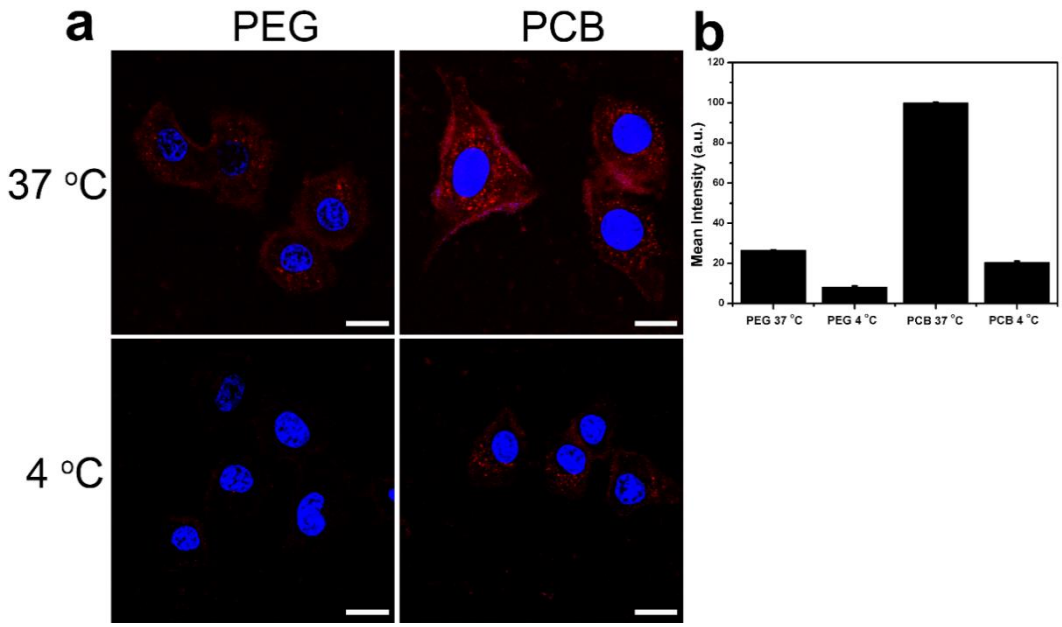


Fig. S9 a) CLSM images of A549 cells after 4 h incubation with PPTPEG and PPTPCB brushes at 37 ºC and 4 ºC, respectively. Scale bars = 20 μm. b) Mean fluorescence intensity in cells measured by flow cytometry after 4 h incubation with PPTPEG and PPTPCB brushes at 37 ºC and 4 ºC, respectively. Data as mean values ± S.D. (n = 3).

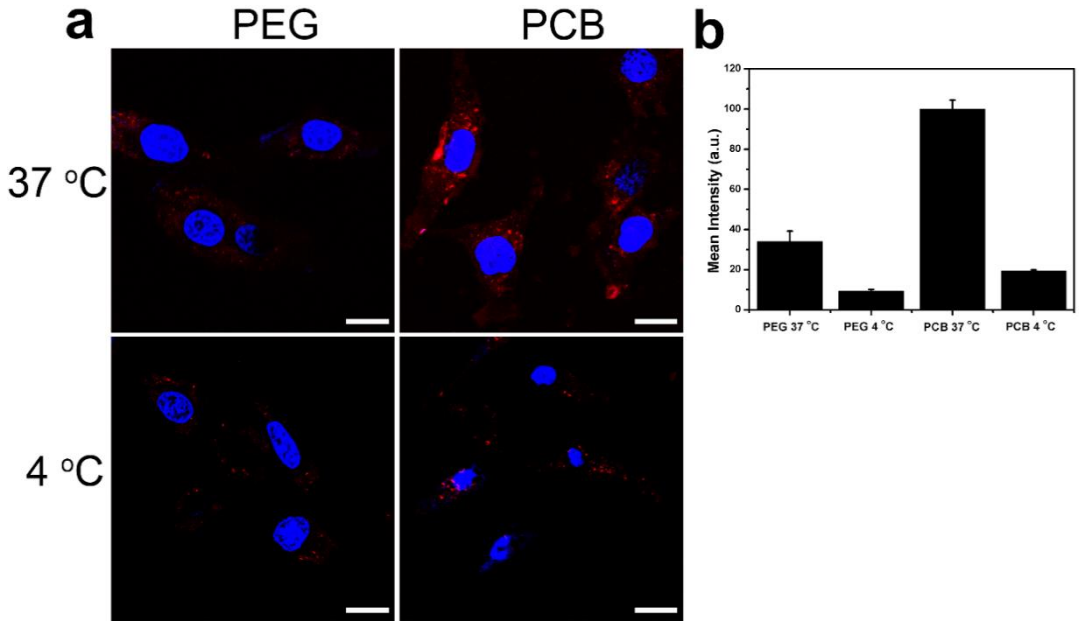


Fig. S10 a) CLSM images of Hela cells after 4 h incubation with PPTPEG and PPTPCB brushes at 37 °C and 4 °C, respectively. Scale bars = 20 μm. b) Mean fluorescence intensity in cells measured by flow cytometry after 4 h incubation with PPTPEG and PPTPCB brushes at 37 °C and 4 °C, respectively. Data as mean values ± S.D. (n = 3).

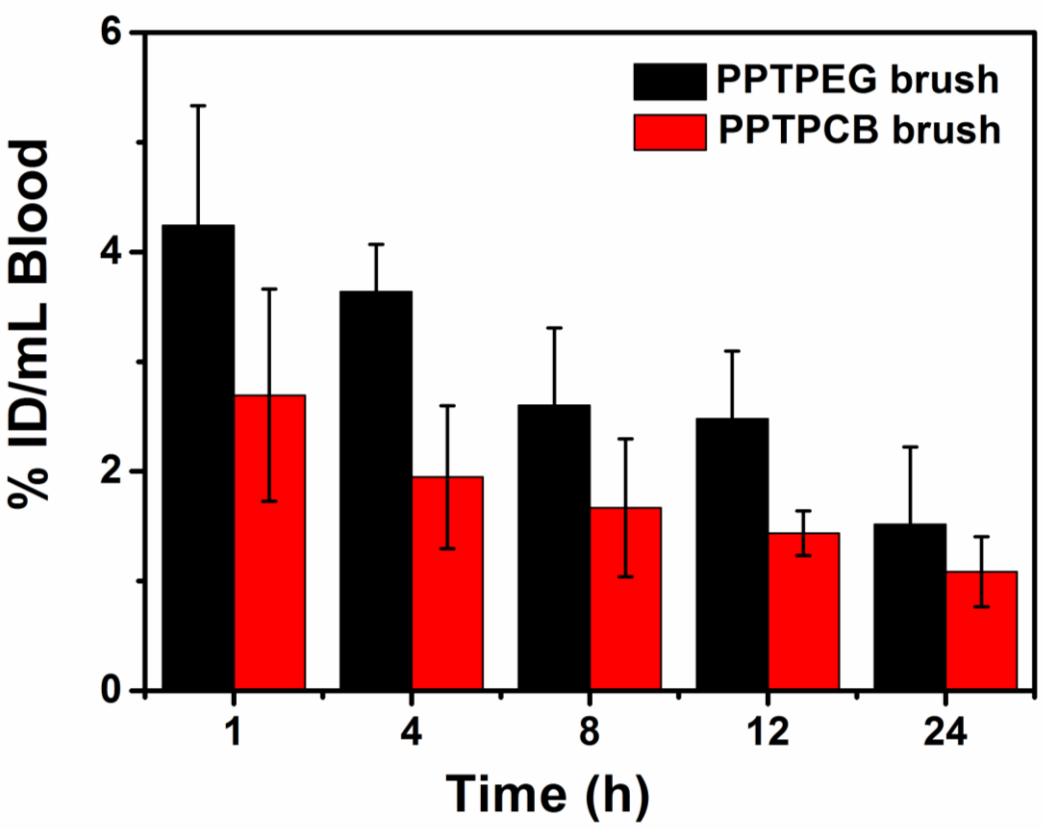


Fig. S11 Molecular brush concentrations in the blood of H22 tumor-bearing mice at different time points after tail-vein injection of PPTPEG and PPTPCB brushes, respectively. The data are expressed as the percentage of injected dose (ID) per milliliter of collected blood and based on three mice per group.

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