Supplemental Information

Assembly of Catechol-Modified Polymer Brushes for Drug Delivery

Kaijie Zhao,[a] Zhiliang Gao,[a] Dongpo Song,*[b] Peiyu Zhang*[a] and Jiwei Cui*[a,c]

[a]*Key Laboratory of Colloid and Interface Chemistry of the Ministry of Education, School of Chemistry and Chemical Engineering, Shandong University, Jinan, Shandong 250100, China*

[b]*Tianjin Key Laboratory of Composite and Functional Materials, School of Materials Science and Engineering, Tianjin University, Tianjin 300350, China*

[c]*State Key Laboratory of Microbial Technology, Shandong University, Qingdao, Shandong 266237, China*

Corresponding authors: dongpo.song@tju.edu.cn, pyzhang@sdu.edu.cn, jwcui@sdu.edu.cn

Experimental Section

Materials and Characterization

Methoxy polyethylene glycol (mPEG, Mw=2000 Da) was purchased from Jenkem Technology Co., LTD (China). All organic solvents engaged in this study were obtained from Sinopharm Chemical Reagent Co., Ltd (China). *N*-Hydroxy succinimide, *exo*-5-norbornenecarboxylic acid (NBCOOH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), 4-dimethylaminopyridine (DMAP), *N*, *N*-diisopropylethylamine (DIPEA), Grubbs catalyst 3rd generation, dopamine hydrochloride, and bortezomib (BTZ) were bought from Sigma-Aldrich (China). Fetal bovine serum (FBS) was commonly obtained from Gibco (Germany) and Dulbecco's modified Eagle's medium (DMEM) was purchased from Beijing Neuronbc Laborataries Co., Ltd. (China).

Nuclear magnetic resonance (NMR) experiments were performed on a 400 MHz device of AVANCE III HD (Bruker, Germany). Ultraviolet visible (UV-vis) absorption spectra were detected on a UV-2600 spectrometer (Shimadzu, Japan). The morphology of nanoparticles (NPs) was characterized by a JEM-1400 (120 kV) transmission electron microscope (TEM) (JEOL, Japan). Hydrodynamic size and zeta potential were measured on a Zetasizer Nano ZS90 instrument (Malvern, UK). Fluorescence images were obtained by an inverted fluorescence microscope (Leica, Germany). The molecular weight (M_w) and polydispersity index (PDI) were determined by gel permeation chromatography (GPC) equipped with a static light scattering detector (Brookhaven, USA). Fourier transform infrared spectra (FT-IR) were scanned by an instrument of Tensor II (Bruker, Germany). The cell association was monitored by a flow cytometry (ACEA, USA).

Synthesis of Macromonomer NB-PEG

The macromonomer of NB-PEG was synthesized according to the published method.[1] mPEG (5 g, 2.5 mmol), NBCOOH (0.55 g, 4.0 mmol), and EDCI (0.92 g, 4.8 mmol) were added into a 100 mL of round bottom flask followed by adding 30 mL of anhydrous dichloromethane (DCM). The resulting solution was immersed into an ice bath for 15 min, followed by the addition of DMAP (24.4 mg, 0.20 mmol). The reaction mixture was allowed to be stirred at room temperature for 48 h. After washing with saturated $NaHCO_3$ and NaCl solutions, the organic phase was dried with Na_2SO_4 powder. After filtration, the solution was concentrated under reduced pressure, followed by precipitation in cold diethyl ether for three times. After vacuum drying, the product of NB-PEG was obtained as a white powder. The structure was confirmed by ^{1}H NMR spectrum (Fig. S3). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.26-6.10 (2H, C$\underline{H}$=C$\underline{H}$), δ 4.25 (2H, COOC$\underline{H_2}$), δ 3.58 (180H, C$\underline{H_2}$C$\underline{H_2}$O), δ 3.31 (3H, OC$\underline{H_3}$), δ 3.08 (1H, CH_2C$\underline{H}$CHCOO), δ 2.81 (1H, CH_2C$\underline{H}$$CH_2$), δ 2.25 (2H, C$\underline{H}$COO), δ 1.92-1.26 (4H, CHC$\underline{H_2}$CH, C$\underline{H_2}$CHCOO).

Synthesis of Monomer NB-NHS

NB-NHS was synthesized according to a reported method with moderate modifications.[2] Briefly, NBCOOH (1.00 g, 7.20 mmol, 1.0 eq.), *N*-hydroxy succinimide (1.12 g, 9.72 mol, 1.4 eq.), and EDCI (1.79 g, 9.36 mmol, 1.3 eq.) were added into a round bottom flask and stirred in DCM (50 mL) for 12 h at room temperature. Most of the solvent was removed from the reaction mixture under reduced pressure using a rotary evaporator, followed by purification with flash chromatography (2:1 hexanes/ethyl acetate as eluent) to obtain the product as a white powder (1.35 g, 5.76 mmol, 80.0% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.21 (1H, C*H*=CH), δ 6.15 (1H, CH=C*H*), δ 3.26 (1H, CH_2C*H*CH), δ 3.00 (1H, CH_2C*H*CH_2), δ 2.79 (4H, C*H*$_2$C*H*$_2$), δ 2.50 (1H, CH_2C*H*CO), δ 2.05-1.32 (4H, CHC*H*$_2$CH, C*H*$_2$CHCOO) (Fig. S4); ^{13}C NMR: (400 MHz, $CDCl_3$) δ 171.6, 169.2, 138.5, 135.3, 47.1, 46.4, 41.8, 40.3, 31.0, 25.6 (Fig. S5). HRMS (ESI^+) m/z 290.2693 calculated for $C_{13}H_{17}NO_5Na$ [M+Methanol+Na^+], 290.1004 found (Fig. S6). The theoretical *Mw* of NB-NHS (M) is 235.2395 Da.

Synthesis of Block Copolymer PEG-*b*-NHS

Macromonomer NB-PEG (200 mg, 100 μmol), NB-NHS (9.4 mg, 40 μmol) and Grubbs 3rd generation catalyst (1.77 mg, 2 μmol) were dissolved in 10, 1, 1 mL of anhydrous DCM in different Schlenk tubes, respectively. The resulting solutions were degassed by freeze-pump-thaw cycles for three times. At room temperature and in N_2 atmosphere, the polymerization of NB-PEG was initiated by adding Grubbs 3rd generation catalyst solution using a syringe. After 1 h polymerization, the second monomer (NB-NHS) was added into the reaction mixture, followed by stirring for another 2 h before termination with ethyl vinyl ether (100 μL). The final solution was poured into 50 mL of cold ethyl ether and centrifuged for three times to get the block copolymer of PEG-*b*-NHS, followed by vacuum drying. The conversion rate was calculated as ~99% by analysis of ^{1}H NMR spectrum. The molecular weight was determined by GPC using THF as eluting agent.

Dopamine Conjugation onto Block Copolymers

PEG-*b*-NHS (100 mg, 1 μmol) was dissolved in DMSO (10 mL) and stirred at room temperature followed by the addition of dopamine hydrochloride (19 mg, 100 μmol). To fluorescently label the copolymer, 10 μg of AF633-NH_2 was added into the mixture. The mixture was stirred under N_2 atmosphere before 20 μL of DIPEA was injected into the solution. The reaction was left stirring for 12 h. Purification was performed by dialysis against HAc/NaAc buffer solution (pH=4.0) in a dialysis bag (MWCO 3500 Da) and the final product was obtained as a light brown powder after freeze drying.

Synthesis of BTZ Prodrug

Generally, BTZ (11.56 mg, 30 μmol, 3 eq.) was added excessively into the solution

of PEG-*b*-DA (60 mg, 10 μmol catechol group, 1 eq.) in 5 mL DMSO, followed by the addition of 10 μL TEA. After 12 h reaction, the mixture was dialyzed in a dialysis bag (MWCO 3500 Da) against methanol and water to remove unconjugated BTZ. Final product of BTZ prodrug was obtained after freeze drying. The prodrug was confirmed by ^{1}H NMR (Fig. S8) and FTIR (Fig. S10).

Preparation and Characterization of BNPs

BTZ-loaded NPs (BNPs) were prepared by solvent exchange method. In brief, BTZ prodrug (10 mg) was firstly dissolved in 2 mL methanol, followed by adding 5 mL water into the solution with an injection pump. Stirring was kept on throughout the whole process. Afterwards, the organic solvent was evaporated under reduced pressure and BNPs were finally obtained with a concentration of 2 mg/mL.

The size and morphology were measured by DLS and TEM. To obtain the CAC value, a series of samples with different concentrations were scanned by using fluorescence spectrophotometer (Hitachi F-7100, Japan) using pyrene (6×10^{-7} M) as the probe.

***In Vitro* Drug Release Behavior Study**

To investigate the pH-responsiveness of BNPs, 1 mL of BNPs (2 mg/mL) was dialyzed (MWCO 3500 Da) against 30 mL PBS buffer at pH 5.0 or 7.4. The device was incubated at 37°C on a shaker at 150 rpm. At different time intervals, 500 μL of medium was withdrawn and replaced with same amount of fresh PBS solution. The collected samples were tested by UV-vis spectrophotometer. The cumulative drug release was calculated according to the standard curve of BTZ in water (Fig. S13 and S14).

Cell Culture and Cytotoxicity Test

All cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 cell line was used for *in vitro* study. Firstly, cells were seeded and incubated in 96-well plates (Cellvis, Mountain View, CA) with a density of 5×10^{4} cells per well. After 24 h incubation at 37°C, the medium was removed and replaced with fresh DMEM containing different concentrations of BNPs or BTZ. The cells were further cultured for 48 h before the cytotoxicity assay was carried out. Finally, MTT was added and cultured for 4 h. After the removal of the medium, DMSO was added and the absorbance at 490 nm was recorded by a microplate reader (Tecan Spark 10M, Switzerland). The cytotoxicity test against 4T1 cells was performed with similar method.

Cell Uptake Analysis

In order to study cellular uptake of BNPs, MDA-MB-231 cells were seeded into 24-well plates with a density of 5×10^{4} cells per well and incubated for 24 h. AF633-labelled NPs were dispersed in DMEM at various concentrations (0, 50, 100, 200, 500, 1000

μg/mL) and incubated with cells for 8 h. To investigate the time dependence of cell uptake, cells treated with BNPs (100 μg/mL) were incubated for different time intervals (0, 0.5, 1, 2, 4 and 8 h). Cells were rinsed 3 times with PBS and resuspended for the analysis using flow cytometry.

Cell Live/Dead Test

MDA-MB-231 cells were cultured and seeded into 24-well plates at a density of 5×10^4 cells per well in DMEM media, followed by 24 h incubation. The medium was removed and replaced with of PEG-*b*-DA (1 mg/mL), BTZ and BNPs (equivalent BTZ concentration of 0.5 μg/mL) for 48 h. Subsequently, the cells were stained with 2 μmol/L of Calcein-Am and 4 μmol/L Propidium Iodide (500 μL). After incubation in dark for 15 min, the cells were observed under an inverted fluorescence microscope.

Establishment of 4T1 Tumor-Bearing Model

4T1 cells were allowed to proliferate in RPMI 1640 medium before they were subcutaneously transplanted to the flank of right leg of Balb/c mice (female, 6-8 weeks) with a dose of 2×10^6 cells per mouse. BALB/c mice were obtained from Vital River Laboratory Animal Technology (China).

Tumor Inhibition and Biodistribution

When the tumor volume was around 100 mm^3, the mice were divided into 3 groups and intravenously injected with BNPs and BTZ with the same drug dose of 1 mg/kg (BTZ was first dissolved into DMSO followed by dilution with PBS). The group treated with PBS was used as control. The injection was repeated twice more on the 3rd and 5th day. The tumor size (length×width^2/2) and body weight of mice were measured and recorded every other day. On the 17th day, all mice were sacrificed and tumors were eventually collected and weighed.

To study the biodistribution *in vivo*, mice with tumor size of around 200 mm^3 were injected with AF633-labelled BNPs (25 mg/kg). After 3 and 12 h, the mice were sacrificed and dissected to collect major organs and tumors. Fluorescence images of organs were obtained using IVIS Spectrum.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of National Institutes and approved by the Animal Ethics Committee of Shandong University.

Statistical Analyses

All data obtained were presented as mean ± standard deviation (SD). Furthermore, student's t-test was engaged to analyze the difference between the groups. *p*-values calculated lower than 0.05 were considered statistically significant.

References:

1. D. Le, M. Dilger, V. Pertici, S. Diabate, D. Gigmes, C. Weiss, G. Delaittre, *Angew. Chem. Int. Ed.,* **2019**, *58*, 4725-4731.

2. X. Lu, E. Watts, F. Jia, X. Tan, K. Zhang, *J. Am. Chem. Soc.,* **2014**, *136*, 10214-10217.

Supplemental Figures

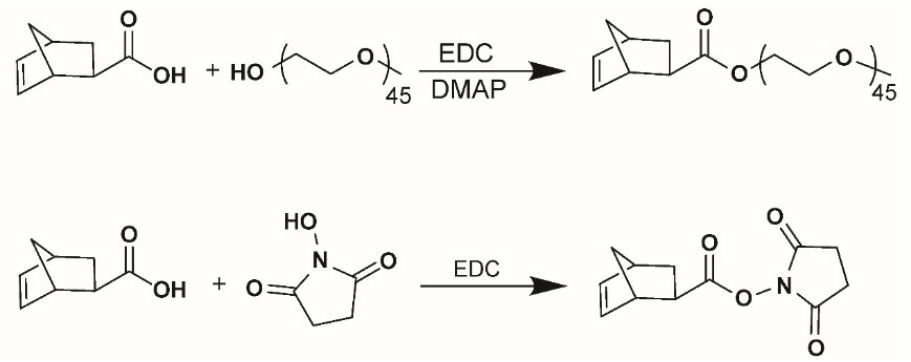

Fig. S1 Synthesis routines of NB-PEG and NB-NHS.

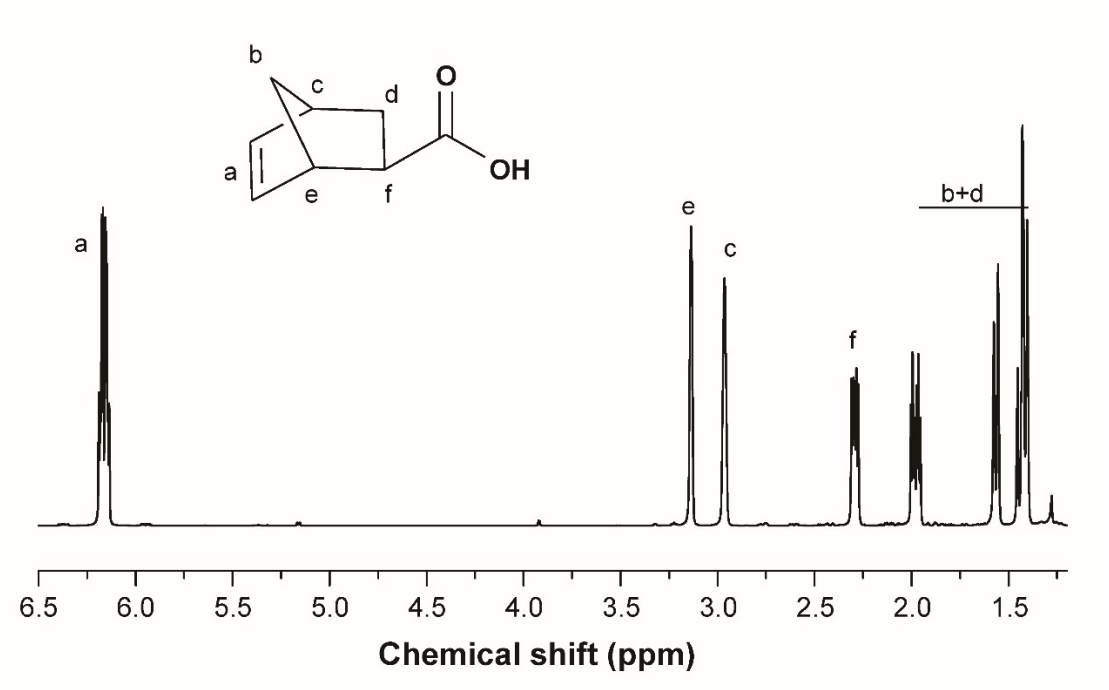

Fig. S2 ^{1}H NMR spectrum of NBCOOH ($CDCl_3$ as solvent).

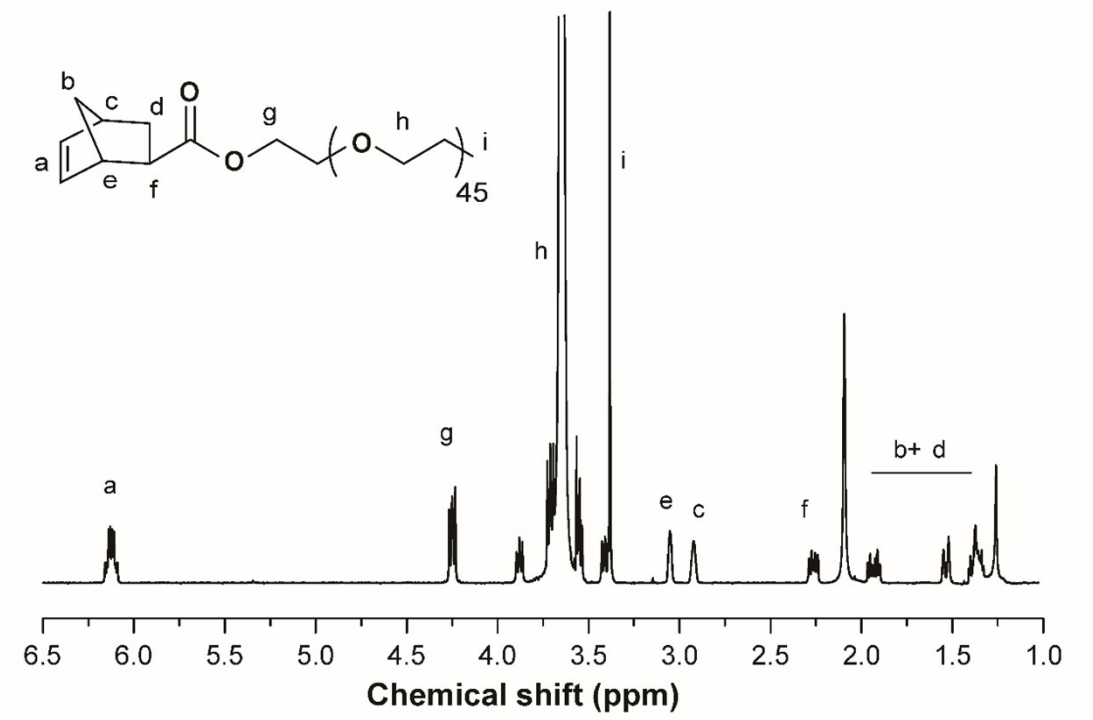

Fig. S3 ^{1}H NMR spectrum of NB-PEG ($CDCl_3$ as solvent).

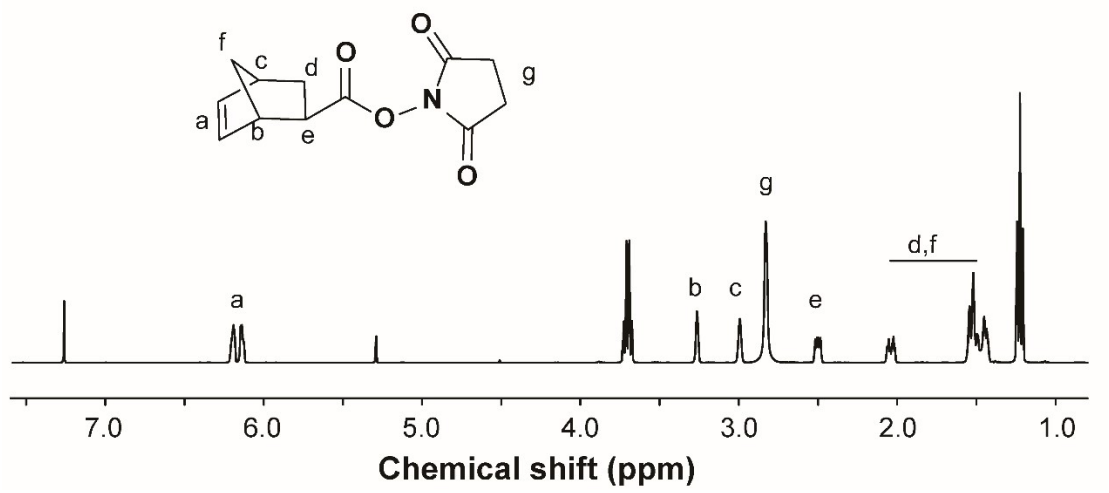

Fig. S4 ^{1}H NMR spectrum of NB-NHS ($CDCl_3$ as solvent).

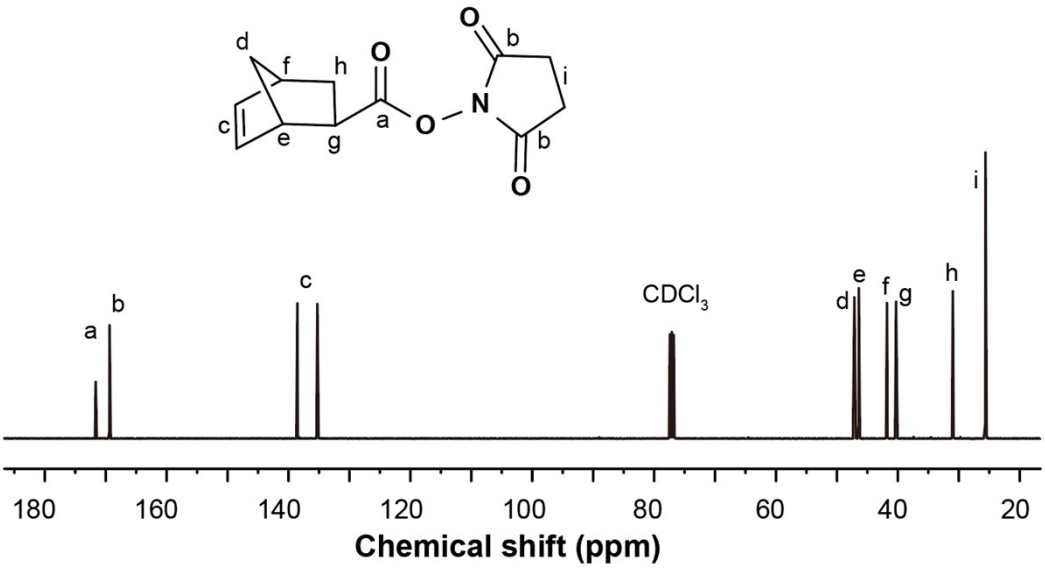

Fig. S5 ^{13}C NMR spectrum of NB-NHS ($CDCl_3$ as solvent).

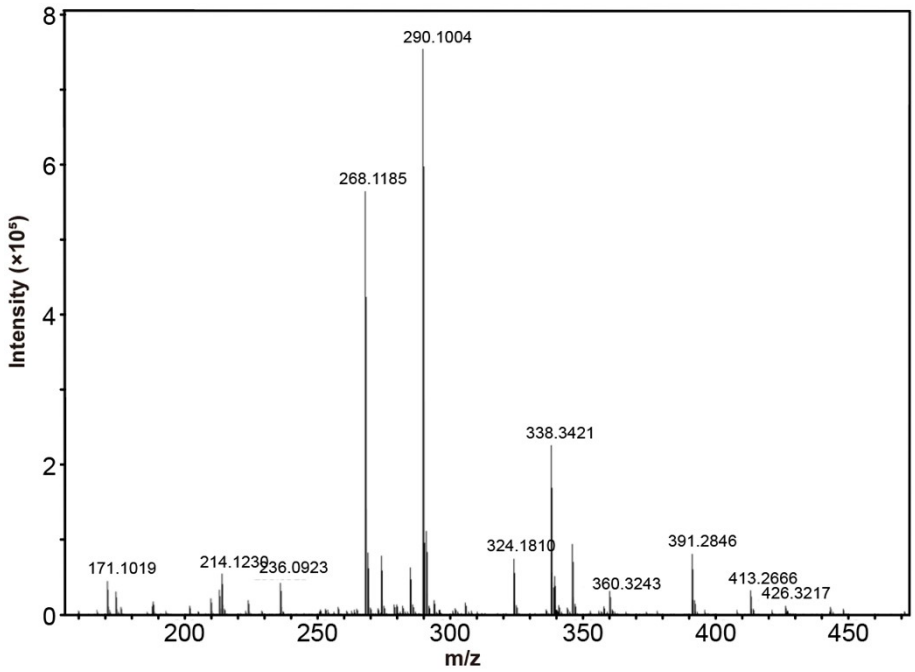

Fig. S6 HRMS of NB-NHS. The theoretical mass calculated for $C_{13}H_{17}NO_5Na$ [M+Methanol+Na^+] is 290.2693.

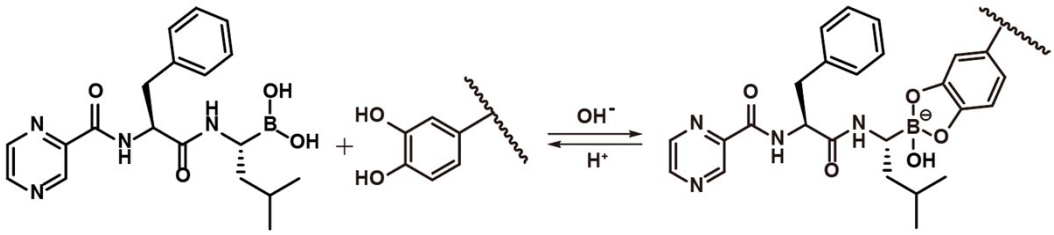

Fig. S7 Conjugation and release behaviors of BTZ based on the pH-responsive borate ester bonds between PEG-*b*-DA and BTZ.

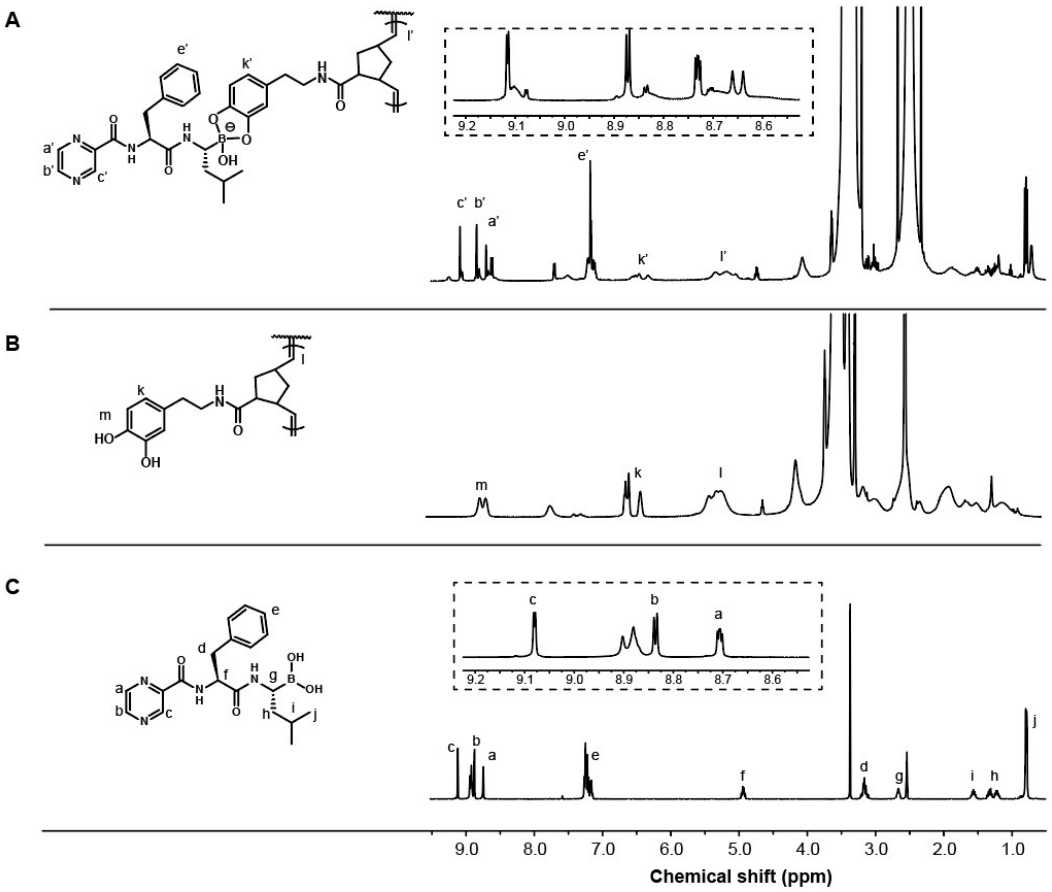

Fig. S8 Characterization of the coordination of BTZ and PEG-*b*-DA. (A) ^{1}H NMR spectrum (400 MHz) of BTZ prodrug (DMSO-d_6 as solvent). (B) ^{1}H NMR spectrum (400 MHz) of PEG-*b*-DA (DMSO-d_6 as solvent). (C) ^{1}H NMR spectrum (400 MHz) of BTZ (DMSO-d_6 as solvent).

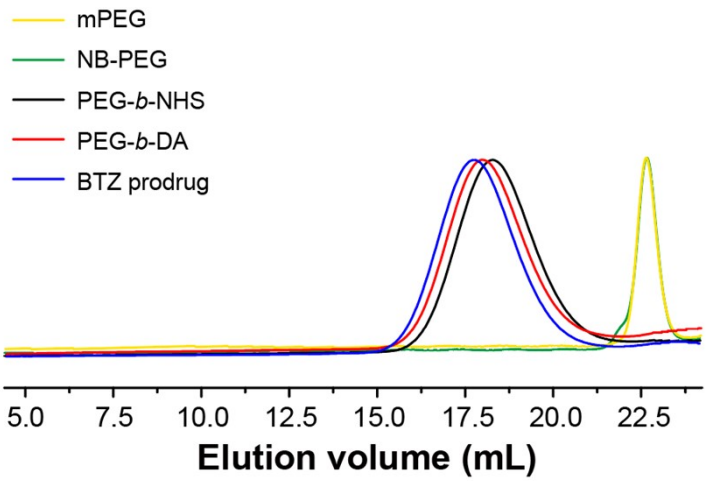

Fig. S9 GPC traces obtained from mPEG, NB-PEG, PEG-*b*-NHS, PEG-*b*-DA and BTZ prodrug using THF as the elution phase.

Table S1 Characterizations of the polymers prepared in this study.

Polymers	M_w (Da)			PDI
	Theoretical[a]	NMR[b]	GPC[c]	
mPEG	2000	1900	2100	1.05
NB-PEG	2130	2200	2250	1.07
PEG-*b*-NHS	111200	110500	109000	1.12
PEG-*b*-DA	111960	111650	112000	1.15
BTZ prodrug	118920	117880	119000	1.21

a Mw (Theoretical) was calculated based on the target DP for each sample and the mass of corresponding monomers. *b Mw* (NMR) was determined according to ^{1}H NMR spectroscopy. *c Mw* (GPC) was obtained via GPC with a static light scattering detector.

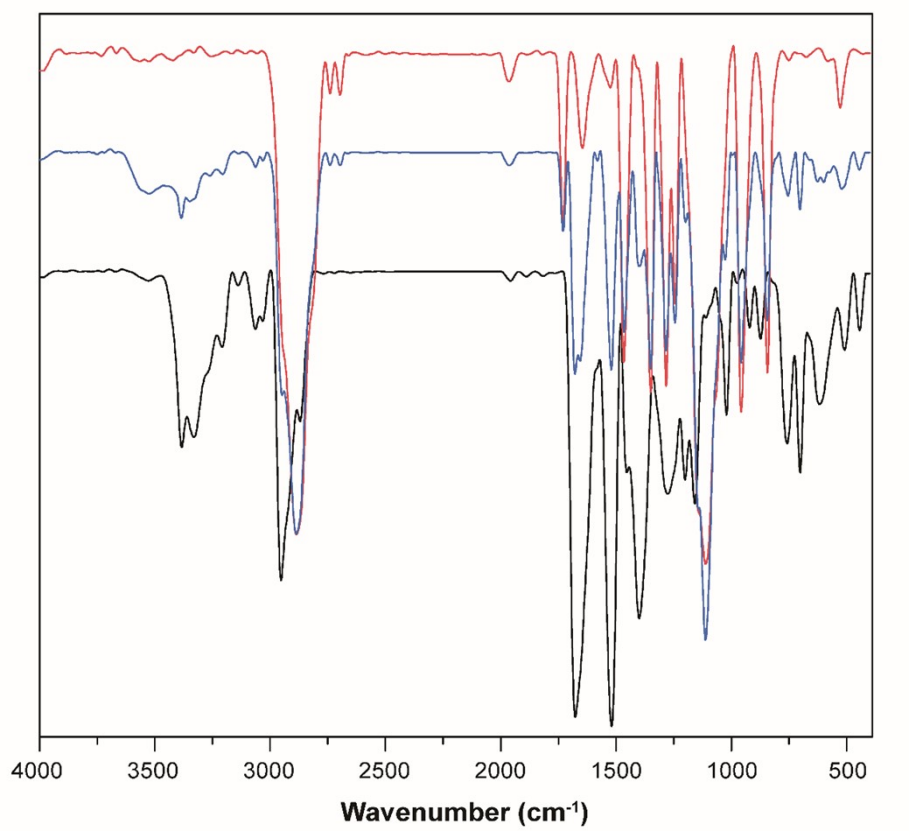

Fig. S10 FT-IR spectra of PEG-*b*-DA (red), BTZ prodrug (blue), and BTZ (black).

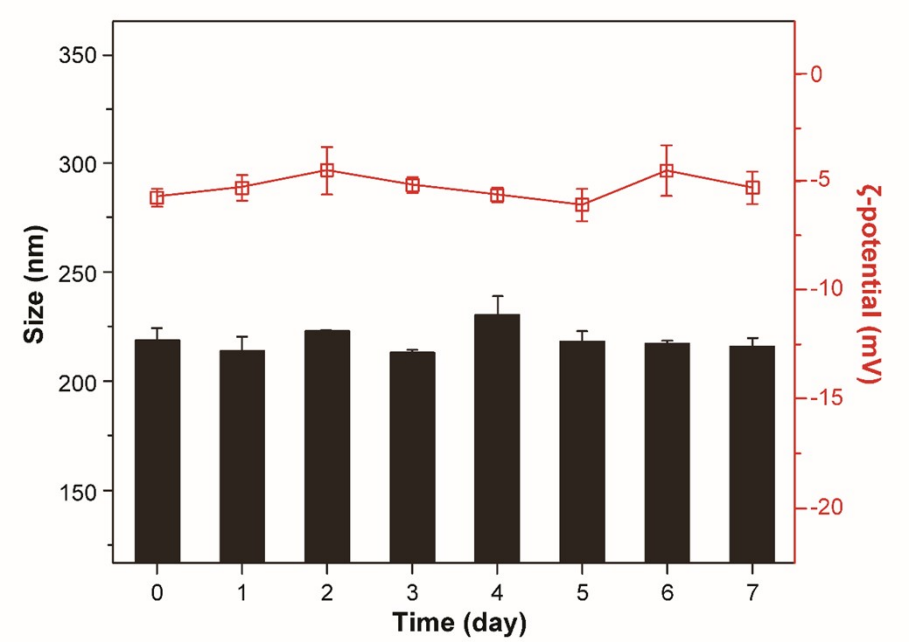

Fig. S11 Size and zeta potential of BNPs in water over a period of 7 days monitored by DLS (n=3).

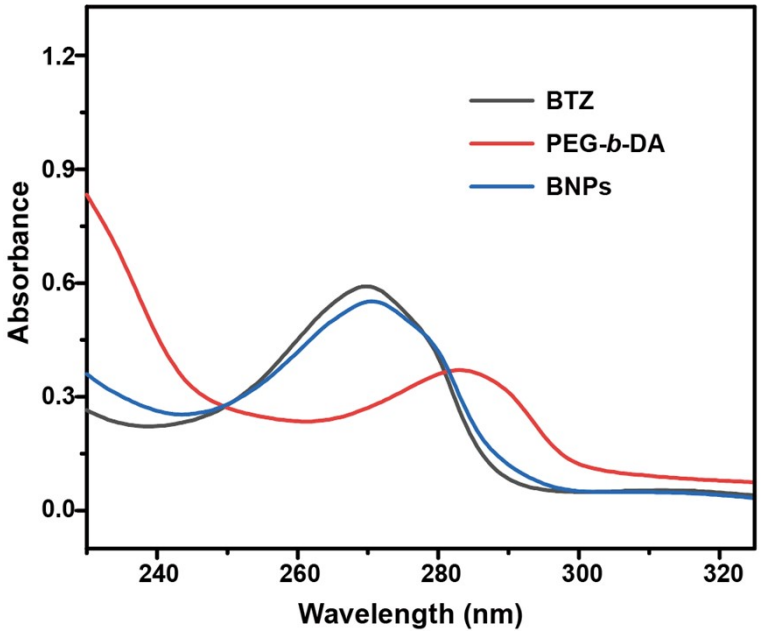

Fig. S12 UV-vis spectra from BTZ, PEG-*b*-DA and BNPs in water.

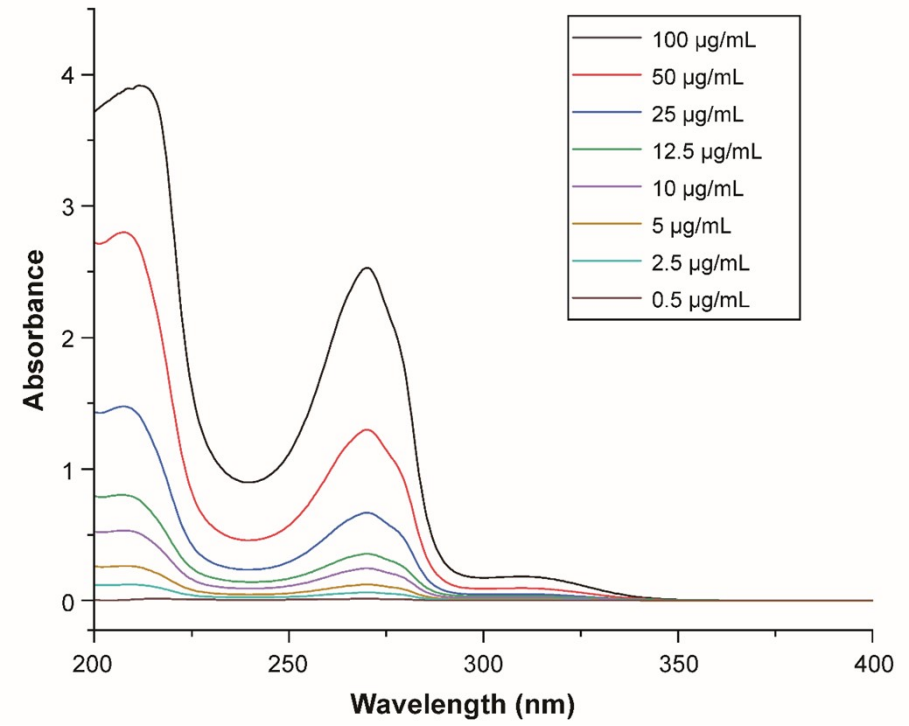

Fig. S13 UV-vis spectra of BTZ with a series of concentrations in water.

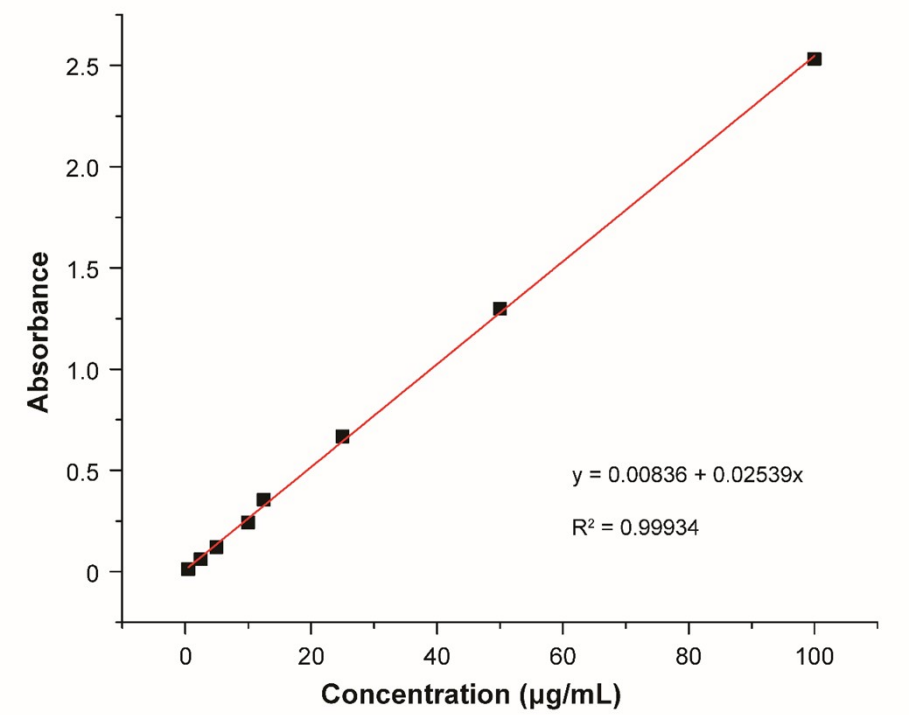

Fig. S14 Standard curve of BTZ in water.

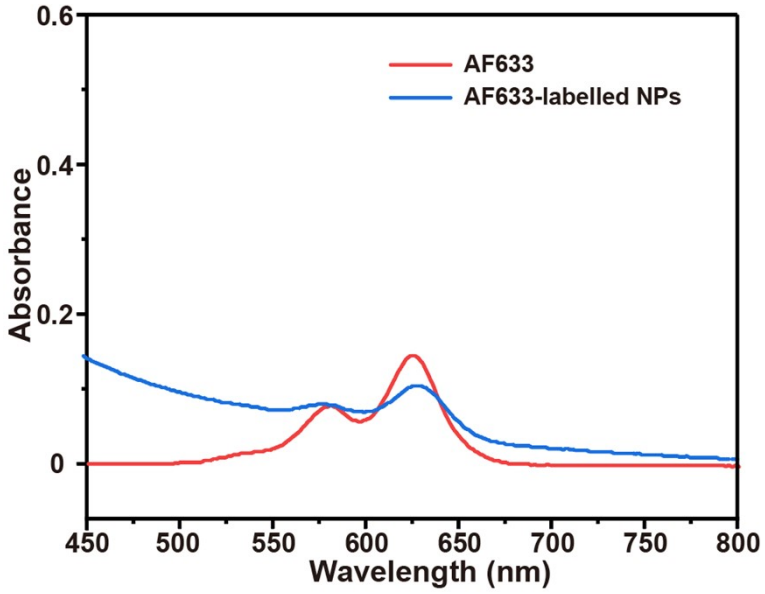

Fig. S15 UV-vis spectra of AF633 and AF633-labelled BNPs.

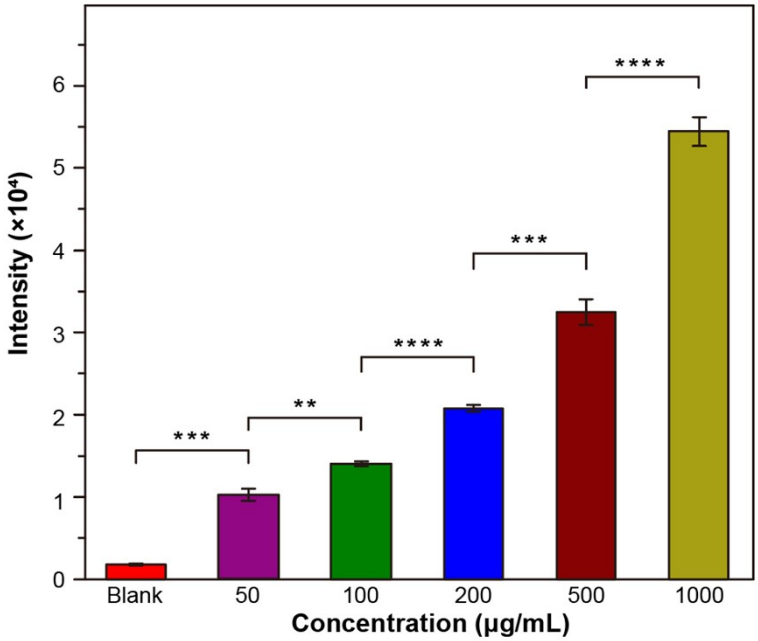

Fig. S16 MFI of MDA-MB-231 cells treated with BNPs of different concentrations for 8 h. Data represent mean ± SD (n=3, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

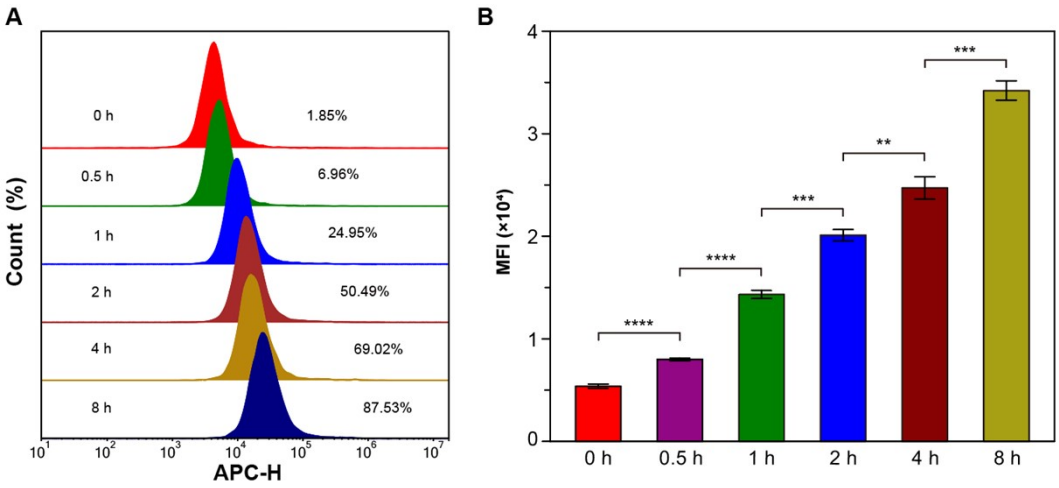

Fig. S17 (A) Flow cytometry results to show MDA-MB-231 cells associated with BNPs for different time. (B) MFI of MDA-MB-231 cells treated with BNPs for different incubation time. The concentration of BNPs was 100 μg/mL. Data represent mean ± SD (n=3, **p<0.01, ***p<0.001, ****p<0.0001).

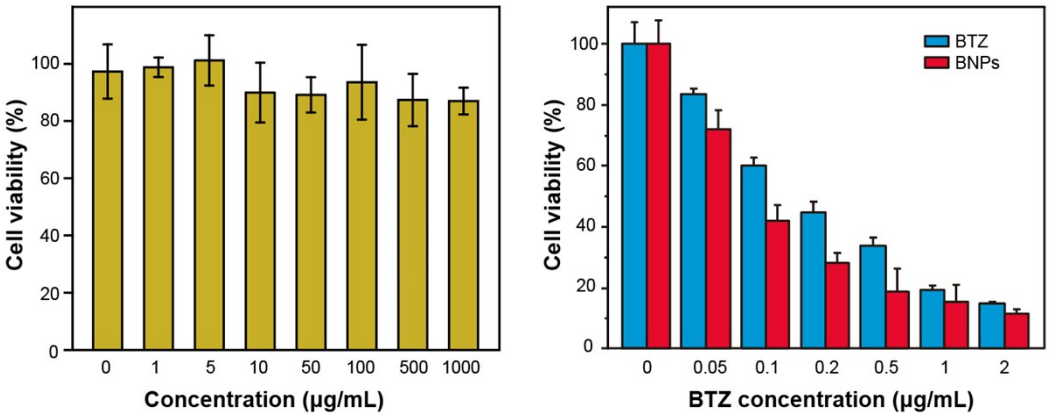

Fig. S18 Cell viability of PEG-*b*-DA after incubation with 4T1 cells for 48 h (left) and cytotoxicity of BNPs containing equivalent BTZ after 48 h incubation (right).

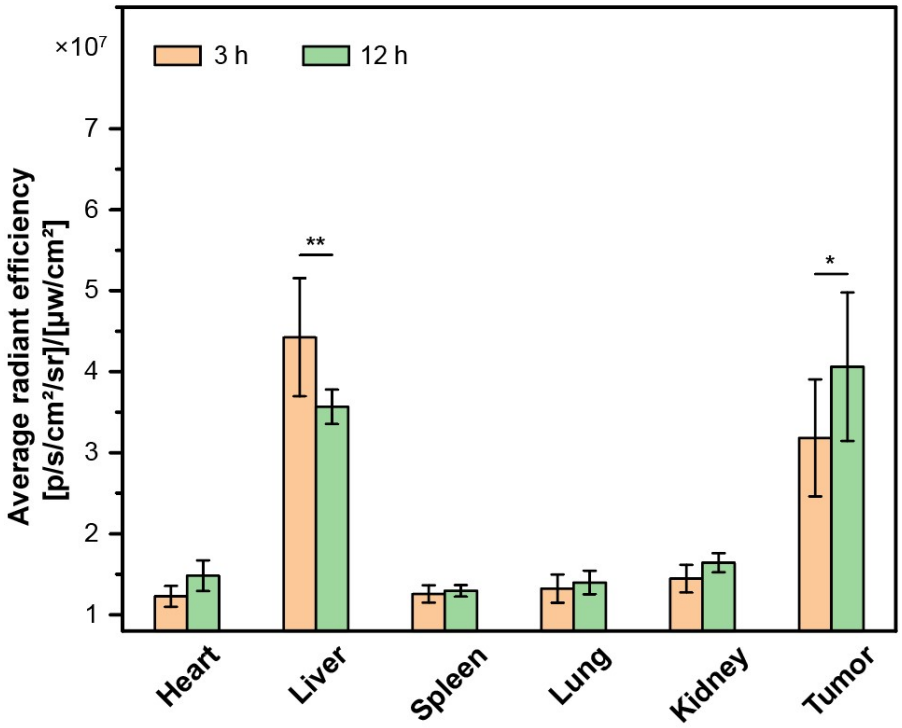

Fig. S19 Fluorescence intensity of major organs (heart, liver, spleen, lung and kidneys) and tumors after injection with AF633-labelled BNPs for 3 and 12 h. Data represent mean ± SD (n=5, *p<0.05, **p<0.01).