Supporting Information

Targeted Ultrasound-Assisted Cancer-Selective Chemical Labeling and Subsequent Cancer Imaging using Click Chemistry

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Supporting Information

General

Materials. D-Mannosamine hydrochloride and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. N-hydroxysuccinimide (NHS) was purchased from Acros Organics (Renningen, Germany). DBCO-Cy5 was purchased from KeraFAST Inc (Boston, MA, USA). DSPE-PEG$_{2k}$-NHS was purchased from Nanocs (New York, NY, USA). Hydro Soy PC L-α-phosphatidylcholine, hydrogenated (HSPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Anhydrous chloroform, dichloromethane (DCM), hexane, and dimethylformamide (DMF) were purified by passing them through alumina columns and kept anhydrous by storing them in the presence of molecular sieves. Perfluorocarbon gas was purchased from FluoroMed, L.P. (Round Rock, TX, USA). Phosphate-buffered saline (PBS) and Dulbecco’s Modified Eagle Medium (DMEM) were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Lonza Walkersville Inc (Walkersville, MD, USA). Becton Dickinson (BD) Falcon 96 well culture plates were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Instrumentation. Nuclear magnetic resonance (NMR) analyses were conducted on a Varian U500 (500 MHz) or a VXR500 (500 MHz) spectrometer. High-performance liquid chromatography (HPLC) analyses were performed on a Shimadzu CBM-20A system (Shimadzu, Kyoto, Japan) equipped with a SPD20A Photodiode Array (PDA) detector (190 nm-800 nm) and a RF10Axl fluorescence detector, and an analytical C18 column (Shimadzu, 3 µm, 50*4.6 mm, Kyoto, Japan). Extrusion of liposomes was conducted with an Avanti mini-extruder (Avanti, Alabaster, AL, USA). The Diameter and diameter distribution of liposomes and microbubbles were
determined by using a ZetaPlus dynamic light scattering (DLS) detector (15 mW laser, incident beam = 676 nm, Brookhaven Instruments, Holtsville, NY, USA). Infrared spectra were recorded on a PerkinElmer 100 serial Fourier transform infrared spectroscopy (FTIR) spectrophotometer. Sonication used in the preparation of MBs was conducted with a 20 kHz Fisher 500 sonic dismembrator (ThermoFisher Scientific, Waltham, MA, USA) using a 1.9 cm diameter sonic horn. Confocal laser scanning microscopy images were taken on a Zeiss LSM 700 Confocal Microscope (Carl Zeiss, Thornwood, NY, USA). Fluorescence intensity of cells was measured on an IN Cell Analyzer 2200 system (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Protein bands were visualized with Image Quant LAS 4010 (GE Healthcare, Little Chalfont, UK). In vivo and ex vivo images were taken with a Bruker Xtreme In-Vivo Imaging System (Bruker, Billerica, MA, USA). Frozen tissues were embedded with optimum cutting temperature (O.C.T.) compound (Sakura Finetek USA, Torrance, CA, USA), sectioned with a Leica CM3050S Cryostat and mounted on a glass slide.

Cell culture. The 4T1 triple negative breast cancer cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM medium containing 10% FBS, 100 units/mL Penicillin G and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO₂ humidified air.

Animals. Female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Feed and water were available ad libitum. Artificial light was provided in a 12-h light/12-h dark cycle. The animal protocol was reviewed and approved by the Illinois Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign.
**Synthesis of AcManAz.** Bromoacetic acid (2.78 g, 20 mmol) was dissolved in deionized (DI) water (30 mL), followed by the addition of sodium azide (2.60 g, 40 mmol). The mixture was stirred at room temperature for 24 h. The resulting solution was adjusted to pH = 1 by adding hydrogen chloride solution dropwise, and then extracted with diethyl ether for three times (100 mL × 3). The organic phase was collected, dried over anhydrous sodium sulfate, and concentrated to yield 2-azidoacetic acid (80% yield, 1.62 g). 2-azidoacetic acid (1.01 g, 10 mmol) and N, N’-dicyclohexylcarbodiimide (2.06 g, 10 mmol) were dissolved in anhydrous DMF, followed by dropwise addition of N-hydroxysuccinimide (1.15 g, 10 mmol) in DMF. The mixture was stirred at room temperature for 24 h. The precipitate was filtrated off and the solvent was removed to yield a yellow solid. The crude product was recrystallized from dichloromethane/hexane to obtain N-(2-azidoacetyl) succinimide as a white solid (70% yield, 1.39 g). D-Mannosamine hydrochloride (539 mg, 2.5 mmol) and triethylamine (253 mg, 2.5 mmol) were dissolved in methanol (40 mL), followed by dropwise addition of N-(2-azidoacetyl) succinimide (545 mg, 2.75 mmol) in methanol. The mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure and the residue was re-dissolved in pyridine. Acetic anhydride (10 mL) was added and the reaction mixture was stirred at room temperature for another 24 h. After removal of the solvent, the crude product was purified by silica gel column chromatography using ethyl acetate/hexane (1/1, v/v) as the eluent to yield a white solid (45% yield, 485 mg). LRMS (ESI) m/z: calculated for C_{16}H_{22}N_{4}O_{10} [M + Na]^{+} 453.1, found 453.1. 1H NMR (CDCl₃, 500 MHz): δ (ppm) 6.58-6.66 (dd, 1 H, C(O)NHCH), 5.88-6.04 (dd, 1 H, NHCHCHO), 5.32-5.35 & 5.04-5.07 (ddd, 1 H, CH₂CHCHCH), 5.16-5.22 (dt, 1 H, CH₂CHCHCH), 4.60-4.63 & 4.71-4.74 (m, 1 H, NHCHCHO), 4.10-4.27 (m, 2 H, CH₂CHCHCH), 4.07 (m, 2 H, C(O)CH₂N₃), 3.80-4.04 (m, 1H, CH₂CHCHCH), 2.00-2.18 (s, 12
$^{13}$C NMR (CDCl$_3$, 500 MHz): $\delta$ (ppm) 170.7, 170.4, 170.3, 169.8, 168.6, 168.3, 167.5, 166.9, 91.5, 90.5, 73.6, 71.7, 70.5, 69.1, 65.3, 65.1, 62.0, 61.9, 52.8, 52.6, 49.9, 49.5, 21.1, 21.0, 21.0, 20.9, 20.9, 20.9, 20.8.

**Preparation of Ac$_4$ManAz-loaded liposomes functionalized with activated carboxylates.**

Hydrogenated L-$\alpha$-phosphatidylcholine (HSPC, 5 mg), cholesterol (1.7 mg) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-$N$-[succinimidyl (polyethylene glycol)-2000] (DSPE-PEG$_{2k}$-NHS, 3.5 mg) were dissolved in anhydrous chloroform (300 $\mu$L), followed by the addition of Ac$_4$ManAz (2 mg) in chloroform (100 $\mu$L). The mixture was left to dry for 12 h to form a lipid film. A hydration buffer (100 mM ammonium sulfate buffer, 300 $\mu$L) was added and the mixture was vortexed for 1 min. After incubating in a 60°C water bath for 15 min, the lipid mixture was further incubated at 30°C for 2 h. The lipid mixture then underwent five freeze-thaw cycles using liquid nitrogen and a 60°C water bath, and was further incubated at 37°C for at least 6 h. The liposomal suspension was then fine sized by extruding successively through a mini extruder equipped with a membrane filter (100 nm pore size) and two syringes for at least 15 times. Ac$_4$ManAz-loaded liposomes were then purified by passing through a Sephadex G100 column using ammonium sulfate buffer (150 mM) as the eluent. Collected liposomal solution was characterized by DLS and stored at 4°C for use. To determine the loading of encapsulated Ac$_4$ManAz, prepared liposome solution (5.0 mg/mL) was added Triton X100 with a final concentration of 10% (v/v). The solution was sonicated for 30 min and further incubated at 37°C for 6 h to completely disrupt liposomes. HPLC measurements were conducted to determine the amount of encapsulated Ac$_4$ManAz in prepared liposomes based on the standard curve of Ac$_4$ManAz.
For the preparation of blank liposomes, the procedures were the same except for not adding the Ac₄ManAz solution during the lipid film formation step.

**Preparation of amine-functionalized MBs.** MBs with a diameter of approximately 1 μm were prepared using the one-step sonication following the literature.¹ Four mL of 5% BSA (w/v) and 12 mL of 15% dextrose (w/v) were mixed in a 50 mL centrifuge tube, infused with perfluorobutane gas to saturate the mixture, and sonicated for 70 seconds using a 20 kHz Fisher 500 sonic dismembrator with a 1.9 cm diameter sonic horn to form MBs. Capped syringes containing MB solution were stored plunger-up in racks at 4°C. Larger MBs rose to the plunger within 6 h to form a distinctly white band. The lower milky fraction containing 1 μm-diameter MBs was collected. Size and size distribution of MBs was characterized by DLS. The MB solution was stored at 4°C.

**Preparation of Ac₄ManAz-loaded MB (Ac₄-MB), blank MB and Cy5-MB.** Ac₄ManAz-loaded liposomes or blank liposomes functionalized with activated carboxylates (20 mg/mL, 0.5 mL) in ammonium sulfate buffer or Cy5-NHS and amine-functionalized MBs (3.6×10⁸/mL, 2 mL) in PBS were mixed in a syringe, and shaken gently on a rotary plate for 24 h. The syringe was placed plunger-up in a rack and stored at 4°C overnight. The milky fraction containing MBs on the top was collected into another syringe. To purify the prepared Ac₄-MB or blank MB, fresh PBS was added to dilute the MB solution and the syringe was stored plunger-up in a rack at 4°C overnight. The milky fraction containing MBs on the top was collected. The purification steps were repeated once to completely remove unconjugated liposomes. To determine the sugar
loading, Ac₄-MB was disrupted under US exposure and treated with Triton X100, followed by HPLC measurement.

**Stability of Ac₄-MB and blank MB in 10% FBS.** Ac₄-MB was dispersed in 10% FBS (3 mL), and incubated at 37°C. The diameter and number density of Ac₄-MB were monitored via DLS at selected time points.

**Sugar release of Ac₄ManAz-loaded liposomes.** Ac₄ManAz-loaded liposomes were dispersed in PBS (pH = 7.4, 1 mL), and then transferred into a dialysis bag. The dialysis bag was immersed in PBS (10 mL) and incubated at 37°C (50 r/min). At selected time points (1 h, 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h), 0.5 mL aliquot of the dialysis medium was withdrawn for HPLC measurement, and the same amount of fresh medium was added.

**Double passive cavitation detection (DPCD) characterization of Ac₄-MB and blank MB.** The double passive cavitation detection (DPCD) experiments involved a transmit 4.6-MHz transducer and two receive 13.8- and 14.6-MHz transducers. Three-cycle tone bursts with a pulse repetition frequency of 10 Hz at the transmit center frequency were generated using a pulse-receive system (RAM-5000, RITEC Inc., Warwick, RI). Several thousand signals (8 series of 500 signals for each tested incident peak rarefractional pressure amplitude (PRPA)) were acquired. The complete setup including the transmit PRPA calibration procedures has been fully described elsewhere.²³ The DPCD analysis of collapsed ultrasound contrast agents (UCAs) (MBs) involved categorizing the UCAs according to a classification scheme based on the presence or absence of postexcitation signals (PESs). PES is defined as a secondary, short, broadband
response typically occurring between 1 and 5 μs after the principle response. In this study, the measurement (MB collapse) criteria were based on the 5% and 50% postexcitation thresholds. These collapse criteria are defined as the level at which a certain percentage of the total population of MBs transiently collapsed with PES for an applied PRPA. It is determined as the ratio between the total number of signals exhibiting PES and the total number of signals associated with the presence of a single bubble in the confocal region. Postexcitation curves were then obtained using a modified logistic regression using Matlab (The MathWorks Inc., Natick, MA) to fit the experimental data, allowing the evaluation of the 5% and the 50% postexcitation thresholds and their 95% confidence intervals.2,3

**In vitro cell labeling experiment.** 4T1 cells were seeded onto coverslips in a 6-well plate with a cell density of 40 k per well and allowed to attach for 12 h. Ac4-MB (50 μM in sugar equivalent) or blank MB (same amount of MB) was then added. Cells incubated with Ac4-MB were treated with focused US: the 6-well cell plate was placed upside down to allow the MBs to rise to the bottom surface of the cell plate where the cells were attached and the US was focused. After treatment with US for 1 min, the cells were incubated at 37°C for 72 h. The medium was removed and washed with PBS for three times. DBCO-Cy5 (25 μM) in Opti-MEM was added and the cells were incubated for another 1 h. Then the medium was removed and the cells were washed with PBS for three times. The cells were fixed for 10 min in 4% paraformaldehyde (PFA) solution, followed by staining of cell nucleus with DAPI (2 μg/mL) for 10 min. The coverslips were mounted on microscope slides and the prepared samples were stored in the dark for imaging.
**In vitro labeling kinetics of Ac₄ManAz-loaded liposomes in 4T1 cells.** 4T1 cells were seeded in black 96-well plates and incubated with Ac₄ManAz-loaded liposomes (50 μM in Ac₄ManAz equivalent) for different time (1 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h). After washing with PBS, cells were incubated with DBCO-Cy5 (20 μM) for 1 h. After cell fixation with 4% PFA, cell nuclei were stained with DAPI. Mean Cy5 fluorescence intensity of cells was measured on IN Cell Analyzer 2200. DAPI fluorescence channel was used for determining cell counts.

**In vivo labeling experiment with intratumoral injection of Ac₄-MB or blank MB:**

1) **Western blot analysis of tumor tissues.** 4T1 tumor models were established in 6-week-old female BALB/c mice by subcutaneous injection of 4T1 cells (1.5 million in matrigel/HBSS, 1/1, v/v) into both flanks. When the tumors reached to a diameter of approximately 5 mm, the hair over the tumor area was shaved, depilated and a layer of gel (to maximize US transmission through the skin surface) was spread over the tumors prior to US imaging. Ac₄-MB (25 mg/kg in sugar equivalent) or blank MB (same amount of MB) was injected into the tumors of Balb/c mice in 1 min, with simultaneous treatment of targeted US. The right tumors were treated with high-amplitude US pressure pulses (the US system’s output was set to a maximum at 100% to collapse MBs), while the left tumors were treated with low-amplitude US pressure pulses (the US system’s output was set to a very low amplitude of 4% so that the MBs would not collapse and yet still provide US imaging capability). US was continuously applied for another 1 min after the removal of the syringe. After three days, mice were euthanized and tumors were harvested and homogenized in lysis buffer (1% SDS, 100 mM Tris-HCl, pH 7.4) containing 1 tablet protease inhibitor (EDTA-free). The lysates were incubated at 4°C for 30 min, followed by centrifugation at 3000 rcf for 10 min to remove insoluble cell debris. The total soluble protein
concentration was determined by bicinchoninic acid (BCA) assay and adjusted to 5 mg/mL. Then 20 μL of the lysate was blocked with 5% bovine serum albumin (BSA) for 2 h and further incubated with DBCO-Cy3 in 5% BSA (20 μM) for 1 h. A loading buffer was added to each sample and samples were loaded onto a 10% SDS-PAGE gel after heating at 95°C for 5 min. After running the gel at 120 V for 120 min, proteins were transferred to a Hybond P membrane. The protein bands were visualized using an Image Quant LAS 4010 system with a Cy3/Cy3 (excitation/emission) channel.

2) In vivo and ex vivo biodistribution of DBCO-Cy5. In a separate study, three days after the intratumoral injection of Ac₄-MB or blank MB and US treatment, DBCO-Cy5 (5.0 mg/kg) was intravenously (i.v.) injected. BALB/c mice were placed on the sample stage equipped with anesthesia input and output ports, and imaged by the Bruker Xtreme In-Vivo Fluorescence Imaging System at 1 h, 5 h, 24 h, and 48 h post injection (p.i.) of DBCO-Cy5, respectively. The excitation filter was set at 630 nm and the emission filter was set at 700 nm. Collected images were analyzed by the Bruker molecular imaging software. Tumors, liver, lung, kidneys, heart, spleen, and brain were harvested at 48 h p.i. of DBCO-Cy5 and imaged ex vivo using the Bruker Xtreme In-Vivo Imaging System. Ex vivo images were quantified by measuring FI at selected region of interest (ROI). All values were expressed as means ± standard deviation (n = 3). After ex vivo imaging, tumors were bisected. Half of the tumor was directly frozen in O.C.T. compound and sectioned on a cryostat (Leica CM3050S) with a thickness of 8 μm. DAPI solution (2 μg/mL) was added to stain cell nucleus. After multiple washing steps, coverslips were mounted onto the microscope slides with the addition of ProLong Gold antifade reagent, and the prepared samples were stored in the dark for confocal imaging. Major organs and the other half of the tumor were placed into separate glass tubes containing 2 mL of lysis buffer (1% SDS, 100
mM Tris.HCl, pH 7.4, 1 tablet of EDTA-free protease inhibitor), homogenized, and incubated at 4°C for 2 h. The lysates were then centrifuged at 3000 rcf for 10 min to remove the insoluble cell debris, and measured on a fluorescence spectrometer to determine the amount of Cy5 retained in the tissues. The amount of Cy5 was calculated based on the standard curve of Cy5 FI and normalized to percentage of injected dose (%I.D.) per gram tissue.

**In vivo labeling experiment with i.v. injection of Ac₄-MB and blank MB:**

4T1 tumors were established in 6-week-old female BALB/c mice by subcutaneous injection of 4T1 cells (1.5 million in matrigel/HBSS, 1/1, v/v) into both flanks. When the tumors reached a diameter of approximately 5 mm, the mice were divided into 4 groups: Ac₄-MB with US treatment on the left tumors (Group 1), blank MB with US treatment on the left tumors (Group 2), Ac₄-MB without US treatment (Group 3), and PBS without US treatment (Group 4). Prior to US imaging, the hair over the tumor area was shaved and a layer of gel (to maximize US transmission through the skin surface) was spread over the tumor. Ac₄-MB (50 mg/kg in sugar equivalent, Group 1) or blank MB (same amount of MB, Group 2) was i.v. injected via the tail vein in 1 min, with simultaneous treatment of targeted US on the left tumors. The US was continuously applied for another 1 min after the removal of the syringe. The right tumors without US treatment were used as a control. A total of three Ac₄-MB injections were given at an interval of 24 h. Group 3 and group 4 mice were i.v. administered with Ac₄-MB (50 mg/kg in sugar equivalent) and PBS, respectively once daily for three days without any US treatment. At 24 h post the last injection of MBs, DBCO-Cy5 (5.0 mg/kg) was i.v. injected. BALB/c mice were placed on the sample stage equipped with anesthesia input and output ports, and imaged by the Bruker Xtreme In-Vivo Imaging System at 1 h, 6 h, 12 h, 24 h, and 48 h p.i. of DBCO-Cy5,
respectively. The excitation filter was set at 630 nm and the emission filter was set at 700 nm. Collected images were analyzed using the Bruker molecular imaging software. Tumors, liver, lung, kidneys, heart, spleen, and brain were harvested at 48 h p.i. of DBCO-Cy5. The ex vivo imaging was taken similarly using the Bruker Xtreme In-Vivo Imaging System. Ex vivo images were quantified by measuring FI at selected ROIs. All values were expressed as means ± standard deviation (n = 3). After ex vivo imaging, the tumors were bisected. Half of the tumor was directly frozen in O.C.T. compound and sectioned on a cryostat (Leica CM3050S) with a thickness of 8 μm. DAPI solution (2 μg/mL) was added to stain cell nucleus. After multiple washing steps, coverslips were mounted onto the microscope slides with the addition of ProLong Gold antifade reagent, and the prepared samples were stored in the dark for confocal imaging. Major organs and the other half of the tumor were placed into separate glass tubes containing 2 mL of lysis buffer (1% SDS, 100 mM Tris.HCl, pH 7.4, 1 tablet EDTA-free protease inhibitor), homogenized, and incubated at 4°C for 2 h. The lysates were then centrifuged at 3000 rcf for 10 min to remove the insoluble cell debris, and measured on a fluorescence spectrometer to determine the amount of Cy5 retained in the tissues. The amount of Cy5 was calculated based on the standard curve of Cy5 FI and normalized to %I.D. per gram tissue.

The control study with a one-injection regimen of Ac₄-MB was conducted similarly, except that BALB/c mice were i.v. injected with Ac₄-MB only for once (Day 1), followed by the i.v. injection of DBCO-Cy5 on Day 2. The left tumors were treated with US, while the right tumors without US treatment were used as controls.
The control study with muscle-targeted US treatment was conducted similarly, except that US was targeted on the right thigh muscles instead of tumors. BALB/c mice were i.v. injected with Ac₄-MB once daily for three days (Day 1-3), with simultaneous US treatment on the right thigh muscles. The left thigh muscles without US treatment were used as controls. DBCO-Cy5 was i.v. injected on Day 4.

**In vivo labeling experiment with i.v. injection of Cy5-MB:**

The experimental procedures are the same as the above-mentioned *In vivo* labeling experiment with i.v. injection of Ac₄-MB, except that Cy5-MB was used instead of Ac₄-MB. Cy5-MB (1 mg/kg in Cy5 equivalent) was i.v. injected once into BALB/c mice bearing subcutaneous 4T1 tumors and its biodistribution was monitored via fluorescence imaging.

**Statistical analysis.** The statistical analysis was performed by one-way analysis of variance (ANOVA) with post hoc Fisher’s Least Significant Difference (LSD) test (OriginPro 8.5) or Student’s t-test (two-tailed) comparisons at 95% confidence interval. The results were deemed significant at 0.01 < *P* ≤ 0.05, highly significant at 0.001 < **P** ≤ 0.01, and extremely significant at ***P* ≤ 0.001.
Fig. S1. Determination of sugar loading of liposomes via HPLC. The red line was the fitted standard curve of $\text{Ac}_4\text{ManAz}$, plotted as the integrated absorbance signal of $\text{Ac}_4\text{ManAz}$ at 220 nm versus the concentration of $\text{Ac}_4\text{ManAz}$. The solution of disrupted liposomes (5 mg/mL) showed an $\text{Ac}_4\text{ManAz}$ concentration of 266.3 μg/mL, as shown by the arrow. The sugar loading was calculated as: $100\% \times \frac{0.2663 \text{ mg/mL}}{5 \text{ mg/mL}} = 5.3\%$ (w/w).
Fig. S2. Sugar release profile of Ac$_4$ManAz-loaded liposomes (Ac$_4$-lipo) in PBS (pH 7.4) at 37°C.
**Fig. S3.** Diameter and diameter distribution of amine-functionalized MB and blank MB. PDI: polydispersity index.
**Fig. S4.** Confocal laser scanning microscopy (CLSM) images of 4T1 cells after incubation with Ac₄-ManAz loaded liposomes (Ac₄-lipo, 50 μM in Ac₄ManAz equivalent) and blank liposomes (same amount of liposomes), respectively for 72 h and subsequent labeling with DBCO-Cy5 (25 μM, red) for 1 h. The cell nucleus was stained with DAPI (blue). Scale bar: 10 μm.
Fig. S5. Mean Cy5 fluorescence intensity of 4T1 cells after incubating with Ac₄ManAz loaded liposomes (Ac₄-lipo, 50 μM in Ac₄ManAz equivalent) for different time and subsequently labeling with DBCO-Cy5 (20 μM) for 1 h. Cy5 fluorescence intensity of cells were measured on IN Cell Analyzer 2200 and normalized over cell counts.
Fig. S6. US imaging of the 4T1 tumors during the intratumoral injection of blank MB. 100% and 4% US pressure levels were applied to the right and left tumors, respectively, upon MB injection (1 min), and continuously applied for another 1 min after MB injection. The yellow arrow indicates the syringe, and the red arrow the MBs.
**Table**

<table>
<thead>
<tr>
<th>Group description</th>
<th># of Mice</th>
<th>Dose of MBs (in sugar equivalent)</th>
<th>Injection route</th>
<th>US treatment</th>
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<tr>
<td>Ac₄-MB</td>
<td>3</td>
<td>25 mg/kg</td>
<td>intratumoral</td>
<td>100% on right tumor, 4% on left tumor</td>
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<tr>
<td>Blank MB</td>
<td>3</td>
<td>0 (same amount of MB)</td>
<td>intratumoral</td>
<td>100% on right tumor, 4% on left tumor</td>
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</tbody>
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**Figure a**
- Schematic of MB injection and tumor growth.

**Figure b**
- Western blot and protein bands.

**Figure c**
- Gel electrophoresis image.

**Figure d**
- Fluorescence images of Ac₄-MB and Blank MB at 1 h, 5 h, and 24 h.

**Figure e**
- Ac₄-MB and Blank MB images.

**Figure f**
- Bar graph showing fluorescence intensity.

**Figure g**
- % ID/g graph showing liver, spleen, lung, heart, kidney, tumor-L, and tumor-R.

**Figure h**
- Images of Ac₄-MB and Blank MB in right and left tumors.
Fig. S7. (a) Time frame of *in vivo* imaging study. When the 4T1 tumors in both flanks of BALB/c mice reached a diameter of approximately 5 mm, the hair over the tumor area was shaved and depilated, and Ac₄-MB (25 mg/kg in sugar equivalent) or blank MB (same amount of MB) was injected into the tumors with simultaneous US exposures. The right tumors were treated with 100% US while the left ones were treated with 4% US as control. On Day 4, the expressed azido groups were detected by western blot analysis of tumor tissues or by monitoring the biodistribution of i.v. injected DBCO-Cy5 (5 mg/kg). (b) Detailed group descriptions of all experimental groups. (c) Western blot analysis of the right (R) and left (L) tumor tissues of the Ac₄-MB group, respectively. Tissue lysates were incubated with 5% BSA for 2 h and DBCO-Cy3 for 1 h at 37°C prior to gel running. Protein bands were visualized under a fluorescence imager. (d) *In vivo* whole body fluorescence imaging of BALB/c mice at 1 h, 5 h, and 24 h p.i. of DBCO-Cy5, respectively. (e) *Ex vivo* imaging of tumors and major organs at 48 h p.i. of DBCO-Cy5. 1-liver, 2-spleen, 3-lung, 4-heart, 5,6-kidneys, 7-left tumor, 8-right tumor. (f) *Ex vivo* Cy5 FI of major organs and tumors extracted from d. (g) Retained Cy5 in tissues of mice at 48 h p.i. of DBCO-Cy5. The Cy5 FI of tissue lysates was measured on a fluorescence spectrometer to determine the %I.D. per gram of tissue. (h) CLSM images of tumor sections from each group. All parameters were kept the same for each sample imaged. The cell nucleus was stained with DAPI (blue). Scale bar: 20 μm. All the numerical data were presented as mean ± SEM (n=3) and analyzed by one-way ANOVA (Fisher; 0.01 < *P* ≤ 0.05; **P** ≤ 0.01; ***P** ≤ 0.001).
**Fig. S8.** (a) Detailed group descriptions of all experimental groups. BALB/c mice were i.v. administered with Ac₄-MB (50 mg/kg in sugar equivalent) or Blank MB (same amount of MB) with simultaneous high amplitude US exposure on the left tumors once daily for three days (Day 1-3). DBCO-Cy5 (5 mg/kg) was i.v. administered via the tail vein on Day 4. (b) *Ex vivo* Cy5 FI of major organs and tumors harvested from BALB/c mice at 48 h p.i. of DBCO-Cy5. (c) Retained Cy5 in tissues of mice from all groups at 48 h p.i. of DBCO-Cy5. The Cy5 FI of tissue lysates was measured on a fluorescence spectrometer to determine the %I.D. per gram of tissue. All the numerical data were presented as mean ± SEM (n=3) and analyzed by one-way ANOVA (Fisher; 0.01 < *P* ≤ 0.05; **P** ≤ 0.01; ***P** ≤ 0.001).
Fig. S9. BALB/c mice bearing subcutaneous 4T1 tumors were i.v. injected once with Ac₄-MB (50 mg/kg in sugar equivalent), with simultaneous US treatment on the left tumors. The right tumors without US treatment were used as controls. At 24 h p.i. of Ac₄-MB, DBCO-Cy5 (5 mg/kg) was i.v. injected. (a) In vivo fluorescence imaging of BALB/c mouse at 48 h p.i. of DBCO-Cy5. The tumors were shown by yellow arrows. (b) Ex vivo imaging of major organs and tumors harvested from BALB/c mice at 48 h p.i. of DBCO-Cy5. (c) Mean Cy5 fluorescence intensity of tissues extracted from (b). Data were presented as mean ± SEM (n=3) and analyzed by Student’s t-test (two-tailed) (P > 0.05 is considered statistically non-significant and denoted as “n.s.”).
**Fig. S10.** BALB/c mice bearing subcutaneous 4T1 tumors were i.v. once with Cy5-MB (1.0 mg/kg in Cy5 equivalent), with simultaneous US treatment on the left tumors. The right tumors without US treatment were used as controls. (a) *Ex vivo* fluorescence imaging of major organs and tumors harvested from BALB/c mice at 48 h p.i. of Cy5-MB. (b) Mean Cy5 fluorescence intensity of tissues extracted from (a). Data were presented as mean ± SEM (n=3) and analyzed by Student’s t-test (two-tailed) (*P* > 0.05 is considered statistically non-significant and denoted as “n.s.”).
Fig. S11. BALB/c mice bearing subcutaneous 4T1 tumors were i.v. injected with Ac4-MB (50 mg/kg in sugar equivalent) once daily for three days (Day 1-3), with simultaneous US treatment on the right thigh muscles. The left thigh muscles without US treatment were used as controls. DBCO-Cy5 (5 mg/kg) was i.v. injected on Day 4. (a) In vivo fluorescence imaging of BALB/c mouse at 48 h p.i. of DBCO-Cy5. The thigh muscles were shown by yellow arrows. (b) Ex vivo fluorescence imaging of major organs and tumors harvested from BALB/c mice at 48 h p.i. of DBCO-Cy5. (c) Mean Cy5 fluorescence intensity of tissues extracted from (b). Data were presented as mean ± SEM (n=3) and analyzed by Student’s t-test (two-tailed) ($P > 0.05$ is considered statistically non-significant and denoted as “n.s.”).
References

