Electronic Supplementary Information:

Bioorthogonal Oxime Ligation Mediated *In Vivo* Cancer Targeting

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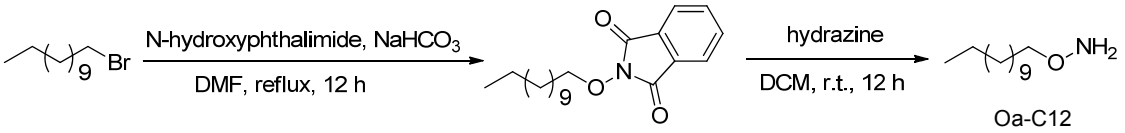
Experimental Section

General

Egg palmitoyl-oleoyl phosphatidylcholine (POPC) and 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). $_{D,L}$-Lactide (LA) was purchased from TCI America (Portland, OR, USA), recrystallized three times in toluene and stored at -30 °C in a glovebox prior to use. (S)-2,2',2",2"'-(2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid (*p*-SCN-Bn-DOTA) was purchased from Macrocyclics, Inc. (Dallas, TX, USA). Butyraldehyde-PEG-Valeric acid (bAld-PEG-VA, M_w=3400) was purchased from Laysan Bio, Inc. (Arab, Alabama, USA). All anhydrous solvents used in this study were purified by being passing through dry alumina columns and kept anhydrous by using molecular sieves. Other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and used as received unless otherwise noted. The NMR experiments were conducted on a Varian U400 (400 MHz), U500 or VXR500 (500 MHz) NMR spectrometer. Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) spectra was collected on an Applied Biosystems Voyager-DE^{TM} STR system. The size and the particle polydispersity of the liposome and the poly(ethylene glycol)-*b*-polylactide nanoparticle (PEG-PLA NP) were determined on a ZetaPALS dynamic light scattering (DLS) instrument (15 mW laser, incident beam = 676 nm, Brookhaven Instruments, Holtsville, NY, USA) and a JEOL 2100 Transmission Electron Microscope (TEM). The ζ-potential of freshly prepared liposomes and PEG-PLA NPs were evaluated by Malvern Zetasizer (Herrenberg, Germany). The 4T1 cells (ATCC, Manassas, VA, USA) used for *in vitro* and *in vivo* studies were cultured in DMEM medium containing 10% Fetal Bovine Serum (FBS), 100 units/mL aqueous Penicillin G and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The confocal microscopy images were taken on a Zeiss LSM700 Confocal Microscope (Carl Zeiss, Thornwood, NY, USA) using a 63×/1.4 oil lens with excitation wavelength set at 405 nm, 555 nm and 633 nm. The flow cytometry analysis of cells was conducted with a BD FACSCanto 6 color flow cytometry analyzer (BD, Franklin Lakes, NJ, USA). Female BALB/c mice were purchased from National Cancer Institute (NCI, Frederick, MD,

USA). Feed and water were available *ad libitum*. Artificial light was provided in a 12/12 hour cycle. The study protocol was reviewed and approved by The Illinois Institutional Animal Care and Use Committee (IACUC) of the University of Illinois at Urbana Champaign. Micro-PET/CT imaging was performed with small animal on a Siemens Inveon PET-CT system (Siemens Healthcare, USA). *Ex vivo* measurement of the radioactivity was conducted on a 2480 Wizard2 Automatic Gamma Counter (Perkin-Elmer). The flash frozen tumor tissue was embedded with optimum cutting temperature (OCT) compound (Sakura Finetek USA, Torrance, CA, USA) and sectioned with a Leica CM3050S cryostat and mounted on glass slides for histological analysis.

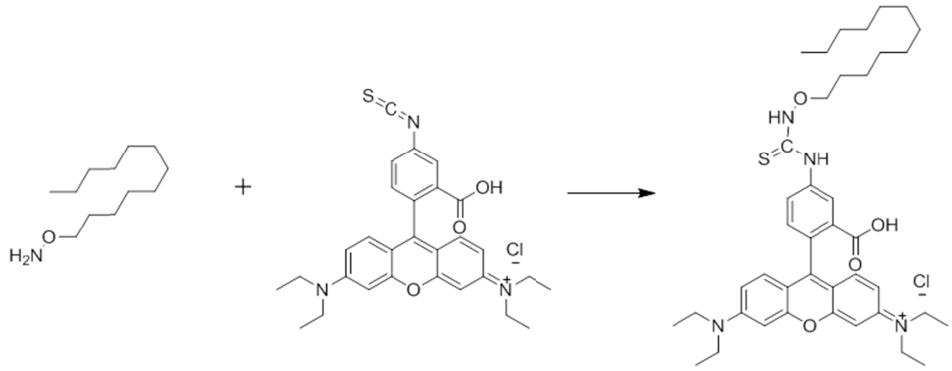
Synthesis of *O*-dodecyloxyamine (Oa-C12)



O-dodecyloxyamine (Oa-C12) was synthesized according to the published procedure.[1] To a solution of N-hydroxyphthalimide (3.92 g, 24.0 mmol) and sodium bicarbonate (2.02 g, 24.0 mmol) in dimethylformamide (DMF) at 80°C was added 1-bromododecene (3.86 mL, 16.0 mmol). The mixture was stirred and refluxed for 12 h. the reaction was diluted with DCM and washed with H_2O (6 × 100 mL), 1 M $NaHCO_3$ (3 × 100 mL) and H_2O (2 × 100 mL), dried over $MgSO_4$, and concentrated to afford a white solid (4.77 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85-7.83 (m, 2H), 7.76-7.73 (m, 2H), 4.20 (t, J = 6.8 Hz, 2H), 1.83-1.75 (m, 2H), 1.51-1.43 (m, 2H), 1.31-1.26 (m, 16 H), 0.88 (t, J = 6.8 Hz, 3H).

To a solution of the above compound (995 mg, 30 mmol) in dry DCM (10 mL) under argon was slowly added hydrazine (0.57 mL, 18.0 mmol). Upon addition, a white precipitate was immediately formed. The mixture was stirred at room temperature (rt) for 12 h. The reaction mixture was diluted with DCM (50 mL) and washed with H_2O (6 × 15 mL), dried over Na_2SO_4, and concentrated to afford a pale yellow oil (452 mg, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.33 (bs, 2H), 3.64 (t, J = 6.8 Hz, 2H), 1.57-1.54 (m, 2H), 1.28-1.20 (m, 18H), 0.88 (t, J = 6.8 Hz, 3H).

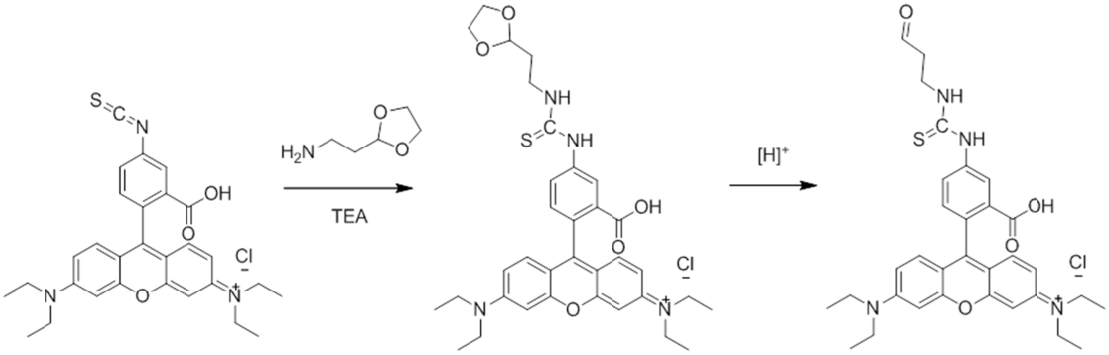
Synthesis of rhodamine labelled Oa-C12 (Rhd-C12)



Oa-C12 Rhd Rhd-C12

To a mixture of Rhodamine B isothiocyanate (Rhd, 28.4 mg, 0.053 mmol) and triethylamine (27 μL, 0.27 mmol) in anhydrous DMF (0.5 mL), Oa-C12 (10.7 mg, 0.053 mmol) was added under nitrogen and stirred at 60 °C overnight. The product is then precipitated by ethyl ether. After the solvent was evaporated, the crude product was purified by preparative TLC (dichloromethane:methanol, 9:1 v/v) and used directly.

Synthesis of Rhd bearing an aldehyde group (Ald-Rhd)

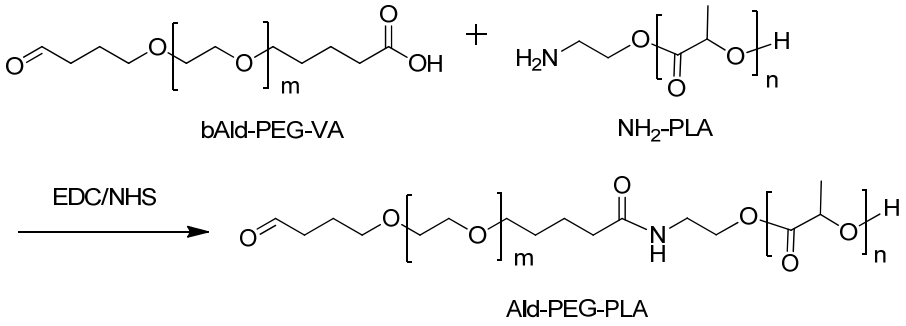


Rhd Ald-Rhd

To a mixture of Rhd (5 mg, 0.0093 mmol) and triethylamine (6 μL, 0.059 mmol) in methanol (0.3 mL), 2-(1,3-Dioxolan-2-yl)ethanamine (10 mg, 0.08 mmol) was added and stirred at 60 °C overnight. The product is then precipitated by ethyl ether. The dark purple precipitate is treated with acidic beads (Amberlyst® 15 hydrogen form) and stirred for 3

h at room temperature. Triethylamine was added until the solution reached pH=7. The reaction mixture was then concentrated to afford a dark purple powder and used without further purification. MALDI (m/z): calcd for $C_{32}H_{37}N_4O_4S^+$, 573.7, $[M]^+$; found, 573.0 $[M]^+$.

Syntheses of PEG-PLA polymer and aldehyde functionalized PEG-PLA (Ald-PEG-PLA)



The PEG-PLA and NH_2-PLA (25-mer) polymer conjugates were synthesized following a similar procedure as published before.[2] In a reaction vial containing anhydrous DMF solution (4.2 mL) of bAld-PEG-VA (85 mg, 0.025 mmol) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (4.8 mg, 0.025 mmol) and *N*-hydroxysuccinimide (NHS) (2.9 mg, 0.025 mmol). The reaction mixture was stirred at rt for 15 min followed by the addition of NH_2-PLA conjugate (146.9 mg, 0.025 mmol). The reaction mixture was stirred overnight under nitrogen at rt. The solvent was removed under vacuum to give Ald-PEG-PLA. The polymer was precipitated with ether (50 mL) collected by centrifugation, dried under vacuum and used directly.

Synthesis of fluorescently labeled Cy5-PLA polymer conjugate

Cy5-PLA (50-mer) polymer conjugate was synthesized following a similar procedure as published before.[3, 4] In a glove box, Cy5 (5.0 mg, 0.01 mmol) was dissolved in anhydrous tetrahydrofuran (THF, 900 μL) and mixed with a THF solution (500 μL) containing (BDI-EI)ZnN(TMS)$_2$ (6.5 mg, 0.01 mmol). The mixture was stirred for 15 min at rt. LA (72.0 mg, 0.5 mmol) was dissolved in THF (1 mL) and added to the stirred mixture of (BDI-EI)ZnN(TMS)$_2$ and Cy5. The reaction proceeded in the glovebox overnight. After

LA was completely consumed, the reaction was stopped by quenching the polymerization solution with cold methanol solution (300 μL). The polymer was precipitated with ether (50 mL), collected by centrifugation and dried under vacuum.

Synthesis of DOTA-PLA polymer conjugate

The DOTA-PLA polymer conjugate was synthesized following the similar procedure as published before.[2] In a reaction vial containing NH_2-PLA (25-mer; 14.4 mg, 0.004 mmol) was added an anhydrous DMF solution (0.5 mL) of *p*-SCN-Bn-DOTA (5.6 mg, 0.008 mmol) and triethylamine (3.6 mg, 0.036 mmol). The reaction mixture was stirred for 4 h under nitrogen at rt. The solvent and triethylamine were removed under vacuum to give DOTA-PLA, which was used directly without further purification.

General procedure for the preparation of liposome bearing Oa group (Oa-Lip)[1]

Oa-C12 (120 μL of 10 mM solution in chloroform, 1.2 μmol) were dissolved with egg-POPC (424 μL of 10 mg/mL in chloroform, 5.58 μmol) and DOTAP (10 μL of 10 mg/mL in chloroform, 0.14 μmol) followed by nitrogen blow to evaporate all the solvent to form a lipid film in the glass vial. The lipid film was further dried under high vacuum for 4 h. The dried lipid film was then rehydrated with PBS buffer (3 mL) and sonicated (15 W) with a tip sonicator for 20 min at 50 °C until the clear solution was formed. The formed liposome solution was brought to a final volume of 3 mL in PBS and sterilized with filter (0.45 μm).

For the preparation of fluorescent Oa-Lip (Rhd-Lip), Rhd-C12 (120 μL of 10 mM solution in chloroform, 1.2 μmol) were dissolved with the same amount of egg-POPC and DOTAP followed by the same preparing procedure.

General procedure for the preparation of PEG-PLA NP (NP) and PEG-PLA NP with surface-modified aldehyde groups (Ald-NP)

NP: The PEG-PLA NPs were prepared using nanoprecipitation method as described in previous publications.[2] A DMF solution of PEG-PLA polymer (200 μL, 10 mg/mL) was

added dropwise to rapid stirring Millipore water (4 mL). The resulting NPs were collected by ultrafiltration (5 min, 3000 × g, Ultracel membrane with 10,000 NMWL, Millipore, Billerica, MA, USA), washed with DI water and then characterized by DLS and TEM for particle sizes and size distributions.

Ald-NP: A DMF solution of the mixture of Ald-PEG-PLA (100 μL, 10 mg/mL) and PEG-PLA polymer (100 μL, 10 mg/mL) was added dropwise to rapid stirring Millipore water (4 mL). The resulting Ald-NPs were collected and washed similarly.

For the preparation of fluorescently labeled NP or Ald-NP, Cy5-PLA (100 μL, 10 mg/mL) was mixed with the DMF solution of PEG-PLA or PEG-PLA/Ald-PEG-PLA polymer for subsequent nanoprecipitation. The resulting fluorescent NPs were collected and washed similarly.

Preparation of radio labeled NP and Ald-NP[2]

A DMF solution of the mixture of Ald-PEG-PLA (100 μL, 10 mg/mL), PEG-PLA polymer (100 μL, 10 mg/mL) and DOTA-PLA (100 μL, 10 mg/mL) was added dropwise to rapid stirring Millipore water (6 mL). The resulting DOTA-Ald-NPs were collected and washed similarly. The DOTA-NPs were prepared similarly using PEG-PLA polymer (200 μL, 10 mg/mL) and DOTA-PLA (100 μL, 10 mg/mL).

The ^{64}Cu chloride (300 μCi, Washington University in at St. Louis, MO, USA) was mixed with NP or Ald-NP (3 mg) in NH_4OAc buffer (pH=5.5, 0.1 M, 0.5 mL). The mixture was incubated for 1 h at 60 °C. The NPs were washed by ultrafiltration (10min, 3000 × g, Ultracel membrane with 10 000 NMWL, Millipore, Billerica, MA, USA). To determine the labeling efficiency, the supernatant was collected and measured on γ-counter for the radioactivity. The purified ^{64}Cu-labeled NP or Ald-NP were used for injection.

ξ-potential measurements.

The ξ-potential of the liposomes or NP was determined with a Malvern Zetasizer. The freshly prepared NPs were dispersed in DI water to a concentration of 0.5 mg/mL.

***In vitro* 4T1 cell surface engineering with Rhd-Lip and Oa-Lip**

4T1 cells (50,000) were seeded in a 4-well chamber slide for 24 h. Cells were washed once with opti-MEM. A PBS solution of Rhd-Lip (300 μL, 2.1 mM) was added to 4T1 cells in 1 mL opti-MEM and incubated at 37 °C for 4 h. After liposome fusion, the cells were washed with PBS (3 × 1 mL), fixed with 4% paraformaldehyde, stained for nucleus with 4',6-diamidino-2-phenylindole (DAPI) and then imaged under a confocal microscope. Free Rhd-C12 at equivalent amount in Rhd-Lip was used as control.

In another study, a PBS solution of Oa-Lip (300 μL, 2.1 mM) was added similarly to 4T1 cells (50,000, seeded in a 4-well chamber slide) in 1 mL opti-MEM and incubated at 37 °C for 4 h. After liposome fusion, the cells were washed with PBS (3 × 1 mL) and incubated with 1 mL opti-MEM containing Ald-Rhd (0.1 mM) or Rhd (0.1 mM) at 37 °C for another 4 h. The cells were then fixed with 4% paraformaldehyde, stained for nucleus with DAPI and imaged under a confocal microscope.

***In vitro* 4T1 cell targeting with Ald-NP**

Confocal study: 4T1 cells (50,000) were seeded in a 4-well chamber slide for 24 h. Cells were washed once with opti-MEM and then incubated for 4 h at 37 °C with opti-MEM (1 mL) containing Oa-Lip (300 μL, 2.1 mM). After liposome fusion, the cells were washed with PBS (3 × 1 mL) and incubated with 1-mL opti-MEM containing Ald-NP (0.12 mg/mL) or NP (0.12 mg/mL) at 37 °C for another 6 h. The cells were then washed by PBS (3 × 1 mL), fixed with 4% paraformaldehyde and subsequently imaged on a confocal laser scanning microscope. Nuclei were stained by DAPI. Cells without the addition of Oa-Lip were imaged as the control.

Flow cytometry study: 4T1 cells (200, 000) were seeded in a 12-well plate for 24 h. Cells were washed once with opti-MEM and then incubated for 4 h at 37 °C with opti-MEM (1

mL) containing Oa-Lip (300 μL, 2.1 mM). After liposome fusion, the cells were washed with PBS (3 × 1 mL) and incubated with 1 mL opti-MEM containing Ald-NP (0.12 mg/mL) or NP (0.12 mg/mL) at 37 °C for another 6 h. The cells were then washed with PBS (3 × 1 mL) and detached via trypsinization. Cells were fixed with 4% paraformaldehyde for flow cytometry analysis (10,000 cells analyzed). The mean fluorescence intensity was assessed. All experiments were performed in triplicate.

***In vivo* tumor cell surface engineering with Rhd-Lip**

BALB/c mice (female, 6-week old) were injected subcutaneously on one flank with 1 × 10^5 4T1 cells suspended in a 1:1 mixture of HBS buffer and matrigel (BD Biosciences, Franklin Lakes, NJ, USA). 4T1 tumor-bearing BALB/c mice were divided randomly into groups of three (n = 3) and were treated when the mean tumor diameter was in the range of ~8.0 mm at Day 12 after inoculation. Each animal received a PBS solution of Rhd-Lip (200 μL per tumor, 2.1 mM) through intratumoral injection. The animals were euthanized 4 h or 24 h post the intratumoral injection and the tumors were collected. The flash frozen tumor tissues were embedded with optimum cutting temperature compound (OCT) (Sakura Finetek USA, Torrance, CA, USA). Tumor sections (5 μm thickness) were collected by cryostat with a Leica CM3050S cryostat and mounted on glass slides for confocal imaging.

***In vivo* biodistribution study with Micro-PET.**

Six female BALB/c mice (female, 6-8 week old) bearing subcutaneous 4T1 murine breast tumors (~8.0 × 8.0 mm) on the both flanks were divided into two groups (n = 3), minimizing the tumor size and body weight differences. In both of groups, the left tumor was injected with Oa-Lip (2.1 mM, 200 μL) and the right tumor was injected PBS (200 μL) as the control. The two groups of mice were then injected intravenously with ^{64}Cu-labeled Ald-NPs or NPs at a dose of 50 μCi 4 h post the intratumoral injections. Mice were placed on the micro-CT imaging bed and kept anaesthetized with a constant isoflurane flow (~2%). The micro-CT scan (80keV/500uA X-rays energy, 360 projections, 360 degrees, 75 μm pixel size) was used for determining the anatomical

localization of different organs. Static micro-PET scans were acquired at three selected time points (1 h, 6 h and 24 h p.i.) together with micro-CT scans for anatomical co-registration. The obtained micro-PET and micro-CT images were reconstructed using ordered subset expectation maximization (OSEM) and cone-beam algorithms with existing commercial software (Inveon Acquisition Workspace and Cobra Exxim, respectively). Micro-PET images were processed using 3-D median filtering and fused with micro-CT images. To quantify the radioactivity of ^{64}Cu in different organs, complex irregular volumes of interest (VOIs) were drawn on the micro-CT images and registered with the micro-PET images to determine mean counts in each VOI. To minimize partial volume effects, the anatomical borders of the organs were not included. The radiotracer activity from each VOI was normalized by injected dose and expressed as percent of the decay-corrected injected activity per cm^3 of tissue, which can be approximated as percentage %I.D./g assuming the density of tissue is ~1 g/cm^3. The initial total injected activity was determined by dose calibrator before the injection.

Radioactivity measurement with γ-counter

Mice were euthanized 24 h post injection. Both of 4T1 subcutaneous tumors were collected, weighed and measured for radioactivity (^{64}Cu) with a γ-counter (Wizard2, Perkin-Elmer, USA) using appropriate energy window centered at photo peak of 511 keV. Raw counts were corrected for background, decay, and weight. Corrected counts were converted to microcurie per gram of tissue (μCi/g) with a previously determined calibration curve by counting the ^{64}Cu standards.

Statistical Analyses

Student T-Test (two tailed) comparisons at 95% confidence interval were used for statistical analysis. The results were deemed significant at $*p < 0.05$.

Supplementary Figures and Tables

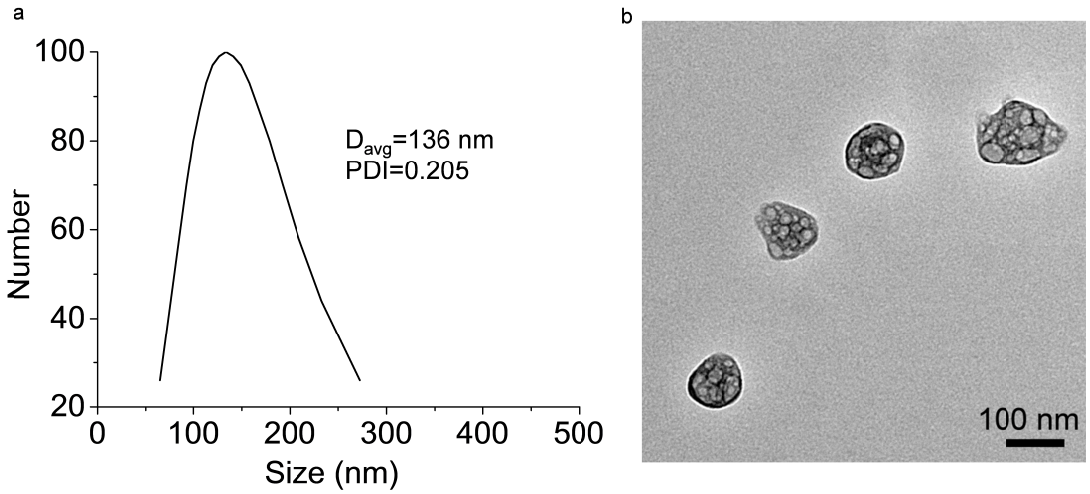


Figure S1. DLS (a) and TEM (b) characterizations of Oa-Lip. Insert is average diameter (D_{avg}) and polydispersity (PDI) in (a).

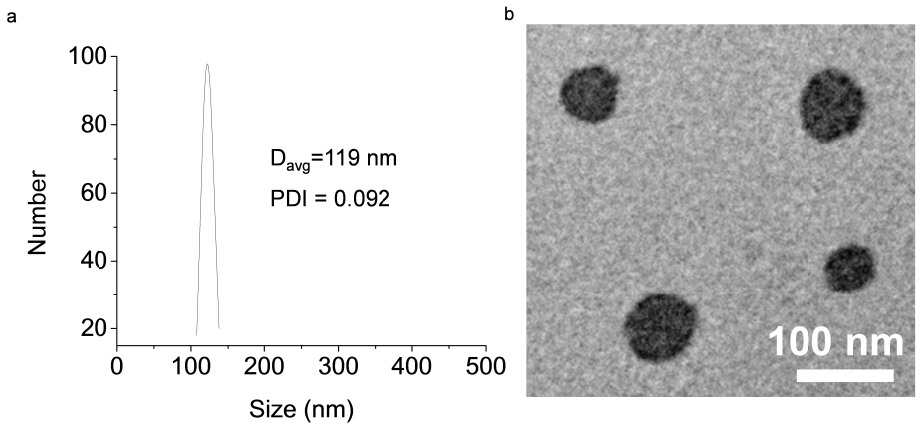


Figure S2. DLS (a) and TEM (b) characterizations of Ald- NPs. Insert is average diameter (D_{avg}) and polydispersity (PDI) in (a).

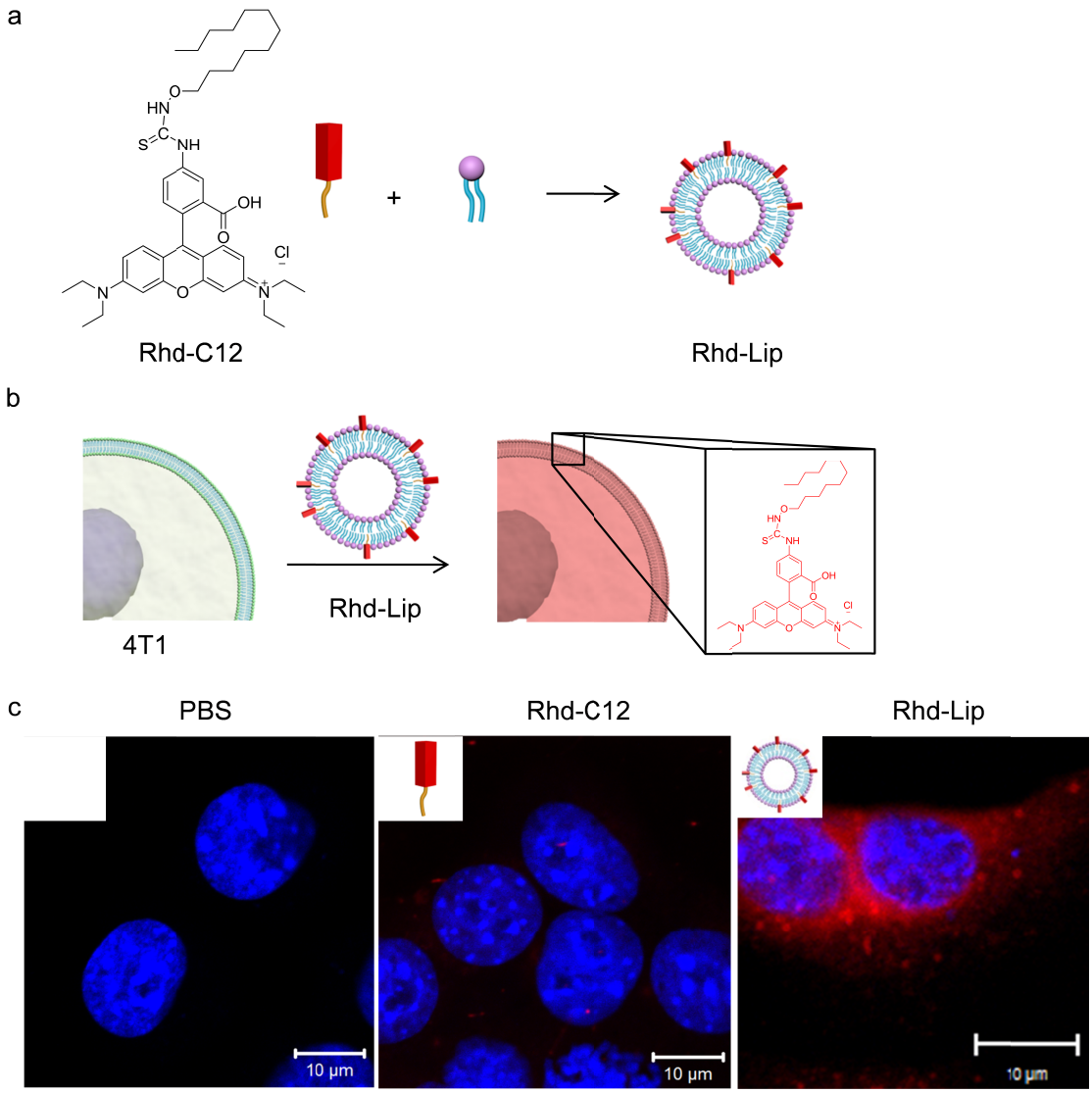


Figure S3. (a) Preparation of fluorescent liposome (Rhd-Lip). (b) Schematic illustration of liposome fusion with 4T1 cells. (c) Confocal microscope imaging of the 4T1 cells after fusion with Rhd-Lip or addition of free Rhd-C12, PBS as controls. Blue, DAPI stained nucleus; red, Rhd-Lip.

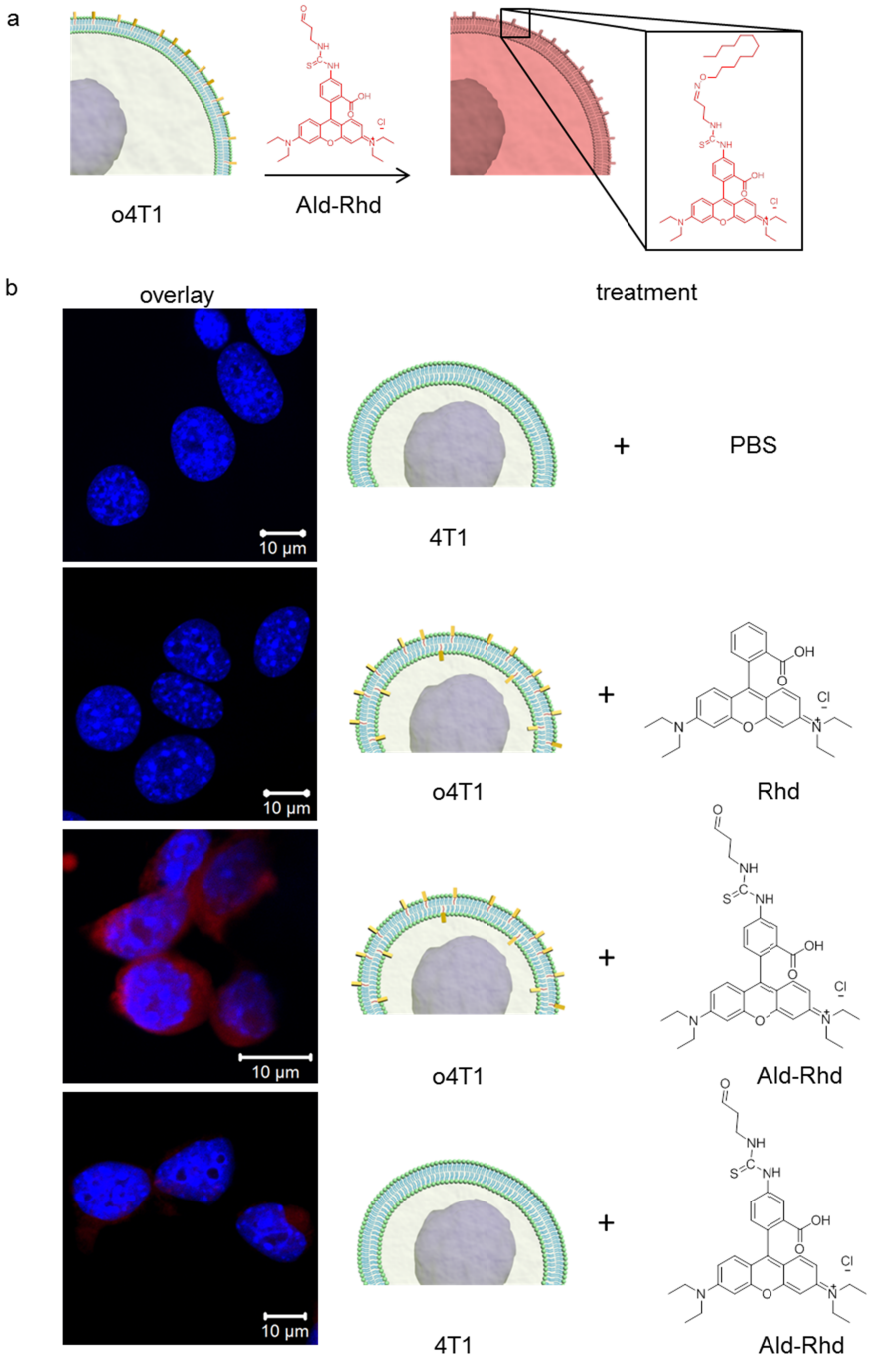


Figure S4. (a) Schematic illustration of oxime ligation between Ald-Rhd and Oa groups on 4T1 cell surface. (b) Verification of the reactivity of cell-surface Oa groups with Ald-Rhd by confocal microscope imaging of the 4T1 cells with different treatments. Blue, DAPI stained nucleus; red, Ald-Rhd.

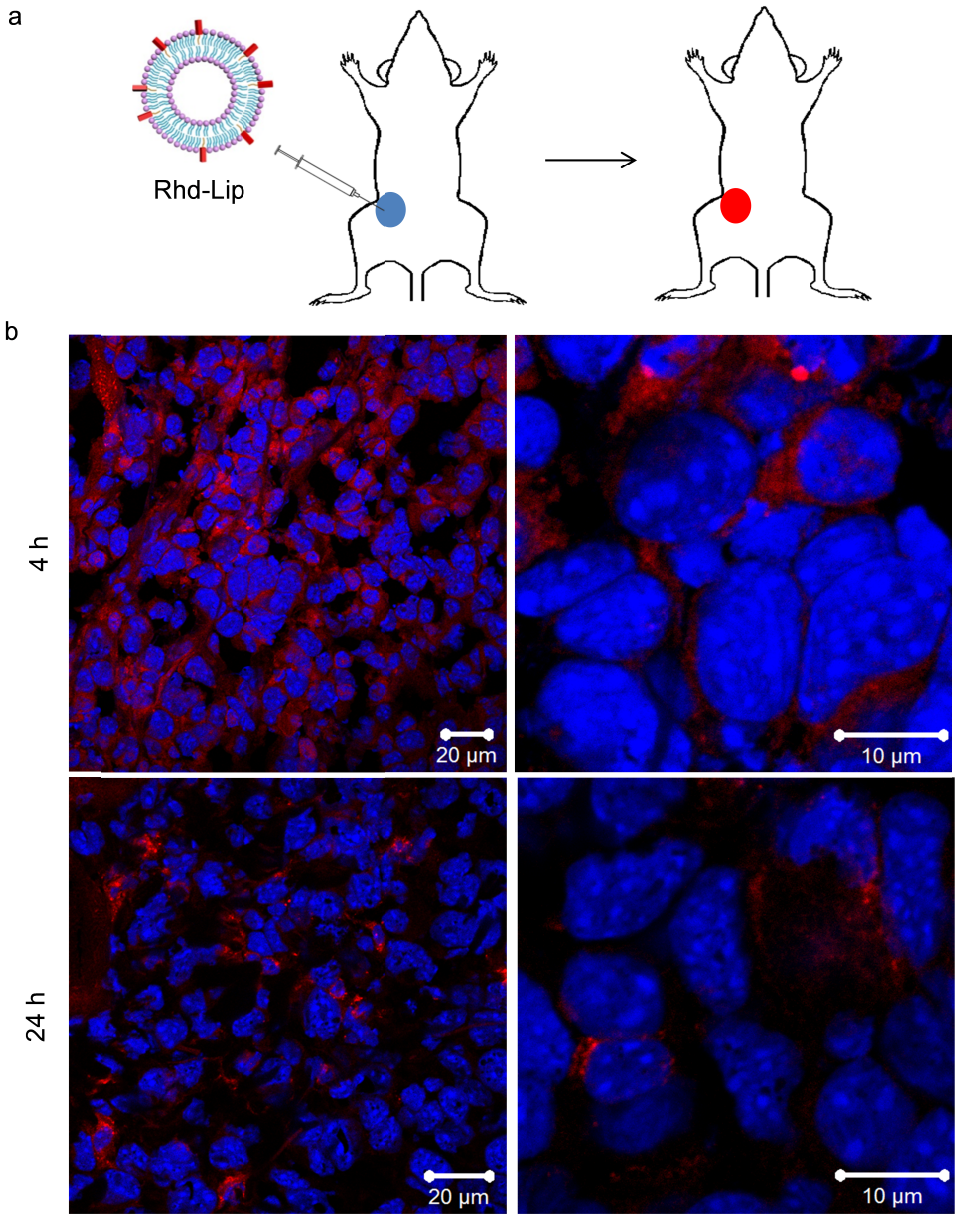


Figure S5. (a) BALB/c mice bearing subcutaneous 4T1 murine breast tumors received intratumoral injections of Rhd-Lip to test *in vivo* labeling of cell membrane in tumor. (b) Tumors were collected, sectioned, stained with DAPI (blue) and then images with confocal microscope to analyze the cell surface labeling by Rhd-Lip (red). Enlarged images are shown on the right.

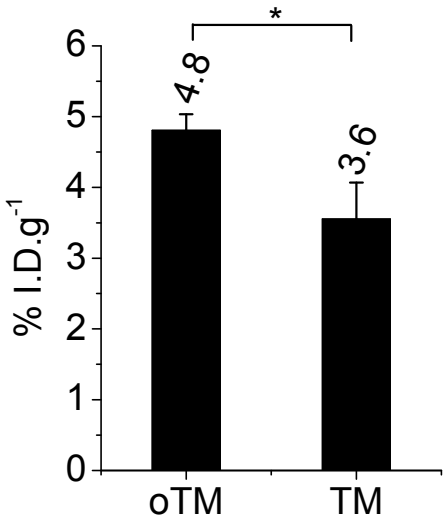


Figure S6. The accumulation of ^{64}Cu labeled Ald-NPs in the excised tumors (24 h p.i.) was measured *ex vivo* with a γ-counter.

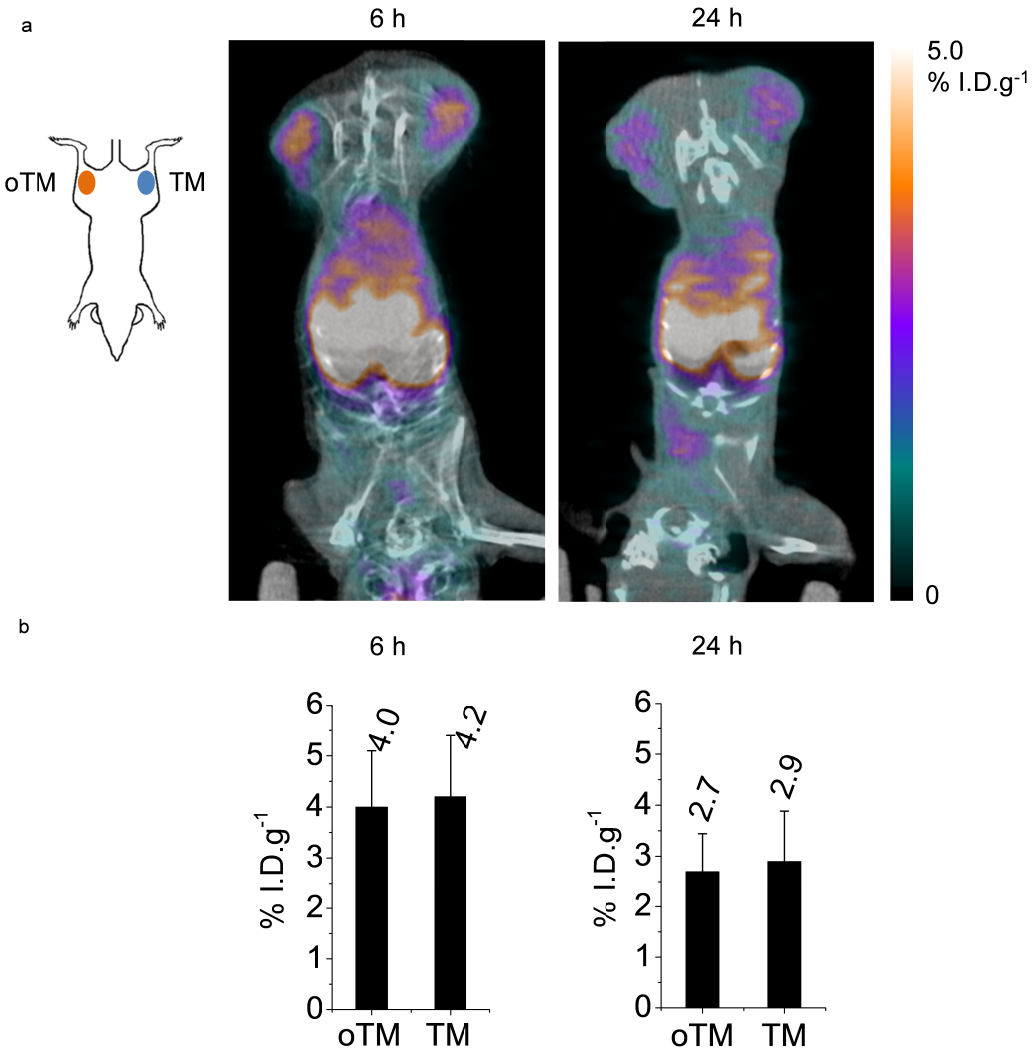


Figure S7. (a) *In vivo* whole-body dynamic PET/CT imaging of mice was performed to assess the tumor accumulation of the NPs without Ald groups on surface. (b) Accumulation of ^{64}Cu labeled NPs in the tumors over time was quantified by selecting the three dimensional regions of interest in the PET images and analyzing with the instrument software.

Table S1. ζ-potential measurements of liposome and NPs.

Particles	ζ-potential (mV)
Oa-Lip	12.3
NP	-10.0
Ald-NP	-9.5

References

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