Stable and Compact Zwitterionic Polydiacetylene Micelles with Tumor-Targeting Properties

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1. Materials

10,12-Pentacosadiynoic acid, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, *N*-hydroxysuccinimide, 3-(dimethylamino)-1-propylamine, 1,3-propanesultone, sodium hydride (NaH), and pyrene were purchased from Sigma-Aldrich and used without further purification. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) was purchased from Invitrogen. All solvents used were purchased locally and purified before use. Dialysis Membrane tubing was purchased from Spectrum Laboratories.

2. Measurements

NMR analysis was performed using a Bruker Avance 400 MHz spectrometer. Dynamic light scattering (DLS) measurements were carried out using a Malvern Nano-S instrument employing a 4 mW He−Ne laser (λ = 632.8 nm) and equipped with a thermostated sample chamber. UV-vis spectra were recorded using Cary 50 Probe UV−vis spectrophotometer. Fluorescence spectra were recorded on a Horiba Jobin Yvon (Fluoromax-4) fluorescence spectrometer. For ultrasonic mixing, an ultrasonic probe (Branson Sonifier 450, Output 4, Duty cycle 30%) was used. Photo-polymerization experiments are carried out using low-pressure mercury UV lamp (Heraeus) emitting light at a wavelength of 254 nm.

3. Synthesis of compound 2

Under N_2, 10,12-pentacosadiynoic acid (1 g, 2.6 mmol) was dissolved in CH_2Cl_2 (15 mL), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.82 g, 4.3 mmol) was added, followed by *N*-hydroxysuccinimide (0.58 g, 5.1 mmol) in 15 mL of CH_2Cl_2. The mixture was stirred at room temperature overnight and poured into water. The aqueous phase was extracted three times with CH_2Cl_2. The organic phases were collected, dried over Na_2SO_4, filtered, and concentrated under vacuum to afford compound **2** as a white powder (1.2 g, yield

95%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.83 (m, 4H), 2.61 (t, J = 7.4 Hz, 2H), 2.24 (t, J = 7 Hz, 4H), 1.74 (m, 2H) 1.51 (m, 4H), 1.25 (m, 26H), 0.88 ppm (t, J = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 169.1, 168.6, 65.2, 31.8, 30.8, 29.6, 29.4, 29.2, 29.1, 28.8, 28.7, 28.6, 28.3, 28.2, 25.5, 24.4, 22.6, 19.1, 14.1 ppm.

4. Synthesis of compound 3

Under N_2, compound 2 (0.40 g, 0.84 mmol) was dissolved in 7 mL of dry DMF. To this 3-(dimethylamino)-1-propylamine (170 mg, 1.7 mmol) in DMF (3 mL) was added dropwise over a period of 15 min, followed by triethylamine (0.6 mL, 4.2 mmol). The mixture was stirred overnight at room temperature, concentrated under vacuum to a minimum volume, and poured into water. The aqueous phase was extracted three times with CH_2Cl_2. The organic phases were collected, dried over Na_2SO_4, filtered, and concentrated under vacuum to afford compound **3** as a white solid (350 mg, yield 90 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.89 (brs, 1H), 3.33 (m, 2H,), 2.42 (t, J = 6.4 Hz, 2H), 2.26 (m, 10H), 2.13 (t, J = 8 Hz, 2H), 1.71 (m, 2H) 1.67 (m, 6H), 1.61 (m, 26H), 0.88 (t, J = 6.8 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.1, 65.1, 57.9, 44.8, 38.6, 36.8, 31.8, 29.5, 29.4, 29.3, 29.2, 29.1, 28.9, 28.8, 28.7, 28.3, 25.7, 25.6, 22.6, 19.1, 14.1 ppm.

5. Synthesis of DA-Zwitt (1)

Under N_2, compound **3** (300 mg, 0.65 mmol) was dissolved in 3 mL of dry CH_2Cl_2 before 1,3-propanesultone (240 mg, 1.96 mmol) in CH_2Cl_2 (2 mL) was added dropwise over a period of 10 minutes. The mixture was stirred at room temperature for 24 h. Solvents were evaporated and the residue was washed with Et_2O three times, dissolved in a minimal amount of $CHCl_3$, and re-precipitated by dropwise addition of Et_2O. The precipitate was dried under vacuum at room temperature to afford a pale pink product (300 mg, yield 80%). ^{1}H NMR

($CDCl_3$, 400 MHz) δ: 7.63 (brs, 1H), 3.68 (brs, 2H), 3.46 (brs, 2H), 3.33 (brs, 2H), 3.15 (brs, 6H) 2.89 (brs, 2H), 2.53 (brs, 2H), 2.23 (m, 6H), 2.05 (brs, 2H), 1.51 (m, 6H), 1.26 (m, 26H), 0.88 (t, J = 6.8 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 174.4, 65.3, 65.2, 50.8, 36.2, 31.9, 29.6, 29.5, 29.4, 29.3, 29.1, 29.0, 28.9, 28.3, 25.7, 22.6, 19.2, 14.1 ppm. MS-EI: 603 (M + Na).

6. Photo-polymerization of amphiphile self-assembly

A solution of amphiphile (10 mg) in saline water (1 mL, 0.9% w/v of NaCl) was sonicated with an ultrasonic probe for 10 min. The solution was then subjected to 254 nm light irradiation for 6 h to yield a yellow solution of photo-polymerized product. Deionized water was added to replace the volume that was lost by evaporation during the photo-polymerization process.

7. Determination of the critical micelle concentration (CMC)

The CMC of the amphiphile was determined by the pyrene florescence method. In a typical experiment, pyrene at a fixed concentration (10^{-6} m) was dissolved by sonication in varying concentrations (from 0.005 to 1 $g\,L^{-1}$) of DA-Zwitt. The colloidal solution was kept undisturbed for 5 h, then fluorescence spectra (excitation at 339 nm) were recorded and the ratio of fluorescence intensity for peaks at 374 and 383 nm (I_{374}/I_{383}) was measured and plotted against the amphiphile concentration (**Figure 2**A).

8. Dynamic Light Scattering (DLS)

DA-Zwitt (10 mg) was dissolved in saline water by sonicating at room temperature, the resulting micelle solution was filtered using 0.22 μm sized membrane filter and photo-polymerized the solution to get PDA-Zwitt micelles. The resulting pale yellow micellar

solution was diluted 10 times and again filtered using 0.22 μm sized membrane filter. Then, the size measurements were carried out using a Nano-S Malvern instrument over 12 runs of 10 s each.

9. Encapsulation of DiR

DiR (0.1 mg) solubilized in chloroform (100 μL) was added to the as prepared 10 mg mL^{-1} photopolymerized PDA-Zwitt micelles (1 mL) and the mixture was tip sonicated for 45 min. The clear colloid was filtered through a 0.22 μm membrane.

10. Payload leaching experiment

Samples of micelle solutions (1.5 mL) were transferred into a dialysis tubing (MWCO = 3 000) and dialyzed for 24 h against 1.5 L of deionised water with gentle stirring. Micelles were collected and the volumes of the different solutions were precisely adjusted to 10 mL by addition of deionised water. The amount of DiR that had leached out of the micelles during dialysis was assessed by absorbance spectroscopy. Measurements of the areas under absorbance curve at 750 nm of the dialyzed samples were compared to those of controls.

11. Cell culture

MDA-MB-231 GFP cells (Human breast cancer MDA-MB-231 cells transformed by a lentivirus to stably express the eGFP) were provided by Daniel Lewandowski (CEA, iRCM). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), l-glutamine (2 mM), penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) at 37 °C in a 5% CO_2 humid atmosphere. All culture reagents were purchased from Life Technologies.

12. Animal Models

All animal use procedures were in strict accordance with the recommendations of the European Community (86/609/CEE) and the French Committee (décret 87/848) for the care and use of laboratory animals. Ethics committee of CETEA – CEA DSV (Comité d'Ethique en Experimentation Animale (CETEA), de la Direction des Sciences du Vivant (DSV) du Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA)) approved the study (ref: 12-093).

All mice that were used for this study were female nude mice, with a weight of approximately 20 g and were housed under standard conditions with food and water *ad libitum*. For the creation of tumor xenograft model, a syringe was prepared containing 3 10^6 MDA-MB-231 GFP cells in a volume of 75 µL of phosphate-buffered saline (PBS) with 75 µL of Matrigel (BD Bioscience) at 0 °C. These cells were subcutaneously implanted between the shoulders of anesthetized mice and allowed to grow for several weeks until a tumor size of 100 mm^3 was reached (*ca.* 3 to 5 weeks). During each injection and imaging experiments, mice were anesthetized with isoflurane (1.25% in a 1:3 mixture of O_2 and air).

13. Blood Kinetic Experiments

Blood samples were collected from the retro-orbital venous sinus using heparin-coated capillary tubes before and after the intravenous administration of the fluorescent micelles. Samples (~30 µL) were collected from two mice at various time points (after 1 min, 5 min, 10 min, 30 min, 1 h, 2 h, 3 h, 6 h and 24 h). Just after blood retrieval, the fluorescence was measured by using the planar imaging option of the TomoFluo3D optical imager. ROIs of the capillaries were selected manually using the ImageJ software for semiquantitative analysis of fluorescence. The $T_{½}\alpha$ and $T_{½}\beta$ were calculated from blood kinetics using a two-compartment model.

14. In vivo Planar near infra-red (NIR) fluorescence imaging

Anesthetized mice were injected intravenously in the tail vein with 150 µL of a solution of DiR-labeled micelles (100 µM for DiR and 10 mg mL^{-1} for the monomer unit of the considered micelles). Planar fluorescence images of dorsal, left lateral and ventral side were obtained before and after the i.v. injection using the planar imaging option of the TomoFluo3D fluorescent tomographic system (developed by CEA/LETI and Cyberstar). Measurements were acquired at different time points (1 min, 30 min, 60 min, 3 h, 1 day and every following day over a period of 8 days post injection) using a 700 long pass filter set (Schott-RG9). For the semi-quantitative analysis of fluorescence planar images, region of interest (ROIs) of the desired zone (tumor and leg) were drawn manually using the ImageJ software (http://rsbweb.nih.gov/ij/). For each image, each ROI value was corrected by subtracting the background ROI's value (at the same zone but before injection) and dividing it by the exposure time.

15. *Ex vivo* Planar near infra-red (NIR) fluorescence imaging of organs

24 h post i.v. injection of DiR-labeled micelles, mice were euthanized and organ resection was performed. Planar fluorescence imaging of organs was performed as described above for *in vivo* imaging. For each organ, the mean of fluorescence signal was subtracted by the autofluorescence of the organ that corresponds to the mean of fluorescence signal measured for the same organ recovered from a mouse that was not injected with micelles.

16. X-ray CT/fDOT Multimodal Imaging

24 h after injection of the DiR-labeled micelles, the anaesthetized animals were positioned on a custom made multimodality mouse supporting plate that allows co-registration of X-ray CT

and fDOT-acquired data, and whole body X-ray computed tomography images were acquired with an Inveon microPET-CT scanner system (Siemens-Concorde Microsystems). The mouse supporting plate was thereafter transported and positioned in the TomoFluo3D apparatus scanner and a transillumination 740 nm-laser scan (5×5 points) was performed at the tumor level. The camera images of the different source positions were used for the reconstruction of the fluorescence signal according to a procedure that is described in details elsewhere.[40] The output of the optical reconstruction was given in 3D matrices of fluorescence signal with a voxel resolution of 0.67×0.67×1 mm^3 (X, Y, Z). 3D scans were done on the tumor zone. Co-registration and analysis of images from X-ray CT and fDOT imaging were performed using a homemade plugin developed in JAVA language and integrated in ImageJ. For co-registration, four landmarks are manually selected in both CT and fDOT images, and a rigid registration is performed using quaternion approach.[1]

17. Histology

Tumor tissues of mice injected with the PDA-Zwitt micelles were collected 24 h post injection. Tumor tissue was embedded in 4% Paraformaldehyde (PFA) and kept overnight at 4 °C. The following day the PFA 4% was replaced by a solution of 4% PFA and 20% sucrose and was also kept overnight at 4 °C. The tissue was removed from the solution, carefully frozen first in liquid isopentane (2-methylbutane), placed in a container and was finally embedded in liquid nitrogen for some seconds before storage at −80 °C.

Prior to frozen sectioning the tumor tissue on a cryostat-microtome, the tissue was embedded and mounted on the cryostat using the Shandon M-1 embedding matrix (Thermo Scientific). Tumor slices obtained had a thickness of 5 µm and were placed on microscope coverslips, rehydrated with H_2O, mounted with ProLong® Gold Antifade Reagent with DAPI (Life

[1] B. K. P. Horn, *J. Optic. Soc. Amer. A*, 1987, **4**, 629.

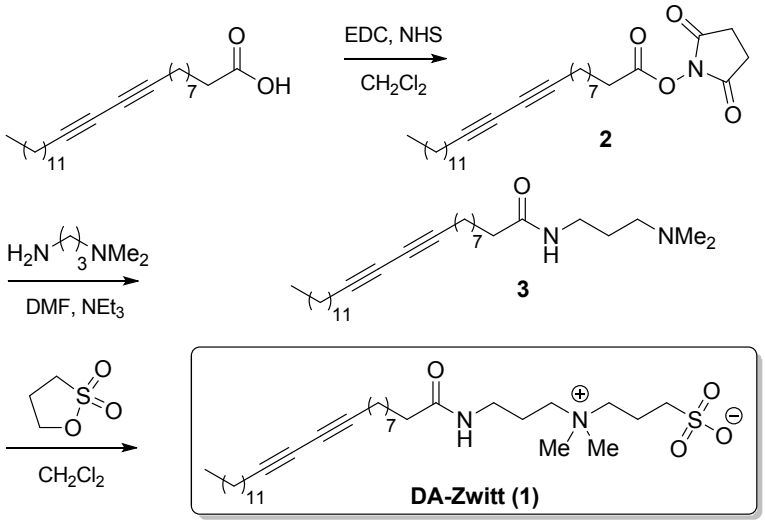
Technologies) and covered with a glass coverslip. Fluorescence microscopy images of the slices were acquired using a wide field AxioObserver Z1 epi-fluorescence microscope (Zeiss, Germany) with a 20× and 63× oil-immersed objective (N.A. 1.40).

18. Investigation of the micelle internalization pathway

The PDA-Zwitt micelles were loaded with 5 wt% of fluorescent DiO dye (λ_{em} = 510 nm) and incubated with MDA-MB-231 cells under various inhibitory conditions: energy depletion (4 °C), inhibition of clathrin- (chlorpromazine) and caveolae-dependent (genistein) endocytosis, and inhibition of micropinocytosis (amiloride).[2] The amount of internalized micelles was assessed by fluorescence-activated cell sorting (FACS). The obtained values were compared to those of a control experiment conducted under standard conditions, without any inhibitor. It appeared that PDA-Zwitt micelles were mainly internalized through an energy dependent caveolae-mediated endocytosis as genistein was the most efficient selective uptake inhibitor (see Figure S5).

[2] E. Gravel, B. Thezé, I. Jacques, P. Anilkumar, K. Gombert, F. Ducongé and E. Doris, *Nanoscale*, 2013, **5**, 1955.

19. Supplementary Scheme



Scheme S1. Synthesis of the DA-Zwitt amphiphile 1.

20. Supplementary Figures

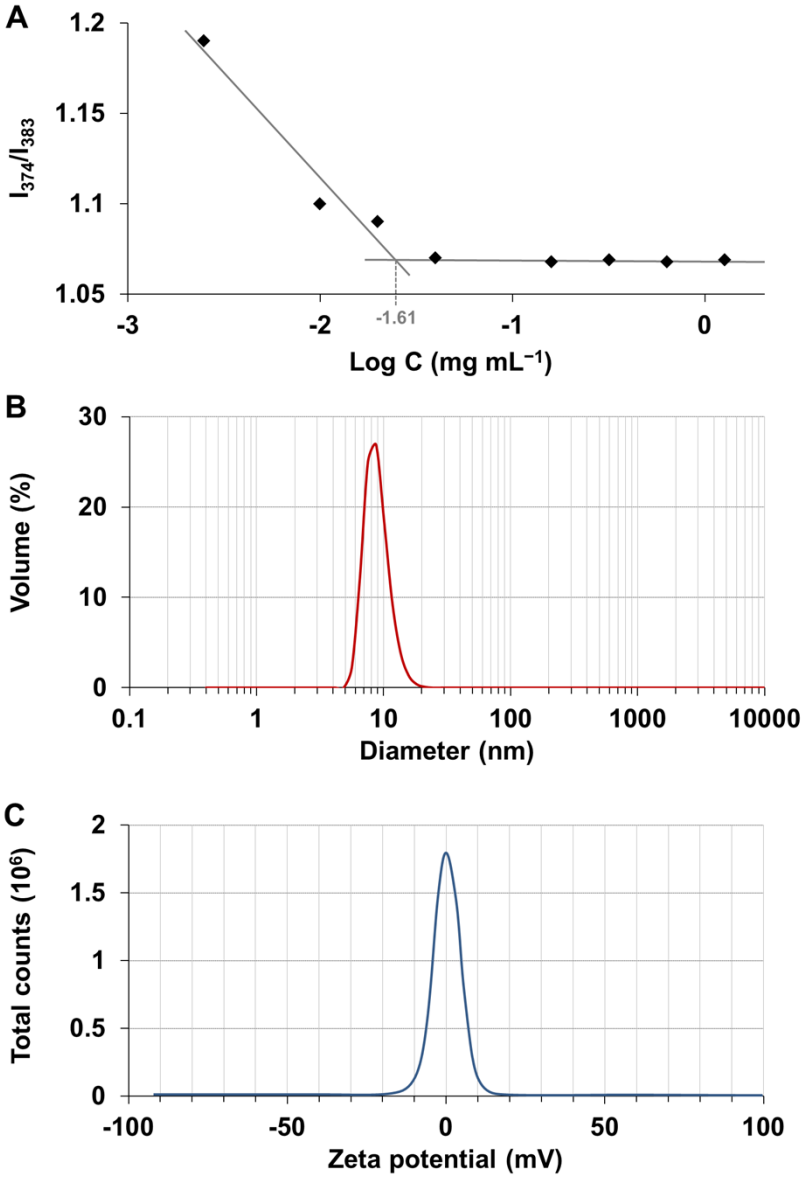


Figure S1. Micellar properties of DA-Zwitt amphiphile. A) Plot of CMC determination, B) size distribution of micelles by DLS, and C) zeta potential measurement.

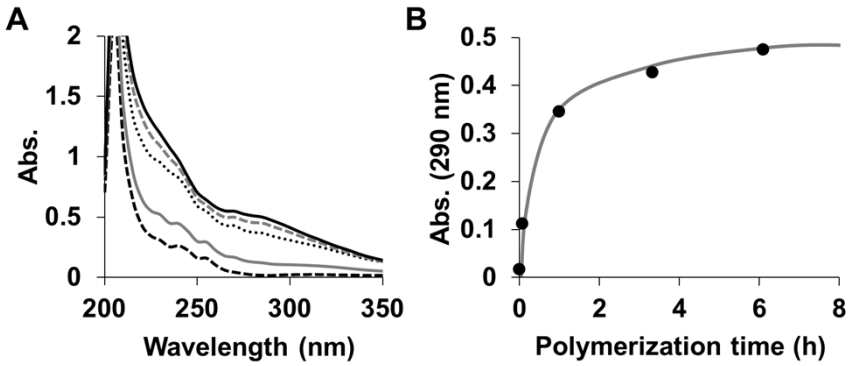


Figure S2. A) Absorption profile of the micelles before irradiation (dashed black line) and after 5 min (plain grey line), 1 h (dotted black line), 3 h (dashed grey line), and 6 h (plain black line) of polymerization; B) Evolution of the micelles' absorbance at 290 nm over the course of the polymerization process.

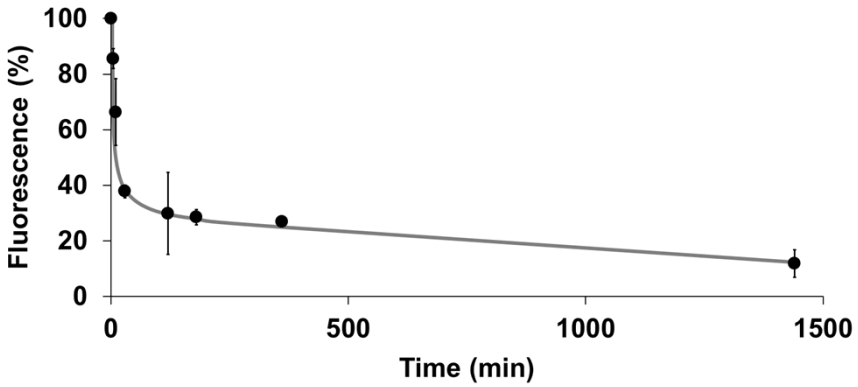


Figure S3. Evolution of PDA-Zwitt micelle concentration in blood after i.v. injection in function of time.

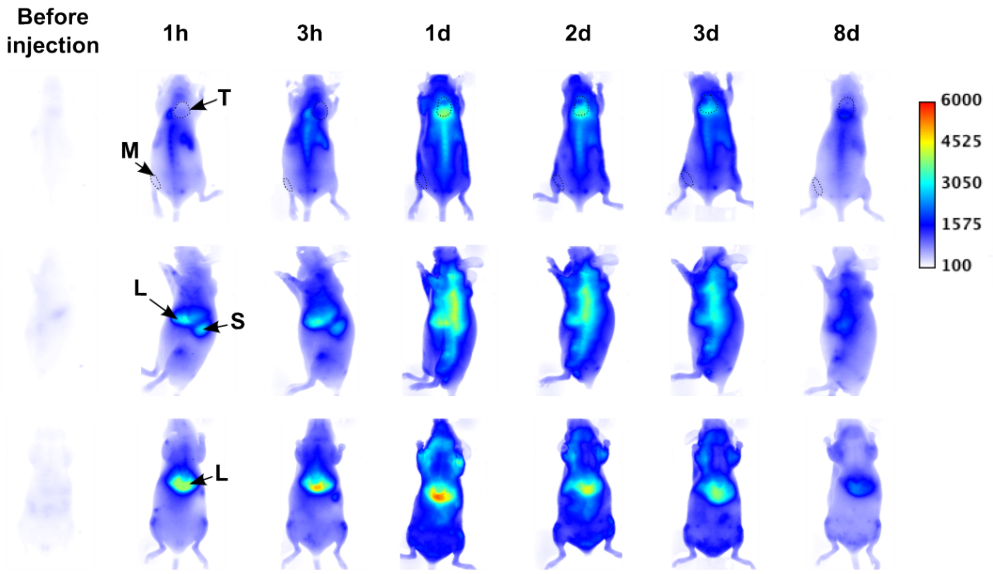


Figure S4. *In vivo* whole body NIR planar imaging of PDA-Zwitt micelles after intravenous injection in mice bearing MDA-MB-231 xenografts. Dorsal, lateral and ventral views are presented from top to bottom. On dorsal view, dashed regions and arrows indicate the areas of tumor (T) and muscle (M). On lateral or ventral view, arrows indicate liver (L) and spleen (S).

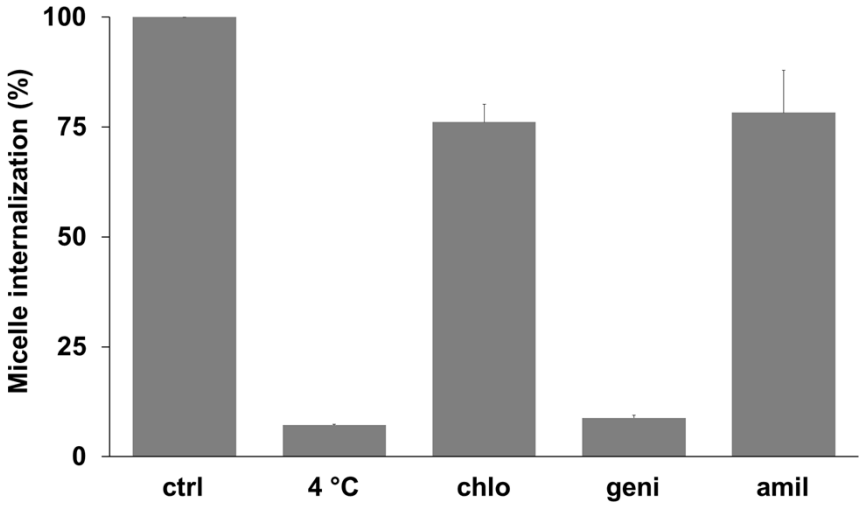


Figure S5. Uptake inhibition tests carried out by incubation of PDA-Zwitt micelles with MDA-MB-231 cells under various conditions (ctrl = control; chlo = chlorpromazine; geni = genistein; amil = amiloride).