Electronic Supplementary Material (ESI) for Nanoscale.

Vibrational Spectroscopy and Imaging Demonstrates Concurrent Cellular Trafficking of Co-localized Doxorubicin and Deuterated Phospholipid Vesicles

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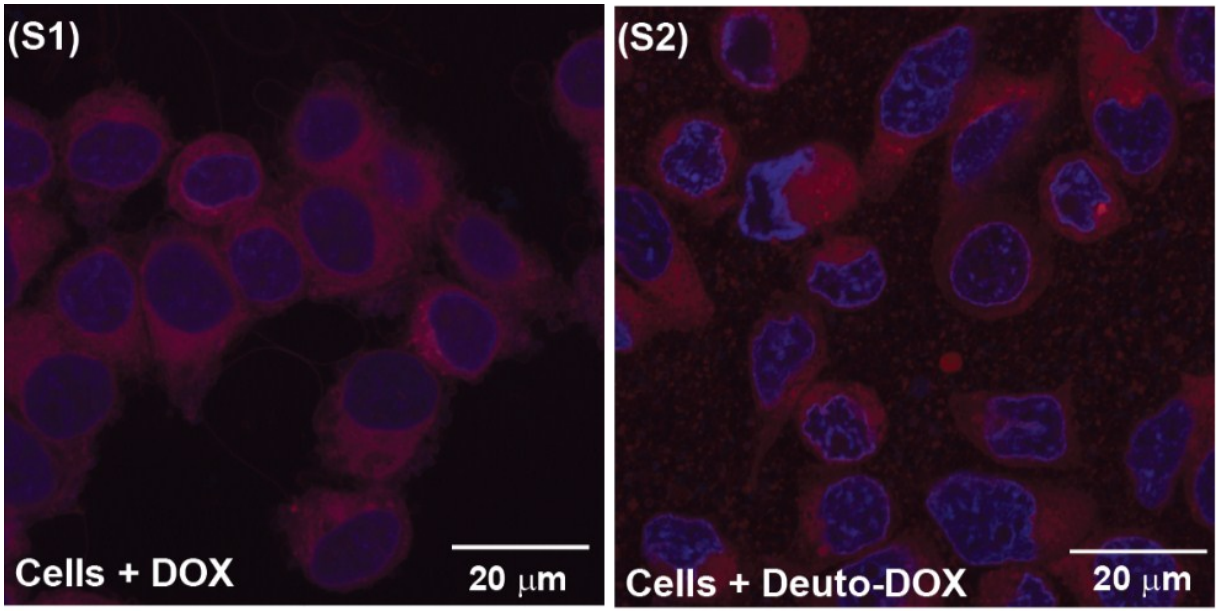

Figure. (S1) and (S2) show the fluorescence confocal images of cells stained with DAPI and loaded with DOX, stained with DAPI and loaded with Deuto-DOX respectively. Blue colors result from the DAPI emission (460 nm) that is concentrated at the nucleus, while the red colors result from the DOX emission at 550 nm. (S4) shows considerable overlap of the blue and red suggesting DOX trafficking at the nuclei.

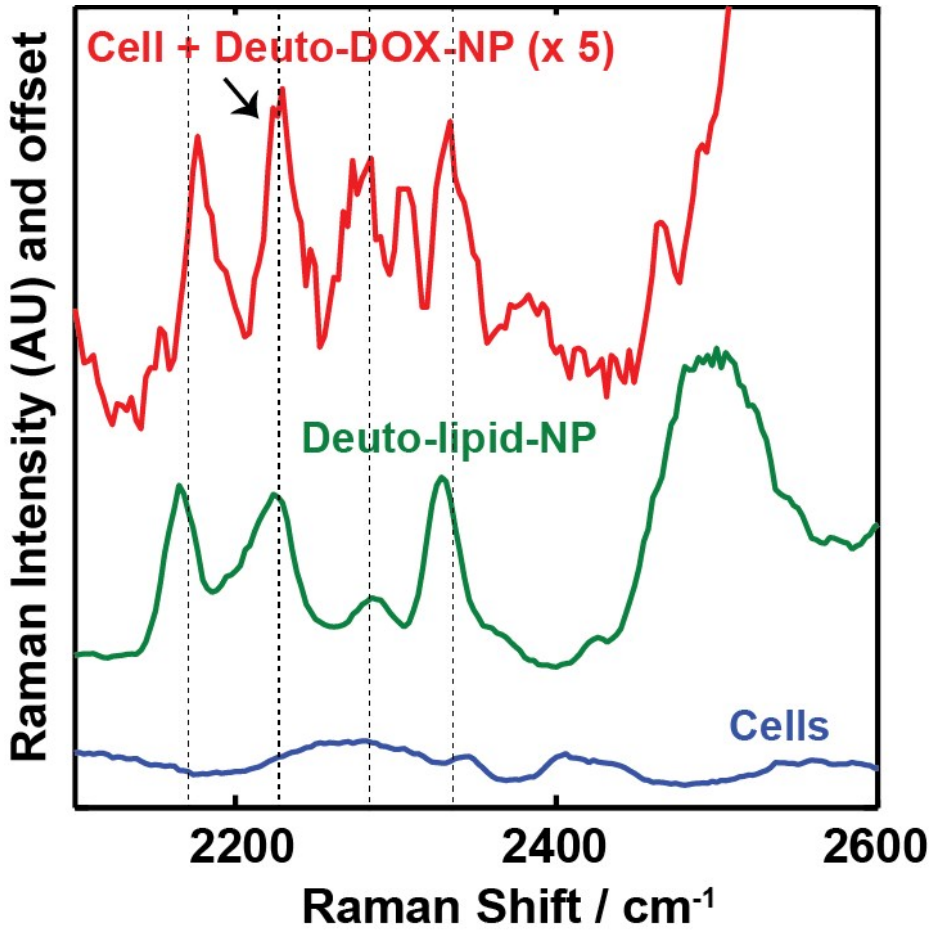

Figure S3. Outline of the C-D spectra in the 2000-2400 cm^{-1} range and highlights the spectral similarity of the Deuto-DOX particles in cells with respect to the control.

Materials

Dodecylphosphocholine (unlabelled) and Dodecylphosphocholine-d38 were purchased from C/D/N Isotopes, Quebec, Canada. Vesicles were prepared using freeze-thaw-sonication protocol with sonicated at required parameters (Q700, Qsonica Sonicators, Newtown, CT). Prepared vesicles were filtered by 0.2 μm filter (Millex, Merck Millipore Ltd., Tullagreen, Carrigtwohill, County Cork, Ireland). DLS measurements were acquired by Zetasizer Nano S, Malvern Instruments, Malvern, PA). Zeta potential measurement was performed on a Malvern Zetasizer instrument, from MRL facility, UIUC. UV−vis spectra were recorded on Genesys 10S UV−vis spectrophotometer machine. Atomic force microscopy was performed on MFP-3D AFM from Asylum Research using Igor Pro software. The TEM images were acquired on a JEOL 2100 Cryo TEM machine and imaged by Gatan UltraScan 2k × 2k CCD. The XRD data was collected on instrument Siemens-Bruker D5000 diffractometer and analyzed using software Jade X-ray analysis. Gel electrophoresis was performed using pBR322 vector DNA (New England Biolabs, Ipswich, MA) and imaged under Universal Hood III, Bio-Rad, Hercules, CA. Bright field images were followed under fluorescent microscope DMI3000 B, Leica Microsystems, Buffalo Grove, IL. Cytotoxicity was measured by MTT reduction using plate reader (Synergy HT, Bio-Tek). Biological experiments were performed in MDA-MB-231 and MCF-7 (ATCC) cells. Flow assisted cell sorting was performed on an iCyt Reflection machine from iCyt Mission Technology equipped with software Win List 3D.

Preparation of Vesicles with and without loaded Doxorubicin

Vesicular nanoparticles were prepared by freeze-thaw-sonication protocol. In a round bottom glass flask, 10 mg of Dodecylphosphocholine (unlabelled) and Dodecylphosphocholine-d38 were dissolved in 1 mL of chloroform (Sigma-Aldrich, St. Louis, MO) before evaporating under

reduced pressure of rotary evaporator at 40 °C with or without co-addition of DOX (MeOH solution) at 1:1 wt ratio. The organic solvents were evaporated in a rotary evaporator and placed under vacuum overnight. Autoclaved water was added to achieve concentrations of 1 mg/mL for the Lipid and Lipid-Dox samples, and 0.5 mg/mL for the Deuto-Lipid and Deuto-DOX samples. The resuspended samples were placed in 4˚C to hydrate the prepared membranes. All the samples were processed through heat-thaw cycles three times. Samples were heated at 60˚C in water bath for 5 min and kept on ice for 5 min followed by vortexing for 2 min in each cycle. Finally samples were sonicated in a bath sonicator for 30 min followed by probe sonication (Q700, Qsonica Sonicators, Newtown, CT) for 5 minutes (Amp:1, On: 2 sec, Off: 1 sec).

Physico-Chemical Characterization

Dynamic light scattering

After filtration with a 0.22 μm syringe filter (Denville Scientific, South Plainfield, NJ), 50 μL of the vesicle formulation was diluted to 1 mL of volume using autoclaved MQ water in plastic cuvettes, and measurements were taken using the Zetasizer Nano S (Malvern Instruments, Malvern, PA) to determine the hydrodynamic diameter of the particles. Three measurements were acquired for each sample.

Zeta potential measurement

After filtration with a 0.22 μm syringe filter (Denville Scientific, South Plainfield, NJ), 50 μL of the vesicle formulation was diluted to 1 mL of volume using autoclaved MQ water in plastic cuvettes, and measurements were taken using the Malvern Zetasizer instrument, from MRL facility, UIUC. Three measurements were acquired with 20 runs for each sample.

UV-Vis absorption spectroscopy

The absorption spectra for DNA, Dox, Lipid, Lipo-Dox, Deuto-Lipid, and Deuto-DOX were taken (GenesysTM 10S UV-Vis Spectrophotometer, Thermo Scientific, Rockford, IL) concentrations of 50 μM. In a 1 mL glass cuvette, the baseline was corrected with 1 mL of autoclaved water. Then, a solution of 125 μL HEPES (40mM), 500 μL NaCl (200mM), and 375 μL water was made and measured. To this solution, 50 μM of free DOX or loaded DOX formulations of Deuto-DOX and Lipo-DOX was added and measurements were taken again. Then, pEGFP-N1 DNA was added to achieve 50 μM of DNA in the solution, incubated at room temperature for 5 min, and measured.

X-ray diffraction studies

The ordered behavior of vesicular assemblies was determined by X-ray diffraction measurement. The aqueous suspensions of each formulation were placed on a pre-cleaned glass plate which, upon air drying, afforded a thin film of the formulations. X-ray diffraction (XRD) of an individual cast film was performed using the reflection method with a Siemens-Bruker D5000 diffractometer. The X-ray beam was generated with a Cu anode, and the Cu Kα beam of wavelength 1.5418 Å was used for the experiments. Scans were performed for 2θ range of 2 to 40.

Fluorescence spectroscopy

The fluorescence emission spectra for free Dox, Lipo-Dox, and Deuto-DOX were acquired using NanoDrop 3300, Thermoscientific fluorospectrometer at concentration of 50 μM of DOX along with controls of Lipid and Deuto-Lipid at same concentration. A 2 μL of formulation was loaded

on Nanodrop and followed the manufacturer's protocol to acquire the emission spectra from λ_{520} to λ_{580} with excitation at λ_{550} nm.

Fluorescence confocal microscopy

Photoluminescence microscopy measurements were carried out using two different instruments: a Zeiss 710 confocal scanner setup for the 460-nm and the 550-nm emission. Here, the sample was pumped at 405 nm and the emission was measured at 460 nm (DAPI) and 550 nm (DOX) using suitable filters.

Gel electrophoretic mobility assay

Samples of DOX in free or loaded form including Deuto-DOX (Dd), Lipo-DOX (Ld), Deuto-Lipid (DL) and Lipid (L) were incubated with duplex plasmid pEGFP-N1 DNA (D) in 1:1, 1:2, 1:3, 1:4, and 1:5 molar ratios of pEGFP-N1 DNA:DOX in free or formulation form at room temperature for 30 min. The samples were run on a 1% agarose gel at 100 V for 30 min. Then, the gel was stained in 3% ethidium bromide solution (10 mg/mL) for 5 min and washed in washing buffer (1x TAE buffer) for 5 min before being imaged (Universal Hood III, Bio-Rad, Hercules, CA).

Transmission Electron Microscopy Studies

For TEM, 2.5 μL of a diluted emulsion was placed on a 300-mesh carbon film supported by a copper grid and allowed to stabilize for 5 min. A filter paper was used to remove liquid for thin liquid films formation. Images were obtained using a Jeol 2010 cryo-electron microscope (Jeol Inc., Peabody, MA, USA) operated at 200 kV, and using different degrees of defocus to obtain an adequate phase contrast. Images were recorded on a Gatan UltraScan 2kx2k CCD (Gatan,

Pleasanton, CA, USA). These CCD images were processed and analyzed with ImageJ version 1.48 and were saved in a .tiff format.

Atomic Force Microscopic Studies

For AFM, four 2.5 μL portions were placed on clean glass and allowed to stabilize for 5 min. A filter paper was used to remove liquid, and the samples were further dehydrated using vacuum chamber. AFM images were obtained using an Asylum Cypher (Asylum Research, Santa Barbara, CA, USA) with tapping mode. The surface of the glass was scanned in air using Tap300Al-G AFM probes (Budget Sensors, Sofia, Bulgaria) at a set point of 0.63 V, a 1 Hz scan rate, and a drive frequency of 750 kHz.

Fourier-Transform Infrared (FTIR) Spectroscopy

As-synthesized particles were repeatedly applied to MirrIR IR-reflective glass slides (Kevley Technologies, Chesterland, OH, USA) to acquire IR spectra using a PerkinElmer Spotlight 400 (PerkinElmer, Waltham, MA, USA) equipped with a thermal source, and a raster-scanning linear array detector was used. Spectra were collected using a 1 cm s^{-1} mirror speed for acquisition, and zero padding was not used. Background scans were taken at 8 cm^{-1} spectral resolution; 150 × 150 μm images were collected at 8 cm^{-1} spectral resolution with 8 scans per pixel and a 6.25 × 6.25 μm pixel size. Data were atmosphere corrected on the spotlight and further processing was done using Matlab R2013 software.

Raman Spectroscopy

Raman spectra were acquired on dried samples in reflection mode (LabRAM Horiba, as discussed above). The excitation wavelength for all measurements was 532 nm and the power

was set to 25 mW at the sample with a 10-s acquisition time. The Raman shift from 400 to 4,000 cm^{-1} was collected at 8 cm^{-1} spectral resolution and the scanning stages were stepped 500 nm on both the x and y axes for image acquisition. Laser light was focused through a 100x, NA 0.8 objective into the sample plane and the scattering was collected in the reflection geometry using the spectrograph coupled with an Andor Newton back-illuminated EMCCD camera.

Processing protocol for separating the fluorescence contribution from the Raman signal

The region from 2000 to 2400 cm-1 has a dominant contribution from the fluorescence, which results in the intensity changes and incorporates an added oscillation (of constant phase) into the spectra. The precise location of the oscillatory peaks coincide with the peaks from the CD_3 and CD_2 symmetric and assymetric stretching vibrations. The entire analysis was carried out using ENVI and homebuilt codes in Matlab R2014. For representative purposes we show the spectral processing for one pixel in the image. To remove the noise we had to apply Minimum Noise Fraction (MNF) tranformations to the entire image. To remove the existing oscillations the following was employed. Since the source of the oscillations were traced to the fluorescence of the drug DOX, Fourier transformations of the Raman spectra with DOX were measured to identify the frequency of the oscillations. These select frequencies were removed from the Raman spectra of the cells with Deuto-DOX. In the resultant spectra the Raman features from the C-D were identified over the broad background and confirmed with second derivative analysis. This justified a rubber band baseline technique to remove any sources of non-oscillatory contributions of fluorescence from the spectra. Further confirmation of the presence of the C-D was obtained upon comparing the Raman spectra arising from blank regions of the slide.

Cell Culture

MD-MB231 cells (ER (−) breast cancer cells) and MCF-7 cells (ER (+) breast cancer cells) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal

bovine serum (FBS) in T25 culture flasks (Cellstar; Germany) and were incubated at 37 °C in a 99% humidified atmosphere containing 5% CO2. Cells were regularly passaged by trypsinization with 0.1% trypsin (EDTA 0.02%, dextrose 0.05%, and trypsin 0.1%) in DPBS (pH 7.4). Nonsynchronized cells were used for all the experiments.

Fluorescence Microscopy and Flow Assisted Cell Sorting (FACS) Analysis

MCF-7 (ER (+) breast cancer cells, ATCC, Manassas, VA) cells (60,000 cells per well) plated in 24-well plates. Deuto-Lipid, Deuto-DOX, and a solution of dox in water were used to treat the cells with concentrations of 0.05 mg/mL. After 4-hour incubation, cells were imaged under microscope for fluorescence imaging. After that media was pipetted out and the wells were washed with DPBS. After pipetting out the DPBS, 50 µL of 1X trypsin was added to each well and immediately pipetted out. The plate was incubated at 37°C and 5% CO_2 for 3 minutes. 300 µL of collection buffer (0.2% FS in DPBS) was added to each well, and the solutions were pooled together in collection tubes for FACS analysis.

Bright Field Imaging and Cell Viability Assay

MCF-7 (ER (+) breast cancer cells) and MDA-MB231 (ER (-) breast cancer cells, ATCC, Manassas, VA) were used for the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated at 10,000 cells/well in a 96-well plate (VWR, Radnor, PA) and incubated for 24 hours before treatments. Formulations of Deuto-Lipid, Deuto-DOX, and Dox were used to treat the cells at concentrations ranging from 0.1 to 0.0125 mg/mL in triplicates. After 44 hours of incubation, cells were imaged under microscope and bright field images were analysed for cell growth density and variation in morphology. After imaging, a 20 µL of MTT solution (5 mg/mL) was added to each well. All media were removed from the wells after an

additional 4 hours, and 200 μL of DMSO was added to each well to dissolve produced formazan crystals. After incubating for 5 min at room temperature, absorbance was quantified on Bio-Tek Synergy HTX Multi-Mode Reader, Winooski, VT. The % absorbance were correlated with % cell viability using following formula considering absorbance from untreated cells as 100%-

% Cell viability = [(A592 treated cells - A592 background)/(A592 untreated cells - A592 background)]x100