

## **Drug-sponge lipid nanocarrier for in situ cargo loading and release using dynamic covalent chemistry**

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## Experimental Section

All starting materials for synthesis were purchased from Alfa Aesar, Sigma-Aldrich unless specifically mentioned. To be specific, Acetic acid (>99.0%, Sigma-Aldrich), Sodium phosphate monobasic (>99.0%, Sigma-Aldrich) and sodium phosphate dibasic dihydrate (>99.0%, Sigma-Aldrich) were used to prepare 20 mM phosphate buffers at pH 7.4 and pH 4. DiD oil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate) (Cy5) were purchased from ThermoFisher Scientific. 4'-dioctylamino-3-octyloxyflavone (F888) were synthesized as described before.<sup>[1]</sup> Labrafac WL1349<sup>®</sup> was purchased from Gattefossé (Saint-Priest, France) Milli-Q water (Millipore) was used in all experiments. Chicken skin were obtained from chicken legs (Tyson industry) purchased in supermarket Lidl. Synthesis and characterization of all new compounds, including LipoHD, Nile Red Ketone (NRK), Nile Red Ketone-LipoHD conjugate (Lipo-NRK) and doxorubicine-LipoHD conjugate (Lipo-Dox) are described in the Supporting Information. Calculated LogP (cLogP) values for reported molecules were obtained using ChemDraw 16.0. All error bars correspond to standard deviation of the mean. Statistical analysis was done based on the one-tailed T-test, using Microsoft Excel. NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer. Mass spectra were obtained using an Agilent Q-TOF 6520 mass spectrometer.

### Optical spectroscopy

The water used for spectroscopy was Milli-Q water (Millipore), and all the solvents were spectral grade. Labrafac (Labrafac WL 1349<sup>®</sup>) is composed of medium-chain triglycerides and caprylic/capric triglyceride and was obtained from Gattefossé (Saint Priest, France). Absorption and emission spectra were recorded on a Cary 4000 Scan ultraviolet–visible spectrophotometer (Varian) and a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon) equipped with a thermostated cell compartment, respectively. For standard recording of fluorescence spectra, the emission was collected 10 nm after the excitation wavelength. All the spectra were corrected for the wavelength-dependent response of the detector. Size distribution was determined by DLS using a Zetasizer Nano ZSP (Malvern Instruments SA). In DLS, measurement statistics by volume was used.

### Formulation of cargo-loaded NCs

NCs were produced by spontaneous nanoemulsification: the cargo (dyes, LipoHD and/or Pro-NRK) were dissolved in Labrafac (50 mg and placed in sonication bath at 40 °C for 10 min. Then, the surfactant Kolliphor ELP<sup>®</sup> was added (50 mg), and the mixture was homogenized under magnetic stirring at 40 °C for 10 min up to complete homogenization. Finally, NCs were generated with the addition of ultrapure (Milli-Q) water (230 mg).

### pH-dependent release of Lipo-NRK

In order to prove that LipoHD modified NRK (Lipo-NRK) is more lipophilic and resistant to leakage than NRK, the conjugate was first synthesized chemically and used for the release model study. For NRK and Lipo-NRK release model study, five formulations were prepared:

NRK with and without F888, Lipo-NRK with and without F888 and F888 alone. The concentration of each dye in labrafac was 10 mM. The condition for testing the dye stability in the presence of empty NCs was created by mixing 100  $\mu$ L 100 times diluted dye-loaded NCs (by mixing 10  $\mu$ L NCs with 990  $\mu$ L distilled water) with 10-fold excess of blank NCs (10  $\mu$ L), which acted as the acceptor medium for the released dyes. The pH dependent release of Lipo-NRK was conducted by using buffers of ranging pH from pH 5 to pH 7.4 prepared by mixing different portion of acetate buffer (pH 4) with phosphate buffer (pH 7.4) during mixing with 10-fold excess of blank particles. Fluorescence of the sample was tested at several time points (5, 10, 15, 30, 60, 120, 240 and 480 min). Fluorescence intensity ratio of F888 and NRK (taken at 453 and 603 nm, respectively) under the excitation wavelength of 405 nm was measured. All the spectra were corrected by subtracting the blank corresponding to the empty NCs.

### ***In situ* formation of Lipo-NRK detected by FRET**

To evaluate the *in situ* reaction inside NCs, NRK was used as a model drug. 300 mL of NCs prepared from Labrafac containing LipoHD (90 mM) and Kolliphor ELP® (see above) was mixed with 12.6  $\mu$ L of 47 mM NRK and 300  $\mu$ L of pH 4 acetate buffer and reacted for 6 h (rt). Then, the emulsion was extracted by 600  $\mu$ L dichloromethane. TLC was used to examine the content in the organic phase compared with pure Lipo-NRK. Dichloromethane:MeOH=95:5 was used as the mobile phase for TLC. With the help of F888 as energy donor, the reaction was monitored by the FRET efficiency between F888, the FRET donor, and NRK, the acceptor. NCs prepared from Labrafac containing F888 (10 mM) and LipoHD (90 mM) were used for the reaction. NCs prepared from Labrafac containing only F888 (10 mM) were used as a control. The reaction was initiated by adding 300  $\mu$ L of pH 4 acetate buffer along with 12.6  $\mu$ L of 47 mM NRK. The reaction was diluted at different time points by mixing 10  $\mu$ L of the reacted solution with 990  $\mu$ L of pH 7.4 phosphate buffer. Then 100  $\mu$ L of this diluted solution was mixed with 10  $\mu$ L empty NCs and 880  $\mu$ L milliQ water, and the emission spectrum was measured under the excitation wavelength of 405 nm. The fluorescence intensity ratio of peak at 450 and 600 nm was calculated from the spectrum. All the spectra were corrected from the blank by subtracting the corresponding spectrum of empty NCs.

### ***In situ* formation of Lipo-Dox conjugate detected by FRET**

Doxorubicine (Dox), an anti-tumor drug, was also tested by this *in situ* pro-drug formation system. Similarly, 300 mL of NCs prepared from Labrafac containing LipoHD (90 mM) were mixed with 300  $\mu$ L acetate buffer (pH 4) along with 10  $\mu$ L of Dox-HCl (150 mM). In the control experiment NCs without LipoHD were used. The mixtures were vortexed for 24 h at 37 °C. The resulting solutions were dialyzed in 500 mL of 0.1 M NaHCO<sub>3</sub> for 24 h. After the dialysis, encapsulation efficiencies were tested based on the absorbance at 500 nm of the NCs solution before and after dialysis using 100 fold diluted reaction solution. Afterwards, NCs containing 90 mM LipoHD and 0.95 mM of DiD (Cy5) in Labrafac were prepared to monitor the reaction by FRET. To start the reaction, 10  $\mu$ L of 150 mM Dox-HCl solution in DMSO were added to 330  $\mu$ L of NCs along with 300  $\mu$ L pH 4 buffer, control was conducted with NCs containing only 0.95 mM of Cy5 in Labrafac. To study the *in situ* reaction kinetics, the reaction

at different time points was quenched with 100 fold of phosphate buffer (pH 7.4). Then, 100  $\mu\text{L}$  of this diluted solution were mixed with 10  $\mu\text{L}$  of empty NCs and 980  $\mu\text{L}$  milliQ water. The reaction was examined by measuring the fluorescence intensity ratio of Cy5 and Dox at 670 and 600 nm, respectively, under the excitation wavelength of 520 nm. After the reaction, the mixture underwent dialysis for 24 hours. UV absorption was used to determine the encapsulation ratio of Dox in the form of Lipo-Dox. NCs with the same concentration of Cy5 but without LipoHD were used as a control in the same protocol above.

### **Lipo-Dox NCs pH dependent release study**

To monitor the pH-controlled release of Dox, we used FRET technique similar to that used for Nile Red derivative NRK. Cy5/Lipo-Dox NCs were prepared by in situ reaction of NCs containing 90 mM LipoHD and 0.95 mM of DiD (Cy5) in Labrafac for 6 h and for dialysis to remove unreacted Dox. The obtained Cy5/Lipo-Dox NCs were mixed with 10-fold excess of blank nano-droplets and the emission spectra were recorded as a function of time at a range of pHs from pH 5 to pH 7.4 prepared by pH 7.4 (phosphate buffer) and pH 4 (acetate buffer). Finally, in order to test the compatibility with cell experiments media, PBS with pH 7.4 and pH 5 adjusted with acetic acid as well as 10% serum were also tested in similar manner. Fluorescence intensity ratio of Cy5 and Dox (peaks at 670 and 600 nm, respectively) under the excitation at 520 nm was measured.

### **Cell experiments**

HeLa cells (ATCC® CCL-2) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-Invitrogen), supplemented with 10% fetal bovine serum (FBS, Lonza) and 1% antibiotic solution (penicillin–streptomycin, Gibco-Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. HeLa cells (ATCC) were seeded on 12 mm coverslips at 30,000 cell per well in DMEM medium with penicillin (100 U/mL), 1% glycan (100 U/mL) and 10 % FBS overnight. The pH of PBS was adjusted with 1 M acetic acid and 1 M disodium phosphate to 5 and 7.4, respectively. Solution of F888/Lipo-NRK NCs (10 mM each in labrafac) or Cy5/Lipo-Dox NCs (sample from in situ reaction) pre-diluted 100-times in milliQ water (100  $\mu\text{L}$ ) was added to PBS of a given pH to a final volume of 1 mL, corresponding to 1000-times total dilution of NCs. The cells were washed 3 times with PBS and then the medium was replaced with a diluted solution of NCs at corresponding pH. Control NCs, containing F888/NRK or DiD/Dox, were also used in PBS medium at pH 7.4 and pH 5 to check the release of free NRK and Dox in cells. Cell imaging were conducted at two different time points: 1 min and 30 min. For both time points, the samples were imaged after washing with PBS.

All cellular imaging studies were done using epi-fluorescence mode with a Nikon Ti-E inverted microscope, equipped with CFI Plan Apo  $\times 60$  oil (numerical aperture = 1.4) objective, and a Hamamatsu Orca Flash 4 sCMOS camera. For Lipo-NRK and NRK imaging, the excitation wavelength was 550 nm, and emission was detected through 600/50 nm filter. For Lipo-Dox and Dox imaging, the excitation wavelength was 470 nm, and emission was detected through 531/40 nm filter. In the colocalization studies, F888 was detected using 390 nm excitation and 470 nm detection filter, whereas DiD was detected using 640 nm excitation and 705/80 nm detection filter.

The *in situ* extraction of molecules from cells was done as follows. Briefly, cells were first incubated with 15  $\mu\text{M}$  of Dox or 1  $\mu\text{M}$  of NRK (concentration determined by the absorption spectrum) for 30 minutes in the cell culture medium. Afterwards, cells were washed with PBS once, the fluorescence images were checked to ensure all the groups of cells were stained with similar amount of Dox and NRK (data not shown). Then, the medium was changed to 100-times diluted LipoHD NCs (above mentioned) in PBS medium. Control group was treated with empty NCs (the same dilution) in PBS, and the PBS buffer without NCs was used as blank control. Images were acquired immediately after the changing of medium defined as time point 1 min ( $<1$  min) and after 1 hour for Dox and NRK in situ uptake study. After imaging, mean intensity from each cell for NRK and Dox channels was analyzed excluding nucleus (the center black part of each cells). The obtained values are the mean fluorescence intensity from  $\sim 15$  cells.

To study the extraction media, HeLa cells were first stained with 1  $\mu\text{M}$  of NRK for 30 min, washed with PBS and then treated with different extraction media (1 mL): 100-fold diluted LipoHD NCs, blank NCs or PBS. The extraction media were collected after 1, 5, 15, 30 and 60 min of incubation with HeLa cells to examine the emission spectrum (each condition was done in triplicate). For PBS group, 100-times diluted LipoHD NCs were added to the extraction solution to ensure the comparable measuring condition for NRK. To quantify NRK concentration in the extraction media, the calibration curve of peak fluorescence intensity vs NRK concentration was built after measuring fluorescence spectra of LipoHD NCs (100-fold dilution) with increasing concentration of NRK. For estimating the total amount of NRK accumulated in HeLa cells, the emission spectra of the 1  $\mu\text{M}$  of NRK (1 mL), used for cell staining, was measured before and after cell staining in the presence of 100-times diluted LipoHD NCs.

To provide chemical identification of Lipo-NRK conjugates in the extract, NRK-stained HeLa cells were treated with the LipoHD NCs for 60 min. Then, the medium was collected and the extraction by dichloromethane was performed. The presence of Lipo-NRK in the extract studied by TLC (Dichloromethane:MeOH = 95:5) using as references pure Lipo-NRK and a the in-situ reaction mixture of NRK with LipoHD NCs extracted by dichloromethane (see above the protocol for the in-situ reaction). Finally, the Lipo-NRK from the same dichloromethane extract (cell medium with LipoHD NCs) was also identified by mass spectrometry.

### **Cytotoxicity study**

Four cancer cell lines were used: D2A1, 4T1 (mouse origin) and HeLa, MDA-MB-231 (human origin). The MTS cytotoxicity assay was used, which is based on the reduction of the MTS tetrazolium compound by viable mammalian cells to generate a colored formazan dye. The formazan dye is quantified by measuring the absorbance at 490-500 nm. Briefly, tumor cells (D2A1, 4T1, MDA-MB-231 or HeLa cells) were seeded at 5000 cell per well in a 96 wells plate. After 24h, the medium was removed and replaced by drug-containing (Lipo-DOX NPCs or DOX alone) medium (DMEM). To study time-dependent effect, HeLa cells were incubated for 12, 24 and 48 hours. For other three cell lines, incubation was done for 48 hours. After incubation, the medium was removed and replaced with MTS containing medium (0.5 mg/mL).

After 4h of incubation, absorbance was read at 490-500 nm. The values of absorbance corresponding to cell viability was normalized to 100% of the non-treated control cells.

### **In vivo experiments on mice**

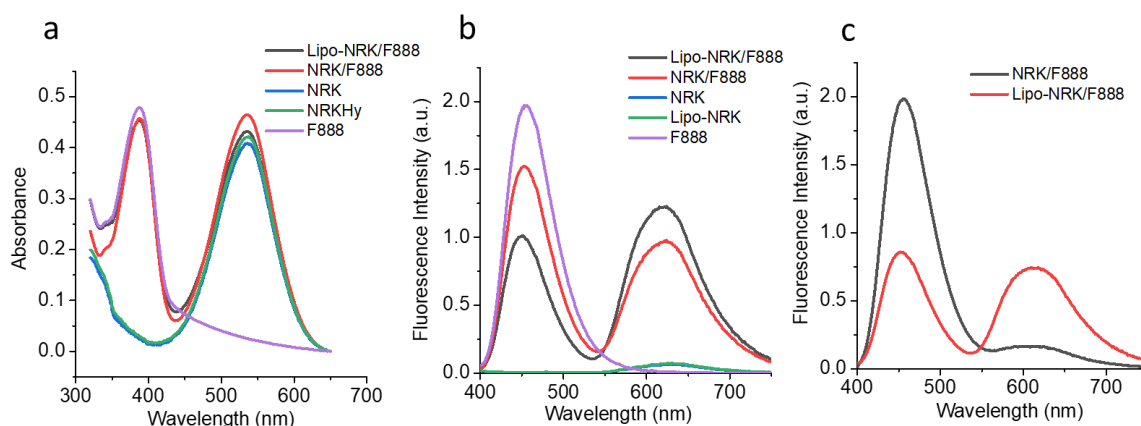
All mice were housed and handled according to the guidelines of INSERM and the ethical committee of Alsace, France (CREMEAS), under the APAFIS agreement number 12780-2017122016498879. For all experiments, 8 weeks-old female Rj:NMRI Foxn1 nu/nu mice were purchased from Janvier Labs.

2 millions of D2A1 or MDA-MB-231 stably expressing Luciferase cells were diluted in 50  $\mu$ l of PBS and injected subcutaneously in the back of 8-week-old female Rj:NMRI Foxn1 nu/nu mice. When tumors reached a tumor volume of  $\pm$  0.5 cm<sup>3</sup> (measured with a caliper using the following formula  $(width^2 \times length)/2$  (mm<sup>3</sup>)), mice were randomized in 4 groups of 8 mice and subjected to the 4 different treatments: PBS, Doxorubicin, LipoHD NCs or Lipo-Dox NCs. Lipo-Dox NCs were prepared in situ as described above by incubating LipoHD NCs with Dox at pH 4, followed by dialysis of the reaction mixture to remove excess of Dox. Final Dox loading in this formulation was 0.7 % with respect to oil. Before the injection, the nanoemulsion was diluted twice in PBS. Mice received the treatments by i.v. injection through the retro-orbital sinus, 2 times per weeks (alternating eyes at each round) of 100  $\mu$ l corresponding solution. For free Dox and Lipo-Dox formulations, the single injection dose of Dox was the same (51 $\mu$ g corresponding to  $\sim$ 2.5 mg/kg). Tumor growth was tracked two times/week either by caliper measurement (D2A1) or by in vivo bioluminescence imaging (MDA-MB-231). For bioluminescence imaging, mice were anesthetized with isoflurane and subjected to i.p. injection of 166  $\mu$ g/g of a luciferin solution (Perkin Elmer). Imaging was done 5 min upon injection on a IVIS Illumina LT (Perkin Elmer). Photon counts (photon/second/cm<sup>2</sup>/sr) were analyzed using Living Image software 4.5 (Perkin Elmer).

### **Experiment with adipose tissue from chicken skin**

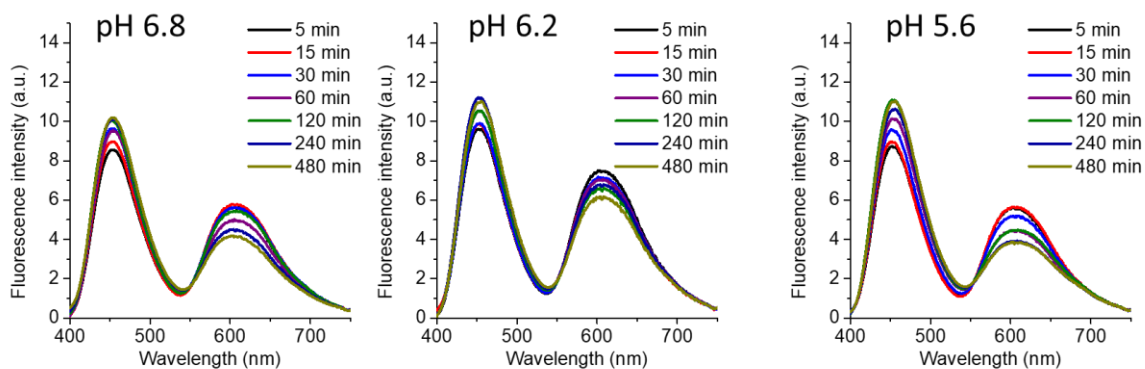
A serial of specially cut chicken skin circles (5 mm diameter) was washed with ethanol for 3 times to remove oil on the surface, followed by washing 3 times with water to remove ethanol. Then, chicken skin was immersed into aqueous solutions of 5  $\mu$ M NRK or 45  $\mu$ M Dox for 10 min. Then, the chicken skin tissue was washed with PBS once. The wet skin then was evenly spread in wells of a Lab-Tek® chamber, and the fluorescence images were acquired. Then, the medium was changed to 100-times diluted LipoHD NCs in PBS medium (300  $\mu$ L). Control group was treated with blank NCs (300  $\mu$ L, the same dilution) in PBS, and the PBS buffer (300  $\mu$ L) without NCs was used as a control. Fluorescence images were acquired immediately after changing the medium, defined as time point 1 min (<1 min). Then the skins with the buffers were removed into a 1.5 mL Eppendorf® tube and shaken for 30 min at speed of 600 r/min on a ThermoMixer at rt. Later on, skins were again flatly spread on the Lab-Tek® wells for the fluorescence microscopy measurements. Fluorescence imaging was done by the Nikon microscope (see above) using Nikon 20x air objective (Nikon CFI Plan Apo, NA = 0.75). For Dox and NRK imaging, the excitation wavelength was 550 nm, and emission was detected

through 600/50 nm filter. After the imaging, mean intensity for NRK and Dox channels was analyzed by selecting the center area (same size) of each images (3 images for each condition).



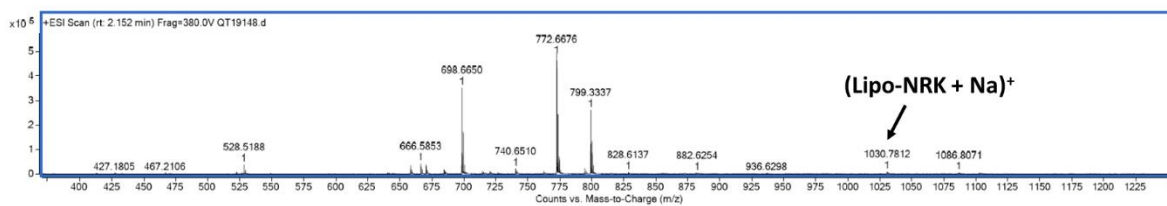
**Fig. S1.** (a) Absorption spectra of NCs loaded with NRK, Lipo-NRK, NRK/F888, Lipo-NRK/F888, F888. (b) Fluorescence spectra of NCs loaded with NRK, Lipo-NRK, NRK/F888, Lipo-NRK/F888 or F888. (c) Fluorescence spectra of Lipo-NRK/F888/ and NRK/F888 NCs after dilution and addition 20 fold-excess of black NCs.

The FRET NCs showed additional absorption band of F888 centered at ~390 nm (Fig. S1a). Control NCs containing F888 only showed strong donor emission, while the NCs loaded with only acceptor (NRK or Lipo-NRK), excited at 405 nm showed poor emission, suggesting inefficient direct excitation of the acceptor (Fig. S1b). These results confirmed FRET inside NCs between co-encapsulated donor (F888) and acceptor (NRK or Lipo-NRK).

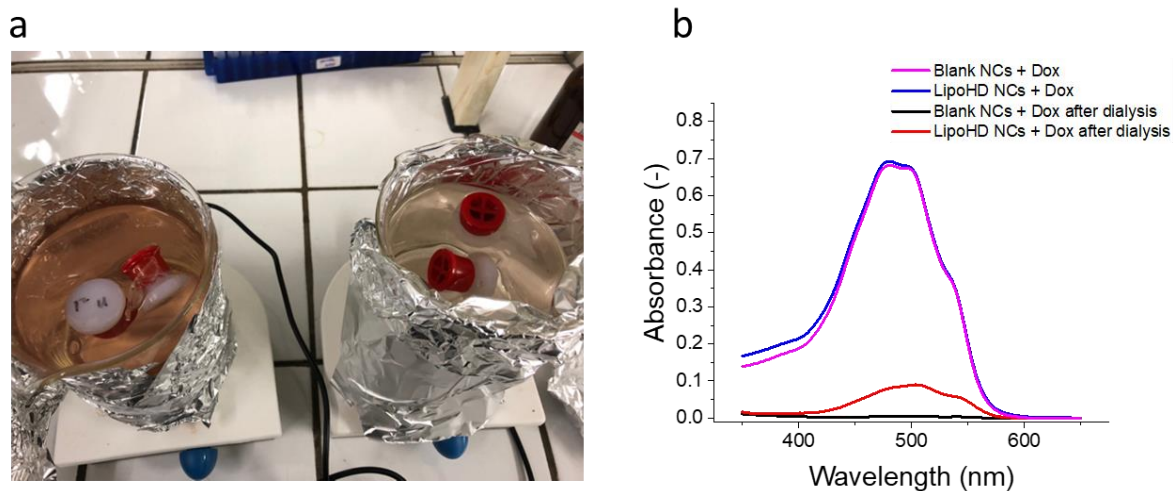


**Fig. S2.** Fluorescence spectra of Lipo-NRK conjugate NCs for different incubation time pH 6.8, pH 6.2 and pH 5.6 after dilution (1000-fold) in the presence of 20-fold excess of blank NCs. (j) Fluorescence intensity ratio of acceptor ( $I_{600}$ ) to donor ( $I_{450}$ ) over time at different pH. Excitation wavelength was 405 nm throughout.

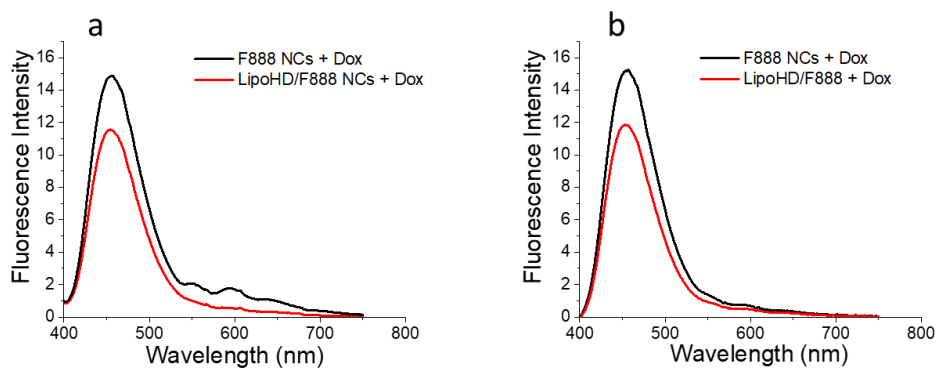




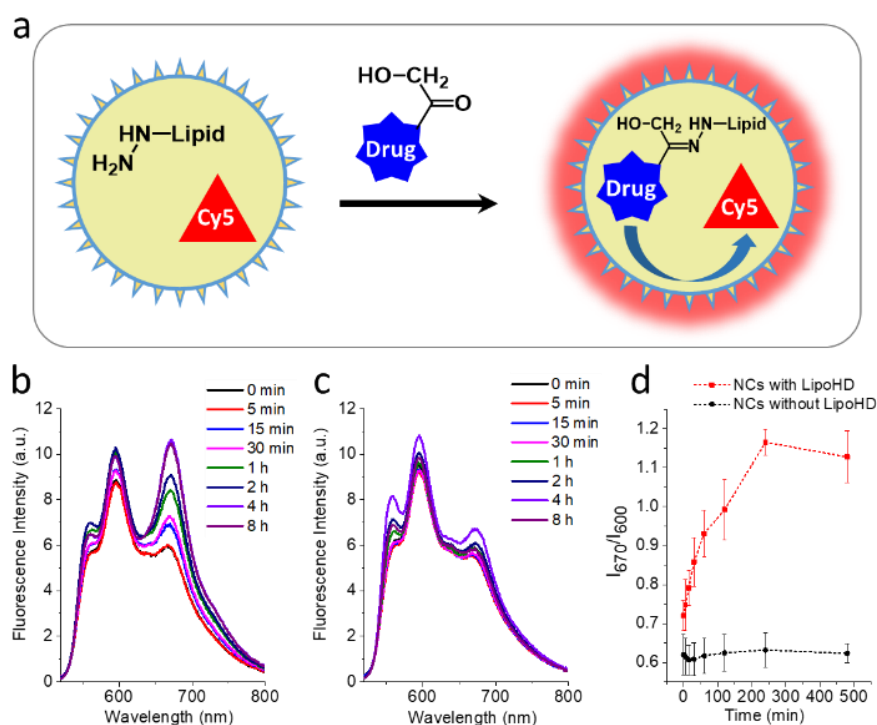
**Fig. S3.** Mass spectrometry of the in situ reaction mixture of LipoHD NCs with NRK after 4 h (40 °C), extracted with dichloromethane. Characteristic pick of (Lipo-NR + Na)<sup>+</sup> can be seen.



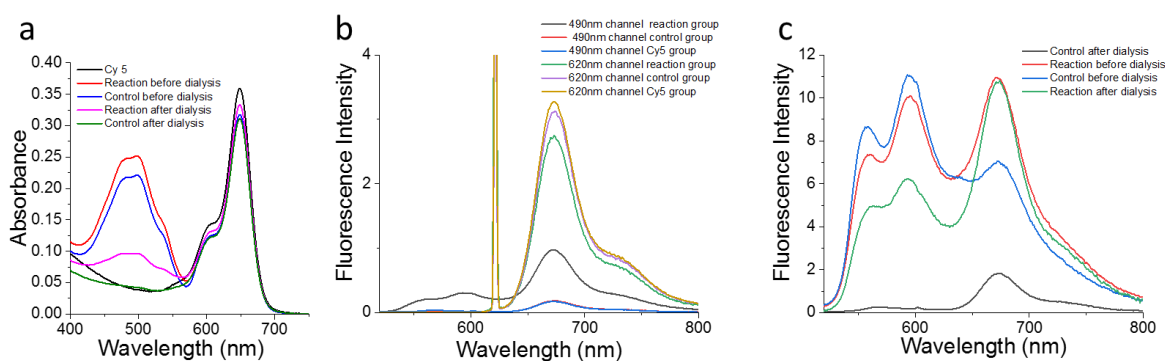
**Fig. S4.** (a) Photo of dialysis for the reaction mixture of Dox with control NCs without LipoHD (left) and with LipoHD NCs (right) after 24 h of dialysis. Deeper color of released Dox for control NCs compared to LipoHD-loaded NCs, suggest that Dox was captured inside LipoHD NCs. (b) Absorption spectra before and after dialysis of reaction mixture Dox with control (blank) NCs and LipoHD NCs incubated for 24h at rt.



**Fig. S5.** Fluorescence spectrum of a reaction mixture of LipoHD/F888 NCs and Dox before (a) and after (b) dialysis, monitored by exciting FRET donor F888 (at 380 nm).

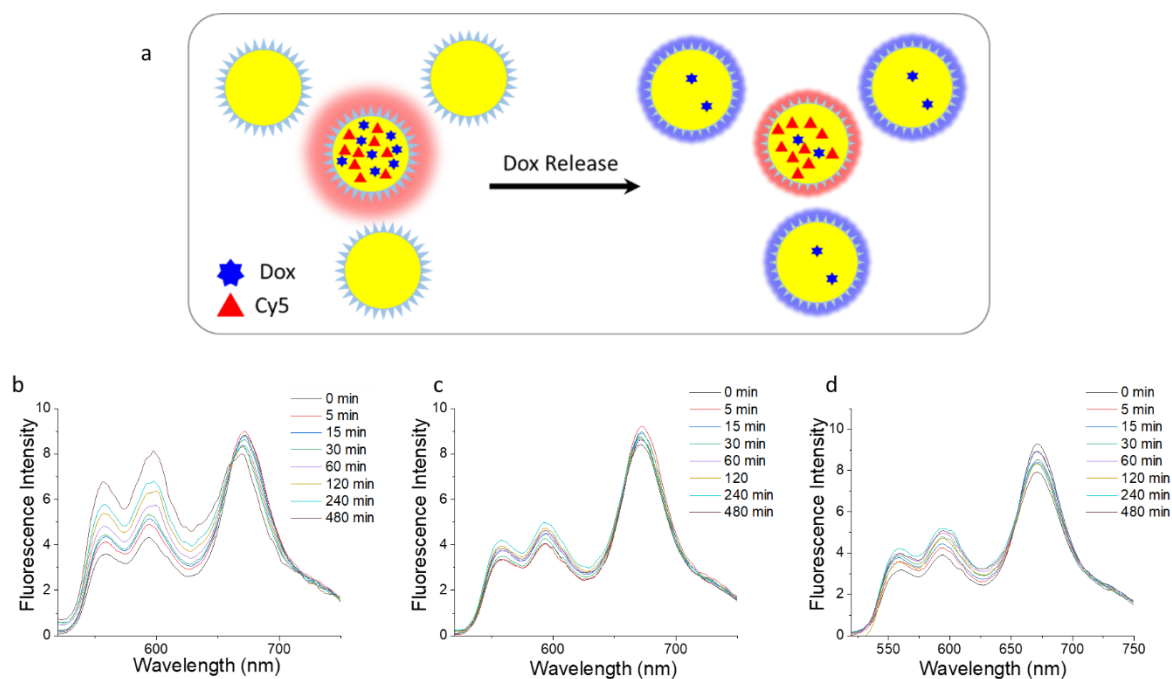


**Fig. S6.** *In situ* reaction of Dox with LipoHD monitored by Cy5. (a) Scheme of *in situ* formation of Lipo-NRK changing FRET of NCs. (b) Emission spectra of reaction mixture of LipoHD/Cy5 NCs with Dox at different reaction times after dilution (1000-fold) with 20-fold excess of blank NCs. Excitation wavelength is 520 nm. (c) The same experiment as (b) for NCs without LipoHD. (d) Fluorescence intensity ratio at fixed wavelengths (674 to 600 nm) for experiments (b) and (c). The *in situ* reaction was done at pH 4.

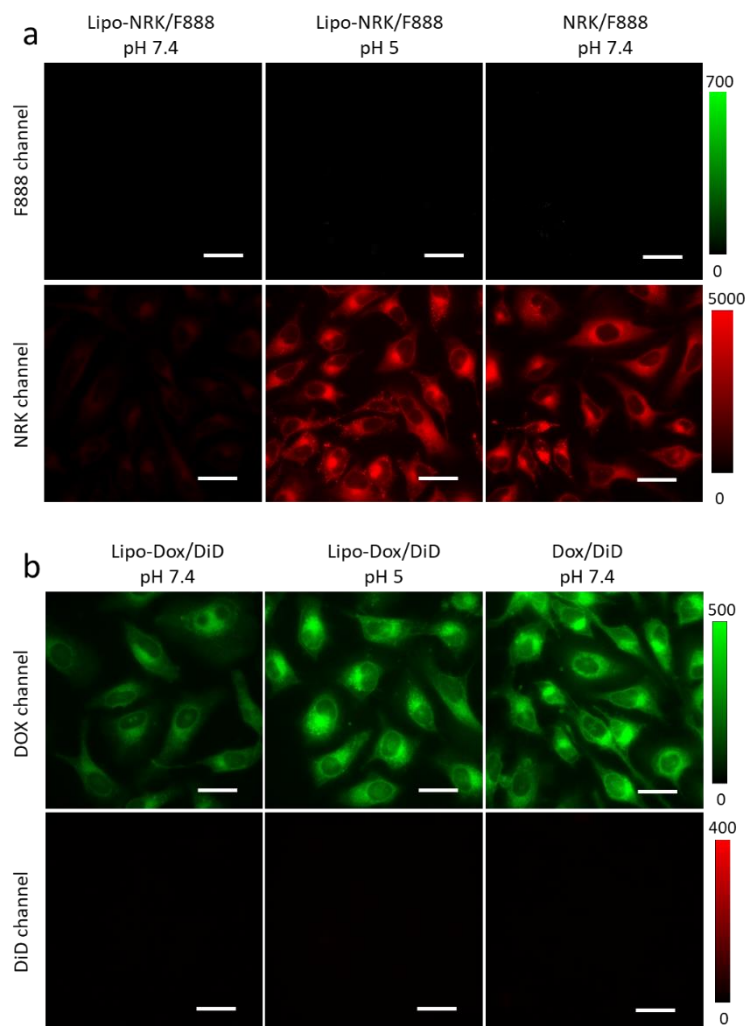


**Fig. S7.** (a) UV absorption of Dox-Cy5 mixture in situ reaction before and after dialysis along with control groups; (b) Fluorescence spectra of NCs exciting at absorption wavelength of DOX and Cy5 after dialysis. All the three NCs types showed similar fluorescence emission from Cy5 excited at 620 nm, confirming that all three samples contained similar amount of FRET acceptor. (c) Comparison of fluorescence spectra exciting at absorption wavelength of DOX of NCs with and without reaction with LipoHD before and after dialysis.

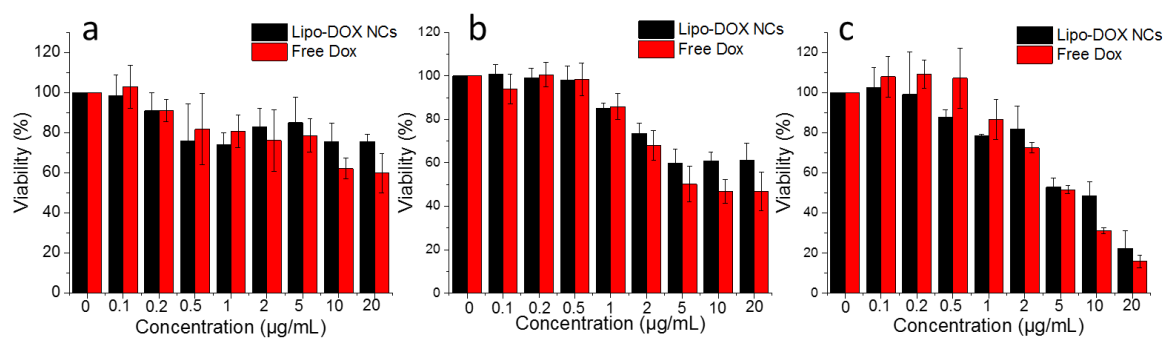
After the dialysis, the acceptor/donor ratio increased (Fig. S7c) because only unreacted Dox was removed, whereas without LipoHD this ratio was very low, corresponding to Cy5 NCs without Dox.



**Fig. S8.** (a) Schematic representation DOX release in Lipo-Dox/Cy5 NCs and Dox/Cy5 NCs in the presence of blank NCs, which is expected to decrease FRET from Dox to Cy5 at given condition. Dox-LipoHD loaded NCs stability test monitored by FRET in physiological conditions: (a) pH 5 PBS; (b) pH 7.4 PBS; (c) 10% Serum. Fluorescence spectra of Lipo-Dox FRET NCs (1000-fold dilution) in the presence of 20-fold excess of blank NCs. Excitation wavelength was 520 nm.

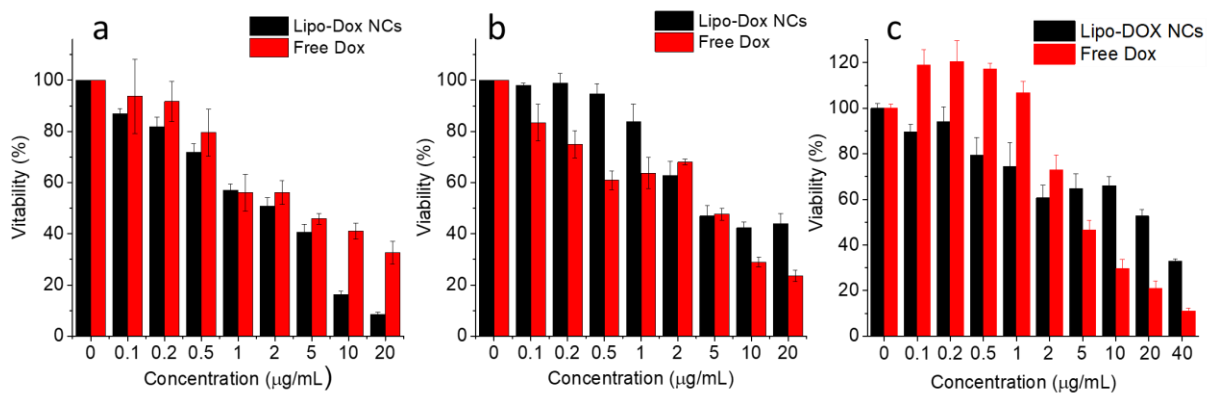


**Fig. S9.** Fluorescence imaging cells incubated for 30 min with (a) LipoNRK/F888 NCs (pH 5 and 7.4) and NRK/F888 (pH 7.4) and (b) Lipo-Dox/Cy5 NCs (pH 5 and 7.4) and Dox/Cy5 NCs (pH 7.4). F888 channel: 390 nm excitation and 470 nm detection filter. NRK channel: 550 nm excitation and 600/50 nm emission filter. DiD channel: 640 nm excitation and 705/80 nm detection filter.

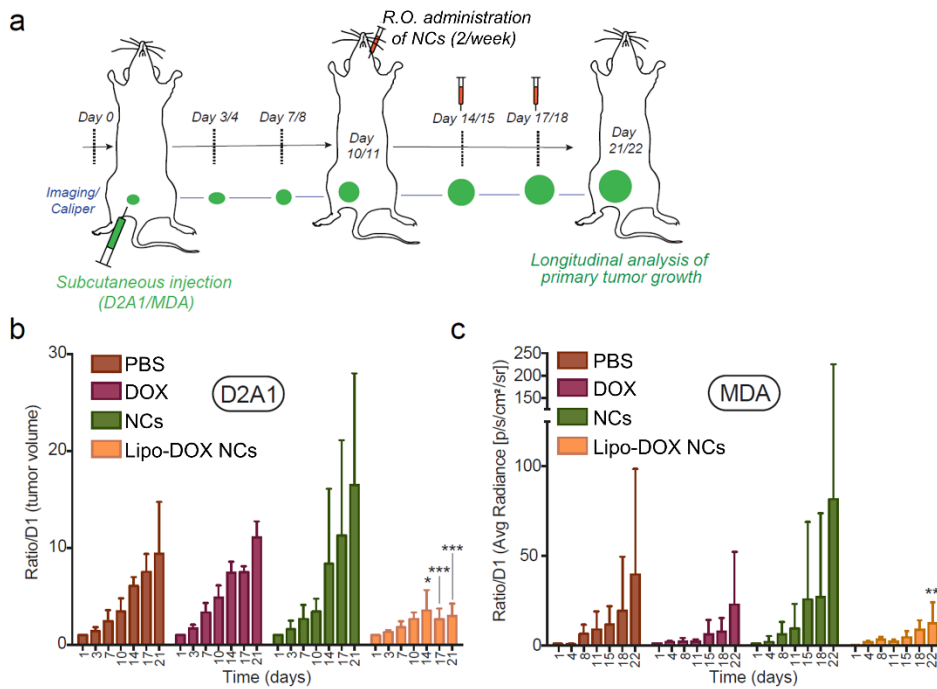


**Fig. S10.** Cytotoxicity of Lipo-DOX loaded NCs compared with free Dox in Hela cell line. Incubation was done for 12 (a), 24 (b) and 48h (c) with Lipo-DOX loaded NCs or free Dox at different drug concentrations. Errors are standard deviation of the mean (n = 6).

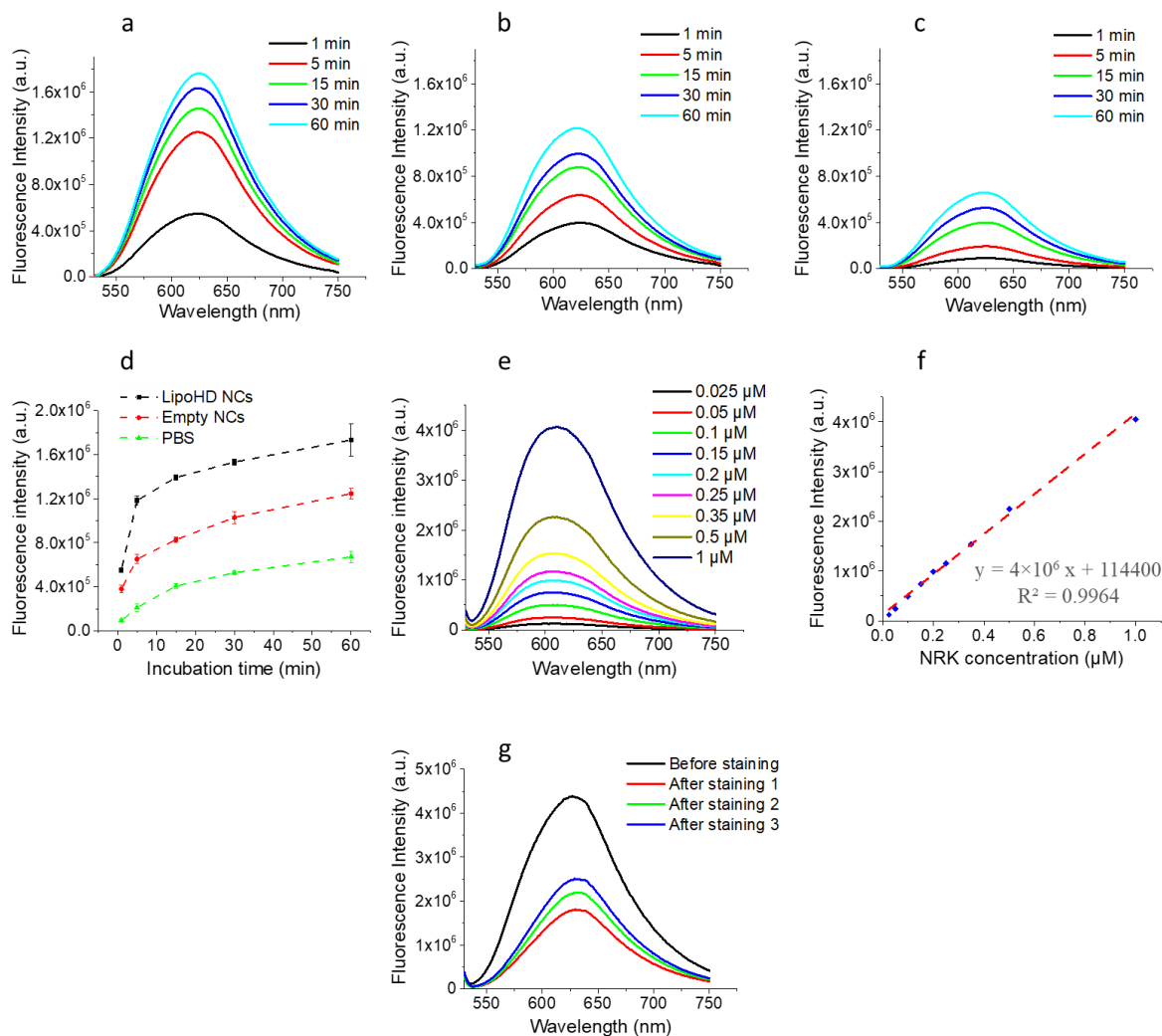




**Fig. S11.** Cytotoxicity of Lipo-Dox loaded NCs compared with free Dox in three cell lines: 4T1 (a), MDA-MB-231 (b) and D2A1 (c). Incubation was done for 48h with Lipo-HD-DOX loaded nanoemulsion or free DOX drug at different concentrations of the drug. Errors are standard deviation of the mean (n = 6).

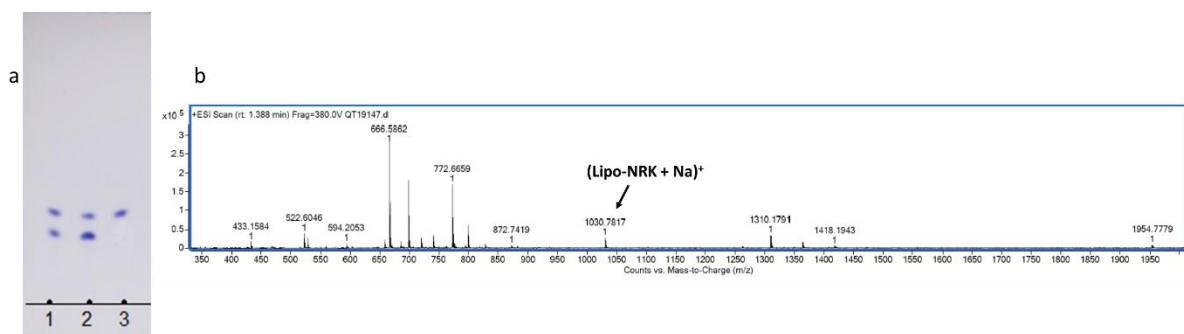


**Fig. S12.** In vivo studies on nude mice bearing D2A1 or MDA-231 tumor xenografts, treated with Lipo-DOX NCs. (a) Tumor implanting and drug administration protocol. (b) Tumor volume ( $\text{mm}^3$ ) assessment for using D2A1 as tumor implanting cell line over 21 days. (c) Tumor bioluminescence assessment for using MDA-231 as tumor implanting cell line over 21 days. Errors are standard deviation of the mean ( $n = 8$ ).

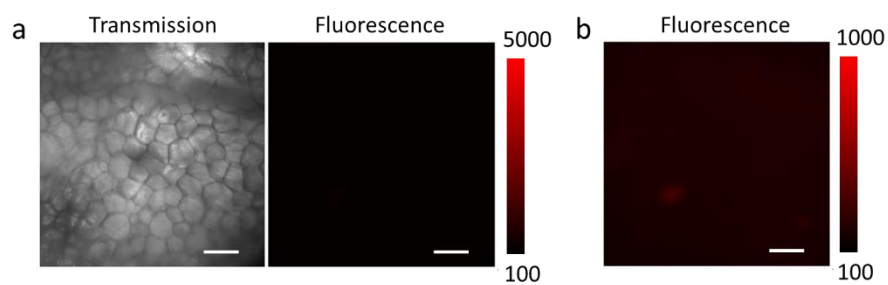


**Fig. S13.** Fluorescence analysis of the extraction medium incubated with HeLa cells stained with NRK (1  $\mu\text{M}$ ). (a-c) Fluorescence spectra of the cell culture (extraction) media, LipoHD NCs (a), blank NCs (b) and PBS (c), after incubation with NRK-stained HeLa cells for different time. 100-times diluted LipoHD NCs and blank NCs or PBS were used. (d) Time-dependent peak fluorescence intensity for three cell culture (extraction) media based on emission spectra presented in (a-c). Errors are standard deviation of the mean ( $n = 3$ ). Quantification of NRK extraction from LipoHD NCs, empty NCs and PBS: fluorescence spectra (e) and a calibration curve of NRK pick fluorescence intensity (f) for different concentrations of NRK in the presence of LipoHD NCs (100-fold dilution). (g) Fluorescence spectra of NRK staining solution before and after staining of HeLa cells for 1h. NRK concentration in the staining medium was 1  $\mu\text{M}$ .

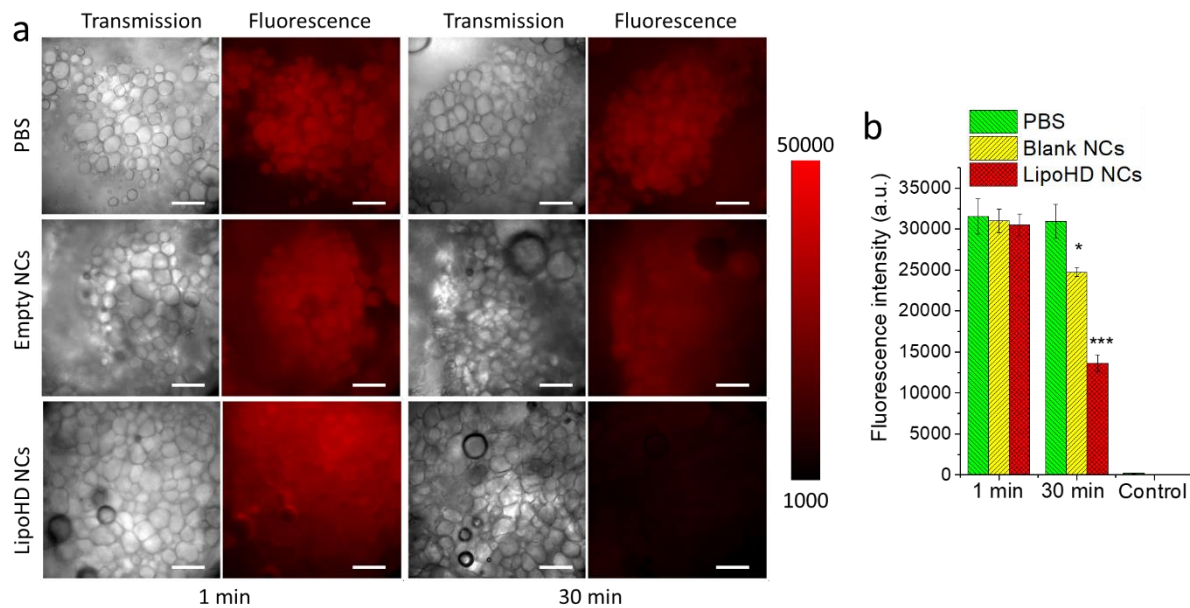
Using pick fluorescence intensity from the cell culture (extraction) media (Fig. S13a-c) and the calibration curve (Fig. S13f), we estimated the amount of extracted NRK from cells. Then, using the loss of the fluorescence intensity during staining (Fig. S13g, corresponding to the internalized NRK) and the calibration curve (Fig. S13f), we could estimate the total amount of the internalized NRK. These data allowed us to further calculate the fraction (%) of the extracted NRK from HeLa cells.



**Fig. S14.** TLC and mass spectrometry data that evidence in-situ formation of Lipo-NRK during the extraction of NRK from HeLa cells. (a) TLC of three samples (eluent dichloromethane:MeOH = 95:5): (1) dichloromethane extract from LipoHD NCs incubated with NRK-stained cells (1h); (2) dichloromethane extract from the in-situ reaction of NRK with LipoHD NCs after 1 h (40 °C); (3) pure Lipo-NRK. (b) Mass spectrum of the dichloromethane extract from LipoHD NCs incubated with NRK-stained cells (1h), presenting a characteristic pick of (Lipo-NR + Na)<sup>+</sup>.



**Fig. S15.** Auto-fluorescence from chicken skin adipose tissue. (a) Transmission and fluorescence images. (b) The same fluorescence image as in (a) but with a different intensity scale. Excitation wavelength was 550 nm and the emission filter was 600 nm.



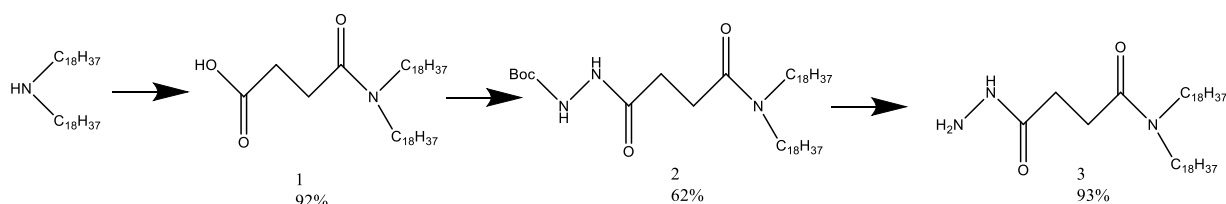
**Fig. S16.** *In situ* extraction of ketone dye NRK by LipoHD NCs from chicken skin adipose tissue. (a) Transmission and fluorescence images of *in situ* extraction of NRK: tissue was pre-incubated with 5  $\mu$ M of NRK for 30 min, washed, treated or 1 or 30 min with 100-fold diluted LipoHD NCs, empty NCs and PBS and then washed with PBS before imaging. Excitation wavelength was 550 nm and the emission filter was 600 nm. (b) Mean fluorescence intensity measured in the center area (3 images analyzed per condition). Statistical analysis (vs. data in PBS, 1 min):  $p < 0.5$  (\*),  $p < 0.01$  (\*\*\*)

**Table 1.** Size measurements of formulated nano-droplets.

Sample	Size (nm)	PDI
Blank NCs	85	0.22
LipoHD	78	0.15
F888	79	0.11
NRK	112	0.36
Lipo-NRK	71	0.10
NRK-LipoHD/F888	77	0.15
NRK/F888	114	0.24
NRK-LipoHD/F888, <i>in situ</i>	79	0.15
NRK-F888, <i>in situ</i>	113	0.23
Dox NCs, before dialysis	134	0.23
Dox-LipoHD NCs, before dialysis	144	0.22
Dox NCs after dialysis	71	0.13
Dox-LipoHD, after dialysis	85	0.06
Dox/Cy5 before dialysis	105	0.22
Dox/Cy5 after dialysis	70	0.10
Dox-LipoHD/Cy5 before dialysis	116	0.19
Dox-LipoHD/Cy5 after dialysis	71	0.10

<sup>a</sup> Dye concentration in labrafac: F888 (10 mM); Lipo-NRK (10 mM); NRK (10 mM); Cy 5 (0.95 mM). Concentration for *in situ* reactions is not shown.

## Synthesis and chemical characterization



**Scheme S1.** Synthesis of LipoHD.

### Synthesis of LipoHD

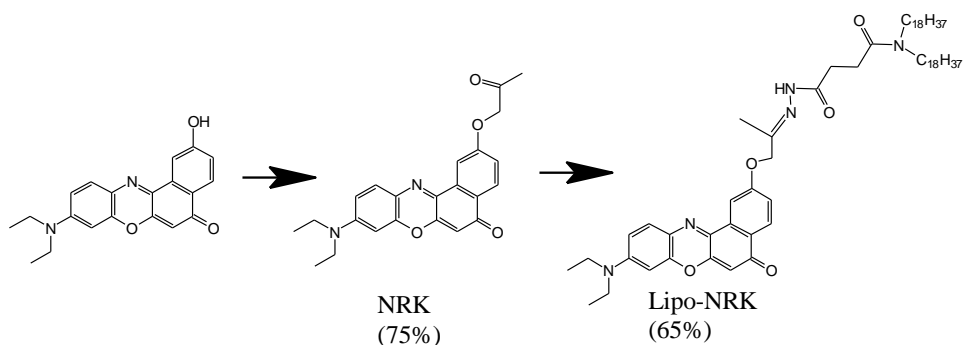
To the solution of succinic anhydride (192 mg, 1.92 mmol) in THF (20 mL) was added dioctadecylamine (500 mg, 0.96 mmol), then mixture was refluxed overnight under argon atmosphere. The solvent was evaporated in vacuo, then resulting residue was purified by recrystallization from dioxane to give desired product as a colorless solid (550 mg, 92%).

**Compound 1.** To the solution of succinic anhydride (192 mg, 1.92 mmol) in THF (20 mL) was added dioctadecylamine (500 mg, 0.96 mmol), then mixture was refluxed overnight under argon atmosphere. The solvent was evaporated in vacuo, then resulting residue was purified by recrystallization from dioxane to give desired product as a colorless solid (550 mg, 92%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ ppm 11.72 (1H, bs), 3.34 (4H, t), 3.19 (4H, t), 1.56 (4H, bs), 1.24 (60H, bs), 0.86 (6H, t). MS for [C<sub>40</sub>H<sub>79</sub>NO<sub>3</sub> – H<sup>+</sup>]<sup>-</sup> m/z (M-1) calc. 620.61, found 620.60.

**Compound 2.** To the solution of **1** (500 mg, 0.080 mmol) in DMF (10 mL), tert-butylcarbazate (159 mg 1.21 mmol), PyBOP (630 mg, 1.21 mmol), and DIPEA (1.11 mL, 6.4 mmol (8 eq)) were added and stirred at room temperature overnight under argon atmosphere. The solvent was evaporated in vacuo, then resulting residue was purified by flash column chromatography on silica gel (eluent EtOAc/Hepane 50:50) to give desired product as a colorless solid (365 mg, yield 62%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ ppm 8.14 (1H, bs), 6.36 (1H, bs), 3.29 (2H, t), 3.21 (2H, t), 2.68 (2H, t), 2.58 (2H, t), 1.53 (13H, bs), 1.20 (60H, bs), 0.88 (6H, t). MS for [C<sub>45</sub>H<sub>89</sub>N<sub>3</sub>O<sub>4</sub>+H<sup>+</sup>]<sup>+</sup> m/z (M+1) calc. 736.69, found 736.69.

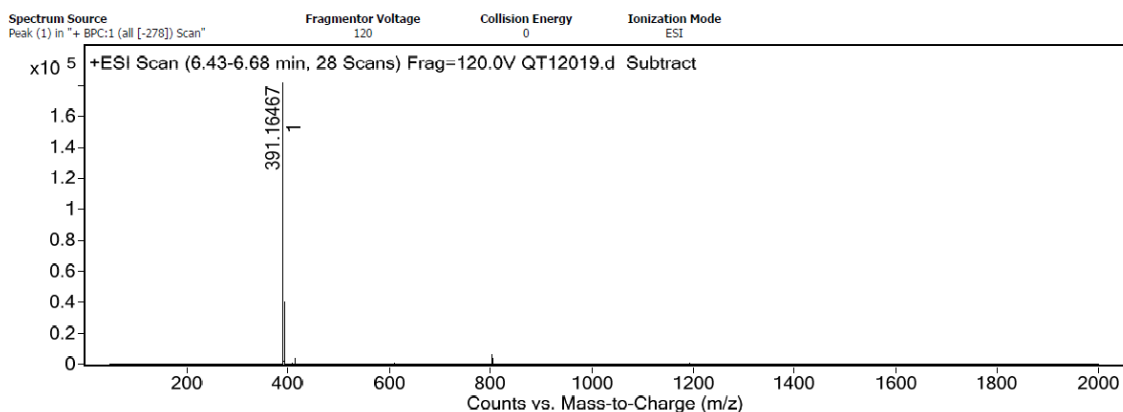
**Compound 3 (LipoHD).** To the solution of **2** (350 mg, 0.48 mmol) in DCM (10 mL), TFA (10 mL) and 2 drops of MiliQ water were added and stirred at room temperature for 2 h. After confirming the reaction completely proceeded with TLC, the solvents were evaporated under reduced pressure, and then the residue was dissolved in MeOH and evaporated 3 times to remove TFA and give desired product **3** as a colorless solid (362 mg). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ ppm 7.77 (1H, bs), 3.27 (2H, t), 3.16 (2H, t), 2.65 (2H, t), 2.49 (2H, t), 1.50 (4H, bd), 1.24 (60H, bs), 0.84 (6H, t). MS for Chemical Formula: [C<sub>40</sub>H<sub>81</sub>N<sub>3</sub>O<sub>2</sub>+H] m/z (M+1) calc. 636.63, found 636.63.



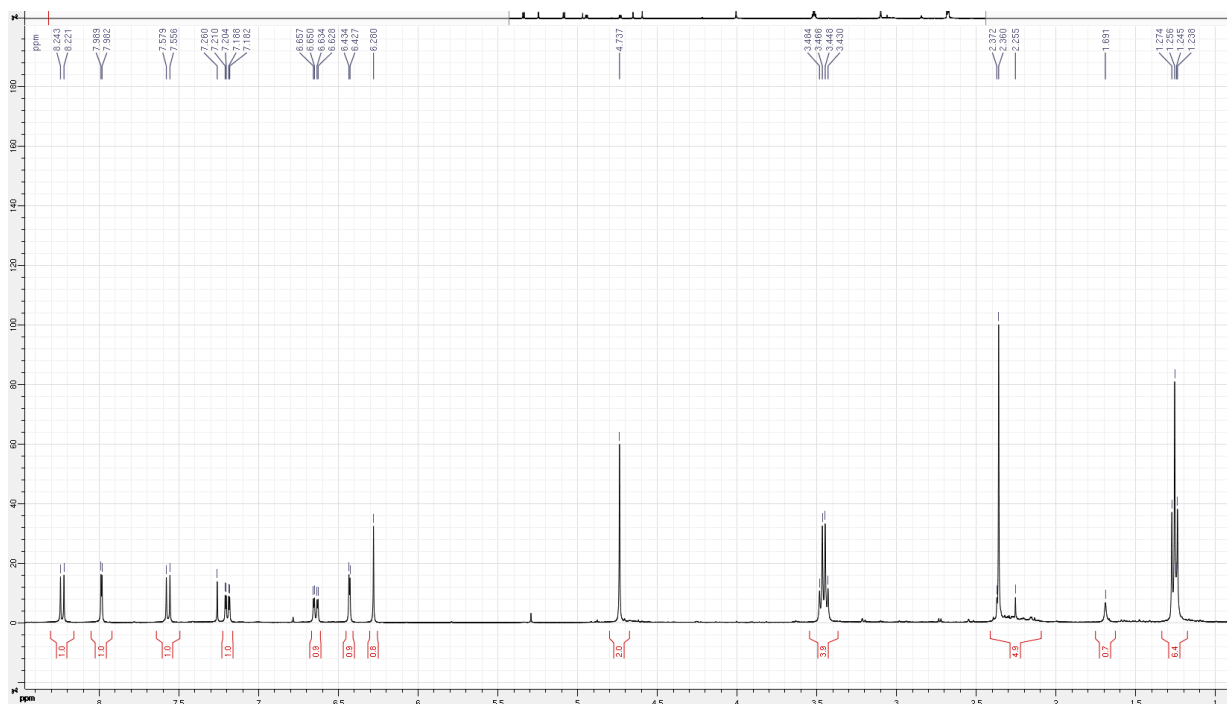


## Scheme S2. Synthesis of LipoHD.

**Compound 4 (NRK).** To the solution of 2-hydroxy-Nile Red (150 mg, 0.45 mmol), potassium carbonate (310 mg, 2.24), catalytic amount of potassium iodide in DMF (10 mL), (159 mg 1.21 mmol), chloroacetone (160  $\mu$ L, 2.24 mmol) was added and stirred at 100  $^{\circ}$ C for 3 h under argon atmosphere. The solvent was evaporated in vacuo, then resulting residue was washed by brine. The organic layer was dried over  $MgSO_4$ , filtered and evaporated in vacuo. The residue was purified by flash column chromatography on silica gel (eluent DCM/MeOH = 98:2) to give desired product as a violet solid (132 g, yield 75%).  $^1H$  NMR (500 MHz,  $CDCl_3$ ) 8.24-8.22 (d,  $J=10.4$  Hz, 1H), 7.99-7.96 (d,  $J = 3.6$  Hz, 1H), 7.21-7.18 (q,  $J=11.6$  Hz, 1H), 6.66-6.63 (q,  $J=10.8$  Hz, 1H), 6.43-6.42 (d,  $J=2.8$  Hz, 1H), 6.28 (s, 1H), 4.74 (s, 1H), 3.46-3.43 (q,  $J=24.3$  Hz, 4 H), 2.36 (s, 3 H), 1.27-1.24 (t,  $J=13.9$  Hz, 6H).MS(ESI+) calcd for  $C_{23}H_{22}N_2O_4H$  ( $M+H$ )+; 391.164 observed.

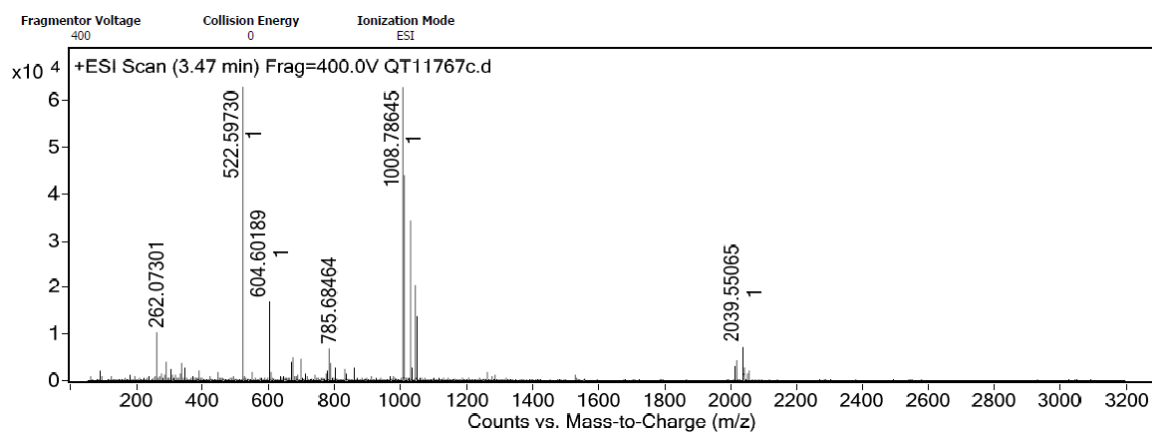


Mass spectrum of **NRK**.

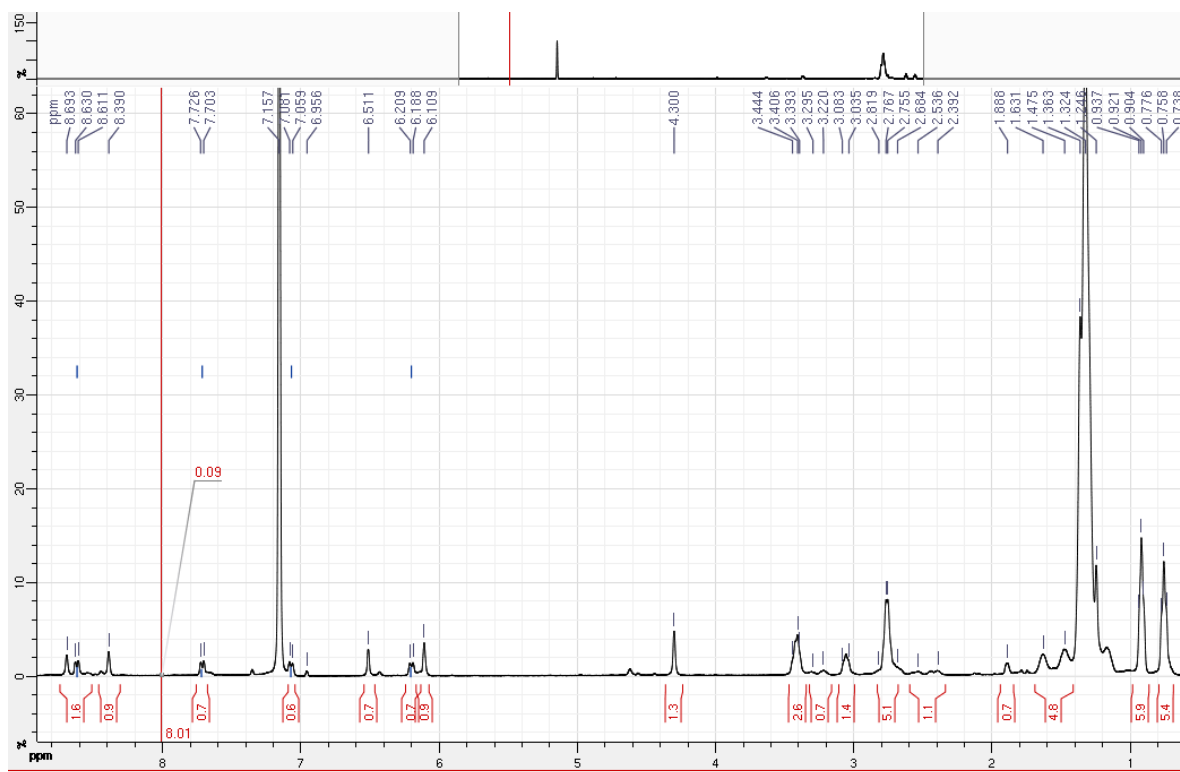


**H-NMR spectrum of NRK.**

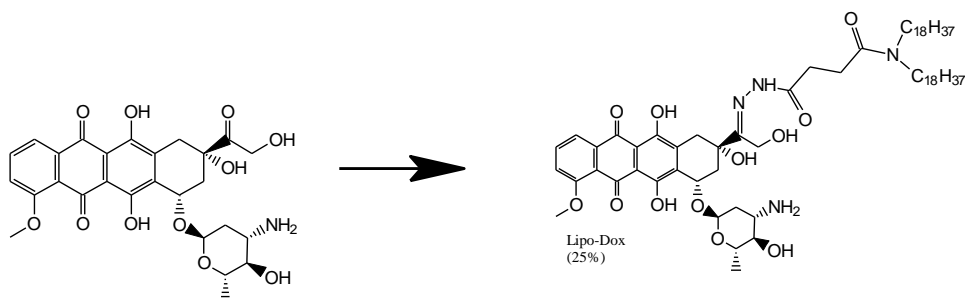
**Lipo-NRK.** To the solution of **LipoHD** (30 mg, 0.077 mmol), in EtOH (2 mL), **NRK** (115 mg 0.15 mmol), triethylamine (22  $\mu$ L, 0.15 mmol) was added and refluxed for 3 h under argon atmosphere. The solvent was evaporated in vacuo, then resulting residue was purified by puri-flash HPLC (eluent DCM/MeOH = 98:2 to 96:4) to give desired product as a violet solid (50 mg, yield 65%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) 8.69 (s, 1H), 8.63 (d, J = 7.0 Hz, 1H), 8.39 (s, 1H), 7.73-7.70 (d, J=8.1 Hz, 1H), 7.08-7.06 (d, J=9.3 Hz, 1H), 6.51 (s, 1H), 6.20-6.19 (d, J = 8.1 Hz, 1H), 6.10 (s, 1H), 4.3 (s, 1H), 3.44 – 3.39 (m, J = 29.1 Hz, 3H), 3.30-3.22 (m, J= 33.7 Hz, 1H), 3.08-3.04 (m, J = 19.8 Hz, 2H), 2.82 – 2.68 (q, J = 47.7 Hz, 5H), 2.54-2.39 (m, J = 57.0 Hz, 1H), 1.89 (s, 1H), 1.63-1.48 (m, J=67.5 Hz, 4 H), 0.94-0.90 (t, J = 12.8 Hz, 6H), 0.78-0.74 (t, J=12.4 Hz, 6H), 1.36-1.25 (m, J=47.7 Hz, 63 H). MS(ESI<sup>+</sup>) calcd for 1009 C<sub>63</sub>H<sub>101</sub>N<sub>5</sub>O<sub>5</sub>H (M+H)<sup>+</sup>; 1008.79 observed, 1008.78 cal.



**Mass spectrum of Lipo-NRK.**



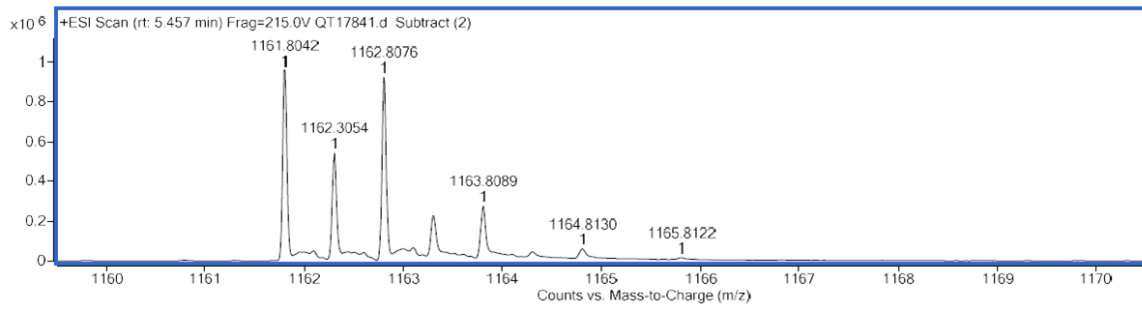
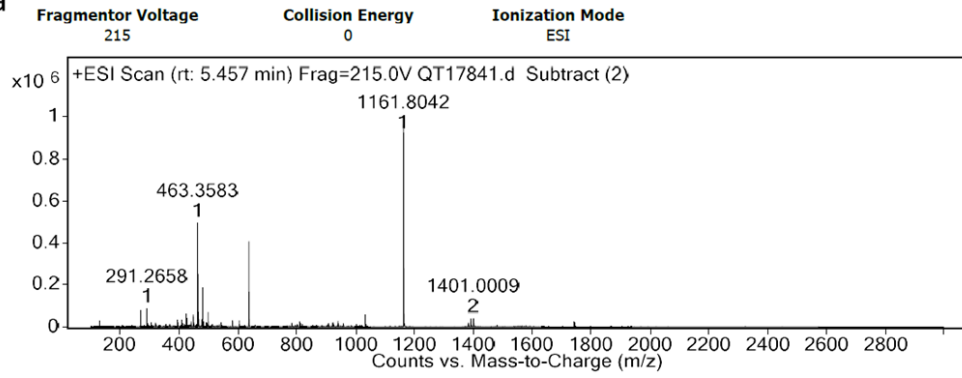
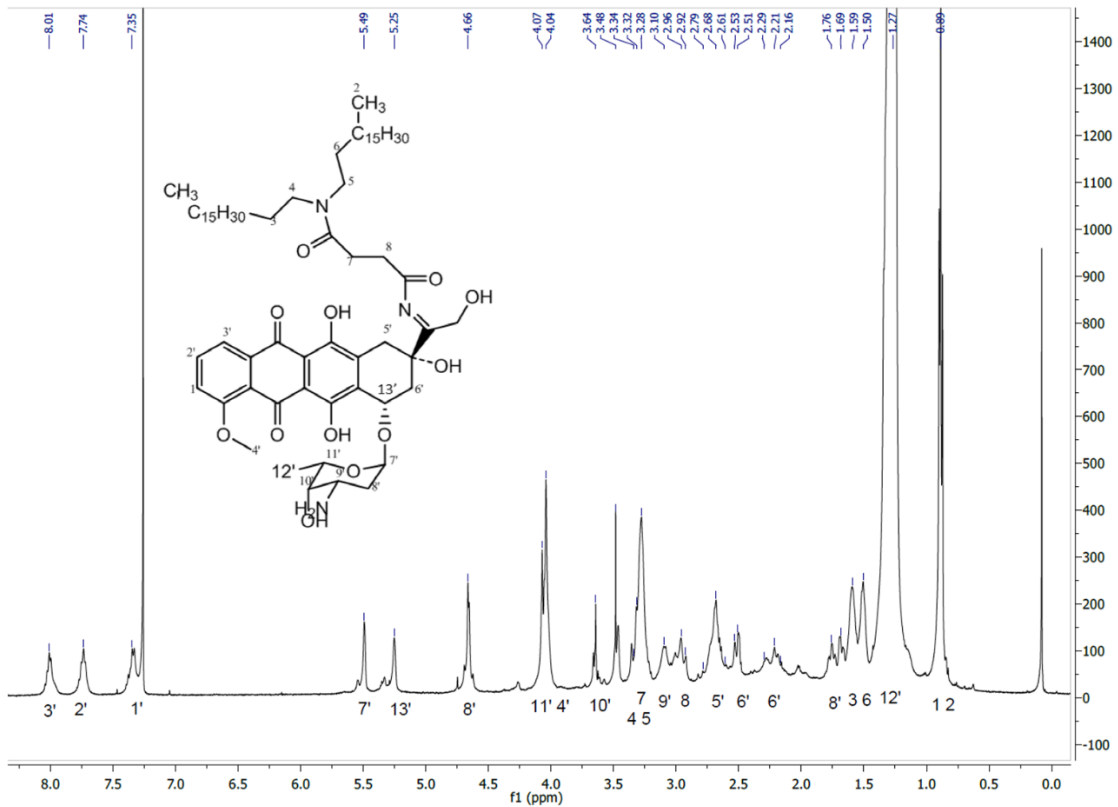
H-NMR spectrum of **Lipo-NRK**.

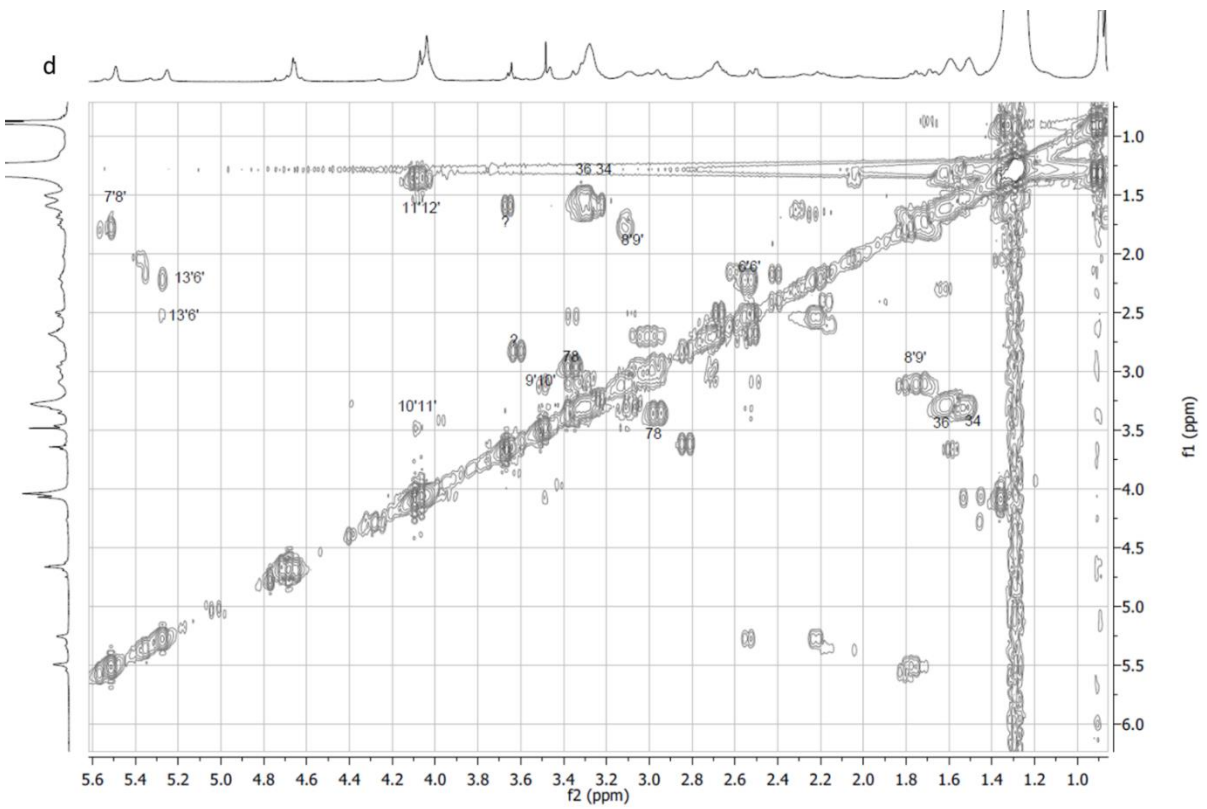
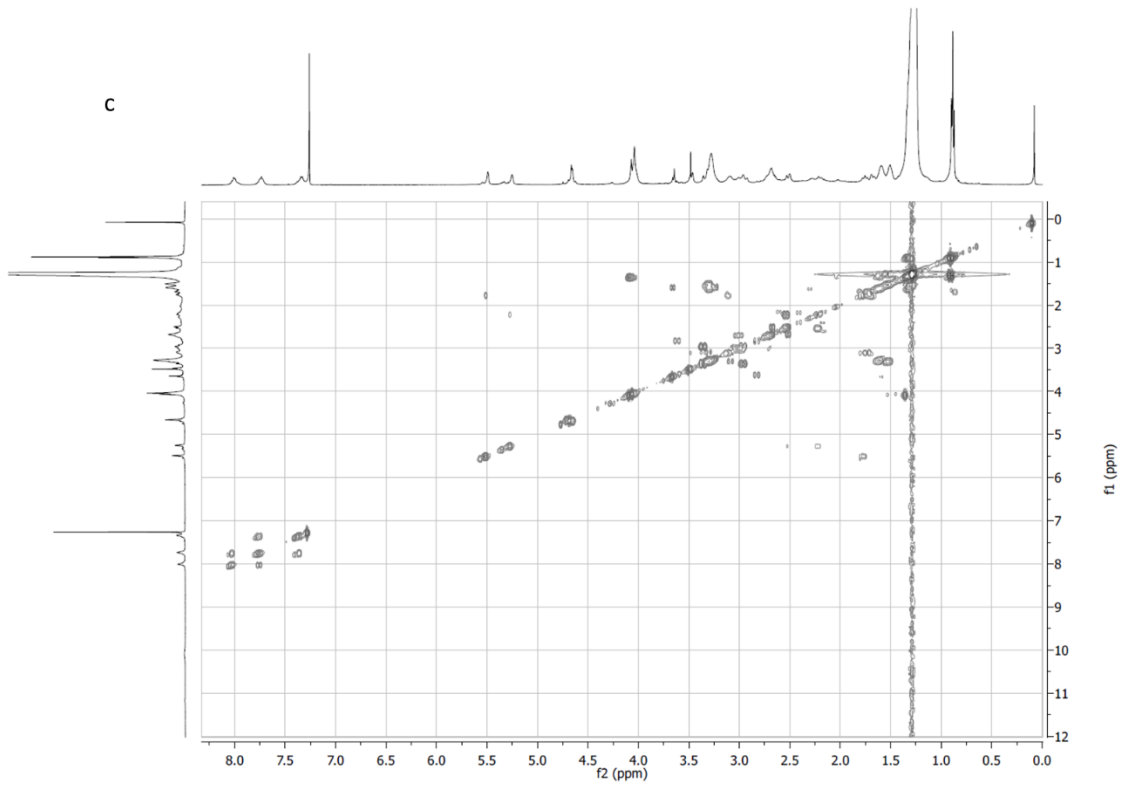


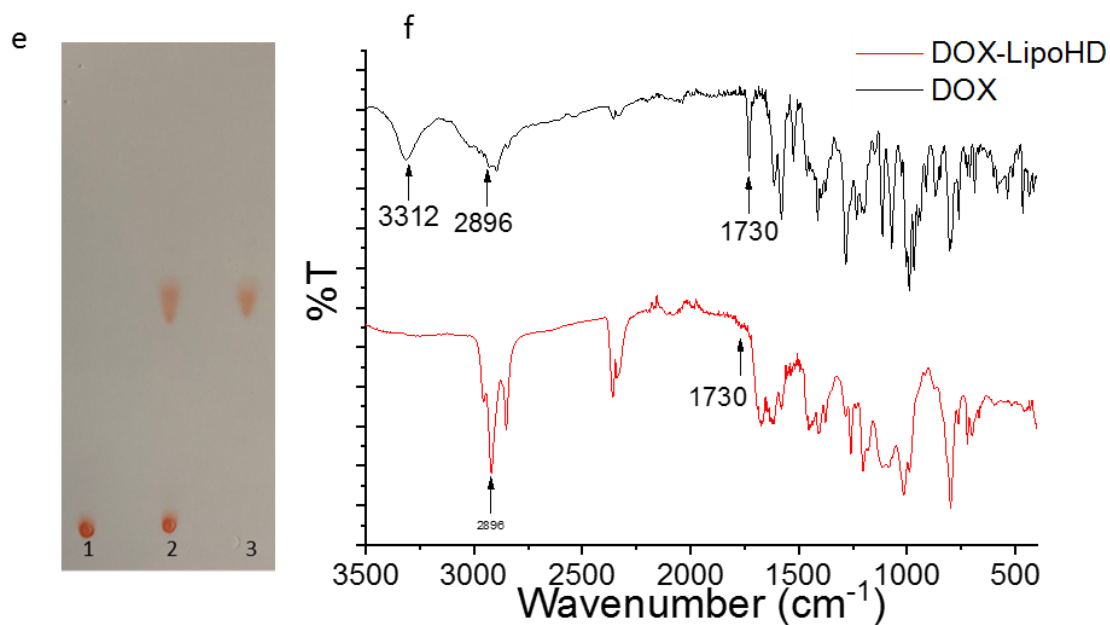
### Scheme S3. Synthesis of **Lipo-Dox**.

**Lipo-Dox.** To the solution of doxorubicin/hydrochloride (Dox, 40 mg, 0.069 mmol), in MeOH (45 mL) and DCM (5 mL), **LipoHD** (57 mg 0.09 mmol), TFA (50  $\mu$ L) was added and stirred overnight under argon atmosphere. The solvent was evaporated in vacuo, then resulting residue was purified by flash column chromatography on silica gel (eluent DCM/MeOH/triethylamine = 85:14: 1) to give desired product as a red solid (45 mg, yield 25%).  $^1\text{H NMR}$  (500 MHz,  $\text{CDCl}_3$ ) 8.01 (m,  $J = 8.0$  Hz, 1H), 7.74 (m,  $J = 7.7$  Hz, 1H), 7.35 (m,  $J = 7.4$  Hz, 1H), 5.49 (s, 1H), 5.26 (s, 1H), 4.75 - 4.57 (m,  $J=4.7$  Hz, 2H), 4.05 (d,  $J = 4.1$  Hz, 4H), 3.48 (s, 1H), 3.38 – 3.18 (m,  $J = 3.3$  Hz, 6H), 3.18 – 2.89 (m,  $J = 3.0$  Hz, 3H), 2.79 - 2.61 (m,  $J= 2.7$  Hz, 2H), 2.52 (d,  $J = 2.52$  Hz, 1H), 2.37 – 2.12 (m,  $J = 2.2$  Hz, 1H), 1.81 - 1.64 (m,  $J = 1.7$  Hz, 2H), 1.55 (d,  $J = 1.6$  Hz, 4H), 1.27 (s, 63 H), 0.89 (t,  $J = 0.9$  Hz, 6H). MS (ESI $^+$ ) calcd for  $\text{C}_{87}\text{H}_{110}\text{N}_4\text{O}_{13}\text{H}$  ( $\text{M}+\text{H}$ ) $^+$ : 1162; observed: 1161.8.

The compound was characterized by  $^1\text{H-NMR}$  and COSY spectrum. The carbon spectrum was difficult to obtain for the reason of compound aggregation at higher concentrations. As shown in the FT-IR spectrum, the peak at  $1730\text{ cm}^{-1}$  which represented the presence of ketone disappeared in the spectrum of Lipo-Dox. The intensity of peaks at  $2896\text{ cm}^{-1}$  increased in comparing to that of Dox. This resulted from the long alkyl chains of Lipo-Dox. Peaks observed in Dox around  $3312\text{ cm}^{-1}$  came from the moisture within the commercially available Dox. This peak disappeared in the synthesized Lipo-Dox, this showed the dryness of Lipo-Dox obtained, which is for stability of Lipo-Dox, sensitive to hydrolysis.

**a****b**





**Figure S17.** Identification of Lipo-Dox (a) Mass spectrum; (b)  $^1\text{H-NMR}$  spectrum; (c) Full spectrum of COSY; (d) COSY spectrum characterization in zoomed areas of interest; (e) TLC after Lipo-Dox purification. 1. Free Dox; 2. Mixture of free Dox and purified Lipo-Dox; 3. Purified Lipo-Dox; (f) FT-IR of Dox and Lipo-Dox.

## References

- [1] A. S. Klymchenko, E. Roger, N. Anton, H. Anton, I. Shulov, J. Vermot, Y. Mely, T. F. Vandamme, *RSC Adv.* **2012**, 2, 11876.