

Supplementary Information

Investigating the impact of 2-OHOA-embedded liposomes on biophysical properties of cancer cell membranes via Laurdan two-photon microscopy imaging

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16 **Investigating the impact of 2-OHOA-embedded Liposomes on biophysical properties of**
 17 **cancer cell membranes via Laurdan two-photon microscopy imaging**

18 This supplementary information contains the following sections:

- 19 1. Liposome DOPC quantification results;
- 20 2. Influence of DOPC on cell membrane fluidities investigation results;
- 21 3. *GP* histograms deconvolution results;
- 22 4. Comparison of the influence of 2-OHOA-embedded liposomes and free 2-OHOA
 23 (fluorescence spectrometer measurements);
- 24 5. Lipid droplet staining and observation;
- 25 6. Illustration of endocytosis-dependent enhancement of 2-OHOA liposome performance;
- 26 7. MTT assay results;
- 27 8. Anticancer performance of 2-OHOA embedded liposome;
- 28 9. G-factor calibration of *GP* images using a reference solution;
- 29 10. Sphingomyelin high-performance thin film chromatography (HPTLC) results

30
 31 **1. DOPC Quantification**

32 **Table. S-1 DOPC quantification result ($n=3$)**

Formulation	DOPC- only	9-1	7-3	5-5	3-7	1-9
DOPC concentration (mg/mL)	3.75 ± 0.16	3.57 ± 0.40	2.79 ± 0.08	2.16 ± 0.04	1.15 ± 0.03	0.42 ± 0.01
DOPC concentration (mM)	5.11 ± 0.21	4.86 ± 0.55	3.81 ± 0.11	2.94 ± 0.06	1.56 ± 0.04	0.57 ± 0.01
Total concentration (mM)	5.11 ± 0.21	5.40 ± 0.61	5.44 ± 0.14	5.88 ± 0.11	5.21 ± 0.13	5.66 ± 0.06

33 Total concentration refers to the combined sum of DOPC and 2-OHOA concentrations.
 34 LabAssay Phospholipid kit was used to quantify the DOPC in liposomes according to the
 35 manual.

36 2. Influence of DOPC on cell membrane fluidities

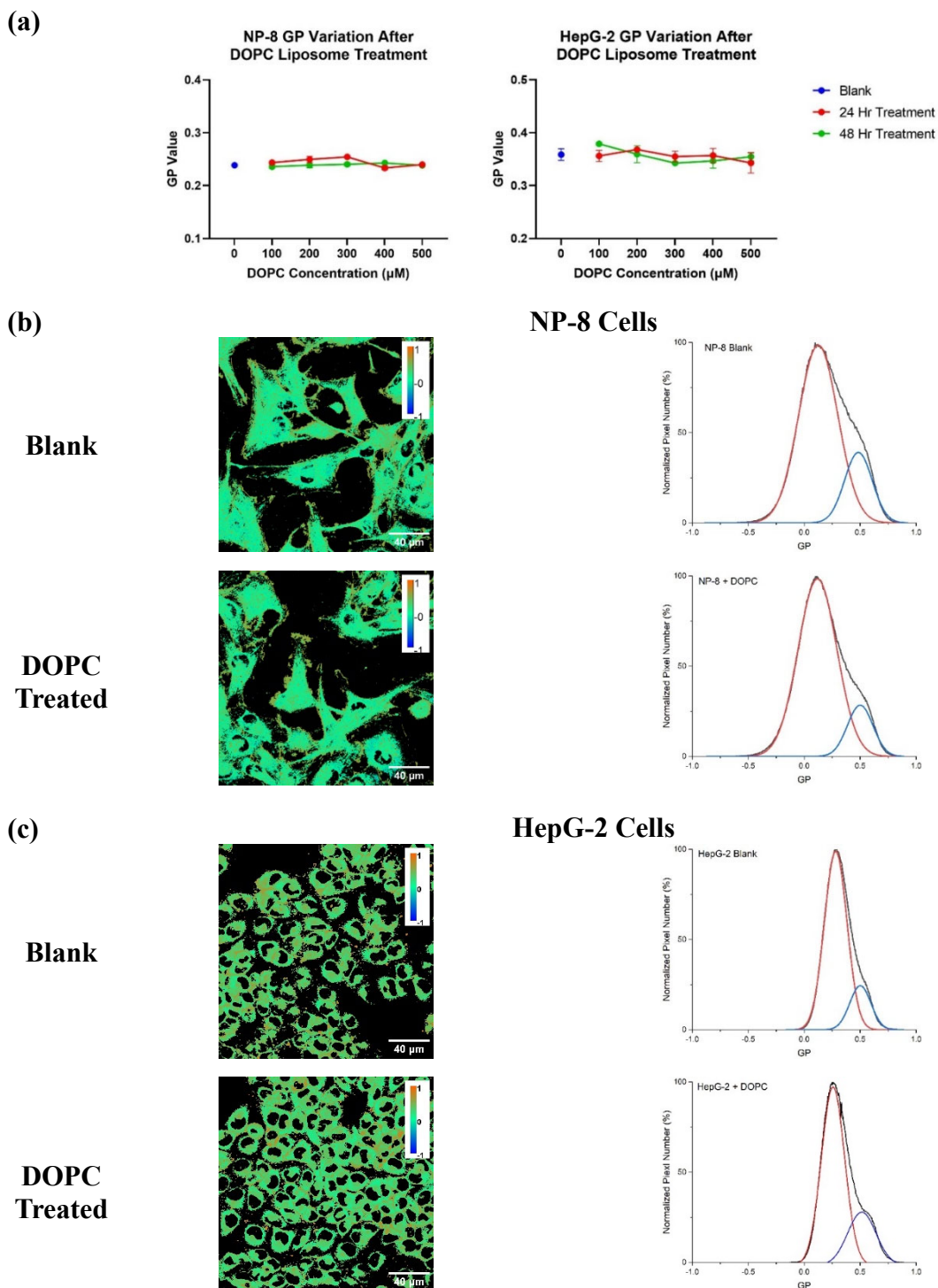


Figure S-1. Influence of DOPC liposome treatment on the HepG-2 and NP-8 cell membrane GP variations. (a) NP-8 and HepG-2 Laurdan GP variations after DOPC liposomes treatments for 24/48 h, GP values were obtained from fluorescence spectrometer. Error bars represent \pm standard deviations, $n=3$. (b) GP images and GP histograms of NP-8 cells with and without DOPC liposomes treatments. (c) GP images and GP histograms of HepG-2 cells with and without DOPC liposomes treatments. Cells were treated with 100 μM DOPC liposomes for 24 h. Images were obtained at 37 $^{\circ}\text{C}$. Scale bars represent 40 μm .

37 **3. GP histograms deconvolution results**38 **Table S-2. Two Gauss distributions of deconvoluted GP histograms**

Cell	Treatment	Low GP Peak center	FWHM 1	High GP Peak center	FWHM 2	High GP coverage (%)
NP-8	Blank	0.116 ± 0.015	0.394 ± 0.048	0.481 ± 0.024	0.291 ± 0.023	20.54 ± 6.98
	DOPC liposome	0.128 ± 0.041	0.427 ± 0.112	0.476 ± 0.043	0.336 ± 0.044	24.25 ± 9.18
	9-1 liposome	0.310 ± 0.037	0.630 ± 0.021	0.596 ± 0.028	0.406 ± 0.027	50.49 ± 6.43
	7-3 liposome	0.266 ± 0.022	0.679 ± 0.018	0.578 ± 0.021	0.414 ± 0.027	45.27 ± 2.68
	5-5 liposome	0.223 ± 0.023	0.678 ± 0.014	0.531 ± 0.026	0.447 ± 0.016	52.95 ± 3.00
	Free 2- OHOA	0.155 ± 0.015	0.710 ± 0.019	0.475 ± 0.008	0.487 ± 0.012	58.93 ± 1.88
	Blank	0.279 ± 0.030	0.237 ± 0.011	0.500 ± 0.058	0.277 ± 0.060	22.32 ± 7.20
HepG-2	DOPC liposome	0.285 ± 0.038	0.244 ± 0.016	0.524 ± 0.058	0.251 ± 0.063	21.67 ± 10.11
	9-1 liposome	0.359 ± 0.020	0.295 ± 0.027	0.593 ± 0.007	0.217 ± 0.015	27.80 ± 7.01
	7-3 liposome	0.325 ± 0.015	0.323 ± 0.056	0.609 ± 0.026	0.261 ± 0.022	22.63 ± 2.13
	5-5 liposome	0.335 ± 0.024	0.297 ± 0.021	0.589 ± 0.003	0.221 ± 0.019	26.07 ± 4.08
	Free 2- OHOA	0.313 ± 0.013	0.296 ± 0.019	0.594 ± 0.022	0.243 ± 0.019	25.03 ± 4.71

39 NP-8 and HepG-2 cells were imaged at 37°C. For the treatment groups, cells were incubated
40 for 24 h with media containing DOPC liposome (100 μM), 2-OHOA-embedded liposome
41 (containing 100 μM 2-OHOA) or free 2-OHOA (100 μM). The obtained GP histograms of GP
42 images, were deconvoluted into two Gauss distributions. The peak center, full width at half
43 maximum (FWHM), and percentage of pixels associated with high GP peak (High GP
44 coverage) were averaged over 5-8 images.

45 **4. Comparison of the influence of 2-OHOA-embedded liposomes and free 2-OHOA**

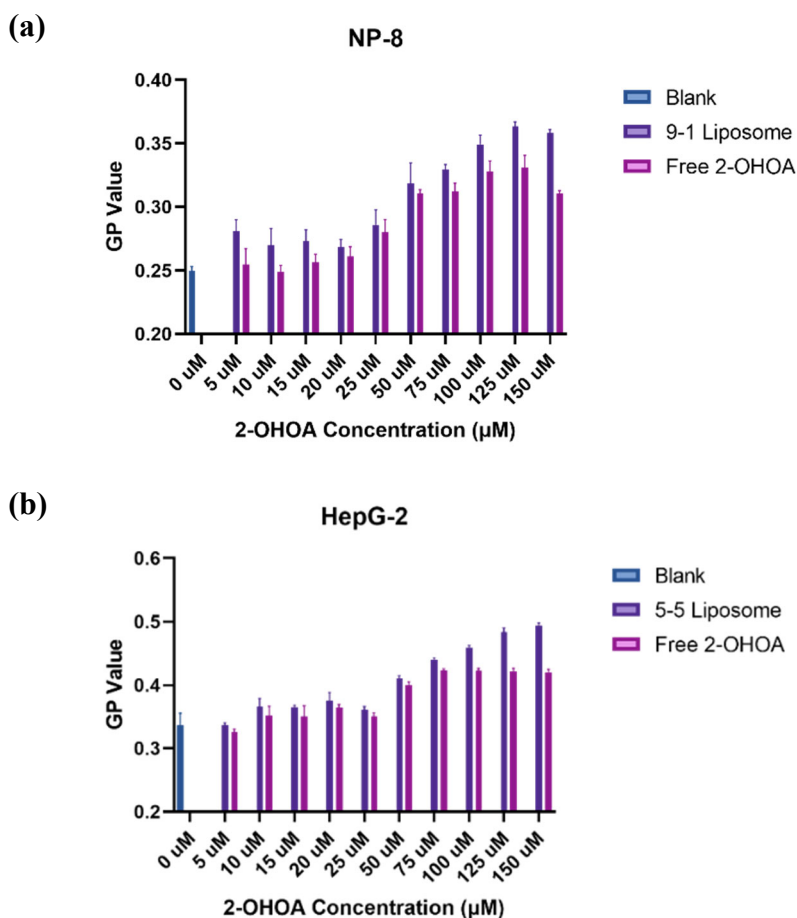


Figure S-2. Comparison of the influence of liposome formulation and free 2-OHOA on the cell membrane *GP* value variations. **(a)** NP-8 cell *GP* values after 24 h treatment using 9-1 liposomes and free 2-OHOA at different concentrations; **(b)** HepG-2 cell *GP* values after 24 hours treatment using 5-5 liposomes and free 2-OHOA at different concentrations. the *GP* values were obtained using fluorescence spectrometer (measured at 37°C, excitation wavelength was set at 345 nm, emission spectra were collected from 400 to 600 nm), *GP* values were calculated according to the equation described in experimental section. Error bars represent $\pm s.d.$ ($n=3-5$)

47 **5. Lipid droplet staining and observation**

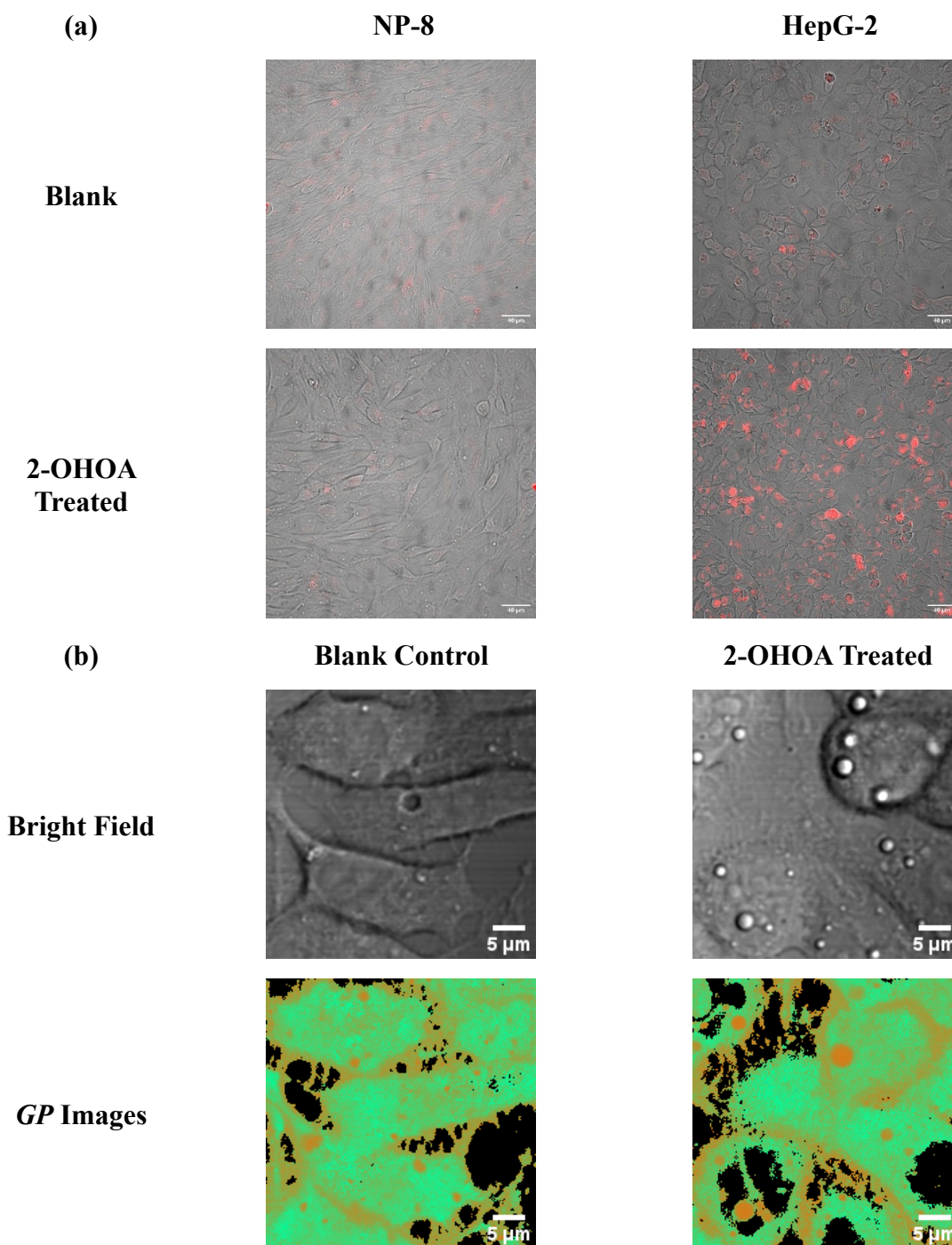
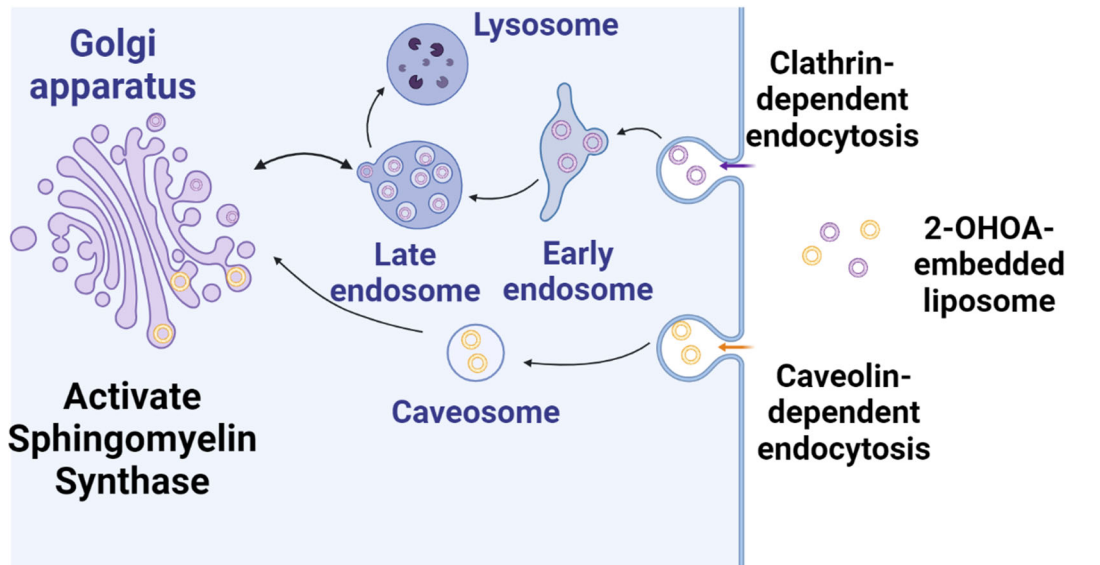


Figure S-3. Lipid droplets images. **(a)** Fluorescence microscopy images of Lipi-Red stained NP-8 and HepG-2 cells. The shown images are merged from bright field image and red channel image. Magnification was 40 times. Scale bars represent 40 μm . **(b)** Bright field and GP images of HepG-2 with and without 2-OHOA treatments, scale bars represent 5 μm . For the treatment group, cells were incubated with 100 μM 2-OHOA for 24 h before staining and imaging.

49 6. Illustration of endocytosis-dependent enhancement of 2-OHOA liposome
 50 performance



51 **Figure S-4.** Schematic illustration depicts a hypothetical scenario of the endocytosis
 process of 2-OHOA-embedded liposomes, enhancing the impact of 2-OHOA on cancer
 cells.

52 7. MTT Assay
 53

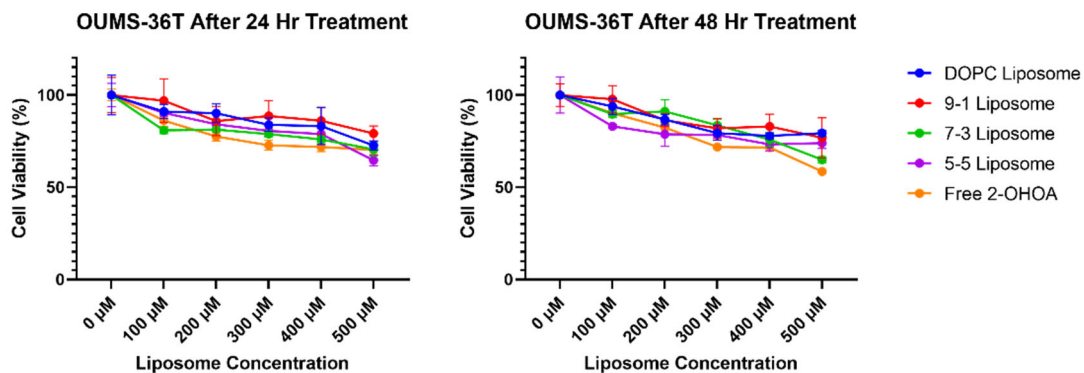


Figure S-5. MTT assay results. OUMS-36T cells were treated with different liposomes as
 well as free 2-OHOA at different concentrations for 24 h and 48 h. Error bars represent \pm
 standard deviations, $n=3$.

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56 8. Anticancer performance of 2-OHOA embedded liposome

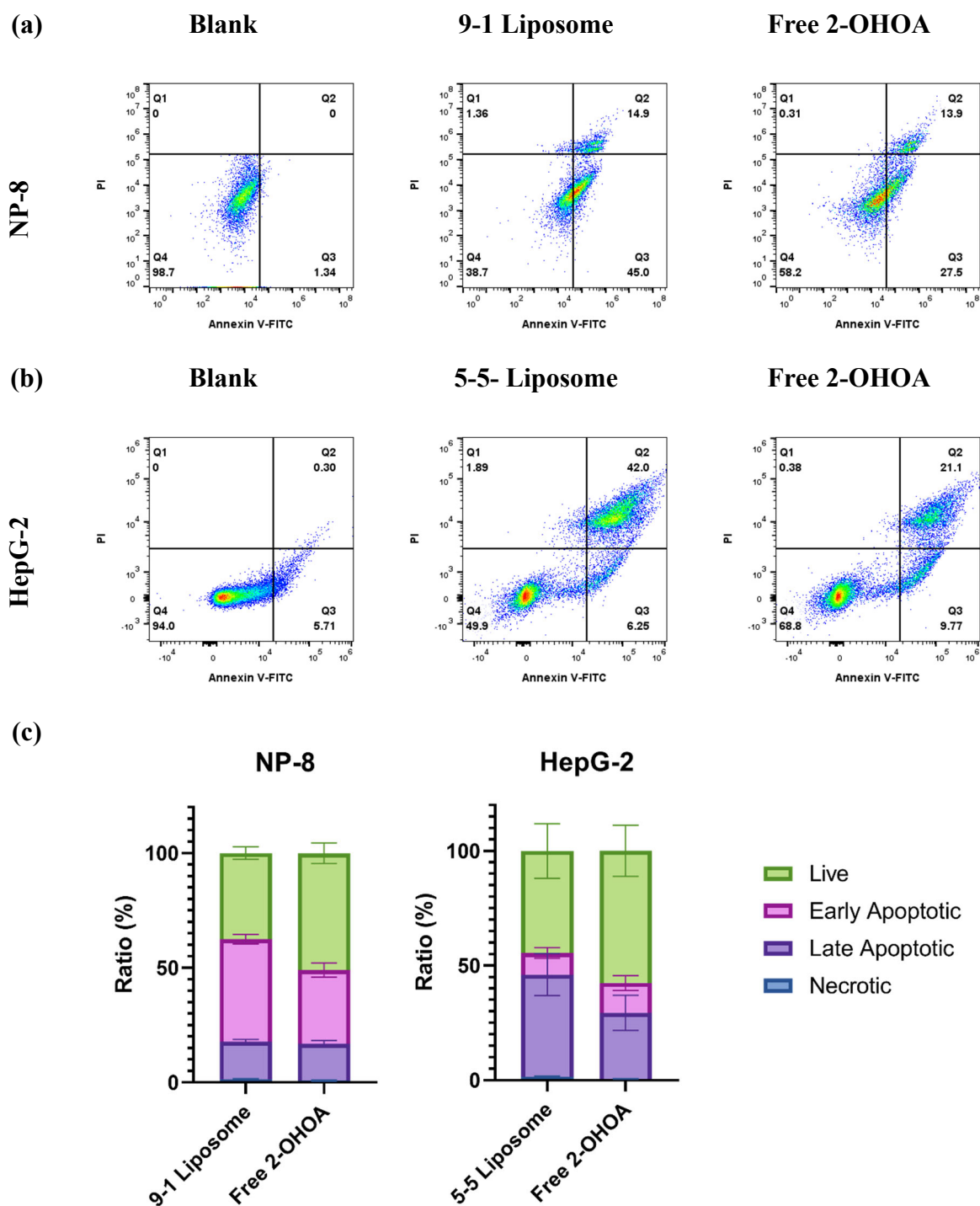


Figure S-6. Apoptosis assay result. Annexin-V/PI assay flow cytometry result dot graph of (a) NP-8 cells and (b) HepG-2 cells. (c) Summarized apoptotic ratio of NP-8 and HepG-2 cells after treatments. NP-8 cells were treated for 48 h with 9-1 liposome (containing 100 μ M 2-OHOA) or free 2-OHOA (100 μ M); HepG-2 cells were treated for 48 h with 5-5 liposome (containing 100 μ M 2-OHOA) or free 2-OHOA (100 μ M). Error bars represent \pm *s.d* (*n*=3).

57 **9. G-factor calibration of GP images using a reference solution**

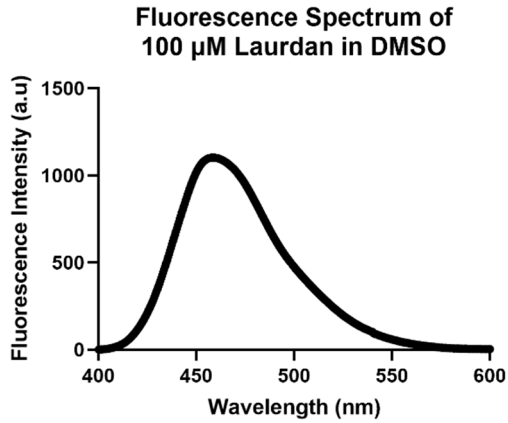


Figure S-7. Fluorescence spectrum of Laurdan (100 μM) in DMSO. Data was obtained using fluorescence spectrometer (FP-8500, Jasco, Japan). The excitation was set as 345 nm, and emission fluorescence intensity was measured from 400 to 600 nm wavelength.

58

59 Laurdan (100 μM) in DMSO was used as the reference solution for calibration. Laurdan
60 fluorescence spectrum in reference solution was measured using fluorescence spectrometer
61 (FP-8500, Jasco, Japan) with an excitation light of 345 nm wavelength. The reference GP
62 value (GP_r) was calculated according to equation (S-1).

63
$$GP_r = \frac{(I_{440} - I_{490})}{(I_{440} + I_{490})} \quad (\text{S-1})$$

64 where I_{440} and I_{490} represent the Laurdan fluorescence intensity at wavelengths of 440 nm
65 and 490 nm, respectively.

66 The reference solution was imaged using two-photon microscopy, and the fluorescence
67 images of the blue and cyan channels were obtained, and the Laurdan calibration factor
68 ($G_{Laurdan}$) was calculated according to equation (S-2).

69
$$G_{Laurdan} = \frac{I_{blue} \times (1 - GP_r)}{I_{cyan} \times (1 + GP_r)} \quad (\text{S-2})$$

70 Where I_{blue} is the is the Laurdan fluorescence intensity of the blue channel and I_{cyan} is the
71 Laurdan fluorescence intensity of the cyan channel;

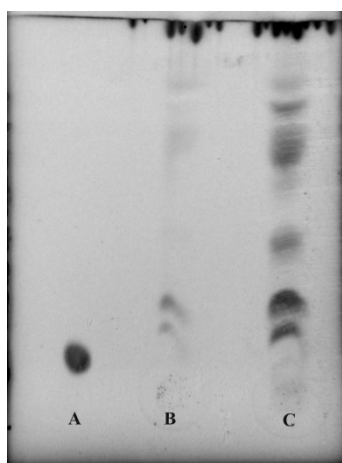
72 The cell GP images were obtained according to equation (S-3).

73
$$GP_m = \frac{I_{blue} - (G_{Laurdan} \times I_{cyan})}{I_{blue} + (G_{Laurdan} \times I_{cyan})} \quad (\text{S-3})$$

74 Where GP_m is the GP value calculated using the two-photon microscopy images; I_{blue} is
75 the Laurdan fluorescence intensity of the blue channel and I_{cyan} is the Laurdan fluorescence
76 intensity of the cyan channel; $G_{Laurdan}$ is the Laurdan calibration factor.

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78 10. High-performance thin film chromatography (HPTLC) results



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80 **Figure S-8.** NP-8 cell membrane lipid HPTLC results. **A:** brain sphingomyelin standard
81 sample (1 mg/mL); **B:** extracted lipid from NP-8 cells (no treatment; phospholipid lipid 1
82 mg/mL); **C:** extracted lipid from NP-8 cells (treated for 24 h using 100 μM 2-OHOA;
83 phospholipid lipid 1 mg/mL).