# Photo-activated drug delivery systems

Kong, L.

# 4

## Light Induced Modulation of the Very Long Chain Fatty Acid Composition of Cell Membranes

**Abstract:** Membrane protein function is highly dependent on the properties of the surrounding lipid bilayer. Herein, we report a chemical and solvent-free method to enrich cellular membranes with very long chain fatty acids (vlcFAs). In this way, we are able to modulate cell membrane lipid composition, thickness and potentially membrane protein activity. Supplementing cell membranes with vlcFAs is notoriously difficult due to their extreme insolubility in aqueous solution. To solve this, we create light sensitive micelles, composed of PEG-nervonic acid (FA24:1) conjugates, which spontaneously disassemble in the presence of lipid bilayers. Once embedded, light is used to cleave off PEG, leaving free nervonic acid within the target membrane. When applied to living cells, released nervonic acid was processed by the cell to generate various species of phospholipids with elevated amounts of incorporated vlcFAs.

Li Kong[1], Edgar Dawkins[2], Frederick Campbell[1], Edith Winkler[2], Rico J.E. Derks[4], Martin Giera[4], Frits Kamp[2], Harald Steiner[2, 3] and Alexander Kros[1]

[1]Leiden Institute of Chemistry, Leiden University, The Netherlands

[2]BMC, Metabolic Biochemistry, Ludwig-Maximilians University, Munich, Germany

[3]German Center for Neurodegenerative Diseases (DZNE), Munich, Germany

[4]Center for Proteomics and Metabolomics, Leiden University Medical Center (LUMC), The Netherlands

## 4.1 Introduction

Phospholipid bilayers, as the main constituent of cellular membranes, act as scaffolds maintaining structural integrity and correct cellular function. Membrane bilayers are involved in numerous cellular processes, regulating bidirectional molecular traffic and supporting numerous membrane associated proteins and receptors (*e.g.* G-protein coupled receptors). The activity and function of membrane proteins is heavily dependent on the local properties of the lipid bilayer in which they are embedded. The transmembrane domains (TMD) of many membrane proteins are highly evolved to prefer a specific lipid environment. In turn, slight changes of bilayer thickness, fluidity, curvature, and/or lipid headgroup chemistry may lead to destabilization of protein structure and affect function and activity.[1,2]

Very long-chain fatty acids (vlcFAs), with a chain-length of ≥22 carbon atoms, play a vital role to many cellular functions including spermatogenesis, skin barrier formation and myelin maintenance.[3] Free, unesterified vlcFA can rapidly diffuse across lipid bilayers and redistribute among various cellular compartments.[4,5] When incorporated within endogenous phospholipids (*e.g.* sphingo- and glycero-phospholipids), vlcFAs are key modulators of cell membrane fluidity and thickness, facilitating the formation of lipid rafts/domains within cellular membranes.[6] Furthermore, vlcFAs are important precursors of inflammation-resolving lipid mediators and several disorders in the synthesis of vlcFAs (*e.g.* elongation of stearic acid to saturated or mono-unsaturated vlcFAs), as well as defects in vlcFA metabolism, can lead to severe diseases such as Stargardt disease and adrenoleukodystrophy.[3]

A change in the thickness of a lipid bilayer can create a mismatch in size with the hydrophobic TMD of an embedded protein.[7] To minimize unfavorable interactions (*e.g.* exposure of hydrophobic amino acids to water), a change in membrane thickness forces a membrane protein to alter its conformation to the most energetically favourable orientation, for instance, by tilting and bending the TMDs in the new membrane landscape. This in turn can alter the ability of the protein to carry out its function and in extreme cases may lead to a complete loss of function.[8] The activity

of ion channels, enzymes and substrate transporters have all been shown sensitive to changes in lipid membrane thickness.[9–12]

Membrane thickness depends on cholesterol content and particularly esterified vlcFA content of endogenous phospholipids. Delivery of vlcFAs to cells is therefore an attractive option to artificially modulate the thickness of cell membranes, and thereby the activity of membrane proteins, as acyl-chain remodeling pathways readily incorporate exogenous fatty acids into cellular phospholipids.[13,14] However, the delivery of vlcFAs to cells is complicated by their extreme insolubility in water. This is particularly problematic in the case of vlcFAs with a low degree of unsaturation, *e.g.* nervonic acid (NA, FA24:1). These species immediately form insoluble aggregates upon dilution in aqueous media. Our motivation for this study was to find ways of solubilizing vlcFAs in aqueous media to enable efficient incorporation into target cellular membranes. Once embedded in the plasma membrane, we hypothesized that delivered vlcFAs would be taken up into cellular compartments, enter membrane remodeling pathways[14] and eventually be incorporated into cellular phospholipids. If successful, this would create a larger hydrophobic domain within the bilayer of cellular membranes and potentially alter the activity of embedded membrane proteins.[1,8]

Herein, we describe a photo-inducible approach to enrich cell membranes with vlcFAs, leading to enrichment of cellular phospholipids with delivered vlcFAs. To achieve this, we conjugated NA to poly-ethylene-glycol (PEG) *via* a photocleavable (*o*-nitrobenzyl) linker (**1**). These amphipathic constructs self-assemble to form close-packed micelles in aqueous solution as illustrated in Figure 1a and 1b.

These micelles spontaneously disassemble in the presence of model and cell membranes, embedding **1** within the target membrane sink (Figure 1c). Once within the membrane, photolysis of PEG leaves unesterified NA within the membrane. By applying this technique to HEK293 cells we demonstrate increased incorporation of esterified vlcFA in cellular phospholipids using lipidomics analysis.

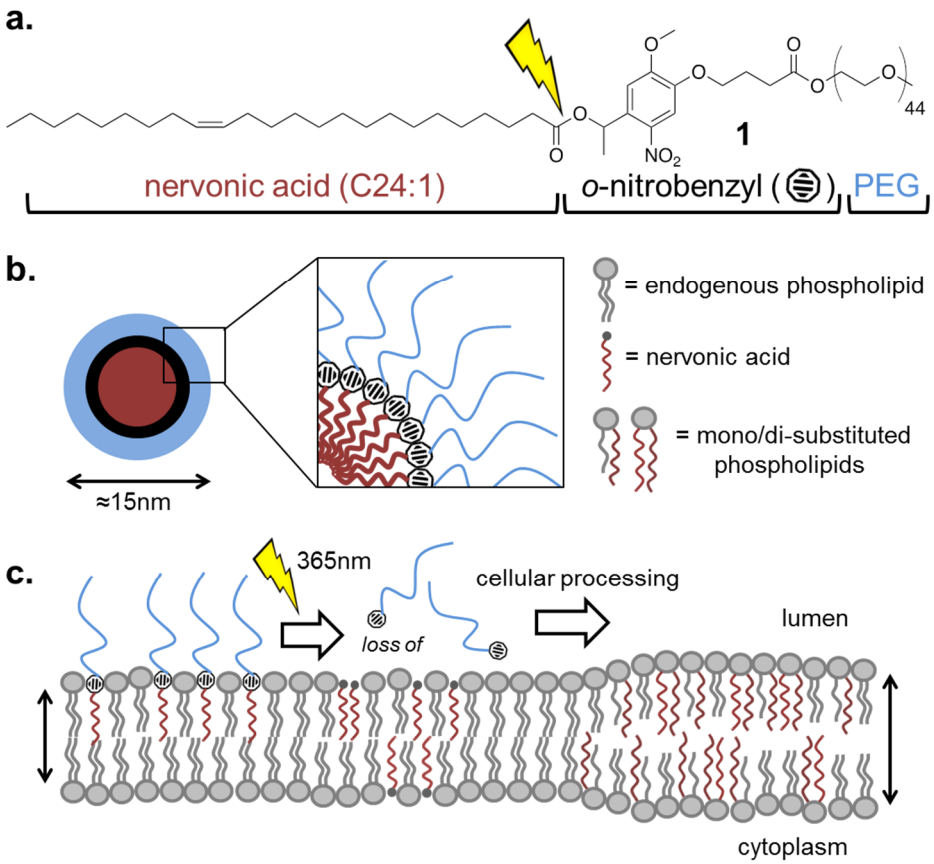



**Figure 1.** Schematic showing the delivery of nervonic acid to cells using light activatable micelles. (a) chemical structure of photo-cleavable nervonic acid-PEG (**1**); (b) the micellar structure of PEGylated nervonic acid; (c) incorporation, light activation and biotransformation of nervonic acid to vlcPLs and ultimately a thicker cell membrane.

## 4.2 Results and discussion

The synthesis and characterization of photolabile, nervonic acid-*o*-nitrobenzyl-$PEG_{2000}$, **1** (Figure 1a), is described in the Supporting Information (Scheme S1, Figures S1, S2). Upon UV light irradiation in PBS, complete photolysis was achieved within 30 min (Figure 2a, b). The appearance of a clear isosbestic point at 320 nm in the UV-Vis absorption spectra indicates clean photoconversion of **1** to its photoproducts. HPLC-ELSD analysis of the photolysis products confirmed the expected release of NA (Figure 2c). Self-assembly of **1** in aqueous media resulted in close-packed micelles. The size of these micelles was approximately 20 nm in size as determined by electron

microscopy (Figure 2d) and dynamic light scattering (DLS, Figure S4). The critical micelle concentration (CMC) of **1** was 2.9 µM (Figure S3) and particles were stable up to at least 1 mM (Figure S4).

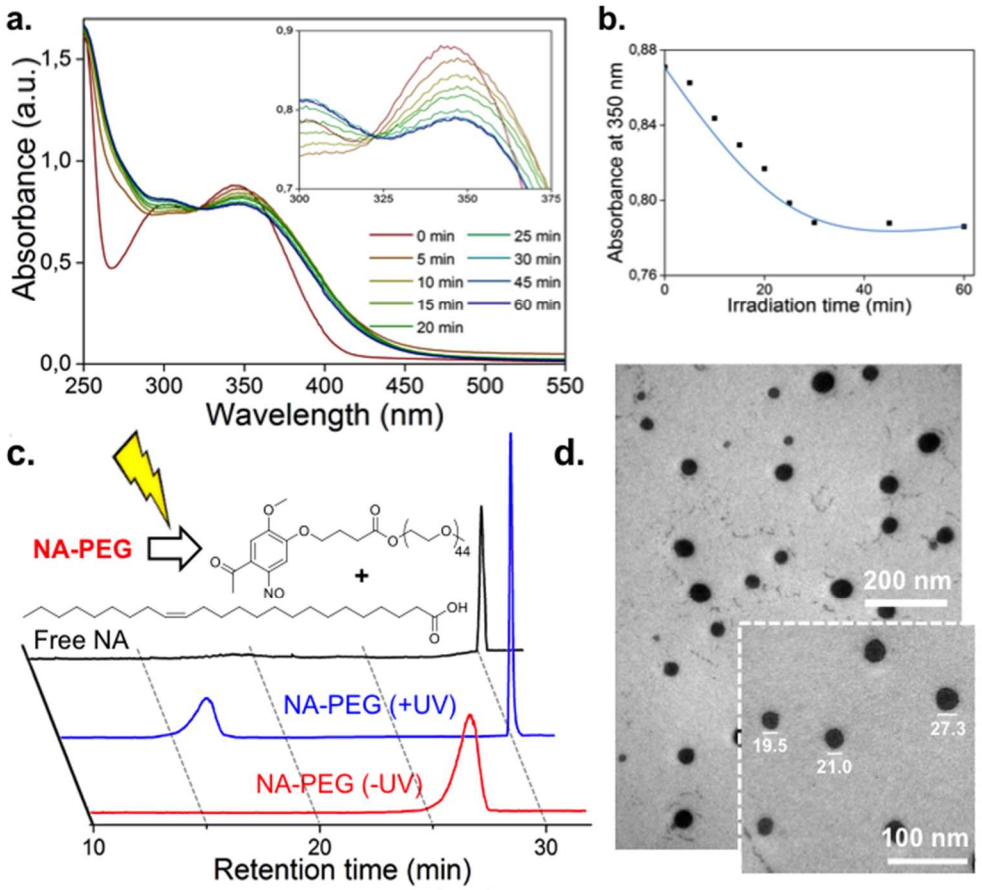



**Figure 2.** (a) Time evolution of the UV-Vis spectra of a solution of **1** during photolysis (365 nm, 3-5 mW/cm2); (b) Time evolution of the UV absorbance at 350 nm during photolysis. (c) HPLC-ELSD analysis of **1** before (red) and after (blue) UV irradiation. HPLC-ELSD analysis of free NA (black) was used to confirm photolysis of **1** to free NA. (d) TEM image (uranyl acetate stain) of micelles of 1 (500 µM, approx. 1.31 mg/mL).

Next, the incorporation of **1** into model phospholipid (POPC) membranes was assessed (Figure 3). An aliquot of concentrated micelles of **1** (1 mM, in PBS) were mixed with preformed POPC liposomes (large unilamellar vesicles) at a 1:10 molar ratio (50 µM **1**, and 500 µM POPC lipid, respectively). Successful incorporation of **1** into the POPC liposome membrane was, in part, confirmed by a small increase in the

hydrodynamic radius ($r_h$) of the liposomes (as measured by DLS) prior to light activation. This we attribute to the additional PEG corona now presented from the outer leaflet of the liposome membrane. As expected, upon light irradiation and cleavage of the PEG corona, the $r_h$ returned to the original size of the parent POPC liposome, and a significant decrease in the surface charge was observed. The latter is attributed to the liberated carboxylate functionality of free NA upon UV irradiation. These observations confirm successful incorporation of NA into model lipid bilayers by our procedure. In contrast, addition of NA (via ethanol injection (*i.e.* addition of an aliquot of a concentrated stock solution of NA in ethanol)) to POPC liposomes resulted in no significant change to the liposome surface charge (Figure 3), highlighting the difficulties in incorporating highly insoluble vlcFAs into lipid membranes by conventional methods.

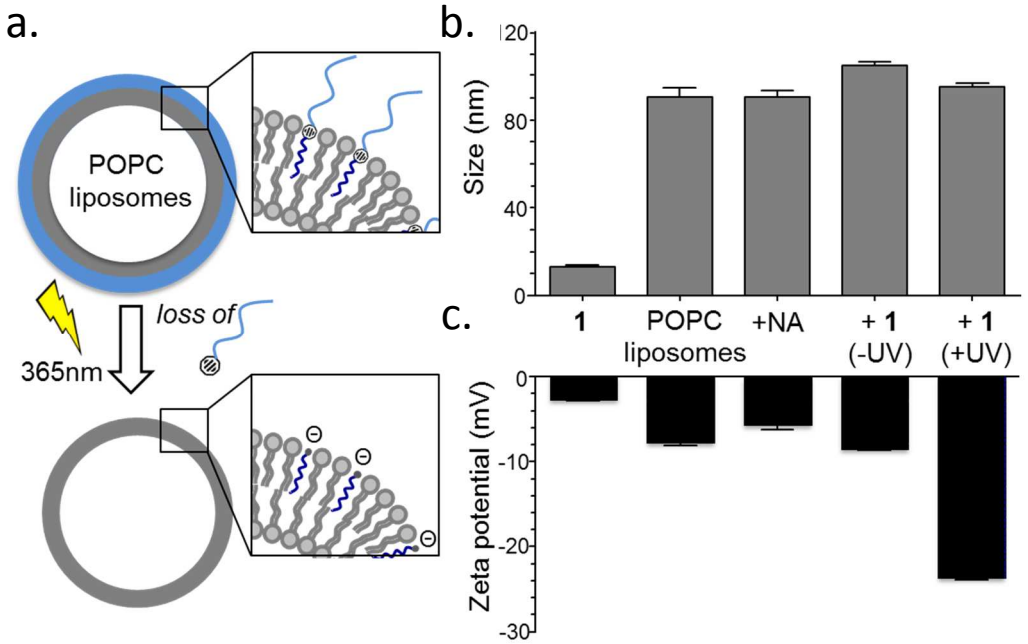



**Figure 3.** Incorporation and activation of **1** in model lipid membranes. (a): Cartoon of the procedure. (b) Measured hydrodynamic radii ($r_h$) and (c) zeta potentials of micelles of **1** and POPC liposomes. From l to r: micelles of **1**, unmodified POPC liposomes, POPC liposomes after ethanol injection of **1**, and, alternatively: POPC liposomes incubated with **1** (10:1 molar ratio), followed with subsequent UV light irradiation and photolytic loss of PEG.

To verify analogous incorporation of **1** into biological membranes, we prepared micelles of **1** containing 1 mol% fluorescently-labeled nervonic acid (**NA-Fluo**, see Supporting Information for synthesis and characterization). When mixed micelles of **1** and **NA-Fluo** were incubated with HeLa cells, a homogenous distribution of fluorescence across all cells was immediately observed (Figure S5 and S6). This indicated **NA-Fluo** had spontaneously incorporated into cellular membranes during the process of dissolution of mixed micelles and subsequent transfer of **1** to cell membranes.

Having established the delivery of NA to cellular membranes by our procedure, we next investigated whether HEK293 cells could process free NA, following photolysis of **1,** to generate elevated levels of specific phospholipids with incorporated vlcFAs. First, in a control experiment, we tested the potential for UV induced cytotoxicity to cells (Figure S8). Interestingly, while untreated cells (in PBS) showed significant UV induced cell death (approx. 80% cell viability following 15 min irradiation; determined by WST cell viability assay), cells modified with **1** showed no significant UV induced cytotoxicity following at least 20 min irradiation. This result is consistent with previous observations showing supplemental monounsaturated fatty acids could protect cells from oxidant injury.[15] Alternatively, quenching of UV light by nitrobenzyl-PEG might have protected the cells. Subsequently, following cell membrane incorporation of **1** (100 µM) and subsequent light activation (10 min), cells were incubated for 72 h to allow for the processing of free NA and incorporation into membrane lipids. After incubation, cells were pelleted and cellular lipids extracted and analyzed by thin layer chromatography (Figure S7). Phosphatidylcholine lipids (PC) are the most common class of endogenous phospholipids in cells[16] and incorporation of vlcFAs into PC lipids, thereby increasing the hydrophobic content, leads to reduced retention on the silica TLC plate. Indeed, for cells incubated with **1** and subsequently irradiated, an upshift in the PC lipid content was observed on the TLC plate (Figure S7). Subsequently, from the same samples the relative amounts of 61 cellular PC, 31 phosphatidyl-ethanolamine (PE), 12 sphingomyelin (SM) and 15 free fatty acid (FA) species were analyzed and compared by LC-MS lipidomics (see SI for methods).

Figure 4a demonstrates the changes, focusing on species with incorporated vlcFA. Remarkably, five PC species (PC (22:1/22:1), PC (24:1/24:1), PC(24:1/16:0), PC(24:1/16:1) and PC (24:1/18:1)) as well as one PE species (PE(24:1/18:1)) were increased significantly at the expense of common PC species (PC(16:0/18:1), PC(18:0/18:1), PC(18:1/18:1)) and PE(18:1/18:1). This redistribution demonstrates that indeed, due to the procedure, liberated NA had been taken up by the cell and incorporated into cellular phospholipids. Thus, part of the NA had been esterified into phospholipids by acyl-transferases and/or in *de novo* synthesis pathways of phospholipids. Moreover, a fraction of NA was shortened to erucic acid (FA22:1) which was subsequently incorporated to yield among others PC (22:1/22:1). Also the profile of SM species had altered. Although these phospholipids represent only a minor (<5%) fraction of membrane lipids, those containing vlcFA accumulate into rafts, thereby increasing locally the membrane thickness.[17] Remarkably, in the treated cells, specific SM species containing vlcFA (*i.e.* SM (d14:2/20:0), SM (d14:1/26:0), SM (d14:1/28:0) and SM (d14:1/28:2) were down-regulated, whereas prevailing SM (d18:1/16:0) and SM (d18:1/24:1) remained the same. Possibly, this redistribution of SM species compensates for increased levels of vlcFA-PC lipids accumulating into membrane rafts, taking over the local membrane thickening function of vlcFA-SM. Interestingly, the distribution of free fatty acids remained shifted dramatically towards vlcFA in the cells to which NA had been delivered, compared to the control (-UV) (Figure 4c). Remarkably, even after 72 h incubation of the cells, still 38 % of the circulating FA were presented by NA (compared to 5% in the control), whereas common FA such as palmitic acid (FA 16:0) and stearic acid were decreased. Interestingly, erucic acid was increased corroborating our above-mentioned observation that a fraction of NA was β-oxidized to erucic acid (FA 22:1). It appears that the initial high levels of released NA were not toxic for the cells and during the incubation only a fraction of the delivered NA had been esterified and/or β-oxidized while the cells down-regulated common long-chain fatty acids. This is in line with known high affinity of fatty acids to bind to cellular membranes, which easily accommodate 2 mole % fatty acids under physiological conditions.[18] In summary, these results demonstrate that the distribution of several of lipid species in cells can be altered significantly by our procedure of delivery of large amounts of NA.

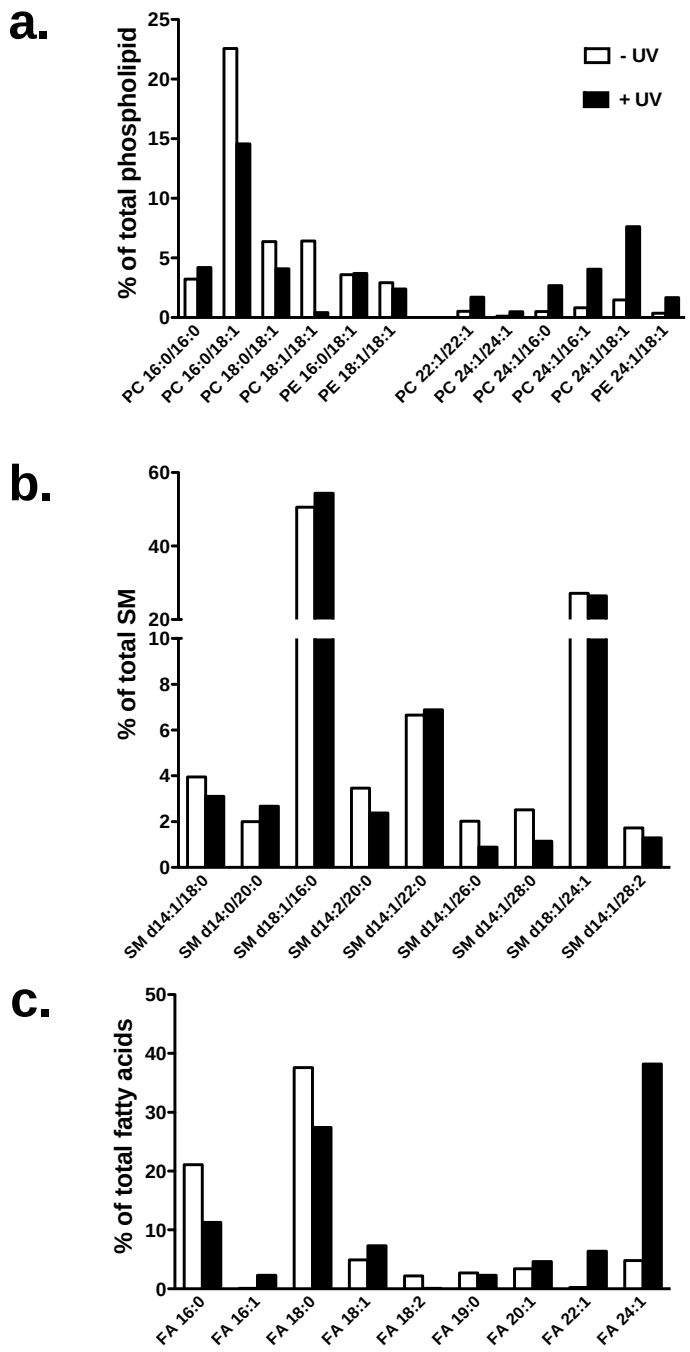



**Figure 4.** Membrane remodeling in HEK293 cells due to delivery of NA by **1**. Changes in the distribution of (a) glycero-phospholipid (PC and PE), (b) sphingomyelin (SM) and (c) free fatty acid (FA) species, after UV release of PEG and subsequent 72 h incubation of the cells at 37 $^{o}$C.

## 4.3 Conclusions

In this chapter, we demonstrate efficient delivery of vlcFAs to cell membranes, through the use of light sensitive micelles, composed of PEG-nervonic acid (FA24:1) conjugates. In the absence of light, PEG-NA micelles spontaneously disassembled in the presence of a cell membrane to leave individual PEG-NA molecules embedded within the membrane. Upon light irradiation, PEG is cleaved, leaving free vlcFAs within the cell membrane. Subsequent cellular processing of vlcFAs led to elevated levels of cellular phospholipids. Our approach overcomes the conventional difficulties associated with supplementing cell membranes with highly insoluble vlcFAs and is expected to be transferable to the delivery of any vlcFA or other very hydrophobic lipid species to cell membranes. This procedure has the potential to open new venues in research ranging from studies of membrane protein activities to cellular mechanisms of disease-related altered levels of vlcFA.

## 4.4 Experimental

### 4.4.1 Materials and Instruments

Phospholipids used for liposomes, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (**POPC**), were purchased from Avanti Polar Lipids. All chemical reagents, including nervonic acid, were purchased from Sigma Aldrich and used without further purification. All solvents were purchased from Biosolve Ltd. Phosphate buffered saline (PBS): 5 mM $KH_2PO_4$, 15 mM $K_2HPO_4$, 150 mM NaCl, pH 7.4. Silica gel column chromatography was performed using silica gel grade 40-63 µm (Merck). TLC analysis was performed using aluminum-backed silica gel TLC plates ($60_F$ 254, Merck), visualization by UV absorption at 254 nm. NMR spectra were measured on a Bruker AV-400MHz spectrometer. Chemical shifts are recorded in ppm. Tetramethylsilane (TMS) is used as an internal standard. Coupling constants are given in Hz. LCMS analysis was performed Jasco HPLC-system coupled to a Perkin Elmer Sciex API 165 mass spectrometer. MALDI-TOF mass spectra were acquired using an Applied Biosystems Voyager System 6069 MALDI-TOF mass spectrometer. α-Cyano-4-hydroxycinnamic acid (CHCA) was used as matrix in all cases. Sample concentrations were ~0.3 mg/ml. HPLC-ELSD analysis was performed using a Shimadzu HPLC setup equipped with two LC-8A series pumps coupled to a Shimadzu ELSD-LT II detection system. Separation (Vydac 214 MS C4 column, 5u, 100 × 4.6 mm, flow rate: 1 mL/min), in all instances, was carried out over a linear gradient of 10-90% **B** over 20 minutes with an initial 5 min hold at 10% **B**. HPLC buffers: **A** – $H_2O$ (0.1% TFA); **B** – Acetonitrile (0.1% TFA). The drift tube temperature for ELSD was set at $37^0$C and the nitrogen flow-rate at 3.5 bar. UV absorption spectra were measured using a Cary 3 Bio UV-Vis spectrometer, scanning from 200 nm to 500 nm at 1 nm intervals. Scan rate: 150 nm/min.

Particle size distributions and zeta potential measurements were obtained using a Malvern Zetasizer Nano ZS equipped with a peltier controlled thermostatic holder. The laser wavelength was 633 nm and the scattering angle was $173^o$. To obtain an estimation of the hydrodynamic radius, $D_h$, the Stokes-Einstein relation was used:

$$D = \frac{K_B T}{3\pi\eta D_h}$$

where, $k_B$ is the Boltzmann constant and η is the viscosity of the solvent. DLS measurements were carried out at room temperature. Zeta potentials were measured at room temperature, at 500 µM total lipid concentration and 10 mM NaCl concentration. All reported DLS measurements and zeta potentials are the average of three measurements.

For experiments not involving cells, UV light irradiation was performed using a hand-held BLAK-RAY B-100AP high intensity UV lamp (365 nm, 5 mW/cm$^2$) encased in a cardboard box. Samples were irradiated in quartz cuvettes at a fixed distance of 10 cm from the UV source. For all cell experiments, UV light irradiation was performed using a high-power LED (365 nm, 15-17 mW/cm$^2$, Roithner Laser Technik, GmbH) mounted at a fixed distance of 1 cm above the cells.

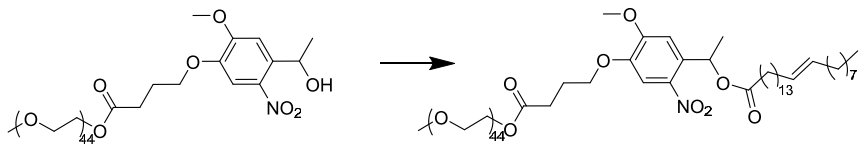
Micelles of **1** were prepared *via* thin hydration (PBS) and bath sonication (Branson 2510 Ultrasonic Cleaner, 50 $^o$C, 5 min). POPC liposomes (10 mM) were prepared by *via* thin hydration and extrusion at room temperature (Mini-extruder, Avanti Polar Lipids, Alabaster, US). Hydrated lipids were passed 11 times through 2 x Confrim 400 nm polycarbonate (PC) membranes (Nucleopore Track-Etch membranes, Whatman), followed by 11 times through 2 x 100 nm PC pores. POPC liposomes were used immediately after formulation.

The structure of the micelles of **1** were characterized using (TEM) operated at 70 kV TEM transmission electron microscopy (TEM, JEOL 1010, USA). The sample was dropped on copper grids with carbon film and washed 3 times with water. All the samples were stained with 0.5% uranyl acetate in water for 2 min. Next, the samples were dried under a $N_2$ atmosphere in the dark.

Cells (HeLa and HEK293) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS, iron supplied), 2% L-glutamine, 1% penicillin and 1% streptomycin. Cells were cultured in an atmosphere of 5% $CO_2$ at 37°C. Medium was refreshed every two days and cells passaged at 70% confluence by treatment with trypsin-EDTA (0.05% trypsin). For fluorescence imaging

(Figure S5), HeLa cells were seeded (1x10$^5$ cells/cm$^2$) in 48-well plates (500 µL, Greiner bio-one, Cellstar®) and cultured (in DMEM+FCS) for a further 24 h. Prior to testing, culture medium was carefully removed and the cells washed once with PBS. Fluorescence microscopy was carried out using an Olympus IX81 fluorescence microscope equipped with a filter cube (Excitation wavelength, 470/40 nm; Emmssion wavelength, 525/50 nm) or visualization of fluorescein-labeled nervonic acid. For lipid analysis, HEK293 cells were seeded 1x10$^6$ cells/cm$^2$ in 12 well plates and cultured (in DMEM+FCS) overnight. Prior to testing, culture medium was carefully removed and the cells washed once with PBS.

### 4.4.2 Synthesis of 1




**2** **1**

**Scheme S1.** Synthetic scheme to **1**.

Methoxy-PEG$_{2000}$-4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate **(2)** was synthesized as reported in Chapter 2.

**Methoxy-PEG$_{2000}$-4-(2-methoxy-5-nitro-4-(1-(tetracos-15 enoyloxy) ethyl) phenoxy) butanoate (1)**

To a stirred solution of **2** (370 mg, 0.16 mmol) in $CH_2Cl_2$ (5 mL) was added DMAP (12.2 mg, 0.1 mmol), EDCI (57.5 mg, 0.30 mmol), DIPEA (78.3 mL, 0.45 mmol) and nervonic acid (92 mg, 0.25 mmol). After overnight stirring under $N_2$, the reaction mixture was diluted by EtOAc (50 mL) and washed with sat. $NaHCO_3$ (3 x 50 mL) and brine (50 mL). The organic fractions were combined, dried ($Na_2SO_4$) and solvent removed under vacuum. Column chromatography (*Gradient*: $CH_2Cl_2$ to 10% MeOH in $CH_2Cl_2$) afforded **1** as a light yellow waxy solid (208 mg, 0.08 mmol, 50%). $\mathbf{R_f}$: 0.38 ($CH_2Cl_2$:MeOH; 10:1). **$^1$H-NMR** ($CDCl_3$, 400 MHz): 7.60 (s, 1H), 7.02 (s, 1H), 6.49 (q, *J = 8 Hz*, 1H), 5.37 (t, *J = 4 Hz*, 1H), 4.28 (m, 2H), 4.13 (t, *J = 4 Hz*, 2H), 4.02 (s, 3H), 3.45-3.95 (m, 174H), 3.41 (s, 3H), 2.60 (t, *J = 4 Hz*, 2H), 2.35 (m, 2H), 2.23 (m, 2H), 2.03 (m, 4H), 1.63 (d, *J = 8 Hz*, 1H), 1.28 (m, 32H), 0.9 (t, *J = 6 Hz*, 3H).

### 4.4.3 Synthesis of fluorescein-labeled nervonic acid, NA-Fluo

To a stirred solution of nervonic acid (20 mg, 0.05 mmol) in $CH_2Cl_2$ (2 mL) was added HCTU (80 mg, 0.2 mmol), DIPEA (70 mL, 0.4 mmol) and fluoresceinamine (70 mg, 0.2 mmol). After stirring for 5h, the reaction solution was evaporated under vacuum. The residue was dissolved in DCM (10 mL) and washed with sat. $NaHCO_3$ (3 x 20 mL) and brine (20 mL). Column chromatography (*Gradient*: $CH_2Cl_2$ to 10% MeOH in $CH_2Cl_2$), afforded fluorescein-labeled nervonic acid as a yellow solid (15 mg, 0.02 mmol, 45%). Purity (>90%) was confirmed by HPLC-ELSD (Figure S5). $\mathbf{R_f}$: 0.27 ($CH_2Cl_2$:MeOH; 10:1).

MS-ESI: (*m/z) found*: 696.2 $[M+H]^+$, *expected*: 695.42.

### 4.4.4 Photolysis of 1

A solution of **1** (100 µM) in PBS was irradiated (365 nm, 3-5 mW/cm$^2$) for 5 min, followed immediately by acquisition of the UV-visible absorption spectra. The same sample was then re-irradiated and this cycle repeated for cumulative irradiation time points of 10, 20, 30 and 60 min. The products of the photolysis reaction were confirmed by HPLC-ELSD analysis.

### 4.4.5 Interaction between micelles of 1 and POPC liposomes

To four solutions of preformed POPC liposomes (10 µL, 10 mM in PBS) were independently added a) 90 µl PBS – to give a 1mM of unmodified POPC liposomes, 2 x b) 10 µL of micelles of **1** (1 mM) followed by 80 µL of PBS – to give get POPC liposomes modified with **1** (1:10 mol ratio; POPC: **1**), c) 10 µL of nervonic acid solution (1 mM in EtOH). The solutions were pipette mixed and left for 15 min at room temperature. UV irradiation (365 nm, 3-5 mW/cm$^2$) of 1 x b) sample was performed for 20 min. DLS and zeta potential measurements were taken immediately.

### 4.4.6 Delivery micelles of 1 and NA-FA to cells

For fluorescence imaging and cell viability assays, HeLa cells were seeded (1x10$^5$ cells/cm$^2$) in 48-well plates (500 µL, Greiner bio-one, Cellstar®) and cultured for a further 24 h. Prior to testing, culture medium was carefully removed and the cells washed once with PBS. Micelles of **1** containing 1% of **NA-Fluo** were prepared by film hydration with PBS and sonication as for non-fluorescent micelles of **1**. Fluorescently

labeled micelles (500 µM total, 5 µM **NA-Fluo**, 500 µL) were added to HeLa cells and incubated for 20 min. The micelle solution was subsequently removed and cells washed with PBS (3x) and re-suspended in DMEM+FCS. Cells were imaged immediately under the fluorescent microscope using an Olympus IX81 fluorescence microscope equipped with a filter cube (Ex. 470/40; Em. 525/50).

### 4.4.7 Lipid analysis of cell lysates with TLC

For analysis of cellular lipids, HEK293 cells were seeded ($8.57x10^5$ cells/$cm^2$) in 12 well plates and cultured for a further 24 h prior to the addition of **1** at a final concentration of 100 µM. Cells were irradiated (10 min, 365 nm, 15-17 mW/$cm^2$) and incubated for a further 72 h. Cells were then scraped and washed twice in ice-cold PBS by centrifugation at 2500 g for 10 min at 4°C. 25-50% of the cell pellet was re-suspended in $CHCl_3$:MeOH (1:2). To extract cellular lipids, pellets were briefly (bath) sonicated before centrifugation at 17000 g for 10 min at 4°C. The supernatant containing cellular lipids was resolved by TLC using a mobile phase of $CHCl_3$:MeOH:$H_2O$ (65:25:4). In this solvent system, the phospholipids phosphatidylcholine, phosphatidylinositol and phosphatidylserine co-migrate as one spot (PL). Synthetic 24:1 PC, 22:1 PC and POPC were used as standards. TLC analysis was performed using Silica HPTLC plates (Millipore Cat no: 1.05644.001), developed by spraying with 10% $CuSO_4$ (w/v) in 8% phosphoric acid (v/v) and charring at 95°C overnight on a hot plate.

### 4.4.8 Cell viability assay

HeLa cells were seeded in 96-well plates at a density of $10^4$ cells per well and incubated overnight. Cells were washed once with PBS, then micelles of **1** (100 µL, varying concentrations in 1:1 PBS:DMEM+FCS), free DOX solutions (100 µL varying concentrations in 1:1 PBS:DMEM+FCS) or DMEM+FCS alone (100 µL) were added and the cells incubated for 12 h. Cells were then washed three times (DMEM+FCS), re-suspended in DMEM+FCS and incubated for a further 24 h. Cell media was removed and 200 µL Cell Proliferation Reagent; WST-1 (Sigma) added to each well. Cells were incubated (37°C) for a further 3 h, according to the supplier guidelines. To determine cell viability, absorbance at 450 nm was measured. All experiments were carried out in quadruplicate.

### 4.4.9 Lipidomics

Lipidomic analysis of HEK293 lipid extracts was performed using a LC-MS/MS based lipid profiling method. A Shimadzu Nexera LC-30 (Shimadzu, 's Hertogenbosch, The Netherlands) was used to deliver a gradient of water/acetonitrile 80:20 v/v (eluent A) and water/acetonitrile/2-propanol 1:90:9 v/v (eluent B). Both eluents contained 5 mM ammonium formate and 0.05% formic acid. The applied gradient, with a column flow of 300 µL/min, was as follows: 0 min 40% B, 10 min 100% B, 12 min 100% B. A Phenomenex Kinetex C18, 2.7 µm particles, 50 x 2.1 mm (Phenomenex, Utrecht, The Netherlands) was used as column. The injection volume was 10 µL. The MS was a Sciex TripleTOF 6600 (AB Sciex Netherlands B.V., Nieuwerkerk aan den Ijssel, The Netherlands) operated in positive (ESI+) and negative (ESI-) ESI mode, with the following conditions: Ion Source Gas 1, 2 and Curtain gas 30 psi, temperature 350˚C, acquisition range *m/z* 100-1200, IonSpray Voltage 5500 V (ESI+) and -4500 V (ESI-), declustering potential 80 V (ESI+) and -80 V (ESI-). An information dependent acquisition (IDA) method was used to identify lipids, with the following conditions for MS analysis: collision energy ±10, acquisition time 250 ms and for MS/MS analysis: collision energy ±45, collision energy spread 25, ion release delay 30, ion release width 14, acquisition time 40 ms. The IDA switching criteria where set as: for ions greater than *m/z* 300, which exceed 200 cps, exclude former target for 2 s, exclude isotopes within 1.5 Da, max. candidate ions 20.

Before data analysis, raw MS data files where converted with the Reifycs Abf Converter (v1.1) to the Abf file. MS-DIAL (v2.74), with the FiehnO (VS27) database, was used to align the data and identify the different lipids. PC and PE lipids where manually curated to confirm their identity. Due to overlap of triglyceride (TG) species, MS-DIAL could not sufficiently identify lipid species, in turn a modified identification approach was applied. Initially, MS-DIAL was used to get the total number of carbons and double bonds of a TG. This information together with the MS/MS spectrum was used to search the glycerolipid MS/MS predicted database on LipidMaps. TG's with all their neutral loss of lipid species fragments matched where assigned as correctly identified.

## 4.5 References

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## 4.6 Appendix

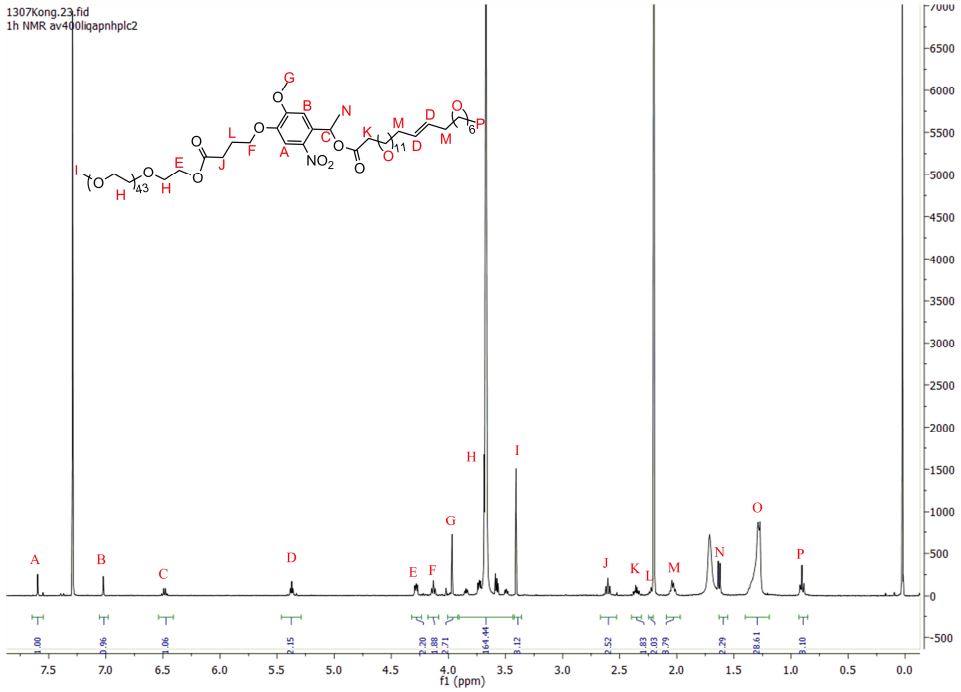


**Figure S1.** $^{1}$H-NMR of **1**.

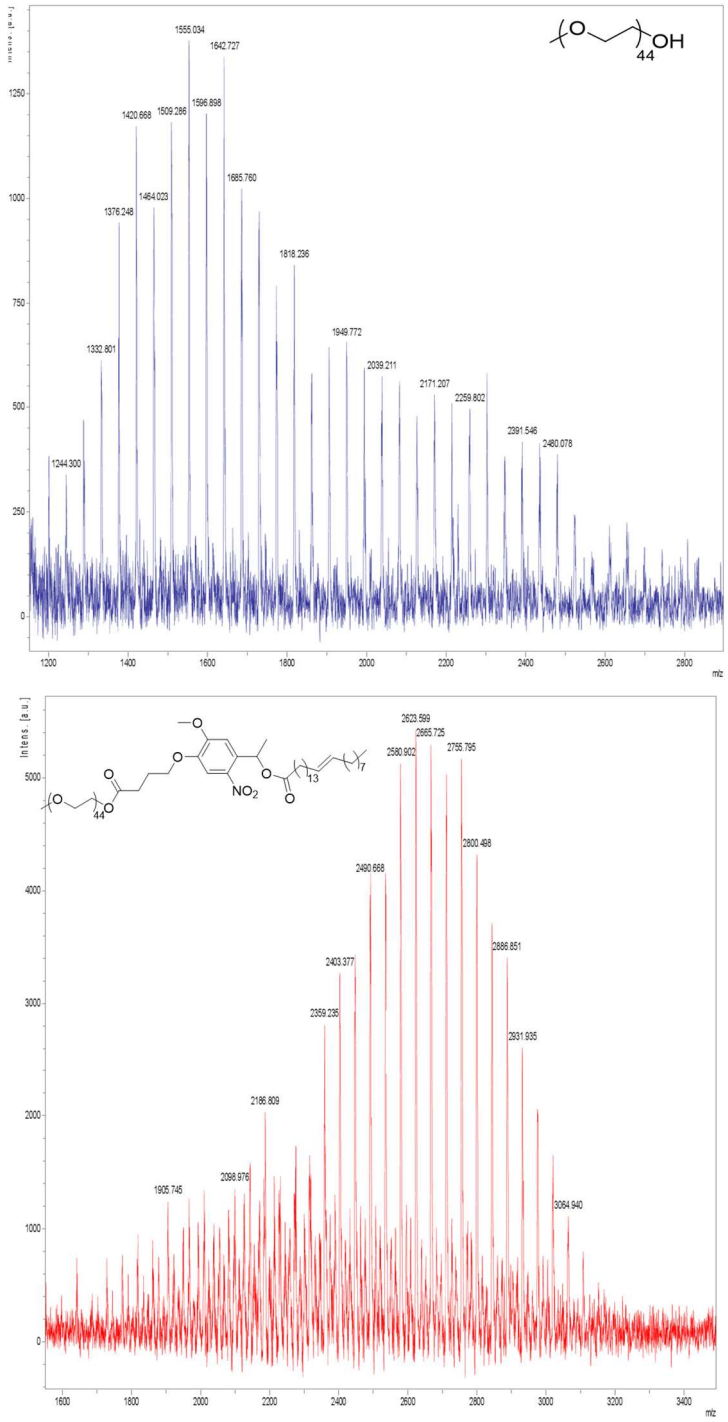


**Figure S2.** Maldi-TOF spectrum of $mPEG_{2000}$ and **1**. (The mass difference between $mPEG_{2000}$ and **1** is 630 g/mol which is the exact mass value of the extra part of nitrobenzyl-nervonic acid).

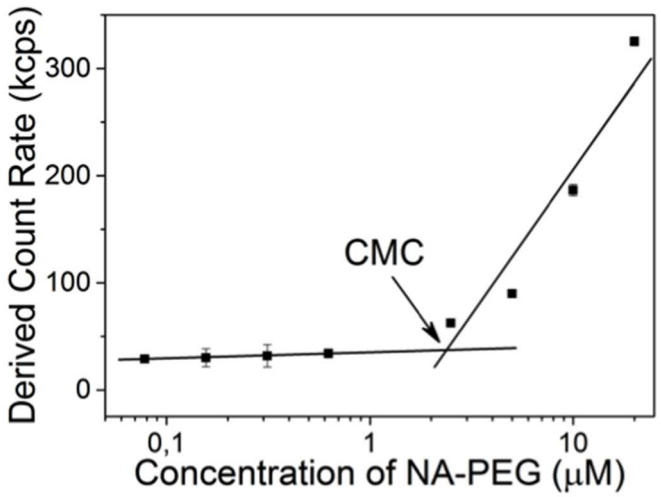


**Figure S3**. CMC determined by measuring light scattering intensity (raw count rate) as a function of concentration of **1** in PBS.

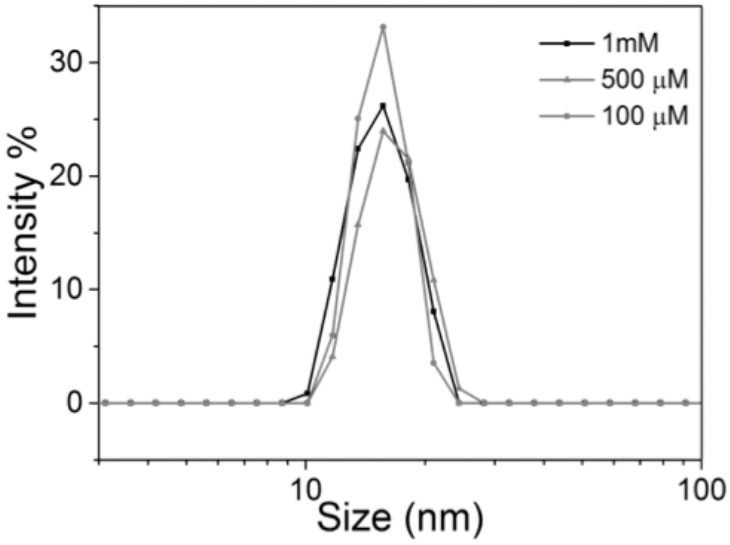


**Figure S4**. DLS size measurement of micelles of **1** at varying concentrations in PBS.

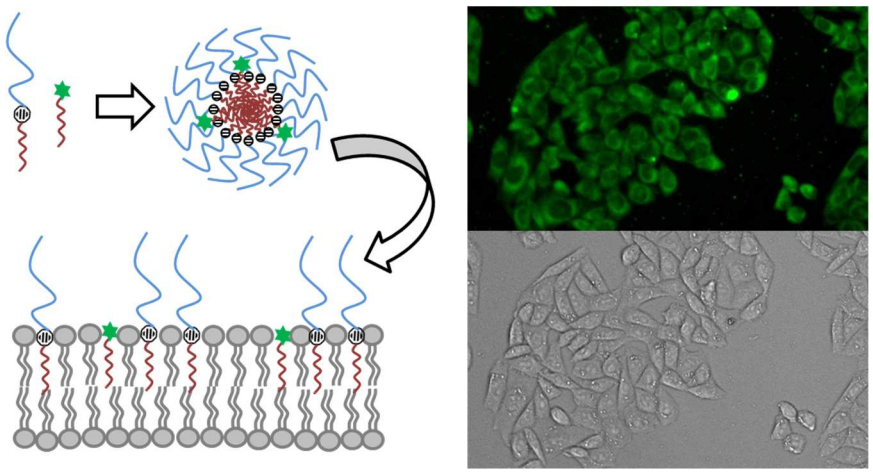

**Figure S5.** Brightfield and fluorescence image of HeLa cells following incubation of **1** micelles (containing 1 mol% **NA-Fluo**).

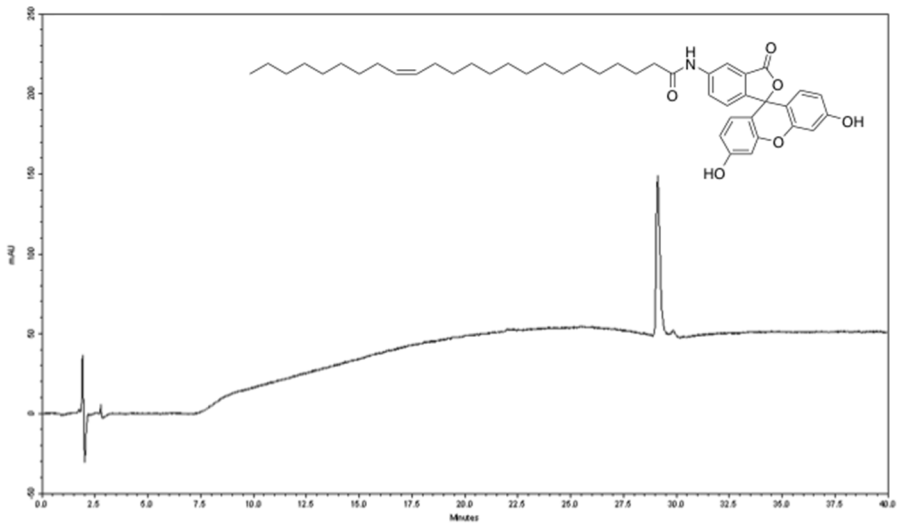


**Figure S6**. Analytical HPLC trace of purified **NA-Fluo**. *HPLC conditions*: Linear gradient of 10-90% **B** over 20 minutes with an initial 5 min hold at 10% **B**. HPLC buffers: **A**: $H_2O$ (0.1% TFA); **B**: Acetonitrile (0.1% TFA). Detection at 254 nm.

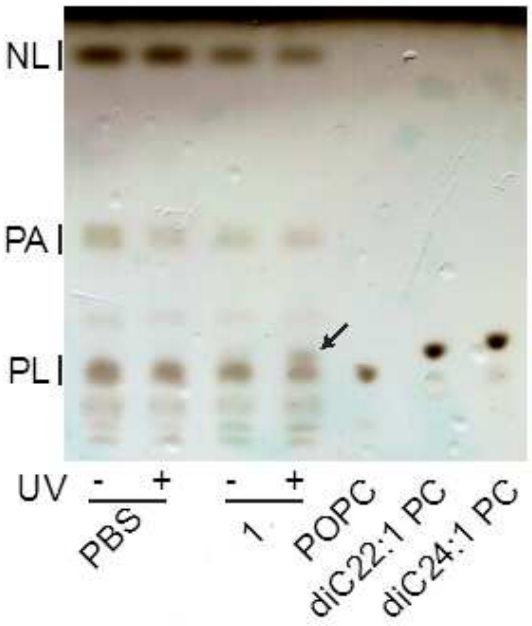


**Figure S7**. Thin layer chromatography of lipids extracted from HEK293 cells following treatment with **1**, In controls, PBS was added. PL: phospholipids (predominantly PC); PA: phosphatidic acid; NL: neutral lipids (predominantly cholesterol). The arrow indicates formation of slightly more hydrophobic phospholipids, *i.e.* species with incorporated vclFA.

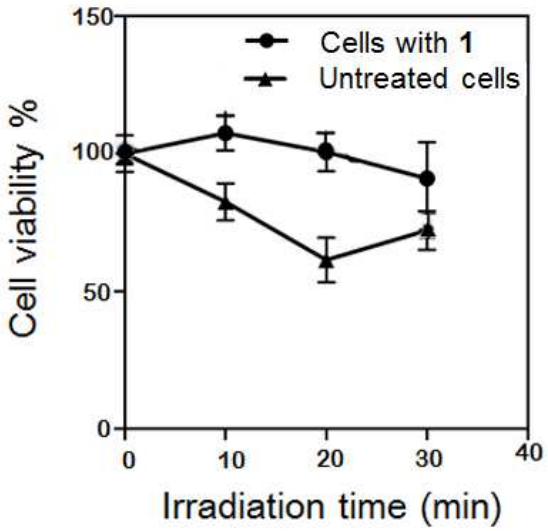


**Figure S8**. Cell viability following UV irradiation.