Photo-activated drug delivery systems

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Temporal Control of Membrane Fusion through Photolabile PEGylation of Liposome Membranes

Abstract: Membrane fusion results in the transport and mixing of (bio)molecules across otherwise impermeable barriers. In this chapter, we demonstrate, for the first time, temporal control of targeted liposome-liposome membrane fusion and contents mixing using light as an external trigger. Our method relies on the steric shielding and rapid, photo-induced de-shielding of complementary fusogenic peptides tethered to opposing liposomal membranes. In an analogous approach, we are also able to demonstrate precise spatiotemporal control of liposome accumulation at cellular membranes *in vitro*.

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2.1 Introduction

Membrane fusion is a fundamental process of life resulting in the highly regulated transport of (bio)molecules both between and within cells.[1] To achieve fusion, energetic barriers associated with bringing opposing membranes together and of subsequent membrane destabilization and merging must be overcome.[2] *In vivo*, large, often multi-component, protein fusion complexes have evolved to carry out this task. [3]

The development of synthetic systems capable of controlled (non-spontaneous) membrane fusion is a tantalizing prospect, not least for applications in vector (liposomal) based drug and gene delivery *in vitro* and *in vivo*. In this context, fusion of drug-loaded vector with target cellular membranes would result in drug delivery directly to the cell cytoplasm. Crucially, this route to intracellular drug and gene delivery minimizes degradative loss of encapsulated payloads associated with hydro- and proteolytic endocytotic uptake.[4]

Given the typical size and complexity of native fusion complexes, significant efforts have been made to develop simplified systems capable of membrane fusion.[5] These can be targeted[6] or non-targeted[7] Towards this goal, we have previously reported a supramolecular system capable of inducing rapid and targeted membrane fusion of distinct liposome populations.[8] Inspired by the native SNARE fusion complex, our targeted fusion system relies on the recognition and binding of complementary coiled-coil forming peptides (E and K) tethered to opposing liposome membranes (Figure 1). In our membrane fusion model, coiled-coil forming peptide pair (E/K) are conjugated to cholesterol via a short polyethylene glycol (PEG) spacer, yielding fusogens, so called CPE/CPK. Upon mixing E- and K-liposomes, membrane fusion and leakage-free, contents mixing occurs spontaneously.

In Nature however, membrane fusion is highly regulated in both time and space, ensuring correct biological function. Likewise, if simplified fusion systems are to be applied to drug and gene delivery systems, the ability to control when and where fusion occurs will be essential in ensuring clinically relevant therapeutic indices. Control of membrane fusion using simplified fusion systems, in either time and/or

space however has yet to be demonstrated. In this communication we first demonstrate precise temporal control of membrane fusion in model (liposome-liposome) systems. This result is achieved through steric shielding and rapid, photo-induced de-shielding of complementary and fusogenic liposome populations (Figure 1).

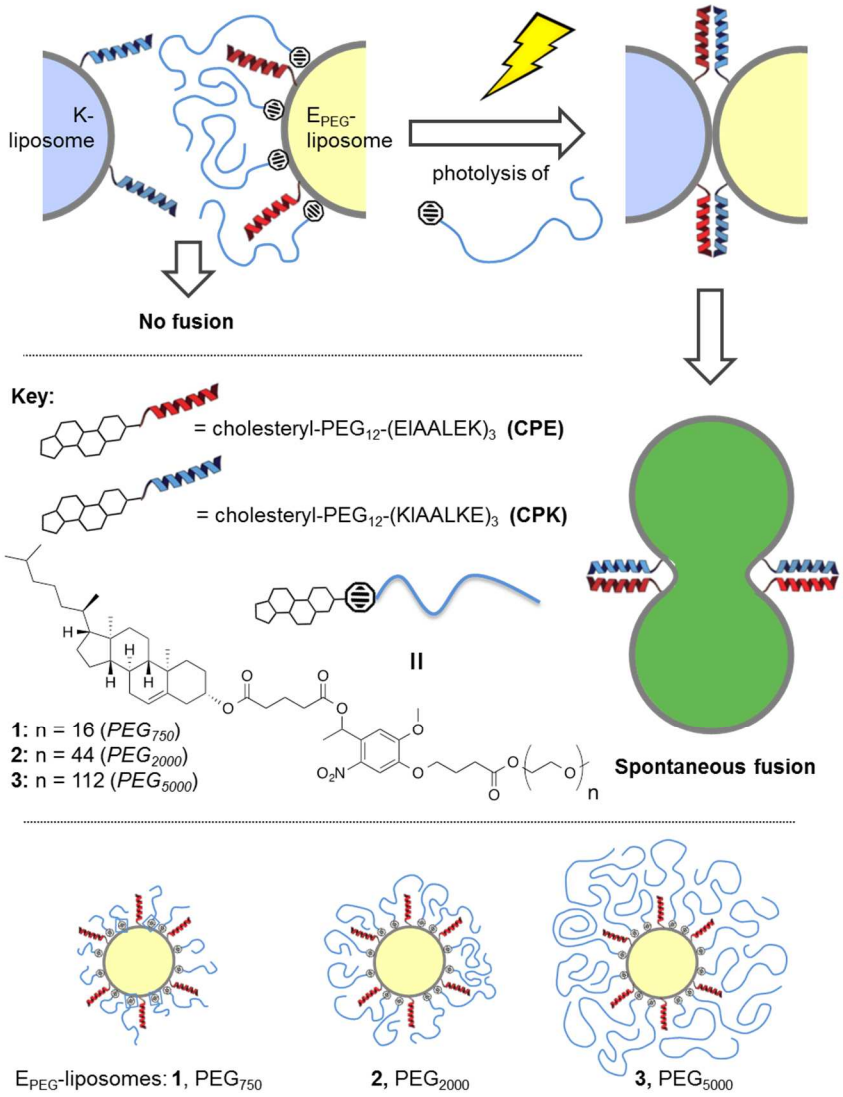


Figure 1. (top) Light induced, temporal control of liposome-liposome fusion through photolabile steric shielding (PEGylation) of fusogenic peptides tethered to opposing liposomal membranes. (bottom) E_{PEG}-liposomes sterically shielded with **1-3**.

Polyethylene glycol (PEG) is chosen as steric 'shield' given its widespread use in improving the pharmacokinetics and dynamics of biomolecules, nanoparticles and liposomes.[9] We have previously shown that 2 mol% PEGylation of both liposomal membranes in our simplified fusion system effectively shuts down membrane fusion through steric shielding of liposome-tethered peptides E and K.[10]

2.2 Results and discussion

The synthesis and characterization of photolabile cholesterol-o-nitrobenzyl-PEG constructs, **1-3**, are outlined in Section 2.4.2 (Figures S1-8). Upon UV light irradiation (365 nm, 3-5 mW/cm^2) in H_2O:MeCN:tBuOH (1:1:1), complete photolysis was achieved within 20 min as shown by UV-Vis spectroscopy (see Figure 2a). The appearance of three clear isosbestic points shows clean photoconversion of **2** to its photoproducts. Comparable spectra and rates of photolysis were found for **1** and **3** under identical irradiation conditions (Figure S9). HPLC-ELSD analysis of the photolysis products following irradiation of **2** in both H_2O:MeCN:tBuOH (1:1:1) (Figure S10b and S11) and PBS (Figure 2b) confirmed the conversion of **2** to cholesteryl hemisuccinate as expected. Conversion of **1** and **3** to their expected photoproducts was similarly observed (Figure S10a and S10c).

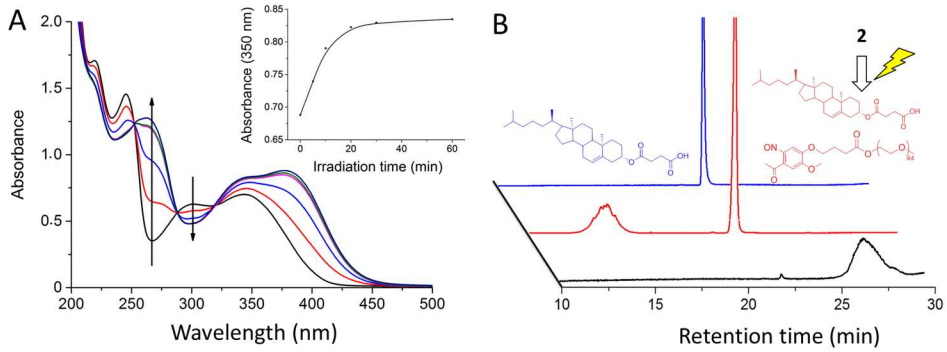


Figure 2. (a) Time evolution of the UV-Vis spectra of a solution of **2** (200 µM; H_2O:MeCN:tBuOH (1:1:1)) during photolysis (365 nm, 3-5 mW/cm^2). Inset: Time evolution of the absorbance at 350 nm. (b) HPLC-ELSD analysis of **2** (200 µM in PBS) before (black) and after (red) 30 min UV irradiation (365 nm, 3-5 mW/cm^2). Cholesteryl hemisuccinate (200 µM in PBS), an expected photoproduct, is shown in blue.

As a next step, liposomes containing 1 mol% CPE or CPK, referred to as E- and K-liposomes respectively, were formulated via lipid film hydration and sonication. Following previously published protocols,[11] *in situ* modification of E-liposomes with **1-3** yielded E_{PEG}-liposomes whose outer membrane leaflet contained between 0 and 10 mol% **1-3**. In all cases, liposomes, both before and after *in situ* modification, were ~100 nm in diameter as shown by dynamic light scattering (polydispersity index <0.2) (Figure S12).

For photolabile PEG constructs **2** and **3**, lipid mixing experiments – between K- and E_{PEG}-liposome populations – revealed that the degree of lipid mixing was inversely correlated to the degree of membrane PEGylation (Figure S13). In both cases, 4 mol% PEGylation of E-liposomes alone was sufficient to completely nullify lipid mixing between E- and K-liposomal membranes. For the shorter (PEG750) construct, **1**, the degree of PEGylation bore no influence on the rate or extent of lipid mixing between E_{PEG}- and K-liposomes (Figure 3). This was confirmed by circular dichroism (CD) measurements which, for mixed K- and E_{PEG}-liposomes (4 mol% **1**), shows a significant increase in helical content (Table S1), indicative of the formation of the expected heterodimeric coiled coil complex between peptides E and K (Figure 3). It should be noted that peptides E and K tethered to the liposome membrane are already ≈50% folded. This is consistent with previous reports on the conformation of these peptides when tethered to a liposome membrane.[8a,c,d]

The inability of the shorter PEG construct, **1**, to sterically shield the interaction between peptides E and K reflects a critical length requirement for the steric shield determined by the molecular size of peptides E and K.[12]

To assess how liposome-liposome fusion was influenced by UV light irradiation, E_{PEG}-liposomes containing 4 mol% **2** and **3** were irradiated for increasing periods of time prior to the addition of K-liposomes. As expected, lipid mixing efficiencies directly correlate with increasing pre-irradiation times (Figure 4). Complete lipid mixing, as compared to E- and K-liposome fusion in the absence of any steric shielding, was achieved following 30 min pre-irradiation of E_{PEG}-liposomes. This result was mirrored in analogous content mixing experiments (Figure S14).

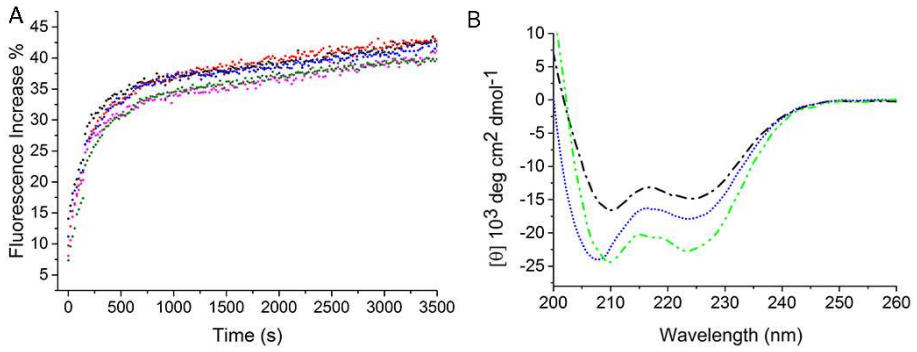


Figure 3. (a) Lipid mixing between E_{PEG}- and K-liposomes with varying amounts of **1** presented from the E-liposome membrane; 0 mol% (---), 2 mol% (---), 4 mol% (---), 8 mol% (---) and 10 mol% (---).Upon mixing E_{PEG}- and K-liposomes, membrane merging results in a decrease in Förster resonance energy transfer between donor and acceptor and an increase in donor fluorescence emission. (b) CD spectra of K-liposomes (---) alone, 4 mol% **1** E_{PEG}-liposomes alone (---) and mixed solutions of K- and E_{PEG}-liposomes (---).

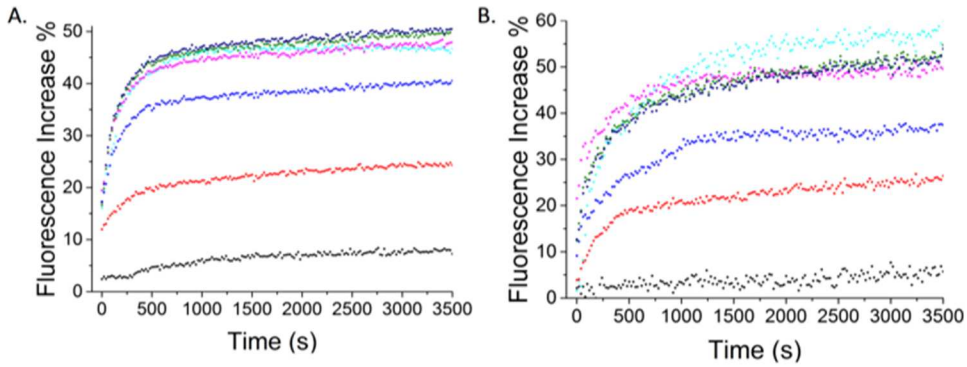


Figure 4. Lipid mixing of E_{PEG}- (4 mol%) and K-liposomes with increasing irradiation times of the E_{PEG}-liposomes prior to mixing with K-liposomes. A: E_{PEG}- (4 mol% **2**); **B**: E_{PEG}- (4 mol% **3**). Time: 0 (---), 5 (---), 10 (---), 20 (---), 30 (---), 60 min (---) irradiation. 0 mol% **2** (---).

To validate that membrane fusion is governed through the interaction of peptides E and K, CD measurements of mixed populations of E_{PEG}- and K-liposomes were taken both before and after irradiation (Figure 5). In both cases, following irradiation, an increase in helical content together with a shift towards an equal ratio of mean residue molar ellipticities at 208 and 222 nm respectively, confirms the formation of

the expected E/K heterodimeric coiled coil complex (see Table S13 for quantitative CD analysis).

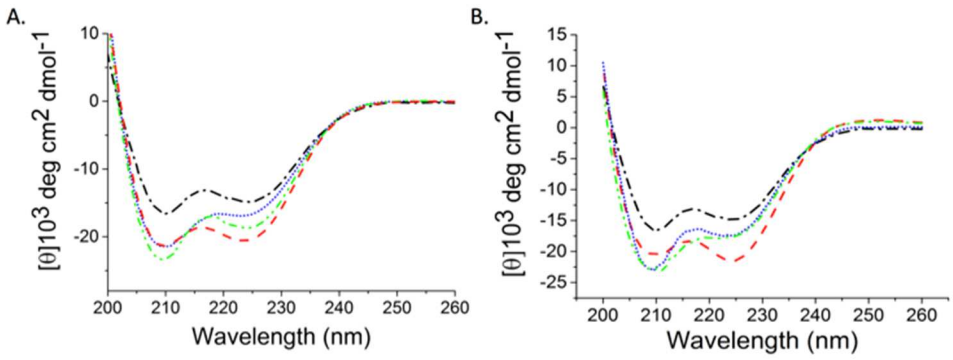


Figure 5. CD spectra: K-liposomes (**---**), 4 mol% E_{PEG}-liposomes (---), mixed solutions of K- and E_{PEG}-liposomes pre- (---) and post-irradiation (---). **A:** E_{PEG}- (4 mol% **2**); **B**: E_{PEG}- (4 mol% **3**).

To monitor the photolysis and subsequent liposome-liposome fusion *in situ*, populations of E_{PEG}-liposomes (containing 4 mol% **2**) and K-liposomes were pre-mixed and contents mixing monitored simultaneously before and during continuous UV irradiation (Figure 6). In the absence of UV light we no content mixing between liposomes was observed, however immediately upon UV irradiation liposome-liposome fusion commences. This demonstrates the direct dependence of liposome-liposome fusion on photolysis of the PEG shield from the E_{PEG}-liposomal membrane enabling precise temporal control of the fusion process. This result was mirrored in the analogous experiment with E_{PEG}-liposomes containing 4 mol% **3** (Figure S16). The influence of continuous UV irradiation upon the rate of fusion between E- and K-liposomes, in the absence of any PEGylation, was found to be insignificant (Figure S15).

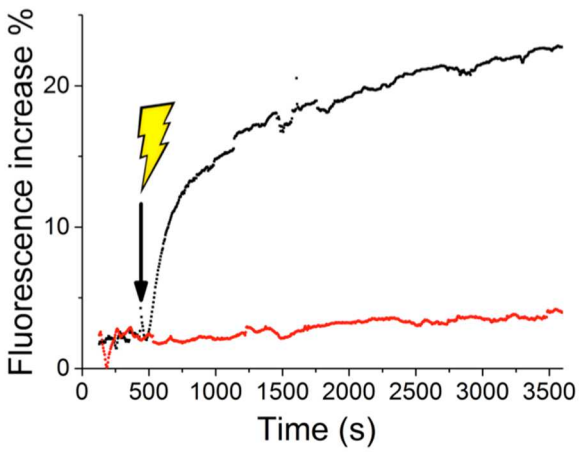


Figure 6. Content mixing following *in situ* photolysis of a 1:1 mixture of E_{PEG}-liposomes (4 mol% **2**) and K-liposomes, upon (---) and in the absence of (---) UV irradiation. Black arrow indicates point at which UV irradiation was turned on. For contents mixing measurements, a self-quenching concentration of a fluorescent dye (sulforhodamine B, 10 mM) is encapsulated within E_{PEG}-liposomes. Upon mixing and fusion with empty K-liposomes, dilution of the dye results in fluorescence dequenching.

Finally, applying this methodology to a biological context, we are able to demonstrate precise spatiotemporal control of liposome accumulation at pre-functionalised cellular membranes (Figure 7). To achieve this, we adapted previously reported protocols,[13] first incorporating lipopeptide K into the membranes of cells then incubating cells with E_{PEG}-liposomes (4 mol% **2**). 1 mol% fluorescent DOPE-LR was added to the E_{PEG}-liposome membrane composition for visualization. Remarkably, following photolysis of the steric shield from the E_{PEG}-liposomes, the interaction between peptides E and K, displayed from the liposome and cell surface respectively, is both specific and strong enough to enable well-defined, light-templated accumulation of liposomes at the cell surface. We have yet to confirm whether this interaction leads to full fusion of liposome and target plasma membranes. However, even if this results in docking alone, we can expect these liposomes, now localized at the cellular membrane, to be internalised over time, most likely via an endocytotic pathway.[14] Subsequent liposome degradation and endosomal escape would result in the intracellular release of liposome encapsulated content.

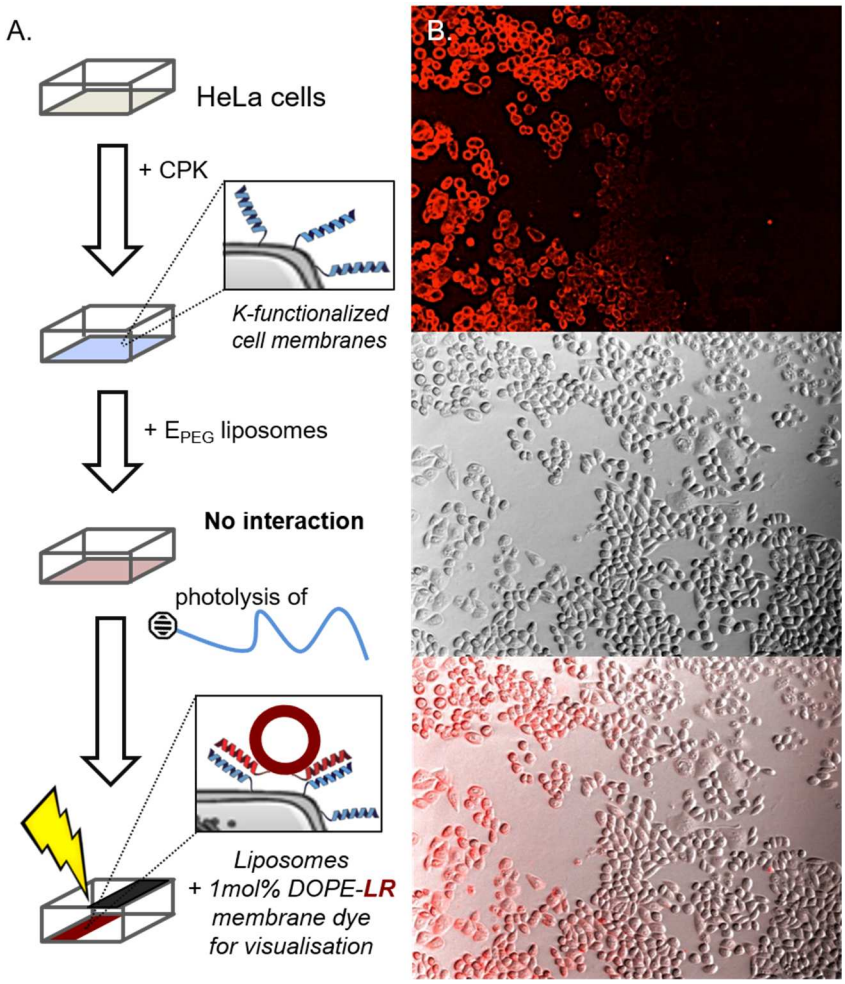


Figure 7. (A) Light directed, spatiotemporal control of liposome accumulation at pre-functionalised HeLa cell membranes *in vitro*. (B) (top) Fluorescence image (10x magnification) of E_{PEG}-liposomes (containing 1mol% DOPE-LR fluorescent probe) docked at pre-functionalised HeLa cell membranes following localised UV irradiation. (middle) Brightfield image (10x magnification). (bottom) Merge. Experimental details: Step 1. CPK solution (5 µM) incubated with cells for 15 min followed by washing. Step 2. E_{PEG}-liposome solution (250 µM containing 1 mol% CPE and 1 mol% DOPE-LR fluorescent probe) incubated with cells for 15 min. Step 3. Localised irradiation (10 min, 10 mW/cm^2) and further incubation for 15 min. Step 4. Wash and image.

2.3 Conclusions

In this study, we successfully synthesized photolabile cholesterol-o-nitrobenzyl-PEG constructs, **1-3** and incorporated these into E-liposomal membranes. We illustrate the need for a minimum PEG length (≥ 2000 g/mol) to effectively shield the interaction between fusogenic peptides, E and K. And we show rapid photo-induced de-shielding of E_{PEG}-liposomal membranes results in spontaneous, and temporal control of, fusion between distinct liposome populations *in situ*. Applying this approach to a biological context, we are also able to demonstrate light directed spatiotemporal control of liposome accumulation at pre-functionalised cellular membranes *in vitro*. It should be noted, no phototoxicity, arising from the use of UV-A (365 nm) light, was observed in cell experiments. In any event, potential issues of phototoxicity can largely be alleviated through the use of longer wavelength, 2-photon excitation sources, to which o-nitrobenzyl functionalities are photosensitive.[15] Likewise, whilst UV-A light suffers from poor tissue penetration, the use of 2-photon excitation sources enables light activation up to tissue depths of 1 cm.[16] In conclusion, the general method described holds significant promise towards non-invasive, user-defined vector based drug and gene delivery both *in vitro* and *in vivo*.

2.4 Experimental

2.4.1 Materials and Instruments

Phospholipids used for liposomes, 1,2-dioleoyl-sn-glycero-3-phosphocholine (**DOPC**), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (**DOPE**), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (**DOPE-NBD**) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (**DOPE-LR**), were purchased from Avanti Polar Lipids. Cholesterol and all other chemical reagents were purchased at the highest grade available 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 40-63µm (Merck). TLC analysis was performed using aluminium-backed silica gel TLC plates (60_F 254, Merck), visualisation by UV absorption at 254 nm and/or staining with $KMnO_4$ solution. NMR spectra (^1H) 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 on a 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. UV absorption spectra were measured using a Cary 3 Bio UV-vis spectrometer, scanning from 200 nm to 550 nm at 1 nm intervals. Scan rate: 120 nm/min. For the pre-irradiation of E_{PEG}-liposomes, UV light irradiation was performed using a hand-held BLAK-RAY B-100AP high intensity UV lamp (365 nm, 100 W) encased in a cardboard box. Samples were irradiated in quartz cuvettes at a fixed distance of 10 cm from the UV source. 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.

CPE (cholesterol-PEG$_{12}$-peptideE) and CPK (cholesterol-PEG$_{12}$-peptideK) were synthesized and purified as previously reported.[11] Peptide sequences were $(EIAALEK)_3$ and $(KIAALKE)_3$ for E and K respectively.

Liposomes were prepared via lipid film hydration and bath sonication using a Branson 2510 Ultrasonic Cleaner. Sonication was carried out at 55^{0}C.

Size exclusion chromatography (SEC) was carried out using illustra™ NAP™ Sephadex™ G-25 DNA grade pre-made columns (GE Healthcare) and used according to the user instructions.

Particle size distributions 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.

Fluorescence measurements for lipid and content mixing using E_{PEG}-liposomes irradiated prior to mixing with K-liposomes were performed on a TECAN Plate Reader Infinite M1000. All experiments were carried out in 96-well plates (PP Microplate, 96 well, solid F-bottom (flat), chimney well). For every experimental well the final experimental volume was 200 μL. Fluorescent measurements were recorded at 25^{o}C.

CD spectra were measured using a Jasco J-815 spectropolarimeter. The observed ellipticity is given in millidegrees, the conversion to the mean residue molar ellipticity is performed by the following equation:

$$[\Theta] = \frac{\Theta\mathrm{obs}}{\mathrm{lC_M N}}$$

where, Θ_{obs} is the observed ellipiticity, C_M, the molar total peptide concentration, l, the path length of the cuvette in cm and N, the number of amino acids per peptide. Spectra were obtained at a sample concentration of 3mM total lipids (1 mol% lipopeptide, 4 mol% photolabile cholesterol-PEG construct) in PBS in a 0.1 cm quartz cuvette. All measurements were made at room temperature. Data was collected at 0.2 nm intervals, at a scanning speed of 20 nm/min and a 1 nm bandwith. Each spectrum was the average of 5 scans.

Helical content was determined using the following formula:

$$\mathrm{rh} = \frac{[\theta]222}{-40\ \ 10^3\,\mathrm{deg\,cm^2\ \ dmol^{-1}}(1-\frac{4.6}{\mathrm{N}})}\ \text{x } 100$$

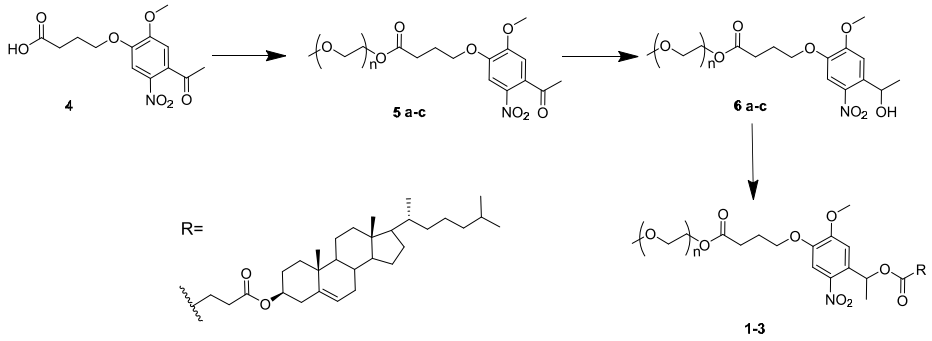
where rh is the helical fraction, Θ_{222} is the ellipticity at 222 nm and N is the number of peptide bonds.

In situ UV irradiation and simultaneous fluorescence measurements were conducted using a custom built setup. All optical parts were connected with FC-UVxxx-2 (xxx = 200, 400, 600) optical fibers from Avantes (Apeldoorn, The Netherlands), with a diameter of 200-600 µm, respectively, and were suitable for the UV-Vis range (200-800 nm). The excitation source was a continuous wave Aries 150 532 nm portable DPSS laser from LaserGlow (Toronto, ON, Canada); the power was controlled using a NDL-25C-4 variable neutral density filter (Thorlabs, Dachau/Munich, Germany) put between the laser and the sample, and was measured using a S310C thermal sensor connected to a PM100USB power meter (Thorlabs). The laser was collimated to a beam of 4 mm diameter to reach an intensity of 80 $mW.cm^{-2}$ (10 mW power). The sample was held in a 104F-QS or 104F-OS semi-micro fluorescence cuvette from Hellma GmbH & Co. KG (Müllheim, Germany) in a CUV-UV/VIS-TC temperature-controlled cuvette holder (Avantes), with the long side of the cuvette perpendicular to the excitation source. Emission measurement was performed by means of a 2048L StarLine CCD spectrometer from Avantes under a 90° angle with respect to excitation. A filter holder with a NF533-17 533 nm notch filter (Thorlabs) was placed between cuvette holder and detector to reject the excitation source. For measurements involving additional illumination with UV light, a 365 nm LED (17 mW) in a custom-made mount was fitted on top of the cuvette.

HeLa cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (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 assays, cells ($2x10^5$ mL^{-1}) were transferred to 48-well cell culture plates (500 µL, Greiner bio-one, Cellstar®) and cultured for a further 24 h. Immediately prior to testing, the 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: 532 – 554 nm, emission: 570 – 613 nm for visualization of DOPE-LR.

2.4.2 Synthesis of 1-3

The right structures are confirmed by both H-NMR and Maldi-TOF (Figure S1-S8).



where **a**, n=16; **b**, n=44; **c**, n=112; **1**, n=16; **2**, n=44; **3**, n=112

Scheme S1. The synthetic scheme to **1**.

4-(4-acetyl-2-methoxy-5-nitrophenoxy)butanoic acid, **4,** was synthesized as previously described.[17] The synthesis and purification of **1-3** were, aside from the variation in PEG chain length, identical. The representative synthesis of **2** is given below. Characterisation of final compounds **1-3** is reported below.

MethoxyPEG$_{2000}$ 4-(4-acetyl-2-methoxy-5-nitrophenoxy)butanoate, 5b

To a stirred solution of **4** (240 mg, 0.81 mmol) in CH_2Cl_2 (5 mL) was added DMAP (98 mg, 0.81 mmol), EDCI (185 mg, 0.97 mmol), DIPEA (209 µL, 1.2 mmol) and

MeO-PEG_{2000}-OH (1.20 g, 0.6 mmol). After overnight stirring, the reaction mixture was diluted with EtOAc (50 mL) and washed with sat. $NaHCO_3$ (3 x 50 mL) and brine (50 mL). The combined organic fractions were dried (Na_2SO_4) and solvent removed in vacuo to yield an orange powder. Column chromatography (Gradient: CH_2Cl_2 to 20% MeOH in CH_2Cl_2) afforded **5b** as a pale yellow powder (0.83 g, 0.49 mmol, 61%). $\mathbf{R_f}$ 0.45 (CH_2Cl_2:MeOH, 9:1). ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.64 (s, 1H), 6.78 (s, 1H), 4.29 (m, 2H), 4.19(t, J=6 Hz, 2H), 3.99 (s, 3H), 3.45-3.89(m, 176H), 3.41 (s, 3H), 2.62(t, J=8 Hz, 2H), 2.53(s, 3H), 2.23 (m, 2H).

MethoxyPEG$_{2000}$ 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate, 6b

$NaBH_4$ (18.9 mg, 0.5 mmol) was added to a suspension of **5b** (0.50 g, 0.22 mmol) in MeOH (5 mL). After 3 h, the reaction was acidified to pH 4 by careful addition of aq. citric acid (5% w/v). The solution was diluted with CH_2Cl_2 (100 mL) and washed with water (50 mL) and brine (50 mL). The combined organic fractions were dried (Na_2SO_4) and solvent removed in vacuo to yield **6b** as a viscous orange oil (0.45 g, 0.2 mmol, 90%). This was used without further purification. $\mathbf{R_f}$ 0.44 (CH_2Cl_2:MeOH, 9:1). ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.59 (s, 1H), 7.34 (s, 1H), 5.58 (q, 1H), 4.28 (m, 2H), 4.16(t, J=6 Hz 2H), 4.00 (s, 3H), 3.45-3.89(m, 176H), 3.41 (s, 3H), 2.61(t, J=8 Hz 2H), 2.23 (m, 2H), 1.57 (d, J=8 Hz , 3H).

1-(5-methoxy-4-(4-(2-methoxyPEG$_{2000}$)-4-oxobutoxy)-2-nitrophenyl)ethyl cholesteryl succinate, 2

To a stirred solution of **6b** (400 mg, 0.15 mmol) in CH_2Cl_2 (5 mL) was added DMAP (36.6 mg, 0.30 mmol), EDCI (57.5 mg, 0.30 mmol), DIPEA (78.3 µL, 0.45 mmol) and cholesteryl hemisuccinate (121 mg, 0.25 mmol). After overnight stirring, the reaction mixture was diluted 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 in vacuo. Column chromatography (Gradient: CH_2Cl_2 to 10% MeOH in CH_2Cl_2) afforded **2** as a colourless waxy solid (195 mg, 0.073 mmol, 50%). $\mathbf{R_f}$ 0.45 (CH_2Cl_2:MeOH, 9:1). ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.60 (s, 1H), 7.06 (s, 1H), 6.49 (q, 1H), 5.37 (t, J=8 Hz, 1H), 4.60 (m, 1H) 4.28 (m, 2H), 4.12 (t, J=6 Hz, 2H), 4.01 (s, 3H), 3.45-3.95 (m, 196H), 3.41 (s, 3H), 0.69-2.66 (m, 56H).

1-(5-methoxy-4-(4-(2-methoxyPEG$_{750}$)-4-oxobutoxy)-2-nitrophenyl)ethyl cholesteryl succinate, 1

^{1}H-NMR ($CDCl_3$, 400MHz): 7.59 (s, 1H), 7.06 (s, 1H), 6.49 (q, 1H), 5.36 (t, J=4 Hz, 1H), 4.58 (m, 1H), 4.27 (m, 2H), 4.11 (t, J=6 Hz, 2H), 4.01 (s, 3H), 3.45-3.89 (m, 62H), 3.39 (s, 3H), 0.69-2.66 (m, 56H).

1-(5-methoxy-4-(4-(2-methoxyPEG$_{5000}$)-4-oxobutoxy)-2-nitrophenyl)ethyl cholesteryl succinate, 3

^{1}H-NMR ($CDCl_3$, 400MHz): 7.60 (s, 1H), 7.06 (s, 1H), 6.49 (q, 1H), 5.38 (t, J=4 Hz, 1H), 4.61 (m, 1H), 4.27 (m, 2H), 4.12 (t, J=6 Hz, 2H), 4.01 (s, 3H), 3.45-3.90 (m, 486H), 3.40 (s, 3H), 0.70-2.75 (m, 51H).

2.4.3 Photolysis of 1

A solution of **1-3** (200 µM) in water:acetonitrile:tert-butanol (1:1:1) was irradiated (hand-held BLAK-RAY B-100AP high intensity UV lamp (365 nm, 100 W, 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 analyzed by HPLC-ELSD.

2.4.4 Liposome preparation

Lipid stock solutions in chloroform (or 1:1 chloroform/methanol for CPE and CPK) were mixed and evaporated to a film under a stream of air. This film was re-hydrated with PBS (containing 10 mM sulphorhodamine B for Content Mixing assays), vortexed briefly and bath sonicated for 1-2 min at 55^{o}C, yielding liposomes of approx. 100 nm diameter with polydispersity <0.2. For all lipid and content mixing assays the total lipid concentration in every well was 100 µM.

Lipid compositions

For lipid mixing assays:

Peptide E decorated: DOPC: DOPE: Cholesterol: DOPE-LR: DOPE-NBD: CPE (49:24.5:24.5:0.5:0.5:1 mol%).

Peptide K decorated: DOPC:DOPE:Cholesterol:CPK (49.5:24.75:24.75:1 mol%).

100% lipid mixing control: DOPC: DOPE: Cholesterol: DOPE-LR: DOPE-NBD: CPE (49.75:24.88:24.88:0.25:0.25:1 mol%).

For content mixing assays:

Peptide E decorated: DOPC:DOPE:Cholesterol:CPE (49.5:24.75:24.75:1 mol%) with 10mM sulphorhodamine B encapsulated.

Peptide K decorated: DOPC:DOPE:Cholesterol:CPK (49.5:24.75:24.75:1 mol%).

100% content mixing control: DOPC:DOPE:Cholesterol: (50:25:25 mol%) with 5 mM sulphorhodamine B encapsulated.

Following liposome formulation by sonication, peptide E decorated and the 100% control liposomes used in contents mixing assays were purified by SEC to remove unencapsulated sulphorhodamine B.

***In Situ* Modification of Peptide E decorated liposomes with PEG**

In situ modification of E-liposomes with **1-3** was carried out following procedures previously described. Briefly, hydrated and sonicated solutions of **1-3** (20 μM) in PBS were added in equal volumes to E-decorated liposomes (200 μM total [lipid]) in PBS and incubated for 30 min to give a final concentration of E-decorated liposomes (100 μM total [lipid]) with varying mol% **1-3** displayed from the outer membrane leaflet. As an example, to formulate E-decorated liposomes displaying 5 mol% **1**, 500 μL E-decorated liposomes (200 μM total [lipid]), 250 μL **1** (20 μM) and 250 μL PBS were mixed to give 1mL of 5mol% **1** E_{PEG}-liposomes (100 μM total [lipid]).

2.4.5 Lipid mixing assay

For lipid mixing assays, NBD fluorescence (excitation wavelength: 465 nm; emission wavelength: 530 nm) was measured upon mixing fluorescent E_{PEG}-liposomes and non-fluorescent K-liposomes every 20 s for 3500 s. The 0% value was determined by measuring NBD emission of E_{PEG}-liposomes to which an equal amount of PBS (in place of K-liposomes) was added. The 100% value was determined using liposomes containing half the probe (DOPE-NBD and DOPE-LR) concentrations *i.e.* 0.25 mol%.

The percentage of fluorescence increase ($\%F_{(t)}$) was calculated as: $\%F(t)=(F(t)-F_0)/(F_{max}-F_0)$ where $F_{(t)}$ is the fluorescence intensity measured at time, t, F_0 is the 0% fluorescence and F_{max} is the fluorescence intensity measured on liposomes with half the fluorescent probe concentrations.

For measuring the effects of UV irradiation on the rate of lipid mixing, E_{PEG}- liposomes were irradiated for various times prior to the addition of K-liposomes.

2.4.6 Content mixing assay

For content mixing assays, the increase in fluorescence emission of sulforhodamine B (**SR-B**, 10 mM (self-quenching), excitation wavelength: 520 nm emission wavelength: 58 nm) encapsulated in E_{PEG}-liposomes was measured every 20 s for 3500 s upon mixing peptide E_{PEG}-liposomes and K-liposomes. The 0% value was determined by measuring SR-B emission of E_{PEG}-liposomes to which an equal amount of PBS (in place of K-liposomes) was added. The 100% value was determined using liposomes containing half the probe (SR-B) concentration (5 mM).

The percentage of fluorescence increase ($\%F_{(t)}$) was calculated as: $\%F(t)=(F(t)- F_0)/(F_{max}-F_0)$ where $F_{(t)}$ is the fluorescence intensity measured at time, t, F_0 is the 0% fluorescence and F_{max} is the fluorescence intensity measured for liposomes containing 5mM encapsulated SR-B.

For measuring the effects of UV irradiation on the rate of lipid mixing, E_{PEG}- liposomes were irradiated for various times prior to the addition of K-liposomes.

2.4.7 *In situ* UV irradiation and simultaneous fluorescence measurement of content mixing

E_{PEG}- and K-liposomes, prepared as above for contents mixing assays, were mixed in a cuvette (total volume; 600 µL) and fluorescence measurements taken continuously. After approximately 5 min, UV irradiation (365 nm, 15-17 mW/cm^2) above the sample was switched on and left on for the remainder of the experiment whilst simultaneously measuring the increase in SR-B fluorescence emission. The laser intensity was recorded simultaneously and all data was corrected for source intensity fluctuations.

The percentage of fluorescence increase ($\%F_{(t)}$) was calculated as: $\%F(t)=(F(t)- F_0)/(F_{max}-F_0)$ where $F_{(t)}$ is the fluorescence intensity measured at time, t, F_0 is the 0% fluorescence and F_{max} is the fluorescence intensity measured for liposomes containing 5mM encapsulated SR-B.

2.4.8 *In vitro* fluorescent assay

A solution of CPK was prepared by 200-fold dilution of CPK (2 mM) in DMSO with PBS, followed by a further 2x dilution with DMEM (without FCS) to give a 5 µM CPK solution in PBS:DMEM(-FCS) (1:1). This solution (500 µL) was added to the cell culture well and incubated for 10-15 minutes at 37 °C (5% CO_2) . The CPK solution was carefully removed and the cells washed 3x with PBS:DMEM (-FCS). E_{PEG}-liposomes (500 µL, 250 µM, PBS:DMEM(-FCS)) containing 1 mol% CPE, 4 mol% **2**, 1 mol% DOPE-LR probe, were then added to the cells. The cells were then irradiated (365 nm, 10 min, 10 $mWcm^{-1}$) from directly above the well, of which half was covered with aluminum foil. Following irradiation, the cells were incubated with the E_{PEG}-liposome solution for a further 15 min. The E_{PEG}-liposome solution was then carefully removed and the cells washed 3x with PBS:DMEM (-FCS). The cells were then immediately analysed under the microscope.

2.5 References

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2.6 Appendix

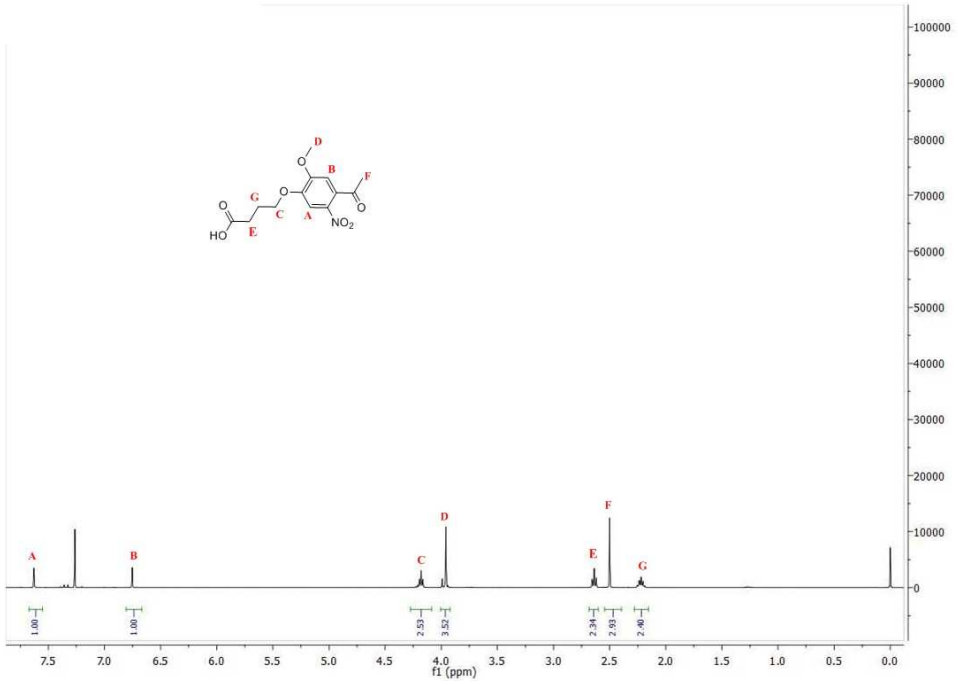

Figure S1. ^{1}H-NMR of **4**.

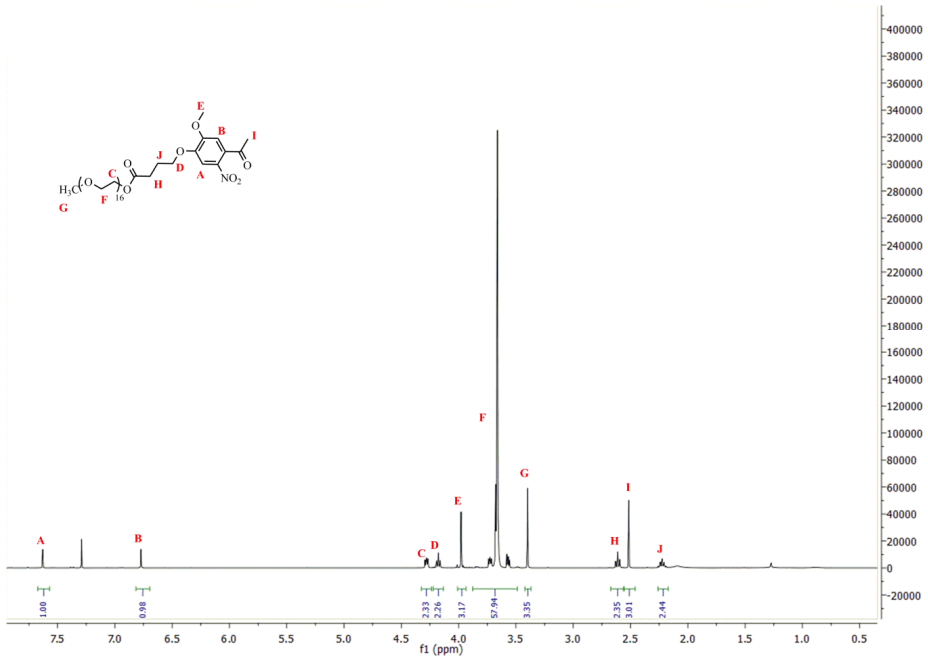

Figure S2. ^{1}H-NMR of **5a**.

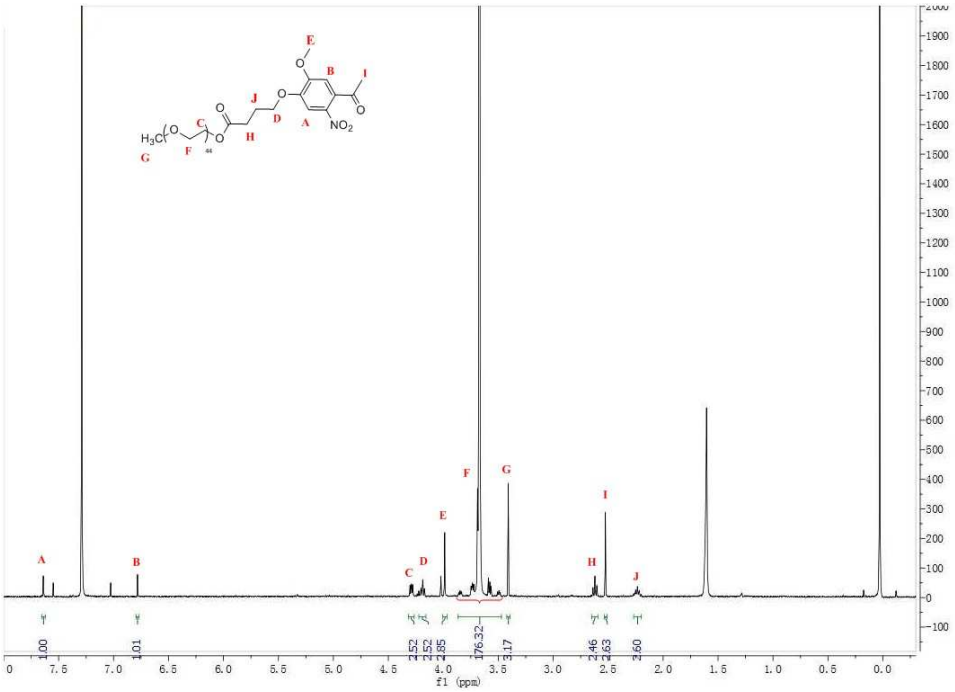

Figure S3. ^{1}H-NMR of **5b**.

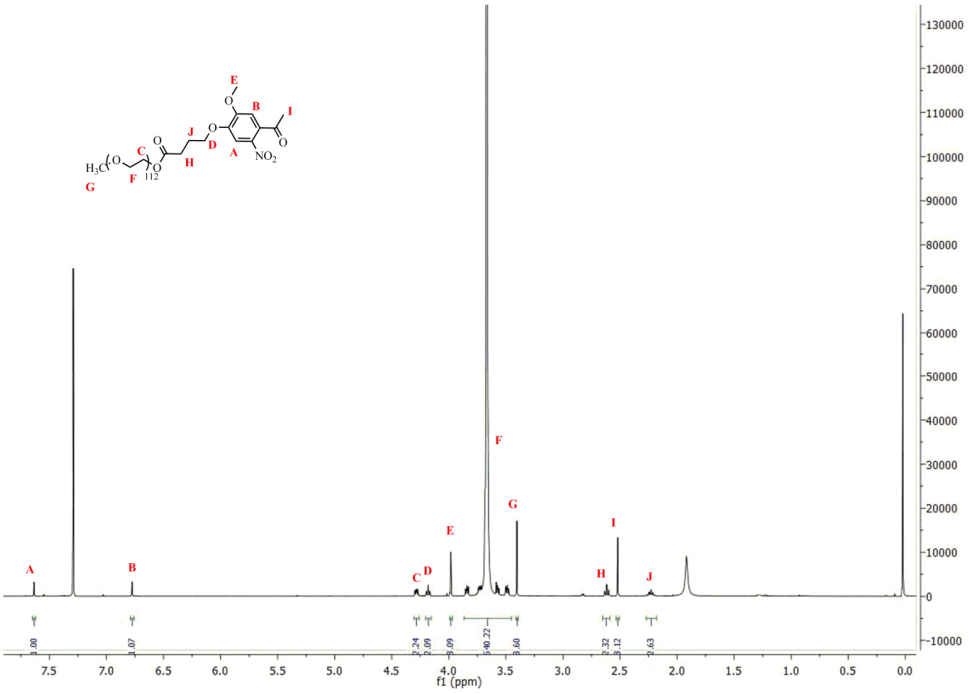

Figure S4. ^{1}H-NMR of **5c**.

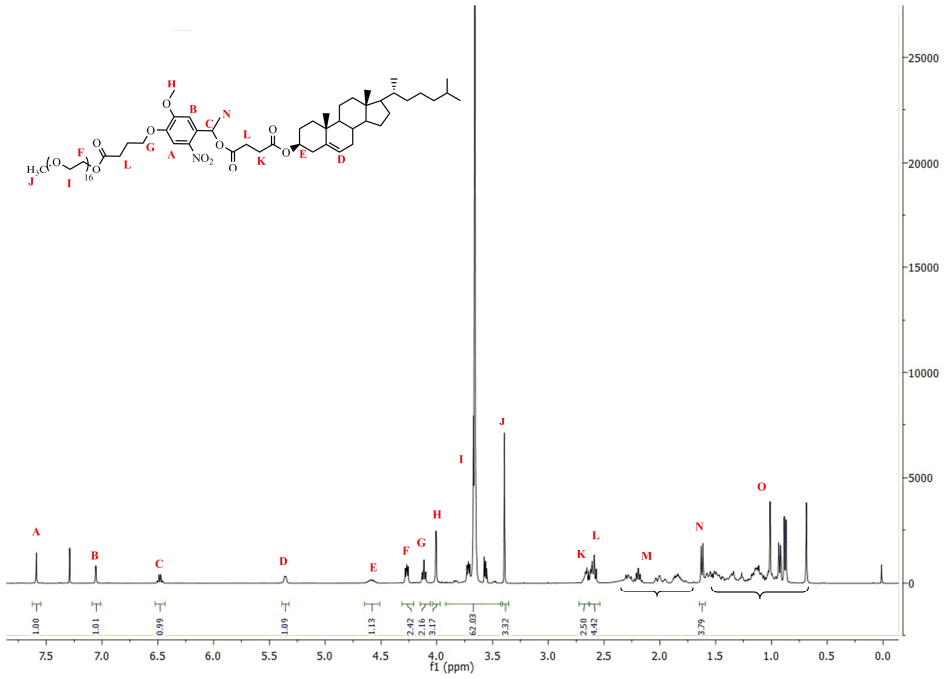

Figure S5. ^{1}H-NMR of **1**.

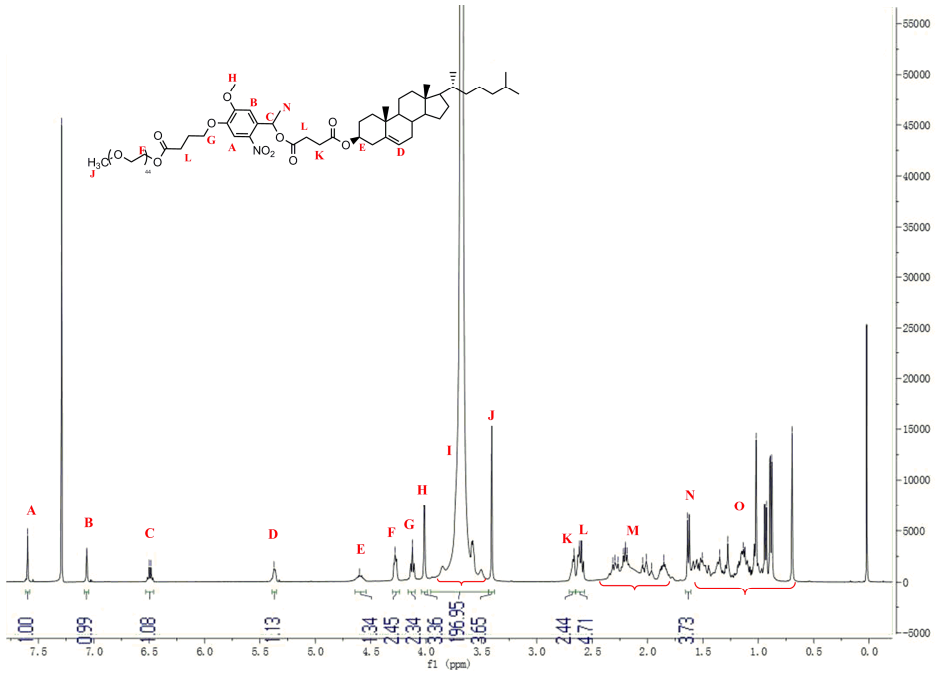

Figure S6. ^{1}H-NMR of **2**.

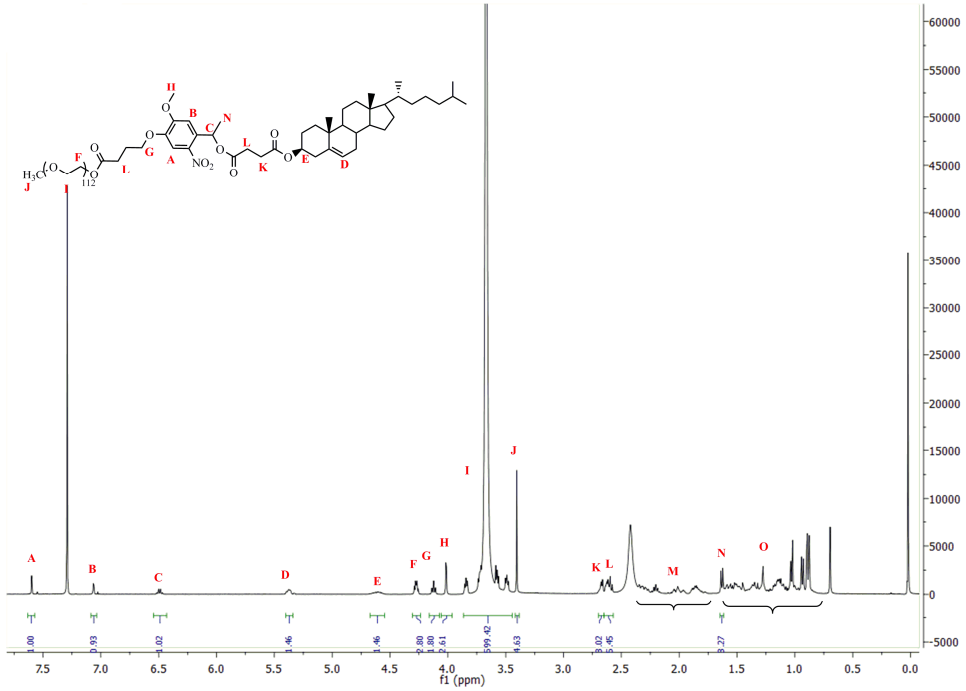

Figure S7. ^{1}H-NMR of **3**.

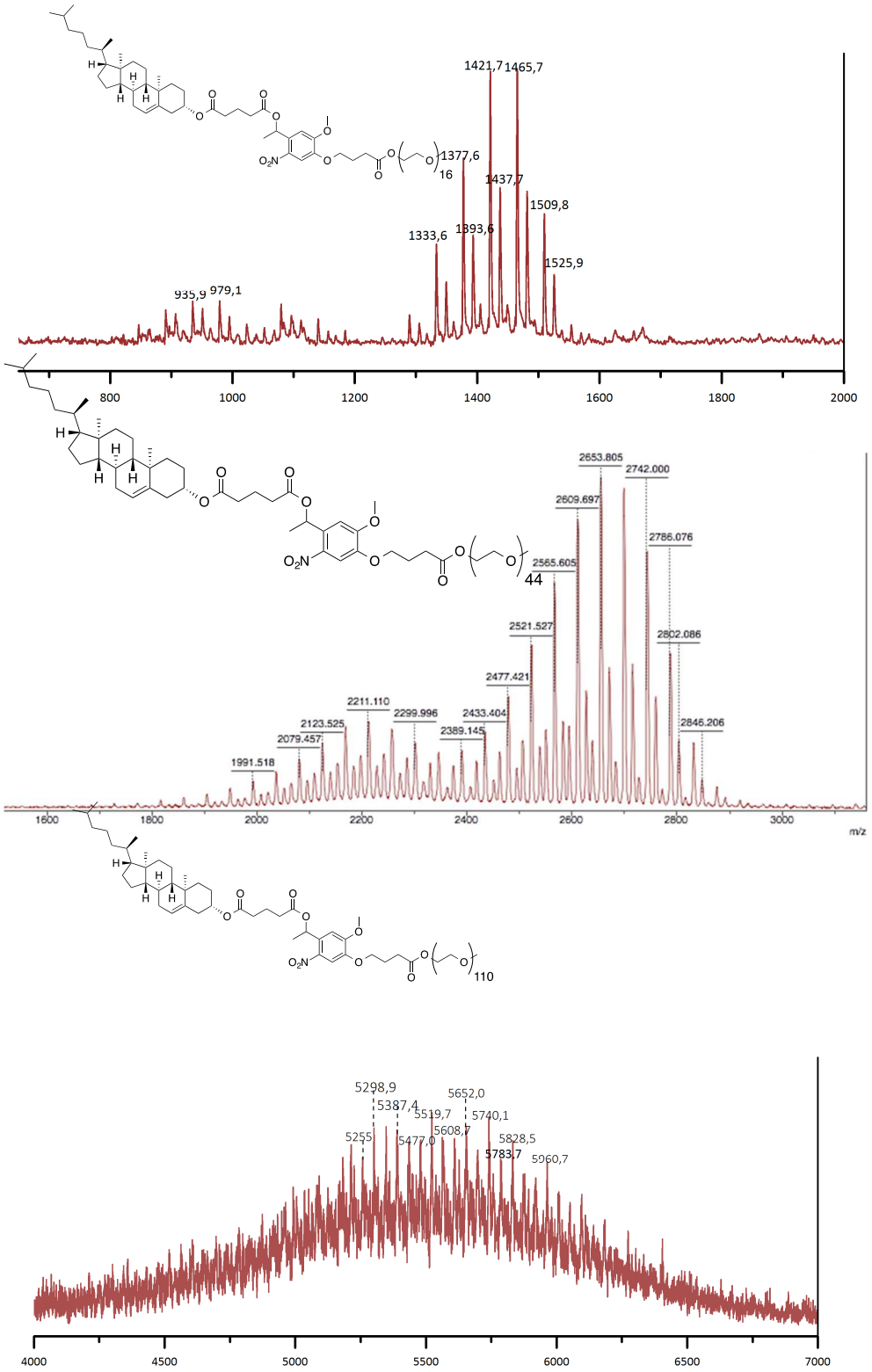

Figure S8. MALDI-TOF spectra of **1-3**.

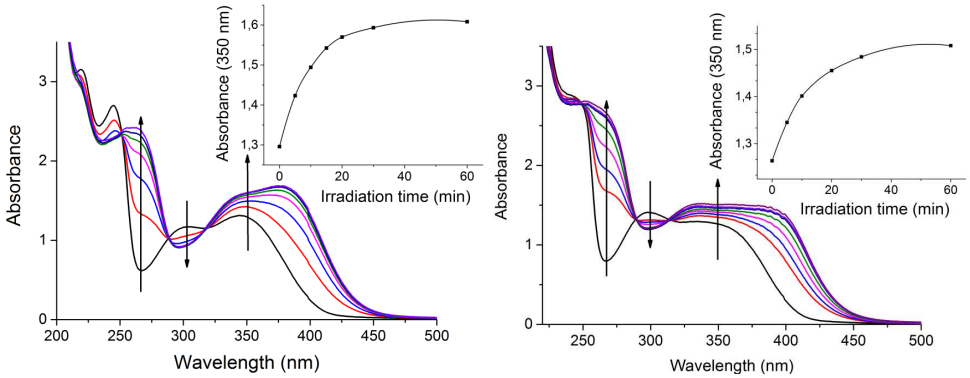

Figure S9. UV-Vis spectra of the photolysis of **1** (left) and **3** (right); all (200 µM) in water:acetonitrile:tert-butanol (1:1:1). Inset: Reaction profile over time as a function of UV absorption at 350 nm.

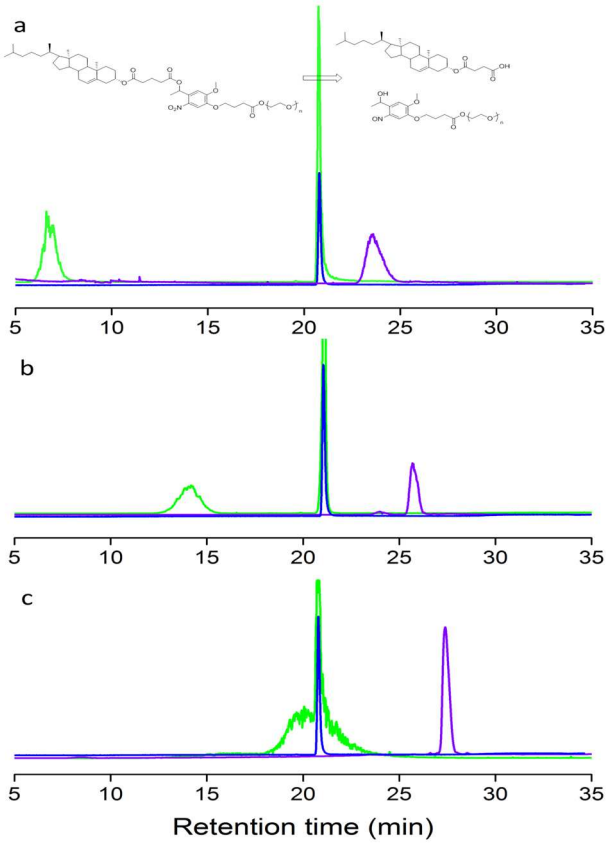

Figure S10. HPLC-ELSD traces of **1**(a), **2**(b) **and 3**(c) in PBS before (**magenta**) and after (**green**) 60 min UV irradiation. HPLC-ELSD of the expected photolysis products, cholesteryl hemisuccinate (**blue**).

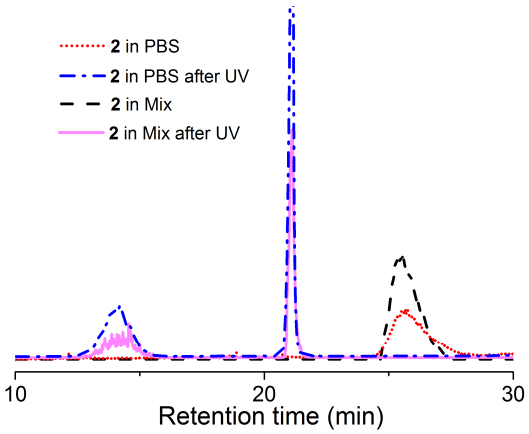

Figure S11. Comparison HPLC-ELSD traces of **2** in PBS and in water:acetonitrile:tert-butanol (1:1:1) before and after 60 min UV irradiation.

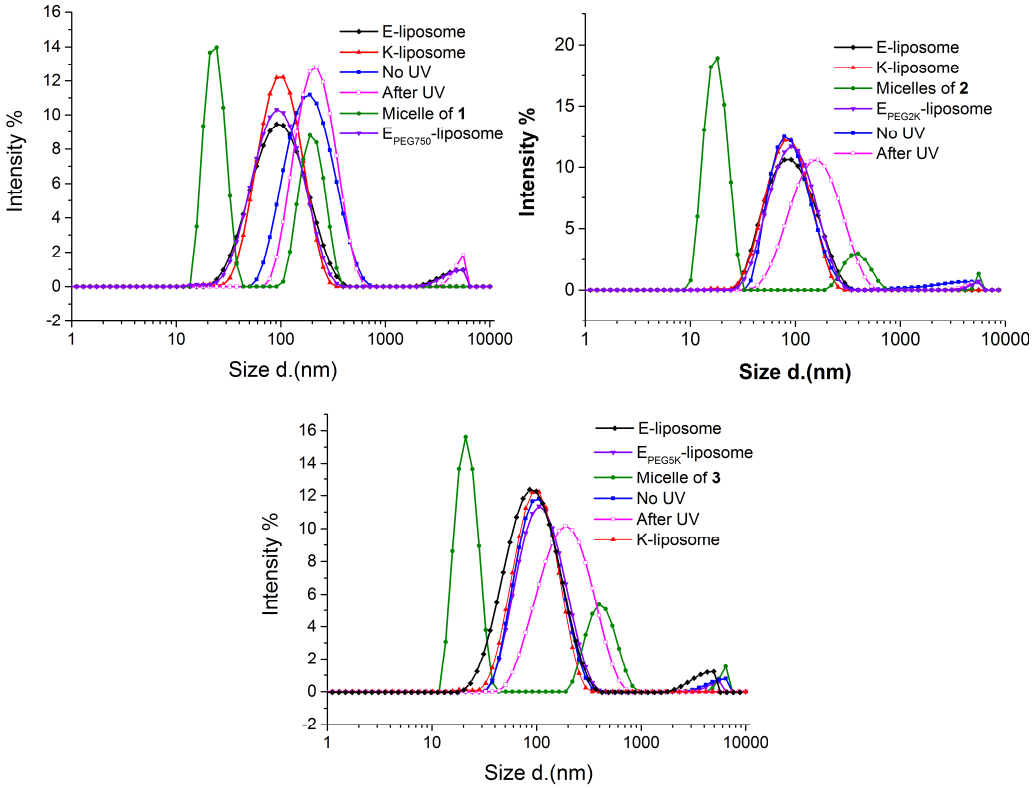

Figure S12. DLS size distributions: **1** (top), **2** (middle), **3** (bottom): **1-3** (20 µM in PBS)**,** E-liposomes (100 µM in PBS) alone (black), K-liposomes (100 µM total [lipid] in PBS) alone, post modified (4mol% **1-3)** E_{PEG}-liposomes (100µM total [lipid]) alone, 1:1 mixtures of (4mol% **1-3)** E_{PEG}-liposomes and K-liposomes (100 µM total [lipid] in PBS) after 60 min, without and following 60 min UV irradiation of E_{PEG}-liposomes prior to mixing with K-liposomes.

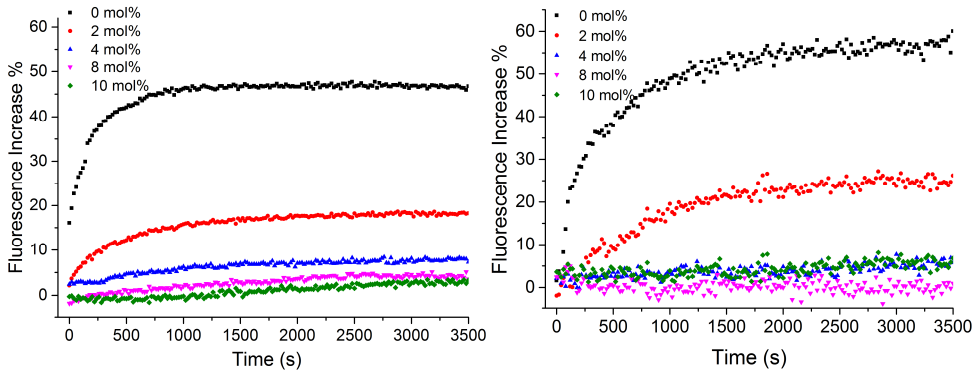

Figure S13. Lipid mixing between E_{PEG}- and K-liposomes with varying amounts of **2** (left) and **3** (right) presented from the E-liposome membrane. 0, 2, 4, 8 and 10 mol%.

Table S1. CD spectra of investigated systems: mean residue molar ellipticities and helical content.

[θ] (10^3 deg cm^2 $dmol^{-1}$)		**[θ] @222 nm**	**[θ] @208 nm**	**Helicity**	**[θ]222/[θ]208**
K-liposomes		14,44674	-15,50488	46.2	0.93
1 (4mol%)	E_{PEG}-liposomes	-17,62973	-23,97608	56.4	0.74
	E_{PEG}- and K-liposomes (pre-irradiation)	-22,37316	-22,9173	71.6	0.97
2 (4mol%)	E_{PEG}-liposomes	-16,881	-20,74584	54.0	0.81
	E_{PEG}- and K-liposomes (pre-irradiation)	-18,37925	-22,73417	58.8	0.81
	E_{PEG}- and K-liposomes (post-irradiation)	-20,40157	-20,62402	65.3	0.99
3 (4mol%)	E_{PEG}-liposomes	-17,84379	-22,45793	57.1	0.79
	E_{PEG}- and K-liposomes (pre-irradiation)	-17,40497	-22,34921	55.7	0.78
	E_{PEG}- and K-liposomes (post-irradiation)	-20,86163	-20,00899	66.8	1.04

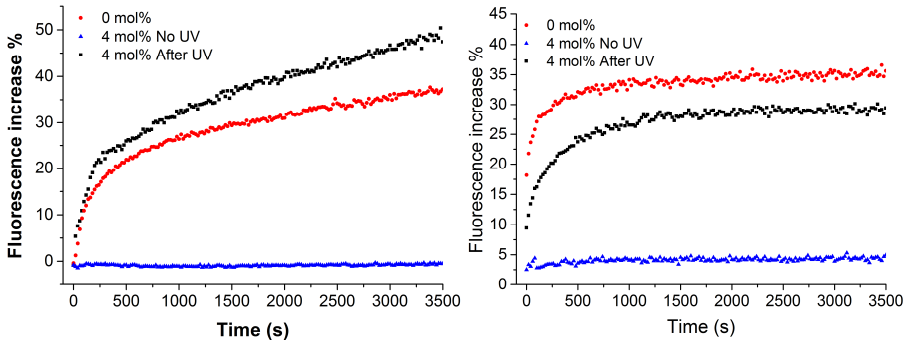

Figure S14. Content mixing of E_{PEG}- and K-liposomes with 0 mol% and 4 mol% **2** (left), **3** (right) presented from the E-liposome membrane, in the absence of UV irradiation and following 60 min prior UV irradiation of E_{PEG}-liposomes.

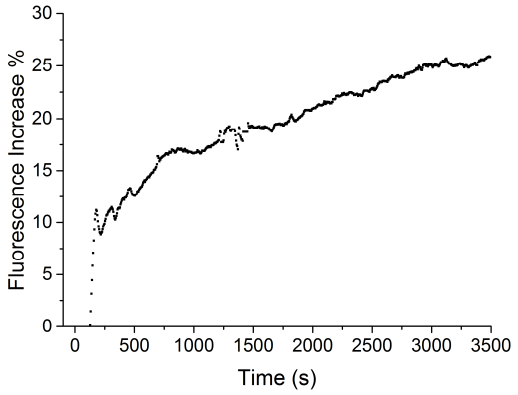

Figure S15. Simultaneous and continuous UV irradiation and monitoring of content mixing between E- and K-liposomes (in the absence of **1-3** presented from the E-liposome membrane).

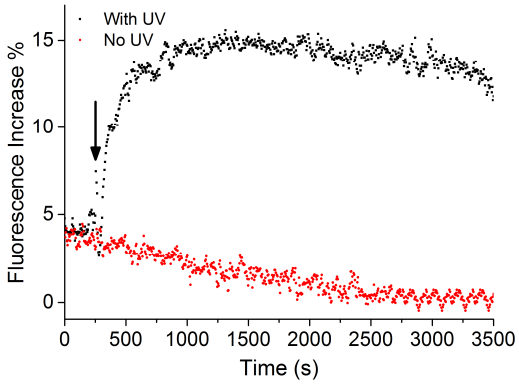

Figure S16. *In situ* photolysis of E_{PEG}-liposomes (4 mol% **3**) and consequent content mixing with K-liposomes, upon (**---**) and in the absence of (**---**) UV irradiation.