Supplementary Information

Photoactivated bioinspired lipoplexes with a chalcone/flavylium photoswitch enhance siRNA delivery—towards precise spatiotemporal control in gene silencing

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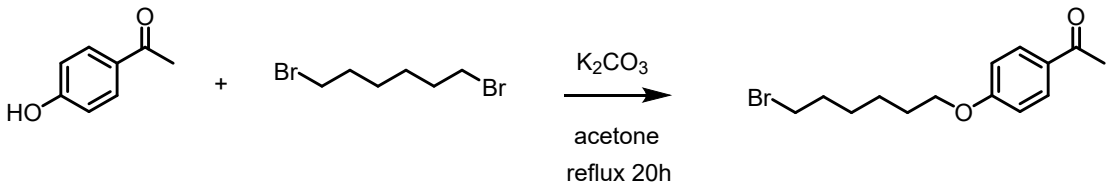
S1. Synthesis of C_6NCh



Scheme 1. Synthesis of acetophenone 1.

Acetophenone **1** was synthesized using a protocol adapted from the literature.[1] A mixture of 4-hydroxyacetophenone (2.8 g, 20.5 mmol), 1,6-dibromohexane (50.0 g, 205.1 mmol) and potassium carbonate (20.0 g, 145.0 mmo1) in acetone (80 mL) was refluxed for 20 hours. The reaction was followed by TLC using chloroform as the eluent. After cooling, the precipitated salts were removed by filtration and the solvent was dried *in vacuo.* The excess 1,6-dibromohexane was separated on a silica gel column eluting with hexane. Then chloroform was used to elute the product. Gave an oil which later solidifies (5.858 g, h = 95%). ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.93 (d, J = 8.5Hz, 2H), 6.92 (d, J = 8.5 Hz, 2H), 4.03 (t, J = 6.3 Hz, 2H), 3.43 (t, J = 6.3 Hz, 2H), 2.55 (s, 3H), 1.90 (m, 2H), 1.83 (m, 2H), 1.52 (m, 4H) ppm.

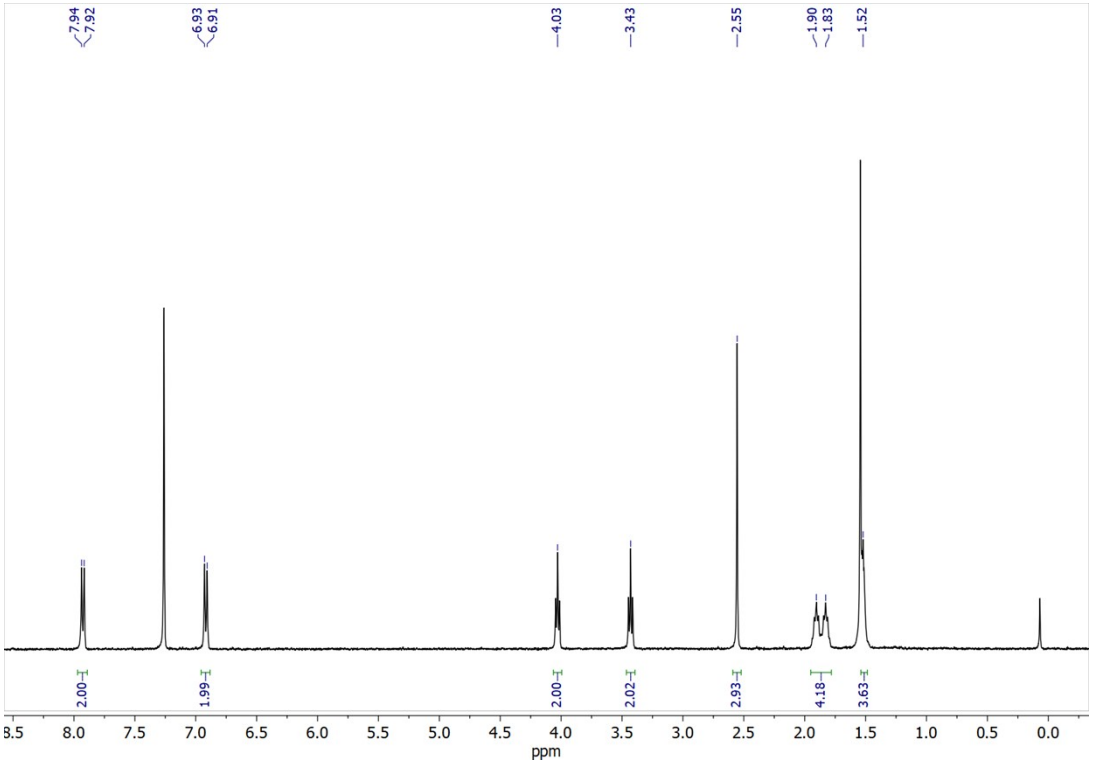
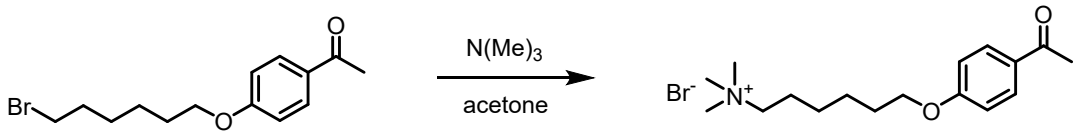


Figure S1. ^{1}H NMR (400 MHz) spectrum of acetophenone 1 in $CDCl_3$.



Scheme 2. Synthesis of acetophenone 2.

Acetophenone **1** (2 g, 6.68 mmol) was dissolved in acetone (48 mL) and $N(Me)_3$ 45% in water (10.4 mL, 66.9 mmol) was added. The mixture was kept stirring at 40ºC for 18h. The mixture was added dropwise to diethyl ether (400 mL). The precipitate was filtered and washed with ether. A white solid was obtained (2.24 g, h = 94%). ^{1}H NMR (400 MHz, CD_3OD), δ (ppm): 8.23 (d, *J* = 8.5Hz, 2H), 7.31 (d, *J* = 8.5 Hz, 2H), 4.40 (t, *J* = 6.3 Hz, 2H), 3.53 (t, *J* = 6.3 Hz, 2H), 3.31 (s, *9*H), 2.84 (s, 3H), 2.05 (m, 4H), 1.76 (m, 2H), 1.66 (m, 2H) ppm.

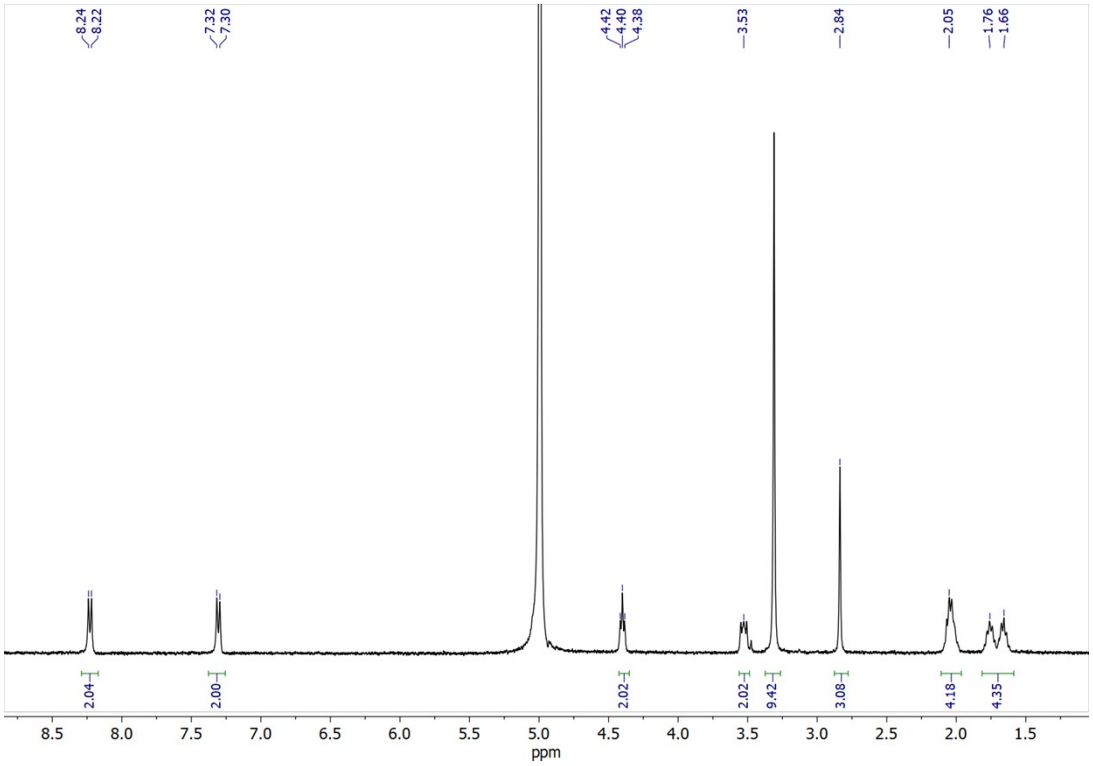
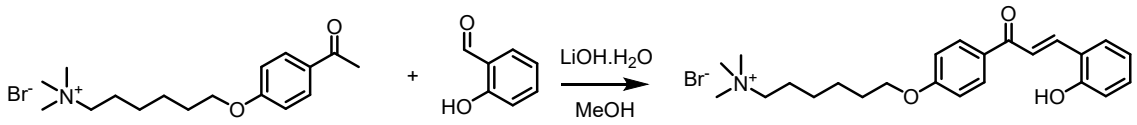


Figure S2. ^{1}H NMR (400 MHz) spectrum of acetophenone 2 in CD_3OD.



Scheme 3:. Synthesis of *trans*-chalcone C_6NCh

$LiOH.H_2O$ (1873 mg, 44.64 mmol) was added to a solution of acetophenone **2** (1 g, 2.79 mmol) dissolved in 20 mL methanol. Salicylaldehyde (1.362 g, 11.16 mmol) was added slowly at RT after which the mixture was kept stirring at 55ºC for 3 days. The mixture was allowed to cool to RT and neutralized with HBr 48% (4 mL). A precipitate formed which was filtered and washed with diethyl ether. The product was further purified by recrystallization from water (0.787 g, 61% yield). ^{1}H NMR (400MHz, CD_3OD), δ (ppm): 8.09 (d, *J* = 15.8 Hz, 1H), 8.06 (dd, 2H), 7.82 (d, *J* = 15.8 Hz, 1H), 7.66 (d, *J*=8.1 Hz, 1H), 7.25 (t, *J*=6.6 Hz, 1H), 7.04 (dd, 2H), 7.66 (t, *J*=6.6 Hz, 1H), 7.25 (d, *J*=8.1 Hz, 1H), 4.12 (t, *J* = 6.4 Hz, 2H), 3.36 (t, *J* = 8.4 Hz, 2H), 3.13 (s, 9H), 1.85 (m, 4H), 1.62 (m, 2H), 1.48 ppm (m, 2H); ^{13}C NMR (101MHz, CD_3OD), δ (ppm): 191.6, 164.6, 158.8, 141.8, 132.9, 132.3, 132.0, 130.4, 123.3, 122.4, 120.9, 117.1, 115.5, 69.1, 67.8, 53.5, 30.0, 27.0, 26.7, 23.9 ppm; HRMS (ESI) m/z calcd for C24H32NO3 [M^+]: 382.2377; found: 382.2395.

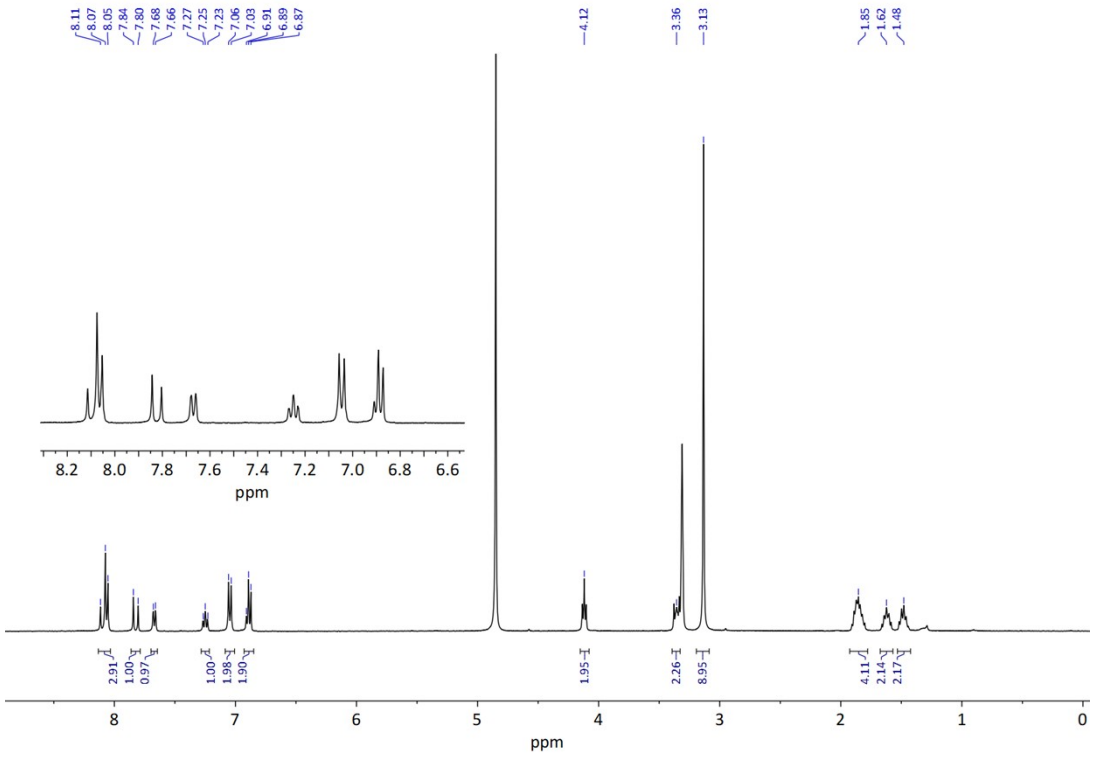


Figure S3. ^{1}H NMR (400 MHz) spectrum of *trans*-chalcone C_6NCh in CD_3OD.

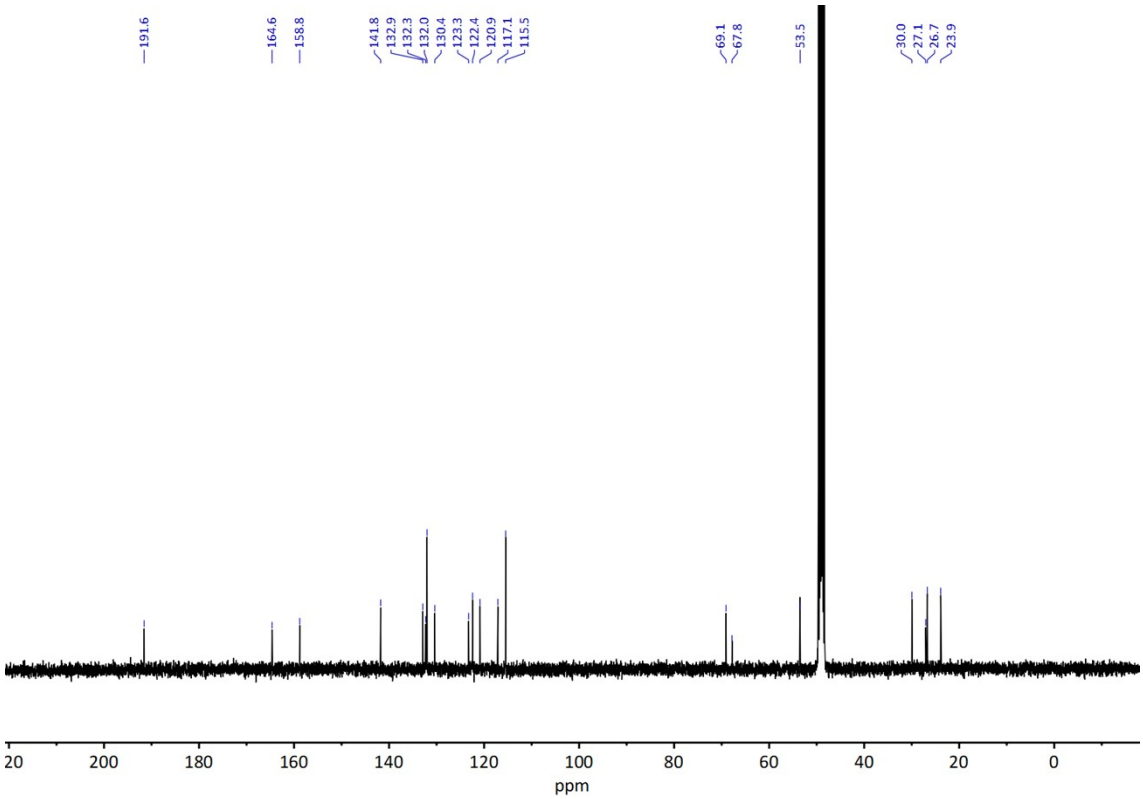
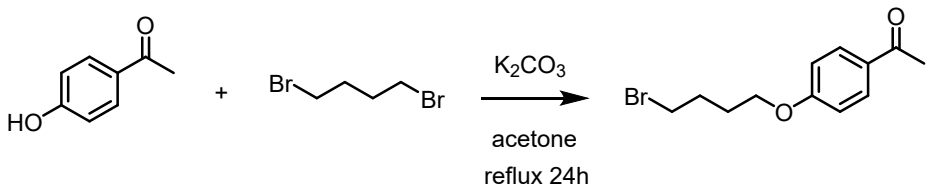


Figure S4. ^{13}C NMR (101 MHz) spectrum of *trans*-chalcone C_6NCh in CD_3OD.

S2. Synthesis of C_4NCh



Scheme 4. Synthesis of acetophenone 3.

To a stirred solution of 4-hydroxy-acetophenone (2.50 g, 18.36 mmol) in 120 mL of acetone, 44.80 g (183.62 mmol, 10 eq.) of 1,4-dibromobutane were added. After homogenization, 12.69 g (91.81 mmol, 5 eq.) of K_2CO_3 were poured into the flask, mostly remaining in suspension. The reaction was left stirring under reflux for 24 hours after which the salts were filtered off and thoroughly washed with acetone. The solution was evaporated until dryness and the residue was redissolved in dichloromethane. This solution was washed 3 times with water to remove remaining salts. The water extracts were then washed with dichloromethane and the concentrated dichloromethane extracts were washed with brine and dried with magnesium sulphate prior to being dried by rotatory evaporation. The crude product (a transparent oil) was subjected to flash column chromatography, first with hexane to remove the starting dibromobutane, and then with chloroform to purify the desired product that, after complete dryness was obtained as a white solid (4.57 g, η = 83.3%). The product was then characterized by ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.93 (d, J = 8.9 Hz, 2H), 6.92 (d, J = 8.9 Hz, 2H), 4.05 (t, J = 6.4 Hz, 2H), 3.48 (t, J = 6.8 Hz, 2H), 2.54 (s, 3H), 2.14 – 2.02 (m, 4H), 2.01 – 1.88 (m, 4H).

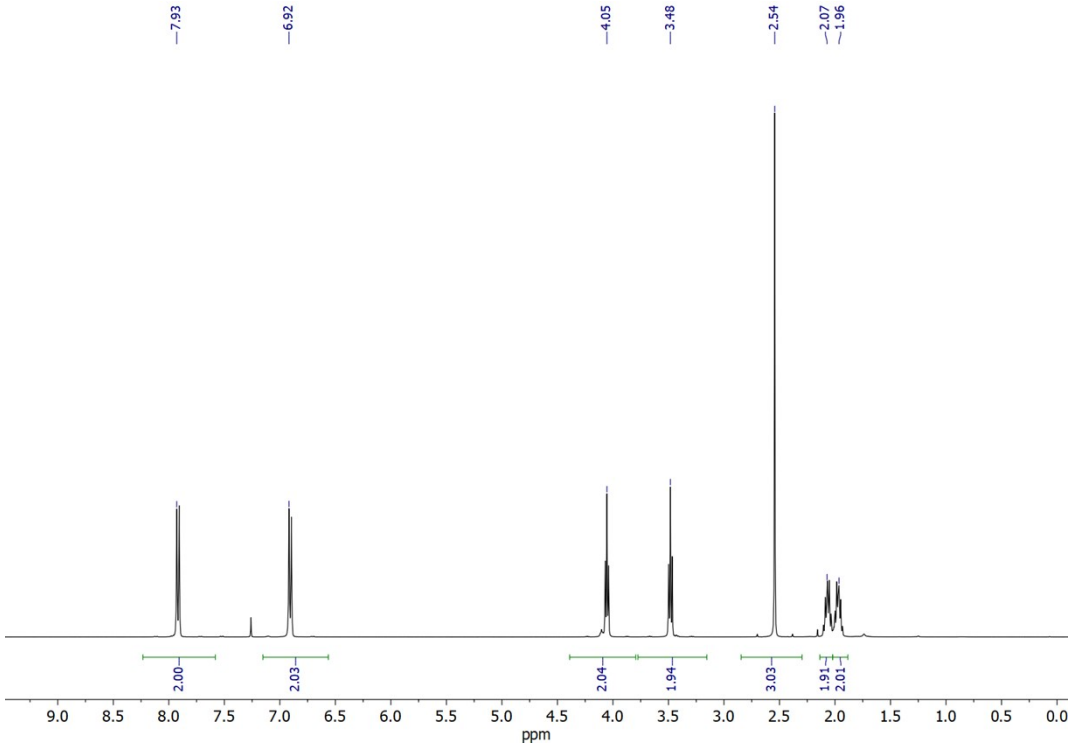
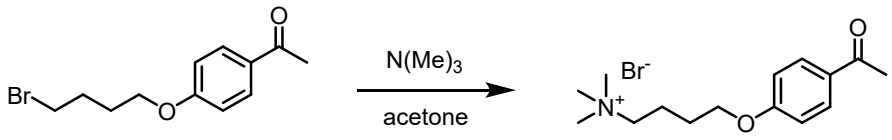


Figure S5. ^{1}H NMR (400 MHz) spectrum of acetophenone 3 in $CDCl_3$.



Scheme 5. Synthesis of acetophenone 4.

1.00 g, 3.69 mmol, of acetophenone **3** was dissolved in 20 mL of acetone on a round bottom flask to which 2.56 mL (10 eq.) of trimethylamine, (45 % in water) were added. The solution was left stirring at 40 °C overnight. The following day all starting material had been consumed and the product was isolated by precipitation in an abundant quantity of diethyl ether which was also used to wash thoroughly the product from leftover trimethylamine. 1.13 g, η = 92.4 %, of a white solid was achieved and was further characterized by ^{1}H NMR (400 MHz, D_2O), δ (ppm): 8.02 (d, J = 8.5 Hz, 2H), 7.10 (d, J = 8.5 Hz, 2H), 4.23 (t, J = 6.0 Hz, 2H), 3.43 (t, J = 7.9 Hz, 2H), 3.15 (s, 9H), 2.63 (s, 3H), 2.09 – 1.96 (m, 2H), 1.95 – 1.82 (m, 2H) and by ^{13}C NMR (101 MHz, D_2O), δ (ppm): 202.60, 162.97, 131.24, 129.68, 114.51, 67.50, 66.17, 52.83, 25.88, 25.14, 19.39.

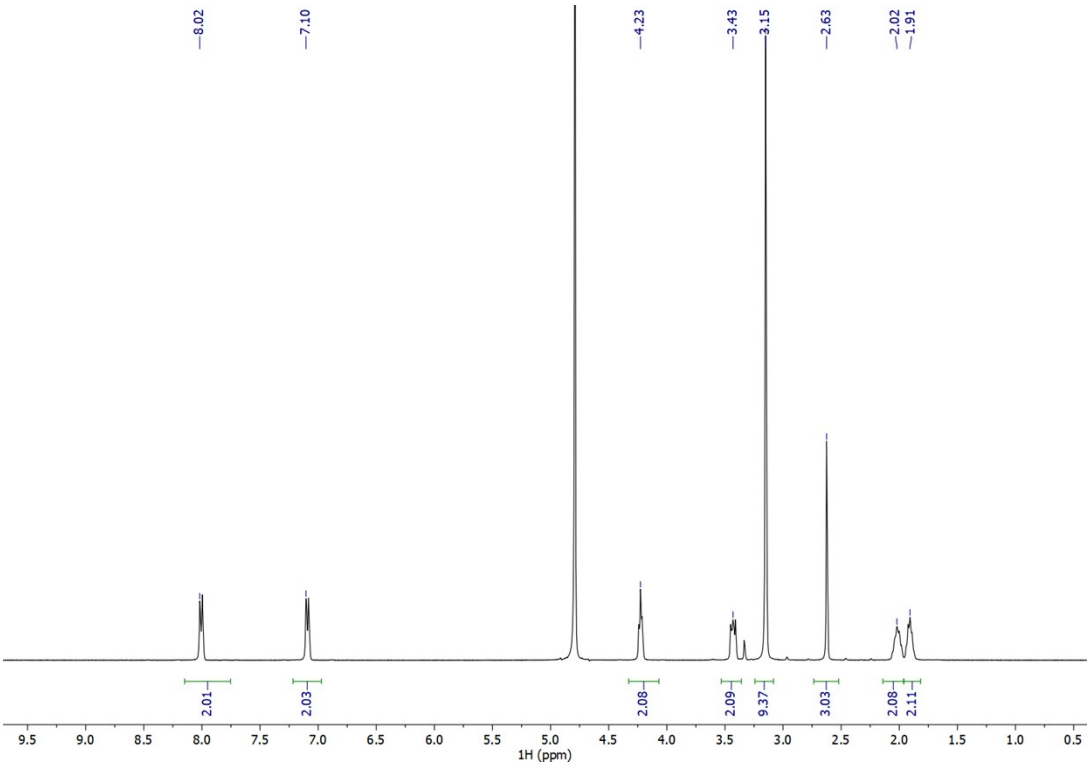


Figure S6. ^{1}H NMR (400 MHz) spectrum of acetophenone **4** in D_2O.

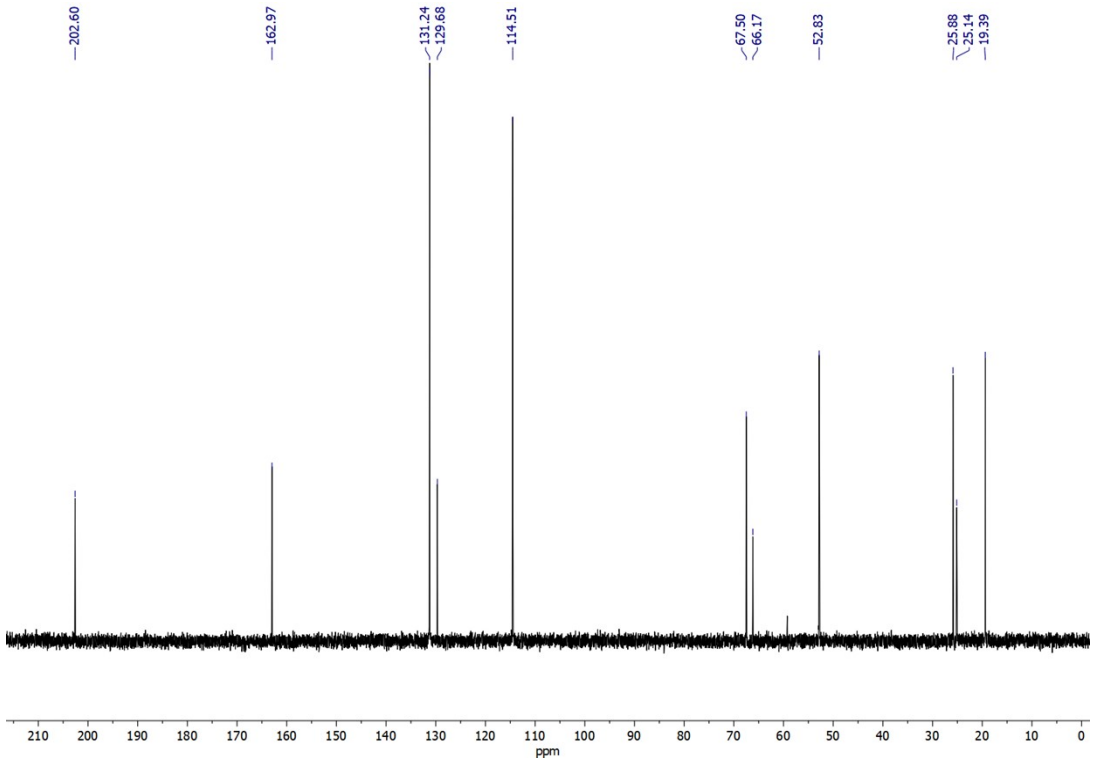
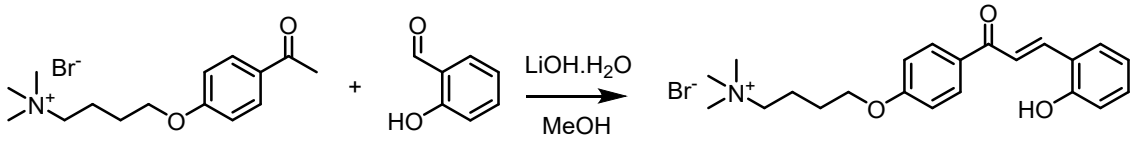


Figure S7. ^{13}C NMR (101 MHz) spectrum of acetophenone 4 in D_2O.



Scheme 6. Synthesis of *trans*-chalcone C_4NCh.

LiOH.H_2O (2.05 g, 48.86 mmol) was added to a solution of acetophenone **4** (1.02 g, 3.09 mmol) dissolved in 5 mL methanol. Salicylaldehyde (1.5 g, 12.28 mmol) was added slowly at RT after which the mixture was kept stirring at 55ºC for 24 hours. The mixture was allowed to cool to RT and neutralized with HBr 48%. The solvent was removed by rotary evaporation and the solid residue was washed with acetone and diethyl ether. The product was further purified by recrystallization from water (0.85 g, 63% yield). ^1H NMR (400 MHz, CD_3OD), δ (ppm): 8.19 – 7.97 (m, 3H), 7.83 (d, J = 15.8 Hz, 1H), 7.69 (dd, J = 8.1, 1.7 Hz, 1H), 7.26 (td, J = 7.8, 1.7 Hz, 1H), 7.16 – 7.03 (m, 2H), 6.98 – 6.83 (m, 2H), 4.19 (t, J = 5.9 Hz, 2H), 3.47 (t, J = 8.6 Hz, 2H), 3.18 (s, 9H), 2.18 – 1.98 (m, 2H), 1.98 – 1.84 (m, 2H) and by ^{13}C NMR (101 MHz, CD_3OD), δ (ppm): 188.64, 161.38, 155.89, 138.91, 130.02, 129.62, 129.11, 127.47, 120.32, 119.50, 117.97, 114.19, 112.61, 65.46, 64.59, 50.66, 46.72, 46.51, 46.30, 46.09, 45.87, 45.66, 45.45, 24.12, 18.14.; HRMS (ESI) m/z calcd for C22H28NO3 [M^+]: 354.2064; found: 354.2062.

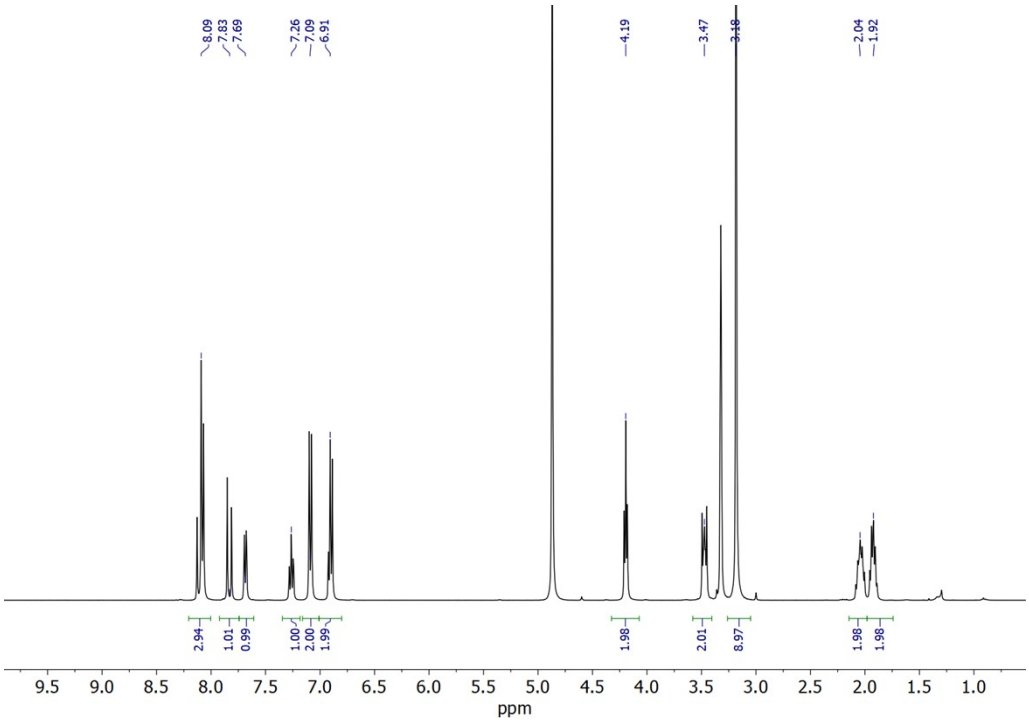


Figure S8. [1]H NMR (400 MHz) of *trans*-chalcone C_4NCh in CD_3OD.

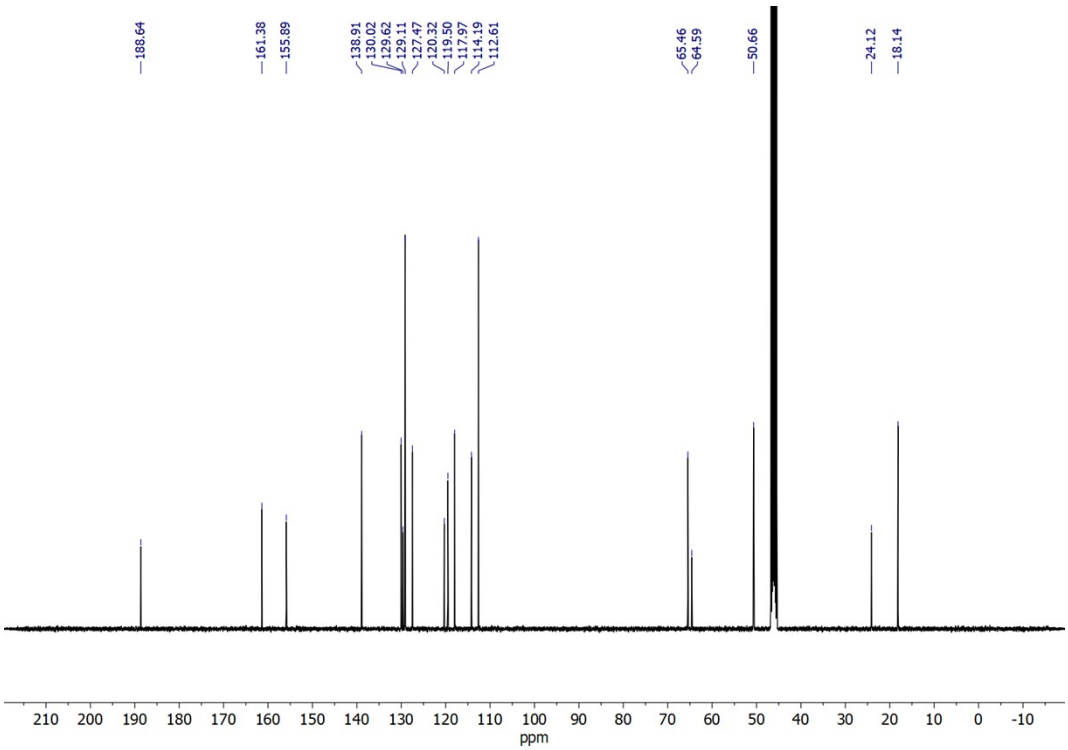


Figure S9. [13]C NMR (101 MHz) of *trans*-chalcone C_4NCh in CD_3OD.

S3. Lipid mixing assay

To prepare biomimicking GUVs, two models were used — cytoplasmatic membrane and endosome/lysosome membrane. All lipid components were dissolved in ethanol (except for 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt (NDB-PE), which was dissolved in methanol). The lipid solutions were then mixed in the desired proportions, and GUVs were formed through the lipid film hydration method. For the cytoplasmic membrane model, PBS (pH = 7.4) was used for hydration, while an acetate buffer (pH = 4.5, containing 0.9% (m/v) NaCl) was used for the endosomal/lysosomal model. After adding buffer so that total lipid concentration was 1.0 mM, the samples were incubated at 50°C without agitation for 6 hours. The final GUV dispersions had the following lipid compositions of 0.45/0.25/0.15/0.15/0.015/0.015 mM and 0.45/0.25/0.20/0.10/0.015/0.015 mM of POPC/DOPE/POPS/Chol/NBD-PE/Rho-PE for the cytoplasmatic and endosome/lysosome membrane models, respectively (Fig. S10a and S10b).

A few key observations are to be made here with regards to the method followed for bio-mimicking GUV preparation.

The choice for composition of both bio-mimicking systems, and more particularly, what differentiated them was based on the fact that endosomal/lysosomal compartments have lower pH than extracellular ones, that endosomal/lysosomal membranes, have lower cholesterol (Chol) content and that they more negative surface charge (hence the higher POPS concentration).[2-5].

The usage of unilamellar vesicles (specifically, GUVs) as biomimicking membranes and their method of preparation was due to unilamellar vesicles representing biological entities more accurately compared to a multilamellar or multivesicular vesicles. The particular conditions used in the film hydration method to favor GUV formation were: low lipid concentration (thin film), high salinity of hydration media and, long hydration time at high temperatures with minimal agitation.[6, 7]

Regarding the lipid mixing assay itself, concentration of fluorophores and the GUV-Lipoplex concentration and mixing ratio were chosen based on literature reports to maximize FRET prior to fusion occurring and to minimize FRET if total fusion was to occur. Similarly, the amount of TX-100 added is ought to simulate total fusion of the membranes present(Fig. S11a).[8]

Finally, emission spectra were collected at 24h post incubation of lipoplexes with GUVs because the AH^+ formed under irradiation in acidic media has an absorption band at λ = 440 nm (Fig S10b and S14), which overlaps the excitation band of the donor fluorophore, thus partially blocking emission that occurs. However, since AH^+ is a product of a pseudo-equilibrium, it can be back-react into the other species of the chalcone/flavylium chemical network if kept under dark hot conditions.[9] We determined that keeping our lipoplexes that had been irradiated in acidic media in the dark at 37ºC for 24h was enough to revert all AH^+ and hence, not affect the FRET measurements (Fig S10b).

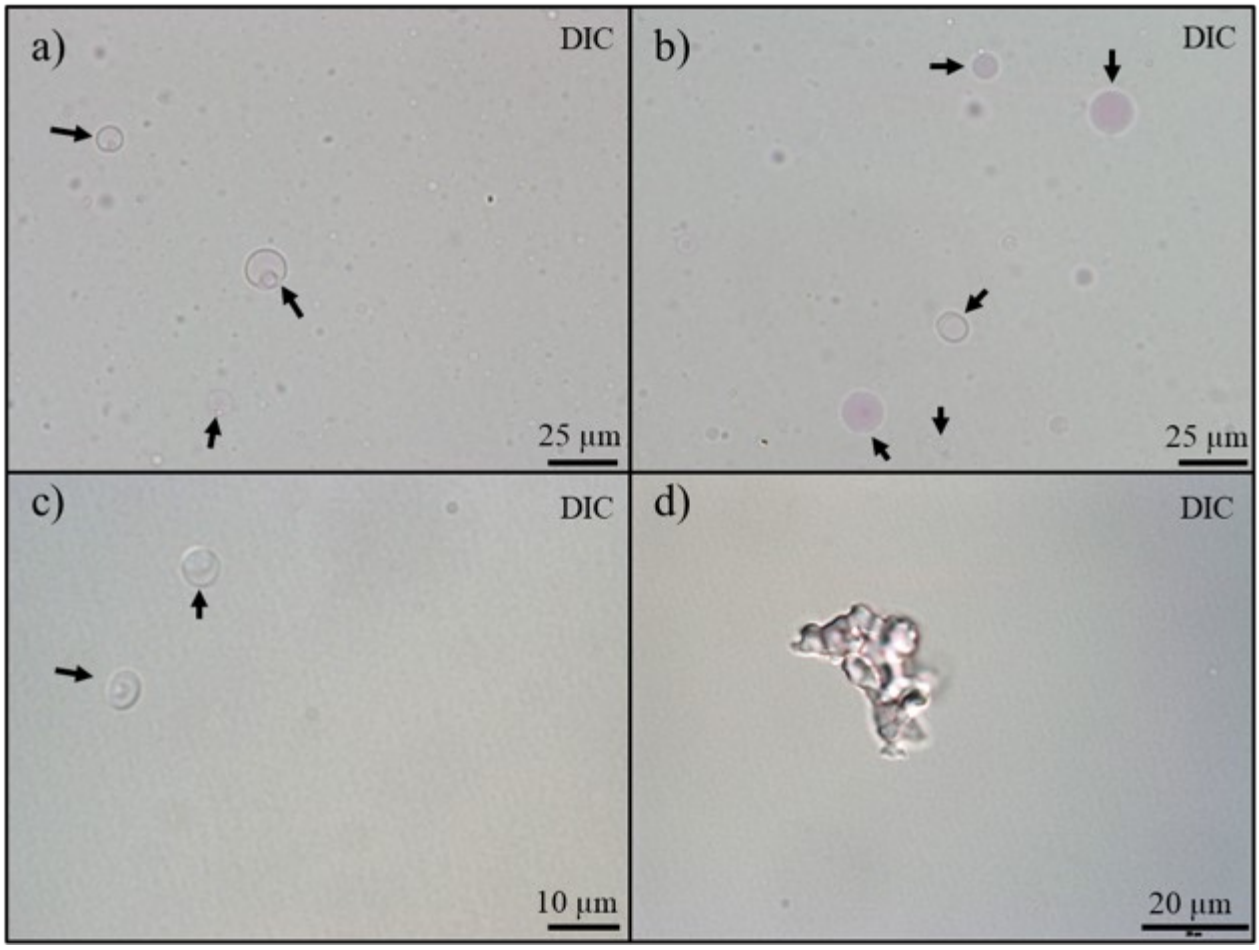


Figure S10. DIC micrographs of a) cell membrane mimicking GUVS containing POPC/DOPE/POPS/Chol = 45/25/15/15 mol % at a total c = 1.0 mM in PBS, pH = 7.4; B) endosome/lysosome mimicking GUVs containing POPC/DOPE/POPS/Chol = 45/25/20/10 mol % at a total *c* = 1.0 mM in acetate buffer (50 mM), pH = 4.5 containing 0.9% (m/v) NaCl; c) Resulting mixture from lipid mixing assay between R_6 = 2/1, N/P = 6 lipoplexes and the endosome mimicking GUVS at a [lipoplex]/[GUVs] = 12 ratio, 21 h after mixing at *T* = 37.0 ºC; d) Resulting mixture from lipid mixing assay between R_4 = 2/1, N/P = 6 lipoplexes and the cell membrane mimicking GUVS at a [lipoplex]/[GUVs] = 12 ratio, 21 h after mixing at *T* = 37.0 ºC.

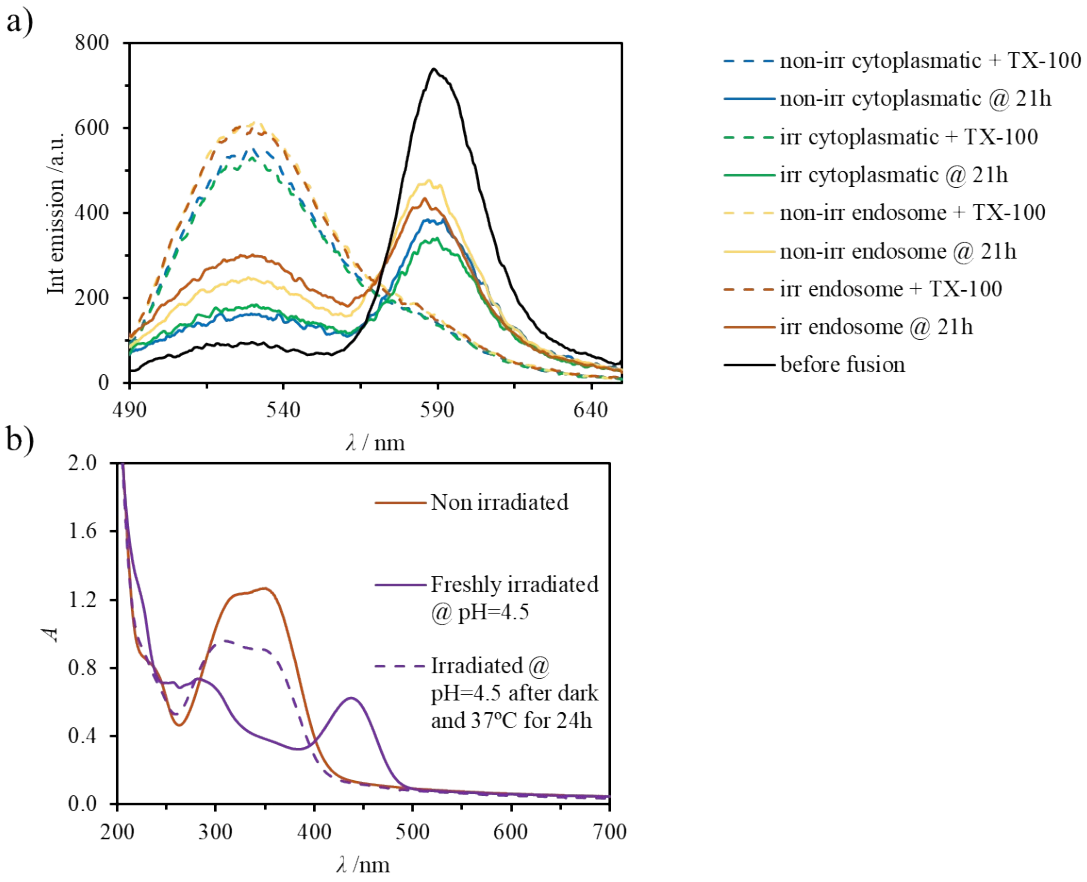


Figure S11. a) Emission spectra of the FRET fluorophore pair (NBD-PE and Rho-PE) under different conditions tested for fusion efficiency calculations. Spectra regarding samples where fluorophore-containing GUVs are mixed with lipoplexes (all except black line) are relative to an assay with R_4 = 2/1 N/P =6 lipoplexes; b) UV-Vis spectra of R_6 = 1/1 N/P =6 lipoplexes at pH = 4.5 before irradiation, right after irradiation until photostationary state has been reached and 24h after irradiation had finished while being and kept under dark condition at T = 37.0 ºC.

S4. Design of CnNCh/MO vesicles: composition, structural features and cytotoxicity – additional data

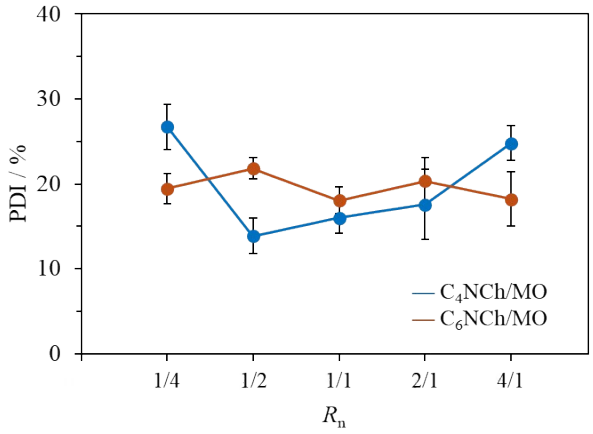


Figure S12. PDI values of the C_nNCh/MO vesicles (3.0 mM) within 24 h after their preparation with different R_n ratios at T = 25.0 ºC.

Table S1. Relative IC_{50} obtained for the neat chalcones and the different R_n mixtures in human tumor cell lines (HCT116, A2780 and MCF7) and in normal primary human fibroblasts. IC_{50} values are expressed as the mean ± SEM of at least two biological independent assays.

Compound / mixture	IC_{50} / µM Tumor cells			Normal cells
	HCT116	A2780	MCF7	Fibroblasts
C_4NCh	> 50.0	> 50.0	> 50.0	> 50.0
C_6NCh	> 50.0	> 50.0	> 50.0	> 50.0
R_4 = 1/4	> 50.0	-	-	> 50.0
R_4 = 1/2	> 50.0	-	-	> 50.0
R_4 = 1/1	> 50.0	-	-	> 50.0
R_4 = 2/1	> 50.0	-	-	> 50.0
R_4 = 4/1	> 50.0	-	-	> 50.0
R_6 = 1/4	> 50.0	-	-	> 50.0
R_6 = 1/2	> 50.0	-	-	> 50.0
R_6 = 1/1	> 50.0	-	-	> 50.0
R_6 = 2/1	> 50.0	-	-	> 50.0
R_6 = 4/1	29.5 ± 0.5	-	-	> 50.0
Doxorubicin	0.5 ± 0.1 [10]	0.1 ± 0.1 [10]	1.2 ± 0.1	12.1 ± 0.2 [10]

S5. Nucleic acid compaction – additional data

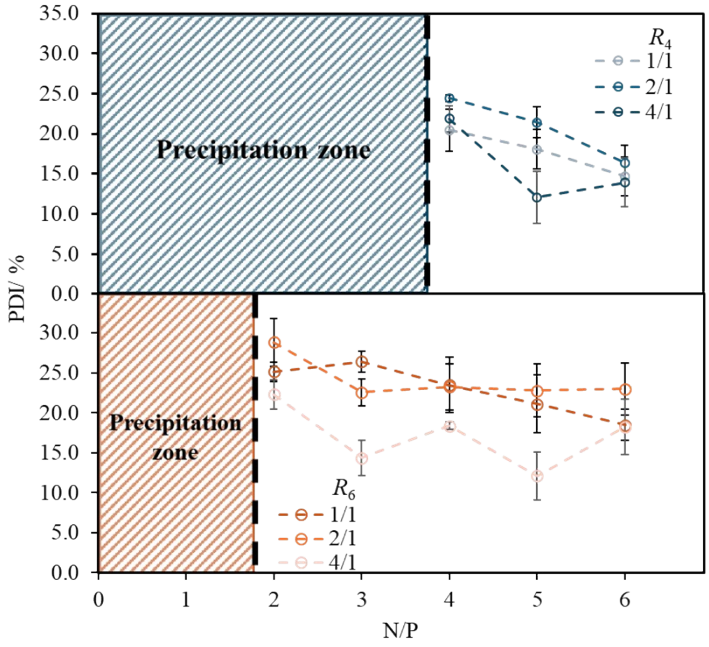


Figure S13. PDI values of the CnNCh/MO/DNA systems at different N/P ratios upon preparation (experiments were performed at a fixed DNA concentration [NA_b] = 2.5 x 10^{-4} M and T = 25.0 ºC).

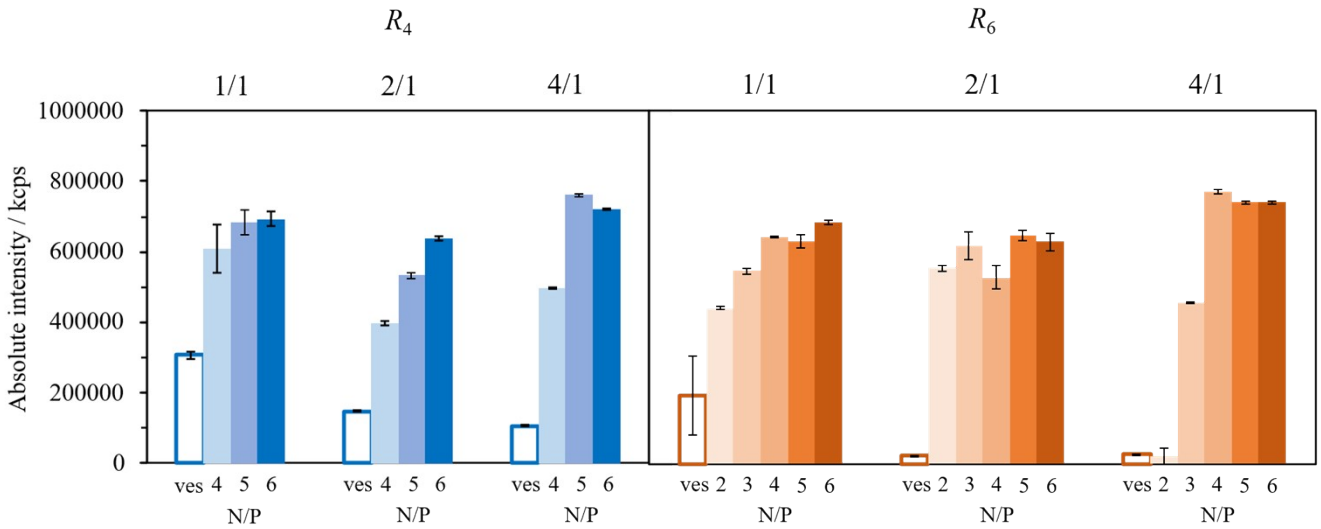


Figure S14. Absolute intensity counts obtained from DLS readings of the CnNCh/MO and CnNCh/MO/DNA systems at different N/P ratios upon preparation (experiments were performed at a fixed DNA concentration [NA_b] = 2.5 x 10^{-4} M and T = 25.0 ºC).

S6. Lipoplex response to stimuli – additional data

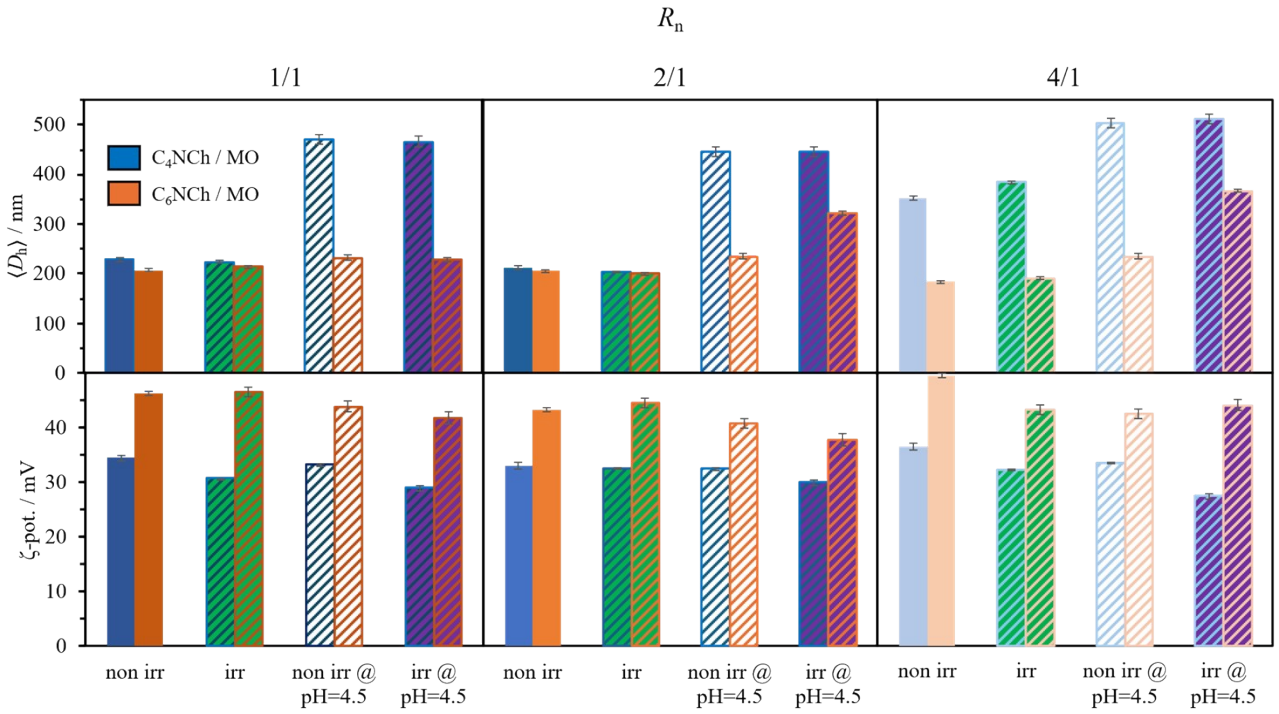


Figure S15. Mean D_h (top panel) and ζ-potentials (bottom panel) of the N/P = 6 lipoplexes ([NA_b] = 2.5x10^{-4} M) with(out) irradiation, at natural (ca. 6) or acidified (pH = 4.5) media (acetate buffer c = 50 mM).

Table S2. DNA released (%) from the N/P = 6 lipoplexes ([NA_b] = 2.5x10^{-4} M) upon irradiation (λ = 365nm) up to the photostationary state at the natural pH of the lipoplex formulation (ca. 6).

	% of DNA released	
R_n	**C_4NCh/MO**	**C_6NCh/MO**
1/1	-2.5	4.0
2/1	-3.9	3.8
4/1	-0.2	1.2

S7. Gene silencing studies – additional data

i) Determination of *cac* of C_nNCh/MO mixtures by surface tension

To ensure that the lipoplexes were still in aggregated form after the extensive dilution occurring in the transfection assays (incubation with cells), we determined the *cac* of the neat C_nNCh/MO systems by surface tension measurements. The 1:1 and 4:1 ratios (the extremes of the C_nNCh/MO range used) were measured, as the 2:1 will fall between (a decrease in MO content will systematically increase *cac*). Surface tension (γ) of the C_nNCh/MO mixtures was determined using a DCAT11 tensiometer, from Dataphysics, by the Wilhelmy method in the static mode (PT11 2,000,323 platinum-iridium Wilhelmy plate). All the experiments were performed at the controlled temperature of 25.0 ± 0.2 °C using a thermostatic circulating water bath Julabo F20. To determine the critical aggregation concentration (*cac*), incremental aliquots of a concentrated C_nNCh/MO aqueous mixture were added to a vessel containing ultrapure water. The surface tension was measured upon each aliquot addition and the value was recorded when γ did not vary for at least 60 s to ensure that equilibrium surface tension was reached.

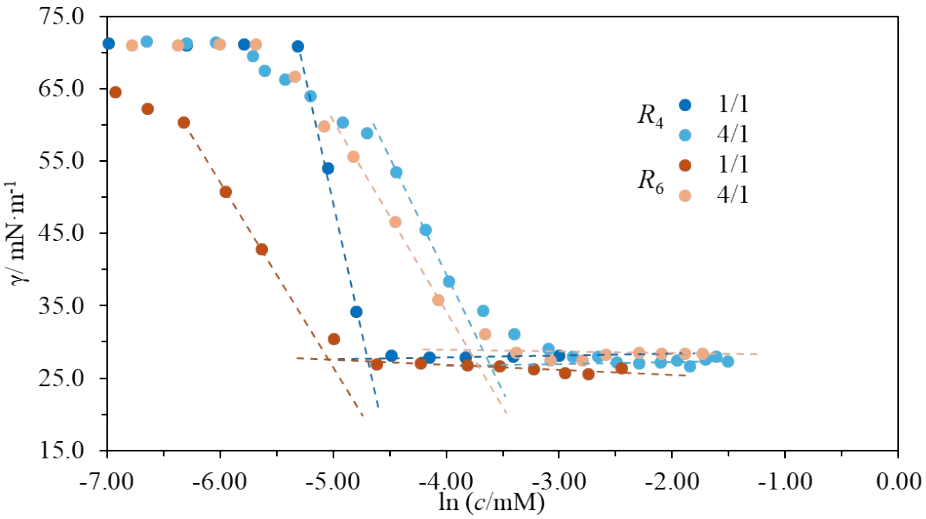


Figure S16. : Surface tension (γ) plots of C_nNCh/MO systems at R_n ratios 1/1 and 4/1 (T = 25.0 ºC).

Table S3. *cac* values of C_nNCh/MO systems at R_n= 1/1 and 4/1 at 25.0 ºC obtained from surface tension plots.

System	R_n	*cac* / μM
C_4NCh / MO	1/1	9 ± 1
	4/1	27 ± 3
C_6NCh / MO	1/1	6 ± 1
	4/1	22 ± 3

ii) Fluorescence microscopy

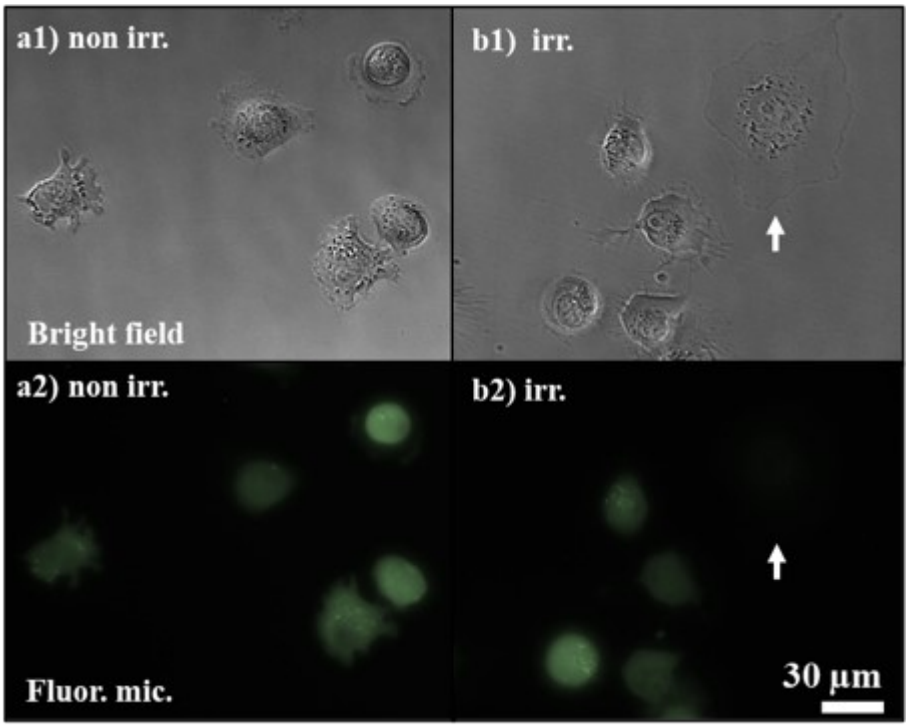


Figure S17. Representative examples of bright field and corresponding fluorescence microscopy micrographs of MCF7-copGFP cells 6h post incubation with R_4 = 1/1, N/P =6 ([NA_b] = 2.5x10^{-4} M) entrapping a copGFP siRNA without (a1 and a2) and with (b1 and b2) irradiation. White arrow is pointing towards a cell with no apparent copGFP expression. Fluorescence microscopy micrographs were acquired on a gray scale, the recoloring was performed only for enhanced visualization.

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