Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information for

Extended photo-induced endosome-like structures in Giant Vesicles promoted by block-copolymer nanocarriers

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1. Materials and Methods

1.1 Materials

All copolymers were bought from Polymer Source Inc. (Dorval Montreal, Canada). They were systematically characterized by ^{1}H NMR and Size Exclusion Chromatography (SEC) and their characteristics are reported in Table 1.1.
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and cholesterol were from Avanti Lipids (Alabaster, AL).
Sucrose (99.5%), glucose (99.5%) and sodium azide (NaN_3) were purchased from Sigma-Aldrich.
3,3'-Dioctadecyloxacarbocyanine Perchlorate (DiO) was from Invitrogen Life Technology (Saint Aubin, France).
Ultrapure water was obtained from an ELGA Purelab Flex system (resistivity higher than 18.2 MΩ cm) and was filtered on 0.2 mm RC filters just before use.

Table SI.1.1 Characterization data for the used copolymers

Block-copolymer	PEO block molecular weight	Second block molecular weight	$Đ=M_w/M_n$
PEO-PS 3.1k-2.3k	3100	2300	1.15
PEO-PCL 5k-2k	5000	4000	1.14
PEO-PDLLA 2.4k-2k	2400	2000	1.18

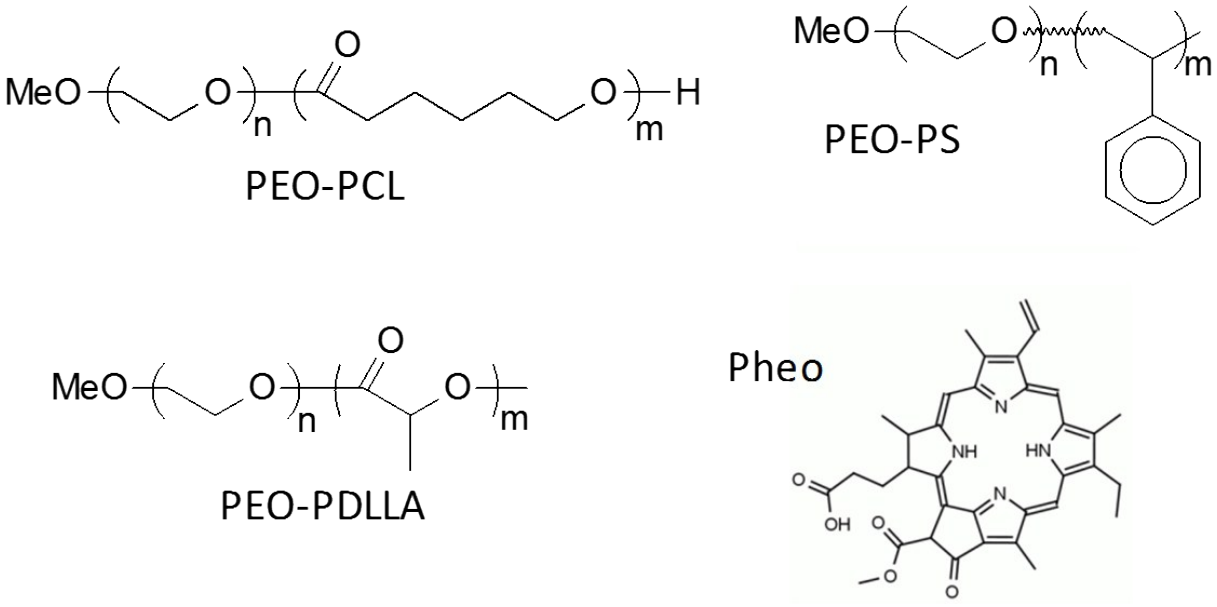

Figure SI.1.1 Chemical structure of the used copolymers and pheophorbide a

1.2 Polymer carrier Preparation

For all block-copolymers, 20 mg of polymer were dissolved in 400 µL of acetone and then slowly added dropwise to 5 mL of filtered ultrapure water under gentle stirring. The solutions were left open 48 hours for acetone to evaporate. Pheophorbide a loading was obtained by adding the desired quantity of the photosensitizer dissolved in acetone to the micellar solution under gentle stirring. The solutions were left open 24 hours for acetone to evaporate.

For all samples loaded with pheophorbide, the molar ratio between the photosensitizer and the polymer was kept constant at 1/30 mol/mol to ensure the complete encapsulation.[1]

1.3 Giant Unilamellar Vesicles (GUV) Preparation

Giant Unilamellar Vesicles (GUV) were prepared through electroformation. Briefly, a DOPC (1,2-Dioleoyl-sn-glycero-3-phosphocholine) or DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) 0.5 mg/ml stock solution in $CHCl_3$ was prepared. 10 µl of the stock solution were deposited on two ITO-coated glass slides, on the conductive side. Chloroform was dried under vacuum for two hours and a dry lipid film on each slide was obtained. The electroformation chamber was prepared sandwiching the slides with an O-ring separating the lipid films. The chamber was filled with 15 mM sucrose solution and a sinusoidal alternating voltage of 10 Hz frequency and 2 Vpp amplitude was applied (the electrical contact between the slides was provided by using copper tape). DOPC GUVs were prepared at 25°C for three hours, DPPC GUVs at 70°C for four hours. GUVs were employed within 24 hours after preparation. Being the total amount of solution in the electroformation chamber approximately equal to 400 µl, the total lipid concentration in the stock solution obtained from electroformation, considering a 100% yield, is approximately 0.025 mg/ml lipid in sucrose 15 mM.

1.4 Preparation of CBF-loaded Large Unilamellar Vescicles (LUV)

CBF-loaded DOPC liposomes were prepared by dissolving 20 mg of DOPC in 1 mL of $CHCl_3$ in a hemolysis tube. The $CHCl_3$ was then evaporated in a rotatory evaporator to form a homogenous film at the bottom of the tube. The film was dried under vacuum for at least 2 hours and subsequently hydrated with 2 mL of CBF at 60 mM in PBS. This suspension was put in a sonication bath at 60°C for 90 minutes. After sonication, the suspension was extruded 19 times through a polycarbonate membrane with pores of 100 nm (Nucleopore Track-Etch Membrane, Whatman, UK) using a hand-held extruder (Avanti Mini Extruder, Avanti Polar Lipids, USA). After extrusion, the LUVs were dialyzed against a PBS buffer solution (11 x 1L) using 12-14 kDa cut-off regenerated cellulose membranes (Cellu Sep T3, Orange Scientific, Belgium). Considering the losses in the extrusion process negligible (a reliable estimate is below 10%[2]) the stock solution is 10 mg/ml of lipid in PBS.
The size of the LUV was measured through DLS (D_h= 120 nm, PDI = 0.22).

1.5 Leakage assays

All samples were directly prepared in the measurement cuvette. First, the LUVs were diluted in PBS (lipid concentration= 125 µM and V=1.85 mL). The fluorescence intensity I_0 was measured; different volumes of the micellar solutions were then added ensuring that the pheophorbide-a concentration was 1 µM for all samples (V=2 mL). A reference containing only LUVs was prepared by adding 0.5 mL of PBS to the LUVs solution. After addition of the micelles, the samples were left 30 minutes in the dark to incubate. The samples were then irradiated during 90 minutes with a mercury-xenon gas-discharge lamp (XBO 200W 2/L1, Osram, Germany) equipped with a long-pass optical filter with a cut-off at 550 nm (1.18 10^{-3} $Em^{-2}s^{-1}$ in the wavelength range 550-700 nm). This ensures the excitation of pheophorbide-a without risking the photobleaching of the CBF. Non-irradiated blanks were made by preparing the same samples as before but keeping them in the dark during the experiment.
At the end of the measurement, 100 µL of Triton X 100 at 10%$_w$ in PBS were added to the sample in order to disrupt the LUVs and release all encapsulated CBF. This gives access to the maximum of the fluorescence intensity I_{max}.

The LUV leakage was measured by following the fluorescence of the CBF when exited at 492 nm using a spectrofluorimeter (Fluorolog 3-2 IHR320, Horiba, Japan). The emission was measured at 518 nm. The leakage was calculated using the following equation:

$$\text{Leakage}(t) = (I(t)-I_o)/(I_{max}-I_0) \quad \text{(eq 1)}$$

For $I_{0\,a}$nd I_{max}, the values were corrected to account for the dilution.

1.6 Dynamic Light Scattering

DLS measurements measurements were carried out at 25°C at θ = 173° on a Malvern (Orsay, France) Zetasizer NanoZS with a 10-20% typical accuracy. Solutions were analyzed in triplicate without being filtered in order to characterize the plain samples. Samples were characterized by a single, monodisperse population, and the auto correlation functions were analyzed through the cumulant fitting stopped to the second order allowing an estimate of the hydrodynamic diameter of particles and of the polydispersity index.

1.7 Zeta Potential

Zeta potentials (ζ) were determined by Electrophoretic Light Scattering (ELS), measurements were carried out on a Malvern (Orsay, France) Zetasizer NanoZS. Zeta potentials were obtained from the electrophoretic mobility u, according to Helmholtz-Smoluchowski equation: $\zeta = (\eta/\varepsilon) \times u$ with η being the viscosity of the medium, ε the dielectric permittivity of the dispersing medium. The Zeta Potential values are reported as averages from ten measurements.

1.8 Confocal Laser Scanning Microscopy (CLSM)

CLSM experiments were carried out with a laser scanning confocal microscope Leica TCS SP2 (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 63x water immersion objective. The 488 nm laser line was employed to detect DiO fluorescence (λ excitation 488 nm, λ emission 498 nm - 530 nm); the 405 nm laser line was employed to detect Pheo fluorescence) (λ excitation 405 nm, λ emission 600nm-700 nm).

For confocal microscopy and FCS experiments 70 µl of GUVs dispersion in sucrose 15mM were diluted in a measurement chamber (Lab-Tek® Chambered # 1.0 Borosilicate Coverglass System, Nalge Nunc International, Rochester, NY USA) with 130 µl glucose 15mM. Different volumes of carrier solutions were then added in order to get a final pheophorbide concentration 1µM (the total volume was completed to 300µl with glucose 15mM). GUVs and NPs were then incubated at r.t. for 10 minutes, before the acquisition of the images.

1.9 Fluorescence Correlation Spectroscopy (FCS)

FCS measurements were carried out with a ISS module (ISS, Inc.1602 Newton Drive Champaign, IL, USA) equipped with two APD with 500-530 nm and 607-683 nm BP. FCS measurements were carried out by exciting the fluorescent probe DiO at 488 nm and acquiring the fluorescence emission between 500 and 530 nm. FCS is based on the analysis of the time fluctuations of the fluorescence intensity of a selected fluorescent probe, due to concentration fluctuations inside a small determined detection volume. The autocorrelation function of the fluorescence intensity (G(τ)) is thus calculated as a function of the fluctuations of the signal from the averaged value, $\delta I(t)=I(t)-\langle I(t)\rangle$, as (S1):

$$G(\tau) = \frac{< \delta I(t) \delta I(t+\tau) >}{< I(t) >^2} \quad \text{(SI 1)}$$

The models employed for the analysis of the autocorrelation functions (ACFs) take into account the shape and the exact size of the detection volume. Generally the volume is approximated as 3D-ellipsoidal Gaussian shape with axial (z0) and lateral (w0) defining parameters. These parameters can be determined through calibration, by employing a reference fluorescent dye with well-known diffusion coefficient. For the calibration, we employed a 50 nM standard solution of Rhodamine 110. For a three-dimensional Brownian diffusion mode in a 3D Gaussian volume shape, the ACFs profiles can be analyzed according to equation (S2):

$$G(\tau) = \frac{1}{< c > \pi^{\frac{3}{2}} {w_0}^2 z_0} \left(1 + \frac{4D\tau}{{w_0}^2}\right)^{-1} \left(1 + \frac{4D\tau}{{z_0}^2}\right)^{-\frac{1}{2}} \quad \text{(SI 2)}$$

With <c> the averaged fluorophore concentration (nM), D the diffusion coefficient of the probe ($\mu m^2 s^{-1}$). The same equation was employed to monitor DiO diffusion inside and outside multiGUVs (Figure 4, manuscript). In the case of a multicomponent system the measured autocorrelation function is a weighted sum of the autocorrelation functions of each component. The general expression for the autocorrelation function of the fluorescence intensity is in this case described by equation (S3)

$$G(\tau) = \frac{1}{< c > \pi^{\frac{3}{2}} {w_0}^2 z_0} \sum_i f_i \left(1 + \frac{4D_i\tau}{{w_0}^2}\right)^{-1} \left(1 + \frac{4D_i\tau}{{z_0}^2}\right)^{-\frac{1}{2}} \quad \text{(SI 3)}$$

being f_i the weight factors of each i diffusing component with diffusion coefficient D_i.

2. Supplementary Results

2.1 Confocal Microscopy Experiments on poly(ethylene oxide)-poly(styrene) (PEO-PS) micelles

Table SI.2.1 Hydrodynamic diameter (intensity weighted), D_h, zeta potential, ζ, of the employed nanocarriers.

Block-copolymer	D_h (nm)	ζ (mV)
PEO-PS 3.1k-2.3k	20	-1.8±0.5

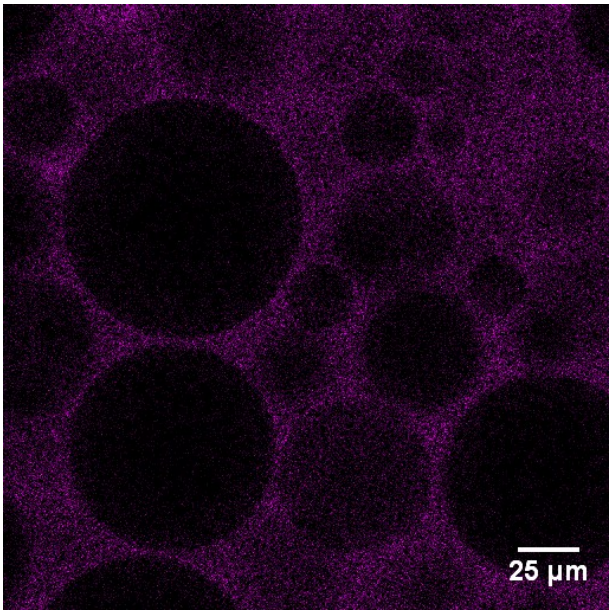

Figure SI.2.1. Representative CLSM images of DOPC GUV challenged with PEO-PS micelles

None of the effects observed for PEO-PCL and PEO-PDLLA were present in the case of PEO-PS micelles (Table SI.2.1). Pheophorbide remained trapped inside the micellar core (see Figure SI 2.1). The GUVs size remained unchanged, no oscillation upon irradiation was observed. This confirms that the effectiveness of the excited photosensitizer in leading to oxidative damage of the model membrane is linked to its location, due to the short half-life (4 µs in water) and limited diffusion length of $^1O_2$ (100 nm in water)[3].

2.2 Confocal Microscopy Experiments on DPPC-Chol GUVs

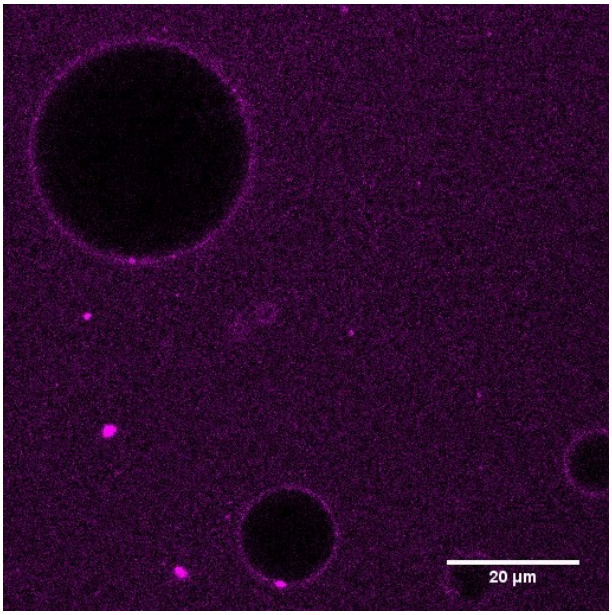

Figure SI.2.2. Representative CLSM images of DPPC:Chol 9:1 GUV challenged with PEO-PCL micelles

2.3 Carboxyfluorescein (CBF) Leakage through Large Unilamellar Vesicles (LUV)

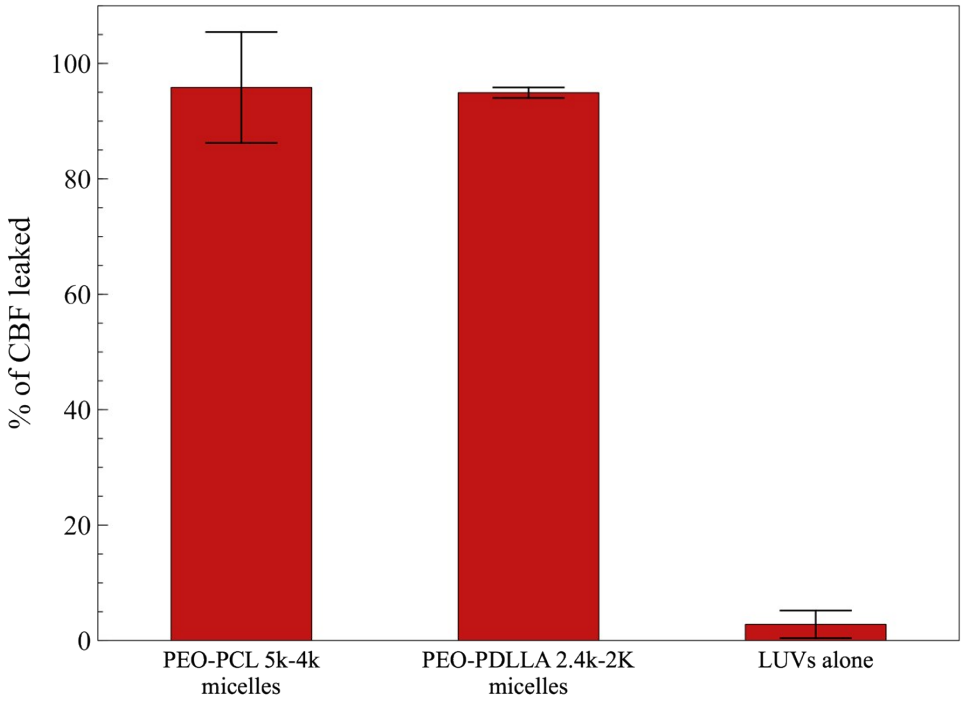

Figure SI.2.3 Normalized leakage of CBF from LUV mixed with PEO-PCL 5k-4k micelles and PEO-PDLLA 2.4k-2k micelles loaded with pheophorbide-a or alone. For each loaded sample, the pheophorbide-a concentration was 1 µM. The measurements were made after 30 minutes of incubation and 90 minutes of light exposure. Error bars are standard deviations.

References

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2. F. Szoka, F. Olson, T. Heath, W. Vail, E. Mayhew, D. Papahadjopoulos, *Biochimica et Biophysica Acta (BBA) – Biomembranes*, **1980**, 601, 559-571 attention

3. E. Skovsen, J. W. Snyder, J. D. Lambert and P. R. Ogilby, *J. Phys. Chem. B*, **2005**, 109, 8570–8573.

Supplementary videos

Video 1. Typical oscillations induced by laser illumination (405 nm, 0.65 mW, 43% intensity) in DOPC GUVs challenged with pheophobide. Total time 39.4 s, 7 fps.

Video 2. Laser illumination of DOPC GUVs challenged with pheophobide loaded PEO-PCL micelles in the presence of 15 mM NaN_3. Total time 67.3 s, 7 fps.

Video 3. Typical oscillations induced by laser illumination (405 nm, 0.65 mW, 35% intensity) in DOPC GUVs challenged with pheophobide loaded PEOPCL micelles. Total time 43.8 s, 7 fps.

All videos are displayed at 7 fps for convenience