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Determination of critical micelle concentrations of Pluronics

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S1. CMC determination by fluorescence of 1,6-diphenyl-1,3,5-hexatriene (DPH)

The CMC of polymers was determined by the change in fluorescence intensity of DPH, which increases when DPH inserts in the hydrophobic core of the polymer micelles [A. Chattopadhyay, E. London, *Anal. Biochem.* 1984, **139**, 408; doi: 10.1016/0003-2697(84)90026-5]. 3-4 mg DPH (Mw 232) was dissolved in 1 ml of acetone, 50 µl of this solution was added dropwise to 50 ml of PBS under vigorous stirring and the resulting solution was stirred for ≈1.5–2 h until the acetone odor disappeared. The polymer to be tested was dissolved in PBS one day before the experiment and diluted to the required concentrations just before use. Usually 600 µL of 10 µM DPH was added to 600 µL of each sample (the final DPH concentration was 5 µM). In some experiments, 0.25 µM DPH was also used. Samples were kept at 37°C for 1 h in a water thermostat. The fluorescence intensity of each sample was measured using a Hitachi 650-10 S fluorimeter (Japan) at an excitation wavelength of 366 nm and an emission wavelength of 433 nm. To minimise tag burn-out, the excitation light slit was kept as small as possible (1–2 nm). To increase the sensitivity of the assay, the transmitted light slit was increased to a maximum of 18–20 nm. Since the micelle formation process of Pluronics is strongly influenced by temperature, it was periodically checked in a thermostatted cell during the experiment. Measurements were made over a wide range of reliably determined emitted light intensity values, *i.e.* from 10 to 100–120 units. The sensitivity of the instrument was selected so that the intensity value in the control samples without polymer was less than 5 arbitrary units. The polymer was diluted in such a way that the fluorescence of the first 3–5 samples was similar to that of the control. These values lie on a straight line parallel to the abscissa axis on the graph of fluorescence intensity versus polymer concentration up to the CMC. Fluorescence intensity increases with increasing polymer concentration. The CMC of the analyzed polymer corresponded to the polymer concentration at which the intersection of the line parallel to the abscissa and the line of increasing fluorescence intensity of DPH was observed.

S2. Examples of Pluronic CMCs determination by DPH fluorescence technique.

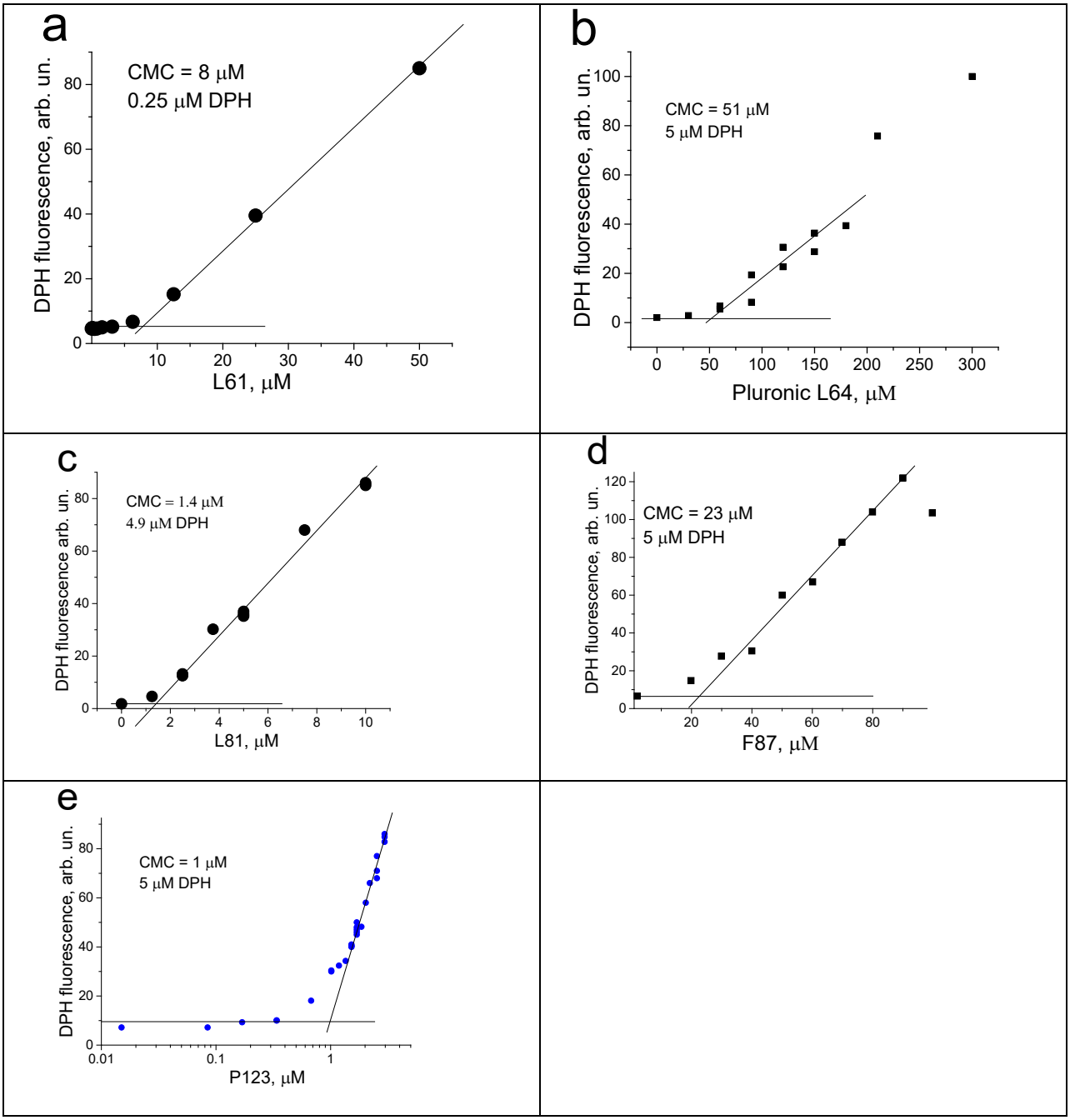

Figure S1 Analyzes of Pluronics (a) L61, (b) L64, (c) L81, (d) F87 and (e) P123. CMCs are indicated on the graphs. CMC of Pluronic L61 was determined with 0.25 μM DPH, while the other polymers were analyzed with 5 μM DPH.

S3. Determination of Pluronic C^{MDR} concentrations that suppress multidrug resistance (MDR) of the NCI/Adr-RES cancer cell line.

NCI/Adr-RES cells (previously designated as MCF7/Res) were grown in DMEM supplemented with 4 mM glutamine, antibiotics and 10% fetal calf serum (culture medium) under standard conditions (CO_2 incubator, 37°C, humidified atmosphere, 5% CO_2). Cells were seeded in a 96-well plate at a density of 3500–4000 cells per well, kept overnight under standard conditions and used the next day. The polymers tested were dissolved in serum-free culture medium overnight at 4°C. The next day, approximately 3 mg of doxorubicin hydrochloride (DOX, Mw 232) was dissolved in 0.3–0.5 ml of water and DOX concentration was determined from its absorbance at 490 nm (ε = 10 500 M^{-1} cm^{-1}). This DOX stock solution was diluted to 10 μg/ml with serum-free medium under sterile conditions. The tested polymers were sequentially 2-fold diluted with serum-free medium. An equal volume of DOX was added to each polymer dilution to achieve DOX final concentration of 5 μg/ml in the samples. The culture medium was aspirated from the wells containing adherent cells and 100 μl of each sample were added per well. Each sample was analysed in triplicate. Cells were incubated with DOX in the absence (control) or presence of polymers for 1.5 h under standard conditions. Then, the test solutions were discarded, 200 μl of the culture medium was added per well and the cells were kept for subsequent three days under standard conditions. The final number of viable cells in the wells was determined by the MTT assay, which is based on the ability of live cell dehydrogenases to reduce MTT and form needle-shaped formazan crystals that are insoluble in aqueous media. The stock MTT aqueous solution, 5 mg/ml, was stored at 4°C and diluted with serum-free medium before use. 50 μL of 1 mg/mL MTT solution was added directly to the medium in the wells and the reaction developed within 3 h at 37 °C in the CO_2 incubator. The medium was then aspirated, violet crystals of formazan were dissolved in 100 μL of dimethyl sulfoxide, and the absorbance at 550 nm was measured on a Multiscan photometer (Titertek, USA) after complete dissolution of the formazan crystals, which occurred within 10–15 min. The percentage of survived cells relative to the controls was plotted against the concentrations of the tested compound. Cell viability was calculated from the ratio of D550 in the wells treated with DOX - polymer mixtures relative to the mean control D550 value.

PS. If the cytotoxicity of the polymer and the drug are unknown, two preliminary experiments are required.

First, cells treatment with various concentrations of the polymer to determine its non-toxic region which precedes the steady decrease in D550 values.

Next, cells treatment with various concentrations of the drug, either alone or mixed with a constant non-toxic concentration of the polymer, chosen on the basis of the results of the first experiment. The drug concentration non-toxic in the absence of the polymer but reducing cell viability to 40–60% in its

presence can be defined from the plot of D550 versus drug concentration. This drug concentration should be used for determination of the polymer's C^{MDR} as described above (**S3**).

All other procedures are the same as described in the main text.

S4. Examples of CMC determination from sensitization of MDR cancer cells.

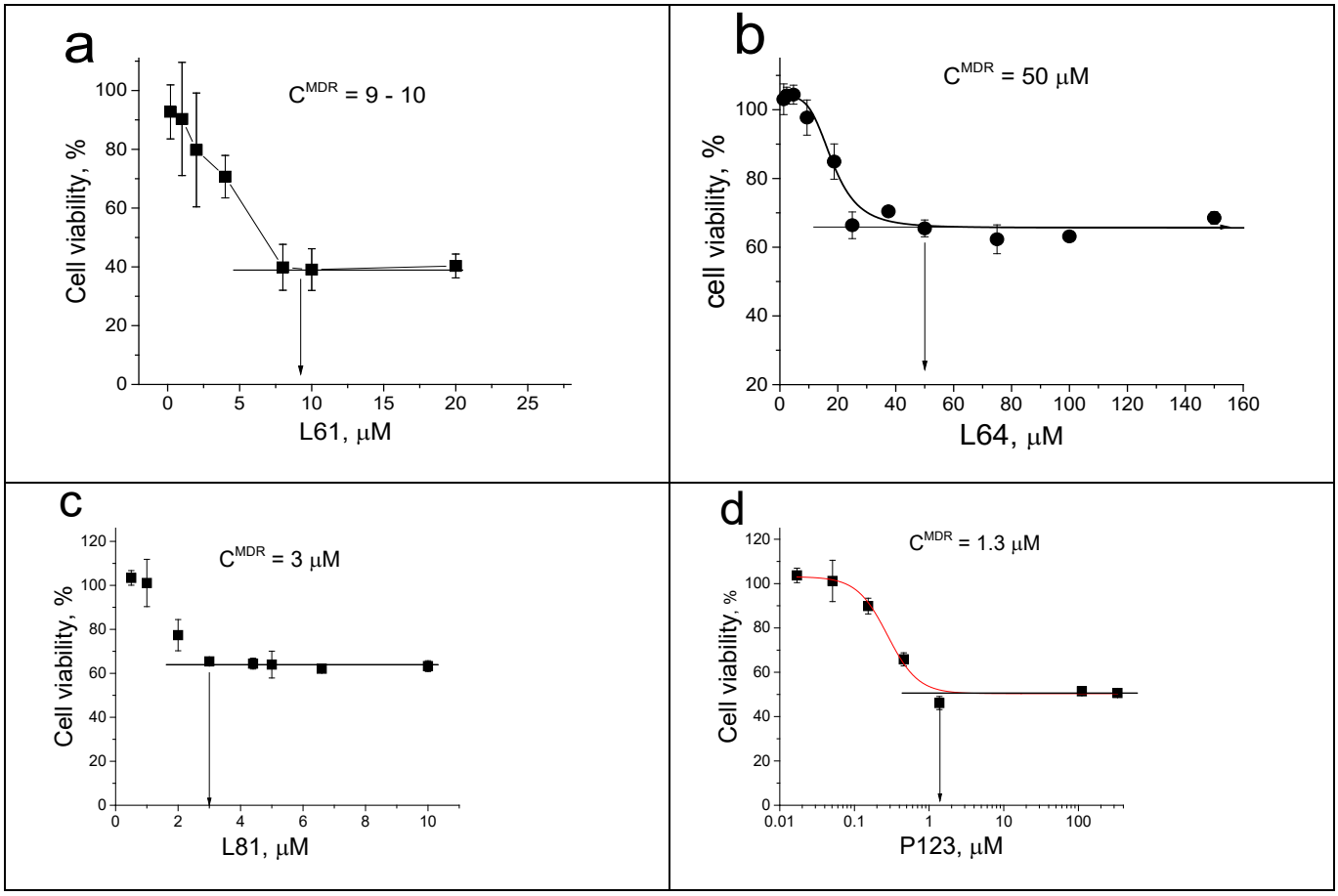

Figure S2 Analyses of Pluronics (a) L61, (b) L64, (c) L81 and (d) P123. In the absence of a Pluronic and at its low concentration the cells remain drug resistant and about 100% of them remain alive. As the polymer concentration increases, MDR become inhibited, the drug acquires cytotoxicity and decreases the final amount of living cells. Their least number reaches minimum at maximum unimers concentration (C^{MDR}), *i.e.* at CMC. Further increase in the polymer concentration leads to formation of micelles while the unimers concentration remains constant forming a plateau. The polymer concentration in the beginning of the plateau = C^{MDR} indicated with arrows. The plateau level depends on DOX concentration: the more DOX, the lower the plateau.

C^{MDR} vales of F68, F87 and F127 were not determined because these highly hydrophilic Pluronics do not inhibit MDR.

S5. Liposome-to-micelle migration of NBD-labelled phosphatidylcholine.

Liposomes were prepared by conventional methods [New, R. R. C. *Liposomes: A Practical Approach*, IRL Press, Oxford, 1990, p. 95]. Specifically, 0.54 mg of egg white lecithin (EL), 1.6 mg of cholesterol in methylene chloride and 0.3 mg of C_{12}-NBD-PC in chloroform were mixed and the solvents evaporated under reduced pressure. The lipid film was hydrated in 1 mL PBS and subjected to three freeze-thaw cycles from liquid nitrogen temperature to +70°C with vortex mixing between cycles. The suspension was sonicated with a Cole-Parmer 4700 Ultrasonic Dispergator (2 × 2 min + 1 × 1 min, 22 kHz, 30 W) keeping the sample in the ice-cold bath and centrifuged to remove titanium dust (Eppendorf microcentrifuge, 9400g, 3 min). The diameter of the liposomes varied from 70–100 nm as determined by photon correlation spectroscopy using Zetasizer Nano ZS (Malvern, UK). Liposomes can be stored at +4°C for 1–2 days. Freezing and storage of the liposomes at −40°C resulted in the formation of a dense precipitate which could not be broken down to the original size of the liposomes even after intensive stirring and repeated ultrasonic treatment. The diameter of such liposomes remained at 140–220 nm. Freezing of liposomes is therefore not recommended.

Polymer–liposome interaction was followed by measuring fluorescence with a Hitachi 650-10S spectrofluorometer at 37 °C (λ_{ex} = 465 nm, λ_{em} = 530 nm) in the 1.5 mL samples obtained by mixing 0.5 mL of a copolymer solution in PBS and 1 mL of 0.01 g/L liposomes. The release of C_{12}-NBD-PC from the liposomes was evidenced by an increase in fluorescence intensity. We determined the polymer concentration corresponding to the onset of the process by taking the intersection of the straight line through the fluorescence intensity values at low polymer concentrations with the straight line through the values of the increasing branch.

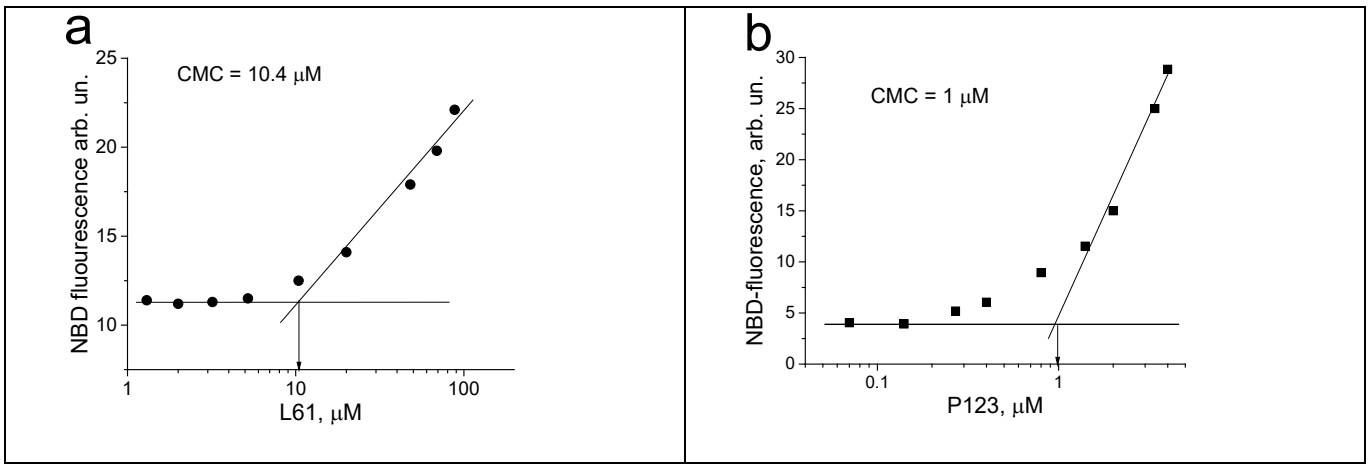

Figure S3 Determination of Pluronics (a) L61 and (b) P123 CMCs from liposome-to-micelle migration of NBD-labelled phosphatidylcholine. CMC values are indicated with arrows.