Determination of the optimal pH for Doxorubicin encapsulation in polymeric micelles

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Appendix A - Supplementary Material

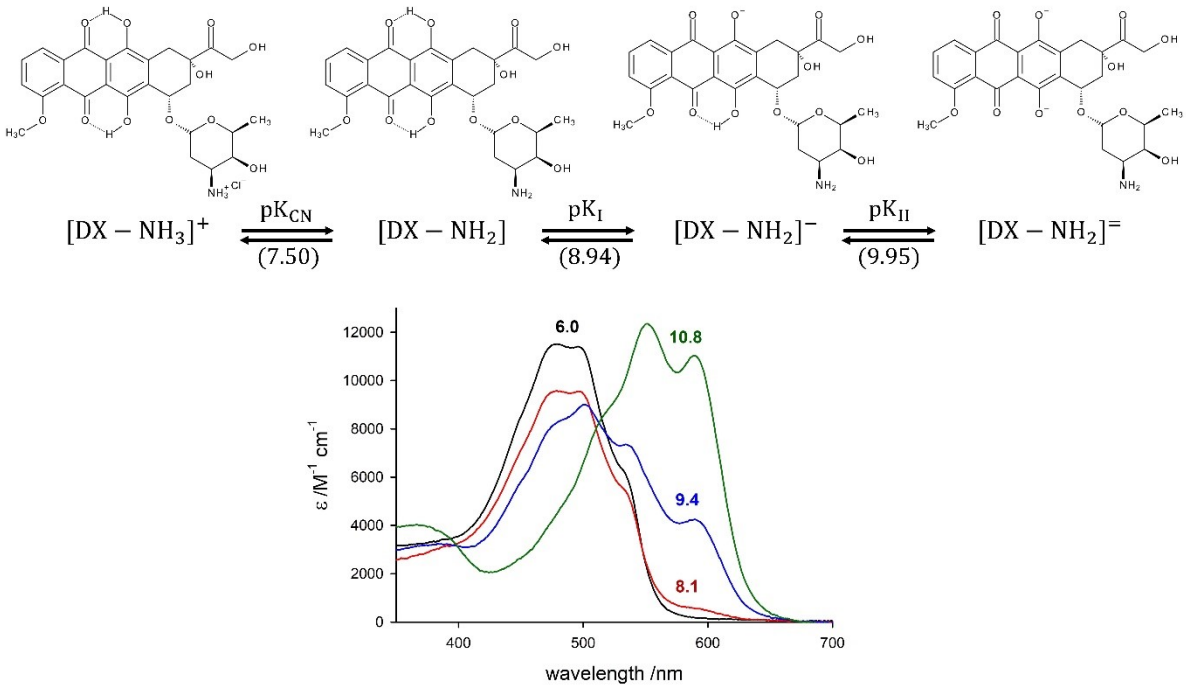

Fig. A1 Doxorubicin deprotonation equilibria and the relevant pKs (values in parenthesis are from ref. 17 of the main manuscript) together with the doxorubicin molar extinction coefficient as a function of wavelength at the different pHs indicated in the graph. Absorption spectra were acquired on doxorubicin solutions $7.8 \cdot 10^{-6}$ M, with l= 1.0 cm and T= 25 °C.

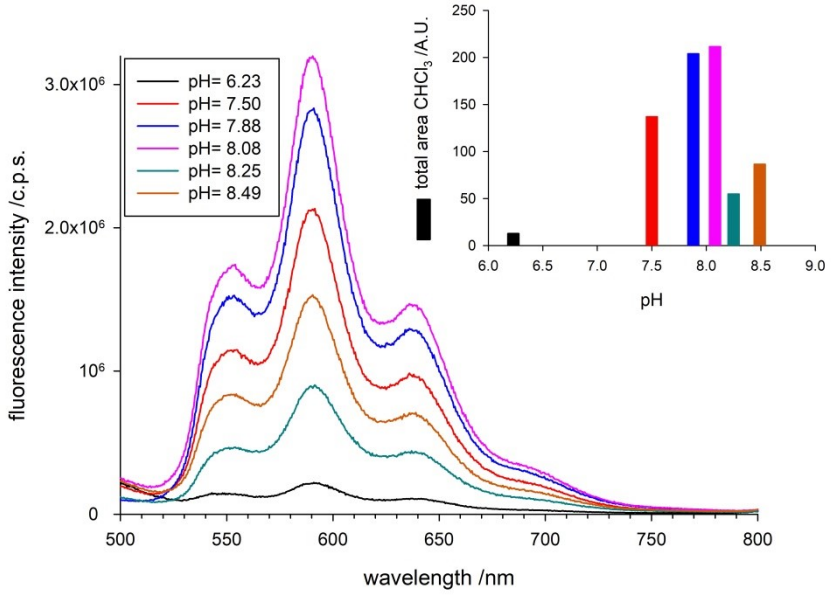

Fig. A2 Fluorescence spectra of the chloroform phase of DX-NH_2 extracted by DX water phases at different pHs (see legend). The inset shows the relevant areas of the fluorescence spectra of the main graph (same colours of the bars as in the main graph). λ_{exc}= 410 nm; slits: 3.0/3.0 nm; 0.3x0.3 cm fluorescence cell; T= 25 °C.

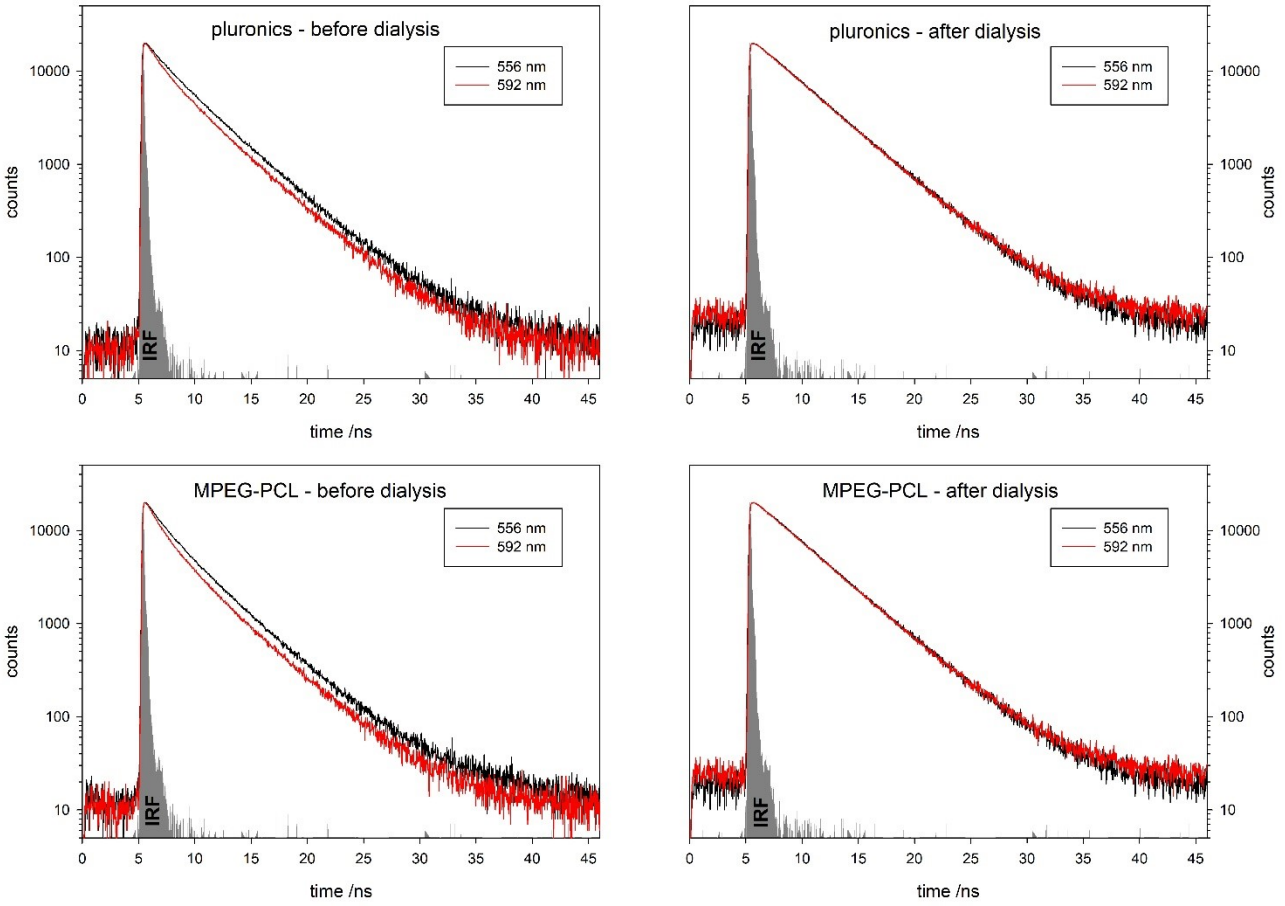

Fig. A 3 Semi-log plot of the Fluorescence decay curves for samples before (left panels) and after (right panels) dialysis (see main manuscript for details). The instrument response function (IRF) has been acquired at 378 nm, using the scattering characteristic intrinsic to the samples measured, through a high-pass filter from Baird Atomic BA400 in emission. λ_{exc} = 378 nm; slits: 0.3/0.3 mm; emission filter: Corning 3391; time resolution 33 ps; cell: 0.3x0.3 cm; T = 45 °C.

The biexponential nature of the drug decays in both polymer systems prior to dialysis is evident to the naked eye. Particularly at the wavelength of 592 nm. This is because the free DX has a maximum in the emission peak at this wavelength while for the encapsulated drug the most intense signal falls at about 556 nm (cfr. Ref. 10 and Fig. 4 of the main manuscript). Furthermore, the fluorescence lifetime of DX in polymer micelles is about four times longer than the free drug in water (on average, 4 ns versus 1 ns – Ref. 10 of the main manuscript). Therefore, even a small amount of unencapsulated drug is highly visible in a fluorescence signal decay curve. After dialysis, however, the fact that at both wavelengths the decay kinetics are monoexponential, with a lifetme of around 4 ns, is an indication that only DX encapsulated in polymer micelles is left in the solution.

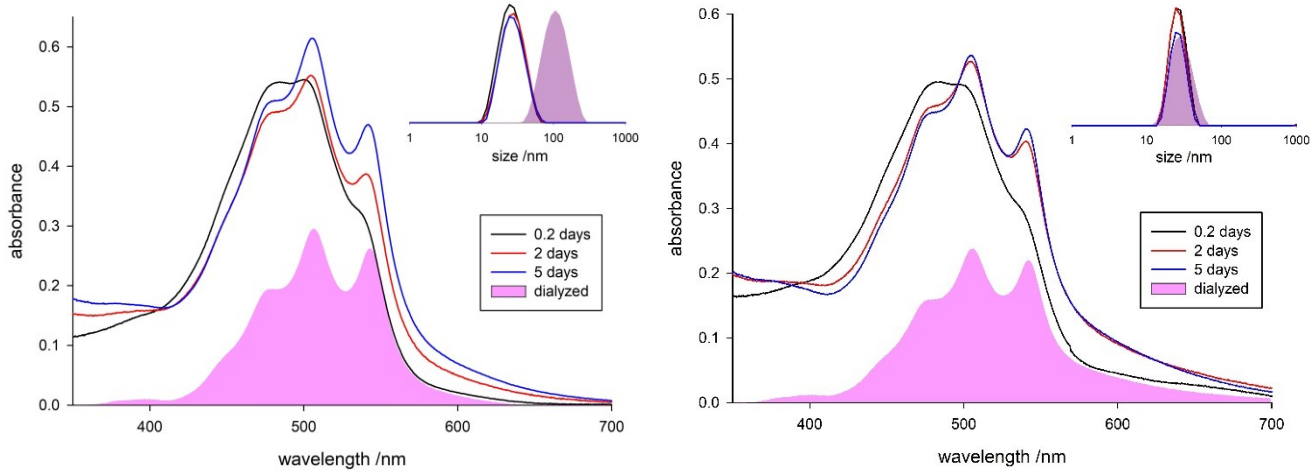

Fig. A4 Time evolution (see legends) of the UV-Vis spectra of DX internalization in the presence of F127/P123 micelles (right panel) and MPEG-PCL micelles (left panel) prepared at pH= 8.10 and at a temperature of 45 °C. In both panels, the inset report the time evolution of the relevant DLS measurements (same colours as in the main graphs). T= 45 °C; cell pathlength= 0.3 cm.

Doxorubicin incorporation kinetics

Apart from the condition explored in the main paper, the study was expanded to include other preparation scenarios.

To determine the role played by NaC and pH in the preparation of the pluronics based polymeric micelles, the following samples have been prepared and the DX incorporation kinetics followed as described in the main paper:

sample S1: F127/NaC (MR= 0.2) @ pH= 8.4

sample S2: F127/P123 (1:2)/NaC (MR= 0.2) @ pH= 8.4

sample S3: F127/P123 (1:2) @ pH= 8.0

Following the behaviour observed with the samples described in the main paper, the kinetics of DX incorporation at pH= 8.4 is significantly faster than those observed at pH= 7.7. It has however to be pointed out that at this more alkaline pH the DX degradation takes place to a larger extent, in agreement with the literature (ref. 32 in the main paper) and with the results shown in Fig. 1 of the main manuscript. This can be well appreciated in Fig. A4 where the behaviour of samples S1, S2 and S3 is compared. In the case of sample S1, the emission spectrum acquired after 1 hour from the addition of DX shows a clear reduction in the intensity of the fluorescent band of the drug due to the exposure to an

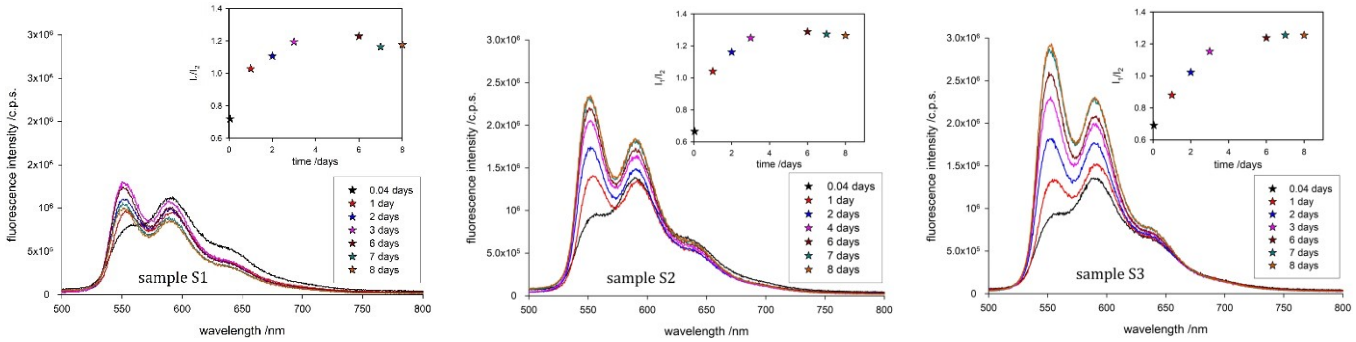

Fig. A5 Emission spectra evolution with time of samples prepared at the same DX concentration, $1.6 \cdot 10^{-4}$ M, but with different composition and pH (see text for the details). λ_{ecc}= 410 nm; slits 1.5/1.5 nm. 0.3x0.3 cm quartz cuvette; T= 45 °C.

alkaline pH. Such a DX degradation is more pronounced with time even though the DX is internalized in the micellar aggregates, as evidenced by the progressive increase of the I_1/I_2 ratio (see the relevant inset). Such degradation effect is reduced in the presence of the mixture of pluronics (sample S2) at the same pH without any appreciable difference in the kinetics of the internalization process. In the case of sample S3, the behaviour is extremely close to the one shown in the main paper for the system at pH= 8.1. In any case, no effect of NaC can be detected (further experiments -not shown- ruled out any direct involvement of NaC in the DX internalization process, apart its role in making the pH slightly alkaline).

Stability of Polymeric micelles

The issue of reproducibility of the micellar preparation and their stability (in terms of amount of DX solubilized and dimensional changes after freeze-drying and after freeze/thaw cycles) has been addressed on freshly prepared polymeric micelles (at pH= 8.1; according to the procedure described in the main manuscript).

In Fig. A6 is shown the incorporation kinetics of DX (limited to three days) for both MPEG-PCL and pluronics based polymeric micelles. According to the results reported in the main manuscript (Fig. 2), reducing the incubation time to three days results in a smaller amount of DX-NH_2 solubilized in the apolar core of both micellar systems. Apart from this, the samples behaviour exactly matches that reported in the main manuscript, also for the micellar's aggregate dimensions.

In the case of MPEG-PCL sample (Fig. A6; lower panel) a significantly increase in the aggregate dimensions was observed. Reasonably this must be due to large extent of the interaction between the polymer and the dialysis membrane. As a matter of fact, by using dialysis membranes made of cellulose ester (such as Spectra/Por® Float-A-Lyzer®G2) instead of regenerated cellulose (such as Sigma-Aldrich Pure-A-Lyzer™) leaded to the complete loss of the incorporate drug from MPEG-PCL based polymeric

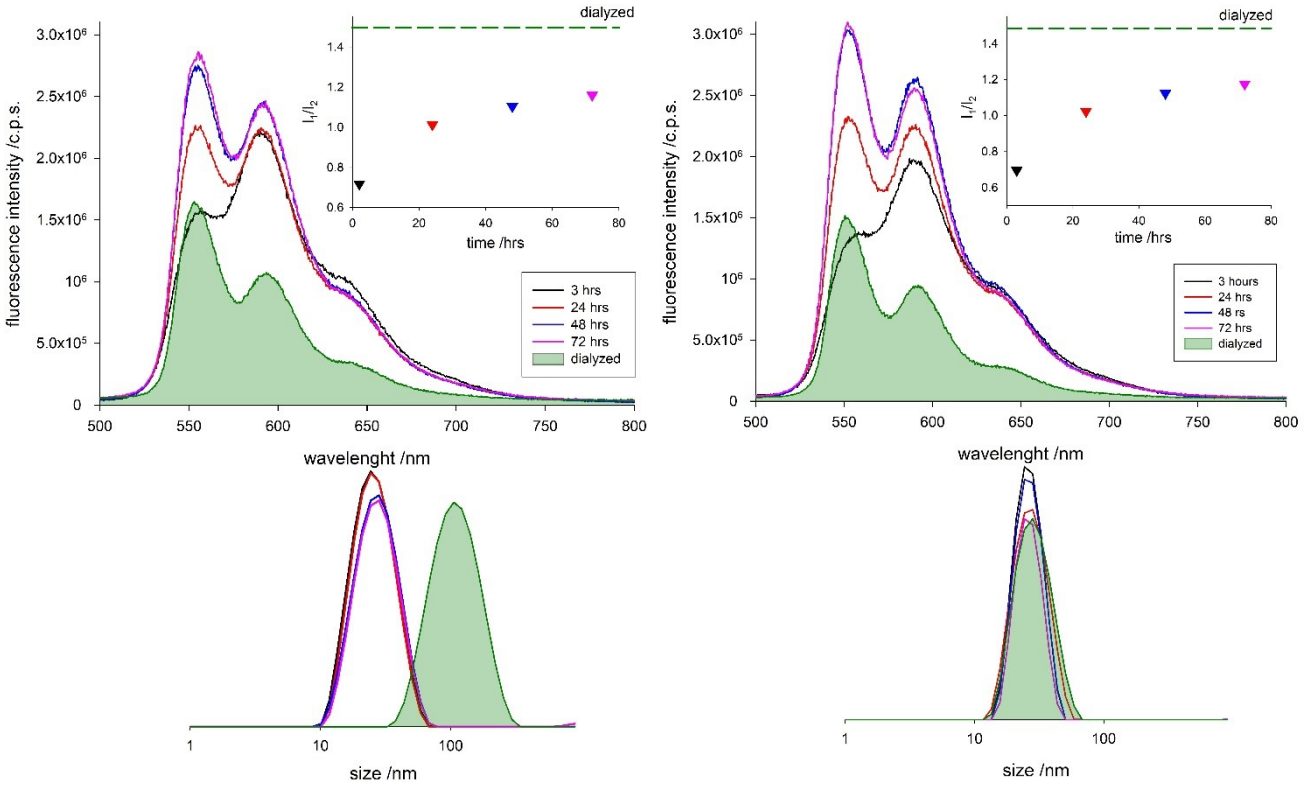

Fig. A6 Time evolution (see legends) of the fluorescence spectra of DX internalization in the presence of F127/P123 micelles (right panel) and MPEG-PCL micelles (left panel) prepared at pH= 8.10 and at a temperature of 45 °C and the corresponding DLS measurements (lower panels). The insets report the relevant time evolution of the I_1/I_2 ratio. Shaded curves refer to samples measured after dialysis. All the measurements were performed at T= 45 °C; cell pathlength= 0.3x0.3 cm.

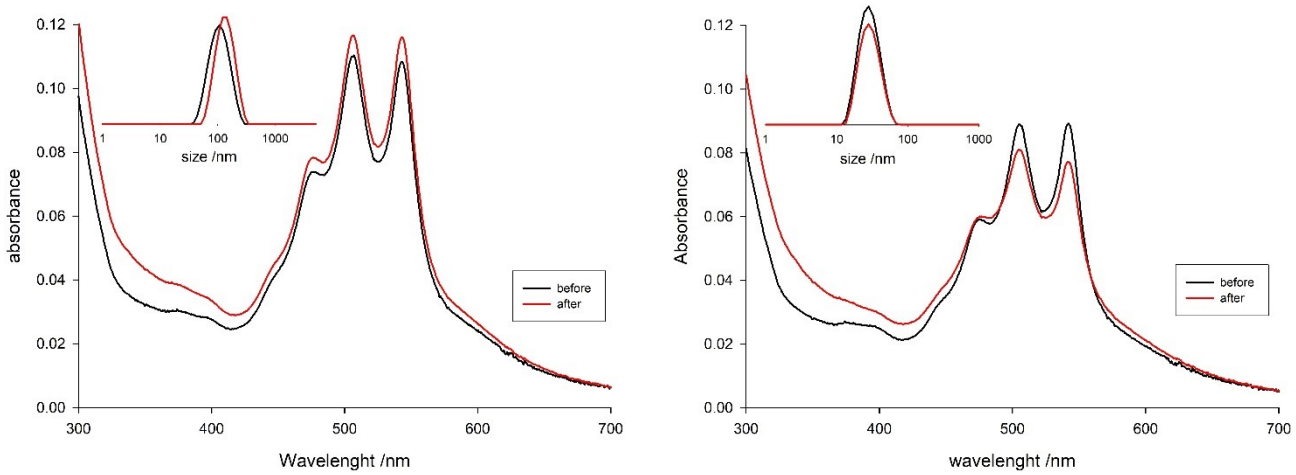

Fig. A7 Effect of freeze-drying on the drug amount loaded into F127/P123 micelles (right panel) and MPEG-PCL micelles (left panel) as detected by UV-Vis spectroscopy (the insets show the relevant DLS measurements). Freeze-dried samples were resuspended in an equal volume of water, prewarmed at 45 °C. T= 45 °C; cell pathlength= 0.3 cm.

micelles (data not shown). Such an effect was not detected for polymeric micelles prepared with pluronics.

Once dialyzed, the samples were freeze-dried and stored at -25 °C.

Fig. A7 shows the effects of freeze-drying in terms of both the amount of DX loaded (UV-Vis spectra - main graphs) and their size distributions (insets).

To carry out an additional check on the stability of the samples, the solutions prepared after freeze-drying were subjected to repeated freeze/thaw cycles (from T= -25 °C to T= 25 °C and then the samples brought to T=45 °C). The results of these tests are collected in Fig. A8.

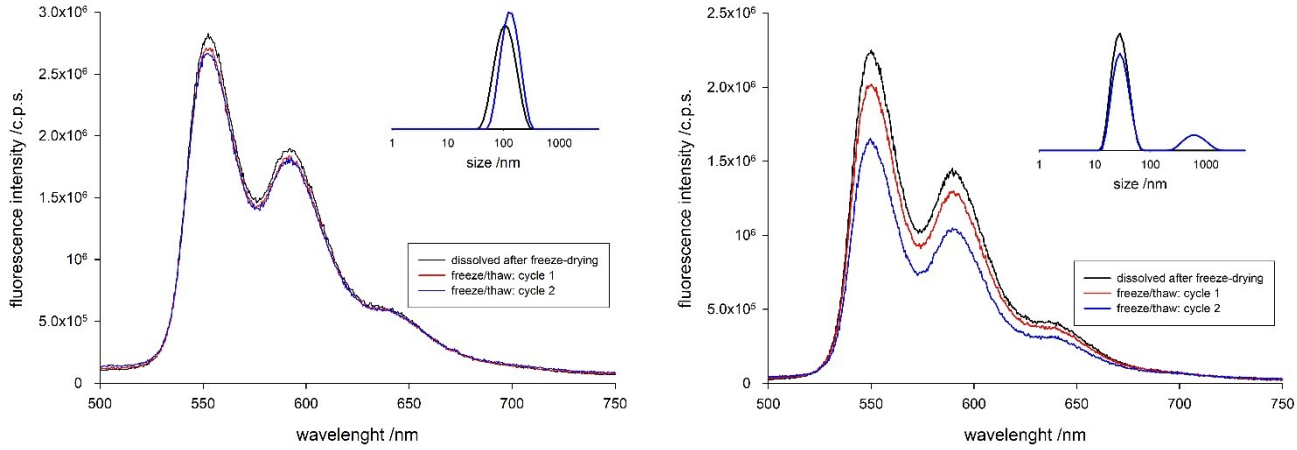

Fig. A8 Effect of freeze/thaw cycles on the fluorescence spectra of F127/P123 micelles (right panel) and MPEG-PCL micelles (left panel) according to the procedure described in the test (the insets show the relevant DLS measurements - same colours as in the main graphs). T= 45 °C; cell pathlength= 0.3 cm.

Loading efficiency

To evaluate the polymeric micelles capacity to host DX (in the form of DX-NH_2, as discussed in the main manuscript), it is mandatory to determine the amount of drug present in the inner, hydrophobic, compartment of the micelles. To do this, the absorption spectra shown in Fig. A3 relevant to the dialyzed samples have been used. In Fig. A8 the molar extinction coefficient of DX in water and of DX-NH_2 in *n*-hexane are shown. By following the procedure described in the main manuscript, the data reported in Table A1 were determined.

By defining the molar drug loading efficiency percentage (DL_M) as:

Table A1

λ /nm	ε /$M^{-1}cm^{-1}$	$[DX]_{PCL32}$ /M	$[DX]_{PLD165}$ /M
510.0	8913±70	$1.87 \cdot 10^{-4}$ ±0.02	$1.68 \cdot 10^{-4}$ ±0.01
524.5	6481±40	$1.91 \cdot 10^{-4}$ ±0.02	$1.78 \cdot 10^{-4}$ ±0.01
533.0	5852±25	$1.89 \cdot 10^{-4}$ ±0.02	$1.78 \cdot 10^{-4}$ ±0.01
<[DX]> /M		$1.89 \cdot 10^{-4}$ ±0.02	$1.75 \cdot 10^{-4}$ ±0.01

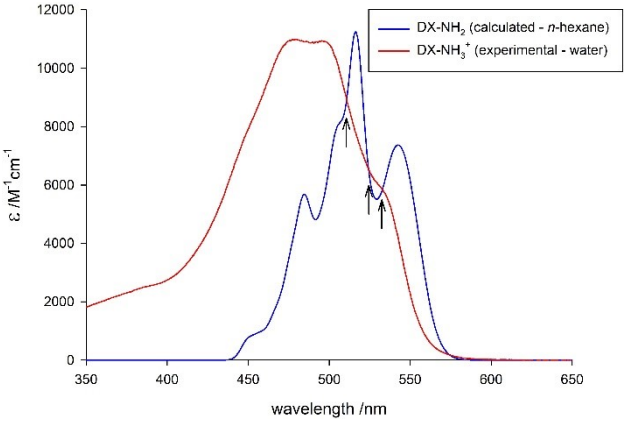

Fig. A9 Molar extinction coefficient of $\mathbf{DX-NH_3^+}$ (red) and of $\mathbf{DX-NH_2}$ (blue - from ref. 26 of the main paper) showing the crossing wavelengths (arrows) at 510.0, 524.5 and 533.0 nm (ϵ_{510}= 8913±70 M^{-1} cm^{-1}; $\epsilon_{524.5}$= 6481±40 M^{-1} cm^{-1} ; ϵ_{533}= 5852±25 M^{-1} cm^{-1}).
The $DX-NH_3^+$ molar extinction coefficient has been determined on a 9.9 µM DX solution in water at 25 °C with the same spectrophotometer used for the other measurements and with a 10 cm cell, obtaining values well in agreement with those reported in the literature.

$$DL_M = \frac{[DX]_{dialyzed}}{[DX]_{starting}} \cdot 100$$

and the mass drug loading efficiency percentage (ML) as:

$$ML = \frac{mass^{DX}_{dialyzed}}{mass^{DX}_{starting} + mass_{polymer(s)} + mass_{buffer}} \cdot 100$$

and from the data shown in Fig.s A4, A9 and A10, the DL_M and ML values reported in Table A2 have been obtained.

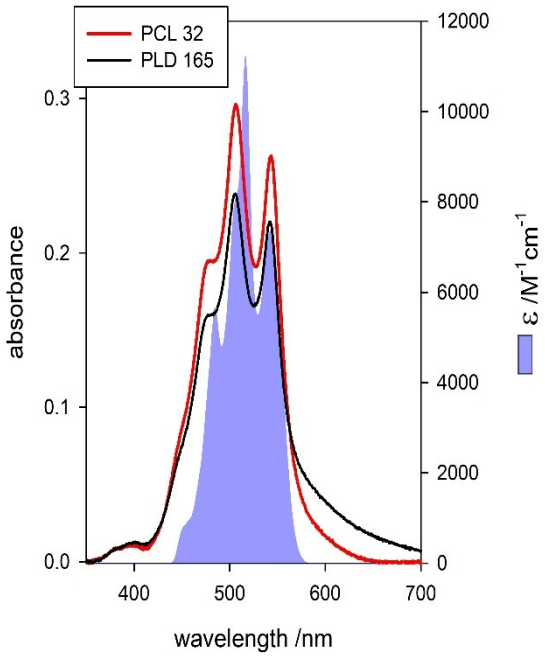

Fig. A10 Absorption spectra of dialyzed samples (see legend - left hand axis) in comparison with the DX-NH_2 spectrum (blue shaded - right hand axis; from ref. 26 of the main manuscript).

Table A2

sample	DL_M %	ML %
PCL32	61.6±0.9	0.40±0.5
PLD165	56.1±1.0	0.20±0.5

Both the DL_M and ML values obtained in this work are more than double compared to the values obtained in the already published work (Ref. 11 in the main manuscript) underlying that not only the drug incorporation kinetics is faster at pH= 8.1 than at pH= 7.7, but also that the amount of drug solubilized is enormously increased.

For the in-vitro tests described in the main manuscript, weighted amounts of the freeze-dried samples were diluted with the proper buffer/culture medium (prewarmed at 45 °C) and the amount of drug present determined by reading the samples absorbances at 541 nm and using the molar extinction coefficient taken from the literature (ref. 26 of the main manuscript).

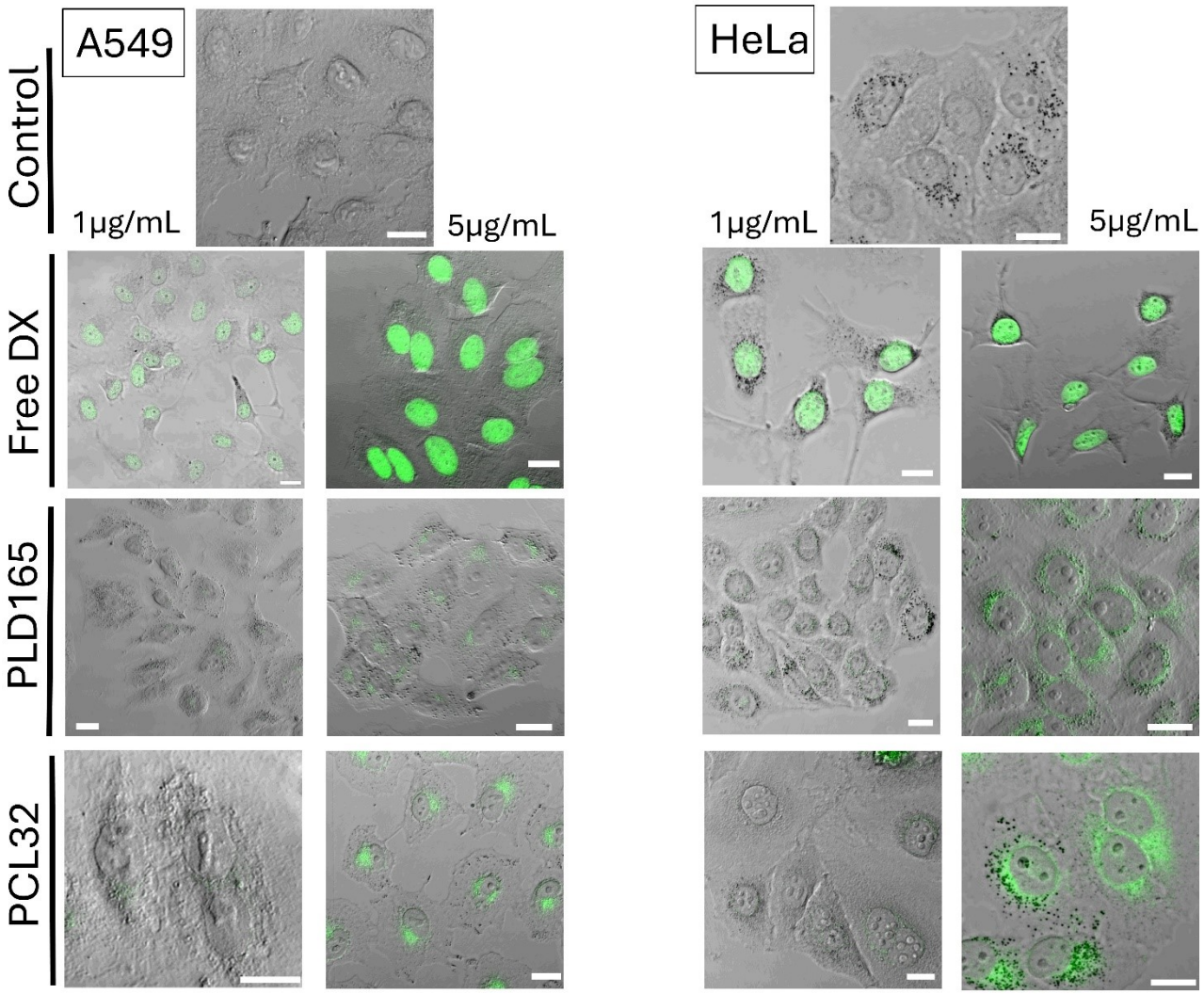

Fig. A 11 Merged transmission and fluorescence CLFM images. Comparison of intensity and distribution of DX for 1 µg/mL and 5 µg/mL drug concentration after 24hrs internalization, as free DX and encapsulated drug, PLD165 and PCL32, respectively. A549 (left) and HeLa (right) cell line; green colour represents DX emission intensity. Bar lengths adjusted to 20 µm.