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*Supporting Information*

# pH-responsive Lyotropic Liquid Crystals and their Potential Therapeutic Role in Cancer Treatment

Renata Negrini[a], Wye-Khay Fong[a,b], Ben J. Boyd[b] , Raffaele Mezzenga*[,a]

[a]*ETH Zürich, Department of Health Science & Technology, Food & Soft Materials Science, Schmelzbergstrasse 9, 8092 Zürich, Switzerland.*
[b]*Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University (Parkville Campus), 381 Royal Parade, Parkville, Victoria 3052, Australia*
**Corresponding author: email: raffaele.mezzenga@hest.ethz.ch*

## Materials and Methods

### Materials

Dimodan U/J (Danisco, Denmark, batch N° 015312) was used as received. This commercial-grade form of monolinolein (ML) contains more than 98 wt% monoglyceride. Glucose, Doxorubicin HCl, hydrochloric acid, ethanol, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), were purchased from Sigma Aldrich-Chemie (Schnelldorf, Germany). Milli-Q water was used for all the experiments. Pyridin-4-ylmethyl linoleate (PML) was synthetized as described previously.[1]

For the cell culture and DOX release studies, Dulbecco's modified Eagle's media (DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAA) and Penicillin-Streptomycin (5,000 U/mL) (pen/strep) were acquired from Gibco, Life Technologies, Switzerland. The pH of the media was modified using 0.1 M hydrochloric acid. The HT29 cell lines were a generous gift from Dr. Tomás de Wouters of the Food Biotechnology group at ETH Zürich.

### Preparation and loading of the lyotropic liquid-crystalline phases

In order to form the lipid phases with different proportions of pyridinylmethyl linoleate (PML) the MLO and the PML were weighed and dissolved in ethanol. The solvent was then removed under vacuum. The mesophases with and without drug were then formed as previously reported[2] in the appropriate buffer system. Samples were then equilibrated at 37 °C and protected from light for one week. At the end of the equilibration period, each LLC mesophase was sampled for the characterization of the mesophase by X-ray diffraction.

### Small Angle X-ray Scattering Measurements

SAXS measurements were used to identify the symmetry of the mesophases at the different conditions. Experiments were performed using a Rigaku MicroMax-002+ microfocused beam X-ray

source operating at 45 kV and 0.88 mA. The Ni-filtered Cu $K_\alpha$ radiation ($\lambda_{Cu\ K\alpha}$ = 1.5418 Å) was collimated by three pinhole collimators (0.4, 0.3 and 0.8 mm in diameter), and the data were collected by a two dimensional argon-filled Triton-200 X-ray detector (20 cm diameter, 200 µm resolution). An effective scattering-vector range of 0.03 $Å^{-1} < q < 0.45\ Å^{-1}$ was probed, where $q$ is the scattering wave-vector defined as $q = 4\pi \sin\theta/\lambda_{Cu\ K\alpha}$, with a scattering angle of $2\theta$. For all measurements, the samples with a sample thickness of ca. 1 mm were placed inside a Linkam HFS91 stage, between two thin mica sheets and sealed by an O-ring. Measurements were performed at 37 °C, and samples were equilibrated for 30 min prior to measurements, while the scattered intensity was collected over 30 min.

**Glucose Diffusion Study**

Glucose diffusion studies were performed in triplicate for the two pH values studied (4.5 and 7.4 respectively) using the diffusion technique developed in our previous work[2]. The concentration of glucose was determined by an optical rotatory dispersion device (ORDE-Y02/15) mounted on a CD spectrometer (Jasco J-815). The measurements were performed at room temperature, and the ORD signal was acquired for each sample three times at a fixed wavelength of 436 nm; a series of solutions of glucose at different pHs were prepared to construct a calibration curve, and the drug concentration was determined by linear interpolation within the linear range (data not shown).

In the first instance, concentration and pH dependent phase behavior of different amounts of PML in MLO was determined by means of SAXS measurements (see Fig. S1). Four different concentrations of PML (4.5, 4.7, 7 and 10 wt%) were tested at pH 4.5 and 7.4. These pHs were chosen as they are either side of the pKa of PML (≈ 5).

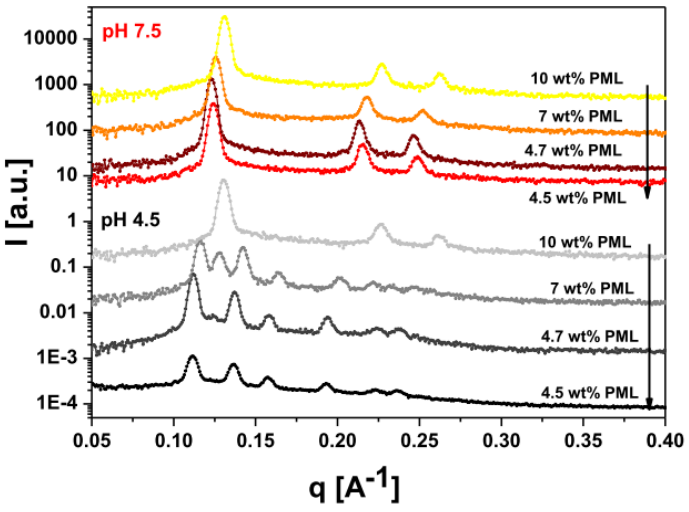


**Fig. S1** Phase behavior of the PML-MLO systems with increasing PML content at pH 4.5 and 7.4. At all PML concentrations, the matrix forms H2 at pH 7.4 whereas the protonation of the pyridine head group results in the formation of Pn3m at pH 4.5.

The system clearly undergoes phase changes in response to a change in pH (see Fig. S1). This is attributed to the pH behavior of the PML, which is neutral at pH 7 and positively charged at acidic pH, thus imposing a change in the critical packing parameter (CPP) of the molecule in the lyotropic liquid crystal matrix.

Fig. S2 shows the diffusion of glucose through the mesophases using our previously established method at the two different pHs considered. It is known that the release of hydrophilic molecules can be controlled, on-demand, by the structure of the mesophase.[2,3] Moreover, the ratio between the square of the diffusion slopes between the cubic and the hexagonal phase was calculated and the value of 16 was obtained, in agreement with previous studies.[2,3]

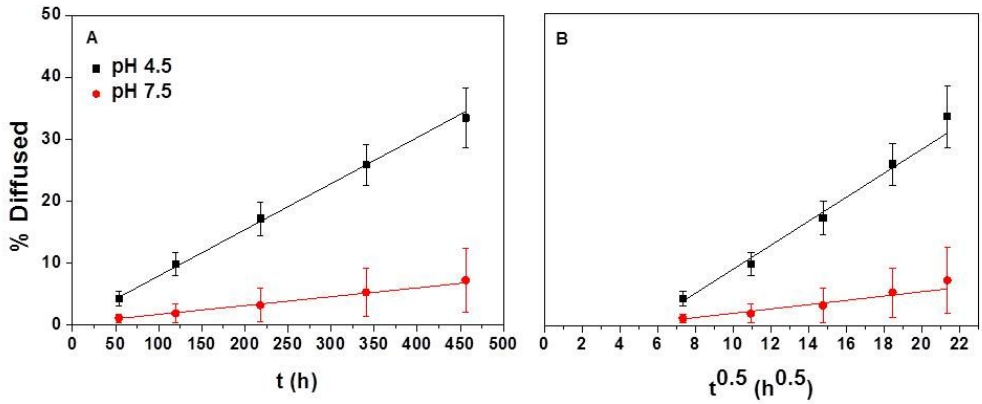


**Fig. S2** Glucose diffusion. pH-induced changes in the diffusion behavior of glucose through the system formed by monolinolein functionalized with 4.5 wt% at 37°C. Percentage of glucose diffused through the bicontinuous cubic phase at pH 4.5 (black) and reverse hexagonal phase at pH 7.4 (red) plotted against time (A) and square root of time (B).

**Doxorubicin Release Studies**

After equilibration, mesophase utilized in the DOX release and cell culture studies were transferred into custom made sample holders printed with a Makerbot Replicator 2 3D printer (Boston, MA, USA) and left to equilibrate overnight immersed in DOX solution in a 24 well plate (see Fig. S3). For the DOX release studies, the sample holder was then transferred into a new 24 well plate containing 1 mL of DMEM medium per well at pH 5.5 and 7.4, respectively. For the experiment at acidic conditions, the pH of the DMEM buffer was adjusted to pH 5.5 by addition of HCl (the buffer turned yellow). The pH was measured before and after the release studies and resulted to be constant, which was also confirmed by visual inspection by the same buffer colour. Sink conditions were maintained by

removing and replenishing the media (1 mL) in each well at each sample point. All DOX studies were conducted at 37 °C in a 10% $CO_2$ humidified atmosphere in order to mimic cell culture conditions.

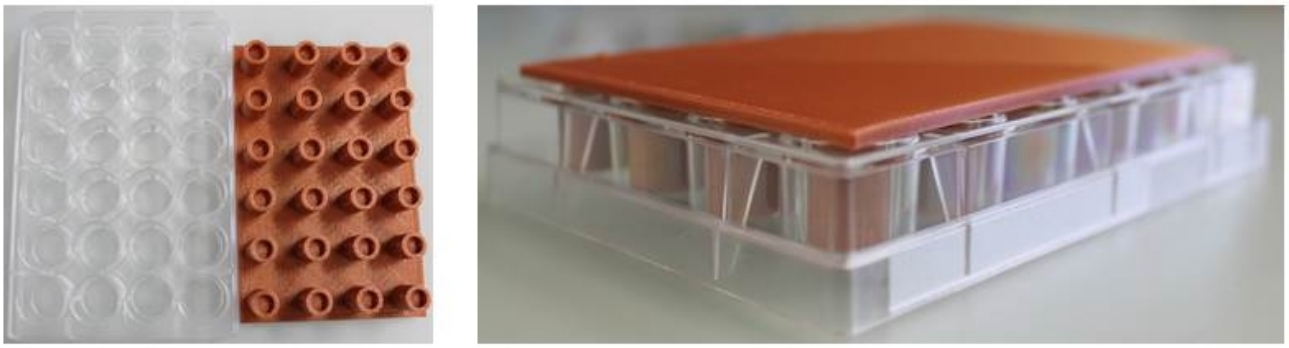

**Fig. S3** custom made sample holders and set up for DOX release and cell culture studies. Custom printed sample holder (Top) and 24 well plate. Mesophase was filled into each well before inverting into the 24 well plate filled with cell media as shown in the bottom panel.

**Doxorubicin Quantification**

The concentration of doxorubicin (DOX) was determined by fluorescence (ex 485/em 590 nm) measurements (FLUROstar Omega) at the two different pHs and 37 °C. A series of DOX solutions with a concentration between 0.4 and 4 μM at different pHs (7.4 and 5.5) were prepared to construct a calibration curve, and the drug concentration was confirmed by linear interpolation within the linear range (data not shown). Data were background subtracted for the cell media.

**Cell Studies**

A HT29 human colorectal adenocarcinoma monolayer was cultured in DMEM, supplemented with 1% v/v NEAA (nonessential amino acids, Gibco), 1% (v/v) Penicillin Streptomycin, and 10% (v/v) fetal bovine serum, (FBS, Gibco) at 37°C with 10% $CO_2$ to 80% confluence. Cells were harvested by trypsinization (2 mL) and then diluted to 10 mL media followed by centrifugation (105 RCF for 5 min). Processed cells were resuspended, counted and then seeded into each well at a density of $10^5$ cells/well in 0.95 mL of media. The plates were then incubated overnight at 37 °C with 10% $CO_2$. After 24 h incubation, non-adherent cells were removed by rinsing the samples in fresh media and replacing the volume with media at pH 7.4 or media with a modified pH of 5.5. The wells were subsequently dosed with mesophase as described for the release study. The Dox dosage was determined as follows: the IC50 of Dox was found to be 6 μmol and the release from the cubic and hexagonal PML-MLO-Dox mesophases were found to be 80% and 20% at 24 h respectively, thus cells were dosed with 14.4 μmol of Dox in order to optimize the difference between the amount release at pH 7.4 and pH 5.5. At 24 h

after dosing, the mesophase and sample holder were removed from the 24 well plate and wells were rinsed with warm PBS, pH 7.4, and replenished with fresh media. At 4 days after seeding, the cells were then prepared for either viability assays or for confocal fluorescence imaging.

**Lactate dehydrogenase assay (LDH)**

Cell viability was investigated utilizing a lactate dehydrogenase (LDH) assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, Switzerland). Briefly, LDH, a stable cytosolic enzyme that is released upon cell lysis, is quantitatively measured with a 30 min coupled enzymatic assay, which results in conversion of a tetrazolium salt into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Visible wavelength absorbance data are collected using a standard 96-well plate reader at 490 nm.

**Confocal fluorescence imaging**

For imaging, cells were stained with calcein AM (Invitrogen, Switzerland) and viewed with a Leica TCS SPE spectral confocal microscope (see Fig. S4).

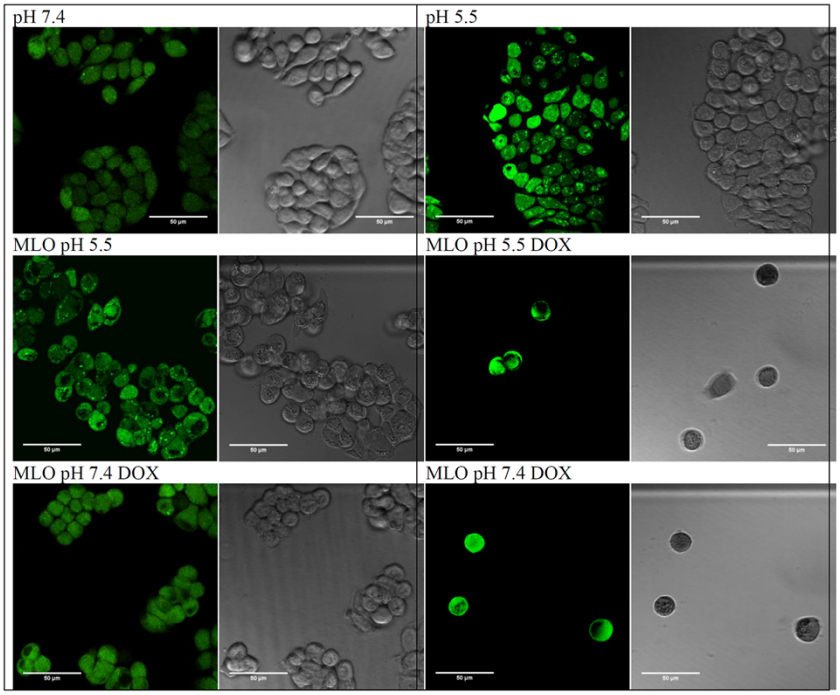


**Fig. S4** Representative fluorescent and greyscale images demonstrating cell viability in the presence of the lipid formulations at the two different pHs, 7.4 and 5.5, with and without doxorubicin (DOX). Scale bars represent 50 µm.

Fluorescent and greyscale images of HT29s exposed to the PML-MLO formulation with and without DOX are shown (see Fig. S5), where cells were stained with calcein fluoresce green to indicate their viability.

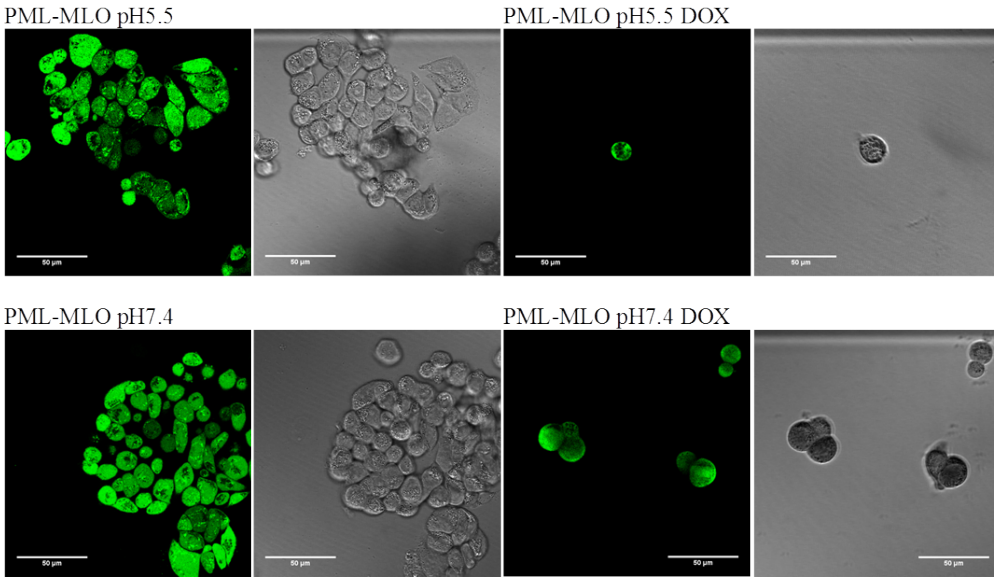


**Fig S5** Representative fluorescent and greyscale images demonstrating cell viability in the presence of the lipid formulation PML-MLO with and without doxorubicin (DOX). Scale bars represent 50 µm.

In agreement with the LDH assay, cells remain viable in the presence of the lipid formulation, but do not in the presence of DOX, where more cells survive at pH 7.4 when the mesophase is H2 than at pH 5.5 when the mesophase has the Pn3m symmetry. The appearance of the cells exposed to the lipid formulation look similar to that of the unexposed cells. In comparison, cells exposed to DOX appear circular and darker but are still intact as demonstrated by imaging with trypan blue (data not shown). The mechanism of action of DOX is twofold; it intercalates with DNA, preventing DNA repair as well as generates free radicals, which results in the irreversible damage to cell membranes.[4] It is concluded that the cellular morphology of HT29s is irreversibly and lethally changed by DOX resulting in their altered appearance. This occurs to different extents in the sample according to the concentration of DOX released from the PML-MLO mesophase at different pH.

**SAXS on the mesophase after release**

Figure S6 shows that the hexagonal to cubic phase transition is not only valid for the binary system PML-MLO, but also occurs when Dox loads the system. The only effect Dox has at pH 5.5 is to maintain a residual coexistence of the hexagonal phase together with the cubic, due to the hydrophobic properties of Doxorubicin. Yet, since the cubic phase is dominant as can be seen from the SAXS, the

order-order transition is unambiguously demonstrated and the release behavior and thus 'killing power' of the system at pH 5.5 becomes three times faster than that at pH 7.4.

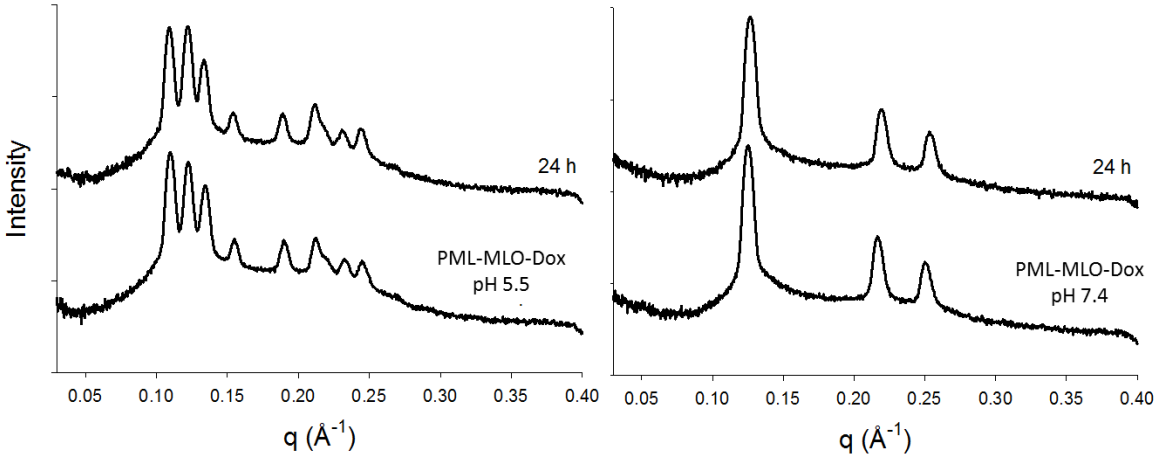


**Figure S6** SAXS profiles of PML/MLO/Dox mesophases before and after HT29 cell culture studies at pH 5.5 (left panels) and pH 7.4 (right panels).

**IC50 for Dox dosage**

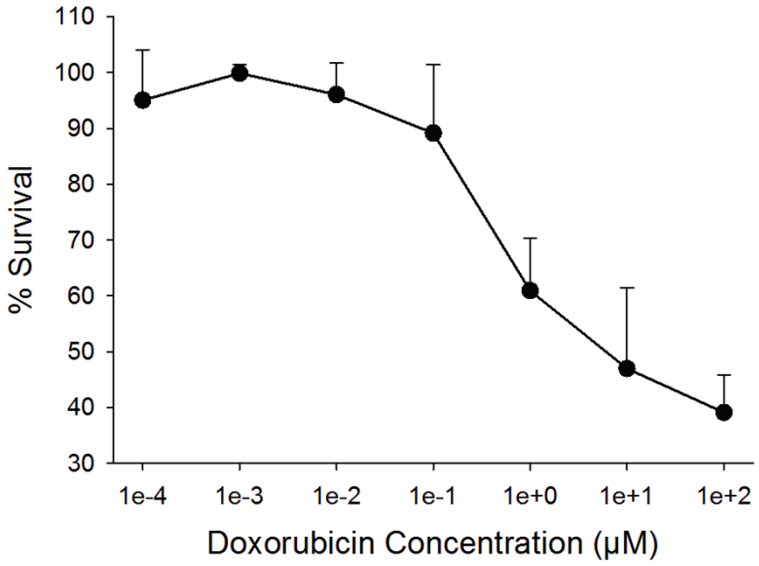


**Figure S7** Determination of optimized DOX dosage for the cell studies.

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