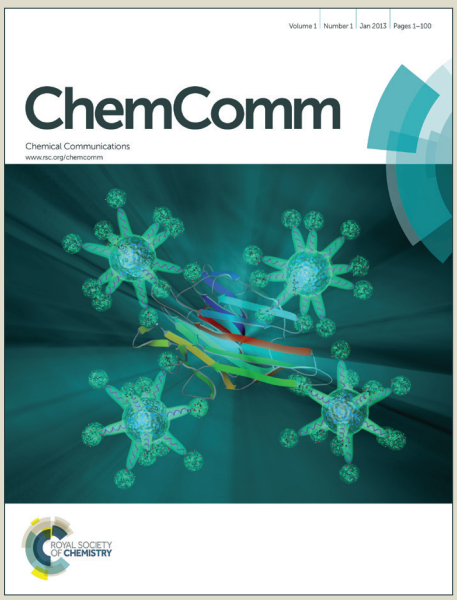
ChemComm

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

Publishing

COMMUNICATION

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

Cite this: DOI: 10.1039/xoxxoooooox

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

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

A weak amphiphilic base, pyridinylmethyl linoleate, is blended to monolinolein, yielding mesophases with a pH-induced hexagonal-to-cubic transition at pH<5.5. We show the potential therapeutic role of this mesophase in treating cancerous tissues exploiting their more acidic pH compared healthy tissues. In-vitro release studies with doxorubicin on HT29 human colon cancer cells show 10-fold faster release and 3-fold increased efficiency in killing cancer cells at pH 5.5 versus pH 7.4, demonstrating the potential of this strategy in cancer treatment.

There has been a flurry of activity towards the development of novel, responsive drug delivery systems for cancer therapy in order to maximize efficacy, whilst reducing toxicity typical of the conventional chemotherapy.[1] As such, targeted and stimuli responsive nanomaterials have emerged as programmable delivery systems for anticancer drugs, in order to free them from their cytotoxic limitations. Targeted materials consist mostly of nano-carriers with ligands bound onto their surface, which are able to induce transport into cancerous cells due to their affinity to specific markers in tumor tissues.[2] On the other hand, stimuli responsive materials can be classified as "externally-activated" or "passive self-triggered" targeting systems.[3] The first class of materials consists of responsive materials activated by external factors such as ultrasound, light, magnetic field or temperature.[4-10] The second class exploits the atypical conditions of disease states like cancer, where factors such as the Enhanced Permeability and Retention effect (EPR),[11] increased temperature[12,13] and acidic local extracellular pH[14-17] in the tumor environment, can be exploited to trigger the release of drugs. Among all these factors, the average lower pH of tumor tissues compared with normal tissues, represents an ideal target for the selective release of anticancer drugs. The pH difference is described by the Warburg phenomenon,[18] which attributes the pH difference to the insufficient vasculature to remove the acidic byproducts of increased anaerobic cellular activity of the rapidly proliferating tumor cells, resulting in the development of an acidic micro-environment. As a result, tumors have a lower extracellular pH than contiguous tissues. Although there is no consensus on the exact pH drop, the extracellular matrix around cancer cells is reported to be in the pH range of 5.5-6.5.[14,15] Until now, the development of pH-responsive materials has largely focused upon polymers and liposomes.[5-7] These materials have shown promise in improving the pharmaceutical efficacy of clinically approved anti-cancer drugs, however, this has been translated into few clinically approved therapies since the first FDA-approved nano-drug, Doxil, in 1995.[19] Therefore, research and development of new drug delivery materials is crucial for the improvement of effective therapies.

Lyotropic liquid crystals (LLC) have attracted great attention in the field of drug delivery, in particular the bicontinuous cubic and reverse hexagonal mesophases.[20] These systems consist of lipidic amphiphilic molecules which self-assemble in aqueous environments to form complex three dimensional structures and which are able to control the release of drugs of varying properties and sizes.[21-27] Their geometry, symmetry and dimension of resulting water nanochannels, primarily determine the rate of drug release,[21-23] and can be modified by the control of lipid self-assembly. This can be achieved through the manipulation of lipid composition, temperature and ionic strength amongst other factors. LLCs also lend themselves to drug delivery as they can be dispersed into colloidal particles which significantly reduces the viscosity of the matrix whilst maintaining the internal nanostructure of the bulk system; colloidal dispersions of the inverse bicontinuous cubic phases of double diamond Pn3m or primitive Im3m symmetries, or hexagonal H_2, named cubosomes or hexosomes respectively,[24,25] are at thermodynamic equilibrium with the surrounding buffer, offering appealing delivery systems.

In this work, we introduce a new model pH-responsive LLC which shows a conceptual promise as a drug delivery system for cancer treatment. The responsiveness was achieved by the addition of a newly in-house synthetized weak base, pyridin-4-ylmethyl linoleate (PML), to the neutral lipid monolinolein (MLO) (see Fig. 1).

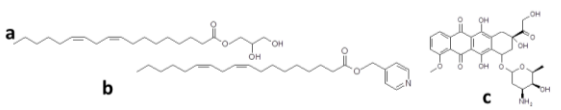


Fig. 1 Molecular structures a) monolinolein (MLO), b) pyridin-4-ylmethyl linoleate (PML) and c) doxorubicin (DOX).

ChemComm Accepted Manuscript

The amphiphilic PML was synthetized expressly to anchor into the mesophase bilayer, offering the possibility of manipulating the structure upon protonation of its head group in response to a change in pH.[26] The phase behavior of PML in MLO is the reverse of our previously reported work on pH responsive LLC, where the responsiveness was achieved through the addition of linoleic acid, a weak acid, to monolinolein.[28] The protonation of the weak acid resulted in the neutralization of the charge of the carboxylic acid, inducing a reduction in hydrophilicity of the linoleic acid at acidic pH and thus decreasing the head group size. The opposite occurs with the weak base employed in this study whereby the protonation of PML results in the expansion of the effective head group size due to the attained positive charge at acidic pH, leading to expected order-order transitions upon protonation of PML.

To efficiently design the system, different concentrations of PML in MLO were investigated at different pH (from 4 to 7.4) and the pH dependent phase behavior determined by means of small angle X-ray scattering (SAXS) measurements. The system clearly undergoes phase changes in response to changes in pH (data not shown). This is indeed attributed to the pH behavior of the PML, which is neutral at pH 7 and positively charged at acidic pH ($\leq$ 5.5). The change in charge upon a change in pH imposes an increase in the critical packing parameter (CPP) of the molecule in the lyotropic liquid crystal matrix, and consequently a change in nanostructure. Thus, the concentration of PML can be exquisitely tuned to switch from bicontinuous cubic phase (Pn3m) at acidic pH and a reversed hexagonal columnar phase (H_2) at neutral pH. By exploiting the different intrinsic transport rates of Pn3m and H_2,[28,29] preliminary diffusion studies at two different pHs simulating cancer and physiological conditions (i.e. 4.5 and 7.4) were conducted by employing glucose as a model drug (see SI). The results showed that the drug diffuses significantly faster in the Pn3m phase at acidic conditions, confirming the opportunity to employ this scheme as controlled drug delivery system for cancer therapy. Towards the creation of an ideal pH-responsive drug delivery material for tumor treatments, the optimization of mesophase behavior was investigated in the cell culture media (DMEM) at different pHs (i.e. 5.5, 6.5 and 7.4) and the final designed composition, namely 3.2% (w/w) PML in MLO (PML-MLO), was selected to mimic the lowest pH (pH 5.5) cancer conditions (see Fig. 2).

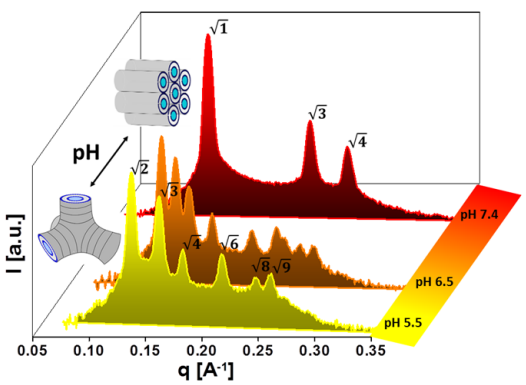


Fig. 2 Phase behaviour of MLO doped by 3.2% (w/w) PML in DMEM (PML-MLO system): pH 5.5 (yellow), 6.5 (orange) and 7.4 (red) at 37°C. The schematic on the left indicates the phase formed with a change in pH, namely Pn3m at pH 5.5 and H_2 at pH 7.4.

SAXS curves reveal the presence of a reverse hexagonal phase H_2 (with reflections spaced at √1, √3 and √4) at pH 7.4 and a bicontinuous cubic phase of Pn3m space group (with reflections spaced at √2, √3, √4, √6, √8 and √9) at pH 5.5. This leads to an ideal order-to-order transition between pH 5.5 and 7.4, although the system can be readily fine-tuned for slightly different pH conditions.

As the system showed the ability to undergo the desired structural changes within a narrow pH range, it was further investigated for controlled release of the anti-cancer drug, doxorubicin (DOX) (see Fig. 1). Release studies were conducted using DOX at two different pHs: 7.4 to simulate physiological conditions, and 5.5 to model the conditions within a tumor. Consistently with expectation based on structural analysis, the release of DOX is faster at pH 5.5 when 80% of the payload is released after 24 h. In contrast, H_2 at pH 7.4 releases only 20% of the initial drug concentration after 24 h (see Fig. 3).

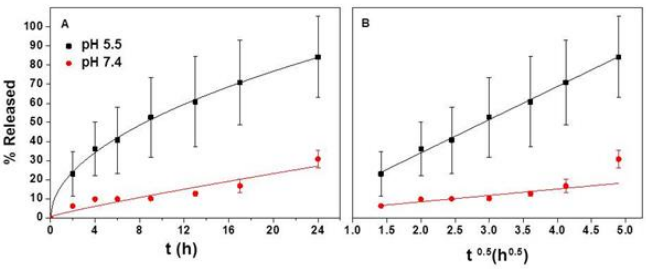


Fig. 3 Controlled release of DOX from PML-MLO mesophases. Doxorubicin released from the bicontinuous cubic phase at pH 5.5 (black) and reverse hexagonal phase at pH 7.5 (red) plotted against time (left) and square root of time (right).

This change in release kinetics is reflected by the diffusion coefficients of the mesophase at the two different pHs: using the Higuchi equation,[30] these were calculated to be $D_{pH7.4}$= 1.28 × 10^{-08} cm^2 s^{-1} and $D_{pH5.5}$= 5.78 × 10^{-07} cm^2 s^{-1}, respectively. The release of the anticancer drug from the Pn3m symmetry at acidic pH is more than one order of magnitude faster than that of the H_2 at physiological conditions. From these results, it can be observed that the release of doxorubicin is accelerated in simulated tumor environment, whilst only minimal release occurs at normal physiological conditions, which has the potential to minimize cytotoxicity in healthy cells. Although some pH-controlled release between guest drugs and amphiphilic molecules doping the host cubic mesophases can be achieved by exploiting electrostatic interactions,[26] as proposed in a recent work for DOX released from Pn3m mesophases,[31] it is only through a symmetry switch of the mesophase upon pH changes that a many-fold increase in transport and release properties can be achieved.[28]

In order to determine how this efficient control in release of DOX may translate into cancer therapy, the study was then extended to in vitro cell culture studies using HT29 human colon cancer cells. Cell survival was quantified in in-vitro drug release studies conducted at pH 7.4 and 5.5 from a bulk matrix using DOX and cell viability assessed utilizing a lactate dehydrogenase (LDH) assay. As bulk mesophases (and not dispersions) were used in excess buffer conditions, this strategy may be particularly suitable for subcutaneous drug release. The HT29 cells appear to be impervious to both the drop in pH for 24 h as well as the presence of the lipid formulations (see Fig. 4A).

ChemComm Accepted Manuscript

This journal is © The Royal Society of Chemistry 2012

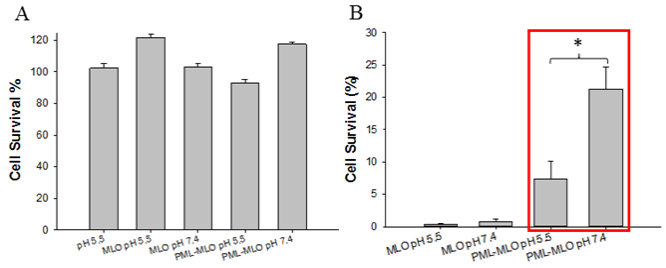


Fig. 4 Panel A: Survival of cells exposed to low pH and/or mesophase without DOX as a percentage of HT29 growth at normal conditions (pH 7.4 in the absence of mesophase). Cell proliferation was >97% of growth at normal conditions. Panel B: Survival of cells exposed to DOX-loaded mesophases as a percentage of HT29 growth in the presence of the mesophase at the same pH.

Importantly, the survival of the cells in the presence of the mesophases shows their cytocompatibility. HT29 survival has been attributed to their ability to secrete mucus, which affords them some protection from the change in cellular growth conditions. Previously reported studies have shown that exposure of HT29 cells to pH down to pH 5 results in the arrest of cellular proliferation; however they remain viable to exposures up to 24 h at low pH.[32-36]

In agreement with the LDH assay, cells remain viable in the presence of the lipid formulation, but do not in the presence of DOX when the pure MLO system is employed. Furthermore, and more importantly, when the PML-MLO pH-responsive system is used, more cells survive at pH 7.4 when the mesophase is H_2 than at pH 5.5 when the mesophase has the Pn3m symmetry, due to the pH-induced symmetry switch. Remarkably, a factor 3 is found between the survival rate of the cells at pH 7.4 and pH 5.5, respectively (highlighted by the red square in Fig. 4). Confocal measurements on cells viability at these two pH in presence of DOX further confirm these findings (see ESI, Fig. S5).

In summary this study demonstrates, that lipid-based lyotropic liquid crystalline systems (LLCs) can be engineered to change their symmetry in response to a change in pH of the extracellular media from 7.4 to 5.5, enabling a pH-controlled release behavior of anticancer drugs. The developed system consists of a blend of monolinolein and pyridinylmethyl linoleate (PML), an amphiphile containing a weakly basic head group able to protonate in response to a change in pH in proximity to its pKa (≈ 5.5). The addition of PML to monolinolein results in LLC mesophases capable of switching the reverse hexagonal phase symmetry (H_2) at physiological pH, characterized by slow transport properties, to a bicontinuous cubic phase (Pn3m) at acidic pH (5.5). From a conceptual standpoint these ex-novo designed pH responsive mesophases can serve as tumor targeting delivery systems, by exploiting the most acidic conditions encountered in tumor tissues, and leading to boosted released of model drugs upon pH decrease. This was exemplified on in-vitro designed experiments. In vitro release studies on cancer cell cultures carried out using the anticancer drug doxorubicin, resulted in a 3-fold increase in the efficiency by which cancer cells are killed at pH 5.5 versus 7.4, as a result of the increased release rate at cancerous conditions, indicating a strong potential of this new route in modern cancer chemotherapies.

Notes and references

[a]*ETH Zürich, Department of Health Sciences & Technology, 8092 Zürich, Switzerland; e-mail:raffaele.mezzenga@hest.ethz.ch;* [b]*Drug Delivery, Disposition & Dynamics, Monash Institute of Pharmaceutical Sciences, Monash Univ., 381 Royal Parade, Parkville, Victoria 3052, Australia*

† Electronic Supplementary Information (ESI) available: experimental details and additional figures. See DOI: 10.1039/c000000x/

‡ Negrini R. and Fong W.K. contributed equally to this study.

1. T. M. Allen and P. R.Cullis, *Science*, 2004, **303,** 1818.
2. Y. H. Bae and K. Park, *J Control Release*, 2011, **153,** 198.
3. M. Yokoyama and T. Okano, *Adv Drug Deliver* Rev, 1996, **21,** 77.
4. M. J. Sailor and J. H. Park, *Adv Mater*, 2012, **24,** 3779.
5. W. Chen, F. Meng, R. Cheng and Z. Zhong, *J Contr Release*, 2009, **142,** 40.
6. Y. Malam, M. Loizidou and A. M. Seifalian, *Trends Pharmacol Sci*, 2009, **30,** 592.
7. J. B. Wolinsky, Y. L. Colson and M. W. Grinstaff, *J Contr Release*, 2012, **159,** 14.
8. C. L. Bayer and N. A. Peppas, *J Contr Release*, 2008, **132,** 216.
9. Z. G. Gao, H. D. Fain and N. Rapoport, *J Control Release*, 2005, **102,** 203.
10. S. S. Agasti, A. Chompoosor, C.-C. You, P. Ghosh, C. K. Kim and V. M. Rotello, *Journal of the American Chemical Society*, 2009, **131,** 5728.
11. H. Maeda, J. Wu, T. Sawa, Y. Matsumura and K. Hori, *J Control Release*, 2000, **65,** 271.
12 S. Cammas, K. Suzuki, C. Sone, Y. Sakurai, K. Kataoka and T. Okano, *J Control Release*, 1997, **48,** 157.
13. O. Soga, C. F. van Nostrum, M. Fens, C. J. F. Rijcken, R. M. Schiffelers, G. Storm and W. E. Hennink, *J Control Release*, 2005, **103,** 341.
14. I. F. Tannock and D. Rotin, *Cancer Res*, 1989, **49,** 4373.
15. M. Hruby, C. Konak and K. Ulbrich, *J Control Release*, 2005, **103,** 137.
16. W. W. Gao, J. M. Chan and O. C. Farokhzad, *Mol Pharmaceut*, 2010, **7,** 1913.
17. J. Connor, M. B. Yatvin, L. Huang, *P Natl Acad Sci-Biol*, 1984, **81,** 1715.
18. O. Warburg, *Science*, 1956, **123,** 309.
19. Y. Barenholz, *J Control Release*, 2012, **160,** 117.
20. C. Guo1, J. Wang, F. Cao, R. J. Lee, G. Zhai, *Drug Discovery Today*, 2010, **15**, 1032.
21. J. Clogston and M. Caffrey, *J Contr Release*, 2005, **107,** 97.
22. R. Negrini and R. Mezzenga, *Langmuir*, 2012, **28,** 16455.
23. B. J. Boyd and W.-K. Fong, Stimuli-Responsive Lipid-Based Self-Assembled Systems. In *Self-Assembled Supramolecular Architectures*, John Wiley & Sons, Inc.: 2012; pp. 257-288.
24. X. J. Gong, M. J. Moghaddam, S. M. Sagnella, C. E. Conn, S. J. Danon, L. J. Waddington and C. J. Drummond, *Acs Appl Mater Inter*, 2011, **3,** 1552.
25. X. Mulet, B. J. Boyd and C. J. Drummond, *J of Colloid and Interface Science*, 2013, **393,** 1.
26. R. Negrini, A. Sanchez-Ferrer and R. Mezzenga, *Langmuir*, 2014, **30,** 4280.
27. B. Angelov, A. Angelova, S. Filippov, M. Drechsler, P. Stepanek, S. Lesieur, ACS Nano, 2014, **8**, 5216.
28. R. Negrini and R. Mezzenga, *Langmuir*, 2011, **27,** 5296.
29. W.-K. Fong, T. Hanley and B. J. Boyd, *J Contr Release*, 2009, **135,** 218.
30. W. I. Higuchi, *J Pharm Sci*, 1967, **56,** 315.

ChemComm Accepted Manuscript

31. E. Nazaruk, M. Szlęzak, E. Gorecka, R. Bilewicz, Y.M. Osornio, P. Uebelhart and E.M. Landau, *Langmuir*, 2014, **30,** 1383.
32. N. Benning, J. Leipziger, R. Greger and R. Nitschke, *Pflug Arch Eur J Phy*, 1996, **432,** 126.
33. R. Nitschke, N. Benning, S. Ricken, J. Leipziger, K. G. Fischer and R. Greger, *Pflug Arch Eur J Phy*, 1997, **434,** 466.
34. R. C. Fitzgerald, M. B. Omary and G. Triadafilopoulos, *J Cell Sci*, 1997, **110,** 663.
35. N. Ben-Dov and R. Korenstein, *Plos One*, 2012, **7,** e35204.
36. D. Maouyo, S. Chu and M. H. Montrose, *Am J Physiol-Cell Ph*, 2000, **278,** C973.

ChemComm Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2012