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Phospholipid-based self-assembled mesophase systems for light-activated drug delivery

Joanne D. Du[1], Wye-Khay Fong[1,2], Stefan Salentinig[1], Suzanne Caliph[1], Adrian Hawley[3] and Ben J. Boyd[1,4]

[1]*Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University (Parkville Campus), 381 Royal Parade, Parkville, VIC, 3052, Australia*

[2]*Food and Soft Materials Science, Institute of Food, Nutrition & Health, ETH Zurich, Schmelzbergstrasse 9, 8092 Zürich, Switzerland*

[3]*SAXS/WAXS beamline, Australian Synchrotron, 800 Blackburn Rd, Clayton, VIC 3168, Australia*

[4]*ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash Institute of Pharmaceutical Sciences, Monash University (Parkville Campus), 381 Royal Parade, Parkville, VIC 3052, Australia*

* Corresponding author details:

Postal Address: Monash Institute of Pharmaceutical Sciences, Monash University (Parkville Campus), 381 Royal Parade, Parkville, VIC 3052, Australia

Telephone: +61 3 99039112; Fax: +61 3 99039583; Email: ben.boyd@monash.edu

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Abstract

The manipulation of the structure of phospholipid-based mesophases to induce a slow to fast drug release profile has potential for use in therapeutic situations where continuous absorption of drug is not desirable and reduce the frequency of injection for short acting or rapidly cleared drugs in treatments for diseases such as macular degeneration. This study had two aims; firstly to confirm the phase behaviour of 20 mol% cholesterol in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), which was previously reported to transition from lamellar (slow release) to bicontinuous cubic (fast release) phase with increasing temperature. Contrary to literature, no bicontinuous cubic phase was observed but a transition to the inverse hexagonal phase occurred at all POPE:cholesterol ratios investigated. The second aim was to render these mesophases responsive to near-infrared laser (NIR) irradiation by incorporation of gold nanorods (GNR) incorporated into the POPE system to induce photothermal heating. The inclusion of 3 nM GNR in POPE systems induced reversible disruption of lipid packing equivalent to increasing the temperature to 55°C when irradiated for 30 s. This study confirmed that although the previously published phase behavior was not correct, GNR and NIR can be used to manipulate the self-assembled mesophases in phospholipid-based systems and highlights the potential for a phospholipid-based light-activated drug delivery system.

1. Introduction

Many amphiphilic lipids such as phospholipids and monoglycerides spontaneously self-assemble to form ordered thermodynamically stable liquid crystalline (LC) mesophases in excess water. Lipid-based LC systems have been gaining interest in the field of drug delivery, the most common being the lamellar (L_α) phase which forms nanoparticles, termed liposomes, when dispersed. Liposomes composed from phospholipids are suitable drug delivery vehicles as they are biocompatible, process low toxicity and can encapsulate both hydrophilic and lipophilic drugs.[1] However, their rapid clearance from circulation by mononuclear phagocyte system (MPS) limits their utility *in vivo*. Polyethylene glycol (PEG) grafted lipids are often used to provide a non-fouling coating to the nanoparticle to inhibit protein binding and reduce non-specific removal from the circulatory system by decreasing uptake by MPS.[2] The consequent long circulation behaviour can assist particle uptake through the leaky vasculature in tumours, termed the enhanced permeation and retention (EPR) effect. The nanoparticles can readily permeate and be retained within the tumour site due to the poorly formed leaky vasculature with gaps up to several hundreds of nanometres in size and compromised or absent lymphatic drainage.[3] There is increasing evidence that the EPR effect may be relevant not only in tumour tissues but also in inflammation.[3, 4] Hence, having a stimuli-responsive element included in stealth particles to activate release at a specific location may provide improved selectivity and reduce toxicity. In order for this LC system to be more responsive, it is critical to actively control the rate of drug release, which may be achieved through the manipulation of self-assembled nanostructures.

Previous studies have shown that the nanostructure of the mesophase in self-assembled lipid systems is important in determining the drug release rate due to the state of the aqueous channels being either open or closed.[5] The inverse hexagonal (H_2) phase displays a slower release than the bicontinuous cubic (V_2) phase due to the smaller water channels and closed rod-like micellar structure.[5, 6] Likewise, the closed nature of the L_α phase when it is dispersed as liposomes, enables the encapsulation of drug, and is expected to provide slow or no drug release in comparison to the V_2 phases. Thus, it is hypothesised that through the manipulation of the self-assembled nanostructure from L_α to V_2 phase, drug release can be triggered to switch "on" and "off", and may have potential in providing external control over drug delivery. Consequently, systems that can be triggered to exhibit this phase behaviour may be particularly useful for on-demand drug delivery.

Temperature has been used as a stimulus to induce transitions between LC structures and has provided an avenue to manipulate drug release.[7] However, there is a major limitation to using temperature directly as the stimulus, as there is no specificity in the heat source which leads to a high potential for accidental activation of drug release. Previous studies have shown that incorporation of gold nanorods (GNR) into the lipid matrix can provide remote heating through the photothermal effect and trigger the phase transitions upon near-infrared (NIR) irradiation.[8] NIR radiation has been reported to penetrate tissues up to 50 mm deep,[9] suggesting a light-sensitive LC system has potential for various applications such as ophthalmic, subcutaneous and deeper tissue applications. Hence the broad aim of this study was to develop LC systems which will undergo the lamellar to cubic phase transition when stimulated with a NIR laser (Figure 1).

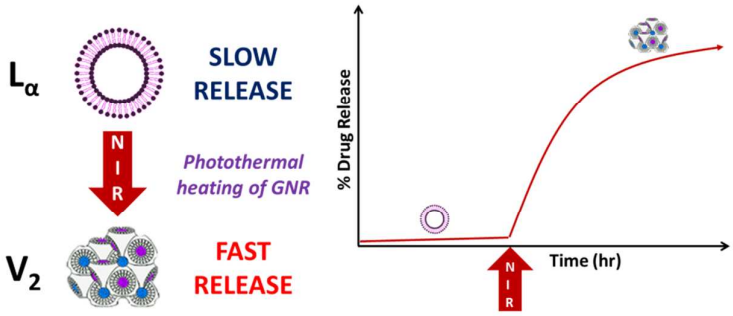


Figure 1 Schematic of desired liquid crystalline system with a phase transition from lamellar phase (L_α) to bicontinuous cubic phase (V_2) and its corresponding drug release profile controlled using NIR laser and GNR.

In previous studies, GNR were embedded in phytantriol cubic phase and irradiated with NIR however, the reversible transition from V_2 to H_2 obtained provides a fast to slow drug release profile, which is not useful for stimulated drug delivery purposes.[8] Therefore, it is necessary to explore other lipid systems. It was reported that the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) + cholesterol system in water transitions from L_α to either H_2 or V_2 phase with increasing temperature depending on cholesterol content (Figure 2).[10] Specifically, as reported in the literature phase diagram in Figure 2, a system containing POPE with 20 mol% of cholesterol was expected to exhibit a L_α to the diamond V_2 phase (Pn3m with the ratio of reciprocal d-spacing of $\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{6}:\sqrt{8}:\sqrt{9}:\sqrt{10}$) at approximately 60°C (represented by the arrow in Figure 2), which should exhibit the desired drug release profile, and PEGylation of the lamellar particles should provide favourable circulation behaviour. Hence in this study, the phase behaviour of these lipid systems was evaluated using small angle X-ray scattering (SAXS) at equilibrium and dynamically using time-resolved synchrotron SAXS observed the effect of photothermal heating via NIR activation of encapsulated GNR. The equilibrium phase diagram for the POPE + cholesterol in water systems was first investigated to determine the robustness of the baseline phase behaviour with different source of POPE and cholesterol. The effect of subsequent addition of PEG-phospholipid to the lipid mixture on the phase behaviour was then evaluated and finally, the responsiveness of the system on incorporation of GNR to NIR irradiation was then demonstrated.

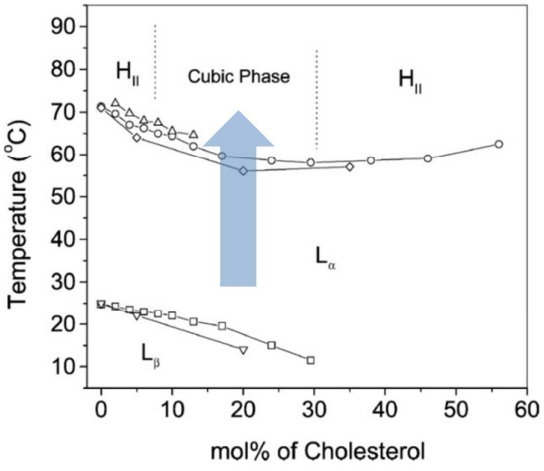


Figure 2 Literature phase behaviour of the POPE + cholesterol system in excess water with increasing cholesterol (mol % cholesterol in POPE + cholesterol mixture). Reproduced with permission from Ref[10].

2. Materials and Methods

2.1. Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (PEG-DSPE) was purchased from Avanti Polar Lipids (Alabaster, USA). Cholesterol was purchased from Sigma-Aldrich (St. Louis, USA). Methyl conjugated Gold Nanorodz™ with an aspect ratio of 4.2 (length, 42 nm; width, 10 nm) were purchased from Nanopartz (Loveland, CO). The measured surface plasmonic resonance (SPR) is 825 nm. Phytantriol (3,7,11,15-tetramethylhexadecane-1,2,3-triol) was a gift from DSM Nutritional Products (Kaiseraugst, Switzerland), with a minimum purity of 95%. All materials were used without further purification. Sodium chloride (Chem-Supply, Australia), di-sodium hydrogen orthophosphate and potassium dihydrogen orthophosphate (APS Ajax Finechem, Australia) and HCl (1M) (Merck, Australia) were of analytical reagent quality. Water used in these studies was obtained from a Millipore Milli-Q purification system (Billerica, USA). Phosphate buffered saline (PBS) was prepared by dissolving 8.0 g of sodium chloride, 0.19 g of potassium dihydrogen phosphate and 2.38 g of disodium hydrogen phosphate in sufficient water to produce 1000 mL and the pH adjusted to 7.4.

2.2. General procedure for LC preparation

Lipids were weighed in the appropriate mole ratios (0-40 mol% cholesterol in POPE ±10 mol% PEG-DSPE) dissolved in chloroform. The mixture was then dried under a stream of nitrogen gas for 3 hr, then under vacuum overnight at 40°C to ensure complete removal of solvent. The lipid mixture was then hydrated in a 20:1 (w/w) ratio (for dispersion) or a 1:1 (w/w) ratio (for bulk) with PBS and was allowed to equilibrate for >24 hr before investigation using SAXS. Gold nanorod solution was diluted with PBS to 0, 0.3 and 3 nM concentration and was added to the dried bulk lipid sample.

2.3. Phase characterisation of POPE and cholesterol systems

The LC structures were assessed using small angle X-ray scattering (SAXS) to qualitatively identify and confirm the internal nanostructure formed from various lipid compositions. SAXS measurements were performed at the SAXS/WAXS beam line at the Australian Synchrotron.[11] The synchrotron X-ray beam was tuned to a wavelength of 1.127 Å (11.0 keV) at a camera to detector distance of 1034.97 mm which gave the q-range $0.0176 < q < 1.016$ Å^{-1}, where q is the length of the scattering vector, defined by $q = (4\pi / \lambda) \sin(\theta/2)$, λ is the wavelength and θ the scattering angle. A silver behenate standard (d-spacing = 58.38 Å) was used for the q range calibration. The 2D SAXS patterns were acquired within 1 s using a Pilatus 1M detector with active area 169 x 179 mm^2 and with a pixel size of 172 µm. Equilibrated dispersion samples were transferred into 1.5 mm diameter glass capillaries, placed in the temperature controlled capillary holder and the SAXS profiles were determined with increasing temperature. Acquisition time for equilibrated samples was 1 s. Bulk samples were used in dynamic activation experiments which allow detection of phase changes when stimulated using NIR in real time. A Class IIIB fiber coupled laser system (MDL-808) with the power output at 400 mW at a wavelength $\lambda = 808$ nm (Changchun New Industries, China) was mounted 20 cm from the sample at a tangential angle to the X-ray beam and samples were illuminated remotely by a computer control system. Scattering patterns were acquired

for 1 s, every 10 s for 2 min. The two dimensional scattering patterns were integrated into the one-dimensional scattering function $I(q)$ using the in-house developed software package scatterBrain. Scattering curves are plotted as a function of relative intensity, I, versus q and phase structures were identified by indexing the Bragg peaks to known relative spacing ratios.[12] The apparent temperature (T_{app}) of the matrix during irradiation in the presence or absence of GNR was determined using a 'calibration' plot of the lattice parameter vs. temperature of the matrix from the equilibrium SAXS results.[8]

3. Results and Discussion

3.1. Phase behaviour of POPE and cholesterol dispersions

The phase diagram for the POPE and cholesterol systems in excess aqueous solution was determined using SAXS (Figure 3). The lamellar (L_α) phase in excess water was found to transition to the inverse hexagonal (H_2) phase in excess water with increasing temperature and the phase transition temperature reduced with increasing cholesterol concentration. From the literature (Figure 2), POPE with cholesterol in the concentration range between 10-30 mol% was reported to transition from L_α to V_2 at approximately 60 °C; in this study, the V_2 phase was not observed.[10] On reflection, there are apparent inconsistencies between the SAXS profiles presented by Wang *et al.* reproduced in Figure 4a. The figure shows coexisting phases from around 50 °C up to the highest temperature, which is not reflected in the derived phase diagram, dominated by the single L_α phase (Figure 2).

Additionally, an unusual peak in Figure 4a can be noticed at high temperature and was indexed as a peak that corresponds to the V_2 phase. However, this peak was not observed in recent data (Figure 4b) suggesting that the unusual peak may be an artefact of impurities in the sample or a detector/integration anomaly, resulting in inaccurate phase identification. The pure POPE sample was found to transition to the H_2 phase above 70 °C, as previously reported.[13] The addition of 20 mol% of cholesterol provided a similar effect to that on addition of 2.5 mol% of vitamin E, such that the H_2 peak first appeared at 44 °C.[13] This implies that cholesterol, like vitamin E, is able to reduce the transition temperature to close to physiological temperature.[13, 14] This is unfavourable in this drug delivery system as an unintentional phase transition may occur *in vivo*. Therefore, subsequent dynamic studies were conducted with POPE alone without added cholesterol, where the phase transition occurs at higher temperature.

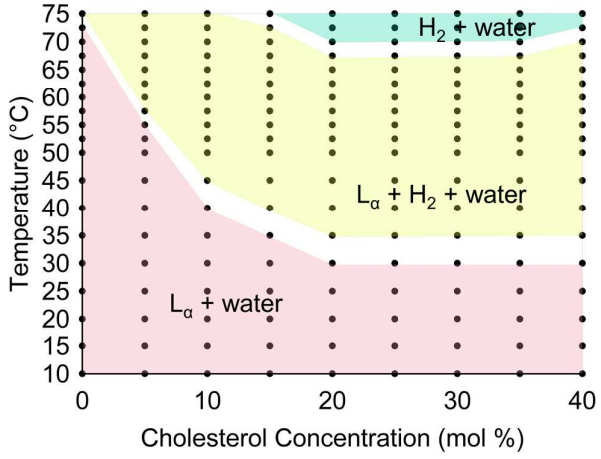
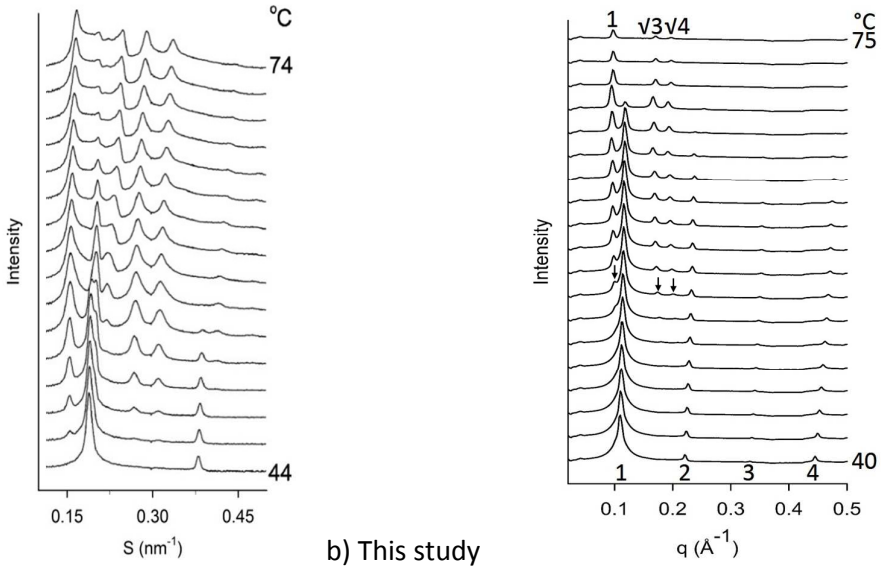


Figure 3 Phase behaviour of the POPE + cholesterol system in excess water with increasing cholesterol (mol % cholesterol in POPE + cholesterol mixture) in this study.



a) Literature b) This study

Figure 4 Small-angle X-ray scattering profiles for 20 mol% cholesterol in POPE from a) literature (reproduced with permission from Ref[10]) and b) this study. The annotations of peaks in Figure 4b indicate the lamellar phase at low temperatures (peaks at spacing 1, 2, 3, 4), and the inverse hexagonal phase at high temperature (1, √3, √4)

3.2. Effect of PEG-lipid in POPE dispersions

As mentioned, polyethylene glycol (PEG) grafted lipids can be incorporated into liposomes to assist in targeted drug delivery and longer circulation of rapidly cleared drugs. Therefore, the effect of addition of PEG-DSPE at 10 mol% to the POPE system was also investigated. This level of PEG-lipid is commonly used to provide a 'stealth' coating for liposomes as it has been shown to decrease MPS uptake by 90%.[1] The addition of PEG-lipid was found to facilitate dispersion and stabilize the L_α phase. POPE with 20 mol% of cholesterol formed the H_2 structure at approximately 30°C, 40°C lower than pure POPE. With the addition of PEG-lipid, both POPE systems with and without cholesterol formed the L_α phase in excess water. Unexpectedly, both systems did not exhibit the phase transition to H_2 phase at high temperatures (Figure 5) despite the differences in phase transition temperature, suggesting that the L_α phase was highly stabilized due to the addition of the PEG-lipid. It should also be noted that the L_α peaks became more defined with increasing temperatures suggesting that multi-lamellar structures were likely to be present. The critical packing-parameter (CPP) concept can be used to understand how the L_α phase was formed using Equation 1,

$$Equation\ 1: CPP = \frac{V_s}{a_0 l}$$

where V_s is the hydrophobic chain volume, a_0 is the head group area and l is the hydrophobic chain length.[15] The geometry of the POPE formed lamellar structure ($1/2 \leq CPP < 1$) due to the double hydrophobic tail, whereas the geometry of the PEG-lipid form more spherical micellar structures ($CPP < 1/3$) since the a_0 is large. At higher temperature, the steric repulsion of the large PEG-lipid head group decreases, increasing the CPP, causing more ordered lamellar structures to form.

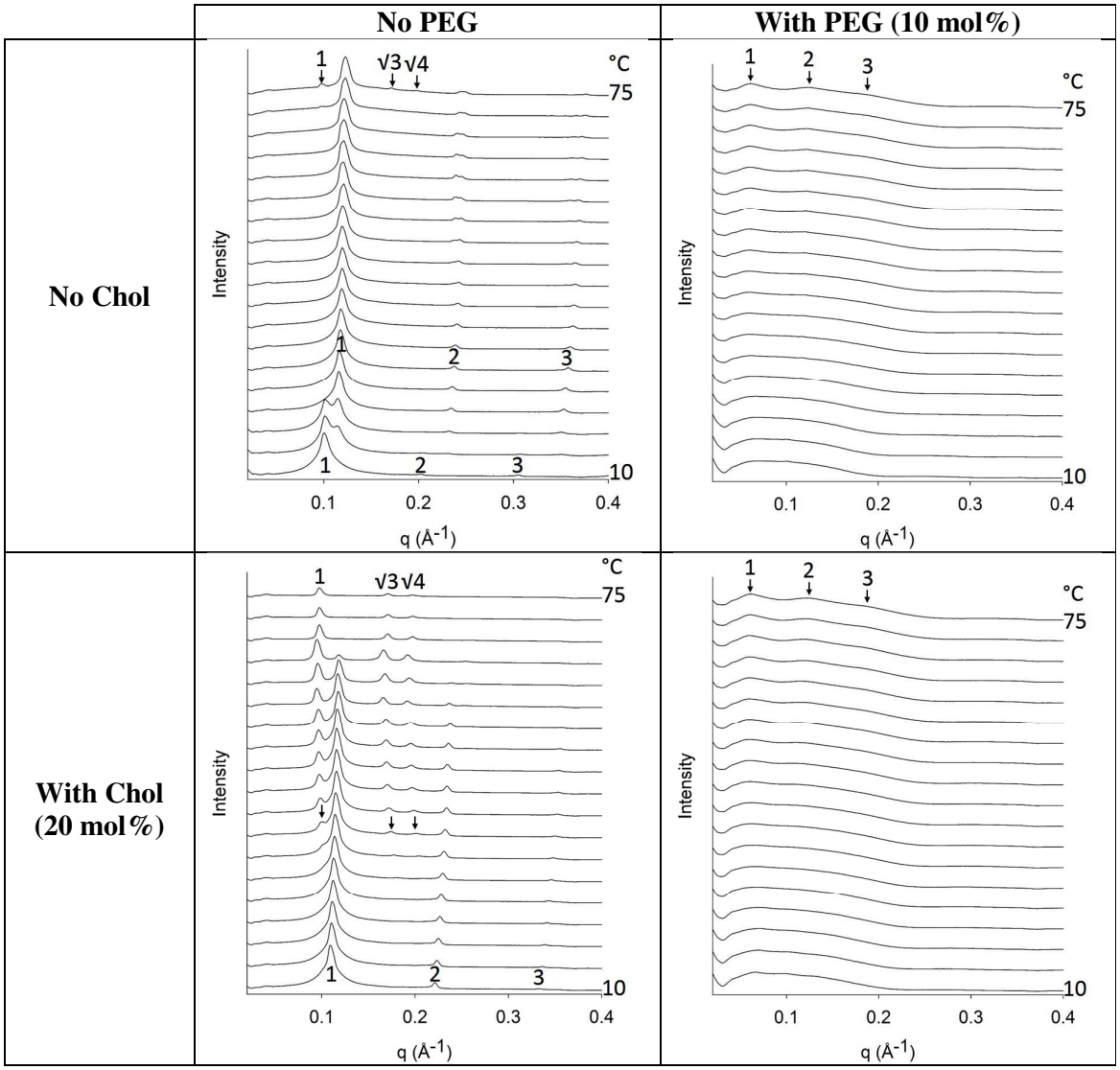
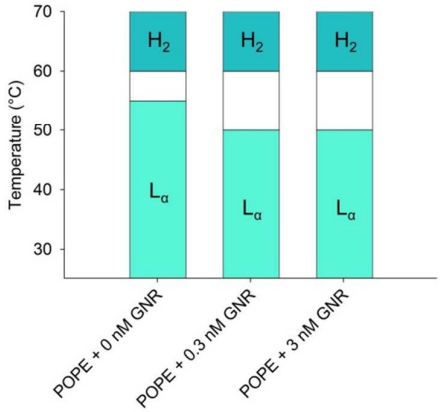


Figure 5 Comparison of SAXS scattering profiles from the POPE system with and without cholesterol (20 mol%) and PEG-DSPE (10 mol%) in excess water

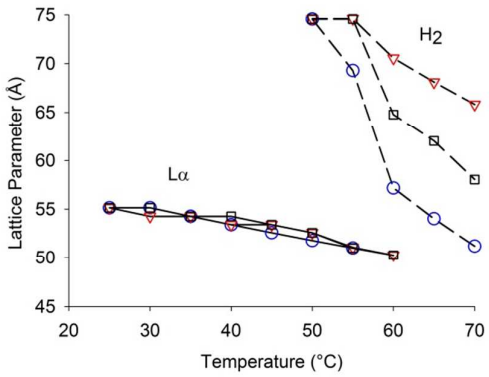
3.3. Effect of GNR on phase behaviour without irradiation in bulk liquid crystalline systems

The effect of GNR incorporation on the equilibrium phase behaviour of the bulk LC systems was evaluated in order to be able to quantify the effect of laser-activated photothermal heating.[8] The phytantriol LC system was used to compare the effect of incorporating the hydrophilic GNR with previous studies using hydrophobic GNR. Consistent with previous literature,[8] the presence of GNR did not substantially change the lattice parameter, phase structure or transition temperature for the phytantriol system despite the difference in hydrophobicity (SI 1 and 2). Likewise, the addition of GNR to the POPE system did not significantly influence the phase behaviour of the matrices as illustrated in Figure 6a, as all three matrices transitioned from L_α to H_2 phase upon an increase in

temperature. The incorporation of GNR did however have a subtle effect on the phase transition temperature causing the formation of a region of coexisting L_α and H_2 phases in excess water to occur at lower temperature (approximately 5 °C lower) and broadening of the temperature range for the mixed L_α + H_2 + water phase region. The lattice parameters vs. temperature calibration plots for determining the apparent temperature on laser irradiation are shown in Figure 6b. A noticeable difference was observed in the lattice parameter for the H_2 phase of the POPE systems at high temperature, but not for the L_α phase (Figure 6). This suggests that the changes in lattice parameter for the H_2 phase are affected by the addition of GNR, although the exact relationship with change in GNR concentration is not yet clear. The calibration curves can thus be applied to any GNR concentration up to 3 nM to estimate the apparent temperature of the matrices during irradiation in the L_α phase region, or at the specific concentrations measured in the H_2 region.



a)



b)

Figure 6 Effect of GNR on equilibrium structure of POPE bulk system in excess water a) Temperature dependent phase diagram and b) the lattice parameter of the lipid systems in the presence of 0 nM (○), 0.3 nM (▽) and 3 nM (□) GNR is plotted against temperature. Corresponding phases are indicated as follow: dashed line for H_2 phase and a solid line for L_α phase.

3.4. Effect of NIR irradiation

The effect of irradiation of the matrices containing GNR on the nanostructure of the LC systems was finally evaluated using time-resolved SAXS. Figure 7 illustrates that incorporation of GNR imparted photo-sensitivity to the lipid systems.

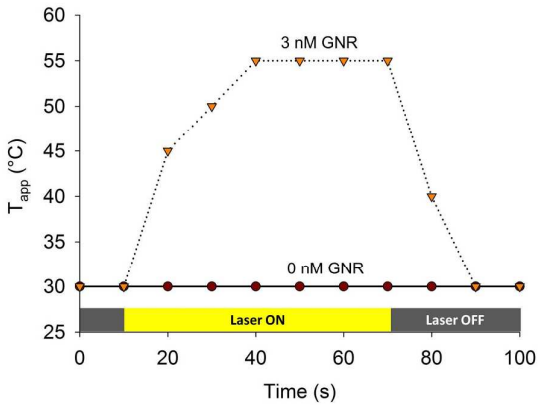


Figure 7 Effect of NIR laser irradiation on apparent temperature (T_{app}) of the POPE system in excess water containing 0 nM (●) and 3 nM (▼) GNR.

In the absence of GNR, the L_α phase was observed for the POPE systems in excess water on irradiation, precluding non-specific heating by the laser, which is consistent with previous studies.[8] Inclusion of a low concentration of GNR (0.3 nM) in POPE did not induce a change in phase structure on irradiation (see Supplementary information) therefore a higher concentration (3 nM) was used in further experimentation. For the POPE system in excess water, a photothermal heating effect was observed, and 30 s of irradiation induced disruption of lipid packing equivalent to increasing the apparent temperature to 55 °C. The duration of irradiation required to achieve this level of disruption was much greater than previous reports for the phytantriol system.[8] This may be due to the use of different GNR functionality and/or difference in heat capacity of the material.[16] It has been reported that the GNR can be tuned by varying the size and aspect ratio of the gold nanoparticles to absorb at a specific wavelength,[17] which will allow optimization of the photo-thermal effect and prevent accidental activation. In this study, the SPR peak of the GNR was measured to be 825 nm (SI 3), meaning that the photo-thermal heating is expected to be less effective when irradiated using the 808 nm NIR diode laser. The reversibility of the photo-thermal effect in the presence of the GNR is also evident in Figure 7, where after a short relaxation period, the apparent temperature returned to that of the starting initial temperature.

The study was further extended to investigate the reversibility of the POPE, PEG-lipid and GNR system in excess water. Figure 8 clearly shows that the nanostructure of the mesophase pre-irradiation is of L_α phase at 30°C. Upon irradiation, the system retained its L_α structure until 20 s of exposure. The peaks are broad in the dynamic study due to the lack of time for the structures to fully equilibrate. It is also evident that the peaks corresponding to the H_2 phase are forming at 20 sec. By utilising the lattice parameters calculated from the equilibrium phase behaviour, the T_{app} of the matrix at different irradiation times were calculated. The presence of H_2 peaks corresponds to a T_{app} of 70°C. Therefore, 20 s of irradiation was shown to induce an increase of about 40°C in the PEGylated POPE and GNR system. Upon cessation of irradiation, the system returned only partially to the initial L_α phase however, complete reversibility of the system was not observed suggesting further equilibration time may be necessary due to a supercooling effect as reported previously.[18]

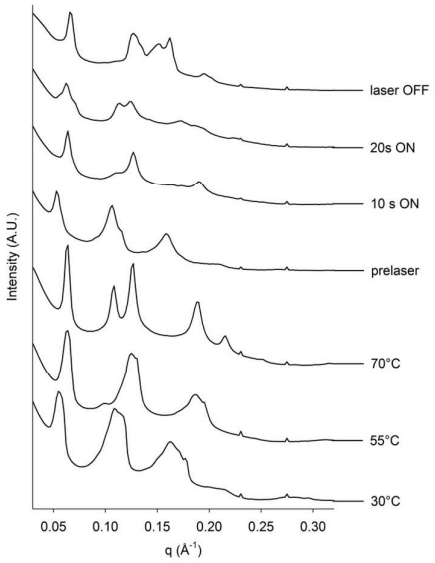


Figure 8 SAXS scattering data for POPE and PEG-lipid system in excess water with 3 nM GNR at equilibrium temperatures (30, 55 and 70°C) and after irradiation with the laser.

Conclusion

This study presents the phase behaviour of the cholesterol / POPE system in excess water. Contrary to previous reports, the H_2 phase was observed rather than the reported L_α to V_2 phase transition between 10-30 mol% cholesterol. The addition of cholesterol (20 mol%) was shown to reduce the transition temperature by 40°C when comparing to the pure POPE + water system. The addition of PEG-lipids to the POPE system in excess water facilitated dispersion and stabilized the L_α phase. Addition of a low concentration of GNR to the phospholipid systems imparted photo-sensitivity without compromising the integrity of the mesophases. The phase transition resulting from the photo-thermal heating was reversible for the non-PEGylated POPE + water system. These findings suggest the potential of this biocompatible composition as a photo-responsive system suitable for drug delivery.

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