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Machingauta, M., McEvoy, A., Karlsson, A. et al (2025). The influence of dioleoylphosphatidylcholine (DOPC) on the lipid sponge phase system. *Frontiers in Soft Matter*, 5. <http://dx.doi.org/10.3389/frsfm.2025.1708264>

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RECEIVED 18 September 2025

REVISED 26 October 2025

ACCEPTED 27 October 2025

PUBLISHED 18 December 2025

CITATION

Machingauta MR, McEvoy A, Karlsson A,
Barauskas J, Nylander T and Gilbert J (2025) The
influence of dioleoylphosphatidylcholine
(DOPC) on the lipid sponge phase system.
Front. Soft Matter 5:1708264.
doi: 10.3389/frsfm.2025.1708264

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The influence of dioleoylphosphatidylcholine (DOPC) on the lipid sponge phase system

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The use of lipid nanoparticles (LNPs) in pharmaceutical and food applications has gained momentum due to their capacity to encapsulate a wide range of biomolecules. Previous studies have demonstrated the effective entrapment of enzymes within lipid sponge nanoparticles, highlighting their potential as versatile delivery vehicles. Similar to inverse bicontinuous cubic phases, the sponge phase features a network of aqueous cavities separated by curved lipid bilayers, but with a more flexible structure and larger water cavities. The objective of this study is to determine how the lipid composition affects the sponge phase properties. Based on food-grade lipid mixtures of the glycerol monooleate-rich lipid mixture (GMO-50), diglycerol monooleate (DGMO), polysorbate 80 (P80), and water, which are known to form sponge phases, we have studied the incorporation of the zwitterionic phospholipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). This is of particular interest due to its potential to increase the biocompatibility of the formulation. Using small-angle X-ray scattering (SAXS) and cryogenic transmission electron microscopy (cryoTEM), we show that DOPC generally promotes the formation of lamellar phases at 25 °C, but sponge phases can be preserved by adjusting GMO-50/DOPC ratios, adding P80, or increasing the temperature to 40 °C. Dispersions in excess water yielded mixtures of sponge nanoparticles and vesicles, while diluting the LNPs in buffers with higher ionic strength (PBS and cell medium) induced multilamellar vesicle formation. These results demonstrate that DOPC provides a tunable handle on lipid nanostructures, enabling temperature and medium-responsive systems, and that the surrounding medium can restructure nanoparticles even after formation. This underscores the importance of

considering both the conditions of nanoparticle assembly and their response to new environments, with direct implications for biopharmaceutical performance.

KEYWORDS

lipid sponge phase, vesicle, dioleoylphosphatidylcholine, acyl glycerides, lipid self-assembly, drug delivery, small-angle X-ray scattering, cryogenic transmission electron microscopy

1 Introduction

Polar lipids self-assemble into a range of structures that depend on the lipid molecular architecture as well as the surrounding medium (Luzzati and Husson, 1962; Larsson, 1989). Here, we will discuss three types of self-assembly structures and their dispersions, namely, the lamellar (L_α) phase, which forms closed bilayer structures in excess of water suitable to confine hydrophilic substances, the bicontinuous cubic phases, and the sponge phases (L_3). The latter phases contain curved bilayers that form aqueous cavities with the capability to include both hydrophilic and hydrophobic compounds. Because the sponge phase has larger water channels than the cubic phase, it can also include larger molecules. Recent advances in the knowledge of the structure–function relationship of both lamellar (L_α) and non-lamellar lipid nanoparticles have expanded their applications in pharmaceutical formulations, surpassing the use of simpler systems such as emulsions. Notably, ground-breaking COVID-19 vaccines such as mRNA-1273 and BNT162b21 employ lipid nanoparticles to deliver antigen-coding mRNA, underscoring the pivotal role of lipid nanoparticles in modern vaccine technology (Hou et al., 2021). Moreover, many lipid nanoparticles for nucleic acid or protein formulations have been developed and are currently undergoing clinical evaluation for the treatment and prevention of diverse viral infections, various cancers, and genetic diseases (Hou et al., 2021). The versatility of lipid liquid crystalline (LLC) phases presents an enticing avenue for encapsulating therapeutic compounds, nutrients, and other complex biomolecules (Barriga et al., 2018; Hou et al., 2021; Chountoulesi et al., 2022; Fornasier and Murgia, 2023). Non-lamellar LLC nano systems are therefore being exploited as delivery systems for therapeutic proteins, peptides, or nucleic acids, given their ability to host a wide variety of hydrophilic, hydrophobic, and amphiphilic small molecules as well as biomacromolecules (Chountoulesi et al., 2022). Functional drug delivery and gene transfection systems require formulations that have low cytotoxicity and good ability to be tailored for targeted delivery and controlled release (Barriga et al., 2018; Chountoulesi et al., 2022; Fornasier and Murgia, 2023; Li J. et al., 2015). Cubosomes, hexosomes, and spongosomes preserve their three-dimensional architecture from the corresponding bulk phase upon dispersion and are capable of entrapping both small and more complex biomolecules (Mathews and Mertins, 2017).

It has previously been observed that mixtures of glycerides enriched in glycerol monooleate (Capmul GMO-50) and diglycerol monooleate (DGMO) can form sponge phases (L_3) in mixtures with water, where the addition of an emulsifier, polysorbate 80 (P80), expands the range of the L_3 phase (Valledeperas et al., 2016). The internal structure of the L_3 phase features a 3D network of water channels delineated by curved lipid–aqueous interfaces, similar to those in bicontinuous cubic

phases, but with bilayers that are less curved and more flexible. Consequently, they have larger aqueous cavities able to entrap large biomolecules. With the addition of P80, the bulk phases can easily be dispersed into well-defined nanoparticles, that is, spongosomes or L_3 nanoparticles (L_3 NPs), in excess water (Valledeperas et al., 2016). Another approach to induce the L_3 phase formation is through modification of the aqueous phase. Brasnett et al. (2023) investigated monoolein/water systems and demonstrated that the addition of butanediol to the aqueous phase promotes the L_3 phase formation. In their study, various dopants, including 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), were evaluated. The inclusion of DOPC increased the butanediol concentration required for the monoolein-based system to transition into the L_3 phase.

The objective of our study is to reveal how the lipid composition affects the L_3 phase properties. For this purpose, the effects of incorporating DOPC to fabricate L_3 phase particles tailored for encapsulating complex biomolecules were investigated. Here, the interaction between the biomolecules and lipid matrix is important, as it can determine the reversibility of the encapsulation as well as changes in the lipid self-assembly structure (Valledeperas et al., 2019a; Gilbert et al., 2019; Gilbert et al., 2022; Gilbert et al., 2023). The zwitterionic DOPC was introduced to modify the physicochemical properties of the lipid–aqueous interface, which has been shown to reduce protein adsorption relative to other lipids (Vermette and Meagher, 2003). Although the effect might not be as universal as previously reported, as discussed in the review by Vermette and Meagher (2003), it opens the possibility to tune the interaction with the lipid matrix. DOPC is generally considered to be biocompatible and forms bilayer structures such as vesicles in excess water (Tristram-Nagle et al., 1998; Li J. et al., 2015), making it a safe and ideal choice for pharmaceutical applications (Li et al., 2015; Mathews and Mertins, 2017; Barauskas et al., 2010; Anderson et al., 1989). In fact, introducing soy phosphatidylcholine into glycerol monooleate (GMO)-based cubosomes was found to reduce the hemolytic potential (Barauskas et al., 2010). Furthermore, it has been suggested that the intestinal mucus barrier to microbiota attack is partially due to the phosphatidylcholine lipids bound to mucins and, therefore, is suggested to prevent inflammation (Stremmel and Weiskirchen, 2024).

This study primarily focuses on elucidating the regions of the phase diagram that correspond to lamellar (L_α) and L_3 structures in the GMO-50/DGMO/DOPC/P80 system and the impact of temperature on the structures. Specifically, we investigate the influence of DOPC on the phase behavior of GMO-50/DGMO and GMO-50/DGMO/P80 systems and further assess how substituting DGMO with DOPC alters the phase characteristics. This system, allows modulation of the lipid water interface in bicontinuous lipid phases and hence the interaction with encapsulated bioactive molecules, as shown in a previous study (Luchini et al., 2025).

TABLE 1 Results showing phases formed in the GMO-50/DGMO (+P80)-based system, hydrated to 60 wt% water, and the calculated unit cell dimension, lattice parameter (a), and water channel diameter (d_{water}). Details of the indexing and values used to calculate the lattice parameters are given in [Supplementary Tables S1–S4](#).

Formulation	Phase	a (Å)	d_{water} (Å)
30/70 GMO-50/DGMO	L_3	109 ± 1	87 ± 1
60/40 GMO-50/DGMO + P80	L_3	120 ± 1	95 ± 1
25/75 GMO-50/DGMO	L_3/L_α	$141 \pm 1/58 \pm 1$	112 ± 1
35/65 GMO-50/DGMO	Pn3m	132 ± 3	87 ± 1

These structures were characterized using small-angle X-ray scattering (SAXS) and visualization under cross-polarized light. Additionally, formulations comprising GMO-50/DOPC/P80 were dispersed in Milli-Q (MQ) water and diluted in various aqueous media, including MQ water, phosphate-buffered saline (PBS), and cell medium (CM). These dispersions were evaluated using synchrotron SAXS and cryogenic transmission electron microscopy (cryoTEM) and compared to reported GMO-50/DGMO/P80 L_3 dispersions ([Valdeperas et al., 2016](#)). The potential of DOPC-based L_3 nanoparticles as versatile platforms for encapsulating a wide range of bioactive molecules is addressed.

2 Materials and methods

Capmul GMO-50 (Lot # 190111-9) was purchased from Abitec (United States) and was composed of 53.8% monoglycerides, 15%–35% diglycerides, and 2%–10% triglycerides with a fatty acid composition of: 86.6% oleic (C18:1), 4.9% linoleic, 4.1% palmitic, and 3.3% stearic. Diglycerol monooleate (DGMO-90V, Lot # 9147) was purchased from Nikko Chemicals (Japan). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) from Merck (Lot No. MBB0006-18) was kindly supplied by Camurus (Lund, Sweden). The polyoxyethylene (20) sorbitan monooleate (P80) (molecular weight $\approx 1,310$ g/mol, Lot # 1102YP0133) was purchased from Croda (Limhamn, Sweden).

The cell medium (CM) was made by mixing 500 mL RPMI-1640 medium HyClone SH30027.01 (Nordic Biolabs), containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4,500 mg/L glucose, and 1,500 mg/L sodium bicarbonate (according to the manufacturer), with 50 mL fetal bovine serum (Life Technologies, USA), 11 mL of 50x Supplement, 5.5 mL 1 M HEPES solution (pH ≈ 7.2 , HyClone SH30237.01 Nordic Biolabs), and 5 mL PenStrep (penicillin–streptomycin (10,000 U/mL, Gibco). The 50x supplement was prepared by mixing 100 mL 100 mM glutamine solution (HyClone SH 30034.01), 100 mL 100 mM Na-pyruvate solution (HyClone SH30239.01), and 35.2 μ L 2-mercaptoethanol (Thermo Fisher Scientific). Phosphate-buffered saline (PBS) was prepared by dissolving one tablet (Sigma-Aldrich, P4417) in 100 mL of MQ water to yield 2X PBS. In 1X PBS, the composition is 10 mM phosphate buffer with 2.7 mM potassium chloride and 137 mM sodium chloride, resulting in a pH of 7.4 at 25 °C. Milli-Q (MQ) water (resistivity of 18.2 M Ω -cm) was used for all solutions in this work.

2.1 Preparation of bulk phases

2.1.1 Preparing lipid mixtures

2.1.1.1 Reference series

The lipid mixtures for the reference series were prepared by gently heating Capmul GMO-50 to 40 °C until it melted and adding it to DGMO (and P80 where relevant) at the ratios (wt/wt) specified in [Table 1](#). The lipid mixtures were left to mix on a rotator table for 24 h before hydration.

2.1.1.2 Samples containing DOPC

The lipid mixtures containing DOPC were prepared by mixing different ratios of DGMO, DOPC, melted GMO-50 and P80 (where relevant) in a round-bottom flask. Ethanol (10–20 wt%) was then added, and the lipids were allowed to dissolve and mix. Once the lipids were completely dissolved, the solvent was removed under vacuum using a rotary evaporator with the water bath set to 40 °C.

For the samples containing DOPC, starting from the lipid composition of the reference samples, we studied the effect of (i) adding DOPC (i.e., addition of DOPC while the GMO-50/DGMO ratio remained constant) and (ii) replacing DGMO with DOPC (i.e., the DGMO/DOPC ratio changed while the amount of GMO-50 remained constant).

2.1.1.3 All samples containing P80

For all samples containing P80, P80 was added at 30 wt% to the lipid mixture (70/30 lipid/P80). For example, the reference sample 60/40 GMO-50/DGMO + P80 has a composition of (60/40)/30 (GMO-50/DGMO)/P80 corresponding to 42/28/30 GMO-50/DGMO/P80.

2.1.2 Hydration of lipid mixtures

From these lipid mixtures, the bulk phases were prepared by adding 60 wt% MQ water (40/60 lipid/water) and centrifuging six times for 2 min at 419 $\times g$ alternating up and down to ensure sufficient mixing. The hydrated samples were equilibrated for 2 weeks on a rotator table. During this period, each vial was centrifuged three times per week, first with the cap facing upward and then with the cap facing downward, as described by [Valdeperas et al. \(2019a\)](#) and [Gilbert et al. \(2019\)](#).

2.2 Preparation of dispersed phases

Additional samples containing (60/40)/30 (GMO-50/DOPC)/P80 (42/28/30 GMO-50/DOPC/P80) were hydrated with 50 wt%, 55 wt%, 60 wt%, 65 wt%, and 70 wt% MQ water, centrifuged, and equilibrated as described in [Section 2.1](#) above. Lipid dispersions were then prepared by adding the equilibrated bulk phases to MQ water to achieve a concentration of 10 wt% of the bulk phase in a glass vial. The glass vials were sealed, hand shaken, and mixed on a mechanical shaker table for 24 h at a speed of 300 rpm ([Gilbert et al., 2019](#)). This produced dispersions (10 wt% bulk phase) with a lipid concentration of 5 wt%, 4.5 wt%, 4 wt%, 3.5 wt%, and 3 wt% lipid/P80, for the respective initial hydrations. These dispersions were then diluted 2-fold and 5-fold to a concentration of 5 wt% and 2 wt% bulk phases, respectively, into MQ water, cell medium (CM), and 1X PBS. For the nanoparticle samples diluted in CM, the final

concentration of CM in the 5 wt% dilutions was 0.5X CM, and in the 2 wt% dilutions, 0.8X CM. For the samples diluted in PBS, 2X PBS and MQ water were added in the required ratios to achieve a final concentration of 1X PBS in both the 2 wt% and 5 wt% dilutions.

2.3 Small-angle X-ray scattering

2.3.1 SAXS of bulk phases

The samples were first visually inspected in cross-polarized light. Then, SAXS measurements to determine the structure of the bulk phases were performed using a SAXSLab Ganesha 300XL instrument (SAXSLAB ApS, Skovlunde, Denmark). The instrument had a microfocus sealed Genix 3D X-ray source and a 2D 300 K Pilatus detector from Dectris. The wavelength of the X-rays used was 1.54 Å, and a q range of 0.023–0.73 Å⁻¹ was investigated. Samples were sandwiched between two thin mica windows separated by a rubber O-ring in a metal sample holder. The samples were measured for an exposure time of 60 min at 25 °C to determine the bulk phase structures and for 45 min at 20 °C, 25 °C, 30 °C, and 40 °C to determine the temperature dependence of the formed structure for selected samples. The temperature was controlled by an external recirculating water bath. The two-dimensional scattering pattern was radially averaged using SAXSGui software to obtain $I(q)$. The results were background-subtracted and analyzed using SasView version 5.05 (<http://www.sasview.org>). Plots were made using Igor Pro 9 software (WaveMetrics, Portland, USA).

2.3.2 SAXS of dispersions

Synchrotron SAXS measurements for the dispersed phases were performed at the CoSAXS beamline at MAX IV (Lund, Sweden). The scattered intensity was recorded at room temperature (25.0 °C ± 0.1 °C) using a wavelength of 0.91 Å and an Eiger2 4M detector at a sample-to-detector distance of 1913 mm. The samples were loaded on a 96-well plate and pipetted into a 1.5 mm flow-through capillary by an autoloader and measured using an exposure time of 20 ms with 600 frames collected. The 2D SAXS data were reduced, frame averaged, normalized, and the background subtracted using the MatFRAIA algorithm provided by MAX IV (Jensen et al., 2022).

2.3.3 Calculating lattice parameter

The d-spacing can be calculated from the q position of the Bragg peak using Equation 1.

$$d = \frac{2\pi}{q} \quad (1)$$

The Miller indices h , k , l , describe the orientation of a set of planes in a (liquid) crystal in three dimensions and can be used to calculate the lattice parameter a from the d-spacings or repeat distances.

For a lamellar stack, $h = k = 0$, as the planes are oriented parallel to each other, and the lattice parameter, a , is calculated as

$$a = d \times l, \quad (2)$$

with $l = 1, 2, 3, 4, \dots$

For a cubic phase, the lattice parameter, a , is calculated as

$$a = d\sqrt{h^2 + k^2 + l^2} = d \times S. \quad (3)$$

For the Pn3m cubic phase (space group no. 224), $S = \sqrt{2}, \sqrt{3}, 2, \sqrt{6}, \sqrt{8}, \dots$, and for the Im3m cubic phase (space group no. 229), $S = \sqrt{2}, 2, \sqrt{6}, \sqrt{8}, \sqrt{10}, \dots$

Equation 4 was applied to estimate the water channel radius (r_{water}) of the bicontinuous cubic phases (Briggs J. et al., 1996):

$$r_{\text{water}} = \left(\frac{A_0}{2\pi\chi} \right)^{1/2} a_{\text{cub}} - d_{\text{mono}}, \quad (4)$$

where a_{cub} is the lattice parameter obtained through the q value from the SAXS diffractogram, d_{mono} is the monolayer thickness, A_0 is the ratio of the infinite periodic minimal surface area to (unit cell volume)^{2/3}, and χ is the Euler–Poincaré characteristic. These parameters are 1.92 and -2 , respectively, for the Pn3m space group and 2.345 and -4 , respectively, for the Im3m space group (Anderson et al., 1989; Turner et al., 1992).

A monolayer thickness with a d_{mono} of 23 Å was assumed in this study, based on previous data for a similar lipid system (Valdeperas et al., 2016).

Unlike the L_α and cubic phases, the L_3 phase does not exhibit a long-range order, and the SAXS results, therefore, do not produce well-defined peaks. The L_3 diffraction pattern features two broad peaks, where the low q peak corresponds to the correlation length, and the high q peak corresponds to the bilayer thickness (Porcar et al., 2013). The water channel radius (r_{water}) can be estimated based on the q value of the first Bragg peak for a phase, allowing a further description of the structure.

The water channel radius for the sponge phase can be calculated if we assume that the increase in the radius from the cubic phase is proportional to the ratio between the d_{space} of the sponge phase and the d_{space} of a cubic phase at the swelling limit before the transition into the sponge phase, as shown in Equation 5 (Valdeperas M. et al., 2016). d_{water} is twice the r_{water} value.

$$r_{\text{water}}(\text{sponge}) = \frac{d_{\text{space}}(\text{sponge})}{d_{\text{space}}(\text{cubic})} r_{\text{water}}(\text{cubic}) \quad (5)$$

2.3.4 Error estimation

The method to estimate the error associated with the calculated lattice parameter depended on the number of visible Bragg peaks to index. For the Pn3m and Im3m cubic phases, the error given was the standard deviation of the lattice parameter calculated from each peak in the SAXS data. For the L_α and L_3 phases, the error given was the instrument resolution, as only one or two peaks were present in the SAXS data for these structures. The error for the lattice parameter based on the instrument resolution was calculated from the error propagation of the q resolution. The errors for the water channel diameters were calculated from the error propagation of the lattice parameter errors.

2.4 Cryogenic-transmission electron microscopy (CryoTEM)

The dispersions were also evaluated by cryoTEM using an optimized JEM-2200FS transmission electron microscope (JEOL). The microscope was equipped with a Schottky field-emission

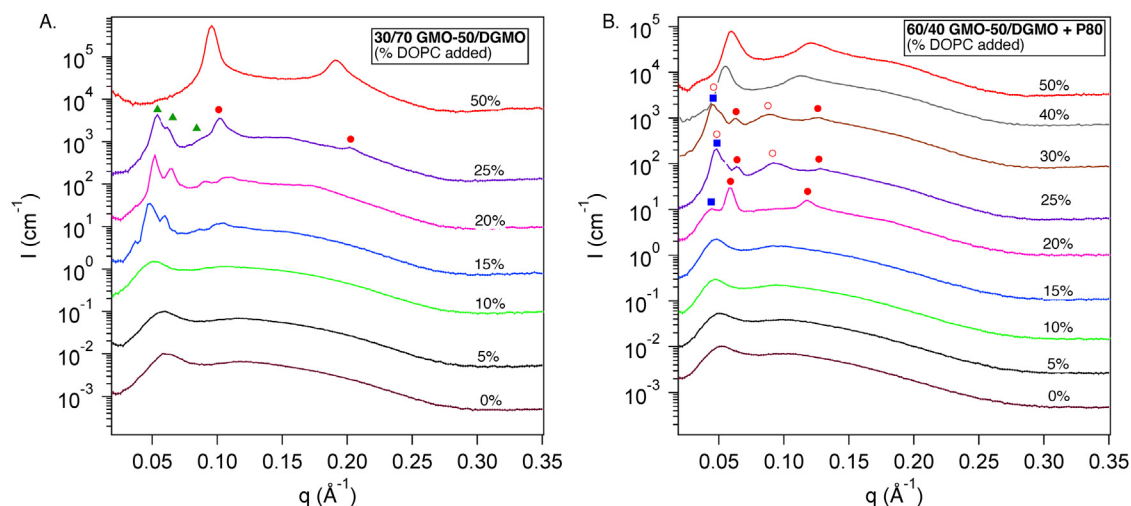


FIGURE 1

SAXS diffractograms showing 0–50 wt% DOPC added to (A) 30/70 GMO-50/DGMO and (B) 60/40 GMO-50/DGMO + 30 wt% P80. All samples were hydrated to 60 wt% with MQ water. In data with coexisting phases, the peaks corresponding to each phase are marked as follows: L_{α} with red circles, L_3 with blue squares, and Pn3m cubic phase with green triangles. Empty vs. filled markers are used to indicate if more than one of the same type of phase is present (i.e., $L_{\alpha,1}$ and $L_{\alpha,2}$). The obtained lattice parameters are shown in Figure 2. The corresponding diffractograms for the addition of 0–50 wt% of DOPC to 25/75 and 35/65 GMO-50/DGMO are shown in Supplementary Figure S2. Curves are offset for clarity.

electron source and was operated at a voltage of 200 kV. An in-column energy (omega) filter and a 25 eV slit were used. The images were recorded using SerialEM software under low-dose conditions onto a bottom-mounted TemCam-F416 camera (TVIPS) operated with 3K by 4K pixels. The samples were prepared using the method described by Dubackic et al. (2022). All formulations were prepared using an automatic plunge-freezer system (Leica EM GP) with the environmental chamber operated at a temperature of 20 °C and relative humidity of 90%. A 4 μ L droplet of each formulation was pipetted onto a lacey Formvar carbon-coated grid (Ted Pella) prior to blotting with a filter paper to get rid of excess fluid. The grid was then plunged into liquid ethane (approximately –184 °C) to ensure the rapid vitrification of the sample in its original state. After this, all prepared samples were kept in liquid nitrogen at a temperature of –196 °C. The cryoTEM imaging was performed under the microscope using a cryotransfer tomography holder (Fischione Model 2550).

3 Results

Valldeperas et al. (2016) determined the phase behavior of the ternary GMO-50, DGMO, and water system, including the effect of adding the surfactant P80. The neat GMO-50/DGMO/water system exhibits a reverse micellar (L_2) phase at low water content. Upon increasing the water content, a transition to the reverse hexagonal (H_{II}) phase occurs at low DGMO concentrations, whereas higher DGMO concentrations promote the formation of inverse bicontinuous cubic phases. Increasing the water content leads to a small region of L_3 phase. A further increase of the DGMO content leads to a large area of L_{α} phase. Introducing a fixed concentration of P80 with respect to the lipid content decreases the area of the H_{II} phase, while it significantly increases the cubic, L_3 , and L_{α} phase regions. It is interesting to note that the

phase diagram for the GMO/DGMO/water system, which makes use of high-purity GMO, is dominated by the L_{α} phase (Pitzalis et al., 2000). Highly swollen cubic phases were observed only at DGMO content below 40 wt% and under conditions of high water content. Given that both DGMO and DOPC are lamellar phase-forming lipids, we hypothesize that DOPC can at least partially replace DGMO within this system. The starting point for this study was a reference series comprising four formulations that, according to the phase diagram reported by Valldeperas et al. (2016), either reside within or close to the L_3 phase region. The key results from the initial structural analysis are summarized in Table 1 based on the SAXS data shown in Supplementary Figure S1. The lattice parameters were calculated for the L_3 , L_{α} and cubic phases using Equations 1–3 respectively. The water channel diameters for the cubic and L_3 phases were calculated using Equations 4, 5 respectively.

3.1 How much DOPC could be added to GMO-50/DGMO without affecting the phase behavior?

The effect of adding DOPC to the reference samples was studied in the first step. To do this, 5–50 wt% DOPC was added to the reference lipid compositions while maintaining constant ratios of GMO-50/DGMO and lipid/P80 (as per Table 1) and the same 60 wt % hydration. The obtained SAXS data are shown in Figure 1 and Supplementary Figure S2.

3.1.1 Samples that initially show a single L_3 phase

The diffractograms are shown in Figure 1, and the calculated lattice parameters (Supplementary Tables S1, S2) are shown in Figures 2A,B.

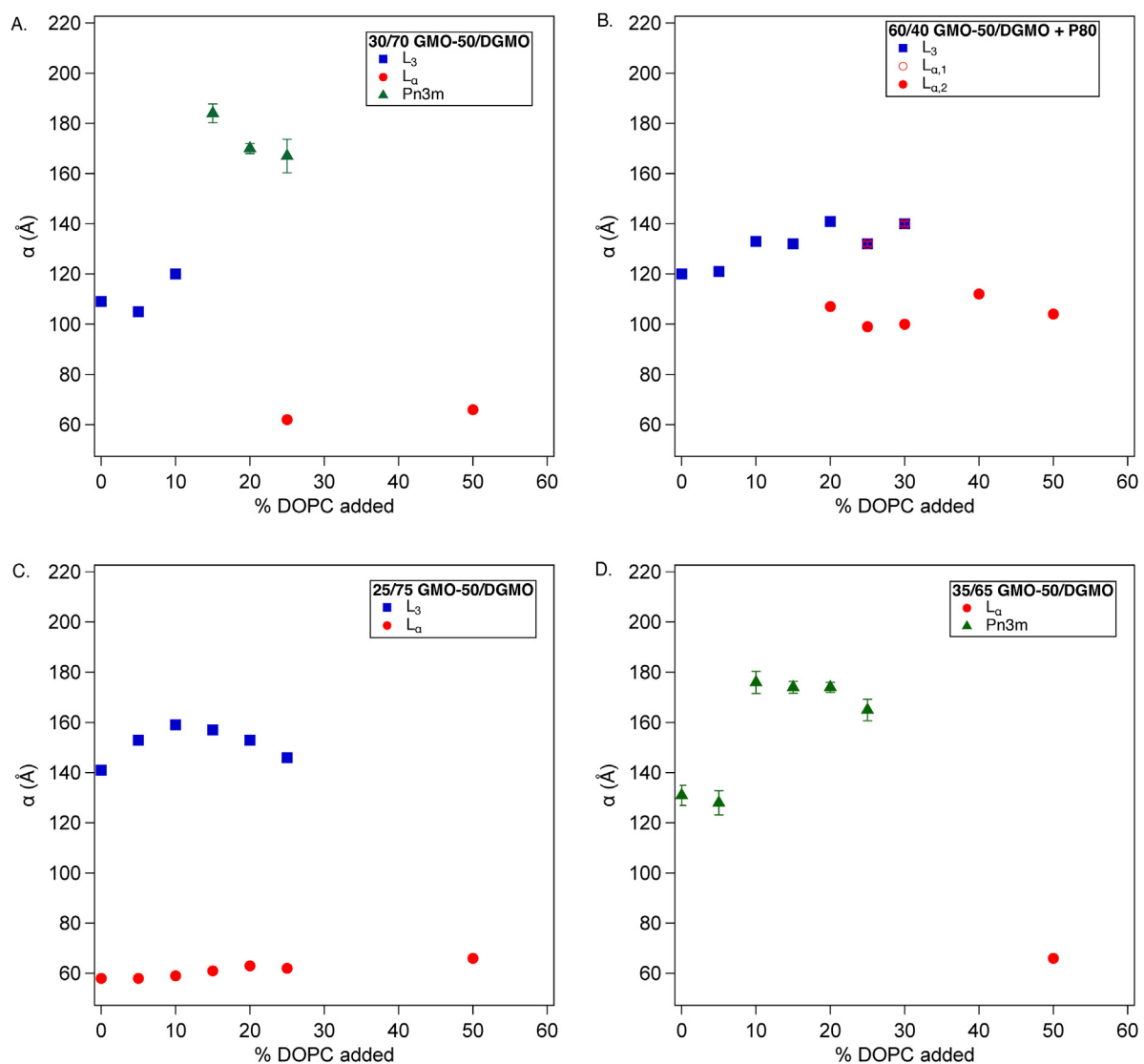


FIGURE 2 Change in the lattice parameter, a , with 0–50 wt% DOPC added to (A) 30/70 GMO-50/DGMO, (B) 60/40 GMO-50/DGMO + 30 wt% P80, (C) 25/75 GMO-50/DGMO, and (D) 35/65 GMO-50/DGMO. The lattice parameter was calculated from the diffractograms, using Equations 2, 3 for the lamellar and cubic phases, respectively. For the L_3 phase, the low- q peak was used as per Equation 1. The tabulated values are shown in the supplementary information, Supplementary Tables S1–S4.

3.1.1.1 Formulations with 30/70 GMO-50/DGMO

This composition maintained the L_3 phase at lower DOPC concentrations (0–10 wt%), while a transition to a mixed L_3 and bicontinuous cubic phase occurred at higher DOPC content (15–25 wt%), as shown in Figure 1A. At the highest DOPC content of 50 wt%, an L_α phase was observed. The lattice parameter (a) values (Figure 2A), derived from the SAXS data and summarized in Supplementary Table S1, exhibited an initial increase followed by a decrease with increasing DOPC fraction.

3.1.1.2 Formulations with 60/40 GMO-50/DGMO + 30 wt % P80

This composition displayed a similar trend to 30/70 GMO-50/DGMO (Figure 1B). Here, the addition of DOPC led to

the coexistence of L_α and L_3 phases, with the dominance of L_α phases at higher DOPC concentrations. The lattice parameter (a) values (Figure 2B), summarized in Supplementary Table S2, exhibited a similar trend of initial increase followed by a decrease, reflecting the phase transition. We note that the lattice parameter for the L_α phase is higher than that observed in the formulations without P80.

3.1.2 Samples that initially are close to the L_3 phase region

The diffractograms are shown in Supplementary Figure S2, and the calculated lattice parameters (Supplementary Tables S3, S4) are shown in Figures 2C,D.

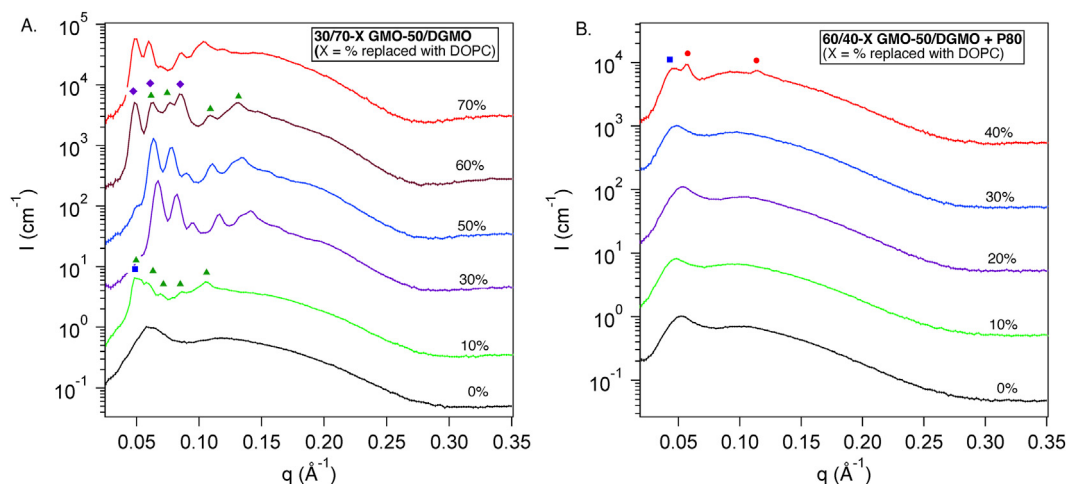


FIGURE 3

SAXS diffractograms showing the replacement of DGMO with DOPC in (A) 30/70-X/X GMO-50/DGMO/DOPC samples ($X = 0-70$ wt%) and (B) in 60/40-X/X GMO-50/DGMO/DOPC samples ($X = 0-40$ wt%) + 30 wt% P80. All samples were hydrated to 60 wt% with MQ water. In data with coexisting phases, the peaks corresponding to each phase are marked as follows: L_α with red circles, L_3 with blue squares, Pn3m cubic phase with green triangles, and Im3m cubic phase with purple diamonds. The obtained lattice parameters are shown in Figure 4. Diffractograms for the replacement of DGMO with DOPC in 25/75 and 35/65 GMO-50/DGMO are shown in Supplementary Figure S3. Curves are offset for clarity.

3.1.2.1 Formulations with 25/75 GMO-50/DGMO

The diffractograms in Supplementary Figure S2A show a coexistence of L_3 and L_α phases from 0 to 25 wt% DOPC. As the DOPC content increases, the L_α phase becomes more distinct, and at 50 wt%, it appears to be a pure L_α phase. A slight shift of the L_α peaks toward lower q values indicates an increase in the lattice parameter of the L_α phase (Figure 2C, derived from values summarized in Supplementary Table S3).

3.1.2.2 Formulations with 35/65 GMO-50/DGMO

The diffractograms in Supplementary Figure S2B show a Pn3m bicontinuous cubic phase up to 25 wt% DOPC with an increase in lattice parameter with DOPC over this range (Figure 2D, derived from values summarized in Supplementary Table S4). We note that the standard deviation when calculating the lattice parameter is relatively large, and therefore, a contribution from other phases cannot be ruled out. At the highest DOPC content studied (50 wt%), an L_α phase was observed.

3.2 Can DGMO be replaced with DOPC in the GMO-50/DGMO/water system?

In the next step, the effect of replacing DGMO in the reference samples with DOPC was studied, specifically to investigate the extent to which DGMO can be replaced with DOPC while maintaining the L_3 phase.

3.2.1 Samples that initially show a single L_3 phase

3.2.1.1 Formulations with 30/70 GMO-50/DGMO

Without DOPC (Figure 3A), a neat L_3 phase persists, but already at 10 wt% DOPC concentration, the L_3 coexists with at least one other phase. A Pn3m cubic phase with a lattice parameter of $182 \pm$

$3 \text{ \AA}$ (Figure 4A) can be identified; however, it is possible that additional phases are present. Even higher DOPC fractions led to a well-defined Pn3m cubic phase for 30 and 50 wt% DOPC with lattice parameters of $132 \pm 1 \text{ \AA}$ and $140 \pm 1 \text{ \AA}$, respectively. Replacement of 60 wt% DGMO with DOPC led to mixed bicontinuous cubic phases exhibiting Pn3m and Im3m space groups, while complete replacement led to a pure Pn3m cubic phase with a lattice parameter of $182 \pm 2 \text{ \AA}$ (Supplementary Table S5).

3.2.1.2 Formulations with 60/40 GMO-50/DGMO + 30 wt % P80

Replacement of 10–30 wt% DGMO with DOPC maintained the characteristic SAXS pattern associated with the L_3 phase (Figure 3B). However, complete replacement of DGMO with DOPC showed peaks indicative of both L_3 and L_α phases. The q_1 values remained largely unaffected by increasing DOPC content, resulting in lattice parameter values for the L_3 phase ranging from 117 to $130 \pm 1 \text{ \AA}$, as shown in Figure 4B. For the sample in which DGMO was completely replaced with DOPC, the calculated L_3 lattice parameter was $123 \pm 1 \text{ \AA}$. This is similar to those observed for the corresponding DGMO-containing samples ($120 \pm 1 \text{ \AA}$, Table 1), indicating that substitution of DGMO with DOPC has a minor effect on the swelling behavior of the L_3 phase.

3.2.2 Samples that initially do not show the L_3 phase

3.2.2.1 Formulations with 25/75 GMO-50/DGMO

For samples with 25/75 GMO-50/DGMO, the SAXS analysis revealed that partial replacement of DGMO with DOPC gave the characteristic Bragg peak patterns of a bicontinuous cubic phase, predominantly featuring the Pn3m space group (Supplementary Figure S3A). However, complete substitution of DGMO with DOPC unveiled distinct peak ratios suggesting an L_α phase with a repeat

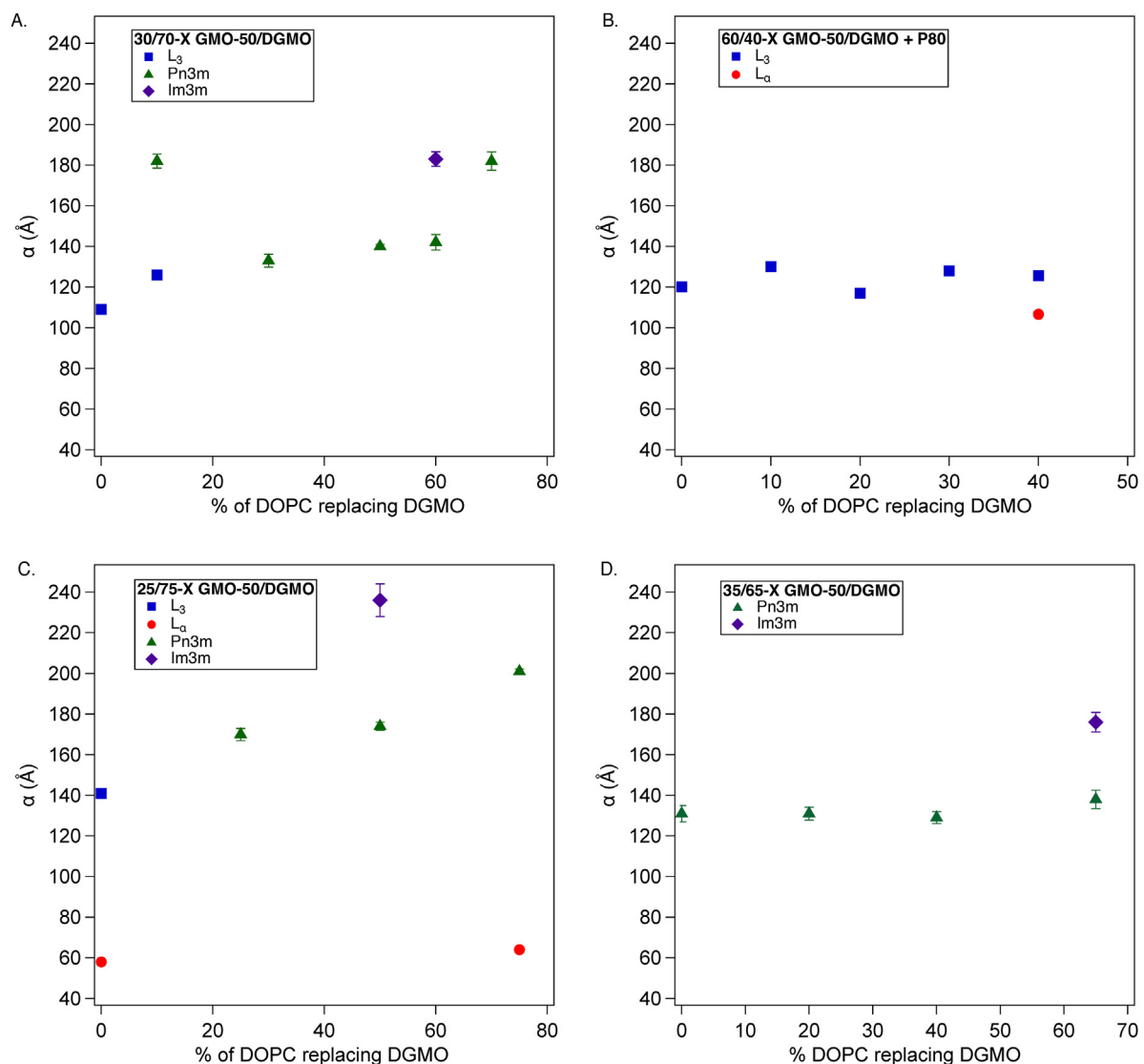


FIGURE 4

Change in the average lattice parameter, a , with replacement of DGMO with DOPC in (A) 30/70-X/GMO-50/DGMO/DOPC samples ($X = 0-70$ wt%), (B) 60/40-X/GMO-50/DGMO/DOPC samples ($X = 0-40$ wt%) + 30 wt% P80, (C) 25/75-X/GMO-50/DGMO/DOPC samples ($X = 0-75$ wt%), and (D) 35/65-X/GMO-50/DGMO/DOPC samples ($X = 0-65$ wt%). The lattice parameter was calculated from the diffractograms, using Equations 2, 3 for the lamellar and cubic phases, respectively. For the L_3 phase, the low- q peak was used as per Equation 1. The tabulated values are shown in the supplementary information, Supplementary Tables S5–S8.

distance of $64 \pm 1 \text{ \AA}$ as well as an additional, Pn3m phase with a lattice parameter of $201 \pm 2 \text{ \AA}$ (Supplementary Table S7).

3.2.2.2 Formulations with 35/65 GMO-50/DGMO

A transition from a pure Pn3m to a mixed bicontinuous cubic phase was observed with increasing DOPC content (Supplementary Figure S3B). The complete replacement of DGMO with DOPC resulted in additional Bragg peaks, indicating a two-phase region with Pn3m and Im3m bicontinuous cubic phases. The lattice parameter of the Pn3m cubic phase was not strongly affected by the replacement of DGMO with DOPC (Supplementary Table S8).

3.2.3 Effect of adding P80

The effect of P80 was further investigated for 75/25, 70/30, and 65/35 GMO-50/DOPC ratios with 30 wt% P80 hydrated to 50 wt%, 60 wt%, and 70 wt% MQ water at 25°C , and the results are shown in Supplementary Figure S4. The diffractograms show that the samples are dominated by the L_3 phase. Increased hydration leads to broader peaks, indicative of disorder in the system, a characteristic of L_3 phases. In addition, the correlation peak is shifted to lower q with increasing hydration. These results show that we can fine-tune the composition of the lipids and surfactant to produce a system with an L_3 phase or mixed L_3 phases.

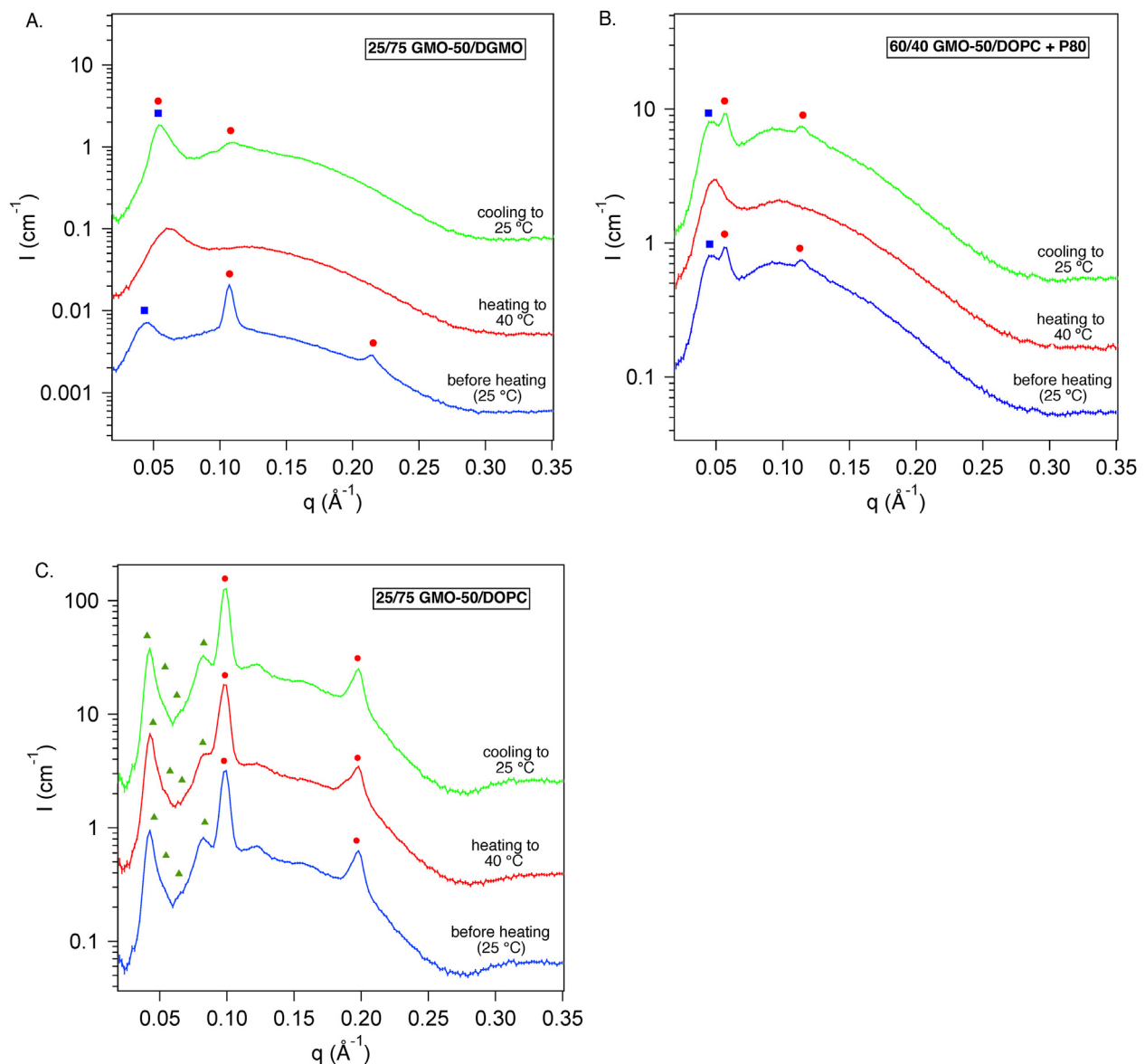


FIGURE 5
SAXS diffractograms showing the effect of temperature on bulk phases (A) 25/75 GMO-50/DGMO, (B) 60/40 GMO-50/DOPC + 30 wt% P80 and (C) 25/75 GMO-50/DOPC. All samples were hydrated to 60 wt% in MQ water. In data with coexisting phases, the peaks corresponding to each phase are marked as follows: L_{α} with red circles, L_3 with blue squares and Pn3m cubic phase with green triangles. Curves are offset for clarity.

3.3 The effect of temperature on bulk phase behavior

The phase behavior of these lipid systems in the temperature range of 25 °C–40 °C is of particular interest due to its relevance in pharmaceutical applications and physiological conditions.

For selected samples, the structure was characterized with SAXS over temperature scans from 20 °C to 40 °C (Figure 5; Supplementary Figure S5; Supplementary Table S9).

3.3.1 Formulations containing either DGMO or DOPC

3.3.1.1 Formulation with 25/75 GMO-50/DGMO

At 25 °C, the SAXS pattern shows the coexistence of L_3 and L_{α} phases (Figure 5A). Upon heating to 40 °C, a transition to a pure L_3 phase was observed. The structural change upon increasing the temperature was partially reversible, as subsequent cooling to 25 °C resulted in the reappearance of the phase coexistence region. However, the L_3 phase was more pronounced after cooling compared to the initial state.

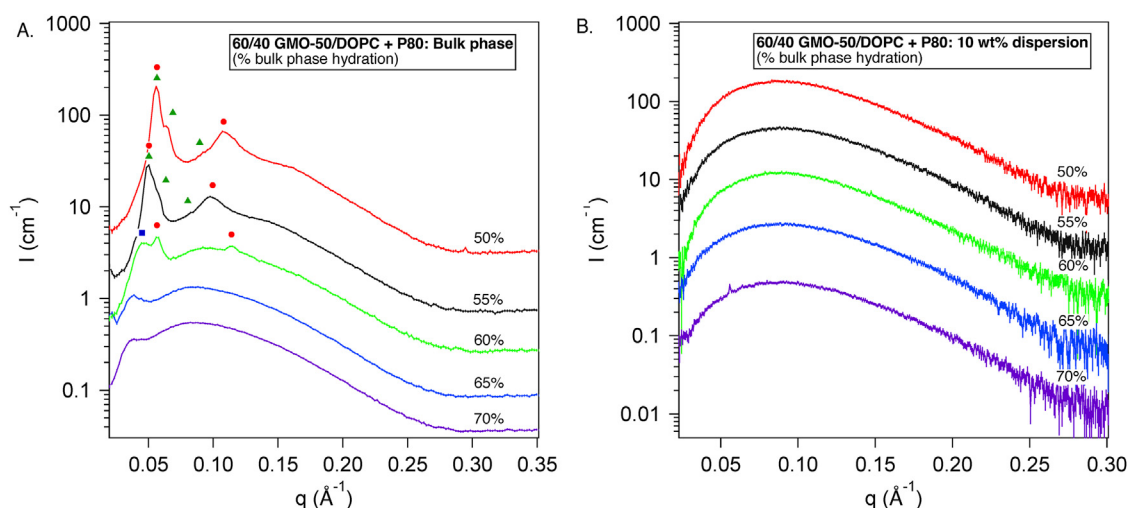


FIGURE 6
SAXS data for **(A)** 60/40 GMO-50/DOPC + P80 bulk phases hydrated with 50–70 wt% MQ water and **(B)** the corresponding bulk phases dispersed to 10 wt% of the bulk phase in MQ water (3–5 wt% lipid/P80). In data with coexisting phases, the peaks corresponding to each phase are marked as follows: L_{α} with red circles, L_3 with blue squares, and Pn3m cubic phase with green triangles. Curves are offset for clarity.

3.3.1.2 Formulation with 60/40 GMO-50/DOPC + 30 wt % P80

A mixed L_3 and L_{α} phase was also observed for 60/40 GMO-50/DOPC with 30 wt% P80 at 25 °C (Figure 5B). Upon increasing the temperature to 40 °C, the system transitioned to a pure L_3 phase. This transition was fully reversible, as the coexistence of the two phases was restored when the sample was cooled back to 25 °C.

3.3.1.3 Formulation with 25/75 GMO-50/DOPC

At 25 °C, the SAXS pattern showed the coexistence of Pn3m cubic and L_{α} phases at 25 °C, which were largely unaffected by heating to 40 °C and cooling back to 25 °C (Figure 5C).

3.3.2 Formulations containing DGMO and DOPC

Supplementary Figure S5A shows the SAXS data for the temperature scan for the 25/60/15 GMO-50/DGMO/DOPC sample. For this formulation with the lowest DOPC content, a transition was observed from a Pn3m bicontinuous cubic phase at 20 °C to an Im3m phase at 30 °C and 40 °C. In this case, the Im3m pattern remained when cooling back to 20 °C. This suggests that in this case, the equilibration time was not sufficient to return to the Pn3m phase.

When the GMO-50 and DOPC content was increased to 30/40/30 GMO-50/DGMO/DOPC (Supplementary Figure S5B) and 30/20/50 GMO-50/DGMO/DOPC (Supplementary Figure S5C), a bicontinuous Pn3m cubic phase, which did not change with temperature, was observed.

For the 15/35/50 GMO-50/DGMO/DOPC (Supplementary Figure S5D), the L_{α} phase did not change upon heating from 20 °C to 40 °C and cooling back to 20 °C.

At the highest DOPC content of 30/0/70 GMO-50/DGMO/DOPC (Supplementary Figure S5E), the SAXS pattern showed a Pn3m cubic phase at 20 °C, which became more well-defined

upon heating to 40 °C. Upon cooling to 20 °C, the structure transitioned to a mixed Pn3m/Im3m cubic phase. Interestingly, the position of the first peak of the Im3m phase appears to coincide with the position of a subtle shoulder in the 20 °C data before heating.

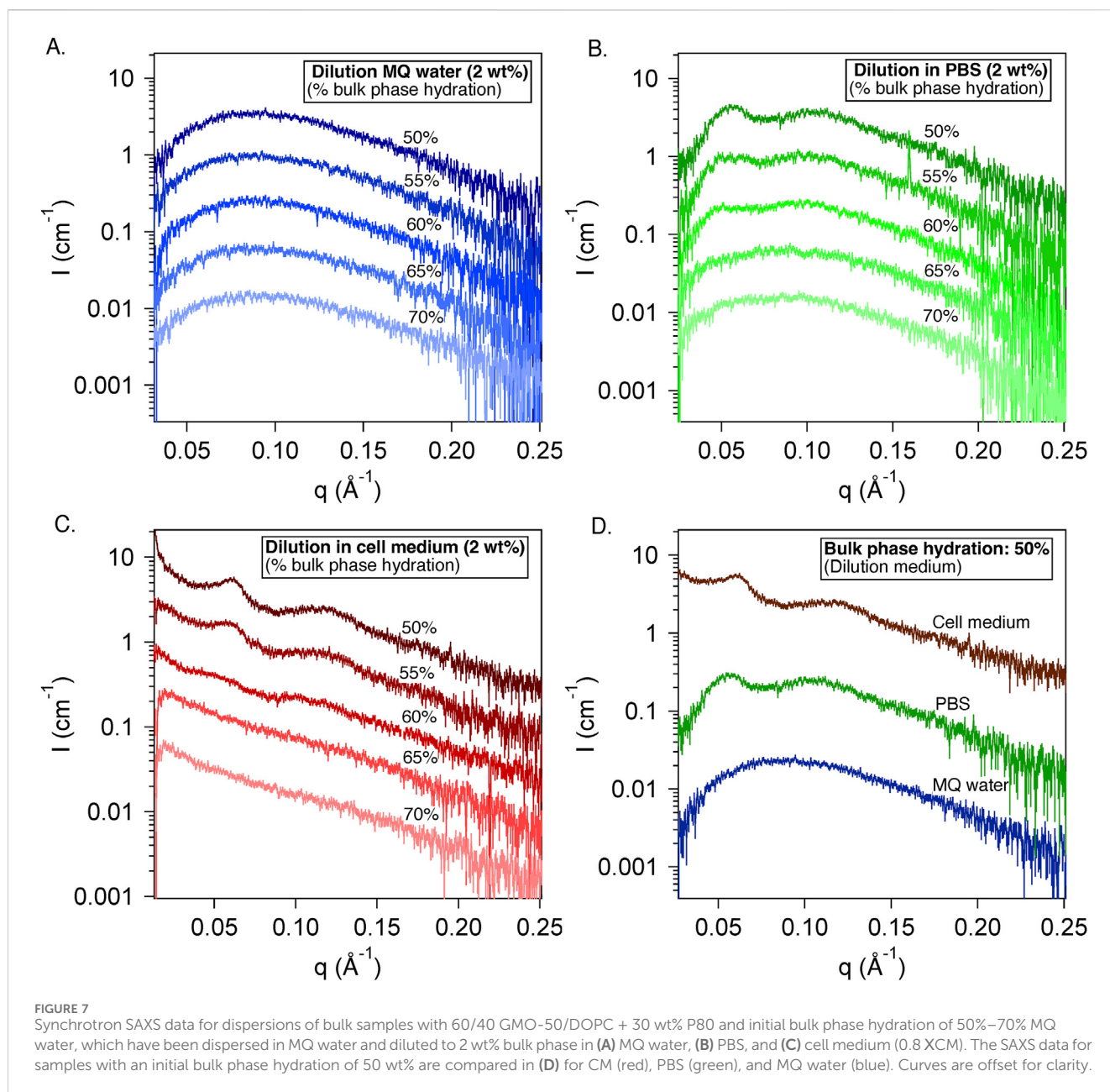
3.4 Dispersions of GMO-50/DOPC/ P80 formulations

From an application perspective, it is important to understand if and how (the structure of) a dispersion changes when diluted into the medium in which the dispersion is prepared, and into other media relevant to the application. For potential pharmaceutical applications, examples of such media include phosphate-buffered saline (PBS) and cell medium (CM).

We, therefore, investigated the effect of initial bulk phase hydration and dilution medium on the structure of formulations with a lipid composition of 60/40 GMO-50/DOPC + 30 wt% P80. Bulk phases with 50–70 wt% hydration (corresponding to 30–50 wt% lipid/P80) were prepared with and subsequently dispersed in MQ water to a final concentration of 10 wt% bulk phase (i.e., lipid/P80 concentration of 3–5 wt%). The 10 wt% dispersions were then diluted 2-fold and 5-fold in PBS and cell medium to bulk phase concentrations of 5 wt% and 2 wt%, respectively, in order to investigate the role of the aqueous media composition upon dilution.

3.4.1 Effect of hydration on the bulk phase

Bulk samples hydrated to 50–55 wt% displayed sharp Bragg reflections with a 1:2 q -ratio, consistent with lamellar (L_{α}) ordering. At 50% hydration, an additional peak (shoulder marked by a green triangle in Figure 6A) suggested the



coexistence of an additional structure. From the definition of the peak and comparison to previous observations for the corresponding GMO-50/DGMO + P80 sample (Valdeperas et al., 2016), it is likely that this is a cubic phase. This shoulder, though less pronounced, can also be seen in the diffractogram for the sample with 55 wt% hydration. Increasing hydration to 60 wt% yielded SAXS features characteristic of mixed L_α and L_3 phases, with the peaks corresponding to the L_α phase marked with red circles in Figure 6A. The samples hydrated to 65–70 wt% exhibited only broad peaks. The loss of the well-defined low- q reflections at higher hydrations indicates reduced long-range order and a more fluid phase. Although the observed broad peaks are in the expected positions for the L_3 phase, the intensity of the low- q peak height is lower, relative to the broader high q peak, than typically observed for L_3 bulk phases.

3.4.2 Dispersion of the bulk phase to 10 wt%

The bulk phases were dispersed to a concentration of 10 wt% bulk phase in MQ water. All samples exhibited a broad peak characteristic of the bilayer form factor, independent of the initial lipid content (Figure 6B). Notably, the L_3 phase correlation peak is not clearly visible after dispersion in excess water. It is present instead as a subtle shoulder on the low- q side of the main peak (highlighted in Supplementary Figure S6).

CryoTEM images comparing the 10 wt% dispersions with initial bulk phase hydrations of 60 wt% and 70 wt% after dilution to 2 wt% are included for qualitative comparison in the Supplementary Information (Supplementary Figure S7). It should be noted that cryoTEM images are included to qualitatively demonstrate the range of structures present and therefore serve as guidance when analyzing the scattering data. As always, artifacts can be

introduced when applying the sample to the grid, which cannot be ruled out. This should be taken into account as both the sponge phases and vesicle structures and morphologies can be sensitive to the shear forces when blotting the grid. Proper quantification of the ratio between different structures and morphologies based on cryoTEM also requires a large number of images and careful analysis, which is beyond the scope of this study.

3.4.3 Dilution in different media

SAXS diffractograms for the five dispersions diluted to 2 wt% in different dilution media (MQ water, PBS, and cell medium) are presented in Figure 7. CryoTEM images of selected samples are included in the [Supplementary Material](#) for qualitative comparison.

The SAXS data for the dispersions in MQ water (Figure 7A) contain a single broad peak independent of the initial bulk phase hydration. CryoTEM images of dispersions prepared from bulk phases initially hydrated to 60 wt% and 70 wt% in MQ water showed the coexistence of predominantly unilamellar vesicles (ULVs) with multilamellar vesicles (MLVs) for 60 wt% ([Supplementary Figure S7A](#)), and the coexistence of ULVs and L₃NPs for 70 wt% ([Supplementary Figure S7B](#)).

In contrast, the SAXS data for the PBS dispersions (Figure 7B) clearly depended on the initial bulk phase hydration. In the lower hydration samples, that is, those initially hydrated in 50–60 wt% MQ water, two broad peaks are present. As the initial hydration increased from 50–60 wt%, the lower q peak ($q \sim 0.055 \text{ \AA}^{-1}$) shifted to lower q and became less intense, until it was not visible at 65–70 wt% and only one broad bilayer form factor peak was present. The positions of the two broad peaks are consistent with L₃ phase nanoparticles; however, the 1:2 ratio of q could also be due to the presence of multilamellar vesicles (MLVs). The cryoTEM images ([Supplementary Figures S11B, S12A](#)) for these samples revealed predominantly ULVs and MLVs.

A similar trend was observed for the dispersions in cell medium (Figure 7C), wherein sharper peaks with a peak position ratio of 1:2 were observed for samples initially hydrated with 50–60 wt% MQ water, also decreasing in intensity with increasing initial hydration. Additionally, a strong low- q upturn is visible in all of the samples, with a slope independent of initial hydration.

A direct comparison of dispersions of bulk phases prepared with 50 wt% MQ water highlights the effect of the dilution medium (Figure 7D). In MQ water, only the bilayer form factor peak is clearly visible, whereas in PBS and CM, additional peaks at $q \sim 0.55 \text{ \AA}^{-1}$ and $\sim 0.1 \text{ \AA}^{-1}$ can be observed. In CM alone, a strong low- q upturn or power law component is visible. This media-dependent structural change was reproduced at both 2 wt% and 5 wt% dispersions ([Supplementary Figures S8–10](#)). [Supplementary Figure S8](#) compares the 2 wt% and 5 wt% of the bulk phase dispersions for an initial bulk phase hydration of 50 wt% MQ water, while [Supplementary Figures S9, S10](#) show the corresponding SAXS data for the 5 wt% dispersions in different dilution media. Altogether, the SAXS and cryoTEM data ([Supplementary Figures S11 and S12](#)) demonstrate that both initial hydration and the composition of the dispersion medium strongly influence the balance between particle morphologies.

4 Discussion

4.1 The phase diagram of the GMO-50/DOPC/DGMO system and the effect of adding P80

The phase behavior of the system is determined by the balance between chain conformation and headgroup interactions. Raman spectroscopy of the GMO-50/DGMO/P80 system has revealed that these are rather similar for bilayer-type structures like the lamellar phase, bicontinuous cubic phase, and sponge phase ([Talaikis et al., 2019](#)). However, careful analyses of the spectra revealed subtle but significant differences, which suggested that the hydrocarbon chains were less densely packed in the sponge phase than in the inverse bicontinuous cubic or lamellar phases, confirming the higher flexibility of the sponge phase.

Based on the SAXS data and visual inspection, the (pseudo) ternary phase diagrams for GMO-50/DGMO/DOPC/(P80) systems with a fixed water content of 60 wt% were constructed. [Figures 8A, B](#) illustrate the phases observed in the GMO-50/DGMO/DOPC system and the DOPC/DGMO/DOPC/P80 system, respectively, highlighting the influence of P80 in expanding the L₃ phase regions. The investigated samples for the GMO-50/DGMO/DOPC system are highlighted in [Supplementary Figure S13](#).

Both DOPC and DGMO promote the formation of the L _{α} phase, although DOPC does this to a larger extent. Notably, as DOPC levels increase, a shift toward more ordered packing ensues, as is indicated by sharp Bragg peaks in the SAXS profiles ([Figures 1, 3; Supplementary Figures S2, S3](#)). This can be related to the differences in their molecular architecture, which can influence their behavior in mixtures. DOPC possesses a relatively large polar headgroup and two hydrophobic tails, giving it a molecular volume of $1,303 \text{ \AA}^3$ and a headgroup area of $72 \text{ \AA}^2$ ([Tristram-Nagle et al., 1998](#)). DGMO has a single hydrocarbon chain with a molecular volume of $735 \text{ \AA}^3$ ([Valdeperas et al., 2019a](#)), which, based on a bilayer thickness of $37 \text{ \AA}$ (monolayer thickness of $18.5 \text{ \AA}$) from X-ray diffraction data ([Holstborg et al., 1999](#)), implies a headgroup area of $40 \text{ \AA}^2$. Thus, per hydrocarbon chain, the headgroup area is slightly smaller for DOPC ($36 \text{ \AA}^2$) than for DGMO, which implies slightly tighter packing. This is consistent with recent neutron reflectometry data, where we compared the properties of bilayers with the lipid compositions (wt/wt) investigated here: 60/40 GMO-50/DGMO, 60/10/30 GMO-50/DGMO/DOPC, and 60/40 GMO-50/DOPC (all containing 30 wt % P80). Here, we found that the DOPC-containing bilayers were slightly more densely packed ([Luchini et al., 2025](#)).

Another important distinction between DOPC and both GMO and DGMO is the markedly lower monomer solubility of long-chain phospholipids, which is sufficiently low that it is challenging to determine accurately ([Buboltz and Feigenson, 2005](#)). DOPC monomer solubility values of 10^{-10} M have been reported ([Ahmed et al., 2023; Tanford, 1976](#)). GMO, the main component of GMO-50, on the other hand, has a significantly higher monomer solubility of 10^{-7} M , as determined from the adsorption kinetics ([Campos et al., 2002](#)). DGMO is expected to have an even higher solubility due to its diglycerol headgroup.

It should be noted that both DOPC and DGMO have the same oleic acid chain, which could be expected to give high miscibility.

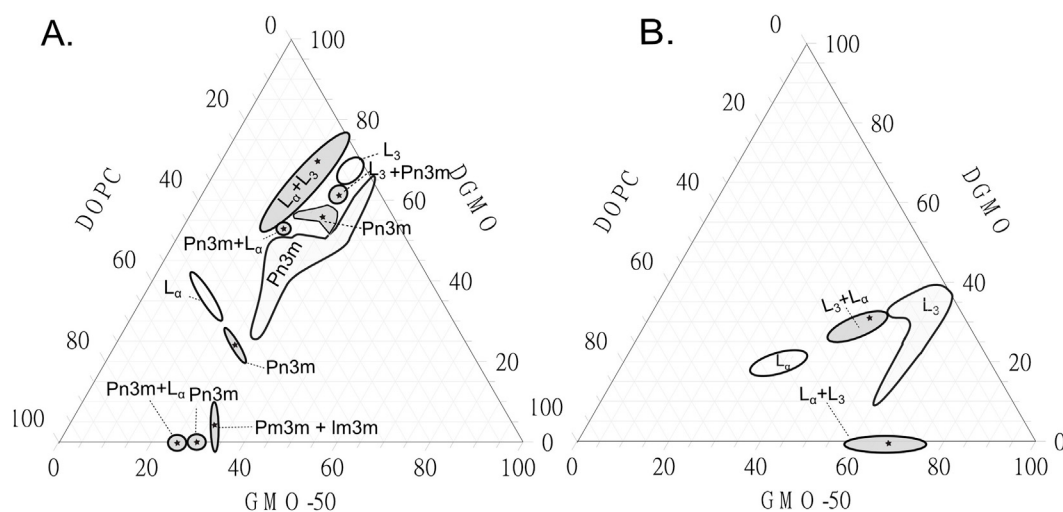


FIGURE 8

(A) Ternary phase map for the GMO-50/DGMO/DOPC system and (B) GMO-50/DGMO/DOPC/P80, where the lipid/P80 ratio is fixed at 70/30. All samples were hydrated to 60 wt% MQ water. The phases present include a sponge phase (L₃), a lamellar phase (L_α), and an inverse bicontinuous cubic phase with the Pn3m and Im3m space groups. Regions with phase coexistence are shaded in gray and marked with an asterisk (*).

However, the phase map reveals substantial regions of phase separation (Figure 8A). For instance, the L₃+L_α phase coexistence regions and the mixed Pn3m cubic phase region are maintained over a relatively wide range of DGMO/DOPC ratio at constant GMO-50 content. The lattice parameter increased slightly with the DOPC content for the L_α phase, provided that the content of DOPC was sufficiently high to enter the one-phase region and for DOPC up to 50 wt% of the lipid (Figure 2C; Supplementary Table S3). It is therefore likely that inter- and/or intra-headgroup interactions, which might include hydrogen bonding, play a role in defining the phase behavior. Here, we note that water forms strong hydrogen bonds and thus promotes hydration. There are a high number of available H bonds for GMO-50 and DGMO due to the hydroxyls on the glycerol headgroup, which are not present on the DOPC headgroup. However molecular dynamics (MD) simulations suggest that water molecules can also penetrate inside the zwitterionic (DOPC) bilayer and even interact with carbonyls of lipids, increasing the lipid molecular headgroup area and thickening the bilayer compared to, for example, the charged DOPS (Polyansky et al., 2005). For the Pn3m cubic phase, we noted that the lattice parameter increased with increased proportions of DOPC, at least in a range of compositions studied (Figure 2D) before transition to an L_α phase that occurs at high DOPC. This is indicated in the phase diagram (Figure 8A).

Interestingly, previous studies of GMO in excess water indicated that the addition of up to 25 mol% of DOPC induced swelling of the Pn3m cubic phase but did not alter the phase behavior, while a transition to a pure L_α phase was observed at 26 mol% DOPC and persisted up to 100 mol% (Cherezov et al., 2002). In the GMO-50/DGMO/DOPC system studied here, we note that the bicontinuous cubic phase persists to a higher fraction of DOPC than in GMO/DOPC, although often in coexistence with an L_α phase. As observed for the GMO-50/DGMO system studied by Valdeperas et al. (2016), in the GMO-50/DGMO/DOPC system, the bicontinuous cubic

phase prevails over a significantly larger composition range than the L₃ phase (Figure 8A), although adding P80 to the system studied here significantly increased the range over which the L₃ phase exists (Figure 8B).

4.1.1 The effect of P80 on the GMO-50/DGMO/DOPC system

It has previously been reported that the addition of P80 expands the L₃ phase region within the GMO-50/DGMO phase diagram (Valdeperas et al., 2016). Here, we also found that the monophasic regions of the L₃ phase are significantly larger (Figure 8B) with the addition of P80. Samples with P80 were less prone to phase transition with increasing amounts of DOPC than those without P80, indicating that it mixes in with the lipid bilayer, which is facilitated by the fact that it also has an oleic acid acyl chain. In fact, for formulations without P80, that is, 30/70, 25/75, and 35/65 GMO-50/DGMO mixtures, a transition away from the L₃ phase to L_α and bicontinuous cubic phases occurred as DGMO was replaced with DOPC. For example, the 60/40 GMO-50/DGMO + 30 wt% P80 formulations retained an L₃ phase even after substituting up to 30 wt% DOPC for DGMO (DOPC fraction of total lipid = 21 wt %), in contrast to the 30/70 GMO-50/DGMO sample, where a transition to Pn3m cubic phase was observed already at 10 wt% DOPC (Figure 3).

A high amount of GMO-50, which has a critical packing parameter (CPP) > 1 (Israelachvili et al., 1976), provides a net negative curvature of the lipid bilayer, and mixing with DOPC and/or DGMO, which have a CPP ≈ 1 and favour the L_α phase, decreases the CPP. With the addition of P80, which has a CPP < 1 and favors a micellar phase, the system can be tuned to form L₃ structures rather than cubic, reverse hexagonal, or L_α phases. This is due to its large, highly hydrated polar headgroup, which likely provides the higher flexibility and swelling that favors the L₃ phase. As concluded in previous studies, the transitions involving L₃ phases are governed

not only by the packing parameter of the lipids but also by the bending modulus (Brasnett et al., 2023).

4.2 Phase behavior at different temperatures

We have so far discussed the influence of lipid/surfactant composition in terms of tuning the phase behavior. Fine tuning of the structure can also be obtained by changing the temperature. An increase in temperature in lipid self-assembly systems usually leads to an increase in the apparent acyl chain volume and an apparent decrease in headgroup size (Evans and Wennerström, 1999). This leads to an apparent increase in the critical packing parameter (Israelachvili et al., 1976). Additionally, this increases the mobility in the system, which can, in turn, promote the formation of more fluid structures, such as the L_3 phase. In this study, increasing the temperature from 25 °C to 40 °C and then decreasing it back to 25 °C had an effect on the structures formed in some of the samples investigated (Figure 5). Data for additional compositions are shown in Supplementary Figure S5.

The most notable observation was that samples containing DOPC and exhibiting cubic and lamellar phases (Supplementary Figure S5) displayed only minimal structural changes with temperature. In contrast, samples with coexisting sponge and lamellar phases at 25 °C (Figure 5) underwent a (partially) reversible transition to pure L_3 upon heating to 40 °C. This is likely due to an increase in membrane curvature and molecular mobility that triggers the transition from the L_α phase to a less ordered L_3 phase. DOPC is less prone to phase changes at elevated temperatures than, for example, GMO-50 and has been observed to form an L_α phase at 10–40 wt% water up to temperatures over 200 °C (Bergensstahl and Stenius, 1987). GMO, which is the main component of GMO-50, on the other hand, transforms from a cubic to an H_{II} phase at above 80 °C. DGMO is not sensitive to temperature changes in the temperature ranges we investigated (Holstborg et al., 1999). Taken together, these observations strongly suggest that the structural transitions observed at 40 °C in our systems are driven primarily by the GMO-50 component, whose higher temperature sensitivity promotes the conversion of L_α phases into L_3 structures. As these temperature-induced structural changes occur between 25 °C (approximately room temperature) and 40 °C (close to physiological temperature), with suitable optimization of the formulation, it could be possible to design a formulation that changes structure and consequently releases its cargo upon administration to the body.

4.3 On the structure and morphology of dispersed phases of GMO-50/DOPC/P80 and the effect of the buffer

The structural response of sponge phase dispersions to dilution is critical for different applications, such as in delivery systems. Bulk phases were prepared at 50–70 wt% hydration with a composition of 60/40 GMO-50/DOPC + 30 wt% P80 and dispersed in MQ water to a bulk phase concentration of 10 wt%. These dispersions were then diluted to 2 wt% and 5 wt% bulk phase in MQ water, PBS, or cell

medium to assess the effect of ionic strength and complex media on their structure.

4.3.1 Presence of mixed populations in starting formulations

There is a strong dependence of the structure of the particle in the dispersion on the structure in the bulk phase. For bulk samples prepared at lower initial hydrations, the SAXS data indicated phase coexistence between an inverse bicontinuous cubic phase and an L_α phase at 50–55 wt% water, and between an L_3 and an L_α phase at 60 wt% water. In contrast, the structural features of the bulk samples hydrated to 65–70 wt% water were less well-defined. As previously noted, although the curves contain two broad peaks indicative of the L_3 phase in the expected positions, the intensity of the lower q correlation peak ($\sim 0.05 \text{ \AA}^{-1}$) compared to the higher q peak ($\sim 0.1 \text{ \AA}^{-1}$) is lower than that usually observed in the bulk L_3 phase (Valledeperas et al., 2016; Valledeperas et al., 2019a). This could suggest that the mechanical energy from mixing and the higher amount of water in these samples has resulted in partial dispersion of the bulk phase, as this decrease in correlation peak intensity is more commonly observed for the L_3 phase upon dispersion. In this case, it is likely that there are also coexisting populations of L_3 particles and vesicles, due to the high proportion of DOPC present.

Upon dispersion of non-lamellar bulk phases stable in excess water, the structure of the bulk phase is retained in the internal structure of the nanoparticle (Fornasier and Murgia, 2023). The bulk phases hydrated to 50–55 wt% were clearly not fully hydrated, as the structure continued to change with increasing hydration to L_3/L_α at 60 wt%. Upon dispersion in excess water, the cubic phase would be further hydrated to L_3 , which, along with the L_3 bulk phase, would form L_3 nanoparticles (L_3 NPs). L_α would be dispersed into uni- or multilamellar vesicles (ULVs/MLVs).

In the SAXS data of the 10 wt% dispersions in MQ water, the broad peak of the bilayer form factor, present in the characteristic SAXS patterns for both L_3 NPs and ULVs, was observed for all five dispersions. A very subtle shoulder can also be observed on the low- q side of this peak, which decreases in intensity with decreasing initial bulk phase hydration (highlighted in Supplementary Figure S6). In SAS data for L_3 dispersions, the low- q correlation peak typically decreases in intensity compared to the bulk phase data, often becoming a shoulder on the main peak, due to an increase in disorder with swollen structures at the surface enriched with P80 upon dispersion and coexistence with vesicles (Valledeperas et al., 2019b; Park et al., 2025). This indicates that the five 10 wt% starting dispersions contain mixed populations of L_3 NPs, ULVs, and MLVs in different proportions, with a higher proportion of vesicles at lower initial hydration due to the larger contribution of L_α in the bulk phase.

4.3.2 Dilution in different media

The five 10 wt% dispersions with different initial hydrations were then diluted 2-fold and 5-fold to concentrations of 5 wt% and 2 wt% bulk phase in MQ water, PBS, and cell medium (CM). In MQ water, the SAXS data were very similar to the 10 wt% starting formulations, although with a lower intensity due to the decreased

concentration (Supplementary Figure S8A). In PBS and CM, however, a clear effect of dilution was observed with the presence of two broad peaks with a 1:2 ratio, which increased in intensity as the initial hydration of the bulk phase. These changes can be rationalized by comparing the different possible responses of the L_3 and vesicle populations present to the change in solution conditions from pure MQ water.

The vesicles present in the dispersions have a closed structure and, therefore, will experience an osmotic pressure gradient across the membrane proportional to the difference in osmolarity between the inner and outer solutions (Zong et al., 2018). It has been observed in the literature that this osmotic pressure can cause deformation of ULVs and, at higher osmotic pressures, formation of MLVs and nested vesicles (Zong et al., 2018; Liu et al., 2022; Sambre et al., 2024; Piccinini et al., 2025). Piccinini et al. (2025) showed that when PBS is spiked into a sample of large unilamellar DOPC vesicles, which have been extruded in D_2O to 50 nm or 100 nm, this yields elongated and/or multilamellar structures. This transition from ULVs, with a single bilayer, to MLVs, containing multiple bilayers stacked concentrically, in response to an increase in osmotic pressure caused by dilution in PBS and CM, would explain the appearance of Bragg peaks in a 1:2 ratio observed in Figure 7. The d spacing of ~ 100 Å corresponding to these peaks is higher than the $d = 63.1$ Å distance expected for a pure DOPC lamellar phase (Tristram-Nagle et al., 1998), indicating that the other lipids were also present.

In work by Liu et al. (2022), studies of the response of giant unilamellar vesicles (GUVs) when exposed to a hypertonic osmotic gradient of sucrose similarly showed increasing deformation with increasing osmotic pressure. At osmotic gradients above 0.2 atm, the DOPC GUV underwent a dramatic morphological change to an elongated shape, which is much lower than the value of 4.7 atm calculated for PBS (Piccinini et al., 2025). Liu et al. (2022) additionally demonstrated that the extent of deformation of the GUVs decreased with increasing bending rigidity by comparing GUVs of DOPC > DMPC + 5 mol% cholesterol > DMPC + 30 mol% cholesterol. Zong et al. (2018) made similar observations by comparing DMPC GUVs containing increasing proportions of cholesterol. The bending rigidity of L_3 NPs composed of GMO-50/DGMO/P80 was previously determined by neutron spin echo (NSE) to be ~ 10 kBT at 25 °C (Gilbert et al., 2022), lower than the calculated bending rigidity of DOPC from MD simulations of 18.3 kBT (Doktorova et al., 2017). It could be speculated that the presence of GMO-50 and P80 in the formulation could decrease the bending rigidity of the vesicles present compared to pure DOPC, thereby making them deform more easily under osmotic stress.

The L_3 NPs present in the formulations, however, are unlikely to experience a strong osmotic gradient to the same extent due to the open water channel network in the structure, allowing access to and much faster equilibration with the bulk solvent upon dilution. In a previous study (Park et al., 2025) of L_3 NPs composed of GMO-50/DGMO/P80, a minor ordering effect was observed when bulk phases hydrated with phosphate or acetate buffers were dispersed into the same buffer compared to dispersion in MQ water. This implies that the presence of (a higher concentration of) buffer ions stabilized the L_3 structure upon dispersion. Only a minor decrease in lattice parameter was

observed by Brasnett et al. (2017) for the bulk cubic phase formed by monoolein (MO), the main component of GMO-50, when comparing the effect of the concentration of different salts (NaCl, LiCl, and $CaCl_2$). Similarly, for disordered cubic nanoparticles composed of MO/cholesterol/DOPC, only a minor decrease in water channel diameter was observed when increasing the concentration of NaCl from 50 mM to 500 mM (Barriga et al., 2022). Although it cannot be ruled out that a potential increase in the order of the L_3 from the addition of ions in solution could contribute to the lower q peak (~ 0.05 Å⁻¹), it would be unlikely that it would result in the formation of the higher q peak (~ 0.11 Å⁻¹).

In summary, it is likely that the proportion of ULVs (compared to L_3 NPs) present in the initial 10 wt% formulations was higher with a lower initial hydration due to the larger proportion of L_α in the bulk phase. These ULVs might have undergone a transition to MLVs in response to the osmotic pressure gradient introduced by dilution in PBS and CM, resulting in more intense Bragg peaks, whereas the L_3 NPs remained mostly unaffected.

It can also be noted in Figure 7 that the peaks observed for the 50–60 wt% initial hydration samples are sharper in CM than PBS. It was not possible to determine the exact cause of this increased order due to the highly complex nature of the CM, which contains fetal bovine serum (FBS). FBS, derived from fetal cow blood, contains over 1,000 components, including a wide variety of proteins, lipids, amino acids, sugars, hormones, and electrolytes, such as calcium (Johnson, 2012).

The influence of lipids and electrolytes has been discussed above and the interactions with the other components can have similarly strong effects. To take the protein component as an example, the effect of protein interactions on the L_3 phase structure is highly dependent on the nature of the protein and the lipid composition of the particle, which is highly likely to vary between the different structural sub-populations. Previous studies of protein interactions with an L_3 phase formed of GMO-50/DGMO/P80 showed a dehydrating/ordering effect for the enzymes β -galactosidase and aspartic protease, but the opposite for myoglobin and phytohemoglobin (Gilbert et al., 2019; Valdeperas et al., 2019b; Gilbert et al., 2023). Conversely, when DOPC was introduced to the formulation, either fully or partially replacing DGMO, the addition of myoglobin to a deposited layer of lipid did not affect the structure, whereas the layer without DOPC was fully removed from the surface (Luchini et al., 2025).

The low-q upturn or power law observed for all CM samples, independent of initial hydration, could be a result of the increased size of the particles, or even aggregation.

4.3.3 Importance of the medium in pharmaceutical applications

In practical environments, it is important to be aware of possible changes in the structure and stability of the formulation when it comes into contact with a different medium. With suitable optimization, this can be leveraged to improve the effectiveness of the formulation. One possible option is to trigger cargo release, that is, release when reaching a specific environment triggered by a change in, for example, pH, temperature, or hydration. The effect of hydration is demonstrated in the FluidCrystal® technology from Camurus AB, in which a transition from an Fd3m micellar cubic to a

hexagonal phase is observed as the lipid depot becomes hydrated, which allows controlled release of an active pharmaceutical ingredient (API) over an extended time period (Engstedt et al., 2025). A commonly discussed example of pH-induced structural changes is the use of ionizable lipids in lipid nanoparticles for nucleic acid delivery, in which pH-induced structural changes are relevant both during formulation, for effective complexation of the nucleic acid, and during cargo release, to interact with the endosomal membrane. Optimization of the structure and pKa of ionizable lipids and understanding the pH-induced changes in LNP formulation structure has been a key area of study for the success of these formulations (Hou et al., 2021). It is also important to consider how the biomolecules in the body will interact with the formulation. When a formulation comes into contact with a biofluid, the particles will tend to acquire a corona of proteins present in that biofluid, which can affect the particle structure, cellular uptake, and cargo delivery efficacy. However, the complexity of the proteins present in biofluids makes this a challenging area to study (Sebastiani et al., 2021; van Straten et al., 2024; Voke et al., 2025).

5 Conclusion

The previous work on L_3 phases composed of GMO-50, DGM0, and P80 has proven useful for the encapsulation of enzymes and other bio-functional proteins for biomolecular delivery applications in, for example, the food industry. Here, we show that adding the zwitterionic phospholipid dioleoylphosphatidylcholine (DOPC) will allow us to further tune the lipid nanostructures and the lipid–aqueous interface that is crucial for the encapsulation capability as well as the biocompatibility of the dispersed particles. DOPC promotes a transition away from the L_3 phase compared to DGM0; however, this can be counteracted by increasing the temperature and tuning the lipid composition. This was demonstrated with 60/40 GMO-50/DOPC + 30 wt% P80 in water, which transforms from a mixed L_α and L_3 phase at 25 °C to a neat L_3 phase at 40 °C. This temperature sensitivity close to physiological temperature offers an opportunity for use in pharmaceutical preparations that rely on temperature-dependent phase transitions. Hence, with suitable optimization, the use of L_α and L_3 phase systems in biopharmaceutical encapsulation of targeted and controlled release can be explored using this system. Our results also showed the importance of dilution after formulation and how this can affect the structures present in the dispersions. This has implications for the performance as delivery vehicles, for example, when lipid nanoparticles are added to cell medium for cell transfection studies or when lipid particles are injected into muscular tissue. Altogether, these results imply that not only is it important to investigate the lipid nanoparticles in their storage dispersion medium but also to investigate how they are affected when added to a new environment with a different medium composition.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Author contributions

MM: Investigation, Data curation, Writing – review and editing, Methodology, Conceptualization, Formal Analysis, Validation, Writing – original draft. AM: Data curation, Methodology, Investigation, Conceptualization, Validation, Writing – review and editing, Formal Analysis, Writing – original draft. AK: Investigation, Data curation, Writing – review and editing, Methodology, Formal Analysis. JB: Supervision, Writing – review and editing, Writing – original draft, Data curation, Conceptualization, Validation. TN: Investigation, Writing – original draft, Funding acquisition, Resources, Conceptualization, Writing – review and editing, Supervision, Formal Analysis, Project administration, Data curation, Validation, Methodology. JG: Investigation, Data curation, Supervision, Methodology, Writing – review and editing, Validation, Conceptualization, Writing – original draft, Formal Analysis.

Funding

The authors declare that financial support was received for the research and/or publication of this article. This study was supported by funding from the Swedish Research Council (Vetenskapsrådet) (grant number 2020-05421) and NanoLund, Lund University (project no: p12-2021). Research conducted at MAX IV, a Swedish national user facility, was supported by the Swedish Research Council under contract 2018-07152, the Swedish Governmental Agency for Innovation Systems under contract 2018-04969, and Formas under contract 2019-02496.

Acknowledgments

The authors are grateful for the skillful assistance of Dr. Crispin Hetherington (cryoTEM) and Dr. Ann Terry (SAXS) and illuminating discussions with Prof. Margaret Holme.

Conflict of interest

Author JB was employed by Camurus AB.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/frsfm.2025.1708264/full#supplementary-material>

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