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Experimental and computational approaches to the lipid L₃ sponge mesophase

Christopher Brasnett

A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy in the School of Physics, Faculty of Science.

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Among a multitude of studies on lipid polymorphism over the last half century, the sponge mesophase remains relatively little-studied, and poorly understood.

The sponge (L₃) mesophase consists of a lipid bilayer separating the system into two bicontinuous water channels. In that sense, it is closely related to the so-called cubic phases, which possess cubic translational symmetry. Unlike the cubic phases, however, the sponge mesophase is aperiodic, its underlying surface often described as being ‘random’. While cubic phases have been the subject of much study due to their potential applications for membrane protein crystallisation, there is increasing evidence to suggest that this occurs via the sponge mesophase, creating a significant gap in our understanding of the process, and how to maximise chances of success. This thesis therefore seeks to further our understanding of the dynamics of the sponge mesophase through a mixture of experimental (X-ray scattering) and computational (molecular dynamics) approaches.

Chapters 1 and 2 introduce the subject of lipid polymorphism, the sponge mesophase, and outlines the theoretical basis of the techniques used to study lipid systems in this work.

In chapter 3, we conduct a systematic type-doping study of the sponge mesophase with common co-crystallisation amphiphiles. We show that the sponge mesophase is significantly less adaptable than the cubic phase, with significant implications for the engineering design of membrane protein crystallisation media.

In chapter 4, we develop computational techniques with which to study lipid mesophases using molecular dynamics. Chapter 5 then utilises the these tools to study the cubic to sponge transition using coarse grained molecular dynamics simulations, showing that the system undergoes the transition on very rapid timescales, and is particularly driven by a combination of a reduction in elastic moduli and increase in interfacial area.

In chapter 6, we study the effect of an interface on the sponge mesophase by combining acoustically levitated droplets with X-ray scattering studies. While the study is overall inconclusive, we present a series of analysis methods which could be utilised to resolve the subject in future.

Chapter 7 summarises the key results of the thesis, and presents an outlook on the future of the topic of lipid sponge mesophases.
I declare that the work in this dissertation was carried out in accordance with the requirements of the University’s Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate’s own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .................................................... DATE: ..........................................
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For my parents
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1.1 Lipids and membranes

Lipids are ubiquitous throughout the biological world, as one of the principal components for cell membranes. As illustrated in fig. 1.1, cell membranes consist of a mixture of lipids and membrane-bound proteins, called membrane proteins.

**Figure 1.1.** A cross-section of a model cell membrane comprised of lipids (coloured with red headgroups and white tails) and proteins. The cytosol with free-floating organelles is coloured in blue.
CHAPTER 1. INTRODUCTION

Figure 1.2. The chemical structure of several common lipids. a) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, b) 1,2-distearoyl-sn-glycero-3-phosphocholine, c) Cholesterol. In a), the head and tail regions of the chemical structure are marked out by blue and red regions respectively. The glycerol linker group can be seen joining them.

The illustration in fig. 1.1 is of course a vast oversimplification of a real membrane: far from the picture presented, cells use around 5% of their genes to synthesize lipids, of several different functional classes not just for cell compartmentalisation, but also energy storage and signalling [1, 2]. That is, lipids play a significant role in the regulation of biological processes, rather than acting as a passive support for proteins - they both play an essential role in regulating each others production and function [3–6]. Such diversity of lipid species can cause lateral phase separation into liquid-ordered and liquid-disordered domains, a process which is often driven by cholesterol (see, eg. [7, 8]). Cholesterol is particularly associated with membrane stiffening and condensing, likely as a result of an increase in the order of phospholipid tails, and a mismatch between the lengths of phospholipid and sterol tails [9–14].

Lipids are able to bring about the remarkable range of structures that they do thanks to their amphiphilic molecular structure: they have domains that are attracted to, and repulsed by, water. The chemical structures of three common membrane lipids are shown in fig. 1.2, where we can see that they consist of a hydrophilic headgroup such as choline or ethanolamine, a
1.2 Self-assembled mesophases

In an aqueous environment, lipids and other amphiphilic molecules will self-assemble into aggregates, driven primarily by the hydrophobic effect: it is energetically unfavourable for hydrophobic regions of the molecules to be exposed to water, so above a critical concentration, an aggregate will form to protect hydrophobic tails from water. The aggregates will usually take on the form of a lyotropic liquid crystal mesophase, with long-range periodicity and short-range disorder. The final self-assembled form of the mesophase will naturally be dependent on a variety of physical factors such as temperature and pressure, as well as physico-chemical properties of the molecules that it comprises of.

Naturally, these properties are principally determined by two of the three chemical sections of an amphiphile previously highlighted: the headgroup and the fatty acid tails. Amphiphiles

hydrophobic carbon tail which - such as in the case of sterols - can contain aromatic rings. These two domains are connected via a linking group such as (in the case of phospholipids) glycerol.
may have 1 or more tails, which could be saturated or unsaturated. Similarly, headgroups could be charged or zwitterionic. Varying either of these factors can result in incredible structural diversity in self-assembled mesophases. Israelachvili proposed that amphiphiles could be described by a geometric packing parameter to encapsulate these factors:

\[ S = \frac{v}{a_0 l_c} \]  

(1.1)

where \( v \) is the volume of the hydrophobic tails, \( l_c \) is their extended length, and \( a_0 \) is the interfacial area per molecule at the polar/non-polar interface [15]. These parameters are illustrated in 2d in fig. 1.3. The type of aggregate that will self-assemble will then depend on the value of \( S \), which will take on a value less than, greater than, or equal to 1.

By inspection of eq. (1.1), we can see that if \( S < 1 \), then we have a molecule with a large interfacial area, a short tail, and a small volume. Therefore, aggregates will tend to form spherical micelles. These type I molecules are often found as detergents (eg. n-Dodecyl β-D-maltoside, DDM) or single-tail lipids. Type 0 molecules will be more ‘cylindrical’ in shape, and tend to form flat bilayers: common membrane phospholipids, such as 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) are type 0 lipids. When \( S > 1 \), the length and volume of the tails dominate over the interfacial area, and induce curved regions into the system. For example, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) has a smaller headgroup (ethanolamine) compared to DOPC, but has identical tails. The reduction in headgroup area results in DOPE spontaneously forming an inverse H_{II} mesophase in water, compared to the flat L_{α} system of DOPC.

Overall, it should be noted that while the molecular packing parameter described in eq. (1.1) is a useful description of molecular geometry, it will vary with other physical forces, such as temperature and pressure. For example, an increase in temperature will result in an increase in the effective volume of the tail region of lipids through greater thermal mobility, and thus increase the magnitude of \( S \) [16, 17]. In the case of the latter, increasing pressure will tend to increase tail ordering and so lead to swelling of mesophases [18, 19].

In fig. 1.3, we have seen several simple examples of possible mesophases that can self-assemble from lipids in water. However, mixtures of lipids and the use of solvents other than water can have significant effects on the self-assembled system observed. In addition to the effect on lateral membrane organisation as discussed above, the inclusion of dopant lipids can cause
packing frustration in membranes, which can be resolved through the system bending away or towards the solvent interface. In doing so, the membrane will exhibit curvature.

### 1.3 Curvature

#### 1.3.1 Defining curvature

Mathematically, curvature is an intrinsic property of a curve or surface. That is, the curvature of a curve or surface depends only on distances measured from point to point within the object, and not on any other external variables. If one were to look at a map of say, the Lake District, then perhaps the most important information required to plan a walk is not so much the grid spacing to describe the scale. Rather the contour lines of the hills describing how steep each one is will tell you the distance you actually travel between successive peaks\(^1\).

Alternatively, as Pressley puts it, for an insect who only readily experiences the flatland of the surface on which they live, they might be interested in the distance between two points which sit on the surface. Importantly, this distance will be different from the linear distance contained in Euclidean space. In the case of either the hiker or the bug, the metric they will both be interested in is the first fundamental form, \(I\), of the surface. From here, we will summarise the derivation of Pressley to explain how measurements of surface curvature arise [20]. At a point \(p\) on a surface \(\mathcal{S}\) with tangent vectors \(t_1\) and \(t_2\) as indicated in fig. 1.4, the first fundamental form is simply the inner product of the vectors:

\[
\langle t_1, t_2 \rangle = t_1 \cdot t_2
\]

(1.2)

This can more conveniently be expressed by considering a parametric form of a surface in the following way. For a surface defined by \(X(a, b)\), any tangent vectors \(w\) are a linear combination of the surface’s partial derivatives \(X_a\) and \(X_b\). So if:

\[
da(w) = \alpha \\
\db(w) = \beta
\]

(1.3)

then we have a tangent vector \(w\) defined by:

---

\(^1\)Unless one has access to a helicopter. But one suspects that might be beyond the scope of most UKRI grant proposals.
\[ w = aX_a + \beta X_b \] (1.4)

Then by using the inner product as before, we see that:

\[ \langle w, w \rangle = a^2 \langle X_a, X_a \rangle + 2\alpha \beta \langle X_a, X_b \rangle + \beta \langle X_b, X_b \rangle \] (1.5)

The first fundamental form is then usually denoted by \( I \) in the form:

\[ I = Eda^2 + 2Fdadb + Gdb^2 \] (1.6)

So by comparison with eq. (1.5), we can see that:

\[ E = \|X_a\|^2 \quad F = X_a \cdot X_b \quad G = \|X_b\|^2 \] (1.7)

To complete the problem of the bug on the curved surface, if \( \zeta \) is a parametric curve lying on \( X(a, b) \), then it can be defined by:

\[ \zeta(t) = X(a(t), b(t)) \] (1.8)

The distance travelled on the surface, \( d \), is then simply defined by the path integral:

\[ d = \int \|\dot{\zeta}\| \, dt \] (1.9)

which, worked through noting that \( \dot{\zeta} = aX_a + \beta X_b \) by the chain rule, shows us the relation to the first fundamental form:

\[ d = \int \langle \dot{\zeta}, \dot{\zeta} \rangle^{\frac{1}{2}} \, dt = \int [Ea^2 + 2F\dot{a}\dot{b} + Gb^2]^{\frac{1}{2}} \, dt \] (1.10)

The first fundamental form is then complemented by the second fundamental form. Together, they can completely define the properties of a surface. The second fundamental form, \( II \), is related to the rate at which the normal vector, \( n \), of the surface changes on a path along the surface:

\[ II = Lda^2 + 2Mdadb + Ndb^2 \] (1.11)

where:

\[ L = X_{aa} \cdot n \quad M = X_{ab} \cdot n \quad L = X_{bb} \cdot n \] (1.12)
1.3. CURVATURE

Using the first and second fundamental forms, we can then look to define the curvatures that describe a surface. The fundamental forms can be written as symmetric 2 x 2 matrices:

\[
M_I = \begin{pmatrix} E & F \\ F & G \end{pmatrix} \quad M_{II} = \begin{pmatrix} L & M \\ M & N \end{pmatrix}
\] (1.13)

To begin, we consider how normal vectors are related to the properties of a surface. If the direction of the normal vector is changing rapidly in a small region of a surface, then it must be highly curved. Conversely, if it varies very little, the surface will be almost flat. The set of normal vectors on a surface \( \mathcal{S} \) is defined by the Gauss map \( \mathcal{G} \), which provides a mapping of the surface normal vectors to a unit sphere. The derivative of the Gauss map from a point \( p \), \( D_p \mathcal{G} \) then naturally describes the rate at which the normal vectors vary across \( \mathcal{S} \). The derivative is then called the Weingarten map or shape operator, \( \mathcal{W}_{p, \mathcal{S}} \), written with a minus sign by convention:

\[
\mathcal{W}_{p, \mathcal{S}} = -D_p \mathcal{G}
\] (1.14)

The shape operator is related to the first and second fundamental forms in the following relation:

\[
\mathcal{W}_{p, \mathcal{S}} = M_I^{-1} M_{II}
\] (1.15)

Most importantly, the eigenvectors and eigenvalues of the shape operator at any point on the surface are the directions and magnitudes of the principal curvatures of the surface at that location. That is, they are a measure of the directions the surface is bending in, and the magnitude of the bending. Principal curvatures can more intuitively be thought of as the reciprocals of the radii of two orthogonal arcs fitted to the surface around a point. The shape operator also has other uses, in its determinant and trace result in the two measures of curvature that describe a surface, the Gaussian (K) and mean (H):

\[
K = \det(\mathcal{W}) \quad H = \frac{1}{2} \text{Trace}(\mathcal{W})
\] (1.16)

Using eq. (1.16), we can combine the definitions we obtained in eq. (1.13) and eq. (1.15) to write the curvatures in terms of the fundamental forms:
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Figure 1.4. At a spot on a curved surface, the eigenvectors and eigenvalues of the shape operator are the directions and magnitudes of the principal curvatures respectively. Intuitively, the curvatures are the reciprocals of the radii of the circular arcs in each direction.

\[
K = \frac{\det(M_{II})}{\det(M_I)} = \frac{LN - M^2}{EG - F^2}, \quad \quad H = \frac{1}{2} \text{Trace}(M_I^{-1}M_{II}) = \frac{LG - 2MF + NE}{2(EG - F^2)} \quad (1.17)
\]

Alternatively, if we consider a tangent plane where the shape operator is simply defined by the directions, \(\{c_1, c_2\}\) and magnitudes, \(k_1\) and \(k_2\), of the principal curvatures, we end up with definitions of \(K\) and \(H\) that are more familiar, showing that they are the product and mean of the two principal curvatures:

\[
K = k_1 k_2 \quad \quad H = \frac{1}{2}(k_1 + k_2) \quad (1.18)
\]

These definitions of the principal curvatures are best illustrated by the surface in fig. 1.4. At a point on the surface, there are two orthogonal great circles in the directions of the eigenvectors of the shape operator, and whose radii are the reciprocals of the principal curvatures. Further examples of the definitions of \(K\) and \(H\) as described by eq. (1.18) are shown in fig. 1.5 and
1.3. CURVATURE

![Figure 1.5](a) (b) (c)

**Figure 1.5.** A saddle surface with negative Gaussian curvature viewed from 3 different perspectives rotated about its origin point. The negative Gaussian curvature is evident from the opposite directions of the circles of greatest curvature at the origin is clear from the orthogonal views of the surface. I.e. At the location of the axes, the surface is bending away from itself in two opposite directions.

![Figure 1.6](a) (b) (c)

**Figure 1.6.** A surface with positive Gaussian curvature viewed from 3 different perspectives rotated about its origin point. The positive Gaussian curvature is clear from the orthogonal great circles at the origin bending in the same direction.

Fig. 1.6, showing surfaces with negative and positive curvatures respectively. In the saddle surface in fig. 1.5, the principal curvatures point in opposite directions, such that at the origin the surface has negative Gaussian curvature and zero mean curvature. The spherical section in fig. 1.6 has both positive Gaussian and mean curvature, as the principal curvatures - uniquely defined in any direction along the surface from the origin - point in the same direction. Other examples of surfaces would include a cylinder, which has positive mean curvature, but zero Gaussian curvature.

For further reading and alternative explanations on the derivation of curvature, readers are referred to *Visual Differential Geometry and Forms: A Mathematical Drama in Five Acts* by Tristan Needham, published since the initial writing of this thesis [21].
1.3.2 Minimal Surfaces

A minimal surface is simply defined by the property of having zero mean curvature at all points. The most trivial example of this is a plane, whose principal curvatures are zero everywhere. A less trivial example would be the catenoid surface, a surface based on revolving the curve \( y = \cosh(x) \) around the x axis \([20]\).

Less trivial still are the triply periodic minimal surfaces. The first examples of these are the so-called Primitive (P) and Diamond (D) surfaces, discovered by H. A. Schwarz. The Diamond is so called because its symmetry is related to the crystal structure of Diamond. The Primitive surface is related to the primitive cubic lattice. These were later added to by Schoen in 1970, who identified many more surfaces, including the closely related Gyroid \([22]\). The three surfaces are shown in fig. 1.7, where they are associated with their crystallographic space groups. At every point on these minimal surfaces, the surface is a saddle surface such as the one seen in fig. 1.5, so it is bending away from itself in two directions, with negative Gaussian curvature.
1.4 Some more exotic self-assembled mesophases

The discovery of the family of minimal surfaces described in section 1.3.2 was quickly related to physical phenomena, as detailed by Schoen in his reflections on their discovery [23]. These initially included the ordering of plastids in leaves, and the crystalline structure of the calcite skeletons of sea urchins [24–26]. Since these, structures based on triply periodic minimal surfaces have been found in a remarkable variety of systems, from fungi to mammalian cells [27]. Perhaps the most well-known examples of cubic phases in nature are the photonic crystals based on gyroids that are responsible for the structural colour of butterfly wings [28–31].

More importantly for studies of lipids, one of the early systems identified by Schoen for its relevance to naturally-formed minimal surfaces was the result of Luzzati and Spegt showing that strontium myristate heated to 235°C formed a cubic crystalline structure with space group Ia3d. Based on X-Ray scattering, they postulated that the molecular arrangement must be based on the polar ends forming two interconnected networks of rods, and the paraffin chains forming an otherwise disordered matrix [32]. The same group also reported cubic phases formed at lower temperatures with lipids and water, showing that it was possible to form cubic phases with either polar headgroups and water inside the rods, or conversely, the paraffin chains [33]. Reporting the result of Luzzati and Spegt and Luzzati et al. in the manner of two interconnected networks of rods seems to have no relevance at first to the topic of minimal surfaces, but it can be rationalised if instead of considering the locations of the headgroups of the lipids used (which, being strontium, would have scattered far more strongly than the paraffin chains), we consider that the paraffin chains must have some connection with the underlying $Q^{G}_{II}$ surface.

The relevance of minimal surfaces to the field of lipid mesophases became more prominent with the work of Larsson et al., who based on the analysis of the data of Lindblom et al. showed that a system of monoolein and water forms a cubic phase, and, for the first time, linked it to the primitive minimal surface [34, 35]. They further posited that the system must consist of a lipid bilayer sitting on the surface in a unit cell, partitioning the system into two distinct water channels. As Longley and McIntosh subsequently showed, however, their analysis was incorrect, and they should have actually found from their X-Ray data that the system showed a symmetry of Pn3m - the Diamond surface [36]. This was further confirmed by Hyde and Andersson, who showed that the primitive surface was inconsistent with the relationship between

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(a) The chemical structure of monoolein.

(b) The water content-temperature phase diagram of monoolein. At room temperature, monoolein will spontaneously form cubic mesophases: a $Q^{G}_{II}$ at low water content, and $Q^{D}_{II}$ at higher- and excess-water content.

**Figure 1.8.** a) The chemical structure of monoolein. b) The water content-temperature phase diagram of monoolein, as measured by Qiu and Caffrey [17].

the lattice parameters measured for low and high water contents. As Schoen showed that the 3 surfaces in fig. 1.7 are related through Bonnet transformations (i.e. with no changes in curvature, only bending), the lattice ratios must be fixed [22]. Using this fact, and by measuring the lattice parameters of the mesophases at different water contents, Hyde and Andersson showed that at a low water weight content the monoolein/water system must have a Gyroid surface bilayer, which becomes a Diamond surface at high water content [37].

1.5 A cubic phase-forming molecule: monoolein

Since the discovery that bicontinuous cubic phases can be formed spontaneously from monoolein, the molecule has been the subject of extensive study. The chemical structure of monoolein is shown in ??, where it can be seen that as a monoacylglyceride, it is comprised of a glycerol
headgroup linked to a single hydrocarbon chain via an ester bond. Like lipids, it is therefore an amphiphilic molecule, and as we have seen, it can form lyotropic liquid crystals spontaneously in water. However, monoolein is not a biological lipid found in cell membranes, although it can be biologically synthesised. Most commonly, monoolein, and other mono-, di-, and triglycerides are used as emulsifiers in the food industry.

The mesophase behaviour of monoolein was first systematically studied by Caffrey et al., who showed that in excess water at room temperature and pressure, it will form a $Q_{DII}^D$ mesophase [16, 17]. By ‘excess water’, we mean the point at which if any more water is added to the lipid, it has no effect on either the mesophase or its size - it sits in coexistence with the mesophase. At higher temperatures the system undergoes a mesophase transition to the $H_{II}$ mesophase driven by an increase in chain splay, while with decreasing water content first a $Q_{II}^G$ and subsequent $L_\alpha$ mesophase are observed as a result of decreased hydration per individual molecule. A similar sequence of mesophase behaviour is exhibited by other monoacylglycerides, with the prominence of the $Q_{DII}^D$ mesophase proportional to the chain splay of the molecule [38]. At room temperature in excess water, the $Q_{DII}^D$ mesophase is relatively stable across a wide range of pressures, exhibiting a transition to the $L_\alpha$ mesophase at pressures of around 1.4 kbar [39, 40].

1.6 Yet more still: the sponge mesophase

In addition to the well-characterised mesophases discussed so far, there is exists a mesophase not formed spontaneously by any lipid system, but has attracted considerable interest for both its interesting structure, and potential applications. This mesophase is the sponge, or $L_3$, mesophase, and appears in lipid systems under particular circumstances. It was first observed and studied, however, in surfactant/brine systems.

1.6.1 Surfactant sponges

The sponge phase was first observed in several different surfactant systems, such pentaethylene glycol monododecyl ether ($C_{12}E_5$) as an unusual isotropic phase [41–43]. It was, however, flow birefringent, and closely associated with a lamellar mesophase, which eliminated the possibility that it was a system based on spherical micelles [44]. Cates et al. subsequently proposed that such a system could therefore conceivably be based on a random surface, which
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Figure 1.9. An illustration of a sponge mesophase

would locally resemble a sheet-like bilayer, but was connected at longer length scales, which was later confirmed experimentally by both Gazeau et al. and Porte et al. [45–47]. Porte et al. and subsequent work further showed that the sponge phase was likely based on a type of minimal surface, so that it locally resembled a saddle surface, but with no long-range symmetry [47, 48]. The classic illustration of such a system is seen in fig. 1.9, where a surfactant bilayer has no translational symmetry, and a bilayer bicontinuously partitions the water in the system.

1.6.2 Lipid sponges

The possibility of lipid sponges was first postulated by Seddon and Templer, who noted that at the time, while the L₃ phase had only been associated with surfactant systems, it may be possible to form a lipid-based L₃ phase by introducing other components - such as co-surfactants - into the system, to reduce bilayer rigidity and enhance thermally-driven fluctuations [49]. Lipid L₃ mesophases were first observed by Engström et al. in 1998, using (as expected) polar solvents, including polyethylene glycol (PEG) with a molecular weight of 400, dimethyl sulfoxide (DMSO), propylene glycol, and ethanol [50, 51]. These additives were added to the monoolein/water system that spontaneously forms a Q₁₁ mesophase. They note that the likely
cause of the formation of the L$_3$ mesophase is the partitioning of these additives between the lipid and aqueous domains of the system, which will reduce the negative curvature of the interface. The family of additives to the monoolein/water system was added to by Cherezov et al., who viewed the sponge-forming problem through the lens of cubic-phase swelling, rather than the formation of the L$_3$ mesophase itself [52].

In addition to the types of additives previously used such as PEG, they show that using the salt KSCN can similarly drive the formation of the mesophase. As KSCN does not interact with water through hydrogen bonding, they speculate that it instead drives the formation of the sponge phase by weakening the hydrogen bonds in the water. The way in which ions affect amphiphilic self-assembly and protein activity can be described by the Hofmeister series [53]. SCN$^-\text{ is catagorised as a strong chaotrope (ie. a water structure breaker), and has been shown to increase interfacial headgroup area [54]. Therefore, as Cherezov et al. say, it is likely that in cubic phases, this will swell the size of the water channels, and eventually result in a mesophase transition to the L$_3$ mesophase. This was later confirmed in $^1$H NMR studies by Evenbratt et al., who showed that in a system of monoolein/water/pentanediol, the pentanediol has a slight preference to reside in the water channels, but partitioning itself between the aqueous and lipid domains of the system [55].

More recent work on lipid L$_3$ mesophases has focused on their relationship with other mesophases, especially the cubic ones. Much of the work in this area has been driven by the result of Seddon et al. showing that by shearing the L$_3$ mesophase, it can be used to form highly oriented cubic phases [56]. Building on this, it has been shown that the rate of shear can control the orientation of the cubic phase observed [57]. These aligned cubic phases, and the sponge mesophase-based methods used to create them have since been used to explain how various cubic phases are related, and the structural pathways that are taken in transitions between them [58–61].

### 1.7 Membrane energetics

The mesophase behaviour of lipid systems can best be understood by considering the free energy of the system. Kirk et al. considered that the total free energy of the system, $g_{total}$, would be made up of contributions from i) membrane curvature elasticity, $g_c$, ii) packing of hydrocarbon tails, $g_p$, and iii) other intermolecular interactions, including hydration and electrostatics, $g_{inter}$ [62]. The total free energy is simply the sum:
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Figure 1.10. The lateral stress profile, $\pi(z)$, across a lipid monolayer. There are attractive forces present in the interfacial region, which are counterbalanced by repulsive ones in the headgroup and tail regions.

In comparison to the first two terms, $g_{\text{inter}}$ can usually considered to be negligible [63].

1.7.1 Membrane curvature elasticity

The second term of eq. (1.19) refers to the energy required to deform the membrane surface. As may be expected, this will depend on the curvatures of the surface. This was first described by Helfrich, who proposed that the free energy could be written as:

$$g_{c} = 2\kappa_c (H - H_0)^2 + \kappa_G K \quad (1.20)$$

where $H$ and $K$ are the surface mean and Gaussian curvatures that we met in section 1.3.1, $\kappa$ and $\kappa_G$ are respectively the bending and Gaussian moduli, and $H_0$ is the spontaneous mean curvature [64]. The two moduli terms are the energy costs per unit area, and the spontaneous mean curvature is the mean curvature of the surface when it is totally relaxed.

$H_0$ itself is determined by the lateral stress profile across a monolayer. A sketch of the profile is shown in fig. 1.10, showing how it varies across a monolayer. In the interfacial region of the monolayer, the dominant force is an attractive interfacial tension, driven by the hydrophobic forces in order to tightly pack headgroups together and minimize the exposure of hydrocarbon tails to the water domains of the system. To counterbalance the attractive forces in this region,
there are repulsive forces in the headgroup and tail regions of the monolayer. In the former, these arise from electrostatic and steric interactions. In the latter, the repulsion is driven by trans-gauche conformational changes among hydrocarbon tails, which will naturally increase with temperature [63, 65]. The monolayer is then at equilibrium when $\int \pi(z) \, dz = 0$, and $H_0$ can be calculated using the first moment of $\pi(z)$:

$$\kappa_c H_0 = \int_0^l z \pi(z) \, dz$$  \hspace{1cm} (1.21)

where $l$ is the length of the monolayer. The elastic moduli can also be calculated in relation to the stress profile [66]:

$$\kappa_c = -\int_0^l \frac{\delta \pi(z)}{\delta H} z \, dz \hspace{1cm} \kappa_G = -\int_0^l \pi(z) z^2 \, dz$$  \hspace{1cm} (1.22)

Importantly, these are the formulations for monolayers of lipids. In bilayers, the three parameters become:

$$H_0^b = 0 \hspace{1cm} \kappa_c^b = 2 \kappa_c \hspace{1cm} \kappa_G^b = 2(\kappa_G - 4 \kappa_c H_0 l)$$  \hspace{1cm} (1.23)

where the superscript $b$ indicates they are bilayered. The first two are perhaps expected as otherwise in the case of $H_0$, energetically unfavourable voids will form in the centre of the bilayer. $\kappa_c^b$ is then simply twice $\kappa_c$ because there are now two monolayers to bend rather than one. The relation for $\kappa_G^b$ is more complex. While it is still related to $\kappa_G$, it is also dependent on $\kappa$ because of the meeting of unit areas at the centre of a bent bilayer: a solid angle from both bilayers must meet in the middle of the bilayer in the same unit area [67].

Measurements of $\kappa_c$ can be made by analysing thermal fluctuations of giant unilamellar vesicles, with common phospholipids having a $\kappa_c / \kappa_B T$ ratio of around 10 [68]. Significantly for this thesis, the same ratio for monoolein, which spontaneously forms cubic phases in water, is only around 2, so can be expected to be much more susceptible to thermal fluctuations [69].

### 1.7.2 Tail packing energy

The free energy due to packing frustration is much more difficult to quantify. In general, models assume that lipid tails act like harmonic springs, so:

$$g_p = k(l - l_r)^2$$  \hspace{1cm} (1.24)
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Figure 1.11. The number of protein crystal structures deposited in the Protein Data Bank using a lipid cubic mesophase technique for their crystallisation. a) is the total number of structure depositions and b) shows the number of structures associated with single publications as indicated by their PubMed IDs. The total number of depositions and unique IDs is 869 and 389 respectively.

where \( k \) is a spring constant, \( l \) is the monolayer width at an interface, and the relaxed monolayer chain length is \( l_r \) [70]. This is perhaps most easy to visualise in a HII phase, where to fill the voids (which consist of around 9% of the system volume [63]), the tails must be in constant motion.

1.8 Applications of lipid mesophases

There is obvious interest in lipid cubic phases because of the cross-disciplinary concepts needed to understand them. However, much motivation for their study now arises from their potential applications. Applications of lipid cubic phases include templating and biosensing [71–73]. Here, we will highlight two of the most significant applications that lipid mesophases have found to date: membrane protein crystallisation, and drug delivery.

1.8.1 Membrane protein crystallisation

Of the applications that have been found for cubic phases, membrane protein crystallisation is perhaps the most significant and widely-known. Membrane proteins are responsible for a phenomenal range of biochemical processes such as ion transport and hormone reception. However, due to their hydrophobic surface and anisotropy, membrane proteins are exceptionally
challenging to crystallise. Often referred to as the ‘Lipic Cubic Phase’ (LCP) technique, the method was first proposed by Landau and Rosenbusch as a way of maintaining the structural integrity of a large quantity of membrane proteins incorporated into a host matrix [74]. That the host matrix of a monoolein cubic phase also has cubic translational symmetry through which the proteins can diffuse was thought to promote the chances of crystallisation, which they successfully demonstrated, solving the structure of bacteriorhodopsin to a 3.7 Å resolution, and improving to a 1.9 Å resolution soon after [74, 75].

To further emphasise the significance of the LCP technique, around 60% of pharmaceutical targets are membrane proteins, and between 20-30% of most genomes encode membrane proteins [76, 77]. Understanding the structure and function of membrane proteins therefore represents a significant challenge for modern biochemistry, partly reflected by the award of the 2012 Nobel Prize to Brian Kobilka and Robert Lefkowitz for their studies of a particular class of membrane proteins, G-Protein Coupled Receptors (GPCRs). Kobilka’s research group was responsible for one of the first successful crystal structures of a GPCR, the human β2 adrenergic GPCR, marking a significant achievement for both the understanding of GPCRs and the use of the LCP technique [78–80].

Figure 1.11 shows the growth in the number of protein crystal structures deposited to the Protein Data Bank annually, as measured on 08/03/21 [81]. These data were acquired searching for a crystallisation method of ‘lipid cubic phase’ or ‘lipidic mesophase’. It is likely to be a slight underestimate, as early LCP structures did not note the new method. For example, deposition 1AP9, the structure of bacteriorhodopsin used to confirm the LCP technique by Pebay-Peyroula et al., does not note the LCP crystallisation technique used [82, 83]. Regardless, it is clear that the use of LCP techniques has increased dramatically in the last 5 years.

1.8.1.1 Have rumours of the success of cubic phases for membrane protein crystallography been greatly exaggerated?

A cursory look at the literature on cubic phases since the work of Landau and Rosenbusch shows that they have long been heralded as a way to solve crystal structures of membrane proteins en masse [63, 84–91]. However, it is possible that while the cubic phase represents the initial state of the mesophase, the system undergoes mesophase transitions during crystalloge-

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2020 notwithstanding, for, you know, obvious reasons.
nessis. Wadsten et al. found that on optical inspection (and confirmed using X-Ray scattering experiments) of the mesophase being used to crystallise the reaction centre of *R. sphaeroides* that it was a sponge phase, and not a cubic one as initially thought [92]. Additionally, the sponge phase has since been used to directly crystallise other membrane proteins [93, 94]. Recent work from Zabara et al. has studied mesophase behaviour in crystallisation trials. The authors show that sponge phases can provide an important intermediary in crystallisation, with sponge phase conditions resulting in larger protein crystals, faster [95, 96]. Sponge phases have also shown promise with the advent of X-Ray Free Electron Laser (XFEL) techniques. With XFEL techniques, protein crystals are pumped into a short and very intense burst of X-Ray radiation. This can be advantageous for LCP crystals, as standard synchrotron methods may otherwise be too cold. However, cubic phases are highly viscous, so using sponge phases as host matrices is advantageous [97, 98].

To add to explicit studies of sponge phase crystallisation, studying reported crystallisation conditions of structures in the PDB suggest that many are also crystallised from the sponge phase, rather than a cubic one. For instance, crystals used to solve the structure of the entire complex of the $\beta_2$ adrenergic receptor were said to be ‘picked from a sponge-like mesophase’ [99]. More recently, structures of complexes of Neurotensin receptor 1 and associated bound peptides (together responsible for a range of processes including blood pressure regulation) were solved using LCP methods [100]. In both cases, interrogation of the optimal LCP conditions for crystallisation reveals that both crystallisation conditions used significant volumetric proportions (20-30 % v/v) PEG400 in their precipitant solutions. PEG400 is at these levels is well known to induce a mesophase transition, so it is highly likely that these crystals - and many others - were in fact grown directly from the sponge phase, rather than a cubic one [50, 101, 102]. The addition of strong buffers in both cases is further likely to have significantly swollen or changed the mesophase used [103].

1.8.2 Cubosomes and drug delivery

Perhaps the most pertinent application of lipid systems is in drug delivery. Using lipid nanoparticles (LNPs) have several advantages, in that they can aid targeting efforts in aiming for a particular cell or tissue, and provide extra protection for the cargo *in vivo* [104–107]. The simplest form of a LNP would be a liposome, forming a single bilayer around the cargo, with no
internal structure. However, LNPs with internal structure can also be formed, using block
copolymers as stabilizers, first shown in cubic phases by Gustafsson et al., who used a poly-
mer based on polypropylene oxide to form individual colloids with an internal structure of the
\(Q_{II}^{P}\) mesophase [108].

One of the areas of significant focus for LNP technology has been in RNA interference (RNAi)
therapies. In short, RNAi uses short sequences of non-coding RNA such as siRNA (short in-
terfering RNA), which can bind to targeted sequences of messenger RNA (mRNA), there-
fore degrading it, and preventing translation and other steps in gene expression or function
[109, 110]. However, to date, only two RNAi have ever been approved for clinical use, reflecting
the challenges in their development [111, 112].

There is then, a natural union between interest in siRNA delivery and lipid mesophases. One
of the major challenges of any encapsulated drug delivery is the timing and efficiency of endo-
somal escape of the cargo - that is, the break down of the host LNPs to release the pharmaceu-
tical. Smith et al. highlight several possible mechanisms related to pH variation, ranging from
the ‘proton sponge’ method of increasing the internal osmotic pressure sufficiently to rupture
the membrane, to engineered destabilisation through pH responsive polymers [113]. However,
perhaps the conceptually simplest mechanism of release is through membrane fusion, where
the LNP fuses to the endosome, creating a pore through which the cargo is released. Mem-
brane fusion, however, is energetically costly, requiring functionalised lipids to fuse success-
fully (see, for example [114, 115]). However, Leal et al. showed that because lipid cubic phases
have a positive Gaussian curvature modulus, they can spontaneously form membrane pores
for successful delivery of siRNA [116]. Kim and Leal further demonstrated that by function-
alising monoolein with polyethylene glycol, cubic phases can both be dispersed in the manner
of Gustafsson et al., and contain siRNA for delivery [117]. As an excellent review by Barriga
et al. points out, while we understand a great deal about how cubic phases behave, and can
be adapted, in bulk systems, it is essential now to understand their behaviour when they are
formed in dispersions, how they can be manufactured, and how their adaptation affects their
function as targeted drug delivery systems [118].

More recently, several authors have investigated the potential for the sponge phase to be used
as a drug delivery system, using similar methods to cubosomes formation and stabilisation to
form nanoparticles with internal sponge-like topologies [119–122].
1.8.3 Applications of lipid mesophases in the SARS-COV-2 pandemic

Writing this thesis in early 2021 provides a particularly pertinent context and motivation for much of the research highlighted in this introduction, and, to a lesser extent, much of the work in what follows. The Coronavirus pandemic is a result of a novel coronavirus first reported in early 2020, later named SARS-COV-2. Coronaviridae are so-called because of their distinctive surface coronas (or spike glycoproteins), which are projected out from a spherical lipid bilayer containing other proteins, and encompassing the viral RNA. The surface spike proteins are perhaps the most important proteins in the virus, as they bind to potential host cells and force viral entry [123, 124].

The first vaccine approved by the UK Medicines and Healthcare products Regulatory Agency (MHRA) was developed by the German company BioNTech in collaboration with the pharmaceutical company Pfizer, following successful phase I/II and III trials [125–127]. At the time of writing, two other vaccines (Oxford/AstraZeneca, Moderna) have also been approved, with yet more under development [128, 129]. The BioNTech vaccine gained particular notoriety in becoming the first approved vaccine in the world based on mRNA delivery technology, for any disease.

Briefly, mRNA vaccines are based on the concept that any piece of mRNA will be transcribed by the ribosome, and so mRNAs encoding an antigen (in the case of the BioNTech and Moderna vaccines, the coronavirus spike protein) can be used to create a harmless antigen (and thus an immune response), in order to promote immunity. In the case of the BioNTech and Moderna vaccines, the mRNA codes for the SARS-COV-2 spike protein - itself harmless - in order to create a recognised immune response.

The concept of using mRNA for vaccines was first described by Jirikowski et al. in 1992, who showed that diabetes in rats can be temporarily reversed when associated mRNAs are injected [130]. In this sense, the use of mRNA as a vaccine is certainly an attractive one: as all proteins are generated by a sequence of mRNA read by the ribosome, it could be used as a platform for either fighting or preventing an incredibly diverse range of diseases [131]. In the context of COVID-19, and particularly mutations to the genetic sequence of the virus, the potential re-writing of the vaccine mRNA sequence in response demonstrates the potential adaptability of mRNA technology for decades to come [132–134].

Since the work of Jirikowski et al., the promise of the success of RNA technology as therapeu-
tics has been long-heralded \[135–138\]. Importantly, this includes the success of Pardi et al. in 2017 of developing an effective vaccine for the Zika virus epidemic, demonstrating for the first time the potential of mRNA vaccines for widespread disease control \[139\]. However, perhaps the most important problem for the future development of mRNA vaccines is the problem of effective delivery of mRNA itself into cells. Outside cells, mRNA is broken down very quickly by enzymes, and so to last sufficiently long in vivo, RNA therapies must use a transfection agent to deliver the mRNA strand \[140\]. Methods that have been most explored use particle encapsulation methods, using polymers, lipids, peptides, or a combination of them to safely deliver mRNA to its intended target \[141–143\]. This concept was first described by Allison and Gregoriadis in 1974, who showed that liposomes could be used for the delivery of vaccines to improve their efficacy \[144\]^3. In particular, while the use of cationic lipids in liposomes leads to spontaneous encapsulation with anionic mRNA, cationic lipids are known to be toxic in vivo, and therefore are not wholly appropriate for mRNA delivery \[146, 147\].

In addition to the problem of cationic lipids, many formulations of LNPs for RNA delivery (either siRNA or mRNA) use polyethyleneglycol (PEG), a polymer included to create a steric barrier between LNPs in order to prevent aggregation, and control the size of the LNPs. The size of vaccines is known to have a significant effect on the eventual uptake by the target host \[148, 149\]. However, potential side effects of PEG acting toxically and causing adverse immune responses has long been noted. Biondi et al. finding chromosomal aberrations in hamster cells exposed to PEG and related chemical derivatives, and Wylon et al. noted the potential for PEG to cause anaphylaxis \[150, 151\].

Although these studies suggest that PEG is inappropriate for use in pharmaceuticals, more recent work has shown that due to the low dosage used, concerns about its effect may well be secondary in comparison to other pharmaceutical factors \[152, 153\]. Indeed, the Pfizer-BioNTech and Moderna vaccines for coronavirus are the first vaccines to ever be approved that use PEG as a steric stabiliser, and studies are ongoing to investigate the possibility that they cause anaphylaxis more than would be expected \[154\]. Moreover, recent work from BioNTech has investigated how the lipids used in LNPs can be functionalised using polysarcosine (pSar) lipids instead of PEGylated ones, as it is known to have a low immunogenicity in several previous mammal experiments. They showed that using pSar - a polymer based on amino acid

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3Fittingly 47 years later, one of the authors received the BioNTech/Pfizer vaccine \[145\]
derivatives - produces less inflammation in comparison to PEG, suggesting that in future, it could be used to make safer mRNA therapeutics [155].

1.9 Thesis outlook

As is evident from section 1.6.2, our understanding of lipid sponge phases (in contrast to surfactant ones) is limited. While other lipid mesophases have been the subject of extensive study for almost half a century, the need to understand the sponge phase remains - and is increasingly important for the applications discussed in section 1.8. Our central question is: what drives the transition between an ordered cubic mesophase, and a disordered one? The work hereafter looks at this problem in the following ways:

• How can we adapt the sponge phase? The construction of mesophase sequence diagrams by the addition of dopant additives to the sponge phase.

• How might the sponge phase look as a microstructure? Coarse grained molecular dynamics simulations of lipid mesophases, and the creation of a model for the simulation of sponge phase.

• How does a cubic phase influence sponge phase structure? Investigations into the confinement of sponge mesophases.
2.1 Materials

For all experiments in this thesis, lipids were acquired in the following ways. Monoolein (MO) was received as gift from Danisco and used without further preparation or purification. Cholesterol and n-Dodecyl β-D-maltoside were purchased from Sigma Aldrich in powdered form. 1,2-Dioleoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, and 1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) were purchased from Avanti Polar Lipids in powdered form.

The sponge-forming additives 1,4-butanol and Potassium thiocyanate (KSCN) were purchased from Sigma Aldrich. These were used in combination with millipore water to form the hydrating solvent added to powdered lipids. Further experimental protocols for specific sample preparation are detailed as required in subsequent chapters.

2.2 X-ray scattering

X-rays are a form of electromagnetic radiation generally categorised as having a wavelength of between 0.01 - 10 nm, well outside of what can be observed by a human eye, and outside of the scope of conventional microscopy techniques. However, biological systems exist on a similar length scale, making X-rays suitable to them. In this section, we will briefly review
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the two methods of generating X-ray radiation used in this thesis, and then outline how their scattering can be used to study the structure of lyotropic lipid systems.

2.2.1 Generation of X-Rays

2.2.1.1 Lab based methods

The oldest method used to generate X-rays uses X-ray tubes. X-ray tubes consist of a heated cathode and a water cooled anode and in a vacuum chamber. As the cathode is heated, it emits electrons, which are accelerated by the electric field towards the anode. When incident on the anode, electrons decelerate and so the anode produces Bremsstrahlung radiation. The radiation given off is further characterised by sharp spectral lines arising from the electronic structure of the anode material. X-ray tubes are further developed for higher intensity beam delivery by rotating anodes, which spin in order to ensure that the anode is not heated excessively in a single place.

The most recent lab based sources use microsources, where electrons are focused onto only a small area of the anode, between 20 and 50 µm in diameter. By focusing on only a small spot of the anode, a narrow beam is produced, and so flux loss through beam collimation is reduced. A microfocus source can therefore increase the beam intensity with a significant reduction of input energy, which also simplifies the necessary cooling mechanism. The SAXSLAB Ganesha 300XL SAXS instrument in Bristol uses a Xenocs GeniX3D microfocus source, which uses a Copper anode. Copper is a very common anode material, with the $K_a$ spectral line producing a 8.04 keV (1.54 Å) X-ray beam.

2.2.1.2 Synchrotron

While modern X-ray tubes can produce X-rays of sufficient brilliance to carry out many experiments in a local lab, the use of synchrotron radiation at a national or international facility can significantly increase the brilliance of the source by many orders of magnitude. The brilliance of a light source is defined by:

$$\text{Brilliance} = \frac{\text{No. photons/second}}{(\text{mrad})^2 \cdot (\text{source area}) \cdot (0.1\% \text{ bandwidth})}$$ (2.1)
2.2. X-RAY SCATTERING

Figure 2.1. An illustration of the structure of a synchrotron. A linear accelerator injects bunches of electrons into a small diameter booster ring, which accelerates them before transfer to the main ring. In the main ring, their path is changed by bending magnets at vertices of the polygon, such that radiation is emitted tangentially. For specific beamlines, insertion devices are used to produce a narrow bandwidth of photons.

where mrad is the angular area over which the beam emerges from the source, the source area is the area of the beam, and the final term in the denominator accounts for the proportion of photons within the central 0.1% of the wavelength spectrum of the source.

An illustration of a synchrotron is shown in fig. 2.1. Oversimplified, it consists of an electron gun source produces bunches of electrons, which are firstly accelerated in the booster ring to speeds near that of light, before being transferred to the main ‘ring’. The main ring is in fact a highly sided polygon, required so that beamlines (individual experiments) can take place tangentially. At every vertex of the polygon, the direction of the electrons is changed by bending magnets. In the frame of the electron, the magnetic dipole field of the bending magnets represents an incoming (virtual) photon. The inelastic collision between the two particles results in the change of direction of electron bunch, and the emission of a forward-moving photon beam [156].

However, the emitted radiation from bending magnets possesses a wide range of energies, and so while the change of direction of electron bunches produces a broad spectrum of energies insertion devices along straight sections of the facility polygon are used to produce narrower beams. 3rd-generation synchrotrons use undulators, which consist of a periodic sequence of dipole magnets changing in polarity. Due to the changing magnetic field, the motion of the electron bunches oscillates and they therefore emit a narrow range of photonic radiation, which is proportional to the length and periodicity of the undulator. For example, the SAXS/WAXS
beamline at Diamond Light Source in Didcot, UK, has a 2m long undulator with a period of 25 mm, which can produce X-rays with energies between 8 keV to 20 keV [157].

2.2.2 Scattering

X-rays then, are used to probe materials in a wide number of ways. This thesis concerns itself with the scattering of X-rays from periodic structures (ie. lyotropic liquid crystals), so in this section we will outline the rules for ascertaining relevant structural parameters. More generally, X-ray scattering (SAXS) can be used to perform structural characterisation on molecules and other soft matter systems, such as (respectively) proteins or gels. For full texts on SAXS and other X-ray techniques, the reader is referred to Als-Nielsen and McMorrow and Sivia [158, 159].

2.2.2.1 The scattering vector

To begin, we consider some probing radiation, with a wavevector $\mathbf{k}$. As illustrated in fig. 2.2, this incoming planar wave is incident on a sample, and is scattered by an angle $2\theta$. This illustration concerns elastic scattering, such that $|\mathbf{k}| = |\mathbf{k}'|$ ie. there is no change in the frequency of the radiation as a result of scattering. We then define the scattering vector $\mathbf{q}$, by the difference between the incoming and outgoing wavevectors:

$$\mathbf{q} = \mathbf{k} - \mathbf{k}'$$  (2.2)
2.2. X-RAY SCATTERING

(a) A real space crystal lattice.
(b) A reciprocal lattice, with dimensions of inverse length.

FIGURE 2.3. A rectangular crystal lattice and its corresponding reciprocal lattice. a) The primitive unit cell of the real space lattice is described by two orthogonal vectors and their magnitudes, \( s_1 \mathbf{a}_1 \) and \( s_2 \mathbf{a}_2 \). Two lattice plane sets, (10) and (11) are illustrated, which have interplane distances \( d_{10} \) and \( d_{11} \) respectively. b) The reciprocal lattice. For a regular 2d rectangular lattice, the reciprocal lattice is also a 2d rectangular lattice, but with new inter-lattice point distances.

Subsequently, by considering the geometry of the scattering event illustrated in fig. 2.2, we can show how \( \mathbf{q} \) is related to the scattering angle:

\[
|\mathbf{q}| = 2k \sin \theta = \frac{4\pi}{\lambda} \sin \theta
\] (2.3)

where \( \lambda \) is the wavelength of the incident radiation, unchanged by the elastic scattering event.

In considering scattering from a lyotropic liquid crystal, we first recall that a crystal is defined by the convolution of a lattice and a basis. Such a 2-dimensional lattice is illustrated in fig. 2.3, where the unit cell - the repeating tessellation unit - is defined by a set of basis vectors:

\[
\mathbf{r} = s_1 \mathbf{a}_1 + s_2 \mathbf{a}_2
\] (2.4)

where in this case, \( \mathbf{a}_1 \) and \( \mathbf{a}_2 \) are orthogonal vectors defining the shape of the unit cell, and \( s_1 \) and \( s_2 \) are their respective magnitudes. As mentioned above, a crystalline structure is then defined by a basis, which is simply a collection of atoms or molecules associated with every lattice site.
Further to the definition of the primitive unit cell illustrated in fig. 2.3, we also note that when considering scattering from periodic structures, it is instructive to consider sets of atoms that are geometrically related. That is, they are connected by planes within the crystal. These are referred to as *Miller planes*, which are defined by their indices (hkl) in reciprocal to the axes they intercept. I.e., the set of (100) - or as illustrated in 2d, (10) - planes intercept one axes exactly, but are parallel to the other. To put this more succinctly, the planes (hkl) have intercepts \((a_1/h, a_2/k, a_3/l)\) for a 3d lattice with basis vectors \([a_1, a_2, a_3]\).

### 2.2.2.2 Scattering from atoms

We can now begin to build our understanding of scattering from a crystal. Consider first a planar wave with amplitude \(\psi_0\) and wavevector \(k\) travelling in the z direction towards a single fixed atom. The incident wave is then described by the function \(\psi_i\):

\[
\psi_i = \psi_0 e^{ikz}
\]

Upon interaction with the atom, the wave is dispersed radially outwards into a final wave \(\psi_f\). As we know that the interaction is elastic, \(|k| = |k'|\), and \(\psi_f\) must be parallel to a displacement vector \(r\), we can say that:

\[
e^{ik' \cdot r} = e^{ikr}
\]

Furthermore, because the scattered beam has lost its collimation, its intensity falls \(\propto r\). Therefore, \(\psi_f\) has a final description:

\[
\psi_f = \psi_0 b e^{ik \cdot |r|} e^{ik' \cdot [r - R_j]}
\]

where \(b\) is the scattering length of the atom, itself proportional to the electron density.

For real systems, we will obviously have far more than a single atom contributing to the overall scattered wave in a particular direction. Every atom \(j\) at a position \(R_j\) will contribute a small bit, \(\delta \psi_f\):

\[
[\delta \psi_f]_j = \psi_0 b e^{ikR_j} e^{ik' \cdot [r - R_j]} [r - R_j]
\]
where \( \mathbf{r} \) is some arbitrary position along the wavefront measured from the origin. Note that for a single particle at the origin, eq. (2.8) simplifies to eq. (2.7). The final wave \( \psi_f \) is then summed across the entire system of \( N \) particles of \( j = 1, 2, 3...N \):

\[
\psi_f = \psi_0 e^{i \mathbf{k}' \cdot \mathbf{r}} \sum_{j=1}^{N} b_j e^{i \mathbf{q} \cdot \mathbf{R}_j} / (\mathbf{r} - \mathbf{R}_j) \tag{2.9}
\]

where we have used the fact that \( \mathbf{q} = \mathbf{k} - \mathbf{k}' \), and removed terms independent of \( j \) from the summation, which excludes the scattering length, as this will vary for different atoms in the system.

We now note that firstly, at the detector, the measured feature is really intensity of the wave, which is dependent on the modulus of the scattered wave, \( |\psi_f|^2 \). Secondly, the detector is a long way from the sample, and so we can use the far-field limit:

\[
|\mathbf{r} - \mathbf{R}_j| = |\mathbf{r}| = r \tag{2.10}
\]

The intensity of the wave is therefore:

\[
|\psi_f|^2 = \frac{\psi_0^2}{r^2} \left| \sum_{j=1}^{N} b_j e^{i \mathbf{q} \cdot \mathbf{R}_j} \right|^2 \tag{2.11}
\]

where we have used the far field limit, and noted that \( |e^{i \mathbf{k}' \cdot \mathbf{r}}|^2 = 1 \). Finally, we consider that the scattered X-rays are deflected towards the detector through a polar angle \((\theta, \phi)\) at a rate \( R_{el} \):

\[
R_{el}(\theta, \phi) = |\psi_f|^2 \delta A = \psi_0^2 \delta \Omega \left| \sum_{j=1}^{N} b_j e^{i \mathbf{q} \cdot \mathbf{R}_j} \right|^2 \tag{2.12}
\]

where \( \Omega = \delta A / \mu^2 \), the solid angle taken up by the detector from the point of the sample. For any scattering experiment, the aim is then to ultimately measure the proportion of the incident photons that have been scattered by some polar angle to arrive at the detector. This is the differential cross-section, \( d\sigma / d\Omega \), which we can establish using eq. (2.12):

\[
\frac{d\sigma}{d\Omega} = \frac{1}{N} \left| \sum_{j=1}^{N} b_j e^{i \mathbf{q} \cdot \mathbf{R}_j} \right|^2 \tag{2.13}
\]

where we have corrected the cross-section for the number of particles in the system.
2.2.2.3 Scattering from a crystal

We can now return to our idea of a crystal, where the structure is defined by a repeating unit cell on a lattice, as described in eq. (2.4). Noting that from eq. (2.4), we can write \( \mathbf{R}_j = \mathbf{r} + \mathbf{R}_j \), we can consider the summation in eq. (2.13), and extend it across the crystal. Once we have done that, we can separate it into terms that arise from the lattice, and terms that arise from the structure of the unit cell:

\[
\sum_{\text{all atoms}} b e^{i \mathbf{q} \cdot \mathbf{R}_j} = \sum_{\text{lattice}} b e^{i \mathbf{q} \cdot \mathbf{r}} \sum_{\text{unit cell}} n e^{i \mathbf{q} \cdot \mathbf{R}_j} \tag{2.14}
\]

We will consider the lattice and unit cell sums separately. The second final decomposed sum is commonly referred to as the unit cell structure factor.

Firstly, from the sum over the lattice, we can see that we will get constructive interference from scattered waves when the following condition is met:

\[
\mathbf{q} \cdot \mathbf{r} = 2\pi n \tag{2.15}
\]

where \( n \) is an integer. To solve this condition, we construct a reciprocal lattice, as illustrated in fig. 2.3b). This lattice, with basis vectors \( \{\mathbf{a}_n^*\} \), is related to the real space crystal lattice by the condition:

\[
\mathbf{a}_i \cdot \mathbf{a}_j = 2\pi \delta_{ij} \tag{2.16}
\]

where \( \delta_{ij} \) is the Kronecker delta:

\[
\delta_{ij} = \begin{cases} 
1 & \text{if } i = j \\
0 & \text{otherwise} 
\end{cases} \tag{2.17}
\]

The reciprocal lattice, then, is a lattice in wavevector space, with units of inverse length. More generally, the reciprocal lattice is the Fourier transform of the lattice function. In fig. 2.3b), we now see that for the regular rectangular lattice as we previously defined, the reciprocal lattice is trivially also a rectangular lattice, with unit cell dimensions corresponding to \( 2\pi / s_n \), where \( s_n \) is the cell axis. This formulation gives us a reciprocal lattice vector of:

\[
\mathbf{G} = h \mathbf{a}_1^* + k \mathbf{a}_2^* \tag{2.18}
\]
2.2. X-RAY SCATTERING

(a) The Bragg condition

(b) The Laue condition

**Figure 2.4.** Illustration of the equivalence of a) Bragg’s law and b) the Laue condition.

For our 2d lattice as illustrated. We can see from this definition that the reciprocal lattice meets the condition set in eq. (2.15), because:

\[ \mathbf{G} \cdot \mathbf{R}_n = 2\pi(hs_1 + ks_2) \]

(2.19)

and, as all of \( h, k, s_1 \) and \( s_2 \) are integers, the bracketed term is also an integer. Therefore, we can say that the amplitude of the scattered wavevector will only be non-zero if the scattering vector \( \mathbf{q} \) coincides with a reciprocal lattice vector, ie. \( \mathbf{q} = \mathbf{G} \). This is known as the Laue condition.

A more familiar picture of this type of scattering is shown in fig. 2.4a), as the Bragg law. The Bragg law can be proved by considering real space wave interference, but we can also consider the reciprocal space picture of the situation in fig. 2.4b). The real space square lattice has planes of atoms separated by a distance \( d \), so the reciprocal lattice has points separated by a factor of \( 2\pi/d \). Because the scattering event is elastic, \( |\mathbf{k}| = |\mathbf{k}'| \), and by the geometry of fig. 2.4b) we have \( \mathbf{q} = 2k \sin(\theta) \), which gives us the familiar version of Bragg’s law.

To extend this formulation to a more general situation, we consider the relationship between Miller planes in the real space lattice and points on the reciprocal space lattice. For each point on the reciprocal space lattice, there are a set of planes such that:

1. \( \mathbf{G}_{hkl} \) is perpendicular to a set of planes with indices \((h,k,l)\)
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2. \(|G_{hkl}| = \frac{2\pi}{d_{hkl}}\), i.e. the magnitude of the reciprocal lattice vector is directly related to the interplane spacing of the real lattice.

Taking the first point, we can consider a plane with Miller indices \((h, k, l)\), such that two vectors in the plane are given by:

\[
v_1 = \frac{a_3}{l} - \frac{a_1}{h}
\]
\[
v_2 = \frac{a_2}{k} - \frac{a_3}{l}
\]

and therefore any point in the plane can be given by a linear sum of \(v_1\) and \(v_2\), i.e. \(v = t_1v_1 + t_2v_2\).

By eq. (2.16), the dot product of \(G\) and \(v\) is:

\[
G \cdot v = (h a_1^* + k a_2^* + l a_3^*) \cdot (t_2 - t_1) \left( \frac{a_3}{l} - \frac{a_1}{h} + t_2 \frac{a_2}{k} \right)
\]
\[
= 2\pi (t_1 - t_2 - t_1 + t_2)
\]
\[
= 0
\]

Therefore, we have shown that the reciprocal space vector, \(G_{hkl}\), is perpendicular to an equivalent Miller plane with indices \((h, k, l)\), meeting the first condition.

Now taking the second point, we can consider the reciprocal lattice unit vector, \(\hat{G}\), and any vector which connects the origin to the plane. The scalar product of these two quantities is necessarily the distance between \((h, k, l)\) planes, which is \(d\). Again using our knowledge of eq. (2.16), we find that:

\[
d = \frac{a_1}{h} \cdot \frac{G}{|G|} = \frac{2\pi}{|G|} \tag{2.20}
\]

which exactly describes the second proposal above. We now note that the Laue condition can be written as \(k = G - k'\). By squaring both sides of this statement, and by again noting the elastic scattering condition \(|k| = |k'|\), we end up with:

\[
G^2 = 2G \cdot k \tag{2.21}
\]

Now, by observing the scattering triangle in fig. 2.3b), we can see that \(G = q = Gk\sin\theta\), and we showed in eq. (2.20) that \(G = 2\pi/d\). Combining these, and noting the definition of \(|k|\) from eq. (2.3), we end up with:
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\[ n_{(hkl)} \lambda = 2d_{(hkl)} \sin \theta \]  
(2.22)

and we end up with the familiar statement of Bragg’s law as before, and where the factor of \( n_{(hkl)} \) indicates that a peak appears due to scattering from the \((hkl)\) plane in the crystal.

To summarise this point, we have shown that Bragg’s law emerges due to the scattering of waves from a lattice. However, if we return to the summations we defined in eq. (2.14), we can now consider the summation over the unit cell. As before, we note that we require constructive interference from the scattered wave as described in eq. (2.15). However, we know from the Laue condition before that for a 3d system:

\[ \mathbf{q} = \mathbf{G} = h \mathbf{a}_1^* + k \mathbf{a}_2^* + l \mathbf{a}_3^* \]  
(2.23)

Secondly, we note that any point \( \mathbf{p} \) in the unit cell can be written in terms of the basis vectors:

\[ \mathbf{p} = u \mathbf{a}_1 + v \mathbf{a}_2 + w \mathbf{a}_3 \]  
(2.24)

and therefore we find that for constructive interference to occur as required, we get:

\[ \mathbf{q} \cdot \mathbf{p} = 2\pi(hu + kv + lw) \]  
(2.25)

When summed across a set of equivalent positions \( \{\mathbf{p}\} \) in the unit cell as required by eq. (2.14), we find that the unit cell structure factor is a real valued function, and dependent on the existence of certain Miller planes. Depending on the form of the function, we will find systematic absences in the measured scattering pattern due to the unit cell structure factor equating to zero. The existence of Miller planes is dependent on the space group of a crystal structure, and as a consequence, different space groups will possess characteristic Bragg peak spacings.

On the length scales of lyotropic liquid crystals then, we observe Bragg peaks in SAXS patterns because the size of the unit cells - and therefore the repetitive spacings of electron densities - are on the same length scales as the wavelength of the X-Rays used to probe them. Our derivation here has shown that Bragg peaks emerge from particular points in the reciprocal lattice, and so for pure crystalline systems we would expect the scattering pattern to produce a series of spots, dependent on the angle of the crystal to the beam. However, lyotropic liquid
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<table>
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<th>Mesophase</th>
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<tr>
<td>L\textsubscript{α}</td>
<td>1, 2, 3...</td>
</tr>
<tr>
<td>H\textsubscript{II}</td>
<td>1, \sqrt{3}, \sqrt{4}...</td>
</tr>
<tr>
<td>Q\textsubscript{D} \textsubscript{II}</td>
<td>\sqrt{2}, \sqrt{4}, \sqrt{6}, \sqrt{8}, \sqrt{10}, \sqrt{12}, \sqrt{14}...</td>
</tr>
<tr>
<td>Q\textsubscript{P} \textsubscript{II}</td>
<td>\sqrt{2}, \sqrt{3}, \sqrt{4}, \sqrt{6}, \sqrt{8}, \sqrt{9}, \sqrt{10}, \sqrt{11}...</td>
</tr>
<tr>
<td>Q\textsubscript{G} \textsubscript{II}</td>
<td>\sqrt{6}, \sqrt{8}, \sqrt{14}, \sqrt{16}, \sqrt{20}, \sqrt{22}, \sqrt{24}...</td>
</tr>
</tbody>
</table>

Table 2.1. The characteristic peak spacings observed for different lipid mesophases by SAXS.

crystals do not have long-range crystalline order, but are instead formed of millions of randomly oriented domains. So while the structure of individual domains can be thought of as homogenous crystals, the entire sample will instead produce isotropic scattering patterns consisting of rings. As we will now see, different structures have different space groups, so we can characterise them from their characteristic ring spacings.

2.3 Analysis of SAXS patterns generated by lipid systems

Following from the formulation of section 2.2.2, it is possible to characterise the lyotropic lipid mesophases that we met in chapter 1 by using SAXS. Because each of the L\textsubscript{α}, H\textsubscript{II}, L\textsubscript{3}, Q\textsubscript{D} \textsubscript{II}, Q\textsubscript{P} \textsubscript{II}, and Q\textsubscript{G} \textsubscript{II} mesophases have characteristic symmetries, they will produce correspondingly characteristic scattering patterns, with Bragg peaks at set ratio spacings. As respective Bragg peaks have set ratio spacings, the measured intensity of the scattering pattern does not have any particular significance for a large number of lipid mesophase SAXS studies. These scattering patterns of characteristic Bragg peaks can then be analysed to determine both the mesophase, and its size.

The characteristic Bragg peak spacings of lyotropic lipid systems are detailed in table 2.1. Using these characteristic spacings, the size of the lattice parameter can be found using the appropriate equation for L\textsubscript{α}, H\textsubscript{II} and cubic mesophase respectively:

\[
a_{[h]} = \frac{2\pi}{q_{[h]}} h \tag{2.26}
\]

\[
a_{[hk]} = \frac{2\pi}{\sqrt{3} q_{[hk]}} \sqrt{h^2 + k^2 - hk} \tag{2.27}
\]

\[
a_{[hkl]} = \frac{2\pi}{q_{[hkl]}} \sqrt{h^2 + k^2 + l^2} \tag{2.28}
\]
Where \([hkl]\) are the sets of Miller indices of the permitted reflections for the correct space group, as detailed in table 2.1 and fig. 1.7. These factors of \([hkl]\) follow naturally from considering the relationship between \(|G|\) and \(d\) that we found in eq. (2.20). For example, in the case of the cubic system, \(G = 2\pi a (h, k, l)\), and therefore \(|G| = \frac{2\pi}{a} \sqrt{h^2 + k^2 + l^2}\). In addition to the mesophases listed in table 2.1, the sponge mesophase has its own characteristic scattering pattern in the form of a broad peak at low \(q\). The centre of the peak, \(q_c\) represents a correlation length, \(\bar{d}\), between so-called ‘necks’ of the system as illustrated in fig. 1.9, such that \(\bar{d} = 2\pi/q_c\) [47].

To determine the mesophase, we start with the detector pattern from fig. 2.5a). This is radially integrated to result in an intensity/scattering vector pattern, as seen in fig. 2.5b). From here, we need to identify the peak positions. This is possible using routine peak finding functions from the SciPy Python library, with some care taken over peak prominence and inter-peak distance to ensure that Bragg peaks - and not background noise - are identified [160]. Typically, this will identify a local maximum in the data, and so a peak fitting routine should be used to ensure that the centre of the peak is fitted. This is indicated by fig. 2.6a) and b). In a), we see firstly that not all the Bragg peaks in the data have been identified, and in b) we can see that the initial peak finding routine has found the highest point within the highest-\(q\) peak.
CHAPTER 2. METHODS AND MATERIALS

Figure 2.6. Using the SAXS pattern from fig. 2.5, we can find approximate positions of peaks using routine Python functions a). In b), we see a focus on the fitting routine of the last peak found: the data (blue points) surrounding the approximate initial peak (solid orange) is fitted to a function composed of a Voigt peak and linear background (dashed purple lines). The resultant fitted peak position is shown as a dashed orange line. c) shows the final position of all of the fitted peaks, which now better represent the peaks than before. Once potential lattice parameters are calculated from the found peaks, a checking routine can be applied in d) to validate how calculated peaks can be assigned to their respective indices.

The peak fitting routine is shown in b), where a convolution of a Voigt peak and a linear background are used to fit the peak, using the LMFIT library [161]. A Voigt peak was found to capture the tails of the peaks better than a Gaussian model, so was used for this purpose. As is then evident from fig. 2.6c), the fitted set of peaks are a better representation of the centres of the peaks than the initial positions.
2.3. ANALYSIS OF SAXS PATTERNS GENERATED BY LIPID SYSTEMS

Table 2.2. Finding the correct cubic mesophase and lattice parameter from a set of Bragg peaks found in SAXS data. The possible peaks in fig. 2.6 are listed across the top. A possible lattice parameter for the first six indices in the three bicontinuous cubic mesophases is calculated, and possible assignments can be made, as highlighted in orange (correctly) or green (incorrectly).

<table>
<thead>
<tr>
<th>Mesophase</th>
<th>Index</th>
<th>Identified peaks (q (Å⁻¹))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.094</td>
</tr>
<tr>
<td>Q_{II}^{II}</td>
<td>\sqrt{2}</td>
<td>93.96</td>
</tr>
<tr>
<td></td>
<td>\sqrt{4}</td>
<td>132.89</td>
</tr>
<tr>
<td></td>
<td>\sqrt{6}</td>
<td>162.75</td>
</tr>
<tr>
<td></td>
<td>\sqrt{8}</td>
<td>187.93</td>
</tr>
<tr>
<td></td>
<td>\sqrt{10}</td>
<td>210.11</td>
</tr>
<tr>
<td></td>
<td>\sqrt{12}</td>
<td>230.17</td>
</tr>
<tr>
<td>Q_{II}^{III}</td>
<td>\sqrt{2}</td>
<td>93.96</td>
</tr>
<tr>
<td></td>
<td>\sqrt{3}</td>
<td>115.08</td>
</tr>
<tr>
<td></td>
<td>\sqrt{4}</td>
<td>132.89</td>
</tr>
<tr>
<td></td>
<td>\sqrt{6}</td>
<td>162.75</td>
</tr>
<tr>
<td></td>
<td>\sqrt{8}</td>
<td>187.93</td>
</tr>
<tr>
<td></td>
<td>\sqrt{9}</td>
<td>199.33</td>
</tr>
<tr>
<td>Q_{II}^{IV}</td>
<td>\sqrt{6}</td>
<td>162.75</td>
</tr>
<tr>
<td></td>
<td>\sqrt{8}</td>
<td>187.93</td>
</tr>
<tr>
<td></td>
<td>\sqrt{14}</td>
<td>248.61</td>
</tr>
<tr>
<td></td>
<td>\sqrt{16}</td>
<td>265.77</td>
</tr>
<tr>
<td></td>
<td>\sqrt{20}</td>
<td>297.14</td>
</tr>
<tr>
<td></td>
<td>\sqrt{22}</td>
<td>311.65</td>
</tr>
</tbody>
</table>

Once a set of peaks has been obtained from a scattering pattern, we can identify the mesophase. This is most conveniently done by considering the sets of possible lattice parameters that can be obtained from the peaks found. This is illustrated in table 2.2, where the \((i,j)\) element of the table corresponds to the lattice parameter that arises from peak \(i\) and the index \(j\) using eq. (2.28) above. The table lists the three cubic mesophases as an example, but can easily be extended to include others. The values highlighted in blue immediately suggest that the mesophase of the sample in fig. 2.5 is a \(Q_{II}^{II}\), because there are the most matching values, with the exception of the \(\sqrt{8}\) peak. However, there are also a set of values, highlighted in red, which suggest that naïvely, there could be a \(Q_{II}^{P}\) instead. To clarify this, we can check how the peaks proposed for the mesophase match to the data. As can then be seen from fig. 2.5, the \(\sqrt{2}\) and \(\sqrt{10}\) peaks for the \(Q_{II}^{P}\) mesophase that has apparently been found don’t actually exist in the data. In comparison, the missing \(\sqrt{8}\) peak from the \(Q_{II}^{II}\) mesophase has now been found, and therefore confirms the mesophase of the sample.
2.4 Molecular dynamics simulations

While SAXS can inform us about the overall structure of a membrane system, it cannot tell us about the dynamics of individual molecules within the membrane. To complement this, we can use computational modelling techniques, which can model molecules. Rapaport points out that formally, the theory behind molecular dynamics (MD) “amount[s] to little more than Newton’s laws of motion” [162]. That is, the fundamental point behind molecular dynamics as a method of studying systems in a classical manner is to solve Newton’s second law for a many-body system. Interest in such a problem is of course historic with reference to the dynamics of the planets of the solar system, which cannot be solved analytically for 3 or more bodies. Therefore, we need numerical methods by which to solve the equations of motion.

If we can’t solve equations for many bodied systems analytically, we can instead do so numerically using the techniques and strategies that follow. Using numerical approximations, we can construct systems with hundreds or thousands of molecules and interrogate their dynamics. In this sense, molecular dynamics provides a ‘computational microscope’ on the behaviour of molecular systems [163]. As an abstract idea, an MD simulation consists of a set of point particles, and definitions of how every particle is related to every other particle in the system. A key challenge for MD investigations is therefore to establish appropriate relationships between the particles. If we were to fully model every molecule at a subatomic resolution, we would soon find ourselves wrapped up in numerically solving computationally expensive quantum mechanical descriptions for atoms. Although this can be done, the size and timescale for which it can be achieved is a significant barrier, and we will not learn anything about the long term structural dynamics of the system. For that reason, this work uses so-called classical potentials to model inter-atomic interactions, neglecting quantum effects.

2.4.1 Equations of motion and potentials

To begin, we return to our idea of a basic simulation box, containing some molecules described by point-like particles. As we have said, physically we know that any motion of the particles making up the molecules - however they have been described - will obey Newton’s second law, relating the acceleration \( \ddot{a} \), to the force that is being exerted on the particle, \( \vec{F} \):

\[
\vec{F} = m\ddot{a}
\]  

(2.29)
2.4. MOLECULAR DYNAMICS SIMULATIONS

(a) Forces between 2 and 3 atoms
(b) A force between 4 atoms: proper dihedrals
(c) Maintaining planar structures with 4 atoms: improper dihedrals

Figure 2.7. Illustration of bonded forces in molecular dynamics simulations. a) Between 2 atoms, there is an equilibrium bond length, $d_{ij}$. Between 3 atoms, there is an angle, $\theta_{ijk}$. For 4 atoms, there are two possible restrictions: in b) we show proper dihedrals, which define the equilibrium angle $\phi_{ijkl}$ between two separate planes of atoms, while improper dihedrals in c) restrict a group of atoms to lying in a plane together, with $\psi_{ijkl}$ defining the equilibrium angle for the plane.

where $m$ is the particle’s mass. To understand the dynamics of the particles, we therefore need to know something about the force that it is experiencing. For this, we know that the force experienced is related to the distance of its origin:

$$\vec{F}(r) = -\nabla \Phi_{total}(r)$$ \hspace{1cm} (2.30)

Our challenge now lies in defining $E_{total}(r)$. Here, we will follow the descriptions of Liguori et al. [164]. Molecules experience forces due to both the bonds that link atoms within them, and forces from other molecules in its vicinity:

$$\Phi_{total}(r) = \sum_{\text{bonded pairs}} \Phi_{\text{bonded}} + \sum_{\text{atom pairs}} \Phi_{\text{non-bonded}}$$ \hspace{1cm} (2.31)

Taking these sums in turn, firstly, $\Phi_{\text{bonded}}$ is itself the sum of the potential energies associated with bonds between atoms within a molecule. These are dependent on the bond lengths, bond angles, and torsional angles between successive groups of 2, 3, or 4 atoms. That is to say:

$$\Phi_{\text{bonded}} = \Phi_{\text{bond}}(d_{ij}) + \Phi_{\text{angle}}(\theta_{ijk}) + \Phi_{\text{dihedral}}(\phi_{ijkl}) + \Phi_{\text{improper dihedral}}(\psi_{ijkl})$$ \hspace{1cm} (2.32)

where the individual terms are defined as:
That is, the potentials mostly have harmonic-like forms, apart from $\Phi_{\text{dihedral}}$. Taking these in turn and as illustrated in fig. 2.7, $\Phi_{\text{bond}}$ accounts for the stretching of the bonds between two atoms directly next to each other, $\Phi_{\text{angle}}$ for the bending of 3 successive atoms, and $\Phi_{\text{dihedral}}$ for the angle between two planes formed by a group of 4 atoms. $\Phi_{\text{improper dihedral}}$ is used to maintain a group of 4 atoms in a single plane, which is useful for ring-like molecular structures.

For each of the forces listed in eq. (2.33), $K_b, K_a, K_d$ and $K_{id}$ are the force constants, and the equilibrium constants are described by $d_b$ (a distance), $\theta_a, \phi_d$ and $\psi_{id}$ (all angles). The $n$ in the expression for $\Phi_{\text{dihedral}}(\phi_{ijkl})$ indicates the multiplicity of the force, as there may be multiple minima through the range of the rotation angle. This is the reason why the potential has a cosine-like form, rather than a harmonic one.

Secondly, the $E_{\text{non-bonded}}$ describes the interaction potential between any given pair of particles in the system. The two most significant interactions are the Lennard-Jones (LJ) and Coulomb potentials:

$$\Phi_{\text{LJ}}(r) = 4\epsilon_{ij} \left[ \left( \frac{\sigma_{ij}}{r} \right)^{12} - \left( \frac{\sigma_{ij}}{r} \right)^{6} \right]$$

$$\Phi_{\text{Coulomb}}(r) = \frac{q_i q_j}{4\pi \epsilon_0 \epsilon_r r}$$

where for $\Phi_{\text{LJ}}(r)$, $\epsilon_{ij}$ is the interaction strength (i.e. depth of the potential well), and $\sigma_{ij}$ is the distance for a pair of particles $\{ij\}$ at which the potential is zero. For $\Phi_{\text{Coulomb}}$, $q_i$ and $q_j$ are the charges of the particles $\{ij\}$, and $\epsilon_0$ and $\epsilon_r$ are the vacuum permittivity and relative dielectric constants respectively. In both potentials, $r$ is the physical distance that $\{ij\}$ are separated by.
2.4. MOLECULAR DYNAMICS SIMULATIONS

This collection of equations then, fully describes what is often referred to as the force field, which can be used to model the interactions and dynamics of the system. The precise description of the forces will depend on the resolution of the simulation. For now, what we are interested in is the matter of how simulations will progress through time using eq. (2.29) and eq. (2.30). A common algorithm to do this is the velocity Verlet one ([165]):

\[
\vec{r}(t + \Delta t) = \vec{r}(t) + \vec{v}(t)\Delta t + \frac{1}{2} \frac{\vec{F}(t)}{m} (\Delta t)^2
\]

(2.36)

\[
\vec{v}(t + \Delta t) = \vec{v}(t)\Delta t + \frac{\vec{F}(t + \Delta t) + \vec{F}(t)}{2m} \Delta t
\]

(2.37)

To summarise, at every time step in the course of a simulation, we calculate the forces on every atom in a system, use that to determine the acceleration, and can then calculate the position using eq. (2.36) and then the velocity of the particle using eq. (2.37) at the subsequent time step. This process is then repeated for a set number of time steps.

That then, is an extremely short summary of how simulations progress. Two further features are worthy of mention: periodic boundary conditions and cutoff lengths. Periodic boundary conditions (pbc) are imposed on systems in order to more effectively simulate bulk systems. In short, if a particle finds itself at the edge of a simulation box and the progression algorithm pushes it ‘outside’ of the box, it will in fact reappear on the ‘other side’ of the box, in whichever axis it leaves. However, this means that every particle in the simulation box experiences not only forces from every other particle in the simulation box, but also forces from particles in periodic images of the simulation box. This naturally results in an extremely large number of interactions that need to be accounted for in progressing a simulation.

Cutoffs are therefore important features in simulations, and they work as they sound. Beyond a distance \( r_c \), potential functions are reduced to zero:

\[
\Phi(r) = \begin{cases} 
\Phi(r) - \Phi(r = r_c) & r \leq r_c \\
0 & r > r_c 
\end{cases}
\]

(2.38)

In this case, the potential is both cutoff and shifted, which ensures that there is no functional discontinuity at \( r_c \). In addition to the potential cutoff, minimum image conventions can be applied, which limits the number of periodic images that a particle can interact with to just the closest one.
CHAPTER 2. METHODS AND MATERIALS

2.4.2 Simulation resolution: coarse-graining and the Martini force field

As mentioned above, the choice of force field is crucial to how a simulation will progress, and the detail that it will provide about the system being probed. Biomolecular simulations naturally involve very large molecules (i.e. sizeable proteins), large numbers of molecules, or both. As previously mentioned, using fully-modelled atomistic detail in quantum mechanical (QM) or molecular mechanical (MM) simulations can provide invaluable insight into the electronic and nuclear degrees of freedom of a system, which advances our understanding of reaction mechanisms and catalysis [166]. However, these simulations are naturally limited to short timescales and intimate detail. Simulating large patches of cell membranes containing lipids and membrane proteins across long timescales naturally forces us to leave some of that molecular detail behind.

The challenges of simulating realistic cell membranes are outlined in an excellent recent review article by Marrink et al. [167]. In short, there are generally two resolutions of force fields which can be employed for biomolecular studies: atomistic, and coarse-grained. Atomistic force fields are either resolved in full molecular detail, or in a ‘united-atom’ way, where hydrogens are incorporated into point particle modelling. That is to say, when we start to ‘blur’ the resolution of our molecular models, we must re-establish the potentials used to determine their subsequent motions as determined throughout section 2.4.1.

If we extend the idea of ‘blurring’ resolution, then we can imagine combining not just hydrogens to atoms, but groups of several atoms into one particle. These systems are coarse-grained, so called because of the coarser level of resolution that we now have. By grouping atoms into beads in this way, we can probe the dynamics of large systems on increased timescales. The foremost force field for simulations involving lipids and proteins is the Martini force field, developed by Marrink et al. [168, 169]. In general, version 2 of the Martini (the version used throughout this thesis) uses a 4:1 mapping procedure for atoms. That is, one Martini particle represents a group of 4 atoms.

The Martini force field then has four classes of particles: P (polar), C (apolar), N (nonpolar), and Q (charged). Each of these has several subtypes, running from 1-5 for P and C (low to high polarity) or a letter for N and Q indicating hydrogen-bonding tendency (0 = none, a = acceptor, d = donor, da = both). The non-bonded interactions between these 18 subtypes in total are described by 10 possible levels of interaction, which change the depth of LJ potential well.
2.4. MOLECULAR DYNAMICS SIMULATIONS

Listing 2.1: The Martini topology file for a DOPC lipid.

Additionally, bond lengths and force constants are defined. The full parameterisation of the model is then based on free energy calculations, and comparison to experimental and all-atom simulation data.

Once a force field is designed, we can construct molecules for use in simulation. An example of this process is demonstrated in fig. 2.8, which shows how the 4:1 Martini mapping is used in creating a model of DOPC. The realisation of this model is written in code in listing 2.1.
CHAPTER 2. METHODS AND MATERIALS

Figure 2.8. The process of coarse graining a lipid for the Martini force field. DOPC (top) is transformed into a coarse-grained molecule (bottom) of Martini types Q0 (light blue), Qa (dark blue), Na (yellow), C1 (light grey), and C3 (dark grey), reflecting the underlying chemical structure of the molecule at each point. This molecule is described by the topology file in listing 2.1. Note that the bonds in the coarse-grained model are illustrative of the particle connections only, rather than reflective of any equilibrium lengths.

showing how the Gromacs simulation software understands the structure. Listing 2.1 is known in Gromacs form as a ‘topology’ file, with the extension .itp. The file defines a name for the molecule, after which there are three sections to describe its structure, working from individual atom - ie. coarse-grained beads - types, the describing the network of bonds, and finally the angles that bonds form. The implementation of the Martini force field is evident from the atom types in the first section.

In addition to its suitability for studying long scale dynamics of membrane systems, the Martini force field was chosen as a result of it being the only force field known to have studied bicontinuous cubic phases stably. Earlier work by Marrink and Tieleman used a united atom force field to study a pre-assembled Q\textsubscript{D}nung mesophase, but additionally found that it was unstable, transitioning to a H\textsubscript{I} mesophase after around 35 ns [170]. More recent work by Khelashvili et al. developed a coarse grained model for monoolein, and used the model to interrogate the hydrophobic mismatch of membrane proteins residing in it [171, 172].
2.4. MOLECULAR DYNAMICS SIMULATIONS

2.4.3 Self-assembly molecular dynamics

The most frequent type of simulation that we will meet in this thesis is a self-assembly simulation, which are used to construct different mesophases \textit{in silico}. For every simulation throughout this thesis, Gromacs 2018.2 was used as the molecular dynamics software [173, 174]. Simulations were prepared using the equilibration technique that was used throughout the simulation Khelashvili et al. used for their cubic phase studies, as illustrated in fig. 2.9 [171].

Firstly as shown in fig. 2.9a), lipid molecules are inserted into a simulation box of size 20x20x20 nm. This is significantly larger than the anticipated final system size, but is necessary to ensure that the molecules are easily inserted. Subsequently, intermolecular forces are minimized using the steepest descent algorithm in Gromacs. Rather than progressing through time, the steepest descent algorithm moves the system to a local equilibrium to remove any excessive forces between atoms. New positions, \( \mathbf{r}_{n+1} \), are calculated by moving particles’ positions away from the source of the force, \( \mathbf{F}_n \) using:

\[
\mathbf{r}_{n+1} = \mathbf{r}_n + \frac{\mathbf{F}_n}{\max(|\mathbf{F}_n|)} h_n
\]

where \( \max(|\mathbf{F}_n|) \) refers to the largest force on any atom in the system, and \( h_n \) is the maximum permitted displacement for a move to be made [175]. After lipids have been inserted, the water molecules are also, and energy minimisation is repeated. Note that in version 2 of the Martini model, water is represented by a P4 bead, which itself represents 4 water molecules in total.

Once the entire lipid and water system is minimized by steepest descent, the equilibration simulation is run. The key difference for an equilibration simulation compared to a production one is that the Lennard-Jones parameters of the Martini interactions are set to the same as water. This is necessary for two reasons. Firstly, the size of the simulation box needs to be optimised for the number of molecules that it contains, and secondly, for self-assembly to occur equally throughout the box, the molecules need to be well mixed. The simulation is then run under NPT conditions at a temperature of 323 K with a reference pressure of 1 bar for 20 ns, using the velocity Verlet methods as previously discussed. Because this essentially turns intermolecular interactions ‘off’, the system becomes randomly mixed as required, so that water and lipids are evenly distributed throughout the box, and the cell size has been reduced to 9.89 nm in each dimension, as shown in fig. 2.9b). Finally, the system is ready to run as a production simulation, using the full proper force field for the molecules in the system. In this
case, we use the standard Martini NPT molecular dynamics parameters, using a temperature of 300K and a pressure of 1 bar. In the case of the illustrated system in fig. 2.9, we finish with the $Q^{D}_{II}$ mesophase in fig. 2.9c), as seen by the bicontinuous water channels running through the cubic unit cell.
2.4. MOLECULAR DYNAMICS SIMULATIONS

(a) A minimized simulation cell

(b) An equilibrated simulation cell

(c) The final self-assembled $Q^D_{II}$ mesophase.

**Figure 2.9.** The process of preparing a simulation cell. The cell is prepared for a 5% mol cholesterol content and a 35% water weight ratio. It contains 902 MO, 48 Cholesterol, and 2543 water molecules. Water is coloured in Blue, monoolein headgroups in Red, and cholesterol headgroups in turquoise. Carbons for each of the molecules are in Grey. a) Molecules are inserted into a simulation cell with initial lengths of each side of the cell of 200 Å. b) In an equilibration simulation, where all LJ interactions are set to that of water-water, the molecules become evenly distributed throughout the cell, the dimensions of which are optimally reduced here to 98.9 Å. c) After a production self-assembly simulation, where the LJ parameters are reset to their proper values, the system has self-assembled into a $Q^D_{II}$ mesophase, evident from the two distinct water channels.
CHAPTER 3

LIPID TYPE-DOPING OF THE SPONGE PHASE

The work in this chapter has been summarised as a paper, at the time of writing deposited to the bioRxiv preprint repository [176].

3.1 Context

In section 1.5, we met monoolein, a cubic phase forming monoacylglyceride that has been extensively studied for a variety of reasons, but perhaps most importantly, for its uses in membrane protein crystallisation as described in section 1.8.1.

One of the principle ways in which monoolein cubic phases have been adapted is through so-called ‘type-doping’, where other lipids have been included in system in order to adapt the mesophase. ‘Type-doping’ specifically refers to how the properties of the Q_{II}^{D} mesophase changes depending on the value of the surfactant packing parameter defined in eq. (1.1). The first extensive systematic study of type-doping was undertaken by Cherezov et al., who investigated a variety of phospholipids and other molecules [177]. In general, they found that the monoolein Q_{II}^{D} mesophase could incorporate up to 20-25% mol of a dopant before the system would either i) undergo a mesophase transition to the preferred mesophase of the dopant molecule or ii) phase separate into a cubic phase + dopant molecule crystal, such as in the case of cholesterol. The dopants had various effects on the size of the mesophase itself, either swelling or contracting it slightly in comparison to an undoped system. As would reasonably be expected, lipids with
CHAPTER 3. LIPID TYPE-DOPING OF THE SPONGE PHASE

charged headgroups (in the case of their particular study, DOPS), both significantly swelled the size of the mesophase and brought about a mesophase change to the \(Q_{II}^D\) mesophase. This latter point can be rationalised in terms of the intra-bilayer electrostatic repulsion from charged headgroups, which will act to flatten the curvature of the membrane. Since this study, the effect of many further such dopant molecules and other additives has been studied, which have been summarised in an excellent review by van ‘t Hag et al. [90].

Doping cubic phases then, has long been seen as a way by which LCP screens can be optimised in order to improve the chances of crystallisation. However as was noted in section 1.8.1.1, several studies have found that membrane proteins can be (directly or indirectly) crystallised using the sponge mesophase. Examining these cases further reveals that for example, the human \(\beta_2\) adrenergic G-protein-coupled receptor was crystallised using a system with 10% mol cholesterol [99]. Similarly crystals of human microsomal prostaglandin E2 synthase 1 were grown under conditions that included a necessary co-crystallised phospholipid, DOPC, at a proportion of 5% mol (Protein Data Bank code: 4UXW) [178]. The role of cholesterol in particular has of increasing chances of successful crystallisation has been noted elsewhere, in studies where membrane proteins have been directly crystallised from a sponge mesophase explicitly, where just 1% weight cholesterol was found to be necessary for producing crystals of a bacterial photosynthetic core complex [93].

While the effect of dopant additives on the \(Q_{II}^D\) mesophase is therefore well understood, the ability of the L\(_3\) sponge mesophase to form while containing dopants has not yet been explored. As the formation of the L\(_3\) mesophase is very sensitive - only appearing in narrow regions of mesophase space - it is not obvious that it should easily form. From both the \(^1\)H NMR studies of Evenbratt et al. and SAXS mesophase sequence ones of Cherezov et al., it is evident that the L\(_3\) mesophase forms as a result of the expansion of the interfacial area of the membrane, along with a lowering of the elastic moduli of the membrane so that it is no longer able to exhibit long-range periodicity [52, 55]. Considering that dopants could variously change the effective mean headgroup area of the interface through type-doping, disrupt tail packing through hydrocarbon length mismatch, or increase membrane stiffness or lateral organisation (for example in the case of cholesterol), it is not trivial that the sponge mesophase should form while monoolein membranes are doped.

In addition to zwitterionic type doping of the \(Q_{II}^D\) mesophase, doping with charged lipids such
as DOPG has often been mooted as a way to increase the lattice parameter of the cubic phase with a view to incorporating significant extra-cellular domains of membrane proteins [88, 179]. The accommodation of large extra-cellular domains is often pointed out as one of the most significant barriers to the LCP technique’s success [180]. However as our recent work has shown, the addition of common crystallisation precipitants to charged mesophases at even low concentrations can immediately screen out any intrabilayer electrostatic repulsion that is responsible for any significant swelling [181]. Furthermore, van ’t Hag et al. have showed using multilinear regression that the most significant component of a membrane protein crystallisation screen was the PEG component [182]. Coupling this together, it could be suggested that by improving our understanding the membrane structural dynamics, we will be able to improve chances of successful crystallisation of membrane proteins.

The aim of this chapter is to systematically test the effect of a variety of dopants of different packing parameters on the ability of the L$_3$ mesophase to form. To that end, we will use a type 0 (DOPC), type I (DDM), and type II (DOPE) to test simple type doping. We will also investigate the effect of cholesterol both on its own and in conjunction with the anionic phospholipid DOPG. This combination of lipids was previously used by Tyler et al. to produce some of the largest cubic mesophases ever observed - with lattice parameters in excess of 450 Å - albeit at elevated (ie. non-crystallisable) temperatures [88]. We will firstly review the dopants used, before presenting the results of a systematic doping study, and outlining the results of a preliminary high-throughput experiment.

### 3.1.1 Dopant molecules

#### 3.1.1.1 DOPC

1,2-Dioleoyl-sn-glycero-3-phosphocholine (18:1 (Δ9-Cis) PC; DOPC) is a type 0 zwitterionic phospholipid with two hydrocarbon tails, and a choline headgroup, shown in fig. 3.1a). It is one of the major components of biological cell membranes, accounting for over 50% of most eukaryotic membranes [1]. When added to MO Q$_{II}$ mesophases, the lattice parameter increases up to around a 26% mol proportion, before the system reverts to the L$_a$ mesophase. The increase is likely due to an increased packing frustration within the membrane as a result of the fact that the tail volume of DOPC is larger than that of MO [177].
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3.1.1.2 DOPE

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (18:1 (∆9-Cis) PE; DOPE) is a type II zwitterionic phospholipid, also with two hydrocarbon tails, but with a smaller ethanolamine headgroup than DOPC, as shown in fig. 3.1b). DOPE is also a major component of eukaryotic membranes. Due to the shape mismatch between DOPE and DOPC, DOPE creates packing and curvature frustration in cell membranes, which is used for membrane fusion [183, 184]. When added to the Q_{II}^{1D} mesophase, the lattice parameter will decrease before a system transition to the H_{II} mesophase at a proportion of around 20% mol [177]. The reduction in lattice parameter can likely be ascribed to the relief of packing stress through the increase of hydrocarbon tails in the membrane.

3.1.1.3 DOPG

1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (18:1 (∆9-Cis) PG; DOPG) is an anionic phospholipid, with its structure shown in fig. 3.1c). Although it is not a common eukaryotic lipid - in mammals it can be found in pulmonary surfactant, see eg. [185] - it is a main component...
(around 20%) of the cell membranes of *Escherichia coli*, and are essential for functions such as protein translocation [186]. Doping the \( Q_{II}^P \) mesophase with charged lipids has been well studied, with Engblom et al. showing that the addition will induce a transition to the \( Q_{II}^P \) mesophase [179]. This is a result of intra-bilayer electrostatic repulsion, which drives a flattening of the membrane into the \( Q_{II}^P \) mesophase.

### 3.1.1.4 DDM

n-Dodecyl \( \beta \)-D-maltoside (DDM) is a detergent, shown in fig. 3.1d), notably common used in membrane protein extraction [187]. As a detergent, its packing parameter is <1. When added to monoolein \( Q_{II}^P \) mesophases in water, DDM has a significant destabilising effect, forcing a mesophase change immediately to the \( Q_{II}^G \) mesophase, and soon after to the \( L_\alpha \) [188–190]. The ultimate mesophase change to a \( L_\alpha \) mesophase can be rationalised by considering the molecular mismatch between the type II monoolein and the type I DDM, which will in effect cancel each other out to average a ‘cylindrical’ type 0 molecule, forming a flat \( L_\alpha \) bilayer. At intermediate concentrations where a \( Q_{II}^G \) mesophase is observed, Ai and Caffrey suggest that the addition of DDM into the bilayer creates osmotic pressure by drawing water away from monoolein headgroups towards the carbohydrate headgroup moiety of the DDM molecules [188]. They suggest that this could consequently further lower the packing parameter of the DDM molecule, and force a mesophase change into the \( Q_{II}^G \) mesophase. Because it is used during membrane protein extraction and purification, it is likely that it is carried to a degree into LCP crystallisation screens, and therefore affect the mesophase used during crystallisation. Therefore understanding the effect of DDM on the formation of the \( L_\alpha \) mesophase has particular interest.

### 3.1.1.5 Cholesterol

As highlighted above, cholesterol, as shown in fig. 3.1e), has significant relevance for LCP applications. Cholesterol is a major sterol component of mammalian membranes, the second most prevalent lipid after phospholipids [1]. Cholesterol is perhaps most well studied for its stiffening effect on membranes, which drive liquid-ordered/liquid-disordered lateral phase separation within bilayers. Liquid-ordered domains rich in cholesterol are often referred to as *lipid rafts*, detailed discussion and controversies over which is best left elsewhere [191–194].
With respect to the $Q_{II}^D$ mesophase, cholesterol is considered a type I molecule, which induces a mesophase change firstly to the $Q_{II}^P$ at around 25% mol, and subsequently a $L_\alpha$ mesophase. Cholesterol monohydrate crystals form in coexistence at sufficiently high concentrations [88, 177]. As Tyler et al. explain, the transition to the $Q_{II}^P$ mesophase can be rationalised with the knowledge that in cubic phases, cholesterol headgroups preferentially forms hydrogen bonds with the carbonyl group of monoolein rather than water. This results in an increase in the lattice parameter of the $Q_{II}^P$ mesophase due to cholesterol molecules thereby forcing monoolein headgroups further apart, reducing the packing parameter of molecules in the bilayer, and ultimately forcing a mesophase change to the $Q_{II}^P$ [12, 88].

### 3.2 Static capillary experiments

#### 3.2.1 Experimental set up

Mixtures of lipids were prepared at either a 0.1 M or 0.05 M concentration in dichloromethane or ethanol. The former was used for monoolein, phospholipid, and cholesterol mixtures, while the latter condition was used for DDM mixtures, as it was insoluble at higher concentrations. The mixtures were then prepared volumetrically at concentrations of 2.5%, 5%, 7.5%, and 10% mol dopant lipid in the case of DOPC, DOPE, DDM, and cholesterol, and 1% and 3% mol for DOPG. 70% of the mixtures were then transferred to 1.5 mm borosilicate X-ray capillaries (Capillary Tube Supplies UK Ltd.). The mixture was left to evaporate for 3 days, before the last of the solvent was removed under vacuum. This left a thin film of dried lipid mixture on the walls of the capillary. 50 µl of the water/butanediol lyotrope was added to hydrate the film. Kim and Leal showed that removal of solvent under vacuum can leave lipid films in metastable states with extremely large lattice parameters, so after the solvent had been added and the capillary sealed, the samples were put through 3 freeze-thaw cycles between -10°C and 60°C to ensure that they were equilibrated [117]. Samples were subsequently measured using SAXS for 600s, mostly in a q range of 0.015–0.35 Å⁻¹. Several samples (DOPG-doped) were measured using a q range of 0.01-0.2 Å⁻¹ instead, in order to capture larger-scale behaviour at low q.

For each concentration of dopant lipid, 9 lyotrope conditions were trialled in which to measure the mesophase, which were: 0%, 20%, 32.5%, 35%, 37.5%, 40%, 42.5%, 45%, 47.5%, indicating
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![Scattering patterns of different lipid/solvent ratios for L₃ mesophase capillary experiments.](image)

Figure 3.2. Scattering patterns of different lipid/solvent ratios for L₃ mesophase capillary experiments.

the v/v ratio of butanediol/water in the solvent. For monoolein alone, the Q¹/₃/L₃ transition occurs at around 30% v/v, and the L₃/L₆ transition at around 50% v/v [52]. The lyotrope ratios selected therefore allows us to investigate the mesophase in 1) water, 2) a point well below the expected transition point to check the effect of the dopant on the swelling behaviour of the Q¹/₃ mesophase, and 3) a fine range of conditions where the L₃ mesophase should form, so any downwards shift the L₃/L₆ mesophase boundary can be observed, and the swelling behaviour of the L₃ mesophase itself can be investigated.

Regarding the choice of 50µl of lyotrope, it should be noted that Cherezov et al. measured mesophase conditions at a fixed ratio of 60:40 w/w lipid:lyotrope, because this is the ratio used during LCP membrane protein crystallisation. In fig. 3.2, we show that above this point - ie. increasing the quantity of solvent in the system, such that it is in excess - there is no substantial effect on the mesophase structure or size. These samples were prepared directly and transferred to capillaries, rather than through evaporation. There is a very minor shift in the position of the L₃ mesophase peak at a ratio of 25:75 w:w lipid:lyotrope, but overall the size of the L₃ mesophase under any of these lyotrope conditions doesn’t change more than ±10 Å in either direction. For all of the lipid mixtures described above, the mass of lipid left in the capillary after evaporation is < 1 mg, so we can be sure that the results throughout this work will be in the excess lyotrope regime, and are therefore relevant for any such conditions.
3.2.2 Results and discussion

3.2.2.1 Monoolein

To begin, we firstly validate the well-known result of Cherezov et al. in fig. 3.3. This result also validates the evaporation method of sample preparation, as the monoolein/water system (ie. 0% v/v lyt trope butanediol) exhibits the expected mesophase and size behaviour of a Q_{II} with a lattice parameter of 97 Å. This is slightly below the usual excess water lattice parameter of the monoolein Q_{II} mesophase of 104 Å, likely as a result of minor impurities (such as triglycerides) in the monoolein used during preparation [16]. Upon the initial increase of butanediol content to 20% v/v, the size of the Q_{II} mesophase swells to 123 Å as expected. Above 30% v/v butanediol, the system has undergone a mesophase transition from the Q_{II} mesophase to the L_{3} mesophase, which grows in size from 100 Å to 154 Å. In fig. 3.3b) a linear trend has been fitted to these data primarily as a guide for comparison to dopant trends. Note that the variation around the approximately linear trend in both this dataset and the subsequent ones in this chapter is most likely a result of small temperature variations between sample measurements, known to affect the lattice parameters of mesophases [16].
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(a) 2.5% mol cholesterol

(b) 5% mol cholesterol

(c) 7.5% mol cholesterol

(d) 10% mol cholesterol

Figure 3.4. 1D scattering patterns for cholesterol-doped systems. a) 2.5% mol, b) 5% mol, a) 7.5% mol, a) 10% mol. The patterns are arranged in ascending order of butanediol concentration.

3.2.2.2 Cholesterol

Doping monoolein with cholesterol presents one immediate distinction from undoped systems. As shown by the sequence of results in fig. 3.5, the addition of cholesterol has shifted the position of the $Q_{II}^{D}/L_3$ mesophase transition from around 30% v/v butanediol in the lyotrope to around 32.5% v/v. The scattering patterns shown in fig. 3.4 in fact nicely demonstrate the soft nature of the $Q_{II}^{D}/L_3$ transition in the broadening of the peaks: note that at 32.5% v/v for a 5% mol doped system, there is likely coexistence between the two mesophases, as the first two $Q_{II}^{D}$ Bragg peaks are still just about visible, but clearly merging towards a single broad
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Figure 3.5. Mesophase sequence results for cholesterol-doped systems. The mesophase type is indicated by the shape of the scatter point, and their colours distinguish the concentration of the dopant in the system. The dashed vertical black line shows the expected point of the mesophase transition in undoped systems as shown in fig. 3.3. For reference, the red dashed trend line from fig. 3.3b) is also shown.

L₃ mesophase peak. Subsequently however, the L₃ mesophase forms relatively easily for most dopant concentrations for the range of lyotrope conditions measured. The only exception to this is that at 10% mol, the position of the L₃/Lα mesophase transition boundary is shifted down to 45% v/v butanediol.

Further to the shift of the mesophase boundary, the addition of cholesterol has reduced the size of the lattice parameter and correlation length in the Q_D II and L₃ mesophases respectively. While in undoped systems, the addition of 20% v/v butanediol in the lyotrope swells the lattice parameter from 97 Å to 123 Å, in cholesterol-doped systems, this is reduced to between 110 Å and 115 Å. At 32.5% v/v butanediol, the 4 dopant proportions all produce Q_D II mesophase with lattice parameters of around 130 Å.

Once the proportion of butanediol in the lyotrope is sufficiently large such that the systems have undergone a mesophase transition, most of the L₃ mesophases observed have shorter correlation lengths in comparison to undoped systems. Notably, there does not appear to be any real systematic variation of the correlation length depending on the proportion of dopant. Although at 10% mol, there is definitely a reduction in correlation lengths compared to other concentra-
3.2. STATIC CAPILLARY EXPERIMENTS

tions, there is little evidence of any variation among them. The fact that cholesterol has such a significant effect on reducing the $L_3$ correlation lengths is perhaps surprising, as it does not have any such dramatic effect on $Q_{II}^D$ in water alone [177]. However, the effect can almost certainly be attributed to the stiffening effect that cholesterol universally has on membranes, increasing the bending modulus [195]. Recent studies by Chakraborty et al. have also pointed towards a cholesterol-driven membrane thickening effect [10]. By increasing the bending modulus, it is likely that the membrane will become flatter, as it is not as susceptible to thermal fluctuations. This in turn will bring bilayers closer together, which will reduce the correlation length measured between them, as we observed.

3.2.2.3 DDM

For clarity, the results measured for DDM-doped systems as shown in fig. 3.6 are split between low concentrations in fig. 3.7a) and higher concentrations in fig. 3.7b). Unlike in other systems, there is no clear change in the lattice parameter of the $Q_{II}^D$ mesophase measured on the initial addition of butanediol to 20% v/v in the lyotrope. However, at 5% mol and 7.5% mol, the size of the mesophase in water has been swollen fairly significantly to 145 and 134 Å respectively. Also unlike other dopants, DDM effects a substantial mesophase change at higher concentrations. However, this is to be expected based on the known destabilising properties that DDM has on cubic mesophases.

The main feature observed for systems doped with DDM is that the $L_3$ mesophase is only present for systems with 2.5% mol dopant, and at one butanediol concentration (32.5% v/v) for 5% mol dopants. Interestingly in both cases, there is a $L_3$ mesophase present at 32.5% v/v butanediol, which suggests that the $Q_{II}^D/L_3$ mesophase boundary may be been lowered slightly to below the 30% v/v point where it is seen in undoped systems. Beyond this point the mesophase behaviour diverges, such that the $L_3$ mesophase persists at 2.5% mol doped concentrations, but disappears immediately as the dopant proportion is increased. That is, at 5% mol proportions or greater, the mesophase observed are coexisting $L_{α}$ and normal-type micellar ones. This same behaviour is also observed for the 2.5% mol doped system, but only when the proportion of butanediol in the lyotrope is increased to 47.5% v/v, the largest measured. That DDM should drive the monoolein membrane towards a $L_{α}$ is unsurprising, as the increase in headgroup area by the dopant alone should be expected to flatten the membrane substantially.
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Further to the mesophase transition observed above 32.5% v/v at a proportion of 5% mol, we also find that at higher doped proportions of DDM that the system is unable to exhibit the L_3 mesophase at all. In fig. 3.7b), while a 7.5% mol doped system can still form a Q\text{II}^D mesophase in water, this is not possible at 10% mol. Upon the addition of butanediol to the lyotrope, both systems form the coexisting L_α and micellar mesophases that we found previously.
3.2. Static Capillary Experiments

(a) 2.5% mol DDM  (b) 5% mol DDM

**Figure 3.7.** Mesophase sequence results for DDM-doped systems. For clarity, the results are split into a) 2.5% and 5% mol doped and b) 7.5% and 10% mol doped systems. The mesophase type is indicated by the shape of the scatter point, and their colors distinguish the concentration of the dopant in the system. The dashed vertical black line shows the expected point of the mesophase transition in undoped systems as shown in fig. 3.3.

3.2.2.4 DOPE

The scattering patterns in fig. 3.8 and the analysed mesophase sequence for DOPE-doped systems shown in fig. 3.9 presents perhaps the simplest trend for any of the doped systems. In the $Q_{II}$ regime, there is not a substantial or systematic variation in the lattice parameters between the doped systems, although there has been a slight reduction in comparison to the undoped MO system shown earlier. Above 30% v/v point, there is no change in where the mesophase boundary lies: at 32.5% v/v, we see a $L_3$ mesophase, as in the undoped system. However, in comparison to the undoped systems, there is a clear reduction in the size of the correlation lengths as the proportion of dopant increases. Note that the undoped trend line is partially obscured by the 2.5% mol trend.

Interestingly, the $L_3/L_\alpha$ mesophase boundary has not shifted downwards, with all the data above the 30% v/v point showing $L_3$ mesophases. There are two opposing competing factors at play here: firstly, the presence of DOPE will drive the system towards the $H_{II}$ mesophase while secondly, the effect of butanediol will be to flatten the interface. It is likely that these competing factors will result in a prolonged stability of the $L_3$ mesophase than might otherwise be anticipated.

The precise reason for the reduction in the correlation length is less obvious, but it is likely
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Figure 3.8. 1D scattering patterns for DOPE-doped systems. a) 2.5% mol, b) 5% mol, c) 7.5% mol, d) 10% mol. The patterns are arranged in ascending order of butanediol concentration.

still linked to an increase in the curvature of the membrane. As the average packing parameter of molecules in the system has been increased by the inclusion of DOPE, the curvature of the membrane will have increased, which in turn will bring the ‘necks’ of the L₃ mesophase closer together. This phenomenon will then only increase when the proportion of DOPE in the system is increased, which explains why the reduction in the correlation length measured is systematic with the four dopant proportions measured.
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Figure 3.9. Mesophase sequence results for DOPE-doped systems as analysed from the scattering patterns in fig. 3.8. The mesophase type is indicated by the shape of the scatter point, and their colours distinguish the concentration of the dopant in the system. The dashed vertical black line shows the expected point of the mesophase transition in undoped systems as shown in fig. 3.3.

3.2.2.5 DOPC

As highlighted above, DOPC can be an essential co-crystallisation component for some membrane proteins, so its effect on the mesophase behaviour of the L$_3$ mesophase should be of particular interest. The scattering data and analysed results are shown in fig. 3.10 and fig. 3.11 respectively. As with DDM, the results are split into lower and higher concentrations of dopant for clarity.

The principal feature of the results is the good accommodation of the L$_3$ mesophase at lower concentrations. For these systems, there is no shift in the position of the Q$_{II}$/L$_3$ mesophase boundary with reference to the undoped system, which remains at around 30% v/v butanediol. Interestingly, many of the correlation lengths for the doped L$_3$ mesophases appear to be not exceptionally dissimilar (cf. cholesterol and DOPE) to those measured in the undoped system shown in 3.3.

The only exception to this is the 2.5% mol system at 47.5% v/v butanediol, which has a dramatically shorter correlation length in comparison to what might be expected. This is highlighted by the inset scattering pattern in fig. 3.10a). Close inspection of this scattering pattern shows a slight sharpening of the broad L$_3$ peak, which suggests that there could be a L$_{α}$ mesophase in
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\[ \text{FIGURE 3.10. 1D scattering patterns for DOPC-doped systems. a) 2.5\% mol, b) 5\% mol, c) 7.5\% mol, d) 10\% mol. The patterns are arranged in ascending order of butanediol concentration. The inset pattern in a) indicates the slight peak sharpening effect at 47.5\% v/v butanediol as the system begins to exhibit L}_{\alpha} \text{ properties.} \]

coexistence. However, this effect can only be slight, considering the absence of any higher order L_{\alpha} scattering peaks. If this were the case, we would expect interbilayer distances to be reduced, as observed. Otherwise, the relatively close agreement between the DOPC doped and undoped correlation lengths is likely to be a result of the increased headgroup area in comparison to the DOPE or cholesterol doped systems.

The trend for the more highly-doped systems in fig. 3.11b) presents a slightly more complex trend. In comparison to the undoped systems, there is a significant shift in the position of the
3.2. STATIC CAPILLARY EXPERIMENTS

![Figure 3.11](image-url)

**Figure 3.11.** Mesophase sequence results for DOPC-doped systems. For clarity, the results are split into a) 2.5% and 5% mol doped and b) 7.5% and 10% mol doped systems. In both figures, the mesophase type is indicated by the shape of the scatter point, and their colours distinguish the concentration of the dopant in the system. The dashed vertical black line shows the expected point of the mesophase transition in undoped systems as shown in fig. 3.3. For reference, the red dashed trend line from fig. 3.3b) is also shown for the lower-doped systems in a).

\(\text{Q}_{II}^D/L_3\) mesophase boundary at both 7.5% mol and 10% mol dopant concentrations. The extension of the \(\text{Q}_{II}^D\) mesophase region is likely to be a result of the change in water distribution around the interfacial region of the system. Molecular dynamics and spectroscopy studies have found that choline headgroups allow comparatively looser packing of water at the interface, and that it is possible for hydrogen bonds to form between choline carbonyl groups and water [196–200]. That is, water can partition at the interface deeper than otherwise allowed by monoolein alone. This could act to reduce the partitioning effect of butanediol that otherwise drives the formation of the \(L_3\) mesophase. We can corroborate this by noting that the lattice parameter of the \(\text{Q}_{II}^D\) mesophase above 30% v/v butanediol steadily increases. The increase in lattice parameter implies a flatter and more hydrated interface, suggesting that there is a comparative increase in the amount of water in this region.

While there is a region in which the \(L_3\) mesophase exists it is comparatively shortened compared to undoped systems, with a downwards shift of the \(L_3/L_α\) mesophase boundary now observed at 47.5% v/v butanediol along with coexisting micelles. This can easily be rationalised because the mean headgroup area will have been significantly increased by the addition of DOPC in these
proportions. The increase in interfacial area will therefore act to reduce membrane curvature and push the system towards the Lα mesophase. As with the final L₃ mesophase at the 2.5% mol proportion, this also explains why the correlation lengths observed for the L₃ mesophase region of the phase sequence are significantly lower than in undoped systems. The formation of micelles can likely be attributed to a degree of phase separation in the system, with small quantities of molecules self-assembling into micelles rather than extended lyotropic liquid crystals.

### 3.2.2.6 DOPG + cholesterol

Some of the largest cubic mesophases ever constructed have been through a ternary mixture of monoolein, cholesterol, and charged lipid: Tyler et al. found that using this mixture at an elevated temperature of 35°C formed Q₊₁₁ mesophases with lattice parameters in excess of 400 Å [88]. To extend that work, and noting the delicacy of L₃ mesophase formation in low concentrations of DOPG alone, we used a fixed molar ratio of 90:10 of monoolein:DOPG/cholesterol, varying the DOPG:cholesterol ratio of 1:9 and 3:7. To note, preliminary experiments investigating doping with DOPG alone encountered solvent removal issues during sample preparation, resulting in reference data incompatible with literature (see, eg. [177, 201, 202]).

The scattering patterns and results of these systems are seen in fig. 3.12. The primary feature observed is that as expected, there is a mesophase transition immediately to the Q₊₁₁ mesophase. Of the two systems, the L₃ mesophase is only observed for the 1:9:90 DOPG:cholesterol:monoolein system. Interestingly, this transition comes about at a butanediol proportion of 32.5% v/v in the lyotrope, which marks a slightly downwards shift in the mesophase boundary compared to cholesterol on its own as we saw in section 3.2.2.2. More importantly, the correlation lengths that we observe for these mesophases grow to be in excess of 200 Å, which mean they are the largest lipid L₃ mesophases that have been observed to date. Notably, the L₃ mesophase persists across an identical range to the undoped system, but with significantly larger correlation lengths. Considering that we know that cholesterol headgroups form strong hydrogen bonds with their monoolein counterparts, it is likely that where before we struggled to observe L₃ mesophases in singly-doped DOPG systems, the addition of a significant quantity of cholesterol has stabilised the bilayer [12]. That is to say, the addition of DOPG makes the system doubly sensitive to both thermal fluctuations as well as intra-bilayer electrostatic repulsion, so
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(a) 1% mol DOPG + and 9% mol cholesterol scattering patterns

(b) 3% mol DOPG + and 7% mol cholesterol scattering patterns

(c) Analysed results for tertiary lipid systems of DOPG, cholesterol, and monoolein.

Figure 3.12. Results for ternary lipid systems of DOPG, cholesterol, and monoolein. a) and b) show the scattering patterns measured. The inset figure in b) shows the 1D SAXS pattern measured for the water (0% butanediol) system, to clarify the existence of the first three Bragg peaks corresponding to the $Q_{II}^P$ mesophase. To clarify the assignment of $L_\alpha$ mesophases in b), the peak order assignments have been included. c) shows the analysed results. In c) the mesophase type is indicated by the shape of the scatter point, and their colours distinguish the concentration of the dopant in the system. The dashed vertical black line shows the expected point of the mesophase transition in undoped systems as shown in fig. 3.3. The dashed red line shows the undoped trend in the growth of the $L_3$ mesophase found in fig. 3.3.

that it is unable to form extended structures. The addition of cholesterol - and note to a proportion less than the $Q_{II}^D/Q_{II}^P$ mesophase boundary in water - increases the bending modulus of the bilayer, so it is less sensitive to thermal fluctuations, so a stable $L_3$ mesophase can form.

With a higher proportion of DOPG and a reduction in cholesterol, the $L_3$ mesophase no longer
forms, and the system exhibits a $L_\alpha$ mesophase instead. In addition, at 0% butanediol (ie. water), the system shows only the first three Bragg peaks, as shown by the inset figure in fig. 3.12b). This is indicative of the loss of long-range order in the $Q_{III}^P$ mesophase, such that only a small proportion of the sample now remains in this mesophase. These $L_\alpha$ mesophases show have remarkable lattice parameters of several hundred ångströms, implying very large inter-bilayer spacings. At these length scales and from SAXS measurements alone, it is not entirely clear what is keeping the bilayers in a stable $L_\alpha$ mesophase, as we would reasonably expect attractive van der Waals forces to have decayed at these distances. This may be resolved in future by further systematic study of the ternary mesophase diagram at $q$ ranges unavailable to most laboratory instruments. To extend this, a quaternary mesophase study, introducing salt screening into the lyotrope component of the system, may also explain the unusual electrostatic stability.

3.3 Attempting high-throughput mesophase diagrams

While the data collected in section 3.2 provides a thorough picture of doped mesophase behaviour of the monoolein $L_3$ mesophase, it is highly labour-intensive to undertake such experiments. As we have shown, the behaviour of the $L_3$ mesophase is not as well understood or necessarily predictable in comparison to the cubic phases, and it is therefore desirable to have a simple method by which to do high-throughput experiments to determine the mesophase behaviour of a wide variety of systems. This experiment undertaken at Diamond Light Source SAXS beamline I22 (experiment sm17767-1) was an attempt as such. Previous work by Squires et al. and Seddon et al. has used a syringe pump to vary hydration conditions of a lipid system, and it was postulated that by slowly increasing the proportion of butanediol in the lyotrope, a mesophase sequence could be observed [57, 58].

3.3.1 Experimental set up

Bulk mixtures of lipids were prepared using the same solvent and concentrations as described in section 3.2.1. The mixtures were prepared in vials and left to evaporate to leave solid lipid mixtures. The mixtures were then heated, and molten lipid was dispersed on the inside of a capillary attached to the syringe pump tubing. The initial sample was then hydrated with water to guarantee that it started in the $Q_{III}^D$ mesophase.
3.3. ATTEMPTING HIGH-THROUGHPUT MESOPHASE DIAGRAMS

The experimental setup is illustrated in fig. 3.13. The syringe pump tubing was loaded with boluses of lyotrope in decreasing order of butanediol proportion, such that the lowest concentration would be pumped into the capillary first. The tubing was connected to the lipid-lined capillary so that the syringe pump could push the boluses through.

The experiment had been envisioned beforehand such that the syringe pump would push each bolus into the capillary individually then stop to allow for equilibration and measurement of the mesophase using SAXS. However, inertia in pump action prevented this method from working: the response of bolus motion to the initial pump motion was nonlinear, and there was no clear calibration by which to fix this issue. It was therefore decided that by loading sufficiently large boluses of 50 µl separated by 50 µl of air, the pump would pump slowly but continuously at a rate of 3 µl/minute.

The sample-detector distance was 5744.2673 mm, and the beam energy was 12.4 keV. I22 has a simultaneous SAXS-WAXS setup, which allowed for detection of discrete boluses entering and leaving the capillary: water will produce a WAXS peak at around 2 Å [203].

3.3.2 Results and discussion

As a result of both the limited time available for the experiment and the calibration error in the syringe pump, only a limited number of conditions could be trialled. In the time available
Table 3.1. Lyotrope conditions used in high-throughput experiments, listed by the dopant lipid used.

<table>
<thead>
<tr>
<th>Dopant molecule</th>
<th>Lyotrope proportions (% v/v butanediol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDM</td>
<td>27.5, 30, 32.5, 35, 37.5, 40, 42.5, 45</td>
</tr>
<tr>
<td>DOPC</td>
<td>27.5, 30, 32.5, 35, 37.5, 40, 42.5, 45</td>
</tr>
<tr>
<td>DOPE</td>
<td>40, 42.5, 45, 47.5</td>
</tr>
</tbody>
</table>

Figure 3.14. Sequential scattering patterns for high throughput syringe pump synchrotron experiments. The proportion of each dopant lipid was 5\% mol. The syringe pump starts at scan number 0, and scans are taken continuously. The bright fringes in the wide angle data correspond descendingly to the concentrations listed in table 3.1. The results here are for a single location along the capillary, with the average stage position across the entire sequence of scans noted for each experiment. In the figures, yellow represents high intensity scattering, and blue low.
3.3. ATTEMPTING HIGH-THROUGHPUT MESOPHASE DIAGRAMS

Dopant molecule | $L_3$ mesophase emergence point (% v/v butanediol)
--- | ---
DDM | 37.5
DOPC | 42.5
DOPE | 45

Table 3.2. Critical lyotropic proportions for lipid type emergence of the sponge mesophase.

three dopants of types 0 (DOPC), I (DDM), and II (DOPE) were used at a fixed proportion of 5% mol.

The sequence of scans for each of these experiments is shown in fig. 3.14. The results show are for a single point along the capillary in the sequence of beam scans made (see fig. 3.13). From top to bottom, the bright fringes in the WAXS region of the scattering data represents increasing exposure of the sample to the boluses of butanediol/water listed in table 3.1. Note that for each of the datasets, the variation in stage position is very small, meaning that the region of the capillary being exposed at the same index of the scanning loop on each successive scan is nearly identical.

For each of the datasets in fig. 3.14, the mesophase at each stage could then be determined by the SAXS region. In all cases, there is an initial $Q_{II}$ mesophase at scan 0, which is expected from the pre-hydration of the lipid film before being loaded into position on the beamline. In each case, the $Q_{II}$ peaks shift towards higher $q$ values in the time period before the first bolus of lyotrope hits the capillary, suggesting that the size of the mesophase is being reduced. This is almost certainly a result of sample dehydration from exposure to air, known to reduce the size of the lattice parameter [16]. Examination of the persistence of the bright fringes in fig. 3.14 also demonstrates the issue with the experimental setup, as although the boluses were made of identical volumes of lyotrope, there is clear inconsistency in how long they last in the capillary.

Once the samples have been exposed to the lyotrope boluses, the mesophase is not ascertainable from the series of peaks that were observed. Once the $Q_{II}$ mesophase has disappeared, there may still be small domains, or other dehydrated mesophases (eg. $L_\alpha$ or $Q_{II}^G$) present, but none could be properly distinguished.

In each of the 3 systems investigated a broad $L_3$ mesophase peak emerges in a single scan at different butanediol lyotrope proportions after several proportion boluses have passed through
CHAPTER 3. LIPID TYPE-DOPING OF THE SPONGE PHASE

(a) DDM  
(b) DOPC  
(c) DOPE

**Figure 3.15.** Capillary scans for high throughput syringe pump synchrotron experiments. Here, the scan number refers to a single location along the horizontal axis of the capillary.

The capillary. These critical proportions are listed in table 3.2. The fact that they are different suggests that the L₃ mesophase region of the mesophase diagram depends on the packing parameter of the dopant lipid. However, because this experiment only used one comparison dopant proportion, we cannot overwhelmingly conclude that this is the case.

While the emergence of the L₃ mesophase clearly depends on the proportion of butanediol in the lyotrope that the lipid film is exposed to, one of the notable features of the data in fig. 3.14 is the subsequent absence of any scattering in the small angle regime. That is, the sample appears to disappear once the L₃ mesophase has emerged. This is perhaps best explained by considering the data in fig. 3.15, which shows that in scanning along the capillary there is
clearly a more intense peak towards the end of the capillary (ie. towards the lyotrope exit as indicated in fig. 3.13). This suggests that the lipid sample is being pushed along the capillary by the action of the syringe pump, and that once the $L_3$ mesophase has formed, the sample itself is pushed out of the capillary, and there is nothing left to hydrate.

### 3.3.3 Comparison to capillary experiments

In comparison to capillary experiments, we have measured the mesophase behaviour of doped monoolein systems under far fewer lyotrope conditions, not least as a result of unintended sample disposal.

The most notable feature that we have observed in this set of experiments is the different quasi-critical points at which the $L_3$ mesophase emerges. As we discussed earlier, the $Q_{DII}/L_3$ mesophase transition is not a sharp transition, but a smooth one. However, the complete absence of coherent SAXS patterns in the sequence of data measured suggest that there is no intermediate mesophase at all, which is evidently not the case when we note the vast array of structures found as such in section 3.2.

That is not to say that the results of section 3.2 do not corroborate the shifting of mesophase boundaries at all. For example it is possible that when doping with DDM, the $L_3$ mesophase does still exist, just at proportions of lyotrope butanediol not measured. However, there is clearly a discrepancy in this sense that fig. 3.7a) shows that for 5% mol doped DDM, the $L_3$ mesophase only exists at 32.5% v/v butanediol in the lyotrope, whereas table 3.2 says the mesophase does not form until this is at 37.5% v/v - by which point the capillary experiment shows a clear $L_\alpha$ mesophase.

### 3.4 Conclusions

In this chapter, we have systematically investigated the effect of different - and common - dopant molecules on the ability of monoolein to form the lipid $L_3$ mesophase. We have found that it is possible to incorporate relatively significant proportions of dopant molecules into the membrane and still successfully form the mesophase. The effects of dopants studied in this chapter are summarised in table 3.3.

More interestingly, we have found that the correlation length of the $L_3$ mesophase are highly
CHAPTER 3. LIPID TYPE-DOPING OF THE SPONGE PHASE

<table>
<thead>
<tr>
<th>Dopant</th>
<th>Can the ( L_3 ) mesophase be formed?</th>
<th>Are there boundary changes at low concentrations?</th>
<th>Are there boundary changes at high concentrations?</th>
<th>Is the correlation length of the sponge mesophase affected?</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPE</td>
<td>yes</td>
<td>none observed</td>
<td>none observed</td>
<td>decrease proportional to dopant quantity</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>yes</td>
<td>slight increase</td>
<td>none observed</td>
<td>decrease; no clear systematic relationship</td>
</tr>
<tr>
<td>DOPC</td>
<td>yes</td>
<td>none observed</td>
<td>slight increase</td>
<td>decrease; no clear systematic relationship</td>
</tr>
<tr>
<td>DDM</td>
<td>yes</td>
<td>none at 2.5% mol</td>
<td>significant increase</td>
<td>where formed, similar to undoped systems</td>
</tr>
<tr>
<td>DOPG</td>
<td>not as observed</td>
<td>-</td>
<td>none observed</td>
<td>-</td>
</tr>
<tr>
<td>DOPG + cholesterol</td>
<td>yes</td>
<td>none observed</td>
<td>immediate ( L_3 ) formation</td>
<td>significant increase</td>
</tr>
</tbody>
</table>

TABLE 3.3. A summary of the effect of different dopants on the formation of the lipid \( L_3 \) mesophase. ‘Low concentrations’ refers to doping at 2.5% or 5% mol, and ‘high concentrations’ refers to doping at 7.5% or 10% mol. The boundary changes are described with respect to the undoped system, shown in fig. 3.3.

adaptable, but extremely sensitive to the dopant used. While DOPE and cholesterol both shorten the correlation length with respect to the undoped system, the larger headgroup area of DOPC enforces the system to remain similar. Where the addition of small proportions of DDM permits the formation of the \( L_3 \) mesophase, the correlation length is similarly reduced.

Further to the addition of zwitterionic and neutral molecules, we have found that electrostatic lipids may significantly increase the \( L_3 \) mesophase correlation length under certain conditions. The addition of DOPG on its own does not appear to permit the formation of the mesophase, although the data collected on the in house instrument are limited by an inaccessible low q regime. It may be the case that such mesophases are measurable on other such instruments. These would likely be synchrotron sources, where both the camera length and source energy can be adapted to allow for measurement in this range. Other methods recently developed by Pauw et al. would also serve this purpose well [204]. While the precise behaviour of these systems remains an open question, we have shown that the combination of anionic DOPG and cholesterol does significantly increase the \( L_3 \) correlation length, and at low proportions of DOPG forms over a wide range of lyotrope conditions.

Overall, we can conclude that the \( Q_{II}^{P} / L_3 \) mesophase transition is particularly sensitive to two
3.4. CONCLUSIONS

quantities in particular: the bending modulus, and the interfacial area of the membrane. In the case of former, we have demonstrated this by finding that cholesterol - known to universally increase membrane elastic moduli - shifts the mesophase boundary upwards to higher proportions of butanediol. Regarding the latter point, sponge-forming agents such as butanediol act to increase the interfacial area of the membrane on their own, and we have found that varying the size of the dopant headgroup area can affect the positions of the mesophase boundaries of the \( Q_{11}^D / L_3 / L_\alpha \) sequence. That is, a larger headgroup (eg. DOPC, DDM) can reduce the proportion of butanediol in the lyotrope required to form the \( L_3 \) mesophase, with knock-on effects on the position of the \( L_3 / L_\alpha \) mesophase boundary.

Returning to the context of this work that we began with, we have demonstrated here that while we understand the effect of a great variety of dopants on monoolein cubic phases in water, there is a significant change in the behaviour of these systems using different lyotropic conditions. In the specific context of LCP crystallisation, we have shown that naïve choice of crystallisation media without considering the effect on the host membrane itself could prove detrimental. Specifically on this point, doping with DDM acts very strongly to prevent the formation of the \( L_3 \) mesophase. This is a significant result for those working to purify and crystallise membrane proteins: recall that section 3.1.1.4, we noted that DDM is used frequently in the process of membrane protein purification. The results of this chapter show that once membrane proteins have been purified, the chance of forming protein crystals should be expected to increase the more DDM that has been removed.

Perhaps most significantly in this chapter, we have shown that tertiary lipid systems of anionic lipid along with cholesterol can result in significant increases in the correlation length of the \( L_\alpha \) mesophase, which could inform future LCP studies where the need for large water channels is essential for successful crystallisation. However, this finding must be balanced against the necessarily high salt concentrations used in LCP trials in order to induce the crystallisation process. Therefore while the use of anionic lipids certainly promotes mesophase swelling, they are likely to have limited effects in LCP applications.
DEVELOPING METHODS FOR MOLECULAR DYNAMICS SIMULATIONS OF LIPID SPONGE PHASES

4.1 Context

In chapter 3, we saw how the $L_3$ mesophase forms in a narrow region of composition space: adding dopant lipids can easily suppress its formation in favour of other mesophases. The questions that have arisen out of that work, which we will now seek to resolve in molecular dynamics studies are how does the $L_3$ mesophase form?, how does it relate to the $Q_{II}$ mesophase?, and why do certain dopants hinder its formation?

While the last several decades have seen many hundreds of experiments conducted on cubic phases to understand their properties, there have been significantly fewer investigations done in silico. In 2001, Marrink and Tieleman performed a united atom simulation of monoolein and water, which principally showed that lipid packing was similar to flatter mesophases, and that the dynamics of water was slowed by a factor of 3 in comparison to flatter mesophases [170]. In addition, they showed that the cubic phase was unstable, transitioning to a $H_{II}$ mesophase after just 35 ns. A separate analysis of this transition showed that the transition occurred, as expected, by the formation of stalks and changes in the arrangement of water channels in the system [205].

To date, no other molecular dynamics work has investigated cubic phases in either atomistic
CHAPTER 4. DEVELOPING METHODS FOR MOLECULAR DYNAMICS SIMULATIONS OF LIPID SPONGE PHASES

![Chemical structure of 1,4 butanediol](image)

**Figure 4.1. The chemical structure of 1,4 butanediol**

or united atom detail. There are three coarse-grained examples, using the Martini force field [168]. The first, by Fuhrmans et al., observed a stable $Q_{II}^D$ mesophase in a self-assembled system of DOPE, water, and fusion peptides [206]. This result is most significant for demonstrating that fusion peptides can alter the topology of the expected system: DOPE would otherwise spontaneously form a $H_{II}$ mesophase. Subsequent work has since used coarse-grained models of monoolein to simulate cubic phases explicitly, with particular interest in the dynamics of membrane proteins in cubic phases, and how hydrophobic mismatch drives the formation of stacked lamellae systems during crystallisation [171, 207].

In this chapter, we will develop and validate several methods to investigate the structural properties of highly curved lipid mesophases modelled using coarse-grained molecular dynamics with a view to being able to probe the $Q_{II}^D/L_3$ transition in a dynamic way. In section 4.2 we will firstly explore how to model a sponge-forming agent to use in modelling a dynamic mesophase transition. Subsequently in section 4.3, we will present a method with which to measure the curvature of membranes in a mesophase-agnostic way. This method will be validated against mesophases of known curvatures before we use it on simulations of $L_3$ mesophase systems. We will then review a method of measuring elastic moduli from simulations using real space fluctuations in section 4.4. Finally in section 4.5, we will show that interfacial areas of mesophases measured using an alpha surface construction matches the analytical results for a $Q_{II}^D$ mesophase.

### 4.2 Modelling butanediol in coarse-grained simulations

#### 4.2.1 Background

1,4 butanediol (as seen in fig. 4.1) is the main sponge-forming diol used throughout this thesis, having been well modelled to induce the sponge phase at sufficient concentrations [52, 56, 57]. However, in modelling the sponge phase in a coarse-grained simulation using the Martini force


### 4.2. MODELLING BUTANEDIOL IN COARSE-GRAINED SIMULATIONS

<table>
<thead>
<tr>
<th>Martini type atom</th>
<th>Suggested building block</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>C₃-OH</td>
</tr>
<tr>
<td>P2</td>
<td>C₂-OH</td>
</tr>
<tr>
<td>P3</td>
<td>HO-C₂=O</td>
</tr>
<tr>
<td>P4</td>
<td>HOH; HO-C₂-OH</td>
</tr>
<tr>
<td>C1</td>
<td>C₄</td>
</tr>
<tr>
<td>C2</td>
<td>C₃</td>
</tr>
</tbody>
</table>

**Table 4.1.** The type types of Martini atoms used to construct diols, and their respective building blocks, taken from reference [168]. ‘P’ atoms replicate polar behaviour, and ‘C’ atoms replicate apolar behaviour.

field, a crucial question arises regarding the coarse-graining procedure. Martini uses a 4-to-1 heavy atom mapping procedure, but butanediol has a total of 6 heavy atoms: 4 Carbons and 2 Oxygens. The Martini building block procedure detailed in Table 4.1 would seem to suggest that the best model would be two atoms of type P2, as the building block for ethanol. However, one of the important features of butanediol as a sponge-forming diol is its symmetric polar ends combined with a non-polar centre, which causes it to partition at the membrane interface, to drive an increase in lipid headgroup area towards the sponge mesophase [55]. Using a two bead model is therefore unlikely exhibit this important partitioning feature, and so an extra non-polar core will have to be introduced to the model.

#### 4.2.2 Measuring diol partition

In order to verify the potential models of a sponge-forming diol, we followed the work of Evenbratt et al., who measured the partition coefficient of pentanediol in lipid mesophases between the lipid and water domains [55]. To begin, they note that sponge phases are a 3 component system: water, a sponge-forming diol (in their experimental case, pentanediol), and monoolein. Their weight fractions, \(x\), are related as:

\[
x_{H₂O} + x_{POL} + x_{GME} = 1
\]

With \(x_{POL}\) indicating the weight fraction for pentanediol, \(x_{GME}\) the monoolein weight fraction, and \(x_{H₂O}\) the water weight fraction. However, as discussed above, because pentanediol has symmetric polar ends and a non-polar core, it is expected that the molecules will distribute themselves between the water channels of the system and the headgroup region of the membrane. Therefore, the weight fraction \(x_{POL}\) is split as:
where $x_{POL}^{aq}$ and $x_{POL}^{lip}$ are the respective weight fractions of pentanediol in the aqueous and lipid domains of the system. That is to say, the total weight fraction of diol in the system is distributed between the aqueous and lipid domains of the system. The proportions of POL in each domain is then described as:

\[ p_{aq}^{POL} = \frac{x_{POL}^{aq}}{x_{POL}} \]
\[ p_{lip}^{POL} = \frac{x_{POL}^{lip}}{x_{POL}} \]

such that:

\[ p_{aq}^{POL} + p_{lip}^{POL} = 1 \] (4.4)

Evenbratt et al. subsequently define the domain partition coefficient of pentanediol between the aqueous and lipid domains as:

\[ K = \frac{x_{POL}^{lip}}{(x_{POL}^{lip} + x_{GME})} / \frac{x_{POL}^{aq}}{(x_{POL}^{aq} + x_{H2O})} \] (4.5)

Ie. the ratio of the proportions of the weights of pentanediol in the lipid and aqueous domains of the system. By measuring the chemical shifts in the $^1$H NMR spectra for the peaks that arise from the 3 different $^1$H atoms in pentanediol in mesophases with different weight fractions of pentanediol, the authors calculate that $K = 0.78 \pm 0.14$. Moreover, they find that this is constant, regardless of the value of $x_{POL}$, showing that it is independent of the mesophase of the system.

However, noting eqs. (4.2) to (4.4) can combine to define:

\[ x_{POL}^{lip} = x_{POL}[1 - p_{POL}^{aq}] \] (4.6)

eq (4.5) can be rearranged to give:

\[ K = \frac{x_{POL}^{1 - p_{POL}^{aq}}}{x_{POL}[1 - p_{POL}^{aq}]} / \frac{x_{POL}^{aq}}{x_{POL}^{aq} + x_{H2O}} \] (4.7)
4.2. MODELLING BUTANEDIOL IN COARSE-GRAINED SIMULATIONS

Figure 4.2. The construction of a Delaunay surface from water beads in a MO/H$_2$O/diol system. a)-c) demonstrates the probe sphere method as explained by Stukowski [208]. A set of input points is firstly tesselated in a), before each one has a circumsphere constructed, whose radius is compared to a set radius of a probe sphere in b). If the radius is smaller that the probe sphere’s, it is accepted into the surface, and otherwise rejected. This is demonstrated by the final constructed surface in c), where the upper edge tesselate has been rejected. In d) and e) two examples of surfaces using probe spheres of radius 10 Å and 15 Å respectively. The water surface (in Blue) is constructed at two different radii, and then the proportion of diol atoms (in Green) within the surface are counted. Other elements of the system are excluded for clarity.

Therefore, by measuring the proportion of butanediol in the water domains in a system of known weight fractions, it is possible to directly measure the partition coefficient. Using the method of Stukowski, we construct an alpha sphere surface through a Delaunay tessellation of inputted atoms [208]. The precise shape of the surface is determined using a probe sphere method, by which elements whose fitted circumspherical radius is smaller than the probe
CHAPTER 4. DEVELOPING METHODS FOR MOLECULAR DYNAMICS SIMULATIONS OF LIPID SPONGE PHASES

Figure 4.3. Illustration of the parameter space considerations for coarse grained diol modelling. In modelling a sponge-forming diol, we need to consider the optimal Martini type bead for the molecule, and how they are linked. Further questions for optimisation modelling are: 1) How many core hydrophobic beads do we need?; 2) What is the optimal bond length; 3) What should the bond equilibrium angle be?

radius are included within the closed surface, and otherwise not, as illustrated in fig. 4.2a) - c). In fig. 4.2d) and e), an example of this surface is shown at two probe sphere radii, 10 Å and 15 Å, and it can be seen that as the probe sphere radius is increased, the surface volume increases also. As implemented in Ovito, it is possible to identify whether a point is within or outside the constructed volume of a surface [209]. We can therefore measure the proportion of the diol atoms that lie either inside or outside the water region of the simulation box, and have a measure of $p_{POL}^{aq}$ with which to calculate K based on the weight proportions of the components.

4.2.3 Designing coarse-grained diols

In considering the design of a coarse-grained diol model, there are therefore several parameters to investigate. As we showed in listing 2.1, the most basic description of a molecule in GROMACS needs to contain information about 1) atom types, 2) bonds between them, and 3) the bond angles. To investigate this parameter space, we should systematically vary this parameter space. An illustration of the problems we face in undertaking this modelling optimisation are shown in fig. 4.3.

Most trivially, we can investigate molecular total and bond length by varying the number of atoms in a diol molecule, and then changing the length of bonds. By molecular symmetry we’ll
4.2. MODELLING BUTANEDIOL IN COARSE-GRAINED SIMULATIONS

assume (but later investigate) an equilibrium angle of 180° between atoms in the molecule.

Using this fixed angle, we can design combinations of the Martini atom building blocks seen in table 4.1 in 2-, 3-, and 4-atom arrangements to replicate the behaviour of a sponge-forming diol additive. To test the hypothesis that a trivial P2-P2 formulation for a diol molecule would not be appropriate, we tested this model only for a 2-atom model.

The combinations of 3-atom tested are detailed in table 4.2. Models a)-d) and g)-h) are the possible combinations of the atoms in table 4.1. Models e) and f) use the same atoms as model c), except use a shorter bond length of 0.43 nm and 0.45 nm respectively. These values are a reduction compared to the standard Martini bond length of 0.47 nm, and have been chosen to shorten the length of the molecule.

As well as ‘normal’ type 3-bead models, Martini ‘S’-type models were tried, as detailed in table 4.3. As Marrink et al. describes, S-type atoms were designed in order to enable ringed molecules to be built from Martini coarse-graining, noting that for ringed molecules in particular, a 4:1 atom mapping is inadequate [168]. The S atoms also have a modified effective non-bonded interaction, such that the strength and zero-potential distance of the LJ interaction are reduced for interactions between S-type atoms only. Interactions between S-type and ‘normal’ atoms remain the same as the non-bonded interaction between the normal atoms.

These four models were chosen in order to use the S-type atoms, which, due to the reduction in effective interaction parameters, can pack together more closely, therefore giving an increased liquid density, which may be a better reflection of the physical characteristics of butanediol.

The models described in table 4.3 are the ‘S’-type equivalents of ‘normal’-type models a)-d) listed in table 4.2.

In addition to the models detailed in table 4.2, we tested six models using the next full iteration of the Martini force field, version 3. Version 3 was under beta release at the time of testing, but already used successfully for a small number of published molecular dynamics experiments [210–212]. Since this work, the full details of the force field have been published [213]. One significant addition in version 3 is the parameterisation of ‘T’ type beads, which allow for finer heavy-atom mapping than before, at a level of 2:1, which allows for a finer coarse-graining of molecules than previously. Six possible models are listed in table 4.4, using two ‘TP’ types and three ‘TC’ types.

To further investigate the effect of molecular length on the measure of the partition coefficient,
CHAPTER 4. DEVELOPING METHODS FOR MOLECULAR DYNAMICS SIMULATIONS OF LIPID SPONGE PHASES

<table>
<thead>
<tr>
<th>a)</th>
<th>P1-C1-P1</th>
<th>b)</th>
<th>P1-C2-P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>c)</td>
<td>P2-C1-P2</td>
<td>d)</td>
<td>P2-C2-P2</td>
</tr>
<tr>
<td>e)</td>
<td>P2-C1-P2</td>
<td>f)</td>
<td>P2-C1-P2</td>
</tr>
<tr>
<td>g)</td>
<td>P3-C1-P3</td>
<td>h)</td>
<td>P3-C2-P3</td>
</tr>
<tr>
<td>i)</td>
<td>P4-C1-P4</td>
<td>j)</td>
<td>P4-C2-P4</td>
</tr>
</tbody>
</table>

**Table 4.2.** The 3-atom Martini models of diols tested in measuring the partition coefficient.

<table>
<thead>
<tr>
<th>a)</th>
<th>SP1-SC1-SP1</th>
<th>b)</th>
<th>SP1-SC2-SP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>c)</td>
<td>SP2-SC1-SP2</td>
<td>d)</td>
<td>SP2-SC2-SP2</td>
</tr>
</tbody>
</table>

**Table 4.3.** The S-type 3-atom Martini models of diols tested in measuring the partition coefficient.

<table>
<thead>
<tr>
<th>a)</th>
<th>TP1-TC1-TP1</th>
<th>b)</th>
<th>TP1-TC2-TP1</th>
<th>c)</th>
<th>TP1-TC3-TP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>d)</td>
<td>TP2-TC1-TP2</td>
<td>e)</td>
<td>TP2-TC2-TP2</td>
<td>f)</td>
<td>TP1-TC3-TP2</td>
</tr>
</tbody>
</table>

**Table 4.4.** The six 3-atom Martini v3 models of diols tested in measuring the partition coefficient.

we can also increase the total length of the diol by using a 4-atom model. The models investigated are listed in table 4.5. All of the models are simple extensions of some of the 3-atom models trialled, by the addition of an extra ‘C’ type atom in the core of the molecule.

Once the effect of molecular length and CG atom types had been investigated, we also used the SP1-SC2-SP1 model in varying the equilibrium bond angle between the 3 atoms, instead using angles of 130°, 160°, and 170°.

### 4.2.4 Simulation details

Using the models listed in tables 4.2 to 4.5, self-assembly simulations were carried out in the standard way detailed in section 2.4.3. A standard ratio system of the components used experimentally detailed in table 4.6 was used, such that the lipid:solvent weight:weight ratio

<table>
<thead>
<tr>
<th>a)</th>
<th>P1-C1-C1-P1</th>
<th>b)</th>
<th>P1-C2-C2-P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>c)</td>
<td>P2-C1-C1-P2</td>
<td>d)</td>
<td>P2-C2-C2-P2</td>
</tr>
<tr>
<td>e)</td>
<td>SP2-SC1-SC1-SP2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.5.** The five 4-atom Martini models of diols tested in measuring the partition coefficient.
4.2. MODELLING BUTANEDIOL IN COARSE-GRAINED SIMULATIONS

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight Proportion (%)</th>
<th>Number of molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoolein</td>
<td>0.6</td>
<td>1000 2000 4000</td>
</tr>
<tr>
<td>Diol</td>
<td>0.16</td>
<td>1055 2110 4220</td>
</tr>
<tr>
<td>Water</td>
<td>0.24</td>
<td>1980 3960 7919</td>
</tr>
</tbody>
</table>

Table 4.6. The 3 components of the self-assembly simulation and their respective weight proportions, and the number of molecules in a simulation box that this corresponds to.

![Graph](image)

**Figure 4.4.** The partition coefficient $K$ for a 2-atom P2-P2 diol model. The plot on the left shows how $K$ changes with both surface construction from the radius of the probe sphere used and time (indicated by scatter point colour). The plot on the right shows how $K$ varies with time.

is 60:40. The solvent consists of 40% diol, a known mid-point in the mesophase sequence as measured by Cherezov et al., so that the system should represent a $L_3$ mesophase [52]. The values in table 4.6 were calculated using real molecular weights, and correcting for the fact that the Martini P4 water atom represents 4 water molecules. After equilibration, the self-assembly simulations were run for 1 $\mu$s. To investigate the effect of the change in diol topology, the system using 2000 molecules was used.

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Figure 4.5. The value of the partition coefficient, K, calculated in self-assembled lipid systems using different possible Martini v2 3-atom models of diols terminated with P1 or P2 types. For each model, the variation with probe sphere radius is plotted, and the time variation for the mean value of K at each value of K is shown as an inset figure. The time variation for probe sphere radii is indicated by the colourbar.
4.2. MODELLING BUTANEDIOL IN COARSE-GRAINED SIMULATIONS

4.2.5 Results and discussion

4.2.5.1 Number of atoms

From each simulation, 33 equally temporally spaced frames were used to calculate $K$. For each frame, a surface was constructed using the water atoms at a range of probe sphere radii, so that for each model of diol, $p_{POL}^{aq}$ was calculated using the method described above and shown.
CHAPTER 4. DEVELOPING METHODS FOR MOLECULAR DYNAMICS SIMULATIONS OF LIPID SPONGE PHASES

**Figure 4.7.** The value of the partition coefficient, $K$, calculated in self-assembled lipid systems using different possible Martini v2 3-atom models of diols using ‘S’ type beads. For each model, the variation with probe sphere radius is plotted, and the time variation for the mean value of $K$ at each value of $K$ is shown as an inset figure. The time variation for probe sphere radii is indicated by the colourbar.

In fig. 4.2. From the known weights in table 4.6 and $p_{aq}^{POL}$, eq. (4.7) was used to calculate $K$ across the different models.

As expected, the result for the P2-P2 model (fig. 4.4) shows an extremely strong preference for residing in the aqueous domains of the system, with $K = 0.197 \pm 0.002$. This is unsurprising, as without any apolar core, the non-bonded interactions are exclusively attractive towards the water domain of the system - recall that in Martini v2, water is modelled using a single P4.
4.2. MODELLING BUTANEDIOL IN COARSE-GRAINED SIMULATIONS

**Figure 4.8.** The value of the partition coefficient, K, calculated in self-assembled lipid systems using different possible Martini v2 4-atom models of diols. For each model, the variation with probe sphere radius is plotted, and the time variation for the mean value of K at each value of K is shown as an inset figure. The time variation for probe sphere radii is indicated by the colourbar.

- a) P1-C1-C1-P1
  - K = 32.060 ± 1.340
- b) P1-C2-C2-P1
  - K = 25.735 ± 0.916
- c) P2-C1-C1-P2
  - K = 10.153 ± 0.453
- d) P2-C2-C2-P2
  - K = 6.211 ± 0.217
- e) SP2-SC1-SC1-SP2
  - K = 3.987 ± 0.050
atom. In fig. 4.4, we can see both how $K$ changes with the surface construction, and what the average value for the range of probe spheres used changes over time. As the probe sphere radius increases, $K$ decreases. This is to be expected, as when the probe sphere radius increases, so too does the volume fraction of the surface. The surface therefore encompasses more of the diol beads in the simulation box, and so $K$ is correspondingly reduced as per eq. (4.7).

There is considerably more variation between models for 3-atom diols, the results for which are in figs. 4.5 to 4.7. In every model, we firstly note from the inset plots that there is not any consistent variation of $K$ with time throughout the simulation. Model c) perhaps shows a slight reduction through the first third of the simulation, but subsequently shows little consistent trend. Either way, the overall value of $K$ is very low compared to the desired value.

The value of $K$ averaged across all times and probe sphere radii varies greatly from model to model, with the most significant cause arising from the changes in the polar end atoms. From examining eq. (4.5), we can see that if diols distribute themselves equally between the lipid and aqueous domains of the mesophase, $K = 1$. The result of Evenbratt et al. of $K = 0.78 \pm 0.14$ shows that the weight fraction of diol in the lipid region is smaller than in the aqueous domain - that is, the diol has a slight preference for the aqueous region of the mesophase.

In fig. 4.5 a) and b), we can immediately eliminate models using P1 polar atoms as useful candidates, as with values of $K$ much greater than 1 they clearly have a strong preference for the lipid region of the system. Models using P2 instead (c) - e)), have a much stronger preference for the aqueous domain. As by definition (seen in table 4.1), P2 has a stronger polarity than P1, this is naturally expected. Similarly, the increase in polar affinity of the molecule through the use of C2 between a) and c), and b) and d) respectively, reduces the value of $K$. The effect of shortening the bond length from 0.47 nm in c) to either 0.45 nm or 0.43 nm in models f) and e) respectively does not have a profound effect on the behaviour of the system, with only a very small increase in $K$ as the bond is shortened. Further increasing the polar affinity of the P-type outer atoms, as shown in fig. 4.6 only serves to dramatically reduce the values of $K$ measured further.

The S-type models trialled in fig. 4.7 produce values of the partition coefficient somewhat closer to the experimentally observed value. The values range from 0.528 to 1.014, suggesting that in comparison to the ‘normal’ type atoms, there is now a much weaker preference to occupy the aqueous domains of the system compared to the lipid ones. This is a result of weaker in-
termolecular interactions between the diol molecules themselves. A weaker intermolecular
interaction between the diols themselves will result in an increased fluid density in the system,
which will likely drive increased occupancy of the lipid domains. Similarly, this would explain
why the variation of K with probe sphere radius is more linear in this case than in others, as
the number of diol molecules that are found around the lipid/aqueous interface is greater than
in other cases.

Increasing the number of atoms in the diol model to 4 produces results shown in fig. 4.8. Inter-
estingly here, there is slightly more temporal variation of the partition coefficient than with
3-atom models, apart from in the S-type model, e). Models a) - d) show an initial increase from
the start of the simulation to a more stable value, again by around a third of the way through.
However, the most obvious result from these trials is that in all cases the partition coefficient
is much greater than 1, indicating a very strong preference to occupy the lipid domains of the
system as opposed to the aqueous ones as seen in 3-atom models. For 4-atom models, all bond
equilibrium bond angles were kept at 180° as before, but these results indicate that with the
increase in the hydrophobic core, the repulsion from the water domains was the dominant force
during the self-assembly simulation. Additionally, we can see from model e), SP2-SC1-SC1-
SP2, that there is no longer a linear relationship between the increase in probe sphere radius
and corresponding decrease in K, indicating that the distribution of diols is no longer around
the lipid/aqueous interface as before.

4.2.5.2 Martini 3

The addition of ‘T’ type atoms in the Martini v3 force field should allow for molecules with
smaller Connolly surfaces, and greater detail in the representation of molecules. Simulations
using the new force field parameters were run under the same conditions as elsewhere. The
topology of monoolein was updated for correct Martini atom names, but otherwise unchanged.
Similarly, Martini 3 uses a new independent model for water, which was used. To confirm that
the slight change in parameterisation did not affect the expected mesophase behaviour, a 1µs
self-assembly simulation was carried out on the MO and water molecules alone at a weight
ratio of 60:40 (950:3135 molecules respectively), which produced a Q
D
II
system as expected.
Subsequent partition coefficient simulations were carried out as before.

The principal change was in the diol models, where we used ‘T’ type based molecules. The par-
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FIGURE 4.9. The temporal variation of the mean of $K$ between different model diols as modelled using version 3 of Martini.
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The temporal variation of the mean of $K$ between different model diols as modelled using the beta release of version 3 of Martini.

Partition coefficients for these molecules are seen in fig. 4.9. Most of the results are far smaller than the physically relevant value of the coefficient. However, they show both a far more consistent variation with time, and a relatively consistent linear decrease in $K$ as the probe sphere size increases. The only system where $K$ is remotely physically relevant is a), where $K = 0.914 \pm 0.009$, showing a very slight preference for the aqueous domain. This is to be expected considering the *intermediate* strength interactions that the diol molecules have with both the lipid headgroup region and water for this model.
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**Figure 4.11.** Histograms showing the distributions of diol angles for the four different models tested.

4.2.5.3 Angles

We further investigated the effect of equilibrium angle on the partition coefficient using just one model with a known result at an equilibrium angle of 180°. The model used was the 3-atom SP1-SC2-SP1, which from fig. 4.7 we note has \( K = 0.834 \pm 0.008 \). The three angles investigated were 170°, 160°, and 130°, the results of which are plotted in fig. 4.10. At angles close to 180°, there doesn’t appear to be a significant effect on the partition coefficient. This is likely due to results we see in fig. 4.11, which shows that while the systems have different equilibrium angles, the distribution of angles across frames shows an average bond angle of around or below 140°, indicating that it is the chemical representation of the diol that drives the partitioning behaviour the most.
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(a) The final frame of the P1C2P1 simulation with an alpha sphere surface constructed from water beads

(b) The final frame of the P1C2P1 simulation with surfaces constructed from both water beads and lipid molecules.

(c) The final frame of the SP1SC2SP1 simulation with a surface constructed over water beads

FIGURE 4.12. Comparing the final simulation frames of the two best diol models trialled, a) and b) P1C2P1 and c) SP1SC2SP1. The water surface is coloured in blue, and the diol atoms in green. In a) and c), lipids have been removed for clarity. In a) there is obvious phase separation between the diol and water atoms, clarified by b), where an additional surface (in red) encompassing all lipid atoms has been constructed.

4.2.6 Choice of diol model

To summarise the above results, we have found the following:

- The optimal number of atoms to use to model a diol is 3.
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Figure 4.13. As the size of the system increases, the value of the partition coefficient remains constant.

- A limited trial with the latest version of the Martini force field shows no comprehensive advantage for our purposes than version 2.

- The length of bonds does not appear to matter; using the standard bond length in the molecule is sufficient.

- The equilibrium angle has a some effect on the value of the partition coefficient: for greatest physical relevance, using an angle of 180° will be appropriate.

The question that then remains is which of the models to use hereafter, considering what we have learned. Most obviously, we will use a 3-atom model which gave us a partition coefficient close to that of Evenbratt et al., $K = 0.79 \pm 0.14$. Two models distinguish themselves in having partition coefficients close to the desired value of $K = 0.79 \pm 0.14$, namely P1-C2-P1, with $K = 0.732 \pm 0.012$, and its S-type equivalent SP1-SC2-SP1 with $K = 0.834 \pm 0.008$. A comparison of the final two frames of these simulations are seen in fig. 4.12, showing that the choice cannot be made entirely naively. In fig. 4.12a) and b), the use of the P1-C2-P1 diol model exhibits a large degree of phase separation between the water and diol domains of the system. One of the fundamental assumptions made about the system - that diols partition themselves between a lipid and aqueous domain - is therefore broken. In comparison, fig. 4.12b) shows that for the
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SP1-SC2-SP1 model, the diols are well encapsulated by the water channels of the system, and do in fact partition themselves between the two domains. As this SP1-SC2-SP1 model does not break the assumptions we have had to make, and has a value of the partition coefficient that is sufficiently close to experimentally confirmed value, it can be used to model a sponge-forming diol for the subsequent work in this chapter.

To further confirm that this model would be appropriate, we conducted 2 further experiments using 1000 and 4000 lipids at the same lipid:water:diol ratio to investigate the effect of system size on $K$. The precise system composition is detailed in Table 4.6. As Fig. 4.13 shows, $K$ remains in a small range around $0.821 \pm 0.005$ across the three systems, indicating that it will be appropriate regardless of the number of lipids used.

4.3 Measuring membrane curvature

The work in this section has been summarised as paper, at the time of writing deposited to the Arxiv preprint repository [214].

One of the major motivations for the work in this chapter is the analysis of the physical properties of lipid sponge mesophases in ‘real’ space, in relation to other simulated lipid mesophases. Of particular interest are the curvature properties of the sponge phase. The topology of the sponge phase is not related to any particular analytical surface (as the cubic phases are), and so an understanding of the curvature will enable us to understand both its topological relevance to other mesophases.

Yesylevskyy and Ramseyer devised a method of measuring the curvature of simulated lipid mesophases using localised surface reconstruction techniques from computer graphics [215]. Using their method on a single L$_\alpha$ mesophase, they investigated the effect of the addition of cholesterol, showing that it made the system more topologically heterogeneous by broadening the distributions of curvature obtained.

Their method works in the following way:

1. From a selected atom find the coordinates of all atoms within a radius, $r$, to form a point cloud of atoms of which to measure the curvature, as illustrated in Fig. 4.14.

2. Perform Principal Component Analysis on the point cloud.
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Figure 4.14. A 2d equivalent of the method of Yesylevskyy and Ramseyer for measuring the curvatures of simulated lipid mesophases. A certain atom in the membrane (striped green, bolded outline) searches for near neighbours of the same green type at 2 radii, \( r_1 \) (blue circle), and \( r_2 \) (orange circle). The green atoms within these circles form point clouds, which are analysed, transformed, and fitted to a quadric surface, whose curvatures are then calculated. In this case, the associated surfaces (blue and orange lines for \( r_1 \) and \( r_2 \) respectively) have very different curvatures, as a result of the different sizes of the point clouds. In this example, the green atoms can be thought of as terminal atoms of individual molecules at the centre of the membrane, while the unfitted purple atoms are associated atoms further towards the molecular headgroups.

3. Select the smallest component as the \( z \) axis, and transform the point cloud’s coordinates accordingly.

4. Translate the point cloud mean to the origin.

5. Fit the point cloud to the quadric surface:

\[
z = Px^2 + Qy^2 + Rxy + Sx + Ty + C
\]  

(4.8)

Using the fitted surface described by eq. (4.8), its first \( (I) \) and second \( (II) \) fundamental forms can be used to calculate the curvature at the origin. To recap section 1.3.1, the fundamental forms of a parametric surface \( f(a, b) \) are:
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\[ I = Eda^2 + Fdadb + Gdb^2 \]
\[ II = Lda^2 + 2Mdadb + Ndb^2 \]  

(4.9)

where:

\[ E = \vec{r}_a \cdot \vec{r}_a \]
\[ F = \vec{r}_a \cdot \vec{r}_b \]
\[ G = \vec{r}_b \cdot \vec{r}_b \]

(4.10)

\[ L = \vec{r}_{aa} \cdot \mathbf{n} \]
\[ M = \vec{r}_{ab} \cdot \mathbf{n} \]
\[ N = \vec{r}_{bb} \cdot \mathbf{n} \]

and \( \mathbf{n} \) is the normal vector:

\[ \mathbf{n} = \frac{\vec{r}_a \times \vec{r}_b}{|\vec{r}_a \times \vec{r}_b|} \]

(4.11)

For the surfaces described by eq. (4.8) fitted to point clouds such that the point cloud mean is at \( x=y=0 \), we therefore arrive at the following values for the parameters:

\[ E = 1 + S^2 \quad F = ST \quad G = 1 + T^2 \]
\[ L = 2P \quad M = R \quad N = 2Q \]

(4.12)

Therefore, the metric tensors of the fundamental forms are:

\[ I = \begin{pmatrix} 1 + S^2 & ST \\ ST & 1 + T^2 \end{pmatrix} \quad II = \begin{pmatrix} 2P & R \\ R & 2Q \end{pmatrix} \]

(4.13)

The mean (\( H \)) and Gaussian (\( K \)) curvatures are then defined by \( I \) and \( II \):

\[ K = \frac{\text{det}(II)}{\text{det}(I)} = \frac{LN - M^2}{EG - F^2} \]

(4.14)

\[ H = \frac{1}{2} \text{Trace}((II)(I^{-1})) = \frac{LG - 2MF + NE}{2(EG - F^2)} \]

By repeating this method across all atoms of a certain type within the membrane, we can build up a distribution of curvature across the entire mesophase. A major advantage of this method is that it does not assume the mesophase of the system to begin with, and so should allow us to measure the curvature of any system presented. However, a limitation of the method to date is
that it has not been used in mesophases with known topologies apart from a simple L\alpha system. To expand the method and apply it to new systems, we evaluate its performance here on the L\alpha, HII, and Q\alphaII mesophases. Note that throughout this section, K indicates the Gaussian curvature of a surface, rather than the partition coefficient of diol molecules.

### 4.3.1 Simulation details

<table>
<thead>
<tr>
<th>Mesophase and lipid</th>
<th>Number of water molecules</th>
<th>Number of lipids</th>
<th>Weight proportion of water</th>
<th>Lattice parameter (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HII (DOPE)</td>
<td>1328</td>
<td>300</td>
<td>0.3</td>
<td>50.3 ± 0.3</td>
</tr>
<tr>
<td>L\alpha (DPPC)</td>
<td>768</td>
<td>128</td>
<td>0.37</td>
<td>63.2 (error &lt; sig. figs.)</td>
</tr>
<tr>
<td>Q\alphaII (MO)</td>
<td>3200</td>
<td>950</td>
<td>0.4</td>
<td>103.6 (error &lt; sig. figs.)</td>
</tr>
</tbody>
</table>

**Table 4.7.** The simulations used in testing curvature fitting.

Three mesophases of known mean and Gaussian curvatures were used, the L\alpha, Q\alphaII, and HII, using Gromacs 2018 and the Martini force field [168]. The L\alpha and HII systems used the Martini 2 topologies for DPPC and DOPE respectively, while the Q\alphaII used the Martini topology for monoolein developed by Johner et al. [207]. The coarse-grained models and the self-assembled mesophases are illustrated in fig. 4.15. The explicit make up of the systems is detailed in table 4.7. All simulations were subject to a short equilibration routine as described in section 2.4.3, before a short initial self-assembly simulation of 150 ns using the full Martini force field was carried out, at a temperature of 300 K and pressure of 1 bar.

The final frame of the initial simulation was then periodically replicated. For the Q\alphaII and HII, this was to a 2x2x2 (x,y,z) cell from the initial single cell, and for the L\alpha, a 3x3x1 cell. The expanded simulation cells were then further simulated for 1 µs in the case of the L\alpha, and HII, or 1 ns in the case of the Q\alphaII system. 11 (Q\alphaII) and 34 (L\alpha, HII) equally spaced frames from the expanded simulations were used to measure curvatures using the point cloud method.

The physical dimensions of the simulated mesophases were determined using the following methods:

- L\alpha: The z dimension of the simulation cell
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**Figure 4.15.** a)-c) The coarse-grained models of DPPC, Monoolein, and DOPE used to form the L\(_{\alpha}\), Q\(_{II}^D\), and H\(_{II}\) self-assembled mesophases shown respectively in d)-f). In all figures, water atoms are blue, lipid headgroup atoms are red, and lipid carbons are grey. In d)-f), a grey surface has been constructed in Ovito from the terminal carbon(s) of the lipids to illustrate the underlying mesophase [209].

- **H\(_{II}\)**: Fitting a cylinder to the coordinates of individual clusters of water atoms using Ovito [209]. The size of the unit cell, \(a\) of is related by the hexagonal geometry:

  \[
  a = \sqrt{\frac{1}{\phi_w} \left( \frac{\pi}{2\sqrt{3}} \right) d_w^2}
  \]  

  (4.15)

  where \(\phi_w\) is the volume fraction of water, and \(d_w\) is the diameter of the water channels. \(\phi_w\) is calculated from:

  \[
  \phi_w = \frac{118 N_w}{V_c}
  \]  

  (4.16)

  where \(N_w\) is the number of water atoms in the system, \(V_c\) is the volume of the simulation cell, and 118 Å is calculated from the volume of a water atom in bulk, and corrected for the 4:1 water atom mapping in Martini [168, 216].

- **Q\(_{II}^D\)**: fitting a Q\(_{II}^D\) surface to the terminal carbon atoms in the unit cell following the method of Khelashvili et al. [171].
In the H_{II} system, the radius of the pivotal plane was additionally measured by using the same clustering construction, using the water, NH_{3}, PO_{4}, GL_{1}, GL_{2}, C_{1A}, C_{1B} atoms and cluster formation cutoff distances of between 5 and 6 Å. The radius of the fitted cylinder was 20.1 ± 0.1 Å, so the expected value of H is 0.049 Å^{-1}.

4.3.2 Comparison of fitting algorithms

The points clouds formed by the search routine will inevitably not lie perfectly on the surface described by eq. (4.8), hence why they are fitted to an optimised surface. However, the curvature measured will depend on several variables: the choice of atoms from the membrane system, and the search radius almost certainly the most important. In addition, for a fitting problem with as many variables as eq. (4.8), the choice of fitting method may also be expected to be important. By that, we mean that in searching for a minimum of an objective function, the failure to find a global minimum may have a significant effect on the results we obtain.

To test this, 5 common minimisation methods from the SciPy Python library were tested on the same 5 frames of the initial (single unit cell) simulated L_{α} mesophase [160]. An L_{α} has both mean and Gaussian curvature equal to zero everywhere, so any serious deviations from this will demonstrate that a method is inappropriate for this problem.

The five fitting methods used were:

1. Least Squares
2. Minimize
3. Differential Evolution
4. Dual Annealing
5. Simplicial Homology Global Optimisation (SHGO)

Least Squares is perhaps one of the most common optimisation methods for modelling data. By default, SciPy uses the Levenberg-Marquardt (LM) method of Least Squares. The LM method uses a trust region strategy for minimization, which defines a small parameter space around the current iterate in which to develop. Using this region of the parameter space, a step size is defined by the size of the trust region, and direction by the gradient from the iterate’s location in the parameter space [217].
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Minimize uses a quasi-Newton method to find minima. By default in SciPy this is the Broyden–Fletcher–Goldfarb–Shanno (BFGS) algorithm \[217\]. Quasi-Newton methods replace the exact Jacobin matrix used for classical optimisation with an approximated Hessian at each iteration. Because unlike the LM method the Hessian is approximated and there are no matrix-matrix operations involved, the cost of calculation is reduced from $O(n^3)$ to $O(n^2)$.

Differential Evolution and Dual Annealing are stochastic methods, designed to find the global minimum of a function. Stochastic methods have the advantage of being able to explore a large parameter space, and, unlike gradient descent methods, do not require the objective function to be differentiable (although that is not of concern here). Differential Evolution is a genetic algorithm designed by Storn and Price to search a large parameter space without making prior assumptions about the optimal solution. In general, genetic algorithms are designed to mimic biological evolutionary processes to find an optimum solution \[219\]. At each iterate, a genetic algorithm will compare a the last iterate to a new candidate solution. The candidate solution is generated randomly from a population of trial vectors across the function space. If the candidate solution evaluates to a lower value of the objective function, it replaces the trial vector for the next iteration of the method, and the routine continues until a tolerance is reached and the global solution is found \[218\].

Dual Annealing is a refined simulated annealing technique \[220, 221\]. Simulated annealing techniques are inspired by metallurgy, where annealing a metal from hot to cold leaves it in a crystalline state. Simulated annealing optimisation methods use a stochastic ‘temperature’ to jump increasingly small distances between points in objective function value space. That is, a set of input parameters is initially chosen for the objective function. At the first iteration, there is a large ‘temperature’, enabling a random move to comparatively far away in the parameter space. If this solution results in lower value for the objective function, it can be accepted at this stage. This process repeats, with increasingly small jumps in parameter space permitted as the process ‘cools’, and until either a minimum temperature has been reached, or when no better solutions can be found. Jumping between positions in parameter space in this way should enable the method to avoid becoming trapped in a local minima.

SHGO is a comparatively recently developed method, which while a global optimisation method, does not use stochastic methods \[222\]. SHGO works instead by finding locally convex domains of the objective function given a set of bounds using concepts from homology group theory. All
the domains are then fitted locally, to ensure that the true global minimum of the objective function is found. This represents an improvement on stochastic methods, which are not necessarily guaranteed to converge to global minimum if the objective function is sampled poorly in the first instance.

To test the fitting methods, point cloud from the Lα mesophase were formed by using the terminal carbon beads, from the centre of the membrane, at a cutoff radius of 25 Å. The surface in eq. (4.8) was then fitted to all point clouds using the 5 fitting methods described. The calculated curvature result of each point cloud is compared in fig. 4.16, showing how each method correlates with each other. For greater comparison, the SHGO and Least Squares methods are plotted in detail in fig. 4.17.

From fig. 4.16, we can see that 4 of the methods have high levels of agreement between the results, with $R^2$ values close to 1 in several cases. Moreover, the medians of most of the individual distributions is very close to 0. Notably, the agreement between the global optimisation methods is very high. Importantly, however, the agreement between the Least Squares method and all other is lower in all cases. This is demonstrated further in fig. 4.17. The overall distributions for Gaussian curvature between the two measurements are almost identical, but with a low agreement on individual measurements ($R^2 = 0.28$). However, for mean curvature, there is considerable divergence between the two methods, most obviously seen by the median of the Least Squares distribution as an order of magnitude different to the other methods, and low agreement between individual measures. From this, we can see that the Least Squares method tends to settle on cylindrical (K=0, H≠0) sections when fitting point clouds from a physically flat membrane. The comparison here demonstrates that in contrast to the work of Yesylevskyy and Ramseyer, using Least Squares to fit the quadric surface of eq. (4.8) to membrane point cloud will not always find the global minimum of the function, and could therefore result in spurious measurements of membrane curvature.

In addition to simple agreement with other fitting methods, fig. 4.18 shows how the methods perform on several metrics when the number of total number of possible iterations for each method is limited to 500. In a) we see that there is substantial variation between the methods and how long they take to terminate. The difference between the total number of evaluations is also substantial. As genetic minimisation methods, Dual Annealing and Differential Evolution naturally evaluate the objective function the most in searching for the global minimum.
4.3. MEASURING MEMBRANE CURVATURE

Figure 4.16. Pair plots of the results of the five fitting methods for a) Gaussian, b) mean curvature. The measurements used a cutoff radius of 25 Å in an Lα mesophase. The R² value for each plot is noted in the title. For each self-paired figure, the median value of the distribution is noted instead.

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However, as we have seen from above, they do typically eventually find it, unlike Minimize or Least Squares. The high agreement between 4 of the functions is further demonstrated here by the identical values for the value of the objective function on termination in fig. 4.18 c), while the Least Squares method is significantly different, indicating a poorer overall fit to the data. Of all the methods, SHGO finds the same value of the objective function, and takes the least time to get there, and therefore is the most appropriate method to fit eq. (4.8) across different mesophases.
4.3. MEASURING MEMBRANE CURVATURE

Figure 4.18. A comparison of various metrics of the different fitting methods, fitted to 1280 individual point clouds in an Lα mesophase across 5 simulation frames at a cutoff radius of 25 Å. The total number of optimisation iterations was limited to 500 for each method, ending at that point if tolerance had not yet been reached. a) The time taken to complete to termination of the routine. b) the total number of evaluations of the objective function during optimisation. c) the value of the objective function on termination.

4.3.3 Effect of atom choice

In measuring the curvature of a Lα mesophase, Yesylevskyy and Ramseyer chose to form point clouds out of both the terminal carbon and associated headgroup atoms of the membrane system, in an attempt to more accurately capture the mid-plane of the system. In Figures 4.19 to 4.21 we measure the curvatures of the three mesophases by forming point clouds out of various selected atoms from within the membrane, averaged across multiple frames from the simulation. For the Lα and HII mesophases, these were the first carbons below the headgroup (labelled C1A, C1B), and the terminal carbons of the lipids (labelled C4A, C4B). In the QDII mesophase, we measured curvature by forming point clouds from all individual carbon atoms in the lipid tails, as well as using the original published method in taking a terminal carbon and associated headgroup bead together (GL1, C1A). Figures 4.19 to 4.21 show this measurement carried out at 3 different cutoff radii for point cloud formation (10 Å, 30 Å, 40 Å). The distribution of Gaussian curvature in the QDII mesophase is known for a given size of unit cell.
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At the smallest cutoff of 10 Å in fig. 4.19, there is little difference between any of the locations where curvature is measured at. Moreover, all the measured distributions show a peak at 0 for both mean and Gaussian curvature, indicating that at such short point cloud radius, the fitted measurement is usually planar.

Increasing the cutoff radius to 30 Å as shown in fig. 4.20 results in some differences between the measured distributions. The $L_α$ distributions for the terminal carbon atoms (C4A/B) are, as expected, distributed about 0. Using the primary carbons instead results in surfaces with slightly non-zero symmetrical distributions of both the Gaussian and mean curvatures. The fitted surface here is likely envisaged by the situation in section 4.3.3. The cutoff radius in this case is longer than the distance between the two adjacent monolayer C1A/C1B atoms, and an erroneously minimally fitted surface is somehow found by a spherical cap. This goes to show that both the radius and the choice of atom are particularly sensitive variables in this

[223]

**Figure 4.19.** The distributions of Gaussian (a-c) and mean (d-f) curvature for the 3 mesophases ($L_α$: a, d; $Q^D_{II}$: b, e; $H_{II}$: c, f) using point clouds formed from different lipid atoms at a cutoff radius of 10 Å

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4.3. MEASURING MEMBRANE CURVATURE

Figure 4.20. The distributions of Gaussian (a-c) and mean (d-f) curvature for the 3 mesophases ($L\alpha$: a, d; $Q_{II}^D$: b, e; $H_{II}$: c, f) using point clouds formed from different lipid atoms at a cutoff radius of 30 Å.

This is further demonstrated in the $H_{II}$ system, where the curvature of the pivotal surface lying at the C1A/C1B atom cylinder is captured very well at a 30 Å radius, and obviously not by using the terminal carbon atoms.

The effect of increasing the cutoff radius is most well demonstrated by the results for the $Q_{II}^D$ system. The results of the mean curvature are once again incidental for all carbon atom choices at 0 as expected for a minimal surface. The Gaussian curvature, however, shows considerable variation. The trend clearly shows that the closer towards the terminal (C5A) carbon, the more closely the measured distribution matches the analytically expected one. Moreover, the proportion of the distribution that is greater than 0 drops significantly. Additionally, following the method of Yesylevskyy and Ramseyer is clearly completely unrepresentative of the expected distribution.

In addition to the improved measurements from an increase in cutoff radius, the proportion
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Figure 4.21. The distributions of Gaussian (a-c) and mean (d-f) curvature for the 3 mesophases (Lα: a, d; QDII: b, e; HII: c, f) using point clouds formed from different lipid atoms at a cutoff radius of 40 Å.

of the data included within the plotted histogram range, noted in Figures 4.19 to 4.21, and plotted in fig. 4.23 show that while the distributions may not precisely match, a remarkably high proportion of measurements of curvature are within the plotted range.

The increase of cutoff radius to 40 Å shown in fig. 4.21 has little effect on either the measured curvature of either the Lα or HII systems. In the case of using the terminal (C4A/B) and primary (C1A/B) carbons respectively, result in the expected distributions.

The effect on the results seen for the QDII measurements are more subtle. Measurements of Gaussian curvature using the C1A, C2A, or a mixture of terminal and headgroup atoms are as bad as before. However, using the D3A, C4A, or C5A carbons has a sharper cutoff at K=0, and a distinct peak that has shifted towards a more negative curvature, close to the expected distribution. While the proportion of data in the plotted range has fallen slightly (see section 4.3.3), the vast majority is still within this range.

From Figures 4.19 to 4.23, it is evident that the atoms chosen to fit using this measurement...
4.3. MEASURING MEMBRANE CURVATURE

(a) bad La fit. C1A atoms (Blue) on both sides of the membrane have been included in the initial point cloud, to which a non-planar surface has been fitted (Orange)

(b) bad C5A/GL1 combined fit. Both head-group (Blue) and terminal carbon (Grey) atoms have been used to fit. In this case, the initial PCA of the cloud have been misidentified, and the fitted surface (Orange) has not captured the curvature of the membrane properly.

**Figure 4.22.** Poor fits of membranes from choosing atoms in the membrane incorrectly

technique have a significant effect on the result obtained.

4.3.4 **Effect of cutoff radius**

To further probe how the cutoff radius can affect the measurement of curvatures, a range of cutoff radii were used to measure curvature using the optimally determined atoms in the membrane, the results of which are in fig. 4.24. As with the considerations in the changing selected atom, the proportion of data included in the plotted range is shown in fig. 4.25.

Trivially, as the radius is increased in measuring the L\textsubscript{α} system, the symmetric peak distributions around K=0 and H=0 both sharpen to a very narrow range. As the radius is increased, the number of atoms used in the formation of a point cloud increases, so the system will very quickly fit to a flat plane.

For the H\textsubscript{II} system, the increase in radius has a similar effect in measuring the Gaussian curvature. However, for mean curvature, peaks of the distribution at the expected value of 0.049 Å\textsuperscript{-1} are only coincidental at r= 25, 30 Å. At slightly lower or higher values of r, the peaks lie at a smaller value of H, so indicating a larger (and so flatter) cylinder radius than expected, a trend
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**Figure 4.23.** Proportion of the data included within the expected range.
4.3. MEASURING MEMBRANE CURVATURE

**Figure 4.24.** The distributions of Gaussian (a-c) and mean (d-f) curvature for the 3 mesophases ($L_α$: a, d; $Q_{II}^0$: b, e; $H_{II}$: c, f) using point clouds formed from the optimum atoms from the membrane at different radii.

**Figure 4.25.** Proportion of the data included within the expected range when the radius of the point clouds is varied.
which continues as the radius is further increased or decreased. As before with atom choice, the effect of the cutoff radius is borne out most by the $Q^{D}_{II}$ data, with only a cutoff radius of 40 Å showing a peak around the expected value. Other radii show a tendency towards $K=0$, or peaks at intermediate values of $K$. These must then be balanced by the proportions included as noted in fig. 4.25. While the most data is included within the expected range is at a cutoff radius of 30 Å, a cutoff of 40 Å is a better representation of the curvature distributions overall, but must exclude more data in doing so.

4.3.5 Strategies for measuring curvatures in simulated mesophases

The method presented here has the advantage of building curvature distributions from local measurements, and without much prior knowledge of the topology of the system. The work builds on that of Yesylevskyy and Ramseyer in showing that the locally fitted surfaces must be found using global optimisation routines rather than local ones. Most significantly in comparison, we have shown that the fitting process cannot be done naively, and that choices must actively be made about how to best represent the mesophase at hand.

To measure the curvature distributions of any given simulated mesophase, the conclusions which we can draw from the analysis here is that firstly, the curvature is most likely best represented by the terminal carbons of the mesophase. This is perhaps not surprising, but it is in contrast to energetics measurements, which must be carried out at the pivotal or neutral plane of the membrane. Secondly, cutoff radii must be chosen judiciously. This is perhaps best done by analysing a range of radii, and analysing the difference between the resulting distributions to ensure there are not significant changes between different radii. However, we have particularly shown that where mean curvature is around zero (for example, the $L_α$ and $Q^{D}_{II}$ systems), a longer cutoff length is preferable.

4.4 Measuring the elastic moduli of membranes from real-space fluctuations

As discussed in section 1.7.1, membrane energetics can be described by the Helfich Hamiltonian, and are proportional to the membrane curvatures. Each curvature has a related moduli, describing the energetic cost of deforming the membrane in that way. In this section, we
4.4. MEASURING THE ELASTIC MODULI OF MEMBRANES FROM REAL-SPACE FLUCTUATIONS

FIGURE 4.26. The membrane vectors required for calculating membrane elastic moduli. Each lipid has a local director, \( \mathbf{n} \), and the membrane interface is described by a vector \( \mathbf{N} \).

will outline how these can be measured from molecular dynamics trajectories, following the methods developed by Khelashvili et al. over the last decade [172, 224–228]. The methods are based on real space fluctuations (RSF) of lipid systems. As they note in reference [228], this has considerable advantages over more traditional spectral analysis methods for determining membrane moduli, which are otherwise restricted to larger, single component systems, with limited topologies. These restrictions are required by the need for global sampling of thermally excited fluctuations across the membrane in order to measure small-wavelength Fourier-space modes of the undulations.

In comparison, the RSF method requires only knowledge about two parameters, shown in fig. 4.26: a director \( \mathbf{n} \) describing the direction of the lipid, and a vector \( \mathbf{N} \) describing the direction of the water/lipid interface. The lipid director of each lipid in the system is determined by a vector pointing from its tail to its head. Following from the analysis procedure in section 4.3, the membrane normal can be determined at each time step by performing point cloud principal component analysis. From these, it is possible to measure both the bending modulus, \( K_c \), and the tilt modulus, \( \kappa_t \). The bending modulus describes the energetic cost of deforming the membrane away from its spontaneous shape, while the tilt modulus describes the force of tilting a molecule within the membrane.
CHAPTER 4. DEVELOPING METHODS FOR MOLECULAR DYNAMICS SIMULATIONS OF LIPID SPONGE PHASES

**Figure 4.27.** Example distributions for calculating the bending ($K_c$, panels a and b) and tilt ($\kappa_t$, panels c and d) moduli. These distributions arise from a $Q^D_{II}$ mesophase using a point cloud cut off of 40 Å. a) and c) show the probability distributions of the splay, $S_i$, and tilt angles $\theta$. These distributions are fitted with a Gaussian to determine a fitting range to within $2\sigma$ of the peak of each function. The $2\sigma$ ranges are indicated on all four plots by dashed yellow lines. Panels b) and d) then show the functions of $S_i$ and $\theta$ found in eq. (4.30) and eq. (4.21) respectively for determining the monolayer values for $K_c$ and $\kappa_t$. The solid red lines are fits to the data contained within the dashed yellow region (extrapolated outside here for clarity). Here, the fits give monolayer values of $K_c = 16.1 \pm 0.8 k_B T$ and $\kappa_t = 3.1 \pm 0.7 k_B T$.

It is important to note that elastic properties should be ideally measured at the neutral surface of the membrane, i.e. the surface where the area remains constant when the unit cell size is increased. However, the neutral surface is not readily measured, and the pivotal surface, which has no change in area for a change in unit cell size, can be used instead to measure the elastic and curvature properties of the membrane [9, 227, 229–231]. Taking this into account, we will now outline the method described based on reference [228], and its implementation.
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4.4.1 Tilt modulus

The free energy of tilt per unit area is simply:

\[ f_t = \frac{1}{2} \kappa_t \langle \vec{t} \rangle^2 = \frac{1}{2} \kappa_t \left( \frac{\vec{n}}{\vec{n} \cdot \vec{N}} - \vec{N} \right)^2 \]  

(4.17)

ie. The free energy of tilt is quadratic in an ensemble-average of the lipid tilt, as determined by the dot product between the lipid and membrane vectors. The dot product therefore really represents an angle between the two vectors, which, assuming they are normalised and we are working in the small angle regime, can be simplified as follows:

\[ \vec{t}^2 = \left( \frac{\vec{n}}{\vec{n} \cdot \vec{N}} - \vec{N} \right)^2 = \frac{1}{\cos^2(\theta)} - 1 = \tan^2(\theta) \approx \theta^2 \]  

(4.18)

We can now write the tilt energy of a single lipid as follows:

\[ E(\theta) = \frac{1}{2} \kappa_t \theta^2 A_l \]  

(4.19)

Because we can expect the energies to be Boltzmann distributed, we can then write:

\[ P(\theta) = g(\theta)C_t\exp\left(-\frac{E(\theta)}{k_B T}\right) = \sin(\theta)C_t\exp\left(-\frac{E(\theta)}{k_B T}\right) \]  

(4.20)

where \( C \) is some normalisation constant, and \( g(\theta) = \sin(\theta) \) accounts for the degeneracy of microstates of any given value of \( \theta \). That is to say, for a vector precessing around an axis with a constant polar angle \( \theta \), the number of states accessible is \( \sin(\theta) \).

Finally, we can combine and rearrange eqs. (4.19) to (4.20). In doing so, we need to include an additional factor of 2 to account for the fact that the tilt modulus is really a two-dimensional tensor with \( \kappa_t \) representing the diagonal components. Our measure \( \kappa_t = \kappa_t^{xx} = \kappa_t^{yy} \). As these are two random variables with Gaussian distributions with variance \( \sigma^2 \), their sum becomes another Gaussian distributed random variable with variance \( 2\sigma^2 \), explaining the extra factor.

All the above consideration leads us to:

\[ -\frac{4k_B T}{A_l} \ln \left( \frac{P(\theta)}{\sin(\theta)} \right) = k_l \theta^2 + \text{constant} \]  

(4.21)

That is, the tilt modulus per lipid can be obtained from \( P(\theta) \) by fitting the left hand side of eq. (4.21) to a quadratic form, of which the tilt modulus will be the quadratic fitted constant. Therefore, once the distribution of lipid tilts is known, the tilt modulus can easily be obtained.
through a simple fitting procedure. Note that this tilt modulus will be the tilt modulus per monolayer, and the tilt modulus per bilayer is $2\kappa_t A_t$.

### 4.4.2 Bending modulus

To take a similar approach to the calculation of the tilt modulus, the quadratic approximation for the bending free energy of the membrane, $f_b$, is:

$$f_b = \frac{1}{2} K_c \langle S \rangle^2 = \frac{1}{2} K_c \left( \nabla \vec{n} - \nabla \vec{N} \right)^2$$  \hspace{1cm} (4.22)

This arises out of the fact that the last time-averaged term, $\nabla \vec{n} - \nabla \vec{N}$, is the lipid splay, $S$ and the bending modulus is associated with the energetic cost of deforming the membrane away from its spontaneous shape. As before, we can then write the energy of the lipid splay for a single lipid:

$$E(S) = \frac{1}{2} K_c S^2 A_l$$  \hspace{1cm} (4.23)

which is distributed Boltzmann-like:

$$P(S) = C \exp \left( \frac{-E(S)}{k_B T} \right)$$

$$= C \exp \left( \frac{-K_c S^2 A_l}{2k_B T} \right)$$  \hspace{1cm} (4.24)

$$= C_s \exp \left( \frac{-1}{2k_B T} K_c (\nabla \vec{n} - \nabla \vec{N})^2 A_l \right)$$

At this point, it would be natural to seek to be able to rearrange eq. (4.24) into something akin to eq. (4.21). However, we are presented with a challenge in measuring the splay: what are the divergences of the lipid and membrane vector fields?

To overcome this, Johner et al. ([227]) note that for any vector field for some point $\vec{p}$:

$$\vec{v}(\vec{p}) = \sum_{i=1}^{3} (\vec{v}(\vec{p}) \cdot \hat{e}_i) \hat{e}_i = \sum_{i=1}^{3} v_i(\vec{p}) \hat{e}_i$$  \hspace{1cm} (4.25)

I.e. a surface can be defined by two tangent vectors and a normal vector. By the finite difference method, the divergence of this is then:
4.4. MEASURING THE ELASTIC MODULI OF MEMBRANES FROM REAL-SPACE FLUCTUATIONS

\[ \nabla \tilde{v}(\bar{p}) = \sum_{i=1}^{2} \frac{v_{i}(\bar{p} + h \tilde{e}_{i}) - v_{i}(\bar{p} - h \tilde{e}_{i})}{2h} \]

\[ = \sum_{i=1}^{2} \frac{v_{i}(\bar{p} + h \tilde{e}_{i}) - v_{i}(\bar{p}) + v_{i}(\bar{p}) - v_{i}(\bar{p} - h \tilde{e}_{i})}{2h} \]

\[ = \frac{1}{2} [v'_{i} + v'_{-i} + v'_{2} + v'_{-2}] \quad (4.26) \]

Where we have said that:

\[ v'_{i} = v_{i}(\bar{p} + h \tilde{e}_{i}) - v_{i}(\bar{p}) \]

ie. \( v'_{i} \) is the covariant derivative of \( v_{i} \) in the direction \( \tilde{e}_{i} \), and \( v'_{-i} \) is the covariant derivative of \( v_{i} \) in the direction of \(-\tilde{e}_{i}\):

Therefore, we can now consider the vector field \( S_{i} = (\vec{n} - \vec{N}) \), and we get:

\[ S_{i} = \frac{n_{i}(\bar{p} + h \tilde{e}_{i}) + N_{i}(\bar{p} + h \tilde{e}_{i}) - n_{i}(\bar{p}) - N_{i}(\bar{p})}{h} \quad (4.28) \]

This allows us to now consider the splay in terms of \( S \), rather than \( S \). \( S \) itself can be constructed by looking at the lipid and membrane normal vectors between pairs of lipids within a small cutoff range. Johner et al. use a cutoff range of 10 Å for this purpose [227]. Now, we can construct a distribution as before:

\[ P(S_{i}) = C \exp \left( \frac{K_{c} S_{i}^2 A_{l}}{2 k_{B} T} \right) \]

\( \Rightarrow -\frac{2 k_{B} T}{A_{l}} \ln(P(S_{i})) = K_{c}(S_{i})^2 + \text{constant} \) (4.30)

Therefore, as before, once we have constructed the distribution of \( P(S_{i}) \), we can fit a quadratic function to the left hand side of eq. (4.30). As before, this will be the monolayer bending modulus, which can be extended to bilayers with reference to eq. (1.23).

4.4.3 Calculating moduli in multi-component membranes

It should be noted that both eq. (4.21) and eq. (4.30) are valid only for single-component lipid systems, where only one lipid is contributing to the elastic properties of the system. For a multi-component system, they become:
\[
\frac{1}{\kappa'_l} = \frac{1}{N_{\text{tot}}} \sum_i N_i \kappa'^{li}_{\text{tot}}
\]
(4.31)

where \(\kappa'_l = \kappa_l A_l\), the tilt modulus per lipid, \(\kappa'^{li}_{\text{tot}}\) is the tilt modulus for the \(i^{th}\) lipid type in the system, and \(N_i\) is the number of that type of lipid in the system. The bending modulus is similarly changed:

\[
\frac{1}{K_c} = \frac{1}{\phi_{\text{tot}}} \sum_{i,j} \phi_{ij} K^{ij}_c
\]
(4.32)

where \(\phi_{\text{tot}}\) is the total number of pairs for which a calculation is done, and \(K^{ij}_c\) is the splay modulus for the \((i^{th},j^{th})\) lipid pair, of which there are \(\phi_{ij}\) in the system.

### 4.4.4 Measuring the moduli of monoolein

As an example of the type of measurement that can be achieved using the RSF method, the bending and tilt moduli of a monoolein \(Q_{II}^D\) mesophase were measured over a short simulation of 1 ns using 11 equally spaced frames. The simulation was run at 298 K and at a pressure of 1 bar. 950 monoolein molecules and 3200 water molecules were pre self-assembled into a \(Q_{II}^D\) mesophase for the purposes of the short production simulation. Experimentally, the bending modulus of monoolein has not been well studied. One study by Vacklin et al. exists, looking at the magnitude of the moduli at 37°C, and with packing stress relieved by 9-cis-tricosene in order for the system to exhibit a \(H_{II}\) mesophase. In this case, \(K_c\) for a monolayer of monoolein was around 3 \(k_B T\). Recalling from section 1.7.1 that a bilayer bending modulus is simply twice the monolayer value, the expected result should therefore be around \(6 k_B T\).

The results for the example measurements here are shown in fig. 4.28. The value for the bilayer bending modulus is \(16.5 \pm 0.5 k_B T\), which is obviously significantly greater than the value that we might expect from experiments. However, these simulations were conducted both at a lower temperature and in a different mesophase. Considering that the experiments of Vacklin et al. were conducted by relieving the packing stress of the membrane and at a higher temperature, it is to be expected that the values measured for the bending modulus here will be greater [232]. Attempts were made to simulate a monoolein/decane/water system at this temperature in order to measure the membrane moduli in a \(H_{II}\) mesophase using molecular dynamics, but no stable conditions could be found. To further comment on the values measured in the
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The interfacial area of a mesophase is simply the surface area of the lipid/water interface. In cubic phases, it is naturally determined by the topology of the underlying surface on which the bilayer sits. Here, we will develop a method to measure the interfacial area using an alpha surface construction, and show that it is comparable to more analytical measures in order to extend the measure to the $L_3$ mesophase.

To measure the interfacial area in a $L_3$ mesophase, we need to establish a method to investigate the effect of diol addition on the interfacial area. This will be particularly significant in considering the effect of additives on the system. However, while we can calculate the interfacial area per molecule easily from topological parameters in the $Q_{II}$ mesophase, we cannot assume...
anything similar in the \(L_3\) mesophase. Instead, we will use the alpha surface construction that we have seen before, and validate it against the known parameters in a cubic phase.

*Turner et al.* (reference [234]) note that, assuming a constant monolayer thickness in the cubic phase, the length of a monolayer, \(l\), in a cubic phase is described in the following way:

\[
\phi_l = 2\sigma \left( \frac{l}{a} \right) + \frac{4\pi}{3} \chi \left( \frac{l}{a} \right)^3
\]  

(4.33)

where \(\phi_l\) is the volume fraction of lipid in the system, \(a\) is the lattice parameter of the cubic phase, and \(\sigma\) and \(\chi\) are topological parameters relating to the underlying surface. \(\chi\) is the Euler characteristic for the surface. \(\sigma\) is also a dimensionless constant relating the area of the minimal surface to the volume that a single repetition of its unit cell occupies, that is (see ref [235]):

\[
\sigma = \frac{Aa^3}{a^2V}
\]  

(4.34)

where \(A\) is the area of the minimal surface in a unit cell, and \(V\) is the volume of the unit cell.

Values of \(\sigma\) and \(\chi\) are listed in table 4.8.

The area of a complete monolayer, \(A_l\) in the cubic phase can then be calculated using:

\[
A_l = \sigma a^2 + 2\pi \chi l^2
\]  

(4.35)

The total interfacial area for the bilayer across the unit cell is then \(2A_l\), and so the interfacial area per molecule, \(a_0\), is:

\[
a_0 = \frac{2A_l}{n_{lipids}}
\]  

(4.36)

where \(n_{lipids}\) is the number of lipids in the unit cell. If \(n_{lipids}\) is unknown, it can be ascertained simply by:

\[
n_{lipids} = \frac{\phi_l a^3}{v_l}
\]  

(4.37)

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<thead>
<tr>
<th>Surface</th>
<th>(\chi)</th>
<th>(\sigma)</th>
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</tr>
<tr>
<td>D</td>
<td>-2</td>
<td>1.919</td>
</tr>
<tr>
<td>G</td>
<td>-8</td>
<td>3.091</td>
</tr>
</tbody>
</table>

*Table 4.8. Topological parameters for cubic phase surfaces, taken from [48].*
4.5. MEASURING INTERFACIAL AREA

Figure 4.29. Calculating interfacial areas from using known equations and alpha surfaces constructed from water atoms. a) How the volume fraction of the constructed surface increases with the radius of the probe sphere. b) How the two measures of interfacial area vary with alpha surface probe sphere. c) How the two measures vary over time, with the shaded regions around a mean line indicating the standard deviations of the data. Note that b) and c) use the same colour scatter points for the alpha surface and calculated areas.

where $\phi_l$ is the volume fraction of lipids as before, and $v_l$ is the molecular volume.

To validate the alpha surface method, a short (10 ns) simulation of a pre self-assembled single unit cell of a Q$_{II}$ mesophase was conducted using 950 monoolein molecules and 3200 waters, giving a weight ratio of 40% water. The simulation was run under standard Martini molecular dynamic parameters, as previously described. The lattice parameter, $a$, was $103.7 \pm 0.1$ Å. 34 equally spaced frames through the simulation were used to measure the interfacial area. For each frame, an alpha surface was constructed using the water atoms at a range of probe radii. The water atoms, and not lipid headgroup ones, were used to ensure that the surface was smooth throughout the unit cell. If headgroup atoms were chosen, because there are comparatively few, and they are separated by a bilayer, the surface cannot be guaranteed to be a useful smooth representation of the lipid/water interface. The surface area per lipid could then simply be calculated from the area of the alpha surface and the number of lipids in the system.
CHAPTER 4. DEVELOPING METHODS FOR MOLECULAR DYNAMICS SIMULATIONS OF LIPID SPONGE PHASES

<table>
<thead>
<tr>
<th>Scenario</th>
<th>$\phi_l$</th>
<th>$l$ (Å)</th>
<th>$a_0$ (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6</td>
<td>17.2</td>
<td>35.5</td>
</tr>
<tr>
<td>2</td>
<td>1-0.33</td>
<td>19.2</td>
<td>33.5</td>
</tr>
</tbody>
</table>

Table 4.9. Using two alternative formulations for $\phi_l$, the interfacial area per molecule in a Q_{II}D mesophase can be calculated using eq. (4.33) and eqs. (4.35) to (4.36). The calculations are made using 950 lipids and 3200 waters per unit cell, and a lattice parameter of 103.6 Å.

To analytically calculate the interfacial areas using eqs. (4.33) to (4.36), the volume fraction of the water atom alpha surface was taken as the water volume fraction, $\phi_w$. The monolayer length can then be determined by solving eq. (4.33) for $l/a$ with the knowledge that $\phi_l = 1 - \phi_w$. The respective values of $l$ and $a$ can then be used to determine the interfacial area per lipid using eqs. (4.35) to (4.36).

The results of this are shown in fig. 4.29. Figure 4.29a) firstly shows that as the probe sphere radius to generate the surface increases, the volume fraction does so correspondingly. The relationship in this range of radii is linear, but the rate of increase increases with larger radii. The uptick outside of the range plotted is a result of the excessively large probe sphere radii resulting in an alpha surface that bridges across the two bicontinuous water channels, and thus becoming an unphysical representation of the interfacial area in the system, hence its exclusion. In fig. 4.29b), the two measures of interfacial area similarly increase as the probe sphere radius increases. Note that to calculate the area based on the topology of the mesophase using eqs. (4.33) to (4.36), the volume fraction is a result of the construction of the surface, so it varies in this way too. The result of perhaps most significance in a) and b) is that the volume fractions measured here are slightly below where we would expect from experimental systems. The excess water point for monoolein to form a Q_{II}D mesophase is around 48% v/v, so the surfaces here occupy slightly less than the expected volume.

To briefly consider alternative ways of determining the volume fraction, there are two options:

1. Declare that $\phi_l = 0.6$ as we would expect from experiments.

2. With reference to eq. (4.16), calculate the expected volume fraction from the number of water molecules that are in the system.

The calculations for both scenarios are worked through in table 4.9, showing that they amount to 35.5 Å² and 33.5 Å² respectively. The result from the calculations arising from the alpha
4.6 Conclusions

In this chapter, we have developed computational methods with which to investigate a variety of structural properties of lipid mesophases, with a view to applying them to the $L_\alpha$ mesophase. To that end, we have explored how a sponge-forming diol can be modelled using the Martini force field, finding that a 3-atom SP1-SC2-SP1 model is both miscible with water, and reproduces partitioning behaviour most closely. Most importantly, we have developed a range of computational analysis tools to measure the membrane curvature, elastic moduli, and headgroup area of lipid mesophases simulated using molecular dynamics. These have been validated against known results and datasets, to ensure that they will be appropriate measures through the course of a mesophase transition. In their open-source implementation, the tools have been parallelised to increase computational efficiency. While the tools may not reproduce experimental results exactly, they have been shown to reproduce them closely, and with few assumptions made about the system they are being used on. This opens up the possibility for further refinement in the future, or highly effective uses when assumptions are made. However, as we will demonstrate in the next chapter, their power can be demonstrated by investigating highly dynamic and highly curved lipid mesophases.
Molecular dynamics simulations of the cubic to sponge transition

5.1 Context

In chapter 4, we developed and validated analysis tools and methods with which we can investigate the L₃ mesophase using molecular dynamics simulations. In this chapter, we will investigate the Q_{II}^D to L₃ mesophase transition using the tools we have developed. This transition is only recently accessible to experimental techniques, so molecular dynamics simulations will provide insights into the nature of molecular rearrangement in detail not previously observed [236, 237]. While theoretical modelling of the L₃ mesophase suggests a close relationship with the Q_{II}^D one, it is not precisely clear how the transition proceeds in lipid membrane systems, and what physical forces and constraints particularly motivate it (see, for example, the work of Stephen Hyde [238, 239]). In addition to investigating the dynamic nature of the cubic to sponge transition, we will explore the question of the curvature preference of cholesterol in the Q_{II}^D mesophase.

5.2 Simulation set up

The basic set up is illustrated in fig. 5.1. A single unit cell of a Q_{II}^D mesophase is first constructed through a self-assembly simulation. The water molecules are then removed, leaving just the
FIGURE 5.1. Setting up a sponge transition simulation. Having simulated a $Q_{II}^{D}$ mesophase (a), the water is removed (b), the unit cell is replicated $2 \times 2 \times 2$ times (c), and a transformation is applied to swell the size (d). Water and diol molecules are then added back in, in the appropriate ratio (e), and a production simulation runs its course to a final state (f). In (f), an alpha surface constructed from lipid terminal carbon atoms has been added in yellow for illustrative purposes. In add panels, monoolein headgroups are coloured in red, their carbons grey, water in blue, and diols in green.
## 5.2. SIMULATION SET UP

<table>
<thead>
<tr>
<th>Diol solvent proportion (% weight)</th>
<th>Number of diol molecules</th>
<th>Number of water molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>201</td>
<td>24826</td>
</tr>
<tr>
<td>10</td>
<td>2005</td>
<td>22569</td>
</tr>
<tr>
<td>15</td>
<td>3007</td>
<td>21315</td>
</tr>
<tr>
<td>20</td>
<td>4009</td>
<td>20061</td>
</tr>
<tr>
<td>30</td>
<td>6014</td>
<td>17554</td>
</tr>
<tr>
<td>40</td>
<td>8018</td>
<td>15046</td>
</tr>
</tbody>
</table>

Table 5.1. The number of water and diol molecules reinserted into an expanded cubic phase for a sponge transition. The number of lipids was 7600, and the lipid:solvent ratio was 60:40.

Lipids. The unit cell is then replicated 8 times in a 2x2x2 array. This step was taken to reduce the effect of periodic boundaries at the centre of the simulation cell. An enlargement transformation was then applied to the simulation cell and molecules within it. Subsequently, water and diol molecules were reinserted into the simulation cell. These last two steps are physically relevant and necessary for several reasons. Physically, the size of the \( \text{Q}_{11}^D \) unit cell will swell on addition of diol, so we can reasonably expect that a new system containing diol molecules will be larger than the one without. The step is also necessary to enable the randomised reinsertion of the diol molecules, which was otherwise not found to be possible. To mitigate the effect of the transformation, a steepest descent minimisation routine was run on the system before the production simulation. After small molecular rearrangement had taken place in this way, the production simulations were run for a time of 1\( \mu \)s under NPT conditions.

This method was used in several ways: Firstly, to continue to investigate the effect of our diol model found in section 4.2 on mesophase structure, 6 setups detailed in table 5.1 were simulated. The initial \( \text{Q}_{11}^D \) mesophase was constructed from a self-assembly simulation of 950 MO lipid molecules, and 3200 water molecules. This initial simulation had a lattice parameter of 103 Å, which was increased to 110 Å as described above - ie. the replicated unit cell simulation box was 220 Å in each dimension. From the replicated simulation cell, the 6 conditions in table 5.1 were set up.

In addition to investigating the transition in pure MO lipid systems, \( \text{Q}_{11}^D \) mesophases doped with 2.5%, 5% and 8% mol cholesterol were constructed, transformed according to the described protocol, and put through a production simulation. Cholesterol was used as a dopant, because of the simple shift in the \( \text{Q}_{11}^D / \text{L}_3 \) phase boundary observed in chapter 3, and its relevance to the
CHAPTER 5. MOLECULAR DYNAMICS SIMULATIONS OF THE CUBIC TO SPONGE TRANSITION

5.3 Changing the proportion of diol in the solvent

5.3.1 Effect of diol concentration on $Q^{D}_{II}/L_3$ phase sequence

The first result that we are able to check returns us to section 4.2. Evenbratt et al. found that in a system of monoolein/water/pentanediol at a fixed ratio of water+pentanediol:monoolein, when the water:pentanediol ratio was increased, two things happened [55]. Firstly, as is reasonably expected, there is a phase transition from a $Q^{D}_{II}$ mesophase to the $L_3$ mesophase. Secondly, they observed that across the transition, the value of the partition coefficient $K$ remains constant at $0.78 \pm 0.14$. The initial experiments described by the conditions in table 5.1 allow us to measure this same feature, and check that it is replicated. Recall that in section 4.2, we...
### Table 5.2. The ratios and numbers of diol and water molecules used in simulations investigating possible phase separation effects in sponge transitions at lower diol proportions in the solvent.

<table>
<thead>
<tr>
<th>diol:water weight ratio</th>
<th>Number of diols</th>
<th>Number of waters</th>
</tr>
</thead>
<tbody>
<tr>
<td>15:85</td>
<td>250</td>
<td>1772</td>
</tr>
<tr>
<td>35:65</td>
<td>250</td>
<td>580</td>
</tr>
<tr>
<td>50:50</td>
<td>500</td>
<td>625</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>1876</td>
</tr>
</tbody>
</table>

5.3. Changing the proportion of diol in the solvent

found that for the SP1-SC2-SP1 diol model that $0.821 \pm 0.005$. The simulations in that section we all carried out on a fixed system of 2000 MO molecules, at a lipid:solvent weight ratio of 60:40, and with a water:diol weight ratio in the solvent of 60:40. Additionally, in systems using either 1000 or 4000 MO molecules instead, the value of the coefficient was not found to vary significantly. In these simulations, the systems will be larger still, while the ratios within the solvent will be varied.

The values of $K$ under different solvent conditions are shown in fig. 5.2, where it is very clear that unlike in experiments, $K$ is not constant. These values were measured using 33 equally spaced frames across the simulation. At low proportions of diol in the system, there is a strong preference for the diol to reside in the lipid domains rather than in the water ones, evidenced by $K$ being much greater than 1. At the other extreme end, with a lyotrope diol proportion of 40% as in the test validation systems, $K = 0.75 \pm 0.02$, residing in the expected range, although slightly lower than previously measured. In between, there is a diminishing decrease in the value of $K$ as the diol proportion is increased, although as the trial fit to the data shows in fig. 5.2, this decrease is not well described by an exponential decay model. Qualitatively, it is better described by the reciprocal model shown in black, although the physical reasons why the relationship should be this way are not clear.

#### 5.3.2 Are anomalous results driven by solvent phase separation?

To ensure that the anomalous partitioning behaviour was not a feature of a phase separation of the type shown in fig. 4.12, several short simulations were run to ensure that no unexpected phase separation was taking place between the water and diol. These were conducted at 3 weight ratios, and, at one of the ratios, 3 different system sizes. The number of molecules used
CHAPTER 5. MOLECULAR DYNAMICS SIMULATIONS OF THE CUBIC TO SPONGE TRANSITION

(a) 250 diols, 15% of system weight
(b) 250 diols, 35% of system weight
(c) 250 diols, 50% of system weight
(d) 500 diols, 50% of system weight
(e) 1500 diols, 50% of system weight
(f) The position of diol molecules in the 1% diol solvent system.

Figure 5.3. (a)-(e) Simulation frames examining possible phase separation in water/diol systems. For clarity, only the diol molecules are shown. None of the systems exhibit the formation of extended diol domains in the simulation cell. (f) Shows the location of diol molecules in the 1% diol solvent system, where alpha surfaces of water (blue) and terminal carbons (red) are shown, with diol molecules (green) sitting between them, in the lipid headgroup region.

is detailed in table 5.2. These systems were run according to standard Martini molecular dynamics parameter conditions, for 1 \( \mu \text{s} \). After molecular insertion and energy minimisation simulations, a random initial configuration of the molecules was obtained in the usual way, with an equilibration simulation where all LJ parameters were the same. The production simulation was then run using standard force field parameters.

The results of these simulations are seen in fig. 5.3a)-e), where it is clear that as expected from section 4.2, there is no phase separation between water and diols. Figure 5.3 shows only the positions of the diol molecules for clarity, but it is clear from these figures that there are no extended domains - as in fig. 4.12 - where there is singularly water, or singularly diol. Therefore, we can be confident that the measures of the partition coefficient presented in fig. 5.2 are
genuine measures of how the diol molecules in the transition simulations partition themselves between the water and lipid domains. Although this result is positive in demonstrating that the diol model remains valid in terms of its miscibility with water, it does not shed any more light on the precise reasons for the significant preference diols have for lipid domains at low concentrations. However, it should be noted from fig. 5.3f) that even though the preference is not explicitly for the water domains, the diols are not any deeper into the lipid domain than the headgroup regions.

That the diols preferentially occupy the headgroup regions of the lipids is most likely a result of the stronger self-interaction that water has with itself in the force field, compared to the marginally weaker (but still attractive) interactions that the diol Martini atoms have with them. In the Martini 2.0 force field, the water-water interaction is a strong polar (level I) interaction, while the P1-water (the terminal diol atoms) interactions are level II. The headgroups of Martini monoolein molecules are a sequence of P4-Na bonded together, which both have level II strength interactions with P1 atoms. The strength and length of the interaction between ‘S’ types and normal types is unchanged, so this level II interaction remains the same between P4 and P1 as for P4 and SP1. Therefore, it is highly likely that this difference in interaction strength between the water self-interactions and the diol attraction to the headgroup regions acts to exclude the diols from the mesophase water channels.

5.3.3 Re-investigating the effect of diol concentration in the solvent

While the partition coefficient is not particularly well represented at a larger range of diol concentrations in the solvent, the phase behaviour at lower concentrations is still relatively well represented. As fig. 5.4a) illustrates, in the system with 1% concentration of diol in the solvent, there is still a very clear \( Q_{II}^D \) system in place. The water channels meet in a 4-way tetragonal junction, and there are two distinct water channels separated by the lipid bilayer. As the proportion of solvent diol is increased to 10% w/w as shown in fig. 5.4b), the arrangement of the water channels changes slightly. There has been no distinct mesophase transition: there are two bicontinuous water channels, albeit slightly flattened in comparison to a \( Q_{II}^D \) mesophase. Moreover, they still meet at a 4-way junction. However, the junction has been slightly elongated in the [111] direction of the cubic unit cell. That is, the system now appears to have two sets of two water channels meeting at a slight distance, but the angle between both pairs are
CHAPTER 5. MOLECULAR DYNAMICS SIMULATIONS OF THE CUBIC TO SPONGE TRANSITION

Figure 5.4. Sections of bicontinuous water channels in the final frames of sponge transition simulations. a) 1% and b) 10% diol in solvent. The water atoms are coloured by cluster in yellow and purple to emphasise the bicontinuity of the systems, with blue alpha surfaces constructed over them. The 1% diol system is still clearly cubic with a clear meeting of the water channels at a 4-way tetragonal junction. The 10% system is less clearly cubic, but the system remains bicontinuous with elongated water junctions.

maintained. This elongation is indicative that the increase in diol content in the system has swollen the size of the cubic phase, even if it is not sufficient to induce a full transition. Additionally, it reinforces the observations in chapter 3 that suggest the increase in diol induces a soft rather than a sharp transition between the $Q_{DII}^D$ and $L_3$ mesophases.

To add to the final frame water networks shown in fig. 5.4, the water networks of the higher proportion diol systems are shown in fig. 5.5. In the cases of the 20% and 30% systems, the water channels appear to be flat, separating the system into several lipid bilayers. This again is likely a result of the preferential partition for the lipid domains seen in fig. 5.2. If as seen the diol model has a stronger preference to occupy the lipid domains over the water ones, this will relieve any chain packing stress that exists. The $Q_{DII}^P$ mesophase is known to have packing stress that can be relieved by the inclusion of a long chain alkane. This was demonstrated by Shearman et al., who showed that the system would undergo a phase transition to a flatter $Q_{DII}^P$, mesophase, or possibly a $L_n$ mesophase at lower temperatures [85]. In comparison the
5.3. Changing the proportion of diol in the solvent

(a) 20% solvent diol  
(b) 30% solvent diol  
(c) 40% solvent diol

**Figure 5.5.** The final frame water networks of a) 20% b) 30% and c) 40% solvent diol systems. An alpha surface has been constructed and shown in blue.

40% solvent diol system in fig. 5.5c) has an aperiodic structure with a single water channel running through it. That it now more closely resembles the image of a L3 mesophase that we first saw in fig. 1.9 is unsurprising, as the diol partition coefficient is now 0.75, as expected from experiments.

**Figure 5.6.** The surface area/volume ratios of water alpha surfaces for different diol proportions in sponge transition simulations. Left: the temporal variation of the surface/volume ratio of the water alpha surface. Right: the time-averaged value of the surface area/volume ratio. A linear regression best fit is plotted as a guide.
5.3.3.1 Interfacial areas

To investigate the systems further, we can apply some of the analytical tools developed. In the case of the interfacial area, we will investigate the surface area per volume of the surface constructed, rather than the area directly. This is because the simulation boxes have different volumes - and therefore different volumes of water alpha surface - as a result of the different numbers of diols and waters used, the surface area per volume is plotted instead to overcome the differences. However, the surface area per volume measurement will still use the same techniques developed in section 4.5.

The results for the surface area to volume ratios for the five systems are shown in fig. 5.6. Temporally, the ratio remains very constant through the course of the simulation with little variation, and increases linearly as the proportion of diol in the system is also increased. The latter would suggest what we already knew, in that the formation of the L₃ mesophase is in part driven by the increase in interfacial area as a result of diol partitioning. Moreover, as all the systems in question have identical numbers of lipids, it tells us that one of the distinguishing features that L₃ mesophases have is that they have a significantly expanded interfacial area in comparison to the Q₁DII mesophase. It is not obvious that the change in topology should necessarily result in a larger molecular interfacial area, so it is significant that it does.

5.3.3.2 Elastic moduli

Figure 5.7 shows the measurements of time-averaged elastic moduli for the five systems. As might be reasonably expected, as the proportion of diol in the solvent is increased, the elastic moduli are reduced. However, the trend does not appear to follow any particular model. Interestingly, the 30% w/w solvent diol system appears to be an outlier in the case of the bending modulus: the modulus otherwise appears to be decreasingly fairly linearly with the increase in the proportion of diol in the solvent. This perhaps explains that while the diol now marginally occupies the aqueous domain more ($K = 0.97$) the system appears to be a L₃ mesophase rather than an aperiodic one, as the energetic cost of deforming the membrane in that way has slightly increased. Furthermore, while the 20% and 30% solvent diol systems have comparable bending moduli, the tilt modulus is significantly reduced in the latter system. This perhaps explains why in fig. 5.5 the 30% solvent diol system shows fewer interconnections between water channels. As the energetic cost of lipids tilting away from the membrane
5.3. CHANGING THE PROPORTION OF DIOL IN THE SOLVENT

Figure 5.7. The time-averaged elastic moduli for sponge transition simulations at increasing solvent diol proportions. Top: bending moduli, bottom: tilt moduli.

normal is reduced, the bilayer thickness decreases, while because the bending modulus has increased, inter-bilayer connections are now harder to form. The system therefore reverts to a series of separated bilayers.

5.3.3.3 Curvatures

To confirm the structures of the 1%, 10%, 20%, and 30% solvent diol simulations, we can use the curvature measuring techniques developed in section 4.3 to build up a picture of curvature across the mesophase, and through the course of the transition. In section 4.3, we established that the optimum atom and cutoff radii for cubic phases was the terminal carbon (labelled C5A), and around 40 Å respectively. These parameters were therefore used in the measurement through the transition, and are plotted in fig. 5.8.

In each case, the distribution of mean curvatures remains centred around zero through the entire course of the simulation, indicating that in each case, the system still resembles a minimal surface of some kind, whether that is Q_{II}^D (or another cubic) or L_α (a plane being the most trivial example of a minimal surface). As we have seen from the previous sections, in the case of the 1% and 10% solvent diol systems, this means that they stay resembling a Q_{II}^D mesophase. In
CHAPTER 5. MOLECULAR DYNAMICS SIMULATIONS OF THE CUBIC TO SPONGE TRANSITION

**Figure 5.8.** Temporal curvature distributions of transition simulations of diol proportions between 1% and 30%. The left column shows the Gaussian curvature distributions, and the right column shows the mean curvature distributions.
5.3. CHANGING THE PROPORTION OF DIOL IN THE SOLVENT

The change in Gaussian curvature is much more profound. In most cases, there is a significant shift away from the initial distribution of Gaussian curvature across the \(\text{Q}_{II}^D\) mesophase. To add to this change, it appears that the shift happens extremely rapidly, as although all the distributions are equally temporally spaced, the initial time frame (in dark purple) stands out from the rest. The least change is seen in the 1% solvent diol case, which is to be expected, as the system remains in the \(\text{Q}_{II}^D\) mesophase throughout the simulation. The 20% and 30% diol systems, however, move very quickly to be distributed around 0, again confirming it as a \(\text{L}_\alpha\) mesophase. The 10% diol case is slightly more complex. The bilayer still has a distinct and broad distribution of negative Gaussian curvature. However, it too is slightly distinct from the initial \(\text{Q}_{II}^D\) mesophase distribution. This corroborates what was speculated earlier, that although the system is still distinctly bicontinuous, the topology of the mesophase has in fact changed slightly with an elongation of the water channels as the bending modulus is slightly reduced, and a degree of diol partition has begun.

To probe the distributions further, two measures of their properties are plotted in fig. 5.9. In

**Figure 5.9.** Fitted parameters of the mean and Gaussian curvature distributions from non-L\(_3\) transition simulations, and their temporal variation. Top: The mean value of the Gaussian curvature probability distribution. Bottom: The fitted full width half maximum of the mean curvature probability distribution.
the case of the Gaussian curvature, the discrete probabilistic mean and standard deviation of the distributions in the left hand column of fig. 5.8 are plotted against time. For the mean curvatures distributions, a Gaussian peak model has been fitted to the broad peaks seen in the right hand column of fig. 5.8. From those fits, the full width half maximum (FWHM) has been extracted, and plotted against time as a measure of the distribution broadness, and therefore homogeneity.

In the case of the Gaussian curvature distributions, they further confirm that the 20% and 30% systems tend towards a L\text{\alpha} mesophase. The mean of the distribution is initially very negative - at the value expected for a Q_{DII} mesophase - and, after and initial jump, both means tend towards zero through the course of the simulation. As expected, the 1% system stays more or less at the same value as the initial one: a less negative mean of the distribution suggests that the lattice parameter of the cubic phase has slightly increased, as would be expected through a diol-induced swelling. The 10% diol system settles at a relatively constant value in the same initial time frame change, and again, the mean value of the distribution remains negative.

The mean curvature FWHM measurements also provide an interesting picture of the eventual transition to the L\text{\alpha} mesophase for the two higher concentration systems. As with the Gaussian curvature measurements, the most significant changes appear to happen on a very rapid timescale; within the first 30 ns of the 1 \mu s simulation. While for the 1% and 10% solvent diol systems, there is very little change in peak broadness, the initially very broad peak upon the phase change in the 20% and 30% systems indicates that the curvature of the bilayers is increasing, and becoming more sphere-like. The slow narrowing of the peaks in these systems is reflective of the fact that there they do not immediately become L\text{\alpha} mesophases, but take a relatively significant period of time to completely rearrange the system, as illustrated by the simulation frames in fig. 5.10.

5.4 The Q_{DII}^{D}/L_{3} mesophase transition

To briefly summarise the preceding results, we have found that for systems with less than 40% diol in the solvent, the coarse-grained simulation does not faithfully reproduce the expected mesophase behaviour as determined by experiment. The values of the diol partition coefficient are greater than 1, indicating a preference for the lipid - and not aqueous - domains. Moreover - and as shown both by visual inspection and quantitative measurements of the membrane
curvatures - this preference results in the formation of the Lα mesophase at intermediate concentrations, which is the opposite of what is expected from experiments. We must therefore ask what are the consequences for the diol model developed in section 4.2 for modelling the L₃ mesophase, and our investigations into the nature of the Q¹⁻⁻/L₃ transition.

Recall that in section 4.2, we used a fixed weight ratio of 40:60 solvent:lipid to determine the best coarse-grained model, and a fixed diol proportion of 40% weight in the solvent, because this is the middle point of the mesophase sequence diagram (see either reference [52] or fig. 3.3). These conditions are therefore optimal for determining the behaviour of a diol model, and so in that sense, our SP1-SC2-SP1 diol model used throughout this chapter is the best model available at this time to bring about the formation of the L₃ mesophase. Therefore, while the diol model we have used may be sub-optimal across the full range of the mesophase diagram, it is still appropriate to use for our transition simulations, where it is present in
FIGURE 5.11. Diol partitioning time variation in the 40% solvent diol system. The mean value is $K = 0.75 \pm 0.02$.

sufficient proportions.

The suitability of the model is demonstrated by the initial results of the 40% solvent diol system, which appear to be a faithful representation of the $L_3$ mesophase: the diol partition coefficient matches the experimental equivalent almost exactly, and by inspection of fig. 5.5c) resembles the complex aperiodic water channels expected. Using this system as a model, in this section we will firstly outline properties of the transition across the full 1 $\mu$s length of the simulation. Secondly, we will investigate the molecular rearrangement in the transition in the time that that happens, within the first c.10 ns.

5.4.1 Properties of the $L_3$ mesophase

5.4.1.1 Diol partitioning

As should be expected over the course of the simulation, the diol partition is extremely constant. This is illustrated by the measures shown in fig. 5.11, where we see that $K = 0.75 \pm 0.002$ when averaged over the entire simulation. This is slightly less than the value of $K = 0.834 \pm 0.008$ we found in model trials in section 4.2, but in fact closer to the experimental value of $K = 0.78 \pm 0.14$ of Evenbratt et al. [55]. Considering the small variation in $K$, we can therefore be confident that the diol partitioning is a fair representation of the $L_3$ mesophase.
5.4. THE Q$_{II}/L_3$ MESOPHASE TRANSITION

5.4.1.2 Interfacial area

The interfacial area across the simulation are replotted in fig. 5.12a) having originally been shown in relation to other systems in fig. 5.6. Taking the system on its own, the equilibrium cell length in the simulation is 217.6 Å. The alpha surface water surface construction uses a range of probe sphere radii between 10 and 16 Å, which give a mean volume fraction of the surface of 0.31±0.05. This is obviously lower than expected, but fig. 5.13 demonstrates the challenge of selecting an appropriate probe sphere radius. At a small cutoff radius (10 Å, fig. 5.13a)), the surface is more closely constructed around the water atoms, but has a very low volume fraction of 0.23. When the probe sphere radius is increased to 15.142 Å (fig. 5.13b)), the volume fraction has risen to a value which would be expected from experiments, at 0.372. Importantly, at this volume fraction, the number of MO headgroup atoms that have been engulfed by the surface has increased significantly, indicating that it is no longer a good representation of the water networks in the system, therefore making the measure of interfacial area a useful one.

Due to the nature of the measurement previously outlined section 4.5, the effect of the increase in volume has a corresponding increase in headgroup area, shown in fig. 5.13c), which shows the average headgroup area measured at each probe sphere radius. The flattening off of the linear pattern at very large radii is a result of the fact that at these radii, the increase can no longer account for a significant increase in volume. However, the most notable result shown in fig. 5.13c) is that the measure of surface area per lipid has increased significantly in compar-
CHAPTER 5. MOLECULAR DYNAMICS SIMULATIONS OF THE CUBIC TO SPONGE TRANSITION

(a) The water alpha surface with a probe sphere of radius 10 Å.

(b) The water alpha surface with a probe sphere of radius 15.142 Å.

(c) The measured headgroup area and its increase with probe sphere radius.

(d) The radial distribution function for the diol atoms with the water and primary carbons of the system.

FIGURE 5.13. Measuring the interfacial area of the 40% solvent diol transition system. a) and b) show the water alpha surface (in blue) constructed using two different probe sphere radii, and how it engulfs the red MO headgroup (ETH) atoms at larger radii. c) shows how the measured area varies with the alpha surface probe sphere. d) the radial distribution functions measured for the diol atoms against the water and primary carbon atoms.
son to the measures made in fig. 4.29, where the calculated area per lipid in a $Q_{II}$ mesophase was 34.5 Å$^2$. Here, we are now observing areas per lipid of between around 66 and 80 Å$^2$.

While it is important to note that in section 4.5 we found that using alpha surfaces overestimated the interfacial area, we would not expect it to be a substantial overestimate. However, an interfacial area study by Balgavy et al. showed that in a $L_\alpha$ mesophase, interfacial areas for a variety of phosphatidylcholines ranged between around 57 and 64 Å$^2$, which were then found to increase with temperature [240]. Therefore, the values measured in this comparatively flat $L_3$ mesophase cannot be completely discounted, as while they almost certainly are overestimated, they are likely to be representative of the scale of growth of the interfacial area as a result of the introduction of diols into the system. Moreover, they demonstrate that the $L_3$ mesophase has a very different geometry to the $Q_{II}$ mesophase. This is significant, as we note from the literature that some authors consider structural calculations for the $L_3$ mesophase may be based on topologically-informed ones for the $Q_{II}$ mesophase [120]. Furthermore, the radial distribution functions measured in fig. 5.13d) show that the locations of the diol are very much associated with the interface, which therefore contributes to the challenge of constructing a precisely smooth surface over the water interface. The strength of the correlation with the diol atoms are clearly linked to their Martini type - note that because BD1 and BD3 are of the same type, they have almost identical $G(r)$ distributions, so only one is plotted for clarity. Most significantly, this shows that the BD2 central hydrophobic bead is most closely associated with the C1A primary carbon atom in the membrane, distinct from the outer atoms, which shows the importance of this atom in the partitioning process.

5.4.1.3 Elastic Moduli

In fig. 4.28, we found that changing the cutoff radii from between 20 to 40 Å to measure elastic moduli had little effect on the magnitude of either the bending or tilt modulus. In fig. 5.14, the three cutoff lengths are again used to measure the time dependency of the membrane moduli. Perhaps most interestingly, over the course of the simulation, there doesn’t appear to be any significant variation of either modulus. Additionally, as before, there is not a clear dependency of the modulus on the cutoff radius used to form the point cloud to measure it.

For the $Q_{II}$ mesophase, the average values across the different radii used were $K_c = 16.5 \pm 0.5 k_B T$ and $\kappa_t = 3.8 \pm 0.1 k_B T$. In comparison, the time- and radius-averaged values for the
CHAPTER 5. MOLECULAR DYNAMICS SIMULATIONS OF THE CUBIC TO SPONGE TRANSITION

Figure 5.14. The elastic moduli from a 40% solvent diol simulation. Moduli measured using different point cloud cutoff radii are shown in different colours, and their average is shown as a black line. Top: the bending modulus, $K_c$. Bottom: tilt modulus, $\kappa_t$. The overall time-averaged values of the moduli are $K_c = 14.59 \pm 0.66 k_B T$ and $\kappa_t = 2.25 \pm 0.81 k_B T$.

$L_3$ mesophase measurements of membrane moduli are $K_c = 14.59 \pm 0.66 k_B T$ and $\kappa_t = 2.25 \pm 0.81 k_B T$. That is to say, for $L_3$ mesophases which are in equilibrium, there is a significant lowering of both the bending and tilt moduli in comparison to their $Q_{II}^D$ mesophase equivalents.

5.4.1.4 Curvature

The time-dependent distributions of curvature for the 40% solvent diol system are shown in fig. 5.15. As previously established, we will use a cutoff radius of 40 Å, and the terminal carbon atoms in the membrane to form the point clouds for measuring curvature, this being the most appropriate measure in curved systems. Similar to the results seen in section 5.3.3.3, both distributions of curvature change extremely rapidly, as neither the Gaussian nor the mean curvature distributions appear to change significantly after the initial change from the $Q_{II}^D$ distributions.

Interestingly as before, the distribution of mean curvature in fig. 5.15 is still centred around zero, indicating that the structure of system is based on a minimal surface as expected by the discussion in section 1.6.1 [47, 48]. However, the distribution is certainly significantly broader.
than before, suggesting that regions of the system are further from the minimal surface than ideal. This is likely mostly driven by thermal fluctuations in a physical system.

That the mean curvature shows we have a minimal surface-like system renders the change in Gaussian curvature more interesting than in the lower solvent diol systems that we encountered earlier. Although there is certainly a change in the overall shape of the distribution of Gaussian curvature, the peak shape obtained is very much skewed towards the negative region: the mean of these equilibrium distributions is \((-1.21 \pm 0.18) \times 10^{-4} \, \text{Å}^2\). As would be necessary for a minimal surface then, the system is mostly locally saddle-shaped, with small regions of spherical caps connecting them.

It is at this point that we can now demonstrate the real power of using a local measuring method of curvature as developed in section 4.3. Because every molecule in the system has its curvature environment measured at every time step, we can begin to understand how the curvature of the system progresses in its transition from the \(Q_{II}^D\) mesophase to the \(L_3\) mesophase. This is demonstrated in fig. 5.16, where we can identify molecules that are either in the initially either highly curved or flatter regions of the \(Q_{II}^D\) mesophase. These molecules are then tracked through the course of the simulation, and the probability distributions of their subsequent curvatures plotted. Interestingly they show that over the course of the entire 1 µs
Figure 5.16. Tracking molecules by their curvature environment through a transition simulation. a) Tracking the subsequent curvature distributions of molecules in an initially highly curved membrane environment. b) Tracks subsequent curvature distributions of molecules in an initially flat membrane environment. c) and d) show the time dependency of the distribution means for a) and b) respectively.
5.4. THE \(Q^{D}_{II}/L_3\) MESOPHASE TRANSITION

<table>
<thead>
<tr>
<th>(\chi^2)</th>
<th>Inverted Exponential</th>
<th>Reciprocal</th>
<th>Logarithmic</th>
<th>Inverted Exponential</th>
<th>Reciprocal</th>
<th>Logarithmic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced (\chi^2)</td>
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<td>55.20</td>
<td>97.47</td>
<td>2.27</td>
<td>20.78</td>
<td>3.48</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>1.84</td>
<td>3.25</td>
<td>0.08</td>
<td>0.69</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 5.3. \(\chi^2\) values for different growth model fits for the partitioning and surface area/volume data in the initial stages of the \(L_3\) transition simulation.

simulation, there is little preference or ‘memory’ of the \(Q^{D}_{II}\) mesophase in the equilibrated \(L_3\) one. As fig. 5.16c)-d) show, both distribution means stabilise after the first 30 ns of the simulation. The time averages of c) and d) from 30 ns onwards are both \((-1.21 \pm 0.03) \times 10^{-4} \, \text{Å}^2\), showing that there is no difference or preference for Gaussian curvature for molecules which begin in the extreme regions of the \(Q^{D}_{II}\) mesophase membrane.

5.4.2 The transition on short timescales

Throughout section 5.4.1, we have investigated the properties of the lipid \(L_3\) mesophase over the full course of a 1 \(\mu\)s simulation. However, in every case from fig. 5.11 to fig. 5.16, we have found that even by the time of the first simulation frame being measured at 30 ns in, the system has reached evolutionary equilibrium. Therefore, to best understand the dynamics of the formation of the \(L_3\) mesophase in relation to its \(Q^{D}_{II}\) predecessor, we should examine the simulation on far shorter timescales.

The swiftness of the evolution of the mesophase is demonstrated by the simulation frames shown in fig. 5.17, where we see six snapshots from within the first 10 ns of the simulation. On this timescale, we can begin to see how the \(Q^{D}_{II}\) system becomes a \(L_3\) mesophase. With reference to fig. 5.18, at 900 ps, the greatest deviations from the initial \(Q^{D}_{II}\) surface appear to come in the flatter regions of the surface, which appear to rupture in the process of the system coming to contain a single water channel. Reflecting on the results from across the whole simulation, this is perhaps surprising given the Gaussian curvature distribution of the system is tending towards becoming flatter overall rather than more curved. However, as we will see, the true picture of the curvature evolution is more complex.
5.4.2.1 Diol partitioning and interfacial area

The results for both the partitioning behaviour of the system diols and the surface area per volume ratio are plotted in fig. 5.19a) and b) respectively. It is instructive to look at the two measures of the system together rather than separately, because on this timescale, they are intrinsically linked. The reason for this is demonstrated by fig. 5.1, where the necessary re-insertion of the water molecules along with the diol molecules means that at the start of the simulation, the system does not have fully constructed water channels. The first action of the production simulation is therefore to recreate the water channels of the system, before undergoing a mesophase transition.

This is demonstrated firstly by the initially very low value of the partition coefficient, as a result of nearly all the diol being in the water channels. As the system evolves, the growth of
5.4. THE Q\textsubscript{III}/L\textsubscript{3} MESOPHASE TRANSITION

**Figure 5.18.** A 2x2x2 array of unit cells of the Q\textsubscript{III} surface, coloured by Gaussian curvature. The yellow regions are where the surface is flatter, and the dark blue regions are the most highly curved.

(a) Diol partitioning time variation. (b) The surface area to volume ratio of the water alpha surface.

**Figure 5.19.** Temporal variation in the first 10 ns of a) the diol partition coefficient and b) the surface area to volume ratio of the 40% solvent diol sponge transition simulation. Goodness of fit values for this model, along with other less successful ones are listed in table 5.3.
the value of $K$ follows an inverted exponential decay with a decay constant of $1033.18 \, \text{ps}^{-1}$. Reciprocal and logarithmic models did not fit to the data successfully, as demonstrated by the $\chi^2$ values listed in table 5.3. Interestingly, the growth in the surface area to volume ratio follows a similar growth trend before stabilising at its final value for the simulation. Comparing the fits to the growth trends of these two parameters reveals that the growth constant for the diol partitioning is smaller than the constant for the increasing surface area to volume ratio. This suggests that the diol partitioning reaches equilibrium value more quickly, which in turn indicates that the rearrangement of the system diols happens faster than the rearrangement of the water channels. Therefore we can conclude that the increase in surface area per lipid at the interface is a direct result of the partitioning behaviour of the diols in the system, rather than any other feature of the system.

5.4.2.2 Moduli

While fig. 5.14 showed that across the simulation there was little change in the magnitude of either the bending or tilt modulus across the course of the simulation once the $L_3$ mesophase had formed, the trend found in the first 10 ns is very different. Figure 5.20 shows the results for the moduli measured over the first 10 ns every 30 ps up for the first 600 ps and also for the first 10 ns every 300 ps.

The trend for the bending modulus (fig. 5.20a, b)) is very clear in that it shows the most significant reduction in magnitude in the first 600 ns, with a fairly steady reduction. This trend subsequently continue in b) up to around 2000 ps, where it appears to level off to its equilibrium value of $K_c = 14.59 \pm 0.66 k_B T$. It should be noted as well that at particularly low timescales in fig. 5.20a), there appears to be a slight relationship between the cutoff radius used for the measurement and the resultant modulus: smaller cutoff radii measure smaller moduli. In these early frames, it is likely that the system is still mostly a $Q_{II}$ mesophase. As shown in section 4.3, a cutoff radius of 20 Å does not capture the curvature of the mesophase particularly well. As the measurement of $K_c$ depends on a measurement of the local membrane normal, this is likely to be the explanation for this correlation.

In comparison, there is not a clear trend in how the tilt modulus is reduced. As we found previously over the full timescale of the simulation, the tilt modulus sees a reduction from $3.8 \pm 0.1 \, k_B T$ to $2.25 \pm 0.81 \, k_B T$. Interestingly, $\kappa_t$ appears to reach this magnitude at around
5.4. THE Q$_{II}$/L$_3$ MESOPHASE TRANSITION

Figure 5.20. Elastic moduli measured on a short transition timescale. a) and b) show the first 600 ps and 10,000 ps of bending modulus measurements respectively. c) and d) show the first 600 ps and 10,000 ps of tilt modulus measurements respectively. The radii used for the measurements are indicated by different colour scatter points, and the black line shows the mean at every time step.

The same time (2000 ps/2 ns) as $K_c$, suggesting that this is the most important timescale for the molecular rearrangement in the transition. However it is not clear that there is a simple quasi-linear reduction in the modulus over this period of time - not least due to a the large intermediate peak seen in fig. 5.20d) at 1200 ps, the precise cause of which is not entirely clear.

Most notably among these results is the step change increase from 0 to 30 ps, which for both the bending and tilt modulus increases slightly after the beginning of the simulation. This is likely due to the initial rearrangement of the water and diol molecules back into the water channels properly, which will have a stiffening effect on the membrane while it is slightly dehydrated without water at the interface. Subsequently however, there is clearly a decrease in both moduli measurements as a result of the transition to the L$_3$ mesophase.
CHAPTER 5. MOLECULAR DYNAMICS SIMULATIONS OF THE CUBIC TO SPONGE TRANSITION

Figure 5.21. Curvature distributions changing on a short transition timescale. a) and b) show respectively the Gaussian and mean curvature distributions changing at very low time scales, between 0 and 600 ps. c) and d) show respectively the Gaussian and mean curvature distributions continuing from that point (600 ps) to the end of the first 10 ns of the simulation.

5.4.2.3 Curvature

In fig. 5.21, we see the distributions of the Gaussian and mean curvatures between both 0 - 600 ps and 600 - 10000 ps. As before, we can see that the former time period is where the most significant changes happen in both measures. In this time, the Gaussian curvature distribution shifts to having a peak closer to zero, while the width of the mean curvature distribution grows.
5.4. THE Q\textsubscript{II}\textsuperscript{D}/L\textsubscript{3} MESOPHASE TRANSITION

Figure 5.22. Tracking how molecular curvature changes on a short transition timescale. a) and b) show how the distributions of curvatures for molecules with initially high and low Gaussian curvature respectively change over the first 10 ns of the simulation. c) and d) show how the mean values of these distributions change over that time.

significantly. In the second time period, both of these trends continue, with a sharper peak just below zero developing for Gaussian curvature, and the mean curvature peak broadening further. These results tell us again that the minimal-surface like structure is retained throughout the transition, but distinctly without the same topology as before.

As before, we can track how the initial curvature environment of the molecules in the Q\textsubscript{II}\textsuperscript{D} mesophase affects their final environment in the L\textsubscript{3} mesophase. These results are shown in fig. 5.22. The more significant change obviously happens when looking at the more molecules in a more
CHAPTER 5. MOLECULAR DYNAMICS SIMULATIONS OF THE CUBIC TO SPONGE TRANSITION

Figure 5.23. From top, the surface area per volume, potential energy difference per molecule, simulation cell dimensions, and pressure during the first 2000 ps of the simulation.

A highly curved environment, which as we can additionally see from fig. 5.22c), take longer to redistribute themselves to have reached the equilibrium distribution for the L₃ mesophase. However, this set of results suggests even more strongly than before that the initial curvature distribution has no significant bearing on the final distribution. That is, the evolution of the L₃ mesophase occurs homogeneously across the unit cell of the Q₁₁ mesophase, and there is no preference for deformation dependent on the curvature of the membrane.

5.4.3 Summary

In section 5.4, we have investigated how the L₃ mesophase comes to form from the Q₁₁ mesophase in a transition simulation. Significantly, we have found that the most important features of the transition are evident after just the first 10 ns of the simulation, and do not exhibit any significant further structural changes after this point. This is perhaps best demonstrated by the data shown in fig. 5.23, which demonstrates that the simulation reaches both pressure and potential energy equilibrium after just 2 ns.
5.5. CHOLESTEROL-DOPED SPONGE TRANSITIONS

We have shown that as a result of the transition, the interfacial area has increased significantly, and that the transition is driven by the partitioning of the diols in the system. Perhaps most significantly, we have found that this partitioning lowers both the bending and tilt moduli of the membrane, which explains why the system no longer exhibits any long-range periodicity. To the author’s knowledge, this represents the first membrane energetics measurement of L\textsubscript{3} mesophase membranes either experimentally or computationally.

Most interestingly, we have shown that the L\textsubscript{3} mesophase as simulated adopts a minimal surface-like topology, without any long range order. By this, we mean that the distribution of mean curvature still averages zero in the L\textsubscript{3} mesophase after a transition, with a change in Gaussian curvature. That the L\textsubscript{3} mesophase is a minimal surface could reasonably be expected, and possible models for surfactant L\textsubscript{3} mesopahses are discussed at length by Schwarz and Gompper [241]. Additionally, we have shown that the evolution of the L\textsubscript{3} phase happens homogeneously across the predecessor Q\textsubscript{D}II mesophase, in that there is no curvature preference for how the L\textsubscript{3} mesophase evolves.

### Table 5.4. Simulation make ups for cholesterol-doped sponge transition simulations.

<table>
<thead>
<tr>
<th>% mol cholesterol</th>
<th>no. MO cholesterol</th>
<th>No. diols</th>
<th>No. waters</th>
</tr>
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<tr>
<td>2.5</td>
<td>7408</td>
<td>192</td>
<td>8035</td>
</tr>
<tr>
<td>5</td>
<td>7216</td>
<td>384</td>
<td>8053</td>
</tr>
<tr>
<td>8</td>
<td>6992</td>
<td>608</td>
<td>8073</td>
</tr>
</tbody>
</table>

5.5 Cholesterol-doped sponge transitions

In chapter 3, we found that the addition of cholesterol to monoolein has relatively little effect on the range of lyotrope/solvent conditions that form the L\textsubscript{3} mesophase. Recall that we found that the Q\textsubscript{D}II/L\textsubscript{3} mesophase boundary is shifted slightly upwards, and at high concentrations (10% mol) the L\textsubscript{3}/L\textsubscript{a} mesophase boundary is shifted slightly downwards. In this section, we will investigate the effect of the addition of cholesterol on the dynamic structure of the L\textsubscript{3} mesophase, such that we can understand why cholesterol can be incorporated so readily.

The protocol for simulation construction was the same as followed for the undoped systems, as described in section 5.2. Single unit cells of Q\textsubscript{D}II mesophases were constructed through self-assembly simulations, using the standard Martini model of cholesterol [242]. For this purpose,
the \texttt{define=-DFLEXIBLE} option was added to the minimization molecular dynamics parameter file during steepest descent energy minimizations as required by the addition of virtual sites in the topology. The construction procedure shown in fig. 5.1 was then followed, with the final quantities of lipids and solvent detailed in table 5.4.

### 5.5.1 Diol partitioning

The results for the diol partitioning in each of the three systems used is shown in fig. 5.24. The results in fig. 5.24a) show that compared to the undoped system we had before, where $K = 0.75 \pm 0.02$, there is a slight reduction in the value of $K$ when cholesterol is introduced. Moreover, the reduction is proportional to the proportion of cholesterol in the membrane, as indicated by the linear fit to the data which tells us there is a reduction of $0.013 \, \text{K/% mol}$.

Importantly, while there is a reduction, the values measured are still within the expected range for the partition coefficient as measured experimentally.

The reduction is likely a feature of the diol model used. The default Martini model of cholesterol comprises almost entirely of ‘S’ type atoms, because of the atomistic ringed structure of...
5.5. CHOLESTEROL-DOPED SPONGE TRANSITIONS

The LJ equilibrium distance and interaction strength between ‘S’ type atoms is reduced to 75% of the full value of the particle type. This is evidenced by comparing fig. 5.24b) and c), which show several pair correlation functions measured for an 8% mol cholesterol and undoped L₃ mesophase respectively. The BD1 atom of the diol clearly has a closer interaction with the headgroup ROH atom of cholesterol than any other atom type in the system. The precise reason for the reduction in K is then slightly unclear: a reduction implies that there is a stronger preference for the water domain of the system rather than the lipid one. However, we note that comparing fig. 5.24b) and c), there is a slight increase in the prevalence of the BD1-W peak, which suggests that more atoms now occupy the water domains, as is directly measured by the partition coefficient.

5.5.2 Interfacial area

The interfacial area measurements for cholesterol-doped L₃ mesophases are shown in fig. 5.25. As with the diol partition, there is a linear reduction in the surface area per volume in the mesophases as the proportion of the diol is increased. As fig. 5.25c) and d) show, the overall reduction in this measure is a result of the decrease in the surface area per lipid, rather than any significant change in the volume of the water channel in the system. It is therefore the
addition of cholesterol to the system has reduced the interfacial area. This reduction in interfacial area may also go some way to explaining why the partition coefficient has been reduced, as there is less interfacial area for the diol molecules to occupy, which reduces the ability for them to enter the headgroup area of the lipid domain.

### 5.5.3 Elastic moduli

The stiffening effect of cholesterol on membranes has been well-documented throughout this thesis (see, eg. [195]). Measurements of the elastic moduli of cholesterol-doped L₃ mesophases are shown in fig. 5.26. The results show that in all cases there is a small increase in both the tilt and bending modulus of the membrane when cholesterol is added. However, the effect is not systematic, and cannot be said to be statistically significant considering the large overlap in data.

With reference to the experimental data seen in chapter 3, we know that cholesterol-doped L₃ mesophases exist fairly stably across a wide range of solvent and dopant proportion conditions. At 40% diol, all dopant proportions exhibited a L₃ mesophase, with relatively little variation in the correlation length (see fig. 3.5). This suggests that we should not necessarily expect cholesterol to have any major structural effect on the L₃ mesophase at this point, so that there is
5.6. DOES CHOLESTEROL HAVE A PREFERENCE FOR GAUSSIAN CURVATURE?

In fig. 5.27, the results for how the distributions of curvatures change with the addition of cholesterol are shown. In the case of the mean curvatures, the addition of cholesterol has tightened the distribution, as noted by the decrease in the size of the full width half maximum of the fitted Gaussian peak around zero. The result on the Gaussian curvature is for the curvature to become less negative, implying that cholesterol has a flattening effect on the membrane. This follows naturally from there being a smaller distribution of mean curvatures around zero, so is to be expected.

5.6 Does cholesterol have a preference for Gaussian curvature?

As has been mentioned several times throughout this work, cholesterol has significant effects on the lateral properties of membranes. One interesting feature that has been investigated on occasion is whether the molecule has a particular preference for curved regions of membranes. Koldsø et al. found that in a complex bilayer, clusters of cholesterol around a protein additionally showed regions of curvature, suggesting that cholesterol itself has an impact on membrane geometry [243]. Conversely, Baoukina et al. found that inducing curvature on a
CHAPTER 5. MOLECULAR DYNAMICS SIMULATIONS OF THE CUBIC TO SPONGE TRANSITION

(a) Measuring surface-carbon distances.

(b) Grouping surface atoms by their Gaussian curvatures.

(c) Counting carbon densities for Gaussian curvature groups

FIGURE 5.28. Measuring the curvature preference of cholesterol in a Q_{II} mesophase. 

plasma membrane has a sorting effect, with cholesterol having a slight preference for flatter regions of the tether [244]. Experimentally, van ’t Hag et al. investigated the curvature preference of peptides using contrast-matched neutron scattering, expecting to find a preference for the flatter regions of the unit cell, in order to minimize elastic deformation of transmembrane proteins [245]. However, the study could not demonstrate any firm conclusions as to a particular preference for the peptides. Further to these studies, I was informed in correspondence with the group of Adam Squires that their recent contrast-matched small angle neutron scattering experimental results suggest that in cubic mesophases doped with 10% mol cholesterol, they were able to observe that the molecule has a preference for flatter regions of the bilayer. This therefore provided motivation to see whether coarse-grained cubic phases demonstrated similar properties.

To attempt to provide complementary simulation data to this, a 10% mol cholesterol cubic phase was simulated using the standard Martini model of cholesterol. The number of monoolein molecules was 945, the number of cholesterols was 105, and the number of waters was 2523, such that the lipid:water weight ratio was 60:40. The self-assembly simulation was run for 6 µs using standard molecular dynamics parameters. 18 equally spaced frames of the self-assembled Q_{II} mesophase were then used for analysis.

To analyse potential curvature preference, a density method illustrated in fig. 5.28 was used.
5.6. DOES CHOLESTEROL HAVE A PREFERENCE FOR GAUSSIAN CURVATURE?

Firstly, a Q_{II} surface was fitted to the terminal carbons of the monoolein and cholesterol molecules. Subsequently, distances between a large collection of points on the surface and the cholesterol terminal carbon atoms were measured. The points on the surface were then binned according to their Gaussian curvature. Using these first two steps, for each collection of surface points, the number of cholesterol terminal carbons were counted within several distances. The results of this process are seen in fig. 5.29, which shows both the analytical distribution of Gaussian curvature across a unit cell of the Q_{II} surface, and the measured distribution at distances of 5 Å, 10 Å, and 15 Å. In each case, the two distributions are remarkably similar. The only point at which it could be suggested there is a difference between the two distributions is in fact at more highly curved points of the surface, rather than in flatter regions. This suggests that - unlike the observations of either van ’t Hag et al. or Baoukina et al. - cholesterol in fact has a preference for more highly curved regions of membranes, rather than flatter ones [244, 245]. This would additionally suggest to the results of Koldsø et al. that it would be membrane proteins, rather than lipids, that have more significant effects on membrane curvature, and that lipids adhere to the protein included in their membrane because there are interaction sites on the transmembrane domain [243].

Regarding the particular discrepancy between the system simulated here and the neutron scattering results of the Squires group, it is not particularly clear as to why the simulation...
results should be in direct opposition. As the work of van ’t Hag et al. demonstrated, investigating curvature effects with neutron scattering is a very challenging task to undertake, and the data must be interpreted carefully. More likely, however, the problems lie with coarse-grained modelling of cholesterol. Issues with the topology of cholesterol as modelled in version 2 of the Martini force field have been frequently noted as a direct result of its aromatic rings and non-planar structure [246, 247]. Future improvements in the coarse-grained modelling of both monoolein and cholesterol in version 3 of the Martini force field will likely make improvements in understanding how curvature can effect the behaviour of both lipids and proteins.

5.7 Conclusions

In this chapter, we have explored how molecular dynamics models of monoolein mesophases change with time, solvent conditions, and the addition of dopants. We have developed a method by which to dynamically investigate the cubic-sponge transition, and shown that the $L_3$ mesophase forms very quickly as a result. Over the course of the entire $1 \mu s$ simulation, our results show that the $L_3$ mesophase exhibits a minimal surface, with a reduction in the elastic moduli compared to the $Q_{II}$ mesophase it evolved from.

Although the models here have provided some useful insight into the structure and dynamics of the $L_3$ mesophase, their limitations should be noted. First and foremost, the transition produces a single water channel in the developed mesophase system, while we should expect bicontinuity to be retained of the $L_3$ mesophase. Because of the timescale of evolution, it has not been possible to track how the initially stable bicontinuous $Q_{II}$ mesophase ruptures and leaves a single water channel. It is not presently clear how this could be overcome. However, it may in future be worth pursuing more atomistic models of both $Q_{II}$ and $L_3$ mesophases, by using united atom topologies of monoolein and butanediol. These should enable faster temporal resolution and detail on the nature of the mesophase transition and associated molecular rearrangements.

Secondly, the reduction in the partition coefficient upon the introduction of cholesterol to the membrane provides a constraint on the significance of the interpretation of the results. Although the systems still exhibit the same structural properties (in terms of curvature distributions measured) as their undoped counterparts, it is likely that the increased preference of the diol molecules for the water domains of the system has had created a systematic error on
the physical measurements made, which cannot be determined without making fundamental changes to the model. These errors further the needed for greater resolution studies on the L$_3$ mesophase in future. At the very least, the methods used here and previously validated in chapter 4 will provide a basis for constructing these simulations.

Regarding efforts to measure the curvature preference of molecules, the simulations do not apparently match experimental results - but do agree with some previous in silico. One explanation for this could be imperfections in the Martini model of cholesterol. Daily et al. have shown that the default Martini model in v2 of the force field suffers from several imperfections that can be improved on [246]. Preliminary studies by other members of our group have shown that using other models of cholesterol can have an effect on the distribution of cholesterol with respect to Gaussian curvature in the Q$_{D_{11}}$ mesophase, which may further shed light on this issue in future. Additionally, future improvements in the coarse-graining of cholesterol in version 3 of the Martini force field, now that ring-types can be modelled in finer detail, should be able to better model the dynamics of cholesterol in highly curved environments, and clarify which way round the curvature sorting mechanism in membranes functions.
POSSIBLE CONFINEMENT EFFECTS ON THE STRUCTURE OF THE LIPID SPONGE PHASE

6.1 Context

Throughout this thesis, we have investigated the structure of the $L_3$ mesophase with reference to its model as being based on a ‘random’ surface. One can reasonably ask of this assumption what we precisely mean by a ‘random’ surface: what makes a surface ‘random’ as opposed to ordered, and what dictates the degree of so-called ‘randomness’ therein?

The modelling of Schwarz and Gompper suggests that Gaussian random fields may provide an answer as to the structure of the underlying surface, whereby space is described by a series of convoluted Gaussian distributions [241, 248]. This then leads us to the question, what is the degree of relation between the $L_3$ and associated bicontinuous lipid mesophases? That is, is there a quasi-epitaxial relation between them? If so, does that control the ‘randomness’ of the surface? After all, Porcar et al. demonstrated the extent to which surfactant $L_3$ mesophases are sensitive to shear, such that on contact with a surface, the system will undergo a transition to the $L_a$ mesophase [249]. Surfactant $L_3$ mesophases, however, do not exhibit any clear relation to a $Q_{II}^D$ mesophase, and so cannot inform us of this relationship. As the $L_3$ mesophase forms an important intermediary structure in the $Q_{II}^D/L_a$ transition, information about structural relationships to either mesophase will significantly elucidate the mechanism of this transition.
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[250].

The work in this chapter therefore investigates the effect of interfaces on the lipid L$_3$ mesophase with a view to better understanding possible confinement effects on changing their structure. Firstly, we investigate this possible structure using cryogenic transmission electron microscopy (cryo-TEM). Secondly, we use acoustic levitation to levitate small droplets of L$_3$ mesophase, and conduct microfocus synchrotron SAXS experiments to probe their interfacial structure.

6.2 Cryo-TEM

As a tool for investigating lipid mesophases, cryo-TEM has mostly been used to probe the structure of lipid nanoparticle dispersions formed by the presence of pluronics, principally motivated by drug delivery applications, or as a real-space complement to SAXS data [119, 251, 252]. However, recent work of Tran et al. has demonstrated that it is also possible to use the technique to probe the nature of a L$_{\alpha}$/Q$_{II}$ mesophase transition, showing evidence for the formation of inter-membrane stalks during the transition [236].

I would like to acknowledge the assistance and generosity of Dr. Rachael Xerri, University of Nottingham, in undertaking these measurements.

6.2.1 Experimental setup and analysis

Holey carbon grids (Agar Scientific, U.K.) were prepared via plasma discharge (Fishione, Model 1020 Plasma Cleaner) for 10 seconds at 5 mA. Grids were loaded with 3 $\mu$l of sample. The sample was then incubated in a humidity-controlled chamber for 1 minute prior to being blotted and freeze plunged in liquid ethane using the Gatan cryo-plunge 3 system. Samples were then stored in liquid nitrogen or immediately transferred to the microscope for analysis.

Sample grids were loaded into a Gatan 626 cryo-TEM holder under liquid nitrogen. The samples were analysed under cryogenic conditions at around -180°C using a US1000 CCD camera and Digital Micrograph GMS 3 operating software on a JEOL 2100Plus instrument at 200 kV. Micrographs were captured at exposure times of 2–64 seconds at doses below 10 e/A$^2$.

Image enhancement for brightness and contrast, and FFTs were done using ImageJ software. FFT analysis was performed using custom scripts written in Python.
6.2.2 Results

The principle challenge for performing cryo-TEM measurements was the bulk nature of the sample. This resulted in an unusually thick sample, presenting a challenge for stable measurement at high resolution. The sample with the clearest results was a 2.5% mol DOPC-doped L$_3$ mesophase, formed using a solvent of 40% v/v butanediol.

Analysis of two areas of this sample are shown in the micrographs of fig. 6.1. Both micrographs observed show a ‘random’ network of lipid membrane throughout, as would be expected for the L$_3$ mesophase. However, it is interesting that this persists even within the thin sample that was formed on the microscope grid, which suggests that the mesophase behaviour has been mostly unchanged by the sample preparation process.

The most notable results are shown in the fast Fourier transforms (FFTs) of the micrographs. As these characterise the reciprocal space structure of the sample image, they should be expected to resemble the characteristic broad ring that we see in SAXS measurements of L$_3$ mesophases. However, if we look at the angular distribution of the FFT intensity, a very different pattern emerges, showing that the broad ring is not completely isotropic. This suggests that on these shorter length scales, the structure of the membrane may in fact be spatially correlated with translational symmetry, and so the surface describing the underlying structure of the L$_3$ mesophase may not be as ‘random’ as previously thought.

This naturally leads to the question as to how the L$_3$ mesophase may be examined to investigate the potential effect of confinement. The samples distributed over microscope grids are necessarily thin. This result suggests that the bulk structure usually measured by SAXS experiments in capillaries (eg. those throughout chapter 3) across billions of L$_3$ mesophase domains may not necessarily reflect the underlying local structure, which could be more closely related to another mesophase than previously thought.

6.3 SAXS investigations of levitated droplets of lipid systems

The results of section 6.2 suggest that on small scales, the surface describing the L$_3$ mesophase may not be as random as previously assumed, exhibiting signs that it may have directional order. To further investigate this topic, we undertook a synchrotron experiment to examine potential orientation effects in the mesophase. This experiment was awarded beamtime at the
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Figure 6.1. Cryo-TEM images of a L₃ mesophase and FFT analysis. Both images a) and b) are from a sample of 2.5%. For each subfigure, i) is the micrograph obtained using cryo-TEM, and ii) is its FFT. iii) is a radial integration of the FFT image, showing how the radial intensity profile varies with angle from the centre of the FFT.
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**Figure 6.2.** Experimental setup for levitation experiments. The beam enters the chamber from the right, scatters through the sample in a node of the acoustic levitator, and exits the chamber towards the detector on the left.

The experiment was proposed to build on previous work on ultrasonically levitated self-assembled mesophases by Seddon et al., which demonstrated for the first time that lipid mesophases could be examined using SAXS in a container-free manner [253]. More significantly for this work however, the authors showed that by levitating the droplets, interfacial orientation is observed in the $Q_{III}^D$ mesophase. Additionally, and as exploited in further work, the work observed hydration-dependent core shell effects, observing different mesophases at the edge of the droplet compared to the centre [254].

These results suggested that when levitated, the $L_3$ mesophase might exhibit interesting behaviour. To consider the two central points of the work described above, we can expect the sponge phase to 1) become ‘aligned’ around the edge of a levitated droplet, and 2) exhibit some sort of ‘core shell’ effect, where the centre of the droplet will exhibit one mesophase (ie. a $L_3$ system) and the edge another.
6.3.1 Experimental setup

The beamline was set up to have a 2802.151 mm sample-detector distance, and a beam energy of 14 keV. The beam used was a microfocus X-ray source, with dimensions 0.3 x 0.3 mm. The experimental setup is seen in fig. 6.2.

As described in previous work, the levitator is based on a modified commercial levitator (tec5, Oberursel, Germany) with a fixed transducer frequency of 100 kHz and a variable HF power of 0.65 to 5 W. A concave reflector is mounted on a micrometer screw for adjustment of the reflector–transducer distance. The distance between the transducer front face and the reflector was set to around 26 mm with a maximum distance variation of ±6 mm. The levitator is enclosed in a custom-built flow-through Pyrex environmental chamber fitted with X-ray transparent windows and access ports for relative humidity and temperature measurements as well as gas supply and removal [253, 254].

This experiment did not require direct control over atmospheric conditions in the manner described, so the chamber was completely sealed while SAXS measurements were taking place. Samples were injected into the nodes of the levitator, using the reflector height adjustment to trap a droplet. Samples were prepared in mixtures of sample and ethanol, in order to ease injection. The ethanol evaporated, leaving the sample.

Once the levitated droplet of the desired sample had been formed, scans were taken across the width or height of the droplet to characterise the mesophase in the interfacial and bulk regions. This is illustrated in fig. 6.3, which shows how we undertook either cross-sectional scans across a single axis of the droplet, or a grid scan. Grid scans were challenging to undertake due to spontaneous instabilities in droplet motion, and software connection issues at the beamline. In either a cross sectional or grid scan, the X-ray source is moved through the droplet in discrete steps, to produce a detector image at each step, building up a complete map of the mesophase behaviour.

6.3.2 Initial results

An example of an initial detector map is seen in fig. 6.4. The sample in this case is an undoped L₃ mesophase, prepared in a standard ratio of 40:60 lipid:solvent, where the solvent itself consists of a 40:60 volumetric ratio of butanediol:water. In fig. 6.4, there are two notable features. The first feature to observe is that the sample has dehydrated, such that there is no broad
To measure interfacial effects on the levitated droplet (in blue), we measured either discrete vertical cross-sectional scans (illustrated in red), or discrete grid mapping scans (illustrated in orange).

characteristic $L_3$ mesophase SAXS ring at any point across the droplet. Simultaneously, the relative humidity meter in the levitator chamber recorded a 99.9% relative humidity. Despite this, water was still driven out of the sample when exposed to air. This effectively increases the proportion of butanediol in the solvent of the sample, and so drives it towards flatter mesophases. This therefore explains the $L_\alpha$ mesophase that we observe. Micellar systems were also observed in several other samples, indicating a very strong dehydration effect under these sample conditions.

The second, more interesting feature that we note in fig. 6.4 is that in the interfacial region, the sample exhibits a strong orientation. This is evidenced by the fact that the sharp first order $L_\alpha$ ring seen has bright spots orientated orthogonally to the direction of the interface. In turn, this suggests that the previous interfacial orientation observations of Seddon et al. are genuinely driven by the droplet interface, as opposed to any effect of the levitator [253].

Figure 6.3. Illustration of scanning SAXS measurements on a levitated droplet. To measure interfacial effects on the levitated droplet (in blue), we measured either discrete vertical cross-sectional scans (illustrated in red), or discrete grid mapping scans (illustrated in orange).
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Figure 6.4. Detector image grid scan map of a levitated $L_3$ mesophase. The interfacial regions have been highlighted in red. In the interfacial regions, the parasitic scattering flare is seen orthogonal to the direction of the interface.

6.3.3 Glycerol replacement results

The initial samples measured dehydrated on very rapid timescales, and so it was necessary to consider methods to prevent this. Richardson et al. found that glycerol prevents the dehydration of lipid mesophases, demonstrating that by replacing water in the solvent, the $Q_{11}^D$ mesophase is observed at lower relative humidity than otherwise possible \([255]\). Therefore, investigations proceeded by investigating the effect on the $L_3$ mesophase of replacing water with glycerol.

Here, we will firstly present results demonstrating that the replacement of water with glycerol has no significant effect on the structure of the $L_3$ mesophase. Subsequently, we will present three analysis methods used to investigate interfacial effects on levitated droplets of $L_3$ mesophase systems as this study intended.
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6.3.3.1 Verification of glycerol inclusion

To begin, we replaced half the water by volume in a reference lipid L₃ mesophase with glycerol. The volume ratio of the sample used was 40:24:18:18 monoolein:butanediol:water:glycerol respectively. This sample was then transferred to a capillary, which, as we show in fig. 6.5a), retains a characteristic L₃ mesophase scattering pattern. Secondly, this sample was mixed in a 3:1 ratio of ethanol:sample, and injected into the acoustic levitator. The scattering pattern obtained from the centre of the droplet is shown in fig. 6.5b), which is also a characteristic L₃ mesophase pattern. The peaks of the broad SAXS ring for the two patterns are at \( q = 0.0658 \text{ Å}^{-1} \) and \( q = 0.0697 \text{ Å}^{-1} \) respectively. These results can be compared to the fig. 3.2, where three reference L₃ mesophases had a mean value of the L₃ mesophase peak at \( q = 0.060 \pm 0.002 \text{ Å}^{-1} \). That these peak positions are so similar indicates that the replacement of water with glycerol has no substantial change on the structure of the L₃ mesophase, while also preventing its dehydration.

This result is further confirmed by the cross-sectional scan seen in fig. 6.6, where a L₃ mesophase has been prepared using full glycerol replacement of water (ie. at a volume ratio of 40:24:36 monoolein:butanediol:water). This vertical scan shows that there is a L₃ mesophase throughout the droplet. Throughout the droplet, the broad peak is centred at \( q = 0.0662 \pm 0.0007 \text{Å}^{-1} \).
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Figure 6.6. Vertical cross-sectional scan for a full glycerol exchange for a levitated droplet. The scans are numbered sequentially as an indication of high to low vertical position, such that the blank scans 1, 2, and 50 are outside the droplet, the flared scans 3-6 and 46-49 are at the interface, and the remainder are in the bulk of the sample. Each detector image is separated vertically by 0.02 mm.

Again, this demonstrates little difference with the partial glycerol replacements, and capillary experiments without, confirming that glycerol has no structural effect on the L$_3$ mesophase.

6.3.3.2 Analysis aims

As described earlier, the motivation for this SAXS study was the anisotropy of the L$_3$ mesophase FFT from cryo-TEM investigations, as shown in fig. 6.1. Furthermore, considering previous results demonstrating that acoustically levitating lipid mesophases in this manner creates an interfacial alignment, we can investigate possible physical epitaxy of the L$_3$ mesophase to other ones. Additionally, capillary-based studies of the L$_3$ mesophase create contact birefringence, and so cannot be relied on to conduct interfacial measurements.

In developing analysis methods, we are therefore looking for ways to quantify the degree of anisotropy in the broad L$_3$ mesophase SAXS ring. This measure can subsequently be used to compare the characteristics of the interfacial and bulk regions of a levitated L$_3$ mesophase droplet, to inform us how the structure of the mesophase changes between them. In addition,
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![Image](image1)
(a) The input detector image

![Image](image2)
(b) The re-formed azimuthal difference image of the detector

![Image](image3)
(c) The cake remapped azimuthal difference image

**Figure 6.7.** Illustration of azimuthal difference analysis. The input detector image in a) is processed to show the azimuthal difference image, b). Here, the detector image is a silver behenate calibrant. The difference image is then cake remapped into azimuthal angle vs. $q$, c).

we will look at any difference in characteristic SAXS patterns between these two regions, in order to further inform us of the differences in mesophase behaviour enforced by the properties of the droplet.

### 6.3.3.3 Analysis method 1: Azimuthal Integration Difference

The first method used is based on azimuthal difference analysis, as implemented in Diamond Light Source’s Dawn analysis software, and as illustrated in fig. 6.7 [256, 257]. Azimuthal difference images are generated by firstly reducing a detector image by azimuthally integrating it, then reforming a new ‘detector’-like image from the reduced data. Finally, the difference
FIGURE 6.8. Comparing azimuthal difference cake remapping analysis at the interface and in the bulk. Two detector images - at the interface, a), and in the bulk, c) - undergo azimuthal difference cake remapping analysis, with their maps plotted in b) and d) respectively. The dashed red line in the b) and d) indicate the location of the centre of the broad L$_3$ mesophase peak.
between the reformed image and the original detector image is made. To complete the analysis, we then perform a cake remap of the data to observe the relationship between the azimuthal angle of scattering and the scattering vector, $q$. This process was developed in order to highlight any significant deviation features in detector images, as a useful feature for detector calibration. In the event that a calibration powder ring is not perfectly isotropic, any angular deviations should be highlighted by the reforming process. As the illustration in fig. 6.7 shows, on a calibrated detector, there are no significant features to be highlighted by the azimuthal difference cake remapping process, because the scattering rings on the original detector are perfectly isotropic.

As this analysis method is designed to highlight anomalous anisotropic scattering features, it should be ideal for investigating potential anisotropy at the interface in the $L_3$ mesophase broad scattering ring. A comparison of this analysis is shown in fig. 6.8, where we have taken a detector image from the bulk region and the interfacial region of the same levitated droplet. The cake remapped azimuthal difference images are then plotted. In the interfacial cake remap plot, there is no significant deviation visible in the region around the broad scattering peak, as indicated by the dashed red line. There is more significant difference at very low values of $q$, due to the parasitic interfacial scattering.

The low $q$ difference is not present in the same analysis for the scattering pattern from the bulk of the droplet, further confirming this. Otherwise, there is similarly no clear deviation from a vertical trend in the results measured for the bulk of the droplet. This suggests that there is no substantial difference in the scattering arising from the interface in comparison to the bulk, at least from this analysis. In turn, this suggests that the interface has no substantial effect on the structure of the $L_3$ mesophase in this region.

### 6.3.3.4 Analysis method 2: Direct detector image analysis

Instead of analysing reduced data, we can instead take an approach of analysing the scattering pattern itself, and looking for anisotropy directly. This method is illustrated in fig. 6.9. To begin, the trough in the azimuthally integrated data between the direct beam and the broad $L_3$ mesophase scattering peak is found, as shown in fig. 6.9c). This is done in order to increase the size of the mask around the beamstop, as seen in fig. 6.9a) and b). Once this mask has been created, points on the detector image are identified with a normalised intensity over a
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Figure 6.9. 2d analysis of detector images. a) shows the raw detector image with red points identified as having a normalised intensity over a threshold of 0.7; b) shows a gaussian-blurred detector image, again with points identified as having a normalised intensity greater than a threshold of 0.7. In both a) and b), the white ellipse has been fitted to the coordinates of the red points. c) shows the azimuthally integrated 1d scattering pattern. The dashed red line identifies the trough below the broad L₃ scattering peak, which defines the masking radius for the analysis in a) and b).

For this purpose, two images are in fact used. Firstly, the direct image as measured, shown in fig. 6.9a), and secondly, an image with a Gaussian blur filter applied to it, shown in fig. 6.9b).

The filter was applied to spread the intensity among neighbouring pixels of the image. This was deemed necessary after finding a significant dearth of points with intensities above the threshold for the raw detector image, as illustrated in fig. 6.9a).

Once the detector coordinates of pixels had been determined, they were fitted to an ellipse. We can therefore measure the eccentricity of the ellipse to investigate the anisotropy of the L₃ mesophase scattering ring both in the bulk of the levitated droplet and at its interface. For an ellipse described by \( \frac{x^2}{a^2} + \frac{y^2}{b^2} = 1 \), the eccentricity, \( e \), is defined as:

\[
e = \sqrt{1 - \left(\frac{b}{a}\right)^2}
\]  

That is, the eccentricity measures the difference between the two axes of the ellipse. For a circle, \( a = b \), and so \( e = 0 \).

Results summarised across a number of droplets are shown in fig. 6.10. These results demonstrate significant distinctions between the interfacial and bulk regions, as well as between the
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**Figure 6.10.** Results of direct detector image analysis. a) and b) show respectively the distributions of eccentricity measurements and their means in the bulk of levitated droplets. c) shows a comparison of the mean eccentricities from the 4 measurements, showing the difference between the two types of detector images at either the interface or bulk of the droplet.

**Figure 6.11.** Illustration of poor detector image fitting. At the interface, it is not systematically possible to distinguish the broad L₃ mesophase peak from the scattering flare, so many of the interfacial regions have extreme eccentricity fits.
two types of detector images used. For both methods used, it is clear that there is a significant reduction in the eccentricity of the fitted ellipse in the bulk of the droplet in comparison to the interface. This difference is far more significant in measurements using a processed detector image, with gaussian blurring, in comparison to the raw intensities as measured. Although this would appear to be a confirmatory result, inspection of many of frames fitted around the interfacial regions, such as that shown in fig. 6.11 shows that in many cases, the data being fitted are not of the broad L₃ mesophase peak itself. Instead, the points which surpass the normalised intensity threshold are found on the parasitic scattering flare. As these are separated by an angle of 180°, an extremely narrow ellipse is fitted, which suggests that the large eccentricities found in interfacial regions shown in fig. 6.11b) and c) are not at all representative of the broad L₃ mesophase peak.

6.3.3.5 Analysis method 3: 1d mapping

The first and second methods used in this analysis are evidently inconclusive, and do not clearly find any significant core shell effect between the interface and bulk regions of the droplets. Both method 1 and method 2 are based on analysing the data using 2d methods, which naturally leads to the question: could a 1d analysis method provide insight into a potential changing structure of the levitated L₃ mesophase instead?

Full mapping scans of levitated droplets were challenging to undertake due to temporal instabilities in the droplet, and limited time allocation for the experiment overall. However, one long-term stable droplet was formed from a sample of 2.5% mol DDM with a solvent consisting of 40:60 volume/volume butanediol:glycerol. This allowed an extensive SAXS map of the droplet to be created, shown fully in fig. 6.12a), and a highlight of which is shown in closer detail in fig. 6.12b). In the highlight around one area of the interface, we can see that in the bulk region, we observe a ‘normal’ broad L₃ mesophase SAXS ring, while at the interface, this is overlaid with a parasitic interfacial scattering flare. More than any other scan, this droplet map can be used to inform us of the differences in structure between the L₃ mesophase at the interface and at the bulk, using a 1 dimensional analysis.

Using our earlier results where we demonstrated that interfacial ordering is really an effect of the interface, rather than of levitation air pressure, if the L₃ mesophase is anisotropic at the interface, the peak profile should be different in the direction tangential to the interface.
Figure 6.12. Map of detector images around a droplet interface. The physical distance between frames is 0.05 mm in both directions. The inset figure a) is the full measured map of the droplet, a highlight of which is shown in full in b). In b), the exterior of the droplet is in the upper left hand corner of the array of detector images, and the interior is seen in the lower right. The interfacial region is in the darker band across the middle. The characteristic broad rings of the L3 mesophase can be seen in the interior of the droplet. In the interfacial region, parasitic scattering flares are seen orthogonally to the direction of the interface.
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Figure 6.13. Example radial intensity profile at the droplet interface. The data have been integrated in a $q$ range of 0.02 - 0.05 Å$^{-1}$. This range was chosen as the L$_3$ mesophase peak is at around 0.06 Å$^{-1}$, so the intensity profile will be dominated by any parasitic scattering peaks in this range. The azimuthal angle is defined clockwise from $0^\circ$ = right, as illustrated in fig. 6.14a).

Therefore, by using the segments as shown in fig. 6.14a), we can measure the peak profile of the segment, and compare it to what we know must by anisotropic broad scattering rings in the bulk of the levitated droplet. To fit the peaks, as illustrated in fig. 6.14c) and d), the LMFIT Python library was used to generate a model of a Voigt peak with a linear background, as described in section 2.3.

The algorithm to undertake this analysis is then as follows:

1. For every detector image measured, find sharp peaks in radially integrated (intensity vs. azimuthal angle) data, as illustrated in fig. 6.13.

2. If two distinct peaks are found, and they are separated by $180^\circ$, then that detector image can be said to be in the interfacial region of the droplet.

3. For all interfacial measurements:
   a) Determine the direction of the flare.
   b) Find a $90^\circ$ segment approximately orthogonal to the flare, as illustrated in fig. 6.14a).
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**Figure 6.14.** Illustration of the partial integration method for interfacial detector images. a) In the interface, integrate a 90° segment in between the parasitic scattering flare. b) In the bulk of the droplet, use the full azimuthal integration around the broad L₃ mesophase peak. The corresponding fitted scattering patterns for a) and b) are shown in c) and d) respectively.

- Use azimuthally integrated (Intensity vs. \( q \)) data from that segment to perform a broad peak fit, as illustrated in fig. 6.14c).

4. For all bulk droplet measurements, fit azimuthally integrated data from a full \( 2\pi \) integration, as shown in fig. 6.14b) and d).

This analysis method is based on the premise that the parasitic scattering flare at the interface will interfere with the azimuthally integrated profile if the segment angle is \( 2\pi \) (i.e. the whole way around the detector). If the segment is instead positioned orthogonally to the direction of the flare as illustrated in fig. 6.14a), we should expect that the scattering from the L₃ mesophase in the interfacial region is otherwise undisturbed. To do this, we can use peak centre and full
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Figure 6.15. Results for peak width and position analysis 1d mapping analysis. a) shows histograms are for the position of the centre of the fitted peak, and b) compares the mean of the two distributions. c) shows histograms for the FWHMs of the fitted peak, and d) shows a comparison bar chart for the FWHM distributions.

width half maximum (FWHM) of the fitted peak to ascertain whether there is anisotropy in the broad L₃ mesophase SAXS pattern. Note that in point 3b) above, we cannot find a directly orthogonal segment for every single flare because of the low-throughput nature of this data processing in the Diamond Light Source Dawn analysis software [256, 257]. Therefore, 90° segments were used at intervals of 45° from 45° to 315°.

The results of this analysis are shown in fig. 6.15. The key results are the comparisons between the bulk and interfacial measurements in fig. 6.15b) and d), which show the mean of the distributions for the centre of the Voigt peak, and the full width half maximums respectively.

The comparison chart in fig. 6.15b) shows that there is very little difference in the position of the peaks in the bulk and at the interface, with average positions of $q = 0.066 \pm 0.001 \text{ Å}^{-1}$ and
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\( q = 0.064 \pm 0.001 \text{ Å}^{-1} \) respectively. On the scale of \( L_3 \) mesophases, this represents remarkably little difference between the two regions of the levitated droplet: recall that in fig. 3.2, we saw a greater - but nonetheless insignificant - variation in correlation lengths for systems of identical lyotropes.

While the peak position remains the same, there is a more marked difference between the mean of the distributions for the full width half maximums, seen in fig. 6.15d). Although there is a significant uncertainty overlap between the two distributions, these results suggest that it is possible that the \( L_3 \) scattering rings are systematically broader than measurements made in the bulk region of the droplet.

From these measurements, it is possible to conclude one of two situations. Firstly, it could be possible that the measurements made in the interfacial region are broader all the way around, which would suggest that at the interface there is a greater distribution of correlation lengths in the \( L_3 \) mesophase. Secondly, it could be possible that a broader scattering peak exists only in the region orthogonal to the parasitic flare. This would suggest that there was in fact a degree of ordering in the \( L_3 \) mesophase as a result of interfacial forces.

6.3.4 Levitation investigations summary

While there was strong motivation to undertake microfocus SAXS experiments on levitated droplets of lipid \( L_3 \) mesophases based on electron microscopy studies, the results presented throughout section 6.3.3 do not corroborate any such confinement effects. We have developed several methods of analysis to investigate the effects proposed, but have been unable to unambiguously conclude that the \( L_3 \) mesophase has any significant ordering imposed on it by the presence of an interface.

The method that demonstrates the most potential for future use is certainly the 1 dimensional direction reduction presented in section 6.3.3.5. However, we are unable to draw any firm conclusions from this, as a result of the fundamental inability to draw any conclusions about the form of the broad scattering ring in the direction parallel to the parasitic scattering flare. Considering that this is the principle barrier to full utilisation of this method, it may be possible in future, with a greater understanding about the behaviour of the flare, to perform a masking procedure in order to refine this method.
6.4 Conclusions

In this chapter, we have explored how the structure of the \( L_3 \) mesophase is affected by physical confinement and interfaces. Firstly, we used cryo-TEM to investigate the \( L_3 \) mesophase using a real-space imaging technique, and found indications that the system may be more spatially correlated than previously anticipated. This motivated a synchrotron SAXS investigation using acoustically levitated droplets of \( L_3 \) mesophases. Combining SAXS and acoustic levitation was proposed as a way to probe the interfacial effects using a known structural method, to examine possible relationships with other mesophases.

In contrast to the anticipated results of the experiment, there is no obvious effect of confinement on the structure of the \( L_3 \) mesophase. None of the three analysis methods developed in section 6.3.3 can be said to conclusively demonstrate the sort of alignment that we observed in the micrographs shown in fig. 6.1. However, the final method developed suggests that the effect of the interface may be to broaden the correlation peak of the \( L_3 \) mesophase. While this could suggest alignment, the complementary result - the structure of the peak in the direction of a parasitic scattering flare - is presently unavailable to us.

While the work in this chapter may be inconclusive, future investigations in this area may shed further light on the effect of the interface on the \( L_3 \) mesophase. Such investigations can certainly build on the knowledge of glycerol replacement for preventing dehydration in the \( L_3 \) mesophase, and our demonstration that cryo-TEM can be used as a tool for structural investigations of the \( L_3 \) mesophase. On this latter point, it may be advantageous in future to develop discrete \( L_3 \) mesophase nanoparticles - such as those used by Chen et al. or Valldeperas et al. - to facilitate higher quality micrographs of the \( L_3 \) mesophase [120, 258]. This could be further complemented by extending the technique to cryo-electron tomography studies of the sort undertaken by [259], who developed techniques to recreate the internal structure of the \( Q^{D}_{II} \) mesophase [259].
7.1 Thesis summary

This thesis has used SAXS and molecular dynamics to investigate the dynamics of the lipid L₃ mesophase. Here, we will briefly re-summarise the key results from each of the chapters, present some preliminary data from two more techniques, and outline some possible future directions for this area of investigation.

In chapter 3, we conducted the first systematic study into type-doping of the L₃ phase, showing that - in comparison to cubic mesophases - molecular packing can have significant adverse effects on the self-assembled mesophase sequence of the L₃ mesophase. Perhaps most importantly, we showed that cholesterol can provide a stabilising effect to accommodate more destructive electrostatic forces to facilitate a significant increase in size of the correlation length. As a sole dopant to the lipid L₃ mesophase, cholesterol changed both the position of Q₁₁₁/L₃ mesophase boundary, and the correlation lengths of the L₃ mesophase subsequently obtained. The addition of cholesterol required more butanediol to be added to the system before the mesophase transition was observed, indicative of the stiffening effect it has on membranes.

On the other hand, the effect of a detergent DDM outright prevented long-scale self-assembly even at low dopant proportions, which is a significant finding for LCP membrane protein crystallisation applications of lipid L₃ mesophases.

Other lipids (namely the phospholipids DOPC and DOPE) were also successfully incorporated
into the L$_3$ mesophase, demonstrating that it is significantly more adaptable than previously anticipated.

Our experimental work was subsequently complemented in chapters 4 and 5 by molecular dynamics studies of how the L$_3$ mesophase can evolve from the Q$_{11}^{D}$ mesophase. This work showed that at a coarse-grained level of study, the transition occurs very rapidly. At equilibrium timescales, our results show that the L$_3$ mesophase forms as a result of the lowering of membrane elasticity driven by the partitioning effect of diols between the aqueous and lipid regions of the system. This work was furthered through the addition of cholesterol to the membrane. Doping with cholesterol slightly increased the elastic moduli of the systems, which goes some way to explaining the shift in mesophase boundaries observed in chapter 3. On shorter timescales, the transition was shown to occur homogeneously across the mesophase unit cell, with no particular preference for Gaussian curvature flattening. Additionally, rapid timescale investigations demonstrated that the principal driver for the transition was a result of the increase in interfacial area, as driven by the partitioning of diols between the aqueous and lipid regions of the system.

Future optimisation of coarse-grained force fields for molecular dynamics - and with particular consideration to the challenges of accurately modelling cholesterol - should provide further clarity on the behaviour of lipids in complex or curved membrane environments. This is especially pertinent for the final question investigated in chapter 5 exploring the Gaussian curvature preference of the lipid, which under current models, seems to contradict the experiments which inspired the investigation.

The final experimental exploration in chapter 6 considered the effect of interfaces on the L$_3$ mesophase. While overall, the cryo-TEM and acoustic levitation SAXS experiments proved inconclusive, they have hinted at interfacial effects not previously observed. A slight broadening of the SAXS peak in the interfacial regions of levitated droplets could suggest with further investigation that the interface may have an aligning effect on the mesophase, but this cannot yet be determined categorically. The preliminary cryo-TEM investigations using the L$_3$ mesophase in bulk demonstrate that it could be a powerful real-space method to use in future characterisation studies for L$_3$ mesophase systems. However, future improvements in investigating the L$_3$ mesophase using real space imaging could almost certainly be found through study the internal structure of nanoparticle dispersions of the L$_3$ mesophase.
7.2 Two preliminary studies

In addition to the SAXS and molecular dynamics work discussed throughout this thesis, two further methods not previously used to investigate the structure of the $L_3$ mesophase. These were pressure-surface area isotherm measurements of monolayers, and NMR.

7.2.1 Langmuir monolayers

Throughout this thesis, we have relied on butanediol as an additive to form the $L_3$ mesophase. This follows in the work of Cherezov et al., who characterised the effect of a number of additives in this way [52]. The additives used in their study fall into two broad categories: small amphiphiles, which partition themselves between lipid and aqueous domains as discussed, and a salt, KSCN. In particular, they find that adding KSCN at concentrations between 0.6 and 2.2 M to monoolein results in a $L_3$ mesophase. The mechanism proposed for this is a so-called 'chaotropic' one, whereby the salt ions weaken the structure of water in the interfacial region of the membrane because they are themselves weakly hydrated.

Although this is a reasonable explanation, it has not been confirmed by any experimental data to date. Moreover, the precise nature of ion effects on membranes remains a topic of outstanding debate, so it is not immediately obvious that it should necessarily be the case [260–262]. Fundamentally, there remains a question as to why a small inorganic molecule has the same effect on a lipid membrane as an amphiphile.

In order to further our understanding of this point, we undertook preliminary measurements of area-pressure isotherms of a monoolein monolayer on water and KSCN subphases using a Langmuir trough. There measurements were used to propose an X-ray reflectivity study, which would be able to further probe the ionic structure around the monolayer/subphase interface. The successful proposal has been scheduled for outside the scope of this thesis, so full results will be forthcoming. Using similar techniques Hallett et al. showed that for thermotropic liquid crystals, a Stern layer forms at the interface, but with significant ion-specific and pressure dependent effects [263]. This suggests in turn that we could expect similar behaviour regarding ionic effects on the structure of monoolein monolayers.

Monoolein monolayers were spread from chloroform solutions on water and 50 mM KSCN subphases in a Langmuir trough (KSV Nima). The monolayers were spread with the barrier
open, at low pressure/high area. The barrier was closed, and the pressure-area isotherm was measured.

The results of these isotherms are seen in fig. 7.1. The primary result from these data is the point of monolayer collapse, ie. the point at which the monolayer buckles from pressure. We can see in fig. 7.1 that with the addition of just 50 mM KSCN, there is a significant increase in the area of the monolayer before this happens. This suggests that the area per molecule within the monolayer has increased as a result of the KSCN. In addition, the pressure/area derivative appears to be markedly different, which could suggest further interaction of the subphase with the monolayer interfacial region. Together, these suggest that there is extensive ionic interaction with monoolein, which may be further elucidated in future by X-ray reflectivity.

7.2.2 NMR

I would like to acknowledge the assistance and generosity of Professor Craig Butts and Paul Lawrence of the Bristol NMR facility in undertaking these measurements.

In the context of lipid mesophases, NMR can be used to study the behaviour of molecules within the membrane. For example, Boyle-Roden et al. first studied monoolein cubic phases using conventional $^1$H NMR to show that it can be used to study the effect of additives to the cubic phase, showing that L-tryptophan is likely to be partially inserted into the membrane when added to the system [264]. As we have highlighted repeatedly through this work, Even-
bratt et al. also used $^1$H to conduct a similar investigation into the mechanism through which pentanediol forms the L$_3$ mesophase in monoolein-based systems, showing that it must similarly partition itself between the lipid and aqueous domains [55]. One further notable result is that of Yang et al., who demonstrated that it was possible to use $^{31}$P NMR to distinguish between the Q$_{II}^{D}$, Q$_{II}^{P}$, and Q$_{II}^{G}$ mesophases [265].

Regarding the L$_3$ mesophase, it was thought that NMR may also be able to clarify the mechanism by which KSCN drives the formation of the mesophase out of the Q$_{II}^{D}$ mesophase. Nilsson et al. previously used $^1$H NMR to study liquid crystal clouding points, showing that the clouding point is driven by changes in head group conformation, and which was particularly driven by the adsorbance of additives to the liquid crystal/water system [266]. Using NMR, we could therefore investigate the effect of KSCN on monoolein headgroup conformation in both the Q$_{II}^{D}$ and L$_3$ mesophases, by varying its concentration.

Samples were prepared directly in 5mm NMR tubes (Fisher Scientific), and $^{13}$C spectra were measured using a Brucker Avance III HD 500 NMR spectrometer. $^{13}$C spectra were measured in order to firstly investigate any structural change in the headgroup carbons, and secondly, to investigate the behaviour of KSCN, which would not be detectable using $^1$H NMR. Four concentrations of KSCN were used to vary the mesophase behaviour of the system. The four concentrations used reflect the concentration-dependent mesophase sequence, as determined by Cherezov et al. [52]. In D$_2$O, the sample remains a Q$_{II}^{D}$ mesophase. At 0.5 M KSCN, the lattice parameter of the Q$_{II}^{D}$ mesophase will have increased due to swelling. At 1 M KSCN, the sample is around the boundary of the Q$_{II}^{D}$/L$_3$ mesophase transition, and will have undergone at full transition at 1.5 M.

The spectra obtained for the four concentrations of KSCN are shown in fig. 7.2. Overall, there is little evidence in the spectra measured to suggest any significant change in the conformation of the monoolein headgroups. There are no significant shifts in the positions of any of the peaks to suggest a significant structural rearrangement upon the addition of KSCN, particularly across the mesophase boundary. One of the principal challenges for these experiments was sufficient and consistent sample preparation, because the volume required by the NMR probe was significant. This inevitably led to sample inconsistencies, which likely did not contribute to clear spectra (eg. we cannot confidently attribute the small degree of peak splitting at 0.5 M KSCN is not to the precise sample description as a result of inhomogeneous samples).
With a particular focus on improvements in sample preparation methods, future work could pursue NMR techniques further to improve the use of this technique and our understanding of membrane-additive interactions. This work would be advised to include utilisation of other active nuclei - for example $^{31}$P through phospholipid doping - in order to build up a more complete picture of these interactions.

### 7.3 Outlook

More than a century has now passed since D’Arcy Wentworth Thompson first published *On Growth and Form*, showing us how a multitude of natural phenomena - from the hexagonal arrangement of beehives to the logarithmic spirals in snail shells - are underpinned by mathematical descriptions [267]. The continuing marvel of lipid polymorphism is that it demonstrates the use of such descriptions on a remarkably small scale, continuing to demonstrate how many biological phenomena are driven by geometrical considerations and constrictions.

While this work has not explicitly explored some of the more involved mathematical models for
7.3. OUTLOOK

the \( L_3 \) mesophase, we have demonstrated through a variety of techniques that it remains an experimentally poorly understood lipid mesophase. In this sense, the most pertinent question we have of the \( L_3 \) phase is a complete description of the underlying surface, and the extent of its so-called ‘randomness’. The two most powerful methods that could be used to tackle this method are almost certainly small angle scattering investigations, and molecular dynamics. In the case of the first, neutron scattering and contrast variation may provide a way to model the water channels of the \( L_3 \) mesophase in a way that has not previously been done. Regarding the latter, atomistic-level molecular dynamics would provide the finest detail yet on the interaction of \( L_3 \) mesophase-forming diols with lipid membranes, which can build on the work undertaken here in chapters 4 and 5. Increased resolution of highly curved membrane systems will also be fertile ground to further study the question of molecular curvature preference.

Turning to potential applications, the most obvious open study from this thesis is a systematic investigation into the use of the lipid \( L_3 \) mesophase to crystallise membrane proteins. Trials could be conducted based on our understanding of additives from chapter 3 using known \( L_3 \) forming formulations, and comparing crystallisation success between them and conditions where the \( L_3 \) mesophase is known not to form.

Ultimately, the work in this thesis has shown that the formation of the lipid \( L_3 \) mesophase is both relatively rare and complex. Moreover, methods by which to study them to provide detailed structural information are presently extremely limited, and the primary focus of any future work in this area will necessarily need to confront that challenge.
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