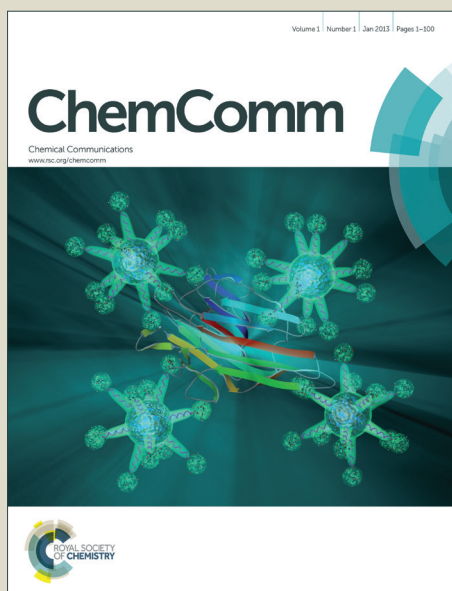


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## Glycerol Prevents Dehydration in Lipid Cubic Phases

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**Lipid cubic phase samples dry out and undergo phase transitions when exposed to air. We demonstrate experimentally and theoretically that adding glycerol controllably lowers the humidity at which cubic phases form. These results broaden the potential applications of cubic phases and open up the potential of a new humidity-responsive nanomaterial.**

Inverse bicontinuous cubic phases ( $Q_{II}$ ) are complex three-dimensional nano-structures formed by self-assembling amphiphilic lipids<sup>1</sup>.  $Q_{II}$  phases are found in three distinct morphologies:  $Q_{II}^G$  (gyroid, space group Ia3d),  $Q_{II}^D$  (double diamond, space group Pn3m) and  $Q_{II}^P$  (primitive, space group Im3m) all consisting of two intertwined water channels separated by a single continuous lipid bilayer. This unique geometry has led to many applications such as the crystallization of membrane proteins<sup>2,3</sup>, drug delivery<sup>4</sup>, sensors<sup>5</sup>, ion conductivity media for batteries and fuel cells<sup>6</sup> and in the templating of nanostructured metals<sup>7</sup>. Typically they are studied either at a fixed water:lipid ratio in a sealed cell<sup>8,9</sup> or else dispersed or submerged in aqueous surroundings<sup>10–13</sup> where they adopt an “excess water” structure, usually a  $Q_{II}^D$  phase<sup>8,9,14</sup>. This is because in an open environment (i.e. in contact with air or other gases) dehydration will reduce the lattice parameter and ultimately cause phase transitions from  $Q_{II}^D$  to  $Q_{II}^G$  and then to lower hydration structures such as the fluid lamellar ( $L_\alpha$ ) or the inverse micellar ( $L_{II}$ )<sup>12</sup>. Maintaining sample hydration is also an issue for existing procedures and applications using  $Q_{II}$  phases, such as membrane protein crystallization and crystallography, especially using the recently developed serial femtosecond approach<sup>15</sup>, topical drug delivery<sup>16</sup>, ion conductance<sup>6</sup> and sample preparation for cryoTEM<sup>17</sup>.

Several groups have reported studies of  $Q_{II}$  phases in a controlled humidity gaseous environment assessing the structure optically<sup>18</sup> as well as by X-ray scattering<sup>6,12</sup> on micron sized droplets or thin films. In these experiments humidity provides precise

control over the lattice parameter and lipid mesophase, allowing for both kinetic and equilibrium properties of the  $Q_{II}$  phases to be studied. The main disadvantage of using humidity control is that  $Q_{II}$  phases require a high relative humidity (RH) in order to remain stable; for example above 90% is needed in monoolein<sup>12</sup>, which is difficult to achieve and measure practically<sup>12,19</sup>.

In this work we demonstrate that the addition of glycerol to thin lipid films will produce samples that spontaneously adopt and maintain cubic phase nanostructures, when exposed to RH values consistent with typical laboratory or outdoor values. Without significantly affecting the cubic phase structure, we were able to maintain a  $Q_{II}$  phase down to RH = 16%, which allows investigation at a more practically manageable level of humidity in a laboratory, as well as being of use for other applications requiring a  $Q_{II}$  phase in an open environment at a typical ambient humidity.

A further value of our work lies in the production of a new class of smart responsive material. Responsive systems have already been reported where the formation and/or lattice parameter of a cubic phase responds to changes in ionic strength<sup>20</sup>, pH<sup>21</sup>, temperature<sup>22–24</sup> and enzymatic lipolysis<sup>25</sup>. These differences themselves change the diffusion properties, and the previous examples were investigated for applications in controlled or triggered drug release. Our work demonstrates a new class of responsive cubic phase where it is humidity that dictates cubic phase formation, as we can control the lower humidity threshold at which cubic phases form by varying glycerol content; we also show that we can model this relationship accurately. This potential, leads to tunable humidity-responsive materials with applications in triggered aroma release<sup>26,27</sup> or sensors.

Thin phytantriol/glycerol and monoolein/glycerol films (0.2 – 1.5  $\mu$ m) were applied to hydrophobic silicon wafer substrate by spin coating. Grazing-incidence small-angle X-ray scattering (GISAXS) was used to investigate the lipid/glycerol films in a controlled-humidity helium environment, full experimental details are given in SI1. This report mainly focuses on the lipid phytantriol with some additional measurements performed upon Rylo. Both phytantriol and Rylo are type II lipids featuring rich polymorphic behavior including the  $Q_{II}^G$  and  $Q_{II}^D$  phases, with the  $Q_{II}^D$  phase

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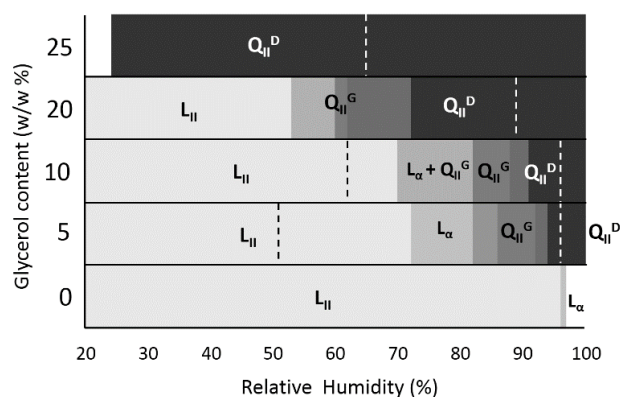
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Electronic Supplementary Information (ESI) available: Ellipsometry data, additional GISAXS data and calculation of humidity phase boundaries. See

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coexisting with excess water, at water contents above saturation<sup>8,14</sup>.

GISAXS images of lipid/glycerol films were taken in series as the humidity was increased. Using YAX<sup>28,29</sup>, custom imageJ macros, the data were reduced and intensity profiles were plotted as stacked scatter plots with the y-axis intercept set to be proportional to the RH as shown in Figure SI3A. This allowed for the emergence of the  $Q_{II}^G$  and  $Q_{II}^D$  phases to be clearly observed. The 2D images featured a relatively low level of mosaicity, indicating well orientated lipid films, which has been discussed in a previous paper<sup>30</sup>. This meant that the diffraction arcs from the  $L_\alpha$  phase were difficult to include in the 1D data without also including the strong on-axis reflection from the main beam. Due to this, the appearance of the lamellar phase was deduced purely from the 2D images as shown in Figure SI3B. A summary of where each phase was observed with respect to RH is shown in Figure 1 with the exact values given in Table 1. The results clearly show that the appearance of each mesophase is seen to occur at lower RH values with increased glycerol content. Of particular note is phytantriol with 25% (w/w) glycerol where the  $Q_{II}^D$  phase was observed across the entire measured humidity range down to RH = 24%. Measurements were stopped during hydration when the lattice parameter of the  $Q_{II}^D$  phase appeared to be saturated (marked by a white dotted line) as no further phase change can be expected according to published phytantriol/water phase diagrams<sup>14</sup> or the maximum achievable humidity (97%) was reached. The black dotted line on 5% and 10% glycerol also indicates where the controlled humidity measurement was halted; the location of the  $L_{II}$  phase below this was extrapolated from phase measurements upon bulk phytantriol/glycerol which is assumed to be equivalent to a film at RH = 0%; see SI4 for details. The order in which phases appear on increasing RH agrees with previously published data<sup>12,14</sup>, apart from at 20% glycerol concentration where the lamellar phase was not observed. This will be discussed later.



**Figure 1:** Identity and location of each phase with respect to RH within phytantriol/glycerol system. Unlabeled grey regions are occupied by a coexistence of the two neighboring phases. White dotted lines indicates the upper limit measured,  $Q_{II}^D$  phase occupation assumed from previous literature<sup>14</sup>. Black dotted lines indicate the lower limit; the existence of the  $L_{II}$  phase below this was inferred from measurements upon bulk phytantriol/glycerol solution (an approximation for RH = 0%); see SI4.

**Table 1:** Measured relative humidity values of phase boundaries in phytantriol/glycerol systems in order of increasing humidity.

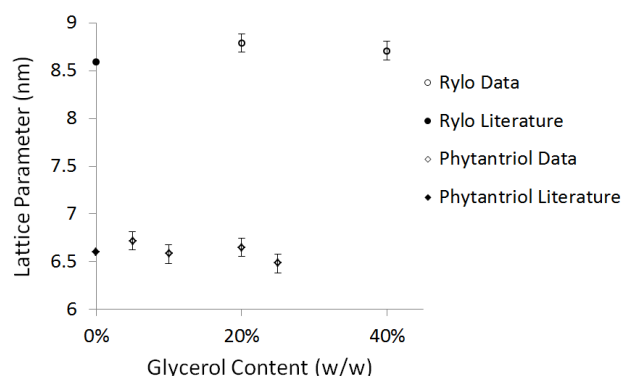
| Glycerol (%w/w) | $L_{II}$ RH (%) | $L_\alpha$ RH (%) | $Q_{II}^G$ RH (%)                     | $Q_{II}^D$ RH (%)     |
|-----------------|-----------------|-------------------|---------------------------------------|-----------------------|
| 0               | 12* - 96        | 96 - 97*          | -                                     | -                     |
| 5               | 51* - 72        | 73 - 86           | 82 <sup>†</sup> ± 1 - 94 <sup>†</sup> | 92 <sup>†</sup> - 96* |
| 10              | 62* - 70        | 70 - 82           | 70 - 91                               | 88 - 96*              |
| 20              | 10* - 60 ± 5    | -                 | 53 ± 7 - 73 ± 1                       | 63 ± 1 - 89*          |
| 25              | -               | -                 | -                                     | 24* - 65*             |

\* indicates where the measurement was halted and not where the phase was observed to appear/disappear. † indicates that a duplicate value of >94.1% from a second wafer has been excluded; see Supporting Information section SI5

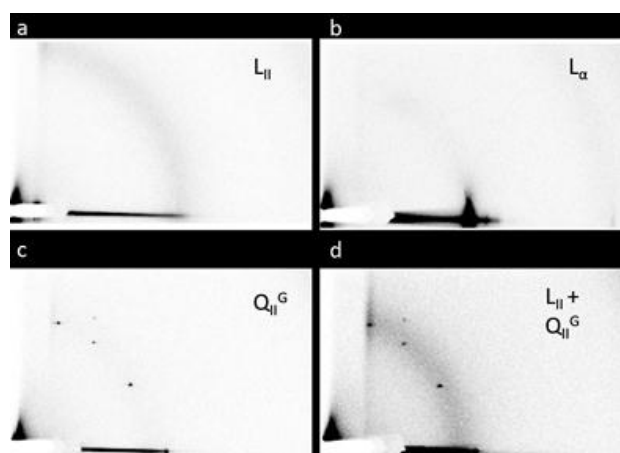
Rylo/glycerol films were found to behave in a similar fashion. For pure Rylo the  $Q_{II}^G$  phase first appeared at RH = 93% and the  $Q_{II}^D$  phase was not observed to appear before the maximum humidity measured of RH = 96% agreeing with observations by Rittman et al.<sup>12</sup>. With the addition of 20% w/w glycerol to Rylo the  $Q_{II}^D$  phase was observed to form at RH = 57% and with 40% w/w glycerol the  $Q_{II}^D$  phase was found to occupy the entire measured range down to RH = 16%. Scattering profiles for Rylo/glycerol films are shown in Figure SI6.

In order to investigate whether the phase structure was modified by the presence of glycerol, the lattice parameter for the saturated  $Q_{II}^D$  phase adopted under limiting high-humidity conditions was plotted at varying levels of glycerol concentration, as shown in Figure 2. For comparison published lattice parameters of the saturated  $Q_{II}$  phase in phytantriol<sup>14</sup> and Rylo<sup>12</sup> have been plotted at 0% glycerol content. No significant variation in lattice parameter is observed as glycerol content is increased. This indicates that glycerol does not enter the bilayer or interact strongly with the lipid headgroups as this would be expected to alter curvature elastic parameters such as relaxed curvature or bending modulus, and thus cause a shift in lattice parameter<sup>31</sup>.

In samples of phytantriol with 20% glycerol the  $L_\alpha$  phase was found to be suppressed. These samples were observed to form the  $L_{II}$  phase at low humidity and then transition to the  $Q_{II}^G$  phase without the  $L_\alpha$  phase being observed. Figure 3 shows a coexistence of the  $Q_{II}^G$  and  $L_{II}$  phases occurring during the transition (radial profiles shown in SI7). This coexistence proves that the  $L_\alpha$  phase is indeed suppressed, and not simply missed experimentally as result of having changed humidity too coarsely on going from  $L_{II}$  to  $Q_{II}^G$ .



**Figure 2.** Lattice parameter of saturated  $Q_{II}^D$  phase of phytantriol/glycerol and Rylo/glycerol films plotted with previously reported literature lattice parameters<sup>12,14</sup>.



**Figure 3.** GISAXS images of phytantriol/glycerol films. Phytantriol and 5% w/w glycerol at RH = 54.2% (a) and RH = 92% (b) showing  $L_{II}$  and  $L_{\alpha}$  phases respectively. Phytantriol with 20% w/w glycerol at RH = 34% showing  $Q_{II}^G$  phase (c) and RH = 45% where a coexistence of  $L_{II}$  and  $Q_{II}^G$  phases was observed without the presence of a  $L_{\alpha}$  (d). Radial profiles shown in SI7

We propose that the glycerol enters the cubic phase water channels, and simply replaces the water with a water/glycerol mixture. The entropy of mixing lowers the chemical potential of the water in the cubic phase, thus lowering its vapor pressure (through Raoult's law), and effectively favoring water being kept within the cubic phase rather than evaporating into the surrounding vapor. This model gives a good quantitative description of the exact extent to which the addition of glycerol lowers the relative humidity at which cubic phases can form, as we show later in this paper. This also explains why the lamellar phase is not observed as, according to measurements by Barauskas<sup>14</sup> the  $L_{\alpha}$  phase is not observed below 89% (v/v) phytantriol (a cubic phase is formed instead) and the maximum lipid volume fraction for the 20% (w/w) glycerol sample is 84% (v/v), even with no water in. However, this does not explain why the micellar phase forms at lower hydration in the 20% (w/w) glycerol sample. The simple model whereby glycerol simply replaces an equivalent volume of water would suggest that, in the absence of water, the system would form a  $Q_{II}^G$  phase; the micellar

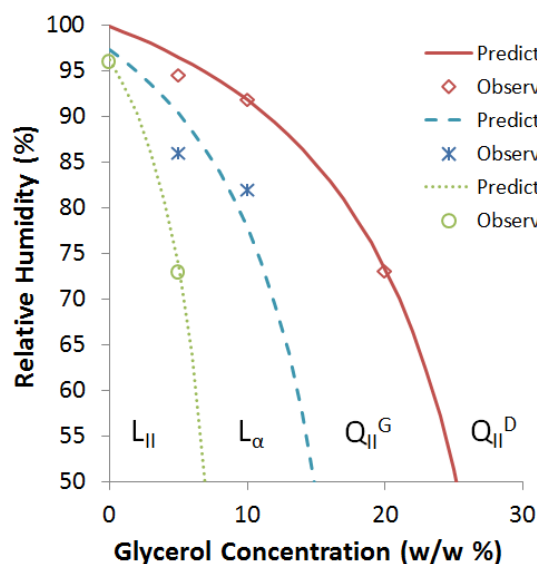
phase in the corresponding phytantriol/water phase diagram only occurs at lipid volume fractions above 95% (v/v).

In order to show the reproducibility of the system, and that we were changing humidity sufficiently slowly for the lipid film to be in equilibrium with its surroundings, a film of phytantriol with 20% glycerol was monitored under decreasing and then increasing humidity. Figure SI8 shows the lattice parameter for the film as humidity is varied. The lattice parameters show good agreement showing the reproducibility of the system, which means that the data can be meaningfully fitted to thermodynamic models.

Phase boundaries were predicated for the phytantriol/glycerol/water vapor system based upon balancing the chemical potential of the water vapor ( $\mu_v$ ) with the sum of the chemical potential of the water inside the cubic phase ( $\mu_w$ ) and the contribution to chemical potential from the bending energy of the lipid bilayer ( $\mu_b$ ),

$$\mu_v = \mu_w + \mu_b$$

The expressions used to determine  $\mu_v$  and  $\mu_w$  were  $\mu_v = \ln(\%RH/100)$  and  $\mu_w = RT \ln(x)$  where  $x$  is the mole fraction of water to glycerol inside the cubic phase.  $\mu_b$  was estimated from the relative humidity or osmotic stress required to induce a phase transition in the absence of glycerol (see SI9) and assumed to be constant at phase transition because the lattice parameters of the lipid phases when they first appear do not vary significantly between samples with different glycerol contents. In addition we assume that volumes are conserved on mixing. The full calculation can be found in SI10. Figure 4 shows the predicted phase boundaries plotted with the observed first appearance of the pure  $Q_{II}^D$ ,  $Q_{II}^G$  and  $L_{II}$  phases. The model shows good agreement with the measured data indicating that the system behaves as we have hypothesized. This raises possibilities of responsive materials where the exact humidity threshold can be chosen by using calculated glycerol content.



**Figure 4.** Lines show predicted first appearance of each phase in the phytantriol/glycerol system with points indicating the observed first appearance of each phase as humidity was increased.

We have demonstrated that adding glycerol progressively lowers the humidity at which  $Q_{II}$  phase films are stable, without affecting their lattice parameter. Furthermore we have used thermodynamic models to explain the systems behavior allowing us to generate predicted phase boundaries dependent upon glycerol content which agree with our observed data. We suggest that, with the addition of glycerol, it becomes possible to use  $Q_{II}$  phases in a much wider range of environments without the occurrence of dehydration. We believe this effect will not be limited to glycerol and may also be observed in other polar high boiling liquids such as ethylene glycol. This work will immediately benefit researchers using  $Q_{II}$  phases in cryo-TEM or membrane protein crystallization; we can envisage further applications, where lipid/glycerol can simply be applied to a surface, and will then spontaneously self-assemble into a cubic phase in contact with ambient humidity, or else will undergo a triggered transition when the humidity reaches a pre-determined threshold. This opens up a world of new possibilities involving smart nanomaterial coatings.

## Notes and references

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