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# Lipid liquid crystalline phase and starch particle mixed formulations

Bachelor thesis

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# Abstract

Lipid liquid crystals (LLC) have been studied widely because of their broad application in both food- and pharmaceutical industry. Inverse bicontinuous cubic phase of LLC have been of particular interest as it has three-dimensional structure and has been proven to be able to incorporate and deliver substances (e.g. topical drug delivery). There is a growing interest to prepare hybrid responsive lipid and polymer system. This study concerns how starch particles influence an LLC system. The system consists of capmul glycerol monooleate (GMO-50) and diglycerol monooleate (DGMO) and are mixed with different water concentration and weight percentage of native or modified starch particles. These has been proven, from small angle x-ray scattering and Cryo-TEM imaging, to form inverse bicontinuous cubic phases. At lower temperatures and water concentration, the system favorably forms gyroid type of bicontinuous cubic phase with space group Ia3d. At high temperatures and water concentrations, the system instead forms cubic crystal symmetry of space group Pn3m. Even at high concentration of starch particles, the system is favoring space group Pn3m. This tells that starch particles regulates the hydration and determines which one of the two crystal symmetries are formed. Further studies should be made in order to fully understand the lipid system with starch. Nevertheless, with this study it has proven new possibilities of using starch for materials based on lipid-polymer responsive layers.

# Acknowledgements

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# Inkorporering av stärkelse i lipid- och vattensystem

Leverering av substanser inom både livsmedels- och läkemedelsindustrin är viktig. Detta för att de aktiva ämnena måste nå dess verkningsställe utan att brytas ned eller påverkas av yttre faktorer. Av dessa anledningar har det har blivit mer populärt att studera nya aggregat, som skall möjliggöra för mer produktiva, effektiva och stabila levereringar från en utgångsplats till målet. Genom att studera olika tillsatser, som i detta fallet är stärkelse, kan levereringarna styras vilket bland annat öppnar upp nya vägar av läkemedelsleverering i kroppen.

Stärkelse är en polymer som består av långa molekylkedjor. Det förekommer naturligt i växter och konsumeras av människor genom råvaror som till exempel potatis. Dess ofarliga förekomst i naturen och dess tillgänglighet är två faktorer till varför det anses vara en bra polymer till att utnyttja för reglering av lipidsystem.

Lipider är fettliknande molekyler som består av en vattengillande (polär) och en icke-vattengillande del (opolär). När lipider befinner sig i vattenhaltig miljö arrangeras de så att den polära delen interagerar med vatten, medan den opolära delen dras och interagerar med andra lipiders opolära del. Dessa kan på så vis packas ihop och bilda olika strukturer. Beroende på lipidernas egenskaper, så samordnas de på olika sätt. Cellen – som är den minsta byggstenen i levande organismer - har ett membran som består av lipider. Dessa har genom tidiga studier visat sig kunna bilda en struktur som en kub, så kallad kubisk fas, som har tredimensionell struktur. Den kubiska fasen har en inre vattenhaltig miljö som möjliggör inkapsling av ämnen. Tanken med att föra in stärkelse bland lipidsystem, är bland annat för att kunna reglera frisläpp av aktiva substanser som har kapslats in för transportering. Genom att använda stärkelse tillsammans med lipidsystemet, blir det möjligt att styra frigivningen. Lipiderna som användes var en blandning av glycerol monooleate, diglycerider, triglycerdier och diglycerol monooleate. Dessa provblandningars struktur studerades med hjälp av röntgenbestrålning (small angle x-ray scattering) där man kunde se att kubisk fas bildades genom att studera spridningen av röntgenstrålarna. Beroende på temperatur, stärkelse- eller vattenkoncentrationen, så bildades det olika kubiska faser. Detta systemet observerades även genom elektronmikroskopi, där systemets struktur kunde ses som bilder.

Denna studie visar att man genom att sätta till stärkelsepartiklar kan kontrollera hydreringen i ett lipid- och vattensystem som kan avgöra hur lipider i ett system samordnas till olika strukturer. Detta öppnar upp nya dörrar för levereringssystem inom både livsmedels- och läkemedelsindustrin.

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# 1. Introduction

At the end of the 19th century a new state of matter was discovered – the liquid crystalline state. Lipids, in liquid crystal phases, have been of big interest in the research field ever since their discovery. The reason is that they are essential building blocks in the cell and can response to external factors like temperature or electric- and mechanical forces. One of their main characteristics is self-assembling into different types of structures, but there are still many unknown aspects of the lipids when in this state of matter. [1]

## 1.1 Lipid liquid crystal and purpose of this work

Lipids are amphipathic molecules consisting of a polar head group and a hydrocarbon chain. The size of the head group (polar region) or the length of hydrocarbon chain (nonpolar region) determines the features of the lipid molecule. When lipids are put in aqueous environment, like water, the polar region will interact with the aqueous environment through hydrogen bonds. As it is unfavorable for the nonpolar region to be in aqueous environment, the lipid will start to self-assemble to form either micelles, lipid bilayer or other aggregates, because of the hydrophobic effect. Due to the hydrophobic effect, London forces promote the aggregation of the hydrocarbon chains, hence the self-assembling. Lipids with larger hydrocarbon chain, or depending on the head group, can form lipid bilayers with an aqueous environment in between the layers. It is then possible for the amphiphilic molecules, depending on their shapes and environment, to self-assemble to form lipid liquid crystalline aggregates. [2,3]

Liquid crystals (LC) are mesophases that have properties similar to both liquid and solid phase. The molecules in liquid crystal has the same orientational long-range order as in a solid crystal, but the molecules can move freely as in a liquid [1]. Lyotropic liquid crystals forms when amphiphilic molecules encounter a certain solvent and are in the right environmental conditions. When lipids self-assemble in a solvent, they can form lyotropic liquid crystals. It is possible for liquid crystalline phases to have different phases at the same time. For the lyotropic lipid liquid crystals, it can be achieved when two different lipids exist in one mixture. By changing the concentration or temperature, one can achieve different phases. [1,2]

Lyotropic lipid liquid crystals has been studied widely because they can form one, two or three-dimensional, stable assemblies in presence of solvent. Non-lamellar lipid liquid crystals - the hexagonal and cubic phase, are good developable candidates for drug delivery. This because it has internal structure where bioactive substances can be held and transferred without being altered by external factors. [4-6]

The cubic phase has three-dimensional lattice. In lipid-water mixture, three different bicontinuous cubic phases where the mid-plane of the curved bilayer can be described by an infinite periodical minimal surface (IPMS). These are termed double diamond (Pn3m

space group), gyroid (Ia3d space group) and primitive/body centered (Im3m space group) shown in fig.1. [1,2]

A few decades ago, it was discovered that it was possible to disperse cubic LC into particles, termed cubosomes, to facilitate drug delivery. This because the increased surface area makes the transportation of drugs go quicker, and they are not as viscous [4]. An example is the use of cubic system for topical delivery of drugs, where studies have revealed that encapsulation of substance inside cubic system can help the drug penetrate through skin [4,5]. Even bio membranes have been seen to have cubic structure, which further increased the interest in using cubic system to facilitate penetration through the cell to deliver drugs [2].

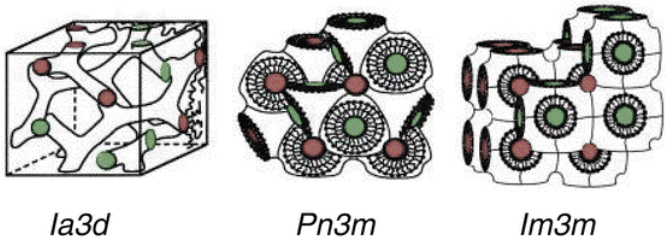



***Figure 1*** *Bicontinuous cubic phase on infinite periodical minimal surface (IPMS); double diamond (Pn3m space group), gyroid (Ia3d space group) and primitive/body centered (Im3m space group).*[7]

Additives have been used to more easily control the release (e.g. of bioactive substances). These should respond to external factors like temperature or electromagnetic radiation to lead to controlled release without disrupting the structure. One approach was previously made (Dabkowska AP et al. 2017) where the polymer poly N-isopropylacrylamide (pNIPAM) was embedded into the lipid matrix of a non-lamellar lipid liquid crystal. The hydration was regulated by temperature as the polymer was thermoresponsive and showed that it is possible to form hybrid lipid polymer films. [8]

In the following paper, the aim is to see whether it is possible to do a similar attempt – embed polymer inside lipid LC-phase and study the bulk phase. The idea is to use the same lipid system as before, but instead of using pNIPAM, starch particles will be used because they are natural polymers that are biodegradable. By starting to study the phase behavior, when trying to embed starch particles into the liquid crystal lipid phase, one can see how the starch particles interact or position themselves in the lipid phase. If possible, it would be interesting to see whether this system can form mixed lipid and biopolymer films of cubic phase - that are able to respond to temperature and possibly control other properties.

The lipid mixture that will be used, consists of capmul glycerol monooleate (GMO-50) and diglycerol monooleate (DGMO) and are of nonionic lipids. GMO-50 consists mainly of glycerol monooleate, that is famous for the ability of forming cubic phases in excess water, and of diglycerides and triglycerides. DGMO mainly assemble to lamellar phase, and it has previously been proven that one can adjust the water channel dimension in the cubic phase

by changing amount of DGMO. The lipid mixture of GMO-50/DGMO at ratio 40/60 can form non-lamellar bicontinuous cubic phase. It was proven by Pitzalis et al.[9] that swelling of the lipid phase in aqueous environment increases with amount of DGMO in mixtures with GMO. [8-10]

## 1.2 Starch particles

The starch particles are of interest because they are natural, nontoxic and biodegradable, which means that they have broader applications compared to PNIPAM nanogel particles.

Starch is a polymeric carbohydrate consisting of the linear amylose and the branched amylopectin. It is not soluble in water, because of hydrophobic interaction and strong hydrogen bonds that holds the starch together, until it reaches high enough temperature. The starch molecules can swell and reach a phase transition, known as initial gelatinization, that usually lie around 55-70 $^{o}$C. When gelatinization starts, water can penetrate into amorphous regions and amylose will flow out. When cooled down, the viscosity will increase again. X-ray diffraction data shows that the sample needs to reach a higher temperature compared to the initial gelatinization (around 55-70 $^{o}$C) to observe changes in the diffraction pattern indicative of the swelling of starch particles. [2, 11]

It is known that certain lipids, such as monoglycerides, do influence properties of starch. Amylose, in the amorphous region of starch, can interact with lipid to form complexes. The complexes are believed to have a lamellar type of structure, and the chain length of amylose or the availability of the lipid determines whether a complex is formed or not. The interaction between starch molecules and lipids in cubic phase is still not well understood and is therefore the focus of this study. [11, 12]

The starch that will be used in this experiment is the native quinoa starch from quinoa seeds, which often are in the size range 0.5-3 µm in diameter [13]. Studies with both unmodified and modified starch were made, where the modified starches have been altered so that they are acetylated, butylated and propylated.

## 1.3 Methods to analyze the samples

To determine the lipid liquid crystal presence, small angle X-ray scattering will be used. When measuring small angle x-ray scattering, the beam is sent through the sample and the sample will send out waves when the x-ray is scattered. The scattering of x-ray will give rise to scattering intensity (I) as a function of angle or as a function of position in the detection plane. Normally the scattering intensity is determined as a function of the scattering vector q, which is the momentum transfer between the beam that hits the sample and the scattered x-rays (eq.1). [14]

$$q = \frac{4\pi \sin\theta}{\lambda} \quad (1)$$

Where $q$ is scattering vector, $\lambda$ *the wavelenght* and $\theta$ half of the diffraction angle.

Bragg peaks results from scattered waves from unit cell that are in phase, this is when constructive interference occur [14]. These peaks will appear if the sample are ordered in a periodic arrangement. For crystalline that has highly ordered arrangement, the symmetry can be calculated from the ratios of q-values at the maximum of peaks. With this, one can identify the space group and determine the distance between the different planes with Bragg´s law (se equation 2). [2, 14, 15]

$$d_{Bragg} = \frac{2\pi}{q_{peak}} \quad (2)$$

Where $d$ is distance, $q$ the moment transfer/maximum of peak.

Miller indices; h, k and l, are reciprocal of intercepts of the planes that give rise to reflection, that is the intercepts of distance in unit cell. In cubic structures, the distance $d_{hkl}$ can be calculated with lattice constant a, and the miller indices (hkl). [15]

$$d_{hkl} = \frac{a}{\sqrt{h^2+k^2+l^2}} \quad (3)$$

To determine size of particles in prepared samples, dynamic light scattering (DLS) will be used. In DLS, the size of particles is measured through the Brownian motion. The Brownian motion is described by the movement of the particles that is made from solvent that is in the surrounding. This movement is measured when light is scattered. The Transmission electron cryomicroscopy (CryoTEM) will be used to analyze the structure of some samples. This works by preparing the sample in cryogenic temperatures (below -150°C) where imaging occurs with electron microscopy (see method).

# 2. Experimental procedure

## 2.1 Starch preparation

The quinoa native starch and the three modified starches (acetylated QA4 3.42%, butylated QB3 3.73% and propylated QP3 3.25%) that were used, were prepared from Food Technology department, Lund University. The procedure used was described as below:

70 g of quinoa starch was dispersed for 20 minutes, whereby the pH was maintained to 6.5 by addition of 0.71 M NaOH. A desired amount of acetic anhydride was added into the solution, where pH was maintained at 8.5±0.2 with addition of 0.71 M NaOH. The solution was mixed, and the process continued for an hour. Thereafter, washing of the solution was made, for two times with distilled water. A final washing with acetone by centrifuging was

done by centrifuging the solution for ten minutes at 1000 G. The final product of SCFA-starch was left to dry in oven overnight until constant weight was obtained. This procedure was repeated for propionic anhydride and butyric anhydride.

## 2.2 Lipid bulk phase preparation

85 wt% of lipid mixture consisting of glycerol monooleate (GMO-50) and diglycerol monooleate (DGMO) at a ratio of 40/60 were mixed with 15 wt% of EtOH in glass vials. Then, the mixtures were left on roller mixer for 24 hours. Afterwards 1, 2.5, 5, 7.5 and 10 wt% of native starch were added to each of the lipid mixtures and mixed again on roller mixer for 24 hours. The samples were centrifuged up and down every day for two weeks. After centrifugation, a quick transfer of 3-5 droplets of sample to a vial with at least 90 wt% of MilliQ water was made.

1 wt% of native starch was prepared with 29, 50 and 60 wt% MilliQ water, 5 wt% of native starch was prepared with 25, 35, 40, 50 respectively 60 wt% MilliQ water in glass vials where the remaining wt% consisted of GMO-50/DGMO at ratio 40/60. The samples were then left to mix on roller mixer overnight and centrifuged up and down a couple of times each day for at least two weeks until they appeared to be homogenous.

The samples of modified starches QA4 (acetylated), QB3 (butylated) and QP3 (propylated), were prepared in glass vials by taking 1 wt% of modified starch with 29 wt% MilliQ water. Further samples were made were 5 wt% of each modified starch were put in separate glass vials with 50 wt% of MilliQ water. The remaining wt% of the modified starch samples also consisted of GMO-50/DGMO at ratio 40/60. The samples were then left to mix on roller mixer overnight and centrifuged up and down a couple of times during the day for at least two weeks until they appeared to be homogenous.

To compare the result of samples with and without starch, samples with 30, 40 and 50 wt% of MilliQ water was prepared with remaining wt% of the sample being GMO-50/DGMO at ratio 40/60. The samples were centrifuged the same way as the ones before.

## 2.3 Lipid dispersion

To further analyze the bulk phases, the sample containing 5 wt% of native starch and 50 wt% of MilliQ water were dispersed in water. This by weighing up 95 wt% of MilliQ water in glass vials, whereby 5 wt% of the bulk phase mixture was put inside with the help of a pipette. Then the samples were further dispersed by sonication. The same process was done with the samples containing lipid mixture and 50 wt% lipid with 50 wt% of MilliQ water respectively the three bulk phases with 50 wt% MilliQ water with modified starch (QA4, QB3 and QP3). To study the starch particles, 0.5 wt% of starch was dispersed in 99.5 wt% MilliQ water by sonication.

## 2.4 Dynamic light scattering (DLS)

2 mL was approximately added to 0.5 mL of 20 mM NaCl in a cuvette. The samples were then measured in the dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) with temperature control. The samples are measured by light that goes through the sample and the scattered lights will give fluctuation due to the mobility of the particles (Brownian motion) that can be converted to a size distribution of particles. All samples were measured at ten different temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C). Each measurement had equilibrium time of five minutes and were measured three times at each temperature.

Another DLS measurement was made on the starches, but they were more diluted. 0.005 g of starch particle (native starch, QA4, QB3 and QP3) was weighed into centrifuge tubes and then diluted in 35 mL of 20 mM NaCl. The solution was then dispersed in a sonicator for ten minutes. These were then measured in the same way as the samples before.

## 2.5 Small angle x-ray scattering (SAXS)

All prepared lipid mixtures/bulk phases with and without starch were measured on the Ganesha 300XL SAXSLAB instrument with configuration 22. The configuration adjusts the beamline according to certain q-range. The samples were put in between two mica sheets in sandwich cells and then sealed. Water bath was used to control temperature, where the samples with water as solvent were measured for 20 minutes at eight different temperatures (25, 30, 35, 40, 45, 50, 55 and 60 °C). This with 45 minutes of equilibrium time in between the measurements. The samples with EtOH as solvent were measured at seven different temperatures (20, 25, 27.5, 30, 32.5, 35 and 40 °C) with the same configuration and measurement time. When the measurements were done, all data was obtained and corrected by SAXSGUI data program.

The dispersed samples were put in capillaries with the help of a syringe and then sealed. Then they were measured with configuration 22, at 25°C for three hours each, with an initial equilibrium time at 45 minutes. All data was obtained and corrected by SAXSGUI data program.

## 2.6 CryoTEM

The three dispersed samples from the bulk phase; 5 wt% native starch 50 wt% MilliQ water with GMO-50/DGMO, 50 wt% MilliQ water with GMO-50/DGMO and 0.5 wt% of starch, were transferred to department of chemistry, Centre for analysis and synthesis - to be prepared for imaging on CryoTEM. 4 µL of the samples were put on copper grid with carbon-coated perforated polymer film that was already put inside the chamber. With a

filter, the sample was blotted and then vitrified when put into liquid ethane, whereby it was held in liquid nitrogen overnight. Afterwards the samples were measured on the transmission electron microscope JEOL JEM-2200FS that has field-emission electron source 200 kV, and the images are taken with TVIPS F416 camera. The TEM has a resolution of 0.28 nm and the whole microscope is operated by computers, with the software Low dose.

# 3. Results and discussion

## 3.1 SAXS analysis of bulk phases

The bulk phases that were made with EtOH had phase separation after mixing in starch with the lipid mixture of GMO-50/DGMO. This phase separation was clear when the samples were left for a while. The reason for this is believed to be because the starch particles are not soluble in EtOH. Therefore, a change of sample preparation was made, where water was used instead. The new choice of solvent was because GMO-50/DGMO have before been proven to form bicontinuous cubic phases when water is used [9].

The bulk phases with water was very viscous, hence the long centrifugation process was needed to make them homogenous as apparent from visual inspection. It is possible that some of the samples were proceeded in the analysis without getting homogenous on a smaller length scale than obvious for the naked eye.

### 3.1.1 Bulk phases with EtOH

Even though the samples with EtOH were not ideal, they were still measured on SAXS. Well-defined Bragg peaks were shown, and the space group could be identified by calculating the ratio between the maximum of well-defined peaks (eq.1). This to get the crystal symmetry. All samples with starch resulted in the space group Pn3m, with spacing ratio $\sqrt{2}$, $\sqrt{3}$, 2 and $\sqrt{6}$, at all seven temperatures (see tab.1 for all spacing ratio). The sample without starch, at 25-27.5 and 35 °C, showed reflection symmetry of Ia3d with the ratio $\sqrt{6}$, $\sqrt{8}$, 4 and $\sqrt{22}$ but had no peak at $\sqrt{14}$ and $\sqrt{20}$. The missed peaks could be due to that the sample was not homogenous enough, or that diffracted intensity was not strong enough to give well-defined peaks (see table 1).

From the SAXS measurement one could see that the phase stays intact as bicontinuous cubic phase, when the temperature is increased (see fig.2). This from 0-10 wt% of starch particle included in the lipid mixture. This means that inclusion of starch particle does not affect the formation of bicontinuous cubic phase. But, without starch the Ia3d was formed at lower temperatures, which means that starch might av influenced the formation of the gyroid (Ia3d) and promote the Pn3m. In fig.2, it can be observed that the scattering vector is shifted

towards higher q-values as the temperature increases from 25 to 40°C, which is a sign that the distance in each unit cell decreases.

**Table 1.** *Lipid mixture consists of GMO-50/DGMO at ratio 40/60 with 15 wt% EtOH. This was mixed with different wt% of starch particles (0-10). These samples were put in excess MilliQ water and measured on small angle x-ray scattering at seven different temperatures (20, 25, 27.5, 30, 32.5, 35 and 40°C). The space group could be calculated by taking the ratio of identified Bragg peaks.*

| Sample (GMO-50/DGMO 40/60) | Space group | Temperature (◦C) |
|---|---|---|
| **Without starch** | Ia3d | 20-27.5, 35 |
| **Without starch** | Pn3m | 30-32.5, 40 |
| **1 wt% native starch** | Pn3m | 20-40 |
| **2.5 wt% native starch** | Pn3m | 20-40 |
| **5 wt% native starch** | Pn3m | 20-40 |
| **7.5 wt% native starch** | Pn3m | 20-40 |
| **10 wt% native starch** | Pn3m | 20-40 |

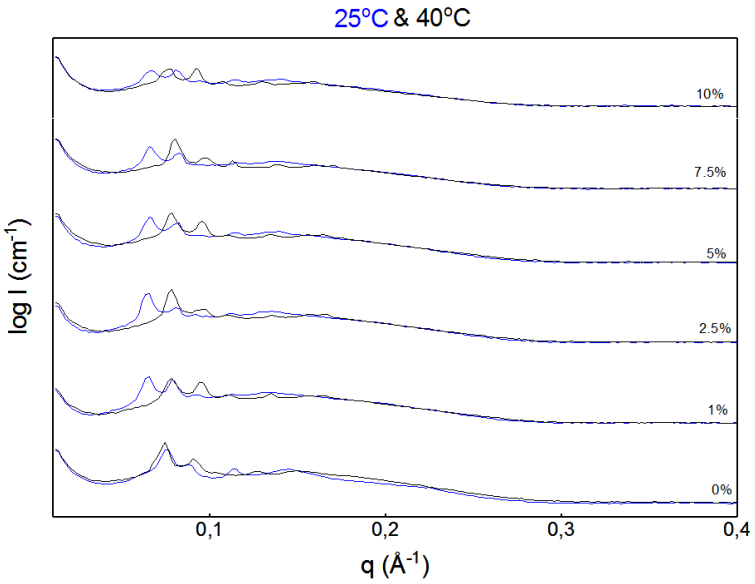



**Figure 2** *Logarithm of Intensity ($cm^{-1}$) as a function of scattering vector q ($Å^{-1}$). The samples consisting of 0, 1, 2.5, 5, 7.5 and 10 wt% of native starch particles in 15 wt% EtOH with remaining wt% of GMO-50/DGMO 40/60, were put in excess water and measured on SAXS. These samples were measured at configuration 22 for 20 minutes, with equilibrium time 45 min, at temperature 25°C (blue) and 40°C (black).*

3.1.2 Bulk phases with water

When preparing the samples containing 1 and 5 wt% of native starch, with different water concentrations, it was only 60 wt% of MilliQ water that seemed to have excess of water as the mixture did not take up all the water. SAXS data showed Bragg reflections that had the symmetry of inverse bicontinuous cubic phases, Pn3m with spacing ratio √2, √3, 2, √6, √8, √9 or Ia3d with spacing ratios √6, √8, √14, 4, √20, √22 (see tab.2 for all values). It seems like lower water concentration in the samples with native starch, promotes the space group Ia3d. When increasing the temperature or the water concentration, Pn3m is instead formed. This can be explained by the fact that, when changing the concentration or temperature, the mobility of hydrocarbon chain changes, which leads to changes in curvature and the packing of the system changes, so that another bicontinuous cubic phase can be formed. In this case, Pn3m seems to form at higher temperatures and concentration of water. From this, one can also assume that native starch particles do not interrupt the lipid mixtures ability to form bicontinuous phase, and their ability to form different phases when altering concentration or temperature.

30 wt% H2O with 1 wt% 29 wt% H2O was plotted in the same graph and it showed that both kept the inverse bicontinuous phase. The q-values for samples without starch, shifted towards higher values, whereas for the sample with starch, it can only be seen to shift at 45°C (see fig.3). The reason for why the shift is at 45°C, could be that a phase transition occurs from 45°C to 50°C. At 45°C it is possible that it is in two-phase area so that the unit cell dimensions have been altered and therefore are smaller.

**Table 2.** *All samples contain GMO-50/DGMO at ratio 40/60 with MilliQ water and native quinoa starch, or only with MilliQ water. The samples were measured in small angle x-ray scattering at eight different temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C) for 20 minutes, with configuration 22, were the intensity was plotted versus scattering vector. Space group was identified by shown Bragg peak reflection where spacing ratio was given using Braggs law.*

| Sample (with MilliQ) | Space group | Temp (◦C) | Spacing ratio |
|---|---|---|---|
| **30 wt% $H_2O$** | Pn3m | 25-30, 40-60 | √2, √3, 2, √6, √8, √9 |
| **30 wt% $H_2O$** | Ia3d | 35 | √6, √8, √14, 4, √20, √22 |
| **40 wt% $H_2O$** | Pn3m | 25-60 | √2, √3, 2, √6, √8, √9 |
| **50 wt% $H_2O$** | Pn3m | 25-60 | √2, √3, 2, √6, √8, √9 |
| **1 wt% native starch 29 wt% $H_2O$** | Ia3d | 20-45 | √6, √8, √14, 4, √20, √22 |
| **1 wt% native starch 29 wt% $H_2O$** | Pn3m | 50-60 | √2, √3, 2, √6, √8, √9 |

| | | | |
|---|---|---|---|
| **1 wt% native starch 50 wt% $H_2O$** | Pn3m | 25-60 | √2, √3, 2, √6, √8, √9 |
| **1 wt% native starch 60 wt% $H_2O$** | Pn3m | 25-60 | √2, √3, 2, √6, √8, √9 |
| **5 wt% native starch 25 wt% $H_2O$** | Ia3d | 25-60 | √6, √8, √14, 4, √20, √22, √24 |
| **5 wt% native starch 35 wt% H2O** | Ia3d | 25-35, 45-60 | √6, √8, √14, 4, √20, √22 |
| **5 wt% native starch 35 wt% H2O** | Pn3m | 40 | √2, √3, 2, √6, √8, √9 |
| **5 wt% native starch 40 wt% H2O** | Ia3d | 25-30 | √6, √8, √14, √22 |
| **5 wt% native starch 40 wt% H2O** | Pn3m | 35-60 | √2, √3, 2, √6, √8, √9 |
| **5 wt% native starch 50 wt% H2O** | Pn3m | 25-60 | √2, √3, 2, √6, √8, √9 |
| **5 wt% native starch 60 wt% H2O** | Pn3m | 25-60 | √2, √3, 2, √6, √8, √9 |

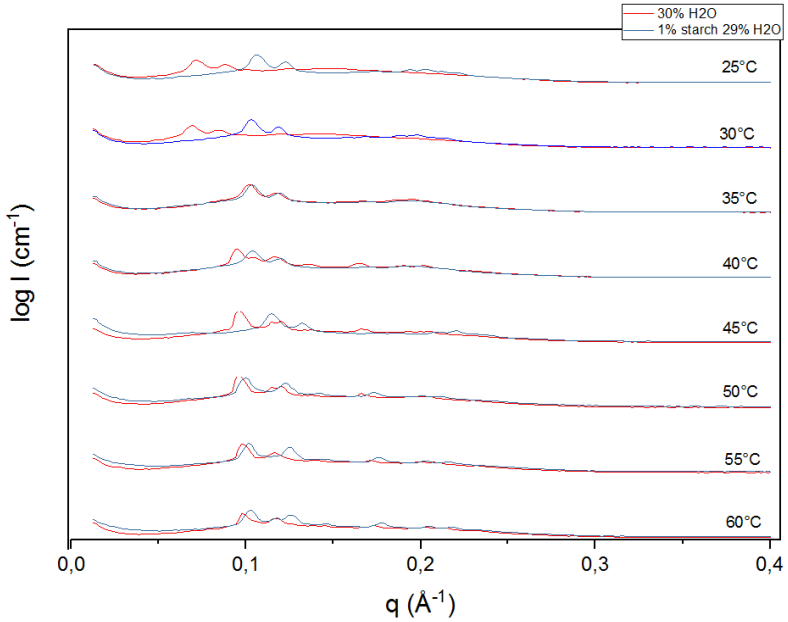



***Figure 3*** *Logarithm of Intensity ($cm^{-1}$) as a function of scattering vector q ($Å^{-1}$). The samples consisting of only 30 wt% water and 1 wt% native starch with 29 wt% water, with remaining wt% of GMO-50/DGMO 40/60, were measured on small angle x-ray scattering (SAXS). These samples were measured at configuration 22 for 20 minutes, with equilibrium time 45 min, at eight different temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C). These were both identified from Bragg peaks to have spacing ratio of inverse bicontinuous space group Ia3d and Pn3m.*

When comparing the SAXS data of 1 wt% native starch 50 wt% $H_2O$, 5 wt% native starch 50 wt% $H_2O$ with the sample containing only 50 wt% $H_2O$, it could be observed that the ones with starch shifted more towards higher q-values at higher temperatures, compared to

the one without (see fig.4). This could be due to that starch take up more water. as the size of starch increase around 50-60°C (see fig.13). All three samples held the inverse bicontinuous phase Pn3m from 25-60°C. This could indicate that starch particles promote lipid bilayer to assemble closer and become more packed while remaining the same inverse bicontinuous phase.

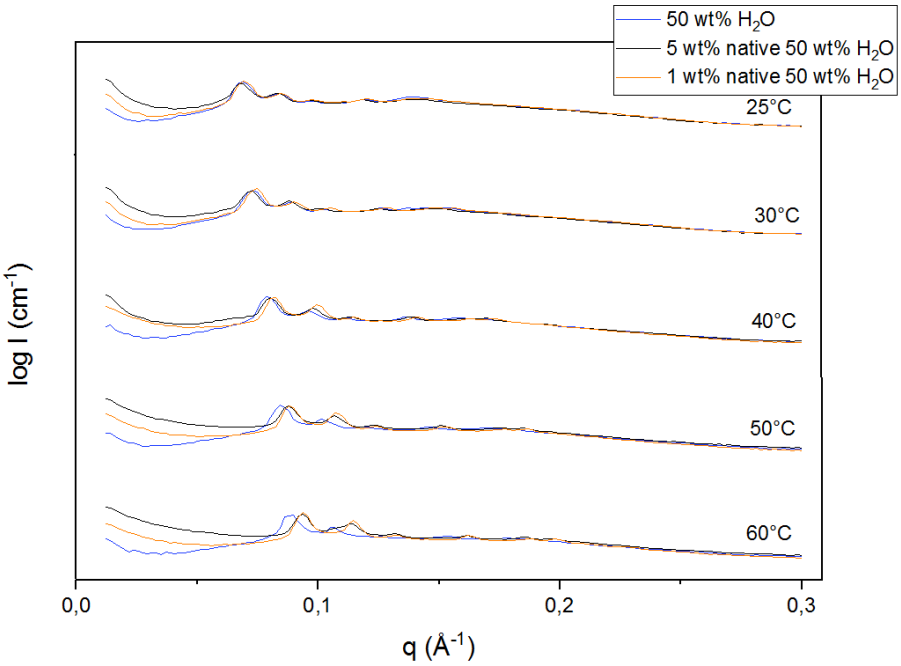



***Figure 4*** *Logarithm of intensity I ($cm^{-1}$) as a function of scattering vector q ($Å^{-1}$). The samples consisting of only 50 wt% water (blue), 1 wt% native starch with 50 wt% water (yellow) and 5 wt% native starch with 50 wt% (black), with remaining wt% of GMO-50/DGMO 40/60, were measured on small angle x-ray scattering (SAXS). These samples were measured at configuration 22 for 20 minutes, with equilibrium time 45 min, at eight different temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C). Only 25, 30, 40, 50 and 60°C are displayed in the graph. These were all identified from Bragg peaks to have spacing ratio of inverse bicontinuous space group Ia3d and Pn3m.*

To see how the water content influenced the unit cell dimension, the samples with 5 wt% native starch with different water content (25, 35, 40, 50 and 60 wt% H2O) was compared in a graph. When plotting intensity versus q, the higher water concentration gave lower q-values. This shift means that the unit cell dimension is increased, which probably is the result of additional uptake of water that makes water channels larger. The samples containing 50 wt% and 60 wt% resulted in very similar Bragg peaks (see fig.5). Since the sample did not take up all 60 wt% $H_2O$, the hydration limit that the system can take, must lie in between 50-60 wt%.

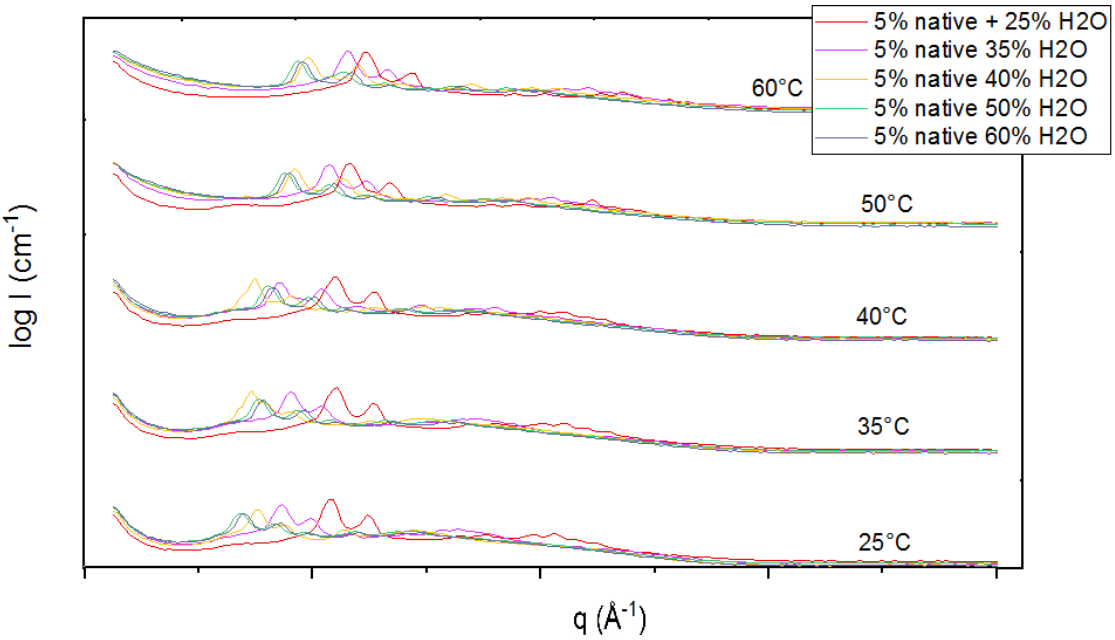



***Figure 5*** *Logarithm of intensity I ($cm^{-1}$) as a function of scattering vector q ($Å^{-1}$). Five different mixtures are shown were they all contain 5 wt% of native starch with different water content (25, 35, 40, 50 and 60 wt%). These were measured on small angle x-ray scattering (SAXS), at configuration 22, for 20 minutes, with equilibrium time 45 min, at eight different temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C). Only 25, 35, 40, 50 and 60°C are displayed in the graph. These were all identified from Bragg peaks to have spacing ratio of inverse bicontinuous space group Ia3d and Pn3m.*

Three samples of modified starch; QA4, QB3 and QP3, also showed distinct Bragg peaks when measured on SAXS. The space group was once again varied between two inverse bicontinuous cubic phases – Ia3d and Pn3m (see tab.3). All samples showed six well-defined Bragg peaks, except for 1 wt% QA4 with 29 wt% $H_2O$ that only had four. Here, all samples with 5 wt% modified starch and 50 wt% $H_2O$ formed Pn3m. But for the ones with only 1 wt% modified starch and 29 wt% starch, gyroid Ia3d also appeared. QP3 with 29 wt% $H_2O$ only formed Ia3d, whereas for QB3, it showed similar behavior as for the native starch – that is, forming Pn3m at higher temperatures and Ia3d at lower. But, for QA4, Pn3m was instead formed at lower temperatures and Ia3d at higher. It seems like the structure of starch does not matter at high concentration of starch, whereas at low concentration it does.

**Table 3.** *All samples contain GMO-50/DGMO at ratio 40/60 with MilliQ water ($H_2O$) and one type of modified quinoa starch. The modified starches used were acetylated (QA4), butylated (QB3) and propylated (QP3). The samples were measured in small angle x-ray scattering at eight different temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C), were the intensity was plotted versus scattering vector. Space group was identified by shown Bragg peak reflection where spacing ratio was given using Braggs law.*

| Sample | Space group | Temperature | Spacing ratio |
|---|---|---|---|
| **1 wt% QA4 29 wt% $H_2O$** | Pn3m | 25-30, 40-50 | $\sqrt{2}$, $\sqrt{3}$, $\sqrt{6}$, $\sqrt{8}$ |

| | | | |
|---|---|---|---|
| **1 wt% QA4 29 wt% $H_2O$** | Ia3d | 35, 55-60 | √6, √8, √14, √16 |
| **1 wt% QB3 29 wt% $H_2O$** | Ia3d | 25-45 | √6, √8, √14, √16, √20, √22 |
| **1 wt% QB3 29 wt% $H_2O$** | Pn3m | 55-60 | √2, √3, 2, √6, √8, √9 |
| **1 wt% QP3 29 wt% $H_2O$** | Ia3d | 25-60 | √6, √8, √14, √16, √20, √22 |
| **5 wt% QA4 50 wt% $H_2O$** | Pn3m | 25-60 | √2, √3, 2, √6, √8, √9 |
| **5 wt% QB3 50 wt% $H_2O$** | Pn3m | 25-60 | √2, √3, 2, √6, √8, √9 |
| **5 wt% QP3 50 wt% $H_2O$** | Pn3m | 25-60 | √2, √3, 2, √6, √8, √9 |

When a comparison was made with the 5 wt% native starch and 5 wt% of the modified starches (QA4, QB3, QP3), they were seen to show similar Bragg reflections and q-values (see fig.6). QA4 was the one that showed deviant result, as it had lower q-values which must be due to that it is more hydrophilic, but they all maintained the inverse bicontinuous cubic phase. The starches shift towards higher q from 25-55°C (all temperatures are not shown in fig.6), which means decreased unit cell dimension. But at 60°C, QA4, QB3 and QP3 shifts towards lower q-values, instead of higher, which is the behavior of the native starch.

The difference of the starches is probably due to their structure. The modified starches are larger in size, which can also be seen in fig. 13 (see below). At 50-60 °C the difference between the size of the particles, can be seen to be clear. Acetylated starch is more hydrophilic compared to native starch, whereas butylated and propylated are more hydrophobic. The shift towards lower q could therefore depend on the size of the particle, as three of them has different properties regarding hydrophilicity.

It is possible for all SAXS analysis that there were two phases. This if the first peak does not have high intensity or that there were some forbidden peaks that occurred. When the spacing ratio was calculated, it did not seem like there were any weird peaks, but there were instead some peaks that were missing – this does not apply to all peaks.

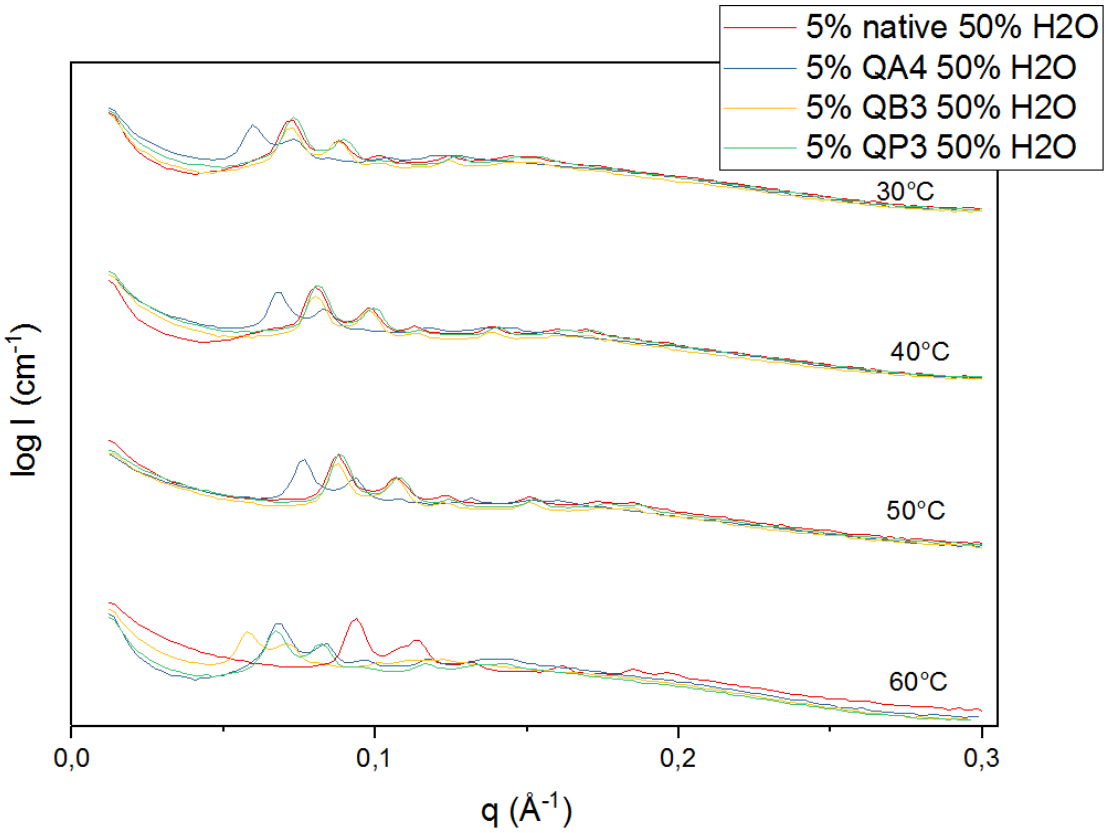



***Figure 6*** *Logarithm of intensity I ($cm^{-1}$) as a function of scattering vector q ($Å^{-1}$). Four different mixtures are shown were they all contain 5 wt% of different quinoa starches with 50 wt% of $H_2O$. Native starch is shown in red, acetylated starch (QA4) in blue, butylated starch (QB3) in yellow and propylated starch (QP3) in green. These were measured on small angle x-ray scattering (SAXS), at configuration 22, for 20 minutes, with equilibrium time 45 min, at eight different temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C). Only 30, 40, 50 and 60°C are displayed in the graph. These were all identified from Bragg peaks to have spacing ratio of inverse bicontinuous space group Ia3d and Pn3m*

From the Bragg reflections, the d-spacing was calculated with Bragg's law. Thereafter 1/d was plotted against square root of Milli indices ($h^2+k^2+l^2$). The lattice parameter, a, could be found from the gradient. The lattice parameter for each measurement from SAXS could then be plotted against the temperature to see how it changes. In fig.7, where 5 wt% native starch with different water content was plotted, one could see that the lattice parameter decreased with increased temperature. This means that the dimension in unit cell is decreasing with temperature. Reason for this is due to increased thermal energy of molecules at higher temperatures, that makes the scattering angle larger.

In fig.8 one can note that the lattice parameter for modified starches is not as linear but is more distributed. The ones that deviates the most is 1 wt% QA4 with 29 wt% $H_2O$ and 1 wt% QB3 with 29 wt% $H_2O$. It could then be concluded that they are not as finely arranged. It can be remarked that the lattice parameter of the two, does not decrease with increased temperature.

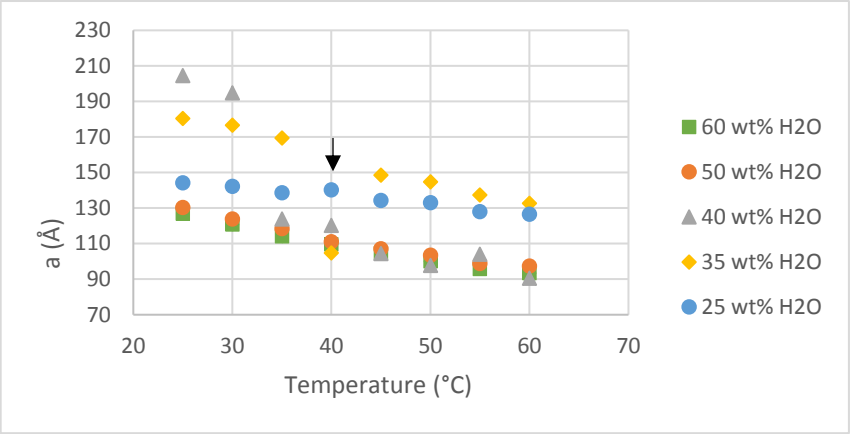



***Figure 7*** *Lattice parameter a (Å) as a function of temperature (°C) for five samples containing 5 wt% native starch with different water concentrations. 25 wt% is shown in blue, 35 wt% as yellow, 40 wt% in grey, 50 wt% in orange and 60 wt% in green. Lattice parameter was obtained from small angle x-ray scattering, measured at 25, 30, 35, 40, 45, 50, 55 and 60°C. The arrow indicates phase transition.*

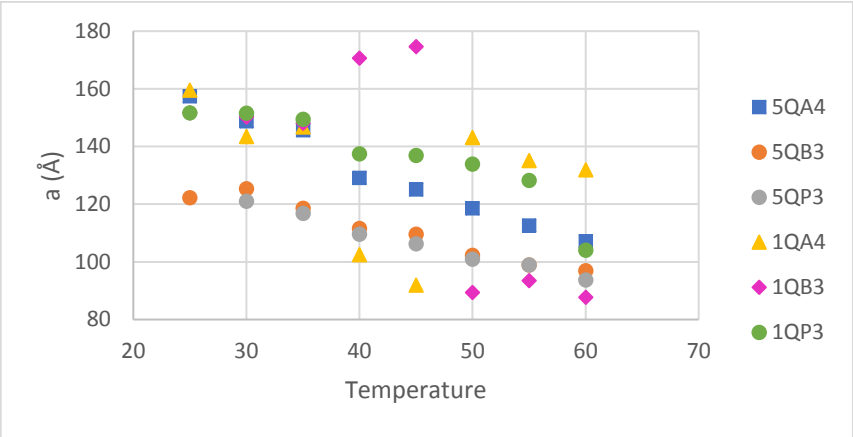



***Figure 8*** *Lattice parameter a (Å) as a function of temperature (°C) for six samples containing modified quinoa starch and water with lipid mixture of GMO-50/DGMO at ratio 40/60. 5 wt% acetylated starch 50 wt% $H_2O$ (5QA450w) is shown in blue, 5 wt% butylated starch 50 wt% $H_2O$ (5QB350w) is shown in orange, 5 wt% propylated starch 50 wt% $H_2O$ (5QP350w) is shown in grey, 1 wt% acetylated starch 29 wt% $H_2O$ (1QA4) is shown in yellow, 1 wt% butylated starch 29 wt% $H_2O$ (1QB3) is shown in purple and 1 wt% propylated starch 29 wt% $H_2O$ (1QP3) is shown in green. Lattice parameter was obtained from small angle x-ray scattering, measured at 25, 30, 35, 40, 45, 50, 55 and 60°C.*

Lattice parameter of 5 wt% native starch was plotted against the water content of the samples. The lattice parameter increased from 25-35 wt% of $H_2O$ for most samples except at 40°C, which could be where the phase transition lies. From 40-60 wt% the lattice parameter was seen to irregularly decrease (See fig.9). This result shows that the water channel dimension increases with water concentration to some extent below 40 wt% water, but after 40 wt% water, it gradually decreases. This reason could be because it is harder to get homogenous samples the more water there is.

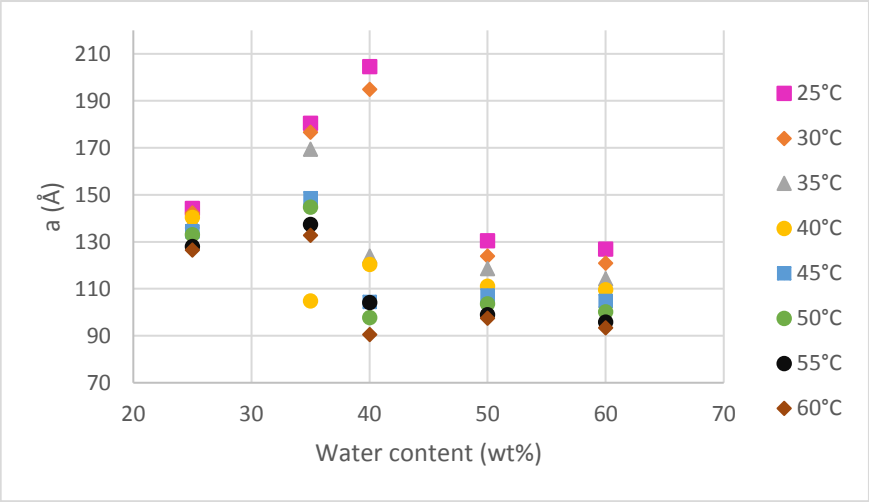



***Figure 9*** *Lattice parameter a (Å) as a function of water content (wt%) in five samples. These were 5 wt% native quinoa starch with 25, 35, 40, 50 and 60 wt% $H_2O$ and were measured on small angle x-ray scattering at eight different temperatures. 25°C is shown in purple, 30°C is shown in orange, 35°C is shown in grey, 40°C is shown in yellow, 45°C is shown in blue, 50°C is shown in green, 55°C is shown in black and 60°C is shown in brown.*

### 3.1.3 Dispersed samples

All dispersion samples that were prepared (see method) did not appear homogenous in water. Chunks of the samples were seen to get stuck on the walls of the glass vials after they were left on roller mixer. When the dispersed samples were measured on SAXS, the intensity (I) was once again plotted against scattering vector (q), and no sharp Bragg peaks were shown due to that the sample is more distributed (see fig.10 and 11). These were measured at 25°C considering that CryoTEM measurements (see below) were measured at this temperature. Lipid LC with starch particles showed spacing ratio √2, √3, √4 and √9 which would correspond to space group Pn3m, but √6 and √8 is not shown. Only the lipid LC showed four peaks, with spacing ratio 1, √3, √4 and √7 which gives the hexagonal phase. As for sample with QA4 and QP3, they also kept the space group Pn3m. QA4 had spacing ratio √2 and √3 whereas QP3 had √2, √3, √4, √6 and √8. QB3 had no good peaks to be identified. For the mixture with starch it kept the symmetry of Pn3m, whereas for only the lipid LC it changed. This could indicate that the starches help to keep the same structure, this is if there is still starch inside the sample. It could also be because the equilibrium time between when the sample was measured in bulk phase, until it was dispersed was long. Then it is possible that it assembled to another phase. Another reason could be that the scattering signals that was recorded, was not of the unit cell dimension as the peaks were very small. It is also possible that the structure of the dispersed samples changed and does no longer contain order as preparation of these samples were hard.

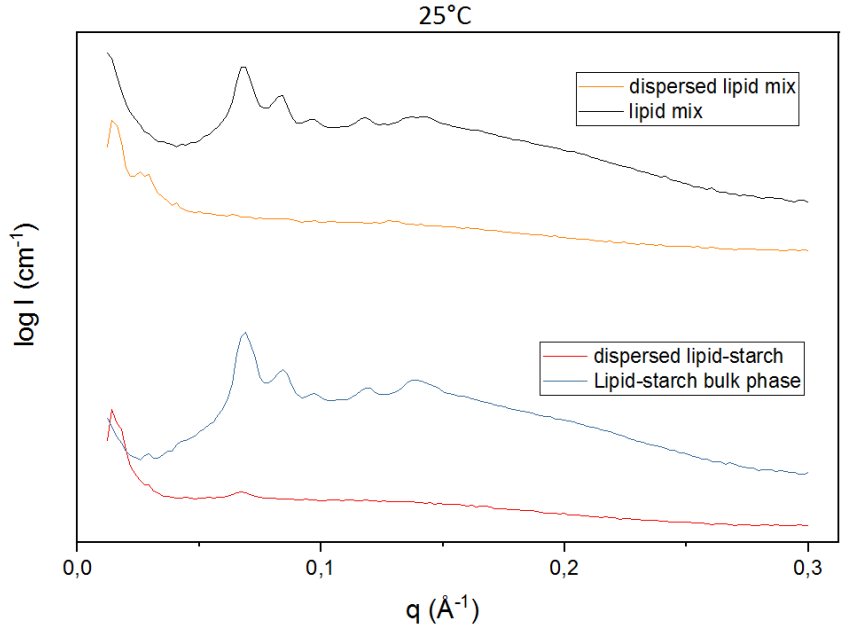



***Figure 10*** *logarithm of intensity I ($cm^{-1}$) as a function of scattering vector q ($Å^{-1}$). Four different mixtures are shown; dispersed lipid mixture (GMO-50/DGMO at ratio 40/60) in yellow, lipid mixture in bulk phase in black, dispersed lipid mixture with starch particles in red and lipid-starch in bulk phase in blue. These dispersed samples were measured for three hours, whereas the bulk phases were measured for 20 minutes. This through small angle x-ray scattering at 25°C with configuration 22.*

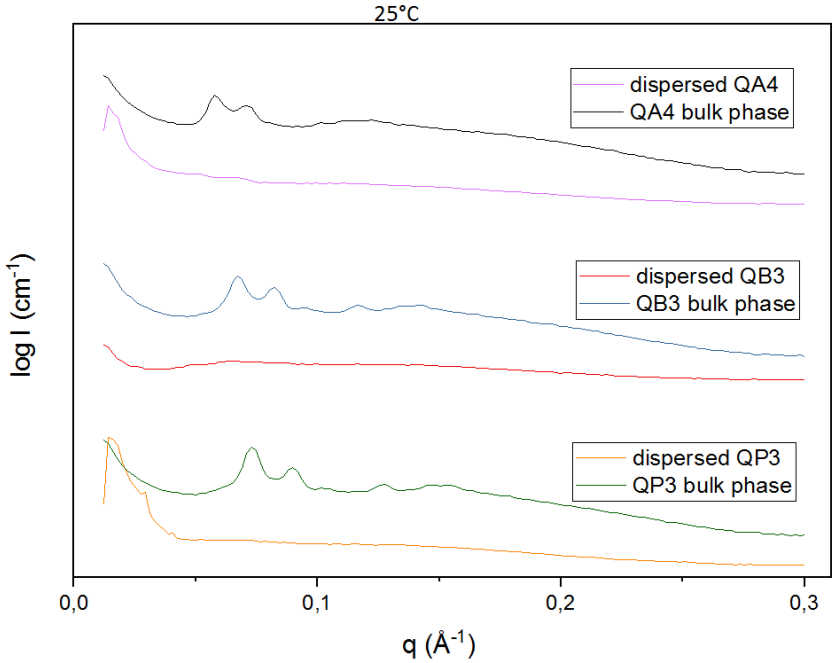
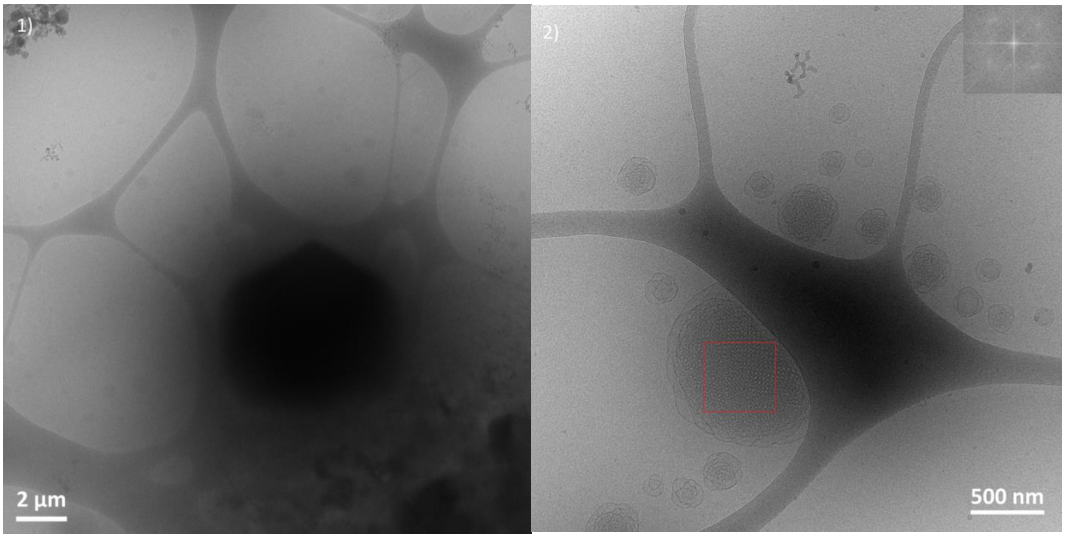



***Figure 11*** *logarithm of intensity I ($cm^{-1}$) as a function of scattering vector q ($Å^{-1}$). Six different mixtures are shown; dispersed lipid mixture (GMO-50/DGMO at ratio 40/60) with acetylated starch (QA4) in purple, lipid mixture with QA4 in bulk phase in black, dispersed lipid mixture with butylated starch (QB3) particles in red, lipid mixture with QB3 in bulk*

*phase in blue, dispersed lipid mixture with propylated starch (QP3) in yellow and lipid mixture with QP3 in bulk phase in green. These dispersed samples were measured for three hours, whereas the bulk phases were measured for 20 minutes. This through small angle x-ray scattering at 25°C with configuration 22.*

## 3.2 CryoTEM of dispersed samples

Three of the dispersed samples; lipid mixture (GMO-50/DGMO 40/60), lipid mixture with native starch and one sample with only native starch, was measured on CryoTEM. The dispersed starch particles are very huge as seen in image 4 (see fig.13). This makes the film, when imaging, very thick whereas less signal is given, and one can see that the particles are very dark. This was done in order to be able to identify the starch particles in the lipid-starch mixture. The sample with only lipid mixture could be seen to have particles that showed some order, but the crystalline ice was bad, and a clear imaging was not made. But when doing the Fast Fourier Transformer (FFT), it does look like hexagonal phase, or it could be something else. The last sample, containing native starch in lipid mixture, showed imaging pictures of finely ordered particles but there was only one starch particle that could be seen (see image 1 fig.12). This could be due to the hard preparation procedure. The starch particles could have been what was stuck on the glass vials, hence not seen in the image. From image 1, it does not seem like the starch particle is interacting with the lipid liquid crystal. But one can see that there are lipid liquid crystal particles around the starch, which means that they can coexist. This result is hard to interpret as only one starch particle was found. When doing the FFT, it seemed to have structure of cubic phase. Bacteria were also found to be present in this sample.



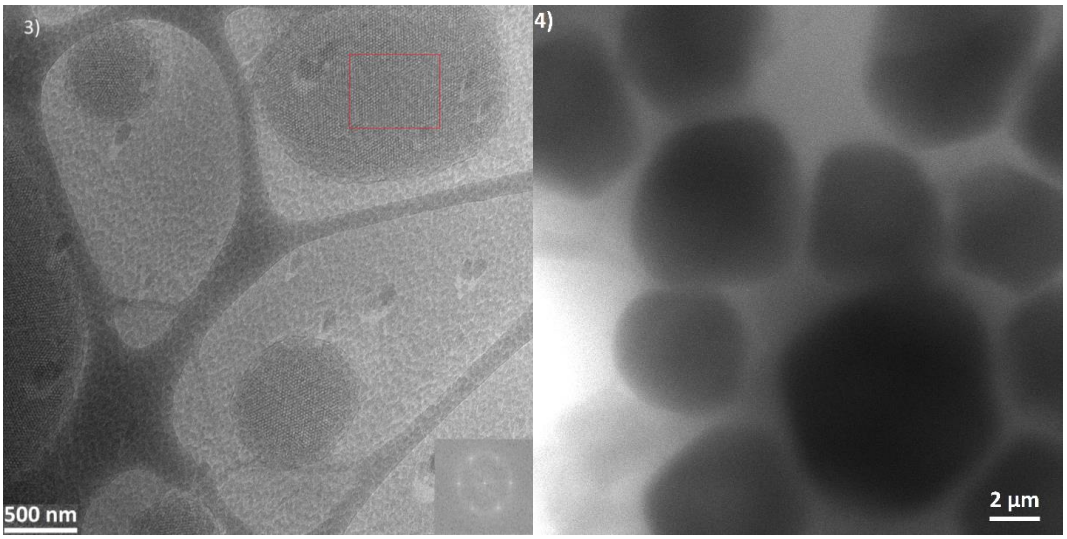



***Figure 12*** *CryoTEM images of dispersed particles. Image 1 and 2 are of lipid mixture with starch, image 3 is lipid mixture only and image 4 are of the starch particles. These samples were prepared through dispersion. Imaging was done through different magnifications, hence the different pixel sizes. Image 2 and 3 had FFT of marked structure shown in the corner.*

## 3.3 Dynamic light scattering of dispersed and starch samples

The starch particles; native, QA4, QB3 and QP3 were measured on dynamic light scattering with temperature control. When 2 mL of starch particle dispersion was put inside the cuvette with 0.5 mL of 20 mM NaCl and measured, it showed that the size of the particles increased with the temperature. This until it reached a certain temperature where it decreased again (see fig. 13). The initial gelatinization probably lies where size suddenly increased - at around 55-60°C. The data showed that the size, given as radius in nm of the starch particles, ranged from approximately 1000-9000 nm.

When an exact weight of the starch particles (0.005 g) was dispersed in 35 mL 20 mM NaCl and measured, the results were different compared to the first measurements. These results showed that the size of particles increased or decreased at different temperatures, without a trend (see fig.14). Results from DLS showed that these starch particles ranged, given as radius in nm, from 500-2200 nm. Each temperature was measured three times, but the standard deviation of this ranged from ±7 to approximately ±700. This standard deviation is quite large, whereas more measurements should have been made at each temperature. Data shows that the largest particles lie around 50-60°C.

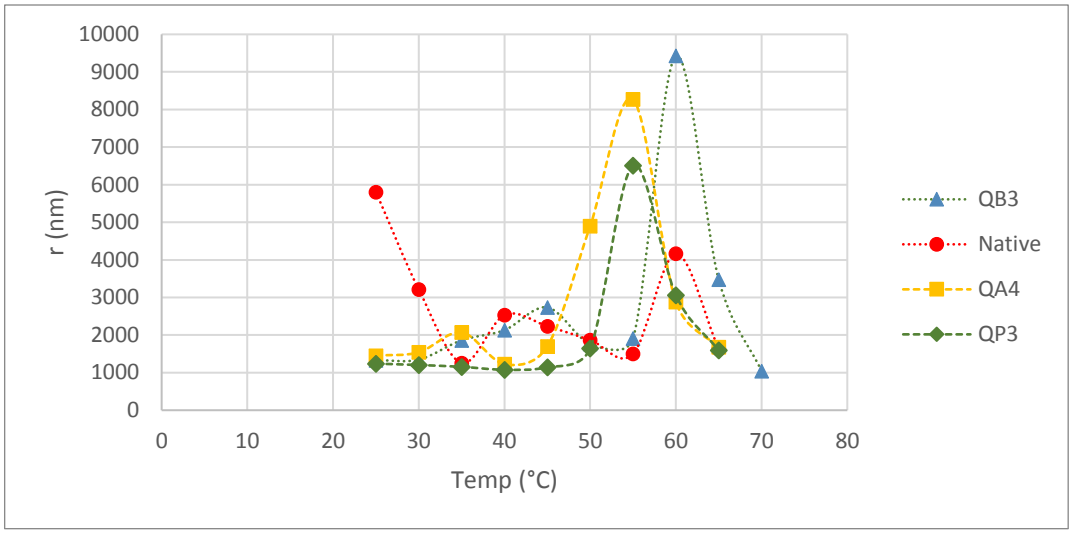



***Figure 13*** *Radius (nm) plotted as a function of temperature (°C) for four different starches; native quinoa starch (native in red), butylated quinoa starch (QB3 in blue), acetylated quinoa starch (QA4 in yellow) and propylated quinoa starch (QP3 in green). These measurements were done by dynamic light scattering in 20 mM NaCl, at ten different temperatures with five minutes of equilibrium time in between.*

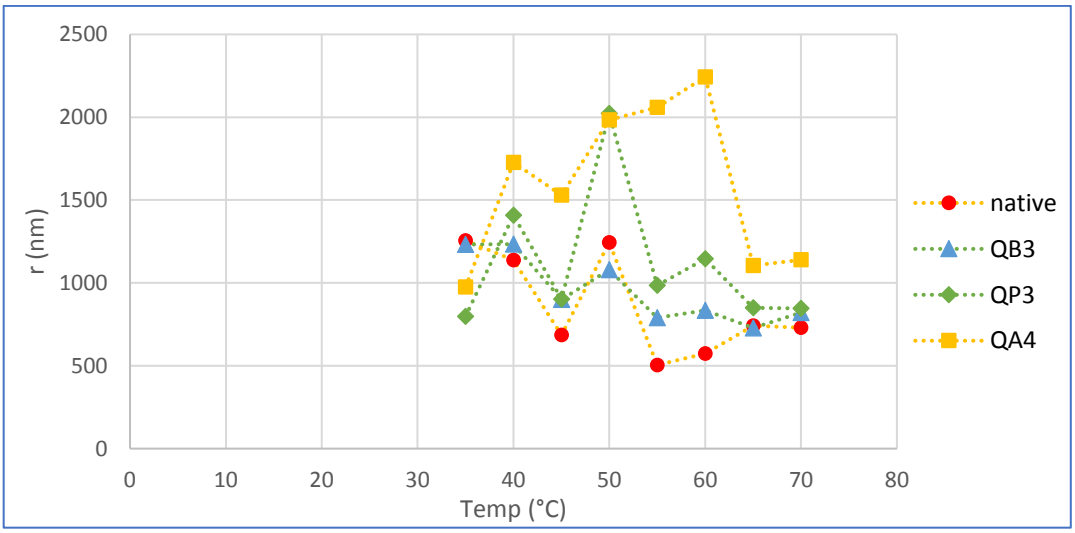



***Figure 14*** *Radius (nm) plotted as a function of temperature (°C) for four different starches; native quinoa starch (native in red), butylated quinoa starch (QB3 in blue), acetylated quinoa starch (QA4 in yellow) and propylated quinoa starch (QP3 in green). These measurements were done by dynamic light scattering in 20 mM NaCl, at ten different temperatures, but only eight are shown, with five minutes of equilibrium time in between.*

From this, a certain size of the particles cannot be remarked. But the initial gelatinization probably lies around 50-60°C. This transition can be crucial, when embedding them into lipid bilayer. With known transition temperature, one could possibly control the release of additives inside lipid matrix. But further studies need to be done in order to recognize the transition and size of these particles.

The dispersed samples of only the lipid mixture GMO-50/DGMO, lipid mixture with native starch, lipid mixture with modified starches and only native starch were also measured on DLS. The average size of the radius of native starch particles were the largest, at 777 ± 32 nm, which is another indication of that the starch particles are large as seen in CryoTEM. The values for the other samples lied around 80-100 nm (see tab.3). Here, it can be assumed that the lipid mixture does not have the large starch particles, as it is around the same size as the sample with only lipid mixture. This is likely due to errors in the preparation of samples. These samples were dispersed longer, compared to the dispersion of starch particles in 20 mM NaCl. An improvement of measuring the size of starch particles, at different temperatures, could be to disperse them the same way as the samples dispersed in water, to possibly decrease the standard deviation.

**Tabel 3.** *All six samples; GMO-50/DGMO 40/60, GMO-50/DGMO 40/60 with 5 wt% native starch, GMO-50/DGMO 40/60 with 5 wt% acetylated starch (QA4), GMO-50/DGMO 40/60 with 5 wt% butylated starch (QB3), GMO-50/DGMO 40/60 with 5 wt% propylated starch and only native starch, were dispersed in 95 wt% MilliQ water. The average size of the particles, given as the radius (nm), was calculated from three measurements in dynamic light scattering. The average size is given with the standard deviation.*

| Sample (dispersed) | Average size (r.nm) |
|---|---|
| **GMO-50/DGMO 40/60** | 85,2 ± 0,54 |
| **GMO-50/DGMO 40/60 with 5 wt% native starch** | 82,7 ± 0,15 |
| **Native starch** | 777 ± 32 |
| **GMO-50/DGMO 40/60 with 5 wt% QA4 starch** | 101 ± 1,7 |
| **GMO-50/DGMO 40/60 with 5 wt% QB3** | 98,1 ± 0,81 |
| **GMO-50/DGMO 40/60 with 5 wt% QP3 starch** | 96,2 ± 0,78 |

# 4. Conclusion

From the following study, it has been shown that starch particles can regulate hydration of lipid liquid crystals when inverse bicontinuous cubic phases with space group Ia3d or Pn3m is formed. What type of bicontinuous cubic phase that forms depends on the concentration of water, temperature or amount of starch particles.

It can be concluded that samples with native starch would assemble to space group Ia3d at low water concentrations and temperatures, whereas with higher temperature and water

concentration, the space group Pn3m was formed. From studies of the influence of water content on the phase-behavior it was found that phase transition occurs at 40°C. With high modified starch concentration (5 wt%), only space group Pn3m was observed. Acetylated and propylated starch showed crystal symmetry of space group Ia3d at high temperatures, which are signs that the polymer possibly influences what type – of the two bicontinuous cubic phase – are formed.

The starch particles, both native and modified, are large and their swelling of size was determined to around 50-60°C. The increase of size is believed to influence the lipid LC system as the unit cell dimension changed. It was also possible to see maintenance of cubic phase when dispersing the samples that have had starch particles in the system. How the starch particles interact or are situated relative to lipid layers requires further studies as based on the few preliminary images obtained from CryoTEM are not enough. One should continue to study the starch particles with lipid liquid crystals for further knowledge of this system. With this, it could be further developed and be applied of using starch for materials based on lipid-polymer responsive layers in, among others, drug- or food industry.

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