Temperature triggering of kinetically trapped self-assemblies in citrem-phospholipid nanoparticles

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Graphical abstract

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Highlights

- Citrem appears to be the stabilizer of choice for the colloidal stabilization of lyotropic non-lamellar liquid crystalline nano-self-assemblies owing to its hemocompatibility and poor activation of the complement system.
- Citrem/SPC nanoparticles are attractive for use in the development of nanocarriers for drug delivery owing to their structural tunability and hemocompatibility
- Batch-to-batch variability in citrem composition is associated with slight alterations in the size, structural characteristics, and surface charge of the produced library of citrem/SPC nanoparticles.
- Citrem inclusion at different temperatures induces lamellar to nonlamellar structural transitions as evident from the appearance of inverse bicontinuous cubic Pn3m and hexagonal (H2) phases upon increasing citrem concentration and varying temperature.

ABSTRACT

Lyotropic non-lamellar liquid crystalline (LLC) nanoparticles are attractive nanocarriers for drug delivery, particularly for the solubilization of poorly water-soluble drugs. Due to the reported problems of complement activation and cytotoxicity of most investigated Pluronic F127-stabilized cubosomes and hexosomes, there is an interest in introducing safe stabilizers for these LLC nanodispersions. Citrem appears to be the stabilizer of choice for the colloidal stabilization of these

LLC nano-self-assemblies owing to its hemocompatibility and poor activation of the complement system. This anionic food-grade emulsifier in combination with soy phosphatidylcholine (SPC) can be used to introduce a library of hemocompatible lamellar and non-lamellar liquid crystalline nanodispersions at different lipid compositions. We found that batch-to-batch variability in citrem composition is associated with slight alterations in the size, structural characteristics, and surface charge of the produced citrem/SPC nanoparticles. Further, we report on the temperature-triggered alterations in these nano-self-assemblies at different lipid compositions by using synchrotron small angle X-ray scattering (SAXS). The addition of citrem at different temperatures induces lamellar to nonlamellar structural transitions as evident from the appearance of inverse bicontinuous cubic $Pn3m$ and hexagonal (H_2) phases, respectively, upon increasing citrem concentration and varying temperature in the range of 5-59 °C. Citrem/SPC nanoparticles are attractive for use in the development of nanocarriers for drug delivery owing to their structural tunability and hemocompatibility.

Keywords: Citrem, cubosomes, hexosomes, inverse lyotropic non-lamellar liquid crystalline phases, nanodispersion, small angle X-ray scattering, nanoparticle tracking analysis, soy phosphatidylcholine

INTRODUCTION

Lyotropic non-lamellar liquid crystalline (LLC) phases are attractive matrices for the solubilization and delivery of hydrophilic and poorly water soluble drugs¹⁻⁶. In particular, the inverse bicontinuous cubic (V_2) and hexagonal (H_2) liquid crystalline phases have a great potential in the development of depots with sustained release of loaded drugs having different physicochemical properties and molecular weights (both small molecules and biomacromolecules).^{5,7-13} These phases

are suitable for the development of platforms for different drug delivery applications that include pain management¹¹, suppression of skin pigmentation¹⁴, and treatments of bacterial infections^{3,15,16}, prostate cancer¹⁷, and respiratory diseases¹⁸. Nevertheless, the high viscosity and irritation on direct contact of these liquid crystalline phases with the biological epithelia lead to difficulties in their handling and limit their use in the development of injectable drug delivery systems, particularly for parenteral applications. Therefore various studies focus on their fragmentation to colloidal aqueous nanodispersions.^{2,4,5,12,19} Among these non-lamellar liquid crystalline (LLC) nanodispersions, cubosomes, hexosomes, and micellar cubosomes are the most investigated nanocarriers for delivery of drugs and essential fatty acids, and for bio-imaging purposes owing to their capability to solubilize hydrophilic, hydrophobic and amphiphilic drugs.^{12,20–25} They consist of nanoparticles enveloping inverse bicontinuous cubic (Q_2) phase, hexagonal (H_2) phase, and micellar discontinuous cubic (I_2) phase of the $Fd3m$ symmetry, respectively.

The most reported LLC nanodispersions, stabilized by the triblock copolymer surfactant Pluronic F127, are prepared by employing a high-energy emulsification method for the fragmentation of the inverse non-lamellar liquid crystalline phases in excess water^{12,19,26–28}. The main lipid constituents are typically unsaturated monoglycerides (e.g. glycerol monooleate (GMO), and monolinolein (MLO)), phytantriol (PHYT), or the combination of these amphiphilic compounds with oil (e.g. vitamin E, medium chain triglycerides (MCT), or oleic acid)^{12,13,19,22,26–29}. Despite the attractiveness of these LLC nanoparticles enveloping tunable nanostructures for pharmaceutical uses, the reported problems of potent complement activation, hemolytic activity, and toxicity of the most investigated F127-stabilized cubosomes, hexosomes, and related LLC nanoparticles inhibit their widespread use for drug delivery and bio-imaging applications^{12,30–33}. In recent reports^{30,33–36}, the anionic food-grade emulsifier citrem, which is citric acid esters of mono- and diglycerides, was introduced as an alternative stabilizer and used to stabilize different LLC nanodispersions. It was found that citrem

modulates the internal nanostructures of GMO/MCT and soy phosphatidylcholine (SPC) nanodispersions and improve their hemocompatibility by bypassing complement activation and overcoming hemolysis.^{30,33}

Among citrem-stabilized nanodispersions, a unique family of citrem/SPC nanoparticles enveloping tunable lamellar or non-lamellar liquid crystalline phases were recently produced by applying either a high-energy or a low-energy emulsification method³⁰. Their structural features are modulated at the investigated temperatures of 25 and 37 °C in excess buffer and also on exposure to human plasma by varying the lipid composition, namely the weight ratio of SPC, a well-known naturally occurring non-toxic phospholipid with negligible hemolytic activity^{30,37}, and citrem, an immune safe stabilizer. In the present work, the main objective is to investigate the temperature-triggered structural changes in citrem/SPC nano-self-assemblies at different lipid compositions and to shed light on the reversibility of the detected phase transitions during a heating-cooling cycle performed in a temperature range of 5-59 °C. Taking into account that a batch-to-batch variability in commercial lipids such as citrem could significantly affect the size and structural characteristics of the produced nanoparticles, we set out to systemically characterise the samples by synchrotron small-angle X-ray scattering (SAXS), and nanoparticle tracking analysis (NTA) and compare our findings with those recently reported by Azmi et al.³⁰ before performing the planned experiments on the temperature-triggered structural alterations in these nano-self-assemblies.

EXPERIMENTAL SECTION

2.1 Materials

Soy phosphatidylcholine (SPC) with 97.6% purity was purchased from Lipoid GMBH (Ludwigshafen, Germany). Grinsted® citrem LR10, which is composed of citric acid monoglycerides and diglycerides was received as a gift from Danisco A/S (Copenhagen, Denmark). It is synthesized from refined high oleic sunflower oil in which oleic acid represents 79.1% of the

total fatty acids³⁸. This food-grade anionic emulsifier mainly consists of the following main monoglycerides and diglycerides: 1,3-diacylglycerides (DAG), 1,2(2,3)-diacylglycerides (DAG), monoacylglycerides (MAG), and glycerol citrate fatty acid esters (GCFE). In accordance with the composition given by the manufacturer, citrem contains 64% of mono- and di-glycerides and 36% of GCFE. In addition, it contains traces of triacylglycerides (TAG) and free fatty acids (FFA)³⁸. 149 mM Dulbecco's Phosphate-buffered solution (PBS) at pH 7.4 was purchased from Sigma Aldrich (Poole, UK). Water was double distilled. All ingredients were used without further purification.

2.2 Preparation of Citrem/SPC Nano-Self-Assemblies

Binary mixtures of citrem and SPC were prepared at the following citrem/SPC weight ratios: 5:0, 4:1, 3:2, 1:1, 2:3 and 1:4, where the total lipid (binary citrem/SPC mixtures) concentration was kept constant at 5.0 wt%. Citrem and SPC were weighed out in a 4 mL glass vial and PBS was then added to the binary lipid mixture and to give 100% of the total nanodispersion weight. The lipid mixture was gently vortexed in excess buffer until obtaining a homogeneous stable milky solution. Further emulsification was done by means of ultrasonication (Ultrasonic Processor Qsonica 500, Qsonica LLC, Newtown, CT, USA) for 5 min in pulse mode (5 s pulses interrupted by 2 s breaks) at 30% of its maximum power (500 W).

2.3 Synchrotron Small Angle X-ray Scattering (SAXS)

Synchrotron small-angle X-ray scattering (SAXS) experiments were performed on the I22 beamline at the Diamond Light Source synchrotron at Harwell Campus (Didcot, UK). An X-ray beam having a wavelength of 0.9998 Å at X-ray energy of 12.4 keV was used. The 2D SAXS patterns were acquired using a Pilatus 2M from Dectris Ltd (Baden, Switzerland). Before carrying out the

continuous temperature scan experiments, static measurements were performed by sealing the samples in the thin-walled glass capillaries at 25 and 37 °C with an exposure time of 5 s per frame with 2 s delay between 3 frames. The temperature ramps during the heating-cooling cycle in the temperature range of 5-59 °C were programmed with scan rates of 2 and 4 °C/min, respectively. The q range was 0.10 to 6 nm⁻¹ ($q=4\pi/\lambda \sin\theta$, where λ is the wavelength and 2θ is the scattering angle). The lattice parameters of the internal inverted-type bicontinuous cubic (V₂) and discontinuous hexagonal (H₂) phases of citrem/SPC nanoparticles were derived from the SAXS diffraction patterns. The software, Dawn version 2.7, available with 2D data reduction pipeline perspective was used for SAXS data reduction. After subtracting the background scattering using PBS buffer, all Bragg peaks were fitted by Lorentzian distributions. We note that in each respective phase regime only the strongest reflections were considered in calculating the corresponding lattice parameters. For the inverted-type micellar phase (the L₂ phase), the scattering profile was characterized by a single broad peak. The characteristic distance d , of this phase was calculated with $d=2\pi/q$.

2.4 Zeta Potential Measurements

The zeta potential of citrem/SPC nanodispersions was measured using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.) equipped with a 633 nm laser and 173° detection optics. The measurements were performed at room temperature on samples diluted 100x in 149 mM PBS and Malvern DTS v.6.34 software (Malvern instruments, Worcestershire, U.K.) was used for data acquisition and analysis. For the viscosity and refractive index, the values of pure water were used.

2.5 Nanoparticle Tracking Analysis (NTA)

Size characterization of the nanoparticles in citrem/SPC nanodispersions was conducted using NanoSight NS300 (Malvern Instruments Ltd, Worcestershire, UK) mounted with a

405 nm laser and a microscope for recording. The Nanoparticle Tracking Analysis (NTA) takes into consideration the properties of both light scattering and Brownian motions of the nanoparticles in order to obtain their size distribution in the nanodispersions. Prior to measurements, the samples were diluted 10^4 times in filtered PBS buffer pH 7.4 prepared from ultra-pure water ($18.2 \text{ M}\Omega\cdot\text{cm}$) to reach particle concentration between 10^8 and 10^9 particles/mL. All samples were size measured in triplicate at room temperature and the data represent the obtained average values of these runs. The measurements were based on individually detected nanoparticles from 5 to 9 videos from different locations in the sample. The recorded videos were analysed using Malvern software (NTA 3.2 Dev Build 3.2.16). PBS buffer was measured as a control at identical instrumental settings.

RESULTS AND DISCUSSION

3.1 Revisiting the size and structural characterization of citrem/SPC nano self-assemblies: batch-to-batch variability in citrem composition

Azmi et al.³⁰ reported on detailed structural, morphological and size characterization of citrem/SPC lamellar and non-lamellar liquid crystalline nanoparticles at different lipid compositions. In addition, a structural mechanism was proposed to explain the role of the embedded citrem molecules at water-SPC interfacial area in the detected lamellar-nonlamellar phase transitions³⁰. In the present study, it was important as a first step to revisit the structural and size characterization of these nano-self-assemblies due to the use of a different batch of citrem but still from the same supplier. It is well known that batch-batch variability could affect the physical properties of the investigated nanoparticles such as size, surface charge and structure, and could particularly lead to a major challenge of maintaining reproducibility during the synthesis of nanoparticles.^{39–43} For

instance, batch-to-batch variation of particle size is one of the major challenges faced in the manufacturing of nanoparticles in pharmaceutical industry.⁴⁴

Table 1 presents the mean and mode nanoparticle sizes as determined by using NTA for citrem/SPC nanoparticles that were prepared at different lipid compositions. Clearly, there was only a slight difference in the mean diameters and mode diameters, which were in the range of 76-133 and 73-112 nm as compared to the mean and mode sizes of those reported by Azmi et al.³⁰, which were in the range of 87-148 and 72-102 nm, respectively. This size and size distribution of the investigated nanoparticles is within the acceptable size range in the development of nanocarriers for drug-delivery applications. In addition to size characteristics, it is important to investigate the surface charge of the nanoparticles that could play an important role in modulating the biodistribution and behaviour of these nanoparticles after administration. The measured zeta potential values ranging from about -15.27 to -32.60 mV were also consistent with those previously reported.³⁰ Citrem is negatively charged food-grade emulsifier and therefore it modulates in a concentration dependent manner the surface charge of the dispersed citrem/SPC nanoparticles. It is typically used for the electrostatic stabilization of emulsions and different lamellar and non-lamellar liquid crystalline nanoparticles.^{30,34,35}

In addition to NTA and zeta potential measurements, it was important to investigate possible structural alterations due to the use of a different citrem batch. **Figure 1A,B** shows SAXS patterns for six citrem/SPC nanodispersions prepared at different lipid compositions and measured at 37 °C and 25 °C, respectively. At 37 °C and citrem/SPC weight ratio of 1:4, two Bragg reflections were detected at q values of 1.05 and 2.09 nm⁻¹ indicating the occurrence of multilamellar vesicles (MLVs) with bilayers having d -spacing of 5.98 ± 0.02 nm (**Table 1**). Increasing citrem/SPC weight ratio to 2:3 led to a structural transition to nanoparticles with biphasic lamellar/inverse bicontinuous cubic $Pn3m$ feature with d -spacing and lattice parameter for these coexisting phases of 5.71 ± 0.01

and 9.39 ± 0.08 nm, respectively. The identification of the coexisting inverse bicontinuous cubic $Pn3m$ phase was based on the detected corresponding first, second, and fourth $Pn3m$ Bragg reflections at q values of about 0.97, 1.10, 1.16 nm⁻¹, respectively. With further increase in citrem/SPC weight ratio to 1:1, the inverse bicontinuous $Pn3m$ phase with lattice parameter of 9.28 ± 0.01 nm was detected based on the detected Bragg reflections at q values of 0.974, 1.122, and 1.70 nm⁻¹. At citrem/SPC weight ratio of 3:2, a SAXS pattern with a Bragg peak spacing ratio of $1:\sqrt{3}:\sqrt{4}$ was detected, representing the first three characteristic reflections for an inverse hexagonal (H_2) phase with lattice parameter of 6.80 ± 0.01 nm. In addition to the newly detected H_2 phase, a shoulder around 0.99 nm⁻¹ and weak peak at 1.70 nm⁻¹ were detected that most likely indicate a coexisting inverse cubic $Pn3m$ phase. At higher citrem content (citrem/SPC weight ratio of 4:1), a transition to a neat H_2 phase was detected with the corresponding Bragg peaks shifted to higher q values of about 1.08, 1.90, 2.2 nm⁻¹ indicating a decrease in the lattice parameter of the H_2 phase to 6.64 ± 0.01 nm with increasing citrem/SPC ratio from 3:2 to 4:1. In absence of SPC, the formation of emulsified L_2 phase (ELP) was detected which is consistent with previous reports^{30,33,34}. A similar phase transition sequence was also observed at 25 °C (**Figure 1B**) and the corresponding lattice parameters and characteristic distances of the identified phases at both temperatures are given in **Table 1**. It should be noted that increasing temperature from 25 to 37 °C did not affect the self-assembled nanostructural features of citrem/SPC nanoparticles (**Table 1**). The only exception was the occurrence of newly coexisting cubic $Pn3m$ phase with a lattice parameter of about 9.39 nm at citrem/SPC weight ratio of 2:3. As compared with the previously reported findings of Azmi et al.³⁰ at 25°C that are also presented in **Table 1**, the use of another batch of citrem was associated with slight changes in the structural features of citrem/SPC nano-self-assemblies.

It is also worth noting that the detected Bragg peaks in all SAXS patterns of nanodispersions containing SPC were depicted on a top of diffuse scattering, indicating the coexistence of the

identified phases with a higher fraction of other vesicular structures (see **Figure 1**). In this respect, the coexistence of uni- and oligo-lamellar vesicles was confirmed by cryo-TEM in the recent report of Azmi *et al.*³⁰.

A structural mechanism was proposed to explain the role of the embedded citrem molecules at water-SPC interfacial area in the detected lamellar to nonlamellar phase transitions³⁰. Briefly, the inclusion of citrem at low concentrations promotes the transition from planar symmetric bilayers with a spontaneous curvature of about zero ($C_0 \sim 0$) to asymmetric bilayers with non-zero spontaneous curvature ($C_0 \neq 0$)³⁰. Therefore, the formation of citrem/SPC vesicles by employing a low-energy emulsification method is attributed to the presence of such a curved state of asymmetric bilayers that enhances the self-closure of citrem and SPC to vesicles in excess buffer³⁰. It was proposed that the occurrence of asymmetric bilayers with lower free energy plays an important role in the spontaneous and low-energy formation of liposomes^{45,46}. At higher citrem concentrations, the transition to an inverse cubic $Pn3m$ phase was suggested to occur through the classical fusion pathway³⁰. A further increase in citrem concentration leads to a higher negative spontaneous curvature that promotes a structural phase transition in the following order: inverse cubic $Pn3m$ phase $\rightarrow$ inverse hexagonal (H_2) phase $\rightarrow$ inverse micelles (L_2 phase)³⁰.

3.2 Effect of temperature on citrem/SPC nano-self-assemblies

Synchrotron SAXS experiments were performed to study the temperature-triggered structural changes in citrem/SPC nano self-assemblies during heating-cooling cycle in the temperature range of 5 to 59 °C and scan rates of 2 and 4 °C/min, respectively. In both the heating and cooling steps, the effect of temperature on the structural features of six citrem/SPC nanodisperisons prepared at different lipid compositions was investigated and representative SAXS patterns are presented in **Figures 2 and 3**. At 35 °C, two selected SAXS patterns for the 1:1 and 3:2 sample are presented in **Figure 4** and show the subsequent diffraction peak fits with the corresponding Miller indices of the

detected H_2 and cubic $Pn3m$ phase marked in blue and green, respectively. In addition, SAXS patterns of citrem/SPC nanodispersions prepared at citrem/SPC weight ratios of 2:3 and 4:1 during the heating step are presented in **Figure 5A,B**.

At citrem/SPC ratio of 1:4, MLVs were formed at 25 and 37 °C as mentioned above. In **Figure 2A**, the two characteristic peaks of the bilayer structure depicted in the SAXS patterns were marked with asterisk. In both heating and cooling steps, these SAXS patterns indicate that variation in temperature did not have significant influence on the bilayer structure in this nanodispersion.

However, at temperatures above 25 °C, a very weak peak was detected at q of about 0.85 nm^{-1} and denoted by x . It was not possible at this lipid composition to use this weak peak for identifying the possible presence of any coexisting phase. Taking into consideration, the tendency of augmenting citrem and varying temperature as discussed below on inducing transition from MLVs to cubosomes with an internal bicontinuous cubic $Pn3m$ phase, we do not exclude that the detected weak peak indicates the presence of traces of coexisting cubic $Pn3m$ phase at temperatures above 25 °C.

At a higher concentration of citrem (citrem/SPC ratio of 2:3), the two characteristic reflections of the bilayer structure are still detected in the diffuse SAXS scattering patterns (**Figure 2B**).

However, at 37 °C, increasing citrem concentration was associated with the detection of additional Bragg peaks at q values of 0.97, 1.10 and 1.66 nm^{-1} indicating a structural transition to inverse bicontinuous cubic $Pn3m$ phase with a corresponding lattice parameter of $9.39 \pm 0.08\text{ nm}$. This phase was still observed in the temperature range of 37 to 59 °C. In the cooling step, It is worth noting that the behavior was different as the inverse cubic $Pn3m$ phase was stable in the temperature range of 27-59 °C and tuned back to a lamellar (L_α) phase with d -spacing of about $6.25 \pm 0.03\text{ nm}$ at about 23 °C. As compared to the temperature-triggered structural changes during heating, the cubic $Pn3m$ - L_α phase transition was lowered from 37 °C to about 23 °C when the temperature was

decreased from 59 to 7 °C. The observed hysteresis is most likely attributed to a longer equilibration time in the transition region than the applied experimental time scales as reported for other lipid-water systems^{22,33,47}. In this respect, the thermal hysteresis may decrease when performing the SAXS experiments at lower temperature scan rates^{22,47}. It is important to note in our study that the applied scan rate during cooling was faster (-4 °C/min) compared to 2 °C/min rise in the heating step. This could contribute to the observed clear hysteresis profile. Moreover, in our present study, the samples were investigated in both heating and cooling steps without having waiting times between the performed experiments; whereas other studies reported on keeping the samples for a certain waiting time (e.g. 600 s) at different respective temperatures when investigating the structural reversibility of the self-assemblies during heating-cooling cycles^{48,49}. In brief, the detected coexisting phases, under the applied experimental conditions, may not represent the equilibrated structures in this system, and can be considered as kinetically trapped self-assemblies in the investigated citrem/SPC nanoparticles.

For both the lamellar and bicontinuous cubic *Pn3m* phases (**Figure 6A**), the *d*-spacing and the lattice parameter, *a*, increased from 6.11 ± 0.05 to 6.25 ± 0.01 nm, and from 9.39 ± 0.01 to 9.74 ± 0.03 nm, respectively, with rates of about 0.004 and 0.01 nm/°C as the temperature increased in the range of 5-59 °C (**Figure 6A**). In the cooling step from 59 to 7 °C, *d*-spacing of the lamellar phase increased slightly from 6.23 ± 0.02 to 6.25 ± 0.01 nm with a rate of 0.005 nm/°C at a temperature range of 7 to 23 °C; whereas a decrease in *a* of the detected cubic *Pn3m* phase from 9.89 ± 0.06 to 9.71 ± 0.01 nm with a rate of about -0.006 nm/°C was found in the temperature range of 27 to 59 °C.

Further increase in citrem/SPC weight ratio to 1:1 led to the formation of bicontinuous cubic *Pn3m* phase that was stable at the investigated temperature range in both heating and cooling steps as seen in **Figure 2C**. The assignment of this phase was based on the recent reported findings of Azmi *et*

al.³⁰ for same nanodispersion at 25 °C, and the detection of its first and fourth Bragg peaks at q values of around 0.95 and 1.60 nm⁻¹, respectively, at temperatures lower than 27 °C (**Figure 2C**). At 25 °C, the corresponding lattice parameter of the cubic $Pn3m$ phase was about 9.27 ± 0.01 nm. Above 27 °C, the second Bragg peak started to be detected at q value of around 1.20 nm⁻¹, which made it possible to detect first, second and fourth Bragg reflections of this phase. Moreover, these detected peaks were shifted slightly to higher q values indicating decrease in lattice parameter from 9.38 ± 0.02 to 9.32 ± 0.05 nm with increase in temperature from 17 to 59 °C.

Coexisting inverse inverse hexagonal (H_2) phase with the cubic $Pn3m$ phase started to evolve at citrem/SPC weight ratio of 3:2 (**Figure 3A**). At 7 °C, the coexisting H_2 phase with lattice parameter of about 6.79 ± 0.02 nm was assessed on the basis of the detection of three Bragg peaks at q values of 1.06, 1.85 and 2.14 nm⁻¹; whereas the corresponding first and fourth Bragg peaks of the bicontinuous cubic $Pn3m$ phase having lattice parameter of 9.08 ± 0.08 nm were detected at q values of 0.98 and 1.68 nm⁻¹, respectively. On increasing temperature, change of the calculated characteristic distance during the heating-cooling cycle was very small and increased from 6.79 ± 0.02 to 6.92 ± 0.04 nm for H_2 phase and decreased from 9.11 ± 0.001 to 9.06 ± 0.04 nm for cubic phase with $Pn3m$ symmetry (**Figure 6B**). In addition to variations in the lattice parameters of the cubic $Pn3m$ and H_2 phases during the heating-cooling cycle, it was interesting to shed light on the effect of temperature change on the first order diffraction peak intensities of these phases. **Figure 7** shows the evolution of SAXS intensities of the first order diffraction peaks of the inverse cubic $Pn3m$ and H_2 phases during the heating-cooling cycle. The first diffraction peak of the inverse cubic $Pn3m$ phase was amplified upon cooling the sample; whereas a decrease in the intensity was observed for the first diffraction peak of the coexisting H_2 phase (**Figure 7A**). In this context, it is interesting to see that 50% of the normalized peak intensity for the cubic phase was observed at lower temperature (about 19 °C) during the cooling stage as compared to about 51 °C in the heating

stage indicating some hysteresis in the temperature behavior of this system (**Figure 7B**). Upon heating from 5 °C to 59 °C, an increase in the normalized peak intensity of the inverse cubic *Pn3m* phase was detected (**Figure 7B**). The intensity of the coexisting H₂ phase on increasing temperature from 5 °C to about 50 °C (**Figure 7C**).

Increasing citrem/SPC weight ratio to 4:1 led to similar results (**Figure 3B**). However, the bicontinuous cubic *Pn3m* phase was stable when heating the sample in the investigated temperature range of 5–21 °C. The lattice parameter for the cubic phase increased only slightly from 8.61 ± 0.02 to 8.66 ± 0.03 nm and for H₂ phase, it increased from 6.44 ± 0.02 to 6.78 ± 0.01 nm as shown in **Figure 6C**. Thus, the lattice parameters were increased with an almost equal rate of about 0.006 nm/°C in both heating and cooling directions for the H₂ phase and about 0.003 nm/°C for the bicontinuous cubic *Pn3m* phase.

Finally, citrem alone formed ELP (L₂ phase) at all the investigated temperature range (**Figure 3C**). The q value decreased from 1.11 to 1.08 nm⁻¹ which resulted in an increase of d from 5.66 ± 0.01 to 5.81 ± 0.02 nm in the temperature range of 5 to 59 °C.

We found that the d -spacing for the L _{α} phase in MLVs and the lattice parameters for both bicontinuous cubic *Pn3m* and H₂ phases slightly increase with increasing temperature. The increase in d -spacing of L _{α} phase is consistent with previous reports on the temperature-triggered structural changes in MLVs based on phospholipids and it was attributed to a simultaneous decrease in the membrane thickness with an increase in the water layer thickness^{50,51}. It was suggested that the temperature increase enhances bilayer undulations and fluctuations that lead to an increase in the d -spacing of the L _{α} phase due to the presence of additional repulsive forces among the adjacent bilayers⁵². Following the same train of thought, the observed increase in the lattice parameters of the internal inverse non-lamellar liquid crystalline phases (the H₂ and cubic *Pn3m* phases) of citrem/SPC nanoparticles is most likely attributed to an increase in the solubilized water in the

hydrophilic nanochannels (an enlargement to water swelling) of these phases with increasing temperature.

CONCLUSIONS

The SAXS investigations on the temperature-triggered structural alterations in citrem/SPC nanoparticles in a range of 5-59 °C revealed structural alterations and eventually to structural transitions to inverse cubic $Pn3m$ phase or H_2 phase depending on citrem content and the investigated temperature. The influence of temperature on the structural parameters of the detected phases and the observation of hysteresis during the applied heating-cooling steps is discussed. We also found using another citrem batch than that used in the recent report of Azmi et al.³⁰ but still from the same supplier led only to slight changes in the size characteristics, and the structural features of citrem/SPC nanoparticles. Briefly, there was only a slight difference in the mean and mode sizes of the produced nano-self-assemblies; whereas zeta potential values were consistent with the previous report³⁰. In agreement with the recent work³⁰, the internal nanostructure of citrem/SPC nanoparticles transformed at 25 °C from MLVs to ELPs *via* cubosomes and hexosomes on augmenting citrem content. This library of aqueous nanodispersions based on the binary lipid mixtures of citrem and SPC are tunable, and promising hemocompatible nanocarriers for drug delivery.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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FIGURE CAPTIONS

Figure 1. Structural characterization of ultrasonicated nanodispersions (CS samples) prepared at different citrem/SPC ratios: 1:4, 2:3, 1:1, 3:2, 4:1 and 5:0. The presented SAXS patterns were measured at 37 °C (A), and 25 °C (B). The Bragg peaks are represented with black and green colours for the corresponding Miller indices of the H_2 and cubic $Pn3m$ phases, respectively. For the lamellar (L_α) phase, the detected two characteristic Bragg peaks are marked with asterisk.

Figure 2. Representative SAXS patterns for the temperature-triggered structural changes in three citrem/SPC nanodispersions prepared at the following citrem/SPC ratios: 1:4 (A), 2:3 (B), and 1:1 (C) during heating-cooling cycle in the temperature range of 5-59 °C and scan rates of 2 and 4 °C/min, respectively, in both heating and cooling steps. In the heating direction, the selected SAXS patterns are represented by red lines and are compared with those taken at same temperatures during cooling the samples (black lines). The Bragg peaks are represented with green colour for the corresponding Miller indices of the cubic $Pn3m$ phase (B-C) and are marked with asterisk for the L_α phase (A-B). The weak peak indicating most likely the presence of traces of cubic $Pn3m$ phase in this citrem/SPC ratio is marked with x (A).

Figure 3. Representative SAXS patterns for the temperature-triggered structural changes in three citrem/SPC nanodispersions prepared at the following citrem/SPC ratios: 3:2 (A), 4:1 (B) and 5:0 (C) during heating-cooling cycle in the temperature range of 5-59 °C and scan rates of 2 and 4 °C/min, respectively, in both heating and cooling steps. In the heating direction, the selected SAXS patterns are represented by red lines and are compared with those taken at same temperatures during cooling the samples (black lines). The Bragg peaks are represented with black and green colours for the corresponding Miller indices of the H_2 and cubic $Pn3m$ phases (A-B).

Figure 4. At 35 °C, two selected SAXS patterns for the citrem/SPC (CS) 1:1 (**Figure 2C**) and 3:2 samples (**Figure 3A**) are presented in (D) and show the subsequent diffraction peak fits with the corresponding Miller indices of the H₂ (blue) and cubic *Pn3m* (green) phases. For the cubic *Pn3m* phase in the 1:1 sample, the characteristic Bragg peaks were detected at q values of about 0.957, 1.170 and 1.658 nm⁻¹; whereas only two characteristic Bragg peaks for this phase were detected in sample 3:2 at about q values of about 0.990, and 1.698 nm⁻¹. The corresponding lattices parameters for the cubic *Pn3m* phases in the citrem/SPC 1:1 and 3:2 samples were 9.29 ± 0.01 and 9.02 ± 0.06 nm. In the CS 3:2 sample, the H₂ phase with a lattice parameter of 6.81 ± 0.01 nm was identified by the detection of its first three characteristic reflections at the following q values: 1.064, 1.846 and 2.137 nm⁻¹.

Figure 5. Representative sample SAXS patterns for the temperature-triggered structural changes in two citrem/SPC nanodispersions prepared at the following citrem/SPC ratios: 2:3 (A), and 4:1 (B) during heating from 5 to 59 °C at a scan rate of 2 °C/min. The Bragg peaks are represented with green colour for the corresponding Miller indices of the cubic *Pn3m* phase (A-B) and with black colour for the H₂ phase (B). For the lamellar (*L_α*) phase (A), the detected two characteristic Bragg peaks are marked with asterisk.

Figure 6. Variation in the d -spacing and lattice parameters of the *L_α* phase, the inverse *Pn3m* cubic phase, and the H₂ phase, respectively, as a function of temperature. The investigated three different nanodispersions were prepared at the following citrem/SPC weight ratios: 2:3 (A), 3:2 (B), and 4:1 (C). The presented results were extracted and calculated from the SAXS experiments performed in the temperature range of 5-59 °C during the applied heating-cooling cycle. During the applied heating and cooling steps, the d -spacing of the *L_α* phase in (A) is represented by full black and open circles, respectively; whereas the lattice parameters of the bicontinuous cubic *Pn3m* (A-C) and H₂ (B,C) phases are represented by full black and open triangles, and full black and open squares,

respectively. In (C), the internal cubic $Pn3m$ phase of the nanoparticles prepared at citrem/SPC weight ratio of 4:1 was only stable in the temperature range of 5-21 °C. The error bars are given in (A-C) for the lattice parameters of the corresponding inverse non-lamellar phases as derived from SAXS experiments.

Figure 7. Variation in the intensities of the first order reflection of the inverse $Pn3m$ cubic phase, and the H_2 phase for citrem/SPC nanodispersion prepared at citrem/SPC ratio of 3:2 in the temperature range of 5-59 °C during the applied heating-cooling cycle (referring to the SAXS data given in **Figure 3A**). (A) Intensity of the first order reflection peaks of the inverse $Pn3m$ cubic phase, and the H_2 phase. The error bars are given as derived from the SAXS experiments presented in **Figure 3A**. (B) and (C) presents the corresponding normalized intensities of the first order reflection peaks of the inverse $Pn3m$ cubic phase, and the H_2 phase, respectively. The intensities in (A), and the normalized intensities for the first order reflection peaks of the bicontinuous cubic $Pn3m$ (B) and H_2 (C) phases are represented by full black and open triangles, and full black and open squares, respectively.

Figure 1

Figure 2

A

B

C

Figure 3**A** **B**

C

Figure 4

Figure 5

A

B

Figure 6

A

B

C

Figure 7

A

B

C

Table 1: Physical parameters of nanodispersions at different citrem/SPC weight ratios

Sample	Citrem :SPC ^a (wt%)	Sample	Space group	<i>a</i> (nm)			Size (nm)		Zeta potential (mV)
				25 °C	37 °C	25 °C ^d	Mean	Mode	
CS 1:4	1:4	MLVs	L_α	6.01±0.01	5.98±0.02	6.28	133±64	112.4±1.6	- 15.27±1.65
CS 2:3	2:3	MLVs ^b	L_α $Pn3m$	6.30±0.03 -	5.71±0.01 9.39±0.08	5.71	123±46	94.3±2.0	- 22.77±1.09
CS 1:1	1:1	Cubosomes	$Pn3m$	9.27±0.03	9.28±0.01	9.10	112±40	95.1±4.1	- 23.41±1.12
CS 3:2	3:2	Hexosomes ^c	H_2 $Pn3m$	6.79±0.04 9.03±0.09	6.81±0.01 8.99±0.07	6.35 -	117±39	103.9±10.3	- 25.37±1.47
CS 4:1	4:1	Hexosomes	H_2	6.53±0.01	6.64±0.01	5.74	119±46	102.4±1.0	- 31.10±2.25
CS 5:0	5:0	ELP	L_2	5.62±0.03 ^e	5.68±0.02 ^e	5.76 ^e	76±34	73.2±36.8	- 32.60±1.21

^aSPC: soy phosphatidylcholine; ELP: emulsified L_2 phase; MLVs: multilamellar vesicles;

a: Lattice parameter of the liquid crystalline (LC) phase.

^bCoexisting cubic $Pn3m$ phase was detected at 37 °C.

^cCoexisting cubic $Pn3m$ phase was detected at 25 and 37 °C.

^dThe recently reported structural parameters of citrem/SPC nanodispersions prepared by using a different batch of citrem³⁰

^e Characteristic distance, $d=2\pi/q$ for emulsified L_2 phase.