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Spectroscopic Determination of Deborah Numbers in Membrane-Mimetic Bolaamphiphile Assemblies

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Abstract

The remarkable stability of Archaeobacteria and their resistance to extreme environmental conditions, such as high temperature (hot springs), high salinity (salt marsh), high acidity (volcanic environment) or low temperature (Arctic or Antarctic) is mostly due to their very stable membranes. Membranes of Archaeobacteria are composed of tetraethers from the lipid family of bolaamphiphiles. The concern in our laboratory is to synthesize bolaamphiphiles that could form vectors with function to carry drugs toward target cells in the body. The new family of bolamphiphiles synthesized has one polar head based on a sugar moiety and the other polar head is glycine betaine, both issued from natural sources (sugar beet, wheat). They are interconnected with a hydrocarbon chain of 12 methylene units. One side hydrocarbon chain, of 8 methylene units is attached to the anomeric position of the sugar moiety. These two molecules are named N-(12-Betainylamino-Dodecane)-octyl β -D-Glucofuranosiduronamide Chloride, abbreviated C8C12 and N-(12-Betainylamino-Dodeca-5,7-diynyl)-octyl β -D-Glucofuranosiduronamide Chloride, abbreviated C8C12X2. These bolaamphiphile molecules organise in membrane-mimetic lamellar structures (L_c , L_{α} , L). They undergo a thermal phase transition upon heating. When cooled back to room temperature, they remain in the undercooled high-temperature phase. In order to follow the relaxation back to the thermodynamically stable phase at 20 °C, the small-angle and wide-angle X-ray spectra were recorded every hour. The Deborah number (De), defined as $De = \text{time of relaxation} / \text{time of observation}$, is a dimensionless number often used in rheology to characterize the fluidity of materials under specific flow conditions. It was evaluated for supramolecular structures of two type of bolaamphiphiles, as the $De(\text{C8C12}) = 35$ and the $De(\text{C8C12X2}) = 62$. These values are typical for solid-like behaviour, which is in accordance with the sticky, paste-like, brownish aspect of these bolaamphiphile assemblies. The time of relaxation was the time for bolaamphiphiles to regain their thermodynamically stable phase at 20 °C and the time of observation was 1 hour, the typical time of recording between two subsequent spectra.

Keywords : bolaamphiphiles, small-angle and wide-angle X-ray scattering, lamellar phases, temperature- and time-driven phase transitions, Deborah number

1. Introduction

The name **bolaamphiphile** describes the molecules with two polar heads connected with a hydrocarbon chain. It is composed of two parts and has two meanings: “bola” and “amphiphile” [1]. “Bola” is referred to two polar heads on two opposite sides of the connecting hydrocarbon chain. The origin of the name is the arm, of South American Indians, for chasing wild cattle. This arm is called “*Bolas*”, and consists of two heavy leather balls inter-connected with a rope. The Indians, while riding their horses, launch this arm between the legs of wild cattle in run. The bolas entangle the legs of the cattle. The cattle drops down and the Indians can approach on their horses and capture it. “*Amphiphile*” means that the molecule has both hydrophobic and hydrophilic character. The hydrophobic part is the hydrocarbon chain, while the hydrophilic part are the two polar heads. Thanks to this amphiphilic character, bolaamphiphiles can spontaneously form supramolecular assemblies, such as vesicles, discs or lamellar phases. The weak intermolecular forces that stabilize these supramolecular assemblies can be hydrophobic interactions, hydrogen forces, π - π interactions or electrostatic interactions [1-8].

The uniqueness of archaeal lipid structures and functions within membranes has prompted a great deal of interest in the use of natural lipids or synthetic analogues as innovative materials for the development of biotechnological applications [9]. Based on these molecules the vectors could be synthesized and used in the field of oral drug delivery, because of their remarkable stability face to intestinal low pH. Bolaamphiphiles may show various membrane-mimetic structures [2].

Cellular membranes are complex assemblies of lipids, proteins and other molecules, and their dynamics includes molecular movements of lipids, such as lateral rotation, transversal rotation (or flip-flop), lateral diffusion and other. In addition, membranes include complex molecules, such as proteins, that may serve as ion-channels and have their own dynamics, connected to their functionality. The complex structure of membranes is even more complex from the point of view of all the processes that happen, on different time-scales, in different parts of the membrane.

The functional importance of the narrow extracellular region acting as an anion selectivity filter was highlighted in the very detailed and well-documented publication of Deneka and co-workers [10]. The authors have elucidated the molecular architecture of volume – regulated anion channels of the LRRC8 family and investigated its relationship to functional properties [10]. Different functions in biological tissues involve time-dependent processes as well. Paulino and co-workers studied structures that reveal the differences between ligand-bound and ligand-free states of a calcium channel in TMEM16A, and when combined with functional experiments suggest a mechanism for

gating [11]. The goal of the present work concerns synthetic lipids that may serve as a model to study the membrane-mimetic behaviour. New lipids of bolaamphiphile family are synthesized, in order to study the relation between the molecular structure and the phase transition between the auto-organised lamellar structures. Further, the relaxation process of molecules involved in phase transitions is examined [12].

Many spectroscopic, diffraction, microscopic and rheological techniques have been employed to better understand and characterize the material properties of supramolecular assemblies [13]. In particular, spectroscopic methods are used to characterize the structure of supramolecular assemblies on the atomic and molecular scales. Diffraction techniques rely on measurements of crystallinity and help in analysing structures of supramolecular assemblies. Small-angle and wide-angle X-ray scattering (SAXS and WAXS) is an universal research tool that may be helpful in the real-time investigation of complex structures while being able to extract structural changes [14-16]. For example, Akkari and co-workers [15] studied the supramolecular structure of the binary thermosensitive hydrogel, which formed micelles. Small-angle and wide-angle X-ray scattering confirmed their sol-gel transition from a lamellar structure at 25 °C to a hexagonal structure at 37 °C [15]. The researches aimed to use these hydrogels for drug-delivery. Both scanning electron microscopy (SEM) and small and wide-angle X-ray scattering analyses displayed similar trends after the incorporation of the drug, with the hydrogel maintaining its supramolecular hexagonal structure. Thus, small-angle and wide-angle X-ray scattering has proven to act as an effective tool to note shapes and structural changes during experimentation. A complementary technique, Small-angle neutron scattering (SANS) is a scattering technique that examines material structures of different types of supramolecular assemblies on the nanometer and micrometer scale, and is particularly useful for better understanding of their microstructure [17-18]. A spectroscopic characterization of optimal conditions necessary for release of liposome contents can be applied to the targeted delivery of therapeutic drugs, as studied in [19]. From the microscopic point of view, Fourier-Transform Infrared Spectroscopy (FTIR) has revealed the molecular orientation and molecular recognition during an order-disorder transition of octadecanoyl ester of 1-(2-carboxyethyl) thymine amphiphilic compounds [20].

Phase transitions of lipids have been observed and characterized by several authors. In 1968 Luzatti and co-workers communicated on X-ray diffraction studies of lipid-water systems [21]. Another study, by Tardieu and co-workers, concerns the structure and polymorphism of the hydrocarbon chains of lipids [22]. Further, the authors have examined cubic phases of lipid-containing systems and

explored elements of a theory and biological connotations [23]. Tristram-Nagle and Nagle have published a very interesting study in 2004, dealing with lipid bilayers: thermodynamics, structure, fluctuations and interactions [24]. Seddon and co-workers studied the polymorphism of lipid-water systems [25] as well as the 3-D hexagonal inverse micellar lyotropic phase [26]. In contrast to lamellar phases, they have studied the structure of the inverted hexagonal (HII) phase and non-lamellar phase transitions of lipids in 1990 [27]. In a recent study, Kulkarni and co-workers have explored the engineering bicontinuous cubic structures at the nanoscale and the role of chain splay [28]. Pabst and co-workers gave a detailed explanation of the X-ray scattering of lipid membranes in reference [8]. Very detailed studies have been performed by Harper and co-workers in their investigation of X-ray diffraction structures of some phosphatidylethanolamine lamellar and inverted hexagonal phases [29]. In their laboratory the authors also studied the intrinsic curvature hypothesis for biomembrane lipid composition and they pointed out the role for nonbilayer lipids [30]. Considering the membrane behaviour, Gruner and co-workers studied the coupling between bilayer curvature elasticity and membrane-protein activity [31].

The variety of the cited studies highlights the importance of lipid membrane studies [32-34]. The small angle and wide angle X-ray scattering is one of the most frequently used methods in investigating the structure of lipid membranes. An alternative technique proposed by Gause and co-workers deals with spin label study of the effects of hydrostatic pressure and temperature on cellular lipids [35].

Membranes often have channels that involve the ion transport over their surfaces. Proteins usually play role of ion channels. In the group of lipids, cholesterol can be involved in membrane transport. In some cases, Bolaamphiphiles can take the role in membrane structure. A certain percentage of bolaamphiphiles in the membrane, may rigidify the membrane. It is known that membranes made uniquely of tetraethers in Archaeobacteria, are particularly stable. This feature is supposed to be the key of the survival of Archeobacteria in very harsh environments such as high temperature (hot springs), high salinity (salt marsh), high acidity (volcanic environment) or low temperature (Arctic or Antarctic).

The Deborah number is a dimensionless number often used in rheology to characterise the fluidity of materials under specific flow conditions. It is defined as the ratio of the time of relaxation of the material over the time of observation, equation (1).

$$De = \frac{\text{time of relaxation}}{\text{time of observation}} \quad (1)$$

It quantifies the observation that given enough time even a solid-like material might flow, or a fluid-like material can act as solid when it is deformed rapidly enough.

Marcus Reiner introduced Deborah number in rheology in 1964 [36]. He chose the name inspired by a verse in the Bible, stating: "*The mountains flowed before the Lord*" in a song by the prophetess Deborah in the Book of Judges 5:5. Together with Eugene C. Bingham, Marcus Reiner was the founder of rheology in 1920. The term "*Rheology*" is inspired by the aphorism attributed to the greek philosopher Heraclitus "*panta rhei*", ("πάντα ῥεῖ") "everything flows". In this context, the mountains would flow before the Lord, because his time of observation is infinite, while for the human, in his lifetime, the mountains appear as solid.

Various studies have used the concept of Deborah number in order to relate relaxation behaviour of the system investigated to the time of observation.

In the recent review article, Hancock discusses recent advances in the understanding of disordered (or "amorphous") materials as potential drug vectors [37]. These concepts have made it feasible for pharmaceutical scientists to confidently predict the properties, performance and stability of high-energy materials so they may be considered to be a viable option for delivering the medicines of the 21st century. An approach that the authors have recently used to assess the likely performance of amorphous pharmaceutical samples is to calculate the Deborah number (De), which is most commonly used in the field of rheology to rank the viscosity of fluids and semi-solids. The Deborah number is considered as the ratio of the time-scale for molecular motion to the desired reference time-scale, and it can be considered to be the index of relative molecular mobility. In the context of pharmaceutical product stability, De values of significantly greater than unity indicate a stable system over the reference time-frame. Typically, a laboratory experiment takes less than 24 h, whereas a typical pharmaceutical stability requirement is 3 years (26280 h). If an amorphous drug sample is determined to have a molecular relaxation time of, for example, 300 h at ambient conditions, it would have a Deborah number of 12.5 (300h/24h) for the laboratory experiment and just 0.01 (300h/26280h) for the stability study. One can see that, using this approach, such a sample would be judged to be stable for the purposes of the laboratory experiment, but that it would not be sufficiently inert to survive long-term storage as a pharmaceutical product [37].

Another study, performed by Babak and Desbrières, considers Deborah number for the viscoelasticity of adsorption layers of amphiphilic polyelectrolytes [38]. In their work, the fluidity of adsorption layers of amphiphilic polyelectrolytes (chitosan derivatives) is characterized by the Deborah number defined as the ratio of the relaxation frequency of the material and the frequency of the applied deformation [38].

In the field of polymers, Bourrigaud and co-workers have studied the draw-ratio – Deborah number diagram as a useful tool for coating applications [39]. The understanding of the basic physical effects of viscoelasticity on drawing performances in the coating process leads to a useful approach to link the rupture of the polymer melt to critical processing conditions. In particular, the authors show that when solving the drawing problem in the air gap with a simple constitutive equation – like the upper converted Maxwell model – a mathematical inconsistency appears for some drawing parameters. This mathematical instability may be experimentally correlated to the occurrence of melt-rupture, giving rise to a discussion on the effect of viscoelastic properties on drawing performances. The authors give the results in terms of a diagram representing the maximum drawing ratio (Dr) with respect to the Deborah number (De). A master curve, obtained from experimental results, accounts for the temperature, melt-index, air-gap height and extrusion output dependences [39].

Kenney and co-workers performed a more mechanistic study and studied large Deborah number flows around confined microfluidic cylinders [40]. Viscoelastic flow around a confined cylinder at high Deborah numbers is studied using microfluidic channels. By varying fluid properties and flow rates, a systematic study of the roles of elasticity and inertia is accomplished. Two new elastic flow instabilities that occur at high Deborah numbers are identified. A downstream instability of disordered and temporally varying streamlines is observed at a Deborah number above 10. This instability is a precursor to an unsteady vortex that develops upstream of the cylinder at higher Deborah numbers. Both instabilities occur at moderate Reynolds numbers but are fundamentally elastic. The Deborah and elasticity number primarily controls the size and steadiness of the upstream vortex [40].

In the present work the Deborah number is evaluated for lipid bolaamphiphile assemblies during their relaxation from one lamellar phase to the other. Concerning the complexity of movements in membranes, this approach can be useful to study the interdependence of various physiological processes, in particularly those that are concerned by the phase transitions.

2. Experimental section

2.1. Materials. Preparation of bolaamphiphiles and formulations. All commercially available chemicals were used without further purification, and solvents were carefully dried and distilled prior to use. Unless otherwise noted, nonaqueous reactions were carried out under a nitrogen

atmosphere. Analytical TLC was performed on Merck 60 F254 silica gel nonactivated plates. A solution of 5% H₂SO₄ in EtOH was used for column chromatography. ¹H and ¹³C NMR spectra were recorded at 400 and 100 MHz, respectively. The procedure of preparation is reported previously in references [41, 4, 42]. Molecules analysed in the present paper are represented in Figure 1. These are the following: **a (C8C12)**: *N*-(12-Betainylamino-dodecane)-octyl β-D-Glucofuranosiduronamide Chloride; **b (C8C12X2)**: *N*-(12-Betainylamino-dodeca-5,7-diyne)-octyl β-D-Glucofuranosiduronamide Chloride. Abbreviations used for different molecules indicate the number of carbon atoms in the side chain and the number of atoms in the main bridging chain as well as the presence or the absence of conjugated triple bonds. Thus, the molecule **a** contains 8 carbon atoms in the side chain and 12 carbon atoms in the main chain (C8C12) and molecule **b** contains the diacetylenic unit in addition designated by X2 (C8C12X2).

The dry samples were prepared by freeze-drying and kept in the refrigerator prior to use. They were thermally equilibrated overnight at room temperature and for 1h at each given temperature to record the spectra [42].

2.2. Methods. Small and Wide Angle X-ray Scattering (SAXS/WAXS). *X-ray Diffraction.* X-ray scattering experiments were performed using a FR591 Bruker AXS rotating anode X-ray generator operated at 50 kV and 50 mA with monochromatic Cu K_α radiation ($\lambda = 1.541 \text{ \AA}$) and point collimation. X-ray patterns were collected with a Mar345 Image-Plate detector (Marresearch, Norderstedt, Germany). The monochromatic Cu K_α radiation ($\lambda = 1.541 \text{ \AA}$) was directed with a 350 $\mu\text{m} \times 350 \mu\text{m}$ focal spot at 323 mm by a double reflection on an elliptic cross multilayer Montel mirror (Incoatec, Geesthacht, Germany). The beam was defined under vacuum by four motorized carbon-tungsten slits (JJ-Xray, Roskilde, Denmark) positioned in front of the mirror (500 $\mu\text{m} \times 500 \mu\text{m}$). Four additional guard slits (750 $\mu\text{m} \times 750 \mu\text{m}$) were placed at the focal point with a 220 mm slit separation distance. The flux after the output mica windows was 3×10^8 photon/s. A 2 mm diameter square lead beam stop was placed under a vacuum at 270 mm; afterward, the sample and the detector were positioned at 420 mm. The X-ray patterns were therefore recorded for a range of reciprocal spacing
 $q = 4\pi \sin \theta/\lambda$ from 0.03 to 1.6 \AA^{-1} , where θ is the diffraction angle. The repetition distances $d = 2\pi/q$ should be between 200 and 3.9 \AA . The samples were placed into 1.5 mm glass capillaries (Glas W. Müller, Germany) and introduced into a homemade capillary holder, which can maintain up to 20 capillaries at a controlled temperature. The procedure is always to take a spectrum after 1 hour of thermal equilibration. We use the programs *Fit 2D* and *IgorPro* to treat the data [43].

The experiment is conducted in such a way that the compound is stabilised at the room temperature (20 °C) and subsequently the temperature is increased up to 70 °C in steps of 10 °C. At each variation of the temperature, the compound is thermally stabilised for an hour before taking a spectrum [42].

Once arrived at 70 °C, the system is cooled down to 20 °C, where it is stabilised for an hour. At this temperature the spectra are recorded in order to observe the evolution of the system in time. Subsequently the relaxation is followed for the system, from the undercooled high-temperature phase to the thermodynamically stable phase at the constant temperature of 20 °C [44].

3. Results

3.1. Description of molecules studied in this work

The bolaamphiphiles in this study are N-(12-Betainylamino-Dodecane)-octyl β -D-Glucofuranosiduronamide Chloride, abbreviated C8C12 (Figure 1a) and N-(12-Betainylamino-Dodeca-5,7-diynyl)-octyl β -D-Glucofuranosiduronamide Chloride, abbreviated C8C12X2 (Figure 1b). One type of bolaamphiphiles (C8C12) has a saturated main hydrocarbon chain, while the other type (C8C12X2) has a diacetylenic unit in the middle of the main hydrocarbon chain. Both type of molecules have one sugar polar head, while the other polar head is based on glycine betaine. The origin of the two polar heads are sugar beet and wheat. Both bolaamphiphiles have a side hydrocarbon chain of eight methylene units that is attached to the anomeric position of the sugar polar head. The supramolecular assemblies studied in this work contain just one type of bolaamphiphiles.

In the Table 1 we give the temperature dependence of interlamellar distances as well as the phase assignation for the dry compounds C8C12 (dry) and C8C12X2 (dry) [42,44]

Table 1 Interlamellar distances and lamellar phases for C8C12 and C8C12X2 upon heating and cooling (Figures 2a and 2b).

C8C12 (dry)		
T /°C	d/Å	phase
heating		
20	26.65	L _c
30	26.21	L _c
40	24.74	L _{α}
50	24.02	L _{α}
60	23.55	L _{α}
70	23.21	L _{α}
cooling		

60	23.44	L_{α}
50	23.67	L_{α}
40	24.01	L_{α}
30	24.37	L_{α}
20	24.76	L_{α}

C8C12X2 (dry)		
T /°C	d/Å	phase
heating		
20	23.8	L + L_{α}
30	23.4	L
40	22.8	L
50	22.4	L
60	22.1	L
70	22.0	L
Cooling		
20	24.1	L

In dry compounds, the change of fluidity of the bridging chain is at the origin of differences in interlamellar distances. At higher temperatures, the chains are more fluid, so the polar heads on the opposite side of the chain are nearer to each other. As the result, the interlamellar distance decreases with increasing temperature. Upon cooling down, the interlamellar distance increases but the high-temperature phase is conserved for hours or days.

3.2. Interpretation of the small angle and wide-angle X-ray scattering spectra

3.2.1. The temperature dependence of the phase transition and spectral analysis

The Small-Angle and Wide-Angle X-ray scattering on the bolaamphiphile C8C12 provides a signature of the lamellar crystalline phase at 20 °C, Figure 2a. Indeed, there are many peaks in the small-angle region, as well as in the wide-angle region. Upon heating, at intervals of 10 °C, up to 70 °C, the signature of the supramolecular structure gradually changes to lamellar fluid phase. There is a distinct difference between the spectrum at 30 °C and the spectrum at 40 °C. In the fluid lamellar phase, there is one strong peak in the small-angle region, and two equidistant harmonic peaks. This phase is present from 40 °C to 70 °C and back to 20 °C. An interesting feature is that for this measurement process (one hour thermal equilibration between two subsequent spectra), the system remains in the undercooled high-temperature phase, that is the lamellar fluid phase, without returning to the starting lamellar crystalline phase, which is the thermodynamically stable phase at 20 °C [44]. In Figure 2b, the analogue investigation of C8C12X2 is represented. The lamellar

crystalline (L_c) and lamellar fluid (L_α) phases of bolaamphiphiles are schematically presented in Figure 3a. Usually the crystalline phase is present at lower temperatures, while the fluid phase is present at elevated temperatures.

The interlamellar distance can be deduced by using the Equation 2, where q is the position of the peak in the spectra (in \AA^{-1}) and d is the interlamellar distance (in \AA). The first peak corresponds to the inverse value of the interlamellar distance in this fluid lamellar structure.

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

For the molecule C8C12X2, the temperature dependence of spectra is given in Figure 2b. For the spectrum at 20 °C we can deduce the coexistence of the lamellar fluid phase (L_α) from the sharp peak, and the lamellar isotropic phase (L), from the large peak. By increasing the temperature, the signature of the lamellar fluid phase is lost, and the isotropic lamellar phase is present all over thermal cycling to 70 °C and back to 20 °C [44]. The large peak is a result of the polydisperse signal on uni-lamellae samples that are not stacked to one another. It can be interpreted as the form factor for one lamellae. The scattering of X-rays results in a large peak. On the contrary, for the multi-lamellar structure of C8C12, the peaks are sharp. The isotropic lamellar phase, L, is schematically represented in Figure 3b. The isotropic lamellar phase may as well be composed of short discs or vesicles, oriented in a random way. Figure 4 is the optical microscopy photograph of the two samples: C8C12 (1a) and C8C12X2 (1b), by the magnification 10x. C8C12 presents the so-called Maltese crosses, that are the signature of the lamellar fluid or lamellar crystalline phase. C12C8X2 shows myeline-like structures due to the presence of uni-lamellar phases or the enrolled structures.

3.2.2. The relaxation following the phase transition

Relaxation from the undercooled high-temperature phase to the initial thermodynamically stable phase takes place at 20 °C. The time of the observation of the relaxation is on the scale of one hour. In Figure 5a we see the relaxation spectra for bolaamphiphiles C8C12. At first, the molecules are in the lamellar fluid undercooled phase. After around 35 hours, they start to relax to the crystalline lamellar phase, which is the initial, thermodynamically stable phase at 20 °C. In Figure 5b the spectra represent the relaxation from the undercooled lamellar isotropic phase to the mixture of phases: lamellar isotropic and lamellar fluid phase, after 62 hours. This mixture of phases was initially present at 20 °C, before the thermal cycle.

Following the thermal cycling of the sample, the relaxation to the initial structural phase is recorded at 20 °C (Figure 5a). For C8C12 the phase transition occurs from the undercooled fluid lamellar phase ($L_{\alpha,u}$) to the thermodynamically stable crystalline lamellar phase (L_c) at 20 °C. The spectrum typical for the lamellar fluid phase has the same shape for over 30 hours. Between 30 hours and 35 hours a phase transition starts to the lamellar crystalline phase [44]. This slow relaxation is a feature of C8C12 bolaamphiphile molecules related to their amphiphilic character. The movements of bolaamphiphile molecules, in order to regain the crystalline lamellar phase, may be slow. They may partially involve the simultaneous transition of two polar heads through the lipophilic domain of hydrocarbon chains in lamellar structures. This transversal rotation, or the flip-flop movement, is particularly slow for bolaamphiphile lamellar structures, due to hydrophobic barrier of hydrocarbon chains to both polar heads.

Figure 2b shows the temperature dependence of the spectra of C8C12X2 supramolecular assemblies, by heating from 20 °C up to 70 °C and cooling back down to 20 °C in steps of 10 °C. Initially, at 20 °C a mixture of two phases can be observed: the lamellar fluid phase with a main sharp peak and the first harmonic and the lamellar isotropic phase represented by a large signal in the small angle region. At 30 °C the compound is in the isotropic lamellar phase that remains present up to 70 °C. Upon cooling back to 20 °C the compound remains in an undercooled isotropic lamellar phase. In Figure 5b, the evolution in time of C8C12X2 is shown. The isotropic lamellar structure remains stable for 62 hours. After 12 h a weak signal appears that is due to the lamellar fluid structure and this signal is more pronounced after 62 hours where the correspondent first harmonic is observed as well. Thus the initial configuration is obtained that is the mixture of the lamellar fluid phase L_{α} and the lamellar isotropic phase, L. Even after 80 hours, this coexistence of two phases remains present.

4. Discussion

In the previous study, the small-angle and wide-angle study of slow relaxation following phase transitions of new bolamphiphiles was reported [44]. The subject of this publication was the investigation of the polymorphism and its time dependence of a new series of bolaamphiphile molecules based on *N*-(12-Betainylamino-dodecane)-octyl β -D-Glucofuranosiduronamide Chloride. The role of the nature of the hydrocarbon chain as well as the role of the diacetylenic unit on the molecular structure and supramolecular assemblies were highlighted. In addition, the relaxation behaviour of six molecules after the thermal cycling was recorded. For the molecules with longer chain lengths, the undercooled high temperature phase is stable over 30 days for C8C22 and for C4C22X2 the corresponding undercooled high temperature phase is stable over 7 days, when we

stopped recording. The compound C4C32X2 evolves from one type of lamellar crystalline structure at 20 °C to the tilted lamellar structure L_{β}' at 60 °C. The evolution to lamellar crystalline structure, with slightly different rearrangement after some 20 hours is recorded as small and wide-angle X-ray scattering spectra. C4C32X2 self-assembly is also observed under polarisation optical microscope as filaments or nanotubes [42].

4.1. Hysteresis upon thermal cycling. The thixotropic behaviour of the material is related to the phase transition.

In Figure 6 the heating and cooling cycle for C8C12 is shown. The phase transition upon heating occurs between 30 °C and 40 °C. We deduce the occurrence of the phase transition from the more pronounced variation of the interlamellar distance. The interlamellar distance is calculated from the small-angle and wide-angle spectrum in Figure 2, by using the equation (2). In the crystalline lamellar phase the interlamellar distance is bigger than in the fluid lamellar phase because in the crystalline lamellar phase the hydrocarbon chains are stretched and thus longer, than the free-moving chains in the lamellar fluid phase, as shown in Scheme 2. The sample is heated from 20 °C to 70 °C in increments of 10 °C and it is thermally equilibrated at a given temperature for one hour, before recording the spectrum. The same protocol is applied during cooling the sample from 70 °C to 20 °C. At the constant temperature of 20 °C, a slow relaxation is monitored, as shown in Figure 5a, for C8C12. The interlamellar distance during the relaxation increases from 24.8 Å to 26.7 Å. In the same table, the phase of the system is indicated as well. We can deduce three cases: the lamellar fluid phase (L_{α}), the lamellar crystalline phase (L_c) and the undercooled lamellar fluid phase ($L_{\alpha,u}$), present during the cooling process. This cooling process is almost linear in interlamellar distance as a function of the temperature, as we can see in Figure 6 [44].

4.2. Relaxation from the undercooled lamellar fluid phase to the crystalline lamellar phase at 20 °C

In Figure 7 the evolution of the interlamellar distance is represented as a function of time, during the relaxation process for C8C12, from $L_{\alpha,u}$ to L_c lamellar phases. The system is in the undercooled lamellar fluid phase at 20 °C after the thermal cycling. With time, it starts to change to the lamellar crystalline phase. This happens after 35 hours from the beginning of the relaxation. An overall

increase in lamellar distance is observed from 24.8 Å (undercooled lamellar fluid phase), to 26.7 Å (lamellar crystalline phase). In a large sense, this process is similar to devitrification, because the system relaxes from a more disordered lamellar fluid phase to the more ordered crystalline lamellar phase, at a constant temperature of 20 °C. One region where the interlamellar distance changes significantly is observed in the time interval from 1 to 15 hours (24,8 Å to 26,0 Å) and between 35 to 50 hours (26.1 Å to 26.5 Å). This is followed by a steady period between 50 and 60 hours followed by the increase of lamellar distance to 26.6 Å between 65 and 70 hours. It is interesting to note that the relaxation is not a linear process because the fluidity of chains is modulated, as well as their arrangement. Figure 7a shows that the relaxation is not at all linear. In some regions, the system is stable over larger time-periods, for several hours. Each spot in the diagram corresponds to a peak maximum characteristic for the lamellar fluid phase that shifts towards larger interlamellar distance, d , as the system adopts the initial lamellar crystalline structure. In Figure 7b the data are fitted to the classical Kolmogorov-Avrami equation (Equation 3) with the accuracy of 95%. For this fit the coefficients k and n are (24.99 ± 0.023) and $(14.0 \pm 2.6)10^{-3}$, respectively.

$$d(t) = 1 - \exp(-kt^n) \quad (3)$$

The Komologov-Avrami model is the most used model for the description of the isothermal phase kinetics, developed in 1940, which relates the kinetic experimentally determined with the growth form and the final structure of the crystalline network [45]. The Komologov-Avrami equation describes how solids transform from one phase to another at constant temperature. Transformations are often seen to follow a characteristic s-shaped, or sigmoidal, profile where the transformation rates are low at the beginning and the end of transformation but rapid in between. The initial slow rate can be attributed to the time required for a significant number of nuclei of the new phase to form and begin growing. In our case this refers more to the alignment of lipid chains and their fluidity. The shape of the curve obtained experimentally does not fit well to the Kolmogorov-Avrami equation because the change of the interlamellar distance is fast at the beginning and rather slow in the middle of the process (Figure 7).

In Figure 8 the time-dependence diagram is represented for the phase transition of the C8C12 lamellar phases. The starting phase is lamellar crystalline phase at 20 °C. Upon heating, the phase transition occurs to lamellar fluid phase. Cooling back from 70 °C to 20 °C the system remains in the

lamellar fluid, undercooled phase. During 35 hours, the system relaxes to lamellar crystalline phase and after 35 hours the signature of the lamellar crystalline phase is recorded. The system regains the thermodynamically stable phase after 35 hours of relaxation.

4.3. Deborah number

The definition of the Deborah number (De) is the ratio of fundamentally different characteristic times, the “relaxation time” and the “time of observation”, typically taken to be the time scale of the process, Equation (1). Materials that have low relaxation times flow easily and as such show relatively rapid stress decay. Consequently, it is important to consider the relaxation time of the process compared to the time of observation in order to state whether the material is fluid or solid. This incorporates both the fluid-like and solid-like aspect of the material. At lower Deborah numbers, the material behaves in a more fluid like manner, with an associated Newtonian viscous flow. At higher Deborah numbers, the material behaviour enters the non-Newtonian regime, increasingly demonstrating solid like behaviour. Following the definition of Deborah’s number, the ratio considered for our investigation is the time of the relaxation of bolaamphiphiles at the temperature of 20 °C, divided by the time of the observation, which is at the timescale of one hour. The relaxation takes place from the undercooled high-temperature phase to the initial, thermodynamically stable phase after the completed thermal cycling of bolaamphiphiles and it is characterised by a constant temperature of 20 °C. The only parameter that changes is the time and the only force applied to the material is the gravity, which is constant for the given experimental setup. We determine the Deborah’s number of two different compounds of the same family, the saturated bolaamphiphile (C8C12) and the unsaturated bolaamphiphiles (C8C12X2), of the chemical structure indicated in Figure 1.

In C8C12, the phase transition, as a function of temperature, from the lamellar crystalline phase, L_c to the lamellar fluid phase, L_α , occurs between 30 °C and 40 °C. After completing the thermal cycle, the system starts to relax back to the initial phase after 35 hours at 20 °C. The saturated bolaamphiphiles C8C12 form crystalline lamellar (L_c) phases at 20 °C, as can be deduced from the small angle and wide-angle X-ray scattering spectrum, Figure 2. Upon increasing the temperature up to 70 °C the structural phase transition progressively occurs to the lamellar fluid phase (L_α). The interlamellar distance in the lamellar fluid phase is shorter because the hydrocarbon chains move more freely and are no more stretched as in the crystalline lamellar phase (L_c).

From the relaxation spectrum, the Deborah number for C8C12 is easily calculated from Equation 1, as shown in Equation 4:

$$De (C8C12) = \frac{\text{time of relaxation}}{\text{time of observation}} = \frac{35 \text{ h}}{1 \text{ h}} = 35 \quad (4)$$

On the contrast, in the unsaturated bolaamphiphile assemblies, C8C12X2 the initial structure is the coexistence of the lamellar fluid phase and the lamellar isotropic phase. By heating to 30 °C the transition to the lamellar isotropic phase, L, is complete. The large peak is observed, as a result of the polydispersed signal on shorter lamellae that are distributed in a random way, typical for the isotropic lamellar phase. After completing the thermal cycle back to the initial temperature of 20 °C, the compound remains in the undercooled isotropic lamellar phase. This structure remains stable for over 62 hours, when the neat signal appears that is due to the lamellar fluid structure, L_α. Even after 80 hours, this coexistence of two phases remains present. The Deborah number for C8C12X2 can be determined by Equation 1, that leads to result presented in Equation 5:

$$De (C8C12X2) = \frac{\text{time of relaxation}}{\text{time of observation}} = \frac{62 \text{ h}}{1 \text{ h}} = 62 \quad (5)$$

Resulting Deborah's numbers of membrane-mimetic bolaamphiphiles are relatively high, which is in accordance with their sticky, brownish, paste-like aspect. In the present work we deduce the order of magnitude of Deborah number, by monitoring the phase transition of the material, that takes place during thermal cycling of the material. For Deborah numbers $De < 0.01$ the material can be considered as fluid, while for high Deborah numbers $De > 10$, it has a solid-like behaviour [46].

4.4. Time-temperature superposition

The Deborah number is particularly useful in conceptualizing the time-temperature superposition principle. Time-temperature superposition has to do with altering experimental time scales using reference temperatures to extrapolate temperature-dependent mechanical properties of polymers. A material at low temperature with a long experimental or relaxation time behaves like the same material at high temperature and short experimental or relaxation time if the Deborah number remains the same. This can be particularly useful when working with materials that relax on a long time scale under a certain temperature. The practical application of this idea arises in the Williams-Landel-Ferry equation. Time-temperature superposition avoids the inefficiency of measuring a polymer's behaviour over long periods at a specified temperature by utilizing the Deborah number.

5. Conclusion

Deborah number is a fundamental number in relating the relaxation time of the system to the time of observation. It can be evaluated for different processes including the dynamic of motion on various scales. Especially in biological systems, such as membranes with different lipid contents it is interesting to study different dynamics of motions. The functions of fundamental proteins and ion-channels are a large field of study at present, with most modern techniques. The need of correlating the functionality of these remarkably complex systems with their dynamics and their lipid environment is a motivation for the future studies.

In the present article, we evaluate for the first time, to our knowledge, the Deborah numbers of bolaamphiphile membrane-mimetic assemblies using the spectroscopic method of Small-Angle and Wide-Angle X-Ray Scattering. The experiment consists of recording the spectra during relaxation, including the phase transition, of the membrane-mimetic bolaamphiphiles. The measuring intervals are on the scale of an hour, and we observe the time it takes for the material to relax to the initial, thermodynamically stable phase, at 20 °C. The ratio of the time of relaxation over the time of observation defines a Deborah number (De) for these substances. This method is innovative because it combines a microscopic structural determination with the macroscopic aspect of the material. The molecular arrangement is related to the macroscopic entity, the Deborah number, using spectroscopy. It enables an insight into the fluid-like ($De < 0.01$) or solid-like ($De > 10$) behaviour of the material. The bolaamphiphiles that we have investigated have relatively high Deborah numbers of $De(C8C12) = 35$ and $(C8C12X2) = 62$. Their behaviour is solid-like, in accordance with their sticky, paste-like behaviour. From this point of view, it is interesting to correlate the relaxation time of the phase transitions of bolaamphiphiles to their macroscopic aspect, using the Deborah number, as proposed in the present work.

The novelty of this manuscript resides in comparing microscopic properties of single molecules, bolaamphiphiles, which is their chemical structure, with macroscopic properties, which is their organization in supramolecular structures as various lamellar phases. The relaxation behaviour from the undercooled high temperature phase to the thermodynamically stable phase at constant temperature of 20 °C is determined for two bolaamphiphiles from the same family, one with saturated bridging chain and the other with unsaturated bridging chain, bearing a diacetylenic unit. We think that this approach is new and that it may inspire further studies of lipids in order to determine their phase transition behaviour and the corresponding Deborah numbers.

Disclosure statement

The authors report there are no competing interests to declare.

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Figure captions

Figure 1 a) Chemical structure of *N*-(12-Betainylamino-dodecane)-octyl β -D-Glucofuranosiduronamide Chloride (C8C12) and b) Chemical structure of *N*-(12-Betainylamino-dodeca-5,7-diynyl)-octyl β -D-Glucofuranosiduronamide Chloride (C8C12X2).

Figure 2 Thermal cycling of the sample C8C12 (Figure 2a) and C8C12X2 (Figure 2b) from 20 °C to 70 °C and back to 20 °C. Figure 2a shows the small-angle and wide-angle spectra typical for the lamellar crystalline phase for at 20 °C and 30 °C, while for the spectra from 40 °C up to 70 °C and during cooling from 70 °C to 20 °C the spectral pattern is assigned to the fluid lamellar phase, L_α . In Figure 2b there is a typical pattern for the fluid lamellar phase, L_α in coexistence with a large peak typical for the isotropic phase, L.

Figure 3a Schematic representation of the lamellar phase. In the crystalline lamellar phase (L_c) the hydrocarbon chains are stretched and ordered, while in the fluid lamellar phase (L_α) they are free to move.

Figure 3b Scattering of light on uni-lamellae of different orientations, results in a wide peak in small angle as well as the wide-angle region of the spectrum, which is typical for the lamellar isotropic phase, L.

Figure 4 Polarised optical microscopy shows Maltese crosses for C8C12 and myeline-like structures for C8C12X2. The magnification is 10x.

Figure 5 Relaxation spectra for C8C12 (2a) and C8C12X2 (2b) at 20 °C, after the thermal cycling. One can observe that after 35 hours (Figure 5a) the spectral pattern changes, which is the signature of the phase transition from L_α to L_c . The Figure 5b shows that the phase transition from L to L_α that takes place after 62 hours.

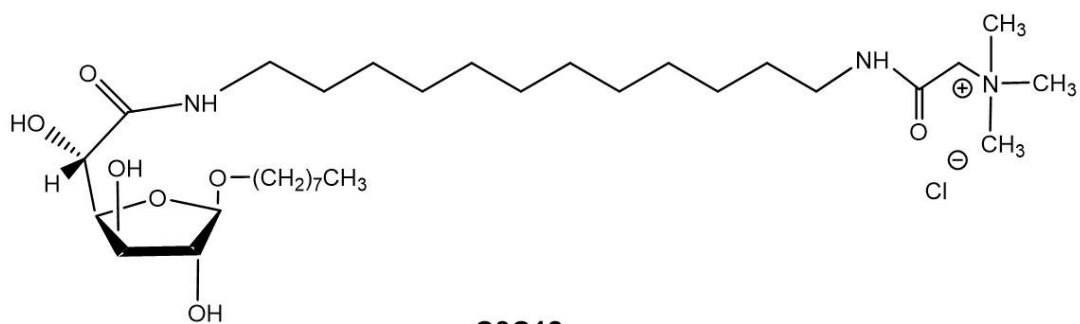
Figure 6 The thermal hysteresis during thermal cycling of the compound C8C12. The variation of interlamellar distances $d(\text{\AA})$ is given as a function of temperature upon heating (\blacktriangle) and during cooling (\blacktriangledown). The relaxation takes place at constant temperature of 20 °C as indicated by the dotted arrow (- - ->).

Figure 7a Evolution of the interlamellar distance with time during relaxation at constant temperature of 20 °C, from the lamellar fluid undercooled phase ($L_{\alpha,u}$) to the lamellar crystalline phase (L_c). This relaxation is a non-linear function of time.

Figure 7b Kolmogorov-Avrami fit (Equation 3) to the experimental data.

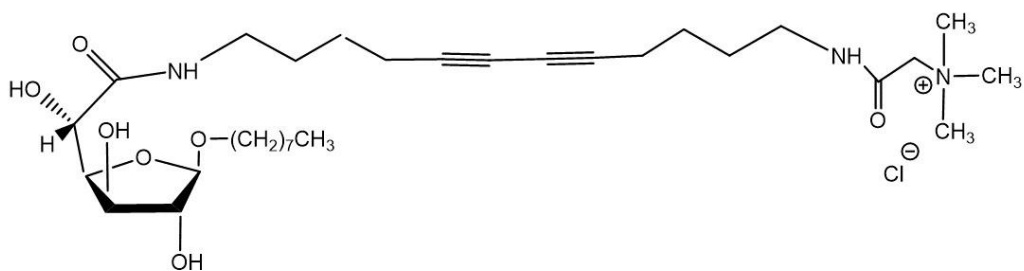
Figure 8 Time – temperature diagram of the phase transition for C8C12. It shows the evolution of lamellar arrangement for C8C12 during thermal cycling as well as the subsequent. Full circles represent the crystalline lamellar phase while circles with pattern represent lamellar fluid phases. It takes 35 hours after the thermal cycle for the system to relax from the undercooled lamellar phase L_{α} to the thermodynamically stable lamellar crystalline phase.

Table 1 Interlamellar distances and lamellar phases for C8C12 and C8C2X2 upon heating and cooling (Figures 2a and 2b).



C8C12

Figure 1a



C8C12X2

Figure 1b

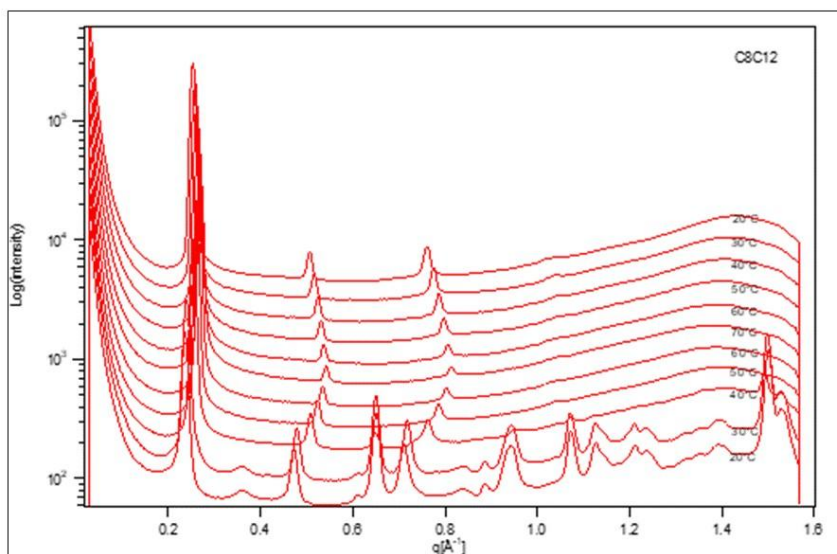


Figure 2a

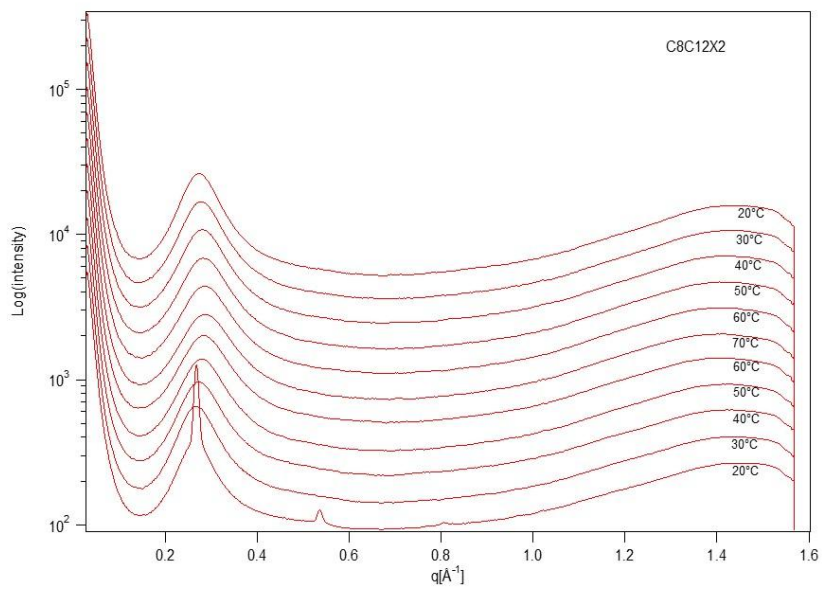


Figure 2b

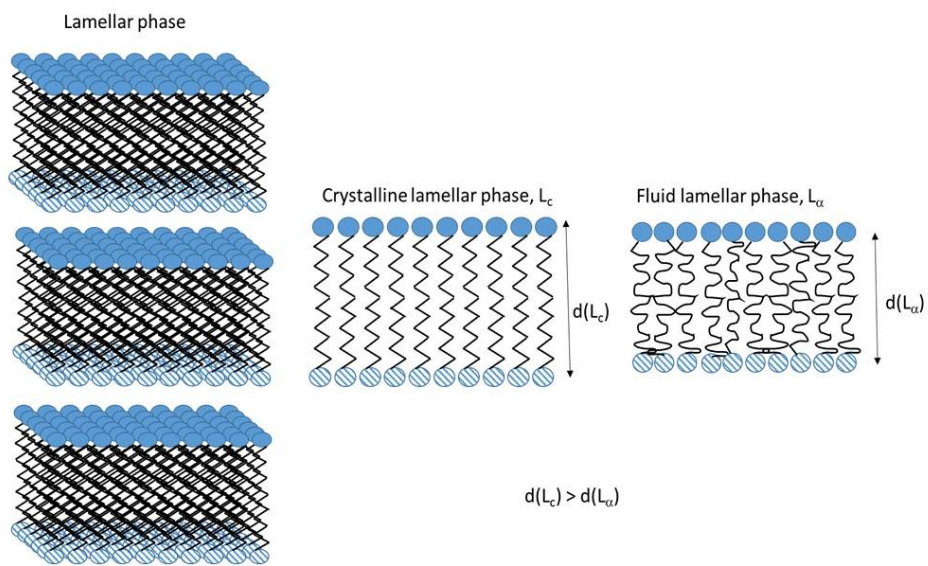


Figure 3a

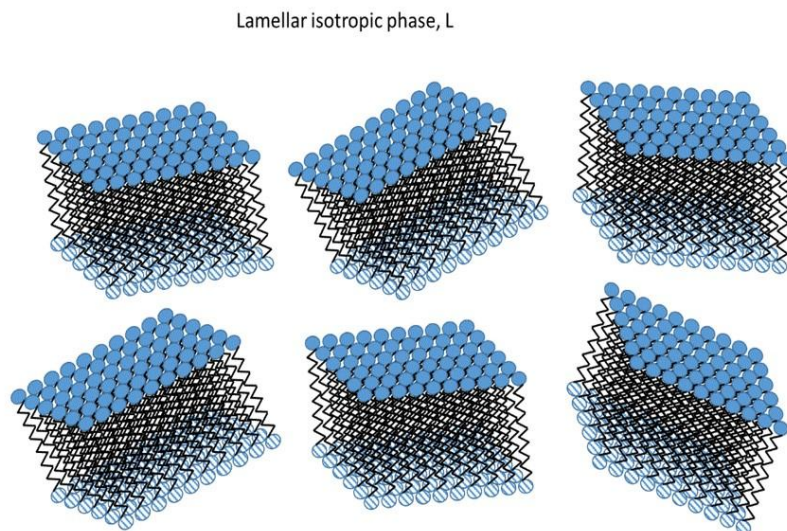


Figure 3b

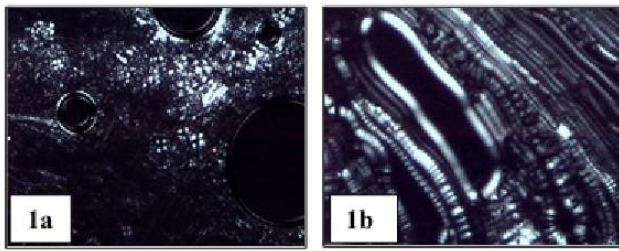


Figure 4

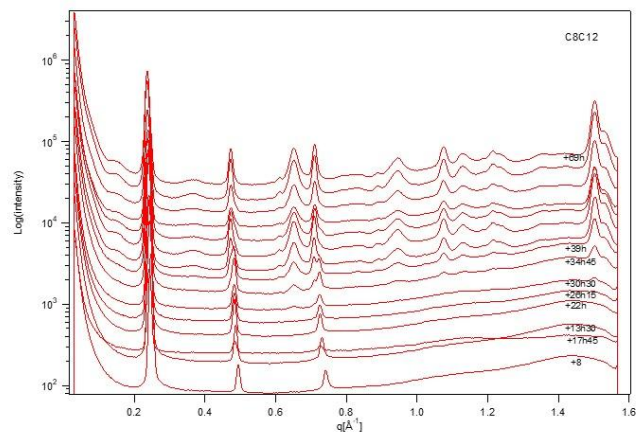


Figure 5a

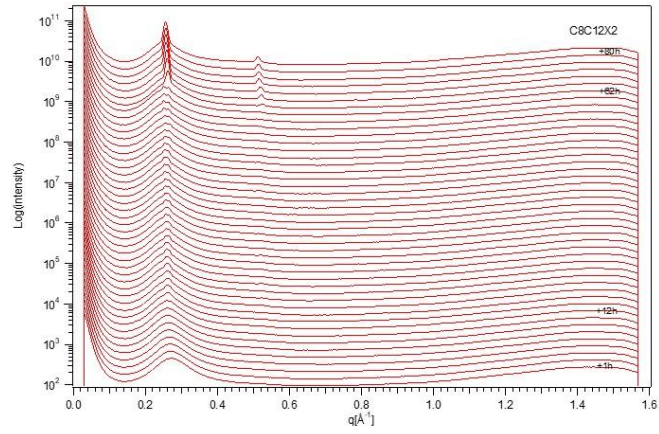


Figure 5b

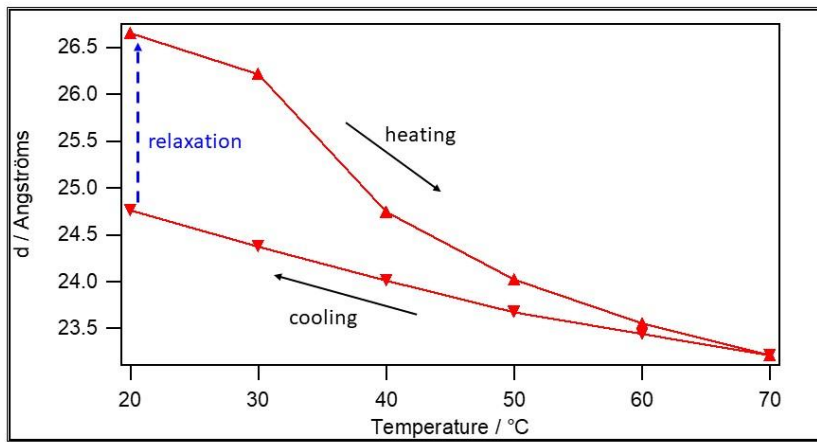


Figure 6

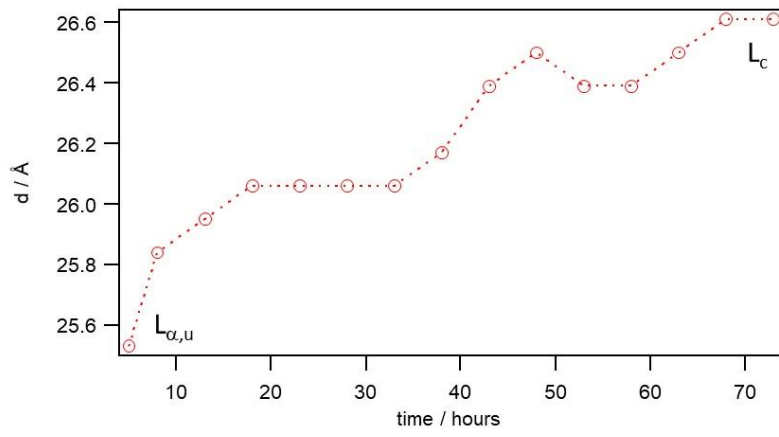


Figure 7a

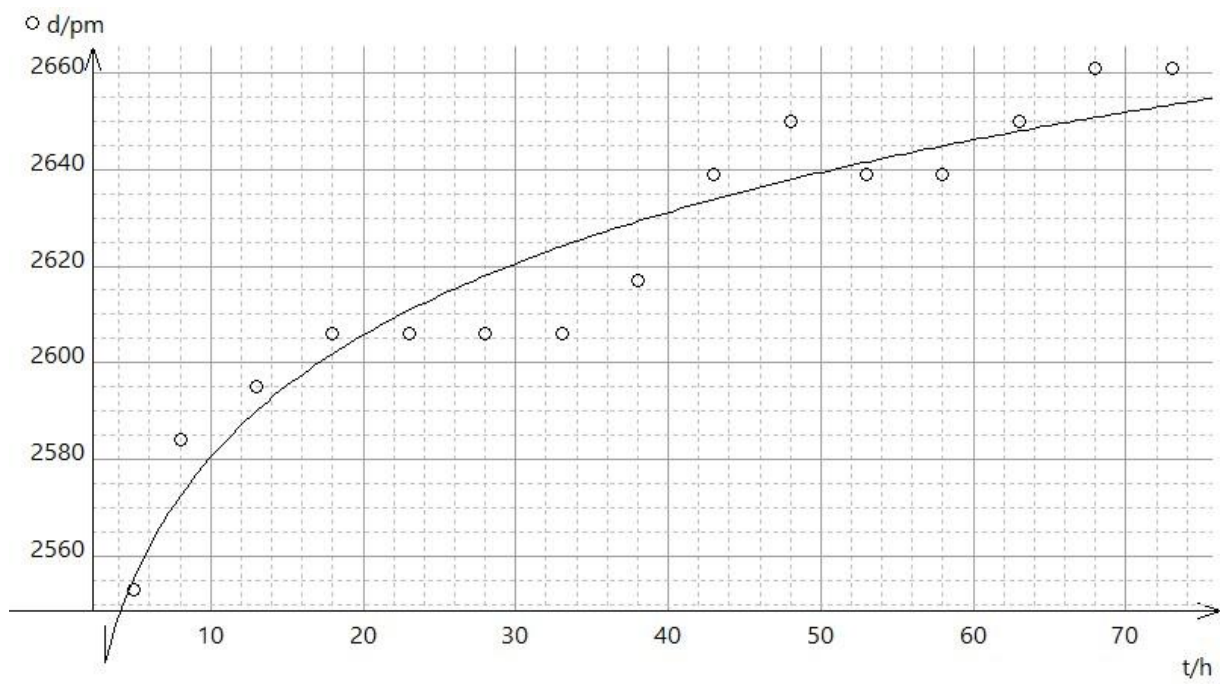


Figure 7b

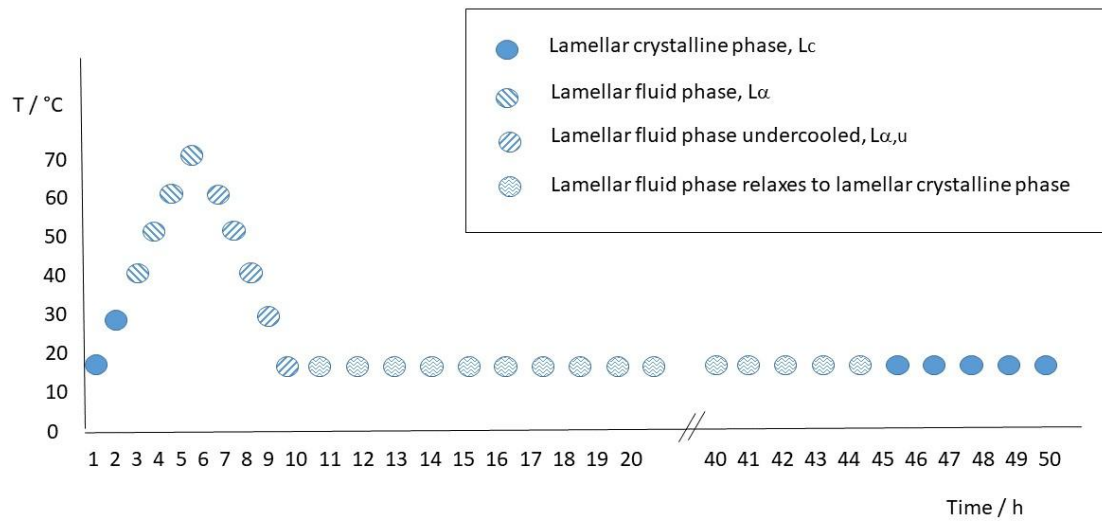


Figure 8