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Experiment Report Form

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- 1st March for experiments carried out up until June of the previous year;
- 1st September for experiments carried out up until January of the same year.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report, in English.
- include the reference number of the proposal to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

ESRF	**Experiment title:** Quantification of local mechanical stresses during freezing and their contribution to freeze-induced protein destabilization	**Experiment number**: **LS-2652**
Beamline: ID-02	**Date of experiment**: from: to:	**Date of report**:
Shifts: 9	**Local contact(s)**: Michael Sztucki	*Received at ESRF:*

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Report:

The main objective of the study is to evaluate molecular probes for measuring local mechanical stresses during freezing (i.e., during water-to-ice transformation). Freeze-induced mechanical stresses have been proposed to be one of the mechanisms of freeze-destabilization of proteins, although there are no direct experimental confirmation of this hypothesis. In this study, two molecular probes are applied, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and glycolide, which undergo significant structural changes at moderate pressures of several kBars; these structural changes result in characteristic small-angle and wide-angle X-ray scattering (SAXS and WAXS) patterns. In addition, mannitol solutions are studied; mannitol is known to form different polymorphs during freezing, and we hypothesize that such freeze-dependent polymorphism could be related to the difference in the local stresses, which are associated with the volume expansion during ice crystals growth. Finally, DPPC-water suspensions with a typical cryo/lyo-protector, sucrose, are also studied.

In the LS-2652 study, solution or suspension is sealed in quartz 1-mm capillary, and SAXS/WAXS measurements are performed during cooling and heating using Linkam temeprature control system. The list of samples is given in Table 1, along with the information on thermal conditions and file names.

Examples of SAXS and WAXS patterns of 1% DPPC solution at different temperatures are shown in Fig. 1. The SAXS patterns in the temperature region of approx. 13°C to -7°C are consistent with the lamellar structure of DPPC (ratio of the peak position is 1:2). Cooling to -11.5°C results in sharpening of the first peak and an appearance of a third peak (ratios of 1:2:3), indicative of the increase in the 1-dimensional lamellar ordering. SAXS patterns obtained after cooling to -15.7°C are characterized by further changes, including a shift in the position of the main peak to higher q (lower d-spacing) and appearance of two additional weak peaks. The experimental peak ratios are 1:1.85:2:2.3, which could potentially indicate the distorted hexagonal phase (the theoretical ratios for the hexagonal phase are $1:\sqrt{3}:2:\sqrt{7}$). The temperature dependence of the position of the first peak for the 1% DPPC sample is plotted in Fig 2, along with the

literature data for both temperature- and pressure-dependence of q for DPPC-water system. The sharp increase in the q in the temperature-dependence coincides with water-to-ice transformation. Importantly, the pressure-dependence of q for DPPC shows an increase in q with pressure (Fig 2, right panel). It is therefore possible that the freeze-induced increase in q could be related to the local pressure due to the water-to-ice transformation. In order to investigate this hypothesis, a detailed data analysis of the temperature- and concentration- dependence of the SAXS patterns will be performed, and additional experiments will also be considered.

Examples of WAXS patterns for 1% DPPC are shown in Fig.3. In the non-frozen sample at 9.5 C (Fig 3, top), a peak is observed at q of approximally 15 1/nm, which corresponds to the intra-lamllar ordering of the hydrocarbon chains in the gel phase. Unexpectidly, the peak is not detected at several other temperatures; the reason for such apparent discrepancy is not currently known, and will be a subject of a future more detail data analysis. In the frozen samples, attentuation was necessary to avoid saturation of the WAXS detector by diffraction from ice. Nevertheless, the WAXS patterns show a peak at q of approx 15 1/nm, which indicates the 2-dimensional ordering corresponding to the gel phase (Fig 3 bottom).

Examples of WAXS patterns for mannitol-water solutions are shown in Fig 4. It is observed that cooling conditions have a major impact on crystallization behavior of mannitol. In particular, crystallization of mannitol takes place during slow cooling (rate 0.5°C/min), whereas mannitol remaines amorphous when a higher cooling rate of 5°C/min is applied. This finding is consistent with the DSC data, as shown in Fig 5. The results indicate that the critical cooling rate for mannitol crystallization is between 0.5 and 5°C/min, which is an important finding from the practical perspectives for development of freeze-drying cycles. Mannitol is a common bulking agent for pharmaceutical formulations, and its crystallization behavior has a major impact on the product yeild and quality.

Representative WAXS patterns for 5% glycolide solution at several temperatures are shown in Fig 6, along with patterns for solid glycolide and ice. Glycolide remains amorphous during cooling and subsequent heating of its aqueous solutions; therefore, this system appears to be not suitable for monitoring mechanical stresses during freezing, at least under the experimental conditions of this study.

Conclusion. The results obtained for DPPC system demonstrate that this molecule has a potential as a molecular probe for freeze-induced mechanical stresses. The next step is to perform in-depth data analysis to separate temperature-dependend changes in the phospholipid phase from the pressure-induced changes. Limited additional experiments, such as measuring the d-spacing of DPPC lamellar at sub-zero temperature is samples with limited ice formation (e.g., DPPC equilibrated via vapor phase at water activity <1), will also be considered. Experiments performed with mannitol/water show a major impact of cooling rate on crystallization pattern of mannitol. This observation has practical significance for freeze-drying of pharmaceuticals, and also would improve fundamental understanding of solute crystallization in partially frozen aqueous systems.

Table 1. List of samples tested during LS-2652 experiment.

Sample	frames/images	temperature range (°C)
DPPC 5%	(2662-2930) + (2962-3227)	10- (-40) - 10
DPPC 1%	(3248-3505) + (3537-3786)	10- (-40) - 10
DPPC 1% sucrose 2.5%	(3824-4078) + (4110-4366)	10- (-40) - 10
DPPC 1%	5483-5580	10- (-30)
DPPC 5%	5785-5983	10- (-30) - 10
DPPC 1%	5985-6081	10- (-30)
DPPC 1%	6158-6352	10- (-30) - 10
DPPC 1% sucrose 1%	6403-6502	10- (-30) - 7.1
DPPC 5%	6511-6556	0-(-30)
DPPC 5%	6557-6677	10- (-40) - 10
DPPC 5% sucrose 1%	6697-6837	10- (-40) - 10
DPPC 5% sucrose 5%	6843-6963	10- (-40) - 10
DPPC 5% sucrose 10%	7053-7183	10- (-40) - 10
Glycolide_Raw	7193	
DPPC 5% 10% sucrose	7534-7676	7.4- (-40) -10
DPPC 10%	7677-7804	10- (-40) - 10
DPPC 10%	7808-7934	10- (-40) - 10
DPPC 10%	7938-8068	10- (-40) - 10
DPPC control	10572-10574	
DPPC suspension 10%*	10576-10745	10- (-30) - 10
DPPC suspension 10%	10750-10769	
DPPC suspension 10%	10771-10839	40- (-20) - 40
DPPC suspension 10%	(10841-10877) + (10895-10933)	40- (-20) - 40
DPPC suspension 10% sucrose 10%	(10935-10995) + (10999-11061)	10- (-40) - 10
DPPC suspension 10% sucrose 1%	11444-11521	10- (-40) - 10
DPPC suspension 10% sucrose 10%	11522-11627	10- (-40) - 10
Mannitol 5% r5	4731-5211	10- (-40) -10
Mannitol 20% r5 c1	(8595-8734) + (8749-9040)	20- (-40)- 20
Mannitol 10% r5 c1	9076-9396	45- (-40)- 12 (?)
Mannitol 10% r0.5	9701-10114	5- (-40)- 5
Mannitol 20% r0.5	10115-10522	5- (-40)- 5
Glycolide 20%	1119-1337	10- (-30)- 10
Glycolide 5%	1647-1851	10- (25)- 10
Glycolide recryst	5266 & 5267	
Glycolide raw	5271-5272	
Glycolide raw	7193	
Glycolide recryst	8092	
Glycolide recryst ground	8094-8112	
water control	10562-10571	

*****"Suspension" refers to preparing the micelles in situ, during SAXS/WAXS freeze-thaw cycles. The samples are prepared by mixing water and DPPC and transfer of the mixture in the capillary.

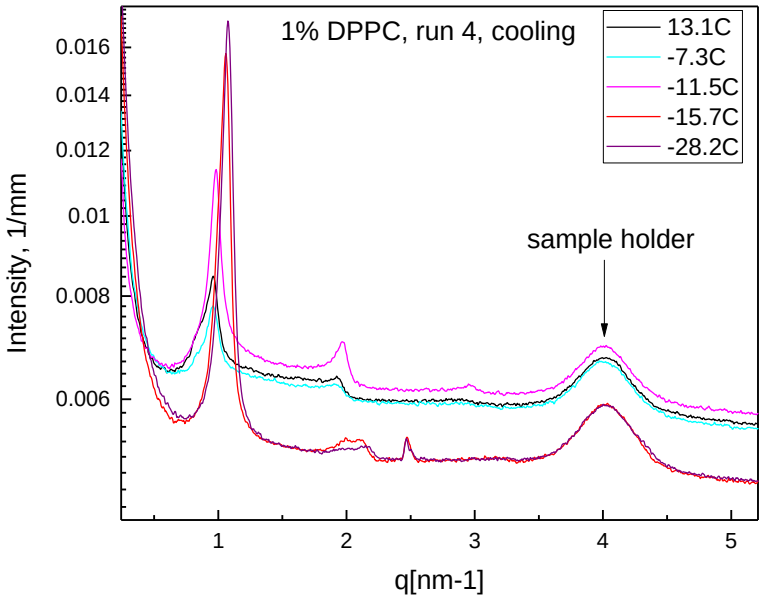


Figure 1. Examples of SAXS patterns for 1% DPPC-water system.

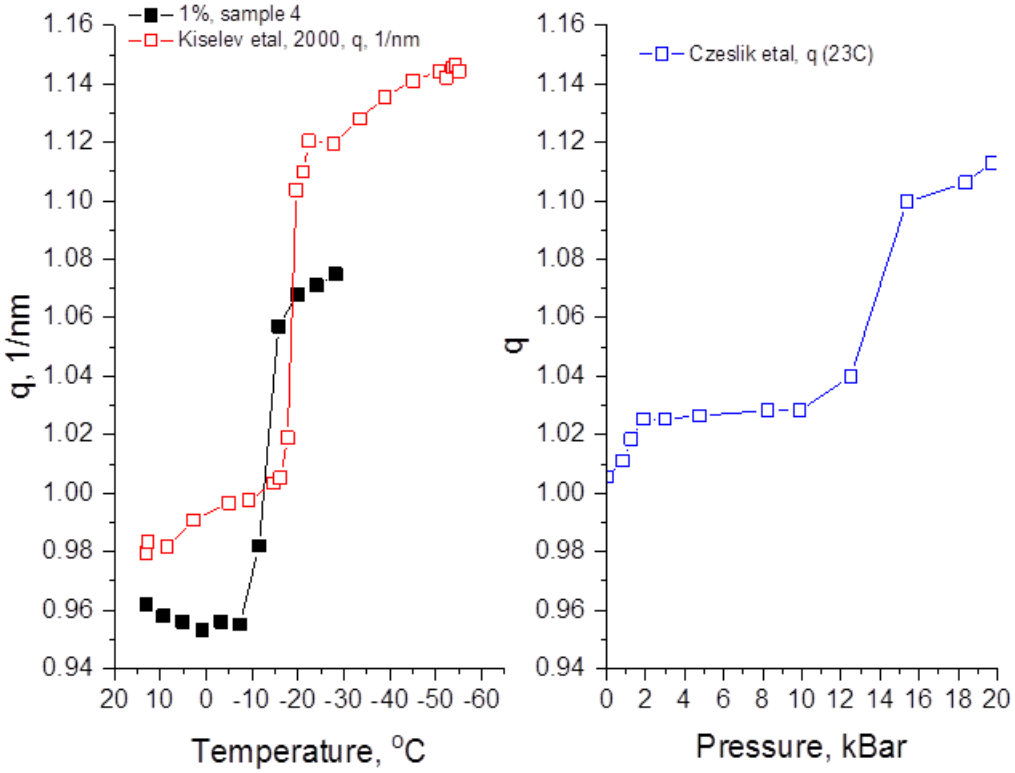
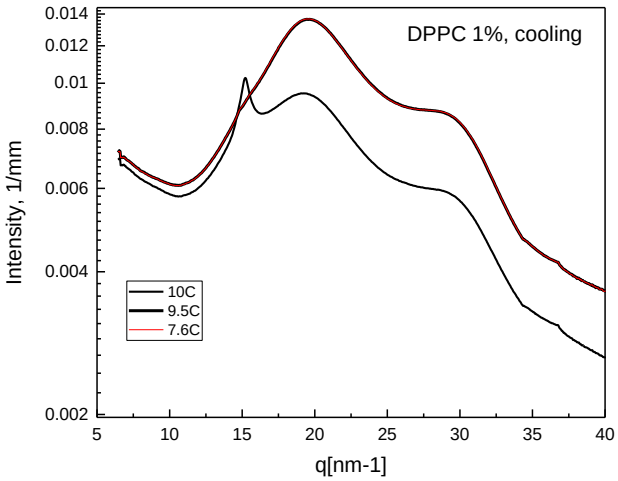


Figure 2. q values as a function of temperature (left) and pressure (right). The literature data are from [M.A. Kiselev, P. Lesieur, A.M. Kisselev, M. Ollivon, Ice formation in model biological membranes in the presence of cryoprotectors, Nuclear Instruments and Methods in Physics Research A 448 (2000) 255-260] (red symbols and line) and [C. Czeslik, O. Reis, R. Winter, G. Rapp. Effect of high pressure on the structure of dipalmitoylphosphatidylcholine bilayer membranes: a synchrotron-X-ray diffraction and FT-IR spectroscopy study using the diamond anvil technique. Chemistry and Physics of Lipids 91 (1998) 135–144] (blue symbols and line).



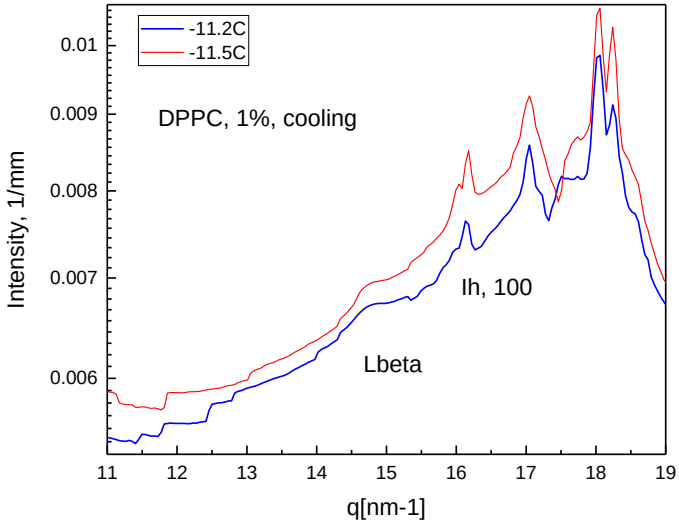
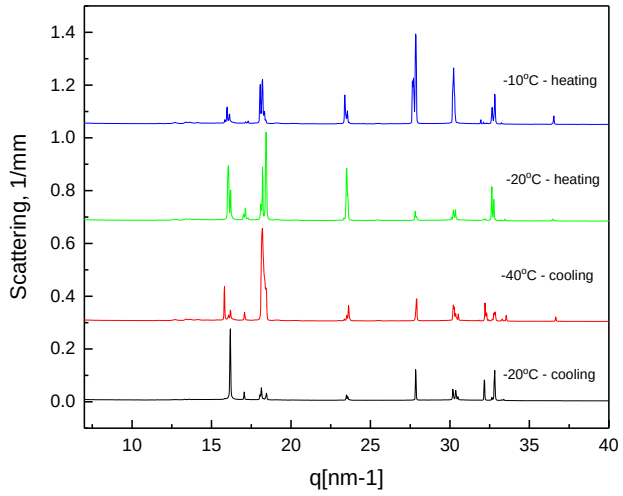
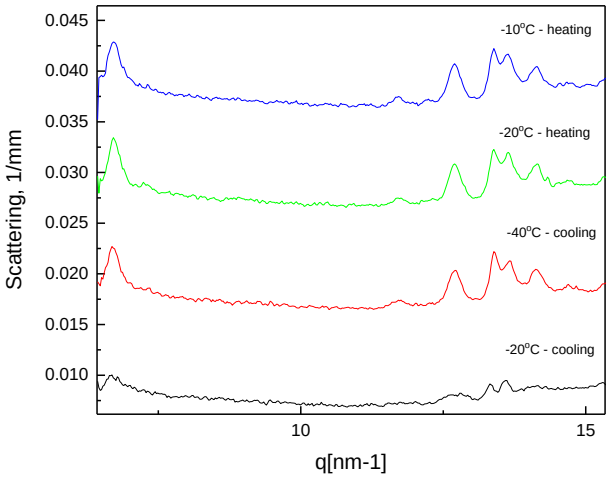


Figure 3. Examples of WAXS patterns of 1% DPPC-water mixture above 0°C (top) and below 0°C (bottom). Ih, 100 corresponds to the hkl 100 for the hexagonal ice, Ih.





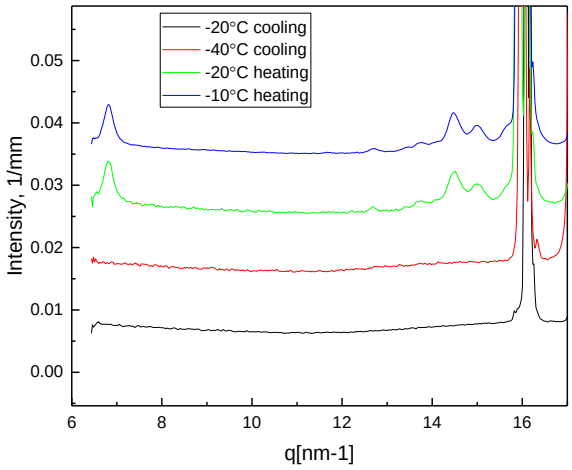
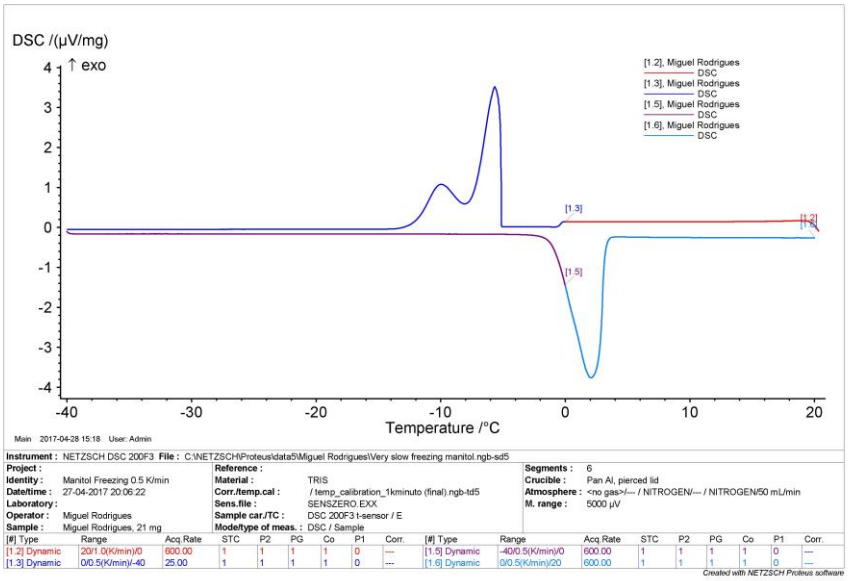


Figure 4. WAXS patterns for 10% mannitol at 0.5 °C/min (top and middle) and 5 °C/min (bottom) cooling rates. The middle and bottom graphs show magnified portion of the WAXS patterns to highlight mannitol peaks.



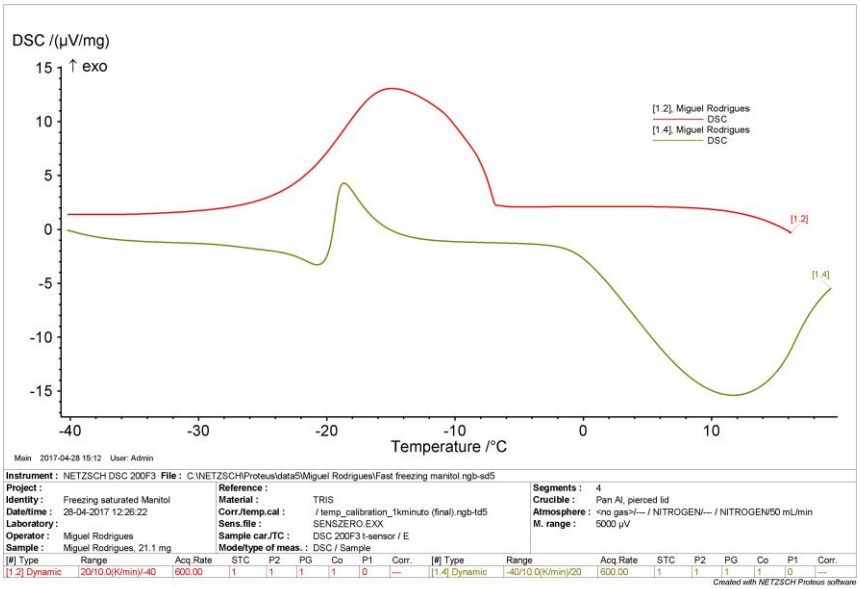


Figure 5. DSC curves of an aqueous solution of mannitol at 0.5 °C/min (top) and 5 °C/min (bottom) cooling rates.

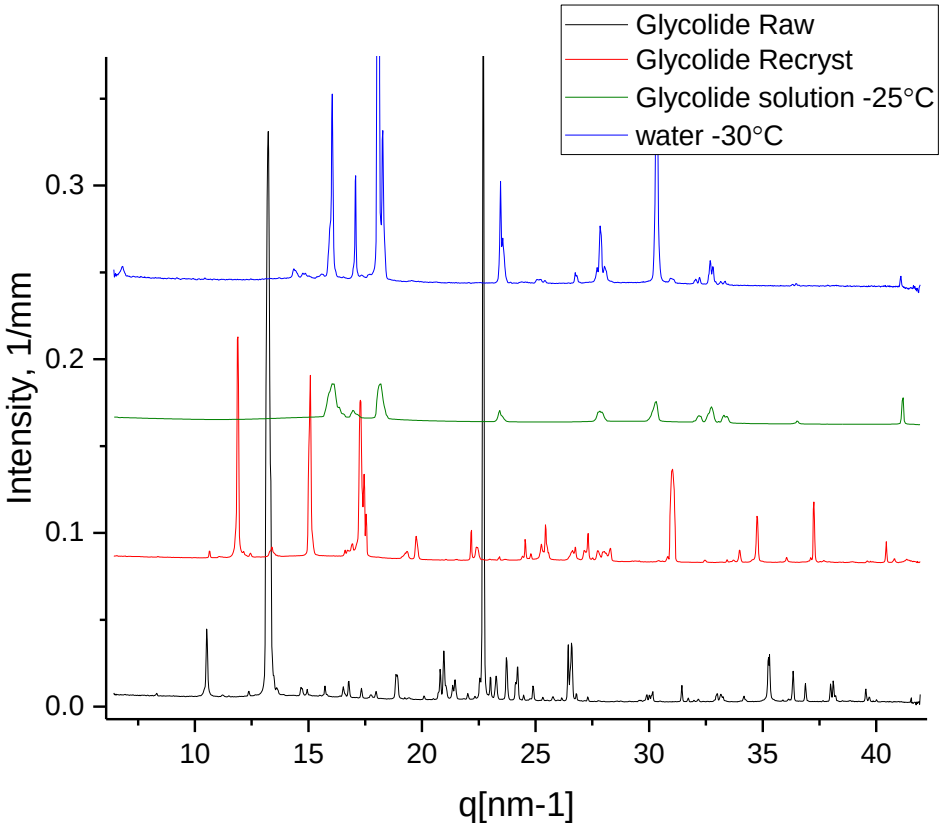


Figure 5. WAXS patterns for water, glycolide powder samples, and aqueous solution of glycolide