EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office via the User Portal:

https://wwws.esrf.fr/misapps/SMISWebClient/protected/welcome.do

Reports supporting requests for additional beam time

Reports can be submitted independently of new proposals – it is necessary simply to indicate the number of the report(s) supporting a new proposal on the proposal form.

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

Proposers awarded beam time for a long term project are required to submit an interim report at the end of each year, irrespective of the number of shifts of beam time they have used.

Published papers

All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Deadlines for submission of Experimental Reports

- 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:** Time-resolved SAXS/WAXS study of protein aggregation during freeze-thaw of aqueous solutions and dissolution of freeze-dried protein formulations	**Experiment number**: **LS-2793**
Beamline: ID-02	**Date of experiment**: from: 7 May 2018 to: 9 May 2018	**Date of report**: 21 February 2020
Shifts: 9	**Local contact(s)**: Michael Sztucki	*Received at ESRF:*

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Report:

The primary objectives of the study were (i) to investigate change in protien/protein interaciton during freeze-thaw of solutions of model pharmaceutical proteins, and (ii) develop experimental set-up to perform SAXS measurements during reconstitution of freeze-dried protein powders. Both freeze-thaw and reconstitution behavior are of primary interest for both applied (e.g., pharmaceutical science and technology) and fundamental science. The main part of the experiment was devoted to freeze-thaw experiments. In-situ SAXS/WAXS was utilized as the main experimental method, with the measurements performed at the ESRF ID02 beamline, both during freeze-thawing of aqueous solutions of proteins and reconstitution of freeze-dried protein powders. Three proteins (GCSF and two mAbs) in different formulations (containing buffer, ±disaccharide, ±sugar alcohol, ±surfactant) were investigated. The solutions were filled into 1 mm O.D. (0.01 mm thickness) quartz capillaries and cooled/heated using a Linkam temperature stage between RT and -50°C. In addition, SAXS patterns were collected during reconstitution of protein powders; where a flow cell was used in an experimental setup comprising of a pump to circulate a reconstitution diluent (water) and a vessel containing the lyophilized sample, and the freeze-dried cakes were powdered and transferred into a bottle for the experiment.

In mAb solutions (5 to 100 mg/mL), a peak attributed to protein-protein interaction (structure factor) was observed by SAXS, whereas it was not detected in **less concentrated solutions** (0.5 mg/mL) (Figure 1). The absence of the peak at 0.5 mg/mL is most likely because of the detection limitThe two shoulders, which are observed at q values of approx. 1 to 2 1/nm in the IgG solutions at 50 and 100 mg/ml, are attributed to the form factor (Figure 1). The posiiton of these shoulders is similar in both 100 and 50 mg/ml solutions. The form factor corresponds to the shape of individual scattering structures (in this case, protein molecules), and therefore can be expected to be independent of the concentration.

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SAXS patterns of the mAb solution (100 mg/ml) during cooling are shown in Figure 2 (top). The shift in the peak position is observed when the sample is cooled from +20 to -10°C (first scan at -10°C, sample is not frozen yet), reflecting an increase in the protein/protein center-of-mass distance from approx. 18.5 to 21 nm (d-spacing ~ 0.3 1/nm) between 20 and -10°C. Similar trend is observed for 50 mg/ml solution, with the

peak interaction peak shifting to lower-q values as the sample is cooled form 20 to -10°C, while the form factor shoulders remaining essentially the same (Figure 2, bottom). For the 5 mg/ml sample, the temperature shift of the protein interaction peak is not detected (SAXS patterns are not shown). Such temperature dependence of the protein interaction peak has not been reported previously. After maintaining -10°C for several minutes, the sample froze as observed by WAXS (WAXS patterns not shown), and major changes are observed in the SAXS patterns (Figure 3). The protein interaction peak is no longer observed, the scattering intensity at higher angles is significantly reduced, and the lower-angle scattering increases with the decrease in the scattering angle. Upon further cooling to -50°C, relatively minor but reproducible changes are observed as follows: (i) Gradual development of a very broad and weak peak at q 1 to 2 1/nm as the sample is cooled from -20 to -30°C, then to -40°C, and finally to -50°C; (ii) small increase in low-q scatter after cooling from -20 to -30°C; no further changes at -40 and -50°C. This observation was reproduced with a second sample of the same composiiton, as shown in Fig 3 (bottom). Representative SAXS patterns during thaw are shown in Fig. 4. The freeze-induced changes in the SAXS patterns were reversed upon thawing of the solution.

Figure 5 (top) shows SAXS patterns obtained for reconstituted protein in the flow cell. A peak attributed to protein-protein interactions (q= 0.2 to 0.6) was detected in solution post reconstitution; the two shoulder peaks are attributed to the form factor. Kratky plot is shown in Figure 5 (bottom). Observation of peaks in the Kratky plot is indicative of a folded protein structure.

Conclusion. The preliminary analysis of the data revealed the following:

- Protein interaction peak(s) were observed in the unfrozen as well as partially frozen solutions.
- The protein interaction peak was not observed in the frozen solution → attributed to lack of sensitivity or due to structural changes in the protein
- In a partially unfrozen samples, immediately post thawing at 0°C, the d-spacing was ≈16.5 nm; it increased to 19 nm on completion of thawing.

In addition, the experimental set-up to collect SAXS data during reconstitution of freeze-dried proteins was established.

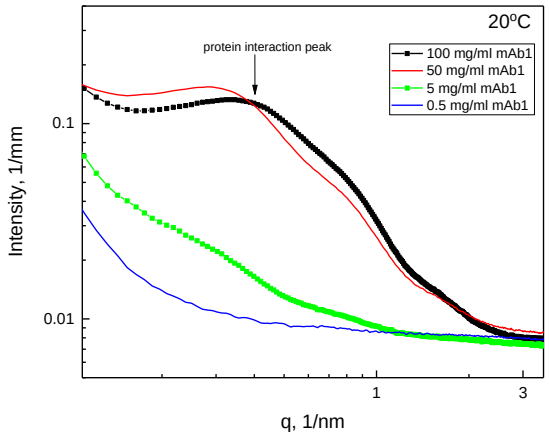

Figure 1. SAXS patterns of mAb solutions with variable concentraiton sof the protein, formulated with 5% Sucrose and PS80 in Histidine buffer

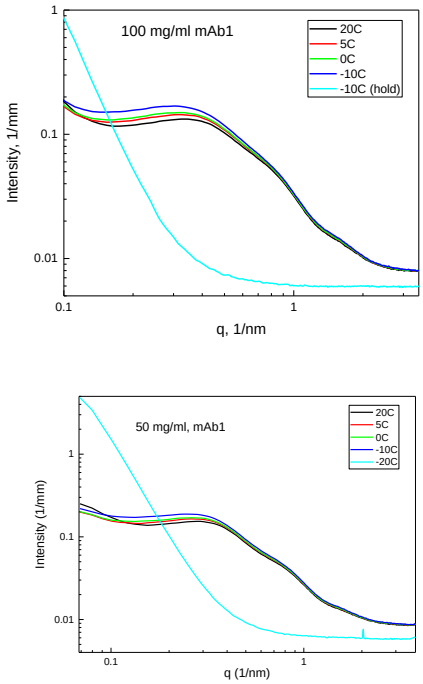

Figure 2. SAXS patterns of mAb1 solutions (100 and 50 mg/ml) during cooling

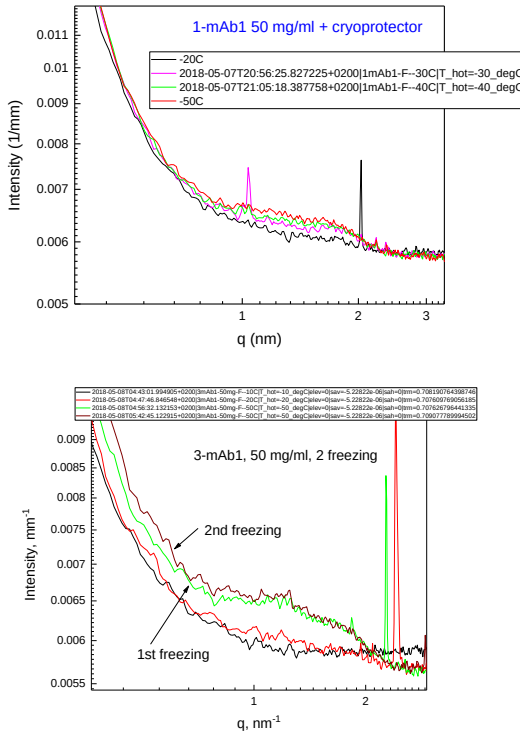

Figure 3. SAXS patterns of 3-mAb-1 solution (50 mg/ml) obtained during cooling.

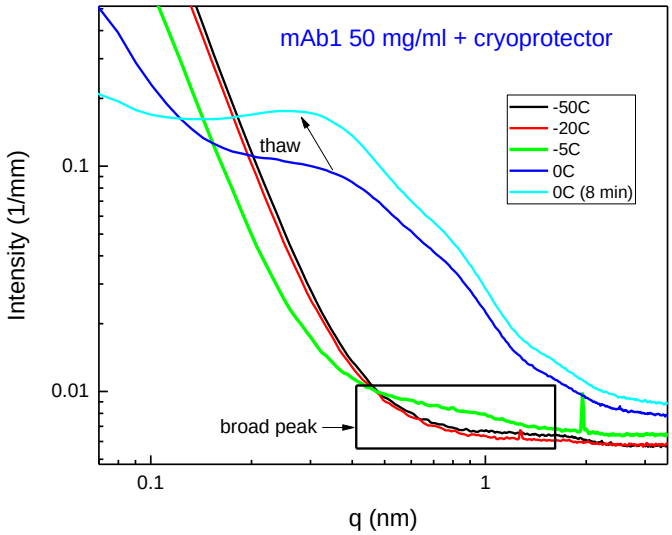

Figure 4. SAXS patterns of mAb1 solution during heating of the frozen sample from -50 to 0C

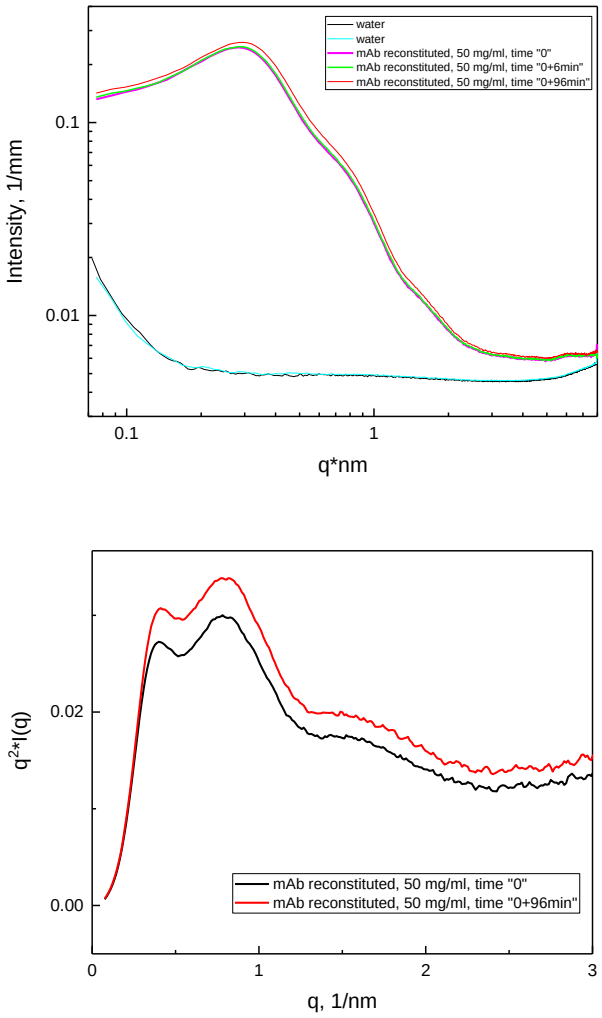

Figure 5. top: SAXS patterns of reconstituted freeze-dried protein formulations, which were obtained using flow cell. Bottom: Kratky plot of the SAXS data.