EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

ESRF

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

Deadlines for submission of Experimental Reports

Experimental reports must be submitted within the period of 3 months after the end of the experiment.

Experiment Report supporting a new proposal ("relevant report")

If you are submitting a proposal for a new project, or to continue a project for which you have previously been allocated beam time, you must submit a report on each of your previous measurement(s):

- even on those carried out close to the proposal submission deadline (it can be a *"preliminary report"),*
- even for experiments whose scientific area is different form the scientific area of the new proposal,
- carried out on CRG beamlines.

You must then register the report(s) as "relevant report(s)" in the new application form for beam time.

Deadlines for submitting a report supporting a new proposal

- 1st March Proposal Round - **5th March**
- 10th September Proposal Round - **13th September**

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.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report in English.
- include the experiment number 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:** SAXS study on protein-protein interactions in freeze concentrates on understanding the impact of formulation and temperature during freezing and frozen storage of biologics	**Experiment number**: LS-3210
Beamline: ID02	**Date of experiment**: from: 12.05.2023 to: 15.05.2023	**Date of report**: 15.08.2023
Shifts: 9	**Local contact(s)**: Michael Sztucki	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

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PRELIMINARY REPORT

Introduction and aim of experiment:

The main aim of the conducted SAXS/WAXS study was to gain a more fundamental understanding of protein behavior in the freeze-concentrated (FC) state in terms of protein-protein interactions (PPI) and protein-protein distances (PPD). Both factors critically influence the colloidal stability of the protein and, as challenging to assess in the frozen state, are still of limited understanding. Expanding this knowledge and investigating the correlation to protein stability will enable a more rational formulation choice for frozen storage.

The main part of the assigned beamtime was dedicated to the analysis of isolated freeze concentrates and frozen aqueous formulations of a model monoclonal antibody (mAb). Formulations included different mAb: sucrose mass ratios and, at a set ratio, different ionic strengths, ion types, and pH values (Tab. 1). Sets of isolated and in-situ generated freeze concentrates were measured to assess if the direct determination of PPD and PPI would be possible in presence of ice interfaces.

Furthermore, we attempted time-resolved SAXS/WAXS measurements during subzero temperatures to monitor potential changes in PPD and folding state to decouple temperature effects in the frozen state from formulation impact itself.

Materials and Methods:

Dilute solutions (0.3-6 mg/ml mAb) for the analysis of P(q), describing single-particle properties, were obtained through the addition of ultrapure water to the freezing solutions and measured in the flow-through cell at RT to obtain exact background subtraction. At investigated concentrations, no interparticle interactions or aggregation have been observed (Fig. 1-2) and a folded mAb is indicated by the Kratky plot (Fig. 3).

Isolated FCs of the model mAb were produced by partial freeze-drying and moisture equilibration prior to measurements. For verification of the amorphous state of all components and the absence of potential crystallization of unbound water, combined SAXS/WAXS was used. Samples were cooled to -40°C on a LINKAM variable temperature stage. *In-situ* FCs were obtained by freezing the same solution as used for freeze drying to -40°C on the Linkam stage. Isolated FCs (75% total solid content) were measured between spaced glass coverslips, using sample cells (1.5mm layer thickness), and *in-situ* generated samples were filled to quartz capillaries (1 mm diameter). The first peak in the S(q) contribution corresponding to the nearest distance between interfering neighbors was monitored as a function of formulation composition. The peak position was fitted polynomially and the shoulder position was determined.

Results and Conclusion:

Preliminary results, as evaluation is ongoing

1. Determination of PPD: A shoulder attributed to S(q), corresponding to the nearest neighbor distance, was observed at an identical q position in corresponding isolated and *in-situ* FC samples (Fig. 4, exemplary: F02, q ($Å^{-1}$) = .
   PPD thus could be determined in the presence of ice interfaces in the Linkam stage set-up. The shoulder became more distinct with increasing mAb content.

2. Impact of formulation on PPD: Formulation dependant changes of center-center distances in the FC could be observed. In the SAXS patterns of different mAb: sucrose mass ratios a q shift of the shoulder from q = 0.0923 $Å^{-1}$, d-spacing of 68 Å, to q = 0.1080 $Å^{-1}$, d- spacing of 58 Å, could be observed with decreasing ratio (Fig. 5-6). Very slight changes were detected for different pH (Fig. 7-8). For ionic strength and different salt types, no differences could be determined.

3. S(q)eff → S(0) analysis ongoing for net interaction behavior in FC and during upconcentration (dilution row of formulations at constant ratio).

Figures and tables:

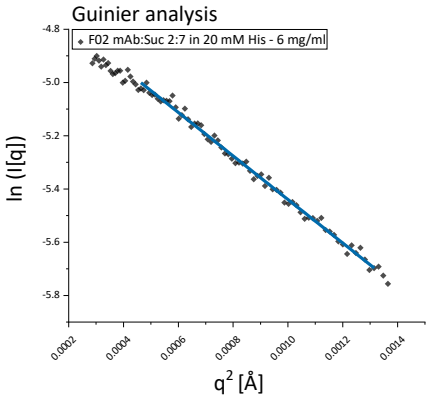

Figure 1. Guinier plot and fit of diluted freezing solution F02 (6 mg/ml mAb).

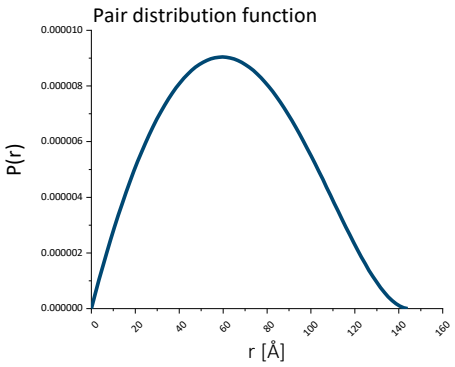

Figure 2. P(r) distribution of diluted freezing solution F02.

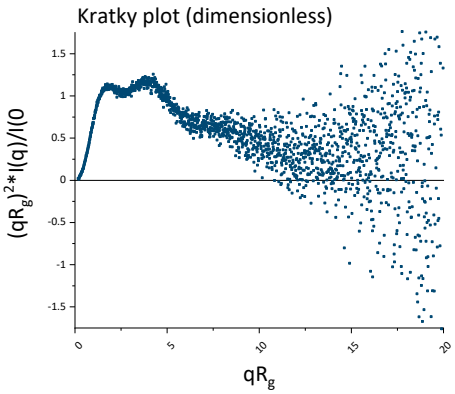

Figure 3. Kratky plot of diluted freezing solution F02, showing a typical curve for a flexible, multidomain but folded antibody structure.

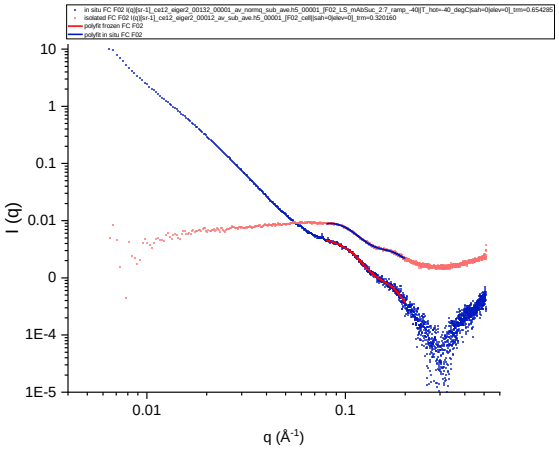

Figure 4. SAXS patterns of in-situ generated and isolated FC of F02.

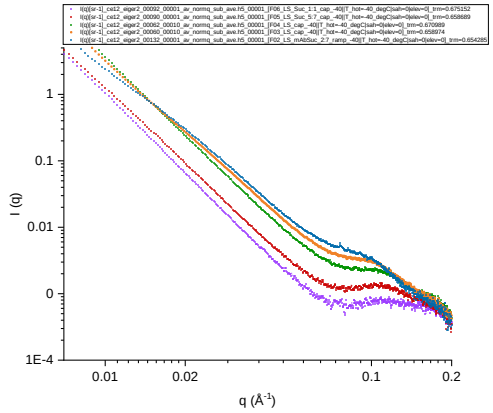

Figure 5. SAXS patterns of mAb: sucrose mass ratio variation.

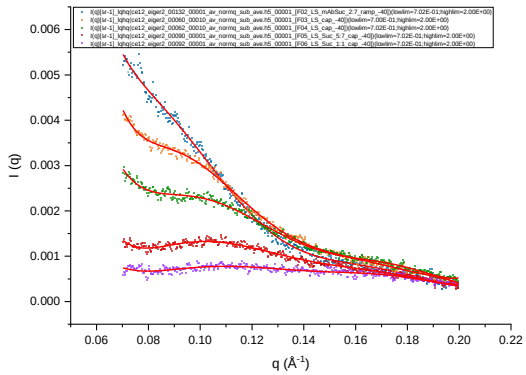

Figure 6. Region of interest and corresponding polynomial fits.

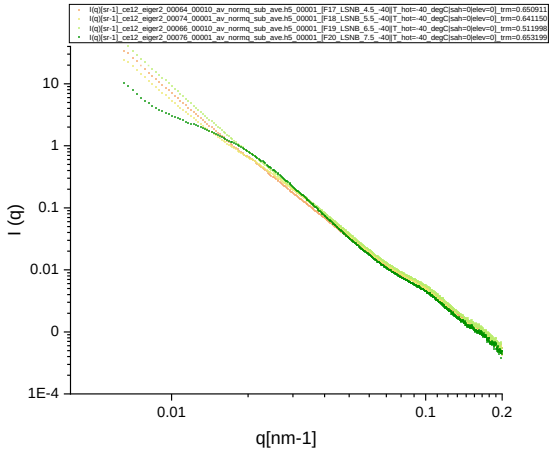

Figure 7. SAXS patterns of pH variation [4.5 to 7.5] at a constant mAb: sucrose ratio [2:7].

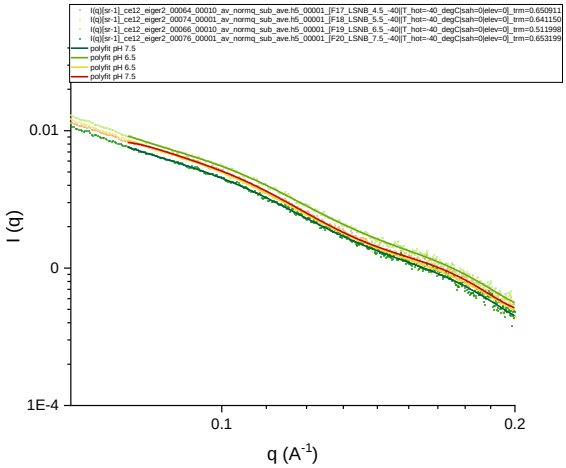

Figure 8. Region of interest and corresponding polynomial fits.