ESRF	**Experiment title:** Investigating the assembly reaction of a novel humanized archeal ferritin with time-resolved SAXS/WAXS	**Experiment number**: LS-2575
Beamline: ID02	**Date of experiment**: from: 09 November 2016 at 08:00 to: 11 November 2016 at 08:00	**Date of report**: 28/02/2017
Shifts: 6	**Local contact(s)**: MOELLER Johannes	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

*LEVANTINO Matteo (ESRF & University of Palermo, Dept. of Physics and Chemistry)
BOFFI Alberto (Sapienza University of Rome, Dept. of Biochemical Sciences & CNR- IBPM)
VALLONE Beatrice (Sapienza University of Rome, Dept. of Biochemical Sciences)
CAMMARATA Marco (Université de Rennes 1, Institut de Physique de Rennes)
*EXERTIER Cécile (Sapienza University of Rome, Dept. of Biochemical Sciences)
*TRABUCO Matilde (Sapienza University of Rome, Dept. of Biochemical Sciences)

Report

Beamtime was requested to investigate the assembly kinetics of the *Archaeoglobus fulgidus* ferritin (AfFt) and of the humanized version of it (AfFt-hum) with X-ray solution scattering. Similar studies have been performed to monitor the assembly of *Escherichia coli* ferritin, induced by extreme pH changes (pH 2.5 to pH 8.0) [1]. Interestingly, AfFt possesses a reversible assembly, from a dimer to a tetraeicosamer, depending on the presence of Mg^{2+} or other divalent cations. These comparatively milder conditions put AfFt as an ideal scaffold for advanced drug-delivery systems, expanding the portfolio of molecules that can be encapsulated [2]. In order to combine these properties with the capability of human ferritin to interact with the Transferrin receptor 1 (CD71), overexpressed in cancer cells, we have mutated a surface exposed loop to resemble the human analogue. Since the application submission, a crystallographic structure of the AfFt-hum was published (Figure 1) [3]. **Detailed understanding of the assembly reaction would provide important information on the effect of modifying the monomer structure, guiding further developments of ferritin based encapsulation systems.**

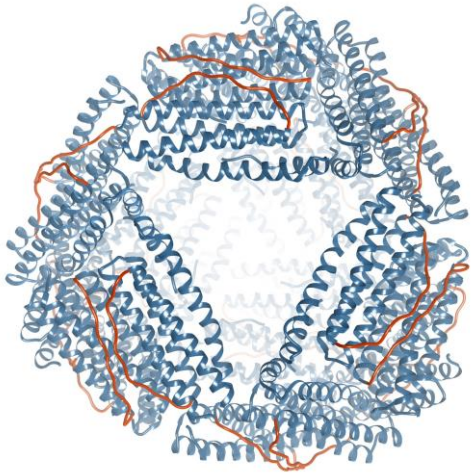

Figure 1 - Crystallographic structure of AfFt-hum (PDBcode 5LS9). Mutated loops are highlighted in red.

Beamtime started with the usual procedures of alignment and beam characteristics optimization for experiments carried in two modes: 1) temperature controlled flow-through cell, for static measurements, and 2) Stopped-flow rapid mixing apparatus, for time-resolved measurements. Several static measurements were performed to establish the optimal conditions for time-resolved experiments. These included testing different

detector-to-sample distances, evaluating the minimal protein concentration required to obtain good S/N data and, in particular, checking the presence of radiation damage as a function of exposure time. The AfFt-wt appeared unfortunately to be either denatured or in a partially assembled state, which conditioned the reliability of the static measurements and precluded any time-resolved experiments on this protein. On the other hand, no pre-assembled ferritin was present in the AfFt-hum sample, which was thus used throughout the rest of the beamtime.

Rapid mixing experiments on AfFt-hum were performed by replacing the hard-stop of the stopped-flow apparatus with a large syringe in order to recover the sample after mixing. Defining an optimal data collection strategy proved challenging due to limitations in defining the time-points with the available data collection macros (e.g., it is currently not possible to have a sequence of delays spaced in time in a logarithmic equidistant fashion). In most cases, combining time points from a linear and a geometric progression was enough to cover the relevant time range. Already from the first few collected time-resolved data, it was clear that the reaction kinetics was faster than expected (in comparison with the pH dependent assembly of *E.coli* ferritin) and that important information was lost at the shorter time delays, at a protein concentration of 2.3 mg/mL and 30 mM $MgCl_2$. The reaction was successfully slowed down by decreasing the $MgCl_2$ concentration. Static SAXS patterns of AfFt-hum at 6 different $MgCl_2$ (0, 1, 3, 6, 15 and 30 mM) concentrations were measured using the flow-through cell in order to verify that a fully assembled state was reached at lower $MgCl_2$ concentrations (6 mM vs. 30 mM, see Figure 2).

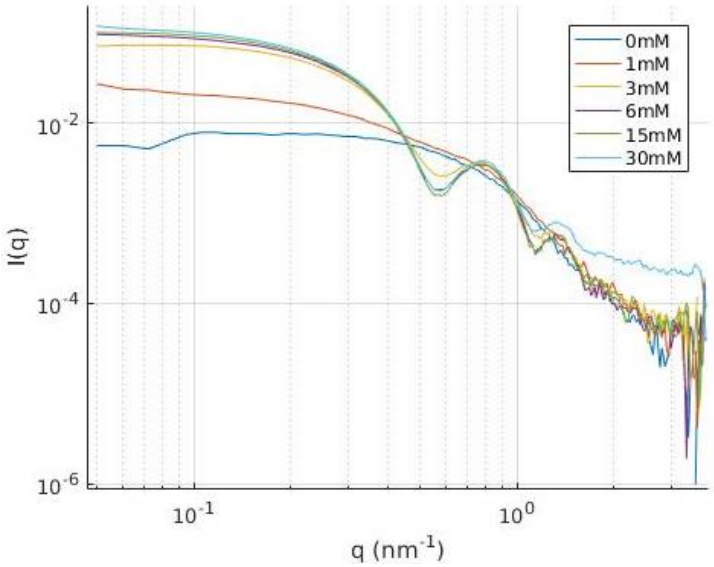

Figure 2 - Static SAXS data on AfFt-hum (2.5 mg/ml) at various $MgCl_2$ concentrations.

The first set of collected time-resolved SAXS data revealed the presence of a small fraction of large disordered aggregates in the AfFt-hum samples. These aggregates were successfully removed by filtration and were absent in the further measurements that we have performed. In order to increase the S/N of our data, measurements at protein concentrations up to ~5 mg/ml were also performed. At the end of the beamtime, we had been able to collect time-resolved scattering data at the 6 different conditions summarized in Table 1, with 42-52 time-delays acquired per condition. The resulting scattering profiles of the 4 best time-resolved datasets are displayed in Figure 3.

Table 1 - Collected time-resolved data

AfFt-hum (mg/mL)	2.3	4.1	2.7	2.7	2.7	5.1
$MgCl_2$ (mM)	30	6	3	6	30	3

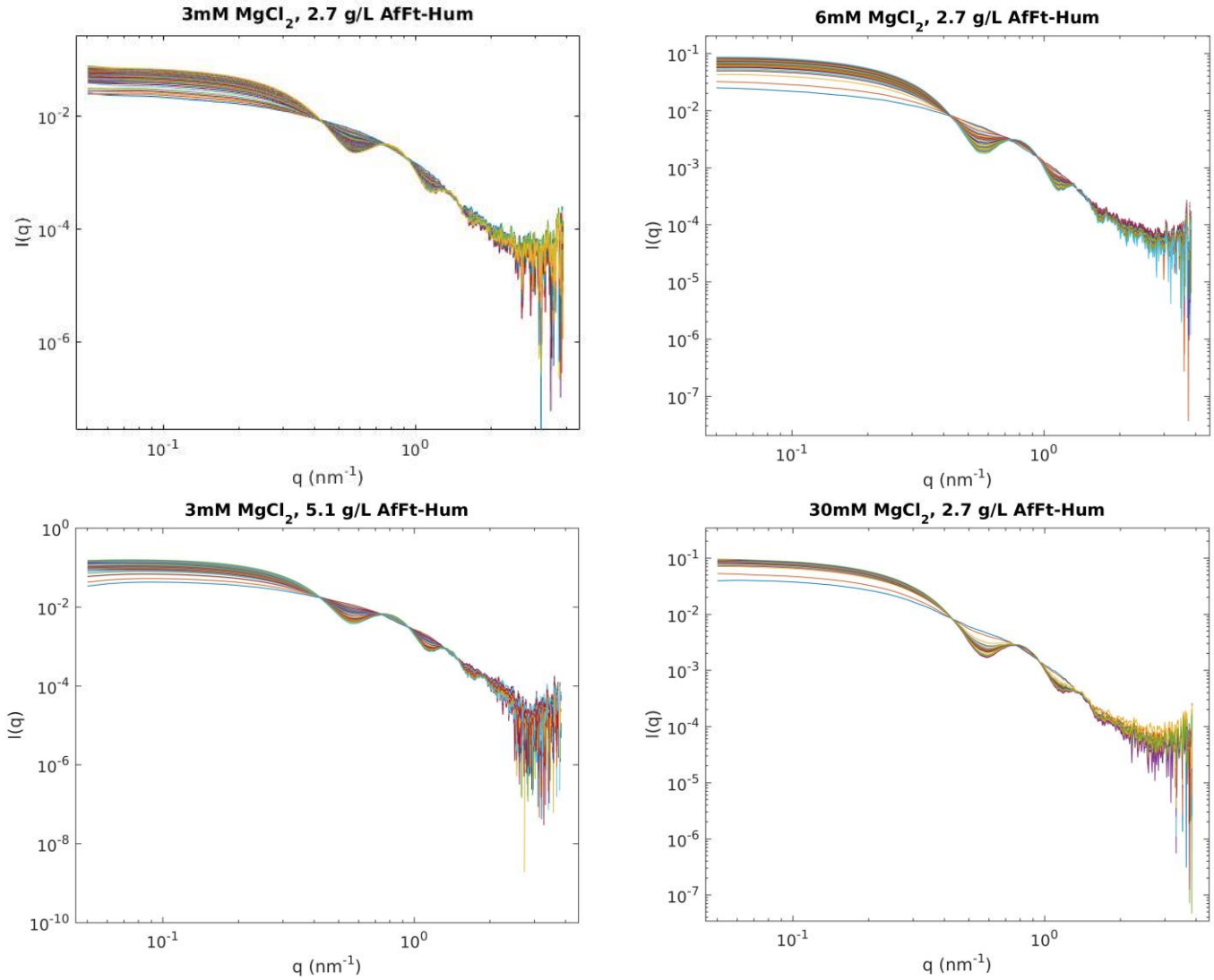

Figure 3 - Scattering curves for the best collected datasets, at 4 different conditions.

Conclusions

Beamtime was rather successful from the point of view of data collection on the assembly kinetics of "humanized" *A. fulgidus* ferritin. Several datasets at different Ferritin/$MgCl_2$ concentration ratios have been measured. Data analysis to extract detailed kinetic information from these datasets is currently in progress. We plan to use our data to test the validity of different models proposed for the ferritin assembly mechanism, which may prove relevant for the design of new protein-based nanodevices for drug delivery and diagnostics. Unfortunately, the available beamtime was insufficient to satisfactorily determine the dependence of the observed rate constants on Ferritin/$MgCl_2$ concentrations, and to determine the reaction order, which may yield clues in the definition of the assembly mechanism. Moreover, it was not possible to carry out measurements on the wild type *A. fulgidus* ferritin which represents the reference system for all the Af-Ft based encapsulating systems we plan to test and use for biotech applications.

References

[1] Sato D, Ohtomo H, Yamada Y, Hikima T, Kurobe A, Fujiwara K, Ikeguchi M. *Biochemistry* 55, 287 (2016)

[2] Falvo E, Tremante E, Arcovito A, Papi M, Elad N, Boffi A, Morea V, Conti G, Toffoli G, Fracasso G, Giacomini P, Ceci P. *Biomacromolecules* 17, 514 (2016).

[3] de Turris V, Cardoso Trabuco M, Peruzzi G, Boffi A, Testi C, Vallone B, Montemiglio LC, Des Georges A, Calisti L, Benni I, Bonamore A, Baiocco P. *Nanoscale* 9,647 (2017)