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New Generation Stopped-Flow Time-Resolved BioSAXS Experiments at the P12 Beamline.

Aleksi Sutinen[1*], Kamil Szpotkowski[2] , Dmytro Soloviov[1] , Sofia Trampari[3], Tilman Donath[3], Marina Nikolova[1], Gleb Bourenkov[1], Clément Blanchet[1]

[1] European Molecular Biology Laboratory Hamburg Outstation, Hamburg,, Germany
[2] Department of Zoology, Poznań University of Life Sciences, Poznań, Poland
[3] DECTRIS Ltd., Baden, Switzerland

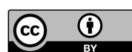
[*]E-mail: aleksi.sutinen@embl-hamburg.de

Abstract. Time-resolved small-angle X-ray scattering enables studying dynamic structural changes in biological macromolecules. Developments in fast mixing devices, such as the significant reduction in sample volume requirements, now allow the technique to be applied to a wider range of biologically relevant macromolecules, often available in limited quantities. Advances in synchrotron radiation sources and detector technology have dramatically increased the time resolution, allowing real-time monitoring of reaction kinetics and folding processes at millisecond and sub-millisecond timescales. Here, we present recent updates to the P12 SAXS beamline at EMBL Hamburg, including integrating the new Micro Stopped-Flow Mixer (μSFM) generation. We demonstrated the kinetics of pH-induced conformational dynamics in two case studies of oxygen-carrying proteins, capturing rapid reorganization of the multimeric state on a millisecond time scale. Based on the experimental findings, the new system significantly improves time resolution and sample consumption, facilitating more efficient TR-SAXS experiments.

1. Introduction

Time-resolved SAXS is essential for understanding dynamic processes in biological macromolecules, including protein folding, conformational changes, and reaction kinetics. Traditional SAXS captures static structural information, while TR-SAXS captures real-time structural changes, often triggered by rapid mixing either with a turbulent mixer such as a stopped-flow or quench-flow, continuous laminar mixing using microfluidics (1,2) temperature shifts or photoactivation. The time scale of the experiment is dependent on the trigger mode. With mixing separate species, the time scale can be flexible from minutes to hours, even milliseconds, with rapid mixing techniques. Faster, sub-millisecond triggering is achieved by probing rapid changes in temperature or light, often utilizing powerful lasers. Furthermore, similar rapid probing can be achieved by pressure in the system.

One of the key challenges in TR-SAXS experiments is high sample consumption, especially when dealing with expensive or limited biological samples. Here, we present recent advancements of TR-SAXS at the P12 beamline. The upgrades include the integration of the BioLogic μSFM Stopped-Flow mixing apparatus (SFM-SAXS) at the P12 beamline (3), improving mixing efficiency and reducing sample consumption by a factor of 4 to 10 compared to the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previous SFM configuration. This improvement enables longer experimental runs and increases the likelihood of obtaining high-quality data from limited sample volumes. To demonstrate improved temporal resolution, we integrated the latest fourth-generation PILATUS4 detector into the beamline, which allowed us to capture structural transitions of protein molecules at millisecond timescales. These developments address challenges such as high sample consumption and limited temporal resolution compared to previous iterations of SFM-SAXS (4).

2. Sample environment description

The µSFM, developed by BioLogic Science Instruments (BioLogic, Seyssinet-Pariset, France), was integrated into the P12 beamline to improve mixing efficiency and reduce sample consumption for TR-SAXS measurements (**Figure 1**). The typical experimental setup involves mixing two solutions with a 1:1 ratio, utilizing 15 µl of sample per acquisition. The µSFM achieves an average flow rate of 1.2 ml/s, facilitating rapid mixing in milliseconds. This setup enables real-time observation of structural changes initiated by mixing, offering time resolutions from millisecond to second-time scale. The P12 beamline configuration for SFM-SAXS experiments includes the installation of the µSFM in the experimental area, aligning the SFM measurement cell in the X-ray beam, and synchronizing the experimental beam shutter, detector and SFM pistons. The control of the beamline hardware is managed through the TINE device server (5). The SFM can be operated with two Hamilton gas-tight syringes. The operating temperature of the observation head can be varied between 4-55 C and is tolerable for most organic solvents. The X-ray observation head houses a quartz 1 mm capillary with a 10 µm wall thickness. Alternatively, the SFM can also be used offline, in combination with a spectrometer, to measure UV-Vis absorption or fluorescence and evaluate the kinetic of the reaction.

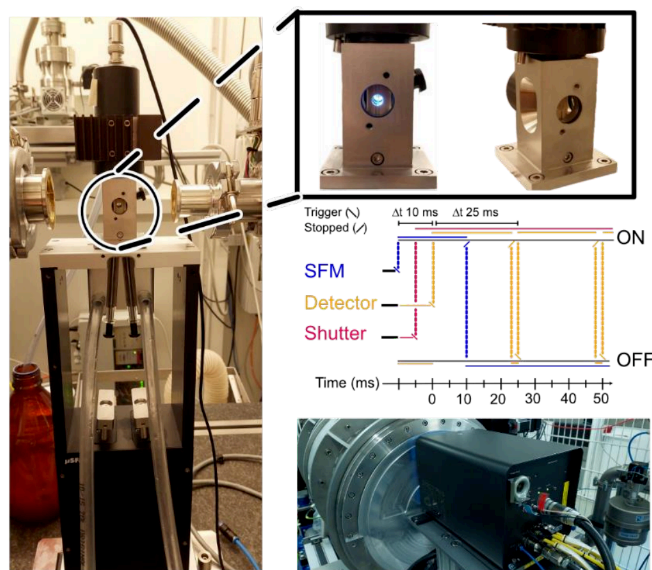

Figure 1. SFM-SAXS operation for TR-SAXS measurements. The data collection is controlled by a synchronization device that triggers the SFM, experimental shutter, and detector. The process is initiated by triggering the mixing sequence on the SFM. The SFM sends a signal 10ms before the flow stops, which synchronizes the shutter opening 5 ms before the first data point is collected, accounting for shutter opening time. Another signal triggers the detector to start data collection. The dead time of the experiment refers to the period between the start of solution mixing and the first reliably measured data point. This is dominated by the time needed to push the solutions through the mixer and reach the measurement point and typically ranges between 1-10 ms, depending on the mixing sequence.

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3. Commissioning and initial studies at P12 Beamline

The SFM-SAXS setup has been available for users since spring 2024. The typical system under investigation is a macromolecule, such as a protein that undergoes a conformational change, changes in the dynamic characteristics, or the order of the molecule's multimerization under the mixing of perturbation species. Mixtures have included rapid changes in pH, salinity, temperature, or a binding ligand. We investigated the two classes of large multimeric proteins susceptible to conformational pH dependence as a case study. The first class was hemocyanin and hemerythrin from species that belong to common and numerous representatives of freshwater malacofauna and are oxygen carriers in the blood (**Case study 1**). Secondly, we studied a well-characterized animal iron storage protein, Apoferritin, extracted from the equine spleen. In native form, the protein is constructed into an octahedral 24-mer unit, susceptible to pH change, prompting the dissociation from a high-ordered multimeric state into smaller subunits. The apoferritin study utilized the rapid data collection capabilities of the PILATUS4 photon-counting detector, which enabled precise time-resolved measurements at millisecond timescales (**Case study 2**).

3.1 Case study 1 – Hemocyanin (Lymneae stagnalis) and Hemerythrin (Planorbarius corneus)

The Hemocyanin and Hemerythrin proteins were purified from the hemolymph of snails. Hemocyanin was isolated from the great pond snail Lymneae stagnalis, and hemoeythrin was isolated from the great ramshorn Planorbarius corneus. The procedure of protein isolation is identical for hemocyanins and hemerythrins. Snails were collected in the Międzychodzko – Sierakowskie lake district in the west–north Wielkopolska Region, Poland. The collected snails were kept in 40L aquariums. Before isolation, snails were cooled down to 1°C. The hemolymph was collected after cutting the foot muscles. The isolated hemolymph was dissolved in buffer 20mM Tris, 150 mM NaCl pH 8.5, 1mM PMSF (Phenylmethylsulfonyl fluoride) in a volume ratio 1:1, and it was centrifuged at 8000 rpm in 4°C for 20 min to remove rough particles and hemolymph cell. After that, the hemocyanin solution was salted out of 80% ammonium sulfate, and then it was centrifuged at 10000 rpm at 4°C for 20 min. After that, the pellet was dissolved in the the buffer of 20 mM Tris and 150 mM NaCl pH 8.5. In the next step, the protein was dialyzed in buffer 20 mM Tris, 150 mM NaCl, 10 mM EDTA pH 8.5 (native buffer), and after 14 days, size exclusion chromatography (SEC) was made. The oligomerization state of hemocyanin was monitored by the dynamic light scattering (DLS) method before the SFM-SAXS measurements (data not shown). The SFM-SAXS measurement of 10 mg/ml hemocyanin and hemerythrin solution was titrated with a 4:1 ratio in the native buffer, where the pH was adjusted to pH 10. The data was recorded at a 1.5 m distance at 10 keV, using the 2.gen PILATUS 6M detector for 1 s, with a 50 ms exposure time interval.

The oligomeric stability of decameric hemocyanin and hemerytrin was perturbed with a rapid pH Jump where the molecule dissociated into smaller subunits in the hundred-millisecond timescale. Interestingly, although the proteins arise from the same family and have a high sequence identity, the Hemerytrin appeared from species with more flexibility. In contrast, hemocyanin retained most of the stable complex structure. (**Figure 2**).

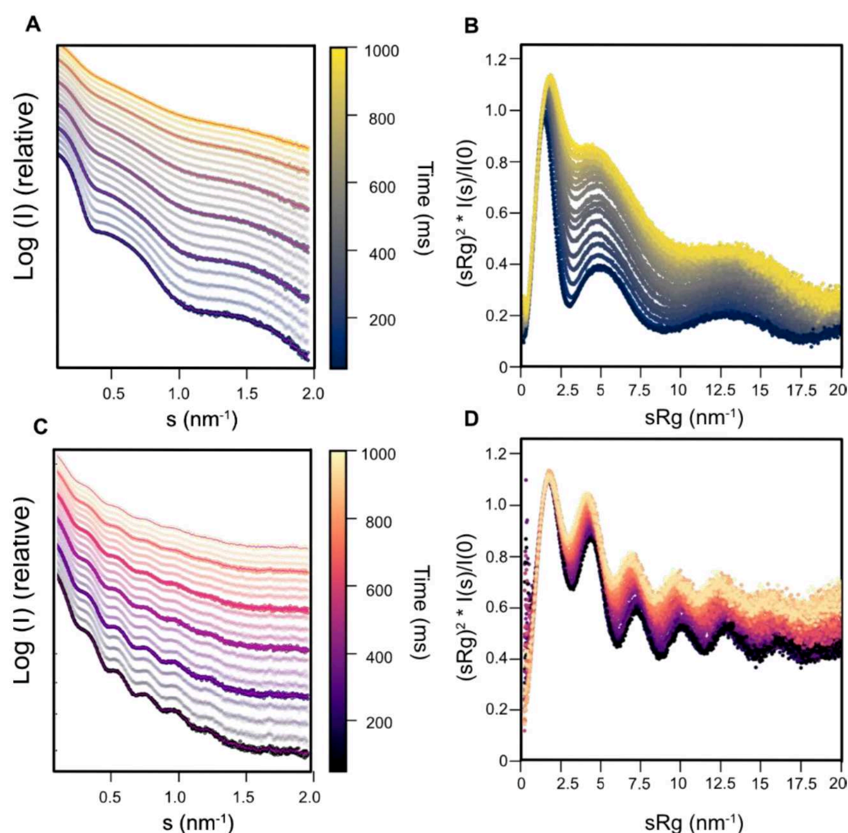

Figure 2. TR-SAXS case study highlighting conformational changes induced by pH in Hemerythrin from *Planorbarius corneus* (great ramshorn) (A, B) and Hemocyanin from *Lymneae stagnali* (Great pond Snail) (C, D). The same time scale applies to both color bars.

3.2 Case study 2 – Apoferritin (Equus ferus caballus)

The Equine spleen Apoferritin was commercially obtained and purchased from Sigma, Darmstadt, Germany (cat# A3660). The sample preparation for Apoferritin for SAXS measurements at P12 has been described previously elsewhere (6). The apoferritin solution was diluted to 4 mg/ml in 50 mM Glycine buffer and titrated to the denaturant buffer with a 4:1 ratio to 50 mM Glycine, 200 mM HCl solution. Data collections studying the dissociation of apoferritin under acidic conditions were conducted using a prototype of DECTRIS Ltd.'s new PILATUS4 photon-counting detector (7), which offers fast framing capability for time-resolved measurements, as used here, as well as modes for gated acquisition that can be employed in laser-triggered measurements (5). The data were collected at a detector distance of 1.5 meters and a photon energy of 10.0 keV. The detector was operated at a 2 kHz frame rate, where the exposure time was 0.5 ms. Post measurement, the frames were summed (resampled) to time intervals ranging from 5 ms to 200 ms to find the optimum between signal intensity and time resolution per frame. The quality of the photon-counting data allows for this retrospective finding of the optimal time interval. In contrast, time resolution does not need to be defined and limited at the time of the measurement (**Figure 3**).

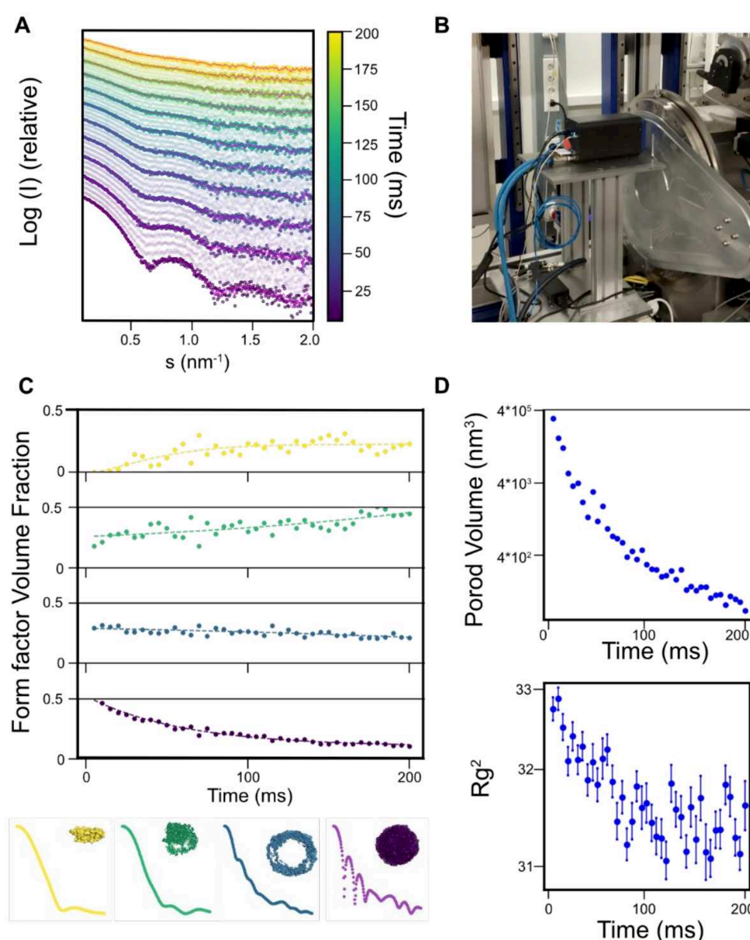

Figure 3. Time-resolved evolution of dissociation of Apoferritin (A), PILATUS4 260K prototype detector installed at P12 (B). The SFM observation head is ~1.6 m from the detector for these experiments. The data shows that over the course of 200 ms, the large 24-mer spherical multicomplex dissociates into smaller oligomers (C). The volume fraction of multimeric species, the evolution of radius of gyration, and Porod volume as a function of time (D).

4. Application and experimental considerations

TR-SAXS experiments present unique challenges, especially compared to static solution bioSAXS experiments. One major difficulty is that stopped-flow experiments are performed in-air, resulting in increased instrument background. Additionally, since the flow is stopped after mixing, the sample is exposed to X-rays for longer periods, leading to more severe radiation damage than in static SAXS, where samples can continuously flow through the beam to limit the damage. The data collection scheme must also be carefully adapted depending on the timescale of the reaction and the required time resolution.

It is highly recommended that static SAXS measurements be performed first on both the initial and final states of the reaction using a standard SAXS setup, which typically produces

higher-quality data. These preliminary measurements help estimate the expected changes in scattering intensity during the reaction and guide the design of the time-resolved experiment [7]. They can provide insights into the necessary exposure time to obtain a strong enough signal and inform the number of repetitions needed for a reliable dataset. Additionally, these measurements offer clues about the radiation sensitivity of the system. This can help determine whether sample modifications, such as adding radical scavengers, are necessary to reduce radiation damage without interfering with the reaction.

Another valuable preparation step, when possible, is to gather kinetic data using the stopped-flow mixer coupled with UV-Vis absorption or fluorescence spectroscopy. Probing different timescales with spectroscopy provides critical information to design the X-ray experiments more efficiently when an estimate of the time scale is known. Even though the μSFM significantly reduces sample consumption compared to traditional setups, we recommend preparing a minimum of 1-2 mg of total protein, particularly for small molecules or experiments requiring high temporal resolution (less than 10 ms).

5. Conclusions

The improvements in time resolution and sample efficiency at the P12 beamline mark a significant advancement in TR-SAXS technology. The combination of the μSFM and rapid photon-counting detectors, such as the EIGER2 [9] and PILATUS4 series, enables the study of rapid structural transitions with unprecedented accuracy and detail. Future developments at the P12 beamline, alongside the upcoming PETRAIV synchrotron source at DESY, will continue to push the boundaries of TR-SAXS research. These advancements position the P12 beamline as a leading facility for high-resolution TR-SAXS experiments, offering deeper insight into the dynamic behavior of biological macromolecules.

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