Using macromolecular-crystallography beamline and microfluidic platform for small-angle diffraction studies of lipidic matrices for membrane-protein crystallization

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Using macromolecular-crystallography beamline and microfluidic platform for small-angle diffraction studies of lipidic matrices for membrane-protein crystallization

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Abstract. Macromolecular-crystallography (MX) beamlines routinely provide a possibility to change X-ray beam energy, focus the beam to a size of tens of microns, align a sample on a microdiffractometer using on-axis video microscope, and collect data with an area-detector positioned in three dimensions. These capabilities allow for running complementary measurements of small-angle X-ray scattering and diffraction (SAXS) at the same beamline with such additions to the standard MX setup as a vacuum path between the sample and the detector, a modified beam stop, and a custom sample cell. On the 21-ID-D MX beamline at the Advanced Photon Source we attach a vacuum flight tube to the area detector support and use the support motion for aligning a beam stop built into the rear end of the flight tube. At 8 KeV energy and 1 m sample-to-detector distance we can achieve a small-angle resolution of $0.01A^{-1}$ in the reciprocal space. Measuring SAXS with this setup, we have studied phase diagrams of lipidic mesophases used as matrices for membrane-protein crystallization. The outcome of crystallization trials is significantly affected by the structure of the lipidic mesophases, which is determined by the composition of the crystallization mixture. We use a microfluidic chip for the mesophase formulation and *in situ* SAXS data collection. Using the MX beamline and the microfluidic platform we have demonstrated the viability of the high-throughput SAXS studies facilitating screening of lipidic matrices for membrane-protein crystallization.

1. Introduction

High-impact membrane proteins such as human G-protein coupled receptors (GPCRs) have been recently crystallized and their structures were solved [1]. In all the cases, lipidic mesophases were used as matrices for crystallization, reviving an interest in this crystallization technique [2]. The structure of the mesophase itself is crucial for the outcome of crystallization trials, and requires measurements of small-angle X-ray scattering (SAXS) for unambiguous identification [3]. While high-throughput measurement procedures have been developed at SAXS-dedicated synchrotron beamlines [4], the ability to use a single beamline to both screen crystallization matrices by SAXS and

perform Macromolecular Crystallography (MX) would facilitate the studies of crystallogenesis. Some MX synchrotron beamlines have SAXS capabilities [5], but significant beamline reorganization may be required for switching between the two techniques. Because lipidic mesophases are highly ordered liquid crystals providing relatively intense powder-diffraction rings on the X-ray patterns, requirements for intensity resolution of the SAXS setup are relatively low. However, sample-to-detector distance must be large in order to avoid overlapping of diffraction rings.

Here we report screening of lipidic mesophases at the 21-ID-D MX beamline at the Advanced Photon Source (APS) and discuss the necessary beamline tuning and transformation for the SAXS experiment. We tested the setup with SAXS measurements of 15 μm-thick mesophase samples of a lipid mixed with aqueous solutions of a detergent. The mesophases were formulated in active-mixing microfluidic platforms [6, 7] that were also suitable for *in situ* data collection. We benchmarked microfluidic results using samples mixed in coupled-syringe mixers [3] and measured in glass capillaries, the current standard for lipidic mesophases.

2. Materials and Methods

Lipid mesophases were formed in microfluidic platforms by mixing lipid monoolein (Sigma Aldrich, 99%) with solutions of detergent β-octylglucoside (ßOG) [6]. Solutions of 5% v/w and 10% v/w ßOG (Anatrace, Anagrade) were prepared by dilution from a 20% v/w ßOG solution in 25 mM NaH_2PO_4, pH 5.5 (NaH_2PO_4 from EMD Chemicals). Monoolein:detergent solution ratio was 55:45 v/v in all samples. For benchmarking, samples with identical compositions were formulated in coupled-syringe mixers [3] and stored in thin-walled 0.9 mm glass capillaries (Charles Supper). All samples were stored at -80 ^{o}C to avoid water evaporation for 1-4 days prior to measurements, then defrosted, and equilibrated at data collection temperature (25 ± 0.5 ^{o}C) for at least 2 hours prior to the measurements.

Microfluidic platforms (chips) were fabricated using a modified soft lithography procedure [6, 7]. The platform consisted of two layers – a control and a fluid one – fabricated in polydimethyl-siloxane (PDMS) (RTV 650, Momentive Performance Adhesives) and sandwiched between 50 μm and 100 μm-thick sheets of cyclic olefin copolymer (COC) (TOPAS Advanced Polymers). The sample thickness in the fluid layer was 15 μm. The total thicknesses of PDMS and COC layers in the path of the X-ray beam were 30 and 150 μm respectively, which made the platforms transparent for X-rays.

SAXS data were collected on 21-ID-D MX beamline at the APS. The 8 KeV beam was focused at 0.5 m distance from the sample position to a size of ~25x50 μm^2 (FWHM) using Kirkpatrick-Baez bimorph mirrors (ACCEL). For beam conditioning, a 20 μm beam-defining aperture and 100 μm beam-cleaning (guard) capillary were chosen from the set of apertures supplied with the microdiffractometer MD2 (MAATEL), a standard part of the beamline MX setup. A vacuum flight tube (300 mm maximal diameter) was installed between the sample and the detector as discussed in the next section. The tube had 25 μm mica (Attwater) and 125 μm Kapton (DuPont) windows at the front and the rear ends respectively. A 5 mm tungsten beam stop (Midwest Tungsten Service) with a built-in PIN photodiode (Fairchild Semiconductor) was attached to the Kapton window by a Neodymium disc magnet (McMaster-Carr). The SAXS patterns were recorded with Rayonix MX-300 detector, a standard part of the MX setup, located at a distance of 885 mm. Sample-to-detector distance was calibrated using a silver behenate standard powder (The Gem Dugout) [4].

Data was collected at beam attenuation up to 76 %, exposure time from 1 to 5 sec and rotation of the sample within 15° which brought more lipidic domains into diffraction condition. MX data-collection software was modified to allow for automated screening in multiple points on the microfluidic chip. For data averaging, the fit2d program (A.P.Hammersley, ESRF) was used in a batch mode. A series of Matlab scripts was developed for phase assignment on the averaged diffractograms. Lattice spacing of the lipidic phases was used for structure identification as described elsewhere [4, 8].

3. Results and Discussion

In order to identify monoolein mesophases, SAXS data need to be collected at a crystal-lattice spacing resolution of up to d~200 Å [4] which corresponds to the resolution over the scattering vector q =

$4\pi\sin\Theta/\lambda \sim 0.03$ Å^{-1} (here λ is the X-ray wavelength and Θ is the scattering angle). To avoid overlapping of powder-diffraction rings on the SAXS images from the mesophases, the sample to detector distance has to be sufficiently long [4]. The best d-resolution immediately reached with the MX setup at the 21-ID-D beamline is d~30 Å (q ~ 0.21 Å^{-1}). It is achieved at the beam energy of 8 KeV (λ=1.55 Å), the MX beam stop of 0.3 mm in diameter located at 5 mm from the sample and the detector at the maximum distance of 1 m. Further lowering of beam energy was not useful for SAXS because of the rapid increase in the background caused by scattering from beamline windows materials and air gaps. To increase the resolution, we used an effectively smaller beam stop as positioned close to the detector. Parasitic scattering around the beam stop was reduced by optimizing the beam-conditioning aperture sizes according to the geometry of a three-pinhole SAXS camera [9]. As a result, the resolution increased to d ~ 500 Å (q ~ 0.013 Å^{-1}) and a good separation of powder-diffraction rings was ensured. Beam intensity on the sample was optimized using ray tracing simulations with SHADOW3 [10]. At high demagnification ratio of the beamline optics, which was ~20, the beam would have lost ~99% of its intensity at the strong collimation if focused on the detector. Focused on the sample, the beam would lose only ~45% of intensity, but would be too divergent to be reliably stopped by the beam stop. We focused the beam half way between the sample and the detector using bimorph mirrors, which speeded up the procedure.

In order to eliminate air scattering on a beam path longer than in typical MX experiments, a vacuum or a helium flight tube is usually installed in SAXS setups [5]. We found a simple way to install a vacuum tube by securing it on the detector support (figure 1a). This enabled precise alignment of the beam stop attached to the rear window of the tube and allowed us to avoid installation of additional table and positioner. The front window of the tube was roughly adjusted vertically by a crane, and horizontally by pushing around a pivot point in a bracing on the detector. The entire process of the MX beamline preparation for SAXS measurements was taking about three hours.

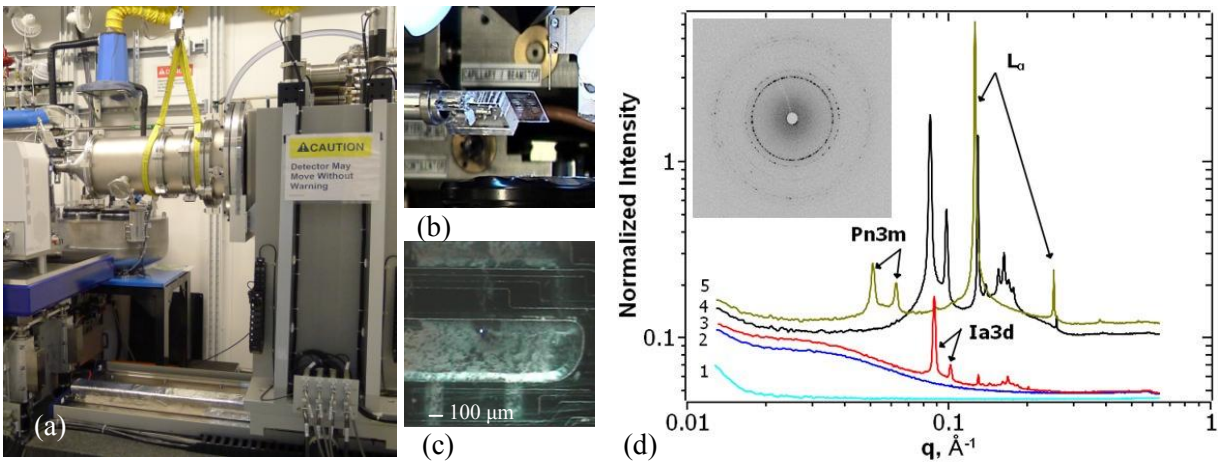

Figure 1. (a) Vacuum flight tube secured on the detector support. (b) Microfluidic chip installed on MD2 spindle and visualized with MD2 built-in on-axis video microscope (c). (d) SAXS curves measured without any sample or holder in the beam (line 1), with an empty microfluidic chip (line 2), and with lipidic mesophases of cubic Pn3m and Ia3d and lamellar Lα structure in the chip (line 3) or glass capillary (lines 4 and 5). The mesophases were obtained by mixing monoolein with a 5% solution of βOG. Inset: a SAXS image from a 15 µm thick lipidic sample in the chip.

Membrane protein crystallization mixtures consist of several components including lipid, precipitant and detergent. The latter is added to solubilize the protein and is known to strongly affect the phase behavior of the mesophases [3]. We tested our SAXS setup by studying the influence of detergent on the structure of mesophases. For SAXS measurements, the microfluidic chip was positioned on a spindle of MD2 using the same cap (Molecular Dimensions) as in MX experiments

(figure 1b). The size of the measurement area was restricted by the MD2 design to 8 x 4 mm^2 and accommodated four sample-formulation units, with each unit consisting of three compartments. The compartments were visualized with the on-axis video microscope of MD2 providing 12x zoom and the smallest field of view 0.18 x 0.12 mm^2 (figure 1c), enabling precise choice of the measurement point.

Figure 1d presents examples of scattering curves of monoolein mesophases with identical compositions prepared and measured in a microfluidic chip (line 3) and a syringe mixer/glass capillary (line 4). The main features of these two scattering curves are identical, proving that the two samples possess the same structure. The inset in Figure 1d reproduces a section of the corresponding SAXS image collected from the sample in the chip. A scattering curve from an empty chip (line 2) confirms the X-ray transparency of the chip. The assignment of phase types was mainly based on the first two diffraction peaks for each phase. Although SAXS signal from the 15 μm thick sample on-chip was much lower in comparison with the signal from the sample in a ~1 mm thick capillary, it was sufficient for structure identification, and we were able to successfully identify the three mesophase types typical of monoolein at room temperature in various samples on-chip. Detailed statistics on formation of the lipidic mesophases in the microfluidic platform will be published elsewhere.

4. Conclusions

We have demonstrated that SAXS screening of lipidic matrices for membrane-protein crystallization can be done on the same MX beamline where crystal diffraction data will be later collected. The SAXS measurements did not require for any substantial modifications of the MX beamline: a vacuum flight tube needed to be installed with a beam stop on its rear window, and beamline alignment parameters needed to be optimized. We showed a simple way of installing the flight tube by securing it on the existing detector support. The d-spacing resolution at a maximal sample to detector distance of 1 m was about 500 Å ($q \sim 0.013$ $Å^{-1}$). Optimized beam intensity was sufficient to collect SAXS data from samples of lipidic mesophases as thin as 15 μm and reliably identify their structure. Active-mixing microfluidic chips facilitated multiple sample preparation and *in situ* data collection.

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