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The application of hybrid pixel detectors for in-house SAXS instrumentation with a view to combined chromatographic operation

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This study analyses the potential for laboratory-based size-exclusion chromatography (SEC) integrated small-angle X-ray scattering (SAXS) instrumentation to characterize protein complexes. Using a high-brilliance home source in conjunction with a hybrid pixel X-ray detector, the efficacy of SAXS data collection at pertinent protein concentrations and exposure times has been assessed. Scattering data from SOD1 and from the complex of SOD1 with its copper chaperone, using 10 min exposures, provided data quality in the range $0.03 < q < 0.25$ Å^{-1} that was sufficient to accurately assign radius of gyration, maximum dimension and molecular mass. These data demonstrate that a home source with integrated SEC–SAXS technology is feasible and would enable structural biologists studying systems containing transient protein complexes, or proteins prone to aggregation, to make advanced preparations in-house for more effective use of limited synchrotron beam time.

Keywords: SAXS; protein–protein complex; home source; chromatography; hybrid pixel X-ray detector.

1. Introduction

Large multi-domain proteins and heterogeneous complexes formed between proteins and other biomolecules are becoming the focus of structural efforts as these species harbour the secrets of the interactome. These high value targets have been successfully investigated using small-angle X-ray scattering (SAXS) (Bernadó *et al.*, 2007; Putnam *et al.*, 2007; Rambo & Tainer, 2010). The synthesis of chromatographic methods, particularly size exclusion chromatography (SEC) with synchrotron SAXS set-ups (SEC–SAXS), has further paved the way to study more intractable protein systems (Mathew *et al.*, 2004; David & Pérez, 2009). These include transient complexes, multiple oligomerization states and aggregation or degradation prone systems. While home-based SAXS instruments have been rigorously tested (Bergmann *et al.*, 2000; Pedersen, 2004) and are widely used, integration with a chromatographic stage has not been reported. The central technical issue in this regard is the quality of the SAXS data produced by the relatively weak home X-ray sources, in conjunction with the low protein concentrations created by the dilution effect of size exclusion chromatography and the short exposure times necessary to capture a protein/complex as it elutes from a column.

A solution to this problem may be found in the use of the 'brightest' home X-ray sources available, such as the Rigaku FR-E+, in combination with a PILATUS single-photon-counting detector (Brönnimann *et al.*, 2001). Hybrid pixel detectors have been characterized in detail and are used widely in synchrotron SAXS endstations (Basolo *et al.*, 2008; Koerner *et al.*, 2011; Blanchet *et al.*, 2012). However, these detectors are also perfectly suited for in-house SAXS instrumentation owing to their high dynamic range, absence of readout or background noise, as well as high quantum efficiency particularly at the Cu $K\alpha$ wavelength (8 keV energy) provided by most home-source X-ray generators. To assess the feasibility of using this set-up for SEC–SAXS applications, we measured SAXS data for copper-zinc superoxide dismutase (SOD1) and its complex formed with the human copper chaperone for SOD1 (hCCS) (Valentine *et al.*, 2005; Wright *et al.*, 2011), both at concentrations that reflect those expected after dilution during chromatography and with exposure times short enough to match protein eluting from a standard preparation-grade SEC column. These measurements are compared with 'gold-standard' data collected using the SEC–SAXS set-up at SOLEIL, a third-generation synchrotron light source.

2. Materials and methods

Expression and purification of SOD1 and hCCS recombinant proteins was performed as described previously (Wright *et al.*, 2011) and purity was assessed by SDS-PAGE. Construction of the hCCS–SOD1 complex was performed as described previously (Winkler *et al.*, 2009). Proteins were stored and measured in Tris buffered saline (20 m*M* TrisHCl, 150 m*M* NaCl) with the addition of 5 m*M* dithiothreitol for the hCCS–SOD1 complex.

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Synchrotron SAXS measurements were carried out using the integrated SEC–SAXS set-up at beamline SWING (David & Pérez, 2009) at the French national synchrotron SOLEIL, Saint Aubin, Paris. Briefly, 1.75 mg of wild-type SOD1 was loaded onto a Shodex 403-4F gel filtration column in a 70 µl volume at 200 µl min^{-1} flow rate. 100 × 1 s buffer frames were averaged and subtracted from 30 × 1 s frames taken over the course of protein elution. The incident beam energy was 12 keV and the sample–detector distance (SDD) was 1.81 m giving an angular momentum transfer range of q_{min} = 0.01 $Å^{-1}$ to q_{max} = 0.6 $Å^{-1}$. The flux density was $\sim10^{13}$ photons s^{-1} mm^{-2}. Scattering was collected on an AVIEX 170 × 170 charge-coupled device detector. hCCS–SOD1 complex measurements were collected in a similar fashion, with 15 mg of complex loaded onto a Superdex 200 16/60 column in a 0.7 ml volume with flow rate 750 µl min^{-1}. SAXS data were averaged for 80 × 3 s exposures with 1.5 s intervening dead-time. Radial integration, data averaging and subtraction were performed with *Foxtrot* (SWING). At the point of exposure to X-rays the protein concentration was approximately 4 mg ml^{-1} for both SOD1 and the hCCS–SOD1 complex, as determined by absorbance at 280 nm.

In-house SAXS measurements were performed at the Barkla X-ray Laboratory of Biophysics at the University of Liverpool, UK. The experimental set-up is pictured in Fig. 1. The scattering instrument combines a Rigaku FR-E+ Superbright rotating copper anode X-ray generator (operated at 45 kV and 55 mA giving a flux density of $\sim10^{11}$ photons s^{-1} mm^{-2}) and a PILATUS 300K-20Hz hybrid pixel detector. The beam size at the sample cell position was 0.8 × 0.8 mm following collimation using a three pin-hole system with aperture sizes 0.8, 0.4 and 1.3 mm. The distances between pinholes were 690 and 485 mm. The SDD was 1.2 m with the detector position offset so scattering was recorded over 180°. The 8 keV beam gave an angular momentum transfer range of q_{min} = 0.03 $Å^{-1}$ to q_{max} = 0.3 $Å^{-1}$. Angular momentum transfer was calibrated using silver behenate. The sample cell consists of a brass holder containing a brass ring sandwiched by two mica windows each of thickness 25 µm. The cell has a sample volume of 80 µl and a thickness of 1 mm. Scattering from solvent was recorded using the buffer used for the final SEC purification step. SAXS from SOD1 and the hCCS–SOD1 complex were recorded from solutions at 4.0 and 3.7 mg ml^{-1}, respectively. In each case 60 × 10 s frames were averaged to yield an overall exposure time of 10 min. Radial averaging was performed using cSAXS MATLAB macros (http://www.psi.ch/sls/csaxs/software) and *FIT2D* (Hammersley, 1997). One-dimensional buffer scattering was subtracted from one-dimensional protein scattering with adjustment of the scale parameter to minimize the sum of squared deviations at high q (0.28–0.3 $Å^{-1}$).

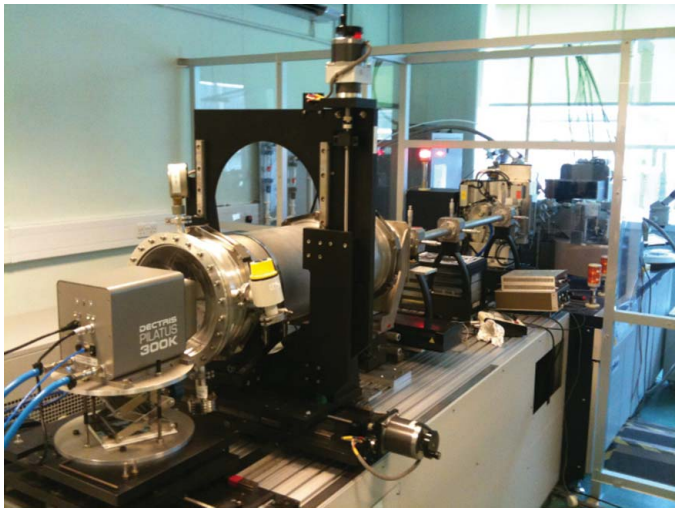

Figure 1
The SAXS instrument at the Barkla Laboratory of Biophysics. The set-up includes a Dectris PILATUS 300K-20Hz detector, three pin-hole optics and Rigaku FR-E+ Superbright X-ray generator.

Table 1
Comparison of SAXS size parameters calculated from home-source and synchrotron data.

Protein	Light source	R_g (Å)	D_{max} (Å)	Estimated molecular mass (kDa)
SOD1	FR-E+/PILATUS 300K	20.8 ± 0.7	62	25.8
SOD1	SWING/SOLEIL	20.6 ± 0.1	61	27.2
hCCS–SOD1	FR-E+/PILATUS 300K	24.3 ± 1.5	80	42.0
hCCS–SOD1	SWING/SOLEIL	24.5 ± 0.1	87	40.8

Radii of gyration (R_g) were estimated using the Guinier analysis by iterative linear regression with q_{min} = 1/3(q_{max}) to q_{max} = 1.5/R_g. Distance distribution functions $P(r)$ were calculated using *GNOM* (Svergun, 1992) and molecular mass estimated with *AUTOPOROD*.

3. Results and discussion

Figs. 2(*a*) and 2(*b*) show experimental SAXS from recombinant human SOD1 and the hCCS–SOD1 complex collected at the synchrotron and home light sources. Home-source measurements were performed at 4.0 and 3.7 mg ml^{-1} protein concentrations for SOD1 and hCCS–SOD1, respectively. This range was chosen to reflect the concentrations at the point of measurement using a SEC–SAXS set-up, approximately 4 mg ml^{-1}. As would be expected, the synchrotron data have very low noise in comparison with home-source data. However, the synchrotron scattering profiles are closely approximated by those acquired on the home source in both cases, up to the characteristic minimum at 0.25 $Å^{-1}$ (Hough *et al.*, 2004) (*R*-factors 12.5 and 22.3% for SOD1 and hCCS, respectively). At low angle ($\leq$0.1 $Å^{-1}$), the in-house data are in good agreement with the synchrotron data (*R*-factors 8.1 and 14.3% for SOD1 and hCCS). This low q region is used for the Guinier approximation and can be seen in Figs. 2(*c*) and 2(*d*) for the two systems. The $P(r)$ function gives the maximum particle dimension and a molecular weight estimation can be made from the Porod volume. Table 1 compares these values for each species and indicates concordance between the values derived from the home-source and synchrotron data.

The low protein concentrations used in these home-source static experiments mirror those found as protein elutes from a gel filtration column when conducting measurements on a synchrotron integrated SEC–SAXS instrument. It is clear that relatively short, in this case 10 min, exposure times are sufficient to deduce reliable common size parameters such as R_g, D_{max} and to estimate the molecular mass. This time frame is practicable given that SOD1 and the hCCS–SOD1 complex elute from a standard-preparation-grade SEC column over a 15 min period at 0.8 ml min^{-1} flow rates. Furthermore, the high frame rate and low read-out time of the PILATUS detector used here are ideal for this application. These characteristics would allow short, in this case 10 s, exposures to finely slice data collection over the course of protein elution while maximizing acquisition time.

The proof-of-principle experiments described above establish the efficacy of using the currently most powerful home X-ray source in conjunction with noise-free single-photon-counting detectors to

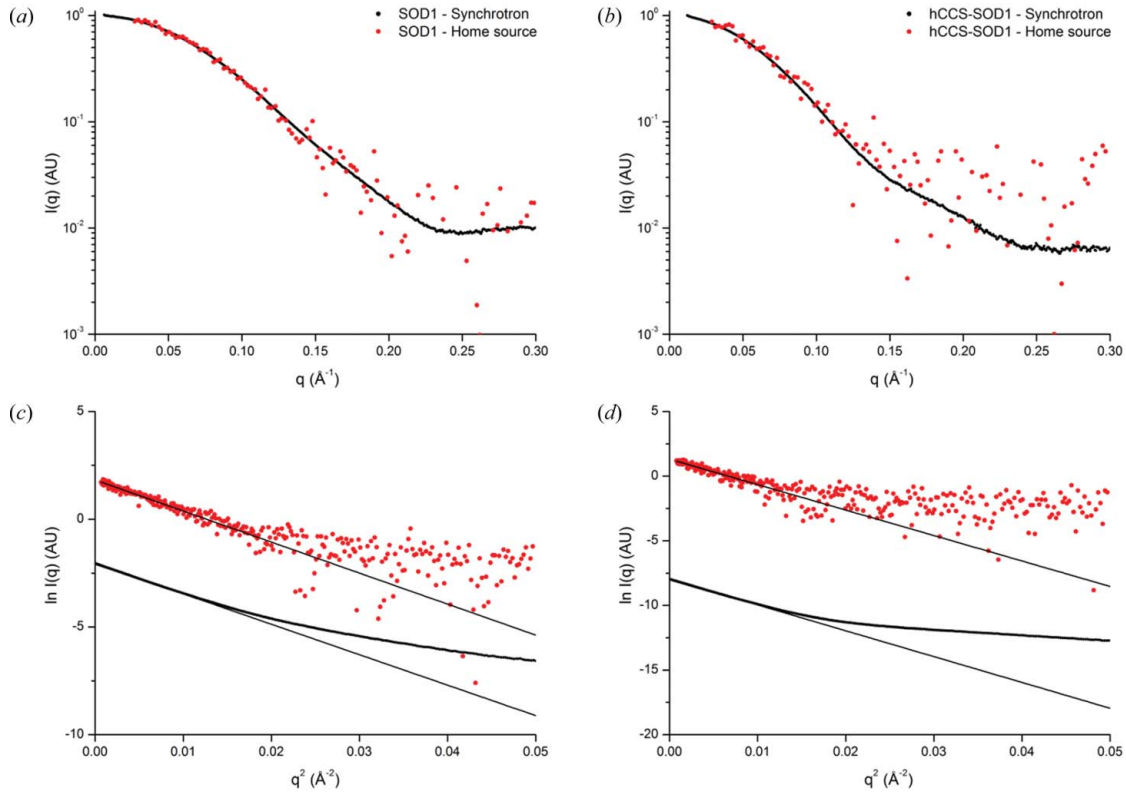

Figure 2
Protein SAXS profiles and Guinier plots collected on a home source with a PILATUS 300K-20Hz detector and on a synchrotron SAXS beamline. (*a*) SOD1 scattering profile. (*b*) hCCS–SOD1 scattering profile. In both cases four adjacent data points were averaged for the home-source data. (*c*) Guinier plot of SOD1. (*d*) Guinier plot of hCCS–SOD1. Grey circles (red online): home source; black circles: synchrotron. Plots were normalized to 1 at $I(0)$.

collect SAXS data from biological macromolecules and their complexes. This set-up would allow users to carefully characterize a protein system without time constraints in their home institutions and foster more effective use of synchrotron beam time.

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