SPINE-compatible 'carboloops': a new microshaped vitreous carbon sample mount for X-ray and neutron crystallography

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A novel vitreous carbon mount for macromolecular crystallography, suitable for neutron and X-ray crystallographic studies, has been developed. The technology described here is compatible both with X-ray and neutron cryo-crystallography. The mounts have low density and low background scattering for both neutrons and X-rays. They are prepared by laser cutting, allowing high standards of production quality, the ability to custom-design the mount to specific crystal sizes and large-scale production.

1. Introduction

Complementary neutron and X-ray studies require data collection from the same crystalline samples and easy exchange between neutron and X-ray sources. Thus, the choice of the sample-mount material is of primary importance in terms of handling, stability and background scattering. A variety of methods have been used to mount crystals for flash-cooling and cryo-crystallographic data collection (Garman & Schneider, 1997; Kriminski *et al.*, 2003). Commercial loop mountings are used as a standard for high-throughput automated cryo-crystallography at synchrotron X-ray beamlines around the world. Unfortunately, none of the materials that are typically used to fabricate these loops are suitable for data collection performed at neutron sources, given the fact that they are usually hydrogen-containing (Thorne *et al.*, 2003) and give rise to incoherent scattering that reduces the precision with which the coherent crystallographic data can be extracted (Blakeley, 2009). The use of such sample mounts is especially problematic given the effort that is now typically taken in preparing perdeuterated protein samples. Another important consideration for neutron experiments is that the use of inappropriate mounts may cause significant radioactive activation. All commercially available crystal mounts are made with stainless-steel supports that contain a minimum of 10.5% chromium by mass. The neutron-capture product life time of ⁵⁰Cr is 27.8 d, necessitating secure storage for a substantial time after neutron experiments.

At present cryo-crystallographic data collection at the Institute Laue–Langevin (ILL) is carried out by mounting protein crystals on the surface of a quartz capillary. Quartz has a good transparency to both neutrons and X-rays, does not give Bragg peaks in the diffraction image and has low diffuse scattering (Nikitin *et al.*, 2007). However, other characteristics make its usage difficult. Quartz capillaries are fragile and difficult to handle: they are difficult to mount on standard SPINE magnetic bases and the process of retrieving large crystals is challenging and prone to crystal loss. A further major problem with the use of pure quartz as a support is the fact that there are problems for storage in liquid nitrogen; quartz and the steel magnetic base have different thermal conductivities and this may result in fracture of the mounting device upon cryocooling.

Here, we describe a laser-fabricated, vitreous carbon alternative to conventional X-ray and neutron crystal-mounting systems. The sample mounts retain all of the major advantages of conventional X-ray mounting systems, including compatibility with standard magnetic goniometer heads, and resolve the problems of activation and hydrogen incoherent scattering in neutron experiments.

Table 1
Data-collection and processing statistics of lysozyme crystals.

A comparison of complete lysozyme diffraction data and statistics between a commercial nylon loop from Hampton Research and the carboloop mounting system is reported. Values in parentheses are for the highest resolution shell. Note that for the two data sets the number of unique observations is essentially the same. The total number of observations differs by around 20% as a result of different crystal orientation.

Loop type	Nylon cryo-loop	Carboloop
Space group	$P4_3 2_1 2$	$P4_3 2_1 2$
Unit-cell parameters (Å)	$a = b = 78.66, c = 37.21$	$a = b = 77.16, c = 37.44$
Resolution range (Å)	55.62–1.60 (1.69–1.60)	54.56–1.60 (1.69–1.60)
Total No. of observations	112940 (16554)	90477 (10126)
No. of unique observations	15936 (2288)	15415 (2191)
Multiplicity	7.1 (7.2)	5.9 (4.6)
Completeness (%)	99.8 (100)	99.7 (99.4)
Mosaicity (°)	0.22	0.51
R_{merge}	0.044 (0.102)	0.056 (0.103)
$R_{\text{p.i.m.}}$	0.018 (0.041)	0.023 (0.053)
Mean $I/\sigma(I)$	28.1 (14.7)	20.2 (9.2)

2. The use of vitreous carbon as a crystallographic mount

Vitreous carbon sheets (SIGRADUR K 60 µm) manufactured by HTW Hochtemperatur-Werkstoffe GmbH (Thierhaupten, Germany) from pure carbon were used to obtain the micro-shaped loops. This material is highly disordered and combines glassy and ceramic properties. Vitreous carbon has a fullerene-related microstructure that consists of discrete fragments of curved carbon planes, in which pentagons and heptagons are dispersed randomly throughout networks of hexagons (Harris, 2004). For the purposes of this paper it is important to note that this material consists exclusively of carbon, which is ideal because of its relatively high transparency to neutrons and X-rays, as well as its low activation in the neutron beam.

In contrast to quartz capillaries, vitreous carbon sheets are robust and can be micro-shaped by laser cutting. The design of the vitreous carbon loops takes into account several aspects: (i) the shape of the crystals that we need to retrieve, (ii) the rigidity of the mount to hold the crystal in place without vibration in the flux of the cryostream, (iii) the need to avoid the presence of a large quantity of liquid near the crystal, ensuring the possibility of removing the excess of this liquid easily and (iv) a comfortable way to handle the loops during the fabrication process.

Figure 1
(a) Design of the carboloops. Each consists of (1) a microshaped vitreous mount, (2) a high-purity thin-walled aluminium tube and (3) a magnetic SPINE goniometer-compatible base. (b) X-ray and neutron cryo-crystallography carboloop mounts. The standard magnetic SPINE base provides compatibility with most X-ray goniometer heads and easy transfer and storage in crystal pucks.

3. Materials and methods

The vitreous carboloop is obtained through the use of a laser-cutting machine that uses a class IV pulsed Nd:YVO₄ laser at a wavelength of 1064 nm, which was selected for its short pulse length below 10 ns. The vitreous carbon sheet is mounted on a motorized *X/Y* and focus stage assembled from standard optical components. The *X/Y* stage has a travel range of 10 × 20 mm and a precision of 20 µm, which is roughly equal to the width of the cut. A *LabView* program converts the computer-drawn images to a series of traces and controls the laser shutter and *X/Y* stage positioning accordingly. The linear cutting speed is 3.5 mm min^{−1}. A sample loop such as those shown in Fig. 1 typically takes about 5 min to cut.

The design of the carboloop mount is shown in Fig. 1(a). A standard SPINE magnetic base (3) is drilled to match the external diameter of a pure aluminium tube at 1.5 mm. The thin (127 µm) aluminium tube (2) (Goodfellow SARL, Lille, France) is cut at a

Figure 2
A lysozyme crystal mounted on a test vitreous glassy carbon pin 60 µm thick (a) and the corresponding X-ray diffraction pattern (b). The crystal was successfully frozen in the Cryostream and a full data set was collected on ID29 at the ESRF at a wavelength of 0.96 Å. There is no evidence of ice rings in the recorded data and diffuse scattering is negligible.

length of 1.5 cm and the profile from one side is V-shaped to ensure a better positioning of the mount. Pure aluminium was chosen because of its short neutron-capture half life (2.3 min), ensuring safe handling of the mount within a short time after the end of an experiment. The aluminium tube is attached to the goniometer-compatible base and the carboloop (1) glued within the V-shaped slot of the aluminium tube using 5 min epoxy glue (Loctite). Fig. 1(b) shows two mounts prepared as explained above. For sample mounting a crystal is retrieved from a drop of solution using the carboloop and the excess liquid is removed. Finally, the crystal is flash-cooled by plunging it directly into liquid nitrogen or placing it in the cryostream. The entire length of the mount varies between 22 and 24 mm, and is compatible with the automatic sample changers available on synchrotron beamlines, so that the crystal can be easily stored and reused as necessary.

4. Results and discussion

In evaluating these carboloop mounts, both synchrotron X-ray and neutron measurements were carried out in a short period of test time. The timescales of these measurements were obviously completely different; whereas a full data set was recorded in the X-ray test, the neutron analysis was based on static single-frame measurements. Hence, whereas a fully tabulated data set is produced for the X-ray data analysis and reported in Table 1, no table is provided for the neutron data.

Fig. 2 shows a tetragonal lysozyme crystal mounted on a square vitreous mount after cryocooling in the cryostream. X-ray data from this sample were collected on ID29 at the ESRF using a wavelength of 0.96 Å. The resolution limit in the outer shell is 1.6 Å. The recorded spots are well defined, the diffuse scattering is minimized

and the presence of ice rings is limited. After data collection the sample was unloaded using the SC3 sample-changer device (Cipriani *et al.*, 2006) and stored in liquid nitrogen for some minutes. Four repeated loading/unloading cycles using the SC3 system did not degrade the mount.

Vitreous carbon mounts were also used to harvest crystals and perform neutron data collection. Data from a 5 mm³ trypsin crystal were collected at 100 K on the single-crystal diffractometer D19 at the ILL using a wavelength of 2.42 Å (Fig. 3). The crystal was stable and did not vibrate in the cryostream at a flux of 5 l min⁻¹ during data collection, suggesting that the mount has sufficient stiffness to hold large crystals needed for neutron crystallography. Ice formation was not observed during the 15 min of data collection. Different neutron diffraction patterns were collected for 1000 s yielding data to 3 Å resolution. Neither ice rings nor significant background scattering were observed (Fig. 3).

4.1. X-ray diffraction experiment

Chicken egg-white lysozyme (Sigma–Aldrich catalogue No. L6876) was dissolved at 50 mg ml⁻¹ in 0.1 M sodium acetate buffer pH 4.6. Crystals were grown at room temperature in 8 µl hanging drops over a reservoir of 0.1 M sodium acetate pH 4.6 with 0.8 M sodium chloride and were found to grow within a week. Crystals were briefly soaked in a cryoprotectant solution composed of the reservoir liquid with the addition of 20% glycerol and then cooled in the cryostream. Crystallographic X-ray diffraction data were collected on beamline ID29 at the ESRF ($\lambda = 0.968$ Å) using a beam size of 30 µm and 4% transmission. The diffraction data were collected with a DECTRIS 6M_F pixel detector at a distance of 233 mm from the sample. Data collection was performed at 100 K using the shutterless collection mode over 100° of rotation in steps of 0.1° oscillation, each exposed for 0.037 s. Data were thereafter indexed and integrated using *iMosflm* (Battye *et al.*, 2011), followed by scaling, merging and conversion of the intensities to structure factors using the CCP4 program *AIMLESS* (Evans, 2011). The analysis of X-ray data collections from a representative set of crystals harvested from the same crystallization drop allows a comparison of carboloops with nylon loops. Parameters including unit-cell parameters, resolution, mosaicity, completeness, multiplicity, R_{merge} and $\langle I/\sigma(I) \rangle$ which were evaluated during data processing are reported in Table 1.

4.2. Neutron diffraction experiment

Trypsin (Sigma–Aldrich catalogue No. T9201) was dissolved at 40 mg ml⁻¹ in 0.1 M Tris–HCl buffer pH 8.5 with 3 mM CaCl₂ and 10 mg ml⁻¹ benzamidine. Crystals were grown at room temperature in a 40 µl sitting drop over a reservoir consisting of 1.7–2.1 M ammonium sulfate, 0.1 M Tris–HCl pH 8.5. To perform isotopic replacement of H₂O by D₂O, crystals were soaked in deuterated buffer consisting of 2.4 M ammonium sulfate, 0.1 M Tris–DCl buffer pD 8.1 for between 1 and 7 d. Crystals were cryoprotected by step-wise soaking with the addition of an increasing concentration of dextrose. The concentration of dextrose was increased in steps of 10% from 30% (w/v) to a final concentration of 70% (w/v). The total soaking time for cryoprotection was about 40 min. Crystals were then retrieved with the carboloop mounting system and cooled by plunging them into liquid N₂. Crystallographic neutron diffraction images were collected on the D19 single-crystal diffractometer ($\lambda = 2.42$ Å) at ILL with a 120 × 30° positive-sensitive gas detector with 640 × 256 pixels. Data collection was performed at 100 K mounting the carboloop on the magnetic head of a four-circle Eulerian cradle goniometer. Diffraction images were collected for 1000 s counts and an

Figure 3
A trypsin crystal (5 mm³) mounted on a carboloop (a) and the corresponding neutron diffraction image (b). Data collection was performed at 100 K on the D19 single-crystal diffractometer at ILL using a wavelength of 2.42 Å.

efficiency correction applied using the diffraction image collected on a vanadium cylinder acquired for 1000 s.

5. Conclusion

In this study, we have presented a novel carboloop mounting system that makes it feasible to retrieve crystals and collect diffraction data using the same mount on neutron and X-ray sources. These novel mounts help to solve the problem of sample exchange between neutron and X-ray facilities, allow easy transfer and storage, and are compatible with all of the automation processes present today on modern beamlines. Crystal mounting and removal are easy and fast, with little risk of crystal damage and with negligible neutron background scattering from the mount and the tubing. The advantages of the new mounting system include reproducibility and shape-adaptable manufacturing, accurate sample positioning, easy removal of excess crystallization mother liquor, low thermal expansion and extreme resistance to thermal shock, easy crystal retrieval and low wettability, high hardness and strength, high purity, low density and low background scattering both with neutron and X-ray sources. Graphene derivatives have been used previously in macromolecular crystallography (Wierman *et al.*, 2013); the vitreous carboloops described here are suitable for mass production because the design of

the crystal mount is made by computer and the fabrication utilizes a laser-cutting machine.

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