Towards long-wavelength protein crystallography: keeping a protein crystal frozen in vacuum

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Abstract. There is growing interest to explore the long-wavelength X-ray domain for macromolecular crystallography (MX) experiments but there are a number of practical issues that make these experiments difficult to perform. In this article we study several aspects related to cooling a protein crystal in a vacuum environment. We investigated thermal contact conductance (TCC) of copper-copper joints and designed a demountable sample holder assembly with a magnetic joint that facilitates good thermal conductivity and reliability over a long time period. The thermal conductivity of amorphous ice formed by a 20% solution of ethylene glycol was studied. It is concluded that the ice thickness is the factor that can compromise the cooling of protein crystals and therefore it should be carefully controlled.

1. Introduction
The long-wavelength macromolecular crystallography (MX) beamline I23 at the Diamond Light Source will be the first beamline dedicated for phasing experiments from native proteins or DNA/RNA crystals using anomalous diffraction signals from sulphur or phosphorous. It will be optimised in the energy range from 3 - 8 keV and provide a beam size of 130 x 130 µm at the sample position. To eliminate adverse absorption/scattering of low-energy X-rays from air, the experimental end station will operate in vacuum. A number of technical challenges must be solved for MX experiments in vacuum to become a viable addition to structural biology. One such challenge is the need to provide efficient cooling of protein samples to 100 K and below. In a vacuum environment cooling is controlled by thermal conductivity. As the protein sample should be mounted on the goniometer in a holder, there will be several dissimilar materials and interfaces (i.e. metal - ice - protein) involved in heat transfer. Recognizing this, we initiated a systematic study aiming to gain insight into the process of thermal conductivity through a system comprising several dissimilar materials and interfaces.

In this work we present the results of our investigations of different aspects of this complex problem. The structure of the paper is as follows. In section 2, we present the results of measurements of the thermal contact conductance of demountable, in vacuum, uncoated and Au-plated copper-copper joints. Using these data we developed a magnetic sample holder / goniometer head interface and carried out extensive tests of long term stability and reliability of this connection (section 3). As a vitreous ice layer is an unavoidable interface between the protein sample and the sample holder, the

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results on measurements of the thermal conductivity of a representative specimen of vitreous ice (20\% solution of ethylene glycol) are discussed in section 4.

2. Thermal contact conductivity of demountable in-vacuum joint

MX experiments at synchrotrons have been optimised over the last years to allow high sample throughputs. Therefore, swift sample changing is an obligatory requirement. This has serious implications for the low-temperature experiments in vacuum. Cooling the sample relies upon good thermal conductance, whereas the interface of two parts joined together creates a break in continuity of the cooling, resulting in a temperature jump across the joint. This is due to the thermal contact conductance (TCC), which in many cases is a primary factor limiting cooling efficiency [1-9].

Knowledge of this parameter is of great significance for the development of a new sample holder that can provide efficient cooling of samples in vacuum. To allow the relatively high throughput of several samples per hour, one of the main challenges is the design of a compact sample holder which can be easily mounted on and demounted from a goniometer and also facilitate high thermal conductance in the temperature range of 30-80 K. Given the lack of quantitative information we instigated systematic studies of TCC for uncoated and Au-plated Cu-Cu joints over the temperature range of interest.

2.1. Experiment

In this study we used the experimental technique of steady-state longitudinal heat flow, a method similar to that applied for measurements of thermal conductivity of solids. In this method, the thermal interface consists of two material pieces sandwiched between a heat source and a heat sink. The assembly is heated at one end and cooled on the other with constant heat flow. When the steady state is reached the temperature distribution is measured across the interface as well as along the heat source or heat sink. The detailed description of this experimental setup and test procedure can be found in [10].

The test pieces are copper blocks made of OFHC-100 copper with diameter of contact surfaces 14.5 mm. The contact surfaces of complementary pairs were prepared with average roughness $R_a = 3.2, 1.6$ and $0.2 \, \mu$m. Initially, we carried out measurements for uncoated test pieces. In this case, to eliminate the effect of copper oxidation, the contact surfaces of the test pieces were cleaned by immersing in 10\% solution of acetic acid for 5 minutes and subsequently washed in water and ethanol. For the second experiment all surfaces of test pieces were electrochemically plated with a ca.1 $\mu$m thick layer of gold.

Each sample pair was tested from 14 to 100 K for a range of loads between 10 and 200 N. For each set of temperatures and heat loads the contact conductance $h$ was calculated as the ratio of the temperature difference between two test pieces $\Delta T$ and the heat flux $q$ across the interface:

$$h = \frac{q}{\Delta T}. \quad (1)$$

The uncertainties of all measurements were analysed and the total error of the measurements of TCC using this method is estimated to be 12\% [10].

2.2. Cu-Cu joint

Figure 1 shows the TCC of uncoated Cu-Cu joints as function of temperature at two applied forces, 25 and 50 N, for contact surfaces with different roughness $R_a$. The common feature of all TCC plots displayed in double logarithmic presentation is a linear rise with temperature from 14 to 40 K that shows the power law temperature dependence of TCC as $h \propto T^\gamma$. The experimental data in this
temperature range are fitted using a power law function $T^\gamma$ with $\gamma=1.25\pm0.02$. For the applied load of 50 N the TCC exhibits a maximum value of $65.9\pm7.8$ mW/K at $T=40$ K for the surface roughness $R_s=0.2$ µm, whereas at higher temperature it decreases. The results of this study also unambiguously evidence an increase of TCC with improvement of surface smoothness.

In order to explain the observed dependencies we used an empirical expression that relates TCC with the other physical quantities as follows [11]:

$$\frac{h}{A} = a \frac{\lambda}{s} \left( \frac{F}{AH} \right)^n$$

(2)

Here $\lambda$ is the thermal conductivity of the material, $s$ is the surface roughness, $F$ is the applied force, $A$ is the contact area, $H$ is the material hardness, $a$ and $n$ are dimensionless correlation coefficients. The formula clearly shows that bigger surface roughness $s$ leads to lower values of TCC. This equation also gives the power law relationship between TCC and applied load $h \propto F^n$ if all other parameters are considered to be invariable.
Inspection of equation (2) shows that the obvious temperature dependant parameter is the bulk thermal conductivity $\lambda$. The temperature dependence of the thermal conductivity of copper rises according to a power law, reaches a maximum value at 30 K and finally decreases to nearly constant value in 100-300 K range [1]. The temperature dependence of TCC exhibits very similar trend in the low temperature domain demonstrating a correlation between $\lambda$ and $h$. Another parameter that contributes to such change is the metal hardness $H$. It is known that hardness of OFHC copper is constant below 85 K and increases fairly linearly with temperature from 85 K to 300 K [12]. The reciprocal dependence of $h$ on $H$ can explain the very mild change of TCC above 40 K. The effect due to the decrease of bulk conductivity is mitigated by the decrease of copper hardness [10].

2.3. Au-plated Cu-Cu joint

Noble metal coating is widely used to protect the surface of copper from oxidation and prevent changes of the thermal conductance with time. However, data on the TCC of gold plated copper-copper contacts are rather fragmentary and not always consistent. Therefore the studies of the effect of gold coating on TCC of Cu-Cu joints are of particular interest. The plots in figure 2 show the temperature variation of TCC of the same Cu-Cu joint before and after Au-plating. Juxtaposition of the data shows that the TCC as a function of temperature roughly follows the same trend for uncoated and Au-plated samples. In general TCC of Cu-Cu joints increased by 1.5-2 times after coating with Au. To the best of our knowledge there are no reference data to be compared with, but published results of studies at T<20 K demonstrates similar level of improvement of TCC for Au-plated Cu-Cu joint [13, 14]. For the applied load of 50 N the maximum value of TCC of a Au-plated Cu-Cu joint (93.0±11.6 mW/K) is observed at 50 K.

Interestingly, the $h = f(T)$ dependence of Au-plated Cu-Cu joint exhibits two maxima around 30 and 50 K with a local minimum between. It is demonstrated for the Cu-Cu joint that the bulk thermal conductivity controls the temperature dependence of TCC. It should be noted that the peak in the temperature dependence of thermal conductivity of gold is shifted to lower temperatures with respect to that of copper [1]. Therefore it is tentatively suggested that the observed feature is due to superposition of thermal conductivities of copper and gold that is subsequently manifested in the shape of the $h = f(T)$ dependence.

3. Long-term reproducibility test of demountable in vacuum joint

Using these results we can now estimate the expected temperature rise of the sample holder. During the experiment the total heat load upon the sample holder assembly due to external blackbody radiation plus X-rays is assessed to be below 40 mW. Feeding the value of TCC of Au-plated Cu-Cu joint at $F=25$ N and $T=30$ K (35 mW/K) into formula (1) we obtain an expected temperature rise of 1.2 K. This is a reasonable number as a temperature step of about 3 K at this interface is considered to be acceptable. This allowed us reduce requirements for load force by a factor of two. Such a pulling force can be achieved using rare-earth magnets.

This basic idea was implemented in the design of the demountable joint. The joint is made of two mating Au-plated copper pieces (plug and socket) with embedded magnets (see insert in fig.3). The plug is the base of the sample holder, while the socket is the matching part of the cryogenic goniometer. The two parts are joined together along the surface created by conical seat that facilitates good contacts and reproducible positioning at pooling force of two magnets 12 N.

To verify the long term stability the demountable vacuum joint was subjected to extensive tests using the modified, previously described, experimental setup. We added a motorised gripper for in-vacuum manipulation of the sample holder. First we examined the variation of temperature of the plug whilst varying the temperature of the socket. As can be seen in fig. 3, the difference between the
temperatures 3.9 K at 30 K, that is consistent with expectations. Then the temperature of the socket
was set at 19 K and contact was repeatedly broken and restored (the plug pulled out and in) while
monitoring temperature changes of the plug. Two runs where completed in which the assembly
performed 4000 and 6080 cycles of connections. At the beginning of the test the plug temperature
quickly settled at 24-25 K but then after 2000-2500 cycles erratic jumps of temperature up to 30 K
started to appear. At this stage a thin layer of ice on the plug and socket were observed. Consequently,
the cryocooler was switched off allowing the setup to warm up. When the test resumed, the
temperature of the sample holder remained stable at 23.5 K until the completion of the run without
further ice formation. Thus, the test proved that this type of demountable joint is very reliable and
reproducible over an extended period of operation.

![Diagram](image1)

**Figure 3.** Variation of absolute temperature of sample holder (1) and temperature rise at
sample holder relative to receptacle (2) as function of receptacle temperature. Inset show the scheme of plug (a) and receptacle (b) with magnets (m).

![Diagram](image2)

**Figure 4.** Temperature dependence of thermal conductivity of vitreous ice, i.e. frozen 20% solution of ethylene glycol.

4. **Thermal conductivity of amorphous ethylene glycol ice**

Solvent around protein crystal has a very detrimental effect upon the signal-to-noise and quality of
data collection statistics. Its elimination is of utmost importance for long-wavelength MX experiments.
However, it is also recognised that frozen solution may be trapped in the gap between the protein
crystal and the tip of a sample holder made from diamond or similar material with low X-ray
absorption cross section and high thermal conductivity. Thus, in vacuum frozen solvent is
inevitably a part of heat transfer link compromising the cooling. To assess the magnitude of this effect
we investigated the thermal conductivity of amorphous ice formed by freezing a typical cryoprotectant; 20% solution of ethylene glycol. The thermal conductivity was measured using steady-state longitudinal heat flow as described above. The sample solution contained in a kapton tube (diameter 28 mm, length 100 mm) was flash frozen in liquid nitrogen, placed in test rig, evacuated and cooled to 30 K. The heater at the bottom of the tube created heat influx while four temperature sensors equally spaced along the tube length monitored temperature distribution to allow calculation of the parameter of interest. The results of the measurements, displayed in Figure 4, show that in the region of interest (30-100 K) thermal conductivity of the vitreous ice is in the range 0.65 to 0.72 W/m·K, close to the value of 0.6 W/m·K reported for high density amorphous ice [15]. Taking into account (conservatively) that total external heat load upon the protein crystal of typical size 0.1 mm might be up to 1 mW we estimate that a 25 µm layer of vitreous ice will contribute 4.2 K to temperature gradient in the whole sample holder assembly.

5. Conclusion
The long-wavelength MX experiment requires development of new methods for handling the protein samples in a vacuum environment. Keeping protein crystals frozen is one of the main challenges to be addressed. In this work we investigated several issues related to the conductive cooling. We carried out systematic investigations of TCC of uncoated and Au-plated Cu-Cu joints and developed a model that consistently explains our observed temperature dependencies taking into account the effects of changes in bulk conductivity and surface hardness of the materials. These results where then used to design a demountable sample holder assembly based on a magnetic joint that ensures good thermal conductivity. The temperature jump at the contact interface is found to be about 3.9 K at 30 K for a contact force of 12 N. Extensive tests have shown that that this type of demountable joint provides an adequate and reliable solution for the applications that require swift and frequent exchange of sample holders in vacuum while preserving high thermal contact conductivity with reproducible characteristics over extended periods of operation. We measured thermal conductivity of amorphous ice formed by a 20% solution of ethylene glycol and estimated that thin (a few 10s µm) layers of such an interposer can lead to a noticeable increase of temperature that will impede the cooling of the protein crystal. Therefore, finding ways to minimise the amount of liquid trapped between the crystal and the tip of the sample holder is a critical point that must be addressed next.

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