Cryopreservation of protein crystals is essential for minimizing radiation damage from brilliant synchrotron beams. However, the treatment is often troublesome, because the crystals are fragile and contain moisture, which expands upon cooling, destroying their crystalline order. To prevent the freezing damage by vitrifying the moisture in the sample, the crystal mother liquor is replaced with an aqueous/organic mixture solution, the so-called "cryoprotectant." This method is quite simple and effective, but it sometimes affects protein structure and/or crystalline quality. Therefore, there is a need for testing it with various kinds and concentrations of cryoprotectant agents. On the other hand, non-cryogenic experiments are also important. For instance, the results of experiments performed at room temperature should be compared with cryogenic ones to estimate crystalline quality. Also, structures at ambient temperature do not have side-effects of cryocooling, such as molecular shrinkage.

However, there has been no versatile method for cryogenic and ambient experiments. The free-mount method, where protein crystals are exposed to controlled humid air, serve possibly the purpose and showed the ability to improve resolution of protein crystals [1]. However, most of protein crystals were heavily damaged, when their mother liquors were removed and the crystals were exposed to air.

To overcome this issue, we have developed a new crystal mounting method: humid air and glue-coating (HAG) mounting method at beamline BL38B1. This method involves a combination of controlled humid air and water-soluble polymer glue for crystal coating [2]. The experimental procedure for HAG method can be briefly described as follows (Fig. 1(a)):

(1) Glue preparation: 8 to 13% (w/w) polyvinyl alcohol (PVA) served as the aqueous glue solutions. The glue solutions vitrified successfully after exposure to wet gas of 92.0% RH or lower. Although the glue was adequate for many types of crystal mother liquors, it formed a physical ion-containing hydrogel in some conditions, especially for high concentrations of multivalent anions such as tartrate, phosphate and sulphate ions. The gel often had trouble thoroughly coating and cryoprotecting the crystals. However, we found that glycerol worked well to prevent gel formation, thus the crystals should be handled with glue or crystal mother liquor containing glycerol.

(2) Crystal handling: a small amount of the glue solution was applied to and spread over a crystal mounting loop with a typical diameter larger than the crystal (Fig. 1(a1)). A crystal was directly picked up from a crystallization droplet by the glue-coated loop, without removal of the mother liquor surrounding the crystal (Fig. 1(a2)). The scooped crystal was kept steady for a few seconds so that it could be thoroughly coated and covered by the glue on the loop (Fig. 1(a3)). Next, the loop was mounted on the diffractometer and exposed to the humid air (Figs. 1(a4) and 1(a5)).

(3) Optimized humidity: to search for a suitable humidity condition, the crystal quality was not only judged by visual inspection but also evaluated by taking diffraction images. We started experiments with an empirically determined humidity of 83.0% RH and changed it in steps of ±2–3% RH. Next, a coarse optimization with the steps of 1–2% RH was performed followed by a finer humidity optimization with the steps of 0.1–0.5% RH. Sample after optimized humidity condition are shown in Fig. 1(a6).

This method can be applied well to many types of protein crystals:

(i) Fragile crystals. The crystals of the bacterial hydrolase RsbQ are mechanically very fragile and sensitive to environmental changes. Thus, the conventional cryoloop mount with traditional cryoprotectant agents does not work (Fig. 1(b1)). Using the HAG method with 13% (w/w) PVA3500, we were able to obtain high resolution (1.4 Å) data after cryocooling without cryoprotectant agents (Fig. 1(b2)).

(ii) Preparation of isomorphous crystals. The changes of the lattice constants in tetragonal lysozyme crystals were reproducible, reversible and gradual for varying humidity, but these amounts were smaller than those in previous experiments without coating. The results suggest that this method can gently control the lattice constants of protein crystals. This advantage will also be useful for multi-crystal data collection.
(iii) Membrane proteins. The crystallization condition of the bacterial membrane protein *Blastochloris viridis* photoreaction center (BvRC) contained high concentrations of sulphate ions (1.9 M). The glue prepared by mixing equal volumes of glycerol and 8% (w/w) PVA4500 was successfully subjected to cryocooling without ice formation. In the conventional cryoloop method, a BvRC crystal soaked in cryoprotectant containing 30% (v/v) glycerol was damaged after only two minutes [3]. However, the glue of PVA and glycerol mixture preserved the crystals for over 20 minutes during a diffraction check at room temperature when mounted with the HAG method.

As stated above, the HAG method can handle crystals stable at room temperature and cryocooled without additional cryoprotectant agents. For users, the humidity control apparatus of HUM-1 (Rigaku Co.) and an automatic switching device of the gas nozzles have been installed at beamline BL38B1.

![Fig. 1. Experimental procedure of HAG method and its practical example.](image)

**Fig. 1.** Experimental procedure of HAG method and its practical example. (a) Overview of the HAG method. (1) Crystal handling procedures. (1) Applying the PVA glue to a cryoloop. (2, 3) Picking up a crystal, and steeping the crystal to be coated by the glue. (4, 5, 6) The crystal mounted on the diffractometer and exposed to humid air. (5) Just before the lysozyme crystal was placed in the apparatus. (6) After the lysozyme crystal was exposed to 73.9% RH air. (b) Diffraction images of bacterial hydrolase RsbQ crystals. (1) The crystal was flash-cooled by a cryoloop mount with a cryosolution composed of the reservoir solution supplemented with 25% (v/v) glycerol. (2) The crystal was mounted by the HAG method, using 13% (w/w) PVA3500 glue at 69.1% RH, then flash-cooled.

Seiki Baba* and Takashi Kumasaka
SPring-8/JASRI

*E-mail: baba@spring8.or.jp

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