

Visitor report.
**Cross-linking - making protein crystals
radiation, temperature and physically robust**

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Report.

Summary

One of the most vital factors for the success of a diffraction experiment is the accurate calibration of the X-ray beam-line in terms of stability, proper alignment, etc. To perform such configuration the ideal sample is well-known and highly stable protein crystal, which can be used multiple times and shipped safely all over the world.

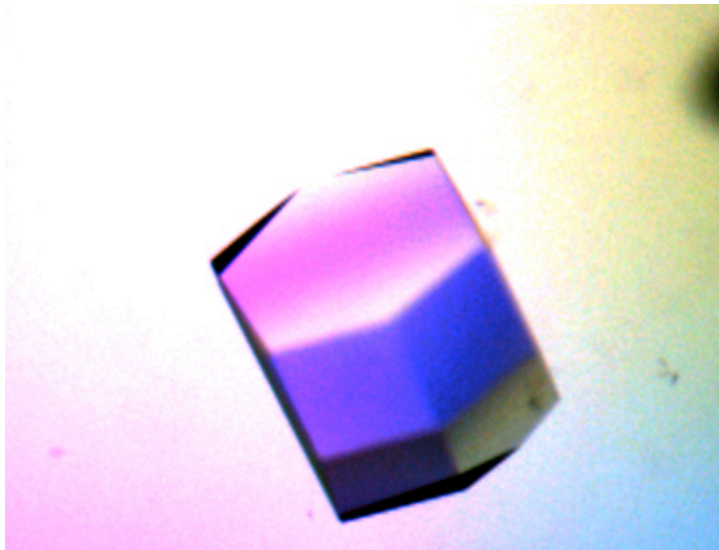
Additionally, radiation damage to biological samples is currently one of the major factors that severely and irreversibly affect the quality of data obtainable. However, this effect can effectively be reduced by cross-linking protein molecules in the crystal, thus making it physically, chemically, radiation and temperature robust.

To cross-link protein crystals we are using *glutaraldehyde*, simple and well-known chemical reagent, frequently used in biochemistry applications as an homobifunctional cross-linker, that reacts predominantly with free amino groups especially that of lysine through the formation of Schiff bases.

During the period of Desy Summer Student program we have developed a new protocol, basing on the “old” protocol by Annette Faust, that seems to have the same effect in relatively short time.

Results

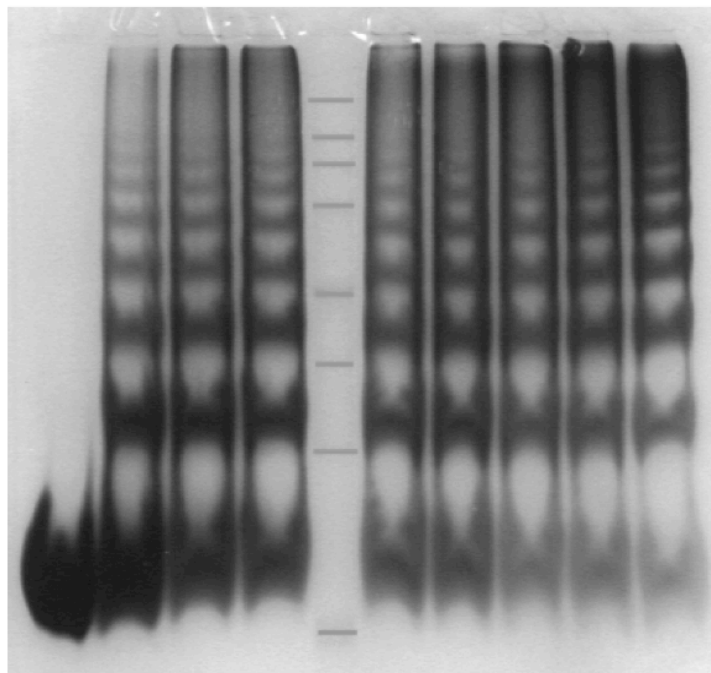
- Tetragonal lysozyme crystals (HEWL, space group $P4_32_12$, average size $400 \times 400 \times 300 \mu\text{m}$, unit-cell parameters $a=b=79.06 \text{ \AA}$, $c=37.54 \text{ \AA}$, $\alpha=\beta=\gamma=90^\circ$, diffracted to beyond 1.3 \AA resolution) were obtained.



Techniques used: Hanging drop crystallization, screening.

Crystallization conditions: equal amounts of protein (35mg/ml in water) and reservoir solution containing 100mM sodium acetate pH 4.5 and 0.9 M NaCl.

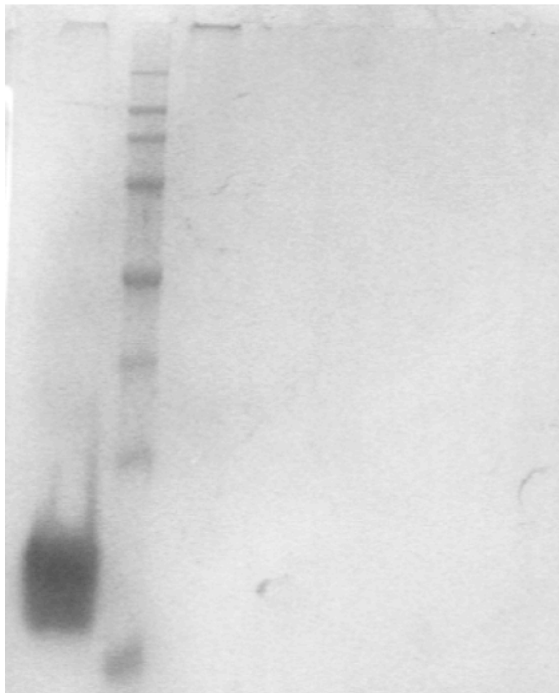
- Glutaraldehyde activity time was assessed in a free solution experiment. Samples of HEWL solution (10 mg/ml) with presence of G.A. (2.25 % v/v) were taken at certain time points (from 0 to 90 minutes). SDS-PAGE analysis of these samples clearly shows that GA starts to crosslink immediately, and is active for the next 60-90 minutes.



C 0 5 10 M 15 20 30 60 90

Techniques used: SDS-PAGE analysis, Gel preparation.

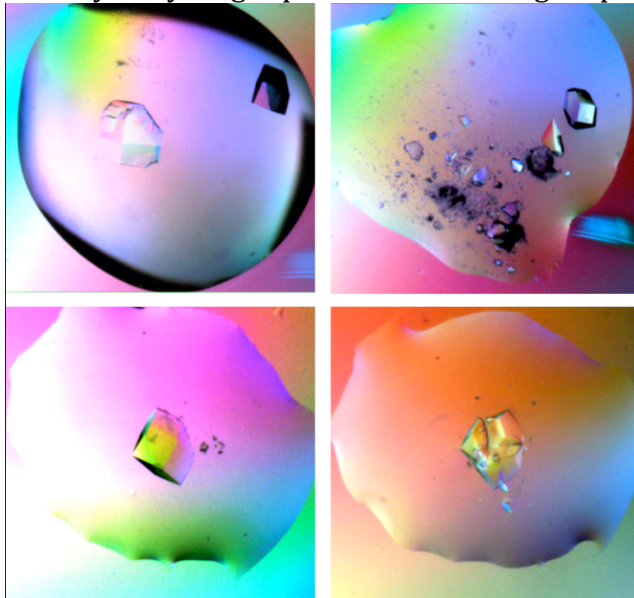
- Glutaraldehyde in-crystal activity depending on its percentage in soaking solution is shown on figure below. As it can be seen, even after 2 partial soaks lysozyme crystals were no longer dissolvable in detergent SDS solution regardless the GA percentage i.e. highly cross-linked.



0 M 0.75 1.5 2.25 3.0 3.75 5.0

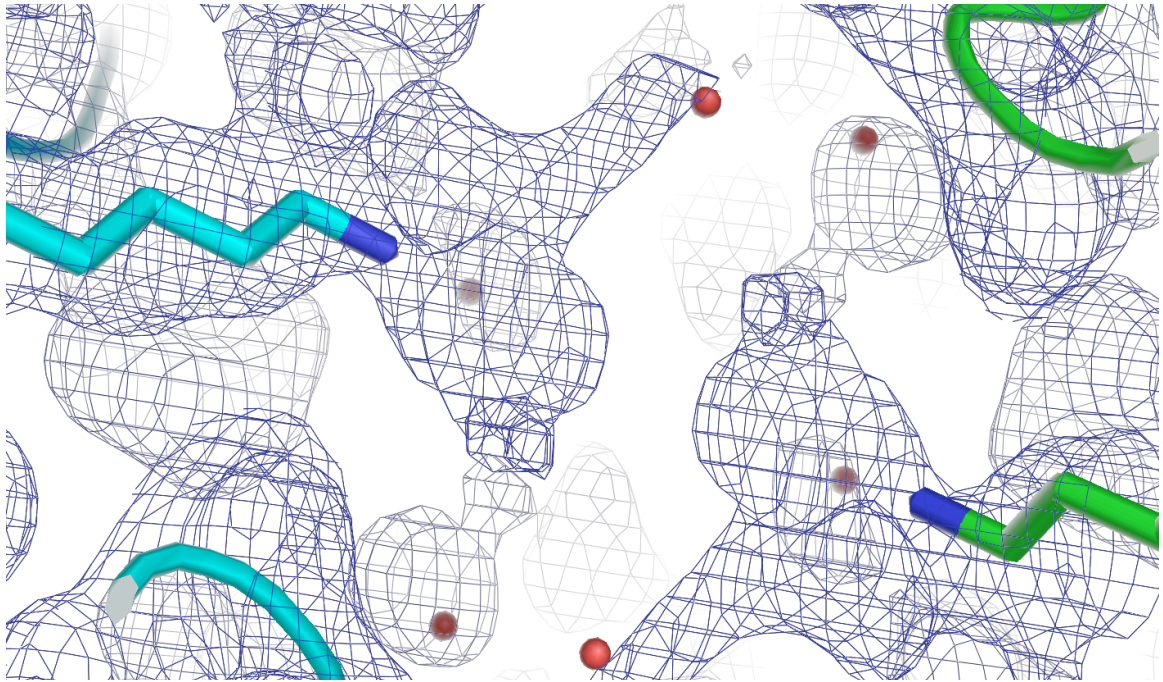
Techniques used: SDS-PAGE analysis, crystal fishing,

- Lysozyme crystals, treated with glutaraldehyde showed high mechanical stability. As it can be seen on figure below, untreated crystals can be easily destroyed by a light pinch with a fishing loop.

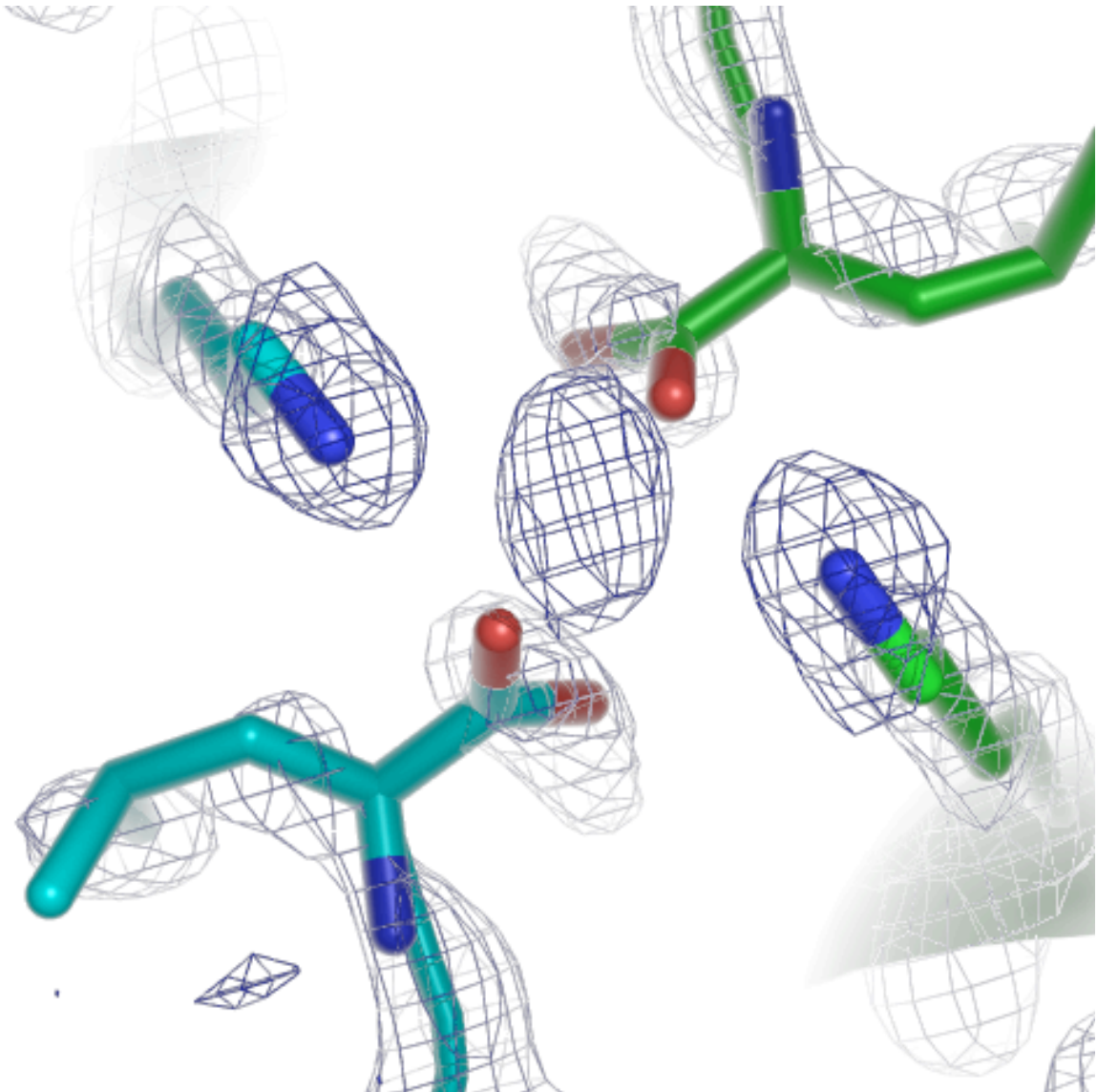


Hewl crystals after 2 pre-soaks were very stable and did not fall apart even after hard pinching.

- Diffraction data
Glutaraldehyde treated crystals diffracted up to 1.5 Angstrom resolution. Electron density maps (figures below) show no cross-linking in awerage between the most affine lysine residues after 3 preparational GA soaks.

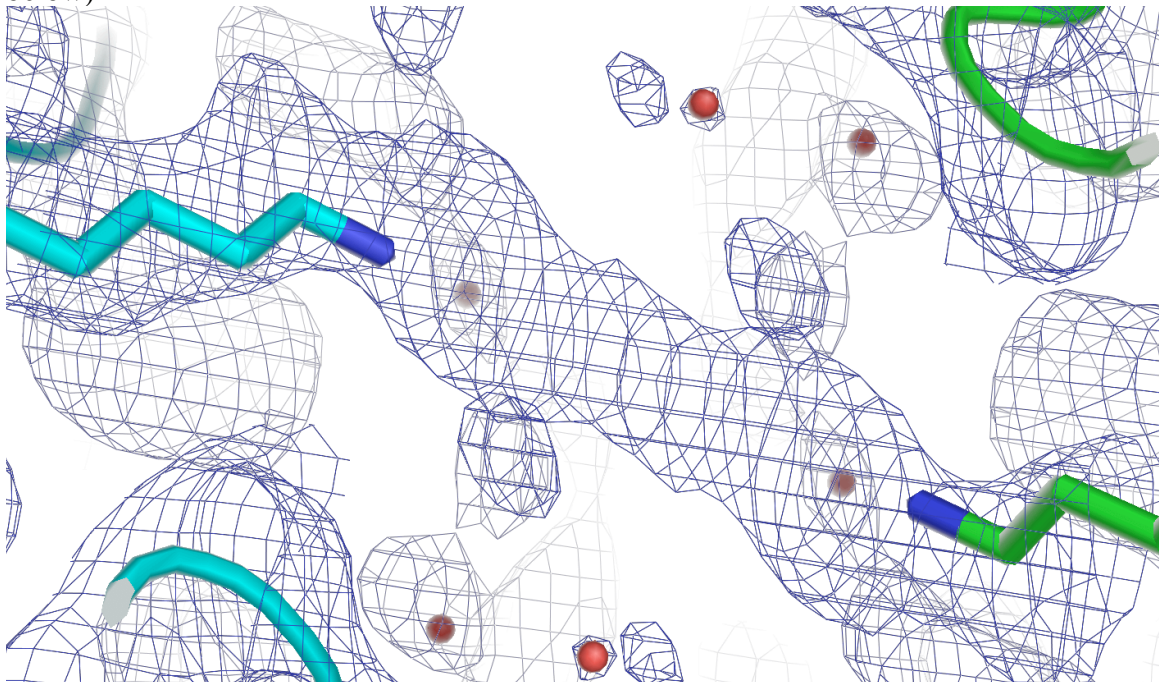


Electron density between lysine residues (K1) before the final soak.

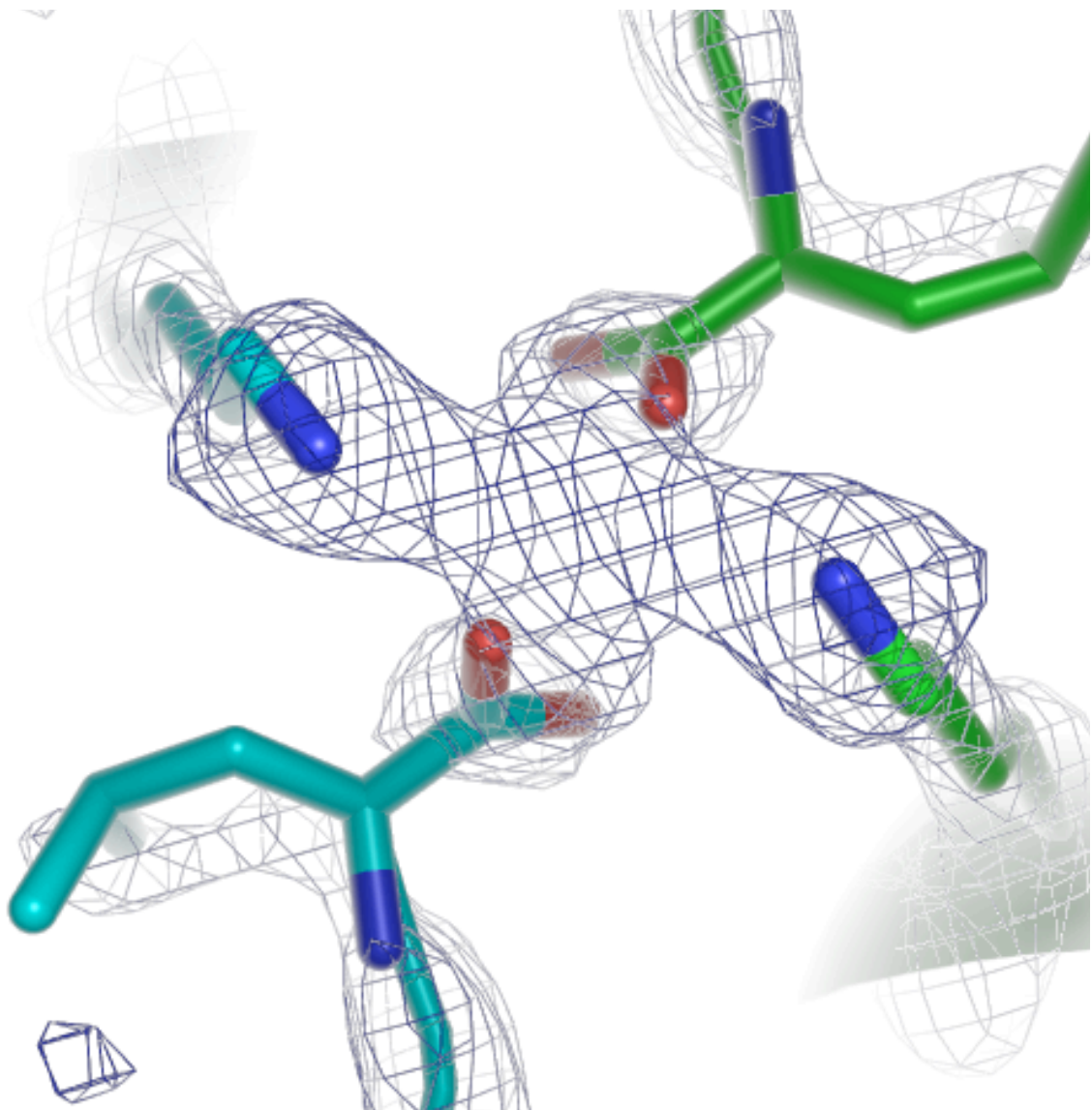


Electron density between lysine residues (K13) before the final soak.

- But, after the final 6 hour soak the clear sign of cross-link can be seen. (see figure below)



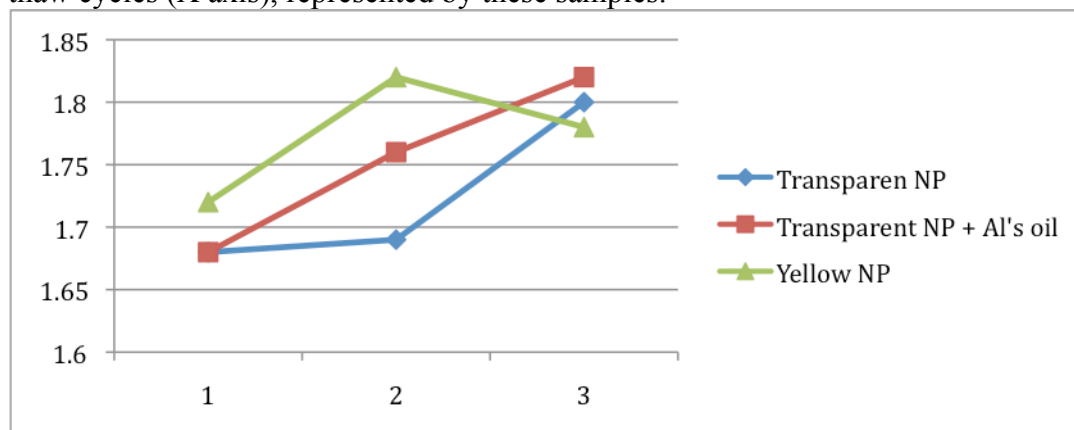
Electron density between lysine (K1) residues after the final soak.



Electron density between lysine residues (K13) after the final soak.

- Final protocol.
 1. Transfer pre-made crystals and their reservoir solutions to a 'screw-top' plate.
 2. Add 3.75%v/v of GA to the reservoirs, transfer crystals to the drop with reservoir solution.
 3. Fish crystals after 24 hours and repeat step 2.
 4. Repeat step 3 twice.
 5. Transfer the crystals to a new drop of 25% v/v GA solution.
 6. Soak 5 hours
 7. Transfer the crystals with suitable litholoops to nail polish (transparent).
(Additional covering with al's oil might have good effect)
 9. Crystals need to be allowed to dry before collecting data.

- Results.
HEWL crystals that were treated according to a "new" protocol with different nail polish materials showed high mechanical, chemical, temperature and radiation stability. Figure below shows resolution (Y axis) change during freeze-thaw cycles (X axis), represented by these samples.



Further freeze-thaw cycles showed seemed to have no significant damage on the samples. Sample with Al's oil still diffracted to 1.6 Angstrom even after 10000 sec. exposure to X-ray radiation.

Further steps

Assessing proper covering material will allow to develop the "final" protocol which can be used to produce "test crystals" capable to resist multiple data collection sets at different beam-lines all over the world.

Acknowledgements

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