Cryopreservation of Biological Samples: From Art to Science (?)

Robert Thorne and Matthew Warkentin
Cornell University
Outline

- Cryocrystallography basics
- Why do we add cryoprotectants?
  - **Ultrafast cooling** – without cryoprotectants
  - **Ultraslow cooling** – without cryoprotectants!
- Mechanisms of radiation damage
- New opportunities for temperature controlled studies of structure and dynamics
Cryocrystallography

Cooling protein crystals (and other biological samples) to $T \sim 100$ K reduces radiation damage and extends sample lifetimes by $\sim 10^2$.

**But** cooling can cause:

- formation of crystalline ice
- disruption of structure and order
- degradation of diffraction properties

Temperature dependent measurements between 150 K and 250 are difficult.
Why does flash cooling create disorder?

- Macromolecular crystals are 20-90% water.
- **Slow cooling**: water transforms to *hexagonal ice* with a 9% specific volume increase.
- **Flash cooling** below $T_g \approx 150$ K: water supercools into an *amorphous* phase.
- **Pure water**: vitrification only with cooling rates of $10^6$ K/s for drops <10 μm.
- **Cryoprotectants allow vitrification with smaller cooling rates.**
Proteins as cryoprotectants

- Proteins also reduce cooling rates required to achieve vitrification (Sartor, Mayer et al., 1992).

Cooling rates for vitrification of hydrated lysozyme powders:

- 23-38% water (1st to 2nd hydration shell): <4 K/s
- 38-41%: <24 K/s

⇒ Penetrating cryoprotectants necessary to prevent internal crystallization only in samples with high solvent content or large solvent channels?
What happens to water during flash cooling?

- Large specific volume change at 0°C eliminated.

Common assumption:

For amorphous ice, \( \rho(T \sim 100 \, \text{K}) \approx 1 \, \text{g/cm}^3 \)

Experiment:

For amorphous ice, \( \rho(77 \, \text{K}) \approx 0.94 \, \text{g/cm}^3 \)

For hexagonal ice, \( \rho(77 \, \text{K}) \approx 0.93 \, \text{g/cm}^3 \)

\[ \therefore \text{Flash cooling and slow cooling produce comparable increases in water's low temperature specific volume.} \]
- Protein crystals are composite materials consisting of interpenetrating water and protein structures.

- Protein crystal unit cell volumes shrink by ~3-6% between 293 and 100 K, while solvent expands.

- Differential contraction of water and protein lattice during cooling causes water to be expelled from some regions and to accumulate in others

  ▸ variation in protein lattice constants.

  ▸ plastic failure of lattice.

  ▸ broadened mosaic.
Before flash cooling:

During flash cooling:

After flash cooling:

• Darker shading = larger water concentration
Rocking image sequence for a triclinic lysozyme crystal:
Fast versus Slow Cooling

All physico-chemical properties of protein crystals and their constituents are temperature dependent. E.g., cell volume, salt solubility, pH, protein conformation.

**Very Fast Cooling**
- no time for crystallization, relaxation and redistribution; only specific volumes change.

**Very Slow Cooling (assume no ice formation):**
- sample remains in quasi-equilibrium as sample constituents relax and redistribute, so low T state is homogeneous.
Conventional Flash Cooling

Teng and Moffat, 1998:

- N₂ stream: ~400 K/s
- Liquid nitrogen: ~400 K/s
- Liquid Propane: ~1000 K/s

Cooling times:
- To below 220 K: ~0.2 s
- To T_g=150 K: ~0.4 s

We are in neither the slow nor fast cooling limit, but in some messy intermediate regime!
How can we cool faster?

• Decrease sample volume V
  Cooling rate $\sim V^{-1/2} \sim L^{-3/2}$
  1 mm to 20 $\mu$m should give a factor of $\sim 350$

• Plunge in liquid rather than gas stream
  $\sim$ factor of 20

• Plunge in liquid propane instead of nitrogen
  $\sim$ factor of 2-4

• Increase plunge velocity in liquid
  $\sim$ factor of 2

But: Comparable cooling rates and diffraction outcomes with gas stream and liquid plunge cooling observed.
WHY?  

A cold gas layer forms above the liquid cryogen

For volumes < 0.1 µl, cooling below $T_g$ occurs in the gas layer, before the crystal reaches the liquid cryogen!
Ultra-Fast Cooling ("Hyperquenching")

Remove the cold gas layer by blowing or sucking.

With gas layer:
\[ \frac{dT}{dt} < 10^3 \text{ K/s} \quad (L<500 \ \mu\text{m}) \]

Remove the gas layer:
\[ \frac{dT}{dt} = 2 \times 10^4 \text{ K/s} \quad (L\sim80 \ \mu\text{m}) \]
\[ \sim10^5 \text{ K/s} \quad (L\sim20 \ \mu\text{m}) \]
Faster cooling ⇒
Less cryoprotectant required to vitrify solvent.

100 μm: 13% w/v
50 μm: 8% w/v
10 μm: 3% w/v
Ultra-Fast Cooling

• 20-1000 times faster than flash cooling – as fast as state-of-the-art cryo-em cooling methods.

• **Successful cooling of all proteins (so far) to $T=100$ K without penetrating cryoprotectants**

• More reliable and consistent cooling
• Improved crystal order and diffraction (?)
• More accurate trapping of room-temperature structure (?)
What about Ultra-Slow Cooling?

- Sample remains in quasi-equilibrium as sample constituents relax and redistribute; low T state is homogeneous.

**Time for ice nucleation**

$\sim 10^{-5}$ s

at $T=200$ K

- Need cooling rates of $10^7$ K/s
Ice formation prevents structural studies at $180 \, K < T < 220 \, K$.

Tilton, Dewan and Petsko, 1992:
Ice formation prevents structural studies at $180 \, K < T < 220 \, K$

Chung and Parak, 2001:
Data collected on cooling from $T=300$ K to $T=100$ K at 0.1 K/s

Thaumatin

56% solvent

No penetrating cryoprotectants
Cooling at 0.1 K/s

Urease
48% solvent
4.9 x10^6 Å^2 cell
No penetrating cryoprotectants
No formation of crystalline ice inside the crystal during cooling at 0.1 K/s

⇒ Crystalline protein is as effective in suppressing ice nucleation as a 70% glycerol solution
Key:
**Completely** remove all surrounding solvent
Data collection at all $T$ between 300 K and 100 K

Thaumatin cooled at 0.1 K/s
# Slow Cooling & Variable Temperature Data Collection: A General Method?

<table>
<thead>
<tr>
<th>Protein</th>
<th>Lowest Mosaicty $T= 100 \text{ K}$</th>
<th>Cryoprotective Mother Liquor Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>0.06°</td>
<td>12.5 % (w/v) ethylene glycol</td>
</tr>
<tr>
<td>Urease</td>
<td>0.45°</td>
<td>1.6 M Li$_2$SO$_4$</td>
</tr>
<tr>
<td>Thaumatin</td>
<td>0.11°</td>
<td>1.5 M Na-K-Tartrate</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.4°</td>
<td>1.2 M NaCl</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.9°</td>
<td>25 % (w/v) PEG 8000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 M ammonium sulfate</td>
</tr>
</tbody>
</table>
Temperature Dependence of Radiation Damage
Thaumatin, no penetrating cryoprotectants
Activation Energies and Mechanisms of Radiation Damage

Radiation sensitivity drops by a factor of 10 between 225 K and 160 K with $E_a \approx 3.7$ kcal/mol.

NMR data for diffusion of lysozyme hydration water:

$E_a \approx 3.5 - 4.1$ kcal/mol.
Urease

$4.9 \times 10^6 \, \text{Å}^2 \text{ cell}$
Structure versus temperature

![Diagram showing structure versus temperature relationship](image)
Structure versus cooling rate

![Graph showing relationship between Cα RMSD and C-Chain Residue for different cooling rates](image)

- 0.1 K/s
- ~100 K/s
- ~100 K/s
- ~10,000 K/s
- ~10,000 K/s
- Room Temperature
Structure versus cooling rate

![Graph showing structure versus cooling rate with different cooling rates represented by different lines and markers. The x-axis represents C-Chain Residue, and the y-axis represents the B-Factor (Å²). The graph includes lines for 0.1 K/s, ~100 K/s, ~100 K/s, ~10,000 K/s, and RT.]
Summary

Differential contraction of solvent and protein creates disorder on cooling.

Ultra-fast cooling using cold-gas removal
• allows most / all samples to be cooled without cryoprotectants.
• can more accurately capture the room temperature structure.

Ultra-slow cooling using external solvent removal
• allows many samples to be cooled without cryoprotectants.
• can yield improved low-temperature sample order.
• allows structural studies at arbitrary temperature; conformational states and energy landscapes.
Broader Implications

• Cryopreservation: Art → Science

• Long term storage of protein solutions

• Cryopreservation of cells (sperm, egg, stem, …) and tissues