## Handling LN2 and the need for a secondary cryogen: the Leidenfrost effect

Leidenfrost effect: a physical phenomenon in which a liquid, close to a solid surface that is significantly hotter than the liquid's boiling point, produces an insulating vapor layer that keeps the liquid from boiling rapidly.

Drop of liquid held up by layer of vapor

0.2 mm — 0.1 mm

Hot surface

Do not do this!
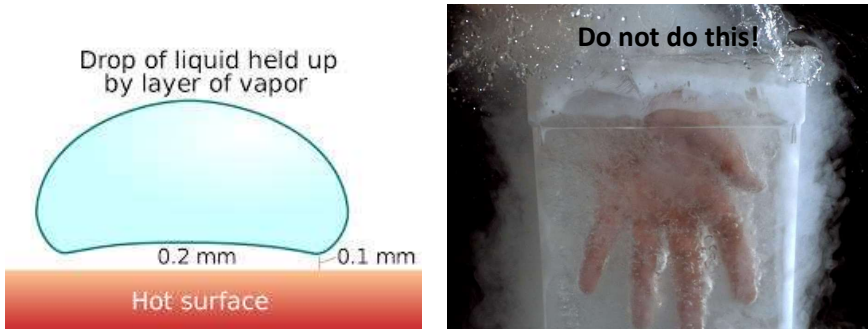
The larger the difference between boiling point of the liquid and the temperature of the surface, the stronger the Leidenfrost effect.

N_2 boiling point: -195.8 °C ← Strong Leidenfrost effect, **poor heat extraction**
Ethane boiling point: -88.5°C   ← Strong Leidenfrost effect, **good heat extraction**
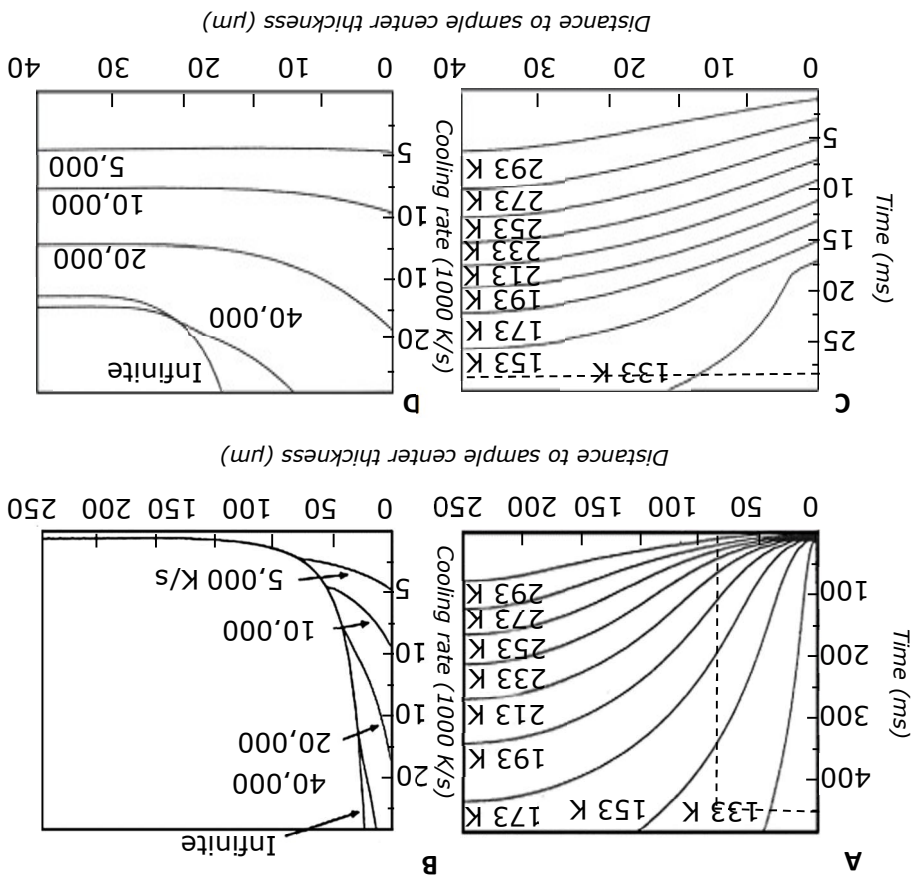

CryoTEM

Introduction

Version 1 – May 2024

Part I: Theory

starts at: the beginning
ends with: Ready to setup the cryoTEM procedure

- Sample thickness

The cooling rate is inversely proportional to the thickness squared. Simulated temperature for slabs with decreasing thickness are shown on the next page.

➔ low thermal conductivity of water
➔ Therefore, cooling rates above 1000 K/s cannot be achieved in the centre of a thick (500 µm) slab (A, B).
➔ Even when infinitely high cooling rates are applied at the surface of the slab (B).
➔ Reducing the sample thickness (C, D), rather than technical improvement is rendering higher cooling rates.
➔ A sample of 100nm plunged into liquid ethane (at -180°C) is reaching theoretical cooling rates of 10^{10}K/s.

Liquid samples can be vitrified to a range of 10-20 µm deep at ambient pressure

Next page: The simulation of cooling rates and temperature distributions in a 500 µm (A, B) and 80µm (C, D) thick water slabs. The X-axis depicts half of the slab (distance from the surface to the centre).

After 400 ms of cooling a 500 µm thick water slab, T_r (about -130°C) is reached only to a depth of about 80 µm (A, dotted lines). Furthermore, about 600 K/s is the maximal achievable cooling rate in the centre, regardless the cooling rate at the surface (B)

Thinner slabs considerably enhance the cooling process in the centre: T_r is reached in the center within 30 ms and a maximum cooling rate at the centre of 15,000 K/s is reached. Source: Studer et al., 1995.

PART I: THEORY

Contents

The phase diagram of water

• Introduction

Phase diagram
Is a plot showing the preferred physical states (Vapor, Liquid and Solid) of matter at different temperatures and pressure.

Phase line
represents the boundary states (= phase boundary) and gives the conditions when two phases may stably coexist in any relative proportions (=having the same Gibbs free energy).

Triple point
Where three phase lines join, there is a 'triple point': where three phases stably coexist (=having identical Gibbs free energies).

• **Solute concentration**
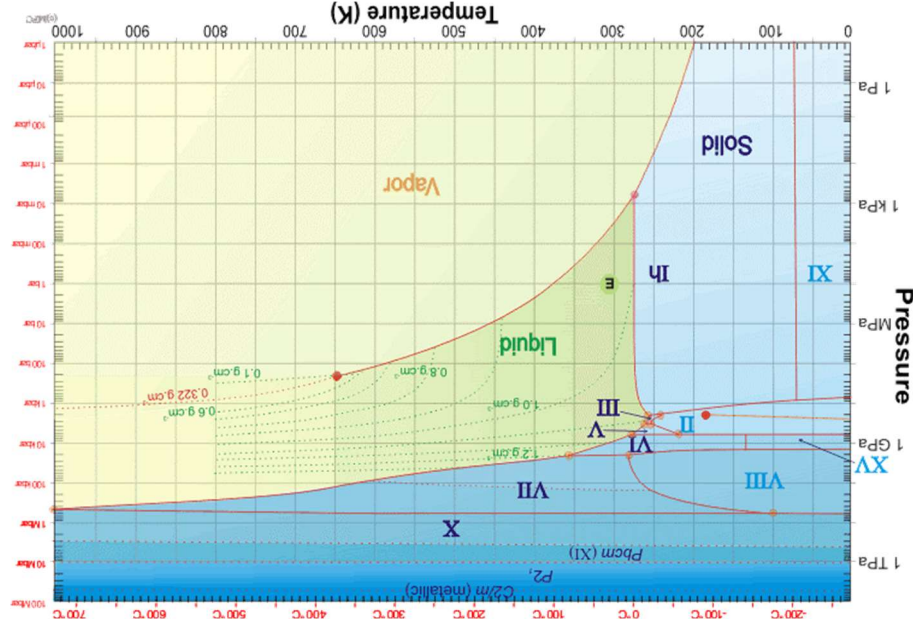
The presence of solutes affects the dynamics and kinetics of ice formation negatively in several ways.

- The crystallisation window is narrowed down due to a decrease of the melting point (T_m) and an increase of the recrystallization point (T_r).
- The ice nucleation rate is decreasing drastically. The minimum (aka "nose") of the curves corresponds to the minimum time for a given volume to crystallize.
- In the extreme case of e.g. Tokuyasu samples, water is replaced by a 2.3M sucrose solution. Here, cooling always leads to vitreous water (Tokuyasu, 1973).

The effect of solute concentration on the escape time (τ).

τ is the time scale for crystallization for a volume fraction, reflecting a combination of nucleation and crystal growth. (From Angell & Choi, 1986)

Compared to pure water (H_2O), solutes (at concentration C_1 and C_2) increase the time needed to form an ice crystal as well as minimize the temperature window wherein crystallization occurs (increase of T_m and decrease of T_r).
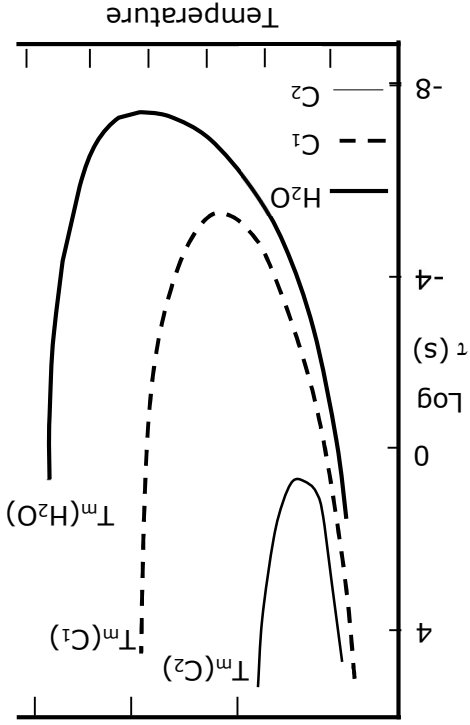
- Cooling rate

The applied cooling rate is the parameter which is the most prone to technical improvements.

Below 1000 Kelvin/second

Ice nucleation and ice formation occurs in regions with lower solute concentration (e.g. the extracellular region). Due to water segregation, the solute concentration in the vicinity rises gradually, eventually leading to osmotic pressure. Due to this pressure, water is extracted from the nearby compartments (e.g. cells, liposomes) and incorporated in the growing ice crystals. Due to the severe dehydration, the morphology of is not preserved.

1000 – 100,000 Kelvin/second

The cooling rate is still to low to surpass the temperature window without the formation of ice, which causes eventually water segregation in the structure and morphological damage. This applies for samples with typical biological solute salt concentrations (about 0.9% or about 0.15M NaCl). For samples at higher solute concentrations (e.g. Tokuyasu method) these cooling rates can be sufficient to avoid morphological disruptions. For lower solute concentrations faster cooling rates are needed.

Above 100,000 Kelvin/second

At high cooling rates, the temperature window is surpassed before ice crystals can form. This is the goal of the vitrification technique. The sample is vitrified, no water segregation occurs and the morphology is preserved. Vitreous samples are required in the case of cryo-TEM. However, when these samples are thawed, they re-enter the temperature window of ice nucleation and ice crystal formation will occur, damaging the morphology.

States may abruptly and totally change into each other given a slight change in temperature or pressure.

- Solid water (ice and vitreous water)

Water has many solid phases AND many metastable phases, divided into two groups.

Crystalline solid water (=ice)

- Low pressure ices (e.g. hexagonal ice (=Ih), cubic ice (=Ic) and ice-eleven)
- high pressure ices (e.g. ice-seven, ice-eight and ice-ten and others)

Non-crystalline solid water (or vitreous water; solid water but not ice)

- Low density amorphous water
- High density amorphous water

The complex central part around 1GPa/250K of the phase diagram is expanded below. Note that water will remain liquid to -22°C at 200 MPa.

Vitrification
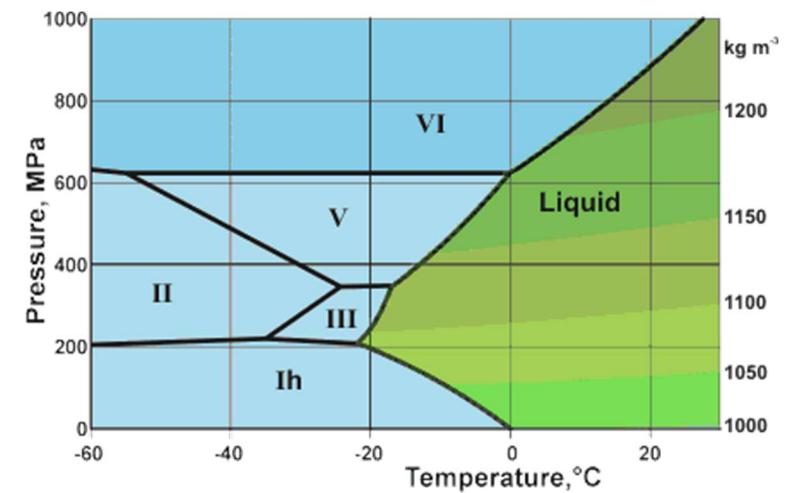
The aim of cryofixation techniques: fix a specimen by immobilizing its water content in a solid, non-crystalline state.

- Non-crystalline state = amorphous state
  - the process is called vitrification.

The non-crystalline state can be regarded as liquid water with an infinite high viscosity.

Formation of ice – the crystalline state of water – is damaging to your structures, especially biological structures (but also NP, liposomes, ...)

Why?

➤ only water molecules are incorporated in the crystal
➤ This leads to phase separation
➤ which leads to segregation, inducing morphological changes.

ice crystal formation develops in two steps:

1. first a nucleus is formed by a thermodynamical process called ice nucleation.
2. Then, as the ice lattice grows, a kinetic growth process plays out that depends on the conditions in the surrounding medium.

ice crystal formation is only occurring within a temperature window bordered by:

- on the upper range by the melting point (T_m)
- on the lower range by the recrystallisation temperature (T_r). At lower temperatures, there is not enough energy to form crystals.

The objective of cryofixation is to surpass this window (beyond the recrystallisation point) faster than the ice crystals can form.

---

physical parameters influencing the outcome of cryofixation

Cooling rate: change in temperature over time, usually expressed in K/s. Very high cooling rates are needed to avoid the growth of nuclei.

Solute concentration: increasing the solute concentrations affects the T_r and T_m (Van 't Hoff factor), hence the window of crystallization, and therefore facilitates cryofixation.

Pressure: the pressure affects the melting temperature (see above). This means, that the range in which crystallization occurs can be reduced.

Sample thickness: due to the particularly bad heat conductivity of water (the main constituent in water-based materials), high cooling rates at the surface do not reflect high cooling rates at the center.

The recrystallisation point is up for debate, but generally, a value of -135 to -140°C is used.

Temperature diagram:

- Axis: Temperature
- Values: 130 · 185 (T_r) · 251 273 (T_m) (T_m@204.5 bar)
- Bars: H₂O · Cytoplasm · 204.5