Tissue and cytoplasm vitrification in cryopreservation monitored by low temperature scanning electron microscopy (cryo-SEM)

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Cryopreservation of cells and tissues frequently relies on inducing cytoplasm vitrification for storage with almost complete stoppage of both chemical reactions and physical processes and without ice crystal biological damage. The most frequently employed technique used for monitoring ice formation and vitrification in these systems is differential scanning calorimetry (DSC). This technique, which was used in this study to observe ice formation in cryoprotecting agent solutions and mint shoot tips, is fairly sensitive for detecting ice formation, but not so for directly observing glass transition. Besides, the possibility of coincident thermal phenomena obscuring the small glass transition signature in DSC and the lack of any spatial resolution, make valuable the information obtained from other sources. Low temperature scanning electron microscopy (cryo-SEM) is able to show ice crystals formed in the cooling process employed to introduce samples into the microscope, very similar to the cooling step of cryopreservation protocols. The images from samples that, after DSC evidence, have no ice crystals and are vitrified, appear completely unetched in cryo-SEM micrographs. Consequently, cryo-SEM is proposed as a suitable method to ascertain cells and tissues effective vitrification in cryopreservation.

Keywords glass-SEM; ice dynamics; DSC; mint tips

1. Introduction

Cryopreservation comprises a family of procedures allowing the long-term storage of viable biological material at low temperature. Currently many different tissue and cell types (of human, animal, plant or microorganisms origin) are cryopreserved [1-3]. Low temperature storage is employed to avoid deterioration or viability loss occurring at room temperature. But the cooling and warming processes involved are also likely to irreversibly damage cells and tissues. Ice crystal formation is known to cause cell death and loss of viability of stored material [4], so the procedures applied are designed to avoid it. Ice formation requires an initial nucleation step, energetically disfavourable, that involves the reorganization of a large number of water molecules driven by stochastic Brownian movement. Only when nuclei have reached a certain size, ice crystal growth becomes energetically favourable, even at temperatures well below the equilibrium freezing point [5].

The protocols developed and the general strategy followed differs greatly, depending on the material type, tissue, and even concrete species. Nevertheless, most protocols aim at reducing the intracellular water content (either by air or osmotic dehydration, or extracted by the extracellular formation of ice), increasing at the same time the intracellular solute concentration. The result is an increase of the cytoplasmic microviscosity, slowing the water reorganization process required for ice nucleation. Viscosity, which is also increasing steeply as the system temperature decreases, can reach such high values that movement is virtually stopped. In this situation, denominated glassy or vitreous state, most chemical reactions and physical processes are considered to be detained, and ice formation is deemed as impossible [6, 7].

Plant cryopreservation is often performed by means of two step procedures in which first, the water content is reduced (increasing, so, both solute concentration and cytoplasm viscosity) and then temperature is quickly reduced, to cross the glassy state thermal border, the glass transition temperature, $T_G$. Although there exist many other fluids with heat transfer and cryogenic properties more favourable than liquid nitrogen (LN), because cryopreservation is, after all, a set of standard preparative procedures, quick plunging into nitrogen is usually applied, as there exist a LN widespread supply system, at reasonable cost. Besides, the glass transition of many cryopreserved specimens often lies within the interval -120 to -90°C, and the temperature of boiling LN (-196°C) provides a large enough temperature difference to ensure a fast cooling drive, as well as a safe storage ambient, well below $T_G$ [5, 8].

Droplet-vitrification protocol is widely applied to different plant species. In it, in order to increase heat transfer rates, the tissue portion to be preserved is included into a small drop of vitrification solution (after a set of previous treatments, causing dehydration but also inducing natural defence mechanisms, useful to protect cells towards both dehydration and low temperature). Several drops of this solution (very concentrated in sugars and related low-molecular weight cryoprotecting compounds) are placed into a small strip of aluminium foil, which is then immersed directly, either into LN for cooling, or, at the recovery stage, into room-temperature or warm culture medium. This protocol is particularly successful when applied to mint shoot tips, and the reported viability reached is high [7, 9].
The transition between liquid and glassy state, not a proper phase transition, can be detected by monitoring a number of properties ultimately related to molecular mobility [10], such as volumetric or mechanical ones. In the context of cryopreservation, the glass transition is most frequently studied by means of differential scanning calorimetry (DSC). This technique allows to detect even small amounts of water freezing (due to its high phase change enthalpy) and also to directly observe glass transition as a step in the heat capacity ($C_p$) baseline. DSC is, however, not a very sensitive technique for this purpose, as the $C_p$ change observed is often very small. Sensitive equipment, yielding a stable baseline, is required, and as the step span depends on the amount of vitrified material, in many occasions its observation is problematic [11].

Low temperature scanning electron microscopy (cryo-SEM) is a user-friendly microscopic technique, allowing an easy, quick and artefact free observation of biological materials. The main drawback of electron microscopy applications to biological materials is their high water content and its incompatibility with the high vacuum required. Cryo-SEM allows the observation of these materials without tedious dehydration procedures, often causing loss or alteration of structural features in specimens. This technique is particularly useful for the observation of the effect of the cryopreservation processes, as the cooling into LN step can be considered equivalent to the final stage of the cryopreservation protocol.

Contrast in this technique is low for high water content biological material, poor in high atomic weight atoms. So, an etching step is included, in which a temperature rise allows the partial sublimation of ice, creating depressions that are visible after gold covering. We are considering in this chapter the employment of cryo-SEM as a tool to visualize glassy state in cells and tissues submitted to cryopreservation processes. Mint shoot tips treated following the droplet-vitrification protocol were used as a test material, and the steps of the protocol with decreasing water content were compared to show examples of specimens with intracellular ice and vitrified.

### 2. Materials and Methods

#### 2.1 Plant Vitrifying Solution 2

Plant Vitrifying Solution 2 (PVS2) was prepared as 30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v dimethyl sulfoxide and 0.4 M sucrose in plant growth medium salts [6]. For some DSC and Cryo-SEM experiments, PVS2 was diluted with milli-Q deionized water to 25%, 50% and 75%.

### Figure 1. Scheme of the steps of the droplet-vitrification cryopreservation protocol employed, see text for more details on media and solutions employed.

**a:** preculture   
Overnight, in MS medium with 0.3 M sucrose  

**b:** loading   
20 min incubation in 2 M glycerol, 0.4 M  

**c:** dehydration   
20 min incubation in PVS2 solution  
LN

#### 2.2 Plant material pre-culture and mint shoot tips extraction

A scheme of the cryopreservation protocol showing the stages in which the specimens were observed is shown in Fig 1. Shoot tips were extracted from Mentha x piperita in vitro plants, maintained by monthly subculture on MS medium [12] with 3% sucrose. Incubation took place at constant temperature (25°C) with a photoperiod of 16 h, and an irradiance of 50 µmol m$^{-2}$ s$^{-1}$ from fluorescent tubes. Shoots were cut into one-node segments, transferred to fresh medium and incubated at alternating temperatures of 25°C (day) and -1°C (dark), always with 16 h photo- and thermoperiod, 50 µmol m$^{-2}$ s$^{-1}$ irradiance, provided by fluorescent tubes. After 3-weeks of culture under these conditions, shoot tips (1-2 mm) were isolated from the axillary buds.

#### 2.3 Mint shoot tips dehydration steps

Shoot tips were pre-cultured overnight in a Petri dish with 2 mL of liquid MS medium containing 0.3 M sucrose, over filter paper, at 25°C (observation stage a). Thereafter, the explants were transferred to a Petri dish with 2 mL of loading
solution (2 M glycerol + 0.4 M sucrose), over filter paper, for 20 min, at room temperature (observation stage b). Finally, they were dehydrated in 2.0 mL PVS2, in a Petri dish, on filter paper, for 20 min at 0ºC (observation stage c).

2.4 Low temperature scanning electron microscopy

Cryo-SEM observations were performed using a Zeiss DSN 960 scanning microscope equipped with a Cryotrans CT-1500 cold plate (Oxford, UK). Cryo-SEM allows sample observations without the need of prior chemical fixing or drying processes.

Three shoot tips in the same stage of the cryopreservation protocol (see above) were fitted on a special bracket with their axes vertically aligned, and this piece was introduced in LN under low pressure, physically fixing tissues for microscopic observation. Then, the holder was introduced in the pre-chamber of the Cryotrans cold plate, at -180 ºC, and specimens were fractured perpendicularly to their axis, to obtain suitable observation surfaces. Finally, samples were inserted in the microscope, etched for three min at -90ºC, gold-covered, and observed at -150/-160ºC under secondary electron mode.

Drops of PVS2 solution and 50% water dilution were deposited on the microscope sample holder and cooled in LN at reduced pressure. They were not fractured, but directly etched for three min at -90ºC, gold-covered, and observed at -150/-160ºC under secondary electron mode.

2.5 Differential scanning calorimetry

DSC experiments were performed with a TA-1000 instrument (TA Instruments, New Castle, DE, USA) using a scanning rate of 10ºC min⁻¹. Five shoot tips in the same stage of the cryopreservation protocol were introduced in a DSC pan, which was sealed and weighed. 10 µl samples of PVS2 and water dilutions were also enclosed in DSC pans. Both sample types were submitted to cooling scans from room temperature to -85ºC, short (1 min) equilibration periods at this temperature, followed by warming scans, back to room temperature. Later, pans with tips were punctured and dried in an oven at 85ºC for 72 hours, when they showed constant weight. Thermograms were analysed using the standard procedures provided in the Universal Analysis Program (TA Instruments). Ice thawing thermal events (more precise than freezing events) were considered. The routine produced the temperature corresponding to the event onset and that to its peak, the former corresponding to the equilibrium freezing temperature. The process associated enthalpy, proportional to the event area, was also obtained.

3. Results and Discussion

3.1 Plant Vitrifying Solution 2 DSC studies

PVS2 solution was employed as a model of a simple vitrifying system. Its composition was adjusted to enable tissue dehydration and protection towards low temperature effects [6]. Some of its components are membrane-penetrating, while some others are not. Its water content is 40,3 % and it is known to vitrify at -112ºC [13].

Figure 2 shows heating DSC thermograms obtained with pure PVS2 solution and several of its water dilutions. The pure PVS2 solution shows no ice formation events either at the cooling (not shown) or heating steps. The diluted mixtures show proof of ice formation. The samples of 25% and 50% PVS2 concentration show melting events corresponding to ice formed in the cooling step. The 75% PVS2 samples had the water mobility so reduced that ice was not formed in the cooling step. However, some ice was formed and quickly melted, during the heating step.

3.2 Plant vitrifying solution 2 cryo-SEM observation

Figure 3 shows the surface of a drop of PVS2 solution and its 50% water dilution deposited on the microscope sample holder without fracture. The clear lumps on the drop surface are volatile substances deposits originated by the lack of a clean surface generated by freeze-fracture. The pure PVS2 drop shows no darker regions that could be associated to ice crystal formation and later sublimation during the etching process. Meanwhile, the 50% PVS2 dilution shows dark holes interpreted as ice crystals formed in the cooling step and later evacuated by sublimation during etching.

The pure PVS2 solution is known to be in glassy state at the -150ºC of the microscope stage [13]. It is shown to form no ice upon cooling, after the presented DSC results. Its 50% dilution however, is forming ice crystals, after these DSC measurements, and this is in good agreement with the microscopic observations, where the dark holes that can be observed after etching would correspond to the ice crystals formed. We conclude that pure, vitrified PVS2 solution undergoes no sublimation and no etching-enhanced contrast. Vitrified water has an amorphous structure [14,15]. Interactions among water molecules and other solution components are strong, though disordered, which may impair cooperative phenomena and may have an important role in phase change kinetics. The null or very slow sublimation of vitreous water has been previously reported [16, 17].
Specimens in the stages a (preculture), b (loading) and c (dehydrated) of the droplet-vitrification protocol were studied, after Fig. 1. Figure 4 shows their typical re-warming DSC thermograms. Table 1 shows the thermal parameters derived from these DSC experiments: \(T_{\text{f(onset)}}\) and \(T_{\text{f(peak)}}\), respectively, the onset temperature of the melting endotherm (corresponding to the equilibrium freezing temperature) and its peak temperature. The freezing enthalpy (\(\Delta H\)) is also showed.

### Table 1. Thermal parameters of the thawing events obtained from mint tips DSC thermograms.

<table>
<thead>
<tr>
<th></th>
<th>(T_{\text{f(onset)}}) (°C)</th>
<th>(T_{\text{f(peak)}}) (°C)</th>
<th>(\Delta H) (J g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>-5.9 ± 1.1</td>
<td>-1.35 ± 0.34</td>
<td>292 ± 16</td>
</tr>
<tr>
<td>b</td>
<td>-15.5 ± 0.1</td>
<td>-7.27 ± 0.21</td>
<td>188 ± 14</td>
</tr>
<tr>
<td>c</td>
<td>-</td>
<td>-</td>
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3.3 Mint tips DSC studies

Specimens both in steps a and b of the cryopreservation protocol undergo freezing (not shown) and melting events in the respective cooling and re-warming scans. Freezing events at cooling scans take place at the nucleation temperature, shifted to lower values by a considerable supercooling degree and are not appropriate to obtain the equilibrium freezing temperature, especially in a situation of high solute concentration and consequent low molecular mobility, favouring supercooling. Both \(T_{\text{f(onset)}}\) and \(T_{\text{f(peak)}}\) decrease from a to b, which is in good agreement with a significant increase in the solute concentration with the second incubation step. The amount of frozen water (proportional to the freezing enthalpy) is consequently reduced also, from a to b, as less water is available for ice formation when the solute concentration increases.

Thermograms corresponding to specimens in step c show a flat baseline, with no freezing or thawing event in either cooling or warming DSC scans, spanning from room temperature -85 ºC (only warming scans from -50 to 15 ºC are shown).

3.4 Mint tips cryo-SEM studies

Figure 5 shows typical cryo-SEM micrographs of mint shoot tips cooled in LN in the microscope cryo-unit, as described. Specimens in both a and b protocol steps showed a clearly visible tissular and cellular structure, with some organelles and vacuolar membrane elements present, among a dark ice crystal mosaic surrounded by a clearer supercooled cryo-concentrated solution matrix. Specimen a shows larger ice crystals while the alteration of cellular
structures by ice is more evident than in b. Meanwhile, specimen c shows a grey continuous mass masking all structural elements in the field, not affected by the etching procedure applied, and ascribed to vitrified solution.

The combined results of DSC and cryo-SEM would be in good agreement, implying that part of the water contained in the system for specimens on steps a and b becomes frozen when cooled under -85ºC (with less amount of ice formation in the latter) while specimens in step c would avoid ice formation, presumably getting vitrified in the cooling process, as they showed no ice trace, either microscopically or calorimetrically. This is also in good agreement with the literature data for viability as a result of applying the droplet-vitrification method to several systems, including mint apices [6].

DSC is the most frequently employed technique used for monitoring ice formation and glass transition in cryopreserved systems. While the high value of the phase change enthalpy for ice melting ensures the detection of even very small amounts of ice, glass transition has a much smaller reflection on DSC thermograms. Besides, glass

Figure 4. Typical DSC thermograms corresponding to re-warming processes of mint shoot tips in different stages of the droplet cryopreservation protocol: a, preculture; b, loading, and c, dehydration. Each experiment was performed with five shoot tips. Scanning rate was 10 ºC min⁻¹. (See Materials and Methods and Figure 1 for more details).
transitions, showed in thermograms as a small baseline displacement, are much less energetic than freezing of thawing processes and are often difficult to be observed in low-mass samples, such as those here considered (total sample mass for each DSC experiment for e-step specimens, about 3.5 mg; total mass of water in these samples, about 2.5 mg).

Besides, many other associated phenomena, causing a stronger distortion on the heat capacity baseline, can take place at the same time [11]. A common example is lipid melting which, being very wide processes can cover underlying glass transition taking place of the aqueous phase [18]. A further difficulty is the global character of the information obtained by calorimetric methods. Although many different types of cells and tissues could be present in cryopreserved specimens, the information on ice formation and/or vitrification given by DSC refers to an average over the whole specimen. The main interest lies, however, in the particular class of cells and tissues that may have been identified as essential for survival.

In these cases, the possible use of a tool such as cryo-SEM could be very valuable. Tissues or cells with ice crystal marks or vitrified could be readily discriminated, no matter their size or water content. Other phenomena contributing to the energetic state of the sample, such as lipid melting or other unrelated phase transition, are not interfering, and besides, each particular type of cell can be identified and its glassy state directly checked.

It must be noted that the cryo-SEM technique is directly reporting the lack of sublimated ice during etching [16, 17]. Ice formation is completely impaired in vitrified solutions, but in regions over \( T_g \) but close to it (at least up to about 20°C over \( T_g \)), although ice has the physical possibility to be formed, its occurrence is very unlikely in a short time period, as the molecular mobility is already very low. So, the cryo-SEM methods would not allow discrimination between these two possible states, both of them not permitting ice formation, at least for short time scales.

4. Conclusions

Low temperature scanning electron microscopy is useful to monitor ice formation in cells and tissues, in conditions very close to that of cryopreservation protocols. The information obtained from this technique is in perfect agreement with the ice formation and vitrification data obtained by differential scanning calorimetry. The combined used of these two techniques yielding complementary information is considered as very promising for further studies concerning the mechanism of both cryopreservation and glass formation and evolution.

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Figure 5. Cryo-SEM micrographs of mint shoot tips in different steps of the droplet cryopreservation protocol: preculture, a; loading, b; dehydrated c. The bar corresponds to 10 µm.
References


