Vitrification of articular cartilage by high-pressure freezing

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Summary
For more than 20 years, high-pressure freezing has been used to cryofix bulk biological specimens and reports are available in which the potential and limits of this method have been evaluated mostly based on morphological criteria. By evaluating the presence or absence of segregation patterns, it was postulated that biological samples of up to 600 μm in thickness could be vitrified by high-pressure freezing. The cooling rates necessary to achieve this result under high-pressure conditions were estimated to be of the order of several hundred degrees kelvin per second. Recent results suggest that the thickness of biological samples which can be vitrified may be much less than previously believed.

It was the aim of this study to explore the potential and limits of high-pressure freezing using theoretical and experimental methods. A new high-pressure freezing apparatus (Leica EM HPF), which can generate higher cooling rates at the sample surface than previously possible, was used. Using bovine articular cartilage as a model tissue system, we were able to vitrify 150-μm-thick tissue samples.

Vitrification was proven by subjecting frozen-hydrated cryosections to electron diffraction analysis and was found to be dependent on the proteoglycan concentration and water content of the cartilage. Only the lower radical zone (with a high proteoglycan concentration and a low water content compared to the other zones) could be fully vitrified.

Our theoretical calculations indicated that applied surface cooling rates in excess of 5000 K/s can be propagated into specimen centres only if samples are relatively thin (<200 μm). These calculations, taken together with our zone-dependent attainment of vitrification in 150-μm-thick cartilage samples, suggest that the critical cooling rates necessary to achieve vitrification of biological samples under high-pressure freezing conditions are significantly higher (1000–100,000 K/s) than previously proposed, but are reduced by about a factor of 100 when compared to cooling rates necessary to vitrify biological samples at ambient pressure.

Introduction
Cryofixation of biological specimens is a thermophysical means of tissue preservation, and as such is endowed with several advantages over conventional chemical fixation. Diffusible ions, as well as molecular components, are not subject to the shifting and extractive effects (Somlyo et al., 1985; Zierold, 1991) which are an inevitable consequence of placing tissue in aqueous chemical fixation media. Immobilization by rapid cooling permits ultrastructural morphology to be preserved close to the nature state (van Harreveld & Fikova, 1975; Dubochet et al., 1983; Müller & Moor, 1984; Studer et al., 1992), and affords a means by which rapid physiological processes may be arrested and characterized at a precise point in time (Heuser et al., 1979; Knoll & Brdicka, 1983). These ideal results can only be realized when cryoimmobilization is optimal, and this entails true vitrification. Solidification of tissue water molecules must occur such that their amorphous liquid nature is retained, and the distortive influences attributable to ice crystal formation and growth are avoided (Dubochet et al., 1988).

A number of rapid cryofixation methods have been developed including spray-freezing (Bachmann & Schmidt, 1971), propane jet freezing (Müller et al., 1980), plunge freezing (Costello & Corless, 1978; Handley et al., 1981) and impact freezing (van Harreveld & Crowell, 1964; Heuser et al., 1979; for review see Sitte et al., 1987). However, the high cooling rates necessary to avoid or at least minimize ice crystal formation are achieved by these means only in very thin samples, of the order of a few micrometres thick. In bulk biological specimens, adequate cryofixation, and in some cases even vitrification, occurs solely in a very thin superficial layer (c. 10 μm, as shown by Richter, 1992).
High-pressure freezing represents the only method by which larger tissue regions may be cryofixed down to a depth of tens or hundreds of micrometres without prior treatment with cryoprotectants (Riehle, 1968; Riehle & Höchli, 1973; Müller & Moor, 1984; Moor, 1987; Studer et al., 1989). A first quantitative approach with aqueous solutions of carbohydrates at different concentrations has shown that vitrification depth in specimens can be increased 10-fold by applying high pressure (c. 2045 bar) during cooling compared to vitrification at ambient pressure (Sartori et al., 1993).

High-pressure freezing was first implemented by Moor and Riehle in the late 1960s (Riehle, 1968; Moor, 1980; for review see Studer et al., 1989), and it was believed that this technique would adequately cryofix biological specimens up to 500–600 μm in thickness. At this time, however, it was not possible to verify directly that true vitrification actually took place; indirect evidence was afforded by morphological inspection of tissue samples after freeze-etching or freeze-substitution; vitrification was assumed to have occurred if segregation patterns, indicative of ice crystal formation and growth, were absent. On the basis of these results, it was claimed that cooling rates of several hundred degrees kelvin per second (theoretically present at the centre of 600-μm-thick samples) were sufficient to vitrify biological specimens under high-pressure conditions (Moor, 1987). The technology for assessing vitrification in biological tissues by electron diffraction was introduced in the early 1980s (McDowell et al., 1982; Chang et al., 1983) but, in the case of high-pressure frozen sample, thus far has only been implemented in 40-μm-thick nematodes entrapped in 200-μm-thick cellulose tubes (Hohenberg et al., 1994), in 200-μm-thick apple leaf discs (Michel et al., 1991), in liver samples (Richter, 1992, 1994) and by using aqueous carbohydrate solutions (Sartori et al., 1993).

Theoretical considerations about cooling rates, freezing times and temperature distributions in samples have been discussed by several authors (Riehle & Höchli, 1973; Kopstad & Elgsaeter, 1982; Jones, 1984; Bald, 1985; Zusadzinski, 1988) each assuming slightly different boundary conditions and being interested in answering different questions. The mathematical basis for these theoretical predictions is contained in the extensive work of Carslaw & Jaeger (1959). We used similar methods in this study to estimate the dependence of achieved cooling rates within specimens as a function of specimen thickness and applied surface cooling rates, since these two parameters may be experimentally controlled.

Materials and methods

Modification of the high-pressure freezing apparatus

In order to increase cooling rates and optimize the coordination of pressure rise and temperature drop, the design of the pressure chamber of the high-pressure freezing apparatus Leica EM HPF was improved. To increase cooling rates the following three measures were taken: (1) the exhaust outlet diameter was enlarged to 1 mm; (2) the volume of liquid nitrogen to be ejected through the pressure chamber was increased to 165 ml; and (3) the pressure chamber volume was reduced by 30%. With these modifications, the coolant speed at the specimen surface attained ≈ 90 m/s. The pressure plateau of ≈ 2045 bar was maintained for about 300 ms.

To achieve optimal pressure-rise/temperature-drop coordination, the pressure chamber was initially filled with ethanol (at 303 K). The pressure chamber outlet was controlled by the addition of an exhaust locking valve, regulated by a mechanical spring mechanism. The valve opens as soon as the pressure reaches the preset value in the chamber. By this mechanism, cooling is only initiated once the preset pressure is achieved within the chamber, opening the valve to allow ejection of the ethanol and the introduction of liquid nitrogen flow.

Tissue preparation

Young adult (10–14 months) bovine humeral head articular cartilage, obtained from a local abattoir, was processed within 12–14 h post exitus (joints being kept at 277 K prior to use). Continuing cell vitality was, as part of another study, confirmed by $^{35}$S-sulphate incorporation in explanted cultured cartilage discs (data not shown). Autoradiography of such specimens showed that the vast majority of chondrocytes still actively incorporated $^{35}$S-sulphate. Cylinders, spanning the entire thickness of articular cartilage, including a layer of subchondral bone, were produced with a 4-mm-diameter hollow myelotomy drill (Institut Straumann EG, Waldenburg, Switzerland). The subchondral bone part was cut off and the remaining cartilage cylinder dissected longitudinally into two halves. A rapid bonding adhesive (Aron Alpha, Borden Inc., U.S.A.) was used to glue one cylinder half with its flat side onto the specimen holder of an OTS-3000-03 Vibratome (Baltec, Balzers, Liechtenstein). Tissue slices, 150 μm in thickness, were cut perpendicular to the joint surface in a bath of 1-hexadecene (Fluka, Buchs, Switzerland) with the vibratome. Discs of 1-7 mm diameter were punched out by use of a precision stainless-steel punch (Grieshaber, Schaffhausen, Switzerland). The cartilage discs were then sandwiched in a specimen holder, consisting of a stainless-steel spacer ring (inner diameter 2 mm; outer diameter 3 mm; thickness according to tissue slice thickness, i.e. 150 μm) and subsequently covered on each side with an aluminium platelet (diameter 3 mm, thickness 200 μm). Remaining small spaces between the biological samples and the metal platelets were still filled with 1-hexadecene (Struder

et al., 1989). The specimen sandwiches were then placed into the 600-μm-deep cavity of the specimen holder (to compensate for the cavity depth, an additional 50-μm-thick spacer ring was placed on top of the specimen sandwich).

Cryofixation protocols
Prior to freezing the samples, optimal functioning of the Leica EM HPF high-pressure freezer was verified by inserting a soldered chromel/alumel thermocouple into the pressure chamber, in place of the biological sample. One freezing cycle was executed and the temperature and the pressure course (measured by a piezoelectric sensor: Burster, Präzisionswerkzeuge, Gernsbach, Germany) were simultaneously recorded using an oscilloscope and printer (Hameg GmbH, Frankfurt, Germany). In order to cryofix tissue discs, the aluminium specimen sandwich was loaded into the high-pressure freezer sample holder and frozen under high-pressure (2045 bar) conditions. Following termination of this step, frozen tissue specimens were immediately placed into liquid-nitrogen (LN₂)-filled containers for storage.

Freeze-substitution
Freeze-substitution of samples was performed in a LN₂-cooled cryostat. The frozen tissue water was substituted by anhydrous acetone, containing 2% osmium tetroxide (van Harreveld & Crowell, 1964), in three stages: 17 h at 183 K, 12 h at 213 K and 12 h at 243 K. Thereafter, the specimens were warmed to 273 K, kept at this temperature for 1 h, then washed three times in anhydrous acetone and embedded stepwise in Epon 812 (30, 70, 100% resin). The infiltration times chosen were 3 h for the first two embedding steps and 3 days for the final resin concentration. Polymerization was carried out with fresh resin at 333 K for 5 days.

Microtomy and microscopy
Thin sections of freeze-substituted samples were cut on a Reichert Ultracut E microtome, equipped with a diamond knife (45° knife; Diatome, Biel, Switzerland). The sections (60–80 nm in thickness) were stained for 7 min with uranyl acetate and lead citrate (Reynolds, 1963). Electron microscopic examination was carried out in a Hitachi H-7100 electron microscope at an acceleration voltage of 75 kV.

Cryosectioning
Frozen cartilage discs were removed from the sandwich in the FC4E-cryo-chamber of a Reichert Ultracut E microtome cooled to 103 K and mechanically fixed in a self-made holder as described earlier (Michel et al., 1991). The frozen cartilage tissue was finely trimmed (with a diamond trimming knife) and then cryosectioned (using a 45° diamond knife; Diatome). Cryosections were transferred to 400-mesh carbon-coated copper grids as described by Michel et al. (1992). The grids were stored under LN₂ until examined in the electron microscope.

Cryomicroscopy
Cryosections were examined at an acceleration voltage of 100 kV in a Hitachi H-7100 transmission electron microscope on a Gatan cryoholder (Gatan Inc., Warrendale, PA, U.S.A.) at a temperature of approximately 103 K. Micrographs were taken at a primary magnification of 10 000× using the low-dose device of the electron microscope. The estimated electron dose was 1500–3000 e/μm² (applied to the region of interest before and during exposure). Focusing was done using the wobbler (no visible movement), setting a defocus of 2–3 μm. Electron diffraction was performed with a camera length of 0.4 m. Image recording was done on Agfa Scientia 23D 56 plates. Brightness was adjusted in order to achieve exposure times of 2 s. The plates were developed for 5 min in Agfa Gevatoine G5c (Agfa-Gevaert AG, Leverkusen, Germany) at 293 K.

Measurement of tissue water content and proteoglycan concentration
From each joint of four individuals, 10 4-mm cartilage—bone cylinders were collected. The cartilage layer was then sliced into three discs of equal thickness (according to the variation in full articular cartilage thickness, these discs were 200–300 μm thick). The top discs corresponded therefore roughly to the tangential and transitional zone, the ones in the middle to the upper radial zone and the ones close to the bone to the lower radial zone (see Fig. 5). The water content of the discs was determined by weighing (Mettler H311; Mettler Instrumente AG, Greifensee, Switzerland) immediately after slicing (fully hydrated) and following complete freeze-drying. Proteoglycans were extracted from the tissue slices with 4 M guanidinium chloride and their concentration measured by the dimethyl-methyleneblue (DMB) method (Chandrasekhar et al., 1987). This method is based on the stoichiometric binding of proteoglycans to the DMB dye, as indicated by the ratio of the absorbance at 550 nm and 610 nm.

Calculated temperature and cooling rate profiles
Temperature and cooling rate profiles within biological specimens were estimated as outlined in the Appendix. Estimates were based on the following assumptions: (1) that tissue thermal properties correspond to those of water at 290 K, that they are homogeneous and do not change during cooling; (2) that the surface temperature, being a
function of time on both sides of the specimen during cooling, is represented as a linear decrease of temperature with time between the initial and final temperatures; and (3) that the cylindrical disc geometry is adequately represented by a slice of infinite lateral dimensions. The validity of the first assumption may be appreciated by noting the difference in thermal diffusivity of water at 290 K, crystallized ice and vitrified water (Bachmann & Mayer, 1987). The thermal diffusivity of crystallized ice is c. 10× larger than that of 290 K water, while that of vitrified water may be c. 10× smaller than that of 290 K water. Our estimations of cooling rates therefore will represent a lower limit in the case of crystallization and an upper limit in the case of vitrification. The validity of the second assumption was ascertained by direct measurements of the temperature vs. time during cooling (Fig. 1). The accuracy of these measurements is evaluated in the discussion.

Results

Performance of the high-pressure freezing apparatus

Measurements of cooling rates using a chromel/alumel thermocouple (c. 50-μm-diameter wires; Fig. 1) revealed that between 303 and 173 K a cooling rate of about 10 000 K/s (Fig. 1a; thick line) was achieved using the modified Leica EM HPP high-pressure freezing apparatus. The pressure vs. time curve (Fig. 1a; thick line) demonstrated that the optimal pressure level of approx. 2045 bar is attained within c. 32 ms. The pressure of 2045 bar lasted for c. 300 ms (Fig. 1b; thick line). If the exhaust valve was reduced from a diameter of 1 to 0·65 mm, the cooling rate decreased to c. 5000 K/s (Fig. 1a; thin line), but the pressure was then maintained for c. 700 ms (Fig. 1b; thin line). The measured surface cooling rate is, thus, dependent on coolant flow velocity. Taking into account the time duration of maximal pressure, the coolant volume and the pressure chamber volume, we can calculate that the velocities of the coolant close to the specimen surface were c. 90 m/s (using the 1-mm-diameter outlet valve) and c. 35 m/s (using the 0·64-mm-diameter outlet).

Comparison of the pressure and temperature curves reveals that initiation of the cooling process is temporarily very well coordinated with the pressurization: cooling is initiated at the moment when the preset pressure level of c. 2000 bar is attained (Fig. 1a).

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Figs. 2–4. Characterization of the central area of three different zones of high-pressure frozen bovine humeral head articular cartilage (Fig. 2 tangential/transitional zones; Fig. 3 upper radial zone; Fig. 4 lower radial zone). In each case (a) represent freeze-substituted samples of extracellular matrix and show collagen fibrils becoming thicker with increasing distance from the articular surface. The interfibrillar space consists mainly of proteoglycans and appears segregated in Fig. 2(a), but not segregated (rough granular appearance) in Fig. 3(a) and of a uniform line and dense granular appearance in Fig. 4(a). The intensity of the cross-banded staining pattern of collagen varied at different locations along a fibril; this seems to depend on where the fibril is cut (centre of periphery of a single fibril). In all cases (b) represent frozen hydrated sections of the same zones. The morphology in the frozen hydrated sections, although impaired in Fig. 2(b) by the section quality, is similar to that of freeze-substituted samples. Note Bragg reflections (black dots) in Figs. 2(b) and 3(b). The insets show the corresponding electron diffraction patterns. It is only the lower radial zone that shows diffuse rings. This zone is therefore fully vitrified (Fig. 4b), whereas in the upper zones (Fig. 2b and 3b), ice crystal formation and growth occurred. Scale bars = 0·5 μm.
Appearance of cartilage matrix

The extracellular matrix of bovine articular cartilage, shown in Figs. 2–4, consists of collagen fibrils, thin in the tangential and transitional zones (Fig. 2) and increasingly thicker fibrils in the radial zones (Fig. 3: upper radial zone; Fig. 4: lower radial zone). The main macromolecular component of the interfibrillar space in the cartilage matrix is proteoglycan (aggrecan, for review see Helnegard & Oldberg, 1989).

In the following, only data from central specimen areas will be presented. Results from peripheral parts closer to the cooled surfaces will not be described here, but are summarized in Fig. 5. In the superficial/tangential zones of freeze-substituted, 150-μm-thick tissue sections, the structural appearance was characterized by thin fibrils and a fine network in the remaining space (Fig. 2a). The frozen hydrated ultrathin section (see Fig. 2b) revealed fibrils surrounded by an uneven proteoglycan moiety; the various black dots are Bragg reflections that are caused by ice crystals present in the section. These findings in the superficial/tangential layer indicate inadequate cryofixation accompanied by damage due to ice crystal formation.

In the upper and lower radial zones the collagen fibrils in freeze-substituted samples are surrounded by proteoglycans having a granular or an almost homogeneous appearance, respectively (Figs. 3a and 4a). In the radial zones, a network-like appearance of the proteoglycan-rich interfibrillar space is not present. In the corresponding cryosections, a network-like appearance in the interfibrillar space is not present either (Figs. 3b and 4b); here, the collagen fibrils are embedded in a more or less uniformly stained background. However, a subtle difference present between the upper and lower radial zones upon examination of the frozen hydrated sections shows that the former (Fig. 3b) exhibits Bragg reflections, whereas the latter (Fig. 4b) does not. This indicates that the lower radial zone is vitrified while the upper radial zone is not.

The state of water in high-pressure frozen samples as a function of tissue water content and proteoglycan concentration

Electron diffraction of frozen hydrated ultrathin sections enables us to determine the state of the water in cryofixed aqueous samples. A broad ring diffraction pattern indicates vitrification, whereas distinct spots or sharp rings are characteristic of a crystalline state of water.

The inserts of Figs. 2(b)–4(b) show electron diffraction patterns of frozen hydrated sections. The sections were taken in the centre of 150-μm-thick samples in each of the three layers (tangential–transitional zone, upper radial zone, lower radial zone). The centres of the two upper layers are crystalline as shown by the distinct spots of the diffraction patterns (insets to Figs. 2b and 3b). The lower radial zone, however, is fully vitrified (inset to Fig. 4b).

It is reasonable to assume that the cooling rate in the centre in the three layers of the sample is similar, and that, in addition to the cooling rate, successful vitrification is dependent on the water content and the solute concentration of an aqueous sample. Our results are consistent with this dependence, considering the measurements of water content and proteoglycan concentration in the cartilage layers. The tangential–transitional zone and the upper radial zone were found to have a water content of 75–80% and a proteoglycan concentration of 0.5–2% and 2–4%, respectively, and are both cryofixed in a crystalline
Table 1. Water content and proteoglycan concentration in the different zones of bovine humeral articular cartilage. The water content determined within each of the different zones was found to have a very low variance (n = 4 measurements per zone). The proteoglycan concentration increases almost linearly from the articular surface to the bone.

<table>
<thead>
<tr>
<th>Zone</th>
<th>Water content (w/w)</th>
<th>Proteoglycan Content (w/w)</th>
</tr>
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<tbody>
<tr>
<td>Tangential and transitional zone</td>
<td>80%</td>
<td>0.5–2%</td>
</tr>
<tr>
<td>Upper radial zone</td>
<td>76%</td>
<td>2–4%</td>
</tr>
<tr>
<td>Lower radial zone</td>
<td>65%</td>
<td>4–8%</td>
</tr>
</tbody>
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state in the centre of a 150-μm-thick sample disc. The lower radial zone, however, which was found to be fully vitrified at this depth, contains c. 65% water and the proteoglycan concentration was 4–8%. These results are summarized in Fig. 5 and Table 1.

Adequate freezing vs. vitrification

Distinct parts of the samples were thus found to be cryofixed in either a vitrified or a crystalline state (insets to Figs. 2b, 3b and 4b). Those parts of the samples which are vitrified always showed excellent structural preservation after freeze-substitution, and would be judged adequately frozen by most investigators (Fig. 4a). However, the term ‘adequately frozen’ is not well defined and its use is very subjective, i.e. it is dependent on the judgement of the experimentors. This is illustrated by Figs. 2(a), 3(a) and 4(a). Many investigators would describe Fig. 2(a) as ‘adequately frozen’, but comparing all aspects of Figs. 2–4, it is evident that the fine network between the collagen fibrils in Fig. 2(a) is a segregation artefact due to ice crystal growth during cryofixation. The structural preservation optimal in the lower radial zone becomes generally worse in regions closer to the articular surface. This inhomogeneity in quality of structural preservation is due to a gradient in the size of ice crystal ramifications, being the largest near the articular surface, becoming progressively smaller in deeper zones until crystals are essentially absent in the lower radial zone. In conclusion, we are not able definitively to evaluate the (previous) state of water in cryofixed and freeze-substituted biological samples on purely morphological criteria.

Calculated temperature and cooling rate profiles

Theoretically calculated temperatures and cooling rates within specimens are shown in Figs. 6–8. The results portray a very strong dependence of these parameters on specimen thickness. This is typical for any diffusion process, where the time for diffusion over a given distance is proportional to the distance squared. For a 600-μm-thick aqueous sample, the maximal cooling rates obtainable in areas deeper than 80 μm from the surface plane are independent of the cooling rate acting along the tissue surface planes, i.e. are the same if the surface cooling rate is 5000 K/s, 10 000 K/s, 20 000 K/s or infinitely high. This indicates that the attainment of surface cooling rates in excess of 5000 K/s will only improve internal cooling rates within a superficial layer of c. 80 μm thickness (Fig. 6b). Hence, no technical improvement of the high-pressure freezing machine, such that it would operate with higher specimen surface cooling rates (above c. 10 000 K/s), would significantly increase the cooling rate in the centre of a 600-μm-thick sample.

This situation changes considerably for thinner aqueous layers (Fig. 7b). An operating surface cooling rate of 5000 K/s at the surface of a 200-μm-thick sample will yield a cooling rate in the specimen centre of c. 3000 K/s. A surface cooling rate of 10 000 K/s, however, results in a cooling rate of about c. 4500 K/s in the specimen centre. Further increases in surface cooling rates (>10 000 K/s) appear, at least theoretically, incapable of increasing the maximal obtainable cooling rate in the specimen centre (Fig. 7b). In an even thinner specimen of 100 μm thickness, a maximal cooling rate of about 17 000 K/s (and potentially optimal cryo-immobilization) could be obtained in its centre, provided that a surface cooling rate of 40 000–50 000 K/s is operating (Fig. 8b). Further increases in surface cooling rates (up to infinitely high rates) would not result in further cooling rate improvements in the specimen centre. However, surface cooling rate variations below 20 000 K/s do significantly influence the central plane cooling rate. Increasing the cooling rate at the surface of the 100-μm-thick sample from 5000 to 10 000 K/s therefore enhances the central plane cooling rate by a factor of 1·7 (i.e. 4500 vs. 7500 K/s). Taken together these results suggest that technical improvements designed to increase surface cooling rates will only be advantageous if the specimen preparation methodology used provides very thin samples (c. 100 μm thick).

A theoretical cooling rate of some thousand kelvin per second is obviously needed to achieve vitrification in the centre of a 150-μm-thick cartilage tissue slice. At ambient pressure, ≈ 10-μm-thick superficial tissue layers (e.g. liver tissue) can be vitrified (Richter, 1992). The theoretical cooling rate at this depth, when using plunging or slamming freezing methods, is of the order of some 100 000 K/s (calculations not shown). The critical cooling rate to achieve vitrification is therefore reduced by a factor of ≈100 under high-pressure freezing conditions (c. 2045 bar) compared to ambient pressure freezing conditions.
Figs. 6–8. Theoretically calculated temperature and cooling rate profiles within tissue slices as a function of sample thickness and imposed surface cooling rates. (a) Temperature contours inside slices subjected to 10000 K/s surface cooling from 303 K to 123 K. (b) Cooling rates calculated when the temperature reaches 253 K inside slices subjected to surface cooling rates of 5000, 10000, 20000, 40000 and infinite K/s. The Leica EM HPF currently achieved c. 10000 K/s.
Since the cooling rate is proportional to the distance squared, this result is consistent with a 10-fold increase in the vitrification depth under high-pressure conditions (as reported by Sartori et al., 1993).

The theoretically calculated temperature distributions within samples (Figs. 6a–8a) suggest that the central plane of a 600-μm-thick sample (Fig. 6a) remains above 253 K for c. 150 ms. The centre of the specimen is therefore exposed to high-pressure conditions for c. 200 ms, before being solidified. The time duration of exposure to high pressure prior to solidification can be reduced only in thinner samples. With a 200-μm-thick specimen, a temperature of 253 K in the specimen centre is achieved within c. 25 ms, and 173 K is obtained after about 60 ms (Fig. 7a). In the case of a 100-μm-thick slab, cooling of the whole sample to 173 K can be accomplished in as little as 20 ms (Fig. 8a).

Discussion

Significance of cooling rate measurements

Cooling rate measurements recorded with a thermocouple are dependent on a number of parameters of the thermocouple, such as its mass density, heat conductivity, heat capacity and its size and geometry. The thermocouple used for our measurements is brazed in a way that the resulting brass tip can be milled to a thickness of c. 400 μm. The thermal diffusivity (see Appendix) of brass is c. 1000× higher than that of water. The small size of the tip and its high thermal diffusivity should allow us to measure cooling rates which are similar to the actual surface cooling rates.

The measurements (Fig. 1) demonstrate that a higher coolant flow results in a higher measured cooling rate. This may prompt the suggestion that enhanced coolant flow increases the measured cooling rate by a more efficient removal of heat from the thermocouple. However, when analysed in detail the cooling process in the high-pressure freezing chamber, this explanation may not be the main reason for the improved measured cooling rates. Before the cooling process is initiated, warm ethanol (303 K) surrounds the specimen. The ethanol must then be substituted by the cold pressurized nitrogen (123 K). Expulsion of the warm ethanol by the cold LN₂ most probably leads to an ethanol–nitrogen mixture that becomes colder and colder. The pressurized LN₂ around the specimen will finally reach 123 K. Cooling may therefore largely be dependent on the expulsion time of the ethanol, and thereby dependent on the coolant flow velocity. This process is, in our opinion, the principal factor determining the cooling rate measured by the thermocouple. We assume also, based on the favourable thermal properties of the thermocouple, that this measured cooling rate reflects closely the effective surface cooling rate operating on a sample.

Influence of water content and solute concentration on cryoimmobilization

Most observations support the fact that vitrification is facilitated if samples have a low water content and a high solute concentration (Riehle & Höchli, 1973; Sartori et al., 1993). Our results obtained with cartilage tissue confirm these findings and support the observations of Duboche et al. (1991).

The cartilage layer which is entirely vitrified throughout its full 150 μm thickness (Fig. 4a) has a water content of ≈ 65% and a proteoglycan concentration of 4–8% (Table 1). Water content may be the deciding factor in the outcome, i.e. if a sample is ultimately vitrified or crystalline. The solute concentration, on the other hand, appears to determine principally the extent to which segregation takes place. Adequate freezing, for example, in the absence of vitrification, can be achieved in the central plane of samples from the upper radial zone, where water content is high (75–80%), but where also the proteoglycan concentration is relatively high (2–4%). Most probably due to the relatively high solute concentration, only very fine ramifications of the ice crystals are formed, so fine in fact that they are hardly detectable morphologically after freeze-substitution. As soon as the proteoglycan concentration is lower (although the water content does not change significantly), i.e. less than 2%, more severe segregation patterns become visible. Due to the low cellularity of mature bovine articular cartilage, we were not able to observe enough cell profiles to determine definitely the state of water in chondrocytes of different layers (the few observed cells of the lower radial zone were vitrified). Our observations on freeze-substituted specimens, however, demonstrate that structural preservation can be very different in adjacent cells and even in compartments of the same cell. The quality of freezing thus seems to be dependent on additional factors, such as the physiological state (potentially transcriptional and/or translational activity), metabolic activity, etc. Moreover, it was previously found, in cryofixed biological samples, that vitrified and crystalline states of water can coexist at topographical sites very close to each other (Duboche et al., 1988; Hohenberg et al., 1994; Richter, 1994).

Limits and potential of high-pressure freezing

Our findings confirm that vitrified samples of cartilage do exhibit an almost optimal ultrastructural preservation (Fig. 4a,b). As previously reported by Keene & McDonald (1993), Studer et al. (1993), Hunziker (1994) and Michel et al. (1994), the extracellular matrix of cartilage tissue does not exhibit a morphologically identifiable network of proteoglycans between collagen fibrils if the samples are adequately frozen.

The term 'adequately frozen' is based on the investigation
of freeze-substituted samples which morphologically do not exhibit obvious segregation patterns (i.e. Fig. 3a). As described in the results, morphological appearance is not a reliable indicator of the state of the water in a sample after cryofixation but vitrified samples are always judged as 'adequately frozen'. The practical difficulty of distinguishing segregation patterns from 'real' native structures is nicely illustrated by the former description of the cartilage extracellular matrix ultrastructure. The previously observed fine filamentous appearance of the interbrillar cartilage proteoglycans of high-pressure frozen samples, judged 'adequately frozen' at the time (Hunziker & Shenk, 1984), cannot be seen in vitrified samples of the present study, or on cryosections (Fig. 4b) or after freeze-substitution (Fig. 4a). This previously observed filamentous proteoglycan appearance is thus now identified as a segregation pattern.

The potential of high-pressure freezing has been overestimated (Moor et al., 1980, 1987; Hunziker et al., 1984; Studer et al., 1989), not with respect to the improved ultrastructural preservation if vitrification does take place, but rather with respect to the sample size that may be vitrified. Most biological samples will probably only be vitrified if the sample thickness does not exceed 200 µm. This statement is supported by the findings of Hohenberg et al. (1994). They showed in 200-µm-thick cellulose capillaries filled with nematodes, the suspension was crystalline, whereas the nematodes were vitrified, mimicking our zone-dependent quality of freezing in cartilage. It is possible that there may still exist special types of biological samples that may be vitrified by relatively low cooling rates (and thus can be thicker than 200 µm), provided they are endowed with very high solute concentrations and unusually low water contents.

Taking together our findings and those of previous investigators, it appears, however, that most specimens require effective internal cooling rates in the range of 10⁻³–10⁻³ K/s to be vitrified under high-pressure conditions. Riehle & Höchli (1973) arrived at a similar lower limit of cooling rates necessary for vitrification (judged by evaluating replicas of freeze-fractured samples showing no obvious segregation patterns). Nevertheless, they concluded that future technical developments could allow the adequate freezing of 100- to 500-µm-thick samples. It was the upper limit of this conclusion that remained subsequently recognized and believed to be correct. Unfortunately not enough attention was given to the limitations of high-pressure freezing, those limitations being the inherent physical properties of the sample, i.e. its thermal properties, size and composition (e.g. water content, solute concentration, etc.).

A concern that is frequently raised with respect to high-pressure freezing is the generation of pressure-induced artefacts. Ding et al. (1992) reported distortion of microfilament bundles during high-pressure freezing. This may occur if relatively long pressure exposures are applied before freezing. For example in the centre of a 600-µm-thick sample the initial pressure rise (about 20–40 ms) is only a small part of the total time of high-pressure exposure before solidification occurs (Fig. 6); this time may be as long as 200 ms. In thinner samples, the exposure time to high pressure before solidification is significantly reduced (e.g. 20 ms for 100-µm-thick samples), again exemplifying the advantages of cooling samples that are as thin as possible.

Our reevaluation of the limitations and the potential of high-pressure freezing may, at first glance, appear somewhat disillusioning. However, it should be emphasized that vitrification does occur at cooling rates 100 times smaller than the ones needed to achieve vitrification at ambient pressure. Furthermore, the parameters used in this study are theoretically optimal for 200-µm samples. According to our calculations, it would only be worth improving the performance of the high-pressure freezing apparatus (e.g. cooling rates ≥ 30 000 K) if samples of ≈ 100 µm were to be used.

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References


Appendix

The temperature distribution in a slice of tissue undergoing rapid cooling was estimated by solving Fourier’s law of heat conduction for a material of thickness 2b in the x-direction and of infinite dimensions in the y–z plane. The temperature, T, as a function of x and time, t satisfies

$$\frac{\partial^2 T(x, t)}{\partial x^2} = \frac{1}{\kappa} \frac{\partial T(x, t)}{\partial t}$$

(1)

where the thermal diffusivity, \(\kappa = K/(C_p\rho)\), \(\kappa\) is taken as a material constant, independent of position and temperature, using values for water at 290 K for the thermal conductivity, \(K = 5.92 \text{ mW/cm/K}\), the heat capacity at constant pressure, \(C_p = 4.2 J/g/K\), and the mass density, \(\rho = 1.0 \text{ g/ml}\). Equation (1) was solved subject to the boundary conditions that the initial temperature of the slice is \(T_0\) and that at time \(t = 0\), both surfaces of the slice, at \(x = \pm b\), are brought immediately to temperature \(T_1\). In this case the cooling rate supplied at the surfaces is infinite since no time is required to change the surface temperature from \(T_0\) to \(T_1\). We also considered the case where a finite time \(t_0\) is required to change the surface temperature from \(T_0\) to \(T_1\). In this case a finite cooling rate of \((T_0 - T_1)/t_0\) is applied to both surfaces of the slice, at \(x = \pm b\). An important parameter for both problems is the characteristic diffusion time, \(\tau = b^2/\kappa\), for heat to diffuse through a distance \(b\). By using the Laplace transformation for the time variable we found the temperature distribution in the case of infinite surface cooling rates to be

$$T(x, t) = T_1 - \frac{2(T_1 - T_0)}{\pi} \sum_{n=0}^{\infty} \frac{(-1)^n}{(n + \frac{1}{2})}$$

$$\times e^{-\left(n + \frac{1}{2}\right)^2 \pi x^2 / \tau} \cos \left(n + \frac{1}{2}\right) \pi x / b.$$  

(2)

For a finite surface cooling rate, and \(t < t_0\), the temperature is

$$T(x, t) = T_0 + \frac{(T_1 - T_0) \tau}{t_0}$$

$$\times \left\{ \frac{t}{\tau} + \left(\frac{x}{b}\right)^2 - 1 + \frac{2}{\pi} \sum_{n=0}^{\infty} \frac{(-1)^n}{(n + \frac{1}{2})^2} \right\}$$

$$\times e^{-\left(n + \frac{1}{2}\right)^2 \pi x^2 / \tau} \cos \left(n + \frac{1}{2}\right) \pi x / b.$$  

(3)

For a finite surface cooling rate, and \(t > t_0\), the temperature is

$$T(x, t) = T_1 + \frac{2(T_1 - T_0)}{\pi^3 t_0} \sum_{n=0}^{\infty} \frac{(-1)^n}{(n + \frac{1}{2})^3}$$

$$\times e^{-\left(n + \frac{1}{2}\right)^2 \pi x^2 / \tau} \left[ 1 - e^{\left(n + \frac{1}{2}\right)^2 \pi x^2 / \tau} \right]$$

$$\times \cos \left(n + \frac{1}{2}\right) \pi x / b.$$  

(4)

By differentiating Eqs. (2)–(4) with respect to \(t\), the cooling rate within the slice in the case of infinite surface cooling rates is

$$-\frac{\partial T(x, t)}{\partial t} = -\frac{2\pi(T_1 - T_0)}{\tau} \sum_{n=0}^{\infty} \frac{(-1)^n}{(n + \frac{1}{2})}$$

$$\times e^{-\left(n + \frac{1}{2}\right)^2 \pi x^2 / \tau} \cos \left(n + \frac{1}{2}\right) \pi x / b.$$  

(5)

For a finite surface cooling rate, and \(t < t_0\), the cooling rate is

$$-\frac{\partial T(x, t)}{\partial t} = \frac{T_1 - T_0}{t_0} \left\{ 1 - \frac{2}{\pi} \sum_{n=0}^{\infty} \frac{(-1)^n}{(n + \frac{1}{2})} \right\}$$

$$\times e^{-\left(n + \frac{1}{2}\right)^2 \pi x^2 / \tau} \cos \left(n + \frac{1}{2}\right) \pi x / b.$$  

(6)

For a finite surface cooling rate, and \(t > t_0\), the cooling rate is

$$-\frac{\partial T(x, t)}{\partial t} = \frac{2(T_1 - T_0)}{\pi t_0} \sum_{n=0}^{\infty} \frac{(-1)^n}{(n + \frac{1}{2})^3}$$

$$\times e^{-\left(n + \frac{1}{2}\right)^2 \pi x^2 / \tau} \left[ 1 - e^{\left(n + \frac{1}{2}\right)^2 \pi x^2 / \tau} \right]$$

$$\times \cos \left(n + \frac{1}{2}\right) \pi x / b.$$  

(7)

Equation (1) is equivalent to eq. (2) of Carslaw & Jaeger (1959, p. 100), and Eq. (2) is equivalent to eq. (4) of Carslaw & Jaeger (1959, p. 104). Numerical values from the infinite series were obtained by estimating the relative error of neglecting succeeding terms and specifying that it be smaller than 10⁻⁶. A program for making these calculations, in the form of a Mathematica package, is available from M. D. Buschmann, Institute of Biomedical Engineering, Ecole Polytechnique, University of Montreal, Montreal, Quebec, Canada.