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Freezing in Sealed Capillaries for Preparation of Frozen Hydrated Sections

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Abstract

We have investigated the freezing of specimens in a confined volume for preparation of vitreous samples for cryosectioning. With 15% dextran as a cryoprotectant, a sample sealed in a copper tube begins to freeze into crystalline ice when plunged into liquid ethane. Crystallization rapidly causes an increase in the pressure to the point that much of the sample freezes in a vitreous state. We used synchrotron X-ray diffraction of samples frozen with various amounts of dextran to characterize the ice phases and crystal orientation, providing insights on the freezing process. We have characterized cryosections obtained from these samples to explore the optimum amount of cryoprotectant. Images of cryosectioned bacteria frozen with various levels of cryoprotectant illustrate effects of cryoprotectant concentration.

Keywords

High pressure freezing; self pressure freezing; cryosection; cryomicroscopy; amorphous ice; SPRF; isochoric subcooling; isochoric freezing

Introduction

Two approaches are commonly used in preparing biological objects in their native, hydrated state for study by electron microscopy: cryo-plunging of the material in a thin layer of water (Adrian *et al.*, 1984), and high pressure freezing (HPF) followed by cryosectioning (Michel *et al.*, 1991). While the first method is the one most commonly used, especially for isolated macromolecular complexes, the second one is attracting more attention as technical problems that have limited its usefulness are overcome. An important advantage of HPF and cryosectioning is the ability to obtain sufficiently thin sections when the biological object (for example, tissue or microbial cells) is too thick to be examined by electron microscopy. Among the disadvantages of the method are possible deformation of the sample during cryosectioning, dependence on cryoprotectant, and need for expensive equipment for freezing. Recently there has been substantial effort devoted to optimizing cryosectioning, demonstrating that stress-induced deformations may be reduced by proper choice of parameters (Al-Amoudi *et al.*, 2005). In this work we address the other two problems. We

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demonstrate cryosections obtained without use of specialized freezing equipment and investigate the possibility of reducing the amount of cryoprotectant for high pressure assisted freezing.

Historically, several methods for rapid freezing have been suggested to preserve large volumes of biological materials, including slamming, plunging in cryogen and jet freezing (Bald, 1985; Escaig, 1982). Each of these methods produces amorphous material only in a thin surface layer (Bald, 1985), which has greatly limited their application. Today high pressure assisted freezing has almost completely replaced these other methods due to its ability to amorphize the sample much more deeply (Satori *et al.*, 1993). The conventional HPF approach employs special equipment in order to build high pressure (about 200 MPa) in the sample in a very short time before freezing (Dahl & Staehelin, 1989). High pressure depresses the crystallization point, which slows the formation of crystalline ice, improving vitrification. Freezing of the sample happens at constant elevated pressure and may be referred to as isobaric. Isochoric freezing has been suggested as an alternative high pressure assisted method, taking advantage of the pressure buildup when water crystallizes in a confined volume (Rubinsky *et al.*, 2005; Szobota & Rubinsky, 2006; Preciado & Rubinsky, 2010). While initially aimed at freezing macroscopic samples, isochoric freezing fits perfectly to the preparation of small electron microscopy samples. Leunissen and Yi developed the “self-pressurized rapid freezing” (SPRF) method, in which samples are frozen in standard HPF capillaries sealed at both ends (Leunissen & Yi, 2009). The fact that pressure buildup in the tube results in high quality cryofixation of biological samples was demonstrated by freeze substitution. However the state of the frozen medium in this work was not evaluated and applicability of the method to preparation of frozen hydrated sections remained unclear.

The essence of isochoric freezing is using the increase in volume as liquid water freezes into crystalline ice to build pressure inside of a confined volume (Szobota & Rubinsky, 2006; Leunissen & Yi, 2009). The density of hexagonal ice is 0.92 g/cm³ while the density of water is 1 g/cm³ (Khan, 2000). Transformation of part of the water into low density crystalline ice inside a constrained volume may cause strong pressure buildup that promotes amorphization of the rest of the sample. Thus the part of the sample that cools first forms crystalline ice, while deeper parts may be well cryopreserved. While both isochoric and conventional HPF employ freezing under high pressure we reserve the term HPF for isobaric high pressure freezing.

Using SPRF for suspensions of cells in growth medium we found that the high water content medium always crystallizes throughout and the sample cannot be successfully cryosectioned. The situation for cryosectioning is different from freeze substitution where crystallization of the medium may not be a problem, as shown by the quality of preservation in the original SPRF report (Leunissen & Yi, 2009). To obtain samples suitable for cryosectioning we investigated the use of a cryoprotectant during freezing. We demonstrate that amorphization of the sample is possible with a lower amount of cryoprotectant than required for the conventional HPF, which may be a significant advantage for some types of specimens. Although cryoprotectants are commonly used in preparing samples for cryosectioning, they might introduce problems including unfavorable osmotic effects and

contrast matching in the images. Additionally the high viscosity and density of cryoprotectant can complicate sample preparation. Reducing the amount of cryoprotectant may thus have multiple benefits. Therefore suggesting a way to reduce the requirement for cryoprotectant is an important achievement of this work.

Experimental methods

In this work we used copper capillary tubes designed for use in high pressure freezing machines. Tubes 16 mm in length were obtained from Leica Microsystems (Leica-Microsystems, Vienna, Austria, part number 16706871). The outside diameter of the tubes was measured to be 600 μm . The internal radius of the tubes was approximately equal to the thickness of the tube wall, i.e. 150 μm . Prior to use, the oxide layer was cleaned from the internal surface of the tube by flushing for 10 minutes with a continuous flow of acetic acid (99.7%) and careful rinsing with deionized water. Removal of the oxide is important to ensure high quality cold welding to completely seal the tube. Tubes were filled with medium and sealed by crimping the ends with flat jaw pliers.

After sealing we employ a procedure that we call prestraining the tube. In a series of steps we continue to crimp the ends of the tube (about a 0.5 mm in each step to about 40 % of the length of the tube) in order to squeeze the liquid into the central part of the tube. The main purpose of this procedure is to increase the pressure inside the tube so that the tube will be strained and cold worked in order to increase its yield strength. The use of this procedure has produced a dramatic effect on the freezing quality.

While prestraining the tube is an important step to obtain high quality ice, applying high pressure may adversely affect some biological materials. In order to avoid this problem we modified the procedure. We first filled the tube with pure water and prestrained the tube in order to cold work it. Then we cut the sealed ends off the tube with a razor blade, filled the tube with the sample and sealed it again by crimping each end just once. We observed that this procedure has the same effect on the freezing quality as regular prestraining.

Sealed tubes were rapidly plunged into liquid ethane using fine tipped tweezers. The temperature of the ethane was kept near the freezing point. Care was taken to insure that tubes were inserted into the cryogen horizontally so the ends were cooled simultaneously. Frozen tubes were transferred to liquid nitrogen where they were stored. For sectioning, the flattened ends of the tubes were cut off with a specially designed guillotine operated in liquid nitrogen, and the tube was mounted in the microtome. We used a glass knife to trim away about a millimeter of the tube so deformation from the guillotine would not influence the ice quality in the examined area. A diamond trimming knife was used to trim away copper and crystalline ice as necessary.

For comparison of self pressure and high pressure freezing techniques, high pressure frozen samples were prepared in the same type of capillary tubes. A Leica EM PACT2 High Pressure Freezer (Leica-Microsystems, Vienna, Austria) was operated in the manual mode to freeze the samples (Studer *et al.*, 2001). Tubes were cut and trimmed similarly to the sealed tubes.

To obtain cryosections, a square area of the ice was exposed by trimming the face of the tube from four sides. The sectioning was done with a 35 degree diamond knife at a temperature of -160°C. Sections were arranged in long ribbons and transferred to lacy carbon support films. Electron microscopy imaging and diffraction were performed on a Philips CM 200 field emission gun microscope operated at 200 keV. Samples were cryo-transferred using a Gatan cryo-transfer stage. Images and diffraction patterns were recorded on a Gatan UltraScan 1000 CCD camera.

For the X-ray diffraction experiments we trimmed the face of the tube from two sides with the trimming knife oriented at 45 degrees to the cylinder face to produce a wedge. The X-ray data were collected from the thin part of the wedge on the SYBILS beamline at the Lawrence Berkeley National Laboratory's Advanced Light Source. The beam was set to a diameter of 30 or 10 μm and directed perpendicular to the axis of the tube and the thin wedge of the sample.

Aqueous dextran solutions were prepared by dissolving 10, 15, 20 or 30 weight percent dextran in deionized water. *Caulobacter crescentus*, wild type strain CB15N, was grown in peptoneyeast extract media to a cell density of $10^8/\text{ml}$. Typically peptone-yeast extract does not significantly affect ice formation due to its low concentration. In our case the samples were centrifuged through or mixed with dextran with average molecular weight 350,000-450,000 (Sigma-Aldrich), greatly decreasing the growth medium concentration, so we can neglect the medium influence on ice formation. The *Caulobacter* culture was pelleted by centrifugation at 1000 xg. With lower dextran concentrations, the pellet was deposited on top of the dextran solution and centrifuged at 1000-16000 xg. Centrifugation through the 20 percent dextran sample required high speed and resulted in many dead cells in the sections. With 30 percent dextran, the sample was obtained simply by mixing the pellet with the concentrated dextran solution.

Results

Freezing quality and diffraction analysis

In order to determine the effects of cryoprotectant with both the self pressure and high pressure freezing methods, we prepared samples of pure water and dextran solutions of various concentrations up to 30 %. The visual appearance of the material as it was sectioned, as well as the ability to obtain useable sections, depend strongly on the cryoprotectant concentration. In several of the samples the appearance varied dramatically across the diameter of the tube. We have performed X-ray scattering experiments on samples trimmed to a thin wedge to characterize the ice at several different locations from the center to the edge of the frozen material, in order to determine whether the ice is crystalline or amorphous.

Sealed capillary freezing of pure water—In our first attempt to prepare vitreous sections we froze tubes filled with pure water. We found that self pressure freezing does not result in vitreous ice that can be cryosectioned, as is also experienced with high pressure freezing. In attempts to section the sample, the ice breaks into a powder rather than staying as a solid section. The surface of the ice block looks opaque and rough, which indicates that

some ice crystals were broken out of the material with the diamond knife. Because the ice we obtained without cryoprotectant was not useful for cryosectioning, we did not perform additional experiments on it.

Sealed capillary freezing of 10 percent dextran solution—The addition of 10 percent dextran appears sufficient to provide enough mechanical flexibility to the material that sections can be cut. Figure 1 shows a cross section of a tube containing 10 % dextran open for cutting and trimmed with the glass knife (Figure 1ia) and trimmed with the diamond knife to a wedge, which allows looking through the ice (Figure 1ib). The surface of the glass knife-trimmed sample looks opaque and similar to the sample without dextran. Looking through the open part of the sample we can see multiple cracks in all parts of the sample. Sometimes these cracks make sectioning difficult but in most cases it is still possible to obtain cryosections that may be easily arranged in ribbons and transferred to the support grid.

We found that X-ray diffraction patterns collected from 10 % dextran samples with the 30 μm beam clearly reflect the polycrystalline structure of the ice (Figure 2a). The crystals are relatively large, producing the grainy structure seen in the diffraction rings due to the limited number of scattering crystals. In higher dextran content samples the sizes of crystals are much smaller so the intensity is more uniformly distributed over the arcs. The diffraction pattern indicates that the ice in this sample has a hexagonal crystal lattice. The three innermost rings in this pattern are of the most interest for us. They arise from spacings of 3.90, 3.67 and 3.44 Å, corresponding to the (100), (002) and (101) planes of hexagonal ice. Previously, calculations (based on hexagonal ice structure with unit cell axes $a = b = 4.516$ Å, $c = 7.354$ Å) reported in (Varshney *et al.*, 2009) resulted in scattering spacings of 3.942, 3.715 and 3.482 Å. These calculations agree with our measurement within one percent; the difference may be due to slight miscalibration of the camera length. We did not observe any preferential orientation of crystals in the 10 % dextran samples.

Sealed capillary freezing of 15 percent dextran solution—The 15 percent dextran samples show very different appearance and mechanical properties. While the surface near the copper tube after glass knife trimming is as rough as the 10 percent sample, in the center of the tube we observe a region with a glossy surface (Figure 1iia). Looking through the trimmed part of the sample (Figure 1iib) we can see that this part does not show any cracks or defects, while the outer part has multiple cracks. The absence of defects makes cryosectioning of this part easier than sectioning of the peripheral part.

X-ray scattering shows that the material in the center of the tube is amorphous. A diffraction pattern obtained from that area with the 30 μm X-ray beam is presented in Figure 2b. The ice in the rest of the sample produces a diffraction pattern that clearly shows crystalline character (Figure 2c). The ice is polycrystalline with sizes of microcrystals so small that the reflections create very uniform arcs. It is very interesting that we can clearly observe the existence of specific orientation of the crystals. This orientation is probably related to the character of sample cooling and its effect on the crystallization process.

More careful examination of the crystalline ice in the 15 percent dextran sample shows variations in the phases and orientation of the crystals which gives some insight on the mechanism of ice formation. We have collected diffraction patterns at several different distances from the center using a 10 μm diameter X-ray beam. We found that the ice near the copper tube does not show any specific orientation. A diffraction pattern from this region is shown in Figure 3a. The integrated intensity as a function of diffraction spacing is shown in Figure 3g. The strong ring at 3.67 $\text{\AA}$ may correspond to the (002) reflection of hexagonal ice. However, the high intensity of this ring is more compatible with the (111) reflection of cubic ice. The weaker ring at 3.90 $\text{\AA}$ may be the (100) reflection of hexagonal ice. The ideal cubic ice structure does not have this reflection. However, X ray diffraction patterns seldom indicate pure cubic ice, but usually include some component of hexagonal ice (Morishige *et al.*, 2009). A very weak ring at 3.44 $\text{\AA}$ that may be the hexagonal lattice (101) reflection can also be observed. When we move the beam 15 μm closer to the center of the tube the diffraction pattern starts to show some preferential orientation (Figure 3b). Now the 3.67 $\text{\AA}$ reflection clearly shows two peaks at an angle about 60 degrees to each other. Reflections at 3.90 $\text{\AA}$ are consistently perpendicular to the reflections at 3.67 $\text{\AA}$ as would be expected for the (002) and (100) planes in hexagonal ice. Reflections at 3.44 $\text{\AA}$ become slightly stronger but remain very blurred. Moving the beam 15 μm closer to the center decreases the angle between the most prominent directions of the 3.67 $\text{\AA}$ reflection to about 45 degrees (Figure 3c). The orientation of the crystals becomes more evident and the intensity of the 3.67 $\text{\AA}$ reflections grows while the 3.44 $\text{\AA}$ reflection remains blurry. Moving the beam still closer to the center continues the same trend. As we see in Figures 3d and 3e the angle between the 3.67 $\text{\AA}$ reflections (and consistently the 3.90 $\text{\AA}$ reflections that are perpendicular to them) decreases eventually to zero. The 3.44 $\text{\AA}$ reflection becomes oriented at about ± 30 degrees to the 3.90 $\text{\AA}$ reflection, as expected for the (101) reflection, and remains blurred. Continuing to move the beam to the center, at some point the crystalline pattern disappears and we see the pattern of high density amorphous ice in its place (Figure 3f).

Sealed capillary freezing of 20 and 30 percent dextran solutions—The 20 percent dextran sample looks very similar to the 15 percent sample. The main difference is in the size of the amorphous area in the center, which increases to fill most of the volume, while the rough ice occupies just a narrow strip along the copper tube wall (Figure 1iiia). Looking through the sample we can see that cracks are concentrated in the periphery (Figure 1iiib), similar to the 15 percent sample. Sectioning such a sample is as easy as the 15 percent dextran sample. X-ray diffraction shows that material in the center is amorphous (Figure 2d) and at the periphery is polycrystalline cubic ice oriented similarly to the ice in the 15 percent dextran sample. Reflections at 3.67 $\text{\AA}$ create an arc and reflections at 3.90 $\text{\AA}$ are oriented perpendicular to the 3.67 $\text{\AA}$ reflections (Figure 2e). The weak reflection at 3.44 $\text{\AA}$ is rotated 30 degrees from the 3.90 $\text{\AA}$ reflection and blurred to the point that no sharp peak is visible. It is difficult to determine whether the crystals are smaller than in the 15 percent dextran sample because in both cases they are significantly smaller than the beam size so that no single reflections are observed.

The 30 percent dextran sample shows homogeneous structure everywhere. After trimming, the surface is glossy and uniform (Figure 1iva). No cracks in the ice are observed (Figure

livb), and we experienced no difficulties with sectioning. The X-ray experiments showed that all ice inside the tube is amorphous (Figure 2f).

Density of amorphous ice in sealed capillary—Careful visual examination of the amorphous ice formed in the center of the 15 and 20% dextran samples reveals that it has a different refractive index than the ice on the periphery, indicating a different density. This effect is easily observed in the optical microscope on the microtome. Figure 4 shows an optical image of a 15 percent dextran sample. One can see the bright region with a regular, round shape in the center of the tube. By tilting the tube we can see that the brightness is due to the different refractive indexes of the crystalline and amorphous ice. In the orientation shown, light reflected from inside the ice block is refracted at the surface of the amorphous ice into the objective of the camera. Because of the different refractive index of the crystalline ice, light is deflected from the crystalline area in a slightly different direction away from the camera objective, so the ice appears much darker. It is interesting that the border between the different ice phases with different refractive indexes appears very sharp and regular. The different refractive indexes of the ices in the sample are an indication of their different densities (Mishima *et al.*, 1984; Browell & Anderson, 1975; Mishima *et al.*, 1985). The density of low density amorphous ice is similar to the density of both cubic and hexagonal crystalline ice while high density amorphous ice is 25 percent more dense, supporting the conclusion that the ice in the center of tube is high density amorphous ice.

Conventional high pressure freezing of 15 and 20 percent dextran solutions—High pressure freezing of a 20 percent dextran solution resulted in a sample consisting of amorphous ice in all parts of the tube, as may be clearly seen from X-ray diffraction patterns of such a sample (Figure 2h). Diffraction patterns collected from different parts of the sample look very similar. However in the optical microscope the ice does not look as uniform as the amorphous ice formed by self pressure freezing (Figure 1va). A few small cracks may be observed (Figure 1vb). These cracks do not affect the quality of sections obtained by cryosectioning, which may be easily performed on such samples.

In contrast to the 20 percent sample, high pressure freezing of a 15 percent dextran solution does not result in amorphous ice in any part of the tube. A typical X-ray diffraction pattern that is shown in Figure 2g indicates cubic ice with small disordered crystallites. We can see the reflection at 3.67 Å forming a sharp ring. Reflections at 3.90 Å and 3.44 Å are blurry to the point that separate peaks are not resolved. Such a pattern can be observed in any part of the sample. Multiple cracks may be seen in the sample in the optical microscope (Figure 1via, 1vib), although sectioning of this sample is possible.

Cryosections

We attempted to cryosection all of the dextran samples that were prepared, in addition to a number of *Caulobacter* samples in various dextran concentrations. The only sample that we found to be not suitable for sectioning was of pure water. Surprisingly, we found that some samples that were clearly seen to be crystalline by X-ray diffraction yielded sections that appeared, by electron diffraction, to be amorphous. A similar observation was made previously (Al-Amoudi *et al.*, 2002) but with a sample containing 17.5 percent dextran. In

our experiments we were able to section even a 10 percent dextran solution. The sections obtained, however, have an appearance different from sections obtained from amorphous ice. Figure 5a shows a bright field TEM image obtained from a sample of *Caulobacter* culture centrifuged through 10 percent dextran. One can see a lot of contrast in the region of the pure ice. Interestingly, this contrast fades with relatively low electron exposure. Figure 5b shows a section after the central part has been irradiated with $20 \text{ e}/\text{\AA}^2$. One can see that in the irradiated part the undesirable contrast from the ice has disappeared. The nature of this contrast is not crystallinity. An electron diffraction pattern obtained with low dose irradiation from a 10 percent dextran section is shown in figure 5c and it clearly proves that the section consists only of amorphous ice. Figure 5d is a diffraction pattern collected from a similar section that was contaminated with small crystals of ice during cryosectioning, providing accurate calibration of the spacings. The contamination is essentially all hexagonal ice. Figure 5e shows the integrated scattering intensity as a function of scattering angle. The peak from the crystalline ice is seen at a lower scattering angle than the amorphous ice peak, indicating that the average spacing is lower in amorphous ice. We have measured the average spacing to be $3.4 \text{ \AA}$ which supports the conclusion that these sections consist of high density amorphous ice (Wright *et al.*, 2006). Thus the contrast observed in the image is primarily due to the rough surface of the sample, and irradiation, causing slight flow of ice, smoothes the surface. However irradiation never makes the section completely uniform, which may be an indication of some phase separation of pure ice and concentrated dextran solution.

Increasing dextran content results in more uniform sections. While some crevasses and knife marks still may be observed, they disappear with irradiation. The higher the dextran content, the more uniform the ice generally appears. However the most dramatic change is observed between 10 percent and 15 percent dextran, corresponding to the change from crystalline to amorphous ice. While both crystalline and amorphous parts of the high dextran content specimen may be cryosectioned, we used only the amorphous part from the center because it does not have cracks or defects.

While increasing dextran content improves the section appearance, the quality of images of bacteria in these sections does not show a corresponding improvement. Figures 6a, 6b, 6c and 6d show typical cryosections of bacteria prepared with 10, 15, 20 and 30 percent dextran content. Comparing the images we can see that sample obtained with 10 percent dextran is strikingly different from the other samples. The S-layer (arrow in image) is clearly visible and has a remarkably granular appearance. None of the higher dextran content samples show nearly as much structure in the S-layer. Magnified parts of the images showing the cell membrane in 10 and 15 percent dextran are shown in Figures 6e and 6f. Wrinkling of the sample prepared with 10 percent dextran affects the appearance of the cell; in higher dextran content samples this effect is much less pronounced. Knife-induced deformations of the membranes are visible in all samples to some degree. For each sample a variety of cells with different deformation levels may be observed. Overall, the appearance of the cells degrades with higher dextran, although it is not clear whether this is a result simply of exposure to the high concentration or cutting artifacts. While we used the same cutting parameters for all samples it is clear that mechanical properties (and thus optimal cutting parameters) of the

samples may be different. More work is necessary to optimize the cutting and to compare the results, as well as to understand the impact of factors such as contrast matching on quality of the images. Removing this ambiguity will show whether the structure of the S-layer is indeed damaged by the high dextran content or masked by its high density. It is clear, though, that the contrast is highest in the 10 percent dextran sample. Profiles of intensity illustrating this effect are presented in supplementary Figure S2.

Discussion

Pressure buildup

We have shown that plunging a sealed capillary into the cryogen results in formation of amorphous ice if the dextran content is properly chosen. The main factor that makes amorphization possible is pressure buildup rather than high cooling speed, as indicated by the fact that no amorphous ice is observed in close proximity to copper where the cooling rate is highest. The pressure increases as a result of the expansion of water as it crystallizes. The maximum pressure reached is determined either by the strength of the copper tube or the value necessary for formation of amorphous ice, which decreases with increasing cryoprotectant. The fraction of the sample that crystallizes depends on the amount of cryoprotectant added.

We next discuss how the pressure relates to the mechanical properties of the tube that holds the sample and show that the pressure will easily reach the yield strength of the tube. The degree of pressure buildup may be estimated based on the properties of ice and the copper tube. The maximum pressure that may be achieved in the copper capillary tube is limited by the strength of copper. For annealed copper, as we believe is the state of the tubes as delivered, the yield strength is about 70 MPa, but this increases to about 200 MPa after work hardening (Davis, 1998). In the tubes that we use the internal radius is approximately equal to the thickness of the tube wall. In this geometry, with equal cross sections of the copper and medium, the pressure inside the tube is equal to the mechanical stress in the copper. Thus the pressure cannot exceed the yield strength. This limitation applies to both high pressure and self pressure freezing. Here we note that when the water starts to freeze near the copper wall it creates a layer of ice that fortifies the tube. However, this layer could not contribute significantly to the pressure resistance of the tube since the toughness of ice is much smaller than that of copper and it is also fractured by multiple cracks in the radial direction.

To estimate the pressure buildup we need to find the combined effect of thermal shrinkage of the copper and expansion of the ice due to crystallization. We assume that the copper tube cools down instantaneously since its thermal conductivity is much higher than conductivity of the sample (copper conductivity is about $401 \text{ W}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$ while it is only $0.6 \text{ W}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$ for water and $2 \text{ W}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$ for ice (Weast, 1988)). The thermal expansion coefficient of copper is $16.5 \mu\text{m}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$ (Weast, 1988) which produces approximately one percent decrease of volume on cooling to liquid nitrogen temperature. The cooling of the water starts from the part that contacts the copper wall and propagates to the center. If we take the case where half of the radius (therefore three quarters of the volume) would become crystalline ice with a density of 0.92 g/ml, the volume increase inside the tube due to crystallization

would be $0.75 \times (1-0.92) = 6\%$. Using half the radius is a useful estimate because it is close to our situation with 15 percent dextran. At this point it is still easy to trim away the crystalline part of sample and use the rest for sectioning. Adding the thermal contraction of copper and expansion of ice we find the total volume of the water and ice in the tube at this point would be 7 percent larger than the internal volume of the copper tube would be without applied stress.

It is straightforward to show that, because water is so incompressible, the pressure required to prevent this expansion is higher than the copper tube can generate. We first consider the case when instant thermal shrinkage of tube is followed by true isochoric freezing, i.e. where the capillary volume stays constant, to estimate the maximum pressure that could be achieved with a very strong tube. The volume compressibility of water is about $5.1 \times 10^{-10} \text{ Pa}^{-1}$ at 0 C and atmospheric pressure. It decreases to $3.9 \times 10^{-10} \text{ Pa}^{-1}$ at 100 MPa (Fine & Millero, 1973). The compressibility of ice at -10 C is $3.7 \times 10^{-11} \text{ Pa}^{-1}$ (Gow & Williamson, 1972) and according to some reports decreases with decreasing temperature (Marion & Jakubowski, 2004). To estimate the minimum pressure in the tube we use the maximum values of compressibility assuming them to be constant. The pressure buildup in the sealed tube would be $P = (\Delta V_I/V_0 + \Delta V_T/V_0)/k$ where $\Delta V_I/V_0$ is the volume change due to ice crystallization, $\Delta V_T/V_0$ is the volume change due to copper thermal contraction and k is the compressibility. It is relatively easy to show that for the case when $\frac{3}{4}$ of the volume is

transformed into ice, the rise in pressure may be expressed as
$$P = \frac{4 \cdot (\Delta V_I/V_0 + \Delta V_T/V_0)}{3 \cdot k_I + k_W}$$
 where k_W is the compressibility of water, k_I is the compressibility of ice and $(\Delta V_I/V_0 + \Delta V_T/V_0)$ is equal to 0.07 as estimated earlier. Putting the values into this equation we find the pressure buildup to be 450 MPa. Previous estimates have shown that pressure 210 MPa may be achieved when 50% of the water crystallizes (Leunissen & Yi, 2009).

This pressure is above the tensile strength of copper, so the tube would show both elastic and plastic deformations. First consider elastic deformation. The modulus of elasticity for copper is $E = 110 \text{ GPa}$ (Davis, 1998) and the yield strength for soft copper is $\sigma = 70 \text{ MPa}$. At the pressure corresponding to the yield stress, elongation is $\epsilon = \sigma/E = 70 \text{ MPa} / 110 \text{ GPa} = 0.63 \times 10^{-3}$. Volume expansion is roughly proportional to the cube of linear expansion. This linear expansion corresponds to volume expansion inside tube of 0.2% which is small enough to neglect compared to the expansion due to crystallization. For cold worked copper the yield strength may be as high as 200 MPa which would correspond to elastic elongation $\epsilon = \sigma/E = 200 \text{ MPa} / 110 \text{ GPa} = 1.8 \times 10^{-3}$ and volume expansion 0.5% which is also negligibly small. Thus only a small amount of elastic deformation will occur before the yield strength is reached and plastic deformation begins.

Plastic deformation presents a serious obstacle to pressure buildup. Starting with annealed copper and straining it, the yield stress of 200 MPa may be reached with ~ 20 percent elongation, according to copper's stress-strain diagram (Joseph & Kundig, 1999). This corresponds to $\sim 70\%$ volume expansion, which is much higher than the expansion due to water crystallization. Thus in annealed copper the pressure would not increase much beyond 70 MPa. This problem can be easily avoided by cold working or prestraining the copper tube as described in the Experimental Methods section. The fact that the inner part of the 15%

and 20% dextran samples vitrifies indicates that the achieved pressure can be comparable to the 200 MP pressure achieved in conventional HPF. On the other hand, the pressure can never reach the level that would be required to convert crystalline ice to amorphous, on the order of 1 GPa (Mishima et al., 1984). It is thus certain that in self pressure freezing, as in HPF, water transforms directly to amorphous ice, rather than crystallizing and then transforming.

The pressure inside of the sealed capillary does not necessary reach 200 MP but probably is somewhat lower, because the pressure rise relies on formation of low density crystalline ice. Increasing pressure leads to formation of high density amorphous (HDA) ice which is known to form in HPF (Richter, 1994). Once high density amorphous (HDA) ice begins to form the pressure stops rising. Thus the pressure buildup within the sealed tube will be automatically limited to the value necessary to continue production of HDA ice. The pressure that is actually reached is clearly a function of both the cryoprotectant concentration and the cooling rate.

It is natural to expect that freezing in the sealed tube would put a lot of stress on the sealed ends of the tube, which are probably the weakest spots on the tube. Thus one might expect that the largest deformations would occur in this exact area and that some liquid could leak through the seal, which would decrease the pressure. Consistent with the original report on SPRF (Leunissen & Yi, 2009) we did not observe leakage or seal deformation when the tube was plunged into ethane parallel to the ethane surface. This observation has been explained by formation of an ice jam in the first moment of freezing, which fortifies the seal (Leunissen & Yi, 2009). The jam forms due to faster heat extraction from the flat sealed ends of the tube, which have a high surface to volume ratio, than from the undeformed tube. This behavior suggests additional steps to avoid even the slightest possible pressure increase as the tube is sealed. Indeed crimping of the tube prior to freezing may cause some level of the pressure build up and possibly damage the biological material even before cooling. To avoid this one could leave a tiny capillary opening along the sealed ends that would ensure atmospheric pressure inside of the tube. If the hole diameter is significantly smaller than the internal diameter of the tube it would be blocked with the ice before pressure builds in the tube. While this procedure looks relatively straightforward we decided that for the freezing of *Caulobacter* it is unnecessary and we did not attempt it.

While unintended pressurizing of the sample during sealing of the tube may be damaging and thus undesirable, the ability to slightly prepressurize the sample may give a substantial advantage for some samples, especially those containing gas bubbles. For example most plant material contains some bubbles. High pressure freezing of such samples is highly unreliable because rapid pressure buildup causes implosion of the gas bubbles that causes sample deformation or even rupture of the tube. In order to avoid those problems plant material is typically treated with mild vacuum and placed in hexadecene that is assumed to fill the voids (Michel et al., 1991). Prepressuring to mild pressure may provide conditions for slow dissolution of gases in aqueous buffer and minimize deformations. We find that slight pre-pressurizing before freezing in a sealed capillary allows reproducible freezing of such materials in water-dextran solution with 100% yield of good samples. Those samples can then be easily sectioned and imaged. One preliminary image of a plant section is shown

in supplementary Figure S1; detailed evaluation of the quality of cryopreservation will require further work.

Ice crystal structure

We next discuss aspects of the ice crystallization inferred from the X-ray diffraction measurements in the context of several recent reports on structures of crystalline ice. The X-ray diffraction data provide information on the phases and orientation of the ice crystals which can be interpreted to give insights on the process of ice formation. The ice nucleating in the beginning of the freezing process in the layer of water near the copper surface does not have any preferred orientation and produces a typical powder diffraction pattern. With 10 % or less dextran, the ice is hexagonal. With higher dextran concentrations, the diffraction pattern is similar to the pattern of cubic ice with a strong (111) reflection, but it also shows the (100) and (101) reflections of hexagonal ice (Morishige *et al.*, 2009). One possible explanation for this phenomenon is that cubic ice contains some fraction of hexagonal ice. Alternatively it has been shown (Hansen *et al.*, 2008a; Hansen *et al.*, 2008b) that diffraction patterns such as we observe may be reproduced by a structure model with a complex stacking sequence composed of cubic and hexagonal ice. It has been reported earlier that cubic ice has a slightly lower surface energy than hexagonal ice (Kieft *et al.*, 1984); thus nucleation of cubic ice may be easier than nucleation of hexagonal ice. Molecular dynamic simulations show that initial nuclei have both hexagonal and cubic features (Moore *et al.*, 2010). It was shown that cubic ice, which is metastable and transforms to hexagonal when heated, may remain stable up to its melting temperature when confined in small pores (Morishige *et al.*, 2009). However, the hexagonal packing of the ice is more stable and dominates when crystallites grow larger (Morishige & Uematsu, 2005).

Crystal growth is generally easier than nucleation of new crystals, so growth of existing crystals will be favored over nucleation as the cooling front advances. Crystal growth consumes the water molecules, leaving a dextran rich layer around the crystal that eventually stops the growth. These conditions promote the growth of thin, plate-like or needle-like crystals because the leading edges of such crystals can propagate beyond the dextran-rich, water-depleted area where they have a better supply of water molecules. If the cooling speed is sufficiently high and diffusion does not have time to transfer enough water through the dextran-rich layer, then the growing crystallites remain very thin with high surface to volume ratio. Under such conditions cubic ice may be more stable than hexagonal.

The small size of the cubic ice crystals can give an idea of the distance over which molecules can diffuse during freezing, which can be compared with predictions based on measured diffusion constants. The diffusion constant for dextran is on the order of tens of microns squared per second (Periasamy & Verkman, 1998), while the freezing front moves at tens of microns per millisecond and whole tubes freeze in tens of millisecond (Yakovlev & Downing, 2011). Thus the distance a dextran molecule may diffuse is much less than one micron and diffusion can not significantly change the dextran concentration over distances on the scale of microns; in particular, dextran can not diffuse ahead of the cooling front to accumulate toward the center of the tube in order to enhance amorphization.

As the cooling front advances toward the center of the tube, crystals that have a growing atomic plane oriented in the cooling direction have an unlimited supply of water molecules so they grow rapidly. This effect favors growth of the crystals oriented perpendicular to the cooling front, and crystals with this orientation are increasingly selected as the cooling progresses producing the orientation effects we see with 15 percent dextran (Figure 3). Crystallization of the 10 percent dextran sample is different from all other samples that we studied. The diffraction patterns clearly indicate that the ice is hexagonal and does not have any preferential orientation. It is noteworthy that the sizes of the crystals in this sample are much bigger than in high dextran content samples. We believe that nucleation may be similar in both low and high dextran samples. However with lower dextran content diffusion puts less restriction on growth and crystals grow larger. Large crystals with smaller surface to volume ratio naturally transform to hexagonal ice. Large crystals grow slowly and may not keep up with the speed of cooling front propagation. This creates conditions for secondary ice nucleation resulting in disordered orientation.

Consistent with previous work (Al-Amoudi et al., 2002) we found that the bulk sample does not have to be completely amorphous in order to obtain sections that appear to be amorphous. In the process of sectioning, the diamond knife applies pressure to the material that may be high enough to cause a transition from the crystalline phase to high density amorphous ice. However the sample has to possess a certain degree of mechanical flexibility for successful sectioning. We envisage a model in which cryoprotectants provide this flexibility by binding crystals together. When pure polycrystalline ice is cut so much strain builds up at grain boundaries that the material just breaks into a powder. Relatively large crystals may be ripped out of the surface of the block and make cutting even more difficult. Adding as little as 10 % dextran significantly changes the situation, because the dextran concentrates at the grain boundaries as it is excluded from the growing ice crystals. Being more elastic than crystalline ice, the amorphous dextran solution in the grain boundaries absorbs a large amount of stress and provides elasticity for the whole sample. It is also important to note that even 10% dextran significantly reduces the size of the crystals due to the necessity for water molecules to diffuse through the dextran-rich layer to the surface of growing crystals in the short freezing time. The network of small crystals connected with vitrified dextran solution provides enough flexibility that sections can be easily cut and bent with a 35 degree diamond knife. Bending of the section during cutting can be one of the most important factors that creates pressure and causes transition of crystals to the high density amorphous state.

Given this mechanism for the formation of high density amorphous ice at the surface of the knife, one may expect that ice formed this way is not uniform. It consists of small volumes of pure high density amorphous ice separated by the solid amorphous solution of dextran and water, which would explain the texture we see in sections of low dextran samples (Figure 5a). This prediction is supported by the fact that irradiation does not completely eliminate this texture. The highest local concentration of dextran in this solution would probably depend on the pressure at the moment of freezing. It is reasonable to expect this concentration to be comparable to 30 percent since this is the concentration that completely impedes crystalline ice formation. At the same time the main contribution to the texture we observe in the sections of 10 percent dextran solution most probably comes from the rough

surface and extensive wrinkling of the sections. This conclusion is supported by observation of the contrast disappearance with irradiation which presumably causes flow of amorphous ice, smoothing the surface (Sartori Blanc *et al.*, 1998). It is not surprising that different viscosities of the crystalline and amorphous regions cause different deformation in those regions and produce the observed pattern.

In spite of these crystallization-induced effects, there may be some benefit to using 10 % dextran for certain specimens. The presence of crystalline ice in biological material by itself does not necessarily cause death to the cell (Sinclair *et al.*, 2009; Studer *et al.*, 2001). Moreover it is highly probable and to some degree supported in our images that, while the medium crystallizes, the interior of the cell vitrifies. It is also known that crystallization increases the cooling rate and may thus minimize cell dehydration due to crystallization (Yakovlev & Downing, 2011). The fact that the cell membrane and S-layer show so much more structure in 10% dextran than in higher dextran samples is surprising and may be a decisive factor for using reduced amount of the cryoprotectant even to the point that the sample crystallizes. The biggest challenge here is to optimize cryosectioning to obtain uniform sections from partially crystallized medium.

While freezing success may depend on the particular cell type, self pressure freezing in 15% percent dextran may be recommended over conventional HPF for a wide range of samples because of lower cryoprotectant requirements. It has been shown that HPF amorphization of water inside of capillary tubes requires adding 20 percent dextran (Al-Amoudi *et al.*, 2002; Al-Amoudi *et al.*, 2004). There are several reasons why it may be desirable to minimize the amount of cryoprotectant used. First, adding cryoprotectant may change the osmotic pressure and cause deformation of cells (Schwarz & Koch, 1995). A second problem is the high viscosity and density of dextran, which complicate preparation of the sample. Indeed, we found that these factors make it difficult to concentrate samples containing relatively light bacteria such as *Caulobacter* by centrifugation even in 20 % dextran while there was no problem with 10% and 15% dextran. Finally, contrast matching by the high density of the dextran solutions may reduce the contrast in electron micrographs of frozen hydrated samples. Some of these reasons still may create problems with 15% dextran. It is possible that using smaller capillaries will further reduce cryoprotectant requirements (Yakovlev & Downing, 2011).

The last issue we discuss in this work is the reason that self pressure freezing requires less cryoprotectant for amorphization than HPF. The maximum achieved pressure is similar for both methods, and cooling in the HPF liquid nitrogen jet is unlikely to be less effective than plunging into cryogen. We believe that the reason is related to formation of the crystalline ice layer in the sealed capillary near the copper wall. Crystalline ice has a higher heat diffusion coefficient than vitreous ice and, as demonstrated in (Yakovlev & Downing, 2011), allows faster cooling. Faster cooling of the central part allows amorphization with lower cryoprotectant amount. While the crystallized part of the sample may not be useful for microscopy it has to be trimmed away for cryosectioning anyway. In HPF a similar effect of boosting the cooling rate could be achieved by reducing the capillary diameter but, because part of the sample still needs to be trimmed, this would result in smaller sections.

Conclusion

In this work for the first time we documented formation of amorphous ice in a confined volume due to pressure buildup induced by formation of low density ice. Our experiments show that although amorphization in a standard HPF capillary tube is only possible in the presence of cryoprotectant, 15% dextran is sufficient for complete amorphization of the central part of the sample. In contrast, conventional HPF requires addition of at least 20% of dextran for amorphization.

X-Ray diffraction shows that samples containing water with more than 15% dextran placed in a sealed capillary and plunged in cryogen starts to freeze into cubic ice that possesses a preferential orientation relative to the radius of the tube. Formation of cubic ice builds up the pressure inside the tube up to the point that vitrification becomes possible. The required pressure depends on the amount of cryoprotectant, with higher amounts resulting in more of the sample being amorphous. When the sample contains less than 10% dextran the whole sample crystallizes into hexagonal ice.

The mechanical properties of the capillary tube have a strong impact on the quality of the ice. Cold working of the tube is essential to provide conditions for sufficient pressure buildup. It is possible that alternative materials and tube geometries may result in achieving even better freezing with lower dextran content.

We found sectioning of the vitreous part of samples frozen in sealed capillary to be similar to the HPF frozen samples. Surprisingly we found that samples with as little as 10% dextran may be successfully cryosectioned. The resultant sections consist of HDA ice. At the same time the appearance of the 10% dextran sections suggests segregation of pure ice and dextran solution within the section.

Use of various concentrations of dextran to freeze cultures of *Caulobacter* in sealed capillaries followed by cryosectioning and imaging showed that the highest contrast images are produced with 10% dextran. However because of excessive wrinkling of the sections and possible crystallization induced artifacts, using 15% dextran may be preferable.

Freezing in sealed capillary tube may be recommended for preparation of various types of biological samples because it does not have obvious disadvantages comparing to conventional HPF. At the same time the method does not require expensive, heavy equipment and is even suitable for field work. However the main advantage of the method is the lower cryoprotectant requirement which reflects faster and more optimal cooling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Optical images of the trimmed copper tubes filled with ice. a) flat trimmed tube, b) wedge for X-ray experiments.

Figure 2.

Diffraction patterns obtained using a 30 μm diameter X-ray beam from self pressure frozen samples of a) 10 % dextran, b) 15 % dextran, center of sample, c) 15 % dextran, periphery of the sample, d) 20 % dextran, center of sample, e) 20 % dextran, periphery of the sample, f) 30 % dextran sample; and patterns from high pressure freezing of g) 15 % dextran sample, h) 20 % dextran sample.

Figure 3.

Diffraction patterns obtained using a 10 μm diameter X-ray beam from 15 % dextran sample a) near the copper tube wall, b) 15 μm from the tube wall c) 30 μm from the tube wall, d) 45 μm from the tube wall, e) 60 μm from the tube wall, f) 100-150 μm from the tube wall, h) azimuthally integrated intensity from (a) with abscissa scaled to spacing.

Figure 4.
Optical image of the frozen 15% dextran solution sample trimmed to a wedge.

Figure 5.

a) Cryosection of *Caulobacter* culture centrifuged through 10% dextran, imaged with $\sim 10 \text{ e}/\text{\AA}^2$; b) same cryosection with area in center pre-irradiated by electron beam at $\sim 20 \text{ e}/\text{\AA}^2$; c) electron diffraction pattern from sample such as (a); d) electron diffraction pattern from sample contaminated with hexagonal ice; e) azimuthally integrated intensity of image (d).

Figure 6.

Cryosections of *Caulobacter* culture in a) 10%, b) 15%, c) 20%, d) 30% dextran; e) magnified part of image (a) arrows indicate: IM – inner membrane, OM – outer membrane, S – S-layer; f) magnified part of image (b).