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Observations on the behavior of vitreous ice at ~82 and~12 K

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Abstract

In an attempt to determine why cooling with liquid helium actually proved disadvantageous in our electron cryotomography experiments, further tests were performed to explore the differences in vitreous ice at ~82 and ~12 K. Electron diffraction patterns showed clearly that the vitreous ice of interest in biological electron cryomicroscopy (i.e., plunge-frozen, buffered protein solutions) does indeed collapse into a higher density phase when irradiated with as few as 2–3 $e^-/Å^2$ at ~12 K. The high density phase spontaneously expanded back to a state resembling the original, low density phase over a period of hours at ~82 K. Movements of gold fiducials and changes in the lengths of tunnels drilled through the ice confirmed these phase changes, and also revealed gross changes in the concavity of the ice layer spanning circular holes in the carbon support. Brief warmup–cooldown cycles from ~12 to ~82 K and back, as would be required by the flip-flop cryorotation stage, did not induce a global phase change, but did allow certain local strains to relax. Several observations including the rates of tunnel collapse and the production of beam footprints suggested that the high density phase flows more readily in response to irradiation. Finally, the patterns of bubbling were different at the two temperatures. It is concluded that the collapse of vitreous ice at ~12 K around macromolecules is too rapid to account alone for the problematic loss of contrast seen, which must instead be due to secondary effects such as changes in the mobility of radiolytic fragments and water.

Keywords: Liquid helium; Radiation damage; Vitreous ice; Amorphous ice; Cryoelectron microscopy

1. Introduction

Most of the water in the universe probably exists in the so-called "high density amorphous" state (Angell, 2004; Jenniskens et al., 1995). This state has become particularly interesting to electron cryomicroscopists because it underlies surprising disadvantages of cooling with liquid helium instead of liquid nitrogen. In electron cryomicroscopy, biological samples are immobilized for imaging in a fully hydrated, near-native state by either plunge or high-pressure freezing (Dubochet et al., 1988; Moor, 1987). In plunge-freezing, thin, aqueous films are rapidly driven into liquid ethane or propane, which removes the heat so quickly that the water "vitrifies" rather than crystallizes. Samples that cannot be spread into thin films can be frozen under high pressures, which inhibits ice crystal formation, and again preserves the specimen in a near-native state within (mostly) vitreous ice (Richter, 1994). High-pressure frozen samples can then be cryosectioned and imaged (Al-Amoudi et al., 2004; Hsieh et al., 2002).

While in the electron microscope, vitreous samples are kept frozen through thermal contact with a cryogen. At first, liquid nitrogen was used, and it was observed that such cooling delayed radiation damage. In hopes that additional cooling would further slow radiation damage and permit the acquisition of statistically better defined images, cryomicroscopes were built that could use liquid helium instead. Disappointingly, we show in the companion paper (Iancu et al., 2006) that at least for the doses and resolutions of interest in electron cryotomography, liquid helium-cooling is actually disadvantageous because at ~12 K, the contrast from proteins and lipid bilayers gradually fades with dose. Surprisingly, iterative temperature cycles up to ~82 K and back to ~12 K prevent this loss of contrast.

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These observations suggested to us that perhaps the vitreous ice was gradually becoming more dense, and thus "contrast-matching" macromolecules. Indeed, nearly three decades ago X-ray diffraction experiments first indicated that there were at least two forms of vitreous ice with different densities (Narten et al., 1976). Since then many other studies have confirmed the existence of the higher density form and shown that it can be obtained at very low temperatures (<50 K) and/or high pressures by various means, including applying pressure to common crystalline ice, condensing water vapor onto surfaces, hyperquenching liquid water under pressure, microtoming ice, and irradiating the lower density form of vitreous ice (Angell, 2004; Jenniskens et al., 1995). Of more particular relevance for cryoelectron microscopy (cryoEM), in a series of papers Heide and Zeitler showed that the ice formed by water vapor condensation at temperatures between 50 and 90 K transformed into a higher density state, when irradiated with electrons below 50 K (Heide, 1982, 1984; Heide and Zeitler, 1985). The exact number and differences between the various phases of vitreous ice and their transitions is still being actively examined (Angell, 2004).

To explore whether the collapse of vitreous ice from a lower to a higher density state could in fact explain the loss of contrast seen in our experiments, here, we report studies on the behavior of plunge-frozen buffers, protein solutions, and bacterial cultures when irradiated at ~82 and ~12 K, and when warmed or cooled between these two extremes. Three different experimental methods were used, including recording electron diffraction patterns, tracking the position of gold fiducials, and measuring the length of tunnels drilled through the ice. Confirming and extending existing literature, our studies show that these specific vitreous ices (i.e., plunge-frozen aqueous biological specimens) do indeed collapse from a lower to a higher density state, when irradiated at ~12 K, and this phase change reverses when the sample is warmed back up to ~82 K. While the collapse is induced by just 2–3 $e^-/Å^2$, the reversal is apparently spontaneous but slow, requiring at least several minutes. Different patterns of ice flow and bubbling are seen at the two temperatures, and certain local stresses generated at ~12 K are relieved by warming. The results show that the loss-of-contrast-effect that makes helium-cooling undesirable for electron cryotomography is not explained by the density change alone, but also by changes in the mobility of radiolytic fragments in the high density state.

2. Results

2.1. Phase transitions as measured by electron diffraction

A dilute phosphate buffer typical for protein solutions was vitrified across Quantifoil electron microscope grids in a "Vitrobot" automatic plunge-freezing device, following standard procedures that produce consistent and thin ice. The specimen was inserted into an FEI G^2 Polara 300 keV electron microscope that had been cooled to ~82 K with liquid nitrogen, and electron diffraction patterns were obtained through various holes spanned by thin, continuous ice. After data collection at ~82 K, the temperature of the specimen was reduced to ~12 K by replacing the liquid nitrogen in the inner cryogen dewar with liquid helium. A second set of diffraction patterns was then recorded from fresh (unexposed) holes in the same grid square.

Both sets of diffraction patterns showed three rings. At ~82 K, the rings were located at 1/3.69, 1/2.12, and 1/1.37 $Å^{-1}$. At ~12 K, the rings were broader, the innermost ring was located further from the center at 1/3.22 $Å^{-1}$, and the outermost ring was less sharp (Fig. 1). For a given temperature, the patterns for all the holes that were analyzed were essentially identical, except that the intensities varied slightly due to small differences in the ice thickness or, possibly, small variations in beam intensity. These changes indicate that after irradiation at ~12 K, the vitreous ice assumed a different and more tightly packed structure.

To explore transitions between these two phases, "dose series" of electron diffraction patterns were recorded at ~82, ~12 K, and through transitions between the two temperatures. First, several series of diffraction patterns were obtained at ~82 K in graded steps from 1 to 1000 $e^-/Å^2$. Intermittent selected area images verified that the entire dose series was obtained within a single hole and that drift was insignificant. Plots of the circularly averaged diffraction patterns showed the same profile throughout each dose series

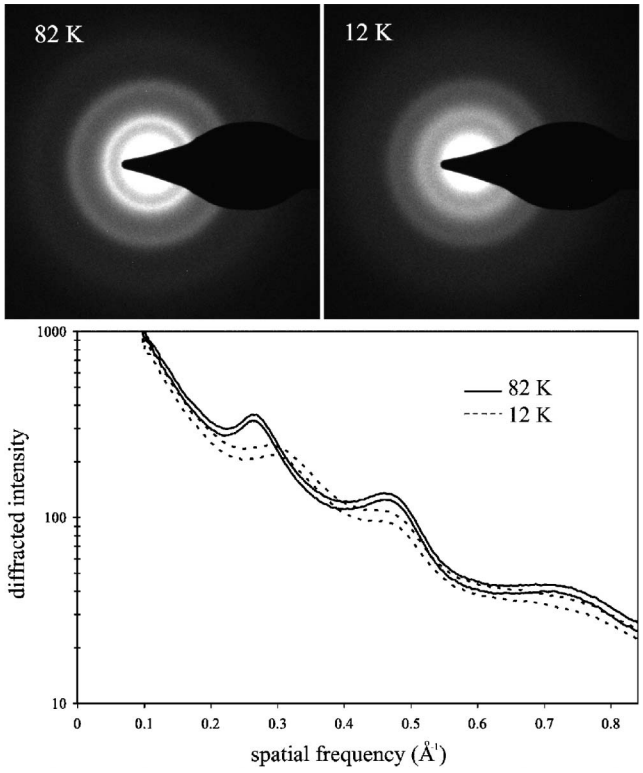

Fig. 1. Electron diffraction patterns from low and high density vitreous ice. Typical electron diffraction patterns of plunge-frozen, dilute buffers at ~82 and ~12 K are shown, evidencing lower and higher density states. Two independent, circularly averaged profiles for each temperature are plotted below.