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**In Situ Transmission Electron Microscopy Study of Bubble Behavior Near the  
Surface of Ice Crystals by Using a Liquid Cell with a Peltier-Cooling Holder**

*Running title:* In situ TEM of ice crystals with Peltier cooling (48/50 characters)

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**Abstract:**

Liquid-cell transmission electron microscopy (LC-TEM) is a unique technique that permits in situ observations of various phenomena in liquids with high spatial and temporal resolutions. One difficulty with this technique is the control of the environmental conditions at the observation area. Control of the temperature in the range from room temperature to minus several tens of degrees Celsius is desirable for controlling the supersaturation in various materials and for observing crystallization more easily. We have developed a cooling TEM specimen holder that uses Peltier devices and we have combined it with a liquid cell to realize accurate temperature control in LC-TEM. We evaluated this system by using water as a specimen. Motionless bubbles, shown to be voids containing pressurized gas, formed in the specimen some time after the temperature had reached  $-12\text{ }^{\circ}\text{C}$ . An electron-diffraction pattern showed that the specimen turned into ice Ih after the formation of these bubbles, confirming that our system works properly and can induce crystallization. In addition, we analyzed the behavior of bubbles formed in the ice Ih, and we discuss the formation of these bubbles and their internal pressure.

29        **Key words:** liquid-cell transmission electron microscopy, in situ observation,

30        crystallization, radiolysis, ice, water, Peltier element, cooling holder

31

## Introduction

Liquid-cell transmission electron microscopy (LC-TEM) is a technique that permits the observation of volatile samples, such as aqueous solutions, by transmission electron microscopy (TEM) (Ross, 2016). Because this technique can be used to observe various phenomena in solution at the nanoscale (de Jonge et al., 2019), it is promising for visualizing the events during the early stages of crystallization, such as the nucleation of crystals from solution. Recent studies using LC-TEM have revealed that the nucleation of crystals is a complex process in which various growth units, such as atoms or molecules, do not assemble one by one to form the nucleus of a crystal but, instead, form a crystal nucleus via intermediates such as amorphous or dense liquid (Nielsen et al., 2014; Patterson et al., 2015; Yamazaki et al., 2017; Ou et al., 2020). These facts clearly indicate that this method is promising for clarifying the previously inaccessible picture of nucleation.

However, it remains difficult to observe the moment of nucleation by LC-TEM. One reason for this is the scarcity of methods available for inducing crystallization within the observation area. To observe the crystallization, it is usually necessary to increase the supersaturation  $\sigma$  (defined as  $\sigma = \ln(C/C_e)$ , where  $C$  is the concentration and  $C_e$  is

the solubility) within the observation area to create the supersaturated conditions that are necessary for crystallization to occur. To increase the supersaturation, it is clearly necessary to increase the concentration of the solute and/or decrease its solubility. Such an operation is difficult to perform within the observation area for LC-TEM. Increasing the concentration of ions in LC-TEM has been achieved for some systems by using radiolysis (Yuk et al., 2016; Yamazaki & Kimura, 2021). Radiolysis is the process by which electrons impart part of their energy to a solution sample, especially a solvent, resulting in the decomposition of the solvent to form active species that undergo secondary chemical reactions with the surrounding solvent and solute molecules to produce various chemical species that are not present in the initial solution (Schneider et al., 2014; Ambrožič et al., 2019). This process permits the production of the chemical species that are necessary for crystallization to occur, thereby generating supersaturated conditions for the target crystals; however, the range of systems to which this technique can be applied is very limited.

Another promising method for increasing the concentration within the observation area involves the use of electrodes. A liquid cell with an amorphous silicon nitride membrane can be equipped with electrodes to which an electrical signal can be applied.

One way in which these electrodes can be used in observing crystallization is to induce an electrochemical reaction on them. This can, for example, permit the visualization of electrodeposition on electrodes during a battery charge–discharge cycle (Mehdi et al., 2015). Another way to use the electrodes is to create an electrical field around them that induces electric-field-related phenomena such as dielectrophoresis (Pohl, 1951). It is possible to induce dielectrophoresis in a liquid cell so that colloidal particles collect around electrodes (Yamazaki et al., 2022). This is a promising method for increasing particle concentrations in the region around the electrodes and could trigger crystallization, although the development of this concept remains a work in progress.

In contrast to methods that increase concentration, methods that decrease solubility generally involve changing the temperature of the observed system, and cooling is particularly effective for most solution systems. Cooling experiments under TEM are generally performed by using a refrigerant such as liquid nitrogen. TEM specimen holders for this technique have heaters to maintain the temperature of the specimen by controlling the balance of heat transfer between the heater and the refrigerant. With this temperature-control system, it is difficult to maintain temperatures that are just below room temperature because of an imbalance between the capacity of the heater and that

of the refrigerant. Such systems are useful in so-called cryo-TEM, where biological samples are frozen and supported in amorphous ice for observation (Dubochet et al., 1988). The range of applications of crystallization from a solution or a pure liquid (crystallization of a melt) through cooling with a liquid cell in TEM experiments is very limited (Tai et al., 2014).

For optical microscopy, cooling stages that use Peltier elements are commonly used in observing crystallization from solution (Dold et al., 2006). Temperature control by using Peltier devices is based on the Peltier effect, in which heat is absorbed or generated when an electric current is applied to a joint between different metals. In practice, when a direct current is applied to a Peltier device, one side of the device is cooled while the other side is heated. By controlling the amount of direct current, the temperature of the specimen and/or its stage can be controlled so that it remains at a desired temperature. This temperature-control system is accurate in principle, and the temperatures that can be controlled by such a system range from several tens to minus several tens of degrees Celsius, with an accuracy of  $\pm 0.1$  °C. The key to successful cooling by using Peltier elements is the release of the heat that is produced by the elements. If insufficient heat is released from the heating side, it can be transferred to

the cooling side, resulting in inadequate cooling. Heat-dissipating fins and/or refrigerant circulated by a pump can be used to remove the heat generated in the heated section of a Peltier element.

We have developed a TEM cooling holder that uses a Peltier device and we have established an experimental system that combines the holder with a liquid cell for performing crystallization experiments on liquid specimen. To demonstrate the effectiveness of our cooling system, we used water, which was expected to form ice crystals upon cooling, as a liquid specimen, and we successfully observed the formation of ice crystals. An initial broad examination also showed that bubbles that formed near the surface of the ice displayed an interesting behavior, analysis of which provided clues as to the conditions present within the ice and the liquid cell.

## **Materials and Methods**

### ***TEM and Electron-Beam Irradiation Conditions***

We used a transmission electron microscope (JEM-2100F; JEOL Ltd., Tokyo) with a field-emission gun operated at an acceleration voltage of 200 kV. The TEM images and

videos were recorded by using a OneView IS TEM camera (Gatan, Inc., Pleasanton, CA). Energy-dispersive X-ray spectroscopy (EDS) for elemental analysis and mapping was performed using an EDS system (JEOL JED2300T) equipped with a silicon drift detector. The electron flux of the electron beam irradiating the specimen was controlled by using an electrostatic shutter (Electrostatic Dose Modulator; IDES, Inc., Pleasanton, CA) to produce a pulsed electron beam. The intensity of the pulsed electron beam followed a square-wave pattern with a frequency of  $10^4$  Hz. To reduce the overall intensity of a continuous electron beam of, for example,  $1.0 \text{ e}^- \text{ nm}^{-2} \text{ s}^{-1}$  to  $0.1 \text{ e}^- \text{ nm}^{-2} \text{ s}^{-1}$  (one tenth), during each 0.1 ms of the experiment, the electron beam is irradiated for 0.01 ms with no irradiation during the remaining 0.09 ms. The irradiation rate of the electron beam is expressed as a percentage along with the electron flux. In the previous example, the electron flux is denoted as  $0.1 \text{ e}^- \text{ nm}^{-2} \text{ s}^{-1}$  (10%). We measured the electron flux when the electrostatic shutter was used without any sample. From this value, we calculated the electron dose rate, and the values of the electron dose rate shown here are those of the electron beam before it penetrated the sample. The electron dose rate was calibrated by using a Faraday cage (JEOL Ltd., Tokyo, Japan). We used *DigitalMicrograph* (Gatan, Inc., Pleasanton, CA) and *imageJ* (Schneider et al., 2012) software to analyze the TEM images. We also used *Recipro* (Seto & Ohtsuka,

2022) to simulate electron-diffraction patterns.

### ***The Peltier-Cooling TEM Holder***

We have developed a Peltier-cooling TEM holder (Figs. 1a–c) that has a double stacked Peltier element inside the handle of the TEM holder (Fig. 1b), together with built-in heat-transfer rods that extend from the Peltier element to just before the sample stage, which can be replaced by other stages, depending on the required purpose (Fig. 1d–h). The holder is equipped with signal lines (Fig. 1b) to apply a dc electric current to the Peltier elements. A thermosensor and a heater are installed immediately before the sample stage inside the holder (not visible in Fig. 1). The thermosensor can measure the temperature near the sample location, including radiant head, and the temperature measured by the thermosensor was used to maintain the temperature at the desired value by balancing the outputs of the Peltier elements and a heater controlled by external signals. A refrigerant-type heat sink was used to dissipate the heat generated in the hot parts of the Peltier elements, and a low-pulsation chiller is used to circulate a refrigerant at  $-20\text{ }^{\circ}\text{C}$  through the heat sink. For this purpose, the holder is also equipped with tubes

(Fig. 1c) for circulating the refrigerant. When this type of chiller is used, vibrations caused by the circulation of the refrigerant are minimal. The actual temperature might differ from that measured by the thermosensor: the difference will depend on the heat capacity of the tip and other factors. We attempted to measure the temperature of the tip in vacuum by using a platinum sensor directly attached to the tip. The temperatures reported in this study are the values corrected by this measurement. As discussed below, the temperature at which ice formed from water was consistent with that previously reported, and the temperature measurement is therefore not significantly incorrect. Note, however, because heat inflow from the platinum sensor was also possible, more-accurate measurements of the temperature at the sample location would be desirable. It is also desirable to calibrate the temperature by observing the phase transition of materials that are less affected by the electron beam.

### ***The Liquid Cell***

The liquid cell, installed at the tip of the holder, consisted of a copper liquid cell holder with large and small O-rings (Fig. 1e), a 3-mm-diameter silicon chip (NT025A;

Norcada Inc., Edmonton, Canada) (Fig. 1f) and a 5-mm-square silicon chip (Fig. 1g) (NX5025A; Norcada), with a 50 nm-thick amorphous silicon nitride (a-SiN<sub>x</sub>) membrane. Both windows measured 250 × 250 μm. The circular silicon chip was placed on the small O-ring, and double-distilled water that had not been degassed was loaded onto the chip as a specimen (Fig. 1f). The square silicon chip was then placed on the circular silicon chip and the larger O-ring (Fig. 1g), and the copper lid was screwed on (Fig. 1h). The water was thus encapsulated between the two a-SiN<sub>x</sub> membranes. The a-SiN<sub>x</sub> membranes were placed so that they overlapped, allowing the electron beam to penetrate through them to permit observation of the encapsulated water specimen.

## Results and Discussion

### *Bubble Generation and Ice Formation*

We observed that ice crystallized in the liquid cell when the temperature was reduced to −12 °C (Fig. 2). Initially, it was difficult to distinguish whether the water had crystallized or not but, as described below, we were eventually able to identify crystallization by means of the behavior of bubbles that formed in the sample. By

subsequently obtaining electron-diffraction patterns, we were able to identify the formation of ice. Note that we use the term ‘bubble’ throughout to refer to a spherical bright-contrast object observed in the ice crystal; this was actually a void, a type of crystal defect, that contained gas, as will be discussed later.

The observation region of the liquid cell was filled with water, and no large air bubbles were generated (Grogan et al., 2014) in the initial sample. The liquid cell was gradually cooled to  $-12\text{ }^{\circ}\text{C}$  and maintained at this temperature. About 3.5 minutes after the temperature reached  $-12\text{ }^{\circ}\text{C}$ , bright spots began to appear (Fig. 2b). The size of these bright spots increased with time (Fig. 2c). The bright contrast of these spots indicated that they were less dense than their surroundings. As we discuss later, we concluded that these spots correspond to bubbles. In general, when such bubbles form in an aqueous solution in a liquid cell they move or instantly grow so large that they cover the entire field of view (Grogan et al., 2014). However, these bubbles grew slowly over time and their centers did not move. We therefore concluded that the water had already crystallized as ice.

When the temperature was raised toward  $6\text{ }^{\circ}\text{C}$  after the formation of these bubbles, the ice crystal in the observation area shrank and the interface of the ice became

obscured; eventually, the observation area filled with water (Fig. 3 and Supplementary Video S1) because all the ice crystals in the liquid cell completely melted into water. When the ice reverted to water, the bubbles disappeared, suggesting that all the bubbles had dissolved in the water because water can dissolve gases, whereas bubbles are not readily incorporated into ice.

A selected-area electron-diffraction (SAED) pattern of the ice was acquired at  $-12\text{ }^{\circ}\text{C}$ , as shown in Fig. 4. Note that it is generally difficult to obtain electron-diffraction patterns when this type of liquid cell is filled with a solvent, because the sample is too thick. We attempted to obtain a diffraction pattern when the liquid cell was filled with ice, but we could not obtain a clear diffraction pattern due to the thickness of the sample, which was probably of the order of several micrometers. We therefore searched for a large air bubble and we obtained a diffraction pattern through it (Fig. 4). The electron-diffraction pattern was consistent with that of ice Ih, as simulated by the *Recipro* software (Seto & Ohtsuka, 2022). We observed the presence of ice Ih at  $-12\text{ }^{\circ}\text{C}$ , the same temperature as that found in a previous study in which ice Ih was observed at  $-12\text{ }^{\circ}\text{C}$  by using a cooling TEM holder with a refrigerant and heater (Tai et al., 2014). Therefore, our temperature-control system combining a Peltier-cooling TEM holder and

a liquid cell worked successfully in the TEM observations.

### ***Formation Routes of the Bubbles***

There are two possible routes for the formation of gas bubbles during ice crystallization: either the gases are excluded from the water during ice crystallization in a degassing process or the gases are produced by radiolysis of the ice after crystallization.

After the ice had crystallized, we were often able to find an area where a large bubble had formed (Supplementary Fig. S1), through which the transmission of the electron beam was sufficient to permit the acquisition of an electron-diffraction pattern (Fig. 4). The EDS mapping images for oxygen and silicon show that neither element could be detected in such bubbles, whereas the surrounding was composed of oxygen, indicating ice (Supplementary Fig. S1). Assuming that the sample water was previously saturated with atmospheric gases, the ratio of the volume of the gas present to that of the water was calculated to be 50:1 (Supplementary Text S1). Because the window area accounted for less than 2% of the total liquid cell (the surface area of the small Si chip

was  $\sim 7 \text{ mm}^2$  whereas the area of the window was  $\sim 0.06 \text{ mm}^2$ ), if a large bubble that covered the entire window is formed, this can be explained by degassing of the water alone. When the ice reverted to water, these bubbles disappeared, suggesting that they had redissolved in the water (Fig. 3, Supplementary Video S1). The bubbles that formed only after the ice crystallization were primarily the result of degassing of the initial water sample as a result of ice crystallization.

As shown in Fig. 2, the bubbles continued to expand when the ice was irradiated with the electron beam. This suggests that radiolysis by the electron beam was the main source of gases after crystallization. When water/ice is irradiated with electrons, water molecules are excited and then undergo chemical reactions with surrounding water molecules. This process, known as radiolysis, results in the formation of various chemical species, including gas molecules such as hydrogen and oxygen (Schneider et al., 2014). The formation of bubbles by radiolysis is also supported by our observation that no bubbles formed in the ice regions not irradiated by the electron beam after crystallization. We therefore conclude that the bubbles formed in the ice crystal were the result of a radiolysis process. A schematic summarizing the two bubble-formation routes involving crystallization and electron-beam effects is shown in Supplementary Fig. S2.

In LC-TEM, radiolysis has significant effects on liquid specimens and on observing phenomena. Calculations of changes in the composition of liquid water resulting from radiolysis have shown that the production of hydrogen and oxygen depends on the electron dose rate (Schneider et al., 2014). Similarly, such gaseous products might be produced by the radiolysis of an ice specimen. The formation of bubbles in both ice and hydrated specimens has also been observed by cryo-TEM (Unwin & Muguruma, 1972; Dubochet et al., 1988). The formation and escape of bubbles (referred to as ‘cavities’ in the literature) under electron-beam irradiation have been observed in ice Ih at  $-80^{\circ}\text{C}$  (Unwin & Muguruma, 1972). Also, similar bubble formation has often been observed in hydrated organic specimens in ice under cryogenic conditions (Dubochet et al., 1988). The presence of hydrogen was confirmed by electron energy-loss spectroscopy (EELS) (Leapman & Sun, 1995). A similar analysis could provide insight into the composition of the bubbles produced in the present study and the chemical reaction pathway for the formation of these bubbles by radiolysis.

#### *A Physical and Chemical View of the Formation of Bubbles in the Ice*

Next, we will consider a physical view of the formation of bubbles in the crystals. During radiolysis, lattice positions occupied by water molecules in an ice crystal become vacant as a result of radiolytic decomposition, resulting in the formation of vacancies. In the formation of a bubble, a plausible picture is that these vacancies diffuse and aggregate to form a void that is filled by the gases generated by radiolysis. The gases could diffuse through the crystal alone or together with the vacancies or voids. Although a detailed model might provide a better account of the effects of gases and defect sinks (Was, 2017), we assumed that the bubble-growth mechanism involves a process of diffusion and aggregation of growth units (vacancies and/or vacancy and gas molecule pairs); we were then able to predict the process that determines the growth rate of the bubbles.

We measured the time dependence of the radius of one bubble (indicated by the white arrow in [Fig. 2](#)) and we found that its radius increased almost linearly with time, indicating that its growth rate  $R$  was constant ([Fig. 5](#)). The growth rate can be described by the equation  $R = K\sigma_{surf}$ , where  $K$  is the kinetic coefficient and  $\sigma_{surf}$  is supersaturation at the surface of the bubble (Chernov, 1984). The kinetic coefficient represents the reaction process at the bubble's surface; this value will therefore be

constant if the environmental conditions, such as temperature, remain unchanged. The supersaturation  $\sigma_{surf}$  can be expressed as  $\sigma_{surf} = C_{surf} - C_{eq}$ , where  $C_{surf}$  is the concentration of growth units in ice at the surface of the bubble and  $C_{eq}$  is the equilibrium concentration of growth units. The equilibrium concentration  $C_{eq}$  will also be constant if the environmental conditions are unchanged. As the particles become larger, the number of sites for the incorporation of growth units increases and the concentration  $C_{surf}$  tends to decrease. In the present case, the constant growth rate during the observation period suggests that bubble growth is rate-limited by the reaction process at the surface of the bubble.

According to the literature (Schneider et al., 2014.), the electron flux in Fig. 2 corresponds to the dose rate of  $10^5 \text{ Gy s}^{-1}$  under our experimental conditions. At this dose rate, the concentrations of hydrogen and oxygen at chemical equilibrium in the irradiated area are approximately  $10^{-5}$  and  $10^{-6} \text{ M}$ , respectively. If all the gas components emerge as a bubble from a water volume of  $1000 \text{ }\mu\text{m}^3$  (this assumes from a volume of  $10 \times 10 \times 10 \text{ }\mu\text{m}^3$ , similar to our observation conditions) at this concentration, the volume of the bubble would be  $\sim 0.2 \text{ }\mu\text{m}^3$  at atmospheric pressure and  $-12 \text{ }^\circ\text{C}$ . Even if the gas is consumed for growing bubbles, the gas concentration is

maintained at this value on the millisecond scale because the solution is in chemical equilibrium. Therefore, the maximum gas production rate is estimated to be  $200 \mu\text{m}^3 \text{s}^{-1}$ , which is sufficient to grow a bubble with a diameter of  $1.8 \mu\text{m}$  (volume of  $\sim 24 \mu\text{m}^3$ ) in 236 s. The value calculated under this assumption is consistent with the results of the present experiment. Note that this gas production rate is calculated for a thin film of water. Because the sample in our experiment is ice and thicker (multiple scattering of electrons should occur), the production rate should be different.

This might provide further insights into the details of the bubble-formation mechanism to measure the bubble growth rate as functions of the temperature and the dose rate.

### ***Estimation of the State of the Ice and Its Environment by an Analysis of the Bubbles***

In this section, we discuss details of the nature of the ice and its surrounding environment obtained by analyzing the bubbles. When all the liquid water had crystallized, the dissolved gases were excluded, and a gas–solid interface formed inside the liquid cell. Observations performed at  $-12^\circ\text{C}$  showed that the interface slowly

315       receded ([Fig. 6 and Supplementary Video 2](#)). While the interface was receding, bubble  
316       growth was not clearly observed. In addition, the temperature increase in LC-TEM has  
317       been estimated to be small (Grogan et al., 2014), and we did not observe any drastic  
318       changes, such as boiling of water, induced by high temperatures. Therefore, this  
319       recession might have been due to decomposition of the ice crystals through radiolysis,  
320       with the radiolysis products, as well as forming bubbles, escaping from the interface  
321       before being incorporated into bubbles.

322             The bubbles formed within the ice crystals remained stationary at constant  
323       positions (the positions of the centers of the spheres did not change), and no movement  
324       of the bubbles inside the crystals was clearly observed. Under these conditions, as the  
325       surface of the ice crystal receded and the surface moved closer to the bubbles, the  
326       bubbles were eventually ejected from the surface of the ice crystal. When bubbles began  
327       to be ejected, they were observed to be moving and their shapes became deformed, so it  
328       appeared that there might have been a liquid layer present on the surface. Here, we  
329       discuss the state of the ice surface by analyzing the behavior of the bubbles.

330             First, let us assume the presence of a liquid layer on the surface of an ice crystal  
331       about the size of a bubble (several hundreds of nanometers), from which the bubble

escapes. This is a similar situation to the case in which bubbles escape from the surface of bulk water at a flat gas–liquid interface. Note that a layer of liquid on the ice surface, commonly called the quasi-liquid layer, is not observed in this temperature region, and its thickness is less than 100 nm (Sazaki et al., 2022).

When a bubble with a size of a few tens of micrometers escapes from bulk water, the relaxation time of the water’s surface has been experimentally determined to be several tens of microseconds (because the water surface generally oscillates during the relaxation, the time for one cycle of oscillation is taken as the relaxation time) (Lee et al., 2011). On the other hand, the relaxation time that we observed before the bubbles escaped from the surface and the surface was roughly flattened was about one second for bubbles with a diameter of 600 nm (Fig. 6). The measured relaxation time was therefore huge compared with that of bulk water. This discrepancy shows that ordinary water was not present on the surface of the ice. If a liquid layer is highly viscous, its relaxation time  $\tau$  can generally be described by the expression  $\tau = \mu h / \gamma$  (Bird et al., 2010), where  $\mu$  is the viscosity,  $h$  is the distance between the interface and the surface of the air bubble, and  $\gamma$  is the surface tension. If we assume that the surface tension of water at  $-12\text{ }^{\circ}\text{C}$  is  $0.077\text{ kg s}^{-2}$  (Kalová & Mareš, 2018), a typical value of  $h$  is about

100 nm (Fig. 4), and the relaxation time is about 1 s, the viscosity of the liquid phase is  $7.7 \times 10^5$  Pa s or  $7.7 \times 10^8$  cP. This is highly viscous for a liquid, and its value is more than eight orders of magnitude higher than that of bulk water. Therefore, it is reasonable to infer that the surface remains in a solid state. It would be interesting to observe the ice surface at closely spaced temperatures around the melting point to identify the boundary between the liquid and solid phases.

If the surface is solid, the mass-transport process involved in restoring a flat surface can be estimated from the relaxation time of the surface. Immediately after bubble rupture, a hole is created in the surface of the ice, and the flat surface is eventually restored because the ice attempts minimize its surface energy. During this recovery process, the supply of molecules to the ice surface can be estimated by applying Herring's scaling law for sintering (Herring, 1950), which can be used to predict the process of mass transfer from the relationship between the relaxation time and the characteristic length power. We measured the dependence of the relaxation time on the bubble size (Fig. 7) and we found that it increased with the bubble size to the power 1.77. According to Herring's scaling law, this corresponds to a process of evaporation and condensation in which molecules that have evaporated into the gas

phase are taken up again at the surface (for which the power is approximately 2), and not volume diffusion (for which the power is approximately 3). This result suggests that the process of surface relaxation involves the release of water molecules from a relatively unstable crystal and their reincorporation from the gas phase to form a surface to minimize the surface energy.

If the ice surface is crystalline, its tensile strength can be used to estimate the pressure inside the bubbles. In Fig. 4, a bubble with a radius of 200 nm ruptured when the ice between the bubble and the environmental phase was 100 nm thick. Rupture occurs when the stress generated by the internal pressure of a bubble exceeds the tensile strength of the ice. In a thick-walled spherical container of thickness  $t$ , the maximum stress due to the internal pressure caused by the enclosed gas is equal to the stress applied at the inner wall of the container, and can be expressed by the following equation:

$$\sigma_{inner} = p \frac{(r + t)^2 + r^2}{(r + t)^2 - r^2} \quad (1).$$

If we assume that the internal pressure inside the liquid cell is equal to atmospheric pressure (0.1 MPa) and that the tensile strength of the ice is 1–2 MPa (Cuda & Ash,

1984), the pressure  $p$  ranges from 0.38 to 0.77 MPa, which is the same order of magnitude as atmospheric pressure. This is small compared with the pressure of  $\sim 10^3$  atmospheres inside bubbles generated in water samples containing organic matter, as measured by EELS (Leapman & Sun, 1995). From a simple ratio, when two water molecules are converted into gases, two hydrogen molecules and one oxygen molecules are produced. The volume occupied by one water molecule in ice Ih is  $0.32 \text{ nm}^3$ , whereas the volume of one ideal gas molecule at one atmosphere is  $41 \text{ nm}^3$ . If three gas molecules are present in the space previously occupied by two water molecules, the local pressure would be about 200 atm. However, the diffusion coefficient of molecular hydrogen in ice Ih under similar condition to those of our study is  $10^{-11} \text{ m}^2 \text{ s}^{-1}$  (Patterson & Saltzman, 2021), a value that is as large as that of macromolecules in aqueous solution. Therefore, interactions might occur between the bubble surface and hydrogen molecules that keep hydrogen within the bubble.

## **Application of the Cooling TEM Holders, and Perspectives**

Our experimental system composed of the Peltier-cooling TEM holder and a liquid cell can be used to study phase transitions of liquids and crystallization from solutions (e.g., salt precipitation from solutions). In the current situation, we can use it to study the dynamics of the early stages of crystallization. Even in a thick solution layer where no bubbles are generated, we have measured the growth rate of crystals using a combination of a liquid cell and machine learning to detect crystallization (Katsuno et al., 2022). In this experiment, we captured a large change in growth rate in the early stages of crystallization. We speculate that this change in growth rate is due to a phase transition from a metastable phase to a stable phase. Our system is useful for studying the nucleation pathways to the most stable phase for various crystallization systems.

Thinning a sample liquid layer for high-resolution imaging is one of the future challenges for realizing the potential of our developed system. There are mainly two methods to produce a thin liquid layer in this type of liquid cell: generation of bubbles and the use of rectangular-slit window frames. The generation of bubbles in this type of liquid cell produces a thin layer of liquid (meniscus) on the membrane (Grogan et al., 2014). This procedure is necessary to attain high-

414 resolution images and electron diffraction patterns. Such a bubble is often  
415 generated without the experimenter realizing it. The presence of a thin liquid  
416 layer on the membrane is supported by indirect evidence such as the growth of  
417 crystals on the membrane. In addition, controlling the thickness of the bubble-  
418 induced liquid layer formed on the membrane is difficult. In practice, the liquid  
419 layer is sufficiently thin for high-resolution images to be acquired. With the  
420 second method (i.e., using rectangular-slit window frames), the thickness of the  
421 sample can be reduced somewhat by using rectangular-slit window frames and  
422 overlapping the window frame in a cross (Li et al., 2021). This type of liquid cell  
423 could be used in our system, and the spatial resolution would be improved at the  
424 corners where the windows overlap. However, controlling the thickness of the  
425 liquid in the entire liquid cell is difficult because of the bulging effect (Holtz et  
426 al., 2013). Controlling the liquid layer thickness over a large-scale area is a  
427 technical challenge not only for this system but also for liquid-cell TEM using a-  
428  $\text{SiN}_x$  membranes.

## 430 Conclusion

We have developed a cooling TEM specimen holder that uses Peltier devices and we have combined it with a liquid cell to establish a temperature-controlled LC-TEM system. We crystallized water by using this system and we showed that the device operates correctly and can promote crystallization by decreasing the temperature. The growth of bubbles formed in ice Ih and their internal pressure have been discussed on the basis of the behavior of the bubbles. Bubbles in ice were formed by radiolysis, and their growth rate suggested that the kinetics of incorporation of growth units at the bubble–ice interface was the rate-limiting step for bubble growth. When the bubbles escaped from the ice surface, the surface appeared to consist of a liquid phase, but the relaxation time of the surface suggested that it remained as a solid. When Herring’s scaling law was applied, the dependence of the relaxation time on the bubble sizes suggested that surface relaxation occurred by a process of evaporation and condensation. Based on the state of ice when the bubbles ruptured, the pressure inside the bubble was estimated to be in the range 0.38 to 0.76 MPa, assuming that the inside of the liquid cell was at atmospheric pressure.

In conclusion, our system can be used to investigate not only crystallization, but also physical properties and interfacial conditions based on dynamic behaviors through

in situ observations. The system will be useful in investigating crystallization from aqueous solutions and, as shown in this study, it will help in achieving an understanding of the properties of materials and phenomena that occur inside liquid cells to elucidate the behavior of materials under observation. It should be possible to understand the observed phenomena more deeply by examining the effect of the dose rate and the temperature dependence.

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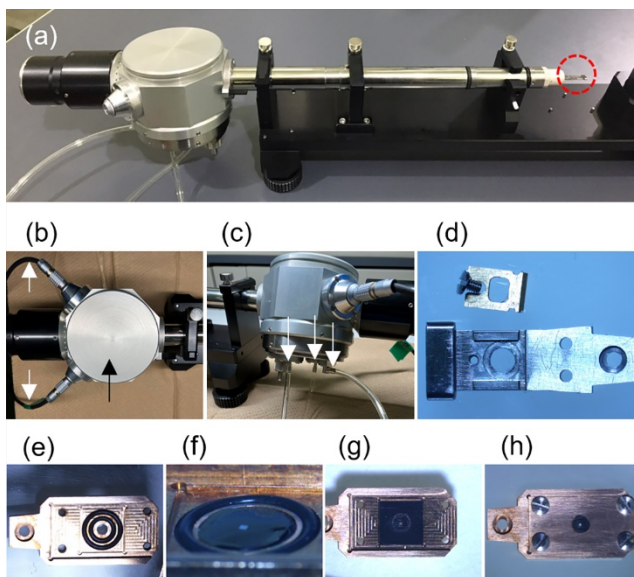
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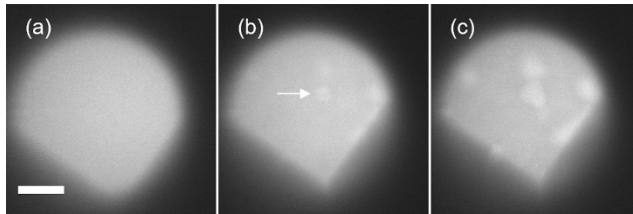
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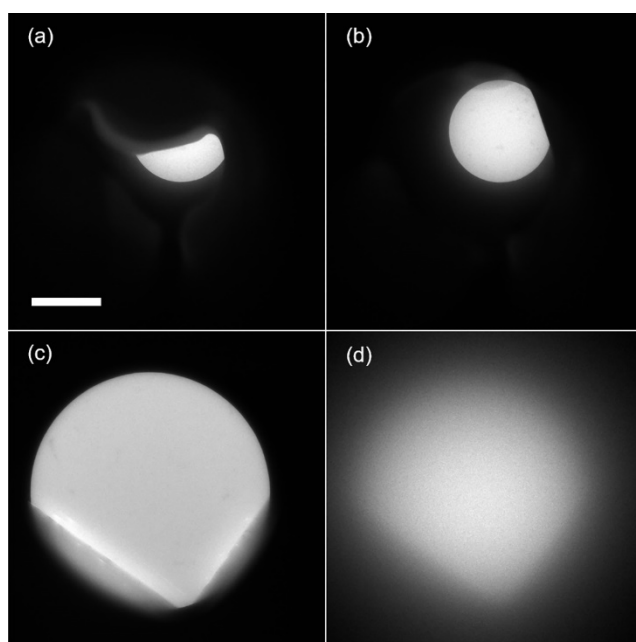


**Figure 1.** The Peltier holder and the procedure for assembly of the liquid cell. The liquid cell is assembled at the tip of the developed Peltier-cooling TEM holder, circled by the red dashed line (a). The Peltier device was installed inside the handle of the holder, as indicated the black arrow in (b), and the current is supplied and the temperature measurements are performed by using the signal lines indicated by the white arrows in (b). For circulation of the refrigerant, there are three ports indicated by white arrows in (c), two for circulation and one for removal of air bubbles by a syringe (c). The sample stage at the tip of the holder can be changed for samples on a conventional 3 mm $\Phi$  grid (d) or for liquid-cell experiments (e–h). The liquid-cell holder is made of copper on which the large and small O-rings are placed (e). A square Si chip

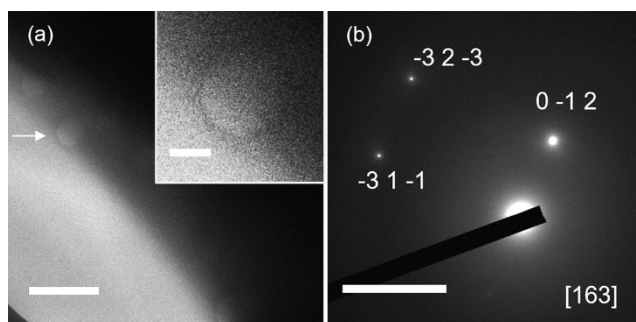
is placed on top of the small Si chip (f) and the large O-ring (g). The copper lid is then positioned and tightened in place by screws (h).



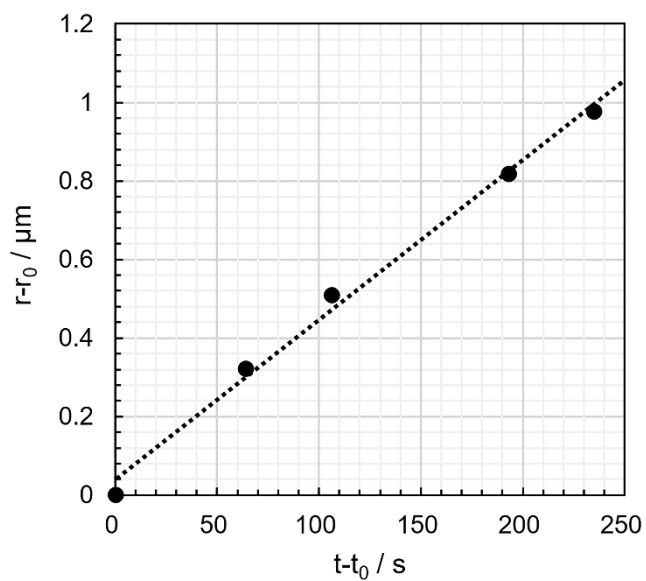
**Figure 2.** A series of bright-field TEM images showing the formation of bubbles in ice. The outer circular dark area is the shadow of the condenser aperture. Nothing is visible in the water sample at  $-8\text{ }^{\circ}\text{C}$  (a). The sample was continuously observed at an electron flux of  $1.8\text{ e}^{-}\text{ nm}^{-2}\text{ s}^{-1}$  (11%). The temperature was lowered from  $-8\text{ }^{\circ}\text{C}$  to  $-12\text{ }^{\circ}\text{C}$  over 3.5 minutes, and after another 3.5 minutes at  $-12\text{ }^{\circ}\text{C}$ , the formation of a bubble (indicated by the white arrow) was clearly observed (b). The bubble grew larger for the next 1.5 minutes (c). The scale bar is  $5\text{ }\mu\text{m}$ , and all images are on the same scale.



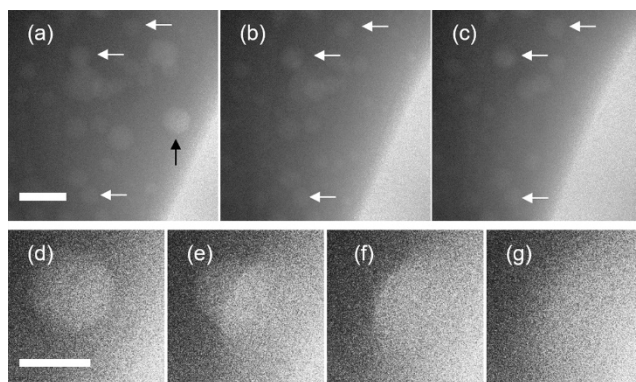
**Figure 3.** Dissolution of ice when the temperature was increasing toward 6 °C. These bright-field TEM images are from frames of Supplementary Video S1. At 0 s, with a selected-area diffraction aperture, ice was observed as a dark object (a). The ice receded and disappeared from the field of view at 2.7 s (b). After removing the selected-area aperture, no ice was present in the entire field of view at 5.2 s (c), and the field of view was filled with water at 7.2 s (d). The circular object in (c) is the condenser aperture. The electron flux was  $17 \text{ e}^- \text{ nm}^{-2} \text{ s}^{-1}$  (100%). The scale bar is 5  $\mu\text{m}$  and all images are on the same scale.



**Figure 4.** Identification of a formed crystal. The SAED pattern was acquired from the region of the crystal with the air bubble indicated by the white arrow in (a). The bright area in the lower left is the region where the large bubble was formed, and the dark area in the upper left corresponds to the ice crystal for which the SAED pattern was recorded. The inset is an enlarged image of the bubble. This bubble was just about to rupture, and these images were acquired at the point where the ice's crystal surface was nearly parallel to the electron beam. The diameter of the bubble was about 400 nm, and the thinnest part of the ice separating the bubble from the gas phase was about 100 nm. The SAED pattern corresponds to the [163] SAED pattern of ice Ih (b). The electron flux was  $65 \text{ e}^- \text{ nm}^{-2} \text{ s}^{-1}$  (50%). The scale bars are 1  $\mu\text{m}$  (a) 200 nm (a, inset), and 5  $\text{nm}^{-1}$  (b).

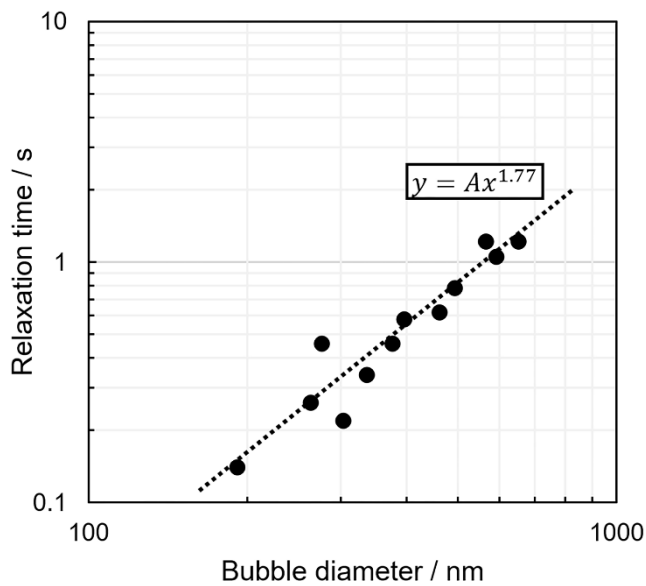


**Figure 5.** Change in the bubble size with time at an electron flux of  $1.8 \text{ e}^- \text{ nm}^{-2} \text{ s}^{-1}$  (11%) at  $-12^\circ\text{C}$ . Because the time of the bubble occurrence was unknown, the plots were produced by subtracting the size and time for the first bubble.



**Figure 6.** Recession of the ice surface and subsequent escape of bubbles. These bright-field TEM images are frames from Supplementary Video S2. Many bubbles were

598 formed in the ice at 0 s (a). The ice surface in the gas phase appears as the straight line  
 599 in the lower right of (a), and this receded after 15 s (b) and 30 s (c), even though the  
 600 bubbles were not moving (indicated by the white arrows). A magnified image of the  
 601 bubble indicated by the black arrow in (a) is shown in (d), and the subsequent escape  
 602 process of the bubble is shown in (e–g). The rupture of the bubble began immediately  
 603 after 0 s (d), progressed through 0.4 s (e) and 0.8 s (f), and was almost complete after  
 604 1.2 s (g). The electron flux was  $89 \text{ e}^- \text{ nm}^{-2} \text{ s}^{-1}$  (100%). The scale bar is  $1 \text{ } \mu\text{m}$  (a) and (a–  
 605 c) are on the same scale. The scale bar is  $500 \text{ nm}$  (d) and (d–g) are on the same scale.



606  
 607 **Figure 7.** Dependence of the bubble size on the relaxation time for bubble rupture. The  
 608 measurements were made on the bubbles shown in Supplementary Video S2. The power  
 609 function  $y = Ax^B$  was fitted with  $A = 1.3 \times 10^{-5}$  and  $B = 1.77$ .