Practical Considerations in the Successful Preparation of Specimens for Thin-Film Cryo-Transmission Electron Microscopy

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Cryo-electron microscopy (cryo-EM) can employ a number of specimen preparation methods such as high-pressure freezing, slam freezing or plunge freezing. These are conventional methods of vitrification and are fine-tuned for larger specimens. However, they all require the use of chemicals at some point during the preparation. The thin-film cryotechnique requires samples to be placed on an electron microscopy grid that is subsequently rapidly frozen in liquid ethane. Thin-film cryo-EM is the ultimate approach where samples in suspensions need to be viewed under near-zero processing artefact conditions and no chemicals are used.

In this contribution, we will discuss the cryo-EM approach illustrated through a variety of samples prepared under fully controlled vitrified aqueous conditions in a thin film using the Vitrobot[TM]. Liposomes, proteins, viruses and metal particles were chosen as reference samples as they have been proven to image relatively easily under cryogenic conditions. Furthermore, artefacts inherent to cryo-EM imaging will be briefly discussed. Together with minimal dose imaging, cryo-TEM opens-up a gateway for future cryogenic EM tomography studies on a wide range of materials.

Keywords: Cryo-TEM, Cryogenic imaging, , Liposomes, Proteins, Thin-film, Viruses, Vitrification, Vitrobot[TM]

1. Introduction

1.1   Benefits of cryotechniques

Freezing is a well-known fixation method of preserving structures. The concept of cryo-EM imaging dates back to the early 80's (for a review, see Dubochet *et al.,* 1988 [1]). As easy and straightforward as freezing can sound, it remains a challenge to preserve ultrastructure in a close to native state. This is due to the fundamental physics of water and ice [2]. Classical electron microscopy (EM) specimen preparation techniques that involve the use of chemicals, drying, staining steps, etc. are the foundation of modern biological electron microscopy. Decades of studies with these procedures have proven their utility for addressing innumerable ultrastructural questions, despite occasional problems with artefacts.

A successful fixation usually entails immobilisation of all structures over a scale smaller than the observation and imaging resolution range. In the case of cryofixation, the rapid cooling of the specimen will result in two sequential scenarios before the water temperature has reached -143°C (i.e., whereby the specimen is considered to be fixed in a stable configuration): (i) destabilisation phase of molecules by thermodynamics and (ii) the preservation of the structure by deprivation of activation energy within the specimen. As destabilisation due to thermodynamics occurs, molecules simultaneously rearrange themselves into a more stable structure depending on the temperature. In the case of water, hydrogen bonds or water crystals will form. As the temperature decreases, activation energy within the specimen lowers to a point where it falls below the threshold for further structural change and the sample is then considered stable.

Modification of the original structure of the specimen may occur during this process of molecular rearrangement. These modifications to the samples' original state are referred to as 'cryo-artefacts', visible as ice crystals. In order to limit the number of cryo-artefacts, the time window between the onset of cooling and the 'safe' temperature (i.e., -143°C) must be minimal. According to the phase diagrams shown in figure 1, only under very specific conditions can water crystal formation be totally avoided to achieve 'liquid-like' or vitreous ice. The vitreous ice is amorphous (has no long-range structure) and this makes it a desirable support phase for cryo-EM. The thermodynamic behavioural characteristics of water is also made use of in the high-pressure freezing method, where it is also possible to form vitreous ice. In this instance, samples are rapidly frozen at a pressure above 200 MPa and at temperatures between -40°C and -100°C.
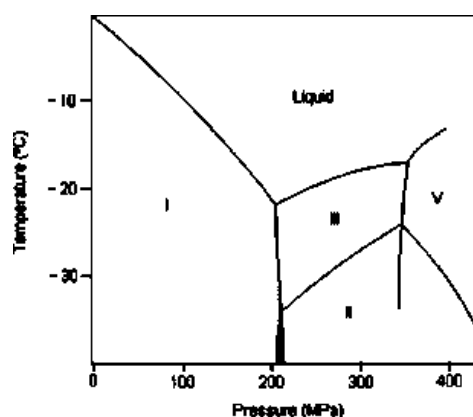
Fig. 1 Phase diagram of ice relative to pressure (MPa) and temperature (°C). Lines represent approximate phase boundaries. I to V represent the stable crystalline forms of ice. The greyed area represents vitreous ice condition. Reprinted from Hobbs PV. *Ice Physics*, Oxford: Clarendon Press. 1974 [3].

In the case of plunge-freezing, water is solidified without crystallization by rapid plunging of the sample into a liquid cryogen at atmospheric pressure [4]. The demonstration of the technique was first reported in 1986 by Bald *et al*. [5]. In this study, a general relationship between ice crystal size formation and critical cooling was reported, whereby the crystal size is inversely proportional to the cooling rate. Typically, crystallisation of ice does not occur under ultra-rapid cooling rates of $10^3 - 10^4$ °C/s [6] and plunge-freezing techniques rely on this [7]. In order to further increase the cooling rate sufficiently much thinner samples are used when compared to other freezing methods. Figure 2 shows how the temperatures at which both heterogeneous and homogeneous nucleation of ice crystallites decreases with sample volume.
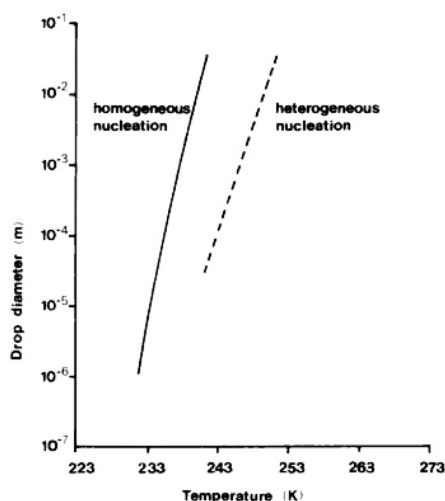

Fig. 2 Homogeneous and heterogeneous nucleation properties of water according to sample volume (drop diameter in m) and temperature (°C). Reprinted from Angell CA. *Ann. Rev. Phys. Chem*. 1983;34:593-630. [6]

Important to plunge-freezing is the choice of cryogen [1]. A range of cryogens exist and the choice thereof is a major contributor to a successful outcome as the cooling efficiencies are dependent on their thermal and physical properties (Table 1). For example, even though the cooling rates for propane and ethane are very similar, ethane sublimes much faster than propane. Volatile cryogens are less likely to leave residue in the specimen after vitrification.

Table 1 Thermal and physical properties of commonly used cryogens. *The cooling efficiencies are relative to propane at 1.0. Data extracted from Sitte H, Edelmann L, Neumann K. Cryotechniques in Biological Electron Microscopy. *R. A. Steinbrecht, and K. Zierold, eds*. Berlin: Springer-Verlag. 1987;88-113. [8]

Cryogens	Melting point (°C)	Boiling point (°C)	Cooling rate (10^3 °C/s)	Relative cooling efficiency*
Ethane	-183	-89	$-260 - -258$	1.3
Liquid nitrogen	-210	-196	-272	0.1
Propane	-189	-42	$-263 - -261$	1.0
Freon 22	-160	-41	$-267 - -265$	0.7

1.2   Thin-film cryo-TEM: The ultimate cryotechnique for samples in suspensions

Nowadays, a variety of cryo-sample preparation and imaging procedures are readily available [1]. Slam-freezing, high-pressure freezing and plunge freezing are a few examples of commonly used cryo-preparation techniques that can be found in the laboratory of the modern chemical and structural biologist. Each of these techniques is designed for a particular purpose. While slam-, and high-pressure freezing can typically cope with 'bulk' material (i.e., ~200 μm sample thicknesses); plunge freezing is the method of choice when one wants to examine solutes or suspensions via 'thin-film' cryo-TEM (i.e., typically ~100 nm film thicknesses).

Often, following freezing, the vitrified specimen undergoes post-processing preparation steps such as freeze-substitution, cryomicrotomy and staining — and even in some instances mild chemical post-fixation steps. Although these steps may appear to be advantageous at first sight, the exposure of the specimen to chemicals and additional preparation steps increases the likelihood of unwanted structural artefacts. In contrast, thin-film cryo-TEM offers an attractive alternative to prepare and view the specimen with a minimum of preparation steps resulting in fine structural data devoid of artefacts. In this approach the entire specimen is rapidly plunge frozen into liquid ethane and remains below the devitrification temperature (-143°C) throughout the TEM observation process. However, the specimen size is the main restriction of the technique. Successful vitrification is achieved for specimens thinner than 1 μm and the spatial resolution decreases for specimens with a thickness over 100 nm [9]. Such thin-film methods have been proven to be an excellent tool to observe specimens in their close-to-native state. Examples of successful thin-film cryo-EM include liposomes, micelles, viruses, metal particles, as well as proteins, gels, organelles or small cells [10] [11] [12].

In the following sections, the materials and methods for freezing samples using the Vitrobot[TM] will be outlined. Special attention will be paid to practical tips and tricks that pave the way for successful thin-film cryo-EM observation. This will be illustrated with examples of a wide range of specimens successfully prepared and imaged under full cryo conditions, including proteins, liposomes, viruses and nanomaterials. Commonly observed artefacts and their proposed solutions are also discussed.

2. Materials

The specimen preparation stage is considered to be by far the most critical part of the experiment. A dedicated cryo-TEM microscope optimised for cryo-imaging will not overcome sample preparation issues. The correct choice of consumables, the selection of cryogen and type of EM grids to be used are all of prime importance.

2.1   Reagents

The following is a list of reagents used to prepare and image thin-film samples for cryo-TEM:
- Phosphate buffer saline
- Distilled water
- Liquid nitrogen
- Ethane gas

As mentioned before, ethane is the preferred cryogen for plunge-freezing as its relative cooling efficiency is higher compared with propane. Precautions should be taken when handling cryogens. Cryogens have a large liquid to gas expansion ratio and therefore it is important to work in a well-ventilated area to avoid potential suffocation. Ethane is an explosive gas and all steps should be performed in an explosion proof fumehood with no possible source of ignition present.

2.1.1   Sample concentration

The concentration of the features of interest (liposomes, viruses, etc.) in the solution must be high enough to allow their location within the TEM specimen, but low enough so the specimen is not overloaded to the point where pile-up of features in projection obscures specimen details. Depending on the molecular weight and viscosity of the sample, the concentration should be optimised by trial and error. A concentration of approximately 10 mg/ml is a good starting point during performance tests with liposomes (up to 100 nm in diameter) [13] and is also a good starting point in optimising the concentration for a new material (see later). If the features of interest are larger than 100 nm, then concentration may be reduced and *vice versa*. In cases where the sample can not be diluted or when dilution would affect the experimental data, the blotting parameters will determine the final sample thickness (Table 2).

2.2   Equipment

Below is the list of equipment used to prepare and image thin-film samples for cryo-TEM:
- FEI Vitrobot[TM], (FEI Company, Hillsboro, OR, USA, www.vitrobot.com)
- JEOL 2100 Cryo-Transmission Electron Microscope (JEOL, Tokyo, Japan)

- Plasma cleaner (South Bay Technology Inc., PC-150)
- 595 Filter paper, punched, for the Vitrobot^{TM}, (ProSciTech, 47000-100, www.proscitech.com.au)
- MICROMAN M10, Precison microliter pipette, Positive displacement pipette (GILSON, France)
- Capillaries and pistons CP10 TIPACK^{TM} (GILSON, France)
- Lacey formvar / carbon on 300 mesh copper grids (ProSciTech, GSCU300FL, www.proscitech.com.au)
- Quantifoils R2/2 (ProSciTech, SQR22-200CU, www.proscitech.com.au)
- Copper grids, 600 mesh hexagonal (ProSciTech, GCU600TBH, www.proscitech.com.au)
- Filter paper
- Storage grid box
- Storage grid box tool
- Typical tools for handling cold objects: liquid nitrogen dewar as a transfer container, long tweezers or forceps, gloves, safety goggles

Note: Cryogens and any tools that come in contact with them should be handled with care, as they can be very cold and cause severe burns: Personal Protective Equipment (PPE) should be worn at all time (e.g., gloves, goggle, etc…).

2.2.1 Choice of grid

The correct choice of grids is critical in the successful preparation of cryo-EM specimens. Different types of grids can be used for preparing thin-film samples for cryo-TEM:

Lacey formvar support films are ideal to prepare specimens during initial experimentation, when trying to determine the optimum parameters. These grids are usually less expensive than Quantifoils® but are more sensitive to the electron dose. Grid holes sizes range from 0.25 μm to 10 μm and allow for support of a large range of sample sizes.

Quantifoils® have a support film of pre-defined holes size, shape and arrangement. Circular holes of 1 or 2 μm (R1/2 or R2/2) are preferred and these types of grids are a better choice when it comes to high-resolution imaging as they are more electron stable. In addition, when imaging using automated low dose techniques, the well-defined spatial relationship of the holes permits the blanked beam to be stepped between regions of interest, without any prior beam exposure.

Commercially purchased support films are usually hydrophobic. Glow discharging them with Argon in a plasma cleaner for 15 s or under a UV light for 5 min will increase their hydrophilicity and improve the adhesion of the specimen to the support film. In cases where the support film is incompatible with the sample, 600 mesh hexagonal copper grids can be used. These grids offer hole sizes of approximately 30 μm.

3. Methods

The method below gives a detailed description of the sequential steps involved in the preparation of thin-film cryo-TEM specimens using the Vitrobot^{TM}.

3.1 Preparation of the reagents

Before attempting to liquefy ethane, it is important to ensure the temperature of the ethane container is below the liquefaction temperature of ethane by cooling down the coolant container (outer ring and inner cup) with liquid nitrogen. The inner cup can, which holds the liquid ethane, can be cooled rapidly by filling it with liquid nitrogen. When the temperature of the container has stabilised and the liquid nitrogen from the inner cup has totally evaporated, the outer ring can be replenished with liquid nitrogen and liquefaction of ethane in the inner cup can begin by placing the ethane delivery tube into the bottom of the cup. The ethane delivery tube must be dry beforehand; otherwise, condensation in the tube can freeze in contact with the cold cup and obstruct the flow, eventually bursting the tube. The regulator of the ethane cylinder is adjusted to approximately 10 kPa (1.5 psi): this provides enough flow for the ethane to liquefy and is low enough so that the outer ring does contaminated with ethane. The level of ethane in the cup should be to the rim of the metal spindle to ensure to that the entire grid will be submerged in the liquid ethane during freezing.

The ideal temperature of the liquid ethane is indicated by the presence of both solidified ethane around the periphery of the cup, while liquid ethane remains in the centre (figure 3). If the ethane becomes too cold and solid ethane is present in the centre of the cup a dry pair of tweezers or similar can be used to provide heat input to melt the ethane. Specimens must never be plunged into ethane that contains excessive solid, as damage to the specimen/tweezers will result. Careful planning is important to ensure that when the ethane reaches the ideal freezing temperature the sample and instrument are ready for plunge freezing (cf. 6.1).

3.2 Sample application

The sample can be applied onto the grid either manually or automatically. Our preference is to use the manual method. In this case, 3 µl of the sample is applied via the side port of the Vitrobot[TM] directly onto the carbon-coated side of the TEM grid by using a positive displacement pipette.

In cases where the sample concentration is too low, sequential application can be used, whereby the sample is applied on the grid then blotted with filter paper by slightly touching the grid at a low angle, through the side port. The sample is then applied a second time on the same grid and finally blotted with the blotting pads and frozen in ethane. This technique increases the amount of sample adhering to the TEM grid.

The sample application can also be performed automatically. This is achieved by dipping the grid into a 1.5 ml Eppendorf vial of the sample, which is placed inside the climate chamber of the Vitrobot[TM]. Blotting parameters would vary if this method were chosen over the manual application for identical samples. More details about this application method can be found in the Vitrobot[TM] user manual [14] and the "How to use the Vitrobot[TM]" manual [15].

3.3 Preparation of the instrument and blotting parameters

The Vitrobot[TM] allows for temperature and humidity control in the climate chamber until immediately prior to freezing. For samples sensitive to evaporation or osmotic effects, the relative humidity (rH) is set above 99% to prevent drying artefacts. The temperature can be adjusted to suit the experiment. For temperatures above 30°C, time must be allowed for the chamber to reach the desired temperature before turning on the humidifier.

Fig. 3 Ethane at its ideal freezing temperature. The cryogen is present in both its solid state (around the rim) and liquid state (centre of the cup). The four-legged metal spindle allows for heat conductivity between the inner cup and the outer ring, which contains the liquid nitrogen.

At the start of each experiment, it is advised to use new blotting filter paper as these can absorb humidity during an experiment and change in shape. One set of filter paper can be used for up to 16 blots in one experiment. Ideally, good vitrification entails specimen film thicknesses below 100 nm, evenly distributed over the entire grid [16]. Aside from the sample concentration, the blotting parameters will be the other critical factor in determining the film thickness and concentration of features of interest in the specimen.

Some typical starting conditions for optimising specimen preparation are listed below. Perform a manual application of a sample (10 mg/ml) to the grid. Set the Vitrobot[TM] parameters as follows: blot time to 2 s, blot total to 1, relative humidity (rH) to 100% and blot offset to -2 mm. The anticipated result would be a thin and clear layer of ice on the grid. If the ice layer is too thick, increase the blotting time. If no sample remained on the grid then decrease the blotting time. Samples with higher viscosity may require longer blots, or even an increased number of blots. If this is not sufficient, the use of a 300 or 400 mesh grid increases the area of thin ice in the centre of the grid. Increase the drain time to even out the thickness of the ice on the surface of the grid when the other blotting parameters have been determined. The latter can also be adjusted to thin the ice if more than 0.5 s (minimum blot time) is required.

Many researchers have prepared samples for cryo-TEM and carefully defined satisfactory sample preparation parameters for a variety of different types of samples. Table 2 provides a summary of some good starting points for vitrifying different types of materials using the Vitrobot[TM].

Table 2 Guideline for blotting parameters for a range of specimens, determined by Iancu *et al.* (2005) [11] and Frederik *et al.* (2009) [12]. Blot time: duration the grid is blotted against the blot pads. Drain time: waiting time after the blotting and before the plunge freezing. Blot total: number of blots. Blot offset: angle between the grid and the blot pads.

Samples	Blot time / drain time (s)	Blot total	Blot offset (mm)
Large protein complexes	3 − 4	1 − 2	2 − 3
Viruses	2 − 3 + drain time 0.5 − 1	1 − 2	2
Organelles	1 − 2	1 − 2	1 − 2
Cells	1 − 2 + drain time 1	1 − 2	1 − 2
Gels (low viscosity)	5 − 6 + drain time 1	1 − 2	2 − 3
Gels (high viscosity)	5 − 6 + drain time 1	2 − 3	2 − 3
Liquid emulsions	2-3	1	2

3.4 Specimen retrieval and storage

The Vitrobot™ plunges the specimen into the ethane held in a pair of tweezers. Once the specimen is frozen, the tweezers are removed from the plunger, and the specimen is blotted to remove excess ethane. Care must be taken so that the specimen does not warm above the critical temperature for devitrification (-143°C). The plunge-frozen specimens are then stored under liquid nitrogen in a receptacle in the baseplate of the liquid ethane holder. The grid can be stored indefinitely under liquid nitrogen, although it is recommended to view it as soon as possible to prevent potential contamination by ice crystals resulting from repeated movements between storage containers.

After each plunge freezing experiment, the outer ring should be replenished with liquid nitrogen to ensure effective cooling of the liquid ethane.

3.5 Imaging under cryo conditions

Fast acquisition and low dose are key towards successful cryo-EM data collection. During imaging, the critical consideration is beam damage. It is unavoidable and irreversible. However, commercial solutions, such as the 'Minimum Dose System (MDS)', significantly reduce beam damage to the sample by using extremely low electron doses when surveying the sample. Focusing and beam convergence are typically set up on a sacrificial region, which is damaged. The beam is then blanked and automatically moved onto a region of interest, which has had negligible beam exposure, and is therefore imaged without any prior beam damage artefacts. Beam damage is most noticeable as large voids, which form in the vitreous ice.

4. Examples & Discussion

4.1 Example 1: Cryo-negative staining of proteins

Negative staining can be applied to samples of very small sizes and/or low densities to increase the contrast. A small quantity of heavy metal stain is applied to the specimen grid (equal volume of stain and sample), during the process. As the exposure time to the stain is very short (few seconds to one minute), the chemical has very limited effect on the specimen.

Figures 4A and 4B are examples of vitrified, negatively stained haemocyanin proteins imaged with cryo-TEM. Haemocyanins are respiratory proteins (oxygen carriers) found in the lymphatic system of shelled organisms [17]. The purified sample concentration is 1:50 and a volume of 1.5 µL was applied to the grid. Additionally, an equal volume (1.5 µL) drop of saturated ammonium molybdate has been added to the grid and left for 10 s in the chamber. The grid was then blotted 3 times for 2 s each preceding vitrification.

4.2 Example 2: Liposomes

Liposomes are vesicles formed by phospholipids self-assembled into a bilayer. Due to their nature, they are very sensitive to dehydration. In the past, they were commonly viewed using negative staining where the specimen was air-dried on a carbon-filmed grid then negatively stained with heavy metals. The resultant liposomes would then have a stacked and crumbled appearance due to the dehydration. Imaging resolution is affected by the presence of stain, and in some cases, the surrounding stain can also conceal some of the liposome layers if present.

Nowadays, liposomes are the most commonly used samples in performance tests for thin-film cryo-TEM samples. Samples vitrified using the Vitrobot™ have a "plump" aspect, as shown in figures 4C and 4D. This is the result of high relative humidity maintained up to the moment the sample was frozen. Dehydration is nearly absent.

Concentrations ranging from 10 mg/ml for large vesicles to 2 mg/ml for smaller vesicles have been tested to give thin and homogeneous thin-films, suitable for cryo-TEM [13] [18]. In this example, the liposomes are approximately 90 nm in diameter and are made of phosphatidyl choline and cholesterol. In the example provided, an antibiotic drug, ciprofloxacin, is encapsulated within the liposomes [19] [20]. Similar cryo-TEM work related to liposomes has been reported by Almgren *et al.* (2000) [21] and Talmon (2007) [22].

4.3 Example 3: Sequential application for low concentration virus samples

Viruses are conventionally observed by negative staining. However, freezing them offers the advantage of omitting the use of chemicals, which can affect the sample morphology.

In some cases, mainly due to availability, it can be difficult to collect a high enough concentration of the sample for thin-film preparation. Sequential application can be useful here as samples can be applied 2 to 3 times on the grid to increase the concentration of sample attached to the grid.

Figures 4G and 4F show examples of tobacco mosaic viruses. The viruses were collected by cutting small pieces of infected tobacco leaves (approximately 5x5 mm) and rinsing them in 1 mL of water. A drop of 3 µL was first applied and manually blotted with a filter paper inserted through the side port. The application was repeated 3 times before automatic blotting of 2 s once followed by plunge freezing.

4.4 Example 4: Iron-gold nanoparticles

Although these gold – iron nanoparticles are not affected by dehydration, they are prone to aggregation and aging within minutes after sonication in water [23]. In order to view them in their fresh and dispersed state, the Vitrobot[TM] can be set up beforehand so the sample can be frozen within seconds after sonication, and within minutes after synthesis. Figure 4G and 4H show vitrified iron-gold nanoparticles, diluted 1:1 in water. The sample was imaged immediately after synthesis. First, they were dried, re-suspended in water and sonicated for 10 min, right before freezing.
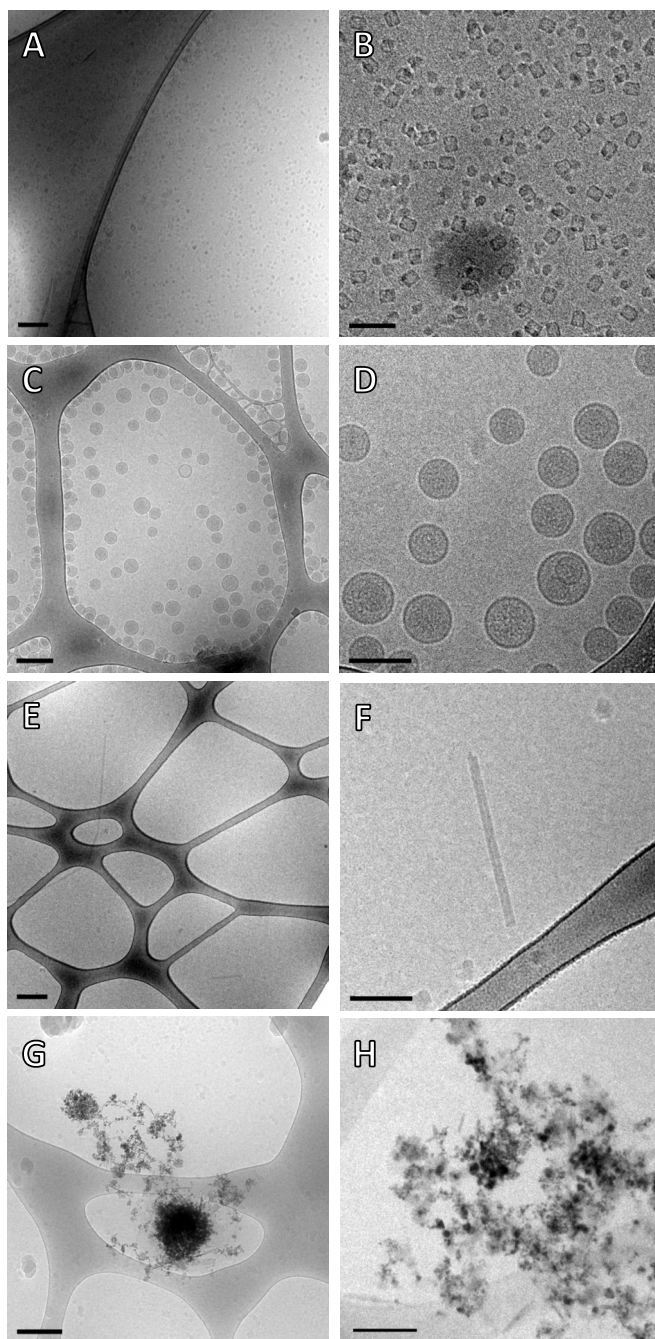

Fig. 4 Examples of various materials imaged using cryo-TEM. **A**, **B**: Haemocyanin proteins negatively stained with saturated ammonium molybdate (sample concentration = 20 mg/mL; sample volume = 1.5 μL; stain volume = 1.5 μL); **C**, **D**: Liposomes (concentration = 5 mg/mL; sample volume = 3 μL); **E**, **F**: Tobacco mosaic virus (sample volume = 3 μL, applied sequentially 3 times); **G**, **H**: Iron-Gold nanoparticles (concentration = 1:1, sample volume = 3 μL). For each of the samples, a lacey formvar 300 mesh copper grid was used and the blotting parameters were: temperature = 22°C, rH= 100%, blot time = 2 s, blot total = 1 and blot offset = -2 mm. Scale bar: 0.2 μm (left) and 0.1 μm (right).

5. Commonly observed artefacts

Specimens that have not been optimally prepared present artefacts inherent to improper sample handling. Below, we summarise the most commonly encountered artefacts that even experienced cryo-EM microscopist will encounter.

5.1 Non-vitreous ice

No matter how well the sample is initially prepared, imprudent handling or transferring the sample between storage vessels or the microscope holder will expose the sample to elevated temperatures, which may potentially exceed the devitrification temperature (-143°C). Devitrification manifests itself as the formation of ice crystals within the vitreous ice. This can arise if the vitreous ice is exposed to temperatures above the devitrification temperature (-143°C) or if the rate of cooling during plunge freezing was inadequate.

Contamination deposited onto the specimen during transfer is often in the form of large hexagonal ice crystals on the surface of the specimen. Large hexagonal ice crystals (Fig. 5A) typically display Fresnel fringe contrast when the main thin film is close to the minimum contrast position, confirming that they are depositions on the outside surface of the film. Such crystals can originate from a contaminated ethane solution and/or any of the intermediate liquid nitrogen containers (transfer container, chamber of the TEM holder transfer station) Ice forms if these vessels are not thoroughly dried before being filled with liquid nitrogen or if frost has been allowed to build up. The hexagonal ice crystals can be small enough (in the range of the 100s nm) to attach themselves on the surface of the grid, as they are free floating in the liquid nitrogen or liquid ethane.

5.2 Sample uniformity and thickness

Glow discharging the grid is important to induce good hydrophilicity to the carbon film. This improves wetting and results in improved attachment and a more even distribution of the sample on the grid. If this is insufficient, the sample will not be evenly distributed and non-uniform film thickness results (Fig. 5B).

If the ice layer is too thick, it can be seen as a layer of cubic ice covering the entire grid (Fig. 4C). Due to the large thickness, the cooling rate is inadequate to form vitreous ice and cubic ice results instead. Increasing the drain time (waiting time between the blotting and the freezing) can produce a thinner layer of ice more evenly distributed across the grid once the sample concentration and other blotting parameters have been optimised.

5.3 Residual ethane on the grid

Some residual ethane will be present on the surface of the frozen specimen if it is not removed from the grid before being transferred to liquid nitrogen; the ethane will freeze *in-situ* and will remain as a contaminant, including during TEM examination. It is therefore important to carry out a blotting step after removal from the liquid ethane to remove excess liquid and allow any residue time to boil away.

It is important to transfer the grid from the ethane cup to the storage container by slowly lifting it up from the liquid ethane and blotting both sides of the grid with pre-cooled pieces of filter paper. The presence of residual ethane can be detected when the grid is placed into the grid storage container as white solid ethane will form on the grid at the instant it touches the liquid nitrogen. This residue can be removed by transferring the specimen back into the liquid ethane to warm it and the process of transferring the grid to the storage container can be repeated. The specimen can then be rechecked for the presence of any white ethane residue. If only small amount of residual ethane remains, it will appear as a layer of cubic ice on the surface of the grid, which evaporates sufficiently fast in the TEM. If a large amount remains, it will appear as a layer of hexagonal ice (Fig. 5D).

5.4 Ice contamination during imaging

The imaging instrument should be ideally equipped with an anti-contamination device (ACD) as well as an oil-free vacuum pumping system (scroll/turbo pumps) to prevent contamination of the microscope column/specimen. Microscope-induced contamination is seen as small ice crystals depositing on the surface of the specimen during imaging. The number and size of these crystals grows with time. Water vapour also evaporates from the specimen during imaging: this is especially true for thick specimens or specimens containing large amount of organic material, as they are more beam sensitive.

The use of a cryo-holder is required to maintain the specimen below the devitrification temperature. The cryo-holder has a small dewar for holding liquid nitrogen coolant. This dewar is equipped with a zeolite sorb to actively pump the dewar's vacuum when cold. The sorb will actively adsorb any gas that leaks into the vacuum space of the holder's dewar. However, once it becomes saturated, the vacuum will degrade rapidly. Under these circumstances, the outside of the dewar will ice up, the nitrogen will boil away quickly and the holder will perform badly in terms of stability and resolution. In extreme cases, the o-rings may freeze and a microscopy vacuum leak can occur. The zeolite sorb should therefore be maintained through baking-out at regular intervals depending on the frequency with which the holder is used.
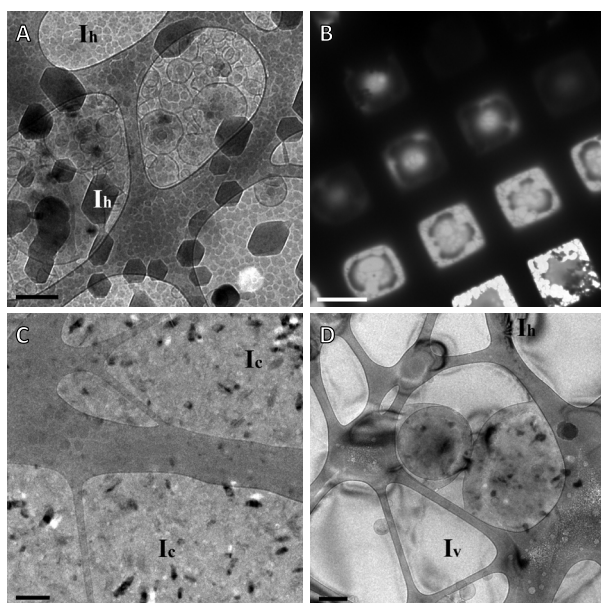
Fig 5. Examples of common artefacts encountered in sample preparation for thin-film cryo-TEM. **A**: the presence of small hexagonal ice crystals (black I_h) suggests that the sample has been warmed up above devitrification temperature or very poorly frozen. Large hexagonal ice crystals (white I_h) present on the surface of the sample: these are depositions, characterised by the presence of a surrounding white Fresnel fringe and are very stable under the beam. **B**: Unevenly distributed and frozen sample across the grid due to poor hydrophilicity of the carbon film. Some areas are too thick when others are empty: the amount of sample area that can be imaged is then limited. **C**: cubic ice crystals (I_c) covering the entire grid. **D**: a thin layer of hexagonal ice (I_h) covering the surface of the grid with "waving-like" electron scattering. The underlying sample contains vitreous ice (I_v). Scale bars: B = 50 µm; A, C and D = 0.2 µm.

6. Tips for successful and consistent results

6.1 Operating protocol

The suggested protocol below is only indicative and has been developed to reduce waiting time in between the different pre-freezing preparation steps. If the steps are followed in this particular order, the Vitrobot™ and grids will all be ready to be used once the ethane reaches the optimal freezing temperature.

1. Prepare the grids for glow discharging by putting them in the plasma cleaner chamber and start the pump.
2. In the meantime, cool down the coolant container. Leave it to cool and wait for the Leidenfrost effect to cease.
3. Start up the Vitrobot™ and set the desired parameters. Close the Vitrobot™ chamber (by clicking on "Place new grid" and "Continue"). By now, the coolant container should be cold enough to liquefy ethane.
4. Replenish the outer ring with LN_2 and start liquefying ethane. Leave the ethane to cool. By now, the plasma cleaner should have reached the right pressure for glow discharge.
5. Glow discharge the grids. By now, the ethane should have cooled down to the optimal freezing temperature. The temperature and humidity of the Vitrobot™ chamber should have reached the desired parameters as well.
6. You are now ready to place a new grid and vitrify your samples.

Note, depending on the stability of the sample, and if it needs to be diluted or not, dilutions can be made-up before step 1 or before step 6.

6.2 Imaging conditions

In case the vitrified sample is contaminated with small amounts of cubic ice or contains excess ethane, exposing the sample to low electron dose usually eliminates this thin layer of ice covering the sample (approximately 1-2 minutes or until the vitreous ice is exposed). These artefacts arise from repeated exposure to room temperature or residual ethane being frozen into the specimen respectively. These are usually very small ice crystals and are less stable under the electron beam compared to the underlying vitreous ice. As shown in figures 6A and 6B, irradiating the sample with low dose would then thin the ice layer and expose the underlying vitreous ice, which contains the features of interest.

As the vitreous ice forms a minuscus in the holes of the support grid, it is also important to acquire images in the defocus range (about -3 μm) from the eucentric position (minimum contrast focus) to optimise the contrast (eg figures 6C and 6D).

Data acquisition must always be done with a view to minimising the electron dose the specimen receives. Depending on the dosage used, the image should be acquired within a couple of minutes (Figures 6E, 6F). It has to be assumed that no matter how fast or how low a dose is used, there will always be some beam damage present in the specimen. Samples, which are completely embedded in vitreous ice, are the most stable compared to samples partially embedded in vitreous ice or in crystalline ice.
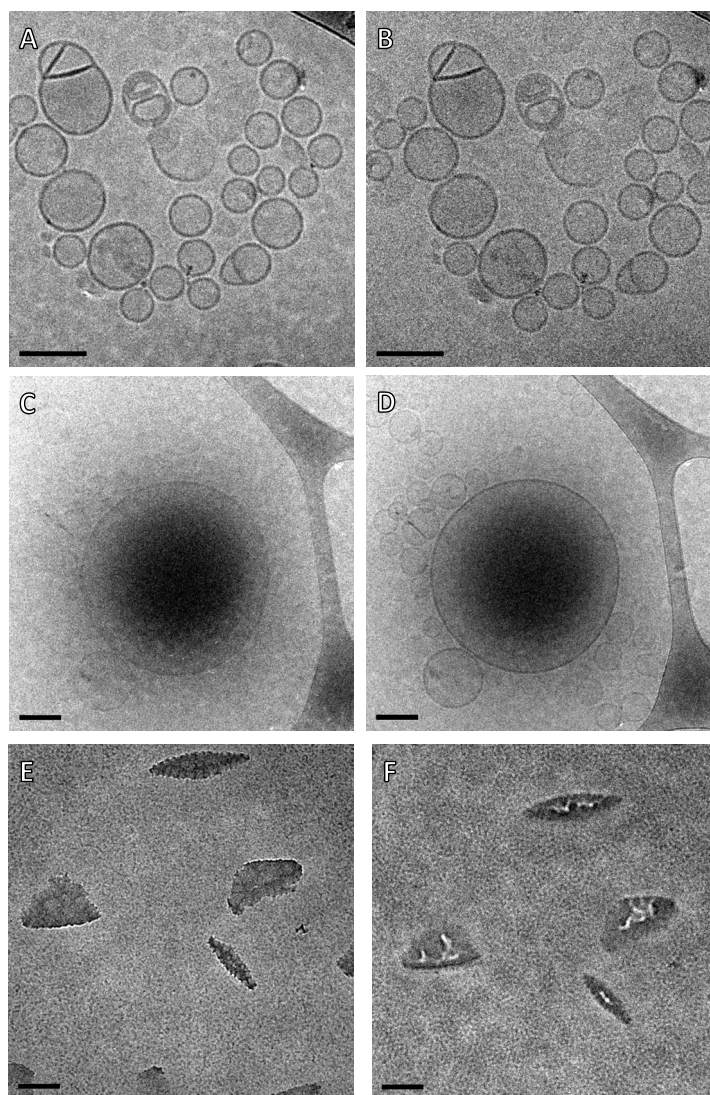
Fig. 6 Optimising microscopy conditions are important to obtain the best images from cryo samples. **A**, **B**: Low dose irradiation of the sample exposes the sample to the surface (**A** = image captured immediately after exposing the sample to the beam; **B** = image captured 5 s after exposing the sample to the beam). **C**, **D**: Defocusing increases the contrast (**C** = at minimum contrast, **D** = -3 μm defocus). **E**, **F**: Imaging should be performed as quickly as possible to reduce beam damage (**E** = imaged within 3 min; **F** = imaged after 5 min). Scale bars = 0.1 μm.

6.3   Note on consumables

Cryo-holders designed for analytical microscopy have beryllium inserts in the specimen holder. These are very expensive and brittle, and may fracture under thermal cycling. Phosphor-bronze screw inserts are generally superior where analytical work is not required, due to their greater robustness and significantly lower cost. Care should be taken when handling cold plastic objects, as they become extremely brittle. The Vitrobot™ filter paper can be substituted with Whatman #1 or 595 filter paper, with a 20 mm centre hole. Punched filter papers are commercially available. Alternatively, the hole can be made with the use of a paper punch. Ensure the filter paper remains flat so it does not affect the blotting offset.

7. Conclusion

Some researchers will still debate whether it is possible to achieve perfect vitrification of water. However, hundreds of research papers clearly show that good results can be obtained and that cryotechniques have brought great insights to the structural and chemical biologist. The cryo-EM specimen preparation techniques are relatively inexpensive. The methods do require some practice to master and optimisation may be necessary for each new material examined. However, with care, good results can be obtained relatively quickly and routinely. The avoidance of involved processing steps and fixatives/stains provides confidence that the results are artefact-free and represent a true snapshot of the material in its native state. Future developments in microscopy instrumentation, such as direct detection cameras [24], may enable routine imaging at much lower doses than is currently possible, greatly reducing the problem of radiation damage.

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