Vitrification of soft matter for cryo- electron microscopy

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Vitrification of aqueous solutions is a prerequisite for cryo- electron microscopy and cryo- electron tomography, and therefore forms the basis for a significant area of modern structural biology. Theoretical and technical considerations mean that structural studies of macromolecules and viruses have excelled, whereas studies of eukaryotic cell biology and hydrogels have been limited. Vitrification has nevertheless had a significant impact in the freeze-substitution methods that have helped to eliminate the artifacts caused by chemical fixation. These methods are more amenable to studies of thicker specimens including hydrogels, and are generally regarded as superior to classical methods. The combination of vitrification and focused ion beam milling has opened up opportunities for exploring cells in their (frozen-)hydrated state. This chapter describes vitreous water, how it is prepared in practice, the physical limitations to this process, and examples of what we have learned since it became practical to prepare vitreous samples for electron microscopy.

Keywords: amorphous ice; biomaterials; cryo- electron microscopy; soft matter; virus structure; vitreous water

1. Vitrification of aqueous solutions

1.1 Background and significance

Electron microscopy of soft matter (biological specimens, polymers) has undergone decades of development aimed at addressing the incompatibility of aqueous specimens with vacuum [1]. At the beginning of the 1980s, the single, unifying and dominant feature of hydrated polymer systems – water – was exploited to achieve solid-state electron microscopy of frozen-hydrated, soft matter [2]. The practical demonstration of vitreous water and its use for electron microscopy sample preparation has revolutionized the field of structural biology.

The concept is remarkably simple: when water is frozen at a sufficiently fast rate, it is transformed into a solid and amorphous phase that functions as an electron-transparent embedding medium for cryo- electron microscopy (cryo-EM). Based on theory and experience with traditional methods, it was predicted that biological matter would not yield sufficient contrast to enable visualization when frozen; however, underfocussing of the objective lens was shown to generate sufficient phase contrast to make the process worthwhile (Figure 1). This meant that the artifacts and inaccuracies of staining as well as the artifacts of aldehyde-induced cross-linking could be avoided altogether.

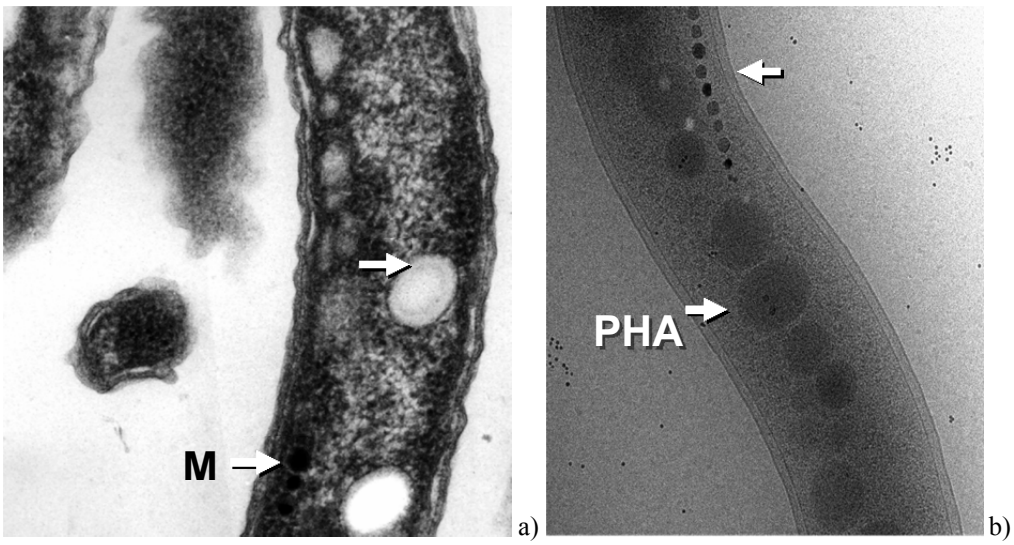

Fig. 1 Comparison of micrographs obtained by (a) classical electron microscopy and (b) cryo-EM. Both micrographs show bacteria belonging to the genus *Magnetospirillum*. In (a), chemical fixation has preserved the overall morphology but the cell wall and membranes appear distorted, especially compared to the chemically unaltered structure visible by cryo-EM. Strong contrast is due to the addition of heavy metal stain. This provides significant amplitude contrast. Note that the contrast of some components is reversed depending on affinity for stain. For example, the polyester inclusions (PHA) are mostly unstained in (a) but appear naturally electron-dense in (b). Iron oxide deposits (magnetosomes, M) and added colloidal gold particles are also comparatively electron-dense in the unstained specimen due to their higher atomic numbers. They appear with remarkable clarity where amplitude contrast is otherwise poor. Contrast can be enhanced artificially but signal-to-noise ratio can only be altered at the stage of acquisition, if the sample can tolerate the extra applied dose or if a more efficient detector is used. Courtesy of Dirk Schuler and Manuela Gruska.

The sensitivity of ice embedded specimens to radiation damage means that only a finite electron dose can be applied, resulting in images with a correspondingly finite signal-to-noise ratio, which ultimately limits the amount of information that can be obtained. Nevertheless, biologists have learned how to make every electron contribute to the end result. Procedures were determined for searching, focussing and positioning features during acquisition without exposing the target unnecessarily. Combined with advances in detector sensitivity and automation, cryo-EM became the standard technique for structure determination in virology as well as for the determination of (multicomponent) supramolecular complexes that are intractable to analysis by crystallographic techniques or those that can only be visualized outside of the cellular context [3]. Vitrification has also been applied successfully to studies using X-ray microscopy and tomography [4-6].

This chapter will first review the physics of amorphous water, and will then describe the most widely used methods for vitrifying aqueous samples: immersion freezing in liquid ethane, and high-pressure freezing. If the specimen cannot be studied by immersion freezing, high-pressure freezing will provide some benefit via cryo-EM of vitreous sections (CEMOVIS) or via the hybrid freeze-substitution route.

1.2 The physical chemistry of solid water

Water is the dominant component in biology. In popular culture, it exists only as liquid, solid and vapor. The phase diagram of water (Figure 2) shows the deceptive complexity of this most important of solvents. It demonstrates that frozen water can be achieved by several routes but that ice exists in many forms, only one of which is known to be suitable for cryo- EM. This amorphous form of solid water is thought to closely resemble liquid water, and it is the reason for its exquisite ability to preserve structure. It is described as hyperquenched glassy water, a low-density phase, and it can be thought of as a viscous form of liquid water. Although vitreous ice is probably metastable, it is stable for experimental timescales (and probably years, as long as it is not allowed to dehydrate in liquid nitrogen). Vitreous ice is characterized by the properties of a homogeneous, non-brittle solid: it can be sectioned at low temperature using a diamond knife, and if the vitreous sections are examined in the frozen-hydrated state, the CEMOVIS technique is analogous to direct examination of vitreous thin films.

Vitreous ice is a glass that also has the properties of a highly viscous fluid: mechanical pressure causes it to flow noticeably during a timescale of seconds to minutes. This is inconceivable for crystalline phases of ice. The phenomenon can be observed when sectioning specimens that have been vitrified by high-pressure freezing (Section 2.3). When the specimen is clamped too tightly in a sample-holder, it begins to ooze from its support. It is also likely that the release of pressure from the confined space contributes to this phenomenon.

Chemically, liquid water and vitreous water have similar Raman spectra [7]. The amorphous visual appearance of vitreous water by cryo-EM is usually sufficient to proceed with an experiment. Extensive crystallinity is easy to recognize, and scattering in the form of Bragg reflections indicates crystalline forms (some Bragg reflections are visible in Figure 1b; these are caused by the iron oxide particles). If there is doubt about the state of the ice, operating the microscope in diffraction mode is useful, since characteristic diffraction spots and sharp rings will be visible. Using the same technique, amorphous water gives rise to diffuse rings with characteristic spacing [8]. Hydrohalite, a crystalline salt hydrate of general formula $NaCl{\cdot}2H_2O$ [9], has been shown to form extracellularly in cryopreserved samples under certain conditions [10, 11], and recently, it was shown to occur intracellularly [12]. This is also an indicator of failure to achieve vitrification.

Water's phase diagram suggests that it cannot exist in more than one phase simultaneously. There is some debate about this. Some authors claim that microcrystalline inclusions can exist in an otherwise amorphous matrix but that it is impossible to resolve them using bright-field cryo-EM (as opposed to diffraction). According to the phase diagram, the ice is either amorphous or crystalline; it cannot be both simultaneously, and there is no evidence for microcrystalline inclusions.

1.3 The role of cryoprotectants

The term vitrification is used to describe several different phenomena involving glass transitions. I refer only to water and aqueous solutions including colloidal suspensions and the cell interior. The term is used in a slightly different context for cell banking, where high concentrations of cryoprotectants help to limit the damage caused by the relatively slow freezing that occurs when a sample is immersed in liquid nitrogen. Liquid nitrogen is a good cryogen if the aim is only to maintain cold temperature. It has very poor heat transfer characteristics, and this is why unprotected cells immersed in liquid nitrogen are damaged. Ice nucleates and grows explosively. The growing crystals consume water in their vicinity, concentrating salts and redistributing material.

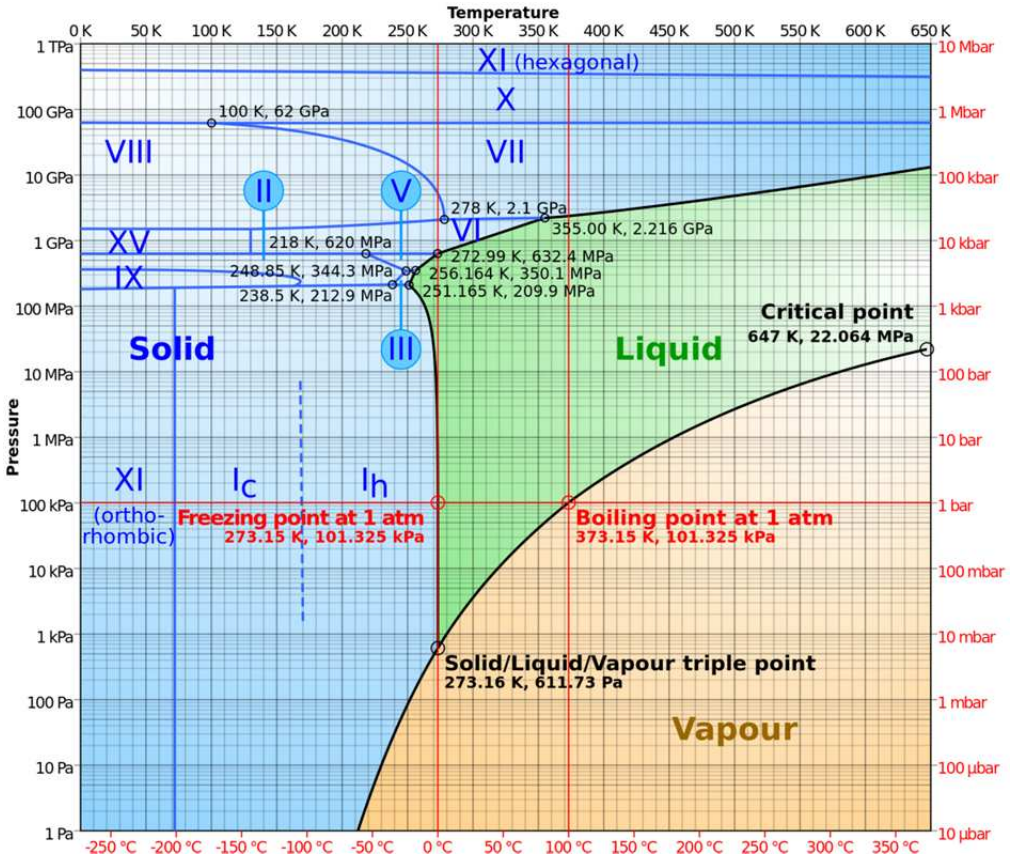

Fig. 2 Phase diagram for water. There are many known phases of solid or metastable water. Vitrification achieves an amorphous phase [13]. Slower cooling from ambient temperature and pressure results in the formation of crystalline ice, as does warming of vitreous ice above the glass transition temperature, which occurs at about -137 °C. Reproduced from author Cmglee (Wikimedia Commons) in unmodified form.

As a rule of thumb, solute concentrations of more than about 20% (w/w) will result in vitrification if the rate of heat exchange is rapid. Secondary cryogens such as ethane and propane (cooled by liquid nitrogen) have far superior heat transfer properties. Alternatively, liquid nitrogen can be effective if the sample is placed briefly under sufficient pressure (see Section 2.3). Pure water can be vitrified to a depth of about 1 µm using thin film vitrification (i.e. immersion in liquid ethane, Section 2.1). This easily surpasses the requirement for an ice film on the order of 100-300 nm suitable for cryo- transmission electron microscopy or tomography. Thicker specimens, which require subsequent sectioning, can be vitrified using high-pressure freezing but only when the thickness does not exceed about 200 µm, and only if water is highly 'impure' in terms of dissolved components.

Intracellular polymer (30-40% v/v) is usually sufficient to guarantee vitrification. The extracellular environment is considerably more dilute and can benefit from the addition of cryoprotectants. These should not be allowed to perturb structure, and distinction is made between penetrating cryoprotectants such as dimethylsulfoxide and non-penetrating cryoprotectants such as high-molecular-weight polysaccharides including dextran. Importantly, dextran does not change the osmotic environment significantly, whereas glycerol and other agents used in cell banking have the potential to induce structural rearrangement (Figure 3).

2. Practical techniques for achieving vitrification

Vitrification is vital to structural studies of viruses and macromolecules using electron microscopy, and it enables 'visual proteomics' studies of cells and tissues [14, 15] because the cells are not perturbed by invasive labeling techniques nor are their contents displaced by solvent substitution. There are only two approaches for introducing soft matter into an electron microscope: converting the water into a stable or (relatively long-lived) metastable form, or removing it entirely. For about 50 years, microscopists pursued the removal route [1]. The discovery of vitrification provided a shortcut that is especially convenient if the sample comprises monodisperse 'particles' with cross-sections smaller than the electron microscope's mean free path.

Two practical methods have gained widespread acceptance for achieving vitrification prior to cryo- electron microscopy: thin-film vitrification, commonly known as 'plunge-freezing' and sometimes as 'immersion freezing,' and

high-pressure freezing, also known as hyperbaric freezing. Two additional methods are worth considering: 'jet' freezing, and impact or metal-mirror freezing, usually known as 'slam' freezing.

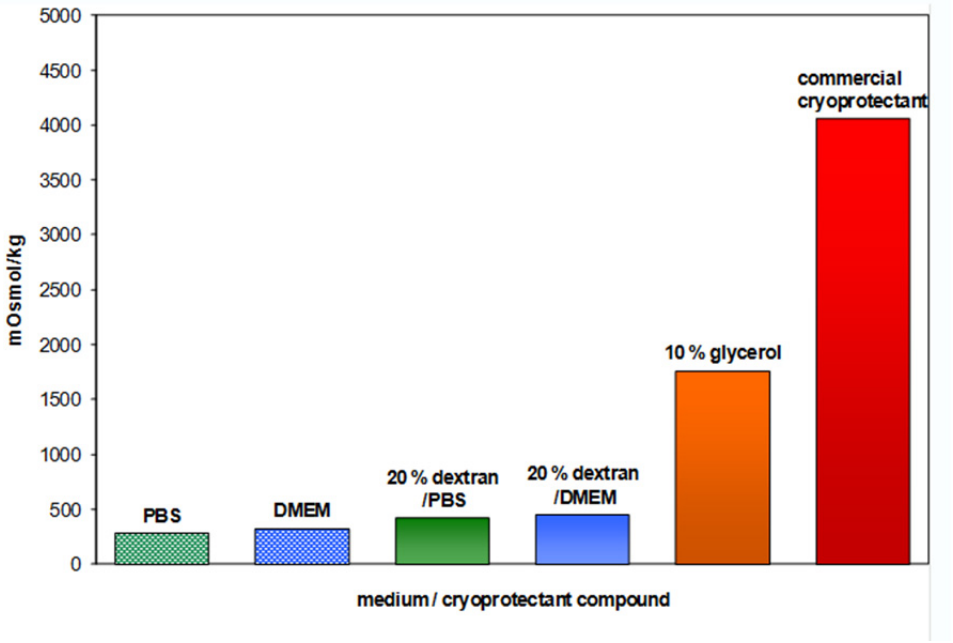

Fig. 3 Osmolality of common cryoprotectant compounds used in cell banking and cryo- electron microscopy of vitreous sections. Values were measured according to the phenomenon of freezing point depression using freezing-point osmometry. The 'commercial' formulation contains glycerol as well as dimethylsulfoxide. The effects of combining these agents appears to be additive for the cases described here. Osmotically neutral formulations simply act as fillers. The minor influence of dextran compared with buffer is probably due to impurities in the form of salts. The tissue culture medium (Dulbecco's Modified Eagle Medium, DMEM) is formulated to match the solution properties of the mammalian cell interior with respect to ionic strength and osmolality.

2.1 Thin-film vitrification

Thin-film vitrification is a common technique for preparing solid state, frozen-hydrated suspensions of colloidal or sub-colloidal-sized particles such as viruses. It makes use of blotting with filter paper to wick excess liquid from a suspension to achieve a thin and almost two-dimensional layer of solution – in reality, a meniscus between the spaces of an essentially two-dimensional, highly porous carbon film. Immediately after the blotting process, the support containing the sample solution is accelerated into a small vessel of a primary cryogen such as liquid ethane or liquid propane 'slush'. The heat transfer characteristics of these solvents results in extremely rapid freezing of the sample solution. It is estimate that, for a 200 nm thick layer, the temperature drops at 10^{11} °C/second [16]. The result is a glass-like layer of frozen solvent with interspersed features of interest. The grid is then transferred to an appropriate cryo-sample holder for microscopy or tomography, or it is stored in liquid nitrogen. Any larger features will result in ice that is too thick for microscopy. The technique is therefore best suited to biochemical, reductionist approaches such as differential centrifugation that give rise to reasonably monodisperse samples. Some excellent overviews are recommended: [17-19]. Although the descriptions of the basic method are similar, these articles contain a wealth of practical information, sample requirements and troubleshooting tips in addition to the basics of vitrification theory.

2.2 Jet freezing and impact freezing

Propane jet freezing is practiced by only a few laboratories, primarily because thin-film vitrification is relatively easy and inexpensive to set up. Jet freezing is better suited to emulsions and aerosolized particles rather than suspensions [20]. Using this method, vitrification is reported to be successful for samples up to 15 µm thick. There is a general consensus in the field that this technique holds significant potential because it makes sense from a thermodynamic perspective.

In impact or slam freezing, the sample (e.g. a small piece of tissue) is brought into contact with a cooled, polished mirror. The depth of vitrification is limited to a few micrometers at the freezing front, although claims of 10-30 µm have been reported [21]. A recent review is provided by Spehner and Edelmann [22]. Perhaps the main reason these techniques are not so widespread is that both require vitreous sectioning for cryo-EM. Slam frozen specimens contain separate amorphous and crystalline regions, which implies that success will be limited.

2.3 High-pressure freezing

It is possible to vitrify nominally larger cells and tissues by high-pressure freezing. Many reference materials claim that this can be done without the addition of cryoprotectants/anti-freeze agents. Experience tells us that this might be the case but it is technically more challenging than it appears at first glance, and it is certainly not the case for hydrogels. If the sample comprises a suspension of bacteria, for example, the interiors of the cells might well be vitreous but the dilute solution in between the cells is typically crystalline. Fillers such as dextran or albumin, or even yeast cells, are used in an attempt to create an entirely vitreous sample that can be sectioned. Crystalline regions will preclude the ability to cut sections. The powdery ice simply crumbles, and besides, amorphous ice is mandatory for cryo-EM.

The ability to vitrify more than 100 μm in depth – still not especially large in relation to tissues – comes at significant cost. It requires that the sample be sectioned prior to cryo-microscopy but at vitreous temperatures to maintain the 'cold chain'. Thinning of vitreous specimens is especially challenging but as explained earlier, it can be achieved by cryo-ultramicrotomy (CEMOVIS, [23]) or by milling with a focussed ion beam [3, 8, 24-26]. Despite concerted research, these nanofabrication methods are still the domain of highly specialized laboratories. Nevertheless, the high-pressure freezing technique is available commercially, and it has resulted in a resurgence of conventional electron microscopy because it circumvents the need for aldehyde-based fixation. Using this method, substitution of ice with solvent typically takes place at low temperature, and the sample is ultimately infiltrated with resin which is polymerized and sectioned in the same manner as traditional epoxy resin techniques. This process typically takes days and requires specialized equipment but more recently, Webb and McDonald showed that the same effect can be achieved in a matter of hours using modest equipment [27]. With freeze-substitution, it can be difficult to assess the quality of freezing objectively. Here, good freezing can only be inferred based on experience [28]. In cryo-EM, the state of solid water can be determined unambiguously.

Finally, it is worth noting that the tendency of liquid water to expand upon freezing has given rise to the idea of containing the sample in a confined space and using the self-pressurization as a means to achieve vitrification [29]. Initial attempts used crimped copper capillaries of the kind used for high-pressure freezing of cell suspensions. A commercial version of the technique uses longer capillaries that are fashioned into a U-tube using a small pipe-bending apparatus. The freezing front is captured in the U and subsequently processed for cryo- electron microscopy of vitreous sections [30, 31] or freeze-substitution [29].

3. Correlative cryo-microscopy

Correlative cryo-microscopy is a powerful way in which to detect and unambiguously identify structures and to combine complementary information over spatial length scales. The sensitivity of fluorescence detection allows the superior survey power of light microscopy to be combined efficiently with the higher resolving power of electron microscopy. This combination is one of the better known examples of correlative microscopy. Importantly, vitrification has additional benefits for correlative microscopy based on fluorescence if both modalities are used on the vitreous specimen [32]. This includes lower rates of photobleaching compared with room temperature fluorescence microscopy, which in turn improves the ability to detect weak signals [33]. Furthermore, it allows acquisition of cryo- electron microscopy data with maximum signal-to-noise characteristics since none of the finite, tolerable electron dose is spent on searching for features.

Approaches for realizing fluorescence and electron microscopy on the same features have included separate fluorescence and electron microscope systems in combination with sample supports ('Finder grids') that provide coordinates for relocating features. An integrated fluorescence and electron microscope instrument has been described [34]. The latter is suitable for room-temperature EM where the sample has been freeze-substituted, i.e. the emphasis is on locating and identifying features rather than exploiting the physical properties of the frozen specimen. More involved workflows have been implemented to achieve this indirectly via separate cryo-fluorescence stages followed by cryo-EM analysis [33]. The efficiency of correlation experiments depends on the precision with which one can recover features. One option is to incorporate fiducial markers into the sample. This allows the establishment of a spatial coordinate system that directs the electron microscope to the feature [35, 36].

4. Time-resolved vitrification: 4D cryo- electron microscopy

Electron microscopy typically provides static snapshots of events. The ability to record three-dimensional datasets by tomography and a further ability to capture this at distinct timepoints ('time-resolved' or 'time-lapse' microscopy) allows the study of dynamic processes. Some observations of dynamic events can be made from large pools of data captured by single-particle analysis. It is recognized that structures can vary not only as a result of extra or missing subunits but also due to the fact that they can be flexible and capable of motion. If enough of these states can be captured, morphing of the different structures provides an animation of how the molecular machine works. An example is the nuclear pore complex solved by cryo- electron tomography [37-39].

Vitrification alone does not constitute time-resolved cryo-microscopy but it is a useful prerequisite [40] because it occurs on the millisecond timescale. Early examples of virus morphogenesis captured by thin-film vitrification showed the power of such approaches [41], while more recent studies have concentrated on reactions induced by so-called 'optogenetics' reactions, where events stimulated by pulses of light are captured by high-pressure freezing [42]. Image capture on this timescale can also determine the kinetics of the processes. The technique has also been reported in combination with electron tomography of freeze-substituted mammalian cells to provide a quantitative, 4D analysis of plasma membrane reshaping during endocytosis [43]. Cryo-EM or tomography would have been poorly feasible for such a detailed study that clearly demonstrates significant progress in our understanding of a key biological phenomenon.

5. The combination of vitrification and cryo-EM has enabled new discoveries

Thin-film vitrification is now employed routinely for structural studies of viruses and macromolecular assemblies by cryo- electron microscopy. This can be achieved by applying single-particle analysis for symmetrical or repetitive structures, electron tomography for pleomorphic structures, or electron tomography and subtomogram averaging where repetitive elements can be selected for statistical averaging and enhancement of resolution.

In conjunction with vitrification, cryo-EM became the method of choice for virus structure determination due to its ability to preserve structure accurately, cope with heterogeneity, and at the same time, avoid chemical fixatives, electron-dense stains and thermal collapse [44]; however, the types of specimen that have benefited most from this approach have properties in common that are readily amenable to analysis by a reductionist approach: small size (typically less than 200 nm), and limited polydispersity. Hence, with some optimization with respect to viscosity, the technique of thin-film vitrification is relatively simple, reproducible and independent of biochemical composition. In fact, it can deal with highly hydrophilic as well as highly hydrophobic samples, provided that they are still 'soluble' [45]. Nevertheless, the possibilities for vitrifying specimens for electron microscopy are not endless. Thermodynamic considerations limit the ultimate practical application of vitrification for cryo- electron microscopy.

In Figure 1, we saw how classical methods can give rise to distortions. Since classical methods have developed empirically, it is easy to suggest that a variation on this protocol – different fixative, different fixation time, different dehydration medium, etc. would have given rise to a better result and that this example is biased. It is true that better micrographs are produced routinely for some types of sample, and that some protocols give rise to what appears to be excellent preservation of membranes, lipid inclusions and other readily identifiable components. It is important to note, however, that bacteria are notoriously difficult to preserve for electron microscopy [28, 46].

The difference between vitrification and the plethora of classical methods is the unifying feature: water. To illustrate this, consider the mycobacterial outer membrane and its discovery by cryo- electron microscopy and tomography [47, 48]. Due to the importance of mycobacteria such as *Mycobacterium tuberculosis* and *M. leprae* as causative agents of debilitating diseases, they have been studied extensively using transmission electron microscopy. In spite of this intensive research, the outer membrane could not be demonstrated using classical methods. It is somehow labile and does not survive dehydration and embedding. In this case, chemical fixation does not serve its intended purpose. Independent knowledge of chemical compatibility had predicted the existence of the membrane but it was not until two research groups applied cryo- electron microscopy to vitreous sections of mycobacteria that the structure was demonstrated [47, 48] (Figure 4).

A detailed and systematic appraisal of the principle EM sample preparation techniques applied to mycobacteria concluded that cryo- electron microscopy of vitreous sections was the reference against which all other methods should be judged [28]. This example is a special, albeit important case but it is certain that cryo-EM has enabled many new discoveries and will continue to do so for decades to come.

Finally, I note that combining vitreous sample preservation with correlative microscopy and 4D electron microscopy is challenging, and that the freeze-substitution route will be preferred by many, at least for initial discovery. It could, however, be used to design more efficient experiments that use vitrification and cryo-EM on a more selective basis.

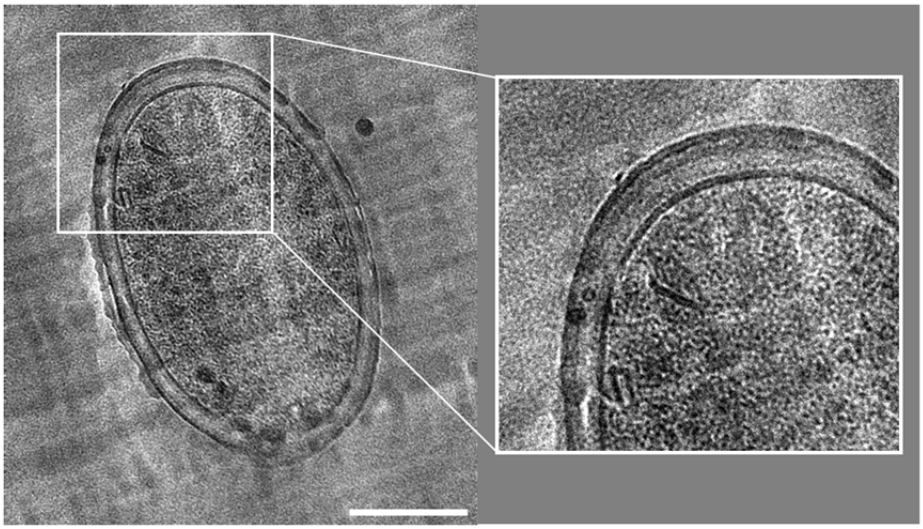

Fig. 4 Demonstration of the mycobacterial outer membrane by cryo- electron microscopy. *Mycobacterium smegmatis* was high-pressure frozen and vitreously sectioned using a diamond knife (nominal thickness: 25 nm). Cryo-EM on the vitreous section (CEMOVIS) was used to reveal the cell wall structure without the addition of stains or chemical fixatives. There can be little ambiguity about the existence of inner and outer bilayers as well as some intermediate layers. Scale bar represents 200 nm.

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