Chapter 6
Techniques Specific to Fine Particles

1 Dispersion of Fine Particle Materials

1.1 Principle

Dispersion of fine particle materials is used to isolate fine particles onto a support grid film so that they can be observed under the microscope.

It consists of dispersing fine particles of a material. Generally, the material is suspended in various liquids, followed by stirring, in order to improve dispersion of the particles that are then deposited onto the film of a support grid (see the techniques on continuous or holey support films in Chapter 2, Sections 13 and 14).

This technique, which is easy to use, can be applied to any type of fine particles whether they are micrometric or nanometric in size.

1.2 Operating Mode

1.2.1 Equipment and Supplies

Usually a mechanical, magnetic, or ultrasound stirrer is used to disperse the particles. A micropipette can be useful for precise dosing when depositing the suspension containing the fine particles onto a support grid holding a continuous or holey film (see the techniques on continuous or holey support films).

1.2.2 Procedure

Fine particles are suspended in a volatile liquid adapted to the specificities of the material. The solvent used must be compatible with the material; usually demineralized, distilled, or even twice-distilled water is used for minerals, ceramics, and biological materials. In some cases, when there is a risk of interaction with water, other solvents are used, e.g., a buffer solution for biological materials undergoing immunological processing. In other cases, alcohol is used because of its rapid evaporation; this is the case for catalysts and nanomaterials in general, which tend to re-agglomerate during the overly slow evaporation of the drop of water. And

J. Ayache et al., *Sample Preparation Handbook for Transmission Electron Microscopy*,
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lastly, hydrocarbons are used to investigate petroleum or other heavy carbonaceous materials containing organic matter.

The suspension is agitated using a mechanical, magnetic, or ultrasound stirrer, in order to separate the agglomerated particles and make the solution homogeneous. Attention must be paid to not to destroy the particles during agitation, in particular nanospheres and biological samples (e.g., viruses). In these cases, dilution is often necessary in order to have a very low material concentration, so that the particles are sufficiently isolated from one another on the support film. Immediately after agitation, a drop of the suspension is sampled using a micropipette or a simple Pasteur pipette, and the drop is placed on a film on a support grid. The preparation is ready after the water or solvent chosen has completely evaporated.

When water is used as dispersion liquid, drying (which can be as long as a few hours) is accelerated by placing the support grid in an oven or under an infrared lamp. Drying can shorten the time the water droplet takes to dry on the support grid and alleviate the related drawbacks. In fact, three concomitant phenomena occur as the diameter of the droplet decreases: increasing surface tension tends to agglomerate the particles; particles tend to stick to one another by flocculation (Figs. 6.1, 6.2; and 6.3) the support film tends to fix particles to its surface via the electric potential effect of the film, when its ionic charge is opposite to that of the particles themselves. Rapid drying helps to limit the artificial formation of aggregates. However, the drying process must be gentle because otherwise there is a risk of breaking the support grid film. In the case of samples composed of light atoms (polymers and biological materials), applying a negative stain before the droplet completely dries can prevent these drawbacks (Chapter 7, Section 2).

The use of alcohol, instead of water, partially eliminates these drawbacks. When the drop of alcohol suspension is placed on the support grid, the drop immediately spreads out and the alcohol evaporates very quickly; the particles do not have time

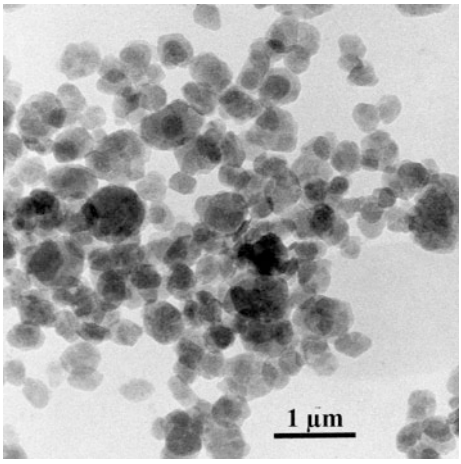

Fig. 6.1 Illite clay. Particles dispersed in distilled water under ultrasound and deposited on a continuous film. Too high a concentration creates aggregates and hinders observation (*J. Duplay, EOST, Strasbourg*)

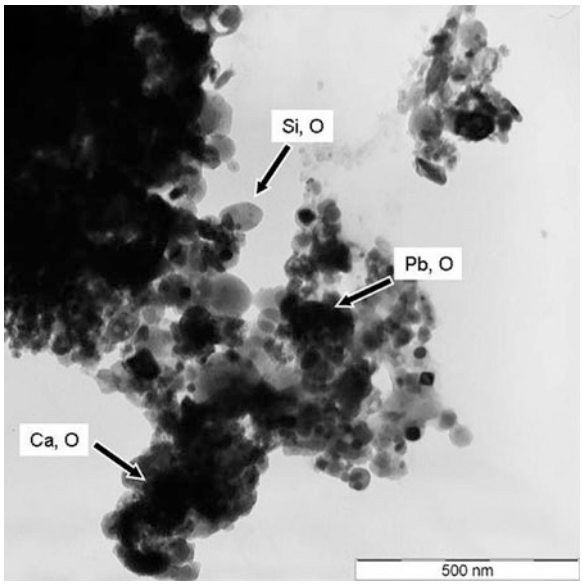

Fig. 6.2 TEM image of grains of dust collected on a filter and washed with water. EDS analysis is possible despite the aggregation of the particles (*J. Boumendil, Université Claude Bernard-Lyon 1*)

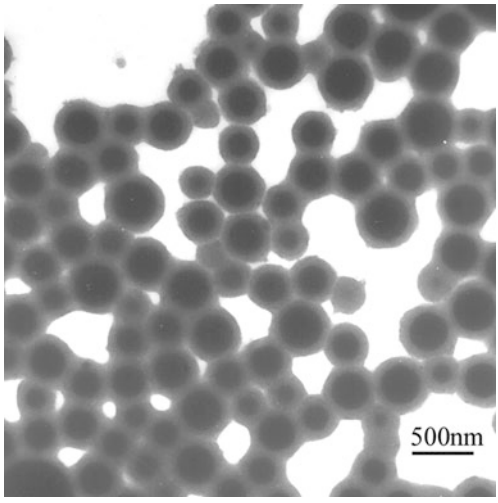

Fig. 6.3 Core–shell: Polymer beads with a core and shell made of different polymers. Wetting agent residues make the beads stick to one another (*J. Boumendil, Université Claude Bernard-Lyon 1*)

to aggregate. *Caution*: The collodion support on the grid is destroyed by alcohol. In this case, a support grid covered with Formvar or carbon should be used.

Films can be processed by ionization in order to promote particle fixation onto the support grids and to prevent aggregates (Chapter 2, Sections 13 and 14).

For some materials, suitable dispersion agents can be used, but they are often poorly eliminated and hinder observation (Fig. 6.3).

1.3 Variants

1.3.1 Dry Dispersion

In the case of an ultrafine powder, samples are taken directly by contact using a support grid carrying a film. Excess powder that does not adhere to the grid is eliminated by lightly tapping the grid. This is the case when preparing nanomaterials, e.g., carbon nanotubes (Figs. 6.4, 6.5, and 6.6). The support grid carrying a holey film is held at the tip of a pair of tweezers, and the side of the film with the fine particles is delicately rubbed on a glass slide. The particles adhere by contact onto the film. The procedure is simple but delicate, because the film will break if rubbed too hard. Excess particles that do not adhere to the film must absolutely be removed in order to prevent the powder from contaminating the microscope.

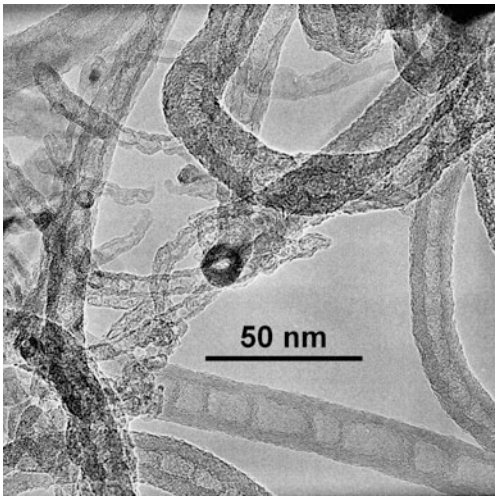

Fig. 6.4 Carbon nanotubes. Dry dispersion of particles on a holey film (*C.S. Cojocaru, IPCMS, Strasbourg*)

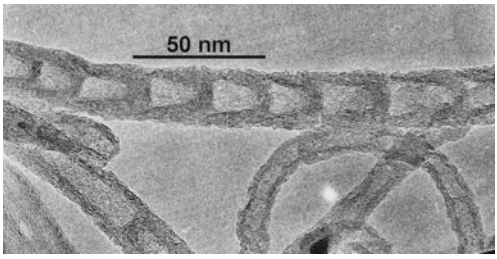

Fig. 6.5 Details on the internal structure of a carbon nanotube (*C.S. Cojocaru, IPCMS, Strasbourg*)

1.3.2 Dispersion on the Surface of Water (Langmuir Film)

The powder is suspended in alcohol. A drop of this suspension is placed on the surface of a crystallizing dish filled with water. An extremely thin film of fine particles forms, which is recovered on a support grid coated with a carbon film. In particular, this technique is used for clay-type minerals that are in the form of platelets

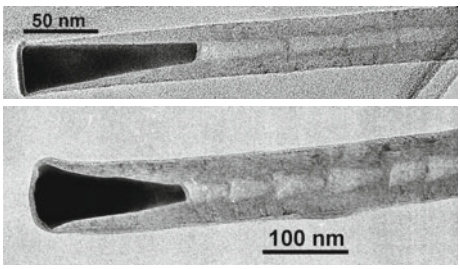

Fig. 6.6 Detail of the ends of carbon nanotubes plugged with a metallic nickel particle when they were produced (*C.S. Cojocaru, IPCMS, Strasbourg*)

(small plates). The surface tension of the water will spread the flakes out, constituting the small-particle platelets, and the resulting film will be a single layer of flakes. Placing a support grid held at the tip of a pair of tweezers, with the film side in contact with the surface of water in the crystallizer, is enough to recover the particles by capillary action. The grid is then quickly dried before its observation under the microscope.

1.3.3 Aerosol Dispersion

When particles are suspended in a gas, e.g., dust floating in the atmosphere, a film-coated support grid just needs to be exposed to a gaseous flow carrying the particles. Grid exposure time is chosen so as to get a suitable concentration of dust on the preparation (Fig. 6.2).

This technique can be used to detect asbestos in a room, as well as to quantify the number of asbestos fibers per liter of filtered air (or to check atmospheric pollution). In this case, the suction of the building's air pumping system over a very long period of time (generally 1 week) is used and the particles are collected on a filter. This filter is then burned, and the asbestos particles are suspended in a known quantity of water and a calibrated drop is placed on a support grid film. Statistical investigation is used to identify and quantify the asbestos fibers contained in the room (Figs. 6.7 and 6.8).

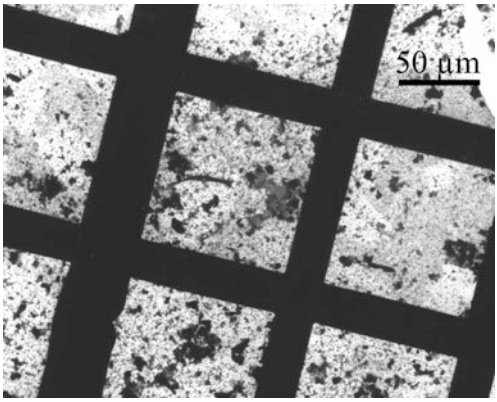

Fig. 6.7 Asbestos fibers dispersed on a carbon film. TEM image at very low magnification showing homogeneous distribution. The support grid mesh measures 100 µm (*G. Ehret, IPCMS, Strasbourg*)

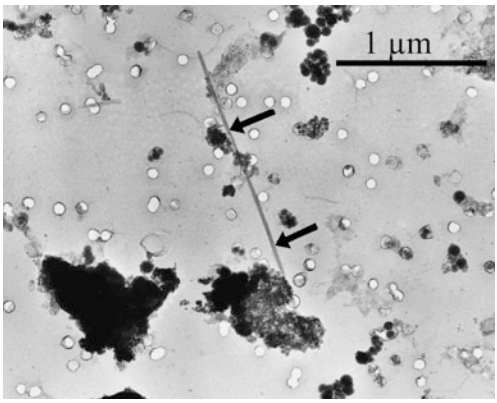

Fig. 6.8 High-magnification image of a chrysotile asbestos particle (indicated by the *arrows*) (*G. Ehret, IPCMS, Strasbourg*)

1.3.4 "Spin Coating" Dispersion

"Spin coating" dispersion is used to disperse particles, while avoiding the successive dilution steps that are often necessary in order to obtain an adequate quantity of particles, i.e., not agglomerated. This technique involves a rotating motorized support (Fig. 6.9) with variable speed, on which a TEM support grid coated with a carbon film or a support for SEM observation can be attached. The rotating support is spun and a drop of suspension is placed on the TEM support grid (or SEM support). The rotation allows the suspension droplet, and thus the fine particle material, to be spread uniformly.

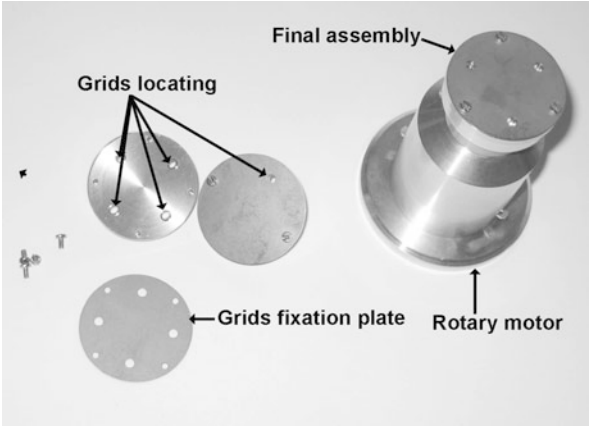

Fig. 6.9 Device and tools for performing the spin-coating-dispersion technique

1.3.5 Dispersion and Spreading Nucleic Acids (DNA, RNA, and Associated Proteins)

There are three spreading methods.

Cytochrome *c* Method

Cytochrome *c* is a basic protein that forms a thin film on the surface of water. When cytochrome *c* is mixed with a nucleic acid, it creates a complex that locks the nucleic acid into the mesh of the film and preserves the spatial conformation of the nucleic acid. This is a classic method for viewing double-chain DNA molecules and DNA–protein complexes. It is necessary to use dispersion agents such as urea or formamide instead so that the single-chain DNA spreads out without forming aggregates. Chemical fixation with aldehydes may be necessary for stabilizing DNA–protein complexes that can dissociate during spreading.

– The film is recovered from the surface of the water in a crystallizing dish according to the Kleinschmidt technique (Fig. 6.10). The film is recovered using a grid covered with collodion carbon. The grid with the cytochrome and DNA film is placed in contact with pure alcohol, which will eliminate the water and stabilize

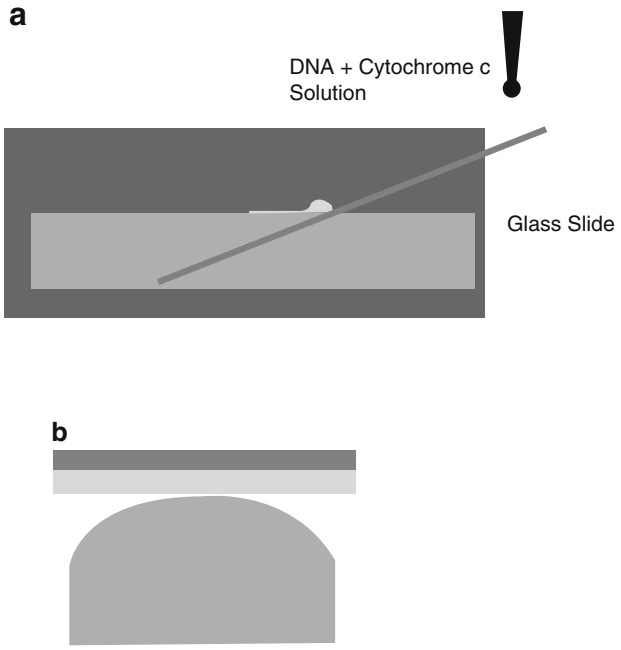

Fig. 6.10 **a** Kleinschmidt method for spreading DNA with cytochrome *c*. **b** Washing with a drop of ethanol

the film. This technique requires a fairly large quantity of cytochrome–nucleic acid solution, which is often hard to obtain.

– In a second method, the film can be recovered on the surface of a 1 ml drop of water (Fig. 6.11) by sliding a microdrop of cytochrome *c*–nucleic acid mixture along a 2-mm-diameter glass rod. The film is recovered by placing a grid covered with collodion carbon over it: This is called the Inman method (Fig. 6.11), which has widely been used to view partially denatured DNA molecules. Formamide is then essential for stabilizing the single-chain segments corresponding to the most easily denatured segments because they are rich in adenine–thymine.
– A third method, the diffusion method (Fig. 6.12), can also be used. This method consists of placing a drop of the cytochrome *c* nucleic acid mixture onto a piece of adhesive tape. The molecule film rises to the surface of the drop and can be recovered on a grid covered with collodion carbon. This method is used to work with small volumes of DNA cytochrome *c* solution.

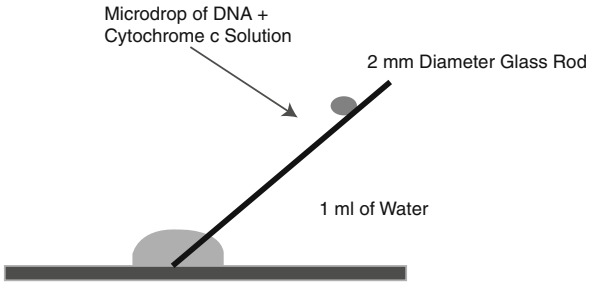

Fig. 6.11 Inman's method for spreading DNA with cytochrome *c*

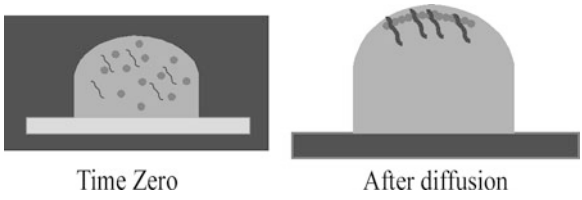

Fig. 6.12 Diffusion spreading method for DNA with cytochrome *c*

It may be necessary to use a glow discharge to treat the carbon films coating the grids in order to improve their wettability.

Detergent Method

This method is more specific to investigating single-strand or double-strand DNA with DNA–protein complexes. These complexes must be stabilized with an aldehyde pre-fixation.

Detergent is used in place of cytochrome *c* to create the film. The detergent used is benzyl alkyl ammonium chloride (BAC). It may be necessary to fix the nucleic acids with aldehydes before placing them into contact with the detergent.

Adsorption Method

There are three different procedures that can be used:

1. Deposit a polylysine spread by "spin coating," which serves as a glue between the carbon film of the grid and the spread-out molecules.
2. Use divalent cations such as Mg^{++} added to the DNA solution to facilitate binding to the grid. This method risks changing the spatial conformation of the molecules. This procedure is widely used to immobilize molecules on mica slides in order to observe them using atomic force microscopy (AFM).
3. Use the Dubochet method, which consists of depositing electrical charges on the surface of the carbon film obtained with a pentylamine plasma. Vaporizing pentylamine will deposit NH^{+} ions, which will bind to the DNA molecules that are negatively charged. The use of an amylamine vapor will make it possible to deposit negative charges.

The lack of contrast between these spread-out molecules next requires a negative or positive contrast or shadowing (Figs. 6.13 and 6.14).

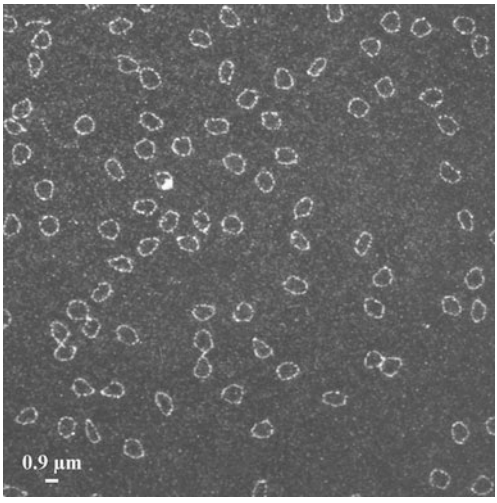

Fig. 6.13 Dark-field image of DNA *minicircles* deposited using the Dubochet method and positive staining (*E. Le Cam, CNRS-UMR8126-IGR, Villejuif*)

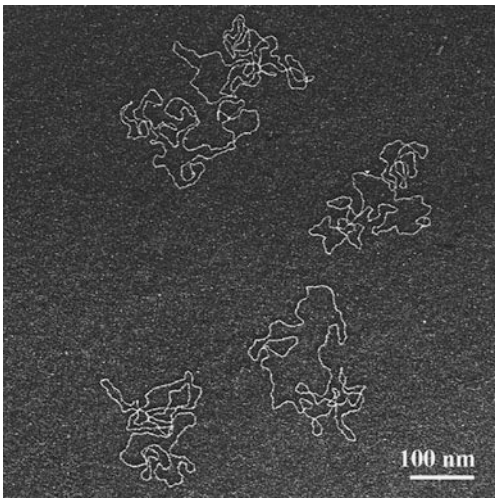

Fig. 6.14 Dark-field image of DNA plasmid deposited using the Dubochet method and positive staining combined with shadowing techniques (*E. Delain, CNRS-UMR8126-IGR, Villejuif*)

1.4 Advantages

This is a very rapid technique. It enables the statistical analysis of fine particles. Knowing the dilution of the suspension and the volume deposited on the grid enables us to increase the amount of initial material. This is the case for the determination and quantification of asbestos fibers in the atmosphere of a room (Fig. 6.8), in order to measure the concentration of viral particles in a vaccine, etc.

1.5 Limitations

The presence of certain additives, such as dispersion agents, can hinder the technique (Fig. 6.3).

1.6 Artifacts

This technique can lead to morphology alteration during desiccation for fragile materials in liquid solution (biological materials, polymers). We can often remedy this by chemical or physical fixation (see Chapter 6, Section 2).

Observation will be hindered when the particle concentration is too high or if agglomerates or dispersion residues are present in the sample (Figs. 6.1 and 6.3).

1.7 Type of Analysis

Structure, crystallography, crystal defects, chemical composition, chemical bonds, properties.

1.8 Risks

The only risk is inhalation of noxious aerosols.

1.9 Conclusion

This is the simplest and fastest technique for fine particle materials.

This technique is particularly suitable for determining concentrations of asbestos fibers, polymers, viruses, etc.

In the case of materials with a low electronic density, the negative-staining and positive-staining contrast techniques can improve observation.

The frozen suspension technique is required for highly brittle nanomaterials.

This technique complements the freeze fracture or ultramicrotomy techniques.

2 Frozen Hydrated Film of Single Particles

2.1 Principle

This technique is based on the direct observation of a thin film of vitreous ice containing fine particles of a biological material in its native environment, i.e., without chemical fixation (DNA, proteins, DNA–protein complexes, viruses, polymers, etc.).

The method consists of performing a physical fixation of a small volume via the ultrarapid freezing of a drop of water (or freezable solvent) deposited on a microscope grid coated with a holey carbon film. Vitrification of the aqueous phase into an amorphous solid phase without crystallization stabilizes heavily hydrated biological structures.

This technique is applied to small-sized fine particles dispersed in liquid solution whose components are composed of light elements (C, O, H, N, etc.). It is widely used for investigating polymers and biological materials. It requires cold TEM observation, i.e., cryomicroscopy. It is often combined with 3D reconstruction of complex molecular systems. This technique can also be suitable for some mixed–composite fine particles.

2.2 Operating Mode

2.2.1 Equipment and Supplies

The ultrarapid freezing equipment used is called a cryo-plunger (Fig. 6.15). It is composed of a cryogenic Dewar (ethane, propane, etc.) filled with liquid nitrogen cooled to 98 K and a system for plunging the TEM grid into the cryogen. The plunger system is used to plunge a clamp holding the support grid and the sample into the cryogenic reservoir. A chamber can be used to control temperature and hydration. These systems are commercially available.

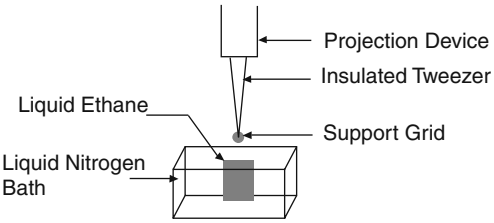

Fig. 6.15 Cryo-plunger diagram, device for freezing with ethane

2.2.2 Procedure

The procedure involves several steps, each of which is very strict, in order to produce an electron-transparent thin vitrified film in a reproducible manner.

Preparing to Deposit the Suspension on the TEM Support Grid

A 4–5 μl drop of an aqueous suspension of previously dispersed particles is spread on a copper grid (300 or 400 mesh), 3 mm in diameter, coated with a holey carbon film (Fig. 6.16).

The concentration of these particles must be adjusted in order to be able to obtain a vitreous ice film with a homogeneous distribution of the particles inside the holes of the carbon film.

Holey films must be thick enough to be resistant and thin enough to provide a layer of ice that "coats" the objects as well as allows for their observation in all possible orientations. The film is usually thinner in the center of the holes. The average size of the holes must be 1–3 μm.

In order to make a proper deposit of the suspension, the holey film must also be sufficiently hydrophilic (polar) so that the drop of suspension moistens the whole grid. Grid wettability can be improved either by first plunging them in ethyl acetate or by treating the grids using an electrical discharge (see Chapter 2, Sections 13 and 14).

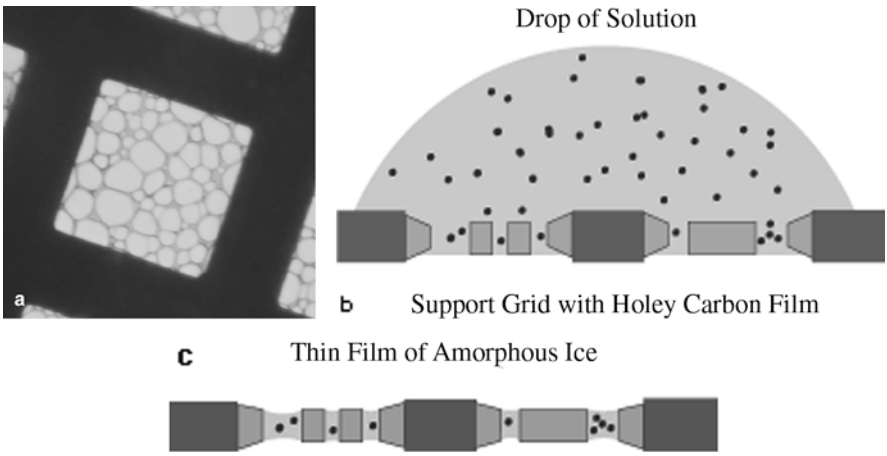

Fig. 6.16 **a** TEM image of a holey carbon support grid; diagram of the particle suspension **b** before and **c** after sponging the drop. The particle suspension can be seen in the holes of the carbon film

Preparation of the Frozen Hydrated Film

The grid holding the drop of particle suspension to be frozen is held between the jaws of a pair of ultrathin tweezers mounted on the plunger. Before cryofixation, excess solution must be removed from the grid in order to leave behind a very small film of the suspension only in the holes of the carbon membrane. To do so, the grid is delicately and quickly blotted using an ashless filter paper placed parallel to the drop of solution on the grid. This step is called "grid blotting" and is the determinant for obtaining a frozen hydrated film of the right thickness in a maximum number of holes in the carbon membrane. This is done either manually by using a single ashless filter paper or mechanically by pressing the grid between two ashless filter papers. Immediately after blotting, the plunger is released. A manual release cryo-plunger is represented in Fig. 6.17. This manual technique has the advantage of being simple and inexpensive, with the drawback of being difficult to implement. The mechanical method using a cryo-plunger device represents an improvement in routinely producing a thin film of ice. Throughout the preparation, humidity and temperature must be monitored so as to prevent excessive dehydration of the suspension drop. Nevertheless, the experimental conditions to obtain a good reproducibility of the film thickness remain difficult to achieve.

Ultrarapid Freezing

This is freezing by projection plunging. When the plunger is released, the suspension of particles or macromolecules remaining on the grid is plunged into the cooling bath and transformed into an amorphous thin frozen hydrated film. This

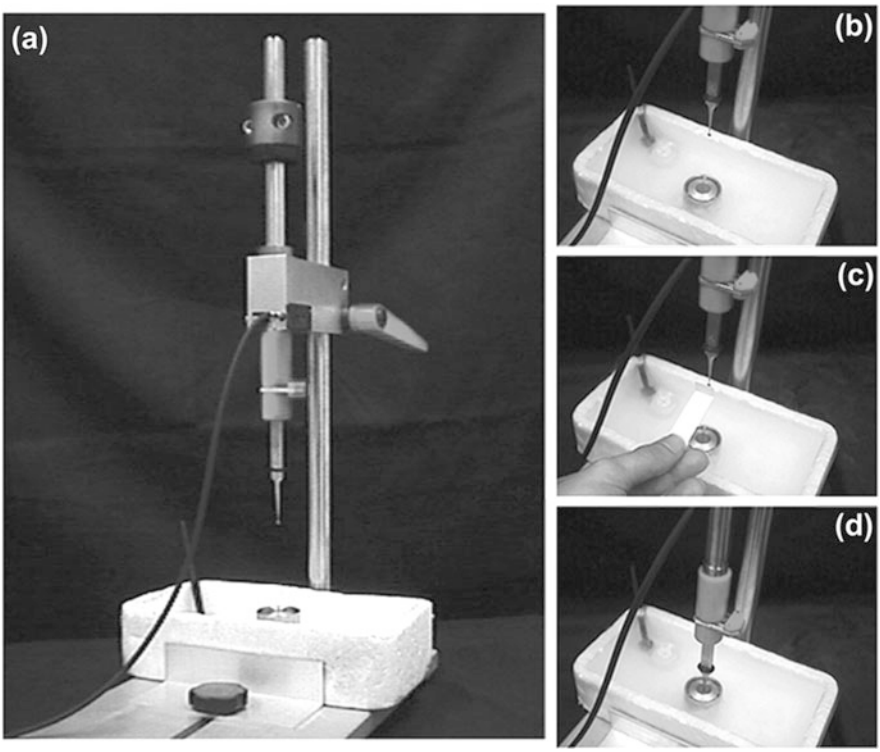

Fig. 6.17 **a** Cryo-plunger and reservoir cooled by a bath of liquid nitrogen; **b, c, d** show the three steps before plunging: **b** placing the grid on the plunger, **c** blotting, and **d** ultrarapid cryofixation in ethane

amorphous ice immobilizes the floating molecules of the solution. After a few seconds, the cryofixed grid is removed from the ethane bath, then transferred into the cold dry vapors of liquid nitrogen in the support for the grid storage, and placed in the liquid nitrogen bath of the cryo-plunger. This support can contain several grids. It is then transferred into the nitrogen vapors, which are either in a nitrogen reservoir or in the cryo-transfer stage of the TEM cryo-specimen holder for observation.

Cryo-Transfer

A second cryo-transfer is carried out to move the sample storage box from the cryo-plunger to the cryo-transfer stage of the cooled TEM specimen holder. This cryo-transfer stage, shown in Fig. 6.18a, includes a chamber cooled with a bath of liquid nitrogen (background) and the cold specimen holder (foreground), which can be inserted into this chamber. Note that at the right end of the specimen holder, there is a Dewar filled with liquid nitrogen that will cool the specimen holder located at the

other end of the shaft. Figure 6.18b shows the details of the chamber in which the grid is transferred from the transport reservoir to the cooled TEM specimen holder. This final step must be performed using a pair of tweezers whose tips are first cooled in liquid nitrogen in order to prevent any change in temperature that would cause the formation of ice crystals in the preparation.

Fig. 6.18 **a** Cryo-transfer stage: The cooled chamber is in the background, and the cold TEM specimen holder is in the foreground. **b** Cooled chamber of the TEM specimen holder for transferring the grid

2.2.3 Observation in Cryo-microscopy

Observation in bright-field mode, which is mandatory in cold conditions, must be performed with a minimum dose of electrons so as not to change the vitreous ice (electron dose < 1,000 e/nm^2/s). This is performed in low-dose mode (a special mode on the microscope). Figure 6.19 shows a bright-field cryo-microscopy TEM image of a frozen hydrated film of a tomato bushy stunt virus (TBSV) in two different conformations.

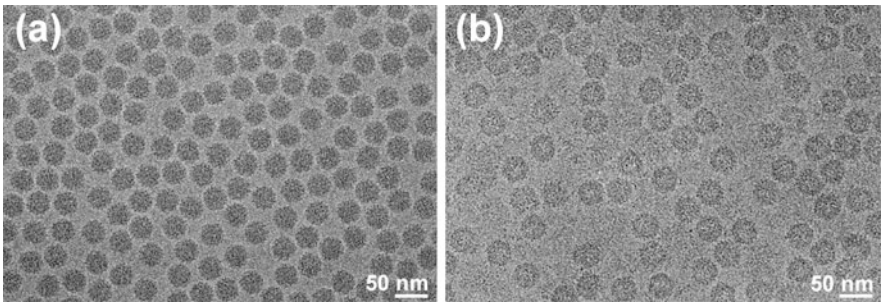

Fig. 6.19 TEM image of a frozen hydrated film of a TBSV virus in two different conformations (*E. Larquet et N. Boisset, IMPMC-UMR CNRS-Université, Paris*)

2.3 Variants

2.3.1 Cryo-plunger in a Controlled Atmosphere Chamber

A new cryo-plunger device developed by F. Livolant and A. Leforestier is supplemented by a chamber allowing for the control of the temperature and humidity and a mechanical system for controlling pressure and sample blotting time. Controlling experimental conditions during cryofixation helps to maintain the ionic concentration of the particles in solution; maintain the temperature in order to prevent evaporation, which ensures the proper preservation of protein conformation, notably thermo-sensitive proteins; and systematically reproduce a frozen hydrated film of particles in suspension, with the same thickness (Fig. 6.20).

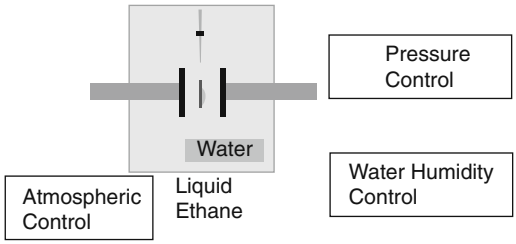

Fig. 6.20 Cryo-plunger in a controlled-atmosphere chamber for controlling temperature and humidity and a mechanical system for controlling blotting time and pressure

2.3.2 Cryo-fixation of a Contrasted Sample

The sample can be contrasted by adding a contrastant, e.g., phosphotungstic acid. However, this method is only applied as a last resort when it is not possible to see the objects in the ice. It helps to preserve the hydrated state but the specimen is no longer native. This type of preparation is called "cryo-negative staining" (Adrian et al., 1998).

2.4 Advantages

As there is no carbon film in the observation areas, nor chemical preparation (addition of fixatives and/or contrastants) or desiccation, it is possible to observe the sample in its native hydrated state. This technique is used to obtain the best resolution for single-particle microscopy analysis.

Using images of several thousand specimens and with the help of image analysis software, it is possible to make 3D reconstructions of specimens and investigate the spatial conformations of complex molecular systems such as viruses (Fig. 6.19) or macromolecules such as proteins.

2.5 Limitations

This technique can be applied only to nanometric-sized single particles in suspension. Surface tension forces on the thin film of water exert an influence on the distribution of molecules but also exert stresses on large objects.

The preparation is very sensitive to the electron beam. Manipulation and successful preparation require a great deal of practice. Producing a very thin film of vitreous ice is difficult. The technique is expensive and requires special equipment on the microscope (cooled stage and transfer system).

2.6 Artifacts

Artifacts usually result from poor freezing. Ice crystals (Fig. 6.21) can form or the sample temperature rises at each transfer step, making observation impossible (Fig. 6.22). If plunging is not fast enough after blotting, the sample might start to desiccate, thus changing the structures or cryofixation. The structures to be observed may also change insidiously during observation under the effect of electron irradiation.

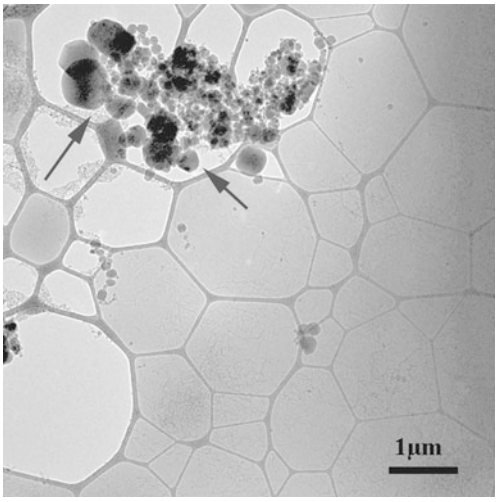

Fig. 6.21 Bright-field TEM image of a frozen suspension film presenting crystals of cubic and hexagonal ice on the carbon holes (*arrows*) without the frozen suspension film (*G. Pehau-Arnaudet, Institut Pasteur CNRS, Paris*)

2.7 Type of Analysis

Topography and structure.

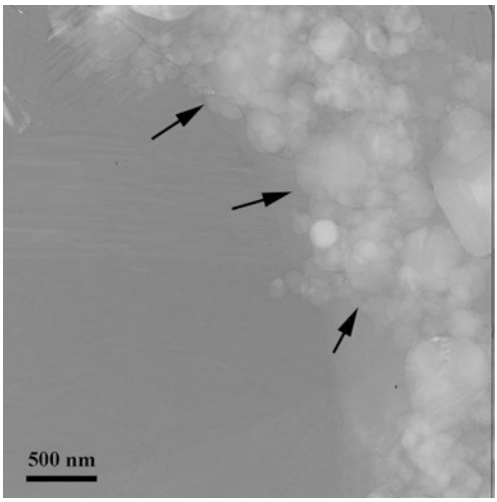

Fig. 6.22 Bright-field TEM image of a frozen suspension film showing the effects of improper freezing. Clusters of segregated crystals (*arrows*) on the edges of the holes of the carbon film can be seen (*S. Baconnais, CNRS-UMR8126-IGR, Villejuif*)

2.8 Risks

There are burn and explosion risks related to the use of liquid nitrogen and ethane gas.

2.9 Conclusion

The observation of a frozen hydrated film of a single particle suspension after cryofixation is the technique used to maintain the best preservation of biological material. It is a very useful technique for fine particle samples of very small sizes, such as single particles that give a low contrast. It is indispensable for samples that are particularly sensitive to desiccation. It complements the simplest negative-staining and decoration-shadowing techniques.

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