Project:

Cryo-TEM

Subtitle

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DOCUMENT HISTORY

Effective Date	Date Revision Required	Supersedes
DD/MM/YYYY	DD/MM/YYYY	DD/MM/YYYY

Version	Approval Date	Description of the Change		Author / Changed by
1.0	09/05/2016	All	Initial Document	Fanny Caputo

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1 Introduction

Cryo-TEM allows direct morphological visualization of nanomaterials/nanoparticles at near native state (e.g. physiological liquid media), reducing the risk of aggregation and of the introduction of artefacts due to sample preparation (e.g. evaporation can lead to aggregation of nanoparticles).The main advantage of using cryo-TEM is the possibility to observe the sample in its native state, since there is no need to use staining or fixation techniques. For this reason, cryo-TEM has been successfully adopted for studying size and morphology of organic nanomaterials such as liposomes, polymeric NPs, lipid nanoparticles as well as for biological samples [1].

This protocol describes sample preparation and sample imaging of organic NPs by cryo-TEM. The specific protocol adopted at PNS laboratory (CEA Grenoble) is described in details. This protocol has to be slightly modified to be applicable to other laboratories.

2 Principle of the Method

Sample preparation for cryo-TEM is based on an ultra-fast conversion of a thin fluid suspension film into vitrified, low vapor pressure specimen for TEM [1]. In the cryo-TEM method a liquid solution containing the sample is deposited on a grid in such a way that a very thin aqueous film is formed. The grid is then plunged into a cooling medium, (liquid ethane) just above its freezing point, thus, the film vitrifies instantaneously. The structures which in this way are captured in the vitrified film, are so quickly vitrified that normally no important reorganization (e.g. crystallization) can take place. Therefore, this technique allows visualization of the sample “frozen” in their native state. The grid with the vitrified film is then transferred into the microscope, and observed at liquid nitrogen temperature (-196°C).

3 Applicability and Limitations (Scope)

The scope of cryo-TEM analysis is to get information on nanoparticle size and morphology in their native state. After the images have been acquired in the microscope, they can be processed to calculate the nanoparticle size distribution.

The contrast in cryo-TEM, especially for organic nanoparticles (e.g. liposomes) is often a limiting factor. To improve it, while acquiring the images, the operator often needs to defocus, putting the defocus of the objective lens to a point of -4-5 µm underfocus (weakening the strength of the objective lens so that the cross-over is actually below the true focal plane of the lens by that amount). The resolution is connected to the defocus through the contrast transfer function in a somewhat complex way, reducing to 4–5 nm the smallest size that can be resolved. Therefore it is often difficult to characterize objects smaller than 5 nm. There are also limitations on the upper size of an object, set by the thickness vitrified film which is formed on the grid. In practice, upper size is limited to about 500 nm; otherwise the scattering of electrons by water gets too large, and the cooling rate during vitrification too slow [2]. With sample preparation with an automatized vitrobot (the one described below), ice thickness is around 100-200 nm, limiting to 100 nm size the NPs that can be observed.

Cryo-TEM procedure significantly reduces artefacts introduced in sample preparation for standard TEM, such as the modification of nanoparticle morphology by negative staining, or the aggregation of nanoparticles during evaporation of the solvent on the grid for conventional TEM. However some artefacts due to sample preparation or to NP interaction with the grid may occur in cryo-TEM and should be taken into account while analyzing the images [1].

For example, cryogen residues can remain from the plunge freezing process. Sometimes, these residues can be removed by careful heating.

Warming of the sample by heating may lead to a phase transition from the vitrified ice into cubic or hexagonal ice. Ice contamination can also occur by a too high content of evaporated water in the column of the TEM.

Since the sample film gets thinner in the middle of a hole of the grid, smaller particles are usually found in the middle part of the film. Larger particles, on the other hand, can be found near the division copper bars of the grid.

Finally, particles on division bars should not be taken into account because the film is not intact in these areas and embedded structures may be deformed.

4 Related Documents

Table 1:

Document ID	Document Title
EUNCL-PCC-001	*Measuring batch mode DLS*
EUNCL-PCC-021	*Measuring NP Aggregation Propensities by batch mode DLS*

5 Equipment and Reagents

5.1 Equipment

- Plasma cleaner (Fischione 1020)
- Carbon coated holey grids by Agar scientific (Agar C-166-3 lacey carbon grid on copper, carbon grid on copper) or Quantifoil grids (e.g. R2/2 or Multi A)
- Automated vitrification system for cryo-TEM: Vitrobot (FEI)
- Sample holder with integrated supplementary sample transfer functions for cryo-TEM (Gatan)
- TEM microscope: Tecnai Osiris (FEI)

5.2 Reagents

- Stock solution/ lyophilized powder of the sample to be imaged.
- Aqueous buffer to disperse the NPs (if a dilution of the stock solution is needed or if NPs needs to be reconstituted).

It is necessary to select a buffer that do not induce aggregation of the nanoparticles. It would be suggested to check size/stability of the NPs in the chosen conditions by DLS prior to TEM analysis.

The buffer selected to prepare a TEM grid should not be too viscous. Viscous sample are not suitable for cryo-EM is their electron transparency. For example, 20% glycerol in solution, won't allow to get good image of NPs (e.g. liposomes) because the contrast difference of the membrane and the 20% glycerol is minimal. Therefore, as a rule, viscosity of the solution should be minimize and checked: an aqueous sample is suitable for the cryo-preparation technique if a drop of sample spreads comparably to water on a filter paper [1].

5.3 Reagent Preparation

- The buffer must be always filtered with 0.2 µm filters prior to its use to avoid contamination of the grid.
- Final sample concentrations of about 1–2 mg/mL (total lipid/polymeric concentration) are recommended for organic NPs (such as liposomes), but the optimal concentration may vary and should be optimized case by case. Usually, it is suggested to follow the specific instructions given by the producers for each specific nanomaterial, to be sure to adopt the most suitable NPs dispersion/reconstitution process.

6 Procedure

6.1 Sample preparation

6.1.1 Plasma cleaner:

Just before the use in the Vitrobot, carbon grids must be cleaned with plasma, using a plasma cleaner machine. The procedure for using our specific equipment is reported below:

- *Put the grid on the specific support*
- *Push on "Vent" to switch on the machine under atmospheric pressure*
- *Insert the support with the grids in the machine*
- *Press on "vacuum" to generate the vacuum*
- *Choose the recipe "cryo" (25% O, 75% Ar for 20s) and press start*
- *When the process is ended press "vent" again*
- *Take the support with the grid and push "Vacuum" to start the vacuum pump again*

6.1.2 Preparation of the grid and automated vitrification of the sample using the vitrobot:

During sample preparation, the TEM grid is fixed into the preparation chamber and then a droplet of sample (approximately 2–3 µL) is applied to the grid with a pipette. Excess of sample is removed by quick blotting (3s) with a filter paper leaving a thin spanned film of the sample in the holes of the grid

and the sample is then after 1s plunge frozen in liquid ethane. A very high cooling rate is required to vitrify the sample (i.e. transform it into a glassy state) and to avoid the formation of crystalline (cubic or hexagonal) ice [1]. An automated vitrification system with controlled humidity is used in our lab to ensure a reproducible sample preparation procedure. The vitrobot (FEI) contains an environmental chamber that allows sample preparation at 100% humidity at room temperature. Below you can find the detailed preparation used with our system. This procedure should be adapted for other equipment.

- *Fill 3 Dewar's with liquid nitrogen,*
- *Change the filter papers into the chamber,*
- *Fill in the MilliQ water tank (60 mL) to obtain the humidification of the chamber,*
- *Switch on the vitrobot station,*
- *From the console put the humidity control "on" and select 100% humidity,*
- *Set the temperature at room temperature or less according to your type of sample*
- *From the console, select "Option", then process parameters and choose the right parameters for the automatized vitrification process: Blot time: 3s, Blot force -5, Wait time 1s, drain time 0s, blot total: 1s,*
- *Start a first cycle without the sample to verify that the process works correctly,*
- *Fill the polystyrene box and the cell represented with liquid nitrogen,*
- *Open the bottle of ethane gas, immerge the gun connected to the ethane tank into the liquid nitrogen. Fill the cell in the center of the polystyrene box with ethane gas as soon as the liquid nitrogen is evaporated. Leave ethane first liquefied and, then solidified,*
- *After ethane has solidified, heat it gently until it liquefy back completely Take one grid and fix it on the tweezer, then push "place new grid" and fix the tweezer on the piston of the vitrobot machine,*
- *Press "continue",*
- *Transfer the polystyrene box with the cell filled with liquid ethane into the external support of the vitrobot and select "place ethane lift container",*
- *Press "process",*
- *Then it's time to deposit the sample into the grid: take 2 µL of the sample solution with a pipette, and deposit a drop on the grid,*
- *Click to "continue" to start the vitrification process,*
- *After the sample has been automatically plunged into liquid ethane and the vitrification process has ended, manually remove the tweezer from the piston, while keeping it into the liquid ethane,*
- *Under cooling, remove the grid with the vitrified sample from the container with liquid ethane and blot excess with a piece of filter paper,*
- *Quickly transfer the grid into a grid box (placed in the polystyrene box into liquid nitrogen), and after move the grid box into a tube filled with liquid nitrogen. Store the tube in a Dewar with liquid nitrogen until TEM observation,*
- *Eliminate the remaining liquid nitrogen and ethane from the polystyrene box,*
- *Press "exit" and switch off the vitrobot,*
- *Remove and clean the humidity chamber,*

6.1.3 Sample transfer into the TEM microscope

The sample is transferred from the Dewar into the cold TEM cryo holder by using specific pre-cooled transfer unit tool, as described in the annex. Subsequently, the TEM cooled holder (Figure 1C) is

quickly inserted into the electron microscope. There is a need to make this step fast in order to reduce water condensation from the atmosphere on the cryo-holder [3].

Below is reported the detailed procedure used in our laboratory:

- *Fill in the transfer station and the TEM cryo holder with liquid nitrogen,*
- *Take the grid with a tweezer which has been pre-cooled and transfer it in the TEM cryo-holder,*
- *Transfer the TEM cryo-holder with the transfer station into the microscope room,*
- *Remove the safety lid of the TEM cryo-holder,*
- *Turn the microscope stage of -70°,*
- *Quickly remove the TEM cryo-holder from the transfer station and insert it into the TEM microscope,*
- *Turn the microscope stage back to 0°*
- *After a suitable vacuum level is reached into the intermediate chamber of the microscope, turn the sample holder to the left and accompany it inside the microscope,*
- *Perform the observation with the TEM microscope.*

6.1.4 TEM observation and processing of the images

- Observation is performed at 200 kV or at lower energy (80-120 kV), depending on the sample. A diaphragm objective is used to reduce the intensity of the beam, in order to avoid sample damages during observation. Moreover, a low beam spot size is used during the observation to improve image contrast.
- The images are processed by a computer program (like Image J), which can subsequently enhance the contrast by several filter techniques. Size distribution of the sample may be calculated by manually or automatically scoring the size of at least 200 NPs. The minimum number of nanoparticles to be scored is very sample dependent. For some sample (e.g. not homogenous ones) scoring of 400-500 particles may be necessary. TEM images only visualize a small part of the whole (more or less complex) sample. A single TEM image should therefore not be overestimated and care should be taken to obtain a series of images that are representative for the whole sample.

6.2 Reporting

The report of the results should include all raw images (with corresponding scalebar) used for statistical evaluation. The report should identify the software applied for image and statistical analysis.

The report should contain the number of particles analysed, the calculated size and –if needed - shape describing parameters (together with short definition) and standard deviations.

Typical size and shape describing parameters are:

Diameter: Equivalent area circle's diameter

Feret's diameter: The longest distance between any two points along the selection boundary

Circularity: $4\pi \times [\text{Area}]/[\text{Perimeter}]^2$

Aspect ratio: The aspect ratio of the particle's fitted ellipse, i.e., [Major Axis]/[Minor Axis].

Roundness:$4 \times [\text{Area}]/(\pi \times [\text{Major axis}]^2)$ or the inverse of Aspect Ratio

Particle size distribution should be reported using a histogram, like the example below. Binning parameters should be also reported.

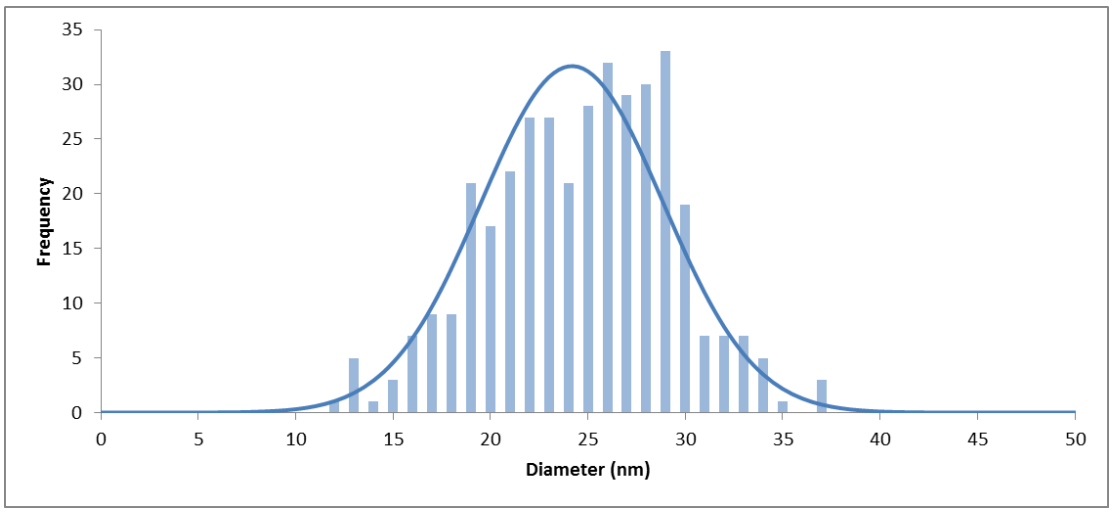
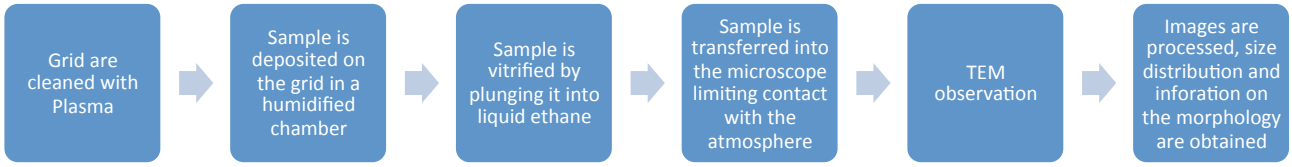


Figure 1. Typical size distribution histogram: result of particle size analysis based on 200 objects using the particle size distribution macro of Image J as image analysis tool and Excel for statistical analysis. The histogram was created with 0-50 nm bin range and 1 nm bins. The curve shows the calculated normal distribution corresponding to the same data set.

6.3 Flow chart

Figure 2: Flowchart of the workflow



7 Health and Safety Warnings, Cautions and Waste Treatment

In case of hazardous materials, samples should be prepared in a safety hood to avoid particulate formation and to minimize exposure. Appropriate safety precautions and protective gear such as gloves, lab coat and goggles must be worn. After the measurement the samples should be discharged as appropriate for nanomaterials.

8 Abbreviations

NPs: Nanoparticles

TEM: Transmission electron microscopy

9 References

[1] J. Kuntsche, J. C. Horst, H. Bunjes, International Journal of Pharmaceutics 417 (2011) 120– 137.

[2] M. Almgren, K. Edwards, G. Karlsson, Colloids and Surfaces A: Physicochemical and Engineering Aspects 174 (2000) 3–21.