Quorum

APPLICATION NOTE

GLOQUBE® PLUS

Glow discharge system for the preparation of TEM grids

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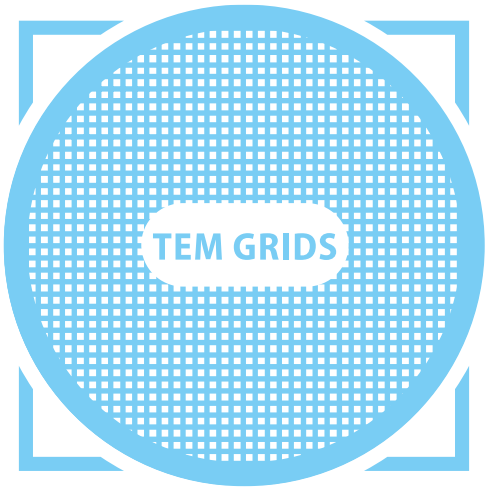


Transmission Electron Microscopy (TEM) is a high-resolution imaging technique that provides information about the structure and morphology of specimens. It uses a high energy electron beam to penetrate samples and renders images using the transmitted part of the beam. Highly accelerated electrons have a small wavelength; hence, they can be used to resolve small features. This capability is essential in structural biology, nanotechnology and material science studies. The sample to be imaged needs to be placed on a special metal grid, typically covered with ultra-thin (2-5 nm) carbon or polymer support. To ensure the surface support is suitable for use, it has to undergo a glow discharge process first.

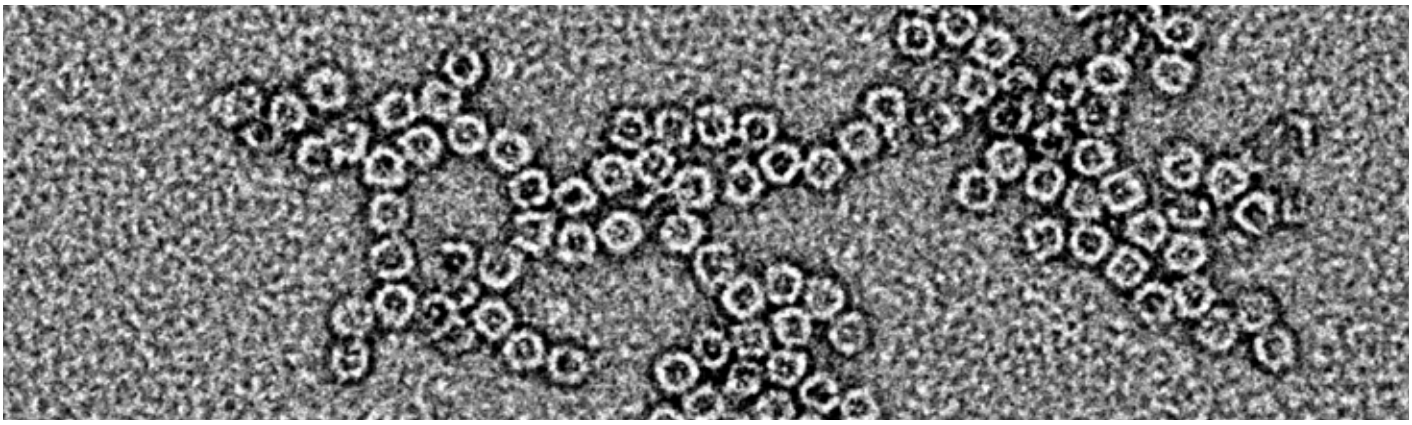


Figure 1 Native ferritin on in-air glow discharged carbon TEM support. Image courtesy of Paul Simpson, Imperial College, London

1 Why do we need to glow discharge TEM grids?

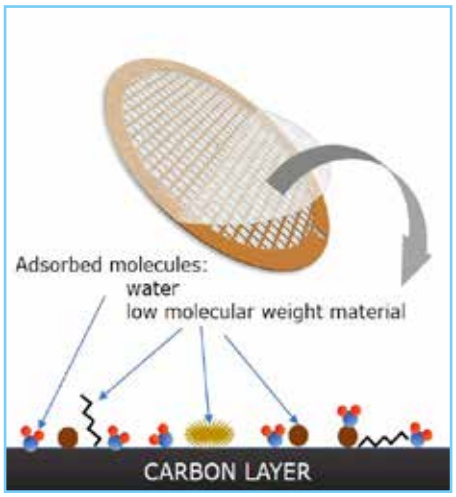


Figure 2 A typical TEM grid with carbon film and representation of surface adsorbates.

Even freshly prepared carbon layers for TEM grids will have unwanted adsorbates like water and low molecular weight material (LMWM) on the surface, typically adsorbed from the air, shown in figure 2. These contaminants need to be removed by a glow discharge before using the grids to ensure the best sample adhesion.

Furthermore, the deposited carbon layer on the TEM grid has a variably charged surface that is usually hydrophobic, thus even spreading of the water-based sample suspension is very difficult, as illustrated in figure 3.

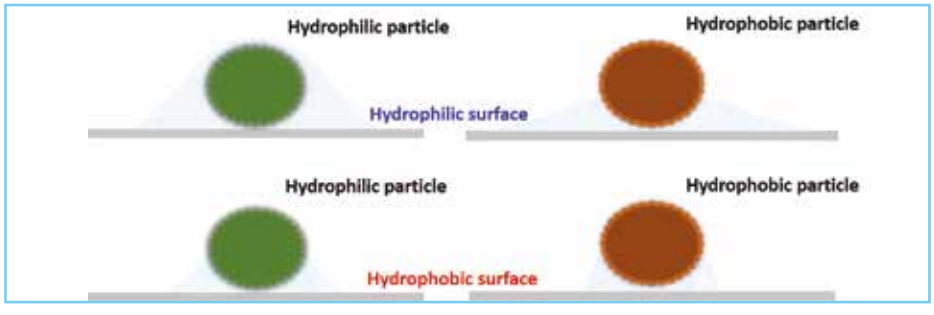


Figure 3 Wetting effect on hydrophilic and hydrophobic particles placed on hydrophilic and hydrophobic surface

2 What is glow discharge?

A glow discharge plasma is a partially ionised low-pressure gas. It contains ions of net-positive and negative charge. That quasi-neutral state is sustained by the presence of energetic electrons. These electrons, when inelastically colliding with gas molecules, excite or ionise them, which results in the formation of free radicals and ions of gas molecules. The characteristic glow observed during the process is caused by photons that are released by neutralised free radicals (i.e. relaxation of electrons). The use of a glow discharge treatment is an acknowledged technique used for cleaning and modifying surfaces. The result of the glow discharge treatment depends on the chosen plasma gas and polarity.

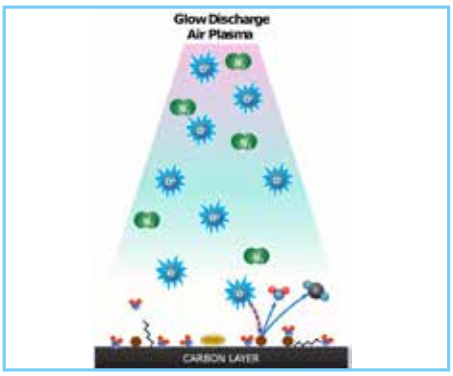


Figure 4 Glow discharge air plasma process

3 Why do we need different types of glow discharge?

Amongst all materials, biological samples are the most challenging to prepare as this process consists of several, equally important steps and a failure of one step can lead to a dramatic consequence of losing the sample entirely. Moreover, due to their specific local charge, the affinity of the molecule to TEM supports might be different, which can be particularly apparent in proteins, where specific parts of the molecule charge differently. Adequately prepared TEM supports will allow the even spread of the sample, increase the number of retained molecules, and also allow the molecules to be oriented explicitly on the TEM support to reveal their sides of interest. Observation of biomolecules in ambient TEM conditions also involves staining the sample with heavy metals. TEM supports that have not been treated with a glow discharge will result in uneven staining and cause poor contrast in the image. This effect is also present in cryo-TEM, where the image can be unstable. Ensuring the TEM grid is uniformly hydrophilic/hydrophobic and of the desired charge is essential for several reasons; the successful placement of the sample on the grid, drying and staining (in ambient TEM), or plunge-freezing (in cryo-TEM) and for imaging. The molecule to be imaged dictates the method of grid surface preparation and modification.

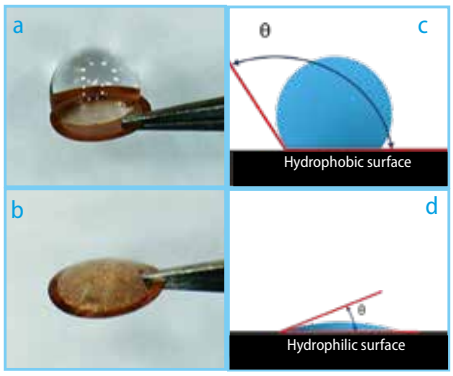


Figure 5 (a) Carbon support TEM grid before glow discharge with a droplet of water showing its hydrophobicity (b) Glow discharge treated TEM grid with a droplet of water showing its hydrophilicity (c), (d) Corresponding contact angle

4 Choosing the glow discharge technique by application

The desired effect of glow discharge is to make the surface of the TEM grid carbon support sufficiently charged and appropriately modified for the application. As a result, a thin liquid film, in which the sample is suspended, will evenly spread and dry over the entire surface. The table below presents examples of possible surface modifications along with their applications.

APPLICATION	ATMOSPHERE	SURFACE TYPE	CHARGE	ADVANTAGES
TEM grids	Air	Hydrophilic	[-]	No aggregation of particles on the grid square boundaries
TEM grids HOPG MICA	Air	Hydrophilic (after subsequent treatment with magnesium acetate or 0.1% w/v polylisine)	[+]	Better binding of nucleic acid to the grid surface
TEM grids (for positively charged proteins)	Hydrocarbons (e.g. Methanol)	Hydrophobic	[-]	Covalent binding to the grid surface for positively charged molecules
TEM grids (for proteins, antibodies and nucleic acids)	Alkylamines (e.g. Pentylamine)	Hydrophobic	[+]	Covalent binding to the grid surface negatively charged molecules

5 Benefits of a two chamber system

A compact, two-chamber system offers a quick and efficient way of trying different types of surface modifications, which is ideal if the most common in-air glow discharge does not give satisfactory results.

When chemical vapour glow discharge is needed, a single chamber system is not as easy or reliable to use as a dual-chamber system. This is due to the chemical contaminants deposited during the process, which then need to be removed entirely by cleaning the chamber and system for in-air plasma treatment.

With two-chambers, the GloQube® Plus system prevents the risk of cross-contamination, when using the chambers separately, and also offers no downtime of the instrument. Additionally, a specially designed purge-pump cycle removes all the remaining vapour of used chemical in the system.

The GloQube® Plus's performance was demonstrated in a series of cycles of in-methanol vapour tests. These were conducted with the use of Hidden 200AMU RGA attached to the backing line and the residual gases were monitored.

The tests showed no presence of contamination with CH_3O^+ ion. The detailed results of the contamination test can be seen in the technical data.

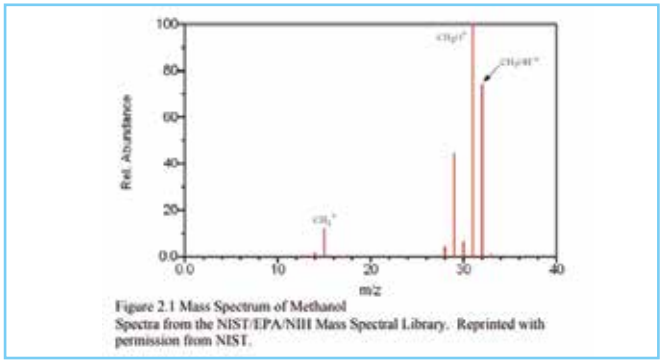


Figure 1
Methanol(CH_3OH) RGA cracking Pattern.
Monitored gasses: CH_3 at 15 amu, CH_3O at 31 amu, CH_3OH and O_2 at 32 amu, CHO at 29 amu

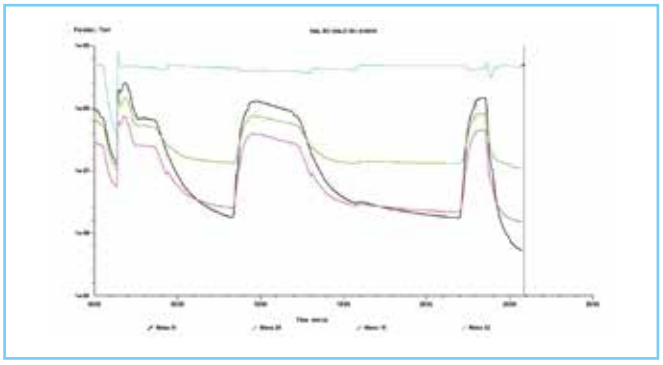


Figure 2
1. Methanol introduced to $1x10^{-1}$ mbar and methanol capsule removed with bleed valve open. Recovery time 4 mins
2. Methanol introduced to $1x10^{-1}$ mbar for 3 mins and methanol removed with bleed valve open. Recovery time 4 mins
3. Methanol introduced to $1x10^{-1}$ mbar for 1 min and methanol removed with bleed valve closed. Recovery time 2 mins

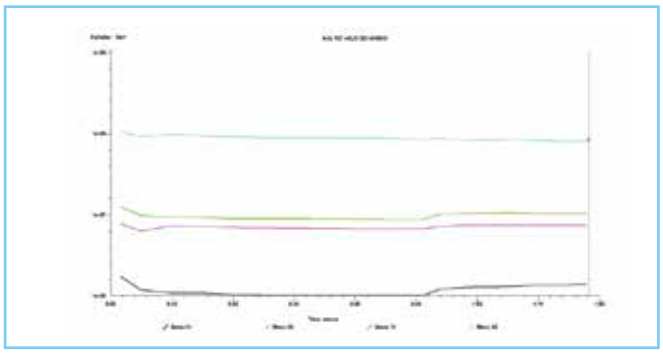


Figure 3
A plasma was run for 30 mins in the vapour chamber with methanol. The chamber was vented and the door for both chambers was opened. The clean chamber was pumped down, and, when 1.5 $x10^{-1}$ mbar was achieved in the clean chamber, the RGA was switched on. Residual gases were monitored as the clean chamber continued to be pumped down. There is a slight decrease in all intensities at the start of this process, which is due to the vacuum level changing, and also due to the adjustment of the RGA operating pressure. No change in the monitored peaks were observed after the initial calibration of pressure. There is no evidence of contamination from the vapour chamber to the clean chamber when methanol is used in the vapour chamber.
It takes approximately two minutes for methanol to be removed, by pumping, from the vapour chamber after gas introduction.

The most responsive gas to monitor is CH_3O with a mass of 31 amu.

Figure 2 shows that if the methanol is removed from the input to the GloQube® Plus injection system, it takes about four minutes for the vapour to be pumped out of the needle and bleed valve assembly.

When the bleed valve is closed, it takes two minutes for the gases to be pumped out of the chamber.

6 Glow discharge: in-air effect

A glow discharge air plasma is used for cleaning and hydrophilization of surfaces. During the process, oxygen from the air is ionized to positive and negative ions, that further react to form clusters. These highly reactive species bombard the surface and remove adsorbates (LMWMs) making the area uniformly, negatively charged and highly oxidized (in majority possessing carboxylic acid and ketone groups). The low concentration of oxygen in the air makes the process non-destructive for subjected surface and allows its easy modification. The majority of macro-molecules are hydrophilic and, hence, they do not like to retain on a hydrophobic surface. This gives a need for in-air glow discharge treatment of a carbon film support before their application on its surface. Such a situation is illustrated in figures 6 and 7. Native ferritin, possessing hydrophilic 3-fold channels, through which iron ions are transferred into the core, does not retain on a non-glow discharged carbon surface when used in low concentrations. In high concentration, we can observe the formation of aggregates. Uneven charge on the carbon support also disturbs correct staining resulting in light patches between protein groups. Modification of the carbon surface by in-air glow discharge makes the surface hydrophilic and negatively charged; thus, retention of native ferritin is much simpler. Furthermore, negative staining of the sample with uranyl acetate works properly giving even staining, and the protein molecules can be seen correctly.

Native ferritin from horse spleen (Sigma Aldrich) of low (6x10^{-4} µg/mL) and high (6x10^{-2} µg/mL) concentration solution was applied onto non-glow discharged and in-air glow discharged TEM grid carbon supports and imaged with use of TEM Tencai F20.

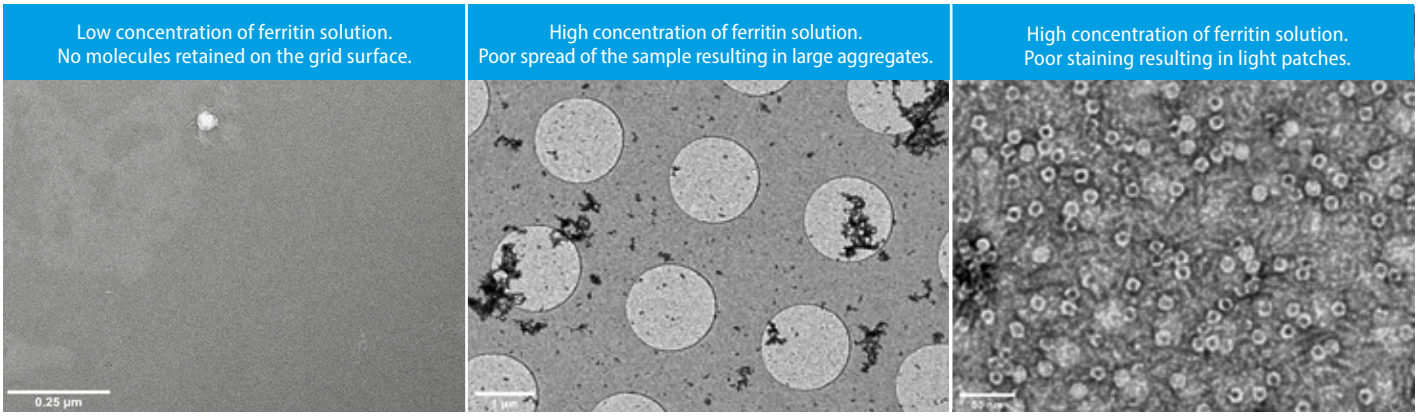


Figure 6 The effect of non-glow discharged carbon support TEM grid on retention, spread and staining quality of native ferritin sample solution. Low (6x10^{-4}µg/mL)and high(6x10^{-2}µg/mL) concentrations of the protein were used.

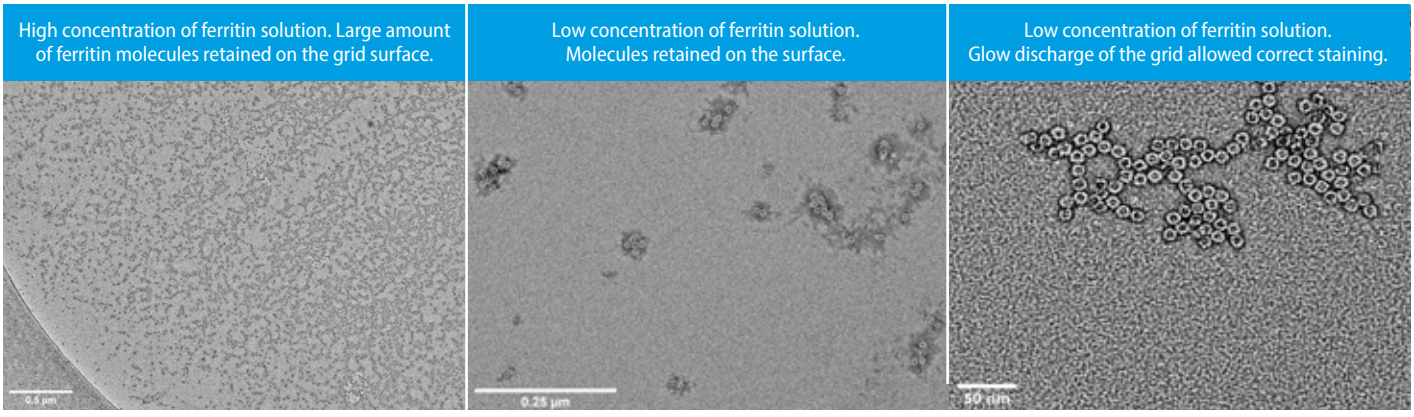


Figure 7 The effect of in-air glow discharged carbon support TEM grid on retention, spread and staining quality of native ferritin sample solutions. Low (6x10^{-4}µg/mL)and high(6x10^{-2}µg/mL) concentrations of the protein were used.

7 Methods of in-vapour glow discharge and advantages of process automation

Manual gas introduction One of the first reported methods of TEM sample preparation was using the manual introduction of vapour into a glow discharge chamber [1]. In this method a glass tube is filled with a few millilitres of chemical and the flow into the glow discharge chamber is controlled by a manual Teflon valve. Once introduced and the glow discharge ignited, this deposits on the surface of the TEM grid.

Filter paper/wool Another well known method of preparing TEM grids for sample dispersion is placing a piece of filter paper or cotton wool saturated in the chemical into a glow discharge chamber. Once the chemical vapour is introduced and the glow discharge ignited, the chemical is deposited on the surface of the TEM grid.

Capillary valve The third method commonly used for chemical introduction into a glow discharge is using a capillary valve attached to an inlet nozzle on the chamber. The bottle of amylamine can then be attached to the capillary valve and the flow of the vapour manually controlled. [2]

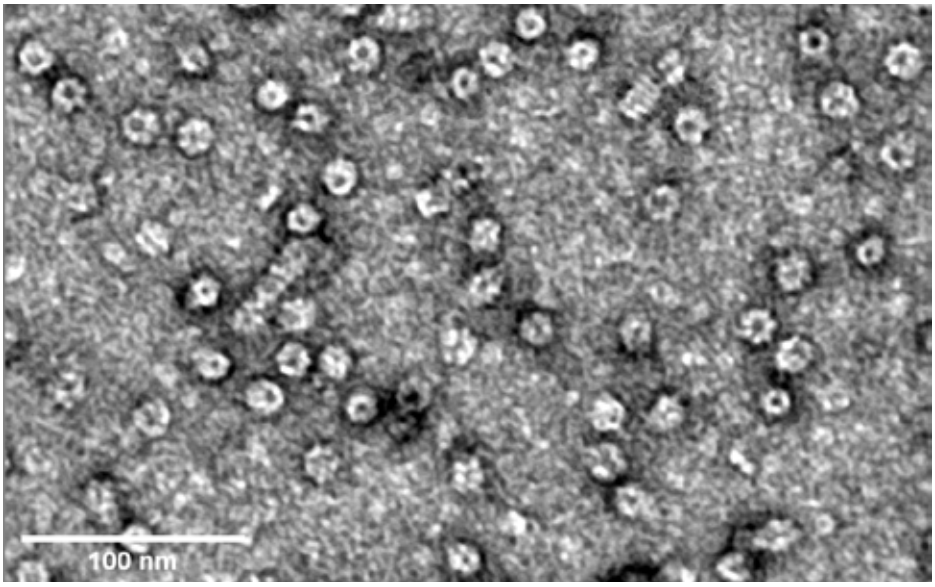
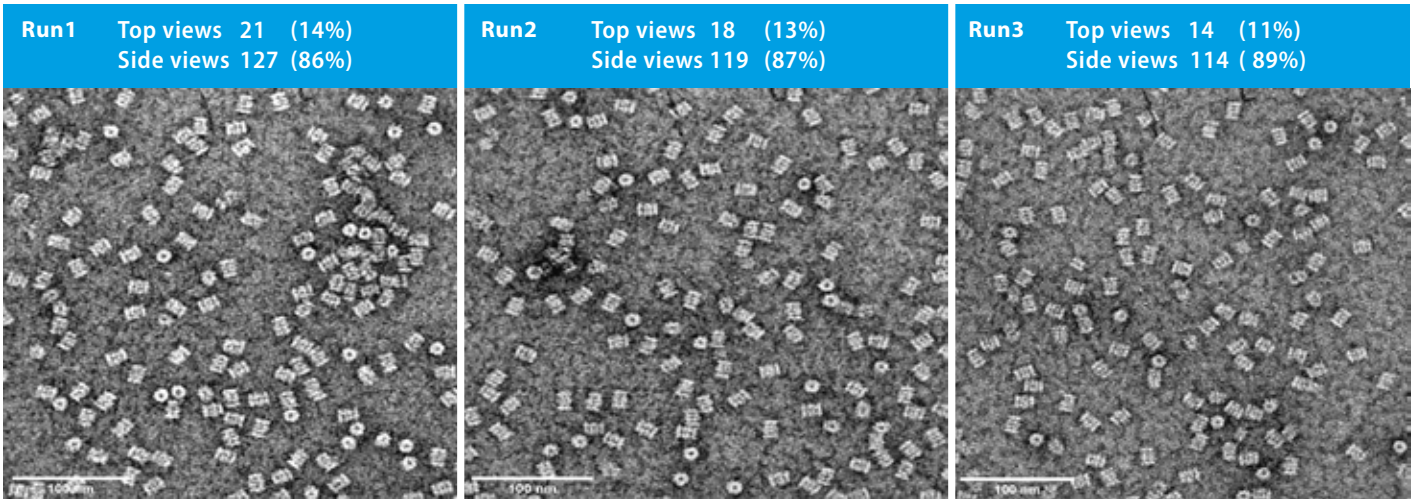


Figure 8 TEM grid carbon support modified by the blotting paper method and used for 20s proteasome sample application. Glow discharge system used: Quorum's Emitech K100X

Disadvantage - the remains of oxygen and water vapour in the chamber disturbs the correct surface modification thus only a few side views appear on the grid.



Automated valve The use of an automated valve reduces the risk of human error, for example, opening the valve at the wrong time or too quickly. The pressure of the chamber is also easily controlled as the valve automatically opens and closes, controlling the flow of the chemical into the system: maintaining stable pressure. It also means process time can be changed, as there is no risk of adding too much or too little chemical into the chamber.

Figure 9 GloQube® Plus with automated valve system, three consecutive runs showing the yield of side views of 20s human proteasome protein complex is over 80%.

8 Glow discharge using chemical vapour

Introducing chemical vapour in the glow discharge process allows the grafting of desired functional groups onto surfaces. This gives a way to not only change the wetting properties and charge of the surface but also can enable complex specimens to be covalently bonded to the grid's surface. This type of preparation is especially crucial for biomolecules, like proteins and nucleic acids. Understanding protein structure and their interactions with other macromolecules are of the highest interest in drug discovery and cancer research fields. High-resolution electron microscopy has to be used to obtain the required information for protein structure analysis and modelling. The orientation of protein molecules on the TEM support grid plays a significant role in this process, as revealing their active subunits is one of the keys for correct and successful data collection. Proteins structure and spatial orientation very often require using advanced methods to direct their adsorption on TEM support grid.

9 Glow discharge using alkylamines

Alkylamines are known to form positively charged, hydrophobic films on carbon surfaces in glow discharge processes. Such films attract negatively charged areas of the protein, allowing for the observation of the desired orientation. Their resulting hydrophobicity also helps to retain the molecules on the surface as the core of proteins is usually also hydrophobic. The 20s proteasome protein complex is a known system for recognition and degradation of misfolded protein. In an essential regulatory mechanism in cells, this is a well-established target for cancer therapy. Amylamine (a homologue of alkylamine group) was used to affect the orientation of 20s proteasome adsorption onto TEM grids. Carbon support TEM grids were modified in a GloQube® Plus using an amylamine vapour glow discharge process to achieve hydrophobic and positively charged surfaces to retain side-views of 20s proteasome complex. In figure 10 the effect of altering the surface charge of the carbon support film on the orientation of the protein complex can be seen. In the first instance, the freshly prepared carbon surface was non-uniformly charged and hydrophobic; therefore, a few of the side-views could be seen. In the second case, after treating the grids with an in-air glow discharge, only top views could be seen as this treatment makes carbon films negatively charged and hydrophilic. This process attracts the positively charged 19s part of the proteasome complex resulting in biased top-views orientation. The last case, in amylamine vapour glow discharge, showed the majority of the 20s proteasome complex molecules having side-view orientation.

The 20s human proteasome complex sample in TRIS buffer solution ($3x10^{-2}$ µg/mL) was applied on three types of carbon support TEM grids: no-glow discharged; glow-discharged in-air, and glow discharged in-amylamine vapour.

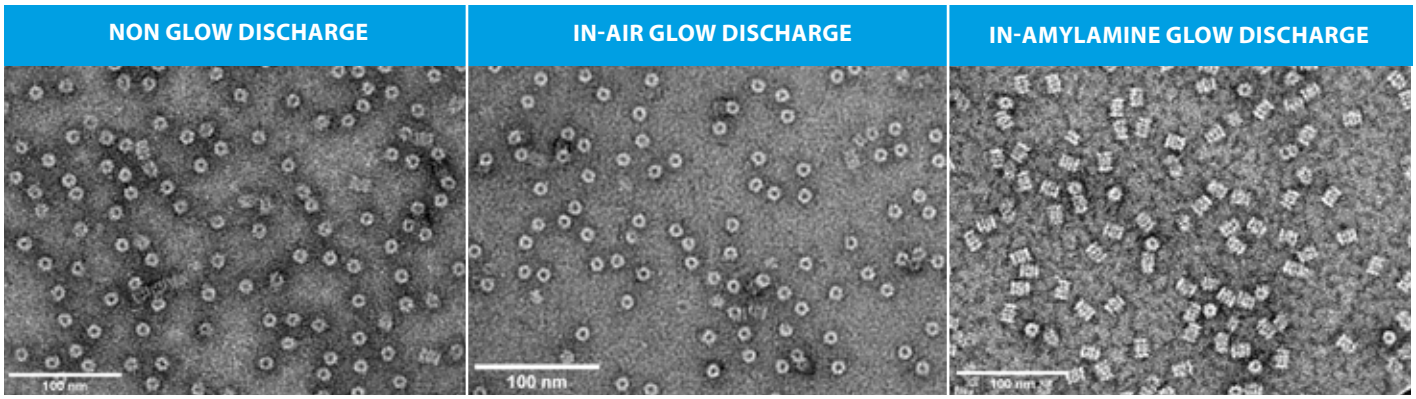


Figure 10 TEM images of 20s human proteasome complex showing the effect of altering the surface charge of the carbon support film on the orientation of the protein molecules. Carbon film of 2.5nm thickness on Quantifoil 1.2/1.3 400 mesh was used as a support for the sample.

10 Glow discharge using alcohols

Alcohols, e.g. methanol vapour, introduced into a glow discharge system will render the carbon support grid negatively charged and slightly hydrophobic. Such a surface will attract positive ions, for example, native ferritin. All ferritin molecules are made of 24 identical peptide subunits that fold into a spherical shell with a water-filled cavity inside. This cavity is connected to the outside through channels with threefold and fourfold symmetry and is thought to provide permeation pathways for iron ions and protons, essential for the proper functioning of ferritin as an iron depository. Apo form of ferritin (an empty shell of ferritin) is also used as an ion cage for the templated synthesis of nanoparticles - ZnSe or CdSe. Imaging of the load of the ferritin cage plays a significant role in studying iron and other metals uptake. An in-methanol vapour glow discharge treatment of carbon support TEM grids will help in the ferritin load investigation and also prevent the loss of ions through the channels. Figure 11 shows that in-air glow discharge treatment of the TEM grid support allows for the iron ions to be lost from the core resulting in empty ferritin (light areas inside the molecule core). Using in-methanol vapour treatment of the grid enables the user to keep the ions inside the core (dark load inside the ferritin molecules), due to the negative charge and hydrophobicity of the surface.

To achieve the images below the same volume of ferritin sample (6x10^{-3} µg/mL) was dispensed in-air and in-vapour (methanol) on a glow discharged TEM grid carbon support.

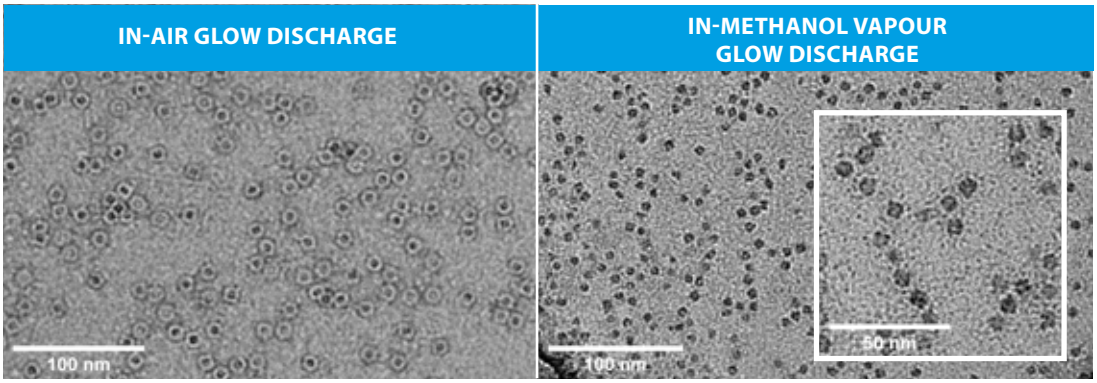


Figure 11 TEM images (Tencai F20 G2) of ferritin protein complex from horse spleen (Sigma Aldrich) applied to in-air and in-methanol vapour glow discharged carbon support TEM grids, courtesy of Imperial College London.

11 Summary

The GloQube® Plus provides all the features necessary for TEM grid preparation when the glow discharge of these grids with air or vapour is required.

The use of a separate chamber for in-air and in-vapour glow discharge is essential to remove the risk of cross-contamination. The GloQube® Plus design ensures no cross-contamination occurs, while also enabling the user to run processes sequentially using the same chemical - without the need for cleaning in between.

Having an automated chemical injection system is also a huge advantage, as the flow rate can be controlled more accurately along with the chamber pressure.

This also provides the ability to run longer process durations without the risk of exhausting the amylamine, as only a fixed amount is used - unlike the filter paper method, for example.

With the automatic valve, there is a continuous flow until the vial runs out.

A visual indication shows the level of chemical left in the vial. The added control provided by the automatic valve ensures repeatability and reproducibility of the TEM grid treatments.

The enclosed vapour delivery system with septum seal vial and automated valve system also minimises operator exposure to the chemicals, increasing the safety of chemical use.

References

[1] Dubochet, Jacques, et al. "A new preparation method for dark-field electron microscopy of biomacromolecules." Journal of ultrastructure research 35.1-2 (1971): 147-167.

[2] Morris, E. P.; da Fonseca, P. C. A. High-Resolution Cryo-EM Proteasome Structures in Drug Development. Acta Crystallogr D Struct Biol 2017, 73 (Pt 6), 522–533.

[3] Aebi, Ueli, and Thomas D. Pollard. "A glow discharge unit to render electron microscope grids and other surfaces hydrophilic." Journal of electron microscopy technique 7.1 (1987): 29-33.

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