Development and Application of the Sample Support in Transmission Electron Microscopy

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Abstract. Cryogenic electron microscopy is becoming an increasingly popular technology that allows us to observe the molecules in high-resolution details. With this refined way to study the structures in scientific researches, the observation of the molecules will be expected to make improvements. The conventional methods, such as the X-ray crystallography and the nuclear magnetic resonance spectroscopy, provide the basic observation of ground-state molecules and the measurement of various small molecules. But both of them contain flaws discovered in the continuing development process. Therefore, the application of cryo-EM becomes a broader area to explore. In this review, we will give an overall understanding of the cryogenic electron microscopy. We will discuss the intention behind the invention of the cryo-EM and introduce the main workflow of the experiments. In addition, we will discuss the methods to prepare the sample in a well-condition and compare them among different groups of experiments. More importantly, we will discuss the application of the graphene in cryo-EM and evaluate possible improvements in future development.

Keywords: Cryo-EM, Graphene, Supporting Film, Amorphous Carbon, Sample Preparation

1. Introduction

Cryogenic electron microscopy (cryo-EM) is a technique using a low temperature to embed the samples into vitreous water, which allows us to observe the tiny molecules with its original state under low temperatures [1]. Before the invention of cryogenic electron microscopy in the 20th century, two traditional ways of observing molecules are often used, which are the X-ray crystallography and the nuclear magnetic resonance spectroscopy [2]. The purpose of the X-ray crystallography is to obtain three-dimensional molecules such as proteins from a crystal state [3]. The backward is later discovered in the experiment that the X-ray crystallography can only observe the ground-state molecules while the observation of the excited state and transitional state molecules are unreachable. On the other hand, the nuclear magnetic resonance spectroscopy is the measurement that is carried out in solution under near-physiological conditions [4]. It is sensitive for observing relatively small molecules but not large molecules. As a result, an advanced technique is required to make improvements in the modern observation of molecules. The invention and development of the cryogenic electron microscopy make the observation more effective and efficient, which allows us to directly observe the molecules using only a small number of samples without crystallization. Aside from the technical advancement of the...
equipment, sample preparation is considered as the most crucial and delicate procedure in the application of cryogenic electron microscopy. The use of different supporting films, along with various samples, has disparate impacts on the result. The incentive of using the graphene as supporting film becomes a significant consideration among a number of recent experiments. In this manuscript, we will discuss and demonstrate some of the outstanding capabilities of graphene and its huge implication in the area of cryogenic electron microscopy.

2. Background about cryo-EM

2.1. Types of electron microscopy
The application of electron microscopy is one of the most crucial parts of scientific study. According to the Electron Microscopy Facility, there are two main types of electron microscopes, which are the transmission electron microscope and scanning electron microscope [5]. The transmission electron microscope uses electrons to project image for observing the protein molecules or thin tissues. High-powered electron beams emitted through the condenser lens towards the objects. Some electrons pass its original trajectory while some electrons deflect or rebound. The modified beam then passes through the objective lens and projector lens onto the fluorescent screen to give the final image [6]. On the other hand, the scanning electron microscope uses the secondary emission electrons to observe a more detailed image and particle distribution of the molecules in a deeper focus [5]. Unlike the concentrated electron beams used in the transmission electron microscope, the scanning electron microscope scans across the object. Different amount of energy is lost according to the different surface during the process. Although it is not as powerful as the transmission electron microscope, the scanning electron microscope can produce images of larger objects to create a three-dimensional picture [6].

The cryogenic electron is a type of transmission electron microscope. And it has been going through a history of development to meet its wider application.

2.2. The history of cryogenic electron microscopy
In the 1960s, the potential damage to the specimen is a major limiting factor for electron microscopic observation. To deal with this problem, scientists must search for alternative or advanced solutions. The creation of the cryogenic electron microscopy was intended to solve this problem based on the comprehension of using low temperatures to prevent damage [7]. As the scientists, Erwin Knappe and Jacques Dubochet published observations related to the beam damage in 1980, thin crystals formed on carbon film were found to be from 30 to 300 times more beam-resistant at 4 K than at room temperature. Under the premise that cryo-protection at 4 K strongly relies on the temperature, most results can be explained [8]. At that time, the results were not reproducible and amendments were published 2 years later, informing that the beam resistance was less significant than predicted for the first time [9]. Then, in 2017, scientists Jacques Dubochet, Joachim Frank and Richard Henderson were awarded the Nobel Prize in Chemistry for developing a technique that would image biomolecules using the cryo-EM technology [10]. Thus, the application of the cryogenic electron microscopy shows excellent significance in observing biomolecules in 3D space.

2.3. The main workflow of cryo-EM
The main workflow of the cryo-EM experiment is fundamental for the development. First, the sample must be well-prepared to fit for observation. Second, the transmission electron microscopy imaging is activated to observe the molecules. Finally, the images taken are processed and analyzed. Sample preparation is one of the most important parts. Several TEM grids are prepared with arrays of holes in the carbon layer attached to the grids. Since the carbon needs to be hydrophilic, the grids are glow discharged. At the same time, a certain container is plunge-freezing with liquid ethane using the liquid nitrogen to keep the temperature low until the Leiden frost effect is achieved, in which an insulating vapor layer is formed that keeps the liquid from boiling rapidly. Then the grids are frozen with the samples at certain blot settings. After that, the grids are attached to the ring and are placed on the
workstation. Finally, using the cassette to put all samples into the capsule and put it under the cryogenic electron microscope. The electron microscope is turned on for observing. It has an electron gun, which is the source for emitting electrons. Below that part, there is a condenser lens to condensing the beam and the objective lens is ready to focus the beam on a certain area. The projective lens and detector are shown at the bottom, which processes the image such as magnifying. The whole device is connected to the computer and therefore, images can be further analyzed. In general, these are what need to be done during the experiment [11]. As shown in Figure 1, the main procedures of cryogenic electron microscopy is introduced.

![Diagram](image)

**Figure 1.** Illustrate the main workflow of the Cryogenic Electron Microscopy [12]

3. The supporting film

3.1. Physical requirement
Since the preparation of the sample will usually be contaminated due to the interaction between the air-water surface, the reduction of interaction is essential before vitrification. One practical solution is the use of supporting film, which attaches to the surface of the holding materials as a contact layer for the samples [13]. Also, they are able to enhance the protein density in the holes of the grids for better observation [14]. Therefore, they provide better conditions for the samples. However, the use of supporting cost the expense of introducing background noise and surface tension [13]. Also, since the observation of molecules requires samples with high resolution, any tiny change could affect the whole process. As a result, improvement has been made during this long period. By changing the material for the supporting film, the observation of the sample is getting more accurate and more convenient.

3.2. Using amorphous carbon as supporting film
One of the classic supporting films is amorphous carbon. Since the carbon family is always one of the most stable elements in the periodic table, we start to consider them as the best surface for the samples to this end. The amorphous carbon is usually applied to support the sample and improve stability. This traditional material allows the attachment and retainment of the initial concentration, especially for the
low concentration or intricate procedures. Additionally, the usage for amorphous carbon is various as well, such as the reduction of the charging and the protection of the stability of the cell sections or tissues. In short, amorphous carbon is a material with extensive application in molecular research [15]. Nevertheless, the initial method using the amorphous carbon as supporting film introduces many fluctuations and make the sample difficult to maintain a certain condition that is ideal for the experiment. The amorphous carbon is usually large, so it is challenging to prepare a favorable supporting film for the experiments. Also, it will easily cause movement along with the sample, which creates problems for the observation. While some protein samples prefer to attach to the carbon film and fail to enter the holes on the grid, others remain at the stage of the air-water interface with the damaged folding process. Additionally, it becomes hard to search across the whole grids for a thin ice area due to the unknown thickness of the ice [14]. Because of its natural amorphous existence, it is more likely to add damage during the preparation of samples.

3.3. Using graphene as supporting film
Urged by the improvement in the accuracy, the scientists have never stopped the pace to explore the new material which suits better for the supporting film and improve the quality of the samples such as the streptavidin, lipid monolayers and double-stranded DNA scaffolds. People begin to search for better materials with excellent electrical and thermal conductivity, greater mechanical strength, chemical and temperature resistance, such as the monatomic layer graphene [16]. It has the highest mechanical strength with its single-atom thickness and highly ordered structure. With the increasing amount of applications of graphene in various areas around the world such as the sensor, transistor, screen, battery, desalination, and so on, the use of graphene becomes an obvious attempt for the supporting film due to its unique properties. Although the graphene is the same kind of element used for supporting the film as the amorphous carbon, they demonstrate different properties due to their different structures. Graphene is a crystalline allotrope of carbon and its carbon atoms are densely packed in a regular hexagonal pattern [17] while the amorphous carbon is one of the most common types of carbon with no fixed shape. Single-layer of graphene is an ideal supporting film since it is a one-atom-thick conductive material, making the supporting film itself invisible in the resolution range of cryo-EM [12]. Compared to the original amorphous carbon as supporting film, the graphene has many more advantages. Graphene demonstrates the high stability and high electrical conductivity 6 orders of magnitude higher than that of amorphous carbon [15]. Since the graphene is thin and light in nature, functional graphene is transparent to 300 kV electrons [14] Besides, because of its single atom structure and tiny thickness, graphene reduces the surface charges and background noise, which provides an assumption for higher resolution of the sample. Moreover, despite the graphene’s experimental advantages, the convenience of its large-scale manufacture by chemical vapor deposition (CVD) and some promising primary work on its use as a substrate for biological molecules also provide a powerful background for wider application [18].

3.4. The use of graphene as supporting material
In fact, a lot of scientists did their own experiment with various parameters in producing a valuable and high-quality sample. The use of graphene as supporting material of the grid is widely supported by modern scientists.

3.4.1. Christopher J. Russo experiment. Scientist Christopher J. Russo published that graphene could be a greater supporting film instead of amorphous carbon due to its multiple functions. The graphene grids were made by chemical vapor deposition (CVD) with a monolayer attached. The size of the grid was 100 um and Thermus Thermophilus 30S Ribosomal Subunit/Human 20S Proteasome/Apol ferritin were imaged to get a resolution of 2.1Å [13]. One potential drawback was that the specimen would have background noise caused by the exposure to the surface of the water and air. Also, this interaction leads to the contamination of the sample. But it reduced the movement of the sample and improved the resolution of the result.
3.4.2. Werner Kuhlbrandt experiment. Scientist Werner Kuhlbrandt deposited the graphene layer on the holey side of the grid, which will be the location for the protein sample on the same side covered with copper after glow discharge. Fatty acid synthase (FAS) from Saccharomyces cerevisiae was applied to the grid with hydrophilized monolayers of graphene and get a resolution of 9.5Å. It was stable under a 300 kV electron beam and almost completely electron-transparent to 2.13 Å resolution and beyond. But unlike Christopher J. Russo’s experiment, hydrophilized graphene-coated grids prevented denaturation at the surface between the air and water [19].

3.4.3. Hong-Wei Wang experiment. Hong-Wei Wang, another scientist who has done a classical experiment. Chemical vapor deposition (CVD) was also applied in this experiment, which used a commercially available streptavidin solution to get a final resolution of 3.2 Å with single-crystalline monolayer graphene [20]. The disadvantage was the denaturation of the macromolecules, which will not take full advantage of the single-particle cryo-EM analysis. However, it is reduced the ice thickness without introducing a strong background noise and attracted protein particles closely. In general, all three scientists take significant advantages from graphene.

4. The use of graphene on the cryogenic electron microscopy

4.1. The production of the graphene
The traditional production method of graphene is the CVD (chemical vapor deposition). CVD is one of the most common ways to produce a thin film through the reaction of gaseous substance and solid substance. The samples provided using Ni foils possess greater graphitic property due to the high carbon solubility of nickel itself at an early stage. In the production of the monolayer graphene, however, the crystalline TEM grids are out of its basic limitation. The application of copper foil with low carbon graphitic solubility and large grain size make the CVD process producing single-layer graphene continuously possible [15]. Indeed, different types of copper will produce different qualities of graphene, such as the various surface condition of holes.

4.2. The transferring of the graphene
Additionally, the graphene needs to be transferred from the copper surface to the gold grids (TEM grids) for the later supporting of samples. As shown in Figure 2, there are two kinds of transferring method. The standard transfer uses the PMMA solution twice before and after the FeCl3 etch and DI rinse. This will produce poorly adhered, contaminated graphene grids. Another method is the direct transfer of the graphene, which use the IPA solution before the FeCl3 etch and DI rinse. The grids will bind to graphene as the IPA evaporates. This will provide greater quality and form a better-adhered grid than the standard method [21].
4.3. The application of graphene in cryo-EM
The use of graphene has been frequently these years due to its various properties. In terms of cryogenic electron microscopy, benefits are apparently shown. As the properties of graphene, it reduces the surface charges and maintains the highest in-plane mechanical strength ever measured [13]. Also, it reduces the movement of the sample and provides a higher resolution of the image [19].

Many experiments also use graphene oxide, which is the compound of the graphene. The primary structure for the graphene oxide shows transmission properties towards that pristine graphene with sparse, nanometer-scale amorphous defects, leading to the gradual decline of the weak phase at the higher resolution level. [15]

4.4. Potential improvement
Under similar conditions, it has space for improvement as well. Since graphene is hydrophobic, we need to make it hydrophilic every time for the preparation of the sample, which is costly and time-consuming. Also, it is susceptible to surface contamination during manufacturing. As for the graphene oxide, it contributes approximately as much background noise as the amorphous carbon. What is more, the graphene oxide is usually an insulator, which creates difficulty to neutralize the surface charges. Additionally, the graphene oxide has decreased mechanical strength, which is less stable than graphene [18]. Overall, the graphene has provided us with a greater view of exploration.

Figure 2. A demonstration and comparison of the standard and direct transfer of graphene from copper surface to TEM grids [21].
5. Conclusion and future direction

Cryogenic electron microscopy is an exceptional technique to observe the biological molecules and a great leap in the development of structural biology. The rapid freezing process allows the initial living sample to fix on position and vitrified samples to be prepared using plunge freezing for the observation in the microscope [22]. In this way, the sample will be immobilized under the microscope, which will make the observation much more accurate. The contribution of cryogenic electron microscopy is significant. It solves the structure determination of complex giant molecules, offering the 3D model with high resolution. It is also a revolutionary advancement to apply the graphene as the supporting film, which provides the sample with high quality. Using a small number of experimental materials while achieving high applicability in the determination of biological structure, the method in cryogenic electron microscopy represents the high efficiency, which can obtain the result using minimum samples and data. From my perspective, the invention of this technology will soon be applied to more observations of various types of samples and aspects of analysis in broader scientific fields in the near future. After all, cryogenic electron microscopy is a technology produced from the knowledge in physics and chemistry to support biology development. I believe that more kinds of interdisciplinary technologies will be invented in the future for a wider purpose and it would be a long way to explore in the future.

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