Structural engineering of graphene for high-resolution cryo-electron microscopy

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Abstract
The revolutionary improvement of hardware and algorithm in cryogenic electron microscopy (cryo-EM) has made it a routine method to obtain structures of macromolecules at near-atomic resolution. Nevertheless, this technique still faces many challenges. The structure-solving efficiency of cryo-EM can be significantly reduced by the biomolecules’ denaturation on the air–water interfaces, the preferred orientation, strong background noise from supporting films and particle motion, and so forth. To overcome these problems, nanomaterials with ultrahigh electronic conductivity and ultrathin thickness are explored as promising cryo-EM specimen supporting films. Herein, we summarize the structural engineering of graphene, for example, surface and interface modification, as supporting films for grids and the application on high-resolution cryo-EM and discuss potential future perspectives.

Keywords
graphene, high-resolution cryogenic electron microscopy, life science, liquid cell, tomography

Jie Xu, Xiaoya Cui, and Nan Liu contributed equally to this study.

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1 | INTRODUCTION

Cryogenic electron microscopy (cryo-EM) was viewed as a “Blob-ology technique” in the structural biology field over a long period of time due to its nanometer-resolution reconstruction performance. The application of cryo-EM to determine the structure of biological macromolecules gained renaissance when Liao et al.1 solved the structure of TRPV1 at near-atomic resolution in 2013. The breakthroughs of cryo-EM benefit from both the improvement of algorithms, for example, the maximum likelihood algorithm, and the development of a direct electron detection device. Classification and refinement based on the maximum likelihood algorithm increase the efficiency and accuracy of structural analysis, whereas the direct electron detection device improves the signal detection efficiency enormously by avoiding the photoelectric conversion.2 All of these make it possible for cryo-EM to perform near-atomic resolution reconstructions of macromolecules without the need for crystallization. Nowadays, the single-particle cryo-EM method is routinely used to analyze structures of many macromolecules.

However, cryo-EM sample preparation is poorly controlled and hardly reproducible. The samples expected to be orientation rich in vitreous ice (Figure 1A) are frequently found to be absorbed at the hole edge or the holey carbon support due to the nonuniform ice thickness (Figure 1B,C), leading to wastage of a large amount of biological samples. In addition, proteins tend to be adsorbed to the air–water interface in the thin layer of solution during preparation on the EM grid (Figure 1D),3,4 which results in two adverse effects: protein denaturation and preferred orientation.5 Moreover, high-energy electron irradiation induces motion of cryo-specimens and therefore results in blurred micrographs and decreased resolution. Several algorithms have been developed to process the micrographs with motion,6 and thin carbon films have been used to anchor macromolecules to avoid the air–water interface. Hence, a thin continuous amorphous carbon film is coated onto the grid to achieve a better particle distribution in ice (Figure 1E). Unfortunately, amorphous carbon leads to new problems, that is, strong background noise and poor conductivity. Two-dimensional (2D) crystals of streptavidin,7 lipid monolayers,8 and double-stranded DNA6 were also used as scaffolds to improve the quality of samples. These materials may require special circumstances or introduce biomass into the imaging.9

In an attempt to solve all these challenges, the two-dimensional nanomaterial graphene with ultrahigh electric/thermal conductivity and mechanical strength drew the attention of cryo-EM scientists. The atomic thin graphene film shows low background noise and generates less motion when imaged under EM, compared with the amorphous carbon film. Hydrogen plasma-treated graphene, functionalyzed graphene, and other graphene materials (e.g., graphene oxide [GO], reduced graphene oxide [rGO], etc.) have been explored to satisfy various characterization demands. In this review, we summarize the fabrication and application of graphene material grids.

2 | GRAPHENE GRIDS

2.1 | Synthesis of graphene nanosheets

The synthesis methods for graphene can be divided into three major categories, that is, mechanical exfoliation, oxidation–reduction, and chemical vapor deposition (CVD).10 The most well-known mechanical exfoliation method to fabricate graphene is to apply scotch tape to peel flakes of graphite repeatedly as reported by Geim and Novoselov in 2004; for this, they won the Nobel prize in Physics in 2010.11 Another popular mechanical exfoliation method to obtain graphene is the ultrasonication of graphite. The CVD method is based on the decomposition of methane at high temperatures to obtain large-scale and solid single-layer graphene on certain substrates with optimal properties.12 According to Hummers’ method,13 sulfuric acid, nitrate, and potassium permanganate are used as oxidants to synthesize GO directly from graphite. There are also other optimized methods to synthesize GO.14,15

Respective pros and cons exist in these methods. It is difficult to prepare large-scale single-layer graphene via the mechanical exfoliation method. The oxidation–reduction method has the limitation of unfavorable reduction efficiency. Graphene synthesized by the CVD method is high
2.2 | Fabrication of a graphene grid

Drop-casting and surface assembly are two simple ways to assemble the graphene EM grids. For drop-casting, a drop of graphene solution with optimized concentration is pipetted onto the grid, and then most of the liquid is blotted off after 1 min. The grid is ready to be used after natural drying. Pantelic et al.\textsuperscript{16} adopted this method to obtain grids covered by graphene. Nevertheless, the number of graphene layers is poorly controlled. Later, Palovcak et al.\textsuperscript{17} developed a novel surface assembly strategy to prepare GO film-coated grids with a high coverage rate. Specifically, graphene solution is pipetted on the water surface above the submerged EM grids. The water level is lowered slowly so that the GO film falls with the water surface and ultimately sticks on the grid. The grids are naturally dried afterward for further use. Obviously, the size of monolayer GO needs to be modified to obtain a fully covered grid.

To fabricate a graphene grid with monolayer graphene, the following three methods are mainly used, that is, indirect transfer, direct transfer, and direct etching (Figure 2). The widely used material in the indirect transfer method is methyl methacrylate (MMA), which aids the transfer of graphene onto the grid, and then it can be dissolved in acetone and removed (Figure 2A).\textsuperscript{18} However, it has some drawbacks such as the incomplete removal of polymers on graphene, causing contaminations in the micrographs of the target sample. Compared with the indirect transfer method, the direct transfer method is a gentle and clean method.\textsuperscript{14} Generally, CVD-prepared graphene adhering onto a copper film can be directly transferred to non-copper EM grids and the copper film is etched away later (Figure 2B).\textsuperscript{19} Another method is the direct etching method (Figure 2C),\textsuperscript{20} which is polymer-free, skipping the transfer procedure completely. The graphene is grown directly on a copper film that can be selectively etched later into the shape of the grid required for transmission electron microscopy (TEM). The graphene grid obtained by direct etching is ultraclean, avoiding polymers and transfer procedure, providing a practical method for batch fabrication of a graphene grid as well.

2.3 | Properties of graphene grids

Graphene is composed of carbon atoms in the sp\textsuperscript{2} hybrid orbital with a hexagonal lattice (Figure 3A) in which one atom forms each vertex.\textsuperscript{22–24} Thus, the hexagonal patterns for one or two layers of graphene or GO can be observed by electron diffraction or Fourier transform of a magnified...
The side length of the hexagonal lattice is 0.142 nm, while the distance between two graphene layers is 0.335 nm in graphite. The single layer of carbon atoms and the ordered pattern contribute to the excellent properties of graphene, especially the superstrong electric conductivity, mechanical performance, and uniformity for cryo-EM.

Graphene has low resistivity and high mechanical strength (e.g., Young’s modulus of 1 TPa). High electron conductivity can weaken the damage from the accumulation of electrons. The relative intensities of Bragg reflections for the graphene membrane and the functionalized graphene membrane show negligible irradiation damage attributed to the excellent conductivity of graphene (Figure 3C). Graphene, as an ultrathin 2D material, absorbs only 2.3% white light and diffracts electrons weakly, and therefore generates negligible background noise. The uniformity makes it easy to remove from the micrograph during image processing if necessary. Graphene is also robust enough to be sonicated, modified, or decorated by chemicals depending on the experimental purposes. In addition, the hydrophilicity of graphene or GO membranes can be adjusted via hydrogen plasma treatment as well (Figure 3D).

3 | APPLICATION OF GRAPHENE IN CRYO-EM

3.1 | Hydrophilicity modification of graphene

Pristine graphene is highly hydrophobic. Several strategies have been used to improve the wettability for
the preparation of cryo-EM specimens, for example, low-energy hydrogen plasma treatment, UV/ozone, glow discharge, and so forth. According to a previous report, graphene can be converted into graphene that is totally hydrogenated. Subsequently, Russo and Passmore discovered that via hydrogen plasma treatment, partially hydrogenated (≤5%) graphene shows good hydrophilicity for anchoring biological samples. On the contrary, Han et al. applied UV/ozone to partially oxidize graphene to generate hydrophilicity, giving rise to a near-atomic resolution cryo-EM reconstruction of a small protein (50 kDa). Moreover, the normal glow discharge can increase the hydrophilicity of the graphene grid. PEG functionalized on graphene also provides sufficient hydrophilicity. Note that the GO film that was explored by Pantelic et al. is naturally hydrophilic for cryo-EM specimen preparation.

The glow discharge method is one of the most facile ways to improve the hydrophilicity of cryo-EM grid supporting films, for example, GO. However, the atomic thin graphene films may be damaged quickly during glow discharge; therefore, UV/ozone is applied to tune the wettability. Moreover, the partially hydrogenated graphene and other multifunctional graphene films can obviously reduce the specimen movement. Unfortunately, these instruments may be unavailable for most conventional structural biology laboratories.

**FIGURE 4** Graphene material grids with reduced background noise and beam-induced motion. (A) Comparison of the background noise of thin carbon and graphene oxide (GO). The inset shows the selected area electron diffraction (SAED) pattern of GO at 245 mm. Reproduced with permission. Copyright 2010, Elsevier Inc. (B) Typical micrograph of streptavidin showing clear particles via a Volta phase plate (VPP)-Cs-corrector-coupled cryogenic electron microscopy. Scale bar = 20 nm. Reproduced with permission. Copyright 2019, Nature Publishing Group. (C) Root mean squared displacement showing the reduced motion of graphene (right, red lines), compared with amorphous carbon (left, black lines) and unsupported ice (middle, blue lines). Reproduced with permission. Copyright 2014, Nature Publishing Group.
3.2 Background noise reduction and motion correction

The background noise of graphene and GO films is negligible compared with other supporting films, for example, amorphous carbon (Figure 4A). For instance, Fan et al. directly used the monolayer graphene grid for sample preparation. In their work, the graphene film shows negligible background noise, benefiting the data processing, thus increasing the final image resolution of samples. With graphene and a Volta phase plate (VPP), a distinct high-contrast micrograph is obtained (Figure 4B) and the structure of 52 kDa streptavidin can be reconstructed at 3.2Å resolution. Even functionalized graphene, for example, Ni–NTA–functioned graphene, has a considerably smaller thickness than amorphous carbon films (~5 nm), therefore decreasing the background noise and increasing the final reconstruction resolution. Another advantage of graphene is its high mechanical strength and high electric conductivity, both of which are beneficial to reduce the electron beam-induced motion of cryo-specimens. Russo and Passmore demonstrated that partially hydrogenated graphene can reduce the motion induced by an electron beam during cryo-EM imaging (Figure 4C).

3.3 Homogenizing distribution and orientation

The uneven distribution and preferred orientation of particles on conventional grids increase the difficulty of data collection and structural determination by cryo-EM. The application of graphene grids exhibits good potential to overcome these possible obstacles. Russo and Passmore showed that hydrogen plasma-treated graphene has the capacity to tune particle distribution by adjusting the hydrogen plasma dose (Figure 5A). Han et al. showed that an ozonated graphene grid can increase the density of various kinds of proteins by at least five times. Moreover, it can alter the distribution of particles. Generally, TRPA1 shows a strong preferred orientation. However, by combining the data obtained from holey carbon grids and amino-GO grids, this issue can be solved, as TRPA1...
showed different orientations on different grids (Figure 5B).

D’Imprima et al. reported that nearly 90% of fatty acid synthase that adhered to the air–water interface was partially denatured on the normal holey carbon grids. The denaturation can be largely prevented by using graphene grids, due to the absorbing tendency of samples onto a graphene film and away from the air–water interface (Figure 5C). This was further confirmed by Liu et al. and Wang et al. through cryogenic electron tomography.

3.4 | Selective capture of target protein

Benjamin et al. modified GO with NTA to selectively capture and enrich target proteins with the His tag. The principle is that NTA can be covalently linked with GO through a dehydration condensation reaction and then activated by nickel ions. Afterward, by using 4-aminobenzoic acid (PABA) and bovine serum albumin (BSA) to block nonspecific sites of graphene, cell lysates containing target proteins with the His tag can be applied to the functionalized GO grid (Figure 6A). The His6-T7 bacteriophage and His6-GroEL were successfully characterized by this method, which demonstrated a reduced background noise and a potential to simplify the protein purification procedure.

Meanwhile, Liu et al. also applied a functionalized graphene-based grid to analyze His-tagged proteasomes (Figure 6B). In their work, the water contact angle of the functionalized graphene grid was 29°, and most His-tagged proteasome particles adhered onto graphene rather than onto the air–water interface. A functionalized graphene grid can increase the efficiency of structural analysis through cryo-EM. At the same time, preferred orientation can be avoided using several different tags.
Another method to reduce the orientation bias is to apply polyethylene glycol (PEG) as a spacer to keep both the GO surface and the air–water interface away from the sample. For instance, Wang et al. introduced PEG on GO to decrease orientation bias while retaining the integrity of the sample simultaneously (Figure 6C). In addition, PEG can be easily combined with tags to selectively capture the target proteins, paving the way for “Purification on Grid.” To introduce a variety of functional groups, an instrument based on helium plasma was developed (Figure 6D) by Naydenova et al. The composition of graphene is monitored through optical spectroscopy.

3.5 In situ observation of cells or proteins

TEM is a powerful technique beyond the diffraction limit to reconstruct the structure or analyze the composition of samples. Nevertheless, it requires high-vacuum conditions for microscopy to minimize the electron scattering effect of the imaging system, indicating that the sample should be solid, as a normal liquid sample evaporates rapidly in high vacuum. Consequently, biological samples in the liquid phase need to be frozen or dried during TEM observation. Sectioning may also be applied for thick samples. Unfortunately, the aforementioned processes unavoidably introduce artifacts onto the target sample and make it difficult to observe dynamic samples in the liquid phase. Optical microscopes, in particular, the phase-contrast microscope and the fluorescence microscope, are widely used to observe living cells. However, the limited imaging resolution of optical microscopes hinders its application, especially for biological macromolecules. Recently, stimulated emission depletion microscopy and stochastic optical reconstruction microscopy provided breakthroughs for optical microscopy with a resolution as high as 2 nm, nonetheless, this is still far from atomic resolution.

A major approach to observe wet samples relies on an electron transparent enclosure (named as liquid cell) in TEM, which seals the sample from the high vacuum to maintain the moisture environment in the sample. As is known, the first modern liquid cell made of silicon nitride was developed to observe electrochemical deposition of copper in 2003. Since then, liquid cells have been widely used in biomimeticization, corrosion, and nanoparticle formation. The silicon nitride used for an enclosed liquid cell has an optimized thickness of ~10 nm. Nevertheless, the thickness and conductivity are still far from adequate for soft materials with low contrast in TEM. Graphene was tested as an ideal candidate to fabricate a graphene liquid cell (GLC) due to its ultrathin structure and superior conductivity. Furthermore, protein-functionalized graphene was adopted to wrap the bacteria. The shape and living status of wrapped bacteria (WB) or unwrapped bacteria (UWB) can be observed by TEM and fluorescence microscopy, respectively (Figures 7A and 7B). Different from a silicon nitride liquid cell that is blocked by glue or clamping, the GLC is sealed through π–π stacking between the two graphene layers (Figure 7C). Although GLC has been used for cell observation, the capture of the dynamic state is still challenging due to influences such as electron radiation damage and low contrast. If the monitoring of the movement of cells and the dynamic process of proteins can be performed in GLC, more biological processes can be visualized at high resolution in TEM. This will inevitably revolutionize structural biology and cell biology.

4 Discussion and conclusion

Despite the superior properties of graphene, the adoption of graphene as a cryo-EM specimen support still faces some challenges in practical applications. First, it is difficult to avoid contaminants during graphene synthesis and transfer, generating extra background noise. Second, it is difficult to guarantee a high coverage of monolayer graphene. It is not easy to maintain and ensure even spreading of the monolayer in a 2D material, and uneven graphene will lead to extra noise as well. On the contrary, currently, the fabrication of clean graphene is expensive, and the extra treatment and functionalization also increase the expense. Therefore, it remains a challenge to optimize the modification procedure and decrease the cost, which are beneficial for not only fundamental investigations but also general industrial applications.

Recently, the discovery of magic-angle graphene with unconventional superconductivity at a twist angle of 1.1° suggests that two sheets of graphene may potentially be useful in cryo-EM to significantly decrease the side effects of electrons. Besides, graphene may also be useful to control the thickness of ice, which is still difficult even with an instrument, by sandwiching ice between two layers of graphene. Other ultrathin 2D nanomaterials such as Mxenes, transition-metal dichalcogenides, and boron nitride (BN), with good conductivity and hydrophilicity, mechanical performance, and even superconductivity, are also promising supporting film candidates for cryo-EM grids. It remains a great challenge to obtain the aforementioned ultrathin or single-layer 2D nanostructures with large sizes that can fulfill the requirements as supporting films. In addition, the strategy for their transfer onto cryo-EM grids
still needs to be developed. We believe that the structural engineering of 2D nanomaterial-based supporting films can provide more opportunities to boost the development of the EM methodology.

In conclusion, graphene, as a 2D material composed of monolayer carbon atoms, shows ultrahigh electric conductivity, superior mechanical strength, and ultrathin thickness, making it an ideal alternative for a grid to replace amorphous carbon films. Several methods have been formulated to regulate the hydrophilicity of graphene materials. Mechanical exfoliation, oxidation–reduction, and CVD are three major methods to produce graphene. CVD can produce good-quality graphene, but the cost is high. Nevertheless, the hydrophobicity of graphene hinders its applications due to the difficulty for proteins to adhere. On the contrary, the application of GO overcomes the disadvantage of hydrophobicity, but the conductivity becomes worse. In addition, by introducing hydrogen plasma, UV/ozone, and glow discharge treatments, graphene becomes hydrophilic and ready to use as a supporting film during cryo-EM. Note that by combining affinity tag with graphene, the selective capture of proteins can be achieved, which shows the potential to simplify the procedure of protein purification. The grid covered with graphene materials has been confirmed to have the capabilities of reducing background noise and motion, homogenizing distribution and orientation, and capturing target protein selectively. Besides the application in cryo-EM as a supporting film, graphene materials can also be applied to fabricate GLC for in situ observation and dynamic processes in many fields like biomineralization, corrosion, and nanoparticle formation. The structural engineering of graphene and even other 2D materials will broaden the application of high-resolution cryo-EM and significantly improve methodology development.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.
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