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Technical Note

A simple and robust procedure for preparing graphene-oxide cryo-EM grids

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ABSTRACT

Graphene oxide (GO) sheets have been used successfully as a supporting substrate film in several recent cryogenic electron-microscopy (cryo-EM) studies of challenging biological macromolecules. However, difficulties in preparing GO-covered holey carbon EM grids have limited their widespread use. Here, we report a simple and robust method for covering holey carbon EM grids with GO sheets and demonstrate that these grids can be used for high-resolution single particle cryo-EM. GO substrates adhere macromolecules, allowing cryo-EM grid preparation with lower specimen concentrations and provide partial protection from the air-water interface. Additionally, the signal of the GO lattice beneath the frozen-hydrated specimen can be discerned in many motion-corrected micrographs, providing a high-resolution fiducial for evaluating beam-induced motion correction.

1. Introduction

Recent technological breakthroughs have made single particle cryogenic electron microscopy (cryo-EM) a versatile and routine method for structure determination of macromolecules at high-resolution (Bai et al., 2015; Cheng, 2015). With automated data acquisition (Mastronarde, 2005; Suloway et al., 2005) enabled by stable high-end electron microscopes equipped with direct electron detection cameras and streamlined image processing software (Kimanius et al., 2016; Punjani et al., 2017), structure determination by single particle cryo-EM has never been easier. What remains more or less unchanged is the plunge freezing technique (Dubochet et al., 1988), which works well for many samples, particularly structurally stable ones. For fragile complexes, however, preparing good frozen-hydrated cryo-EM grids with intact and monodispersed particles is often a challenging task and a main bottleneck to cryo-EM structure analysis. It has been suggested that exposing protein samples to an air-water interface during plunge freezing can damage fragile protein complexes or induce preferred orientations in thin vitreous ice (Dubochet et al., 1988; Glaeser and Han, 2017; Glaeser, 2018). Additionally, some samples may prefer to stick to the carbon matrix of holey grids instead of being suspended in the vitreous ice spanning the holes (Zhao et al., 2015; Snijder et al., 2017). A common approach to mitigate these problems is to apply a continuous thin layer of substrate to the holey carbon grid, making the distribution of particles in the holes more even and holding protein samples away from the air-water interface (Williams and Glaeser, 1972; Russo and Passmore, 2014; Han et al., 2016; Glaeser, 2018).

Amorphous carbon is the most commonly used substrate (Grassucci et al., 2007), but it adds significant background noise to particle images, limiting its use to relatively large particles such as ribosomes (Gao et al., 2007). Other substrates include monolayer sheets of graphene (Pantelic et al., 2012; Russo and Passmore, 2014) and two-dimensional crystals of streptavidin (Wang et al., 2008; Han et al., 2017). More recently, thin sheets of graphene oxide (GO) were introduced as a substrate (Pantelic et al., 2010). In two subsequent studies, GO coated grids were used on challenging macromolecular targets to ameliorate a tendency to aggregate at high concentrations and a bad preferred orientation problem, respectively (Bokori-Brown et al., 2016; Boland et al., 2017).

Compared to other options, GO sheets are nearly electron transparent, hydrophilic enough to adhere macromolecules from dilute solutions, inexpensive to purchase or synthesize, and amenable to functionalization (Pantelic et al., 2010; Chen et al., 2012). However, obtaining EM grids evenly covered with one or several layers of GO sheets has not been easy. In our experience, our attempts to use the previously reported drop-casting method (Pantelic et al., 2010) mostly produced grids with irregular coverage of GO sheets over the holes. Typically, fewer than twenty percent of holes were covered by one or a

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few layers of GO sheets, while the majority of the grids were either covered with multi-sheet aggregates or lacked GO entirely. Because we regularly need to screen tens of cryo-EM grids before finding conditions suitable for automated data acquisition and high-resolution structure determination, reproducibility and ease of manufacture are key considerations for any substrate.

To improve the usable area on GO covered grids, we established a simple and robust surface assembly procedure for evenly covering holey carbon EM grids with one to very few layers of GO sheets. These GO-covered grids are suitable for high-resolution single particle cryo-EM studies of biological macromolecules. We prepared frozen-hydrated archaeal 20S proteasomes using such GO-covered Quantifoil EM grids and collect a dataset resulting in a 2.5 Å resolution 3D reconstruction, comparable to our best previous results in freestanding vitreous ice (Li et al., 2013; Zheng et al., 2017). We also used cryogenic electron tomography experiments (cryo-ET) to confirm that particles are concentrated close to the GO sheet, with about 90% of particles closer to the GO substrate than the air-water interface. In addition, lattice images of the graphene oxide film recorded together with frozen-hydrated 20S proteasome particles provide information for evaluating beam-induced motion correction.

2. Fabricating GO-covered holey carbon cryo-EM grids

Ideally, a GO covered grid would be completely covered in a single monolayer GO sheet without any wrinkled regions or GO aggregates. Commercial GO are available in the form of small flakes. Direct application of an aqueous suspension of GO flakes to a glow-discharged EM grid (drop-casting) (Pantelic et al., 2010) tends to leave many regions of the grid uncovered and deposits high-contrast multi-flake aggregates over many others. Initially we speculated that our commercial GO suspension might have deteriorated with age, so we used bath sonication to break up weakly aggregated sheets followed by centrifugation to isolate mostly large single GO flakes. This treatment reduces the presence of aggregates but does not greatly improve coverage uniformity.

GO surface is sufficiently hydrophobic to be enriched at air-water interfaces (Kim et al., 2010). We used this property to assemble a thin, mostly continuous film of GO flakes at the surface of a dish of pure water. By draining the water, the assembled GO film is then slowly lowered onto submerged holey carbon EM grids with their holey carbon film sides facing up (Fig. 1A). In agreement with previous reports (Kim et al., 2010; Cote et al., 2011), we found that methanol-dispersed GO flakes spread and float easily on a pure water subphase. Once completely dried, GO flakes stably adhere to holey carbon films and remain adhered during blotting. The boundaries of individual GO flake on EM grids can be directly observed in the transmission electron microscope (TEM) (Fig. 1B).

According to the vendors, such as Sigma-Aldrich, their GO flakes are predominantly monolayer sheet, based on atomic force microscope height measurements. Selected area electron diffraction patterns taken from a single flake shows a single hexagonal lattice of Bragg peaks (Fig. 1C), suggesting that such flake either contains a single layer of GO sheet, or multiple sheets stacking together coherently as a thin three-dimensional (3D) crystal. Because GO is exfoliated through chemical oxidation of graphite, it is possible that some flakes contain more than one layer of GO sheet coherently stack together. We did not attempt to experimentally determine how many layers of GO sheets are coherently stacked together in each flake (Meyer et al., 2007), since the flakes are sufficiently thin that we did not observe any noticeable influence of image contrast. As a result, we assume that most of flakes contain a single layer of GO sheet. Diffraction patterns with sharp high-order spots indicates a long-range periodicity of the GO lattice over the hole (Fig. 1C). While GO grids can acquire surface contaminants which render them less hydrophilic, they can be cleaned without damage by brief glow-discharge in air (five to ten seconds) immediately before use. A detailed protocol for fabricating GO grids by surface assembly is provided in the Supplementary methods.

Suspensions of GO sheets can vary significantly in the lateral sheet size and degree of oxidation. The cryo-EM experiments in this study were performed on grids coated with GO sheets from a commercial source (Sigma-Aldrich). We have also recently produced grids with home-made suspensions of GO with larger sheet size. These GO suspensions were made according to an established protocol (Marcano et al., 2010). Larger GO sheets coat grids more evenly and produce fewer high-contrast 'edges' in images, increasing the efficiency of data collection. Even so, surface assembly is robust to these variations and works well with both commercially-available GO suspensions and home-made GO sheets. The only important parameter to optimize with our protocol is the amount of GO applied to the surface. We typically make one or two test grids and ensure satisfactory coverage by screening in a transmission electron microscope before producing a large batch of grids.

3. Single particle cryo-EM of archaeal 20S proteasome on a GO grid

Using the archaeal 20S proteasome as a test specimen, we evaluated the practicality of using GO grids for high-resolution single particle cryo-EM. As previously reported, the commonly used plunge freezing procedure works well for GO grids, though we have found that longer blotting times (10–30 s) are often preferred. For the 20S proteasome, achieving an optimal particle distribution required a specimen concentration approximately ten times lower with GO (0.05 mg/mL) than without (0.5 mg/mL) (Fig. 2A, Supplementary Fig. 1A). By cryo-ET, we confirmed that 20S proteasome particles directly adhere to the GO substrate, as almost 90% of particles were closer to the GO substrate than the air-water interface (Fig. 2B, Supplementary Fig. 1C and D). Based on the locations of the few proteasomes and gold nanoparticle

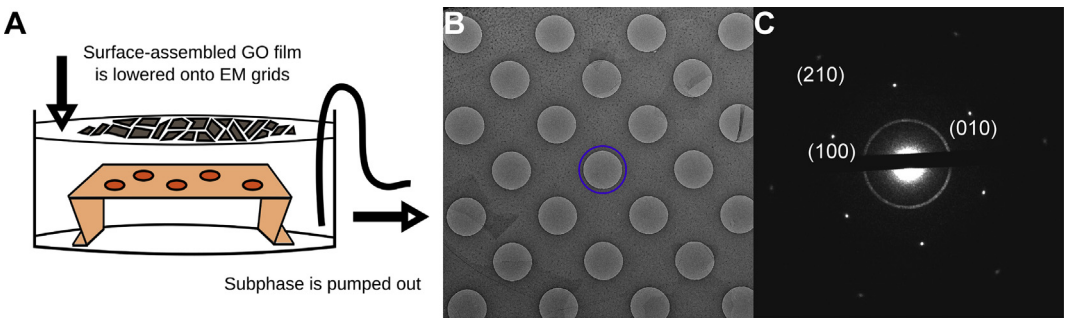

Fig. 1. EM grids can be evenly coated with GO sheets using surface assembly. (A) Schematic of the apparatus used for surface assembly of thin GO films and subsequent deposition onto holey carbon EM grids. (B) Low magnification image of a holey carbon (Quantifoil) grid covered with a thin film of GO sheets. A hole covered by a single GO sheet is circled in blue. (C) Electron diffraction pattern of the hole in B. Without a scrolled edge, it is hard to discern by image contrast alone whether a hole has a GO sheet spanning it. Instead, the diffraction pattern shows unambiguously the hexagonal lattice of each GO sheet.