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Superb resolution and contrast of transmission electron microscopy images of unstained biological samples on graphene-coated grids

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ABSTRACT

Background: In standard transmission electron microscopy (TEM), biological samples are supported on carbon films of nanometer thickness. Due to the similar electron scattering of protein samples and graphite supports, high quality images with structural details are obtained primarily by staining with heavy metals.
Methods: Single-layered graphene is used to support the protein self-assemblies of different molecular weights for qualitative and quantitative characterizations.
Results: We show unprecedented high resolution and contrast images of unstained samples on graphene on a low-end TEM. We show for the first time that the resolution and contrast of TEM images of unstained biological samples with high packing density in their native states supported on graphene can be comparable or superior to uranyl acetate-stained TEM images.
Conclusion: Our results demonstrate a novel technique for TEM structural characterization to circumvent the potential artifacts caused by staining agents without sacrificing image resolution or contrast, and eliminate the need for toxic metals. Moreover, this technique better preserves sample integrity for quantitative characterization by dark-field imaging with reduced beam damage.
General significance: This technique can be an effective alternative for bright-field qualitative characterization of biological samples with high packing density and those not amenable to the standard negative staining technique, in addition to providing high quality dark-field unstained images at reduced radiation damage to determine quantitative structural information of biological samples.

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1. Background

TEM has been a popular tool for structure characterization of biological samples at sub-micrometer scale since the first prototype built by Ruska and Knoll in 1931. The sizes of many biological samples fall in this range, such as bacteria [1], viral particles [2–4], and various macromolecular assemblies [5,6]. In the preparation steps for TEM observations, normally samples are deposited on a thin layer of substrate, usually made of amorphous carbon film [7,8]. The thickness of the substrate is usually about a few nanometers or more. The image resolution and contrast are limited by the differential scattering of electrons in the electron beams of TEM from different areas of the samples and supporting substrate [9]. In order to enhance image contrast to reveal detailed structure features, negative staining the samples with agents containing heavy metallic elements is commonly practiced [10,11], exploiting their significantly stronger electronic scattering cross sections than C, N and O, the most prevalent elements in biological materials. However, artifacts associated with the application of these staining agents are also well-known, due to the distinct pH values of the staining buffers, or the artificial aggregation caused by the heavy metal binding with phosphate and carboxyl groups [12,13]. In addition, some of the commonly used staining agents pose health and environment hazards, for example, uranyl acetate, the most widely-adopted staining agent that provides the highest image contrast, is both toxic and weakly radioactive.

Graphene is a single layer of graphite constructed by carbon atoms via sp^2 bonding [14]. It possesses extraordinary electronic, thermal and mechanical properties [14]. The pure 2D lattice structure and weak scattering with electrons make graphene nearly transparent under TEM [15,16]. The resolution of a single hydrogen atom in a graphene membrane under aberration-corrected and monochromatic TEM has been previously demonstrated [15,16]. In addition, the ultra high electronic and thermal conductivities of graphene are more than 10,000 fold better compared to graphite carbon film of nanometer thickness [17], which in theory will prevent or greatly relieve the charge accumulation and electron beam damage to biological samples. These facts suggest that graphene can be an optimal sample supporting substrate for TEM in comparison to the standard materials, as predicted by Sousa, A.A. and Leapman, R.D. according to theoretical estimation [18]. Only a

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few brief examples have been reported using graphene for biological sample characterization by TEM. Nair et al. and Pantilec et al. showed that unstained Tobacco Mosaic Virus (TMV) could be observed on a graphene-coated TEM grid [19,20]. Mohanty et al. demonstrated that graphene can be used to preserve the structure of complete cells for TEM analysis [21]. In the work by Pantelic et al., they adopted single-layered graphene oxide films to exploit its better binding affinity to protein samples and showed unstained images of vitrified 26S proteasome [22]. The resolution and contrast of these images are better than unstained images on carbon film. However, they are still far from satisfactory in comparison to the negatively-stained images. The practical value of graphene as a TEM support substrate for biological samples seemed very limited.

In this article, we examine unstained images of a wide range of protein assemblies on graphene-coated TEM grids, obtained under a normal TEM without aberration correction. By directly comparing to the unstained and negatively-stained images on carbon film of nanometer thickness, we show for the first time that the quality of unstained bright-field images on graphene support can be comparable or even superior to the negatively-stained images for samples with high packing density. The negligible background from graphene provides a dramatic enhancement of image contrast and enables the resolution of the structural features at remarkable detail. Our results suggest that high quality images of unstained biological samples can be obtained on graphene-coated TEM grids as an effective alternative for samples with high packing density to conventional staining techniques. In addition, we also demonstrate that high quality unstained dark-field images obtained in the dark-field mode of TEM at reduced radiation damage for quantitative characterizations of biological samples [23], which proves the potential application for graphene coated grids for the measurements of mass density by similar techniques such as Scanning Transmission Electron Microscopy (STEM) and Energy-Filtered TEM (EF-TEM) [18,24,25].

2. Methods

2.1. Sample preparation

$A\beta_{1-40}$ was kindly provided by Dr. Robert Tycko at NIDDK, NIH and the fibrils were prepared as previously described [26,27].

Recombinant Sup35NM (residues 1–253 of Sup35 with a c-terminal histidine tag) was expressed in BL21-CodonPlus® (DE3) *Escherichia coli* cells as previously described using the pET expression vector pFPS167 [28]. Cells were grown in LB broth supplemented with 50 μg/mL ampicillin and protein expression was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). Protein purification and amyloid formation were respectively performed as previously described using Ni-NTA purification [28] and subsequent incubation in phosphate buffer.

Full-length Ure2p was similarly expressed in BL21-CodonPlus® (DE3) cells using the pET vector pKT41 [29]; protein expression, purification and amyloid formation were performed as previously described [29].

A-synuclein amyloid was kindly provided by Dr. Jennifer Lee and Thai Leong Yap at NHLBI and was prepared as previously described [30].

Carbon nanotubes were purchased from Sigma-Aldrich (cat number 724769). They were suspended in chloroform and sonicated for dispersion and debundling before being deposited onto graphene-coated grids for TEM observation.

2.2. Graphene preparation and coating on perforated 300 mesh copper grids

Graphene was grown on copper by chemical vapor deposition (CVD) following a procedure described previously [31]. Briefly, copper foil (99.8% pure, 25 μm thick) was pretreated in a bath of 50% by volume glacial acetic acid and 50% deionized (DI) water solution for 15 min. Then it was rinsed with DI water. CVD graphene was synthesized inside a 1-inch quartz tube furnace by first evacuating the tube (~10 mTorr base pressure) and back-filling it with hydrogen gas at a flow rate of 2 sccm. The furnace was heated to a temperature of ~1045 °C and the copper foil was kept at this temperature for 20 min to allow the temperature to stabilize. Then, methane gas was flowed into the tube at a flow rate of 20 sccm for 15 min, after which the methane flow was stopped and the furnace was cooled. After the temperature of the foil reached ~70 °C, as measured using an infrared thermometer, the foil was removed from the tube. The cool-down process took 1 h. Raman spectra performed on the prepared graphene show that they are primarily single-layered and free from defects, as shown in Fig. 1*E*.

To transfer the graphene onto the TEM grids (300 copper mesh grids from SPI covered by Quantifoil holey films with 2 μM holes, cat number 4330C-XA), we spin-coated a ~300 nm thick PMMA support layer onto the graphene. The copper foil was removed using a 1.6 M solution of $FeCl_3$. After the copper removal, we used a two-step cleaning procedure using 10:1:1 H_2O:H_2O_2:HCl and 10:1:1 H_2O:H_2O_2:NH_4OH to remove residual metal ions and organic residues from the graphene/PMMA stack [32]. The graphene/PMMA stack was then transferred to a bath of DI water for 20 min. The stack was then scooped onto the TEM grids and baked at 70 °C for 1 h to remove the water. The PMMA was then removed by placing the grid in an acetone bath for ~15 min, with a final rinse in isopropanol. We expect only a few nanometers of resist residue to be remaining on our graphene TEM grids and we get clean graphene coating reproducibly by following this procedure [33], as proved by our Raman spectroscopy measurements.

2.3. Sample deposition on grids

To prepare TEM samples, a 5 μL droplet of the sample solution was deposited on graphene, or on carbon film-coated grids (the thickness of carbon film is 3 nm to 5 nm). A 1 μL droplet of TMV solution (0.08–0.23 mg/mL concentration) was added for mass per length (MPL) dark-field images. After 5 min, excessive solutions on the grid were blotted and the grid was then washed three times or more with 5 μL protein buffer solution and the grid was dried before TEM observation. For negatively-stained samples, the sample was stained for 20 s with 5 μL of 3% uranyl acetate solution before being washed twice with 5 μL DI water.

2.4. Image acquisition and processing

All images except those in Fig. 1 were acquired on an FEI Morgagni operating at 80 kV, equipped with a side-mounted, 1 megapixel AMT Advantage HR CCD camera. Bright-field images were acquired at magnifications from 22,000×, to 180,000×. Images were taken with gain set to 1 with 400 ms exposure time, 10 integrations and 1024 × 1024 pixels. Dark-field images were acquired and analyzed as previously described [23] with TEM setting of spot 5, bias 3 and filament current around 14 μA. Electron dose was around 1500 to 3000 e/nm^2 as previously estimated using empty grids [23]. Images in Fig. 1 were acquired on a Joel 1011 TEM operating at 100 kV, equipped with a side-mounted, 1 megapixel AMT advantage HR CCD camera. Images of α-synuclein fibers were acquired on a JEOL TEM-1011 operated at 80 kV using custom grids or commercial carbon-coated grids (Ted Pella ultrathin carbon (<3 nm) on holey carbon support, product #1824).

2.5. Raman spectroscopy

Raman spectroscopy was performed using a Renishaw micro-Raman system with a 514 nm laser at 6.25 mW. In order to characterize the number of graphene layers used for TEM grid, we transferred a graphene film, prepared using the same CVD procedure, on 280 nm thermal oxide