Multifunctional graphene supports for electron cryomicroscopy

Katerina Naydenova¹,¹, Mathew J. Peet¹,¹, and Christopher J. Russo¹,²

¹Medical Research Council Laboratory of Molecular Biology, Cambridge, CB2 0QH, United Kingdom

Published online May 24, 2019.

Edited by Eva Nogales, University of California, Berkeley, CA, and approved April 17, 2019 (received for review March 20, 2019)

With recent technological advances, the atomic resolution structure of any purified biomolecular complex can, in principle, be determined by single-particle electron cryomicroscopy (cryoEM). In practice, the primary barrier to structure determination is the preparation of a frozen specimen suitable for high-resolution imaging. To address this, we present a multifunctional specimen support for cryoEM, comprising large-crystal monolayer graphene suspended across the surface of an ultrastable gold specimen support. Using a low-energy plasma surface modification system, we tune the surface of this support to the specimen by patterning a range of covalent functionalizations across the graphene layer on a single grid. This support design reduces specimen movement during imaging, improves image quality, and allows high-resolution structure determination with a minimum of material and data.

cryoEM | structure determination | graphene functionalization | low-energy plasma

High-resolution structural studies by electron cryomicroscopy (cryoEM) require the biological specimen to be embedded in a thin layer of amorphous water ice (1, 2). During preparation and cryoplungering, the specimen is exposed to the surfaces of this thin film of water and thus to potentially detrimental interactions with an air–water interface just before vitrification (1, 3). These interactions can be ameliorated with the aid of a thin film covering the specimen support (4), which provides an adherent surface for the specimen—but usually at the expense of introducing background noise, movement during imaging, contamination, and uncontrolled specimen–surface forces. Monolayer graphene is a near perfect support film since it is a conductive material that is only one atom thick, making it effectively invisible in the resolution range of interest to cryoEM (5–8). Pristine graphene, due to its hydrophobicity, is not a suitable substrate for preparing cryoEM specimens; previous work introduced partial hydrogenation with a low-energy plasma as a way of rendering the graphene hydrophilic without damaging the lattice, making it appropriate for cryoEM (8). Still, partially hydrogenated graphene provides only a single type of adherent surface, which is not sufficient for all possible specimens. To address this problem we developed a method to produce multifunctional and ultrastable graphene supports with tunable surface properties for cryoEM (Fig. 14).

Results

Design and Production of Multifunctional Graphene Supports. We optimized a chemical vapor deposition (CVD) graphene growth process (9) to produce 20-cm² scale sheets of monolayer graphene where the average crystal was larger than one grid square, ~50 μm across (SI Appendix, Fig. S1). We aimed to reduce the number of graphene crystal grain boundaries in the film to increase the mechanical and chemical robustness during subsequent functionalization and imaging. To combine the stability of all-gold specimen supports (10) with the benefits of a controllable graphene surface (8), we devised a clean and scalable graphene transfer procedure (Fig. 1 B and C). This method preserves both the structural integrity and tensile strength of the suspended gold foils, covering them with a continuous monolayer film of large graphene crystals, while minimizing contamination of the graphene surface (SI Appendix, Figs. S2–S4).

We developed an instrument for creating a range of covalent functionalizations of graphene using a low-energy helium plasma. The instrument comprises an inductively coupled plasma generator, attached to a high-vacuum specimen chamber, where vapors of chemical precursors for graphene functionalization are introduced at a controlled rate (Fig. 24). By using helium (which is inert and has a low atomic number) as the primary plasma gas with a remote plasma generator, sputter damage and other changes to the graphene are prevented while chemical reactions between the graphene and the chemical precursors are enabled. This is in contrast to typical residual air glow discharge systems used for cryoEM which create a plasma with sufficient energy to destroy the suspended monolayer graphene even with short exposures (SI Appendix, Fig. S5). We tested four precursor chemicals with different terminal reactive groups (SI Appendix, Table S1); in principle this method of functionalization can be used with any small-molecule compound provided it has a vapor pressure sufficient to inject into the chamber. We note that the orientation and length of the functional groups are not specifically controlled by this technique, implying there will possibly be a mixture of functional fragments on the surface. Similar

Significance

Single-particle electron cryomicroscopy (cryoEM) has now proved to be the method of choice for determining the structure of biological macromolecules and complexes. Yet success in determining a structure by cryoEM depends on being able to prepare a frozen specimen on a small metal support called a grid. This process is poorly controlled at present because of molecule–surface interactions. Here we used a modified form of graphene, in conjunction with a stable grid made of gold, to control these surface effects. Functionalized graphene-on-gold grids improve the reliability of specimen preparation and enhance image quality. This technology has the potential to take specimen preparation for cryoEM from a trial and error art to a controlled and reproducible process.
We illustrate the potential of multifunctional ∼ of an amine group. Of all 

plasma-assisted chemical modification and deposition methods are often used in the semiconductor manufacturing industry (11). To create reproducible and well-defined surface reaction conditions we monitored the atomic composition of the plasma in real time via optical spectroscopy (Fig. 2D).

The covalent functionalization of the graphene was confirmed by X-ray photoelectron spectroscopy (Fig. 2E). An initial hydrogen plasma treatment (8) allowed for subsequent functionalization of the graphene surface with potentially hydrophobic compounds while preserving the macroscopic surface wetting necessary for the formation of a thin ice layer (SI Appendix, Fig. S6). The integrity of the graphene lattice was preserved after these treatments, as demonstrated by electron diffraction (SI Appendix, Fig. S4); the graphene lattice is also directly visible in electron micrographs at suitably high magnification (SI Appendix, Fig. S7). The surface properties of functionalized grids are preserved when stored in vacuum; contamination in air occurs in 1–10 h, depending on the storage vessel (SI Appendix, Fig. S8).

We also designed a noncontact method to pattern multiple functionalizations across the graphene surface of a 3-mm support (Fig. 2 B and C), allowing one to simultaneously test multiple conditions for the same biological specimen on a single grid. Multiple exposures allow multiaxis patternning (SI Appendix, Fig. S6) and serial patternning of the same region with multiple chemicals. At the typical plasma process pressure of ∼1 Torr, and with ~50-μm mask-to-surface spacing, the resolution of this patternning method is ∼50 μm or 1 grid square (SI Appendix, Fig. S5A). With more complicated masks, a distinct function- 

Control of Specimen–Surface Interactions on Multifunctional Graphene Supports. We illustrate the potential of multifunctional graphene supports for optimizing the specimen orientation distribution using the 30S ribosomal subunit as a test specimen (12, 13). We prepared graphene functionalized with amylamine, hexanoic acid, 1-pentanethiol, and 4-pentylphenol by applying each of these chemicals to one-half of a partially hydrogenated graphene-coated gold grid. We analyzed the orientation distribution of the 30S particles on each of these surfaces (Fig. 3 A and B and SI Appendix, Fig. S10 A–D). We also compared these distributions to those on partially hydrogenated graphene and in unsupported ice (SI Appendix, Fig. S10 E and F). We were able to identify a combination of functionalized surfaces which increased the efficiency of the orientation distribution (14) to 0.8, enabling us to calculate a high-resolution reconstruction of the 30S subunit alone (SI Appendix, Fig. S11). The clear differences between all observed distributions indicate that the particles are interacting with the functionalized graphene; a tomographic reconstruction shows that the particles are in a monolayer on the graphene surface in a layer of ice just thicker than the particle diameter (SI Appendix, Fig. S12 and Movie S1) (15).

Interestingly, these experiments reveal a functional interaction map of the surface of the 30S ribosomal subunit. This allows us to create a physical model of the particle–surface interaction which accounts for the observed orientation distributions. On the carboxylated support, the dominant view indicates that the protein-rich exterior side of the 30S faces the surface (Fig. 3D). Several positively charged amino acid residues, including Arg-S226, Lys-S1079, and Lys-S4106,1070, are exposed on this side and likely stabilize contact with the surface. The same orientation is more favored on the thiol-functionalized surface (SI Appendix, Fig. S10B), consistent with the low pK_a of a thiol group. The next most frequent orientation on this side facing the surface (Fig. 3C). The interactions that cause this orientation are likely similar to the ones occurring at the 30S–50S interface in vivo. The putative anchor points include the Arg/Lys-rich S13 chain near the head of the 30S and the 16S rRNA 5′ domain at the surface of the body. This orientation is strongly favored on partially hydrogenated graphene (SI Appendix, Fig. S10E). In contrast, it is underrepresented on amylamine-treated partially hydrogenated graphene which is consistent with the high pK_a of an amine group. Of all
surfaces tested here, the amylamine/hydrogen-functionalized graphene minimized orientation bias for the specimens tested. Still, some acidic amino acid residues (Glu-S6, Glu-S13) can be identified as putative interaction points of the 30S subunit contacting the amine–graphene surface (Fig. 3 E and F). The Debye screening length in the buffer used is ~10 Å, and therefore we consider only electrostatic interactions with amino acid side chains that come to closer contact with the graphene surface. The spread of views around each preferred direction is determined by the shape of the particle and how it limits rotation around the fixed interaction point.

**High-Resolution Structure Determination on Multifunctional Graphene Supports.** To demonstrate that these supports are suitable for high-resolution structure determination, we used an amylamine-functionalyzed graphene on gold support to determine the structure of horse spleen apoferritin. The reconstruction reached 2.1 Å resolution (0.143 Fourier shell correlation [FSC] (16)) from 41,202 particles with standard data collection and processing (SI Appendix, Fig. S7 and Table S2 and Fig. 4B). The resolution of the reconstructed map, which is equivalent to the highest reported to date for this specimen (17), but required about half as many data, is demonstrated by clear densities for side chains in the map (Fig. 4). This includes complete aspartate and glutamate residues, for which densities beyond the Cβ are often absent from EM maps (18).

The decay rate of high-resolution information with fluence after the first 5–10 e−/Å² of irradiation in this dataset (Fig. 4D) is similar to measurements of radiation damage in 2D protein crystals (19, 20). We compare the movement of particles on multifunctional graphene-on-gold supports to previous measurements on several other supports (SI Appendix, Figs. S13 and S14) (4). Adding a graphene layer to an all-gold support reduced particle movement by a factor of 2 during electron irradiation. Compared with graphene-on-carbon supports, graphene-on-gold supports reduced particle movement by a factor of 3. The reduction in particle motion was also verified by tracking the movement of individual gold nanoparticles on an all-gold support with and without an additional graphene layer (Fig. 4C and SI Appendix, Fig. S15). Importantly, besides reducing the rate of random (uncorrelated) particle motion, the addition of the graphene layer also reduces the movement during the first few electrons of irradiation, improving the quality (B-factor) of these initial frames (Fig. 4 C and D), in which the molecules are less damaged by the beam.

**Discussion**

Multifunctional graphene-on-gold supports provide several improvements over previously described graphene oxide and graphene-on-carbon supports (6, 8, 21, 22). The graphene
growth, transfer onto all-gold grids, functionalization, and patterning methods presented here are all scalable; more than 100 grids can be processed in one day and this could be easily increased for commercial manufacture. Besides being a tunable surface for improving specimen orientation in cryoEM, functionalized graphene provides a method of mapping and quantifying the interaction surface of a particular biomolecule. Knowledge of how a purified protein interacts with other well-defined surfaces, like those present in ion exchange, hydrophobic interaction, or other affinity purification columns, will help guide the choice of appropriate functionalization and buffer conditions for a particular cryoEM specimen. This includes small proteins which would benefit from the reduced background signal and movement on graphene and membrane-bound molecules prepared by a range of solubilization and purification methods. With the spatial resolution of the noncontact plasma patterning method described here, grids on which every square has a distinct functionalization and surface property or linker chemistry are possible. Reactive chemical groups bound to the graphene surface, in particular thiol groups, can serve as a versatile platform for further covalent modifications, including to display ligands for specific binding on short flexible hydrocarbon linkers.

Graphene-on-gold specimen supports reduce the motion of the specimen, improving electron micrograph quality. We posit that the twofold reduction in the initial movement of the particles in ice is due to the graphene layer restricting the bulk movement of the thin film of ice within each hole. This occurs as mechanical stress—likely caused by the differential contraction of the gold foil and the water during vitrification—is released at the initiation of irradiation with the electron beam. This bulk movement of ice is evident from the correlation between the trajectories of neighboring particles in the first $e^-/A^2$ (SI Appendix, Fig. S15 B and E), which rapidly become uncorrelated. The reduction of the movement in the second, uncorrelated phase may be due to a reduction in the degrees of freedom of the particle adhered to the graphene surface. Still, complete elimination of the initial movement of the specimen would improve the images even further, reducing the movement to the limit set by pseudodiffusion of the particles in ice (23) (SI Appendix, Fig. S14 E and F). One strategy to reach the pseudodiffusion limit is to improve motion correction algorithms by using the graphene lattice as a fiducial, as suggested previously for graphene oxide (24). This approach is now more likely to succeed when starting from the sub-2-Å movement afforded by this specimen support design, but still relies on the movement of the lattice and the particle being correlated. We have made an entire raw dataset publicly available via the EMPIAR database to allow others to improve data-processing algorithms for micrographs on ultrastable graphene supports. We envision that patterned, multifunctional graphene-on-gold supports will be instrumental both in rapidly finding optimal surface conditions for cryoEM specimen preparation with a minimum of effort and microscope use and in determining high-resolution structures with the fewest possible data.

**Materials and Methods**

**Graphene Growth and Transfer.**

**Chemical vapor deposition of graphene on copper.** Graphene was grown on copper foil (Alfa Aesar 46365) in our in-house–constructed graphene CVD system with a 25-mm-diameter reaction tube. Copper foil was cut to 65-mm × 23-mm strips and cleaned by exposing each side to 120 s of UV–Ozone, before submersion into 200 mL 20% hydrochloric acid, followed by washing in deionized 18 MΩ H₂O (Millipore) and drying with nitrogen gas. The copper foil was then placed in the quartz tube of the CVD system under a flow of argon and heated to 1,050 °C for annealing, during which the copper grain size increased from ~10 μm to ~1 cm scale. This was followed by annealing under hydrogen flow at 20 scm and pressure 0.3 Torr for 1.5 h. At the end of the hydrogen anneal, oxygen gas (BOC, N6 purity) was applied for 120 s at pressure of 1.8 mTorr (9). After this, graphene was grown at 1,035 °C under a flow of 0.072 sccm methane, 20 scm hydrogen, and 30 sccm argon, at total pressure of 500 mTorr for a duration of 8 h. Typical growth conditions for the graphene used in this work are shown in SI Appendix, Fig. S1A. During the postgrowth cool down, oxidation was applied at 180 °C for 1 h to make the graphene-coated copper regions easily distinguishable from the uncoated ones. By varying the graphene growth conditions and increasing the growth time, we could in principle produce grains as large as 1 mm and control the extent of coverage of the substrate. The target crystal size was chosen at 100 μm, sufficient to cover an entire grid square while minimizing growth time.
High-resolution structure determination using multifunctional graphene-on-gold supports. (A) Contoured density maps of apoferritin, showing amino acid side chains within the structure. (B) Fourier shell correlation (FSC) plot for the apoferritin structure. (C) Mean-squared displacement of gold nanoparticles unsupported in ice (yellow circles) or in ice on graphene (red crosses), on all-gold supports. The addition of the graphene film reduces both the movement at the beginning of irradiation and the diffusion-like movement during the later frames by 2x. The dashed lines are linear fits to the data excluding the first frame. The slope decreases from 0.13 ± 0.1 A^2/Å^2 to 0.088 ± 0.005 A^2/Å^2 with the addition of the graphene to the all-gold support sample. (D) Calculated B-factors as a function of electron fluence for the apoferritin dataset. The B-factor in the first 3 e/Å^2 corresponds to the initial nondiffusive movement phase. The B-factor decreases at an approximately constant rate of 7.7 ± 0.8 A^2/Å^2 with fluence in the range 10–20 e/Å^2 (dashed line shows linear fit).

Graphene transfer to all-gold grids. Graphene was transferred onto all-gold grids (UltrAuFoil R0.6/1; Quantifoil) using a compliant form of collodion polymer. This method (SI Appendix, Fig. S2A) was chosen after many tests since it preserves the structural integrity of the gold foil, while keeping the large graphene sheet intact, and provides optimal plastic adhesion for the transfer, conformal coating of the surface of the grid, and separation of the grid from the polymer. An aqueous solution (2% cellulose nitrate in amyl acetate; Sigma) was pipetted onto the meniscus of a 135-mm-diameter crystallization dish filled with deionized 18 MΩ H2O, and the graphene-covered copper was gently placed on top. Within 5 min, as the solvent evaporated, the collodion solidified and the foil could be picked up. Any excess collodion around the foil area was removed, and the foil was turned over (with the collodion side up) and placed flat on the surface of the copper etchant (FeCl3 based; Sigma) in another 135-mm-diameter crystallization dish. The etchant was partially drained and the dish was refilled with 100 mL 20% HCl; this wash was usually repeated 10 times. This was followed by 5 washes with 2% HCl, 5 washes with 0.2% HCl, and 10 washes with deionized 18 MΩ water. Then the water was siphoned out and the collodion-supported graphene was lowered onto the batch of all-gold grids, arranged on the bottom of the dish. From one copper foil we could obtain 150–200 graphene-coated all-gold grids in a single transfer. The collodion was removed from the grids before use by immersing each grid in amyl acetate, 2-ethoxyethanol, chloroform, acetone, and isopropanol (all high-purity semiconductor grade; Sigma) in this order.

Graphene quality control. Examination by eye, optical microscopy, and scanning electron microscopy was applied to monitor the degree of graphene growth and surface contamination. Scanning electron images of graphene on copper and suspended graphene were acquired on a Scios DualBeam FEG SEM (FEI) in secondary electron mode using an Everhart–Thornley detector (SI Appendix, Figs. S1C, S2B, and S3). Transmission electron diffraction experiments were performed on the suspended graphene to assess the structure of the crystals. Weak and monotonic variation of the intensity of the graphene diffraction spots with tilt unambiguously identified suspended monolayer graphene (SI Appendix, Figs. S4A and S4B) (25). The mean linear intercept grain size of the graphene was measured to be 60 ± 10 μm (SI Appendix, Fig. S4C), meaning one could expect to encounter only one to two grain boundaries per grid square. For this measurement, the number of different grain orientations observed in the selected-area electron diffraction pattern was counted while traversing the whole grid in the TEM. We and others have previously characterized the background signal of graphene and hydrogenated graphene vs. thin layers of amorphous carbon and graphene oxide (7, 8). We have also measured the background signal in power spectra from grids transferred and functionalized as reported here and found it was 6–7x less than for a 46-A-thick layer of amorphous carbon, whose thickness was measured accurately by atomic force microscopy. Thus, the background signal is slightly more than that of pristine graphene (3-A-thick crystalline lattice) but much lower than 6 Å of amorphous carbon. We note that residual polymers, including nitrocellulose, left on the graphene can align with the lattice and can be observed in the low-dose diffraction pattern, as reported previously for PMMA (26). We observed this as well when the collodion was not completely removed from the graphene layer. Still, unlike for PMMA, for collodion this residual contamination can be avoided through the careful use of semiconductor grade solvents and the plasma treatment method described here.

Graphene Functionalization.

Covalent functionalization using a low-energy helium carrier plasma. Plasma treatments were performed in an extensively modified commercial plasma cleaner (Fischione 1070), equipped with a custom grid and mask holder, a custom gas-injection system (Fig. 2A), and a custom fiber-coupled sapphire viewport attached to a UV-vis spectrometer (Thorlabs). The vacuum chamber was evacuated to <10^-5 Torr by a turbo pump. A carrier plasma of N6.0 grade helium was produced at 28 scm flow rate and typical pressure 5 x 10^-1 Torr. The 18-MHz RF coil was operated at 40% power, yielding typically 9 W forward power and 0.6 W reverse power. The vapors of the chemicals used for functionalizations were introduced through an evacuated five-channel manifold. The chemicals were stored in sealed, evacuated stainless steel vials, and the vapor of the desired chemical was supplied through a precision micrometer needle valve and a shutoff valve into the plasma chamber. The effect of the introduced chemical was observed in the real-time spectrum of the plasma. Spectra were acquired with a 6-s exposure time, which saturated the helium lines and made the additional peaks from the precursors visible. The most prominent feature in the optical spectra due to the introduction of organic molecules was the Χν peak at 606 nm (Fig. 2D). We hypothesize that this is due to the high probability of liberating terminal hydrogens from the molecule; whereas the separation of larger fragments or nonterminal moieties, like the atomic sulfur from a thiol, would require two or more bonds to be broken, and therefore is less likely. The actual amount of chemical present in the plasma depends on the vapor pressure of each compound; this can be adjusted by controlling the temperature of the liquid container and the setting on the leak valve. Once the plasma composition was established, a shutter was placed over the grid and mask holder was used to control the exposure time. Between the use of different chemicals, the chamber was cleaned using a pure helium plasma at 70% power for 30–60 min, until no signs of trace hydrogen were visible in the optical spectrum.

X-ray photoelectron spectroscopy of functionalized graphene. X-ray photoelectron spectroscopy (XPS) was used to study the covalent modifications to graphene. The beam from an Al Kα source (1,486.68 eV; ESCALAB 250 Xi) was focused in a 900-μm-diameter probe on the sample. Graphene on copper (as grown) and graphene transferred to UltrAuFoil gold grids were used for these measurements, and charge compensation was applied. Partially hydrogenated and non-treated graphene specimens were used as controls to compare against functionalized graphene. The pressure in the XPS chamber during data acquisition was 5 x 10^-9 mbar. First, spectral scans at 1 eV sampling were acquired in the 136- to 1,361-eV range with 200-eV pass energy, and then 30–50 scans with 30- to 50-eV pass energy at 0.1-eV sampling were acquired around the regions of interest and averaged to produce the spectra plotted in Fig. 2E. The peaks were identified using data from the NIST XPS Database (27). Some of the observed peaks were attributed to signal from the metal substrate supporting the graphene.

Contact angle measurements. Contact angle measurements were performed to evaluate the surface properties of the support after varying doses of functionalization (SI Appendix, Fig. S5F) and to determine the effect of various storage conditions on these properties over time (SI Appendix, Figs. S5A–S5E).
The whole-micrograph movies were motion corrected in SI Appendix 2±µmbar. The grids were 2µm) which become contaminated and lose their hydrophilicity within immediately after ˚E

Grid preparation. Purified Thermo m philus 305 ribosomal subunits (in 5 mM Hepes, 50 mM KCl, 10 mM NH4Cl, 10 mM Mg(OAc)2) were provided by the Ramakrishnan laboratory. The concentration was adjusted to 8 mg/mL except for the specimen on partially hydrogenated graphene, where the concentration was 1.7 mg/mL. All graphene-coated grids used were first exposed to H2 plasma for 180 s to render the whole surface hydrophilic, followed by a 30-s treatment of half of the grid with He plasma carrying the vapor of the corresponding functionalizing chemical and another 30-s treatment for the other half. Plain UltrAofi grids for the control experiments without graphene were treated with a 9:1 Ar:O2 plasma mixture for 60 s. Grids were plunged using an FEI Vitrobot equilibrated at 4°C and 100% relative humidity; the liquid ethane was kept at a fixed temperature of 93 K. A 3- to 4-µL volume of the protein solution was pipetted onto the graphene-coated side of the grid, double-blotted for 5 s, and immediately plunged into the ethane. Typically less than 10 min elapsed between the plasma treatment and the vitrification. The grids were stored in liquid nitrogen until they were transferred into the electron microscope for imaging.

Imaging and data collection. Micrographs of 305 ribosomes in ice on functionalized graphene were acquired on a Tecnai Polara microscope operated at 300 keV using a Falcon 3 detector in integrating mode. The nominal magnification was 93,000×, corresponding to 1.17 Å per pixel (calibrated using 2.7 Å cryo-EM reference of 305 ribosomes) at a 120°C temperature. The local sharpness of the pattern depends on and can be controlled by varying the following: the distance between the mask and the graphene surface, the sharpness of the mask edge, and the mean free path of the species in the plasma. Under the typical conditions used here (1 Torr, nonthermal remote plasma), the mean free path in the chamber is much larger than the characteristic length scales; we therefore estimate the patternning resolution to be approximately equal to twice the distance between the grid and the mask, i.e., 50 µm. This is confirmed by the width of the transition region in SI Appendix, Fig. S5A for a knife-edge mask placed 20 µm above the surface of the foil.

Motion Tracking in Vitreous Ice Using Gold Nanoparticles. Grid preparation. Specimens were prepared by manual plunge freezing in a 4°C cold room. All-gold supports (UltrAofi R0.661, 300-mesh; Quantifoil) with 800-nm hole diameter, with and without graphene, were used. Specimen supports were plasma treated to render them sufficiently hydrophilic with 800-nm hole diameter, with and without graphene, were used. The grids were positioned in a custom slotted holder, analogous to the one used for plasma treatments, and covered by a mask plate. Single-slot apertures with 100-µm hole diameter (EMS GA100-Au) were used for masks to expose only the center of the grid to the evaporated carbon. The grid-to-mask distance was ~100 µm. The localization deposition of a continuous amorphous carbon film on the graphene was verified by TEM imaging (SI Appendix, Fig. S9).

Amorphous carbon deposition onto graphene-on-gold supports. Amorphous carbon was deposited directly onto graphene-coated gold grids in an Edwards 306A evaporator evacuated to 10−5 mbar. The grids were positioned in a custom slotted holder, analogous to the one used for plasma treatments, and covered by a mask plate. Single-slot apertures with 100-µm hole diameter (EMS GA100-Au) were used for masks to expose only the center of the grid to the evaporated carbon. The grid-to-mask distance was ~100 µm. The localization deposition of a continuous amorphous carbon film on the graphene was verified by TEM imaging (SI Appendix, Fig. S9).

Motion analysis. The whole-micrograph movies were motion corrected in MotionCorr (32) to remove stage drift. The motion-corrected stacks were then visually inspected. The trajectories of all particles during the first 20 e−/Å2 of irradiation were used to calculate the mean-squared and root-mean-square displacements of the particles over the ensemble.

CryoEM of Thermus thermophilus 305 Ribosomal Subunit. Grid preparation. Single-particle data analysis. The sectors of the functionalized grids were identified using the asymmetric center mark (SI Appendix, Fig. S6B). The micrographs in Relion 3 (17) were first inspected for 800-nm diameter (EMS GA100-Au) were used for masks to expose only the center of the grid to the evaporated carbon. The grid-to-mask distance was ~100 µm. The localization deposition of a continuous amorphous carbon film on the graphene was verified by TEM imaging (SI Appendix, Fig. S9).

Amorphous carbon deposition onto graphene-on-gold supports. Amorphous carbon was deposited directly onto graphene-coated gold grids in an Edwards 306A evaporator evacuated to 10−5 mbar. The grids were positioned in a custom slotted holder, analogous to the one used for plasma treatments, and covered by a mask plate. Single-slot apertures with 100-µm hole diameter (EMS GA100-Au) were used for masks to expose only the center of the grid to the evaporated carbon. The grid-to-mask distance was ~100 µm. The localization deposition of a continuous amorphous carbon film on the graphene was verified by TEM imaging (SI Appendix, Fig. S9).

Motion analysis. The whole-micrograph movies were motion corrected in MotionCorr (32) to remove stage drift. The motion-corrected stacks were then visually inspected. The trajectories of all particles during the first 20 e−/Å2 of irradiation were used to calculate the mean-squared and root-mean-square displacements of the particles over the ensemble.

CryoEM of Thermus thermophilus 305 Ribosomal Subunit. Grid preparation. Single-particle data analysis. The sectors of the functionalized grids were identified using the asymmetric center mark (SI Appendix, Fig. S6B). The micrographs in Relion 3 (17) were first inspected for 800-nm diameter (EMS GA100-Au) were used for masks to expose only the center of the grid to the evaporated carbon. The grid-to-mask distance was ~100 µm. The localization deposition of a continuous amorphous carbon film on the graphene was verified by TEM imaging (SI Appendix, Fig. S9).

Amorphous carbon deposition onto graphene-on-gold supports. Amorphous carbon was deposited directly onto graphene-coated gold grids in an Edwards 306A evaporator evacuated to 10−5 mbar. The grids were positioned in a custom slotted holder, analogous to the one used for plasma treatments, and covered by a mask plate. Single-slot apertures with 100-µm hole diameter (EMS GA100-Au) were used for masks to expose only the center of the grid to the evaporated carbon. The grid-to-mask distance was ~100 µm. The localization deposition of a continuous amorphous carbon film on the graphene was verified by TEM imaging (SI Appendix, Fig. S9).

Motion analysis. The whole-micrograph movies were motion corrected in MotionCorr (32) to remove stage drift. The motion-corrected stacks were then visually inspected. The trajectories of all particles during the first 20 e−/Å2 of irradiation were used to calculate the mean-squared and root-mean-square displacements of the particles over the ensemble.
then rotating the mask by 90°, exposing one-half of the grid to amylamine under helium plasma for 30 s, and rotating the mask by 180° to expose the other half of the grid to hexanoic acid under helium plasma for 30 s. Vitrification was done in the same way as above.

**Imaging and data collection.** Micrographs were acquired on a Titan Krios microscope operated at 300 kV using a Falcon 3 detector in integrating mode. The flux was set to 17 e⁻/Å²/s and the exposure time was 2 s; a 70-μm objective aperture was used. The nominal magnification was 59,000×, corresponding to 1.34 Åpix, calibrated using the (101) reflections from anatase (TiO₂) nanoparticles dispersed on a separate calibration grid. The quadrants of the grids were identified with respect to the orientation of the asymmetric grid center mark as above (**SI Appendix, Fig. 56**).