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Charge accumulation in electron cryomicroscopy

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ABSTRACT

When irradiated in a transmission electron microscope, plunge-frozen, amorphous water ice specimens accumulate a pattern of static charge that changes dynamically as the specimen is irradiated, and which can deflect the transmitted electrons and blur the resultant micrographs. Here we provide a physical description of this charge accumulation and characterise its dynamic behaviour in the context of low-dose electron cryomicroscopy (cryoEM). We observe the accumulation of positive charge in the primary irradiation area as expected from earlier work. To our surprise, we also observed a build-up of negative charge in nearby unirradiated regions of the specimen. Using a standard carbon support foil containing a pure water ice specimen, we collect a portion of this negative charge in the micrometer sized specimen holes which act as electrostatic lenses. These unusual, diverging micro-lenses are extremely sensitive charge detectors that allow us to directly measure the magnitude and dynamics of charge accumulation and neutralisation that occur during cryoEM imaging. Using these measurements, we find that the build-up of charge on the specimen saturates to a dynamic equilibrium at an electron fluence which is orders of magnitude lower than required for a typical low-dose micrograph. The measurements here will guide the development of optimal imaging conditions for biological specimens and contribute to a complete theory of information loss in electron cryomicroscopy.

1. Introduction

When irradiated with electrons of sufficient energy to traverse its thickness, a biological molecule suffers rapid and irreversible damage that destroys its structure [1,2]. This limits the amount of information available in an electron micrograph of a biological specimen and ultimately determines the resolution at which its undamaged structure can be determined. It is important to consider all forms of image blurring or contrast loss that might occur during the acquisition of a low-dose image of a biological specimen under cryogenic conditions. The thin foils of amorphous carbon used in cryoEM are poor conductors at liquid nitrogen temperatures (resistivity of $10^{-2} - 10^{-1}$ Ωcm) [3] and the suspended ice is an insulator which is many orders of magnitude less conductive (10^{9} Ωcm for crystalline pure water ice at 190K, 10^{8} Ωcm for crystalline 0.1 M NaCl ice at 190K) [4], which is part of the motivation for quantifying the amount of charging that occurs during imaging. Here and in an accompanying paper [5], we investigate one source of image blurring in cryoEM – the build-up and fluctuations of electric charge in, and on, the specimen during imaging.

Much is known about charging effects in the transmission electron microscope [6–9]. When irradiated with high energy electrons in a localised area, semiconducting and insulating materials undergo ionisation events by several inelastic scattering processes and lose energetic electrons to the vacuum [10]. These take the form of emitted secondary electrons, most having an energy of about 1 – 100 eV, and which leave behind regions of positive charge that create electric fields in and around the specimen which can deflect the electron beam [10]. Over time, the positive charge builds up in the irradiated layer and can then shift the phase of subsequent electron waves. Sometimes called the Berriman effect [11,12], this build-up of semi-static charge in the specimen occurs when an insulating or semiconducting material, like an amorphous carbon foil, is irradiated in a small region (1 – 10 μm in diameter) with an electron beam and then subsequently imaged with a broad, low-intensity beam (10^{-2} $e^{-}/Å^{2}$) and high defocus (~ –10 mm). A region corresponding to the intense beam then appears dark in the highly defocused image; under continuing low flux imaging, the dark contrast then slowly decays away until it virtually disappears. This distinguishes it from the beam-induced build-up of hydrocarbons or other contaminants on the surface of the foil [13], which is permanent after irradiation. High-defocus renders the image sensitive to small deflections in electron path, like those that occur when a specimen becomes charged [7]. Models

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have been proposed to account for how charge builds up on semi-insulating specimens at or above room temperature [14–16] and even cryoEM specimens on carbon films [17], but no direct measurements of the charging phenomenon on cryoEM specimens under typical irradiation conditions have been reported. Here we use these high defocus conditions in conjunction with standard low-dose imaging methods to investigate the build-up of charge on a standard cryoEM specimen, consisting of vitrified water spanning the micron sized holes in an amorphous carbon foil, during typical imaging at 300 keV. Using an interesting observation of a micro-lensing effect, we are now able to quantify this build-up of charge and characterise the time and length scales involved in the build-up of charge during low-dose imaging in cryoEM.

2. Materials and methods

2.1. Specimen preparation

Specimens were prepared by plunge freezing using the Dubochet method [18,19] and a manual plunger of the Talmon design [20]. Briefly, 3 µL of deionised, filtered water (resistivity $>18\,M\Omega cm$) was applied to a specimen support (a patterned amorphous carbon foil on a gold grid, Quantifoil Au 1.2/1.3 [21]) in a cold room at 4 °C. The grid was blotted manually with filter paper (Whatman No. 1) from one side for 10 s in a humid chamber and then was plunged immediately into liquid ethane held at a temperature of 93K using a precision cryostat [22]. The specimen supports were rendered hydrophilic by exposure to a residual air plasma (glow discharge) in an Edwards S105B for 30 s, just prior to use. The frozen specimens were transferred to small plastic grid storage boxes and kept in liquid nitrogen until they were imaged in the electron microscope.

2.2. Electron microscopy

Frozen specimens were transferred to the load lock system of an electron cryomicroscope (FEI Polara) and loaded into the specimen position. All micrographs were recorded using 300 kV bright field transmission mode, and detected with a 2048 × 2048 phosphor-coupled CCD camera (Gatan Orius). Micrographs were collected using a pre-specimen beam blanker using the data collection software attached to the camera (Gatan Digital Micrograph), which allows capture of a series of micrographs using a set exposure time and a fixed delay between images. A diagram of the typical experiment is presented in Fig. 1. A low-dose experiment scheme was set up with two modes: *mode 1*: Low flux (1.2×10^{-4} $e^-/Å^2/s$), low magnification (350 ×), broad, parallel beam ($2000\,\mu m^2$) and high defocus (−60 mm) *mode 2*: High flux (1.4 $e^-/Å^2/s$) focused beam (diameter = 5 µm) centred in the low flux region, exposed continuously for anywhere from 1 m sec to 100 s depending on the experiment. To conduct an experiment as diagrammed in Fig. 1, the specimen was moved to a new, unirradiated square on the support with the beam off, and then a single 0.2 s image in *mode 1* was collected, which becomes the first image in the movie. The microscope was then switched to *mode 2* and the centre region of the square was irradiated for the specified time corresponding to a particular fluence of electrons. The microscope was then switched back to *mode 1*, and a movie was collected by taking a series of 10–300 images (depending on the particular experiment) with a set delay between each image to allow the files to be written out. The delay for the supplementary movie and Fig. 2 was 5 s, and was limited by the bandwidth of writing the files to disk. Thus the total elapsed time was between 1 and 25 min depending on the experiment.

This experimental scheme was tested on holey amorphous carbon foils having an additional carbon film on top of the foil, to establish the appropriate conditions, and then conducted on cryo-specimens prepared as described above. To determine the focal length of the microlenses we repeated the experiment several times under the same conditions until the maximum intensity for the focused microlenses was found; in this case - 60 mm. The error in determining this maximum is estimated to be ±5 mm. The beam current was measured using the current amplifiers attached to the two phosphor viewing screens, which had been previously calibrated against a picoammeter attached to a Faraday cup in the projection chamber. The total error in the measurement of the beam current, which is the dominant source of error in the reported flux, is estimated to be less than 30%. All other errors are less than 10%.

2.3. Data analysis and processing

Images were converted to MRC format and cropped, converted to 8 bit depth and saved as TIFF files for creating the panels in Figs. 2 to 3. In both figures, higher electron intensity is shown as white. Linear sections in intensity along lines were taken from the raw, unprocessed micrographs and used to create the plots in Fig. 4. To quantify the charge on a local region of the specimen, like the suspended ice within the holes of the foil, the normalised intensity vs. radius from the centre of the hole was found by integration about the azimuth, and the difference between the maximum at the centre of the hole and the minimum at the edge was taken as the intensity, as plotted in Fig. 4c. Although the thickness of the ice is not necessarily the same across the entire width of each hole, the image contrast due to charging is much greater than any variation due to ice thickness. This was repeated three times on each of three squares for three separate experiments, and the mean and standard deviation were taken as the representative data point and error bar, respectively. Supplemental movies of the charging process were created by combining the series of micrographs into a stack, which was drift corrected [23] and converted and compressed to .avi format using ImageJ [24].

3. Results

3.1. Imaging charge build-up and neutralisation

To investigate the kinetics of charge build-up and neutralisation, it was important to use specimens consisting of amorphous ice on a holey carbon film, which is the specimen used in most single particle cryoEM experiments [18]. We used electron-optical conditions for illumination and imaging that are equivalent to typical low-dose conditions used for single particle cryoEM, and which are similar to those used originally [11] to describe the Berriman effect. In particular, we used high defocus (−20 to −80 mm) at low magnification (350 ×) to amplify the small electrostatic deflections of the illumination that are caused by the charging. We found that −60 ± 5 mm defocus maximised the intensity of the bright spots in the centre of the holes with amorphous ice. This can be used to estimate the focal length of the electrostatic lens and thus the lateral electric field at the hole.

3.2. Quantifying the charge

For a 300 keV electron to be displaced by 0.6 µm (r, the radius of the hole) in $f = 60$ mm, this corresponds to a deflection angle of $\theta = \arctan r/f = 10$ µrad. Using this information, an order of magnitude approximation for the electrical potential in the centre of the hole can be determined, in a way that follows previous estimates [6,7]. A 300 keV electron has a velocity in the lab frame of $v_e = 0.77c$ where c is the speed of light in vacuum. Thus the lateral velocity, v_s required to give a deflection angle of 10 µrad is