Transmission electron microscopy (TEM) of biological specimens requires the use of support films deposited on 3 mm specimen grids. For cryo electron microscopy (cryo EM), having a surface that is both clean and hydrophilic is critical to allow uniform distribution of biomolecular complex in aqueous solution onto the surface [1]. The most common supporting material is amorphous carbon, because it induces adsorption of particles across the holes in the grid and minimizes the effect of the support on TEM images. Unfortunately, these grids tend to be hydrophobic, which may alter the particle concentration and orientation in a grid hole such that it is no longer representative of the original particle concentration and orientation. Because of this, it is necessary to modify the grid surface to make it hydrophilic before placing the biomolecular complex on it.

Hydrophilicity and hydrophobicity are terms that describe how water interacts with a surface. If water beads up into droplets and does not spread evenly across the surface, the surface is said to be hydrophobic. The contact angle between the water droplet and surface will be large. In contrast, if a droplet placed on the surface spreads out evenly and has a low contact angle with the surface, the surface is said to be hydrophilic. While hydrophilicity and hydrophobicity are relative terms, the contact angle of water on a substrate has been shown to be a good indicator of the relative hydrophilicity or hydrophobicity of the surface [2, 3].

A common way to create hydrophilic grids is to expose them to a low-power plasma; O\(_2\) and Ar are two gases frequently used for plasma treatment of surfaces. An O\(_2\) plasma will remove biological contaminants through chemical reaction with the surface, which removes hydrocarbons and allows adsorption of functional groups that change the surface properties. In addition, these functional groups can themselves be modified by the plasma. For most surfaces, treatment with an O\(_2\) plasma will increase hydrophilicity due to the presence of hydrophilic groups, such as hydroxyl (OH), carboxyl (COOH), and carbonyl (CO), on the surface that attract water molecules [2, 3]. In contrast, an Ar plasma will not chemically react with the surface; however, depending upon the plasma energy, it will remove surface contaminants through ablation, resulting in increased surface roughness. This change in surface roughness changes the surface energy, which affects the hydrophilicity of the surface [2].

Figure 1a shows a water droplet placed on a 3 mm grid before plasma processing. The droplet did not spread across the entire grid and has a large contact angle (Figure 1c), indicating that the grid is hydrophobic. A droplet placed on the same grid after plasma processing (Figure 1b) spread across the entire grid and had a much smaller contact angle (Figure 1d), which indicates that the grid is now hydrophilic.

Model 1070 NanoClean
Preparing hydrophilic, carbon-supported grids for cryo electron microscopy

Using plasma technology to make up to 10 grids hydrophilic without degrading the carbon support

Figure 1. A water droplet placed on the grid before plasma processing (a, c) did not spread across the entire grid, indicating that the grid is hydrophobic. In contrast, the water droplet placed on the grid after plasma processing (b, d) spread across the entire grid and had a much smaller contact angle, indicating that the grid is hydrophilic.
Preparing hydrophilic, carbon-supported grids for cryo electron microscopy

The most common use for a plasma cleaner is to remove hydrocarbon contamination from the surfaces of specimens. However, as previously discussed, one can also use a plasma cleaner to alter the surface properties of grids for cryo EM so that they become hydrophilic. Because grids commonly used for cryo EM have a very thin (< 3 nm thick) carbon support film onto which the biomolecular complex is placed, care must be taken to ensure that the grid support is not damaged during plasma processing. Parameters that are ideal for plasma processing of standard TEM specimens may not be ideal for processing carbon-supported grids for cryo EM.

A single 3 mm grid loaded on a standard TEM specimen holder that is plasma processed will become hydrophilic. However, the grid’s carbon support structure will degrade after prolonged exposure to the plasma, so one must determine how long it takes to make the grid hydrophilic and how long it takes for the carbon support structure to degrade to the point that the grid is no longer usable. When the holder is inserted into the Model 1070 NanoClean, the specimen holder tip is positioned in the plasma. Both sides of the grid are exposed to the plasma during processing. To minimize the deleterious effects of the energetic plasma on the carbon support structures and to minimize the rate of thinning of the carbon support, a low power (10 watts), low oxygen content (5% O₂ or less) plasma is used to treat the grids.

When using a standard specimen holder and a less aggressive plasma (low power, low oxygen content), processing times resulting in a hydrophilic surface are very short – 45 to 60 seconds (Table 1). Exposure times required to degrade the carbon support are on the order of 90 seconds; thereby, creating a time window in which the grid becomes hydrophilic without damaging the carbon support film.

<table>
<thead>
<tr>
<th>Standard TEM specimen holder</th>
<th>Minimum time required to make grid hydrophilic</th>
<th>Carbon support degradation time</th>
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<td></td>
<td>45 to 60 seconds</td>
<td>90 seconds</td>
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Some cryo EM facilities wish to prepare multiple grids simultaneously, which requires the use of a multi-grid holder. Fischione Instruments’ Grid Specimen Holder (part number 011-0002), which holds up to 10 grids, positions the grids within the plasma. When combined with the use of low power (10 watts), low oxygen content (5% O₂ or less) plasma, the plasma acts less aggressively on the carbon-support structures, allowing better control over the plasma process. In addition, the multi-grid holder allows both single- and double-sided plasma processing, which gives an added degree of control over the plasma process.

One can expect that the amount of time required to make grids hydrophilic without damaging the grids’ carbon support will differ when comparing single- and double-sided exposure. As it is the goal to batch process up to 10 grids at a time, the ideal plasma processing time is one that is long enough to ensure that all 10 grids become hydrophilic, but short enough to avoid degradation of the grid support on all the grids.

This application note presents high-throughput plasma processing methods for rendering hydrophilic, carbon-supported grids for cryo EM applications without excessively degrading the grids’ carbon-support structure.
Experiment design

Using a 10-grid specimen holder (Grid Specimen Holder, part number 011-0002), experiments were performed to determine the plasma processing time required to:

- make multiple carbon support grids hydrophilic (single- and double-sided exposure)
- degrade the carbon film to the point that it is unusable

Copper mesh grids with ultrathin carbon film (< 3 nm thick) on lacey carbon support were used. All grids were first examined with an optical microscope; grids with visible defects or significant contamination were discarded. The Model 1070 NanoClean parameters for all tests were:

- gas mixture: 5% O₂ / 95% Ar
- power: 10 watt
- gas flow rate: 30 sccm

Rendering grids hydrophilic

To determine the time needed to make grids hydrophilic when using the multi-grid holder, experiments were performed using a single carbon-supported grid. The grid was imaged in the TEM prior to plasma processing and then loaded into the multi-grid holder. The grid was then plasma processed, after which a drop of water was placed onto the surface to ascertain hydrophilicity. It was then dried and the carbon support was imaged in the TEM to determine if there were any changes to the carbon support morphology after plasma treatment. This experiment was repeated using additional grids that were plasma processed for increasing amounts of time. Experiments were done for both single-sided and double-sided plasma exposure.

Once the ideal plasma processing time was determined, 10 new grids were selected and imaged in the TEM to verify their state before plasma processing. These 10 grids were then loaded into the multi-grid holder and plasma processed using the optimal plasma exposure time. The hydrophilicity of each grid was then checked using a water droplet placed on the grid, after which TEM imaging was performed to determine if there were any significant morphological changes to the carbon support after plasma processing.

Once it was determined that all 10 grids were hydrophilic and that there were no significant changes to the morphology of the carbon support, a recommended plasma processing time was established: a plasma processing time of 3 minutes for double-sided exposure and 6 minutes for single-sided exposure will yield hydrophilic grids.

This recommended processing time was derived from experiments performed using two different lots of specimen grids. Due to variability in the grid manufacturing process, significant variations in the thickness of the carbon support can occur; this will result in variations in the exposure time needed to make the grids hydrophilic. Fischione Instruments recommends that a grid from each new batch of grids be tested to determine the ideal exposure time needed to make it hydrophilic before processing an entire batch of grids.
Degradation of the grid’s carbon support
Experiments were performed in the same manner as the hydrophilicity tests to determine the plasma exposure time at which the carbon support showed signs of degradation.

<table>
<thead>
<tr>
<th>Plasma exposure times and carbon-support degradation</th>
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<tr>
<td>Single-sided exposure</td>
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<tr>
<td>7 minutes</td>
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**Table 2.** NanoClean plasma exposure time at which the grid’s carbon support showed signs of degradation while using the multi-grid holder.

**Figure 4.** TEM images of grid before (a, c) and after (b, d) 6 minutes of single-sided plasma exposure in the NanoClean. This plasma processing time is sufficient to render the grid hydrophilic, but does not cause undo carbon support degradation.

**Figure 5.** TEM images of grid before (a, c) and after (b, d) 3 minutes of double-sided plasma exposure in the NanoClean. This plasma processing time is sufficient to render the grid hydrophilic, but does not cause undo carbon support degradation.
Preparing hydrophilic, carbon-supported grids for cryo electron microscopy

Figure 6. TEM images of a 3 mm carbon-supported grid before plasma processing (left) and after 4 minutes of double-sided plasma exposure (right). Degradation of the carbon is circled (right).

The results determined that degradation of the carbon support began after 4 minutes of double-sided exposure and 7 minutes of single-sided exposure.

Based on the results, the time required to degrade the 10 grids’ carbon supports to the point where the grids were unusable was longer than the time required to render the grids hydrophilic.

Discussion

It is possible to make 10 grids hydrophilic without degrading the support on any of the grids if a plasma processing time of 3 minutes for double-sided processing and 6 minutes for single-sided processing are used.

Table 3. Recommended NanoClean plasma processing times required to render ultra-thin carbon support grids hydrophilic when using the multi-grid holder.

<table>
<thead>
<tr>
<th>Exposure (single- or double-sided)</th>
<th>Recommended time to make grids hydrophilic</th>
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<tbody>
<tr>
<td>Single</td>
<td>6 minutes</td>
</tr>
<tr>
<td>Double</td>
<td>3 minutes</td>
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References