Effect of surface roughness of carbon support films on high-resolution electron diffraction of two-dimensional protein crystals

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The surface roughness of carbon films, which are used as a specimen support in high-resolution electron microscopy, has been investigated by atomic force microscopy. Carbon films were prepared by evaporating carbon onto mica. We found that the carbon surface that had been in contact with the mica was between 3 and 9 times smoother than the surface which had faced the carbon source. Surfaces of carbon films prepared by multiple evaporation were smoother than films evaporated in a single step. The surface roughness on the mica side of these films was close to that of mica. Two-dimensional crystals of plant light-harvesting complex yielded isotropically sharp high-resolution electron diffraction patterns at high tilt angles only when supported on the smoothest carbon films, produced by multiple evaporation.

1. Introduction

Carbon films are routinely used as specimen support in electron microscopy. Recently, the surface properties of carbon support films have come under scrutiny because of their crucial role in electron crystallography of biological macromolecules [1]. Two-dimensional (2D) crystals are particularly susceptible to distortion by uneven supporting films. Even small deviations from planarity can cause severe blurring of diffraction spots at high tilt angles, thus making it difficult or impossible to collect high-resolution electron diffraction data [21]. Thorough understanding and control of the interaction of 2D crystals with the support film are necessary before electron crystallography can become a routine method for high-resolution structure determination of biological specimens [3].

To investigate the surface structure of support films and in order to find a method for producing smooth carbon films we examined carbon films evaporated onto mica with the atomic force microscope [4,5]. The atomic force microscope (AFM) yielded topographic images of the carbon film surface from which the roughness was calculated. The surface facing the evaporation source (referred to as “top surface”) was imaged on mica. After stripping the mica off the carbon we were also able to image the surface of the carbon film that had been in contact with the mica (referred to as “mica side”). Our results show that the mica side is much smoother than the top side. By varying the method of carbon evaporation we were able to reduce the roughness of the carbon surface close to that of a freshly cleaved mica surface. These films proved to be indispensable for collecting high-resolution electron diffraction data of 2D crystals of light-harvesting chlorophyll a/b-protein complex (LHC-II) at high tilt angles.

2. Materials and methods

2.1. Preparation of carbon films

Carbon films were deposited onto freshly cleaved mica in three different ways, referred to as
methods 1, 2, and 3. For method 1 we used an Edwards model 306 evaporator. The chamber was evacuated by an oil-diffusion pump equipped with a liquid-nitrogen cold trap. Carbon rod (Agar Aids) was evaporated at a voltage of 10 V in one step at a high evaporation rate (few seconds) when the vacuum had reached \((2-5) \times 10^{-6}\) Torr. For method 2, a Baltzers BAE 250 evaporator evacuated by a turbo-molecular pump was used. Carbon was evaporated in a single step by passing a current through a tightly twisted rope of thin carbon fibres of sufficient thickness (approximately 2 mm), at a vacuum similar to that in method 1. Methods 1 and 2 are referred to as standard techniques. Method 3 was the same as method 1, except that the evaporation was slow and stepwise, turning the current down as soon as the tip of the carbon rod became white hot and the vacuum deteriorated to roughly \(10^{-5}\) Torr. The heating current was turned up again when the tip had cooled down and the vacuum had recovered. This process was repeated until the desired thickness of the carbon film had been reached. The final thickness of all carbon films was about 10 nm.

2.2. Specimen preparation

To image the top surface of carbon films with the AFM, a piece was cut off the carbon-coated mica sheet and glued with epoxy adhesive to a steel disk. For imaging the mica side, a piece of mica was attached with the carbon side to the steel disk. When the adhesive had set, the mica was peeled off to expose the surface of the carbon film that had been in contact with the mica during evaporation.

2.3. Electron diffraction

Specimens for electron crystallography were prepared as described in detail by Wang and Kühbrandt [6]. Electron diffraction patterns of specimens tilted by 60° were recorded in a JEOL 2000 EX electron microscope with a side-entry Gatan cryo-transfer specimen holder cooled with liquid nitrogen at a specimen temperature of about -130°C [6].

2.4. The atomic force microscope

The AFM used for this study is similar to the one described by Drake et al. [7]. The sample was placed on an \(xyz\) translator and scanned below a stylus which had been mounted on a cantilever. While scanning, surface features caused the stylus to deflect the cantilever. We used 100 μm long microfabricated Si₃N₄ cantilevers with a spring constant of 0.37 N/m (Park Scientific Instruments, 476 Ellis St., Mountain View, CA 94043). The force applied to the sample was typically 10 nN.

The deflection of the cantilever was measured by detecting the angular deflection of a laser beam reflected off the back of the cantilever [8–10]. Our AFM used a light beam from a laser diode. The reflection from the back of the cantilever was picked up by two segments of a photodiode and the difference in intensity measured by the two segments served as a deflection signal. This signal was used by a feedback loop to control the \(z\) motion of the single tube piezoelectric \(xyz\) translator (Digital Instruments, Cortona Drive, Santa Barbara, CA 93117). For all experiments the cantilever was kept at a constant deflection so that the force applied by the stylus to the sample was kept constant. The variable voltage applied to the \(z\) segment was used as the image contrast and hence a measure of the surface topography. An image was obtained by plotting the vertical motion of the \(xyz\) translator as a function of its lateral position. Scanning was activated by applying voltages to the \(xyz\) translator from Nanoscope II electronics (Digital Instruments). The scanning time for a single image ranged from 20 to 80 s for scan widths of 10 nm to 15 μm, respectively. All images were recorded in air.

To calibrate the height scale of the \(xyz\) translator, purple membranes were imaged. Purple membranes were prepared from Halobacterium halobium strain JW3 according to Oesterhelt and Stoeckenius [11]. The purple membrane suspension was sonicated for 20 s in a bath sonicator before each experiment. Purple membranes were
diluted in distilled water to a protein concentration of 4 μg/ml. 50 μl were applied to freshly cleaved mica and the samples were dried in vacuum.

2.5. Image processing

First the best-fit plane was subtracted from each digitized AFM image. For all images the roughness σ was calculated:

\[
\sigma = \left[ \frac{1}{n} \sum_{i=1}^{n} (z_i - Z_i)^2 \right]^{1/2},
\]

where \( z_i \) is the observed height at a certain point \( i \), \( Z_i \) is the best-fit base plane at the point \( i \), and \( n \) is the total number of data points (400 × 400) in one image. In addition, the vertical difference between the highest and lowest point in an image, the peak-to-valley distance \( P-V \), is given. When measuring the roughness or peak-to-valley distance, only areas without large-scale imperfections such as dust were used. The images were processed with software from Digital Instruments.

3. Results

Dried purple membranes were imaged to calibrate the \( xyz \) translator of the AFM in the \( z \) (height) direction (fig. 1). X-ray diffraction [12–14] and scanning tunneling microscopy [15,16] have shown that purple membranes are 4.9 nm thick. We used the edges of overlapping purple membranes to calibrate the \( z \) direction, as indicated by arrows in fig. 1, and not the edge of a purple membrane directly in contact with mica. In this way we avoided height corrugation which may result if materials with different elasticities are scanned. The \( xy \) direction of the translator was calibrated with images of the hexagonal surface lattice of mica (fig. 2) as a standard which has a lattice constant of 0.519 nm [17].

All samples were imaged with various scan sizes, ranging from 15 μm down to 10 nm. Fig. 3 shows AFM images of freshly cleaved mica. We measured a surface roughness \( \sigma \) of 0.03 nm (table 1) comparable to the value of 0.02 nm reported by Nakagiri and Kaizuka for graphite [18], measured by scanning tunneling microscopy. With an experimentally determined \( P-V \) value of 0.29 nm,
the ratio $P-V/\sigma$ was close to 10. This was considerably higher than the expected value which we estimated, as an example, for a cubic crystal described by

$$z(x, y) = A \sin(2\pi x/a) \sin(2\pi y/a),$$  \hspace{1cm} (2)$$

where $a$ is the lattice constant and $A$ is the corrugation amplitude of the crystal surface. The roughness of such a surface is $A/2$, the peak-to-valley value $2A$, yielding a ratio $P-V/\sigma$ of 4. The discrepancy between expected and measured ratio $P-V/\sigma$ was probably caused by adsorbants on the sample. Adsorbed material would affect the $P-V$ value more than the roughness.

Fig. 4 shows AFM images of carbon evaporated onto mica from a carbon rod in a single step (method 1). The top surface (fig. 4a) appeared rather rough, covered with randomly distributed peaks measuring approximately 5–8 nm diameter and up to 1 nm in height. The peak-to-valley distance was 2.9 nm (see table 1) and the roughness, calculated from eq. (1), was 0.44 nm. An AFM image of the mica side of a carbon film prepared by method 1 (fig. 4b) indicated that this side was much smoother than the top surface. At the same vertical scale as in fig. 4a, no peaks were visible. The average roughness was 0.07 nm, and the peak-to-valley distance was 0.52 nm. The variation of the surface roughness from one experiment to another was estimated from multiple experiments with specimen prepared in the same way to be about 30%. The variation of the peak-to-valley distance was also 30% as long as there were no large-scale imperfections such as dust on the surface. The presence of such visible imperfections increased the peak-to-valley parameter dramatically. A wider scan range of both surfaces of the same film is shown in figs. 4c and 4d. No large bumps were evident on the top surface (fig. 4c). The long-range curvature of this surface therefore appeared to be similar to that of the mica substrate which we estimated from AFM images to be at most 0.006°.

We satisfied ourselves in two ways that the surface we examined was actually carbon rather than mica. First, at a scan range of 8.4 nm, the AFM image of a carbon surface (fig. 5) looked entirely different from the hexagonal mica lattice shown in fig. 2. Second, a drop of water was placed on the sample after each experiment. Water spreads on the hydrophilic mica surface, whereas...
its contact angle with carbon is 50° to 75° [1]. The observed contact angle of approximately 50° to 60° was characteristic of carbon films freshly floated off mica [1]. Surfaces of films prepared by evaporating carbon fibre in an oil-free vacuum (method 2) appeared very similar to those shown in fig. 4. However, the roughness and the peak-to-valley distance of the top surface were both slightly higher than for films prepared from carbon rod, whereas the mica side of these films appeared to be somewhat smoother (table 1).

Films prepared by multiple evaporation of carbon rod onto mica (method 3) looked strikingly different from standard carbon films prepared by method 1 or 2. The top surface (figs. 6a and 6c) had a roughness of 0.13 nm and a peak-to-valley distance of 1.2 nm. Therefore it was smoother than the surface of films prepared by method 1 or

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**Table 1**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$\sigma_{\text{top}}$</th>
<th>$\sigma_{\text{mica}}$</th>
<th>$P-V_{\text{top}}$</th>
<th>$P-V_{\text{mica}}$</th>
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<td>0.07</td>
<td>2.9</td>
<td>0.52</td>
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<tr>
<td>Method 2</td>
<td>0.57</td>
<td>0.06</td>
<td>4.1</td>
<td>0.38</td>
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<tr>
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<td>0.04</td>
<td>1.2</td>
<td>0.30</td>
</tr>
<tr>
<td>Mica</td>
<td>-</td>
<td>0.03</td>
<td>-</td>
<td>0.29</td>
</tr>
</tbody>
</table>

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Fig. 3. AFM image of freshly cleaved mica. Image size: (a) 100 × 100 nm, (b) 2 × 2 μm.
Fig. 4. AFM image of a carbon film prepared by evaporating carbon rod onto mica in a single step (method 1). (a) Top surface; image size: 100 x 100 nm. (b) Mica side; image size: 100 x 100 nm. (c) Top surface; image size: 2 x 2 μm. (d) Mica side; image size: 2 x 2 μm.
2. The mica side (figs. 6b and 6d) was almost perfectly smooth; roughness and peak-to-valley distances of this side were almost the same as corresponding values of freshly cleaved mica. Figs. 6c and 6d show that these films were also planar over areas measuring several μm in diameter.

Fig 7a shows an electron diffraction pattern of a 2D crystal of LHC-II, supported on the mica...
Fig. 6. AFM image of a carbon film evaporated in several steps (method 3). (a) Top surface; image size: 100 × 100 nm. (b) Mica side; image size: 100 × 100 nm. (c) Top surface; image size: 2 × 2 μm. (d) Mica side; image size: 2 × 2 μm. Imperfections can be seen in (d).
side of the carbon film shown in fig. 6 and tilted by 60° in the electron microscope. Good patterns such as this one, with reflections that are almost uniformly sharp, were obtained routinely and reproducibly with films made by multiple evaporation but never with standard support films (fig. 7b), irrespective of treatment by glow discharge or heating, and of the age of the carbon film. At tilt angles above 45°, crystals supported on the mica side of carbon films made by methods 1 or 2 yielded diffraction patterns without any visible reflections beyond about 1.5 nm in the direction perpendicular to the tilt axis, even though the same specimens gave excellent results at 0° tilt.

4. Discussion

4.1. Surface roughness of carbon films

In their study of carbon evaporated onto graphite by scanning tunneling microscopy, Nakagiri and Kaizuka [18] observed features very similar to the ones we found on the top surface of carbon films evaporated onto mica. The two techniques of surface imaging therefore seemed to perform equally well on this type of material. However, unlike the scanning tunneling microscope which works only on conductive materials, the atomic force microscope does not rely on the conductivity of the sample. This enabled us to image not only the top surface of carbon films but also the surface which had been in contact with the mica.

Films produced by methods 1 and 2 were similar in terms of surface roughness. The technique of electron beam evaporation used by Nakagiri and Kaizuka [18] produced films that were somewhat rougher, with a distribution of grain sizes that gave rise to peaks in the Fourier spectrum at 5 and 8 nm. By contrast, there were no peaks in the Fourier spectra of any of our carbon film images, indicating a more or less even distribution of grain sizes.

Table 1 shows that the mica side of all films was very smooth compared to the surface facing the evaporation source. This was not surprising since the first carbon atoms or clusters reaching the mica at the beginning of the evaporation can probably diffuse on the mica surface and thus form a flat, continuous interface. A similar effect has been reported for metal atoms on graphite [19].

The top surface of carbon films prepared by multiple evaporation was significantly smoother than that of standard carbon films. To our surprise, this was also true of the mica side. Even though the differences were small, they were observed reproducibly with several specimens. We believe that the somewhat greater roughness of the mica side of standard films is due to the higher temper-
was turned down as soon as the tip became white hot and the pressure did not rise above $10^{-5}$ Torr. We believe that carbon clusters emitted under these conditions were comparatively small and that as a result, both surfaces of carbon films prepared in this way were smoother than those of standard films.

The process of multiple evaporation can be controlled easily with rod, but we were unable to use carbon fibre for this purpose because individual strands are thin and break within about one second of reaching the required temperature. Below this temperature evaporation was too slow and became impractical.

Carbon films prepared by multiple evaporation are, in fact, laminated, consisting of successive layers of carbon and hydrocarbon contaminants (J. Berriman, personal communication, and ref. [20]). It is possible that this laminated structure contributes to the greater smoothness and planarity of these films and provides extra mechanical strength.

4.2. Implications for electron crystallography

Carbon films prepared by multiple evaporation differed greatly from standard films with respect to high-resolution electron diffraction of 2D protein crystals of LHC-II. In our experiments, crystals deposited on the mica side of standard films never gave isotropically sharp reflections at tilt angles above $45^\circ$, whereas crystals deposited on the mica side of films prepared by multiple evaporation did so reproducibly. In both cases, diffraction patterns of untilted crystals were of equally high quality. We therefore concluded that the roughness of the supporting surface was critical for sharp diffraction patterns at high tilt angles. The observed difference in roughness on the mica side of all types of carbon films seemed too small to account for this effect. However, the surface exposed by dry stripping of the mica from a carbon film stuck with adhesive to a solid support is almost certainly smoother than that of a film floated off on water and deposited on an electron microscope grid. Our results led to the conclusion that the relief on the top surface of unsupported film is transmitted to some extent to

Fig. 7. Electron diffraction pattern of a 2D crystal of LHC-II deposited on the mica side of a carbon film prepared by method 3 (a) and method 1 (b). The specimen was tilted by $60^\circ$ in the electron microscope.
the mica side, presumably by surface tension which would tend to equalize the roughness of both sides. Unfortunately, vibrations of unsupported films made it impossible to measure the roughness of carbon films on grids for electron microscopy directly by atomic force microscopy.

The long-range curvature of carbon support films is an equally important aspect for recording sharp electron diffraction patterns of highly tilted 2D crystals. Since the films examined with the AFM were all firmly attached to solid supports, their curvature was minimal. A much higher curvature may result when carbon films are mounted on specimen support grids. This is confirmed by the observation that, at best, about 30% of the diffraction patterns of LHC-II crystals recorded at 60° tilt angle showed sharp spots in all directions, even with films that looked perfectly smooth in the AFM. The other patterns had blurred spots in the direction perpendicular to the tilt axis, indicating local deviations from planarity.

2D crystals of bacteriorhodopsin [2] and PhoE porin [21] may be less sensitive to the roughness of the support film for two reasons. First, the average intensity of diffraction spots is higher by a factor of about 7 for bacteriorhodopsin and about 3 for PhoE porin compared to LHC-II [2,6,21]. Second, the protein packing density of 2D crystals of LHC-II is roughly two times lower, making them more susceptible to distortion in the z direction.

For electron diffraction with purple membranes the long-range curvature of the specimen needed to be flat to within about 1° [2]. At these conditions, the diameter of diffraction spots at 0.3 nm resolution and 60° tilt angle was about one half of the distance between the spots. Due to the large unit cell of 2D crystals of LHC-II, diffraction spots are spaced twice as closely. This means that LHC-II crystals need to be at least twice as flat (to within 0.5°) to achieve the same separation. The low signal-to-noise ratio of most high resolution spots of LHC-II makes the requirement for flat support films even more stringent. From the slight blurring of diffraction spots far from the tilt axis in fig. 7a we estimate that our best films were flat to within 0.2°. The maximum lack of flatness is probably 0.3°. For 2D crystals of PhoE porin which also have a large unit cell, flatness seems to be less critical, since diffraction patterns can be recorded with a higher dose [21] and, hence, from a smaller area.

The chemical properties of the supporting surface which are an important factor in specimen preparation with purple membrane [1,3] seem to play only a minor role for 2D crystals of LHC-II. Pretreatment of the carbon films by glow-discharging, ageing, or baking had no detectable effect on specimen flatness.

From the blurring of diffraction spots far from the tilt axis it is clear that, with one exception, most published electron diffraction patterns of highly tilted purple membranes [1,2,22,23] have been obtained with specimens that were distinctly less flat than the ones described here. Although flat specimens of PhoE porin [21] and purple membrane [23] have been obtained in other ways, we hope that the use of smooth carbon films prepared by multiple evaporation will help to prepare flat specimens routinely.

5. Conclusion

(1) The surface roughness of carbon films depends on the method of carbon evaporation. The top surface of films prepared by standard, single step evaporation had a roughness of about 0.5 nm whereas the roughness of films prepared by multiple evaporation was 0.13 nm. These measurements were obtained by atomic force microscopy which is an appropriate method for studying the roughness of surfaces at an atomic scale.

(2) The mica side of all carbon films we investigated was much smoother than the top surface. The mica side of carbon films made by multiple evaporation was almost as smooth as mica, having a roughness of 0.04 nm.

(3) Isotropically sharp electron diffraction patterns of highly tilted 2D crystals of LHC-II could be recorded only with carbon films exhibiting a minimal surface roughness on both sides. Standard films could not be used for this purpose, even when the crystals were deposited on the mica side.
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