Graphite: A Mimic for DNA and Other Biomolecules in Scanning Tunneling Microscope Studies

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Highly ordered pyrolytic graphite (HOPG) is the substrate often used in scanning tunneling microscope (STM) studies of biomolecules such as DNA. All of the images presented in this article are of freshly cleaved HOPG surfaces upon which no deposition has occurred. These images illustrate features previously thought to be due to biological molecules, such as periodicity and meandering of "molecules" over steps. These features can no longer be used to distinguish real molecules from features of the native substrate. The feasibility of the continued use of HOPG as a substrate for biological STM studies is discussed.

SINCE BINNIG AND RÖHRER FIRST REPORTED THE ABILITY OF the scanning tunneling microscope (STM) (*1*) to image DNA molecules by depositing them onto a conducting substrate (*2*), researchers have continued to investigate and improve the biological applications of the technique. With the exception of a few researchers (*3, 4*), most of the initial results in this field were obtained with highly ordered pyrolytic graphite (HOPG) as the conducting substrate. For example, several researchers have presented images illustrating the capability of the STM to resolve the major and minor grooves of DNA with a wide range of periodicities (*5–8*). The possibility of using the STM to distinguish between the purine and pyrimidine bases of DNA has been illustrated by studies of poly(dA) molecules deposited onto HOPG and imaged in air (*9*). Recently, DNA deposited onto HOPG and imaged in vacuum revealed near-atomic detail with close correspondence to molecular models (*10*).

Most likely because of the initial successes with HOPG, the field of biological STM studies has experienced a dramatic increase in the number of researchers presenting results of their STM studies made with HOPG as a substrate both in air and liquid (*11, 12*). However, only a few of these groups (*13*) addressed the problem of HOPG surface features interfering with their ability to distinguish biological features from HOPG features. Other researchers, who have previously presented results obtained on HOPG or who have investigated the surface and found it unfeasible for this type of work, have discussed this problem and their growing concern that all researchers need to be made aware of the surface features associated with HOPG (*14–16*).

We have completed an in-depth study of DNA molecules deposited electrophoretically onto HOPG and imaged in situ with the STM (*17*). The images we present in this article are taken from several hundred images of HOPG blanks that we completed concurrently with our in situ DNA studies. We present images illustrating regular periodicity from features that appear to meander across the surface. We examine HOPG steps and the confusion that they can bring to an image containing biological deposits.

A significant percentage of the previous work in this field has appeared in this and other highly visible, broadly viewed journals. It is not our intent to disprove or discount these previous results, but rather to enlighten the broader scientific community. A knowledge of the controversial interpretation of HOPG surface features will allow the broader scientific community to view past and present results (including results from our own lab) with a more critical eye.

The STM used in this study consisted of a coaxial double-tube piezo design built in our labs. The design has been described previously in detail (*18*). *All* of the images that we present in this paper are freshly cleaved HOPG surfaces (*19*) involving no deposition of any kind. The tips were made of a Pt/Rh (10% Rh) alloy wire (0.051-cm diameter) and were prepared by two methods: ac etching (*20*) or mechanical cutting with scissors. All of the images are raw unprocessed data.

The most common HOPG surface features we have found in our studies are steps. In most cases, the steps are featureless and straight for many thousands of angstroms. We have found steps that make sharp 60° and 120° angles with one another, with the confirmed HOPG geometry. Difficulty arises in using cross sections to distinguish between an HOPG step or adsorbed molecule, as Fig. 1 illustrates. Both of these images represent HOPG features, as no other molecules have been deposited onto the surface. Figure 1A is consistent with our conventional notion of an HOPG step, as evidenced by both the topographical image and the cross-section data (Fig. 1C). However, in Fig. 1B, it would be impossible to assign this feature based on the symmetrical cross section of the topographic data (Fig. 1C) if biological molecules had been deposited onto the surface.

On occasion we have discovered regions of HOPG that have steps traversing in all directions with angles other than the expected 60° and 120°, as Fig. 2 demonstrates. Perhaps the most confusing aspect of HOPG steps and other surface features is that they quite often exhibit periodicity along the step edge. This could have been confused in the past with DNA pitch or packing of oligonucleotides, since a range of periodicities can be seen along a step edge, depending on the angle the step makes with the major symmetry

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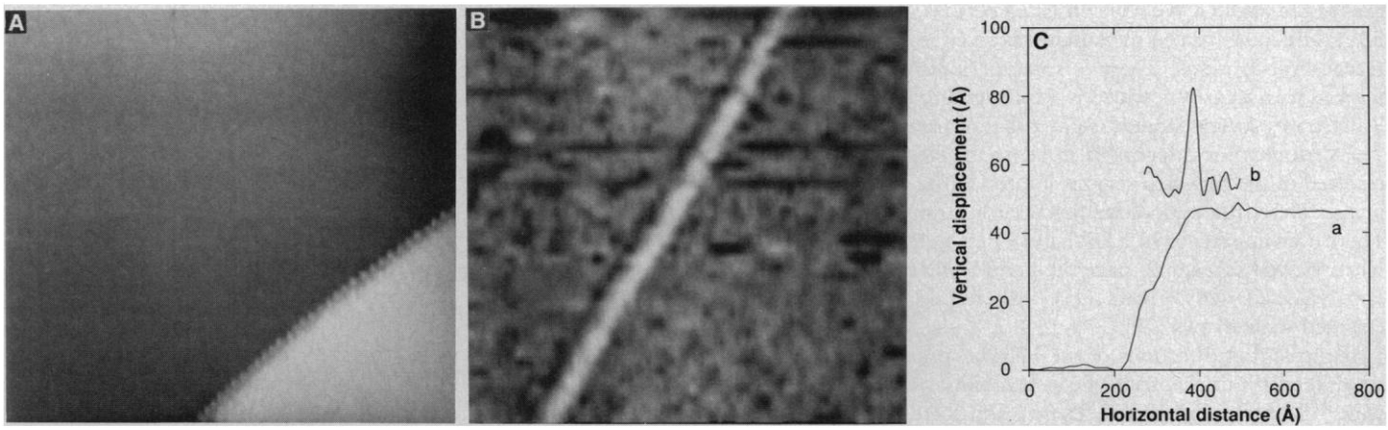

Fig. 1. This series of topographic images and cross sections illustrate the difficulty experienced with distinguishing HOPG features from deposited molecules. (**A**) This image measures 2965 Å by 2956 Å by 73 Å (x, y, z). The sample bias was +0.298 V, and the gap resistance was 295 MΩ. (**B**) This image measures 698 Å by 698 Å by 63 Å (x, y, z) and was collected with a sample bias of −0.200 V and a gap resistance of 132.5 MΩ. The feature has an apparent width of 36 ± 3 Å, full-width at half-maximum. (**C**) This plot illustrates the noticeable difference between the cross section corresponding to what is more obviously an HOPG step (a) and to what is some other HOPG feature (b). The topographic view (A) and corresponding cross section of the first HOPG feature (a) are easily identified as an HOPG step. However, the topographic view (B) and cross section of the second HOPG feature (b) demonstrate the problem that would be created if one were also looking for biological molecules on the HOPG surface, since the cross section of the feature is symmetrical.

Fig. 2. This image illustrates a region of HOPG in which HOPG steps are traversing the surface in all directions instead of the straight, 120° HOPG geometry nominally represented by steps. Notice the periodicity marked with an arrow. It measures 36 ± 6 Å, which could easily be confused with DNA pitch. This image measures 2002 Å by 2005 Å by 138 Å (x, y, z). The sample bias was +0.477 and the gap resistance was 795 MΩ.

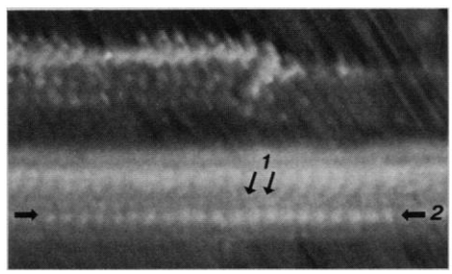

Fig. 3. This image demonstrates the periodicity that we have found in many of our examinations of steps. Although straight and parallel as expected, the periodicity along a step presents a problem when actually trying to determine whether these features are due to HOPG or biological molecules. The periodicity measures 18 ± 1 Å. This image measures 537 Å by 315 Å by 21 Å (x, y, z), and was collected with a sample bias of −0.099 V and a gap resistance of 28.9 MΩ. There even appears to be a left-handedness to the periodic structure when viewed as a spiral, since the periodic features are aligned at a slant [indicated with arrows marked (1)] with respect to the long chain [indicated with arrows marked (2)].

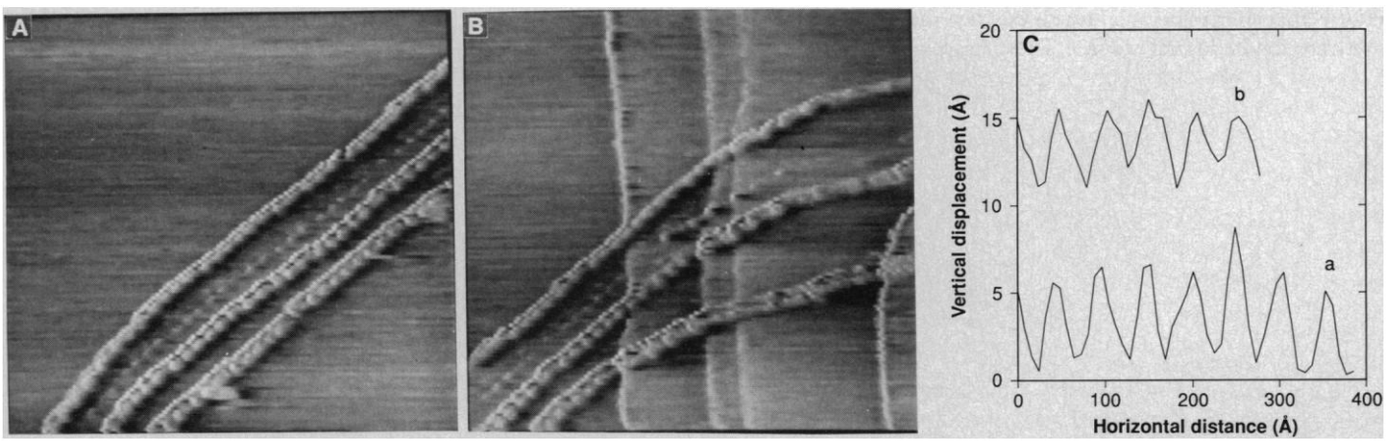

Fig. 4. This series of images and cross sections illustrate HOPG features that closely resemble biological molecules even more so than the preceding image illustrating periodicity. Here, the surface features not only appear to possess periodicity but also to meander across other HOPG steps. The average periodicity was determined to be 53 ± 12 Å (obtained from 48 measurements from 15 images). Both images were obtained with a sample bias of −0.213 V and a gap resistance of 219.6 MΩ. (**A**) This image measures 1500 Å by 1500 Å by 47 Å (x, y, z). (**B**) This image measures 1500 Å by 1500 Å by 46 Å (x, y, z). (**C**) This diagram displays the periodicity of these HOPG features. The average periodicity in (A) is 53 ± 10 Å and is illustrated in the cross section labeled a. The average periodicity in (B) is 51 ± 6 Å and is illustrated in the cross section labeled b.

axes of the HOPG. We have observed periods in the range of 18 to 53 Å (obtained from 61 measurements taken from 20 images) in our recent investigations. There is some periodicity visible in Fig. 2, marked with an arrow, which was measured to be 36 ± 6 Å. Figure 3 illustrates several straight steps running parallel to one another. The periodicity is discernable in all of the steps but appears most resolved in the two-step regions located in the center region of the image. Here, the periodicity has been measured to be 18 ± 1 Å. There even appears to be a left-handedness to the periodic structure when viewed as a spiral, since the periodic features are aligned at a slant [labeled with arrows (1)] with respect to the long chain [marked with arrows (2)].

We have found features of the HOPG surface that have further strengthened our skeptism of the feasibility of this surface for use with STM biological studies. These features are presented in Fig. 4. They demonstrate not only periodicity but also meander across HOPG steps and appear to have a symmetric cross section, suggestive of a molecule that has been deposited onto the surface. The periodicity is 53 ± 12 Å (obtained from 48 measurements taken from 15 images). Following these features to their ends in either direction showed that they were representative of a disrupted area of the surface and appeared to be associated with flaking of the surface.

Other than HOPG steps, we have found that the surface can exhibit HOPG flakes, typically located in areas of surface disruption. These features are nonuniform in size and have been found in a multitude of shapes, some of which may resemble known or proposed structures of proteins and other biological molecules. When working with HOPG, it is therefore important to recognize that a surface feature may closely resemble the anticipated shape of a deposited biological molecule. Unfortunately, this approach of hunting for the expected structure has been used in the past when assigning image features to biological structures.

In addition to HOPG surface features complicating biological STM studies, large-range periodicity in the form of large hexagonal arrays has been reported on clean HOPG (*21, 22*). These superperiodicities have been interpreted as moiré patterns caused by slight rotations of the top HOPG layer with respect to its underlayers. Thus discretion should be used when interpreting images of similar appearance as two-dimensional arrays of protein molecules that have been deposited onto HOPG.

In summary, we have presented various STM images from our study of HOPG. These images have demonstrated what may actually be only a small subset of the surface features associated with HOPG that contribute to ambiguities when this surface is used as a substrate for biological studies. These have included problems of distinguishing molecules from HOPG steps, periodicity, flakes, moiré patterns, and HOPG features that meander across the surface. Based on these results, we strongly suggest that investigators use another substrate for biological STM studies.

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