Point projection imaging of unstained ferritin molecules on tungsten*

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SUMMARY

Point-projection microscopy has been used to obtain the first images of closely spaced (but isolated) unstained ferritin molecules on a tungsten substrate. The microscopy, in conjunction with a digital processing algorithm, produces images which display a striking three-dimensional quality usually associated with scanning electron micrographs. From these images, an apoferritin diameter of 13 nm has been deduced. This is identical to the diameter of apoferritin obtained from a reconstruction of the molecule using X-ray coordinate data, but almost 70%, larger than that deduced from TEM images of ferritin in which only its iron rich core can be seen.

INTRODUCTION

The morphology of organic species interacting with a metal surface is of considerable interest. The nature of corrosion resistant coatings, polymer adhesion, and fundamental properties of metal–molecule interactions could all be studied in much greater detail if organic molecules could be directly observed on a metallic substrate. The electron microscope can observe unstained organic molecules deposited on very thin carbon or dielectric substrates (Ottensmeyer, 1982). Unfortunately, image contrast depends upon the ability of a molecule to scatter electrons more effectively than the substrate on which it is placed. This means that metallic substrates cannot be used. Although cryogenic cooling can reduce the problem of radiation damage by the probing electron beam, volatilization of organic species during imaging remains a serious problem (Klug, 1978).

In order to image organic species deposited on a metallic surface and eliminate the radiation damage problem, a new point-projection imaging technique is being developed at Sandia National Laboratories. It has been used to obtain the first images of submonolayer coverages of ferritin on a tungsten surface (Panitz, 1982a). The imaging procedure can be repeated without destroying the ferritin layer provided a critical electric field strength is not exceeded (Panitz, 1982b). In this paper we extend these observations to include closely spaced, but isolated, molecules of ferritin. We also describe a simple digital algorithm which can process point-projection images so that they display a three-dimensional quality usually associated with conventional scanning electron micrographs.

THE FERRITIN MOLECULE

Ferritin is an organic macromolecule which is found in the liver and spleen of a number of higher organisms (including man). It is an essential part of haemoglobin-based respiration in mammals. The most thoroughly studied ferritin (from horse spleen) consists of twenty-four identical protein subunits, or monomers, as shown in Fig. 1 (Banyard et al., 1978). The mono-

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Fig. 1. A computer generated, space filling model of the apoferritin monomer. The monomer was reconstructed by Richard Feldman of the National Institutes of Health in Bethesda, Maryland, from X-ray coordinate data supplied by Pauline Harrison of Brookhaven National Laboratories.

Fig. 2. A computer generated, space filling of ferritin. The molecule is viewed along a four-fold axis of symmetry where a 1 nm wide channel provides access to a large, interior cavity. The cavity acts as a storage reservoir for iron where it is found as a complex ferric hydroxyphosphate polymer. Ferritin is assembled from twenty-four identical apoferritin monomers (one of which is highlighted). The ferritin molecule was reconstructed by Richard Feldman, the National Institutes of Health, Bethesda, Maryland.

Fig. 3. A TEM image of three ferritin molecules deposited on a thin carbon substrate. Only the iron rich core of each molecule is easily seen. Figure courtesy of S. Iijima, the National Center for High Resolution Electron Microscopy, Arizona State University, Tempe, Arizona.

Fig. 4. A TEM image of five ferritin molecules deposited on the same substrate, and near to the triplet shown in Fig. 3. The expected spherical shape of the core is distorted in this image. Figure courtesy of S. Iijima, the National Center for High Resolution Electron Microscopy, Arizona State University, Tempe, Arizona.

The apoferritin monomers are arranged in the form of a nearly spherical, hollow protein shell known as apoferritin. Apoferritin is approximately 3 nm thick and has a molecular weight of about 460,000 Daltons. Six channels, about 1 nm in diameter, lead into the interior of the molecule. One such channel appears in the graphical reconstruction of apoferritin shown in Fig. 2. The diameter of apoferritin as shown in Fig. 2 is approximately 13 nm. The large interior cavity of apoferritin acts as a storage reservoir for iron. It can contain almost five thousand iron atoms concentrated in a microcrystalline array as a complex ferric hydroxyphosphate polymer (Chrichton, 1971). Apoferritin which contains iron is called ferritin, a molecule which can have a molecular weight as high as 960,000 Daltons.

If ferritin is deposited onto a sharply curved metal surface which is then imaged in profile in the transmission electron microscope, a distribution of iron-rich ferritin cores can be directly observed (Panitz & Giaever, 1981). However, such images do not clearly show the outer protein
shell of each ferritin molecule. If ferritin is placed on a thin carbon substrate and imaged in the TEM, a similar problem is encountered (Iijima, 1977). For example, Fig. 3 shows a TEM image of unstained, horse spleen ferritin deposited on a very thin, homogeneous carbon substrate. The iron rich core of each imaged molecule is visible as a nearly circular, opaque spot. However, the apoferritin shell of each molecule is essentially invisible. By measuring the average, centre-to-centre distance of each core, ferritin’s diameter can be deduced. From the TEM micrograph of Fig. 3, a value of approximately 9 nm is obtained. This is roughly 70% of the diameter obtained using X-ray crystallographic data to reconstruct the molecule (Fig. 2). It is interesting to note that not all TEM images of ferritin display the expected core symmetry shown in Fig. 3. One example is shown in Fig. 4. Here, a cluster of five ferritin molecules has been selected for imaging from another region of the substrate shown in Fig. 3.

**Point-projection imaging**

In order to image the ferritin molecule by point-projection microscopy, ferritin is deposited onto the spherically curved apex of a sharply pointed wire, known as a field emitter tip (Panitz & Giaever, 1981). After placing the tip in an ultra-high vacuum environment, it is cooled to cryogenic temperatures in order to maximize image resolution (Panitz, 1982a). The imaging procedure involves embedding each deposited molecule within an immobile multilayer of benzene which is condensed onto the cold tip surface. By controlled field-desorption (Müller & Tsong, 1969) the benzene layer can be slowly removed, and the resulting benzene ion image recorded at a suitable detector placed several centimetres from the tip surface. The detector image is a highly magnified map of benzene ionization probability at the surface of the condensed benzene layer.

Provided the benzene layer covers the deposited ferritin molecules during the desorption event (and provided the ionization probability of the layer is relatively high and isotropic) the detector image will appear bright and relatively uniform. However, as soon as enough of the layer is removed to expose an embedded molecule, a dark region will appear in the image. As the field-desorption process proceeds, more of the benzene layer will be removed, and successive molecular contours (defined by the intersection of the molecule with the surface of the remaining benzene layer) will be revealed in the detector image.

Figure 5 shows a typical detector image integrated over a 400 V change in tip potential. The integration was started just as the ferritin molecules were exposed at the surface of the receding benzene layer. We can obtain a rough estimate of the maximum number of molecular contours in this image if we assume that the condensed benzene layer is crystalline. A previous study (Lehwald et al., 1978) indicates that the benzene molecule will lie flat (with its plane parallel to the substrate) if benzene is chemisorbed onto macroscopic platinum or nickel surfaces at temperatures below 150 K. If condensed benzene multilayers behave similarly on our tungsten tip surface and if (on the average) the desorption process removes only one layer of benzene at a time, each contour will be equal to the thickness of a benzene molecule, or 0.34 nm. Since we completely uncover a ferritin molecule during the time a detector image is recorded, the total thickness of benzene removed will be equal to the diameter, *d*, of the ferritin molecule. As a result, the maximum number of contours contained within the integrated image shown in Fig. 5 is just \( N = d/0.34 \). From the X-ray reconstruction of Fig. 2, *d* = 13 nm, so that \( N = 28 \). From the TEM micrograph of Fig. 3, *d* = 9 nm or \( N = 26 \). The important point is that each integrated image contains a reasonably large number of molecular contours. These appear in the image as a variation in contrast (or image spot density) across the edge of each dark image feature. In principle, this contrast variation contains all of the three-dimensional information about the molecule’s morphology inherent in the microscopy. The problem is to display this information so that the three-dimensional detail can be easily recognized.

**Image reconstruction**

The interpretation of any image is made easier if sensory clues are present. Perhaps the most important of these is an impression of depth. It is known that the three-dimensional appearance
of an object can be enhanced if the object appears as though it were illuminated by an oblique beam of light from above. Our visual experience tells us that under such illumination the upper half of a protrusion will be highlighted while its lower half will remain in shadow. Since molecules which appear in our integrated images protrude from the tip surface, adding highlights to the upper half of each image feature will enhance the shape of a molecule and accentuate the three-dimensional detail inherent in the image.

The digital procedure which has been developed for adding highlights and shadows to an integrated image (Ghiglia of Flickner, 1982) is a variation of a standard processing technique known as 'unsharp masking' (Pratt, 1978). We begin by digitizing an integrated image, and then smoothing a copy of the image by averaging the pixels within a \(5 \times 5\) pixel window. We have found that the final image is not appreciably affected by the size of the averaging window. If the resulting image is shifted with respect to the original image by several video lines, and subtracted from it, highlights and shadows will be added in the direction of the shift. We have found that the size of the shift is not particularly important (we usually shift the image six video lines vertically downward to highlight the top of each image feature). As the shift is increased, the most noticeable effect on the image is a slight loss in resolution, and an increase in image contrast (which can be mimicked for smaller shifts by contrast stretching the image). Figures 6 and 7 show the result of processing the image of Fig. 5 using two different shifts: six video lines and twelve video lines, respectively. Image contrast has been adjusted so that the two shifted images display the same contrast range.

The theoretical resolution of the original image shown in Fig. 5 is estimated to be \(\approx 1.5\) nm, using an effective tip radius of 240 nm and a tip temperature of 20 K (Panitz, 1982a). Since detail much smaller than the resolution of the image is not physically meaningful, it is convenient to smooth the processed image such that smaller detail is strongly attenuated. The result is shown in Fig. 8. The features which appear roughly spherical are identified with the apoferritin shell of individual ferritin molecules. The identification is based on the size and shape of the features and the fact that smaller spherical features have never been observed.

The average diameter of the spherical features seen in Fig. 8 is approximately 13 nm. This is identical to the diameter of apoferritin deduced from the X-ray reconstruction of the molecule shown in Fig. 2. Since both point-projection microscopy and TEM images are obtained in a vacuum environment, the effect of specimen denaturing on the size and shape of a ferritin molecule should be roughly equivalent in both techniques. This means that if our measurement of ferritin's diameter is correct, the smaller diameter deduced from TEM images may reflect the effect of radiation damage induced by the probing electron beam. With the electron dose required for high resolution imaging at room temperature, most biological molecules can lose 20–80% of their mass (Dubochet & Knapke, 1978), presumably by volatilization in the probing beam. If the resulting molecular residues in a cluster coalesce and move closer together, the smaller apparent diameter of ferritin obtained from Fig. 3 would be explained. Radiation damage could also help to explain the distorted shape of the ferritin core seen in Fig. 4.

It is important to emphasize that our measurement of ferritin's diameter is based on the calculated magnification of a point-projection microscope: \(M = D/\beta R\) (Panitz, 1982a). The tip-to-detector distance, \(D\), and the tip radius, \(R\), can both be measured to within a few per cent. However, the image compression factor, \(\beta\), is not accurately known. For most normal tip geometries \(1.5 \leq \beta \leq 1.8\) (Müller & Tsong, 1969). This means that a magnification of our images can be uncertain by 20%. As a result, a more convincing argument for radiation damage in TEM images of ferritin can only be made if we can find a more accurate way to calibrate the magnification of our microscope.

Conclusions

A new point-projection microscope has been used to image the morphology of closely spaced, but isolated ferritin molecules. In conjunction with a digital processing algorithm, it has produced the first images of individual, unstained ferritin molecules deposited on a metal
Fig. 5. A linear addition of four contour slice images taken during point-projection imaging of a ferritin coated field-emitter tip. Dark regions indicate the absence of benzene or the presence of a molecular feature. Contrast variations which define the edge of each dark region reflect the fact that the integrated image contains many molecular contours which increase in size during the imaging event. \( T = 30 \, \text{K}, \text{Tip radius} = 240 \, \text{nm} \).

Fig. 6. A digitally processed micrograph obtained from the image shown in Fig. 5. Highlights and shadows result from a six video line shift (vertically downward) which is part of the processing procedure discussed in the text.

Fig. 7. The same image as shown in Fig. 6, but incorporating a twelve video line shift during processing. The contrast of the processed image was adjusted to match that of Fig. 6.

Fig. 8. The processed image of Fig. 6 after smoothing by averaging the pixels within a \( 15 \times 15 \) pixel window. The window size was chosen to correspond to a linear dimension of \( \approx 1.5 \, \text{nm} \) in the image. This is equal to the theoretical resolution of the original image shown in Fig. 5.

surface. From these images an apoferritin diameter of 13 nm has been deduced. This is identical to the diameter of apoferritin obtained from a reconstruction of the molecule using X-ray coordinate data, but almost 70% larger than that deduced from TEM images in which only ferritin’s core can be seen. It is suggested that radiation damage caused by the probing electron beam could account for the smaller apoferritin diameter deduced from TEM images.

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REFERENCES