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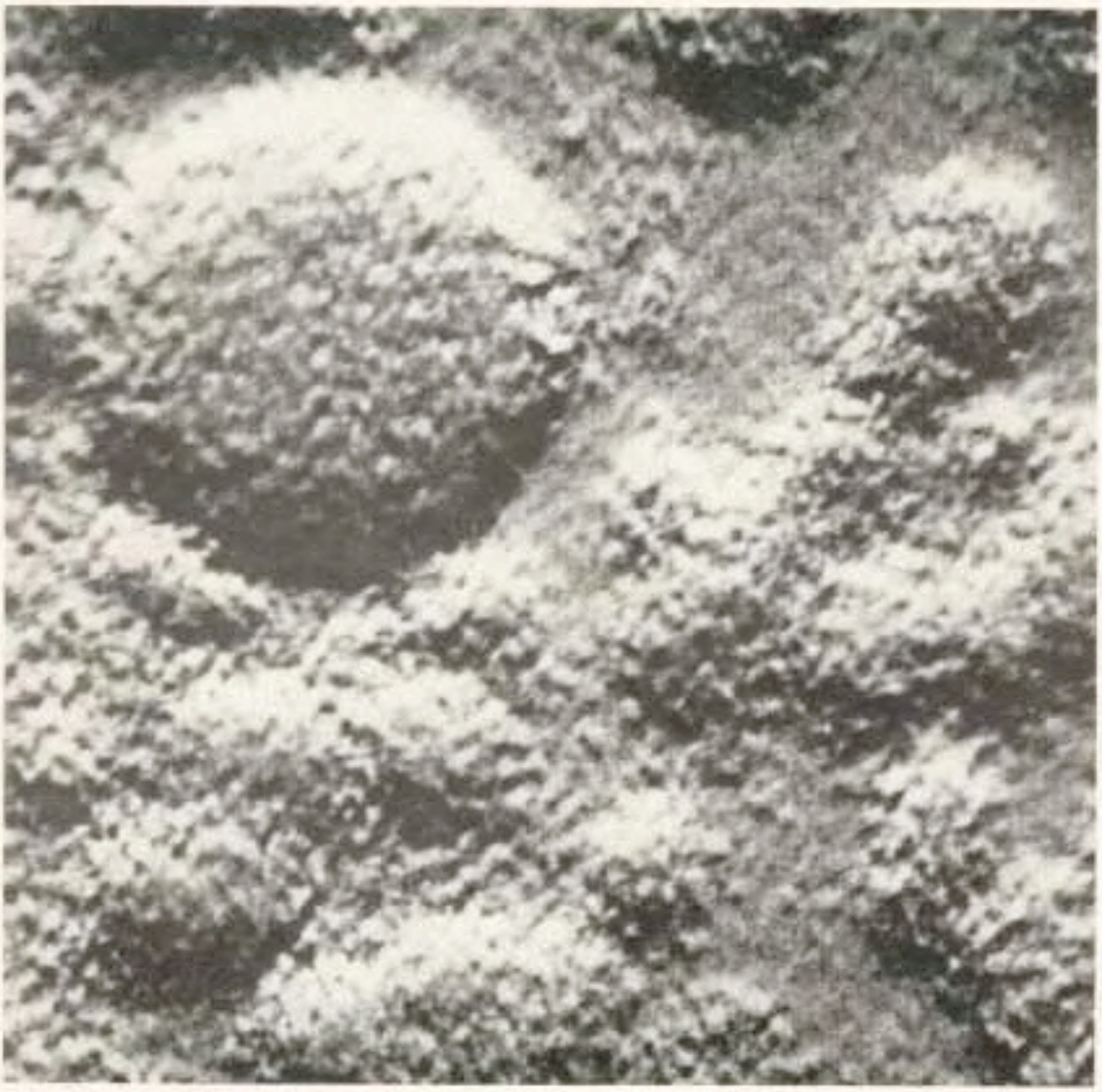

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POINT-PROJECTION IMAGING OF UNSTAINED FERRITIN CLUSTERS

J.A. PANITZ

*Sandia National Laboratories *, Albuquerque, New Mexico 87185, USA*

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A point-projection microscope is described which has been used to obtain images of isolated, unstained ferritin clusters on a tungsten substrate. The microscope images the contour of the collection of protein shells associated with each cluster, rather than the iron core of each ferritin molecule accessible to TEM imaging. Quasi, three-dimensional images of cluster morphology are obtained by controlled field-desorption of an immobile, condensed benzene multilayer which initially surrounds and covers each cluster. The imaging process appears to be nondestructive provided a critical electric field strength, F_c, is not exceeded. For large, weakly bound ferritin clusters $F_c \approx 4$ V/nm, a value well above that required for imaging.

For more than thirty years, attempts have been made to image molecular species in the field-electron emission microscope (FEEM), and the field-ion microscope (FIM). These attempts [1–6] were inspired by the simplicity of the techniques, and their potential for achieving high image contrast, magnification, and resolution. Since the FEEM and FIM are point-projection microscopes, they do not require lenses and are essentially immune to specimen vibration [7]. As a result, both techniques offer the hope of achieving high quality molecular images with a minimum of effort.

Unfortunately, attempts to use the FEEM and FIM to image molecular contours have not been particularly successful [8,9]. Images did not usually reflect the known morphology of the imaged species; most images were transient, and few were reproducible. Although there is no satisfactory explanation for all of the imaging difficulties which were encountered, two particular problems seem to emerge from the literature: (1) no method *independent of imaging* was used to determine if molecules had been placed on the specimen surface, and (2) there was no assurance that the electric field required for imaging would not distort or destroy a molecule's conformation.

In two previous publications [10,11], we addressed the first of these problems and demonstrated that the transmission electron microscope (TEM) could be used as an aid in determining criteria for effective molecular deposition on field-emitter tips. Recently, we described a new point-projection imaging technique for unstained macromolecules which appeared to be nondestructive [12]. In this communication, we wish to provide additional information to support this position.

In our point-projection imaging technique, molecules of characteristic dimension, S_0, are deposited onto a field emitter tip by deposition from aqueous solution [11]. The apex radius of the tip, R, is chosen such that $R/S_0 \gg 1$. After placing the tip in an ultra-high vacuum environment ($\approx 10^{-9}$ Torr) it is cooled to a temperature below 30 K. An infrared laser pulse briefly raises the tip temperature to ≈ 300 K in order to thermally desorb any contaminant species which have adsorbed on the cold tip surface. A precise quantity of benzene is then condensed onto the tip apex from the gas phase. The benzene acts as an immobile "blanket", completely covering and surrounding each deposited molecule or molecular cluster. If a potential, V_0, is applied to the tip, an electric field will be established at the surface of the condensed benzene layer. As V_0 is increased, the benzene layer will begin to field-desorb [13], gradually exposing the embedded species. This stage in the

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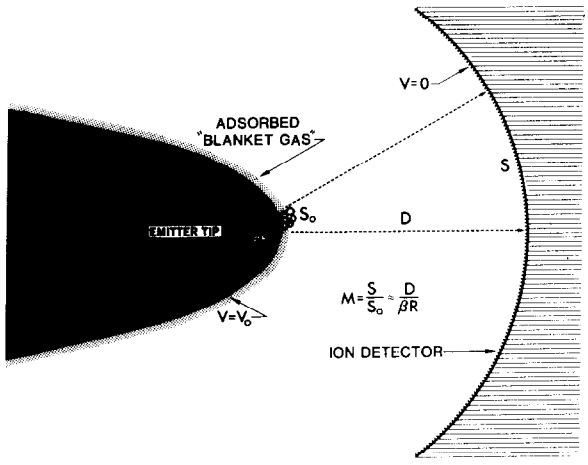

Fig. 1. Point-projection imaging of molecular contours. Molecules of characteristic dimension S_0 are deposited from aqueous solution onto a field emitter tip of apex radius R. As the tip potential (V_0) is increased, the resulting electric field gradually removes an immobile layer of benzene which originally surrounded and covered the molecules. Benzene ions formed during the field-desorption process are accelerated to a sensitive ion detector where they form a highly magnified image of their relative positions within the layer. As a molecule is exposed, a dark region appears in an almost contrastless, benzene background. By increasing V_0, more of the molecule's contour (closer to the tip surface) is revealed in the image.

imaging process is shown schematically in fig. 1.

Benzene ions produced by the field-desorption process will be accelerated along electric field lines directed outward from the tip apex. If the ion trajectories far from the tip are extrapolated back toward its apex, they will converge at a focus inside the tip, a distance βR from its surface; β represents an "image compression factor" which accounts for any deviation from pure radial projection ($\beta = 1$) which would occur if the tip apex was a sphere of radius R isolated in space. If the desorbed benzene ions are intercepted by a suitable detector placed several centimeters from the tip apex, they will produce a highly magnified image of their relative positions in the condensed benzene layer prior to desorption. Typically, $R \approx$ 150 nm and $\beta = 1.5$, so that tip-to-detector distances of only a few centimeters will produce magnifications greater than 10^5.

Provided the benzene layer covers the adsorbed molecules during the desorption event, and the ionization probability is relatively high and isotropic, the detector image will appear bright and relatively contrastless. However, as soon as enough of the layer is removed to expose a molecule or a molecular cluster, a dark region will appear in the image. If the electric field in the vicinity of the exposed molecule is undistorted, the contour of the dark region will accurately reflect the contour of the molecule at some well defined elevation above the tip surface. As the field-desorption process proceeds, more of the molecule's contour (lying closer to the tip surface) will be revealed in the detector image. By consecutively recording the detector image during the desorption process, a series of images can be obtained which will reflect the three-dimensional morphology of the deposited species.

The imaging process described in the last paragraph appears to explain the main features of the ferritin images which we have observed. Ferritin was chosen to demonstrate the principle of the microscopy because its coverage on a field-emitter tip can be observed in the TEM before and after point-projection imaging. Such observations have suggested that the imaging process is nondestructive. Further evidence for the nondestructive nature of the process is provided by the reproducibility of the images. Identical images can be obtained provided a tip is not mechanically damaged or exposed to organic contamination. It does not even seem to matter if a tip is stored in vacuum or laboratory ambient; the resulting images are apparently the same.

Reproducible images can only be obtained if the deposited molecules are unaffected by the elecric field strength required for imaging. Since an early study [14] suggested that relatively low field strengths could cause desorption of molecules from the tip apex, it is important to determine the effect of the apex field on adsorbed ferritin. We began a preliminary investigation of this problem in 1979 [15]. At that time we could only examine the gross effect of the average electric field by examining ferritin covered tips in profile in the TEM. Since it is now possible to obtain point-projection images of ferritin with a resolution of $\approx$ 2–3 nm [12], we can examine the effect of the local electric field over distances on the surface which are compara-

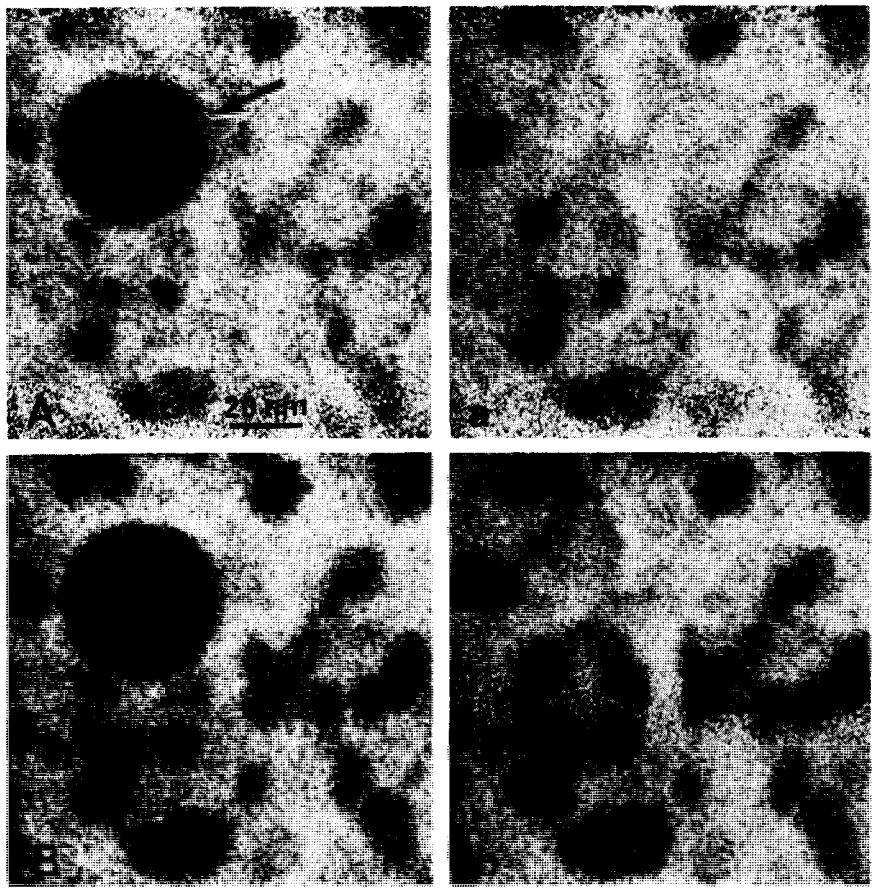

Fig. 2. Consecutive point-projection images of ferritin on tungsten at 30 K: Before applying a critical field strength (A–B), and after applying a critical field strength (a–b). Each image is the integrated sum of three desorption sequences as follows: (1) laser pulse to 300 K to thermally desorb contaminants; (2) dose with benzene at 30 K to form a condensed layer >12 nm thick; (3) linearly ramp the tip bias from 0–3000 V; (4) image over a 100 V increment of the ramp as follows: (A) 3500–3600 V, (B) 3600–3700 V, (a) 3400–3500 V, (b) 3500–3600 V. The arrow points to a large molecular cluster extending many tens of nanometers from the surface.

ble to the diameter of a ferritin molecule.

Figs. 2 and 3 compare two point-projection image sequences of ferritin taken before and after a critical field-strength, F_c, was applied to the tip. In the sequence taken before F_c was applied, a large circular dark feature can be seen (indicated by an arrow in fig. 2A). This feature first appeared as a very small dark region, in images taken earlier in the desorption sequence. The dark region gradually increased in diameter as the benzene layer was field desorbed. Since the feature appeared in the image before other features had developed, it was interpreted as a relatively large molecular cluster extending many tens of nanometers from the surface. TEM micrographs taken of the tip apex before and after F_c was applied, supported this hypothesis [16]. Figs. 2 and 3 clearly indicate that the large, circular feature disappeared after the desorption event, but all of the other features remained essentially intact. This observation suggests that large clusters which extend far from the surface are more susceptible to field desorption than smaller, neighboring clusters. It appears that the electric field acts as a highly specific probe which selectively removes from the surface those species which are more weakly bound (or more

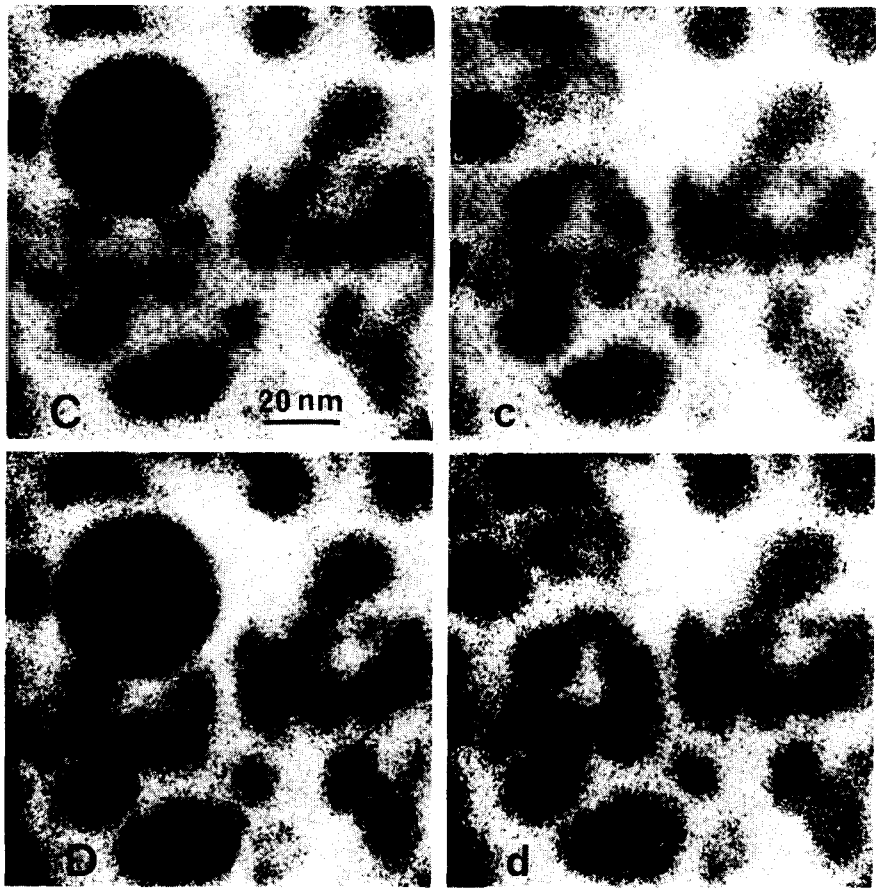

Fig. 3. A continuation of the imaging sequence of fig. 2 during point-projection imaging of ferritin on tungsten at 30 K: Before applying a critical field strength (C–D), and after applying a critical field strength (c–d). The imaging voltages were as follows: (C) 3700–3800 V; (D) 3800–3900 V, (c) 3600–3700 V, (d) 3700–3800 V.

exposed to the field) than their neighbors. The average electric field strength required to remove the large cluster shown in figs. 2 and 3 can be estimated from the average tip voltage, V_c, which was required to desorb it. That is:

$$F_c = V_c/kR = 4000/1.02 \times 10^4 \approx 3.9\ \mathrm{V/nm}, \quad (1)$$

where F_c is a critical field strength which is expected to be species (and, perhaps substrate) dependent. In eq. (1), R is the apex radius of the tip and k is a proportionality factor which depends on the shape of the emitter [17]. Fortunately, the quantity kR can be obtained from an analysis of the current-voltage (or Fowler–Nordheim) characteristic of the clean tip in ultra-high vacuum [17].

The field strength calculated in eq. (1) is more than 10% larger than the field required to produce the images of the cluster shown in fig. 2A. It is also high enough to allow the cluster to be repeatedly imaged over a relatively large range of tip voltages without visible change. The critical field strength required to desorb small clusters and isolated ferritin molecules [15] is almost a factor of two greater than the critical field for large clusters calculated in eq. (1). These observations appear to confirm our belief that point-projection microscopy can be used to nondestructively image the

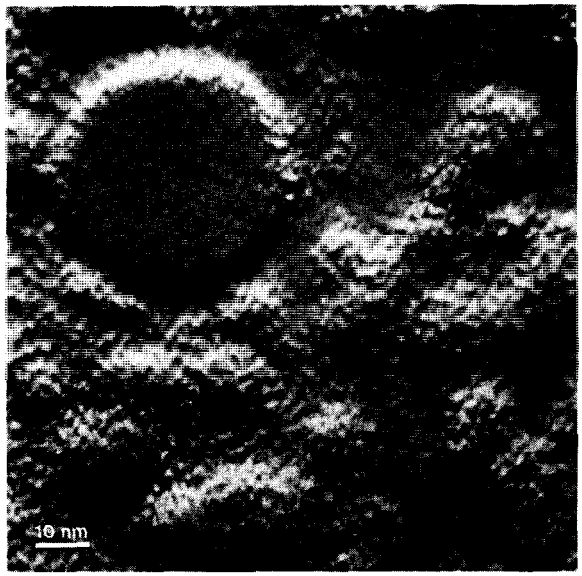

Fig. 4. A quasi three-dimensional, point-projection image of ferritin on tungsten at 30 K *before* applying a critical field strength, F_c. The image is a digital composite of the four images shown in figs. 2A–2D and figs. 3 C–3D.

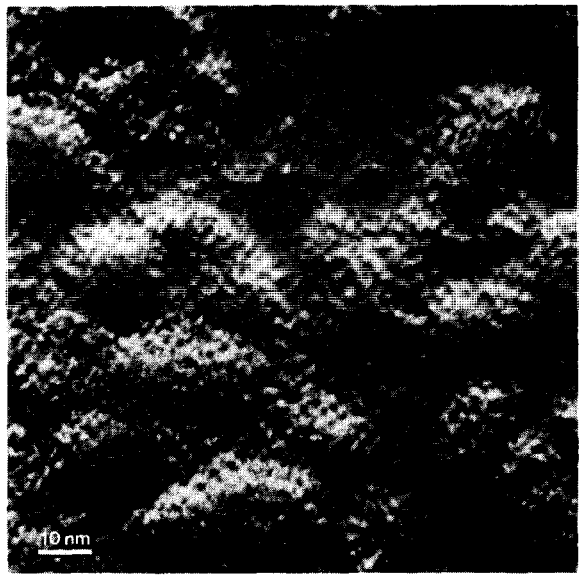

Fig. 5. A quasi three-dimensional, point-projection image of ferritin on tungsten at 30 K *after* applying a critical field strength, F_c. The image is a digital composite of the four images shown in figs. 2a–2b and figs. 3c–3d.

ferritin molecule. However, a word of caution is in order. The value of F_c which was calculated in eq. (1) may not be entirely reliable because kR was obtained for a clean tip and not for a tip partially covered with benzene.

Although figs. 2 and 3 display all of the relevant information within each of the two desorption sequences, we have found a more instructive way to present our data. Each of the images within a desorption sequence represent a slice through the molecule at some elevation above the tip apex. If we add several images together, we will obtain a quasi three-dimensional view of the ferritin distribution on the tip apex as viewed along the tip axis. The three-dimensional detail inherent in this type of composite image can be accentuated by adding highlights and shadows to the image with a simple image processing technique [18].

We first digitize a higher contrast copy of each image shown in figs. 2 and 3. The enhanced contrast minimizes structure in the benzene background which tends to add "noise" to the final image. The images within each sequence are then digitally added to produce a linear superposition of four images. This composite image is duplicated, low pass filtered, shifted by several video lines, and subtracted from the original image. Since each of the four separate images within a sequence contains molecular contours of decreasing size, the resulting composites (shown in figs. 4 and 5) display a striking three-dimensional quality [19]. The large cluster appears as a relatively flat plateau in fig. 4 because only four images were used for the reconstruction. Since the feature extends far from the surface we must add several more images to the composite in order to obtain a more accurate indication of its true shape [20]. The result of adding three more images to the composite of fig. 4 is shown in fig. 6.

The appearance of the images presented in this paper is consistent with an image resolution of ≈ 2.5 nm as calculated for a tip of radius $R = 18$ nm at a tip temperature of 30 K [12]. The calculations suggest that we may be able to improve the image resolution by a factor of two for this tip radius if we can lower the tip temperature to 5 K [12].

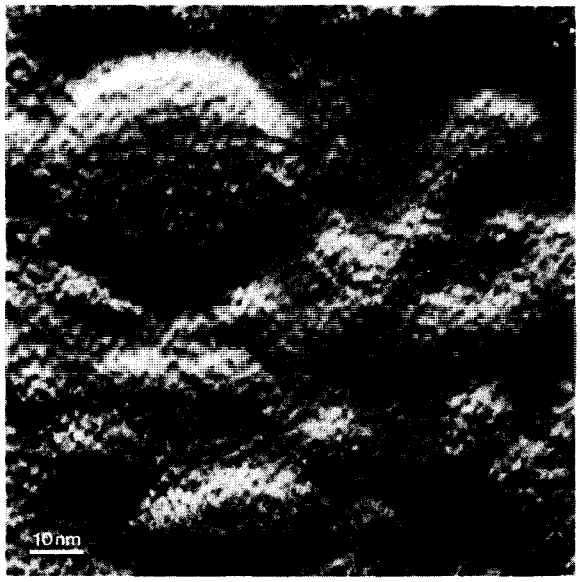

Fig. 6. The same reconstruction as shown in fig. 4 with three additional images (corresponding to greater distances from the tip surface) included in the digital sum. A comparison of this image with that shown in fig. 4 indicates that the additional images have the effect of developing the morphology of the large circular features while leaving the other image feature intact.

Conclusions: A point-projection microscope has been described which uses field-desorbed benzene to provide shadow images of the contour of macromolecular clusters of ferritin deposited from aqueous solution onto a field-emitter tip apex. Identical images have been consistently obtained even after repeated exposure of the tip to laboratory ambient. If a critical electric field strength, $F_c \approx 4\,V/nm$ is exceeded at the tip apex, large clusters which extend far from the surface will disappear from the image. However, individual ferritin molecules (and one molecule high clusters) within 10 nm of the larger features seem to be unaffected by the desorption event. This observation is consistent with a previous measurement of the desorption field of isolated ferritin molecules (and one molecule high ferritin clusters) observed in the TEM before and after desorption [15]. Our results confirm that point-projection imaging of the ferritin molecule is nondestructive, provided a critical field strength is not exceeded.

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[16] Before F_c was applied, a feature of approximately the correct diameter and unusual height appeared in the TEM images of the tip apex at approximately the correct position. After F_c was applied, the feature disappeared. Although we have associated this feature with a molecular cluster of ferritin, individual molecules within the cluster were not observed. As a result, we could not actually distinguish between a cluster of ferritin molecules and some other unknown protein contaminant as the source of this feature.

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[19] Since we do not know the ionization efficiency of benzene in condensed multilayers, we cannot determine the precise quantity of benzene removed from the surface at any given time. As a result, we do not accurately know the spacing between each image in each sequence. However, measurements of the desorption rate of benzene *ions* as a function of a linearly increasing tip potential have been made (see ref. [12]). These show that the rate is essentially constant for a series of images taken close to the tip surface where isolated ferritin molecules (and one-molecule-high clusters) are imaged. In other words, it appears that over distances from the surface comparable to a ferritin molecule (10 nm), the images we obtain are linearly spaced from each other. For this reason, the four images in each sequence shown in figs. 2 and 3 probably produce a reasonable reconstruction of cluster morphology.

[20] Since several more contours displaying the gradual development of the large circular feature were included in this image, the height of the feature may be distorted (see ref. [19]).