Field-electron emission microscopy as a probe of biomolecular adsorption from solution

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The field-electron emission microscope (FEEM) has been used as a probe of biomolecular adsorption from aqueous solution. Image contrast in vacuum has been correlated with the adsorption of tris buffer, and with ferritin/anti-rabbit goat IgG conjugate in tris buffer. If the imaging current is kept below 100 nA, tip morphology is unchanged. At higher imaging currents, in the presence of conjugate, a change in the surface morphology of the tip apex is seen by transmission electron microscopy. Control specimens exposed only to buffer and/or laboratory ambient do not change their morphology. We find that low-current FEEM imaging in vacuum can be used as a qualitative detector of biomolecule adsorption from solution. Reliable estimates of the size, the shape, and the number of adsorbates per unit area cannot be obtained with this technique.

INTRODUCTION

In recent years there has been a growing interest in developing systems for detecting very low concentrations of biological molecules in aqueous solution. The implementation of novel solid-state devices such as a penicillin-sensitive transistor,[1] and the need to verify suspected deployment of biological and chemical warfare agents[2] has provided the impetus for developing new sensor technologies. This paper explores the possibility of using the field-electron emission microscope (FEEM) to detect biological species adsorbed onto metallic substrates from aqueous solution in laboratory ambient. The ability of the FEEM to visualize monomolecular films deposited by sublimation in high vacuum is well documented.[3]

A field-electron emission microscope[4] (FEEM) consists of an evacuated chamber containing a fluorescent screen placed several centimeters in front of a sharply curved specimen "tip." An electron emission image appears on the screen when the tip is biased to a sufficiently high negative potential. The image reflects variations in the local electric field strength and the local work function at the tip apex. Radial projection of field-emitted electrons from the tip apex to the screen produces an image with a magnification and a resolution that is capable of observing work function changes on a nanometer scale.

More than twenty years ago, attempts were made to use the FEEM to visualize small organic molecules placed on the apex of a field-emitter tip by sublimation in high vacuum.[5–6] These experiments were probably motivated by the ability of the FEEM to observe molecular aggregates,[7,8] and by the speculation that even single atoms could be seen.[6,8,9] At the time, electron microscopy was still in its infancy. The FEEM was the only microscope that had demonstrated the high magnification and the nanometer resolution required to image single molecules.

Molecular imaging in the FEEM appeared to be a simple task. Molecules of interest were sublimed in high vacuum onto the apex of a field-emitter tip. A high vacuum environment insured that the adsorbate was the only "contaminant" on the tip surface. An adsorbed species will change the work function and/or the electric field strength in its immediate vicinity. Since the field-emission current depends exponentially on these parameters,[4] the resulting image should reflect the number, the size, and the shape of adsorbed species.

The first FEEM images of molecules sublimed onto a tip apex were convincing. A planar, fourfold symmetric molecule (copper phthalocyanine) produced striking patterns with local contrast reflecting a fourfold symmetry. A twofold symmetric molecule (flaventhrene) produced twofold symmetric variations in image contrast.[10,11] Unfortunately, it soon became apparent that essentially any molecule sublimed onto the tip produced similar images.[12–16] Various explanations were advanced to explain the effect,[12–18] but none were completely satisfactory.

The accumulated evidence of the past thirty years suggests that a FEEM image can be used to detect small organic molecules sublimed onto a tip in high vacuum, but cannot be used to determine their morphology. Under exceptional conditions, single atoms may become visible in a FEEM image.[19] It has been demonstrated that time variations in the field-electron emission current (rather than contrast variations in the FEEM image) can be used to monitor the adsorption of single metal atoms.[20]

From the preceding discussion, it is clear that the field-electron emission phenomenon is a sensitive probe of molecular adsorption in high vacuum. A high vacuum environment insures that a preselected adsorbate will be the major contaminant on the surface of a field-emitter tip. As a result, it will produce the major contrast variation in a FEEM image of the surface. If a biological molecule is adsorbed from a liquid environment in laboratory ambient, other species at higher concentrations (such as salt and buffer molecules) will adsorb and could dominate the appearance of a FEEM image. On the other hand, biological molecules are generally much larger (and fewer in number) than these contaminants. As a result, biological molecules deposited from solution might be visible in an image whose average contrast is determined by a saturation coverage of smaller contaminants.

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The experiments described in this paper were designed to investigate this possibility.

MOLECULAR DEPOSITION

In order to adsorb molecules from solution onto a field-emitter tip, we used a deposition procedure which we described previously.[21] The protocol evolved from a study of ferritin adsorption on tungsten.[22] Several tips are immersed in an appropriate buffer. A dilute solution of the molecule of interest is added in order to obtain a typical concentration of 10–50 $\mu g/m\ell$. The tips are not immersed directly into a dilute solution of the molecule. This prevents the adsorption of an uncharacterized monolayer then the air-liquid interface is traversed. Monolayer adsorption at an interface is encouraged by the well-known Langmuir–Blodgett effect, first studied for soaps and fatty acids.[23]

The coverage of molecules on the emitter tip apex is diffusion limited. It depends on the concentration of molecules in solution, the adsorption time, the degree to which the solution is stirred, and the sticking coefficient. The effect of these parameters can be estimated prior to deposition by using the Giaever Slide Assay.[24] Molecules in solution will tend to deposit on all exposed surfaces until a saturation coverage is obtained. On Lexan, a saturation coverage of ferritin occupies only 50%–80% of the surface area available for adsorption.[25] A similar coverage has been observed for DNA on tungsten.[26]

Following deposition, the tips are rinsed by immersing them in a volume of organic-free distilled water. Provisions must be made to insure that the tips remain wet during transfer into the rinsing volume. If the tips are allowed to dry, salt and buffer may irreversibly deposit on their surface. Repeated traversals through a water-air interface must also be avoided. During each traversal, surface tension forces can rearrange the coverage of molecules on the surface, or can desorb them back into the liquid.

FEEM IMAGING OF BIOLOGICAL ADSORBATES

Ferritin/goat anti-rabbit IgG conjugate was selected for the FEEM imaging experiments. It was obtained at a concentration of 1–2 mg/mℓ in 100-mM tris-Cl buffer containing 0.04% sodium azide at pH 7.5.[27] A dilution of approximately 120:1 (in 20-mM tris-Cl, 0.15-mM NaCl, pH 7.6) produced a saturation coverage on the tip apex in 3 min as judged by the Giaever Slide Assay. Since ferritin contains iron, the coverage of the conjugate on the tip could be independently verified by transmission electron microscopy (TEM). Figure 1 shows a TEM image of a saturation coverage of this species on the apex of a field-emitter tip. Figure 2 shows a saturation coverage of the conjugate and three other biological molecules visualized by field-ion tomography.[28] Both figures indicate that a saturation coverage of protein does not completely cover the tip apex. The effect appears to be independent of the molecule that is adsorbed, and may be a general characteristic of molecular adsorption onto metals in a liquid environment.

Field-electron emission images were obtained in an unbaked stainless-steel vacuum chamber evacuated to about 10^{-9} Torr using turbomolecular and ion pumping. Eight

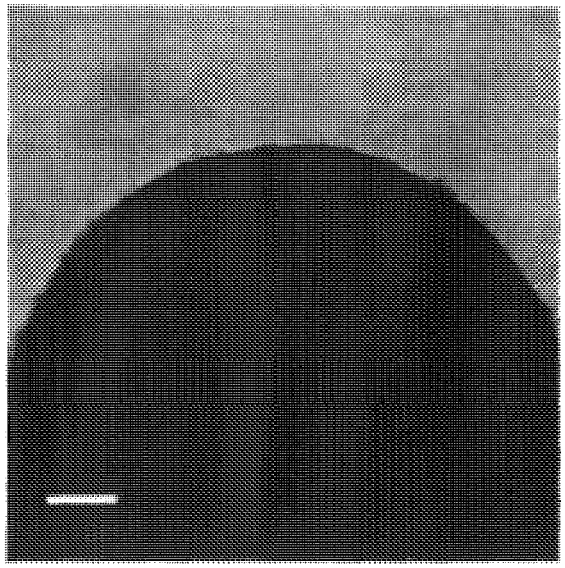

FIG. 1. A TEM image of a tungsten field-emitter tip after adsorption of a saturation coverage of ferritin anti-rabbit goat IgG conjugate (12 $\mu g/m\ell$ in 0.15-M NaCl, 20-mM tris-Cℓ, pH, 7.6 for 3 min). Marker = 90 nm.

field-emitter tips could be inserted into the vacuum chamber at one time. Imaging pressures were achieved in about 12 h. Image intensification was not used, and Fowler–Nordheim characteristics were not recorded.

Thirty-two tungsten tips were examined. The following protocol was repeated 16 times: (1) FEEM images of two clean tips were obtained immediately following thermal annealing at ~1800 °C for several seconds. (2) Both tips were removed from the vacuum environment. One tip (which we will call Tip 1) was placed in 20-mM tris-Cl buffer containing 0.15-M NaCl and pH 7.6 for 180 sec. The other tip (which we

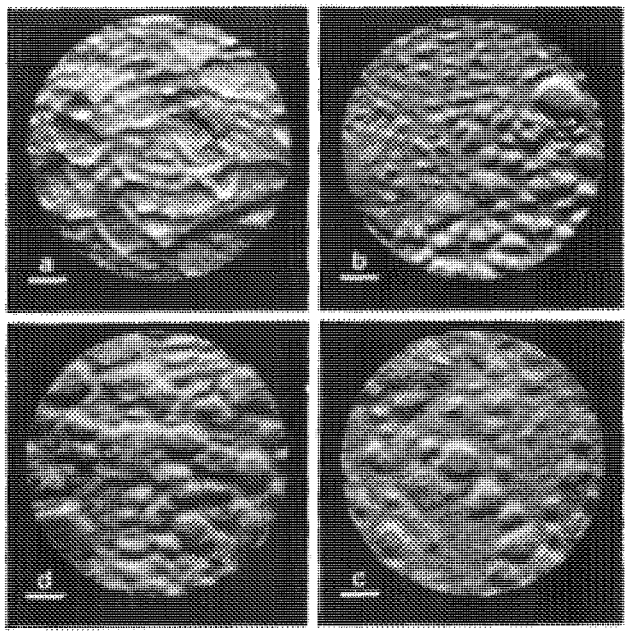

FIG. 2. Saturation coverage of various biomolecular species on tungsten field-emitter tips. (a) Poly G-C DNA. (b) Ferritin. (c) Ferritin anti-rabbit goat IgG conjugate. (d) Haemocyanin. Marker = 40 nm.

will call Tip 2) acted as a "control." Tip 2 was exposed to laboratory ambient during the time that tip 1 was exposed to the liquid environment. (3) Both tips were returned to the vacuum system and a FEEM image was taken of each tip after a 12-h pumpdown. Tip 1 produced an image that reflected ambient gas adsorption, and buffer adsorption from solution. Tip 2 (the control) produced an image that reflected only the adsorption of gas phase contaminants from laboratory ambient.

After imaging, both tips were removed from the vacuum environment and the protocol continued as follows: (4) Tip 1 was placed in buffer containing ferritin/IgG conjugate at a concentration of about 12.5 $\mu g/m\ell$. After 3 min tip 1 was rinsed in pure water, and transfered wet into a mixture of 90% ethanol in water for 15 sec, and then dried in air. (5) Both tips were returned to the vacuum environment and a FEEM image was taken of each tip. The image of tip 1 reflected the adsorption of gas phase contaminants during tip transfer in air, buffer adsorption from solution, and the adsoption of ferritin/IgG complexes from solution. The image of tip 2 (the control tip) reflected gas phase adsorption from laboratory ambient.

The protocol described above resulted in three FEEM images of each tip. The first image documented the appearance of the clean tip surface. The second image of tip 1 reflected the adsorption of buffer molecules from solution, and gas-phase contaminants adsorbed during tip transfer in laboratory ambient. The third image of this tip reflected the adsorption of ferritin/IgG complexes, buffer molecules from solution, and inevitable gas-phase contamination. The second and the third FEEM images of tip 2 (the control tip), taken after successive exposures to laboratory ambient, were used to assess the effect of gas phase contamination of the tip apex.

Figure 3 shows a sequence of FEEM images (a–c) obtained by exposing a clean tungsten tip (tip 1) to aqueous solution in laboratory ambient using the deposition protocol described above. The corresponding control tip images (tip 2) are shown in (d–f) for comparison. The control tip images indicate that the effect of ambient gas adsorption on the apex of a tungsten tip is negligible. This is somewhat surprising since at least chemisorbed oxygen must be present on the tip surface. Perhaps oxide formation was minimal, and other less reactive species were weakly bound so that most of the adsorbed layer was desorbed during pumpdown, before the images were obtained.

Figure 3 (a) is a FEEM image of tip 1 taken at -2.3 kV after thermal annealing in vacuum. Figure 3 (b) shows the same tip imaged at -1.5 kV after exposing it to laboratory ambient and to buffer. The localized regions which appear bright in the image are characteristic of buffer adsorption. It is tempting to associate each bright region with an adsorbed buffer molecule, but the number would be well below a saturation coverage. A small number of bound buffer molecules would be reasonable if the rinsing procedure used in the deposition protocol was very effective. TEM imaging of the coverage of bound buffer molecules is not feasible because of their small size.[21]

Figure 3 (c) shows the effect of exposing the same tip to a

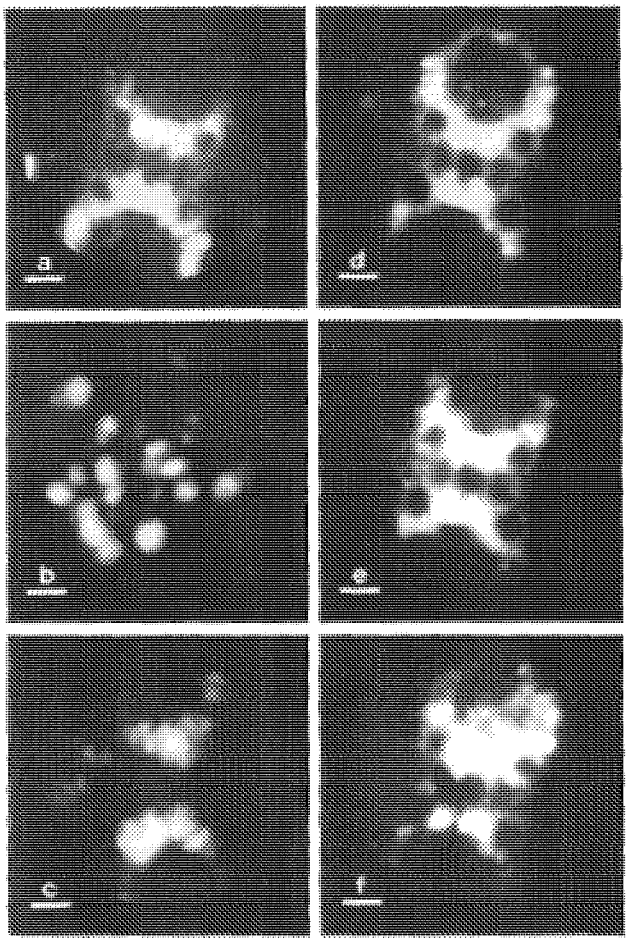

FIG. 3. Field-electron emission imaging in vacuum. Exposure to laboratory ambient (d–f). Deposition from aqueous solution (a–c). See text. Marker = 125 nm.

solution of ferritin-IgG complexes in buffer, and to laboratory ambient for a second time. A comparison of the FEEM image shown in Fig. 3 (c) with the clean tip image of Fig. 3 (a) suggests that the surface morphology of the tip has changed. This conclusion has been supported by TEM imaging. Figure 4 (a) is a TEM micrograph taken of the clean tip immediately after the FEEM image shown in Fig. 3 (a). Figure 4 (b) shows a TEM micrograph taken of the tip after the image shown in Fig. 3 (c) was recorded. The surface morphology of the tip apex has changed. This phenomenon has been attri-

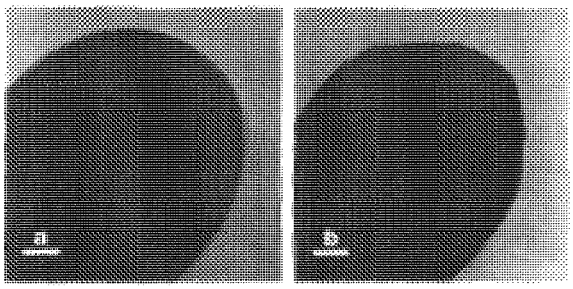

FIG. 4. TEM images of a tungsten control tip. (a) Before the image of the clean (control) tip shown in Fig. 3 (a). (b) After two exposures to laboratory ambient [after the image shown in Fig. 3 (c)]. Marker = 151 nm.

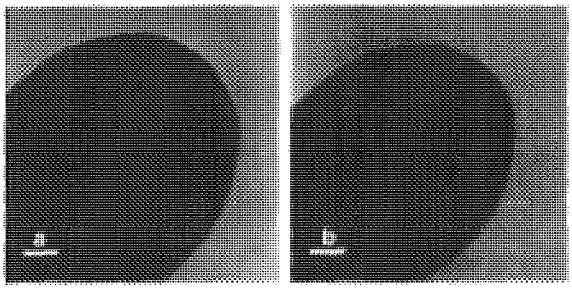

FIG. 5. TEM images of biomolecule deposition. (a) Before the image of the clean tip shown in Fig. 3 (d). (b) After biomolecule deposition [after the image shown in Fig. 3 (f)]. Marker = 151 nm.

buted to the combined effects of FEEM imaging at a current of approximately 1 μA, and exposure to an aqueous buffer containing ferritin-IgG complexes. The tip morphology only changes if both the ferritin-IgG complex is present in the buffer, and the FEEM imaging current is higher than 1 μA. If either condition is not satisfied the tip morphology does not change, and the image contains bright spots similar in appearance to those recorded after buffer adsorption [Fig. 3 (b)]. It is impossible to visually distinguish ferritin-IgG adsorption from buffer adsorption in a FEEM image.

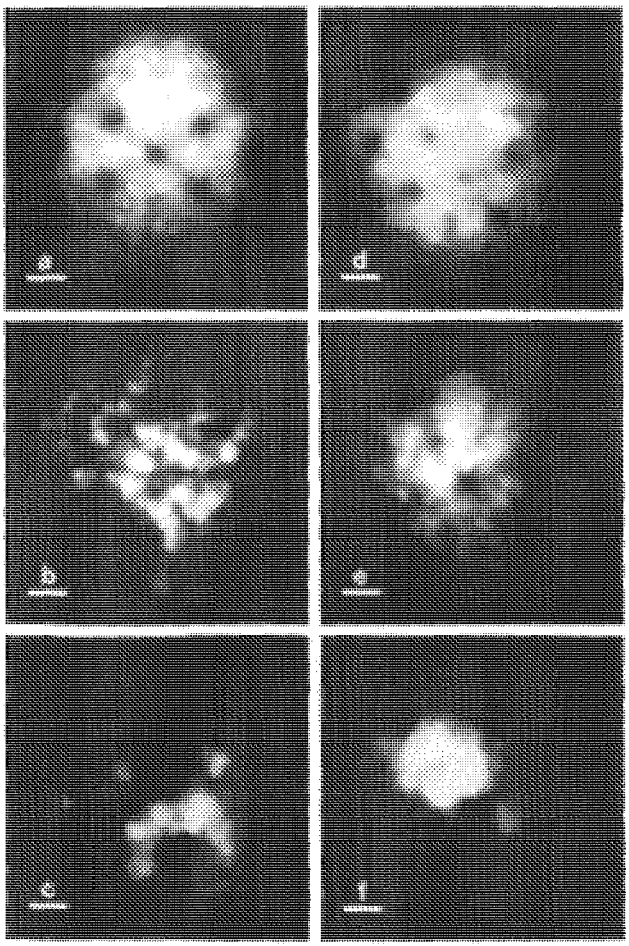

FIG. 6. Field-electron emission imaging in vacuum. Exposure to laboratory ambient (d–f). Deposition from aqueous solution (a–c). See text. Marker length unknown.

Figures 5 (a) and 5(b) are TEM micrographs taken of tip 2 (the control tip) before the FEEM images shown in Figs. 3 (d) and 3 (f) were recorded. Figures 5 (a) and 5 (b) demonstrate that the morphology of a tip does not change as a result of imaging after repeatedly exposing the tip to laboratory ambient.

Figure 6 shows another sequence of FEEM images taken after molecular deposition (a–c), and the corresponding control images (d–f). Although quantitative differences exit between the FEEM images shown in Fig. 3 and 6, the same general features are observed. Figures 3 and 6 reflect the maximum range of image contrast which we have observed with the thirty two tips used in this study.

CONCLUSIONS

Sixteen experiments have been performed under identical conditions in order to assess the ability of the FEEM to detect biomolecular adsorption from solution. Each experiment employed one field-emitter tip that was exposed to liquid environments, and one (control) tip that was only exposed to laboratory ambient. The following conclusions were reached.

(1) Repeated exposure of a tip to laboratory ambient does not appreciably alter the clean tip image which is obtained after thermally annealing the tip in high vacuum. Since the tip surface must be covered with a monolayer of gas phase contaminants as a result of each air exposure, the adsorbed species must either not affect FEEM image contrast, or must desorb during the pumpdown cycle prior to imaging. Occasionaly, localized regions of increased image brightness are seen after air exposure, but these do not survive minor increases in field strength (of the order of 0.5%). After the field strength is increased and then reduced, the clean tip image is essentially reproduced.

(2) If a clean tip is exposed to a buffer consisting of 20-mM tris-Cl and 0.15-M NaCl at pH 7.6, a characteristic FEEM image is recorded in vacuum. Image features consist mainly of bright regions of increased contrast, often superimposed on a weak background image. The background image is qualitatively similar in appearance to the image of the clean tip. Unlike the bright regions which are occasionally observed in a control tip image after air exposure, the bright spots in these images are stable, even after increasing the field strength by several percent.

(3) If a tip is exposed to an aqueous solution of buffer containing ferritin-IgG complexes, a subsequent FEEM image recorded in vacuum at a current greater that 100 nA will generally show some change in substrate morphology. TEM images of the tip confirm that the radius and orientation of the tip has changed. If the imaging current is kept below 1 μA the substrate will remain unchanged, and the image will be indistinguishable from those obtained after dosing with buffer in laboratory ambient. Since TEM imaging confirms that ferritin-IgG complexes are present on the tip apex, we conclude that they are responsible for producing the same FEEM image features (bright regions of increased contrast) seen after exposure to buffer, alone. At high imaging currents (much greater than 1 μA) the tip surface appears to change its morphology, an effect apparently influenced by

the presence of the ferritin-IgG conjugate. FEEM images of tips exposed to laboratory ambient and/or buffer alone do not show this effect.

Our results suggest that FEEM imaging in vacuum can be used to detect molecular adsorption from solution provided the FEEM imaging current is kept well below 1 μA. Since low imaging currents produce a very weak FEEM image on a fluorescent screen, some form of image intensification is usually required. Our results are consistent with previous FEEM studies of small organic adsorbates sublimed onto the tip surface in high vacuum. These investigations and the present series of experiments support the contention that a FEEM image can be used as a qualitative detector of molecular adsorption, but cannot be used to determine the size or shape of the adsorbate.

It is interesting to extend our results to the problem of imaging biological molecules in the scanning tunneling microscope[29] (STM). The STM is a brilliant, high-resolution adaptation of Russel Young's "topografiner."[30] In these devices, a biased field-emitter tip is laterally scanned across a substrate while the resulting tunneling current from the tip is held constant. Changes in local surface morphology or work function cause the tip to move in a direction perpendicular to the surface, mapping surface topography in three dimensions. If the tip is scanned at a distance greater than the width of the tunneling barrier, field-electron emission will dominate the tunneling process. At smaller distances from the surface, metal-vacuum-metal tunneling will become dominant. This is the usual mode of operation of the STM. It is conceivable that the physical processes responsible for distorting the appearance of biological molecules in a field-emission microscope image will also influence their STM image. For example, it has been suggested that FEEM images may reflect the orbital symmetry associated with a molecule-substrate complex.[17] Accordingly to this hypothesis, an electron emitted from a metal substrate has a high probability of tunneling into an unoccupied orbital of a molecular adsorbate from which it is subsequently reemitted. One can hope that interpretable STM images of biological molecules will lead to a clearer picture of tunneling in the vicinity of macromolecular adsorbates on a metal surface so that field-electron emission images of these species will finally be explained.

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