

A Deposition Technique for the Imaging and Analysis of Protein Interactions With Metal and Semiconductor Surfaces

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ABSTRACT A new technique for placing biological molecules on metal, insulator, and semiconductor surfaces is described. The procedure requires only 10 μ l of solution containing molecules at a concentration of 0.1–10 μ g/ml. The use of a buffer that does not affect metal substrates, the possibility of fixing the molecules in solution prior to deposition, and the ability to minimize surface tension forces during air drying are other features of the new protocol. Simultaneous deposition on TEM grids and highly curved substrates permits biomolecular adsorption on technologically interesting materials to be visualized in the transmission electron microscope.

INTRODUCTION

The use of the electron microscope in molecular biology has been greatly facilitated by the development of techniques for the adsorption of proteins and nucleic acids onto thin insulating substrates (plastic and carbon films). With the advent of new imaging techniques such as photoelectron microscopy (Griffith et al., 1981), scanning tunneling microscopy (Binnig and Rohrer, 1983), and field ion tomography (Panitz, 1982a), it has become essential to develop reproducible and nonartificial methods for the deposition of proteins and nucleic acids onto metals and semiconductors.

In order to image macromolecules by present methods, the sample must be first securely bound to the substrate in order to minimize artifacts introduced when the substrate is removed from liquid, air dried, and placed in a high-vacuum environment. During the last 20 years, electron microscopists have improved the quality of protein and nucleic acid images by minimizing artifacts associated with the adsorption, adherence, and drying of these species on thin films of carbon and plastic (Griffith and Christiansen, 1978). Our goal has been to adapt some of these techniques to the deposition of proteins and nucleic acids on field-emitter tips.

Field-emitter tips are useful substrates for the characterization of macromolecule-sur-

face interactions because they can be made from technologically interesting materials, including most metals and semiconductors (Müller and Tsong, 1969). The tip can be imaged in profile in the transmission electron microscope (TEM), and molecules adsorbed on its surface can be readily visualized (Panitz and Giaever, 1980). This procedure, referred to as "edge-projection" TEM (EPTEM), is a powerful diagnostic tool for the study of macromolecular adsorption on materials that would normally be unsuitable as a substrate for conventional TEM imaging. Tips can be prepared with rough or atomically smooth surfaces. If necessary, the apex can be imaged at atomic resolution by field-ion microscopy, and its chemical composition can be determined by atom-probe mass spectroscopy (Panitz, 1982b).

In this paper we describe a deposition procedure that has been used to place protein molecules onto field-emitter tips and carbon coated TEM grids. Our protocol can also be used to deposit nucleic acids and other macromolecular species onto these substrates. Novel features of the new protocol include: 1) the simultaneous deposition of biological molecules onto a field-emitter tip and a car-

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bon-coated TEM grid for comparison of relative adsorption characteristics, 2) the minimization of surface tension artifacts, and 3) the requirement of only very small sample volumes (10 μ l).

MATERIALS AND METHODS

The field-emitter tip and holder

A field-emitter tip is typically fashioned by chemically polishing a fine wire until its apex radius of curvature is of the order of a few hundred nanometers. To obtain a clean and atomically smooth surface, the tip can be thermally annealed near its melting point in high vacuum (Gomer, 1961). Vacuum annealing removes surface impurities by thermal desorption, and smooths the tip contour by surface self-diffusion (Boling and Dolan, 1958). During this process, the tip apex assumes a surface of minimum free energy, which is approximately spherical, and characterized by broad, flat, crystal planes, which are smoothly joined together.

The small size and the highly curved surface of the tip apex cause it to dry immediately after it is removed from an aqueous medium (Panitz and Giaever, 1980). Surface tension forces during the drying process can seriously affect the structure of the adsorbed macromolecules. In order to prevent the tip apex from drying during various stages in the deposition protocol, a fixture was designed to keep the tip immersed within a 10- μ l volume of liquid at all times. If the end of the fixtures is lightly touched to the surface of a TEM grid, the grid will adhere to the liquid in the fixture by surface tension forces. The grid acts as one wall of the captivated liquid volume, allowing its surface to sample the same deposition volume as the tip.

A drop of liquid is captivated in the fixture by a three-turn loop of tungsten wire. The loop, 1.2 mm in diameter, is formed at one end of a 0.5-mm diameter tungsten wire. The other end of the wire is supported by a small block of Teflon. The Teflon block can be moved manually along a stainless steel rod, 1.5 mm in diameter. Figure 1 shows a field-emitter tip being placed in a socket that is attached to the end of the rod. The tip is held in the socket by friction. After tip insertion, the rod is translated with respect to the loop in order to position the tip approximately at its center. The Teflon block electrically insulates the tip from the wire loop in order to prevent galvanic activity in solution from

influencing molecular deposition on the tip apex (Panitz and Giaever, 1980).

An important feature of the deposition fixture is its ability to restrict solution contact to the tip apex and a small portion of its shank. In a previous deposition technique (Panitz and Giaever, 1981), the entire tip assembly was immersed in liquid. In order to prevent galvanic activity in solution, the tip and its metallic support had to be fabricated from the same material. With the new fixture, the tip can be prepared from a fine wire of an appropriate material and then fastened to a support rod of a different material. Since both materials are not immersed into the deposition volume, galvanic activity will not occur.

The use of a different material for the tip and for the support rod can greatly simplify tip preparation. Previously, tips had to be prepared by machining or grinding a support rod of the appropriate material to a very small diameter. The rod was cut to length, and the reduced diameter chemically etched to form the tip. With the new fixture, a tip can be etched from small diameter, commercially available wire and then connected to a support rod.

Deposition protocol

In order to deposit molecules on the tip, the tip is immersed in a 10 μ l solution of buffer containing the molecule at a concentration of 0.15–51 μ g/ml. For protein deposition, we found that a simple buffer consisting of 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), and 150 mM NaCl at pH 7.5 is satisfactory. For nucleic acids and nucleoprotein complexes, we use a buffer consisting of 50 mM HEPES, 0.1 mM DTT (dithiothreitol, Cleland's reagent), 1 mM $MgCl_2$, and 50 mM KCl at pH 7.5. Neither buffer appears to attack tungsten field-emitter tips, as judged by TEM imaging.

Deposition begins by touching the wire loop at the end of the deposition fixture to a 10 μ l drop of solution resting on a sheet of Parafilm¹ as shown in Fig. 2A. A TEM grid can be exposed to the solution by moving the fixture close to a grid placed on the Parafilm sheet (Fig. 2B). Surface tension forces will pull the grid onto the end of the wire loop without damage, as shown in Fig. 2C.

¹A product of the American Can Company (Dixie/Marathon) Greenwich, CN.

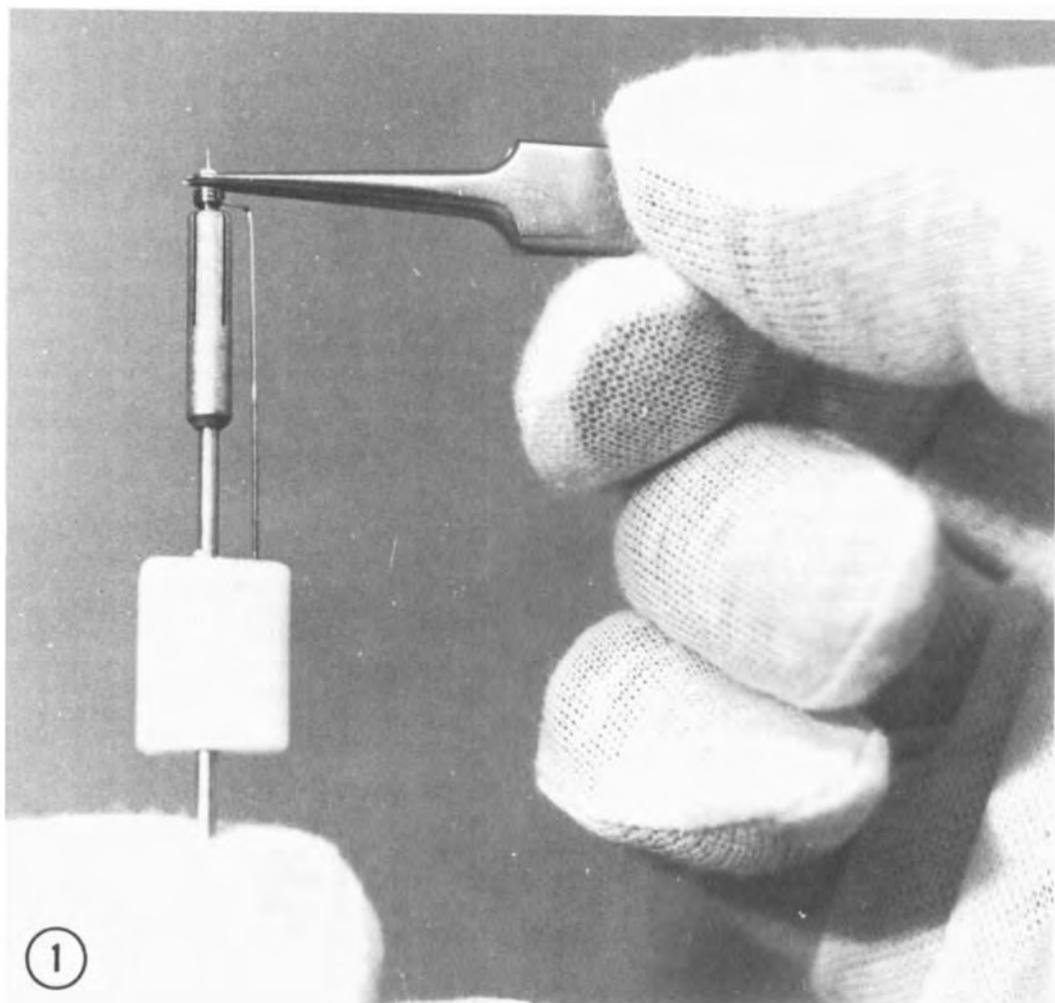


Fig. 1. A field-emitter tip being inserted into the deposition fixture described in the text.

We have found that the final coverage of protein on the field emitter tip depends on the concentration of macromolecules in solution, the degree to which the solution is stirred, the adsorption properties of the macromolecule, and the deposition time. These parameters must be empirically determined for each macromolecule, each substrate, and the experimental conditions. For example, we found that a saturation coverage of ferritin forms on carbon films or tungsten tip in about 2 min at a concentration of 100 $\mu\text{g}/\text{ml}$.

Many other proteins seem to behave in a qualitatively similar manner.

To stop deposition, the end of the deposition fixture is quickly placed in 10 ml of fresh triple-distilled water as shown in Fig. 2D. The grid will drop to the bottom of the rinsing volume if the surface is quickly broken. At this point in the protocol, the concentration of molecules in the original droplet has been reduced by the ratio of its volume to that of the rinse water, or by a factor of approximately 1,000:1.

Rinsing presumably cleans the surface of a substrate of loosing adhering molecules and soluble adsorbates (such as salt). The time taken for rinsing is a compromise between the time required to clean its surface of unwanted contaminants, and the time at which a significant amount of the previously bound adsorbates will desorb from the surface back into the liquid. The optimum rinsing time can be considered the shortest time which that produce an acceptably "clean" background in a TEM image. Under our conditions, this time is of the order of 1 or 2 min for protein adsorption on carbon films and tungsten field-emitter tips.

Surface-tension considerations

It is known from TEM images that surface tension forces can alter the deposition characteristics of biological molecules deposited on carbon films when the films are moved through an air-water interface. For example, ferritin (a structurally rigid protein) can be removed from a surface or moved about (Feder and Giaever, 1980), while nucleic acids (which are very flexible polymers) are known to "tangle," like spaghetti, if surface tension forces are not minimized (Fisher and Williams, 1979). The same observation has been made for DNA deposited onto tungsten tips (Panitz, 1983). The general conclusion is that the binding of a biological molecule to a substrate is so weak that significant artifacts can be introduced by crossing an air-water interface.

In order to minimize artifacts associated with crossing an air-water interface, a substrate can be removed into air from pure ethanol (or from an ethanol-water mixture) where surface tension forces are considerably lower (Adamson, 1960). The pure aqueous environment of an adsorbed molecule can be gradually equilibrated with that of the final ethanol rinse by placing the substrate (for 1 min) into each of a series of increasingly concentrated aqueous ethanol solutions (Griffith, 1973). We typically transfer the tip (and then the TEM grid) for 1 min into 20%, 50%, 70%, and then into 100% ethanol.

Our deposition fixture insures that the tip is always immersed in liquid during its transfer into each successive alcohol rinse. As the TEM grid is removed into air from each alcohol rinse, a stream of rinse solution is directed onto its surface. The grid retains a drop of the solution, so that its surface also remains wet during transfer in air. These

procedures insure that only weak interfacial tension forces will act on adsorbed molecules as the tip and grid are rinsed.

After the last rinse, the tip (in the deposition fixture) and the TEM grid are removed into air. The tip is translated out of the droplet of rinse solution adhering to the deposition fixture by reversing the insertion procedure shown in Fig. 1. When the tip and grid are dry, they are placed in high vacuum and rotary stained with tungsten in order to increase the visibility of molecules on their surface during subsequent TEM imaging. The procedure developed for rotary staining a tip with tungsten is based on an existing method for rotary staining carbon-coated TEM grids (Griffith, 1973), and is described elsewhere (Panitz, 1985).

Fixation

Fixation of proteins in solution helps to preserve their structure during adsorption and desiccation (Bullock, 1984). The fixation is achieved by the addition of glutaraldehyde (6% solution in HEPES buffer) to the deposition solution in order to obtain a final concentration of 0.6% (Griffith, 1973). The solution is incubated for 5 min prior to molecular deposition.

RESULTS AND DISCUSSION

Figure 3 shows TEM images taken of a control tip, dosed only with buffer (3A), a tip dosed with rho protein² (3B), and a carbon-coated, TEM grid (3D) dosed simultaneously with the tip. Rho is a hexameric protein composed of six identical (46,000-dalton) subunits (Platt and Bear, 1983; von Hippel et al., 1984). The tips and the carbon film were rotary shadowed with tungsten at a low angle (11°) in order to improve image contrast (Panitz and Bear, 1985).

In order to assess the importance of the rinsing schedule, a saturation coverage of ferritin³ was placed on several tungsten field-emitter tips. Some of the tips were rinsed in water for 1 min, and then for 1 min in each of the graded series of ethanol-water mixtures (ending with pure ethanol) described above. Other tips were rinsed for 1 min in water and then for 1 min in a mixture of 90%

²10 µg/ml for 1 min in the nucleic acid/nucleoprotein buffer described in the text. The tip was rinsed in fresh triple-distilled water for 1 min and then in 90% ethanol for 15 s.

³100 µg/ml for 2 min. Miles Laboratories, Elkhart, IN. Cat. No. 96-027-2.

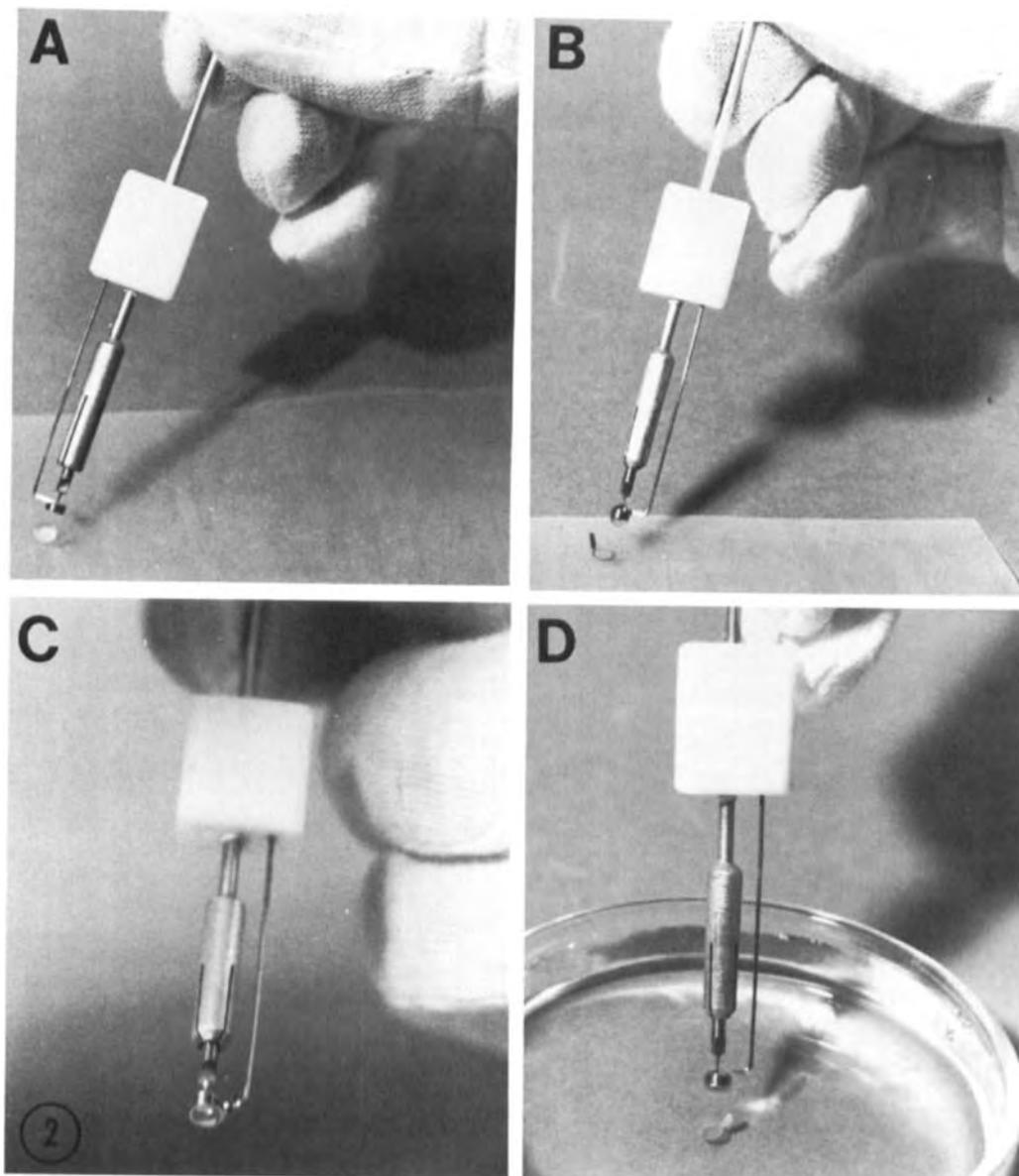


Fig. 2.(A) prior to tip deposition; (B) prior to grid deposition; (C) simultaneous deposition on tip and grid; (D) rinsing.

ethanol in water. The remaining tips were rinsed in water for 1 min and then removed directly into air, through the air-water interface. The TEM images shown in Fig. 4 are typical of the results obtained. The nominal saturation coverage of ferritin obtained with

the graded series of ethanol rinses (20%, 50%, 70%, 100% ethanol) is essentially retained when a single 90% ethanol rinse was used for rinsing. When the tip is rinsed in water and removed into air, the ferritin coverage is significantly altered. Apparently, the surface

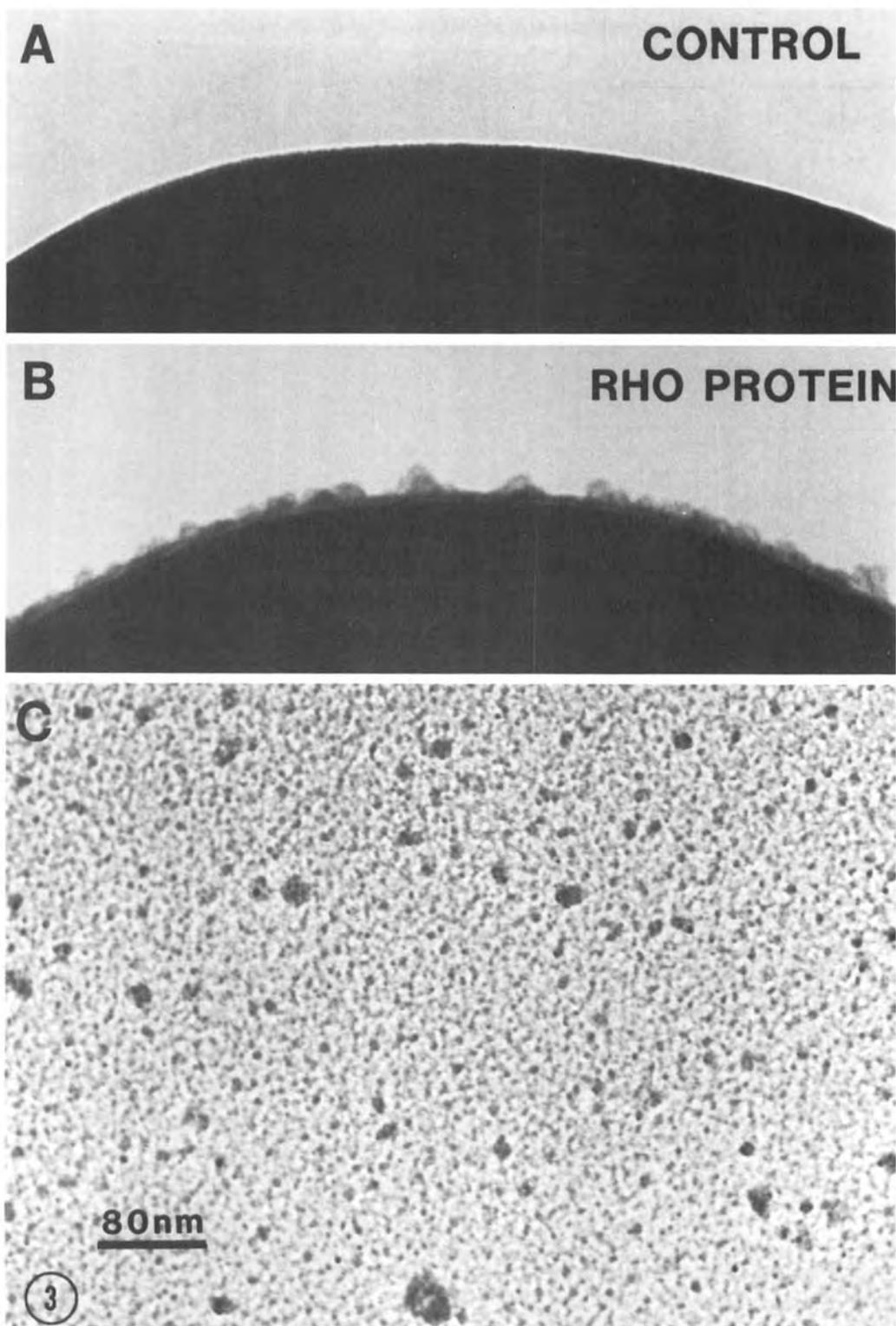


Fig. 3. TEM imaging at 200 kv; (A) control tip, buffer deposition only; (B) rho protein on tip; (C) rho protein on grid (50 kv image). The tip and grid were simultaneously dosed with rho at a concentration of 10 $\mu\text{g}/\text{ml}$ for 2 min, rinsed in water for 1 min, and rinsed in 90% ethanol in

water for 15 s. A nucleic acid buffer, (described in the text) was used for deposition. The tips and the grid were rotary shadowed with tungsten at a low angle (11°) in order to improve image contrast.

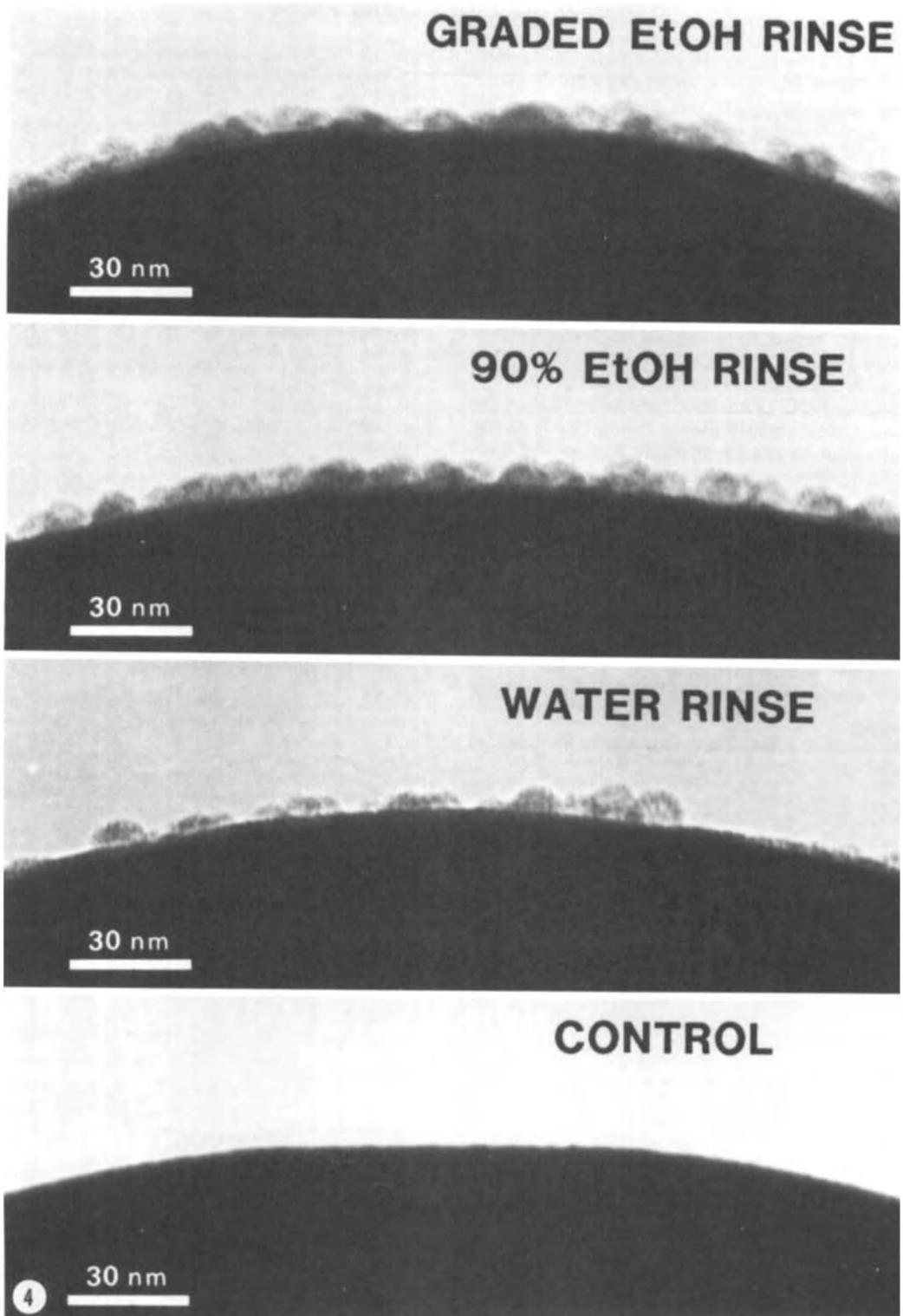


Fig. 4. 200 kV TEM images of thermally annealed tungsten field-emitter tips exposed to a saturation coverage of ferritin (100 $\mu\text{g}/\text{ml}$ for 2 min), and buffer only (control tip). A buffer consisting of 50 mM HEPES, 150

mM NaCl, pH 7.5 was used with the indicated rinse schedule and the deposition protocol described in the text.

tension forces at an air-water interface cause adsorbed molecules to rearrange on the surface, and desorb from the substrate back into the liquid phase as the interface is crossed. A TEM image of a control tip, dosed only with buffer, is shown in Fig. 4 for comparison.

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