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Robert P. Apkarian
Emory University

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ANALYSIS OF HIGH QUALITY MONATOMIC CHROMIUM FILMS USED IN BIOLOGICAL HIGH RESOLUTION SCANNING ELECTRON MICROSCOPY

Robert P. Apkarian

Integrated SEM & Microanalytical Facility, Yerkes Research Center
Emory University, Atlanta, GA 30322
Telephone No.: (404) 727-7766 / FAX No.: (404) 727 7845

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Abstract

During the recent employment of field emission (FE) in-lens scanning electron microscopes (SEMs), refractory metal deposition technology has co-evolved to provide enhanced contrast of 1-10 nm hydrocarbon based biological structures imaged at high magnifications (> 200,000 times). Pioneer development employing the Penning sputter system in a high vacuum chamber proved that imaging of chromium (Cr) coated biological specimens contained enriched secondary electron (SE)-(I) contrasts. Single nanometer size fibrillar and particulate ectodomains within the context of complex biological membranes were accurately imaged without significant enlargement using the high resolution SE-I mode (HRSEM). This paper reports the transmission electron microscopy (TEM) testing of ultrathin (0.5-2.0 nm) Cr films deposited by planar magnetron sputter coating (PMSC). Essential parameters necessary to reproduce quality sputtered films of refractory metals used in HRSEM studies were described for the vacuum system and target operation conditions (current, voltage, and target distance). HRSEM imaging of biological specimens is presented to assess contrast attained from ultrathin fine grain Cr films deposited by PMSC. High magnification images were recorded to illustrate high quality contrasts attainable by HRSEM at low (1-5 kV) and high (10-30 kV) voltages. Dispersed molecules on formvar coated grids were sputter coated with a 1 nm thick Cr film before employing scanning transmission (STEM)/SEM modes of the FESEM to establish non-decorative image accuracy in the transmitted electron mode.

Key Words: Planar magnetron sputtering, chromium, chemical fixation, high resolution scanning electron microscopy, capillary fenestral diaphragms, bulk biological samples.

Introduction

Advances in ultrathin metal film deposition techniques and high resolution scanning electron microscopy (HRSEM) provide recordings of molecular dimension from biological structure containing topographic contrasts that surpass transmission electron microscope (TEM) images of sections and platinum-carbon replicas. Given the inherent limitations of chemical fixation, solvent dehydration, and drying procedures on biological samples, and recognizing the future prospects for rapid freeze fixation and drying methods, molecular immobilization must be properly conducted to provide microscopic imaging of an unimpaired structure.

High quality imaging of molecular features in biological samples is achieved with HRSEM when topographic features of the specimen are not significantly enlarged by the metal coating and are resolved accurately. The central importance of an ultrathin metal film coating for HRSEM is to attain the highest resolution from bio-organic samples of low atomic number. Enhanced contrast of small specimen features is attainable by creating a greater yield of secondary electrons (SE)-I generated by the electron probe at the point of impact with the metal coated surface (Apkarian *et al.*, 1990).

Scanning electron microscope (SEM) operation in the SE-I signal mode for maximal resolution necessitates restricted secondary electron collection of specimen specific SE-I and SE-II components only and the signal quality will depend on the ratio of SE-I/SE-II collection (Peters, 1982; Apkarian, 1986). Because the yield of SE-I signal from hydrocarbon samples is low, an ultrathin continuous fine grain metal film may be applied to enhance the SE-I signal and enrich the recorded contrast of a bio-organic feature.

The HRSEM imaging of molecular features within the context of complex biological compartments such as organelles, cell membranes, or as isolated structures was made feasible with commercially available in-lens SEMs fitted with high brightness LaB_6 or field emission (FE) sources (Apkarian, 1987a,b). Operated at high acceleration voltages (15-30 kV; HV-HRSEM), in-lens FESEMs

can produce beam diameters of 0.5-1.0 nm and ultimate resolution of appropriately coated particulate features from biological specimens in the range of a few nanometers (Apkarian and L'Hernault, 1990; Apkarian *et al.*, 1990). Even at accelerating voltages as low as 1-5 kV, (LV-HRSEM) FESEMs can produce 2-3 nm beam diameters and particle resolution on biological materials in the 5-10 nm range (Joy, 1991).

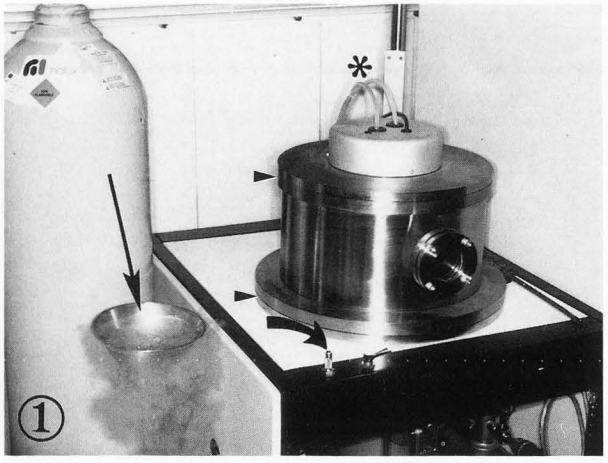
The ideal metal film would be composed of fine grains (smaller than the probe diameter) and have low or no surface mobility to achieve a non-decorative continuous coat at a minimal thickness. The high resolution signal capable of imaging 1-10 nm particle contrasts are generated by the specimen specific SE-I component. SE-I originate within the cross-sectional area of the probe and from an escape depth within the sample of 1-2 nm (Joy, 1984, 1991). Strategic generation and collection of a sufficient number of SE-I will result in imaging small surface features (1-5 nm) provided that a single or a minimal number of scattering events occur within the metal coated sample surface. A high grain density film will restrict the interaction volume thus reducing the number of scattering events (reduced SE-II signal component) occurring at a distance from probe impact. The image quality will have high fidelity because the total collected signal would have a high SE-I/SE-II ratio.

Precious metals (gold, silver, platinum) are deposited onto samples for conventional SEM to render them conductive. However the grain size, surface mobility and critical thickness necessary to produce a continuous monolayer film of these metals will decorate or completely blanket fine structure one wishes to resolve at high magnification (for a complete review, see Peters, 1986a). Deposited metals with grain sizes > 1-2 nm are unsuitable for 1-5 nm particle contrasts of biologically significant structures by HRSEM because their large grain size will cover the fine structure and create multiple scattering upon beam impact thereby generating a higher SE-II signal component. Fine grain metals such as tungsten (atomic number Z = 74), tantalum (Z = 73), chromium (Z = 24) and Titanium (Z = 22) have been used successfully to coat biological specimens and produce SE-I enriched topographic contrasts with HV-HRSEM (Peters, 1984, 1985, 1986b). Chromium is the primary metal of choice because the low Z metal would create lower background signal (Peters, 1989).

Several deposition methods for refractory metals such as Cr and Ta have been successfully employed in biological HV-HRSEM studies (Peters *et al.*, 1985; Peters, 1986a,b; Apkarian and Curtis, 1986; Apkarian, 1987b, 1991, 1993; Apkarian and Joy, 1988; Apkarian *et al.*, 1990; Goldberg and Allen, 1992; Hermann *et al.*, 1988; Müller and Hermann, 1991). Penning sputtering of ultrathin refractory metals in a high vacuum (10^{-5} torr range) was introduced over a decade ago (Peters, 1980, 1984). Manufacture of a commercially available high resolution refractory metal magnetron sputter system, employing a low vacuum (10^{-3} torr) was introduced by Denton Vacuum (Specht and Lutz, 1986). Nockolds (1982) employed a planar magnetron design to produce a fine grain Ta film thereby demonstrating that a quality refractory metal coating suitable for HRSEM studies could be deposited by PMSC. Parameters for ultrathin films produced at low vacuum by magnetron sputter deposition were defined and shown to evenly coat cell membrane features (Apkarian and Curtis, 1986; Apkarian, 1987, 1991, 1993). Others have chosen to deposit ultrathin continuous Cr films and reduce the self shadowing effect between metal atoms by using a double axis rotary shadowing system (DARS) (Hermann *et al.*, 1988). Recently, magnetron sputtered Cr and W on frozen hydrated yeast has produced quality SE-I contrasts. Frozen specimens staged in-lens, HV-HRSEM, could be imaged at 100,000 x and displayed fine particle contrasts of membrane domains (Hermann and Müller, 1993).

Magnetron sputter deposition is a more desirable method for creating ultrathin Cr or other refractory metal films because it has the least specimen exposure to radiant heat. Heat generated during the deposition process would subject the specimen to radiant energies that may compromise molecular integrity of surface features. Metals deposited with high radiant energies usually result in implanting metal grains into the specimen surface. Sputtering also has the potential for greater repeatability, coverage and control of metal deposition rates (Specht and Lutz, 1986). Regardless of the deposition method chosen, the quality of Cr or other refractory metal films useful for HRSEM in biological studies should be tested via deposition onto a coated grid for TEM analysis. SE-I contrast enhancement and coating accuracy on test samples of known molecular dimension from TEM studies should be conducted with HRSEM.

Specific criteria for the vacuum environment is essential in establishing a sputter system useful for repetitive deposition of quality ultrathin refractory metal films. Unlike the precious metals, refractory metals form oxides readily and if oxygen is present in a system in which gas plasmas are used, then certainly radical oxygen will attack the metal target resulting in deposition of a metal oxide layer. The compactness of the oxide film and the yield of the SE-I signal component could be quite different than for a pure metal film. Argon sputtering under specific vacuum and target conditions has high dispersion and secondary collision properties thereby increasing the adherence of Cr atoms total coverage with a precise mass average thickness. The bench-top sputter systems used to deposit precious metals with an average metric thickness of 2-20 nm and employing only



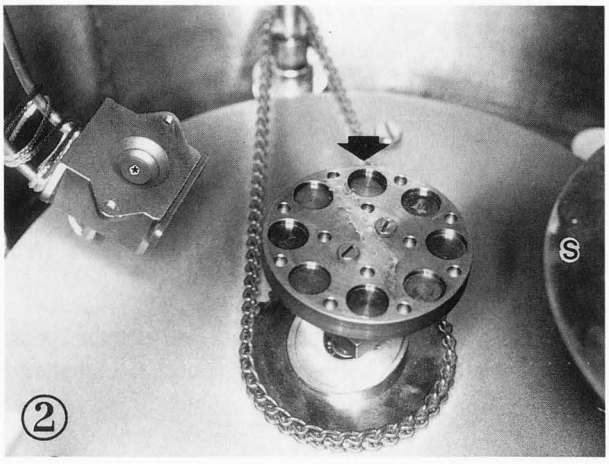


Figure 1. The chamber of the Denton DV-602 sputter coater is sealed to the baseplate and magnetron top plate by "O" rings (arrowheads). Water cooling lines (*) converge on the magnetron head. The LN_2 trap (long arrow) is used to help attain ultimate vacuum and the precision leak valve (curved arrow) is used to regulate exact Ar sputter pressures.

Figure 2. The water cooled QCM (*) and specimen stage (arrowhead) are isolated from the target during pre-sputtering by the shutter (S).

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a rotary pump clearly lacked the sophistication necessary to reproducibly deposit refractory metal films 0.5-2.0 nm thick. The methods and strategies for sputter system and vacuum design is provided to produce and ultimately test fine grain ultrathin refractory metal films.

Methods

The prototype Denton DV-602 turbo-pumped magnetron sputter system located at the Integrated SEM Facility of Yerkes Research Center was used to establish and test these Cr coating procedures. All subsequent discussion will provide design requirements and rationale based on this instrument. These parameters may serve as a model for establishing the necessary conditions to planar magnetron sputter ultrathin refractory metal films in any system.

Sputter chamber

The sputter chamber should be capable of sustaining a very high vacuum, in the 10^{-8} torr range. The system is routinely purged to an ultimate vacuum for removal of atmospheric gases, water vapor, and volatiles emerging from the "dried" sample. Safety considerations would dictate that a metallic implosion proof chamber is a preferable design. The DV-602 chamber is a stainless steel 7 inches (~ 18 cm) tall cylinder, 12 inches (~ 30 cm) diameter, and fitted with a 1 inch (~ 2.5 cm) quartz window. Neoprene "O" rings are used to seal the cylinder from the baseplate and the integral magnetron sputter head and top plate (Fig. 1).

The rotary-tilt, tumbling stage inside the chamber is at the same distance from the target as the Maxtek quartz crystal film thickness monitor (QCM; Fig. 2). Internal water lines provide constant temperature cooling of the target and quartz crystal. Computer controlled QCM provides accurate film thickness measurements and control of deposition via control of the shutter which shields the sample from the target during pre-sputtering. Below the chamber and base plate is the 5 3/4 inches (~ 15 cm) high vacuum valve (HVV) and cold cathode discharge vacuum sensor. Just below the HV valve is a liquid nitrogen (LN_2) cold trap. A metallic line within the chamber extends from the baseplate and delivers argon to the cathode vicinity.

Magnetron sputter cathode

Low voltage sputter coating of precious and refractory metals is known to produce finer grain size metal films than conventional diode sputter coating with only a high voltage (1-2 kV dc) power source (Nockolds, 1982; Echlin, 1985). The ionizing efficiency of the sputter system is greatly enhanced by trapping high energy electrons in a torroidal magnetic field in close proximity to the target metal. Increased collisions between the electrons and the argon gas atoms create a more efficient plasma at a lower cathode voltage (300 V dc). As positive ions strike the cathode, they liberate electrons by secondary electron emission. These emitted electrons accelerate toward the anode and ionize more neutral argon atoms creating more electron pairs in the interelectrode gap. Denton Vacuum utilizes an electrically

neutral grid 1.25 inches (about 3 cm) below the cathode to contain the ionization avalanche created along the magnetic lines of flux. The concentrated charged particle "sheath" remains at the cathode and away from the specimen. A quasi-neutral plasma fills the inter-electrode region in which the electron and ion number densities are equal (Singh and Denton, 1981). The high energy argon plasma sheath can more efficiently erode the target metal for rapid deposition rates.

The DSM-300 magnetron sputter head is located on the underside of the stainless steel lid. Coolant water lines converge onto the backing plate of the magnetron cathode from the top of the lid. Once the chamber is vented with dry nitrogen gas, the lid is hoisted up over the chamber for specimen loading. The south pole annular ring and the north pole center are constructed of powerful Samarium-Cobalt magnets. The path of erosion can be clearly seen on the 4 inch (~ 10 cm) diameter metal target surface (Fig. 3). As stated previously, the patented Denton grid located 1.25 inches (~ 3 cm) below the target confines stray electrons in the magnetic field and away from the sample stage. The true magnetron field strength measured 0.5 inches (~ 1.25 cm) below the target half way between the north and south poles is 200 gauss. The Cr and Ta targets are a 99.9% pure metal plate supplied by Demetron Inc.

Vacuum requirements

The most critical requirement of a sputter system for creating reproducible quality ultrathin refractory metal films is cleanliness. This is also taken to mean that residual atmospheric gases cannot be tolerated. The construction of the chamber and cathode described here provide high vacuum sealing against the atmospheric gases for compatibility with the high vacuum system. Chamber contaminants may still arise from the vacuum pumps, the argon gas, or the specimen. The design and specifications of the vacuum system dictate the ultimate vacuum which can be achieved on a chamber prior to the introduction of the specimen or argon. However, in a successful coating run, the specimen and argon must also be thoroughly purged.

Major hydrocarbon contaminants in the chamber originate within vacuum pump oils. It is therefore logical to design as oil free a system as possible. The manufacturers of several high vacuum deposition systems have avoided oil-diffusion pumps and have chosen to employ either turbo-molecular or cryogenic high vacuum pumps. The obvious advantage to cryopumping is that it is oil free. This method may have long pump down times and usually will consume large quantities of LN_2. Magnetic bearing turbo-molecular pumps are also oil free but are substantially more expensive than oiled bearing type turbo-pumps.

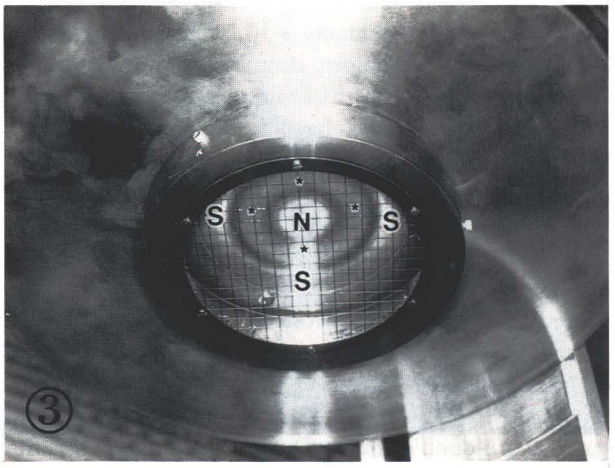


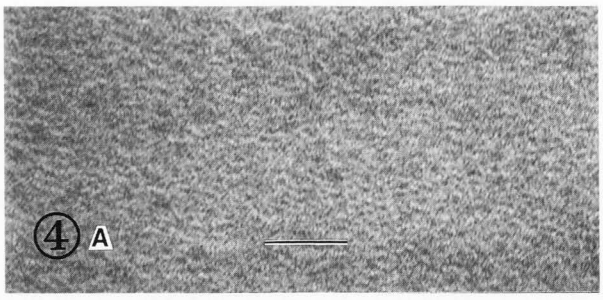
Figure 3. The north (N) central and south (S) annular poles of the Samarium-cobalt cathode create a torroidal zone of errosion (*).

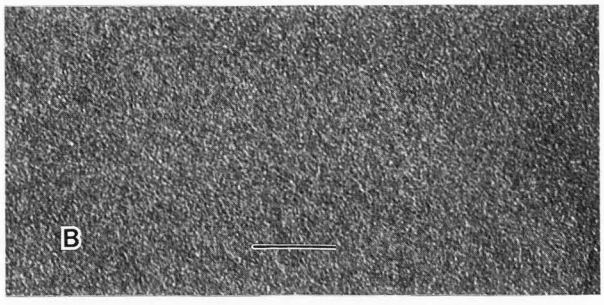
The DV-602 turbo-pumped vacuum system is designed to ensure low contamination levels. An oil-bearing 150 l/sec Leybold turbo-molecular pump (TP) is separated from the main chamber by a 5 3/4 inches (~ 15 cm) diameter high vacuum valve (HVV) and a LN_2 cold trap. The chamber is indirectly rough pumped by a two stage rotary mechanical pump (MP) through an in-line molecular sieve filter and a valved foreline to the chamber. The foreline is then valved off and the HVV is opened to the high vacuum turbo-pump. The backing valve couples the MP to the TP. During the initial instrument set up, copper tubing, which is used to deliver argon from the gas cylinder to the specimen chamber is evacuated back to the tank regulator by the vacuum system. The copper tubing ensured that only argon (99.99% Grade A) is bled into the chamber and atmospheric gases which can leak through tygon tubing were avoided.

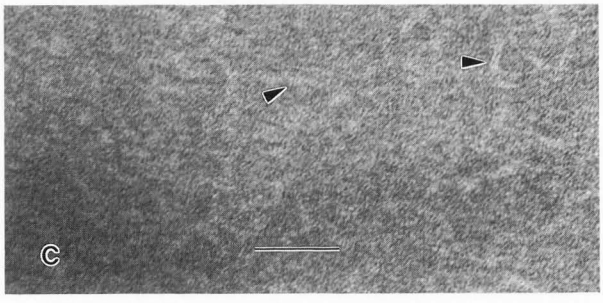
Sputter conditions and system operation

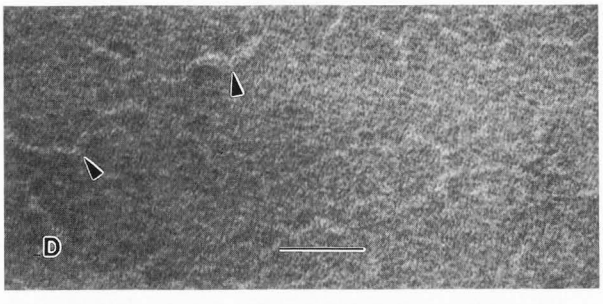
It is prudent to previously degas all specimens to 10^{-3} torr in a system other than the one used for refractory metal deposition. Although critical point or freeze dried specimens are theoretically devoid of moisture or trapped gases, volatiles from within the sample or the adhesive used to mount the specimen may outgas directly into the coating chamber. Outgassed hydrocarbons may remain adsorbed to the chamber walls and under subsequent high vacuum purging of the sample and chamber, may volatilize to contaminate the specimen or argon plasma.

The sputter chamber is vented with dry nitrogen gas for specimen loading and to avoid moisture entering from the room. After lowering the magnetron top plate









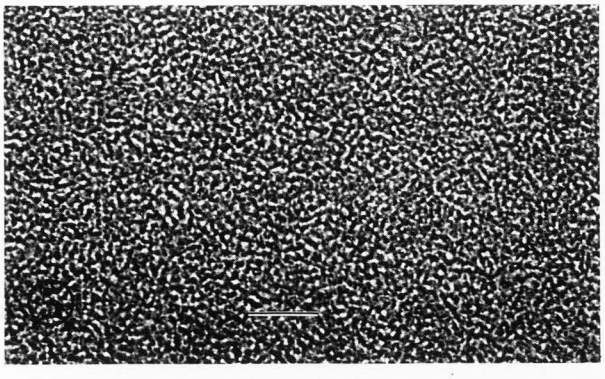


Figure 4 (at left). QCM thickness reading of 1 nm Cr films sputtered at (**A**) 25 mA; (**B**) 50 mA; (**C**) 75 mA; and (**D**) 100 mA. Films were sputtered on formvar coated grids and imaged at 80 kV on a JEOL 100 CX TEM. Note the film thickness irregularities at higher sputter currents. Bars = 20 nm.

Figure 5 (at bottom left). High magnification transmission electron micrograph (Topcon 002B at 200 kV) of 0.5 nm thick Cr film sputtered onto carbon film at 50 mA. Grain sizes were on an atomic scale and a continuous film was produced that was suitable as a quality coat at an ultrathin "critical thickness". Bar = 3 nm.

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onto the chamber, the system is evacuated. Because the argon leak valve into the chamber is opened during the chamber roughing sequence, the constant positive pressure eliminates the risk of oil back-streaming from the MP. Set to 200 millitorr, the DV-602 vacuum system cycles its valves to test for adequate chamber pressure before opening the HVV. The MP continues to evacuate the chamber against the Ar leak so long as the valve is left open. After the Ar leak valve is closed, the chamber pressure is met, the HVV is opened, and the TP rapidly attains 2x 10^{-6} torr vacuum pressure. The LN_2 cold trap is then filled and an ultimate vacuum in the 10^{-8} torr range is attained.

This high vacuum outgassing is important on two accounts. It provides a dry and stable specimen for metal film deposition and the specimen will not outgas further under the FESEM column vacuum. Specimen stability ensures against volatiles contaminating the sputtered metal and microscope column. The stabilized and metal coated specimen reduces risk of charging and drift during specimen observation.

A Hastings vacuum gauge may be used to regulate argon pressure by adjusting a precision needle valve. To ensure reproducible sputter conditions, the turbo-pumped chamber was always back-filled to 5 x 10^{-3} torr. The cathode voltage is 300 V dc and is powerful enough to touch off a plasma discharge at 6 x 10^{-4} torr. The initial plasma sheath created when current is applied has a sky blue center with violet trim indicating the presence of some metal oxides. The shutter remains closed until the entire plasma is sky blue. The lower vacuum setting was chosen and precisely maintained in all experiments because at this vacuum range adequate scattering within the Ar plasma was achieved for producing uniform films. The advantage of this vacuum setting is that the lowest plasma current and short deposition time can be used without overloading the turbo-pump. Target distance ($\sim$ 5 cm), cathode voltage, and Ar partial pressure are established as constant sputter conditions for this system.

Table 1. Deposition rates for a 1 nm thick Cr film.

Sputter Current	Time	Deposition Rate
25 mA	100 sec	0.1 Å/sec
50 mA	32 sec	0.3 Å/sec
75 mA	20 sec	0.5 Å/sec
100 mA	14 sec	0.7 Å/sec

Results

The two remaining parameters that regulate the sputter deposition are current and time. A study was conducted to determine what current to apply to produce a uniform and continuous film in the least amount of time (Table 1). Cr deposition rates were determined for plasma currents ranging from 25 to 100 mA and film quality was directly assessed by TEM. A through focus series, imaged by TEM at 80 kV (JEOL 100CX) and 200 kV (Topcon 002B), was performed on Cr films (continuous metric thickness of 1.0 nm) which were deposited on formvar or carbon coated grids.

Although 1 nm thick films appeared continuous and fine grained in the TEM at all sputter currents, thickness variations or undulations were apparent when sputter currents above 50 mA were applied (Figs. 4c and 4d). High magnification TEM employing a 200 kV source revealed an amorphous or fine-grain image (Fig. 5). Based on these results, it was concluded that for sputtered Cr films in the 1.0-2.0 nm thickness range, a 50 mA current (0.3 Å/sec) would produce a smooth, continuous, fine grain film in the shortest time. The short deposition time reduces the risk of hydrocarbon contamination of the film (Peters, 1986a).

Non-biological hydrocarbon samples of polyvinylstyrene were mounted onto aluminum stubs and coated with 1 nm of Cr. HRSEM was performed in-lens on a DS-130F Schottky field emission (SFE) SEM operated at 20 and 30 kV (Fig. 6). Photographic recordings at a cathode ray tube (CRT) magnification of 800,000 x resolved 3.75 and 2.5 nm styrene particles at 20 and 30 kV, respectively. The image was recorded at a 16 second scan rate and no digital enhancement was performed. The lack of Cr metal granularity attests to the accuracy of the image.

Uranyl acetate stained synthetic amphiphiles (Apkarian *et al.*, 1993) were sprayed onto formvar coated grids and Cr coated with a 1 nm thick film. STEM images of globular and rod-like structures were recorded from the DS-130F SEM operated at 25 kV (Fig. 7). Particulate fine structures, 3.5 nm in diameter, were resolved within the rods while Cr grain structure was not resolved. The 1 nm Cr film on the formvar supported negatively stained amphiphile, served to enhance electron density and STEM contrast. The interaction of the 1 nm diameter 25 kV low voltage SFE source when scanned across dispersed particles on a formvar support grid created very low radiation damage. A 16 second scan was used for CRT recording on P-55 film. Cr coated organic specimens were useful to determine image quality of hydrocarbon based samples similar to bio-membranes in the HRSEM and STEM modes.

Figure 6 (at right). Polyvinylstyrene organic samples were coated with 1 nm Cr and imaged at (**A**) 30 kV and (**B**) 20 kV by HRSEM. These hydrocarbon test specimens were very stable under a 25 kV FE source. At magnifications over 1,000,000 x, particulate features (arrowheads) 2-4 nm on elevated regions and depressions were equally resolved. The granularity of the metal film was not resolved by HRSEM. Bars = 10 nm.

Isolated macromolecules and viral fragments dispersed onto supports, Cr coated, and imaged with HV-HRSEM contain SE-I particle contrast in the 4 nm range (Müller *et al.*, 1993). We have attained macromolecular resolution from Cr coated soft and hard biological tissue specimens by HV-HRSEM (Apkarian and L'Hernault, 1990; Apkarian *et al.*, 1990). Both LV-HRSEM and HV-HRSEM studies utilizing in-lens FESEMs greatly benefited from enhanced SE-I contrasts when specimens are properly coated with Cr.

An application in which LV-HRSEM was determined to produce the best SE-I topographic contrasts involved multi-layered cultured cells. Neural cells (astrocytoma) grown on silicon chips in culture flasks were fixed, dried, Cr coated (1 nm), and ultrastructural studies were performed at 5 kV (Fig. 8). Low magnification survey of the cultured cells showed that they were multi-layered. Regions of surface cell bodies which were adjacent to one another were examined for characteristic plasma membrane (PM) features. At intermediate magnification (50,000 x CRT), the recorded SE-I signal from a thin continuous 1 nm Cr coated cell surface contained topographic contrasts of fine particulate ectodomains. Frequently, these cells contained clusters of 400 nm blebs which appeared to be vesicles pushing outward from the cytoplasmic surface of the PM. At high magnification (200,000 x CRT), quality SE-I imaging provided surface information that contained particle contrast in the 5-10 nm range. The region of the PM which had been stretched by an underlying organelle displayed particulate ectodomains of the same dimension as on the flat regions of the cell surface. The self shadowing effect between metal atoms was diminished and PMSC operated at a high enough energy so that the metal atoms had low mobility and did not decorate individual free standing PM glycoproteins.

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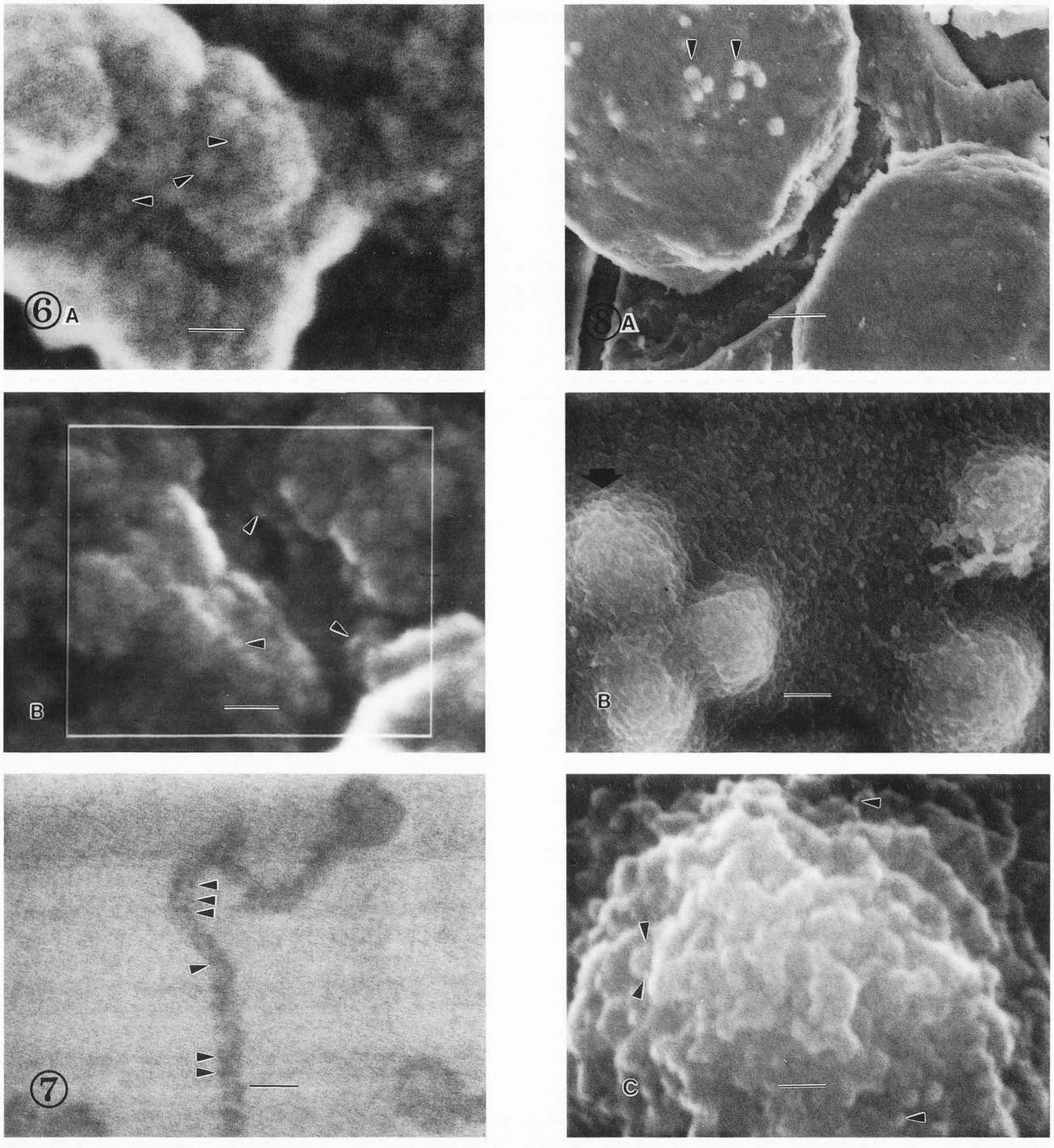

Figure 7. Synthetic amphiphiles sprayed onto formvar-grids and Cr coated with a 1 nm film were imaged on the STEM mode of the DS-130 FESEM at 25 kV. Rod-like regions, 10 nm wide, displayed rows of adjacent particles (arrowheads) which appeared zippered together. Bar = 20 nm.

Figure 8. LV-HRSEM of astrocytoma multicell layered cultures coated with 1 nm Cr. (**A**). Membrane surfaces of adjacent cells reveal vesicular shaped elevations (arrowheads). (**B**). The entire cell surface appeared covered by particulate fine-structure; an elevated region marked by arrow is magnified in (C). (**C**) Cr coated ectodomains (arrowheads) on elevated membrane surfaces < 10 nm were imaged. Bars = 2 μm (A), 200 nm (B), and 50 nm (C).

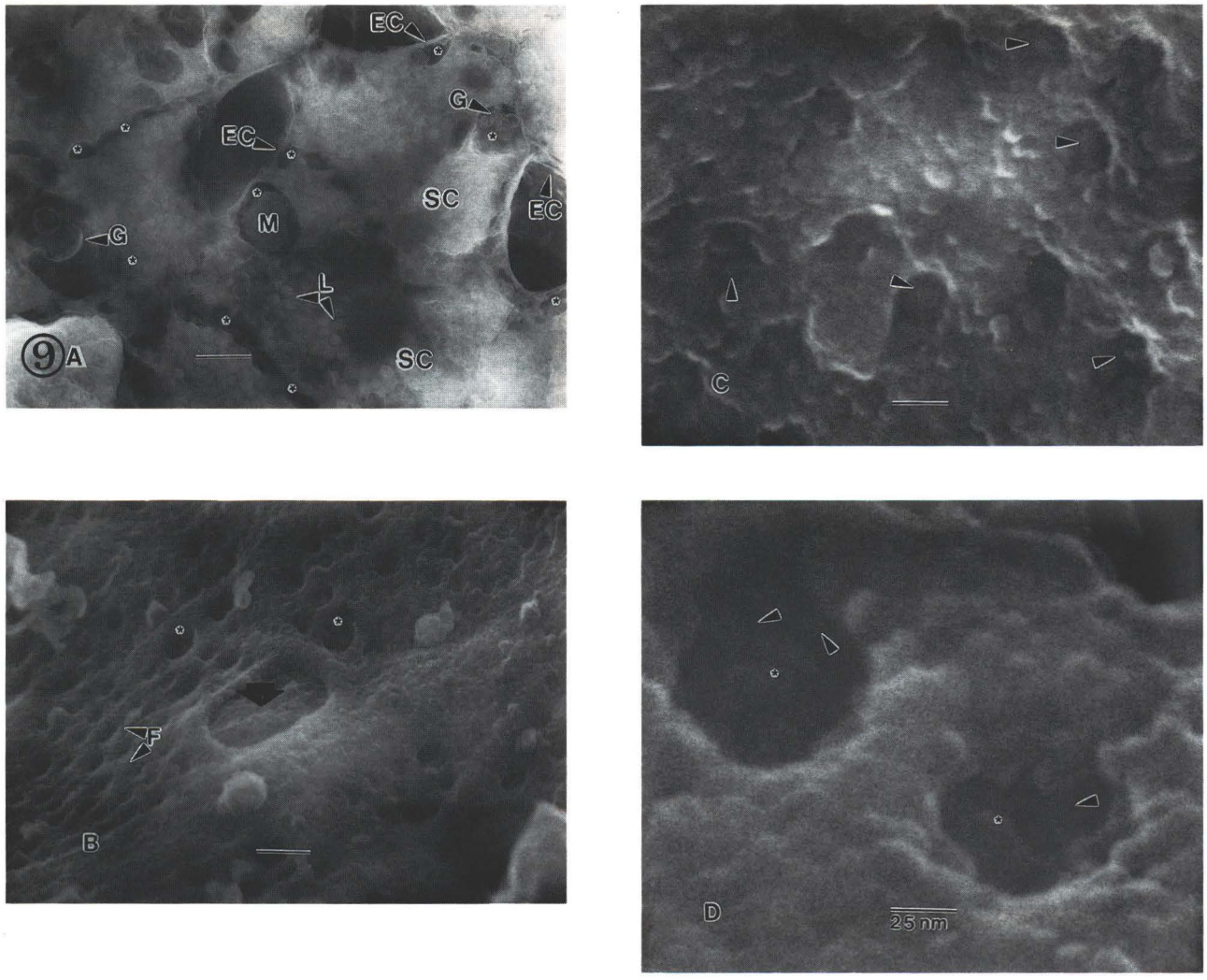

Figure 9. HV-HRSEM of cryofractured and Cr coated rabbit adrenocortical tissue. (**A**). Thin endothelial cell (EC) expanses and steroid cells (SC) form perivascular and intercellular spaces (*) for exchange. These spaces contain membrane ghosts (G) and an occasional wandering macrophage (M). Note retention of intracellular lipid droplets (L). (**B**). EC lumenal surfaces contained clusters of fenestrae 50-60 nm (F), larger 120 nm transendothelial openings (*), and a rarely seen depression (arrow) whose base appeared continuous with basement membrane components. (**C**). 50-60 nm diaphragmed openings with a central density (arrowheads) appeared to be fenestrae do to their clustered seive plate like arrangement. (**D**). Diaphragms of 80-90 nm openings contained a central density (*) and what appeared to be radiating spokes (arrowheads). (**E**). The regions between transendothelial openings contained many membrane particles (arrowheads) that represent the ectodomains of glycoproteins. Bars = 5 μm (A); 200 nm (B); 50 nm (C), and 30 nm (D and E).

Cryofractured adrenocortical tissue fragments contain the lumenal aspect of fenestrated capillary endothelium recessed below the cryofractured plane of the specimen surface. These cells, which express a variety of different type openings of similiar dimension and ectodomains of membrane glycoproteins, have been studied by HV-HRSEM and TEM (Apkarian and Curtis, 1986; Apkarian, 1987a, 1993; Apkarian and L'Hernault, 1990). Perfuse fixed adrenocortical fragments were treated with tannic acid and *para*-phenylenediamine (Robinson and Apkarian, 1992). This effective mordant/stain retained tissue lipids and preserved lipid droplet and membrane moieties in inter- and intracellular spaces. Dried specimens were ultrathin coated with 1 nm Cr. Adrenal fragments were in-lens staged on the DS-130 FESEM at 25 kV (Fig. 9). Intermediate magnification SE-I enriched images revealed clusters of small 50-60 nm openings, 60-90 nm openings, and an occasional large 200 nm opening.

High magnification images revealed the smallest openings, presumed to be fenestrae, containing a diaphragm with a central particle. Minute particulate features depict glycoprotein PM ectodomains that border the openings and cover the cell surface not occupied by an opening. Larger 80 nm openings also appear to have a diaphragmatic entity and may represent diaphragmed vesicles or double diaphragmed channels while the largest openings correspond in dimension to non-diaphragmed coated pits.

Discussion

High magnification TEM provided the necessary image resolution to appreciate the continuity and uniformity of fine grain Cr films produced by PMSC on carbon and formvar supported grids. Cr films were measured at 0.5 and 1.0 nm thickness by quartz crystal monitor (QCM) and contained 1-2 Å grain size when measured directly from high magnification TEM micrographs. Water cooled QCMs with computer control can be trusted to oscillate and reach a resonant frequency that can be translated into a film thickness reading. The monitor reading is a very close approximation of an actual metric measurement provided that a "tooling factor" is programmed into the sputter system. Regardless of QCM accuracy, readouts should not be taken to represent a precise measurement.

The PMS deposition strategy employed ultraclean vacuum methods to ensure stable specimens and the production on large specimens of a pure ultrathin continuous refractory metal film coating . Sputter currents and deposition rates were determined to establish parameters that eliminated even the finest undulations and created reproducible sub-nanometer thick films on formvar and carbon support. The powerful PMS cathode with electron retaining grid created a high energy plasma capable of eroding the pure Cr target without thermal radiation and damage of the specimen.

The description of adrenocortical capillary studies illustrated the ability to attain quality SE-I enriched HV-HRSEM images of glycoprotein membrane ectodomains < 10 nm and intact fenestral diaphragms when a 1 nm ultrathin fine grain Cr film is deposited by PMSC onto a bulk biological specimen. Diaphragm components from within fenestrae, located on the luminal surface of adrenocortical capillaries, were visualized directly by HV-HRSEM. The contrast quality of a molecular membrane component, such as the fenestral diaphragm, is a result of beam interaction with a Cr coated lumenal surface of the capillary recessed 2-5 μm from the surrounding fractured cortical cell surface. Although field emission sources are intense electron emitters, the SE-I yield from fenestral diaphragms composed of hydrocarbon on a recessed specimen surface would be poor if it were not for the even Cr coat.

Polyvinylstyrene is a bulk hydrocarbon specimen which when Cr coated provided imaging of 2-3.5 nm particles at 20-30 kV on the in-lens stage of the DS-130 FESEM. Undoubtedly, computerized digital image capture and processing will provide greater signal quality and clear delineation of nanometer size low atomic number particulate features.

Astrocytoma, neuroblastoma, and co-cultures of both cell types (unpublished results) were also imaged with enriched SE-I contrasts when PMS coated with a 1 nm Cr film. Substance "P" receptor is thought to be expressed on this cell type. Imaging of ectodomains in the 5-10 nm range on the surface of these cells was possible using LV-HRSEM but digital image processing is a practical necessity due to weak particle delineation. Because FESEMs can produce small beam diameters when operated at low or high voltage, Cr restricts the interaction volume and will enrich SE-I signal contrasts at what ever voltage is suited. Often, the specimen mass and mounting support will influence the interaction volume and selection of what accelerating voltage to apply in order to attain maximal resolution at high magnification.

Chromium films were used to coat synthetic amphiphiles (Fig. 8) and isolated lipoproteins for simultaneous SE-I and STEM recordings (Apkarian *et al.*, 1993). The STEM and upper secondary electron detector collected their respective signals from the DS-130 FESEM operated at 25 kV. This operational strategy created simultaneous images with SE-I topographic contrasts (HV-HRSEM) and scanning transmission bright field imaging (LV-HRSTEM). The fine granularity Cr coat did not obscure the particulate features which gave the

100 Å wide rod-like structure a zippered appearance in the STEM mode.

In summary, cleanliness and a quality vacuum system combined with a powerful and shielded planar magnetron sputter cathode produced depositions of ultrathin fine grain Cr films useful for contrast enhancement in HRSEM and STEM. Biological studies will be directed toward molecular, cellular and tissue specimens which combine immunolabelling for membrane glycoprotein identification and Cr coating for contrast enhancement. Further application of ultrathin refractory film deposition methods may provide useful coatings for atomic force, electron energy loss, and other microscopies.

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Discussion with Reviewers

M. Malecki: Can you analyze grain geometry you obtained with PMS at room temperature as compared to that obtained at low temperatures (Hermann and Müller, 1991a,b; Muller *et al.*, 1990)?

W. Wergin: Would you comment/speculate on the advantages and/or disadvantages of using PMSC to deposit ultrathin Cr films on frozen biological specimens for low temperature FESEM observation?

Author: The growth conditions of an ultrathin metal film are dependent on surface coverage and average mass thickness (Peters, 1986). During argon plasma sputtering of Cr in the turbo-pumped Denton system maintained at $5x10^{-3}$ torr, the "secondary energy" transferred to the metal atom is very low because of the "field" containment by the cathode grid (see Fig. 3). A powerful magnetic force created close to the metal target results in removal of Cr atoms with a high primary energy but low substrate surface mobility so that nucleation of the transitional atoms was increased and surface coverage was accomplished with a minimal amount of metal in a short time (< 1 minute). Sputtered Cr atoms have low lateral diffusion properties on the specimen surface, are of low atomic number, and have low backscatter coefficients (Chopra, 1966). A frozen specimen would further lower the secondary energy of the metal atoms on its surface as compared to a specimen at ambient temperature, resulting in a higher nucleation density and a reduced critical film thickness (Neugebauer, 1970). However, the Cr films are nearly amorphous when sputtered at ambient temperature, due to high adherence properties of tightly packed small atom clusters to the specimen surface that coalesce and form a continuous ultrathin film.

Cryo-specimens coated with Cr by PMS (Müller *et al.*, 1990; Hermann and Müller, 1991a,b) and observed with low temperature (LT) HRSEM revealed quality topographic contrasts. Cryo-fixed and staged specimens do yield images with improved structural accuracy of specimen features over HRSEM observation made from chemically fixed specimens, but the metal film would serve to localize the signal in any case. It is my experience that the compact nature of a fine-grain Cr film deposited by this PMS system would reduce specimen self-contamination, and mass loss during electron irradiation, whether the sample has been chemically fixed and dried or cryo imaged.

PMS coating with Cr for LT-FESEM imaging of frozen biological specimens would be adventageous in terms of its technical quality; superior morphological preservation of the living non-fixed state, so long as high magnification imaging (> 50,000 x) was required.

Lower magnification images from Cr coated specimens lack contrast because of the low backscatter yield from Cr and since the area being scanned is beyond the signal range of the SE-I component (see Fig. 9A).

M. Malecki: Can you comment on coating uniformity of complex structures obtained with PMS and double-axis rotary shadowing?

T.D. Allen: What about the possibility of directional coating, and consequently shadowing artefacts from the use of relatively high vacuum conditions for sputtering?

Author: Mass thickness contrast effects from DARS deposition of Cr onto structures has been estimated to be three times higher on top of a structure as on it's sides (Hermann *et al.*, 1988). Procedures were developed for DARS to produce a more homogeneous film capable of generating high resolution SE-I particle contrasts on freeze-dried, beam transparent T-phage capsomere proteins with image quality at least as good as images formed with transmitted electrons. These ultrathin test

specimens have very small interaction volumes due to their physical dimensions and naturally low SE-II background signal. The same authors achieved similar image quality after PMS coating with Cr.

PMS is better than evaporation methods for eliminating shadowing and uneven film thickness artefacts and allows for bulk specimen analysis. PMS coatings with the Denton system (1 nm Cr film) produced images with quality SE-I particle contrasts from cell membrane surfaces within a large 1 mm^3 piece of adrenal tissue. High primary energy sputtering (low surface diffusion) evenly coated the specimen surface including the lumenal aspect of fenestrated capillary walls (Apkarian, 1993). Previous freeze-fracture and etched platinum replica preparations of fixed tissue for TEM studies (Bearer and Orci, 1985) revealed internal structures of fenestral diaphragms. SE-I particle contrast of fenestral diaphragms and membrane ectodomains extends the three-dimenional appreciation and understanding of these macromolecular complexes. Cr coated adrenal fragments allow for hundreds of capillary lumen to be viewed in a single specimen. Molecular resolution of Cr coated fenestral structures within the context of a large bulk sample provided by HRSEM greatly extends aquisition of fenestral morphology limited by TEM replica size.

The high background pressure of the PMSC system (10^{-8} torr) helps reduce metal aggregation and scattering. If the Cr target is properly etched to remove gas atoms before specimen coating, then argon vacuum pressures of 10^{-3} torr are suitable for the PMC cathode with grating to deposit Cr at a moderate rate. The position of the cathode directly over the entire specimen stage insures film formation is dominated by the Cr atoms high primary energy; shadowing has not been observed. Penning sputtering is done with a high energy gun, in a higher argon vacuum (10^{-5} torr) than for PMSC, and results in a very low deposition rate ultrathin film without shadowing so long as the specimen is in a tumbling motion (Peters, 1980).

M. Malecki: What is your experience in the safe sample transfers from dryer to coater, and from coater to a microscope, as compared to multifunctional or directly linked units?
Author: Isolated molecules and cultured cells on silicon chips were removed from the dryer, silver pasted onto aluminum supports and immediately degassed prior to coating. Bulk adrenal tissue were also pasted onto the aluminum supports. It was important to avoid solvent absorption into the bulk tissue fragments by employing a thick silver paste. Cr coated specimens endure transfer to the microscope or to storage dessicators without noticable effect to image quality as long as low humidity is maintained. Experiments with multifunctional, rapid transfer, and coating chamber linked devices have not been performed.

T.D. Allen: What exactly causes the wrinkles in the 1 nm coatings deposited at higher energy? Is it contraction of the chromium film?
Author: I do not believe the wrinkling is due to film contraction but rather due to vertical stacking of high secondary energy atom clusters. Although I do not have empirical evidence, it is possible that higher cathode currents promote cohesion of metal atoms to one another rather than adhesion to the substrate surface, such that atoms may cluster and stack to form these minute wrinkles.

T.D. Allen: Did you try imaging uncoated amphiphiles?
Author: Uncoated amphiphiles are beam sensitive and the high accelerating voltage necessary to resolve these hydrocarbon specimens at high magnification rapidly caused radiation damage. Cr coated specimens were greatly stabilized and had adequate contrast.

T.D. Allen: In practical usage, total plasma time (30-60 seconds for removal of Cr Oxide layer on target, and 20 seconds for deposition) does not warm the surface perceptibly in our own system. No cooling of the target means no condensation on the target at the end of each run. Is this a worthwhile consideration?
Author: Cooling the cathode serves to maintain the target close to ambient temperatures during sputter deposition thus decreasing the secondary energy transferred to the Cr atoms. The cooling water temperature is only a few degrees below room temperature and dry nitrogen gas is vented constantly into the chamber during specimen transfer to keep condensation to a minimum.

T.D. Allen: You quote cold trapped turbo-pumping as a good system for reducing vacuum contaminants. How does this cope with water vapour?
Author: The routine procedure for the sputter chamber pump down is to turbo-pump the chamber to the low 10^{-6} Pa range without LN_2 in the cold trap so that the turbo-pump removes the bulk of the residual water vapor. Once this vacuum level is achieved, then LN_2 is added and another order of magnitude of vacuum is achieved. The residual water molecules along with hydrocarbons condense onto the trap. Once the system is turned off and the cold trap returns to ambient temperatures, there is negligible amounts of water that can be reintroduced into the chamber.

T.D. Allen: What fixation did you use for optimal preservation of cell surface? Do you think that standard preservation protocols, as used in TEM, are automatical-

ly optimal for HRSEM? Did you try similiar preservations to that incorporated for your cryofractured adrenocortical preparations on tissue culture cells?

Author: I have used a standard glutaraldehyde-osmium tetroxide fixation for both HRSEM and TEM adrenal studies (Apkarian and Curtis, 1986; Apkarian and L'Hernault, 1990). More recently, tannic acid and *Para*-phenylenediamine (TA-PDA) and ruthenium red have been added to provide improved preservation of glycocalyx and extracellular lipids associated with membranous structures. Each tissue or cell system may have their own optimal fixation protocols for any image mode and should be assessed accordingly. Cultured cells were aldehyde fixed and osmicated for HRSEM analysis similar to adrenal tissue but for shorter time periods.

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