Photoelectron Imaging of Viruses and DNA: Evaluation of Substrates by Unidirectional Low Angle Shadowing and Photoemission Current Measurements

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ABSTRACT Photoelectron imaging (photoelectron emission microscopy, PEM or PEEM) is a promising high resolution surface-sensitive technique for biophysical studies. At present, image quality is often limited by the underlying substrate. For photoelectron imaging, the substrate must be electrically conductive, low in electron emission, and relatively flat. A number of conductive substrate materials with relatively low electron emission were examined for surface roughness. Low angle, unidirectional shadowing of the specimens followed by photoelectron microscopy was found to be an effective way to test the quality of substrate surfaces. Optimal results were obtained by depositing ~0.1 nm of platinum-palladium (80:20) at an angle of 3°. Among potential substrates for photoelectron imaging, silicon and evaporated chromium surfaces were found to be much smoother than evaporated magnesium fluoride, which initially appeared promising because of its very low electron emission. The best images were obtained with a chromium substrate coated with a thin layer of dextran derivatized with spermidine, which facilitated the spreading and adhesion of biomolecules to the surfaces. Making use of this substrate, improved photoelectron images are reported for tobacco mosaic virus particles and DNA-recA complexes.

INTRODUCTION

There are a number of new or improved ways of imaging DNA and DNA-protein complexes (Howard and Griffith, 1993; Jett and Bear, 1994; Rees et al., 1993; Lyubchenko et al., 1993; Habliston et al., 1993; Bazett-Jones, 1992). The unique advantage of photoelectron imaging (PEM or PEEM) is that it can provide spectroscopic information in addition to a topographical map. The specimen surface is illuminated by a broad beam of UV light resulting in the emission of electrons (the photoelectric effect). These low energy electrons are accelerated and then imaged by a series of electron lenses in a photoelectron microscope (Fig. 1). It is known that there are differences between the ionization energies of the four bases of DNA (Yu et al., 1978; Urano et al., 1989), and modeling experiments show that it should be possible, in principle, to utilize these differences to provide a physical map of DNA modulated by the base composition along the double helix (Griffith et al., 1990). This map, although not at base-sequencing resolution, would permit an identification of the regions of DNA being imaged. A second advantage of photoelectron imaging is that large regions of a genome or cell surface could be examined simultaneously because of the large field of view. However, in order to achieve the potential of photoelectron imaging, substrates must be improved. We recently initiated a search for substrates based on relative photoemission (Habliston et al., 1993). Here we combine two criteria for the selection of substrates: photoemission current and surface topography. We find that unidirectional shadowing of substrate materials at low angle followed by photoelectron imaging is a useful tool to assess the suitability of substrates from the standpoint of surface roughness. We report here the first photoelectron images of shadowed DNA and use this technique to evaluate substrates and to obtain improved photoelectron images of unshadowed recA-DNA complexes.

MATERIALS AND METHODS

Substrate preparation

All substrates with the exception of silicon were on no. 1 glass microscope coverslips 5 mm in diameter (Belco Glass, Inc., Vineland, NJ). The glass coverslips were carefully cleaned before use by first sonicating in dilute aqueous detergent (MICRO; International Products Corp., Trenton, NJ) followed by extensive rinsing in deionized water and overnight soaking in 3 M HCl-3% H2O2. Finally, after additional rinses in deionized water, the discs were air-dried from distilled acetone in a laminar-flow clean air cabinet. Silicon discs (5 mm diameter, polished on one side, N type (1–1–1)) were obtained from International Wafer Service (Portola Valley, CA). The silicon wafer from which the discs were cut had been highly doped with phosphorus (bulk resistance of 0.008–0.02 ohm-cm) to provide surface conductivity to prevent buildup of a surface charge as electrons are emitted.

Before use the wafers were cleaned by sonicating in a dilute aqueous solution of MICRO, sonicated in deionized water, and air-dried from distilled acetone. Coatings of gold, chromium, or magnesium fluoride were obtained by resistive heating in an oil-free Varian Vacuum Products (Lexington, MA) FC-12 vacuum evaporator at a pressure of ~1 × 10^-7 mm Hg. Film thickness was monitored with a Leybold Inficon (East Syracuse, NY) XTM thin film thickness monitor employing a cooled quartz crystal oscillator. All evaporated films were 5 nm thick. Spermidine-derivatized dextran was prepared by the procedure of Hicks and Molday (1985), except that spermidine (Calbiochem, La Jolla, CA) was used in place of ethylenediamine. Silicon and chromium-coated discs were coated with spermidine-derivatized dextran by floating the discs on aqueous solutions (10 μg/ml) of this material on a Teflon block for 10 min. The discs were rinsed using a continuous flow procedure in which water was rapidly added to the drop of derivatized dextran and aspirated off over the course of 1–2 min followed by air drying.
DNA preparations

Relaxed, circular, double-stranded φX174 (RF II form) and virion (single stranded) φX174 were obtained from New England BioLabs (Beverly, MA). Complexes of virion φX174 and recA were prepared as follows. To a 1.5-ml microfuge tube were added 12.5 μl of 4X buffer (100 mM HEPES, 4 mM magnesium acetate, pH 7.2), 25.5 μl deionized water, 4 μl (0.4 μg) virion φX174, and 8 μl recA protein (2 mg/ml; New England BioLabs) followed by incubation for 1 h at 37°C. Then 12.5 μl of 0.5% glutaraldehyde (Polysciences, Warrington, PA) was added for fixation, and the mixture was incubated for 20 min at 37°C followed by addition of glycerine (200 μl) to react (30 min at 37°C) with excess glutaraldehyde. Unbound recA protein was separated from the recA-DNA complexes on a 1-ml Sepharose 4B column prepared in a Pasteur pipette. 150 μl fractions were collected and were examined for the presence of recA-DNA complexes by transmission electron microscopy after negative staining. Photoelectron microscopy samples were prepared by floating the 5-mm-diameter substrates first on a 40 μl drop of either recA-φX174 (at a DNA concentration of 0.1 μg/ml) or naked DNA (1 μg/ml) for 30 min and then on a 40-μl drop of water containing ~10^9 tobacco mosaic virus particles (TMV; American Type Culture Collection, Rockville, MD) per ml for 10 min. The samples were rinsed using the continuous flow procedure described above, after which the added water was replaced with ethanol. Aspiration and addition of more ethanol continued until the final concentration of ethanol was 100%, after which the sample was air-dried.

Shadow casting

A schematic diagram of the shadowing procedure used in this study is shown in Fig. 2. The prepared PEM discs (silicon or chromium-coated glass) were mounted on a goniometer stage 20 cm from the evaporative source (0.89 mm diameter tungsten wire, bent into a V-shaped filament, and wrapped with 1.5 cm of 0.20 mm diameter platinum: palladium: 80:20 wire; Ted Pella, Inc., Redding, CA) at an angle of ~3°. The quartz crystal oscillator was mounted normal to the source and at the same distance as the PEM discs. The electrical current to the source was increased to ~40 A to maintain a deposition rate of 0.05 nm/s until a thickness of 1.6 nm was measured on the quartz crystal. The actual deposition on the sample at 3° is then ~0.1 nm. The evaporation was carried out on a specially constructed fixture connected to the Varian FC-12 vacuum evaporator described above. Upon completion of the evaporations, the sample discs were immediately transferred to the sample chamber of the photoelectron microscope and placed under oil-free high vacuum.

Photoelectron imaging

The photoelectron microscope used in this study is of oil-free design and utilizes the output from two Osram (Berlin, Germany) HBO 100 W/2 short-arc mercury lamps to stimulate photoemission. The instrument has been illustrated elsewhere (Rempfer et al., 1991; Habliston et al., 1991) and is illustrated schematically in Fig. 1. In the previous version of the microscope the UV light was reflected off the anode of the objective lens and onto the specimen. The instrument has been redesigned to allow direct illumination of the specimen as shown diagrammatically in Fig. 1, resulting in more light striking the sample and less contamination of the anode. Exposure times were 2 min or less for all photoelectron micrographs.

Photoelectron emission measurements

Relative substrate photoelectron emission was measured by collecting the electrons emitted from a variety of potential substrate materials. The substrates were prepared on 5-mm diameter round glass coverslips with the exception of silicon, which was purchased in the form of 5-mm round discs. For these measurements the electron optics of the PEM were adjusted as follows. The projector and intermediate electron lenses were turned off and the objective lens was adjusted to near crossover with the cathode at ~30 KV. The effect is to put the microscope in a very low magnification mode to collect the maximum number of electrons over the largest sampling area. The electrons were collected on an aluminized fiber optic and measured with a Keithley Instruments (Cleveland, OH) model 26000 picoammeter connected to the fiber optic through a BNC feed-through and cable. The relative photoelectron currents are reproducible to within 20%. Electron emission from evaporated gold is stable over extended periods of time and was used as a reference standard for all substrate emission measurements.

RESULTS

Substrate electron emission

Relative photoelectron emission data from a number of potentially useful PEM substrates are shown in Fig. 3. On this logarithmic scale plot, all of the substrates tested are at least 40-fold lower in photoelectron emission than the highly photoemissive gold standard (Pt/Pd is not a potential substrate,
FIGURE 3 Relative photoelectron emission of the substrate materials. Included also are measurements on uniform layers of Pt/Pd at the thickness (0.1 nm) used for shadowing and of the protein, recA. All measurements were made relative to 5-nm-thick films of evaporated gold which served as a stable standard. The emission currents are plotted on a log scale with the emission from gold arbitrarily set at 100. SD, spermidine-derivatized dextran.

but is included because it is used in shadowing). A 5-nm layer of MgF₂ evaporated over chromium had the lowest photoemission of any substrate tested, being only 25% of that of the next lowest material, uncoated chromium. Electron emission from silicon was slightly higher than from chromium. Chromium and silicon substrates tend to be quite hydrophobic and bind some biomolecules poorly. To improve the binding, a layer of dextran derivatized with spermidine was applied to chromium and silicon discs. The coating makes the discs distinctly hydrophilic and improves the attachment of TMV and DNA. As shown in Fig. 3, electron emission from chromium and silicon coated with spermidine-derivatized dextran is only slightly higher than that of the uncoated substrates. The beam current of recA is included to indicate the relative photoemission to be expected of recA-coated DNA and as a reference point for proteins in general. All proteins examined thus far produce similar photoelectron emission currents (Habliston et al., 1993).

Detection of surface roughness using low angle shadowing and PEM

As with substrate photoemissivity, substrate surface roughness can have a major impact on image quality, especially when the object being imaged is similar in dimensions to the surface irregularities. Surface roughness is difficult to detect in normal photoelectron images when the substrate has low photoemissivity. An example is shown in Fig. 4a, which shows a normal unshadowed photoelectron image of TMV on a MgF₂-coated chromium substrate. The TMV particles are easily visible, showing up brightly against what appears to be a relatively smooth, dark background, consistent with the low electron emission of MgF₂ seen in Fig. 3. However, a different picture of the background emerges if a similar preparation is unidirectionally shadowed with metal at low angle before photoelectron microscopy as shown in Fig. 4b. From Fig. 4b, it is clear that the surface of the metal-coated MgF₂ substrate is so rough that shadows cast by the surface topographical features almost completely obscure the TMV particles. The reason that the TMV particles show up so well in Fig. 4a is due to the material contrast. That is, the TMV particles give off many more electrons than does the substrate. This material contrast is lost in Fig. 4b because the entire surface is coated with metal. Only topographical contrast remains in Fig. 4b. Thus, the MgF₂-coated substrate is quite satisfactory for imaging TMV but Fig. 4b provides an explanation as to why it is difficult to image DNA on this substrate. The surface of the shadowed substrate, and therefore presumably the unshadowed substrate, is too rough for imaging the much smaller DNA, which is only 2 nm in diameter, compared to TMV which is 15 nm in diameter (Brenner and Horne, 1959). It would, however, be worthwhile to explore other methods of preparing thin layers of MgF₂ with the goal of obtaining a smoother surface, since this substrate offers the lowest relative photoemission of the
substrates listed in Fig. 3 and provided excellent contrast in photoelectron images of unshadowed TMV.

**Photoelectron imaging of DNA preparations**

The other substrates listed in Fig. 3 appear to be much smoother than the MgF₂-coated chromium surface, based on the images of shadowed specimens. For example, Fig. 5 a is a photoelectron micrograph of low angle-shadowed TMV particles and recA-DNA complexes on a chromium-coated glass disc. The virus particles apparently do not bind well to chromium, so only a few virus particles are visible in the field of view. However, recA-coated DNA attaches to the chromium and can be easily distinguished from the substrate. Interestingly, the shadows are more pronounced from the virus particles than from the recA-DNA complexes even though the reported diameters of TMV (15 nm) and recA-single stranded DNA complexes (12 nm; Dunn et al., 1982) are quite similar. Compared with the MgF₂ substrate shown in Fig. 4 b, evaporated chromium appears much smoother, although somewhat grainy. Fig. 5 b is a photoelectron image of a similar preparation of TMV and recA-DNA complexes on a spermidine-derivatized dextran-coated silicon disc after low angle shadowing. It was necessary to coat the silicon because biomolecules do not attach well to native silicon. The spermidine-derivatized dextran molecules contain free amino groups which can interact electrostatically with charged groups on the surfaces of the virus particles and DNA-protein complexes, providing better attachment. The number of virus particles attached to the derivatized dextran substrate is higher (Fig. 5 b) than attached to uncoated chromium (Fig. 5 a) and the recA-DNA complexes appear to be somewhat better spread on derivatized dextran. In addition, in comparison to the chromium surface, the background of the silicon disc coated with derivatized dextran appears to be significantly smoother.

Although the protein-coated DNA molecules are easy to distinguish from the background in these shadowed preparations, naked DNA is more difficult to see. Fig. 6 shows the photoelectron image of a low angle shadowed preparation of TMV particles and naked, relaxed φX174 double stranded circular DNA on a derivatized dextran-coated chromium disc. Compared to the uncoated chromium disc in Fig. 5 a, many more virus particles are bound to the derivatized dextran surface. In addition, the derivatized dextran-coated surface in Fig. 6 appears to be less grainy than that of bare
the similarity in images, data from only one of the substrates is presented here (Fig. 7) in which the substrate was chromium coated with derivatized dextran. Photoelectron images recorded with the other two substrates were of slightly lower quality. Those of recA-DNA complexes on silicon coated with derivatized dextran were not quite as sharp as those in Fig. 7, suggesting that the conductivity of the silicon used is a possible limiting factor. Images obtained on uncoated chromium were comparable to those in Fig. 7, but the recA-DNA complexes did not appear to be quite as well spread on this hydrophobic surface.

**DISCUSSION**

Photoelectron imaging is the electron optical analog of fluorescence microscopy. According to Einstein’s photoelectric equation $KE = hv - \phi$, where $KE$ is the maximum kinetic energy of the emitted electron, $h$ is Planck’s constant, $v$ is the frequency of the exciting light, and $\phi$ is the work function of the surface. The highest resolution in present instruments (e.g., 5–10 nm) is achieved near threshold because this limits the chromatic aberrations in the imaging system. In this mode, the energy of the emerging electrons is very low ($\sim 1$ eV) and material contrast in photoelectron imaging is determined by the differences in work functions. In the case of organic and biological molecules, the work functions are closely related to the first ionization potentials, but are systematically lower due to polarization effects on the surface. It is the differences in ionization energies that enrich the information content of the images. A second contrast mechanism is provided by specimen topography: high topographic contrast and surface selectivity are a direct consequence of the very low energies of the emitted electrons. Both of these contrast mechanisms, material contrast and topographic contrast, are essential to the imaging of small, uncoated biological specimens such as DNA and viruses. The specimens must also be sufficiently conductive so that the photoejected electrons can be replaced with electrons from the cathode. Conductivity of nucleic acids and viruses is sufficient, perhaps because of the increased photoconduction under the intense UV illumination required for high resolution images in a photoelectron microscope and because the electrons are collected over the entire specimen, rather than in a small region as in a scanning microscope.

Photoelectron microscopy is a surface imaging technique. As in all surface imaging methods, the choice of substrate can determine the success or failure of the experiment. Two universal criteria are: 1) the substrate must be as smooth as possible, with roughness significantly smaller than the details of the biological specimen; and 2) the substrate must facilitate the binding and spreading of the specimen. These criteria are perhaps most readily understood by briefly discussing their roles in the development of more widely used surface imaging techniques. For example, continued progress in imaging with scanning tip microscopes has been dependent on these two criteria. Early work in scanning
tunnelling microscopy (STM) utilized cleaved graphite substrates. Cleaved graphite is relatively flat, but does not have optimal binding and spreading properties. Gold has also been tested as a substrate for STM with mixed success (Clemmer and Beebe, 1992). Another widely used substrate is mica. Mica is readily available, and when freshly cleaved forms an extremely smooth surface capable of binding DNA and proteins. Because of this, mica is used in the preparation of replicas of specimens for transmission electron microscopy (Portmann and Koller, 1976; Burkardt and Lurz, 1984) and, more recently, in atomic force microscopy (Bustamante et al., 1992; Lyubchenko et al., 1992, Droz et al., 1993). However, mica has the drawback of being a relatively poor conductor of electrons, ruling out its use in STM. Conductivity is also a requirement for substrates for photoelectron microscopy because the photoejected electrons must be replaced by electrons from the cathode via the substrate to prevent the buildup of a positive charge on the surface.

The only criterion that is unique to photoelectron imaging is that the substrate must be relatively non-photoemissive, compared with the specimen. This is analogous to selecting a background in fluorescence microscopy which has low fluorescence compared to the specimen. Because of the importance of this criterion, it has been the focus of our search for substrates. The easiest conductive substrates to prepare are those that can be readily vacuum evaporated, including most metals. However, almost all metals we have tested, although conductive, are also highly photoemissive (e.g., see gold in Fig. 3). The one exception we have found is chromium, which is initially much less photoemissive than gold, and becomes even less so with time as it is exposed to air, presumably reflecting the slow buildup of a surface oxide layer. After 1–2 days in air, the photoemission from chromium reaches a stable level, which does not change with further exposure to air. At the same time, the initially hydrophilic chromium surface becomes increasingly hydrophobic, making it somewhat more difficult to attach biological molecules. For this reason we often apply a derivatized dextran coating to the chromium substrates. This treatment makes the surfaces hydrophilic, and the amino groups of spermidine apparently help in the attachment and spreading of the DNA molecules. The derivatized dextran treatment also appears to reduce some of the surface imperfections. Like chromium, mechanically polished heavily doped silicon has relatively low electron emission and between the polishing scratch marks, the silicon surface appears to be quite smooth. Also like chromium, silicon surfaces can be used with biomolecules if a layer of derivatized dextran is first applied to the disc. However, the final test of the suitability of a substrate is the quality of the images obtained, and the quality of the images we obtained are slightly sharper on chromium than on silicon, and the chromium substrates are less expensive and easier to prepare.

Magnesium fluoride, like mica, is a poor conductor, but can be used as a PEM substrate because it is possible to prepare films of this material that are thin enough that electrons emitted from the surface are replaced rapidly enough to avoid buildup of a surface charge. The relative electron emission is also low, as shown in Fig. 3, as predicted by the low absorption of UV light (MgF2 is used in optics because of its high transmission in the UV). In a previous study we examined MgF2 over chromium, among other possibilities, as substrates (Habliston et al., 1993). Although we were able to obtain images of DNA plasmids on this substrate, we subsequently observed considerable variation in image quality and, in many cases, we were unable to obtain good images of specimens that were within the resolution limits of the photoelectron microscope. Photoelectron microscopy is very sensitive to surface topography and imperfections when the surface is photoemissive, because the slow moving electrons are easily deflected as they emerge into the electric field between the cathode and the anode. However, when the surface is not photoemissive, it is difficult to determine the degree of surface roughness, and additional information is required. As shown here, unidirectional shadowing of the substrate at low angle with small amounts of Pt/Pd (and presumably other metals) followed by PEM examination can provide very useful information about the condition of the substrate surface. Metal shadowing can, of course, contribute to the surface roughness. For this reason, we do not claim that this is the best method of determining surface roughness of an uncoated specimen. However, for our purposes, the present approach is satisfactory and has the advantage that two criteria (photoemission and surface roughness) can be evaluated quickly in the photoelectron microscope on the same specimen. Based on these two criteria, we conclude that improvements in methods of depositing thin layers of MgF2 over chromium are required before this can become a reliable substrate for the photoelectron imaging of biological specimens. We have also used this information for substrate optimization and have presented the first PEM images of shadowed biological samples.

On a relatively smooth substrate, as shown in Figs. 5 and 6, viruses and DNA can be observed quite clearly by PEM after low angle shadowing. This is primarily due to the difference in photoemission between the highly photoemissive Pt/Pd shadowing material and the much less photoemissive substrate. This is the case only if the shadowing is unidirectional. When preparations similar to those of Fig. 5 were rotary shadowed at low angle, the result was a uniformly bright image with nearly complete loss of contrast making it impossible to detect even virus particles. We also found the shadowing angle and amount of shadow to be important. The sharpest PEM images were obtained at an angle of 3° with deposition of very light (0.1 nm thick) amounts of Pt/Pd. A 3° shadowing angle also seems to be optimal for exposing substrate surface imperfections and visualization of DNA. (The shadow from a 2-nm-high DNA molecule is 40 nm long when shadowed at an angle of 3°.) Even though the amount of shadow used is minimal compared with that used in TEM, it greatly increases the number of electrons emitted from the sample as can be seen from the beam current measurement of Pt/Pd in Fig. 3. Thus,
significantly shorter exposure times are required for micrographs of shadowed preparations compared with unshadowed samples.

We conclude that photoelectron microscopy combined with low angle unidirectional shadowing is effective for the evaluation of substrate surface roughness. The combination of electron emission measurements with images of low angle shadowed substrates provides an improved criteria for substrate selection for PEM. Using these criteria, several substrates have been evaluated. The first photoelectron images of shadowed viruses and DNA preparations are reported. We also show that high quality images of unshadowed recA-DNA can be obtained by photoelectron imaging.

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