BIOMOLECULAR ADSORPTION AND THE LIFE DETECTOR

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Résumé: Ce mémoire traite du dépôt de molécules biologiques sur la surface de pointes métalliques à émission de champ. Une nouvelle procédure de dépôt et une nouvelle méthode de coloration permettent de déposer des protéines avec précision sur l'extrémité d'une pointe, pour les visualiser ensuite dans le microscope électronique à transmission. Ces techniques permettent la préparation et la caractérisation de pointes à émission de champ immunologiquement actives. En combinant la spécificité chimique de ces pointes aux possibilités d'amplification moléculaire propres au processus d'émission par effet de champ, on peut alors envisager la création d'un détecteur chimique d'une conception entièrement nouvelle.

Abstract: The placement of biological molecules on the surface of metal field-emitter tips is discussed. A new deposition protocol and staining procedure allows precise coverages of proteins to be placed on the apex of a tip and then visualized in the transmission electron microscope. These techniques provide a means for preparing and then characterizing immunologically active field-emitter tips. By combining the chemical specificity of these tips with the molecular amplification capabilities of the field-electron emission process, a novel chemical sensor concept can be envisioned.

Introduction: For several years we have discussed methods to place biological macromolecules on the apex of a metal field-emitter tip [1-4]. These studies were motivated by the development of a field-ion Tomographic (FIT) microscope that used point-projection imaging to reveal the three-dimensional appearance of unstained biological molecules [5]. The images that we obtained demonstrated the feasibility of the technique [6-7], but image reproducibility was poor, presumably because the nature of the deposition process encouraged the binding of molecular aggregates of random size and shape -- rather than single molecules -- to the region of the tip apex accessible to imaging. We demonstrated that ferritin could be visualized on the surface of metal field-emitter tips by imaging the tips in profile in the TEM [4]. The core of an individual ferritin molecule could be seen because it contains a high concentration of iron, an electron opaque element. Clusters of ferritin were always observed which seemed to confirm our conclusion that the reproducibility of a FIT image was related to the size, the shape and the distribution of ferritin clusters on the tip apex.

Attempts to image DNA by field-ion tomography were moderately successful [7], even though DNA could not be independently visualized

on a tip by TEM imaging because DNA is not electron opaque. Without TEM imaging, trial and error, and an a priori knowledge (and belief) in the appearance of a FIT image was required to develop a successful deposition strategy.

Rotary staining: The problem of depositing a biological molecule on a tip apex is complicated by the interaction of many parameters. Biological molecules must be deposited from aqueous solution. The type of buffer that is used, its pH and the concentration of other constituents (such as salt), the advisability of "fixing" the molecules in solution[8], and even the temperature of the deposition solution can have an effect on the coverage and the morphology of the molecules that are placed on the tip surface. In order to evaluate the importance of these parameters, it was essential to develop a generic procedure for visualizing the coverage of any molecule on the tip surface by direct imaging in the TEM. This meant that the image contrast of an adsorbed molecule had to be increased. The procedure that we developed to accomplish this task is shown schematically in Figure 1[9].

Fig. 1. A procedure for rotary staining field-emitter tips.

After placing molecules on the tip surface, the tip is rotated in vacuum and covered with a thin layer of evaporated metal. The 1 nm - 2 nm thick layer enhances the visibility of individual molecules in a TEM image by enclosing them within an electron opaque shell that faithfully preserves their shape. Our procedure is actually an extension of the well known rotary staining process used by molecular biologists to enhance the contrast of proteins and nucleic acids placed on thin carbon films and imaged in the TEM[10].

Figure 2 shows the result of rotary staining a partial monolayer of ferritin molecules with tungsten. The protein shell of individual ferritin molecules is clearly seen, delineated by a thin layer of evaporated tungsten. Individual molecules that are behind one another in the imaging direction are also visualized. The 200 kV electron beam used for imaging easily penetrates each molecule, reproducing detail from each species encountered on its way to the photographic emulsion. Electron beam damage is minimized by the short dwell time of the electrons in an adsorbed species.

Fig. 2. Ferritin molecules rotary stained with tungsten.

Rotary staining has allowed us to develop a deposition protocol that is capable of placing a reproducible coverage of molecules on the surface of a field-emitter tip[11]. The essential steps in the protocol are as follows:

1. Molecules are deposited onto a tip for two minutes from a 10 μ l droplet of buffer that contains the molecule at the desired concentration. A typical buffer for protein deposition consists of 50mM HEPES, 150mM NaCl (pH 7.5).
2. The tip is rinsed for two minutes in 10ml of buffer. At this point, deposition stops because the concentration of molecules in solution has been reduced by the droplet /buffer volume ratio ($10/10000 = .001$).
3. Molecules that are adsorbed on the tip surface are fixed for five minutes in a 20 μ l droplet of a 0.6% solution of gluteraldehyde in buffer.
4. The tip is rinsed for one minute in 10ml of fresh, double (glass) distilled water.
5. The tip is rinsed for one minute in 10ml of a 20% mixture of double (glass) distilled ethanol in double distilled water.
6. The tip is rinsed for one minute in 10ml of a 50% mixture of double distilled ethanol in double distilled water.
7. The tip is rinsed for one minute in 10ml of a 70% mixture of double distilled ethanol in double distilled water.
8. The tip is rinsed for one minute in 10ml of 100% double distilled ethanol, removed into air, and dried.

It is essential to insure that the tip remains wet until the rinse in 100% ethanol has been completed. A special fixture has been designed for this purpose[11]. With our protocol, the ability to adsorb isolated protein molecules on the tip apex depends upon the lack of molecular aggregation in solution prior to deposition. Aggregation on the tip surface is minimized by the rinse schedule outlined above.

The sequence of ethanol-water mixtures gradually replaces the initial aqueous environment of the molecule with pure ethanol. This reduces the surface tension forces that act on the adsorbed molecules as the tip is removed into air and dried. If a tip is removed into air from pure water, the number and the distribution of molecules deposited on its apex will be severely perturbed, and lateral aggregation will increase[11].

Immunologic detection: Several years ago, we used the TEM to visualize the morphology of thick protein layers placed on a tip by the immune reaction[3]. Unstained protein layers could be seen covering the tip surface because the density of the deposit and its thickness insured that sufficient electron scattering would occur, and adequate image contrast would be obtained. Recently, we decided to use our new deposition and shadowing techniques to investigate, in more detail, protein multilayers formed on the tip surface by the immune reaction. In particular, we wanted to see if a chemically specific surface could be prepared by adsorbing a selected protein antigen on the tip apex, and then selectively capturing its specific protein antibody from solution by the immune reaction. Figure 3 shows a tip on which a monolayer of antigen (ferritin) has been placed. Figure 4 shows the multilayer formed when a ferritin coated tip is exposed to a solution containing a low concentration of anti-ferritin rabbit antibodies of the IgG type.

Fig. 3. A saturation coverage of ferritin.

Fig. 4. Anti-ferritin rabbit IgG bound to ferritin.

The difference in layer morphology between Figure 3 and Figure 4 is caused by the formation of immune complexes after exposing the antigen covered tip to antibody. A series of such experiments has demonstrated that it is possible to prepare an immunologically active tip that will recognize and irreversibly bind antibody from a solution containing other contaminant proteins.

The exact mechanism that is responsible for the highly specific recognition phenomena between antigen and antibody molecules is largely unknown. Chemical and steric interactions over angstrom dimensions, coupled with morphological changes in one or both species, are probably responsible for the tight binding which is generally observed [12]. The rate constant for the dissociation of an antigen-antibody complex in solution is typically six orders of magnitude smaller than the rate constant that is associated with its formation [12].

The LIFE Detector: Our experience in preparing immunologically active field-emitter tips has suggested a novel chemical sensor concept. It combines the chemical specificity of an immunologically active field-emitter tip with the single molecule detection capability of the field-electron emission process. Field-electron emission in vacuum can be used as a nonspecific detector of biomolecular adsorption from aqueous solution [13]. It is also known that field-electron emission can be observed in very pure, cryogenic liquids [14]. By operating a field-emission diode in a biologically compatible fluid at room temperature, with an immunologically active field-emitter tip, a chemical sensor might be created with a single molecule detection capability. The sensor would be very small, lightweight, and require almost no power for operation. It could be tailored to detect almost any chemical species in solution by binding its complementary antigen (or antibody) to the surface of a field-emitter tip. The resulting Liquid Field-Emission (or, LIFE) detector would be a biologically active diode. The concept is shown schematically in Figure 5.

Fig. 5. The LIFE (LIquid Field-Emission) detector concept.

Figure 5 depicts an antigen covered tip that has irreversibly bound antibody from solution. Inactive regions of the tip surface are passivated against nonspecific adsorption. When the tip is biased to a negative potential, a current is drawn that will reflect the coverage of molecules bound to the tip apex. We are evaluating several liquids in which stable electron currents can be drawn at room temperature, and in which immunological activity can be maintained. Promising candidates are mixtures of dimethyl sulfoxide (DMSO) and water. Stable electron currents have been measured at room temperature from tips coated with bovine serum albumin (BSA), and with ferritin conjugated to goat anti-rabbit IgG. A tip imaged in the TEM before, and after a typical current measurement is shown in figure 6A and Figure 6B, respectively. The fixture that we used to make the current measurements is shown in Figure 6C. An electrometer connected to the anode measured the current injected into the

Figure 6. Current injection into a liquid.

liquid from the molecule-coated tip. Figure 6D shows a typical current profile measured by applying a bias of -273 Vdc to an antigen (BSA) covered tip in a 10% mixture of DMSO in water. Figure 6A and Figure 6B demonstrate that the morphology of the tip and the appearance of the BSA layer on its surface did not change as a result of drawing current from the tip for several minutes. After drawing current for about ten minutes, the current profile became erratic; after about twenty minutes the average current increased catastrophically, and the tip was quickly destroyed by electrochemical erosion. The same effect has been observed on several tips, and has been associated with a gradual decomposition of the BSA layer that initially coats its surface. The random current spikes seen in Figure 6C may reflect a series of minor electrochemical breakdown events prior to complete destruction of the protein layer. On the other hand, they may reflect the adsorption and desorption of molecular contaminants from the liquid at the BSA-liquid interface. Future studies will attempt to clarify the mechanism that produces the measured current, and the sensitivity of the measured current to contaminant binding at the surface of the tip. Current profiles will also be measured as a function of antibody concentration in solution.

Conclusions: A new deposition protocol and a new rotary staining process have resulted in the preparation and the characterization of immunologically active field-emitter tips. A novel chemical sensor concept has been introduced in which the chemical specificity of the immune reaction is combined with the single molecule detection capability of the field-electron emission process. Stable electron currents have been drawn from tips covered with a protein layer and immersed in a biologically compatible liquid at room temperature. Experiments are planned to test the sensitivity of the detector to the adsorption of antibody from solution, and to optimize the liquid environment in which the detector operates.

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