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Scanning Tunneling Microscopy Studies of Glucose Oxidase on Gold Surfaces

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Three immobilization methods have been used for scanning tunneling microscopy (STM) studies of glucose oxidase (GOD) on gold. They are based on (a) physical adsorption from solution, (b) microcontact printing, and (c) covalent bonding onto self-assembled monolayers (SAMs) of 3-mercaptopropionic acid (MPA). The STM images are used to provide information about the organization of individual GOD molecules and more densely packed monolayers of GOD on electrode surfaces, thus providing information on the role of interfacial structure on biosensor performance. The use of atomically flat gold substrates enables easy distinction of deposited enzyme features from the flat gold substrate. Microcontact printing is found to be a more reliable method than adsorption from solution for preparing individual GOD molecules on the gold surface. STM images of printed samples reveal two different shapes of native GOD molecules. One is a butterfly shape with dimensions of 10 ± 1 nm $\times$ 6 ± 1 nm, assigned to the lying position of the molecule, while the second is an approximately spherical shape with dimensions of 6.5 ± 1 nm $\times$ 5 ± 1 nm assigned to a standing position. Isolated clusters of five to six GOD molecules are also observed. With monolayer coverages, GOD molecules exhibit a tendency to organize themselves into a two-dimensional array with adequate sample stability to obtain high-resolution STM images. Within these two-dimensional arrays repeating clusters of five to six enzyme molecules in a unit are clearly seen. Cluster structures are observed at both high and low coverages despite the fact that native GOD is a negatively charged molecule under physiological conditions. GOD monolayers covalently immobilized onto SAMs (MPA) are considerably more difficult to image than when the enzyme is adsorbed directly onto the metal.

Introduction

The behavior of enzymes on surfaces has been the subject of extensive research in recent years using a wide variety of solid surfaces.[1−3] Study of spatial organization, ordering, morphology, and activity of enzyme molecules in artificial molecular systems is an important target for the development of high-performance enzyme biosensors.[4−6] Molecular level fabrication of enzyme biosensors is based on the ability to immobilize monolayers, or submonolayers, of enzymes on the surface while retaining enzyme activity. Recently, alkanethiol self-assembled monolayers (SAMs) have received attention as a possible route for fabrication of enzyme biosensors because a monolayer of the enzyme can be immobilized with a high degree of control over the molecular architecture of the recognition interface.[7−10] This approach provides a number of advantages including the ability to mimic biological membranes in addition to fabricate enzyme interfaces with a high degree of reproducibility and the immobilization of the enzyme close to the electrode surface allowing direct electron transfer without denaturation.[11−13] However, classical immobilization methods performed by physical adsorption produce unstable biosensor devices with poor reproducibility.[1,3,14−16] By far the most commonly studied enzyme is glucose oxidase (GOD) due to its importance as a model enzyme, thus allowing comparison with other enzyme immobilization protocols.[17]

In our previous work, we have studied many aspects of glucose biosensors based on self-assembled monolayers.[5−6,18−22] The primary focus was to determine the important parameters for the biosensor response with regard to fabricating reproducible devices and tuning the

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response to a particular application.[5,19] Using a quartz crystal microbalance (QCM), we have studied the immobilization of GOD covalently bonded onto SAMs to find the optimal enzyme concentration and deposition time.[5,19] Using atomic force microscopy (AFM) in a similar way, we studied the enzyme surface and the influence of the roughness of underlying gold surfaces using different gold substrates.[20,23] AFM was able to resolve the spatial distribution of the GOD monolayer on a molecular level,[20,24] but image resolution was insufficient to investigate submolecular features.

Scanning tunneling microscopy (STM) can yield molecular or even atomic resolution images of molecules that may allow additional insights in the study of biological surfaces and/or interfaces.[25−27] Although direct tunneling through adsorbed biomolecules is not clearly understood, a number of STM studies of various biomolecules such as proteins, enzymes, and DNA have been reported in the past 10 years.[28−30] A few of these STM studies have involved GOD immobilized on different surfaces (highly oriented pyrolytic graphite, polypyrrole and SAM-modified gold).[31,32] There is, however, a lack of systematic AFM or STM investigation of GOD immobilization directly onto gold or modified gold surfaces.[16,33,34]

In this work, we have extended our previous AFM studies of immobilized GOD on SAM-modified gold surfaces using STM. The purpose of this study is to image GOD molecules on gold surfaces and SAM-modified gold surfaces to provide an indication of how surface immobilization influences the conformation of immobilized enzymes. Three different immobilization methods are used: adsorption from solution onto bare gold, microcontact printing onto bare gold, and covalent bonding onto SAMs. Atomically flat gold is used as an immobilization platform since it represents a superior surface to other gold substrates.[35−37] Using STM, we have investigated the three-dimensional molecular structure of individual GOD molecules and their orientation on gold surfaces. These STM studies give insights into the structure and assembly of GOD as part of a biosensor recognition element.

Experimental Section

Materials. Gold foil (99.95%), 25 × 25 mm, was supplied from Peter W. Beck Pty. Ltd., Australia. The mica used was muscovite mica from one of two sources: either natural samples from South Australia or commercial samples from SPI Supplies, West Chester, PA. The enzyme glucose oxidase from *Aspergillus niger* (type VII-S), β-D-glucose, 3-mercaptopropionic acid (MPA), *N*-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were from Sigma (Sydney, Australia). Potassium chloride, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, and ethanol were supplied by Ajax Chem Pty. Ltd (Sydney, Australia). All chemicals were of the highest quality commercially available and were used without further purification. All aqueous solution was prepared with Milli-Q grade reagent water.

Preparation of Flat Gold Substrates. Thin gold film substrates were prepared by thermal vacuum evaporation of about 2000 Å of gold (measured using a film thickness monitor) onto freshly cleaved, heated muscovite mica. The turbomolecular-pumped evaporator's base pressure was below 1×10^{-5} Torr. For the heating of the mica sheets (∼50 mm × 50 mm), a special heater block was used with temperature feedback control through a thermocouple placed on the mica face. The mica is heated at 300 °C for a minimum of 4 h before the evaporation and held at 300 °C during the evaporation, and the resulting gold films on mica were annealed for between 4 and 16 h at 300 °C. The sample was cooled under vacuum to less than 60 °C prior to removal from the evaporation chamber. The gold film on mica is used as a precursor for preparation of flat gold using a stripping method as described[35,36] and improved by Shapter and co-workers.[37] The basic idea is to use the first gold atomic layer, which has been deposited directly on the mica, rather than the top gold surface. Using the gold layer contacting the mica takes advantage of the atomic flatness of the mica. After each stripping, the conductivity of the surface was checked and finally the surface was examined using a standard stereomicroscope. The flat gold substrates are used immediately after stripping.

Immobilization of GOD on the Gold Surface. *Adsorption from Solution.* Flat gold substrates were immersed in freshly prepared GOD solution in 0.05 M phosphate buffer, pH 5.5. Enzyme concentrations are varied from 1×10^{-2} to 10 mg L^{-1}, and the deposition time ranges between 0.5 and 30 min. Immediately after adsorption, samples were rinsed with buffer followed by three rinses with deionized water to remove any residual salt or weakly adsorbed enzyme. The samples were then dried with nitrogen for 2 min before STM imaging.

Microcontact Printing. Unpatterned poly(dimethylsiloxane) flat stamps were prepared from Silgard 184 (Dow Corning, Midland, MI) and cured for at least 12 h at 60 °C on flat mica sheets as described.[38] Inking of the stamps was performed by applying 1 μL of GOD solution per cm^{-2} of stamp. Concentrations of GOD were varied from 1×10^{-1} to 10 mg L^{-1} in 0.05 M phosphate buffer solution, pH 5.5. The drop of ink solution was removed after 30 s under a stream of nitrogen. The nitrogen flow was maintained for 1 min after removal. The stamp was then used 2 min after drying. The stamp with GOD was placed by hand horizontally on the flat gold surface for 2−3 s.

Covalent Immobilization onto Self-Assembled Monolayers. Freshly prepared flat gold substrates were immersed in a solution of 20 mM MPA in ethanol−water solution (75:25). SAM formation was allowed to proceed for 12−24 h (overnight). Before further modification steps, SAM-modified substrates were rinsed with 75:25% ethanol/water solution and then with water. The terminal carboxylic acid groups of MPA self-assembled on gold were activated by immersion in a pH 5.5 buffer solution containing 0.002 M EDC and 0.005 M NHS for 1 h. The electrodes were rinsed with pH 5.5 buffer and immediately placed in a solution of glucose oxidase (490 mg dm^{-3} in pH 5.5 buffer) for 90 min. After rinsing with buffer and copious amount of water, electrodes were dried under nitrogen for 2 min. All preparations were performed at room temperature.

Instrumentation. *Scanning Tunneling Microscopy.* STM measurements of the gold substrates were made under ambient conditions using a Digital Instruments Nanoscope II. All substrate images were obtained in the constant current mode using commercial (DI) or mechanically cut Pt/Ir tips. The bias voltage was typically +0.2 V with a tunneling current set point of 3 nA for bare flat gold. *Z*-Calibration was confirmed by

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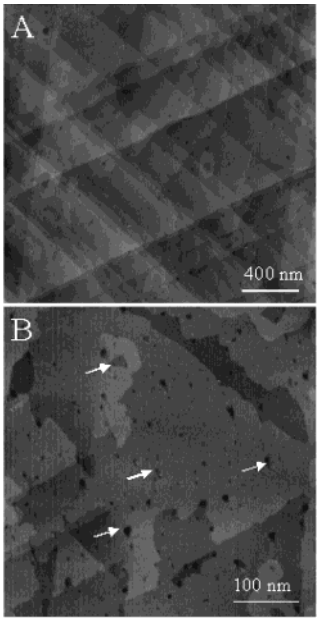

Figure 1. STM images of typical flat gold surfaces stripped from a mica template (1 nA, 500 mV): (A) image showing triangular features expected for the Au(111) plane and (B) higher resolution image of bare gold. Arrows indicate some of the observed imperfections.

measurement of Au(111) step heights. For optimal imaging of substrates with GOD adsorbates, a variety of tunneling conditions were used: tip biases from 0.1 to 1.0 V and tunneling currents of 60−200 pA. Images from 2 μm × 2 μm down to 100 nm × 100 nm were typically obtained for each substrate. Five different areas on at least three samples of each type of substrate were examined.

Results and Discussion

Evaluation of Flat Gold Surfaces. To resolve the structure of GOD molecules on gold surfaces by STM, the starting point is the preparation of an atomically flat substrate with few defects. From our previous study, we found that stripped gold film from a mica template is superior to other gold surfaces.[20,23,37] A large number of STM images were obtained to confirm flatness and topography of flat gold as the blank substrate before deposition of enzyme.

Two typical topographic STM images (2 μm × 2 μm, 500 nm × 500 nm) of flat gold are shown in Figure 1. The surfaces exhibit atomically flat triangular terraces with 60° angles characteristic of epitaxial Au(111) planes. Occasionally, some surface variation is observed with a mixture of deformed triangular plates and irregular stepped areas caused by lower annealing temperatures and use of poorer quality mica sheets. The height of each step is about 2.4 Å in accordance with the monotonic step height of 2.35 Å for Au(111). The terraces or triangular plates shown in images A and B are absolutely flat resulting in a very stable tunneling current. The average size of these atomically flat areas is found to have linear dimensions of the order of about 200−500 nm (generally the atomically flat regions have areas of about ∼50 000 nm^2) which is easily large enough to allow resolution of biomolecules with sizes of 10 nm or less. Roughness analysis shows average roughness R_{rms} of 0.12 ± 0.03 nm, R_a of 0.09 ± 0.03, and R_{max} of 1.2 nm ± 0.2 for a 500 × 500 nm typical flat region.

We have characterized imperfections of flat gold to distinguish enzyme features from the gold surface. Three main groups of defects are observed. Very frequently, small holes with a size of 2−20 nm in diameter, which are discontinuities in the gold film, exist as seen in Figure 1B (arrows). These small holes probably originate from incomplete coalescence during annealing in the deposition chamber. Our STM experiments have shown that adsorption of enzyme molecules at low concentration shows some minor preference for these holes but generally molecules are adsorbed on the terraces concurrently. Larger scale STM and AFM images (5−50 μm) reveal deep (10−20 nm), sparsely spaced large holes (100−300 nm across) in the gold substrate.[37] These holes result from the stripping process and correspond to observed impurities on the mica substrate from which the sample is separated. The third type of imperfections observed very infrequently are steps or deeper rows (3−6 monotonic steps). Such surface areas are not used in any STM imaging described here which focuses only on flat areas. As sample flatness is one of the crucial factors for imaging biomolecules, these results show that flat gold is a highly suitable gold substrate for SPM of GOD molecules or any other biomolecules, deposited by either adsorption or covalent immobilization.

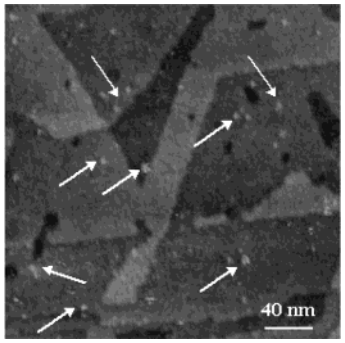

Figure 2. Typical STM image of GOD on a flat gold surface adsorbed from solution. The enzyme concentration was 0.05 mg dm^{-3} in solution. Arrows indicate GOD features.

STM Visualization of Individual GOD Molecules. Two approaches were used to adsorb low concentrations of GOD on gold in an attempt to image individual molecules: adsorption from solution and microcontact printing. Initially, using adsorption from solution with higher enzyme concentrations (1−10 mg L^{-1}), we observe irreproducible STM images with considerable molecular aggregation. A large population distribution of enzyme features is seen ranging from smaller isolated clusters to uniform layers or larger three-dimensional aggregations. Using lower enzyme concentrations of 0.01−0.1 mg dm^{-3}, STM images with broadly spaced enzyme features, some of which are individual molecules, are obtained. A typical STM image is shown in Figure 2. A number of nanometer-size features (indicated by the arrows) on the characteristic triangular background of the Au(111) surface are visible. These images indicate that bonding to the substrate is reasonably strong as the position of the enzyme features is quite stable. Different shapes from spherical to ellipsoidal are observed for the individual GOD molecules. The average dimension of spherical features is 6−8 nm long and 5−7 nm wide in agreement with crystallographic data.[40,41] Small clusters with two or more linked molecules are also observed. The sizes of the features quoted through this discussion are meant as rough guides, and given the uncertainties in tunneling mechanisms in imaging of large biomolecules, precise dimension determination of such

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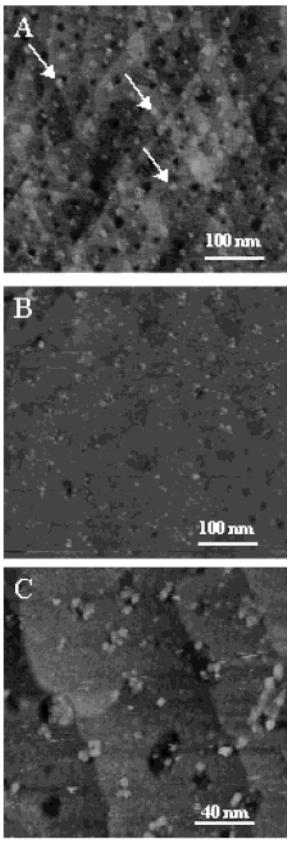

Figure 3. STM images of *printed* GOD on flat gold. (A) The enzyme concentration in the printing solution was 10 mg dm^{-3}. Arrows indicate clusters of GOD. (B and C) The enzyme concentration in the printing solution was 0.1 mg dm^{-3}.

features is difficult. All of the assignments of features are based on the observed differences in the electron densities as measured by STM.

Solution preparation of appropriate coverage of GOD to give isolated GOD molecules is possible but is a difficult and irreproducible process. For this reason, we have investigated the microcontact printing technique as an alternative approach. It has been reported that this method could be successfully used for transferring biomolecules and cells onto solid surfaces.[42] Typical STM images of microcontact-printed GOD molecules on flat gold are shown in Figure 3. Figure 3A shows the initial attempt using a higher concentration of enzyme in the printing solution (10 mg dm^{-3}). Three-dimensional clusters of five to six GOD molecules with an average size between 20 and 35 nm in diameter (indicated by arrows) are observed randomly distributed on the surface. A lower population of clusters and an increasing number of isolated individual GOD molecules are observed using lower concentrations of enzyme (0.1−1 mg dm^{-3}) as shown in Figure 3B,C. All of the same features from Figure 2 are observed in Figure 3C, but given the ease of preparation of the surface via printing, this approach was used for all low enzyme concentration depositions on bare gold.

To verify structures and orientation of individual enzyme molecules on the gold surface, a series of STM images are summarized in Figure 4. We observed various groupings of GOD molecules into both clusters, usually containing five or six molecules, and dimers in addition to isolated GOD molecules (monomers).

Figure 4A shows an image of an isolated GOD molecule with two linked units that can be described as butterfly shape characteristic for the native GOD molecule. The average size of this molecular structure is a length of 10 ± 1 nm and a width of 6 ± 1 nm. Cross sections through the bridge between two wings show that the width of the bridge point varies from 2 to 4 nm. The calculated cross-sectional area for this molecule is found as 45−50 nm^2. Figure 4B shows an image of a GOD molecule with an approximately spherical shape. The lateral dimensions were determined as 6.5 ± 1 nm along the long axis and 5 ± 1 nm along the perpendicular direction. The corresponding space occupation on the surface for the molecule with this shape is found as 37−42 nm^2.

To interpret observed GOD molecular structures from STM images, a model of the native GOD molecule was considered. Glucose oxidase from *Aspergillus niger* is a dimeric protein with a molecular weight of 160 kDa, containing one tightly bound flavin adenine dinucleotide (FAD) per monomer as a cofactor. The dimeric protein displays an ellipsoidal shape with a high content of secondary structure (28% helix, 18% sheet). The overall dimensions of the dimer are 7.0 nm × 5.5 nm × 8.0 nm according to X-ray crystallography data.[40,41] The monomers are connected noncovalently via a long but narrow contact area. There are 120 contact points between the dimers centered around 11 residues, which form either salt linkages or hydrogen bonds. Two possible orientations of the adsorbed native GOD molecule are reported: parallel to the major axis (lying position) and along the perpendicular direction (standing position).[16,31] The third is the unfolded form of the enzyme, which can vary in length from 20 to 50 nm.

Figure 5 shows the comparison of the STM images of two observed GOD monomers with a corresponding model of GOD positions on the surface. Cross-sectional analysis of many examples of each form of the monomer allows matching of the shape and the lateral dimensions of the observed enzyme features to the expected geometries. The shape is used as the primary criterion to distinguish the appearance of the standing and lying positions of the enzyme on the surface, where the spherical shape presents the standing position and the butterfly shape the lying position. Furthermore, this analysis allows the identification of various forms of dimers observed.

Our results show good agreement with expected dimensions of the native GOD molecule and the model of arrangements on the surface. Molecular sizes determined by STM are typically about 80−90% of the values for native molecules observed crystallographically. This difference is simply attributable to the convolution of the tip and molecule electronic states that are at the core of STM.[26,27]

We have observed hundreds of features in various STM images. Generally, a similar number of small clusters and spherically shaped molecules was found, but surprisingly, fewer molecules in the butterfly orientation are observed. The numbers of cysteine groups and gold−sulfur bonds mean that the favored adsorption geometry is the lying position of the GOD molecule.[27−29] This orientation allows greater bonding to the substrate and is energetically more stable.

If we accept that the spherical features represent the standing position, the present results indicate that this position is more favorable, contradicting previous reports.[16] One possible reason for the difference could be the different deposition methods.

The unfolded form of GOD is observed very rarely. These features appear as two parts (20 × 20 nm) fragmented into many (10−20) small, linked fragments. This means

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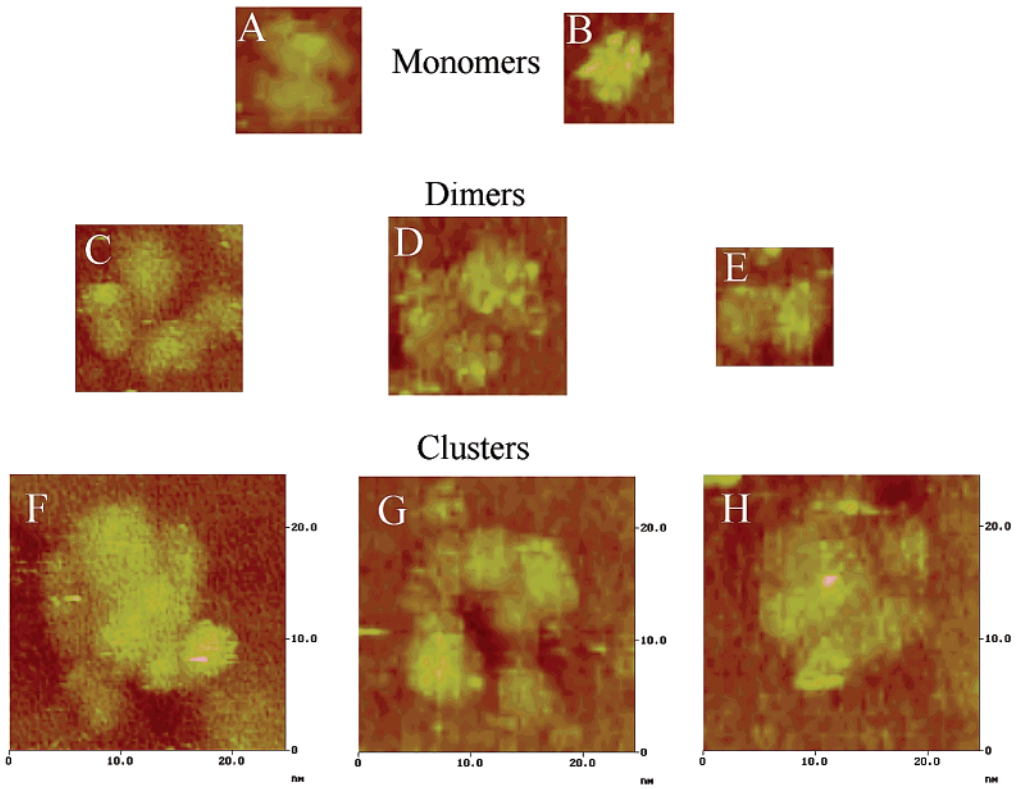

Figure 4. Summary of observed GOD features: (A and B) individual GOD molecules; (C−E) dimers of GOD; (F−H) clusters of GOD.

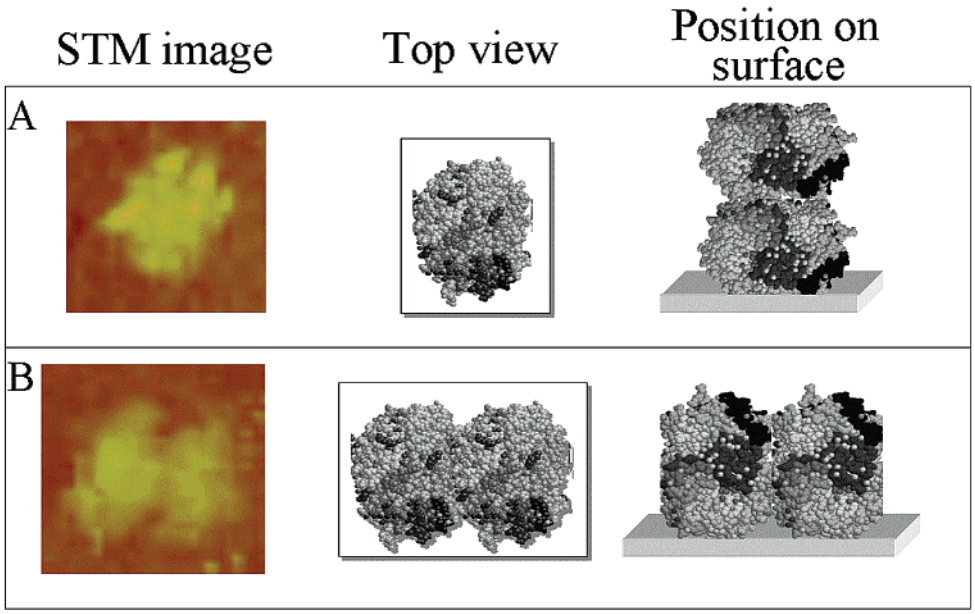

Figure 5. Typical shapes of individual GOD molecules (sphere and butterfly) observed in STM images compared with the top view of the GOD model and the corresponding position on the surface: (A) sphere shape vs model of the standing position of GOD on the surface and (B) butterfly shape vs model of the lying position of GOD on the surface.

that the enzyme denaturation does not occur during the printing process, making the printing approach a viable alternative for deposition of intact biomolecules.

Figure 4C−E shows another class of enzyme features that have been observed. These dimer features have three distinct internal structures: two butterfly molecules (C), two spherical molecules (E), and one of each (D). Cross-sectional analysis of the molecules in each of these dimers confirms that two molecules are present. One interesting observation is that in the dimer with two spherical molecules, one molecule always appears higher than the other.

Three distinct cluster structures have also been found. These clusters are rarely found using a low enzyme concentration but at higher concentrations form the majority of the enzyme features observed in Figure 3A. The first cluster is a three-dimensional cluster (Figure 4F). Cross sections through these clusters show molecules

adsorbed in at least two layers. All of the other clusters observed are two-dimensional only (that is, they consist of a single layer of molecules adsorbed directly to the surface). The second structure has four to six GOD molecules linked to each other on the surface in a tight cluster with a hole in the center (Figure 4G). One of the interesting features of this particular cluster is that while normally the molecules observed in clusters are of the spherical type, one of the molecules in this cluster is in the butterfly orientation. The third cluster is very similar to the second except that the central hole has been filled by another enzyme molecule (Figure 4H). These clusters are relatively rare compared to the other two forms of clusters. The central molecule in these structures seems to sit in the hole but is raised slightly with respect to the molecules directly bound to the surface. This is most probably due to the fact that the donut structure forms initially and then the central molecule fills the hole but cannot fit completely within the hole and hence sits slightly above the plane of the other enzyme molecules in the cluster. The size of the individual molecules in the cluster group has been determined to be about 6−7 nm in diameter on average. The overall dimension of tight clusters is found to be about 15−20 nm in diameter.

It is interesting to note that features with three or four molecules are never observed. This is certainly an indication of the stability of the observed ring structure with five or six molecules. This is confirmed further by our AFM work on the covalently attached GOD where similar ring structures are also observed.[24]

STM Imaging of GOD Monolayers. To study monolayers of GOD molecules, two approaches for immobilization are used: adsorption from solution onto bare gold and covalent attachment of the enzyme to self-assembled monolayers. The STM imaging of both of these enzyme surfaces was difficult. With regard to the enzyme adsorbed directly onto gold, visible distortions and movement of molecular material under the influence of the STM tip were occasionally observed. Large-scale images (>1 μm × 1 μm) of the enzyme layer show visible triangular features of underlying flat gold. The robustness of the enzyme-modified surface was probed using higher tunneling currents in an attempt to produce regions where enzyme molecules are removed. Re-examination of these regions at lower tunneling currents showed the underlying triangular face of flat gold confirming that only one layer of enzyme molecules is present. This is further supported by the fact that the enzyme layer is sufficiently thin to allow significant electron tunneling and hence images with molecular resolution can be obtained.

Figure 6A−D shows several STM images of GOD immobilized on gold from enzyme solution. The STM images show close-packed spherical GOD molecules in a layer structure with considerable long-range order. This provides evidence that GOD molecules exhibit a tendency to spontaneously organize themselves into two-dimensional arrays with adequate sample stability for high-resolution images to be obtained. The black spots visible in the image are believed to be holes which result from discontinuities as seen in the bare gold surface in Figure 1.

Two distinct regions are observed in the monolayer structure, one very ordered and the other quite disordered. The ordered regions show closely packed GOD molecules in a standing orientation. The geometry in these ordered regions is typically of the donut-shaped clusters observed at lower concentrations (Figure 4G). Smaller scale STM images of one of these ordered regions are shown in Figure 6C,D. The other region observed in the GOD monolayer

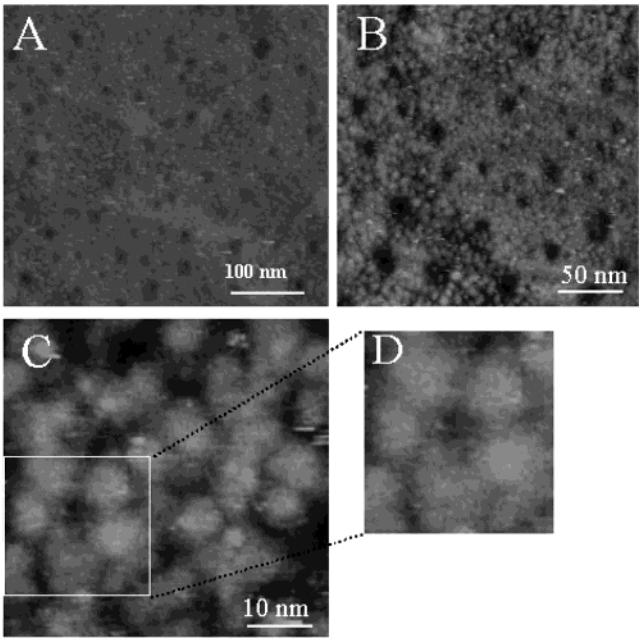

Figure 6. STM images of a GOD monolayer adsorbed from solution: (A) size 500 × 500 nm, (B) size 200 × 200 nm, (C) size 100 nm × 100 nm, and (D) zoom of (C). Bias from 500 to 50 mV; current from 0.2 to 0.07 nA.

is disordered. Few holes between the molecules are observed in contrast to the ordered regions. The disordered regions correspond to the second type of two-dimensional cluster observed at low concentration (Figure 4H). The disorder in these regions may well arise from the molecules nominally sitting just above the plane of the cluster. As stated earlier, these molecules do not fit completely within the holes, and hence we expect them to be quite mobile. This motion will lead to random adsorption in various sites and hence a disordered structure.

Individual molecules of GOD in the monolayer images were clearly resolved. However, because of the closeness of their packing it was difficult to accurately determine dimensions of individual molecules. The dimensions of the spherical form are estimated to be 5−6 nm in diameter which is on the order of the expected size. All of the molecules in the monolayer appear to be in the standing position as opposed to the lying position observed for isolated molecules. This is hardly surprising given that this orientation allows better two-dimensional molecular packing and also greater interaction between the enzyme molecules.

The observation of GOD clustering at both low and high coverages might seem somewhat surprising given that in solution, the molecules are nominally negatively charged. In solution at low concentrations, we would not expect any cluster formation and clusters were not detected by light scattering experiments.[20] Therefore, the clusters must be formed on the surface after adsorption indicating that there must be attractive forces between the molecules. This force may simply be thermodynamically driven. The formation of ring structures seems to be highly favored as we have observed this structure in several dramatically different environments. It is likely that the negative charges are not localized on the molecules following adsorption, as one would expect at least some partial transfer of electrons to the substrate. This transfer must leave the molecules with little residual charge allowing aggregation into the observed ring clusters.

STM images were used to calculate the number of GOD molecules in the monolayer simply by counting the number

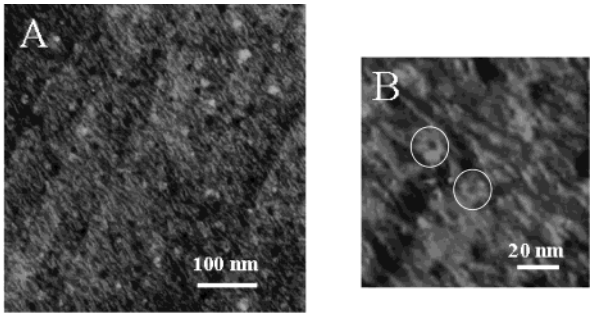

Figure 7. STM images of a GOD monolayer covalently bonded onto a SAM. (A) The hexagonal clusters of GOD molecules are visible on the triangular structure of the underlying gold surface. (B) A zoomed image with more details of GOD features is shown.

of molecules observed in an image. The number of molecules is found to be 1.16×10^{12} molecules cm^{-2} ($\sim$1.9 pmol cm^{-2}). This surface density of glucose oxidase molecules represents a fractional coverage of between 0.43 and 0.52. This coverage is determined simply by counting the number of molecules observed in a 100×100 nm image of the monolayer surface. The coverage determined from this calculated approach certainly represents a lower limit of the number of molecules on the surface for two reasons. First, there are holes in the images at this scale and we cannot see molecules at the bottom of the holes. Our other work,[23] as well as smaller scale imaging in this work, indicates that these holes are filled with molecules. Second, only well-defined molecules have been included in the count. In the disordered regions, molecules tend not to be well resolved and hence these have been omitted.

Previous work has used a QCM to determine the coverage of GOD covalently attached to MPA SAMs.[43] That work determined a coverage of 1.3 ± 0.5 pmol cm^{-2}. The slight difference in these two coverages is traceable to the different substrates used. Our AFM work[24] has shown that the clusters observed on the MPA SAM are much bigger than those observed on the bare gold, and hence, given this lower density, it is hardly surprising that the coverage on the bare gold is higher.

With a view to relating interface structure to biosensor function, the GOD-modified gold surfaces were used as electrodes for the detection of glucose. The electrodes responded to additions of glucose which indicated that the activity of the enzyme was retained despite being adsorbed directly onto the gold. However, the poor stability of the interfaces resulted in electrode response being unstable and irreproducible. In biosensor applications, where the stability of the enzyme layer is essential, immobilization by covalent bonding onto self-assembled monolayers has been shown to be an effective approach for reproducible enzyme immobilization on gold surfaces.[8] These more stable enzyme interfaces were prepared for imaging by covalently attaching GOD onto SAMs of 3-mercaptopropionic acid on gold using carbodiimide coupling reagents (EDC/NHS). As expected, sweeping experiments indicated that the GOD is more strongly bound to the MPA-modified substrate than in the case of physisorption directly to gold. STM images of immobilized GOD on MPA are shown in Figure 7. The underlying triangular face of flat gold and black spots is observed to be similar to the monolayer of physisorbed GOD. Resolution of the adsorbates is extremely difficult, however. This is attributable to two things. First, electrochemical measurements[20] show that the MPA SAM is not well-ordered which is not too surprising due to the short hydrocarbon chain. This will lead to dramatic variation in topography in different parts of the base substrate. Second, having added GOD to the top of this base substrate means that the tip is quite far from the conducting surface making it very difficult to obtain and keep a reliable tunneling current.

Despite these difficulties, we have managed to obtain some rough images of GOD on MPA. The ring cluster structure observed for GOD physisorbed directly onto gold is observed here as well although the structures are difficult to see (see inside circles of Figure 7). Other AFM work in our group shows these clusters much more clearly[20,24] with the enzyme interface looking very similar to Figure 6 with interconnecting clusters over the entire surface. In the STM work reported here, the cluster size on the two substrates (bare gold or MPA-modified gold) appears to be about the same but definitive statements are very difficult to make given the quality of the MPA images. The AFM images suggest that the clusters are larger than those observed with STM. This difference is attributable to the different imaging mechanisms.

Although the interfaces prepared by adsorbing GOD directly onto the gold were too unstable for looking at biosensor function, despite the greater resolution of the STM images being ideal for investigating structure–function relationships, the apparent similarity in the enzyme organization on the bare gold and the SAM surface does allow some conclusions to be drawn from the SAM-based enzyme electrodes. What is clear is that the organization of the enzyme molecules into clusters does not compromise the enzyme activity. QCM studies coupled with electrochemical performance of the interfaces show that at the higher surface concentration of enzyme, where the clusters dominate the interface, the electrodes exhibit high enzyme activity.[5]

Conclusions

We have compared two immobilization methods for the preparation of individual GOD molecules on flat gold: adsorption from solution and the microcontact printing method. This comparison revealed a greater preponderance of individual molecules using deposition from solution, while clusters were more prevalent with printing surfaces. However, more successful imaging of individual native GOD molecules was obtained using the microcontact printing method. Results show that it is possible to image the molecular structure of individual GOD molecules and resolve their orientation on the gold surface using STM. Two different structures of GOD molecules are observed: the butterfly-shaped form with two symmetrical wings corresponding to a lying position and the spherical shape that corresponds to the standing position of the GOD molecule on the gold surface. Larger enzyme features are attributable to different clustering of molecules. The size of the GOD molecule determined by STM is in good agreement with the native size of GOD molecules described in the literature.

STM images with molecular resolution of monolayers of GOD prepared by physical adsorption have been obtained. A close-packed structure of spherical molecules is observed in a monolayer structure showing that GOD molecules exhibit a tendency to spontaneously organize themselves into two-dimensional arrays. Images of GOD immobilized on an MPA SAM are possible, but the image quality is poor.

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