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Immobilized Enzymes as Catalytically-Active Tools for Nanofabrication

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The miniaturization of molecular arrays produces many useful effects, some stemming purely from the greater density of information,[1] some arising from new material properties that arise when the dimensions of the components approach the dimensions of the constituent molecules.[2,3] One efficient strategy for creating nanostructures on surfaces is to use surface molecules to template the construction of self-assembled arrays.[4] This strategy takes advantage of the *binding* properties of the template molecules. Another potential strategy is to use the *catalytic* properties of a surface molecule. Such a strategy would benefit from the amplification and chemical specificity inherent in catalysis. In this report, we describe a demonstration of the key step of such a strategy: the surface trapping of a product generated by a nanometer-scale patch of surface-bound enzyme.

When one reduces the dimensions of a catalyst patch, one also decreases the amount of product that can be produced within a given time, and therefore, one increases the difficulty in analyzing for the product. Even given turnover rates as high as 1000 reactions per second, a patch of 100 catalytic sites produces only 10 billion product molecules per day. If these molecules diffuse into a volume of 100 μL, then there is an analytical difficulty in accurately finding the product in the resulting 1.5×10^{-10} M solution. We solve the dilution problem by trapping some of the product at the surface in a second reaction before it can diffuse away. Subsequent reaction of the catalysis product is more than an analytical necessity, it also offers a new route to nanometer-scale fabrication: the enzyme is used for the further fabrication of the thin film. In this way, a set of enzymes catalyzing different reactions could be used to build up additional complexity on a nanometer scale without human intervention on the nanometer scale.

We have investigated reactions on gold surfaces. In this case, a simple method for trapping molecules is to create a product that reacts directly with the gold surface. The enzyme acetylcholinesterase (AChE) can be used to cleave acetylthiocholine (ATCh) to produce a free thiol (thiocholine).[5] This free thiol can react with gold to form a stable structure (Figure 1). Thiocholine is a particularly attractive analyte because it produces high contrast for atomic force microscope (AFM) imaging (see Figure 2).

We created nanometer-scale reaction and trap sites using the procedures of nanoshaving and nanografting that were developed by Liu and co-workers.[6,7] In nanoshaving, a monolayer of thiol-compound is first attached to a gold substrate. An AFM tip is then used to create a pattern in the film by applying a force to a selected area of the film. The applied force removes the adsorbed thiol. In nanografting, a second thiol is attached to the patterned area during the nanoshaving. It is hypothesized that the gold surface is exposed by the AFM tip during the scratching procedure. If the first and second thiols have different ω-groups, then a chemical pattern is

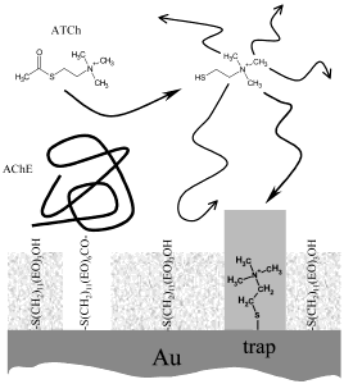

Figure 1. Schematic of the procedure used to trap the catalysis product. A nanometer-sized trap is located near a nanometer-sized enzyme patch, while the remainder of the surface is coated in a film that resists the adsorption of the enzyme, reactant, and product. Some of the product of catalysis is captured in the trap before it can diffuse into bulk solution

created on the surface. Liu's group[8] and others[9] have succeeded in attaching molecules, including proteins, to nanografted surfaces. Adsorption of the protein has been confirmed through attachment of antibodies that recognize the primary sequence, but confirmation of tertiary protein structure or activity in these patches has not been reported.

In our experiments, we first coat a very smooth gold sphere with a monolayer of $HS(CH_2)_{11}(OCH_2CH_2)_3OH$[10] (EOthiol) by deposition from ethanol solution for 24 h. The ω-ethylene oxide groups are known to resist the adsorption of protein molecules.[10] We then use nanografting to create a small patch of carboxylate groups on the surface by deposition of an acid-terminated thiol ($HS(CH_2)_{11}(OCH_2CH_2)_6OCH_2CO_2H$).[10] Note that the carboxylic acid has a longer oligo(ethylene oxide) segment than the alcohol, so the carboxylic acid should protrude from the surrounding area. AChE (*Electrophorus electricus* Type V-S, Sigma) is covalently attached to the carboxylic acid via amide bonds with a standard *N*-hydroxysuccinimidyl ester protocol.[11] The surface is thoroughly washed with phosphate buffer (pH 7), with water, and then with ethanol to remove both unwanted reactants and nonspecifically bound enzyme.[12] We create a trap for the reaction product, thiocholine, by using nanoshaving to create a region of exposed gold. The final result is shown by the AFM image in Figure 2A. Note that the enzyme is localized to a small patch. Nanografting can be used to draw much smaller patches, but a large patch was drawn to raise the probability of obtaining active enzyme.[13]

A 9 mM aqueous solution of ATCh in phosphate buffer (pH 7) at 33 °C was introduced for 2 h, and then the reaction vessel was rinsed with buffer, water, and then ethanol (see Figure 2B). The very bright spots in the trap show the adsorption of the thiocholine. The bright patch where the enzyme is attached is also larger and brighter. We attribute this to weakly bound ATCh or thiocholine.

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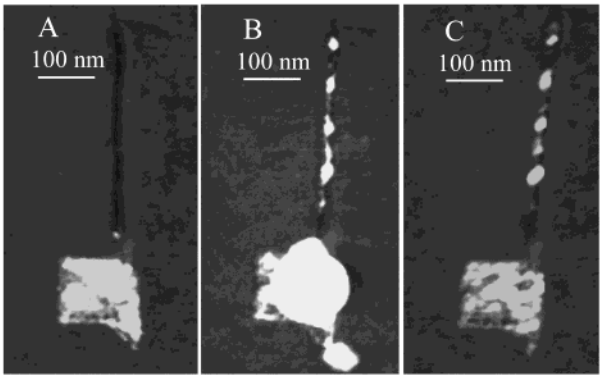

Figure 2. Tapping mode AFM images in ethanol of the enzyme and trap. Both are on the surface of a smooth gold ball that was produced by melting a 1-mm radius gold wire in a H_2/O_2 flame. (A) Before addition of ATCh, the AChE (white patch) is seen at the end of the product trap (dark line). The product trap is exposed gold. The remaining area is covered in EOthiol. (B) After addition of ATCh, most of the trap is filled with material (white), which we attribute to adsorption of thiocholine. There is also a large "plume" of material around the enzyme, which is either reactant or product. (C) The original shape of the enzyme patch is recovered by rinsing with 15 mM NaBr. This salt rinse promotes the removal of electrostatically bound material. All images have a 5-nm *Z*-range. Note that the contrast in the *z*-scale is a combination of height, chemical, and elasticity differences.

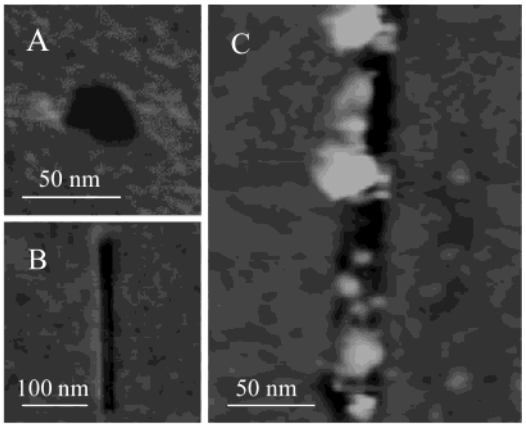

Figure 3. AFM images in ethanol of control experiments when the enzyme was not present. (A) A small hole was scratched in the EOthiol and left for 16 h in water. The size and shape are stable over this period (5-nm *Z*-range). (B) A line-trap was left in 9-mM ATCh and phosphate buffer (pH 7) solution at 33 °C for 2 h. The trap is not filled by ATCh (10-nm *Z*-range). (C) A line-trap was exposed to a solution of thiocholine at pH 9 for 2 h. The free thiol adsorbs in the trap (10-nm *Z*-range).

This weakly bound material was removed by rinsing with NaBr solution (Figure 2C). In other experiments it was removed by gentle scraping of the surface with the AFM tip.

Comparing the trap before and after the addition of ATCh, it is clear that most of the trap contains patches of adsorbed molecules. In some experiments there is a gradient of patches, with a greater density near the enzyme patch (see Supporting Information). This supports the idea that the filling material comes from the physical location of the enzyme and thus is consistent with a product that diffuses from the enzyme.

Two alternate explanations of the observed line-filling need to be addressed before we attribute our observations to enzyme catalysis of ATCh. (1) The remainder of the film consists of thiol molecules. Did the EOthiol simply migrate to fill the hole? Consistent with the original claims from Liu's group, in control experiments in the absence of enzyme and ATCh, a nanoshaved hole in an EOthiol film was stable for at least 16 h (Figure 3A). Thus, lateral migration of the EOthiol does not fill the hole. (2) Does the reactant, ATCh, fill the hole? Figure 3B shows that the hole was also stable in the presence of ATCh when the enzyme was absent. Additional controls are described in the Supporting Information.

As a further check for the consistency of our hypothesis that the filling material is actually thiocholine, we observed the adsorption of thiocholine that was produced by homogeneous inorganic catalysis in aqueous solution. ATCh is easily hydrolyzed to thiocholine by OH^-.[14] Figure 3C is an AFM image of a nanoshaved line that was exposed to a 5 mM solution of thiocholine at pH = 9. The bright spots that appear are very similar to those observed in the presence of enzyme and ATCh.[15,16]

In summary, we have produced a nanometer-scale patch of enzyme and demonstrated that it is catalytically active through a surface-trapping experiment. The localization of active biological material enables the fabrication of high-density biological assays. In addition, the localization of an active catalyst is a step in the evolution of nanostructures that can be used as an aid in the fabrication of additional or more complex nanostructures.

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Supporting Information Available: Image showing distribution of thiocholine within the trap, and additional control experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) We confirmed the activity of the enzyme after immobilization by immobilizing the enzyme on the entire surface of a gold ball, and exposing a solution of 7-acetoxy-1-methylquinolinium iodide (AMQI) to the gold ball. The hydrolysis product of AMQI was visible by fluorescence microscopy.
(13) We use the highest purity of AChE that is commercially available (Sigma, Type V-S, 60% protein, 1000−2000 units AChE/mg protein). The turnover rate of AChE is approximately 16 000 s^{-1}, and the molar mass is about 70 kDa per catalytic site (Rosenberry, T. L. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1975**, *43*, 103−218), so that the proteinaceous material contains 7−15% active AChE. The exterior of *Electrophorus electricus* AChE presents eight lysines that have amino groups available for tethering (Bourne Y.; Grassi J.; Bourgis, P. E.; Marchot, P. *J. Biol. Chem.* **1999**, *274*, 30370−30376, PDB codes 1C2B and 1C2O). The effect of tethering on catalytic activity is unknown.
(14) Green, T. W.; Wuts, G. M. *Protective Groups in Organic Synthesis*, 3rd ed.; Wiley: New York, 1999; pp 482−484. Our solution was initially at pH 10 and was kept at or above pH 9 during the hydrolysis through the addition of NaOH.
(15) The thiocholine molecules produce high contrast in AFM images in ethanol. When imaged using tapping mode in ethanol solution, the thiocholine molecules are about 3.9 nm "higher" than the surrounding EOthiol, even though the extended van der Waals length of the EOthiol is about 1.7 nm longer. Contrast in tapping mode arises from differences in height, force, and elasticity. In this case the contrast is probably high because we contrast the weak tail of a long-range electrostatic force of the quaternary ammonium to a shorter-range solvation force of the ethylene oxide groups. We observe similar high contrast for carboxylate groups imaged under the same conditions.
(16) The facile adsorption of thiocholine to gold was confirmed by exposing a 1 cm^2 thin film of gold to thiocholine and monitoring the adsorption by SPR (Leica).

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