

An Electrochemical Redox Couple Activated by Microelectrodes for Confined Chemical Patterning of Surfaces

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Microelectrodes, printed as an array on the surface of a silicon chip, generate chemically active species in a solution of electrolyte held between the electrode array and a glass plate. The active species induce chemical change in molecules coupled to the surface of the glass plate, which is separated from the electrode array by a gap of several micrometers. This paper explores the nature and pattern of the induced chemical change. The patterning is discussed with respect to the electrolyte composition and the magnitude and duration of current applied to the microelectrodes. We show that under suitable conditions the active species is confined to micrometer-sized features and diffusion does not obscure the surface pattern produced.

The techniques that revolutionized the manufacture of electronic components have recently been applied to biological and chemical systems. Small devices carrying molecules of DNA^{1–6} or peptides⁷ have been fabricated on glass, silicon, and plastic substrates. These chemical and biological “chips” have been used in genomic analysis,^{8,9} gene expression profiling,¹⁰ drug analysis,^{11,12} and environmental and biological sample analysis.^{13–17}

Although several in situ fabrication techniques have been used to make these devices, there remain many significant technical challenges.^{18,19}

There are two requirements for any in situ molecular fabrication method. First, it must be able to apply the methods of chemical synthesis to make molecules of defined structure on a solid substrate. Second, it must be capable of creating the molecular features in spatially defined regions. Many organic syntheses follow a stepwise path in which an active group is exposed by removal of a protecting group and then coupled with an active reagent.^{20–22} Nucleic acid and peptide syntheses are examples of this approach;²³ these chemistries were first adapted to solid-state synthesis and then to patterned synthesis on planar substrates. In these and other syntheses, protecting groups are typically removed by acid or base.

Methods that have been used for patterned in situ synthesis include ink-jet application of deprotection agents,²⁴ application of precursors by physical masking^{25,26} or ink-jet printing,^{27–30} application of physical masks by photolithography,³¹ and removal of photolabile protecting groups by photomasking.^{18,32} Although these methods have proven useful, each has certain disadvantages. Physical masking is only suitable when the synthetic pattern may be formed by overlapping placements of the mask; the resolution of the ink-jet method is limited by the accuracy in aiming droplets of reagents and by their spread when they hit the surface; the

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photolithographic methods require special photosensitive reagents and expensive fabrication of mask sets for each pattern produced.

In this work, we explore a method of patterning a surface using electrochemically generated reagents. Reagents that can be produced electrochemically include acids, bases, radicals, reactive gases or ions, metals, and many types of reducing and oxidizing species.^{33–39} The amount and reactivity of reagents can be controlled by the choice of electrolyte solution or the applied voltage. This fine regulation of the chemical conditions may thus permit a degree of control of the reaction not possible with other fabrication methods.

Electrochemical methods described previously used single active electrodes³⁷ and are not suitable for creating large numbers of small features. We describe a new approach which uses an array of individually addressable electrodes placed a short distance from the surface to be treated. The electrodes generate active reagent that reacts with molecules on the surface. In the examples described here, active reagent is an acid, generated at anodes.

The elements of the microelectrode array are individually addressable, so independent features of the pattern can be generated in parallel. Furthermore, using anodes and cathodes in close proximity introduces a means of controlling diffusion of the reactants. In systems that use a single isolated electrode to generate an active species, such as scanning electrochemical microscopy, reactants diffuse rapidly from the vicinity of the electrode. A number of electrical and chemical means have been used to confine the reagents generated at the microelectrode,^{40,41} but diffusion is intrinsic to any reagent in solution. For example, a small 1.7- μm electrode generates a 250- μm pattern after 20 s which grows to the relatively macroscopic dimension of 400 μm after 80 s.⁴² We have designed a chemical system that limits these effects.

A “quenching” reagent generated at the cathodes destroys acid everywhere but in regions close to the anode that generated it. This constraining effect of the counter electrodes creates features that approach the size of the microelectrodes themselves. Unlike methods such as scanning electrochemical microscopy patterning, where the tool must be manipulated to “write” a surface feature, this method allows “printing” on a surface, with printed pattern determined simply by the microelectrode arrangement.

EXPERIMENTAL SECTION

Materials. Silicon wafers were used for creating the microelectrodes and as the solid supports for the electrochemical

patterning. Wafers were purchased from Aurel GmbH (Landsberg, Germany) as P-type (boron doped), (100) orientation, 7–21 $\Omega\text{ cm}$ resistivity, 100-mm diameter, 518–532 μm thick, CZ Silicon Prime Wafers, SEMI standard. The wafers were thermally oxidized to yield a $124 \pm 0.4\text{-nm}$ surface thickness of silicon dioxide. (Glycidioxypropyl)trimethoxysilane (Sigma-Aldrich, Poole, England) was used as supplied. Poly(ethylene glycol) (200 average molecular weight, Sigma-Aldrich) was used without further purification. Benzoquinone, hydroquinone, and tetrabutylammonium hexafluorophosphate (Sigma-Aldrich) were dissolved in anhydrous acetonitrile (supplied as “phosphoramidite diluent”, Cruachem, Glasgow, Scotland) under dry argon immediately before use. Dimethoxytrityl (DMT) was attached to the substrate as a thymidine β -cyanoethyl phosphoramidite (Cruachem) using standard DNA synthesis reagents (Cruachem). Equal volumes of acetic anhydride and (dimethylamino)pyridine (Cruachem) were used in solution for the acetylation. “Cy5 phosphoramidite” used for fluorescent reporting was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England) and diluted with anhydrous acetonitrile (100 mg/mL) before use. Iridium metal used for microelectrodes was 99.9% purity (Johnson Matthey Noble Metals, London, England).

Microelectrode Array. The fabrication of an array of microelectrodes resistant to reduction, oxidation, chemical attack, and mechanical destruction presented special challenges and will be described in detail elsewhere. Briefly, 96 linear electrodes were fabricated by electron beam evaporation of 50-nm iridium metal onto silicon wafers previously patterned with an organic photoresist using conventional UV light photolithography. After the photoresist was removed in acetone, the iridium was annealed by heating at 350 $^{\circ}\text{C}$ for 30 min in air and then cleaned by reactive ion etching (in oxygen and argon). The resulting microelectrodes, each measuring $40 \pm 0.1\text{ }\mu\text{m}$ wide by 7500 μm long and separated by 40- μm gaps, were connected to separate printed circuit board tracks via 20- μm gold wire bonds.

Preparation of Glass Substrate. Polished, oxidized silicon wafers were used as the patterned surface supports. Before electrochemical patterning, the wafer surface was functionalized with a linker molecule to which the organic reagents were attached.⁴³ Wafers were placed in a 18.1-L vacuum furnace chamber with an ampule containing 5 mL of (glycidioxypropyl)-trimethoxysilane. After the furnace was heated to 185 $^{\circ}\text{C}$, the ampule was heated to 205 $^{\circ}\text{C}$ and the chamber evacuated to 25–30 mBar. After ~ 2.5 mL of the silane had evaporated, the chamber was allowed to cool under vacuum (10^{-3} Torr). A “linker molecule” was attached by immersing the (glycidioxypropyl)trimethoxysilane-derivatized wafers in 200 mL of poly(ethylene glycol) containing 100 μL of sulfuric acid. DMT-containing phosphoramidite was then covalently attached to the free hydroxyl on the poly(ethylene glycol) by conventional oligonucleotide synthesis techniques^{23,44} employing 3% dichloroacetic acid deblocking. The wafer substrate surface thus prepared was cut into 1-cm squares for use in patterning.

Reagent Delivery and Substrate Positioning. Reagents used for the electrochemically directed synthetic steps were flushed across the entire substrate surface in an apparatus designed to

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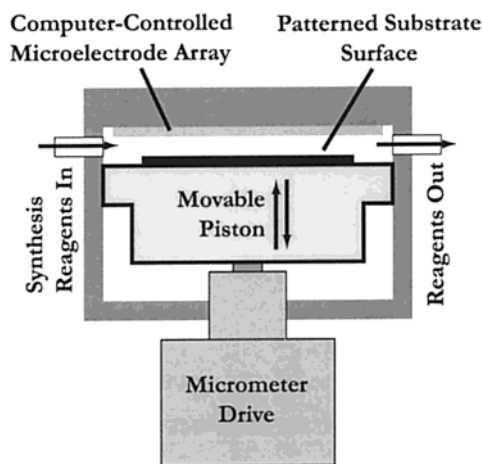


Figure 1. Chemical patterning reactions on a surface carried out in an enclosed chamber through which appropriate reagents could be delivered. A digital micrometer head precisely manipulated a gap between a microelectrode array and the surface so that chemical products generated at the microelectrodes could diffuse to the patterned surface, where synthetic chemical steps were performed. A specially designed computer control board controlled the flow of reagents, applied external potentials to each of 96 individual microelectrodes, and measured the resulting currents.

hold the substrate a specified distance ($20 \pm 1 \mu\text{m}$ in these experiments) from the microelectrode set (Figure 1). This distance could be increased by adjusting a digital micrometer head piston to allow large volumes of solvent or other reagent through the system as appropriate and then reestablished during the electrochemical acid generation.

Acid Generation and Current Measurement. A custom electronic circuit applied current in a parallel fashion to each of the electrodes independently and is described in detail elsewhere. Any electrode could be disconnected from the voltage source (made "floating") by analog multiplex switch integrated circuits. Voltages were applied at the anodes with respect to the cathodes (the electrochemical system is a "two-electrode cell"). As the current delivered to each individual electrode was very small (nanoamps), instrumentation amplifiers were employed in its measurement. A computer software program controlled this electronic circuit and also automated delivery of reagents to the substrate.

Acetylation and Fluorescent Reporter. The loss of the acid-sensitive trityl protecting group on conventional phosphoramidites indicated regions where acid reached the substrate during the electrochemical deblocking step. After electrochemical patterning of a microscope slide coated with trityl-containing phosphoramidite, the entire surface was treated with a mixture of equal volumes of acetic anhydride and (dimethylamino)pyridine using an ABI 394 DNA synthesizer. This reagent renders the regions previously subject to acids unreactive toward further chemical modification. Remaining trityl groups were removed by treating the whole surface with DCA, and a subsequent final modification of the entire surface with Cy5 phosphoramidite resulted in fluorescence everywhere except those regions patterned by electrochemically generated acids.

Fluorescence Detection. Fluorescent molecules were detected using a Leica TCS NT confocal microscope. Confocal microscopy allowed examination of fluorescence in a single focal

plane, thus eliminating background fluorescence while the monolayer was observed. Before each measurement, the focal plane was adjusted to the height of the substrate on the microscope stage and the photomultiplier tube (PMT) voltage adjusted to maximum sensitivity without saturation. The fluorescent units recorded for each image are arbitrary and not directly comparable across experiments, as microscope adjustments were independent for each sample.

Cyclic Voltammetry. Voltammetric experiments were performed with an Autolab potentiostat system (Eco Chemie) in a conventional three-electrode cell with a platinum gauze counter electrode and a saturated calomel (SCE) reference electrode. The cyclic voltammograms were obtained for the reduction and oxidation of an acetonitrile solution (0.1 M NBu_4PF_6) of 2.5 mM benzoquinone and 2.5 mM hydroquinone at a 1-mm platinum disk electrode ($T = 22^\circ\text{C}$).

RESULTS AND DISCUSSION

Interaction of Acid at the Surface. As an example of a step used widely in organic synthesis, we chose to study the thermodynamically and kinetically favorable removal of a DMT group by mild acid to form a primary hydroxyl (an overall depiction of the process is shown in Figure 2). A glass chip was first derivatized with a linker to which a deoxyribothymidine (dT) phosphotriester was attached by conventional phosphoramidite coupling.²³ The dT carried a DMT group on the 5'-hydroxyl. We had previously verified that the DMT group could be efficiently removed by dilute sulfuric acid in acetonitrile.

The objective was to remove this group using acid generated at the anodes of a microelectrode array placed against the glass chip by a substrate positioning apparatus allowing fine control of the distance between the electrodes and chip (Figure 1) and to acetylate the hydroxyl groups in the exposed regions by treatment of the whole surface with acetic anhydride. The DMT groups not removed by the electrochemical step were then removed by treating the whole surface with a solution of dichloroacetic acid in dichloromethane. The hydroxyl groups thus exposed were coupled to Cy5, a fluorescent dye, so that the pattern produced by the electrochemical generation of acid was revealed by observing the fluorescence of the Cy5 in a confocal microscope.

In the following sections, we discuss the processes that generate active species at the electrodes, the reaction that takes place on the substrate, and the interactions that take place in the solution between the electrodes to destroy the species generated at the anode and cathode. We discuss the stoichiometry and kinetics of these processes.

Acid Generation. We have explored the effects of varying the electrolyte solution, and the results presented here were obtained with an electrolyte system optimized for the acetylation patterning process. Acid was generated at the anode by the oxidation of hydroquinone (HQ) to benzoquinone (Q) in acetonitrile. Although the mechanism was subject to some controversy in the early literature,^{45–50} the oxidation half-reaction yields a clean source of protons at the anodes, as shown below:

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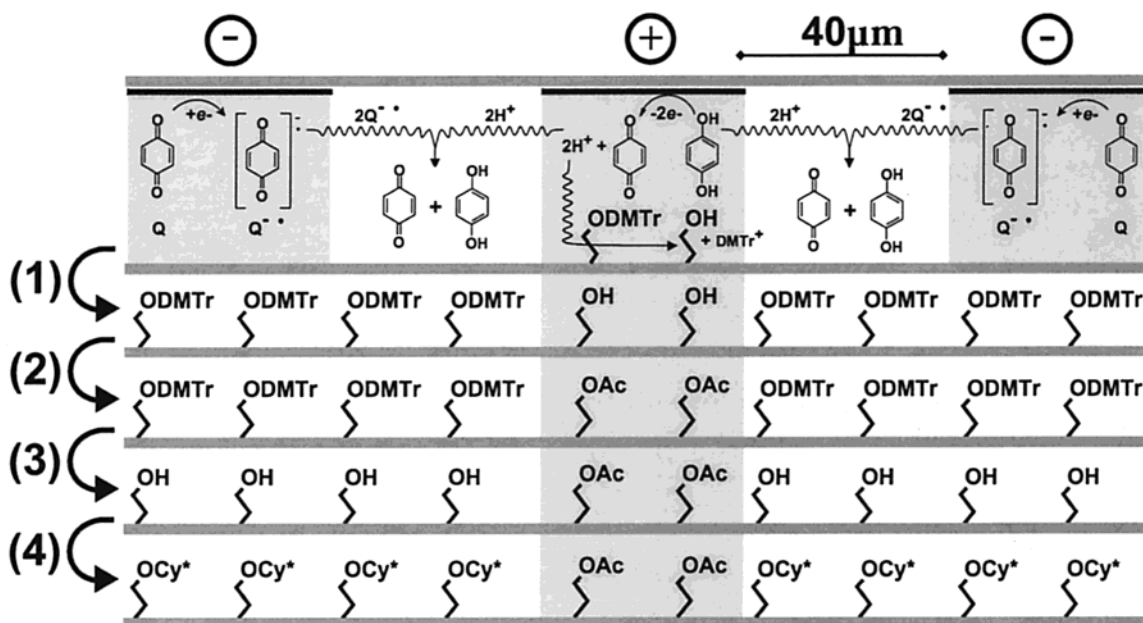
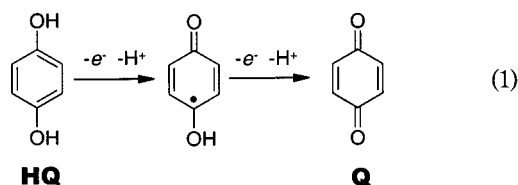
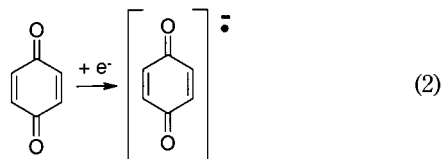


Figure 2. Oxidation process, Electrochemical oxidation of hydroquinone at the anodes (+) on an array of microelectrodes delivers acid to localized regions on a surface (the gray shading symbolizes surface regions near the anode). The acid removes a dimethoxytrityl (DMTr) group (1) to expose a primary hydroxyl (OH), which is then exposed to an acetylating reagent (2) to attach an acetyl group (Ac). Subsequent treatment of the entire surface with an acid solution (3) followed by coupling of a fluorescent (Cy*) dye (4) allows imaging by confocal microscopy so that regions of acetylation are revealed by diminished fluorescence. The counter electrode process at the adjacent cathodes (−) is the reduction of benzoquinone, which yields a radical anion reactive with protons, thus acting to deplete acid in the cathode region and regenerate hydroquinone.



This oxidation of hydroquinone and the reduction of benzoquinone in these experiments was characterized by cyclic voltammetry in bulk electrolyte solution (see Figure 3). The two main processes detected under these conditions are the oxidation of hydroquinone ($P2_{ox}$) at +1.2 V versus SCE peak potential (eq 1) and the reduction of benzoquinone ($P1_{red}$, eq 2) at −0.47 V vs



SCE peak potential. Two minor signals in the voltammogram may be attributed to the oxidation of hydroquinone in the deprotonated state ($P4_{ox}$) and the reduction of benzoquinone in the presence of electrogenerated acid ($P3_{red}$). Overall, the reaction scheme is complex but in agreement with previous reports.⁵⁰

The process at the cathodes is the reduction of benzoquinone and yields a radical anion as shown in eq 2.⁵¹ The radical anion is relatively stable but may undergo followup chemical reactions in the presence of protons. We therefore speculated that acid in

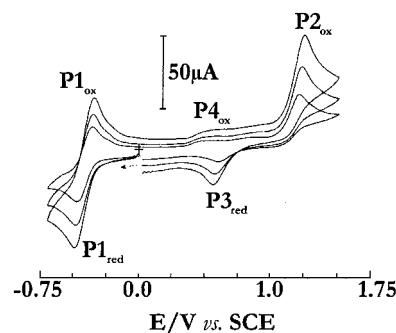
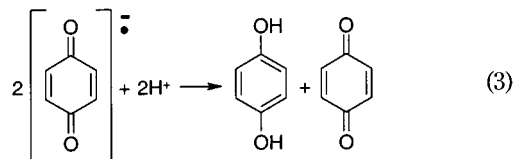


Figure 3. Reversible oxidation of hydroquinone and reduction of benzoquinone. The chemical system comprising this process generates well-defined, electrochemically induced pH gradients, with proton generation at the anode and proton consumption at the cathode. This figure shows cyclic voltammograms for the reduction and oxidation of an acetonitrile solution (0.1 M NBu_4PF_6) of 2.5 mM benzoquinone and 2.5 mM hydroquinone at a 1-mm platinum disk electrode (scan rates 200, 500, and 1000 mV/s, $T = 22^\circ\text{C}$). The peak $P1_{red}$ corresponds to the reversible reduction of benzoquinone to yield a radical anion, and $P2_{ox}$ corresponds to the oxidation of hydroquinone. Minor signals $P4_{ox}$ and $P3_{red}$ have been assigned tentatively to the oxidation of deprotonated hydroquinone and the reduction of protonated benzoquinone, respectively.

solution would be consumed by the cathodic radical anion as shown in eq 3. As the radical anion and proton are of opposite



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charge, we considered that electric field effects in depleted concentrations of supporting electrolyte may also act to enhance the annihilation of acid.⁵²

We speculated that the acid generated in the anodic process (P2) would be strong enough to remove the acid-labile DMT group. The voltage (applied at the anodes with respect to the cathodes) required to establish the pH gradient was expected to be between 0.9 (difference between potentials for reduction P1 and oxidation P4) and 1.67 V (difference between reduction P1 and oxidation P2). Observations described below support this prediction and demonstrate that the rate of production of acid at the anodes and the rate of diffusion across the gap to the surface leads to complete removal of the DMT in a few seconds.

Stoichiometry and Kinetics. The rate of the patterning reaction is determined by the probability that protons in solution reach the immobilized DMT at the surface. We therefore found it instructive to compare the total number of hydroquinone molecules in solution to the number of DMT groups on the surface.

Before voltage is applied, the amount of DMT on the glass chip is ~ 10 pmol/mm² surface area.⁵³ The quantity of hydroquinone in a 20- μ m depth of electrolyte solution overlying the surface is 500 pmol/mm². Thus, oxidation of all the hydroquinone in this area would result in a 100-fold molar excess of acid, assuming all protons transverse the electrode-surface gap and arrive evenly over the chip surface.

A true estimate of the number of protons generated at the anode can be obtained from measurement of the current. The number of protons (N_p) produced during patterning of duration t is equal to the number of electrons (N_e) removed from the anodes by the external voltage source and was calculated from the applied current (i) as follows:

$$N_p = N_e = \frac{\int_0^t i dt}{F} \quad (4)$$

where F is Faraday's constant (we assumed 100% current efficiency for these calculations). We designed an amplifier circuit to record current at each of the microelectrodes to nanoampere precision, with negligible measured background current. We then used the electrodes to generate patterns and considered the amount of acid produced under various conditions.

We applied a range of dc potentials to anodes (with respect to cathodes) which were 7.5 mm long by 40 μ m wide and separated from adjacent cathodes by 40 μ m (the significance of the electrode arrangement is discussed later), with a depth of solution of 20 μ m between the electrode array and substrate. The rate of protons generated is very small at cell potentials below 1.2 V and increases exponentially above 1.3 V (Figure 4), with a corresponding increase in the rate of the patterning at the substrate (Figure 5). The current is high directly after the voltage is applied while the hydroquinone near the anodes is oxidized (as in a potential step experiment). It then reaches a diffusion-limited steady state near 1 μ A for a cell potential of 1.33 V after ~ 2 s, when the rate of

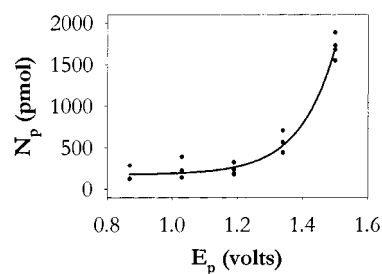


Figure 4. Total protons liberated after 20 s as a function of the applied electrical potential (E_p). The number of protons (N_p) produced was calculated from electrical current measurements. The voltage and microelectrode geometry for these experiments is as shown in Figure 5. The plot shows that N_p varies exponentially with potential. Thus, the rate of acid production and therefore surface patterning may be manipulated by small changes in applied potential. The total number of protons liberated at high voltages exceeds 1000 pmol (the maximum amount that can be generated without regeneration of reactant), thus demonstrating the regeneration of hydroquinone from benzoquinone and free protons in solution.

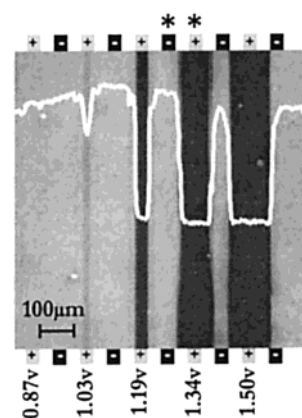


Figure 5. Fluorescent image of a surface after patterning with electrochemically generated acid. White (fluorescence) appears where the surface was left unchanged by the acid generated at the anodes; dark areas represent formation of an ester bond at the surface where the dimethoxytrityl was removed. The active patterning reagents are generated at the anodes (+) and confined by the cathodes (−). The potential applied to the microelectrodes greatly affects the surface patterning reaction. Five different potentials were applied for 20 s to electrodes 20 μ m from the surface. A potential of 0.87 V generated insufficient quantities of acid for reaction at the surface. At 1.03 V, reaction is detectable but incomplete. In contrast, at 1.50 V, reaction is complete everywhere inside the flanking cathodes. The overlying white plot indicates average fluorescent intensity (arbitrary units) across the image.

anodic hydroquinone oxidation is balanced by its regeneration from benzoquinone and protons at the cathode.

We consider here the total amount of acid generated over an anode versus the amount required to pattern the surface in a time course experiment where the electrodes were 7500 μ m long by 40 μ m wide and separated from adjacent cathodes by 40 μ m, with a depth of solution of 20 μ m between the electrode array and the substrate. At 1.33 V, 2.0 ± 0.1 pmol of protons are generated at each anode in 0.2 s, rising to 800 ± 40 pmol in 80 s. This total quantity of acid generated after 80 s is in vast excess to the DMT groups in the region of the substrate opposite the anode.

Analysis of the reaction at the surface of the substrate shows that reaction is essentially complete after ~ 4 s at 1.33 V, resulting in a 75- μ m-wide pattern (Figure 6) and the complete detritylation

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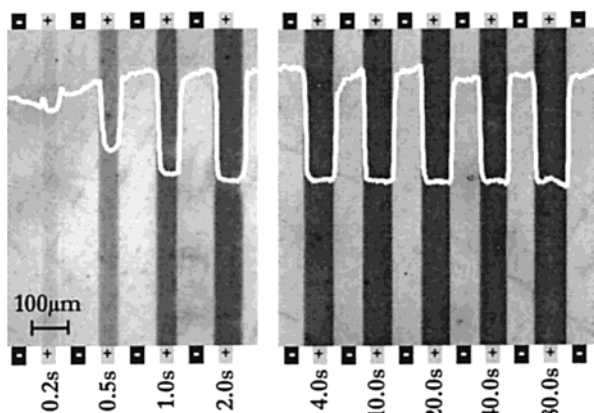


Figure 6. Surface reaction following timed applications of current. The extent of reaction at the surface may be controlled and investigated by delivering timed applications of current to the electrodes; the patterning reagent is confined to the anodic region over very long times. This image shows the patterns formed over a time course, using a fixed potential of 1.33 V applied to electrodes 20 μm from the surface. Diffusion does not blur the edges of the stripe formed after 80 s. Instead, the sharply defined stripes are limited to regions between the cathodes and show that patterning reagents may be directed to strictly confined areas. After 0.2 s, detritylation is incomplete; it is near completion at 1.0 s and complete by 2.0 s. With longer times, the width of the stripe increases slightly until it is stable at ~ 10 s. There is no detectable change between 10 and 80 s, when the reaction was terminated.

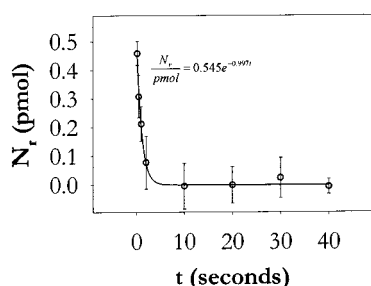


Figure 7. Initial extent and rate of the chemical reaction at the surface determined by the amount of acid reaching the surface. This plot shows the rate of patterning reaction, measured as the consumption of unreacted surface molecules (N_t), and is taken from area measurements of the stripes in replicates of the experiment shown in Figure 6. In these experiments, protons were liberated at the anode at a measured, constant rate of 10 pmol/s. The reaction rate at the surface is determined by both this rate of proton production and the proportion of these protons that reach the patterned surface. If the protons reach the surface, they act to remove remaining DMT groups; if they travel elsewhere, they are consumed by the cathodic products (reaction shown in Figure 2). The plot shows that reaction is complete by ~ 4 s (additional protons liberated after this time elicit no further chemical change).

of 30 pmol of DMT. Figure 7 shows stoichiometry calculated from replicates of the experiment in Figure 6, where N_t was calculated by measuring the surface area of the completely reacted stripe and multiplying by the known initial density of unreacted DMT on the surface, as described above. The amount of acid generated after 4 s is 40 ± 2 pmol. Some of this acid does not reach the substrate because it is consumed by an interaction with products diffusing locally from the cathode into the region between the electrodes.

Diffusion and Proton Neutralization. We believe the electrode arrangement presented in this paper causes consumption

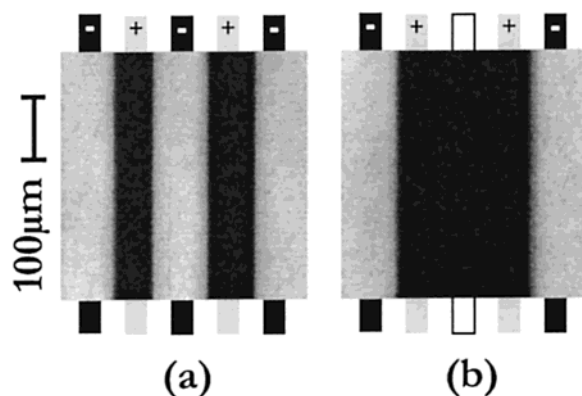


Figure 8. Ability of the cathodes to confine the acid. (a) Current (fixed at 1.33 V for 16 s) applied to two anodes and three cathodes produced two dark stripes. (b) Without a cathode separating the anodes (outline \square shows where the cathode was disconnected from the circuit), anodic products flood the middle area, resulting in a single, wide stripe. The patterned surface was 40 μm from the electrodes in this experiment.

of anode-generated protons near the cathodes by the reaction shown in eq 3. Because we used cathodes situated only 40 μm from the anodes, the products liberated at the cathodes diffuse rapidly to mix with the protons generated at the anodes allowing regeneration of hydroquinone and benzoquinone in the solution between the electrodes (complete reaction system as mentioned previously in Figure 2). These products can then diffuse into the region of the electrodes where they will take place in further electrochemical reactions.

Figure 8 is a dramatic demonstration of this effect; a single cathode placed between two adjacent anodes prevents any acid from reaching the surface in its vicinity upon application of 1.33 V for 20 s. Subsequent removal of the cathode allows protons to flood the area.

That the width of the line in Figure 6 does not increase with prolonged generation of acid demonstrates the confinement of protons over very long time periods. As the patterning technique presented here utilizes free protons in solution, the lines generated at the surface would become wider if protons were free to diffuse over time. For example, an unconfined collection of 40 pmol of protons released from the 40- μm -wide anodes would reach the surface to produce a stripe over 400 μm wide after 40 s. Figure 6 shows that this does not occur; the radical anions near the cathode consume protons in this region and thus limit the stripe to ~ 75 μm , and even after 80 s the width of the stripe is unchanged.

The ability to deliver sharp pH gradients over a long time period provides significant advantages in inducing chemical reactions to completion on a surface. Although this particular reaction was relatively fast so that the surface was patterned after ~ 4 s, less kinetically favorable reactions may be driven to completion by delivering anodic products to confined regions for any duration required, without diffusion outside the regions.

CONCLUSIONS

In this work, we have demonstrated a number of advantages of the use of arrays of microelectrodes for performing synthetic reactions on a surface.

First, the proximity of anodes and cathodes restricts diffusion of the active species generated at one electrode by interaction

with the products of the electrode of opposite charge. Thus, the pattern of change induced on the substrate closely mirrors the pattern of the electrode array, and features with dimensions of a few micrometers are readily generated.

Second, fine control of the reaction is permitted by regulating the voltage and duration of the pulse and by measurement of current.

Third, a high degree of parallelism permits many different reaction conditions to be applied to different regions of the substrate surface; the number is limited only by the number of electrodes in the array.

We illustrate the method by the removal of an acid-labile protecting group that is commonly used in organic synthesis. Elsewhere we will describe the extension of this reaction to the synthesis of oligonucleotides, but the method has wider potential. Electrochemical methods can be used to generate acids and bases or other reactants of different strengths by changing the electrolyte, solvent, or applied potential. There are many examples of

organic synthesis steps that use acids, bases, or other reactants that could be generated at electrodes. Thus, the method could be applied to a very diverse collection of chemical syntheses.

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