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Electrochemically directed synthesis of oligonucleotides for DNA microarray fabrication

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ABSTRACT

We demonstrate a new method for making oligonucleotide microarrays by synthesis *in situ*. The method uses conventional DNA synthesis chemistry with an electrochemical deblocking step. Acid is delivered to specific regions on a glass slide, thus allowing nucleotide addition only at chosen sites. The acid is produced by electrochemical oxidation controlled by an array of independent microelectrodes. Deblocking is complete in a few seconds, when competing side-product reactions are minimal. We demonstrate the successful synthesis of 17mers and discrimination of single base pair mismatched hybrids. Features generated in this study are 40 μm wide, with sharply defined edges. The synthetic technique may be applicable to fabrication of other molecular arrays.

INTRODUCTION

DNA microarrays are increasingly used in the biological sciences to help interpret the data emerging from large-scale genome sequencing. They are used for the analysis and comparison of gene expression levels (1–3), resequencing genes to identify mutations (4–6), analysis of sequence polymorphism on a large scale (7,8), optimization of antisense oligonucleotides (9), basic studies of molecular hybridization (10,11) and analysing DNA–protein interactions (12). Several methods have been developed to fabricate arrays. One set of methods deposits spots of presynthesized nucleic acids on the surface of the support (3), or on microbeads (13). In others, probes are synthesized *in situ* (14–17). Each method has its strengths and weaknesses. An ideal method of array fabrication would be rapid so that an array could be designed and made within a few hours, would have high spatial resolution to reduce the size and hence the amounts of reagents used in hybridization, would allow the use of different chemistries so that probes could be made with different backbones and bases, would allow 3′ or 5′ attachment so that probes could be used in enzymatic extension as well as hybridization based tests and would be programmable so that a computer file would direct defined sequences to defined sites on the array. No existing method of array fabrication meets all these criteria. A photolithographic method provides high spatial resolution (15) and has proven successful for repeat manufacture of the same oligonucleotide sets. But as each oligonucleotide set requires a new mask set, the method is less suitable for custom designs. A new light-directed method (18), which uses programmable microarrays of mirrors rather than masks, may eliminate this problem and new photolabile protecting groups may improve coupling yields (19), which are reported to be lower than conventional oligonucleotide synthesis yields at present (20,21). Physical masking using mechanical flow cells and conventional synthetic chemistry gives high coupling yields and also provides high resolution, but is best used for sets of probes with related sequences, such as all sequences of a predetermined length (22), or tiling paths of all oligonucleotides complementary to a gene of known sequence (23). Ink jet fabrication is rapid, highly flexible and has a high throughput (24–26). However, its resolution is limited by the accuracy in aiming droplets of reagents and by their spread upon surface impact (17).

Ideally, all molecules at each location in an array should have the same predefined base sequence. For *in situ* synthesis, whereby nucleotides are coupled one at a time to the growing chain, non-quantitative yield at any nucleotide addition results in a defective oligonucleotide probe at that location on the array. This problem places heavy demands on the chemistry for oligonucleotide synthesis, which must have high stepwise yields with a minimum of side reactions for each chemical step required to add a nucleotide.

In this paper, we describe a new method for directing the synthesis of oligonucleotides on the surface of a solid support, which uses electrode arrays to induce electrochemical reactions in highly localized regions. The microarray ('DNA chip') is made on a planar substrate of either glass or silicon dioxide, the face of which is placed against the electrode array for the duration of synthesis. At each step of synthesis, acid is produced in confined regions by application of current to individual electrodes, directing patterned removal of protecting

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groups on the adjacent substrate. We demonstrate the rapid synthesis of 17mers, in small, confined regions and show discrimination of single base mismatches by hybridization.

MATERIALS AND METHODS

Electrode and reaction chamber assembly

Electrode materials were chosen to exhibit high conductivity and chemical stability under the harsh redox conditions employed during deblocking. Positive resist photolithography was used to produce iridium metal (50 nm thickness) electrodes on oxidized high-resistivity silicon wafers. After heat annealing and cleaning, each electrode was individually connected by ultrasonic gold wire bonding to a printed circuit board, where digitally controlled analogue switch integrated circuits activated electrodes chosen for a given deblocking step. Currents were applied as constant voltage sources regulated by independent operational amplifiers. Parallel low-noise instrumentation amplifier feedback circuits continuously measured nanoamp-precision current at each of the electrodes. A computer, programmed for this work, controlled all voltages, timing and electrode switching, and collected current measurement data.

The piston and cylinder of the synthesis chamber assembly was machined from PTFE, chosen to provide a good seal against the atmosphere and be resistant to chemical attack by the harsh reagents used during synthesis. A fixed force of 500 N, applied to the synthesis chamber assembly with a stepper motor-driven clamp, sealed the reaction chamber assembly against the electrode array before each experiment.

Synthesis chemistry

A modified DNA Synthesiser (ABI 394 DNA Synthesiser, ABI) delivered all reagents to the synthesis chamber assembly. Delivered volumes of freshly prepared electrolyte (25 mM hydroquinone and 25 mM benzoquinone with 25 mM tetrabutylammonium hexafluorophosphate in anhydrous acetonitrile), phosphoramidite solution (0.1 M with 0.5 M tetrazole activator solution; Cruachem), oxidizing solution (0.1 M iodine in 20% pyridine, 10% water and 70% tetrahydrofuran; Cruachem) and washing solvent (acetonitrile) were individually optimized to completely fill and rinse the reaction chamber cavity and allow sufficient time for complete chemical reaction. The piston was routinely separated 800 μm from the electrode during delivery of all reagents. For nucleotides added using conventional deblocking, 3% dichloroacetic acid in dichloromethane was used in place of the electrolyte solution.

The solid support used for synthesis was a glass chip derivatized with (3-glycidoxypropyl)-trimethoxysilane and a polyethylene glycol linker (27). An initial dimethoxytrityl (DMT)-protected dA residue was then added across the entire support surface to present a DMT group to the first deblocking step.

Preliminary experiments established that, holding all other variables constant, decreasing the voltage at the electrodes, shortening the current application time or increasing the separation between the electrode array and substrate decreased the quantity of acid reaching the substrate. In optimizing the acid exposure for all work described here, we fixed the voltage of the anodes at 1.33 V with respect to the cathodes and maintained a separation distance of 40 μm, while varying only current application time (ranging from 0.5 to 90 s).

Hybridization to synthesized probes

Probes were deprotected according to the method of Polushin *et al*. (28) by immersing the entire substrate in a 50% aminoethanol, 50% ethanol solution for 25 min at 60°C. Hybridization targets were 5′ labelled with Cy5 (Amersham) and purified by high-pressure liquid chromatography (HPLC). Hybridization reactions were carried out at 25°C in solutions (5 μl) containing 50 pmol/μl target, 0.1% w/v ficoll, 0.1% w/v polyvinylpyrrolidone, 0.1% BSA pentax fraction V, 20 mM Tris–HCl, 50 mM KCl, 10 mM $MgCl_2$ and 1 mM EDTA, in distilled water, (pH 8.3) by introducing the solution between a glass coverslip and the substrate. A brief room temperature wash in hybridization buffer was used to remove any non-specific interactions without significant dissociation of the target. Hybridization to the substrate was analysed by a Leica TCS NT confocal microscope with maximal pinhole aperture, in photon counting mode. As substrate alignment in the microscope focal plane is difficult to achieve, but important to measured readings, each signal was maximized by adjusting the microscope stage before measurement. Because the full range of hybridization signals exceeded the dynamic range of the microscope, each sample was observed with three or more photomultiplier tube (PMT) voltages to ensure measurement of the full signal range of each sample. Images from the confocal microscope were captured in a digital format and quantified using 'The Image Processing Toolkit' (Reindeer Software) in Adobe Photoshop 6.0.

RESULTS

Others have made arrays by attaching presynthesized nucleic acids to electrodes (29), and it is possible to make arrays by *in situ* synthesis on electrodes (30). In these systems, the array of electrodes becomes incorporated into the nucleic acid array. However, oligonucleotides synthesized directly on electrode surfaces may be subject to destructive electrolysis products, and there are advantages to using an electrode array as a printing tool so that one such tool can make many arrays. Our objective was to make a device similar to a printing tool, with addressable, individually controlled, electrodes, so that the same tool could make arrays with different sets of oligonucleotides.

To evaluate this method of making microarrays, we addressed the following five areas:

(i) manufacture of suitable, robust microelectrodes
(ii) development of a programmed power supply to deliver predetermined potential to each electrode independently for a preset duration with means to measure current at each electrode
(iii) construction of an automated reaction system, including a sealed chamber in which to carry out the electrochemistry and nucleotide coupling reactions
(iv) choice of electrolyte
(v) optimization of reaction parameters.

The apparatus and its construction (summarized in Figure 1) will be described in detail elsewhere. The most important

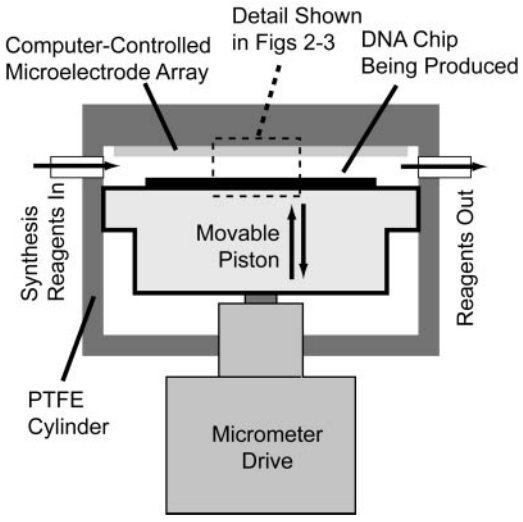

Figure 1. Apparatus used for electrochemical treatment and oligonucleotide synthesis. Synthesis is accomplished in a reaction chamber in which an array of electrodes directs acid deblocking to confined regions on a solid support, a glass chip. The surface of the electrode array is sealed against the end of a PTFE cylinder to create a cavity. The glass chip is mounted on the end of a PTFE piston positioned by a micrometer drive. During the electrochemical deblocking step, the chip is presented to the electrode array by moving the piston forward to a 40 μm gap. The piston is then moved back to widen the gap to 800 μm to allow the cavity to be flushed with solvent, electrolyte or solutions of reagents at the different stages of oligonucleotide synthesis, delivered from an ABI 394 DNA Synthesiser. A computer coordinates and controls the overall process: delivery of reagents from the synthesizer and application, switching and measurement of current to the electrodes. Oligonucleotides of predetermined sequence can be made at any desired positions on the chip by appropriate programming of the electrochemical deblocking and nucleotide coupling steps.

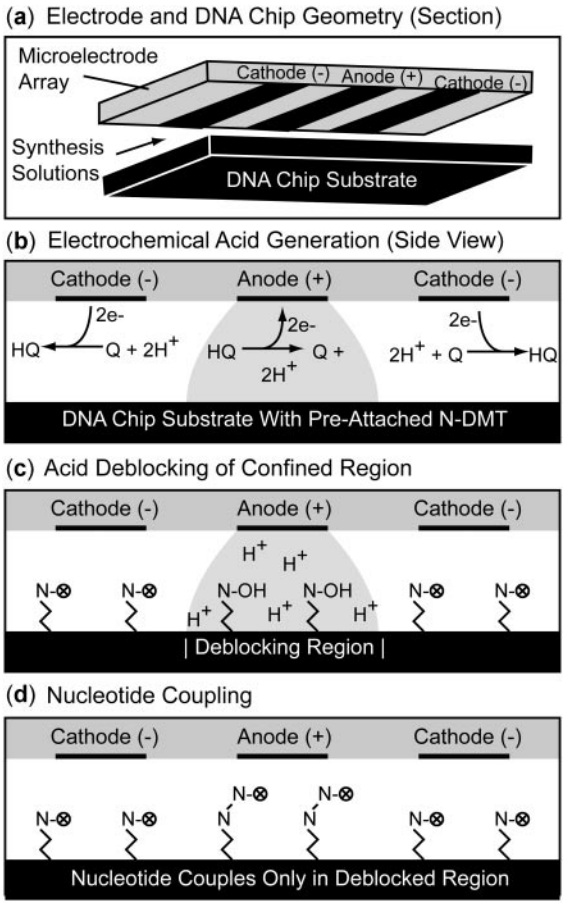

Figure 2. Chemical reaction cycle. (**a**) Alternating electrodes of the array were connected as cathodes and anodes and placed adjacent to a glass chip with the apparatus in Figure 1. (**b**) The electrolyte held between the surface of the chip and the electrode array comprises a solution of hydroquinone (**HQ**) and benzoquinone (**Q**) in acetonitrile. Oxidation at the anode produces acid (in the shaded region) and regenerates the benzoquinone consumed by cathodic reduction. Acid (**H**+) migrates away from the anode. Acid that migrates sideways reacts with cathodic products. (**c**) Acid that migrates downwards will meet the surface of the chip where it induces deblocking [removal of the DMT group (encircled cross, ⊗) from the end of the oligonucleotide chain], thus selectively determining where the next nucleotide (**N**-⊗) additions will occur (**d**). The amount of reactant produced and therefore the extent of modification of the surface depends on the strength and duration of the current.

element of the apparatus is the array of metallic electrodes. These corrosion-resistant electrodes were linear, 40 μm wide, separated by 40 μm gaps, and fabricated by thin-film photolithography. The accompanying control circuitry allowed independent addressing and current measurement at each electrode. The electrodes were durable and used for over 500 cycles without mechanical or electrical deterioration (as determined by scanning electron microscopy and resistivity measurements). This paper is mainly concerned with the choice of reaction conditions to give the maximum oligonucleotide yield in well-defined microscopic areas on a glass or silicon support.

Electrochemical generation of acid by arrays of microelectrodes and interaction with an apposed substrate

Oxidation of an electrolyte solution after the application of current to microelectrodes liberates acid at the anodes; concomitant reduction at the cathodes consumes acid (Figure 2). The ions and radicals generated by these redox reactions at the electrode surfaces move away from the electrodes and to the substrate through a combination of diffusion, migration and convection effects. During this transit time the electrode products may further react. Once the primary or secondary products reach the substrate, they may either react to remove protecting groups (deblock), facilitating coupling of the next nucleotide or depurinate and thereby destroy the oligonucleotides already synthesized. This complex nature of the system precludes modelling without unreasonable assumptions and so we have developed an empirical approach, which allows us to see the end result of acid reacting with groups on the surface of a glass substrate placed against the electrode array.

We used fluorescent dyes as reporter groups. In an extensive set of experiments, fluorescent Cy5 groups were attached to any regions where the electrochemical reactions exposed free hydroxyl residues using otherwise conventional DNA synthesis chemistry. These preliminary experiments (data not shown) allowed us to find a range of conditions, which gave high deblocking yields in well-defined regions. They also established the basic shapes and dimensions of the patterns of deblocking on the adjacent surface, and showed the effects

of varying the voltage, the duration of the pulse, the spacing between electrodes and the distance separating the microelectrode array and the surface of the substrate. One unexpected result of this series of experiments was the high edge definition of the pattern generated by the action of the protons on the substrate surface. We have shown that this effect is due to annihilation of protons by the electrode products of the cathodes that flank the anodes (31). Another desirable outcome of this effect is that diffusion of reactive protons to adjacent regions is suppressed to an insignificant level.

The total yield of oligonucleotide on the surface is likely to be of the order of 1–10 pmol per mm^2—too small to measure via direct methods or via HPLC analysis after cleavage from the surface (32). To overcome this measurement difficulty, we estimated the synthesis yield of oligonucleotides, and hence the extent of the electrochemical reactions, from the level of hybridization to a complementary fluorescent probe. For semi-quantitative analysis, we took three sets of measurements for each experiment at different PMT voltages so as to view all intensity values of the sample in a linear response region of the PMT. Given likely fluorescence self-quenching, we further verified an approximately linear fluorescent intensity response in the small range of probe densities used in these experiments (calibration curve data not shown). This approach was sufficient to optimize stepwise coupling yield, as it was easy to determine the set of conditions that produced the highest yield. An indirect approach to estimating the rates of deblocking and depurination is described below.

Synthesis of oligonucleotides using electrochemical deblocking

The yield and quality of oligonucleotides are of great importance for any *in situ* method of making oligonucleotide arrays. In the method presented here, only the deblocking step differs from conventional phosphoramidite oligonucleotide synthesis chemistry. Coupling yields are close to quantitative for synthesis on columns of controlled pore glass, with moderate excess of reagent over the large number of active sites on the solid support (33). For the planar glass substrate used in this work, the excess is orders of magnitude larger. We therefore assume coupling efficiency is approximately quantitative (as in conventional phosphoramidite synthesis) and consider how the overall yield of oligonucleotide is affected by incomplete deblocking and losses due to depurination. In a series of experiments, a six base sequence, 3′-CGCATC-5′, was synthesized on top of a lawn of dA_{10}, introduced to maximize detection of any electrochemically induced depurination [dA is the most acid-depurination sensitive base (34) and deprotection may lead to probe cleavage during deprotection]. In one set of early range-finding experiments using electrochemical synthesis for two of the six residues, time was varied over a wide range (Figure 4), and in another experimentation set, the voltage was varied (data not shown). The synthesis of the hexanucleotide was observed by hybridizing to a Cy5-labeled target containing the complement of the above sequence (detailed in Materials and Methods where the full hexamer was synthesized electrochemically as shown in Figure 3b). This target hybridizes only to the full six base sequence of the probe; absence of any one of the six bases abolishes hybridization (31).

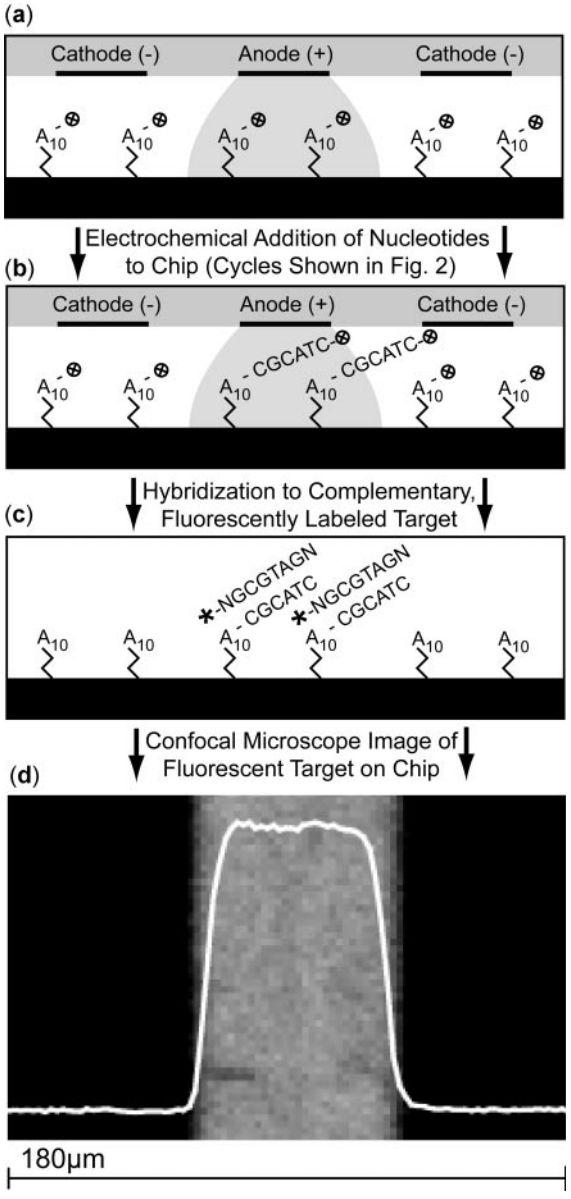

Figure 3. Evaluation of electrochemical deblocking by hybridization to a fluorescent complementary hexamer. (**a**) The hexamer was synthesized by adding six bases on to a presynthesized 'lawn' of 10 dA nucleotides. (**b**) Electrochemical deblocking, followed by base addition was repeated six times (for the result shown) to produce the hexamer. (**c**) This electrochemically synthesized oligonucleotide was detected by hybridizing to a complementary target known to hybridize in high yield only to the complete hexamer probe sequence. (**d**) The resulting fluorescent image of the complementary target; the high signal of the complementary target with respect to background fluorescence shows that synthesis of the hexamer was in high yield. The shaded region shows where electrochemical deblocking was performed as in Figure 2b and c and the white plot indicates fluorescent intensity across the image (arbitrary units).

Rates of deblocking and depurination and synthesis yields

Deblocking in the region of the substrate opposite the anode is rapid. The distribution of hybridization intensity produced by electrochemically deblocking for 3–5 s (Figure 4) suggests that acid has reached the substrate surface as a cylindrical

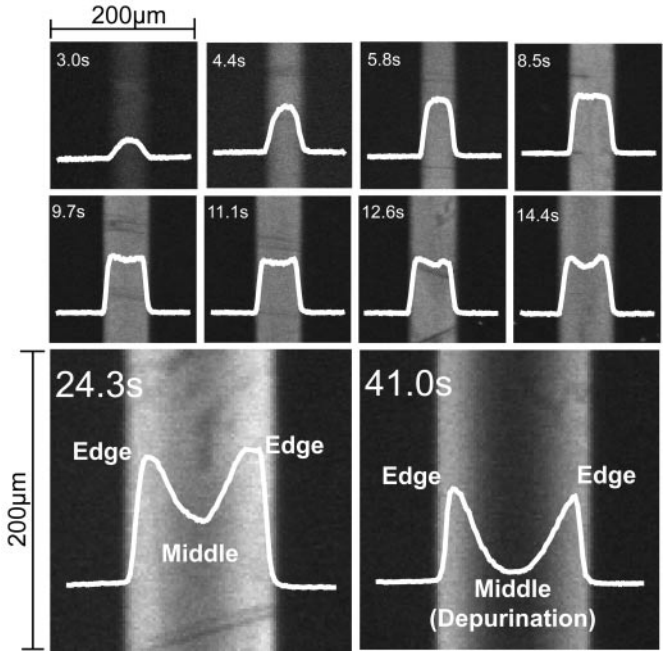

Figure 4. A time course experiment with synthetic steps as shown in Figure 3. The images show hybridization to stripes of hexadecanucleotide, AAAAA-AAAAACGCATC, made by coupling the first 14 nt over the whole surface. For the final two couplings, deblocking was carried out electrochemically. In each image, the duration for each deblocking step (in seconds) is shown in the upper left. The white plot indicates fluorescent intensity across each image; the units are arbitrary but consistent for all images. At short times (~3 s at each of the electrochemical deblocking reactions) the yield is low, because deblocking is incomplete. Intensity of hybridization increases to a maximum between 5.8 and 8.5 s. With prolonged exposure to acid, depurination becomes more pronounced in the middle of the stripe where acid is most concentrated (11.1–24.3 s), but the edges still show high intensity, indicative of quantitative yield. Only after extremely long exposure to acid (41.0 s, bottom) does edge intensity decrease, after almost all of the middle has been depurinated. Even after such long exposures to acid, the edges remain sharp, indicating that the acid generated at each electrode is strictly confined and demonstrating minimal cross-talk between adjacent electrodes.

diffusion layer 'wave front.' At 3 s, the intensity at the centre of the stripe is higher than the intensity at the edges. The overall intensity has increased at 5 s, and by 8.5 s the profile has a flat top, indicating complete deblocking. After 11.1 s, the intensity in the middle of the stripe decreases, indicating loss of oligonucleotide by depurination in this region most exposed to acid.

After 11.1 s deblocking for each of two steps, fluorescent intensity in the middle decreased by 4.2%, relative to the 'peak' edge intensity, which we assume to represent complete deblocking. This analysis suggests a small average loss of yield of ~2% per step in the middle of the stripe at 11 s. Losses by depurination at earlier times, when deblocking is complete, are much smaller, as the rates of both deblocking and depurination accelerate rapidly over the early time period as acid begins to reach the surface. Thus, loss of oligonucleotide in the middle of the stripe is not detectable at 8.5 s, is 4.2% at 11.1 s, 50% at 24.3 s and ~100% at 41.0 s.

Other experiments show that losses in the middle of the stripes, where acid is strongest, are <1% for conditions that give complete deblocking over the width of the stripe. Syntheses of the 6mer (Figure 3) and a 17mer (Figure 5),

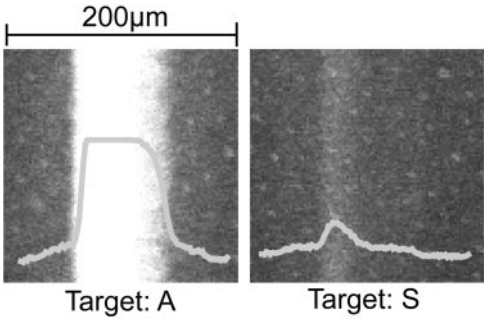

Figure 5. Hybridization of fluorescently labelled wild-type 'A' human β-globin oligonucleotides to electrochemically synthesized 17mer. This confocal micrograph shows images of hybridization to the matched target 'A' human haemoglobin mRNA (left) and mismatched target 'S' type sickle cell mutant mRNA (right). All 17 residues in the probe were coupled after electrochemically deblocking the previous residue. The peak signal for the matched target on the left is ~4.5 times that of the mismatch on the right. These images demonstrate high fluorescent intensity with good discrimination, indicating high synthetic yields in these preliminary experiments.

with 9 s of electrochemical deblocking at each step, show no measurable intensity losses in the middle of the stripes. If there were 1% loss at each step, there should have been 6 and 15% cumulative losses during the 5 and 16 deblocking steps used in the synthesis of the 6mer and 17mer, respectively. Such high losses would produce stripes with decreased intensity in the middle, resembling those seen in the later images from the time course experiment (described above and Figure 4). However, the intensity across the 6mer and 17mer stripes is uniform, showing that the loss during 9 s of deblocking must be <1% and may be <0.5%.

In addition to demonstrating minimal depurination, these syntheses confirm that deblocking is essentially complete at 9 s. If deblocking were incomplete in the multi-step synthesis of the 6mer and the 17mer, accumulated low yields would produce profiles with low, rounded tops and diffuse edges, similar to those seen in the early steps of the time course experiment (Figure 4, 3.0–5.8 s). Instead, the intensity profiles have flat tops and sharp boundaries (Figures 3 and 5), indicating that deblocking is essentially complete in each of the steps.

Finally, these results show that diffusion of acid into adjacent regions is confined by the cathodes. As the overall width of the stripe continues to increase with time (a manifestation of the cylindrical 'wave front' mentioned above), the effects of the cathodes maintain the sharpness of the edges; these effects, discussed in detail elsewhere (31), result from the efficient annihilation of protons by species produced at the cathodes and act to prevent synthesis cross contamination between adjacent electrodes.

DISCUSSION

This study explored the potential of a novel method for generating patterned surfaces by an electrochemical printing process. The printing tool was an array of microelectrodes patterned on the surface of a silicon chip. By directing the current to selected electrodes, chemical reactions were induced on the surface of a substrate held close to the electrode array.

The deblocking step in oligonucleotide synthesis demands precise control. Too much or too little deblocking over multiple couplings has a dramatic effect on yields of long sequences. The independent control of the electrodes of the array and the adjustable reaction synthesis chamber apparatus allowed us to explore multiple conditions, such as separation between electrode array and substrate, voltage and duration of pulse. We were quickly able to establish a set of conditions that gave well-defined stripes of chemical transformation on the substrate, with near complete deblocking and without serious depurination. Creating a 17mer in good yield illustrates that this system is capable of precise and reproducible control.

The electrodes used in this study were directly connected to a switchable power supply. This configuration lends itself well to preliminary studies described here. We aim to adapt standard fabrication methods, e.g. those that have been used in the manufacture of visual displays and demonstrated in related electrochemical applications (35), to develop a system in which the electrodes form a 2D array of small points, each switchable by indirect addressing. This will allow for random access, with flexible addressing so that the design of the array can be rapidly and easily changed from one run to another. The sharpness of the edges of the chemical image suggests that 10–20 μm features should not be difficult to achieve. In addition, in other experiments we have made features as small as 2 μm using a multilayer electrode (Figure 6).

A number of features distinguish this new method from alternative ways of patterning surfaces, such as photolithography, ink-jet printing or the use of stencil masks. As there is potentially no movement of parts between coupling steps, loss of registration between the printing tool and the printed surface does not lead to degradation of the chemical image. The method uses standard chemistries, so it is fast and can be applied to the synthesis of oligonucleotides in either chemical orientation or to nucleotide analogues for which standard reagents are available. Electrochemical processes can generate a variety of active species, including ions, radicals and radical ions, depending on the electrolyte. In this study, we chose an electrolyte that generates acid and used this reaction to remove an acid-labile protecting group during the synthesis of oligonucleotides. However, the method is not limited to this application and could be used to generate arrays of other types of molecules and to pattern materials by etching or deposition.

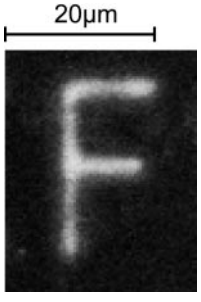

Figure 6. Demonstration of small feature sizes. A multilayer microelectrode device with 2 micron microelectrodes was used to attach a single fluorescently labelled phosphoramidite to a solid surface using the electrochemical deblocking methods as shown in Figure 2. Confocal microscopy of the fluorescent molecule reveals features approximating the size and shape (the letter 'F') of the microelectrode used for synthesis. The relatively sharp and small features demonstrate the potential of electrochemical syntheses for high-density molecular array fabrication.

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Conflict of interest statement. None declared.

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