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Patterning Enzymes Inside Microfluidic Channels via Photoattachment Chemistry

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We have developed a general method for photopatterning well-defined patches of enzymes inside a microfluidic device at any location. First, a passivating protein layer was adsorbed to the walls and floor of a poly(dimethylsiloxane)/glass microchannel. The channel was then filled with an aqueous biotin-linked dye solution. Using an Ar^+/Kr^+ laser, the fluorophore moieties were bleached to create highly reactive species. These activated molecules subsequently attached themselves to the adsorbed proteins on the microchannel walls and floor via a singlet oxygen-dependent mechanism. Enzymes linked to streptavidin or avidin could then be immobilized via (strept)-avidin/biotin binding. Using this process, we were able to pattern multiple patches of streptavidin-linked alkaline phosphatase inside a straight microfluidic channel without the use of valves under exclusively aqueous conditions. The density of alkaline phosphatase in the patches was calculated to be ∼5% of the maximum possible density by comparison with known standards. Turnover was observed via fluorogenic substrate conversion and fluorescence microscopy. A more complex two-step enzyme reaction was also designed. In this case, avidin-linked glucose oxidase and streptavidin-linked horseradish peroxidase were sequentially patterned in separate patches inside straight microfluidic channels. Product formed at the glucose oxidase patch became the substrate for horseradish peroxidase, patterned downstream, where fluorogenic substrate turnover was recorded.

The use of microfluidic strategies for investigating the catalytic properties of immobilized enzymes has gathered significant attention in recent years.[1−9] Indeed, these techniques should enable the rapid testing of a library of biocatalysts as well as allow for the screening of an array of small-molecule inhibitors against substrate binding in systems that require only minimum concentrations of analyte. Such impetus has led to the development of various lab-on-a-chip platforms for heterogeneous assays. For example, Kim et al. immobilized trypsin in sol−gel filled PDMS microchannels for monitoring fluorogenic substrate cleavage.[4] Similarly, Peterson et al. used in situ polymerization to form porous polymer monoliths into which trypsin was covalently attached.[5] Eteshola and Leckband demonstrated the feasibility of ELISA assays inside microfluidic devices,[2] while Crooks and coworkers provided several examples of heterogeneous enzyme catalysis employing hydrogels[3] and weir-dammed enzyme beads.[6,7] Endogluconase activity has been regulated using a photoresponsive polymer by Shimoboji et al.[9] In our own laboratory, we have immobilized enzymes on supported lipid bilayers to create one-shot Lineweaver−Burke plots as well as for demonstrating the first example of a two-step enzyme reactor.[8] Additionally, we have exploited temperature gradient microfluidics to make one-shot Arrhenius plots and abstracted the corresponding activation energies for enzyme turnover.[10]

While the above strategies are quite promising, a large issue remains unsolved. Namely, it can be time-consuming and tedious to place enzymes at the appropriate locations on-chip. Since there is usually only limited control over positioning, it would be advantageous to find a route to enzyme immobilization that would rapidly pattern proteins at any desired location in a microfluidic network. Ideally, this could even be done from an aqueous solution after the device has been fully fabricated and enclosed. One potential route for accomplishing these tasks is the employment of a photoattachment strategy, whereby enzymes or linkers would bind to a channel surface from solution only at locations that are being exposed to light of a particular frequency. This would allow complex protein patterning networks to be created in situ inside a microfluidic device in a manner such that the enzyme would not have to be exposed to air, organic solvent, or other denaturing conditions. The challenge, of course, is that this method would necessarily require the photolinker chemistry to work in water-based solutions under mild conditions.

As a step toward this goal, we recently developed a simple, but widely applicable method for the attachment of photoactive molecules to protein-coated interfaces from aqueous solution near neutral pH and room temperature.[11] The process works by photobleaching fluorophore-linked species to generate short-lived,

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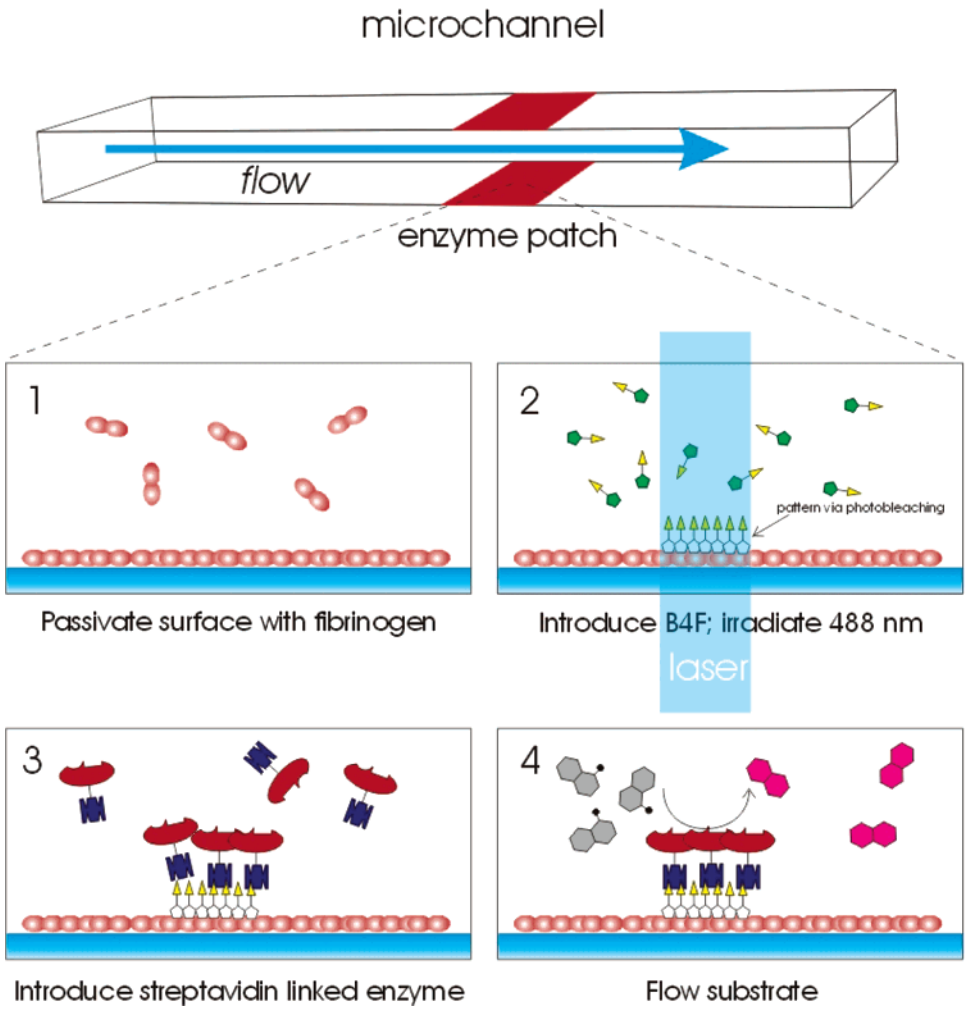

Figure 1. Schematic diagram of the photoimmobilization process. (Top) Enzyme patches are formed on the top and bottom of a microchannel using the following procedure: (1) Passivation of the surface with a fibrinogen monolayer is followed by (2) biotin-4-fluorescein surface attachment. This is accomplished by photobleaching with a 488-nm laser line. (3) Next, the binding of streptavidin-linked enzymes that can be exploited to immobilize catalysts and (4) monitor reaction processes on-chip.

highly reactive free radicals capable of attacking electron-rich surfaces. For example, bovine serum albumin (BSA)-coated glass coverslips could be patterned with a variety of proteins from phosphate-buffered saline (PBS) with micrometer-level precision. Herein, we expand on this immobilization method to create well-defined patches of (strept)avidin-linked enzymes inside microfluidic channels (Figure 1). For this purpose, bovine fibrinogen has replaced BSA as the surface coating layer due to its superior ability to present a large number of binding sites while preventing nonspecific adsorption of the three enzymes investigated herein: glucose oxidase (GO), horseradish peroxidase (HRP), and alkaline phosphatase (AP). The addition of fibrinogen to the enzyme patterning solution also aided in the prevention of nonspecific adsorption by out-competing enzyme-linked streptavidin for defect sites.[1,12]

EXPERIMENTAL SECTION

Microfluidic Device Fabrication. Poly(dimethylsiloxane) (PDMS)/glass microfluidic devices were prepared as previously described.[13] Briefly, 50 mm × 75 mm soda lime microscope slides were spin coated with Microposit S1813 photoresist (Shipley, Marlborough, MA) to a thickness of 10 μm and baked in a convection oven at 90 °C for 1 h. Photomasks were prepared as reduced negatives on photographic film with simple geometries consisting of three independent microchannels 32 mm long × 290 μm wide. A second pattern consisting of a 12-channel microarray with 130-μm widths was also made. Samples were exposed using a Quintel 6000 mask aligner and developed in a 1:1 solution of Microposit developer concentrate (Microchem) and DI water. Glass barriers were glued around the photopattern, and degassed PDMS was poured and cured in a convection oven at 70 °C for at least 3 h. Molds were peeled away and inlets were reamed at the channel termini using a hollow flat-tipped syringe needle. Next, the PDMS mold and a clean 25 mm × 50 mm microscope coverslip were placed in a plasma cleaner and oxidized for 20 s. The two pieces were brought immediately into contact to form an irreversible bond. The passivating protein solution was injected into the microchannels as quickly as possible to ensure the surface of the PDMS remained hydrophilic.

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Streptavidin Immobilization Assay. Fibrinogen, lysozyme, BSA, and IgGs (all from Sigma) were tested individually for their ability to form a passivating thin-film coating on glass slides. In addition, they were probed for their number densities of specific binding sites for photoactivated fluorophores. Each protein was dissolved at various concentrations in pH 7.2, 150 mM PBS. The protein solutions were injected into individual microchannels of a 12-channel PDMS/glass array and allowed to incubate for 30 min. After incubation, fresh PBS was flushed through the channels to remove unadsorbed protein. Next, a solution of biotin-4-fluorescein (B4F, Molecular Probes), 0.05 mg/mL in PBS, was injected into all the channels. To surface pattern the B4F, the 488-nm line from an Ar^+/Kr^+ laser beam (Spectra Physics) was passed through a reducing telescope to produce beam diameters of ~300 μm (full width half-maximum). The PDMS microfluidic device was scanned through the laser beam by mounting it on a syringe pump pusher block moving at a constant speed. This ensured that each channel was uniformly exposed to the same amount of light across the entire width. Typically, laser power of 150 mW and a scan rate of 1 mm/s were sufficient to maximize the amount of B4F that could be photoattached. The photoattached patches were roughly 500−800 μm long. PBS was used to rinse out excess B4F. At this point, ~200 μL of a 0.1 mg/mL Alexa 594-labeled streptavidin solution in PBS (which also contained 2.7 mg/mL unlabeled fibrinogen) was rinsed through the channel. The fibrinogen was added to compete against streptavidin for any remaining nonspecific adsorption sites on the surface. The solution was rinsed out of each microchannel after less than 1 min. The resulting pattern of immobilized streptavidin could be imaged under a Nikon E800 epifluorescence microscope with a CCD camera (Princeton Instruments) using MetaMorph software.

Immobilized Single-Enzyme Assay. Using the laser scanning procedure described above, B4F was patterned in consecutive patches inside PDMS/glass microfluidic devices that were previously incubated with 2 mg/mL bovine fibrinogen for 30 min. To make more than one patch along a single channel, a motion stage attachment was added to the syringe pump pusher to allow for movement normal to the direction of travel of the pusher block. This technique was employed to pattern various patches of alkaline phosphatase-linked streptavidin (APLS) (Molecular Probes), which was done from a solution containing 0.1 mg/mL APLS and 1.9 mg/mL fibrinogen. The enzyme-containing solution was injected and rinsed out with PBS after less than 1 min. The immobilized enzyme was used to dephosphorylate the substrate molecule, 4-methylumbelliferyl phosphate (MUP). MUP was chosen because it is only weakly fluorescent, but becomes strongly fluorescent in the blue upon dephosphorylation. Experiments were performed with 0.195 mM MUP in pH 9.8 carbonate buffer (150 mM). The molecule was flowed into the enzyme-containing channels using a modified syringe pump fitted with 100-μL syringes (Hamilton) via Teflon lines. Enzyme turnover was monitored by epifluorescence under the E800 microscope.

Immobilized Two-Enzyme Assay. Using the same laser scanning procedure described above, B4F was again patterned in patches inside fibrinogen-coated microchannels. Glucose oxidase-linked avidin (GOLA) (Vector Labs) and horseradish peroxidase-linked streptavidin (HRPLS) (Molecular Probes) were immobilized either sequentially (two patches) or in the same patch. GOLA was prepared in PBS at 0.025 mg/mL in a solution also containing 2.7 mg/mL fibrinogen. On the other hand, 0.020 mg/mL HRPLS was immobilized from a solution that contained 2.7 mg/mL fibrinogen in PBS. When GOLA and HRPLS were patterned together as one patch, 100 μL of each solution was added to 800 μL of 3 mg/mL fibrinogen and roughly 200 μL of the mixture was injected into the microchannel. In all cases, enzymes were allowed no more than 1 min to bind to the biotin-presenting surfaces before being washed out with buffer. This was done to minimize nonspecific adsorption.

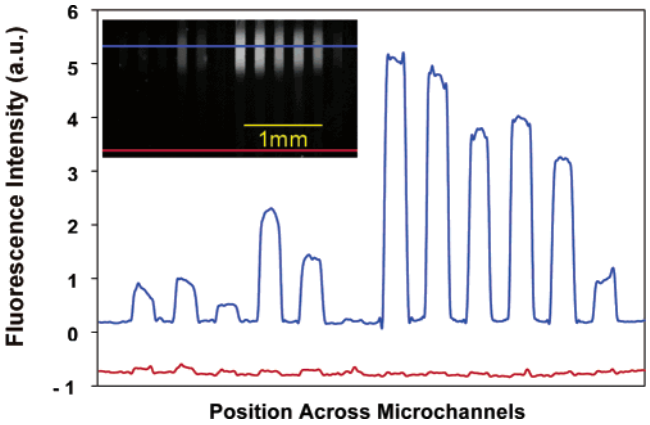

Figure 2. Intensity line scan of fluorescence (blue) emanating from a protein patterned region across a series of microchannels and a second line scan (red) below this (an unpatterned region). The latter was used as a measure of nonspecific adsorption. The red and blue scans have been offset by one intensity unit for visual clarity. From left to right, channels 1−3 were incubated with BSA at 9.4, 1.8, and 0.38 mg/mL respectively, channels 4−6 with lysozyme at 4.1, 0.82, and 0.16 mg/mL, channels 7−9 with fibrinogen at 3.0, 0.6, and 0.12 mg/mL, and channels 10−12 with IgG at 4.4, 0.88, and 0.18 mg/mL. Inset: Fluorescence micrograph of the patterned 12-microchannel PDMS/glass array.

Glucose oxidase catalyzes the conversion of β-D-glucose and O_2 to gluconolactone and releases H_2O_2. The peroxide can combine with Amplex Red (Molecular Probes) in the active site of horseradish peroxidase to produce resorufin, which is highly fluorescent. A stock solution of Amplex Red was prepared by dissolving 5 mg of the molecule in 0.5 mL of DMSO. The final solution injected into the microchannels was prepared by mixing 1 μL of stock Amplex Red and ~1 mg of β-D-glucose (Sigma) into 1 mL of PBS. This solution was flowed into the channel and enzyme turnover was monitored via epifluorescence microscopy.

RESULTS

Streptavidin Immobilization Assay. The binding of B4F to BSA-, fibrinogen-, IgG-, and lysozyme-coated channels was investigated in a first set of experiments. Figure 2 shows data from a 12-microchannel PDMS/glass device. From left to right, the channels were first incubated with three concentrations each of BSA, lysozyme, fibrinogen, and IgG, respectively. The fluorescence emanated from Alexa 594-labeled streptavidin that was subsequently flowed over the surface after B4F attachment and allowed to bind to the biotin moieties. A line scan across the patterned area (blue) reveals that the fibrinogen coating led to the greatest density of specifically bound streptavidin molecules

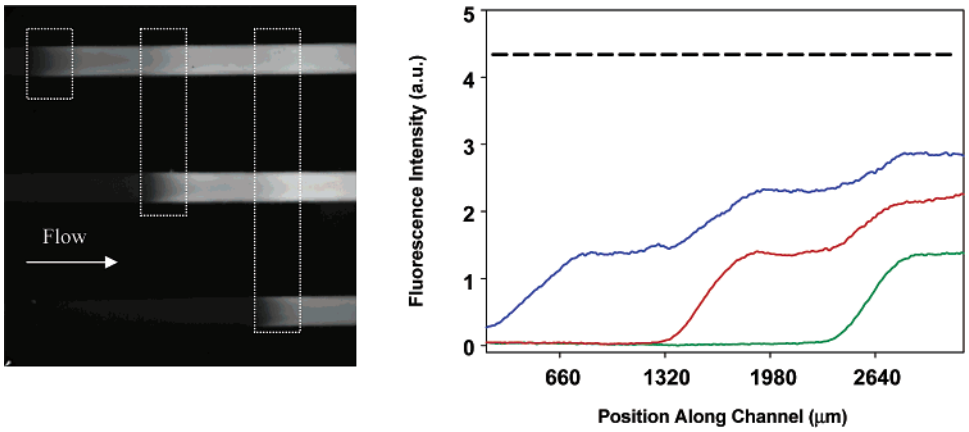

Figure 3. Left: fluorescence micrograph (3.2 × 3.2 mm) of a three-channel PDMS/glass device patterned with APLS. The top channel has three patches, the middle has two patches, and the bottom has one patch. The patch positions are outlined with white rectangles. Right: fluorescence line scans along each individual channel (blue top, red middle, and green bottom). The dashed line represents the maximum product intensity achieved in the microchannels when the flow was stopped.

and the lowest nonspecific adsorption (red). Lysozyme and BSA yielded comparatively moderate patterning densities and suffered from somewhat more nonspecific adsorption. IgG was nearly as good as the fibrinogen but seemed to have slightly fewer specific binding sites. The amount of binding to any particular protein-coated surface probably depended on the number of free amines available at the surface.[14] On the other hand, the prevention of nonspecific binding by streptavidin should be related at least in part to a coat protein's ability to spread at the surface and cover the bare glass. On the basis of the above results, 2.5 mg/mL fibrinogen solutions were chosen for forming the passivation coating in all subsequent photopatterning experiments.

Immobilized Single Enzyme Assay. To demonstrate the ability of this process to create well-defined patches of enzyme, APLS was patterned in three parallel microchannels. The first channel contained one patch, the second contained two consecutive patches, and the third contained three consecutive patches. Next, MUP was infused into the channels at 20 nL/min and imaged by fluorescence microscopy. A weak background could be observed from all three channels even before the patches because MUP itself is weakly fluorescent. As expected, control experiments verified that this intensity was not flow rate dependent. On the other hand, when the MUP encountered an enzyme patch, the fluorescence signal increased strongly. In the bottom channel, there was only a single patch so the increase occurred only once and then leveled off immediately downstream from it (Figure 3). By contrast, two intensity increases could be observed in the middle channel and three from the top channel. In other words, the fluorescence intensity rose as the substrate passed over each subsequent enzyme patch. To determine the fluorescence signal from fully equilibrated substrate, the pump was stopped and the fluorescence was monitored directly over any given patch. Eventually, a maximum intensity was achieved and is represented by the dotted line at the top of the graph in Figure 3. From the single patch data it can be seen that ~31% conversion was achieved at a flow of 20 nL/min. Each subsequent downstream patch turned over less substrate, because there was a lower concentration of it left to convert. Approximately 67% of the maximum substrate turnover could be achieved after three patches. It was possible to speed up or slow the flow rate to modulate the fluorescence intensity jumps; however, it should be noted that, below 20 nL/min, the flow rate became uneven in the present setup. This was caused by slip stick friction in the individual syringes. It is also of importance to note the ease with which consecutive patch geometries can be achieved. This is often difficult to accomplish with other immobilization strategies.

To obtain more quantitative information from the reactor, it was necessary to estimate the number density of immobilized APLS enzymes in a given patch. Control experiments were performed with streptavidin, which has a roughly 33.6-nm^2 footprint on a membrane surface.[15] Known densities, 3×10^4 streptavidin molecules/μm, of this protein were bound to biotinylated supported lipid bilayers following our previously reported procedures.[8] By comparison of those results with the present experiments, it was revealed that streptavidin coverage could reach ~5% of the close-packed density for photoattachment to fibrinogen. Since the area of a patch was at most 0.8 mm × 0.29 mm, this meant that no more than 7×10^8 streptavidin molecules were bound to the channel surfaces under the conditions described above. It is reasonable to assume that immobilized APLS would have approximately the same number density as streptavidin alone since the distance between the surface attached species was quite large compared to the average footprint size for this linked protein complex (134 nm^2).[8]

At a flow rate of 20 nL/min, it should take a substrate molecule roughly 4.2 s to cross a 0.8-mm patch. From the data in Figure 3, the first patch of each channel converted ~31% of substrate to product. Employing a substrate concentration of 0.195 mM, we found that 5.13×10^{10} molecules of substrate were converted by 7×10^8 APLS enzymes in 4.2 s to yield a turnover number of ~20 molecules per enzyme per second. Several assumptions were made in this estimation of turnover number: (1) the top and bottom of the channel contained the same enzyme density, (2) every site of patterned biotin was available for enzyme binding, (3) the cross sectional area of the PDMS/glass device was

(14) In a separate experiment, polyelectrolytes were adsorbed in PDMS/glass microchannels and photopatterned with B4F. Poly(diallyldimethylammonium chloride), poly(styrenesulfonic acid) poly(acrylic acid), and polyethylenimine (the only polymer with primary amine substituents) were used. Only the last species gave rise to a visible pattern when labeled streptavidin was introduced. All surfaces suffered from nonspecific adsorption.

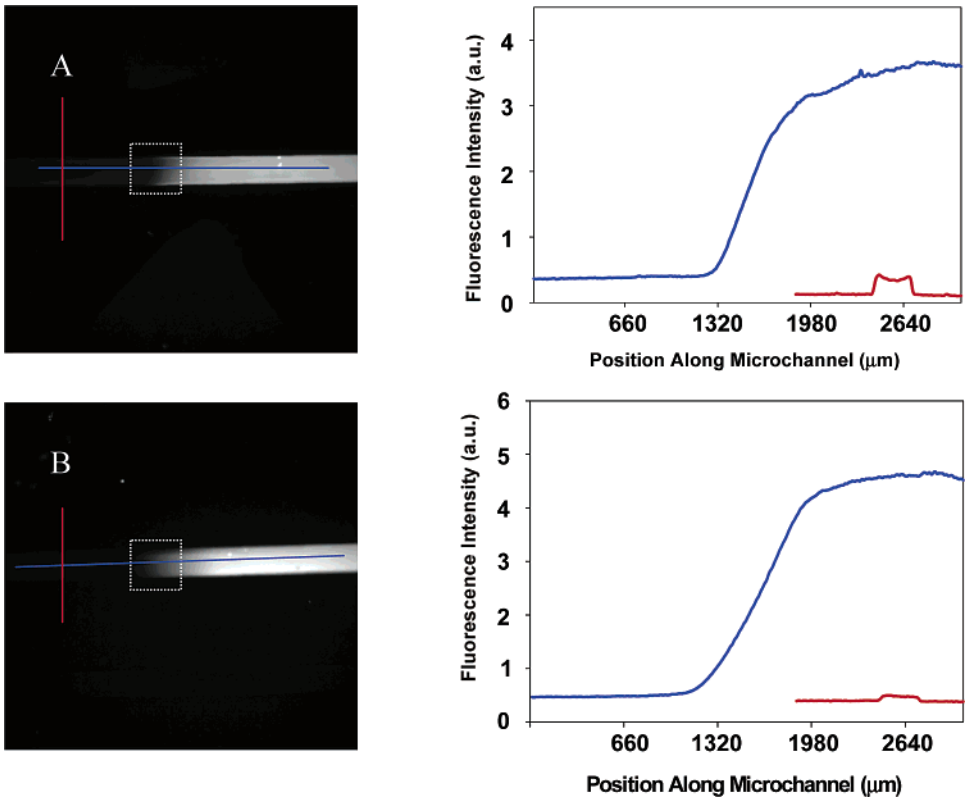

Figure 4. Left: fluorescence images of PDMS/glass microchannel devices patterned with GOLA and HRPLS imaged near the HRPLS patch (boxes). (A) A sequentially patterned enzyme channel and (B) a single patch with both enzymes placed in the same location. Right: The blue lines represent the product intensities along the channels. The red lines show the background intensity taken across the microchannels ~1 mm before the final enzyme patch. The flow rate was 50 nL/min in both reactors.

constant, and (4) the enzymes were uniformly patterned. Such assumptions lead to a lower bound to the actual turnover rate. The APLS patch enzyme reactor was very robust and capable of operating for a minimum of several hours of continuous substrate turnover. In fact, one APLS reactor was kept hydrated at room temperature for 2 days, and when substrate was reintroduced, the turnover rate was more than 90% of the initial value.

Immobilized Two-Enzyme Assay. Using the methodology described above, we sequentially patterned glucose oxidase and horseradish peroxidase in a single microchannel to perform a two-step enzyme reaction. HRPLS and GOLA were patterned such that the horseradish peroxidase was downstream from the glucose oxidase. In the first step, GOLA oxidized glucose to form gluconolactone and hydrogen peroxide. Next, the peroxide combined at the active site of HRPLS with Amplex Red to form resorufin, which fluoresces strongly near 580 nm upon excitation with green light. Amplex Red was prepared in PBS buffer with β-D-glucose as described in the Experimental Section. It was found that sufficient oxygen was already dissolved in PBS just from exposure to ambient air; therefore, it was not necessary to purge with additional oxygen.

The substrate was pumped into the device at 50 nL/min and the fluorescence intensity jumped dramatically after the HRPLS patch, as can be clearly seen from the image and accompanying line profile (blue line, Figure 4A). It should be noted, however, that there was already a small rise in intensity[16] directly before the HRPLS patch (red line, Figure 4A). In fact, 6.7% of the ultimate fluorescence intensity was achieved before the second patch. This was the case because HRPLS suffers from a relatively high degree of nonspecific adsorption. H_2O_2 is produced as soon as the GOLA patch is encountered. Amplex Red is, therefore, turned over by even minute quantities of HRPLS that are nonspecifically adsorbed downstream from this location. The reason for the nonspecific adsorption problem almost certainly stems from the fact that horseradish peroxidase has a p*I* very close to neutral pH (7.2). This can potentially lead to aggregation and surface adsorption problems when patterning the protein from standard buffer solutions. To reduce this interaction, the HRPLS photopatterning was repeated (data not shown), at a pH of 9.8. This reduced the background to 4.5% of the ultimate turnover before the final patch under the same conditions.

In an effort to lower the background turnover even further, a separate experiment was performed with an equimolar mixture of GOLA and HRPLS patterned together in the same patch at pH 7.2 (Figure 4B). The background was reduced to 2.2% of the eventual total turnover, meaning that some nonspecific adsorption of both enzymes was occurring before the patch.

DISCUSSION

The enzyme immobilization method presented herein has several advantages over previous enzyme reactors. The method

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(16) Amplex Red can spontaneously oxidize to the fluorescent product, resorufin; however, we found that the substrate used for HRPLS had little if any background fluorescence when introduced into a microchannel with no enzyme. Therefore, we attribute all prepatch fluorescence to nonspecifically adsorbed enzyme.

is general, allows precise control over enzyme placement, and could be used in conjunction with virtually any microfluidic geometry. Patterning enzymes requires no UV irradiation[17−20] and is quite fast. Furthermore, patterning can be done from an aqueous solution without ever exposing the protein molecules to air,[21,22] which should help maintain their integrity, although this was not a major concern with rugged enzymes such as alkaline phosphatase, horseradish peroxidase, or glucose oxidase. More than one enzyme can be patterned in a single straight channel to run multistep reactions without valves or additional complexities.

There are also some disadvantages to the present strategy. For example, this patterning process relies on a biotin−streptavidin linkage, which limits flexibility. While it is relatively easy to biotinylate almost any protein with commercially available linking kits, some enzymes may be more sensitive to environmental conditions of pH, ionic strength, and temperature. This could lead to the loss of activity under linking conditions. There may, of course, be ways around this. Indeed, we have previously shown that the photoattachment process works for a variety of fluorophore dyes,[11] and it should be possible to create functionalized dyes that can be used to directly capture proteins from solution using specific chemistry. For example, maleimido groups tethered to dyes are typically used to label cysteine residues. It should therefore be possible to photoattach dyes to create patches of maleimido groups, which could subsequently react with free cysteines on the surface of engineered proteins. The sulfhydryl reaction chemistry could provide a generic linking chemistry for capturing novel enzymes for study in such systems. Also, by combining a variety of fluorophores, which photobleach at different wavelengths, with antibody-specific ligands, it should be possible to create more complicated sequences of antibody-linked enzymes simultaneously, rather than sequentially.

Another concern in these experiments was the nonspecific adsorption of the HRPLS and GOLA enzymes. This caused some background fluorescence before the enzyme patch, whether patterned separately or together. This background level may be acceptable in the framework of combinatorial analysis since sharp changes in enzyme activity can be easily recorded in parallel. Applications requiring <1% background, however, need to be optimized by varying salt concentration, surface chemistry, and solution additives to minimize the nonspecific problem. Further experiments will be needed to explore this issue, and the particular strategy employed may ultimately depend on the particular enzyme that is being patterned.

CONCLUSION

We have developed a basic strategy for the photoimmobilization of multiple, well-defined enzyme patches in simple PDMS/glass microfluidic channels. This method allowed us to estimate enzyme turnover number for a single-enzyme system. The technique also proved to be viable for multistep enzyme reactions by patterning proteins in sequence. Nonspecific adsorption of enzymes was reduced by careful selection of the surface passivating layer and by adding competitive nonspecific adsorbers to the patterning solutions.

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