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Hydrogel-Based Microreactors as a Functional Component of Microfluidic Systems

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A simple two-step method for fabricating poly(ethylene glycol) (PEG) hydrogel-based microreactors and microsensors within microfluidic channels is described. The intrachannel micropatches contain either a dye, which can report the pH of a solution within a fluidic channel, or enzymes that are able to selectively catalyze specific reactions. Analytes present within the microfluidic channel are able to diffuse into the micropatches, encounter the enzymes, and undergo conversion to products, and then the products interact with the coencapsulated dye to signal the presence of the original substrate. The micropatches are prepared by photopolymerizing the PEG precursor within the channel of a microfluidic system consisting of a poly(dimethylsiloxane) mold and a glass plate. Exposure takes place through a slit mask oriented perpendicular to the channel, so the size of the resulting micropatch is defined by the channel dimensions and the width of the slit mask. Following polymerization, the mold is removed, leaving behind the micropatch(es) atop the glass substrate. The final microfluidic device is assembled by irreversibly binding the hydrogel-patterned glass slide to a second PDMS mold that contains a larger channel. Multiple micropatches containing the same or different enzymes can be fabricated within a single channel. The viability of this approach is demonstrated by sensing glucose using micropatches copolymerized with glucose oxidase, horseradish peroxidase, and a pH-sensitive dye.

Cells and other natural systems use sequential reactions that occur at spatially well-defined reaction zones to carry out complex chemical syntheses.[1] We envision that microfluidic devices can also be used to execute multistep reactions that would be difficult or impossible to achieve by alternative means. Applications of this approach include multistep synthesis of small amounts of biological reagents or sensing of multiple targets. As in cells, this approach will require, at a minimum, the delivery of reagents to these reaction sites and the placement of catalysts. Here we focus on the catalytic aspects, specifically, immobilization of biocatalysts within the channels of microfluidic devices. Our strategy involves the immobilization of enzymes within hydrogels via photopolymerization,[2,3] anchoring these hydrogel micropatches onto glass slides using silane coupling chemistry,[4−6] and finally fabrication of microfluidic channels that surround the gel micropatches.[7,8]

Biological materials can be immobilized onto a surface or within a medium using one or more of the following approaches: physisorption, chemisorption, covalent binding, entrapment, or cross-linking.[9−11] For many applications, biological materials must be positioned with 10−1000-μm spatial resolution. The length scale of photolithography is well-matched to this range so it is not surprising that it has been used extensively for this purpose.[12,13] Typically, photolithography is used in one of two modes. In the first case, a pattern of a photopolymerizable material that either increases or decreases the interaction energy between the biomaterial of interest and the surface is transferred to a surface from a mask.[14,15] In the second case, the biological material of interest is polymerized directly, or in the presence of a polymerizable encapsulant, in the desired configuration.[2,16,17] Another family of methods based on soft lithography can be used to directly place biomaterials on surfaces or, in a two-step process, restrict immobilization to locations previously patterned.[7,18,19] There are

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several related methods in this family, including: microcontact printing,[7,20] microfluidic patterning,[21,22] and laminar flow patterning.[23,24] Immobilization of biological reagents by jet-printing and robotic spotting have also been pursued because of their promise of high efficiency and automation.[11,25−29]

Bioimmobilization within microchannels presents some special considerations related to their small scale and also postimmobilization processing of the device (chemical and thermal treatments associated with sealing the channel, for example). Some successful strategies that have been reported include direct immobilization of reagents onto the inside wall of the channel via conjugation reactions[21,30] and microbead immobilization.[31] These methods allow straightforward placement of biomaterials within a channel, but it is more difficult to prepare spatially distinct micropatches containing different reagents without addition to the device of extra fluidic channels.[31] In the approach described here, the microfluidic channel is placed over the immobilized biomaterials (enzymes in this case) *after* they are positioned and photopolymerized. This approach can significantly simplify microfluidic architectures, and thus, it is worth exploring.

Here, we integrated poly(ethylene glycol) (PEG) hydrogel micropatches into the channels of microfluidic devices. Hydrogel micropatches of different sizes are conveniently prepared by controlling the dimensions of the microfluidic channels and photomasks. The complete device is prepared by replacing the poly(dimethylsiloxane) (PDMS) channels used to fabricate the hydrogel micropatches with a somewhat larger channel that permits fluid to flow over the micropatches. The intrachannel micropatches contain either a dye, which can report the pH of a solution within a fluidic channel, or enzymes that are able of selectively catalyzing specific reactions. Experimentally, sensing of pH is realized by first conjugating SNAFL-1 (a pH-sensitive dye) with glucose oxidase and then physically entrapping the resulting complex, within the hydrogel matrix. Quantitative results of the pH measurements are obtained by monitoring the fluorescence intensity changes of the dye. Glucose sensing is made possible by co-photopolymerization of two enzymes (glucose oxidase and horseradish peroxidase) within the gel precursor.

EXPERIMENTAL SECTION

Chemicals. Glucose and glucose oxidase (GOX, EC 1.1.3.4, type X-S, 128 units/mg of solid from *Aspergillus niger*) were obtained from Sigma Chemical Co. (St. Louis, MO). Poly(ethylene glycol) diacrylate (PEG-DA) having an average molecular weight of 575 and 2-hydroxy-2-methylpropiophenone (HOMPP) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Before use, PEG-DA was purified using an alumina (ICN Biomedicals, Germany) column to remove stabilizer and other polymeric impurities.[32] 3-(Trichlorosilyl)propyl methacrylate (TPM) was obtained from Fluka Chemicals (Milwaukee, WI). Biotin-conjugated horseradish peroxidase (HRP, 1:1 stoichiometry), Amplex Red, and succinimidyl ester (mixed isomers of 5- and 6-carboxy SNAFL-1) were purchased from Molecular Probes (Eugene, OR). Phosphate buffer solutions (PBS) were prepared immediately before use by titrating a pH 7.0 buffer with either 0.5 M NaOH or 0.5 N HCl. 18 MΩ·cm water (Milli-Q reagent water system, Bedford, MA) was used to prepare all aqueous solutions.

The conjugate of SNAFL-1 and glucose oxidase (GOX−SNAFL-1) was prepared by reacting the succinimidyl ester-activated SNAFL-1 (1 mg of dye dissolved in 100 μL of DMSO) with the lysine residues present on the enzyme dissolved in 100 mM PBS (pH 8.2). Unreacted dye was removed by dialysis.

Gel Lithography and Device Fabrication. Microfluidic devices were prepared using a previously reported procedure.[8] Briefly, patterns designed using a computer graphics program were transferred photolithographically to PDMS molds (Dow Corning Sylgard Silicone Elastomer-184, Krayden, Inc.) through a positive photoresist (AZ P4620, Clariant Co., Somerville, NJ). Glass microscope cover slips (100 μm thick; Fisher Scientific) were used to cover the open PDMS channels. Prior to attachment, the slides were first sonicated in a 1% (v/v) detergent solution (Micro-90, International Products Co., Burlington, NJ) for 30 min and washed thoroughly with water. To enhance adhesion between the hydrogel micropatches and the glass slides, a monolayer of TPM was covalently attached to the glass. This was accomplished by dipping the dry slides into a 5 mM TPM solution (4:1 heptane and carbon tetrachloride) for 1 min and then washing with hexane and water.[5] Subsequent photopolymerization covalently links the gel to the glass slide via the silane-coupling agent.

The hydrogel precursor solutions were made by mixing PEG-DA, the photoinitiator (HOMPP), and the SNAFL-1-tagged enzyme solutions at different volumetric ratios. The resulting mixtures were vortexed for 5 min and kept in the dark prior to use. A syringe pump (Sage M365, Orion Research, Beverly, MA) was used to deliver the precursor solution and for all subsequent introduction of fluids. Alternatively, capillary force could be used to fill the microchannels with PEG precursor solution.

Photopolymerization of the enzyme/PEG-DA precursor solution was initiated through a mask using a 365-nm light source (EFOS Lite, UV spot lamp, Mississauga, ON, Canada) having an approximate power/area-of-exposure ratio of 300 mW/cm^2. The chrome mask had open slits ranging in width from 20 to 100 μm. These slits were oriented perpendicular to the microfluidic channel and placed in direct contact with the glass slide. Prior to polymerization, the emitting end of the UV light guide was fixed ∼1 mm above the slit in the chrome mask. The sizes of the hydrogel microstructures and the microchannels were determined using a Veeco Dektak3 profilometer (Veeco Instruments, Plainview, NY).

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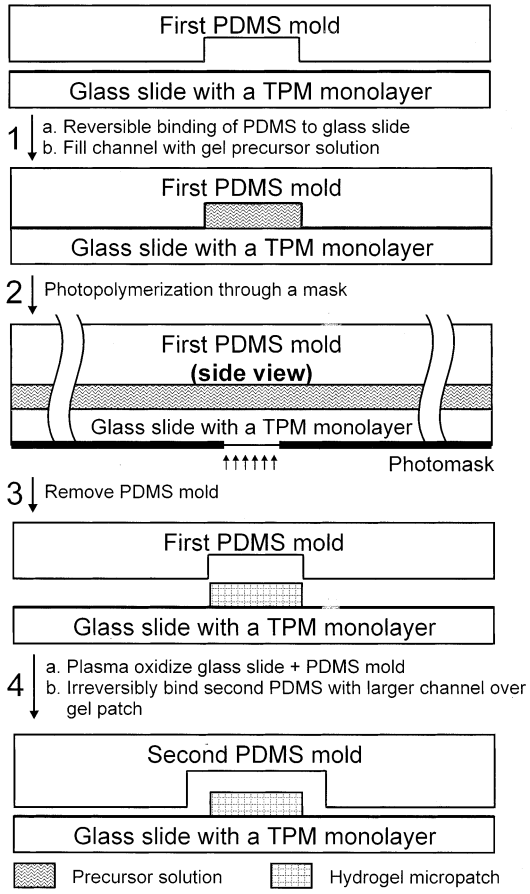
Scheme 1



After the gel micropatches were prepared, the glass slide and a second PDMS mold having a channel wider than the micropatches were exposed to an oxygen plasma (60 W, PDC-32G, Harrick Scientific, Ossining, NY) for 30 s. Alignment of the hydrogel micropatches and the second PDMS mold was performed either manually or with the aid of a three-axis micropositioner. In the latter case, the PDMS mold was affixed to a steel arm connected to the micropositioner with double-stick tape. The microchannel and hydrogel micropatch were aligned by adjusting the stage ring of the microscope, and then the PDMS mold was lowered onto the glass slide. Upon contact, the two pieces formed an irreversible bond.[8]

Data Acquisition. Images of the microchannels and hydrogel micropatches were obtained using an optical/fluorescence-inverted microscope (Eclipse TE300, Nikon). Photographs were captured using a scientific-grade CCD camera (Synsys, Photometrics, Tucson, AZ) installed at the front port of the microscope. At the side port of the microscope, a liquid nitrogen-cooled CCD-based (LN/CCD, Roper Scientific, Tucson, AZ) fluorescence spectrometer (SpectraPro-300i, Acton Research Co., Acton, MA) enabled acquisition of the emission spectra of hydrogel-encapsulated SNAFL-1. Fluorescence spectra were obtained using an excitation wavelength of 475 nm (from a mercury arc lamp). Depending on the size of the hydrogel micropatches, 4×, 10×, and 40× objective lenses were used to obtain data.

RESULTS AND DISCUSSION

Fabrication of Hydrogel Micropatches within Microchannels. Intrachannel hydrogel micropatches were prepared as shown in Scheme 1. First, a PDMS microfluidic channel having a height of 17 μm and a width of 135 μm was fabricated. This PDMS channel was covered with a glass slide previously modified with a monolayer of an acrylate-terminated silane coupling agent (TPM). TPM was used to ensure good adhesion between the hydrogel micropatch and the glass slide.[5] Reversible binding between the TPM-treated glass slide and the PDMS mold took place instantaneously after they were put into conformal contact. Next, the channel was filled with a solution containing the PEG hydrogel precursor and either GOX−SNAFL-1 or enzymes. An opaque mask having a linear transparent feature 20−100 μm in width was placed atop the glass side with the transparent feature oriented 90° with respect to the channel. The exposed precursor solution within the channel was then photopolymerized with a UV source; polymerization was complete within 1−2 s. Following polymerization of the hydrogel, the first PDMS mold was removed from the glass slide, leaving the hydrogel micropatches covalently bound on the glass slide via the TPM monolayer. The unpolymerized solution was removed with several drops of water, and the slide was dried under flowing nitrogen.

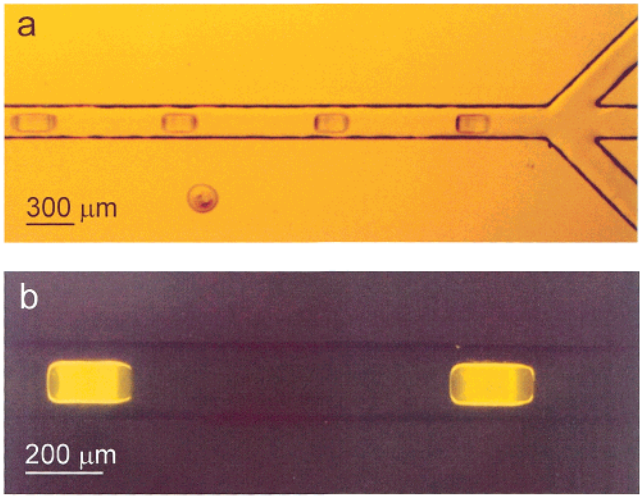


Figure 1. Micrographs of hydrogel micropatches within a microfluidic channel: (a) optical micrograph; (b) fluorescence micrograph of the same micropatches shown in (a). Fluorescence arises from the dye SNAFL-1 entrapped within the hydrogel. To prevent the dye, which is a relatively small molecule (MW = 523.5), from leaching out of the hydrogel matrix, it was conjugated to GOX prior to PEG-DA polymerization.

Figure 1a shows an optical micrograph of several hydrogel micropatches fabricated on a glass slide using the procedure described in the previous paragraph. The micropatches were prepared with a 50-μm-wide slit mask, and the resulting features are approximately 135 μm wide, 200 μm long, and 15 μm high. Light scattering from the glass channel cover led to a 3−5-fold increase in the length of the gel feature. The width and thickness, however, are well replicated by the channel template. A fluorescence micrograph of the micropatches is shown in Figure 1b. The fluorescence was produced by exciting GOX−SNAFL-1 embedded within the hydrogel at 470 nm.

To complete device fabrication, the glass slide supporting the hydrogel micropatches and a second PDMS mold having a larger channel (height, 32 μm; width, 200 μm) were treated with an oxygen plasma for 30 s and then pressed together, resulting in

an irreversible bond (bottom of Scheme 1).[8] The resulting structure consists of a hydrogel micropatch contained within a larger channel in such a way that fluid within the channel flows over and around the micropatch.

Entrapment of Enzymes within Hydrogel Micropatches and Diffusion of Analytes within Micropatches. By monitoring the swelling of PEG hydrogel beads in 0.1 M PBS buffer, Russell et al. previously estimated that a hydrogel composed of PEG-DA-575 has a porosity of ~1 nm.[33] Accordingly, we expected that polymerization of PEG-DA in the presence of an enzyme, such as the GOX used in this study (dimensions, $\sim 7 \times 5.5 \times 8$ nm), would result in physical entrapment of the enzyme. This assumption was confirmed by monitoring fluorescence intensity as a function of time for experiments similar to that described for Figure 1b. On the time scale of the experiments reported here, we did not observe a significant decrease in fluorescence from the gel-encapsulated GOX−SNAFL-1 conjugate under constant buffer flow, although extensive UV irradiation did result in photobleaching. In contrast, when unconjugated SNAFL-1 was encapsulated within the micropatches, the fluorescence began to decrease as soon as the buffer flow was initiated (data not shown).

This last experiment gave us confidence that substrates present in solution would be able to move in and out of the hydrogel on a technologically useful time scale (seconds to minutes). It is also possible to estimate the time required for substrate permeation through the gel using information available in the literature. For example, it has been shown that the diffusion coefficient (D_s) of tetramethylrhodamine (MW = 444) in a hydrogel matrix (prepared from 40% v/v PEG-575 and 60% v/v water) is 2.3×10^{-7} ($cm^2 \cdot s^{-1}$).[33] By adopting this value of D_s, eq 1 approximates the

$$\delta = 2(D_s t)^{1/2} \qquad (1)$$

time necessary for substrates of the type used in this study to diffuse through the 15−17-μm-thick hydrogel micropatches.[34] Here, δ is the Nernst diffusion layer thickness and t is time. The calculation indicates that the hydrogel can be fully penetrated by substrates within 3−5 s.

Intrachannel pH Sensing Using SNAFL-1 Fluorescence. SNAFL-1 is a seminaphthofluorescein dye that has pH-dependent fluorescence intensity. Moreover, SNAFL-1 is a ratiometric dye, which means that errors arising from instrumental and environmental drift are minimized.[35] To demonstrate the usefulness of hydrogel micropatches for intrachannel operations, we incorporated SNAFL-1 into a hydrogel micropatch and used it to determine the pH of fluids.

To prevent SNAFL-1, which is a relatively small molecule (MW = 523.5), from leaching out of the hydrogel matrix, it was conjugated to GOX prior to polymerization of the PEG-DA precursor. Figure 2 shows emission spectra obtained from hydrogel micropatches containing SNAFL-1 as a function of the pH of the buffer solution present within the channel. The spectra

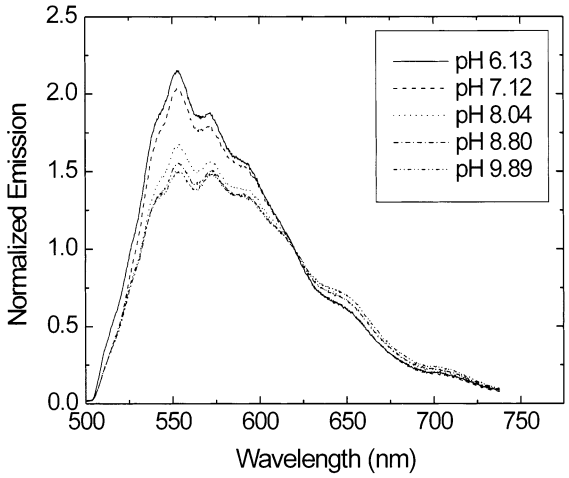


Figure 2. Normalized emission spectra for GOX−SNAFL-1 entrapped within a hydrogel micropatch upon exposure to different pH buffer solutions. The dye was excited at 470 nm, and emission spectra were collected by filtering the signal through a 500-nm long-wavelength-pass filter. The spectra were normalized at the isoemissive wavelength (620 nm).

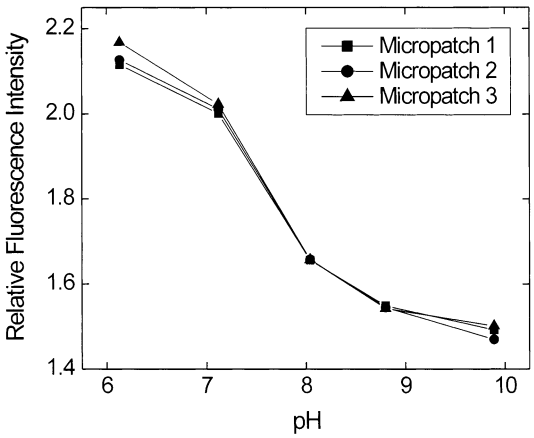


Figure 3. Relative fluorescence (I_{550}/I_{620}) intensity as a function of pH for three different GOX−SNAFL-1-containing micropatches placed within a single microfluidic channel. pH was controlled by exposing the patches to phosphate buffer saline solutions (0.2 M NaCl and 50 mM phosphate buffer having the indicated pH).

were normalized to the emission intensity at the isoemissive wavelength, 620 nm.[36] The data show that the normalized fluorescence intensity at 550 nm, which is the wavelength of maximum emission, decreases as the solution pH increases. As is found for free SNAFL-1 in solution,[35] the largest change of the relative emission intensity ($I_{550\ nm}/I_{620\ nm}$) appears between pH 7 and 8, the optimum working range for this dye. Figure 3 shows how the relative fluorescence intensity of GOX−SNAFL-1 in individual hydrogel micropatches changes when the pH value of the solution is varied from 6 to 10. These data indicate that precise sensing of pH can be reproducibly obtained from different gel micropatches.

We also tested the time response for pH sensing using micropatches. The results of this experiment are summarized in Figure 4. Here, a pH 8.04 phosphate buffer was pumped (flow rate, 1 μL/min) through a microchannel hosting a micropatch

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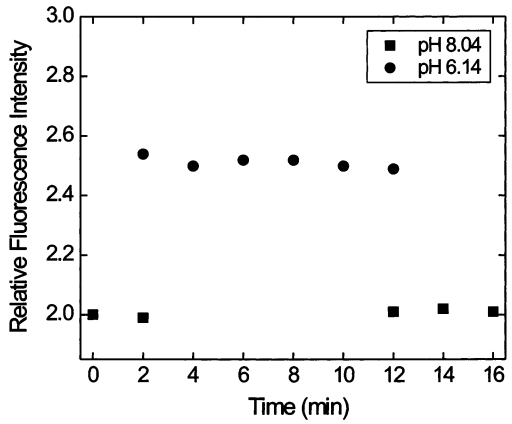

Figure 4. Relative fluorescence intensity (I_{550}/I_{620}) as a function of time. A pH 8.04 phosphate buffer was pumped (flow rate, 1 μL/min) through a microchannel hosting a micropatch containing GOX−SNAFL-1, and the relative fluorescence was measured. After 2 min, this solution was replaced by a second buffer solution having a pH of 6.14. After 10 min, the flow was changed back to the original pH 8.04 buffer. Spectra were acquired only every 2 min to prevent photobleaching.

containing GOX−SNAFL-1 for 2 min, and the fluorescence was measured. After 2 min, this solution was immediately replaced by a second buffer solution having a pH of 6.14 and a new spectrum was obtained. The time required to change the buffer solution and acquire the new spectrum was ~5 s, and as shown in the figure, the relative fluorescence intensity achieved its maximum value within this time period. This confirms our earlier estimate of the time response of the hydrogel micropatch. After 10 min at pH 6.14, the pH was changed back to 8.04, and within the 5 s necessary to change the buffer solution and acquire a spectrum, the relative fluorescence intensity from the hydrogel micropatch attained its original, limiting value. This finding indicates that GOX−SNAFL-1 does not leach out of the gel and that its pH response is reversible.

Sensing of Glucose Using Hydrogel Micropatches Containing Glucose Oxidase and Horseradish Peroxidase. To demonstrate how enzymes entrapped within hydrogel micropatches can be used for chemical sensing, we implemented an established glucose-sensing protocol.[37] The approach is outlined in Scheme 2. First, the reaction between glucose and oxygen is catalyzed by GOX to produce gluconolactone and H_2O_2. Second, in the presence of HRP, the peroxide further reacts with the dye Amplex Red to generate a fluorescent product, resorufin. The presence of glucose is, therefore, signaled by the appearance of resorufin fluorescence in the wavelength range of 600−750 nm. For this approach to work, the two enzymes (GOX and HRP) must be entrapped within the hydrogel and the substrates, glucose, oxygen, and Amplex Red, must be able to diffuse into the hydrogel on a reasonable time scale. Accordingly, this is a very good system for testing the viability of this hydrogel-based sensing system.

The hydrogel micropatches were prepared by mixing 0.1 mg/mL GOX and 0.1 mg/mL HRP in 50 mM Tris-HCl buffer (pH 7.4) and then combining this solution with the PEG-575 precursor in a 1:1 volumetric ratio. Subsequent photopolymerization resulted in the entrapment of the enzymes within the

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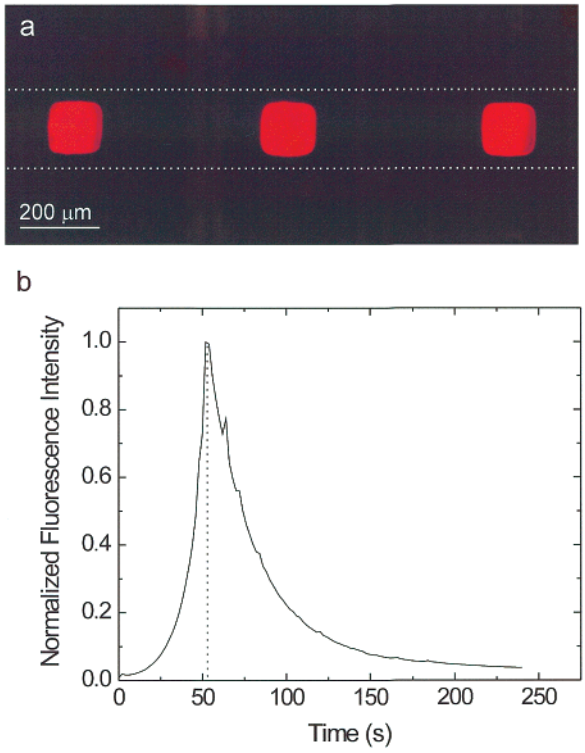
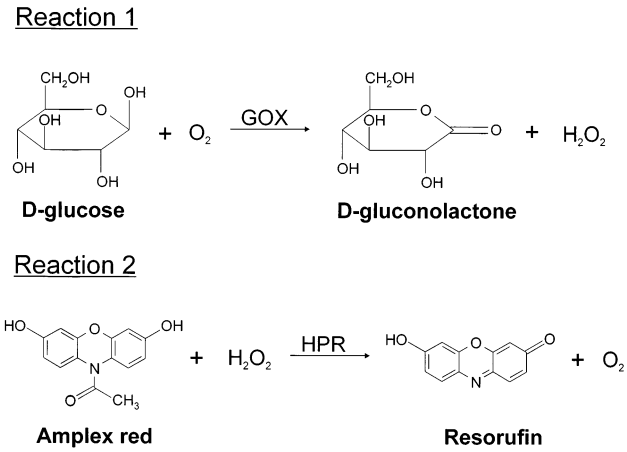


Figure 5. (a) Fluorescence image of intrachannel hydrogel micropatches (prepared by polymerization of a PEG-DA solution containing GOX and HRP in Tris-HCl buffer (pH 7.4)) after being exposed to a solution containing 10 mM glucose and 10 μM Amplex Red in 50 mM Tris-HCl (pH 7.4). The fluorescence arises from resorufin (Scheme 2, reaction 2). The dashed lines indicate the edges of the microchannel. (b) Normalized fluorescence intensity at 650 nm as a function of time measured for one of the micropatches shown in (a). The dashed line indicates the time at which a solution containing only buffer was pumped into the channel. Emission intensity data were acquired at 2-s intervals. The flow rate was 1 μL/min.

Scheme 2



hydrogel. When a glucose solution was pumped into the microchannel, strong fluorescence arising from conversion of Amplex Red to resorufin (reaction 2) was observed (Figure 5a). This result unambiguously confirms that both enzymes are present and active within the hydrogel micropatches and that all necessary substrates are able to diffuse into the interior.

The time response of this microfluidic sensor is shown in Figure 5b. A pH 7.4 solution containing 10 mM glucose and 10

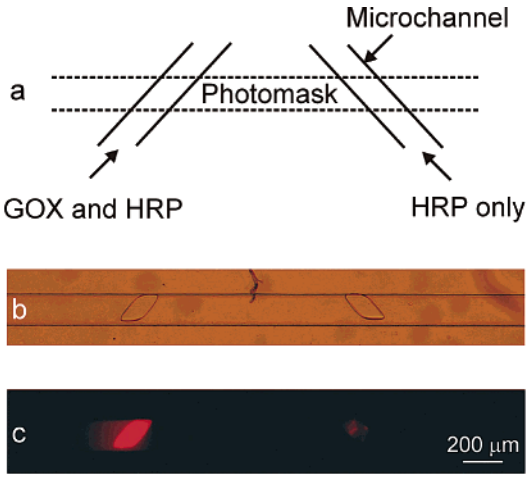


Figure 6. Control experiment confirming the reaction sequence shown in Scheme 2. (a) Schematic diagram illustrating how two micropatches containing different enzymes can be immobilized within a single channel. See text for complete explanation. (b) Optical micrograph of the two hydrogel micropatches. The one on the left contains GOX and HRP, and the one on the right contains only HRP. (c) Fluorescence micrograph of the two micropatches shown in (b) in the presence of glucose, oxygen, and Amplex Red. Strong fluorescence is apparent only in the micropatch containing both enzymes. The glucose solution was pumped from right to left at 1 μL/min.

μM Amplex Red was introduced into the channel at a flow rate of 1 μL/min. Red fluorescence was clearly observed within 20 s after pumping was initiated, which provides an estimate of the time required for glucose to diffuse into the gel and react with GOX and for the peroxide generated in that reaction to further react with Amplex Red in the presence of HRP (Scheme 2). Note that maximum fluorescence is not obtained within 1 min. We believe this to be a consequence of fluorescent resorufin accumulating within the relatively hydrophobic hydrogel. After ∼1 min (dashed line in Figure 5b), the substrate flow was replaced with a solution containing only buffer. The fluorescence falls to 90% of the maximum value after ∼ 90 s. The turn-off time is probably governed by diffusion of the relatively hydrophobic resorufin dye from the gel.

We performed a control experiment to demonstrate that the micropatches fluoresce only when both enzymes are encapsulated within the hydrogel. The channel shown in Figure 6 contains two micropatches: the one on the left contains both GOX and HRP, while the one on the right contains only HRP. When a 10 mM glucose solution containing Amplex Red flows over the micropatches (from right to left), a significant level of fluorescence is observed only at the micropatch containing both enzymes.

This experiment demonstrates another important aspect of this sensing approach. It is possible to place two or more micropatches, each containing a different enzyme, other catalyst, or reporter, within a single microfluidic channel. The approach for doing this is shown in Figure 6a. During the first two steps of the fabrication procedure shown in Scheme 1, we used a PDMS mold having two separate channels. The slit photomask was placed across both channels to ensure alignment of the two micropatches after photopolymerization. The second PDMS mold is then aligned over both micropatches and irreversibly attached to the underlying glass substrate.

SUMMARY AND CONCLUSIONS

We have shown that PEG-based hydrogels containing active enzymes can be immobilized within microfluidic channels. The pores within the hydrogels are sufficiently small to retain the enzymes and the necessary reporter dyes, but they are sufficiently large to admit substrates on a time scale of seconds. The operation of these micropatches was demonstrated by sensing intrachannel pH and glucose concentrations. We also showed that it is possible to place micropatches containing different enzymes within a single channel. The strategy described in this paper should be sufficiently general to accommodate a number of sensing and synthetic applications. For example, it seems reasonable to assume that active cells,[38] DNA,[39] and proteins other than those described here could be entrapped within intrachannel hydrogels. Perhaps in the future it will be possible to combine such components within a single fluidic system to carry out more complex sensing operations or to perform multistep syntheses.

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