Microfluidic Peroxidase Biochip for Polyphenol Synthesis

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Abstract: An enzyme-containing microfluidic biochip has been developed for the oxidative polymerization of phenols. The biochip consists of a simple T-junction with two feed reservoirs 20 mm apart and a microreaction channel 30 mm long. The channel is 15 μm deep and 200 μm wide at the center, giving a reaction volume of 90 nL. The biochip was fabricated using conventional photolithographic methods on a glass substrate etched using a HF-based solution. Fluid transport was enabled using electroosmotic flow. Soybean peroxidase was used as the phenol oxidizing catalyst, and in the presence of *p*-cresol and H_2O_2, essentially complete conversion of the H_2O_2 (the limiting substrate) occurred in the microchannel at a flow rate of ca. 290 nL/min. Thus, peroxidase was found to be intrinsically active even upon dramatic scale-down as achieved in microfluidic reactors. These results were extended to a series of phenols, thereby demonstrating that the microfluidic peroxidase reactor may have application in high-throughput screening of phenolic polymerization reactions for use in phenolic resin synthesis. Finally, rapid growth of poly(*p*-cresol) on the walls of the microreaction channel could be performed in the presence of higher H_2O_2 concentrations. This finding suggests that solution-phase peroxidase catalysis can be used in the controlled deposition of polymers on the walls of microreactors. © 2003 Wiley Periodicals. *Biotechnol Bioeng* **81:** 563–569, 2003.
Keywords: peroxidase; microfluidics; polyphenol synthesis; biochips

INTRODUCTION

Inspired by advancements in microelectronic fabrication techniques, chemical and biochemical devices have been designed with sub-millimeter fluidic channel network feature sizes etched into planar substrates using standard photolithography, wet chemical etching, and bonding techniques (Brouse et al., 2000; Stone and Kim, 2001; Kakuta et al., 2001). Chemical analysis (e.g., micro total analytical systems (μTASs)) on microchips containing integrated flow channels and reaction chambers represent a key development in the ability to speed-up, automate, and miniaturize traditional benchtop analytical tools with enhanced performance and reduced sample size (Harrison et al., 1993; Jacobson and Ramsey, 1996).

Biochemical microscale devices have been applied in biosensing (Laurell and Rosengreen, 1995), DNA hybridization (Fan et al., 1999), drug delivery (Santini et al., 1999), and enzyme reactions (Hadd et al., 1997; Moser et al., 1995; Nagy et al., 1998; Laurell et al., 1996). Enzymatic reactions on a chip can take place either in discrete reservoirs/spots or within microfluidic channels. The former has led to arrays prepared using nanoliter pipetting techniques. For example, MacBeath and Schreiber (2000) attached protein kinases to a glass substrate to study protein–protein and protein–small molecule interactions, and Arenkov et al. (2000) fabricated extremely small protein-containing polyacrylamide gel "pads" with 100 × 100 × 20 μm dimensions each with a volume of 0.2 nL. Assays involving horseradish peroxidase and alkaline phosphatase were developed.

Microfluidic analysis using enzymes within the microchannels has also been explored. For example, Drott et al. (1997) used porous silica as a material for glucose oxidase immobilization within long and deep channels (10 mm long × 50 μm wide × 250 μm deep to give a $V_{channel} = 6.3$ μL) for use in the measurement of glucose concentrations. Highly sensitive analysis of 0.5 μL sample volumes was possible using such a microfluidic device. Recently, several trials have used enzymes other than glucose oxidase, including β-galactosidase (Hadd et al., 1997), protein kinase A (Cohen et al., 1999), and trypsin, the latter in sol–gel-encapsulated PDMS-based microfluidic channels (Kim et al., 2001). The kinetic constants (kcat, Km, and Ki) from the microscale enzyme reactors were comparable to those from solution-based, conventional reactions in vials, indicating that active biocatalytic microscale reactors could be constructed. Channel specific immobilization of β-galactosidase on microchips and subsequent monitoring of activity has also been reported (Xiong and Regnier, 2001). Several pertinent reviews have summarized the state of the art in microscale biological systems (Haswell and Skelton, 2000; Blawas and Reichert, 1998; Sanders and Manz, 2000).

Despite these studies, the catalytic activity of enzymes on microfluidic biochips remains largely unexplored and has

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not been demonstrated for synthetic applications. Nevertheless, enzymes offer an array of tantalizing opportunities at the microscale, in areas as diverse as new reaction discovery, parametric optimization of enzyme activity under synthetically relevant conditions, and new compound discovery using techniques such as combinatorial biocatalysis (Michels et al., 1998). The high-throughput capability available on the microscale has been exploited in gas- and liquid-phase heterogeneous and homogenous catalysis, catalytic oxidation, heterocyclic synthesis, and photochemical reactions (DeWitt, 1999; Fletcher et al., 1999). Such synthetic discovery efforts devoted to microreactors have thus far adapted existing chemical synthetic routes. This has been extended to combinatorial chemistry for parallel reactions for lead discovery that can reduce the time scale for chemical synthesis and assist in rapid synthesis and screening (Haswell and Skelton, 2000).

In the current work, we have developed the first microfluidic reactor designed for synthetic biotransformations using soybean peroxidase (SBP) as the catalyst for polyphenol synthesis. Peroxidase-catalyzed phenolic oxidation, ultimately yielding oligo- and polyphenols, is a well-studied example of enzymatic catalysis (Dordick et al., 1987). The enzyme has extremely broad substrate specificity and can accept most phenols (Kim et al., 1998). These attributes make peroxidases ideally suited for high-throughput biocatalysis on microfludic biochips.

EXPERIMENTAL PROCEDURES

Materials

SBP and fluorescein isothiocyanate (FITC)-labeled horseradish peroxidase (HRP) were purchased from Sigma (St. Louis, MO). Phenols (*p*-cresol, *p*-methoxyphenol, *p*-hydroxyphenylacetic acid, and *p*-hydroxyphenethyl alcohol), calcein, and dimethylformamide (DMF) were purchased from Aldrich (Milwaukee, WI). Hydrogen peroxide (H_2O_2), ammonium hydroxide (NH_4OH), and sodium hydroxide (NaOH) were obtained from Fisher Scientific (Pittsburgh, PA). Buffered oxide etch was purchased from Doe and Ingalls, Inc. (Boston, MA). All other solvents and reagents were obtained commercially at the highest purity available and used without further purification.

Device Fabrication

Borosilicate microscope slides obtained from Fisher Scientific were cleaned with isopropyl alcohol and acetone in a class-100 clean room. A "biochip" with a T-channel was fabricated using standard photolithographic techniques (Fig. 1). Briefly, the pattern from a photomask was transferred onto a glass slide spin-coated with a layer of Shipley 1813 positive photoresist (Microlithography Chemical Corp., Watertown, MA). Wet etching was performed using 10:1 buffered oxide etch for 45 min. The dimensions of the channel, as measured using a profilometer (Alpha-Step 2000, Tencor Instruments, Mountain View, CA), were 15 μm deep and 200 μm wide at the center. The microreaction channel was 30 mm long, and each arm of the T channel was 10 mm long. Holes were drilled at the end of the channels to act as reservoirs for sample withdrawal and addition of substrate and enzyme solutions. The drilling dust was removed in an ultrasonic bath before cover plate bonding. The silanol groups on the surfaces of the etched glass slide and the cover plate were activated by treating with a 1:1 solution of NH_4OH and H_2O_2 at 70°C for 25 min. After the glass slide and cover plate were rinsed with distilled water and dried using a nitrogen gun, bonding was performed in an oven at 590°C for 6 h by placing the cover plate and glass slide between stainless steel plates. Subsequently, additional glass tubes of ≈80 μL volume served as reactant, buffer, and product reservoirs, and platinum electrodes were attached to the drilled holes using epoxy glue.

Enabling Fluid Flow

The surface of the channels was prepared for electroosmotic flow (EOF) by flowing 1 *N* solution of NaOH solution for 20 min (via vacuum) and subsequent washing with buffer (0.1 *M* sodium phosphate buffer, pH 7.0, containing 20% (v/v) DMF) for 15 min. A high-voltage power supply (Model 215, Bertan Associates, Inc., Syosset, NY) was used to maintain a constant voltage difference between the reservoirs. Calcein (10 μg/mL) in buffer was used to image the flow and confirm fluid velocity through the microchannels. FITC-HRP (10 μg/mL) was used for mixing studies and to estimate the diffusion coefficient for the largest reaction component under EOF conditions. The diffusion coefficient was calculated by measuring the time for calcein or FITC-HRP to diffuse across the channel when the voltage was switched off. Images were collected using a Spot RT camera attached to a Nikon Eclipse TE 200 inverted microscope with a TE-FM epifluorescence attachment (Micro Video Instruments, Avon, MA).

Soybean Peroxidase-Catalyzed Reactions

Unless otherwise specified, the solvent used was 0.1 *M* sodium phosphate buffer, pH 7.0, containing 20% (v/v) DMF to aid in dissolution of the phenols. Spectrofluorophotometry was used to determine the activity of SBP. We took advantage of the intrinsic fluorescence of oligo- and polyphenols (monomeric phenols have minimal fluorescence) to monitor the formation of product on the microscale. Reactions on the biochip were performed with 0.125 m*M* H_2O_2 (limiting reactant) and a large excess of a phenol. Samples (20 μL) were withdrawn from the product reservoir at specified reaction times and analysis was performed in a 384-well plate (Simport Plastics, Quebec, Canada). The reactions were monitored by measuring the fluorescence of the products using a Bio Assay Reader HTS 7000 Plus (Perkin-Elmer, Norwalk, CT) at an excitation wavelength of

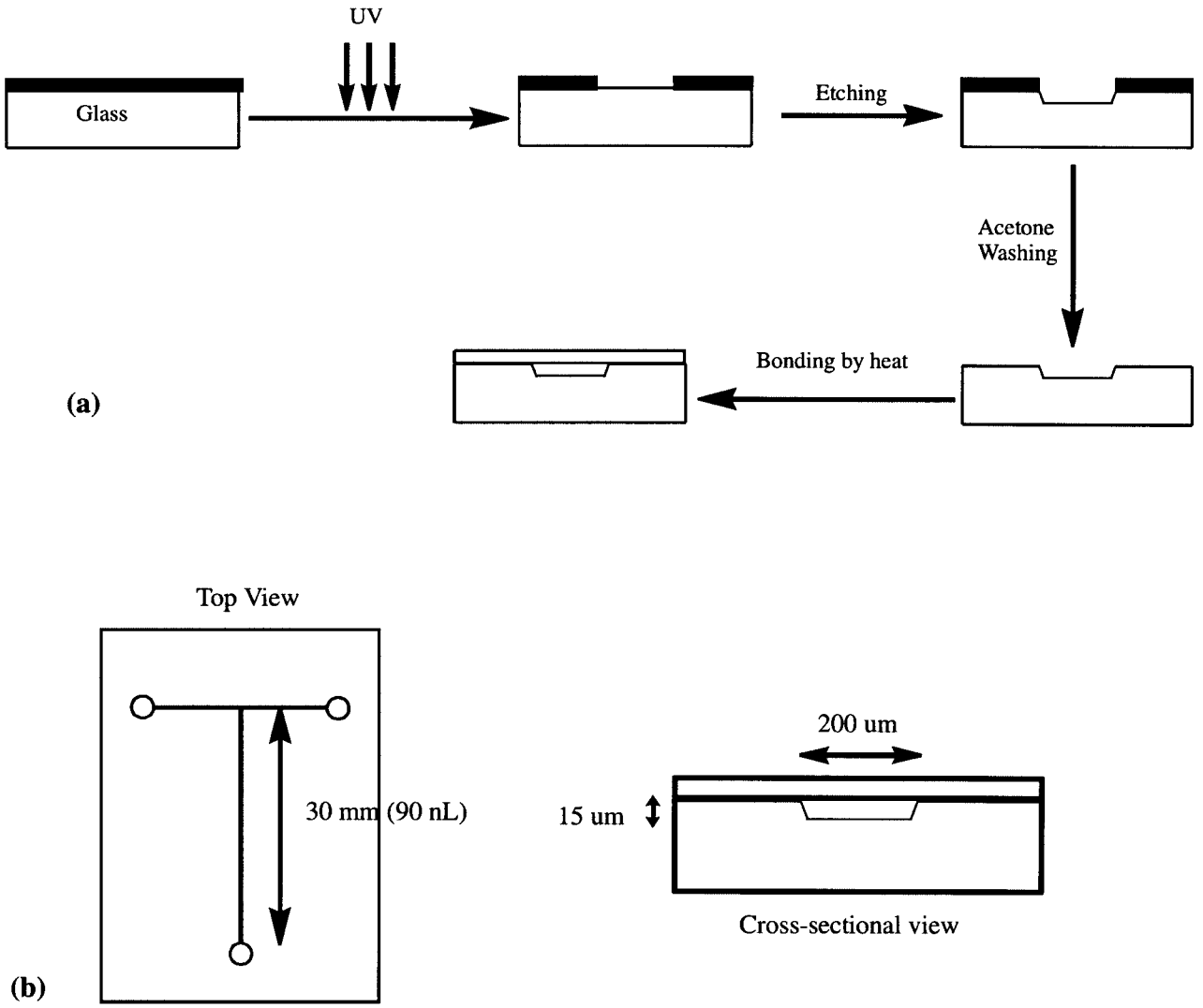

Figure 1. (**a**) Schematic representation of steps involved in fabrication of microfluidic biochip used in this study. (**b**) Top and cross-sectional views of fabricated biochip.

325 nm and emission wavelength of 405 nm (Wu et al., 2000).

To convert from fluorescence values to actual H_2O_2 consumed, we standardized the fluorescence measurements. To that end, we added 0.125 m*M* H_2O_2 to 10 m*M* of each phenol and 25 μg/mL SBP in 384-well plates in volumes of 20 μL (containing 20%, v/v, DMF) and the reactions were allowed to go to completion, i.e., no further increase in fluorescence, thereby representing 0.125 m*M* H_2O_2 consumed. A series of dilutions of these solutions were then performed to establish the correlation between fluorescence intensity and the concentration of H_2O_2 consumed.

Mass Spectral Acquisition

Electrospray ionization (negatively charged ion mode) mass spectra (ES-MS) were obtained on an Agilent 1100 series LC/MSD ion trap instrument. Samples were introduced into the ion source using a syringe pump at a flow rate of 300 μL/h or using the autosampler (methanol, flow rate 200 μL/min). Samples for ES-MS were prepared as follows: for the vial reaction sample, the poly(*p*-cresol) mixture was dried and then dissolved at a concentration of 0.2 μg poly(*p*-cresol)/μL in dioxane or acetonitrile. The biochip reaction involved the combination of three runs of 2 h each (ca. 60 μL), drying via passing a nitrogen gas stream over a small Eppendorf centrifuge tube, and extracting the solids with 600 μL dioxane to give a concentration of ca. 0.2 μg/μL polymer for ES-MS analysis.

Flow Velocity

To compute the flow velocity at a particular voltage, a known concentration of the fluorescent *p*-cresol oligomeric product was flowed using EOF. The product reservoir was filled with 40 μL of buffer. After 1 h of flow, 20-μL

samples were withdrawn from the product reservoir and the fluorescence was measured as described earlier. The flow velocity was calculated by correlating the fluorescence intensity with known oligomeric concentration.

RESULTS AND DISCUSSION

The goal of this study was to demonstrate synthetically useful biotransformations on a microfluidic template. To this end, soybean peroxidase (SBP) was used to catalyze the oxidation of phenols. We utilized four phenols in this work; *p*-cresol, *p*-methoxyphenol, *p*-hydroxyphenyl acetic acid, and *p*-hydroxyphenethyl alcohol. This reaction is of moderate complexity, requiring two substrates, one of which (H_2O_2) must be segregated from the enzyme prior to reaction due to potential enzyme inactivation. Figure 1 shows a schematic view of the fabrication of the microchip with the T channel. Initially, the channel was filled with buffer and Reservoir A was filled with a solution containing the enzyme and a phenol. Reservoir B was filled with 0.25 m*M* H_2O_2. When voltage was applied to the platinum leads, the solutions from reservoirs A and B flowed by EOF toward reservoir C. The polymerization reaction occurred in the reaction channel once mixing of the enzyme and the two substrates took place, and the resulting polyphenols accumulated in reservoir C. The enzymatic reaction was assumed to terminate once the reaction entered reservoir C, as this resulted in an ca. 50-fold dilution of the channel reaction.

Fluid Flow in the Biochip

The fluid flow velocity under experimental conditions was found to be linear with voltage intensity, as shown in Fig. 2. Thus, upon applying 2,000 V across the biochip from reservoirs A and B to reservoir C, a linear flow rate of ca. 290 nL/min was obtained. On the basis of the dimensions of the microchannel and the fluid flow rate, we calculated the Reynolds number to be ca. 0.05 at 2,000 V. Under such laminar flow conditions, any mixing in the microchannel would be due to diffusion of the enzyme and substrate molecules. Hence, we proceeded to evaluate the mixing lengths that are represented by substrate (using calcein as a model low molecular weight substrate mimic) and enzyme (using FITC-labeled HRP, an enzyme of nearly equivalent size and structure to SBP). From visual inspection (data not shown), the mixing length for calcein was found to be 1–2 mm, while that for FITC-HRP was found to be ca. 10 mm. These mixing distances resulted in calculated diffusion coefficients of 3.0×10^{-5} and 3.0×10^{-6} cm^2/s, for calcein and enzyme, respectively. The former agrees well with the values reported in literature for fluorescein, another phenolic-based fluorophores (Hadd et al., 1997). Because of the rapid diffusion of the small molecule (which will be similar to the diffusion of the phenolic substrate), we expected the observed reactivity of peroxidase in the microchannel to be free from diffusional limitations.

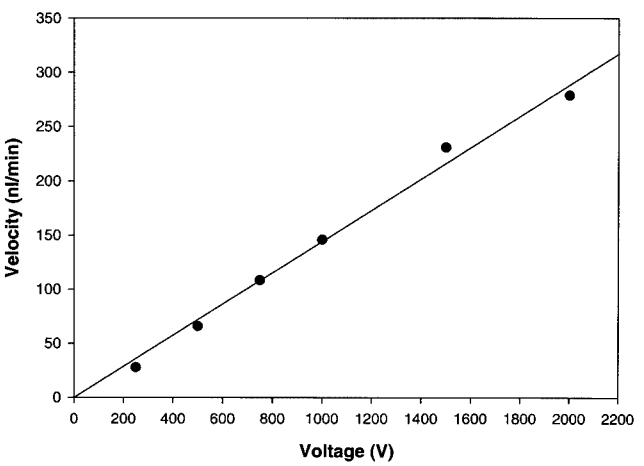

Figure 2. Flow velocity plotted as a function of potential applied across the reservoirs. Flow rate was measured by flowing a known concentration of oligophenol and measuring the fluorescence of diluted sample from reservoir C. Electroosmotic flow was carried out with 0.1 *M* sodium phosphate buffer, pH 7.0, containing 20% (v/v) DMF.

Peroxidase Catalysis on the Microscale

Because of the need to convert the SBP-catalyzed oxidation of phenols from fluorescence values to the more practical units of concentration of H_2O_2 consumed, reactions were first performed in 384-well plates with reaction volumes of 20 μL. In addition to this dramatic scale-down from typical small-scale reaction volumes of several milliliters, as used in several of our previous studies with SBP (Kim et al., 1998), this approach enabled us to assess the correlation between fluorescence intensity of oligo- and polyphenols and the consumption of the limiting substrate, H_2O_2.

Reactions were performed in aqueous buffer, pH 7.0, containing 20% (v/v) DMF with 5 m*M* *p*-cresol, 0.125 ng/μL SBP, and 0.06 or 0.125 m*M* H_2O_2. Progress time-courses for these two H_2O_2 concentrations are shown in Fig. 3. In both cases, the reactions reached completion in less than 10 min (as determined by the maximum change in fluorescence intensity due to phenolic oxidation), and this was confirmed by adding either more SBP or more H_2O_2. In the case of the former, no additional reaction took place; however, addition of 0.125 m*M* H_2O_2 resulted in further *p*-cresol oxidation (data not shown). Thus, we concluded that the fluorescence intensity change directly correlated with the concentration of H_2O_2 consumed. This conversion technique was used throughout this study to obtain absolute reactivity and conversion data based on H_2O_2 consumed. We also carried out identical experiments with the other phenols and obtained information necessary to convert change in fluorescence into concentrations of H_2O_2 consumed.

SBP, therefore, was active on the 20 μL microplate scale, with an initial rate of 0.062 μmol/(mg SBP-min) at 0.125 m*M* H_2O_2. Interestingly, this rate is similar to that obtained

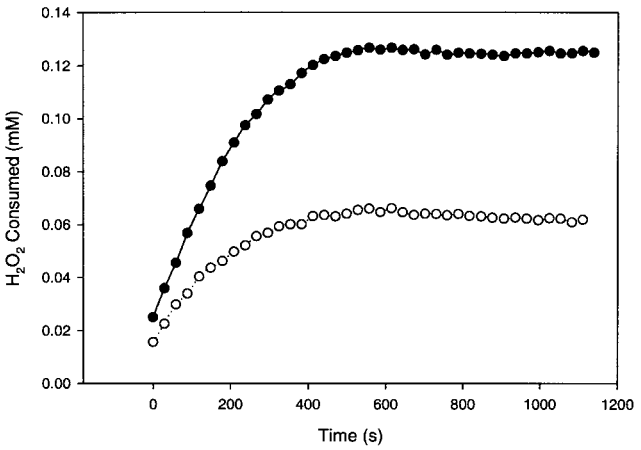

Figure 3. Time course of H_2O_2 consumption for *p*-cresol polymerization reaction performed in a 384-well plate carried out in 0.1 *M* sodium phosphate buffer, pH 7.0, containing 20% (v/v) DMF with 0.125 μg/mL SBP and 5 m*M* *p*-cresol; (●) 0.125 m*M* H_2O_2; (○) 0.062 m*M* H_2O_2.

in reactions conducted in 5-mL reactions (in 20-mL scintillation vials) under identical reaction conditions Kim et al. (1998), indicating that SBP catalysis scales down effectively. Using the 384-well plate scale, we determined the kinetic constants for SBP-catalyzed oxidation of *p*-cresol to be $V_{max} = 0.20 \pm 0.03$ mmol/mg SBP-min and $K_m = 0.95 \pm 0.14$ m*M*. These values are also similar to those obtained in larger-scale reactions (Kim et al., 1998). Therefore, we proceeded to scale-down the reaction volumes to ca. 90 nL by performing SBP catalysis on the biochip.

Peroxidase Catalysis on the T-Channel Biochip

Reactions on the biochip were initially performed with *p*-cresol using EOF driven by 2,000 V. The concentration of H_2O_2 was set at 0.25 m*M* in reservoir B, and this was diluted to 0.125 m*M* in the reaction microchannel; a concentration that is known to avoid enzyme deactivation. Samples (20 μL) were withdrawn after 5, 10, and 15 min from reservoir C, and were transferred to a 384-well plate for product analysis. This resulted in different dilutions of the oligomeric fluorescent product in reservoir C as a function of time. Because fluorescence does not linearly correlate to fluorophore concentration (Chen and Hayes, 1965; Nemet et al., 1983), fluid flow rate will affect the fluorescence intensity of the reaction product that we remove from reservoir C for analysis in the 384-well plate. For example, in 15 min at 1,000 V, the total volume of product entering reservoir C is 4.35 μL. This results in a dilution ratio of 17.1 as reservoir C initially contains 70 μL of buffer. Using a serial dilution of the *p*-cresol oligomeric product, we established an expression that accurately corrected for the effect of dilution on the fluorescence of the reaction product [Eq. (1)]; where, *X* is the dilution ratio in reservoir C, *Y* is the measured fluorescence of the diluted sample (20 μL) from reservoir C, and *A* is the fluorescence of the sample after correcting for dilution. This equation was used throughout this work.

$$\log Y = -0.85 * \log X + \log A \quad (1)$$

Figure 4a depicts the effect of SBP concentration on the rate of *p*-cresol oxidation, in terms of amount of H_2O_2 consumed. The reaction rate was strongly influenced by enzyme at concentrations below 10 ng/μL. Above this concentration, however, the reaction rate was largely unaffected by enzyme concentration, indicating that enzyme activity was not rate limiting and that H_2O_2 flux through the microchannel was the rate limiting factor in the essentially plug–flow reactor. The conversion of H_2O_2 in the microreaction channel at enzyme concentrations above 10 ng/μL was calculated to be ca. 100% (Table I) based on 5-min flow times.

The influence of *p*-cresol concentration on SBP catalysis is shown in Fig. 4b using 10 ng/mL SBP. Apparent kinetic constants were obtained $-V_{max}$ of 0.45 ± 0.08 mmol/mg SBP-min and K_m of 2.35 ± 0.89 m*M*. Thus, the maximal reactivity of the enzyme in the 90-nL microchannel close to

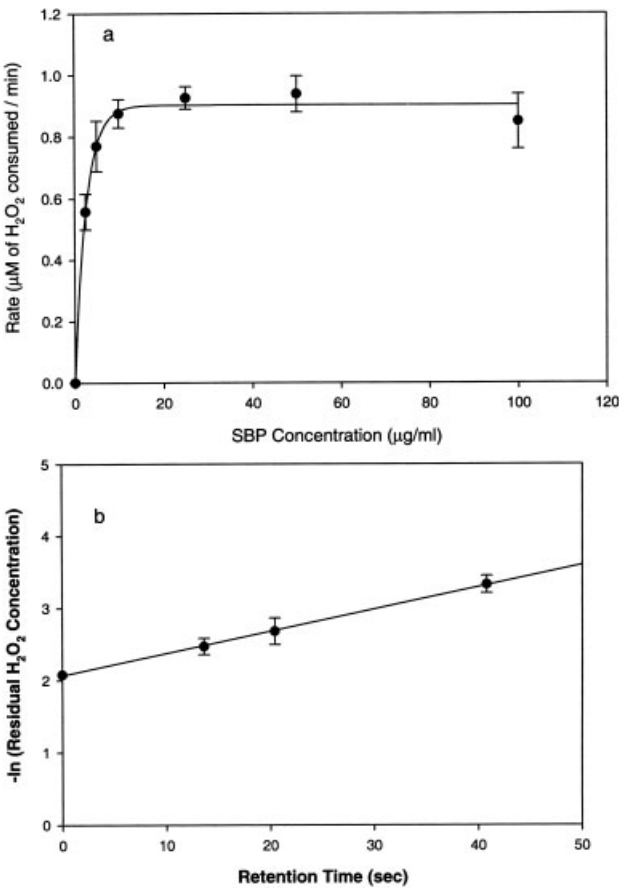

Figure 4. (**a**) Rate of reaction of *p*-cresol polymerization catalyzed by SBP, as measured by [H_2O_2] consumed, as a function of SBP concentration. Concentration of *p*-cresol was maintained at 10 m*M*. EOF was achieved by applying 2,000 V to platinum leads attached to reservoirs. (**b**) Influence of residence time of the SBP in the microchannel on H_2O_2 conversion. The concentration of SBP was maintained at 10 μg/mL and the initial concentration of H_2O_2 was 0.125 m*M*.

Table I. SBP-catalyzed oxidation of phenols on the biochip.[a]

Phenol	Conversion
p-Cresol	100%
p-Hydroxyphenylacetic acid	58.6%
p-Hydroxyphenethyl alcohol	50.7%
p-Methoxyphenol	26.5%

[a]The final concentrations of enzyme and substrates in the reaction channel were 10 ng/μL SBP, 0.125 m*M* H_2O_2, and 5 m*M* of a phenol.

that achieved in the 20 μL microwell. The differences observed are not too distinct given the intrinsic error that is likely in the calculation of corrected fluorescence measurements at the dilution levels encountered in this experimental design.

SBP catalysis leads to polyphenol synthesis in the microchannel, with an $M_n = 349$, representing mainly trimers, although electrospray MS analysis indicates the presence of 11-mers (data not shown). This is half that for the polymer formed in 5-mL reaction mixtures ($M_n = 712$), although again 11-mers were generated. The lower polymer size may have been due to the higher enzyme concentration in the microchannel and the shorter reaction times available than in more conventional size reactions. Such an increase in biocatalyst concentration favors lower molecular weight oligomers (Ryu et al., 1993). In addition to *p*-cresol, we examined the oxidative polymerization of several other phenols (Table I). After 5-min flow times on the microfluidic biochip, >50% conversion was achieved for *p*-hydroxyphenylacetic acid and *p*-hydroxyphenethyl alcohol, and lower conversion (indicating a substantially slower reaction rate) was obtained for *p*-methoxyphenol.

Polymer Growth on Microchannel Walls

Peroxidase can generate higher molecular weight polymers that fall out of solution even in the presence of 20% (v/v) DMF. We reasoned that this property of peroxidase catalysis could be used to selectively deposit polymers onto the microchannel walls, essentially providing an in situ biocatalytic approach to polymer deposition. To that end, we pushed the polymerization reaction toward higher molecular weight by increasing the concentration of H_2O_2. With 10 m*M* *p*-cresol as substrate and 10 ng/μL SBP concentration, low H_2O_2 concentrations (e.g., 0.125 m*M* as was used in the kinetic studies) did not result in polymer deposition onto the microchannel walls (Fig. 5a). However, increasing the H_2O_2 concentration to 1.25 and 3.12 m*M*, respectively, resulted in clear polyphenol deposition onto the walls of the microchannel (Fig. 5b,c). The approximate thickness of the deposited polyphenol on the channel wall was found to be 25 and 75 μm, respectively. As expected, the polymer deposition was mainly on the side from which SBP entered the microchannel, likely because H_2O_2 diffuses much faster across the microchannel than enzyme resulting in formation of poly(*p*-cresol) only on one side of the channel. We did not determine the molecular weight of the polymers deposited on the channel walls; however, we expect them to be larger than those oligomers that are soluble in 20% (v/v) DMF and that are carried into the product reservoir for downstream analysis. This is the first report of enzyme-catalyzed polymer growth and deposition in microchannels. The inherent control of peroxidase catalysis (e.g., reaction rate by enzyme and substrate concentrations) and amounts of polymer generated (via H_2O_2 concentration) may be useful in selective deposition of polymer layers containing phenols with different properties (e.g., hydrophobicity). Such

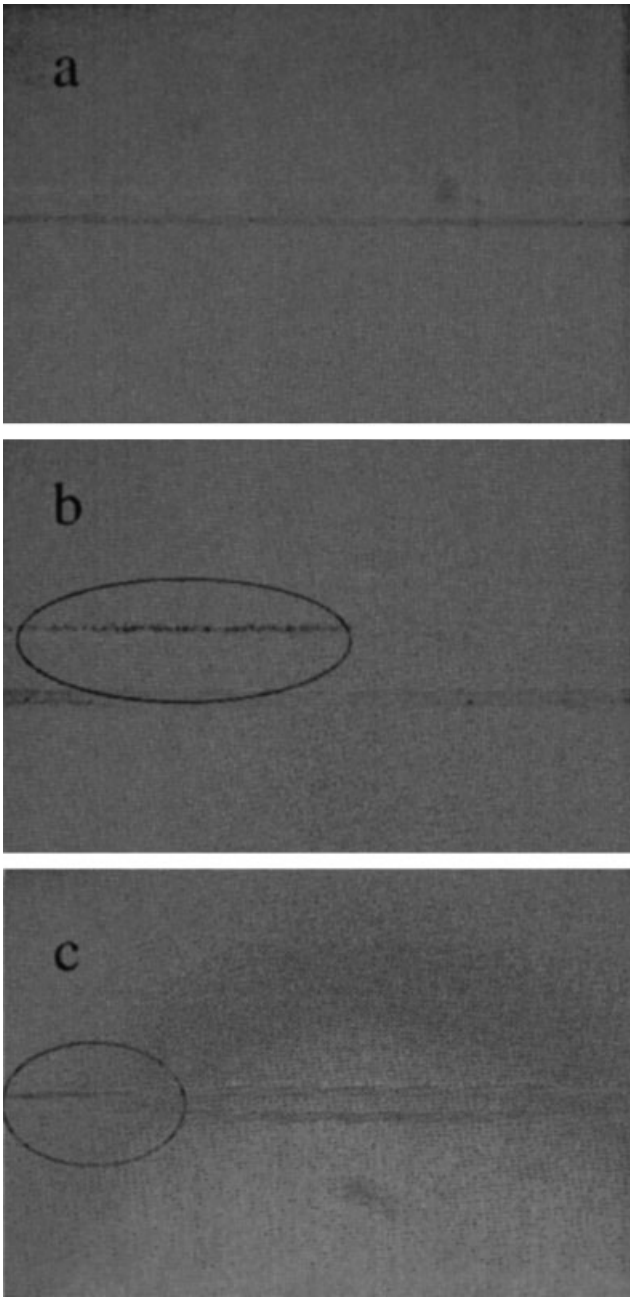

Figure 5. Microscopic images of poly(*p*-cresol) deposited on the walls of the microreaction channel. (**a**) [H_2O_2] of 0.125 m*M;* (**b**) [H_2O_2] = 1.25 m*M;* (**c**) [H_2O_2] = 3.12 m*M*. Ovals indicate locations of polymer wall growth.

controlled deposition may be used to control EOF-based flow properties for biochips and non-biochips. Moreover, the deposition of layers may be useful in the incorporation of organic and biological molecules, as well as microbial cells, for synthesis and screening operations on a chip. We are currently pursuing these techniques, as well as improving the uniformity of polymer deposition onto miocrochannel surfaces, which may result in a number of potential applications ranging from chromatography (e.g., as a capillary packing) to enzyme-containing coatings for chip-based biotransformations.

In conclusion, we have demonstrated that SBP is catalytically active on a microfluidic biochip with a reaction volume of 90 nL, and catalyzes the oxidative polymerization of a number of synthetically relevant phenolic monomers. To our knowledge, this is the first report of an enzyme-catalyzed polymer synthesis reaction performed on a microfluidic device. The design of multichannel biochips is underway, and this will facilitate the high-throughput, simultaneous synthesis of phenolic polymers and copolymers under a variety of reaction conditions and with a large number of phenolic substrates. One may envision that such high-throughput transformations on the microscale may be useful in rapidly identifying phenolic polymers for electronic materials (Khobragade and Gupta, 1995) or for sensor elements (Kim et al., 1998; Wu et al., 2000), as well as other biosynthetic products beyond those generated by peroxidase using a more complete biocatalytic repertoire.

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