Measurement of Enzyme Kinetics Using a Continuous-Flow Microfluidic System

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This paper describes a microanalytical method for determining enzyme kinetics using a continuous-flow microfluidic system. The analysis is carried out by immobilizing the enzyme on microbeads, packing the microbeads into a chip-based microreactor (volume ~1.0 nL), and flowing the substrate over the packed bed. Data were analyzed using the Lineweaver-Burk equation and compared to values obtained from conventional measurements based on the Michaelis-Menten equation. The two different enzyme-catalyzed reactions studied so that the substrate would be nonfluorescent and the product fluorescent. The first reaction involved the horseradish peroxidase-catalyzed reaction between hydrogen peroxide and N-acetyl-3,7-dihydroxyphenoxazine (amplex red) to yield fluorescent resorufin, and the second the \( \beta \)-galactosidase-catalyzed reaction of nonfluorescent resorufin-\( \beta \)-D-galactopyranoside to yield D-galactose and fluorescent resorufin. In both cases, the microfluidics-based method yielded the same result obtained from the standard Michaelis-Menten treatment. The continuous-flow method required ~10 µL of substrate solution and 10³ enzyme molecules. This approach provides a means for rapid determination of enzyme kinetics in microfluidic systems, which may be useful for clinical diagnostics, and drug discovery and screening.

Microfluidic chip-based devices have been developed for a number of important assays, including DNA analysis, protein analysis, and immunoassays. During the past decade it was demonstrated that, compared to traditional analytical instruments, the microfluidic platform provides a means for reducing both analysis time and the amount of reagent necessary to perform each analysis. However, profitable commercialization of microanalytical systems awaits development of methods that capitalize upon these enhancements without introducing complex problems that outweigh the advantages. Here, we address this need by describing an analytical method for measuring enzyme kinetics using a continuous-flow microfluidic system. In this approach, a microfluidic channel is filled with enzyme-modified microbeads, and then a nonfluorescent substrate is moved over the bead bed by convective flow. It is possible to correlate the enzyme reaction rate to the appearance of the fluorescent product of the enzyme-catalyzed reaction. Accordingly, this method is potentially useful for high-throughput screening of enzymatic activity.

The first report of using a microfluidic system for analyzing the kinetics of an enzymatic reaction was provided by Hadd et al., who used computer-controlled electrokinetic transport for diluting and mixing reagents. Specifically, precise concentrations of a fluorogenic substrate were mixed with \( \beta \)-galactosidase, and the kinetics of the reaction was obtained by monitoring the fluorescence of the hydrolysis product. More recently, Duffy et al. developed a centrifugal microfluidic system capable of carrying out multiple simultaneous homogeneous enzymatic assays using colorimetric detection.

Most previous quantitative reports of enzyme activity measured using microfluidic systems have been carried out in homogeneous solutions. However, heterogeneous assays using immobilized enzymes have the potential for providing the following advantages: ease of enzyme placement within arrays, ability to assay membrane proteins (which of course is not possible in homogeneous solution), simplification of enzyme recycling, and continuous-flow analysis. As discussed later, a potential drawback of our approach is that immobilization of enzymes may alter their activities.


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intrinsically, only a few reports describe the use of biomolecules immobilized either directly on the walls of microchannels or on a support contained within the channel. For example, a very early report from Karube and co-workers described a sensor for glucose detection based on immobilization of glucose oxidase on the walls of a micromachined capillary. More recently, Harrison et al. showed that a microfluidic device containing trypsin immobilized on beads could be used to digest proteins prior to analysis by mass spectrometry. Mao et al. reported a method for immobilizing enzymes on fluid bilayers supported on the walls of microfluidic channels and then evaluating their kinetics in one shot using laminar flow-controlled dilution. Finally, Peterson et al. reported the preparation of reactive porous monoliths within microchannels, their use as supports for the immobilization of trypsin, and the characterization of the resulting conjugates for the digestion of proteins.

In previous reports, we described microbead-based microfluidic systems for carrying out multiple, sequential enzymatic reactions and DNA hybridization analyses. A key finding of these studies is that microbeads offer the convenience of fluidic handling, a high degree of synthetic and characterization flexibility, very high surface-to-volume ratio compared to open-channel microfluidic devices, and the capacity to efficiently mix reagents even under laminar flow conditions.

The intrinsic kinetics of soluble enzymes in homogeneous solution are generally described by the Michaelis–Menten relationship. However, the kinetics for immobilized enzymes may be different from those measured for the same enzyme in solution because of diffusional restrictions or interactions with the support. The kinetics of immobilized biocatalysts are often referred to as the apparent kinetics. Under steady-state conditions, the apparent kinetic parameters in packed-bed enzyme reactors are usually calculated using a formalism known as the Lilly–Hornby model.

In the experiments reported here, horseradish peroxidase (HRP) was immobilized on microbeads via streptavidin–biotin conjugation, and the kinetics of the HRP-catalyzed reaction between hydrogen peroxide and N-acetyl-3,7-dihydroxyphenoxazone (amplex red) was monitored under continuous-flow conditions. The results compare favorably to literature values and to values we obtained using conventional analysis methods. To investigate the generality of this approach, the β-galactosidase (β-Gal) catalyzed hydrolysis of resorufin β-D-galactopyranoside (RBG) was also studied.

**EXPERIMENTAL SECTION**

**Chemicals.** Positive photoresist (AZP4620) and developer solution (AZ421K) were obtained from the Clariant Co. (Somerville, NJ). Poly(dimethylsiloxane) (PDMS) microfluidic devices were fabricated by curing the prepolymer components of Sylgard 184 (Dow Corning, Midland, MI). Microbeads were ProActive Streptavidin-Coated Microspheres (Bangs Laboratories, Fishers, IN) that were found by optical microscopy to be 15.5 μm in diameter. Biotin-labeled horseradish peroxidase, biotin-labeled β-galactosidase, and sodium resorufin (7-hydroxy-3H-phenoxazin-3-one) were purchased from the Sigma Chemical Co. (St. Louis, MO). Amplex red (N-acetyl-3,7-dihydroxyphenoxazone) and resorufin (β-D-galactopyranoside) were obtained from Molecular Probes (Eugene, OR). Hydrogen peroxide (30%) and dimethyl sulfoxide (DM SO) were obtained from EM Science (Gibbstown, NJ). All chemicals were of reagent grade quality or better. M III-Q (Millipore) water (18 M Ω-cm) was used to prepare aqueous solutions.

**Fabrication of Microfluidic Devices.** The fabrication of microfluidic devices and the associated weir structures were described in our previous reports. Briefly, positive photoresist was spin-coated onto a glass slide and illuminated with UV radiation through a mask for 5 min to create a master. A slit-type photomask having a width of 100 μm was aligned on the glass master to fabricate weirs within the microchannels. After UV exposure for 5 min, the glass master was developed in 60%AZ421K solution for 30 s. PDMS polymer molds were prepared by casting a PDMS prepolymer against a positive photoresist master and curing at 65 °C for 2 h. The PDMS mold was irreversibly sealed to a clean glass substrate after both were treated with an oxygen plasma (60 W, model PDC-32G, Harrick Scientific, Ossining, NY) for 60 s. The depth and width of the microchannel and weir were determined using a Veeco Dektak 3 profilometer (Veeco Instruments, Plainview, NY).

**Enzyme Immobilization on Microbeads.** HRP and β-Gal were conjugated to biotin via amide coupling of an aminocaproyl spacer. HRP–biotin (240 units/mg) and β-Gal-biotin (350–700 units/mg) were used for the immobilization of HRP and 2–4 mol of biotin of β-Gal, respectively. The conjugation of streptavidin-coated microbeads with the biotinylated enzymes was carried out using the following procedure. A 300 μL sample of stock beads (4.8 × 106 bead/mL) were rinsed in 200 μL of phosphate buffered saline (PBS) solution (pH 7.4, 150 mM NaCl, 4 mM KCl, 8.1 mM Na2HPO4, and 1.47 mM KH2PO4) and centrifuged at 4000 rpm for 5 min. The microbead pellet was resuspended in 30 μL of PBS, and then 30 μL of the biotinylated enzymes (1 mg/mL) in PBS (pH 7.4) was added to the microbeads to yield the microbead–enzyme complex. The mixture of microbeads and biotinylated enzymes was incubated for 2 h at room temperature (18–25 °C) with gentle mixing. After conjugation, the mixture was centrifuged to remove unreacted biotinylated enzymes and resuspended in 500 μL PBS. These microbead–enzyme complexes were stored at 4 °C prior to use.

**Enzyme Kinetic Assays.** To carry out enzyme assays in continuous-flow microfluidic systems, microbead–enzyme complexes were packed into on-chip microreactors using pump-driven pressure. Stock solutions of amplex red and RBG were prepared in DM SO and stored at −20 °C. Immediately before use, the thawed stock solutions were diluted in buffer solution. Various concentrations of H2O2 (in 50 mM Tris-HCl plus 10 mM amplex red, at pH 7.4) and RBG (in 100 mM Tris-HCl, 2 mM KCl, and 0.1 mM MgCl2, at pH 7.8) were introduced into the microchannels at various flow rates using a microsyringe pump. Optical and fluorescence images of the enzyme reactions were acquired with
RESULTS AND DISCUSSION

A schematic illustration of the microfluidic device used in this study is shown in Figure 1A. The device was fabricated from PDMS using standard photolithographic and replica molding methods (fabrication details are provided in the Experimental Section). The key components of the device are the microreactors (labeled M in Figure 1A) and weirs (labeled W in Figure 1A). The width and depth of the weirs ranged from 40 to 60 ìm and 7 to 12 ìm, respectively. Therefore, microspheres having a diameter of 15.5 ìm are retained within microreactors (Figure 1B). Microspheres were coated with streptavidin, a tetrameric protein having four biotin binding sites, so that nucleic acids or proteins modified with biotin could be conjugated to the microsphere surface. Microspheres were introduced into microreactor using pump-driven pressure. The volume of the microreactor was 1.0 nL, and 250–300 microbeads could be packed into each microreactor.

Oxidase enzymes, and particularly HRP, are used in a wide variety of bioassays. Accordingly, we chose to benchmark this continuous-flow study of enzyme kinetics using the HRP-catalyzed reaction between H2O2 and amplex red. In the presence of HRP, amplex red reacts with H2O2 in a 1:1 stoichiometry to produce highly fluorescent resorufin (Figure 2A).28

Before carrying out the continuous-flow study of enzyme kinetics, the inner walls of the microdevice were passivated against nonspecific adsorption of proteins with 1 mg/mL BSA in PBS solution for 3 h at room temperature. Biotin-labeled HRP was then conjugated to the streptavidin-coated microspheres, and these modified beads were packed into the microreactor. Aqueous solutions containing 5 ìM H2O2 and 10 ìM amplex red in 50 mM Tris-HCl buffer (pH 7.4) were introduced into the main channel at a flow rate of 0.5 ìL/min from the inlet reservoir. Figure 2B shows a fluorescence image of the resulting HRP-catalyzed reaction in the continuous-flow microreactor. Fluorescence line scans (Figure 2C) taken across the inlet and outlet streams (obtained at positions a and b, respectively, in Figure 2B) indicate that amplex red is a fluorogenic substrate having very low background fluorescence but that its fluorescent product, resorufin, exhibits strong fluorescence.

Enzyme kinetics were evaluated using the Lilly–Hornby model, which was developed for packed-bed reactor systems.26 This model is embodied in eq 1. Here, f is the fraction of substrate converted to product during the reaction, Q is the flow rate of the substrate, [A0] is the initial substrate concentration, C is the reaction capacity of the microreactor, and K(m(app)) is the apparent M ichalis constant. The value of f was determined experimentally by subtracting the fluorescence signal in the inlet stream from the fluorescence signal in the outlet stream of the microreactor.

\[ f[A_0] = C/Q + K_{m(app)} \ln(1 - f) \]
Because the appearance of resorufin and the disappearance of H$_2$O$_2$ are related by a 1:1 stoichiometry (Figure 2A), the turnover of H$_2$O$_2$ could be evaluated from calibration curves obtained from known concentrations of resorufin and its corresponding fluorescence intensity. In this study, the initial H$_2$O$_2$ concentrations ranged from 1 to 10 $\mu$M and the amplex red concentration was held constant at 10 $\mu$M. These solutions were flowed through the microreactor at rates ranging from 0.2 to 1.5 $\mu$L/min.

Panels A and B of Figure 3 show substrate concentration and flow rate effects, respectively, on the HRP-catalyzed hydrolysis of H$_2$O$_2$. From these data, linear plots of $f[A_0]$ versus $-\ln(1-f)$ were obtained. The flow rates ranged from 0.20 to 1.5 $\mu$L/min.

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C_{\text{max}} \text{ value of } 9.03 \text{ pmol/min. The value of } K_{\text{m(app)}} \text{ should approach the value of the true Michaelis constant of the free enzyme, } K_{\text{m}}, \text{ at zero flow rate if only mass-transfer effects are responsible for masking the intrinsic enzyme kinetics. This assumption was tested by plotting the } K_{\text{m(app)}} \text{ values obtained against } Q \text{ (Figure 4B). The intercept on the vertical axis yields a zero-flow } K_{\text{m(app)}} \text{ value (} K_{\text{m(app)}}^{0} \text{) of } 1.51 \mu \text{M. As discussed next, this value compares favorably with measurements of } K_{\text{m}} \text{ determined using standard methods.}

Enzyme kinetics in homogeneous solution were evaluated using the Michaelis–Menten equation (eq 2). This equation

\[ V_0 = \frac{V_{\text{max}}[S]}{(K_{\text{m}} + [S])} \quad (2) \]

relates the initial rate of the enzyme reaction, } V_0, \text{ to the concentration of substrate, } [S], \text{ a maximum rate, } V_{\text{max}}, \text{ corresponding to the velocity of the reaction when the active sites of the enzyme are saturated with substrate, and the Michaelis constant, } K_{\text{m}}. \text{ The values of } K_{\text{m}} \text{ for HRP were determined using the following procedure. First, the background fluorescence was measured 100 s after mixing various concentrations of } H_2O_2 \text{ with } 10 \mu \text{M amplex red. Second, } 0.01 \text{ unit/mL HRP in } 50 \text{ mM Tris-HCl (pH 7.4)} \text{ was added to these solutions, and then fluorescence intensity was measured for } 500 \text{ s. } V_0 \text{ was determined for each } H_2O_2 \text{ concentration (0.1–5.0 } \mu \text{M) from the initial slopes of plots of normalized fluorescence intensity as a function of time (Figure 5A). A plot of the Lineweaver–Burk equation (eq 3) then yields } K_{\text{m}} \text{ and } V_{\text{max}}.

\[ \frac{1}{V_0} = \frac{K_{\text{m}}}{V_{\text{max}}} / [S] + 1 / V_{\text{max}} \quad (3) \]

(Figure 5B). The results of this treatment yield } V_{\text{max}} = 1.90 \mu \text{M/min} \text{ and } K_{\text{m}} = 1.55 \mu \text{M. This value of } K_{\text{m}} \text{ is very close to the value of } K_{\text{m(app)}}^{0} (K_{\text{m(app)}} = 1.51 \mu \text{M}) \text{ determined using the continuous-flow approach described earlier.}

To demonstrate the generality of this approach, the } \beta\text{-Gal-catalyzed hydrolysis of nonfluorescent RBG to } D\text{-galactose and fluorescent resorufin was also investigated (Figure 6A). To validate the microfluidic-based method for measuring enzyme kinetics, the value of } K_{\text{m}} \text{ was first determined in homogeneous solution. Figure 6B shows plots of fluorescence intensity, arising from the appearance of resorufin, as a function of time for RBG concentrations ranging from 50 to 250 } \mu \text{M. The initial slopes from these plots and the Lineweaver–Burk equation (Figure 6C) were used to determine a Michaelis constant, } K_{\text{m(app)}}, \text{ of } 363 \mu \text{M.}

Next, the kinetics for RBG hydrolysis by } \beta\text{-Gal \text{ were evaluated using the continuous-flow approach. RBG solutions varying in concentration from 25 to 200 } \mu \text{M (buffer: } 100 \text{ mM Tris-HCl, } 2 \text{ mM KCl, and } 0.1 \text{ mM MgCl}_2 \text{ at pH 7.8)} \text{ were pumped into the inlet of the microfluidic device at flow rates of 10, 30, 50, 70, and } 100 \text{ nL/min. As before, the reaction rates were determined by capturing fluorescence images at the inlet and outlet of the microreactor (Figure 7A) and then converting the corresponding fluorescence intensities (determined from line scans, Figure 7B) to RBG concentrations using a calibration curve constructed by flowing resorufin only through the channel. From data obtained using various RBG concentrations and flow rates, primary plots of } [A_{\text{0}}] \text{ against } -\ln(1 - f) \text{ were used to generate } K_{\text{m(app)}} \text{ values. A secondary plot of } K_{\text{m(app)}} \text{ versus } Q \text{ yields a } K_{\text{m(app)}}^{0} \text{ value of } 409 \mu \text{M (Figure 7C). This value compares reasonably well with that determined in homogeneous solution (} K_{\text{m}} = 363 \mu \text{M) and with literature values (} K_{\text{m}} = 380 \mu \text{M and } 500 \pm 200 \mu \text{M}).}

**SUMMARY AND CONCLUSIONS**

We have described a continuous-flow method for measuring enzyme kinetics in microfluidic systems. The approach is conveniently implemented using bead-immobilized enzymes contained within microreactors having a liquid volume (the difference between the total microreactor volume and the beads) of 0.45 mL. It is possible to estimate the total number of enzyme molecules required for the kinetic analysis by assuming each enzyme has a projected area of 80 nm², that the total surface area of the 275 beads in each microreactor is } 2.1 \times 10^5 \mu \text{m}^2, \text{ and that the enzymes are close-packed on the bead surface. This treatment suggests a maximum of } 3 \times 10^9 \text{ enzyme molecules are required for the analysis.}


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**Figure 5.** Analysis of data obtained under homogeneous reaction conditions for the HRP-catalyzed reaction between } H_2O_2 \text{ and amplex red to yield fluorescent resorufin. (A) Plot of normalized fluorescence intensity vs time. } H_2O_2 \text{ concentrations ranged from 0.1 to 5.0 } \mu \text{M. Conditions: } 0.01 \text{ unit/mL HRP plus } 10 \mu \text{M amplex red in } 2.0 \text{ mL of } 50 \text{ mM Tris-HCl buffer (pH 7.4). (B) Lineweaver–Burk plot of the reciprocal initial reaction rates obtained from (A) vs the reciprocal of the } H_2O_2 \text{ concentration.}
The kinetic analysis relies on the Lilly–Hornby model, which has been previously used to evaluate apparent kinetic parameters in packed-bed enzyme reactors under steady-state conditions. The Michaelis constants determined using the new approach reported here and traditional methods based on the Lineweaver–Burk relationship are indistinguishable within the estimated precision of the methods. A significant outcome of this study was the finding that the kinetics of both β-Gal and HRP were the same in solution and after immobilization on the microbeads. Similar findings have previously been reported for enzymes immobilized in fluid lipid bilyaers.[21]

Because of the reduced cost, reagent economy, and increased throughput associated with microfluidic-based assays, it is quite likely that many analytical determinations presently carried out in microtiter plates will be performed using microfluidic systems in the not-too-distant future. This paradigm shift will necessitate development of new standard methods for measuring kinetic and thermodynamic properties of reactions. The results reported here contribute to this important goal, and they are therefore likely to...
be of value for clinical diagnostics, and drug discovery and screening.

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