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Accelerated Articles

Microchip Device for Performing Enzyme Assays

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An automated enzyme assay was performed within a microfabricated channel network. Precise concentrations of substrate, enzyme, and inhibitor were mixed in nanoliter volumes using electrokinetic flow. Reagent dilution and mixing were controlled by regulating the applied potential at the terminus of each channel, using voltages derived from an equivalent circuit model of the microchip. The enzyme β-galactosidase (β-Gal) was assayed using resorufin β-D-galactopyranoside (RBG), a substrate that is hydrolyzed to resorufin, a fluorescent product. Reaction kinetics were obtained by varying the concentration of substrate on-chip and monitoring the production of resorufin using laser-induced fluorescence. Derived Michaelis−Menten constants compared well between an on-chip and a conventional enzyme assay. Bias in the derived K_m and k_{cat} was primarily due to the limited solubility of RBG and the associated lack of measurements at substrate concentrations exceeding the K_m. A K_i of 8 μM for the inhibitor phenylethyl β-D-thiogalactoside (PETG) was determined from plots of initial rate versus substrate concentration obtained at three concentrations of PETG. The relative inhibition of β-Gal by lactose, *p*-hydroxymercuribenzoic acid, and PETG was determined by varying the inhibitor concentration with constant enzyme and substrate concentration. An enzyme assay performed on the microchip within a 20-min period required only 120 pg of enzyme and 7.5 ng of substrate, reducing the amount of reagent consumed by 4 orders of magnitude over a conventional assay.

Microfabricated devices have been recently developed for performing a variety of chemical measurements.[1,2] A class of these devices, known as microchips, are designed with a channel network etched into a planar glass substrate using standard photolithography, wet chemical etching, and bonding techniques. Microchips represent the ability to miniaturize current "benchtop" experiments with the advantages of speed, automation, and volumetric reduction of sample and waste. Analytical applications using microchips have been primarily demonstrated with electrically driven separation techniques, such as capillary electrophoresis,[3−8] synchronized cyclic electrophoresis,[9] free-flow electrophoresis,[10] open-channel electrochromatography,[11] and capillary gel electrophoresis.[12−14] Monolithic integration of a chemical reaction and electrophoretic separation on single devices has been demonstrated using pre- and postseparation derivatization.[15,16] Biochemical applications of integrated microchips have been demonstrated with a DNA restriction digest combined with electrophoretic sizing[17] in PCR amplification with product analy-

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sis,[18] and multichannel restriction fragment separations.[19]

In these microchip systems, fluid flow and reagent mixing is achieved using electrokinetic transport phenomena (electroosmosis and electrophoresis). Electrokinetic flow is controlled by regulating the applied potentials at the terminus of each channel of the microchip. Within the channel network, cross intersections and mixing tees are used for valving and dispensing fluids with high volumetric reproducibility (0.3% RSD). The mixing tee can also be used to mix proportionately two fluid streams in any ratio from 0 to 100% from either stream simply by varying the relative field strengths in the two channels. As an example, dilution of disodium fluorescein with buffer at a microchannel T-intersection has been demonstrated.[6] This precise fluid control can be applied to the study of reaction systems in which reagent concentrations are dynamically changed within a single experiment.

This paper describes the use of electrokinetic flow to control the dilution and mixing of the reagents used in an enzyme assay. Precise concentrations of a fluorogenic substrate, resorufin β-D-galactopyranoside (RBG), are mixed with β-galactosidase (β-Gal), and the kinetics of the reaction are obtained by monitoring the fluorescence of the hydrolysis product, resorufin, using laser-induced fluorescence. Michaelis−Menten constants are derived for the hydrolysis reaction in the presence and absence of a competitive inhibitor, phenylethyl β-D-thiogalactoside (PETG). These results are compared to values obtained using conventional measurements. An experiment comparing the relative inhibition of three compounds is also described. These results are applicable to the development of microfabricated devices for use in drug discovery, medical diagnostics, and biochemical detection methods.

EXPERIMENTAL SECTION

Chip Design. The microchip design used for this study is shown in Figure 1. The fabrication of the microchips using standard photolithographic, wet chemical etching, and cover plate bonding techniques has been described.[1,2,20] Cylindrical reservoirs cut from glass tubing were bonded to the edge of the cover plate at the end of each channel using Epo-tek 353ND epoxy (Epoxy Technologies, Inc., Billerica, MA). The channels had a depth of 9 μm and a width at half-height of 35 μm, measured using a Tencor P-10 surface profiler (Tencor, Mountain View, CA). The microchip was prepared daily by washing the channels with 0.1 N sodium hydroxide followed by analysis buffer using a vacuum line applied to one of the channel reservoirs.

Chemicals. Sodium resorufin (7-hydroxy-3*H*-phenoxazin-3-one) and resorufin β-D-galactopyranoside ($\epsilon_{469} = 1.9 \times 10^4$ L mol^{-1} cm^{-1}) were purchased from Molecular Probes (Eugene, OR). Tris(hydroxymethyl)aminomethane (Tris), *o*-nitrophenyl β-D-galactopyranoside (ONPG), phenylethyl β-D-thiogalactoside (PETG), *p*-hydroxymercuribenzoic acid (PHMB), D-lactose, β-galactosidase (β-Gal, *Escherichia coli*, 780 units/mg, 540 kDa), rhodamine B (90%), disodium fluorescein, and mercaptoethanol were obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade. Stock solutions of β-Gal, 1.0 mg/mL, and RBG, 340 μM, were prepared in an analysis buffer of 100 mM Tris, 2.0 mM KCl, and 0.10 mM $MgCl_2$ at pH 7.8 using filtered Barnstead Nanopure water.

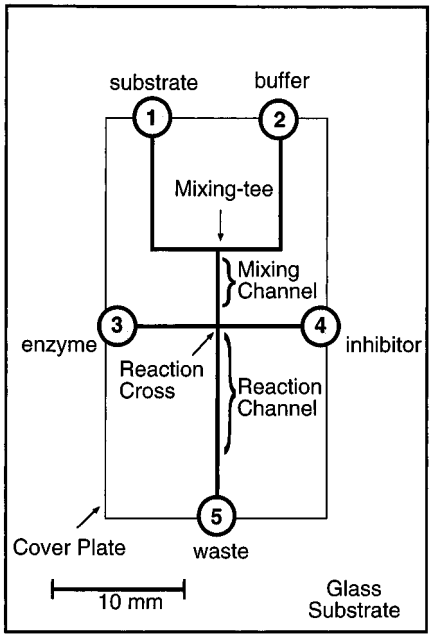

Figure 1. Schematic of the enzyme analysis chip. The channels terminate at reservoirs containing the indicated solutions.

Apparatus. Platinum electrodes in each reservoir provided electrical contact between each buffer solution and an individual Ultravolt 10A12-P4 high-voltage power supply (Ultravolt, Ronkonkoma, NY). An electrode attached to ground was submersed in the buffer solution of the waste reservoir. The resistance in each segment of the channel network was obtained by applying a high voltage between each of the 10 reservoir pairs and measuring the current using a Keithley 617 programmable electrometer (Keithley Instruments Inc., Cleveland, OH). Analysis of the equivalent circuit was performed using the software P-SPICE (Microsim Corp., Irvine, CA).

Imaging of fluid flow within the channels was obtained using laser-induced fluorescence of a 100 μM disodium fluorescein solution. An argon ion laser (Omnichrome, 488 nm, 100 mW) was expanded to a 10 mm spot at the chip surface using a lens. Laser-induced fluorescence was measured using a charge-coupled device (CCD) mounted on an optical microscope. The fluorescence signal was spectrally filtered using a 540DF30 band-pass filter (Omega Optical, Brattleboro, VT). On-chip reaction kinetics were monitored using single-point laser-induced fluorescence detection as described previously.[17] A helium−neon laser beam (Melles-Griot, 543.5 nm, 1.5 mW) was focused on the channel 20 mm downstream from the four-way reaction cross using a planoconvex fused-silica lens and a mirror to impinge the beam onto the chip 50° from normal. The fluorescence signal was collected using a 20× objective lens (NA = 0.42), followed by spatial filtering (0.8-mm-diameter pinhole) and spectral filtering with a 570-nm cutoff filter (Oriel 51310, Stratford, CT). Collected fluorescence emission was measured using a photomultiplier tube (PMT, Oriel 77340). A Labview (National Instruments Corp., Austin, TX) program written in-house was used to control the high-voltage power supplies and data acquisition.

On-Chip Enzyme Assay. A continuous-flow assay, monitored with the single-point setup, was used to observe the product of the on-chip reaction of β-Gal and RBG. The general reaction is

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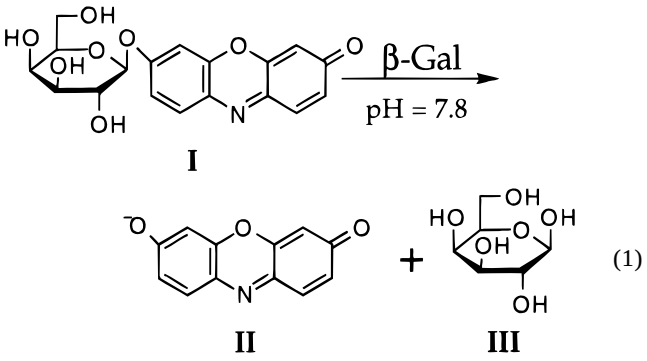
shown in eq 1 for the β-Gal catalyzed hydrolysis of the nonfluo-



I

II + III (1)

rescent RBG (**I**) to resorufin (**II**, λ_{em} = 585 nm) and D-galactose (**III**). Reagents were mixed using electrokinetic pumping. In a typical experiment, a stock solution of 340 μM RBG added to reservoir 1 (Figure 1) was mixed in the mixing channel with various amounts of buffer. The flow of diluted substrate was continuously mixed at the reaction cross with a flow of β-Gal (0.625−2.50 μg/mL in reservoir 3) and either inhibitor or buffer from reservoir 4. Fluid flow from the mixing channel and the two side channels (channels 3 and 4) was calculated using current measurements as described in the results section. A mixing ratio of 40% flow from the mixing channel and 30% flow from each of the side channels (3 and 4) was used for all experiments. Detection of resorufin was obtained 20 mm downstream from the reaction cross, which corresponded to a reaction channel volume of 6.5 nL. The reagent flow rate and mixing time were controlled by modifying the electric field strength in the reaction channel, which was 220 V/cm for most experiments. Initial reaction rates were calculated by subtracting the fluorescence signal from the background signal (the signal of RBG without enzyme present) and dividing by the transit time. The fluorescence signal was calibrated using a standard solution of resorufin. Enzyme assays were performed at an ambient temperature of 21 °C.

Standard Enzyme Assay. In order to compare results of the on-chip assay with a conventional method, hydrolysis kinetics of β-Gal and RBG were obtained by monitoring the absorbance of resorufin at 571 nm ($\epsilon_{571} = 4.7 \times 10^4$ L mol^{-1} cm^{-1}) with a Cary 1E UV−visible spectrophotometer. The reaction was initiated by adding 50 μL of 10.4 μg/mL β-Gal to 950 μL of a prepared RBG solution for a final enzyme concentration of 520 μg/L. The linear portion of the absorbance versus time plot was used to determine the initial reaction rate. The activity of β-Gal was calibrated by monitoring the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside at 410 nm (3500 L mol^{-1} cm^{-1}) in 80 mM phosphate, 20 mM mercaptoethanol, pH 7.3, buffer at 37 °C.

RESULTS AND DISCUSSION

Microfluidic Control. A circuit model of the microchip was used to derive the voltages applied at each channel reservoir necessary for controlling fluid flow. The channels in the microchip function as electrical resistors, and when measured, the potential at any point within a channel can be estimated by application of Kirchoff's rules and Ohm's law.[21] Since the current through a channel approximates the electrokinetic transport of material through that channel, microfluidic control is realized by adjusting the applied potentials at each channel reservoir. With the channels of the microchip filled with the Tris analysis buffer, the resistances (in MΩ) for each channel section were R_1 = 60.3, R_2 = 75.0, R_{mix} = 5.5, R_3 = 23.5, R_4 = 20.0, and R_{rxn} = 83.0, with an average error of 0.1 MΩ. The subscripts 1−4 designate the channel section connected to the reservoirs, and "mix" and "rxn" designate the mixing and reaction channels as indicated in Figure 1. The placement of the cover plate affected the actual channel length, contributing to the slight differences between R_1 and R_2 and R_3 and R_4.

In order to accurately partition fluid flow in the channels, the junction potentials at the mixing tee, V_{mix}, and reaction cross, V_{rxn}, were maintained at constant values. By setting V_{rxn} to 625 V, for a field strength of 220 V/cm, the current in the reaction channel, I_{rxn}, was defined, and the currents in the other channels were calculated as a percent of I_{rxn}. For the enzyme assay, the currents were partitioned such that $I_{mix} = 0.40I_{rxn}$ and $I_3 = I_4 = 0.30I_{rxn}$. The voltages applied to each reservoir according to those current settings were calculated using Ohm's law.[22] Incremental changes in V_1 and V_2 were used to change the concentration of substrate in the mixing channel. The values for V_1 and V_2 were constrained by keeping V_{mix} and I_{mix} constant and choosing current values for I_1 and I_2 from −3 to +103% I_{mix}. The 3% "over-shoot" in the current values compensated for diffusional and hydrodynamic bleeding of solution from either channel at 0 and 100% flow.

Microfluidic control was verified using fluorophore-doped buffer. A qualitative view of the flow of solution at the two channel junctions is shown in Figure 2. White-light photomicrographs of the mixing channel T-intersection and the reaction cross are shown in Figure 2a and c. The voltages applied at each channel reservoir are indicated. Dilution of a disodium fluorescein doped buffer is shown in Figure 2b, with 50% flow from channels 1 and 2 into the mixing channel. Figure 2d shows the flow of disodium fluorescein-doped buffer from channels 3 and 4 into the reaction channel. The channels in Figure 2b and d are shown labeled with the reagents used for the enzyme assays.

Mixing of the reagents and buffer streams at channel intersections was diffusion controlled due to the very low Reynolds numbers ($<10^{-2}$) associated with electrokinetically induced flow. The 34-μm channel width allowed for rapid diffusion of reagents downstream from an intersection. In the case of fluorescein, with an estimated diffusion coefficient of 3×10^{-6} cm^2 s^{-1},[23] diffusion across 17 μm of the mixing channel occurred in ~0.5 s. In the reaction channel of the microchip, diffusion of β-Gal ($D \approx 2.7 \times 10^{-7}$ cm^2 s^{-1})[24] across 70% of the channel width required ~11 s. The slower diffusion of β-Gal relative to the substrate had a minimal effect on the observed reaction kinetics.

Controlled fluid flow within the microchip channels was evaluated by diluting a stock rhodamine B solution on-chip and monitoring the fluorescence signal 20 mm downstream from the reaction cross. The fluorescence signal of a rhodamine B solution stepped in equal increments from 0.32 to 1.68 μM is shown in Figure 3 for three runs. Each plateau in the signal versus time plot represents the increased amount of flow from channel 1 containing the fluorophore-doped buffer. The average signal of

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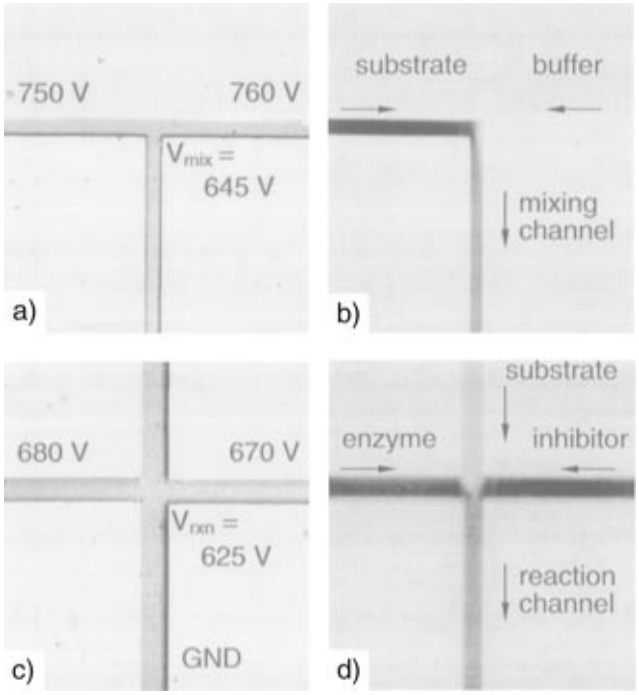

Figure 2. CCD images of (a) the mixing tee and (c) the four-way reaction cross under white-light illumination. The voltages applied to the reservoirs of each channel section are indicated; the two junction potentials are $V_{max} = 645$ V and $V_{rxn} = 625$ V. (b) The fluorescence signal of the electrokinetic flow and dilution of disodium fluorescein (shaded areas) into the mixing channel. (d) The mixing of disodium fluorescein solution from channels 3 and 4 with buffer from the mixing channel into the reaction cross. The arrows indicate the direction of electroosmotic flow, and the channels are labeled with the reagents used in the enzyme assay.

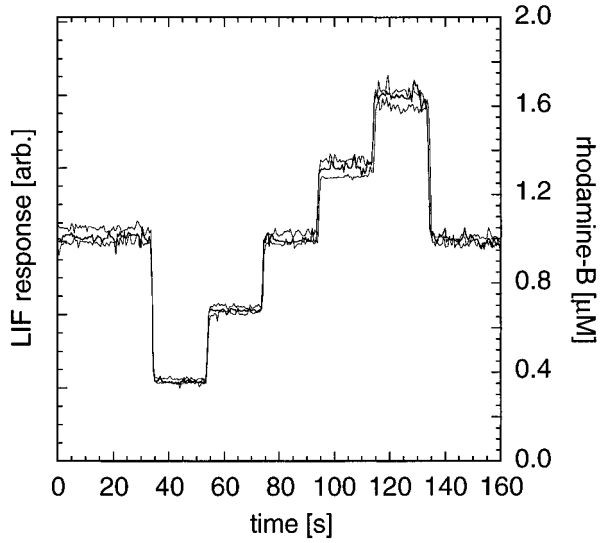

Figure 3. Fluorescence intensity versus time for three runs of a step-gradient increase from 0.32 to 1.68 μM rhodamine B. The left axis shows the fluorescence signal collected 20 mm downstream from the mixing cross, and the right axis shows the concentration of rhodamine B corresponding to each signal plateau.

each step in Figure 3 was compared with the fluorescence signals obtained by a manual calibration method. For the manual calibration curve, solutions of rhodamine B prepared off-chip were added to reservoirs 1 and 2 and the fluorescence signal was measured as in the on-chip dilution method. The two calibration curves agreed within an average of 2% RSD and confirmed the ability to control the flow of fluid within a channel network by changing the voltage applied at each reservoir.

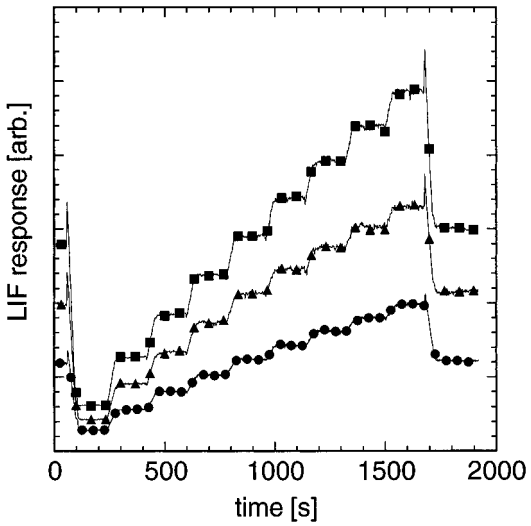

Figure 4. Fluorescence signal of resorufin obtained by mixing stepped-increments of RBG with (●) 190 , (▲) 370, and (■) 740 μg/L β-Gal. Each plateau represents a step increase of RBG from 14 to 122 μM. Every fiftieth data point is indicated with a symbol. Conditions: 100 mM Tris, 2.0 mM KCl, and 100 μM $MgCl_2$ pH 7.8 buffer at 21 °C.

Enzyme Assays. Enzyme kinetics were evaluated using the Michaelis−Menten equation:

$$v_o = \frac{V_{max}[S]}{K_m + [S]} \tag{2}$$

which relates the initial rate of the enzyme reaction, v_o, to the concentration of substrate, [S], a maximum rate, V_{max}, and an equilibrium constant, K_m. The equation describes a rectangular hyperbola in which the initial rate is first-order in substrate concentration when $[S] \ll K_m$ and zeroth order in substrate concentration when $[S] \gg K_m$. The K_m of the reaction is defined as the amount of substrate required for half-maximal velocity. The k_{cat} of an enzyme, a first-order rate constant, is related to the total enzyme concentration, $[E]_t$, by

$$k_{cat} = V_{max}/[E]_t \tag{3}$$

A Lineweaver−Burk plot of $1/v$ versus $1/[S]$, a linear form of eq 2, was used to calculate k_{cat} and K_m from initial rate versus substrate concentration data.

The enzyme kinetics were obtained by measuring the fluorescence signal of resorufin after a fixed mixing time determined by the electrokinetic flow rate in the reaction channel. In a typical assay (Figure 4), the final concentration of RBG was increased in equal increments from 14 to 122 μM RBG and mixed with enzyme for a final β-Gal concentration of 190, 370, or 740 μg/L. The enzyme concentration was changed by manually refilling reservoir 3 with a new enzyme concentration. . An increase in substrate concentration resulted in the gradual increase of product reaching a new plateau, the height of which was proportional to the rate of enzyme turnover for that substrate concentration. Each increment of RBG was monitored for 150 s, and the assay was completed in 20 min. At a flow rate of 14 nL/min, the assay consumed an

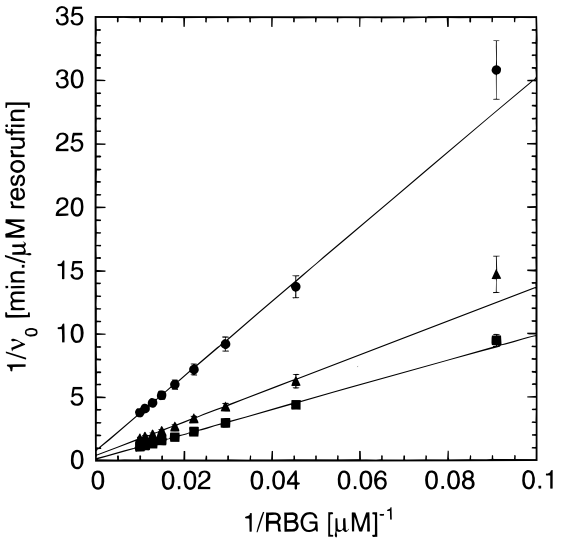

Figure 5. Lineweaver−Burk plot of the initial rate versus substrate concentration for (●) 190 , (▲) 370, and (■) 740 μg/L β-Gal.

Table 1. Derived Michaelis−Menten Constants Measured On-Chip with Three Concentrations of ***β***-Gal Compared with a Cuvette-Based Measurement

	concentration of β-Gal, μg/L			
	190	370	740	absorbance[a]
K_m (μM)	360 ± 100	320 ± 80	660 ± 200	550 ± 200
k_{cat} (s^{-1})	54 ± 20	54 ± 20	80 ± 20	70 ± 30

[a] Initial rates obtained by conventional absorbance assay of resorufin at 571 nm using 520 μg/L β-Gal.

average of 120 pg of β-Gal and 7.5 ng of RBG. The dwell time at each substrate concentration and the corresponding reagent consumption can be further reduced with additional refinement.

A Lineweaver−Burk plot using the initial rates calculated from the data in Figure 4 is shown in Figure 5. For a Lineweaver−Burk plot, the slope of the line is K_m/V_{max}, the y-intercept is $1/V_{max}$ and the extrapolated 1/[S] intercept is $-1/K_m$. Values for K_m and k_{cat}, derived from a weighted least-squares fit to the points shown in Figure 5, are summarized in Table 1 and compared with constants obtained using a conventional method. The average on-chip K_m value was 450 ± 200 μM, which compared well with the 550 ± 200 μM obtained using cuvettes. The turnover rate for the enzyme, k_{cat}, also agreed between the microchip and cuvette measured reaction. The values in Table 1 compare reasonably well with a literature value K_m of 380 μM but differ by a factor of 10 from a literature value k_{cat} of 700 s^{-1}.[25] For β-Gal, different rate constants using similar substrates have been obtained, due to differences in the pH, ionic strength, and the presence of activators,[24] which may account for the difference between the literature k_{cat} value and those listed in Table 1.

Reproducibility in the microchip enzyme assay was mainly affected by the solubility of the substrate, with minor contributions from protein absorption to the channel walls. In an ideal enzyme assay, a substrate concentration range is chosen to encompass values above and below the approximate K_m. In this experiment, saturation kinetics, in which the initial reaction rate approaches

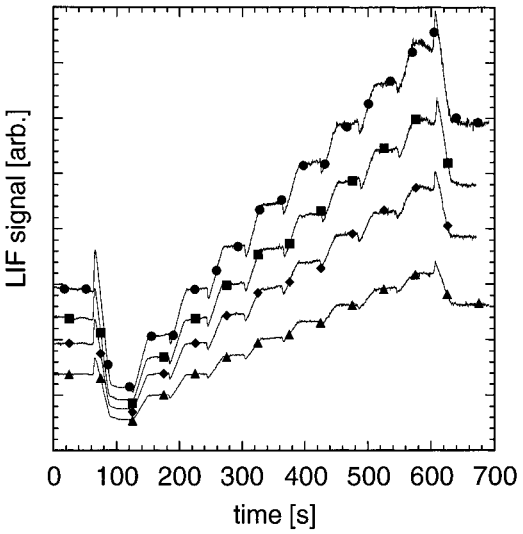

Figure 6. Fluorescence signal of resorufin produced in the PETG-inhibited hydrolysis of RBG and β-Gal. Each plateau represents increments of 14 μM RBG from 28 to 115 μM mixed with (●) buffer, (■) 6 μM PETG, (◆) 12 μM PETG, and (▲) 30 μM PETG. Symbols are shown for 0.5% of the data points. Conditions: 570 μg/L β-Gal for each reaction in 100 mM Tris, 2.0 mM KCl, and 100 μM $MgCl_2$ pH 7.8 buffer at 21 °C.

the maximum rate, could not be observed, because the maximum concentration of RBG was at least 3 times lower than the derived K_m. Therefore, the range of RBG concentration used in this study limited the run-to-run precision in the derived Michaelis−Menten constants.

Protein absorption to the channel walls contributed to an increase in the background signal of RBG. At low substrate concentrations, 28−42 μM RBG, the background signal was 50% of the signal produced in the enzyme-catalyzed reaction. The background signal increased from a starting value by 10−20% during the course of three runs, depending on the concentration of the enzyme and length of analysis time. An increase in the background signal during the course of an assay may have contributed to the observed deviation of the results for the lower substrate concentrations in the Lineweaver−Burk plot shown in Figure 5.

Inhibition of *β*-Galactosidase. Enzyme inhibition was evaluated using phenylethyl β-D-thiogalactoside, a competitive inhibitor of β-Gal. Three concentrations of PETG were mixed in the reaction cross with β-Gal, and the resulting decrease in resorufin production was monitored over a range of RBG concentrations. As the concentration of PETG increased, the initial reaction rate at each RBG concentration decreased, as shown in Figure 6. The PETG concentration was changed by manually refilling reservoir 4 with a new solution of PETG. Average values for the rate of resorufin produced for each concentration of RBG and PETG are shown in Figure 7 as a set of Lineweaver−Burk plots. The plots shown in Figure 7 are diagnostic for competitive inhibition and a K_i of 8 μM was derived by plotting the slope of each line versus the inhibitor concentration.[26] The K_i derived using RBG and the microchip assay is larger than the reported literature values, which range from 0.98 to 2.5 μM PETG.[27−29] A limited substrate

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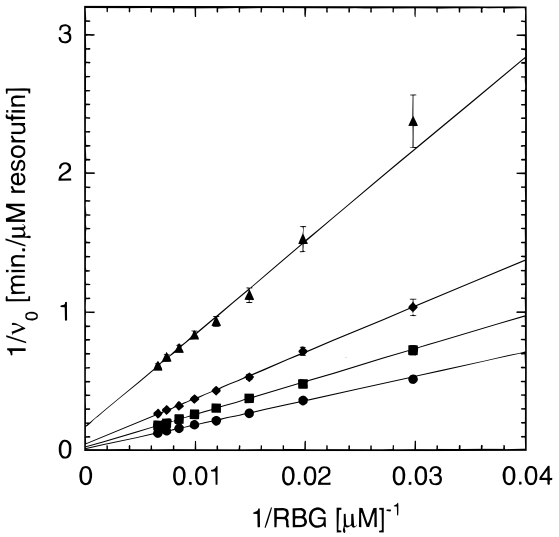

Figure 7. Lineweaver−Burk plot of the PETG-inhibited hydrolysis of RBG showing initial rate and substrate concentration for (●) no, (■) 6, (◆) 12, and (▲) 30 μM PETG.

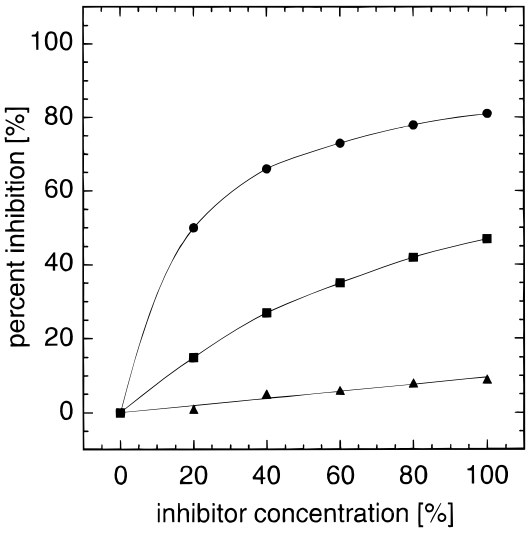

Figure 8. Effect of (●) 0−40 μM PETG, (■) 0−4.0 mM lactose, and (▲) 0−2 mM PHMB on the observed relative inhibition of the β-Gal-catalyzed hydrolysis of RBG. Each data point represents a 20% increase in the concentration of inhibitor ranging from 0 to 100%. Conditions: 100 μM RBG; 370 μg/L β-Gal.

concentration range, with the corresponding uncertainty in the derived V_{max}, may account for the discrepancy between this experiment and the reported values.

The relative effect of PETG, lactose, and PHMB on the inhibition of β-Gal was evaluated by varying the inhibitor concentration with constant substrate and enzyme concentration. The inhibitor was added to reservoir 1, diluted in the mixing channel, and mixed with a constant concentration of enzyme and substrate in the reaction cross. Signal versus time profiles similar to those in Figure 4 were obtained using 20% increments from 0 to 100% of the inhibitor concentration contained in reservoir 1. Percent inhibition, % I, was defined as

$$\% I = \frac{S_o - S_i}{S_o} \times 100 \qquad (4)$$

where S_o was the signal produced in the absence of an inhibitor, and S_i, the signal at the various inhibitor concentrations. Plots of % I in the range 0−4 mM lactose, 0−2 mM PHMB, and 0−40 μM PETG are shown in Figure 8 as a function of inhibitor concentration. By fitting the lactose and PETG inhibition curves to the Michaelis−Menten equation (eq 2), values for i_{50}, the inhibitor concentration required to give 50% inhibition, were determined. With 100 μM RBG, the i_{50} values were 7.2 μM for PETG and 4.2 mM for lactose. Since PHMB deactivates the enzyme by reacting with SH groups, only slight inhibition over the 0−2 mM range was observed due to the slower rate of inhibition. Previous inhibition studies of β-Gal have observed similar effects and concentration ranges for these compounds.[24]

CONCLUSIONS

An integrated microfabricated device that performs automated enzymatic assays was evaluated. Active and precise microfluidic control of reagent transport throughout the interconnected channel network was achieved using electrokinetic-induced motion. Computer control of the applied potential at each channel terminus allowed the various reagents to be combined in varying proportions while the extent of the reaction was monitored under continuous-flow conditions. The β-galactosidase-catalyzed hydrolysis of resorufin β-D-galactopyranoside was used as a model system for enzyme kinetic and inhibition determinations. This approach to enzyme assays allows automated studies to be completed with significant time savings and reduction of reagent consumption by more than 4 orders of magnitude while delivering results consistent with conventional approaches. Further decreases in measurement time and reagent consumption are possible. Highly parallel assays are also imaginable due to the small footprint of the microchip device. The channel network of the microchip also allowed for a rapid assessment of relative inhibition over a range of inhibitor concentrations. This technique represents the ability to rapidly study enzyme inhibition with small amounts of potentially expensive enzymes, substrates, or inhibitors.

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