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PAPER

Non-linear and linear enhancement of enzymatic reaction kinetics using a biomolecule concentrator†

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In this work we investigate concentration-enhanced enzyme activity assays in nanofluidic biomolecule concentrator chips which can be used to detect and study very low abundance enzymes from cell lysates and other low volume, low concentration samples. A mathematical model is developed for a mode of operation of the assay (J. H. Lee, B. D. Cosgrove, D. A. Lauffenburger and J. Han, *J. Am. Chem. Soc.*, 2009, **131**, 10340–10341) in which enzyme and substrate are concentrated together into a plug on chip which results in a non-linear enhancement of the reaction rate. Two reaction phases, an initial quadratic enzyme-limited phase and a later, linear substrate-limited phase, are predicted and then verified with experiments. It is determined that, in most practical situations, the reaction eventually enters a substrate-limited phase, therefore mitigating the concern for non-specific reactions of biosensor substrates with off-target enzymes in such assays. We also use this mode to demonstrate a multiplexed concentration-enhanced enzyme activity assay. We then propose and demonstrate a new device and mode of operation, in which only the enzyme is concentrated and then mixed with a fixed amount of substrate in an adjacent picolitre-scale reaction chamber. This mode results in a linear enhancement of the reaction rate and can be used to perform mechanistic studies on low abundance enzymes after concentrating them into a plug on chip.

Introduction

Enzyme catalyzed reactions are ubiquitous in nature and enzyme assays and the study of enzyme kinetics are essential in a wide range of scientific and technological domains such as biochemistry, medical diagnosis and biochemical engineering. Well-established methods for enzyme reactions such as micro-titer-plate based colorimetric and fluorimetric assays usually require large sample volumes ($\sim 100 \mu\text{L}$) and use relatively large amounts of the enzyme ($\sim 1 \text{ ng}$) per reaction. This presents a significant bottleneck in studying the kinetics of enzymes from precious samples such as those obtained directly from patients as well as from very low concentration samples such as a lysate from a single or a few cells. Monitoring the activities of various protein kinases, which play key roles in the cell signaling network, in

single cells can help in understanding the heterogeneity in their levels in cell populations.² Such cellular heterogeneity, which cannot be studied by the usual ensemble average measurements, is thought to cause incomplete sensitivity to chemotherapy in cancer.^{3,4} However none of the frequently used methods provide the sensitivity needed to measure activities from molecules contained in a single cell, especially once they are diluted in a volume ($\sim 1 \mu\text{L}$) that can be physically handled by micropipettes.⁵

Microfluidic systems significantly reduce required sample volume and assay time and also increase throughput of biochemical assays in general. Microfluidic enzyme kinetics studies that explore these opportunities have been reviewed earlier.⁶ Homogeneous reactions using reactants mixing while in flow^{7,8} or with stationary reactants mixed in isolated chambers⁹ or in water-in-oil droplets have been performed.¹⁰ In addition, heterogeneous reactions using surface-immobilized enzymes¹¹ or gel-immobilized enzymes¹² have also been studied in microfluidic systems.

Most microfluidic approaches to enzyme kinetics successfully reduce the sample volume required but still have limitations in terms of the minimum enzyme concentration/activity they can probe. This is due to unfavorable scaling stemming from the limited reaction volumes in these devices. The optical path length available in these devices for use with any optical detection method is at least an order of magnitude lower ($\sim 10 \mu\text{m}$ to $50 \mu\text{m}$ typically) than that in a micro-titer plate ($\sim 1 \text{ mm}$ or more). The

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high surface to volume ratio in microfluidics also results in relatively high non-specific surface-binding reactions, which can compete with or even overshadow the bulk reaction rate in very low volumes. Also, when using small sample volumes and with low analyte concentrations, the statistical variation in number of enzyme molecules in a given volume can be significant resulting in irreproducible results.

Our group has earlier demonstrated a novel nanofluidic concentrator¹³ that can be used to collect and trap charged molecules from a larger sample volume (~ 1 to $10\ \mu\text{L}$) into very small volume plugs (~ 10 to $100\ \text{pL}$) on chip using the electric field gradient formed due to ion concentration polarisation¹⁴ at the interface of a microchannel and a nanochannel¹³ or nanoporous membrane¹⁵ across which a voltage is applied. This technique can be used to tackle the above mentioned scaling problems in microfluidics as it results in a large increase in the local analyte concentration. Previously, we have employed such concentration-enhanced assays for protein immunoassay¹⁶ and enzyme assays^{1,15} yielding significant (~ 100 to 1000 fold) sensitivity gains without changing the biochemistry involved (*e.g.* quality of antibody) in the assay. In enzyme assays, the enhancement was obtained by mixing the sample containing the target enzyme with a fluorogenic substrate off-chip and then trapping both molecules from the mixture into such a plug on the chip using the concentrator.¹⁵ In these devices, the operation scheme of which is depicted in Fig. 1a, at the very low enzyme concentrations of interest, a significant reaction rate is observed only in the trapped plug. This plug, in effect, acts like a reaction chamber to which more reactant molecules are being continuously added by the incoming flow. Using this concentration-enhanced enzyme assay, two cellular kinase activities (MAPKAPK2 and Protein Kinase A) were measured directly from unfractionated cell lysates yielding a sensitivity good enough to measure the activity from a few cells.¹

While the sensitivity gains in this concentration-enhanced enzyme assay are desirable, this mode raises two valid questions. First, can one still extract important reaction parameters from this experiment, since the reaction in the accumulated plug would not be directly comparable to the usual isolated chamber reaction format as both reactants would be accumulating and reacting simultaneously with a potentially continuous turnover

of substrate. Second, in complex samples like cell lysates (especially in the kinase activity assay using the chemosensor Sox-substrates¹⁷) would there be interference or non-specific reaction between the fluorogenic substrate and non-target kinases, especially when the substrate might possibly get accumulated to higher levels than starting conditions. These questions necessitate a careful characterization to understand the differences between standard equilibrium reaction kinetics and concentration-enhanced enzyme reaction kinetics.

In this work we first study this unique concentration-enhanced reaction kinetic regime, which results in a non-linear enhancement of product formation rate. We develop a simple model for the reaction kinetics in the plug based on a modification of the standard Michaelis–Menten model and present an experimental verification of our model using a concentration-enhanced reaction of the widely used reporter enzyme β -Galactosidase with a fluorogenic substrate. We show that while the product formation rate is non-linear in time, a linear calibration curve from initial reaction rate to enzyme concentration is in fact expected and is experimentally obtained. This mode of enhancement is thus suitable for detecting very low activity levels with maximum amplification.

We also then propose and demonstrate a new scheme (depicted in Fig. 1b) which linearly enhances the enzymatic reaction rate by accumulating only the enzyme molecules and mixing the concentrated enzyme plug with a fixed amount of substrate and placing and observing the reaction mixture in a closed picolitre-scale reaction chamber on chip. We demonstrate that reaction kinetics in this scheme obeys the standard Michaelis–Menten model while still benefitting from the increased enzyme concentration. This mode of enhancement is suitable for mechanistic studies with low abundance enzymes such as cellular kinases as well as for applications such as studies of inhibitory action of drugs on them where fixed amounts of other agents can be introduced *via* the substrate inputs into the reaction chamber.

Experimental

Device fabrication

The integrated concentrator and reaction chamber device was fabricated using a standard two layer soft lithography protocol

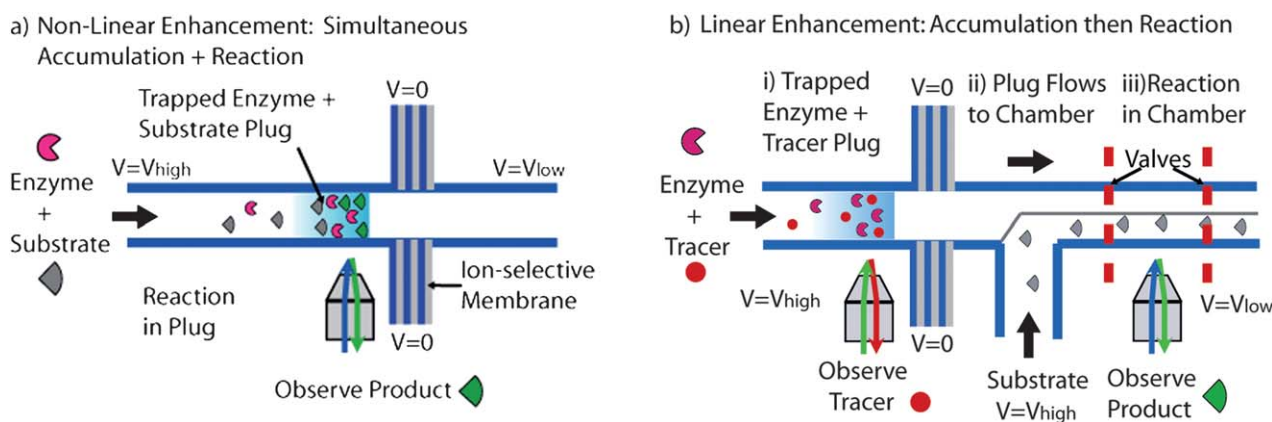


Fig. 1 (a) Non-linear enhancement mode of concentration-enhanced enzyme assay with simultaneous accumulation and reaction of reactants in the trapped plug. (b) Linear enhancement mode where only enzyme is accumulated into a plug and then mixed with a fixed amount of substrate in an integrated chamber where reaction occurs.

for making the PDMS channels and valves and a Nafion surface-patterning and sealing method reported earlier¹⁵ for making the concentrator membrane. These processes are described here in brief for completeness. A 10 μm tall AZ4620 positive photoresist was patterned on a 6 inch silicon wafer to make the mold for the flow layer of the PDMS device. This photoresist layer was reflowed for 30 minutes at 150 $^{\circ}\text{C}$ to yield rounded channels. A 15 μm tall SU-8 (SU-8 2015, Microchem Inc, Newton, MA) mold was made on another 6 inch silicon wafer for the pneumatic valve control layer of the device. Both the molds were silanized by placing them inside desiccators with a drop each of heptadecafluoro-1,1,2,2-tetra-hydrodecyl trimethoxysilane (Gelest Inc, Morrisville, PA) and venting the desiccators for 1 minute and maintaining the vacuum for 1 hour. A thin layer of mixed and degassed PDMS (Dow Corning Inc, Midland, MI, Sylgard 184, 20 : 1) was spin-coated at 2500 rpm on the flow-layer mold. A thicker layer of PDMS with a higher amount of curing agent (5 : 1) was poured on the valve-layer mold. Both PDMS layers were partially cured for 30 minutes at 65 $^{\circ}\text{C}$. The thicker valve-layer PDMS was then peeled from its mold and aligned to the flow layer on its mold under a microscope and brought into contact with it. After ensuring proper bubble-free contact, the mold was further cured overnight at 65 $^{\circ}\text{C}$ to obtain monolithic devices. The Nafion membrane was cast using a PDMS piece with a 50 μm tall and 50 μm wide straight microchannel with access holes which was reversibly sealed to a solvent-cleaned glass piece. A 20% alcohol suspension of Nafion (Sigma Aldrich Inc) was filled into this channel by venting one of the access holes. The casting PDMS piece was then carefully removed and the Nafion pattern was allowed to dry for 1 hour at room temperature. The cured monolithic PDMS piece with channels and valves was cut and peeled from the flow layer mold and access holes were punched into it using a biopsy punch. It was then exposed to plasma along with the glass piece bearing the patterned, dried Nafion membrane and both were aligned and brought into contact. The assembled devices were baked at 65 $^{\circ}\text{C}$ for at least an hour and were degassed for 15 minutes under vacuum before use. The active area of a fabricated device is shown in Fig. 2. Plastic pipette tips were attached to the access holes to act as reservoirs.

Materials

To demonstrate the use of the devices, the enzyme β -Galactosidase (β -Gal) (from *E. coli*) and the fluorogenic substrates fluorescein di- β -galactopyranoside (FDG) and resorufin β -D-galactopyranoside (RDG) (all from Sigma-Aldrich Inc) were used. B-Phycoerythrin (BPE) and Alexa-488 tagged bovine serum albumin (Invitrogen Inc) were used as fluorescent tracers for enzyme accumulation. $1\times$ PBS (pH = 7.4) and magnesium chloride were obtained from Sigma-Aldrich Inc and a reaction buffer with final concentration of 10 mM magnesium chloride in $0.01\times$ PBS was prepared. Stock solutions of both enzyme and substrate were diluted before use to the concentrations needed into this buffer. A 1% w/v solution of Bovine Serum Albumin (BSA) (Sigma-Aldrich Inc) in the same buffer was used for coating the channel surfaces before the experiment to reduce non-specific binding of proteins.

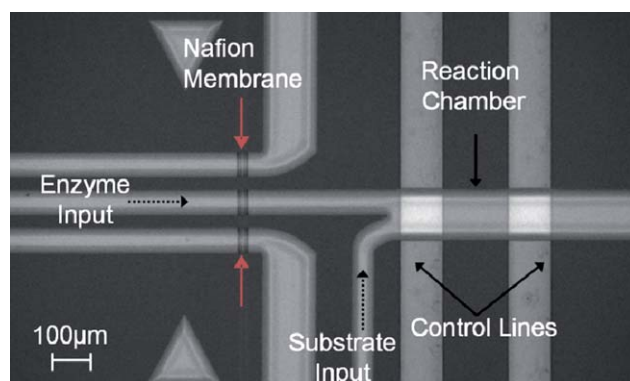


Fig. 2 Micrograph of the fabricated concentration-enhanced enzyme assay device which is used to implement both non-linear and linear enhancement mode experiments.

Measurement

An inverted epifluorescence microscope IX71 (Olympus, Melville, NY) equipped with a LED-based light source and electronic shutter (CoolLED Ltd, UK) and a thermoelectrically cooled CCD camera (Hamamatsu Co., Japan) was used for imaging. Chroma C38229 (Fluorescein) and Omega XF108-2 (Rhodamine) filter sets were used to observe green and red fluorescence emission respectively. Open source microscopy software, μ Manager (www.micromanager.org), was used for image acquisition and NIH ImageJ was used for image analysis. Fitting and plotting was done using MS Excel and programs written in MATLAB.

Results and discussion

Reaction kinetics model and simulation

We consider an enzyme catalyzed reaction where a substrate S is irreversibly converted to a product P in the presence of the enzyme E going through the bound intermediate state ES . With k_1 , k_{-1} and k_2 as the rate constants of the reactions, this reaction can be represented as:



In order to understand the kinetics of product formation in the trapped plug in the concentrator device and to compare it with standard closed system enzyme kinetics, we make a few simplifying assumptions about the device operation. The concentrator device is assumed to be able to trap all the incoming reactant molecules into the stationary plug without any losses while letting all carrier fluid *i.e.* water flow past the plug without a significant change in plug volume. The trapped plug is thus assumed to act as a reactor of constant volume to which enzyme and substrate molecules are continuously added by the incoming flow from the reservoir. The plug is also assumed to act as a well-mixed reactor. Further, the reaction rate in the input reservoir is assumed to be negligible so that the incoming reactant concentration remains constant. Note that this is expected to be valid at low enough input enzyme concentration which is the domain of application of this device.

Under these assumptions, representing the concentration of the species E by $[E]$ and so on, with $[E_0]$ and $[S_0]$ as the input concentrations of enzyme and substrate respectively in the reservoir and α as a proportionality factor representing the accumulation rate,[‡] the rate equations and mass conservation for total amount of enzyme can be written as:

$$\frac{d[E]}{dt} = -k_1[E][S] + (k_{-1} + k_2)[ES] + \alpha[E_0] \quad (1)$$

$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES] + \alpha[S_0] \quad (2)$$

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_2)[ES] \quad (3)$$

$$\frac{d[P]}{dt} = k_2[ES] \quad (4)$$

$$[E_T] = [E_0](1 + \alpha t) = [E] + [ES] \quad (5)$$

Here the terms that appear due to the accumulation into the plug are highlighted in bold font. We explore this model first by simplifying arguments to obtain the initial and final rates and then by numerical simulations to obtain the complete product formation curve.

For $\alpha = 0$, the above set of equations reduce to the standard model of enzyme kinetics in a closed system.¹⁸ As proposed by Briggs and Haldane,¹⁸ the enzyme–substrate binding step can be assumed to be quick and bound intermediate can be assumed to quickly reach a quasi-steady state ($\frac{d[ES]}{dt} \approx 0$). So, the initial product formation rate can be expressed in the standard Michaelis–Menten form:

$$\left. \frac{d[P]}{dt} \right|_{t=0} = k_2[E_0] \frac{[S_0]}{(k_{-1} + k_2)/k_1 + [S_0]} = v_{\text{MAX}} \frac{[S_0]}{K_M + [S_0]} \quad (6)$$

using eqn (3) and (4) where K_M and v_{MAX} are the Michaelis constant and the maximum reaction velocity respectively.

With non-zero accumulation rate α , we can similarly estimate the initial rate by assuming that enzyme–substrate binding is quick relative to the accumulation. The usual high initial substrate concentration ($[S_0] \gg [E_0]$) results in a proportionally high substrate accumulation rate which is expected to result in a quick rise of substrate concentration in the plug initially. So, although free enzyme is continuously added to the plug from the reservoir by the flow, almost all enzyme can be assumed to quickly bind with excess substrate in the plug and end up in the bound intermediate state as soon it arrives in the plug. Thus, in the plug:

$$[E] \approx 0 \Rightarrow [ES] \approx [E_T] = [E_0](1 + \alpha t) \quad (7)$$

Then using eqn (4):

$$\frac{d[P]}{dt} \approx k_2[E_0](1 + \alpha t) = v_{\text{MAX}}(1 + \alpha t) \quad (8)$$

Integrating this:

$$[P] \approx [P_0] + v_{\text{MAX}}t + \frac{1}{2}\alpha v_{\text{MAX}}t^2 \quad (9)$$

Thus, initially the product formation curve is expected to have a quadratic shape. In this initial phase, the rate of reaction is limited by the arrival rate of the free enzyme and we call this an *enzyme-limited* phase. In this phase:

$$\left. \frac{d[P]}{dt} \right|_{t=0} \approx v_{\text{MAX}} \quad \left. \frac{d^2[P]}{dt^2} \right|_{t=0} \approx \alpha v_{\text{MAX}} \quad (10)$$

Note from eqn (8) and (10) that in this phase the product formation rate is independent of substrate concentration but is linear in enzyme concentration with a slope which increases with the time. This is expected to make the device operating in this mode effective in detecting very low enzyme activities by using this amplification with time.

At later times as the enzyme molecules recycle while the substrate is irreversibly converted to product, an eventual excess of enzyme is expected to build up in the plug and all arriving substrate molecules quickly bind to excess enzyme as they arrive in the plug. So in this *substrate-limited* phase:

$$\frac{d[S]}{dt} \approx 0, \quad \frac{d[ES]}{dt} \approx 0 \quad (11)$$

Using eqn (11) along with eqn (1)–(5):

$$\left. \frac{d[P]}{dt} \right|_{t \rightarrow \infty} \approx \alpha[S_0] \quad (12)$$

This implies that at later times a linear product formation curve is expected.

We verify the validity of these simplifications by numerically solving the system eqn (1)–(5) of ordinary differential equations. Here, we integrated them as an initial value problem in MATLAB using the Runge-Kutta-(4,5) solver ode45. The results of these simulations for no accumulation, $\alpha[s^{-1}] = 0$ (with reaction parameters $k_1[\mu\text{M}^{-1}\text{s}^{-1}] = k_{-1}[s^{-1}] = 0.01$, $k_2[s^{-1}] = 1$) and initial values $[S_0] = 20\ \mu\text{M}$ and $[E_0] = 0.01\ \mu\text{M}$, $0.1\ \mu\text{M}$, which are experimentally reasonable starting conditions are shown in (red and magenta) product curves in Fig. 3a. The effect of initial enzyme concentration can be seen in this plot as at low enzyme concentration, product curve [P2] rises slow and remains linear within the simulation time window while at the higher enzyme concentration, product curve [P1] rises faster and levels off due to substrate depletion.

With accumulation, setting $\alpha = 1$,[‡] the results for the same initial conditions and reaction parameters as above are shown in Fig. 3b. Note from the different y-axis scale that the reaction product concentration rises much faster in this case. Also the (red) product curve clearly shows the quadratic and linear phases as argued above. Further, the initial high substrate concentration in the enzyme-limited phase and the later higher enzyme concentration in the substrate-limited phase can be clearly observed in the (blue) enzyme and (green) substrate concentration curves respectively (see Fig. S1† for further simulated curves at different initial substrate concentrations). This simulation

[‡] α , neglecting dispersion effects, is same for all species. It can be estimated as the ratio of the average linear flow velocity to the accumulated plug length and is expected to be ~ 0.5 under the experimental conditions used in the concentration-enhanced assay.

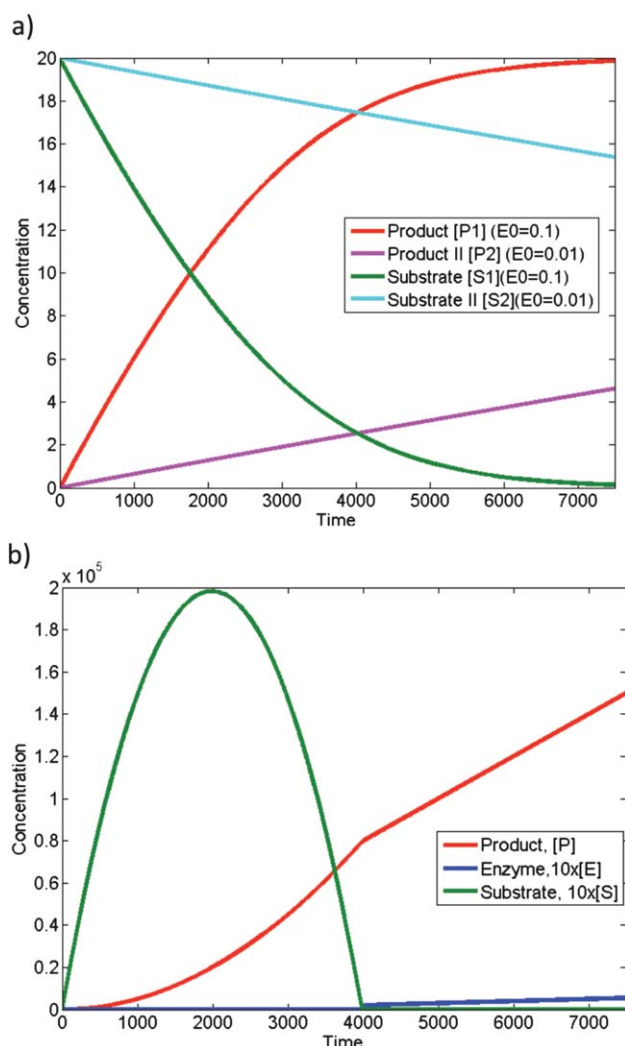


Fig. 3 (a) Numerical simulation results for an enzyme-substrate reaction modeled in the system of eqn (1)–(5) with *no accumulation* i.e. $\alpha = 0$, with parameter values $k_1 = k_{-1} = 0.01$ and $k_2 = 1$ and initial values $[E_0] = 0.01, 0.1$ and $[S_0] = 20$. (b) Enzyme-substrate reaction in the trapped plug with a *constant accumulation rate*, $\alpha = 1$ due to fluid bringing additional unreacted enzyme and substrate—with same parameter and initial values. Note the different y-axis scale and that in this case, for easier visualization, enzyme and substrate concentrations are scaled 10-fold and plotted.

result clearly agrees in expected shape—with an initial quadratic and later linear phase—with the analytical approximations made earlier. This further justifies our earlier simplifying assumptions and in fitting the experimental data we use the initial and final rate expressions (10) and (12) derived above.

Non-linear enhancement mode experiments

Non-linear enhancement experiments were performed in the device shown earlier in Fig. 2 by using only the enzyme input reservoir and channel and introducing enzyme and substrate mixed off-chip. In this experiment, the enzyme β -Galactosidase was mixed at a final concentration of $0.5 \mu\text{g mL}^{-1}$ with fluorogenic substrate fluorescein di- β -galactopyranoside (FDG) and the mixture was loaded into the enzyme input reservoir and

a gravity driven flow was established, controlled by a difference in fluid height between the enzyme input reservoir and the common output reservoir. A potential difference was then applied across the Nafion membrane by applying a voltage (10–25 V) to platinum electrodes dipped in the input and output reservoirs while grounding both the adjoining buffer channels. Both β -Galactosidase ($pI = 5.1$)¹⁹ and FDG²⁰ are expected to be negatively charged at this pH and hence expected to be trapped and accumulated in the electric field gradient zone formed near the membrane. β -Galactosidase catalyses the hydrolysis of non-fluorescent FDG to form the green fluorescent product fluorescein. A green fluorescent plug formed upstream of the membrane as shown in Fig. 4a. This region was observed at fixed intervals and the average fluorescence of the plug was measured and is plotted in Fig. 4b.

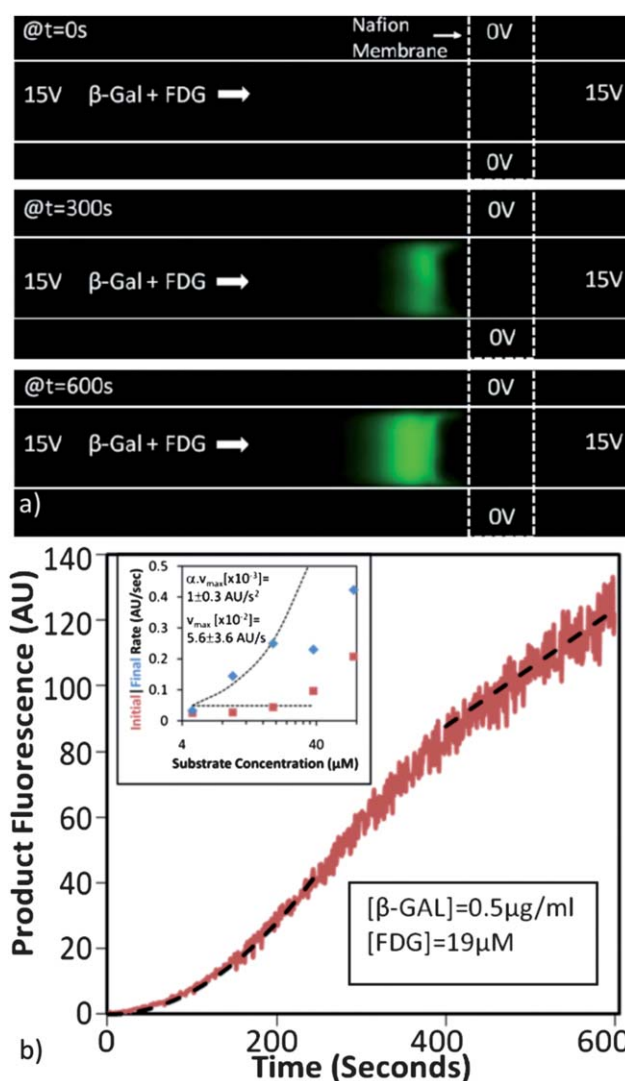


Fig. 4 (a) Fluorescence micrographs of trapped reaction plug at different time intervals after starting the reaction and accumulation. (b) Variation of the mean fluorescence of the plug with time. The two black dotted lines are quadratic and linear fits to the segments of data on which they are shown. The inset shows the variation of initial and final reaction rates with substrate concentration.

This product curve shows an initial non-linear phase, which was found to fit well to a quadratic polynomial in time, and a later linear phase. The plot in Fig. 4b also shows that the experimental product curve is in broad agreement to the model and simulation described above. Note that the final saturation phase that is expected in closed-chamber enzymatic reactions with fixed amounts of reactants, due to substrate depletion, is not observed even at long times in this case. This is because unconverted substrate is continuously flowed in from the reservoir which gets added to and converted in the plug without significantly diluting the existing enzyme concentration in the plug. This feature results in a continuously rising, very high amount of product fluorescence from the trapped enzyme which can be very useful in studies from very small amounts of enzyme such as from a single or few cells. The final limit on this continuous amplification may appear due to effects such as product inhibition that occur at very high product concentrations.

To further verify our understanding of the kinetics and the applicability of the asymptotic analytical model proposed for the initial enzyme-limited and final substrate-limited rates in eqn (9) and (12) respectively, the variation of these rates was studied with varying initial substrate concentrations (data shown in Fig. S2†). As seen in the inset of Fig. 4b, for a range of substrate concentrations ($[S_0] = 4.75 \mu\text{M}$ to $38 \mu\text{M}$), the initial rate (0–10 s) remains independent of substrate concentration as predicted by eqn (10) while the final rate (550–600 s) increases linearly with substrate concentration as predicted by eqn (12). Quadratic polynomials in time were fitted to the initial non-linear phases (0–200 s) of the product curves at different substrate concentrations and these were found to have constant curvatures as predicted by eqn (10). These polynomial fits (along with a fluorescence (AU) to product concentration (μM) calibration shown in Fig. S3†) were used to obtain the value of the parameters $\alpha\nu_{\text{MAX}} = 1.0 \pm 0.3 \times 10^{-3} \text{ AU s}^{-2} = 4.6 \pm 1.4 \mu\text{Ms}^{-1}$ from the quadratic term and $\nu_{\text{MAX}} = 5.6 \pm 3.6 \times 10^{-2} \text{ AU s}^{-1} = 21.2 \pm 16.6 \mu\text{Ms}^{-1}$ from the linear term. The higher error in the linear term is possibly due to differences in starting observation times in the different reactions. Similarly the later linear phase can be used to obtain $\alpha = 6.75 \times 10^{-2} \text{ s}^{-1}$. This is lower than the estimated value of $\alpha \approx 0.5$, possibly due to dispersion of molecules or a lower than estimated flow velocity due to channel height variation. This along with the earlier quadratic term then yields a lower estimate of $\nu_{\text{MAX}} = 1.5 \pm 0.4 \times 10^{-2} \text{ AU s}^{-1} = 6.9 \pm 1.8 \times 10^{-2} \mu\text{Ms}^{-1}$. At higher substrate concentrations ($[S] > 38 \mu\text{M}$) the product curves deviate significantly from this predicted form and become completely linear in time and the initial rates rise linearly with substrate concentration too. This could be because the assumption of very low reaction rate in input reservoir might break down at such high substrate concentrations and there may be a significant contribution of direct accumulation of product already formed in the reservoir into the observed plug adding to or overshadowing the reaction in the plug itself. This can be compared with the product accumulation device reported recently by Cheow *et al.*²¹ where the reaction always happens upstream of the plug at enzyme-coupled beads and linear product accumulation curves are obtained.

An important conclusion from this model and the above observations verifying it is that the concentration-enhanced

reaction in the accumulation plug ultimately enters a substrate-limited linear regime where adverse effects of excessive substrate accumulation—such as reactions with non-target kinases in cell lysates due to an uncontrolled substrate concentration—are not expected to happen.¹ Also the simple model still provides an opportunity to find reaction parameters such as ν_{MAX} from experimental data although within limits of relatively low substrate concentrations.

We also directly verified the linearity of initial reaction rates with enzyme concentration as predicted in eqn (8). To avoid variability due to different starting observation times and other device and experimental variations, we ran five simultaneous reactions using a 5-channel multiplexed version of the device reported earlier.¹² The results from this experiment are shown in Fig. 5. The input reservoirs of the five channels are loaded with the same volume of enzyme–substrate mixtures at the same substrate concentration but differing enzyme concentrations and the same potential is applied to all the channels and the five

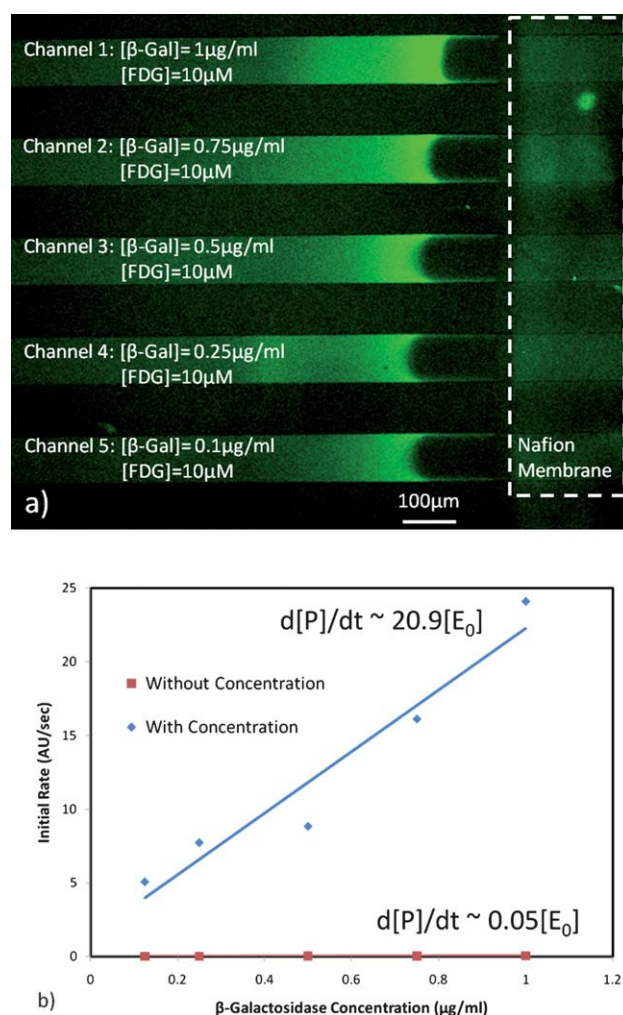


Fig. 5 (a) Five simultaneous reactions in the non-linear enhancement mode running in a multiplexed device with different enzyme concentrations. (b) Initial reaction rates *versus* enzyme concentration show that the rate rises linearly with enzyme concentration. The initial rate is enhanced $\sim 400\times$ at all the five enzyme concentrations when the potential is applied to accumulate the reactants and the product.

reaction plugs are observed simultaneously as shown in Fig. 5. The five product curves are analyzed as earlier and the initial rates of product formation with and without application of the trapping voltage are plotted in Fig. 5. A 400-fold enhancement in reaction rate is observed while linearity with enzyme concentration is maintained.

Linear enhancement mode experiments

To demonstrate the proposed linear enhancement mode, the enzyme and substrate solutions were loaded into separate input reservoirs attached to the two input channels of the device shown in Fig. 2. Only the enzyme would be accumulated into a plug in this case so the enzyme solution was spiked with a fluorescent tracer to make the plug visible. These experiments were performed with two different substrates: Fluorescein β -D-galactopyranoside (FDG) as described above and Resorufin β -D-galactopyranoside (RDG) which gets hydrolysed to red fluorescent product Resorufin. The tracer was chosen to have a different emission color than the fluorescent reaction product to avoid interference with later observation of the reaction progress. A red fluorescent protein, β -Phycoerythrin, was used as tracer for the FDG reaction while a green fluorescent protein, Alexa® 488 tagged BSA, was used as a tracer in the RDG reaction. Gravity-driven flows of enzyme and substrate were established in the device with identical pressure heads and the mixing of the two reactant streams could be observed in the reaction chamber both in the tracer fluorescence and in the fluorescence of the product as it formed at the mixing interface. As a control experiment without accumulation of the enzyme, the valves were closed in this condition by applying and holding air pressure using tubing attached to a syringe and the reaction was observed in the closed chamber using the product fluorescence filter set.

For observing the reaction with accumulation of the enzyme—a potential difference was applied across the Nafion membrane as earlier. To maintain identical flow of enzyme and substrate, the same voltage was also applied to the substrate input reservoir. During the accumulation, the region upstream of the membrane was observed using the tracer fluorescence filter set. A tracer plug formed near the membrane (Fig. 6a.i) and it grew in brightness over time as more of the tracer accumulated in the plug. After 20 minutes of accumulation, the plug was released by turning the voltage off and it was pushed downstream by the flow (Fig. 6a.ii). The plug met the substrate flow at the junction and moved into the reaction chamber region. When the plug was positioned in the reaction chamber, the valves were closed (Fig. 6a.iii) and the image acquisition was started under the product filter set with the same exposure time and frame rate as in the control experiment. The time variation of product fluorescence in the chamber is plotted in Fig. 6b along with that from the control experiment. We assume here that the enzyme plug is formed at the same position as the tracer plug and hence gets transferred to chamber along with it. This was experimentally verified by changing the position of the tracer plug relative to the valves before closing them and it was observed that the maximum reaction rate was obtained when the tracer plug was centered in the chamber. A 50-fold increase in the initial product formation rate is observed due to the increased enzyme

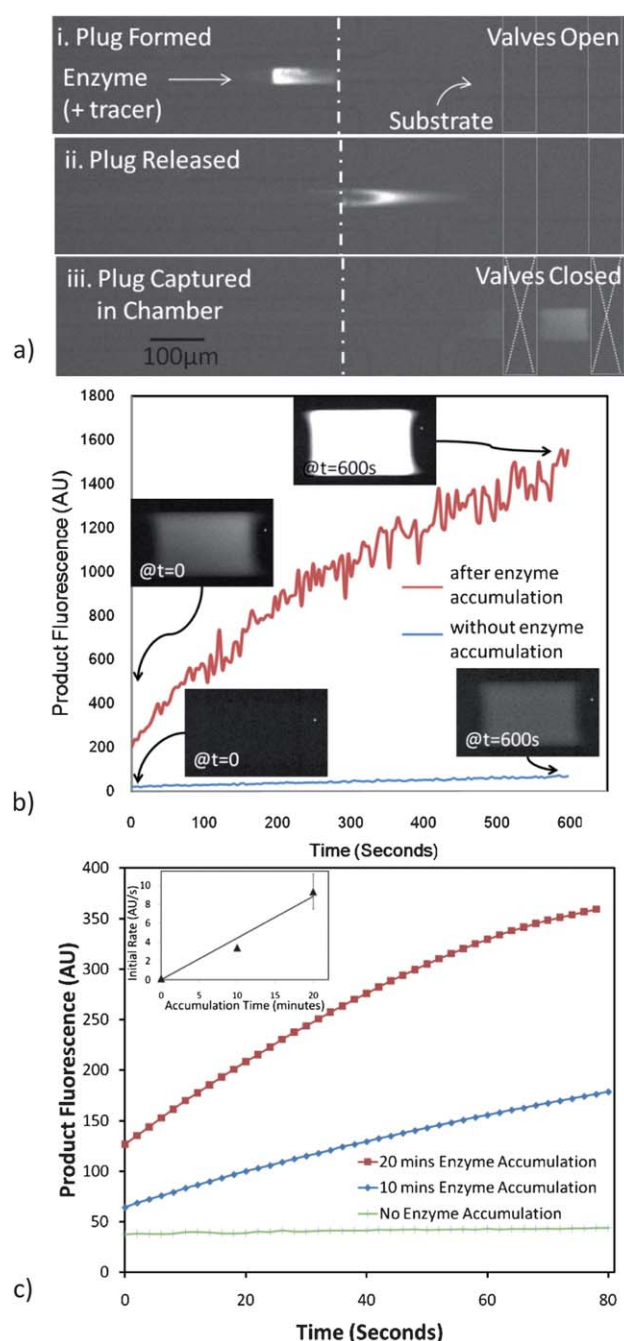


Fig. 6 (a) Fluorescence micrographs showing the operation of the device. (i) The enzyme and the tracer protein, B-Phycoerythrin, is concentrated into a plug by applying the voltage. (ii) The plug released by turning the voltage off and is driven by gravity driven flow to the reaction chamber. (iii) The pneumatic valves are actuated to capture the plug. (b) Variation of peak green fluorescence in the reaction chamber with time for the reaction between β -Galactosidase and FDG with and without accumulation of the enzyme. The insets show the reaction chamber at the beginning and end of the observation period. (c) Variation of peak red fluorescence in the reaction chamber with time for the reaction between the enzyme β -Galactosidase and RDG with different accumulation times of the enzyme.

concentration from the trapped plug. The control experiment has a very low amount of enzyme present in the chamber and hence the product curve obtained remains in the initial linear region (as

predicted in simulated product curve [P2] in Fig. 3a) till the end of the 10 minute observation period. However with the accumulated enzyme plug, the product curve shows the effect of higher enzyme concentration and we observe substrate depletion and the resultant reduction in reaction rate with time (as predicted in simulated curve [P1] in Fig. 3a). In this mode product curves are found to fit the standard Michaelis–Menten kinetics model (see Fig. S4† for details) as derived in eqn (6) from which the parameter K_M was extracted as $\sim 12 \mu\text{M}$ and $v_{\text{MAX}} \approx 6.9 \times 10^{-2} \mu\text{Ms}^{-1}$ which is within the range of reported values.²²

The enzyme concentration in the captured plug and hence the level of enhancement of the reaction rate can be tuned by changing the time for which the enzyme is accumulated before releasing it into the reaction chamber. The effect of accumulation time on the reaction rate is shown in Fig. 6b. Longer accumulation times yield higher reaction rates in the chamber due to higher concentration of enzyme in the incoming plug. The reaction rate variation with enzyme accumulation time is shown in the inset of Fig. 6c. Each data point here indicates the mean and standard deviation from three experiments. A 73-fold enhancement of reaction rate is obtained for 20 minutes of enzyme accumulation time. For a given accumulation time, the product curve obtained is repeatable over experiments as evidenced in the small variation in initial rates measured over three experiments.

A current limitation of this device in this mode is the reduction in enhancement due to the dispersion during transfer from the concentrator region to the reaction chamber which is evident in the tracer plugs seen in Fig. 6a. The dispersion is expected to decrease with the distance between these regions which is dependent on the alignment accuracy between the two-layers of PDMS during device fabrication which can be reduced by optimizing the fabrication process further.

Conclusions

In summary, we have demonstrated here that the nanofluidic biomolecule concentrator can be used in multiple ways to perform enzyme assays from low volume and/or low abundance samples. The enzyme reaction kinetics in these devices was studied and was found to obey simple models within certain limits of reactant concentrations. We showed that the simultaneous accumulation and reaction mode gives a high amplification factor with a product formation curve that is initially non-linearly rising in time but is linear in enzyme concentration. Finally at long enough times, this reaction mode enters a linearly rising phase which is explained in our model as a substrate-limited phase. The substrate concentration in the accumulated plug is maintained low in this phase and this enables ruling out non-target reactions of chemosensor substrates in complex mixtures such as cell lysates which might arise due to uncontrolled substrate concentrations. This allows maintaining the performance of the assay within designed limits in terms of cross-reactivity while continuously forming a large amount of product over time with a limited amount of trapped enzyme. This mode is thus well suited for detection of very low enzyme activities where a high reaction rate enhancement is the most desirable feature. We also showed the ability to multiplex these assays in the same

chip which leads to the possibility of higher throughput in concentration-enhanced assays. The new separate accumulation and reaction device offers a linearly enhanced reaction rate with a simpler product curve that replicates the standard kinetics observed in macro-scale isolated chamber assays. It offers the opportunity to perturb this kinetics—for example by adding drugs or other inhibitor molecules to the reaction chamber—and study the mechanism of reaction of low abundance enzyme molecules while maintaining an easy comparison with data from existing non-microfluidic reaction formats. It also offers the opportunity to study more complex mixtures where addition of fixed amounts of substrate maybe critical to the assays. More broadly, we have demonstrated the use of the biomolecule concentration technique as a world-chip interface which can be used to bring molecules from low concentration samples into highly concentrated very small volume plugs on chip and then manipulate and study them using flow and reaction.

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