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High-throughput enzyme assay on a multichannel microchip using optically gated sample introduction

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Abstract

To meet the requirements for high-throughput screening for drug discovery research, it is very important to develop techniques with the ability of performing multiple enzyme assays simultaneously. Using optically gated sample introduction on a multichannel microchip, multiple enzyme assays have been demonstrated in four parallel channels. The hydrolysis of fluorescein mono- β -D-galactopyranoside by β -galactosidase and the inhibition of this reaction by the competitive inhibitor phenylethyl β -D-thiogalactoside were initially studied to determine the effect of system movement using the voice coil actuator on the enzyme assay reaction. The results from these two studies are consistent with the results from the assay using a single-channel microchip, and they demonstrate that the system using optically gated sample introduction on multichannel microchip can be used to perform multiple enzyme assays. Three unique enzyme assays were also performed in different channels, which show this technique could be competitive for high-throughput screening in drug discovery with other traditional techniques.

Keywords

Enzyme reactions; Microchip; Multiple lanes

1 Introduction

Since their introduction in the 1990s, micro CE chips have become widely accepted and applied in different areas, including genomic analysis and proteomics [1-7]. Current microfabrication techniques allow multiplexing of microstructures, and thus different analysis steps on a single small device [8-12]. This ability has allowed the advancement of high-throughput analysis on CE chips. The low sample volume required for analysis on a CE chip also reduces analysis cost and makes the technique applicable to the analysis of extremely small volumes, such as that of a single cell. With all of these advantages, CE chips are ideal for high-throughput analysis of biological samples. Another area where sample volume is important is high-throughput screening for drug discovery following combinatorial synthesis.

In drug discovery, combinatorial libraries are commonly used to search potential drug candidates for future possible use [13]. Thousands of drug candidates are generated, which need to be tested for their inhibition properties against target enzymes or receptors and also for their interactions with other drug candidates for the same enzyme.

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Traditional enzyme assay methods for high-throughput screening of these drug candidates, including microtiter plate assay with detection on the plate or microtiter plate assay coupled to HPLC separations, have the ability to perform parallel analyses of enzyme assays. However, these methods are labor intensive and time consuming [14,15]. Additionally, the direct-plate assay method has signal interference problems, especially when a fluorescent substrate and product are involved [16].

Recently, CE chips have been applied to different enzyme assays using a single-separation channel [17-21]. These applications have shown the potential of the CE chip for enzyme assay. However, there are still challenges associated with this technique, such as signal interference between fluorescent substrate and fluorescent product generated during the assay, and noncontinuous injections on CE chips, which prevent continuous monitoring of the assays. Although high-throughput analysis for genetic analysis on CE chips has been widely applied [6,10,12], there has been no parallel for enzyme assays performed on microchips to meet the requirement for high-throughput screening.

Optically gated sample introduction has been developed as an alternative sample injection method on CE chips and has been successfully applied to rapid analysis of amino acids and oligonucleotides [22,23]. This method involves use of a split laser beam, one beam strong to photobleach material entering the channel and the other less intense beam to detect solutes by fluorescence following separation. The first beam acts to “inject” sample solutes by its brief (millisecond to second) removal allowing unbleached solutes to pass. This provides a very rapid injection time which is repeatable at high repetition rate. Recently, this sample introduction method also has been used in a single channel on a CE chip to perform rapid monitoring for the hydrolysis of fluorescein mono- β -D-galactopyranoside (FMG) by β -galactosidase (β -Gal) and to evaluate the effect of specific inhibitors on enzyme activity [24]. With the advantage of serial injections and separations, optically gated sample introduction has been used to continuously monitor enzyme reaction rates with little intervention from an operator and also avoiding signal interference between FMG and the reaction product, fluorescein. Results from the single-channel system are comparable to data in the literature, thus demonstrating the potential of optically gated sample introduction for enzyme assays. Another important advantage to the use of optically gated sample introduction on CE chips is that it allows for the fabrication of a single-chip device with a large number of separation channels. High-throughput analysis of amino acids and oligonucleotides has been successfully performed using parallel channels with this sample introduction method [11,25]. Thus, it is possible to perform multiple enzyme assays simultaneously with optically gated sample introduction to meet the requirement for high-throughput screening.

β -Gal is a very important tool in many biological applications [26,27]. FMG is a common fluorogenic substrate for β -Gal. The hydrolysis products of FMG catalyzed by β -Gal are fluorescein and galactose. Phenylethyl β -D-thiogalactoside (PETG) and lactose are two competitive inhibitors for the hydrolysis of FMG by β -Gal. As both the substrate, FMG, and the product, fluorescein, are fluorescent, interference when detecting either has been a problem for plate assay methods used to study this hydrolysis. This problem can be overcome by determining substrate or product concentrations after on-chip electrophoretic separation of FMG and fluorescein with optically gated sample introduction and LIF detection for FMG and fluorescein, as shown previously [24].

In this paper, the hydrolysis of FMG by β -Gal is used as a model to demonstrate the potential of taking the single-channel measurements to carry out multichannel microchip electrophoresis using optically gated sample introduction. Parallel enzyme assays on a single microchip are simultaneously performed using this technique. Initially, the hydrolysis and the inhibition of the hydrolysis by PETG are explored to examine the effect of the system on the enzyme

reactions. The results are compared with the results from a single-channel microchip. Additionally, multiple unique enzyme assays are demonstrated on a CE chip.

2 Materials and methods

2.1 Chemicals

All chemicals were obtained from Sigma Chemical (St. Louis, MO, USA) unless otherwise noted. FMG, fluorescein, and PETG were first dissolved in acetone (Burdick & Jackson, Muskegon, MI, USA). Stock solutions of β -Gal, fluorescein, FMG, PETG, and β -lactose were prepared in an analysis buffer of 100 mM phosphate and 1 mM MgCl_2 (pH 7.2; filtered; degassed). FMG and the enzyme solution were diluted with a solution of 10 mM phosphate and 1 mM MgCl_2 to the necessary concentrations daily before use.

2.2 Instrument and microchip device

The instrument setup and the microchip device were the same as the one used previously [25]. The beam from a 488-nm argon ion laser (Innova 70, Coherent, Santa Clara, CA, USA) was passed through a coated mirror (02WBK003/006 Melles Griot, Irvine, CA, USA) to be split into two beams, the gating beam and the probe beam. The gating beam (320 mW) was directed through an electronic shutter (UniBlitz, Vincent Associates, Rochester, NY) and focused by a lens ($f = 96.71$ mm, Newport, Irvine, CA) onto a microchip. The probe beam was passed through a collimator/expander (Melles Griot) and an objective (20 $\times$, Oriel, Stratford, CT, USA), which focused the beam onto the channel for LIF detection. The resulting fluorescence was collected by the objective and passed through a dichroic mirror (Chroma Technology, Brattleboro, VT, USA). The detection signal with a maximum of 540 nm was optically filtered with a 530 nm cut-off filter (51300, Oriel) and a bandpass filter (530DF30, Omega Optical, Brattleboro, VT, USA), then collected using a photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA). The collected signal was then amplified by a current amplifier (Keithley Instruments, Cleveland, OH, USA) and sent to a computer controlled data acquisition board (National Instruments, Austin, TX, USA).

The voice coil actuator (BEI, San Marcos, CA, USA) was used to move microchips back and forth at 10 Hz, as illustrated previously [11]. Microchip fabrication was based on the techniques discussed before [11]. The depth of each separation channel on the microchip was ~ 42 μm . The channel width was approximately 100 μm . The channel length for the straight area in the middle was 3 cm and the total length for the middle channel (third channel) was 8 cm. The diameter of the reservoirs was 2 mm. In the experiments discussed in this paper, the middle channel (third channel of five) on the microchip was left empty as the alignment channel.

2.3 Procedures

The enzyme assay procedures were similar to those described before [24]. The samples for analysis were mixed well before transferring to the reservoirs on microchip. After each experimental run, the separation channels on the microchip were electrophoretically cleaned for 10 min using NaOH (0.5 mM) followed by a 5-min cleaning with analysis buffer. The data analysis for multiple channel analysis was based on the method shown previously [11]. An in-house program written in Visual Basic was used to select and sort the data collected into groups to represent individual channels. Peak areas were integrated and analyzed using PeakFit v4.1 (SPSS, Chicago, IL, USA).

3 Results and discussion

3.1 Rapid enzyme assay

A schematic of the system setup for multichannel enzyme assay using optically gated sample introduction is shown in Fig. 1a. In this system, the two laser beams, the gating and the probe beam, are scanned through all the separation channels to perform multiple injections and detection by moving the microchip back and forth at 10 Hz using a voice coil actuator, as discussed before [11]. Therefore, the analytes in the reservoirs are vibrating during the whole experiment. The hydrolysis of FMG by β -Gal has been performed to check the effect of this kind of vibration on the enzyme reactions on a microchip.

Figure 1b shows separations obtained during parallel monitoring of the hydrolysis of FMG by β -Gal in four channels. To demonstrate different hydrolyses in different channels simultaneously, two different concentrations of FMG were used with the same enzyme concentration for each channel. A substrate concentration of 47.5 μ M FMG was used in channels 1 and 4, while 31.6 μ M FMG was used as substrate in channels 2 and 3. Nine serial injections were performed and nine separations were conducted for each channel. Similar to what was observed using the single-channel microchip, the height of the peak corresponding to the hydrolysis product, fluorescein, increases with reaction time [24]. It is also clear that the fluorescein peak height in channels 1 and 4 is relatively higher than the fluorescein peak height in channels 2 and 3 at the same reaction time. This is expected as a higher concentration of FMG was used in channels 1 and 4.

The peaks for fluorescein have been used to follow the rate of the enzymatic reaction. Fluorescein peak areas were integrated and corresponding initial reaction rates have been calculated for each channel. As expected, the average initial reaction rates for channels 1 and 4, which contained a higher concentration of FMG, were higher than the initial reaction rates for channels 2 and 3, which contained a lower concentration of FMG. The hydrolysis of FMG at different concentrations of substrate has been examined on the multichannel microchip using optically gated sample introduction. Initial reaction rates were plotted against the substrate concentration using the Lineweaver–Burk approach (Fig. 2). The kinetic constant, K_m , was obtained from this linear regression of $1/[\text{FMG}]$ versus $1/V_o$. The average K_m from three individual hydrolysis studies using a multichannel microchip and optically gated sample introduction was calculated to be 20.0 ± 2.2 μ M. Although this result appears slightly greater than the result from the single-channel microchip (17.2 ± 2.1 μ M), it is not significantly different based on a paired Student's *t*-test (*p* value = 0.3) [24]. This simple test demonstrates that the vibration of analytes due to the scanning of a microchip during the monitoring process does not affect the hydrolysis of FMG by β -Gal, and that other factors associated with the multichannel analysis system, including optical gating mechanisms, the enzyme reaction, and data analysis, are not fundamentally different from the single-channel device.

Inhibition studies also were explored with multichannel microchip to examine the effect of vibrations on enzyme reactions. Figure 3 shows parallel monitoring of enzyme reactions in four channels using optically gated sample introduction. In this figure, the hydrolysis of FMG by β -Gal without inhibitor has been monitored in channels 1 and 2. Channels 3 and 4 were used to monitor the hydrolysis of FMG in the presence of a competitive inhibitor of β -Gal, PETG. The evaluation of initial reaction rates for the reactions in these four channels shows that these rates decrease when the inhibitor PETG is present in the reaction mixture, as expected. The reaction rates at different substrate concentrations were plotted using the Lineweaver–Burk approach, and the result is shown in Fig. 4. The Michaelis–Menten constant, K_m , calculated with the enzyme inhibitor, PETG, present in channels 3 and 4 is 45.3 μ M. This is significantly higher than that observed for the uninhibited hydrolysis observed in channels 1 and 2 ($K_m = 21.6$ μ M) (*p* = 0.0006). This result is consistent with what has been reported

before for single-channel experiments [24]. In fact, the average value of K_m from three individual trials containing $4.56 \mu\text{M}$ PETG was $44.4 \pm 3.7 \mu\text{M}$. Statistical analysis demonstrates that this value is not significantly different ($p = 0.2$) from that obtained with the single-channel microchip ($K_m = 40.0 \pm 3.2 \mu\text{M}$) [24].

Interestingly, the amounts of the reaction compounds including enzymes, substrates, and inhibitors used for each reaction were all in the nanogram range, which is similar to other microchip studies and considerably lower than the amounts of reaction compounds used with direct-plate assays [17].

3.2 Multiple enzyme assays on a microchip

In Section 3.1, the potential for parallel monitoring of enzyme reactions with optically gated sample introduction on microchips was demonstrated. To meet the requirement for high throughput screening in drug inhibition studies, it is important to develop the ability to rapidly screen the effects of multiple inhibitors (drug candidates) on the action of a single enzyme or the effect of multiple enzymes with a single inhibitor simultaneously. Here, the uninhibited hydrolysis of FMG and the hydrolysis reaction in the presence of two different inhibitors were monitored simultaneously using optically gated sample introduction to demonstrate the potential of this technique (Fig. 5).

In this parallel analysis, three separation channels were used to continuously monitor the reactions for 7 min and 13 injections were performed in each separation channel. In Fig. 5, the first channel shows data from the uninhibited hydrolysis of FMG by β -Gal. The inhibition of the hydrolysis of FMG by lactose is studied in the second channel. The inhibition of the hydrolysis of FMG by PETG is studied in the third channel. It is very clear from Fig. 5 that more fluorescein is produced when the reaction is not inhibited (channel 1) than when compared to the fluorescein detected in channels 2 and 3 at same reaction time. This result is expected since there is no inhibitor present in the hydrolysis monitored in the first channel. For the hydrolysis inhibited by PETG and monitored in channel 3, fluorescein is not observed in the separation until the third injection and the area of the detected fluorescein peak is relatively lower than that detected in channels 1 and 2 for the corresponding separations. The relative initial reaction rates for these three channels are 0.0002 (channel 1), 0.00012 (channel 2), and 0.000095 (channel 3) Abs U/s, respectively. These results demonstrate that inhibition by both lactose and PETG in the reaction of the hydrolysis of FMG by β -Gal can be monitored with this type of analysis system. It is also very clear from these results that PETG is a better inhibitor of β -Gal than lactose, which is consistent with results presented in the literature [17,28] and also the results shown before [24].

4 Concluding remarks

In summary, rapid parallel monitoring of multiple enzyme assays has been successfully performed on a microchip with optically gated sample introduction. The hydrolysis of FMG by β -Gal in the presence of PETG provides kinetic data consistent with that reported previously [24]. These data show that moving the microchip to perform multiple injections and detection with optically gated sample introduction does not significantly affect enzyme reactions run on-chip. Additionally, multiple parallel inhibition studies have been used to demonstrate the potential of this technique for high-throughput screening of drugs as enzyme inhibitors. The ability to carry out parallel enzyme analyses will make this technique competitive with traditional plate-based assay methods.

Abbreviations

FMG, mono- β -D-galactopyranoside; β -Gal, β -galactosidase; PETG, phenylethyl β -D-thio-galactoside.

5 References

1. Manz A, Fetting JC, Verpoorte E, Ludi H, Widmer HM, Harrison DJ. Trends Anal. Chem 1991;10:144–149.
2. Auroux PA, Iossifidis D, Reyes DR, Manz A. Anal. Chem 2002;74:2637–2652. [PubMed: 12090654]
3. Figeys D, Pinto D. Anal. Chem 2000;72:330A–335A.
4. Khandurina J, Guttman A. J. Chromatogr. A 2002;943:159–183. [PubMed: 11833638]
5. Regnier FE, He B, Lin S, Busse J. Trends Biotechnol 1999;17:101–106. [PubMed: 10189715]
6. Carrilho E. Electrophoresis 2000;21:55–65. [PubMed: 10634470]
7. Chovan T, Guttman A. Trends Biotechnol 2002;20:116–122. [PubMed: 11841863]
8. Burns MA, Mastrangelo CH, Sammarco TS, Man FP, Webster JR, Johnson BN, Foerster B, Jones D, Fields Y, Kaiser AR, Burke DT. Proc. Natl. Acad. Sci. USA 1996;93:5556–5561. [PubMed: 8643614]
9. Shi YS, Peter C, Scherer JR, Wexler D, Skibola C, Smith MT, Mathies RA. Anal. Chem 1999;71:5354–5361. [PubMed: 10596215]
10. Medintz IL, Paegel BM, Blazej RG, Emrich CA, Berti L, Scherer JR, Mathies RA. Electrophoresis 2001;22:3845–3856. [PubMed: 11700713]
11. Xu H, Roddy TP, Lapos JA, Ewing AG. Anal. Chem 2002;74:5517–5522. [PubMed: 12433082]
12. Smith E, Xu H, Ewing AG. Electrophoresis 2001;22:363–370. [PubMed: 11288906]
13. Geysen HM, Schoenen F, Wagner D, Wagner R. Nat. Rev. Drug Discov 2003;2:222–230. [PubMed: 12612648]
14. Donato MT, Gomez-lechon MJ, Castell JV. Anal. Biochem 1993;213:29–33. [PubMed: 8238878]
15. Kennedy SW, Jones SP. Anal. Biochem 1994;222:217–223. [PubMed: 7856852]
16. Huang Z. Biochemistry 1991;30:8530–8534. [PubMed: 1909565]
17. Hadd AG, Raymond DE, Halliwell JW, Jacobson SC, Ramsey JM. Anal. Chem 1997;69:3407–3412. [PubMed: 9286159]
18. Hadd AG, Jacobson SC, Ramsey JM. Anal. Chem 1999;71:5206–5212.
19. Starkey DE, Han A, Bao JJ, Ahn CH, Wehmeyer KR, Prenger MC, Halsall HB, Heineman WR. J. Chromatogr. B 2001;762:33–41.
20. Wang J. Electrophoresis 2002;23:713–718. [PubMed: 11891703]
21. Cohen CB, Chin-Dixon E, Jeong S, Nikiforov TT. Anal. Biochem 1999;273:89–97. [PubMed: 10452803]
22. Lapos JA, Ewing AG. Anal. Chem 2000;72:4598–4602. [PubMed: 11028616]
23. Roddy ES, Lapos JA, Ewing AG. J. Chromatogr 2003;1004:217–224.
24. Xu H, Ewing AG. Anal. Bioanal. Chem 2004;378:1710–1715. [PubMed: 14634707]
25. Xu H, Roddy TP, Roddy ES, Lapos JA, Ewing AG. J. Sep. Sci 2004;27:7–12. [PubMed: 15335051]
26. Wallenfels, K.; Well, R. The Enzymes. 7. Academic Press; New York: 1972. p. 617–663.
27. Huang Z, Haugland RP, Szalecka D. Bio-Techniques 1992;13:543–546.
28. Lehninger, AL.; Nelson, DL.; Cox, MM. Principles of Biochemistry. Worth Publishers; New York: 1993.

Figure 1.

(a) Schematic showing a three-channel multichannel microchip (experiments in this manuscript use a five-channel chip) with a voice coil actuator to translate the chip under the laser beam. (b) Parallel monitoring of the hydrolysis of FMG at different concentrations. For channels 1 and 4, 47.5 μM FMG was used and 31.6 μM FMG was used for channels 2 and 3. Fluorescein peaks are identified by asterisks. Separation conditions: 500 V/cm separation field; 1 s injection time; 30 s delay between injections; and 3.35 mg/L β -Gal from bovine liver. Separation conditions were the same for all experiments shown in this chapter unless otherwise noted.

Figure 2. Lineweaver–Burk plot for the parallel hydrolysis of FMG by β -Gal from bovine liver. Fluorescein peak areas were integrated and plotted against reaction time. Slopes were calculated as the initial reaction rates (V_o). Kinetic constant, K_m , calculated from this plot is 20.9 μ M.

Figure 3.

Parallel monitoring of regular hydrolysis of FMG with or without the inhibitor PETG. Reactions monitored in channels 1 and 2 are without PETG and the reactions in channels 3 and 4 are the hydrolysis inhibited by PETG. Fluorescein peaks are identified by asterisks. Separation conditions: 63.1 μ M FMG used in all four channels, 4.56 μ M PETG in channels 3 and 4, no PETG in channels 1 and 2. Enzyme concentrations (3.35 mg/L) in each channel were the same. All reactions were monitored for 6 min simultaneously and 12 injections were performed for each separation channel.

Figure 4.

Lineweaver–Burk plots for the parallel hydrolysis of FMG and the enzymatic hydrolysis inhibited by PETG. Uninhibited hydrolysis (data shown with solid “◆” symbols) is shown in the bottom trace and the kinetic constant calculated from this line is $K_m = 21.6 \mu M$. Data for the hydrolysis of FMG inhibited by PETG (shown with solid “■” symbols) is shown in the top trace and the kinetic constant calculated from this plot is $K_m = 45.3 \mu M$.

Figure 5.

Parallel monitoring of three different enzyme assay conditions. First channel is the uninhibited hydrolysis of FMG (15.8 μM) by β -Gal from bovine liver. Second channel is the hydrolysis inhibited by 155.7 μM lactose. Third channel is the hydrolysis inhibited by 4.56 μM PETG. Fluorescein peaks are identified by asterisks.