MULTIPLEX ANALYSIS OF ENZYME KINETICS AND INHIBITION BY DROPLET MICROFLUIDICS USING PICOINJECTORS

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ABSTRACT

We present a novel microdroplet-based device for extensive characterization of the reaction kinetics of enzyme-inhibitor systems in a single experiment, for the first time utilizing droplet picoinjectors for bioanalysis. This device enables the scanning of multiple inhibitors, inhibitor concentrations and substrate conditions in a single, highly time resolved experiment yielding the Michaelis constant (K_m), the turnover number (K_{cat}) the mode of inhibition and the inhibitor enzyme binding constants (K_i, K_i'). Using this device we determine K_m and K_{cat} for β-galactosidase and the fluorogenic substrate Resorufin β-D-galactopyranoside (RBG) to 252 μM and 477 s^{-1}, respectively. Furthermore, we examine the inhibitory effects of Phenylethyl β-D-thiogalactopyranoside (PETG) on this system.

KEYWORDS

Enzyme kinetics, Enzyme inhibitors, Droplet microfluidics, High throughput.

INTRODUCTION

Enzyme kinetics and inhibition are important to a wide range of medical and industrial applications including drug development and biosustainable chemicals processing. Droplet microfluidics has many advantages for enzyme kinetic measurements as it enables high time resolution, large numbers of reactions and low sample volumes. A variety of droplet-based systems for enzyme kinetics characterization have been demonstrated[1,2]. Our system is unique in that it will enable characterization of enzyme-inhibitor systems over a range of substrate and inhibitor concentrations in picoliter reaction volumes concurrently.

EXPERIMENTAL

Microfluidic devices are manufactured in glass and Polydimethylsiloxane using standard soft lithography techniques with injected metal electrodes. The workflow includes two circuits (Figure 1A). The first circuit generates 23 pL droplets at rate of 1200 droplets per second, combining enzyme and various inhibitor concentrations fluorescently encoded by a red dye.

The second circuit includes an electric field controlled picoinjector module [3] where enzyme-inhibitor droplets are injected with a defined amount of fluorogenic substrate (Figure 2B). Droplets are subsequently incubated in a 170 s incubation channel with droplet-shuffling and measurement constrictions at regular intervals[4] (Figure 1B-C). Droplet fluorescence measurements utilize a 50 mW 491 nm laser and photomultiplier tubes detecting at at 525 and 593 nm.

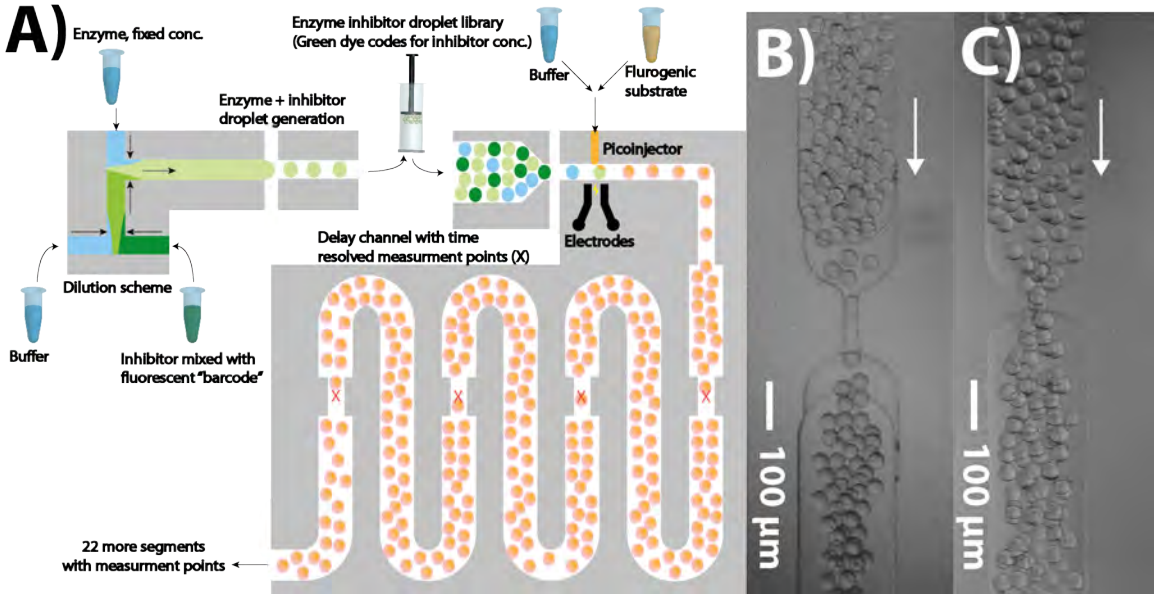


Figure 1. A. *Workflow schematic of enzyme kinetics and inhibition analysis. A library of droplets with a fixed enzyme concentration and a range of green dye barcoded inhibitor concentrations is generated. Droplets are transferred into the second device and injected with fluorogenic enzyme substrate. Droplet fluorescence (525 nm and 593 nm) is measured at defined intervals in the device (labeled x).* ***B-C.*** *Microscope images of the 90 μm deep and 200 μm wide incubation line.* ***B.*** *One of the 26 measurement points (width and depth 30 μm) , which constrict flow to allow single droplet resolution.* ***C.*** *One of 125 constrictions that mix droplets and thereby reduce spread in incubation time between droplets.*

RESULTS AND DISCUSSION

The syringe pump actuated substrate picoinjector unit was characterized by injecting pre-generated droplets containing the red fluorescent dye rhodamine B with the green fluorescent dye fluorescein and measuring droplet fluorescence post injection. Analysis of data confirms that all droplets which passed the picoinjector nozzle were injected with a defined quantity of fluorescein. By varying the flow rate on the picoinjector channel, different volumes of fluorescein solution could be injected into the droplets. The nominal injected volume fraction agreed well with the resulting fluorescence (Figure 2A-B). Furthermore, picoinjection was shown to be stable over time (>30 min) with a coefficient of variation of 4.4% in the resulting fluorescence when operating at ca 450 Hz.

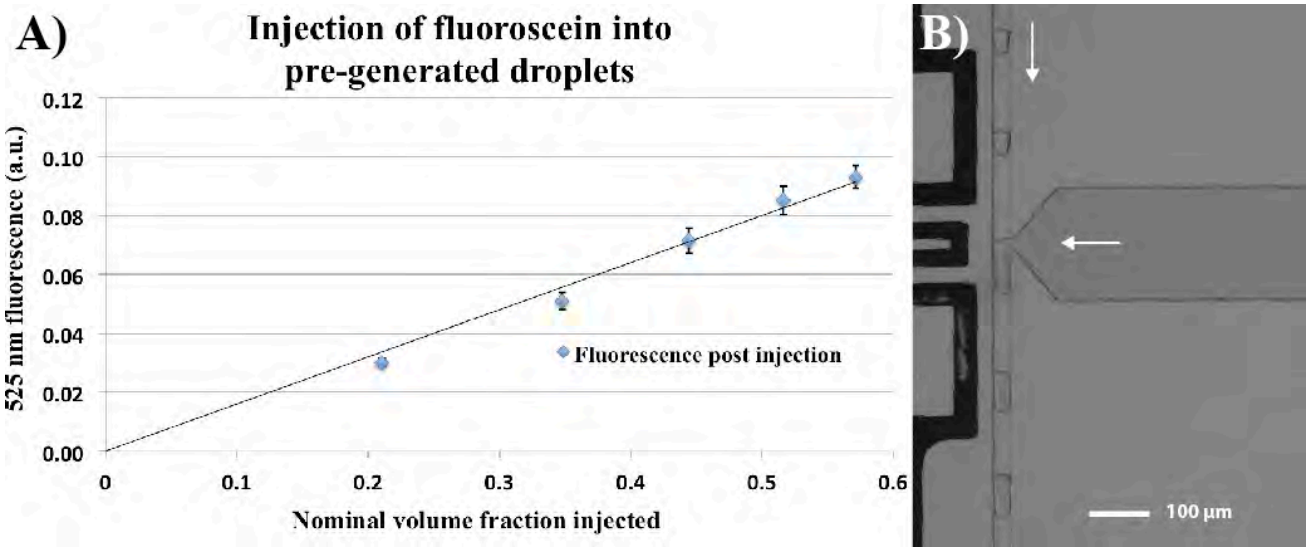


Figure 2. A. *The picoinjector was validated by injecting five different volumes of fluorescein solution into pre-generated droplets and measuring fluorescence post injection. Graphs shows nominal volume fraction injected (derived from the relative flow rates of the emulsion and the fluorescein solution) vs the fluorescence of the droplets post injection. Each data point represents the average fluorescence of ca 40 000 droplet events and error bars indicate one standard deviation.* ***B.*** *Microscope image of picoinjector. Droplets flow through the center channel (top to bottom) and a controlled amount of liquid is injected into each droplet from the side channel as they pass the nozzle.*

The multiplexing capabilities of droplet microfluidics for enzyme kinetics analysis was demonstrated by simultaneously assaying three green fluorescently barcoded droplet populations for the red fluorescent assay product (Figure 3).

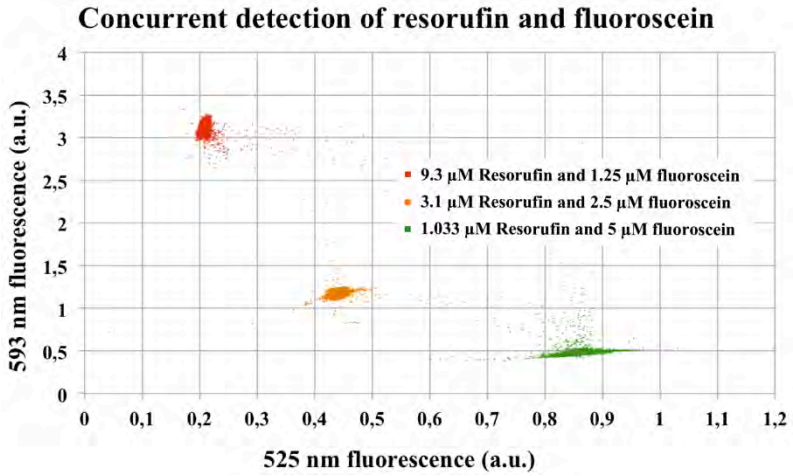
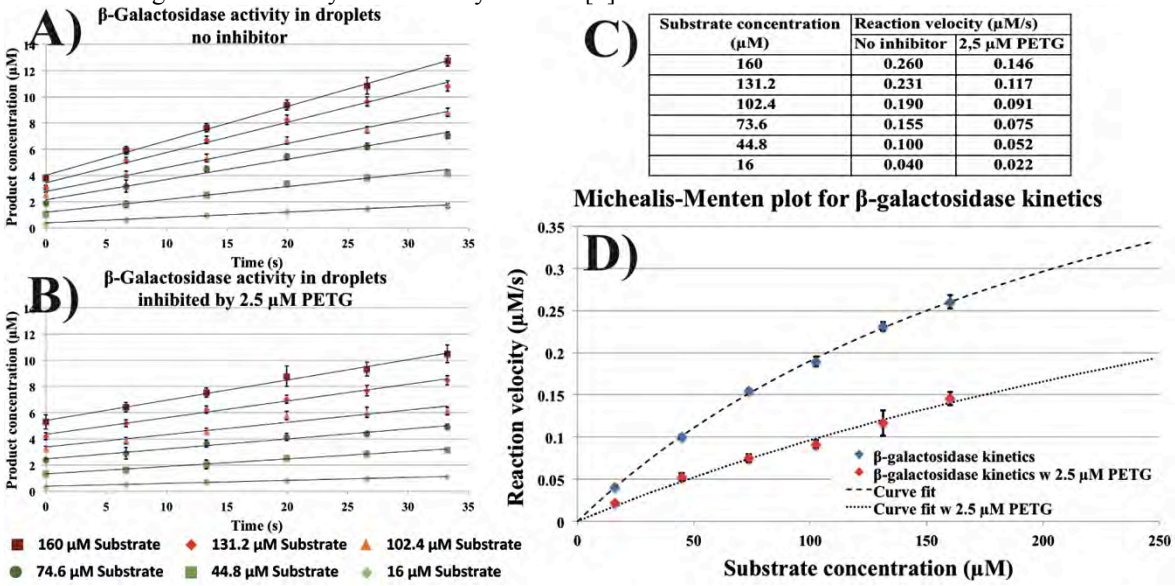


Figure 3. *Graph shows concurrent detection of three populations of droplets containing 9.3, 3.1 and 1.033 µM resorufin (red fluorescent) and 1.25, 2.5 and 5 µM fluoroscein (green fluorescent) respectively. Each dot represents one droplet event and the graph displays a total of 60 000 events.*

Droplets containing β-galactosidase were generated on-chip and enzyme kinetics measurements were performed using the microfluidic circuit with or without PETG, an inhibitor of β-galactosidase activity. The reaction progress was analyzed by droplet fluorescence interrogation at 6 points along the incubation channel i.e. every 6.7 seconds (Figure 4A-B). Reaction velocities were determined for six different concentrations of the enzyme substrate RBG.

Reaction velocity data was fit to the Michaelis-Menten equation (r^2=0.998) and enzyme kinetics parameters K_m and K_{cat} were found to be 252 µM and 477 s^{-1}, respectively, at a pH of 6.9 and room temperature. The experiment was repeated in presence of PETG and the value of K_i and K_i' was estimated as 2 µM and 20 µM (r^2=0.96), respectively, indicating that PETG is a competitive inhibitor (Figure 4C-D). One limitation in the determination of the kinetic parameters for the β-galactosidase and RBG system is the limited solubility of the enzyme substrate. Ideally, the assay should be performed at a range of substrate concentrations both above and below the K_m of the substrate but the concentration range was limited by the solubility of RBG [5].



Substrate concentration (µM)	Reaction velocity (µM/s)	
	No inhibitor	2,5 µM PETG
160	0.260	0.146
131.2	0.231	0.117
102.4	0.190	0.091
73.6	0.155	0.075
44.8	0.100	0.052
16	0.040	0.022

Figure 4. A-B. *Graph showing β-galactosidase reaction progress in droplets without inhibitor* ***(A)*** *and with 2.5µM PETG* ***(B)****. Each data point was derived from the average fluorescent intensity of >1500 droplets and error bars denote one standard deviation.* ***C****. Enzyme reaction velocities for the different substrate concentrations extracted from reaction progress data.* ***D.*** *Michaelis- Menten equation fit for β-galactosidase activity. Velocity measurement was done in triplicates and error bars denote one standard deviation. Enzyme constants K_{cat}=477 s^{-1}, K_m=252 µM, k_i=2 µM and k_i'=20 µM were extracted from fit.*

CONCLUSION AND OUTLOOK

We demonstrate a system for highly multiplex enzyme kinetics and inhibition analysis in picoliter microfluidic droplets. A picoinjector is used to add enzyme substrate to droplets and thereby initiate the enzymatic reaction at a specific location in the device. Using this system, we characterize β-galactosidase enzyme kinetic parameters and the inhibitory action of PETG on this enzyme. This system aims to increase the throughput of enzyme kinetics and inhibition measurements and to allow for massive multiplexing using several fluorescently barcoded inhibitors and inhibitor concentrations to benefit pharmaceutical research.

We are expanding the analysis to multiple enzyme-inhibitor systems, such as the effects of acarbose on α-amylase, performing enzyme kinetics characterization using libraries of dye-barcoded droplets with a range of inhibitors and inhibitor concentrations.

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