SINGLE-MOLECULE ENZYMATIC ANALYSIS IN A DROPLET-BASED MICROFLUIDIC SYSTEM

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ABSTRACT

The kinetic activity of individual enzyme molecules was determined in aqueous droplets generated in a nano- and microfluidic device. To avoid high background noise, the enzyme and substrate solution was confined into femtolitre carriers to achieve single-molecule encapsulation. The tiny droplets (φ~2.5-3 µm) generated from this fluidic system were highly monodisperse, beneficial for an analysis of single enzyme activity. Single-enzyme kinetics has previously been demonstrated in the microfluidic format in PDMS containers [1], surface-immobilized droplets [2], or liposomes [3]. Single-enzyme analysis in the droplet-based microfluidics was reported before [4] by encapsulating highly-diluted enzyme solution (110 fM) into large droplets (φ~40 µm). But to our knowledge this is the first demonstration of the direct method of single enzyme encapsulation and analysis at high enzyme concentration in tiny droplets in a microfluidic system.

KEYWORDS: Microfluidics, Nanofluidics, Single Molecular Analysis, Enzyme, Droplet.

INTRODUCTION

In this work, we employ a droplet-based microfluidic technology for single-enzyme analysis by means of the encapsulation of molecules of enzyme (β-glucosidase) and substrate (fluorescein-β-D-glucopyranoside; FDGlu) into tiny aqueous carriers in oil phase to produce fluorescence product molecules. The increase in fluorescence intensity was used to determine the kinetic activity of this enzyme. To reduce the background noise from the solvent and oil molecules and the substrate material, the aqueous solution containing enzyme and substrate molecules was compartmented as femtolitre carriers by use of a T-junction nanofluidic network. The increasing product molecule was later observed from the array of droplets in an outlet channel, consisting of a nanochannel and microchannel section (Fig.1). When the generated droplets, which were squeezed by the top and bottom walls of nanochannels, entered the outlet microchannel they transformed to spherical shape which is more stable than the squeezed shape for prolonged measurement.

Figure 1: Schematic of the droplet generator using a micro-and nanofluidic network. The depths of nano channel and microchannel are around 500 nm and 3 µm, respectively.

EXPERIMENTAL

The micro- and nanofluidic system was produced from Borofloat glass substrate by a standard etching technique and hydrophobized using a published procedure [4]. A dispersed phase containing β-glucosidase (0.1 nM), FDGlu (200 µM) in PBS solution (pH 7) and a continuous oil phase (silicone oil with 4% Span80) were individually loaded into the fluidic system by a syringe pump (Fig.1). After a large amount of generated droplets entered the outlet channel, the flows of aqueous and oil solutions were stopped. Subsequently, the array of droplets was imaged by using a high sensitive EMCCD camera. The fluorescence intensities in each droplet were later determined by software. The data from droplets ranging from 2.5–3 µm in diameter were counted for this analysis. In the experiment in the presence of an anti-oxidant reagent, (n-propyl gallate; nPG, 5% w/v) was added to the aqueous solution before injection to the fluidic device.

RESULTS AND DISCUSSION

Enzyme kinetic activity in the presence of n-propyl gallate:

After droplet formation, images of the array of droplets containing the enzyme and substrate solution after the reaction had proceeded were exemplified in Fig.2 B and C. The fluorescence intensity of a single droplet is plotted against time in Fig. 2 A. The signal from the droplets initially remained constant during a time labeled as “delay time”. After this delay time, the fluorescence intensity increased proportionally to the incubation time. The rate of increase of fluorescence intensity was then used to determine the kinetic activity of the enzymatic reaction. The increasing fluorescence intensity in

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each droplet between minutes 1200 and 1335 is plotted in a histogram (Fig. 2D) revealing the increasing intensity is periodically enhanced and can be categorized into four peaks which we interpreted as 0, 1, 2 or 3 enzyme molecules encapsulated in the droplets. The resulting $K_{\text{cat}}$ is around 1.6 molecule of product per second. The histogram of the enzyme occupancy fits well to a Poisson distribution (Fig. 2D: Inset), with a mean occupancy $\lambda$ of 0.7 molecules/droplet while the occupancy calculated from the droplets is around 0.9 molecules/droplet. These data strongly suggest a successful encapsulation of single enzymes into the droplets. The $K_{\text{cat}}$ obtained from our droplet-based device (1.6 sec$^{-1}$) is significantly lower than that from the bulk experiment (5.7 sec$^{-1}$) on the same solution determined by a fluorescence spectrometer.

**Figure 2.** (A) The evolution of the fluorescence intensity from the enzymatic reaction in the presence of nPG. In the first part labeled as ‘delay time’, the signal almost remains constant with a few fluctuation. In the second part, the fluorescence intensity increases proportionally to the incubation time and is used to determine the enzyme kinetics. Bright-field image of the array of droplets (B); Fluorescence image of the same system after incubation for 1335 minutes (C). (D) Histogram of the increasing fluorescence intensity from the 1200th to the 1335th minute. Four peaks indicate the occupancy of droplets by (0, 1, 2 and 3) enzymes. (D: Inset) Distribution of the occupancy of enzyme in each droplet (bar chart) corresponding to the Poisson distribution with a mean occupancy $\lambda = 0.7$ (dash line).

**Enzyme kinetic activity in the absence of n-propyl gallate**

In this experiment, the aqueous solution without added nPG was loaded into a fluidic platform to generate droplets. The evolution of the fluorescence intensity recorded is illustrated in Fig. 3A. A shorter delay time was observed as compared to the previous experiment. The increasing fluorescence intensity and hence the enhancement of fluorescence product in each droplet from the 240th to the 260th minute is plotted in a histogram (Fig. 3D) showing the fluorescein concentration was seen to be periodically increased in four peaks which can be interpreted as corresponding to different numbers of enzymes encapsulated into the droplets. The obtained $K_{\text{cat}}$ from these experiments was around 3.8 sec$^{-1}$.

**Figure 3.** The evolution of the fluorescence intensity from the enzymatic reaction in the absence of nPG (A). Snapshots of the array of droplets containing enzyme and substrate molecules without the addition of nPG at minute 240 (B), and 260 (C) are shown. (D and E) The histograms of increasing fluorescence intensity when the enzyme solution was 0.1 and 0.2 nM, respectively. Four peaks can be interpreted as indicating the occupancy of droplets by (0, 1, 2 and 3) enzymes. (F) Two histograms express the distribution of the occupancy of enzymes (0, 1, 2 and 3) per droplet. The dark grey bar and the light grey bar depict the data distribution when the enzyme concentration was 0.1 and 0.2 nM, respectively, which were fitted to a Poisson distribution as shown by a dotted line and a solid line, respectively, with a mean occupancy $\lambda$ of 0.62 and 1.24, respectively.
To confirm this single-enzyme encapsulation and our interpretation, another experiment was performed using a two-fold greater concentration of the enzyme (0.2 nM) than that in the previous experiment. The histogram of the increasing concentration of fluorescein in each droplet is plotted in Fig.3 E and the resulting $K_{cat}$ was around 4.5 sec$^{-1}$. In Fig.3 F, the experimental data are plotted as fractional occurrence of droplets with enzyme occupancy (0, 1, 2 or 3) using bars of dark grey and light grey for the 0.1 and 0.2 nM of enzyme solution, respectively. As shown, the histograms for both experiments can be well fitted to Poisson distributions with mean occupancies $\lambda$ of 0.62 and 1.24. Considering the amount of enzyme encapsulated into one droplet by calculation, we found the averaged enzyme occupancies are around 0.7 and 1.4 molecules for the 0.1 and 0.2 nM of enzyme solution, respectively. These calculated values correspond well to the Poisson fitting curve. These data indicate that single-enzyme encapsulation was obtained into the tiny aqueous droplets generated in our device. The kinetic activity of the encapsulated enzymes (3.8-4.5 sec$^{-1}$) was lower than the kinetic activity observed in bulk experiments (5.7 sec$^{-1}$), but much higher than the kinetic activities observed in the presence of nPG as will be further discussed in the following section.

DISCUSSION

The kinetic activity obtained in droplets in our device is somewhat lower than the value obtained from a bulk experiment. This observation corresponds to several works reported before mentioning the kinetic activity obtained in droplets in microfluidic devices were marginally lower than in bulk experiments [5-6]. Considering the cause of the observed delay time, it was initially speculated to be the accumulation time of the fluorescein molecules until it overcame the background noise. A rough estimate of the duration of this delay time from the lowest signal of fluorescein solution and resulting $K_{cat}$ (3.8 sec$^{-1}$) results however in a delay time of 30 minutes. However, it prolonged to 230th minute in our experiment. Therefore the main cause of the delay must be another phenomena. A possible explanation is that this delay time occurs because the produced fluorescein molecules vanished due to some process which in time becomes saturated resulting in an increasing fluorescence. From our knowledge, two possible processes are (i) the adsorption of fluorescein molecule at the water and oil interface and (ii) the diffusion of the fluorescein into the oil phase.

The measured kinetic activity in the presence of nPG (1.6 sec$^{-1}$) was lower than that in its absence (3.8-4.5 sec$^{-1}$). We speculate that nPG might inhibit or disturb the interaction between the enzyme and the substrate molecules since the structure of nPG is somewhat similar to one side of the structure of cellubiose which is a natural substrate of beta-glucosidase. On the other hand, in the experiment without nPG, the fluorescein molecules might undergo photobleaching. Indeed, the photobleaching effect might be less pronounced in our experiment since the life time of the fluorescein molecule under illumination from our setup is around 5 minutes (data not shown). The UV light was, however, illuminated for a few seconds for every single snapshot taken. Else, this photobleaching effect might be the reason of the relative low value of the kinetic activity from our experiment. The effect of photobleaching should be further investigated by experiments with other anti-oxidant reagents.

CONCLUSION

We have demonstrated a micro- and nanofluidic platform for creating droplet arrays encapsulating single enzyme molecules. Femtolitre droplets need to be generated to reduce the background noise from environment. The histogram of the rate of fluorescence intensity increase and thus the turnover number of the enzymatic reaction in each droplet showed periodically-increased peaks revealing the occupancy of droplets by multiples of a single enzyme. The distributions fitted well to a Poisson distribution verifying the achieved encapsulation of single enzymes. The resulting kinetic activity was somewhat lower than that from the bulk experiment.

REFERENCES


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