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A Digital Microfluidic Approach to Homogeneous Enzyme Assays

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A digital microfluidic device was applied to a variety of enzymatic analyses. The digital approach to microfluidics manipulates samples and reagents in the form of discrete droplets, as opposed to the streams of fluid used in channel microfluidics. This approach is more easily reconfigured than a channel device, and the flexibility of these devices makes them suitable for a wide variety of applications. Alkaline phosphatase was chosen as a model enzyme and used to convert fluorescein diphosphate into fluorescein. Droplets of alkaline phosphatase and fluorescein diphosphate were merged and mixed on the device, resulting in a 140-nL, stopped-flow reaction chamber in which the fluorescent product was detected by a fluorescence plate reader. Substrate quantitation was achieved with a linear range of 2 orders of magnitude and a detection limit of $\sim 7.0 \times 10^{-20}$ mol. Addition of a small amount of a nonionic surfactant to the reaction buffer was shown to reduce the adsorption of enzyme to the device surface and extend the lifetime of the device without affecting the enzyme activity. Analyses of the enzyme kinetics and the effects of inhibition with inorganic phosphate were performed, and K_m and k_{cat} values of 1.35 μM and 120 s^{-1}, respectively, agreed with those obtained in a conventional 384-well plate under the same conditions (1.85 μM and 155 s^{-1}). A phototype device was also developed to perform multiplexed enzyme analyses. It was concluded that the digital microfluidic format is able to perform detailed and reproducible assays of substrate concentrations and enzyme activity in much smaller reaction volumes and with higher sensitivity than conventional methods.

Enzymatic assays are a key component in a variety of applications, such as glucose monitoring in serum[1] and screening for phenols[2] or organophosphates[3] in drinking water. Relative to other analytical techniques such as separations and mass spectrometry, enzyme assays can be used to detect or quantify the concentrations of small molecules in a format that is far more compact, portable, and easy-to-use. In addition, enzyme assays are uniquely suited for analyzing the activity of proteins (instead of merely detecting them). A challenge for enzyme assays is in analytical capacity and sensitivity—several different analyses may be required to characterize a highly complex sample (e.g., a cellular extract), and many replicates of each analysis must be performed to ensure the reproducibility of the data. As the number of analyses being performed increases, the cost of reagents and the amount of sample required (often available in limited quantities) become important factors. The technology of microfluidics, which can be used to examine many samples in parallel while consuming smaller amounts of samples and reagents than is required for conventional-scale methods, has the potential to make enzyme assays indispensable for a wide range of applications.[4]

Most microfluidic enzyme assays have been implemented via the channel microfluidic format, in which reagents and samples are transported through a system of enclosed micrometer-dimension channels as streams of fluid. Hadd et al.[5,6] performed some of the first enzyme assays in microchannels in a continuous-flow format and demonstrated a 4 order of magnitude reduction in enzyme and substrate consumption over conventional methods. However, this system relied on diffusional mixing, and reaction times of up to 20 min were required to obtain accurate kinetic data. With the help of a microfabricated mixer, Burke et al.[7] conducted a stopped-flow enzyme assay that consumed only 6 nL of enzyme and required less than 60 s to complete. While this approach greatly reduces reagent consumption and analysis times over macroscale methods, fabrication methods for such systems are often expensive and time-consuming.[8] Once a particular arrangement of channels and reaction chambers is designed, a great deal of time and expense is required to change the device to suit a different application. The fabrication of multiplexed devices becomes a daunting prospect when features such as mixers must be incorporated, as even the simplest mixing schemes require an integrated network of many smaller features such as channels[9] or raised ridges.[10]

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An alternative paradigm for microfluidics has recently emerged, in which small volumes of fluid are manipulated in the form of droplets on an open platform instead of as streams in enclosed channels. This approach, called digital microfluidics (DMF), exploits the electrowetting and dielectrophoresis forces generated when an electrical potential is applied to an electrode in an array.[11−14] By applying electrical potentials to sequential electrodes, a droplet of fluid can be dispensed from a reservoir, transported to any position on the array, and merged with other droplets to perform reactions.[15,16] This technique has been used to actuate a wide range of volumes (nL to μL), and unlike channel devices, there is no sample wasted in creating a small plug for analysis. In addition, each droplet is isolated from its surroundings rather than being embedded in a stream of fluid—a simple method of forming a microreactor in which there is no possibility that products will diffuse away. Perhaps most importantly, the geometry of the array ensures that any device design is reconfigurable—since a greater number of paths through the device are possible, a variety of functions can be performed without redesigning the device. There is currently much enthusiasm for applying DMF to multiplexed assays;[17] however, it has only been applied to a few biological applications.[18−25] One reason for the lack of applications for this technique is that fabrication of DMF devices is arduous and time-consuming; we recently developed a suite of rapid prototyping methods for DMF devices,[26−28] which should make the method available to all who wish to use it.

A wide variety of channel microfluidic devices has been developed for enzyme studies,[5−7,29] but the potential of digital devices has been largely unexplored. Taniguchi et al.[18] demonstrated the movement and merging of droplets of luciferase and luciferin on an electrowetting-based device, but this system required a hydrophobic liquid (oil) medium surrounding the droplet, and no quantitative data were obtained. A two-enzyme assay for the detection of glucose was reported by Srinivasan et al.[19, 20] that also required a silicone oil medium to achieve droplet movement and prevent analyte adsorption to the device surface. While this method proved to be effective for the quantitation of glucose in a variety of biological fluids, a detailed analysis of enzyme activity was not achieved. It would be desirable to develop an assay that does not require oil, as it can make working with some solvents such as ethanol or methanol impossible; it is also likely that hydrophobic analytes may partition out of the aqueous droplet and into the oil, resulting in incomplete reactions, inaccurate quantitation, and cross-contamination. Jary et al.[21] developed a microfluidic microprocessor for DNA repair enzyme analysis that was just as sensitive as macroscale methods, but with a more limited dynamic range. An ideal microfluidic assay system would have high sensitivity, yet retain the flexibility and dynamic range of its large-scale counterpart.

Here, we demonstrate the first application of digital microfluidic methods to the detection and quantification of small molecules via an enzyme assay, as well as for the study of enzyme kinetics, with no need for liquid suspending media (e.g, silicone oil). We show that the DMF assay has better sensitivity than macroscale methods, but without sacrificing dynamic range. This technique has great potential as a simple yet versatile analytical tool for implementing enzymatic analyses on the microscale.

EXPERIMENTAL SECTION

Reagents and Materials. Alkaline phosphatase (type VII-L from bovine intestinal mucosa), diethanolamine, magnesium chloride, Fluorinert FC-40, sodium phosphate dibasic, and Pluronic F-127 were purchased from Sigma Chemical (Oakville, ON, Canada). Fluorescein diphosphate (FDP) was purchased from Molecular Probes (Invitrogen Canada, Burlington, ON, Camada). Parylene-C dimer was from Specialty Coating Systems (Indianapolis, IN), and Teflon-AF was purchased from DuPont (Wilmington, DE).

Clean room reagents and supplies included Shipley S1811 photoresist and developer from Rohm and Haas (Marlborough, MA), solid chromium and gold from Kurt J. Lesker Canada (Toronto, ON, Canada), CR-4 chromium etchant from Cyantek (Fremont, CA), hexamethyldisilazane (HMDS) from Shin-Etsu MicroSi (Phoenix, AZ), AZ-300T photoresist stripper from AZ Electronic Materials (Somerville, NJ), and conc. sulfuric acid and hydrogen peroxide (30%) from Fisher Scientific Canada (Ottawa, ON, Canada). Piranha solution was prepared as a 3:1 (v/v) mixture of sulfuric acid and hydrogen peroxide.

Device Fabrication. Digital microfluidic devices were formed using conventional methods in the University of Toronto Emerging Communications Technology Institute (ECTI) fabrication facility. Glass wafers were cleaned in piranha solution (10 min) and then coated with chromium (10 nm) and gold (100 nm) by electron beam deposition. After rinsing and baking on a hot plate (115 °C, 5 min), the substrates were primed by spin-coating with HMDS (3000 rpm, 30 s) and then spin-coated with Shipley S1811 photoresist (3000 rpm, 30 s). Substrates were prebaked on a hot plate (100 °C, 2 min) and exposed through a photomask using a Suss Mikrotek mask aligner. Substrates were developed in MF321 (3 min) and then postbaked on a hot plate (100 °C, 1 min). After photolithography, substrates were immersed in gold etchant

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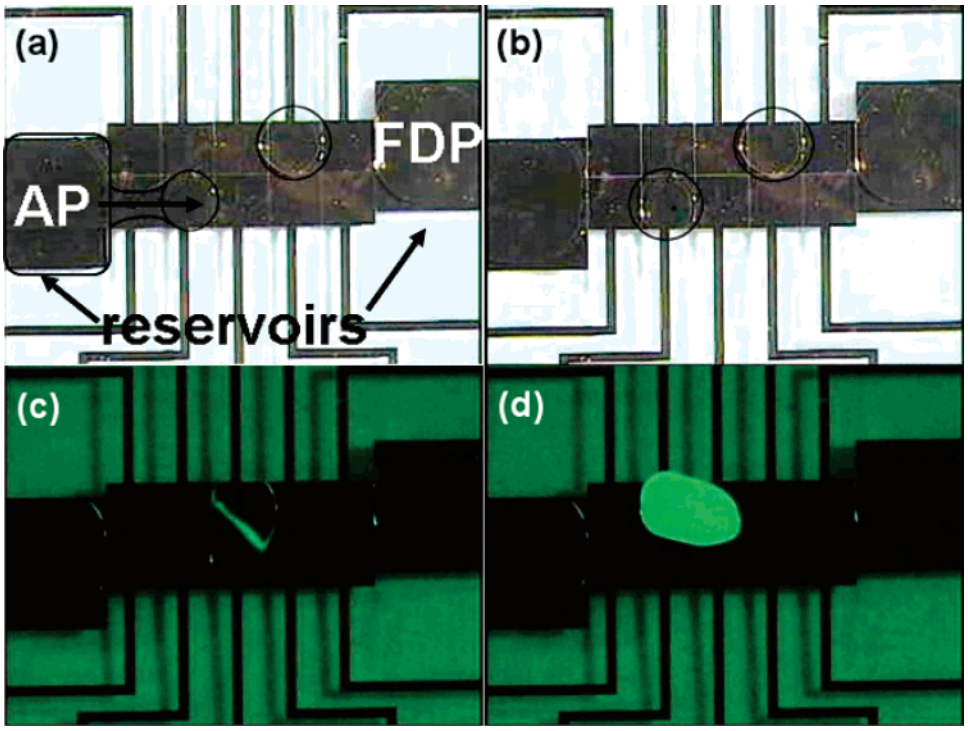

Figure 1. Fluorescent enzymatic assay on a DMF device. A droplet containing FDP was dispensed from the reservoir on the right (a) and a droplet of AP was dispensed from the reservoir on the left (a, b). When the droplets were merged under fluorescent illumination, the product was observed at the interface of the droplets (c). After active mixing, the reaction proceeded to completion (d). Lines have been added to the images in (a, b) to indicate the location and movement of droplets.

(60 s) followed by chromium etchant (60 s). Finally, the remaining photoresist was stripped in AZ300T (5 min) in an ultrasonic cleaner.

After forming electrodes, devices were coated with 2 μm of parylene C and 50 nm of Teflon-AF. Parylene C was applied using a vapor deposition instrument (Specialty Coating Systems), and Teflon-AF was spin-coated (1% w/w in Fluorinert FC-40, 2000 rpm, 60 s) and then postbaked on a hot plate (160 °C, 10 min). To actuate droplets, the polymer coatings were locally removed from the contact pads by gentle scraping with wafer tweezers. In addition to patterned devices, unpatterned indium-tin oxide (ITO)-coated glass substrates (Delta Technologies Ltd, Stillwater, MN) were coated with Teflon-AF (50 nm, as above).

Droplet Actuation. Each device was assembled with an unpatterned ITO/glass top plate and a patterned bottom plate separated by a spacer formed from one piece of double-sided tape (~70 μm thick). The 140-nL droplets in devices with this arrangement had a diameter of ~1.6 mm (~40% of the size of a well in a 384-well plate). As described previously,[11,16,24] droplets were sandwiched between the two plates and actuated by applying electric potentials between the top electrode and sequential electrodes on the bottom plate. Applied potentials (60−80 V_{RMS}) were generated by amplifying the output of a function generator operating at 18 kHz. Driving potentials were applied manually to exposed contact pads on the bottom plate surface. Droplet actuation was monitored and recorded by a CCD camera mated to a stereomicroscope with fluorescence imaging capability (Olympus Canada, Markham, ON, Canada). All devices had 1 mm × 1 mm actuation electrodes, with an interelectrode gap of 40−75 μm.

Analysis of Enzyme Activities and Kinetics. Alkaline phosphatase solutions were prepared in 10 mM diethanolamine (DEA) buffer, pH 10.1, containing 1 mM $MgCl_2$ and 0.1% (w/v) Pluronic F-127. Enzyme concentrations were 6 U/mL for standard curve experiments and for observation through the microscope and 0.21 U/mL for kinetic analyses. Fluorescein diphosphate solutions of various concentrations were prepared in 10 mM DEA buffer. For enzyme inhibition experiments, fluorescein diphosphate solutions containing various concentrations of inorganic phosphate were also prepared. For all quantitative experiments, a digital microfluidic device was manually positioned on the top of a 384-well microtiter plate, and a 650-nL reservoir droplet of enzyme or substrate solution was pipetted onto each of two large electrodes (2 mm × 2 mm). As depicted in Figure 1, 70-nL droplets of each reagent were actively dispensed from the reservoirs (as described previously[16]). Briefly, two electrodes adjacent to the reservoir were actuated sequentially to form a finger of fluid extending onto the array; upon simultaneous actuation of the target electrode and the reservoir electrode; the finger was split, resulting in a dispensed droplet with a volume defined approximately by the electrode and spacer dimensions (1 mm × 1 mm × 70 μm). The two dispensed reagent droplets were then merged and mixed by moving the coalesced droplet around a loop of six actuation electrodes for 15 s. After mixing, the device (still positioned on the microtiter plate) was inserted into a PheraStar multiwell plate reader (BMG Labtech, Durham, NC) for fluorescence detection (λ_{ex} = 485 nm; λ_{em} = 520 nm; focal height, 15.0 mm; gain, 1462 or 90 for substrate quantitation and kinetics experiments, respectively). After each assay, devices were rinsed in DEA buffer and DI water and allowed to dry. Because of the finite time required

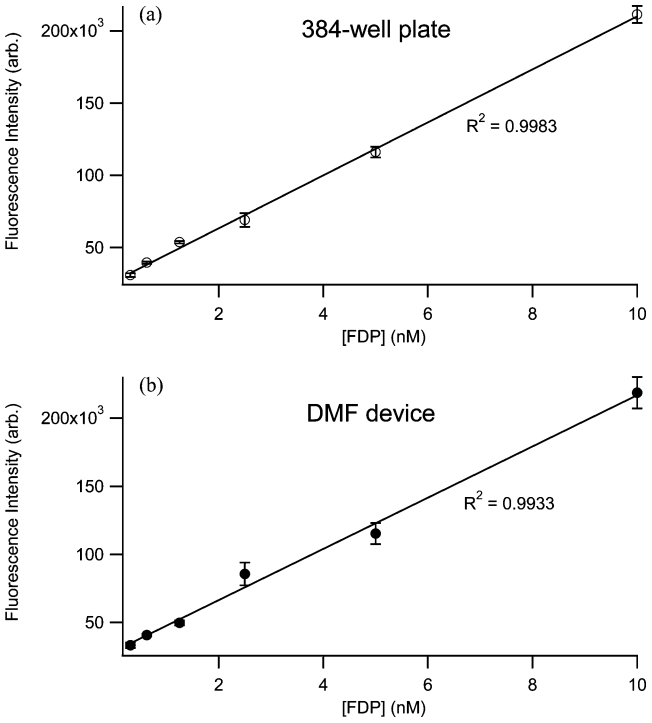

Figure 2. FDP calibration curves. On a 384-well plate (a) and a DMF device (b), the fluorescence response was linear over a range of 2 orders of magnitude of substrate concentration. Error bars are ±1 SD. In both experiments, the error in the data points ranged from <2% to ~10% RSD.

for mixing of the reagents, insertion of devices into the plate reader, and measurement of the fluorescent signals, kinetic assays were conducted at low concentrations of enzyme and substrate, making the linear approximation of the initial rate more accurate. The concentration detection limits were determined by sequentially diluting the substrate solution until the measured signal intensity was equal to the background plus 3× the standard deviation of the background measured from 10 droplets not containing the fluorophore. For all experiments, 3−5 trials were conducted to evaluate reproducibility of the method.

For comparison, all assays implemented by digital microfluidics (in 140-nL droplets, as described above) were also carried out in 384-well microtiter plates. For these conventional-scale assays, 10 μL each of the enzyme and substrate solutions were pipetted into wells and the plate was shaken by the plate reader to ensure mixing of the reagents before detection. All experiments in microtiter plates used the same enzyme and substrate solutions used on digital microfluidic devices to ensure that each assay had similar dilution and mixing conditions, and 3−5 replicates of each measurement were performed for error analysis.

RESULTS AND DISCUSSION

Device Optimization. A common difficulty in the application of DMF devices to proteomic assays lies in achieving consistent actuation of droplets of protein solutions. Droplet actuation by means of electrowetting requires a hydrophobic electrode surface, and protein deposition on this hydrophobic surface reduces the efficiency of actuation and the lifetime of the device.[30] The alkaline

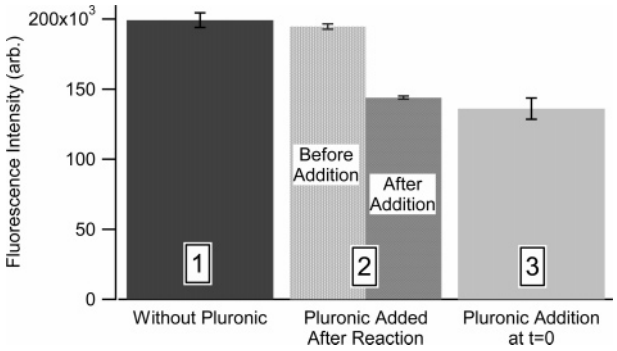

Figure 3. Effect of Pluronic F-127 on enzyme assays. An AP−FDP reaction was allowed to proceed to completion in a well plate under three sets of conditions: (1) without any Pluronic added to the reaction mixture, (2) with Pluronic added to the wells after the reaction was completed, and (3) with Pluronic added to the wells at the start of the reaction. Error bars are ±1 SD. While the presence of the polymer does reduce the fluorescent signal at the detector, the reduction is the same whether the Pluronic is added at the beginning of the reaction or at completion. The activity of the enzyme appears to be unaffected.

phosphatase (AP) used here had sufficient activity such that substrate turnover was observed when the enzyme was present at concentrations of 6 U/mL (2 μg/mL) or less—a concentration range for proteins that has been reported to be compatible with DMF.[20,30]

In preliminary work, we noticed that, even when using low concentrations of alkaline phosphatase, some enzyme adsorbed to the Teflon surface during the course of experiments, which caused the devices to fail (i.e., failed devices could no longer support droplet movement) after a small number of assays. In an attempt to extend the device lifetimes and reduce cross-contamination, we evaluated the effect of supplementing the carrier buffers with Pluronic F-127, a triblock copolymer and nonionic surfactant that has been shown to reduce nonspecific adsorption of proteins to hydrophobic surfaces.[31−33] In initial trials, we observed that droplets containing low concentrations of Pluronic F-127 were reliably moveable by DMF, although they moved less rapidly than droplets not containing the surfactant. When used in enzyme assays, the strategy was remarkably successful at extending device lifetimes—in one experiment using alkaline phosphatase at 6 U/mL (2 μg/mL) with 0.1% w/v Pluronic F-127, a single device was used for 21 consecutive assays, with no noticeable degradation of device performance. Between assays, a simple rinse with buffer was sufficient to maintain droplet actuation, and no contribution to the error in subsequent trials was observed (i.e., no evidence of cross-contamination). In ongoing work, we are evaluating the optimal conditions (concentration and type of Pluronic, etc.) for reducing unwanted adsorption in DMF;[34] we used 0.1% w/v F-127 for all of the results reported here.

Enzyme Activity. In order to determine the potential of the new DMF method for substrate quantitation and other enzymatic analyses, a standard curve was generated and compared to a well-

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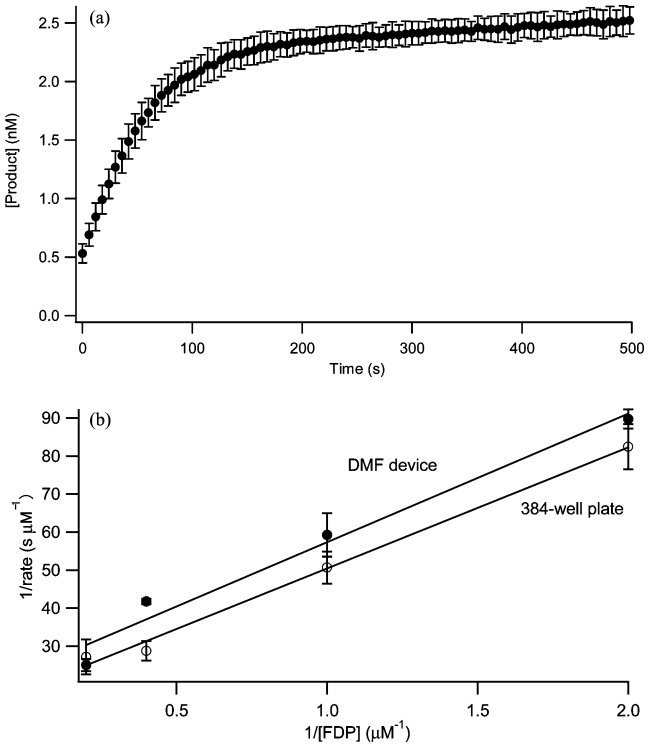

Figure 4. Study of enzyme kinetics on a DMF device. After droplets containing enzyme and substrate (2.5 nM) were merged and mixed, the fluorescent product was monitored over time (a). The initial rate was measured at several substrate concentrations and reported as a Lineweaver−Burk plot (filled circles) (b) and compared to a macroscale experiment in a 384-well plate (open circles). Error bars are ±1 SD.

Table 1. Michaelis−Menten Kinetic Variables for Alkaline Phosphatase

	384-well plate	DMF device
V_{max}	54.8 ± 5.7 nM/s	42.5 ± 7.8 nM/s
K_m	1.85 ± 0.2 μM	1.35 ± 0.3 μM
k_{cat}	155 ± 16 s^{-1}	120. ± 20 s^{-1}

plate experiment under the same conditions (Figure 2). Both methods had a dynamic range of ~2 orders of magnitude, which appears to be limited only by the detector—there is no marked difference between the data generated by the two methods. The concentration detection limits of the DMF and well-plate methods were determined to be approximately 1.0 and 0.50 pM FDP, respectively. These concentration data represent mass detection limits of 7.0×10^{-20} and 5.0×10^{-18} mol, respectively—an improvement of nearly 2 orders of magnitude in the microfluidic system. This increase in sensitivity is likely a function of the placement of the device within the plate reader—the DMF device is closer to the detector than the fluid at the bottom of the well. In addition, the gold surface of the electrodes on DMF devices reflects the emitted light rather than absorbing it (as in the black wells), so more fluorescence is likely collected from the DMF device. The dispensing of 70-nL droplets on the microscale device appears to be nearly as reproducible as dispensing 10-μL aliquots via micropipet on the macroscale; the error in each data point is within ~10% RSD in both experiments.

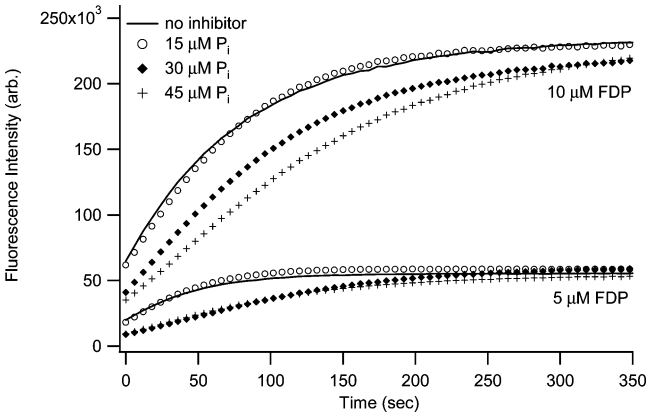

Figure 5. Inhibition of AP with P_i. The concentration of FDP was held constant at either 10 or 5 μM and the concentration of P_i varied from 0 to 45 μM. The rate of reaction is reduced with each increase in inhibitor concentration, as the inorganic phosphate competes with the substrate for the active sites on the enzyme.

The effect of the Pluronic additive on the assay was also examined. An AP−FDP reaction was carried out in a 384-well plate under three sets of conditions: (1) without any Pluronic added to the reaction mixture, (2) with Pluronic added to the wells after the reaction was completed, and (3) with Pluronic added to the wells at the start of the reaction. As shown in Figure 3 (comparing conditions 1 and 3), the presence of the additive decreases the fluorescent signal measured by the detector by ~30%. As shown by comparing conditions 2 and 3, the decrease in the fluorescent signal is identical for cases when Pluronic is added at the beginning or end of the reaction, which indicates that this effect is not caused by a decrease in the amount of product. Therefore, the effect is likely caused by interaction of the fluorescent product with Pluronic molecules rather than by a reduction in enzyme activity caused by the surfactant. Regardless, the effect is small and does not cause problems for conducting enzyme assays.

Enzyme Kinetics. After demonstrating the compatibility of DMF with fluorogenic enzyme assays, we applied the technique to measuring Michaelis−Menten enzyme kinetics. Since ~30 s elapses from the time the reagent droplets are merged to the time that the first fluorescence data points can be collected, experimental conditions were optimized such that the rate at 30 s was similar to the initial rate. As shown in Figure 4a, it was determined that an AP concentration of 0.21 U/mL met these conditions. A series of experiments was then performed, implemented on DMF devices and in 384-well plates, in which the enzyme concentration was held constant at 0.21 U/mL while the substrate concentration was varied and initial velocities were calculated. Five replicates were run at each substrate concentration to determine the precision of each method. The initial velocities were then plotted against substrate concentrations in a double-reciprocal Lineweaver−Burk plot (Figure 4b).

For both formats (macro- and microscale), the Lineweaver−Burk plot was linear over a range of ~1 order of magnitude. The DMF data appear slightly more reproducible than the 384-well plate (errors in the data from the DMF device ranged from 2 to 10% RSD, while errors in the data from the macroscale method varied from 7 to 17% RSD). The values of K_m, V_{max}, and k_{cat} calculated from these plots are shown in Table 1. In both cases, the calculated constants are similar to those typically reported

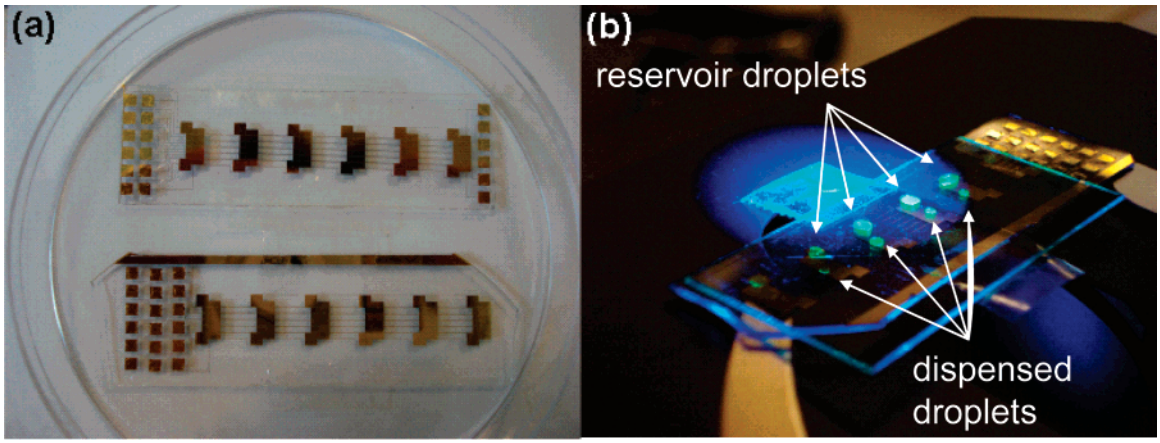

Figure 6. Multiplexed DMF device. Picture of a device designed for simultaneous control of six independent DMF-driven assays (a). A droplet of fluorescein is dispensed from a reservoir on several platforms via a single set of electrical contacts (b).

for alkaline phosphatase (k_{cat} ~100).[35] The DMF-determined value of k_{cat} is slightly lower than that obtained by using well plates—this is likely due to the longer time lag between mixing and measurement. However, there is excellent overall agreement between the values calculated from the micro- and macroscale systems, which suggests that there is great potential for using DMF for the analysis of enzyme kinetics.

In addition to Michaelis−Menten analyses, we evaluated the suitability of DMF for implementing enzyme inhibition studies. In these experiments, inorganic phosphate (P_i) at various concentrations was added to the droplet containing FDP. P_i is a well-known competitive inhibitor of alkaline phosphatase,[36] and as shown in Figure 5, the rate of reaction is slowed with each increase in P_i concentration. These qualitative results are particularly exciting, as in the future, we plan to use DMF to carry out quantitative enzyme inhibitor screens to identify new reagents with pharmaceutical activities. As described above, the advantages for digital microfluidics of reduced reagent consumption and increased assay sensitivity make this plan a promising one.

Multiplexed Devices. Recently, we designed and fabricated a DMF device for multiplexed analysis, shown in Figure 6. This device contains six identical reaction platforms connected in series, which facilitates simultaneous control of six separate reactions using a single program of driving potentials. The spacing between platforms is 9 mm, the same as the interwell pitch on a 96-well plate, allowing all six devices to be simultaneously monitored within the plate reader. We are currently optimizing the operating parameters for this device; when completed, we expect the new design (or a similar one) will be characterized by improved speed and precision of this method. Each measurement will have a built-in redundancy, eliminating the need to run replicates and reducing the amount of time required to perform an analysis. Devices such as the one pictured in Figure 6 are moving us one step closer to realizing a high-throughput microfluidic platform for multiplexed screening.

CONCLUSION

Enzymatic assays are widely used in carrying out chemical analyses on the microscale. We have demonstrated that the digital microfluidic paradigm is well-suited to these assays, without the flow control, mixing, and fabrication problems encountered in channel microfluidic devices. In terms of small-molecule detection and quantitation, the digital microfluidic device performed comparably to the macroscale method, with standard errors of 10% or less. An increase in sensitivity of nearly 2 orders of magnitude was achieved, while decreasing sample and reagent consumption by 2.5 orders of magnitude. Reproducible and accurate kinetic assays were implemented via DMF, yielding kinetic constants that agree with those reported in the literature. Protein adsorption to device surfaces can be controlled with the addition of a Pluronic polymer to the reaction buffer—a silicone oil medium is not required. Finally, we conclude that screening of inhibitors and multiplexed analyses are possible, which will decrease analysis times and enable us to achieve a truly high-throughput enzymatic assay on the microscale.

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