Enzyme Chemotaxis on Paper-based Devices

Grenalynn C. ILACAS,* Alexis BASA,* Ayusmen SEN,** and Frank A. GOMEZ*†

*Department of Chemistry and Biochemistry, California State University, Los Angeles, 5151 State University Drive, Los Angeles, California 90032, United States
**Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802, United States

Microfluidics has served as a technology for the design and development of a myriad of devices owing to their reduced reagent consumption rate and short sampling-to-result time. Chemotaxis is the movement of materials, particularly biological species, in response to the influence of chemical stimulation. Herein, we describe, for the first time, chemotactic behavior on a microfluidic paper-based analytical device (µPAD) to afford a distribution of products not obtainable under other (non-µPAD) experimental conditions using as a model enzyme-substrate system glucose oxidase (GOx) and glucose. µPADs are easily fabricated by patterning hydrophobic materials in hydrophilic paper. They are low cost, compatible with biological samples, and have shown promise as platforms for various applications and in resource-limited settings.

Keywords Microfluidics, molecular chemotaxis, paper-based analytical device, glucose oxidase

(Received August 29, 2017; Accepted November 15, 2017; Published January 10, 2018)

Introduction

Microfluidics is the design, development, and use of devices that apply to fluidic flow to channels smaller than 1 mm in one or more dimensions. The field of microfluidics has yielded important platforms for lab-on-chip systems, LOC, since microfluidic devices can decrease sample and reagent consumptions considerably, shorten the time of experiments, allow for the controlled mixing and manipulation of minute particles, integrate and automate multiple assays, and reduce the costs of applications. In addition, miniaturization and automation inherently improve the precision of the experiments, lowers the limits of detection, and allow for multiple analyses to be run simultaneously.

Recently, microfluidics has been used to conduct a wide array of low-cost diagnostics, ranging from chemical-analysis systems to optofluidic technology. Within microfluidics, there is an interest in chemical analysis focusing on a specific analyte. Chromatography and chromatographic techniques have for many years been a means to separate species in a variety of mixtures. Paper has historically been the stationary phase of choice due to its cellulosic structure and ability to interact with molecules of varied types due to its fibers nature, thereby, creating a complex of theoretical plates. It also easily transports fluids via capillary action without the need of an external power source, and has a high surface area-to-volume ratio.

In 2007, the use of chromatography paper as a platform in microfluidic chips was first described for chemical analyses, which were termed microfluidic paper-based analytical devices (µPADs). Since then µPAD technologies have resulted in applications in the pharmaceutical, defense, biotechnology, bioterrorism detection, and point-of-care (POC) diagnostic areas. As the development of µPAD technology has progressed, their potential use as diagnostic assays for underdeveloped countries has increased. In response, the World Health Organization (WHO) has directed research towards µPADs to fulfill the ASSURED criteria: affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, and deliverable to end-users.

Laboratory techniques required in the separation of chemicals or substances in complex mixtures are limited in resource-limited countries due to such factors as the cost of labeling, reagent consumption, and instrumentation. Hence, challenges exist in developing viable techniques that also require low-cost instrumentation in the separation of biomolecular species. Sen et al. recently reported enzymatic separation utilizing molecular chemotaxis of an enzyme towards an imposed substrate gradient in microfluidic devices fabricated from poly(dimethylsiloxane) (PDMS). The use of PDMS as a substrate in microfluidics is well-known due to its transparent property at optical frequencies, ability to bond to glass or other PDMS layers via simple plasma treatment, and ease of mass-production. Still, there are a number of drawbacks to PDMS, including its ability to absorb small, hydrophobic molecules, its tendency to swell in the presence of small hydrocarbon solvents, its hydrophobic nature, and the fact that water evaporates through it. A µPAD utilizes capillary action vis-a-vis the movement of fluid as a result of interactions between the fluid molecules and the adhesive forces with the surface, whereas, external pumps are needed to drive the fluid through the channels in PDMS-based microfluidic chips. Hypothetically, chromatography paper should be an alternative to PDMS to examine chemotaxis and provide cost and time efficiency in fabricating the devices.

To date, there have been only a few examples documenting
cell chemotaxis on paper-based devices. In one of these works, a low cost, quasi-stable, chemokine gradient system capable of examining cell migration response over short times was described. Using human pan-T cells as a proof-of-concept, directed migration to the chemokine gradient over the control condition was shown. Besides the obvious advantages of using paper (low-cost and pumpless function), competing gradients caused by wicking action may also result. Herein, we describe molecular chemotactic behavior in an optimized μPAD using colorimetric detection using the enzyme-substrate model system, glucose oxidase (GOx) and glucose. Here, a Y-shaped μPAD with multiple outlets and of varying mixing lengths is subjected to two streams of solution (GOx in one, and glucose in the second) to afford the products gluconic acid and hydrogen peroxide which, in the presence of horseradish peroxidase (HRP), forms iodine from KI. Analysis of the extent of product distribution compared to controls and fluorescent studies prove chemotactic behavior on paper-based platforms.

Experimental

Materials
Glucose oxidase (GOx) from Aspergillus niger, HRP, D-(+)-glucose, sodium acetate anhydride, sodium phosphate monobasic, and acetic acid were purchased from Sigma Aldrich Co. (Saint, Louis, MO). Sodium phosphate dibasic and KI were purchased from Fischer Scientific Co. (Hampton, NH). Whatman Grade 1 CHR chromatography paper was purchased from GE Healthcare Life Sciences (Pittsburgh, PA).

Solutions
Acetate buffer (0.2 M, pH 5.1) was prepared by the addition of 71.5 mL sodium acetate anhydride (0.2 M) and 28.5 mL acetic acid (0.2 M). GOx and glucose solutions were prepared with acetate buffer at concentrations of 120 units/mL and 6 mM, respectively. PBS buffer (0.1 M, pH 6.0) was prepared by addition of 87.7 mL of sodium phosphate monobasic (0.2 M) and 12.3 mL of sodium phosphate dibasic (0.2 M). A HRP solution was prepared with PBS at 30 units/mL. KI (1.0 M) was prepared with DI water.

Fabrication, methods, and detection
μPADs were fabricated via design using Inkscape 0.91 software (Boston, MA, USA) and printed on Whatman Grade 1 CHR chromatography paper using a Xerox ColorQube 8580 wax printer (Norwalk, CT, USA) at mixing size areas of 3–22, 25, and 30 mm. To ensure the viability of the hydrophobic boundaries of the μPADs, the chips were heat pressed on both sides to melt the wax into the pores of the paper using a Hotronix® Auto Open Clam Heat Press at 350°F for 120 s. Prior to chemotactic behavior runs, outlets of the μPAD were spotted with a KI:HRP solution (0.8 μL) and dried. Chips were then positioned on a platform attached to a laboratory jack and lowered into solutions of GOx (120 units/mL), glucose (6 mM), or acetate buffer (0.2 M, pH 5.1) in volumes respective to the chip length (Fig. 1). To initiate the reaction between the enzyme and substrate, the chips were lowered above either solution to ensure equivalent fluid flow. Colorimetric detection was scanned using a Canon CanoScan LIDE 220 at 600 dpi high resolution and chips were analyzed via Adobe Photoshop Ver. 2015.1.1.

Fluorescence measurements
Alexa Fluor™ 488 Protein Labeling Kit was purchased from Invitrogen by Thermo Fischer Scientific (Eugene, OR, USA). Fluorescently tagged GOx (0.25 mg/mL) was prepared as described in the labelling kit. Inactivation of GOx was confirmed and details are provided in Supporting Information (SI). Fluorescent enzyme activity was conducted using even μPAD mixing lengths of 6 – 16 mm. Observations and data was acquired using a Nikon Eclipse Inverted Microscope equipped with Nikon fluorescence filter cube B-2A with excitation at λ = 495 nm and emission at λ = 519 nm (Minato, Tokyo, Japan). SPOT Imaging Microscopy Imaging Software 5.2 (Sterling Heights, MI, USA) was used for capturing images and quantitative analysis of the μPAD was carried out via ImageJ.

Results and Discussion

Chemotactic behavior on μPADs
To demonstrate chemotaxis on μPADs, devices with mixing area lengths of 5 – 9 mm were initially examined with GOx and glucose at concentrations of 120 units/mL and 6 mM,
respectively, spotted under each inlet (Fig. 2A). Minimal-to-no color detection was observed in the left or right outlet of the 5 mm chip when compared to the center outlet of μPADs of similar lengths. The coloration in the center outlet is attributed to the limited distance GOx is given to migrate toward glucose. The allotted time for GOx and glucose to react in the center region of the mixing area and travel toward the center outlet is limited by the mixing length. It is not until the mixing length is 6 mm that a larger color intensity is observed in the left outlet as compared to the right outlet. At this length, GOx has sufficient time to migrate toward the solution of glucose, ultimately collecting in the left outlet. To validate the observed behavior of GOx and its movement toward glucose, a control was conducted for each mixing length of the μPAD. In lieu of having the glucose solution flow through the left inlet, acetate buffer (0.2 M, pH 5.1) was run alongside GOx (120 units/mL). At each outlet, a cocktail of KI:HRP (0.80 μL) was spotted. After flowing GOx in acetate buffer through μPADs of varying mixing area lengths, the chips were dried. The series of chips were then cut (post-mixing area) and 6 mM glucose (2 μL) was added to each outlet “lane” to determine glucose independent of GOx movement. All relative lengths for the control displayed a similar trend where both the center and right outlets displayed brown color (Fig. 2B). Since glucose did not flow through the mixing area simultaneously with GOx, the enzyme did not migrate toward the left area of the chip, due to lack of affinity to acetate buffer. In the presence of glucose, GOx molecules migrate towards the left region of the μPAD producing color in increasing amounts in the left outlet as length of mixing area increases.

For both the glucose and control runs, μPADs with mixing area lengths of 3 – 22, 25, and 30 mm (and using similar conditions to the aforementioned) were conducted in triplicate. Each outlet (left, center, and right) was analyzed via yellow color mean where each color mean was averaged and graphed against length (Fig. 3). As the length of the mixing area increased, color intensity increased in all outlets. Similarly to the visual detection in Fig. 2A, this occurs due to the chemotactic behavior that the enzyme exhibits in response to the imposed substrate concentration gradient. As length increases, a decrease in color is seen in the right outlet due to GOx having more time to migrate toward the left side of the chip, ultimately surpassing the color intensity of the center outlet, as seen in the 17 mm length mixing area. Control chips displayed a similar trend to visual detection (Fig. 2B) where all relative lengths for the control displayed colorimetric detection in the center and right outlets.

Fluorescent enzymatic activity

We utilized fluorescently labeled activated and inactivated...
GOx to assess the movement of enzyme towards an imposed substrate gradient. Fluorescent studies of molecular chemotaxis can provide detailed fluorescently tagged enzyme movement, whereas, colorimetric detection can be limited in providing only the presence of products (oxidation of KI to I₂). Due to limitations of instrumentation, lateral flow assay (LFA) architecture was utilized when running solution through the μPADs (Fig. 4). μPADs were encased in customized laser-cut PVA casings for stability; holes were cut out for image capture. Similar to non-fluorescent methods, solutions of active/inactive GOx (0.25 mg/mL) and glucose (6 mM) were introduced into two inlets simultaneously. Solutions were then allowed to run and sequential images were taken of the outlet lanes and subsequently analyzed on ImageJ.

Even chip lengths of 4 – 16 mm were utilized to examine chemotactic behavior using two GOx enzyme conditions and one control: Alexa Fluor dye-tagged activated GOx, Alexa Fluor dye-tagged inactivated GOx, and 1X PBS buffer (pH 7.4). Fluorescence captured images (Figs. 5A - 5C) of the 14 mm chip length was chosen as a representative example to definitively show chemotactic movement. A control experiment was designed using 1X PBS buffer as a substrate solution with Alexa Fluor dye-tagged activated GOx (Fig. 5A). Visual analysis provided no fluorescent detection in the left lane, indicating no migration of GOx. Similar to the acetate buffer controls run previously, GOx has no affinity to 1X PBS buffer and, therefore, will not display movement towards the left lane of the μPAD. A solution of glucose (6 mM) was then used as the substrate with Alexa Fluor dye-tagged activated GOx (Fig. 5B). As predicted, fluorescence was detected in the left lane, supporting previous data reported (Fig. 3A) where chips of mixing length 14 mm displayed chemotactic movement. Finally, a solution of glucose (6 mM) was used with Alexa Fluor dye-tagged inactivated GOx (Fig. 5C). Fluorescent imaging provided definitive similarities between the control chip (Fig. 5A) with the inactivated GOx chip (Fig. 5C).

The quantification of fluorescent chips conducted using even chip mixing lengths of 4 – 16 mm was graphed using all three conditions mentioned above (Fig. 6). Data analysis shows fluorescence from chip length greater than 8 mm which differ from the colorimetric detection data. Whereas Fig. 3A shows a larger color intensity analyzed in the left outlet at the length of 6 mm, fluorescent studies show increased fluorescence after 8 mm. Explanations for this difference in observable outcomes are due to differences in detection and analysis methodologies. Colorimetric detection is carried out using the detection of oxidation of KI to I₂, which is an indirect method to measure the presence of enzyme. It is also important to note that KI exhibits high sensitivity to light exposure, such that the solution becomes colored when exposed to light. This coloration can also be attributed to the lower detection as displayed in chip lengths greater than 6 mm (Fig. 3A), as colorimetric detection experiments were not conducted in a dark room and, therefore, not shielded from light. Nonetheless, data provided in Fig. 6 support our claim that as chip length is increased, movement of enzyme towards an imposed substrate also increases.
Conclusions

We have demonstrated, for the first time, the use of paper-based microfluidic devices to examine chemotactic behavior of enzymes. Here, a Y-shaped µPAD containing multiple outlets of varying mixing lengths is used and is subjected to enzyme and substrate streams forming product ratios that are quantitated colorimetrically and that are dependent on the extent of chemotaxis. There is a direct correlation between the increase in mixing area length and enzyme chemotactic behavior. Fluorescent studies further support the colorimetric results. We also demonstrated the extent of affinity of active enzyme towards its substrate relative to the inactive form. Future studies can be directed to utilizing chemotactic activity in the separations of mixtures of enzymes as well as the purification of active/inactive species.

Acknowledgements

The authors gratefully acknowledge financial support for this research from the National Science Foundation (DMR-1523588, HRD-1547723) and the W. M. Keck Foundation.

Supporting Information

Heat Inactivation of Alexa Fluor® 488 dye-tagged GOx. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References

1. G. M. Whitesides, Nature, 2006, 442, 368.
2. W. Jung, J. Han, J. W. Choi, and C. H. Ahn, Microelectron. Eng., 2015, 46.
3. E. Fu, T. Foley, J. Weinstein, and P. Yager, Rev. Sci. Instrum., 2004, 75, 2300.
4. D. Janasek, J. Franzke, and A. Manz, Nature, 2006, 442, 374.
5. A. Lenshof and T. Laurell, Chem. Soc. Rev., 2010, 39, 1203.
6. A. M. Foudeh, T. F. Didar, T. Veresa, and M. Tabrizian, Lab Chip, 2012, 12, 3249.
7. D. Psaltis, S. R. Quake, and C. Yang, Nature, 2006, 442, 381.
8. P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, Proc. Natl. Acad. Sci. U. S. A., 1968, 61, 636.
9. J. Porath, J. Protein Chem., 1997, 16, 463.
10. S. J. Gerberding and C. H. Byers, J. Chromatogr. A, 1998, 808, 141.
11. M. Das and D. Dasgupta, Prep. Biochem. Biotechnol., 1998, 28, 339.
12. M. A. Firer, J. Biochem. Biophys. Methods, 2001, 49, 433.
13. J. A. Queiroz, C. T. Tomaz, and J. M. Cabral, J. Biotechnol., 2001, 87, 143.
14. D. M. Cate, J. A. Adkins, J. Mettakoonpitak, and C. S. Henry, Anal. Chem., 2014, 87, 19.
15. A. W. Martinez, S. T. Phillips, M. H. Butte, and G. M. Whitesides, Angew. Chem. Int. Ed., 2007, 46, 1318.
16. Y. Xia, J. Si, and Z. Li, Biosens. Bioelectron., 2016, 77, 774.
17. J. C. Cunningham, N. Brenes, and R. M. Crooks, Anal. Chem., 2014, 86, 6166.
18. P. Rattanarat, W. Dungchai, D. M. Cate, W. Siangproh, J. Volckens, O. Chailapakul, and C. S. Henry, Anal. Chim. Acta, 2013, 800, 50.
19. T. S. Park, W. Li, K. E. McCracken, and J. Y. Yoon, Lab Chip, 2013, 13, 4832.
20. A. Pesenti, R. V. Taudte, B. McCord, P. Doble, C. Roux, and L. Blanes, Anal. Chem., 2014, 86, 4707.
21. A. W. Martinez, S. T. Phillips, E. Carrilho, S. W. Thomas, H. Sindri, and G. M. Whitesides, Anal. Chem., 2008, 80, 3699.
22. A. W. Martinez, S. T. Phillips, and G. M. Whitesides, Proc. Natl. Acad. Sci. U. S. A., 2008, 105, 606.
23. A. W. Martinez, S. T. Phillips, B. J. Wiley, M. Gupta, and G. M. Whitesides, Lab Chip, 2008, 8, 2146.
24. W. Zhao and A. van der Berg, Lab Chip, 2008, 8, 1988.
25. A. W. Martinez, S. T. Phillips, G. M. Whitesides, and E. Carrilho, Anal. Chem., 2009, 82, 3.
26. P. Yager, T. Edwards, E. Fu, K. Helton, K. Nelson, M. R. Tam, and B. H. Weigl, Nature, 2006, 442, 412.
27. K. K. Dey, S. Das, M. F. Poyton, S. Sengupta, P. J. Butler, P. S. Cremer, and A. Sen, ACS Nano, 2014, 8, 11941.
28. Y. Xia and G. M. Whitesides, Angew. Chem. Int. Ed., 1998, 37, 550.
29. A. Meta, A. J. Fleischman, and S. Roy, Biomed. Microdevices, 2005, 7, 281.
30. D. I. Walsh, M. A. Lalli, J. M. Kassas, A. R. Asthagiri, and S. K. Murthy, Anal. Chem., 2009, 87, 5505.
31. R. M. Kenney, M. W. Boyce, A. S. Truong, C. R. Bagnell, and M. R. Lockett, Analyst, 2016, 141, 661.
32. B. Mosadegh, M. R. Lockett, K. T. Minn, K. A. Simon, K. Gilbert, S. Hillier, D. Newsome, H. Li, A. B. Hall, D. M. Boucher, B. Eustace, and G. M. Whitesides, Biomaterials, 2015, 52, 262.