JOURNAL OF CHEMICAL EDUCATION

Laboratory Experiment

pubs.acs.org/jchemeduc

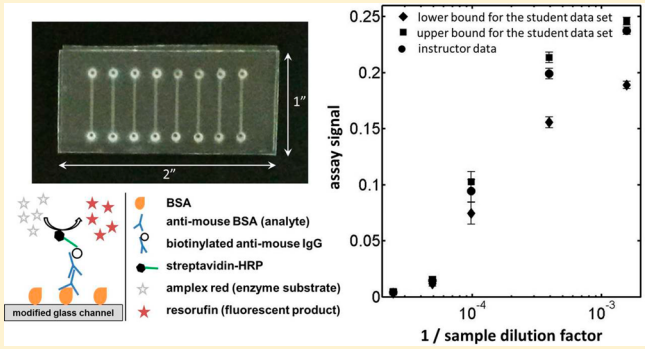
Undergraduate Laboratory Module for Implementing ELISA on the High Performance Microfluidic Platform

Basant Giri, Ravichander R. Peesara, Naoki Yanagisawa, and Debashis Dutta*

Department of Chemistry, University of Wyoming, Laramie, Wyoming 82071, United States

Supporting Information

ABSTRACT: Implementing enzyme-linked immunosorbent assays (ELISA) in microchannels offers several advantages over its traditional microtiter plate-based format, including a reduced sample volume requirement, shorter incubation period, and greater sensitivity. Moreover, microfluidic ELISA platforms are inexpensive to fabricate and allow integration of analytical procedures, such as sample preconcentration, that further enhance the performance of the immunoassay. In view of the scientific potential of microfluidic ELISAs, inclusion of this technique into an undergraduate curriculum is valuable in preparing the next generation of scientists and engineers. Here, an experimental module is presented for this immunoassay method that can be completed in an undergraduate laboratory setting within two 3-h periods (including all incubation and data analyses procedures) using only a microliter of sample and reagents per assay. In addition to acquainting students with the microfluidic technology, the reported module provides training in quantitating ELISAs using the kinetic format of the assay. Furthermore, it offers a useful educational tool for introducing undergraduates to basic image analysis techniques, as well as signal-to-noise ratio and limit of detection calculations that are valuable in characterizing any analytical method.



KEYWORDS: *Analytical chemistry, Biochemistry, Hands-On Learning, Laboratory Instruction, Microscale Lab, Nanotechnology, Upper-Division Undergraduate*

INTRODUCTION

Enzyme linked immunosorbent assay (ELISA) is an important bioanalytical method that allows the quantitation of a variety of molecular targets in clinical diagnostic,[1] food inspection,[2] and environmental applications,[3] among others. The key advantage to using the ELISA technique is its high sensitivity, which arises from signal amplification by the enzyme label conjugated to the detection antibody in the system.[4] Moreover, the high selectivity/specificity of this immunoassay often allows one to work with raw samples without the need to process them laboriously prior to an analysis. Today the ELISA method is commonly practiced on polystyrene-based microtiter plates, which, in spite of being a convenient assay platform, suffers from two important drawbacks. Interestingly, both these drawbacks originate from the relatively large size of its assay compartments, leading to sample/reagent volume requirements of about 100 μL and incubation times ranging from several hours to several days. Although the long incubation periods can pose serious challenges in high throughput applications, it is usually not as debilitating in designing an undergraduate laboratory experiment due to the flexibility available in choosing the sample for the curriculum. On the other hand, the relatively large sample/reagent consumption in microwell plates can render a laboratory ELISA module considerably expensive in the long run. For example, although the use of 100 μL of antibodies and other expensive reagents may not be financially burdening for a single assay, this material cost can be significant to an educational laboratory curriculum where the analysis needs to be performed hundreds of times over extended periods. In any case, the time and cost involved in an ELISA experiment can be significantly reduced by miniaturizing this bioanalytical technique.

Microfluidic devices have emerged as a powerful platform in this regard by allowing the implementation of this immunoassay in channels that are about 10 μm deep, 100 μm wide, and a few centimeters long.[5,6] The shorter dimensions of the microfluidic assay compartment have not only led to significant reductions in sample/reagent consumption and incubation period but also moderate improvements in the smallest detectable analyte concentration.[6,7] In addition, the smaller amounts of sample/reagent used in microfluidic ELISAs reduce the generation of biohazardous wastes, as well as operator exposure to these materials. Although several journal articles focused on teaching the ELISA technique in an undergraduate laboratory setting have been previously published,[8−13] there is no report of training students on this immunoassay using the microchip platform. In particular, the surface chemistry used for immobilizing capture antibodies, and the procedures employed

DOI: 10.1021/ed4009107
J. Chem. Educ. XXXX, XXX, XXX−XXX

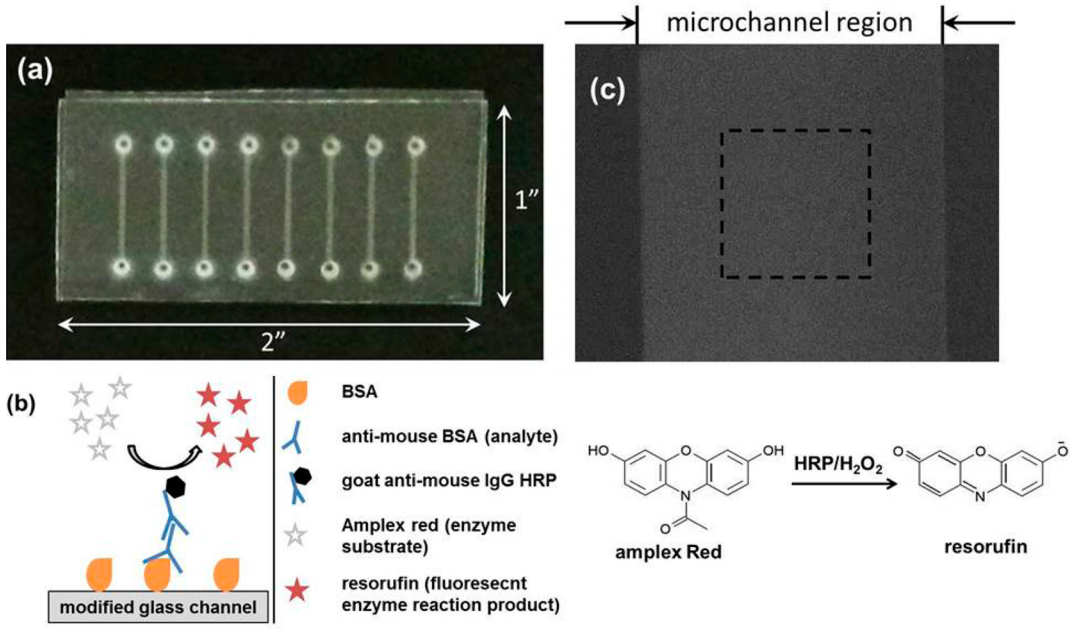


Figure 1. (a) Glass microfluidic chip used in the reported ELISA experiments. (b) Schematic of the biomolecular complex formed on the glass microchannel surface for the reported assay. The modified glass channel here refers to a glass surface reacted with (3-aminopropyl)triethoxysilane followed by glutaraldehyde. The chemical structures of Amplex Red and resorufin are shown. The enzyme reaction product (resorufin) was detected in the experiments based on its fluorescence properties yielding the ELISA signal used in quantitating the assay. (c) Fluorescence image of the ELISA microchannel after an enzyme reaction time of 30 min while assaying a 1.05×10^4-fold diluted antimouse BSA sample. The greater brightness of the microchannel channel region seen in the image is a result of the fluorescence signal being generated in it. The square box with the dotted boundaries (~500 × 500 pixels) here represents a typical window over which the fluorescence signal is quantitated using Adobe Photoshop software. The fluorescence values reported in the experimental data are the average brightness over this window as measured by Photoshop software.

for introducing/purging sample/reagents into the assay chamber, as well as the methods applied for quantitating the experimental outcomes in a microfluidic ELISA, can be significantly different from those used in its traditional microtiter-plate-based counterpart.

The current experiment fills this gap in the undergraduate educational curriculum by developing a module for performing ELISAs in glass microchannels and quantitating them using a standard epifluorescence microscope system within two 3-h laboratory periods. Moreover, the module introduces students to basic image analysis techniques, as well as signal-to-noise ratio and limit of detection calculations, to characterize this bioanalytical method, which makes it an ideal educational tool for training students in science and engineering majors. The microfluidic ELISA experiment described was successfully introduced into an upper-division undergraduate chemistry course titled "Instrumental Methods of Chemical Analysis" recently, demonstrating its effectiveness as a hands-on approach for educating the practice of this miniaturized immunoassay method.

EXPERIMENTAL PROCEDURE

Students work in groups of two. Glass microfluidic devices produced on a 2 × 1 in. footprint using standard photolithographic and wet-etching techniques[14,15] are provided to the students. These devices comprise an array of 8 microchannels, each 30 μm deep, 500 μm wide, and 1.5 cm long, that serve as the assay compartments in the experimental design (Figure 1a). The microchannels are prepared for an ELISA by students by sequentially derivatizing them with solutions of (3-aminopropyl)triethoxysilane and glutaraldehyde at room temperature to prepare a surface that can be covalently bonded to amine groups on a protein molecule. The microfluidic conduits are reacted with a 1% (by weight) bovine serum albumin (BSA) solution prepared in a 0.1 M carbonate–bicarbonate buffer, pH 9.4, and later incubated with a chosen dilution of an antimouse BSA (analyte) sample prepared in a 0.1 M phosphate buffer, pH 7.4. The ELISA surface is completed by treating the analysis channels with goat antimouse IgG horseradish peroxidase (HRP) conjugate (Figure 1b) where IgG refers to the immunoglobulin G protein complex. The enzyme reaction is initiated by introducing a solution containing 10 μM Amplex Red and 5 μM hydrogen peroxide prepared in a 0.1 M phosphate buffer, pH 7.4 into the fluidic ducts. The assays are quantitated by analyzing the fluorescent images of the microchannels taken at different time points over the enzyme reaction period (Figure 1c) using Adobe Photoshop software. Blank assay measurements are made by preparing the analysis channel in exactly the same way except the sample solution is replaced with 0.1 M phosphate buffer, pH 7.4, during the incubation step. Although ELISAs are performed in different microchannels for assessing the level of the analyte in six standard samples (five dilutions and a blank) during the first period of the laboratory, the students focus on analyzing the recorded fluorescence images in order to quantitate their microfluidic assays in the second period. A detailed experimental procedure is in the Supporting Information.

HAZARDS

(3-Aminopropyl)triethoxysilane is corrosive and causes eye and skin burns. This chemical may also lead to severe respiratory and digestive tract irritation with possible burns if inhaled or swallowed. Glutaraldehyde is hazardous in the case of skin

DOI: 10.1021/ed4009107
J. Chem. Educ. XXXX, XXX, XXX−XXX

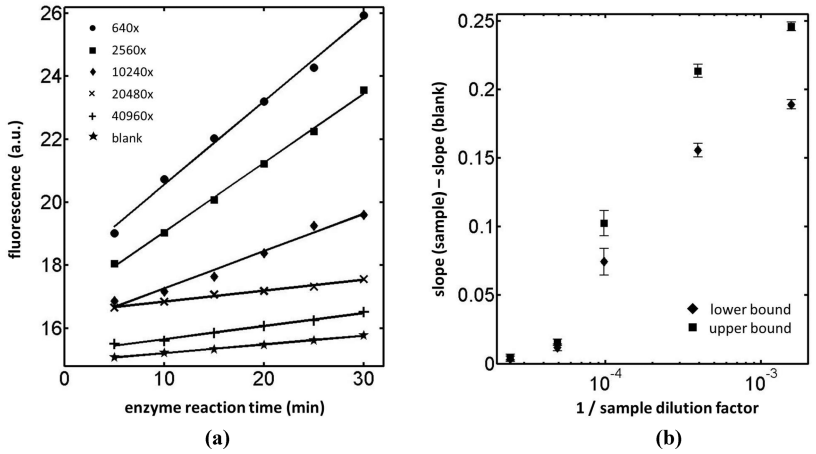


Figure 2. (a) Temporal variation in the observed fluorescence in microfluidic analysis channels over the enzyme reaction period for different dilutions of the antimouse BSA sample as reported by a representative student group. (b) Upper and lower bounds for the ELISA response curves reported by the student groups. The sample dilution factor here refers to the factor by which the original analyte solution (antimouse BSA) obtained from the commercial source was diluted to prepare the sample used in the assay.

contact (sensitizer, permeator). Liquid or spray mist may produce tissue damage particularly on mucous membranes of eyes, mouth, and respiratory tract. Inhalation of the spray mist may produce severe irritation of respiratory tract characterized by coughing, choking, or shortness of breath. Severe overexposure can result in death. Phosphate and carbonate buffers may cause irritation in eyes, skin, or the respiratory system. Amplex Red is known to cause only minor discomfort upon contact with skin or eyes but can be harmful if swallowed. Hydrogen peroxide is highly corrosive upon ingestion or contact with skin and eyes. Inhalation of the spray mist may produce severe irritation of respiratory tract characterized by coughing, choking, or shortness of breath. Prolonged exposure may result in skin burns and ulcerations. Eye protection, hand gloves, and laboratory coats are recommended while performing this experiment. Contaminated materials should be disposed appropriately as hazardous chemicals. The edges of the glass microfluidic devices pose a small cutting hazard.

RESULTS AND DISCUSSION

The antimouse BSA ELISA described in this article is quantitated by monitoring the change in fluorescence signal in the analysis channel over a 30 min long enzyme reaction period. This temporal variation recorded for different dilutions of the sample shows a linear rise at a rate that increased with an increase in the analyte concentration (Figure 2a). A calibration curve is generated for the microfluidic ELISAs by plotting the slope of the lines in Figure 2a minus the corresponding blank against the reciprocal of the dilution factor for the antimouse BSA sample (a measure of the analyte concentration) (Figure 2b).

The smallest detectable analyte concentration (limit of detection (LOD)) for the assay is subsequently determined by plotting the signal-to-noise ratio (S/N) for these measurements as a function of the analyte concentration and estimating the value of the latter quantity when S/N = 1 (Figure 3). Using this approach, an LOD value in the range of $2.88-3.90 \times 10^4$ was obtained by the student groups in terms of the dilution factor for the original antimouse BSA sample. The approach to determining the detection limit using samples with small analyte concentrations rather than a blank solution as described

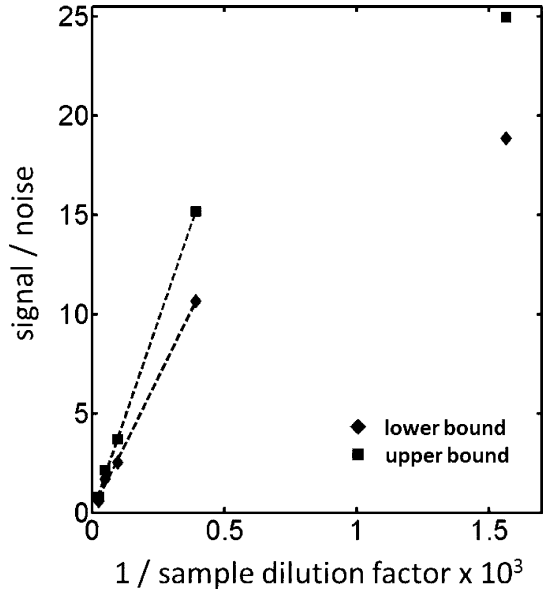


Figure 3. Estimation of LOD for the reported microfluidic antimouse BSA ELISA. The dotted lines depict the range of measurements reported by student groups. Only the smallest four dilutions of the antimouse BSA sample were used in estimating the LOD in the present experimental module as the signal-to-noise ratio yielded by them was seen to grow linearly with an increase in the analyte concentration. The solid square and diamond symbols located furthest along the *x* axis are experimental measurements that lie outside the linear range of the response curve for the reported assay and have therefore been ignored in the LOD calculations.

above is reported to yield more realistic values for LOD and has been therefore adopted in this experiment.[16]

The experiment was done in an upper-division undergraduate laboratory course, "Instrumental Methods of Chemical Analysis" during the 2012 and 2013 fall semesters. For the 2013 class of 10 students, the ELISA experiment was performed in groups of two. These groups were rotated between five different experiments over the course of the semester, which meant that the epifluorescence microscope was available to each group for the entire two periods during their turn. The range of data obtained by the student groups in these

DOI: 10.1021/ed4009107
J. Chem. Educ. XXXX, XXX, XXX−XXX

experiments in Figures 2b and 3 was observed to deviate by <20% from the idealized data set obtained by the authors. The corresponding limit of detection estimates (in terms of the dilution factor for the antimouse BSA sample) reported by these groups were also found to lie within 20% of the corresponding idealized value.

Overall, all student groups were able to complete the reported ELISA experiment within their allotted laboratory periods and had a very positive feedback on this experiment in terms of its educational value. Moreover, results from the course final examination showed that the students were able to learn the underlying principles for the microfluidic ELISA method effectively through the reported experimental module (see Supporting Information for questions used). On the fall 2012 final examination, 8 out of the 11 students were able to solve a numerical problem related to the rate of signal generation in a hypothetical ELISA experiment. Two of the remaining students who attempted this problem followed a sound approach, but were not able to arrive at the correct numerical answer due to calculation errors. One student did not demonstrate a proper understanding of the concepts involved in the problem. Similar results (seven out of the nine students answering correctly) were obtained in fall 2013 for a problem that focused on the Michaelis–Menten rate equation for enzyme kinetics as it related to the ELISA technique.

CONCLUSIONS

An experiment for practicing the ELISA method in microfluidic channels has been successfully developed for an upper-division undergraduate chemistry laboratory course. The experiment can be completed in two 3-h periods (including all incubation and data analyses procedures) using only one microliter of sample and reagents per assay, which makes it significantly more time and cost-effective compared to its traditional microtiter plate-based counterpart. Furthermore, it introduced a modern nanotechnology-based bioanalytical platform to undergraduate students, and at the same time served as a powerful educational tool for teaching some of the important data analyses techniques relevant to science and engineering majors. Although the present microfluidic immunoassay allowed the quantitative determination of antimouse BSA in a sample, it can be readily applied to detecting other biomolecules of practical interest. Moreover, it can be adapted to train students on the competitive version of the immunoassay as well as integrating the ELISA method to other analytical procedures, such as preconcentration of the sample[17] or the enzyme reaction product,[18] for improving the assay sensitivity.

ASSOCIATED CONTENT

Supporting Information

A student handout providing more background material on the reported ELISA module and a detailed step-by-step procedure for the students to follow to complete this experiment. Instructor's Notes that describe the device fabrication procedure, list the supplies/reagents needed for the experiment along with the names of their vendors, and present a couple of sample assessment questions on the topic. This material is available via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Corresponding Email: ddutta@uwyo.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research work was supported by a grant from the National Science Foundation (DBI 0964211) and the National Institutes of Health (1R15AG045755-01A1). B.G. also acknowledges graduate assistantship through the Wyoming INBRE program (grant # P20RR016474 and P20GM103432). The authors would like to thank Dale Isaak and Robert Corcoran for their guidance on the reported assay.

REFERENCES

(1) Mor, G.; Visintin, I.; Lai, Y.; Zhao, H.; Schwartz, P.; Rutherford, T.; Yue, T.; Bray-Ward, P.; Ward, D. C. Serum protein markers for early detection of ovarian cancer. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7677–7682.

(2) Van Hengel, A. J. Food allergen detection methods and the challenge to protect food-allergic consumers. *Anal. Bioanal. Chem.* **2007**, *389*, 111–118.

(3) Sherry, J. P. Environmental chemistry – The immunoassay option. *Crit. Rev. Anal. Chem.* **1992**, *23*, 217–300.

(4) Kemeny, D. M.; Challacombe, S. J. *ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects*; John Wiley & Sons: New York, 1998.

(5) Eteshola, E.; Leckband, D. Development and characterization of an ELISA assay in PDMS microfluidic channels. *Sens. Actuators, B* **2001**, *72*, 129–133.

(6) Yanagisawa, N.; Dutta, D. Kinetic ELISA in microfluidic channels. *Biosensors* **2011**, *1*, 58–69.

(7) Yanagisawa, N.; Mecham, J. O.; Corcoran, R. C.; Dutta, D. Multiplex ELISA in a single microfluidic channel. *Anal. Bioanal. Chem.* **2011**, *401*, 1173–1181.

(8) Anderson, G. L.; McNellis, L. A. Enzyme-linked antibodies: A laboratory introduction to the ELISA. *J. Chem. Educ.* **1998**, *75*, 1257–1277.

(9) O'Hara, P. B.; Sanborn, J. A.; Howard, M. Pesticides in drinking water: Project-based learning within the introductory chemistry curriculum. *J. Chem. Educ.* **1999**, *76*, 1673–1677.

(10) Weck, R.; VanPutte, R. A simple and rapid ELISA for detecting aflatoxin contamination in corn. *Am. Biol. Teach.* **2006**, *68*, 492–495.

(11) Brokaw, A.; Cobb, B. A. A simple test tube-based ELISA experiment for the high-school classroom. *Biochem. Mol. Biol. Educ.* **2009**, *37*, 243–248.

(12) Botasini, S.; Luzuriaga, L.; Cerda, M. F.; Mendez, E.; Ferrer-Sueta, G.; Denicola, A. Multiple experiments and a single measurement: Introducing microplate readers in the laboratory. *J. Chem. Educ.* **2010**, *87*, 1011–1014.

(13) Powers, J. L.; Rippe, K. D.; Imarhia, K.; Swift, A.; Scholten, M.; Islam, N. A direct, competitive enzyme-linked immunosorbent assay (ELISA) as a quantitative technique for small molecules. *J. Chem. Educ.* **2012**, *89*, 1587–1590.

(14) Bien, D. C. S.; Rainey, P. V.; Mitchell, S. J. N.; Gamble, H. S. Characterization of masking materials for deep glass micromachining. *J. Micromech. Microeng.* **2003**, *13*, S34–S40.

(15) Toh, G. M.; Yanagisawa, N.; Corcoran, R. C.; Dutta, D. A low molecular weight cut-off polymer-silicate membrane for microfluidic applications. *Microfluid. Nanofluid.* **2010**, *9*, 1135–1141.

(16) Armbruster, D. A.; Tillman, M. D.; Hubbs, L. M. Limit of detection (LOD)/limit of quantitation (LOQ): Comparison of the empirical and statistical methods exemplified with GC-MS assays of abused drugs. *Clin. Chem.* **1994**, *40*, 1233–1238.

DOI: 10.1021/ed4009107
J. Chem. Educ. XXXX, XXX, XXX–XXX

(17) Yanagisawa, N.; Dutta, D. Enhancement in the sensitivity of microfluidic enzyme-linked immunosorbent assays through analyte preconcentration. *Anal. Chem.* **2012**, *84*, 7029−7036.
(18) Giri, B.; Dutta, D. Improvement in the sensitivity of microfluidic ELISA through field amplified stacking of the enzyme reaction product. *Anal. Chim. Acta* **2014**, *810*, 32−38.

DOI: 10.1021/ed4009107
J. Chem. Educ. XXXX, XXX, XXX−XXX