analytical chemistry

Article

pubs.acs.org/ac

Paper-Based Enzyme Immobilization for Flow Injection Electrochemical Biosensor Integrated with Reagent-Loaded Cartridge toward Portable Modular Device

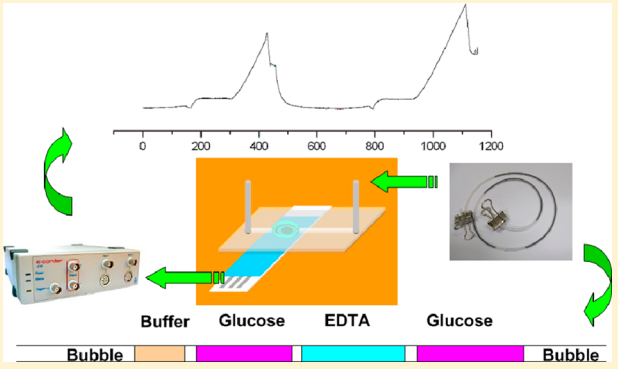
Swee Ngin Tan,[†] Liya Ge,[†] Hsih Yin Tan,[‡] Weng Keong Loke,[‡] Jinrong Gao,[§] and Wei Wang*[,§]

[†]Natural Sciences and Science Education Academic Group, Nanyang Technological University, 1 Nanyang Walk, 637616, Singapore

[‡]DSO National Laboratories, 20 Science Park Drive, 118230, Singapore

[§]School of Chemical and Biological Engineering, Yancheng Institute of Technology, 9 Yingbin Road, Yancheng, 224051, China

ABSTRACT: Paper-based enzyme immobilization for a flow injection electrochemical biosensor integrated with a reagent-loaded cartridge toward a portable device was developed. A paper disk was immobilized with enzyme, then it was integrated in a flow cell as an electrochemical biosensor. A silicon tube reagent-loaded cartridge was integrated into the system, a complicated procedure was simplified as a one-click operation toward development for point-of-care applications. In this research, glucose oxidase (GOx) was employed as a model enzyme, silver ion as an inhibition reagent for GOx, and EDTA as a regeneration reagent. When GOx was inhibited by silver ions, glucose was introduced for electrochemical measurements before and after inhibited enzyme regeneration and the difference was caused by silver inhibition. The modular device has great potential for other applications, e.g., detection of enzyme activity and substrate. The platform based on double-test mode provided accurate results due to elimination of an average or control value in comparison with classical routine approaches.



Analytical methodologies based on enzymes have increased exponentially and gained popularity and significance over the last few decades. This is mainly related to the high selectivity and specificity of enzymes that enable the determination of single analyte species in complex samples.[1] The implementation of biocatalytic procedures in flow systems as an alternative to conventional batch assays can effectively control the reaction conditions and allow the maximization of enzyme activity.[2]

Electrochemical biosensors combine the sensitivity of electrochemical transducers with the high specificity of biological recognition processes. These devices contain a biological recognition element (e.g., enzymes, proteins, antibodies, nucleic acids, cells, tissues, or receptors) that selectively reacts with the target analyte and produces an electrical signal that is related to the concentration of the analyte being studied. Electrochemical biosensors can be divided into two main categories based on the nature of the biological recognition process, i.e., biocatalytic and affinity modes.[3] Enzyme electrodes are electrochemical probes with a thin layer of immobilized enzyme on the surface of the working electrode.[4−6] The enzyme is the most critical component of the enzyme electrode, and the shelf life and stability of the enzyme generally determine the lifespan of the biosensor.

Screening-printing technology can be easily applied to the mass production of inexpensive, reproducible, and sensitive disposable electrodes,[7,8] and screen-printed electrodes (SPEs) have been applied in portable devices. Paper represents a great and useful supporting material for developing sensing devices.[9−18] A dry filter paper can sorb more than its dry mass of aqueous solution when immersed in a bath, removal of the paper and drying will leave all the nonvolatile components of the aqueous solution in the paper structure. Printing and coating technologies allow for the applications of almost any fluid onto dry paper. Aqueous solutions are particularly easy because capillary forces and the hydrophilic nature of cellulose promote rapid sorption. Antibodies, enzymes, aptamers, and phages can be spotted or printed onto dry filter paper without denaturation. The bioreceptor adheres to the paper surface because of physical immobilization, van der Waals, and electrostatic forces. Such desirable properties have been applied on paper-based enzyme-linked immunosorbent assay (ELISA).[19]

A simple method for handling multiple reagents is the bottleneck for portable devices. Whitesides' group reported a cartridge for storing reagents and delivering sequentially the reagents in a microfluidic device to carry out a bioassay.[20]

Motivated by the inspired applications mentioned above, based on the advantages of microfluidics, we proposed paper-

Received: September 3, 2012
Accepted: November 1, 2012
Published: November 1, 2012

based enzyme immobilization for flow injection electrochemical biosensor integrated with reagent-loaded cartridge toward portable modular device. A paper disk is applied for prestorage of enzyme and integrated with screen printed electrode SPE in a flow system, and the modular enzyme immobilization paper can be replaced conveniently. Glucose oxidase (GOx) is applied as a model enzyme, and Ag^+ inhibition is tested as demonstration of concept. The products of catalytic reaction were measured before and after enzyme regeneration, and the difference resulted from enzyme inhibition by Ag^+ ions. Because of the combination of the plug-in cartridge and the activity of enzyme, inhibition analyte, and substrate could be determined accurately with one simple click. This simple technology will open up a new avenue for rapid point-of-care applications to widen the applicability of biosensors.

EXPERIMENTAL SECTION

Materials and Reagents. All chemicals used for the preparation of stock and standard solutions were of analytical grade. Glucose oxidase (type VII from *Aspergillus niger*; 192 000 units/g) and β-D-glucose were obtained from Sigma. $AgNO_3$ was analytical grade reagents obtained from Aldrich. Stock glucose solutions (freshly prepared, in a phosphate buffer pH 7.0, 0.1 M) were allowed to mutarotate at room temperature overnight before use. Glucose standards were prepared by serial dilution of the stock solution. The inhibitor standards were prepared by serial dilution from their respective stock solutions, and EDTA 2.5 mM in water was employed unless otherwise stated.

Tygon 3350 silicone tubing 1/32 × 3/32 (0.8/2.4 mm internal/external diameter) was applied for storing solutions, and 200 μL of solution in the tubing is about 40 cm length, with a 4 cm air space for an interval between solutions. The dye for plug visualization was bought from Waterman (Janesville, WI). Whatman filter paper no. 1 was obtained from Cole-Parmer (Vernon Hills, Illinois).

Apparatus. All electrochemical characterizations and calibrations were performed using a four channel system (eDAQ QuadStat, e-Corder 8 and Echem software, eDAQ Europe, Poland). A disposable screen-printed carbon electrode (SPCE) (Dropensens, Spain) was employed as the working electrode (4 mm diameter disk) and a Ag/AgCl and a carbon ring as the reference and counter electrodes, respectively. Solutions were pumped with a syringe pump (New Era Pump Systems, Inc. NE-1000 programmable single syringe pump).

Fabrication of Paper Disk for Enzyme Immobilization and Assembly of Detection Chip. Whatman filter paper no. 1 was carefully cut into round sheet with ~7 mm diameter. Then, 5 μL of 200 U/mL GOx (pH 4.5) solution was dropped on the paper and allowed to dry at room temperature. The flow chip was shown in Figure 1a. The channel width and depth were 1 and 0.4 mm, respectively. The detection round chamber diameter and depth were 7 and 1 mm, respectively. Detection device was assembled as described in Figure 1, and it was integrated with the order of flow chip (a), poly(dimethyl siloxane) (PDMS) shim (b), paper disk impregnated with enzyme(c), and SPCE (d). The device was clamped for operation as shown in Figure 1e.

Preparation of Reagent-Loaded Cartridge. Liquids/air was aspirated into the cartridge using a manually operated syringe. We prepared the cartridges by cutting commercially available Tygon 3350 silicone tubing 1/32 × 3/32, polyethylene (PE) tubing into 200-cm-long units. Volumes of 200

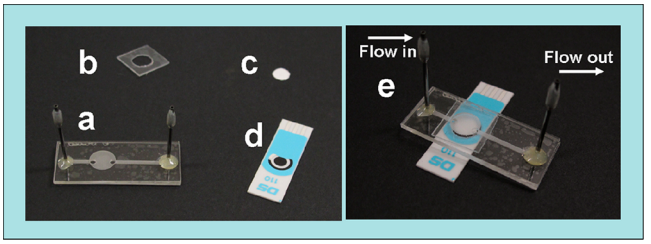


Figure 1. Assembly of detection chip: flow chip (a), PDMS shim (b), paper disk impregnated with enzyme (c), and SPCE (d), and the sections were assembled (e) for application with a simple clamp.

μL of buffer, 200 μL of 10 mM glucose, 200 μL of 2.5 mM EDTA, and 200 μL of 10 mM glucose were aspirated into the tube sequentially with 4 cm air spacers. When the cartridge preparation was completed, we sealed the tubing end with clamps. Finished reagent-loaded cartridge with dye for visualization was shown in Figure 2.

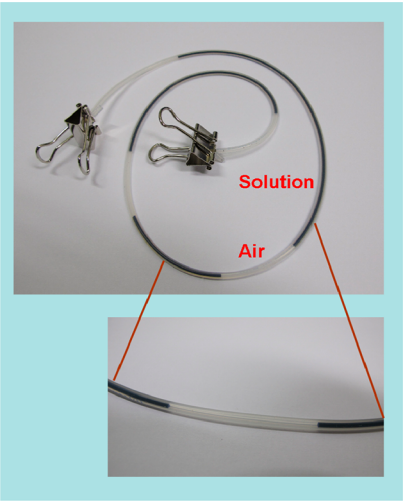


Figure 2. Reagent-loaded cartridge. Dye was employed for plug visualization.

Portable Modular Device for Enzyme Inhibition Detection. Measurements were performed in the portable device at room temperature. Typically 200 μL of Ag^+ (0.15−1.2 μM) inhibition solution was pumped through the detection cell with a flow rate of 100 μL/min, after inhibition, a reagent-loaded cartridge was connected to a detection chip and the reagents were pumped with a flow rate of 100 μL/min, when the detection cell was full of reagents, namely, glucose and EDTA, Typically the reagents were stopped 3 min for complete reaction, respectively. The amperometric response was recorded, and the applied potential is 0.30 V vs Ag/AgCl. The inhibition degree (percentage) is evaluated as $(I_0 - I)/I_0 \times 100$, where I_0 and I are the glucose responses before and after inhibition, respectively.

RESULTS AND DISCUSSION

Motivation of the Design. The aim of this work is to develop a miniaturized, self-contained, single-use, portable modular enzyme assay device for quantitative determination of enzyme, enzyme inhibition, and substrate with one simple click. In the current enzyme assays, a control or baseline is needed because the measured value of enzyme activity has to be

dx.doi.org/10.1021/ac302537r | *Anal. Chem.* 2012, 84, 10071−10076

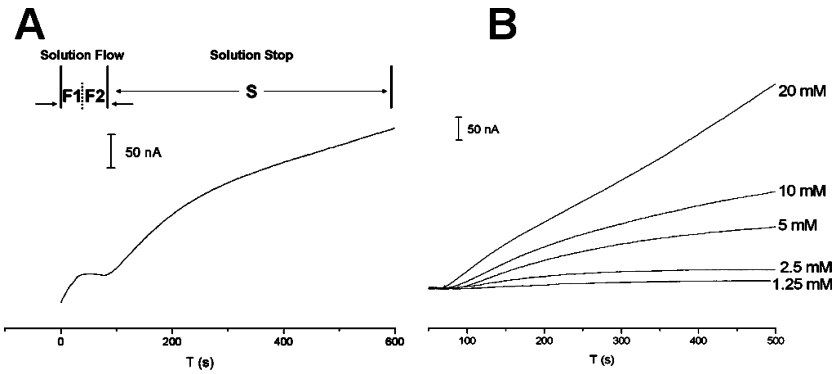


Figure 3. Amperometric response of 5 μL of 200 U/mL GOx in the paper disk, and 10 mM glucose was pumped through the reaction cell at a flow rate of 100 μL/min (A). Electrochemical response for different concentrations of glucose in stop flow (B). Detection conditions: applied potential was 0.3 V vs Ag/AgCl.

compared with the unexposed normal (baseline) values. To circumvent this problem, a statistically derived value of enzyme activity measured from a large sample size of population generally serves as the control. However, these methods are not accurate because of the large variability in baseline values derived from the variation of enzyme activity between individuals and the deviation of measurement methods from different laboratories.[21−24] Moreover, this current approach is rather time-consuming and costly.

Toward the direction of point-of-care application, we propose a portable modular device, which is a flow injection electrochemical biosensor with paper-based enzyme immobilization and integrated with reagent-loaded cartridge. In this approach, at first, a double-test mode detects the variation of enzyme activity before and after reactivation. This method provides advantages since a control enzyme is not needed and it excludes inter- or intraindividual variation in the normal levels of enzyme. Therefore, this measurement is an accurate and reliable determination. Also, a paper platform is suitable for enzyme assay as it has been demonstrated by paper-based ELISA.[19] In this research, a paper disk is employed as a carrier matrix for enzyme storage and the component in the modular device is inexpensive and thus can be easily replaced. This paper-based enzyme immobilization electrochemical biosensor combines the sensitivity and specificity of the enzyme assay with the convenience, low cost, and ease-of-use of paper-based platforms. Furthermore, reagent-loaded cartridge is employed for storage of liquid reagents which eliminates the cumbersome requirement of reagent dispensing during a typical conventional measurement. It has numerous potential advantages for point-of-care applications. In general, this method is inexpensive, is portable, and provides real time results. For a point-of-care device, an ideal application is to provide a direct answer with only a simple sample introduction leading to the desired analytical results. The proposed device exactly meets the key requirement of practical applications.

Enzyme Immobilization and its Catalytic Reaction. First, we tested the feasibility of GOx catalytic reaction in the flow system: 5 μL of 200 U/mL GOx was spotted in a paper disk and allowed to dry under ambient conditions, and the enzyme adsorbed on the paper disk was positioned in the detection cell of the portable device. Phosphate buffer was pumped through the detection cell, and then 10 mM glucose was pumped in as the subsequent follow up solution. The amperometric response of GOx enzymatic catalytic reaction was investigated as shown in Figure 3A. The substrate glucose solution was pumped through the detection cell (sections F1 and F2), then the glucose solution was stopped in the detection cell (section S) for the enzymatic reaction. In the beginning of the F1 section, the substrate glucose solution flowed through the detection cell and replaced the previous phosphate buffer progressively and the signal thus increased gradually; obviously, this increase was attributed to the oxidation of hydrogen peroxide, an enzymatic product of the glucose substrate which was catalyzed by active GOx. When the detection cell was full of substrate glucose solution, as shown in section of F2, the concentration of enzymatic product was kept at a steady state level under the stable flow velocity, and the signal did not change with time. After the substrate solution was stopped in the detection cell, as in section S, the concentration of enzymatic product increased with the reaction time; it follows the Michaelis−Menten kinetic equation. On the basis of the amperometric response, the current data for the reaction 3 min in stop-flow status was collected for analysis due to its steep slope. Different concentrations of glucose were applied in the system (Figure 3B), in order to maintain a proper sensitivity and avoid big errors in operation; thus, 10 mM of glucose was selected for the enzyme inhibition study.

Second, we tested the stability of GOx adsorbed in the paper. The substrate solution of 10 mM glucose was pumped through the detection cell and stopped for reaction repeatedly; on the basis of the data from Figure 3, the glucose solution was pumped for 2 min and then stopped for 3 min. The electrochemical signals were recorded in Figure 4, and good repeatability was achieved from the low relative standard deviation (RSD) 2.7%, obtained for a series of five repetitive measurements. The results demonstrated that the GOx was absorbed well on the paper matrix and remained stable in the paper disk and met the requirements for multiple analyses. Paper disk with absorbed GOx was tested after 1 month for shelf life investigation (stored in an airtight plastic bag at 4 °C until assay), and the signal decreased about 9.6% and good repeatability was still obtained with RSD 4.2%.

Enzyme Inhibition and Regeneration. Enzyme inhibition and regeneration efficiency were investigated: initial enzyme activity was tested, then the inhibition solution 0.3 μM Ag^+ was pumped through the detection cell for 3 min (inhibition step), then amperometric signal decreased; after treating with 2.5 mM EDTA for 5 min (regeneration step), 96% of the enzymatic activity could be regained (Figure 5). For each reaction for three repetitive measurements, the RSD was less than 4.3%. On the basis of the nearly complete

dx.doi.org/10.1021/ac302537r | Anal. Chem. 2012, 84, 10071−10076

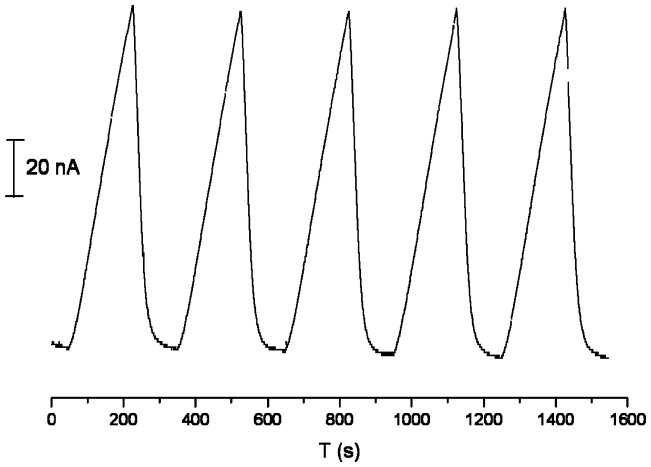


Figure 4. Repeatitive detection with electrochemical response for enzyme catalytic reaction in the flow chip. A volume of 5 μL of 200 U/mL GOx was immobilized in the paper disk, and 10 mM glucose was pumped through the reaction cell with 100 μL/min for 2 min and then flow was stopped for 3 min reaction. Detection conditions: applied potential was 0.3 V vs Ag/AgCl.

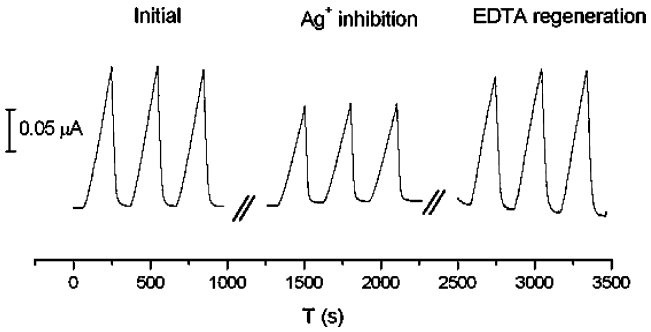


Figure 5. Reactivation efficiency of GOx treated with 2.5 mM EDTA for 6 min after 0.3 μM Ag^+ inhibition, and each reaction was repeated 3 times. Other experimental details were as in Figure 4.

regeneration, the proposed technique can be applied for the detection of inhibition analyte. On the basis of previous research,[25] GOx can be inhibited by Ag^+ in 2 min and regenerated within 3 min; in this investigation, 3 min for inhibition and 6 min for regeneration were applied for complete reaction.

Reagent-Loaded Cartridge and Flow Velocity. For a disposable and portable device, storing and delivering a sequence of reagents is a critical key step in the evaluation of a device's performance. In this method, cartridges made of commercially available Tygon 3350 silicone tubing were filled by sequentially injecting plugs of reagents separated by air spacers. They were 200 μL of buffer, 200 μL of 10 mM glucose (40 cm length), 200 μL of 2.5 mM EDTA (40 cm length), and 200 μL of 10 mM glucose (40 cm length) were aspirated into the tube sequentially with 4 cm air spacers. The air spacers can prevent the reagents from mixing with each other during cartridge preparation, storage, and usage. In the process of analysis, the cartridge was connected with the detection chip and the reagents were delivered with a constant flow pump.

Plug-to-plug contamination was tested. Using dyed plugs, we observed that the plugs left small amounts of residues behind them as they moved through the tubing when the flow rate reached 120 μL/min; since the subsequent plugs in the sequence collected the residue, this process resulted in plug-to-plug contamination. In this research, 100 μL/min flow rate was applied for delivering reagents and plug-to-plug contamination could be avoided.

Paper-Based Enzyme Immobilization for a Flow Injection Electrochemical Biosensor Integrated with a Reagent-Loaded Cartridge for Silver Detection. We demonstrated that an integrated biosensor could be used for detection of silver ion. The biosensor included two parts, namely, the enzyme reaction cell (Figure 1e) and the reagent-loaded cartridge (Figure 2). After 200 μL of Ag^+ inhibition solution was pumped through the detection cell with a flow rate of 100 μL/min, the reagent-loaded cartridge was connected to the detection chip, the reagents were pumped with a flow rate of 100 μL/min, and the amperometric response was recorded. We carried out a series of different concentrations of Ag^+ inhibition solutions. The signals and the reagents matched quite well, as shown by representative amperometric response in Figure 6. In step 1, when the buffer was pumped into the

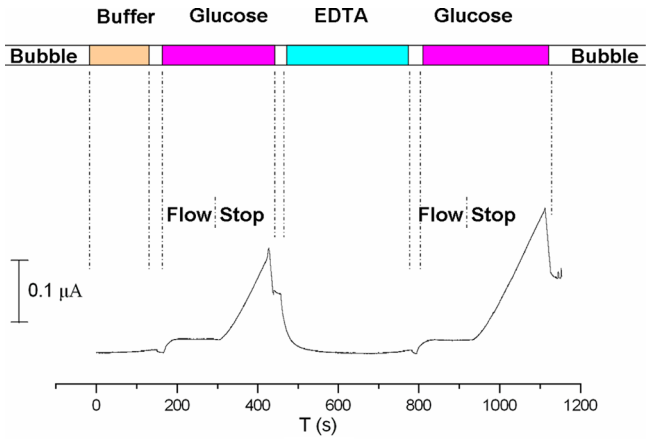


Figure 6. Current−time response curve with portable modular device applied for the detection of enzyme inhibition. Reactivation of GOx was treated with 2.5 mM EDTA for 6 min after 0.3 μM Ag^+ inhibition. Other experimental details were as in Figure 4.

detection cell, a baseline electrochemical response was recorded. In step 2, the substrate glucose was pumped into the detection cell and stopped for 3 min; the phenomenon observed was the same as that shown in Figure 3, then when the solution started to flow again, the signal decreased. In step 3, the regeneration solution EDTA was pumped into the detection cell, electrochemical signal decreased to baseline again, and in order to keep the enzyme regeneration step complete, the flow of EDTA solution was stopped and the reaction time was kept at 6 min. In step 4, the substrate glucose was pumped into the detection cell again, and a stronger electrochemical signal occurred due to GOx enzyme regeneration. The difference between two signals results in the inhibition efficiency. Interestingly, when each bubble reached the detection cell, the signal became noisy, and after subsequent following solution entered the detection cell, the signal resumed to be normal again. It indicated that the air spacers did not cause interferences in the process of analysis. When the bubbles reaching the electrode surface after the glucose solution had entered into the detection cell, the signals did not decrease steeply but were maintained at the same level, because the enzymatic product H_2O_2 was neither increasing like the stopped reaction nor decreasing like the flowing status; it is

dx.doi.org/10.1021/ac302537r | Anal. Chem. 2012, 84, 10071−10076

caused by the wetting layer of glucose solution on the electrode surface becoming thinner with the passage of an air bubble.[26] The signal decreased steeply until the next solution replacement on the electrode surface took place.

Additionally, we observed that the electrochemical signal after EDTA solution treatment could reach the same level of baseline generated from buffer under the applied conditions (Figure 6). It implied that no glucose solution residue remained in the reaction cell and demonstrated that the initial solution could be replaced thoroughly by the following one with the flow rate of 100 μL/min for 2 min in this design. Because of the presence of a paper disk in the round cell, the flow profile and the mechanism of replacement of different solutions are complex. On the basis of the research about segmented gas–liquid flow,[20,26,27] flow-based enzyme kinetics,[28] and our previous experience about paper disk,[16] the proposal for enzyme inhibition detection with plug-to-plug reagent-loaded cartridge has practical applications.

Silver was detected in the range between 0 and 1.5 μM. The relationship of inhibition degree of GOx and silver concentration was shown in Figure 7.

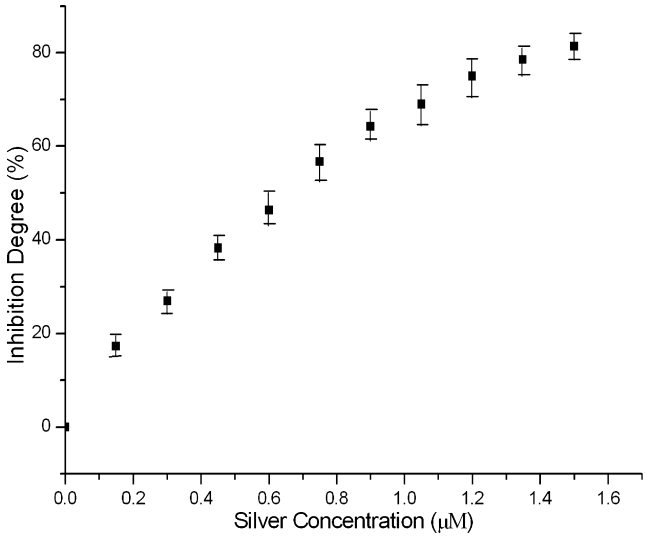


Figure 7. Relationship of inhibition degree of GOx and silver concentration. Experimental details were as in Figure 6.

The design can be easily extended for enzyme activation detection and substrate detection. For enzyme activation detection, a series of known substrates were pumped through the detection cell and the changes of enzymatic product concentrations can be used to deduce enzyme activation. Moreover, for substrate detection, the comparison of enzymatic products from known substrate and unknown sample can be used to deduce the concentration of sample. In this experimental approach, the double-test mode excludes inter- or intraindividual variation avoiding an average or control value.

CONCLUSIONS

We have developed here for the first time paper disk impregnated with enzyme applied in a flow system integrated with a reagent-loaded cartridge toward disposable device. We demonstrate that its low-cost, simplicity, reproducibility, and stability for silver ion detection with a double test mode via regeneration of GOx. The ease of fabrication of the paper disk impregnated with enzyme and a reagent-loaded cartridge which is suitable for mass production also opens opportunities for a wide range of applications in the areas of medical health, food safety, and environmental monitoring.

AUTHOR INFORMATION

Corresponding Author

*Phone: +86-515-88298848. Fax: +86-515-88298959. E-mail: wangw@ycit.edu.cn.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We wish to thank the Agency for Science, Technology and Research of Singapore (Grant SOP-BMRC300/F01-02), the National Natural Science Foundation of China (Grant 20875080), International Cooperation Project of Jiangsu Province (Grant BZ2010053), and Key Laboratory for Advanced Technology in Environmental Protection of Jiangsu Province (Grant AE201017) for financial support.

REFERENCES

(1) Illanes, A. *Enzyme Biocatalysis: Principles and Applications*; Springer Science/Business Media B.V: New York, 2008.
(2) Hansen, E. H. *Anal. Chim. Acta* **1989**, *216*, 257–266.
(3) Wang, J. *Analytical Electrochemistry*; John Wiley & Sons VCH: Hoboken, NJ, 2006.
(4) Savizi, I. S. P.; Kariminia, H. R.; Ghadiri, M.; Roosta-Azad, R. *Biosens. Bioelectron.* **2012**, *35*, 297–301.
(5) Wang, J. *Anal. Chem.* **1995**, *67*, 487–492.
(6) Ronkainen, N. J.; Halsall, H. B.; Heineman, W. R. *Chem. Soc. Rev.* **2010**, *39*, 1747–1763.
(7) http://www.dropsens.com (accessed July 2012).
(8) http://www.gwent.org/Gem/index.html (accessed July 2012).
(9) Ali, M. M.; Aguirre, S. D.; Xu, Y.; Filipe, C. D. M.; Pelton, R.; Li, Y. *Chem. Commun.* **2009**, 6640–6642.
(10) Martinez, A. W.; Phillips, S. T.; Whitesides, G. M. *Anal. Chem.* **2010**, *82*, 3–10.
(11) Lankelma, J.; Nie, Z. H.; Carrilho, E.; Whitesides, G. M. *Anal. Chem.* **2012**, *84*, 4147–4152.
(12) Zhao, W.; Ven den Berg, A. *Lab Chip* **2008**, *8*, 1988–1991.
(13) Martinez, A. W.; Phillips, S. T.; Carrilho, E.; Thomas, S. W., III; Sindi, H.; Whitesides, G. M. *Anal. Chem.* **2008**, *80*, 3699–3707.
(14) Zhao, W.; Ali, M. M.; Aguirre, S. D.; Brook, M. A.; Li, Y. *Anal. Chem.* **2008**, *80*, 8431–8437.
(15) Martinez, A. W.; Phillips, S. T.; Butte, M. J.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **2007**, *46*, 1318–1320.
(16) Tan, S. N.; Ge, L.; Wang, W. *Anal. Chem.* **2010**, *82*, 8844–8847.
(17) Wang, W.; Wu, W.; Wang, W.; Zhu, J. J. *J. Chromatogr., A* **2010**, *1217*, 3896–3899.
(18) Delaney, J. L.; Hogan, C. F.; Tian, J.; Shen, W. *Anal. Chem.* **2011**, *83*, 1300–1306.
(19) Cheng, C. M.; Martinez, A. W.; Gong, J.; Mace, C. R.; Phillips, S. T.; Carrilho, E.; Mirica, K. A.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **2010**, *49*, 4771–4774.
(20) Linder, V.; Sia, S. K.; Whitesides, G. M. *Anal. Chem.* **2005**, *77*, 64–71.
(21) Jun, D.; Bajgar, J.; Kuca, K.; Kassa, J. Monitoring of Blood Cholinesterase Activity in Workers Exposed to Nerve Agents. In *Handbook of Toxicology of Chemical Warfare Agents*; Gupta, R. C., Ed.; Academic Press: Amsterdam, The Netherlands, 2009.
(22) Lotti, M. *Clin. Chem.* **1995**, *41*, 1814–1818.
(23) Du, D.; Wang, J.; Smith, J. N.; Timchalk, C.; Lin, Y. *Anal. Chem.* **2009**, *81*, 9314–9320.
(24) Du, D.; Wang, J.; Wang, L.; Lu, D.; Lin, Y. *Anal. Chem.* **2012**, *84*, 1380–1385.

(25) Guascito, M. R.; Malitesta, C.; Mazzotta, E.; Turco, A. *Sens. Actuators, B* **2008**, *131*, 394−402.
(26) Fethi, K.; Michael, E. R. *Int. J. Numer. Meth. Fluids* **2002**, *38*, 407−427.
(27) Kreutzer, M. T.; Gunther, A.; Jensen, K. F. *Anal. Chem.* **2008**, *80*, 1558−1567.
(28) Seong, G. H.; Heo, J.; Crooks, R. M. *Anal. Chem.* **2003**, *75*, 3161−3167.