Anal. Chem. **2010,** *82,* 4283–4287

Integrated Self-Powered Microchip Biosensor for Endogenous Biological Cyanide

Liu Deng, Chaogui Chen, Ming Zhou, Shaojun Guo, Erkang Wang, and Shaojun Dong*

State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied, Chemistry, Chinese Academy of Sciences, Changchun, Jilin, 130022, P. R. China

In this work we developed a fully integrated biofuel cell on a microchip, which consisted of glucose dehydrogenase supported (carbon nanotubes/thionine/gold nanoparticles)$_8$ multilayer as the anode, and the (carbon nanotubes/polylysine/laccase)$_{15}$ multilayer as the cathode. The as-obtained biofuel cell produced open circuit potential 620 mV and power density 302 μW cm^{-2}, showing great potential as a small power resource of portable electronics. Most importantly, for the first time we demonstrated the feasibility of developing a self-powered biosensor based on the inhibitive effect on microchip enzyme biofuel cell. With cyanide employed as the model analyte, this method showed a linear range of 3.0×10^{-7} to 5.0×10^{-4} M and a detection limit with 1.0×10^{-7} M under the optimal conditions. The detection limit was lower than the acceptable cyanide concentration in drinking water (1.9×10^{-6} M) according to the World Health Organization (WHO). This self-powered sensor was successfully used to detect the cyanide concentration in a real sample, cassava, which is the main carbohydrate resource in South America and Africa. This presented biosensor combined with a resistor and a multimeter demonstrated the general applicability as a fast and simple detection method in the determination of endogenous biological cyanide.

Cyanide is highly toxic to humans and almost all other forms of life. Uptake of CN^- often results in the cellular hypoxia, with severe damage to the medullar respiratory center and vasomotor center, by inhibiting the terminal respiratory chain enzyme, e.g., cytochrome c a3. It also increases the level of reactive oxygen species (ROS) and inhibits antioxidant defense systems by increasing the concentration of intracellular Ca^{2+} to trigger a cascade of enzymatic events. Cyanide is widespread in nature, either produced and further metabolized by cyanogenic microorganisms or integrated and stored as cyanogenic glycosides in plants like sorghum, flax, giant taro, bamboo, and cassava. Cassava is the main carbohydrate sources for about 500 million people in South America and parts of Africa.[1] The enzymatic release of cyanide from cyanogenic glycosides after

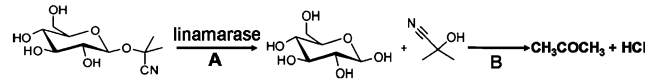


Figure 1. Structural formula and schematic representation of β-aquo-α-cyanocobyrinic acid 2 and β-aquo-α-cyanocobyrinic acid heptamethyl ester 3[a].

cell ruptures (Figure 1) exhibits great danger to the consumer and can cause severe chronic as well as significant acute public health problems. On the other hand, the widespread use of cyanide in industrial settings (1.5 million tons per year)[2] and potential threats for terrorism continue to engender significant research efforts directed toward its detection under biologically relevant conditions. Various methods have already been developed to detect and quantify cyanide. They include either the direct estimation of cyanogenic glycosides by gas chromatography,[3] potentiometric,[4] amperometric,[5] fluorometric,[6,7] or enzymatic techniques.[8] Recently, a novel concept of developing biosensor devices based on chemical-to-electrochemical energy transformations in biofuel cell (BFC) elements was developed.[9] The biofuel cell is similar to conventional fuel cells, except the precious metal catalysts are replaced with a biological catalyst.[10−12] The biofuel cells reported are usually limited in application as energy supplies due to their low efficiency. However the biocatalyst utilized in BFC, such as microorganism, organelles and enzyme, can be adjusted by the kinds of modulators (inhibitors or activators). BFC power output is related to the biocatalyst activity as well as the biocatalyst substrate. So the BFC can be regarded as a sensitive and selective method for the detection modulator or substrate

* Corresponding author. Phone: +86-431-5262101. Fax: +86-431-5689711. E-mail: dongsj@ciac.jl.cn..

(1) World Health Organization. *Background Document for Development of WHO Guidelines for Drinking-Water Quality*; World Health Organization: Geneva, Switzerland, 2007.

(2) Bhattacharya, R.; Flora, S. J. S. In *Handbook of Toxicology of Chemical Warfare Agents*; Gupta, R. C., Ed.; Academic Press: Boston, MA, 2009; pp 255−270.

(3) Nahrstedt, L. P. In *Cyanide in Biology*; Vennesland, B., Conn, E. E., Knowles, C. J., Westley, J., Wissing, F., Eds.; Academic Press: London, 1981; pp *145*−181.

(4) Yeoh, H. H.; Truong, V. D. *Food Chem.* **1993**, *47*, 295–298.

(5) Tatsuma, T.; Tani, K.; Oyama, N.; Yeoh, H. H. *Anal. Chem.* **1996**, *68*, 2946–2950.

(6) Männel-Croisé, C.; Probst, B.; Zelder, F. *Anal. Chem.* **2009**, *81*, 9493–9498.

(7) Shang, L.; Dong, S. J. *Anal. Chem.* **2009**, *81*, 1465–1470.

(8) Martinez-Manez, R.; Sancenon, F. *Chem. Rev.* **2003**, *103*, 4419–4476.

(9) Katz, E.; Bückmann, A. F.; Willner, I. *J. Am. Chem. Soc.* **2001**, *123*, 10752–10753.

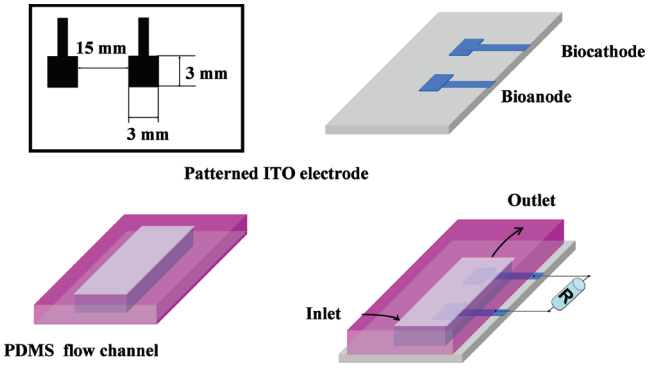
(10) Tasca, F.; Gorton, L.; Harreither, W.; Haltrich, D.; Ludwig, R.; Noll, G. *J. Phys. Chem. C* **2008**, *112*, 13668–13673.

(11) Deng, L.; Wang, F. A.; Chen, H. J.; Shang, L.; Wang, T.; Dong, S. J. *Biosens. Bioelectron.* **2008**, *24*, 329–333.

(12) Toninaga, M.; Ohtani, M.; Taniguchi, I. *Phys. Chem. Chem. Phys.* **2008**, *10*, 6928–6934.

10.1021/ac100274s
Published on Web 04/19/2010

Scheme 1. Formation of the Microchip Biofuel Cell



because of the amplifying nature imparted by substrate turnover and the specificity of the biological recognition. In fact, the low electrical current of the BFCs has advantages in the sensing processes, since redox transformation of interfering agents at the electrode can be eliminated. The success in applying self-powered glucose sensor based on integrated NAD^+-dependent enzyme BFC was reported.[9] Minteer's group used a mitochondrial-catalyzed pyruvate/air biofuel cell to detect nitroaromatic compounds;[13] however, this sensor has no quantitative relationship with the compound's concentration. The inhibitive effect of the enzyme has never been employed in a self-powered biosensor. Our work is to fabricate a self-powered biosensor based on the cyanide inhibitive effect toward the enzyme.

The development of microfluidic devices has been spurred by the desire to produce low-cost point-of-care diagnostics and environmental monitoring devices.[14] A microchip based bioanode combined with an external platinum cathode to construct a microfabricated biofuel cell was reported.[15] Our technical note focuses on the development of a biofuel cell fully integrated on a microchip and application as a self-powered biosensor for cyanide sensing. Recently, our group has fabricated an efficient membraneless enzyme biofuel cell.[16] The glucose dehydrogenase (GDH) was immobilized on the multilayer of (carbon nanotubes-(CNTs)/thionine/gold nanoparticles (AuNPs))$_8$ and acted as the bioanode catalyst. The multilayer (CNTs/polylysine (PLL)/laccase)$_{15}$ worked as the biocathode. This membraneless structure would simplify the chip fabrication, allow the cell stack up, and eliminate the cost. Accordingly, a microchip based biofuel cell with both the anode and cathode on a glass slide was designed, as shown in Scheme 1. More than three cells could be stacked up easily to increase the power output based on this design. The direct catalyzed four-electron reduction of O_2 to water was realized in laccase of this BFC cathode. The cyanide can affect the type 2 (T2) Cu of the laccase active center and inhibit the O_2 consumption but no effect on GDH activity. The decrease of O_2 reduction at the cathode of the biofuel cell in combination with the glucose oxidation at the bioanode will result in a power decrease that can be measured as the signal in the presence of cyanide, as shown in Figure 2.

EXPERIMENTAL SECTION

Reagents. Laccase (EC 1.10.3.2, from Trametes versicolor), glucose dehydrogenase (E.C. 1.1.1.47, *Thermoplasma acidophilum*, recombinant expressed in *E. coli*, initial activity 183 U/mg^{-1}), thionine, PLL, and branched-poly(ethylenimine) (B-PEI) were obtained from Aldrich Chemical Co. NADH was obtained from Beijing Ding Guo Chemical Reagent (Beijing, China). Glucose, $HAuCl_4 \cdot 3H_2O$, trisodium citrate, potassium cyanide, and other salts were purchased from Beijing Chemical Co. (China). They were used as received, without further purification. Multiwalled carbon nanotubes (95%, 20−50 nm) were purchased from Shenzhen Nanotech. Port. Co. Ltd. (China) and purified according to ref 17. The Au NPs were prepared according to ref 17. Phosphate buffer solutions (PBS, 0.2 M) consisted of Na_2HPO_4 and NaH_2PO_4 and were employed as the supporting electrolyte. All other chemicals were of analytical grade, and the solutions were prepared with the doubly distilled water.

Biofuel Cell Microchip Fabrication and Measurements. The chips containing the electrodes were prepared using standard microfabrication on indium-doped tin oxide (ITO) glass plates. For the assembly of a glucose/O_2 BFC, the (CNTs/thionine/AuNPs)$_8$GDH film modified ITO electrode acted as a bioanode, and the (CNTs/PLL/laccase)$_{15}$ film modified ITO electrode acted as a biocathode. The preparation of a multilayer modified ITO electrode was according to our previous work,[16,17] with little modification. ITO glass plates were cleaned by ultrasonication in a series of solvents, Milli-Q water, ethanol, ethanol aqueous solution saturated with NaOH, and ethanol to provide a clean, negatively charged surface. The negatively charged substrate ITO was first immersed in B-PEI aqueous solution (2 mg mL^{-1}) with 0.1 M NaCl for 15 min. The B-PEI terminated ITO electrode was formed by alternately dipping the electrode into the CNT solution (0.25 mg mL^{-1}), PLL aqueous solution (1 mg mL^{-1}) with 0.1 M NaCl, and then immersed into a laccase aqueous solution and PLL aqueous solution for 15 min. The membranes were carefully washed with distilled water. This sequence was repeated until the desired layer number of CNTs/PLL/laccase was obtained. On the other hand, the CNTs/thionine/Au NPs multilayers were grown on the B-PEI-terminated film by alternately dipping the modified ITO electrode into the CNT solution (0.25 mg mL^{-1}) and thionine aqueous solution (0.1 mM) for 15 min, respectively, and then immersed into the AuNPs solution (0.1 mM) and thionine solution for 1 h, respectively. This sequence was repeated until the desired layer number was obtained. The bioanode was completed by immersing the (CNTs/thionine/AuNPs)$_8$ multilayer film modified electrode into the GDH aqueous solution (1 mg/mL, pH 3.0) for 2 h. All modification processes were carried out in a PDMS cell with 3 mm ×3 mm (the same size as the electrode surface) × 60 mm (height). Then a PDMS flow cell with dimensions of 30 mm × 5 mm × 60 mm covered the two modified electrodes, and then the electrolyte was added into the cell to generate the power output. The electrochemical measurements of bioelectrodes were performed using an EG&G 273A electrochemical system (Princeton Applied Research). Coiled platinum wire and a Ag/AgCl (saturated KCl) electrode were used as

(13) Germain, M. G.; Arechederra, R. L.; Minteer, S. D. *J. Am. Chem. Soc.* **2008**, *130*, 15272–15273.
(14) Zhang, Q.; Xu, J. J.; Chen, H. Y. *J. Chromatogr., A* **2006**, *1135*, 122–126.
(15) Moore, C. M.; Minteer, S. D.; Martin, R. S. *Lab Chip* **2005**, *5*, 218–225.
(16) Deng, L.; Shang, L.; Wang, Y. Z.; Wang, T.; Chen, H. J.; Dong, S. J. *Electrochem. Commun.* **2008**, *10*, 1012–1015.
(17) Deng, L.; Wang, Y.; Shang, L.; Wen, D.; Wang, F.; Dong, S. *Biosens. Bioelectron.* **2008**, *24*, 951–957.

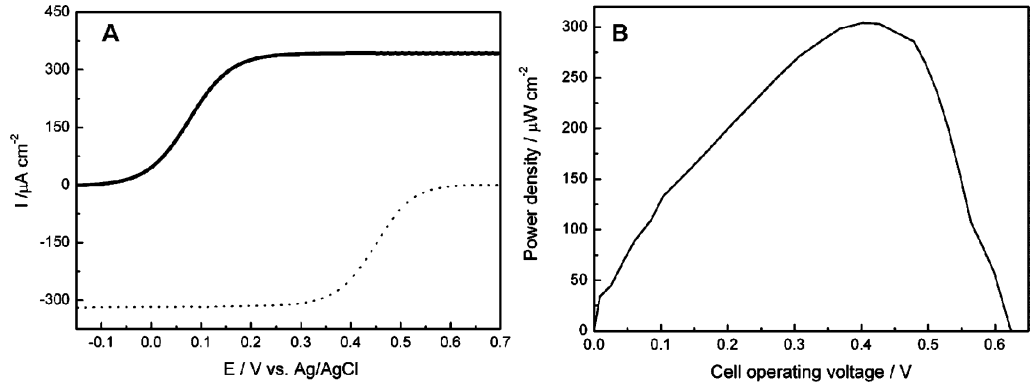


Figure 2. (A) Polarization of the anode (solid line) and of the cathode (dashed line). (B) Dependence of the power density on the cell voltage. Quiescent solution in O_2, 10 mM NADH, 40 mM glucose, and 0.2 M PBS (pH 6.5) solution.

the counter electrode and the reference electrode, respectively. Current and potential output of the biofuel cell system was measured by using a digital multimeter (Keithley 2700). We connected the two electrodes through an external 50 Ω resistor for assement of the BFC performance. All tests were conducted in a 25 °C temperature-controlled room.

Self-Powered Detection of Cyanide. A 0.1 M stock solution of KCN was prepared in a 0.01 M NaOH−$NaHCO_3$ buffer of pH 9.5, from which various CN^- concentrations were prepared by serial dilutions. The stock solutions were used within 3 days. Under the quiescent mode, the BFC record was performed after 2 min waiting time after each addition of concentrated cyanide solution in 10 mM NADH, 40 mM glucose, and 0.2 M PBS (pH 6.5). Under the flowing mode test, the variable concentrations KCN were added to 10 mM NADH, 40 mM glucose, and 0.2 M PBS (pH 7.0). The 0.2 M PBS (pH 5.0) was used to regenerate the laccase activity. To probe the selectivity, the following salts were used: KCN, CH_3COONa (NaAc), $Na_2B_4O_7$, NaBr, $KBrO_3$, NaCl, $NaClO_4$, Na_2CO_3, $K_2C_2O_4$, KF, NaI, KIO_3, $NaNO_2$, $NaNO_3$, Na_3PO_4, Na_2S, Na_2SO_3, Na_2SO_4, $K_2S_2O_8$, sodium citrate, $Fe_2(SO_4)_3$, $FeSO_4$, $CuSO_4$, $CaSO_4$, $MnSO_4$, $ZnSO_4$, and $MgSO_4$. A 0.1 M stock solution was prepared. Subsequently, the salt solution with appropriate volume was mixed with the above mixture solution.

Application of Self-Powered Biosensor. To assess the practical applicability of the present system, the detection of total cyanide in cassava was carried out. Enzymatic hydrolysis of cyanogenic glycosides from the cassava root extract is according to ref 6. A total of 2 g of fresh peeled cassava roots were ground with a zest grater and subsequently homogenized with a mortar and pestle. The cassava extract was stored in a sealed tube for 60 min at room temperature, then diluted with 5 mL of water and centrifuged for 10 min at 6000 U. The solution pH was adjusted to 7.0 with sodium hydroxide. Aliquots of 10 μL of the supernatant were used for further analysis.

RESULTS AND DISCUSSION

Figure 2A shows the polarization curves of the (CNTs/thionine/AuNPs)$_8$GDH film modified ITO anode and of the (CNTs/PLL/laccase)$_{15}$ film modified ITO cathode, in a quiescent 15 mM glucose solution in air at 25 °C in 0.2 M PBS (pH 6.5). Catalytic electrooxidation of glucose is observed at 0.0 V, and it reaches a plateau with 341 μA cm^{-2} near 0.2 V. Catalytic electroreduction of O_2 is observed at +0.55 V and reaches a plateau with 313 μA cm^{-2} near +0.35 V. These two microchip based bioelectrodes were combined to fabricate a biofuel cell. The current-output of this BFC was controlled by concentrations of glucose and O_2 in the system. The current-output was increased as the concentration of glucose increasing and reached plateau when glucose concentration rising to 30 mM in air or 40 mM in O_2. At low O_2 concetration, where electroreduction of O_2 to water at biocathode is mass transport limited in the quiescent solution, the power increases with the O_2 concentration increasing until the kinetic limit of the cathodic electrocatalysis is reached. The pH dependence of the power density was also investigated. The power density was increased with pH increasing, reached a plateau at pH 6.5, and then declined slowly above pH 8.0. The current density of the anode was near its maximum between pH 7−8.2. The current density of the cathode was nearly independent of pH in the pH 5−6.5 range. At pH < 6.5, the power was limited by kinetics of the anode. As the pH was rising, the kinetics of the anode improved and the power density increased. Above pH 6.5, the current of the cathode was lower than that of the anode, the power was controlled by the cathode. Thus, the optimal operating pH for the presented cell was 6.5. Above pH 8.0, the power decreased because of denaturation of laccase. So we chose 10 mM NADH, 40 mM glucose, 0.2 M PBS (pH 6.5) solution for the BFC operation. Figure 2B displays the polarization curve of the assembled glucose/O_2 BFC in a quiescent solution in O_2 at 25 °C in the solution mentioned above. The open-circuit voltage is 620 mV, and the maximum power density is 302 μW cm^{-2}. Compared with the results in our previous work, this integrated biofuel cell power output decreased by 8%. This results suggested that our designation of this microchip biofuel cell was applicable, since the power almost retained that of the large biofuel cell. Compared with the reported miniaturized enzyme biofuel cell, the maximum power density was almost 10 times higher than that of the integrated bioanode combined with an external platinum cathode.[15] This miniaturized biofuel cell shows great potential in powering the small autonomous sensor-transmitter systems in animals and in plants, since its work condition is similar to the physiological condition.

To investigate the feasibility of cyanide detection with this microchip biofuel cell, 0.1 mM KCN was added into the cell

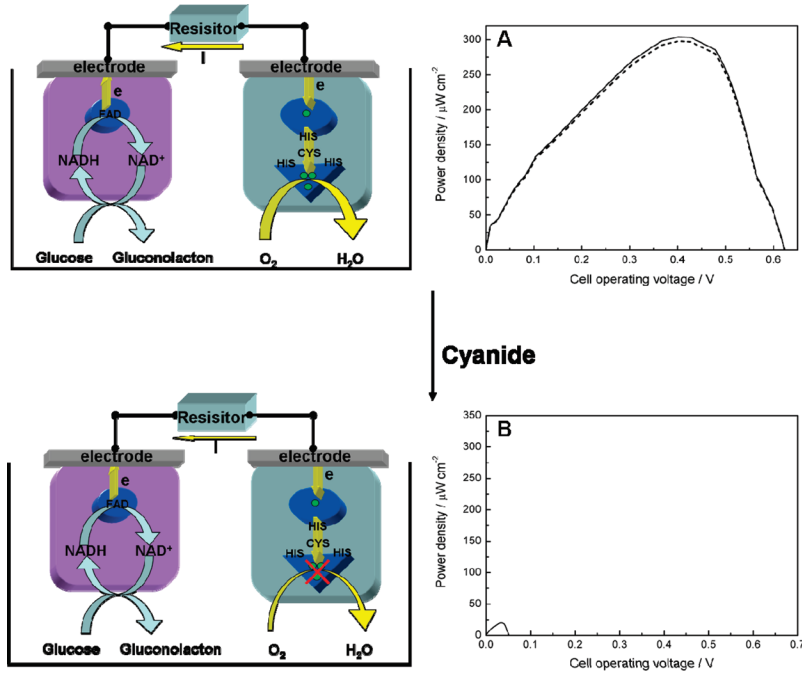


Figure 3. Bioelectrocatalysis mechanism of the cyanide inhibitive effect at the laccase immobilized electrode. (A) Polarization of the uninhibited control and regeneration with 30 min fresh PBS washing of the glucose/O_2 biofuel cell. (B) Polarization of 1 mM cyanide inhibition of the glucose/O_2 biofuel cell. The electrolytes are 10 mM NADH, 40 mM glucose, and 0.2 M PBS (pH 6.5) solution.

electrolyte. Figure 3B shows the polarization curve of the glucose/O_2 biofuel cells in the presence of cyanide. Its power decreases over 1 order of magnitude than that of cyanide absence, obviously, the cyanide can be used to inhibit the power response from the biofuel cell. When the BFC could be regenerated by washing with the fresh PBS, showing the power is not obviously different from the original value under the uninhibited control. It is believed that the catalysis involves laccase at the type 1 (T1) Cu site, internal electron transfer from the T1 Cu to type 2/type 3 (T2/T3) trinuclear Cu cluster, and O_2 reduction at the T2/T3 site. The binding and reduction of O_2 in laccase and other multi-Cu oxidases (such as ascorbate oxidase and ceruloplasmin) take place at the T2/T3 Cu sites.[18] The binding of CN^- onto the T2 Cu is well-known,[19] and its negative effect on oxygen reduction is attributed to a perturbed T2/T3 Cu cluster unfavorable toward the internal electron transfer from T1 Cu. Thus the catalysis of laccase is significantly affected by cyanide addition, as shown in Figure 3. Figure 4 shows the current-output with constant overload of the glucose/O_2 biofuel cell upon the injection with the cyanide concentration at flow conditions. A rapid current decrease is observed immediately after cyanide addition, and after 60 s reached a plateau. Upon increasing cyanide concentration, the current is decreased due to the reduced laccase activity.

It is shown in Figure 3A as the dashed line that the recovery of the power output from 1 mM cyanide inhibition by fresh PBS washing is achieved, but the total power density occurred after 1 h. However, with low concentration of cyanide from 0.1 to 300 μM, the recovery time was less than 10 min. To accelerate the recovery time under a high concentration of cyanide inhibition,

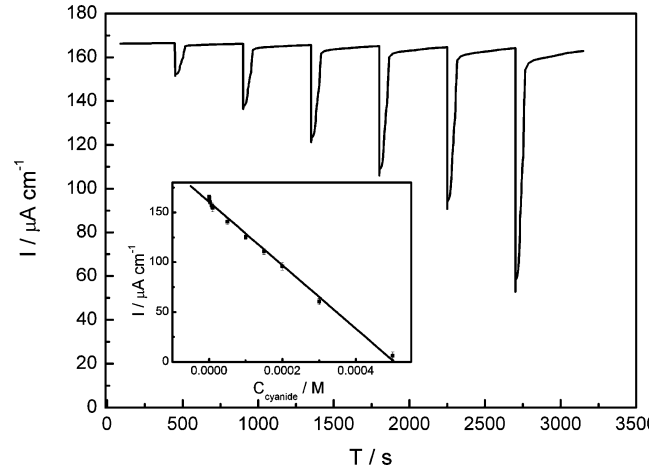


Figure 4. Current-output at variable concentration of the cyanide added into the biofuel cell-based sensor devices. The flowing solution was 10 mM NADH, 40 mM glucose, and 0.2 M PBS (pH 6.5); the cyanide was added at the concentrations 10, 50, 100, 150, 200, and 300 μM, respectively.

0.1 M $CuSO_4$ was added to facilitate the regeneration process and the total recovery time was eliminated to 30 min. Further analytical study under the optimal conditions suggested a linear range of 3.0×10^{-7} to 5.0×10^{-4} M with a detection limit of 1.0×10^{-7} M. The detection limit was lower than the acceptable cyanide concentration in drinking water (1.9×10^{-6} M) according to the World Health Organization (WHO). The reproducibility of the present method was then evaluated. The relative standard deviation for five repeated measurements of 2.0×10^{-4} M CN^- was 3.8%. The self-powered biosensor was stable for at least 1 month upon storage in the PBS at 4 °C. Solutions containing 7 common inhibitor cations and 19 anions (Fe^{3+}, Fe^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, CN^-, Ac^-, $B_4O_7^{2-}$,

(18) Shleev, S.; Tkac, A.; Christenson, A.; Ruzgas, T.; Yaropolov, A. I.; Whittaker, J. W.; Gorton, L. *Biosens. Bioelectron.* **2005**, *20*, 2517–2554.
(19) Xu, F. *Appl. Biochem. Biotechnol.* **2001**, *95*, 125–133.

Br^-, BrO_3^-, ClO_4^-, CO_3^{2-}, $C_2O_4^{2-}$, citrate, F^-, I^-, IO_3^-, NO_2^-, NO_3^-, PO_4^{3-}, S^{2-}, SO_3^{2-}, SO_4^{2-}, and $S_2O_8^{2-}$) and were then tested under the same conditions to evaluate the selectivity of the presented system. All cations exhibited no obvious inhibitive effect on BFC performance up to 0.3 mM at least. Among the tested anions, F^- could be allowed to be at least 7.0×10^{-5} M. There was no obvious decrease of the current-output by the other anions up to 0.1 M at least. Concentrations above 0.1 M were not investigated, due to the higher concentration of other salts added that will make the ionic strength increase, thus the results could not be limited by anion effect only. For a real sample, however, it showed that the interferences from Fe^{3+}, Fe^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, and F^- ions were not significant.

To demonstrate the possibility of this self-powered biosensor on endogenous biological cyanide sensing, the cassava was employed as a model plant. The endogenous enzymes could sufficiently hydrolyze the cyanogenic glycosides in 60 min.[6] The contents of total cyanide by enzyme hydrolysis of the finely ground biological samples were determined directly with the presented self-powered biosensor. The average contents of total cyanide of the finely ground cassava were 126 ± 3 mg/kg from three measurements. The results were in good agreement with the determination of cyanide by gas chromatography, which indicated that our method can be employed as endogenous biological cyanide sensing. Two tests were carried out to demonstrate the general applicability of the self-powered biosensor as a fast and simple detection method in the determination of total cyanide during food processing. Two different methods were conducted in cassava processing to study the removal of hydrogen cyanide, the boiling and washing of a cassava extract. Figure 5 shows the decrease of cyanide during boiling of 1 g of finely ground cassava in 8 mL of water. A total of 45% of the cyanide is released in the first 10 min, further boiling has only a minor influence, suggesting that the enzyme linamarin denatured at high temperatures. On the other hand, washing removed cyanide more efficiently. After only three washings, no cyanide could be detected anymore in the sample. These phenomena are consisted with the reported results;[6] the presented self-powered biosensor could be applied as a fast and simple detection method in the determination of total cyanide during food processing.

CONCLUSIONS

In this technical note, we developed an integrated biofuel cell microchip with both the anode and cathode on the chip, and open circuit potentials of 620 mV and a maximum power density of 302 $\mu W\ cm^{-2}$ were produced. Though the power output of the presented BFC is still low, improvement attempts at serially stacking up multiple biofuel cells together are under way in our lab. This BFC integration with other electronics has the potential to fit the requirement of the totally self-sufficient lab-on-a-chip devices. Most importantly, we demonstrated the feasibility of developing a self-powered biosensor based on the inhibitive effect on the miniaturized enzyme biofuel cell for the first time. With the use of cyanide as the model analyte, the sensor produced power without cyanide, but in presence of cyanide, it inhibited the laccase's oxygen reduction ability and induced the power output decrease. This method showed a linear range of 3.0×10^{-7} to 5.0×10^{-4} M and a detection limit of 1.0×10^{-7} M. Possible applications of the presented sensor during food manufacturing were demonstrated. Compared with the conventional cyanide detection, the present strategy is independent of those relatively large and expensive instruments in laboratories. This self-powered biosensor chip allows the design of portable, economical, analytical tools with minute volume.

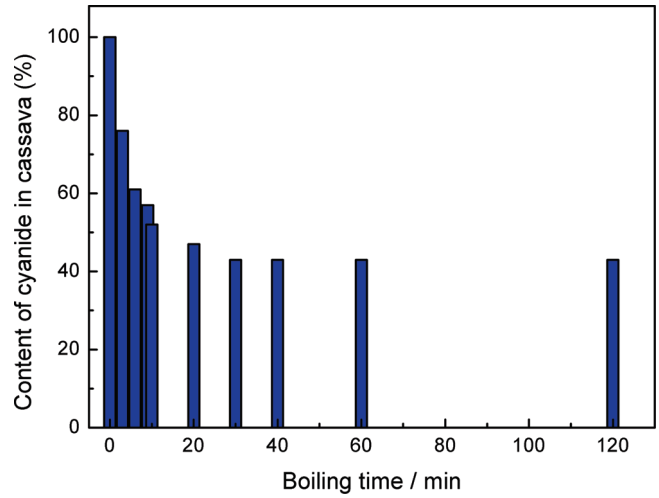


Figure 5. Relative decrease of cyanide during stirring of 1 g of finely ground cassava in 8 mL of water at 100 °C.

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (Grants 20820102637 and 20935003, 973 Projects, Grants 2009CB930100 and 2010CB933600). The authors are also grateful to the reviewers for helpful suggestions to improve the work.

Received for review January 31, 2010. Accepted April 12, 2010.

AC100274S